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Sequence Variations of the Latent Membrane Protein 1 of Epstein-Barr Virus Isolates from Cases of Malaysian Nasopharyngeal Carcinoma: A Preliminary Study

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ABSTRACT The nucleotide sequences of the Epstein-Barr virus (EBV) latent membrane protein 1 (LMP1) genes from throat washing samples of six Malaysian nasopharyngeal carcinoma (NPC) patients were determined. The full-length LMP1 gene was amplified by PCR in two segments; exon A to exon B (exons AB) and exon C, and cloned into pcDNA3.1/V5-His©TOPO®TA plasmid for DNA sequencing. Full length LMP1 DNA sequences were obtained from three throat wash samples while in the remaining three samples, only the exons AB was successfully sequenced. DNA sequence alignment showed that the three completely sequenced LMP1 genes analyzed had, on average, 96% homology with the LMP1 sequence of the EBV B95.8 wild-type strain. The majority of the point mutations identified were common to those reported for the EBV isolates from China and Taiwan. These mutations were mainly clustered at the transmembrane region. However, there were a few base substitutions, which were unique to individual samples. All six samples displayed a loss of *Xho*1 restriction site at the amino terminal of the LMP1 gene. In three of the six samples (P8, P17 and P58), the 15 bp and 30 bp deletions at the carboxyl terminal were detected. As a result of the 15 bp deletion, the LMP1 genes from two of the three samples (P17 and P58) displayed four perfect repeats while in the remaining sample (P8), five perfect repeats were detected. Overall, of the amino acid sequences of the six LMP1 genes analyzed showed the highest homology to the LMP1 gene derived from the China 1 EBV strain.

ABSTRAK Jujukan nukleotida gen "latent membrane protein 1" (LMP1) virus *Epstein-Barr* (EB) yang berasal dari hasil kumuh pesakit kasinoma nasofarinks (NPC) telahpun ditentukan. Gen LMP1 diamplikasikan secara PCR dalam dua segmen; exon A hingga exon B (exon AB) dan exon C, dan diklonkan ke dalam plasmid pcDNA3.1/V5-His©TOPO®TA untuk proses penjujukan DNA. Jujukan DNA gen LMP1 yang lengkap dapat ditentukan dari tiga sampel kumuh manakala tiga sampel yang lain hanya dapat meghasilkan jujukan DNA untuk exon AB. Penjajaran urutan DNA tiga gen LMP1 lengkap tersebut menunjukkan 96% homologi dengan urutan gen virus EB B95.8. Sebilangan besar daripada mutasi yang dikesan merupakan mutasi umum yang juga didapati pada virus EB dari Negara China dan Taiwan. Kebanyakan daripada mutasi tersebut berkelompok pada bahagian trans-membran. Tetapi terdapat juga beberapa mutasi yang unik pada sampel-sampel yang tertentu. Setiap enam sampel tersebut mempunyai kehilangan tapak enzim restriksi *Xho*1 pada bahagian terminal amino gen LMP1. Tiga daripada sampel tersebut (P8, P17 dan P58) mempunyai pencoretan DNA sepanjang 15 bp dan 30 bp pada bahagian terminal karboksi. Pencoretan DNA 15 bp meghasilkan empat unit pengulangan DNA di dalam dua sampel (P7 dan P58) dan lima unit pengulangan DNA pada satu sampel yang lain (P8). Secara keseluruhannya, jujukan asid amino enam gen LMP1 yang telah dikaji menunjukkan homologi yang ketara dengan gen LMP1 yang berasal dari EBV jenis *China 1*.

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a malignant tumor derived from epithelial cells at the posterior part of the nasopharynx, the site of origin of which is the Fossa of Rosenmuller (FOR) [1]. NPC occurs in all parts of the world but its frequency is geographically distinct. It is relatively rare in Europe and North America but prevalent in Southeast Asia especially in Southern China, Hong Kong, Taiwan, Singapore and Malaysia [2, 3]. According to the Malaysian National Cancer Registry Report, 2003 [4], the incidence of NPC, among all types of cancer, was ranked the second highest among Malaysian males (8.8%) and ranked twelfth among females (2.5%).

One of the etiological factors of NPC is the Epstein-Barr virus (EBV). Biochemical and immunological evidence have demonstrated, indirectly, the close association between EBV and NPC [5]. EBV is a ubiquitous human herpesvirus, which infects approximately 90% of the world's adult population [6]. It infects human B-lymphocytes and epithelial cells [7, 8]. EBV is known to cause infectious mononucleosis [9] and is associated with several human cancers namely Burkitt's lymphoma [10], NPC [11, 12] and lymphoma in immunocompromised patients [13]. In NPC, the EBV infection is characterized by latency type II where only the less-immunogenic proteins such as EBNA 1, LMP 1 and LMP 2 are being expressed in the infected tumor cells [14]. Of all the EBV gene products, LMP1 has been postulated to be the major oncogenic protein in NPC as it is able to inhibit differentiation and induce transformation of human epithelial cells.

