[BIO14] Genotypic and functional comparisons of *Epstein-barr* virus latent membrane protein-1 from different stages of nasopharyngeal carcinoma

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Introduction

Epstein-Barr virus (EBV) 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. One of the most important EBV latent genes expressed in NPC is the viral oncogene, latent membrane protein-1 (LMP1). Its expression is known to occur early in the pre-invasive stage. (Pathmanathan *et al*., 1995). Under the influence of such host selective pressure, this present study hypothesized 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 full malignant stage but does not compromise malignant growth. In other words, the hypothesis states that a dynamic equilibrium process governing the expression of LMP1 in NPC carcinogenesis does so by constantly selecting for the LMP1 genotypes that are not in conflict with but instead facilitate the progress of tumorigenesis.

Materials and Methods

Polymerase Chain Reaction

DNA was extracted from throat wash samples collected from 120 NPC patients and 14 healthy controls. DNA template (100 ng) was amplified in a total reaction volume of 50 µL. Each reaction contained 1.25 U HotStar® Taq polymerase, 1 µM of each primer, 10 µM of each deoxynucleotide, 2 mM $MgCl_2$ and 1× PCR Reaction Buffer. The primer sequence as well as amplification conditions and expected the amplimer size for each analysis are described in Eng-Lai *et al*., (2003). For *Xho*1 polymorphism, the amplified PCR products were subjected to *Xho*1 restriction enzyme digestion.

Reverse-transcription PCR

RT was performed using the Superscript™ II One-step RT-PCT System following the manufacturer's recommendations. Gene-specific primers in 10 µM concentrations were used in a total reaction volume of 50 µL. Amplified product were analysed by agarose gel electrophoresis and fragments of the correct size were cloned into the pcDNA3.1 vector.

Cloning of cDNA

cDNA of genes of interest were synthesized, amplified and cloned into the pcDNA3.1 TOPO-TA plasmid. The plasmid was a linearized vector with single 3'-thymidine overhangs that facilitates efficient TA-cloning of PCR products which normally have one overhanging adenosine residue at the 3' ends. Due to the incorporation of a termination codon in the LMP1 cDNA, there was no fusion of the poly-histidine region at the C-terminus. Freshly amplified cDNA (1 µg) was mixed with 10 ng of pcDNA3.1 vector in a final volume of six µL reaction mix containing 0.2 M NaCl and 0.01 M $MgCl_2$. The cloning reaction was incubated at room temperature for five minutes to allow for the insertion of cDNA into the plasmid. The circularized plasmid with the cDNA insert of interest was added into a tube containing 100 µL of competent *Escherichia coli* and transformed by heat-shock treatment at 42°C for one minute. Immediately, 250 µL of SOC medium was added and the transformed *E .coli* was incubated in a shaking incubator at 37°C for one hour. The *E. coli* transformants were spread onto agar plates containing the selection medium (LB + 100 µg/mL amplicillin). Plates were incubated at 37°C for 48 hours to allow for the growth of bacterial colonies.

Real-time Quantitative PCR

The mRNA transcript levels of E-cadherin 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). The quantification 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. E-cadherin mRNA was quantified using the primers, E-cadRTF and E-cadRTR, and detected with the TaqMan® probe. GAPDH was used as an internal control to normalize the E-cadherin transcript levels between different samples using primer and JOE-labeled probe supplied in TaqMan® GAPDH Control Reagents Kit. One-step RT-PCR was performed in a 50 µL reaction volume that contained the following components; 1.25 U MultiScribe™ reverse transcriptase, 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 minutes, UNG activation at 50°C for 2 minutes, initial denaturation at 95°C for 8 minutes, followed by 40 cycles of 95°C for 30 seconds and 56°C for 1 minutes. 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 correlate 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.

