รายงานวิจัยฉบับสมบูรณ์

โครงการ

" อณูพันธุศาสตร์ของมะเร็งโพรงหลังจมูก : ลักษณะทางพันธุกรรมที่เสี่ยงต่อการเกิดโรค ขบวนการของการกลายพันธุ์และการประยุกต์ใช้ทางคลินิค "

รองศาสตราจารย์ นายแพทย์อภิวัฒน์ มุทิรางกูร

เสร็จสิ้นโครงการ 31 ตุลาคม พ.ศ. 2545

BRG/9/2542

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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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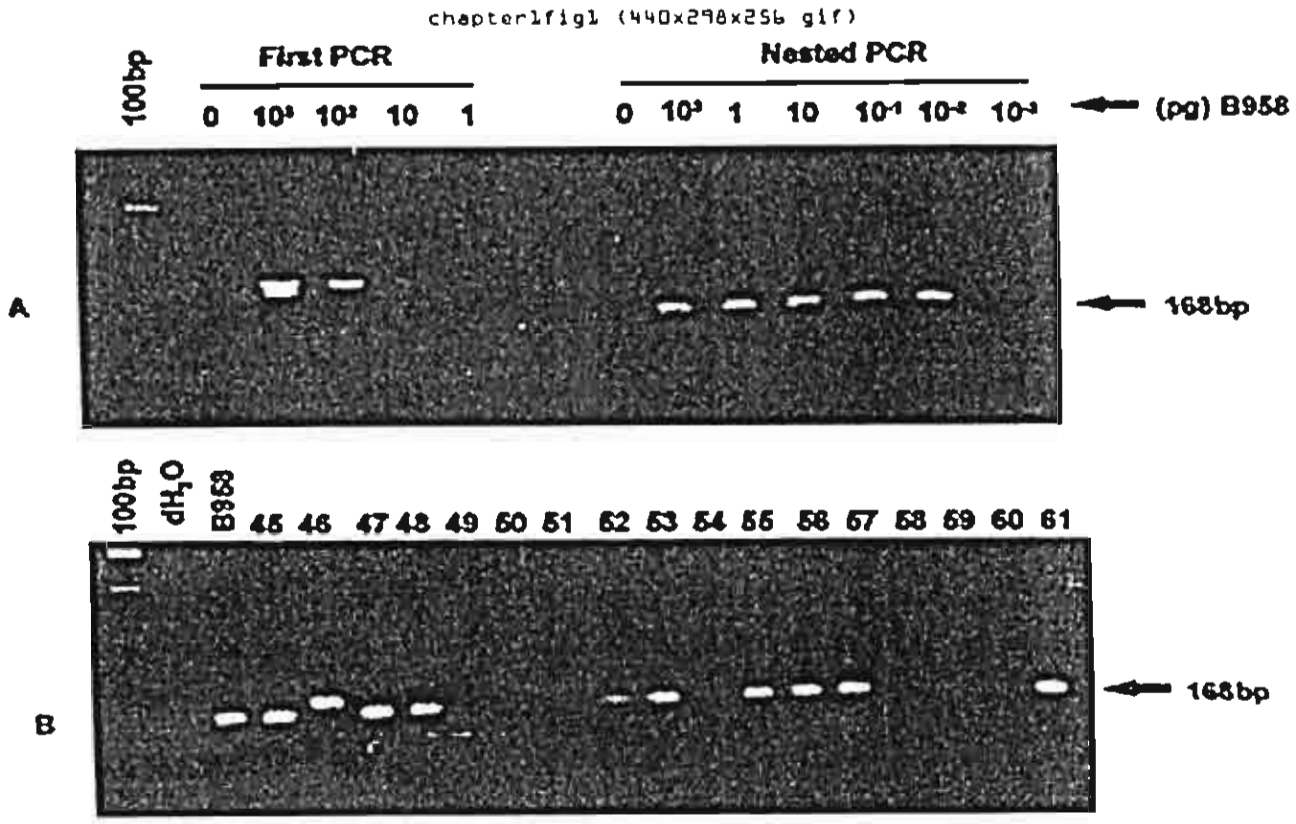

Fig. 1. Sensitivity of nested PCR for EBV DNA. A 100-bp ladder, distilled H_2O (dH_2O), and DNA from B958 cell lines were used as size standard, negative control, and positive control, respectively. *A*, compare sensitivity between single PCR and nested PCR for EBV DNA. *B*, examples of nested PCR for 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.

