ORIGINAL ARTICLE

Green Fluorescent Proteins (GFP) Induce Oxidative Stress in Normal Nasopharyngeal Cells Expression

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ABSTRACT

Objective: **To evaluate the effect of Green fluorescent proteins (GFP) expression on oxidative stress generation in normal nasopharyngeal cells.**

Material and Methods: **pTracer vector that contained GFP expression and pcDNA transfected 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 GFP induce oxidative stress level in normal nasopharyngeal cell were calculated by comparing mean of pTracer and pcDNA gene by use independent Sample T test.**

Results: **The association between oxidative stress and pTracer expression are statistically significant compared to pcDNA vector control cells.**

Conclusion: **Our results show that, there are association of GFP expression in pTracer vector with oxidative stress production in normal nasopharyngeal cell thus support that GFP expression induce production of oxidative stress that mediated pathways in normal nasopharyngeal cell.**

KEY WORDS

GFP, pTracer, pcDNA, oxidative stress, nasopharyngeal cell

INTRODUCTION

Oxidative stress act as neutralization ability of antioxidants in human body counteract the free radicals harmful effects. Example of free radical is reactive oxygen species (ROS) which is highly reactive with other molecules due to an oxygen pair with one or more unpaired electrons. Reactive oxygen species participate in cellular functions such as intracellular signal transduction, proliferation, cell apoptosis, control over internal homeostasis of calcium Ca^{2+}, regulation of gene expression, as well as protein phosphorylation processes. They may also activated transcription factors such as nuclear factor-kappa B—NF-κB, which causes disorders in the normal functioning of cells[1].

Reactive oxygen species and reactive nitrate species (RNS) have a dual nature, on the one hand, they are necessary for normal cellular functions, but on the other hand, in excess they can damage cells and can lead to cancer development[2]. Free radicals may also damage biomolecules found in the body at the molecular level and cell organelles. Imbalance between the formation of reactive oxygen species and the body's antioxidant capacity are called oxidative stress[1]. Oxidative stress plays a role in many diseases, including inflammation states, diabetes, cardiovascular diseases, or cancer. The role of free radicals and ROS in many cancers has been documented. Intermediate products of oxygen reduction attack DNA and other cellular components, such as lipids, proteins, leaving reactive compounds that in turn can react with DNA bases[2].

Epstein-Barr Virus reactivation as well as expression of the latency genes could play an important role in inducing chromosome rearrangements perhaps via the production of reactive oxygen species (ROS) and the involvement of apoptosis. It is known that ROS induces apoptosis which resulted in chromosome breaks within regions that are commonly rearranged in NPC[3]. Therefore, it is possible that during repeated EBV reactivation and establishment of latency, ROS is produced, and the cells' genomes are subjected to oxidative damage repeatedly, leading to apoptosis. In the effort of DNA repair, erroneous repair occurs resulted in cells bearing rearranged chromosomes survived the apoptosis, continue to replicate, and eventually contribute partly to NPC carcinogenesis.

Green fluorescent protein (GFP) was used for protein tracking and enzyme activity detection, enhance fusion protein folding and is relatively small[4]. The GFP ability to quantitatively monitor gene expression has been demonstrated in different organisms[5]. Green fluorescent protein (GFP) was the first and has been the most used fluorescent protein[6]. The fluorescent chromophore of fluorescent proteins is formed through an intramolecular reaction between the side chains of certain amino acids localized inside the barrel structure of the protein[7]. It has been calculated that one molecule of hydrogen peroxide (H_2O_2) is generated independently of NAD(P)H during the maturation of each chromophore[8]. Current study design to evaluate the effect of GFP expression on oxidative stress generation in normal nasopharyngeal cell.

