รายงานวิจัยฉบับสมบูรณ์

โครงการ ชีววิทยาจีโนมมะเร็ง การวิจัยและการนำไปใช้

โดย ศาสตราจารย์ นายแพทย์อภิวัฒน์ มุทิรางกูร

กรกฎาคม 2551

สัญญาเลขที่ RTA4880001

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สังกัด

ภาควิชากายวิภาคศาสตร์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

สัญญาเลขที่ RTA4880001

โครงการ: ชีววิทยาจีโนมมะเร็ง การวิจัยและการนำไปใช้

ชื่อหัวหน้าโครงการวิจัยผู้รับทุน ศ. อภิวัฒน์ มุทิรางกูร

รายงานในช่วงตั้งแต่วันที่ 29 กค 2548

1.2 กิตติกรรมประกาศ

ขอขอบคุณความร่วมมือที่ดีเยี่ยมจากสมาชิกผู้วิจัยที่เป็นอาจารย์ แพทย์ นักวิทยาศาสตร์ ส่วนหนึ่ง ได้แก่ รศ.นพ. ภาคภูมิ สุปิยพันธุ์ รศ.พญ. เสาวณีย์ เย็นฤดี รศ.นพ. นรินทร์ วรวุฒิ ผศ.พญ. กาญจนา โชติเลอศักดิ์ รศ.นพ. วีรชัย ศิริกาญจนะรงค์ รศ.นพ. สมชัย นิรุตติศาสน์ รศ.นพ. ดำรง ตรีสุโกศล รศ. นพ. ประเสริฐ ตรีวิจิตรศิลป์ รศ.พญ. สุรางค์ ตรีรัตนชาติ ผศ.นพ. วิชัย เติมรุ่งเรืองเลิศ ศ.นพ. นเรศร สุขเจริญ รศ.นพ. ประเสริฐ เลิศสงวนสินชัย รศ.นพ. สังคม จงพิพัฒน์วณิชย์ ศ.นพ. วรศักดิ์ โชติเลอ ศักดิ์ อ.พญ. รัศมน กัลยาศิริ อ.ดร.ทพญ อรนาฏ มาตังคสมบัติ รศ.นพ. พิสิฐ ตั้งกิจวานิชย์ ศ.นพ. ยง ภู่วรวรรณ ผศ.นพ. ชลเกียรติ ขอประเสริฐ อ.ดร.พญ. จงกลนี วงศ์ปิยะบวร น.ส. สายรุ้ง ศักดิกุล นาย ประกาศิต รัตนตันหยง อ.นพ. สรภพ เกียรติพงศ์สาน รศ.นพ. อิศรางค์ นุชประยูร น.ส. นุสรา หัวไผ่ Ms Petra Hirsch, Dr. J. Silvio Gutkind, น.ส. ชุติภา พวงไพโรจน์ น.ส. ธัชวรรณ ธนาศุภวัฒน์ น.ส. โชติกา สุญาณเศรษฐกร นาย ศุภกิจโชวุฒิธรรม ศ.ทพญ. กอบกาญจน์ ทองประสม ดร แสงสูรย์ เจริญวิไลศิริ รศ.ดร.พญ. ณัฏฐิยา หิรัญกาญจน์ ศ.นพ. ชนพ ช่วงโชติและ ผศ.ดร.นพ. วิโรจน์ ศรีอุฬารพงศ์ ให้คำปรึกษาทางคลินิกและร่วมทำวิจัย

ขอขอบคุณความร่วมมือที่ดีเยี่ยมจากสมาชิกนิสิตระดับบัณฑิตศึกษา บัณฑิตที่จบการศึกษาส่วนหนึ่ง ได้แก่ ดร. วิชัย พรธนเกษม น.ส. วันเพ็ญ พลเยี่ยม และ ดร. นริศร คงรัตนโชค ศึกษาหมู่เมททิลของ EDSBs. น.ส. พิชญาณีน ชลิตชากร พญ. ฐิติวรรณ ลมดี อ.ทพญ. ดร. เกศกัญญา สัพพะเลข พญ. จีริชุดา ปัทมดิลก และ น.ส. จุรีรัตน์ โพธิ์แก้ว ศึกษาปริมาณและลักษณะการกระจายตัวของหมู่เมททิล ที่ไลน์ 1 ดร. เกรียงศักดิ์ ฤชุศาศวัต น.ส. รุ่งทิวา วัฒนวราภรณ์ ดร. ปฐมวดี ญาณทัศนีย์จิต ดร. จิรนันท์ วราชิต และ อ.ดร.ทพ. นครินทร์ กิตกำธร ศึกษาหมู่เมททิลของยีน ดร.ชูพงศ์ อิทธิวุฒิ และ ดร. รุ่งนภา หิรัญสถิต ศึกษาพันธุศาสตร์ของประชากร ผศ.ดร.พญ. มนตกานต์ ตันสถิตย์ ศึกษาพันธุศาสตร์ของเซลล์ ดร. วิชัย พรธนเกษม ดร. นริศร คงรัตนโชค ดร. รุ่งนภา หิรัญสถิต และ อ.พญ. จันทร์ทรา ดนันท์ยุทธวงศ์ ศึกษามะเร็งโพรงหลังจมูก

1.3 บทคัดย่อทั้งภาษาไทยและภาษาอังกฤษ

1) โครงการศึกษาปริมาณหมู่เมททิลที่ทรานโปซอน

สภาวะเหนือพันธุกรรมของจีโนมที่เกิดขึ้นโดยการเติมหมู่เมททิลในเซลล์ของมนุษย์ส่วนใหญ่พบว่าเป็นการเติมหมู่เมททิลที่ดีเอ็นเอบริเวณที่เป็นทรานโปซอนและดีเอ็นเอที่มีจำนวนชุดซ้ำๆหลายๆครั้ง (ซึ่งเป็นดีเอ็นเอที่พบได้เป็นหมื่นเป็นแสนชุดในแต่ละเซลล์ในขณะที่ยีนทั่วไปพบได้สองชุดโดยแต่ละชุดมาจากพ่อและแม่) เชื่อกันว่าเซลล์จะเติมหมู่เมททิลที่ทรานโปซอนนี้เพื่อปกป้องจีโนม ในเซลล์มะเร็งปริมาณหมู่เมททิลนี้จะลดลงและส่งผลให้เซลล์มะเร็งกลายพันธุ์เร็วขึ้นโดยไม่ทราบกลไกที่แน่นอน คณะวิจัยได้พัฒนาเทคนิกเพื่อตรวจวัดหมู่เมททิล ได้มีการศึกษาเปรียบเทียบการนำการวัดหมู่เมททิลของไลน์1 สำหรับการวินิจฉัยมะเร็ง โดยศึกษามะเร็งปากมดลูก มะเร็งรังไข่ และ ซีรั่มของผู้ป่วยมะเร็งตับ นอกจากนี้ ได้รายงานการคงค้างของดีเอ็นเอที่ฉีกขาดที่เกิดขึ้นเองในเซลล์ เพราะมีหมู่เมททิลเกาะอยู่ รายงานการศึกษาหมู่เมททิลของดีเอ็นเอที่ฉีกขาดที่เกิดขึ้นเองนี้ ได้นำเสนอแนวทางที่สำคัญในการค้นหากลไกที่ทำให้เกิดความไม่เสถียรของจีโนมมะเร็ง เพราะมีหมู่เมททิลน้อย การศึกษานี้จึงเป็นแนวทางที่สำคัญเพื่อทำให้เกิดความเข้าใจต่อกลไกที่ทำให้เซลล์มะเร็งเกิดกลายพันธุ์ในอัตราที่เร็ว ซึ่งความรู้ดังกล่าวอาจนำไปสู่วิธีการป้องกันการเกิดโรคมะเร็งได้อย่างมีประสิทธิภาพในอนาคต

2) โครงการศึกษาการเติมหมู่เมททิลที่โปรโมเตอร์ของยีน

โครงการนี้เป็นแนวทางหนึ่งของการค้นหา tumor markers ตัวใหม่ ๆ ได้แก่การค้นหายีนที่มีการเติมหมู่เมททิลที่โปรโมเตอร์ของยีนนั้นๆ ในเซลล์มะเร็ง การค้นพบดังกล่าวจะบ่งบอกว่ายีนนั้นๆน่าจะมีความสำคัญเป็นยีนต้านมะเร็งในเซลล์มะเร็งชนิดนั้นๆ ดังนั้นนอกจากจะมีประโยชน์ในการวินิจฉัยแล้ว ยังเป็นข้อมูลที่สำคัญต่อการทำวิจัยเพื่อการรักษาแบบมุ่งเป้า (targeted therapy) อีกด้วย แนวทางหลักของงานวิจัยนี้ใช้ข้อมูลจากการศึกษา การแสดงออกของยีนทั้งจีโนมของเซลล์มะเร็งโพรงหลังจมูก และเลือกยีนที่มีความเป็นไปได้มาศึกษาในเซลล์ชนิดต่างๆ และพิสูจน์การเติมหมู่เมททิลและการแสดงออกของยีนในเซลล์มะเร็งโพรงหลังจมูก ในขณะที่พยายามศึกษาเซลล์มะเร็งโพรงหลังจมูก ได้มีการค้นพบการเติมและสูญเสียหมู่เมททิลของยีนต่าง ๆในเซลล์และโรคต่าง ๆ ได้แก่ *CCNA1* methylation ในมะเร็งปากมดลูก, *TTC12* methylation ในมะเร็งเม็ดเลือดแบบ ALL, และ การมีหมู่เมททิลของยีน *SHP-1* ในเซลล์เยื่อบุผิวและการสูญเสียในโรคผิวหนังสะเก็ดเงิน

3) โครงการสนับสนุนการศึกษาวิจัยทางอณูพันธุศาสตร์

จากการเป็นเมธีวิจัยอาวุโสของ สกว. ทำให้มีหน้าที่ช่วยเหลือนักวิจัยรุ่นใหม่และทำวิจัยทางอณูพันธุศาสตร์ร่วมกับสถาบันอื่นๆ ผลงานวิจัยเหล่านี้ได้แก่ การร่วมมือกับมหาวิทยาลัย Yale ในการศึกษาพันธุศาสตร์ของการติดยาและประชากร และ การศึกษาร่วมกับนักวิจัยไทยเพื่อศึกษาการกลายพันธุ์ของยีน *EGFR* ในมะเร็งปอด และการถ่ายทอดโครโมโซมที่ผิดปกติจากพ่อหรือแม่เท่านั้น (uniparental disomy)

คำหลัก 5 คำ หมู่เมททิล ไลน์1 จีโนมไม่เสถียร โปรโมเตอร์ของยีน ดีเอนเอที่ฉีกขาดเอง

1) Study methylation of transposon derived elements project

Majority of DNA methylation in human genome is localized in interspersed transposon derived elements. It is generally believe that eukaryotic cell uses this mechanism to protect its genome. In cancer, these methyl groups usually decrease and consequently lead to genomic instability (faster rate of mutations). The mechanism is unknown. We developed a new technique to measure the DNA methylation level. We evaluated long interspersed nuclear element-1 (LINE-1 or L1) methylation level by our PCR technique, namely COBRALINE-1. We evaluated LINE-1 methylation levels of cervical cancer, ovarian cancer and sera of patients with hepatoma. Moreover, we reported LINE-1 methylation status of endogenous DNA double strand breaks (EDSBs) and proved that EDSB methylation levels are higher than genomes across all cell types. This finding is crucial for better understanding the global hypomethylation leading genomic instability mechanism. We also hope this will be an important clue for future cancer prevention method.

2) Gene promoter methylation project

Discovering new genes with promoter hypermethylation is another approach to discover new tumor markers. The findings, indicating the possibility of tumor suppressor genes, are not only benefit cancer diagnosis but also important for future targeted therapy. Our first approach was to screen for nasopharyngeal carcinoma (NPC) tumor suppressor genes using our previous micro array information. We hypothesized that down regulated genes that localized within loss of heterozygosity regions or known to be other cancer tumor suppressor genes should possess promoter hypermethylation in NPC. In addition to three new genes in NPC, we discovered *CCNA1* methylation in cervical cancer, *TTC12* methylation in acute lymphoblastic leukemia and also *SHP-1* methylation in normal epithelium and demethylation in psoriasis.

3) Molecular genetics support projects

We collaborated with several young Thai investigators and Yale university. With Yale University, we produced several articles in population genetics and genetics of drug addiction. We also help several young Thai investigators to report EGFR

Keywords: DNA methylation, Genomic Instability, long interspersed nuclear element-1, Promotor methylation, Endogenous DNA double strand breaks

1.4 เนื้อหางานวิจัย

1) โครงการศึกษาปริมาณหมู่เมททิลที่ทรานโปซอน

- General Concept

Cancer cells are frequently characterized by hypomethylation of the genome including repetitive sequences. This epigenetic process is believed to be associated with several biological causes and consequences in cancer. Therefore, long interspersed nuclear element-1 (LINE-1 or L1) repetitive sequences demethylation in cancer should result in different clinical outcomes. Recently, we have developed an improved quantitative combined bisulfite restriction analysis PCR protocol that efficiently evaluates the methylation status of LINE-1s; the method is referred to as PCR "COBRALINE-1". This article reviewed what have been learned by applying this technique to study methylation level of repetitive sequences from several sources of genomic DNA. We have found that LINE-1 methylation patterns among normal tissues are distinct. Therefore, this epigenetic event may be continuously altered in adult tissues by the process of cellular differentiation. Moreover, we confirmed that global hypomethylation is an ongoing process that develops during tumor progression, in addition to previous evidence of genomic and LINE-1 hypomethylation occurring as an early event in carcinogenesis. COBRALINE-1 is a highly effective technique for evaluating the genome-wide level of methylation, in particular from tissue samples with minute amounts of low quality DNA. The technique has been applied to study samples from micro-dissected archived paraffin-embedded tissues and sera of several types of cancer. The COBRALINE-1 technique demonstrated its potential to be a tumor marker and a great tool to explore the biology of global hypomethylation.

- LINE-1 methylation status of endogenous DNA double-strand breaks

DNA methylation and the repair of DNA double-strand breaks (DSBs) are important processes for maintaining genomic integrity. Although DSBs can be produced by numerous agents, they also occur spontaneously as endogenous DSBs (EDSBs). In this study, we evaluated the methylation status of EDSBs to determine if there is a connection between DNA methylation and EDSBs. We utilized interspersed repetitive sequence polymerase chain reaction (PCR), ligation-mediated PCR and combined bisulfite restriction analysis to examine the extent of EDSBs and methylation at LINE-1 sequences nearby EDSBs. We tested normal white blood cells and several cell lines derived from epithelial cancers and leukemias. Significant levels of EDSBs were detectable in all cell types. EDSBs were also found in both replicating and non-replicating cells. We found that EDSBs contain higher

levels of methylation than the cellular genome. This hypermethylation is replication independent and the methylation was present in the genome at the location prior to the DNA DSB. The differences in methylation levels between EDSBs and the rest of the genome suggests that EDSBs are differentially processed, by production, end-modification, or repair, depending on the DNA methylation status.

- Detection of LINE-1s Hypomethylation in Oral Rinses of Oral Squamous Cell Carcinoma Patients

This study aimed to (i) investigate long interspersed nuclear element-1 (LINE-1) methylation levels of oral squamous cell carcinomas (OSCCs), the major type of oral malignancies; (ii) investigate whether the hypomethylation of LINE-1s can be detected in oral rinses of OSCC patients. The combined bisulfite restriction analysis polymerase chain reaction (PCR) of LINE-1s (COBRALINE-1) was used. We found that tissues from OSCC specimens had lower methylation levels of LINE-1s than cells collected from oral rinses of normal volunteers. Interestingly, cells collected from oral rinses of OSCC patients also revealed hypomethylated LINE-1s at the same level as OSCC tissues. There was no difference in the level of hypomethylation among tumors with different stages, locations, histological grades, history of betel chewing, smoking and/or alcohol consumption. In conclusion, OSCCs possessed global hypomethylation and this alteration could be detected from oral rinses of OSCC patients by a simple PCR technique, COBRALINE-1. Therefore, COBRALINE-1 of oral rinses may be applied for non-invasive detection of oral malignancies.

- Serum LINE-1 hypomethylation as a potential prognostic marker for hepatocellular carcinoma

We investigated the clinical implications of global hypomethylation, one of the most consistent epigenetic changes in cancer, in the sera of patients with hepatocellular carcinoma (HCC). Combined bisulfite restriction analysis PCR was used to assess the methylation status of LINE-1 repetitive sequences in genomic DNA derived from sera of 85 patients with HCC, 73 patients with cirrhosis, 20 healthy carriers of hepatitis B virus (HBV) and 30 healthy controls. Serum genome hypomethylation, the percentage of unmethylated LINE-1, was significantly increased in patients with HCC ($P<0.001$). The levels of serum LINE-1 hypomethylation at initial presentation correlated significantly with the presence of HBsAg, large tumor sizes, and advanced tumor stages classified by the CLIP score. Multivariate analyses showed that serum LINE-1 hypomethylation was a significant and

independent prognostic factor of overall survival. Serum LINE-1 hypomethylation may serve as a prognostic marker for patients with HCC.

-Line-1 hypomethylation in multistage carcinogenesis of the uterine cervix

To evaluate characteristics of global hypomethylation in evolution of cervical cancer. Eight cases of squamous cell carcinoma (SCC) and seven cases of carcinoma in situ (CIS) were studied. Each of the SCC samples contained CIS, and all SCC and CIS samples contained normal ectocervical epithelium. Microdissection was performed to separate normal epithelium, CIS and SCC prior to DNA extraction. Hypomethylation levels of long interspersed nuclear elements (LINE-1 or L1) were measured with a combined bisulfite restriction analysis (COBRA) PCR (polymerase chain reaction) protocol. The percentage of L1 hypomethylation for SCC, CIS and normal epithelium was compared. RESULTS: In the SCC cohort, the L1 hypomethylation level showed progressive increase comparing normal epithelium (59.4 +/- 8.86%) to CIS (64.37 +/- 7.32%) and SCC (66.3 +/- 7.26%) (repeated measurement ANOVA, P = 0.005). A significantly greater L1 hypomethylation level was found in CIS (62.06 +/- 3.44 %) compared to normal epithelium (60.03 +/- 3.69 %) (paired t-Test, P = 0.03). No significant difference in L1 hypomethylation level was noted between CIS of the two sample groups (unpaired t-Test, P = 0.2). In our study, there was a significant correlation between the degree of hypomethylation and progression from normal ectocervical mucosa to CIS and invasive cancer. Laboratory assessment of biopsies for this molecular event may have clinical significance.

2) โครงการศึกษาการเติมหมู่เมททิลที่โปรโมเตอร์ของยีน

- Promoter hypermethylation of CCNA1, RARRES1, and HRASLS3 in nasopharyngeal carcinoma

In search for putative tumor suppressor genes critical of nasopharyngeal carcinoma (NPC), we analyzed the available information from the expression profiling in conjunction with the comprehensive alleotyping published data relevant to this malignancy. Integration of this information suggested eight potential candidate tumor suppressor genes, CCNA1, HRASLS3, RARRES1, CLMN, EML1, TSC22, LOH11CR2A and MCC. However, to confirm the above observations, we chose to investigate if promoter hypermethylation of these candidate genes would be one of the mechanisms responsible for the de-regulation of gene expression in NPC in addition to the loss of genetic materials. In this study, we detected consistent hypermethylation of the 5' element of CCNA1, RARRES1, and HRASLS in NPC tissues with prevalence of 48%, 51%, and 17%, respectively. Moreover,

we found a similar profile of promoter hypermethylation in primary cultured NPC cells but none in normal nasopharyngeal epithelium or leukocytes, which further substantiate our hypothesis. Our data indicate that CCNA1, RARRES1, and HRASLS3 may be the putative tumor suppressor genes in NPC.

- Promoter hypermethylation of CCNA1, RARRES1, and HRASLS3 in nasopharyngeal carcinoma

In search for putative tumor suppressor genes critical of nasopharyngeal carcinoma (NPC), we analyzed the available information from the expression profiling in conjunction with the comprehensive alleotyping published data relevant to this malignancy. Integration of this information suggested eight potential candidate tumor suppressor genes, CCNA1, HRASLS3, RARRES1, CLMN, EML1, TSC22, LOH11CR2A and MCC. However, to confirm the above observations, we chose to investigate if promoter hypermethylation of these candidate genes would be one of the mechanisms responsible for the de-regulation of gene expression in NPC in addition to the loss of genetic materials. In this study, we detected consistent hypermethylation of the 5' element of CCNA1, RARRES1, and HRASLS in NPC tissues with prevalence of 48%, 51%, and 17%, respectively. Moreover, we found a similar profile of promoter hypermethylation in primary cultured NPC cells but none in normal nasopharyngeal epithelium or leukocytes, which further substantiate our hypothesis. Our data indicate that CCNA1, RARRES1, and HRASLS3 may be the putative tumor suppressor genes in NPC.

- Hypermethylation of TTC12 gene in acute lymphoblastic leukemia

Human chromosome 11q23 may locate an important tumor suppressor gene associated leukaemia. This study proposed methylation of CpG island in tetratricopeptide repeat domain 12 (*TTC12*) gene located in this region was specific to leukaemogenesis. Whereas the gene was methylated in some leukaemic cell lines, Daudi, Jurkat and Molt4, no methylation was observed in epithelial cell lines, HeLa, SW480 and HN12. Moreover, we tested 29 acute lymphoblastic leukaemia (ALL), normal white blood cells (WBC) and eight remission bone marrows. The *TTC12* is hypermethylated in most ALL but not in WBC and the bone marrows. The *TTC12* methylation level of ALL, mean 59.41, 73.45 and 44.87% of B-ALL, T-ALL, and ANLL, respectively, was more than normal WBC, mean 9.42%, ($p<0.0001$). The methylation level also inversely correlated with expression of the gene in both in cell lines and clinical samples. We found significant increase in expression of *TTC12* in hypomethylated but not in hypermethylated cases. TTC12 possesses two

conserved domains have been found including the tetratricopeptide repeat domain and the armadillo repeat domains and may be important ranging from cell cycle control to signal transduction, kinase regulation and tumorigenesis. Therefore, methylation of *TTC12* may be important in leukaemogenesis.

- SHP-1 promoter 2 methylation in normal epithelial tissues and demethylation in psoriasis

SHP-1 promoter hypermethylation has been studied in hematopoietic cells and observed only in various types of lymphoma and leukemia. This study reports a contrasting situation in normal epithelial tissues and an association with skin pathogenesis, particularly in psoriasis. We investigated several cell lines, five of them were epithelial and six were hematopoietic, white blood cells from normal, healthy donors, and normal microdissected epithelium of kidney, liver, breast, cervix, lung, prostate, bladder, and skin. Interestingly, promoter 2 hypermethylation was apparent in all epithelial cell lines and tissues. However, distinctive degrees of demethylation were noted in some skin samples. The methylation patterns of each cell line corresponded to their mRNA isoforms, in that isoforms I and II could not be detected with either promoter 1 or 2 hypermethylation, respectively. We further explored whether an enhanced degree of demethylation could be observed in various dermatopathology lesions. While the promoter 2 methylation levels of squamous cell cancers, eczemas, and normal skins were not different, a significant degree of demethylation can be observed in psoriasis ($p<0.005$). In addition, psoriasis displays a higher level of SHP-1 isoform II than normal skin ($p<0.05$). In conclusion, this study discovered an unprecedented role of SHP-1 methylation in tissue-specific expression and its alteration in a nonmalignant human disease besides the transcription inhibition in leukemia and lymphoma. Furthermore, the promoter demethylation may play an important role in skin pathogenesis by enhancing SHP-1 isoform II transcription in psoriatic skin lesions.

- Cyclin A1 promoter hypermethylation in human papillomavirus-associated cervical cancer

The aim of this study was to evaluate epigenetic status of cyclin A1 in human papillomavirus-associated cervical cancer. Y. Tokumaru et al., Cancer Res 64, 5982-7 (Sep 1, 2004)demonstrated in head and neck squamous-cell cancer an inverse correlation between cyclin A1 promoter hypermethylation and TP53 mutation. Human papillomavirus-associated cervical cancer, however, is deprived of TP53 function by a different

mechanism. Therefore, it was of interest to investigate the epigenetic alterations during multistep cervical cancer development. In this study, we performed duplex methylation-specific PCR and reverse transcriptase PCR on several cervical cancer cell lines and microdissected cervical cancers. Furthermore, the incidence of cyclin A1 methylation was studied in 43 samples of white blood cells, 25 normal cervices, and 24, 5 and 30 human papillomavirus-associated premalignant, microinvasive and invasive cervical lesions, respectively. We demonstrated cyclin A1 methylation to be commonly found in cervical cancer, both in vitro and in vivo, with its physiological role being to decrease gene expression. More important, this study demonstrated that not only is cyclin A1 promoter hypermethylation strikingly common in cervical cancer, but is also specific to the invasive phenotype in comparison with other histopathological stages during multistep carcinogenesis. None of the normal cells and low-grade squamous intraepithelial lesions exhibited methylation. In contrast, 36.6%, 60% and 93.3% of high-grade squamous intraepithelial lesions, microinvasive and invasive cancers, respectively, showed methylation. This methylation study indicated that cyclin A1 is a potential tumor marker for early diagnosis of invasive cervical cancer.

3) โครงการสนับสนุนการศึกษาวิจัยทางอณูพันธุศาสตร์

- Maternal uniparental disomy of chromosome 16 resulting in hemoglobin Bart's hydrops fetalis

We describe HbBart's hydrops fetalis with $^{-SEA}$ deletion in a fetus who was a product of a Thai woman affected with HbH disease ($^{-SEA}$ and $^{3.7\ kb}$ deletions) and a man with heterozygous HbE. In conjunction with the presence of fetal anomalies (single umbilical artery, bilobed right lung, and meckel diverticulum), maternal uniparental disomy for chromosome 16 (matUPD16) was suspected. DNA fingerprint analysis indicated no conflict of paternity. Microsatellite marker analysis confirmed maternal heterodisomy for chromosome 16 with segmental isodisomy of 16p13.3 resulted from meiosis I error with recombination between the homologous chromosomes, as a cause of the HbBart's disease. Other mutations of alpha1 globin gene previously reported to cause fetal hydrops were also excluded. Despite being an uncommon genetic event, matUPD16 should be considered as a possible mechanism for HbBart's hydrops fetalis, especially in populations with high frequency of alpha1 globin gene deletions.

- Interpopulation linkage disequilibrium patterns of GABRA2 and GABRG1 genes at the GABA cluster locus on human chromosome 4

GABRA2 and GABRG1, which encode the alpha-2 and gamma-1 subunits, respectively, of the GABA(A) receptor, are located in a cluster on chromosome 4p. The GABRA2 locus has been found to be associated with alcohol dependence in several studies, but no functional variant that can account for this association has been identified. To understand the reported associations, we sought to understand the linkage disequilibrium (LD) patterns and haplotype structures of these genes. With close intergenic distance, approximately 90 kb, it was anticipated that some markers might show intergenic LD. Variation in 13-SNP haplotype block structure was observed in five different populations: European American, African American, Chinese (Han and Thai), Thai, and Hmong. In the Hmong, a 280-kb region of considerably higher LD spans the intergenic region, whereas in other populations, there were two or more LD blocks that cross this region. These findings may aid in understanding the genetic association of this locus with alcohol dependence in several populations.

- Demographic changes and marker properties affect detection of human population differentiation

Differentiating genetically between populations is valuable for admixture and population stratification detection and in understanding population history. This is easy to achieve for major continental populations, but not for closely related populations. It has been claimed that a large marker panel is necessary to reliably distinguish populations within a continent. We investigated whether empirical genetic differentiation could be accomplished efficiently among three Asian populations (Hmong, Thai, and Chinese) using a small set of highly variable markers (15 tetranucleotide and 17 dinucleotide repeats). RESULTS: Hmong could be differentiated from Thai and Chinese based on multi-locus genotypes, but Thai and Chinese were indistinguishable from each other. We found significant evidence for a recent population bottleneck followed by expansion in the Hmong that was not present in the Thai or Chinese. Tetranucleotide repeats were less useful than dinucleotide repeat markers in distinguishing between major continental populations (Asian, European, and African) while both successfully distinguished Hmong from Thai and Chinese. CONCLUSION: Demographic history contributes significantly to robust detection of intracontinental population structure. Populations having experienced a rapid size reduction may be reliably distinguished as a result of a genetic drift -driven redistribution of population allele frequencies. Tetranucleotide markers, which differ from dinucleotide

markers in mutation mechanism and rate, are similar in information content to dinucleotide markers in this situation. These factors should be considered when identifying populations suitable for gene mapping studies and when interpreting interpopulation relationships based on microsatellite markers.

- Sequence variation and linkage disequilibrium in the GABA transporter-1 gene (SLC6A1) in five populations: implications for pharmacogenetic research

GABA transporter-1 (GAT-1; genetic locus SLC6A1) is emerging as a novel target for treatment of neuropsychiatric disorders. To understand how population differences might influence strategies for pharmacogenetic studies, we identified patterns of genetic variation and linkage disequilibrium (LD) in SLC6A1 in five populations representing three continental groups. RESULTS: We resequenced 12.4 kb of SLC6A1, including the promoters, exons and flanking intronic regions in African-American, Thai, Hmong, Finnish, and European-American subjects (total n=40). LD in SLC6A1 was examined by genotyping 16 SNPs in larger samples. Sixty-three variants were identified through resequencing. Common population-specific variants were found in African-Americans, including a novel 21-bp promoter region variable number tandem repeat (VNTR), but no such variants were found in any of the other populations studied. Low levels of LD and the absence of major LD blocks were characteristic of all five populations. African-Americans had the highest genetic diversity. European-Americans and Finns did not differ in genetic diversity or LD patterns. Although the Hmong had the highest level of LD, our results suggest that a strategy based on the use of tag SNPs would not translate to a major improvement in genotyping efficiency. CONCLUSION: Owing to the low level of LD and presence of recombination hotspots, SLC6A1 may be an example of a problematic gene for association and haplotype tagging-based genetic studies. The 21-bp promoter region VNTR polymorphism is a putatively functional candidate allele for studies focusing on variation in GAT-1 function in the African-American population.

- High frequency of mutation of epidermal growth factor receptor in lung adenocarcinoma in Thailand

Recent reports have suggested influences of racial difference on the frequency of mutation of EGFR in lung cancer. We therefore sought to characterize the frequency and pattern of mutation of EGFR in lung adenocarcinoma in Thai patients. Overall, EGFR catalytic domain mutations were detected in 35/61 (57.4%). We found 29/60 (48.3%) of exon 19 deletions, 5/54 (9.3%) of exon 21 point mutations, and 1/54 (1.9%) of double-

mutation of both exons. The presence of these mutations was significantly associated with non-smoking habit. In summary, we report a strikingly high prevalence of mutation of EGFR in Thai lung adenocarcinoma, which may explain the high response rate to the treatment with TKI among Asian populations.

1.5 output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

- ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุค่า impact factor)/ หนังสือ / สิทธิบัตร

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สิทธิบัตร

จุรีรัตน์ โพธิ์แก้ว และ อภิวัฒน์ มุทิรางกูร. กรรมวิธีการตรวจวัดหมู่เททิลของยีน LINE-1 ในแต่ละตำแหน่ง. วันที่รับคำขอ 25 เม.ย. 2551, เลขที่คำขอ 0801002098.

หนังสือ

1. อภิวัฒน์ มุทิรางกูร และ สุทัศน์ ฟู่เจริญ "แนวทางของการเขียนรายงานเพื่อให้ได้รับการตีพิมพ์: มุมมองของนักวิจัยสาขาวิทยาศาสตร์การแพทย์" ใน ยอดหทัย เทพธรานนท์ ประมวล ตั้งบริบูรณ์รัตน์ บรรณาธิการ มกราคม 2548 การเขียนบทความวิจัยระดับนานาชาติด้านวิทยาศาสตร์และเทคโนโลยี ISBN 974-92744-4-X
2. หนังสือเฉลิมพระเกียรติ ของ สกว งานวิจัยพื้นฐาน: ฐานรากของการพัฒนาประเทศตามปรัชญาเศรษฐกิจพอเพียงด้านการวิจัยพื้นฐานเพื่อพัฒนาสุขภาพ 2 บท

- การประชุมวิชาการนานาชาติ

30 ก.ค. 2547 วิทยากรบรรยายเรื่อง "Molecular Genetics of Epstein-Barr virus associated Nasopharyngeal Carcinoma: Chulalongkorn University Experience" และเป็น Chairman ในหัวข้อ Beyond Diversity: EBV Related Malignancies in Asia-Pacific Region ณ การประชุม The 63rd

Annual Meeting of the Japanese Cancer Association — Program of the Asia-Pacific Session ณ Kyushu University, JAPAN

11 พ.ย. 2548 วิทยากรบรรยายเรื่อง "L1 methylation in cancer" ณ Kyushu University, JAPAN

20 มิ.ย. 2550 วิทยากรบรรยายเรื่อง "Study DNA methylation: Chulalongkorn University Experience" ในการประชุม Thailand-Virginia Tech Forum on Applications of Biotechnology in Modern Agricultural and Medical Research Focusing on Plant Science Research and Cell & Developmental Biology Research" ณ ศูนย์พันธุวิศวกรรมและเทคโนโลยีแห่งชาติ

11 ม.ค. 2550 วิทยากรบรรยายเรื่อง "Global DNA hypomethylation and cancer diagnosis" ในการประชุม Thai and Japanese workshop in diagnostic cytopathology ณ โรงแรมสยามซิตี้

26 พ.ย. 2550 วิทยากรบรรยายเรื่อง "The possible roles of intersperse repetitive sequence hypomethylation in cancer" ในการประชุม The Sixth Princess Chulabhorn International Science Congress: The Interface of Chemistry and Biology in the "Omics" Era: *Environment & Health and Drug Discovery* November 25 - 29, 2007, Bangkok, THAILAND ณ โรงแรมแชงกรีลา

5 ส.ค. 2551 วิทยากรบรรยายเรื่อง "The Possible Roles of Long Interspersed Element-1 (LINE-1) Sequence Hypomethylation in Cancer" ณ Department of Chemical Pathology of The Chinese University of Hong Kong.

- การนำผลงานไปใช้ประโยชน์ในเชิงพาณิชย์ เชิงสาธารณะ หรือเชิงนโยบาย

ผลงานทางวิชาการได้รับการอ้างอิงอย่างกว้างขวางจาก ตำราต่างประเทศ (Textbook) และวารสารระดับนานาชาติ (International Journal) โดยการอ้างอิงเกิดขึ้นอย่างต่อเนื่องนับตั้งแต่เวลาที่ผลงานได้รับการตีพิมพ์เผยแพร่ต่อเนื่องมาจนถึงปัจจุบัน โดยจากฐานข้อมูล ISI web of science แสดงจำนวนครั้งของการได้รับการอ้างอิงจากผลงานของทุนวิจัยนี้รวมทั้งหมด 16 ครั้ง ส่วนการอ้างอิงตลอดชีวิตของหัวหน้าโครงการคือ 1265 ครั้ง ผลงานที่นำไปใช้ประโยชน์ในทางการแพทย์ ได้แก่ การค้นพบดีเอ็นเอของ EBV ในน้ำเหลืองในกระแสเลือดของผู้ป่วยด้วยโรคมะเร็งโพรงหลังจมูก การค้นพบนี้ได้นำไปสู่การตรวจวัดปริมาณดีเอ็นเอของไวรัสเพื่อติดตามผลการรักษาผู้ป่วยมะเร็งโพรงหลังจมูกซึ่งเป็นวิธีการที่มีประสิทธิภาพสูงและใช้กันทั่วไปในปัจจุบัน นอกจากนี้ยังได้ยื่นจดสิทธิบัตร 1 ใบ

- ความก้าวหน้าของทีมวิจัย ฯลฯ

1. ศ.ดร.นพ. อภิวัฒน์ มุทิรางกูร ได้รับรางวัลผลงานวิจัยดีเด่น (หัวหน้าโครงการ) และนักวิจัยดีเด่นแห่งชาติ ของสภาวิจัยแห่งชาติ และนักวิทยาศาสตร์ดีเด่นประจำปี 2551 ของมูลนิธิส่งเสริมวิทยาศาสตร์และเทคโนโลยีในพระบรมราชูปถัมภ์
2. ผศ.ดร.นพ วิโรจน์ ศรีอุพารพงศ์ ทุนพัฒนาอาจารย์ใหม่ สกว และ สกอ และทุนเมธีวิจัย
3. ศ.นพ. ชนพ ช่วงโชติ ทุนพัฒนาอาจารย์ใหม่ สกว และ สกอ รางวัลนักวิจัยรุ่นเยาว์ของมูลนิธิส่งเสริมวิทยาศาสตร์และเทคโนโลยีในพระบรมราชูปถัมภ์ ปัจจุบันดำรงตำแหน่งศาสตราจารย์
4. พญ. จีริชุดา ปัทมดิลก รางวัลวิทยานิพนธ์ดีเด่น ของ ราชวิทยาลัยสูติศาสตร์-นรีเวชวิทยา อนุสาขามะเร็งนรีเวชวิทยา ปัจจุบันประจำที่ สถาบันวิจัยจุฬาภรณ์
5. อ. นพ. สรภพ เกียรติพงศ์สาน รับพระราชทานทุนทุนอานันทมหิดล
6. ดร. แสงสูรย์ เจริญวิไลศิริ ทุนวิจัยจาก BIOTEC
7. อ. ดร. ปฐมวดี ญาณทัศนีย์จิต ทุนการศึกษาระดับ PhD ของ สกอ ปัจจุบันเป็นอาจารย์ประจำจุฬาลงกรณ์มหาวิทยาลัย และรับทุนพัฒนาอาจารย์ของ สกว และ สกอ
8. อ.ดร.ทนพ นครินทร์ กิตกำจร ทุน RGJ ปัจจุบันเป็นอาจารย์ประจำมหาวิทยาลัยมหิดล และรับทุนพัฒนาอาจารย์ของ สกว และ สกอ
9. ดร. วิชัย พรธนเกษม ทุน RGJ ปัจจุบันประจำ BIOTEC
10. ดร. นริศร คงรัตนโชค ทุน RGJ ปัจจุบันประจำโครงการนี้และมีแผนการจะต่อ Postdoctoral Training ที่ Emory University
11. ศุภกิจ โขวุฒิธรรม ทุน RGJ
12. ดร. เกรียงศักดิ์ ฤชุศาศวัต ทุน RGJ ปัจจุบันประจำกรมวิทยาศาสตร์การแพทย์
13. โชติกา สุญาณเศรษฐกร ทุน RGJ
14. ดร. รุ่งนภา หิรัญสถิตย์ ทุนการศึกษา PhD จาก NIH, USA ปัจจุบันประจำโครงการนี้
15. ดร. ชูพงศ์ อิทธิวุฒิ ทุนการศึกษา PhD จาก NIH, USA ปัจจุบันประจำโครงการนี้
16. ผศ.ดร.พญ. รัศมน กัลยาศิริ ทุนการศึกษา fellow (postdoc) จาก NIH, USA ปัจจุบันเป็นอาจารย์ประจำจุฬาลงกรณ์มหาวิทยาลัย และรับทุนพัฒนาอาจารย์ของ สกว และ สกอ

1.6 รายชื่อคณะผู้ร่วมวิจัยในกลุ่มวิจัยของท่าน ตั้งแต่เริ่มโครงการจนถึงปัจจุบัน โดยแบ่งเป็น นักวิจัย ผู้ช่วยวิจัย นักศึกษาปริญญาโท นักศึกษาปริญญาเอก โดยระบุตำแหน่งวิชาการ และสถาบันต้น
สังกัด รวมทั้งสถานะภาพปัจจุบัน
ผู้ร่วมวิจัยเมื่อร่วมโครงการ

1. วิโรจน์ ศรีอุพารพงศ์ พบ., Ph.D. (VIROTE SRIURANPONG M.D., Ph.D.)
อาจารย์ ภาควิชา อายุรศาสตร์ คณะ แพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
โทรศัพท์ 022564533 โทรสาร 022564534
2. ชนพ ช่วงโชติ พบ (SHANOP SHUANGSHOTI M.D.)

ผู้ช่วยศาสตราจารย์ ภาควิชา พยาธิวิทยา คณะ แพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

3. วีระชัย คีรีกาญจนะรงค์ พบ (VIRACHAI KEREKHANJANARONG M.D.)

รองศาสตราจารย์ ภาควิชา โสตนาสิกลาริงซ์วิทยา คณะ แพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

4. กาญจนา โชติเลอศักดิ์ พบ (KANJANA Shotelersuk M.D.)

ผู้ช่วยศาสตราจารย์ ภาควิชา รังสีวิทยา คณะ แพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

5. มนตกานต์ ตันสถิตย์ พบ (MONTAKARN TANSATIT M.D.)

ผู้ช่วยศาสตราจารย์ ภาควิชา กายวิภาคศาสตร์ คณะ แพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

6. พิสิฐ ตั้งกิจวาณิชย์ พบ Ph.D. (PHISIT TANGKITVANIT M.D., Ph.D.)

ผู้ช่วยศาสตราจารย์ ภาควิชา ชีวเคมี คณะ แพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

7. สรภพ เกียรติพงศ์สาน พบ (Sorapop Kiatpongsan, M.D)

อาจารย์ ภาควิชา สูตินรีเวช คณะ แพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

8. สมชัย นิรุตติศาสน์ พบ (SOMCHAI NIRUTHISARD, M.D)

รองศาสตราจารย์ ภาควิชา สูตินรีเวช คณะ แพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

9. ดำรง ตรีสุโกศล พบ (DAMRONG TRESUKOSOL, M.D)

รองศาสตราจารย์ ภาควิชา สูตินรีเวช คณะ แพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

10. สุรางค์ ตรีรัตนชาติ พบ (SURANG TRIRATANACHAT, M.D)

รองศาสตราจารย์ ภาควิชา สูตินรีเวช คณะ แพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

11. วิชัย เติมรุ่งเรืองเลิศ พบ (WICHAI TERMRUNGRUANGLERT, M.D)

ผู้ช่วยศาสตราจารย์ ภาควิชา สูตินรีเวช คณะ แพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

12. ภาคภูมิ สุปิยพันธุ์ พบ (PAKPOOM SUPIYAPHUN M.D.)

รองศาสตราจารย์ ภาควิชา โสตนาสิกลาริงซ์วิทยา

คณะ แพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

13. นรินทร์ วรวุฒิ พบ (NARIN VORAVUD M.D.)

รองศาสตราจารย์ ภาควิชา อายุรศาสตร์

คณะ แพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

14. แสงสูรย์ เจริญวิไลศิริ Ph.D. (Saengsoon Charoenvilaisiri Ph.D.)

ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ

113 อุทยานวิทยาศาสตร์ ประเทศไทย ถนนพหลโยธิน ตำบลคลองหนึ่ง

อำเภอคลองหลวง จังหวัดปทุมธานี 12120

15. ดนัย ทิวาเวช Ph.D. (DANAI TIWAWECH Ph.D.)

สถาบันมะเร็ง กรุงเทพ 10400

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อาจารย์ ภาควิชา สูตินรีเวช คณะ แพทยศาสตร์ มหาวิทยาลัยขอนแก่น

17. ศิริวรรณ สัพพะเลข ทบ (SIRIWAN SAPPALAEK DDS)

อาจารย์ ภาควิชาศัลยศาสตร์ คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

18. อาทิพันธุ์ พิมขาวขำ ทบ Ph.D. (ATIPHAN PINKHAOKHAM DDS, Ph.D.)
ผู้ช่วยศาสตราจารย์ ภาควิชาศัลยศาสตร์ คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
19. ประสิทธิ ภวสันต์ ทบ Ph.D. (Prasit Pavasant DDS, Ph.D.)
รองศาสตราจารย์ ภาควิชากายวิภาคศาสตร์ คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

นิสิตระดับปริญญาโทและเอก จุฬาลงกรณ์มหาวิทยาลัย
20. จุรีรัตน์ โพธิ์แก้ว (CHUREERAT PHOKAEW)
นิสิตปริญญาโท
หลักสูตรมหาบัณฑิตวิทยาศาสตร์การแพทย์
21. รุ่งทิวา รัตนวราภรณ์ (ROONGTOWA WATTANAWARAPORN)
นิสิตปริญญาโท
หลักสูตรมหาบัณฑิตวิทยาศาสตร์การแพทย์
22. วันเพ็ญ พลเยี่ยม (WANPEN PONYEAM)
หลักสูตรมหาบัณฑิตวิทยาศาสตร์การแพทย์
23. นครินทร์ กิตกำจร (NAKARIN KITKUMTHORN)
หลักสูตรดุษฎีบัณฑิตสหสาขาวิชาจุลชีววิทยา
24. วิชัย พรธนเกษม (WICHAI PORNTHANAKASEM)
หลักสูตรดุษฎีบัณฑิตสหสาขาวิชาจุลชีววิทยา
25. นริศร คงรัตนโชค (NARISORN KONGRUTTANACHOK)
หลักสูตรดุษฎีบัณฑิตสหสาขาวิชาชีวเวชศาสตร์
26. ปฐมวดี ญาณทัศนีย์จิต (PATTAMAWADEE YANATATSANEEJIT)
นิสิตปริญญาเอก
หลักสูตรดุษฎีบัณฑิตสหสาขาวิชาชีวเวชศาสตร์
27. ศุภกิจ โชวุฒิธรรม (SUPAKIT KOWUDTITHAM)
หลักสูตรดุษฎีบัณฑิตสหสาขาวิชาชีวเวชศาสตร์
28. เกรียงศักดิ์ ฤชุศาศวัต (KRIANGSAK RUCHUSATSAWAT)
หลักสูตรดุษฎีบัณฑิตสหสาขาวิชาชีวเวชศาสตร์
29. โชติกา สุญาณเศรษฐกร (CHOTIKA SUYARNSESTAKORN)
หลักสูตรดุษฎีบัณฑิตสหสาขาวิชาชีวเวชศาสตร์
30. ธัชวรรณ ธนาศุภวัฒน์
หลักสูตรมหาบัณฑิตวิทยาศาสตร์การแพทย์
31. ประกาศิต รัตนดันหยง

ผู้ร่วมวิจัยในปัจจุบัน

ชื่อ (ไทย) นพ.วิโรจน์ ศรีอุพารพงศ์

(อังกฤษ) VIROTE SRIURANPONG M.D., Ph.D.

ตำแหน่งทางวิชาการ ผู้ช่วยศาสตราจารย์

ภาควิชา อายุรศาสตร์ คณะ แพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

โทรศัพท์ 022564533 โทรสาร 022564534

หน้าที่หรือความรับผิดชอบในโครงการ ออกแบบการทดลอง แปรผลการทดลอง เขียนรายงาน โดยเฉพาะในส่วน ทุนเมธีวิจัย สกว. ที่เป็นหัวหน้าโครงการ

โครงการที่ดำเนินอยู่ ทุนเมธีวิจัย สกว.

ชื่อ (ไทย) นพ. ชนพ ช่วงโชติ

(อังกฤษ) SHANOP SHUANGSHOTI M.D.

ตำแหน่งทางวิชาการ รองศาสตราจารย์

ภาควิชา พยาธิวิทยา คณะ แพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

หน้าที่หรือความรับผิดชอบในโครงการ คัดเลือกชิ้นเนื้อและวิเคราะห์ข้อมูลทางพยาธิ

เวลาที่ใช้ในโครงการวิจัย 30% ของเวลาทำงาน

ชื่อ (ไทย) พญ.มนตกานต์ ตันสถิตย์

(อังกฤษ) MONTAKARN TANSATIT M.D.

ตำแหน่งทางวิชาการ ผู้ช่วยศาสตราจารย์

ภาควิชา กายวิภาคศาสตร์ คณะ แพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

หน้าที่หรือความรับผิดชอบในโครงการศึกษา fluorescence insitu hybridization

เวลาที่ใช้ในโครงการวิจัย 5% ของเวลาทำงาน

ชื่อ (ไทย) พญ. จงกลนี วงศ์ปิยะบวร

(อังกฤษ) Jongkonnee Wongpiyabovorn ,M.D, Ph.D

ตำแหน่งทางวิชาการ ผู้ช่วยศาสตราจารย์

ภาควิชา จุลชีววิทยา คณะ แพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

โทรศัพท์ 02-25641132 โทรสาร 02-2525952

หน้าที่หรือความรับผิดชอบในโครงการ ออกแบบการทดลอง แปรผลการทดลอง เขียนรายงาน โดยเฉพาะในส่วน ทุนเมธีวิจัย สกว. ที่เป็นหัวหน้าโครงการ

โครงการที่ดำเนินอยู่ ทุนเมธีวิจัย สกว.

ชื่อ (ไทย) นพ.อมรพันธ์ เสรีมาศพันธุ์

(อังกฤษ) Amornpun Sereemaspun M.D., Ph.D.

ตำแหน่งทางวิชาการ อาจารย์

ภาควิชา กายวิภาคศาสตร์ คณะ แพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

โทรศัพท์ 022564281 โทรสาร 022564281

'หน้าที่หรือความรับผิดชอบในโครงการ ดำเนินการวิจัยในโครงการทุนพัฒนาอาจารย์ สกว สกอ
การจัดทำอนุภาคนาโนลูกผสมของทองคำและ Polyethylinimine เพื่อใช้ตรวจหาโรคฉี่หนูที่เห็น
ด้วยตาเปล่าโดยอาศัยแอนติบอดี
เวลาที่ใช้ในโครงการวิจัย 50% ของเวลาทำงาน

ชื่อ (ไทย) ดร. ศิษเฎศ ทองสิมา
(อังกฤษ) Dr. Sissades Tongsima
ตำแหน่ง นักวิจัย 2 (Senior Researcher) คุณวุฒิ ปริญญาเอก (Ph.D.)
หน่วยงานต้นสังกัด สถาบันจีโนม, ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ, สำนักงาน
พัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ (Genome Institute, BIOTEC, NSTDA)
โทรศัพท์ 02-5646700 ต่อ 5551 โทรสาร 02-564 6704
หน้าที่หรือความรับผิดชอบในโครงการศึกษา Bioinformatics
เวลาที่ใช้ในโครงการวิจัย 15% ของเวลาทำงาน

ชื่อ (ไทย) นาย ประกาศิต รัตนตันหยง
(อังกฤษ) PRAKASIT RATTANATANYONG
ตำแหน่งทางวิชาการ เจ้าหน้าที่วิทยาศาสตร์
ภาควิชา กายวิภาคศาสตร์ คณะ แพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
โทรศัพท์ 022564281 โทรสาร 022564281 E-mail
หน้าที่หรือความรับผิดชอบในโครงการดำเนินการวิจัย EDSB-LMPCR และ cell culture
เวลาที่ใช้ในโครงการวิจัย 90% ของเวลาทำงาน

ชื่อ (ภาษาไทย) รศ.ทพ.ดร.ประสิทธิ์ ภวสันต์
(ภาษาอังกฤษ) Prasit Pavasant
ตำแหน่งวิชาการ รองศาสตราจารย์
ที่ทำงาน ภาควิชากายวิภาคศาสตร์ คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
เวลาที่ใช้ในการทำวิจัย 2%

ชื่อ (ไทย) แสงสูรย์ เจริญวิไลศิริ
(อังกฤษ) SAENGSOON CHAROENVILAISIRI Ph.D.
ตำแหน่ง นักวิจัย 1 คุณวุฒิ ปริญญาเอก
หน่วยงานต้นสังกัด หน่วยปฏิบัติการวิจัยกลางไบโอเทค
สถานที่ติดต่อ ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ
113 อุทยานวิทยาศาสตร์ประเทศไทย ถนนพหลโยธิน ตำบลคลองหนึ่ง อำเภอคลอง
หลวง
จังหวัดปทุมธานี 12120 โทรศัพท์ 0 2564 6700 (3480) โทรสาร 0 2564 6707
หน้าที่หรือความรับผิดชอบในโครงการ ดำเนินการวิจัยในโครงการ AM-SEREX
เวลาที่ใช้ในโครงการวิจัย 90% ของเวลาทำงาน

ชื่อ (ไทย) ดร. พรรัศมิ์ จินตฤทธิ์

(อังกฤษ) DR. PORNRUTSAMI JINTARIDTH

ตำแหน่ง อาจารย์ คุณวุฒิ ปริญญาเอก

หน่วยงานต้นสังกัด ภาควิชาโภชนศาสตร์เขตร้อนและวิทยาศาสตร์อาหาร คณะเวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล

โทรศัพท์ 02-3549100 ต่อ 1582-5 โทรสาร 02-6447934

หน้าที่หรือความรับผิดชอบในโครงการ ดำเนินการวิจัยในโครงการทุนพัฒนาอาจารย์ สกว สกอ Association between genome-wide methylation level and cellular senescence and aging

เวลาที่ใช้ในโครงการวิจัย 50% ของเวลาทำงาน

ชื่อ (ไทย) ดร .เกรียงศักดิ์ ฤชุศาศวัต

(อังกฤษ) DR. KRIANGSAK RUCHUSATSAWAT

ตำแหน่ง นักวิจัย คุณวุฒิ ปริญญาเอก

หน่วยงานต้นสังกัด สถาบันวิจัยวิทยาศาสตร์ สาธารณสุข กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข 88/7 ซ. รพ. บำราชนราดูร ถ. ติวานนท์

โทรศัพท์ 02-5899850-8 ต่อ 99313 โทรสาร 02-5915449

หน้าที่หรือความรับผิดชอบในโครงการ ดำเนินการวิจัยในโครงการทุนพัฒนาอาจารย์ สกว The effect of DNA demethylation/methylation to candidate genes in Psoriasis

เวลาที่ใช้ในโครงการวิจัย 50% ของเวลาทำงาน

ชื่อ (ไทย) ดร ปฐมวดี ญาณทัศนีย์จิต

(อังกฤษ) Pattamawadee Yanatatsaneejit

ตำแหน่ง อาจารย์ คุณวุฒิ Ph.D.

หน่วยงานต้นสังกัด ภาควิชา พฤกษศาสตร์ คณะ วิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

หน้าที่หรือความรับผิดชอบในโครงการ ออกแบบการทดลอง แปรผลการทดลอง เขียนรายงาน โดยเฉพาะในส่วน ทุนพัฒนาอาจารย์ สกว. สกอ ที่เป็นหัวหน้าโครงการ

เวลาที่ใช้ในโครงการวิจัย 50% ของเวลาทำงาน

ชื่อ (ไทย) ผศ.พญ รัศมน กัลยาศิริ

(อังกฤษ) Rasmon Kalayasiri

ตำแหน่ง ผศ คุณวุฒิ M.D.

หน่วยงานต้นสังกัด ภาควิชา จิตเวชศาสตร์ คณะ แพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

หน้าที่หรือความรับผิดชอบในโครงการ ออกแบบการทดลอง แปรผลการทดลอง เขียนรายงาน โดยเฉพาะในส่วน ทุนพัฒนาอาจารย์ สกว. สกอ ที่เป็นหัวหน้าโครงการ

เวลาที่ใช้ในโครงการวิจัย 50% ของเวลาทำงาน

ชื่อ (ไทย) อ.ทพ.ดร .นครินทร์ กิตกำธร

(อังกฤษ) DR. NAKARIN KITKUMTHORN

ตำแหน่ง อาจารย์ คุณวุฒิ D.D.S., Ph.D.

หน่วยงานต้นสังกัด ภาควิชาทันตพยาธิวิทยา คณะทันตแพทยศาสตร์ มหาวิทยาลัยมหิดล

โทรศัพท์ 02-203-6470 โทรสาร6470-203-02

หน้าที่หรือความรับผิดชอบในโครงการ ดำเนินการวิจัยในโครงการทุนพัฒนาอาจารย์ สกว สกอ อณูพันธุศาสตร์ของเนื้องอกเซลล์ต้นกำเนิดฟัน

เวลาที่ใช้ในโครงการวิจัย 50% ของเวลาทำงาน

ชื่อ (ไทย) อ. ดร . อรจิรา อารักษ์สกุลวงศ์

(อังกฤษ) DR. ORNJIRA ARUKSAKUNWONG

ตำแหน่ง อาจารย์ คุณวุฒิ Ph.D.

หน่วยงานต้นสังกัด ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยรังสิต ปทุมธานี 12000

โทรศัพท์ 081-454-8420

หน้าที่หรือความรับผิดชอบในโครงการ ดำเนินการวิจัยในโครงการทุนพัฒนาอาจารย์ สกว สกอ การศึกษาหาโครงสร้างที่สมบูรณ์และกลไกในการยึดจับกับ DNA ของโปรตีน p53 โดยใช้แบบจำลองพลวัตเชิงโมเลกุลและข้อมูลทางโครงสร้างผลึกการศึกษาหาโครงสร้างที่สมบูรณ์และกลไกในการยึดจับกับ DNA ของโปรตีน p53 โดยใช้แบบจำลองพลวัตเชิงโมเลกุลและข้อมูลทางโครงสร้างผลึก

เวลาที่ใช้ในโครงการวิจัย 50% ของเวลาทำงาน

รายชื่อ นิสิตปริญญาโท หลักสูตรมหาบัณฑิตวิทยาศาสตร์การแพทย์

- ธัชวรรณ ธนาศุภวัฒน์ ปีที่เริ่มศึกษา 2549
- ชุดิภา พวงไพโรจน์ ปีที่เริ่มศึกษา 2549

รายชื่อนิสิตปริญญาเอก หลักสูตรดุษฎีบัณฑิตสหสาขาวิชาจุลชีววิทยา 2 คน และ หลักสูตรดุษฎีบัณฑิตสหสาขาวิชาชีวเวชศาสตร์ 4 คน

หัวข้อวิทยานิพนธ์ การเติมหมู่เมททิลในยีน *CCNA1* ของมะเร็งปากมดลูก

- ศุภกิจโชวุฒิธรรม

หัวข้อวิทยานิพนธ์ การลดหมู่เมททิลของไลน์-1 และการควบคุมการทำงานของยีนข้างเคียง

- โชติกา สุญาณเศรษฐกร

หัวข้อวิทยานิพนธ์ การศึกษา หน้าที่ของ PPP2R2B

- จุรีรัตน์ โพธิ์แก้ว

หัวข้อวิทยานิพนธ์ การศึกษา L1 methylation pattern และ การแสดงออกของยีน

- อ.ท.พญ. เกศกัญญา สัพพะเลข

หัวข้อวิทยานิพนธ์ การศึกษา L1 methylation pattern ในมะเร็งของช่องปาก

1. รายชื่อคณะผู้ร่วมวิจัย

ชื่อ-นามสกุล	เริ่มเข้าร่วมโครงการ			ปัจจุบัน		
	ตำแหน่งวิชาการ	สังกัด	ตำแหน่งในโครงการ	ตำแหน่งวิชาการ	สังกัด	ตำแหน่งในโครงการ
วิโรจน์ ศรีอุฬารพงศ์	อ.นพ.	คณะแพทย์ จุฬาฯ	ผู้วิจัย	ผศ.นพ.	คณะแพทย์ จุฬาฯ	ผู้วิจัย
วีระชัย คีรีกาญจนะรงค์	รศ.นพ.	คณะแพทย์ จุฬาฯ	ผู้ร่วมวิจัย	รศ.นพ.	คณะแพทย์ จุฬาฯ	ผู้ร่วมวิจัย
ชนพ ช่วงโชติ	รศ.นพ.	คณะแพทย์ จุฬาฯ	ผู้วิจัย	รศ.นพ.	คณะแพทย์ จุฬาฯ	ผู้วิจัย
กาญจนา โชติเลอศักดิ์	ผศ.พญ.	คณะแพทย์ จุฬาฯ	ผู้ร่วมวิจัย	ผศ.พญ.	คณะแพทย์ จุฬาฯ	ผู้ร่วมวิจัย
มนตกานต์ ตันสถิตย์	ผศ.พญ.	คณะแพทย์ จุฬาฯ	นิสิต	ผศ.พญ.ดร.	คณะแพทย์ จุฬาฯ	ผู้ร่วมวิจัย
พิสิฐ ตั้งกิจวาณิชย์	ผศ.นพ.	คณะแพทย์ จุฬาฯ	ผู้วิจัย	รศ.นพ.	คณะแพทย์ จุฬาฯ	ผู้วิจัย
สรภพ เกียรติพงศ์สาน	อ.นพ.	คณะแพทย์ จุฬาฯ	ผู้วิจัย	นักเรียนทุนอานันท มหิดล	Harvard U	
สมชัย นิรุตติศาสน์	รศ.นพ.	คณะแพทย์ จุฬาฯ	ผู้ร่วมวิจัย	รศ.นพ.	คณะแพทย์ จุฬาฯ	ผู้ร่วมวิจัย
ดำรง ตรีสุโกศล	รศ.นพ.	คณะแพทย์ จุฬาฯ	ผู้ร่วมวิจัย	รศ.นพ.	คณะแพทย์ จุฬาฯ	ผู้ร่วมวิจัย
สุรางค์ ตรีรัตนชาติ	รศ.นพ.	คณะแพทย์ จุฬาฯ	ผู้ร่วมวิจัย	รศ.นพ.	คณะแพทย์ จุฬาฯ	ผู้ร่วมวิจัย
วิชัย เติมรุ่งเรืองเลิศ	ผศ.พญ.	คณะแพทย์ จุฬาฯ	ผู้ร่วมวิจัย	ผศ.พญ.	คณะแพทย์ จุฬาฯ	ผู้ร่วมวิจัย
ภาคภูมิ สุปียพันธุ์	รศ.นพ.	คณะแพทย์ จุฬาฯ	ผู้ร่วมวิจัย	รศ.นพ.	คณะแพทย์ จุฬาฯ	ผู้ร่วมวิจัย
นรินทร์ วรวุฒิ	รศ.นพ.	คณะแพทย์ จุฬาฯ	ผู้ร่วมวิจัย	รศ.นพ.	คณะแพทย์ จุฬาฯ	ผู้ร่วมวิจัย

Malison, R	รศ.นพ.	Yale U	นักวิจัย	รศ.นพ.	Yale U	นักวิจัย
Gelernter, J	ศ.นพ.	Yale U	นักวิจัย	ศ.นพ.	Yale U	นักวิจัย
Listman, J	นิสิต	Yale U	นิสิต	นิสิต	Yale U	นิสิต
แสงสูรย์ เจริญวิไลศิริ	นักวิจัย	BIOTEC	นักวิจัย	นักวิจัย	BIOTEC	นักวิจัย
ดนัย ทิวาเวช	นักวิจัย	สถาบันมะเร็ง	นักวิจัย	นักวิจัย	สถาบันมะเร็ง	นักวิจัย
จีริชุดา ปัทมดิลก	พญ	คณะแพทย์จุฬาฯ	Clinical fellow	พญ	CRI	ผู้ร่วมวิจัย
ฐิติวรรณ ลมดี	พญ	คณะแพทย์จุฬาฯ	Clinical fellow	พญ	รพ ชลบุรี	
ศิริวรรณ สัพพะเลข	อ.ทญ	คณะทันตแพทย์จุฬาฯ	นิสิต	อ.ทญ	คณะทันตแพทย์จุฬาฯ	นิสิต
อาทิพันธุ์ พิมขาวขำ	ผศ.ทพ	คณะทันตแพทย์จุฬาฯ	ผู้ร่วมวิจัย	ผศ.ทพ	คณะทันตแพทย์จุฬาฯ	ผู้ร่วมวิจัย
ประสิทธิ ภวสันต์	รศ.ทพ	คณะทันตแพทย์จุฬาฯ	ผู้ร่วมวิจัย	รศ.ทพ	คณะทันตแพทย์จุฬาฯ	ผู้ร่วมวิจัย
ปฐมวดี ญาณทัศนีย์จิต	นิสิต	บัณฑิตวิทยาลัยจุฬาฯ	นิสิต	อ. ดร	คณะวิทย์จุฬาฯ	**Mentee** อาจารย์ใหม่
จุรีรัตน์ โพธิ์แก้ว	นิสิต ป โท	คณะแพทย์จุฬาฯ	นิสิต ป โท	นิสิต ป เอก	บัณฑิตวิทยาลัยจุฬาฯ	นิสิต ป เอก
รุ่งทิวา รัตนวราภรณ์	นิสิต ป โท	คณะแพทย์จุฬาฯ	นิสิต ป โท	นิสิต ป เอก	CRI	นิสิต ป เอก
วันเพ็ญ พลเยี่ยม	นิสิต ป โท	คณะแพทย์จุฬาฯ	นิสิต ป โท	นิสิต ป โท	คณะแพทย์จุฬาฯ	นิสิต ป โท

นครินทร์ กิตกำจร	นิสิต ป เอก	บัณฑิต วิทยาลัย จุฬาฯ	นิสิต ป เอก	อ.ดร.ทพ	คณะทันตแพทย์ มหิดล	**Mentee** อาจารย์ใหม่
วิชัย พรธนเกษม	นิสิต ป เอก	บัณฑิต วิทยาลัย จุฬาฯ	นิสิต ป เอก	ดร	BIOTEC (applying)	postdoc
นริศร คงรัตนโชค	นิสิต ป เอก	บัณฑิต วิทยาลัย จุฬาฯ	นิสิต ป เอก	ดร	Just graduated	
ศุภกิจ โชวุฒิธรรม	นิสิต ป เอก	บัณฑิต วิทยาลัย จุฬาฯ	นิสิต ป เอก	นิสิต ป เอก	บัณฑิต วิทยาลัย จุฬาฯ	นิสิต ป เอก
เกรียงศักดิ์ ฤชุศาศวัต	นิสิต ป เอก	บัณฑิต วิทยาลัย จุฬาฯ	นิสิต ป เอก	ดร	กรมวิทย์	**Mentee** อาจารย์ใหม่
จีรวัฒน์ นาคขนนทด				นิสิต ป เอก	บัณฑิต วิทยาลัย จุฬาฯ	นิสิต ป เอก
โชติกา สุญาณเศรษฐกร	นิสิต ป เอก	บัณฑิต วิทยาลัย จุฬาฯ	นิสิต ป เอก	นิสิต ป เอก	บัณฑิต วิทยาลัย จุฬาฯ	นิสิต ป เอก
ธัชวรรณ ธนาศุภวัฒน์	นิสิต ป โท	คณะแพทย์ จุฬาฯ	นิสิต ป โท	นิสิต ป โท	คณะแพทย์ จุฬาฯ	นิสิต ป โท
ชุติภา พวงไพโรจน์	นิสิต ป โท	คณะแพทย์ จุฬาฯ	นิสิต ป โท	นิสิต ป โท	คณะแพทย์ จุฬาฯ	นิสิต ป โท
ดวงฤดี วัฒนศิริชัยกุล				รศ.ดร	คณะแพทย์ รามา มหิดล	ผู้ร่วมวิจัย
ตวงพร สุทธิพงษ์ชัย				รศ.ดร	คณะวิทย์ มหิดล	ผู้ร่วมวิจัย
สิทธิสม วิจิตรบูรพัฒน์				นิสิต ป โท	คณะวิทย์ มหิดล	นิสิต ป โท

รัศมน กัลยาศิริ				อ.พญ	คณะแพทย์ จุฬาฯ	**Mentee** อาจารย์ใหม่
อมรพันธ์ เสรีมาศพันธ์				อ.ดร.นพ	คณะแพทย์ จุฬาฯ	**Mentee** อาจารย์ใหม่
พรรัศมิ์ จินตฤทธิ์				อ.ดร.	เขตร้อน มหิดล	**Mentee** อาจารย์ใหม่
รุ่งนภา หิรัญสถิตย์	นิสิต ป เอก	บัณฑิต วิทยาลัย จุฬาฯ	นิสิต ป เอก	นิสิต ป เอก	บัณฑิต วิทยาลัย จุฬาฯ	นิสิต ป เอก
ชูพงศ์ อิทธิวุฒิ	นิสิต ป เอก	บัณฑิต วิทยาลัย จุฬาฯ	นิสิต ป เอก	นิสิต ป เอก	บัณฑิต วิทยาลัย จุฬาฯ	นิสิต ป เอก
ศิฏษเดช ทองศิมา				นักวิจัย	BIOTEC	นักวิจัย
นิสิต ป ตรี **27** คน ใน 3 ปี	ป ตรี	คณะแพทย์ คณะวิทย์ คณะสหเวช ศาสตร์	นิสิต ฝึกงาน			
ประกาศิต รัตนตันหยง	ป ตรี	คณะแพทย์ จุฬาฯ	นักวิจัย	ป ตรี	คณะแพทย์ จุฬาฯ	นักวิจัย

1.7 ภาคผนวก ประกอบด้วย reprint หรือ manuscript และรางวัลวิจัยที่ได้รับของหัวหน้าโครงการและทีมวิจัยแต่ละท่าน รวมทั้งกิจกรรมที่เกี่ยวข้องกับการนำผลจากโครงการไปใช้ประโยชน์

ประวัติการได้รับรางวัลหรือการประกาศเกียรติคุณของหัวหน้าโครงการ

2539 นักวิทยาศาสตร์รุ่นใหม่
มูลนิธิส่งเสริมวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทยในพระบรมราชูปถัมภ์

2542 รางวัลผลงานเกียรติยศ
สำนักงานกองทุนสนับสนุนการวิจัย (สกว.) สำหรับผลงานวิจัยของ สกว. ที่มี impact factor สูงสุด

2542 รางวัลผลงานเกียรติยศ
สำนักงานกองทุนสนับสนุนการวิจัย สำหรับผลงานวิจัยของ สกว. ที่ได้รับการอ้างอิง สูงสุด

2543 Eminent Scientist & IRPC International Award Winner
(IRPC= International Research Promotion Council Asia-Pacific Chapter)

2543 The Takeo Wada Outstanding Cancer Researcher
(Faculty of Medicine, Chiang Mai University and Takeo Wada fund)

2548 รางวัลผลงานวิจัยระดับดีเยี่ยม
สำนักงานคณะกรรมการวิจัยแห่งชาติ เรื่อง "อณูพันธุศาสตร์ของมะเร็งโพรงหลังจมูก"

2548 เมธีวิจัยอาวุโส สกว. สำนักงานกองทุนสนับสนุนการวิจัย

2549 ผลงานวิจัยได้รับการอ้างอิงสูงสุด
จุฬาลงกรณ์มหาวิทยาลัย

2549 นักวิจัยดีเด่น
จุฬาลงกรณ์มหาวิทยาลัย

2549 นักวิจัยดีเด่นแห่งชาติ
สำนักงานคณะกรรมการวิจัยแห่งชาติ

2551 นักวิทยาศาสตร์ดีเด่น
มูลนิธิส่งเสริมวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทยในพระบรมราชูปถัมภ์

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Nucleic Acids Research

LINE-1 methylation status of endogenous DNA double-strand breaks

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ABSTRACT

DNA methylation and the repair of DNA double-strand breaks (DSBs) are important processes for maintaining genomic integrity. Although DSBs can be produced by numerous agents, they also occur spontaneously as endogenous DSBs (EDSBs). In this study, we evaluated the methylation status of EDSBs to determine if there is a connection between DNA methylation and EDSBs. We utilized interspersed repetitive sequence polymerase chain reaction (PCR), ligation-mediated PCR and combined bisulfite restriction analysis to examine the extent of EDSBs and methylation at long interspersed nuclear element-1 (LINE-1) sequences nearby EDSBs. We tested normal white blood cells and several cell lines derived from epithelial cancers and leukemias. Significant levels of EDSBs were detectable in all cell types. EDSBs were also found in both replicating and non-replicating cells. We found that EDSBs contain higher levels of methylation than the cellular genome. This hypermethylation is replication independent and the methylation was present in the genome at the location prior to the DNA DSB. The differences in methylation levels between EDSBs and the rest of the genome suggests that EDSBs are differentially processed, by production, end-modification, or repair, depending on the DNA methylation status.

