Exp Oncol 2007
29, 3, 166–174

Experimental ONCOLOGY

BIOLOGICAL PROPERTIES OF TW01 CELLS EXPRESSING LATENT MEMBRANE PROTEIN-1 GENE OF EBV-DERIVED FROM NASOPHARYNGEAL CARCINOMA CELLS AT DIFFERENT STAGES OF MALIGNANCY

E.-L. Tan, C.-K. Sam*

NPC Laboratory, Institute of Postgraduate Studies, University of Malaya, 50603 Kuala Lumpur, Malaysia

Background and Aim: **Epstein—Barr virus (EBV), a human gammaherpesvirus is intimately associated with nasopharyngeal carcinoma (NPC), with the incidence of the virus detected in malignant tissues being close to 100% in NPC endemic areas. The viral latent gene, latent membrane protein 1 (LMP1), has all the typical characteristics of an oncogene and extensive studies have shown beyond doubt its abilities in cellular transformation giving rise to malignant phenotypes. The present study compares the gene sequence and biological properties of *LMP1* gene derived from two patients with different stages of NPC – one presented with dysplastic, pre-malignant lesion and the other with malignant lesion.** ***Methods:*** **The sequences of the *LMP1* genes derived from pre-malignant (NORLMP1) and malignant (NPCLMP1) tissues were compared. *NORLMP1* and *NPCLMP1* were expressed in TW01 cells and their biological properties conferred were assessed in terms of *in vitro* invasion capability, ability to resist apoptosis and regulation of E-cadherin (CDH1) expression.** ***Results:*** **NORLMP1 and NPCLMP1, possessed numerous amino acid changes with respect to the wild-type B95.8 *LMP1*. Both *LMP1* variants demonstrated distinct biological properties when expressed in TW01 cells. NORLMP1-expressing TW01 cells exhibited more aggressive phenotypes than the NPCLMP1-expressing counterpart in terms of invasive ability, resistance to stimuli-induces apoptosis and regulation of CDH1 expression.** ***Conclusion:*** **It could be inferred from the present study that *LMP1* genes derived from two patients with different stages of NPC differed in their gene sequences that manifested in distinct biological properties.**

Key Words: **Epstein—Barr virus, *LMP1* gene, nasopharyngeal carcinoma, biological properties, malignancy.**

Epstein—Barr virus (EBV), a human gammaherpesvirus is intimately associated with nasopharyngeal carcinoma (NPC), with the incidence of the virus detected in malignant tissues being close to 100% in NPC endemic areas. The viral latent protein, latent membrane protein 1 (LMP1), has all the typical characteristics of an oncogene and extensive studies have shown beyond doubt its abilities in cellular transformation giving rise to malignant phenotypes. NPC carcinogenesis, as typical as any other solid tumours, is a multi-step process. EBV infection that culminates in malignant undifferentiated NPC is known to occur early in pre-malignant or pre-invasive lesions, presumably preceded by aberrant genetic events such as loss of heterozygosity (LOH) [1]. The expression of EBV latent genes in NPC is subjected to the consequences of host immune response in which cytotoxic T-lymphocytes (CTL) plays an important role [2]. It is thus postulated that there exist dynamic viral-specific immune-responses at different tumour stages and EBV adapts accordingly to the tumour microenvironment. One of the most important EBV latent genes expressed in NPC is the viral *LMP1*. Its expression is known to occur early in the pre-invasive stage. The expression of *LMP1* in NPC has an interesting feature. It was shown by immunohistochemistry (IHC) that LMP1 was expressed in most cells during the pre-invasive stage but its expression was detected in only a fraction of cells in the malignant stage [3]. These observations invoke the question of whether the difference in the LMP1 expression pattern in the pre-invasive and malignant lesions is the result of genotypic selection imposed on LMP1 in the course of tumourigenesis. Under the influence of such host selective pressure, this present study hypothesizes that there exist a selection process in the progress from the pre-malignant to the malignant stages of NPC that culminates in the selection for the *LMP1* genotype that is expressed in the malignant stage. In other words, we hypothesized that *LMP1* genes derived from patients with different stages of NPC differ in their sequences and consequently, in their biological properties.

Received: March 15, 2007.
*Correspondence: Fax: +603-79674509
E-mail: englai.tan@gmail.com
Abbreviations used: EBV – Epstein – Barr virus; LMP1 – LMP1 gene; NPC – nasopharyngeal carcinoma.

MATERIAL AND METHODS

Cell lines. TW01 is an EBV-negative epithelial NPC cell line established from biopsy specimens of NPC patients in Taiwan, kindly provided by Prof S.W. Tsao of the Hong Kong University, China SAR [4]. TW01 was used as the host cell for the expression of different *LMP1* gene variants. As it was our interest to assess the biological properties conferred by different *LMP1* genes derived from two patients with different histological stages of NPC, it thus justified the use of an EBV-negative NPC cell line instead of a normal epithelial line. B95.8 and AG876 were cultured in RPMI-1640, while TW01, in DMEM/F-12. Both media were supplemented with 10% v/v foetal calf serum (FCS). All cells were maintained in an incubator at 37 °C, 5% CO_2 and sub-cultured in 1 : 10 dilutions in the same medium unless for specific applications.

Clinical samples. The collection of all clinical samples was subjected to prior approval by the Ethics Committee of the University of Malaya Medical Centre (UMMC) and after informed consent has been obtained from each individual. Fresh PNS biopsies were collected from the ENT Clinic, UMMC, under the supervision of Prof. U. Prasad. Two biopsy samples were collected from patients suspected of NPC — one for RNA extraction and other for histological examination. Subsequently, two biopsy tissues from two unrelated patients were used for further laboratory investigations. These consisted of one pre-malignant tissue with high-grade dysplasia and one malignant, stage II NPC tissue (UICC/AJCC classification 1997), as confirmed by a qualified pathologist. The specimens meant for RNA extraction were immediately kept in RNAlater® (Qiagen, USA) and stored in –80 °C to preserve the RNA.