MATERIALS AND METHODS

Plasmid DNA

pTracer plasmid DNA was sub-cloning into pTracerTM-EF/V5-His

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Table 1: Mean and Standard Deviation of Oxidative Stress in Transfected NP69

Transfection Plasmid DNA	N	Mean	Std. Deviation	Std. Error Mean
pcDNA	100	55463.32	6165.988	616.599
pTracer	100	18043.47	11527.655	1152.765

Table 2: Association of Oxidative stress with Vector Control Transfected Cells

Plasmid DNA		t value	Df		95% Confidence Interval of the Difference	
				Sig. (2-tailed)	Lower	Upper
pcDNA & pTracer	Equal variances assumed	28.624	198	.000	34841.810	39997.890
	Equal variances not assumed	28.624	151.362	.000	34836.916	40002.784

B (pTracer). Recombination of pTracer-LMP1 then transformed by One Shot ® Electrocomp™ *E.coli* by electroporation. Subsequently, the restriction of the pTracer-LMP1 was done prior to the glycerol deposition into the -80°C freezer. Recombinant plasmid with the GFP expression and Zeocin resistance gene was used in the respective transfection efficiency determination and stable transfectant selection experiments.

The plasmid pcDNA was then isolated by using the High Pure Plasmid Isolation Kit (Roche). Subsequently, restriction mapping of the pcDNA was done prior to the glycerol stock deposition into the -80°C freezer.

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°C.

Transfection

In this study, transfection using Lipofectamine ® 3000 Reagent. The transfection of all cell line consisted of pTracer 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°C 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°C 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°C. 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 GFP expression with oxidative stress were derived using Statistical Package for Social Sciences (SPSS) version 23 by performed Independent Sample T test.

RESULTS

To measure the role of green fluorescence protein (GFP) with oxidative stress that express together in pTracer expression by compare vector control of pTracer and pcDNA transfected cell. Table 1 shows the mean and standard deviation of oxidative stress in cells transfected with pTracer and pcDNA. For pcDNA vector control transfected cells, the mean is 55463.32 with standard deviation of 6165.988. Mean of 18043.47 and standard deviation of 11527.655 were observed in pTracer vector transfected cell.

Table 2 shows the association of oxidative stress with pTracer and pcDNA transfected cells. The association between oxidative stress and pTracer expression are statistically significant with value of t (28.624), p value less than 0.05 compared to pcDNA vector control cells.

DISCUSSION

Reactive oxygen species (ROS) are highly reactive oxygen containing radicals expressed mainly by neutrophils and phagocytes as part of host defence mechanisms against pathogens[9]. Excessive intracellular ROS may result in cell damages, inducing somatic cell mutation and tumorigenesis[10]. Infection of some viruses, including EBV, is frequently accompanied by the elevated level of ROS in cells, which might be associated with viral pathogenicity[11]. There were also reports that EBV reactivation occurred after oxidative stress induction[12]. 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[13]. ROS is also involved in the progression — at this stage, the generation of large amounts of ROS may contribute to mutation, increase the amount of matrix metalloproteinases (MMPs) and damage local tissues. Increased levels of DNA modified due to oxidation can contribute to genetic instability and may be crucial in tumour metastases in developed cancer[14].

In current study, comparing vector control pTracer and pcDNA, pTracer shows induce oxidative stress compared to pcDNA with statistically significant differences value. Our study supported by study of the

formation of the reactive oxygen species by enhanced green fluorescent protein (eGFP) shows eGFP induces oxidative stress in cells[8]. Study shows that, in initiation apoptosis cascade, reactive oxygen production induced by GFP has been linked to cellular toxicity and eventual death in GFP expressing cells[15]. In addition, neuroblastoma cell lines, showed increased sensitivity to cytotoxic agents when transduced with GFP, eGFP, and YFP[16].

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 effects. In current study, GFP expression shows contribution in oxidative stress production in normal nasopharyngeal cells. Thus, vector that contain GFP expression not suitable to use in study of oxidative stress measurement.

CONCLUSION

Our results show that there is association of GFP expression and oxidative stress production in normal nasopharyngeal cell thus support that GFP expression involve in production of oxidative stress.

