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รายงานวิจัยฉบับสมบูรณ์

โครงการ

" อณูพันธุศาสตร์ของมะเร็งโพรงหลังจมูก : ลักษณะทางพันธุกรรมที่เสี่ยงต่อการเกิดโรค ขบวนการของการกลายพันธุ์และการประยุกต์ใช้ทางคลินิค "

รองศาสตราจารย์ นายแพทย์อภิวัฒน์ มุทิรางกูร

เสร็จสิ้นโครงการ 31 ตุลาคม พ.ศ. 2545

BRG/9/2542

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โครงการ

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รองศาสตราจารย์ นายแพทย์อภิวัฒน์ มุทิรางกูร
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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

รหัสโครงการ BRG/9/2542

ชื่อโครงการ อณูพันธุศาสตร์ของการเกิดมะเร็งโพรงหลังจมูก: ลักษณะทางพันธุกรรมที่เสี่ยงต่อการเกิดโรค ขบวนการของการกลายพันธุ์ และการประยุกต์ใช้ทางคลินิค

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เนื้อหา

วัตถุประสงค์ของงานวิจัยนี้มี 3 ข้อ ข้อแรกเป็นการศึกษาความเป็นไปได้ในการใช้ EBV DNA เป็นตัววัดมะเร็งโพรงหลังจมูกและธรรมชาติของ DNA นี้ในน้ำเหลืองของผู้ป่วยมะเร็งโพรงหลังจมูก ข้อที่สองเป็นการศึกษาหาพันธุกรรมเสี่ยงของการเกิดโรคนี้โดยการศึกษาเปรียบเทียบความหลากหลายทางพันธุกรรมระหว่างกลุ่มผู้ป่วยและกลุ่มควบคุม สุดท้ายนี้เราได้พยายามสำรวจวิธีการเกิดมะเร็งโพรงหลังจมูกโดยการศึกาาวิธีการจำเพาะของมะเร็งโพรงหลังจมูกในการเกิดความไม่เสถียรของจีโนม

วัตถุประสงค์แรกประสงค์แรกเสร็จสิ้นและตีพิมพ์ในวารสารระดับนานาชาติหลายฉบับได้แก่ Shotelersuk, K et al., Clin Cancer Res. *6:* 1046-51., 2000, Kerekhanjanarong, V et al., Otolaryngol Head Neck Surg. *123:* 624-9., 2000, Pornthanakasem, W et al., BMC Cancer. *1:* 2, 2001, and a review article Mutirangura, A. Serum/plasma viral DNA: mechanisms and diagnostic applications to nasopharyngeal and cervical carcinoma, Ann N Y Acad Sci. *945:* 59-67., 2001. หมายความว่าเราประสพความสำเร็จในการศึกษาวิธีการใช้ EBV DNA ในหลายรูปแบบ ข้อแรกเป็นการศึกษาต่อเนื่องจากการค้นพบ EBV DNA ในน้ำเหลืองของผู้ป่วย โดยตีพิมพ์งานวิจัยต่อเนื่องเพื่อพัฒนาความไวโดยใช้เนสเต็ดพีซีอาร์ และพิสูจน์ว่า DNA หายไปหลังจากการรักษาด้วยการฉายแสงเสร็จสิ้น นอกจากนี้บางรายที่ไม่ตอบสนองดีต่อการรักษาหรือมีการกลับเป็นใหม่พบมี DNA ให้ผลบวก สุดท้ายนี้ EBV DNA นี้มีความสัมพันธ์ใกล้ชิดกับ DNA ก่อนการฉายแสง นอกจากการตรวจ DNA ในน้ำเหลืองแล้วยังพบอีกว่าสามารถใช้การตรวจหา EBV DNA จากการป้ายโพรงหลังจมูกมีความเป็นไปได้ในการนำมาใช้ตรวจกรองประชากรและการกลับเป็นใหม่ที่บริเวณโพรงหลังจมูก โดยที่ Kerekhanjanarong, V ตรวจพบเซลล์มะเร็งจากการป้ายด้วยความไวร้อยละร้อยเมื่อนับผลการตรวจ DNA ด้วยการทำ nested EBV PCR หรือ TRAP เพื่อตรวจการทำงานของเทอโลเมอเรส สุดท้ายนี้ด้วยแนวคิดการตรวจหา DNA ของไวรัสคณะผู้วิจัยได้พัฒนาการตรวจหา DNA ของไวรัสฮิวแมนแปปิโลมา (HPV) ในน้ำเหลืองของผู้ป่วยมะเร็งปากมดลูก Pornthanakasem, W และคณะพบว่าการตรวจหา HPV DNA ให้ผลไม่เหมือนกับ EBV DNA โดยพบ HPV DNA ในน้ำเหลืองของผู้ป่วยมะเร็งปากมดลูกได้น้อย โดยพบ 6% และมีความสัมพันธ์กับการกระจายของมะเร็ง บทสรุปการตรวจ DNA ของไวรัสในมะเร็งได้รายงานใน Mutirangura Ann N Y Acad Sci. *945:* 59-67, 2001.

เพื่อที่จะสืบหาความเสี่ยงทางพันธุกรรมของผู้ป่วยมะเร็งโพรงหลังจมูกของกลุ่มประชากรไทยและจีนในประเทศไทยคณะผู้วิจัยได้ศึกษาความสัมพันธ์ระหว่างความหลากหลายของ DNA ของยีน

หลายยีน (เพื่อหาความแตกต่างของความถี่ของความหลากหลายของ DNA) ระหว่างกลุ่มผู้ป่วยและกลุ่มเปรียบเทียบ ยีนที่เคยได้รับการศึกษาแล้ว (ในประชากรอื่น) สองยีนได้แก่ HLA และ CYP2E1 ได้นำมาศึกษาและความสำคัญในกลุ่มประชากรในประเทศไทยได้รับการยืนยันและตีพิมพ์ (Kongruttanachok, N et al., and Pimtanothai, N et al.). เรายังได้ศึกษายีนใหม่อีก 3 ยีน ได้แก่ *PERB11*, *CR2* และ *PIGR*. เฉพาะ *PIGR* เท่านั้นที่มีความสัมพันธ์กับความเสี่ยงของการเกิดมะเร็งโพรงหลังจมูกอย่างมีนัยสำคัญ เราศึกษา *CR2* และ *PIGR* โดย SNP 3 SNPs ได้แก่ *PIGR*IVS3-156G⟶T, *PIGR*1093G⟶A and *PIGR*1739C⟶T ในผู้ป่วย 175 ราย และ กลุ่มควบคุม 317 ราย โดยแบ่งเป็น ไทย จีน และ ไทย-จีน ตามบรรพบุรุษ พบว่าความเสี่ยงจะสูงในทุกกลุ่ม adjusted OR(95%CI) of 2.71(1.72-4.23) และ p <0.00001. นัยสำคัญนี้ยังยืนยันได้ด้วย แฮปโปรไทปของ 1093G⟶A และ 1739C⟶T โดยที่ *PIGR*1739C⟶T เป็นการกลายพันธุ์ที่เปลี่ยนกรดอมิโนจากอลานีนเป็นวาลีนใกล้กับบริเวณที่เป็นตำแหน่งที่โปรตีนถูกตัดในเซลล์เยื่อบุผิวเพื่อปล่อย IgA-EBV การกลายพันธุ์อาจส่งผลถึงประสิทธิภาพของขบวนการนี้ทำให้ส่งผลถึงความเสี่ยงของการเกิดมะเร็งโพรงหลังจมูกนี้ ข้อมูล *PERB11* ส่งตีพิมพ์ที่ Chula Med J 2001 March 45:207-214. ส่วน CR2 และ PIGR ส่งตีพิมพ์รวมกันและอยู่ในช่วงพิจารณา

วัตถุประสงค์ที่สามเป็นการศึกษาวิธีการกลายพันธุ์ของจีโนมของมะเร็งโพรงหลังจมูก เราได้ศึกษาความเป็นไปได้ที่ขบวนการกลับรวมตัวแบบ V(D)J จะเป็นวิธีการกลายพันธุ์ดังกล่าวโดยการศึกษาการแสดงออกของยีน *RAG1&2* จากเนื้อเยื่อมะเร็งที่ถูกตัดจากชิ้นเนื้อสด และ จากเซลล์มะเร็งที่ถูกเลี้ยงเพื่อกำจัดเซลล์เม็ดเลือดขาวและเซลล์อื่นๆที่ปนเปื้อน ซึ่งการปราศจากการปนเอปื้นได้รับการพิสูจน์โดย flow-cytometry, RT-PCR amplifying CD3 and CD20, และ microsatellite analysis ทั้งเซลล์มะเร็งจากชิ้นเนื้อสดและจากเซลล์ที่เลี้ยง จะพบมีการแสดงออกของทั้ง *RAG1* และ *RAG2* งานวิจัยที่กำลังดำเนินอยู่ใกล้สำเร็จแล้วเป็นการพิสูจน์หน้าที่ของยีนดังกล่าวในเซลล์มะเร็งโพรงหลังจมูก

คำสำคัญ EBV DNA ในน้ำเหลือง, *PIGR*, *RAG1&2* และ มะเร็งโพรงหลังจมูก

Project Code: BRG/9/2542

Project Title: Molecular Genetics of Nasopharyngeal Cancer: Genetic Susceptibility, Mechanism of Mutation and Clinical Application.

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Project Period: November 1st, 1999 to October 31, 2002

Content: There are three objectives of this project. The first is to study the feasibility of Epstein-Barr viral (EBV) DNA as a nasopharyngeal cancer (NPC) marker and study nature of the DNA in NPC patients' plasma. Secondly, the study will explore genetic susceptibility of this disease using association studies by comparing DNA polymorphisms between cases and controls. This is to both prove several characterized genes from other populations and also identify new candidate susceptible genes. Finally, we attempted to explored mechanism of NPC development by characterizing the NPC unique genomic instability.

The first objective was completed and published in several highly impact international journal including, Shotelersuk, K et al., Clin Cancer Res. *6:* 1046-51., 2000, Kerekhanjanarong, V et al., Otolaryngol Head Neck Surg. *123:* 624-9., 2000, Pornthanakasem, W et al., BMC Cancer. *1:* 2, 2001, and a review article Mutirangura, A. Serum/plasma viral DNA: mechanisms and diagnostic applications to nasopharyngeal and cervical carcinoma, Ann N Y Acad Sci. *945:* 59-67., 2001. In the otherword, we, Shotelersuk, K et al., succeeded in studying EBV DNA as NPC tumor marker in several aspects. First, after our first discovery of EBV DNA in patients' NPC serum, we published a second article to improve the sensitivity of the test by nested PCR and prove that the DNA disappeared after complete remission of tumor after radiotherapy. In addition, some cases with resistance to treatment or relapse, the DNA remained or reappeared. Finally, the presentation of plasma EBV DNA was considered strongly relating to the present of DNA prior radiotherapy. Besides EBV DNA in patients' circulation, we proved that EBV DNA has potential to be used to screen for early NPC patients both in general population and early local relapse patients. Kerekhanjanarong, V et al has tested NPC cells from swabs and demonstrated sensitivity of 100% when tested the DNA with nested EBV PCR or TRAP assay, a test for telomerase activity. Finally, we have applied the same viral DNA in circulation concept to study plasma human papillomavirus (HPV) DNA in cervical cancer (CC) patients. Pornthanakasem, W et al. Demonstrated that in contrast to EBV DNA in NPC, plasma HPV DNA in CC was rare, only 6%, presented as naked DNA and significantly correlated with metastasis. Conclusion of

both plasma EBV DNA and HPV DNA was reported in Mutirangura Ann N Y Acad Sci. *945:* 59-67, 2001.

To search for genetic susceptibility of NPC of Thai and Chinese population living in Thailand, we applied association studies of several DNA polymorphic marker of several genes between cases and controls. Two known genes, HLA and CYP2E1, were evaluated the feasibility of the test and their significant to Thai population. The results of both genes were as significant as reported elsewhere and published, Kongruttanachok, N et al., and Pimtanothai, N et al. We also investigated three candidate genes, *PERB11*, *CR2* and *PIGR*. Only *PIGR* as a nasopharyngeal epithelial EBV receptor was associated with NPC susceptibility significantly. We evaluated two candidate genes, complement receptor 2 (*CR2*) and polymeric immunoglobulin receptor (*PIGR*) by using 3 SNPs, *PIGR*IVS3-156G⟶T, *PIGR*1093G⟶A and *PIGR*1739C⟶T, to genotype 175 cases and 317 controls, divided into Thai, Chinese and Thai-Chinese based on their respective ethnic origin. The results obtained indicated that *PIGR* is an NPC susceptible gene. The risk association pertaining to each ethnic group was detected for homozygous *PIGR*1739C with a significant ethnic group adjusted OR(95%CI) of 2.71(1.72-4.23) and p <0.00001. Haplotype of the two missense *PIGR* SNPs, 1093G⟶A and 1739C⟶T, and sequence analyses have confirmed the role of the nucleotide *PIGR*1739 and excluded possibility of an additional significant nonsynonymous NPC susceptibility SNP. The *PIGR*1739C⟶T is a missense mutation changing alanine to valine near endoproteolytic cleavage site. This variant could alter the efficiency of PIGR to release IgA-EBV complex and consequently increase the susceptibility of populations in endemic areas to develop NPC. The *PERB11* negative data was published at Chula Med J 2001 March 45:207-214. The *CR2* and *PIGR* data are submitted together as an original article.

The third goal was to study mechanism of NPC genome mutation. We investigated if V(D)J recombination might serve as a mechanism of tumor development in Epstein-Barr virus (EBV)-associated nasopharyngeal cancer (NPC) by examining primary biopsy samples as well as NPC cell cultures derived thereof for expression of *RAG1&2*. Lymphocyte and non-transformed cell infiltration was excluded by applying a particular culture technique and confirmed by flow-cytometry, RT-PCR amplifying CD3 and CD20, and microsatellite analysis. All samples harbor EBV and of those most fresh biopsy and NPC cell culture samples expressed *RAG 1* and/or *2* hinting at the site-specific recombination as a potential mechanism of carcinogenesis in EBV-associated NPC. Our ongoing research has been attempting to identify mechanical evidence of this new theory.

Keywords: Plasma EBV DNA, PIGR, RAG1&2, nasopharyngeal cancer

Chapter 1
Epstein-Barr Virus DNA in Serum/Plasma as a Tumor Marker for Nasopharyngeal Cancer

Summary

Nasopharyngeal cancer (NPC) constitutes a type of carcinoma encountered frequently in Southern China, among Eskimos of the Arctic region, and to a lesser extent in Southeast Asia. Because EBV DNA present in plasma or serum of NPC patients has proven to represent a promising noninvasive tumor marker, the present study was designed to determine the incidence of serum/plasma EBV DNA by nested PCR during various disease management stages. By this method, we could detect EBV DNA in plasma/serum of 98 of 167 NPC patients prior to treatment, compared with 10 of 77 samples derived from healthy blood donors serving as controls, with a similar prevalence observed in plasma *versus* serum. Investigation of 13 patients subjected to radiotherapy revealed plasma EBV DNA to persist in the plasma of one case, whereas among the remaining patients, it had vanished during the early phase of treatment. Finally, with 52 samples derived from 37 NPC patients during follow-up, we established 100% specificity and 0% false-positive rate for plasma DNA detection by nested PCR. Moreover, we subjected 24 known EBV DNA-positive serum samples to DNase digestion prior to DNA extraction and amplification to differentiate between free and encapsulated viral DNA, which demonstrated complete absence of the human ß-globin genomic DNA in contrast to EBV DNA detectable in 14 samples. In conclusion, applying this noninvasive method, serum/plasma EBV DNA constitutes a reliable tumor marker prior to, during, and after treatment of NPC.

Introduction

The discovery of tumor-derived DNA in the circulation of cancer patients raises the possibility of a new strategy for noninvasive cancer detection and monitoring (1, 2, 3) . In NPC,[4] we have previously demonstrated that EBV DNA is not only detectable in tumor tissue but is also detectable in the patients' cell-free sera (4) . In addition, comparison between EBV typing of primary tumors and their sera showed identical results, suggesting that serum EBV DNA represents tumor DNA. In a recent study, Lo *et al.* (5) have further explored this issue with respect to sensitivity and quantity of the plasma EBV DNA derived from NPC patients. A very high incidence of plasma EBV DNA was shown prior to treatment. Furthermore, the presence of plasma EBV DNA 1 month after completion of radiotherapy was associated with the disease persisting, either because of partial response or distant metastasis. Hence, serum/plasma EBV DNA represents a promising tumor marker for noninvasive cancer detection and monitoring of NPC.

NPC constitutes an important cancer in Asia encountered frequently in Southern China and among Eskimos of the Arctic region. An intermediate incidence is observed in Southeast Asia (6, 7, 8) . Therefore, the attempt at understanding how serum/plasma EBV DNA could be used for diagnosis and monitoring of NPC is crucial. In the present study, we applied nested PCR to analyze the incidence of serum/plasma EBV DNA during several phases of NPC, prior to as well as in the course of treatment and during follow-up. In addition, we determined whether the viral DNA was encapsulated. The data presented here not only demonstrate sensitivity and specificity of serum/plasma EBV DNA in each phase of the disease but also assist in an increasing comprehension as to its biological significance.

Materials and Methods

Sample Collection.

Upon informing the patients about the purpose of the study and obtaining their

consent, primary NPC tissues were collected before onset of treatment at Chulalongkorn University Hospital. The tissues were divided into two parts. The first part was sent for routine histological examination. The second part was immediately stored in liquid nitrogen for future DNA extraction and PCR analysis for the presence of the EBV genome. All tumors were histologically ascertained to be undifferentiated NPC according to the WHO classification.

Blood samples were obtained by venipuncture from several groups of patients selected on the grounds that the EBV genome was present in their tumor tissues. The first group, 146 serum and 21 plasma samples, comprised patients prior to treatment. The second group included plasma samples obtained from 13 patients at the weekly complete blood count evaluation in the course of radiotherapy. The last group constituted 52 plasma samples from 37 patients, who after completion of treatment came to the hospital every 3 months for follow-up. The DNA samples extracted from the sera of healthy blood donors serving as controls in a previous study were used again for the same purpose (1) . To obtain cell-free sera and plasma, clotted and EDTA blood specimens were centrifuged at low speed for 5 min within 1 h after venipuncture. Both sera and plasma samples were stored at -20°C until further analysis.

DNA Isolation and DNase Treatment.

NPC tissue was incubated in Tris/HCl buffer containing SDS and proteinase K at 50°C overnight, followed by phenol/chloroform extraction and ethanol precipitation of DNA (9) . As for serum or plasma, 200 µl were purified for DNA extraction on Qiagen columns (Qiamp blood kit; Qiagen, Basel Switzerland) according to the "blood and body fluid protocol." Ten sera and 10 plasma samples, respectively, were reanalyzed for the presence of the EBV genome to compare the efficiency of the Qiamp blood kit with that of reextracting the nucleic acid with the QIAamp viral RNA mini kit (Qiagen). One-tenth of the DNA extracted was then used for nested PCR analysis.

To distinguish free EBV DNA molecules from virions, 400 µl of 24 known positive EBV DNA serum samples were divided into two parts. The first part was twice digested extensively with DNase I (37°C for 1 h; Ref. 10). Both parts were then subjected to DNA extraction and nested EBV DNA PCR analysis. Seminested PCR for amplification of ß-*globin* DNA was used to determine whether free DNA had been digested completely.

EBV Detection by Nested PCR.

For the detection and typing of EBV DNA in tumor tissue and serum/plasma samples, nested PCR protocols were used, modifying those described previously for amplification of the *EBNA-2* (11 , 12) . DNA extracted from the cell line B958, EBV-transformed lymphocytes (American Type Culture Collection), was used as positive control and double-distilled water as a negative control.

The first PCR amplified the EBNA-2, generating a DNA fragment of 237 bp for EBV type A and of 253 bp for EBV type B, respectively. With nested primers, the PCR product comprised 168 bp for EBV type A and 184 bp for EBV type B, respectively. The nucleotide sequences for these first PCR primers were 5'-GCGGGTGGAGGGAAAGG-3' (E3–44mer) and 5'-GTCAGCCAAGGGACGCG-3' (E5–25mer). The nested PCR primers were E3 and E5 primers for *EBNA2* (11 , 12) . Amplification of the *ß-globin* gene by seminested PCR was used to determine the presence of amplifiable human DNA in all samples tested for EBV DNA. The primers GH20 and PCO4 were used for the first-round primary PCR, generating a DNA fragment of 260 bp (13) . The *ß-globin*-specific seminested primers were 5'-ACCTCACCCTGTGGAGCCA-3' (ß-globin 62028) and PCO4, generating a 231-bp PCR product. The sequences of the primers used for *EBNA-2* and *ß-globin* PCRs were identical to those reported previously (11, 12, 13) .

The first-round PCR reactions were performed in a total volume of 20 µl using one-tenth of the extracted DNA in a reaction mixture containing 200 µmol of each

deoxynucleotide triphosphate, 1.5 mM magnesium chloride, 50 mM potassium chloride, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 0.5 unit of *Thermus aquaticus* DNA polymerase, and 0.2 μM for EBV or 0.5 μM for *β-globin* primers. The PCR amplification was performed as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94° C for 30 s, annealing at 57°C for 30 s, with an extension at 72°C for 1 min, and a final extension at 72°C for 7 min. One μl of each PCR product was used as the template for nested and seminested PCR, respectively. The 35 cycles of second-round PCR reactions were performed in a manner identical to that applied for the first-round PCR, except for using different sets of primers, 0.5 μM for EBV or 1 μM for *β-globin* primers, and adjusting the annealing temperature to 50°C. The PCR products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide upon preparation.

Statistical Analysis.

Data regarding histology, tumor staging, EBV detection, and response to treatment were collected in a double-blind fashion until analyzed. The ^{2} test was used to compare the results obtained from serum/plasma analysis with clinical and tumor parameters.

Results

Sensitivity and Specificity of Nested PCR to Detect EBV DNA in Serum and Plasma.

In this study, nested PCR was applied to improve the sensitivity for identifying EBV DNA. For the control cell line, nested PCR for *EBNA-2* still yields a positive result with 10 fg DNA (Fig. 1)F1F1 . This suggests a much higher sensitivity of nested PCR. We then reevaluated sensitivity and specificity of EBV DNA detection in sera and plasma of NPC patients, as well as of normal blood donors (Table 1)T1T1 . Whereas higher sensitivity, 9[illegible] of 167 cases (58.68%), was demonstrated, fewer cases of normal blood donors, 10 of 77 cases (13%), showed positive results. The positive EBV DNA incidence from plasma samples, 71.4%, was slightly higher than from serum, 56.85%. Twenty previously analyzed samples, 10 serum and 10 plasma samples, were reextracted for nucleic acid by QIAamp viral RNA mini kit. With these samples, testing for the EBV genome was repeated by nested PCR and yielded identical results. There is no significant statistical correlation between serum EBV DNA and clinical parameters, staging, histological classification, or response to treatment at 3 and 6 months.

To evaluate whether the EBV DNA detected in serum was present as a free nucleic acid or within intact virions, 24 EBV DNA-positive sera from different patients were subjected to DNase I treatment prior to DNA extraction. After extensive DNase I treatment, nested PCR did not yield any detectable product of the control *β-globin* DNA, whereas nested PCR for EBV DNA remained positive for 14 cases (Fig. 2 ; Table 1).

Plasma EBV DNA during Radiotherapy.

Plasma samples were collected from 13 cases, and the EBV DNA status was determined on a weekly basis in the course of radiotherapy (Fig. 3 ; Table 1). Five of these cases showed no serum/plasma EBV DNA prior to treatment and remained negative during treatment. With the others, serum/plasma EBV DNA was detectable prior to treatment. In seven of them, plasma EBV DNA disappeared between the first and third week of radiotherapy. One case showed persistence of plasma EBV DNA throughout the course of radiotherapy.

Sensitivity and Specificity of Plasma EBV DNA for Patients' Follow-Up.

Fifty-two blood samples of 37 patients after treatment of between 3 and 42 months duration were evaluated at follow-up for the presence of the EBV genome in plasma (Fig. 4 ; Table 1). Using WHO criteria for response, five cases presented with evidence of NPC, three with recurrence, and two with partial response, =" src="/math/ge.gif" border=050% decrease in total tumor size of the lesions and no appearance of new lesions or progression of any

lesion. Plasma EBV DNA was detectable in three cases, one partial response, patient 365, and two recurrence cases, patients 72 and 106. Interestingly, whereas the plasma of both recurrence cases, patients 72 and 106, were positive for EBV DNA in their serum EBV DNA absent prior to treatment. Furthermore, repeated evaluation of the plasma for the EBV DNA status prior to further treatment twice in patient 106 and four times in patient 240, respectively, still showed identical results. The other 32 cases were in complete remission at the time of evaluation. Case 106R was patient 106 after the second course of radiotherapy. In the 32 plasma samples tested from all complete remission cases, some of which were examined more than once, no plasma EBV DNA was detectable. This suggested 100% specificity and 0% false positive rate, respectively, for serum/plasma EBV DNA to be used as an NPC tumor marker for follow-up after completion of treatment.

Discussion

The discovery of tumor-derived DNA in the circulation of cancer patients implies the possibility of a new strategy for noninvasive cancer detection and monitoring (1, 2, 3) . For NPC, using EBV DNA for this purpose should prove advantageous because virtually all cases of NPC have been found associated with EBV (14 , 15) . Because a comparison between two studies has shown much divergence as to the prevalence of EBV DNA in serum/plasma samples, we improved the sensitivity for further analysis by using nested PCR (4 , 5) . This technique should prove to be the one with the highest sensitivity. Several positive cases (13%) were identified among normal blood donors infected previously with EBV, compared with not a single case identified previously by one-round PCR or with 7% established by the quantitative PCR study. The present study revealed serum/plasma EBV DNA to be present in 58.68% of NPC cases. Although plasma samples demonstrated a slightly higher prevalence of EBV DNA, there was no significant difference of DNA identification between plasma and serum samples or between two different DNA extraction methods, Qiamp blood kit and Qiamp viral RNA mini kit. The continuous presence or absence of EBV DNA in serum/plasma was confirmed by repeatedly evaluating the plasma for EBV DNA status in two patients. The difference in prevalence of the tumor DNA in the circulation of NPC patients was similar to that reported for other types of cancer. For example, the frequency of identifying serum/plasma colorectal cancer DNA can vary between studies from 40 to 90% (16, 17, 18) . Although differences in serum/plasma EBV DNA between EBV DNA prevalence reported by real time PCR and nested PCR may be attributable to different PCR primers and detection system. Nevertheless, as yet unsuspected biochemical and/or environmental mechanisms may be responsible for this finding.

Serum/plasma EBV DNA has been studied in several conditions, such as infectious mononucleosis, acute lymphoproliferative disorder, and EBV-associated lymphoma (19, 20, 21, 22, 23, 24) . In addition to the association with EBV-associated cancers, serum/plasma EBV DNA was found to be an indicator for active infection rather than latent virus (19 , 21, 22, 23, 24) . The purpose of the DNase digestion experiment was to establish whether the presence of serum EBV DNA was attributable to lytic replication or release of latent episomal DNA. The results suggest that there might be two simultaneously present populations of serum EBV DNA, one encapsulated in the viral particle and the other the free nucleic acid probably released from NPC as episomal DNA. The presence of virions in the circulation of NPC patients is surprising because the majority of EBV in NPC cells should be in the latent phase, as shown by EBV clonal studies (25 , 26) . Consequently, serum/plasma EBV DNA should all be present as free nucleic acids. Nevertheless, our data invite the hypothesis that some EBV in NPC should enter lytic replication. This could explain why antibody titers to lytic cycle antigens, such as VCA and ZEBRA, rise in NPC patients (27 , 28) . In addition, expression of the immediate early *BZLF1* and *BHLF1* genes or ZEBRA protein is frequently detectable in NPC (29 , 30) . Because there may be only few cells entering lytic viral

replication, studying the clonal progression of EBV by analyzing terminal repeat lengths by Southern blot and hybridization may not be sensitive enough to commonly detect the lytic replication or might be interpreted as impurities and hence ignored. Definite proof, such as electron microscopic analysis, is required to identify virions in NPC circulation.

Studying plasma EBV DNA during radiotherapy not only suggests a direction to further explore the behavior of EBV DNA present in the circulation of patients receiving radiotherapy but may also lead to clinical implications. EBV DNA positive in serum/plasma before treatment disappeared early in the course of radiotherapy, whereas plasma EBV DNA initially negative remained negative. This suggests two important findings: (*a*) serum/plasma EBV DNA is short lived; and (*b*) cell death as a consequence of radiation does not promote the presence of EBV DNA in the patients' circulation. On the contrary, because EBV DNA could disappear from plasma as early as during the first week of treatment, radiation may in addition to its ability to decrease the number of NPC cells use a specific mechanism that prevents the tumor from releasing EBV DNA. The biological effect of ionizing radiation at the cellular and molecular level appears to be DNA double strand break and a subsequence activation of DNA-dependent protein kinase (31 , 32) . It will be interesting to further explore whether DNA-dependent protein kinase plays any role in the release of EBV DNA from NPC into the patients' circulation. Whereas most cases of previously positive serum/plasma EBV DNA disappeared during radiation, one case showed persistence of plasma EBV DNA. This NPC case may not respond to the radiation induced inhibition of virus release, or it may indicate micrometastases. This persistence of serum/plasma EBV DNA during radiotherapy may be crucial for future clinical treatment modalities.

The other important clinical application of serum/plasma EBV DNA with NPC suggested here is as a tumor marker for patients' follow-up. NPC is a form of cancer with a high success rate of radiochemotherapy (33) . However, many cases may recur, even after very long periods of latency (34) . Consequently, most NPC patients require very consistent and long follow-up studies. Some of them may require expensive investigations, such as computed tomography scan or invasive methods, especially punch biopsy. Analyzing serum/plasma EBV DNA is an inexpensive and noninvasive technique suitable for clinical application. Upon using plasma EBV DNA as a marker for follow-up, the sensitivity (60%) shown in this study was similar to the prevalence detected prior to treatment. In addition, whereas only patients with evidence of disease showed plasma EBV DNA, the DNA was not detectable in any of the cases with complete remission. This suggested 100% specificity and a 0% rate of false positives. Interestingly, whereas we detected 13% of serum EBV DNA in 77 healthy individuals, no EBV DNA was identified in 42 tests of 32 NPC cases with complete remission. The usefulness of serum/plasma EBV DNA as a molecular marker for NPC patient monitoring was emphasized recently by Lo *et al.* (35) . They demonstrated a close relationship between plasma/serum EBV DNA quantity and tumor recurrence. In addition, significant elevations in serum EBV DNA were observed in the patients who subsequently developed tumor recurrence.

In conclusion, this study has shown how frequently serum/plasma EBV DNA can be discovered in the course of NPC prior, during, and after treatment. Approximately 59% of NPCs prior to treatment were positive for serum/plasma EBV DNA. The presence and/or absence of serum/plasma EBV DNA is likely to depend on each individual and remain persistent as long as there is no change in the tumor status. Radiotherapy cannot induce but rather prevents NPC from releasing EBV DNA into the patients' circulation. Finally, investigating serum/plasma EBV DNA after treatment suggested its potential as a tumor marker.