LMP1 cDNA cloning. Reverse transcription (RT) followed by Polymerase Chain reaction (PCR) was performed using the Superscript™ III One-step RT-PCT System (Invitrogen, USA) following the manufacturer's recommendations. *LMP1* gene-specific primers (M-AR: GCC ACC ATG GAA CAC GAC CTT GAG AGG GGC, EBV co-ordinates: 169207–169230; and CF: TTA GTC ATA GTA GCT TAG CTG AAC TGG, EBV co-ordinates: 168163–168186) at 0.2 μM concentrations were used in a total reaction volume of 50 μL containing the following components; 0.2 mM of each dNTP, 1.2 mM $MgSO_4$, RT/Platinum Taq polymerase mixture and 1 μg of RNA. Primers M-AR and CF contained the Kozak's fragment and termination codon respectively as underlined in bold). The RT was performed at 50 °C for 30 min and terminated by incubating at 94 °C for two min. Immediately, the completed RT reaction was subjected to 40 cycles of PCR amplifications consisting of the following steps; 94 °C, 15 s; 55 °C, 30 s and 72 °C, two min. Amplified product were analysed by agarose gel electrophoresis and fragments of the correct size were cloned into the pcDNA3.1 TOPO-TA vector (Invitrogen, USA) following the supplier's protocol. As an LMP1-negative control vector, the human *β-globin* gene was amplified and ligated to the pcDNA3.1 TOPO-TA vector in order to produce a covalent-closed circular plasmid and the *β-globin* gene was subsequently deleted by *BstX*1 digestion and the empty plasmid re-ligated. This LMP1-negative control vector was used to transfect the TW01 cells to establish to the *LMP1*-negative clone.

DNA sequencing. DNA sequencing of the cloned *LMP1* cDNA in pcDNA3.1 vector was performed using a set of three primers; S1 (5'-TAA TAC GAC TCAC TAT AGG G-3'), S3 (5'-TCA CCC TCC TGC TCA TCG C-3') and S5 (5'-ACG GAC CCC CAC TCT GCT CTC AAA-3'). The S1 primer corresponded to the T7 promoter sequence located upstream of the plasmid multiple cloning site. The S3 primer corresponded to the first 19 residues of exon b of the *LMP1* mRNA (EBV coordinate: 169110–169128), while primer S5 corresponded to the internal sequence of exon c of LMP1 mRNA (EBV Coordinate: 168600–168623). Sequencing was performed by Research Biolabs Sdn. Bhd., Kuala Lumpur, at their facility using the ABI Prism 373 DNA Sequencer. The sequenced DNA results for each *LMP1* clone was aligned and compared with the B95.8 *LMP1* sequence (Gene Bank Acc. No.: V01555) using the Megalign® program of the Lasergene® v.5 suite (DNASTAR Inc., USA).

Establishment of LMP1-expressing TW01 clones. LMP1-expressing TW01 cell clones were established by transfecting the cells with pcDNA-LMP1 plasmids that carried *LMP1* genes derived from the pre-malignant (NORLMP1) and malignant (NPCLMP1) tissues as well as the wild-type gene from B95.8 cells. The fluorescence protein expression plasmid, pECFP, was used to optimize the transfection efficiencies. Transfection of the epithelial cells was performed using Lipofectamine™ 2000 (Invitrogen, USA) according to the supplier's recommendation. Transfected cells were incubated for 48 h at 37 °C with 5% CO_2 and transgene expressing cells were selected by passaging at 1 : 10 dilutions in fresh selection medium, DMEM-10 supplemented with 200 μg/mL Geneticin (G418). Stable clones were obtained by routine passaging in the selection medium for up to four weeks.

In vitro invasion assay. The invasive ability of LMP1-expressing TW01 cells was assessed using the BD Biocoat™ Tumour Invasion System (BD Biosciences, USA) following the supplier's recommendations. Sub-confluent cells were harvested by trypsinization and suspended in DMEM/F-12 at a cell density of 1.0×10^5/mL for 2 h at RT. The suspended cells, in 0.5 mL aliquots, were added to each well containing the Matrigel®-coated insert and were incubated for 48 h at 37 °C, with 5% CO_2. Non-coated membrane inserts as well as TW01 transfected with the empty vector were also seeded to serve as controls. Following incubation, the upper membrane surfaces of the inserts were gently scrubbed with a cotton swab to remove all the non-invading cells. The invading cells that had traversed across the membrane to the bottom of the well was counted using an improved Neubauer hemocytometer after staining with 1% w/v Evans blue solution.

Apoptosis assays. LMP1-transfected and empty vector-transfected TW01 cells were assessed for their resistance to apoptosis induced by staurosposine. Cells were grown to 80% confluence in a 96-well culture plate and the growth medium was replaced by DMEM/F-12, 10% v/v FCS and supplemented with either 500 nM staurosporine or 62.5 ng/mL of FasL (Sigma, USA). Two types of assays were performed to detect apoptosis in cells — cytochrome c release and caspase-3 activation. Cytochrome c release assay was used to assess the integrity of the apoptotic mechanisms in TW01 cells and was performed using the reagents supplied in the cytochrome c Release Apoptosis Assay Kit® (Oncogene™ Research Products, USA) following the supplier's recommendations. Total cellular protein contents in both the cytosolic and mitochondrial fractions were determined using the

method by Bradford and 10 μg protein was analysed in 10% SDS-PAGE. Cytochrome c was detected using the mouse monoclonal anti-cytochrome c IgG supllied by the kit. Caspase-3 activity in staurosporine-induced cells was determined using the Caspase-3 Assay Kit (Sigma, USA) following the supplier's recommendations. Briefly, staurosporine-induced cells grown in a 96-well culture plate were washed once in PBS and incubated on ice for 20 min in 25 μL lysis buffer supplied with the kit. Following incubation, 200 μL of assay buffer containing Ac-DEVD-AMC was added to each well. The plate was incubated at 37 °C to allow sufficient time for substrate digestion. The fluorescence intensity in each well resulting from the release of the AMC moiety was determined using an image analyzer with the scanning parameters set to excite at 360 nm and detect at 460 nm.

Regulation of E-cadherin (CDH1) expression. The mRNA transcript levels of CDH1 in transfected TW01 cells were quantified using reverse-transcription coupled to real-time quantitative PCR using iCycler iQ™ Real Time PCR system (Bio-Rad, USA). In developing the real-time quantitative assay, the E-cadherin gene plasmid construct was first generated by cloning the full length *E-cadherin* (Gene Bank Acc. No. AB025106) amplified from LMP1-negative TW01 cells. The 2.8 kb reverse transcribed amplimer was cloned into pcDNA-TOPO-TA vector. This CDH1 plasmid construct, pcDNA-Ecad, was used as standards in the quantitative assay. The standards consisted of serially diluted pcDNA-Ecad plasmids ranging from 1.0×10^2 to 1.0×10^7 copies in 10-fold increments. A five μL aliquot containing 250 ng of RNA extracted from cells were subjected to reverse-transcription (RT) followed by conventional amplification. The combined RT-PCR was done using the TaqMan® One-Step RT-PCR Master Mix Reagents (Applied Biosystems, USA). CDH1 mRNA was quantified using the primers, E-cadRTF (5'-CCG CGT CCT GGG CAG AGT GAA T-3') and E-cadRTR (5'- TCC CAG GCG TAG ACC AAG AAA TG-3'), and detected with the TaqMan® probe (5'- FAM-CGA TTC AAA GTG GGC ACA GAT GGT-TAMRA). GAPDH was used as an internal control to normalize the *E-cadherin* transcript levels between different samples. One-step RT-PCR was performed in a 50 μL reaction volume that contained the following components; 1.25 U MultiScribe™ reverse transcriptase (supplied with the kit), 300 nM of each primer, 25 nM of TaqMan probe, 4 mM $MgCl_2$, 200 μM of each dATP, dCTP and dGTP; 400 μM of dUTP; 0.5 U AmpErase uracil N-glycosylase (UNG) and 1.25 U AmpliTaq Gold. Amplification parameters consisted of the following step in sequential order; cDNA synthesis at 42 °C for 30 min, UNG activation at 50 °C for 2 min, initial denaturation at 95 °C for 8 min, followed by 40 cycles of 95 °C for 30 s and 56 °C for 1 min. Amplification for each sample and standard was performed in duplicates. A standard curve was only accepted if the correlation coefficient is 0.996 or higher and its slope ranged between –3.74 to –3.32, which correlates to amplification efficiency of between 85 to 100% respectively. The fluorescence detection threshold value was set at 10 × the mean standard deviation of fluorescence in all reactions.

