

# Consistent Sequence Variation of Epstein-Barr Virus Nuclear Antigen 1 in Primary Tumor and Peripheral Blood Cells of Patients with Nasopharyngeal Carcinoma<sup>1</sup>

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## ABSTRACT

**Purpose:** Nasopharyngeal carcinoma (NPC) has been proven as a cancer associated with Epstein-Barr virus (EBV). This study was performed to examine sequence variations of the EBV nuclear antigen 1 gene (*EBNA-1*) in primary tumor and peripheral-blood cells of NPC patients from Taiwan.

**Experimental Design:** DNA extracted from freshly frozen tumor tissues and corresponding peripheral-blood cells of 13 previously untreated NPC patients were subjected to PCR and direct sequencing using *EBNA-1*-specific primers. We compared the sequence data and analyzed the clinical outcomes.

**Results:** We obtained a 100% positive-detection rate of EBV DNA in the primary tumors of all patients irrespective of the degree of differentiation. The *EBNA-1* gene of all tumor samples was the “V-val” strain, showing the same clustered point mutations. They included 21 nucleotide exchanges, leading to 14 amino-acid mutations and 6 silent exchanges, relative to B95-8 cell line. Two of 13 tumors exhibited an additional point mutation at codon 585. EBV DNA was also detected in peripheral-blood cells of 9 of 13 patients under our experimental conditions. Direct-sequencing data showed match alterations of *EBNA-1* gene between the primary tumor and peripheral-blood cells. Tumor relapse was observed in four of nine patients with detectable *EBNA-1* DNA in their peripheral-blood cells, whereas none of the four patients without detectable *EBNA-1* DNA in their peripheral-blood cells developed tumor relapse.

**Conclusions:** Results of the current study represents the first demonstration of consistent sequence variation of *EBNA-1* in primary tumors and peripheral-blood cells. Clinical observations support that the presence of EBV DNA in the peripheral-blood cells may arise from disseminated cancer cells, resulting in a higher relapse rate and poor prognosis.

## INTRODUCTION

EBV<sup>3</sup> is a ubiquitous human  $\gamma$ -herpes-virus that infects >90% of the population worldwide. It has been demonstrated that EBV infection is associated with a variety of epithelial and lymphoid neoplasms (1, 2).

*In vitro*, EBV infects and transforms B lymphocytes. Lymphoblastoid cell lines derived from EBV-infected B cells express six EBV nuclear antigens (*EBNA-1*, -2, -3A, -3B, -3C, and *EBV-LP*), two EBV latent membrane proteins (*LMP-1* and -2), as well as two small untranslated RNAs (*EBER1* and *EBER2*). Infection in EBV-associated tumors is often latent, with only a limited range of viral genes being expressed by cells (3). *EBNA-1* is essential for the persistence and replication of the EBV genome in latently infected cells. It is the only viral protein required for the maintenance of latent EBV infection (4) and is expressed in all EBV-associated malignant tissues (5). *EBNA-1* may, therefore, play a critical role in the onset, progression, or maintenance of these tumors (6). This hypothesis was supported further by the fact that two independent lines of *EBNA-1* transgenic mice succumbed to monoclonal B-cell lymphoma (7).

The predicted aa sequence of *EBNA-1*, which consists of 641 aa, can be separated into unique N- and COOH-terminal domains joined by internal, glycine/alanine-rich short repeat sequences (8). Several studies have been carried out to map functional domains within *EBNA-1*, including dimerization, transactivation, nuclear localization, DNA looping, and RNA binding (9–12). Most of the domains associated with these activities were localized to the COOH-terminus of *EBNA-1*, except for RNA binding, which involved the RGG motifs at aa 34–55, 330–349, and 355–376 (12).

Recently, sequence variations within the functionally important unique domains of *EBNA-1* were reported by various groups (13–22). It was proposed that this polymorphism could be critical for the apparent evasion of host cytotoxic T-lymphocyte responses by *EBNA-1*, and for disease progression, or it might be important for *EBNA-1* transactivation within different

