Polymerase Chain Reaction Study of Epstein-Barr Virus in High Risk Group of Nasopharyngeal Carcinoma : A Preliminary Report

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Abstract

The incidence of nasopharyngeal carcinoma (NPC) is relatively high in Thailand. Early diagnosis which leads to early treatment is crucial, in order to obtain a high salvage rate. Epstein-Barr virus (EBV) has long been known to be associated with nasopharyngeal carcinoma. Polymerase chain reaction (PCR) is a sensitive and specific technique for the detection of EBV genome in NPC. The first generation of members in the families of NPC patients is considered a high risk group. This study utilized the PCR technique as a screening test for early detection of EBV DNA in this group and also attempted to compare the result with that of IgA antibody level. The data was collected from July 1995 to 1996, this included 35 high risk volunteers, 15 males and 20 females. All subjects underwent telescopic examination and biopsies were performed to obtain specimens for PCR. Blood samples were also taken to determine serum titer of EBV IgA. All specimens studied were negative for EBV DNA and low IgA antibody titer obtained. The negative results implied that either the sample studied was inadequate to detect the low percentage of positivity of EBV in a high risk population or prolonged infection of EBV in nasopharyngeal mucosal cells is not necessary for initiation of NPC.

Key word : Polymerase Chain Reaction, Epstein-Barr Virus, Nasopharyngeal Carcinoma

Epstein-Barr virus (EBV) is found to be the cause of infectious mononucleosis and Burkitt's lymphoma[1,2]. However, in humans the malignancy most consistently associated with the virus is nasopharyngeal carcinoma (NPC)[2]. Association between EBV and NPC was demonstrated by the detection of antibodies to EBV in the serum and identification of viral genomes in the epithelial tumor cells by Polymerase chain reaction (PCR) technique from formalin-fixed paraffin-embedded tissues[2-4].

The incidence of NPC is high in the population of South-East-Asia, Southern China as well as in Thailand[3,5,6]. The incidence predominates

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in male by 3:1 and starts to peak at 40 years of age[7]. Heriditary factors have been noted relating to the disease. In addition to a general racial susceptibility, a positive family history is present in 1-4 per cent of all patients[8]. Early diagnosis in this high risk group is important. PCR technique has been used to detect EBV genomes, and when compared with IgA antibodies to EBV, it is more sensitive and specific in detection of NPC patients[9-14]. This study was designed to utilize the PCR technique as a screening tool for detecting early nasopharyngeal carcinoma in a high risk group. Moreover, this study attempted to compare the sensitivity of PCR technique with that of EBV IgA antibody.

MATERIAL AND METHOD

Subjects

Candidates considered as a high risk group for nasopharyngeal carcinoma were those in the families of NPC patients. This included the first generation of members in the families i.e. mothers, fathers, younger and elder brothers or sisters. Their marriages and cousins were excluded. Their natural and medical histories from July 1995 to 1996 were collected including age, sex, occupation, history of smoking and alcohol intake, and relationship to the NPC patients. Thirty five volunteers were included in the study, there were 15 males and 20 females whose ages ranged from 16 to 62 years (Mean 31.9 yrs). Relationship to the NPC patients was as follows; fathers 19, mothers 5, elder sisters 2, younger sisters 4, elder brother 1, and younger brothers 4. Two patients had a history of smoking 3-4 cigarettes per day and four of them were moderate alcohol drinkers. All of them had occasionally included salted fish in their menu.

Every candidate was informed about the procedure and all were willing to join the project. Each underwent a complete physical examination and telescopic nasopharyngeal examination was performed. Tissue specimens were obtained from Rosenmuller fossa on both sides of every volunteer, using cup forceps. The specimens were then preserved in buffered formalin solution, and subsequently submitted for routine paraffin-embedded procedure. Pathological diagnosis was made from part of these specimens. Serum for EBV IgA antibody titre was obtained from each candidate immediately after tissue biopsy.

DNA preparation

Ten tissue sections of 5 mm thick, were sliced from each tissue block with microtome and placed in 1.5 mL microfuge tubes. The sections were deparaffinized and extracted twice with xylene and 100 per cent ethanol by the method of Wright and Manos[15]. Tissues were digested in 0.3 mL. digestion buffer (200 µg/mL proteinase K in 50 mmol/L TRIS-HCL pH 8.5, 1 mmol/L EDTA and 0.5 per cent Tween 20), then incubated for 24 hours at 37°C. The proteinase K was subjected to heat inactivated at 95°C for 10 minutes. The tissue sections were then purified using phenol chloroform isoamylalcohol (25:24:1). DNA was precipitated at -80°C in 3 mol/L sodium acetate and 100 per cent cold ethanol.