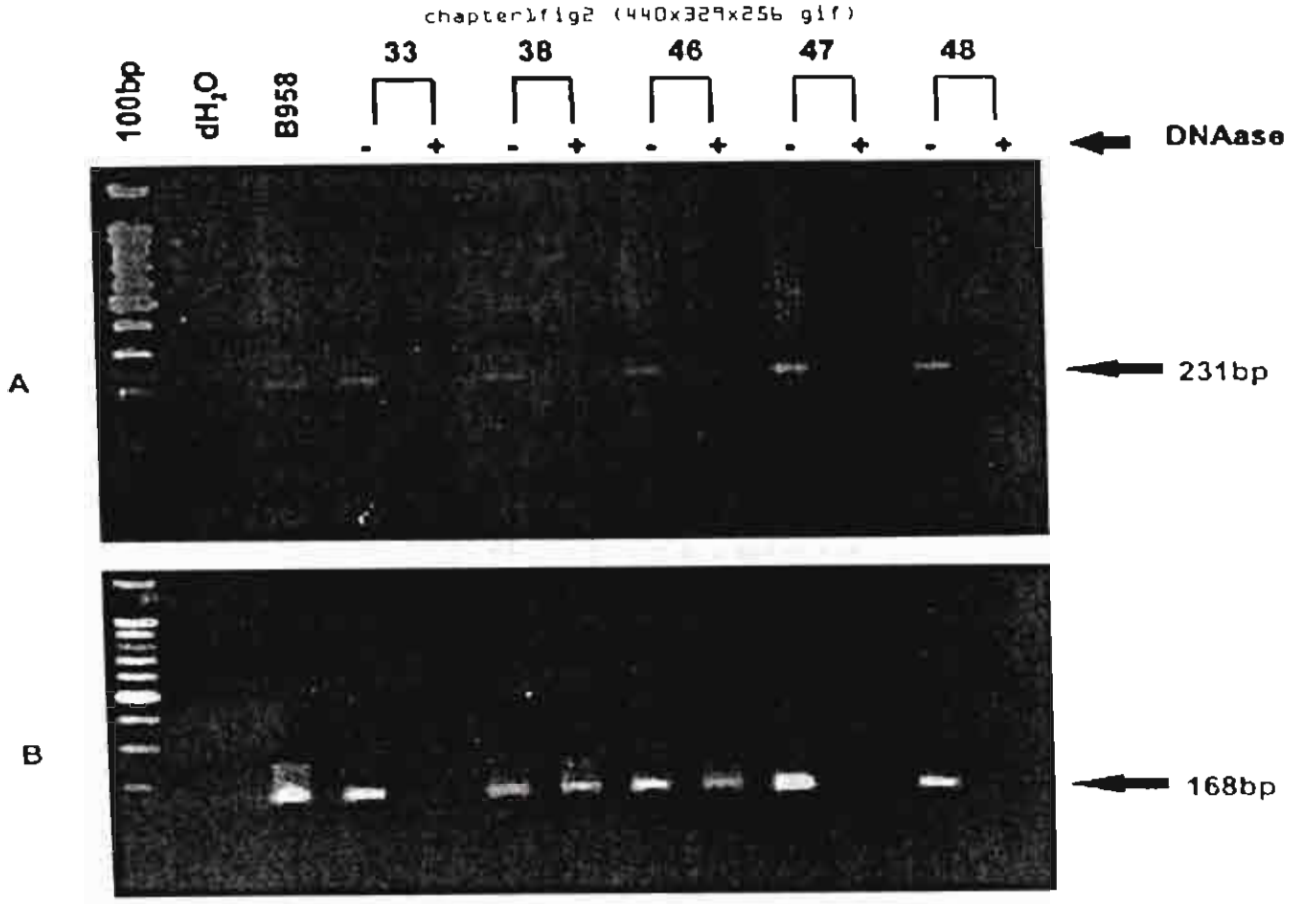

Fig. 2. Nested and seminested PCR from known EBV DNA-positive serum samples after DNase treatment. DNA. A 100-bp ladder, distilled H_2O (*dH_2O*), and DNA from B958 cell lines were used as size standard, negative control, and positive control, respectively. Lanes with (+) and without (-) DNase treatment 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.

