Epstein-Barr Virus EBNA-2 Polymorphic Patterns in Nasopharyngeal Carcinoma in Southern China

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Key Words

Epstein-Barr virus · Nasopharyngeal carcinoma · Nuclear antigen 2 · Polymorphism

Abstract

Objectives: The aim of this study was to characterize the specific Epstein-Barr virus (EBV) polymorphisms from nasopharyngeal carcinoma (NPC) patients and healthy donors in Southern China, and to explore the relationship between EBV genotypes and NPC. **Methods:** The EBNA-2 gene of 32 EBV-positive NPCs and 39 EBV-positive throat washing (TW) samples from healthy individuals from Southern China was analyzed using PCR and sequencing. **Results:** We found E2-A to be the predominant subtype in Southern China. The distribution of EBNA-2 subtypes between NPCs and TWs was significantly different ($p < 0.01$) and the E2-A subtype was overly represented in NPCs. **Conclusions:** E2-A is the predominant subtype in Southern China, and the distribution of EBNA-2 subtypes between NPCs and TWs is significantly different. In Southern China, the E2-A subtype is significantly more frequent in NPCs than TWs, and is also more common than in Northern China. This suggests that the E2-A subtype may play an important role in the pathogenesis of NPC and that EBNA-2 gene variations are tumor-specific polymorphisms.

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Introduction

Epstein-Barr virus (EBV) is a human herpesvirus that infects over 90% of the human population and has been linked to numerous malignant/benign diseases, including Burkitt's lymphoma, posttransplant lymphoproliferative disorders, undifferentiated nasopharyngeal carcinoma (NPC), Hodgkin's lymphoma and infectious mononucleosis [1–3]. NPC is a squamous cell carcinoma of the head and neck that is closely associated with EBV infection, with a high prevalence in certain regions of Southern China [4–6]. The exact role of EBV in the pathogenesis of EBV-associated malignancies remains to be determined. The fact that EBV infection is ubiquitous around the world yet the incidence of EBV-associated malignancies differs geographically raises the possibility that certain EBV strains may contribute to the development of specific EBV-associated malignancies [7–11].

EBV nuclear antigen protein 2 (EBNA-2) is one of the first latent proteins detected after an EBV infection [12]. EBNA-2 primarily upregulates the expression of viral and cellular genes, including CD23, CD21, c-myc and the viral EBNA-C promoter [13–15]. EBNA-2 is also known to interact with other transcription factors involved in the Notch signaling pathway [16, 17]. This pathway is important in cell fate determination in the fruit fly and

may play a role in the development of T-cell lymphoma in humans.

In our previous studies on EBNA-2 gene polymorphism in NPC and EBV-associated gastric carcinomas from Northern China, 4 variations (AA417, 470, 476 and 486) were detected only in NPC, raising the possibility that strains with specific variations may associate with the development of NPC [18]. Here, we aimed to elucidate the variations of the EBNA-2 gene and potential association of EBNA-2 with the development of NPC in Southern China, as well as the potential differences between Southern and Northern Chinese samples. To do this we further collected 32 NPC specimens and 39 throat washing (TW) samples from Southern Chinese healthy donors in order to sequence and characterize the sequence variation of EBNA-2, which we then compared with the previously analyzed variation patterns in the NPC samples from Northern China.

Materials and Methods

Specimens and DNA Extraction

Paraffin-embedded NPC tissues were collected from Guangzhou province in Southern China, which is an endemic NPC area. Infection with EBV in NPC tissues was determined by EBER-1 (EBV-encoded small RNA 1) in situ hybridization, as described previously [19]. The cases with EBER1-positive signals in carcinoma cells were classified as EBV-positive NPCs and the DNA was extracted using the QIAamp DNA FFPE tissue kit (Qiagen GmbH, Hilden, Germany). TWs were collected from 135 age- and gender-matched healthy blood donors in the same geographic regions by gargling with 10 ml of phosphate-buffered saline. DNA was extracted from TWs using standard methodology employing proteinase K digestion and phenol-chloroform purification. The EBV-positive TWs were determined by positive BamHI W fragment signals using PCR with a BamHI W-specific primer pair [20]. All extracted DNA was stored at -20°C . All the study subjects gave their informed consent for the study, which was approved by the Medical Ethics Committee at the Medical College of Qingdao University, China.