INTRODUCTION

Vilenchik and Knudson (1) estimated the existence of endogenous double-strand breaks (EDSBs) and suggested that EDSBs could account for a substantial fraction of oncogenic events in human carcinomas. If EDSBs do not arise uniformly or are not processed at equal rates across the genome, mutation hot spots should be present (1). Our study helps to elucidate if DNA methylation influences EDSBs processing.

Several pieces of evidence suggest that DNA methylation may play an important role in maintaining genomic integrity. Genome-wide decreases in DNA methylation levels commonly occur in cancer (2–5), which leads to higher rates of mutations and genomic instability (6–8). In addition to alterations in the number of chromosomes, hypomethylation can result in chromosomal rearrangements and deletion of DNA, suggesting that DSBs are the intermediate products (6–8). Moreover, because the mutations occur spontaneously, the DSBs should occur endogenously. Studies in ICF syndrome (immunodeficiency, chromosomal instability and facial anomalies) (9), which is characterized by loss-of-function mutations in the cytosine DNA methyltransferase *DNMT3B*, and Wilm's tumor (10) demonstrated a direct association between loss of DNA methylation and rearrangements in the pericentromeric heterochromatin. Therefore, hypomethylation could lead to spontaneous mutations in *cis*, which are the epigenetic and genetic events occurring on the same chromosome. Consequently, evaluating methylation status of EDSBs may provide clues to better understanding how DNA methylation helps maintaining genomic integrity.

To determine whether DNA methylation affects EDSBs, we first developed a set of novel techniques to analyze the extent of methylation in genomic EDSBs. These techniques were devised from interspersed repetitive sequence (IRS) polymerase chain reaction (PCR) (11), ligation-mediated (LM) PCR-based assays (12–14) and combined bisulfite restriction analysis (COBRA) for

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genome-wide methylation level analysis (4,5,15). LMPCR is a commonly used PCR technique designed to analyze locus-specific EDSBs during lymphoid development, such as V(D)J recombination (14,16,17) and hypermutation (18). LMPCR has also been used to detect DNA-associated proteins or chromatin accessibility to such proteins (19,20), and this technique has shown previous utility in genome mapping research (21,22). With these new assays, we evaluated the relationship between DNA methylation and EDSBs.

MATERIALS AND METHODS

Cells, cell lines and culture

Cell lines used were HeLa (cervical cancer), SW480 (colorectal adenocarcinoma), K562 (erythroleukemia), Daudi (B lymphoblast), Jurkat (T-cell leukemia) and Molt4 (T lymphoblast) (ATCC, Manassas, VA, USA). HeLa cells were synchronized at G0 phase by culture in serum deprivation medium, Dulbecco's modified Eagle's medium plus 0.2% fetal bovine serum, for 48 h. HeLa cells in G1/S and S phase cells were synchronized by the thymidine block method, and were cultured with 2 mM thymidine (Sigma-Aldrich, St Louis, MO, USA) to obtain cells at G1/S phase (23). Flow cytometry was used to determine stages of the cell cycle as well as fragmented and apoptotic cells. One millimolar of H_2O_2 was added for 24 h to induce apoptosis. For radiation treatment, the medium of G0 phase HeLa cells was replaced with 15 ml of ice-cold medium, and cells were exposed to γ- with a ^{60}Co source (Eldorado78).

High-molecular weight DNA preparation

To prepare high-molecular weight (HMW) DNA, 5×10^5 cells were embedded in 1% low-melting point agarose, lysed and digested in 400 µl of 1 mg/ml proteinase K, 50 mM Tris, pH 8.0, 20 mM EDTA, 1% sodium lauryl sarcosine. The plugs were rinsed four times in Tris-EDTA (TE) buffer for 20 min. To polish overhang or cohesive-end EDSBs, T4 DNA polymerase (New England Biolabs, Beverly, MA, USA) and dNTPs were added and later inactivated by adding EDTA to a concentration of 20 mM for 5 min followed by rinsing four times in TE buffer for 20 min. To analyze blunt-end EDSBs, LMPCR was performed without T4 DNA polymerase. The modified LMPCR linkers (24) were prepared from the oligonucleotides 5′-AGGTAACGAGTCAGACCACCGATCGCTCGGA AGCTTACCTCGTGGACGT-3′ and 5′-ACGTCCAC GAG-3′. The linkers (50 pmol) were ligated to HMW DNA using T4 DNA ligase (New England Biolabs) at 25°C overnight. DNA was extracted from agarose plugs using a QIAquick gel extraction kit (Qiagen, Basel, Switzerland). For liquid DNA preparation, cells or HMW DNA were incubated in 1% sodium dodecyl sulfate/proteinase K (0.5 mg/ml), at 48°C overnight and subjected to phenol–chloroform extraction and ethanol precipitation. The precipitated DNA was resuspended in 20 µl of TE buffer.

IRS–EDSB–LMPCR

The quantity of IRS–EDSB was measured by real-time PCR using a Lightcycler™ instrument (Roche Applied Science, Indianapolis, IN, USA) with the IRS primers, including long interspersed nuclear elements-1 (LINE-1s or L1s) primers 5′-CTCCCAGCGTGAGCGAC-3′ (outward), 5′-AAGCCGGTCTGAAAAGCGCAA-3′ (inward), Alu, Alu-CL2 5′-ACTGCACTCCAGCCTGG GC-3′ or Tigger1 5′-CTCGCTGAAGGCTCAGATGAT C-3′), the linker primer 5′-AGGTAACGAGTCAGA CCACCGA-3′ (24), and the Taqman probe homologous to the 3′ linker sequence (6-fam) ACGTCCACGAGGT AAGCT TCCGAGCGA (tamra) (phosphate). Amplification was performed with 0.5 µM of each primer, 0.4 µM Taqman probe, 2 U of HotStarTaq (Qiagen, Valencia, CA, USA), 1× PCR buffer and 10 ng of ligated DNA for up to 40 cycles, with quantification after the extension steps. Two types of control DNA were used. The first was a 100-bp oligonucleotide sequence with the 5′ linker sequence and 3′ homology to L1 oligonucleotide sequences. The second was DNA digested with EcoRV and AluI and ligated to the LMPCR linkers. The amounts of EDSBs were compared with the ligated control digested DNA and reported as L1-EDSB–LMPCR templates per nanogram of DNA.

COBRA–IRS and COBRA–IRS–EDSB

Ligated HMW DNA was modified with bisulfite using a standard protocol (25). Bisulfite-modified DNA was recovered using a Wizard DNA clean-up kit (Promega, Madison, WI, USA) and desulfonated before PCR amplification. For PCR COBRA (15) of L1s (COBRA-L1) (4,5), bisulfite-treated DNA was subjected to 35 cycles of PCR with two primers, B-L1-inward 5′-CGTAAGG GGTTAGGGAGTTTTT-3′ and B-L1-outward 5′-RTAA AACCCTCCRAACCAAATATAAA-3′ (4). Applying a hot-stop technique to prevent heteroduplex amplicons, α^{32}P-labeled-bisulfite-L1-outward oligo was added in the last PCR cycle. The amplicons were doubly digested in a 10 µl reaction volume with 2 U of TaqI and 8 U of TasI in 1× TaqI buffer (MBI Fermentas, Vilnius, Lithuania) at 65°C for 4 h. The PCR is designed to detect unmethylated and methylated L1 sequences of 98 and 80 bp, respectively. The intensity of DNA fragments was measured with a PhosphorImager using Image Quant software (Molecular Dynamics, GE Healthcare, Slough, UK). The LINE-1 methylation level was calculated as the percentage of TaqI intensity divided by the sum of TaqI- and TasI-positive amplicons. For COBRA-L1-EDSB, the B-L1-inward oligo was replaced with B-LMPCR oligo, 5′-GTTTGGAAGTTTATTTTGTGGAT-3′, and 40 PCR cycles were carried out according to the same protocol. Bisulfite-treated Daudi, Jurkat and HeLa DNAs digested with EcoRV and AluI and ligated LMPCR linker were used as positive controls to normalize the inter-assay variation of all COBRA experiments. HeLa DNA without ligation was used as a negative control.

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Southern blot hybridization

Southern blot was performed to compare between 5 μg of HpaII- and MspI-digested HeLa DNA. L1-LMPCR amplicons from HMW and AluI–EcoRV-digested HeLa DNA were used as probes. L1-most-outward primer sequence was 5′-TATTCGGCCATCTTGGCTCCT-3′. Competitor DNA, COT-1 DNA, was used to prevent non-specific DNA hybridization, including sequence from L1s. Intensities in the >4 kb regions were measured with a PhosphorImager using Image Quant software (Molecular Dynamics). Semiquantitative methylation percentage was reported as the proportion of probes bound to HpaII-digested DNA to HpaII plus MspI-digested DNA.

Statistical analyses

Statistical significance was determined according to an independent sample *t*-test, a paired sample *t*-test or ANOVA using the SPSS program version 11.5 as specified.

RESULTS

Detection of genomic EDSB and methylation

First, we developed a new assay for the detection of EDSBs. This assay is based on a LMPCR, originally designed for the analysis of locus and cell-specific EDSBs (14,16–18). General EDSBs are believed to occur rarely and arbitrarily throughout the genome (1). Using repetitive sequences that randomly scatter throughout the human genome, we can detect genome-wide EDSBs in proximity to these repetitive sequences. We, therefore, combined LMPCR with IRSPCR or inter-Alu PCR (11) using the widely distributed L1s human retrotransposons (26) into a new assay called 'L1-EDSB-LMPCR'. In this assay, linker oligonucleotides are ligated to EDSBs in HMW DNA preparation and quantitatively analyzed by real-time PCR using an L1 primer and a Taqman probe complementary to the linker (Figure 1A). L1-PCR using a primer in the outward direction will amplify DNA sequences located outside the repetitive sequences.

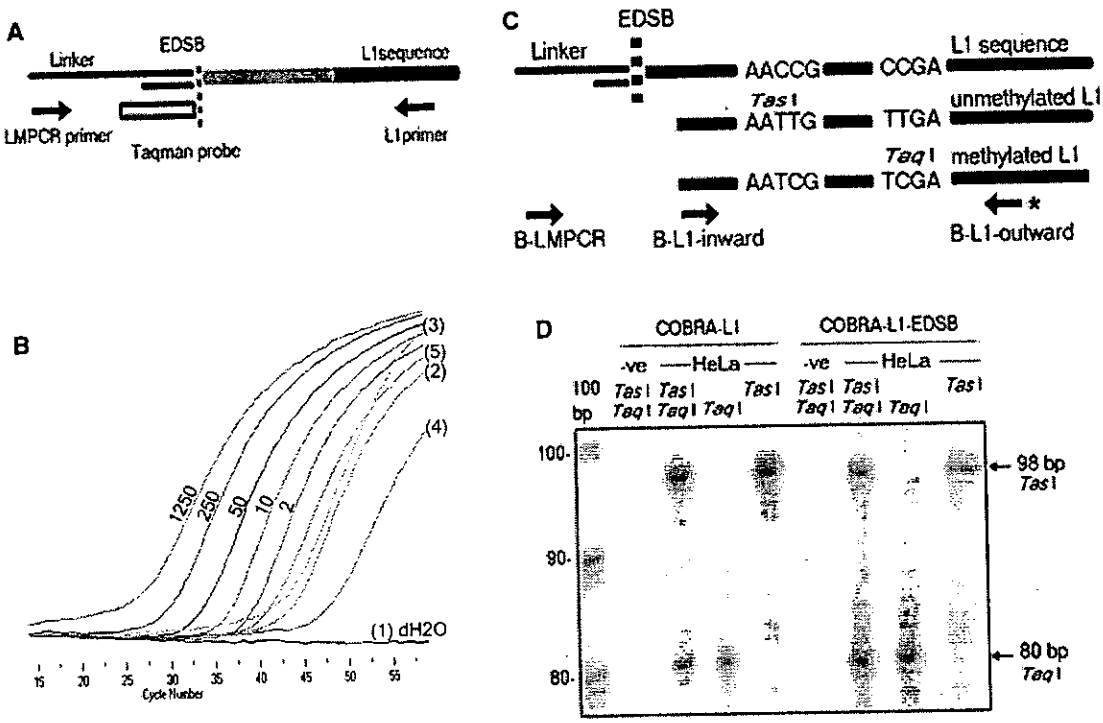

Figure 1. L1-EDSB–LMPCR and COBRA-L1–EDSB. (**A**) Schematic representation of L1-EDSB–LMPCR showing L1 sequence and the ligated linker at a nearby EDSB. The white rectangle is a Taqman probe complementary to the LMPCR linker. Arrows are PCR primers. (**B**) The quantity of EDSBs detected with this method increased directly with the amount of experimentally induced DSBs, and significant amounts of EDSBs were detected in all tested cells but not in cells without ligation. An example of results of real-time L1-EDSB–LMPCR with tests and controls was demonstrated. The values 2, 10, 50, 250 and 1250 are quantities of restriction enzyme-digested (EcoRV and AluI) HeLa genomes ligated with LMPCR linker. dH2O is water. Tested templates are HMW DNA from (1) HeLa without ligation, (2) Daudi blunt-end-EDSBs ligation, (3) Daudi polished-end-EDSBs ligation, (4) Jurkat blunt-end-EDSBs ligation and (5) Jurkat polished-end-EDSBs ligation. (**C**) Schematic representation of COBRA-L1 and COBRA-L1-EDSB, showing L1 sequence ligated by linker at an EDSB. Arrows are PCR primers, with asterisk indicating α-^{32}P-labeled primer for COBRA. AACCG and CCGA are L1 sequences; when treated with bisulfite and after undergoing PCR, unmethylated AACCG will be converted to AATTG (TasI site) and methylated CCGA to TCGA (TaqI site). (**D**) A typical example of results from COBRA-L1 and COBRA-L1-EDSB experiments indicating that the intensity between methylated, TaqI and unmethylated, TasI, bands of EDSBs were higher than the matched pair genomes. The arrow at 98 bp indicates TasI-digested unmethylated L1 sequences and the arrow at 80 bp indicates TaqI-digested methylated L1 sequences. −ve is dH2O for COBRA-L1 and non-ligated HMW DNA for COBRA-L1-EDSB. TasI and TaqI are restriction enzymes added in each experiment.

Therefore, the sequences yielded from IRSPCR will represent human genome-wide sequences, including both unique and repetitive sequences (11,21). Increases in the L1-EDSB–LMPCR products corresponded with the amount of control DSBs generated by restriction enzyme digesting DNA. Without ligation, no L1-EDSB–LMPCR could be detected. Finally, significant amounts of EDSBs were detected in all tested cells (Figure 1B).

Next, we developed an assay to analyze the methylation level of EDSBs. Previously, we had extensively studied methylation status of L1s in several cancers and normal tissues by COBRA-L1. Treatment with bisulfite converts unmethylated cytosines, but not methylated cytosines, to uracils and then thymines after PCR. Therefore, this bisulfite treatment generates detectable methylation-dependent changes in the restriction pattern of PCR-amplified L1 sequences. Methylation level is then calculated and presented as a percentage of total DNA. We thus combined L1-EDSB–LMPCR with COBRA-L1 by treating linker-ligated DNA with bisulfite before PCR with L1/linker primers and restriction analysis (Figure 1C). With this new 'COBRA-L1-EDSB' assay, we can measure the methylation level of L1s near EDSBs, which reflects the methylation level of EDSBs in a genome-wide fashion. The degree of methylation between genomic L1 and L1-EDSB sequences was examined by COBRA-L1 and COBRA-L1–EDSB, respectively (Figure 1D).

With these new assays, we first evaluated what types of cells possess EDSBs and if the quantity of EDSBs reflects carcinogenic potentials. Using L1-EDSB–LMPCR, significant amounts of EDSBs were detected in all samples from several cancer cell lines, including Daudi, Jurkat, Molt4, K562, SW480 and HeLa cells, as well as in normal cells, including sperm and white blood cells (WBCs) from several individuals (Figure 2A). These data suggest that EDSBs can commonly be found in all cells both normal and cancer.

This assay prepared HMW DNA by in-gel preparation. This technique has been reported to decrease the number of DSBs generated during DNA preparation (27) and did not generate false positives in the LMPCR assay for the analysis of locus-specific EDSBs (14,18,27). To evaluate if in-gel HMW DNA preparation breaks genomic DNA, we compared the yield of the LMPCR from several sources of DNA, including, in-gel HMW DNA, liquid DNA, liquid DNA extracted from in-gel HMW DNA (gel–liquid) and in-gel DNA prepared from liquid DNA (liquid–gel). The quantities of L1-EDSB–LMPCR from liquid DNA were higher than in-gel HMW DNA (Figure 2B). Extracting liquid DNA from a gel increased the amount of LMPCR template over HMW DNA (Figure 2B). This result indicates that more DNA breaks were generated during the liquid DNA preparation process. Embedded liquid DNA into gel following by HMW DNA preparation protocol did not increase the amount of LMPCR template over liquid DNA alone (Figure 2B). Therefore, in-gel preparation did not generate significant additional DNA breaks. Moreover, the quantities of L1-EDSB–LMPCR from gel–liquid and liquid–gel were clearly lower than when L1-EDSB–LMPCR

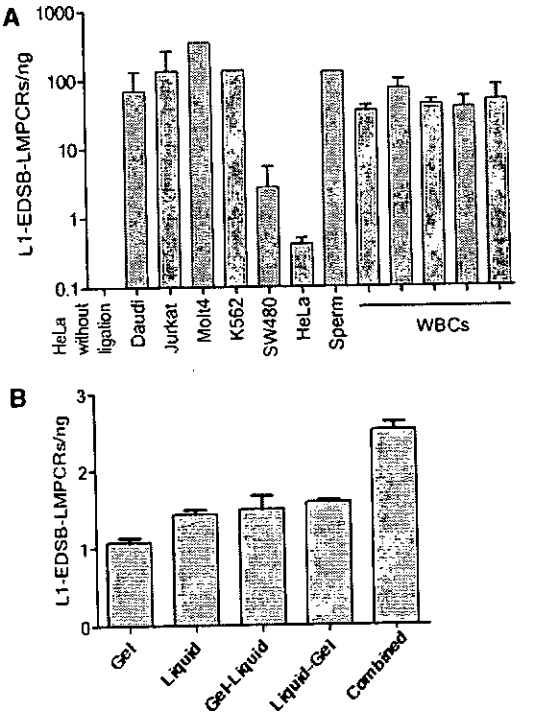

Figure 2. Discovery and specificity of EDSB-LMPCR. (A) Using L1-EDSB-LMPCR, significant amounts of EDSBs were detected in all samples, including, cancer cell lines, sperm cells and WBCs from several individuals. (B) L1-EDSB-LMPCR of DNA from HeLa cells using different DNA-extraction methods. Gel, liquid, gel–liquid, liquid–gel and combined are L1-EDSB–LMPCR from DNA prepared in gel, liquid, extracted liquid DNA from gel-embedded HMW DNA, embedded liquid DNA into gel following by HMW DNA preparation protocol and 2 × admixture between 1:1 of HMW DNA and liquid DNA, respectively. Data represent means ± SEM.

products from HMW DNA and liquid DNA were combined (Combined) (Figure 2B). Therefore, the significant amounts of LMPCR products from HMW DNA were not derived from DNA preparation. This experiment supported the presence of EDSBs.

General characteristics of EDSB–LMPCR

Repetitive sequences have been commonly recognized as locations of general recombination that destabilize human cancer genomes (28,29). It is interesting to investigate if the quantities of EDSBs differ between repetitive and unique sequences. Full length L1 is ~6 kb (26). L1-EDSB–LMPCR using the L1-inward primer will amplify L1 sequences, while the L1-outward primer should amplify unique sequences near L1 (11). We observed L1-EDSBs at similar levels regardless of the directions of L1 primers used (Figure 3A). The data indicate that the amount of EDSBs does not differ based on the nature of DNA sequences, between L1 repetitive and unique sequences. We found that IRS–LMPCR using primers for other types of repetitive sequences, including Alu and Tigger1, also yielded significant EDSBs in direct proportions to their copy numbers in the human genomes (30) (Figure 3B).

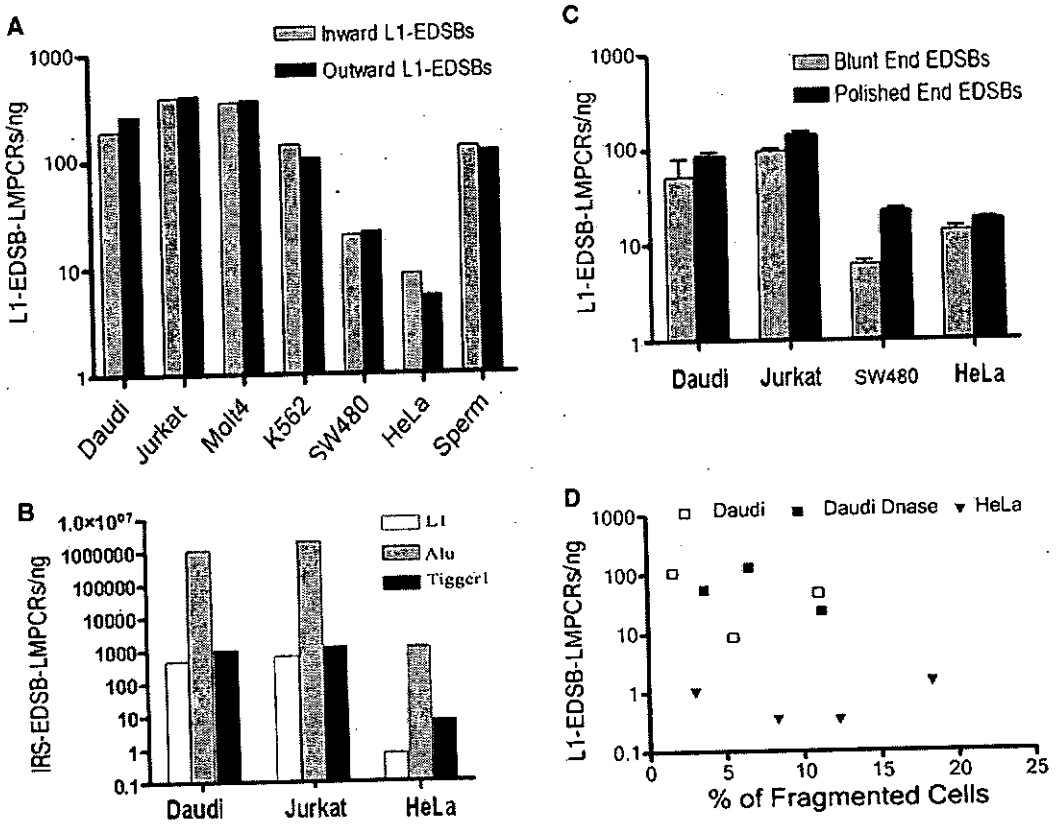

Figure 3. General characteristics and distribution of EDSB–LMPCR in the human genome. (A) L1-EDSB–LMPCR, using L1-inward and L1-outward primers, was performed in several cell types, and products were observed at similar levels regardless of the nature of linked EDSB sequences, L1, or unique sequences. Inward L1-EDSB–LMPCR was normalized by the proportion of L1 sequence copies in the human genome (www.ncbi.nlm.nih.gov). (B) Comparison of the amount of EDSB–LMPCRs using L1, Alu and Tigger1 primers indicated EDSBs in direct proportions to their copy numbers in the human genomes. (C) EDSBs were measured from HMW DNA with and without T4 polymerase treatment, respectively. Significant amount of L1-EDSB–LMPCRs of blunt and polished-end EDSBs from cancer cell lines were identified. Therefore, L1-EDSB ends are heterogeneous. (D) The quantity of EDSBs was not related to the proportion of fragmented cells. L1-EDSB–LMPCR quantity related to the percent of fragmented cells, documented by flow cytometry. Daudi DNase represents Daudi cells treated with DNase I before HMW DNA preparation.

L1s are AT rich, whereas Alu sequences are frequently located in CG-rich regions (31). Therefore, EDSBs are widely distributed in the human genome. In addition, because EDSBs are thought to occur rarely and randomly throughout the genome, LMPCR with primers specific to unique sequences yielded no positive locus-specific amplicons (data not shown). EDSB ends are heterogeneous, as significant quantities of L1-EDSB–LMPCR products were obtained, including two EDSB types: blunt- and polished-, blunt plus cohesive, end EDSBs (Figure 3C). Blunt-end DSBs are DNA that both strands terminate in a base pair. A cohesive or overhang is a stretch of unpaired nucleotides in the DNA end (14,16,17).

The quantity of EDSBs was not related to the proportion of fragmented cells (Figure 3D). Our EDSB–LMPCR protocol, particularly that for epithelial cells, minimizes contamination with apoptotic cells because these dying cells with fragmented DNA (32) usually lose attachment (33) and are thus washed off before cell collection for EDSB analysis. While there was positive L1–EDSB–LMPCR amplification, we were unable to detect any fragmented DNA or apoptotic cells, as determined by LMPCR ladder (34) and flow cytometry (35), respectively (data not shown). Moreover, the apoptotic genome possesses normal levels of methylation and may not be detectable by COBRA-L1–EDSB. Fragmented DNA, as documented by electrophoresis, was collected from floating apoptotic HeLa cells after treatment with 1 mM H_2O_2 for 24 h (36). COBRA-L1 shows that apoptotic HMW DNA has similar L1 methylation to living cells, but COBRA-L1–EDSB yielded amplicons with multiple sizes that interfere with interpretation (data not shown).

L1-EDSB–LMPCR under different conditions

We utilized L1-EDSB–LMPCR on cells under different conditions known to associate with DSBs. Specifically; we used radiation and cell cycle synchronization. Radiation, which directly causes DNA damage, increased L1-EDSB–LMPCR levels directly correlating with the dosages of radiation used to treat the cells (Figure 4A). Nevertheless, there were wide ranges of the amount of

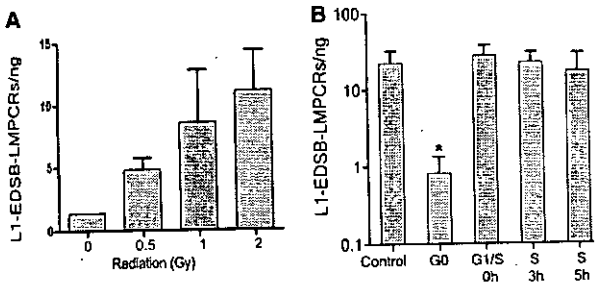

Figure 4. L1-EDSB–LMPCR under different conditions. L1-EDSB–LMPCR of (A) irradiated HeLa. (B) HeLa cells at G0, G1/S and 0, 3 and 5 h after the release into S phase from thymidine block. Control is without cell synchronization. G0 bore the least amount of EDSBs when compared with control, $^{*}P < 0.05$ (independent one-tailed t-test). Data represent means ± SEM.

EDSBs (Figure 4A). This result may be the consequence of several factors, including DSB end modifications and DSB repair rate. Therefore, even though L1-EDSB–LMPCR can detect radiation-induced DSBs, the technique lacks efficiency in evaluating the precise number of DSBs generated by radiation.

Because EDSBs were hypothesized to be preferentially produced in S phase from the conversion of single-strand lesions (1), we used HeLa cells to assess the frequency of EDSBs and their methylation status during various cell cycle stages: G0, G1/S and S. We found the impact of cell cycle effect is on the borderline of significance and EDSBs can be found in G0 phase (Figure 4B). Interestingly, there was a minor decrease in S phase cells (Figure 4B). Therefore, cell cycle stage is one of the conditions that may alter the amount of EDSBs. Nevertheless, similar to radiation the amount of EDSBs during cell phases may be influenced by several factors in addition to the production rate.

Specificity of COBRA-L1–EDSB

Figure 5A demonstrates a reconstitution experiment of EDSB–LMPCR. Because Daudi cells shows significantly higher level of genomic methylation than HeLa cells, we used DNA prepared from Daudi and HeLa cells as representative DNA with high and low methylation, respectively. We added varying ratios of HeLa and Daudi DNA digested with EcoRV and AluI (ligated to LMPCR linker) into HeLa genomic DNA. As expected, a COBRA-L1–EDSB analysis of the digested DNA, regardless of the presence of the HeLa genome, yielded equivalent levels of methylation to COBRA-L1. Furthermore, the methylation levels in these samples measured by both COBRA-L1 and COBRA-L1–EDSB are increased, which correlates with the proportion of highly methylated Daudi DNA contained in that sample (Figure 5A). The higher levels of methylation, compared to the rest of the genome, are a characteristic of EDSBs but not DSBs randomly generated by DNA shearing during sample preparation. Lower DSB methylation levels of liquid DNA, gel–liquid DNA and liquid–gel DNA were demonstrated when compared to HMW DNA (Figure 5B). In contrast to gDNA

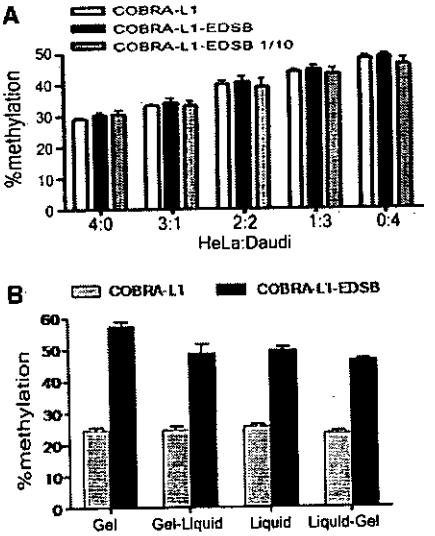

Figure 5. Specificity of COBRA-L1–EDSB. (A) A reconstitution experiment of COBRA-L1–EDSB. COBRA-L1 and COBRA-L1–EDSB of the mixture between HeLa and Daudi DNA digested with AluI and EcoRV and ligated to EDSB–LMPCR linker. The 4:0, 3:1, 2:2, 1:3 and 0:4 are proportions of recombinant HeLa:Daudi DNA. COBRA-L1–EDSB 1/10 was the mixture between 2 ng of linker-ligated DNA and 18 ng of HeLa genomic DNA. Because Daudi cells shows significantly higher levels of genomic methylation compared to HeLa cells, we used DNA prepared from Daudi and HeLa cells as representative DNA with high methylation and low methylation, respectively. We added varying ratio of HeLa and Daudi DNA digested with EcoRV and AluI ligated to the LMPCR linker into HeLa genomic DNA. As expected, the methylation levels of the digested DNA, regardless of the presence of the HeLa genome, were measured by COBRA-L1-EDSB and COBRA-L1, and increases in final product correlated with the proportion of highly methylated Daudi DNA. (B) COBRA-L1 and COBRA-L1–EDSB methylation levels in a comparison of DNA preparation methods. Gel, gel–liquid, liquid and liquid–gel refer to DNA analyzed via L1-EDSB-LMPCR from DNA prepared in-gel, extracted liquid DNA from gel embedded HMW DNA, liquid and embedded liquid DNA into gel following by HMW DNA preparation protocol, respectively. COBRA-L1–EDSB methylation levels of HMW DNA were higher than liquid DNA. COBRA-L1–EDSB methylation levels of in-gel DNA prepared from liquid DNA, and liquid DNA prepared from HMW DNA, were lower than HMW but not different from liquid. Therefore, HMW DNA preparation does not cause hypermethylated DSBs. Data represent means ± SEM.

prepared from cells cast into low melting point agarose plugs, the preparation protocol for liquid DNA generates DSBs (Figure 2B). Therefore, COBRA-L1–EDSB levels of liquid DNA should be derived from combination between EDSBs and DNA preparation producing DSBs. The lower DSB methylation levels of liquid DNA confirmed that the DNA preparation producing DSBs possess lower methylation levels than EDSBs. Moreover, DSB methylation levels of gel–liquid DNA and liquid–gel DNA are not different from levels of liquid DNA alone (Figure 5B). Therefore, higher methylation levels of COBRA-L1–EDSB, detected from HMW DNA, were unlikely to be derived from HMW DNA preparation.

EDSBs are ubiquitously hypermethylated

To compare the degree of methylation between L1 and L1-EDSB sequences, we examined by COBRA-L1 and

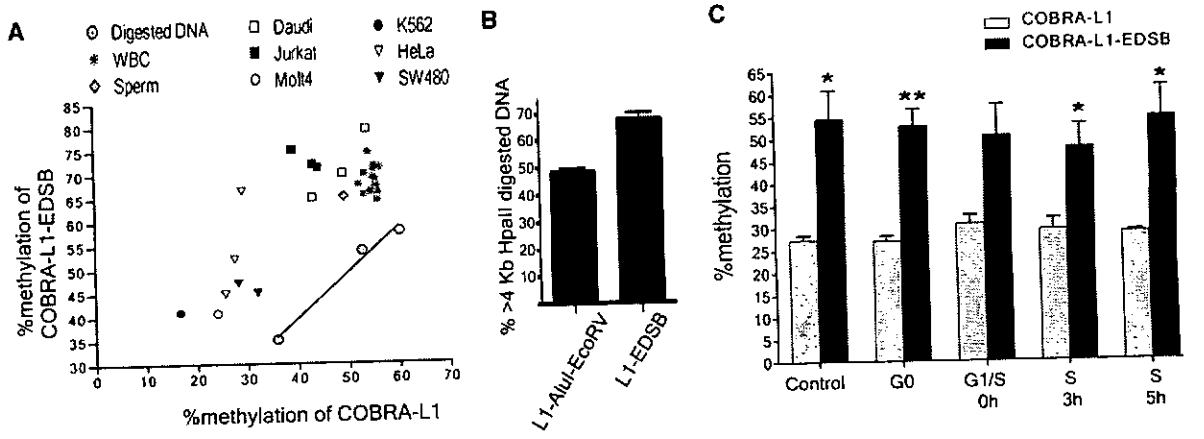

Figure 6. Methylation status of EDSBs. (A) EDSBs possess higher methylation levels than the rest of the genome, across all tests. A comparison of COBRA-L1 and COBRA-L1–EDSB among cell types demonstrated universally higher COBRA-L1-EDSB methylation levels than the matched pair COBRA-L1. Digested DNA is from HeLa, Jurkat or Daudi cells, and after being treated with AluI and EcoRV, it was used for a control. Each dot represents an individual test result of COBRA-L1 and COBRA-L1–EDSB. (B) Methylation of EDSBs pre-existed at the break sites. A representation of results from Southern blot hybridization of HpaII and MspI digested DNA demonstrates that EDSBs are located in larger HpaII digested HeLa DNA fragments than experimentally induced methylation-independent DSBs. L1-EDSB are L1-LMPCR probes from HMW DNA, representing EDSBs, while L1–AluI–EcoRV contains AluI plus EcoRV-digested DNA, representing methylation-independent DSBs. The bar graphs reports the percentage of hybridization intensities in the >4 kb regions of HpaII-digested DNA divided by the summation of those of HpaII and MspI-digested DNA. (C) COBRA-L1 and COBRA-L1–EDSB of HeLa cells at G0, G1/S, and 0, 3 and 5 h after the release into S phase from a thymidine block. The control is without cell synchronization. G0 cells contained the most significant hypermethylation level of EDSBs when compared between COBRA-L1 and COBRA-L1-EDSB, $^{*}P < 0.05$, $^{**}P < 0.001$ (pair two-tailed t-test). (B) and (C) data represent the mean ± SEM.

COBRA-L1–EDSB, respectively. We found that, EDSBs are hypermethylated. Specifically, these data show that the EDSBs from all tested cells possess higher methylation levels than the rest of the genome. In our analysis, we included several cancer cell lines, along with normal sperm and WBCs (Figure 6A). In contrast, restriction enzyme-digested DSBs show the same level of methylation compared to genomic DNA (Figure 6A). The COBRA-L1-EDSB levels reflect the rate of EDSB synthesis, EDSB end modification and repair at a given time. Therefore, the EDSB hypermethylation means that methylated and unmethylated DNA may possess distinctive EDSB processing (i.e. repair) pathways.

The methylation of EDSBs may have pre-existed at the break sites

As shown in Figure 6A, the levels of methylation at EDSBs and the rest of the genomes are positively correlated in all cell lines ($P = 0.01$; Pearson $r = 0.873$). We further proved the source of DNA methylation around EDSBs by Southern blot hybridization of HeLa genomic DNA digested with HpaII or MspI. The activity of HpaII is blocked by CpG methylation, while that of the isoschizomer MspI is insensitive to methylation. Therefore, hypermethylated DNA is not digested with HpaII and electrophoretically co-migrates with long DNA fragments. We arbitrarily used the length of >4 kb to represent long DNA fragments concentrated in hypermethylated DNA for subsequent calculations. We determined the percentage of hybridization intensities in the >4 kb regions of HpaII-digested DNA divided by the sum of those digested with HpaII and MspI. The L1-EDSB-LMPCR products hybridized to >4 kb HpaII-digested DNA fragments in higher proportions than the control, restriction enzyme-generated DSB-LMPCR products (Figure 6B). Therefore, EDSBs are hypermethylated, and the methylation was present in the genome at this location before the break itself.

EDSB hypermethylation is replication independent

Because EDSBs can be detected at different levels in all phases of the cell cycle (Figure 4B), we evaluated the level of EDSB methylation during cell cycle progression. EDSBs were hypermethylated in most stages, with G0 being the most significant (Figure 6C). This result implies that the increase levels of methylation at EDSBs are DNA replication independent.

DISCUSSION

Even though molecular characteristics of locus-specific EDSBs during lymphoid development, such as V(D)J recombination (14,16,17) and hypermutation (18), have been described by LMPCR, general EDSBs have not (1). Conventionally, radiation-induced DSBs can be visualized as fragmented DNA that migrate out of cells or chromosomes via electrophoresis, using the comet assay (37) and/or pulsed field gel electrophoresis (38). Nevertheless, because detection using these techniques requires extensive damage of DNA and because EDSBs are rare, the sensitivity is likely not high enough for analyzing EDSBs.

The IRS-EDSB-LMPCR technique is able to detect EDSBs because of the extensive distribution of the IRS sequences and the sensitivity of real-time PCR. This study

demonstrates that EDSBs are not only detectable but also widely distributed. Positive results were obtained from all types of selected IRS sequences. Nevertheless, LMPCR may not be able to detect some subset of EDSBs. LMPCR preparation requires ligation of a blunt-end DNA linker to a blunt and phosphorylated DSB end. In this study, we polished cohesive-end EDSBs by T4 polymerase. We found that both blunt and overhang ends are present and that the majority of EDSB ends are blunt. LMPCR fails, however, to detect some complex ends that cannot be polished such as a hairpin loop. Unlike studying V(D)J recombination (17,27), in this study, we did not apply Mung Bean nuclease to screen for these end-types because the enzyme can convert single-strand lesion into DSBs.

IRS–EDSB–LMPCR is the first method characterizing EDSBs. Consequently, there is no report of cells with different levels of EDSBs. Therefore, even though it is commonly accepted that in-gel HMW DNA preparation does not significantly affect DNA breaks (27), we performed additional experiments to disregard the possibility of error. The comparison among liquid DNA preparation, HMW DNA and combined experiments suggested that there are no detectable DSBs generated by the HMW DNA preparation process. Finally, cells treated under different conditions, including, serum deprivation and temperature (data not shown), altered EDSB levels. Because the differentially treated cells were processed for HMW DNA simultaneously, the possibility that the same DNA preparation method led to bias shearing of the DNA depending on different prior cellular conditions is remote.

LMPCR detects DSBs directly, while γ-H2AX foci staining aims to detect a cellular response to DSBs. Therefore, L1-EDSB–LMPCR analysis and staining for γ-H2AX foci may not yield 'the same information. For example, a recent report demonstrated that some genomic regions, particularly heterochromatin, are devoid of radiation-induced γ-H2AX foci (39). Consequently, L1-EDSB–LMPCR and γ-H2AX foci should assess radiation-induced DSBs differently depending on chromatin structures. In this study, we have demonstrated that, even though L1-EDSB-LMPCR products increased after radiation, the correlation to radiation dosage was not as precise as using γ-H2AX foci for detection (40). One explanation for the broad range of results when using L1-EDSB-LMPCR to detect radiation-induced DSBs could be that there is a wide variety of biological processes involved in radiation-induced DSB repairs (41,42).

This study demonstrates that EDSBs are present in all cell types. Moreover, EDSBs normally possess higher levels of methylation compared to the cellular genome, and this methylation pre-exists at the break sites. These findings were not only unprecedented but also not generally expected. Vilenchik and Knudson suggested that the causes of EDSBs are oncogenic events in human carcinomas (1). Moreover, genomic instability can be observed while cancer genomes are hypomethylated (6–8). Therefore, it is tempting to hypothesize that EDSBs should occur more frequently at hypomethylated sequences and, consequently, that COBRA-L1-EDSB should have been hypomethylated. Nonetheless, IRS-EDSB-LMPCR and COBRA-L1-EDSB are methods to measure the extent of methylation at EDSBs during a given time. Therefore, the LMPCR levels reflect not only the rate of EDSB synthesis but also EDSB end modification and repair. Therefore, discovery of DSBs does not exclusively indicate DSB formation, but, the EDSB hypermethylation finding leads us to conclude that methylated and unmethylated DNA possess distinctive EDSB processing (i.e. repair) pathways. If these pathways have different precision, methylated and unmethylated DNA should have unequal rate of spontaneous mutations. In the future, it would be interesting to explore if and how cells process or repair EDSBs depending on DNA methylation. This finding may yield an important clue to prevent global hypomethylation-induced chromosomal rearrangements.

There may be several other mechanisms by which DNA methylation prevents IRS from inducing DNA rearrangements. Repetitive sequences have been commonly recognized as locations of general recombination that destabilizes human cancer genomes, particularly when genetic recombination occurred between different loci (28,29,43). A deletion at *Xist* alters chromatin conformation, usually associated with DNA methylation, of the inactive X chromosome and this change destabilizes both X chromosomes (44). In the future, it would be intriguing to explore if EDSBs play a role in these mechanisms and whether or not DNA methylation is important in preventing these chromosomal rearrangements.

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ORAL ONCOLOGY

Detection of LINE-1s hypomethylation in oral rinses of oral squamous cell carcinoma patients

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Summary This study aimed to (i) investigate long interspersed nuclear element-1 (LINE-1) methylation levels of oral squamous cell carcinomas (OSCCs), the major type of oral malignancies; and (ii) investigate whether the hypomethylation of LINE-1s can be detected in oral rinses of OSCC patients. The combined bisulfite restriction analysis polymerase chain reaction (PCR) of LINE-1s (COBRALINE-1) was used. We found that tissues from OSCC specimens had lower methylation levels of LINE-1s than cells collected from the oral rinses of normal volunteers. Interestingly, cells collected from oral rinses of OSCC patients also revealed hypomethylated LINE-1s at the same level as OSCC tissues. There was no difference in the level of hypomethylation among tumors with different stages, locations, histological grades, and with a case history of betel chewing, smoking and/or alcohol consumption. In conclusion, OSCCs possessed global hypomethylation and this alteration could be detected from oral rinses of OSCC patients

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Abbreviations: LINE-1, long interspersed nuclear element-1; OSCC, oral squamous cell carcinoma; COBRALINE-1, combined bisulfite restriction analysis polymerase chain reaction of LINE-1; WBC, white blood cell; HNSCC, head and neck squamous cell carcinoma; LOH, loss of heterozygosity

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by a simple PCR technique, COBRALINE-1. Therefore, COBRALINE-1 of oral rinses may be applied for non-invasive detection of oral malignancies.

Introduction

The incidence of oral cancers is increasing, especially in young adults (less than 40 years of age).[1,2] In 2002, cancers of the oral cavity were found to be the 8th ranking among global cancers of various sites. They occurred in about 2,74,000 patients, not only in developing countries but also in the developed ones.[3] Despite the arrival of molecular biology leading to effective treatment in many types of cancers, the treatment results of oral cancers are still not satisfactory. The standard treatment, surgery, not only causes facial disfiguration and difficulty in eating and speaking, but also achieves a low 5-year survival rate, which improved by only 5% (from 54% in 1974–1976 to 59% in 1995–2000).[4] Although OSCCs are easily diagnosed, most patients were detected in advanced stages which results in poor survival rates.[5] Early detection and diagnosis is important and results in better outcome of the treatment.[6,7] Then, many studies tried to develop techniques for easy and early detection including (i) clinical examination with toluidine blue,[8–10] methylene blue,[11] tolonium chloride staining[12] and the use of chemiluminescence[13] (ii) exfoliative cytology studies by brush biopsy[14–16] or oral scraping[17] and (iii) studies of salivary biochemistry[18,19] and also molecular biology including search for tumor markers.[20–23] However, histological study which requires surgical biopsy is still the "gold standard" for diagnosis. Therefore, it is important to further explore and to improve non-invasive methods for reliable early detection of oral malignancies.

Epigenetic alterations including global (genome-wide) hypomethylation were reported in many types of cancers.[24–27] Although there were evidences that supported the epigenetic involvement in oral malignancies, global hypomethylation has not been reported.[28–31] Global loss of methylation is a decrease in methylated CpG dinucleotides, which are dispersed throughout the whole genome, in both noncoding repetitive sequences and genes.[32] Long interspersed nuclear element-1s (LINE-1s) are highly repetitive mobile DNA sequences which distribute randomly across the entire human genome with up to 6,00,000 copies and 2000 copies are of full length.[33] Thus, methylation levels of LINE-1s can reflect genome-wide methylation levels.[27,34] Hypomethylation of LINE-1s has been reported in several malignancies, including neuroendocrine tumors,[35] carcinomas of the breast, lung, liver, esophagus, stomach, colon, urinary bladder prostate, and head and neck.[34,36–43] Moreover, hypomethylation levels of LINE-1s can be used as a prognostic marker for epithelial ovarian cancers,[44] cervical cancers[45] and hepatocellular carcinoma.[46] From our previous study, the combined bisulfite restriction analysis polymerase chain reaction (PCR) of LINE-1s (COBRALINE-1) can efficiently evaluate the genome-wide methylation status of LINE-1s in genomic DNA and it represents the whole genome methylation status.[34,47] We also found not only that cancers exhibited significantly increased levels of hypomethylation compared to their normal tissue counterparts, but also that normal tissues from different organs had tissue-specific levels of methylated LINE-1s. Although oral cancers are included in the head and neck cancers, these malignancies originate from different tissue types and may possess different levels of LINE-1 methylation. The global methylation status of oral cancers, originating from oral mucosa, has not been clarified.

The aims of this study were (i) to elucidate global methylation levels of oral malignancies, and (ii) to investigate if aberrant global methylation levels can be detected in oral rinses of oral cancer patients. We studied oral squamous cell carcinomas (OSCCs), the most common type of oral malignancies occurring in oral mucosa.[7,48] In order to investigate aberrant methylation levels in oral rinses of OSCC patients, oral rinses of normal individuals were used as controls. We studied methylation levels of LINE-1s, which are highly repeated and widely interspersed throughout the genome and represent genome-wide methylation levels.[27,33,34,47] The methylation levels of LINE-1s in OSCC tissues collected from primary lesions and in cells collected from oral rinses of OSCC patients were analyzed. Relation of methylation levels to clinical tumor stages, histological grades, tumor locations and risk factors including smoking, alcohol consumption and betel chewing was also investigated.

Materials and methods

Patients and samples

Normal oral rinses were collected from 37 volunteers who had no pathology of oral mucosa. They were used as controls. Patients histologically confirmed to have OSCCs were included in this study. OSCC oral rinses were collected from 38 OSCC patients before they received any treatment. Twenty millilitre of sterile 0.9% NaCl solution was gargled for 15 s then spat into a sterile 50-ml closed container and kept at 4 °C until processed within 24 h. OSCC tissues were collected from 69 OSCC patients at the same time when they had surgical excision or biopsy. The specimens were immediately frozen in liquid nitrogen and stored at −80 °C until processed to collect DNA. White blood cells (WBCs) from 12 normal healthy individuals were also collected. Nine archival paraffin-embedded tissues derived from OSCCs were obtained and prepared into 5 μm-thickness sections on slides for micro-dissection as previously described.[34] All the samples were obtained with informed consent under protocols approved by the Ethics Committee, Faculty of Dentistry, Chulalongkorn University. Genomic DNA were extracted from all the samples and then applied for COBRALINE-1.

Table 1 Numbers, age, and LINE-1 methylation levels among groups of samples

	Normal oral rinses			OSCC oral rinses			OSCC tissues		
	Male	Female	Total	Male	Female	Total	Male	Female	Total
N	13	24	37	24	14	38	32	37	69
Age range (years)	20–75	20–75	20–75	25–77	35–88	25–88	26–82	35–90	26–90
Average age (years)	50.23	41.33	41.78	57.12	63.21	59.37	60.78	68.57	64.96
Range of % methylation	37.69–49.46	36.83–46.97	36.83–49.46	31.60–43.36	32.24–39.54	31.60–43.36	22.82–48.47	7.62–47.13	7.62–48.47
Average % methylation (SD)	42.61 (3.50)	41.33 (2.36)	41.78 (2.84)	37.87 (2.98)	36.95 (1.75)	37.53 (2.61)	35.73 (6.36)	36.01 (6.88)	35.88 (6.60)
p-value (*t* -test)			0.191			0.297			0.862

Genomic DNA extraction

DNA from WBCs and microdissected paraffin-embedded tissues was prepared as previously described.[34] Cells from oral rinses were collected by centrifugation at 2500*g*, at 4 °C for 15 min. The supernatant was discarded, and cell pellets were washed with sterile phosphate buffered saline (PBS). The pellets were then incubated in 1% SDS/proteinase K (0.5 mg/ml) at 48 °C for 72 h.[15] OSCC tissues were thawed on ice and washed twice in sterile PBS, then cut into small pieces and incubated in 1% SDS/proteinase K (0.5 mg/ml) at 48 °C for 72 h. The digested cell pellets or tissue and fluid were then subjected to phenol-chloroform extraction and ethanol precipitation. The precipitated DNA was resuspended in 20 μl of Tris-EDTA buffer.

COBRALINE-1

The method of COBRALINE-1 methylation assay as described previously was employed.[34] Briefly, the 5′ UTR of LINE-1.2 from NCBI Accession number M80343 was used. Genomic DNA was treated with sodium bisulfite[49] and then subjected to 35 cycles of PCR with a primer couple, 5′-CCGTAAGGGGT-TAGGGAGTTTTT-3′ and 5′-RTAAAACCCTCCRAACCAAATA-TAAA-3′at an annealing temperature of 50 °C. The LINE-1 amplicons (160 bp) were digested in 10 μl reaction volume with 2 U of *Taq*I and 8 U of *Tas*I in 1× *Taq*I buffer (MBI Fermentas, Glen Burnie, MD) at 65 °C overnight, and were then electrophoresed in 8% nondenaturing polyacrylamide gel. The methylated amplicons, *Taq*I positive, yielded two 80 bp DNA fragments; whereas the unmethylated amplicons, *Tas*I positive, yielded 63 and 97 bp fragments.

Methylation levels

Intensities of DNA fragments were measured by PhosphorImager using Image Quant Software (Molecular Dynamics, Sunnyvale, CA). LINE-1 methylation level was calculated as a percentage of the intensity of methylated LINE-1 digested by *Taq*I divided by the sum of the unmethylated LINE-1 digested by *Tas*I and the *Taq*I-positive amplicons. The same preparation of genomic DNA from HeLa, Daudi, and K562 cell lines was used as positive controls in all the experiments and to adjust for interassay variation.

Statistical analysis

Statistical analyses were performed using SPSS software for Windows 11.5 (SPSS Inc., Chicago, IL). Values were calculated using Student's *t*-test, analysis of variance (ANOVA), Brown–Forsythe test or Kruskal–Wallis test as indicated. A p-value of <0.05 was considered significant.

Results

Comparing the methylation levels of LINE-1s between genders in different groups of samples

Even though males and females possess different sets of sex chromosome, our previous study exhibited that levels of LINE-1 methylation in WBC did not differ between males

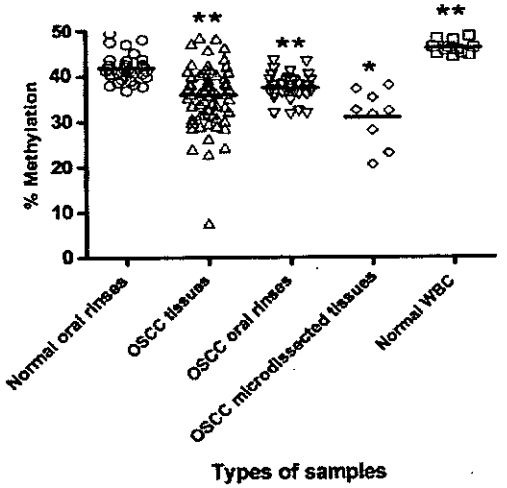

Figure 1 Methylation levels of LINE-1s in each group of samples. The horizontal line indicates the mean of methylation levels. Normal oral rinses ($n = 37$), OSCC tissues ($n = 69$), OSCC oral rinses ($n = 38$), OSCC microdissected tissues ($n = 9$) and normal WBCs ($n = 12$) had mean methylation levels ± SD of 41.78% ± 2.84, 35.88% ± 6.60, 37.53% ± 2.61, 30.95% ± 6.03, and 46.15% ± 1.48, respectively. ** and * are $p < 0.001$ and ≤0.005, respectively, when compared with normal oral rinses.

and females.[34] In order to clarify this phenomenon in oral tissues, methylation levels between genders in each group were compared. Normal oral rinses were collected from 37 volunteers (13 males and 24 females); mean methylation levels ± SD were 42.61% ± 3.50 and 41.33% ± 2.36 in males and females, respectively. OSCC tissues were obtained from 69 OSCC patients (32 males and 39 females); mean methylation levels ± SD were 35.73% ± 6.36 and 36.01% ± 6.88 in male and female, respectively. OSCC oral rinses were collected from 38 OSCC patients (24 males and 14 females); mean methylation levels ± SD were 37.87% ± 2.98 and 39.95% ± 1.75 in males and females, respectively. When comparing the LINE-1s methylation levels between genders in normal oral rinses, OSCC oral rinses and OSCC tissues, there was no significant difference (p-value = 0.191, 0.297, and 0.862, respectively using Student's t-test) (Table 1). Therefore, LINE-1 methylation levels in normal and malignant oral tissues between males and females are not different.

Methylation levels of LINE-1s in OSCCs

In order to clarify whether there is hypomethylation in OSCCs, levels of COBRALINE-1 were analyzed. The contamination of normal mucosal cells and leukocytes in OSCC tissues and OSCC oral rinses may affect the levels of methylation. We therefore also included genomic DNA from nine OSCC microdissected samples which had more homogeneity of cancerous cells, and 12 normal WBCs in the analysis. Mean methylation levels ± SD of OSCC tissues, OSCC oral rinses, OSCC microdissected samples, normal oral rinses, and normal WBCs were 35.88% ± 6.60, 37.53% ± 2.61, 30.95% ± 6.03, 41.78% ± 2.84, and 46.15% ± 1.48, respectively (Fig. 1). The Brown–Forsythe tests of equality of means were used, and revealed a significant difference among groups, p-value ≤0.001. Normal WBCs showed significantly higher methylation levels than normal oral rinses, OSCC tissues, OSCC oral rinses and OSCC microdissected samples (p-value ≤0.001, <0.001, <0.001 and ≤0.001, respectively). OSCC tissues, OSCC oral rinses, and OSCC

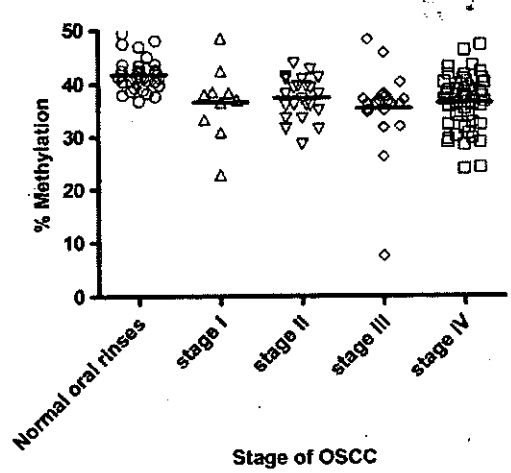

Figure 2 Methylation levels of LINE-1s in each cancer stage. All stages showed hypomethylation levels. The mean methylation levels did not statistically differ among stages (ANOVA p-value = 0.681). Normal oral rinses ($n = 37$), OSCC stage I ($n = 10$), II ($n = 23$), III ($n = 21$) and IV ($n = 53$) had mean methylation levels ± SD of 41.78% ± 2.84, 36.62% ± 6.81, 37.47% ± 3.84, 35.40% ± 7.78, and 36.43% ± 4.96, respectively.

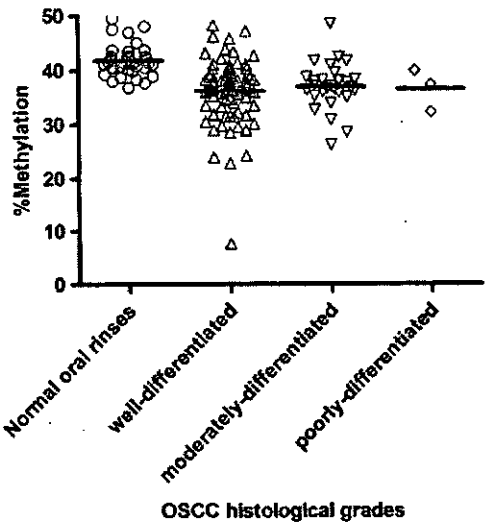

Figure 3 Methylation levels of LINE-1s in each histological grade. All the three grades of malignant cells had hypomethylation. There was no statistical difference in methylation levels among grades (Kruskal–Wallis test p-value = 0.924). Means methylation levels ± SD of normal oral rinses ($n = 37$), well-differentiated ($n = 75$), moderately-differentiated ($n = 28$), and poorly-differentiated ($n = 3$) were 41.78% ± 2.84, 36.19% ± 6.06, 37.05% ± 4.28, and 36.50% ± 3.86, respectively.

microdissected samples had significantly lower methylation levels than normal oral rinses with p-value < 0.001, <0.001, and = 0.005, respectively. Moreover, methylation levels of OSCC oral rinses did not differ from those of OSCC tissues (p-value = 0.518) and OSCC microdissected samples (p-value = 0.106). Besides, there were no different levels of methylation between OSCC tissues and OSCC microdissected samples (p-value = 0.361). These experiments indicated that, similar to other cancers, LINE-1 methylation levels in OSCCs were lower than in normal oral mucosa. Interestingly, even though normal WBCs and normal oral mucosal cells had higher methylation levels, their contamination in OSCC samples seems not to affect the hypomethylation. Therefore, COBRALINE-1 levels of OSCC tissues and OSCC oral rinses could reflect the methylation levels of cancerous cells.

Methylation levels of LINE-1s and clinicopathological correlations

Mean methylation levels of OSCCs stages I (36.62% ± 6.81), II (37.47% ± 3.84), III (35.40% ± 7.78) and IV (36.43% ± 4.96) did not differ from each other, p-value = 0.681 (one-way ANOVA) (Fig. 2). Tumors which had histological features of well-differentiated, moderately-differentiated, and poorly-differentiated cells had no different levels of methylation, p-value = 0.924 (Kruskal–Wallis test), mean methylation levels were 36.19% ± 6.06, 37.05% ± 4.28, and 36.50% ± 3.86, respectively (Fig. 3). Moreover, OSCCs that occurred in tongue, gum, buccal mucosa, floor of the mouth, palate, lip, or oropharynx had no statistical difference in methylation levels, p-value = 0.464 (Kruskal–Wallis test), mean methylation levels were 36.33% ± 6.57, 35.88% ± 5.88, 36.37% ± 4.42, 35.67% ± 5.74, 39.07% ± 2.00, 37.30% ± 5.74, and 43.11, respectively. Patients, who did not smoke, drink alcohol or chew betel had no significant difference in methylation levels from patients who were exposed to any of these risk factors, p-value = 0.427 (Brown–Forsythe test) (Table 2).

Discussion

Detection of cancers in the oral cavity still requires expertise. Uptil now, the accurate diagnosis of oral cancers depends on surgical biopsy and histological studies which are difficult to apply in large populations. However, attempts are are being made develop simple and reliable tools for the early discovery of oral cancers. During this decade, the use of saliva or mouthwashes/oral rinses for malignancy detection has been a focus of interest. Evidence suggested that epithelial cells in saliva provide suitable materials for the head and neck squamous cell carcinomas (HNSCCs) genetic analysis.[50] Exfoliated oral mucosal cells and also

Table 2 LINE-1 methylation levels in different tumor stages, histological grades, tumor locations, and risk factors

	N (%)	% Methylation			
		Mean	SD	95% CI	p-value
OSCC samples[a]	107 (100)				0.681
Stage					
I	10 (9.35)	36.62	6.81	(31.75 – 41.49)	
II	23 (21.50)	37.47	3.84	(35.81 – 39.13)	
III	21 (19.63)	35.40	7.78	(31.85 – 38.94)	
IV	53 (49.53)	36.43	4.96	(35.07 – 37.80)	
Histological grading[b]					0.924
Well-differentiated	75 (70.09)	36.19	6.06	(34.87 – 37.63)	
Moderately-differentiated	28 (26.17)	37.05	4.28	(35.39 – 38.71)	
Poorly-differentiated	3 (2.80)	36.50	3.86	(26.91 – 46.10)	
Location					0.464
Tongue	30 (28.04)	36.33	6.57	(33.88 – 38.78)	
Gum	24 (22.43)	35.88	5.88	(33.40 – 38.37)	
Buccal mucosa	21 (19.63)	36.37	4.42	(34.35 – 38.38)	
Floor of mouth	16 (14.95)	35.67	5.74	(32.61 – 38.73)	
Palate	8 (7.48)	39.07	2.00	(37.40 – 40.73)	
Lip	7 (6.54)	37.30	5.74	(31.99 – 42.61)	
Oropharynx	1 (0.93)	43.11	[c]	[c]	[c]
Risk factors					0.427
No	18 (16.82)	36.84	6.63	(33.54 – 40.13)	
Betel	30 (28.04)	35.54	4.60	(33.82 – 37.25)	
Smoking	13 (12.15)	38.77	4.62	(35.98 – 41.57)	
Alcohol	6 (5.61)	32.21	12.77	(18.81 – 45.61)	
Smoking + alcohol	34 (31.78)	36.55	4.08	(35.12 – 37.97)	
Smoking + betel	5 (4.67)	39.70	2.95	(36.03 – 43.36)	
Smoking + alcohol + betel	1 (0.93)	34.47	[c]	[c]	[c]

[a] OSCC samples include OSCC tissues (n = 69) and OSCC oral rinses (n = 38)
[b] Data were not available in one case
[c] Data could not be obtained due to n < 2 and were excluded from the statistical analysis

malignant cells can be easily collected via saliva or oral rinses. This procedure is not invasive, not expensive, and does not require expertise. Moreover, the shed cancer cells in saliva and primary cancerous tissues had the same results of microsatellite alterations,[15] and aberrant promoter methylation.[51] Saliva or oral rinses of HNSCC patients exhibited telomerase activity,[52] increased mitochondrial DNA content,[53] and promoter hypermethylation.[54] Loss of heterozygosity (LOH) was found in the mouthwashes of OSCC patients but was not found in those of healthy individuals.[55] Comprehensive salivary analysis revealed an overall altered salivary composition in OSCCs[18,19] and also an increase in tumor markers including Cyfra 21–1, tissue polypeptide antigen, CA125, and IL-8.[20,21] Three species of bacteria in saliva were found to be increased in OSCC patients.[56] Salivary transcriptome study revealed the elevation of seven transcripts including DUSP1, H3F3A, IL1B, IL8, OAZ1, SAT, and S199P in OSCCs.[57,58] However, cancer development is a multistep process; the use of specific markers may be insufficient for detection. Thus, we try to seek for a biomarker which can detect malignant change at any step.

The alteration of global methylation levels can reflect the presence of malignancy. Though some studies revealed global hypomethylation in HNSCCs which included carcinomas in oral cavity, nose, sinuses, pharynx, and larynx.[42,43] But our previous study pointed out that methylation levels of LINE-1s, which reflect global methylation levels, have tissue specification.[34] Furthermore, tumors of larynx/hypopharynx and oral cavity had different levels of soluble CD44[22], as well as the incidence of LOH.[59] Accordingly, OSCCs which originate from mucosa in oral cavity may have different methylation levels from carcinomas which originate from other locations in the head and neck regions. Therefore, we studied LINE-1 hypomethylation in oral rinses of OSCC patients by using oral rinses of normal individuals as controls. Our study of this epigenetic alteration could detect OSCCs not only at an early occurrence but also at any hidden site of the oral cavity or in any histological type. It is independent of smoking, alcohol consumption and betel chewing. The hypomethylation could be detected in oral rinses of OSCC patients and did not statistically differ from that in primary OSCC tissues and OSCC microdissected samples. Thus, COBRALINE-1 of OSCC oral rinses could reflect the majority of OSCC methylation levels. Consequently, COBRALINE-1 of oral rinse appears to have a role in oral cancers screening.

While the degree of global hypomethylation of HNSCCs was associated with smoking history, alcohol use and stage,[42] we could not find any statistical difference in the hypomethylation levels in each stage, histological grade, location or risk factor of OSCCs. Therefore, our LINE-1 methylation study suggested that OSCCs may have different natures from HNSCCs, and that methylation levels of LINE-1s are specific to types of tissues. In the case of oral cancer, the loss of LINE-1 methylation may occur more significantly at the early onset of carcinogenesis. Consequently, more limit alterations can be found during the later stage of the tumor progression.

In conclusion, this study revealed that OSCCs had hypomethylation of LINE-1s and this aberration could be found in oral rinses of the patients. Our findings suggested the potential use of COBRALINE-1 of oral rinses as a non-invasive tool for OSCCs detection. However, this tool needs further development to improve sensitivity and eventually become a reliable investigation technique.

Conflict of interest statement

None declared.