Latent membrane protein 1 (LMP1) has been found in 65% of biopsy specimens from NPC patients [15]. The EBV-encoded LMP1 is known to transform rodent fibroblasts cell lines [17], B-lymphocytes [17] and human epithelial cells [18]. Although the EBV LMP1 gene is highly conserved among different isolates, a few studies in the past had postulated the existence of NPC-specific LMP1 genes that were characterized by several consistent amino acid changes [19, 20]. One of the commonly reported polymorphism among the NPC isolates is the loss of *Xho*1 restriction site at codon 17.

Cheung *et al.* [21] reported that over 90% of the primary tumor in Hong Kong has the 30 bp deletion. This deletion has also been observed in Taiwan and Northern China [22]. By contrast, LMP1 genes from the Alaskan EBV strains do not harbour the 30 bp deletion [23]. In addition, the carboxyl terminal domain of LMP1 gene of EBV isolates derived from NPC specimens were also reported to display a number of 11 amino acid internal repeats that are distinct from the wild-type [22]. We hypothesize that there exists NPC-specific EBV LMP1 variants. In this study, the EBV LMP1 genes derived from throat wash samples of patients with NPC were compared to the LMP1 from the wild-type EBV, B95.8.

MATERIALS AND METHODS

Clinical samples and cell lines

Throat wash samples were collected from 20 nasopharyngeal carcinoma patients who were in remission and from 19 healthy individuals. The NPC patients who sought treatment at University of Malaya Medical Centre (UMMC), Kuala Lumpur, consisted of 15 males and five females aged between 22 to 69 years. The 19 healthy individuals consisted of six males and 13 females aged between 22 to 50 years. Throat wash samples were obtained by gargling with 10 ml of PBS for one min. The collected throat washes were centrifuged at 3000 rpm for 15 minutes and may contain EBV derived from exfoliated epithelial cells. The cell pellets were resuspended in 200 µl of PBS. DNA was extracted using QIAamp DNA Mini Kit (QIAGEN, Germany) following the manufacturer's protocol. B95.8 and AG876 cell lines were used as controls.

PCR and Cloning

Polymerase chain reaction (PCR) was performed with 100 – 200 ng of DNA in a 50.0 µl reaction volume containing PCR buffer, 2.5 mM $MgCl_2$, 1 µM of each primer, 200 µM of each dNTP (Promega, USA) and 1.25 units of Hot Star Taq DNA Polymerase (QIAGEN, Germany). Primers AR and BF were used to amplify the fragment from exon A to exon B (exons AB) whereas primers CF and CR were used to amplify exon C (Table I). The cycle profile consisted of initial denaturation at 95°C for 10 minutes, amplification for 35 cycles with denaturation at 95°C for one minute, annealing at 55°C for one minute and polymerization at 72°C for one minute, and followed by final extension at 72°C for five minutes. The PCR reaction was carried out using Perkin-Elmer-Cetus Thermal Cycler

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Table 1. Primers used in PCR and sequencing reactions

PRIMERS	SEQUENCE 5' – 3'	B95.8 COORDINATES[a]
AR	ATGGAACACGACCTTGAGAGGGGC	169474 – 169451
BF	GGGAATGTTAGATCTTACCAAG	169024 – 169045
CR	CCACACCCCCACTTTTCCAACCC	169010 – 168987
CF	GTCATAGTAGCTTAGCTGAACTGG	168163 – 168186
S1	TAATACGACTCACTATAGGG	
S6	TAGAAGGCACAGTCGAGG	

[a] Sequence coordinates correspond to the B95.8 sequence published by Baer *et al.* [24].

DNA Sequencing

DNA sequencing was carried out using the MegaBACE 1030 DNA Analysis System (Amersham Biosciences, UK) at the Cancer Research Initiatives Foundation (CARIF), Subang Jaya Medical Centre. This is a fluorescence-based system utilizing capillary electrophoresis. The sequencing reagent mix consisted, in addition to the standard buffer components, 5 µM primer and 300 – 350 ng of plasmid DNA. The primers used in LMP1 sequencing were S1 (forward primer) and S6 (reverse primer) (Table I). The 30 sequencing cycles consisted of denaturation at 95°C for 20 seconds, annealing at 50°C for 15 minutes and extension at 60°C for 1 min 30 seconds. The sequencing data were analyzed using DNASTAR© Sequence Analysis Software (Lasergene, USA).