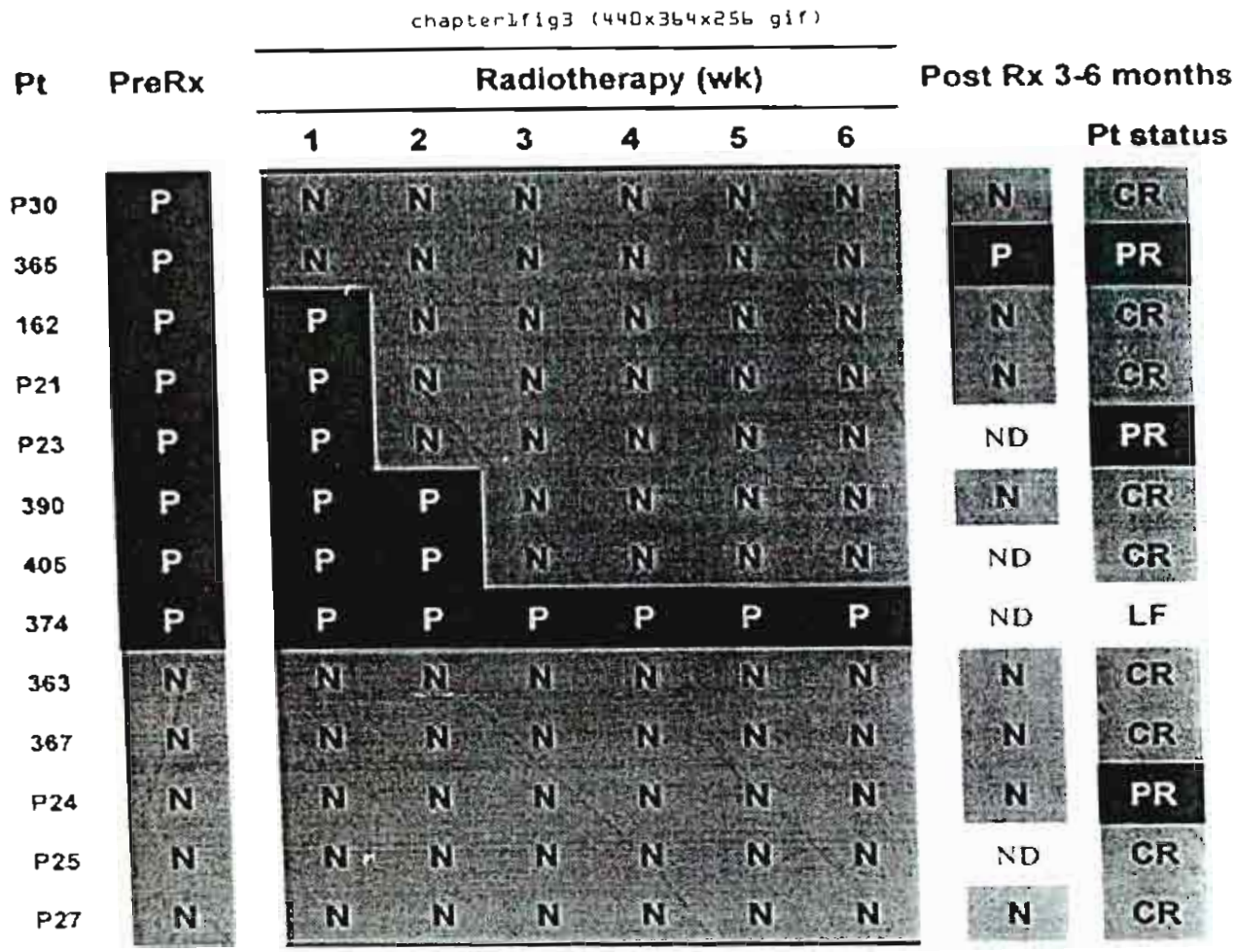

Fig. 3. Plasma EBV DNA at the end of each week during radiotherapy. Present (*P*) and absent (*N*) EBV DNA are shown prior (*PreRx*) and during treatment. NPC status of each patient at 3–6 months after treatment is demonstrated as complete remission (*CR*) and partial response (*PR*). *LF*, loss to follow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

