GENE EXPRESSION

No Association of Latent Membrane Protein 1 (LMP1) Gene Expression of Epstein-Barr Virus and Oxidative Stress Production in Normal Nasopharyngeal Cell

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ABSTRACT

Objective: **To evaluate the effect of *LMP1* gene expression on oxidative stress generation in normal nasopharyngeal cell.**

Material and Methods: **LMP1 gene were established in pTracer and pcDNA form. The cell then transfection into normal nasopharyngeal cell line. Level of reactive oxygen species (ROS) will be assessed by measuring the fluorescence generated by the chemical 2',7'-Dichlorohydrofluorescein diacetate (DCFH-DA) for pcDNA and cell rox for pTracer. Association of LMP1 gene expression and oxidative stress level were calculate by comparing mean of pTracer and pcDNA with LMP1 gene and without LMP1 gene by use independent Sample T test.**

Results: **There were no association between LMP1 gene expression in both pTracer LMP1 and pcDNA LMP1 compare to vector control cells in normal nasopharyngeal cell.**

Conclusion: **Our results show that, there are no association of LMP1 gene expression and oxidative stress production in normal nasopharyngeal cell thus support that LMP1 gene expression not involve in production of oxidative stress that mediated pathways in the process of nasopharyngeal carcinoma.**

KEY WORDS

latent membrane protein 1, Epstein-Barr virus, oxidative stress, nasopharyngeal cell

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is an epithelial malignancy with highly variable incidence rates around the world. About 84,400 incident cases of NPC and 51,600 deaths occurred in 2008 with the highest incidence in South-Eastern Asia, relative to the Americas, Europe, Africa, and Central and Eastern Asia (Baer *et al*., 1984). NPC is a unique malignancy that arises from the epithelium of the nasopharynx and has a restricted prevalence in certain regions of the world. The remarkable geographical variations in NPC prevalence are the result of the complex development of this carcinoma (Chang & Adami, 2006). Lo et al. 2012 proposed that the loss of chromosomes 3p and 9p regions is an early event for the transformation of the normal nasopharyngeal epithelium (K.-W. Lo, Chung, & To, 2012).

Epstein-Barr Virus has long been known to be associated with NPC (Niedobitek, 2000), however, its direct role in the pathogenesis of NPC is still under investigation. More recently, it has been shown that recurrent chemical reactivation of EBV promotes genome instability and thus enhances tumour progression of NPC cells (Fang *et al*., 2009). In addition, a N-nitroso compound, N-methyl-N'nitro-N-nitrosoguanidine (MNNG) was found to cooperate with 12-O-tetradecanoylphorbol-1,3-acetate (TPA)/sodium butyrate to enhance EBV reactivation (Huang *et al*., 2010). Interestingly the same treatment increased the levels of reactive oxygen species (ROS), and enhanced genome instability in NPC cells. Furthermore, expression of the Epstein-Barr virus latency gene, the latent membrane protein (LMP1) gene also promotes genome instability, detected as nonclonal chromosomal aberrations in Burkitt's lymphoma cell line (Gruhne, Sompallae, & Masucci, 2009).

Expression of these EBV latent proteins will constitutively activate multiple signalling pathways, enhance genetic instability, induce epigenetic changes, modulate microenvironment and erase host immune response during early stage of cancer development (K.-W. Lo *et al*., 2012). Other clonal genetic and epigenetic changes will be accumulated under the continual selection process. LMP1 and LMP2A expression are repressed by various mechanisms such as EBV miRNAs, DNA methylation, and transcription factors to avoid its cytotoxic effect and host immune response in most of the primary tumours (Takacs *et al*., 2010). Their oncogenic function will be maintained by various permanent genetic alterations developed during progression.

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 one study, all 6 early, preinvasive NPC (NPC in situ) lesions analysed expressed the LMP1 protein, arguing for a crticial role of LMP1 in the early pathogenesis of NPC (Pathmanathan, Prasad, Sadler, Flynn, & Raab-Traub, 1995). LMP1 expression was shown to inhibit the AMPK/LKB1 signaling pathways to promote cellular growth and survival (A. K. Lo, Lo, Ko, Young, & Dawson, 2013). Current study design to evaluate the effect of LMP1 gene expression on oxidative stress generation in normal nasopharyngeal cell.