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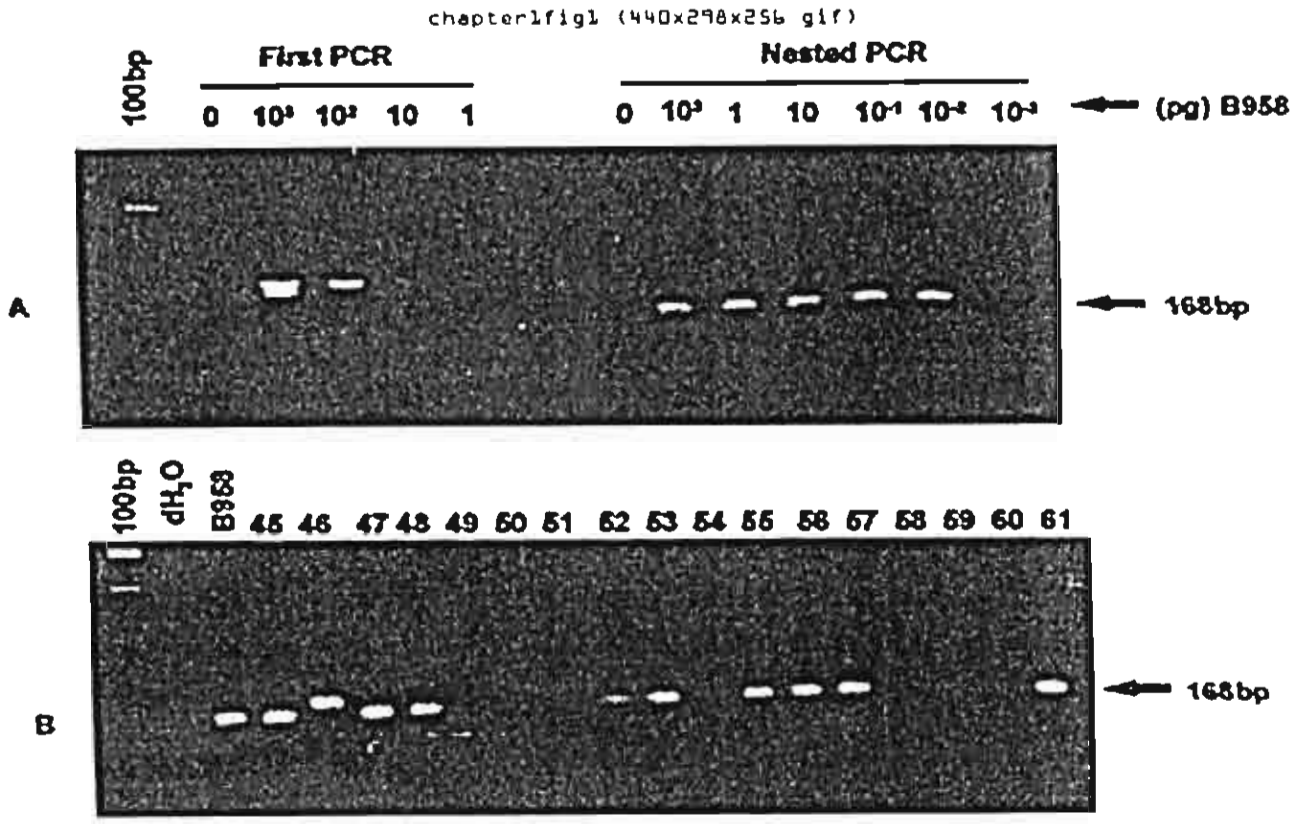

Fig. 1. Sensitivity of nested PCR for EBV DNA. A 100-bp ladder, distilled H_2O (dH_2O), and DNA from B958 cell lines were used as size standard, negative control, and positive control, respectively. *A*, compare sensitivity between single PCR and nested PCR for EBV DNA. *B*, examples of nested PCR for EBV DNA from NPC sera. Positive results were cases 45–48, 52, 53, 55–57, and 61. Most positive cases were EBV type A, whereas case 46 was EBV type B.

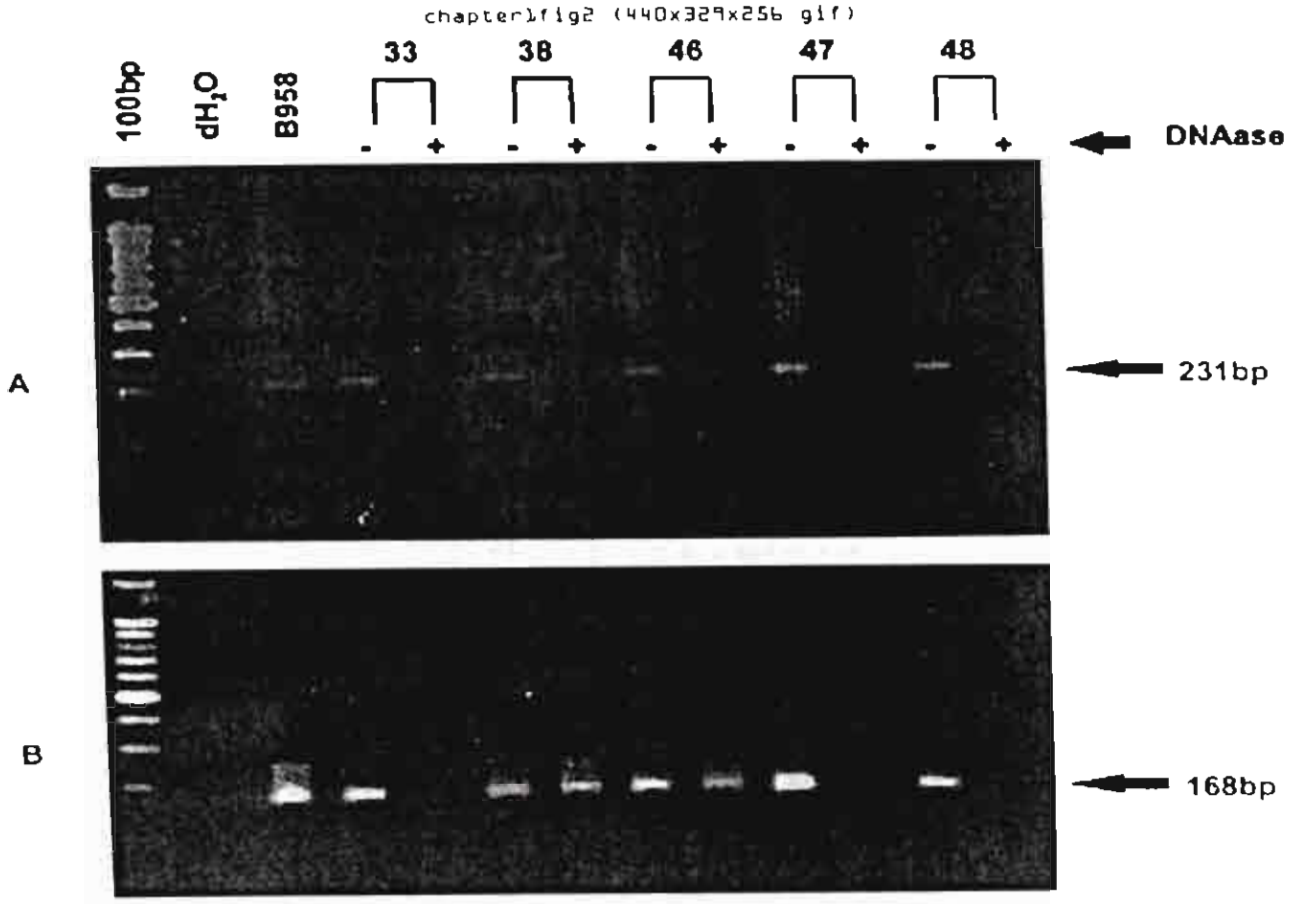

Fig. 2. Nested and seminested PCR from known EBV DNA-positive serum samples after DNase treatment. DNA. A 100-bp ladder, distilled H_2O (*dH_2O*), and DNA from B958 cell lines were used as size standard, negative control, and positive control, respectively. Lanes with (+) and without (-) DNase treatment are shown. *A*, seminested PCR for human *ß-globin*. *B*, nested PCR for EBV DNA. All of these cases showed complete DNase treatment. Whereas cases 38 and 46 demonstrated resistant of EBV DNA from DNase, sera from cases 33, 47, and 48 were digested.

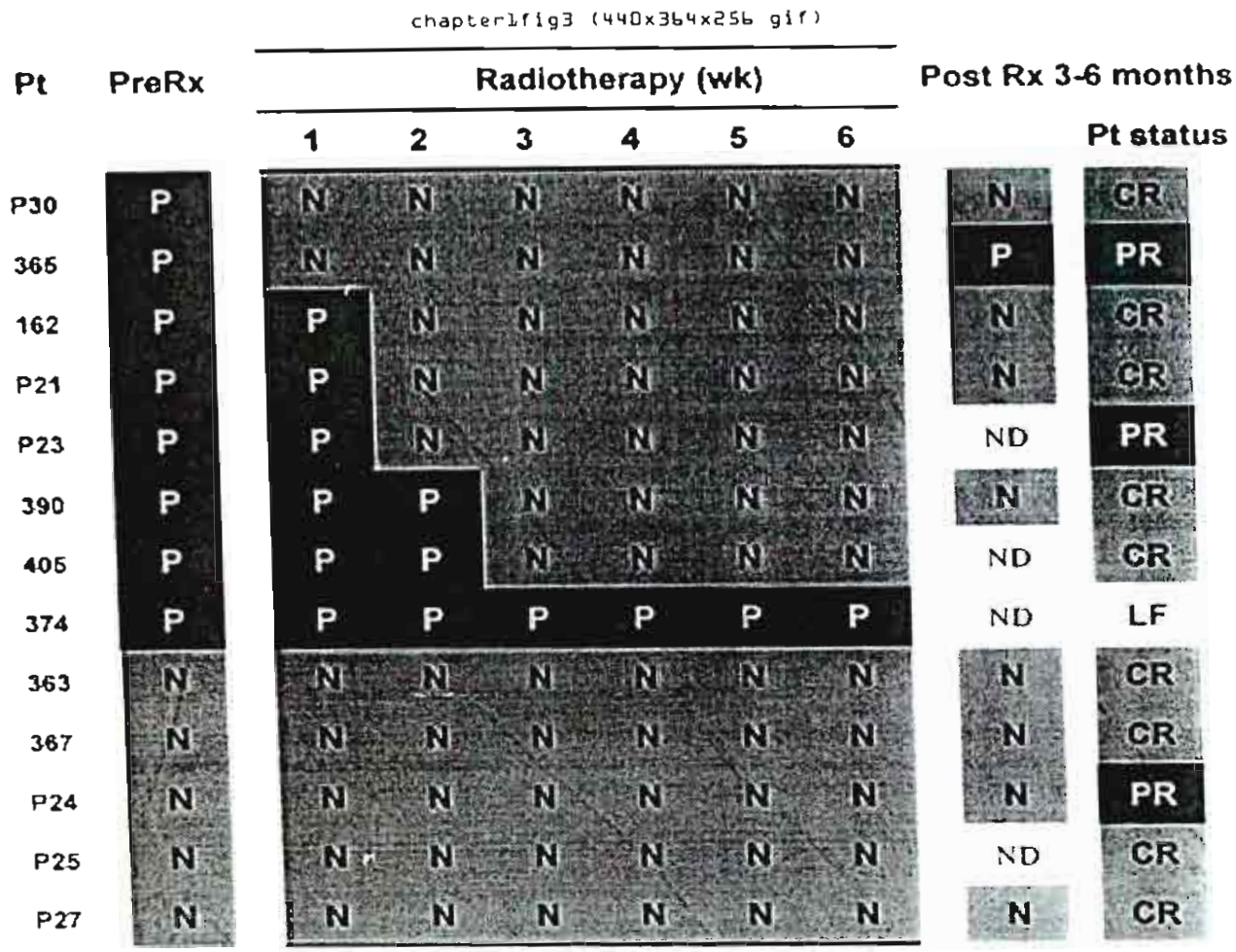

Fig. 3. Plasma EBV DNA at the end of each week during radiotherapy. Present (*P*) and absent (*N*) EBV DNA are shown prior (*PreRx*) and during treatment. NPC status of each patient at 3–6 months after treatment is demonstrated as complete remission (*CR*) and partial response (*PR*). *LF*, loss to follow up; *ND*, not done.

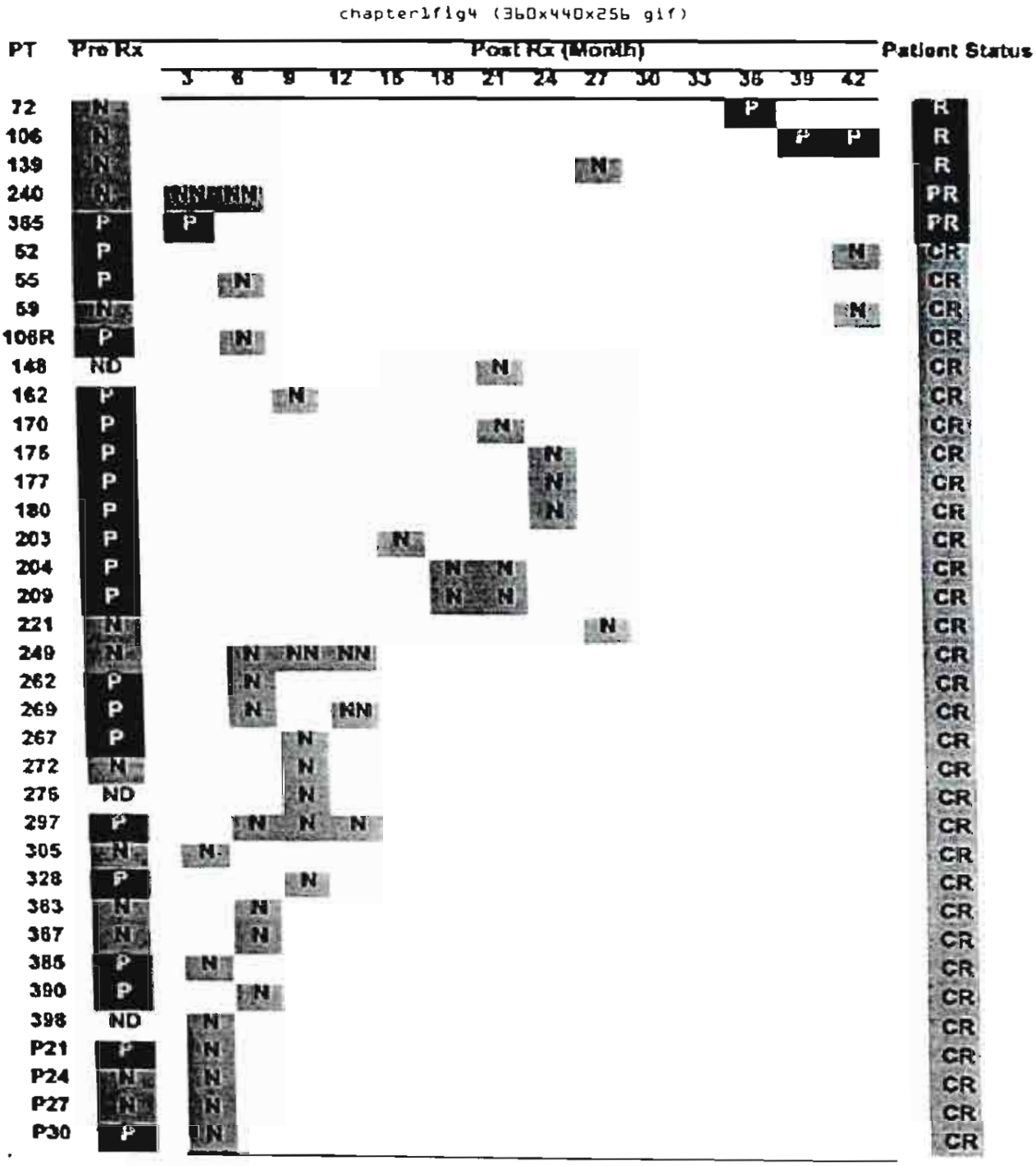

Fig. 4. Serum/plasma EBV DNA during follow-up after complete treatment. NPC status of each patient is demonstrated as recurrence (*R*), partial response (*PR*), and complete remission (*CR*). Present (*P*) and absent (*N*) of EBV DNA are shown. *ND*, not done.

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Table 1 Incidence of serum/plasma EBV DNA during various NPC management stages

Diagnosis	Status	Sample	Positive	Total (case)	%
NPC	Before treatment	Serum/plasma	98	167	58.7
		Serum	83	146	56.9
		Plasma	15	21	71.4
	Known positive EBV DNA in serum	Serum treated with DNAase	14	24	58.3
	During radiotherapy	Plasma (before Rx, 1, 2, 3–6 wks)[a]	(9, 6, 3, 1)	13	(69, 46, 23, 7)
	Follow-up	Plasma	3	37	8.1
	Recurrence or partial response	Plasma	3	5	60
	Complete remission	Plasma	0	32	0
Normal control		Serum	10	77	13.0

[a] (before Rx, 1, 2, 3–6 wks) incidence of plasma EBV DNA before treatment at first, second, and third to sixth weeks during radiotherapy.

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Chapter 2
Polymeric Immunoglobulin Receptor Polymorphisms and Risk of Nasopharyngeal Cancer

Summary

Epstein-Barr virus (EBV) associated nasopharyngeal cancer (NPC) is an important squamous cell cancer endemic in Southeast Asia and the Far East and can be considered a multifactorial genetic disease. This research explores potential associations between nasopharyngeal epithelial EBV receptor and NPC susceptibility. To prove the hypothesis, we evaluated two candidate genes, complement receptor 2 (*CR2*) and polymeric immunoglobulin receptor (*PIGR*) by using 4 SNPs, *CR2*IVS2-848C→T, *PIGR*IVS3-156G→T, *PIGR*1093G→A and *PIGR*1739C→T, to genotype 175 cases and 317 controls, divided into Thai, Chinese and Thai-Chinese based on their respective ethnic origin. The results obtained indicated that *PIGR* is an NPC susceptible gene. The risk association pertaining to each ethnic group was detected for homozygous *PIGR*1739C with a significant ethnic group adjusted OR(95%CI) of 2.71(1.72-4.23) and p <0.00001. Haplotype of the two missense *PIGR* SNPs, 1093G→A and 1739C→T, and sequence analyses have confirmed the role of the nucleotide *PIGR*1739 and excluded possibility of an additional significant nonsynonymous NPC susceptibility SNP. In conclusion, we present genetic evidence supporting PIGR to function as the EBV nasopharyngeal epithelium receptor via IgA-EBV complex transcytosis failure. The *PIGR*1739C→T is a missense mutation changing alanine to valine near endoproteolytic cleavage site. This variant could alter the efficiency of PIGR to release IgA-EBV complex and consequently increase the susceptibility of populations in endemic areas to develop NPC.

Introduction

Nasopharyngeal cancer (NPC [MIM 161550]) constitutes an endemic multifactorial genetic disease. [1] Whereas the disease is quite rare in the Western world, it occurs at high frequencies in Southern China, Southeast Asia, and among the Greenland Inuit. The highest aged-adjusted incidence has been reported in South China to be 30-50/100,000. Intermediate incidence has been noted in SouthEast Asia with a ratio of 3/100,000 among Thais and 10/100,000 among Thais of Chinese extraction, respectively. [2] Regarding environmental carcinogenesis, by both viral and chemical carcinogens contribute to NPC with Epstein Barr virus (EBV) as the most important etiological factor. The single clonally derived viral genome can be found in all endemic NPC cells. [3-5] On the other hand, several reports indicate that consuming salty fish or preserved food and thus concentrating chemical carcinogens such as nitrosamine, can promote the development of NPC. [6,7] Interestingly, two NPC susceptibility genes, *HLA* and cytochrome P450 2E1 (*CYP2E1*), were discovered based on their hypothesized function to interact with environmental factors contributing to NPC etiology. HLA was first studied in Singapore as this gene would be responsible for patients' immune response to cancer or EBV infected cells. [8] In particular HLA A2 and B46 conferred a high relative risk as to NPC development according to various reports in Asia. [9] In addition, NPC development was proposed to correlate with patients' history of consuming preserved food and hence *CYP2E1*, which metabolises nitrosamine, was studied revealing a risk association in Taiwan [10] and Thailand. [11] This research aims at exploring the genetic aspect of EBV entry into the nasopharyngeal epithelium (NE). This process not only requires specific host factors but also differentiates between the mechanisms of EBV carcinogenesis originating in epithelial and/or lymphoid cells. Whereas EBV-associated Burkitt's lymphoma is prevalent in Africa, populations demonstrating a higher risk for developing NPC do not show any significant increase in the lymphoma incidence. [12]

The mechanism of EBV entry into the NE has not yet been conclusively elucidated but at least two receptors, complement receptor type 2 (CR2) and polymeric immunoglobulin receptor (PIGR), have been proposed. CR2 is an integral membrane glycoprotein to which EBV can bind and thus infect B-lymphocytes. [13] EBV can infect recombinant epithelial cells expressing CR2 especially when in contact with virus-producing lymphocytes. [14,15] In addition, there has been a study reporting CR2 expression in embryonic NE cell by RT-PCR. [16] As for PIGR, the protein can be discovered on NE, interestingly as a pneumoccocus

receptor,[17] where it mediates endocytosis and transcytosis of IgA-EBV complexes to deliver EBV into the luminal surface. [18] The viral translocation process can fail and consequently cause EBV infection if the epithelium loses its polarity or has mutated *PIGR*. [19,20] In addition to NE, EBV was reported to enter NPC in vitro depending on the presence of viral specific IgA. [21] Interestingly, serology studies indicate indirectly that PIGR is involved in NPC development. High EBV-IgA titers can be detected specifically in patients with NPC or people who will develop the disease. [22] Based on specific endemic distribution, NPC susceptibility genes can be hypothesized to originate from ancestor alleles. Hence, this study has been designed candidate-gene approaches for studying complex genetic traits. [23] The specific aim has been to explore whether *CR2* or *PIGR* might be NPC susceptibility genes by determining the risk association between their DNA polymorphisms and haplotypes among NPC patients and controls from several individuals of high-risk ethnic origin.

Subjects and Methods

Sample Collection

After having obtained the subjects' informed consent as to the purpose of the study blood samples were collected by venipuncture from NPC patients and controls. The patients and controls were interviewed and then separated into three groups, Thai, Chinese, and Thai-Chinese, respectively, based on the ethnic origins of their grandparents. If their ancestors, including their great grandparents, originated from China, they were considered Chinese. On the other hand, if their ancestors originated from Thailand, they were defined as Thai. In addition, if their ancestors originated from Thailand and China, they were defined as Thai-Chinese. Conclusively, 104 Thai, 107 Chinese and 106 Thai-Chinese were enrolled in the control group whereas the NPC patients comprised 110 Thai, 42 Chinese and 23 Thai-Chinese. The 175 patients were recruited at King Chulalongkorn Memorial Hospital between 1994 and 2001. They were permanent residents in Bangkok or the central part of Thailand. All cases were Thai, Chinese or Thai-Chinese. The tumors of every patient was histologically ascertained as NPC type II or III, according to WHO classification. The 317 controls were healthy blood donors from Thai Red Cross Society, locating in the King Chulalongkorn Memorial Hospital. All had permanent resident in Bangkok or the central part of Thailand and those previously diagnosed with NPC or belonging to an ethnic group other than Thai. Chinese or Thai-Chinese had been excluded. The male:female ratio among the cases was 1.8:1 and among the controls 1.6:1, respectively. Their DNA was isolated by proteinase K and incubated overnight at 50°C, followed by phenol/chloroform extraction and ethanol precipitation.

Single Nucleotide Polymorphisms (SNPs) information

Four SNPs, *CR2*IVS2-848C→T, *PIGR*IVS3-156G→T, *PIGR*1093G→A and *PIGR*1739C→T, were chosen. *CR2*IVS2-848C→T located in intron 2 [24] and *PIGR*IVS3-156G→T in intron3, [25] had previously been published as RFLP polymorphisms, whereas *PIGR*1093 and *PIGR*1739 were selected from a SNPs database. (http://www.ncbi.nlm.nih.gov/SNP/)

PCR-RFLP

PCR reactions were performed, using Perkin-Elmer/DNA Thermal cycle480, in a total volume of 50 µl to amplify *CR2*IVS2-848C→T, *PIGR*IVS3-156G→T, and *PIGR*1739C→T. The reaction mixtures consisted of 100 ng of genomic DNA and the following set of primers at the respective concentrations: 0.1 µM of *CR2*IVS2-848C→T primers (5'-CTTTCTGTGCAGACCACGTT-3' and 5'-GATCTATGGTAGCCAGTTGG-3' *PIGR*IVS3-156G→T primers (5'-TCAGCCAGGGTAAGGATCC-3' and 5'-TGATGGTCACCGTTCTGCC-3'), or 0.2 µM of *PIGR*1739C→T primers (5'-GGGTCCCGCGATGTCAGCCTAG-3' and 5'-TTCTCCGAGTGGGGAGCCTT-3'). The DNA samples were amplified in the presence of 200 µmol dNTPs, 5 µl of 10X PCR buffer (20 µM Tris-HCl pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$ and 4U Taq DNA polymerase (Gibco). The PCR condition for *CR2*IVS2-848C→T comprised an initiation denaturation step at 94°C for 4 minutes, followed by 35 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes and final extension step at 72°C for 7 minutes. *PIGR*IVS3-156G→T was amplified 40 cycles at an annealing temperature of 60°C for 1 minute, and extension at 72°C for 3 minutes. The conditions for *PIGR*1739C→T was 35 cycles at 60°C annealing temperature for 1 minute and extension at 72°C for 1 minute. Subsequently, RFLP analysis was performed on 20 microliters each of the respective PCR products by subjecting them to the following restriction enzymes (New England Biolabs) at a 10U concentration: *TaqI* for *CR2*IVS2-848C→T, *PvuII* for *PIGR*IVS3-156G→T, and *HgaI* for *PIGR*1739C→T with

overnight incubation at 65°C for *TaqI* and at 37°C for both of *PvuII* and *HgaI*. The resulting products were further analyzed by 2% agarose gel electrophoresis.

ARMS of *PIGR*1093G→A

The Amplification Refractory Mutation System (ARMS) [26] was used to detect SNP *PIGR*1093G→A. The primers can be divided into 2 sets, A and B. Set A primers comprised of 1093Fa and 1093R (GCCCCACTGTGGTGAAGGGGGTGGCAGGTG and ACTGGGCCTTAACCCACCCC), whereas 1093Fb (GCCCCACTGTGGTGAAGGGGGTGG CAGGTA) and 1093R were mixed in set B. For each sample, the PCR reaction was performed in duplicate, set A and B, in a total volume of 50 µl containing 100 ng of genomic DNA, 1.5 mM $MgCl_2$, 5 µl of 10X buffer (20 µM Tris-HCl pH 8.4, 50 mM KCl), 4U Taq Gold DNA polymerase (Perkin Elmer) and 0.3 µM of primer set A or set B. PCR conditions included initial denaturation at 95°C for 10 minutes followed by 30 cycles of 95°C for 1 minute, 65°C for 1 minute, 72°C for 1 minutes and final extension for 72°C for 7 minutes in a Perkin-Elmer/DNA thermal Cycle 480. Each SNP was investigated by 2% agarose gel electrophoresis for complementarity of the PCR product with the 3' nucleotide of primers set A and/or set B.

Statistical analysis

Both cases and controls were subdivided based on ethnic, sex and age at sampling (i.e., <30, 30-49, 50-69, ≥70 years). The association between certain alleles of the *CR2* or *PIGR* and NPC development was estimated by the statcalc from Epi info 2000 program (http://www.cdc.gov/epiinfo/ei2000.htm) to calculate the odds ratio (OR) and 95% confidence interval (CI), Mantel-Haenszel chi squares and associated p values. Mantel-Haenszel weighted OR, summary chi square and p value were adjusted for the confounding effect of ethnic, and/or age and sex by combining stratified 2X2 tables. The effect exerted by genotype was estimated as if autosomal inheritance according to actual number of alleles contributed to a significant OR.[27]. Haplotype frequencies for pairs of alleles, as well as Chi square values for allele associations were estimated by the Estimating Haplotype-frequencies (EH) software program. [28] The number of each haplotype from each ethnic were estimated based on the allele frequencies multiplied by the number of cases. The haplotype numbers of all the cases as well as controls were summation of the haplotype numbers of Thai, Chinese and Thai-Chinese groups. The association between each haplotype and NPC development was calculated based on the OR using Epi info 2000 program. The haplotype differences between groups were estimated by $T(x^2/2)$ = ln(L, group1)+ln(L, group2)-ln(L, group1+group2) as previously described [28].

Sequencing

All 11 exons of *PIGR* genomic DNA were amplified from DNA samples of 16 unrelated patients. Each exon was amplified by a pair of primers locating on the following positions in relation with nucleotide number AL359089: 93732C-93751G and 94163A-93782C, 99668C-99687T and 100025A-100044C, 100724C-100743T and 101427C-101446T, 102578A-102597G and 103454C-103473A, 104403C-104422A and 105039T-105058A, 105584C-105604G and 106080G-106099G, 107094C-107113A and 107518G-107537C, 107737G-107756C and 108066C-108085C, 108524A-108543T and 109039G-109059T, and 109935G-109954G) and 110656G-110675C. Subsequently, PCR products were purified and directly sequenced in both directions by dye terminators into cycle-sequencing products.

Results

***PIGR* and *CR2* SNPs NPC case-control study**

The study explored the risk of association between *CR2* or *PIGR* DNA polymorphisms and NPC phenotype. Both the 175 patients and 317 controls were separated into Thai, Chinese and Thai-Chinese, according to their grandparents' ethnic origin so that

each group would have a higher probability of shared ancestors. The genotype of four SNPs, *CR2*IVS2-848C→T, *PIGR*IVS3-156G→T, *PIGR*1093G→A and *PIGR*1739C→T, were investigated. *CR2*IVS2-848C→T located near exon 1, 2 [24], and *PIGR*IVS3-156G→T in intron 3 [25], have previously been published as RFLP polymorphisms. *PIGR*1093G→A and *PIGR*1739C→T, on exon 5 and 7, respectively, were selected from a SNP database (http://www.ncbi.nlm.nih.gov/SNP/). *PIGR*1093G→A displays an amino acid alteration from glycine to serine and *PIGR*1739C→T from alanine to valine, respectively. No missense SNP from *CR2* has recently been reported by genbank. The amplicons of *CR2*IVS2-848C→T, *PIGR*IVS3-156G→T and *PIGR*1739C→T were genotyped by PCR-RFLP, and *PIGR*1093G→A by ARMS (fig 1).

Upon comparison between the frequency of these alleles in patients and controls of identical ethnic origin, Thai or Chinese, a significant risk association to *PIGR*1739C→T was detected but neither to *PIGR*IVS3-156G→T nor *PIGR*1093G→A (table 1). The OR(95%CI) of *PIGR*1739C→T in the Thai group was 1.70(1.03-2.82) and 2.35(1.11-5.07) among Chinese NPC cases with 1739C as the susceptible allele. When we analyzed all three ethnic groups and used Mantel-Haenszel stratification method to correct for ethnic matched control, the difference between crude and adjusted OR of *PIGR*1739C→T was less than 15%. The significant race adjusted OR(95%CI) is 2.26(1.51-3.25) with the *p* value was less than 0.0001. The NPC susceptibility conferred by *PIGR* requires homozygous 1739C to increase the likelihood of NPC development, with the race adjusted OR(95%) = 2.71(1.72-4.23) and p <0.00001(table 2). The similar significant contribution can be demonstrated with all subgroups (table 2). Regarding *CR2*, *CR2*IVS2-848C→T demonstrated no considerable correlation with NPC when analyzing Thai, Chinese or including all cases. Nevertheless, significant OR(95%CI) = 1.80(1.14-3.03) with p<0.05 could be demonstrated upon adjusting for age, sex and ethnic of all cases (table 1). This data suggests that the role of *CR2* as an NPC susceptibility gene need further evaluation while the role of *PIGR* is crucial.

Haplotype analysis of two missense *PIGR* SNPs, 1093 G→A and 1739 C→T

To further elucidate the relevance of the two missense SNPs as for NPC development, genotype data of 1093G→A and 1739C→T were tabulated into four haplotypes, 1093G-1739T (GT), 1093G-1739C (GC), 1093A-1739T (AT), and 1093A-1739C (AC) (table 3). The frequency of each haplotype based on its ethnic group was estimated by the EH program and distribution was compared between groups. Among the controls, there were no relevant difference between the Thai and the Chinese, yet the *p* values for the cases and the controls of both subgroups and the total were below 0.05 and 0.005, respectively (table 3).

Comparison among haplotypes confirmed 1739C→T as NPC susceptible mutation and excluded any other particular *PIGR* allele (table 3). First, whereas 1739C→T did, no haplotype was associated with significant OR in all Thai, Chinese and Thai-Chinese populations. Second, there were haplotypes with 1739C as susceptible alleles such as GC in the Chinese and total and 1739T as protective alleles such as AT in the Thai, GT in the Chinese and both haplotypes in the total population. In addition, there was no significant haplotype with 1739C as a protective allele or 1739T associated with significant higher relative risk. Finally, the relationship between each haplotype was measured and the data obtained supported the importance of 1739C→T (table 4). Whereas the ORs of the same 1739 nucleotides, between GC and AC or GT and AT, were not statistically significant, both GC and AC haplotypes conveyed a higher OR than GT and AT.

***PIGR* sequences of NPC patients**

By complete sequencing the coding region of the *PIGR* gene from 16 NPC patients with homozygous 1739C and upon comparison with the genomic DNA sequence AC098935 and AL359089 as well as with *PIGR* SNPs reported in genbank, the 2 SNPs at base pairs 1093 and 1739 were confirmed. No additional nonsynonymous mutation was identified.

However, there were six positions of new synonymous SNPs, IVS1-59G→A, IVS1-35G→A, IVS4-3C→T, 373C→T, 549G→A, and 1773C→T.

Discussion

The SNPs and haplotypes case control association study implicated that the probability to develop NPC might altered by DNA variation of *PIGR*, with nucleotide 1739 playing this crucial part. *PIGR* has been assigned to 1q31 and comprises of 11 exons. [18] The 1093G→A and 1739C→T are located on exon 5 and 7, respectively. The physical distance between nucleotide *PIGRIVS3*-156, 1093 and 1739 were 25 and 2.5 kb, respectively. Thus the three SNPs were closely linked but they distinctly contributed to NPC development. Whereas *PIGR*1739C→T exhibited a remarkably significant relative risk of NPC development among all Thai and Chinese populations, association of *PIGR*IVS3-156G→T and 1093G→A was not shown. Our data have provided an example that conclusions drawn from association studies aimed at identifying a susceptibility gene without related functional SNP should be interpreted with extreme caution. [23] If 1739C→T had not been chosen for this study, the importance of *PIGR* as an NPC susceptibility gene would have been misconstrued.