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REFERENCES

1. Kulbacka J, Saczko J, Chwiłkowska A. Oxidative stress in cells damage processes. Polski merkuriusz lekarski: organ Polskiego Towarzystwa Lekarskiego. 2009; 27(157): 44-7.
2. Choudhari SK, Chaudhary M, Gadbail AR, Sharma A, Tekade S. Oxidative and antioxidative mechanisms in oral cancer and precancer: a review. Oral oncology. 2014; 50(1): 10-8.
3. Tan S-N, Sim S-P, Khoo ASB. Potential role of oxidative stress-induced apoptosis in mediating chromosomal rearrangements in nasopharyngeal carcinoma. Cell & Bioscience. 2016 05/25 09/25/received 05/10/accepted;6:35. PubMed PMID: PMC4880972.
4. Stepanenko OV, Stepanenko OV, Kuznetsova IM, Verkhusha VV, Turoverov KK. Beta-barrel scaffold of fluorescent proteins: folding, stability and role in chromophore formation. International review of cell and molecular biology. 2013; 302: 221-78.
5. Bolhassani A, Taheri T, Taslimi Y, Zamanilui S, Zahedifard F, Seyed N, *et al*. Fluorescent Leishmania species: development of stable GFP expression and its application for in vitro and in vivo studies. Experimental parasitology. 2011; 127(3): 637-45.
6. Tsien RY. Constructing and exploiting the fluorescent protein paintbox (Nobel Lecture). Angewandte Chemie International Edition. 2009; 48(31): 5612-26.
7. Zhang L, Patel HN, Lappe JW, Wachter RM. Reaction progress of chromophore biogenesis in green fluorescent protein. Journal of the American Chemical Society. 2006; 128(14): 4766-72.
8. Ganini D, Leinisch F, Kumar A, Jiang J, Tokar EJ, Malone CC, *et al*. Fluorescent proteins such as eGFP lead to catalytic oxidative stress in cells. Redox biology. 2017; 12: 462-8.
9. Torres MA, Jones JD, Dangl JL. Reactive oxygen species signaling in response to pathogens. Plant physiology. 2006; 141(2): 373-8.
10. Khandrika L, Kumar B, Koul S, Maroni P, Koul HK. Oxidative stress in prostate cancer. Cancer letters. 2009; 282(2): 125-36.
11. Lassoued S, Ameur RB, Ayadi W, Gargouri B, Mansour RB, Attia H. Epstein-Barr virus induces an oxidative stress during the early stages of infection in B lymphocytes, epithelial, and lymphoblastoid cell lines. Molecular and cellular biochemistry. 2008; 313(1-2): 179.
12. Lassoued S, Gargouri B, El Feki AeF, Attia H, Van Pelt J. Transcription of the Epstein–Barr virus lytic cycle activator BZLF-1 during oxidative stress induction. Biological trace element research. 2010; 137(1): 13-22.
13. Wiseman H, Halliwell B. Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. Biochemical Journal. 1996; 313(Pt 1): 17.
14. Katakwar P, Metgud R, Naik S, Mittal R. Oxidative stress marker in oral cancer: a review. Journal of cancer research and therapeutics. 2016; 12(2): 438.
15. Gambotto A, Dworacki G, Cicinnati V, Kenniston T, Steitz J, T ing T, *et al*. Immunogenicity of enhanced green fluorescent protein (EGFP) in BALB/c mice: identification of an H2-Kd-restricted CTL epitope. Gene therapy. 2000; 7(23): 2036-40.
16. Goto H, Yang B, Petersen D, Pepper KA, Alfaro PA, Kohn DB, *et al*. Transduction of green fluorescent protein increased oxidative stress and enhanced sensitivity to cytotoxic drugs in neuroblastoma cell lines. Molecular cancer therapeutics. 2003; 2(9): 911-7.