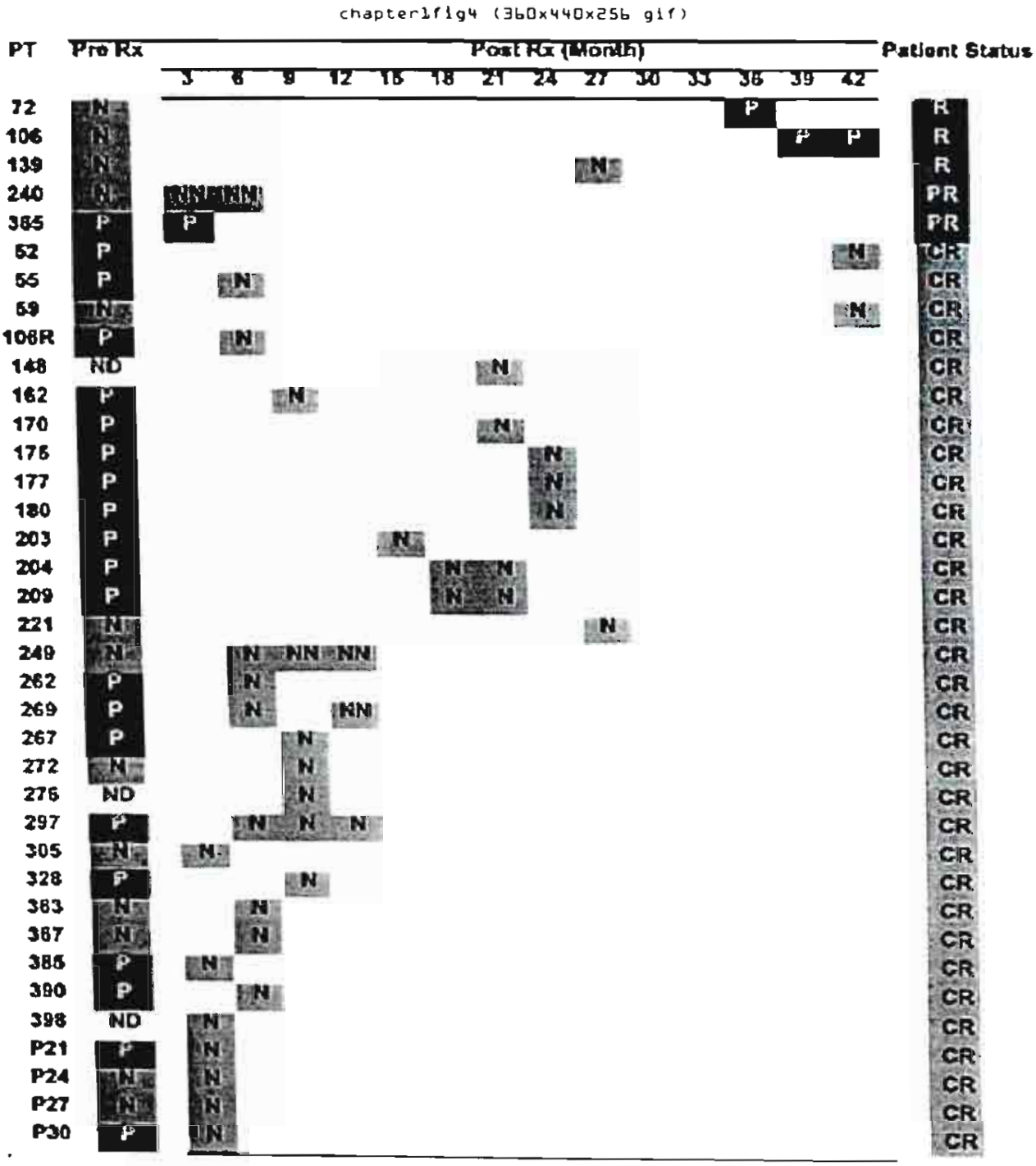

Fig. 4. Serum/plasma EBV DNA during follow-up after complete treatment. NPC status of each patient is demonstrated as recurrence (*R*), partial response (*PR*), and complete remission (*CR*). Present (*P*) and absent (*N*) of EBV DNA are shown. *ND*, not done.

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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.

