Epstein-Barr Viral DNA in Serum of Patients with Nasopharyngeal Carcinoma[1]

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ABSTRACT

This study evaluated Epstein-Barr virus (EBV) DNA in sera of 42 patients with nasopharyngeal carcinoma (NPC) and 82 healthy individuals who had been infected previously with EBV. Thirteen of 42 NPC samples were positive for EBV DNA in their sera, whereas all 82 normal controls were negative. In addition, EBV typing between primary tumors and sera showed identical results, suggesting that serum EBV DNA represented tumor DNA. To evaluate the importance of the serum NPC DNA, clinical data and tumor phenotypes including age, sex, WHO type, EBV type, stage, tumor invasion, metastasis, and apoptosis were correlated with serum EBV DNA, and only apoptosis was found statistically significant. In conclusion, EBV DNA was detectable in the serum of some patients with NPC, represented tumor DNA, and might have clinical implications in the future.

INTRODUCTION

Nasopharyngeal carcinoma is a common cancer in Southern China and among Eskimos in Arctic regions, where it occurs at a frequency of 20–50 per 100,000 men. An intermediate incidence is observed in Southeast Asia (1, 2). As to the etiology of NPC,[3] several factors have been identified, of which EBV appears to be the most important (3). EBV has been considered crucial for NPC clonal evolution (4). From a diagnostic viewpoint, the consistent presence of EBV in NPC allowed EBV DNA to be a genetic marker for clinical diagnosis. For example, positive detection of EBV by PCR from a neck node with a metastatic tumor of unknown origin can be diagnosed as NPC (5).

Recent studies demonstrated that tumor DNA was detectable in plasma or serum of several cancers including lung, head and neck, colorectal cancer, and leukemia (6–9). This finding may relate to tumor cell death such as necrosis and apoptosis. In addition, tumor DNA in serum may reflect some clinical significance and serve as a potential diagnostic marker in the future. These previous studies identified tumor DNA using microsatellite analysis for lung, head and neck cancers, or mutation analysis of *RAS* oncogenes for colorectal cancer and leukemia. Because latent EBV infection was almost always found in NPC, EBV DNA may serve as a specific and sensitive genetic marker for this type of cancer (10). In the course of this study, we tested whether EBV DNA could be discovered in the serum of NPC patients and whether it originated directly from tumor cells. Moreover, we determined whether the presence of serum EBV DNA had any association with clinical or tumor phenotypes, such as staging, invasion, metastasis, and tumor cell death.

MATERIALS AND METHODS

Sample Collection. Primary NPC tissues were collected from 46 patients before treatment at Chulalongkorn University Hospital from January 1996 to June 1997. The tissues were divided into two parts. The first part was sent for routine histological examination. The second part was immediately stored in liquid nitrogen until further use. All of the tumors were histologically ascertained to be undifferentiated NPC according to the WHO classification. The 46 tumors included stages ranging from I to IV. All patients were being followed for treatment outcome and survival.

Blood samples were obtained by venipuncture from the same patients and 84 healthy blood donors. To obtain sera, clotted blood specimens were centrifuged at low speed for 5 min, and the sera were stored at −20°C before use.

DNA Isolation. NPC tissue was treated with SDS and proteinase K at 50°C overnight, followed by phenol/chloroform extraction and ethanol precipitation of DNA (11). Serum DNA was purified on Qiagen columns (Qiamp blood kit; Qiagen, Basel, Switzerland) according to the "blood and body fluid protocol." Serum (2–3 ml) was passed on the same column. One-tenth of DNA extracted was then used for PCR analysis.

ELISA Test. Serum from healthy blood donors was examined for previous EBV infection. For detection of antibody of the IgG class to EBV viral capsid antigen (anti-EBV-VCA IgG), a commercially available ELISA kit (human; Gesellschaft fur Biochemica und Diagnostica) was used.

EBV Detection and Typing by PCR. For the detection and typing of EBV DNA in the tumor tissues, three previously described PCR protocols were used (5, 12, 13). DNA from cell

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[3] The abbreviations used are: NPC, nasopharyngeal carcinoma; EBV, Epstein-Barr virus; EBNA, EBV nuclear antigen.