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ORAL ONCOLOGY

Promoter hypermethylation of *CCNA1*, *RARRES1*, and *HRASLS3* in nasopharyngeal carcinoma

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Summary In search for putative tumor suppressor genes critical of nasopharyngeal carcinoma (NPC), we analyzed the available information from the expression profiling in conjunction with the comprehensive alleotyping published data relevant to this malignancy. Integration of this information suggested eight potential candidate tumor suppressor genes, *CCNA1*, *HRASLS3*, *RARRES1*, *CLMN*, *EML1*, *TSC22*, *LOH11CR2A* and *MCC*. However, to confirm the above observations, we chose to investigate if promoter hypermethylation of these candidate genes would be one of the mechanisms responsible for the de-regulation of gene expression in NPC in addition to the loss of genetic materials. In this study, we detected consistent hypermethylation of the 5′ element of *CCNA1*, *RARRES1*, and *HRASLS* in NPC tissues with prevalence of 48%, 51%, and 17%, respectively. Moreover, we found a similar profile of promoter hypermethylation in primary cultured NPC cells but none in normal nasopharyngeal epithelium or leukocytes, which further substantiate our hypothesis. Our data indicate that *CCNA1*, *RARRES1*, and *HRASLS3* may be the putative tumor suppressor genes in NPC.

Abbreviations: *CCNA1*; cyclin A1; *RARRES1*, retinoic acid receptor responsive element; *HRASLS3*, h-RAS like suppressor; NPC, nasopharyngeal carcinoma.

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Introduction

The development of nasopharyngeal carcinoma (NPC) appears to be associated with the *Epstein-Barr* virus (*EBV*) infection together with multiple genetic and epigenetic alterations of the nasopharyngeal epithelium.[1–3] Regarding the latter, many studies have now demonstrated that the epigenetic changes may underlie the molecular basis of these lesions. For example, the extensive and high resolution alleotyping studies revealed potential tumor suppressor gene loci in NPC on chromosomes 3p, 9p, 9q, 11q, 12q, 13q, 14q, and 16q.[1,4] In addition, the recent studies have identified that certain genes residing within these chromosomal loci are aberrantly hypermethylated, such as RASSF1A (3p21.3), RARβ2 (3p24), and p16INK4A (9p21).[5–7] However, for most of the loss of heterozygosity (LOH) loci, the presence of candidate tumor suppressor genes (TSG) has not been thoroughly investigated.

Previously, we have analyzed the mRNA expression profile of a small sub-set of NPCs by using laser capture microdissection coupled with high density cDNA microarrays and identified several aberrantly expressed genes which may play a role in NPC pathogenesis.[8] Here, we have applied the integrated information acquired from both our mRNA expression profile together with the genome-wide alleotyping data, to further define whether any of these molecules might represent potential TSG candidates. Genes whose expressions was down-regulated, as detected by the microarray experiments, were matched to the critical regions for LOH of either chromosomes 13 or 14, as there are some evidences suggesting that these may be the locations of TSG.[1,4,9,10] In addition to the above criteria, we further investigated if the hypermethylation of 5′ regulatory element of these genes would be an additional mechanism of the gene silencing in NPC. Our data indicate that in NPC but not in normal samples, there are the promoter hypermethylations of certain critical genes (*CCNA1*, *HRASLS3*, and *RARRES1*), which were shown to be down regulated in our previous microarray study, suggesting that these genes may potentially function as TSG in NPC.

Materials and methods

Tumor samples and DNA extraction

Primary NPC tumor samples were surgically obtained from patients with appropriate informed consent at The King Chulalongkorn Memorial Hospital. Samples were then divided into two portions. The first portion was fixed in formalin and was submitted for routine histopathological examination, and the second portion was immediately snap-frozen and stored in liquid nitrogen until use in further experiments. Control nasopharyngeal epithelium was obtained from nasopharyngeal swabs of unrelated patients in the Department of Otolaryngology who had clinically defined normal nasopharyngeal mucosa. Control blood samples were collected from unrelated healthy donors. All samples were tested to confirm the presence of the genetic material of EBV following the method previously described.[11]

Extraction of genomic DNA was performed with proteinase K (Amersham, Aylesbury, UK) digestion in the presence of SDS at 50 °C overnight, followed by phenol/chloroform extraction and ethanol precipitation.[11]

Cultured NPC cells

Primary NPC tissue was finely chopped, suspended in 0.25% collagenase (Life Technologies, Gaithersberg, MD), and incubated at 37 °C, 5% CO_2 for 4–5 h. After centrifugation, the collagenase was discarded and the pellet subjected to two washing steps with 5 ml DMEM (Dulbecco's Modified Eagle Medium; Life Technologies, Gaithersberg, MD) each. Subsequently, the pellet was resuspended in serum and growth factor free DMEM with 1% penicillin/streptomycin added, and incubated at 37 °C, 5% CO_2 until the cell layer had spread sufficiently to be further sub-cultivated. Pellets of NPC cells with limited passages were collected for DNA extraction.

Presence of NPC cells was confirmed by the detection of LOH of chromosome 3, 9, and 14, using microsatellite analyses for the following markers; D3S1038, D14S283 and TCRD, in order to confirm the complete loss of heterozygosity as well as showing the absence of contaminating normal tissue. Briefly, one strand of each primer pair was end-labeled at 37 °C for 1–2 h in a total volume of 10 μl containing 10 μM primer, 0.025 mCi [^{32}P] γATP (Amersham, Aylesbury, UK) at 3000 Ci/mmol, 10 mM MgCl, 5 mM DTT, 70 mM Tris–HCl (pH 7.6) and 10 units of T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The kinase reaction was then added to the PCR buffer mix. The PCR reactions were performed in a total volume of 10 μl using 50 ng of genomic DNA in 200 μM dNTP each, 10 mM Tris–HCL (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 units of *Thermus aquaticus* DNA polymerase (Perkin–Elmer Cetus, Norwalk, CT) and primer concentrations were 0.05–0.5 μM each. The PCR reactions were optimized as follows: an initial de-naturation step at 95 °C for 4 min, 25 cycles of de-naturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min, and a final extension at 72 °C for 7 min. The PCR products were analyzed with electrophoreses in 6% polyacrylamide and 7 M urea gel.

Sodium bisulfite modification

The DNA samples were subjected to bisulfite treatment.[5–7] Briefly, 2 μg of genomic DNA were denatured in 0.2 M sodium hydroxide. Subsequently, 10 mM hydroquinone (Sigma–Aldrich, St. Louis, MO) and 3 M sodium bisulfite (Sigma–Aldrich, St. Louis, MO) were added and incubated at 50 °C for 16–20 h. The modified DNA was then purified using Wizard DNA purification resin (Promega, Madison, WI) followed by ethanol precipitation.

Duplex MSP and COBRA

We used the duplex methylation specific PCR (MSP) to determine the methylation status of *CCNA1*, *TSC22*, *CLMN*, and *EML1* and performed the combined bisulfited restriction analysis (COBRA) to analyze the promoter hypermethylation of *HRASLS3*, *RARRES1*, *LOH11CR2A*, and *MCC*. The PCR reactions contained 1× PCR buffer (Qiagen, Chuo-ku, Japan), 0.2 mM deoxynucleotide triphosphates, 0.4 μM of appropri-

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ate PCR primers (Table 2), 1u of hotstarTaq (Qiagen, Chuoku, Japan), and 80 ng of bisulfited DNA. Cycles of PCR amplification were as follows: an initial de-naturation step at 95 °C for 10 min, followed by 30 cycles of de-naturation at 95 °C for 1 min, annealing at 48–55 °C for 1 min (Table 2), extension at 72 °C for 2 min, and the final extension at 72 °C for 5 min. In the COBRA analysis, all PCR products were digested with *TaqI* enzyme and subjected to electrophoresis in 8% native polyacrylamide gel. Methylation of the TCGA sequence in gel was visualized using phosphoimager after staining with Syber Green.

Bisulfite genome sequence analysis

We performed additional analysis of the bisulfited DNA by subcloning the fragments into pGEM – t Easy Vector System I (Promega, Madison, WI), which was then subjected to sequence analysis using the ABI prism 3100 Genetic Analyzer (Applied Biosystems, CA) for the presence of methylated nucleotides in all the samples.

Statistical analysis

Fisher's exact and Chi-square tests were used to determine the association between clinical parameters and the presence of promoter hypermethylation of the studied genes.

Results

Selection of tumor suppressor gene candidates from the genome-wide expression analysis of NPC

From our previous microarray analysis, we re-evaluated the data in order to search for potential candidate TSG. We first retrieved a list of genes whose expression was shown to be differentially down-regulated in NPC when compared to the normal nasopharyngeal epithelium. We next applied two additional selective criteria including the genes that have previously been shown to be tumor suppressor genes in other cancer types as well as the genes residing within the critical regions of LOH in chromosomes 13 and 14, which are also thought to be the important areas in NPC.[1,4,9,10] From the 238 down-regulated genes in NPC,[8] we found four genes that have been indicated to be putative tumor suppressor genes in other tumor types: *HRASLS3*,[8,12] *RARRES1*,[8,13–16] *LOH11CR2A*,[8,17] and *MCC*.[8] Furthermore, we selected *CCNA1* and *TSC22* whose genetic locations are within the critical LOH region of chromosome 13 at position 35,948–35,949 kb and 44,010–44,020 kb, respectively. Similarly, we selected *EML1* and *CLMN*, whose expression was differentially down-regulated, residing within the critical region of LOH in chromosome 14 at position 98,320–98,330 kb and 93,710–93,720 kb. Under the above selection algorithm, we were able to identify eight putative genes with potential tumor suppressor activity (Table 1). Fold difference of geometric means (T/N) were calculated by using fractionate with geometric mean of ratios in class 1:N and geometric means of ratios in class 2:T as described previously.[8] The ratios of less than 1 indicate a relative down-regulation of gene expression in NPC lesions.

Table 1 Ratios of eight selected candidate genes from microarray data and their chromosomal region

Gene	Chromosomal location	Fold difference of geometric means (T/N)
CCNA1	13q12.3–q13	0.404
TSC22	13q14	0.355
EML1	14q32	0.308
CLMN	14q32.2	0.237
HRASLS3	11q12.3	0.261
RARRES1	3q25.32	0.124
LOH11CR2A	11q23	0.448
MCC	5q21–q22	0.204

Analysis of the methylation status of promoter regions of candidate genes

We further investigated the potential mechanism which might be responsible for the down-regulation of all eight candidate genes. The 5′ elements of all eight genes were analyzed for methylation status by either MSP or COBRA technique (Table 2) in several NPC tissues and control normal peripheral blood leukocytes. All NPC were tested positive for *EBV*, whereas the control tissues were absence of *EBV* genetic materials. The presence of promoter hypermethylation in NPC was detected on *CCNA1*, *RARRES1* and *HRASLS3* promoter but not in the control leukocytes (Figs. 2 and 3, and Table 3). However, there was no hypermethylation detected on *MCC*, *TSC22*, *EML1* and *CLMN* upstream regulatory regions. Additional experiments demonstrated a partial methylation of *LOH11CR2A* promoter which could be observed in both NPC and control leukocytes (Figs. 2 and 3 and Table 3). The above findings in conjunction with the under-expression of these genes found by the microarray experiments suggest that the promoter hypermethylation of *CCNA1*, *RARRES1* and *HRASLS3* may be crucial in regulating expression of these genes especially in NPC.

Promoter hypermethylation of *CCNA1*, *RARRES1* and *HRASLS3*

To further validate the crucial role of the promoter hypermethylation of these TSG candidates in NPC carcinogenesis, we determined the frequency of methylation of *RARRES 1*, *HRASLS 3* and *CCNA1* in approximately 40 NPC tissue samples either by COBRA or MSP PCR. An additional 10 primary cultures of relatively pure population of NPCs with no normal cell contamination as indicated by our LOH study (Fig. 1), were also analyzed for the methylation status of these genes. We used several controls in this study including 20 normal nasopharyngeal swabs and 30 peripheral blood leukocytes from healthy subjects or non-cancer patients. Strikingly, in all normal cells including nasopharyngeal epithelium and WBC, we could not demonstrate the presence of promoter hypermethylation of all the studied genes (Table 4). In contrast, NPC tissues displayed frequent promoter hypermethylation of for *RARRES1*, *HRASLS3*, and *CCNA1* at 51%, 17%, and 48%, respectively (Table 4). Moreover, we confirmed the frequent promoter hypermethylation in isolated NPC cells to avoid normal cells contamination and found 83%

Table 2 Selected candidate genes and primers used to detect methylation

Gene	Detection	Primer	Sequence	Product size/Tm (bp/°C)
CCNA1[a]	MSP	*CCNA1* met (F)	TTTCGAGGATTTCGCGTCGT	46/53
		CCNA1 met (R)	CTCCTAAAAACCCTAACTCGA	
		CCNA1 unmet (F)	TTAGTGTGGGTAGGGTGTT	67/53
		CCNA1 unmet (R)	CCCTAACTCAAAAAAACAACACA	
TSCC22[a]	MSP	*TSCC22* met (F)	TTTAGTGTTTTGAGGTCGTC	87/48
		TSCC22 met (R)	TAAAATAAACTAACCTAACGCGA	
		TSCC22 unmet (F)	GGGATATAGTTTTGGGGATT	190/48
		TSCC22 unmet (R)	ATAAACTAACCTAACACAAACCA	
CLMN[a]	MSP	*CLMN* met (F)	AAACCTAACTAACAAACGCG	185/55
		CLMN met (R)	TCGTATTCGTCGTTTCGC	
		CLMN unmet (F)	TGTTGTGATTTTAGTTTTGTGGT	96/55
		CLMN unmet (R)	CCAACACAACACAAACAACA	
EML1[a]	MSP	*EML1* met (F)	TCGAGGTCGTTTTTTCGC	130/55
		EML1 met (R)	ACGCTAACGCTAAAACCG	
		EML1 unmet (F)	TTTGGGTTTTGTGGTTGT	162/55
		EML1 unmet (R)	ACCCAACCACAACCAACA	
HRASLS3[b]	COBRA	*HRASLS3* (F)	GGTGTTTGGGATGTTTTTTT	109/55
		HRASLS3 (R)	AAC (A/G) CCCAAAAAACTACAAA	
MCC[b]	COBRA	*MCC* (F)	AAAATGTGGTAGAAGGGATT	86/53
		MCC (R)	AAACTCTCAATCCCCCAAA	
RARRES1[b]	COBRA	*RARRES1* (F)	GGGAGG (C/T)GTTTTTATTGTTTT	115/53
		RARRES1 (R)	CTACCC (A/G)AACTTAATACTAAA	
LOH11CR2A[b]	COBRA	*LOH11CR2A* (F)	TTGAGGAAATGAGGTTGTAAGTT	129/48
		LOH11CR2A (R)	AATCCTAAATTTCCAATATCCAC	

[a] Genes locating within the critical regions of LOH.
[b] Genes shown to be tumor suppressor gene in other cancers.

Table 3 Frequency of methylation status of candidate genes promoter in cultured NPC cells and control leukocytes

	NPC cells		Normal leukocytes	
	Presence	Absence	Presence	Absence
CCNA1	16	11	0	20
RARRES1	2	8	0	20
HRASLS3	2	8	0	20
EML1	0	10	0	10
CLMN	0	10	0	10
TSC22	0	10	0	10
MCC	0	10	0	10
LOH11CR2A	28	0	10	0

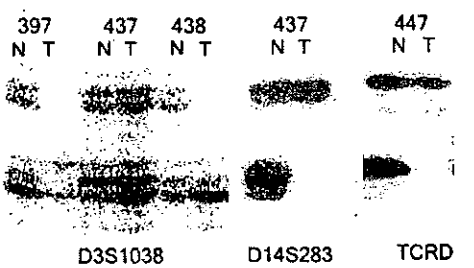

Figure 1 LOH study. Three microsatellite markers, D3S1038, D14S283, and TCRD, are used to study the LOH pattern in normal leukocytes (N) and tumor cells (T) of primary cultured NPC cells. In normal leukocytes, there were two bands of PCR products observed, whereas only single band of PCR products was detected in tumor cells.

hypermethylation of *RARRES1*, 17% of *HRASLS3* and 100% of *CCNA1*. Additional sequence analyses of the promoter of all three genes to prove the presence of promoter hypermethylation were performed in each sample (Fig. 4). The above findings suggest a possible tumor suppressive role of these genes. When analyzing for an association between hypermethylation and clinical parameters, we found no significant correlation with the tumor stage (data not shown).

Discussion

Currently, comprehensive gene expression profiling of individual cancer type has become one of the most popular ways to analyze individual cancer. We here apply the information derived from the gene expression analysis and to expand this knowledge to identify potential tumor suppressor genes in

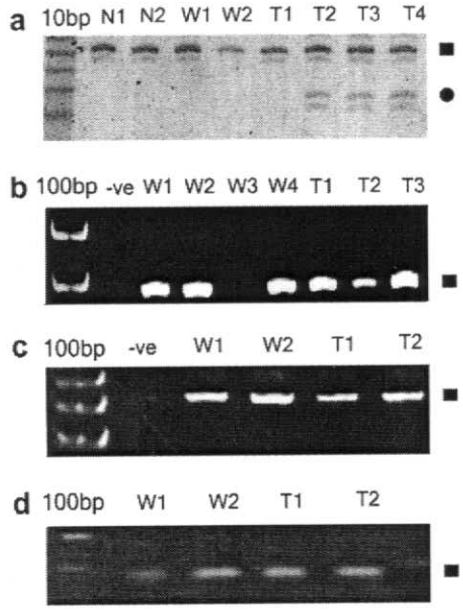

Figure 2 MSP analysis. (a) Typical results of a MSP analysis of *CCNA1* show frequent methylation of 5′ element of *CCNA1* in NPC but not in normal leukocyte and nasopharyngeal epithelium. (b–d) The results of MSP analysis of *EML1*, *CLMN*, and *TSCC22*, are shown. There was no methylation observed in these set of genes. Square: unmethylated band; circle: methylated band; -ve: control lane (water); T: NPC tissue; W: leukocytes; N: normal nasopharyngeal swab.

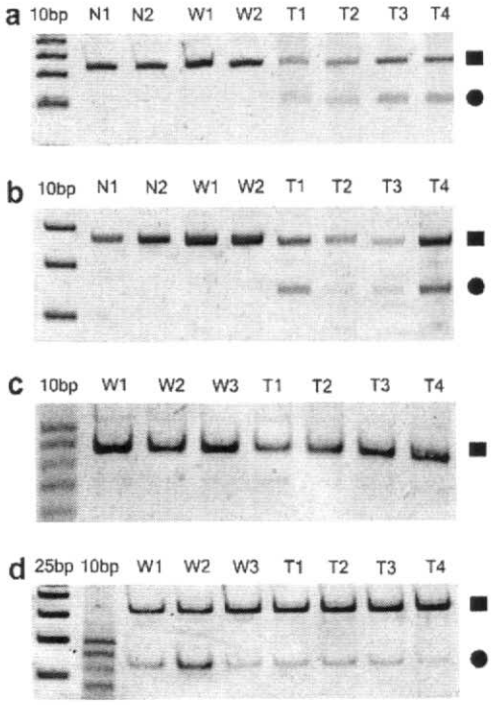

Figure 3 COBRA analysis. Methylation status of *HRASLS3*, *RARRES1*, *MCC*, and *LOH11CR* 2A promoter were analyzed by COBRA technique. Panels a and b showed frequent methylation of *HRASLS3* and *RARRES1* upstream elements in NPC but not in normal tissues. Methylation of *MCC* was not detected in both normal and tumor cells. In panel d, methylation of *LOH11CR2A* DNA sequence are invariably presence in both normal and tumor cells. Square: unmethylated band; circle: methylated band; -ve: control lane (water); T: NPC tissue; W: leukocytes; N: normal nasopharyngeal swab.

NPC. Integrating one known feature of tumor suppressor genes, namely LOH to the genome-wide expression analysis, allowed us to identify three potential TSGs in NPC, *CCNA1*, *HRASLS3*, and *RARRES1*. These genes have been proven to be relatively under-expressed and preferentially methylated at the promoter in NPC comparing to normal tissue. Furthermore, these three genes have been shown previously to be putative TSGs in other cancer type, for example *HRASLS3* in lung cancer,[8,12] *RARRES1* in lung cancer, prostrate cancer, and head and neck cancer.[8,13–16] The above findings not only corroborate the results from our previous microarray study but also help in elucidating the molecular epigenetic changes underlying the NPC.

Table 4 Frequency of methylation status of candidate tumor suppressor genes in NPC comparing to control nasopharyngeal epithelium and leukocytes

Gene	Sample	Promoter hypermethylation		Total
		Absence	Presence	
CCNA1	Normal leukocytes	30 (100%)	0	30
	Normal nasopharyngeal epithelium	20 (100%)	0	20
	NPC biopsy	24 (52.1%)	22 (47.8%)	46
	Cultured NPC cells	0	9 (100%)	9
RARRES1	Normal leukocytes	30 (100%)	0	30
	Normal nasopharyngeal epithelium	20 (100%)	0	20
	NPC biopsy	22 (48.9%)	23 (51.1%)	45
	Cultured NPC cells	1 (16.7%)	5 (83.3%)	6
HRASLS3	Normal leukocytes	30 (100%)	0	30
	Normal nasopharyngeal epithelium	20 (100%)	0	20
	NPC biopsy	34 (82.9%)	7 (17.1%)	41
	Cultured NPC cells	5 (83.3%)	1 (16.7%)	6

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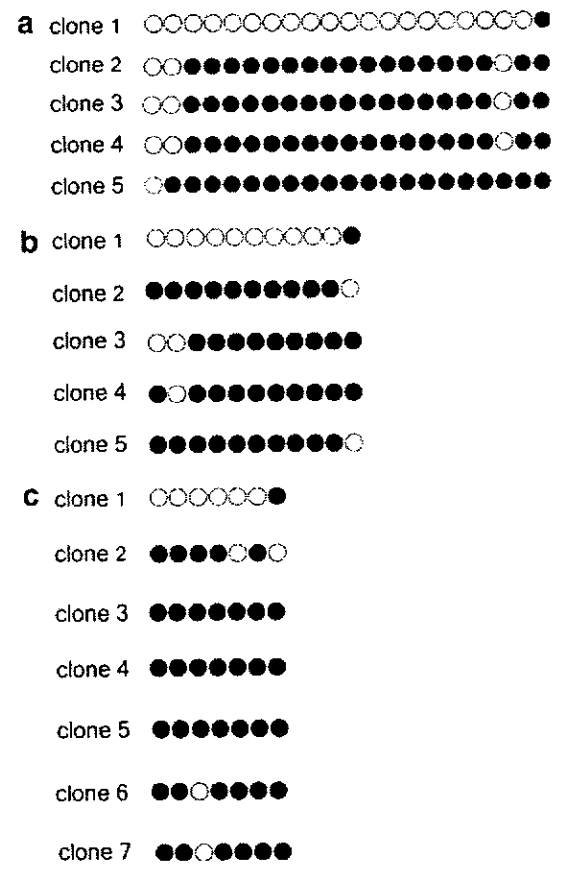

Figure 4 Bisulfite sequencing of candidate genes promoter. Results of bisulfite sequencing display the anatomical pattern of promoter hypermethylation of the three tumor suppressor gene candidates; *CCNA1* in panel a, *RARRES1* in panel b, and *HRASLS3* in panel c. Closed or opened circles are the methylated or unmethylated CpG or TG dinucleotides, respectively.

Function of cyclin A1 is unclear, whether it would act as an oncogene or a TSG is currently unknown. However, cyclin A1 is thought to play a role in oncogenesis because of its involvement in cell cycle progression.[18,19] Moreover, high levels of cyclin A1 have been found in acute leukemia cell lines and myeloid leukemia samples.[20] More recently, it has been implicated that cyclin A1 might be involved in DNA-double-strand-break repair by interacting with Ku70.[21] In addition, there are several reports documenting this gene to be down regulated and hypermethylated in squamous cell carcinomas of the head and neck and the cervical locations.[22,23] Here, we retrieved the data from global gene expression profile of NPC to search for candidate TSG and found *CCNA1* to be one of the candidates as its expression was down-regulated in NPC. Furthermore, we found that its promoter region was methylated in a sub-set of NPC tissues but not in normal nasopharyngeal epithelial tissue and leukocyte. Because transcriptional silencing by promoter methylation is a common mechanism for an inactivation of tumor suppressor gene, in the above regards, cyclin A1 may play a role as a tumor suppressor gene in squamous cell cancers including NPC.

HRASLS3 (H-ras like suppressor 3) is a class II tumor suppressor gene which is down-regulated in various cancer cell lines derived from melanoma, neuroblastoma, adenocarcinoma, and fibrosarcoma. There is evidence that promoter methylation of this gene in lymphoma cells can result in gene silencing.[24]

From our microarray data, *HRASLS3* is the gene, which is down regulated in NPC and in this study, we detected promoter methylation of this gene in NPC, which might be responsible for the inactivate of this putative tumor suppressor gene in NPC.

RARRES1 (retinoic acid receptor responsive element) is a candidate tumor suppressor gene of human prostate cancer, head and neck cancer and lung cancer, and recent evidence suggesting that the promoter of this gene is methylated in several of these cancers such as esophageal cancer, endometrial cancer, head and neck cancer, and lung cancer. [13–15] Indeed, promoter methylation of this gene has been detected in head and neck squamous cell carcinoma but not in normal tissue.[25] Recently, other groups studying this gene detected 90.7% promoter methylation in primary NPC,[16] here we also detected 51% promoter methylation in primary tumor and 83% in NPC cells. In addition, our microarray data revealed that this gene was down-regulated in NPC, therefore *RARRES1* may be the important TSG in this cancer type.

In conclusion, we report three putative tumor suppressor genes, *CCNA1*, *RARRES1* and *HRASLS3*, in NPC identified from the analysis of available gene expression profiling and alleotyping database, and the status of promoter hypermethylation. These three genes might well be the molecular markers for NPC since their promoters are methylated in NPC but not in normal cells. Further expanding the analysis in broader panel of genes from the database and functional study is warranted in the future.

Conflict of Interest Statement

None declared.

Acknowledgements

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Letter to the Editor

Maternal uniparental disomy of chromosome 16 resulting in hemoglobin Bart's hydrops fetalis

To the Editor:

Since the first cases of maternal uniparental disomy for chromosome 16 or UPD(16)mat was described by Kalousek et al. in 1993, a handful of cases have been diagnosed in newborn infants and terminated fetuses (1–4). Most cases are associated with confined placental mosaicism, with high incidence of adverse pregnancy outcome (4, 5). The most common findings include intrauterine growth retardation, single umbilical artery, imperforate anus, and pulmonary and cardiac malformations (1, 2, 4, 6, 7). Also, UPD(16)mat individual with normal phenotypes has been reported (8). The only confirmed case with UPD(16)pat was isodisomic and identified through pre-natal detection for abnormal maternal serum screening; eventually, the child was quite normal (9). Herein, we describe a hemoglobin (Hb) Bart's hydroptic fetus and fetal malformations caused by UPD(16)mat.

The counselee is a 34-year-old G1P0 pregnant Thai woman affected with Hb H disease caused by heterozygous Southeast Asian (–SEA) and the 3.7-kb deletions of the alpha1 and 2 globin genes, respectively. Her husband is Hb E heterozygote. Ultrasound performed at a gestation of 23 weeks showed a hydroptic fetus. Fetal blood from cordocentesis showed Hb Bart's hydrops fetalis with –SEA globin1 gene deletion but not the other types of alpha1 globin gene mutations, suggesting the following possibilities: non-paternity, another rare but as yet unidentified alpha-thalassemia 1 mutation inherited from the father, or UPD(16)mat. Fetal autopsy showed characteristic features of Hb Bart's hydrops including interstitial edema of skin, cardiomegaly, and hepatosplenomegaly with extramedullary hemopoiesis. Single umbilical artery, bilobed right lung and ileal Meckel's diverticulum were found. Retrospectively, a two-vessel cord was noted on pre-natal ultrasonogram. Peripheral blood culture for fetal karyotype analysis was unsuccessful.

It is unlikely that the husband has an unidentified alpha-thalassemia 1 gene deletion, given normal hematocrit, mildly small red cell size, and percent Hb E as expected in a heterozygous Hb E individual. Normally, heterozygote for Hb E with co-inheritance of alpha-thalassemia 1 will

Table 1. Hb typing and genotyping of α-globin genes[a]

Hb typing and α-globin gene genotype	Mother	Father	Abortus (fetus)
Hb (g/dl)	10	14	NA
Hct (%)	31.7	42	NA
MCV (80–99 fl)	61.6	78.1	NA
Hb typing	A_2A Bart's H	EA	Bart's (100%)
Hb A (%)	76.4	61.3	
Hb A2 (%)	1.1		
Hb F (%)	<0.5	0.8	
Hb E (%)		33.5	
Alpha globin gene analysis			
α-thalassemia 1 deletions [–MED, –SEA, –THAI, –FIL, and -(α)20.5]	SEA	Not found	SEA
α-thalassemia 1 point mutations (cd 14G>A and Hb Adana)	NA	NA	Not found
αααanti-3.7 gene triplication	NA	NA	Not found
α-thalassemia 2 deletions (-α3.7 and -α4.2 kb)	3.7 kb	Not found	Not found
α-thalassemia 2 point mutations (initiation cd T>C, cd 19-G, IVS1-5nt, cd 59 G>A, Hb Quong Sze, Hb Constant Spring, Hb Icaria, Hb Pakse, Hb Koya Dora, polyA-1, and polyA-2)	NA	NA	Not found

–FIL, Filipino mutation; Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; –MED, Mediterranean mutation; NA, no data available; –SEA, Southeast Asian deletion; –THAI, Thai mutation.

[a]Analysis for additional uncommon mutations of alpha1 and 2 globin genes were carried out by reverse -hybridization assay for simultaneous detection of 21 α-globin mutations using the α-globin StripAssay™ from Vienna Lab (12).

have a lower Hb E level (around 20.7 ± 1.2%) (10). Analysis for additional uncommon mutations of alpha globin genes in the fetal DNA revealed negative findings (Table 1) (11, 12).

Microsatellite analyses were conducted after informed consent given, following approval by the Institutional Review Board. Paternity testing using markers on 10 different chromosomes indicated no conflict of paternity (AmpFLSTR® Profiler Plus™ ID PCR Amplification Kit, Applied Biosystems, Foster City, CA). UPD16 was analyzed by using five microsatellite markers, D16S2618, D16S2622, D16S748, D16S287, and D16S511. Primers for polymerase chain reaction (PCR) amplification of the markers were obtained from Research Genetics (Huntsville, AL). PCR and gel electrophoresis were conducted as previously described with the exception of gel visualization in which after electrophoresis, the wet gel was transferred to filter paper (Whatman, Maidstone, Kent, UK), wrapped with Saran wrap and visualized by PhosphorImager using IMAGEQUANT software (Molecular Dynamics, Sunnyvale, CA) instead of overnight X-ray film exposure (13). The results of UPD study showed maternal heterodisomy delineated by marker D16S511 and isodisomy segment at 16p13.3 delineated by marker D16S2618 (Fig. 1a,b).

In the present case, we proposed that recombination occurring between the maternal homologous

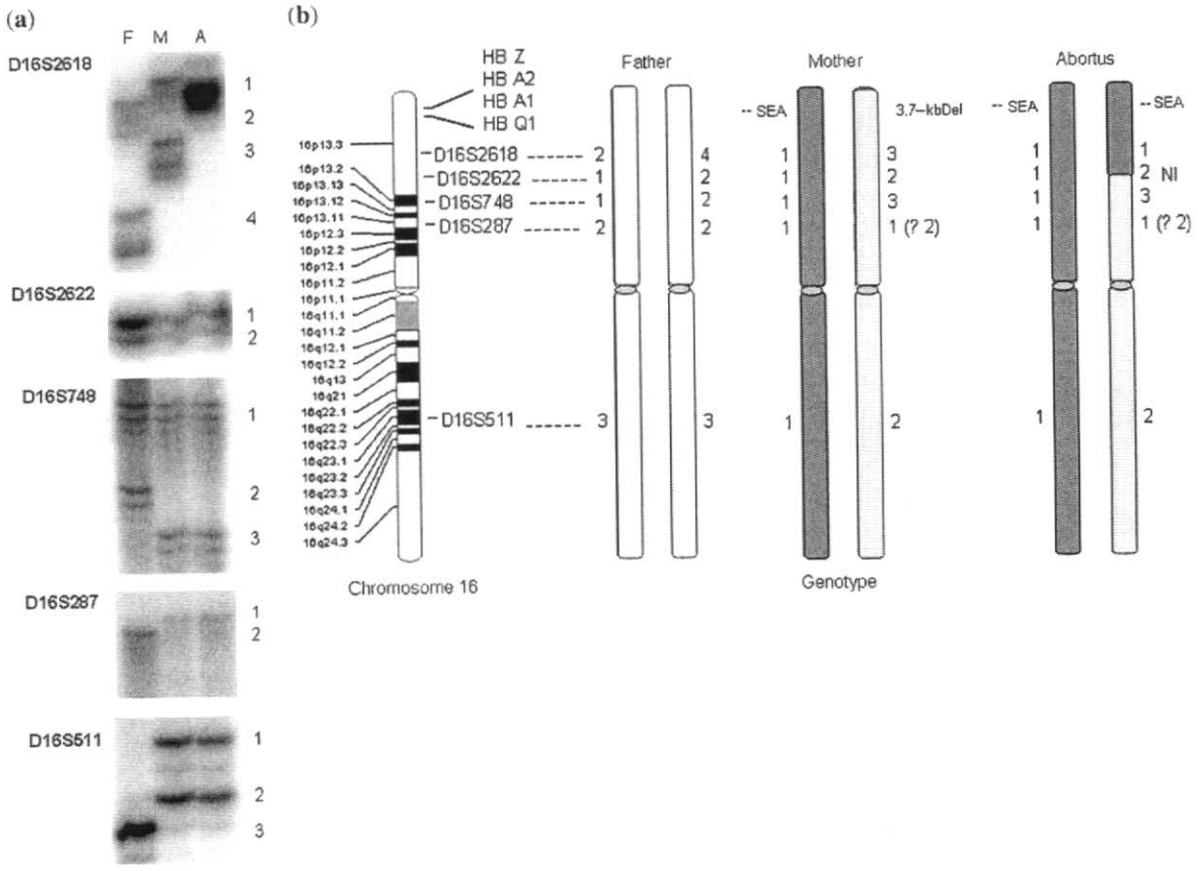

Fig. 1. UPD16 analysis and mechanism resulting in UPD(16)mat. (**a**) Results of UPD16 analysis shown on 6% polyacrylamide/7 M urea gel and (**b**) cytogenetic map of chromosome 16 and locations of α-globin gene cluster and microsatellite markers. Note lack of transmission of the paternal allele of the markers D16S2618 (mat isodisomy) and D16S511 (mat heterodisomy) to the abortus. D16S2622 was non-informative, while D16S287 was difficult to interpret if it was mat isodisomy or heterodisomy. Although a UPD(16)mat cannot be delineated from D16S748, the finding of UPD by D16S511 and D16S2618 were indirect evidence indicating maternal heterodisomy at this site. (**c**) Proposed mechanism leading to maternal uniparental heterodisomy with disomic segment of 16p13.3-pter as a result of recombination and meiosis I error followed by trisomy rescue. A, abortus/fetus; F, father; M, mother; NI, non-informative; –SEA, Southeast Asian mutation. Genetic locations of the markers are from the following database: the Genome Database (http://ncbi.nlm.nih.gov), the National Institutes of Health/CEPH Collaborative Mapping Group (http://www.genlink.wustl.edu), and the Marshfield Map (http://research.marshfieldclinic.org). Physical map was from the GDB Human Genome Database MAPVIEW 2.4 (http://www.gdb.orgx).

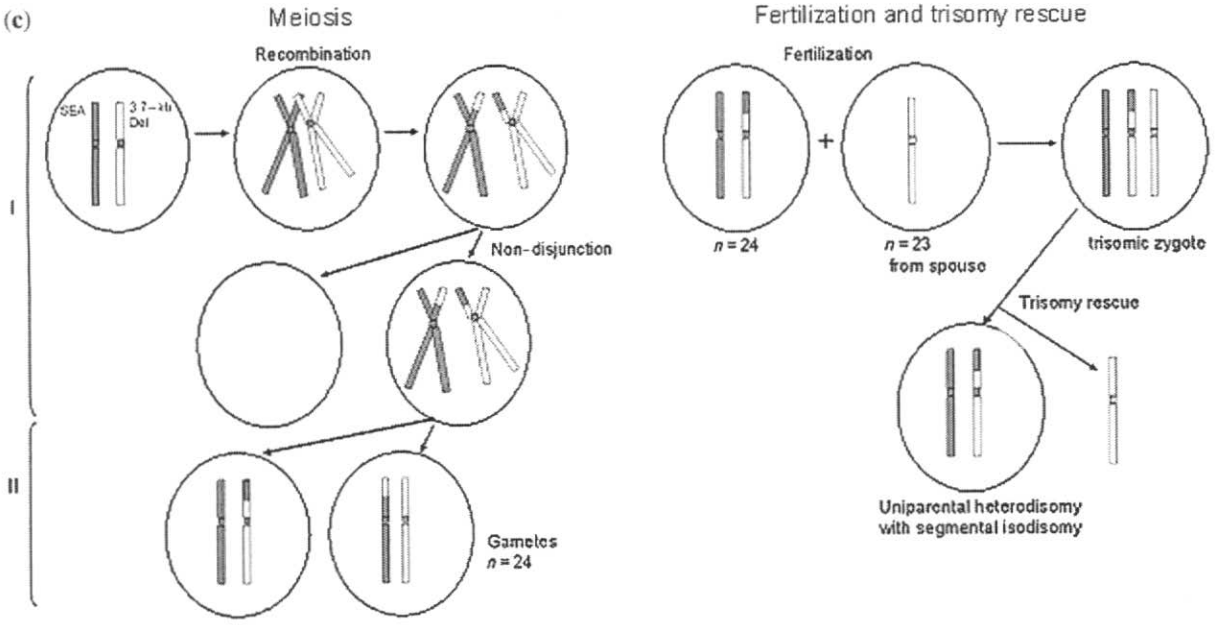

Fig. 1. Continued

chromosomes at 16p13.3 and non-disjunction at maternal meiosis I resulted in gamete containing extra chromosome 16 with isodisomic segment at 16p13.3-pter, which later fertilized by normal gamete from her spouse, leading to zygote with trisomy 16 followed by trisomy rescue with the removal of the paternal chromosome (Fig. 1c). The recombination might have occurred between the markers D16S748 and D16S2618 that are 30.2 cM apart (Fig. 1b) (14). Without such recombination, the fetus should have had UPD(16)mat-related anomalies and Hb H disease.

To date, more than 40 examples of recessive trait transmission by UPD have been observed (15). Among these cases, UPD for chromosomes 1, 2, and 7 have contributed to the majority of the recessive conditions (15). The studies of UPD in cystic fibrosis (chromosome 7), Herlitz junctional epidermolysis bullosa (chromosome 1), and cartilage-hair hypoplasia (chromosome 9) have indicated that UPD accounts for nearly 2–4% of the cases (16–18). Of note, the present case is one among 49 Hb Bart's hydrops fetalis thus far studied in thalassemia pre-natal diagnosis laboratory at Ramathibodi Hospital.

Our data suggest that in the absence of a history of non-paternity, Hb Bart's hydrops fetalis with homozygous mutation in a fetus of a couple in whom only one parent is heterozygote of the alpha1 globin gene deletion should be further evaluated for possible UPD(16)mat, initially by looking for two-vessel cord and fetal malformations identified through pre-natal ultrasonography or fetal autopsy followed by genotyping analysis for UPD(16)mat and/or non-paternity.

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LINE-1 hypomethylation level as a potential prognostic factor for epithelial ovarian cancer

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Abstract. Pattamadilok J, Huapai N, Rattanatanyong P, Vasurattana A, Triratanachat S, Tresukosol D, Mutirangura A. LINE-1 hypomethylation level as a potential prognostic factor for epithelial ovarian cancer. *Int J Gynecol Cancer* 2008;**18**:711–717.

A genome-wide hypomethylation is a common and crucial event in cancer. This study was to evaluate common epithelial ovarian cancer (EOC) if long interspersed element-1 (LINE-1) repetitive sequences methylation levels are progressively decreased during multistage carcinogenesis and there are the correlation between LINE-1 methylation levels and clinicopathologic characteristics. A total of 59 pairs of microdissected EOC tissues obtained from patients with EOC were examined for the methylation levels of LINE-1 repetitive sequences by a COBRALINE-1 (combined bisulfite restriction analysis of LINE-1) PCR protocol. The methylation levels were correlated with clinicopathologic parameters to determine the potential role of global hypomethylation as a prognostic marker for EOC. The LINE-1 methylation levels of 59 EOCs, 34.87 ± 7.39%, were lower than in representative normal ovarian tissues (46.89 ± 8.31%; 95% CI: 9.42–14.62; $P < 0.001$, paired-two-tailed t test). A decrease in the LINE-1 level of methylation was correlated with histological subtypes, higher FIGO and advanced tumor grade. Patients with greater hypomethylation (i.e., a methylation level ≤34.87%) had poorer mean overall survival ($P = 0.003$) and a lower mean progression-free interval ($P < 0.001$). Therefore, progressive decrease in LINE-1 methylation level is a common and important epigenetic process in ovarian multistep carcinogenesis. Moreover, the COBRALINE-1 method has the potential to be used as a tumor marker for EOC.

KEYWORDS: COBRALINE-1, epithelial ovarian cancer, global hypomethylation, LINE-1 methylation, tumor marker.

Epithelial ovarian cancer (EOC) is one of the most significant gynecologic malignancies. The majority of patients with EOC presents in an advanced stage and eventually dies of disease persistence or recurrence a short period after relapse. In fact, the 5-year survival rate for patients with advanced disease is less than 20%[1–3]. Many factors, such as age, FIGO stage, tumor grade, and residual tumor[4], are suspected to play a role in the prognosis of EOC, and recently, much interest has been focused on the molecular and genetic changes which occur in EOC[5–7]. Over the past 30 years, there has been little change in the incidence and mortality rates associated with ovarian cancer, a likely manifestation of the lack of effective diagnostic tools, which may play a role in the early detection of the disease.

Global hypomethylation, demonstrated by the downregulation of genome-wide methylated CpG dinucleotides, is one of the most common molecular events in cancer[8–11]. Genome-wide losses of methylation in cancer may lead to carcinogenesis[12,13] and may facilitate chromosomal instability[14–16]. In addition, the extent of genomic hypomethylation appears to correlate with tumor progression and invasiveness

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in several cancer types[10], including EOC[17]. Recently, we developed an improved quantitative combined bisulfite restriction analysis (COBRA) PCR protocol, referred to as COBRALINE-1, that efficiently evaluates the methylation status of LINE-1 repetitive sequences[18] in genomic DNA derived from microdissected tissues[10]. LINE-1 sequences represent genome-wide DNA since LINE-1s are highly repeated and widely interspersed human retrotransposon sequences. LINE-1s constitute about 17% of the human genome[19]. There are up to 600,000 copies of LINE-1s in the human genome. Approximately 2000 copies remain full length[20]. We applied a modified COBRA PCR protocol to efficiently and quantitatively evaluate LINE-1 methylation status in the entire genome of isolated cell populations. We demonstrated that most cancers exhibit significantly increased levels of hypomethylation compared with their normal tissue counterparts[10,21]. Recently, the technique has been used to evaluate clinicopathological correlation with prostate cancer[22]. In addition, we evaluated the sera of patients with hepatocellular carcinoma and proved that serum LINE-1 hypomethylation may serve as a prognostic marker for patients with hepatocellular carcinoma[23].

In this study, we described LINE-1 hypomethylation in relation to multistage carcinogenesis. Consequently, this relation should reflect how the genome-wide methylation levels correlate with clinicopathologic characteristics of EOC[1,24,25]. This study evaluated the correlation between LINE-1 methylation levels and histologic subtypes, tumor grade, FIGO stage, and disease recurrence. Moreover, we studied the potential role of aberrations of DNA methylation in LINE-1 as an independent prognostic marker for the survival of EOC patients. The outcome of this study may provide additional epigenetic information regarding the different pattern of multistage carcinogenesis in EOC. Finally, the COBRALINE-1 technique has a high potential to be used as a tumor marker for EOC.

Materials and methods

Patients and samples

The tumor registry of patients with EOC treated in the Gynecologic Oncology Unit of King Chulalongkorn Memorial Hospital between January 1, 1997 and December 31, 2004 were retrospectively reviewed. After preliminary study[10] and calculation to yield adequate number for further statistical comparison among EOC classes, a total of 59 patients with EOC who underwent primary surgical resection were randomly selected to enroll our protocol. The patient's data, including medical history, initial laboratory profile with preoperative CA125 level, preoperative and postoperative ultrasonographic scans or CT scan of the whole abdomen, operative record, pathology report, adjuvant treatment regimen, clinical outcome, and clinical status at the time of the last follow-up evaluation were recorded. All of the enrolled patients were reevaluated according to the surgical staging criteria classified by FIGO of 1985[1]. The surviving patients were reevaluated within 24 months and the last follow-up was offered on December 31, 2005. The progression-free interval was defined as the time from primary surgery to the onset of recurrence of disease, as detected by physical examination, imaging, or tumor markers. Overall survival was considered to begin at the time of primary surgery until the patient died from any causes or at the last follow-up examination.

The histopathologic diagnosis, including cell type, tumor grade, and organ metastases of all resected specimens, were reviewed by a gynecologic pathologist (S.T.). The paraffin-embedded ovarian specimens from the Gynecologic Cytopathology Unit were collected and prepared in 5-μm thickness sections on slides for microdissection[26]. The hematoxylin and eosin stained (H&E) slides were reviewed and mapped to unstained paraffin-embedded slides to confirm the areas of malignant and normal tissues needed for study. After the unstained slides were deparaffined, microdissection of cancer tissues was performed under an inverse microscope. For the control group, the tissue from the normal ovarian cortex of the same patient (i.e., from the contralateral ovary or regions of the ipsilateral ovary shown to be negative for malignancy using histopathologic techniques) was microdissected and mixed with lysis buffer solvent in micropipette tubes.

Exclusion criteria included two primary cancers, mixed histologic subtypes of EOC, tumors of low malignant potential, neoadjuvant chemotherapy treatment, or lack of availability of comparative normal ovarian tissue.

DNA extraction and COBRALINE-1 PCR

COBRALINE-1 was performed as previously described, with some modifications[10,27]. Briefly, genomic DNA was denatured in 0.22 M NaOH at 37°C for 10 min. Thirty microliters of 10 mM hydroquinone and 520 μL of 3 M sodium bisulfite were added for 16–20 h at 50°C. The DNA was purified and incubated in 0.33 M NaOH at 25°C for 3 min, ethanol precipitated, then washed with 70% ethanol and resuspended in 20 μL of H_2O. Two microliters of bisulfited DNA was subjected to 35 cycles of PCR with

two primers (5′-CCGTAAGGGGTTAGGGAGTTTTT-3′ and 5′-RTAAAACCCTCCRAACCAAATATAAA-3′) at an annealing temperature of 50°C. The amplicons were digested in 10 μL reaction volumes with 2 U of *Taq*I or 8 U of *Tas*I in 1× *Taq*I buffer (MBI Fermentas, Burlington, ON, Canada) at 65°C overnight and then electrophoresed in 12% non-denaturing polyacrylamide gels. The intensities of DNA fragments were measured by PhosphorImager, using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The LINE-1 amplicon size is 160 bp. The methylated amplicons, *Taq*I positive, yielded two 80 bp DNA fragments; whereas the unmethylated amplicons, *Tas*I positive, yielded 63 and 97 bp fragments (Fig. 1). The LINE-1 methylation level was calculated as a percentage (the intensity of methylated LINE-1 digested by *Taq*I, divided by the sum of the unmethylated LINE-1 digested by *Tas*I- and the *Taq*I-positive amplicons). The same DNAs from HeLa, Daudi, and Jurkat cell lines were applied as positive controls in all COBRALINE-1 experiments and to adjust for interassay variation.

Statistical analysis

Statistical analysis was performed using SPSS software, version 11.5. The mean differences in the percentage level of methylation between EOC and normal ovarian tissue and between subgroups of EOC were analyzed by a paired sample *t* test and an independent sample *t* test, respectively. The analysis of overall survival and progression-free survival time was calculated using the Kaplan–Meier method and the differences in survival between the groups were compared using a log-rank test. The Cox regression analysis was performed to identify which independent factors had a significant influence on overall survival. The results were considered significant when the *P* value was <0.05.

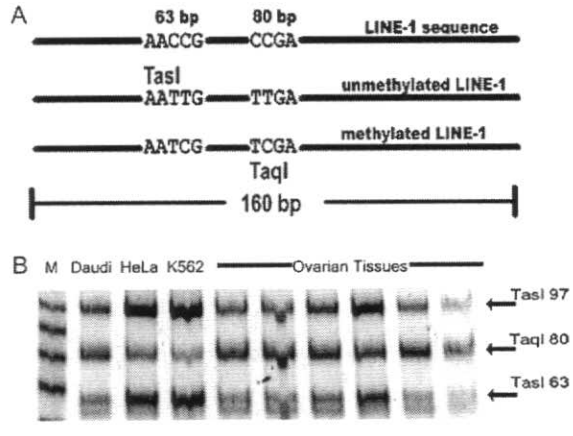

Figure 1. Combined bisulfite restriction analysis of LINE-1 (COBRALINE-1). (A) Schematic illustration of COBRALINE-1. The LINE-1 amplicon size is 160 bp. Methylated amplicons, *Taq*I positive, yielded two 80 bp DNA fragments, whereas unmethylated amplicons, *Tas*I positive, yielded 63 and 97 bp fragments. (B) An example of COBRALINE-1. M, 10-bp DNA size marker; Daudi, HeLa and K562 are positive control and for adjusting interassay variation. Ovarian tissues are ovarian cancer and normal cortex of EOC patients. *Tas*I 63 and 97, unmethylated amplicons; *Taq*I 80, methylated amplicons.

Results

LINE-1 methylation and clinicopathologic correlations

Fifty-nine patients with EOC were enrolled in the study. The mean age at the time of diagnosis was 49.6 ± 11.9 years. Of the 59 patients, 30 (50.8%) had early stage ovarian cancer and 29 (49.2%) had advanced stage ovarian cancer. To determine LINE-1 methylation levels in patients with EOC, we performed the COBRALINE-1 method in both cancerous and normal ovarian tissues. The mean percentage level of methylated amplicons was 34.87 ± 7.39% and 46.89 ± 8.31% in the cancer and normal groups, respectively. The cancers were hypomethylated, having lower methylation levels when compared with the normal tissue counterparts (95% CI: 9.42–14.62, $P < 0.001$; Table 1). From this study, the LINE-1 methylation pattern was hypomethylated in EOC specimens from 56 of 59 patients (94.9%). Even though there were less striking differences in LINE-1 methylation level between most cell types, mucinous possessed higher methylation levels than endometrioid (Table 1).

Table 1. LINE-1 methylation in different histology, FIGO stage, and tumor grade

		% Methylation (±SD)		
	n	Cancer	*P* value	Normal
Histology				
Mucinous	12	38.39 (5.16)	0.05	47.88 (5.05)
Serous	14	36.02 (4.47)	0.722	50.65 (7.78)
Clear cell	15	32.66 (5.91)	0.192	48.24 (5.15)
Endometrioid	18	33.47 (6.60)	Reference	42.18 (5.21)
FIGO stage				
I–II	30	36.62 (5.21)	0.035	45.64 (5.17)
III–IV	29	31.70 (7.09)	Reference	47.18 (10.24)
Tumor grade				
Grade 1	19	36.72 (6.32)	0.020	45.57 (6.35)
Grade 2	10	37.51 (5.06)	0.025	45.41 (8.58)
Grade 3	30	31.97 (6.76)	Reference	47.54 (8.96)

Statistics were determined by independent two-tailed *t* test.
% methylation, mean of percentage level of LINE-1 methylation.

When we compared LINE-1 methylation levels with a reference group, the data demonstrated that loss of genome methylation may be associated with more advance or aggressive EOC phenotypes. There were significant differences between combined EOC stages I and II and III and IV ($P < 0.05$; Table 1). In addition, grade 3 EOC possessed lowest LINE-1 methylation level ($P < 0.05$; Table 1). This data, comparing between EOCs and normal tissues and between advancement of EOCs, concluded that genome-wide methylation levels are progressively decreased during multistage carcinogenesis.

Prognostic value of LINE-1 hypomethylation

By univariate analysis, FIGO stage, optimal surgery, 12-month recurrence of disease, and LINE-1 hypomethylation were associated with overall survival (Table 2). By multivariate analysis, LINE-1 hypomethylation was analyzed with other variables that may influence prognosis, such as patient age, FIGO stage, histologic subtype, tumor grade, optimal surgery, and disease recurrence. A Cox regression model revealed that excessive LINE-1 hypomethylation, clear cell carcinoma histologic subtype, and advanced FIGO stage independently had tendency to be associated with a shortened overall survival (Table 3).

We then examined the potential prognostic value of LINE-1 hypomethylation in ovarian cancer. The median follow-up period was 24.6 months (range: 2.3–93.9 months). The patients with EOC were divided into two groups based on the mean level of LINE-1 methylation (34.87%). The mean overall survival time of patients with less hypomethylation (methylation >34.87%) was 50.49 ± 1.6 months, which was significantly better than the patients with greater hypomethylation, who had a median overall survival time of 30.83 ± 4.46 months and a mean overall survival time of 52.70 ± 8.65 months ($P = 0.003$ by the log-rank test; Fig. 2). The mean progression-free survival time of patients with less hypomethylation was 45.03 ± 3.23 months, which was significantly better than patients with greater hypomethylation (median progression-free survival, 24.38 ± 3.66 months; mean progression-free survival, 21.23 ± 3.23 months; $P = 0.0008$ by the log-rank test; Fig. 3). Therefore, the lower genome-wide methylation levels correlate with more advanced clinicopathologic characteristics. Moreover, the COBRALINE-1 method has the potential to be used as a tumor marker for EOC.

Table 2. Patients' status and clinicopathological features

	Status (cases)				
	Alive	Death	Risk ratio	95% CI	*P* value
Age					
<50 years old	24	7			0.900
≥50 years old	19	6	1.063	0.409–2.761	
Histological subtype					
Mucinous	10	2			
Endometrioid	14	3	1.059	0.208–5.401	0.698
Serous	9	5	1.500	0.303–7.432	
Clear cell	10	5	2.000	0.467–8.557	
Grade					
Well-differentiated	15	3			
Moderately differentiated	8	2	1.200	0.239–6.025	0.702
Poorly differentiated	20	8	1.714	0.523–5.621	
Tumor stage					
I and II	28	1			<0.001
III and IV	15	12	12.889	1.795–92.561	
Optimal surgery					
No residual tumor	30	2			
Resident tumor ≤1 cm	2	3	9.600	2.098–43.929	0.023
Residual tumor >1 cm	11	8	6.737	1.593–28.484	
Recurrence in 12 months					
No	42	2			<0.001
Yes	1	11	20.167	5.151–78.947	
LINE-1 hypomethylation					
Methylation level >34.87%	26	1			0.001
Methylation level ≤34.87%	17	12	11.172	1.556–80.235	

Table 3. Multivariate Cox regression model of prognostic factors of survival in patients with epithelial ovarian cancer

	Risk ratio	95% CI	*P* value
Histological subtype			
Mucinous			
Endometrioid	0.131	0.018–0.979	0.040
Serous	0.190	0.028–1.278	
Clear cell	1.235	0.215–7.101	
Tumor stage			
I and II			0.038
III and IV	11.014	1.140–106.429	
LINE-1 hypomethylation			
Methylation level >34.87%			0.087
Methylation level ≤34.87%	6.997	0.755–64.841	

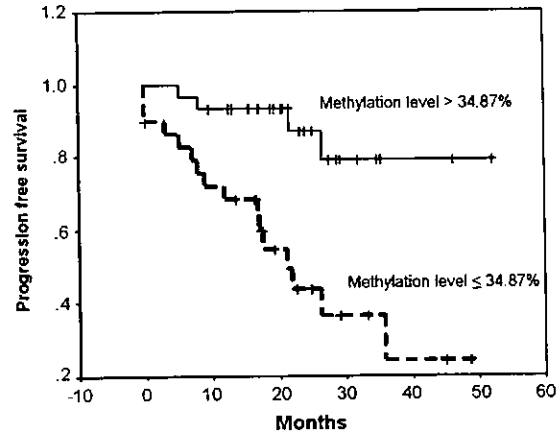

Figure 3. Progression-free survival of EOC patients with difference LINE-1 hypomethylation status. Spot and black line represented more LINE-1 methylation level ≤34.87% and >34.87%, respectively.

Discussion

An increasing number of studies have described the critical roles and clinical applications of epigenetic alterations in carcinogenesis, though most reports have focused on the function of DNA hypermethylation in silencing tumor suppressor genes[9,28,29]. Cancer cells, however, are frequently characterized by hypomethylation of the genome that largely affects the intergenic and intronic regions of DNA, particularly repeat sequences and transposable elements. This epigenetic process is believed to result in chromosomal instability, increased mutation events, and altered gene expression[12,30]. Moreover, LINE-1s are human retrotransposable interspersed repetitive sequences in which some have retained the promoter and transposable activities. Consequently, there are several possible specific biological roles of LINE-1 retrotransposons, and consequently, their methylation: (1) LINE-1 are linked to genomic instability; not only are some LINE-1 still retrotranposable[20], but also upregulation of LINE-1 creates DNA double-stranded breaks[31], (2) promoter hypomethylation of LINE-1 retrotransposable elements activate antisense transcription, as previously demonstrated in the *c-met* gene of chronic myeloid leukemia[32], (3) the reverse transcriptase of LINE-1 was demonstrated to possess a significant role in controlling cancer cell proliferation and differentiation[33], and (4) the insertion of expression-modulating LINE-1 sequences into host gene introns was proposed to fine-tune gene expression[34]. Consequently, LINE-1 methylation may control this activity, and the consequences of LINE-1 demethylation may be locus dependent. Therefore, the level of LINE-1 hypomethylation should be associated with the cellular functions and clinicopathologic statuses of malignancies[23].

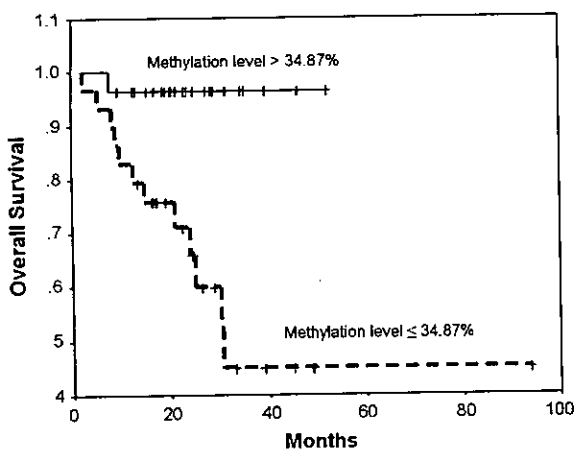

Figure 2. Overall survival of EOC patients with difference LINE-1 hypomethylation status. Spot and black line represented more LINE-1 methylation level ≤34.87% and >34.87%, respectively.

Less is known how global hypomethylation evolves in cancer. Vogelstein placed a genome-wide demethylation step in premalignant colon cancer lesions[8]. We found that histological subtypes and cancer progression determined global methylation levels. Mucinous possessed higher methylation levels than endometrioid. This may be the result of different multistep processes, similar to the differences in global methylation levels between microsatellite stable and instable colon cancers[35]. Higher levels of LINE-1 hypomethylation in the late stages of malignant progression than in early stages and a prominent lower global methylation levels in EOC than normal tissues.

Therefore, in EOC, LINE-1 hypomethylation is a progressive process developing during tumor progression, in addition to previous evidence of genomic and LINE-1 hypomethylation occurring as a common event in carcinogenesis[12,17,30].

In the current study, we applied a modified COBRA PCR protocol[10] to efficiently and quantitatively evaluate LINE-1 methylation status in the whole genome of isolated cell populations. This new protocol targets shorter amplicon sizes of the widely distributed LINE-1 sequences, which greatly improves the yield when amplifying genetic material derived from microdissected paraffin-embedded tissue. In this study, four histological subtypes of EOC and non-neoplastic ovarian tissues from patients who were treated in our department were analyzed for patterns of LINE-1 methylation. Using the PCR-based technique on LINE-1, the higher copies of sequences have allowed much more efficient and rapid analysis of larger tumor sets. The results of our study indicated that significant decrease in LINE-1 methylation level occurs during epithelial ovarian carcinogenesis. These results are also consistent with recent data that have demonstrated LINE-1 hypomethylation in other cancers[10] and global hypomethylation in ovarian cancer[17,36,37]. Moreover, this study is the first that demonstrated that global hypomethylation influences histology of EOC and survival of patients.

In conclusion, the significance of LINE-1 hypomethylation in EOC was demonstrated. Furthermore, there was an association between genome-wide hypomethylation and some clinicopathologic characteristics, including FIGO stage and tumor grade. LINE-1 hypomethylation also influences the prognosis of EOC. Therefore, LINE-1 hypomethylation is an important epigenetic process that is associated with ovarian carcinogenesis and tumor progression.

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Interpopulation linkage disequilibrium patterns of *GABRA2* and *GABRG1* genes at the GABA cluster locus on human chromosome 4

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Abstract

GABRA2 and *GABRG1*, which encode the α-2 and γ-1 subunits, respectively, of the $GABA_A$ receptor, are located in a cluster on chromosome 4p. The *GABRA2* locus has been found to be associated with alcohol dependence in several studies, but no functional variant that can account for this association has been identified. To understand the reported associations, we sought to understand the linkage disequilibrium (LD) patterns and haplotype structures of these genes. With close intergenic distance, ~90 kb, it was anticipated that some markers might show intergenic LD. Variation in 13-SNP haplotype block structure was observed in five different populations: European American, African American, Chinese (Han and Thai), Thai, and Hmong. In the Hmong, a 280-kb region of considerably higher LD spans the intergenic region, whereas in other populations, there were two or more LD blocks that cross this region. These findings may aid in understanding the genetic association of this locus with alcohol dependence in several populations.

Keywords: Linkage disequilibrium; Alcohol dependence; *GABRA2*; *GABRG1*; Haplotype

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the human brain. It is widely involved in the modulation of neurotransmission, particularly via regulation of neuronal excitability [1,2]. GABA exerts its effects through interaction with GABA receptors. Functionally, fast synaptic inhibition in the mammalian CNS is mediated largely by activation of the γ-aminobutyric acid type A ($GABA_A$) receptor [3], a ligand-gated receptor that is expressed in many regions of mammalian brain. The working receptor is composed of five protein subunits that form a chloride channel that remains closed until it binds its ligand. Each subunit is a long-chain polypeptide with four putative α-helical cylinder domains embedded within the cell membrane, with the N-terminal end being extracellular [4,5]. The 19 distinct $GABA_A$ receptor subunits have been classified into α, β, γ, δ, ∈, π, and ρ types [6]. Two α and two β subunits are typically part of the pentameric assemblies of $GABA_A$ receptors. The particular subunit composition varies widely among brain regions and species. GABA binds specifically to

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the recognition site in the $GABA_A$ receptor and forms a chloride ion-selective channel that mediates neuronal membrane potential [7–9]. Other pharmacologically important molecules, for example, barbiturates, benzodiazepines, ethanol, and the anesthetic steroids, also interact with these receptors, either directly or indirectly.

Among the 19 distinct subunits of mammalian $GABA_A$ receptors identified to date [6], the γ-aminobutyric acid α-2 receptor (encoded by *GABRA2*) (OMIM 137140) is the subtype that has received the greatest attention in relation to alcohol dependence risk. Based on its physiological role and its direct interactions with ethanol [10], as well as evidence from genetic linkage studies, it was suggested that the gene encoding the $GABA_A$ receptor α-2 subunit should be considered a candidate locus for influencing risk for alcohol dependence [11–13]. Results from a genome-wide linkage scan by the Collaborative Study on the Genetics of Alcoholism showed suggestive linkage to alcohol dependence in the region of the GABA receptor gene cluster on chromosome 4p13–p12. This region contains the *GABRG1, GABRA2, GABRA4,* and *GABRB1* genes, encoding γ-1, α-2, α-4, and β-1 subunits of the $GABA_A$ receptor, respectively. Association studies considering some of these loci indicated that a genetic predisposition to alcohol dependence is related to polymorphic variation at or near *GABRA2* [14–17]. Edenberg et al. [14] reported a significant association between SNPs in the *GABRA2* flanking genes and alcohol dependence (AD). They found that the region of strongest association with AD extended from intron 3 to 58 kb beyond the 3′ end of the gene, spanning 164 kb. This study included 31 SNPs within or closely flanking *GABRA2* that were significantly associated with alcohol dependence. They also found that all consecutive 3-SNP haplotypes within *GABRA2*—1 of the 5 haplotypes at the 5′ end of the gene and all 43 of the haplotypes starting within exon 3 and extending to the 3′ end of the gene—were significantly associated with AD. The association was confirmed by Covault et al. [15], who compared allele frequencies of 10 *GABRA2* SNPs spanning the coding region in European American (EA) controls and subjects with AD. The strongest evidence of association was shown across a region encompassed by 7 of the 10 SNPs, from rs279837 in intron 3 through rs567926 in the 3′ downstream region. This location provided the best evidence for association in all studies published to date. Subsequent studies have shown association of these *GABRA2* SNPs and alcohol dependence in populations of European and American ancestry; including Russians, Germans, Finns, and American Indians [16–19]. These replicated findings established the importance of *GABRA2* variation—or variation at loci mapping close enough to *GABRA2* to be in linkage disequilibrium (LD) with associated markers—in discovering genetic risks for AD.

GABRA2 spans approximately 140 kb. There are 10 transcribed exons present in human *GABRA2* mRNA (NM_000807), with four major isoforms, consisting of combinations of 2 alternative 5′ and 3′ exons caused by alternative splicing and (potentially) alternative promoter use [20]. Previous studies have employed sets of markers that extended only through the 3′ end of the *GABRA2* gene to obtain LD data; these data showed strong pairwise LD between the last two markers at the 3′ end of the haplotype block of each study, consistent with the possibility of an extended LD block in the 3′ direction [15–18]. Across the intergenic region in the 3′ direction, the gene encoding the γ-1 subunit (*GABRG1*) of the $GABA_A$ receptor is located. This gene spans approximately 83 kb and consists of 9 exons [21]. A consistent pattern of LD was previously constructed in EAs; this study [19] also showed an extended (193-kb) LD region, with D' 0.6–1.0. This region of LD spans from rs279867 in *GABRA2* intron 6 across the intergenic region to rs1391168, which is located in intron 1 of *GABRG1* [19].

We analyzed 13 SNPs, located either within these genes or in the intergenic region. We aimed to provide sufficient SNP density such that our SNP set would be informative for most other nongenotyped SNPs that map to the region (Fig. 1). To evaluate whether the effect of differing genetic background among populations with different ethnicities is reflected in distinct patterns of LD blocks, six populations were included: European American, African American, and Han Chinese American samples from the United States and Hmong, Thai, and Chinese Thai from Thailand.

In this study, we consider the LD relationships within and between *GABRA2* and *GABRG1,* to improve our understanding of the previously reported associations between *GABRA2* and AD. The extent of LD in the populations of African, European, and Chinese descent, represented by African American, European American, and Chinese American (USC), presented in this study is comparable to that observed in the YRI, CEU, and CHB populations in the International HapMap projects. These data are complementary; some of our samples of unrelated subjects are larger than those included in HapMap and although the samples are of comparable ethnicity they are not identical. Understanding LD patterns and the *GABRA2/GABRG1* haplotype structure by extending it to multiple populations also will facilitate inferring the evolutionary history of any variant that increases AD risk.

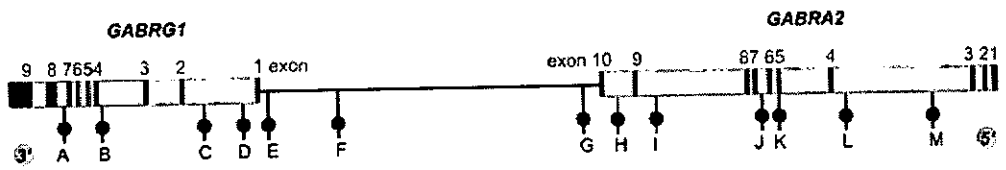

Fig. 1. Locations of SNP markers genotyped across *GABRG1* and *GABRA2* in the GABA cluster on chromosome 4p13–p12. The order of the genes is shown from telomere to centromere (distances not to scale).

Results

Allele frequency differences between populations

Allele frequencies in each population are presented in Table 1. The genotype distributions of all SNPs were consistent with Hardy–Weinberg equilibrium expectations, in all populations.

Analysis of LD and tagging SNPs

LD and haplotype block structure results are summarized in Fig. 2. Blocks were defined by LD analysis function and the confidence-interval method [22–24] selected within the parameter list. In the Hmong (HM) population, high LD ($D'>0.85$) extended from rs1497571 in intron 7 of *GABRG1* to rs279837 in intron 3 of *GABRA2*, a 280-kb segment, whereas in other populations, there were two or more LD blocks across this region. The LD block boundary observed in other populations corresponds to lower D' between rs10033451 and rs567926, which are located 89 kb apart in an intergenic region. Based on the SNP tagging function in Haploview, we determined that the largest number of haplotype-tagging SNPs (htSNPs) required was 7, in the African American (AA) population, whereas the smallest number required was 3, in the HM population (Fig. 2).