Statistical analysis. All statistical analyses were performed using the program SPSS (Statistical Program for Social Sciences; SPSS Inc, USA). Means of paired samples were analysed using the Paired Sample *t*-test. Means of independent samples were analysed using Independent Sample *t*-test. Samples from two or more groups with a factor having two or more levels were analysed using One-way Analysis of Variance (ANOVA). The ANOVA *F* test was used to evaluate whether the group means on the dependent variable differ significantly from each other.

RESULTS

NORLMP1 and NPCLMP1 differed in their amino acid sequences. DNA sequencing of the pre-malignant tissue-derived NORLMP1 and the malignant tissue-derived NPCLMP1, revealed numerous base substitutions, in addition to deletions, along the *LMP1* gene. The corresponding amino acid sequences are presented in Fig. 1. NORLMP1 had a total of 20 mutations detected with respect to the wild-type B95.8 *LMP1*. Of these, 18 were single base substitutions with 13 of them being non-conservative and resulted in amino acid substitutions. Some of these mutations were also reported in CAOLMP1 (from China), 2117-LMP1 (from Hong Kong) and 1510-LMP1 from Taiwan [5, 6, 7]. The remaining two mutations were the 30-bp and 15-bp deletions that resulted in deletions of their corresponding 10 and 5 amino acid residues, respectively. The 30-bp deletion which resulted in the deletion of 10 amino acids (H345_G354del) was also evident in CAOLMP1, 2117-LMP1 and 1510-LMP1. By comparison, NPCLMP1 harboured a significantly higher number of mutations. A total of 45 mutations were detected in NPCLMP1 to be at variant with the B95.8 LMP1. Of these, 42 were single base substitutions with 24 of them being non-conservative. Similar to NORLMP1, the 30-bp deletion was detected in NPCLMP1 but not the 15-bp deletion. Some of the 24 non-conservative mutations were also reported in the Asian isolates as mentioned [5, 6]. The presence of the 30-bp deletion (168266_168295del) and the loss of *Xho*1 restriction site (169425C > A) in NORLMP1 and NPCLMP1 were also shown independently by PCR (results not shown). The prevalence of the 30-bp deletion in NORLMP1, NPCLMP1 and other NPC-associated LMP1 variants derived from China, Hong Kong and Taiwan underscored the intimate association of this deletion with NPC. Of note, the 15-bp deletion (168479_168493del) detected in NORLMP1 but not in NPCLMP1 was also detected in CAOLMP1 and 1510-LMP1 but not in 2117-LMP1. *In silico* analyses of sequence data using Lasergene v.5.0 (DNASTAR Inc., USA) for NORLMP1 and NPCLMP1 indicated a protein length of 371 and 382 amino acids respectively. With a few exceptions, most of the amino acid changes in both NORLMP1 and NPCLMP1 did not occur in the im-