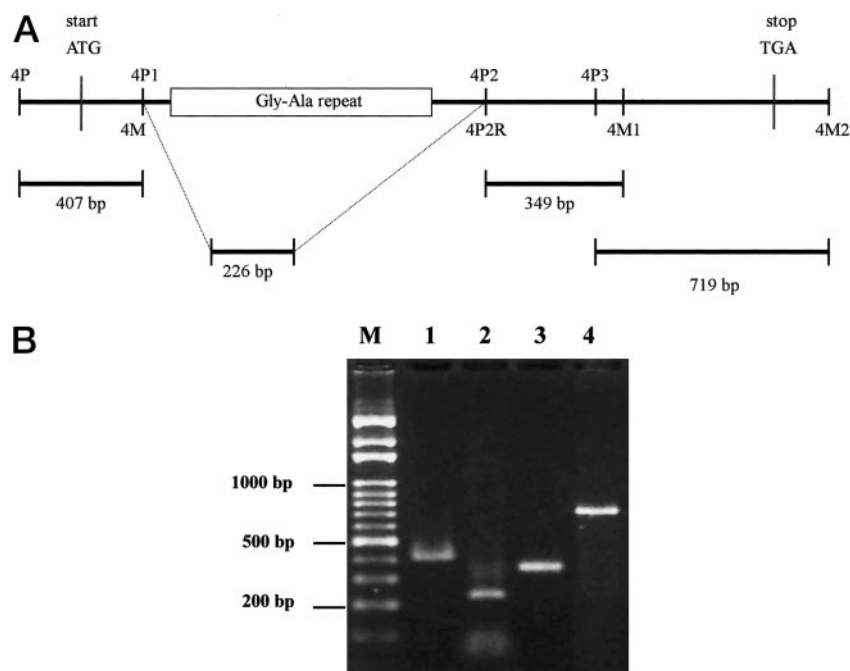
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<sup>3</sup> The abbreviations used are: EBV, Epstein-Barr virus; EBNA, EBV nuclear antigen; aa, amino acid(s); NPC, nasopharyngeal carcinoma.



*Fig. 1* Schematic representation of the position of primer pairs on the *EBNA-1* gene (A) and agarose gel electrophoresis of the PCR products of each fragment (B). M, 100-bp DNA marker; Lanes 1–4, 407-, 226-, 349-, and 719-bp products.

cell types. However, no obvious functional differences between these variants have been documented.

In this study, we examined sequence variations of *EBNA-1* gene from the freshly frozen tumor tissue of NPC and their corresponding peripheral-blood cells by PCR and direct sequencing. We observed that all tumor cells of NPC patients from Taiwan and their corresponding peripheral-blood cells harbored the same subtype, V-val.

## MATERIALS AND METHODS

**Sample Collection and DNA Extraction.** A small piece of primary tumor from 13 patients with previously untreated, biopsy-confirmed NPC was collected under nasopharyngoscopy after obtaining informed consent. The specimens were freshly frozen in liquid nitrogen until DNA extraction. Venous blood (10 ml) was collected before treatment from each patient. After erythrocytes were lysed, the pellet of peripheral-blood cells was washed and subjected to DNA extraction with TRIzol (Life Technologies, Bethesda, MD) according to the manufacturer's instructions.

**PCR and DNA Sequencing for Tumor Tissue.** *EBNA-1* gene was amplified as four subfragments (Fig. 1): a 407-bp fragment including the N-terminal coding region upstream of the Gly-Ala repeat region; a 226-bp fragment including nucleotides 57 to 82 upstream of the Gly-Ala repeat region and nucleotides 235 downstream of the Gly-Ala repeat region, excluding the whole Gly-Ala repeat; and two overlapping fragments (349 and 719 bp) that cover the entire carboxyl terminus downstream of the Gly-Ala repeat region.

The primer sequences and PCR conditions are summarized in Table 1. In brief, PCR for the N-terminal coding region and two overlapping COOH-terminal regions of *EBNA-1* gene were carried out using 100 ng of extracted DNA in a 50- $\mu$ l mixture

containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.04% (w/v) gelatin, approximately 1.5–6.5 mM MgCl<sub>2</sub>, 0.1% Triton X-100, 50  $\mu$ M deoxynucleotide triphosphate, 2 units of *Taq* polymerase (Protech), and 10 pmol of each primer. The DNA solution was preheated to 94°C for 5 min with a final extension for 5 min at 72°C.

However, PCR for the Gly-Ala repeat region of *EBNA-1* gene was amplified under the following conditions: 43 cycles of 94°C for 30 s then 60°C for 60 s. The DNA solution was preheated to 95°C for 9 min with a final extension for 10 min at 60°C. The reaction was carried out using 100 ng of extracted DNA in a 50  $\mu$ l mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001% (w/v) gelatin, 3 mM MgCl<sub>2</sub>, 200  $\mu$ M deoxynucleotide triphosphate, 1.25 units of *Taq* polymerase (AmpliTaq Gold), and 10 pmol of each primer.