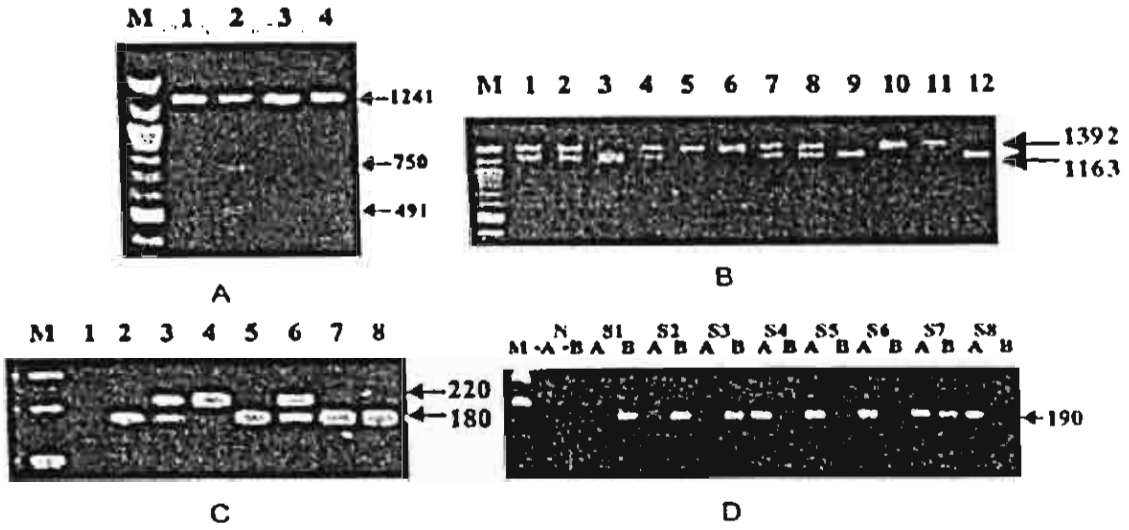

Figure 1 SNPs analysis of *CR2* and *PIGR*. (A) The *CR2*IVS2-848C→T was distinguishable by *TaqI* restriction. Digestion of the 1241 bp amplicon yielded two DNA fragments, 750 and 491 bp. Lane 1, 3 and 4 were homozygous -/- and lane 2 was heterozygous +/-. (B-D) *PIGR* polymorphisms was investigated 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

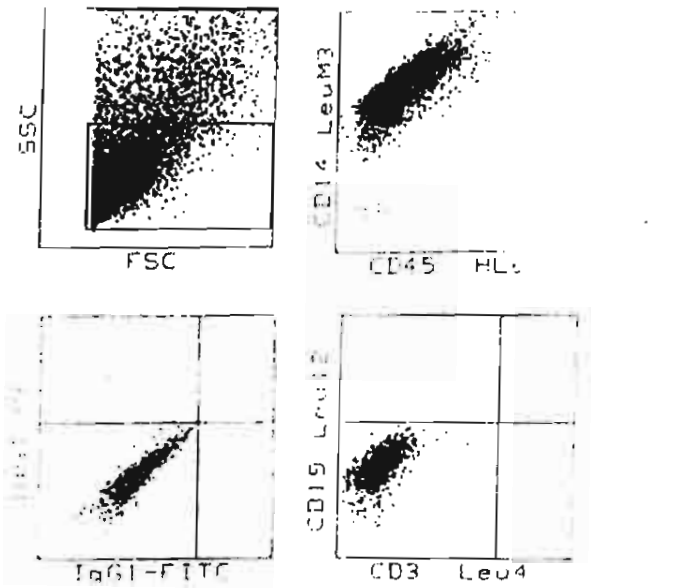
Patient code	Age	Sex	Tumor Stage	WHO	PCR	RT-PCR			
					EBNA1	*RAG1*	*RAG2*	*LMP1*	*LMP2A*
304	32	M	III	II	+	-	-	+	+
381	49	F	III	III	+	-	+	-	+
384	42	F	III	II	+	+	+	+	+
397	50	M	IV	III	+	+	+	-	+
405	48	M	III	III	+	+	-	+	+
424	57	M	IV	II	+	+	+	-	+
433	35	F	III	II	+	+	+	+	+
436	45	M	I	III	+	+	+	+	+
437	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