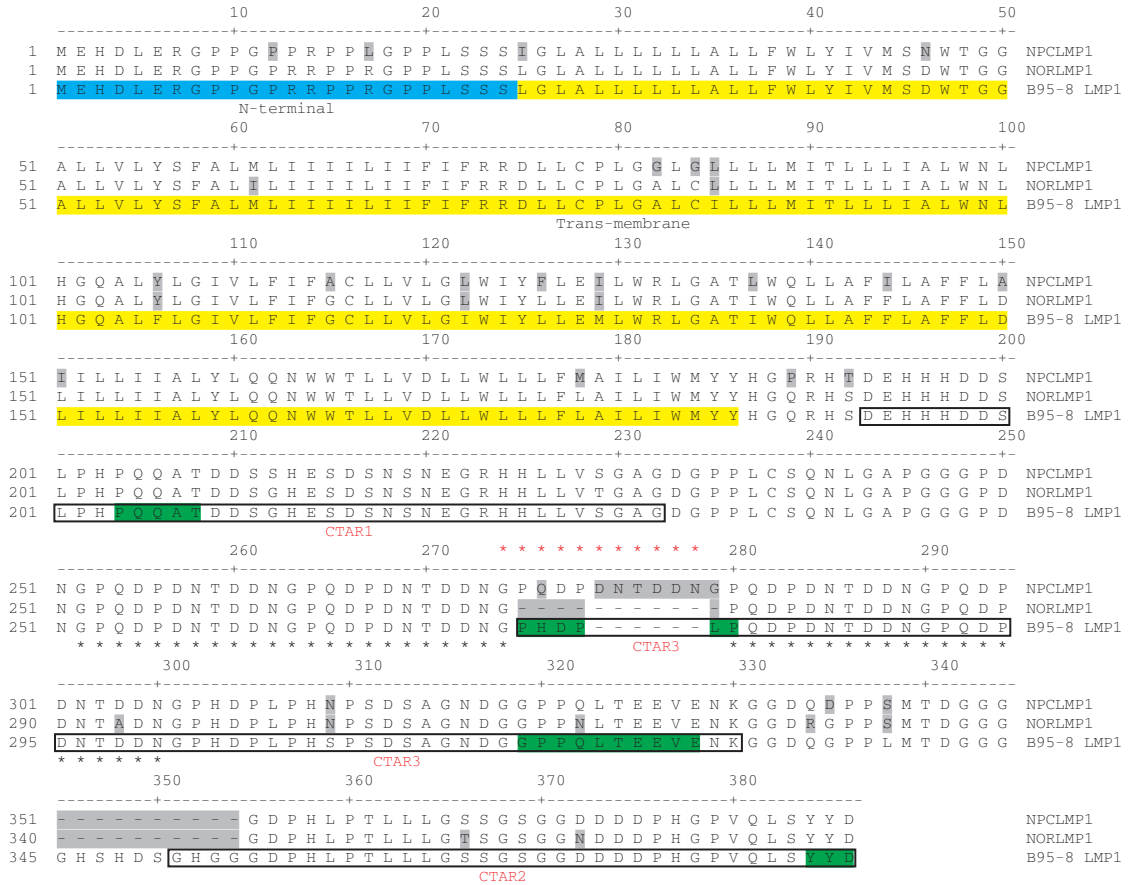

Fig. 1. Amino acid sequence of NPCLMP1 and NORLMP1. Graphical representation on the comparison of amino acid sequences of NPCLMP1, NORLMP1 and B95.8 LMP1 proteins. The B95.8 LMP1 sequence was obtained from Swiss-Prot Protein Database (Acc. No.: P03230). Amino acid residues are represented in the single letter code and numbered with reference to B95.8 LMP1. Residues in NORLMP1 and NPCLMP1 that differ from B95.8 LMP1 are highlighted in grey. The domains of LMP1 are indicated in the B95.8 sequence. N-terminal (aa 1–24) is highlighted in blue, trans-membrane (25–186) is highlighted in yellow. The C-terminal activating regions are boxed and labeled while the respective motifs are highlighted in green; CTAR1 (aa 194–232), CTAR3 (aa 275–330) and CTAR2 (351–386). Asterisks indicate the internal repeats

portant functional motifs along the different functional domains of LMP1. In NORLMP1, the only mutation that directly affected a functional motif was the deletion (P275_L279del) in one of the two CTAR3 motifs, $_{275}$PXXPXT$_{208}$. This deletion also resulted in the loss of one internal 11-bp repeat unit. On the other hand, amino acid changes were detected in the N-terminal (R13P and R17L) and trans-membrane (I173L) domains of NPCLMP1. In addition to these, an insertion of six amino acid residues (P278_L279insDNTDDN) and a single residue substitution (L279G) were detected in motif box 1 of the CTAR3 domain. The insertion of six amino acid residues added one unit of internal repeat thus giving a total of five complete 11-bp repeats in NPCLMP1 compared to the typical four repeats present in both NORLMP1 and the B95.8 LMP1. NORLMP1 and NPCLMP1 shared six non-conservative mutations; I85L, F106Y, I122L, M129I, S309N and L388S, in addition to the deletion of 10 amino acids (H345_G354del). All these mutations with the exception of S309N were also detected in CAOLMP1, 2117-LMP1 and 1510-LMP1 [5,6,7]. Although not detected in CAOLMP1, S309N has been reported in 2117-LMP1, 1510-LMP1 and LMP1 of other EBV strains such as China-1, China-2 and Mediterranean [8, 9]. Of note, four and two mutually exclusive non-conservative mutations were detected in NORLMP1 and NPCLMP1, respectively that were not present in CAOLMP1, 2117-LMP1 and 1510-LMP1. The four mutations specific to NORLMP1 were M61I, S229T, D298A and D372N. The two specific mutations detected in NPCLMP1 were G115A and H276Q. M61I, S229T and D298A were reported in AG876 EBV [10]. S229T was also detected in the LMP1 of C15 strain [11]. G115A was reported in the RV EBV stain isolated from an NPC biopsy [6].

NORLMP1 and NPCLMP1-transfected TW01 cells demonstrated different biological properties. Success of transfection and expression of LMP1 in TW01 clones was examined by RT-PCR targeting the LMP1 gene (Fig. 2) and immunofluorescence microscopy using the CS1-4 monoclonal antibody specific to LMP1 (Fig. 3). Both methods confirmed the expression of LMP1s in TW01 cells. These LMP1-expressing TW01 clones — TW01-B95LMP1, TW01-NORLMP1 and TW01-NPCLMP1; and the LMP1-negative TW01 control were used in subsequent experiments.

In vitro invasion assay. LMP1-expressing NPC cells were reported to be more progressive and showed a greater tendency of lymph node metastasis than the LMP1-negative counterpart [12]. The invasiveness of the TW01 cells that expressed NORLMP1, NPCLMP1 and B95LMP1 recombinant proteins were compared

using an *in vitro* invasion assay. Highest invasive capability was demonstrated by TW01-NORLMP1 with the highest number of invasive cells (3.0×10^4 invading cells/mL) followed by TW01-NPCLMP1 (5.0×10^3 invading cells/mL) and TW01-B95LMP1 (2,500) (Table). None of the LMP1-negative control cells were able to invade across the matrix into the lower-side of the wells. These results demonstrate that not all EBV LMP1 genes conferred the same invasive potential and TW01 that expressed the LMP1 derived from the dysplastic tissue was more invasive than its counterpart that expressed the malignant tissue derived LMP1.

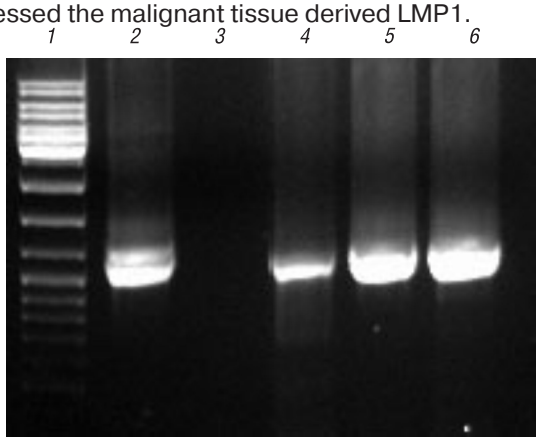

Fig. 2. Reverse-transcript PCR of LMP1. RT-PCR of the LMP1 gene derived from the newly established LMP1-expressing TW01 clones. RT-PCR was performed on RNA derived from each cell line using LMP1-specific primers — M-AR and CF. Lanes 1: Molecular Markers, 2: B95.8 control, 3: LMP1-negative TW01, 4: TW01-B95LMP1, 5; TW01-NORLMP1 and 6: TW01-NPCLMP1

Table. *In vitro* invasion assay. Each cell line was seeded in duplicates and the number of invasive cells in each well was determined after 48 h

Cell Line	Number of invasive cell / mL		
	Replicate 1	Replicate 2	Mean
TWO1	0	0	0
TWO1-B95LMP1	2500	2500	2500
TWO-NPCLMP1	5000	5000	5000
TWO1-NORLMP1	36250	23750	30000

Apoptosis assay. EBV LMP1 is known to protect B cells from apoptosis by up-regulating anti-apoptosis factors such as A20 and Bcl-2 [13, 14]. However, the knowledge of the roles of LMP1 in protecting epithelial NPC cells from apoptosis is currently incomplete. At present, one study has established the susceptibility of NPC to *Fas*L-induced apoptosis through the caspase-dependent pathway [15]. In the time-course staurosporine induction study, cytochrome c could be detected in both the mitochondrial and cytosol fractions at every time interval tested (Fig. 4). This observation indicates the preservation of an intact apoptotic pathway and sensitivity of TW01 cells to staurosporine-induced cell death.