ELISA

Purified and concentrated LMP1 protein was diluted to 2µg/mL in PBS. Each well of a microtitre plate was coated with 50µL of the diluted antigen solution. The coated plate was incubated for two hours at room temperature (RT). The coating solution was removed and the plate was washed twice, each time with 300 µL PBS per well. Each well of the antigen-coated plate was incubated overnight in 300 µL blocking buffer at 4ºC. The following day, the plate was washed twice with PBS and challenged with 50 µL serum samples that had been diluted accordingly in blocking buffer (200× dilutions for IgG; 100× dilution for IgA), and incubated at RT for two hours. Negative control wells were incubated with 50 µL blocking buffer only. The plate was washed four times with PBS. The bound human antibody was detected by the addition of either 50 µL AP-conjugated goat anti-human IgG or IgA (both diluted 2000× in blocking buffer) according to the manufacturer's recommendations. The plate was incubated at RT for two hours and stringently washed four times as described. Bound phosphatase conjugate was detected using Bluephos® Microwell Phosphatase Substrate Reagent (KPL Inc, USA: Product No.: 50-88-02) and the chromogen was allowed to develop for 30 minutes. Colour development was stopped by adding 100 µL stop solution (2.5% EDTA). Absorbance at 630 nm was measured using an ELISA plate reader.

Tumorigenicity in Nude Mice

Six to eight-week-old male Balb/c nu/nu mice maintained in pathogen-limited conditions at Institute of Medical Research, Kuala Lumpur, Malaysia, were used throughout the studies. Exponentially growing TW01 clones with viability greater than 90% were used for injection. A total of 5.0×10^6 cells in 200 µL RPMI-1640 medium were subcutaneously injected into the right flank a mouse using a 25-gauge needle attached to a 1000 µL plastic syringe. Three mice were injected with each of the three LMP1-expressing TW01 clones (TW01-NORLMP1, TW01NPCLMP1 and TW01-B95LMP1) and the empty vector-transfected control. All mice were observed weekly and tumour size was estimated as the product of three dimensions (width × length × height) and expressed as mm^3. Final observation was done on the seventh week and mice that had developed tumours were sacrificed to have the growth removed. Excised tumours were immediately kept in RNAlater®, and stored at -80°C until use.

Differential Gene Expression Assays

The cancer related genes that were differentially-expressed in LMP1-expressing TW01 clones (*in vitro* study) and in and their resulting nude mice tumours (*in vivo* study) were investigated using the Panorama™ Human Cancer Oligoarray™. Each array comprised of two fields where each gene was represented by duplicate of spots. Each spot consisted of three 70-bp oligonucleotides for a particular gene. Also included on the nylon array were spots representing a number of 'housekeeping' genes as positive controls and for signal normalization and non-human genes that served as negative controls. The signal to noise ratio in this array experiment was significantly improved by the use of Human Cancer cDNA Labeling primers. The labeling primers contained all the necessary primers for amplifying cDNA species that have their complimentary pair on the membrane.

Results

LMP1 30-bp Deletion and Xho1 Polymorphism

Throat washes from 120 NPC patients and 14 healthy subjects were genotyped for the *Xho*1 polymorphism and 30-bp deletion. Of the 49 amplifiable NPC samples, 10 (20%) possessed the deletion at exon C of LMP1 (156-bp product) whereas 38 (78%) showed the 30-bp retention variant (186-bp product) (Figure 1). One NPC sample displayed a deletion larger than 30-bp. All amplimers from the seven throat washes of healthy subjects showed the retention variant. The primers, LMP1 and LMP-Pro2, amplified the *Xho*1 polymorphic region in exon A of LMP1 in 21 (18%) of the 120 NPC samples. Following restriction digest, the 113-bp undigested fragments corresponding to the loss of *Xho*1 restriction site in amplimer were detected in 17 (81%) of the 21 samples (Figure 2). All five of the throat washes amplifiable from the 14 healthy subjects retained the *Xho*1 restriction site and displayed the 67 and 46-bp digested products following *Xho*1 digestion.

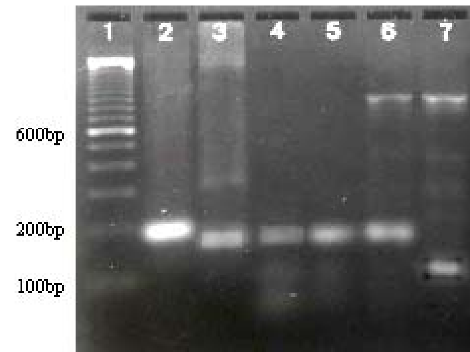

FIGURE 1 EBV LMP1 30-bp deletion analysis of throat wash samples. Lane 1: 100-bp molecular ladder. Lane 2: B95.8 control without the deletion (186-bp). Lane 3: AG876 control that possessed the 30-bp deletions (156-bp). Lanes 4 – 6: Samples from NPC patients with the 30-bp deletion. Lane 7: One NPC sample possessing a deleted fragment larger than 30-bp.