MATERIALS AND METHODS

Cell Line

For cell line use in current study, pTracer LMP1, pTracer, pcDNA LMP1 and pcDNA. LMP1 gene (Strain B95-8) is an EBV genome-posi-

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Table 1: Mean and Standard Deviation of Oxidative Stress in all pTracer and pcDNA cell line

Cell Line	N	Mean	Std. Deviation	Std. Error Mean
pTracer LMP1	100	17981.12	11996.745	1199.674
pTracer	100	18043.47	11527.655	1152.765
pcDNA LMP1	100	49426.75	7489.795	748.979
pcDNA	100	49168.78	9034.687	903.469

tive marmoset cell line that obtained from Professor Sam Choon Kook from Universiti Malaya, Malaysia. For the 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 cell line, pcDNA3.1/V5-His-TOPO-B95 was obtained from Professor Sam Choon Kook from Universiti Malaya.

Cell Culture

Cell line NP69 is an immortalized nasopharyngeal epithelial cell line was use in current study. 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. This cell line retains its normal nasopharyngeal epithelial cells and is non-tumourigenic. The cell line was culture in Keratinocyte-SFM medium supplemented with recombinant Epidermal Growth Factor (rEGF) (4-5 ng/ml), Bovine Pituitary Extract (BPE) (40-50 ug/mL), 2% (v/v) heat-inactivation fetal bovine serum and 1% penicillin-streptomycine (GIBCO, Invitrogen, USA). Then, the medium contained cell were incubated in a humidified 5% CO_2 incubator at 37℃.

LMP1 Transfection

In this study, transfection using Lipofectamine ® 3000 Reagent. The transfection of all cell line consisted of pTracer LMP1, pTracer, pcDNA LMP1 and pcDNA confirmed by Western Blot to ensure the transfection contained LMP1 gene before proceed to analysis. After third times passage, 5 x 10^2 cells of NP69 cell was added into 100 ul seeding medium and incubate 37℃ for overnight. Then, replaced seeding medium with 100 ul transfection/plain medium. Added 0.2 ug plasmid DNA into 10 ul optimum, 0.2 ul p3000 reagent and 0.3 ul 3000 reagent in 0.2 ml centrifuge tube. Then the mixture incubated at room temperature for 10 minutes. After incubation, the mixture was added into 96 well plate that already contain with NP69 cell with transfection medium and incubate for 4 hours. After 4 hours, transfection medium was replaced with 100 ul culture medium. The transfection completed after 48 hours.

Measurement of Oxidative Stress in LMP1 Transfection

After proceeded with transfection for 48 hours, for pTracer cell line the medium replaced medium with 100 ul fresh clear medium (Keratinocyte-SFM medium supplemented with recombinant Epidermal Growth Factor (rEGF) (4-5 ng/ml), Bovine Pituitary Extract (BPE) (40-50 ug/mL)). Then, incubate cell with 5 uM CellRox reagent and incubate 37℃ for 30 minutes. To prepare 5 µM of CellRox, need to add 50 ul of 2.5 mM from CellRox stock solution into 500 ul dMSO. After incubation, the cells were washed two times with 100 ul of 1X PBS. Lastly, fluorescence intensity measure by a multimode microplate reader with excitation at 640 nm and emission at 665 nm. Replicated 100 sample of each cell line.

For pcDNA cell line, after transfection process, the medium replaced medium with 100 ul fresh clear medium (Keratinocyte-SFM medium supplemented with recombinant Epidermal Growth Factor (rEGF) (4-5 ng/ml), Bovine Pituitary Extract (BPE) (40-50 ug/mL)). Then, transfected cell incubated cell with 2 ul of 10 uM* of 2', 7 '-Dichlorofluorescin diacetate (DCFH-DA) for 30 minutes (34 minutes at the machine) at 37℃. Prepared 0.243 mg/ml working stock by added 12.15 mg DCFH-DA with 50 ml DMSO. After incubation, the cells were washed two times with 100 ul of clear media. The fluorescence intensity measure by a multimode microplate with excitation at 485 nm and emission at 538 nm. Replicated 100 sample of each cell line.

Statistical Analysis

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

RESULTS

Hundred replications of each cell line used in order to measure the oxidative stress in all cell line. Table 1 show mean and standard deviation of all pTracer and pcDNA cell lines. Mean 17981.12 and standard deviation of 11996.745 is observed in pTracer LMP1. For pTracer vector control cells, mean observed was 18043.47 and standard deviation 11527.655. The mean for pcDNA LMP1 observed is 49426.75 and 7489.795 for standard deviation.

Table 2 show association between pTracer LMP1 and pcDNA LMP1 with production of oxidative stress by compare by pTracer and pcDNA vector control cells. On observed of association between pTracer LMP1 and pTracer vector control cells with production of oxidative stress not statistically significant with value of t (0.37), p value of 0.970. Similarly for pcDNA LMP1 and pcDNA vector control cells, the association value not statistically different with value of t (-0.220), p value of 0.826.