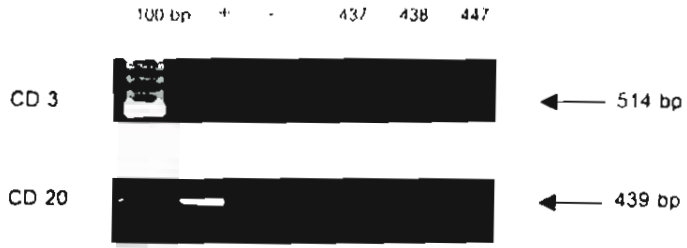
FIGURE LEGENDS

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

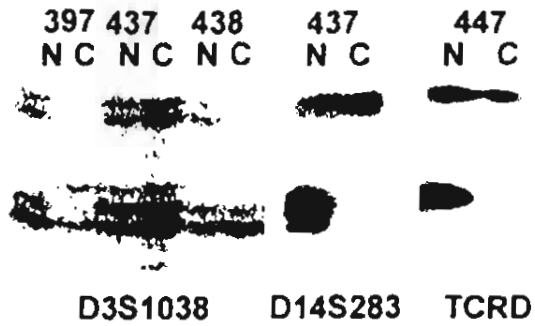
397 437 438 437 447
N C N C N C N C N C
D3S1038
D14S283
TCRD

Fig 1

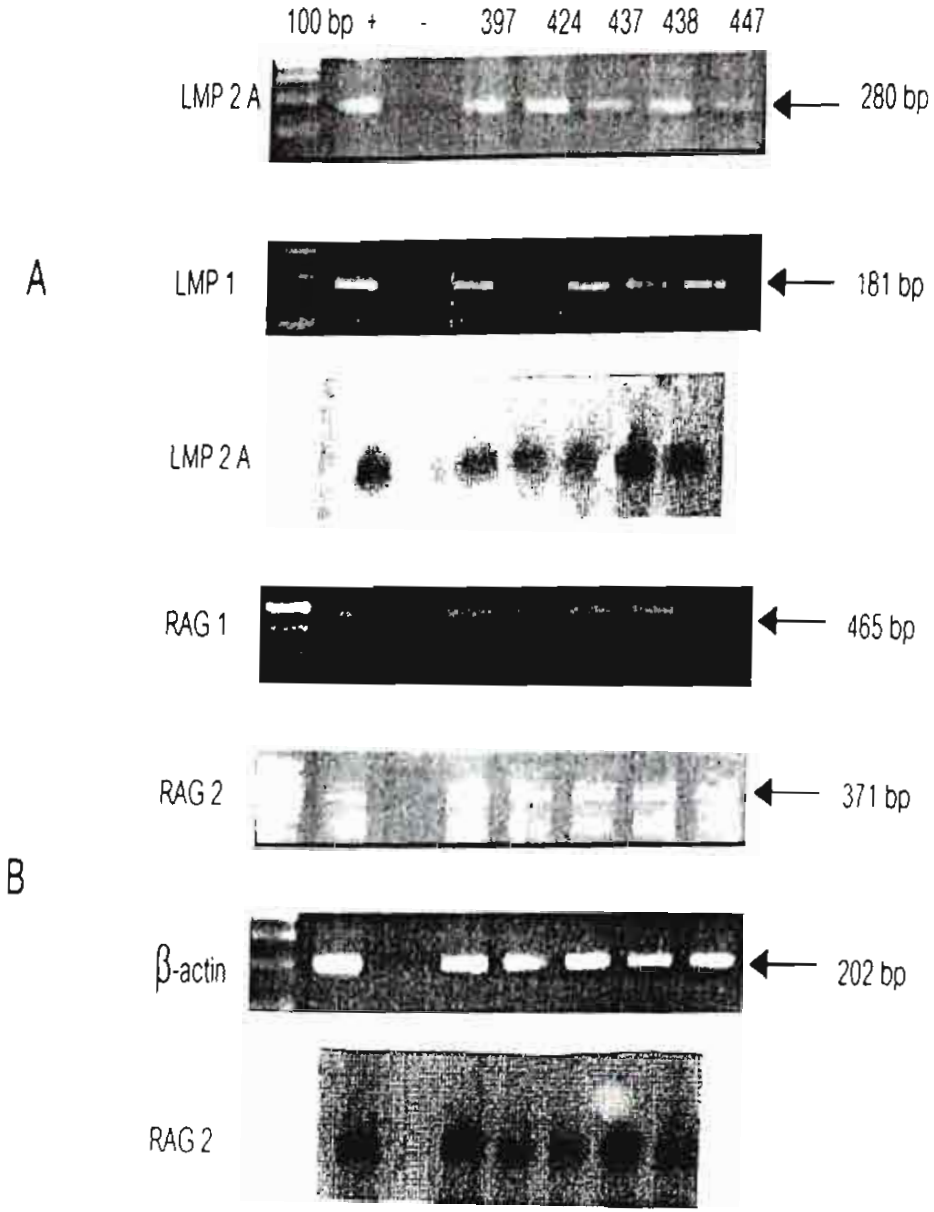
100 bp
+
-
397
424
437
438
447
LMP 2 A
280 bp
A
LMP 1
181 bp
LMP 2 A
RAG 1
465 bp
RAG 2
371 bp
B
β-actin
202 bp
RAG 2