RESULTS

The EBV LMP1 gene was amplified in two separate segments. The first segment consisted of exons A and B and the second segment consisted of exon C. Of the 20 throat washes, LMP1 was amplifiable only in six samples (30%) for either one or both of the segments. Figure 1 shows amplimers of exons AB. The exon C amplimers in five of the six samples (P7, P17 P58, P75 and P82) had bands corresponding to the amplimer from AG876 (803 bp) while the remaining sample (P8) had an amplimer with the size similar to B95.8 (848 bp) (Figure 2). The LMP1 gene was not detected by PCR in all the throat washes from healthy individuals. The LMP1 amplimers from the six NPC samples were cloned into plasmids and subjected to sequencing. The sequence of the entire LMP1 coding region was successfully sequenced in three (50%) of the six clones (from samples P8, P17 and P58). The other three clones (from samples P7, P75 and P82) were only able to yield the sequences of exons AB.

Sequence variation in the amino terminal cytoplasmic domain of LMP1

The amino terminal cytoplasmic domain of LMP1 spans from codon 1 to codon 24 of exon A. This region was generally conserved among the six NPC samples sequenced. There were only three nucleotide substitutions in this region with respect to the B95.8 strain. Two of these nucleotides changes (169437 G→C and 169425 G→T) were found in the LMP1 genes of samples P7, P8, P17, P58, P75 and P82. The other nucleotide substitutions at position 169428 C → T was found only in the LMP1 gene from P8 isolate (Figure 3). These changes had led to amino acid changes at positions 13 R→P, 16 P→L and 17 R→L respectively (Figure 4). The loss of *Xho*1 restriction site was detected in all six samples.

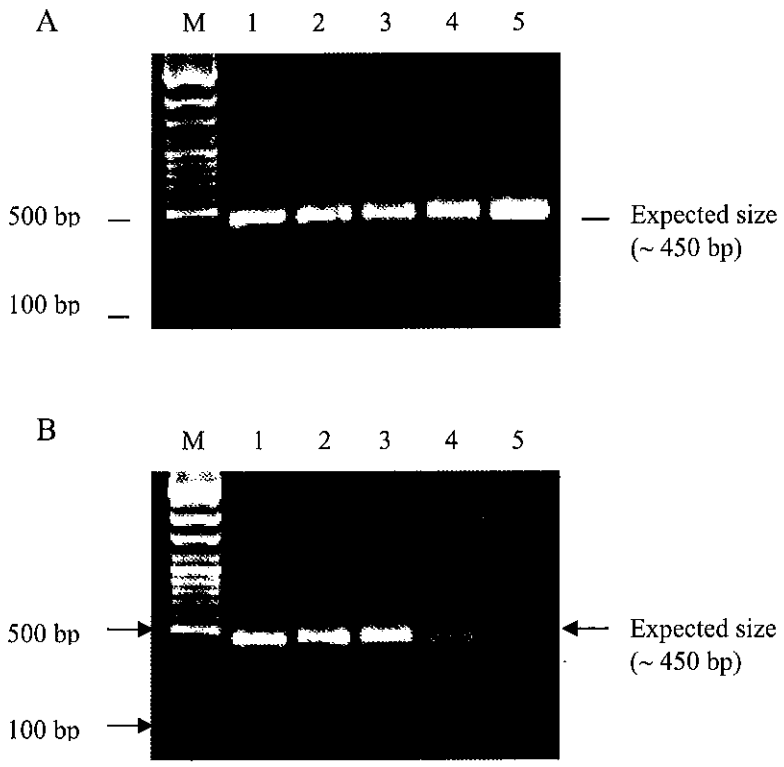


Figure 1. Amplimers of exons AB from throat wash samples of six NPC patients. M: molecular marker. Lane 1: B95.8 (positive control). Lane 2: AG876 (positive control). (A) Lanes 3 – 5: Samples P7, P8 and P17, respectively. (B) Lanes 3 – 5: Samples P58, P75 and P82, respectively.