Amplification of the EBNA-2 Gene

Nested PCR was used to amplify the DNA sequence coding 331–487 aa of EBNA-2. The sequences used in this research were described in our previous study [18]. The first round of PCR was performed in a total volume of 25 μl containing 1 $\times$ PCR reaction buffer, 100 ng of genomic DNA, 0.5 μM of each primer, 200 μM of each deoxyribonucleotide triphosphate, and 1 unit Pfu of Taq polymerase (TaKaRa Biotechnology Co. Ltd., Kyoto, Japan). PCR amplification was performed with an initial denaturation at 94°C for 5 min. Then, 35 cycles were carried out with denaturation at 94°C for 30 s, annealing at 53°C for 30 s, with extension at 72°C for 1 min. A final elongation step at 72°C for 10 min was also conducted. The products of the first round of PCR were amplified in the second round with the primers EBNA-2-N/E; identical condi-

tions were used in both rounds. DNA from the EBV-positive B95-8 and EBV-negative Ramos cell lines were used as positive and negative controls, respectively. The PCR products were separated by electrophoresis through 1.5% agarose gel and photographed under a UV light transilluminator after the gels were stained with ethidium bromide.

Sequencing Analysis of the PCR Products

The PCR products were purified using a gel extraction kit (QIAEX II; Qiagen GmbH) according to the directions of the manufacturer. For sequencing, the purified products were sent to Shanghai Mapbiotech Co. Ltd. Bidirectional sequencing was carried out to ensure that any variations were not due to experimental variability.

Data Analysis

All sequences were aligned and categorized based on their consensus sequence. One sequence was chosen to represent samples with identical sequences. Alignments between representative sequences were performed using MegAlign in DNA Star software (DNASTAR Inc., version 5.0), and a phylogenetic tree was constructed using the Clustal W method.

The distribution differences of EBNA-2 subtypes between NPCs and TWs were compared and analyzed by Fisher's exact test. The variant subtype distributions between the NPC samples from Southern and Northern China were compared using Fisher's exact test. Differences were considered significant at $p < 0.05$. SPSS 15.0 statistical software (SPSS, Chicago, Ill., USA) was used for the statistical analyses.

Results

Sequence Variation Patterns of EBNA-2 in Southern China

All of the samples (32 NPCs and 39 TWs) were successfully amplified and sequenced. A phylogenetic tree was created according to the representative unique sequences (fig. 1). Based on the variation patterns and our previous studies on EBNA-2, 3 subtypes were identified: E2-A, E2-C and B95-8-like (fig. 2) [18].

The most common subtype was E2-A (represented by NPC1). In this subtype, 5 common mutations were identified, including 2 missense mutations at residues 476 (Glu $\rightarrow$ Gly/Trp) and 486 (Ile $\rightarrow$ Thr), and 3 same-sense mutations at residues 370 (cct $\rightarrow$ cca), 417 (acg $\rightarrow$ aca) and 470 (tca $\rightarrow$ tct). Additional sporadic mutations were observed at 6 residues, 331 (Pro $\rightarrow$ Leu), 383 (cct $\rightarrow$ cca), 390 (ggg $\rightarrow$ gga), 411 (Val $\rightarrow$ Ile), 438 (Ile $\rightarrow$ Leu) and 484 (Pro $\rightarrow$ His).

The second most common subtype was E2-C (represented by NPC3). In this subtype there was 1 common mutation (AA370, cct $\rightarrow$ cca) and 2 AA deletions (AA357 and 358). Additional sporadic mutations were observed

Fig. 1. Phylogenetic tree based on the comparison of EBNA-2 amino acid sequences of 13 representative isolates using the Clustal W method. The analyzed regions were 331–487 aa of the EBNA-2 gene.

Subtype	South China	B95-8															
		331 P c c g	357 K a a g	358 G g g c	370 P c c t	378 S t t c	383 P c c t	390 G g g t	411 V g t t	417 T a c g	438 I a t t	451 P c c c	470 S t c a	476 E g a g	484 P c c c	486 I a t c	
		49194	49196	49197	49198	49199	49200	49201	49202	49203	49204	49205	49206	49207	49208	49209	
E2-A (23/15)	NPC1 (20/10)				a					a			t	G		T	
	TA21 (0/1)				a		a			a	L		t	G	H	T	
	TA52 (0/1)				a			g		a	L		t	G	H	T	
	NPC22 (1/0)				a					a			t	W		T	
	NPC24 (1/1)				a			a	I	a			t	G		T	
	NPC-A11 (1/2)	L t			a					a			t	G		T	
E2-C (9/14)	NPC3 (8/11)		*	*	a												
	T2 (0/1)		*	*	a	Y a											
	TA32 (0/2)		*	*	a						H a						
	NPC-A13 (1/0)		*	*	a				I a								
B95-8- like (0/10)	T39 (0/2)																
	TA56 (0/8)				a												

