SERPINB2 overexpression inhibited cell proliferation, invasion and migration, led to G2/M arrest, and increased radiosensitivity in nasopharyngeal carcinoma cells

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(Received 17 September 2018; revised 21 December 2018; editorial decision 22 January 2019)

ABSTRACT

The aim of this study was to evaluate the effect of SERPINB2 on cell proliferation, cell cycle, epithelial–mesenchymal transition (EMT), invasion, migration, and radiosensitivity in nasopharyngeal carcinoma cells. Both CNE2R and CNE2 cells were transfected with pEGFP-N1-SERPINB2. Cell proliferation was measured by MTT assay, cell cycle by flow cytometry, and \textit{SERpINB2} expression by quantitative real-time polymerase chain reaction (qRT-PCR). Western blot was carried out to detect the protein expression. In addition, SERPINB2 and \textit{β}-catenin were located intracellularly using an immunofluorescent assay, and cell migration and invasion were measured by wound healing and Transwell assays, respectively. Radiosensitivity was assessed using colony formation and MTT assays. SERPINB2 expression was downregulated in CNE2R cells. After transfection with pEGFP-N1-SERPINB2, the OD values were decreased, and there was an increased fraction in the G2/M phase. Moreover, SERPINB2 overexpression could inhibit the invasion and migration capabilities of CNE2R and CNE2 cells, with downregulation of vimentin, N-cadherin, nuclear-\textit{β}-catenin, matrix metalloproteinase (MMP)-2 and MMP-9, and upregulation of E-cadherin. Moreover, transfection with the SERPINB2 plasmid reduced the growth rate of CNE2R cells at doses of 2, 4 and 6 Gy, and also decreased the surviving fractions. Overexpression of SERPINB2 could reduce the proliferation, invasion and migration capabilities of CNE2R and CNE2 cells, and led to G2/M arrest via EMT inhibition, and this may be a potential strategy for enhancing the radiation sensitivity of nasopharyngeal carcinoma cells.

Keywords: SERPINB2; nasopharyngeal carcinoma; radiotherapy; CNE2; CNE2R

INTRODUCTION

Nasopharyngeal carcinoma (NPC), one of the most life-threatening malignancies for human health, ranks top in both incidence and mortality out of all head-and-neck malignant tumors [1]. NPC has a typical geographical and racial distribution, and is frequently seen in Southern China and Southeast Asia [2]. So far, radiotherapy has been regarded as the major method for the treatment of NPC because of its anatomic location in the nasopharynx [3]. Unfortunately, evidence has shown that radiotherapy can also induce radioresistance in various tumors, including NPC, leading to the recurrence and metastasis of tumors, which can be a major obstacle in NPC treatment [4, 5]. Thus, better understanding of the specific mechanism concerning the induction of tumor radioresistance in NPC is urgently needed in order to improve the survival rate of NPC patients. Epithelial–mesenchymal transition (EMT) refers to the specific biological process of the phenotypic transition of epithelial cells into mesenchymal cells [6],...
which can occur during the growth and development of normal tissues, but can also be involved in the regulation of the malignant process of tumor growth [7, 8]. It should be noted as well that radiotherapy can affect the expression of a group of genes associated with the promotion of EMT in tumor cells [9]. In addition, a number of studies have reported that the phenotypic changes arising from EMT are related to the increased resistance to radiotherapy; thus, it represents a potentially critical mechanism for acquired radioresistance in tumor cells, including those in NPC [10–12].

SERPINB2, also named PAI-2 (plasminogen activator inhibitor type-2), is the second member of the clade B serine protease inhibitors in the Serpin family, with unique properties similar to those of a novel plasminogen activator inhibitor [13]. To date, many studies have been focused on the effect of SERPINB2 on tumors. For example, SERPINB2 has been found to be upregulated in breast cancer, and has been linked to unfavorable outcome and increased lymph node metastasis in breast cancer patients [14], but SERPINB2 has also been linked with a favorable prognosis in some other cancers [15], suggesting that there are different roles for SERPINB2 in different tumors. Moreover, SERPINB2 was observed to be located on chromosome 18q21 (the known location of the serpin gene cluster), and this region has been reported to have important roles in oral squamous cell carcinoma (another common malignancy in the head-and-neck region) [16], implying a potential role for SERPINB2 in head-and-neck tumors, including NPC. Notably, there is evidence demonstrating that upregulation of SERPINB2 enhances the sensitivity of NPC cells to chemotherapy [17]; however, whether SERPINB2 affects the sensitivity of NPC cells to radiotherapy remains unclear. Furthermore, SERPINB2 is indispensable for extracellular matrix remodeling [18], which plays a key role in the initiation of EMT in tumors [19].