Next, the sensitivity of the NORLMP1, NPCLMP1 and B95LMP1-expressing TW01 cells to staurosporine-induced apoptosis was investigated. Induction of apoptosis was assayed based on the activation of the protease, caspase-3. Fluorescence intensity, corresponding to the caspase activity was determined one hour after the addition of substrate and was expressed as percentage increase over the blank reactions devoid of the apoptotic stimulants ($\%\Delta_{460nm}$ Fluorescent Unit or $\%\Delta_{460nm}$ FU). Thus, higher $\%\Delta_{460nm}$ FU value, indicated

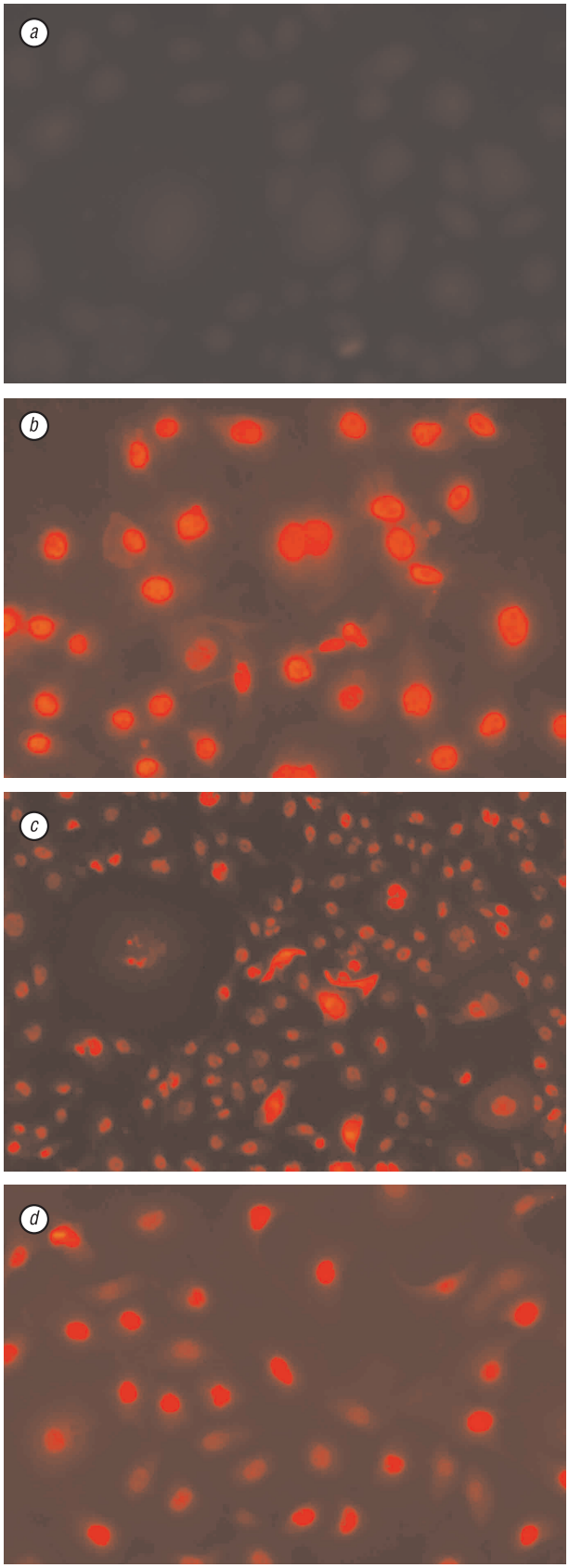

Fig. 3. Immunofluorescence staining of LMP1-expressing TW01 cells. Immunofluorescence staining of the LMP1 protein in TW01-B95LMP1, TW01-NORLMP1 and TW01-NPCLMP1. CS1-4 (Dako) at a 1 : 200 dilution was used as the primary antibody followed by Alexa-Fluor 594 conjugated-chicken anti-mouse IgG (Molecular Probes) at a 1 : 200 dilution as the secondary for fluorescence detection. *(a)* LMP1-negative TW01, *(b)* TW01-B95LMP1, *(c)* TW01-NORLMP1, *(d)* TW01-NPCLMP1. All images were taken at 100 × magnification

higher caspase activity and hence greater the extent of apoptosis and vice versa. Strong indication of cell death was observed in the LMP1-negative TW01 cells following stimulation by staurosporine (57%Δ_{460nm} FU). This was followed by TW01-NORLMP1 (21%Δ_{460nm} FU), TW01-B95LMP1 (6%Δ_{460nm} FU) and TW01-NPCLMP1 (4%Δ_{460nm} FU) (Fig. 5). These differences in mean percentage increase in fluorescence unit among the different cell lines were significant except between TW01-B95LMP1 and TW01-NPCLMP1 (One-way ANOVA, $p \geqslant 0.05$) (Fig. 5).

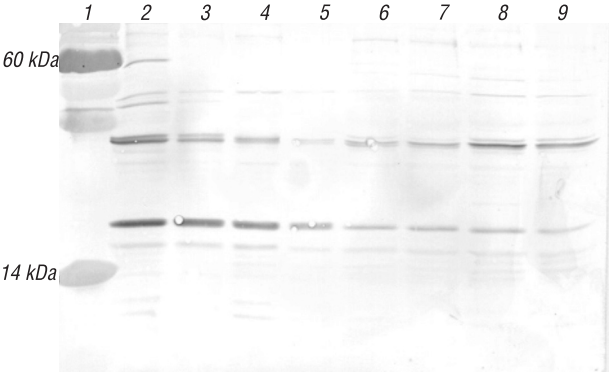

Fig. 4. Time-course induction of cytochrome c using anti-cytochrome c monoclonas release in LMP1-negative TW01 cells. Cytochrome c was detected. The cytochrome c proteins were detected as bands corresponding to circa 20 and 40 kDa. Cells were treated with DMEM/F-10 medium supplemented with 500 nM staurosporine, an apoptosis inducer. The detection of cytochrome c in the mitochondrial fractions (Lanes 2 to 5) and in its cytosolic fractions (Lanes 6 to 9) indicated the positive induction of cell death by apoptosis. Lanes 2 and 6: 3 h; Lanes 3 and 7: 6 h; Lanes 4 and 8: 9 h; Lanes 5 and 9: 18 h