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

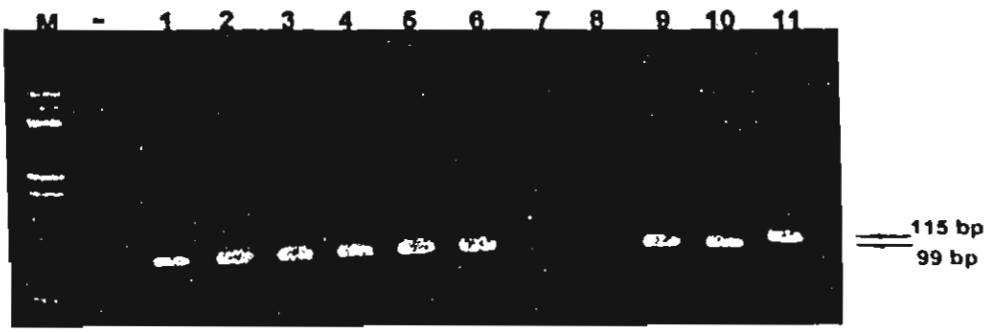

Fig 1. Examples of nested PCR products from NPC swab in agarose gel electrophoresis. The 99- or 115-bp fragments indicated the presence of EBV DNA; 100-bp DNA ladder *(lane M)* was used for DNA size standard. Distilled water *(-)* was used as a negative control. Numbers *1* through *11* are PCR products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

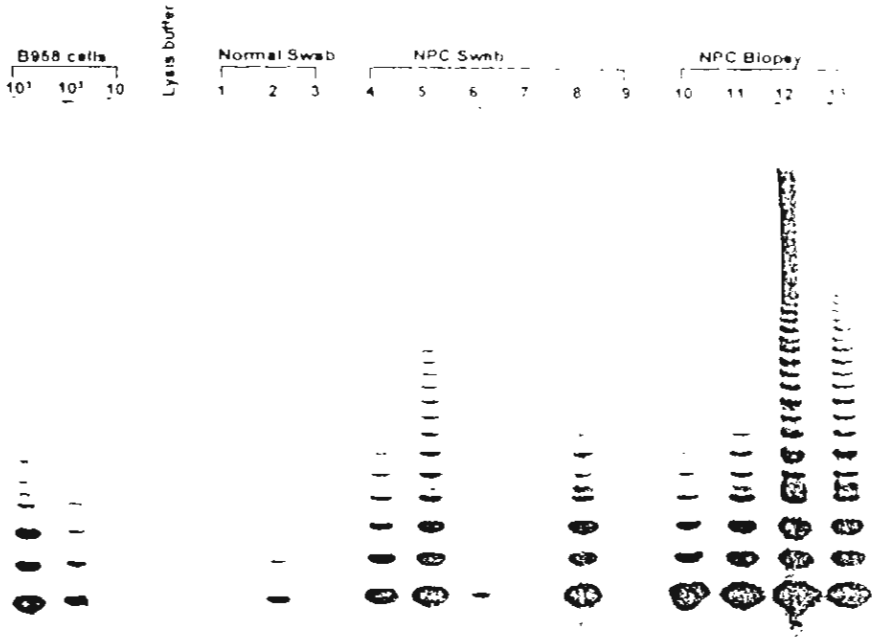

Fig 2. [illegible] EBV-transformed human lymphocytic cell line. [illegible] 10^3, 10^2, and 10 cells represented levels of [illegible] ative control.

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