BIOMEDICINE

Association of Epstein-Barr Virus Latent Membrane Protein 1 (LMP1) Gene Expression and Caspase Activity in Normal Nasopharyngeal Cell

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ABSTRACT

Objective: **To assess *LMP1* gene expression-induced apoptosis in normal nasopharyngeal cells by measuring caspase activity.**

Material and Methods: ***LMP1*** **gene was subcloned into pTracer and pcDNA vector, producing pTracer-*LMP1* and pcDNA-*LMP1* expression plasmids. The plasmids were then transfected into normal nasopharyngeal cells. *LMP1* gene expression-induced apoptosis was accessed by measuring Caspase activities using Caspase-Glo ® 3/7 Assay kit following the manufacturer's protocol. The luminescence intensity was measured by microplate reader. Association of *LMP1* gene expression with caspase activation was analysed by independent Sample T test.**

Results: **LMP1 gene expression in normal nasopharyngeal cells is significantly associated with higher caspase activity of apoptosis compare to the vector control with t(-2.142), p value of 0.03.**

Conclusion: **Our results show that, there is association of *LMP1* gene expression and caspase activity level in normal nasopharyngeal cell thus support that *LMP1* gene expression is involved in apoptosis induction. This in turn suggests that the apoptosis process is potentially involved in the carcinogenesis of nasopharyngeal carcinoma.**

KEY WORDS

latent membrane protein 1, Epstein-Barr virus, caspase activity, apoptosis, nasopharyngeal cell

INTRODUCTION

Latent membrane protein 1 (LMP1), which is encoded by EBV, is the most important oncoprotein in EBV-related malignancies. Constitutive expression of *LMP1* contributes to the initiation and progression of NPC. *LMP1* is one of the seven informative genes that can accurately predict the survival of NPC patients (Wang *et al*., 2011). However, the exact mechanism of how *LMP1* contributes to carcinogenesis is largely unclear. The oncogenic potential of *LMP1*, which results in B cell transformation, is suggested by its high functional similarity to the tumor necrosis factor receptor (TNFR) family members, CD40 and TNFR1 (Kulwichit *et al*., 1998).

EBV infection occurs mostly during childhood and remains asymptomatic due to immune-related activities. Nearly 90% of the adult population worldwide is currently EBV infected, and the most common route of infection is through intimate contact with saliva from an EBV-infected person (Kgatle, Spearman, Kalla, & Hairwadzi, 2017). In NPC, EBV is harboured in a latent stage with restricted viral gene expression of *LMP1/2*, *EBNA1*, *EBER1/2*, and *BARTs*. *LMP1* can activate cellular DNA methyltransferase via c-Jun NH2-terminal kinase signaling (Tsai *et al*., 2006) and upregulate BMI1 expression (Dutton *et al*., 2007), which is associated with epigenetic changes in NPC.

Although chromosomal abnormalities are commonly found in NPC, the detail molecular mechanisms leading to these abnormalities remain elusive. However, there are increasing evidences that the apoptotic nuclease is responsible for the initial event of chromosome translocation, that is the breakage of the chromosome (Betti, Villalobos, Diaz, & Vaughan, 2003; Sim & Liu, 2001). Although apoptosis is a cell death process, it has also been implicated in chromosome rearrangement. Chemotherapeutic drug-induced apoptosis has been implicated in the introduction of chromosome break within the Mixed Lineage Leukaemia (MLL) gene, a gene frequently involved in chromosome translocations (Sim & Liu, 2001). More recently, the apoptotic nuclease was suggested to be involved in oxidative stress-induced and bile acid-induced chromosome breaks in normal nasopharyngeal epithelial cells (Tan, Sim, & Khoo, 2016). Apoptosis is a naturally occurring programmed cell death process that is characterised by a series of distinct morphological changes which result from the activity of caspases, a class of cycteine protease (Hengartner, 2000). The current study was designed to assess *LMP1* gene expression-induced apoptosis by measuring caspase activity in nasopharyngeal cells expressing *LMP1* and com-

Table 1: Mean and Standard Deviation of Caspace Activity in transfected cell line

Plasmid Transfection in Cell Line	N	Mean	Std. Deviation	Std. Error Mean
pTracer-LMP1	100	513.18	413.327	41.333
pTracer	100	529.99	322.257	32.226
pcDNA-LMP1	100	304.30	119.322	11.932
pcDNA	100	271.52	95.827	9.583

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Table 2: Association of caspase activity with LMP1 expression

Plasmid Transfection in Cell Line		t value	df	Sig. (2-tailed)	95% Confidence Interval of the Difference	
					Lower	Upper
pTracer and pTracer-LMP1	Equal variances assumed	.321	198	.749	-86.545	120.165
	Equal variances not assumed	.321	186.885	.749	-86.583	120.203
pcDNA and pcDNA-LMP1	Equal variances assumed	-2.142	198	.033	-62.959	-2.601
	Equal variances not assumed	-2.142	189.187	.033	-62.968	-2.592

pare with that without *LMP1* gene expression.