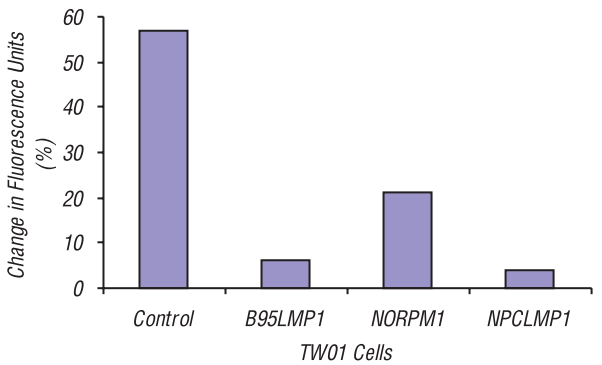

Fig. 5. Fluorogenic assay of staurosporine-induced caspase-3 activity in TW01 clones. Fluorogenic assay of staurosporine-induced caspase-3 activity in TW01 clones. Caspase activity measured as the mean percentage change in fluorescence unit (%Δ460nm FU) indicative of cells undergoing apoptosis are presented as solid bars with standard deviations. There were no differences in mean caspase activities between TW01-B95LMP1 and TW01-NPCLMP1 in response to staurosporine (One-way ANOVA, $p \geqslant 0.05$)

Regulation of E-cadherin (CDH1) expression. *LMP1* has lately been documented to down-regulate the expression of E-cadherin, the effect of which leads to enhancement in metastatic potential [16]. However, no studies have so far investigated the regulatory effect of this gene by LMP1 derived from different histological stages of NPC. The E-cadherin protein, encoded by the CDH1 gene, is a classical cadherin from the cadherin superfamily and loss of function is thought to contribute to progression in cancer by increasing proliferation, invasion, and/or metastasis [17]. The CDH1 transcript was detected in all LMP1-expressing TW01 clones as well as in the LMP1-negative control. In order to compare the levels of CDH1 in different samples, the transcript level of this gene was normalized against the level of a ubiquitous house-keeping gene, GAPDH. CDH1 was detected at 3.67×10^4 copies/μg RNA in the LMP1-negative TW01 control. However, significantly lower levels of CDH1 transcripts were observed in LMP1-expressing cells (Fig. 6). Of the TW01 clones tested, the levels of CDH1 in NORLMP1-expressing cells (956 copies/μg RNA) was ~ 2.5 times lower than in the clone expressing NPCLMP1 (2360 copies/μg RNA). Reduction in the level of CDH1 transcripts were also detected in TW01 clone expressing the wild-type B95LMP1 gene (1916 copies/μg RNA).

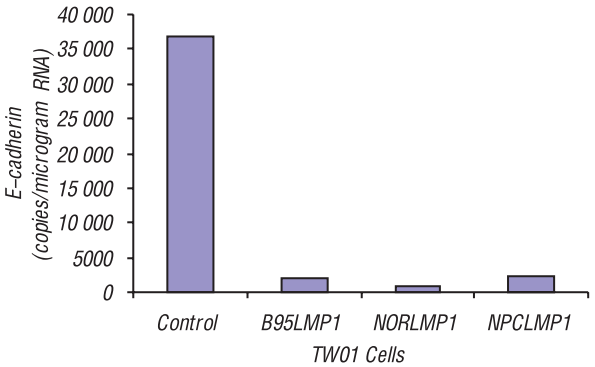

Fig. 6. *LMP1* downregulates E-cadherin (CDH1). *LMP1* downregulates E-cadherin as determined using real-time quantitative PCR. TW01 cells transfected with *NORLMP1* and *NPCLMP1* recorded a significant reduction in CDH1 expression. Each bar represents the mean value and standard errors for assays conducted in duplicates and normalized to the levels of *GAPDH* transcript

DISCUSSION

This study compares the sequence and biological properties of EBV LMP1 derived from two NPC patients — one presented with a pre-invasive lesion with high grade dysplasia and the other presented with malignant NPC stage II (UICC/AJCC classification 1997). It was hypothesized that there exist specific variants of LMP1 genes expressed at different histological stages in the progress from pre-malignant to malignant NPC. This hypothesis was based on the findings of Pathmanathan et al. [3], whereby the authors showed that EBV infection of the Fossa of Rosenmüller (FOR), the site known for its predilection to malignant NPC, was an early event with the viral gene products including EBERs and LMP1, being detected as early as in pre-invasive, dysplastic lesions. The procurement of the pre-invasive lesion from which the NORLMP1 gene was isolated was an arduous task as patients were often presented at the ENT clinic with clinical symptoms only after malignancy had developed. This problem was confounded by the fact that the progression from the pre-invasive to the malignant NPC is a rapid one. The patients from whom the NORLMP1 gene was isolated succumbed to stage II NPC within one year.

Sequence analyses made on the cDNAs derived from the pre-malignant (NORLMP1) and malignant tissue-derived LMP1 (NPCLMP1) identified DNA se-

quences that were at variant with each other. Both the 15-bp and the 30-bp deletions reported in *CAOLMP1* [5] were found in *NORLMP1*. *NPCLMP1* has only the 30-bp deletion but base insertion in the internal repeat regions of the gene resulted in an additional unit of repeat. These deletions and insertions translated into NORLMP1 and NPCLMP1 proteins that were respectively, 371 and 382 amino acid (aa) residues in length as compared to 386 aa in the wild-type. Both LMP1 variants also possessed numerous base substitutions along the gene with respect to the wild-type B95.8 LMP1 [18]. The six non-conservative point mutations shared by NORLMP1 and NPCLMP1 were also reported in NPC-derived LMP1 variants from China (China 1 and 2), including Hong Kong (2117-LMP1), and Taiwan (1510-LMP1). Of particular interest was the presence of M129I mutation in both NORLMP1 and NPCLMP1. This mutation has been reported recently to be associated with increased half-life of the LMP1 protein [19]. The sequence data (see Fig. 1) show that the NORLMP1 and NPCLMP1 proteins, resembled their counterpart derived from the nude mice passaged NPC, CAOLMP1 [5] based on the number of identical mutation, notably the 30-bp deletions and base substitutions.

The establishment of stable TW01 clones expressing NORLMP1, NPCLMP1 and the prototypical LMP1 from B95.8 EBV, B95LMP1 (as LMP1 positive control), represented the first step in which the functions of these LMP1 variants were subsequently tested and compared against the background of EBV-negative TW01 NPC cells. The use of TW01 line allowed the biological assessment of EBV LMP1 that was not confounded by the presence of other EBV genes and hence the functional implication of LMP1 in NPC could be exclusively implicated.