FIGURE 2 *Xho*1 restriction digest analysis of EBV LMP1 from throat wash samples. Lane 1: 100-bp molecular ladder. Lane 2: B95.8 control that retained the restriction site (two bands of 67 and 46-bp after *Xho1* digestion). Lane 3 -5: NPC samples that displayed loss of *Xho*1 restriction site yielded the undigested 113-bp product.

Antigenicity of Recombinant LMP1 Protein

Serum samples from three study groups comprising of; 1) 20 untreated NPC patients, 2) 20 healthy controls and 3) 20 NPC patients who had been treated by radiotherapy were assayed against a panel of four recombinant LMP1s – AGLMP1, B95LMP1, NPCLMP1 and NORLMP1. All study groups had significantly higher mean IgG levels against NORLMP1 as compared to NPCLMP1. The difference in mean IgG level against these two LMP1 proteins was especially pronounced in untreated NPC patients (Figure 3). Differences in IgG levels between B95LMP1 and AGLMP1 were not significant in all three

study groups at the p = 0.05 level (One-way ANOVA).

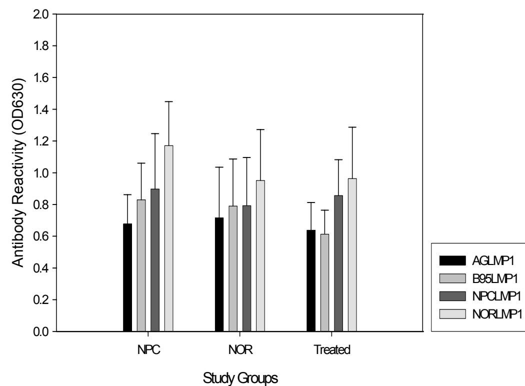

FIGURE 3 Bar charts with standard deviations representing mean IgG levels in three study groups comprising of untreated NPC patients (NPC), healthy controls (NOR) and treated NPC patients (Treated) against a panel of recombinant LMP1 derived from type 2 EBV (AGLMP1), type 1 EBV (B95LMP1), malignant tissue (NPCLMP1) and dysplastic tissue (NORLMP1). Differences in mean IgA levels against the panel of four different recombinant LMP1 proteins were not significant in all three study groups. Differences in mean IgG levels between NORLMP1 and NPCLMP1 were significant and consistent in all three study groups (One-way ANOVA, $p \leq 0.05$).

Down-regulation of E-cadherin Expression

E-cadherin was detected at 3.67×10^4 copies/µg RNA in the LMP1-negative TW01 control. However, significantly lower levels of E-cadherin transcripts were observed in LMP1-transfected cells. Of the two variants of LMP1 tested, the levels of E-cadherin in NORLMP1-transfected 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 E-cadherin transcripts were also detected in TW01 clone expressing the wild-type B95LMP1 (1916 copies/µg RNA).

Tumorigenicity in Athymic Nude Mice

Tumour volume was determined at the end of the seventh week. Sizable tumours with irregular shapes began to appear five weeks after inoculation in all mice inoculated with both the LMP1-expressing cells and the LMP1-negative TW01 clone. Sizes of tumour arising from different inoculums at the seventh week were at variance and on average, largest mean tumour volumes (132 mm^3) were observed in mice inoculated with the dysplastic tissue-derived LMP1 (NORLMP1). It could be inferred that the contribution of LMP1 to tumorigenesis was negligible as the LMP1-negative control developed tumours in the absence of LMP1 expression. Thus, the TW01 line was proven to be tumorigenic in its own right and the direct tumourigenic contribution of the different LMP1 variants tested could not be concluded from the observations made here. However, as mentioned, the variations in mean tumour volumes between mice injected with different LMP1-expressing clones were noteworthy.