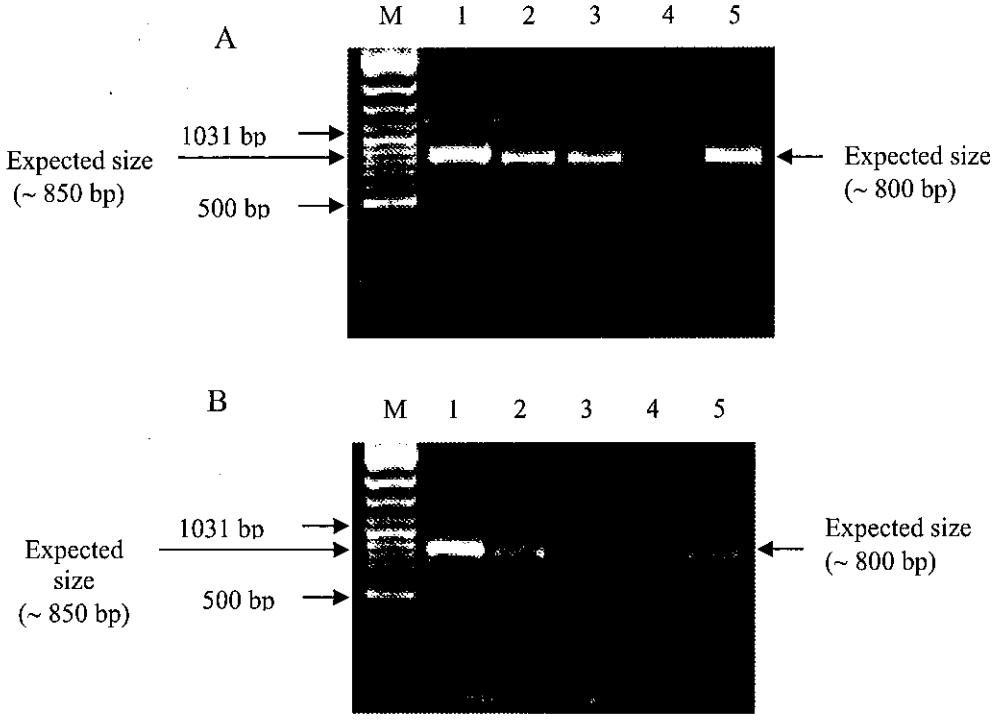
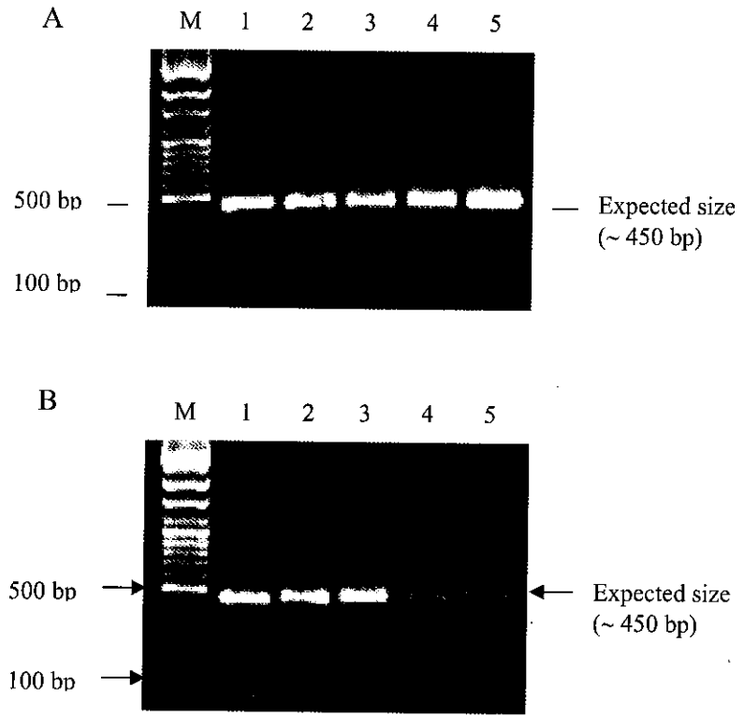


Figure 2. Amplimers of exon C from throat wash samples of six NPC patients. M: molecular marker. Lane 1: B95.8 (positive control). Lane 2: AG876 (positive control). (A) Lanes 3 – 5: Samples P7, P8 and P17, respectively. (B) Lanes 3 – 5: Samples P58, P75 and P82, respectively.



