

## Original Article

# RNAi silencing targeting LMP2A enhances the radiosensitivity of nasopharyngeal carcinoma cell line C666-1

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**Abstract:** Latent membrane protein 2A (LMP2A) is found to play an important role in development of nasopharyngeal carcinoma (NPC), to which the main current approach was radiotherapy. However, the role of silencing LMP2A in the radiosensitivity of NPC cells has not been clarified. In this study, we inhibited LMP2A expression by lentivirus-mediated RNAi, to explore the effects of silencing LMP2A on the radiosensitivity of NPC cell line C666-1. A lentivirus-mediated RNAi technology was employed to specifically knock down the LMP2A gene in C666-1 cells, whose expression was detected by Western blotting. Clonogenic survival assay and flow cytometry were performed to evaluate the clonogenic cell survival, cell cycle distribution and apoptosis of C666-1 cells after irradiation. We successfully construct a highly efficient and stable lentivirus vector, which efficiently down-regulates the expression of LMP2A gene in the infected cell line C666-1. Down-regulation of LMP2A significantly decreases the radiobiological parameters ( $SF_2$ ,  $D_0$ ,  $D_q$ ), and the specific down-regulation arrests cells in G0/G1 phase of cell cycle and increases cell apoptosis after irradiation. Our findings suggest that lentivirus-mediated RNAi silencing targeting LMP2A enhanced the radiosensitivity of NPC cell line C666-1, and LMP2A may be a potential target for radiotherapy in treatment of NPC.

**Keywords:** Latent membrane protein 2A, nasopharyngeal carcinoma, RNA interference, radiosensitivity

## Introduction

Nasopharyngeal carcinoma (NPC) arising from epithelial cells that cover the surface and line of the nasopharynx is one of the most common tumor in head and neck [1]. To which NPC are sensitive, radiotherapy is the main current treatment modality for this disease. Although the great technological advances have been made in radiotherapy such as three-dimensional conformal radiotherapy and intensity-modulated radiotherapy [2, 3], some of patients with NPC present local recurrences and distant metastases after radiotherapy due to irradiation (IR) resistance, which remains a serious obstacle to successful treatment for NPC [4]. Therefore, increasing the sensitivity of NPC cells to radiotherapy would provide a significant advancement in the effective treatment for NPC.

Epstein-Barr virus (EBV), which is a human gamma herpes virus that exists in humans for a

long time without producing any symptoms, is closely associated to NPC, particularly the undifferentiated type [5]. In NPC, EBV expresses a well defined set of latent viral genes, including latent membrane protein 2A (LMP2A), whose functions similarly lead to the activation of the PI3K/Akt, NF- $\kappa$ B, mTOR, Wnt/ $\beta$ -catenin, STAT, and Notch pathways [6-10]. In our previous study, we demonstrated that inhibition of Notch could enhance radiosensitivity of NPC cells [11], and reduce the proportion of side population (SP) cell, which contributed toward irradiation resistance [12]. Thus, we hypothesize that silencing LMP2A might enhance the radiosensitivity of NPC cell.

RNA interference (RNAi) has emerged as a powerful tool to induce loss-of-function phenotypes by posttranscriptional silencing of gene expression [13]. Lentivirus vectors have provided a huge advancement in technology and seemingly offered the means to achieve significant levels of gene transfer in vitro [14]. We also suc-

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successfully constructed a highly efficient and stable lentivirus vector, which efficiently down-regulate the expression of LMP2A gene in the infected cell line C666-1 [15]. In this study, we treated the infected cell line C666-1 with irradiation, to investigate the effect of silencing LMP2A on the radiosensitivity of NPC cell line C666-1. The results reveal that lentivirus-mediated RNAi silencing targeting LMP2A promoted IR-induced G<sub>0</sub>/G<sub>1</sub> arrest and apoptosis of C666-1 cells, which result in enhancing the radiosensitivity of NPC cell line C666-1. Our studies indicate that LMP2A may serve as a potential target for radiotherapy in treatment of NPC. RNAi silencing targeting LMP2A may have promising clinical applications as radiosensitizers in the treatment of NPC.

## Materials and methods

### *Cell line and culture*

Human NPC cell line C666-1 was obtained from the Xiangya Central Experiment Laboratory, Central South University, China. Cells were cultured in RPMI-1640 (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; Sijiqing, China), 50 U/ml penicillin G and 50 U/ml streptomycin (Gibco, Carlsbad, CA, USA) in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C. The medium was changed every 3 days.