Polymerase chain reaction

The primer pair that amplified EBV nuclear antigen gene (5' GTAGAAGGCCATTTTTCCAC 3' and 5' CTCCATCGTCAAAGCTGCA 3')[5] was used for the amplification of EBV genomes and the primer pair of human ß-globin gene (5' ACACAAC TGTGTTCACTAGC 3' and 5' CAACTTCATCCA CGTTCACC 3')[5] was used to assess the quality of DNA in PCR. Ten microlitres from the prepared DNA sample was separately amplified using each primer pair. Each reaction contained, a total of 100 µL, 10 µL sample DNA ; 0.1 µg of each primer; 200 µmol/L of each dNTP; 2.5 units of Taq DNA polymerase (Boehringer, Mannheim, Germany) ; 50 mmol / L KCL; 4 mmol/L $MgCl_2$; and 10 mmol/L TRIS-HCL (pH 8.5). All PCR assays were performed for 30 cycles at 94°C for 1 minute for denaturation, 53°C for 1 minute for primer annealing and at 72°C for 1.30 minutes for extension and with an additional 5 minutes at 72°C in DNA Programmable Thermal Controller (MJ Research Inc.,Mass., U.S.A.). Purified DNA from formalin-fixed Raji cells was used as EBV-genomic-positive template as well as for human DNA control template. Sterile distilled water was used as negative control. After amplification, 10 µL of each amplification product was electrophoretically separated on 2 per cent agarose gel containing 0.5 µg ethidium bromide per millilitre and GelMarker-I™ (Research Genetic, Canada) was used as DNA size marker.

Southern blot hybridization analysis

The ampliflied products were transferred on to nylon membrane (Boehringer, Mannheim, Germany) from the agarose gel after electrophoresis.

The DNA fragments on the membrane were hybridized with digoxigenin labeled EBV genome DNA fragments. A 270 bp EBV genome DNA fragment obtained from the amplification using inner primer pair (5' TAGCCAGGAGAGCTCTTAAA 3' and 5' CTCCATCGTCAAAGCTGCA 3') of the amplified product of EBNA-1 gene was labeled with digoxigenin-11-dUTP by PCR[14,16-18] and used as a probe for PCR products. The hybridization condition followed the procedure as previously described[19].

Immunofluorescence for IgA antibody

Serum for each candidate was detected for EBV IgA antibody by indirect immunofluorescence. The suspension of 80,000 cells/ml of B95-8 cells, the EBV infected lymphoblastoid cell line, was smeared and fixed onto slides and used as the substrate for the antibody detection. Each serum was serially diluted and overlaid onto the cells. Fluorescein-conjugated rabbit immunoglobulin to human IgA (DAKO,DK-2600 Glostrup, Denmark) was used as the secondary antibody. Sera known to be positive for EBV IgA antibody with the dilution of 1:10 and 1:160 were used as positive antibody control. Slides were examined under a fluorescent microscope. The validity of immunofluorescent staining was assessed with the 4+ of B95-8 fluorescence with the 1:10 dilution of the control sera. The titre of EBV IgA antibody was obtained as the highest dilution of each tested serum showing fluorescence of B95-8 cells.

RESULTS

The pathological results of all specimens were negative for malignancy. Most reports were compatible with chronic inflammation. Polymerase chain reaction for the detection of EBV genome in all the specimens studied revealed negative results. None of the extracted DNA from the thirty-five formaline-embedded tissues contained visible PCR product of 610 bp of EBV genome on agarose gel after electrophoresis. The PCR products of 110 bp generated by the amplification using the primer pair of human ß-globulin gene were clearly visible in all of the thirty-five specimens studied. The amplified products of 610 bp as well as 110 bp could be clearly identified using extracted Raji DNA as the positive templates.

The hybridization using the 270 bp EBV DNA fragment as the probe was carried out in all the specimens studied as well as the extracted DNA from Raji cells. Only the amplified products of Raji DNA gave positive signals.

The results of EBV IgA antibody titre in all candidates were negative (below 1:160 titer level) as shown in Table 1.

Table 1. Result of EBV IgA antibody titre in high risk candidate.