The NORLMP1-expressing TW01 clone was shown to confer the greatest overall invasive capability (see Table). As none of the LMP1-negative cells invaded the Matrigel®-coated membrane, it was clear that this invasive property was conferred by LMP1. This is in agreement with a report using a closely related EBV-negative NPC epithelial cell line, TW03. By infecting the otherwise EBV-negative TW03 cells through co-cultivation with EBV-positive Akata cells, Teramoto *et al*. [20] were able to demonstrate the greatly enhanced *in vitro* invasive ability of the EBV-infected cell over its parental counterpart. Their observations postulated that EBV infection restored the invasive capability of EBV-negative NPC cells and this present study indicated that this capability was specifically conferred by LMP1.

There was a correlation between invasive capability and the cellular levels of E-cadherin (CDH1) in LMP1-expressing cells. In comparison to the LMP1-negative cells, the expression of NORLMP1 and NPCLMP1 significantly suppressed the transcript level of *CDH1* by several orders of magnitude (see Fig. 6). Moreover, the extent of suppression induced by NORLMP1 was 2.5-fold greater than that induced by NPCLMP1. This observation was consistent with the sequence data of the two LMP1 variants (see Fig. 1) which show that the integrity of the CTAR2 domain motif critical for CDH1 repression through the NF-κB signaling pathway [21] was maintained and unaffected by mutations. However, the two mutations specific to NORLMP1, S366T and D372N, though they did not reside within the CTAR2 motif, might be responsible for its enhanced ability over NPCLMP1 in suppressing E-cadherin expression. The ability to down-regulate CDH1 may thus explain the invasive nature of NORLMP1 and NPCLMP1-expressing TW01 clones and showed that the suppression of CDH1 may be one of the several ways by which LMP1 induces metastasis in an otherwise non-invasive TW01 NPC cell. This confirms an earlier study which reported an enhanced invasive capability across type-1 collagen gel as a result of decreased cellular expression of CDH1 in LMP1-expressing RHEK-1 cells [22].

The apoptotic mechanism in TW01 cells was shown to be intact as they were sensitive to staurosporine-induce cytochrome c release (see Fig. 4). Cytochrome c release from the mitochondria to the cytosol is a well-known early event leading to the caspase activation cascades and ultimately apoptosis. Assay for caspase-3 indicated that NPCLMP1 conferred significantly higher anti-apoptotic potential than NORLMP1 in TW01 cells (see Fig. 5). This observation could be corroborated with clinical data whereby early but not advanced stage NPC is known to be sensitive to both radio and chemotherapies [23]. Interestingly, the 2117-LMP1 that bears amino acid sequence homology to NPCLMP1 was also shown to enhance the resistance of epithelial cells against apoptosis induced by growth factor deprivation [24].

This present study is the first in pursuing the notion that LMP1 derived from lesions of different histological stages are also at variant in their biological properties. The discovery of sequence and functional variations between the *LMP1* derived from the pre-malignant lesion (NORLMP1) and the malignant lesion (NPCLMP1) implied that there existed a dynamic selection pressure on the viral oncogene during the progress of NPC. This observation was consistent with the report on the dynamic shifts in cytotoxic T-cell epitopes that select for a particular LMP1 variant during the development of NPC and render the viral oncogene non-immunogenic [25, 26]. Both 30-bp deletion and *Xho*1 polymorphism detected in both *NORLMP1* and *NPCLMP1* indicated an early selection event for these mutations in NPC. However, the presence of these mutations alone could not account for their manifested differences in biological functions. Hence the important contributions of non-conservative point mutations that have not been fully addressed to date.

ACKNOWLEDGEMENTS

The authors are indebted to Prof. U. Prasad of the ENT Department, University of Malaya Medical Centre, for providing the fresh post-nasal space biopsies. This

work was supported by the Ministry of Science, Technology and Innovation (MOSTI) of Malaysia. Project No.: 06-02-03-0000-PR0054/05.

REFERENCES

1. **Yi HM, Li H, Peng D, Zhang HJ, Wang L, Zhao M, Yao KT, Ren CP.** Genetic and epigenetic alterations of LTF at 3p21.3 in nasopharyngeal carcinoma. Oncol Res 2006; **16**: 261–72.

2. **Rickinson AB, Moss DJ.** Human cytotoxic T lymphocyte responses to Epstein—Barr virus infection. Ann Rev Immunol 1997, **15**: 405–31.

3. **Pathmanathan R, Prasad U, Sadler R, Flynn, K, Raab-Traub N.** Clonal proliferations of cells infected with Epstein—Barr virus in preinvasive lesions related to nasopharyngeal carcinoma. New Engl J Med 1995; **333**: 693–8.

4. **Lin CT, Chan WY, Chen W, Huang HM, Wu HC, Hsu MM, Chuang SM, Wang CC.** Characterization of seven newly established nasopharyngeal carcinoma cell lines. Lab Invest 1993; **68**: 716–27.

5. **Hu LF, Zabarovsky ER, Chen F, Cao SL, Ernberg I, Klein G, Winberg G.** Isolation and sequencing of the Epstein—Barr virus BNLF-1 gene (LMP1) from a Chinese nasopharyngeal carcinoma. J Gen Virol 1991; **72**: 2399–409.

6. **Cheung ST, Leung SF, Lo KW, Chiu KW, Tam JS, Fok TF, Johnson PJ, Lee JC, Huang DP.** Specific latent membrane protein 1 gene sequences in type 1 and type 2 Epstein—Barr virus from nasopharyngeal carcinoma in Hong Kong. Int J Can 1998; **76**: 399–406.

7. **Chen ML, Tsai CN, Liang CL, Shu CH, Huang CR, Sulitzeanu D, Liu ST, Chang YS.** Cloning and characterization of the latent membrane protein (LMP) of a specific Epstein—Barr virus variant derived from the nasopharyngeal carcinoma in the Taiwanese population. Oncogene 1992, **7**: 2131–40.

8. **Sung NS, Edwards RH, Seillier-Moiseiwitsch F, Perkins AG, Zeng Y, Raab-Traub N.** Epstein—Barr virus strain variation in nasopharyngeal carcinoma from the endemic and non-endemic regions of China. Int J Cancer 1998; **76**: 207–15.

9. **Edwards RH, Seillier-Moiseiwitsch F, Raab-Traub N.** Signature amino acid changes in latent membrane protein 1 distinguish Epstein—Barr virus strains. Virology 1999; **261**: 79–95.

10. **Sample J, Kieff EF, Kieff ED.** Epstein—Barr virus types 1 and 2 have nearly identical LMP-1 transforming genes. J Gen Virol 1994; **75***:* 2741–6.

11. **Miller WE, Edwards RH, Walling DM, Raab-Traub N.** Sequence variation in the Epstein—Barr virus latent membrane protein 1. J Gen Virol 1994; **75**: 2729–40.