### *Irradiation procedure*

Irradiation was performed with single doses of X-rays ranging from 1 Gy to 8 Gy using a linear accelerator (Trilogy, Austin, TX, USA) with 6 MV photons/100 cm focus-surface distance at room temperature. The dose rate was 4.0 Gy/min, which was determined by Fricke's chemical dosimeter.

### *LMP2A depletion by lentivirus-mediated RNAi*

To produce lentivirus expressing RNAi specific for the LMP2A gene (GU979791), the RNAi sequence for human LMP2A (CTC CCA ATA TCC ATC TGC T) was identified by using the manufacturer's RNAi Designer programme, and the negative control construct (control RNAi) was created by a scrambled sequence (TTC TCC GAA CGT GTC ACG T). Both sequences were annealed and inserted into the AgeI and EcoRI enzyme sites of pGCSIL-GFP vector (Genechem, Shanghai, China), respectively. After confirma-

tion of the constructed plasmids by DNA sequencing, lentiviral vector DNAs and packaging vectors (pHelper 1.0 and pHelper 2.0, Genechem) were then transfected into 293T cells. Supernatants containing lentiviruses such as pGCSIL-LMP2A-shRNA-LV and pGCSIL-neg-shRNA-LV were harvested, respectively. Then, purification was performed using ultracentrifugation and the titer of lentivirus was determined. The lentivirus stock was added to C666-1 cells at a multiplicity of infection (MOI) of 10. Total protein was isolated and the expression of LMP2A was detected by Western blotting. The efficiency of infection was observed by fluorescence microscopy. Three groups of C666-1 cells were used in subsequent assays: CON group (blank control group, with no infection), NC group (negative control group, infected with pGCSIL-neg-shRNA-LV), KD group (LMP2A RNAi group, infected with pGCSIL-LMP2A-shRNA-LV).