The manner, in which this *PIGR*1739C→T alters cellular movement of the IgA complex and consequently increases the possibility of EBV associate NPC development, remains to be elucidated. The polymorphism is a missense mutation altering the amino acid alanine to valine. The codon is located on exon 7, which is adjacent to the endoproteolytic cleavage site of the PIGR extracellular domain. [29,30] As a result, homozygous 1739C of the PIGR of individuals from high-risk ethnic origin may alter efficiency to release IgA-EBV complex and consequently, their NEs would have higher possibility to be infected by EBV.

It is important to note that the 1739C→T mutation can also be found in other lower risk ethnic groups, such as Caucasian. Hence, this *PIGR* nucleotide, despite a remarkably association with NPC, cannot account for its unique endemic distribution. On the other hand, this gene may be critical only for members of high-risk populations, to develop NPC as a consequence of other endemic genetic and/or environmental risk factors.

PIGR plays a crucial role in mucosal immunity not only against EBV, but also pneumococcal infection by facilitating transporting polymeric immunoglobulin transport across the mucosal epithelium. Human PIGR can bind to a major pneumococcal adhesion and enhanced pneumococcal adherence and invasion. [17] In other words, two important human pathogens, EBV and pneumococcus, employ the same human antibody transport protein, PIGR, to cause two common human diseases at the same tissue type, NE. The manner in which these two organisms interact with host (DNA) variation in vivo and what are the consequences might be on a global evolutionary scale will be very interesting subject for further investigation.

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Table 1 *CR2* and *PIGR* polymorphisms in NPC cases and control subjects.

	THAI		CHINESE		TOTAL	
	case	control	case	control	case	control
Number of tests	110	104	42	107	175	317
*CR2*IVS2-848C⟶T						
+/+	0	1	1	1	2	2
+/-	34	20	7	21	48	63
-/-	76	83	34	85	125	252
+ allele frequency	0.15	0.11	0.11	0.11	0.15	0.11
Crude OR (95%CI)	1.55(0.84-2.85)		1.00(0.41-2.39)		1.48(0.98-2.22)[1]	
Ethnic group adjusted OR(95%CI)					1.47(0.96-2.26)	
age, sex and ethnic group adjusted OR(95%CI)					1.80(1.14-3.03)[1]	
*PIGR*IVS3-156G⟶T						
+/+	32	30	9	42	49	114
+/-	61	58	26	48	99	155
-/-	17	16	7	17	27	48
+ allele frequency	0.57	0.57	0.52	0.62	0.56	0.60
Crude OR(95%CI)	1.00(0.67-1.50)		0.68(0.40-1.17)		0.84(0.64-1.11)	
Ethnic group adjusted OR(95%CI)					0.88(0.66-1.18)	
Age, sex and ethnic group adjusted OR(95%CI)					0.93(0.67-1.29)	
*PIGR*1093G⟶A						
GG	53	48	17	52	79	142
GA	49	46	24	42	82	137
AA	8	10	1	13	14	38
G allele frequency	0.70	0.68	0.69	0.68	0.69	0.66
Crude OR(95%CI)	1.11(0.72-1.71)		1.04(0.58-1.86)		1.10(0.83-1.47)	
Ethnic group adjusted OR(95%CI)					1.03(0.76-1.40)	
Age, sex and ethnic group adjusted OR(95%CI)					0.96(0.68-1.36)	
*PIGR*1739C⟶T						
CC	79	58	32	60	132	170
CT	26	40	9	38	37	130
TT	5	6	1	9	6	17
C allele frequency	0.84	0.75	0.87	0.74	0.86	0.74
Crude OR(95%CI)	1.70(1.03-2.82)[1]		2.35(1.11-5.07)[1]		2.14(1.49-3.09)[3]	
Ethnic group adjusted OR(95%CI)					2.26(1.51-3.25)[3]	
Age, sex and ethnic group adjusted OR(95%CI)					2.06(1.36-3.30)[2]	

Total is Thai, Chinese and Thai-Chinese cases and controls.

*CR2*IVS2-848C⟶T and *PIGR*IVS3-156G⟶T allele+ and allele- are digested and not digested with the restriction enzyme, respectively. G and A are nucleotide at *PIGR*1093, and C and T are nucleotide at *PIGR*1739, respectively.

OR (95%CI) = odd ratios and 95% confidence interval of allele CR2+, *PIGR*IVS3-156G, *PIGR*1093G, *PIGR*1739C when compared with the other alleles of the same SNPs. [1] $p<0.05$, [2] $p<0.001$, [3] $p<0.0001$

Table 2 Risk of nasopharyngeal carcinoma associated with *PIGR*1739C→T genotype according to different models of inheritance.

	THAI OR(95%CI)	CHINESE OR(95%CI)	TOTAL OR(95%CI)	ETHNIC GROUP ADJUSTED OR(95%CI)	AGE, SEX AND ETHNIC GROUP ADJUSTED OR(95%CI)
C codominance, T wild type					
CC	1.63(0.42-6.55)	4.80(0.57-105.53)	2.20(0.79-6.44)	2.59(0.85-7.48)	2.53(0.68-13.43)
CT	0.78(0.18-3.35)	2.13(0.22-50.65)	0.81(0.27-2.48)	1.08(0.34-3.53)	1.24(0.23-8.00)
TT	1.00	1.00	1.00	1.00	1.00
C dominance, T wild type					
TT	1.00	1.00	1.00	1.00	1.00
CC or CT	1.29(0.33-5.04)	3.77(0.46-81.87)	1.60(0.58-4.62)	1.96(0.66-5.70)	1.99(0.51-10.40)
C recessive, T wild type					
CT or TT	1.00	1.00	1.00	1.00	1.00
CC	2.02(1.10-3.17)[1]	2.51(1.05-6.09)[1]	2.65(1.73-4.08)[1]	2.71(1.72-4.23)[1]	2.42(1.51-4.29)[2]

CC and TT are the homozygous C and T at nucleotide 1739, respectively. CT is the heterozygous at nucleotide 1739.

Total is Thai, Chinese and Thai-Chinese cases and controls.

OR (95%CI) is odd ratios and 95% confidence interval between allele and compared allele. [1] $p<0.05$, [2] $p<0.001$. [3] $p<0.00001$

Table 3 Haplotype frequencies of *PIGR*1093-1739 , crude OR and ethnic group adjusted OR.

Haplotype	THAI		CHINESE		TOTAL		ETHNIC GROUP ADJUSTED
	case	control	case	control	case	control	
GC frequency	0.556169	0.525147	0.590097	0.447343	0.543478	0.451985	
OR(95%CI)	1.13(0.76-1.68)		1.18(1.05-3.12)		1.37(1.04-1.79)		1.36(1.02-1.80)
GT frequency	0.148376	0.171968	0.100379	0.234900	0.043478	0.175374	
OR(95%CI)	0.84(0.49-1.46)		0.35(0.14-0.80)		0.64(0.43-0.93)		0.55(0.37-0.84)
AC frequency	0.280194	0.224853	0.278951	0.286302	0.413043	0.283864	
OR(95%CI)	1.34(0.85-2.13)		0.95(0.52-1.72)		1.23(0.91-1.66)		1.28(0.93-1.76)
AT frequency	0.015260	0.078032	0.030573	0.031455	0.000000	0.088777	
OR(95%CI)	0.17(0.04-0.62)		1.10(0.22-4.85)		0.12(0.03-0.40)		0.26(0.09-0.63)
case vs case			0.085				
case vs control	<0.05		<0.05		<0.005		
control vs control			0.192				

Each haplotype frequency was calculated by the Estimating Haplotype-frequencies (EH) software program[23].

Total is Thai, Chinese and Thai-Chinese cases and controls.

OR (95%CI) = odd ratios and 95% confidence interval between the tested haplotype and the other three alleles, The OR(95%CI) calculation used the estimated number from each haplotype number of cases and controls. The number of haplotypes in the total category were calculated from the summation of estimated haplotype number from each ethnic. GC, AC, GT, and AT are 1093G-1739C, 1093A-1739C, 1093G-1739T, and 1093A-1739T haplotypes, respectively.

Case vs case = p value comparing haplotype frequency between Thai case and Chinese case.

Case vs control = p value comparing haplotype frequency between case and control in each ethnic.

Control vs control = p value comparing haplotype frequency between Thai control and Chinese control.

Table 4 Ethnic group adjusted odd ratio between each *PIGR*1093-1739 haplotype.

	GC	AC	GT	AT
GC	1.00	0.97(0.70-1.36)	1.93(1.24-2.91)	4.26(1.68-11.85)
AC		1.00	2.02(1.23-3.23)	4.31(1.76-13.18)
GT			1.00	2.18(0.83-7.34)
AT				1.00

Number of cases and controls of each haplotype in the first column were calculated for ethnic group adjusted odd ratios by comparing with haplotype listed in the upper row.

Numbers before () are odd ratios and within ( ) are 95% confidence interval.

GC, AC, GT, and AT are 1093G-1739C, 1093A-1739C, 1093G-1739T, and 1093A-1739T haplotypes, respectively.

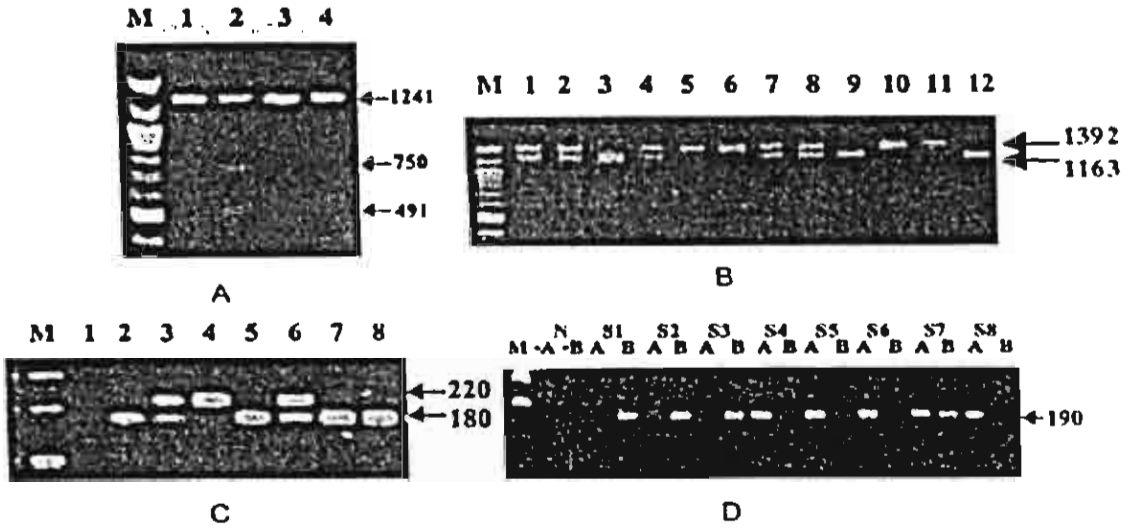

Figure 1 SNPs analysis of *CR2* and *PIGR*. (A) The *CR2*IVS2-848C→T was distinguishable by *TaqI* restriction. Digestion of the 1241 bp amplicon yielded two DNA fragments, 750 and 491 bp. Lane 1, 3 and 4 were homozygous -/- and lane 2 was heterozygous +/-. (B-D) *PIGR* polymorphisms was investigated by PCR-RFLP and ARMS. (B) RFLP analysis of 1392 bp *PIGR*IVS3-156G→T PCR products with *PvuII* digestion yielded 1163 and 229 bp DNA fragments. Lane 1,2,4,7 and 8 were heterozygous +/-, samples 3,9 and 12 were homozygous + +, and samples 5,6,10 and 11 were homozygous -/-. (C) RFLP of *PIGR*1739C of the 220 bp PCR product was analyzed by *HgaI* digestion and yielded two fragments of 180 and 40 bp, respectively, whereas 1739T remained as 220 bp DNA product. Heterozygous CT yielded 220,180 and 40 bp fragments. Negative control in lane1; homozygous CC in lanes 2,5,7 and 8; heterozygous CT in lanes 3 and 6, and homozygous TT in lane 4. (D) *PIGR*1093G→A was detected by ARMS. Negative control in lanes 1 and 2 for primer sets A and B. Samples S1, 2, 3, were homozygous 1093G. Samples S4, 5, 6 were homozygous 1093A, and S7 was heterozygous.

Chapter 3
Expression of Recombination Activating Genes (*RAG1* and *RAG2*) in Epstein-Barr Virus associated Nasopharyngeal Cancer[1].

SUMMARY

We investigated if V(D)J recombination might serve as a mechanism of tumor development in Epstein-Barr virus (EBV)-associated nasopharyngeal cancer (NPC) by examining 6 primary biopsy samples as well as 16 NPC cell cultures derived thereof for expression of *RAG1&2*. Lymphocyte and non-transformed cell infiltration was excluded by applying a particular culture technique and confirmed by flow-cytometry, RT-PCR amplifying CD3 and CD20, and microsatellite analysis. All samples harbor EBV and of those 5 fresh biopsy and 15 NPC cell culture samples expressed *RAG 1* and/or *2* hinting at the site-specific recombination as a potential mechanism of carcinogenesis in EBV-associated NPC.

INTRODUCTION

The purpose of the present study has been to investigate if V(D)J recombination, a site specific recombination serving to assemble antigen receptor genes in T and B lymphocytes, represents one potential mechanism responsible for the development of Epstein-Barr virus (EBV) associated nasopharyngeal cancer (NPC). NPC carcinogenesis has been shown to require latent EBV infection and subsequent genomic alterations of the nasopharyngeal epithelium (1-3). Along these lines, cell growth might be promoted by some EBV-encoded proteins as for example the latent membrane protein 1 (LMP1) which acts as a signal transducer thus resulting in the activation of nuclear factor-κB responsive elements (4). Nevertheless, whether EBV can directly effect mutation has to date remained unknown. During the life-long viral carrier state, EBV infection has been traced not only to epithelial but also lymphoid and myocytic origins (5). The presence of chromosome translocations involving both antigen receptor genes and oncogenes, along with the expression of recombination activating genes 1 and 2 (*RAG1&2*) in EBV associated lymphoma, invites the compelling hypothesis that the mutation may have arisen from virally induced and hence, probably faulty V(D)J recombination (6-8). In nearly all cases of endemic EBV positive Burkitt's lymphoma translocations involving *C-MYC* have been implicated mostly affecting the *IgH* locus in t(8;14) (9). Under normal physiologic conditions, *RAG1&2* are simultaneously expressed during a limited phase of lymphocyte precursor development and this concerted expression has been shown sufficient for V(D)J recombination (10). Interestingly, *RAG1&2* RNAs have been detected in some endemic EBV positive as well as sporadic EBV negative Burkitt's lymphoma lines experimentally infected with EBV in vitro (7,8). Thus, EBV infection may reactivate *RAG1&2* expression and result in genetic rearrangement in B lymphocyte tumors. We have demonstrated in a recent study frequent allelic loss and homozygous deletion at the T cell receptor delta locus occurring in NPC suggesting a specific genomic rearrangement mechanism (11). The present study has been designed to establish if and at what prevalence the *RAG1&2* genes are expressed in NPC and moreover, if erroneous V(D)J recombination might serve as a mechanism responsible for EBV-associated NPC development.

MATERIALS AND METHODS

Tissue Samples & Culture

Primary NPC biopsy samples were collected before the onset of treatment at Chulalongkorn University Hospital. The samples were divided into two pieces, with one sent for routine histological examination, and the second providing the source for further DNA and RNA analysis, and/or tissue culture.

NPC was finely chopped, suspended in 0.25% collagenase, 0.5% BSA in PBS (60 x 15 mm^2 tissue culture plate; Nalge Nunc International, Denmark) and incubated at 37° C, 5% CO_2 for 4-5 hours. After centrifugation, the collagenase was discarded and the pellet subjected to two washing steps with 5 ml DMEM (Dulbecco's Modified Eagle Medium, Gibco BRL, Gaithersburg, MD, USA) each. Subsequently, the pellet was resuspended in 7 ml DMEM devoid both growth factors and fetal calf serum, with the appropriate antibiotics added, and incubated at 37° C, 5% CO_2 until the resulting cell layer had spread sufficiently to be passaged. After four to six passages, cells were removed from the tissue culture plate by directly scraping the cell layer off the plate using a cell scraper. Before DNA and RNA extraction, cells were loaded onto a 50% (vol/vol) Ficoll Hypaque gradient and spun at 1500 rpm for 20 min. The supernatant layer was discarded and the pellet proceeded as described for NPC cells.

DNA and RNA preparation

Total cellular RNA and total genomic DNA were extracted from nasopharyngeal cancer biopsies or cells using a commercially available kit (Qiagen RNA/DNA kit,Qiagen,Valencia, California,USA). The EBV cell line B958, EBV-transformed lymphocytes (American type culture collection), was used as a positive EBV DNA and RNA control. Bone marrow and WBC served as positive controls for the *RAG1* and *RAG2* genes, as well as CD3 and CD20 expression, respectively. Distilled water was used as negative control.

Flow Cytometry

Cell suspensions were stained with the following antibodies: FITC-anti-CD14, PE-anti-CD45, FITC-anti-CD3 and PE-anti-CD19 Simultest™ (Becton-Dickinson). Data were analyzed by flow cytometry.

Reverse Transcription and PCR amplification

PCR for EBV DNA detection and microsatellite analysis of chromosomes 3, 9 and 14 were performed essentially as previously described (2). Applying RT-PCR, several expression studies of the *RAG1&2*[8], *LMP1*[12], *LMP2A*[13], *CD3*[14] and *CD20*[14] genes have been performed. *β actin* expression served as an RNA quality control. All experiments were performed applying previously described primer sequences and PCR reactions with minor modifications as in ref 8, 12-14. Each reverse transcription reaction was carried out in a 10- µl reaction volume containing 690 ng total RNA, a 60-ng oligo-(dT) primer, 40 mM dNTP, 75 mM KCl, 50 mM Tris-HCl (pH 8.3), 3 mM $MgCl_2$ and 10 units of Moloney murine leukemia reverse trancriptase. After annealing the primers to the RNA at 65 °C for 5 minutes reverse transcription was performed at 37°C for 15 minutes. Subsequently, 2-5µl of cDNA were used as a template for further amplification. PCR was performed in a total volume of 50 µl comprising 2.5 units of *Pfu Turbo* DNA polymerase, 200 µM dNTP, 0.15-0.2 µmol of each primer pair, and 5 µl of 10x ultra HF PCR buffer (MMLV-RT, Strategene, La Jolla, California, USA). The PCR conditions have been optimized as described in ref 8, 12-14. Upon electrophoresis in a 2% agarose gel stained with ethidium bromide on preparation the amplified products were visualized under UV light as distinct bands. RT-PCR for *RAG1&2* was repeated for some samples using rTth DNA polymerase (Perkin Elmer, Branchburg, NJ, USA).

To confirm the presence of *RAG1*, *RAG2* and *LMP2A* PCR products, the gels were subsequently transferred to a Hybond N+ membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK), applying a routine Southern blot protocol. The membrane was hybridized to the (γ^{32}) P-dATP end-labeled internal oligonucleotide sequences, *RAG1*[15] (GCTGAGAAGGTACTTCTGCCAGGC), *RAG2*[16] (GGGGATTCCACTCCCTTTGAAGAC) or previously described *LMP2A*[13].

RESULTS AND DISCUSSION

The preliminary results obtained from 6 fresh biopsy samples confirmed as NPC demonstrated unambiguous expression of *RAG 1&2*. All samples were positive for EBV DNA and of those 5 showed simultaneous expression of *RAG 1&2*. Identical results were observed upon using either MMLV reverse transcriptase or rTth DNA polymerase for RT-PCR. Since cells derived from fresh biopsies are heterogeneous by nature, we established a tissue culture to exclude tumor infiltrating lymphocytes and other non-transformed epithelial as well as connective tissue cells. NPC was harvested and cultured in media devoid of fetal calf serum or growth factors. After at least four passages (approximately 4-6 weeks) the cells were harvested and analyzed for the presence of B or T cells by flow cytometry, using CD3 and CD19 antibodies, respectively. 18 out of 22 showed no lymphocytes altogether and the remainder demonstrated a minimal lymphocyte contamination amounting to a mere 1% of the total cell count. In addition, those NPC samples without lymphocyte were further purified by centrifugation on a Ficoll Hypaque gradient, and the RNA obtained was re-evaluated in a subsequent RT-PCR using primers specific for the B- and T- cell markers CD3 and CD20, respectively (fig 1). 16 NPC cultured samples devoid of contaminating lymphocytes were further analyzed in order to confirm the absence of other normal cells by showing complete loss of heterozygosity, absence of contaminating normal allele, of at least one microsatellite from chromosomes 3, 9 or 14 (fig 1). All 16 samples were proven to harbor the EBV genome. The RNA obtained from cancer cells was then further analyzed for the presence of latent EBV infection and all RNA samples were found positive. Whereas 13 showed both *LMP1* and *LMP2A* RNA, the other 3 samples expressed *LMP2A* only. *RAG1* or *2* expression was detectable in 15 cases, whereas 9 samples showed simultaneous expression (fig 2, table 1). To exclude a possibility of false positive from genomic DNA contamination, PCR using *RAG1* and *2* as primers and NPC cells' RNA as template were performed and none showed detectable PCR product (data not shown). No statistically significant correlation between *RAG1&2* expression and various tumor parameters such as tumor size, local involvement, lymph node metastasis, and histology type has been observed (table 1).

The discovery of *RAG1&2* expression in NPC is surprising since apart from the discovery of *RAG1* transcripts in the murine central nervous system (17), this study is so far the only other report of naturally occurring *RAG1&2* transcripts in tissue not particularly primed for lymphocyte development. More importantly, their active expression suggests V(D)J recombination as a potential mechanism responsible for NPC development. Although several genes are involved in this process, a previous study has shown co-transfection of *RAG1&2* expression plasmids to induce the recombination in fibroblasts (10). Thus, *RAG1&2* are the only lymphoid-specific genes apparently required for V(D)J rearrangement in non-lymphoid cells (10). Taken together, combined expression of *RAG1&2* in NPC should be adequate, as well. In lymphocytes, because of cell type specific control especially chromosome packaging and methylation, the site-specific recombination only exists in antigen receptor genes (18). Since chromatin organization in NPC cells is different from that in lymphocytes, inappropriate diversion of V(D)J rearrangement to a transpositional pathway in NPC may be linked to some oncogenes or tumor suppressor genes (19). Consequently, this might constitute an important mechanism for DNA rearrangement associated with NPC development.

The mechanism EBV employs to activate *RAG 1&2* expression is unknown. Previous experiments have shown that in addition to co-transfection of *RAG 1&2* expression plasmids, there have been at least two other methods to activate those genes in vitro. For one, EBV

infection induces sustained expression of *RAG 1&2* in B cells. Epstein-Barr virus nuclear antigen 1 (*EBNA1*) and *LMP1* may be EBV gene products crucial in this process (8). The expression of *EBNA1* in transfection assays has been sufficient to transiently induce both *RAG 1&2* in mature lymphocytes (8). In EBV positive Burkitt's lymphoma lines, transcription of *LMP1* has been correlated with down-regulation of *RAG 1&2* (7). The second approach has succeeded in stimulating V(D)J recombination and steady expression of *RAG 1&2* in nonlymphoid cells, 3T3 fibroblasts, by a second round of DNA transfection (20). Thus, it will be interesting to further explore whether specific EBV genes or the presence of foreign DNA in the form of replicating virus are responsible for the induction of *RAG 1&2* transcripts in NPC.

ACKNOWLEDGEMENTS

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Table 1 RAG 1&2 expression, EBV status, tumor staging and WHO type of 16 NPCs

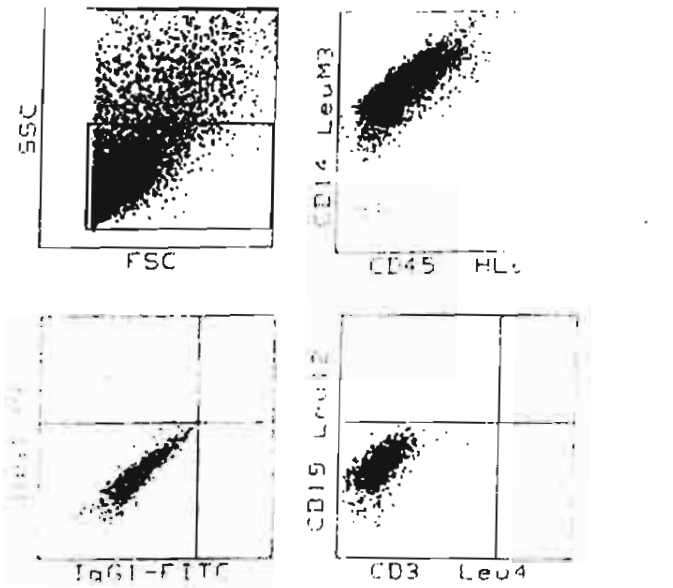
Patient code	Age	Sex	Tumor Stage	WHO	PCR	RT-PCR			
					EBNA1	*RAG1*	*RAG2*	*LMP1*	*LMP2A*
304	32	M	III	II	+	-	-	+	+
381	49	F	III	III	+	-	+	-	+
384	42	F	III	II	+	+	+	+	+
397	50	M	IV	III	+	+	+	-	+
405	48	M	III	III	+	+	-	+	+
424	57	M	IV	II	+	+	+	-	+
433	35	F	III	II	+	+	+	+	+
436	45	M	I	III	+	+	+	+	+
437	37	M	III	III	+	+	+	+	+
438	52	M	IV	II	+	+	+	+	+
440	48	F	IV	III	+	+	+	+	+
443	58	M	IV	II	+	+	+	+	+
446	43	F	II	II	+	-	+	+	+
447	48	F	II	II	+	-	+	+	+
453	30	F	II	III	+	-	+	+	+
454	36	M	IV	II	+	-	+	+	+
Total					16	10	14	13	16

M, male; F, female; +, positive; -, negative

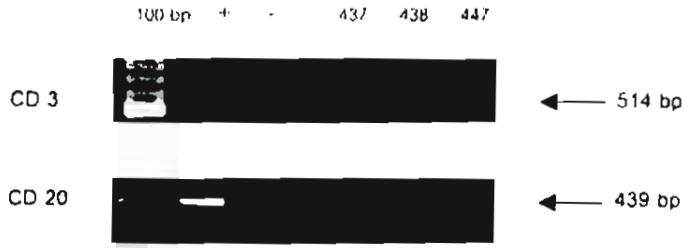
FIGURE LEGENDS

Fig 1. Excluding lymphocytes and other infiltrating normal cells from NPC by devoiding serum and growth factors from cell culture. A, Flow cytometry using antibody CD3 and CD19. The example is NPC cell 437. B, RT-PCR products of *CD3* and *CD20*. From left to right: 100 bp ladder marker, + and -. Positive control (bone marrow RNA) and negative control (distilled water), respectively, numbers indicate corresponding RT-PCR products of NPC cells. No positive RT-PCR was observed. C, Microsatellite analysis in selected NPC cell culture samples showing no normal allele contamination. Representing NPC cell culture samples (C) and corresponding normal leucocytes (N) are shown with microsatellite markers indicated at the buttom. Absent of normal allele was demonstrated in all cases, except marker D3S1038 showed no LOH from case 437.

Fig 2. Expression of *RAG 1&2* in EBV associated NPC cell cultures. From left to right: 100 bp ladder marker, + and -, positive control (A. B958 RNA, B. bone marrow RNA) and negative control (distilled water), respectively, numbers indicate corresponding RT-PCR products of NPC cells. A, RT-PCR generating 280 and 181 bp DNA fragments of *LMP2A* and *LMP1*. The bottom is hybridization using *LMP2A* PCR products as template and its internal primer as probe. B, RT-PCR generating 465, 371 and 202 bp DNA fragments of *RAG1*, *RAG2* and *β-actin*, respectively. The bottom is hybridization using *RAG2* PCR products as template and its internal primer as probe.


SSC
FSC
CD14 LeuM3
CD45 HL
IgG1-FITC
CD19 Leu12
CD3 Leu4

B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437
438
447
CD 3
514 bp
CD 20
439 bp

C

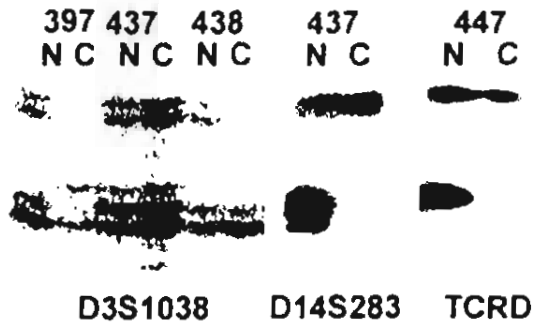
397 437 438 437 447
N C N C N C N C N C
D3S1038
D14S283
TCRD

Fig 1

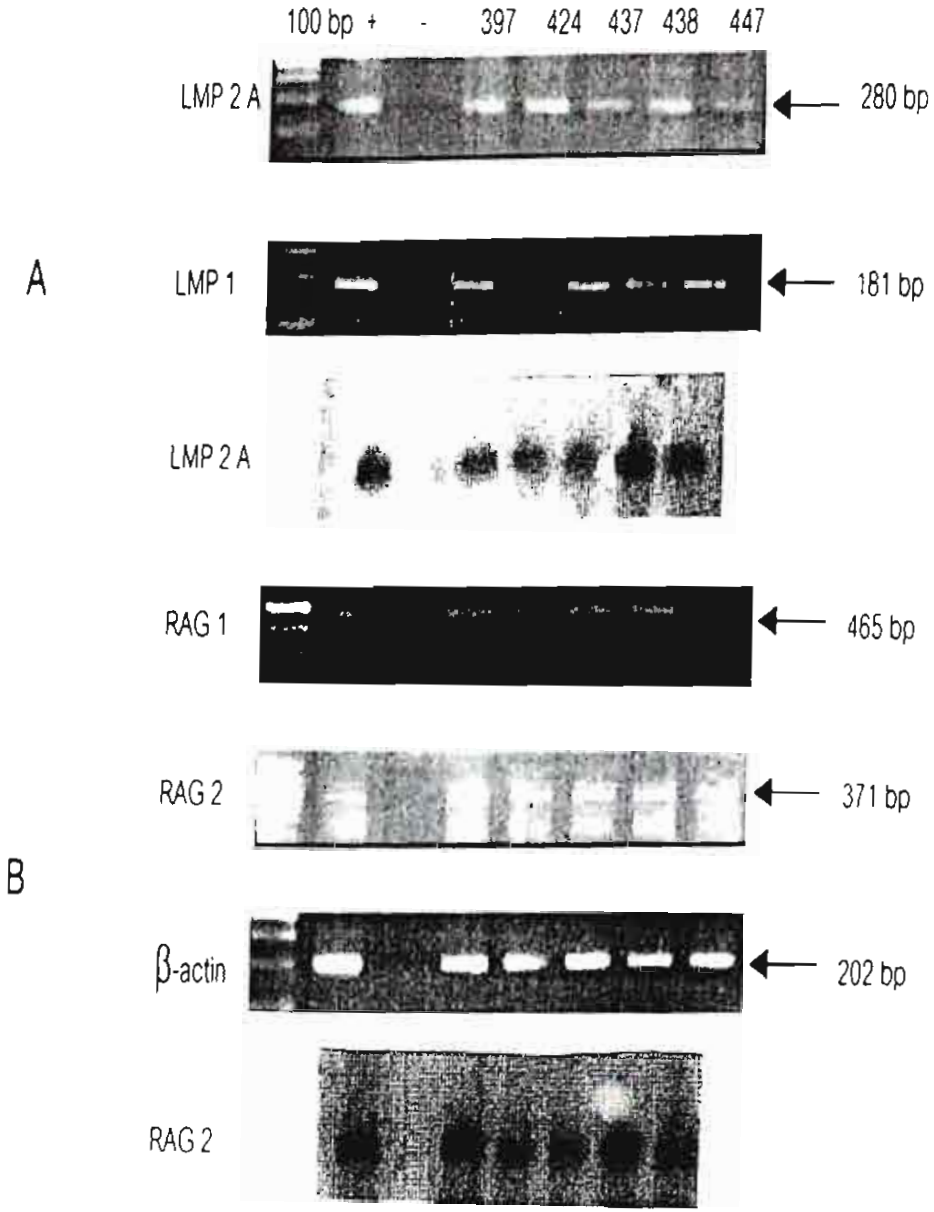
100 bp
+
-
397
424
437
438
447
LMP 2 A
280 bp
A
LMP 1
181 bp
LMP 2 A
RAG 1
465 bp
RAG 2
371 bp
B
β-actin
202 bp
RAG 2

Fig 2

Output

1. Kerekhanjanarong, V., Sitawarin, S., Sakdikul, S., Saengpanich, S., Chindavijak, S., Supiyaphun, P., Voravud, N., and **Mutirangura, A.** Telomerase assay and nested polymerase chain reaction from nasopharyngeal swabs for early noninvasive detection of nasopharyngeal carcinoma, Otolaryngol Head Neck Surg. *123:* 624-9., 2000.
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ภาคผนวก

Reprint

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Distribution of HLA-B alleles in nasopharyngeal carcinoma patients and normal controls in Thailand

Key words:
HLA-B, nasopharyngeal carcinoma

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Abstract: HLA-B frequencies in 54 unrelated nasopharyngeal carcinoma (NPC) patients and 49 healthy random controls in Thailand were investigated by direct DNA sequencing. Similar to previous reports in Chinese NPC patients, HLA-B*4601 was observed at a greater frequency in patients (21/54 (40‰)) compared to controls (7/49 (14‰)). An increase in HLA-B*51012 was also demonstrated. B*51012 was present in 6/54 (11‰) NPC patients but was not observed (0‰) in controls. B*44032 was associated with a decreased risk. Five out of 54 (9‰) NPC patients had B*44032 compared to 14/49 (29‰) in the control group.