12. **Hu LF, Chen F, Zhen QF, Zhang YW, Luo Y, Zheng X, Winberg G, Ernberg I, Klein, G.** Differences in the growth pattern and clinical course of EBV-LMP1 expressing and non-expressing nasopharyngeal carcinomas. Eur J Cancer 1995; **31**: 658–60.

13. **Laherty CD, Hu HM, Opipari AW, Wang F, Dixit VM.** The Epstein—Barr virus LMP1 gene product induces A20 zinc finger protein expression by activating nuclear factor kappa B. J Biol Chem 1992; **267**: 24157–60.

14. **Rowe M, Peng-Pilon M, Huen DS, Hardy R, Croom-Carter D, Lundgren E, Rickinson, AB.** Upregulation of bcl-2 by the Epstein—Barr virus latent membrane protein LMP1: a B-cell-specific response that is delayed relative to NF-kappa B activation and to induction of cell surface markers. J Virol 1994; **68**: 5602–12.

15. **Li HP, Chang YS.** Epstein—Barr virus latent membrane protein 1: structure and functions. J Biomed Sci 2003; **10**: 490–504.

16. **Tsai CN, Tsai CL, Tse KP, Chang HY, Chang YS.** The Epstein—Barr virus oncogene product, latent membrane protein 1, induces the downregulation of E-cadherin gene expression via activation of DNA methyltransferases. Proc Natl Acad Sci USA 2002; **99**: 10084–9.

17. **Mansouri A, Spurr N, Goodfellow PN, Kemler R.** Characterization and chromosomal localization of the gene encoding the human cell adhesion molecule uvomorulin. Differentiation 1998; **38**: 67–71.

18. **Baer R, Bankier AT, Biggin MD, Deininger PL, Farrell PJ, Gibson TJ, Hatfull G, Hudson GS, Satchwell SC, Séguin C, Tuffnell PS, Barrell BG.** DNA sequence and expression of the B95-8 Epstein—Barr virus genome. Nature 1984, **310**: 207–11.

19. **Pandya J, Walling DM.** Epstein—Barr virus latent membrane protein 1 (LMP-1) half-life in epithelial cells is down-regulated by lytic LMP-1. J Virol 2004; **78**: 8404–10.

20. **Teramoto N, Maeda A, Kobayashi K, Hayashi K, Oka T, Takahashi K, Takada K, Klein G, Akagi T.** Epstein—Barr virus infection to Epstein—Barr virus-negative nasopharyngeal carcinoma cell line TW03 enhances its tumorigenicity. Lab Invest 2000; **80**: 303–12.

21. **Yoshizaki T, Sato H, Murono S, Pagano JS, Furukawa M.** Matrix metalloproteinase 9 is induced by the Epstein—Barr virus BZLF1 transactivator. Clin Exp Metastasis 1999; **17**: 431–6.

22. **Fahraeus R, Chen W, Trivedi P, Klein G, Obrink B.** Decreased expression of E-cadherin and increased invasive capacity in EBV-LMP-transfected human epithelial and murine adenocarcinoma cells. Int J Cancer 1992; **52**: 834–8.

23. **Prasad U.** Nasopharyngeal Carcinoma. Kuala Lumpur: University of Malaya Press, 2000.

24. **Lo AK, Huang DP, Lo KW, Chui YL, Li HM, Pang JC, Tsao SW.** Phenotypic alterations induced by the Hong Kong-prevalent Epstein—Barr virus-encoded LMP1 variant (2117-LMP1) in nasopharyngeal epithelial cells. Int J Cancer 2004; **109**: 919–25.

25. **Edwards RH, Sitki-Green D, Moore DT, Raab-Traub N.** Potential selection of LMP1 variants in nasopharyngeal carcinoma. J Virol 2004; **78**: 868–81.

26. **Jansson A, Johansson P, Li S, Rymo L.** Activity of the LMP1 gene promoter in Epstein—Barr virus-transformed cell lines is modulated by sequence variations in the promoter-proximal CRE site. J Gen Virol 2007; **88**: 1887–94.

БИОЛОГИЧЕСКИЕ СВОЙСТВА КЛЕТОК TW01, ЭКСПРЕССИРУЮЩИХ ГЕН ЛАТЕНТНОГО МЕМБРАННОГО БЕЛКА 1 ВЭБ, ВЫДЕЛЕННОГО ИЗ КЛЕТОК РАКА НОСОГЛОТКИ РАЗЛИЧНОЙ СТЕПЕНИ ЗЛОКАЧЕСТВЕННОСТИ

Обоснование и цель: **вирус Эпштейна—Барр (ВЭБ), герпесвирус человека, ассоциирован с развитием рака носоглотки (РНГ): вирус выявляют в малигнизированной ткани практически в 100% случаев. Ген, кодирующий латентный мембранный белок 1 (*LMP1*), обладает типичными характеристиками онкогена; в ряде исследований продемонстрирована его способность вызывать трансформацию клеток и развитие злокачественного фенотипа. В данной работе проведено сравнение нуклеотидной последовательности генов *LMP1*, выделенных из ткани РНГ двух больных (с дисплазией/предраком и РНГ II стадии соответственно) и биологических свойств клеток, трансфецированных этими генами. *Методы:* сравнивали нуклеотидные последовательности генов *LMP1* из образцов предраковой *(NORLMP1)* и опухолевой *(NPCLMP1)* тканей и соответственно аминокислотные последовательности кодируемых ими белков. Гены *NORLMP1* и *NPCLMP1* встраивали в клетки ВЭБ-отрицательной линии TW01 и исследовали биологические свойства трансфецированных клеток, в частности их способность к инвазивному росту *in vitro*, устойчивость к апоптозу и экспрессии Е-кадгерина (CDH1). *Результаты:* оба белка — NORLMP1 и NPCLMP1 — значительно отличались по аминокислотной последовательности от белка LMP1 дикого типа (B95,8), и клетки линии TW01, в которых они экспрессировались, различались по биологическим свойствам. Клетки TW01, экспрессирующие NORLMP1, обладали более злокачественным фенотипом, чем таковые, экспрессирующие NPCLMP1, по показателям инвазивной способности, устойчивости к апоптозу и регуляции экспрессии CDH1. *Выводы*: полученные данные свидетельствуют о том, что гены *LMP1*, выделенные из образцов биопсии двух больных с различными стадиями РНГ, отличаются по нуклеотидной последовательности, что проявляется в различиях биологических свойств клеток, трансфецированных соотвествующими генами.**

Ключевые слова: **вирус Эпштейна—Барр, ген *LMP1*, рак носоглотки, биологические свойства, злокачественность.**