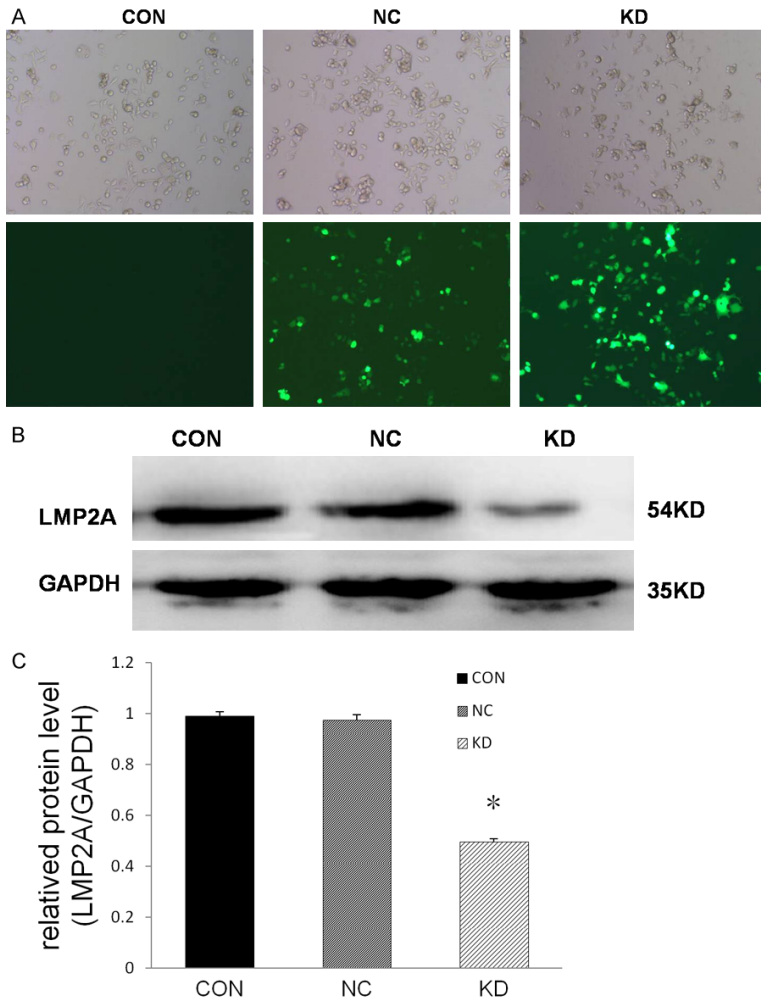
### *Western blot analysis*

Cell extracts were prepared in radioimmuno-precipitation assay buffer (150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, and 50 mM Tris, pH 8.0), with the addition of 2 mM phenylmethylsulfonyl fluoride. Lysis buffer was freshly prepared and added to infected cells in six-well plates (100 µL/well) on ice, which were then incubated for 10 min. Protein concentrations were determined by protein assay kit. Equal amounts of proteins (10 µg per condition) were boiled for 10 min in loading buffer before being separated on 15% SDS-PAGE gels. Separated proteins were transferred to polyvinylidene difluoride membranes at 100 V for 1 h before membrane was blocked in PBS with 5% skim milk powder and Tween 20. Anti-LMP2A primary antibody (Cell Signaling Technology) and secondary antibodies (Abcam) were diluted to 1/1,000 with PBST buffer and incubated for 60 min at room temperature. Membrane was washed 3 times by PBST before each step. Protein bands were visualized by enhanced chemiluminescence.

### *Clonogenic cell survival*

Cells were seeded into culture dishes, irradiated the next day at the distinct doses (0, 1, 2, 3, 4, 6, and 8 Gy). Plates were incubated for 14 days, fixed with methanol, stained with Giemsa, and colonies containing at least 50 cells in size were counted. The surviving fraction (SF) was

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**Figure 1.** Lentiviral vectors encoding LMP2A RNAi or control RNAi were constructed and transfected into C666-1 cells, with untreated C666-1 cells (Con) served as a control. A. Three groups of C666-1 cells are observed in bright and fluorescent fields of microscope ( $\times 100$ ). B. The expression of LMP2A protein is detected by Western blotting. C. The protein levels of LMP2A in the KD group are significantly reduced, compared with the CON and the NC group (\* $P < 0.05$ ).

calculated as: [(mean colonies counted)/(cells plated)  $\times$  (plating efficiency)]; plating efficiency was defined as: [(mean colonies counted)/(cells plated)] for unirradiated controls (0 Gy). The data were fit into the multi-target single-hit mathematical model, and survival curves for three groups were drawn. Radiobiological parameters, such as the survival fraction at 2 Gy ( $SF_2$ ), the mean lethal dose ( $D_0$ ), the quasi-threshold dose ( $D_q$ ), and the extrapolate number (N) were calculated according to the survival curves.

## Cell cycle analysis

Twenty four hours after irradiated with 4 Gy X-rays, cells were harvested by trypsinization,

then were fixed with 70% ethanol, and resuspended in 20 mg/mL propidium iodide (PI). DNA content was detected by a flow cytometer (FACSCalibur™, America BD). The relative proportions of cells in the individual cell-cycle phase fraction were determined from the flow cytometry data.

## Apoptosis assay

Twenty four hours after exposed to 4 Gy X-rays, cells were stained with annexin V-APC to differentiate intact cells from apoptotic cells. A total of  $1.0 \times 10^6$  cells were washed twice with ice-cold PBS and were incubated for 30 min in binding buffer (1 mg/mL annexin V-APC). FACS analysis for annexin V-APC staining was performed by flow cytometer (FACSCalibur™, America BD).

## Statistical analysis

All experiments were performed in triplicate, and data were shown as the mean  $\pm$  SD where applicable. Statistically significant differences between groups were determined by One-way ANOVA or Student's t-test with using SPSS 17.0 software (SPSS, Chicago, IL, USA), and  $P < 0.05$  was considered statistically significant.

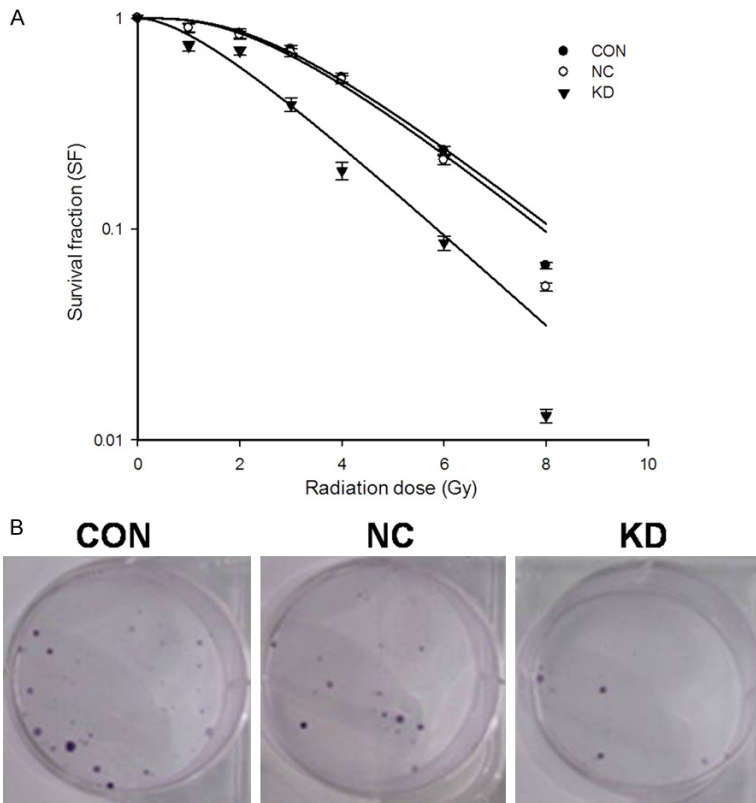
The survival curve was drawn with SigmaPlot 12.0 (Systat Software Inc., San Jose, CA).