Haplotype frequencies

Haplotype frequencies (for those >0.05) are summarized in Table 2. The number of common haplotypes in populations varied between 4 (HM) and 8 (AA). Haplotype diversity also varied among populations. Four common haplotypes in the HM population accounted for 88% of the total information of all haplotypes. For the other populations, the set of population-specific common haplotypes represented 62–77% of the total information.

In most of the populations studied, there are two major haplotype blocks. Block I includes SNPs A–F and spans a region of 93 kb, and block II includes SNPs G–L and extends 98 kb. The LD break (between these blocks) observed in most populations corresponds to lower D' between SNPs F and G, which are located 89 kb apart in an intergenic region. The populations that showed different LD structure were the HM, in which there was a single block spanning both of these blocks (i.e., SNPs A through L), and the AA, in which we observed four main blocks. In the AAs, block I was divided into Ia (comprising SNPs A and B) and Ib (SNPs D–F), and block II was divided into blocks IIa (SNPs H and J) and IIb (SNPs K and L), as shown in Fig. 2. The EA and Thai (THT) populations also showed a small difference in block definition in which smaller blocks were defined rather than the two main haplotype blocks. Three main haplotype blocks were observed in these populations. The definition of blocks in EAs is as seen in AAs, in which LD breaks between SNPs B and C create subblocks Ia and Ib, whereas block I in THT is divided into block Ia and Ib by LD breaks between SNPs C and D (Figs. 2a and 2e).

Since it was previously reported that in EAs the LD block spanning from SNP G to SNP L contains haplotypes that are associated with alcohol dependence [15], the two major haplotype blocks (block Ib and block II) examined in this population across *GABRA2* and *GABRG1* in this study were used as a standard to compare haplotype frequencies among populations. Three haplotype blocks were reconstructed in the EA population. We analyzed frequencies of the four-SNP haplotypes (block Ib) and the six-SNP haplotype (block II) compared to other populations. Block I, with SNPs C–F, covers part of the 5′ region, a 46-kb segment of the *GABRG1* gene, and block II, comprising SNPs G–L, spans the 5′ flanking region through exon 3 of the *GABRA2* gene. Block II corresponds to that identified in a previous study [15] starting at rs567926 (SNP G) and extending to rs279837 (SNP L), covering a 97-kb region.

Two major block I haplotypes, T-A-C-T and A-G-T-C, were observed in all populations except AA (Table 3). These two common haplotypes accounted for 78 (Chinese Thai; THC), 97

Table 1
Location and prevalence of allele frequencies of *GABRG1* and *GABRA2* polymorphisms

Marker information					Populations					
	Marker	Position	Gene	NT	THC (N=38)	HM (N=48)	THT (N=59)	AA (N=48)	USC (N=100)	EA (N=160)
A	rs1497571	45,900,178	GABRG1 intron 7	[C/T]	0.66/0.34	0.73/0.27	0.62/0.38	0.38/0.62	0.57/0.43	0.56/0.44
B	rs2350438	45,912,197	GABRG1 intron 3	[C/T]	0.66/0.34	0.72/0.28	0.62/0.38	0.37/0.63	0.57/0.43	0.56/0.44
C	rs1391166	45,946,795	GABRG1 intron 1	[A/T]	0.39/0.61	0.28/0.72	0.35/0.65	0.77/0.23	0.45/0.55	0.50/0.50
D	rs10938426	45,959,163	GABRG1 intron 1	[A/G]	0.66/0.34	0.72/0.28	0.59/0.41	0.30/0.70	0.56/0.44	0.51/0.49
E	rs7654165	45,967,995	Intergenic	[C/T]	0.62/0.38	0.73/0.27	0.64/0.36	0.30/0.70	0.55/0.45	0.53/0.47
F	rs10033451	45,993,260	Intergenic	[C/T]	0.28/0.72	0.24/0.76	0.34/0.66	0.29/0.71	0.38/0.62	0.44/0.56
G	rs567926	46,082,697	Intergenic	[A/G]	0.46/0.54	0.43/0.57	0.49/0.51	0.81/0.19	0.54/0.46	0.61/0.39
H	rs534459	46,097,733	GABRA2 intron 9	[A/G]	0.50/0.50	0.55/0.45	0.48/0.52	0.71/0.29	0.46/0.54	0.39/0.61
I	rs529826	46,112,580	GABRA2 intron 8	[C/T]	0.48/0.52	0.55/0.45	0.47/0.53	0.70/0.30	0.42/0.58	0.39/0.61
J	rs279869	46,148,923	GABRA2 intron 6	[G/T]	0.50/0.50	0.43/0.57	0.48/0.52	0.28/0.72	0.50/0.50	0.60/0.40
K	rs279858	46,155,521	GABRA2 exon $5_{(synonymous)}$	[C/T]	0.48/0.52	0.57/0.43	0.52/0.48	0.19/0.81	0.53/0.47	0.39/0.61
L	rs279837	46,180,251	GABRA2 intron 3	[A/G]	0.48/0.52	0.36/0.64	0.45/0.55	0.78/0.22	0.44/0.56	0.61/0.39
M	rs9291283	46,212,761	GABRA2 intron 3	[A/G]	0.13/0.87	0.18/0.82	0.17/0.83	0.33/0.67	0.04/0.96	0.26/0.74

Position is given according to the numbering in GenBank database entry NT_006238 (*Homo sapiens* chromosome 4 genomic contig, reference assembly). NT represents nucleotide change from allele 1, GenBank NT_006238 strand, to allele 2. Both are presented in brackets [allele 1/allele 2]. Abbreviations: THC, Chinese Thai; HM, Hmong; THT, Thai; AA, African American; USC, Chinese American; EA, European American.

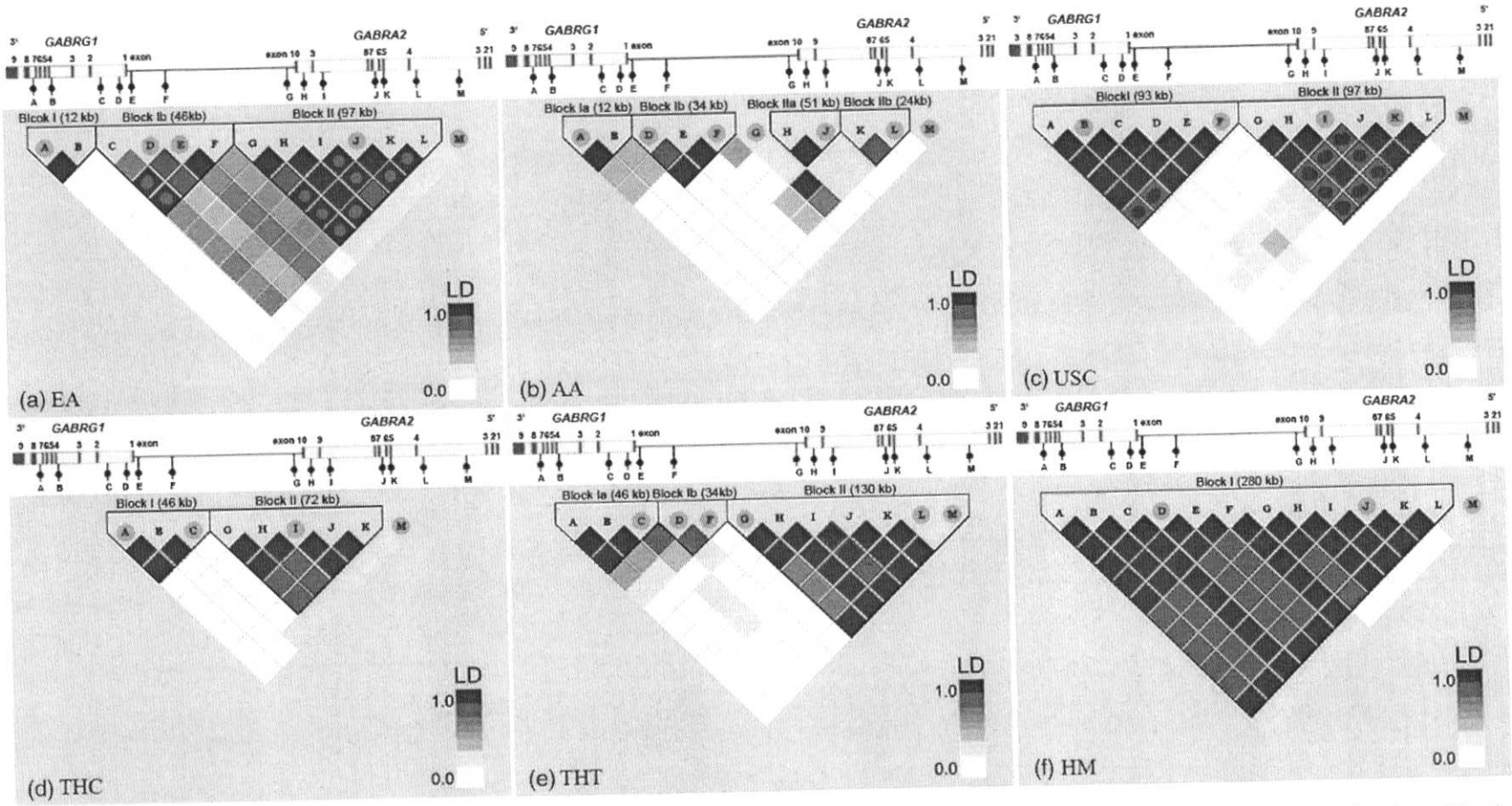

Fig. 2. Comparisons of LD structure and htSNPs over the *GABRG1/GABRA2* region on chromosome 4p13–p12 across five different ancestral populations. Blue marks represent htSNPs shown in each population. Some markers with >25% missing genotypes are excluded as they are not informative for haplotype block definition. (a) EA, European American; (b) AA, African American; (c) USC, Chinese American; (d) THC, Chinese Thai; (e) THT, Thai; (f) HM, Hmong.

Table 2
Distribution of common haplotypes among populations: 13 SNPs (A-M)

Haplotype: SNPs A-M	THC	HM	THT	AA	USC	EA
C-C-T-A-C-T-A-G-T-G-T-A-G	0.27	0.35	0.25	0.09	0.3	0.2
T-T-T-A-C-T-A-G-T-G-T-A-G						0.16
C-C-A-G-T-C-A-G-T-G-T-A-G						0.08
C-C-A-G-T-C-G-A-C-T-C-G-A						0.08
T-T-A-G-T-C-G-A-C-T-C-G-G	0.07	0.24	0.12		0.21	0.08
C-C-A-G-T-C-G-A-C-T-C-G-G						0.07
T-T-A-G-T-C-G-A-C-T-C-G-A			0.05	0.06		0.05
C-C-T-A-C-T-A-A-C-T-T-A-A				0.06		
C-C-T-A-C-T-A-A-C-T-T-A-G				0.05		
C-C-T-A-C-T-A-G-T-G-T-G-G					0.05	
C-C-T-A-C-T-G-A-C-T-C-G-A		0.17	0.07			
C-C-T-A-C-T-G-A-C-T-C-G-G	0.14	0.12	0.11		0.14	
C-C-T-A-C-T-G-G-T-G-T-A-G	0.06					
T-T-A-G-T-C-A-G-T-G-T-A-G	0.06		0.06	0.05	0.07	
T-T-A-G-T-T-A-A-C-T-T-A-G				0.15		
T-T-A-G-T-T-A-G-T-G-T-A-G				0.06		
T-T-A-G-T-T-G-A-C-T-C-G-G	0.08					
Total	0.68	0.88	0.66	0.52	0.77	0.72

(HM), 79 (THT), 93 (USC), and 86% (EA) of those observed. In AA, however, the most common haplotypes were T-A-C-T and A-G-T-T (Table 3). For block II (six-locus LD analysis for SNPs G–L), haplotypes A-G-T-G-T-A and G-A-C-T-C-G represented the common haplotype in other populations with 83–96% of chromosomes; there was only 45% (26 and 19% for A-G-T-G-T-A and G-A-C-T-C-G, respectively) in African Americans (Table 4). The haplotype A-A-C-T-T-A was uniquely found in African Americans, with 48% of total chromosomes.

Discussion

With methodological developments in high-throughput genotyping, and the development of large-scale genotyping projects such as the international HapMap project, huge quantities of data are being generated, making it possible to measure correlations between SNP genotypes and create better marker maps for association studies. Improved understanding of underlying LD-based mapping approaches has been helpful in elucidating the relationship between common genetic variation and heritable risk for common diseases [25–27]. Polygenic and oligogenic effects are considered to play important roles in influencing complex traits and behaviors. Further, it is likely that the genetic architecture of common, complex traits will differ, at least to some extent, among major

Table 3
Haplotype frequencies reconstructed by comparing block Ib (SNP C-D-E-F) in European Americans to other populations

Haplotype: SNPs C-F	THC	HM	THT	AA	USC	EA
T-A-C-T	0.56	0.73	0.53	0.26	0.55	0.46
A-G-T-C	0.22	0.24	0.26	0.29	0.38	0.40
T-G-C-T						0.05
A-G-T-T	0.16	0.03	0.07	0.40	0.07	
T-G-T-C			0.06			
Total	0.94	1.00	0.92	0.95	1.00	0.91

Table 4
Haplotype frequencies reconstructed by comparing block II (SNPs G-L) in European Americans to other populations

Haplotype: SNPs G-L	THC	HM	THT	AA	USC	EA
A-G-T-G-T-A	0.40	0.35	0.40	0.26	0.40	0.62
G-A-C-T-C-G	0.43	0.55	0.44	0.19	0.44	0.34
A-G-T-G-T-G	0.02	0.05	0.01	0.02	0.06	0.01
G-G-T-G-T-A	0.08	0.01	0.03		0.01	
A-G-T-T-C-G	0.02	0.02	0.06		0.07	
A-A-C-T-T-A				0.48		
Total	0.95	0.99	0.95	0.95	0.98	0.97

population groups. To understand the genetic basis of these traits, we need to understand linkage disequilibrium profiles and haplotype diversity in genomic regions of interest in multiple populations.

In the present study, we focused on two adjacent $GABA_A$ receptor subunit genes, one of which has been associated with risk for alcohol dependence in several previous studies. To define extensively linkage disequilibrium in the chromosomal region, more SNPs covering the *GABRG1* gene located telomeric to *GABRA2* on chromosome 4p were studied. This made it possible to investigate whether these two genes are located within the same LD block and, more broadly, to ascertain the possibility that the effect observed at *GABRA2* could actually be mediated through a variant mapped to *GABRG1*. We conclude that this is in fact the case, and associations observed with *GABRA2* might be attributable to functional genetic variation at the *GABRG1* locus, or there may be disease-related variants at both loci; this may facilitate our understanding of reported associations between *GABRA2* polymorphisms and AD and permit a more informed search for the functional variant or variants underlying this association—a search that must now extend into the intergenic region and the *GABRG1* locus. It is not known how well genetic information from the four HapMap populations (CEU, YRI, JPN, and CHB) represents that of other populations around the world; it is thus useful to answer this question directly, especially for loci of great interest.

Allele frequencies for the 13 SNPs of *GABRA2* and *GABRG1* that were genotyped in this study in six different populations from three continents provide a measure of allele distribution and different LD patterns among populations. These results should allow investigators studying populations similar to those characterized herein, or admixed population derivatives, to explore associations between these genes and substance dependence and other phenotypes.

This study provides the first comprehensive analysis of patterns of LD spanning these two important genes. In general, LD block size might vary between different populations in which differences in the degree of admixture and ancestry are found. Thus, from generation to generation, differences in LD between markers from the initial populations due to LD decay can be observed by changes in LD block and haplotype structure and long stretches of LD that can reflect haplotype diversity [28,29]. That is clearly the case for these loci in the populations studied. We have systematically analyzed SNPs

covering the range of *GABRA2* and *GABRG1* in the GABA receptor cluster on human chromosome 4p, estimating haplotypes in a total of 453 unrelated healthy individuals. The more complex LD structure found in the African American population is consistent with what is known about the population's history, i.e., that it is older than the Asian and European populations, which show less complex LD patterns. This is consistent with the observation of Gabriel et al. [22] that haplotype blocks found in African populations showed a set of shorter genomic LD blocks than samples from Europe and Asia. While haplotype blocks defined in African Americans indicated the presence of four main LD regions spanning all 13 of the SNPs examined, we found that Chinese populations both in Thailand and in the United States and the Thai population were characterized by a two-block structure in this genomic region. In contrast, the Hmong population, which is an isolated minority hill tribe dwelling in the northern part of Thailand, was found to have a unique long-ranged haplotype block structure with a single block encompassing the two blocks observed in the others. This interesting finding in the Hmong population may be attributed to the occurrence of recent selection that can be reflected in long-range haplotypes [30]. Alternatively, the Hmong population may be relatively new or have undergone a recent bottleneck [31]. A small number of htSNPs are generally sufficient to capture most of the haplotype structure in high LD regions [29,32]; consistent with this expectation, only three htSNPs were required to capture the haplotype block diversity in the Hmong population, whereas four htSNPs were necessary for THC, five for EA and USC, and six for THT. In the AA population, there were four haplotype blocks in the same region; 7 SNPs were sufficient to capture most of the genetic information of the total 13 SNPs. As can be seen in Fig. 2, rs9291283 (SNP M) showed very low LD with the other SNPs examined, falling outside the limits of the haplotype block. Thus, it was tagged as a htSNP for all groups.

LD analysis based on HapMap data with a denser SNP map (Fig. 3) shows that LD definitions in our sample are consistent with those based on well-characterized HapMap populations. The LD regions observed in this study, European American, African American, and Chinese American, are similar to those observed in CEU, YRI, and CHB, respectively, from the HapMap project (Fig. 3), whereas the LD pattern in our Thai populations, compared to the HapMap CHB population, is slightly different in THT and THC and very different in the Hmong population. This suggests a degree of genetic differentiation among these populations. The Thai population may have unique genetic characteristics, especially the minority Hmong population (recruited in Thailand but present in other Asian countries, including China and Laos, as well).

Because each population has its own evolutionary history in which distinct allele frequencies, LD patterns, and haplotype structures can develop, a number of studies show that htSNPs must be defined within specific populations to identify optimal sets of markers for association studies [33–35]. Previously, *GABRA2* haplotype structure was reported only in EA, AA, and Russian populations [15,16,19]; the present study documents LD patterns and haplotypes in three different Asian populations. As shown in European Americans by Covault et al. [15], seven markers spanning the *GABRA2* gene defined an LD block associated with AD. Our study, which used six of those seven markers (we omitted rs279844), also shows a haplotype block defined by SNPs G (rs567926) to L (rs279837) that confirms the same region of strong LD in European Americans.

Haplotype frequencies in both LD blocks found in African Americans were uniquely different from those observed in other populations. In the Hmong population, we observed a small number of 13-SNP haplotype patterns indicative of low diversity, commonly found in an isolated population.

In the analysis of the four-SNP (C–F) and six-SNP (G–L) haplotypes, the two haplotype blocks observed in EAs differed from those in AAs. Specifically, the six-SNP haplotype defined by SNPs G–L identified two complementary common haplotypes, A-G-T-G-T-A and G-A-C-T-C-G, as reported by Covault et al. [15], which together accounted for 92.8% of chromosomes (in the control sample), that represented 96% of chromosomes in the European American, 90% in the Hmong, 84% in the THT and USC, 83% in the THC, and, interestingly, only 45% in the African American populations. The African American population showed a specific haplotype, A-A-C-T-T-A, which was not observed in the other populations. In the case of both the four-SNP and the six-SNP haplotypes, only one recombination event is required to explain the presence of the third unique but common haplotype in the AA sample. The lack of these two common haplotypes in non-African populations may be explained by one of two most likely scenarios: (1) The recombination events took place in Africa after humans migrated out of Africa and subsequently rose to high frequency either from genetic drift or positive selection. (2) The recombination events predate migrations out of Africa, but due to a bottleneck effect were not represented in founding populations in either Europe or Asia or were present at such low frequencies that they were lost through genetic drift.

Only small differences were observed between haplotype frequencies in the European American and Asian populations. Further, no significant differences were found in haplotype frequencies between any two Asian groups except in comparisons involving the Hmong.

In conclusion, this study presents patterns of specific htSNPs and LD block structure in six different populations: European American, African American, Chinese American, and three from Asia (i.e., Thai, Chinese Thai, and Hmong). LD extended from most of the *GABRA2* gene through the *GABRG1* locus in the same $GABA_A$ cluster region on chromosome 4p, suggesting the possibility of association (and interaction) of both of these two genes with alcohol dependence. Differences in genetic architecture observed in these populations may help to define the physical and genetics regions of *GABRG1* and *GABRA2* that contain an as yet unidentified alcohol dependence-related functional change.

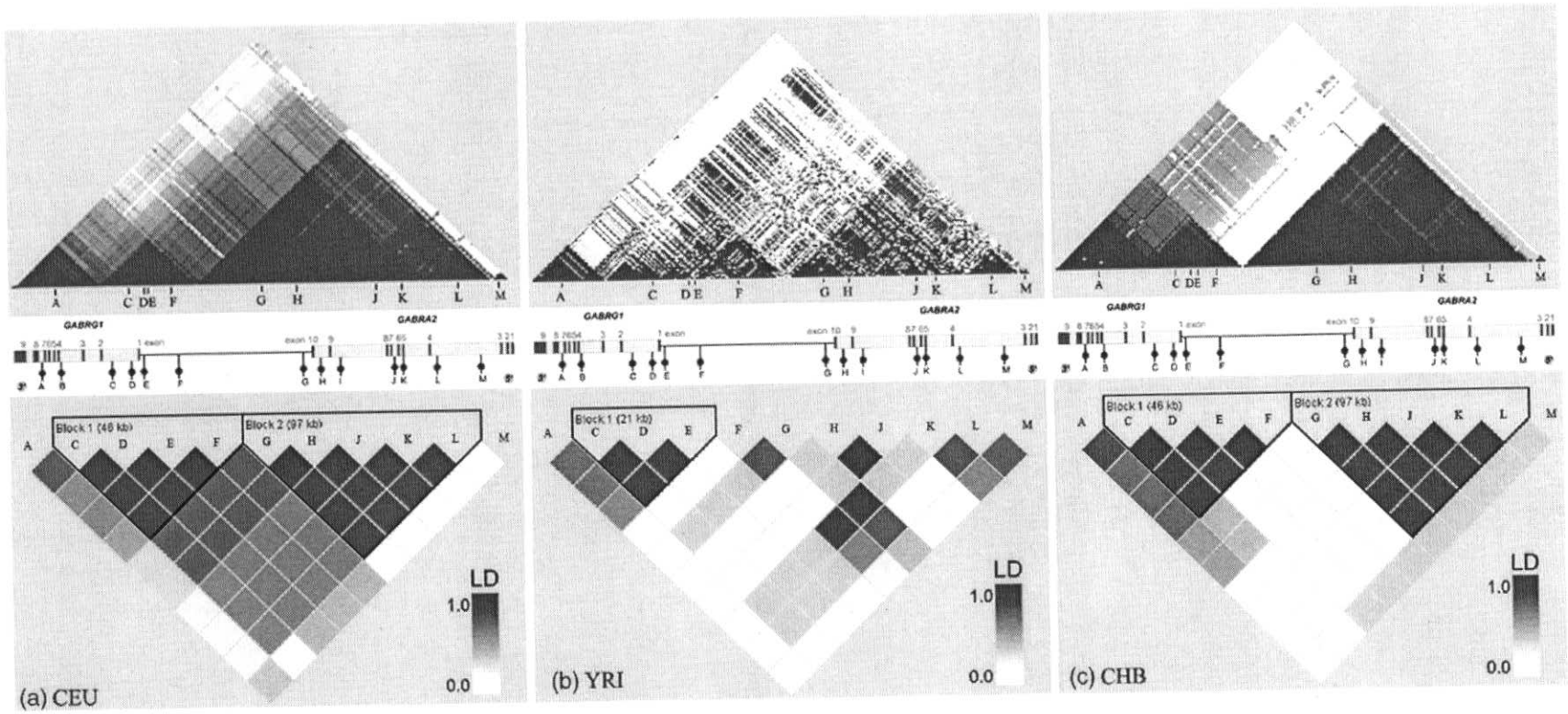

Fig. 3. LD patterns computed based on genotype data from the HapMap CEU, YRI, and CHB populations for 11 SNPs used in our study. Note that the 2 SNPs unavailable in HapMap, rs2350438 and rs529826, are not included.

Materials and methods

Populations

A total of 450 unrelated subjects were included in this study, from Thailand and the United States. The study sample of Thai populations was recruited as adult blood donors ages 18 years and older from the Thai Red Cross. Only individuals who reported that all four grandparents were of either Thai or Chinese Thai ethnicity were included, resulting in panels of 56 THTs and 38 THCs. Forty-eight subjects of HM descent were recruited from a hill tribe population near Chiang Mai, Thailand. Related subjects were identified and excluded based on an ML-Relate analysis [36], as described elsewhere [31]. Samples from the United States were from three different sources. USC DNA samples were obtained from a Han Chinese sample panel (Catalog No. HD100CHI) available from Coriell Cell Repositories (Camden, NJ, USA). This sample comprises 50 males and 50 females, all of whom reported that all four of their grandparents were born in Taiwan, China, or Hong Kong. The 160 EA and 48 AA subjects were recruited at the University of Connecticut Health Center or at the VA Connecticut Healthcare System, West Haven Campus. All subjects enrolled in the study provided informed consent as approved by the institutional review board at the appropriate institution.

Selection of SNP markers

We selected 13 SNPs with minor allele frequency >0.15, based on their use in previous studies and/or map position. These SNPs span the 312.6-kb region including *GABRA2* and *GABRG1* and were genotyped in the six populations described above. Six SNPs that map to *GABRA2* (rs567926, rs534459, rs529826, rs279869, rs279858, and rs279837) are a subset of the 10 SNPs reported previously by Covault et al. [15] and are designated here as SNPs G to L, respectively. The other 7 SNPs include 3 intergenic SNPs and 4 that map to *GABRG1*, selected from the NCBI database based on LD and intermarker distance. No SNPs resulting in amino acid change are known to map in this region; only 1 known nonsynonymous SNP (rs279858) in the *GABRA2* coding region was genotyped (Table 1). All of these SNPs were polymorphic in all populations.

Genotyping

The TaqMan method, a fluorogenic assay based on 5′-nuclease activity [37], was employed for genotyping. All 13 SNPs were identified in all subjects by using specific assays synthesized and designed by Applied Biosystems (Foster City, CA, USA). All of these TaqMan probe primers are available as ABI "Assays-on-Demand." Each PCR was performed with a reaction volume of 2 μl including 1× concentration of TaqMan 2× Universal PCR Master Mix (Applied Biosystems), 1× concentration of 20× assay mix, distilled water, 1× concentration of 100× BSA, and 2 ng DNA. PCR amplification was accomplished using an ABI 9700 thermocycler at 95 °C for 10 min, followed by 15 s at 92 °C, and then 60 s at 60 °C for 40 cycles, before detection in the ABI Prism 7900HT sequence detection system and analysis using software available from Applied Biosystems.

Statistical analysis

Allele frequencies for each SNP marker were calculated using Powermarker [38]. The Hardy–Weinberg equilibrium exact test was applied. The extent of LD was estimated using the confidence-interval method in Haploview software version 3.32 (available at http://www.broad.mit.edu/mpg/haploview) [39], based upon pairwise $|D'|$ calculation between markers. LD measures and haplotype block structure were obtained using the program Haploview. Minimum percentage of genotype was set to exclude markers in which fewer than 75% of individuals were genotyped. Most markers exceeded this ratio, except rs1391166, rs10938426, and rs7654165 in the THC population and rs1391166 and rs529826 in the AA population. Genotype data for SNPs in the same region ranging from chromosome 4 nucleotide 45,874,178 to nt 46,226,602, which spans these two genes and covers all of the 13 SNPs, were downloaded from the HapMap project Web site (http://www.hapmap.org) and were analyzed by Haploview to compare LD block structures between our populations and the three HapMap populations of comparable ethnicities (EA comparable to CEU, AA comparable to YRI, and Asian populations USC, Thais (THT, THC), and HM comparable to CHB from HapMap). Comparisons were made in two scales. The 11 SNPs presented in our study that were also genotyped in the HapMap population were selected for Haploview analysis, but there were no available genotypes for SNPs B and I (rs2350438 and rs529826, respectively) in the HapMap dataset. LD patterns, including all informative SNPs (with minor allele frequency >0.15), were selected from HapMap for analysis. We also analyzed subsets of our dataset defined as htSNPs selected from LD blocks using the Tagger function within Haploview. Analysis was performed using both "pairwise" and "aggressive tagger" Haploview functions. This function is an extension algorithm developed by Carlson et al. [23] and based on de Bakker's tagger [24]. To examine haplotype frequencies of the htSNPs, Phase 2.0.2 software [40], based on Bayesian statistical methods, was employed to reconstruct and estimate haplotype frequencies.

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ORAL ONCOLOGY

Promoter hypermethylation of *CCNA1*, *RARRES1*, and *HRASLS3* in nasopharyngeal carcinoma

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Promoter hypermethylation;
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RARRES1;*HRASLS3*

Summary In search for putative tumor suppressor genes critical of nasopharyngeal carcinoma (NPC), we analyzed the available information from the expression profiling in conjunction with the comprehensive alleotyping published data relevant to this malignancy. Integration of this information suggested eight potential candidate tumor suppressor genes, *CCNA1*, *HRASLS3*, *RARRES1*, *CLMN*, *EML1*, *TSC22*, *LOH11CR2A* and *MCC*. However, to confirm the above observations, we chose to investigate if promoter hypermethylation of these candidate genes would be one of the mechanisms responsible for the de-regulation of gene expression in NPC in addition to the loss of genetic materials. In this study, we detected consistent hypermethylation of the 5′ element of *CCNA1*, *RARRES1*, and *HRASLS* in NPC tissues with prevalence of 48%, 51%, and 17%, respectively. Moreover, we found a similar profile of promoter hypermethylation in primary cultured NPC cells but none in normal nasopharyngeal epithelium or leukocytes, which further substantiate our hypothesis. Our data indicate that *CCNA1*, *RARRES1*, and *HRASLS3* may be the putative tumor suppressor genes in NPC.

Abbreviations: *CCNA1*; cyclin A1; *RARRES1*, retinoic acid receptor responsive element; *HRASLS3*, h-RAS like suppressor; NPC, nasopharyngeal carcinoma.

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Introduction

The development of nasopharyngeal carcinoma (NPC) appears to be associated with the *Epstein-Barr* virus (*EBV*) infection together with multiple genetic and epigenetic alterations of the nasopharyngeal epithelium.[1–3] Regarding the latter, many studies have now demonstrated that the epigenetic changes may underlie the molecular basis of these lesions. For example, the extensive and high resolution alleotyping studies revealed potential tumor suppressor gene loci in NPC on chromosomes 3p, 9p, 9q, 11q, 12q, 13q, 14q, and 16q.[1,4] In addition, the recent studies have identified that certain genes residing within these chromosomal loci are aberrantly hypermethylated, such as RASSF1A (3p21.3), RARβ2 (3p24), and p16INK4A (9p21).[5–7] However, for most of the loss of heterozygosity (LOH) loci, the presence of candidate tumor suppressor genes (TSG) has not been thoroughly investigated.

Previously, we have analyzed the mRNA expression profile of a small sub-set of NPCs by using laser capture microdissection coupled with high density cDNA microarrays and identified several aberrantly expressed genes which may play a role in NPC pathogenesis.[8] Here, we have applied the integrated information acquired from both our mRNA expression profile together with the genome-wide alleotyping data, to further define whether any of these molecules might represent potential TSG candidates. Genes whose expressions was down-regulated, as detected by the microarray experiments, were matched to the critical regions for LOH of either chromosomes 13 or 14, as there are some evidences suggesting that these may be the locations of TSG.[1,4,9,10] In addition to the above criteria, we further investigated if the hypermethylation of 5′ regulatory element of these genes would be an additional mechanism of the gene silencing in NPC. Our data indicate that in NPC but not in normal samples, there are the promoter hypermethylations of certain critical genes (*CCNA1*, *HRASLS3*, and *RARRES1*), which were shown to be down regulated in our previous microarray study, suggesting that these genes may potentially function as TSG in NPC.

Materials and methods

Tumor samples and DNA extraction

Primary NPC tumor samples were surgically obtained from patients with appropriate informed consent at The King Chulalongkorn Memorial Hospital. Samples were then divided into two portions. The first portion was fixed in formalin and was submitted for routine histopathological examination, and the second portion was immediately snap-frozen and stored in liquid nitrogen until use in further experiments. Control nasopharyngeal epithelium was obtained from nasopharyngeal swabs of unrelated patients in the Department of Otolaryngology who had clinically defined normal nasopharyngeal mucosa. Control blood samples were collected from unrelated healthy donors. All samples were tested to confirm the presence of the genetic material of EBV following the method previously described.[11]

Extraction of genomic DNA was performed with proteinase K (Amersham, Aylesbury, UK) digestion in the presence of SDS at 50 °C overnight, followed by phenol/chloroform extraction and ethanol precipitation.[11]

Cultured NPC cells

Primary NPC tissue was finely chopped, suspended in 0.25% collagenase (Life Technologies, Gaithersberg, MD), and incubated at 37 °C, 5% CO_2 for 4–5 h. After centrifugation, the collagenase was discarded and the pellet subjected to two washing steps with 5 ml DMEM (Dulbecco's Modified Eagle Medium; Life Technologies, Gaithersberg, MD) each. Subsequently, the pellet was resuspended in serum and growth factor free DMEM with 1% penicillin/streptomycin added, and incubated at 37 °C, 5% CO_2 until the cell layer had spread sufficiently to be further sub-cultivated. Pellets of NPC cells with limited passages were collected for DNA extraction.

Presence of NPC cells was confirmed by the detection of LOH of chromosome 3, 9, and 14, using microsatellite analyses for the following markers; D3S1038, D14S283 and TCRD, in order to confirm the complete loss of heterozygosity as well as showing the absence of contaminating normal tissue. Briefly, one strand of each primer pair was end-labeled at 37 °C for 1–2 h in a total volume of 10 μl containing 10 μM primer, 0.025 mCi [^{32}P] γATP (Amersham, Aylesbury, UK) at 3000 Ci/mmol, 10 mM MgCl, 5 mM DTT, 70 mM Tris–HCl (pH 7.6) and 10 units of T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The kinase reaction was then added to the PCR buffer mix. The PCR reactions were performed in a total volume of 10 μl using 50 ng of genomic DNA in 200 μM dNTP each, 10 mM Tris–HCL (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 units of *Thermus aquaticus* DNA polymerase (Perkin–Elmer Cetus, Norwalk, CT) and primer concentrations were 0.05–0.5 μM each. The PCR reactions were optimized as follows: an initial de-naturation step at 95 °C for 4 min, 25 cycles of de-naturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min, and a final extension at 72 °C for 7 min. The PCR products were analyzed with electrophoreses in 6% polyacrylamide and 7 M urea gel.

Sodium bisulfite modification

The DNA samples were subjected to bisulfite treatment.[5–7] Briefly, 2 μg of genomic DNA were denatured in 0.2 M sodium hydroxide. Subsequently, 10 mM hydroquinone (Sigma–Aldrich, St. Louis, MO) and 3 M sodium bisulfite (Sigma–Aldrich, St. Louis, MO) were added and incubated at 50 °C for 16–20 h. The modified DNA was then purified using Wizard DNA purification resin (Promega, Madison, WI) followed by ethanol precipitation.

Duplex MSP and COBRA

We used the duplex methylation specific PCR (MSP) to determine the methylation status of *CCNA1*, *TSC22*, *CLMN*, and *EML1* and performed the combined bisulfited restriction analysis (COBRA) to analyze the promoter hypermethylation of *HRASLS3*, *RARRES1*, *LOH11CR2A*, and *MCC*. The PCR reactions contained 1× PCR buffer (Qiagen, Chuo-ku, Japan), 0.2 mM deoxynucleotide triphosphates, 0.4 μM of appropri-

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ate PCR primers (Table 2), 1u of hotstarTaq (Qiagen, Chuo-ku, Japan), and 80 ng of bisulfited DNA. Cycles of PCR amplification were as follows: an initial de-naturation step at 95 °C for 10 min, followed by 30 cycles of de-naturation at 95 °C for 1 min, annealing at 48–55 °C for 1 min (Table 2), extension at 72 °C for 2 min, and the final extension at 72 °C for 5 min. In the COBRA analysis, all PCR products were digested with *TaqI* enzyme and subjected to electrophoresis in 8% native polyacrylamide gel. Methylation of the TCGA sequence in gel was visualized using phosphoimager after staining with Syber Green.

Bisulfite genome sequence analysis

We performed additional analysis of the bisulfited DNA by subcloning the fragments into pGEM – t Easy Vector System I (Promega, Madison, WI), which was then subjected to sequence analysis using the ABI prism 3100 Genetic Analyzer (Applied Biosystems, CA) for the presence of methylated nucleotides in all the samples.

Statistical analysis

Fisher's exact and Chi-square tests were used to determine the association between clinical parameters and the presence of promoter hypermethylation of the studied genes.

Results

Selection of tumor suppressor gene candidates from the genome-wide expression analysis of NPC

From our previous microarray analysis, we re-evaluated the data in order to search for potential candidate TSG. We first retrieved a list of genes whose expression was shown to be differentially down-regulated in NPC when compared to the normal nasopharyngeal epithelium. We next applied two additional selective criteria including the genes that have previously been shown to be tumor suppressor genes in other cancer types as well as the genes residing within the critical regions of LOH in chromosomes 13 and 14, which are also thought to be the important areas in NPC.[1,4,9,10] From the 238 down-regulated genes in NPC,[8] we found four genes that have been indicated to be putative tumor suppressor genes in other tumor types: *HRASLS3*,[8,12] *RARRES1*,[8,13–16] *LOH11CR2A*,[8,17] and *MCC*.[8] Furthermore, we selected *CCNA1* and *TSC22* whose genetic locations are within the critical LOH region of chromosome 13 at position 35,948–35,949 kb and 44,010–44,020 kb, respectively. Similarly, we selected *EML1* and *CLMN*, whose expression was differentially down-regulated, residing within the critical region of LOH in chromosome 14 at position 98,320–98,330 kb and 93,710–93,720 kb. Under the above selection algorithm, we were able to identify eight putative genes with potential tumor suppressor activity (Table 1). Fold difference of geometric means (T/N) were calculated by using fractionate with geometric mean of ratios in class 1:N and geometric means of ratios in class 2:T as described previously.[8] The ratios of less than 1 indicate a relative down-regulation of gene expression in NPC lesions.

Table 1 Ratios of eight selected candidate genes from microarray data and their chromosomal region

Gene	Chromosomal location	Fold difference of geometric means (T/N)
CCNA1	13q12.3–q13	0.404
TSC22	13q14	0.355
EML1	14q32	0.308
CLMN	14q32.2	0.237
HRASLS3	11q12.3	0.261
RARRES1	3q25.32	0.124
LOH11CR2A	11q23	0.448
MCC	5q21–q22	0.204

Analysis of the methylation status of promoter regions of candidate genes

We further investigated the potential mechanism which might be responsible for the down-regulation of all eight candidate genes. The 5′ elements of all eight genes were analyzed for methylation status by either MSP or COBRA technique (Table 2) in several NPC tissues and control normal peripheral blood leukocytes. All NPC were tested positive for *EBV*, whereas the control tissues were absence of *EBV* genetic materials. The presence of promoter hypermethylation in NPC was detected on *CCNA1*, *RARRES1* and *HRASLS3* promoter but not in the control leukocytes (Figs. 2 and 3, and Table 3). However, there was no hypermethylation detected on *MCC*, *TSC22*, *EML1* and *CLMN* upstream regulatory regions. Additional experiments demonstrated a partial methylation of *LOH11CR2A* promoter which could be observed in both NPC and control leukocytes (Figs. 2 and 3 and Table 3). The above findings in conjunction with the under-expression of these genes found by the microarray experiments suggest that the promoter hypermethylation of *CCNA1*, *RARRES1* and *HRASLS3* may be crucial in regulating expression of these genes especially in NPC.

Promoter hypermethylation of *CCNA1*, *RARRES1* and *HRASLS3*

To further validate the crucial role of the promoter hypermethylation of these TSG candidates in NPC carcinogenesis, we determined the frequency of methylation of *RARRES 1*, *HRASLS 3* and *CCNA1* in approximately 40 NPC tissue samples either by COBRA or MSP PCR. An additional 10 primary cultures of relatively pure population of NPCs with no normal cell contamination as indicated by our LOH study (Fig. 1), were also analyzed for the methylation status of these genes. We used several controls in this study including 20 normal nasopharyngeal swabs and 30 peripheral blood leukocytes from healthy subjects or non-cancer patients. Strikingly, in all normal cells including nasopharyngeal epithelium and WBC, we could not demonstrate the presence of promoter hypermethylation of all the studied genes (Table 4). In contrast, NPC tissues displayed frequent promoter hypermethylation of for *RARRES1*, *HRASLS3*, and *CCNA1* at 51%, 17%, and 48%, respectively (Table 4). Moreover, we confirmed the frequent promoter hypermethylation in isolated NPC cells to avoid normal cells contamination and found 83%

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Table 2 Selected candidate genes and primers used to detect methylation

Gene	Detection	Primer	Sequence	Product size/Tm (bp/°C)
CCNA1[a]	MSP	*CCNA1* met (F)	TTTCGAGGATTTCGCGTCGT	46/53
		CCNA1 met (R)	CTCCTAAAAACCCTAACTCGA	
		CCNA1 unmet (F)	TTAGTGTGGGTAGGGTGTT	67/53
		CCNA1 unmet (R)	CCCTAACTCAAAAAAACAACACA	
TSCC22[a]	MSP	*TSCC22* met (F)	TTTAGTGTTTTGAGGTCGTC	87/48
		TSCC22 met (R)	TAAAATAAACTAACCTAACGCGA	
		TSCC22 unmet (F)	GGGATATAGTTTTGGGGATT	190/48
		TSCC22 unmet (R)	ATAAACTAACCTAACACAAACCA	
CLMN[a]	MSP	*CLMN* met (F)	AAACCTAACTAACAAACGCG	185/55
		CLMN met (R)	TCGTATTCGTCGTTTCGC	
		CLMN unmet (F)	TGTTGTGATTTTAGTTTTGTGGT	96/55
		CLMN unmet (R)	CCAACACAACACAAACAACA	
EML1[a]	MSP	*EML1* met (F)	TCGAGGTCGTTTTTTCGC	130/55
		EML1 met (R)	ACGCTAACGCTAAAACCG	
		EML1 unmet (F)	TTTGGGTTTTGTGGTTGT	162/55
		EML1 unmet (R)	ACCCAACCACAACCAACA	
HRASLS3[b]	COBRA	*HRASLS3* (F)	GGTGTTTGGGATGTTTTTTT	109/55
		HRASLS3 (R)	AAC (A/G) CCCAAAAAACTACAAA	
MCC[b]	COBRA	*MCC* (F)	AAAATGTGGTAGAAGGGATT	86/53
		MCC (R)	AAACTCTCAATCCCCCAAA	
RARRES1[b]	COBRA	*RARRES1* (F)	GGGAGG (C/T)GTTTTTATTGTTTT	115/53
		RARRES1 (R)	CTACCC (A/G)AACTTAATACTAAA	
LOH11CR2A[b]	COBRA	*LOH11CR2A* (F)	TTGAGGAAATGAGGTTGTAAGTT	129/48
		LOH11CR2A (R)	AATCCTAAATTTCCAATATCCAC	

[a] Genes locating within the critical regions of LOH.
[b] Genes shown to be tumor suppressor gene in other cancers.

Table 3 Frequency of methylation status of candidate genes promoter in cultured NPC cells and control leukocytes

	NPC cells		Normal leukocytes	
	Presence	Absence	Presence	Absence
CCNA1	16	11	0	20
RARRES1	2	8	0	20
HRASLS3	2	8	0	20
EML1	0	10	0	10
CLMN	0	10	0	10
TSC22	0	10	0	10
MCC	0	10	0	10
LOH11CR2A	28	0	10	0

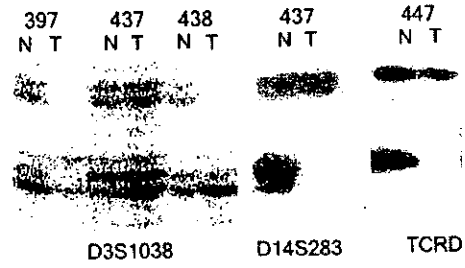

Figure 1 LOH study. Three microsatellite markers, D3S1038, D14S283, and TCRD, are used to study the LOH pattern in normal leukocytes (N) and tumor cells (T) of primary cultured NPC cells. In normal leukocytes, there were two bands of PCR products observed, whereas only single band of PCR products was detected in tumor cells.

hypermethylation of *RARRES1*, 17% of *HRASLS3* and 100% of *CCNA1*. Additional sequence analyses of the promoter of all three genes to prove the presence of promoter hypermethylation were performed in each sample (Fig. 4). The above findings suggest a possible tumor suppressive role of these genes. When analyzing for an association between hypermethylation and clinical parameters, we found no significant correlation with the tumor stage (data not shown).

Discussion

Currently, comprehensive gene expression profiling of individual cancer type has become one of the most popular ways to analyze individual cancer. We here apply the information derived from the gene expression analysis and to expand this knowledge to identify potential tumor suppressor genes in

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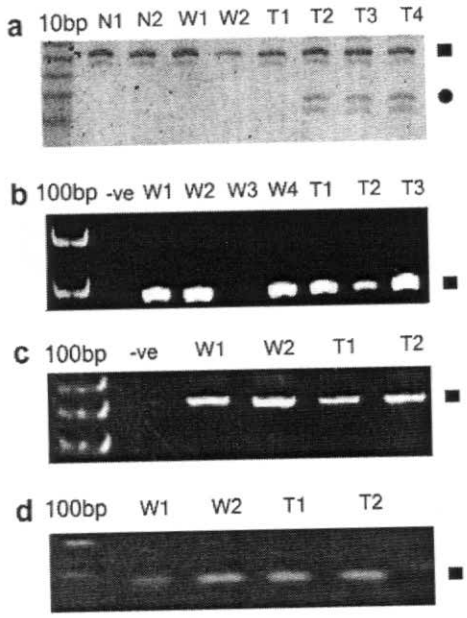

Figure 2 MSP analysis. (a) Typical results of a MSP analysis of *CCNA1* show frequent methylation of 5′ element of *CCNA1* in NPC but not in normal leukocyte and nasopharyngeal epithelium. (b–d) The results of MSP analysis of *EML1*, *CLMN*, and *TSCC22*, are shown. There was no methylation observed in these set of genes. Square: unmethylated band; circle: methylated band; -ve: control lane (water); T: NPC tissue; W: leukocytes; N: normal nasopharyngeal swab.

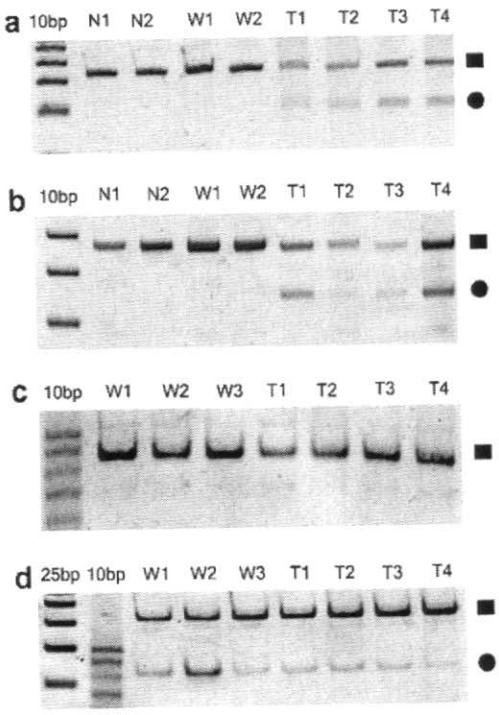

Figure 3 COBRA analysis. Methylation status of *HRASLS3*, *RARRES1*, *MCC*, and *LOH11CR* 2A promoter were analyzed by COBRA technique. Panels a and b showed frequent methylation of *HRASLS3* and *RARRES1* upstream elements in NPC but not in normal tissues. Methylation of *MCC* was not detected in both normal and tumor cells. In panel d, methylation of *LOH11CR2A* DNA sequence are invariably presence in both normal and tumor cells. Square: unmethylated band; circle: methylated band; -ve: control lane (water); T: NPC tissue; W: leukocytes; N: normal nasopharyngeal swab.

NPC. Integrating one known feature of tumor suppressor genes, namely LOH to the genome-wide expression analysis, allowed us to identify three potential TSGs in NPC, *CCNA1*, *HRASLS3*, and *RARRES1*. These genes have been proven to be relatively under-expressed and preferentially methylated at the promoter in NPC comparing to normal tissue. Furthermore, these three genes have been shown previously to be putative TSGs in other cancer type, for example *HRASLS3* in lung cancer,[8,12] *RARRES1* in lung cancer, prostrate cancer, and head and neck cancer.[8,13–16] The above findings not only corroborate the results from our previous microarray study but also help in elucidating the molecular epigenetic changes underlying the NPC.

Table 4 Frequency of methylation status of candidate tumor suppressor genes in NPC comparing to control nasopharyngeal epithelium and leukocytes

Gene	Sample	Promoter hypermethylation		Total
		Absence	Presence	
CCNA1	Normal leukocytes	30 (100%)	0	30
	Normal nasopharyngeal epithelium	20 (100%)	0	20
	NPC biopsy	24 (52.1%)	22 (47.8%)	46
	Cultured NPC cells	0	9 (100%)	9
RARRES1	Normal leukocytes	30 (100%)	0	30
	Normal nasopharyngeal epithelium	20 (100%)	0	20
	NPC biopsy	22 (48.9%)	23 (51.1%)	45
	Cultured NPC cells	1 (16.7%)	5 (83.3%)	6
HRASLS3	Normal leukocytes	30 (100%)	0	30
	Normal nasopharyngeal epithelium	20 (100%)	0	20
	NPC biopsy	34 (82.9%)	7 (17.1%)	41
	Cultured NPC cells	5 (83.3%)	1 (16.7%)	6

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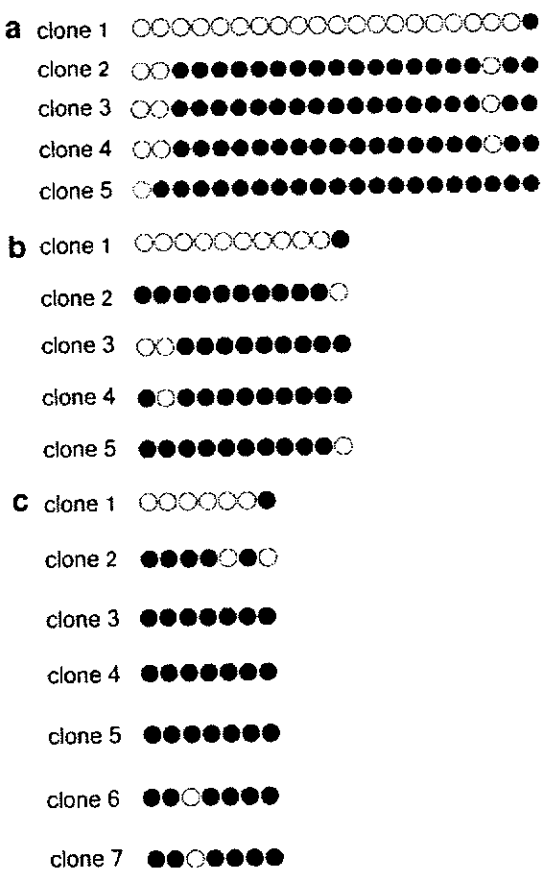

Figure 4 Bisulfite sequencing of candidate genes promoter. Results of bisulfite sequencing display the anatomical pattern of promoter hypermethylation of the three tumor suppressor gene candidates; *CCNA1* in panel a, *RARRES1* in panel b, and *HRASLS3* in panel c. Closed or opened circles are the methylated or unmethylated CpG or TG dinucleotides, respectively.

Function of cyclin A1 is unclear, whether it would act as an oncogene or a TSG is currently unknown. However, cyclin A1 is thought to play a role in oncogenesis because of its involvement in cell cycle progression.[18,19] Moreover, high levels of cyclin A1 have been found in acute leukemia cell lines and myeloid leukemia samples.[20] More recently, it has been implicated that cyclin A1 might be involved in DNA-double-strand-break repair by interacting with Ku70.[21] In addition, there are several reports documenting this gene to be down regulated and hypermethylated in squamous cell carcinomas of the head and neck and the cervical locations.[22,23] Here, we retrieved the data from global gene expression profile of NPC to search for candidate TSG and found *CCNA1* to be one of the candidates as its expression was down-regulated in NPC. Furthermore, we found that its promoter region was methylated in a sub-set of NPC tissues but not in normal nasopharyngeal epithelial tissue and leukocyte. Because transcriptional silencing by promoter methylation is a common mechanism for an inactivation of tumor suppressor gene, in the above regards, cyclin A1 may play a role as a tumor suppressor gene in squamous cell cancers including NPC.

HRASLS3 (H-ras like suppressor 3) is a class II tumor suppressor gene which is down-regulated in various cancer cell lines derived from melanoma, neuroblastoma, adenocarcinoma, and fibrosarcoma. There is evidence that promoter methylation of this gene in lymphoma cells can result in gene silencing.[24]

From our microarray data, *HRASLS3* is the gene, which is down regulated in NPC and in this study, we detected promoter methylation of this gene in NPC, which might be responsible for the inactivate of this putative tumor suppressor gene in NPC.

RARRES1 (retinoic acid receptor responsive element) is a candidate tumor suppressor gene of human prostate cancer, head and neck cancer and lung cancer, and recent evidence suggesting that the promoter of this gene is methylated in several of these cancers such as esophageal cancer, endometrial cancer, head and neck cancer, and lung cancer. [13–15] Indeed, promoter methylation of this gene has been detected in head and neck squamous cell carcinoma but not in normal tissue.[25] Recently, other groups studying this gene detected 90.7% promoter methylation in primary NPC,[16] here we also detected 51% promoter methylation in primary tumor and 83% in NPC cells. In addition, our microarray data revealed that this gene was down-regulated in NPC, therefore *RARRES1* may be the important TSG in this cancer type.

In conclusion, we report three putative tumor suppressor genes, *CCNA1*, *RARRES1* and *HRASLS3*, in NPC identified from the analysis of available gene expression profiling and alleotyping database, and the status of promoter hypermethylation. These three genes might well be the molecular markers for NPC since their promoters are methylated in NPC but not in normal cells. Further expanding the analysis in broader panel of genes from the database and functional study is warranted in the future.

Conflict of Interest Statement

None declared.

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IPI were available only in 116/215 samples analyzed. PIK3CA mutations were associated with a higher IPI score although this was not statistically significant ($P=0.0834$), which might be attributed to the relatively small number of DLBCL cases that were positive for the *PIK3CA* mutation. In addition, it was significantly associated with involvement of more than one extra nodal site ($P=0.0497$) and with tumor stage ($P=0.0103$). As a read out of *PIK3CA* functional activation, we tested AKT phosphorylation in DLBCL by IHC. Virtually all of the samples harboring PIK3CA mutations showed some staining (scored +1 to +3) for p-AKT (17 out of the 17 cases). However, only 10 of the 17 (59%) mutated cases showed increased p-AKT level by IHC (defined as DLBC samples scored as +2 or +3).[5] Furthermore, our findings that *PIK3CA* mutations correlate with tumor stage, suggest that increased PIP3 production and activation of PI3K/AKT may enhance tumor progression. PIK3CA mutations showed association with the non-germinal-activated B-cell type subtype, although this association was not statistically significant ($P=0.074$).

Given the significant association of *PIK3CA* mutations to two clinical markers (tumor stage and involvement of more than one extra nodal site) we performed survival analysis. No significant associations were found between *PIK3CA* mutational status and overall survival within DLBCL patients. We further stratified all our DLBCL cases into two groups depending on the presence of *PIK3CA* mutation and *PTEN* expression status: normal *PIK3CA* and normal *PTEN* ($n=114$) and DLBCL with either mutated *PIK3CA* or absent/reduced *PTEN* expression ($n=91$). Such a stratification showed a significant correlation with 10-year overall survival ($P=0.0249$) and remained significant even in multivariate analysis that included IPI, stage, number of extranodal sites and the above mentioned two groups. The relative risk for death was 1.9 for the DLBCL group with abnormal *PIK3CA* or *PTEN* (95% CI 1.2–2.9, $P=0.0017$) and 2.4 for high-risk IPI group (95% CI 1.4–4.2, $P<0.0011$).

To our knowledge, this is the first report on the presence of *PIK3CA* mutations in DLBCL patients. Furthermore, the ability to stratify DLBCL patient outcome based on the deregulation of PIP3 production gives further support of putative role of PI3K pathway in the regulation of DLBCL progression, which makes this pathway an attractive target for pharmacologic interventions. In particular, directed small molecule antagonism of *PIK3CA* proteins may be an effective measure for the treatment of DLBCL. Altogether, our study shows that a large proportion of DLBCL have either an inactivation of *PTEN* or activation of *PIK3CA* and this group is associated with poor overall survival. This loss of PIP3 homeostasis due to *PTEN* inactivation and *PIK3CA* activation in DLBCLs is remarkably similar to that reported in other human cancers. These findings indicate that mutation in *PIK3CA* is likely to function as an oncogene in DLBCL and argue for the role of *PIK3CA* targeting in the treatment of DLBCL patients.

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Hypermethylation of *TTC12* gene in acute lymphoblastic leukemia

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11q23 may house several genes that are important for the process of leukemogenesis. Structural aberrations involving 11q are among the most common aberrations in a number of hematological malignancies and often harbor a breakpoint at 11q23.[1] One of the common aberrations is deletion, that is associated with a poor clinical outcome.[2] Chromosome deletion suggests that the region might contain at least a tumor suppressor gene important for the genesis of lymphoproliferative disorders.[3,4] Investigating cancer-specific methylation is a preferential approach for identifying genes that are important in cancer development.[5] There have been few reports regarding methylation involving 11q23 in leukemia. Therefore, we further investigated whether, in leukemia, there exists a gene that is controlled by the epigenetic modification in this region.

We screened several candidate genes on chromosome 11q23,[3] for example, *ZW10*, *NCAM1*, *ANKK1* and tetratricopeptide repeat (TPR) domain 12 (*TTC12*), by combined bisulfite

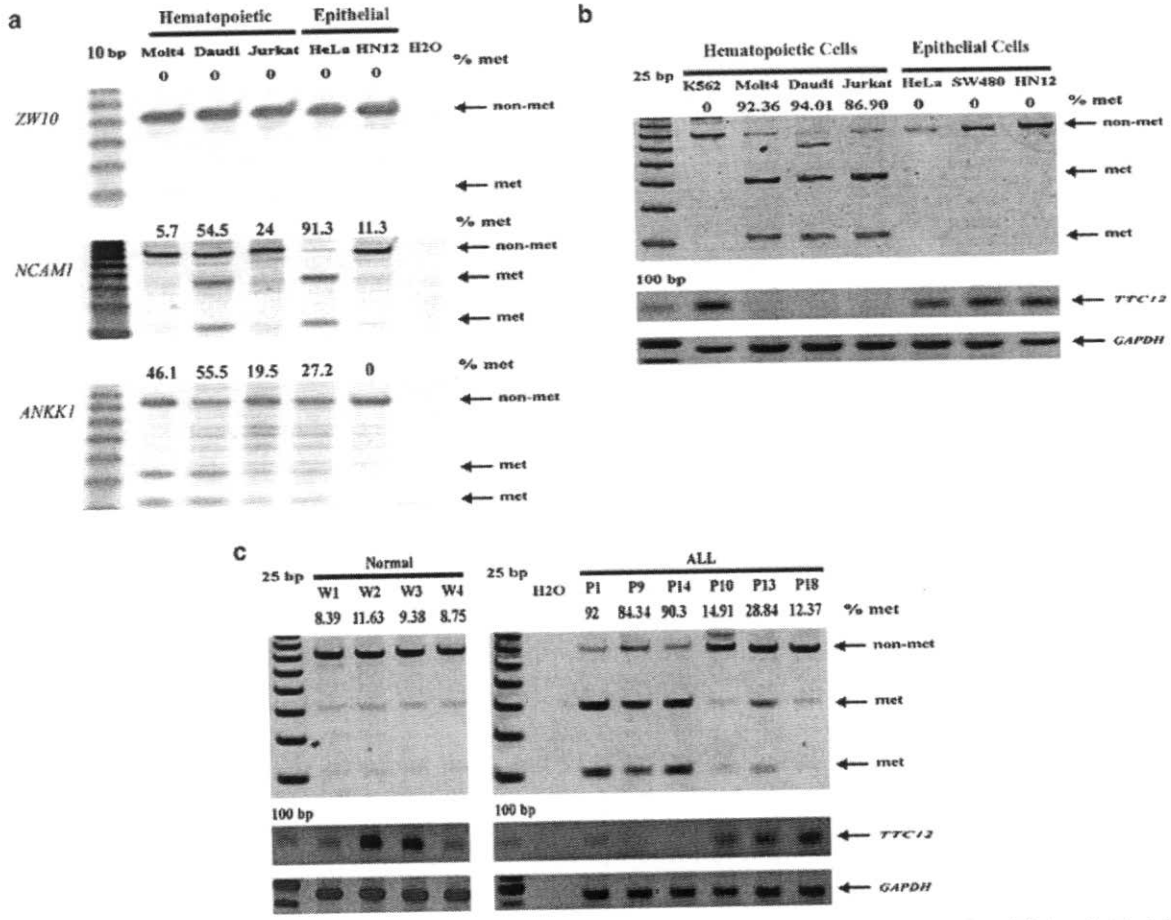

Figure 1 *TTC12* methylation and expression in cell lines and leukemia. Molt4 (acute lymphoblastic leukemia), Daudi (B lymphoblastoid line), Jurkat (acute T-cell leukemia), HeLa (cervical cancer), HN12 (head and neck squamous cell cancer), K562 (chronic myelogenous leukemia) and SW480 (stomach cancer) were investigated. Whereas no methylation or only partial methylation levels of *ZW10, NCAM1* and *ANKK1* were detected in leukemic cell lines, *TTC12* was hypermethylated. In leukemia, *TTC12* was methylated and showed a decrease in expression. (**a**) COBRA analysis in cell lines of indicated genes, *ZW10, NCAM1* and *ANKK1*. The primers were as follows: *ZW10*, AAATAACCAATCAA TAAATCCTATATATC and ATAATATGGGAGTTTTGATAAAAGAG; *NCAM1*, GTGTTGAGGTTGGGATTGTTA and TTTTTACAAAATTATTTCC TACCAAC; and *ANKK1*, GGTAGGGTcgGTTTGTTTTTTTA and CGCTATAACTACTACCTAAATAACC. (**b**) The primers for the *TTC12* COBRA set were TTTGGGAG(C/T)GGTTGTTGGG and CAATACCCCCCTAC(G/A)CTAAA. After PCR, the amplicons of (**a**) *ZW10, NCAM1* and *ANKK1* and (**b**) *TTC12* are digested by *Taq*I. The (**a**) 82, 86 and 92 bp and (**b**) 200 bp non-methylated sequences, respectively, will not be digested. If methylated, *Taq*I can digest the bisulfited PCR product at (**a**) 53, 55 and 51 bp and (**b**) 123 bp, respectively. Parts **b** and **c** demonstrate analyses of *TTC12*, Part **b** COBRA and RT-PCR analysis in cell lines and Part **c** COBRA and RT-PCR analysis in clinical samples. W and P are normal WBC and ALL sample, respectively. DNA size standard markers are of 10 and 25 bp. %met indicates the level of DNA methylation. The arrows in (**a–c**) indicate non-methylated and methylated amplicons, *TTC12* and *GAPDH* cDNA, respectively. Abbreviations: ALL, acute lymphoblastic leukemia; COBRA, combined bisulfite restriction analysis; RT-PCR, reverse transcription-polymerase chain reaction; WBC, white blood cells.

restriction analysis (COBRA) assay[6] (Figure 1). *TTC12* is located in between *NCAM1* and *ANKK1* (www.ncbi.nlm.nih.gov). We examined the methylation status of CpG dinucleotides around the 5′ end from the transcriptional start site. Treatment with bisulfite converts unmethylated cytosines, but not methylated cytosines, to uracils and then thymines after PCR.[7] This generates detectable methylation-dependent changes in the restriction pattern of PCR-amplified sequences from CCGA to TCGA in case of methylation and TTGA in case of non-methylation. Whereas methylation levels of *ZW10, NCAM1* and *ANKK1* in leukemic cell lines were none to partial (Figure 1a), *TTC12*[8] was shown to be hypermethylated in several leukemic cell lines (Figure 1b).

We investigated if *TTC12* methylation is directly correlated with the inhibition of expression in leukemia. Hypermethylation was demonstrated in all leukemic cells, except K562, (Figure 1b). In contrast, epithelial cells were not methylated (Figure 1b). The cDNA was amplified by PCR and real-time PCR with the TTC12-RT primers, TGGGCGAATCAGGGATTCC and GCATTCATCCTCCTCCTGGT, and the GAPDH primers, GTGGGCAAGGTATCCCTG and GATTCAGTGTGGTGGGG GAC. *GAPDH* was used as control and to calculate the relative quantity of TTC12 mRNA. Non-methylated and hypermethylated cell lines expressed and did not express TTC12 mRNA levels, respectively (Figure 1b).

Furthermore, we evaluated 30 bone marrow samples from acute lymphoblastic leukemia (ALL) cases, normal white blood cells (WBC) from 10 healthy volunteers and eight samples of complete remission bone marrows. The ALL diagnosis was based on the criteria of the World Health Organization (WHO) classification of tumors. All bone marrows were subjected to immunophenotyping and immunomagnetic selection.[9] The

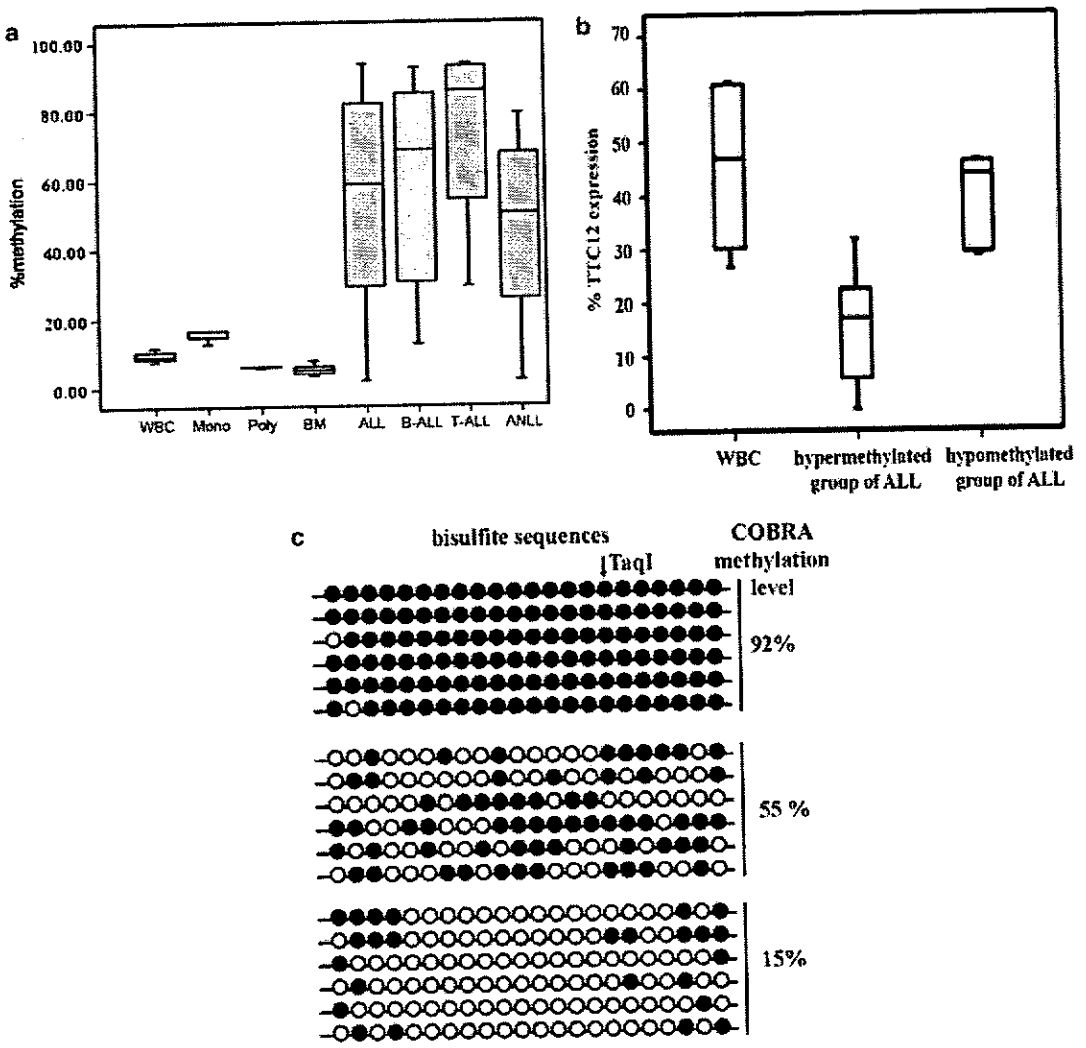

Figure 2 *TTC12* methylation and expression levels, and bisulfite sequencing in ALL. (a) Higher methylation level is demonstrated specifically in ALL. WBC is white blood cells, mono is mononuclear cells, poly is polymorphonuclear cells, BM is remission bone marrow, and ALL includes B-ALL, T-ALL and ANLL. (b) *TTC12* expression of WBC and between hypermethylated and hypomethylated ALL showed that methylation levels of ALL were inversely correlated with the level of expression in ALL. The expression unit is relative percentage in comparison with *GAPDH*. The box plots show mean and error bar of each group. (c) Bisulfite sequences of three ALL cases at the CpG islands of *TTC12* are shown: methylated CpG dinucleotides and non-methylated CpGs of each clone are shown by closed and open circles, respectively. Methylation levels of each ALL, as detected by COBRA, are shown on the right. Abbreviations: ALL, acute lymphoblastic leukemia; BM, bone marrow; COBRA, combined bisulfite restriction analysis; WBC, white blood cells.

results indicated the association between epigenetic regulation and leukemogenesis. While in normal WBC *TTC12* was hypomethylated, hypermethylated *TTC12* was usually detectable in ALL ($P<0.0001$; Figures 1c and 2a). Whereas the mean and the highest methylation levels were, respectively, 9.42 and 11.63% in WBC and 5.12 and 6.62% in remission bone marrow, the mean *TTC12* methylation in ALL was 56.33%. In addition, 93% of all ALL cases showed methylation levels higher than 12% (Figure 2a).