MATERIALS AND METHODS

Cell Line

For cell line use in current study, normal nasopharyngeal epithelial cell line NP69 obtained from Professor Sam Choon Kook from Universiti Malaya, Malaysia. For the plasmid pTracer *LMP1* cell line, *LMP1* gene (Strain B95) is sub-cloning into pTracer™-EF/V5-His B (pTracer). Recombination of pTracer *LMP1* then transformed by One Shot ® Electrocomp™ *E.coli* by electroporation. For control vector, pTracer was prepared without *LMP1*. For pcDNA *LMP1* plasmid, pcDNA3.1/V5-His-TOPO-B95 was obtained from Professor Sam Choon Kook from Universiti Malaya.

Cell Culture

Cell line NP69 was used in the current study. It is an immortalised nasopharyngeal epithelial cell line. The cell line was provided by Prof. Tsao Sai Wah and Prof. Lo Kwok Wai from University of Hong Kong and The Chinese University of Hong Kong. This cell line was established by transfection with SV40 large T oncogene (Tsao *et al*., 2002). This cell line retains its normal nasopharyngeal epithelial cells and is non-tumourigenic. The cell line was cultured in Keratinocyte-SFM medium supplemented with recombinant Epidermal Growth Factor (rEGF) (4-5 ng/ml), Bovine Pituitary Extract (BPE) (40-50 µg/mL), 2% (v/v) heat-inactivated fetal bovine serum and 1% penicillin-streptomycin (GIBCO, Invitrogen, USA). Cell were incubated in a humidified 5% CO_2 incubator at 37°C.

LMP1 Transfection

In this study, transfection was performed using Lipofectamine ® 3000 Reagent following the manufacturer's protocol with modification. NP69 cells were transfected with pTracer-LMP1, pTracer, pcDNA-LMP1 and pcDNA. After third times passage, 5 x 10^2 cells of NP69 cell was added into 100 µl seeding medium and incubated at 37°C for overnight. Then, seeding medium was replaced with 100 µl transfection/plain medium. Added 0.2 µg plasmid DNA into 10 µl Opti-MEM I Reduced Serum Media, 0.2 µl p3000 reagent and 0.3 µl 3000 reagent in 0.2 ml centrifuge tube. Then the mixture was incubated at room temperature for 10 minutes. After incubation, the mixture was added into 96 well plate that already contains NP69 cell with transfection medium and incubated for another 4 hours. After 4 hours, transfection medium was replaced with 100 µl culture medium. The transfection was completed after 48 hours.

Measurement of Caspases Activity in LMP1 Transfection

NP69 cells of 70% confluency in a T-25 flask were with Trypsinized with TrypLE express, incubated for 4 minutes and add with 4 ml of culture medium. Transfection of LMP1 into NP69 was carried out for 48 hours in 100 µl growth medium per well in a white-walled 96-well plate with clear bottom (Corning). Caspase activity was measured by using Caspase-Glo ® 3/7 Assay kit according to manufacturer's manual. Caspase-Glo ® 3/7 Buffer and the lyophilized Caspase-Glo ® 3/7 substrate were equilibrated to room temperature prior to use. Caspase-Glo ® 3/7 Buffer was transferred into an amber bottle containing the Caspase-Glo ® 3/7 substrate. Solution was mixed by inverting the bottle until dissolved to form Caspase-Glo ® 3/7 reagent. Seeding medium from the plate and cells were washed with 1x PBS buffer (room temperature) once. A mixture of 100 µl of 1x PBS buffer and 100 µl of Caspase-Glo ® 3/7 reagent was added to the cells in the plate. The mixture was incubated at room temperature for 30 minutes. The plate was shake at amplitude of 1 mm (~174 rpm) for 30 seconds before and after incubation. The luminescence was measured by microplate reader.

Statistical Analysis

Association of caspase activity with LMP1 expression were derived using Statistical Package for Social Sciences (SPSS) version 23 by performing Independent Sample T test.

RESULTS

Hundred replications of cell transfected with the vectors pTracer, pcDNA and *LMP1* expression plasmids pTracer-*LMP1* and pcDNA-*LMP1* used to measure the caspase activity. Table 1 shows the mean and standard deviation of caspase activity in normal nasopharyngeal cells transfected with the vectors pTracer, pcDNA and *LMP1* expression plasmids pTracer-*LMP1* and pcDNA-*LMP1*. Mean of 513.18 and standard deviation of 413.327 is observed in pTracer-*LMP1* transfected cells. For pTracer vector transfected control cells which without *LMP1*, mean observed was 529.99 and standard deviation of 322.257. On the other hand, the mean for pcDNA-*LMP1* transfected normal nasopharyngeal cell observed is 304.30 and 119.322 for standard deviation. Mean of 271.52 and 95.827 for standard deviation was observed in pcDNA transfected cells without *LMP1*.