## Results

### Knockdown of LMP2A by lentivirus-mediated RNAi in C666-1 cells

To knock down LMP2A gene, we used a lentivirus vector system derived from HIV-1 to express short hairpin RNAs (shRNAs) directed against LMP2A. In addition, GFP is incorporated as a reporter gene. After a single exposure of C666-1 cells to the constructed lentivirus, high percentage (more than 90%) of infectants express-

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**Figure 2.** Clonogenic assay to obtain the single-dose radiation survival curves of C666-1. A. Radiation survival curves for C666-1 cells pretreated with lentivirus for 72 h and irradiated with the doses indicated. Survival curves represent data fitting into the multi-target click model. B. C666-1 cells radiated at 8 Gy show few visible colonies after 2 weeks.

**Table 1.** Survival curve parameters fitting the data into a multi-target single-hit model

Group	$D_0$	$D_q$	N	$SF_2$
CON	2.271	2.138	3.734	0.865
NC	2.236	2.088	3.607	0.850
KD	1.998	1.348	1.926	0.586

$D_0$ , mean lethal dose;  $D_q$ , quasi-threshold dose; N, extrapolation number;  $SF_2$ , surviving fraction at 2 Gy.

es GFP 72 h after the infection (**Figure 1A**), indicating that a highly efficient and stable lentivirus vector is successfully constructed. The effect of LMP2A-specific RNAi-expressing lentivirus on the expression of LMP2A was examined by Western blot analysis (**Figure 1B**). Compared with the NC group, the protein levels of LMP2A in the KD group are sharply reduced ( $P < 0.05$ ,  $n=6$ , **Figure 1C**). No significant difference is found between the CON group and the NC group. These data demonstrate that the expression of LMP2A gene is efficiently down-regulated in infected cell line C666-1.

### Knockdown of LMP2A improves radiosensitization of C666-1 cells

To determine the radiosensitizing effect of LMP2A depletion on the NPC cell line C666-1, a clonogenic formation assay was performed. The shoulder area of the survival curves is significantly narrowed and the surviving fractions at each dose (1, 2, 3, 4, 6 and 8 Gy) decrease in KD group (**Figure 2A**). The values of  $SF_2$ ,  $D_0$ ,  $D_q$ , and N in the KD group are all lower than that in the CON group and the NC group (**Table 1**).  $SF_2$  is reduced to 58.6% in the KD group from 86.5% in the CON group and the sensitization enhancement ratio (SER) is 1.48. There are few visible colonies in cells that were radiated with 8 Gy, which can be easily seen with the naked eye (**Figure 2B**). Thus, we conclude that down-regulation of LMP2A can radiosensitize C666-1 cells.

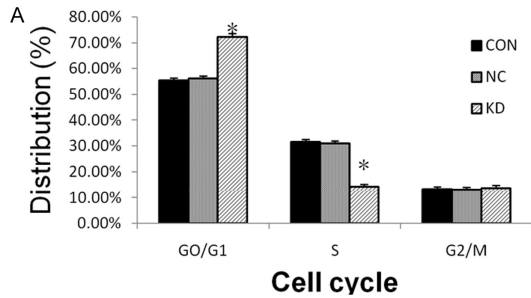
### Knockdown of LMP2A promotes IR-induced G0/G1 arrest of C666-1 cells

To further evaluate potent reasons for radiation sensitivity induced by knockdown of LMP2A, we tested cell cycle distribution by flow cytometry assays. The analysis showed an increase in the proportion of G0/G1 phase ( $56.09 \pm 1.07\%$  vs.  $72.21 \pm 1.38\%$  in the NC group and the KD group,  $P < 0.05$ ,  $n=6$ , **Figure 3**). The frequency of LMP2A RNAi cells in the G0/G1 phase is significantly increased while the frequency of cells in the S-phase is decreased, as compared with the control group. These data indicate down-regulation of LMP2A expression promotes IR-induced G0/G1 arrest of C666-1 cells.