Fig. 2. EBNA-2 sequence variations in NPCs and TWs of healthy donors in Southern China. The numbers across the top correspond to the amino acid positions under which the B95-8 prototype amino acid and nucleotide sequence are listed. Different patterns are indicated in the left column while the specimens showing sequences identical to each other are represented by a single isolate in the second column. The numbers in parentheses indicate the number of identical sequences from NPCs and TWs, respectively. Only sequences that differed from B95-8 are indicated. For example,

NPC1 (20/10) indicates that 20 NPCs and 10 TWs had an identical sequence, and we chose NPC1 as the representative isolate. The lowercase letters indicate the nucleotide, and the amino acids are represented by capital letters. The consensus mutations of each pattern are shaded. Asterisks (*) indicate the deletion of an amino acid. T and TA represent the TW samples, and NPC and NPC-A represents the NPC tissues. A 6-bp insertion of gcaagg between nucleotide 49566 and 49567 was identified in TA21.

Subtype	<div><div>B95-8</div><div>Previous results</div></div>	357	358	370	411	417	470	474	476	486
		K	G	P	V	T	S	Y	E	I
		a a g g c c t g t a c g t c a t g a g a t c	a a g g c c t g t a c g t c a t g a g a t c	a a g g c c t g t a c g t c a t g a g a t c	a a g g c c t g t a c g t c a t g a g a t c	a a g g c c t g t a c g t c a t g a g a t c	a a g g c c t g t a c g t c a t g a g a t c	a a g g c c t g t a c g t c a t g a g a t c	a a g g c c t g t a c g t c a t g a g a t c	a a g g c c t g t a c g t c a t g a g a t c
E2-A	GD2			a		a	t		G	T
	C666-1			a		a	t		G	T
	HKNPC3			a		a	t		G	T
	HKNPC4			a		a	t		G	T
	HKNPC5			a		a	t		G	T
	HKNPC8			a		a	t		G	T
	HKNPC9			a		a	t	H	G	T
	HKNPC1			a		a	t		G	T
E2-C	GD1	*	*	a						
	HKNPC2	*	*	a						
	HKNPC6	*	*	a	I					
	HKNPC7	*	*	a	I					

Fig. 3. Results from the study by Kwok et al. [21] and other previously published NPC strains. The numbers across the top correspond to the amino acid positions under which the B95-8 prototype amino acid and nucleotide sequence are listed. Different patterns are indicated in the left column. Only sequences that differed from B95-8 are shown. The lowercase letters indicate the nucleotide, and the amino acids are represented by capital letters. Asterisks (*) indicate the deletion of an amino acid. GD2 is the nucleotide 49551–49566 deletion.

Table 1. Subtype distributions of sequence variations in NPC samples and TWs from Southern China

	NPCs (n = 32), %	TWs (n = 39), %	Total, n
E2-A	71.9 (23/32)	38.5 (15/39)	38
E2-C	28.1 (9/32)	35.9 (14/39)	23
B95-8-like	0 (0/32)	25.6 (10/39)	10

at 3 residues, including AA378 (Ger → Tyr), 411 (Val → Ile) and 451 (Pro → His).

The least common subtype, B95-8-like (represented by TA56), only had 1 mutation at residue 370 (cct → cca).

Sequence Variant Subtype Distribution in NPCs and TWs

The frequency of EBNA-2 subtypes in the NPC samples and TWs of healthy donors is summarized in table 1. E2-A was the predominant subtype in the tested speci-

mens (38/71, 52.1%) of the total samples, including 22/32 (71.9%) NPCs and 15/39 (38.5%) TWs. E2-C was found in 32.4% (23/71) of the specimens, including 9/32 (28.1%) NPCs and 14/39 (35.9%) TWs (table 1). The B95-8-like subtype was only identified in a quarter of the TWs (10/39; 25.6%).