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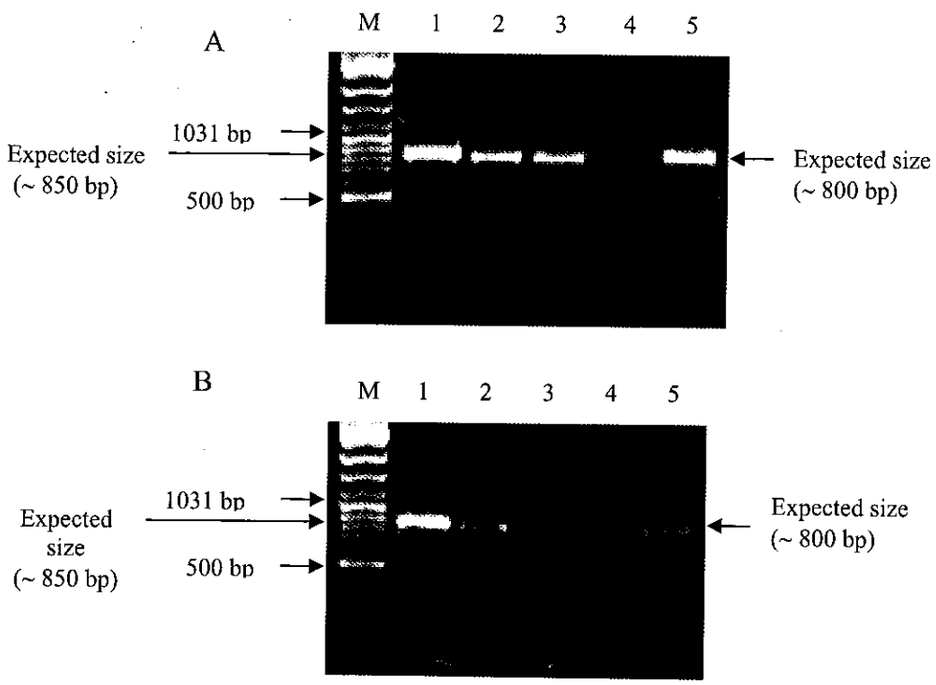

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Sequence variation in the transmembrane domain of LMP1

In the transmembrane domain of the LMP1 gene, all the six NPC samples shared the same nucleotide substitutions at 28 positions with respect to the B95.8 sequence (Figure 3). Fourteen of these nucleotide changes resulted in amino acid changes at their respective positions: 25 L → I, 46 D → N, 82 A → G, 84 C → G, 85 I → L, 106 F → Y, 122 I → L, 126 L → F, 129 M → I, 144 F → I, 150 D → A, 151 L → I and 178 L → M (Figure 4). The other 14 nucleotide substitutions were silent mutations (Figures 3 and 4). In addition, there were some nucleotide changes unique to individual samples. A nucleotide substitution at position 168912 A → C was unique to sample P8 and resulted in amino acid change at position 137 I → L. Unique nucleotide changes 169068 T→C and 169277 T→C was found in samples P58 and in P17, respectively.

Sequence variation in the carboxyl terminal cytoplasmic domain of LMP1

The carboxyl terminal cytoplasmic domain is the third domain of the LMP1 and functionally, the most important. It is encoded in exon C and comprises of amino acids 187 to 386. However, sequencing of this region was only successfully conducted for three of the six samples (samples P8, P17, and P58). Sequence analysis of this region revealed 11 nucleotides substitutions at different positions that were detected in all the three NPC-derived LMP1 sequences (Figure 3). Of these 11 base substitutions, eight resulted in amino acid changes corresponding to the following positions: 189 Q → P, 192 S → T, 212 G → S, 309 S → N, 322 Q → N, 334 Q → R, 338 L → S, 366 S → T (Figure 4). Sample P8 harboured an additional two base substitutions at positions 168338 A → G and 168317 G → A that resulted in amino acid changes at positions 328 E → G and 335 G → D respectively. Base substitutions at positions 168601 A → C and 168736 C → T were unique to samples P17 and P58 respectively. The former resulted in amino acid substitution at position 240 Q → H while the latter was a silent mutation (Figures 3 and 4).

In addition to base substitutions, there were also deletions detected along the carboxyl terminal. The most frequent deletion detected was the 30 bp deletion, which was found near the 3' end of the LMP1 gene. All of the isolates had this deletion that resulted in 10 amino acid deletions corresponding to codons 343 to 352 of the B95.8 reference sequence (Figure 4). In addition, there were different numbers of 11 amino acid repeat units between amino acids 250 and 305. Sample P8 had five perfect repeats whereas samples P17 and P58 had four (Figure 4).

DISCUSSION

The EBV LMP1 genes that are expressed in NPC are thought to represent mutated variants that differ from the B95.8 EBV wild-type sequence. The LMP1 gene was only amplifiable in six of the 20 throat washing samples. This low frequency of detection was expected of this latent EBV gene as the sequestration of the viral particle from the epithelial cells into the saliva is a rare occurrence. Sequence analyses of the LMP1 gene of NPC-derived EBV isolates are an initial step towards the elucidation of amino acid changes with respect to the wild-type that are responsible for its tumour-promoting activities. This study represents a preliminary investigation carried out to analyze the sequence variations of the entire coding region of LMP1 gene isolated from Malaysian NPC patients. The LMP1 isolated from the throat washes of three NPC patients (P8, P17 and P58) had an average of 96% homology at the nucleotide level and 93% homology at the amino acid level to the LMP1 sequence of the reference B95.8 strain. This is close to the 95% homology to the B95.8 strain reported for the EBV isolates from China and Taiwan isolates [19, 20]. There are 50 nucleotide substitutions that resulted in 30 amino acid alterations along the entire coding region of the NPC-derived LMP1 gene. Overall, the LMP1 genes derived from six of the 20 NPC patients recruited in this study (P7, P8, P17, P58, P75 and P82), displayed high sequence homology to EBV isolates from China and Taiwan. In comparison, the LMP1 derived from an African isolate (C15) and the AG876 strain, displayed a 99% homology to the B95.8 [23, 27]. These findings clearly indicate the existence of specific mutations in the LMP1 gene that were unique to NPC.