CNE2 is a poorly differentiated NPC epithelioid cell line derived from a primary tumor biopsy [20], and it has been used in a multitude of NPC-related studies [21–23]. CNE2R, a radioresistant NPC cell line, was established from CNE2 cells that had undergone 400 cGy 60Co γ-radiation repeated 16 times for a total dose of 64 Gy for 1 year [24], and its tumor-suppressing capabilities are naturally lower than that of CNE2 cells [25]. Thus, in this study, we first compared the expressions of SERPINB2 in the radioresistant human NPC cell line CNE2R and its parental cell line (CNE2), and then, via transfection with the pEGFP-N1-SERPINE2 plasmid, we investigated the effects of SERPINB2 on cell proliferation, cell cycle, EMT, invasion, migration and radiosensitivity in NPC cells.

**MATERIALS AND METHODS**

**Cell lines and culture**

The NPC CNE2 cell lines were provided by the Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China), and a radioresistant human NPC cell line (CNE2R) was constructed according to the previously described methods [24]. Next, both of these cell lines (CNE2 and CNE2R) were cultured regularly in RPMI-1640 medium (Hyclone, Logan, UT, USA) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA) (5% CO₂, 37°C), in the presence of penicillin (100 U/ml) and streptomycin (100 μg/ml).

**Construction of recombinant plasmids and cell grouping**

In this experiment, CNE2 and CNE2R cell lines were divided into three groups: the blank group (cells with no treatment); the vector group (cells transfected with the empty vector plasmid, enhanced green fluorescent protein (EGFP) gene pEGFP-N1); and the SERPINB2 group (cells transfected with pEGFP-N1-SERPINE2). The corresponding cDNA of SERPINB2 was provided by Genebank. Primers for SERPINB2 cDNA: upstream, 5′-GGGGCCTCGAGATGGGATCTTTGTTGAGCAACACAC3′; downstream, 5′-CCCGAATTCTGGTTGAGAAATCTGCCGAAAATAAGTG-3′. Then, cDNA was inserted into the restriction site of pEGFP-N1 between XhoI and EcoRI, followed by transient transfection, using FuGENE® HD (Promega) according to the manufacturer’s instructions.

**qRT-PCR**

According to the kit instruction (QIAGEN, Valencia, CA), the total RNA was extracted from cells and subjected to the concentration measurement using an ultraviolet spectrometer to calculate the OD (optical density)260/OD280 ratio, which, in this experiment, was >1.8, suggesting that the extracted RNA could be applied in the following test. Reverse transcription of cDNA was also performed in accordance with the instruction (QIAGEN, Valencia, CA). Primers were designed based upon the published genes in Genebank, and synthesized by Sangon Biotech Co., Ltd (Shanghai, China). qRT-PCR was carried out in 20 μl of the reaction system: including SYBR PremixExTaq (10 μl), Forward Primer (0.4 μl), Reverse Primer (0.4 μl), ROX Reference Dye II (0.4 μl), DNA template (2 μl), and ddH2O (6.8 μl), and the reaction conditions were set as follows: 40 cycles of 95°C for 30 s, 95°C for 5 s and 60°C for 30 s. Results were normalized to the GAPDH, and the relative expressions of targeted genes were calculated using the 2−ΔΔCt method.