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Nasopharyngeal carcinoma (NPC) is a tumor affecting the epithelial lining in the head and neck region. It is one of the most common cancers in Asia, with the highest incidence rate in South China and intermediate incidence rate in South-east Asia (1). This tumor is quite rare in the Western population. Multiple factors have been reported to be involved in the pathogenesis of this disease including EBV infection, environmental carcinogens (e.g., cigarette smoke, certain foods), and genetic factors (2). HLA is one of the genetic factors reported as having a significant association with NPC (1). Certain HLA antigens or haplotypes, mostly identified using serological techniques, have been associated with either increased or decreased risk in various studies. For example, HLA-A2/B46 haplotypes were consistently reported to be positively associated with NPC in Chinese populations living in different countries (e.g., Singapore, China, Hong Kong, Malaysia, California, U.S.A) (1, 3-6). HLA-B58 is another NPC-associated antigen observed at a higher frequency in Chinese and Malay patients (7) while HLA-A11 was observed at a lower frequency among Chinese NPC patients (6, 7). Studies of HLA in other low incident populations gave more variable results (8-12).

The underlying mechanisms of these associations have not been elucidated. One explanation is the existence of an NPC susceptible gene closely linked to the HLA region, which was suggested from an

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HLA-B allele frequencies in patients with NPC and healthy controls from Thailand

HLA-B Allele	NPC Patients Count (2N = 108)	%	Controls Count (2N = 98)	%
B*0705/6	1	1.8	2	4.0
B*1301	4	7.4	4	8.1
B*1302	1	1.8	1	2.0
B*1402	0	0.0	1	2.0
B*1501	3	5.5	1	2.0
B*1502	6	11.1	8	16.3
B*1504	1	1.8	1	2.0
B*1511	1	1.8	1	2.0
B*1517	1	1.8	3	6.0
B*1518	0	0.0	1	2.0
B*1521	0	0.0	1	2.0
B*1525	1	1.8	2	4.0
B*1532	0	0.0	1	2.0
B*1801	1	1.8		0.0
B*1802	4	7.4	1	2.0
B*2704	3	5.5	3	6.1
B*2706		0.0	1	2.0
B*3501	6	11.1	2	4.0
B*3503	1	1.8	1	2.0
B*3505	1	1.8	1	2.0
B*3701	1	1.8	1	2.0
B*38021	6	11.1	2	4.0
B*3804	0	0.0	1	2.0
B*3901	1	1.8		0.0
B*3909	1	1.8	3	6.1
B*4001	13	24.1	7	14.2
B*4002	3	5.5	2	4.0
B*4006	0	0.0	2	4.0
B*44032	6	11.1	14	28.5
B*4601	25	46.3	8	16.3
B*4801	1	1.8	0	0.0
B*51011	5	9.2	3	6.1
B*51012	7	12.9	0	0.0
B*52011	3	5.5	5	10.0
B*5502	1	1.8	2	4.0
B*5604	1	1.8	0	0.0
B*5801	7	12.9	6	12.2
B*5401	1	1.8	3	6.1
B*5701	0	0.0	3	6.1
B*7021	0	0.0	1	2.0

N = the total number of individuals studied in the patient or control group

Table 1

HLA-B alleles that demonstrated significant associations with NPC

HLA-B*	NPC (N = 54) n	%	Controls (N = 49) n	%	* association	P-value
44032[a]	5	9	14	29	-	0.01
4601[b]	21	39	7	14	+	0.005
51012[c]	6	11	0	0	+	0.02

N = the total number of individuals studied in either patient or control group

n = the number of individuals positive for each allele

[a] χ^2 = 6.4, P = 0.01, OR = 0.26, 95%CI = 0.07–0.85

[b] χ^2 = 7.9, P = 0.005, OR = 3.8, 95%CI = 1.34–11.82

[c] Fisher's exact, P = 0.02

Table 2

HLA study of 30 sibling pairs (13). A more recent study, using microsatellite markers, predicted that this putative NPC susceptible gene, not yet identified, is close to the D6S1624 marker (14). However, the ability of certain HLA molecules to present EBV antigens might also contribute to the observed HLA associations. Moreover, with the increasing interest in epitope-based immunotherapy in EBV-related tumors including NPC, the distribution of HLA alleles in NPC populations will provide useful information for epitope selection i.e., by including the epitopes restricted to common HLA allelic products in the target population. In this study, we investigated the distribution of HLA-B alleles in Thai NPC patients compared to normal controls to identify the HLA-B alleles associated with NPC in Thailand.

The study population included 54 unrelated Thai patients with histologically confirmed NPC diagnosed at King Chulalongkorn Memorial Hospital in Bangkok. There were 34 men and 20 women, with a median age of 48 years (range 16–81 years). All of these patients were positive for the EBNA1 gene in tumor cells as detected by PCR, as reported previously (15, 16). Forty-nine healthy unrelated Thai individuals served as ethnically and geographically matched controls. All patients and controls were typed for the HLA-B locus by direct sequencing of the PCR products using primers BIN1-TA, BIN1-CG and BIN3, which amplified exons 2 and 3 and intron 2 as previously described (17, 18). The allele frequencies were determined by direct counting based on the assumption that individuals carrying a single allele were homozygous. The significance of differences between the two groups was analyzed by the chi-square test. Fisher's exact tests were applied if the expected frequency was less than 5.

The distribution of HLA-B alleles between the two groups is shown in Table 1. A total of 34 HLA-B alleles was observed in the Thai control group, including one new allele (B*3804) (19). The five most common alleles in Thai controls were B*44032 (28.5%), B*4601 (16.3%), B*1502 (16.3%), B*4001 (14.2%) and B*5801 (12.2%). Twenty-nine alleles were detected in NPC patients with the same five common alleles

observed at high frequencies (11.1–46.3%). In addition, the allele frequencies of B*38021 (11.1%) and B*51012 (12.9%) were high in the patient group compared to controls. This information is useful as vaccine development including T cell epitopes restricted to these common alleles will benefit the majority of affected population. When the frequency of HLA-B alleles in NPC patients and normal individuals was compared, significant associations between NPC and three HLA-B alleles were observed, as summarized in Table 2. Specifically, a lower incidence of B*44032 was noticed in the patient group (9 vs 29%, $P < 0.01$) which was similar to a previous report in East Africa, in which B44, characterized by serological typing, was reported as a resistance antigen in NPC (10). Interestingly, some CTL epitopes from EBV antigens have been identified as restricted by B44 (20). Studies of these epitopes that contribute to the protective effect of the protective allele might be useful in vaccine development. The frequencies of another two alleles, B*4601 and B*51012, were significantly increased in NPC patients (39 and 11% vs 14 and 0%, $P < 0.005$ and $P < 0.02$, respectively). As mentioned above, the association with B46 has been consistently observed in Chinese populations (1, 3–6). One previous report of 20 Thai NPC patients also demonstrated a positive association of NPC with B46 ($P < 0.05$) (6). Our study has confirmed that genetic susceptibility of NPC to in the Thai population is likely to be similar to the Chinese population. The restricted-antigen binding properties of the B*4601 molecule (21) might be one explanation for its link to susceptibility to NPC. Interestingly, no EBV epitopes restricted by B46 have been reported so far and further studies are required to prove this hypothesis. No association with B58 was demonstrated in this study; however, in a previous study, B58 association was only seen in newly diagnosed patients and its frequency is very low in long-term survivors (1), which were not identified in this study.

In conclusion, this study reported a protective B*44032 allele and two susceptible alleles, B*4601 and B*51012, for NPC in Thai population.

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Telomerase assay and nested polymerase chain reaction from nasopharyngeal swabs for early noninvasive detection of nasopharyngeal carcinoma

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The main purpose of this study was to analyze sensitivity and specificity of combining nested polymerase chain reaction for detection of Epstein-Barr virus (EBV) genome and telomerase assay for identifying nasopharyngeal carcinoma (NPC). Eighty patients with NPC and 27 healthy control subjects were included in this study; 97.5% and 94.9% of NPC patients were positive for EBV genome and telomerase activity, respectively. When nasopharyngeal swabs were tested, 95.7% presented the EBV genome and 85.5% were positive for telomerase expression. The sensitivity for counting either positive result of these two techniques was 100%. Among the 27 control subjects, only 6 and 5 cases were positive for EBV DNA and telomerase activity, respectively. This indicated a specificity of 92.6% when both positive results were included. At present, early diagnosis of NPC requires multiple biopsy specimens, especially to identify subclinical cases. Because this study showed a very high sensitivity for detecting NPC from swabs when combining the telomerase assay and nested polymerase chain reaction technique, this noninvasive technique may be a good candidate for screening of subclinical NPC, especially before multiple biopsy specimens are obtained. (Otolaryngol Head Neck Surg 2000;123:624-9.)

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Nasopharyngeal carcinoma (NPC) is a common cancer among various populations. High-risk groups include those in South Eastern China, those in Taiwan, and native Alaskans. An intermediate risk has been reported for several countries such as Thailand, Vietnam, the Philippines, and certain North African regions.[1-3] As with all cancers, the prognosis is very good if the cancer is diagnosed and treated at an early stage. Unfortunately, because of minimal discomfort and factual lack of symptoms in the early stages, most patients visit the hospital when the cancer has already advanced. For example, the most common symptom of NPC patients in Thailand is neck node enlargement.[4] Proper cancer screening or better methods for early diagnosis would heighten the chances to detect the cancer at an early stage and consequently the treatment outcome of NPC patients.

NPC can develop as a consequence of chronic Epstein-Barr virus (EBV) infection with subsequent genetic alterations of the epithelial cells.[5-7] Screening methods currently available are based on the presence of EBV antibody. EBV viral capsid antigen specific IgA antibody response has been the one most commonly used. However, specificity and sensitivity of this technique vary depending on the definition and determination of the cutoff point.[8-11] To improve early detection methods, several groups studied the feasibility of detecting EBV DNA from exfoliated NPC cells by washing, swab, or brush biopsy. Different results have been reported, especially with respect to the detection rate of EBV DNA from non-NPC samples as a consequence of the chronic carrier state.[12-15]

Telomerase is a ribonucleoprotein complex synthesizing telomeres and thus preventing their being shortened with every DNA replication.[16] The activation of this enzyme is thought to be responsible for preventing cellular senescence and developing cancer.[17,18] Telomerase activity has been demonstrated in most cancer cells, including NPC, associated with several advanced premalignant lesions, especially stratified squamous cell epithelium.[18-21] Hence, telomerase can serve as an important marker for screening and diagnosing a wide variety of carcinomas. Nevertheless, telomerase activity

could be detected in some benign conditions, such as inflammation of the oral epithelium.[22] Therefore, as with applying the polymerase chain reaction (PCR) for the detection of EBV DNA alone, the telomerase assay on its own would likewise yield some false-positive results. The objective of this study was to determine sensitivity and specificity for diagnosis of NPC on combination of screening for EBV DNA and telomerase activity from nasopharyngeal swab.

METHODS AND MATERIAL

The patient population included 80 NPC and 27 non-NPC patients from the outpatient department of King Chulalongkorn Memorial Hospital and the National Cancer Institute, Bangkok, Thailand. All patients received counseling and signed informed consent forms. The patients were anesthetized by application of 2% Xylocaine spray in the nasal cavity and nasopharynx. Under endoscopy, the nasopharynx was swabbed with cotton wool at multiple sites, and the samples were soaked in normal saline solution and sent for telomerase and EBV genome analysis. In cases where NPC was suspected, biopsy was performed at the respective lesions for histology, telomerase, and EBV genome analyses. These procedures have been reviewed and approved by the hospital review board.

Telomeric Repeat Amplification Protocol Assay

Telomeric repeat amplification protocol (TRAP) was performed as previously described with some modification.[17,23] In brief, each sample was first centrifuged and washed in 500 µL ice-cold phosphate-buffered saline solution and then homogenized in 20 to 200 µL of ice-cold 3[3-cholamino-propyl diethyl-ammonio]-1-propane sulfonate (CHAPS) lysis buffer, according to sample size, with a manual homogenizer. After a 30-minute incubation on ice, the lysate was centrifuged at 14,000*g* for 30 minutes at 4 C. The supernatant was divided into aliquots, flash-frozen in liquid nitrogen, and stored at –80°C until further analysis. An aliquot of the extract containing 6 µg protein was used for each TRAP assay. EBV-transformed human lymphocytes (American Type Culture Collection Cell Line, B958) were used as positive controls.

An aliquot of 1 µg TS substrate primer was end-labeled in a 10-µL reaction mixture with 10 µCi [γ-^{32}P]-dATP (3000 Ci/mmol). The PCR-based assay was carried out in a 25-µL reaction mixture containing 6 µg protein from the lysate, 1× TRAP buffer,[17] 50 mmol/L dNTPs, 0.1 µg labeled TS primer, 2 units Taq polymerase, and DEPC H_2O in a 0.5-mL tube containing 0.1 µg ACX sealed at the bottom by a wax barrier.

After a 10-minute incubation at 23°C to allow telomerase-mediated extension of the TS primer, the reaction mixture was subjected to 31 PCR cycles at 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute. Aliquots (5 µL) of the PCR products were analyzed on an 8% nondenaturing polyacrylamide gel. The gel was subsequently exposed to a phosphorus screen, and the bands were visualized on a PhosphorImager with Image Quant software (Molecular Dynamics, Sunnyvale, CA). Duplicate assays were performed on all positive samples with RNAase pretreatment at a final concentration of 0.05 mg/mL for 10 minutes at room temperature.

The samples exhibiting negative results were subjected to 2-step TRAP assay (Shay, personal communication, 1996). The first part consisted of TS primer extension in a 25-µl reaction mixture containing 1× TRAP buffer, 50 mmol/l dNTPs, 0.1 µg TS, and DEPC H_2O. The reaction mixture was incubated in the thermocycler at 23°C for 15 minutes. The product was then subjected to standard phenol-chloroform DNA extraction and ethanol precipitation. The precipitate was dissolved in DEPC H_2O and amplified in the second round. The second-round reaction mixture was identical to that described for 1-step TRAP, with omission of the 15-minute incubation at 23 C. The amplification cycle was the same as in the original protocol.

All negative TRAP results were tested for the possible presence of a telomerase inhibitor. Lysates from 10^3 cells of the B958 lymphocyte cell line were added, and the mixed samples were subjected to 1- and 2-step TRAP assay.

EBV Detection by Nested PCR

Pellets derived from the previous extraction with CHAPS lysis buffer were subjected to standard DNA extraction. The DNA was used for subsequent PCR amplification of EBV DNA with nested primers. For the detection and typing of EBV DNA in tumor tissues and swabs, nested PCR protocols were used, modifying those previously reported for amplification of the EBV nuclear antigen 2 (EBNA-2).[5,24] DNA extracted from the cell line B958, EBV-transformed human lymphocytes (American Type Culture Collection), was used as the positive control, and double-distilled water was used as the negative control.

The first PCR amplified the EBNA-2, generating a DNA fragment of 168 base pairs (bp) for EBV type A and 184 bp for EBV type B. With nested primers, the PCR product comprised 99 bp for EBV type A and 115 bp for EBV type B. The nucleotide sequences for these nested primers are 5'-GCGG-GTGGAGGGAAAGG-3' (E3-44mer) and 5'-GTCAGCCA-AGGGACGCG-3' (E5-25mer). Primers GH20 and PC04 were used to amplify β-globin to determine the presence of amplifiable human DNA in all samples tested for EBV DNA, generating a DNA fragment of 260 bp.[25] The nucleotide sequences of the primers used for the first EBNA-2 and β-globin PCRs were identical to those previously reported.[24,25]

The primary PCR reactions were performed in a total volume of 20 µL with 50 ng of the corresponding tumor DNA or swab in 200 µmol/L dNTP each, 1.5 mmol/L magnesium chloride, 50 mmol/L potassium chloride, 10 mmol/L Tris-HCl (pH 9.0), 0.1% Triton X-100, 0.5 units of Thermus aquaticus

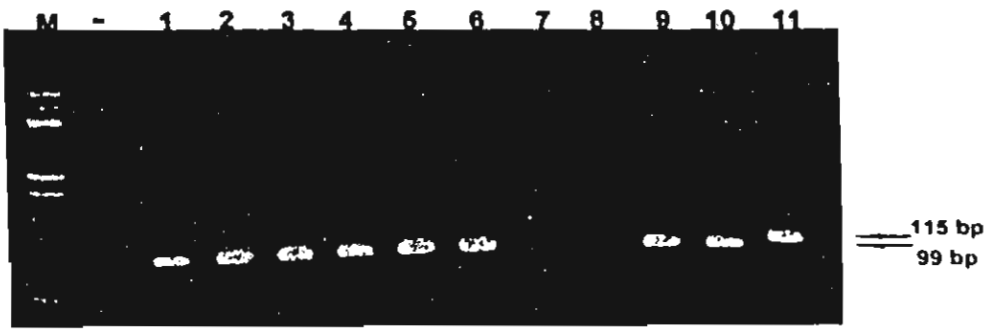

Fig 1. Examples of nested PCR products from NPC swab in agarose gel electrophoresis. The 99- or 115-bp fragments indicated the presence of EBV DNA; 100-bp DNA ladder *(lane M)* was used for DNA size standard. Distilled water *(-)* was used as a negative control. Numbers *1* through *11* are PCR products from NPC swabs.

Table 1. EBV and telomerase activity in NPC and noncancerous nasopharyngeal tissues

	Nested PCR for EBV DNA		Telomerase activity	
	Swab (positive/total)	Biopsy (positive/total)	Swab (positive/total)	Biopsy (positive/total)
NPC	66/69 (95.7%)	78/80 (97.5%)	59/69 (85.8%)	75/79 (95%)
Nonkeratinizing carcinoma, differentiated	48/50	57/59	42/50	55/59
Nonkeratinizing carcinoma, undifferentiated	18/19	21/21	17/19	20/20
Skull or nerve involvement	17/17	18/19	13/18	16/19
No skull or nerve involvement	49/52	59/60	46/51	59/60
LN metastasis	52/54	61/63	44/51	59/63
No LN metastasis	14/15	16/16	15/18	16/16
Distance metastasis	4/4	6/6	3/4	6/6
No distant metastasis	62/65	73/73	56/65	69/73
Noncancerous tissues	6/27		8/27	
With inflammation	5/14		6/19	
Without inflammation	1/6		2/8	

LN, Lymph node

DNA polymerase, and 0.2 μmol/L of each primer. The PCR amplification was performed as follows: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds; and annealing at 57°C for 30 seconds, with an extension at 72°C for 1 minute and a final extension at 72°C for 7 minutes. One microliter of each PCR product was used as a template for nested PCR. The nested PCR reactions were performed in a manner identical to that applied in primary PCR, except for using different sets of primers and adjusting the annealing temperature to 50°C. The PCR products were analyzed after electrophoresis on a 2% agarose gel stained with ethidium bromide on preparation.

Statistical Analysis

Data regarding histology, tumor staging, EBV detection, and telomerase activity were collected in a double-blind fashion until analysis. The χ^2 test was used to compared the results gained from serum analysis with clinical and tumor parameters

RESULTS

Eighty NPC and 27 non-NPC patients were enrolled in this study. Both groups had swabs taken for TRAP assay and EBV genome analysis. Telomerase activity and EBV genome from tissue biopsy specimens were studied only in cases of confirmed NPC. Detailed histopathology, stage, and clinical manifestations are shown in Table 1.

Detection and Correlation of EBV Genome in Nasopharyngeal Swabs

Nested PCR proved highly sensitive for detecting EBV DNA extracted from NPC biopsy tissues (Fig 1). Among 80 NPC patients, 78 (97.5%) cases tested positive for EBV DNA in tumor tissue. Eleven NPC swabs were excluded from the study because of negative β-globin PCR products. The nested PCR demonstrated similar sensitivity (ie, 66/69 cases [95.7%]) when

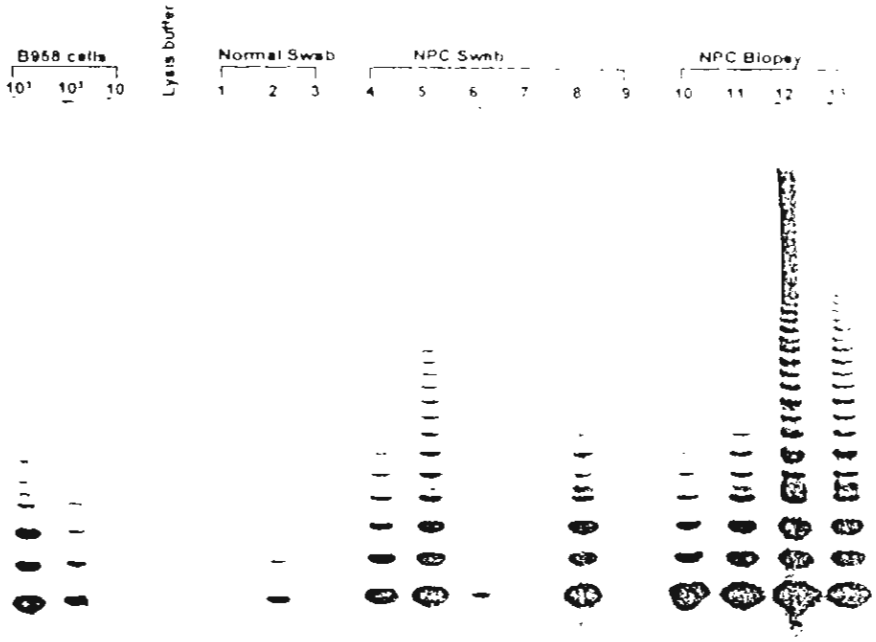

Fig 2. [illegible] EBV-transformed human lymphocytic cell line. [illegible] 10^3, 10^2, and 10 cells represented levels of [illegible] ative control.

Table 2. [illegible]

	Nasopharyngeal swab				[illegible]			
	PCR	TRAP	PCR and TRAP	PCR or TRAP	PCR	TRAP	PCR and TRAP	PCR or TRAP
[illegible]								
[illegible]								

applied to detect EBV DNA from nasopharyngeal swabs. Two of 3 cases with no EBV presenting from swab, were negative for EBV among the biopsy specimens. The presence of EBV DNA was less frequent among non-NPC tissue. Among 27 cases, 6 cases were positive for EBV DNA. This suggested a [illegible] specificity for the presence of EBV DNA for NPC. As expected, this data revealed a high correlation between NPC and the presence of EBV DNA (Table 2).

Telomerase Activity in Nasopharyngeal Biopsy Specimens and Swabs

Telomerase activity was present in [illegible] of 29 NPC biopsy specimens (94.9%) (Fig 2). The enzyme activity was found in all histology subtypes and all stages. There was no statistically significant correlation between telomerase expression and clinical manifestation such as skull or nerve involvement, lymph node, and distant metastasis.

[illegible]

Comparison Between Nested PCR for EBV DNA and TRAP Assay

Table 2 shows [illegible] using EBV genome analysis and TRAP assay. The sensitivity was very high on combination of PCR and TRAP assay. All biopsy specimens were positive for either EBV genome or telomerase activity. The sensi

tivity was also as high as 100% on screening for EBV genome or telomerase activity from swabs and amounted to 81.2% when both EBV and telomerase were simultaneously assessed. Six and 8 of 27 non-NPC swabs were positive for EBV genome and telomerase activity, respectively. Only 2 cases of non-NPC swabs showed both EBV DNA and telomerase activity. These 2 cases, acute rhinitis and sinusitis, also presented with inflammation. This suggests that the specificity on combination of both techniques was 92.6% and could be as high as 100% when analyzing only patients without signs and symptoms of inflammation.

DISCUSSION

Screening to obtain early and definite diagnoses of NPC is an important issue among high-risk populations. For countries with intermediate risk, such as Thailand, this population includes middle- or advanced-aged men of Chinese origin or siblings of patients with the tumor.[1] At present, the screening method most commonly available is based on anti-EBV serology. Nevertheless, the specificity of this method is low, especially when the cut off point is set at a low level to increase the sensitivity. Consequently, cancer develops in only a few positive cases. In addition, a substantial proportion of patients with subclinical NPC would not be detected with conventional nasopharyngeal examination. For identification of such patients, endoscopy and multiple biopsy specimens of the nasopharynx have proved to be an effective method for diagnosing submucosal growth of NPC.[26]

Because virtually all cases of NPC are associated with EBV, a number of studies identified the EBV genome from nasopharyngeal epithelium with noninvasive methods such as wash, swab, and brush biopsy. The purpose of such studies was to help improve sensitivity and specificity for NPC screening and early diagnosis. In addition, these techniques may help reduce the number of multiple nasopharyngeal biopsies, which are invasive techniques. However, those studies showed different results with normal controls. Approximately 81%, 35%, and 47% of normal populations from Greenland, Denmark, and Taiwan, respectively, were found to be positive for EBV DNA from throat washes.[13,14] Twenty-five percent of normal cases in Taiwan were positive by nasopharyngeal swab.[15] Interestingly, only 2 of 157 control cases were positive for EBV DNA when brush biopsy was performed among Chinese immigrants in North America.[16] Our study used nested PCR of nasopharyngeal swabs from Thai patients and identified EBV in 6 of 27 cases. On the contrary, nasopharyngeal swab and brush biopsy showed higher frequencies of detecting the EBV genome from NPC than the wash.[13-16] These data suggested EBV in the throat could be identified from 2 sources. The first source is premalignant or early lesions of NPC. The second is chronic EBV infection. As a result, use of the EBV genome as a marker for identifying NPC exfoliated cells should yield a wide range of sensitivity and specificity depending on the methods and source of specimens.

Telomerase is a good candidate marker for cancer screening because it is usually expressed in most cancers, including NPC, but not in most normal tissues.[18,19] These data were confirmed in this study because 93.7% of NPC biopsy specimens were positive. Our recent studies performed among stratified squamous epithelial tissues, such as oral epithelium and cervix, indicated that telomerase activity is associated not only with cancer development but also with certain benign conditions (especially with inflammation), such as lichen planus.[20-22] This may cause false-positive results when telomerase is used as a marker to screen cancer from squamous cell origin, including NPC. However, because the mechanism causing false-positive results is distinct compared with identifying the EBV genome and telomerase, combination of both markers should improve sensitivity and specificity for NPC diagnosis from nasopharyngeal exfoliated cells. Telomerase assay and nested PCR showed high sensitivity and specificity when applied to nasopharyngeal swabs. On combination of both techniques, the sensitivity was improved to 100% if either one of the positive results was counted, and the specificity was 92.6% if both positive results were analyzed together.

CONCLUSION

Analyzing NPC markers from nasopharyngeal exfoliated cells with a method such as swab could help improve diagnosis, especially for cancer screening and early diagnosis. As different frequencies of EBV genome detection were reported between normal controls, depending on the methods applied and the source of cases, an additional marker should be helpful for identifying NPC cells. Telomerase activity, according to this study, is a good candidate for use in conjunction with nested PCR for detection of the EBV genome. Because the sensitivity was 100% when counting either positive result obtained by these techniques, only high-risk populations with either EBV genome or telomerase activity identified from nasopharyngeal swabs should be indicated for multiple biopsies. More case control studies, especially from subclinical NPC, should be performed to indicate the sensitivity of nested PCR and TRAP assay when applied to this group.

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Epstein-Barr Virus DNA in Serum/Plasma as a Tumor Marker for Nasopharyngeal Cancer[1]

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ABSTRACT

Nasopharyngeal cancer (NPC) constitutes a type of carcinoma encountered frequently in Southern China, among Eskimos of the Arctic region, and to a lesser extent in Southeast Asia. Because EBV DNA present in plasma or serum of NPC patients has proven to represent a promising noninvasive tumor marker, the present study was designed to determine the incidence of serum/plasma EBV DNA by nested PCR during various disease management stages. By this method, we could detect EBV DNA in plasma/serum of 98 of 167 NPC patients prior to treatment, compared with 10 of 77 samples derived from healthy blood donors serving as controls, with a similar prevalence observed in plasma *versus* serum. Investigation of 13 patients subjected to radiotherapy revealed plasma EBV DNA to persist in the plasma of one case, whereas among the remaining patients, it had vanished during the early phase of treatment. Finally, with 52 samples derived from 37 NPC patients during follow-up, we established 100% specificity and 0% false-positive rate for plasma DNA detection by nested PCR. Moreover, we subjected 24 known EBV DNA-positive serum samples to DNase digestion prior to DNA extraction and amplification to differentiate between free and encapsulated viral DNA, which demonstrated complete absence of the human β-globin genomic DNA in contrast to EBV DNA detectable in 14 samples. In conclusion, applying this noninvasive method, serum/plasma FBV DNA constitutes a reliable tumor marker prior to, during, and after treatment of NPC.

INTRODUCTION

The discovery of tumor-derived DNA in the circulation of cancer patients raises the possibility of a new strategy for noninvasive cancer detection and monitoring (1–3). In NPC,[4] we have previously demonstrated that EBV DNA is not only detectable in tumor tissue but is also detectable in the patients' cell-free sera (4). In addition, comparison between EBV typing of primary tumors and their sera showed identical results, suggesting that serum EBV DNA represents tumor DNA. In a recent study, Lo *et al.* (5) have further explored this issue with respect to sensitivity and quantity of the plasma EBV DNA derived from NPC patients. A very high incidence of plasma EBV DNA was shown prior to treatment. Furthermore, the presence of plasma EBV DNA 1 month after completion of radiotherapy was associated with the disease persisting, either because of partial response or distant metastasis. Hence, serum/plasma EBV DNA represents a promising tumor marker for noninvasive cancer detection and monitoring of NPC.

NPC constitutes an important cancer in Asia encountered frequently in Southern China and among Eskimos of the Arctic region. An intermediate incidence is observed in Southeast Asia (6–8). Therefore, the attempt at understanding how serum/plasma EBV DNA could be used for diagnosis and monitoring of NPC is crucial. In the present study, we applied nested PCR to analyze the incidence of serum/plasma EBV DNA during several phases of NPC, prior to as well as in the course of treatment and during follow-up. In addition, we determined whether the viral DNA was encapsulated. The data presented here not only demonstrate sensitivity and specificity of serum/plasma EBV DNA in each phase of the disease but also assist in an increasing comprehension as to its biological significance.

MATERIALS AND METHODS

Sample Collection. Upon informing the patients about the purpose of the study and obtaining their consent, primary NPC tissues were collected before onset of treatment at Chulalongkorn University Hospital. The tissues were divided into two parts. The first part was sent for routine histological examination. The second part was immediately stored in liquid nitrogen for future DNA extraction and PCR analysis for the presence of the EBV genome. All tumors were histologically ascertained to be undifferentiated NPC according to the WHO classification.

Blood samples were obtained by venipuncture from several groups of patients selected on the grounds that the EBV genome was present in their tumor tissues. The first group, 146 serum and 21 plasma samples, comprised patients prior to treatment. The second group included plasma samples obtained from 13

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[4] The abbreviations used are: NPC, nasopharyngeal cancer; EBNA, EBV nuclear antigen.