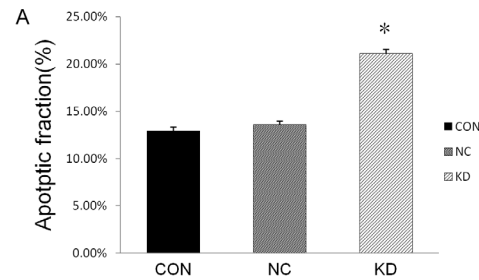
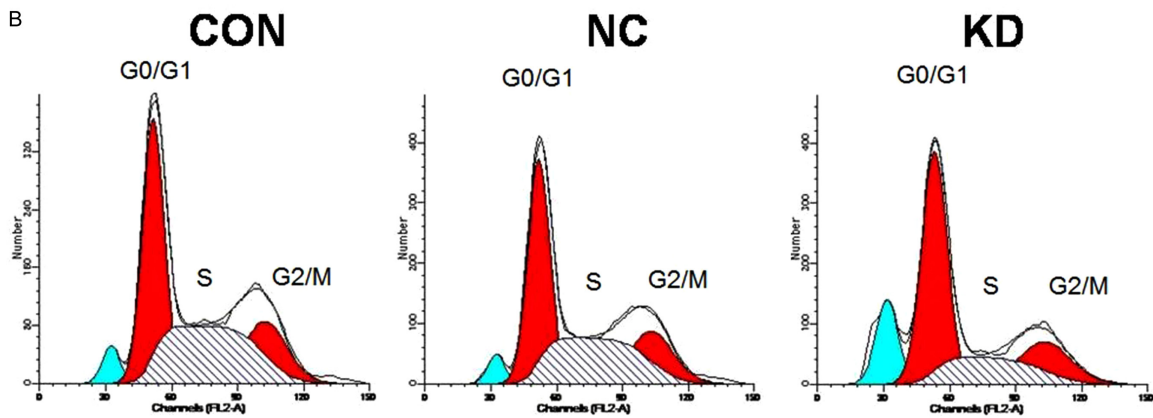
### Knockdown of LMP2A increases IR-induced apoptosis of C666-1 cells

To further investigate the reasons for down-regulation of LMP2A radiosensitizing C666-1 cells, the rate of apoptosis was also evaluated

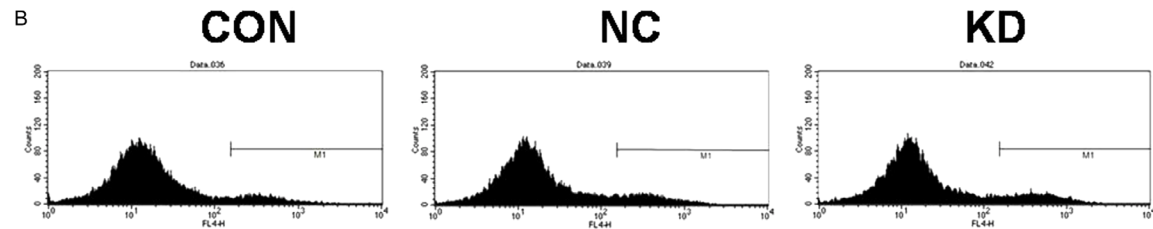
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**Figure 3.** Changes of cell cycle distribution in C666-1 cells treated with *LMP2A* RNAi after irradiation. A. *LMP2A* RNAi significantly increases the proportion of cells in G0/G1 phase and decreases the S phase in the KD group, compared with the CON and the NC group (\* $P < 0.05$ ). B. FCM analysis show that *LMP2A* RNAi increases IR-induced G0/G1 arrest of C666-1 cells.