The PCR products were separated on a 1.5% agarose gel and purified with Amicon Microcon-PCR centrifugal filter devices (Millipore Corporation). The sequencing reactions were performed with a dye-labeled terminators kit (Perkin-Elmer). Sequence analysis was performed in an ABI PRISM Genetic Analyzer, Model 377 (Perkin-Elmer, Foster City, CA).

**Nested PCR and DNA Sequencing for Peripheral-Blood Cells.** Because the relative proportion of EBV DNA extracted from the peripheral-blood cells was extremely low, nested PCR was used to amplify a 308-bp fragment of COOH-terminal part of the *EBNA-1* gene. The primer sequences and PCR conditions were the same as described previously (23) and are summarized in Table 1. Positive samples were purified and subjected to DNA sequencing.

The negative control (consisting of reaction mixture with no added DNA) and positive control (DNA extracted from an EBV-positive cell line, B95-8) were processed in parallel with patient samples in every PCR run. In addition, several measure-

Table 1 Primer sequences and PCR conditions

Primers	Sequences	B95-8 coordinates	Product size (bp)	PCR condition
For fresh frozen tumor tissue				
4P	5'-TCCGGGCTGCGAGTAATTGG-3'	107754–107773		94°C for 5 min: 1 cycle
4M	5'-GGACACCATCTCTATGTCTTGCC-3'	108137–108160	407	94°C for 30 s, 58°C for 30 s, 72°C for 60 s: 30 cycles
				72°C for 5 min; final extension
4P1	5'-GGGCCAAGACATAGAGATGGTGTCC-3'	108136–108160		95°C for 9 min: 1 cycle
4P2R	5'-GCGGTGGAGACCCGGATGATGATGA-3'	109111–109135	226	94°C for 30 s, 60°C for 60 s: 43 cycles
				60°C for 10 min; final extension
4P2	5'-TCATCATCATCCGGGTCTCCACCGC-3'	109111–109135		94°C for 5 min: 1 cycle
4M1	5'-CCCAAGTTCCTTCGTCGGTAGTCC-3'	109436–109459	349	94°C for 30 s, 61°C for 30 s, 72°C for 60 s: 30 cycles
				72°C for 5 min; final extension
4P3	5'-AGATGACCCAGGAGAAGGCCCAAGC-3'	109266–109290		94°C for 5 min: 1 cycle
4M2	5'-GGCCAGAAATACACCAACAGCACGC-3'	109960–109984	719	94°C for 30 s, 64°C for 30 s, 72°C for 60 s: 30 cycles
				72°C for 5 min; final extension
For peripheral-blood cells				
Outer	5'-GTAGAAGGCCATTTTCCAC-3'	109151–109170		94°C for 5 min: 1 cycle
	5'-CTCCATCGTCAAAGCTGCA-3'	109741–109759	609	94°C for 30 s, 58°C for 30 s, 72°C for 40 s: 25 cycles
				72°C for 10 min; final extension
Inner	5'-AGATGACCCAGGAGAAGGCCCAAGC-3'	109266–109290		94°C for 5 min: 1 cycle
	5'-CAAAGGGGAGACGACTCAATGGTGT-3'	109549–109573	308	94°C for 30 s, 58°C for 30 s, 72°C for 40 s: 25 cycles
				72°C for 10 min; final extension

ments were taken to prevent contamination during each experiment, such as frequently changing gloves and cleaning the equipment, using aerosol-resistant pipette tips for PCR, and performing different procedures in separate areas.

## RESULTS

**Detection of EBNA-1 from NPC Tumor Tissue and Sequence Variation.** All tumor tissues from 13 NPC patients (one keratinizing squamous cell carcinoma, nine nonkeratinizing squamous cell carcinoma, and three undifferentiated carcinoma) harbored EBV DNA. All tumor samples had the same clustered point mutations. They differed in the sequence pattern when compared with the B95-8 strain. The sequence variation included 21 nucleotides exchange, leading to 14 aa mutations and 6 silent exchanges (Table 2). Two of 13 patients exhibited an additional nucleotide exchange at position 109703 (C→T), resulting in an aa exchange at codon 585 (Thr→Ile).