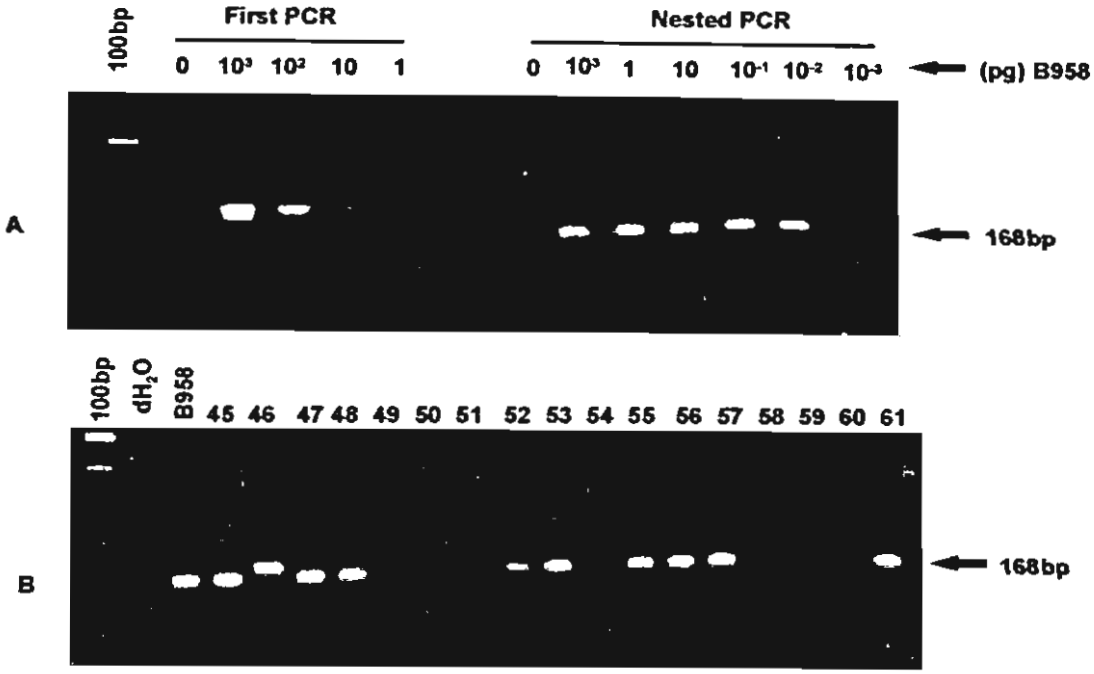

Fig. 1 Sensitivity of nested PCR for EBV DNA. A 100-bp ladder, distilled H_2O (dH_2O), and DNA from B958 cell lines were used as size standard, negative control, and positive control, respectively. *A*, compare sensitivity between single PCR and nested PCR for EBV DNA. *B*, examples of nested PCR for EBV DNA from NPC sera. Positive results were cases 45–48, 52, 53, 55–57, and 61. Most positive cases were EBV type A, whereas case 46 was EBV type B.

Table 1 Incidence of serum/plasma EBV DNA during various NPC management stages

Diagnosis	Status	Sample	Positive	Total (case)	%
NPC	Before treatment	Serum/plasma	98	167	58.7
		Serum	83	146	56.9
		Plasma	15	21	71.4
	Known positive EBV DNA in serum	Serum treated with DNAase	14	24	58.3
	During radiotherapy	Plasma (before Rx, 1, 2, 3–6 wks)[a]	(9, 6, 3, 1)	13	(69, 46, 23, 7)
	Follow-up	Plasma	3	37	8.1
	Recurrence or partial response	Plasma	3	5	60
	Complete remission	Plasma	0	32	0
Normal control		Serum	10	77	13.0

[a] (before Rx, 1, 2, 3–6 wks) incidence of plasma EBV DNA before treatment at first, second, and third to sixth weeks during radiotherapy.

patients at the weekly complete blood count evaluation in the course of radiotherapy. The last group constituted 52 plasma samples from 37 patients, who after completion of treatment came to the hospital every 3 months for follow-up. The DNA samples extracted from the sera of healthy blood donors serving as controls in a previous study were used again for the same purpose (1). To obtain cell-free sera and plasma, clotted and EDTA blood specimens were centrifuged at low speed for 5 min within 1 h after venipuncture. Both sera and plasma samples were stored at −20°C until further analysis.

DNA Isolation and DNase Treatment. NPC tissue was incubated in Tris/HCl buffer containing SDS and proteinase K at 50°C overnight, followed by phenol/chloroform extraction and ethanol precipitation of DNA (9). As for serum or plasma, 200 μl were purified for DNA extraction on Qiagen columns (Qiamp blood kit; Qiagen, Basel Switzerland) according to the "blood and body fluid protocol." Ten sera and 10 plasma samples, respectively, were reanalyzed for the presence of the EBV genome to compare the efficiency of the Qiamp blood kit with that of reextracting the nucleic acid with the QIAamp viral RNA mini kit (Qiagen). One-tenth of the DNA extracted was then used for nested PCR analysis.

To distinguish free EBV DNA molecules from virions, 400 μl of 24 known positive EBV DNA serum samples were divided into two parts. The first part was twice digested extensively with DNase I (37°C for 1 h; Ref. 10). Both parts were then subjected to DNA extraction and nested EBV DNA PCR analysis. Semi-nested PCR for amplification of *β-globin* DNA was used to determine whether free DNA had been digested completely.

EBV Detection by Nested PCR. For the detection and typing of EBV DNA in tumor tissue and serum/plasma samples, nested PCR protocols were used, modifying those described previously for amplification of the *EBNA-2* (11, 12). DNA extracted from the cell line B958, EBV-transformed lymphocytes (American Type Culture Collection), was used as positive control and double-distilled water as a negative control.

The first PCR amplified the EBNA-2, generating a DNA fragment of 237 bp for EBV type A and of 253 bp for EBV type B, respectively. With nested primers, the PCR product comprised 168 bp for EBV type A and 184 bp for EBV type B,

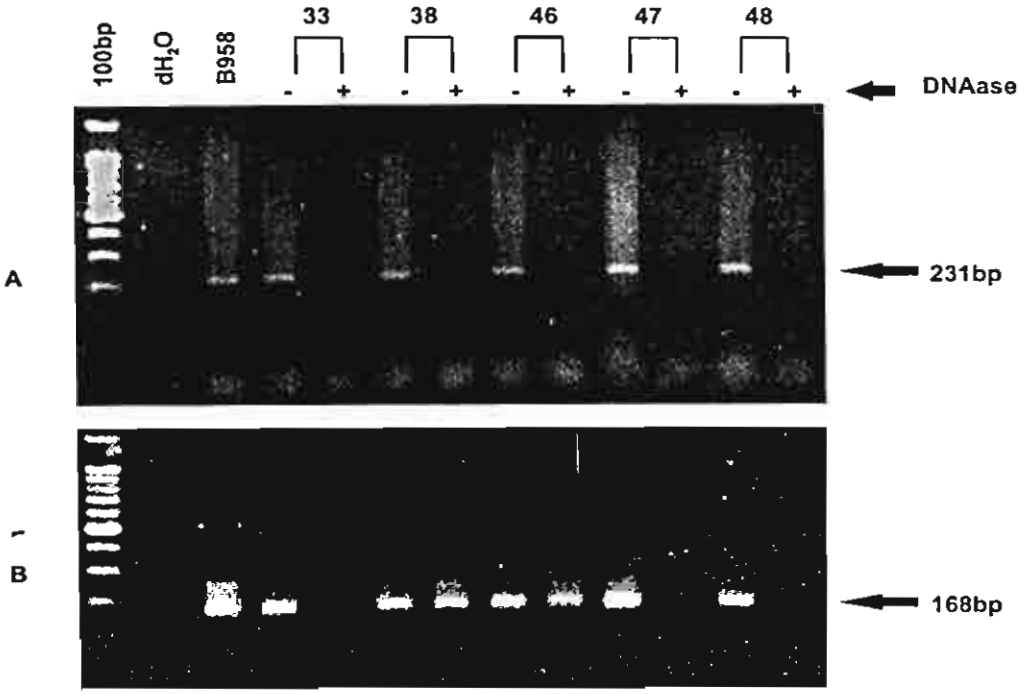

Fig. 2 Nested and seminested PCR from known EBV DNA-positive serum samples after DNase treatment. DNA. A 100-bp ladder, distilled H_2O (dH_2O), and DNA from B958 cell lines were used as size standard, negative control, and positive control, respectively. Lanes with (+) and without (−) DNase treatment are shown. *A*, seminested PCR for human *β-globin*. *B*, nested PCR for EBV DNA. All of these cases showed complete DNase treatment. Whereas cases 38 and 46 demonstrated resistant of EBV DNA from DNase, sera from cases 33, 47, and 48 were digested.

respectively. The nucleotide sequences for these first PCR primers were 5′-GCGGGTGGAGGGAAAGG-3′ (E3–44mer) and 5′-GTCAGCCAAGGGACGCG-3′ (E5–25mer). The nested PCR primers were E3 and E5 primers for *EBNA2* (11, 12). Amplification of the *β-globin* gene by seminested PCR was used to determine the presence of amplifiable human DNA in all samples tested for EBV DNA. The primers GH20 and PCO4 were used for the first-round primary PCR, generating a DNA fragment of 260 bp (13). The *β-globin*-specific seminested primers were 5′-ACCTCACCCTGTGGAGCCA-3′ (β-globin 62028) and PCO4, generating a 231-bp PCR product. The sequences of the primers used for *EBNA-2* and *β-globin* PCRs were identical to those reported previously (11–13).

The first-round PCR reactions were performed in a total volume of 20 μl using one-tenth of the extracted DNA in a reaction mixture containing 200 μmol of each deoxynucleotide triphosphate, 1.5 mM magnesium chloride, 50 mM potassium chloride, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 0.5 unit of *Thermus aquaticus* DNA polymerase, and 0.2 μM for EBV or 0.5 μM for *β-globin* primers. The PCR amplification was performed as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, with an extension at 72°C for 1 min, and a final extension at 72°C for 7 min. One μl of each PCR product was used as the template for nested and seminested PCR, respectively. The 35 cycles of second-round PCR reactions were performed in a manner identical to that applied for the first-round PCR, except for using different sets of primers, 0.5 μM for EBV or 1 μM for *β-globin* primers, and adjusting the annealing temperature to 50°C. The PCR products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide upon preparation.

Statistical Analysis. Data regarding histology, tumor staging, EBV detection, and response to treatment were collected in a double-blind fashion until analyzed. The χ^2 test was used to compare the results obtained from serum/plasma analysis with clinical and tumor parameters.

RESULTS

Sensitivity and Specificity of Nested PCR to Detect EBV DNA in Serum and Plasma. In this study, nested PCR was applied to improve the sensitivity for identifying EBV DNA. For the control cell line, nested PCR for *EBNA-2* still yields a positive result with 10 fg DNA (Fig. 1). This suggests a much higher sensitivity of nested PCR. We then reevaluated sensitivity and specificity of EBV DNA detection in sera and plasma of NPC patients, as well as of normal blood donors (Table 1). Whereas higher sensitivity, 98 of 167 cases (58.68%), was demonstrated, fewer cases of normal blood donors, 10 of 77 cases (13%), showed positive results. The positive EBV DNA incidence from plasma samples, 71.4%, was slightly higher than from serum, 56.85%. Twenty previously analyzed samples, 10 serum and 10 plasma samples, were reextracted for nucleic acid by QIAamp viral RNA mini kit. With these samples, testing for the EBV genome was repeated by nested PCR and yielded identical results. There is no significant statistical correlation between serum EBV DNA and clinical parameters, staging, histological classification, or response to treatment at 3 and 6 months.

To evaluate whether the EBV DNA detected in serum was present as a free nucleic acid or within intact virions, 24 EBV DNA-positive sera from different patients were subjected to DNase I treatment prior to DNA extraction. After extensive DNase I treatment, nested PCR did not yield any detectable product of the control *β-globin* DNA, whereas nested PCR for EBV DNA remained positive for 14 cases (Fig. 2; Table 1).

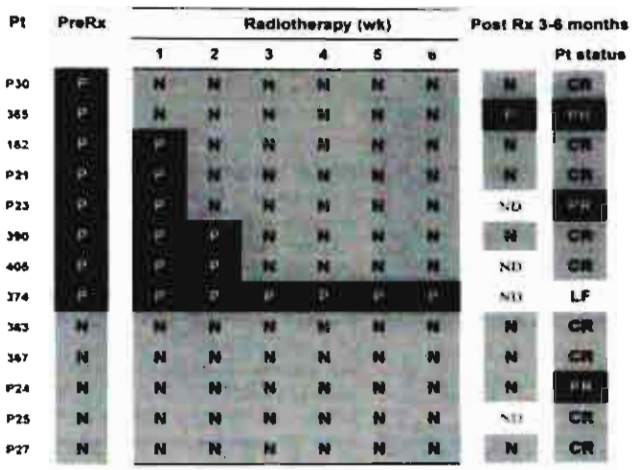

Fig. 3 Plasma EBV DNA at the end of each week during radiotherapy. Present (*P*) and absent (*N*) EBV DNA are shown prior (*PreRx*) and during treatment. NPC status of each patient at 3–6 months after treatment is demonstrated as complete remission (*CR*) and partial response (*PR*). *LF*, loss to follow up. *ND*, not done.

Plasma EBV DNA during Radiotherapy. Plasma samples were collected from 13 cases, and the EBV DNA status was determined on a weekly basis in the course of radiotherapy (Fig. 3, Table 1). Five of these cases showed no serum/plasma EBV DNA prior to treatment and remained negative during treatment. In the others, serum/plasma EBV DNA was detectable prior to treatment. In seven of them, plasma EBV DNA disappeared between the first and third week of radiotherapy. One case showed persistence of plasma EBV DNA throughout the course of radiotherapy.

Sensitivity and Specificity of Plasma EBV DNA for Patients' Follow-Up. Fifty-two blood samples of 37 patients after treatment of between 3 and 42 months duration were evaluated at follow-up for the presence of the EBV genome in plasma (Fig. 4, Table 1). Using WHO criteria for response, five cases presented with evidence of NPC, three with recurrence, and two with partial response, ≥50% decrease in total tumor size of the lesions and no appearance of new lesions or progression of any lesion. Plasma EBV DNA was detectable in three cases, one partial response, patient 365, and two recurrence cases, patients 72 and 106. Interestingly, whereas the plasma of both recurrence cases, patients 72 and 106, were positive for EBV DNA in their serum EBV DNA absent prior to treatment. Furthermore, repeated evaluation of the plasma for the EBV DNA status prior to further treatment twice in patient 106 and four times in patient 240, respectively, still showed identical results. The other 32 cases were in complete remission at the time of evaluation. Case 106R was patient 106 after the second course of radiotherapy. In the 32 plasma samples tested from all complete remission cases, some of which were examined more than once, no plasma EBV DNA was detectable. This suggested 100% specificity and 0% false positive rate, respectively, for serum/plasma EBV DNA to be used as an NPC tumor marker for follow-up after completion of treatment.

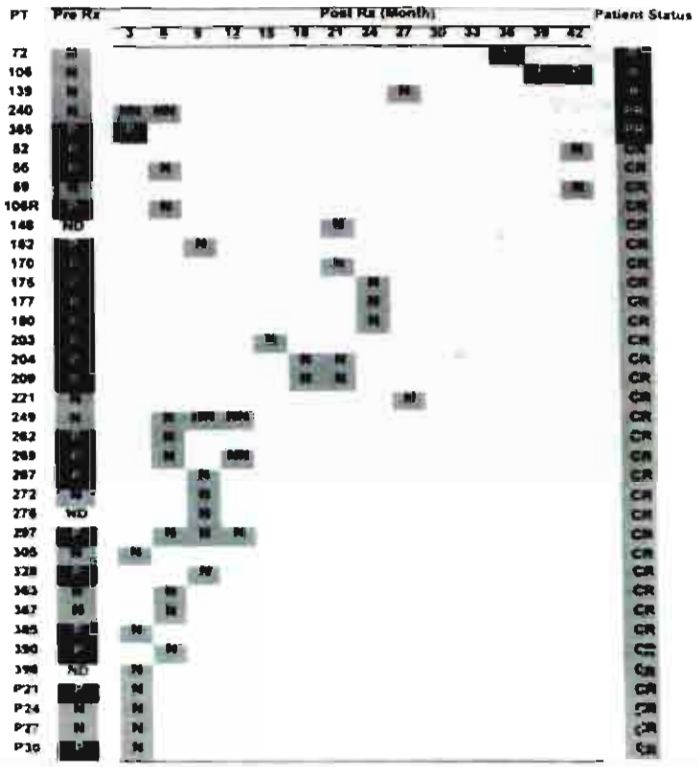

Fig. 4 Serum/plasma EBV DNA during follow-up after complete treatment. NPC status of each patient is demonstrated as recurrence (*R*), partial response (*PR*), and complete remission (*CR*). Present (*P*) and absent (*N*) of EBV DNA are shown. *ND*, not done.

DISCUSSION

The discovery of tumor-derived DNA in the circulation of cancer patients implies the possibility of a new strategy for noninvasive cancer detection and monitoring (1–3). For NPC, using EBV DNA for this purpose should prove advantageous because virtually all cases of NPC have been found associated with EBV (14, 15). Because a comparison between two studies has shown much divergence as to the prevalence of EBV DNA in serum/plasma samples, we improved the sensitivity for further analysis by using nested PCR (4, 5). This technique should prove to be the one with the highest sensitivity. Several positive cases (13%) were identified among normal blood donors infected previously with EBV, compared with not a single case identified previously by one-round PCR or with 7% established by the quantitative PCR study. The present study revealed serum/plasma EBV DNA to be present in 58.68% of NPC cases. Although plasma samples demonstrated a slightly higher prevalence of EBV DNA, there was no significant difference of DNA identification between plasma and serum samples or between two different DNA extraction methods, Qiamp blood kit and Qiamp viral RNA mini kit. The continuous presence or absence of EBV DNA in serum/plasma was confirmed by repeatedly evaluating the plasma for EBV DNA status in two patients. The difference in prevalence of the tumor DNA in the circulation of NPC patients was similar to that reported for other types of cancer. For example, the frequency of identifying serum/plasma colorectal cancer DNA can vary between studies

from 40 to 90% (16–18). Although differences in serum/plasma EBV DNA between EBV DNA prevalence reported by real time PCR and nested PCR may be attributable to different PCR primers and detection system. Nevertheless, as yet unsuspected biochemical and/or environmental mechanisms may be responsible for this finding.

Serum/plasma EBV DNA has been studied in several conditions, such as infectious mononucleosis, acute lymphoproliferative disorder, and EBV-associated lymphoma (19–24). In addition to the association with EBV-associated cancers, serum/plasma EBV DNA was found to be an indicator for active infection rather than latent virus (19, 21–24). The purpose of the DNase digestion experiment was to establish whether the presence of serum EBV DNA was attributable to lytic replication or release of latent episomal DNA. The results suggest that there might be two simultaneously present populations of serum EBV DNA, one encapsulated in the viral particle and the other the free nucleic acid probably released from NPC as episomal DNA. The presence of virions in the circulation of NPC patients is surprising because the majority of EBV in NPC cells should be in the latent phase, as shown by EBV clonal studies (25, 26). Consequently, serum/plasma EBV DNA should all be present as free nucleic acids. Nevertheless, our data invite the hypothesis that some EBV in NPC should enter lytic replication. This could explain why antibody titers to lytic cycle antigens, such as VCA and ZEBRA, rise in NPC patients (27, 28). In addition, expression of the immediate early *BZLF1* and *BHLF1* genes or ZEBRA protein is frequently detectable in NPC (29, 30). Because there may be only few cells entering lytic viral replication, studying the clonal progression of EBV by analyzing terminal repeat lengths by Southern blot and hybridization may not be sensitive enough to commonly detect the lytic replication or might be interpreted as impurities and hence ignored. Definite proof, such as electron microscopic analysis, is required to identify virions in NPC circulation.

Studying plasma EBV DNA during radiotherapy not only suggests a direction to further explore the behavior of EBV DNA present in the circulation of patients receiving radiotherapy but may also lead to clinical implications. EBV DNA positive in serum/plasma before treatment disappeared early in the course of radiotherapy, whereas plasma EBV DNA initially negative remained negative. This suggests two important findings: (*a*) serum/plasma EBV DNA is short lived; and (*b*) cell death as a consequence of radiation does not promote the presence of EBV DNA in the patients' circulation. On the contrary, because EBV DNA could disappear from plasma as early as during the first week of treatment, radiation may in addition to its ability to decrease the number of NPC cells use a specific mechanism that prevents the tumor from releasing EBV DNA. The biological effect of ionizing radiation at the cellular and molecular level appears to be DNA double strand break and a subsequence activation of DNA-dependent protein kinase (31, 32). It will be interesting to further explore whether DNA-dependent protein kinase plays any role in the release of EBV DNA from NPC into the patients' circulation. Whereas most cases of previously positive serum/plasma EBV DNA disappeared during radiation, one case showed persistence of plasma EBV DNA. This NPC case may not respond to the radiation induced inhibition of virus release, or it may indicate micrometastases. This persistence of serum/plasma EBV DNA during radiotherapy may be crucial for future clinical treatment modalities.

The other important clinical application of serum/plasma EBV DNA with NPC suggested here is as a tumor marker for patients' follow-up. NPC is a form of cancer with a high success rate of radiochemotherapy (33). However, many cases may recur, even after very long periods of latency (34). Consequently, most NPC patients require very consistent and long follow-up studies. Some of them may require expensive investigations, such as computed tomography scan or invasive methods, especially punch biopsy. Analyzing serum/plasma EBV DNA is an inexpensive and noninvasive technique suitable for clinical application. Upon using plasma EBV DNA as a marker for follow-up, the sensitivity (60%) shown in this study was similar to the prevalence detected prior to treatment. In addition, whereas only patients with evidence of disease showed plasma EBV DNA, the DNA was not detectable in any of the cases with complete remission. This suggested 100% specificity and a 0% rate of false positives. Interestingly, whereas we detected 13% of serum EBV DNA in 77 healthy individuals, no EBV DNA was identified in 42 tests of 32 NPC cases with complete remission. The usefulness of serum/plasma EBV DNA as a molecular marker for NPC patient monitoring was emphasized recently by Lo *et al.* (35). They demonstrated a close relationship between plasma/serum EBV DNA quantity and tumor recurrence. In addition, significant elevations in serum EBV DNA were observed in the patients who subsequently developed tumor recurrence.

In conclusion, this study has shown how frequently serum/plasma EBV DNA can be discovered in the course of NPC prior, during, and after treatment. Approximately 59% of NPCs prior to treatment were positive for serum/plasma EBV DNA. The presence and/or absence of serum/plasma EBV DNA is likely to depend on each individual and remain persistent as long as there is no change in the tumor status. Radiotherapy cannot induce but rather prevents NPC from releasing EBV DNA into the patients' circulation. Finally, investigating serum/plasma EBV DNA after treatment suggested its potential as a tumor marker.

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Genetic heterogeneity and progression in different areas within high-grade diffuse astrocytoma

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Abstract. The primary objective of this study was to determine loss of heterozygosity (LOH) in various portions of 9 high-grade diffuse astrocytomas, 8 glioblastomas and 1 anaplastic astrocytoma. LOH was observed on chromosomes 9, 10, 17 and 19 in 8, 3, 4 and 2 cases, respectively. Genetic heterogeneity and a multistep process were identified in 4 glioblastomas explaining the diverse morphological characteristics, a common feature of diffuse astrocytomas. In 2, 2, 3 and 1 cases, the allele losses were found within part of grade IV astrocytomas but not grades II or II/III, on chromosomes 9, 10, 17 and 19, respectively. In one of these, while genetic heterogeneity was observed on chromosome 17 between the area of grade II and grade IV, 9pLOH was found within both areas and occurred on the same allele. The other 5 cases did not demonstrate genetic heterogeneity and the LOH was on the same allele, irrespective of grade, suggesting clonal origin. In conclusion, at the molecular level, the diverse morphological features of astrocytoma develops by a multistep mechanism of genetic alterations from one cell via low-grade and more malignant tumors towards glioblastoma.

Introduction

Diffuse astrocytoma is the most commonly occurring intracranial tumors, accounting for more than 60% of all neoplasms of the brain (1). Various evidence suggested the development of this cancer to proceed by a multistep process (2-4). Some of the most malignant forms, glioblastoma, arises secondarily from a number of lower grade gliomas. Secondly, some genetic alterations, such as loss of heterozygosity on chromosomes 9p, 10q and 19q, are found more frequently in anaplastic astrocytoma and glioblastoma, but are rare in low-grade astrocytoma (2-5). Finally, based on morphological criteria, heterogeneity is frequently found in this cancer. It should be noted that within a high-grade tumor, areas displaying low-grade tumor growth can be found (1,6,7). In addition heterogeneity has been observed with respect to the expression of tumor antigens (8), cell proliferation kinetics (9), cytogenetics (10) and telomerase activity (11).

Diffuse astrocytoma arises after multiple genetic aberrations. Molecular genetic studies implicate several oncogenes and tumor suppressor genes to be involved as for example, *TP53*, *EGFR*, *CDK4*, *MDM2*, *CDKN2A* and *PTEN* (12-17). Loss of heterozygosity was also found on a number of chromosomes, 9p, 10q, 13q, 17p and 19q to date (18). Interestingly, not every glioblastoma shows all the genetic changes described suggesting several independent pathways leading to the common end point (18). Hence, the morphological heterogeneity observed would be a result of the multistep carcinogenesis. Genetic distinction between lower- and higher-grade stages of the same tumor would provide more insight into astrocytoma development. Contrasting this hypothesis, a study on genetic heterogeneity in 1992 demonstrated all genetic chances analysed in 4 cases to be identical between low-grade and high-grade tumors (19). We propose to re-evaluate the genetic abnormalities characteristic for high-grade diffuse astrocytomas in areas of different gradation as to whether there is genetic heterogeneity in the individual tumor which could explain the genetic pathway of astrocytoma development.

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Abbreviations: WHO, World Health Organization; LOH, loss of heterozygosity; MSI, microsatellite instability

Key words: loss of heterozygosity, astrocytoma, genetic heterogeneity, multistep process, glioblastoma

Materials and methods

Tissues and DNA extraction. Nine large diffuse astrocytoma with distinct areas of different gradation, according to guidelines of the WHO (World Health Organization), were obtained from paraffin-embedded specimens (20). These comprised 8 glioblastomas (astrocytoma grade IV) with lower-grade astrocytoma areas (grade II and grade II/III in 4 cases each) and 1 anaplastic astrocytoma (grade III) containing grade-II portion. Grade II/III areas represented a distinct

Table I. Loss of heterozygosity in different areas of 9 astrocytomas.

Case		1			2			3			4			5			6			7			8			9		
Chro	STR	N	II	III	N	II	IV	N	II	IV	N	II	IV	N	II	IV	N	II/III	IV	N	II/III	IV	N	II/III	IV	N	II/III	IV
	D9S1748		U		2	2	1		U			U		2	2	2		U		2	2	1	2	1	1		U	
9	RPS6		U		2	2	1		U		2	1	1	2	1	1	2	1	1	2	2	1		U		2	2	2
	IFNA	2	1	1	2	0	1	2	1	1	2	1	1	2	1	1		U			U			U		2	2	2
	D10S196	2	2	2		U		2	2	2	2	2	2		U		2	1	1		U			U		2	2	1
10	D10S249	2	2	2	0	1	1	0	0	2	0	1	1		U		2	1	1	2	2	2	2	1	1	2	0	1
	D10S187	2	2	2	2	2	2		U		2	2	2		U		2	2	2	2	2	1		U		2	2	2
17	D17S1176		U		2	2	2	2	2	2	0	2	2	2	2	1	2	2	2	2	2	1	1	M	M	2	2	M
	TP53	2	1	1		U		2	2	2	0	1	1	2	2	1	2	2	2	2	2	M	2	2	2	2	2	1
19	D19S47	2	2	2		U			U		2	2	2		U		2	2	2	2	2	2	2	2	2	2	2	2
	ERCCI		U		2	2	1	2	1	1	2	2	2	2	0	2	2	0	2	2	2	2		U			U	

Chro, chromosome; STR, microsatellite; N, normal; II, astrocytoma WHO grade II; II/III, astrocytoma WHO grade II/III; IV, astrocytoma WHO grade IV (glioblastoma); 2, heterozygosity; 1, one allele detected; 0, could not be amplified; M, microsatellite instability; U, uninformative.

region in 4 glioblastomas consisting of low to moderate cellularity of well differentiated tumor cells retaining the astrocytic appearances but began to show a few mitotic figures. Fifteen to twenty 4 µm sections per sample were stained with hematoxylin and eosin and left uncovered. Under an inverted light microscope, the differently graded areas in each tumor were microdissected for molecular analysis using 27-30 gauge steel needles (21). In this context, the term 'high-grade diffuse astrocytoma' means either anaplastic astrocytoma or glioblastoma.

Blood samples collected by venipuncture served as constitutional controls in cases 1, 5, 6 and 7. Since all tumor samples included in our study were large and contained normal tissue such as neurons in the cerebral cortex, these normal cells were microdissected and used as internal controls. DNA was extracted from paraffin-embedded tissue and circulating leukocytes as described elsewhere (21).

Allelotyping. Ten microsatellite markers on chromosomes 9p, 10q, 17p and 19q used for PCR analyses are listed in Table I. Information regarding cytogenetic localization of the markers was provided by the Genome Data Base.

Prior to microsatellite analyses, DNA extracted from paraffin-embedded tissue was amplified by DOP-PCR in a total volume of 50 µl as previously described (22,23). One strand of each primer pair was end-labeled as described elsewhere (24). The PCR reactions were performed in a total reaction volume of 10 µl comprising 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), primer concentrations between 0.05-0.1 µM each, and 50 ng of genomic DNA obtained from both leukocyte extraction and DOP-PCR of extracted paraffin-embedded specimens.

Several PCR reactions have been optimized for each primer set as follows: an initial denaturation step at 95°C for 4 min, followed by 40 cycles of denaturation at 94°C for 1 min, with 1 min annealing at 55°C, extension at 72°C for 2 min and a final extension at 72°C for 7 min.

Aliquots (2 µl) of each reaction were mixed with 1 µl formamide-loading buffer, heated at 95°C for 2 min, chilled on ice for 30 sec, then loaded onto a 6% polyacrylamide 7 M urea gel. DNA fragments were size fractionated at 70 W until the tracking dye had covered the appropriate distance on the gel. After electrophoresis, the wet gel was transferred to filter paper (Whatman, Maidstone, UK), covered with Saran wrap and exposed to a phosphorus screen and the bands were visualized on a PhosphorImager using ImageQuaNT™ software (Molecular Dynamics, Sunnyvale, CA).

Results

Table I shows microsatellite alterations observed in 9 high-grade diffuse astrocytomas. Allelic losses were scored when at least one microsatellite locus on each chromosome examined was informative and revealed loss of one allele in comparison with matched normal DNA. Genetic heterogeneity was recorded when areas within the same tumor exhibiting the lowest and the highest gradation were genetically different. Microsatellite instability (MSI) as reflected by variations in length of microsatellite repeats in tumor DNA was also recorded. On the example of case 5, representative histopathology with corresponding loss of heterozygosity (LOH) is shown in Figs. 1 and 2.

Frequencies of LOH corresponding to the highest histopathological grade of each tumor were found in 8/9 (89%) of chromosome 9p, 4/8 (50%) of chromosomes 10q and 17p, and 2/9 (22%) of chromosome 19q, respectively. The

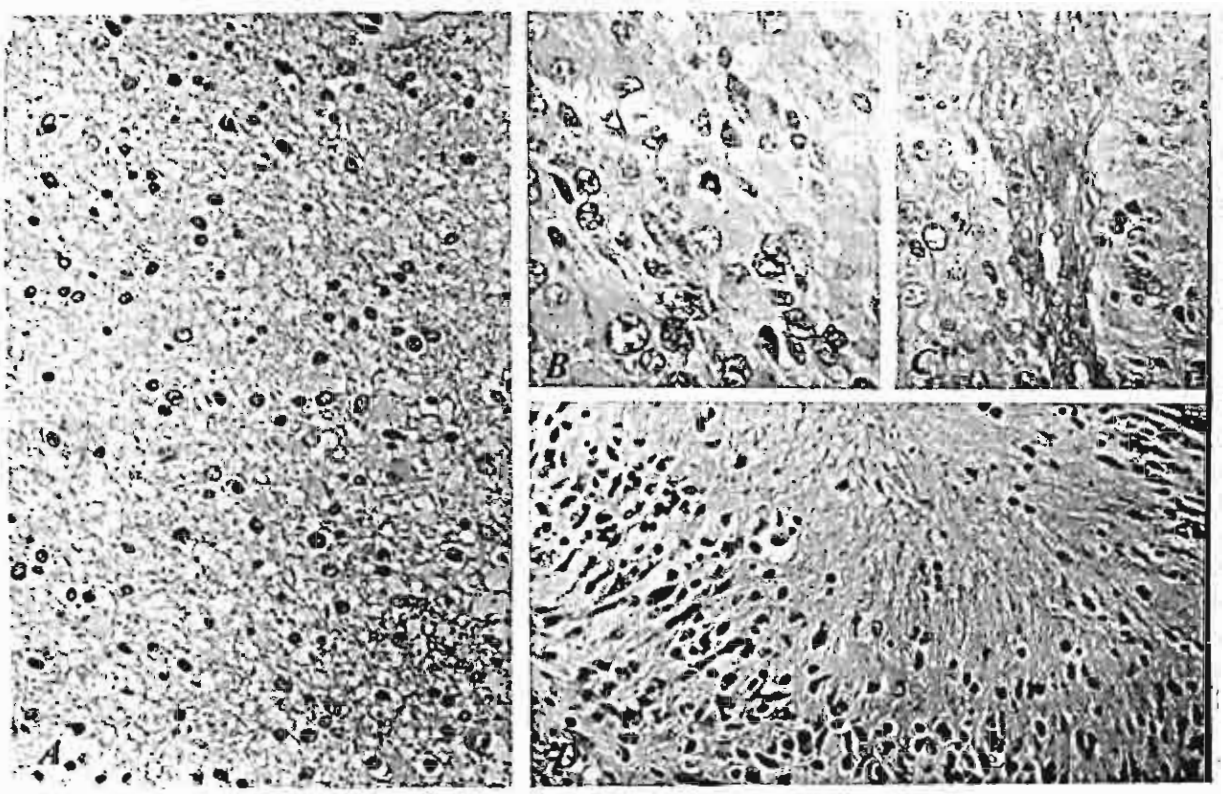

Figure 1. Histopathology of astrocytoma grade IV (glioblastoma) from case no. 5. A, Low magnification reveals grade II astrocytoma area consisting of neoplastic astrocytes with nuclear atypia. B-D, Medium and high magnifications display astrocytoma grade IV area as evidenced by marked nuclear pleomorphism with mitosis (B), vascular proliferation (C), and pseudopalisading necrosis (D).