**Figure 4.** Apoptosis ratio of C666-1 cells treated with *LMP2A* RNAi after irradiation. A. *LMP2A* RNAi significantly promotes cell apoptosis in the KD group, compared with the CON and the NC group (\* $P < 0.05$ ). B. FCM data show that down-regulation of *LMP2A* significantly increases IR-induced apoptosis of C666-1 cells.



by flow cytometry analysis. Compared with the CON and the NC group, the percentage of apoptosis in the KD group is increased ( $P < 0.05$ ,  $n=6$ , **Figure 4**). Apoptosis in the CON and the NC group are  $12.91 \pm 0.41\%$  and  $13.61 \pm 0.38\%$ , while apoptosis in the KD group is  $21.12 \pm 0.45\%$ . These data show that the apoptosis rate of down-regulation of *LMP2A* is significantly increased compared with the control group, 24 h after irradiation. The results demonstrate that *LMP2A* RNAi sensitize C666-1 cells to radiation-induced apoptosis.

## Discussion

*LMP2A* has been detected in the majority of NPC samples [16], which has been reported to correlate with a poor survival outcome [17]. It has been evidenced that activation of *LMP2A* was capable of promoting nasopharyngeal carcinoma cell growth and protecting the tumor cell from apoptosis, which were modulated through a variety of signaling pathways. Recent studies propose a model in which two ubiquitin-mediated signaling pathways are involved in

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these processes, namely the Wnt and Notch pathways [18]. LMP2A activates and stabilizes  $\beta$ -catenin in epithelial cells through PI3K/Akt activation, which negatively regulates glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ). GSK-3 $\beta$ , in turn, is tightly regulated by Wnt signaling [19]. Notch signaling pathways influence a variety of cellular processes, including lineage specification, cell survival and proliferation [20]. Our previous studies demonstrated that down-regulation of Notch signaling could enhance radiosensitivity of NPC cells. These findings thus raised the possibility that silencing LMP2A might serve as a potential radiosensitizer for radiotherapy in treatment of NPC. However, the role of silencing LMP2A in the radiosensitivity of NPC cells has not been clarified. In this study, we inhibited LMP2A expression by lentivirus-mediated RNAi, to investigate the effect of silencing LMP2A on the radiosensitivity of NPC cell line C666-1 and its possible mechanism.

To investigate the effects of silencing LMP2A on radiosensitivity of nasopharyngeal carcinoma cells, we genetically down-regulated the expression of LMP2A in human nasopharyngeal carcinoma cell line C666-1 by infecting with LMP2A-specific RNAi-expressing lentivirus, and characterized its effects on the clonogenic formation, cell cycle distribution and cell apoptosis after irradiation. We find that down-regulation of endogenous LMP2A decrease the radiobiological parameters ( $SF_2$ ,  $D_{01}$ ,  $D_{02}$ ), and the specific down-regulation arrests cells in G0/G1 phase of cell cycle and increases cell apoptosis. The radiosensitization mechanism might be correlated with its effect on G0/G1 arrest and apoptosis induction. Numerous studies have shown that down-regulation of Notch signaling increased cell population in G1 phase and resulted in the induction of apoptosis [21, 22]. It has been also found that high Notch activity contributed to radiation resistance [23, 24]. So, we deduce a conclusion that silencing LMP2A results in down-regulation of Notch expression in C666-1 cells, which exhibits the G0/G1 arrest. This could explain why silencing LMP2A altered the cell cycle with G0/G1 phase arrest in this study. The present study demonstrates that silencing LMP2A promotes G0/G1 phase arrest, which is also seen in combination with IR. However, we did not detect expression of Notch in this study. So, we need further study to explain this mechanism and discover other mechanisms in which inhibition

of LMP2A enhances the radiosensitivity in C666-1 cells.

RNAi was a useful tool for gene functional analysis, and might be a potential therapeutic strategy for various diseases including cancers [25]. Lentivirus has been applied for siRNA delivery not only in vitro, but also in vivo for many years [26]. Evaluation of targets for effective inhibition of nasopharyngeal carcinoma cell growth should precipitate clinical application of gene silencing therapy. In this study, we successfully constructs a highly efficient and stable lentivirus vector system, which efficiently knock down the LMP2A gene in infected cell line C666-1. Our studies also show that lentivirus-mediated RNAi silencing targeting LMP2A increases IR-induced G0/G1 arrest and apoptosis of C666-1 cells. Therefore, a new strategy, reducing expression of LMP2A mediated by lentivirus, may prove useful try in treatment of human nasopharyngeal carcinoma.

In summary, our studies demonstrate that lentivirus-mediated RNAi silencing targeting LMP2A promotes IR-induced G0/G1 arrest and increased IR-induced apoptosis, which result in enhancing the radiosensitivity of NPC cell line C666-1. This provides an attractive adjuvant to the radiotherapy of human nasopharyngeal carcinoma.

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### Disclosure of conflict of interest

None.

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