Survivin silencing enhances radiosensitivity in oral squamous cell carcinoma cell

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Abstract. – OBJECTIVE: Survivin is a member of the inhibitor of apoptosis protein (IAP) family. It is overexpressed in most cancer tissues and induces resistance to radiation therapy. In this study, we investigated whether knockdown of survivin by siRNA could induce apoptosis and enhance radiosensitivity in oral squamous cell carcinoma cells (OSCC).

MATERIALS AND METHODS: Oral squamous cell carcinoma cell lines KB was subjected to radiotherapy, small interfering RNA (siRNA) targeting survivin was transfected into KB cells in vitro, then subjected to radiotherapy. After irradiation or/and siRNA transfection, viable and dead cells were counted to determine radiation sensitivity by MTT assay, proliferation by colony-forming ability and apoptosis was analyzed by flow cytometry. Tumor-bearing mice were irradiated with 6 Gy of 60 Co γ radiator.

RESULTS: The results showed knockdown of survivin in KB cells showed reduced cell proliferation and increased number of radiation-induced apoptosis. Apoptosis was increased by survivin silencing alone and increased further in combination with irradiation. Colony formation was significantly reduced by survivin silencing in combination with irradiation.

CONCLUSIONS: Survivin silencing sensitizes KB cells toward irradiation. Survivin silencing in combination with radiation inhibits cell proliferation and colony formation significantly and increases apoptosis more than each single treatment alone. In addition, survivin silencing significantly enhanced inhibition of tumor growth and potentiated cell apoptosis by irradiation in KB xenografts. In conclusion, survivin silencing could enhance sensitivity of human KB cells to radiotherapy in vitro and in vivo.

Key Words:
Oral squamous cell carcinoma cells, Survivin, Small interfering RNA, Radiosensitivity.

Introduction

Radiation therapy has played an important role in controlling tumor growth in many patients with cancer. In patients with oral squamous cell carcinoma (OSCC), radiation therapy is currently the standard adjuvant treatment. However, radiation therapy is sometimes ineffective as cancer cells can be resistant to radiation. Hence for successful radiotherapy, it is crucial to understand the mechanisms involved in the development of radiation resistance in tumor cells.

Survivin (BIRC5), is a member of the family of inhibitors of apoptosis proteins (IAPs) of which eight members are known, including X-linked inhibitor of apoptosis (XIAP), cIAP1, cIAP2, NAIP (NLR family, apoptosis inhibitory protein), livin, ILP2 (IAP-like protein-2), BRUCE and survivin. Survivin, the smallest family member, is a 142-amino acid, 16.5 kDa protein encoded by a single gene located on the human 17q25 chromosome, consisting of three introns, and four exons and exists physiologically as a functional homodimer.

Survivin is upregulated in almost all human tumors. Biologically, survivin has been shown to inhibit apoptosis, and enhance proliferation. Over the last years, research studies have shown that the role of survivin in cancer pathogenesis is not limited to apoptosis inhibition but also involves the regulation of the mitotic spindle checkpoint and the promotion of angiogenesis and chemoresistance.
Survivin is a radiation-inducible protein in Ewing’s sarcoma and its down-regulation sensitizes cells toward irradiation. Survivin knockdown in combination with radiation inhibits cell proliferation, repair, and colony formation significantly and increases apoptosis. In RCC patients, the status of survivin protein expression may be an independent factor for predicting the prognosis and survivin knockdown significantly increases radiation sensitivity.

Survivin is overexpressed in oral SCC, and patients with low survivin expression had statistically significant better survival rates than the group with high survivin expression, and survivin plays an important role during oral carcinogenesis. It has recently found knockdown of survivin by shRNA or siRNA induced apoptosis and enhanced chemosensitivity to cisplatin or 5-FU in tongue squamous cell carcinoma cell lines. Roberg et al. has reported, survivin was overexpressed in irradiation resistant oral squamous cell carcinoma cell lines. Whether knockdown of survivin could increase the radiosensitivity in these cells is uncertain.