Table 1 Serum EBV DNA, apoptosis, and clinical staging of 42 NPC patients

		Serum EBV DNA			Apoptosis		
	Total (x)[a]	+	−	*P*	+	−	*P*
NPC patient	42 (4)	13	29		10	32	
EBV type (A:B)	40:2	11:2	29:0	0.09	9:1	31:1	0.42
Age <40:40–60:>60[b]	13:18:11	2:6:5	11:12:6		3:5:2	10:13:9	
Sex (M:F)	23:19	9:4	14:15	0.32	8:2	15:17	0.08
Stage (I:II:III:IV)	2:5:9:26	0:2:1:10	2:3:8:16		0:1:3:6	2:4:6:20	
WHO typing (II:III)	34:8	10:3	24:5	0.69	9:1	25:7	0.65
Local invasion (+:−)	17:25	7:6	10:19	0.31	6:4	11:21	0.27
Skull or nerve involvement (+:−)	8:34	5:8	3:26	0.08	3:7	5:27	0.37
LN metastasis (+:−)	28:14	10:3	18:11	0.48	7:3	21:11	1.00
Distance metastasis	0						
Serum EBV DNA (+:−)	13:29				6:4	7:25	0.046
Apoptosis (+:−)	10:32	6:7	4:25	0.046			

[a] (x), number excluded because of negative PCR for β globin.
[b] <40:40–60:>60, age less than 40, between 40 and 60, and above 60 years, respectively.

line B958, EBV-transformed human lymphocytes (American Type Culture Collection), was used as positive control; double-distilled water was used as negative control.

Duplex PCR was performed to detect EBV using two sets of primers. The first amplified the nonpolymorphic EBNA-1, generating an ~610-bp DNA fragment. The second amplified a human β-actin genomic sequence, generating an ~310-bp DNA fragment (5).

Two sets of PCR primers were used for EBV typing. The first primer amplified the EBNA-2, generating a DNA fragment of 168 bp for EBV type A and of 184 bp for EBV type B. The second one amplified the EBNA-3c, generating a DNA fragment of 153 bp for EBV type A and of 246 bp for EBV type B (12, 13). Only EBNA-2 was used for detection and typing of EBV DNA in serum due to the quality of the PCR product. Primers GH20 and PCO4 were used to amplify β-globin to test for the presence of amplifiable human DNA in all sera (14), generating a DNA fragment of 260 bp. The oligonucleotide sequences for all sets of PCR primers were identical to the ones reported previously.

The PCR reactions were performed in a total volume of 20 μl using 50 ng of the corresponding tumor DNA or 1/10 total DNA extracted from 3 ml of serum in 200 μM deoxynucleotide triphosphates each, 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 0.5 μM of each primer. The PCR amplification was performed as follows: initial denaturation at 94°C for 5 min, followed by 40 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. The PCR products were then analyzed using 2% agarose gel electrophoresis.

To confirm the presence of EBNA-2 PCR product in the serum DNA tested, the gels were subsequently transferred to a Hybond N^+ membrane (Amersham Corp.) applying a routine Southern blot protocol, and the membrane was hybridized to the EBNA-2 common probe that had been end-labeled with ^{32}P using T4 polynucleotide kinase (13).

DNA Fragmentation. Ten μg of tumor DNA were treated with 50 ng of RNase A for 30 min. The DNA was then ethanol precipitated, resuspended in 10 μl of TE [10 mM Tris.Cl (pH 7.4), 1 mM EDTA (pH 8.0)], and analyzed on a 1.8% agarose gel run in 1× TBE (0.09 M Tris-borate, 0.002 M EDTA) at 50 V for 3 h. Apoptosis was detected by the presence of a DNA ladder with each band separated by a distance representing between 180 and 200 bp.