**Western blot**

The total proteins were extracted from cells and subjected to the measurement of protein concentrations using the BCA kit (Boster, Wuhan, China). Nuclear proteins were extracted using an extraction kit (Fermentas, Pittsburgh, PA, USA) according to the manufacturer’s instructions. Then, proteins, together with the loading buffer, were boiled at 95°C for 10 min, and in each well, 30 μg of sample was loaded for electrophoresis in 10% SDS-PAGE to separate the proteins, followed by transferring the proteins on the PVDF membrane and blocking at temperature using 5% bovine serum albumin (BSA). Proteins on the PVDF membrane were probed with the primary antibodies, including anti-SERPINE2 (ab47742, 1 μg/ml), anti-E-cadherin (ab1416, 1/50), anti-vimentin (ab137321, 1/50 μg/ml), anti-N-cadherin (ab18203, 1 μg/ml), anti-β-catenin (ab32572, 1/5000), anti-MMP-2 (ab37150, 0.5 μg/ml), anti-MMP-9 (ab73734, 1 μg/ml) and anti-GAPDH (ab181602, 1/10 000) [which were all purchased from Abcam (USA)] and they were incubated at 4°C overnight. Following three washes in Tris-buffer saline with Tween 20 (TBST) (5 min/time), immunoblots were then incubated with the corresponding secondary antibodies for 1 h at room temperature. Again, PVDF membrane was washed three times in TBST (5 min/time). Results were normalized to the GAPDH, and immunoblots were developed in the Bio-Rad Gel Doc EZ imaging system (GEL...
DOC EZ IMAGER, Bio-rad, California, USA); the gray values of targeted bands were analyzed using Image J software.

**MTT assay**
Cells after transfection were diluted to a density of $5 \times 10^4$/well and then inoculated into the 96-well plate for incubation. When the cell confluence reached 80%, the medium in each well was removed and washed with serum-free medium, respectively, at 0, 12, 24, 36 and 48 h (or irradiated with 6 MV X-ray irradiation at doses of 2, 4 and 6 Gy for 24 h), and in each well, cells were incubated in the presence of 20 μl of MTT [3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide] reagent (Sigma, USA) for 4 h at 37°C. Thereafter, MTT reagent was replaced by 150 μl dimethyl sulfoxide (DMSO) (Sigma, USA), and cells were incubated on a shaker at room temperature for 10 min. OD values were then detected at a wavelength of 490 nm using a microplate reader (Thermo MK3).

**Flow cytometer**
Cells, following digestion and collection, were centrifuged at 1000 rpm for 5 min to collect the cell sediment, which was then rinsed with phosphate buffer saline (PBS) and centrifuged at 1000 rpm for 5 min. Cells were fixed in 70% ethanol–PBS solution at 4°C. Thereafter, the ethanol–PBS was removed, and the cells were incubated with 100 μl RNase A at 37°C in a water bath for 30 min, and then mixed with 400 μl propidium iodide (PI). Following 30 min of reaction at 4°C in a dark room, the red fluorescence was detected at a wavelength of 488 nm using a flow cytometer. This experiment was conducted in triplicate.

**Immunofluorescence assay**
Cells in logarithmic growth were collected for digestion, and then inoculated into a 96-well plate at a cell density of $2 \times 10^4$/well. Before permeabilization by addition of 0.5% triton, the cells were fixed with 4% paraformaldehyde (PFA) for 20 min followed by three PBS washes. Then, cells were incubated with primary anti-SERPINEB2 (sc-166539, 1:1000; Santa Cruz Biotechnology, Inc., CA, USA) and anti-β-catenin (ab16051, 1:100; Abcam, Cambridge, UK) at 4°C overnight, and then incubated with diluted CP 488A-labeled secondary antibody (SAB4600045, 1:50; Sigma-Aldrich, St Louis, MO, USA) for 1 h at room temperature in a dark place. The nuclei were stained using DAPI (4′-6-diamidino-2-phenylindole) (C1002, 1:1000; Beyotime Institute of Biotechnology, Jiangsu, China) for 3–5 min. The section was mounted for observation under a fluorescence microscope (Olympus IX71, Olympus Corp., Tokyo, Japan).

**Wound healing test**
Prior to the inoculation on the plate, three or four parallel lines were drawn on the bottom of the 24-well plate. NPC cells (CNE2 and CNE2R) in logarithmic phase were digested and inoculated into the 24-well plate; we attempted to ensure that the cell quantity in each well satisfied the condition that cells could spread over the bottom of the plate on the second day. On the surface of the plate, a 10 μl pipette was used to draw a line perpendicular to the lines that were drawn prior to the inoculation, and the residues were rinsed using PBS. Then, the plate was placed under the inverted microscope for photographing at 0 and 24 h to observe the cell migration.