DISCUSSION

EBV is a double strand DNA gamma herpesvirus of the Lymphocryptovirus genus, and it is associated with Burkitt's lymphomas, Hodgkin's lymphomas, nasopharyngeal and gastric carcinomas (Ayadi *et al*., 2008; Deyrup, 2008; Thompson & Kurzrock, 2004). Epstein-Barr virus (EBV) infection is a significant factor in the pathogenesis of nasopharyngeal carcinoma, which is the dominant histopathological type in high-risk areas (Banko *et al*., 2016). Upon viral infection, the EBV genome enters into the nucleus and circularises into an episomal form to establish persistent latent infection, an important step in initiating EBV-related tumorigenesis (Fingeroth *et al*., 1984).

EBV latency genes play an important role in gastric cancer by independently promoting genomic instability that triggers DNA damage by abnormally regulating cell cycle machinery and DNA repair of infected cells (Kgatle, Spearman, Kalla, & Hairwadzi, 2017). Exposure of EBV-infected cells correlates with increased DNA damage induced by ROS-induced NOX and NADPH oxidase (Chen, Kamranvar, & Masucci, 2016). Several studies demonstrated that oxidative stress facilitates

Table 2: Association of Oxidative Stress with pTracer and pTracer LMP1

Cell Line		t value	df	Sig. (2-tailed)	95% Confidence Interval of the Difference	
					Lower	Upper
pTracer	Equal variances assumed	.037	198	.970	-3218.608	3343.308
	Equal variances not assumed	.037	197.686	.970	-3218.640	3343.340
pcDNA	Equal variances assumed	-.220	198	.826	-2572.236	2056.296
	Equal variances not assumed	-.220	191.423	.826	-2572.725	2056.785

EBVinduced B-cell transformation through posttranscriptional regulation of viral (LMP1) and host (STAT3) genes, which are critical for promoting B-cell immortalisation, malignant transformation, and tumorigenesis (Chen *et al*., 2016; Frappier, 2012; Park *et al*., 2016).

The major EBV oncogene is latent membrane protein 1 (LMP1). LMP1 gene shows variability with different tumorigenic and immunogenic potentials. In current study, association of pTracer LMP1 (t(0.37), p value of 0.970) and pcDNA LMP1 9 t (-0.220), p value of 0.826) cell line not show statistically significant with production of oxidative stress when compare with vector control cells. But, in other study shows EBV-encoded latent membrane protein 1 (LMP1) found to be associated with tumor relapse and poor prognosis of nasopharyngeal carcinoma (NPC) (Yang *et al*., 2014). LMP1 intermittently expressed, and this usually correlates with induction of uncontrolled cell growth and proliferation while suppressing programmed cell death (Gastaldello, Chen, Callegari, & Masucci, 2013).

Chronic inflammatory responses triggered by persistent viral infection usually lead to unceasing production of reactive oxygen species (ROS). ROS are highly reactive oxygen containing radicals expressed mainly by neutrophils and phagocytes as part of host defence mechanisms against pathogens (Torres, Jones, & Dangl, 2006). Excessive intracellular ROS may result in cell damages, inducing somatic cell mutation and tumorigenesis (Khandrika, Kumar, Koul, Maroni, & Koul, 2009). Infection of some viruses, including EBV, is frequently accompanied by the elevated level of ROS in cells, which might be associated with viral pathogenicity (Lassoued et al., 2008). There were also reports that EBV reactivation occurred after oxidative stress induction (Lassoued, Gargouri, El Feki, Attia, & Van Pelt, 2010). DNA viruses may encode oncogenic genes that are also capable of hijacking host cellular mechanisms to regulate cell survival and propagation. When these oncogenic genes overcome the ability of host cell machinery to control homeostasis, they trigger the tumour microenvironment associated with an elevated level of mutations that cause malignant transformation and ultimately cancer (Wiseman & Halliwell, 1996).

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. Despite the opposite findings in the present research, it still could be presumed that differences in signaling and biological properties of the LMP1 variants contribute to differences in pathogenicity. It also shows that, LMP1 gene expression not contribute in oxidative stress production that can cause cancer in NPC.

CONCLUSION

Our results show that, there are no association of LMP1 gene expression and oxidative stress production in normal nasopharyngeal cell thus support that LMP1 gene expression not involve in production of oxidative stress that mediated pathways in the process of nasopharyngeal carcinoma.

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