EBV IgA titre	Number of candidates
< 1 : 10	28
1 : 20	2
1 : 40	4
1 : 80	1
> 1 : 160	–

DISCUSSION

Nasopharyngeal carcinoma is one of the most common cancers found in South East Asia. Currently, the mainstay treatment for this malignancy is radiotherapy alone or in combination with chemotherapy. As for all malignancies, the prognosis of NPC is more favorable in the early stage than in the late stage. Unfortunately, most patients usually present in the late stage of the disease because they usually have no symptoms in the early stage. It would be advantageous if a sensitive mean for detecting an asymtomatic group of patients was available. Detection occurs at the advanced stage of the disease in the vast majority of individuals, despite wide public awareness of the prevalence of the tumor. Since the best results can be achieved from treatment in the early stage of NPC with simpler techniques and lower doses of radiotherapy rather than the advanced stage, screening for this cancer is an attractive proposition[7]. Many techniques have been advocated for screening symptom less patients. EBV IgA titre and PCR have proved to be sensitive and specific in detection of EBV genomes from patients with NPC[13,14]. However, in the high risk population there are quite a few reports[20,21]. In this study, we considered EBV IgA titre of 1:160 or more to be positive[22]. From this preliminary report, athough we obtained negative results of EBV DNA as well as IgA antibody and a definite conclusion could not be drawn, this

study may give rise to some implications concerning NPC. The number of candidates studied was inadequate to detect the low percentage of positivity of EBV in the high risk population. In addition to this, prolonged infection of EBV, especially in nasopharyngeal mucosal cells, is not necessary for initiation of NPC and the biopsy sites may not represent a tumor prone location in this high risk group.

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การศึกษาไวรัสเอ็นสกาย-บารร์ โดยวิธี พีซีอาร์ ในกลุ่มประชากรที่เสี่ยงต่อโรคมะเร็งหลังโพรงจมูก : การรายงานเบื้องต้น

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อุบัติการณ์ของโรคมะเร็งหลังโพรงจมูกพบได้บ่อยในประเทศไทย การวินิจฉัยโรคในระยะเริ่มแรกมีความสำคัญต่อผลการรักษา เป็นที่ทราบกันมานานแล้วว่า Epstein-Barr ไวรัส (EBV) เกี่ยวข้องกับโรคมะเร็งหลังโพรงจมูก และการตรวจหา EBV genome โดยวิธี polymerase chain reaction (PCR) เป็นวิธีที่มีความไวและความจำเพาะสูง ในกลุ่มครอบครัวที่เป็นญาติสายตรงกับผู้ป่วยโรคมะเร็งหลังโพรงจมูกจัดเป็นกลุ่มที่มีความเสี่ยงสูงต่อการเกิดโรคนี้ การศึกษาจึงใช้การตรวจหา EBV genome โดยวิธี PCR ในการตรวจหามะเร็งหลังโพรงจมูกระยะเริ่มแรกในกลุ่มดังกล่าว และยังศึกษาเปรียบเทียบกับผลของ EBV IgA antibody อาสาสมัครที่อยู่ในกลุ่มศึกษาดังกล่าว 35 คนเป็นชาย 15 คนและหญิง 20 คนเก็บรวบรวมข้อมูลตั้งแต่เดือนกรกฎาคม พ.ศ. 2538–2539 ทุกรายจะได้รับการตรวจบริเวณหลังโพรงจมูกด้วยกล้องกำลังขยาย (telescope) และตัดชิ้นเนื้อเพื่อตรวจหา EBV DNA และเจาะเลือดหาระดับของ EBV IgA antibody ผลการตรวจไม่พบว่ามีอาสาสมัครรายใดที่ให้ผลบวกของ EBV DNA และ EBV IgA antibody มีค่าต่ำกว่า 1 : 160 แสดงให้เห็นว่ากลุ่มทดสอบดังกล่าวมีปริมาณน้อยเกินไปที่จะตรวจพบผลบวกของ EBV ในกลุ่มเสี่ยงดังกล่าวซึ่งมีเปอร์เซ็นต์ที่ต่ำ หรือการติดเชื้อ EBV ของเยื่อบุผิวหลังโพรงจมูก (nasopharyngeal mucosal cells) เป็นระยะเวลานานอาจไม่จำเป็นต่อการเกิดมะเร็งหลังโพรงจมูก

คำสำคัญ : การตรวจด้วยวิธี พีซีอาร์, ไวรัสเอ็นสกาย–บารร์, มะเร็งหลังโพรงจมูก

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