The distribution of the EBNA-2 subtypes between the NPCs and TWs was significantly different ($p < 0.01$). The proportion of E2-A in NPCs (71.9%; 22/32) was significantly higher than in TWs (38.5%; 15/39), whilst the proportion of the B95-8-like subtype in TWs (25.6%; 10/39) was significantly higher than in NPCs (0%; 0/32).

Discussion

In this study, 32 NPC biopsies and 39 TWs of healthy donors in Southern China were successfully sequenced for the EBNA-2 gene. The DNA extraction methods used for the NPC samples and the TWs were both standard procedures and had no implication on the results. To the best of our knowledge, this is the first analysis of EBNA-2 sequence variations in Southern China, an endemic area of NPC. Based on the phylogenetic tree and the variation patterns of EBNA-2, all isolates could be subdivided into three subtypes, namely E2-A, E2-C and B95-8-like.

E2-A (53.5%; 38/71) was the predominant subtype in Southern China. The common sequence changes for this subtype were 370 (cct → cca), 417 (acg → aca), 470 (tca → tct), 476 (Glu → Gly/Trp) and 486 (Ile → Thr). These mutations were also identified in our previous studies on NPC in Northern China, in the study by Kwok et al. [21] on NPC in Hong Kong (fig. 3), and in the study by Walling et al. [22], with the exception of 486 (Ile → Thr).

Subtype E2-C (32.4%; 23/71) was the second most common subtype in Southern China. The common sequence changes for this subtype were AA370 (cct → cca) and two AA deletions (AA357 and 358). Similar results were also detected in our previous studies on NPC in Northern China [18] and in the study by Kwok et al. [21]. The AA370 (cct → cca) mutation was identified in the study by Walling et al. [22], but not the AA357 and 358 deletions. The consensus sequence of this subtype was identical to that of GD1, which is an EBV strain that was isolated from an NPC patient in Southern China [23]. The B95-8-like subtype (14.1%; 10/71) was the least common subtype in Southern China, with only 1 mutation at 370 (cct → cca).

Additionally, the synonymous variant at residue 370 (cct → cca) was detected in nearly all the isolates of this

Fig. 4. EBNA-2 sequence variations in NPCs and TWs in Northern China. The numbers across the top correspond to the amino acid positions under which the B95-8 prototype amino acid and nucleotide sequence are listed. Different patterns are indicated in the left column while the specimens showing sequences identical to each other are represented by a single isolate in the second column. The numbers in parentheses indicate the number of identical sequences from NPCs and TWs, respectively. Only sequences that differed from B95-8 are shown. The lowercase letters indicate the nucleotide, and the amino acids are represented by capital letters. The consensus mutations of each pattern are shaded. Asterisks (*) indicate the deletion of an amino acid. T represents the TW samples, and N represents the NPC tissues.

Subtype	<div><div>B95-8</div><div>Northern China</div></div>	357	358	370	383	408	413	417	438	470	476	484	486											
		K	G	P	P	A	R	T	I	S	E	P	I											
		a a g	g g c	c c t	c c t	g c c	c g a	a c g	a t t	t c a	g a g	c c c	a t c											
		49573	49574	49576	49612	49651	49652	49726	49727	49740	49741	49742	49753	49754	49815	49816	49817	49818	49819	49820	49821	49822	49823	49824
E2-A (14/0)	N116 (3/0)			a			a		a		t													T c
	N36 (5/0)			a					a															T c
	N107 (3/0)				a	a			a															T c
	N104 (1/0)				a	a		g		a c														T c
	N38 (2/0)				a			a		a														T c
E2-C (31/13)	N11 (31/12)	*	*		a																			
	T392 (0/1)	*	*		a			g	G															
B95-8-like (11/19)	T257 (5/9)				a			g	G															
	T63 (6/10)				a																			

Table 2. Distribution of variant subtypes between the NPC samples from Southern and Northern China

	NPCs in Northern China (n = 56), %	NPCs in Southern China (n = 32), %	χ^2
E2-A	25.0 (14/56)	71.9 (23/32)	p < 0.01
E2-C	55.4 (31/56)	28.1 (9/32)	p < 0.05
B95-8-like	19.6 (11/56)	0 (0/32)	p < 0.01

Table 3. Subtype distributions of sequence variations in NPC samples and TWs from Northern China

	NPCs (n = 56), %	TWs (n = 32), %	Total, n
E2-A	25.0 (14/56)	0 (0/32)	14
E2-C	55.4 (31/56)	40.6 (13/32)	44
B95-8-like	19.6 (11/56)	59.4 (19/32)	30

study (97.2%; 69/71), and was also identified in all of the NPC, gastric carcinoma and healthy adult TW samples in our previous study [18]. This variant was also reported in 6 human immunodeficiency virus-infected patients and the NPC samples from Hong Kong or Guangzhou [22], in the work of Kwok et al. [21] (fig. 3) and in oral hairy leukoplakia samples from the USA [22, 24], indicating that 370 (cct → cca) might be a common allele around the world.