Methylation of a gene is directly correlated with the inhibition of expression in ALL. Higher expression of *TTC12* was also detectable in WBC (Figures 1c and 2b). We categorized ALL into two groups as hypermethylated and hypomethylated *TTC12*, depending on the level of methylation in relation the mean methylation level (56.33%). The mRNA level of hypermethylated ALL was less than in hypomethylated ALL ($P<0.05$; Figure 2b). In conclusion, *TTC12* methylation associated with leukemogenesis, and methylation may be important in this gene inactivation.

We further confirm and characterize the *TTC12* methylation status by cloning and sequencing hypomethylated, partially methylated and hypermethylated COBRA amplicons from ALL (Figure 2c). Methylation of the CpG island was directly correlated with methylation levels of COBRA. We re-evaluated the methylation status of *TTC12* at 408 bp, 5′ end from the previous COBRA, by TT(C/T)GATTTTGGTTTTTAATTTGG and AAC(G/A)AAACCAAAAACCAAAAACC, generating 187 bp methylation-dependent *BstUI*-digestible amplicons, and compared the methylation levels with the previous COBRA. The methylation levels from both locations were directly associated ($P=0.0004$; Pearson $r=0.7974$). This confirmed the previous COBRA results.

This study shows that the *TTC12* gene was specifically hypermethylated in ALL. Moreover, TTC12 expression was

inhibited in association with the epigenetic modification. Therefore, depletion of *TTC12* activity may be important for the process of leukemogenesis. Although the function of *TTC12* has not been identified, *in silico*, two conserved domains have been found, including the TPR and the armadillo repeat (ARM) domains.[8] Both ARM and TPR are implicated in a variety of cellular processes, ranging from cell cycle control to signal transduction, kinase regulation and tumorigenesis. Therefore, downregulation of *TTC12* may contribute to the promotion of a multistep process of ALL. Further investigations will provide new knowledge of hematologic malignancy biology that could be applied to diagnosis as well as therapeutic modalities.

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Portal venous thrombosis in a young patient with idiopathic myelofibrosis and intrahepatic extramedullary hematopoiesis: a difficult diagnosis, prognosis and management

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Chronic myeloproliferative syndromes (MPSs) occur much less commonly in children than in adults. Idiopathic myelofibrosis (IMF) is characterized by stem cell-derived clonal myeloproliferation and shows constitutional symptoms, increasing splenomegaly, bone marrow fibrosis, extramedullary hematopoiesis, progressive anemia and cytopenias; hemorrhages and above all thrombosis are frequent complications.[1]

Portal vein thrombosis (PVT) is associated with liver cirrhosis, hepatocellular carcinoma, intra-abdominal infections, hemodynamic factors, hypercoagulability states and thrombophilic disorders, including MPS.[2] Chronic PVT can be often recognized casually, after a fortuitous diagnosis of hypersplenism or portal hypertension or while having biliary symptoms related to cholangiopathy or only initial ascites; moreover, acute PVT can present with life-threatening events such as gastrointestinal hemorrhage and intestinal infarction. The PVT can result from the concurrence of several acquired factors, with or without the presence of genetic ones. Cirrhotic PVT patients usually have an advanced liver disease; in these patients, the concomitant mutation G20210A of the prothrombin gene increases more than fivefold the risk.[3] About 70% of patients with PVT have a general risk factor, most commonly MPS, while local precipitating risk factors, such as an abdominal inflammatory focus, can be identified in about 30%. A moderate hyperhomocysteinemia is associated with heightened risk,[4] and PVT has been reported in patients with antiphospholipid syndrome.[5] PVT in children or young adults is reported anecdotically.[4]

A girl was referred for isolated thrombocytosis (median $700 \times 10^3\,\mu l^{-1}$) since she was 2 years old. The bone marrow was hypercellular, with numerous clusters of megakaryocytes; EPO-independent BFU-E colonies were demonstrated; the karyotype was normal and bcr-abl rearrangements were absent. A childhood essential thrombocythemia (ET) was initially diagnosed, while excluding a familial disease. At the age of 9, the spleen was at the upper normal limit, while showing an increase afterwards: at 16 and 20 years, there was a slight splenomegaly (surface 75 and 80 cm^2, respectively), while showing only a moderate and steady thrombocytosis. The thrombophilic study (lupus anticoagulant, anticardiolipin, antiphospholipid, anti-β 2 glycoprotein I antibodies, lipoprotein a, ATIII, protein S and C, activated protein C resistance, factor V Leiden G1691A and prothrombin gene G20210A mutations) resulted negative; she was only heterozygous for MTHFR C677T mutation without hyperhomocysteinemia. A watch-and-wait strategy seemed appropriate in this asymptomatic case, platelet count remaining only moderately elevated, and low-dose aspirin was started. In January 2006, when she was 23 years old, the girl showed increasing thrombocytosis ($1500 \times 10^3\,\mu l^{-1}$), slight leukocytosis ($18 \times 10^3\,\mu l^{-1}$) with neutrophilia ($13.1 \times 10^3\,\mu l^{-1}$) and without basophilia and eosino-

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Serum LINE-1 hypomethylation as a potential prognostic marker for hepatocellular carcinoma

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Abstract

Background: We investigated the clinical implications of global hypomethylation, one of the most consistent epigenetic changes in cancer, in the sera of patients with hepatocellular carcinoma (HCC).
Methods: Combined bisulfite restriction analysis PCR was used to assess the methylation status of LINE-1 repetitive sequences in genomic DNA derived from sera of 85 patients with HCC, 73 patients with cirrhosis, 20 healthy carriers of hepatitis B virus (HBV) and 30 healthy controls.
Results: Serum genome hypomethylation, the percentage of unmethylated LINE-1, was significantly increased in patients with HCC ($P<0.001$). The levels of serum LINE-1 hypomethylation at initial presentation correlated significantly with the presence of HBsAg, large tumor sizes, and advanced tumor stages classified by the CLIP score. Multivariate analyses showed that serum LINE-1 hypomethylation was a significant and independent prognostic factor of overall survival.
Conclusion: Serum LINE-1 hypomethylation may serve as a prognostic marker for patients with HCC.

Keywords: Global hypomethylation; Prognostic marker; Hepatocellular carcinoma; COBRA LINE-1; Hepatoma

1. Introduction

Hepatocellular carcinoma (HCC) represents one of the most common cancers worldwide, accounting for >500,000 new cases annually [1]. The prevalence of HCC is geographically variable, with the highest frequencies observed in sub-Saharan Africa and Southeast Asia where hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are endemic. Despite remarkable improvements in surgical and ablative therapies, the overall prognosis of patients with HCC remains unsatisfactory because of its aggressiveness and high recurrence rates [2]. As a result, a reliable serum marker is needed and important for monitoring tumor progression, treatment responsiveness, and predicting the prognosis. Although several molecular biological factors related to HCC have been studied in recent years, a prognostic marker for this cancer in routine clinical practice is not yet available.

Recent advances in molecular biology have shown that genetic and epigenetic alterations accumulated through repeated destruction and regeneration of hepatocytes are responsible for multistage hepatocarcinogenesis [3]. Among these changes, widespread global DNA hypomethylation accompanied by region-specific hypermethylation is a common feature found in HCC. Global hypomethylation has been demonstrated by downregulation of methylated CpG dinucleotides, which are dispersed throughout the whole genome, in both noncoding repetitive sequences and genes [4]. Global losses of methylation in cancer may lead to alterations in the expression of proto-oncogenes critical to carcinogenesis and may facilitate chromosomal instability [5]. Previous studies have described the hypomethylation of genomic repetitive sequences, a marker of

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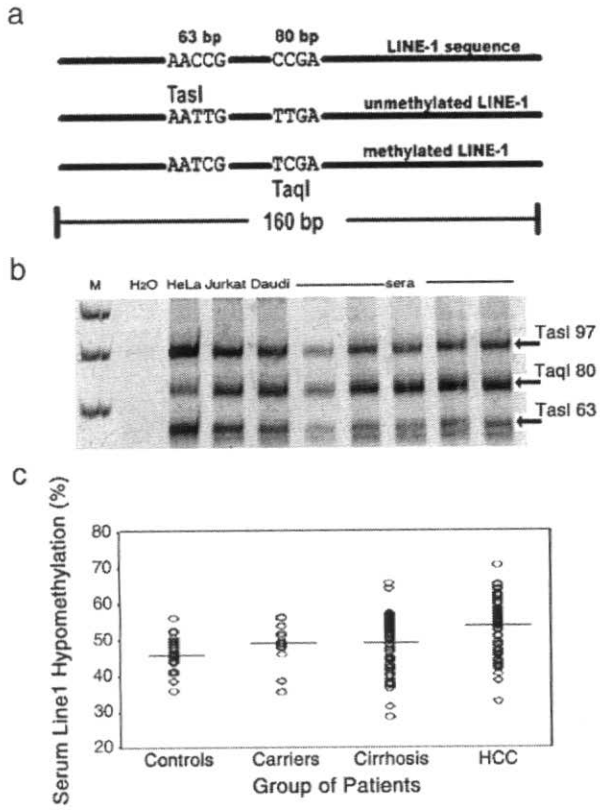

Fig. 1. Combined bisulfite restriction analysis of LINE-1 (COBRA LINE-1) in sera. (a) Schematic illustration of COBRA LINE-1. The LINE-1 amplicon size is 160 bp. Methylated amplicons, *TaqI* positive, yielded two 80 bp DNA fragments, whereas unmethylated amplicons, *TasI* positive, yielded 63 and 97 bp fragments. (b) An example of COBRA LINE-1. M, 10-bp DNA size marker; H_2O, HeLa, Jurkat and Daudi are positive control and for adjusting inter-assay variation. Sera of HCC patients. *TasI* 63 and 97, unmethylated amplicons; *TaqI* 80, methylated amplicons. (c) Serum LINE-1 hypomethylation levels in each group of patients. The horizontal line indicated the mean levels of the patients' subgroups.

global genomic hypomethylation, in several malignancies including carcinoma of the breast, lung, head and neck, esophagus, stomach, urinary bladder, prostate, colon, and liver [6–12]. In addition, the extent of global hypomethylation appears to correlate with tumor progression and invasiveness in several cancer types, including HCC, indicative of its role in tumor development and progression [7,13–16].

Previous studies have demonstrated that tumor-derived nucleic acids can be detected in the sera of patients with cancers, and appear to be relevant surrogate markers for genetic alterations present in the primary tumors [17]. Based on these observations, circulating tumor-specific methylation has become an emerging serum or plasma marker for cancer diagnosis and prognosis [18]. Recently, we developed an improved quantitative combined bisulfite restriction analysis (COBRA) PCR protocol that efficiently evaluates the genome wide methylation status of LINE-1 repetitive sequences in genomic DNA derived from microdissected tissue and serum samples [7]. The LINE-1 methylation level measured by this technique has been shown to represent the genome wide methylation status by demonstrating linear correlation with conventional global hypomethylation assay by Southern blot and hybridization. Unlike conventional qualitative reports, COBRA LINE-1 not only confirmed that most cancers exhibited significantly increased levels of hypomethylation, compared with their normal tissue counterparts, but also normal tissues from different organs showed tissue-specific levels of methylated LINE-1. Furthermore, DNA derived from sera of patients with stomach cancer displayed significantly higher LINE-1 hypomethylation levels. These findings suggest the potential value of COBRA LINE-1 as a marker of various cancer types, including HCC. Nonetheless, whether serum LINE-1 hypomethylation level can serve as a molecular marker for HCC has not yet been investigated. To address this issue, we examined the methylation status of LINE-1 in serum samples of patients with HCC, and compared them with those of healthy individuals and patients with cirrhosis. We also determined the clinicopathological correlations and prognostic significance of this epigenetic alteration in sera of patients with HCC.

2. Materials and methods

2.1. Patients and blood samples

Eighty-five patients who were diagnosed with HCC in King Chulalongkorn Memorial Hospital (Bangkok, Thailand) between April 1999 and December

Table 1
Relationship between serum LINE-1 hypomethylation levels and clinicopathological features in patients with HCC

Variables	Serum LINE-1 hypomethylation		*P*
	<53.17%	≥53.17%	
Age (y)			
≥60 (n=27)	13	14	NS
<60 (n=58)	23	35	
Gender			
Male (n=69)	29	40	NS
Female (n=16)	7	9	
Etiology of liver disease			
HBV positive (n=58)	19	39	0.011
HBV negative (n=27)	17	10	
Serum AFP level (ng/ml)			
≥400 (n=47)	18	29	NS
<400 (n=38)	18	20	
Tumor cell differentiation			
Well (n=13)	9	4	NS
Moderately and poorly (n=39)	17	22	
Tumor number			
Solitary (n=53)	24	29	NS
Multiple (n=31)	11	20	
Tumor size			
≤5 cm (n=15)	13	2	<0.001
>5 cm (n=70)	23	47	
Venous invasion			
Presence (n=30)	14	16	NS
Absence (n=55)	22	33	
Extrahepatic metastasis			
Presence (n=8)	1	7	NS
Absence (n=77)	35	42	
CLIP score			
Score 0–2 (n=41)	23	18	0.017
Score 3–5 (n=44)	13	31	

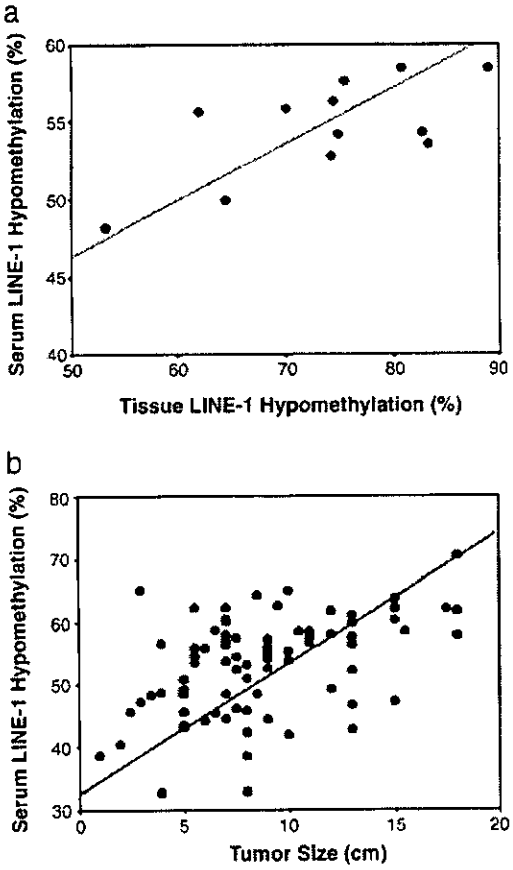

Fig. 2. Serum LINE-1 hypomethylation levels in patients with HCC exhibited a positive correlation with a) corresponding tumor tissue levels and b) tumor size.

2003 were recruited into the study. HCC was diagnosed based on liver tumor characteristics detected by imaging studies (ultrasonography or computed tomography), serum α-fetoprotein (AFP) >400 ng/ml, and/or histopathology. The clinicopathological data of the patients at initial diagnosis were collected, and included sex, age, liver function test, Child-Pugh classification, serum AFP concentration, tumor size, number of tumors, tumor cell differentiation, venous invasion, extrahepatic metastasis, and HCC staging classified by the CLIP score [19]. Overall survival time of the patients was defined as the period from initial presentation to the time of last follow-up (December 2004) or until they expired.

The patients with HCC consisted of 69 males and 16 females, with the mean age of 53.6±12.7 y. All patients had cirrhosis as underlying liver disease. In regard to predisposing etiologic factors, 58 patients (68.2%) were positive for serum hepatitis B surface antigen (HBsAg), 8 patients (9.5%) were positive for hepatitis C virus antibody (anti-HCV), and 12 patients (14.1%) were associated with alcohol-dependence. For the remaining 7 patients (8.2%), the etiology could not be determined. According to the CLIP score at initial presentation, there were 6 patients (7.1%) with score 0, 14 patients (16.5%) in the score 1 subgroup, 21 patients (24.7%) in score 2, 19 (22.4%) in score 3, 19 (22.4%) in score 4, and 6 (7.1%) in the score 5 subgroup. Thirty patients (35.3%) had venous invasion, while extrahepatic metastasis was found in 8 patients (9.4%). Twelve patients (14.1%) had undergone surgical resection, 25 patients (29.4%) had been treated with transarterial chemoembolization (TACE), and the remaining 48 patients (56.5%) had received no specific treatment because of an advanced tumor stage or refusal of the therapy.

The control groups comprised 30 healthy individuals (20 males, mean age 30.2±8.7 y), 20 healthy carriers of hepatitis B (14 males, mean age 31.5±10.3 y) and 73 patients with cirrhosis (55 males, mean age 50.3±11.7 y). Healthy carriers were defined as individuals who were positive for HBsAg, had persistent normal serum aminotransferase activities, and had normal abdominal ultrasonography. Cirrhosis was diagnosed based on histological examinations and/or imaging studies. With the patients' written consent, all serum samples were collected at the time of the diagnosis and stored at −70 °C until they were assayed.

2.2. DNA preparation

Peripheral blood (3 ml) was collected from each subject for the isolation of serum. Sera were centrifuged at 1600 ×*g*, transferred into plain polypropylene tubes and stored at −20 °C until further processing. DNA from 800 μl of serum samples was extracted using a QIAamp Blood Kit (Qiagen, Hilden, Germany) using the blood and body fluid protocol as recommended by the manufacturer. Twelve HCCs were microdissected from paraffin-embedded tissues.

After extraction, all DNA samples were treated with sodium bisulfite as previously described [20]. Briefly, genomic DNA was denatured in 0.22 mol/l NaOH at 37 °C for 10 min. Thirty microliters of 10 mmol/l hydroquinone and 520 μl of 3 mol/l sodium bisulfite were added for 16–20 h at 50 °C. The DNA was purified and incubated in 0.33 mol/l NaOH at 25 °C for 3 min, ethanol precipitated, then washed with 70% ethanol and resuspended in 20 μl of H_2O.

2.3. Measurement of serum LINE-1 hypomethylation

The COBRA LINE-1 assay of the serum was performed as described previously (13). We used 5′UTR of LINE-1.2 sequence from NCBI Accession Number M80343. DNA was isolated and extracted from serum, and treated with bisulfite. Two microliters of bisulfited DNA was subjected to 35 cycles of PCR with 2 primers, 5′-CCGTAAGGGGTTAGGGAGTTTTT-3′ and 5′-RTAAAACCCTCCRAACCAAATATAAA-3′, with an annealing temperature of 50 °C. The amplicons were digested in 10 μl reaction volumes with 2U of *TaqI* or 8U of *TasI* in 1× *TaqI* buffer (MBI Fermentas) at 65 °C overnight and

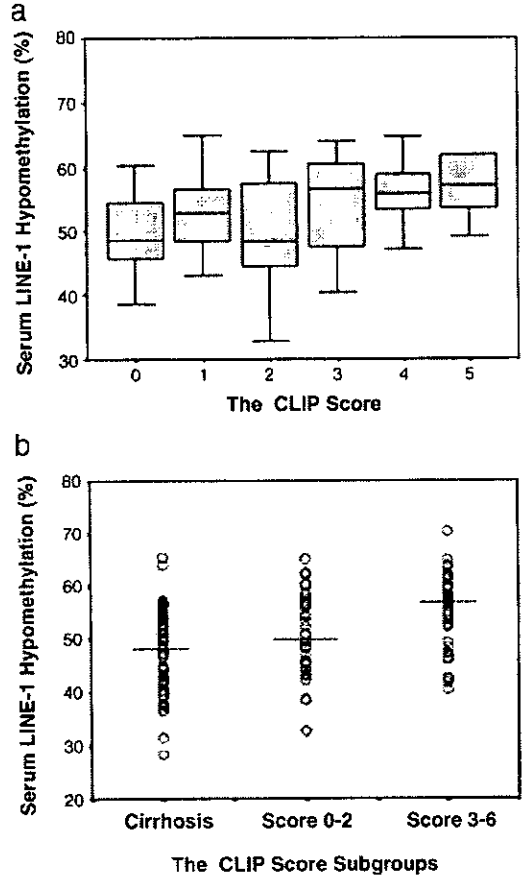

Fig. 3. Association between serum LINE-1 hypomethylation levels and HCC staging classified by the CLIP score. The horizontal line indicated the mean levels of the patients' subgroups.

then electrophoresed in 12% nondenaturing polyacrylamide gels. Intensities of DNA fragments were measured by PhosphorImager, using ImageQuant software (Molecular Dynamics). The LINE-1 amplicon size is 160 bp. Methylated amplicons, *TaqI* positive, yielded 2 80 bp DNA fragments, whereas unmethylated amplicons, *TasI* positive, yielded 63 and 97 bp fragments (Fig. 1). LINE-1 hypomethylation level was calculated as a percentage: the intensity of unmethylated LINE-1, digested by *TasI* divided by the sum of methylated LINE-1, digested by *TaqI*- and the *TasI*-positive amplicons. The same DNAs from HeLa, Daudi, and Jurkat cell lines were applied as positive controls in all COBRA LINE-1 experiments and to adjust for inter-assay variation.

2.4. Statistical analysis

All data are expressed as mean±S.D., and percentages as appropriate. Comparisons between groups were analyzed by the χ^2 or Fisher's exact test for categorical variables and by the Mann–Whitney test or Student's *t*-test for quantitative variables. The analysis of overall survival was calculated by the Kaplan–Meier method and the differences in survival between the groups were compared using the log rank test. The Cox regression analysis was performed to identify which independent factors have a significant influence on overall survival. A $P<0.05$ was considered significant. All statistical analyses were performed using the SPSS software for Windows 10.0 (SPSS Inc., Chicago, IL).

3. Results

Serum LINE-1 hypomethylation in 85 patients with HCC obtained at the time of diagnosis ranged from 32.73% to 70.42%, with a mean of 53.17±7.74%. The average level of serum LINE-1 hypomethylation in these patients was significantly different from that of healthy controls (46.55±4.29%; range 35.94–56.01%), HBV carriers (48.07±6.20%; range 35.49–56.31%), and patients with cirrhosis (48.78±8.01%; range 28.51–65.75%) ($P<0.001$, $P=0.003$ and $P=0.001$, respectively). There was no significant difference in serum LINE-1 hypomethylation level between non-HCC individuals ($P>0.05$) (Fig. 1).

To evaluate the association between serum LINE-1 hypomethylation levels and clinicopathological features, the patients with HCC were divided into 2 groups based on the mean value (53.17%) of the whole HCC group. Accordingly, there were 36 patients with serum LINE-1 hypomethylation levels of <53.17% and 49 patients with serum levels of ≥53.17%. The correlations between hypomethylation in these 2 groups and various clinicopathological parameters listed in Table 1 were analyzed. There was no significant correlation between serum LINE-1 hypomethylation level and patient age, gender, AFP concentration, tumor cell differentiation, number of tumors, the presence of venous invasion, or extrahepatic metastasis. However, high serum LINE-1 hypomethylation levels were significantly associated with HBsAg positivity ($P=0.011$), tumor size >5 cm ($P<0.001$), and high CLIP score ($P=0.017$).

To determine the correlation between serum LINE-1 hypomethylation level and tissue expression, 12 cases of the

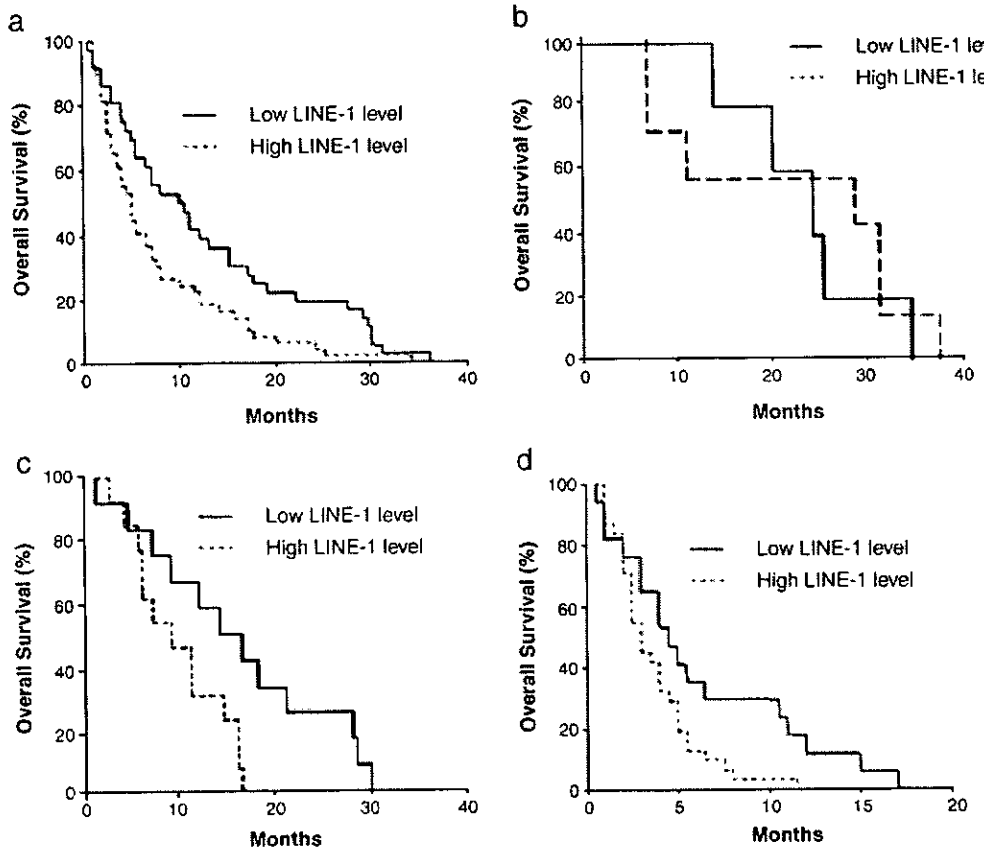

Fig. 4. Overall survival of HCC patients with serum LINE-1 hypomethylation less than or greater than 53.17% a) all patients, b) patients who were treated with surgery, c) patients who were treated with TACE and d) untreated patients. LINE-1 level, serum LINE-1 hypomethylation levels.

Table 2
Multivariate analysis of prognostic factors of survival with Cox's proportional hazards model in patients with HCC

Factors	Risk ratio	95% confidence interval	P
High serum LINE-1 hypomethylation level	1.742	1.087–2.793	0.021
Presence of vascular invasion	2.094	1.179–3.719	0.012
High CLIP score (score 3–5)	2.101	1.433–3.081	<0.001
No receiving therapy	5.712	3.116–10.471	<0.001

corresponding tumor tissue samples were analyzed. A positive correlation between serum and tumor LINE-1 hypomethylation was observed ($P=0.017$; Pearson $r=0.670$) (Fig. 2a). Serum LINE-1 hypomethylation levels also exhibited a positive correlation with tumor size ($P<0.001$; Pearson $r=0.439$) (Fig. 2b), and CLIP score ($P=0.005$; Pearson $r=0.304$) (Fig. 3a). In addition, serum LINE-1 hypomethylation levels in patients in the CLIP score 3–6 subgroup (55.56±6.83%) were significantly higher than those of patients in the score 0–2 subgroup (50.61±7.92%) and patients with cirrhosis (48.±8.01%) ($P=0.003$ and $P<0.001$, respectively). However, serum LINE-1 hypomethylation levels in patients in the score 0–2 subgroup were not significantly different from those with cirrhosis ($P=0.241$) (Fig. 3b).

We also examined the potential prognostic value of serum LINE-1 hypomethylation. The median overall survival of patients with LINE-1 hypomethylation levels of <53.17% was 10.5 months, which was significantly better than that of patients whose serum levels were ≥53.17% (median overall survival, 5.5 months; $P=0.012$ by log rank test) (Fig. 4a). Among patients who were treated with surgery, the median overall survival times for the low and high LINE-1 hypomethylation groups were 27.5 and 24.0 months, respectively ($P=0.670$) (Fig. 4b). Among patients who were treated with TACE, the median overall survival times for the low and high LINE-1 hypomethylation groups were 15.5 and 10.0 months, respectively ($P=0.024$) (Fig. 4c). In untreated cases, the median overall survival times for the low and high LINE-1 hypomethylation groups were 4.5 and 3.0 months, respectively ($P=0.030$) (Fig. 4d).

Serum LINE-1 hypomethylation level was entered into the multivariate analysis together with other variables that may influence prognosis. These factors included age, gender, AFP concentration, HBsAg status, Child-Pugh classification, tumor size, tumor number, venous invasion, extrahepatic metastasis, the CLIP score, and therapy for HCC. The multivariate analysis using the Cox proportional hazards model revealed that high serum LINE-1 hypomethylation level, presence of vascular invasion, high CLIP score, and absence of therapy for HCC were independent prognostic factors of overall survival (Table 2).

4. Discussion

An increasing number of studies have described the critical roles of epigenetic alterations in hepatocarcinogenesis, though most reports have focused on the function of DNA hypermethylation in silencing tumor suppressor genes [4,21]. Previous data have shown that CpG island-specific hypermethylation is already detectable in potentially precancerous lesions, including cirrhosis and dysplastic nodules, indicating its contribution to early stages of hepatocarcinogenesis [22]. Recently, the presence of cell-free-circulating DNA of hypermethylation of candidate tumor suppressor genes has been described in serum or plasma samples from patients with various types of cancers [21], including HCC [23,24]. Although the clinical significance of this epigenetic alteration in sera of patients with HCC is unclear, these data suggest the potential use of this molecular phenomenon in cancer diagnosis and monitoring. Conversely, relatively little is currently known concerning the status and clinical implications of global hypomethylation in sera of patients with HCC, as well as other cancer types.

In this study, we first demonstrated that serum LINE-1 hypomethylation levels were significantly higher in patients with HCC than in healthy individuals and patients with cirrhosis. These results were consistent with recent data, including our previous report, demonstrating that global hypomethylation is significantly increased in tumor tissues compared with cirrhotic and non-cirrhotic tissues [7,12]. Indeed, we have previously shown that though serum samples possess a wide distribution of LINE-1 methylation levels, there is a high correlation between the presence of a primary tumor and serum LINE-1 hypomethylation [7]. Thus, the increase in serum LINE-1 hypomethylation likely reflected the global methylation level in the tumor tissues. Notably, there was no significant difference in serum LINE-1 hypomethylation levels among non-HCC samples, suggesting that there might be no sequential increase of global hypomethylation between normal controls and cirrhosis. These results confirmed previous data that indicated that the levels of hypomethylation in normal liver tissues are comparable with those detected in chronic liver disease [12].

In addition to the tumor global methylation level, the increase in serum LINE-1 hypomethylation level likely reflected the malignancy status. While global hypomethylation is one signature of most cancer types, it often displays considerable specificity with regard to tumor type, stage, and the sequences affected [25]. Furthermore, the COBRA LINE-1 quantitative assay demonstrated that instead of being abruptly altered, genome wide hypomethylation in the tumor evolves progressively in multistage carcinogenesis [7]. In addition, several studies have reported that the amount of serum tumor DNA correlates directly with the advancement of the cancer; for example, serum or plasma Epstein Barr viral DNA level correlates with tumor size in nasopharyngeal carcinoma [1], and plasma human papilloma viral DNA level correlates with cervical cancer metastasis [26]. Nonetheless, in contrast to most tissue samples, a relatively wide range of LINE-1 hypomethylation levels in the sera could be anticipated, even within the same group of patients or tumor stages. One explanation for this observation could be the distinct LINE-1 methylation levels seen in different tissues and the high variability between individuals in tissues such as esophagus and thyroid [7].

Moreover, little is currently known about the mechanisms determining the amount of cellular DNA released into the circulation and whether there is eradication bias between methylated and unmethylated DNA [17]. With better understanding of such mechanisms, the specificity and sensitivity of serum LINE-1 hypomethylation as an indicator will improve.

General consequences of global hypomethylation are genomic instability and possible upregulate genes [4]. There are 3 possible specific biological roles of LINE-1 retrotransposons and consequently their methylation. First, LINE-1s are linked to genomic instability. Not only some LINE-1s are still retrotransposable [27] but also upregulation of LINE-1s creates DNA double-strand breaks [28]. Second, promoter hypomethylation of LINE-1 retrotransposable element activates antisense transcription as previously demonstrated in c-met gene of chronic myeloid leukemia [29]. Finally, reverse transcriptase of LINE-1s was demonstrated to possess a significant role in controlling cancer cells proliferation and differentiation [30].

In this study, analysis of serum LINE-1 hypomethylation in relation to clinicopathological features showed that a high level of LINE-1 hypomethylation was significantly correlated with more aggressive tumor behavior in patients with HCC. Specifically, a high serum LINE-1 hypomethylation level was observed more frequently in patients with tumors >5 cm in diameter and in advanced disease stages (CLIP score 3–6 subgroup). This is in accordance with previous reports demonstrating that there is a positive correlation between the expression levels of global hypomethylation in liver tissues and advanced stages of disease [16]. Similar findings of global hypomethylation in tissue specimens in association with tumor progression have been observed in a variety of cancers [14,15]. These data suggest an important and active role of global hypomethylation in the progression of HCC and other tumor types. As shown in this study, measurement of COBRA LINE-1 in the serum has several advantages over tissue-based techniques, including its rapidity, reproducibility, and noninvasiveness. Thus, in clinical settings, the quantitative serum LINE-1 hypomethylation assay may be more useful and feasible for predicting tumor progression than the measurement of hypomethylation in tumor specimens.

Several clinicopathological factors related to the prognosis of HCC have been reported recently. Of these, the CLIP score is an important prognosticator among the conventional pathological features of HCC [19]. In our study, a high serum LINE-1 hypomethylation level at initial diagnosis was a significant prognostic factor in terms of overall survival in patients who were treated with TACE, as well as in untreated cases. Furthermore, multivariate analyses revealed that this molecular marker was an independent, unfavorable predictor of long-term survival in patients with HCC. These findings strongly suggest that the prognosis of HCC is influenced by the extent of global hypomethylation in the cancer. Although the mechanisms remain to be elucidated, we speculate that progressive loss of genomic methylation might provide incremental survival advantages for tumor cells, and thus enhance tumor progression and aggressiveness. If our results are confirmed in large-scale prospective studies, the detection of serum LINE-1 hypomethylation may serve as a promising prognostic marker for patients with HCC.

In conclusion, we demonstrated the clinical implications of serum LINE-1 hypomethylation in HCC. A high level of LINE-1 hypomethylation was significantly associated with tumor progression and invasiveness. Moreover, a high serum LINE-1 hypomethylation level at initial presentation was an unfavorable prognostic marker for HCC. Apart from its prognostic role, it will be interesting to determine the usefulness of serum LINE-1 hypomethylation in monitoring tumor progression or treatment response in patients with HCC.

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RESEARCH COMMUNICATION

Line-1 Hypomethylation in Multistage Carcinogenesis of the Uterine Cervix

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Abstract

Objective: To evaluate characteristics of global hypomethylation in evolution of cervical cancer. Materials and Methods: Eight cases of squamous cell carcinoma (SCC) and seven cases of carcinoma in situ (CIS) were studied. Each of the SCC samples contained CIS, and all SCC and CIS samples contained normal ectocervical epithelium. Microdissection was performed to separate normal epithelium, CIS and SCC prior to DNA extraction. Hypomethylation levels of long interspersed nuclear elements (LINE-1 or L1) were measured with a combined bisulfite restriction analysis (COBRA) PCR (polymerase chain reaction) protocol. The percentage of L1 hypomethylation for SCC, CIS and normal epithelium was compared. Results: In the SCC cohort, the L1 hypomethylation level showed progressive increase comparing normal epithelium (59.4 ± 8.86%) to CIS (64.37 ± 7.32%) and SCC (66.3 ± 7.26%) (repeated measurement ANOVA, P = 0.005). A significantly greater L1 hypomethylation level was found in CIS (62.06 ± 3.44 %) compared to normal epithelium (60.03 ± 3.69 %) (paired t-Test, P = 0.03). No significant difference in L1 hypomethylation level was noted between CIS of the two sample groups (unpaired t-Test, P = 0.2). Conclusions: In our study, there was a significant correlation between the degree of hypomethylation and progression from normal ectocervical mucosa to CIS and invasive cancer. Laboratory assessment of biopsies for this molecular event may have clinical significance.

Keywords: Uterine cervical cancer - LINE-1 methylation - global hypomethylation - carcinogenesis

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Introduction

Carcinoma of the cervix uteri is an important cause of death of women worldwide, especially in developing countries (Cannistra and Niloff, 1996; Pornthanakasem et al., 2001). Invasive squamous carcinoma develops through a multistage process of carcinogenesis, and sexually transmitted human papillomavirus is the major etiological factor for cancer development (Cannistra and Niloff, 1996; Pornthanakasem et al., 2001; Tavassoli and Devilee, 2003). Viral oncoproteins E6 and E7 have been shown to interact with tumor suppressor gene products, induce chromosomal abnormalities, and change cellular phenotypes (Pornthanakasem et al., 2001; Tavassoli and Devilee, 2003; Duensing and Munger, 2004). Identification of genetic events during cancer progression is important, not only because it provides a basic knowledge of the disease, but may also have clinical implications given that some of the genetic alterations precede morphological changes in biopsies.

Genome-wide losses of DNA methylation have been demonstrated in several human cancers in which there is downregulation of methylated CpG dinucleotides, which are dispersed throughout the genome both in genes and noncoding repetitive sequences (Sugimura and Ushijima, 2000; Kaneda et al., 2004; Chalitchagorn et al., 2004). Although it remains controversial as to whether the epigenetic change occurs at the initial step or during progression of carcinogenesis (Chalitchagorn et al., 2004; Feinberg et al., 1988), Kim et al (1994) has demonstrated that global hypomethylation increased progressively during the evolution of cervical cancer. Assessment of this epigenomic alteration may have clinical significance, but technical difficulty with conventional methods, such as Southern blotting, has made the evaluation impractical for routine usage.

Using combined bisulfite restriction analysis (COBRA) polymerase chain reaction (PCR), we have recently developed a quantitative method to analyze the methylation status of long interspersed nuclear elements (LINE-1 or L1), called COBRALINE-1 (Chalitchagorn et al., 2004). Our modified PCR protocol targets short amplicon sizes of the widely distributed L1, and is thus highly suitable for formalin-fixed paraffin-embedded material. The purpose of this study was to evaluate L1 hypomethylation status during cervical carcinogenesis using this technique, and to assess the degree of hypomethylation in relation to the morphological changes.

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Materials and Methods

Samples

Two groups of samples were used in this study. The first group included eight cases of invasive squamous carcinoma (SCC) stage I of the cervix uteri obtained from radical hysterectomy. The second group included seven cases of carcinoma in situ (CIS) with free resection margins, obtained from conization or total abdominal hysterectomy. All SCC specimens contained CIS and normal ectocervical epithelium; all CIS samples contained normal ectocervical epithelium.

Laboratory Investigations

Formalin-fixed paraffin-embedded unstained tissue sections were manually microdissected under an inverted light microscope to separate normal epithelium, CIS and SCC prior to DNA extraction. COBRALINE1 analysis was performed as previously described (Chalitchagorn et al., 2004). Briefly, unmethylated cytosines in DNA samples were changed to uracil by treatment with sodium bisulfite. The modified DNA was, then, amplified by 5'UTR L1 bisulfited sequence primers as follows:
5'-CCGTAAGGGGTTAGGGAGTTTTT-3'
5'-RTAAAACCCTCCRAACCAAATATAAA-3'
and digested by TaqI and TasI restriction enzymes, which recognize methylated and unmethylated sequences, respectively. TaqI or TasI-positive amplicons had been previously cloned and sequenced, revealing complete methylation and unmethylation of all linked CpG dinucleotides, respectively (Chalitchagorn et al., 2004). Reproducibility of the test was confirmed by our previous and other subsequent studies (Chalitchagorn et al., 2004; Matsuzaki et al., 2005). In this study, the level of L1 hypomethylation in each sample was expressed as a percentage, dividing the measured intensity (PhosphoImager) of TasI digestible amplicons by the sum of TasI and TaqI products.

Statistical analysis

The L1 hypomethylation level among the three different tissues (normal, CIS and SCC) in the carcinoma group was assessed by repeated measurement ANOVA. To evaluate the differences in L1 hypomethylation level between CIS and normal epithelium in the CIS group, and between CIS in the two groups, paired and unpaired T-tests were used, respectively.

Results

Results are summarized in Figure 1. Of the eight invasive cancers, the degree of L1 hypomethylation level progressively increased from normal epithelium (59.4 ± 8.86%) to CIS (64.37 ± 7.32%) and SCC (66.3 ± 7.26%) (repeated measurement ANOVA, P = 0.005). In the CIS group, the CIS portion showed a significantly greater L1 hypomethylation level (62.06 ± 3.44 %) compared to normal epithelium (60.03 ± 3.69 %) (paired t-Test, P = 0.03). No significant difference in L1 hypomethylation level was noted between CIS of the two sample groups (unpaired t-Test, P = 0.2).

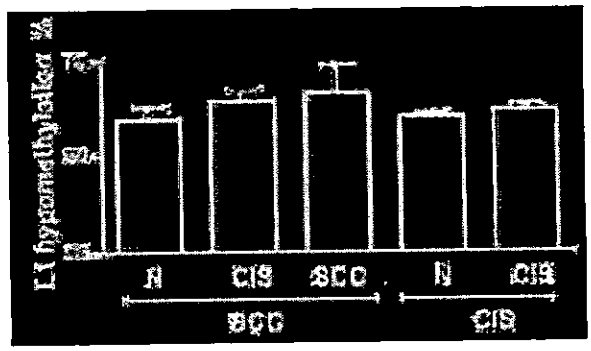

Figure 1. L1 Hypomethylation in Multistage Cervical Carcinogenesis. In samples showing invasive squamous cell carcinoma (SCC), the degree of L1 hypomethylation level progressively increases from normal epithelium (N) (59.4 ± 8.86%) to CIS (64.37 ± 7.32%) to SCC (66.3 ± 7.26%) (repeated measurement ANOVA, P = 0.005). In samples with carcinoma in situ (CIS) without nearby invasive cancer, significantly greater L1 hypomethylation was found in the CIS portion (62.06 ± 3.44 %) compared to normal epithelium (60.03 ± 3.69 %) (paired t-Test, P = 0.03). No significant difference in L1 hypomethylation status was noted in CIS with or without adjacent invasive carcinoma (unpaired t-Test, P = 0.2).

Discussion

LINE-1 (L1), a highly repeated interspersed human retrotransposon, is ubiquitous and constitutes approximately 17% of the human genome. Its methylation status, therefore, reflects the genome-wide methylation level (Chalitchagorn et al., 2004; Gilbert et al., 2004). Although the role of this epigenetic loss in cancer development has not been conclusive, hypothetical mechanisms include increasing genomic instability and interfering with the expression of linked genes (Chalitchagorn et al., 2004; Gilbert et al., 2004; Florl et al., 1999). With COBRALINE-1, we have shown previously that most human carcinomas possessed significantly greater L1 hypomethylation levels, when compared with their normal counterparts (Chalitchagorn et al., 2004). While hypermethylation takes place at specific genes, most cancers are globally hypomethylated, and L1 has been shown to be responsible for the overall losses of DNA methylation (Chalitchagorn et al., 2004; Florl et al., 2004; Feng et al., 2005).

In the present study, we first evaluated how global hypomethylation evolves during the multistep process of carcinogenesis of the cervix uteri. SCC specimens containing CIS and normal epithelium were microdissected and separately analyzed with COBRALINE1. In parallel with the previous investigation (Kim et al., 1994), which utilized a different technique, the degree of global hypomethylation increased progressively during the progression from normal cervical epithelium to CIS and then invasive cancer. Although differences in the hypomethyletion level between CIS (64.37 ± 7.32%) and SCC (66.3 ± 7.26%) in the SCC group and between normal cervical epithelium (60.03 ± 3.69 %) and CIS (62.06 ± 3.44 %) in the other appears to be minimal, considering the fact that L1 segments constitute a significant component of the genome (17%), such magnitude of change would be critical to cellular functions.

Next, we determined whether the precancerous lesion

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Review Article

Quantitative PCR analysis for methylation level of genome: clinical implications in cancer

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Background: Cancer cells are frequently characterized by hypomethylation of the genome including repetitive sequences. This epigenetic process is believed to be associated with several biological causes and consequences in cancer. Therefore, LINE-1 repetitive sequences demethylation in cancer should result in different clinical outcomes.
Objective: Recently, we have developed an improved quantitative combined bisulfite restriction analysis PCR protocol that efficiently evaluates the methylation status of LINE-1s; the method is referred to as PCR "COBRALINE-1". This article reviewed what have been learned by applying this technique to study methylation level of repetitive sequences from several sources of genomic DNA.
Results: We have found that LINE-1 methylation patterns among normal tissues are distinct. Therefore, this epigenetic event may be continuously altered in adult tissues by the process of cellular differentiation. Moreover, we confirmed that global hypomethylation is an ongoing process that develops during tumor progression, in addition to previous evidence of genomic and LINE-1 hypomethylation occurring as an early event in carcinogenesis. COBRALINE-1 is a highly effective technique for evaluating the genome-wide level of methylation, in particular from tissue samples with minute amounts of low quality DNA. The technique has been applied to study samples from micro-dissected archived paraffin-embedded tissues and sera of several types of cancer.
Conclusion: The COBRALINE-1 technique demonstrated its potential to be a tumor marker and a great tool to explore the biology of global hypomethylation.

Keywords: Cancer, LINE-1, COBRALINE-1, DNA methylation, genomic hypomethylation, global hypomethylation, LINE-1 demethylation, retrotransposon, tumor marker.

Cancer cells are frequently characterized by hypomethylation of the genome [1-5]. This epigenetic process is believed to result in chromosomal instability [6-9], increased mutation events [10], and altered gene expression [11]. Recently, we developed an improved quantitative combined bisulfite restriction analysis (COBRA; [12] PCR protocol that efficiently evaluates the methylation status of LINE-1 repetitive sequences in genomic DNA derived from micro-dissected tissue and serum samples), the so-called PCR "COBRALINE-1" [3]. We demonstrated that most cancers exhibit significantly increased levels of hypomethylation compared with their normal tissue counterparts [3]. This article reviews the current knowledge of measurement of LINE-1 level of methylation and the clinical implications in cancer diagnosis.

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Biologic consequence of global and LINE-1 hypomethylation

DNA methylation is one of the main forms of epigenetic control of cells. In cancer, there are two main alterations in epigenetic control [2]: 1) promoter hypermethylation of tumor suppressor genes [13-16] and 2) genome-wide or global hypomethylation [1]. Global hypomethylation has been demonstrated by down-regulation of methylated CpG dinucleotides, which are dispersed throughout the entire genome, both in non-coding repetitive sequences and genes. Global losses of methylation in cancer may lead to

alterations in the expression of proto-oncogenes critical to carcinogenesis [17, 18], may facilitate chromosomal instability [8] and alteration of host defense from mobile genetic elements, viral DNA and transgene [19]. Moreover, loss of methylation at LINE-1 may have additional consequences. LINE-1s are highly repeated and widely interspersed human retrotransposon sequences [20]. There are up to 600,000 copies of LINE-1s in the human genome. Approximately, 2,000 copies remain full length, some may be transcriptionally active, and less than 50 copies are retrotransposible [21].Therefore, changes in the LINE-1 level of methylation may possess additional consequences regarding global hypomethylation. First, insertion of LINE-1 in the intron may result in down-regulation of the linked gene [22, 23]. Second, methylation in the intron may result in down-regulation of the linked gene [24]. Third, up-regulation of LINE-1 may result to genomic instability by increasing generalized DNA double strand breaks [25] and retrotransposition [26, 27]. Moreover, LINE-1s may have a similar function to oncogenes, whereas LINE-1 up-regulation may promote tumor phenotype [28, 29]. Finally, LINE-1 promoter may function to control transcription in both the upstream antisense sequence [30] and in LINE-1 with alternative splicing, causing LINE-1 chimeric RNA [31]. Consequently, a difference in level of LINE-1 methylation among cancers may reflect several biologic consequences, depending on the location of demethylated LINE-1s. Therefore, LINE-1 demethylation in cancer should result in different clinical outcomes.

History of global hypomethylation

Loss of DNA methylation at CpG dinucleotides was the first epigenetic abnormality to be identified in cancer cells and was reported at a symposium at Johns Hopkins in 1982 on tumour-cell heterogeneity by Feinberg and Vogelstein [1]. Since then, genome-wide hypomethylation has been reported in several malignancies [2, 3]. In addition, the extent of global hypomethylation appears to correlate with tumor progression and invasiveness in several cancer types, thus indicative of its role in tumor development and progression [32, 33].

What do we learn from COBRALINE-1?

We developed a PCR-based analysis for quantifying the genome-wide level of methylation to improve upon the conventional Southern blot and hybridization approach [3]. Southern blot requires large amounts of DNA and consequently it is difficult to analyze tumor DNA derived from micro-dissection. We applied a modified COBRA PCR protocol to efficiently and quantitatively evaluate LINE-1 methylation status in the entire genome of isolated cell populations. This new protocol targets shorter amplicon sizes of the widely distributed LINE-1 sequences, which greatly improves the yield when amplifying genetic material derived from micro-dissected paraffin-embedded tissue. Interestingly, based on our experience, DNA from paraffin-embedded tissue is difficult to be amplified by conventional PCR methods [34, 35] because DNA is usually degraded by formalin [34]. Interestingly, we experienced a very limited failure rate of PCR by COBRALINE-1, which may be due to the large number of repetitive sequence in the genome [20].

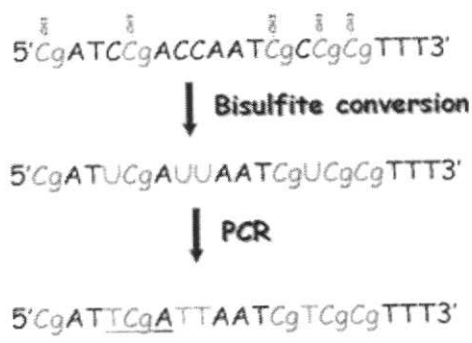

Fig. 1 Schematic representation of DNA sequences. From above to below, the sequences were methylated DNA, bisulfited DNA and bisulfited DNA after PCR, respectively. Bisulfite reaction converted unmethylated cytosines to uracils but methylated cytosines were not changed. After PCR, uracils were amplified as thymine. The underline nucleotide represents sequence that was digestible by *Taq*I.

Before PCR, to distinguish between methylated and unmethylated sequences, the DNA is treated with bisulfite [14]. Treatment with bisulfite converts unmethylated cytosines, but not methylated cytosines, to uracils and then thymines after PCR (**Fig. 1**). This generates detectable methylation-dependent changes in the restriction pattern of PCR-amplified LINE-1 sequences. The modified DNA was amplified by 5'UTR LINE-1 bisulfited sequence primers and then digested with *Taq*I and *Tas*I restriction enzymes, which recognize methylated and unmethylated sequences, respectively. The level of LINE-1 hypomethylation in each sample was calculated by dividing the measured intensity of *Tas*I digestible amplicons with the sum of the *Tas*I and *Taq*I products [3] (**Fig. 2**).

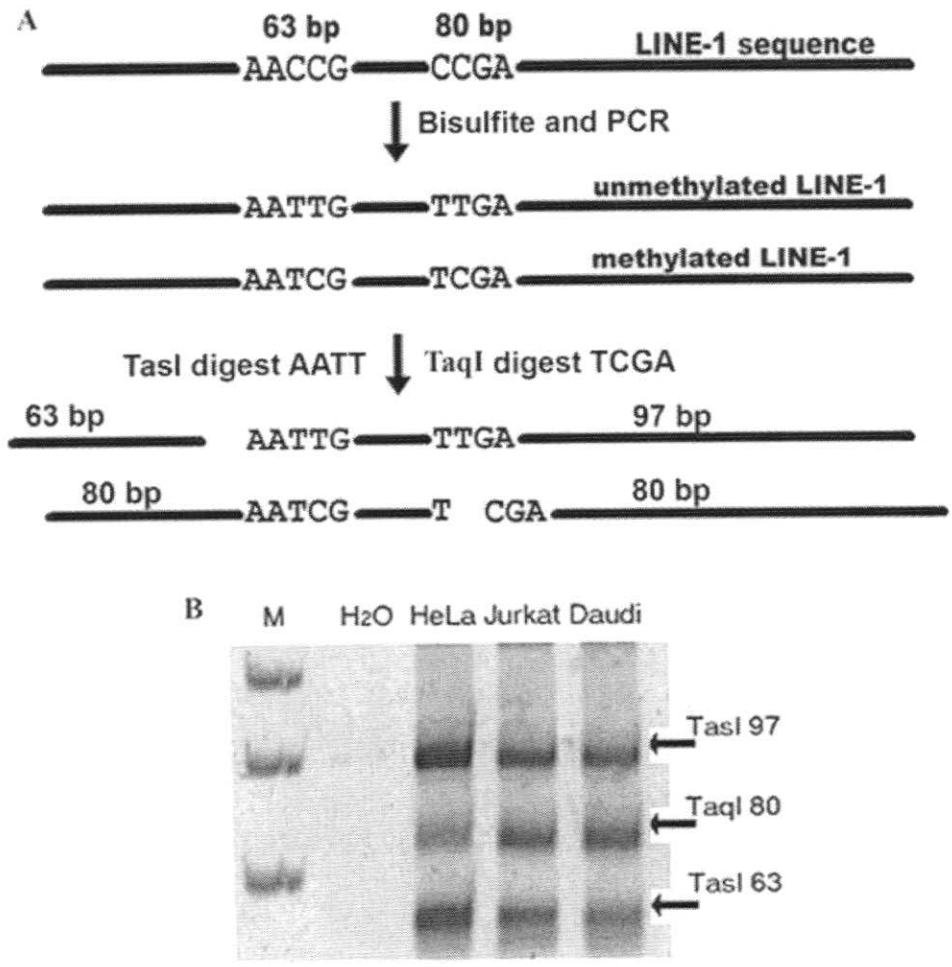

Fig. 2 Combined bisulfite restriction analysis of LINE-1 (COBRA LINE-1) [3]. LINE-1 methylation level was calculated as a percentage of intensity of *Taq*I divided by the sum of *Taq*I-and *Tas*I-positive amplicons. (**A**) Schematic illustration of COBRA LINE-1. The LINE-1 amplicon size is 160 bp. Methylated amplicons, *Taq*I positive, yielded two 80 bp DNA fragments, whereas unmethylated amplicons, *Tas*I positive, yielded 63 and 97 bp fragments. (**B**) An example of COBRA LINE-1. M, 10-bp DNA size marker; numbers under each sample, methylation levels; TasI 63 and 97, unmethylated amplicons; TaqI 80, methylated amplicons. H2O is water, HeLa, Jurkat and Daudi are cell lines. HeLa LINE-1s are the most hypomethylated among the three.

Interestingly, there are distinctive LINE-1 methylation patterns among normal tissues [3]. In most cases, the distribution of detectable LINE-1 hypomethylation within normal tissue of the same type was consistently clustered, whereas the levels of methylation in thyroid and esophageal tissues were widely distributed. Strikingly, the levels of LINE-1 methylation were significantly different among different tissue types. Our data suggest that this epigenetic event may be continuously altered in adult tissues by the processes of cellular differentiation (**Fig. 3A**). Moreover, this finding implies the physiologic function of LINE-1 level of methylation, which would be new knowledge regarding how human genes are controlled.

For cancer, our finding is consistent with the previous notion that global hypomethylation is a common epigenetic event in cancer. Carcinomas, including breast, colon, lung, head and neck, bladder, esophagus, liver, prostate, and stomach, reveal a greater percentage of hypomethylation than their normal tissue counterparts [3] (**Fig. 3B**). Only lymphomas and carcinomas of the kidney and thyroid do not exhibit significant LINE-1 methylation alterations (**Fig. 3B**). Collectively, with findings from other approaches, global hypomethylation has been found in almost all types of cancers, including colon [1], lung [3], breast [36, 37], stomach [38], prostate [3], ovary [32], urinary bladder [39], and head and neck [3]. It is interesting to note that global hypomethylation is detectable in those cancer types known to have premalignant lesions (Shuangshoti S, *personal communication*). Therefore, global hypomethylation evolves through a multistep process during carcinogenesis. This hypothesis is consistent with our finding that LINE-1 hypomethylation increases in direct relation with tumor progression. Therefore, global hypomethylation is an ongoing process developing during tumor progression, in addition to previous evidence of genomic and LINE-1 hypomethylation occurring as an early event in carcinogenesis [3].

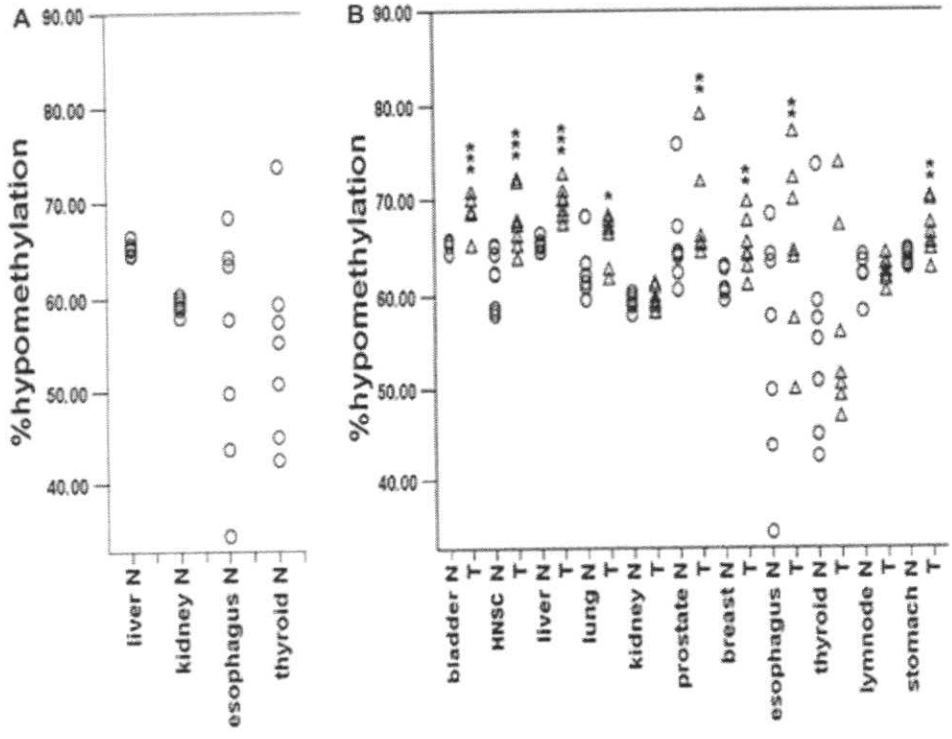

Fig. 3 LINE-1 hypomethylation levels in several tissue types [3]. Circles and triangles are levels of COBRA LINE-1 from normal and malignant, respectively. The vertical axis displays percentage levels of LINE-1 hypomethylation. Sample types are labeled. (A) is the hypomethylation levels of adjacent normal tissues and (B) is normal and cancers. Single, double, and triple asterisks indicate significant differences in hypomethylation levels between normal tissues and the tested samples at $P<0.05$, <0.01, and <0.001, respectively. N and T are normal and malignant tissues, respectively. The figure was modified from Chalitchagorn K *et al.* [3].

Current clinical application

Applying COBRALINE-1 in analyzing the genome-wide level of methylation has the advantage over conventional Southern blot and hybridization techniques in that minute quantities of low quality of DNA can be analyzed. Therefore, several sources of DNA can be analyzed, in particular DNA sources from body fluids. Moreover, the analysis can be performed in a quantitative fashion. Consequently, the correlation among large sample sizes can be effectively determined.

Cancer DNA is released into a patient's circulation by an unknown mechanism [40]. The amount of tumor DNA depends on the type and severity of the malignancy. For example, whereas Epstein Barr viral DNA is found in serum/plasma from the majority of patients with nasopharyngeal carcinoma, few patients with cervical cancer have human papilloma viral DNA in their plasma [41]. Moreover, patients with cervical cancer in whom the plasma is HPV-positive are strongly associated with metastasis [41]. Because LINE-1 hypomethylation is a common event in cancer and the level may directly correlate with clinical severity, we evaluated the use of COBRALINE-1 in the serum of cancer patients. Our first systematic approach was to evaluate the sera of patients with hepatocellular carcinoma (HCC) [42]. Serum genome hypomethylation, the percentage of unmethylated LINE-1, was significantly increased in patients with HCC. The levels of serum LINE-1 hypomethylation on initial presentation correlated significantly with the presence of HBsAg, large tumor sizes, and advanced tumor stages, as classified by the CLIP score. Multivariate analyses showed that serum LINE-1 hypomethylation was a significant and independent prognostic factor of overall survival. Therefore, serum LINE-1 hypomethylation may serve as a prognostic marker for patients with HCC [42].

Our ongoing approach was to evaluate whether LINE-1 hypomethylation is a potential prognostic factor for epithelial ovarian cancer (EOC). We found that LINE-1 levels of methylation in EOC were lower than in representative normal ovarian tissues. The mean percentage of the level of methylation in the cancer group was lower than in the control group. In addition, the LINE-1 levels of methylation among specific histologic subgroups were different. The mean LINE-1 levels of methylation ranked higher-to-lower were as follows: mucinous, serous, endometrioid, and clear cell carcinomas. An increase in the level of hypomethylation was correlated with a higher FIGO stage, advanced tumor grade, elevated CA 125 level, and tumor recurrence. Patients with greater hypomethylation had poorer mean overall survival and a lower mean progression-free interval. Therefore, LINE-1 hypomethylation is a common and important epigenetic process in ovarian carcinogenesis. Moreover, the COBRALINE-1 method has the potential to be used as a tumor marker for EOC.

Cho et al. [43] studied hypermethylation of CpG island loci and hypomethylation of LINE-1 and Alu repeats in prostate adenocarcinoma and their relationship to clinicopathologic features. They found that prostate adenocarcinoma with hypermethylation of ASC, COX2, RARB, TNFRSF10C, MDR1, TIG1, RBP1, NEUROG1, RASSF1A, and GSTP1 showed a significantly lower level of methylation of Alu or LINE-1 than prostate adenocarcinoma without hypermethylation. In addition, hypomethylation of Alu or LINE-1 was closely associated with one or more of the above prognostic parameters. These data suggest that with respect to tumor progression, a close relationship exists between CpG island hypermethylation and the hypomethylation of repetitive elements, and that CpG island hypermethylation and DNA hypomethylation contribute to cancer progression [43].

Similarly, the PCR-based method has been used to evaluate the significance of methylation of repetitive sequences in particular Alu and LINE-1 in association with chromosomal instability, and microsatellite instability (MSI) remains unclear in colorectal cancer. Matsuzaki et al. [44] investigated the relationship between global methylation status, loss of heterozygosity (LOH), and MSI in sporadic colorectal cancer. They found LOH was observed more frequently in microsatellite stable (MSS) cancers than in MSI cancers at all loci. MSS cancers showed significantly lower global methylation levels when compared with MSI cancers. Tumors with higher global hypomethylation had a significantly increased number of chromosomal loci with LOH than did tumors without global hypomethylation. This suggests that global hypomethylation plays an important role in inducing genomic instability in colorectal carcinogenesis [44].

Interestingly, the level of global hypomethylation from white blood cells may reflect head and neck

cancer risk. Hsiung et al. [45] determined whether global methylation in DNA derived from whole blood was associated with head and neck cancer. They found that hypomethylation lead to a significant 1.6-fold increased risk for disease in models controlled for other head and neck squamous cell cancer (HNSCC) risk factors. Smoking showed a significant differential effect on blood levels of methylation between cases and controls. Therefore, DNA hypomethylation in non-target tissues was independently associated with HNSCC and had a complex relationship with the known risk factors associated with the genesis of HNSCC [45]. The mechanism to account for this finding is not known. However, it is interesting to speculate that changes in the genome-wide level of methylation are systemic rather than local. Chronic feeding of a methyl-donor (methionine, choline, folic acid, and vitamin B12) deficient diet induces hepatocellular carcinoma in rats. Asada et al. [46] determined cytosine methylation status in the LINE-1 repetitive sequences of rats fed a choline-deficient (CD) diet of various durations and compared the cytosine methylation status with rats fed a choline-sufficient diet. Progressive hypomethylation was observed in LINE-1 liver of rats fed a CD diet as a function of feeding time [46].

Other applications

LINE-1 methylation may be continuously altered in adult tissues by the processes of cellular differentiation [3]. Therefore, it is interesting to explore the role of LINE-1 methylation during human developmental process. Perrin D et al [47] studied global DNA methylation of trophoblastic tissues. Partial hydatidiform mole and normal placenta have identical global levels of DNA methylation. Surprisingly, LINE-1 sequences are hypermethylated in partial hydatidiform mole tissues. This confirmed the physiologic roles of LINE-1 methylation.

Several causes and consequences of global hypomethylation appear to occur in cis that is linked to nearby unmethylated DNA. For example, studies in ICF syndrome (immunodeficiency, chromosomal instability, and facial anomalies) with loss-of-function mutations in the cytosine DNA methyltransferase *DNMT3B* [48] and Wilm's tumor [49] demonstrated the direct association between loss of DNA methylation and rearrangements in the pericentromeric heterochromatin. Therefore, hypomethylation could lead to spontaneous mutations in cis. Moreover, LINE-1 methylation may influence the linked gene expression [22, 30, 31]. Consequently, LINE-1 demethylation may lead to an alteration of linked gene expression. Therefore, COBRALINE-1 should be a great tool for evaluating the biology of these phenomena. Finally, alteration of DNA methylation should not only affect tumor phenotype, but a potential role of changes in human DNA methylation patterns in other conditions, such as atherosclerosis and autoimmune diseases (e.g., multiple sclerosis, psoriasis, and lupus) has been recognized [50-52]. Therefore, in addition to cancer, COBRALINE-1 should be a great tool to study fundamental biologic processes and may be a diagnostic tool in the future.

Further improvement

The potential clinical application of the COBRALINE-1 approach is illustrated by a significantly higher serum LINE-1 hypomethylation levels in HCC patients when compared to normal individuals [42]. However, the two levels are, as expected, considerably overlapped. Further improvements of the current method in order to show a more distinct LINE-1 hypomethylation pattern for tumor versus normal tissues, if possible, are crucial. Our previous study [3] revealed that there are differences of LINE-1 methylation levels not only among normal tissues but also cancer types and stages. Our ongoing researches indicate that the differences may be due to the distinctive patterns of methylation among LINE-1 loci. Therefore, LINE-1 hypomethylation from several loci may serve as potential prognostic markers for certain cancers.

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Demographic changes and marker properties affect detection of human population differentiation

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Abstract

Background: Differentiating genetically between populations is valuable for admixture and population stratification detection and in understanding population history. This is easy to achieve for major continental populations, but not for closely related populations. It has been claimed that a large marker panel is necessary to reliably distinguish populations within a continent. We investigated whether empirical genetic differentiation could be accomplished efficiently among three Asian populations (Hmong, Thai, and Chinese) using a small set of highly variable markers (15 tetranucleotide and 17 dinucleotide repeats).