	169437	169428	169425	169402	169379	169361	169352	169339	169322	169286	169280	169277	169276	169274	169230	169225	169222	169118	169080	169068	169055	169053	169052
B95.8	G	C	G	C	C	T	C	G	C	A	A	T	T	G	C	T	A	G	T	T	C	G	G
P7	**C**	C	**T**	**A**	**G**	**C**	**T**	**A**	**G**	**T**	**C**	T	**C**	**C**	**G**	**G**	**C**	**A**	**A**	T	**T**	**C**	**C**
P8	**C**	**T**	**T**	**A**	**G**	**C**	**T**	**A**	**G**	**T**	**C**	T	**C**	**C**	**G**	**G**	**C**	**A**	**A**	T	**T**	**G**	**C**
P17	**C**	C	**T**	**A**	**G**	**C**	**T**	**A**	**G**	**T**	**C**	**C**	**C**	**C**	**G**	**G**	**C**	**A**	**A**	T	**T**	**G**	**C**
P58	**C**	C	**T**	**A**	**G**	**C**	**T**	**A**	**G**	**T**	**C**	T	**C**	**C**	**G**	**G**	**C**	**A**	**A**	**C**	**T**	**G**	**C**
P75	**C**	C	**T**	**A**	**G**	**C**	**T**	**A**	**G**	**T**	**C**	T	**C**	**C**	**G**	**G**	**C**	**A**	**A**	T	**T**	**C**	**C**
P82	**C**	C	**T**	**A**	**G**	**C**	**T**	**A**	**G**	**T**	**C**	T	**C**	**C**	**G**	**G**	**C**	**A**	**A**	T	**T**	**C**	**C**

	168957	168943	168934	168925	168912	168891	168872	168870	168859	168789	168784	168763	168755	168746	168736	168694	168687	168631	168601	168495 to 168481
B95.8	A	A	G	A	A	T	A	C	C	C	G	C	A	G	C	T	G	A	A	Retention
P8	**C**	**C**	**T**	**G**	**C**	**A**	**C**	**A**	**T**	**A**	**C**	**T**	**C**	**C**	C	**C**	**A**	**G**	A	15 bp deletion
P17	**C**	**C**	**T**	**G**	A	**A**	**C**	**A**	**T**	**A**	**C**	**T**	**C**	**C**	C	**C**	**A**	**G**	**C**	15 bp deletion
P58	**C**	**C**	**T**	**G**	A	**A**	**C**	**A**	**T**	**A**	**C**	**T**	**C**	**C**	**T**	**C**	**A**	**G**	A	15 bp deletion

	168574 to 168406	168395	168357	168355	168338	168320	168317	168308	168295 to 168266	168225
B95.8	4.5 repeats	G	C	A	A	A	G	T	Retention	T
P8	5 repeats	**A**	**A**	**T**	**G**	**G**	**A**	**C**	30 bp deletion	**A**
P17	4 repeats	**A**	**A**	**T**	A	**G**	G	**C**	30 bp deletion	**A**
P58	4 repeats	**A**	**A**	**T**	A	**G**	G	**C**	30 bp deletion	**A**

Figure 3. Sequence variations of LMP1 genes from 6 NPC patients with respect to the wild-type B95.8 sequence. Numbers across the top row correspond to the EBV genome coordinates according to Baer et al., [24]. Numbers across the bottom row correspond to amino acid coordinates. The base pair substitutions are indicated in boldface.

	169437	169428	169425	169402	169379	169361	169352	169339	169322	169286	169280
B95.8	G	C	G	C	C	T	C	G	C	A	A
P7	C	C	T	A	G	C	T	A	G	T	C
P8	C	T	T	A	G	C	T	A	G	T	C
P17	C	C	T	A	G	C	T	A	G	T	C
P58	C	C	T	A	G	C	T	A	G	T	C
P75	C	C	T	A	G	C	T	A	G	T	C
P82	C	C	T	A	G	C	T	A	G	T	C