Table 2 Prevalence of anti-EBV-VCA IgG and serum EBV DNA among healthy Thai controls

Age (yr)	No.	No. of positives, Anti-EBV	% positive	No. of positives, serum EBV DNA	% positive
19–29	41	39	95.1	0	0
30–39	23	23	100	0	0
40–49	13	13	100	0	0
50–59	7	7	100	0	0
Total	84	82	97.6	0	0

Correlation between Clinical Data, Apoptosis, and Serum EBV DNA. Data regarding histology, tumor staging, EBV detection and typing, and apoptosis were collected in a double-blind fashion until analyzed. Fisher's exact test and logistic regression were used to compare the results gained from serum analysis with clinical and tumor parameters.

RESULTS

EBV DNA in Serum. To determine whether EBV DNA can be detected in sera of NPC patients and whether it represents tumor DNA, DNA from sera of two groups was studied. The first one comprised 46 NPC patients, and the other one comprised 84 healthy blood donors (Tables 1 and 2). The control marker could not be amplified in four of the 46 sera; therefore, they were excluded from this analysis. The average age of NPC patients was 43.2 years, ranging from 16 to 81 years. Twenty-six of them were male and 20 were female. All of the primary tumors and sera of these patients were tested for EBV infection (Figs. 1, 2, and 3). All of the tumors showed positive results, with 40 cases infected with type A and the other two cases with type B, respectively (Table 1 and Fig. 1). Nevertheless, only 13

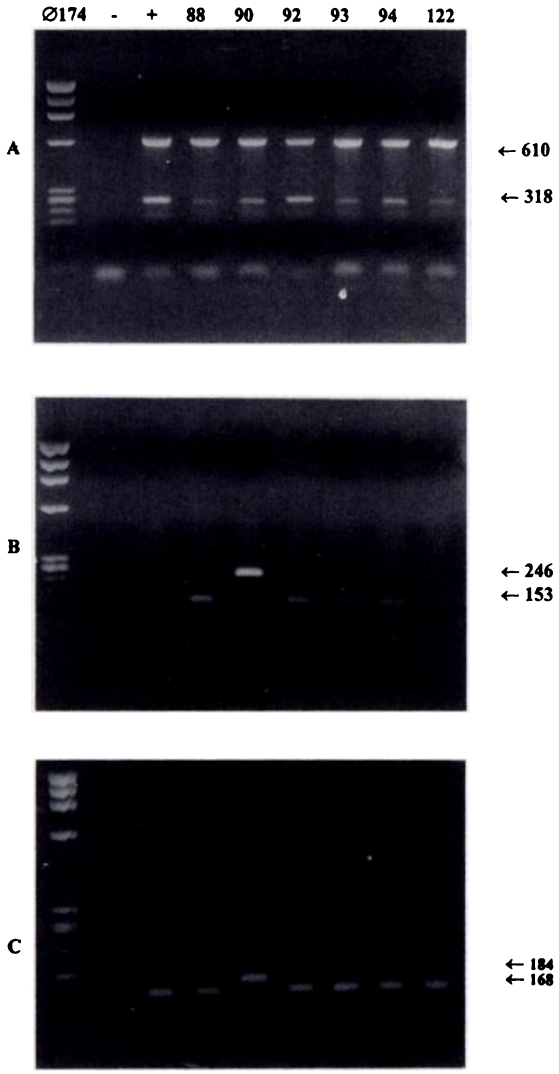

Fig. 1 PCR genotyping of EBV-infected NPC on a 2% agarose gel stained with ethidium bromide. From *left* to *right*: ϕ*174*, *Hae*III standard DNA size marker; − and +, PCR products of the negative control (double-distilled water) and positive control (B958 cell line), respectively; numbers indicate corresponding PCR products of NPC patients. *A*, duplex PCR generating 610- and 318-bp DNA fragments of EBNA-1 and human β-actin genomic sequence, respectively. *B*, PCR generating 246- and 153-bp DNA fragments of EBNA-3C of EBV types B and A, respectively. *C*, PCR generating 184- and 168-bp DNA fragments of EBNA-2 of EBV types B and A, respectively.