Results: Hmong could be differentiated from Thai and Chinese based on multi-locus genotypes, but Thai and Chinese were indistinguishable from each other. We found significant evidence for a recent population bottleneck followed by expansion in the Hmong that was not present in the Thai or Chinese. Tetranucleotide repeats were less useful than dinucleotide repeat markers in distinguishing between major continental populations (Asian, European, and African) while both successfully distinguished Hmong from Thai and Chinese.

Conclusion: Demographic history contributes significantly to robust detection of intracontinental population structure. Populations having experienced a rapid size reduction may be reliably distinguished as a result of a genetic drift -driven redistribution of population allele frequencies. Tetranucleotide markers, which differ from dinucleotide markers in mutation mechanism and rate, are similar in information content to dinucleotide markers in this situation. These factors should be considered when identifying populations suitable for gene mapping studies and when interpreting interpopulation relationships based on microsatellite markers.

Background

Genetic characterization and differentiation of populations are often necessary for the conduct of valid case-control association studies [1-5], determining the role of ancestry in phenotypic differences [6,7], assigning population groups for valid linkage analysis [8], examining the distribution of neutral genetic variation among populations, and inferring migration histories [9-11]. Such differentiation has been accomplished with relative ease between major continental populations [10,12-15], but it has been asserted that population differentiation *within* a continent may not be possible; and when it appears to be so, may actually be an artifact of study design [16].

The ubiquity and frequently highly variable nature of short tandem repeat polymorphisms (STRs or microsatellites) have made them desirable markers for measuring population stratification. Commercially available marker sets such as those used for forensic purposes make STR genotyping cost effective, eliminating the time and effort required to develop multiplex marker panels. Panels developed for forensic purposes are designed to identify or exclude an individual as a match for another sample and were compiled, in part, for their high levels of variation in many populations [17]. Such panels have been adopted for non-forensic purposes such as inference of population phylogenies [18-21] and quantification of levels of population differentiation [1,2,5,8].

Homoplasy, as applied to STRs, refers to the situation where alleles of the same length have arisen from different mutation events, such that alleles identical-by-state are not necessarily identical-by-descent. Simulations of STR evolution using the stepwise mutation model (SMM) have indicated that homoplasy in STR genotypes may cause individuals or populations to appear to be more genetically similar than they really are. Point mutations, insertion or deletion events (indels), or complex repeat motifs can generate additional forms of size homoplasy that are sometimes revealed by sequencing but are not detectible through size fractionation (electrophoresis) [22-24]. These forms of homoplasious alleles have been observed in a number of the tetranucleotide repeats that are standard in forensic panels (some of which are included among the markers used in this study; see below) [25].

However, it has also been shown that even in the presence of homoplasy, multi-locus genotypes (the combined genotypes from multiple loci) of highly variable STR markers are effective in assigning individuals to known or unknown populations [26-33]. Again, this has typically been true for large continental populations. Population differentiation *within* a continent has been successful, but only with large numbers of markers when applied to population isolates [10]. Here, we used a small set of markers and, in contrast to the majority of past studies, addressed the properties of the markers used.

Conditions such as small population size or recent founding of a population may enable statistical differentiation using a small panel of highly variable markers, due to increased effects of genetic drift and decreased incidence of homoplasy. To evaluate this possibility, we investigated whether empirical genetic differentiation could be accomplished efficiently among three closely related Asian populations (Hmong, Thai, and Chinese) using a small set of STRs that includes both tetranucleotide and dinucleotide markers. In addition, we studied the relative information content of tetranucleotide versus dinucleotide markers for discriminating among these three Asian populations, as well as European Americans (EA) and African Americans (AA). We then evaluated the populations for evidence of recent changes in effective population size.

Results

Population differentiation

The program STRUCTURE 2.1 [32,33] uses Bayesian clustering of multilocus genotypes to assign individuals to populations, estimate admixture proportions for individuals, and infer the number of parental populations (K) for a sample. For STRUCTURE runs which included the three East Asian populations only and all 32 markers, the Hmong were allocated into a cluster distinct from a single Thai/Chinese cluster with 86.0% estimated ancestry for K = 2 with a posterior probability (Pr(K = 2)) of 1, indicating K with the best fit for the data (Figure 1a). Separate Thai and Chinese clusters were not inferred with K = 3 and Pr(K = 3) was effectively zero (3.3×10^{-156}) (Figure 1b). When the three East Asian populations were analyzed with EA and AA samples, the Hmong were then allocated to a separate cluster with an average of 90.0% estimated ancestry when K = 4 and Pr(K = 4) = 1 (Figure 1c). Under these same conditions, the Thai and Chinese were assigned together to a single cluster with 86.5% and 84.2% estimated ancestry, respectively. When K was increased to 5, the Thai and Chinese populations continued to form a single cluster (Figure 1d) and Pr(K = 5) was 1.7×10^{-48}. For K = 2, K = 3, or K = 6, Pr(K) was similarly effectively zero (barplots not shown).

When the markers were separated by repeat size the extent of successful population assignment differed greatly between the two panels; for STRUCTURE runs using the 15 tetranucleotide markers alone Pr(K = 3) was 1, while for the 17 dinucleotide markers alone Pr(K = 4) was 1. In addition, although K = 3 had the best fit for the data for tetranucleotide markers, assignment of individuals to major continental populations was not robust (Figure 1e) (EA 63.2%, AA 75.3%, Thai 54.1%, Chinese 48.7%,

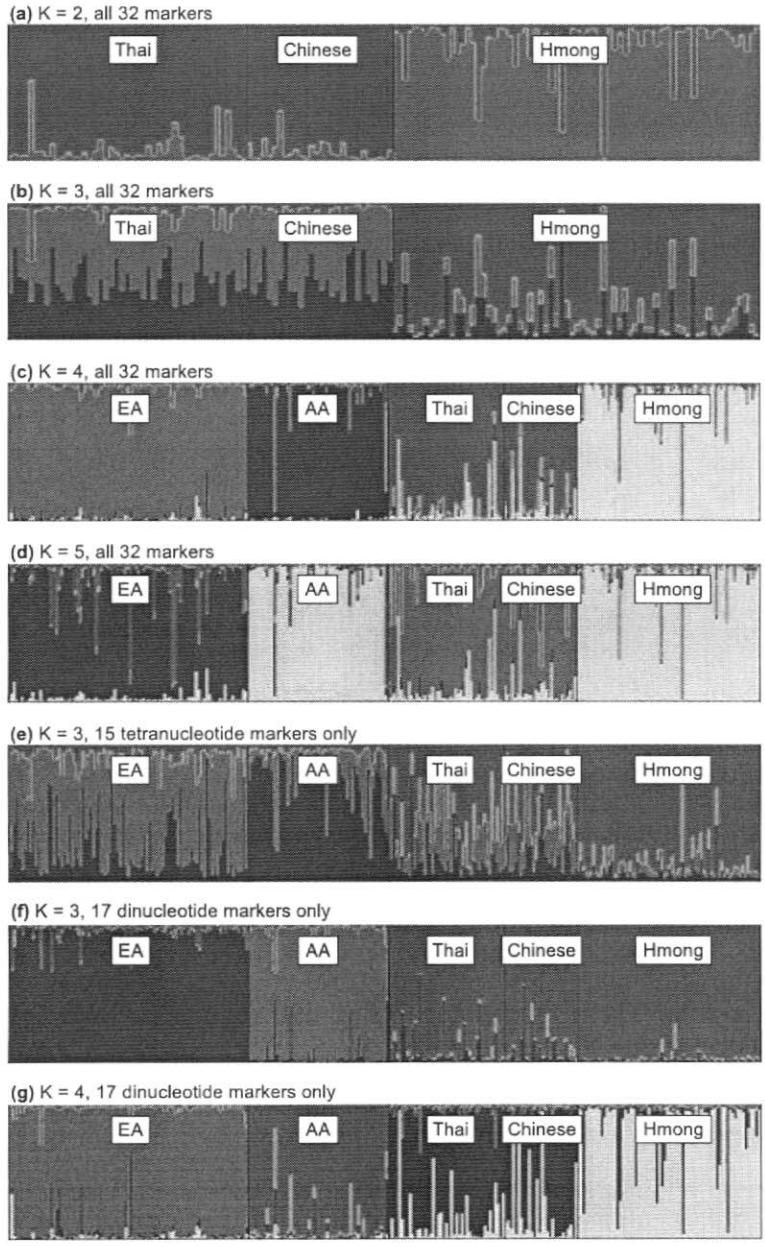

Figure 1
Hmong populations are consistently and reliably distinguished from all others in STRUCTURE analyses using a small number of either dinucleotide or tetranucleotide markers while for other populations successful assignment of individuals varies with marker type. In **(a)** and **(b)** Chinese, Hmong, and Thai samples were assigned by STRUCTURE to 2 or 3 populations respectively. In **(c)** and **(d)** European-American (EA), African-American (AA), Chinese, Hmong and Thai samples were assigned to 4 or 5 populations respectively. Finally, tetranucleotide markers **(e)** are less useful for differentiating among EA, AA, Thai and Chinese populations than dinucleotide markers **(f & g)**. These plots were produced using the STRUCTURE software; each individual is represented by a vertical line depicting the estimated percent assignment of the individual into K assumed populations. Each assumed population is represented by one color. Vertical black lines separate individuals by self-reported ancestral population.

Hmong 86.1%). Dinucleotide markers alone resulted in higher assignment rates than those of the tetranucleotide markers when K = 3 (Figure 1f) (EA 94.0%, AA 91.4%, Thai 88.3%, Chinese 88.4%, Hmong 97.3%) or under the best fit for the data, K = 4 (Figure 1g) (EA 91.4%, AA 90.4%, Thai 81.0%, Chinese 73.6%, Hmong 82.8%).

Out of concern that each Hmong village in which samples were collected could consist of its own apparent cluster due to close relatedness within each village, the villages were analyzed initially as separate populations in STRUCTURE under the same conditions as all STRUCTURE runs reported here. In all cases, the two source villages formed one cluster and the average assignment values for all population samples, including Hmong, were no different than assignment values reported here when the two villages were combined and assumed to be one population (data not shown).

Effective population size

The Hmong sample was found to have a heterozygosity deficiency ($p = 0.004$), based on a sign test in BOTTLENECK [34], indicating a possible recent population expansion. Given the number of observed alleles, if the Hmong population was at equilibrium heterozygosity is expected to be higher than that which is observed. All other samples had neither excess nor deficiency for this measure.

Relatedness

Based on maximum-likelihood estimates of pair-wise relationships, potential parent-offspring pairs and sibling pairs were discovered in the Chinese and Hmong samples. In each case, one individual was then deleted from the sample and excluded from all other analyses.

Hardy Weinberg Equilibrium (HWE)

No population showed significant deviation from HWE over all loci (EA $p = 0.07$, AA $p = 0.82$, Chinese $p = 0.57$, Thai $p = 0.87$, Hmong $p = 0.99$) (Table 1). If a Bonferroni correction is applied to correct for multiple testing, (requiring a p value of $0.05/32 = 0.00156$ for significance) none of these p-values for individual loci are significant (Additional File 1).

Heterozygosity

The mean observed heterozygosity (H_o) (Table 2) for all loci was not statistically different for any of the Asian population pairs, based on paired two-sample t-test (Chinese/Hmong $p = 0.07$, Thai/Chinese $p = 0.34$, Thai/Hmong $p = 0.27$). With the exception of EA/Chinese, mean observed heterozygosity was significantly different for all other population pairs (EA/AA $p = 0.01$, EA/Thai $p = 0.03$, EA/Chinese $p = 0.37$, EA/Hmong $p < 0.01$, AA/Hmong $p < 0.01$, AA/Thai $p < 0.01$, AA/Chinese $p = 0.04$).

Mean tetranucleotide H_o was not significantly different from mean dinucleotide H_o for any population other than Chinese based on a two-sample t-test (Table 3) (AA $p = 0.49$, EA $p = 0.17$, Thai $p = 0.30$, Chinese $p = 0.05$, Hmong $p = 0.57$).

Marker information content

The mean Hmong/Thai and Hmong/Chinese δ values are nearly equivalent, and the Hmong were similarly differentiated from these two populations (delta values for each locus and mean delta values for all loci and by repeat size are reported for each population pair in Additional File 2). The low mean Chinese/Thai δ appears to explain the inability of this marker panel to assign the Thai and Chinese to separate clusters. Overall, the dinucleotide markers provide more information than the tetranucleotide markers, but this difference is not as great for population pairs that include the Hmong; for the Hmong/Chinese and Hmong/Thai population pairs, the difference in the average dinucleotide δ and the average tetranucleotide δ is negligible (Figure 2).

Discussion

In this study, we successfully differentiated between closely related populations using a marker set much smaller than that previously suggested to be minimally necessary for such studies. We used a set of highly polymorphic microsatellite markers of which some were specifically selected for high δ between EA, AA, and Asian populations [15], however, the value of this marker set for differentiating populations within Asia was previously unknown. To explain our results, we investigated the evolutionary histories of the samples, and found evidence for changes in N_e for the Hmong population, based on an excess of rare alleles. This tribal population has a recent history of repeated fractioning and migration throughout Southeast Asia as well as loss of numbers due to military conflict, which is consistent with our results [35]. Further suggestive evidence of a recent Hmong population bottleneck followed by expansion can be found in the delta values of tetranucleotide markers compared to that of dinucleotide markers. Delta measures absolute values of allele frequency differences which can arise over time via accumulated mutations or through deviations from neutral conditions such as drift caused by a bottleneck.

If time since divergence determines differences in allele frequencies delta should be correlated with time since divergence. The tetranucleotide markers consistently provide as much information for the Hmong as the dinucleotide markers provide, while this is not so for any other population. We propose that this suggests forces other than mutation as measured by divergence time contributing to differences in population allele frequencies between Hmong and other populations examined here.

Table 1: Results from Fisher's test for deviation from HWE for all 32 loci combined

	EA	AA	Thai	Chinese	Hmong
chi square	81.3	53.6	51.5	61.3	41.6
df	64	64	64	64	64
p-value	0.07	0.82	0.87	0.57	0.99

The effects of this can be seen in the differences in clustering behavior using STRUCTURE when either tetranucleotide or dinucleotide genotypes are analyzed alone – specifically, the difficulty in using the tetranucleotide panel to assign individuals to major continental groups for all populations, with the exception of the Hmong. Rosenberg et al [30] found dinucleotide markers to be more informative than tetranucleotide markers for population assignment in a larger study based on a different measure of marker informativeness. In their study, populations from the Americas or Oceania were exceptions to this pattern. The authors proposed genetic drift during founding events as one explanation for their results.

Mean tetranucleotide H_o was not statistically different from mean dinucleotide H_o for any population other than Chinese. Although these markers suggest high intrapopulation variation for all populations in this study, this does not provide information on differences in the sources of that variation either within or between populations for each type of marker.

The decrease in difference between δ values between the two subsets of markers for population combinations that include the Hmong indicates that genetic drift (random changes in allele frequencies from one generation to the next that are more likely to affect small populations) rather than mutation has been a major force of evolution contributing to observed allele frequencies in this population. Differences in marker information content between the tetranucleotide and dinucleotide panels for all other populations in this study indicate that mutation rate and mechanism have shaped allele frequency distributions in these populations more than genetic drift, as would be expected for large populations at mutation-drift equilibrium.

The dinucleotide markers were previously selected for differentiation between European and African populations and high variation [15] and the tetranucleotide markers were chosen for forensic purposes for their high rates of variation in multiple populations.

Total sample size, unequal sample size between populations, and number of markers can affect the stability of clustering in STRUCTURE [36,37]. We cannot exclude biases introduced through these study design elements influencing our observations, however, stable clustering patterns were inferred in this case by repeated STRUCTURE runs. Although increasing the number of markers or population sample size can strengthen clustering patterns where clustering exists, the number of individuals in a sample or the minimum number of markers necessary to differentiate between all populations is dependant on the evolutionary histories of the population samples. Sample sizes similar to ours have been demonstrated previously to be generally sufficient for stable and accurate clustering [36].

Some of the tetranucleotide markers in this study have been shown to consist of complex repeats including more than one repeat motif, as well as insertions or deletions of partial repeats [17] (structure of observed alleles and their amplicon sizes can be found for tetranucleotide repeats typically used for forensic purposes and in this study at [25]. These factors, as well as historically large effective population size such as those of the EA and AA populations, increase the likelihood of size homoplasy. We hypothesize that the accumulation of homoplasious alleles of tetranucleotide loci may contribute to their lower information content when compared to that of the dinucleotide markers in populations other than Hmong.

Table 2: Mean Nei's gene diversity (H_z)and mean observed heterozygosity (H_o)for all markers for each population

	N	H_z	H_z SD	H_o	H_o SD
EA	91	0.77	0.01	0.76	0.01
AA	54	0.81	0.01	0.81	0.01
Thai	45	0.74	0.02	0.72	0.01
Chinese	28	0.75	0.03	0.74	0.01
Hmong	70	0.71	0.02	0.69	0.01

Table 3: Mean observed heterozygosity (H_o) for each marker type for each population

	EA	AA	Thai	Chinese	Hmong
H_o tetranucleotide	0.78	0.80	0.74	0.80	0.71
H_o dinucleotide	0.75	0.82	0.69	0.68	0.68

Conclusion

When STR loci are used either to detect association, linkage, or population substructure, population history and marker choice both affect study results. Demographic history and marker properties are often overlooked when determining population or marker suitability for gene mapping studies (i.e. to identify variants that affect traits), but have bearing on the efficiency and feasibility of such studies.

The three Asian populations in this study have mean H_o values which are not significantly different from each other. It is likely that genetic drift, in conjunction with long-standing endogamy, allow the Hmong to be statistically differentiated from the Thai and Chinese using multilocus genotypes, despite the high level of within-population variation of the Hmong. Potential homoplasy in populations at equilibrium warn against the use of STRs (particularly those with known homoplasious alleles) for phylogenetic analysis or linkage or association mapping purposes in large populations, other than quantifying population stratification. Since the tetranucleotide repeats used in commercially available kits designed for forensic purposes have been well-characterized and have been demonstrated to contain many instances of size homoplasy, these markers should not be relied upon for phylogenetic analyses. Risks of homoplasy interfering with association or linkage analysis, in which identical-by-state is often assumed to mean identical-by-descent,

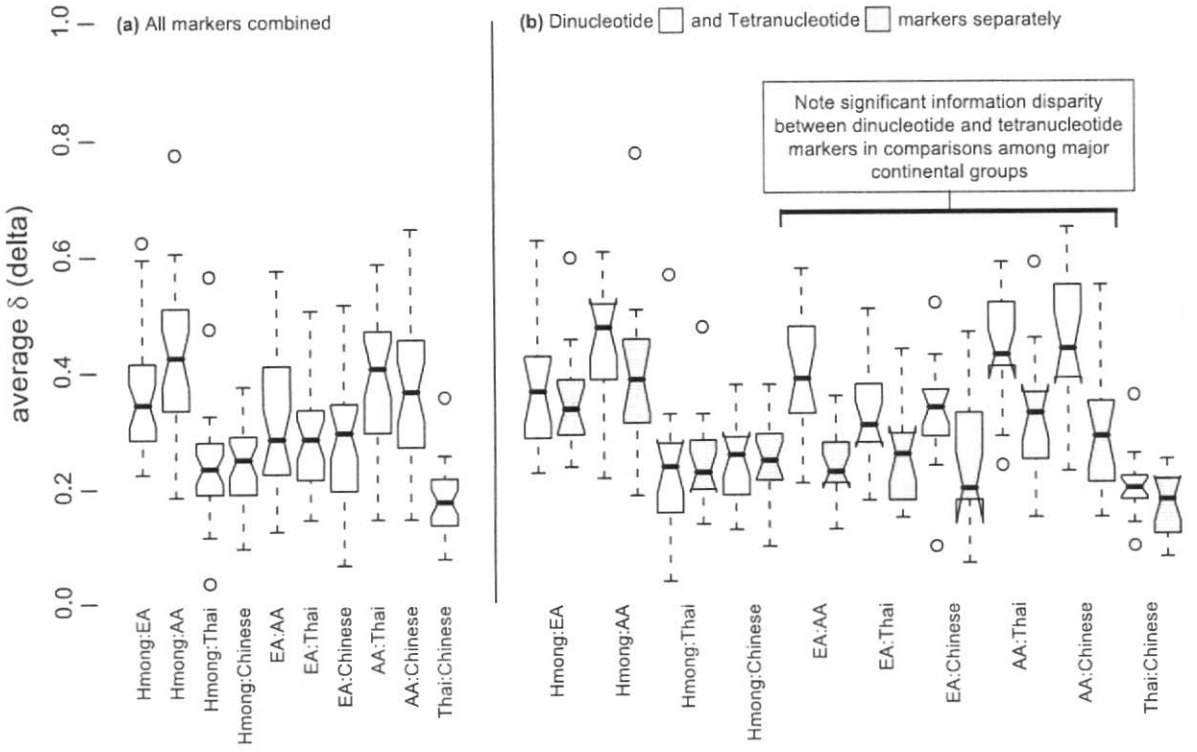

Figure 2
The left side of the figure shows the average delta for all 32 loci for each population pair and the right side of the figure shows the average delta for 15 tetranucleotide (shaded) and 17 dinucleotide (unshaded) markers, separately, for each population pair.

should be less of a concern in populations that have undergone recent bottlenecks.

In addition, a history of migrations or bottlenecks in an isolated population is expected to initially reduce levels of intrapopulation variation, and increase interpopulation differentiation [38]. Linkage disequilibrium (LD) will be higher in such populations [39,40]. A subsequent population expansion will recover allelic variation faster than LD will degrade for a given genomic region. Low intrapopulation variation, the corresponding increase in interpopulation variation, and higher LD, have been identified as desirable characteristics in a population for gene mapping and admixture detection [41,42], making geographically or culturally isolated populations with a history of bottlenecks potentially more valuable for gene mapping than populations whose size has remained stable and large. In addition to reducing genetic heterogeneity for the trait of interest, use of such populations also could reduce the costs of association mapping studies because the number of subjects needed for a specified power level is inversely related to the population's level of LD [43]. It would therefore be useful to identify such populations prior to designing a study.

Existence of a large number of rare alleles within a population can be the result of a bottleneck followed by expansion [44-46]. It is possible to infer these events from a significant heterozygosity deficiency in the Hmong sample based on results from BOTTLENECK. The data from the EA, AA, Chinese and Thai samples do not violate the assumptions of mutation-drift equilibrium. These data suggest that the populations in this study have been large and at equilibrium for a relatively long period, with the exception of the Hmong population (as represented by the individuals we sampled). Unknown migrants or recent admixture can introduce new alleles into a population at initial low frequencies, mimicking the pattern caused by population expansion. Such recent admixture is not a likely explanation of the data in this case in light of successful clustering of the Hmong sample when analyzed with samples from the two populations most likely to contribute to hypothetical admixture: Thai and Chinese.

STRUCTURE, and other clustering algorithms, detect admixture and quantify population differentiation through differences in population allele frequencies. These differences which allow for successful clustering arise through various evolutionary forces and are shaped by ascertainment processes which must also be considered when identifying populations suitable for gene mapping studies or interpreting estimates of inter or intrapopulation genetic distance. The ability to differentiate between East Asian populations that have diverged recently relative to major continental populations indicates that it may also be possible to use more easily-accessible closely related populations, such as European, for admixture mapping if marker choice and population history are taken into account.

Methods

Populations and sampling

The Asian populations in this study were collected as part of an ongoing gene mapping study. Samples of self-identified Thai (N = 45) and Chinese (N = 29) were obtained from a blood drive in Bangkok, Thailand. The Thai and Chinese samples used in this study were selected to include only subjects for whom all four grandparents were reported to have the same self-identified ethnicity as the subject. The Hmong, a Miao-Yao-speaking group of the Austro-Thai language family, are an endogamous tribal population with an estimated total population throughout China, Laos, Vietnam, and Burma of eight million, approximately 120,000 of whom reside in Thailand. Chinese written history documents the presence of Hmong in Central China at least 2,300 years ago and their migration to Southern China several hundred years later. Migrations farther south have occurred since the seventeenth century [47]. Hmong refugees fleeing military conflict in Laos have periodically been resettled since 1975 in the U.S., France, and Australia. Hmong samples (N = 103) were obtained in two Hmong villages in northern Thailand. Data on grandparents' reported ethnic affiliation were not available for the Hmong subjects. The dataset also included samples of unrelated African Americans (AA, N = 54) and European Americans (EA, N = 91), a subset of a sample described elsewhere [15]. Both EA and AA samples were self-identified as such, and these identifications were previously confirmed via Bayesian marker clustering [15]. After immediate relatives were discovered and excluded from analysis (see below), sample sizes were reduced as follows: Hmong (N = 70) and Chinese (N = 28). No close relative pairs were found within the remaining three population samples. All subjects provided informed consent as approved by the appropriate institutional review boards.

Markers and genotyping

For the three East Asian populations, DNA was extracted directly from blood using PaxGene materials and the manufacturer's specified protocol (Qiagen, Valencia CA, USA) (Hmong) or standard phenol/chloroform methods (Thai and Chinese). All samples were genotyped for thirty-two unlinked autosomal STR markers. The panel is comprised of the 15 tetranucleotides in the AmpF/STR Identifiler PCR Amplification kit (PE Applied Biosystems, Foster City, CA, USA) (D8S1179 [GenBank:AX412206], D21S11 [GenBank:AJ550387], D7S820 [GenBank:NC_000007], CSF1PO [GenBank:AF076965], D3S1358 [UniSTS:148226], TH01 [UniSTS:240639],

D13S317 [GenBank:G09017], D16S539 [GenBank:AF249681, D2S1338 [GenBank:G08202], D19S433 [GenBank:G08036], vWA [UniSTS:240641], TPOX [GenBank:M25706], D18S51 [GenBank:L18333], D5S818 [GenBank:G08446 and FGA [GenBank:G3347]) and an additional 17 dinucleotide repeats (D17S799 [GenBank:Z16830], D8S272 [GenBank:Z17250], D7S640 [GenBank:Z23671], D8S1827 [GenBank:Z50970], D22S274 [GenBank:Z16730, D5S407 [GenBank:Z16723], D2S162 [GenBank:Z17035], D10S197 [GenBank:Z16611], D11S935 [GenBank:Z17148], D9S175 [GenBank:Z17021], D5S410 [GenBank:Z16825], D7S2469 [GenBank:Z53000], D16S3017 [GenBank:Z52036], D10S1786 [GenBank:Z51854], D15S1002 [GenBank:Z53249], D6S1610 [GenBank:Z53131], and D1S2628 [GenBank:Z52173]). The amelogenin locus, included in the AmpF/STR Identifiler PCR Amplification kit for sex identification, was not included in any analyses. All STR markers were analyzed on an ABI PRISM 3100 semiautomated capillary fluorescence sequencer. Data were scored using Genemapper (ABI). We have previously used this marker panel to determine and statistically correct for ancestry in case-control studies and genome-wide linkage studies [1,2,5,8].

Statistical analyses

Population differentiation

Because variance of STRUCTURE results increases with small sample sizes [15], each run was repeated five times. However, results did not vary notably for each of the five runs given a set of conditions. For analysis of the three East Asian populations alone, the parameters used were K = 2 and K = 3, 50,000 burn-in and 50,000 Markov chain Monte Carlo (MCMC) iterations. For analysis of all five populations in this study, the parameters used were K = 2, K = 3, K = 4, and K = 5, with 50,000 burn-in and 50,000 MCMC iterations. These STRUCTURE runs were each carried out with all 32 markers and then with the 15 tetranucleotide markers and the 17 dinucleotide markers separated into two marker panels. The posterior probability for each value of "K" was calculated to determine the "K" that best fit the data for each set of populations and markers. The self-reported population of origin was not used as additional data by STRUCTURE and the presence of admixture was assumed.

Effective population size

The program BOTTLENECK evaluates populations for evidence of a recent rapid change in effective population size, according to differences between Nei's gene diversity, or unbiased expected heterozygosity (H_z) based on observed allele frequencies versus expected equilibrium gene diversity (H_{eq}), simulated based upon an assumed mutation model, number of alleles, and number of gene copies (2N for a diploid system) for each locus. Based on simulations of a coalescent process in which observed alleles at a locus are traced back to a hypothetical common ancestral allele, BOTTLENECK predicts present-day allele frequencies assuming constant population size. This results in a H_{eq} value for the present-day population. Significant deviations from this predicted value are used to infer drastic changes in effective population size which have occurred in the recent past. A significant heterozygosity excess ($H_z > H_{eq}$) indicates a possible bottleneck while a significant deficit ($H_z < H_{eq}$) indicates a possible expansion. Observed heterozygosity is the percentage of heterozygous individuals in a sample for a locus and is based on observed genotypes while (H_z) is the probability that two alleles chosen at random from the population sample will not be identical, correcting for sample size, and is an indirect measure of the extent to which allele frequencies for a locus are evenly distributed.

Significance of deviations from H_{eq} was tested under the two-phased model of mutation (TPM) which assumes that the majority of mutations are single step mutations, as in the stepwise mutation model but allows for some multi-step mutations, which are more likely to be observed in dinucleotide repeats and may be a more accurate model for microsatellite mutation than the SMM [48]. BOTTLENECK allows the user to specify the percent of multi-step mutations assumed and the variance of allele size for the mutation model. BOTTLENECK authors suggest a percent of multi-step mutations between 5 and 10. It has been shown that incidence of type I error (detecting a bottleneck when a population has been at equilibrium) for the algorithm used in BOTTLENECK increases when assumed parameters are overestimated [49]. Therefore, based on detection of predicted bottlenecks for the AA, Chinese and EA populations when larger values were used, variance was set conservatively at 20 and percent of multi-step mutations was set at 5. The number of iterations of the simulated coalescent process under the TPM was 1000.

Relatedness

We used marker genotypes to identify, and then exclude from the analysis sample, closely related subjects who may not have identified themselves as such. The admixture model in STRUCTURE assumes HWE and linkage equilibrium within subpopulations; the use of close relatives within a sample would violate those assumptions and possibly result in false cluster detection [33]. Similarly, BOTTLENECK software assumes no close relatives in a population sample [34]. Although potential subjects may be instructed that multiple family members should not participate, cultural differences in kin definitions, lack of understanding of instructions, or financial compensation of subjects may result in individuals disregarding such instructions. Maximum likelihood estimates of pair-

wise relationships (parent-offspring, full sib, half-sib, or unrelated) were produced using the program ML-Relate [50] for all possible pairs within each population. ML-Relate does not require pedigree information and therefore can be applied to a large anonymous sample.

Hardy Weinberg Equilibrium (HWE)

Tests for deviation from Hardy-Weinberg equilibrium were conducted for each locus within each population using the exact test for HWE based on a Markov chain method implemented in the web-based version of GENEPOP [51]. The parameters used were 5000 dememorizations, 500 batches, and 5000 iterations per batch. The parameter values were increased from defaults until the observed standard error for p-values was less than 0.01.

Heterozygosity

Allele frequencies, observed heterozygosity (H_o) values, and Nei's gene diversity (H_z) for each locus were calculated using MStools [52]. For a diploid system, H_z is calculated as $H_z = 2N(1-\Sigma pi^2)/2N - 1$, where N is the number of individuals sampled, and pi is the frequency of the i^{th} allele [53].

Marker information content

Markers were evaluated for delta (δ) [54], a measure of marker information content, reflecting the ability of a marker to statistically differentiate between populations. To arrive at δ, the absolute values of allelewise frequency differences between two populations are added and this sum is divided in half, $\delta = \frac{1}{2}\sum_{i=1}^{L}\left|p_i^A - p_i^B\right|$ where p_i^A and p_i^B are the allele frequencies for the i^{th} allele in population A and B. The more effective the marker is at differentiating between populations, the higher the value for δ [15]. In comparison to F_{ST}, the measure δ is easily calculated and independent of mutation model assumptions.

Authors' contributions

JBL designed the study, carried out statistical analyses and drafted the manuscript. BZY participated in the design and execution of statistical analyses. AS, NT, ST, AM, and KS participated in sample collection in Thailand. HRK carried out sample collection in the United States. RLR and TRD assisted in the writing of the manuscript and TRD is JBL's advisor. RTM participated in project coordination and sample collection, and the writing of the manuscript. JG participated in study design and supervision, project coordination, sample collection, and the writing of the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1

Results from Fisher's test for deviation from HWE for each marker. The data provided represent, for each marker, for each population, the probability of the observed sample given the conditions for HWE are met and the standard error of the probability.

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Additional file 2

δ (delta) for each marker, for each population combination. The data provided show, for each marker, the values for δ (delta), a measure of marker informativeness between each pair of populations.

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Sequence variation and linkage disequilibrium in the GABA transporter-1 gene (*SLC6A1*) in five populations: implications for pharmacogenetic research

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Abstract

Background: GABA transporter-1 (GAT-1; genetic locus *SLC6A1*) is emerging as a novel target for treatment of neuropsychiatric disorders. To understand how population differences might influence strategies for pharmacogenetic studies, we identified patterns of genetic variation and linkage disequilibrium (LD) in *SLC6A1* in five populations representing three continental groups.

Results: We resequenced 12.4 kb of *SLC6A1*, including the promoters, exons and flanking intronic regions in African-American, Thai, Hmong, Finnish, and European-American subjects (total n = 40). LD in *SLC6A1* was examined by genotyping 16 SNPs in larger samples. Sixty-three variants were identified through resequencing. Common population-specific variants were found in African-Americans, including a novel 21-bp promoter region variable number tandem repeat (VNTR), but no such variants were found in any of the other populations studied. Low levels of LD and the absence of major LD blocks were characteristic of all five populations. African-Americans had the highest genetic diversity. European-Americans and Finns did not differ in genetic diversity or LD patterns. Although the Hmong had the highest level of LD, our results suggest that a strategy based on the use of tag SNPs would not translate to a major improvement in genotyping efficiency.

Conclusion: Owing to the low level of LD and presence of recombination hotspots, *SLC6A1* may be an example of a problematic gene for association and haplotype tagging-based genetic studies. The 21-bp promoter region VNTR polymorphism is a putatively functional candidate allele for studies focusing on variation in GAT-1 function in the African-American population.

Background

γ-aminobutyric acid (GABA) is the most ubiquitous inhibitory neurotransmitter in brain. Abnormal function of the GABA system has been implicated in almost every common neurological and psychiatric disorder. Augmentation of brain GABA function is the presumed therapeutic mechanism of several classes of medications, including the benzodiazepines, gabapentin, and pregabalin, which are used in treatment of many psychiatric and neurological disorders, addictions, and pain [1,2]. Identifying genetic factors responsible for variation in clinical response holds promise as a way to improve the clinical application of these medications; it may be possible to identify those who are most likely to respond to treatment and those who are at risk to develop adverse effects. For these kinds of studies, it will be important to identify polymorphisms in genes encoding the components of the brain GABA system. Although the HapMap project [3] will identify the major basic blocks of linkage disequilibrium in these genes, detailed patterns of genetic variation, including the discovery of novel functional variants, can only be accomplished through resequencing.

A novel class of medications was recently developed that act by blockade of GABA transporters (GAT), thereby inhibiting GABA uptake. Four main subtypes of GABA transporters, the GAT-1, GAT-2, GAT-3 and Betaine/GABA transporter-1 (BGT-1), have been identified through molecular cloning. The first medication in the GABA reuptake inhibitor class to become available for clinical use was tiagabine [(-)-(R)-1-(4,4-Bis(3-methyl-2-thienyl)3-butenyl) nipecotic acid hydrochloride], which selectively blocks GAT-1 sites [4]. Tiagabine was developed for treatment of seizure disorders but is being used by many psychiatrists to treat a variety of conditions in which augmentation of brain GABA function is thought to be desirable to alleviate clinical symptoms. Preliminary studies suggest that tiagabine is effective in treatment of anxiety [5], sleep disorders [6], depression [7] and addictions [2,8,9]. Animal studies suggest that most of the tiagabine's common adverse effects, such as muscle twitching and sedation, arise from specific inhibition of GAT-1 [10]. Tiagabine has a rare, but serious adverse effect – non-convulsive status epilepticus – which has raised concerns about its off-label use in treatment of psychiatric disorders [11]. Pharmacogenetic study could plausibly help to identify those subjects who are at greatest risk for developing side-effects to tiagabine and other medications that inhibit GAT-1.

The *SLC6A1* gene, which encodes the GAT-1 protein, is an obvious candidate for pharmacogenetic studies of tiagabine and other GAT-1 inhibitors [12]. In anticipation of larger pharmacogenetic studies, we aimed to identify novel genetic variation and examine linkage disequilibrium in the *SLC6A1* gene in five populations representing three major continental groups [European (EA and Finnish), African (AA) and Asian (Thai and Hmong)]. Populations considered isolated, Finnish and Hmong, and two mixed populations, EA and AA, were examined, to understand how population differences should influence planning for pharmacogenetic studies. By comparing isolated and mixed populations, we hoped to shed further light on the purported benefits of isolated populations in mapping complex genetic traits [13,14]. Furthermore, our goal was to identify a set of haplotype tagging markers for studies focusing on response to GAT-1 inhibition. However, very low levels of LD was discovered in *SLC6A1* gene suggesting that traditional haplotype-based approaches of examining this gene in pharmacogenetic studies would be of limited utility.

Results

Algorithmic promoter prediction

The sequence for *SLC6A1*, located in contig NT_022517, which contains the sequence for human chromosome 3, was used as a reference. ElDorado software [15] located two putative *SLC6A1* promoter regions in the contig at positions 10973950–10974553 and 10998399–10998999 (+ strand). The first region is located in an area surrounding the boundary between the 5' region and exon 1 of *SLC6A1* and isestimated to be 604 bp long. Hereafter, we refer to this region as the upper promoter region. The second putative promoter region of 601 bp was located in the area flanking the boundary between intron 2 and exon 3. This region extends 70 bp across the *SLC6A1* start codon in the 3' direction in exon 3. We refer to this region as the lower promoter region.

Nucleotide diversity in SLC6A1 *in five populations*

The *SLC6A1* promoter regions, 16 exons, and the flanking intronic regions were amplified and resequenced in 7–9 samples from each of the five populations. A total of 61 SNPs were found in the sequencing screening sample, including 5 SNPs in the promoter regions, 3 synonymous coding sequence SNPs, and 53 non-coding region SNPs. No non-synonymous SNPs were found. The identified *SLC6A1* variants, including frequency in the screening sample, location, and flanking sequence are provided in the additional data file 1.

In the screening sample, 33 of the 61 SNPs discovered (54%) were present in more than one population. More SNPs unique to a single population or continental group were found in the AAs than in all of the other populations combined (counting Finns and EAs, and Thai and Hmong, together). Sixteen population-specific SNPs were observed in the AAs, one in the EAs, 6 in the Finnish, 4 in the Thai, and 2 in the Hmong (see additional data file 1); i.e., a total of 13 in the populations other than AAs. Three

common population-specific SNPs were found in AA population (-24794A/G, -24126G/T and 17885 A/C, minor allele frequencies = 0.39, 0.33 and 0.22) (These estimates of population allele frequencies have to be interpreted with caution since the data were derived from sequencing only 14–18 chromosomes per population). Analysis of nucleotide diversity based on the sequencing data indicated that AAs had the greatest number of polymorphic sites (n = 41), highest nucleotide diversity per bp (2.77×10^{-4}) and highest Watterson's estimator of theta (θ) (2.55×10^{-4}). Inspection of nucleotide diversity in the Hmong, Finnish, EA, and Thai populations revealed no marked differences among these four populations (Table 1). As expected, nucleotide diversity was lower in exons as compared to intronic regions ($\pi_{EX} = 5.5–7.0 \times 10^{-5}$, $\pi_{INT} = 1.1–2.3 \times 10^{-4}$). Interestingly, nucleotide diversity was not higher in the AAs when exonic sequence only was considered ($\pi_{AA} = 5.9 \times 10^{-5}$, $\pi_{EA} = 6.4 \times 10^{-5}$, $\pi_{Finn} = 7.1 \times 10^{-5}$, $\pi_{Thai} = 5.6 \times 10^{-5}$, $\pi_{Hmong} = 5.6 \times 10^{-5}$). The average number of heterozygous SNPs per person in the approximately 12.4 kb of the *SLC6A1* sequence was the highest in AAs (12.22) and was lowest in the Hmong population (3.75). In Finns, the mean number of heterozygous SNPs was 8.63, in EAs it was 6.57, and in Thai it was 8.38 (Table 1). The number of heterozygous SNPs differed significantly by population (ANOVA $p < 0.0001$). *Post hoc* comparisons showed that the Hmong had fewer heterozygous SNPs than the other populations (each comparison $p < 0.009$), with the exception of EAs ($p = 0.11$). AAs had more heterozygous SNPs than each of the other populations (each comparison $p < 0.032$).

Novel length polymorphisms in the promoter region

Two novel length polymorphisms were identified in the upper promoter of *SLC6A1*. The largest, and perhaps the more interesting polymorphism, is a 21-bp VNTR polymorphism, which is present either as a single element or as two tandem repeats, hereafter referred to as the *SLC6A1* short and *SLC6A1* long allele, respectively. The more common *SLC6A1* short allele contains an A/G SNP in its 16th base (-24794 A/G) (Figure 1). In our sample, the A allele of the -24794 SNP is in perfect linkage disequilibrium with the long allele, which contains G in its 16th base. In other words, the A allele always coincides with the long allele.

A novel two-base-pair deletion (GG/-GG) at positions (-24780 to -24781) was present in all 5 populations studied. This polymorphism is located only 8 bp 3' to the last base pair of the short/long polymorphism. The -GG allele occurs only in the context of the short allele.

The *SLC6A1* short/long and GG/-GG polymorphisms were genotyped in 46 EA, 60 AA, 59 Thai, 47 Finnish and 48 Hmong individuals. The allele frequency of the long allele was 0.39 in the AA sample but it was not found in other populations. The GG/-GG polymorphism occurred in all 5 populations. The -GG allele frequency was 0.30 in EA, 0.23 in AA, 0.33 in Finnish, 0.22 in Thai and 0.16 in Hmong.

Linkage disequilibrium in the SLC6A1 *gene*

For analysis of linkage disequilibrium in *SLC6A1*, a total of 16 SNPs were genotyped in the AA, Thai, Hmong, EA, and Finnish samples. The SNPs were selected with the goal of encompassing the *SLC6A1* gene with SNPs with allele frequencies > 10% in most populations studied in order to allow comparison of LD patterns between populations. Twelve of the 16 SNPs studied met this criterion. SNP rs1710879 was virtually monomorphic in AA population. The allele frequencies of all 16 SNPs were in Hardy-Weinberg equilibrium (HWE). The LD structure of the *SLC6A1* gene, as detected by this set of SNPs, was evaluated by calculating D' and r^2 using the HAPLOVIEW program [16]. The LD structure of the *SLC6A1* gene is presented in Figure 2. Fragmentation of LD into several poorly-defined blocks was noted in all 5 populations studied (Figure 2). There were two short blocks of LD (D' = 0.8–1) observed in all five populations. The first block is located between the markers -29477 (Marker 1, Figure 2) and -24321 (Marker 2, Figure 2). The second LD block, which is located between markers -17590 (Marker 3, Figure 2) and -9765 (Marker 5, Figure 2), was also found in all populations studied, although the level of LD was lower in AAs. The third LD block was observed in the EA and Finnish populations between markers -1529 (Marker 6, Figure 2) and 3164 (Marker 8, Figure 2).

Table 1: Indices of *SLC6A1* nucleotide diversity in five populations.

Populations	Number of polymorphic sites	Nucleotide diversity per bp (π) (10^{-4})	Watterson's estimator of theta (θ) (10^{-4})	Mean number of heterozygous SNPs per person
European-American	21	1.72	1.41	6.57
African-American	41	2.77	2.55	12.22
Finnish	29	2.11	1.87	8.63
Thai	31	2.14	2.00	8.38
Hmong	24	1.62	1.55	3.75

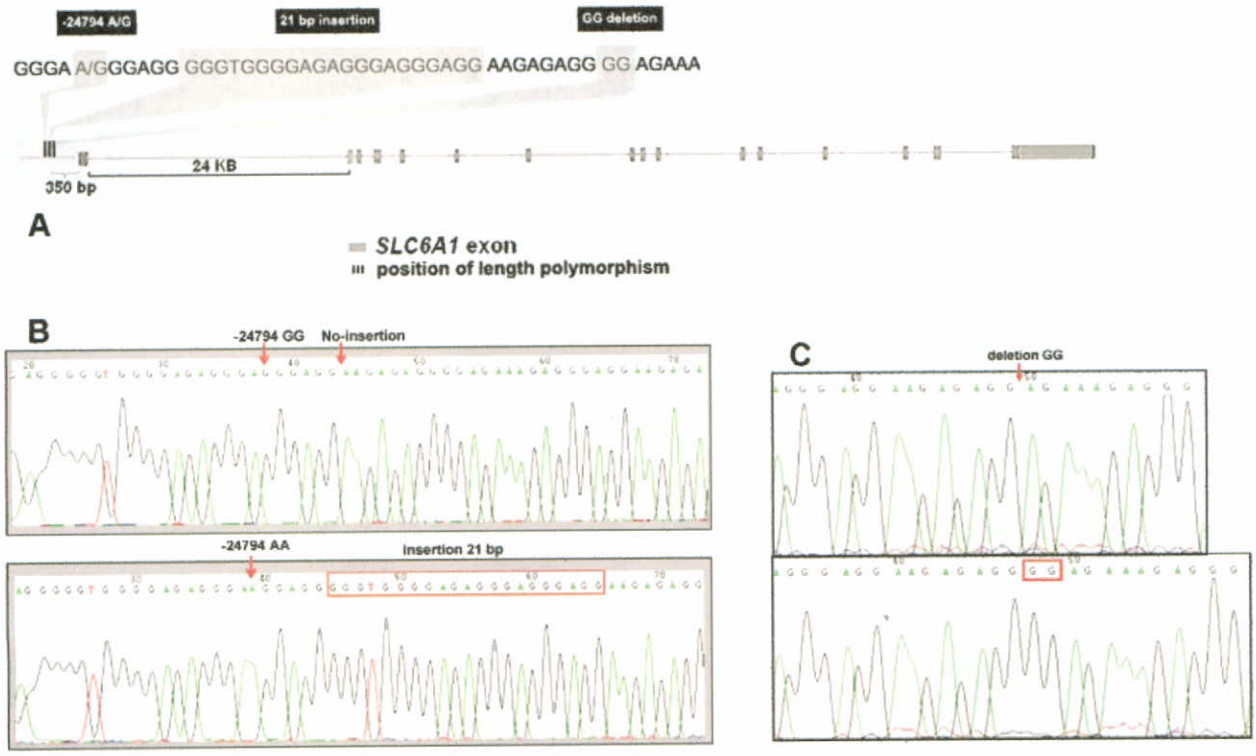

Figure 1
Illustration of the 21 bp insertion/deletion polymorphism (*SLC6A1* long and short alleles) and the insertion/deletion GG allele in the *SLC6A1* gene. Picture (A) shows position of -24794 A/G SNP, 21 bp insertion and GG deletion. Picture (B) shows the sequence for the *SLC6A1* short and long alleles and the -24794 A/G SNP. The A allele of the -24794 always coincided with the long allele in our populations. Sequence for the GG insertion/deletion polymorphism is presented in picture (C).

In accordance with low levels of LD, *Tagger* identified few haplotype tagging SNPs [17]. A SNP tagging approach would have allowed omission of three SNPs each in the EA, Finnish, and Thai populations and omission of two SNPs in the Hmong population. None of the 16 SNPs examined in the AA population was identified as a haplotype tagging SNP. We estimated the span of LD in different populations using r^2/distance as an index. The index of LD span was about twofold higher in the Hmong population than in any of the other populations. The differences were greatest in the distance bins < 10 kb and 30–40 kb, where the median r^2/distance value was two-to-three fold higher in the Hmong than in the other populations (Figure 3).

Haplotype diversity in SLC6A1

To further elucidate the *SLC6A1* haplotype structure, we used PHASE [18,19] to estimate haplotype frequencies in the five populations. Consistent with the observation of low levels of LD, no common *SLC6A1* haplotypes spanning the entire gene were identified in any of the populations. Haplotype dispersion varied depending on the number of SNPs included in the haplotype. When all 16 SNPs were included in the analysis, no common haplotypes were observed. The most common haplotype had a frequency of 0.048 in the EA, 0.039 in the AA, 0.076 in the Finnish, 0.049 in the Thai and 0.097 in the Hmong populations. Common haplotypes were observed when the analysis was restricted to narrower segments of the gene, i.e., when there were computationally fewer possibilities. We noted that there were differences between populations in the composition and number of common *SLC6A1* haplotypes. To examine this further, we reconstructed each consecutive three-SNP haplotype in each population using a sliding window analysis across the panel of 16 SNPs. The four most common three-SNP haplotypes in each window and in each population were identified. We then calculated how many times each of the common three-SNP haplotypes in each window was disjoint (i.e., not shared) between the populations. A summary pairwise score was calculated for each of the populations, which is presented in Table 2. For example, of all "top-four" three-SNP *SLC6A1* haplotypes, 26.8% were disjoint between AAs and EAs.

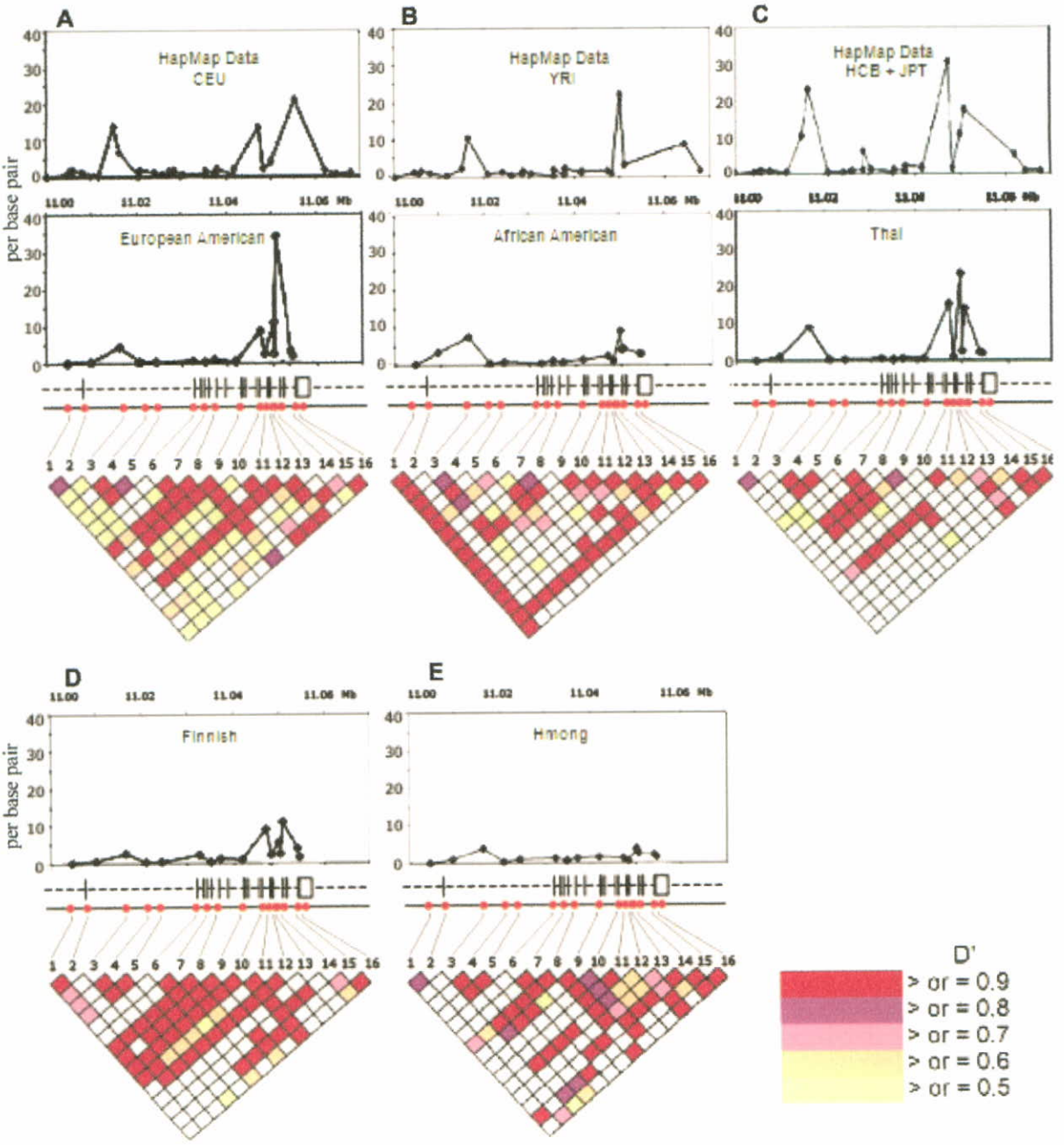

Figure 2
Illustration of the *SLC6A1* LD structure and recombination hotspots in the 5 populations. Upper graphs illustrate elevations from the background recombination rates across the *SLC6A1* gene. Y axis represent recombination rate and X axis represents physical distance between the markers [19, 20]. In Figure A, recombination rates for HapMap CEPH Western Europeans (CEU) and European-Americans of the present study are presented. In Figure B, recombination rates for HapMap Yoruban (YRI) and African-Americans of the present study are presented. In Figure C, recombination rates for HapMap combined Han Chinese and Japanese populations (HCB+JPT) and the Thais of the present study are presented. In Figures D and E, recombination rates for the Finns and Hmongs of the present study are presented. In the middle, the exon-intron structure of *SLC6A1* and location of the markers is presented. LD (D') between the SNPs in *SLC6A1* is illustrated in the lower triangular graphs.

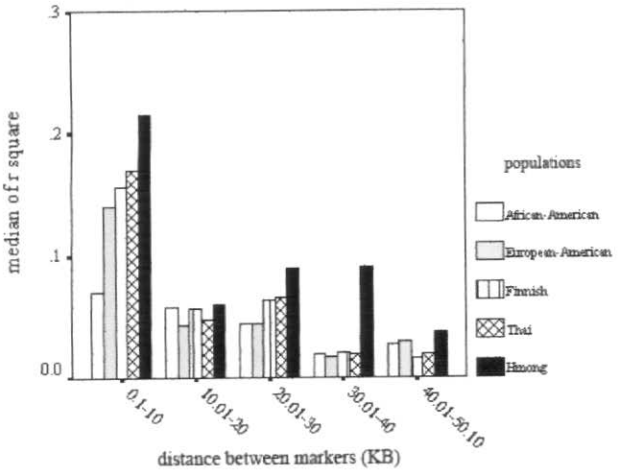

Figure 3
This graph illustrates LD span (expressed as median r^2) in different populations. Median r^2 calculated for SNP pairs in different distance bins (0.1–10 kb, 10.01–20 kb etc) is presented.

Recombination hotspots in SLC6A1

We considered recombination hotspots as an explanation for low level LD in *SLC6A1*. Recombination rates were calculated for the genotype data using the -MR and -X10 options of the PHASE program [20,21] and genotype data for all 16 *SLC6A1* SNPs. Average recombination rates in the five populations are shown in Figure 2. These data show two areas in *SLC6A1* with elevated recombination rates. The first area is demarcated by markers -29477 (Marker 1) and -13071 (Marker 4) and the second one by markers 7772 (Marker 9) and 20172 (Marker 15), hereafter referred to as the upper and lower hotspots, respectively. There were population differences in the amplitude of the hotspots (Figure 2). In the upper hotspot, which is located in the areas of intron 1 and exon 1, an increase in the recombination rate was observed in all five populations. The Hmong and AA populations showed lower rate and width of the lower hotspot, located in the area between exon 8 and 16, than did the EAs, Finns or Thai (Figure 2). To further elucidate these findings, we analyzed *SLC6A1* genotypes available through HapMap using PHASE, as described above. Genotyping data for the combined group of Japanese and Han Chinese, Yoruba, and Western Europeans were analyzed and compared to our results. We compared genotype data from our 16 SNPs and genotype data from 27–31 SNPs in HapMap. Two sets of SNPs, our and the HapMap SNPs, are not exactly the same but they are all located within the *SLC6A1* gene (one dot on X-axis in Figure 2 represents one SNP). It appears that genotype data from the two samples (the present study and HapMap) are consistent, both showing evidence for two areas of increase in recombination rate within *SLC6A1*. These results support our findings (Figure 2) regarding two recombination hotspots in *SLC6A1*.

Table 2: A summary pairwise percentage score representing the degree to which the four most common haplotypes were disjoint between the five populations.

E					
A	26.8				
F	16.1	26.8			
T	12.7	23.6	16.3		
H	26.8	23.2	30.3	17.9	
	E	**A**	**F**	**T**	**H**

E = European-American, A = African-American, F = Finnish, T = Thai, H = Hmong. For example, of all consecutive "top-four" 3-SNP *SLC6A1* haplotypes, 26.8 % were disjoint between African-Americans and European-Americans.

Discussion

The goal of the present study was to comprehensively analyze sequence variation and linkage disequilibrium in the *SLC6A1* gene in anticipation of larger pharmacogenetic studies of tiagabine and other GAT-1 inhibitors. Resequencing 12.4 kb of the *SLC6A1* gene, including all 16 exons and the two putative promoter regions, revealed numerous novel genetic variants. Perhaps the most interesting polymorphism identified was a 21 bp VNTR polymorphism located in the upper promoter sequence of the *SLC6A1* gene (Figure 1). We have termed the alleles as the "*SLC6A1* short," which has one copy of the allele and "*SLC6A1* long," in which the allele is duplicated (Figure 1). Interestingly, the long allele was common in AAs (39%), while in the other populations, it was absent. A likely explanation for the lack of this allele in non-African populations is genetic drift. However, other explanations, such as natural selections, are also possible. Functional studies focusing on understanding whether the short and long allele lead to differential expression of the GAT-1 protein are clearly warranted; these studies may also help in elucidating whether the allele frequency discrepancy between African-Americans and other populations is due to genetic drift or selection. Previously, a comparable promoter region VNTR polymorphism was described in the serotonin transporter gene, which is known to influence the expression of the gene [22]. Several studies have shown that this polymorphism partially accounts for differences in the therapeutic response to serotonin selective reuptake inhibitors (SSRIs) [23-26], susceptibility to depression [27,28] and alcohol dependence [29]. The *SLC6A1* short/long is a candidate polymorphism for moderating the response to tiagabine and susceptibility to neuropsychiatric disorders in which GABA dysfunction may play a role, albeit only in AA populations (and any other populations where this variant is present).

Genetic diversity in *SLC6A1* revealed through *SLC6A1* resequencing showed interesting results. Although a limited number of chromosomes was examined, certain trends were found. First, no non-synonymous SNPs were discovered in the 80 chromosomes sequenced, suggesting that the coding sequence of *SLC6A1* has been conserved against common amino-acid altering substitutions through active background selection. Consistent with these results, the nucleotide diversity was much lower in the *SLC6A1* exons compared to the intronic regions examined [30-32]. Comparison of the sequence data between populations revealed higher nucleotide diversity in AAs than in other populations (Table 1). In accordance with these data, the only population in which we found common population specific SNPs, were AAs. In exons, however, nucleotide diversity was no higher in the AA population than in the other populations. In other populations, the SNPs observed in only one population were rarer. Although Finns and Hmong are considered to be isolated populations, nucleotide diversity in these two populations was not different from that observed in EAs or Thais. Overall, no major differences in nucleotide diversity were observed among the Hmong, Thai, EA, and Finnish populations. Together, these findings most likely reflect the older age of the African population relative to the other populations, which had allowed more intronic variation to accumulate in this population, founder effects in non-African populations, and selection pressure conserving the *SLC6A1* exonic sequence. The extent of conservation of GAT-1 amino acid sequence suggests an important role of this protein in normal brain function. The Hmong had a significantly lower number of heterozygous SNPs in comparison to the other populations. One explanation for this finding is that the Hmong subjects may have been distantly related. We postulate that differences in the degree and age of population bottlenecks between the Hmong and the other populations are less likely explanations for the lower heterozygosity observed in the Hmong; we feel that this explanation is less likely because all non-African populations had a low observed frequency of population-specific SNPs and because Hmong nucleotide diversity was not significantly lower than that of the other non-African populations [33]. A caveat of this study is that the sample size per ethnicity was small. Consequently, rare non-synonymous SNPs, specific to a population, would have not been identified. It may be useful to resequence larger samples to identify these kinds of variations, at least in the primary target populations of clinical trials. Laboratory methods established for the present study should facilitate such analyses. Another limitation of the study is that the resequencing effort focused on exons. If deep intronic variation in *SLC6A1* contributes to functional variation at the protein level, those variants would have been missed in the present study. A low level of LD in *SLC6A1* was observed in all five populations (Figure 2). Consistent with these results, in the EA, Thai, Hmong, and Finnish populations, only two or three haplotype tagging SNPs in the areas of preserved LD were identified. In the AAs, no haplotype tagging SNPs were found in the 16 SNPs genotyped. These results suggest that very dense SNP panels would be required to capture common variation in this gene. Using r^2/distance as the index, a longer LD span was observed in the Hmong population than in the other populations (Figure 3). However, higher LD probably would not translate to significant practical improvement in genotyping efficiency overall, because there were no major differences in the number of haplotype blocks in Hmong than in the Finnish, Thai and EA populations. Considering the low level of LD, *SLC6A1* may pose special challenges for association studies both in isolated and mixed populations. The common *SLC6A1* 3-SNP haplotypes were largely the same in the five populations (Table 2), but were not completely overlapping. These results suggest a certain degree of, but not absolute, portability of SNP genotyping sets between the populations. It would interesting to study larger EA, AA, Hmong, Thai and Finnish population samples to further refine the structure and frequencies of the common overlapping and population-specific haplotypes [34]. In addition, it will be interesting to study whether haplotype and SNP profile characteristics, such as absence of common non-synonymous substitutions extends to patient populations suffering from various neuropsychiatric disorders, which were not studied here. The present study primarily focused on non-clinical samples and therefore no data were available to assesses whether disease associated variants are present in human populations.

Intrigued by these findings, we examined whether recombination hotspots could explain low levels of LD in *SLC6A1*. Two hotspots were identified using PHASE; the first is located in the areas of exon 1 and intron 1. The second hotspot is located in the area demarcated by exons 8 and 16. As expected, within the hotspots, D' fell off rapidly. For example, in the Finns, in the area of the distal hotspot, D' was only 0.184 between markers 12 and 13, which are spaced 107 bp apart and D' was 0.044 between 13 and 14, which are spaced 489 bp apart. Areas of high recombination, such as seen in *SLC6A1*, potentially limit large-scale association studies, as it would be exceptionally difficult to find risk alleles relying on linkage disequilibrium if the alleles were located inside a recombination hotspot.

Conclusion

SLC6A1 is a complicated target for pharmacogenetic studies because low levels of LD and recombination hotspots would make it difficult to establish an association between genetic markers and response to GAT-1 modula-

tion. Furthermore, our study suggests that focusing on isolated populations, such as Finns or Hmong, would not provide major benefits to genotyping efficacy of *SLC6A1*. An interesting 21-bp VNTR polymorphisms (*SLC6A1* short and *SLC6A1* long) was discovered in the promoter sequence of the *SLC6A1* gene, which is common in African-Americans. This new polymorphism is a novel candidate for studies focusing on genetically influenced differences in GAT-1 expression, and hence, response to medications that inhibit GAT-1 function.

Methods

DNA samples

For re-sequencing of the *SLC6A1* gene, 40 genomic DNA samples were collected from unrelated individuals representing 5 different populations: EA (n = 7), AA (n = 9), Finnish (n = 8), Thai (n = 8), and Hmong (n = 8). The Finnish subjects were unrelated parents of adolescent subjects who were participating in an epidemiological study focusing on the identification of risk factors for early-onset mental illness and substance dependence in Finland [35]. The Thai and Hmong populations were collected in Thailand as part of an ongoing genetic association and population genetic study. The Thais selected for resequencing had grandparents and parents of Thai ancestry (Thai-Thai) or had mixed Thai and Chinese ancestry (Thai-Chinese), by subject report. These samples were obtained from a blood drive in Bangkok, Thailand. The Hmong subjects were recruited in a Hmong village in the northern part of Thailand. The AA and EA samples have been described earlier elsewhere [36]. Both EA and AA samples were self-identified and confirmed as such by Bayesian marker clustering [36]. All subjects provided informed consent as approved by the appropriate institutional review boards. In addition, 46 EA, 60 AA, 59 Thai, 47 Finnish and 48 Hmong individuals were genotyped for 16 *SLC6A1* SNPs to examine linkage disequilibrium (LD) in this gene. The Thai subjects selected for examination of linkage disequilibrium were Thai-Thai. Recruitment and population characteristics of subjects selected for *SLC6A1* genotyping were identical of subjects selected for resequencing [35,36]. The participants were recruited from non-clinical populations. No detailed medical information was available for most of the participants. Therefore biases deriving from undetected medical conditions of the control sample could not be controlled. All studies described in this article were conducted according to the Declaration of Helsinki. The studies were approved by the institutional review boards of Yale University School of Medicine, West Haven VA Hospital, Northern- Ostrobothnia Hospital District (University of Oulu, Finland) and Chulalongkorn University (Bangkok, Thailand). All subjects signed a written informed consent for participation in this study.

Promoter prediction

The ElDorado program of the Genomatix software package was used to predict the location of the *SLC6A1* promoter region [15]. The sequence of the *SLC6A1* gene submitted to the promoter region analysis was obtained from the National Center for Biotechnology Information (NCBI) [37].

Amplification and sequencing

For sequencing of *SLC6A1*, the upper and lower promoter regions, all 16 *SLC6A1* exons (total of 4.4 kb) and 7.3 kb of flanking intronic regions were amplified. About 70 bp of the predicted 601 bp of the lower promoter region were not included in the sequence analysis. Approximately 12.4 kb of the *SLC6A1* gene was amplified, corresponding to about 25% of the total length of the gene. All primers were designed with the PRIMER3 software [38]. Primers were obtained from Invitrogen (Carlsbad, CA). PCR amplification was optimized before sequencing by testing different cycling conditions. Betaine (Sigma Aldrich, St. Louis, MO) at 0.5–1 M final concentrations was added to the reactions, as needed, to enhance specificity and yield of PCR amplification. PCR reactions were carried out in 15 μl volumes containing 20 ng genomic DNA, 200 μM of dNTPs mix (Stratagene, La Jolla, CA), 1 μM of mixed primers forward and reverse, 1X PC2 buffer, 0.75 U of KlenTaq1™ (Ab Peptides, St Louis, MO) and 0.5–1 M betaine when needed. Thermocycling conditions consisted of an initial denaturation step at 95°C for 5 min, 30 cycles of denaturation step at 95°C for 30 sec, an annealing step at 60–65°C 30 sec, and an extension step at 72°C. The duration of the extension step varied from 30 sec to 2 min depending on the length of the amplicon. After optimization, genomic DNA samples from each population were PCR amplified followed by purification with MinElute PCR purification columns (Qiagen, Valencia, CA) or the reaction mixtures were treated with ExoSAP-IT (USB, Cleveland, OH) to remove excess nucleotides and primers. Purified PCR samples were sequenced in the forward and reverse directions at Yale University W.M Keck Foundation Biotechnology Resource Laboratory. Sequencing reactions were conducted using the BigDye Terminator v3.1 cycle sequencing kit and an ABI 9800 Thermocycler (Applied Biosystem, Foster city, CA). Sequencing reactions were analyzed on an ABI 3730 xl DNA Analyzer (Applied Biosystems, Foster city, CA). Owing to technical problems, approximately 300 bp in exon 7 and intron 8 (2.4% from total 12.4 kb sequenced region) is missing in the sequencing data in Thai and Hmong populations (see additional data file 1).

Owing to the repeat elements contained in the identified upper promoter sequence and homologous sequences within the *SLC6A1*, the upper promoter region and parts of exon 1 were amplified using nested PCR. In addition,

for amplification of a 180 bp fragment located in the junction of the 5' upstream region and exon 1, a region which is very high in CG content, 7-deaza-dGTP (New England BioLabs, Beverly, MA) was added to the reactions.

Genotyping and linkage disequilibrium study

A total of 16 SNPs were chosen for genotyping in population samples to examine haplotype structure of the *SLC6A1* gene. Nine SNPs chosen for genotyping were identified through resequencing: -24321A/C, -1529A/G, 949A/G, 3164C/T, 14351A/G, 16009A/G, 16116C/T, 20172C/T and 20622A/G. The remaining seven SNPs, -29477C/T, -17590C/T, -13071A/G, -9765C/T, 7772A/G, 13269C/T, and 16605C/T, were chosen from the NCBI dbSNP [39] collection. Of the 16 SNPs studied, 14 were available through Applied-Biosystem's Assay-On-Demand service (Applied Biosystems, Foster city, CA). One assay was custom designed and obtained through the ABI's Assay-by-Design service (Applied Biosystems, Foster city, CA). PCR amplification of the 5' nuclease assays were conducted using 1 ng of DNA, 1X TaqMan universal PCR master mix (Applied Biosystems, Foster city, CA), 0.5X SNP genotyping assay mix [Applied Biosystems, Foster city, CA]. PCR conditions were as follows: denaturation step of 95°C for 10 min, followed by 50 cycles of 95°C for 15 sec and 60°C for 1 min. Amplification was performed on PTC-200 cyclers (MJ Research, Hercules, CA) and data were analyzed using the ABI Prism 7900HT Sequence Detector System and software version 2.1 (Applied Biosystem, Foster city, CA). All samples were run in duplicate for quality control purposes. Based on comparison of the duplicate runs, we estimated the genotyping error rate to be less than 0.05%. The -24321A/C SNP was genotyped using 7-deaza-dGTP sequencing because its location inside a GC-rich region made it very difficult to design a 5' nuclease assay for this SNP.