In vitro and In vivo Gene Expression Studies

A total of 22 genes were down-regulated in LMP1-expressing TW01 clones irrespective of the LMP1 variants. Some of the known functions of these genes include control of cell proliferation (FES, TGFB1, LOC55908, FXYD3, BCAR1, FGFR4 and JUND), angiogenesis (BAI2 and ECGF1), and cellular differentiation (MYOG and GBX2). Some were structural proteins (ANK1 and ITGB4) and some were involved in protein transport and signal transduction (GNAS1, TRA1 and LGALS3BP). The TW01 cells expressing NORLMP1 had 17 genes that were up-regulated with respect to LMP1-negative control and these were also specific genes in that they were not detected in other clones expressing other variants of LMP1. Nine of these 17 genes were also detected in the corresponding nude mouse tumour that formed after the injection of this cell clone. By contrast, only the 10 house-keeping genes were detected in TW01 clone expressing NPCLMP1 while all other genes detected in the LMP1-negative control cells were down-regulated. TW01 clone expressing the wild-type LMP1, B95LMP1, had 11 genes detected on the array, out of which, only one was up-regulated with respect to the control. In this *in vitro* gene expression study, the TW01-NPCLMP1 and TW01-B95LMP1 clones were very similar in their expression profiles based on the number of identical genes that were down-regulated with respect to the control. There were five genes that were down-regulated in these two clones. Moreover, most of the genes up-regulated in the TW01-NORLMP1 clone were not detected in these

two clones. Five genes were up-regulated in all tumours arising from LMP1-expressing clones irrespective of variants.

Discussion

The identification of EBV infection in the pre-invasive or pre-malignant lesion underscored the important role of the virus in early tumorigenesis and the expression of the viral oncogene, LMP1, implicated its functional significance in early transformation events leading to malignancy. The discovery of sequence, anitigenic and functional variations in 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. Of note was the differential antigenicity manifested by these two LMP1 variants in untreated NPC patients. 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. These epitope shifts were known to render the LMP1 non-immunogenic (Edwards *et al*., 2004). The presence of both 30-bp deletion and *Xho*1 polymorphism in both NORLMP1 and NPCLMP1 suggested an early selection event for these mutations and their specificity to NPC as has been demonstrated in the laser-microdissected NPC cells. As pointed out in this present study, the presence of these mutations alone could not account for their manifested differences in biological functions important for tumorigenesis, i.e. oncogenicity, gene regulations and resistance to apoptosis. Hence the important contributions of non-conservative point mutations that have not been fully addressed to date. 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 consequential effect of NORLMP1 being more antigenic and thus theoretically more likely to be recognized by the host immune system, was countered by its more aggressive nature of being better enhanced for growth in soft agar, conferred higher resistance to stimuli-induced apoptosis and more tumorigenic as it gave rise to relatively larger tumours in nude mice. Moreover, in comparison to NPCLMP1, NORLMP1-expressing TW01 cells regulated a larger number of genes *in vitro* and *in vivo*, again suggesting their differences in the intricacies of molecular interactions. The differential gene regulation profiles induced by NORLMP1 and NPCLMP1 in EBV-negative TW01 cells were not only an indication of functional dissimilarities but also represented a molecular 'snap-shot' on the complex molecular interactions at work in the progress of NPC that were specifically regulated by different LMP1 variants. It could be inferred from the present study that EBV has culminated a successful strategy that enabled it to adapt to changing histological conditions by specifically allowing its oncoprotein, LMP1, to be a subject of selection process imposed by the tumour microenvironment while at the same time, LMP1 contributes to the success of tumorigenesis in ways that are most appropriate in a given histological stage. The persistence of the *Xho*1 polymorphism in the N-terminus and the C-terminal 10 aa deletion and their manifestation in early NPC suggested their roles in the early tumorigenic events while the point mutations were more likely to be responsible for determining the biological characteristics manifested in the NPC epithelial cells, TW01. The striking resemblance in sequence homology of NPCLMP1 to other reported NPC-derived LMP1 variants suggested the existence of universally conserved mutations befitting malignant NPC while the NORLMP1, expressed in the pre-malignant stage, may represents an intermediate variant that was of temporal benefit in the course to malignancy.

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