**Transwell invasion test**
ECM matrigel at a density of 50 mg/l, diluted using serum-free medium (1:8), was evenly spread in the upper chamber of the Transwell and dried at 4°C. Residual liquid in the upper chamber was then discarded; 50 μl of RPMI-1640 was added for hydration and it was then placed in a thermostat incubator for 30 min; then 600 μl of complete medium was added to the lower chamber. Thereafter, 100–150 μl of cell suspension was added to the upper chamber of the Transwell before incubation for 24 h, followed by washes in PBS. The cell suspension was then fixed in 95% ethanol for 15 min at room temperature, followed by staining with 0.1% crystal violet. After 30 min, the Transwell plate was placed under the inverted microscope to observe the bottom of each chamber, from which four fields of view were selected randomly for cell counting.

**Colonies formation assay**
CNE2R cells were exposed to different doses of radiation (2, 4, 6 and 8 Gy), and then cultured for 14 days at 37°C. The colonies were fixed and stained with 0.1% Giemsa. The numbers of single colonies containing >50 cells were scored as survivors. Grapad Prism 5.0 software was used to create a fit curve. Plating efficiency (PE) = Number of colonies formed/Number of cells seeded; Surviving fraction (SF) = Number of colonies formed after irradiation/Number of cells seeded × PE; Mean lethal dose (D0) = 1/slope (K) [26].

**Statistical methods**
All the data was presented as mean ± standard deviation (SD). The comparisons among multiple groups were analyzed with one-way ANOVA followed by Tukey’s post-hoc test using SPSS 22.0 (SPSS Inc., Chicago, IL, USA). The comparisons between two groups were analyzed by Student’s t test. The surviving fractions were analyzed by the multi-target single-hit model in GraphPad Prism Version 5.0 (GraphPad Software, San Diego, CA, USA). A value of $P < 0.05$ was taken to indicate a significant difference.

**RESULTS**

**Comparison of SERPINB2 expression in CNE2R and CNE2 cells**
The mRNA and protein expressions of SERPINB2 in CNE2R and CNE2 cells were measured by qRT-PCR and western blot assays, respectively. We found that SERPINB2 was significantly lower in CNE2R cells than in CNE2 cells (all $P < 0.05$, Fig. 1A), and the protein expression of SERPINB2 presented a similar expression
SERPINB2 overexpression inhibited CNE2 and CNE2R cell proliferation, and led to an increased fraction in the G2/M phase

As shown by MTT assay in Fig. 2, the OD values of the three groups showed no statistically significant difference at 0 or 12 h (all \( P > 0.05 \)), but the OD values in the SERPINB2 group were significantly lower than those in the blank group and the vector group (all \( P < 0.05 \)). As shown in Fig. 3, we carried out flow cytometry to determine the effect of SERPINB2 on the distribution of cells in the cell cycle in NPC cells, and found that the fraction of cells in the S phase in the SERPINB2 group were lower, while the fraction in the G2/M phase was higher than in the blank group and the vector group (all \( P < 0.05 \)).

SERPINB2 overexpression suppressed the EMT of CNE2 and CNE2R cells

E-cadherin (the epithelial marker) was downregulated, but there was significantly increased expression of vimentin and N-cadherin (the mesenchymal markers) in CNE2R cells, as compared with CNE2 cells (data not shown). The results of the western blot assay are shown in Fig. 4A–D. SERPINB2 overexpression led to an increase in SERPINB2 and E-cadherin, but a decline in vimentin, N-cadherin and nuclear \( \beta \)-catenin in CNE2 and CNE2R cells, as compared with those cells in the blank group and the vector group (all \( P < 0.05 \)). In addition, we found that the nuclear translocation of \( \beta \)-catenin was prevented in the CNE2 and CNE2R cells overexpressing SERPINB2 (Fig. 4E).