Genotyping of the length polymorphisms

Amplification of the region containing the 21 bp short/long VNTR and 2 bp GG/-GG insertion/deletion polymorphisms was accomplished using primers 5'AAGGAGAGAGATTGGAGCG 3' and 5'CTTCTTTCCTCTCGCATTC 3' (Invitrogen, Carlsbad, CA). PCR reactions were conducted in 15 μl volumes containing 20 ng genomic DNA, 200 μM of dNTPs mix (Stratagene, La Jolla, CA), 1 μM of mixed reverse and forward primers, 1X PC2 buffer, 0.75 U of KlenTaq1™ (Ab Peptides, St Louis, MO) and 1 M Betaine. The thermocycling conditions consisted of an initial step at 95°C for 5 min, 30 cycles of denaturation at 95°C for 30 sec, annealing 60°C 30 sec, and extension 72°C 30 sec. The lengths of the PCR products corresponding to the long and short alleles are 166 bp and 145 bp. The long and short alleles were separated using 3% metaphore agarose and gel electrophoresis (ISC BioExpress, Kaysville, UT). The GG/-GG insertion/deletion polymorphism was genotyped using direct sequencing of the PCR product as described in above.

Statistical analysis

Indices of sequence variation in *SLC6A1* were calculated using a web application SLIDER [40]. These indices included the number of polymorphic sites, nucleotide diversity per base pair (π) and the Watterson's estimator of theta (θ). Nucleotide diversity per base pair (π) describes the mean number of differences per site between two sequences chosen at random from a sample of sequences. The Watterson's estimator of theta (θ) is the observed number of SNPs adjusted for the sample size and new mutation rate expected to occur in each generation [41]. In addition, for each subject we calculated the number of heterozygous SNPs observed in the sequence data. The number of heterozygous SNPs was compared between populations using ANOVA followed by post hoc Fisher's Least Significant Difference-test.

PHASE software, which implements a Bayesian algorithm for haplotype reconstruction, was used to estimate haplotype frequencies [18,19]. PHASE's options -X10 and -MR were used to estimate recombination rates across *SLC6A1* [20,21]. The value on the Y-axis of Figure 2 shows changes in recombination parameter (ρ) per base pair of *SLC6A1* exceeding the background recombination rate [20,21]. The average recombination rate was estimated based on 1,000 burn-ins and 1,000 iterations. Recombination frequencies at *SLC6A1* were compared visually between our and HapMap data. No statistical analyses were performed. To evaluate haplotype diversity among populations, we studied how often the most common haplotypes were shared or disjoint. The haplotypes were identified using a sliding window analysis across every three consecutive *SLC6A1* SNPs. The four most common three-SNP haplotypes in each window and in each population were identified. The rationale for choosing the four most common haplotypes for this analysis was that visual inspection of the haplotype frequencies told us that in each window and in each population virtually all variation in haplotype diversity was captured by the four most common haplotypes. The average percent of all haplotypes captured by the top four haplotypes was 96%. We then calculated how many times each of the common three-SNP haplotype was disjoint between the populations. A summary pairwise score derived for the populations is presented in Table 2.

LD patterns in *SLC6A1* were visualized using HAPLOVIEW version 3.2[16] We used the *Tagger* algorithm, implemented in HAPLOVIEW, to search for haplotype tagging SNPs in *SLC6A1*. [17] We used the default *Tagger* thresholds $r^2 > 0.8$ and LOD score > 3. POWERMARKER [42] was used to calculate allele frequencies and examination

of Hardy-Weinberg equilibrium (HWE). To illustrate differences in the span of LD in the five populations, r^2 was plotted against physical distance. To do this, r^2 was calculated for all SNP pairs. Because these values were not normally distributed, median values are presented. Physical distance (bp) was divided into distance bins to illustrate population differences in LD span across a range of physical distances. Median r^2 in distance bins (0.1–10 kb, 10.01–20 kb etc) in different populations is presented in Figure 3. No statistical analyses were performed on these data. Software CENSOR was used to search for repeat elements within the recombination hotspots [43,44].

Abbreviations

GABA γ-aminobutyric acid

GAT GABA transporter

BGT-1 betaine/GABA transporter-1

SLC6A1 solute carrier family 6 (neurotransmitter transporter, GABA), member 1

LD linkage disequilibrium

SNPs single nucleotide polymorphisms

ANOVA analysis of variance

-MR recombination model

-X10 run 10 times

π nucleotide diversity

θ Watterson's estimator of zeta

EA European-American

AA African-American

HWE Hardy-Weinberg equilibrium

LOD log of the likelihood odds ratio

Competing interests

The author(s) declares that there are no competing interests.

Authors' contributions

JL (Jaakko Lappalainen) initiated and planned to study *SLC6A1* gene. RH did the experiment and drafted the manuscript. Finnish samples in this project were collected by RI, PR and EI. African-American and European-American samples were collected by HK, TK and JG. RM, JG, AS, NT, ST, JL (Jennifer Listman) and AM participated in collecting samples in Thai and Hmong populations. All authors have read and approved the final version of this manuscript.

Additional material

Additional file 1

List of SNPs discovered in SLC6A1 by resequencing 40 individuals from Thai (n = 8), Hmong (n = 8), European-American (n = 7), African-American (n = 9) and Finnish (n = 8) populations. The data provided descriptions of all SNPs in SLC6A1 in the population samples.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2156-8-71-S1.doc]

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High frequency of mutation of epidermal growth factor receptor in lung adenocarcinoma in Thailand

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Abstract

Recent reports have suggested influences of racial difference on the frequency of mutation of *EGFR* in lung cancer. We therefore sought to characterize the frequency and pattern of mutation of *EGFR* in lung adenocarcinoma in Thai patients. Overall, *EGFR* catalytic domain mutations were detected in 35/61 (57.4%). We found 29/60 (48.3%) of exon 19 deletions, 5/54 (9.3%) of exon 21 point mutations, and 1/54 (1.9%) of double-mutation of both exons. The presence of these mutations was significantly associated with non-smoking habit. In summary, we report a strikingly high prevalence of mutation of *EGFR* in Thai lung adenocarcinoma, which may explain the high response rate to the treatment with TKI among Asian populations.

Keywords: EGFR; Adenocarcinoma; Lung cancer; Mutation; Tyrosine kinase inhibitor; Gefitinib

1. Introduction

EGFR is an important membrane receptor protein, which plays crucial roles in multiple cellular functions. Binding of one of its ligands to the extracellular domain leads to phosphorylation and activation of EGFR. Activated EGFR then conveys signals to the downstream targets, which participate in multiple cellular processes involving cell proliferation, differentiation, migration, and survival [1]. Deregulation of EGFR signaling either by overexpression or amplification of the EGFR has been described in many cancers. This supports the idea that the functions of EGFR are critical to abnormal tumor growth and metastasis [2,3]. Also in line with these

Abbreviations used EGFR, epidermal growth factor receptor; NSCLC, non small cell lung cancer; TKI, tyrosine kinase inhibitor; bp, base pair.

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notions, recent developments of molecularly targeted therapy for cancer have been focusing on blocking critical molecules that determine malignant phenotypes in EGFR pathways in many types of malignancy that are difficult to be treated, including non-small cell lung cancer (NSCLC) [4]. Several reports indicate that NSCLC utilizes EGFR pathways, which could be demonstrated either by over-expression or amplification, detected in more than 80% of the tumors [5]. Two recent multi-institutional phase II trials showed certain clinical benefits but only in a small number of advanced and chemotherapy refractory NSCLC patients who were treated with a tyrosine kinase chemical inhibitor (TKI), gefitinib [6,7]. The objective response rates in these studies were differentially demonstrated in NSCLC according to their ethnicity, namely, 9–12% in Caucasian [6] versus 18.4–19.0% in Japanese patients [7]. Additional experiences with currently available TKIs, gefitinib and erlotinib, consistently show an association between the responses and certain clinical parameters like the female gender, adenocarcinoma, and non-smoking habit [8–11]. However, molecular predictors of the response are needed for better clinical guidance and insight in the biology of this highly malignant neoplasm related to the response to TKIs.

Several attempts to identify other potential predictor of the response like the expression pattern of EGFR did not clearly demonstrate a significant correlation between EGFR expression and the response to treatment with TKIs [12,13]. Recently, a few studies have identified a convincing molecular marker that predicts the response to TKIs of NSCLC, the mutation of the *EGFR* catalytic domain [14–16]. With systematic mutation analyses, the majority of mutations were detected exclusively within the tyrosine kinase domain of EGFR encoded in exon 18 to 21 and only in lung adenocarcinoma. Characteristically, 88% of the reported mutations are in-frame deletions of exon 19 and point mutations of exon 21, which lead to functioning mutated EGFR proteins [16]. Almost all patients who showed the responses to TKI harbored EGFR mutations in the tumor cells whereas mutations were rarely detected in known TKI resistant patients. The prevalence of mutations coincided well to the described clinical predictors of the response to TKIs including racial background, the female gender, adenocarcinoma, and non-smoking habit [14,15]. The higher number of Japanese patients who governed mutations of *EGFR* in these studies raises an important question: whether or not NSCLC patients with Asian ethnicity follow a similar pattern and prevalence of *EGFR* mutation.

Our experience with a compassionate expanded access program of gefitinib treatment in Thailand showed an overall objective response rate of 20% reminiscent to those of Japanese patients (N.V. and P.L. unpublished observations). The data prompted us to investigate the prevalence and pattern of *EGFR* mutation in Thai NSCLC patients. Hence, we chose to screen for exon 19 and exon 21 mutation of *EGFR* in lung adenocarcinoma patients according to the previous findings that indicated clustering of mutations in these specific regions of the *EGFR* gene and the tumor histology. In principle, the association between the EGFR mutations and the clinical response were demonstrated in a limited set of patients previously treated with gefitinib with known responses. We observed a strikingly high rate of mutations in our patients. In addition, a new pattern of exon 19 mutation, 9-base deletion, and double mutations of both exons were detected in our study.

2. Materials and methods

2.1. NSCLC tissue sample

The samples were paraffin-embedded tissue samples of lung adenocarcinomas taken from the archieve of the Department of Pathology, Faculty of Medicine, Chulalongkorn University, Thailand. The study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University. All samples were reviewed and marked by a pathologist (C.C.) for an area, approximately 3–8 mm at the greatest diameter which contained more than 70% of the tumor cells. The first panel comprised of five samples with prior treatment with gefitinib and known response. Additional 56 samples were screened for the mutation of *EGFR* gene.

2.2. Mutation analysis

Tumor samples were mapped with hematoxylin-eosin sections, manually microdissected with needle, and subjected to DNA isolation as previously

described [17]. Isolated DNAs were PCR amplified for the coding regions of *EGFR* exon 19 and exon 21. Primers for exon 19 amplification are: 5′ tggatcccagaaggtgagaaag and 3′ gcaaagcagaaactcacatcgag; and exon 21 are: 5′ cgtactggtgaaaacaccgc and 3′ cctt-actttgcctccttctgc. For exon 21, we used an additional 5′ nested primer of which the sequence is tggtgaaaacaccgcagcatg to enhance the efficiency of the amplification. PCR products were then electrophoresed in 12% native polyacrylamide gel in Tris-borate-EDTA buffer and visualized for positive PCR products. We submitted the positive PCR products to the Bioservice Unit, National Institute of Biotechnology, Bangkok, Thailand for direct sequencing of both exons. Both PCR and sequencing were individually repeated to confirm the presence of the mutation.

2.3. Statistical analysis

Statistical analyses were performed with SPSS software using binary logistic regression analysis. The dependent variable was either the presence or the absence of mutation. Individual variables were analyzed with both univariate and multivariate methods.

3. Results and discussions

Recent studies indicated that the majority of the mutations of *EGFR* in NSCLC cluster in exon 19 and exon 21 were within the catalytic domain which is encoded from exon 18 to exon 21 [14–16]. Based on these results, we then concentrated on analyzing mutation within the common regions for mutation, exon 19 and 21. We first confirmed the association between the response to gefitinib treatment and the mutations of the EGFR catalytic domain in our lung cancer population. Genomic DNA isolated from tissue samples of a small panel of lung adenocarcinomas consisting of three patients who displayed objective responses to gefitinib and two patients who showed resistance to their treatment. Initially, we amplified exon 19 from isolated genomic DNA and then electrophoresed the PCR products in polyacrylamide gel to screen for deletions. Under gel visualization, we could detect smaller amplicons, which represented amplified deleted exon 19 in conjunction with the expected size of wild type amplicon *EGFR* exon 19 in all three tumor tissues derived from gefitinib responders. In contrast, the two non responders showed only amplicons of wild type of *EGFR*

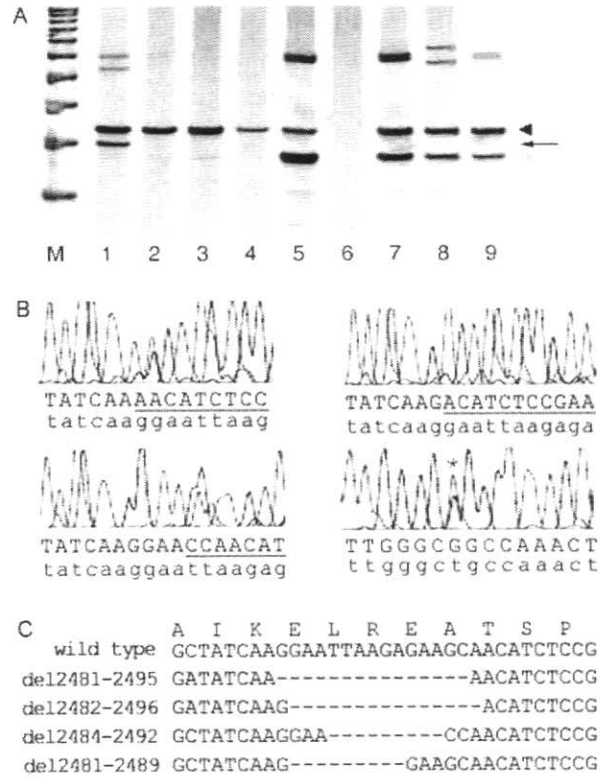

Fig. 1. Mutation analysis of *EGFR* in lung adenocarcinoma. (A) A typical example of gel electrophoresis shows amplicons from exon 19 PCR. We used 12% native polyacrylamide gel for electrophoreses. Wild type exon 19 bands are illustrated across all samples, which serve as internal positive control for PCR (dark arrow head). Faster migrate bands as approximately 15-bp deletion amplicons are shown in lane 5, 7, 8, and 9 (gray arrow head). A 9-bp deletion amplicon is detected in lane 1 sample (arrow line). Lane M is DNA ladder. Lane 6 is water serving, a negative control for PCR. (B) Chromatograms of DNA sequencing demonstrate different types of *EGFR* mutations. The upper right panel is an example of exon 19 del 2481–2495. The upper left panel shows exon 19 del 2482–2496. The lower right panel is exon 19 del 2484–2492. The lower left panel is exon 21 point mutation at 2189 T>G, indicated with an asterisk. The upper DNA sequences in each panel demonstrate the mutated sequence where underlined portions are the frame-shifted segments. The lower annotated sequences are of wild type. (C) Aligned wild type and mutated DNA sequences of exon 19 for comparison are demonstrated. Amino acid sequence of a wild type EGFR is on the top showing the critical region of deletion, which spans the common LRE amino acid sequences (For interpretation of the reference to colour in this legend, the reader is referred to the web version of this article).

Table 1
Frequency and pattern of *EGFR* mutation in lung adenocarcinoma

Type of mutation	Frequency (%)	Cumulative percent (%)
Exon 19 del 2481–2495	21 (34.4)	34.4
Exon 19 del 2482–2496	5 (8.2)	42.6
Exon 19 del 2484–2492	2 (3.3)	45.9
Exon 19 del 2181–2489	1 (1.6)	47.5
Exon 21 2189 T>G	5 (8.2)	55.7
Exon 19 del 2481–2495/Exon 21 2189 T>G	1 (1.6)	57.4
WT	26 (42.6)	100
Total	61 (100)	

(Fig. 1(A)). Direct sequencing from all three samples revealed a similar 15 bp deletion of nucleotide 2481–2495 (reference nucleotide sequence is from Genbank NM_005228). All amplified exon 21 from this set of tissue showed no mutation. Consistent with other reports, at least it appears that the presence of exon 19 deletions correlates well with the response to gefitinib treatment [14–16]. We then screened additional 56 lung adenocarcinomas under the same approach. Presence of exon 19 deletions was screened initially by visualizing the smaller mutated amplicons and then confirmed by direct sequencing and characterizing the breakpoint location. All samples were analyzed for exon 21 mutations by direct sequencing. Overall yields of the detection were excellent and suitable for formalin fixed paraffin embedded tissue. We could amplify exon 19 successfully: 60/61 (98.4%) samples and 54/61(88.5%) for those of exon 21. In the second set of patients without prior gefitinib treatment, we detected 33/56 (58.9%) mutations which were 23/55 (41.8%) of exon 19 deletions, 6/49 (12.2%) exon 21 point mutations, and 1/56 (1.8%) dual exon 19 deletion and exon 21 point mutation. The spectrum of exon 19 deletions consisted of three patterns. The deletion of nucleotide 2481–2495 which caused an in-frame deletion resulting in the loss of amino acid E746–A750 which could be identified in 22/30 (73.3%) (Fig. 1(B) and Table 1). The second most common pattern was the deletion of nucleotide 2482–2496, 5/30 (16.7%), which resulted in a similar E746–A750 deletion (Fig. 1(B,C), and Table 1). We found two patterns of 9 bp deletion of exon 19, nucleotide 2484–2492 deletion in 2/30 (6.7%) and nucleotide 2481–2489 deletion in 1/30 (3.3%) (Fig. 1(B,C), and Table 1). We found exon 21 nucleotide 2819 T>G point mutations resulting in L858R amino acid change in 5/49 (10.2%). Moreover, we detected one dual-mutation of the exon 19 nucleotide 2481–2495 deletion in a combination with the exon 21 L858R point mutation (Fig. 1(B) and Table 1). Overall, we describe a very high frequency of EGFR catalytic domain mutation in lung adenocarcinoma in Thai patients. In our studied population, the exon 19 deletions resulting in the loss of E746–A750 amino acids are the most common pattern of mutation which is similar to the previous reports in the Caucasian and the Japanese populations [14–16]. These findings thus raise a practical approach for mutation analysis in our populations that have high prevalence of exon 19 deletions. Screening for exon 19 deletion by distinguishing smaller PCR amplicon size before direct sequencing may be a suitable approach in our country which has limited resources. Additionally, we describe a few new patterns of *EGFR* somatic mutations including 9-bp in-frame deletions of exon 19 and dual mutations of both exon 19 and exon 21. These findings further support the previous notions of high variability of *EGFR* mutation patterns.

Early clinical trials with gefitinib in NSCLC described clinical predictors of response including the female gender, adenocarcinoma with bronchioloalveolar feature, non-smoking habit, and the Japanese ethnicity [6,7]. We further analyzed clinical characteristics of our patients to correlate these clinical parameters and the status of EGFR mutation. Under binary logistic regression univariate analysis, the only clinical parameter showing statistical significance was never less than 10 packs of whole-life smoking ($P=0.022$) (Table 2). Other factors considered in the univariate analysis including gender, age, stage, presence of nodal metastasis, presence of distant metastasis, and the amount of bronchioloalveolar carcinoma within tumor did not reveal any association to the mutation status. The significance of negative correlation between the smoking history and *EGFR* mutation persisted under multivariate analyses including all variables ($P=0.024$) except the gender. The influence of the gender may be partly explained by the rarity of smokers among our female patients, one out of 21 (4.8%). The above data suggest an alternate, yet unknown, etiology other than cigarette smoking in the

Table 2
EGFR mutation status correlates with clinical parameters

	Mutation		*P* value*
	No	Yes	
Sex			0.112
Male	19	18	
Female	7	16	
Smoking			0.022
< 10 packs	4	18	
> 10 pack	12	11	
Stage			0.438
I	5	8	
II	3	7	
III	5	7	
IV	4	5	
Nodal metastasis			0.432
No	7	10	
Yes	12	15	
Extrathoracic metastasis			0.336
No	13	22	
Yes	4	5	

**P* value bases on univariate binary logistic regression analysis.

pathogenesis of many lung adenocarcinomas. In this regard, several reports have demonstrated the effects of smoking to lung cancer especially a strong association of K-*ras* mutations, a downstream target of the EGFR signaling pathway, with lung cancer patients who had smoking history [18–20]. We hypothesize that activated K-*ras* mutation would bypass the necessity of the presence of *EGFR* mutation. It is interesting to note that a recent study that analyzed *EGFR* mutations in Taiwan NSCLC did not reveal any similar negative association to the smoking history [21]. This discrepancy may arise from different pattern of mutations detected, higher rate of exon 21 point mutation in Taiwan, or sampling bias. Further investigations are needed to elucidate the exact relationship between cigarette smoking, the pattern of *EGFR* and K-*ras* mutation, and the response to TKIs.

Through our approach and population, the actual prevalence of *EGFR* mutation in adenocarcinoma would be underestimated since we characterized only the hotspot areas of mutation, exon 19 and exon 21. However, these mutation patterns have been reported to be tightly correlated with the response to gefitinib or erlotinib. Our patients who had known response to gefitinib revealed a similar association to the mutation status. Additionally, three patients who were known carriers of exon 19 deletions in tumor from our analysis showed objective responses to the subsequent treatment with gefitinib. Uncommon or different pattern of mutations beyond the critical regions, L745–A748 deletion of exon19 and exon 21 L858R point mutations, have been identified in exon 18 and exon 20 or other regions of exon 19 and exon 21. The relationship between these uncommon mutation patterns to the sensitivity to TKI treatment is less well characterized. A very high frequency of *EGFR* mutations at the critical regions in our current study thus supported the better clinical outcomes with TKI treatment in Asian NSCLC patients, which are consistent with other recent reports from the Japanese and Taiwanese populations [15,21]. Furthermore, the significant association between *EGFR* mutation to non-smokers and Asian ethnicity suggests a unique biology of NSCLC adenocarcinoma in this population against other types of NSCLC, which display less sensitivity to TKI. It will be important to characterize this unique adenocarcinoma further in separation from other types of NSCLC in the future.

In summary, we report a very high frequency of *EGFR* mutation in lung adenocarcinoma in Thailand. The presence of the mutation displays a tight association with the response to TKI treatment in NSCLC and with non-smokers. Our data corroborate the presence of the unique NSCLC in Asian populations and explains the higher response rate to TKI found in Asian lung adenocarcinoma.

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Cyclin A1 promoter hypermethylation in human papillomavirus-associated cervical cancer

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Abstract

Background: The aim of this study was to evaluate epigenetic status of *cyclin A1* in human papillomavirus-associated cervical cancer. Y. Tokumaru *et al.*, *Cancer Res* **64**, 5982-7 (Sep 1, 2004)demonstrated in head and neck squamous-cell cancer an inverse correlation between *cyclin A1* promoter hypermethylation and *TP53* mutation. Human papillomavirus-associated cervical cancer, however, is deprived of TP53 function by a different mechanism. Therefore, it was of interest to investigate the epigenetic alterations during multistep cervical cancer development.

Methods: In this study, we performed duplex methylation-specific PCR and reverse transcriptase PCR on several cervical cancer cell lines and microdissected cervical cancers. Furthermore, the incidence of *cyclin A1* methylation was studied in 43 samples of white blood cells, 25 normal cervices, and 24, 5 and 30 human papillomavirus-associated premalignant, microinvasive and invasive cervical lesions, respectively.

Results: We demonstrated *cyclin A1* methylation to be commonly found in cervical cancer, both in vitro and in vivo, with its physiological role being to decrease gene expression. More important, this study demonstrated that not only is *cyclin A1* promoter hypermethylation strikingly common in cervical cancer, but is also specific to the invasive phenotype in comparison with other histopathological stages during multistep carcinogenesis. None of the normal cells and low-grade squamous intraepithelial lesions exhibited methylation. In contrast, 36.6%, 60% and 93.3% of high-grade squamous intraepithelial lesions, microinvasive and invasive cancers, respectively, showed methylation.

Conclusion: This methylation study indicated that *cyclin A1* is a potential tumor marker for early diagnosis of invasive cervical cancer.

Background

Cervical cancer (CC) is an important health problem and is a leading cause of cancer mortality worldwide in women. [1] When exposed to and infected by one of the high-risk human papillomaviruses (HPV), vulnerable cervical epithelium may enter a complex multistep process and develop an invasive carcinoma. [2-4] The spectrum of histologic alterations during the intricate processes of multistep carcinogenesis can be classified as premalignant lesions, including low-grade and high-grade squamous intraepithelial lesions (SILs), and malignant invasive cervical cancers. [5] Despite its strong association with CC, HPV infection alone is not sufficient for the cervical epithelium to fully develop an invasive cervical cancer. Persistent HPV infection contributes to the development of SILs, with viral oncoproteins facilitating the dysregulation of cellular proliferation and the apoptotic process. However, additional accumulation of mutations, as well as epigenetic alterations in the crucial oncogenes and tumor suppressor genes, is required before these premalignant lesions fully transform into invasive cancers. [6]

The aim of this study was to evaluate DNA methylation status of *cyclin A1* (*CCNA1*) in HPV-associated CC. *CCNA1*, a second A-type cyclin, has been shown to be essential for entry into metaphase of male meiosis I[7,8] Consistent with this function, *CCNA1* is highly expressed in testis and hematopoietic progenitor cells, but is present at low levels in most other tissues. [9] No phenotype other than male infertility has been reported in mice lacking *CCNA1*. [10] Surprisingly, several lines of evidence suggest that *CCNA1* may be a potential epithelial tumor suppressor gene. First, the expression of *CCNA1* has been demonstrated to be downregulated in several cancers, such as nasopharyngeal carcinoma and head and neck squamous-cell cancer (HNSCC). [11-13] Second, CCNA1 plays an important role in DNA double-strand break repair following radiation damage by activation of the non-homologous end-joining process that confers DNA stability. [14] Finally, the promoter, similar to several key tumor suppressor genes, is frequently hypermethylated in colon cancer and HNSCC. [13,15]

Expression of *CCNA1* has been shown to be correlated with the activation of *TP53*. In a HNSCC model, there is an inverse relationship between *CCNA1* promoter methylation and *TP53* mutation status in HNSCC tissues. [13] Similar to HNSCC, the majority of CC is of squamous cell origin and its molecular carcinogenesis strongly correlates with impaired TP53 function. [16-18] However, unlike HNSCC, the functional loss of TP53 in CC is not ascribed to gene mutation, but is processed by viral and host protein-protein interaction. CC is strongly associated with infection by high-risk HPV types and its oncoprotein E6 has the ability to associate with and neutralize the function of TP53. [17,18] E6 binds to TP53 and catalyzes multi-ubiquitination and degradation of TP53. Consequently, the majority of CC cells have a wild-type *TP53*, but the protein levels are decreased. Therefore, in comparison with HNSCC, it was of interest to determine if *CCNA1* is methylated in HPV-associated squamous cell CC.

Methods

Cell lines and tissue samples

SiHa and two HeLa CC cell lines from different sources were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. All three cells were purchased from ATCC. SiHa, HeLa (S), and HeLa (K) were grown and maintained in laboratories of Dr. Ponglikitmongkol M, Mahidol University, Dr. Gutkind JS, NIH, USA and Dr. Ruxrungthum K, Chulalongkorn University, respectively.

With approval of ethical committee, faculty of medicine, Chulalongkorn university, normal cervical tissues, cancer tissues and blood samples were obtained and prepared as previously described. [19,20] Cervical tissues were obtained by punch biopsy of lesions under direct visualization or under colposcopic examination. Specimens were divided in two. The first sample was submitted to routine histological examination, and the second was reserved for DNA isolation. Blood samples were obtained by venipuncture from CC patients and healthy blood donors. All HPV-positive premalignant lesions were exfoliated cells, selected from routine cytological screening. In brief, cervical cells were collected with a cervical sampler (Digene Corporation, Gaithersburg, MD, USA) using the cervical cytobrush technique, and were divided into three parts. The first was reserved for routine cytological diagnosis. The second was tested for the presence of high-risk HPV (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) DNA by Hybrid Capture 2 (Digene Corporation, Gaithersburg, MD, USA). [21] In cases of positive high-risk HPV and complete histological tissue evaluation, the third part was subjected to *CCNA1* methylation analysis. DNA extraction was performed using Tris/SDS and proteinase K at 50°C overnight, followed by phenol/chloroform extraction and ethanol precipitation.

Cervical biopsy specimens and Papanicolaou smears were examined and reviewed by at least two gynecologic pathologists to ensure good quality control of the final pathology results. All CCs contained 20–95% malignant cells. The histological diagnoses distinguished among normal epithelium, low-grade SILs, high-grade SILs, microinvasive and invasive cancer. In case of invasive cancer, only those samples classified as squamous-cell lesions were used for further analysis.

Table 1: Oligonucleotide sequences and conditions for PCR analyses

Primer	Sequence	Amplicon size (bp)	Annealing temperature (°C)
CCNA1metF	TTTCGAGGATTTCGCGTCGT	46	53
CCNA1metR	CTCCTAAAAACCCTAACTCGA		
CCNA1unmetF	TTAGTGTGGGTAGGGTGTT	67	53
CCNA1unmetR	CCCTAACTCAAAAAAACAACACA		
CCNA1cloningF	TGGGTAGGGCGTCGTAGTT	196	55
CCNA1cloningR	GCCCCCGACCTAAAAAAA		
CCNA1cDNAF	ATTCATTAAGTGAAATTGTGC	170	47
CCNA1cDNAR	CTTCCATTCAGAAACTTATTG		
GAPDHF	GTGGGCAAGGTATCCCTG	460	52
GAPDHR	GATTCAGTGTGGTGGGGGAC		

Additional six OTC-embedded frozen CCs and five normal cervices, obtained from hysterectomy specimens, were microdissected as previously described.[22] Histologically normal epithelium, connective tissue and malignant cells were subjected to *CCNA1* methylation and expression studies.

HPV detection and typing

HPV *L1*, *E6* gene amplification and dot blot hybridization were performed as previously described[19,22,23] Briefly, each L1 amplification reaction contained the L1 degenerate primers MY11 and MY09. The E6 reactions contained WD72, WD66, WD154, WD67 and WD76. Both reactions were used to amplify genomic DNA during 40 PCR cycles. To analyze the amplicons for the presence of high-risk HPV, we applied dot blot hybridization using the HPV type-specific oligo probes, WD170, WD132, RR1, RR2, WD103, WD165, WD, consensus L1, MY12/13, WD126, WD128, MY16, WD133/134, MY14 and WD174. The membranes were subjected to analysis by a phosphoimager. Results for L1 and E6 dot blots were scored independently. Duplicate filters were prepared for all specimens.

Sodium bisulfite modification and duplex methylation-specific PCR (MSP)

The DNA samples were subjected to bisulfite treatment. [24,25] Briefly, 2 μg of genomic DNA was denatured with NaOH (final concentration 0.2 M). Subsequently, 10 mM hydroquinone and 3 M sodium bisulfite were added and incubated at 50°C for 16 h. The modified DNA was then purified using Wizard DNA purification resin (Promega, Madison, WI, USA) followed by ethanol precipitation. Duplex MSPs were performed to identify the *CCNA1* methylation status of all samples. The duplex PCR mixtures contained 10× PCR buffer (Qiagen, Chuo-ku, Tokyo), deoxynucleotide triphosphates (0.2 mM), primers CCNA1metF, CCNA1metR, CCNA1unmetF and CCNA1unmetR (final concentration 0.4 μM each per reaction) (Table 1), 1 U of HotStarTaq (Qiagen, Chuo-ku, Tokyo) and bisulfited DNA (80 ng). The amplification reaction was carried out for 30 cycles in a 2400 Perkin Elmer thermal cycler. Then 10-μl aliquots of the PCR products were stained with cyber green, run on an 8% non-denaturing polyacrylamide gel. The band intensity was visualized and measured by using a phosphoimager.

RNA preparation and analysis

Expression of *CCNA1* in the CC cell lines was examined by RT-PCR. Total RNA was extracted using the TRIZOL reagent (Invitrogen, Singapore) according to the manufacturer's specifications and 5 μg of each sample was subjected to cDNA synthesis using MMLV reverse transcriptase (Fermentas, Hanover, MD, USA). PCR mixtures contained 10× PCR buffer, 0.2 mM dNTPs, 0.4 μM each of primers CCNA1cDNAF and CNA1cDNAR, 1 U of HotStartaq and 80 ng cDNA. *GAPDH* served as the internal control (Table 1). Aliquots of 10 μl of the PCR products were subjected to electrophoresis on a 2% agarose gel stained with ethidium bromide on preparation, and were visualized by a UV trans-illuminator.

Bisulfite genome sequence analysis

Some *CCNA1* methylation-positive CCs were selected for sequence analysis. The bisulfited DNAs were amplified using CCNA1cloningF and CCNA1cloningR (Table 1). The amplified fragments were cloned using the PGemT easy vector and sequenced.

Results

The aim of this study was to determine if the *CCNA1* promoter is methylated in CC and to elucidate how the epigenetic alteration occurs during multistep CC development. The experiments conducted comprised of: first, establishment of *CCNA1* MSP; second, identification of the methylation status and correlation with expression in CC cell lines, normal cervix and CC; and finally, investigation of the frequency of methylation in normal tissues, high-risk HPV-associated low SILs, high SILs, microinvasive and invasive squamous cell CC.

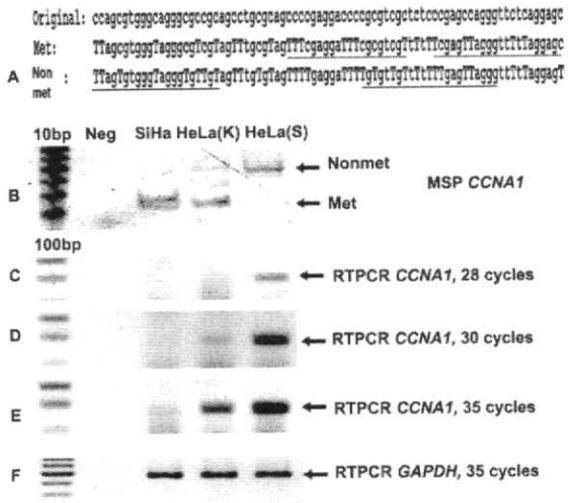

Figure 1
Schematic representation of inverse correlation between promoter methylation and expression of *CCNA1* in CC cell lines. (A) Diagram of methylated and non-methylated sequences after bisulfite modification covering the area of both primers (underlined) in the promoter region of *CCNA1*. M, DNA size marker. Top panel, 10-bp ladder; bottom four panels, 100-bp ladder. Neg, negative. (B) Duplex MSP analysis of cell lines. Upper and lower arrows indicate non-methylated and methylated amplicons, respectively. MSP, methylation-specific PCR. (C-E) RT-PCR of the *CCNA1* gene after 28, 30 and 35 cycles, respectively. (F) RT-PCR of the *GAPDH* gene as an internal control.

CCNA1 methylation in CC cell lines

Duplex MSP for *CCNA1* was designed according to the sequence in Figure 1A. The methylated sequence comprised of 46 bp and the non-methylated sequence, 67 bp, shown as the lower and the upper amplicons, respectively.

Previously, Carsten Müller-Tidow et al. [26,27] extensively studied the role of *CCNA1* methylation and found that *CCNA1* was methylated in several non-expressing tumor cell lines, including HeLa. To confirm this particular finding in CC cell lines, we investigated methylation and expression in HeLa and SiHa cells. Our preliminary study in HeLa, HeLa(S), revealed complete non-methylation, which contradicts the previous report (Fig. 1B). To settle this controversy, we attempted to further evaluate additional CC cell lines, including HeLa(K) grown in a different laboratory, and SiHa. The result confirmed the Carsten Müller-Tidow et al. [26,27] finding, in that the majority of Hela(K) cells, as well as all SiHa cells, were hypermethylated. CCNA1 RT-PCR confirmed the inverse relation between DNA methylation and gene expression. CCNA1 RNA levels were high, intermediate and low in HeLa(S), HeLa(K) and SiHa cells, respectively (Fig. 1). These data indicate that *CCNA1* methylation is common in CC cell lines and its physiological role is to decrease gene expression. The absence of methylation in HeLa(S) might indicate a demethylation process that occurs under different cell culture and maintenance conditions.

We validated the reliability of this duplex MSP by performing calibration experiments using SiHa mixed with HeLa(S), CCNA1 completely hypermethylated and non-methylated cells, respectively (Fig. 2A). With at least three replicates for each experiment, the result demonstrates the consistency of the current approach, with minimal intra- and inter-assay variations (Fig. 2B). It is noteworthy that the correlation between measured and actual CCNA1 methylation percentages was not linear, but exponential.

CCNA1 methylation and expression in cervical tissues

The discovery of an inverse correlation between CCNA1 methylation and expression in CC lines suggested possibility of the same situation in vivo. To test this hypothesis, we evaluated the epigenetic control in vivo. Six frozen OTC-embedded CCs and five normal cervices were microdissected and subjected to duplex MSP and CCNA1 RT-PCR. Figure 3 shows examples of typical in vivo results. First, whereas no methylation could be observed, CCNA1 mRNA was discoverable by RT-PCR in normal cervix from both epithelium and connective tissue cells (Fig. 3A). In contrast, epigenetic control was detectable in cervical epithelia of CC patients from both malignant cells and adjacent histologically normal cervical epithelia. Nonetheless, in matched cases, a higher degree of methylation could be demonstrated in cancer than in normal cells. From all CCs, no CCNA1 mRNA was detectable. Interestingly, even if methylation was detected, CCNA1 was expressed in malignancy-adjacent histologically normal cervical tissues. Moreover, an inverse correlation between the methylation level and mRNA quantity was observed. CCNA1 expression in methylated malignancy-adjacent histologically normal cervical epithelium may be due to normal cell contamination or partial methylation at the promoter according to CC multistep progression. Whereas complete methylation could be observed in most cancer cells, partial and non-methylated CCNA1 was discovered in the adjacent epithelia (Fig. 3B). In conclusion, this experiment evaluating cervical tissue in vivo led to three conclusions. First, *CCNA1* methylation was exclusively associated with cervical carcinogenesis. Second, the epigenetic alteration occurred earlier than morphological transformation of the cellular phenotype. Finally, methylation may play a role in this gene inactivation.

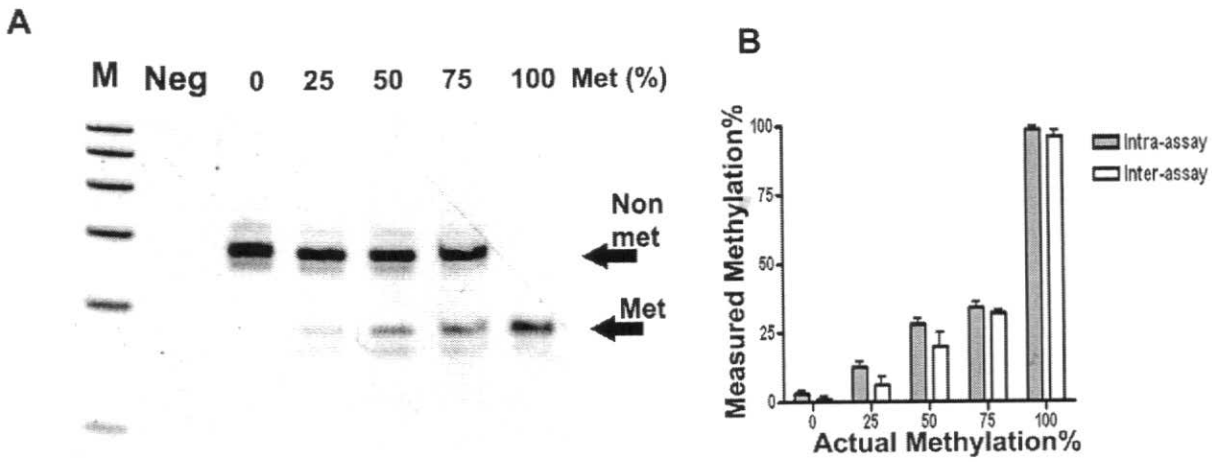

Figure 2
Intra- and inter-assay variation of the duplex MSP. (A) Duplex MSP of a mixture of *CCNA1* complete and non-methylated CC cell lines, SiHa and HeLa(S), respectively. M, DNA size marker; Neg, negative; 0, 25, 50, 75, 100 Met (%) represent the proportion of SiHa DNA in the mixture, varied from 0 to 100%, respectively. The upper and lower bands are non-methylated and methylated bands, respectively, indicated by labeled arrows. (B) Graphical comparison between measured *CCNA1* methylation, percentage intensity of methylation amplicon (x-axis), and actual methylation, the proportion of SiHa DNA (y-axis). The bar height indicates the mean and error bars, T, represent standard deviation (SD) across experiments.

CCNA1 methylation incidence during multistep cervical carcinogenesis

Cervical intraepithelial neoplasia provides a crucial model to study the multistep process of carcinogenesis. Therefore, we evaluated the frequency of CCNA1 methylation in several cervical epithelial tissues with a distinctive degree of malignant transformation, normal cervix, CIN, microinvasive and CC, respectively. We selected 43, 25 and 30 cases of white blood cells (WBC), normal cervical biopsies and invasive CCs, respectively (Table 2). Among these samples, 13 WBC samples and 6 normal cervical samples, located at least 3 cm from the tumor margin and showing the absence of HPV DNA, originated from CC patients. For all cases, when a methylated amplicon was visible and the methylation percentage measured exceeded 5%, the test was deemed positive. All selected CCs were squamous and positive for HPV. Of the cases, 24 harbored HPV type 16, 4 had HPV type 18 and 2 cases displayed unclassifiable HPV types. Interestingly, a high frequency of methylation was exclusively present in CCs, i.e., 28 cases or 93.3% (Fig. 4A,B and Table 2). To reveal multistep carcinogenesis, we included 24 cases of SILs and 5 microinvasive cancers from exfoliated cervical cells. All cases were positive for oncogenic HPV, analyzed by Hybrid Capture 2. Whereas 60% and 36.6% of the microinvasive cancers and high SILs, respectively, demonstrated *CCNA1* methylation, none of the HPV-associated low SILs exhibited these epigenetic changes (Fig. 4B and Table 2).

Discussion

This study demonstrated that: (i) *CCNA1* promoter hypermethylation in HPV-associated squamous cell CC is unusually common; (ii) it is specific to CC; and (iii) the methylation is more common in invasive phenotypes compared to other histopathological stages during multistep carcinogenesis. This finding identifies both the interesting biology of CC and a potential clinical application of *CCNA1* methylation as an additional molecular marker for the early diagnosis of invasive CC.

Annual cytology screening has dramatically increased the effectiveness of early CC detection. Nonetheless, additional tests will help to improve the sensitivity and specificity of a single Papanicolaou smear for histological

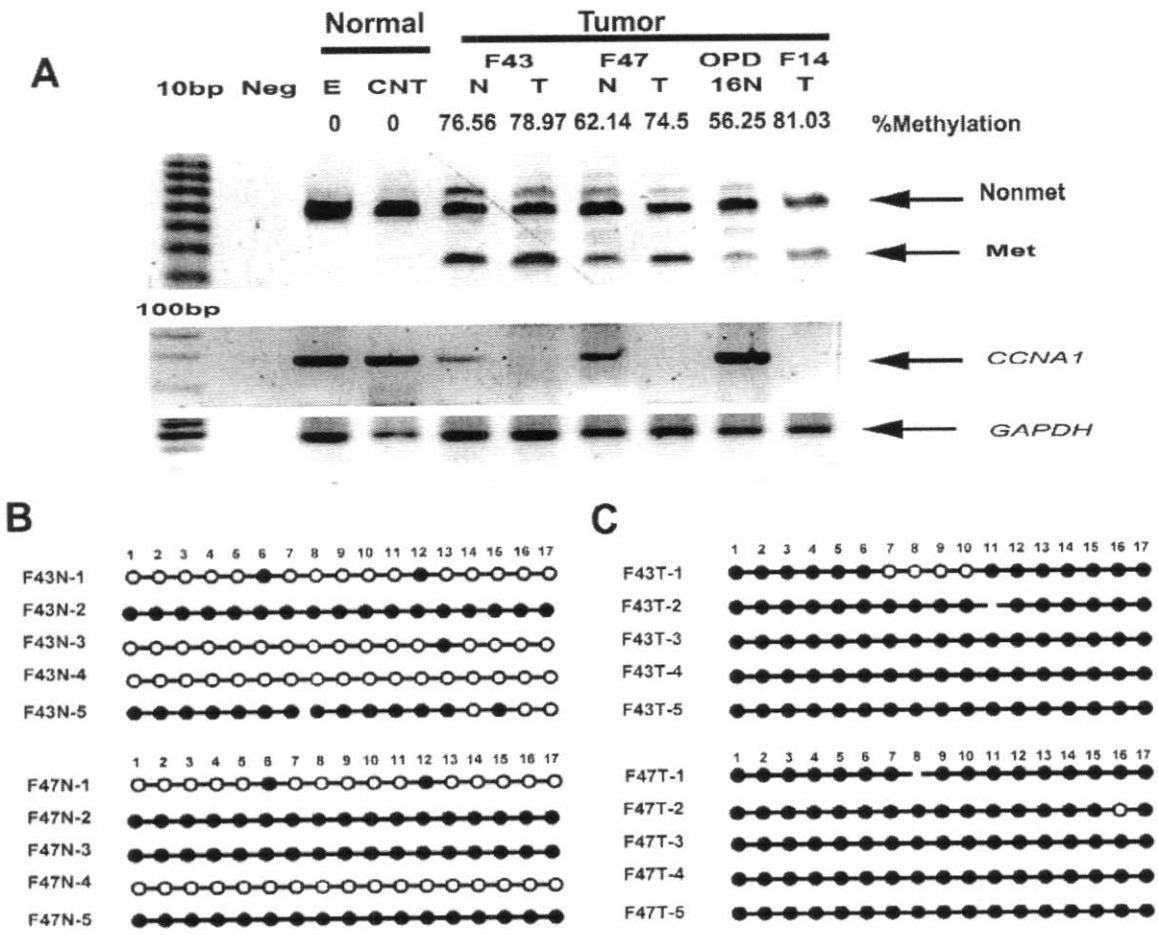

Figure 3
CCNA1 methylation and expression in microdissected cervical tissues. (A) Duplex MSP and *CCNA1* PCR; E and CNT are epithelium and connective tissue cells from normal cervix; N and T are adjacent histological normal and cancer cervical epithelium from CC, respectively. Arrows indicate non-methylated, methylated, *CCNA1* cDNA and *GAPDH* cDNA, respectively. (B) Bisulfite sequencing at the *CCNA1* promoter, with circles denoting the methylation status of each selected clone. Black and white circles are methylated CG dinucleotides, and non-methylated CpG dinucleotides and TG dinucleotides, respectively.

analysis. Recently, testing for oncogenic HPVs has been introduced to aid in the triage of women with atypical squamous cells of undetermined significance (ASCUS). [28] However, because the majority of patients with HPV-associated lesions do not progress to invasive cancer, several studies have attempted to add a panel of tumor suppressor gene methylations to improve the effectiveness of molecular cytological diagnosis. [29,30] Since the frequency of *CCNA1* methylation is high and specific to invasive CC, this gene should be a good candidate to increase the coverage rate for early cancer detection.

In HNSCC, *CCNA1* promoter hypermethylation is inversely related to *TP53* mutation. [13] Nonetheless, the frequency of *CCNA1* promoter hypermethylation in CC is high, whereas the function of TP53 in CC is usually impaired as a consequence of protein degradation induced by binding of the viral E6 protein. [18] This observation may be due to either differences in tissue types or pathophysiological outcomes of *TP53* between mutations and diminution of the protein function subsequent to E6 binding. We prefer the latter hypothesis, since *TP53* and *CCNA1* have been shown to augment each

Table 2: *CCNA1* methylation and clinico-pathological correlation

Histological characteristics	Total number of cases	*CCNA1* promoter hypermethylation	
		Absent	Present
WBC	43	43	0
Normal cervix	25	25	0
Low-grade SIL	13	13	0
High-grade SIL	11	7	4
Microinvasive cancer	5	2	3
Squamous cell CC	30	2	28
FIGO stage I-IIA	6	0	6
FIGO stage IIB-IV	24	2	22
Grade 1, keratinized type	9	0	9
Grade 2, non-keratinized type	21	2	19

FIGO, International Federation of Gynecology and Obstetrics.

other's expression. [13,14] Consequently, the CCNA1 protein could help to increase physiologic TP53 to counter the function of E6, except for cases of *TP53* mutation. In other words, alterations of both *CCNA1* and *TP53* in HNSCC will be redundant. In contrast, in CC, a decrease in CCNA1 protein should prevent the increment of TP53 that would have compensated for the protein destruction by E6.

Multistep process analysis revealed that *CCNA1* methylation is remarkably specific for cervical carcinogenesis. The biological function of CCNA1 is to activate DNA breakage repair by mechanisms depending on CDK2 activity and Ku proteins. [14] It is interesting to hypothesize why the genomic instability, triggered by impairment of the CCNA1 function, is crucial as an early event in CC development. Perhaps the rate of spontaneous mutations in cervical epithelial cells is too low to accumulate sufficient malignancy-transformation-dependent oncogene and

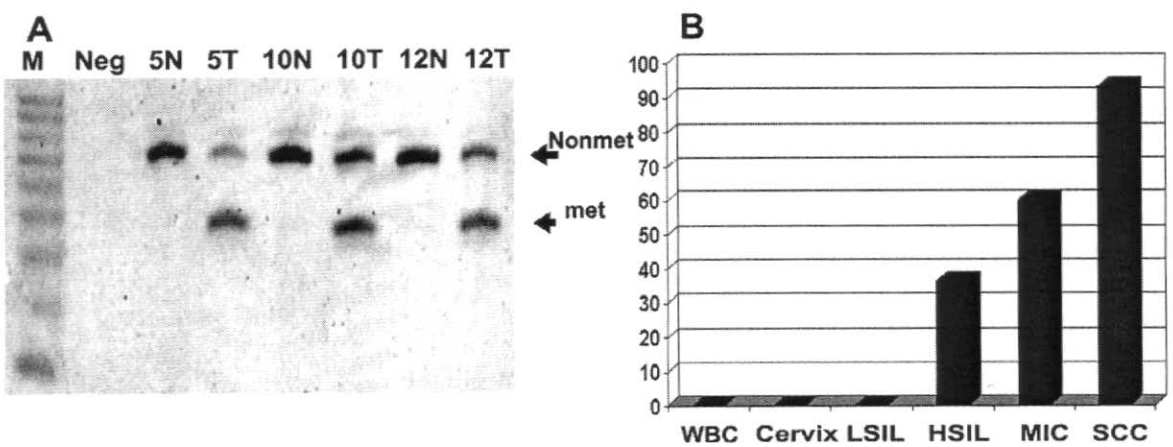

Figure 4
Schematic representation of methylation-specific PCR in CC. (A) PCR analysis of CC: M, DNA size marker; Neg, water; N and T, matched normal cervices and tumors, respectively. (B) Bar graph demonstrating the frequency of DNA methylation. Numbers on the y-axis are the percentage of positive methylation cases. Sample types are on the x-axis. WBC, normal cervix, Low-grade SIL, High-grade SIL, microinvasive cancer and squamous cell CCs number are 43, 25, 13, 11, 5, and 30, respectively. The methylation frequencies of each tissue type are represented by the height of each rectangular bar.

tumor suppressor gene mutations if the cells possess fully functional CCNA1. Therefore, the frequency of invasive CC devoid of *CCNA1* methylation is limited.

Conclusion

This study demonstrates the strong association between *CCNA1* promoter hypermethylation and invasive HPV-associated CC indicates that this gene could serve as an effective molecular marker. Moreover, our finding, in comparison with previous reports, [13,14] also suggests that there is a possible molecular link between oncogenic HPVs, TP53 and *CCNA1* promoter hypermethylation.

Abbreviations

CC: cervical cancer, CCNA1: cyclin A1, SILs: squamous intraepithelial lesions, HPV: *Human papillomavirus*, WBC: white blood cell

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

NK: Perform all experiments, data analysis and write the article. PY: set up duplex MSP experiment, CP: collecting and HPV analysis of CIN, SK, ST, PT, WT, DT and SN: collect clinical samples and data, AM: Hypothesize, design and analyze the experiments and write the article

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ORIGINAL ARTICLE

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SHP-1 promoter 2 methylation in normal epithelial tissues and demethylation in psoriasis

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Abstract *SHP-1* promoter hypermethylation has been studied in hematopoietic cells and observed only in various types of lymphoma and leukemia. This study reports a contrasting situation in normal epithelial tissues and an association with skin pathogenesis, particularly in psoriasis. We investigated several cell lines, five of them were epithelial and six were hematopoietic, white blood cells from normal, healthy donors, and normal microdissected epithelium of kidney, liver, breast, cervix, lung, prostate, bladder, and skin. Interestingly, promoter 2 hypermethylation was apparent in all epithelial cell lines and tissues. However, distinctive degrees of demethylation were noted in some skin samples. The methylation patterns of each cell line corresponded to their mRNA isoforms, in that isoforms I and II could not be detected with either promoter 1 or 2 hypermethylation, respectively. We further explored whether an enhanced degree of demethylation could be observed in various dermatopathology lesions. While the promoter 2 methylation levels of squamous cell cancers, eczemas, and normal skins were not different, a significant degree of demethylation can be observed in psoriasis ($p<0.005$). In addition, psoriasis displays a higher level of *SHP-1* isoform II than normal skin ($p<0.05$). In conclusion, this study discovered an unprecedented role of *SHP-1* methylation in tissue-specific expression and its alteration in a nonmalignant human disease besides the transcription inhibition in leukemia and lymphoma. Furthermore, the promoter demethylation may play an important role in skin pathogenesis by enhancing *SHP-1* isoform II transcription in psoriatic skin lesions.

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Keywords *SHP-1* · DNA Methylation · Psoriasis · *PTPN6* · Demethylation

Abbreviations SCC: Squamous cell carcinoma · COBRA: combined bisulfite restriction analysis · MSP: methylation-specific PCR

Introduction

SHP-1 or *PTPN6* has been considered a tumor suppressor gene in hematopoietic cells due to its promoter hypermethylation in various types of lymphoma and leukemia [1–4]. Surprisingly, during our study, while *SHP-1* methylation would have been detectable in other malignant genomes, instead, the epigenetic modification was discovered in normal epithelial tissues. This information is very interesting not only because it presents a paradox situation but also because identification of new tissue-specific methylation control genes is crucial for a better understanding of human development and tissue differentiation processes [5, 6]. The aim of this study was to explore *SHP-1* methylation and transcription profile in various cell and tissue types and to elucidate if this epigenetic modification could be altered in any epithelial pathological conditions.

SHP-1, a nonreceptor protein tyrosine phosphatase containing an SH2 domain in tandem and protein tyrosine phosphatase, plays an important role in regulating the growth and proliferation process depending on cell types [2, 7–9]. The gene comprises two promoters, directing the expression of two isoforms [10]. Promoter 1 is located approximately 7 kb upstream from promoter 2. Previous studies distinguished levels and types of *SHP-1* mRNA isoforms in various cell types. Studies in cell lines have shown that isoform I is normally expressed at lower level in epithelial and barely discovered in hematopoietic cells, whereas isoform II is exclusively and vigorously expressed in hematopoietic cells. Interestingly, the SHP-1 protein is located in the cytoplasm of hematopoietic and in the nucleus of epithelial cells [11]. *SHP-1* promoter hypermethylation, discernible in various lymphomas/leukemias, increases the lymphoma development potential by silencing *SHP-1* transcription. The epigenetic regulation has been attributed to promoter 2 [3, 4]. It is important to note that the mechanism inducing the methylation has been described. A recent study conducted on T-cell lymphoma indicates that STAT3, in part, transforms cells by inducing the epigenetic silencing in cooperation with DNMT1 and, apparently, histone deacetylase 1 [12].

Interestingly, transgenic mice with keratinocytes expressing a constitutively active *Stat3* (K5.Stat3C mice) develop a skin phenotype either spontaneously or in response to wounding, which closely resembles psoriasis [13]. Psoriasis is a benign chronic inflammatory skin disease. The skin lesions are characterized by sharply demarcated erythematous plaques of various sizes covered with silvery scaling. These changes are caused by intense skin inflammation with infiltration of inflammatory cells into the dermis and epidermis and strongly enhanced keratinocyte proliferation [14]. This information indicates that both *SHP-1* methylation and psoriasis are linked to STAT3 functions, pointing at a possible association between *SHP-1* methylation and psoriasis.

Psoriasis is a common chronic skin disease affecting up to 2.0% in many populations. It is believed to be a multifactorial genetic disorder with the phenotype depending on the patient's genetic background and environmental factors. The disease is characterized by keratinocyte hyperproliferation and early differentiation as a consequence of autoimmune reaction [15]. Management of psoriasis ranges from topical therapies for limited disease to systemic therapies for more widespread disease. Despite various available treatments, the adverse effect or inadequate efficacy of the therapies involved has invoked a necessity for safer and more effective treatment. The advent of therapies based on mechanisms that target critical molecular pathways has evoked considerable interest [16]. Our novel finding in psoriatic molecular pathophysiology, the epigenetic modification of *SHP-1* promoter 2, may assist in developing new and effective targeted therapies.

Materials and methods

Sample

Cell lines and patient samples

Hematopoietic cell lines, Daudi, Jurkat, Molt4, U937, K562, and epithelial cell lines, Hela, HEp2, SW480, HepG2, and HaCaT, were used. Additionally, two Epstein–Barr virus transformed B-lymphoblastoid cell lines (BLCLs) were generated from two donors. All cells were cultured in Dulbecco's modified Eagle's medium or RPMI1640 (Gibco BRL, Life Technologies, Pairly, UK) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO, USA) and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin). Cells were incubated at 37°C in 5% CO_2.

Several archival paraffin-embedded tissues derived from normal and adjacent normal tissues of several tumor patients of various locations of kidney, liver, breast, prostate, cervix, lung, bladder, and OTC-embedded frozen normal cervical sections were obtained [17]. Nine to ten each of normal skins, collected from normal skin removed during plastic surgery, and various pathological skins, including chronic stable plaque type psoriasis, guttate psoriasis, squamous cell carcinoma (SCC), and chronic eczema, were chosen for the study. All patients with dermatologic condition and who were free from any skin therapies 4 weeks prior to taking biopsy samples were diagnosed clinically by an experienced dermatologist. The diagnosis was confirmed by skin biopsy and was revealed by an experienced dermatopathologist as typical pathology for each skin condition. The tissues were microdissected as previously described [18]. Additional cases of chronic plaque type psoriasis were microdissected to separate between upper and lower section of their psoriatic epidermis. Several samples of white blood cells (WBCs) from normal healthy individuals were also included. An additional seven cases each of healthy donor and chronic stable plaque type psoriasis pathological skin conditions were obtained by 6-ml-wide punch biopsies under local anesthesia for RNA analysis. All of the psoriasis patients were of the chronic stable plaque type and did not receive any treatment except the two cases who sporadically used low-potency topical

corticosteroids at least 4 weeks prior to taking biopsy samples.

Bisulfite treatment for DNA

After extraction, all DNA samples were treated with sodium bisulfite as previously described [19]. Briefly, 1 μg of genomic DNA was denatured in 0.22 M NaOH at 37°C for 10 min. Thirty microliters of 10 mM hydroquinone and 520 μl of 3 M sodium bisulfite were added for 16–20 h at 50°C. The DNA was purified and incubated in 0.33 M NaOH at 25°C for 3 min, ethanol precipitated, then washed with 70% ethanol, and resuspended in 20 μl of H_2O.

Methylation analysis of the promoter regions by the COBRA and MSP method

All *SHP-1* bisulfite oligonucleotide sequences were derived from GenBank (accession number U47924.1). DNA methylation in the promoter 2 region of *SHP-1* was detected by duplex methylation-specific polymerase chain reaction (PCR) (MSP) using methylation-specific primers and nonmethylation-specific primers. The methylation-specific primer sequences were identical to those previously published [20]. The methylation-specific primer sequences were 5′-TGT-GAA-CGT-TAT-TAT-AGT-ATA-GCG-3′ and 5′-CCA-AAT-AAT-ACT-TCA-CGC-ATA-CG-3′, and the nonmethylation-specific ones were 5′-GTG-AAT-GTT-ATT-ATA-GTA-TAG-TGT-TTG-G-3′ and 5′-TTC-ACA-CAT-ACA-AAC-CCA-AAC-AAT-3′. A duplex MSP reaction, containing bisulfite-treated DNA and 375 nM each of all oligonucleotides, was performed for 35 cycles with the annealing temperature set at 58°C. The PCR products, 174 and 162 bp for methylated and nonmethylated sequences, respectively, were separated by electrophoresis on a 6% nondenaturing polyacrylamide gel. The amplicons were analyzed by staining with cyber green and calculating the percent methylation upon visualization on a phosphor imager using the Image Quant software (Molecular Dynamics, Pharmacia Amersham). The *SHP-1* promoter 2 methylation level was calculated as a percentage of intensity of the methylated sequence divided by the sum of methylated and nonmethylated amplicons.

On the microdissected, paraffin-embedded tissues, seminested PCR was performed. Two microliters of the duplex MSP product was added to a solution containing a nested forward primer, 5′-AGY-GTG-GGT-TAG-GGA-GGG-3′ (bisulfited nucleotides 163031-163048), and both of the reverse duplex PCR oligonucleotides. The amplification reaction was carried out as described above. The duplex amplicons, 94 and 83 bp for methylated and nonmethylated sequences, respectively, were analyzed by the phosphor imager as previously described.

Combined bisulfite restriction analysis (COBRA) [21] by seminested PCR was applied for detection of methylation in the promoter 1 region. The forward (5′-GTT-TTT-GTA-GTG-TTA-TTG-GTT-3′) and reverse (5′-AAA-CAA-CAT-CTC-TCT-ATA-AAA-A 3′) primers (bisulfited nucleotide number 158572-158592, 158743-158722, respectively) were used to generate a 172-bp amplicon during 35 cycles of PCR with the annealing temperature set at 44°C. One microliter was transferred for seminested PCR with 5′-AAC-CCA-AAC-CAA-ATA-AAA-3′ (bisulfited nucleotide number 158657-158640) as the reverse primer. Amplification conditions were identical to those in the primary PCR and generated an 86-bp amplicon. The PCR products were digested by *Taq I* (MBI Fermentas, Flamborough, Ontario, Canada) at 65°C for 16 h and separated by 6% acrylamide gel electrophoresis.

Bisulfite DNA sequencing

For promoter 1, bisulfite-treated genomic DNA was amplified by using primers 5′-GTT-TTT-GTA-GTG-TTA-TTG-GTT-3′ and 5′-AAA-CAA-CAT-CTC-TCT-ATA-AAA-3′ (bisulfited nucleotides 158572-158592 and 158743-158722, respectively). For promoter 2, bisulfite-treated genomic DNA was amplified using primers 5′-GTT-TTA-TAG-GGT-TGT-GGT-GAG-AAA-TT-3′ and 5′-ACA-CAT-ATA-TAC-CTT-ACA-CAC-TCC-AAA-3′ (bisulfited nucleotides 162912-162937, 163153-163127, respectively). The PCR products were cloned into the pGEM-T easy vector (Promega, Santhan, UK) and sequenced.

RT-PCR and real-time RT-PCR

Total RNA was extracted from cell lines as well as skin biopsies using the Trizol reagent (Life technologies, Inc.) according to the manufacturer's instructions. The RNA preparation was dissolved in 20 μl of RNase-free distilled water containing 40 U of ribonuclease inhibitor (Promega), and 50 ng of the RNA was incubated for 5 min at 70°C followed by 45 min at 42°C with 15 pmol of oligo deoxythymidine primer. To each sample, we added 19 μl of a reaction mixture containing 1× ImProm-II buffer (10 mM Tris–HCl, pH 8.3, 1.5 mM MgSO4, 50 mM KCl, and 0.001% gelatin), 5 U of reverse transcriptase (RT) (Promega), and 0.2 mM deoxynucleotide triphosphates. cDNA was amplified using the exons 3–4 primer 5′-CCC-ACC-CTG-ACG-GAG-AGC-3′ and either the exon 1 primer 5′-CTC-CCT-ACA-GAG-AGA-TGC-TGT-CC-3′ or the exon 2 primer 5′-ACT-GGG-AGC-TGC-ATC-TGA-GG-3′. All *SHP-1* RT-PCR oligo sequences were obtained from GenBank (accession number NM080549.2). Glyseraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control as previously published [22]. Both PCRs were performed for 35 cycles with the annealing temperature set at 60°C.

Real-time RT-PCR was performed in a Light Cycler machine (Roche Molecular Biochemicals, Indianapolis, IN, USA) using QuantiTect SYBR Green I (Qiagen, Hilden, Germany), according to the manufacturer's instructions in a total volume of 20 μl. Cycling conditions

were 95°C for 15 min, 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s for 40 cycles, followed by melt analysis from 65 to 95°C. The *SHP-1* (II) primers were same as above. PCRs were conducted in parallel to normalize for differences in cDNA synthesis. To correlate the threshold (Ct) values from the amplification plots to copy number, a standard curve was generated using the pGEM-T *SHP-1*(II) plasmid. PCR product melt curves were analyzed for a specific peak, and the products were visualized by the agarose gel electrophoresis and ethidium bromide staining to ensure and proportionate specific amplicons. A non-template control was run with every assay, and all determinations were performed to achieve assay.

Statistical analysis

Levels of *SHP-1* promoter 2 methylation and isoform II among skin lesions were compared, and significant differences were determined by unmatched two-tail *t* test using the SPSS software for windows 10.0 (SPSS Inc., Chicago, IL, USA).

Results

SHP-1 methylation and isoforms in human cell lines

Cell-type-specific *SHP-1* isoforms, distinguishable by promoter transcriptional activity, have been reported [11]. Whereas most hematopoietic cells express high levels of isoform II, the majority of epithelial cell line transcripts display lower levels of isoform I. Hence, the aim of our first experiment was to determine if this tissue-specific transcription process is regulated by promoter hypermethylation. A new COBRA approach was designed to analyze promoter 1, and the published MSP [20] was modified to study promoter 2 (Fig. 1a). The results demonstrate distinctive methylation patterns for hematopoietic and epithelial cell lines. While promoter 2 hypermethylation was constantly detected in all epithelial cell lines (Hela, HEp2, SW480, HaCaT, and HepG2), promoter 1 without exception was nonmethylated. In hematopoietic cells, a variety of methylation patterns could be observed. Whereas methylation was not detected in WBC, it was discernible in some hematopoietic cell lines. Promoter 1 was nonmethylated in both BLCLs and methylated in Daudi, K562, and U937. Both promoters were incompletely methylated in Jurkat and Molt4. Limited or absent amounts of promoter 2 methylation were demonstrated in both BLCLs, Daudi, and U937. Finally, promoter 2 of K562 was hypermethylated. Bisulfite, PCR, cloning, and sequencing were applied to confirm both MSP and COBRA results. Figure 1b shows examples of bisulfite sequencing results of HEp2, Daudi, and Molt4, confirming complete, absent, and partial methylation, respectively.

We observed a striking inverse correlation between promoter methylation and transcriptional activity. As for promoter 1, DNA methylation was discovered in Daudi, Jurkat, Molt4, K562, and U937, while isoform I cDNA was not detectable. In contrast, promoter 1 of all epithelial cells and both BLCLs was nonmethylated and transcribed. WBCs, however, were devoid of both methylation and isoform I, suggesting another transcriptional silencing mechanism (Fig. 1a,c). cDNAs, 239 bp, derived from isoform II were observed when amplifying *SHP-1* RNA between exons 2 and 3–4 (Fig. 1c). All human epithelial cell lines, including HeLa, HEp2, SW480, HaCaT, and HepG2, were promoter 2 hypermethylated and *SHP-1* mRNA isoform II deficient. In contrast, *SHP-1* isoform II was strongly expressed in all promoter-2-nonmethylated human hematopoietic cells, BLCLs, Daudi, and U937 (Fig. 1a,c). Both promoters 1 and 2 were methylated in K562, and consequently, we found limited amounts of both *SHP-1* mRNA isoforms. The high isoform II levels from promoter 2 of Jurkat and Molt4 were noteworthy since they displayed incomplete promoter 2 methylation (Fig. 1a,b). These findings confirmed the strong association between methylation of both promoters and their activities.