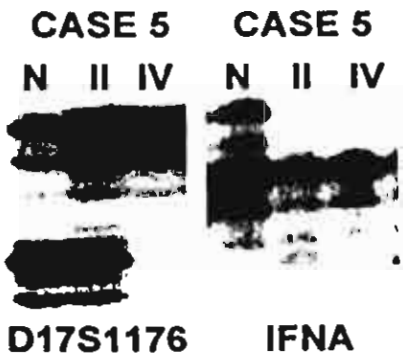

Figure 2. Microsatellite analysis in selected diffuse astrocytomas, case 5, showing genetic heterogeneity of chromosome 17, D17S1176, but not chromosome 9, IFNA. Representative astrocytoma grade II, IV and corresponding normal tissue (N) are shown with microsatellite markers.

areas with the lowest gradation in each sample exhibited a lesser incidence of LOH, that is, 6/9 (67%) on chromosome 9p, 2/8 (25%) on chromosome 10q, and 1/8 (12%) on both chromosomes 17p and 19q. In case of LOH present in both the lower- and higher-grade areas, the loss had originated from the same allele.

Genetic heterogeneity could be identified in 4 tumors (44%), that is, in cases no. 2 (chromosomes 9p and 19q), no. 5 (chromosome 17p), no. 7 (chromosomes 9p, 10q, and 17p), and no. 9 (chromosomes 10q and 17p). In case no. 5, whereas chromosome 17p showed genetic heterogeneity, chromosome 9p originating from either a grade II or grade IV area demonstrated identical genetic components (Fig. 2). The remaining samples displayed no genetic differences.

MSI was observed in 3 tumors, more specifically, in grade IV areas of cases nos. 7 and 9, and both grade II/III and IV portions of case no. 8.

Discussion

High-grade diffuse astrocytomas, like other malignant neoplasms, are histopathologically heterogeneous tumors (6,7). This phenomenon might be explained by the tumor evolution hypothesis that all neoplastic cells are derived from the same clonal origin but differ by stages of genetic alterations, resulting in diverse morphological appearance of tumor cells within the same lesion. However, a previous study has not been able to demonstrate such genetic heterogeneity on chromosomes 9p, 10, and 17p in 2 glioblastomas and 2 anaplastic astrocytomas, regions presumed to be related to tumor progression in diffuse astrocytoma (19). In our study, additional application of microdissection technique, as well as an increased number of analyzed glioblastomas and chromosomes have established the existence of genetic heterogeneity as evidenced by retention of both alleles in the grade-II or grade-II/III areas and LOH in the glioblastomatous area of the same tumor. The varying genetic alterations correspond well to the histopathological gradation. Our findings strongly support abnormal genetic events to increasingly occur during the multistep process of glioblastoma development.

The detection of LOH in grade II or grade II/III areas on chromosome 9p (cases nos. 1, 3-6, and 8), chromosome 10q (cases nos. 6 and 8), and chromosome 19q (case no. 3) was of interest since LOH in these regions has been unequivocally associated with malignant progression of diffuse

astrocytomas. Their frequencies of allelic loss are directly proportional to an increase in histopathological severity (2-5). Furthermore, chromosomes 9p21 and 10q23.3, respectively, harbor the candidate tumor suppressor genes *CDKN2A* and *PTEN*, both genes also play a role in malignant transformation of astrocytomas (5,18). LOH detected at the 3 chromosome loci in the low grade area implies that some genetic alterations in diffuse astrocytoma have already occurred before morphological changes could be discerned by conventional methods. These molecular markers may be employed as additional criteria or unfavorable prognostic indicators in cases of diffuse astrocytoma where histological features alone are insufficient to diagnose grade III or grade IV tumors. However, large series of well-controlled trials are required in order to fully evaluate the applicability of this molecular tool.

The *TP53* gene located on the short arm of chromosome 17 is involved not only in the initiation but also in the progression of gliomas (25,26). Apparently, in case no. 1 the former role has been favored as LOH 17p could be demonstrated in both grade II and grade II/III portions. Contrasting that, LOH 17p appeared to be a late event in the malignant progression from grade II and III astrocytomas toward glioblastomas in cases nos. 5, 7, and 9 as evidenced by the presence of LOH only in the grade IV area. Hence, our data differ from all three molecular subtypes of glioblastomas recently proposed (18). However, we suggest that all glioblastomas included in our study belong to the 'secondary' form as defined by Scherer (27) as each one contained a distinct area of lower grade lesion. There might be other, as yet undiscovered genetic pathways responsible for the development of glioblastomas.

MSI, a result of replication errors, has been suggested as a possible mechanism in the initiation of cancer. Since the frequency of MSI has been reported between 30-50% in glioblastomas but is very rare in low grade diffuse astrocytomas, this phenomenon is considerred an evolution in astrocytoma progression rather than the initial event (28,29). The occurrence of MSI in 3 cases of grade IV tumor opposed to none of the grade II portions supports these previous observations.

To summarize, our current study has demonstrated genetic heterogeneity to be discernible in diffuse astrocytoma which provides an explanation for the variety in morphological appearance of neoplastic astrocytes within the same lesion. In addition, some genetic alterations already exist in morphologically low-grade areas of a high-grade tumor. These antecedent molecular changes may be utilized as additional molecular diagnostic criteria or prognostic markers. Finally, the microdissection technique may unravel the complex molecular pathways employed in tumorigenesis and malignant progression of diffuse astrocytomas.

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Research article

Human papillomavirus DNA in plasma of patients with cervical cancer

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Abstract

Background: Human papillomavirus (HPV) is a crucial etiological factor for cervical cancer (CC) development. From a diagnostic view-point, the consistent presence of HPV in CC allows the viral DNA to be used as a genetic marker. The aims of this study were to evaluate the presence, physical status and clinical significant of HPV DNA in circulation of CC patients.

Results: Whereas 6 out of 50 (12%) HPV positive CC patients revealed plasma HPV DNA, it was detected in none of 20 normal controls or 13 HPV negative CC cases. The plasma DNA exhibited an HPV type identical to the HPV in the primary tumors and the DNA from both sources was integrated into host genome. Interestingly, several findings suggested an association between plasma HPV DNA and metastasis. First, three of the HPV DNA positive cases were CC patients with clinical stage IVB or recurrence with distance metastases ($P = 0.001$, RR = 15.67). Second, the amount of plasma HPV DNA from metastatic patients to be three times more than three other patients without metastases. Finally, the later cases had tendency to develop recurrence distant metastases within one year after complete treatment when compared with other HPV associated CC patients with the same stage but without the present of plasma HPV DNA.

Conclusions: The plasma HPV DNA originated from the CC, was associated with metastasis and could be used as a marker representing the circulating free CC DNA.

Background

Cervical cancer (CC) is one of the most common malignancies in women worldwide, especially in developing countries [1]. Several studies have suggested that human papillomavirus (HPV) initiates and causes endogenous genetic alterations in the progression of CC [2]. First, epidemiological studies have shown that most human CCs harbor the "high risk" HPV types 16, 18, 31 and 33 [3,4]. Second, some HPV proteins such as E6 and E7 interact with human tumor suppresser gene products and change cellular phenotypes [5]. Finally, the integration linearizes HPV DNA between E1 and L1 genes and dis-

rupts the viral E2 gene, which consequently induces expression of E6 and E7 genes [6,7]. This genomic rearrangement is thought to be critical for the transformation and proliferation of the early precursors to these cancers [1]. From a diagnostic viewpoint, the consistent presence of HPV in CC allows the viral DNA to be used as a genetic marker. For example, cervical pre-malignant lesions can be screened for highly sensitive HPV DNA detection technology in cell scrapings [8].

Accumulating lines of evidence have elucidated that there is tumor DNA in patients' circulation. Such DNA can be detected in plasma or serum via specific genetic and epigenetic alterations of the primary tumor. Though the mechanism of this phenomenon is not clear, the presence of tumor DNA in blood may have diagnostic and prognostic value [9,10,11,12]. Interestingly, viral DNA has been documented to occur as tumor DNA in the circulation of patients with primary tumors caused by viral infection. For example, there is a high frequency of hepatitis viral genomes and Epstein-Barr viral (EBV) DNA in the circulation of patients with hepatoma and nasopharyngeal cancer (NPC), respectively [13,14,15]. In addition, the circulating EBV DNA may be an invaluable tool for patient monitoring [15,16]. Since HPV DNA serves as a genetic marker for CC, we tested whether HPV DNA could be detected in the plasma of CC patients and whether it originated directly from tumor cells. Moreover, we determined whether the circulating HPV DNA has any diagnostic and prognostic clinical potential for patients with CC.

Materials and methods

Sample Collection

Primary CC tissues were collected from 63 patients before treatment at King Chulalongkorn Memorial Hospital. The tissues were divided into two parts. The first part was sent for routine histological examination. The second part was immediately stored in liquid nitrogen until further use. All of the tumors were histologically ascertained to be CC and the staging was done according to FIGO criteria. The 63 tumors included stages ranging from I to IV. All patients have been followed for treatment outcome and survival.

Blood samples were obtained by venipuncture from the same patients and 20 healthy blood donors. To obtain plasma, blood specimens in EDTA anticoagulant were centrifuged at 3,300 rpm for 10 min, and the plasma were stored at -20°C before use.

DNA Isolation

CC tissue was treated with SDS and proteinase K at 50°C overnight, followed by phenol/chloroform extraction and ethanol precipitation of DNA. Plasma DNA was purified on Qiagen columns (Qiamp blood kit; Qiagen, Basel, Switzerland) according to the "blood and body fluid protocol". Plasma (200 µl) was processed using the column and one-tenth of the extracted DNA was then used for PCR analysis.

HPV Detection, Quantitation and Typing

For analysis of the HPV DNA in the plasma from CC patients, HPV E6 gene amplification was performed using a protocol previously described with some modifications [17]. The PCR reaction was in a total volume of 20 µl containing 200 µM of each dNTP, 10 µM Tris-HCl (pH 8.4), 50 mM potassium chloride, 4.0 mM magnesium chloride, 0.5 units of AmpliTaq Gold (Perkin Elmer Cetus), 0.4 µM of WD76 (CGGTTSAACCGAAAMCGG) and WD67 (WGCAWATGGAWWGCYGTCTC), 0.1 µM of WD66 (AGCATGCGGTATACTGTCTC), WD 72 (CGGTCGGGACCGAAAACGG) and WD154 (TCCGTGTGGTGTGTCGTCCC). Additionally, another PCR using 0.3 µM of primers specific for *ZP3*, a gene on the human X chromosome, were performed to test the quality of DNA [18]. For testing DNA and screening for HPV incidence, one ZP3 primer, and WD72 and WD76, respectively, were end labelled at 37°C for 1-2 h in a total volume of 10 µl containing 10 µM primer, 0.025 mCi [γ-^{32}P] ATP (Amersham-Pharmacia) at 3000 Ci mmol^{-1},10 mM magnesium chloride, 5 mM DTT, 70 mM Tris-HCl (pH 7.6) and 10 units of T4 polynucleotide kinase (New England Biolabs). Without further separating of the unincorporated nucleotides, the kinase reaction was added to the PCR buffer mix. The PCR amplifications were performed as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°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. Reaction products were mixed with loading buffer and loaded onto a 4% non-denaturing polyacrylamide gel. DNA fragments were size fractionated at 800 V until the tracking dye had covered the appropriate distance of the gel. After electrophoresis, the wet gel was transferred to filter paper (Whatman), wrapped with Saran wrap and exposed to a phosphorus screen; the bands were visualized on PhosphoImager using ImageQuaNT software (Molecular Dynamics). E6 and ZP3 primer pairs yielded 243 and 177 bp PCR products, respectively. For HPV typing, 50 µl of unlabeled PCR product was prepared for dot blot experiments. All HPV DNA positive plasma and some tumor DNA samples were measured semiquantitatively by performing duplex radiolabeled E6 and ZP3 primer PCR and repeating the above PCR reaction [18]. The difference between viral and host genome copy numbers was calculated from the relative intensity of PCR bands by the imageQuaNT software (Molecular Dynamics).

Table 1: Plasma HPV DNA in tumor, Clinical staging, and treatment outcome of 50 HPV associated CC patients

		Total	Plasma HPV DNA		
			Positive		Negative
Cervical Cancer patient		50	6		44
Age[a]					
	<50	25	3		22
	>50	25	3		22
HPV type					
	U[b]	4	0		4
	16	28	2		26
	18	16	4		12
	33	2	0		2
Stage					
	IB	2	0		2
	IIB	17	3		14
	IIIB	28	0		28
	IVB	1	1		0
	R[c]	2	2		0
IIB response to radiotherapy					
	PR[d]	3	2		1
	CR[e]	15	1		13
IIB clinical outcome within one year after complete treatment					
R[c]		6	2		4
NED[f]		5	0		5
Metastases					
	Positive	3	3	P = 0001	0
	Negative	47	3	RR = 15.67	44
Histopathology type					
	Squamous	42	5		37
	Adenomatous	8	1		7

[a]<50 and >50 age less than 50, and above 50, respectively; [b] U, unknown; [c] R, recurrence; [d] PR, partial response; [e] CR, complete response; [f] NED, no evidence of disease

E6 type-specific probes were used for HPV typing. Positive controls of HPV type 6, 11, 16, 18, 31 and 33 from each PCR amplification were included [17]. Products obtained from another unlabeled E6 PCR reaction were heated to 95°C and thereafter 1 volume of 20 X SSC was added. Aliquots of 40 µl were applied to a Hybond-N$^+$ nylon membrane (Amersham Pharmacia Biotech) under vacuum, prewetted in denaturing solution (1.5 M NaCl, 0.5 M NaOH). The membranes were transferred to a filter paper soaked in neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 0.001 M EDTA) for 1 min. The membranes were air dried at room temperature, soaked in 0.4 M NaOH for 20 min for fixation and washed with 5 X SSC. Prehybridization at 65°C for 1 h was carried out using 6 X SSC, 5 X Denhardt's solution, 0.5% SDS and 100 µg of single-stranded sheared salmon sperm DNA per ml. Replicate membranes were separately hybridized with denatured ^{32} P-labelled, type-specific probes in prehybridizing solution for 1 hr at 55°C. Probes WD170 (GCAAGACATAGAAATAA) required hybridization at 45°C. Filters were rinsed briefly in 2 X SCC and 0.1% SDS at room temperature and then twice for 10 min at 45°C (WD170), 50-52°C (WD132 (GACAGTATTGGAACTTACAG), RR1 (GTACTGCACGACTATGT) and RR2 (ACCTTTGCAACGATCTG)), 55-56°C (WD103 (CAACAGTTACTGCGACG), WD165 (AAATCCTGCAGAAAGACCTC) and WD166(CCTACAGACGCCATGTTCA)), or 56-57°C (WD133 (ACACCTAAAGGTCCTGTTTC) and WD134 (ACACTCTGCAAATTCAGTGC)). The membranes were exposed to a

phosphorus screen and the signals were visualized on a PhosphoImager using ImageQuaNT software (Molecular Dynamics).

Detection of HPV Integration by PCR

All DNA samples from plasma and tissue that were positive for HPV type 16 and 18 were analyzed in two PCR reactions. One used 0.5 μM of each primer (5' E2 consensus primer HPV-16/18 5'-ATGAAAATGAYAGTAMAGAC-3', 3' E2 primer HPV-16 5'-CCAGTAGACACTGTAATAG-3' and HPV-18 5'-CATTGTCATGTATCCCACC-3'), with ^{32}P-labelled HPV-16/18 to detect 1026 and 1028 bp PCR products from HPV 16 and 18 episomal forms, respectively [7]. The other used 0.5 μM of each primer of *PIGR* gene (PIGR-F 5'-TCAGCCAGGGTAAGGATCC-3' and *PIGR*-R 5'-TGATGGTCACCGTTCTGCC-3') to amplify a 1392 bp fragment of human genomic DNA as a control. The PCR reactions contained 200 μM dNTP each, 10 μM Tris-HCl (pH 8.4), 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.5 units of *Thermus aquaticus* DNA polymerase (Perkin Elmer Cetus), and the PCR amplifications were performed as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 45°C for E2 gene or 55°C for*PIGR* for 1 min, extension at 72°C for 3 min and a final extension at 72°C for 7 min. Reaction product was mixed with loading dye and loaded onto 4% non-denaturing polyacrylamide gel. DNA fragments were size fractionated at 800 V until the tracking dye had covered the appropriate distance of the gel. After electrophoresis, the wet gel was transferred to filter paper (Whatman), wrapped with Saran wrap and exposed to a phosphorus screen. The bands were visualized on a PhosphoImager using ImageQuaNT software (Molecular Dynamics).

Correlation between Clinical Data and Plasma HPV DNA

Data regarding histology, tumor staging, clinical phenotypes, treatment outcome, and HPV PCR and typing were collected in a double-blind fashion until analyzed. The Fisher's exact test was used to compare the results obtained from plasma analysis with those of HPV and clinical parameters.

Results

HPV DNA in Plasma

To determine whether HPV DNA could be detected in the circulation of CC patients and represented tumor DNA, DNA from the plasma of three groups was studied. The first two comprised 63 positive and negative HPV CC patients, and the other 20 healthy female blood donors. The patients included in this study, were classified by age, pathological structure, and stage (Table 1). The control marker, ZP3, was positive in all samples. As a result, all were further analyzed for the presence of HPV DNA by PCR using labeled E6 degenerate primers. Plasma HPV DNA was a specific genetic marker for HPV associated CC, since it was detectable from neither HPV negative CC nor normal controls. Nevertheless, the circulating DNA had low sensitivity. Only 6 out of 50 (12%) with HPV associated CC patients were demonstrated to have circulating HPV DNA (Fig 1A and Table 1).

The frequency and type of HPV DNA were reevaluated from all plasma and tumor samples by PCR using the E6 degenerate primers and dot blot hybridization using type specific oligonucleotides (Fig 1B and Table 1,2). The presence of HPV DNA status was confirmed and plasma HPV DNA showed the same viral type as the tumors. Furthermore, DNA from both plasma and tumor from the 6 plasma-positive cases were differentiated between integrated and episomal. Since the integration process disrupts the HPV E2 gene, PCR using radioactive labeled E2 primer would show positive result from episomal form of HPV but negative if the DNA integrated (Fig 1C). Whereas E2 PCR product was detectable in 20.75% of the HPV positive tumors, none of the plasma or tumor samples from plasma HPV DNA positive CC patients was positive (Fig 1D). In consideration with the absence of episomal HPV DNA from tumor, it is most likely that the negative E2 PCR result was due to plasma HPV DNA derived from integrated HPV genome. This observation suggested that the circulating free HPV DNA originated from the CC and thus could be used as a marker for tumor DNA.

Plasma HPV DNA and Clinical Correlation

To elucidate the importance and meaning of CC DNA present in plasma, the correlation between the presence and amount of plasma HPV DNA and clinical data was investigated (Table 1). Clinical data including age, staging, metastasis, response to treatment, and site of recurrence of plasma HPV DNA positive patients were included inTable 2. Interestingly, there was an association between plasma HPV DNA and metastasis. Three out of six plasma HPV DNA positive patients but none of the other patients with HPV associated cancers had distant metastasis at the time of blood analysis ($P < 0.001$, RR = 15.67 ($5.24<RR<46.83$)). One of the three was stage IVB and two had a recurrence distant metastasis, one of whom had previously been plasma HPV DNA negative at stage IIIB. In addition, we evaluated the amount of HPV DNA in circulation by semi-quantitative PCR, comparing the intensity of PCR products between HPV E6 gene and *ZP3*, a gene on the human X chromosome. The average intensity ratio between these two bands from tumor DNA and plasma DNA samples was one and 0.08, respectively (Fig 1E and Table 2). Plasma from one of the patients, 106, was collected twice at dif-

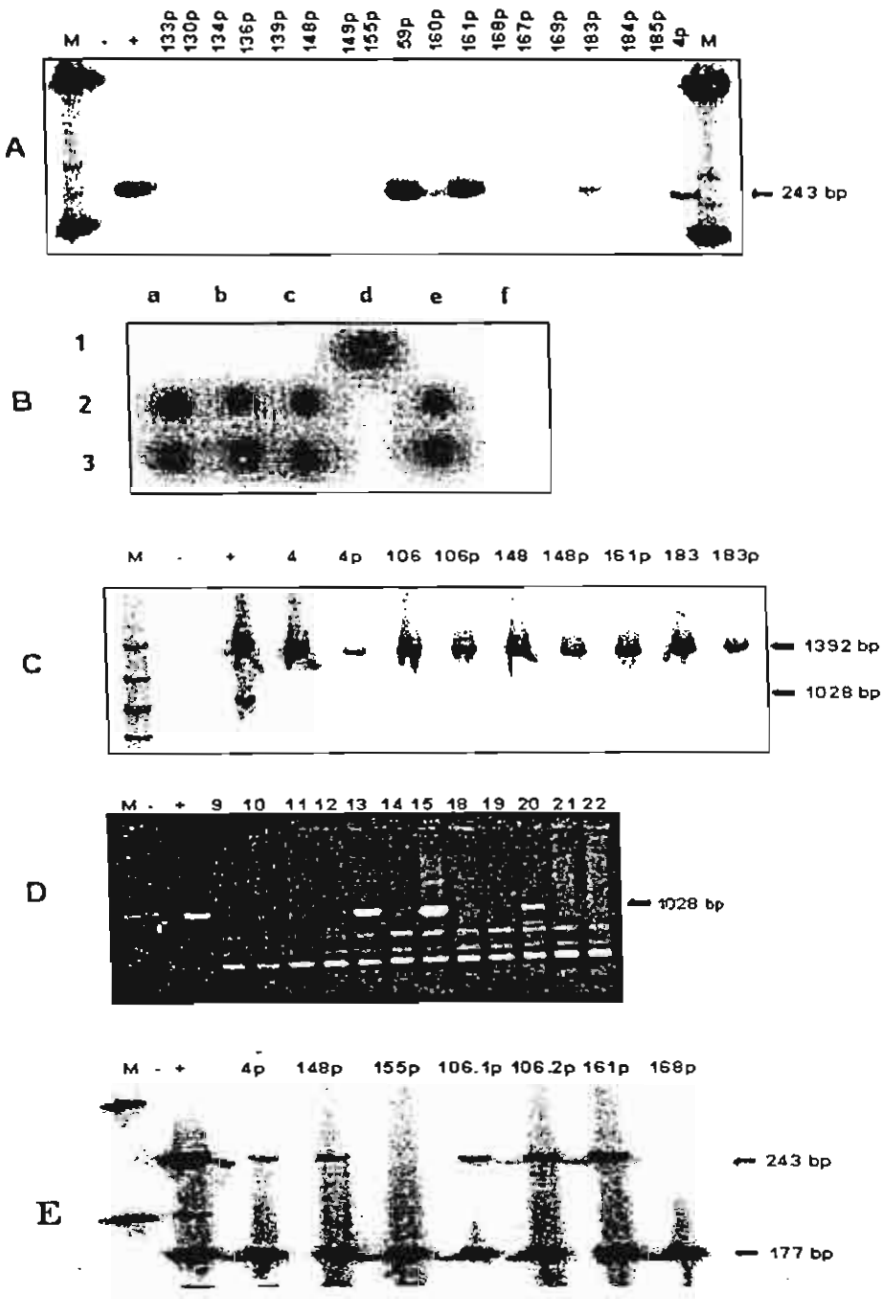

Figure 1

HPV DNA in plasma of patients with cervical cancer. (A) PCR generating 243-bp DNA fragments of HPV E6 gene (B) Dot-blot hybridization for HPV 18 probe Lanes a1-f1 were positive controls using purified plasmids of HPV type 6, 11, 16, 18, 31 and 33, respectively Lanes a2-f2 were plasma DNA from patients number 4, 106, 148, 161, 183, and 59 respectively Lanes a3-f3 were tumor DNA from the same patients as lanes a2-f2 (C) PCR generating 1392- and 1028-bp DNA fragments of human *PIGR* genomic sequence and HPV-18 E2 (D) PCR generating 1026, 1028-bp DNA fragments of HPV 16 and 18 E2 gene, respectively, from tumor DNA on a 2% agarose gel stained with ethidium bromide (E) Duplex PCR generating 243- and 177-bp DNA fragments of HPV E6 gene and human *ZP3* genomic sequence, respectively From left to right of A, C-E: M, 100-bp ladder standard DNA size marker; - and +, PCR products of the negative control (double-distilled water) and positive control (Hela cell line for A and E; purified plasmids for D; and for C, DNA from human leukocyte for *PIGR* and purified HPV 18 DNA plasmid for E2 gene), respectively; numbers without p indicate PCR products of tumor DNA; numbers with p indicate PCR products of plasma DNA of CC patients Number 1061p and 1062p were plasma DNA from patient 106 but the samples were collected at different time

ferent time and demonstrated the identical ratio (Fig 1E). Furthermore, the ratios from the three plasma samples of patients with metastases, 0.05, 0.14, and 0.19, was higher, though not statistically significant, p = 0.098, than from the other three non-metastasis patients, 0.02, 0.04, and 0.05. Interestingly, the three plasma HPV DNA positive patients who were stage IIB did not have as good a clinical outcome when compared with the other plasma HPV DNA negative patients with the same stage. Whereas 13 out of 14 plasma negative cases had a complete response to radiotherapy, 2 out of the 3 circulating viral DNA positive cases were partially resistant. However, formal comparison did not yet show significant statistical value, p = 0.063. In addition, we found that plasma HPV DNA positive patients may have tendency to develop recurrent disease. In patients who was followed up for more than one year, 2 out of 2 and 4 out of 9 plasma HPV DNA positive and negative patients, respectively, suffered reappearance of CC.

Discussion

The results of the present investigation confirm that it is possible to identify HPV DNA in DNA extracted from the plasma of patients with CC. More importantly, there are at least three findings suggesting that the viral DNA most likely originates from the tumor itself. First, circulating HPV DNA was not present in normal controls or HPV negative CC. In addition, all HPV DNA derived from HPV-associated CC patients' circulation was type 16 or 18. These high-risk HPV types are frequently associated with high-grade cervical intraepithelial neoplasia and CC [4,19]. Second, the plasma DNA exhibited an HPV type identical to the primary tumors in the patients. Thus the possibility that HPV DNA in circulation is derived from another latent epithelial infection was remote. Finally, the HPV genomes from both tumor and plasma were revealed the same physical status by integrating into the host genome. This was a unique characteristic for CC development and confirmed that the circulating viral DNA was from cancer cells [6,7].

Though both NPC and CC are viral associated lymphoepithelioma, squamous epithelial tumor with heavily lymphocyte infiltration, the incidence and physical status of viral DNA in the circulation and in tumors suggests that the DNA is released into the circulation of NPC and CC patients via a different mechanism. A much higher frequency of circulating EBV DNA has been discovered in NPC [15,16]. In addition, serum EBV DNA is resistant to Dnase treatment [15]. In conjunction with the expression and high antibody titer of lytic replication genes in NPC patients, it is likely that some circulating EBV DNA in NPC is virion associated [15,20,21]. In contrast to EBV, at least two lines of evidences indicated that plasma HPV DNA was free DNA, not virion-associated. First, the clinical phenotype associated with circulating DNA was similar between head and neck squamous cell cancer (HNSCC) and CC. A recent study used human promotor hypermethylation as a serum marker and demonstrated an association between this epigenetic marker and metastasis [22]. In addition, circulating HPV DNA could be found in a low percentage and associate with metastasis not only of CC cases but also of HNSCC cases where the primary tumor harbors HPV DNA [23]. The other reason that argues against the possibility that circulating HPV DNA is virion-associated is that the viral genome has integrated into the host genome with consequent deletion of part of the HPV genome. This physical characteristic is unique to HPV carcinogenesis and prevents viral ability to undergo complete lytic replication. All this evidence suggested that the mechanism by which viral DNA is released into the circulation of NPC and CC, although both are viral associated squamous epithelial cancers, is distinct.

Table 2: Clinical parameters and HPV characteristics of 6 plasma HPV DNA positive CC patients

Patient Code	Age	Stage[a]	Pathology Type	Tumor Size (cm)	Response to Radiotherapy	Time Recur (month)[b]	Metastatic Organ	HPV type	Relative amount of E6:ZP3 PCR[f]
4	39	IIB	Adenomatous	7	Partial			18	0.02
148	65	IIB	Squamous	3	Complete	8	Pleura, Peritoneumc	18	0.04
183	27	IIB	Squamous	6	Partial	11	Bone[c]	18	0.05
106	51	IVB	Squamous	>4			Liver, Pleurad	18	0.14
161	34	R[e]	Squamous	7			Umbilicus[d]	16	0.19
59	46	R[e]	Squamous	5			Liver, Lung[d]	16	0.05

[a] Stage at the time of blood examination; [b] Time for recurrence after complete treatment; [c] Metastasis at time of recurrence; [d] Metastasis at time of plasma DNA analysis; [e] R, recurrence; [f] Average amount from two experiments

This study indicated that plasma HPV DNA was a specific, but not a sensitive, genetic marker for CC diagnosis. More importantly, there was a strong association between plasma HPV DNA and metastasis, both when considering incidence and amount of the circulating viral DNA. Plasma HPV DNA positive non-metastatic CC patients had tendency toward poor clinical outcome and development of recurrent distant metastasis. This was not surprising. Although a variety of studies have shown that patients with very early stage disease can harbor free circulating tumor DNA, several reports have revealed plasma tumor DNA to be more commonly detectable in advanced stage disease, to correlate inversely with clinical outcome and to correlate with a tendency to fail with effective treatment [9,22,23,24,25,26]. There were at least three possible explanations for the association between plasma CC DNA and metastasis. The most common hypothesis is that circulating cancer DNA due to the lysis of circulating cancer cells or micrometastasis shed by the tumor. HPV-specific mRNA has been found in peripheral blood cells, circulating micrometastatic CC cells, and lymph nodes of advanced-stage CC patients with metastasis [27 ,28]. Nevertheless, a study of the relative amount of DNA in the plasma of pancreatic cancer patients indicated that a number of circulating cancer cells was insufficient and rejected this hypothesis [29]. Second, some in vitro and vivo studies have suggested that circulating tumor DNA in plasma might play a role in metastasis because of its high transforming activity [30, 31]. Finally, because there is a tendency toward poor clinical outcome for plasma HPV DNA positive CC patients, the mechanism to actively release tumor DNA into circulation might occur late during the multistep process of CC development when metastasis is more likely [10]. Regardless of the mechanisms, our data show that plasma HPV DNA in viral associated CC should be an independent genetic marker to predict disease progression and clinical outcome.

Conclusions

The results of the present investigation indicated that it was possible to identify HPV DNA in DNA extracted from the plasma of some patients with CC. More importantly, The HPV genomes from both tumor and plasma were revealed the same type and physical status by integrating into the host genome. Thus the viral DNA most likely originated from the tumor itself and the plasma HPV DNA represented the circulating free cell CC DNA. Finally, plasma HPV DNA was a specific, but not a sensitive, genetic marker in which strongly associated with CC metastasis.