SERPINB2 overexpression reduced the invasion and migration of CNE2 and CNE2R cells

According to the wound healing and Transwell invasion assays, the migration and invasion rates of CNE2R cells were elevated significantly when compared with those of CNE2 cells (data not shown). Moreover, SERPINB2 overexpression resulted in a significant decrease in the migration and invasion capability of CNE2 and CNE2R cells in comparison with the cells in the blank group and the vector group (all \( P < 0.05 \), Figs 5 and 6). In addition, the western blot assay was performed to measure the expressions of metastasis-related proteins (MMP-2 and MMP-9), and CNE2 and CNE2R cells in the SERPINB2 group showed significant downregulation of MMP-2 and MMP-9 when compared with cells in the blank group and the vector group (all \( P < 0.05 \), Fig. 7).

SERPINB2 overexpression enhanced the radiosensitivity of CNE2R cells

Transfection with the pEGFP-N1-SERPINB2 plasmid reduced the growth rate of the CNE2R cells following X-ray irradiation at doses

Fig. 1. The mRNA and protein expressions of SERPINB2 in CNE2R and CNE2 cells as determined by qRT-PCR (A) and western blot (B–C), respectively. * \( P < 0.05 \) vs CNE2 cells.

Fig. 2. SERPINB2 overexpression inhibited the proliferation of CNE2 and CNE2R cells as shown by MTT assay. * \( P < 0.05 \) vs the blank group or the vector group.
of 2, 4 and 6 Gy (all \( p < 0.05 \), Fig. 8A). In addition, as detected by colony formation assay (Fig. 8B), SERPINB2 overexpression \( (k = 0.951, D_0 = 1.052) \) enhanced the radiosensitivity of CNE2R cells, resulting in decreased surviving fractions compared with cells in the blank group \( (k = 0.446, D_0 = 2.250) \) and those in the vector group \( (k = 0.477, D_0 = 2.09) \).

**DISCUSSION**

With the developments in radiotherapy and chemotherapy, there has been a significant increase in the local control rate of NPC in recent years [27], but some patients continue to suffer recurrence or metastasis even after radiotherapy, and radioresistance might be a potential reason for failure in NPC treatment [28].

EMT has been implicated in multiple malignant biological behaviors in tumor cells, with the loss of polarity of epithelial cells and acquisition of a mesenchymal phenotype being the major features. At the same time, EMT is also characterized by the downregulation or deletion of E-cadherin (the epithelial marker) and the upregulation of vimentin and N-cadherin (the mesenchymal markers) [29]. Currently, it has been well documented that EMT is involved in the development of tolerance of tumor cells to treatment, including radioresistance, chemoresistance, and drug resistance to the molecularly targeted drugs [30, 31]. In this study, we found CNE2R cells exhibited downregulated E-cadherin and upregulated vimentin and N-cadherin when compared with CNE2 cells, which is in agreement with previous results [21], suggesting the alteration of EMT during radiotherapy, and verifying the involvement of EMT in the development of acquired radioresistance in NPC from another aspect. After EMT, the migration and invasion of NPC cells is usually induced due to the weakened or lost intercellular adhesion [32]. Thus, further experiments were conducted in this study, and the results showed obvious increases in the migration and invasion rate of CNE2R cells, with significant elevations of MMP-2 and MMP-9. As we know, MMP-2 and MMP-9 are secreted in form of zymogens, and once activated, they are transformed into collagenase IV, which can degrade or destroy Type IV or V collagen or gelatin in the extracellular matrix spread on the surface of tumor cells, thereby enabling the tumor cells to infiltrate along the damaged basal membrane to the surrounding tissues, and giving rise to the invasion and metastasis of tumors [33, 34]. These results indicate that radiation was able to induce EMT to participate in the development of acquired radiotherapy resistance in NPC cells.