SHP-1 methylation in normal epithelia in vivo

The complete promoter 2 methylation in epithelial cell lines prompted us to hypothesize that the epigenetic control was tissue specific rather than indicative for carcinogenesis. Accordingly, we screened the methylation in normal epithelial tissues of several organs. Several stratified epithelial tissues, including skin, bladder, and cervix, and large solid organs mainly comprising epithelial tissues, such as prostate gland, breast, lung, kidney, and liver, were investigated. Whereas no promoter 2 methylation was found in WBCs from healthy donors, complete methylation was demonstrated in almost all epithelial cells (Fig. 2a,b). The methylation status is not depended on tissue preservation (Fig. 2c). It is noteworthy that incomplete methylation was sporadically identifiable in one out of nine skin cases (Figs. 2 and 3).

SHP-1 promoter 2 demethylation in psoriasis

To explore the significance of promoter 2 demethylation in skin, we investigated if and which types of pathological conditions possessing larger degrees of loss of methylation could be observed. Keratinocytes from psoriasis, SCC, and eczema lesions were collected using microdissected, paraffin-embedded tissues, and their methylation levels were compared with those of normal skins (Fig. 3a). In addition to eight out of nine normal skins, hypermethylation was discernible in the majority of eczema and SCC cases. In contrast, demethylation was found in almost all, eight out of nine, cases of psoriasis. The average methylation level of normal skin is 94.8%, whereas in psoriasis, it is 68.1%. From this, it can be concluded that the levels of promoter 2 methylation in psoriasis are distinct from those in healthy skin lesions ($p<0.005$) (Fig. 3b). Since promoter activity and expression of many proteins change with the

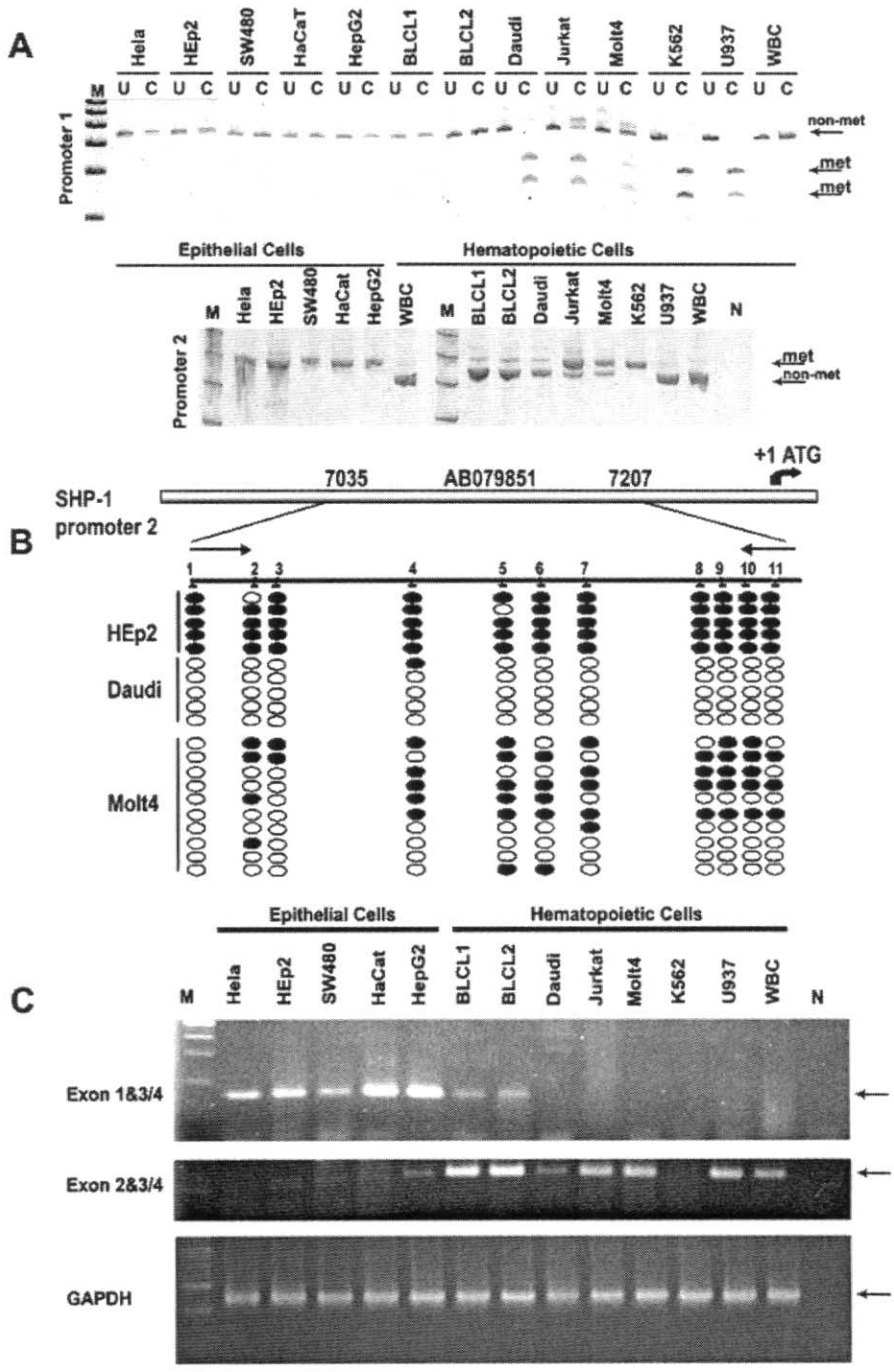

Fig. 1 *SHP-1* promoter methylation and RNA isofom in various epithelial and hematopoietic cell lines. **a** Promoter 1 and 2 methylation patterns. Promoter 1 methylation is detectable by COBRA. The PCR products, indicated by *non-met arrow*, are 86 bp. Methylated amplicons, indicated by *met arrow*, are digestible to 52 and 34 bp. *M* is standard size DNA marker, *U* is uncut, and *C* is digested amplicons. Cell sources of genomic DNA are listed above each lane. For promoter 2, duplex MSP yield 2 DNA fragments. Methylated amplicons, indicated by *met arrow*, are 174 bp, and nonmethylated products, indicated by *non-met arrow*, are 162 bp, respectively. *N* is negative control. **b** Methylation status of CpG nucleotides at promoter 2 of HEp2, Daudi, and Molt4. The uppermost *vertical bar* locates the relative nucleotide sequence in GenBank (accession number AB079851). The *numbers* and *arrows* indicate the start and end of sequenced nucleotide in relation to the trancriptional start codon, ATG (nucleotide 7604). The *numbers* of the line below indicate CpG nucleotides. *Each circle* exemplifies the methylation status of each selected clone. *Black* and *white circles* are methylated and nonmethylated CpG dinucleotides, respectively. **c** *SHP-1* RT-PCR result of epithelial and hematopoietic cell lines. Cells are listed on the top of each lane. *M* is standard DNA marker, and *N* is negative control. *Arrows* indicate the locations of expected amplicons. *Exon1&3/4* is RT-PCR between exon 1 and exons 3–4, and the amplicon size is 156 bp. *Exon2&3/4* amplifies 239 bp of isoform II. *GAPDH* was used in the RT-PCR as the control

course of keratinocyte terminal differentiation and the pattern of epidermal differentiation is altered in psoriatic skin, we investigated the demethylation in distinctive compartments. Several psoriatic skins were microdissected and divided into upper and lower half of the epidermis, and the promoter methylation levels were compared. No significant demethylation differences between the compartments were identified (Fig. 3b). In addition, the demethylation level between psoriasis with different clinical sign, chronic stable plaque type, and guttate psoriasis were not distinguishable (Fig. 3b).

We also compared mRNA expression *SHP-1* isoforms in fresh biopsies of psoriatic lesion and normal skin by Real Time RT-PCR. Interestingly, the association between promoter 2 methylation and the amount of isoform II mRNA in psoriatic skins was similar to that observed in the previously analyzed promoter 2 incompletely methylated cell lines, Jurkat and Molt4. Psoriatic skin lesions predominantly expressed isoform II, identifiable from all cases, whereas promoter 2 activities were absent or decreased in the majority of normal skin samples (Fig. 4a). The isoform II mRNA level of psoriatic skin

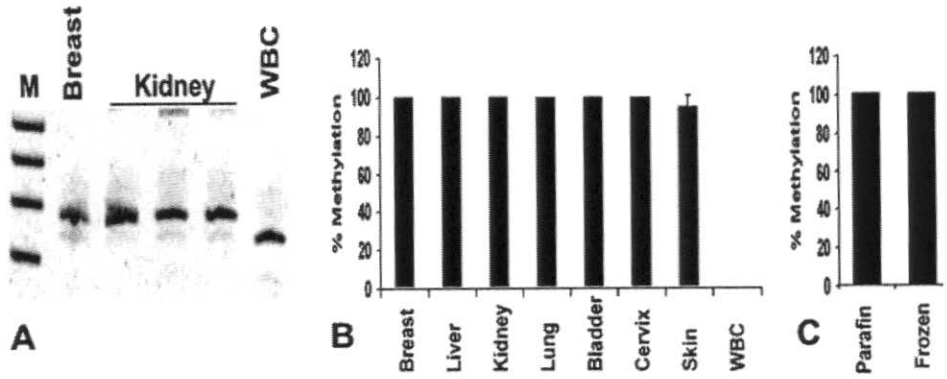

Fig. 2 *SHP-1* promoter 2 methylation in epithelia. **a** Representative result of seminested duplex MSP of epithelial tissue obtained from microdissected, paraffin-embedded tissue of breast, kidney, and WBC. *M* is standard DNA size marker. **b** Average level and standard deviation (SD) of promoter methylation in epithelial tissues of several organs and WBC, as listed below. **c** Epithelial DNA from paraffin-embedded tissues and frozen section. The *heights* of each bar represent percentage of promoter 2 methylation of each tissue types. *T* indicates the methylation SD within individual cell types. Note that 0 SD can be observed in most cases

lesions as measured by real-time RT-PCR of exons 2 and 3–4. The RT-PCR was average at 58 copies, whereas in normal skin, they amounted to 18 copies per 1 μg of total RNA (Fig. 4b). This indicated that *SHP-1* promoter 2 activity of psoriatic lesions was significantly higher than in normal skins ($p<0.05$). This finding confirms the increment of promoter 2 activity in psoriasis by a demethylation mechanism compared to normal skin.

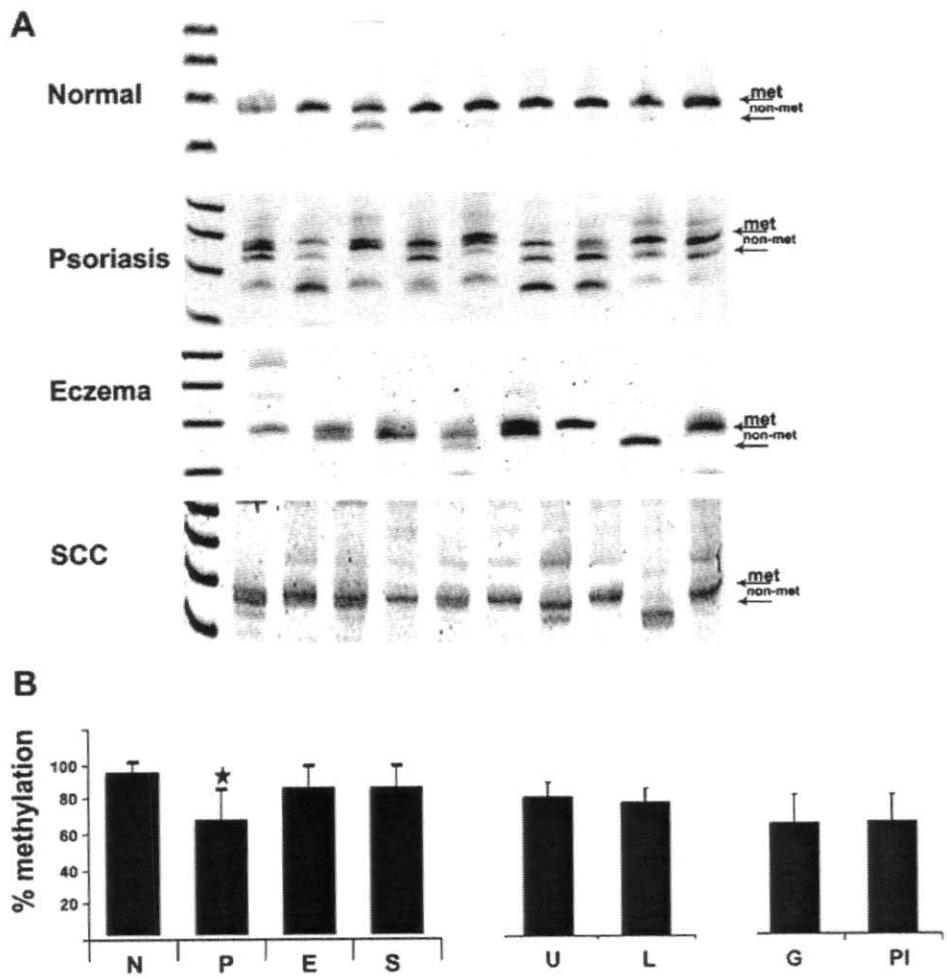

Fig. 3 *SHP-1* promoter 2 methylation in various skin lesions, normal, psoriasis, eczema, and SCC. **a** Examples of seminested duplex PCR results. *Met* and *non-met arrows* are expected 94-bp methylated and 83-bp nonmethylated amplicons, respectively. **b** Methylation percentages and SD; *N*, *P*, *E*, *S*, *U*, *L*, *G*, *Pl* are the levels of normal skins, psoriasis, eczema, SCC, upper and lower part of microdissected psoriatic skin epidermis, and guttate and chronic plaque type psoriasis, respectively. The *star* indicates significant differences of $p<0.005$ between psoriasis and normal skins

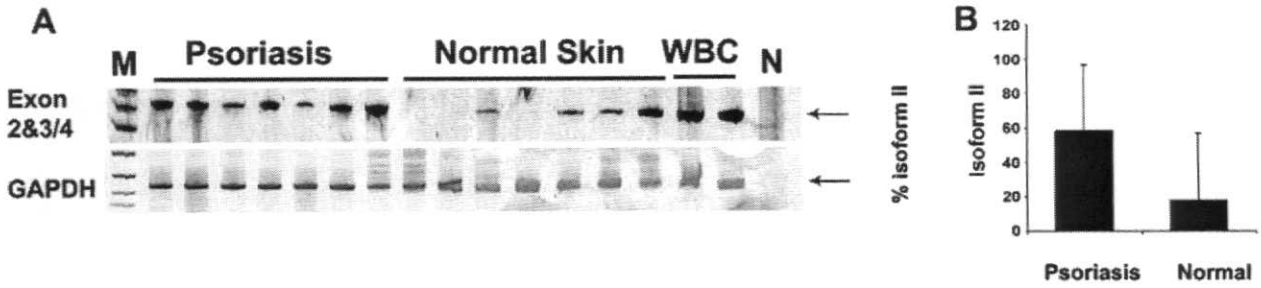

Fig. 4 *SHP-1* RT-PCR of psoriatic skin lesions and normal skins. **a** Cell types are listed on the top of each lane. *M* is standard DNA marker, and *N* is negative control. *Arrows* indicate the locations of expected amplicons. Exon 1 and 3/4 is RT-PCR between exons 1 and exon 3–4, and the amplicon is 156 bp. *Exon2&3/4* amplifies 239 bp of isoform II. *GAPDH* is RT-PCR results located at the bottom of **a**. **b** The mean and SD of isoform II level of psoriasis and normal skin proportion to 1 μg total RNA are the height of each bar and T, respectively. The real-time RT-PCR showed significant difference at $p<0.05$

Discussion

Alteration of gene expression is at the center of cellular pathogenesis. Accordingly, DNA methylation should be associated with human diseases since epigenetic modification usually silences gene transcription. Normally, DNA methylation is important in several biological processes, such as X chromosome inactivation and genomic imprinting [23–27]. As for abnormal conditions, promoter hypermethylation of several tumor suppressor genes and generalized hypomethylation have been reported to promote the potential for malignant transformation [20, 27–30]. To the best of our knowledge, this study has been the first to prove that the tissue-specific promoter methylation status can be altered in a nonmalignant human disease condition. In contrast to previously described cases of lymphomas/leukemias, the *SHP-1* promoter 2 is hypermethylated in normal epithelia, and its activity is consequently limited. Finally, we selectively analyzed several pathological skin lesions and demonstrated that promoter 2 demethylation usually arose in psoriatic skin lesions and consequently triggered up-regulation of *SHP-1* isoform II.

According to previous data, mRNA derived from promoter 2 is distinctive from promoter 1 transcripts in both structure and quantity [11]. The sequence of isoform II differs from isoform I by embracing few different amino acids at the N-terminal. Additionally, a strikingly large amount of mRNA is usually generated from promoter 2 [11]. Moreover, the molecular physiological responses to up-regulate cell growth of *SHP-1* in hematopoietic and epithelial cells are distinct. *SHP-1* isoform II has been projected as a negative regulator for signaling of cell proliferation and differentiation of hematopoietic cells via cytokine and non-cytokine receptors such as the cKit/stem cell factor receptor, the interleukin-3 receptor, the colony-stimulating factor–1 receptor, and the erythropoietin receptor [31–35]. Conversely, it has been reported to be a positive regulator of cell signaling via the mitogen-activated pathway (MAP) kinase pathway in nonhematopoietic cells [11]. In the course of this study, we found demethylation of the promoter 2 region of *SHP-1* correlated with up-regulation of *SHP-1* isoform II mRNA in psoriatic skin lesions. Hence, up-regulation of the promoter 2 in psoriatic skin lesions should result in a downstream *SHP-1* signal transduction pathway different from that in normal skin. Interestingly, psoriasis is a T-cell-mediated disease [36], involving many defects in the regulation of the transcription factors: signal transducer and activator of transcription (STAT-1 alpha), interferon-regulated factor 1, and nuclear factor-kappaB. Moreover, the pathogenesis leads to alteration in intracellular signal transduction pathways, including MAP kinase and JAK-STAT pathways, which lead to loss of growth and differentiation control when the cells are subjected to physicochemical and immunologic stress [37]. Consequently, it is crucial to further elucidate if and which particular role *SHP-1* isoform II plays in the pathological psoriasis signal transduction network.

This association study does not indicate that *SHP-1* demethylation is specific to psoriasis. In contrast, some other skin lesions possessed the epigenetic loss; therefore, we presume that promoter 2 demethylation is a dynamic physiological process and that its prolongation or sensitization implicates psoriatic pathogenesis. In this study, we could observe a variety of methylated sequences in some hematopoietic malignant cell lines such as Jurkat and Molt4. This indicates that epigenetic modification is mosaic even if the cancer cells are subject to selective clonal expansion. Accordingly, the variation should result from a gradual loss of methylation in vitro. Furthermore, in addition to most psoriasis samples, some cases of skin lesions also sporadically disclosed demethylation. Finally, a recent study on lymphoma has proven that down-regulation of *STAT3* mRNA results in *SHP-1* demethylation [12]. Since several cytokines and growth factors are responsible for STAT3 activity, *SHP-1* methylation being controlled by STAT3 supports our hypothesis. However, STAT3 may act differently on the *SHP-1* promoter in epithelial cell. A study reported by Sano et al. [13] has demonstrated that STAT3 up-regulation is an important mechanism in psoriatic pathogenesis. Hence, if on a molecular level, STAT3 activity is related to *SHP-1* methylation in epithelial cells, there must be additional tissue-specific factors to trigger the opposite outcome, demethylation instead of hypermethylation.

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คำขอรับสิทธิบัตร/อนุสิทธิบัตร

☐ การประดิษฐ์
☐ การออกแบบผลิตภัณฑ์
☐ อนุสิทธิบัตร

ข้าพเจ้าผู้ลงลายมือชื่อในคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ ขอรับสิทธิบัตร/อนุสิทธิบัตร ตามพระราชบัญญัติสิทธิบัตร พ.ศ. 2522 แก้ไขเพิ่มเติมโดยพระราชบัญญัติสิทธิบัตร (ฉบับที่ 2) พ.ศ. 2535 และพระราชบัญญัติสิทธิบัตร (ฉบับที่ 3) พ.ศ. 2542

สำหรับเจ้าหน้าที่

วันรับคำขอ 25 ก.พ. 2551	เลขที่คำขอ 0801002098
วันยื่นคำขอ	
สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ	
ใช้กับแบบผลิตภัณฑ์	
ประเภทผลิตภัณฑ์	
วันประกาศโฆษณา	เลขที่ประกาศโฆษณา
วันออกสิทธิบัตร/อนุสิทธิบัตร	เลขที่สิทธิบัตร/อนุสิทธิบัตร
ลายมือชื่อเจ้าหน้าที่	

..ชื่อที่แสดงถึงการประดิษฐ์/การออกแบบผลิตภัณฑ์
"กรรมวิธีการตรวจวัดหมู่เมทธิลของของยีน LINE-1 ในแต่ละตำแหน่ง"

2.คำขอรับสิทธิบัตรการออกแบบผลิตภัณฑ์นี้เป็นคำขอสำหรับแบบผลิตภัณฑ์อย่างเดียวกันและเป็นคำขอลำดับที่
ในจำนวน - คำขอ ที่ยื่นในคราวเดียวกัน

3. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร และที่อยู่ (เลขที่ ถนน ประเทศ)
สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ 111 อุทยานวิทยาศาสตร์ประเทศไทย ถ.พหลโยธิน ต.คลองหนึ่ง อ.คลองหลวง จ.ปทุมธานี 12120 และสำนักงานกองทุนสนับสนุนการวิจัย และจุฬาลงกรณ์มหาวิทยาลัย

3.1 สัญชาติ ไทย
3.2 โทรศัพท์ 02-564-7000 ต่อ 1314 – 1350
3.3 โทรสาร 02 5647003
3.4 อีเมล์ tlo@tmc.nstda.or.th

4.สิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร
☐ ผู้ประดิษฐ์/ผู้ออกแบบ ☐ ผู้รับโอนสิทธิ ☐ ผู้ได้รับสิทธิโดยเหตุอื่น

25 FEB 2551

5. ตัวแทน (ถ้ามี) ที่อยู่ (เลขที่ ถนน จังหวัด รหัสไปรษณีย์)
.อรุณศรี ศรีธนะชิทธิพล และ/หรือ นายชาญชัย นิรพัฒนกุล และ/หรือ น.ส.อรกนก พรรณรักษา อยู่ที่ สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ 111อุทยานวิทยาศาสตร์ประเทศไทย ถ.พหลโยธิน ต.คลองหนึ่ง อ.คลองหลวง จ.ปทุมธานี 12120

5.1 ตัวแทนเลขที่ 1463,1731,1513
5.2 โทรศัพท์ 02 5647000
5.3 โทรสาร 025647003
5.4 อีเมล์ tlo@tmc.nstda.or.th

6.ผู้ประดิษฐ์/ผู้ออกแบบผลิตภัณฑ์ และที่อยู่ (เลขที่ ถนน ประเทศ)
1.นายอภิวัฒน์ มุทิรางกูร 2. น.ส.จุรีรัตน์ โพธิ์แก้ว อยู่ที่ จุฬาลงกรณ์มหาวิทยาลัย

7.คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิม
ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้ถือว่าได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ ในวันเดียวกับคำขอรับสิทธิบัตร
เลขที่ วันยื่น เพราะคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิมเพราะ
☐ คำขอเดิมมีการประดิษฐ์หลายอย่าง ☐ ถูกคัดค้านเนื่องจากผู้ขอไม่มีสิทธิ ☐ ขอเปลี่ยนแปลงประเภทของสิทธิ

<u>หมายเหตุ</u> ในกรณีที่ไม่อาจระบุรายละเอียดได้ครบถ้วน ให้จัดทำเป็นเอกสารแนบท้ายแบบพิมพ์นี้โดยระบุหมายเลขกำกับข้อและหัวข้อที่แสดงรายละเอียดเพิ่มเติมดังกล่าวด้วย

8. การยื่นคำขอนอกราชอาณาจักร

วันยื่นคำขอ	เลขที่คำขอ	ประเทศ	สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ	สถานะคำขอ
8.1				
8.2				
8.3				

8.4 ☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอสิทธิให้ถือว่าได้ยื่นคำขอนี้ในวันที่ได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรในต่างประเทศเป็นครั้งแรกโดย
☐ ได้ยื่นเอกสารหลักฐานพร้อมคำขอนี้ ☐ ขอยื่นเอกสารหลักฐานหลังจากวันยื่นคำขอนี้

9. การแสดงการประดิษฐ์ หรือการออกแบบผลิตภัณฑ์ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรได้แสดงการประดิษฐ์ที่หน่วยงานของรัฐเป็นผู้จัด
วันแสดง วันเปิดงานแสดง ผู้จัด

10. การประดิษฐ์เกี่ยวกับจุลชีพ

10.1 เลขทะเบียน	10.2 วันที่ฝากเก็บ	สถาบันฝากเก็บ/ประเทศ

ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอยื่นเอกสารภาษาต่างประเทศก่อนในวันยื่นคำขอนี้ และจะจัดยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ที่จัดทำเป็นภาษาไทยภายใน 90 วัน นับจากวันยื่นคำขอนี้ โดยขอยื่นเป็นภาษา
☐ อังกฤษ ☐ ฝรั่งเศส ☐ เยอรมัน ☐ ญี่ปุ่น ☐ อื่น ๆ

12. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้อธิบดีประกาศโฆษณาคำขอรับสิทธิบัตร หรือรับจดทะเบียนและประกาศโฆษณาอนุสิทธิบัตรนี้
หลังจากวันที่ เดือน พ.ศ.
☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอให้ใช้รูปเขียนหมายเลข ในประกาศโฆษณา

13. คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ประกอบด้วย
ก. แบบพิมพ์คำขอ 2 หน้า
ข. รายละเอียดการประดิษฐ์
หรือคำพรรณนาแบบผลิตภัณฑ์ 7 หน้า
ค. ข้อถือสิทธิ 1 หน้า
ง. รูปเขียน 3 รูป 2 หน้า
ภาพแสดงแบบผลิตภัณฑ์
☐ รูปเขียน - รูป - หน้า
☐ ภาพถ่าย - รูป - หน้า
จ. บทสรุปการประดิษฐ์ 1 หน้า

14. เอกสารประกอบคำขอ
☐ เอกสารแสดงสิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร
☐ หนังสือรับรองการแสดงการประดิษฐ์/การออกแบบผลิตภัณฑ์
☐ หนังสือมอบอำนาจ
☐ เอกสารรายละเอียดเกี่ยวกับจุลชีพ
☐ เอกสารการขอนับวันยื่นคำขอในต่างประเทศเป็นวันยื่นคำขอในประเทศไทย
☐ เอกสารขอเปลี่ยนแปลงประเภทของสิทธิ
☐ เอกสารอื่น ๆ เอกสารประกอบการยื่นคำขอรับสิทธิบัตร

15. ข้าพเจ้าขอรับรองว่า
☐ การประดิษฐ์นี้ไม่เคยยื่นขอรับสิทธิบัตร/อนุสิทธิบัตรมาก่อน
☐ การประดิษฐ์นี้ได้พัฒนาปรับปรุงมาจาก .....

16. ลายมือชื่อ (☐ ผู้ขอรับสิทธิบัตร / อนุสิทธิบัตร; ☐ ตัวแทน)

(นางสาวอรุณศรี ศรีธนะอิทธิพล)
ตัวแทนผู้รับมอบอำนาจ

หมายเหตุ บุคคลใดยื่นขอรับสิทธิบัตรการประดิษฐ์หรือการออกแบบผลิตภัณฑ์ หรืออนุสิทธิบัตร โดยการแสดงข้อความอันเป็นเท็จแก่พนักงานเจ้าหน้าที่เพื่อให้ได้ไปซึ่งสิทธิหรืออนุสิทธิบัตร ต้องระวางโทษจำคุกไม่เกินหกเดือน หรือปรับไม่เกินห้าพันบาท หรือทั้งจำทั้งปรับ

รายละเอียดการประดิษฐ์

ชื่อที่แสดงถึงการประดิษฐ์

กรรมวิธีการตรวจวัดหมู่เมททิลของของยีน LINE-1 ในแต่ละตำแหน่ง

สาขาวิทยาการที่เกี่ยวข้องกับการประดิษฐ์

พันธุวิศวกรรมด้านการแพทย์

ภูมิหลังของศิลปวิทยาการที่เกี่ยวข้อง

การเติมหรือการลดหมู่เมททิลที่ดีเอ็นเอ (DNA methylation) มีความสำคัญในการควบคุมการทำงานของยีนและจีโนมในการเกิดมะเร็ง เนื่องจาก DNA methylation เป็นการเปลี่ยนแปลงของสายดีเอ็นเอที่ไม่ถาวร การควบคุมยีนหรือจีโนมโดย DNA methylation จึงถูกจัดอยู่ในการควบคุมแบบเหนือพันธุกรรม (epigenetics) ในการเกิดมะเร็ง DNA methylation มีบทบาทในสองลักษณะ ได้แก่ การเติมหมู่เมททิลที่ promoter ของยีนต้านมะเร็ง (tumor suppressor gene) เพื่อมีส่วนในขบวนการยับยั้งการทำงานของยีนต้านมะเร็งนั้นๆ อีกลักษณะหนึ่งคือการลดหมู่เมททิล (demethylation) โดยส่วนใหญ่จะพบว่าโดยรวมทั้งเซลล์ เซลล์มะเร็งจะมีปริมาณของหมู่เมททิลลดลง เรียกว่า global hypomethylation[1,2] พบว่าการลดหมู่เมททิลนี้มักจะเกิดขึ้นที่ repetitive DNA sequences เช่น LINE-1 หรือ Alu intersperse repetitive sequences การลดของหมู่เมททิลน่าจะมีบทบาทสำคัญในการทำให้เกิดสภาวะไม่เสถียรของจีโนม (genomic instability) แบบมีการกลายพันธุ์ของโครโมโซมมากกว่าปกติ (chromosomal instability, CIN)[3] นอกจากนี้การการลดหมู่เมททิลของยีนมะเร็ง (oncogene)[2] เป็นอีกสมมุติฐานหนึ่งในการเกิดเซลล์มะเร็ง

DNA methylation เกิดขึ้นได้หลายตำแหน่งในขบวนการเกิดเซลล์มะเร็งได้แก่ที่ยีนต้านมะเร็ง จีโนมไวรัส และ ทั่วไปทั้งจีโนม จากการศึกษาเดิมที่คณะผู้ประดิษฐ์ได้ศึกษาโดยวิธีการ combined bisulfite restriction analysis (COBRA LINE-1)[4] มาแล้วและพบว่าการลดหมู่เมททิลเป็นการเปลี่ยนแปลงของภาวะเหนือพันธุกรรมที่พบบ่อยที่สุดในมะเร็ง และยังพบว่าระดับของการเกิด DNA methylation มีความจำเพาะสูงระหว่างเนื้อเยื่อชนิดต่างๆ แต่การวัดค่าการลดหมู่เมททิลทั้งหมดพร้อมกันยังไม่สามารถใช้เป็นมาตรฐานในการคำนวนความเสี่ยงของการเกิดโรคมะเร็งได้ดี เพราะว่าค่าการลดหมู่เมททิลของยีน LINE-1 ในแต่ละตำแหน่งมีค่าไม่เท่ากัน และการลดหมู่เมททิลในแต่ละตำแหน่งมีลักษณะสำคัญในทางชีววิทยาของยีนในตำแหน่งนั้นๆ ด้วย ขึ้นอยู่กับบทบาทต่อการก่อมะเร็งของจีโนมในตำแหน่งนั้น ดังนั้นการวัดค่าการลดหมู่เมททิลของยีน LINE-1s ในแต่ละตำแหน่งจะเป็นการบ่งชี้มะเร็ง (tumor marker) ที่แม่นยำกว่า

คณะผู้ประดิษฐ์ได้ตั้งสมมุตติฐานว่ายีน LINE-1 ในแต่ละตำแหน่งมีการเกิด DNA methylation ที่แตกต่างกันขึ้นกับชนิดของเซลล์และการเป็นมะเร็ง ดังนั้นคณะผู้ประดิษฐ์ได้คิดกรรมวิธีเพื่อเปรียบเทียบระหว่างเนื้อเยื่อว่าการลดหมู่เมททิลของยีน LINE-1 ในตำแหน่งต่างๆ ของเนื้อเยื่อต่างชนิดกัน และเปรียบเทียบระหว่างเซลล์ปกติกับเซลล์มะเร็งว่ามีความต่างกันหรือไม่

การประดิษฐ์นี้เกี่ยวกับบทบาทของการลดหมู่เมททิลต่อการเกิดมะเร็งโดยเฉพาะการลดหมู่เมททิลที่ยีน LINE-1 ในการประดิษฐ์นี้จะพิสูจน์ว่าการลดหมู่เมททิลของยีน LINE-1 โดยส่วนใหญ่จำเพาะต่อเซลล์มะเร็งแต่มีบางตำแหน่งที่ไม่จำเพาะ ดังนั้นหมู่เมททิลที่ลดลงของยีน LINE-1 ในแต่ละตำแหน่งจะให้ข้อมูลทางชีววิทยาที่สัมพันธ์กับลักษณะทางคลินิคที่แตกต่างกัน กรรมวิธีการพิสูจน์โดยใช้เทคนิกพีซีอาร์ เพื่อหาค่าของ DNA methylation ในตำแหน่งต่างๆ ของยีน LINE-1 ที่ประดิษฐ์คิดค้นขึ้นนี้เรียกว่า combine unique and LINE-1 sequences for PCR หรือ CU-L1 นอกจากวิธีการตรวจหาปริมาณหมู่เมททิลที่ยีน LINE-1 ในแต่ละตำแหน่งแล้ว การประดิษฐ์นี้แสดงนัยสำคัญในการใช้กรรมวิธี CU-L1 สำหรับการตรวจแยกระหว่างเซลล์มะเร็ง และเซลล์ปกติด้วย แสดงถึงการใช้กรรมวิธี CU-L1 เป็นการบ่งชี้มะเร็งที่แม่นยำได้ด้วย

ลักษณะและความมุ่งหมายของการประดิษฐ์

การประดิษฐ์นี้เกี่ยวกับกรรมวิธีการตรวจวัดหมู่เมททิลของยีน LINE-1 แต่ละตำแหน่งโดยวิธีพีซีอาร์ เนื่องจากหมู่เมททิลของยีน LINE-1 ในจีโนมของเซลล์ปกติจะสูงกว่าเซลล์มะเร็ง เพราะผลรวมของหมู่เมททิลโดยรวมของมะเร็งจะน้อยลง ดังนั้นความแตกต่างของระดับของหมู่เมททิลระหว่างเซลล์ปกติและเซลล์มะเร็งโดยรวมจะต่างกันและสามารถนำมาใช้ในการคำนวนความเสี่ยงที่จะเกิดโรคได้[4-5] ค่าความแตกต่างที่แคบทำให้ยากต่อการนำไปใช้จริง เนื่องจากหมู่เมททิลของยีน LINE-1 แต่ละตำแหน่งและในเซลล์แต่ละชนิดจะไม่เท่ากัน ดังนั้นการตรวจวัดหมู่เมททิลในแต่ละตำแหน่งบางตำแหน่งจะมีความแตกต่างระหว่างดีเอ็นเอของเซลล์ปกติและเซลล์มะเร็งอย่างชัดเจน การประดิษฐ์นี้จึงมุ่งหมายที่จะคิดค้นวิธีตรวจวัดค่าของหมู่เมททิลที่ยีน LINE-1 ในแต่ละตำแหน่ง ความแตกต่างของค่าหมู่เมททิลในแต่ละตำแหน่งของยีน LINE-1 จะสามารถนำมาใช้เป็นข้อมูลในการตรวจกรอง ตรวจวินิจฉัยและคำนวนความเสี่ยงที่จะเกิดโรคมะเร็งในอนาคต

คำอธิบายรูปเขียนโดยย่อ

รูปที่ 1 แผนผังโมเลกุลวิธีการศึกษา CU-L1 และ COBRALINE-1[4] รูปแสดง LINE-1 และ ลำดับเบสเฉพาะที่อยู่ 5' (5' unique sequence) AACCG and CCGA คือลำดับเบส เมื่อถูกปรับแต่งโดยไบซัลไฟท์และพีซีอาร์ จะเปลี่ยน unmethylated AACCG เป็น AATTG (*Tas*I site) และ methylated CCGA เป็น TCGA (*Taq*I site) ขนาดพีซีอาร์ของ CU-L1

ประมาณ 300 ถึง 500 คู่เบส และของ COBRALINE-1 คือ 160 คู่เบส หลังจากตัดด้วยเอนไซม์ *Tas*I และ *Taq*I แล้ว COBRALINE-1 มีขนาด 62 คู่เบส unmethylated LINE-1 98 คู่เบส และ methylated LINE-1 80 คู่เบส

รูปที่ 2 ตัวอย่างผลการศึกษาวิธี CU-L1 และ COBRALINE-1[4] ขนาดลูกศรดีเอ็นเอที่เป็น unmet, met และ control หมายถึง unmethylated, methylated และ control LINE1s, ตามลำดับ M คือ standard size marker –ve คือ dH2O รูปแสดง WSU-HN หลายตัวอย่าง

รูปที่ 3 ระดับของหมู่เมททิลจากการตรวจ CU-L1 ของเซลล์มะเร็งศีรษะและคอ (head and neck squamous cell cancer, HNSCC) ได้แก่ WSU-HN[6], HNSCC ที่ตัดแบ่งเฉพาะเนื้อมะเร็ง (microdissected HNSCC tissues), เซลล์เยื่อบุช่องปาก (normal oral epithelium, NOE) และเซลล์เม็ดเลือดขาว (white blood cells, WBC)

ตารางที่ 1 ตำแหน่งของ LINE-1

ตารางที่ 2 ลำดับเบสของไพรเมอร์ และ ลำดับเบสที่ใช้แปลผล

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การเปิดเผยการประดิษฐ์โดยสมบูรณ์

ในการวัดค่าการลดหมู่เมททิลทั้งจีโนมในการศึกษาที่ผ่านมาได้ออกแบบไพร์เมอร์สำหรับ PCR ทั้ง 2 เส้นให้อยู่บนยีน LINE-1 ส่วนการวัดค่าการลดหมู่เมททิลในการประดิษฐ์นี้ได้ออกแบบให้ไพร์เมอร์ของ PCR เส้นที่ 1 อยู่บน unique sequence และเส้นที่ 2 อยู่ในยีน LINE-1 และเรียกวิธีการนี้ว่า COBRA for unique to L1 sequence (CU-L1)

1. กรรมวิธีการคัดเลือกตำแหน่ง

ยีน LINE-1 มีทั้งหมดกว่า 6 แสนตำแหน่ง การศึกษาหมู่เมททิลของยีน LINE-1 ในแต่ละตำแหน่ง (COBRA for unique to LINE-1 sequence, CU-L1) เน้นที่ลำดับเบสของยีน LINE-1 ที่ 5' ซึ่งมีรายงานส่วนหนึ่งในลักษณะที่เป็นยีน LINE-1 ทั้งเส้นอยู่ประมาณมากกว่าพันตำแหน่ง[7] การใช้วิธี CU-L1 สามารถเลือกทำได้จากตำแหน่งดังกล่าว

เพื่อที่จะศึกษาปริมาณของหมู่เมททิลที่ยีน LINE-1 ในแต่ละตำแหน่ง 17 ตำแหน่งได้ถูกเลือกเพื่อการศึกษา โดยทั้ง 17 ตำแหน่งเป็นยีน LINE-1 ที่มีขนาดเต็มและมีตำแหน่งที่อยู่ในอินทรอน แผนผังโมเลกุลของวิธีการได้บรรยายที่ รูปที่ 1 และตัวอย่างผลการศึกษาแสดงที่ รูปที่ 2

2. กรรมวิธีการปรับสภาพด้วยสารไบซัลไฟท์

การปรับสภาพโดยสารไบซัลไฟท์[9] จะทำให้ลำดับเบสของดีเอ็นเอเปลี่ยนจากไซโตซีนเป็นยูราซิล ไซโตซีนที่มีหมู่เมททิลเกาะอยู่จะคงสภาพไม่เปลี่ยนแปลง ทำให้เมื่อทำพีซีอาร์ลำดับเบสเดิมที่เป็นไซโตซีนและไม่มีหมู่เมททิลจะกลายเป็นไทมีน (เนื่องจากไทมีนมีโครงสร้างที่คล้ายกับยูราซิลและใช้แทนยูราซิลในพีซีอาร์) แต่ไซโตซีนที่มีหมู่เททิลจะเป็นไซโตซีนเช่นเดิม ลำดับเบสจากพีซีอาร์ของสายดีเอ็นเอที่มีหมู่เมททิลและไม่มีหมู่เมททิลจะแตกต่างกัน และในวิธี CU-L1 นี้จะมีลำดับเบสที่สามารถแยกได้โดยเอนไซม์ที่ใช้ตัดสายดีเอ็นเอ (restriction enzymes) ดังแสดงในรูปที่ 1

ผสมดีเอ็นเอประมาณ 1 ไมโครกรัม กับ 0.22 M NaOH ที่อุณหภูมิ 37°ซ เป็นเวลา 10 นาที แล้วเติมสาร hydroquinone (10nM) ประมาณ 30 ไมโครลิตร และ สารไบซัลไฟท์ (sodium bisulfite) (3M) 520 ไมโครลิตรทิ้งไว้เป็นเวลา 16-20 ชม ที่อุณหภูมิ 50°ซ ดีเอ็นเอจะถูกทำให้บริสุทธิ์แล้วแช่ใน 0.33 M NaOH ที่อุณหภูมิ 25°ซ เป็นเวลา 5 นาที หลังจากนั้นจึงตกตะกอนด้วยสารเอทธานอลล้างด้วย 70% สารเอทธานอลแล้วละลายตะกอนดีเอ็นเอด้วยสารละลาย TE

ประมาณ 20 ไมโครลิตร ตารางที่ 1 แสดงตำแหน่งของ LINE-1 ลำดับเบสของไพรเมอร์ และ ลำดับเบสที่ใช้แปลผลได้แสดงอยู่ที่ ตารางที่ 2

3. **กรรมวิธีการทำพีซีอาร์**

ออกแบบให้ไพร์เมอร์ของ PCR เส้นที่ 1 อยู่บน unique sequence และเส้นที่ 2 อยู่ในยีน LINE-1 โดยไพรเมอร์ในตำแหน่งที่ 5'UTR (ส่วนต้น) ของ LINE-1 ดีเอ็นเอที่ถูกปรับเปลี่ยนจะถูกเพิ่มปริมาณ ตัดดีเอ็นเอที่ได้จากพีซีอาร์ด้วย *Taq*I และ *Tas*I ซึ่ง *Taq*I จะตัดเฉพาะที่ลำดับเบส TCGA แสดงว่าตัดสายดีเอ็นเอที่มีหมู่เมททิล ส่วนเอนไซม์ *Tas*I จะตัดเฉพาะที่ลำดับเบส AATT โดยลำดับเบสของ 5'UTR ของ LINE-1 จะมีตำแหน่งหนึ่งที่มีลำดับเบส AACCG โดยจะถูกปรับเป็น AATTG ในกรณีที่ไม่มีหมู่เมททิล ดังนั้นในตำแหน่งนี้ *Tas*I จะตัดเฉพาะดีเอ็นเอที่ไม่มีหมู่เมททิล หลังจากสกัดดีเอ็นเอแล้วนำตัวอย่างไปผ่านกรรมวิธีการปรับสภาพด้วยสารไบซัลไฟท์ตามวิธีข้างต้นอีกครั้ง

นำตัวอย่างที่จะถูกตรวจสอบมาทำพีซีอาร์โดยใช้ดีเอ็นเอ 2 ไมโครลิตร จำนวน 35 รอบ ให้ไพรเมอร์จับติดที่อุณหภูมิ 53°ซ ตัดดีเอ็นเอที่ได้จากพีซีอาร์ 30 ไมโครลิตรด้วย *Taq*I 2 หน่วย และ *Tas*I 8 หน่วยใน 1xTaqI buffer ที่อุณหภูมิ 65°ซ ตลอดเวลาข้ามคืน นำผลดีเอ็นเอมาแยกสายตามขนาดโดยกระแสไฟฟ้าใน 8% non-denaturing polyacrylamide gels ย้อมดีเอ็นเอด้วยไซเบอร์กรีน ปริมาณของดีเอ็นเอในขนาดต่างๆ วัดได้จากความเข้มของสีย้อมโดย PhosphorImager, ImageQuant software (Molecular Dynamics) ดีเอ็นเอที่ถูกตัดโดย *Taq*I จะมี 1 เส้นที่มีขนาด 80 คู่เบส ส่วนดีเอ็นเอที่ไม่มีหมู่เมททิลที่ถูกตัดด้วย *Tas*I จะมีขนาดเท่ากับ 97 คู่เบส

4. **การวัดค่าการลดจำนวนหมู่เมททิล**

ค่าการลดจำนวนหมู่เมททิลที่สาย LINE-1 คำนวนจากความเข้มของหมู่เมททิลที่สาย LINE-1 ที่ตัดด้วยเอนไซม์ *Taq*I หารด้วยผลรวมของสายที่มีหมู่เมททิลที่ตัดด้วยเอนไซม์ *Taq*I และที่ไม่มีหมู่เมททิลที่ตัดด้วยเอนไซม์ *Tas*I

5. **การใช้ประโยชน์ของกรรมวิธีตรวจวัดจำนวนหมู่เมททิลของยีน LINE-1 ในแต่ละตำแหน่ง**

กรรมวิธี CU-L1 จะเป็นเครื่องมือที่สำคัญในการวินิจฉัยมะเร็ง เนื่องจากการลดลงของหมู่เมททิลทั้งจีโนมพบได้ทั่วไปและพบลดลงมากขึ้นเมื่อมะเร็งพัฒนารุนแรงขึ้น ลักษณะการลดลงของหมู่เมททิลที่เกิดขึ้นใน LINE-1 ในแต่ละตำแหน่งจะสามารถวัดเห็นความแตกต่างระหว่างดีเอ็นเอของเซลล์ปกติและเซลล์มะเร็งได้มากกว่าการศึกษาทั้งจีโนมโดย COBRALINE-1[4] หมู่เมททิลของ LINE-1s แบบ genome-wide เป็นผลรวมของหมู่เมททิลของ LINE-1s โดย COBRALINE-1 ซึ่งจะพบว่าเซลล์มะเร็งมีหมู่เมททิลของ LINE-1s น้อยกว่าเซลล์ปกติ เมื่อเปรียบเทียบระหว่างเซลล์ปกติ จะพบว่าเซลล์เม็ดเลือดมีหมู่เมททิลของ LINE-1s มากกว่าเซลล์เยื่อบุผิวของช่องปาก จากผลการศึกษานี้แสดงว่า COBRALINE-1 สามารถใช้แยกเซลล์มะเร็งจากเซลล์ปกติได้ แต่มีความน่าเชื่อถือต่ำ ทั้งนี้

เพราะระดับของร้อยละที่แตกต่างอยู่ในระดับที่แคบประมาณ 5-10% ในการนำไปใช้ถ้าดีเอนเอของเซลล์มะเร็งมีเม็ดเลือดขาวมาปนเปื้อนก็จะทำให้ผลการตรวจคลาดเคลื่อนได้มากขึ้น นอกจากนี้ COBRALINE-1 ยังแยกความแตกต่างของเซลล์มะเร็งได้ยากเพราะระดับร้อยละของหมู่เมททิลของเซลล์มะเร็งในแต่ละตัวอย่างมีความห่างกันไม่มากเพียงพอ นอกจากนี้ค่าที่คำนวนจากวิธี CU-L1 ที่แตกต่างกันในแต่ละตำแหน่งจะมีความหมายที่แตกต่างกัน เช่น บางตำแหน่งจะมีจำนวนหมู่เมททิลเพิ่มขึ้นในเซลล์มะเร็งบางราย ดังนั้นหมู่เมททิลของ LINE-1 ในแต่ละตำแหน่งจะส่งผลทางชีววิทยาที่ต่างกัน ส่งผลให้การวัดจำนวนหมู่เมททิลโดยวิธี CU-L1 จะเป็นการศึกษาลักษณะของมะเร็งเพื่อการวินิจฉัย (Tumor Marker) หลายตำแหน่งโดยใช้วิธีการเดียวกัน ซึ่งจะทำให้สามารถนำเทคนิคนี้มาใช้ประกอบการวินิจฉัยได้อย่างมีประสิทธิภาพ

ตัวอย่าง

ปริมาณของหมู่เมททิลจากการตรวจด้วยวิธี CU-L1 ของเซลล์มะเร็งศีรษะและคอ (head and neck squamous cell cancer, HNSCC) ได้แก่ WSU-HN[8], HNSCC ที่ตัดแบ่งเฉพาะเนื้อมะเร็ง (microdissected HNSCC tissues) เซลล์เยื่อบุช่องปาก (normal oral epithelium, NOE) และเซลล์เม็ดเลือดขาว (white blood cells, WBC) แสดงที่ รูปที่ 3 สามารถสังเกตุได้ว่าระดับปริมาณของหมู่เมททิลของวิธี CU-L1 ในแต่ละตำแหน่งไม่เท่ากันและไม่เหมือนกัน อย่างไรก็ดีโดยทั่วไประดับปริมาณของหมู่เมททิลของวิธี CU-L1 จากมะเร็งจะน้อยกว่าเซลล์ปกติ โดยระดับความแตกต่างของหมู่เมททิลของแต่ละตำแหน่งจะหลากหลาย

หมู่เมททิลของ LINE-1 ในแต่ละตำแหน่งจากการตรวจแบบ CU-L1 จะให้ผลการตรวจที่แตกต่างกันในแต่ละตำแหน่ง จากการศึกษาทั้งหมด 17 ตำแหน่ง พบว่า 15 ตำแหน่ง จะมีหมู่เมททิลน้อยลงในเซลล์มะเร็ง โดยที่ L1-SPOCK3 และ L1-MGC42174 ไม่มีค่าเฉลี่ยของหมู่เมททิลของ LINE-1 ลดลงในเซลล์มะเร็ง ในบางตำแหน่งของบางตัวอย่างจะพบว่าหมู่เมททิลของ LINE-1 มีค่าเพิ่มขึ้นมากกว่าเซลล์ปกติได้ เช่น L1-CNTNAP5, L1-MGC42174 และ L1-ADAMTS20 เป็นต้น ที่สำคัญการลดลงของหมู่เมททิลโดย CU-L1 มีระยะห่างของร้อยละการลดที่มีนัยสูง เช่น ระยะค่าเฉลี่ยของหมู่เมททิลของ L1-CDH8 ระหว่าง WSU-HN กับ NOE มีค่าถึงกว่า 60% ทำให้ง่ายและจำเพาะต่อการแยกเยะเซลล์มะเร็งออกจากเซลล์ปกติ นอกจากนี้การลดลงของแต่ละตำแหน่งมีค่าที่แตกต่างกัน บางตำแหน่งเซลล์มะเร็งส่วนใหญ่มีหมู่เมททิลของ LINE-1 ลดลง เช่น L1-CDH8 และ L1-CNTNAP5 เป็นต้น ในขณะที่บางตำแหน่งมีมะเร็งที่หมู่เมททิลบางตำแหน่งมีตัวอย่างที่หมู่เมททิลลดลงในจำนวนที่น้อยกว่า เช่น ทำให้เมื่อนำไปใช้สามารถนำข้อมูลมารวมกันได้ เช่น ถ้าตัวอย่างนั้นมี CU-L1 เพียง 1-2 ตำแหน่งจาก 17 ตำแหน่ง ที่มีหมู่เมททิลลดลงอย่างมีนัยสำคัญ ก็

อาจจะบ่งชี้ได้ว่าตัวอย่างนั้นเป็นมะเร็ง ดังนั้นการตรวจดีเอนเอโดยใน CU-L1 หลายๆ ตำแหน่งจะเป็นการเพิ่มความเป็นไปได้ในการตรวจวินิจฉัยเพิ่มขึ้น นอกจากนี้ค่า CU-L1 ในแต่ละตำแหน่งอาจแสดงถึงชีววิทยาของยีนหรือจีโนมในตำแหน่งนั้นๆ เช่น การเพิ่มขึ้นของหมู่เมททิลของ L1-CNTNAP5 น่าจะแสดงถึงเฉพาะของชีววิทยาของจีโนมในตำแหน่งนี้ ถ้าระดับของหมู่เมททิลในแต่ละตำแหน่งแตกต่างกันระหว่างตัวอย่าง แสดงว่าชีววิทยาในระดับโมเลกุลของมะเร็งนั้นๆ ต่างกัน แสดงว่า CU-L1 มีความเป็นไปได้ที่จะเป็น tumor marker ที่ให้ข้อมูลที่หลากหลายขึ้นกับตำแหน่งที่ทำการศึกษา

วิธีการประดิษฐ์ที่ดีที่สุด

ดังได้แสดงไว้แล้วในหัวข้อการเปิดเผยการประดิษฐ์โดยสมบูรณ์

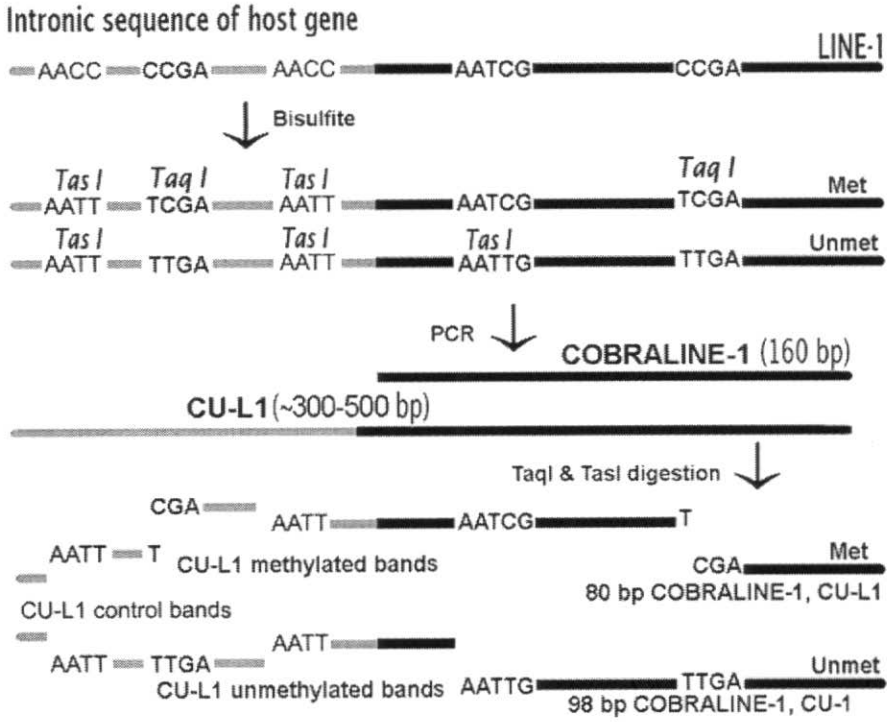
ข้อถือสิทธิ

1. กรรมวิธีตรวจวัดหมู่เมททิลของยีน LINE-1 ในแต่ละตำแหน่ง ที่ประกอบด้วย การปรับลำดับเบสของดีเอ็นเอ การออกแบบให้ไพร์เมอร์อยู่ข้างนอกและข้างใน LINE-1 และการตรวจวัดปริมาณหมู่เมททิล
2. กรรมวิธีตรวจวัดหมู่เมททิลของยีน LINE-1 ในแต่ละตำแหน่ง ในข้อถือสิทธิที่ 1 ที่ซึ่งยีน LINE-1 เป็นยีนมีขนาดเต็มและมีตำแหน่งที่อยู่ในอินทรอน
3. กรรมวิธีตรวจวัดหมู่เมททิลของยีน LINE-1 ในแต่ละตำแหน่ง ในข้อถือสิทธิที่ 1 ที่ซึ่งการปรับลำดับเบสของดีเอ็นเอ มีลักษณะเฉพาะคือ ปรับลำดับเบสจากไซโตซีนที่ไม่มีหมู่เมททิลให้เป็นยูราซิล
4. กรรมวิธีตรวจวัดหมู่เมททิลของยีน LINE-1 ในแต่ละตำแหน่ง ในข้อถือสิทธิที่ 1 ที่ซึ่งการปรับลำดับเบสของดีเอ็นเอทำโดยใช้สารไบซัลไฟท์
5. กรรมวิธีตรวจวัดหมู่เมททิลของยีน LINE-1 ในแต่ละตำแหน่ง ในข้อถือสิทธิที่ 1 ที่ซึ่งการออกแบบให้ไพร์เมอร์อยู่ข้างนอกและข้างใน LINE-1 มีลักษณะเฉพาะคือออกแบบให้เส้นที่ 1 อยู่บน unique sequence และเส้นที่ 2 อยู่ในยีน LINE-1
6. กรรมวิธีตรวจวัดหมู่เมททิลของยีน LINE-1 ในแต่ละตำแหน่ง ในข้อถือสิทธิที่ 1 ที่ซึ่งการตรวจวัดปริมาณหมู่เมททิล มีลักษณะเฉพาะคือ เซลล์มะเร็งมีหมู่เมททิลของ LINE-1s น้อยกว่าเซลล์ปกติ
7. กรรมวิธีการตรวจวัดปริมาณหมู่เมททิล ในข้อถือสิทธิที่ 6 มีลักษณะเฉพาะคือ ตรวจวัดจากปริมาณดีเอนเอที่ถูกตัดโดยเอนไซม์ TasI และ TaqI มากกว่า 1 ตำแหน่ง
8. กรรมวิธีการตรวจวัดปริมาณหมู่เมททิล ในข้อถือสิทธิที่ 6 มีลักษณะเฉพาะคือ ค่าการลดหมู่เมททิลที่สาย LINE-1 คำนวนจากความเข้มของหมู่เมททิลที่สาย LINE-1 ที่ตัดด้วยเอนไซม์ *TaqI* หารด้วยผลรวมของสายที่มีหมู่เมททิลที่ตัดด้วยเอนไซม์ *TaqI* และที่ไม่มีหมู่เมททิลที่ตัดด้วยเอนไซม์ *TasI*

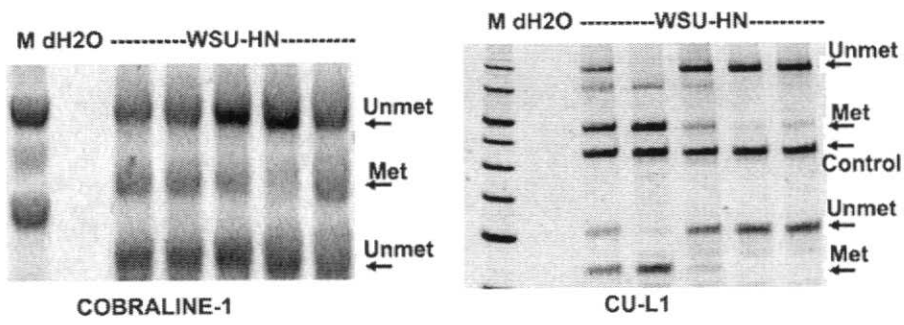
บทสรุปการประดิษฐ์

ผู้ประดิษฐ์ได้คิดค้นกรรมวิธีตรวจวัดหมู่เมททิลของ LINE-1 ในแต่ละตำแหน่ง และตั้งชื่อวิธีการตรวจนี้ว่า CU-L1 วิธีการตรวจวัดทำโดยประยุกต์ใช้การตรวจดีเอนเอด้วย COBRA โดยออกแบบให้ไพร์เมอร์อยู่ข้างนอกและข้างใน LINE-1 และตรวจวัดปริมาณหมู่เมททิล การประดิษฐ์นี้ทดลอง LINE-1 ทั้งหมด 17 ตำแหน่ง และตรวจสอบเปรียบเทียบเซลล์มะเร็งศีรษะและคอ กับ เซลล์ปกติในช่องปาก และเซลล์เม็ดเลือด โดยพบ LINE-1 16 จาก 17 ตำแหน่งมีหมู่เมททิลลดลงในเซลล์มะเร็ง ดังนั้น CU-L1 จึงเป็นสิ่งประดิษฐ์ที่สามารถตรวจหาและวินิจฉัยมะเร็งได้

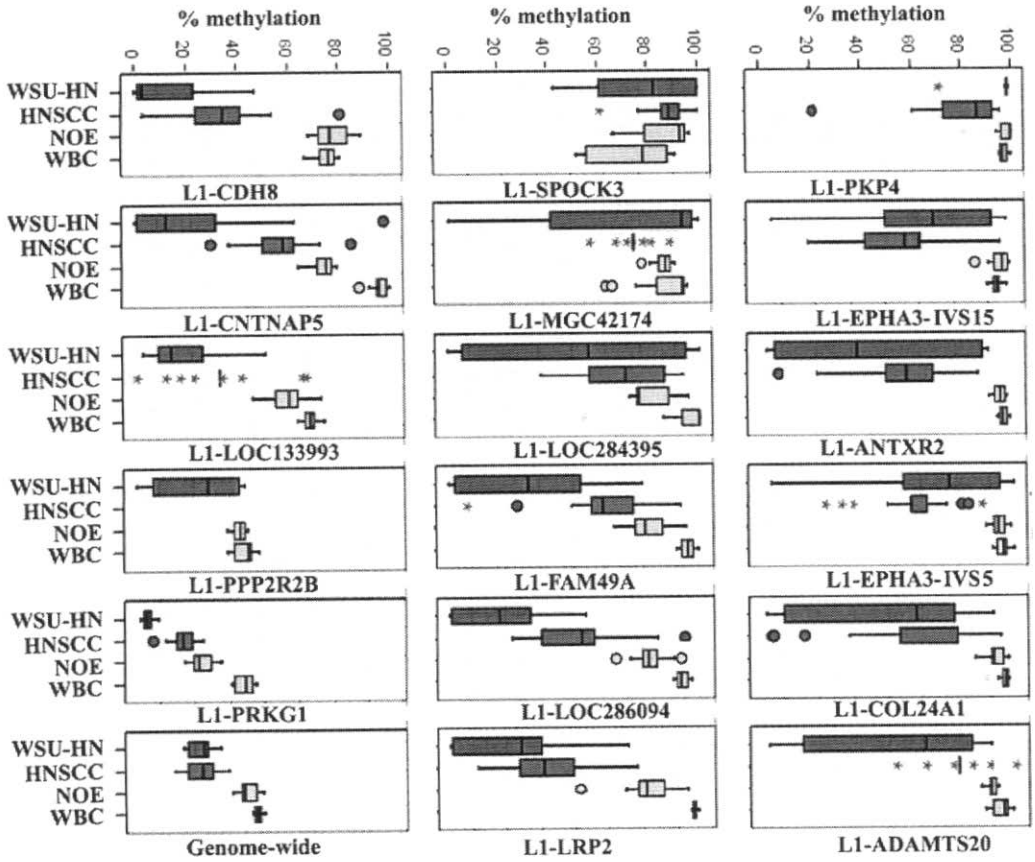
รูปที่ 1


Intronic sequence of host gene
AACC CCGA AACC AATCG CCGA LINE-1
Bisulfite
Tas I Taq I Tas I Taq I
AATT TCGA AATT AATCG TCGA Met
Tas I Tas I Tas I
AATT TTGA AATT AATTG TTGA Unmet
PCR
COBRALINE-1 (160 bp)
CU-L1 (~300-500 bp)
TaqI & TasI digestion
CGA AATT AATCG T
AATT T CU-L1 methylated bands
CGA Met
80 bp COBRALINE-1, CU-L1
CU-L1 control bands
AATT
AATT TTGA
CU-L1 unmethylated bands
AATTG TTGA Unmet
98 bp COBRALINE-1, CU-1

รูปที่ 2


M dH2O ----------WSU-HN----------
Unmet
Met
Unmet
COBRALINE-1
M dH2O ----------WSU-HN----------
Unmet
Met
Control
Unmet
Met
CU-L1

รูปที่ 3


% methylation
0
20
40
60
80
100
WSU-HN
HNSCC
NOE
WBC
L1-CDH8
L1-SPOCK3
L1-PKP4
L1-CNTNAP5
L1-MGC42174
L1-EPHA3-IVS15
L1-LOC133993
L1-LOC284395
L1-ANTXR2
L1-PPP2R2B
L1-FAM49A
L1-EPHA3-IVS5
L1-PRKG1
L1-LOC286094
L1-COL24A1
Genome-wide
L1-LRP2
L1-ADAMTS20

ตารางที่ 1

Gene	Gene location	LINE-1 location	LINE-1 orientation
COL24A1	1p22.3	intron 24	Antisense
FAM49A	2p24.3-2p24.2	intron 2	Sense
CNTNAP5	2q14.3	intron 11	Antisense
PKP4	2q24.1	intron 1	Sense
LRP2	2q31.1	intron 19	Antisense
MGC42174	2q37.1	intron 8	Antisense
EPHA3	3p11.1	intron 5	Antisense
EPHA3	3p11.1	intron 15	Antisense
ANTXR2	4q21.21	intron 16	Antisense
SPOCK3	4q32.3	intron 7	Antisense
LOC133993	5q12.3	intron 3	Antisense
PPP2R2B	5q32	intron 8	Antisense
LOC286094	8q24.22	intron 1	Sense
PRKG1	10q21.1	intron 9	Sense
ADAMTS20	12q12	intron 7	Antisense
CDH8	16q21	intron 7	Antisense
LOC284395	19q12	intron 1	Antisense

ตารางที่ 2

Gene	COBRA unique and LINE-1 sequence oligoes	Size (bp)	Methylated bands (bp)	Unmethylated bands (bp) & Control bands (bp)
COL24A1	GTTAAAGGGTTAAGAATGTGTGTAG GTAAAACCCTCCGAACCAAATATAAA	336	47,151,60,54,80	294,98
FAM49A	GTTTTAAAAAAAAATAAAGTTGG GTAAAACCCTCCGAACCAAATATAAA	385	41,151,113,80	287,98
CNTNAP5	GATTAAATTTTAATTGAATTAGAG GTAAAACCCTCCGAACCAAATATAAA	403	43,151,60,53,80	5,6,5,289,98
PKP4	GGTATGATTTTAAAAAAAGAGAT GTAAAACCCTCCGAACCAAATATAAA	392	48,211,53,80	294,98
LRP2	GGTATATAATTTTTATGGTGTTG GTAAAACCCTCCGAACCAAATATAAA	435	44,150,60,53,80	7,27,14,289,98
MGC42174	ATTGAGGTGTATTAAGAGATGGA GTAAAACCCTCCGAACCAAATATAAA	553	181,60,53,80	25, 154, 276, 98
EPHA3-IVS5	TGTTATTGGAATATATGGAGATT GTAAAACCCTCCGAACCAAATATAAA	386	42,151,60,53,80	288,98
EPHA3-IVS15	TAAGGATAAAAATTTTTGAAGTT GTAAAACCCTCCGAACCAAATATAAA	464	60,150,60,53,80	10,33,18,305,98
ANTXR2	TATTGAGTATTAATTATGTATTTAGTAT GTAAAACCCTCCGAACCAAATATAAA	416	28,150,60,53,80	11,34,273,98
SPOCK3	GTGTAATTTTTTTAGATTTTGTAG GTAAAACCCTCCGAACCAAATATAAA	492	300,60,36,17,80	6,22,23,37,46,262,98
LOC133993	TTAGGATATTTTTTATTTTGGGA GTAAAACCCTCCGAACCAAATATAAA	446	101,264,80	347,98
PPP2R2B	GGGGAAAAAATTGAAAGTT GTAAAACCCTCCGAACCAAATATAAA	590	8,24,151,60,53,80	94,28,20,42,21,9,270,98
LOC286094	TATGTAAGTATGGAAATTTGAGG GTAAAACCCTCCGAACCAAATATAAA	429	43,151,60,53,80	16,20,290,98
PRKG1	AAAATTTTTAGTTGTTAAATGG GTAAAACCCTCCGAACCAAATATAAA	374	152,60,53,80	2,27,247,98
ADAMTS20	AAGTTGTGTGGTTTTTTGTAAAT GTAAAACCCTCCGAACCAAATATAAA	468	81,151,60,36,17,80	22,328,98
CDH8	GGATTTGGGAGTTGGATAGTTAG GTAAAACCCTCCGAACCAAATATAAA	405	21,211,53,38	30,10,276,56,42
LOC284395	GAGAAATAGAATAGGTATGATTGATAA GTAAAACCCTCCGAACCAAATATAAA	473	23,151,60,53,80	27,5,33,36,270,98
Genome Wide	CCGTAAGGGGTTAGGGAGTTTTT RTAAAACCCTCCRAACCAAATATAAA	160	80	62, 98

bp, base pairs