	169277	169276	169274	169230	169225	169222	169118	169080	169068	169055	169053	169052
B95.8	T	T	G	C	T	A	G	T	T	C	G	G
P7	T	C	C	G	G	C	A	A	T	T	C	C
P8	T	C	C	G	G	C	A	A	T	T	G	C
P17	C	C	C	G	G	C	A	A	T	T	G	C
P58	T	C	C	G	G	C	A	A	C	T	G	C
P75	T	C	C	G	G	C	A	A	T	T	C	C
P82	T	C	C	G	G	C	A	A	T	T	C	C

	168957	168943	168934	168925	168912	168891	168872	168870	168859	168789
B95.8	A	A	G	A	A	T	A	C	C	C
P8	C	C	T	G	C	A	C	A	T	A
P17	C	C	T	G	A	A	C	A	T	A
P58	C	C	T	G	A	A	C	A	T	A

	168784	168763	168755	168746	168736	168694	168687	168631	168601	168495 to 168481
B95.8	G	C	A	G	C	T	G	A	A	Retention
P8	C	T	C	C	C	C	A	G	A	15 bp deletion
P17	C	T	C	C	C	C	A	G	C	15 bp deletion
P58	C	T	C	C	T	C	A	G	A	15 bp deletion

	168574 to 168406	168395	168357	168355	168338	168320	168317	168308	168295 to 168266	168225
B95.8	4.5 repeats	G	C	A	A	A	G	T	Retention	T
P8	5 repeats	A	A	T	G	G	A	C	30 bp deletion	A
P17	4 repeats	A	A	T	A	G	G	C	30 bp deletion	A
P58	4 repeats	A	A	T	A	G	G	C	30 bp deletion	A

Figure 3. Sequence variations of LMP1 genes from 6 NPC patients with respect to the wild-type B95.8 sequence. Numbers across the top row correspond to the EBV genome coordinates according to Baer et al., [24]. Numbers across the bottom row correspond to amino acid coordinates. The base pair substitutions are indicated in boldface.

	Exon A (Codon 1 – 90)								Exon B (Codon 90 – 119)			
	N-cytoplasmic		Transmembrane domain									
	13	16	17	25	46	82	84	85	106	110	115	119
B95.8	R	P	R	L	D	A	C	I	F	V	G	V
P7	**P**	P	**L**	**I**	**N**	**G**	**G**	**L**	**Y**	V	**A**	-
P8	**P**	**L**	**L**	**I**	**N**	**G**	**G**	**L**	**Y**	V	G	V
P17	**P**	P	**L**	**I**	**N**	**G**	**G**	**L**	**Y**	V	G	V
P58	**P**	P	**L**	**I**	**N**	**G**	**G**	**L**	**Y**	**A**	G	V
P75	**P**	P	**L**	**I**	**N**	**G**	**G**	**L**	**Y**	V	**A**	-
P82	**P**	P	**L**	**I**	**N**	**G**	**G**	**L**	**Y**	V	**A**	-

	Exon C (Codon 119 – 386)											
	Transmembrane domain								C-cytoplasmic domain			
	122	126	129	137	144	150	151	178	189	192	212	240
B95.8	I	L	M	I	F	D	L	L	Q	S	G	Q
P8	**L**	**F**	**I**	**L**	**I**	**A**	**I**	**M**	**P**	**T**	**S**	Q
P17	**L**	**F**	**I**	I	**I**	**A**	**I**	**M**	**P**	**T**	**S**	**H**
P58	**L**	**F**	**I**	I	**I**	**A**	**I**	**M**	**P**	**T**	**S**	Q

	Exon C (Codon 119 – 386)									
	C-cytoplasmic domain									
	276 – 280	250 – 305	309	322	328	334	335	338	343 – 352	366
B95.8	Retention	4.5 repeats	S	Q	E	Q	G	L	Retention	S
P8	5 a.a deletion	5 repeats	**N**	**N**	**G**	**R**	**D**	**S**	10 a.a deletion	**T**
P17	5 a.a deletion	4 repeats	**N**	**N**	E	**R**	G	**S**	10 a.a deletion	**T**
P58	5 a.a deletion	4 repeats	**N**	**N**	E	**R**	G	**S**	10 a.a deletion	**T**

Figure 4. Amino acid variations of LMP1 in NPC patients. Numbers across the top row correspond to the amino acid position. Names in the left column refer to the individual isolates. The amino acid changes are in boldface.

In the amino terminal cytoplasmic domain of LMP1, two non-conservative changes at amino acid positions 13 and 17 were identified in all the six NPC samples analyzed in this study. These changes were also reported in the CAO and 1510 isolates [19, 20]. These changes resulted in two non-polar residues and correspondingly, a transition from hydrophilic to hydrophobic domain in this region is predicted. This transition may affect the folding and function of the LMP1 protein in the plasma membrane especially the anchoring of its first transmembrane domain into the plasma membrane and its protein turnover by the ubiquitin-proteasome degradation pathway [25, 26].