Table 2 shows the caspases activity association between pTracer-*LMP1* and pTracer, pcDNA-*LMP1* and pcDNA transfection. Association analysis of the caspase activity of pTracer-*LMP1* transfection and pTracer transfection was performed. The caspases activity was found not statistically associated with value of t(0.321), p value of 0.749. However, in pcDNA-*LMP1* and pcDNA transfection, the caspase activity association with *LMP1* expression was found to be statistically significant, with value of t(-2.142), p value of 0.033.

DISCUSSION

The role of LMP1 in the pathogenesis of NPC remains speculative. There is considerable variation in the reported expression of LMP1 in NPC biopsies and a consensus that approximately 20%—40% of tumours express LMP1 at the protein level (Tao, Young, Woodman, & Murray, 2006). In stably EBV-infected cells, various latent gene proteins, such as LMP1 and LMP2A, will drive rapid clonal expansion and transformation. Discoveries in LMP1 gene variability and LMP1 functions could be critical for the definition of EBV responsibility for carcinogenesis mechanism. Expression of these EBV latent proteins will constitutively activate multiple signaling pathways, enhance genetic instability, induce epigenetic changes, modulate microenvironment and erase host immune response during early stage of cancer development (Lo, Chung, & To, 2012).

Caspase-activated DNAase (CAD) seems to be playing multiple roles. On one hand it is the apoptotic nuclease, one the other hand, it was also found to play a role in chromosome rearrangement commonly found in leukaemia (Nicholas & Sim, 2012). In addition, CAD was also shown to promote cell differentiation by inducing DNA strand breaks (Larsen *et al*., 2010). CAD is one of the enzymes involved in apoptosis and play an important role in chromosome rearrangement mostly found in leukaemia (Sim & Liu, 2001) as well as in nasopharyngeal carcinoma

(Boon & Sim, 2015; Tan *et al*., 2016). The finding also suggested that, CAD may play an important role in chromosomal cleavages mediated by oxidative stress-induced apoptosis as well as bile-acid induced apoptosis (Tan & Sim, 2018; Tan, Sim, & Khoo, 2018).

In the current study, we assess the induction of apoptosis by measuring caspase activity in normal nasopharyngeal cells expressing LMP1 and compare with nasopharyngeal cell without *LMP1* gene expression. Our results showed the association value is statistically associated with value of t(-2.142), p value of 0.033 when comparing the mean of caspases activity in normal nasopharyngeal cell transfected with pcDNA-LMP1 and pcDNA without LMP1. Similarly, other study showed that EBV-encoded latent membrane protein 1 (LMP1) to be associated with tumour relapse and poor prognosis of nasopharyngeal carcinoma (NPC) (Yang *et al*., 2014). LMP1 is intermittently expressed, and this usually correlates with induction of uncontrolled cell growth and proliferation while suppressing programmed cell death (Gastaldello, Chen, Callegari, & Masucci, 2013). The apoptotic nuclease, caspase activated DNase (CAD) was suggested to play a direct role in mediating chromosome translocation in leukaemia (Hars, Lyu, Lin, & Liu, 2006; Sim & Liu, 2001).

Although chromosome rearrangements are commonly found in nasoparyngeal carcinoma (NPC), the mechanism leading to these rearrangements is yet unclear. Data showed that expression of Epstein-Bar virus (EBV) latent membrane protein 1 (LMP1) induced cleavage of the mixed lineage leukemia (MLL) gene at 11q23, which was significantly reduced by caspase inhibitor (Yee & Sim, 2010). Since 11q23 is a commonly deleted site in NPC, thus it was suggested that virus-induced CAD activation could be involved in the initiation of chromosome rearrangement in NPC.

Association study is the most applicable tool to access the gene susceptibility to complex diseases that involve high interaction between genetic and environmental factors. Many complex diseases have a variety of genetic variants that affect the disease risk even though with minimal effect. It also shows that, LMP1 gene expression in normal nasopharyngeal cell contributes to caspase activation leading to apoptosis. Although apoptosis is a cell death process (Kerr, Wyllie, & Currie, 1972), upcoming literature had also supported the role of apoptosis in leukaemia (Sim & Liu, 2001) as well as in NPC (Tan & Sim, 2018; Tan *et al*., 2016; Tan *et al*., 2018).

CONCLUSION

Our results show that, there is an association of LMP1 gene expression in normal nasopharyngeal cell and caspase activity of apoptosis. This support that LMP1 gene expression is involved in apoptosis activation that may mediate pathways in the process of nasopharyngeal carcinoma pathogenesis.

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