The present study was undertaken to evaluate whether radiosensitivity of oral cancer cells is determined by cellular survivin by using siRNA targeted against survivin gene.

Materials and Methods

Cell Lines and Culture Conditions

The human OSCC-derived cell line KB (ATCC, Shanghai, China) were prepared for this study. The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% heat-inactivated foetal bovine serum and 50 U/ml penicillin and streptomycin. All cultures were grown at 37°C under a humidified atmosphere of 5% CO₂ for routine growth.

siRNA Transfection

Survivin silencing was achieved using survivin specific siRNA (sc-29499) along with a control siRNA (sc-44233) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The siRNA duplexes were transfected using Lipofectamine-2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The medium was changed after 16 hrs of transfection and 24 hrs post-transfection the cells were assessed for knockdown by RT-PCR. The specific silencing of surviving was confirmed in three independent experiments. For optimal siRNA transfection efficiency, Santa Cruz Biotechnology’s siRNA Transfection Reagent: sc-29528 (0.3 ml), siRNA Transfection Medium: sc-36868 (20 ml) and siRNA Dilution Buffer: sc-29527 (1.5 ml) are used. Control siRNAs is available as 10 µM in 60 µl.

G418 (400 ng/ml) (Life Technologies, Carlsbad, CA, USA) was added to the medium after 48h of culture, and the cells were cultured for two weeks to permit selection.

RT-PCR

Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. For RT-PCR, the following DNA primers were synthesized by Invitrogen: Human survivin, sense primer 5’-AAATGACCTTGGCTCGATGCT-3’ and anti-sense primer 5’-TCCATCATCTTACCGAGACT-3’; human glyceraldehyde 3-phosphate dehydrogenase (GAPDH), sense primer 5’-TGGCTTCCTCTGACTTCAAC-3’ and anti-sense primer 5’-GTGGGTCTCCTCTTCTCCT-3’. Human GAPDH was used as an internal control.

Radiation Treatment

The exponentially growing cells (1 × 10⁴ cells) and stably transfected cells (1 × 10⁴ cells) were treated with doses of IR 6 Gy using ⁶⁰Co-γ radiot along with an untreated control. Cells were incubated up to 48 h, harvested and stored in -80°C until use.

Cell-proliferation Assay

Cells were seeded into 24-well plates at a density of 1 × 10⁴ per well and treated with survivin siRNA and/or IR as described above. Mock-transfected cells were treated with phosphate buffered saline (PBS) as vehicle controls but not siRNA. At 48 h, cells were trypsinised and counted using a haemocytometer in triplicate samples.

Clonogenic Assay

Cells were transfected as above with the vehicle and siRNA for 24 h. After transfection, the cells were trypsinised, counted, and the appropriate number of cells were plated in 60 mm dishes and allowed to attach for 24 h. After 24 h, the cells were irradiated 6 Gy and incubated for 14 days to form colonies which were fixed and stained with a mixture of glutaraldehyde (6.0% v/v) and crystal violet (0.5% w/v) and colonies (≥ 50 cells) were counted using a microscope.
The percent plating efficiency and fraction surviving a given radiation dose were calculated based on the survival of non-irradiated cells.

**Apoptosis Detection**

The Annexin V-FITC apoptosis detection kit (Santa Cruz Biotechnology Santa Cruz, CA, USA) was used for the detection of apoptotic cells in the cells, as per the manufacturer’s specifications. Briefly, cells were collected by trypsinization at 48 h post-IR/siRNA treatment. Cells were washed; 2 µg Annexin-V FITC and 10 µl PI were added, incubated in the dark for 15 min and analyzed on a flow cytometer (FACS Caliber, BD, San Jose, CA, USA).