In addition, the mRNA and protein expressions of SERPINB2 in CNE2R cells in our study were determined to be significantly lower than those in CNE2 cells, which was consistent with the findings of Li XH et al. [22]. Mounting data has shown that radiotherapy can induce the release of inflammatory cytokines and oxidative stress markers, and these mediators can lead to the significant upregulation of SERPINB2 in multiple cells [35, 36]. More importantly, evidence has confirmed a high correlation between SERPINB2 and EMT alteration in cells; for example, the absence of SERPINB2 enhanced cell migration via regulating EMT in tumor cells, as indicated by Longhin E et al. [37]. Thus, we supposed that SERPINB2 may play a critical role in the development of radiation-induced EMT in NPC. To further characterize the role of SERPINB2 in radiation-induced EMT in NPC, we carried out the transfection in CNE2R cells using the plasmids to induce the overexpression of SERPINB2, and subsequently, E-cadherin in CNE2R cells was upregulated, while vimentin and N-cadherin were downregulated, with a significant reduction in the nuclear translocation of β-catenin after SERPINB2 overexpression. In general, E-cadherin was able to bind to β-catenin to form the compound on the membrane of epithelial cells [38], suggesting the possibility that SERPINB2 increases radioresistance in NPC, possibly through inhibition of the nuclear translocation of β-catenin.

Then, we also discovered that SERPINB2 overexpression could inhibit the migration and invasion abilities of CNE2R cells. Mechanically, SERPINB2 is a specific inhibitor of urokinase-type
plasminogen activator (uPA), while uPA, as a kind of serine protease, can activate the transformation of plasminogen into the plasmin, thus degrading the extracellular matrix and triggering the cell migration [39]. In addition, it can also activate the MMP system to increase the activity of vascular endothelial growth factor and angiogenesis [40]. Furthermore, multiple domestic and international studies have demonstrated a close correlation between low expression of SERPINB2 and the metastasis and poor prognosis of tumors, like pancreatic cancer [18], gastroesophageal cancer [41], and breast cancer [42]. During radiotherapy, tumor cells may experience alteration in the distribution of cells in the cell cycle. In particular, cells in the G2/M phase have been found to have the highest sensitivity.

Fig. 4. SERPINB2 overexpression suppressed the EMT of CNE2 and CNE2R cells. (A–D) The expressions of SERPINB2, E-cadherin, vimentin, N-cadherin and nuclear β-catenin in CNE2 cells (A–B) and CNE2R cells (C–D) from three groups, as shown by western blot assay; *P < 0.05 vs the blank group or the vector group. (E) Subcellular localization of β-catenin in CNE2 and CNE2R cells (magnification: ×1000), as shown by immunofluorescent assay.
Fig. 5. SERPINB2 overexpression reduced the migration of CNE2 cells (A–B) and CNE2R cells (C–D), as shown by the wound healing assay. *P < 0.05 vs the blank group or the vector group.

Fig 6. SERPINB2 overexpression inhibited the invasion of CNE2 cells (A–B) and CNE2R cells (C–D), as shown by the Transwell invasion assay. *P < 0.05 vs the blank group or the vector group.
Indeed, SERPINB2 was able to increase the proportion of G2/M phase in CNE2R cells, as shown by our flow cytometry results. SERPINB2 is mainly distributed in the cells in the non-glycosylated form, and, through interaction with RB1 in the cytoplasm and the nucleus, the PENF domain in the CD-loop can bind to the domain of the C-terminal of RB1 to protect RB1 from hydrolysis by calpain, thus increasing the intracellular level of RB1. Non-phosphorylated RB1 can further bind to E2F1, and the resultant loss of transcription activity can further affect the cell cycle and inhibit cell proliferation. In this study, we found that with the transfection of SERPINB2-overexpression plasmids, the proliferation of CNE2R cells was obviously inhibited, suggesting that SERPINB2 can influence cell proliferation through regulation of the cell cycle distribution, thereby increasing the sensitivity of NPC cells to radiotherapy.

In conclusion, lower mRNA and protein expression of SERPINB2 were observed in CNE2R cells than in CNE2 cells, and SERPINB2 overexpression can inhibit the proliferation, invasion and migration of CNE2R and CNE2 cells, thus triggering cell cycle arrest in the G2/M phase via inhibition of EMT, as well as increasing radiosensitivity in NPC cells.

ACKNOWLEDGEMENTS
This work was supported by a Major Research Project of Shandong Province (2015GSF12102S). We would like to give our sincere appreciation to the reviewers for their helpful comments on this article.

CONFLICT OF INTEREST
No potential conflicts of interest were disclosed.
FUNDING

None.

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