**Detection of EBNA-1 from Peripheral-Blood Cells and Sequence Variation.** The positive rate of EBNA-1 DNA from peripheral-blood cells of NPC patients by our nested PCR assay was 69.2% (9 of 13). Sequencing data of this COOH-terminal part (codons 440–541) of the *EBNA-1* gene revealed that all positive blood samples have the same point mutations as their primary tumor. These included one silent nucleotide exchange at position 109509 (A→C) and six clustered point mutations at position 109409 (C→T), 109446 (C→G), 109454 (C→A), 109520 (C→T), 109531 (A→G), and 109546 (C→A). The resulting aa exchanges were at codons 487 (Ala→Val), 499 (Asp→Glu), 502 (Thr→Asn), 524 (Thr→Ile), 528 (Ile→Val), and 533 (Leu→Ile). One of nine EBNA-1-positive peripheral-blood samples exhibited an additional nucleotide exchange at position 109472 (T→C), resulting in an aa exchange at codon 508 (Phe→Ser).

**Clinical Outcomes.** All patients received combined radiotherapy and chemotherapy. After a median follow-up period of 29 months (range, 14–40 months), there were four failures:

Table 2 The nucleotide and aa changes observed in the *EBNA-1* gene sequence from the primary tumor of all 13 NPC patients relative to B95-8 cell line

Nucleotide no.	Nucleotide change	aa no.	aa change
107995	G→C	16	E→Q
108002	G→A	18	G→E
108007	A→T	20	T→S
108057	T→G	36	no change
108117	C→T	56	no change
108972	T→C	341	no change
109039~109041	GGA→AGG	364	G→R
109080	A→T	377	no change
109182	A→T	411	E→D
109202	A→T	418	H→L
109264	G→A	439	A→T
109409	C→T	487	A→V
109446	C→G	499	D→E
109454	C→A	502	T→N
109509	A→C	520	no change
109520	C→T	524	T→I
109531	A→G	528	I→V
109546	C→A	533	L→I
109608	G→A	553	no change
109703 <sup>a</sup>	C→T	585	T→I
109730	G→A	594	R→K

<sup>a</sup> Only 2 of 13 tumor samples exhibit this point mutation.

two distant metastases alone, one primary recurrence, and one distant metastasis plus neck recurrence. Four of nine patients with EBV-carrying cells and none of the four patients without EBV-carrying cells in the peripheral blood developed tumor relapse (Table 3).

## DISCUSSION

EBNA-1 is the only EBV antigen consistently expressed in all EBV-infected cells studied to date and is essential for the persistence and replication of the virus in latently infected cells

Table 3 Summary of clinical data

Case no.	Age (years)/Sex	Pathology (WHO)	Stage <sup>a</sup>	Treatment	Failure site	EBV	
						Tumor	PBC <sup>b</sup>
1	59/M	Type II	T2bN1M0	CRT		+	+
2	56/M	Type II	T4N3bM1	CRT	distant	+	+
3	57/F	Type II	T2bN1M0	CRT		+	–
4	31/M	Type I	T2bN3bM0	CRT	distant, neck	+	+
5	27/F	Type III	T3N1M0	CRT	primary	+	+
6	39/M	Type II	T2bN1M0	CRT	distant	+	+
7	57/F	Type II	T2aN2M0	CRT		+	–
8	60/F	Type II	T2bN0M0	CRT		+	+
9	37/M	Type II	T3N2M0	CRT		+	+
10	31/F	Type II	T4N2M0	CRT		+	–
11	41/F	Type II	T4N2M0	CRT		+	+
12	55/M	Type III	T2bN2M0	CRT		+	+
13	38/F	Type III	T2bN1M0	CRT		+	–

<sup>a</sup> 1997 American Joint Committee on Cancer staging system.

<sup>b</sup> PBC, peripheral-blood cells; CRT, combined chemoradiotherapy.

(4). Recent reports have described several variants of EBNA-1 relative to the prototype virus B95-8 (13–22). Each of these EBNA-1 variants has been identified as the exclusive virus subtypes in tumor biopsies and/or tumor or lymphoblastoid cell lines known to carry monoclonal EBV, strongly suggesting that these variants represent distinct virus subtypes. Five subtypes, based on the EBNA-1 sequence in the COOH-terminal region were identified. There were two prototypes (P-ala and P-thr) and three variant subtypes (V-pro, V-leu, and V-val) according to the aa at position 487 in EBNA-1, because this aa residue is highly predictive of the pattern of mutations observed.