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Serum/Plasma Viral DNA

Mechanisms and Diagnostic Applications to Nasopharyngeal and Cervical Carcinoma

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ABSTRACT: **Following reports describing circulating tumor DNA, serum/plasma viral nucleic acid has shown its potential as a new diagnostic target in cancer. In the majority of examples of viral carcinogenesis, the viral genome is consistently present in certain tumors and serves as an effective marker. This article reviews recent findings, proposes possible mechanisms, and examines the potential clinical application of serum/plasma Epstein-Barr virus (EBV) DNA in nasopharyngeal cancer (NPC) and human papillomavirus (HPV) DNA in cervical carcinoma (CC). These tumors share a DNA viral etiology and present similar histopathological findings. However, plasma EBV and HPV DNA are distinct in several aspects, including incidence, mechanism of release from tumor, and clinical application. Both circulating cell-free EBV and HPV DNA reveal the same viral type as their matched tumors, indicating both are derived from the neoplastic tissue. Plasma viral DNA incidence and copy number are high in NPC, but low in HPV-associated cancers. Whereas much EBV DNA in NPC is episomal, the resistance to DNase treatment of serum EBV DNA and evidence confirming lytic EBV replication in NPC suggest that a reasonable proportion of plasma EBV DNA is virions. On the contrary, plasma HPV genomes, as in CC, integrate into host chromosome. Plasma EBV DNA copy number, by quantitative PCR, is related to tumor mass, predicts prognosis, measures immediate response to treatment, and is useful in early detection of recurrence. Plasma HPV DNA, on the other hand, is associated with and can be considered as an early tumor marker for distant metastasis.**

KEYWORDS: **Serum/plasma viral DNA; Nasopharyngeal cancer; Cervical cancer; Human papillomavirus; Epstein-Barr virus**

INTRODUCTION

Plasma or serum DNA has become the most promising circulating tumor marker during the last decade.[1,2] However, the genetic heterogeneity of cancer complicates its use since a number of different genetic markers are required to evaluate each patient.[1,2] Viral DNA, on the other hand, is more simple since viruses are the cause of particular types of neoplastic development and progression. Consequently, viral

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TABLE 1. Correlation between nasopharyngeal and cervical carcinoma

		Nasopharyngeal cancer	Cervical cancer
Incidence		High in southern China, but rare in most other parts of the world	Worldwide
Histology	Normal tissue lining	Transitional zone (junction changing from pseudostratified columnar to stratified squamous epithelium)	Transitional zone (junction changing from simple columnar to stratified squamous epithelium)
	Histopathology	Mostly poor or undifferentiated squamous cell cancer with heavy lymphocytic infiltration	Mostly squamous cell cancer and some with heavy lymphocytic infiltration
Etiological factor	DNA virus	Epstein-Barr virus	Human papillomavirus
	Viral life cycle	Systemic (infects B lymphocytes and nasopharyngeal epithelium)	Local (infects, replicates, and reinfects cervical epithelium)
	Viral physical status in cancer cells	Mostly episomal form	Mostly integrated form
	Viral oncogenesis	Expresses viral oncogenes	Expresses viral oncogenes

DNA is a specific and sensitive marker for viral-associated cancers.[3] Nasopharyngeal cancer (NPC) and cervical cancer (CC) are important epithelial malignancies with DNA viral etiology and present similar histopathology findings (TABLE 1). Most cases of NPC and a majority of CC are squamous cell carcinoma. In addition, some CCs are lymphoepitheliomas, a squamous epithelial tumor with heavy lymphocytic infiltration that is strikingly similar to NPC.[4] This article reviews recent reports of circulating cell-free Epstein-Barr virus (EBV) DNA in NPC and plasma human papillomavirus (HPV) DNA in CC (TABLE 2). In addition, possible distinct mechanisms and potential uses as circulating tumor markers of both viral DNA types will be discussed.

NPC AND CC

Both NPC and CC are significant problems in world health (TABLE 1). NPC, although rare in most parts of the world, constitutes an important cancer in Asia, most frequently encountered in southern China (30–50 cases per 100,000 people/year). An intermediate incidence is observed in Southeast Asia (3–10 cases per 100,000 people/year). Other ethnic groups showing a higher incidence are Eskimos of the Arctic region and some North and East African populations.[5] EBV appears to be the most important etiological factor in NPC.[6] The viral genome is clonal in origin and detectable in almost all NPC tissues.[7] CC, on the other hand, is one of the

most common tumors affecting women worldwide, in both incidence and mortality, with approximately 471,000 new cases/year diagnosed globally.[8,9] Almost all CCs are associated with some specific types of HPV.[9,10] From a diagnostic viewpoint, the consistent presence of EBV in NPC and HPV in CC allows viral DNA detection to be used in clinical diagnosis. For example, the positive detection of EBV by PCR from a cervical lymph node with a metastatic tumor of unknown origin can be confidently diagnosed as a case of NPC.[11] Regarding CC, cervical premalignant lesions can be screened for HPV DNA in cell scrapings using highly sensitive DNA detection technology.[12]

BOTH PLASMA EBV AND HPV DNA ARE DERIVED FROM TUMOR

There are several potential sources of EBV DNA in the circulation since EBV can latently infect a number of cell types, particularly B lymphocytes.[3,6] However, plasma EBV DNA in NPC has been shown to be derived from the tumor. In general, primary infection with EBV occurs early in life. The virus primarily infects the oropharyngeal epithelium. The EBV then replicates, lyses, and infects B lymphocytes. After infection, the virus enters the lytic phase and causes diseases ranging from a mild self-limiting illness to infectious mononucleosis. However, in some cells, the virus enters the latent phase. These latently infected B lymphocytes have the ability to lead to malignancies, such as B cell lymphomas of the immunosuppressed, Burkitt's lymphoma, and Hodgkin's disease. This latent virus can play another important role in the dissemination of EBV infection within the nasopharynx. With subsequent host genome mutations, NPC may develop.[6,13] Thus, plasma EBV DNA can be derived from lymphocytes and detectable in some people as an indicator for active EBV infection.[14] Nevertheless, several lines of evidence suggest that EBV DNA discovered in the plasma of NPC patients derives from the tumor.[15] First, EBV DNA is frequently detectable in the plasma of NPC patients (70% and 96%), but rare in normal people (13% and 7%) by nested and quantitative PCR, respectively.[16,17] Second, in contrast to that due to lytic replication from lymphocytes, EBV DNA from NPC patients is continuously present before treatment.[16] Third, comparison between EBV typing in primary tumors and matched patient sera shows identical results.[15] Finally, plasma EBV DNA disappears from patients showing complete response to radiotherapy.[16,17]

HPV, on the contrary, can be considered as a local viral infection.[3] The papillomaviruses are highly species-specific and have a specific tropism for squamous epithelial cells. The viruses also induce squamous epithelial tumors and fibroepithelial tumors in their natural hosts.[3] Viral replication can persist within the basal cell layer of the epithelium. High-risk HPV types (16, 18, 31, and 33) will synthesize viral oncoproteins, especially E6 and E7, usually by integrating its 8-kb genome into host DNA. This process linearizes HPV DNA between the E1 and L1 genes and disrupts the E2 gene. As a result, expression of the E6 and E7 genes are not suppressed.[3,9] Recently, we reported HPV DNA typing in the plasma of CC patients in comparison with their matched tumors.[18] Whereas no HPV DNA could be detected from the circulation of normal controls and HPV-negative CC, an incidence of 12% was demonstrated in the plasma of HPV-associated CC patients. In addition, all positive cases revealed the same HPV type as found in the primary cancers. Thus, plasma

HPV DNA could be identified from HPV-associated CC and the DNA was shown to originate from the cancer cells.

DISTINCT VIRAL DNA RELEASING MECHANISMS BETWEEN NPC AND CC

A high incidence (70–96%) and copy number (4000–90,000 copies/mL) of plasma EBV DNA from patients with NPC suggest a unique mechanism for tumor release.[16,17] There are at least three possible mechanisms for this process, in which all may be responsible together in the presence of plasma EBV DNA. Two lines of evidence support cell death as one mechanism. There is a weak correlation between the presence of serum EBV DNA and apoptosis,[15] and there is a rise in plasma EBV DNA concentration after initiation of radiotherapy-induced cell death.[19] The second possible mechanism would be a consequence of the EBV genome remaining in NPC in an episomal form. Chen *et al.* demonstrated that extrachromosomal DNA could be induced in human lymphocytes by treating with phytohemagglutinin *in vitro*.[20] More importantly, this DNA could be detected not only in the nucleus, but also in the cytoplasm. This finding has led to the proposition that induction of such small fragmented DNA might be another mechanism of tumor DNA release because of the small size and its lack of specific structures, such as the centromere, for it to be retained in the nucleus. This hypothesis is very interesting since genome of cancer cells is prone to contain small fragmented DNA caused by double-strand breaks[21] or double minute chromosomes from oncogene amplification.[22] In more than 76% of NPC, the viral genome is not integrated and remains in an episomal form.[23] Consequently, the EBV DNA may be more easily released from the NPC cells.

The third possible mechanism for EBV DNA to be released into NPC patient circulation is lytic replication. In this case, a reasonable proportion of the circulating EBV DNA should be in the form of virions. Most EBV DNA from NPC patients' sera, unlike human genomic DNA, resists DNase treatment.[16] Thus, at least some of the viral DNA is encapsulated. The presence of virus in the circulation of NPC patients is surprising because the majority of EBV in NPC cells should be in the latent phase, as shown by EBV clonal studies.[7] Consequently, serum/plasma EBV DNA should present solely as free nucleic acid. Nevertheless, our data invite the hypothesis that some EBV in NPC has entered lytic replication. This could explain why antibody titers to lytic cycle antigens, such as the viral capsid antigen and BZLF1 immediate-early gene product ZEBRA, rise in NPC patients.[24,25] A recent study has shown that IgG antibodies directed against the BRLF1 immediate-early gene protein product, Rta, are detectable in 83% of NPC plasma samples.[26] In addition, expression of the immediate-early BZLF1 and BHLF1 genes or ZEBRA protein is frequently detectable in NPC.[26–28] Our ongoing research has discovered mRNA of the late lytic replication gene, BLLF1, by reverse transcriptase PCR in these cases. This confirms that there are active genes from all the viral lytic replication phases required for replicating EBV in NPC. There may only be a small proportion of cells entering lytic replication or most of virions may be released from NPC cells. Thus, studying the clonal progression of EBV by analyzing terminal repeat lengths using Southern blot hybridization may not be sensitive enough to detect lytic replication or the results might be interpreted as contaminants and hence be ignored.

Definitive proof, such as electron microscopic analysis and characterization of the physical appearance of EBV in patients' plasma is required.

Circulating free-cell HPV DNA from CC is different from plasma EBV DNA from NPC in both physical status and incidence. We have recently reported that a low proportion of HPV-positive CC patients, 6 out of 50 (12%), revealed plasma HPV DNA, and the DNA from both plasma and tumor was integrated into the host genome.[18] The low plasma HPV DNA incidence is controversial. Three groups (Sidransky *et al.*,[29] Pornthanakasem *et al.*,[18] and Duenas *et al.*[30]) reported the discovery of plasma HPV DNA, but with different detection rates. Studies in the United States and Thailand demonstrated 6% and 12% of plasma HPV DNA from CC patients, respectively.[18,29] However, an incidence of over 70% was shown from a study in Mexico.[30] This difference may have been due to technical factors. Nevertheless, a number of lines of evidence support the low incidence. First, two groups, in the United States and Thailand, showed the same finding by different experimental approaches. Second, in addition to incidence, our data have demonstrated a strong statistical correlation between the presence of plasma HPV DNA and distant metastasis ($p = 0.001$).[18] This correlation would be unlikely if positive results were obscured by poorly sensitive or erroneous techniques. In addition, this correlation with metastasis was also supported in head and neck squamous cell cancer patients with positive plasma HPV DNA.[31] Finally, the presence or absence of plasma HPV DNA is not altered when repeatedly evaluating the same patient using different times of sample collection before treatment. This consistency would not be likely without an accurate detection method.

Regarding our recent study, among the 50 patients with HPV-associated CC, 20.75% revealed episomal HPV genome in their tumors.[18] Interestingly, in all 6 cases with positive plasma HPV DNA, no episomal DNA could be demonstrated in both tumor or plasma. Hence, all plasma HPV DNA was in an integrated form and thus should be free DNA. This lack of plasma HPV DNA from tumors with episomal virus does not support the extrachromosomal hypothesis discussed above. In addition, the low incidence of HPV DNA in circulation and the low copy number (1–35 copies/mL) of head and neck cancer plasma HPV DNA suggest that,[31] unlike EBV, there is no or a less efficient mechanism for releasing HPV DNA into the circulation.

VIRAL DNA IS A CIRCULATING TUMOR MARKER

High sensitivity and specificity have led to circulating EBV DNA becoming a useful tumor marker, especially for patient follow-up (TABLE 2). NPC is a form of cancer with a high success rate of radiochemotherapy. However, many cases may recur, even after very long periods of latency. Consequently, most NPC patients require very consistent long-term follow-up. Some of them may require expensive investigations, such as computed tomography scanning or invasive methods, especially punch biopsy. Analyzing serum/plasma EBV DNA is an inexpensive and noninvasive technique suitable for clinical monitoring. Whereas a very high incidence and amount of plasma EBV DNA are shown prior to treatment, the DNA disappears after completion of radiotherapy in patients with a complete response. The plasma DNA reappears or persists in cases of recurrence or incomplete response to treatment.[16] Circulating EBV DNA may thus be used to determine the effectiveness

TABLE 2. Correlation between serum/plasma EBV DNA in NPC and HPV DNA in CC

	EBV DNA in NPC	HPV DNA in CC
Origin	Tumor cells	Tumor cells
Incidence	70–96%	6%, 12%, 70%[a]
Copy number	4000–90,000 copies/mL	1–35 copies/mL[b]
Physical status	Virus and episome	Integrates into host genome
Proposed releasing mechanism	Lytic replication, cell death, and episomal DNA	Tumor DNA and associated with metastasis
Diagnostic application	Quantitative PCR for prognosis, immediate response to treatment, and early detection of recurrence	Tumor marker for distant metastasis

[a]References 29, 18, and 30, respectively.
[b]Plasma HPV DNA in head and neck squamous cell cancer [31]

of treatment. For most patients, circulating viral DNA disappears within three weeks after initiation of radiotherapy. A few patients show persistent or reappearing DNA during the later period of treatment. These later groups may reflect tumor resistance or metastasis.[16] The usefulness of serum/plasma EBV DNA as a molecular marker for patient monitoring is most effective when analyzed quantitatively. This PCR technique provides more detailed information of viral load, which is related to tumor mass. Thus, quantification of plasma EBV DNA yields better information not only regarding tumor recurrence, but also concerning the immediate response of treatment and prognosis.[17,19,32,33]

Unfortunately, the lower incidence of plasma HPV DNA makes the test less attractive. However, the strong association with distant metastasis ($p = 0.001$, RR = 15.87) may allow this marker to be useful in this aspect (TABLE 2).[18] This association is not surprising since several reports have revealed plasma tumor DNA to be more commonly detectable in advanced stage disease and metastasis, to correlate with unfavorable clinical outcome, and to be associated with a tendency to fail in treatment.[1,2,31,34,35] There are at least three possible explanations for the association between plasma CC DNA and metastasis. The most common hypothesis is that circulating cancer DNA is due to the lysis of circulating cancer cells or micrometastases shed by the tumor. Circulating micrometastatic CC cells have been found by analyzing HPV-specific mRNA in peripheral blood cells of advanced stage CC patients with metastasis.[36] Second, some *in vitro* and *in vivo* studies have suggested that circulating tumor DNA in plasma might play a role in metastasis because of its high transforming activity.[37] Finally, there is a tendency towards poor clinical outcome for plasma HPV DNA–positive CC patients. The mechanism to actively release tumor DNA into the circulation might occur late during the multistep process of CC development when metastasis is more likely.

Detection of plasma HPV DNA may thus be an early sign of distant CC metastasis. In our recent study,[18] 3 out of 6 CC patients found to have plasma HPV DNA had distant metastasis at the time of evaluation. Interestingly, the remaining 3 plasma

HPV DNA–positive patients, who were at stage IIB, did not have as good a clinical outcome when compared with the other plasma HPV DNA–negative patients and developed recurrence of distant metastasis within one year after treatment. Although CC distant metastasis is rare at the time of first admission, it is frequently discovered if the disease recurs. Consequently, plasma HPV DNA may be a useful marker to screen for undetectable metastasis or to monitor patients after treatment.

APPLICATION TO OTHER VIRAL-ASSOCIATED CANCERS

Evaluating circulating viral DNA as a tumor marker can be applied to other malignancies. A similar incidence and condition with treatment of plasma viral DNA to NPC has been reported in EBV-associated lymphoma.[38] Thus, the same concepts might be applied to this and other viral-associated cancers. There are other EBV-associated malignancies, that is, gastric cancer or HPV-associated cancers, and malignancies of the upper airway, skin, and anogenital organs. In addition, other DNA and RNA viruses that can cause cancers, such as hepatitis B and C virus and HTLV-1, may also be studied.[3] A high frequency of the hepatitis viral genome has been discovered in hepatoma patients.[39] Unfortunately, the viral genome is not distinguishable between patients with hepatoma and chronic hepatitis, limiting the usefulness of detecting viral hepatitis DNA as a tumor marker. HTLV-1 infects circulating T lymphocytes;[40] thus, there is no obvious advantage in using plasma nucleic acid as a diagnostic target for leukemia associated with this virus. Nevertheless, better understanding of how tumors release viral genomes into the circulation may provide a better understanding of the biology of viral carcinogenesis and may lead to future clinical applications.

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Research article

Cytochrome P450 2E1 polymorphism and nasopharyngeal carcinoma development in Thailand: a correlative study

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Abstract

Background: Nasopharyngeal carcinoma (NPC) is a rare tumor in most parts of the world but occurs at relatively high frequency among people of Chinese descent. The cytochrome P450 2E1 enzyme (CYP2E1) is responsible for the metabolic activation of nitrosamines, and has been shown to be a susceptibility gene for NPC development in Taiwan [RR = 2.6; 95%CI = 1.2-5.7]. Since there has been only one report of this link, it was decided to investigate the susceptibility of *CYP2E1* to NPC development in other populations. Therefore, the correlation between the *RsaI* polymorphism of this gene and NPC was studied in-patients including Thai and Chinese in Thailand. The present study comprised 217 cases diagnosed with NPC and 297 healthy controls.

Results: Similar to the result found in Taiwanese, a homozygous uncut genotype demonstrated a higher relative risk both when all cases were analyzed [RR = 2.19; 95%CI = 0.62-8.68] or individual racial groups, Thai [RR = 1.51; 95%CI = 0.08-90.06] or Chinese [RR = 1.99; 95%CI = 0.39-10.87]. The ethnicity-adjusted odds ratio is 2.39 with 95%CI, 0.72-7.89.

Conclusions: Though our finding was not statistically significant due to the moderate sample size of the study, similarity to the study in Taiwan with only a slight loss in precision was demonstrated. The higher RR found for the same genotype in distinct populations confirmed that *CYP2E1* is one of several NPC susceptibility genes and that the RsaI minus variant is one mutation that affects phenotype.

Background

Nasopharyngeal carcinoma (NPC) is a rare tumor in most parts of the world, with annual age-standardized incidence rates typically below 1 per 100,000 people/year in both sexes [1]. The tumor occurs most often in Southern Chinese who reside in Guangdong Province, at an incidence rate 30-50 per 100,000 people/year, in contrast with <1 per 100,000 people/year in white Europeans [2,3,4,5]. The disease also occurs at moderate frequencies (3-10 per 100,000 people/year) in several non-

Chinese ethnic groups such as Malay, Thai and Vietnamese [6]. Numerous factors, both environmental and genetic, have been associated with the risk of developing NPC. The environmental factors include infection with the Epstein-Barr virus (EBV), as well as frequent consumption of high levels of nitrosamine from preserved food such as salted fish [7,8,9]. In addition, host factors also play a major role in NPC development. Unique alleles of the human leukocyte antigen (*HLA*) and cytochrome P450 2E1 (*CYP2E1*) have been shown to be associated with high relative risk in several Asian ethnic groups, including the Chinese in Taiwan [10,11,12].

CYP2E1, an enzyme involved in the metabolic activation of procarcinogens into reactive intermediates capable of forming adducts and damaging DNA, is believed to play an essential role in chemical carcinogenesis [13,14]. Nitrosamine is a substrate of CYP2E1. It is believed that nitrosamine, once activated can lead to the development of numerous cancers [15]. Studies have also demonstrated that *CYP2E1* is expressed in the nasal epithelium of human [16]. Evidence from previous epidemiological studies has suggested that salted fish is a food preferred by Chinese people and contains nitrosamines and nitrosamine precursors [9]. Therefore, *CYP2E1* is believed to render the nasopharyngeal epithelium susceptible to NPC development. A previous study in Taiwan employed a PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) assay using the restriction enzyme *RsaI* in order to compare wild-type (+/+) and variant forms (-/-) of the *CYP2E1* gene between NPC patients and the general population [10,11]. The variant form of contains *CYP2E1* polymorphic mutations in the distal 5'-flanking region of the gene, causing a marked difference in its transcriptional activity, as shown by CAT (chloramphenicol acetyltransferase) [17]. The Taiwanese association study showed that individuals homozygous for the variant allele (-/-) were at an increased risk for NPC development (relative risk [RR] = 2.6; 95% confidence interval [CI] = 1.2-5.7) [11]. There are many Chinese people who have immigrated and permanently lived in Thailand for 2 to 3 generations, resulting in a mixed population of Thai and Chinese people. From clinical observation, we had observed at least one-third of NPC patients were Chinese in origin. Since the susceptibility of *CYP2E1* gene to develop NPC had only been reported from Chinese people in Taiwan, it was decided to investigate whether this allele played the same role in other populations. Therefore, we studied the correlation of the polymorphism of the *CYP2E1* gene with NPC in Thai and Chinese populations in Thailand.

Materials and Methods

Sample collection

Blood samples were obtained by venipuncture from 217 NPC patients at Chulalongkorn Hospital and 297 healthy blood donors. All subjects were interviewed and separated into two groups, Thai and Chinese, based on their grandparents' ethnic origin. When their ancestors, including their great grandparents, originated from China, the patients were considered Chinese. When their ancestors originated from Thailand, the patients were considered Thai. There were 99 Thai and 98 Chinese in the control group. The NPC patient group included 132 Thai and 56 Chinese.

PCR-RFLP analysis

Genomic DNA (0.1 µg) extracted from leukocytes was used for each PCR analysis. The amplification was performed with primers as described previously by Hayashi et al. The total reaction volume of 50 µl consisted of 20 µM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 125 µM deoxynucleoside triphosphate, 0.2 µM primers, 4 U Taq DNA polymorase (Gibco), and 0.2 µg of template DNA. The PCR conditions were 40 cycles at 92°C for 1 minute, 60°C for 1 minute and 72 for 2 minutes in a Perkin-Elmer/DNA thermal Cycle 480. Genotypes of *CYP2E1* gene were determined by RFLP analysis. Twenty microliters of PCR products were digested with 10 U *RsaI* restriction enzyme (New England Biolabs) overnight at 37°C. The restricted products were analyzed by electrophoresis on 2% agarose gel. Bands were visualized with an ultraviolet transilluminator after ethidium bromide staining.

Statistical Analysis

The relative risk (RR) was estimated by the odds ratio method, to determine the correlation between genotype of the *CYP2E1* gene and NPC development. In addition, the RR was used to estimate the association of the pattern of genetic inheritance of the *CYP2E1* gene and NPC phenotype. The 95% confidence interval (CI) was computed to determine the statistical significance of the findings. The RR and 95%CI was calculated by using Exact method from Epit info version6 program.

Results

In the present study, we investigated the correlation between the polymorphism of the *CYP2E1* gene and NPC on a total of 217 patients and 297 controls. The diagnosis of NPC was confirmed histologically and by the presence of EBV DNA in the tumor. PCR-RFLP analysis was used to evaluate *RsaI* polymorphism in the *CYP2E1* gene (fig 1). The distribution of alleles in all, Thai and Chinese were found to be in Hardy-Weinberg equilibrium. The calculated frequencies of heterozygous using $2x(+/+)^{1/2} x (-/-)^{1/2}$ were 0.32, 0.26, 0.42 from all, Thai, and Chi-

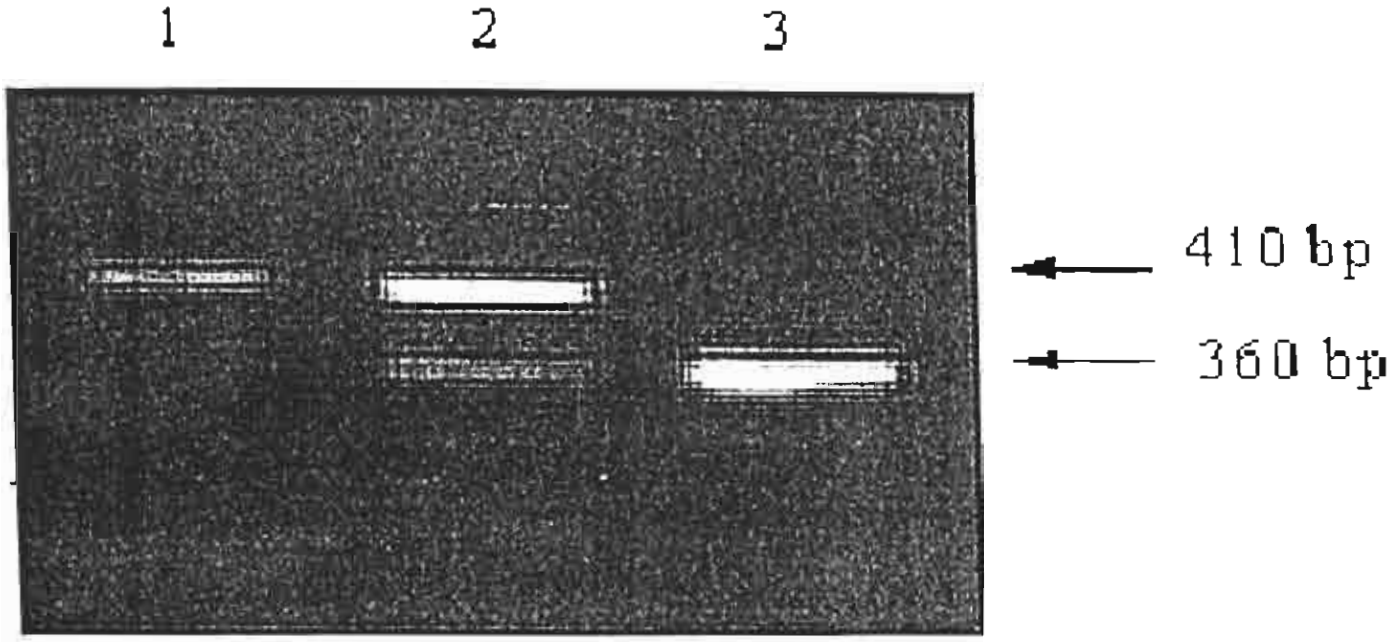

Figure 1
PCR-RFLP assay to detect the polymorphism of the *CYP2E1* gene by *RsaI* enzyme digesting the 410 bp PCR product into 360 bp and 50 bp fragments. Lane 1, homozygous variant (-/-); Lane 2, heterozygous (+/-); Lane 3, homozygous wild type (+/+).

nese NPC patients, and 0.32, 0.26 and 0.42 from all, Thai and Chinese control, respectively. These numbers are similar to actual frequencies of heterozygous from all groups, 0.32,0.28,0.42,0.35,0.28,0.52 from all, Thai and Chinese patients and all, Thai and Chinese control, respectively. We found the relative risk of the variant form (-/-) of the *CYP2E1* gene at a high risk [RR = 2.19]. However, this result had no statistical significance [95%CI = 0.62-8.68]. To evaluate whether the lack of significance of the trend was due to a mixed genetic background, both patients and controls were analyzed according to the origins of their ancestors (Thai or Chinese). A slightly increased risk of the variant from (-/-) of the *CYP2E1* gene could be demonstrated in both the Thai and Chinese sample groups [RR = 1.51; 95%CI = 0.08-90.06, RR = 1.99; 95%CI = 0.39-10.87, respectively]. Nevertheless, no statistical significance could be established. The estimated crude odds ratio lies outside of two ethnicity-specific odds ratios for Thai and Chinese indicates ethnicity is a confounder in this genotype-phenotype association. Odds ratio for the combined group of subjects with ethnicity information can be calculated with adjustment for ethnicity. The ethnicity-adjusted odds ratio (-/- vs +/-) is 1.86 (95%CI = 0.55-6.31). A similar adjustment using the whole sample of three groups, Thai, Chinese, and ethnicity unspecified showed an odd ratio of 2.39, with only a slight loss in precision (95%CI = 0.72-7.89). This is closer to the odds ratio of 2.6 from the study in Taiwan. Furthermore, Thai-Taiwanese comparison was done quantitatively by testing Ho:OR(Thai)=OR(Taiwan). Using Mantel-Haenszel test for odds ratio homogeneity, the p-value was 0.81. Thus there is no statistical different for the role of *CYP2E1* on NPC development between these two populations.

In this study, we further analyzed the association of the pattern of genetic inheritance of *CYP2E1* gene and NPC phenotype by calculating the relative risk if the genotype were either autosomal dominant (AD) or autosomal recessive (AR). In autosomal dominance, the contribution of a single variant allele would show a higher RR. Thus the RR for the combination of the heterozygous (+/-) and the variant form (-/-) compared with wild type (+/+) were computed. There was no association between AD heredity and NPC risk in the total, Thai, and Chinese sample groups [RR = 1.00; 95%CI = 0.68-1.47, RR = 1.01; 95%CI = 0.55-1.87, RR = 0.84; 95%CI = 0.41-1.71, respectively] (Table 2). By contrast, AR inheritance requires an abnormality in both alleles of the *CYP2E1* gene. The RR were calculated by comparison between the wild type (+/+), the heterozygous (+/-) or the combination of the wild type and heterozygous (+/+ and +/-) and the variant form (-/-). A higher RR value in all comparisons was shown for AR heredity in all sample groups (Table

3). However, these results showed no statistical significance for either the AD or AR pattern.

Table 1: Frequency distribution and relative risks associated with genotype variants of*CYP2E1* detected RFLP using RsaI

CYP2E1	Frequency		RR	95%CI
	Cases	Controls		
Total	**217***	**297**		
+ / +	138	189	1.00	
+ / -	71	103	0.94	0.64 - 1.39
- / -	8	5	2.19	0.62 - 8.68
Thai	**132**	**99**		
+ / +	93	70	1.00	
+ / -	37	28	0.99	0.54 - 1.86
/ -	2	1	1.51	0.08 - 90.06
Chinese	**56**	**98**		
+ / +	27	43	1.00	
+ / -	24	51	0.75	0.36 - 1.57
- / -	5	4	1.99	0.39 - 10.87

* 29 cases lack precise information regrarding ethnicity +, The allele could be digested with *RsaI* enzyme -, The allele could not be digested with *RsaI* enzyme

Table 2: Correlation between Autosomal Dominant (AD) pattern of genetic*CYP2E1* gene and NPC phenotype

CYP2E1	Frequency		$RR_{(AD)}$	95%CI
	Cases	Controls		
Total				
+ / +	138	189	1.00	
+ / - and - / -	79	108	1.00	0.68 - 1.47
Thai				
+ / +	93	70	1.00	
+ / - and - / -	39	29	1.01	0.55 - 1.87
Chinese				
+ / +	27	43	1.00	
+ / - and - / -	29	55	0.84	0.41 - 1.71

+, The allele could be digested with *RsaI* enzyme -, The allele could not be digested with *RsaI* enzyme

Table 3: Correlation between Autosomal Recessive (AR) pattern of genetic*CYP2E1* gene and NPC phenotype

CYP2E1	Frequency		$RR_{(AR)}$	95%CI
	Cases	Controls		
Total				
+ / +	138	189	1.00	
- / -	8	5	2.19	0.62 - 8.68
+ / -	71	103	1.00	
- / -	8	5	2.32	0.64 - 9.36
+ / + and + / -	209	292	1.00	
- / -	8	5	2.24	0.63 - 8.80
Thai				
+ / +	93	70	1.00	
- / -	2	1	1.51	0.08 - 90.06
+ / -	37	28	1.00	
- / -	2	1	1.51	0.07 - 92.44
+ / + and + / -	130	98	1.00	
- / -	2	1	1.51	0.08 - 89.84
Chinese				
+ / +	27	43	1.00	
- / -	5	4	1.99	0.39 - 10.87
+ / -	24	51	1.00	
- / -	5	4	2.66	0.51 14.48
+ / + and + / -	51	94	1.00	
- / -	5	4	2.30	0.47 - 12.08

+, The allele could be digested with *RsaI* enzyme -, The allele could not be digested with *RsaI* enzyme

Discussion

We have shown an increased risk of developing NPC associated with the homozygous variant form of the *CYP2E1* gene. This higher RR was demonstrated in both the Thai and Chinese populations in Thailand. This finding was similar to the result reported from Taiwan. However, these results were marginal statistical significance, which may well be due to the small sample size employed in the present study. Thus the *CYP2E1* gene appears to be a susceptibility gene for NPC development regardless of the patient's genetic background. Patients of both Thai and Chinese ethnic origin revealed a higher relative risk from the same allele, despite their distinct ancestry. Thus it is more likely that the *RsaI* negative allele affects the phenotype directly rather than being a consequence of linkage disequilibrium from another mutation or gene. This confirms the previous finding that the polymorphic *RsaI* site was essential for a marked difference in transcriptional activities [17]. A higher level of expression in the variant form would result in larger amounts of procarcinogens being changed into carcinogens, that then produce DNA damage. The affect of the distinct expression level of a metabolic gene should be reduced if the

person with abnormal genotype is not exposed to the substrate. For example, Phenylketonuria (PKU) patients would not demonstrate mental retardation if they were prevented completely from exposure to tyrosine [18]. In other words, a mutation can not cause the phenotype without interaction from environmental factors. Regarding NPC development, the role of *CYP2E1* variant may be varied upon the amount of consumed salted fish and/or preserved foods that contain nitrosamine and nitrosamine precursors.