Whether a particular EBV strain contributes to the development of specific EBV-associated malignancies is still debatable. In our previous studies of EBNA-2 gene polymorphisms in NPC, gastric carcinoma and TWs from Northern China, E2-A was detected only in NPC, which suggests the strain with specific mutations plays an important role in the pathogenesis of NPC (because the sequence regions were not the same, we redivided the Northern Chinese results according to the variation patterns of Southern China; fig. 4; table 3) [18]. Similarly, in this study we found that the E2-A subtype was signifi-

cantly more common in NPCs than in TWs (p < 0.01; table 1), and it was also more frequent than in the samples from Northern China (p < 0.01; table 2). E2-A was also identified as the predominant subtype in the Hong Kong NPC study conducted by Kwok et al. [21] (fig. 3). As an endemic NPC region, the high frequency of E2-A in Southern China suggests that the E2-A subtype may play an important role in the pathogenesis of NPC and that EBNA-2 gene variations are tumor-specific polymorphisms. However, genetic variations in the small subsets of the genes investigated were not sufficient to assess the geographical distribution of EBV variants and their precise associations with diseases. Whole genome sequences and a genome-wide comparison of the variations found in EBV genomes isolated from NPC and normal subjects are needed to determine the role of EBV genomic variations in the pathogenesis of NPC, which will be the focus of our future studies.

Other factors may influence the occurrence of cancers, such as host genetics and the environment. For example,

5p13.1 was identified as a subset of hereditary risk for NPC [25], and 3p21 may play a critical role in the tumorigenesis of familial NPC [26]. Salted fish containing volatile nitrosamines, cigarette smoking and the consumption of alcohol are environmental factors associated with the development of NPC [27]. DNA methylation is known to contribute to interindividual phenotypic variations and has recently been linked to the pathogenesis of cancer and complex disease phenotypes [28, 29].

EBNA-2 contains three domains critical for its transcription regulation function: the self-association domain (122–232 aa), trans-activation domain (TAD; 431–487 aa) and nuclear localization signals (NLS; 244–378 aa and 466–483 aa) [30, 31]. TAD (431–487 aa) and NLS (244–378 aa and 466–483 aa) were sequenced in this study.

TAD is a strong transcriptional activator, particularly in B lymphocytes, and is essential for the EBNA-2 activation of cells and viral genes [32]. Additionally, it has been previously shown that the amino acid composition of TAD is similar to that of a number of human and viral TADs, including the human tumor suppressor protein p53 and the herpes simplex viral protein 16 [33]. Meanwhile, it has been reported that TAD binds to the pleckstrin homology domain of the Tfb1/p62 subunit of TFIIH and determines the three-dimensional structure of a complex between EBNA-2 and Tfb1/p62 [34]. The AA476 (Glu → Gly/Trp) and 486 (Ile → Thr) mutations in this study were detected in this domain.

AA335–362 in the NLS domain is a protein-protein and protein-nucleic acid interaction domain, important

for efficient cell growth transformation, and a downregulator of the LMP1 promoter [32]. Meanwhile, AA357–363 has been proven to be the protein kinase C phosphorylation site, which could reduce the formation of the EBNA-2/CBF1 complex [35]. The AA357/358 deletions and the AA476 (Glu → Gly/Trp) mutation in this important region are likely to affect the function of EBNA-2, which warrants further functional studies.

Considering the shortcomings of this work, in future studies direct comparison of TWs and NPC tissue from the same population and on the same patients should be included. This may be difficult but would be very useful in indicating the tissue specificity of the genotypic changes.

In conclusion, subtype E2-A was the predominant subtype in Southern China. The distribution of EBNA-2 subtypes between NPCs and TWs was significantly different ($p < 0.01$). The E2-A subtype is significantly more frequent in NPCs than in TWs, and is also more common than in Northern China, which suggests that the E2-A subtype may play an important role in the pathogenesis of NPC and that EBNA-2 gene variations are tumor-specific polymorphisms.

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