NPC has been proven as an EBV-associated cancer for a long time. It has been demonstrated that EBV is harbored in almost every NPC tumor irrespective of the degree of differentiation and geographic distribution. Thus far, EBNA-1 sequence data in NPC biopsy is seldom reported (13–15). Snudden *et al.* (13) determined the sequence of the *EBNA-1* gene except for the internal glycine/alanine repeat region in seven NPC patients from Hong Kong Chinese and identified several mutations (V-val) relative to B95–8 (P-ala). Many of the nucleotide changes would lead to substantial aa alterations in apparently functionally significant regions of the protein. In our study, identical sequence variations were demonstrated in 13 biopsy samples from NPC patients of Taiwanese. We further found a new aa mutation at codon 364 (Gly→Arg) attributable to nucleotide exchange at positions 109039–109041 (GGA→AGG).

Gutierrez *et al.* (14) reported that EBV with an EBNA-1 subtype (V-val) frequently associated with NPC in 9 of 13 tumor tissue samples from patients from Hong Kong and France. The remaining four samples belonged to a P-thr subtype. However, the authors did not indicate whether or not a V-val subtype had a predilection for Hong Kong Chinese. Chen *et al.* (16) from Taiwan determined the COOH-terminal part of EBNA-1 sequence (codons 363–641) from several EBV-containing cell lines and two NPC biopsies. These two NPC samples also expressed a V-val subtype. Sandvej *et al.* (15) sequenced the COOH-terminal part of the *EBNA-1* gene (codons 438–547) in Danish and Chinese NPC patients. They found that two of three Chinese NPC samples were of a V-val subtype but

none of the Danish NPC samples (0 of 11) harbored a V-val subtype. Thus, we deduce that EBV containing a V-val subtype of EBNA-1 is frequently associated with Chinese NPC.

Sandvej *et al.* (15) also showed that two of three Hodgkin's disease, both the nasal T-cell lymphoma, and four of five throat wash samples from Chinese patients contained a V-val EBNA-1 subtype, which was never seen in Danish patients. In Danish patients, P-thr was the most common EBNA-1 subtype irrespective of source (Hodgkin's disease, NPC, and throat wash samples). Chen *et al.* (19) from the United States recently reported V-val EBNA-1 subtypes in tested cases of EBV-positive gastric carcinoma (25 of 25) and EBV-positive reactive lymphoid follicular hyperplasia (8 of 8) in Japanese patients. Only 1 in 28 cases of known EBV-positive American gastric carcinoma or reactive lymphoid tissue was of a V-val subtype. MacKenzie *et al.* (20) investigated tumor samples of EBV-associated lymphoma from Brazil and the United Kingdom, and no V-val subtype could be found. Chang *et al.* also reported that no V-val subtype occurred in Brazilian and American patients with Hodgkin's disease and reactive lymphoid tissue. These findings may suggest that V-val is the dominant subtype of EBV latent infection in the Asian region.

NPC is distinguished from other cancers of the head and neck by its epidemiology, histopathology, clinical characteristics, and therapy. Radiotherapy is the primary treatment for NPC. Recent trials support the use of combined chemoradiotherapy for patients with advanced NPC. Because of recent advances in radiation oncology, the failure pattern has predominantly changed to distant metastasis (23–25). Distant metastasis is the most important and urgent issue to be solved in the management of NPC. Using real-time quantitative PCR, Lo *et al.* (26, 27) have suggested that EBV DNA load in cell-free plasma can be used as a prognostic indicator for tumor relapse. Instead of using plasma samples, we demonstrated that the presence of EBV DNA in the peripheral-blood cells was a poor prognostic factor for NPC patients with a significantly higher risk of developing distant metastasis, in addition to a lower survival rate (23). EBV has been proven in metastatic cancer cells of NPC patients with neck nodes or distant organ metas-

tases by *in situ* hybridization (28–30). Thus, the circulating EBV-carrying cells we detected may arise from disseminated cancer cells. Sequencing data in the current study showing the match mutation pattern in codons 487, 499, 502, 524, 528, 533 from both primary tumor and peripheral-blood cells give further support to this hypothesis. These finding and clinical observations reflect a higher relapse rate for patients with the presence of EBV DNA in the peripheral blood and should be considered in treatment planning of future patients.

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