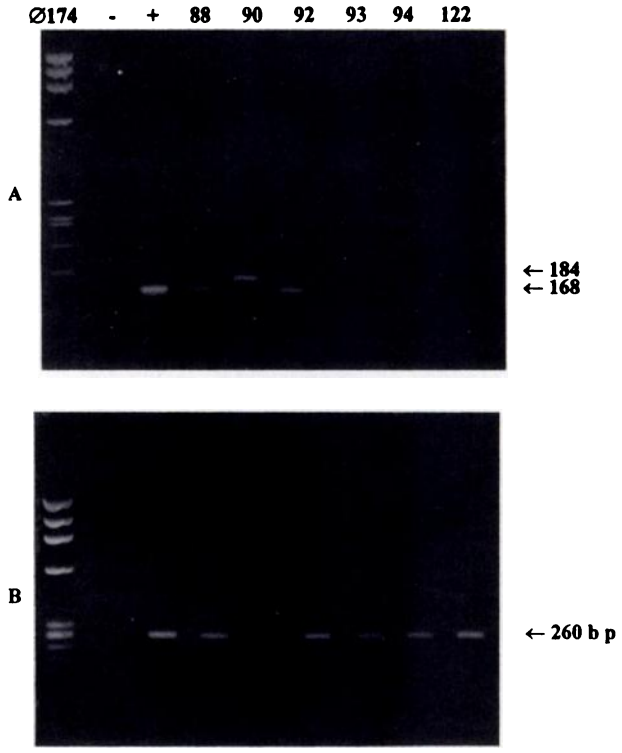

Fig. 2 PCR genotyping of sera of EBV-infected NPC patients on a 2% agarose gel stained with ethidium bromide. From *left* to *right*: ϕ*174*, *Hae*III standard DNA size marker; − and +, PCR products of the negative control (double-distilled water) and positive control (B958 cell line), respectively; numbers indicate corresponding PCR products of NPC patients. *A*, PCR generating 184- and 168-bp DNA fragments of EBNA-2 of EBV types B and A, respectively. *B*, PCR generating 260-bp DNA fragments of β-globin.

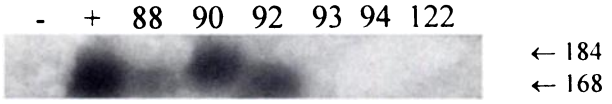

Fig. 3 Hybridization after PCR genotyping sera of EBV-infected NPC patients using EBNA-2 PCR products as template and its internal primer as probe. Samples are loaded in the same order as shown in Fig. 2.

serum samples were positive for EBV. Interestingly, these 13 cases exhibited an EBV type identical to the one encountered in the primary tumors, 11 of which were type A and 2 type B, respectively (Table 1; Figs. 1 and 2). This observation suggests that serum EBV DNA originates from NPC and thus can be used as a marker for the tumor DNA.

To exclude the possibility of having obtained serum EBV DNA from other latently infected cells, sera of 84 blood donors were tested (Table 2). Almost all of them, 82 of 84 cases, tested positive for anti-EBV-VCA IgG, suggesting previous infection. The average age was 31.8 years, ranging from 19 to 59 years. Thirty-two individuals were female, and the others were male. However, none of them tested positive for serum EBV DNA.

Serum EBV DNA, Apoptosis, and Clinical Correlation. To elucidate the importance and meaning of NPC DNA present in serum, the correlation between the presence of serum EBV DNA, clinical data, and programmed cell death was established (Table 1). Clinical data including age, sex, staging, invasion, and metastasis are included in Table 1. None of them seemed to have a statistically significant correlation with the presence of EBV DNA in serum. Because serum or plasma DNA has been hypothesized to be due to the leaking of DNA from dead cells (15), we explored whether apoptosis relates to the presence of tumor DNA in the serum of NPC patients. All 42 tumor DNAs were tested for DNA fragmentation, and 10 of them were positive (Table 1 and Fig. 4). Interestingly, the apoptosis was