Not surprisingly, the LMP1 gene of all the NPC samples displayed a loss of the *Xho*1 restriction site resulting from a base substitution at nucleotide position 169425 G→T. This point mutation has been previously reported in Malaysian NPC [28, 29]. This well-known polymorphism was also observed in the LMP1 genes derived from NPC samples from Hong Kong, Taiwan and China [22] and also in Alaska [29]. The loss of the *Xho*1 has been suggested to be specific to Asian NPC LMP1 [28].

The present study demonstrated that most of the amino acid alterations are clustered at the transmembrane domain. An important non-conservative amino acid change occurred at position 129 that was observed in the three NPC samples that were successfully sequence for this region (Figure 4). This mutation resulted in the substitution of methionine for isoleucine and thus the abolishment of the lytic-LMP1 translation initiation site, the expression of which is a crucial component of the viral lytic cycle [30]. Therefore, this mutation may reinforce the maintenance of latent EBV infection in NPC.

There are several functional epitopes in LMP1. The most notable was the A2-restricted epitope that was located at amino acid 125 to 133 in the transmembrane region [31]. Interestingly, a change in amino acid at position 126 L → F was observed in three samples (Figure 4). This position is critical for peptide binding by major histocompatibility complex class I (MHC-I) as it is the anchoring position within the LMP1 epitope [32]. Chromium release assay has demonstrated a much weaker induction of LMP1-specific cytotoxic T lymphocytes response resulting from the change in amino acid 126 L → F [31]. This may result in the expression of the non-immunogenic LMP1 variant in NPC that contribute to host immune evasion.

The well established 30 bp deletion in the carboxyl-terminus of the LMP1 was also detected in all three NPC samples analyzed. This deletion is known to be closely related to NPC and has been reported in LMP1 genes derived from NPC cases in China and Taiwan [19, 20]. However, it was not found in the Alaskan isolates [23]. Sandvej *et al.* [33] have proposed that the 30 bp deletion is caused by homologous recombination between short repeats that flank the deleted region during viral replication. The LMP1 gene of the Alaskan EBV isolates possess nucleotide changes at positions 168288 and 168260 that flank the repeat region and thus disrupt the site for homologous recombination [23]. On the other hand, most Asian EBV isolated such that reported here, do not possess any point mutation in the repeat region of the LMP1 gene. A few studies have indicated that the 30 bp deletion was able to increase the oncogenicity of LMP1 [34] and produce higher metastatic activity with reduced immunogenicity [16, 19, 35] Other studies failed to show any importance of the 30 bp deletion in functional analyses of the LMP1 gene [36, 37, 38].

There are repeats consisting of 11 amino acids in the carboxyl terminal of LMP1. The number of repeats varies between different viral isolates and is caused by recombination during virus replication [23]. The LMP1 genes from two samples in this study, P17 and P58, had four repeats (Figure 4), which were similar to the Guangzhou isolates [39]. Interestingly, one of the samples, P8, had five repeats (Figure 4), as has been reported in the Taiwan, Hong Kong and African isolates [22]. However, there was no correlation between the number of repeats and the geographical region [23]. Besides, Li *et al.* [34] have suggested that these repeats have no effect on the transformation activity of LMP1. In addition to different number of repeats, the P8 isolate had a unique non-conservative amino acid change at position 335 (Figure 4), which is identical to DV-Asp 335 LMP1 variant prevalent in Hong Kong [22]. As the altering position laid between codons 322 to 364, it may affect the turnover regulation of LMP1 [16].

With the exception of a non-conservative change at amino acid 189 in the CTAR1 region all the LMP1 genes from the samples analyzed in this study displayed a conserved CTAR1 and CTAR2 regions. There are two critical functional motifs within the CTAR 1 and CTAR 2 regions. The PXQXT motif in CTAR 1 located at amino acid 204 to 208 and the PXQXS motif in CTAR 2, which is located at amino acid 379 to 383. These motifs are important regulators of down-stream signaling functions involving tumor necrosis factor (TNF) receptor-associated factor (TRAF) [40], epidermal growth factor receptor expression (EGFR) [41] and TNF receptor-associated death domain-containing protein (TRADD) [42]. Both of the motifs are also conserved in EBV isolates from different geographic regions [43] although silent mutations that do not alter the functions of the motifs may accumulate [44].

In conclusion, sequence analyses of all the LMP1 genes derived from Malaysian NPC samples in this study demonstrated high homology to the China 1 strain. This finding is consistent with a previous study which reported that 63% of LMP1 genes of Asian EBV isolates belonged to the China 1 EBV strain [44].

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