Conclusion

Result of the reported crude odds ratio is 2.19 [95%CI = 0.62-8.68]. If the result is adjusted odds ratio, it will be 2.39 [95%CI = 0.72-7.89], which is closer to the Taiwanese odds ratio of 2.6, with only a slight loss in precision. Thus, this study confirmed a previous study in Taiwan that *CYP2E1* appears to be one of a number of NPC susceptibility genes and the RsaI minus variant is not just a polymorphism but directly influences the development of the phenotype.

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Competing interests

None declared

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ยีน FERB11 (MIC) กับการเกิดโรคมะเร็งโพรงหลังจมูก

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PERB11 (MIC) as a possible susceptibility gene for nasopharyngeal cancer development

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Background : *There have been many studies indicating that a gene located near HLA A2 and HLA B46 is a tumor susceptibility gene involved in nasopharyngeal cancer (NPC) development. PERB11 (MIC) is a candidate gene since it is linked to HLA B.*

Objective : *To investigate the association between PERB11 (MIC) and the probability of having NPC.*

Materials and Methods : *The frequencies of six alleles from a triplet repeat polymorphism of the transmembrane region of MICA (PERB11.1) were analyzed by PCR from 300 healthy blood donors' and 171 NPC patients' DNA samples. The relative risk was estimated by odds ratio to determine the allele association with NPC patients in comparison with normal controls.*

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Result : *The frequency of the A6 allele, but not others, was increased in the patient group, as compared with the control group (RR = 1.6, P < 0.05, OR = 1.60, 95 % CI = 0.98-2.63).*

Conclusion : *One particular allele (A6) of the PERB11 (MIC) gene presents at a higher frequency in NPC patients than in controls. This suggests a possible association of NPC development with the PERB11 gene.*

Key words : *PERB11 (MIC) gene, Polymorphism, Nasopharyngeal cancer.*

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ปัญหาของการทำวิจัย : *มีการศึกษาพบว่ายีนที่อยู่บริเวณ HLA A2 และ HLA B46 เป็นยีนที่ส่งเสริมให้เกิดมะเร็งชนิดนี้ เนื่องจากยีน PERB11 (MIC) เป็นยีนที่อยู่ใกล้กันกับยีน HLA B ดังนั้น PERB11 (MIC) อาจจะเกี่ยวข้องกับการเกิดโรคมะเร็งโพรงหลังจมูก*

วัตถุประสงค์ : *เพื่อศึกษาหาความสัมพันธ์ระหว่างยีน PERB11 (MIC) กับการเกิดโรคมะเร็งโพรงหลังจมูก*

วัสดุและวิธีการวิจัย : *คณะผู้วิจัยได้ทำการศึกษาความสัมพันธ์ระหว่างยีน PERB11 (MIC) กับการเกิดโรคมะเร็งโพรงหลังจมูกโดยเปรียบเทียบระหว่างดีเอ็นเอจากเลือดคนปกติ 300 ราย และ 171 รายจากคนผู้ป่วยที่ได้รับการตรวจวินิจฉัยว่าเป็นโรคมะเร็งโพรงหลังจมูกโดยหาความสัมพันธ์ระหว่างความถี่ของอัลลีลทั้งหกของ triplet repeats ในส่วนของยีนที่สร้างโปรตีนอยู่ในเยื่อบุเซลล์ของยีน PERB11 (MIC) ในกลุ่มของผู้ป่วยกับคนปกติโดยดูถึง*

ผลการศึกษา : *พบว่าความถี่ของยีนเฉพาะอัลลีล A6 เพิ่มขึ้นในกลุ่มของผู้ป่วยเมื่อเปรียบเทียบกันกับกลุ่มคนปกติ (R.R.=1.6, P < 0.05, OR = 1.60, 95% CI = 0.98-2.63) แต่ไม่พบการเพิ่มขึ้นอย่างมีนัยสำคัญในอัลลีลอื่น ๆ ของยีน PERB11 (MIC)*

สรุป : *ข้อมูลของคณะผู้วิจัยพบว่ามีความถี่ของอัลลีล A6 เพิ่มขึ้นในกลุ่มผู้ป่วยที่เป็นโรคมะเร็งโพรงหลังจมูกเมื่อเปรียบเทียบกันกับกลุ่มคนปกติ แสดงให้เห็นถึงความเป็นไปได้ของความสัมพันธ์ระหว่างการเกิดโรคมะเร็งโพรงหลังจมูกกับยีน PERB11 (MIC)*

The etiology of nasopharyngeal carcinoma (NPC) has opened an interesting field of study concerning the interplay between genetic and environmental factors combined with Epstein – Barr virus (EBV) infection. NPC is rare among Caucasians, with incidence rates below 1 per 100,000 persons/ year. Among Chinese, with a high incidence rate (30 - 50 per 100,000/year) and Southeast Asians with an intermediate rate (3 -10 per 100,000 people/year), the possibility of a genetic contribution becomes apparent. There have been many reports indicating various human leukocyte antigen (HLA) alleles are associated with NPC. The link between HLA and NPC was first reported in Singapore [1] and this finding has subsequently been confirmed in several countries in Asia. [2] All have demonstrated the association with HLA-A2 and HLA-B46 (relative risk = 2.35). However, HLA-A2 subtyping studies have shown that it is unlike' to play a role in the EBV clearance hypothesis. In addition, in a causative association the risk would increase if both HLA-A2 and B46 were inherited on the same chromosome, i.e. haplotype. In contrast, this HLA haplotype in non-Chinese patients is not associated with NPC. These data suggest that HLA is not the susceptibility gene *per se*, but the NPC susceptibility gene locus is most likely to reside within the HLA region.

PERB11 (*MIC*), a major histocompatibility complex (MHC) class I chain-related gene, is located in the HLA region. *PERB11.1* (*MICA*), an expressed *PERB11*, has recently been identified to be located near the HLA-B locus [3,4] and displays 6 distinct alleles of microsatellite polymorphism in the transmembrane (TM) region.[5,6] *MICA* is frequently expressed in epithelial tumors. Upon interaction between MICA and its receptor NKG2D, diverse innate anti-tumor NK cell and antigen-specific T-cell responses are triggered. [7] Therefore, *PERB11* (*MIC*) is a candidate tumor susceptibility gene.

In this study, we have investigated the correlation between short tandem repeat polymorphisms in the TM of *PERB11.1* (*MICA*) and NPC development in a total of 171 cases diagnosed with NPC and 300 controls. The hypothesis was that if one of the alleles was significantly increased in the patients, as compared with the control group, the *PERB11* (*MIC*) gene might be a NPC susceptibility gene.

Method

Samples and DNA extraction

Blood samples were obtained by venipuncture from 300 healthy blood donors (Thai Red-Cross Society) and 171 NPC patients (Chulalongkorn Hospital and National Cancer Institute). The diagnosis had been confirmed histologically and by the presence of EBV DNA in the tumors. DNA was extracted from blood leukocytes by methods previously described. [8]

PCR

For analysis of the microsatellite repeat polymorphism in the TM region of the *MICA* gene, PCR primers flanking the TM region were used (MICA5F, 5'-CCTTTTTTTCAGGGAAAGTGC-3'; MICA5R, 5'-CCTTACCATCTCCAGAAACTGC-3'). [5] The PCR reactions were performed in a total volume of 10 µl using 50 ng of gemomic DNA, 200 µM each dNTP, 10 µM Tris-HCl (pH 8.4), 50 mM potassium chloride, 2.5 mM magnesium chloride, 0.5 units of *Thermus aquaticus* DNA polymerase (Perkin Elmer Cetus) and

0.1 μM of each primer. One of each primer pair was end labelled at 37°C for 1-2 h in a total volume of 10 μl containing 10 μM primer, 0.025 mCi [γ^{32}P] ATP (Amersham Pharmacia Biotech) at 3000 Ci mmol^{-1}, 10 μM magnesium chloride, 5 mM DTT, 70 mM Tris-HCl (pH 7.6) and 10 units of T4 polynucleotide kinase (New England Biolabs). Without further separating of the unincorporated nucleotides, the kinase reaction was added to the PCR buffer mix. The PCR amplifications were performed as follows: initial denaturation at 94°C for 5 min, followed by 25 cycles of denaturation at 95°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.

Two microlitres of each reaction were mixed with 1 μl of formamide-loading buffer, heated at 95°C for 2 min, put on ice for 30 s and then loaded onto 6 % polyacrylamide/7 M urea gel. DNA fragments were size fractionated at 70 W until the tracking dye had covered the appropriate distance of the gel. After electrophoresis, the wet gel was transferred to filter paper (Whatman), wrapped with Saran wrap and exposed to a phosphorus screen; the bands were visualized on PhosphoImager using ImageQuaNT software (Molecular Dynamics).

Statistical Analysis

Gene frequencies were estimated by direct counting. The significance of the distrubution of alleles between NPC patients and normal controls were tested by chi-square (χ^2) method with continuity correction and Fisher's extra probability test (P value test).[9] Comparison between two groups was made with a 95 % confidence interval to estimate statistical significance.

Result and Discussion

To address the possibility that *MICA* is a susceptibility gene for NPC development, triplet repeat polymorphisms in the TM region of *PERB11.1* (*MICA*) were investigated in 171 cases diagnosed with NPC and 300 healthy blood donors as a control (Fig 1).

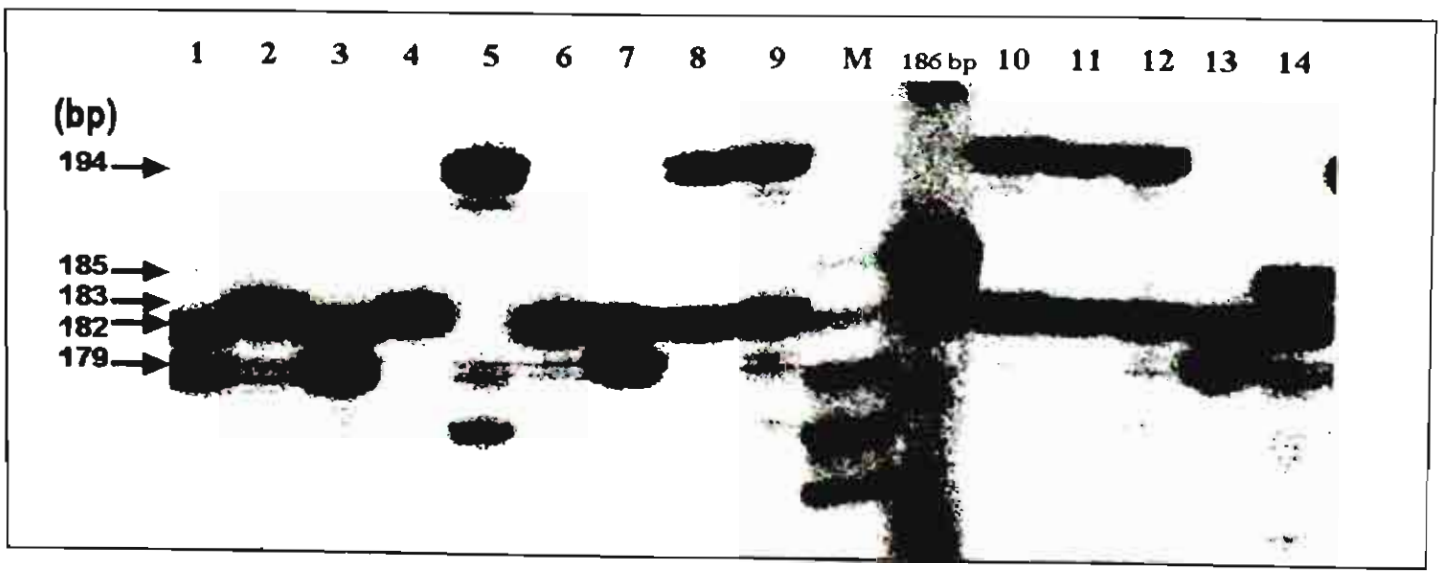

Figure 1. Microsatellite analysis of PCR-amplified products of triplet repeat polymorphism in the TM region of the PERB11.1 (MICA) gene. Cases 1-14: nasopharyngeal cancer patients. M, molecular marker.

PERB11 displays 6 distinct alleles of microsatellite polymorphism at the TM region. The frequency of A6 allele was significantly increased in the patient group, as compared with the control group (R.R.=1.6, $P < 0.05$, O.R. = 1.60, 95 % CI = 0.98 - 2.63) (Table 1) but none of the other alleles showed any significant association. These data suggest a possible important role for this allele in the development of NPC. Interestingly, A6 is the same allele found to be associated with Beh?et disease in the Japanese population. [6]

In conclusion, we have found that one particular allele (A6) of the microsatellite is present at a higher frequency in NPC patients than in controls. Yet, since the 95 % CI contains the nominator 1 the OR value dies not achieve significance. This may well be due to the small sample size employed in the present study. Since the TM polymorphism is not well correlated with polymorphisms of the extracelluiar domains, [7,8] it is essential to further evaluate this polymorphism to establish the relevance of this gene family in NPC development.

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Table 1. Gene frequencies of the microsatellite polymorphism in the TM region (exon 5) of the PERB11.1 (MICA) gene in nasopharyngeal carcinoma

Microsatellite allele	Amplified product (bp)	Control (n = 600)	Patient (n = 342)	P value	R.R.	95% CI
A4	179	87	40			
A5	182	220	129			
A5.1	183	138	70			
A6	185	41	36	0.046	1.60	0.98 - 2.63
A9	194	114	67			

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Case Report

Astroblastoma: Report of a case with microsatellite analysis

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A 5-year-old girl who developed progressive headache, vomiting, and left hemiparesis was found to have a cystic tumor with an enhanced mural nodule in the right frontoparietal region on a computed tomography examination. The lesion was histologically and ultrastructurally verified as an astroblastoma, an uncommon neuroepithelial tumor of uncertain origin. Molecular analysis using 17 microsatellite markers on chromosomes 9, 10, 11, 17, 19, and 22 showed loss of heterozygosity at the D19S412 locus on the long arm of chromsome 19. This observation suggests that there is a tumor suppressor gene in this chromosomal region, which plays a role in the pathogenesis of astroblastoma.

Key words: astroblastoma, glioma, loss of heterozygosity, tumor suppressor gene.

INTRODUCTION

Bailey and Busy originally described astroblastomas in 1930 and considered their prognosis to be intermediate between that of astrocytoma and glioblastoma.[1] Exact epidemiological data of astroblastomas are not available but, in general, they are uncommon and most frequently affect young individuals, usually as circumscribed supratentorial tumors.[1,2]

Because of their rarity, information regarding the genetics of astroblastomas is scant. Cytologic analysis of one case demonstrated an abnormal hypodiploid karyotype with 45 chromosomes and monosomies of chromosomes 10, 21, and 22 and two additional marker chromosomes in all cells studied.[3] Molecular analysis of another example disclosed allelic loss on chromosomes 4p, 9p, 14q, 17p, 17q, 22q and Y. The purpose of the present paper is to report a case of astroblastoma in which we have investigated the loss of tumor suppressor genes in oligodendrogliomas by loss of heterozygosity (LOH) on the chromosomes 9, 10, 11, 17, 19, and 22 which occurs commonly in human gliomas.

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CLINICAL SUMMARY

A 5-year-old girl presented with a 2-month history of progressive headache and vomiting. Weakness of left extremities was noticed 1 month prior to admission. Neurological examination revealed left hemiparesis and bilateral papilledema. A contrast-enhanced computed tomography (CT) scan of the brain (Fig. 1) revealed a well-demarcated cystic mass measuring 6.3 × 5.2 × 4.5 cm with hyperdense mural nodule in the right frontoparietal region. The lesion involved the right basal ganglia and thalamus and extended to the right posterior limb of the internal capsule as well as into the deep white matter of the parietal lobe. The right lateral ventricle was compressed by the mass effect. There was evidence of right uncal and subfalcine herniations.

Intraoperatively, the tumor was totally removed. Clear yellow fluid was found in the cystic component. The patient's postoperative course was uneventful. The patient received megavoltage radiation therapy.

METHODS

Sections from formalin-fixed paraffin-embedded tissue were histologically examined with hematoxylin and eosin. Immunohistochemical studies were performed using the following antibodies: glial fibrillary acidic protein (GFAP), S-100 protein, vimentin, epithelial membrane antigen (EMA) and neurofilament protein (NF). Details of the antibodies used and results are given in Table 1. Elec-

Table 1 Antibodies used in the immunohistochemical study of astroblastoma

Antibodies	Species	Clonality	Source	Dilution	Result
Anti-GFAP	Rabbit	Polyclonal	Dako	1:1000	+
Anti-S-100 protein	Rabbit	Polyclonal	Dako	1:400	+
Anti-vimentin	Mouse	Monoclonal	Dako	1:200	
Anti-EMA	Mouse	Monoclonal	Dako	1:50	
Anti-NF	Mouse	Monoclonal	Dakos	1:100	

GFAP, glial fibrillary acidic protein; EMA, epithelial membrane antigen; NF, neurofilament; Dako, Japan

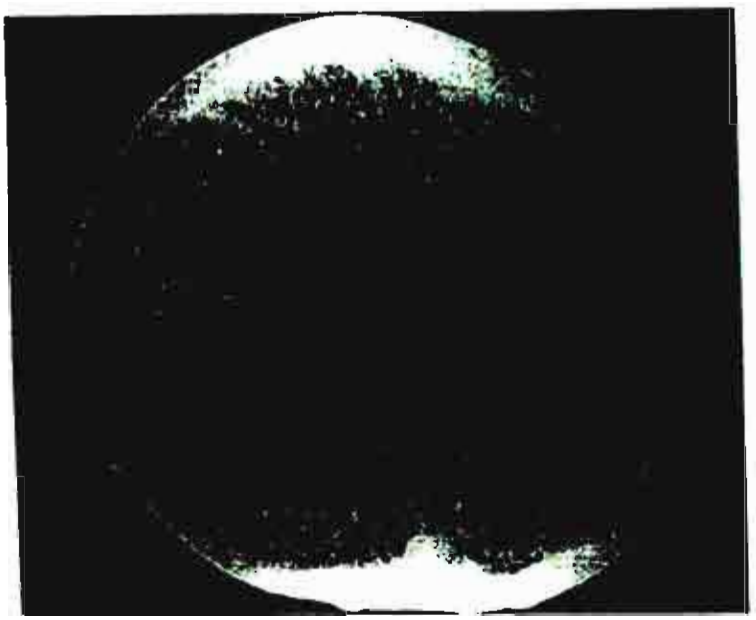

Fig. 1 An axial postcontrast computed tomography scan showing a well-circumscribed cystic mass with a moderately enhanced mural nodule in the right frontoparietal region.

microsposy was carried out using representative tissue extracted from a paraffin block.

LOH was determined using 17 microsatellite polymorphic markers on chromosomes 9 (D9S1748: 9p21, RPS6: 9p21, IFNA: 9p22), 10 (D10S196: 10q11.2, D10S178: 10pter-10qter, D10S249: 10pter-10qter), 11 (D11S554: 11p12-11p11.2, WT1: 11p13), 17 (D17S1176: 17p13.1, TP53: 17p31.1, D17S1876: 17pter-17qter), 19 (D19S47: 19q13.1, ERCCII: 19q13.2, D19S412: 19q13.3) and 22 (D22S281: 22q12.2-12.3, D22S284: 22q13.1-13.2, D22S282: 22q13.2-13.3). Information regarding the cytogenetic localization of the markers was obtained from the Genome Data Base.

Before microsatellite analyses, tumor DNA extracted from 20 paraffin sections of 5 µm thickness from a tissue area of 2.0 × 1.5 cm was amplified by degenerated oligonucleotide-primed polymerase chain reaction (DOP-PCR) in a total volume of 50 µL as previously described.[8] One strand of each primer pair was end-labeled as described elsewhere.[9] The PCR reactions were performed in a total volume of 10 µL consisting of 200 µmol/L deoxyribonucleoside triphosphate, 10 mmol/L tris-HCL (pH 8.4), 50 mmol/L KCL, 1.5 mmol/L $MgCl_2$, 0.5 U *Thermus aquaticus* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT, USA), primer concentrations between [illegible] each, and 50 ng of genomic DNA extracted from both leukocyte extraction and DOP PCR extracted paraffin-embedded tissue.

Several PCR reactions have been optimized for each primer set as follows: an initial denaturation step at 95°C for 4 min, followed by 40 cycles of denaturation at 92°C for 1 min, with 1 min annealing at 55°C, extension at 72°C for 2 min, and a final extension at 72°C for 7 min.

Aliquots (2 µL) of each reaction were mixed with formamide-loading buffer, heated at 95°C for 2 min, chilled on ice for 30 s, and then loaded onto a polyacrylamide 7 mol/L urea gel. DNA fragments were size-fractionated at 70 W-unit until the tracking dye had reached the appropriate distance on the gel. After electrophoresis the wet gel was transferred to filter paper (Whatman, Maidstone, UK) covered with Saran wrap and exposed to a phophorous screen; the bands were visualized on a PhosphorImager using ImageQuaNT™ software (Molecular Dynamics, Sunnyvale, CA, USA).

PATHOLOGICAL FINDINGS

The lesion predominately consisted of perivascular pseudorosettes formed by neoplastic cells with coarse non-tapering cell processes centrally radiating to central vascular channels (Fig. 2a). The tumor cells were round to oval and showed brisk mitotic figures. There was marked sclerosis of the blood vessels and stroma. Several foci of necrosis and occasional vascular proliferation were encountered. Immunostainings for GFAP (Fig. 2b), S-100 protein and vimentin were positive, whereas negative results were noted on EMA and NF.

Electron microscopy showed tumor cells with ill-defined cell borders (Fig. 3a). There were abundant bundles of intermediate filaments within the cytoplasm (Fig. 3b). Basal lamina were seen separating these neoplastic cells from blood vessels. The pathological diagnosis of astroblastoma was made.

The D19S412 locus was the only informative marker on the long arm of chromosome 19 that showed LOH

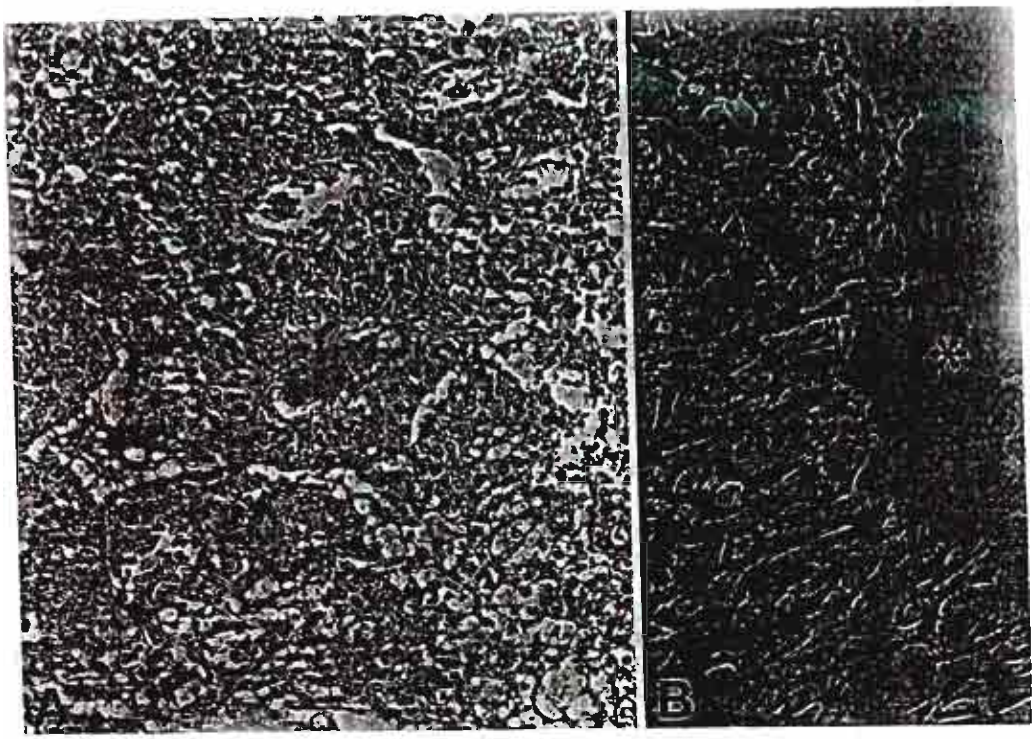

Fig. 2 Histopathologically, astroblastoma predominately consists of (a) perivascular pseudorosette (HE). (b) Glial fibrillary acidic protein immunostain highlights thick non-tapering cell processes radiating toward a central blood vessel (asterisk; immunoperoxidase with hematoxylin counterstain).

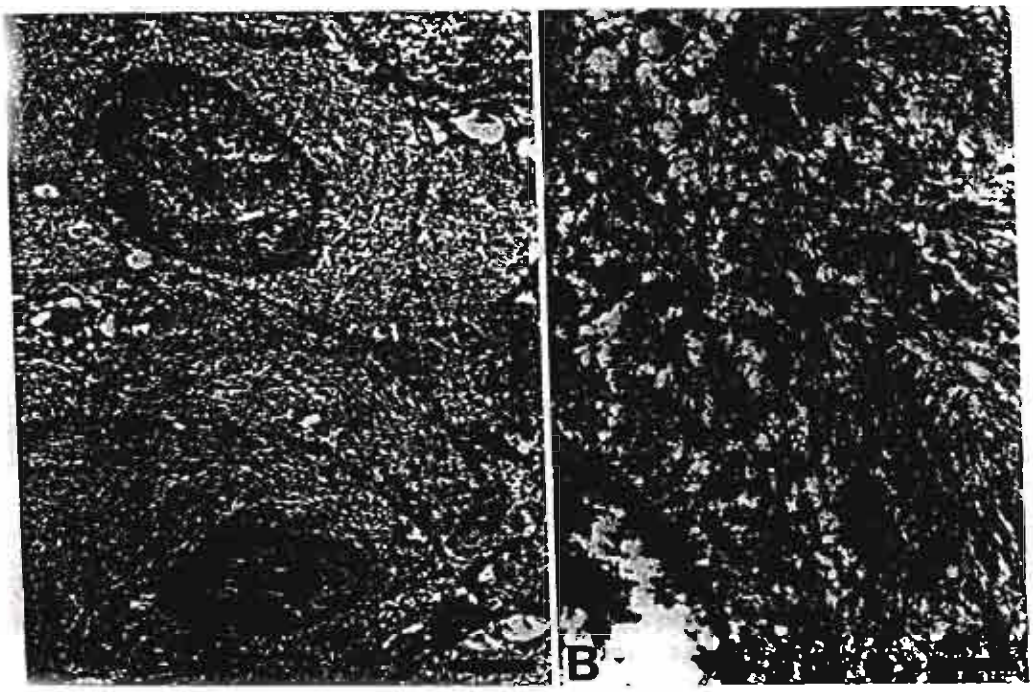

Fig. 3 (a) Ultrastructurally, tumor cells possess a well-demarcated cell border. (Bar = 2 μm). (b) Abundant 6–9-nm intermediate filaments are demonstrated in the cytoplasm. (Bar = 0.5 μm).

4b). The other microsatellite markers on chromosomes 9, 10, 11, 17 and 22 demonstrated either maintenance of both alleles or uninformative data. At least one informative locus in each chromosome, however, was tested. A representative microsatellite locus (D11S554) showing maintenance of both alleles was also depicted (Fig. 4a).

DISCUSSION

In the recent World Health Organization classification of the central nervous system tumors, astroblastomas appear in the group of neuroepithelial tumors of uncertain origin, reflecting their controversial histogenesis.[10] The

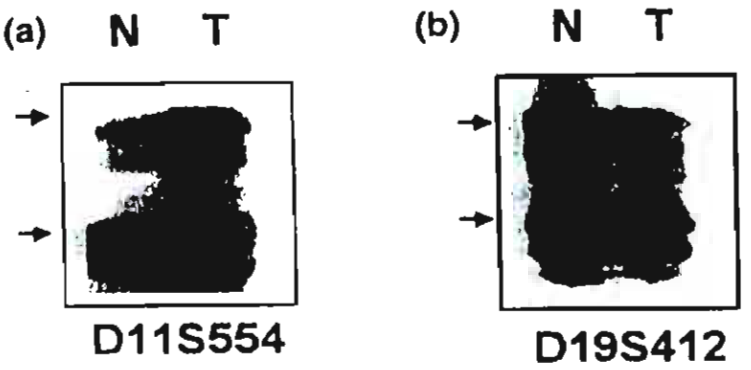

Fig. 4 Autoradiographs show (a) maintenance of both alleles at D11S554 on chromosome 11p and (b) loss of the upper allele at the D19S412 locus on chromosome 19q. N, normal leukocyte DNA; T, tumor DNA.

positivity to GFAP, S-100 protein, and vimentin immunostains, together with the presence of intermediate filaments on electron microscopy and the absence of ependymal or neuronal features favor a putative astrocytic origin.[2] Based on an electron microscopic, immunohistochemical, tissue and organ culture study showing a glial cell with features intermediate between those of ependymocytes and astrocytes, however, the tanycyte, a glial precursor cell normally found scattered along the ependymal lining of the embryonal and neonatal mammalian brain, was also considered to be the cell origin of astroblastomas.[11] Although the entity is not now universally accepted, a series of 17 astroblastomas showing distinct clinicopathological features has recently been described.[17]

A well-demarcated cystic mass with an enhancing mural nodule could be one of several lesions that have a similar radiological feature, including pilocytic astrocytoma, ganglioglioma, pleomorphic xanthoastrocytoma (PXA) and hemangioblastoma.[13–16] Pilocytic astrocytomas are low-grade astrocytic neoplasms that commonly occur in childhood. Their most common locations are the midline structures including the visual system, the hypothalamus and the cerebellum, but not the cerebral hemispheres.[13,14] Although they are frequently encountered in the cerebral hemispheres, gangliogliomas and PXA favor the temporal lobes and most patients with these tumors present with seizures.[13,16,17] Hemangioblastomas may also demonstrate the same radiological finding but they are rare in children.[13,15]

The histological differential diagnosis of astroblastomas include ependymomas, papillary meningiomas and diffuse astrocytomas. Ependymomas are common intraventricular tumors but occasionally occur in the brain parenchyma. The problem is more complicated by the fact that both tumors show identical immunoreactivity, being positive for GFAP, vimentin and S-100 protein.[18] Nevertheless, cytoplasmic processes in ependymoma are typically fine and taper towards central vascular channels, whereas those in astroblastoma are coarse and differ further in terminating on their target vessels as expanded footplates.[1,2,18] Extensive vascular sclerosis is another finding suggestive of astroblastoma.[18]

Vascular cores surrounded by neoplastic cells in papillary meningiomas are distinctly similar to perivascular pseudorosettes of astroblastomas.[18] Most meningiomas, however, are dural-based lesions.[1] The lack of [illegible] meningothelial differentiation together with immunohistochemistry showing reactivity for [illegible] negativity for EMA excludes this possibility.

It should be kept in mind that diffuse astrocytomas, especially the gemistocytic variant and glioblastomas, frequently contain focal areas of perivascular pseudorosettes. Therefore the diagnosis of astroblastoma should be reserved for well-demarcated gliomas that are or mainly composed of the characteristic glioblastic structure mentioned here.[1,13,18]

Electron microscopy is essential to confirm the diagnosis of astroblastoma even though there is no ultrastructural feature that can be regarded as characteristic of this entity.[3] The presence of cytoplasmic intermediate filaments is a consistent finding that was also observed in the present case.[3,19]

LOH 19q in the present study of astroblastoma suggests that there may be a tumor suppressor gene (TSG) in this chromosomal region that plays a role in astroblastoma oncogenesis. The locus D19S412 also lies among the common sites of allelic loss in diffuse astrocytomas and oligodendroglial tumors.[6,7] Because the TSG in the q arm of chromosome 19 that is responsible for the development of these central nervous system neoplasms has not been identified, it remains to be elucidated whether or not these tumors are caused by mutations in the same gene. Microsatellite analysis on chromosome 1p in astroblastoma is also of interest because oligodendrogliomas frequently show LOH in this chromosomal region.[7]

A question may be raised as to whether LOH 19q in the present case is true or whether it has resulted falsely from allele dropout because the tumor DNA was extracted from paraffin-embedded tissue while the constitutional DNA came from blood leukocytes. We do believe, however, that such a possibility is unlikely because a minimally contaminated normal tissue (e.g. endothelial cells around the tumor) was amplified, as reflected by a weak normal band in the tumor (T) lane (Fig. 4a). Both the contaminating normal and the tumor DNA were derived from the same paraffin blocks. Furthermore, it has been suggested that the incidence of allele dropout during DOP-PCR is low if provided a large amount of tissue is used.[5]

In summary, an astroblastoma is reported in a 5-year-old girl. The presence of allelic loss on the long arm of chromosome 19 indicates that this chromosomal region harbors a putative tumor suppressor gene related to astroblastoma oncogenesis.

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