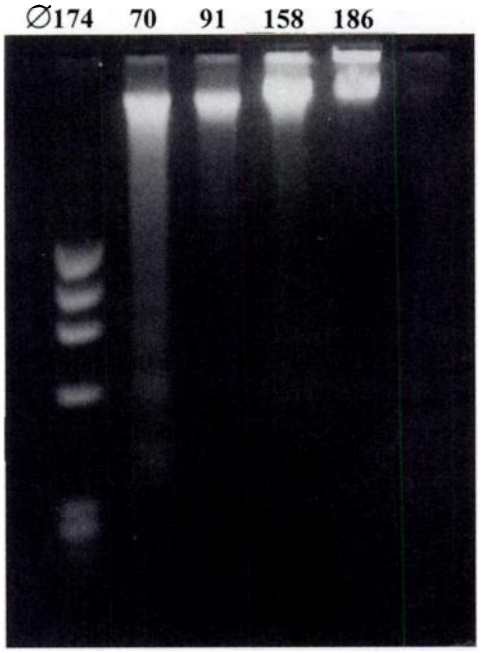

Fig. 4 DNA fragmentation of EBV-infected NPC on a 1.8% agarose gel stained with ethidium bromide. From *left* to *right*: ϕ*174*, *Hae*III standard DNA size marker, followed by numbers indicating corresponding tumor DNA of NPC patients.

independently correlated with the presence of EBV DNA in the serum in a statistically significant manner (P = 0.046 from Fisher's exact test; odds ratio, 5.36; 95% confidence interval, 1.47–24.4 from logistic regression).

DISCUSSION

Although the presence of EBV in tumor tissue is unique to NPC, especially in Southern China and Southeast Asia, EBV infection is very common worldwide (16). In addition, a recent study showed that EBV infection is very common in the Thai population and that it happens early in life (17). Primary infection with EBV usually leads to clinical manifestations ranging from mild, self-limited illness to infectious mononucleosis. Most EBV infection in humans originates in the oropharyngeal epithelium. These cells are permissive for virus replication. A persistent, active lytic infection can continue at some level for many years. After the primary infection, EBV can diffuse across the basal membrane and cause latent infection in B lymphocytes. This infection is important regarding the dissemination of infection to distal epithelial surfaces such as the nasopharynx (3). This study identified EBV DNA in the sera of 13 of 42 NPC patients. In addition, EBV typing from sera and primary tumor DNA yielded identical results, suggesting the EBV DNA in sera originated from tumors. To prove that serum EBV DNA did not originate from other latently infected cells, such as the oral epithelium or B lymphocytes, we tested a number of healthy subjects infected previously with EBV. None of their sera was positive for EBV DNA; consequently, the EBV DNA found in serum must have originated from NPC cells.

How tumor DNA comes to be present in serum is not yet known. Nevertheless, plasma or serum DNA could originate from dead cells (15). To investigate whether NPC DNA in serum is associated with tumor cell death, we studied the correlation between apoptosis and serum EBV DNA. Although these two factors were correlated, 40% of cases with apoptosis were negative for serum EBV DNA. In addition, almost 54% with serum positive for EBV DNA were negative for apoptosis. Thus, there may be other mechanisms, such as necrosis or active release, involved. More detailed studies are needed to clarify whether and how apoptosis and other mechanisms are related to the presence of viral DNA in serum.

Viral carcinogenesis is a prevalent etiological factor for cancer in the Far East, for example EBV and NPC, human papillomavirus and cervical carcinoma, and hepatitis B virus and hepatoma (2). Similar studies can be performed by looking at viral DNA in serum. Interestingly, our preliminary results showed a high incidence of hepatitis B virus in sera of seropositive hepatoma patients but failed to identify human papillomavirus in sera of patients with cervical carcinoma.

This study showed no correlation between serum EBV DNA and/or apoptosis and other clinical phenotypes including sex, age, staging, invasion, and metastasis. Prospective trials will be necessary to determine whether serum EBV DNA is an independent prognostic factor, especially for treatment outcome and patient's survival. Additionally, because the serum EBV DNA may be associated with tumor cell death, this PCR method could be used for follow-up regarding the rate of tumor regression or as a marker to determine the response to radiation and chemotherapy.

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