**Western Blot Analysis**

Whole cell lysates were prepared in SDS lysis buffer (2% SDS, 50 mM Tris-HCl pH 6.8, 0.1% BME and 10% glycerol) with protease inhibitors cocktail (Calbiochem, San Diego, CA, USA). Equal amount of protein was loaded and run on SDS-PAGE. The gels were transferred on PVDF membrane (Hybond Amersham, Piskataway, NJ, USA) and probed with the primary antibody followed by secondary antibody conjugated with horseradish peroxidase (HRP) (Amersham). The primary antibodies used were as follows: Survivin (working dilution 1:2000). The signals were detected using ECL plus detection system (Amersham) according to manufacturer’s protocol.

**Animal Experiments**

All experiments were carried out in accordance with China Animal Welfare Legislation and were approved by the Affiliated Hospital of Medical College Committee on Ethics in the Care and Use of Laboratory Animals. KB or KB/siRNA cells (5 x 10⁶) in 100 ml phosphate-buffered saline (PBS) were injected subcutaneously into the right flank of nude mice, respectively. When the tumors grew to approximately 50-100 mm³ (10 days) after cell injection, Tumor-bearing mice were randomized into the following six treatment groups (n =14 animals per group): (1) KB cells; (2) KB/siRNA survivin; (3) KB/siRNA mock; in every group, 7 animals were irradiated with 6 Gy of ⁶⁰Co-γ radiator. On day 26, xenografts from each group were completed isolated and tumor volumes were then examined exactly. The maximum diameters (Dmax) and minimum diameters (Dmin) of xenografts were measured before each treatment and after mice killed, and tumor size was calculated according to the formula: tumor size (mm³) = (Dmax x Dmin²)/2. Inhibition ratio was calculated at day 26 after mice killed by the formula [1-(treated tumor average volume/untreated tumor average volume)] x 100%.

**TUNEL Staining in Mouse Xenograft**

TUNEL staining was performed according to the manufacturer’s protocol. The slides were stained with 3,3-diaminobenzidine reagent and counterstained with hematoxylin.

**Statistical Analysis**

All quantitative data were obtained from three independent experiments and expressed as mean±standard deviation values. The statistical significance of differences was determined by one-way analysis of the variance (ANOVA) using computer SPSS software SPSS 10.0 (SPSS Inc., Chicago, IL, USA). A value of p < 0.05 was considered statistically significant.

**Results**

**Transfection of siRNA on Survivin mRNA and Protein**

To ascertain whether conditions for RNA inhibition were optimal and that transfection efficiency was satisfactory, siRNA-survivin was used as a positive silencing control. In RT-PCR analysis, survivin mRNA expression decreased by 95% with siRNA-survivin 48 h after transfection in KB cells (Figure 1A). In western blot analysis, survivin was detected as a single band. In cells transfected with siRNA-survivin, the band also diminished significantly (p < 0.01, Student’s t-test), confirming high transfection efficiency 48 h after transfection compared with the vehicle and the non-transfected control cells (Figure 1B).

**Effect of Survivin siRNA on KB Cell Growth**

To determine the effect of survivin siRNA on the growth of the KB cell lines, the cell growth experiment was carried out. The growth curves of KB cells showed that treatment with survivin siRNA inhibited cell growth over a period of 5 days, but cell growth was not inhibited by siRNA control (Figure 2A). After 3 days, a great number of survivin siRNA cells floated in the medium, while the siRNA control transfected cells appeared well attached (Figure 2A). The effect of
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**Figure 1.** Effect of siRNA. **A,** Expression of survivin mRNA in non-transfected KB cells (control) and KB cells transfected with siRNA survivin (p < 0.01, Student’s t-test). **B,** Western blot analysis of survivin protein (positive control) in KB. The cells were transfected with 2 ug siRNA survivin and analyzed at 24 and 48 h. Survivin and β-actin bands were scanned and quantified. The values obtained from densitometric analysis of each survivin protein first were normalized to β-actin protein levels and then expressed as the percentage of the values of control. Survivin proteins were significantly inhibited (p < 0.01, Student’s t-test) in cells transfected with siRNA survivin at 48 h. There is no change in survivin in KB cells transfected with control siRNA.

**Figure 2.** Effect of survivin expression on cell growth and clonogenic survival. **A,** KB cells were transfected with 2 ug survivin siRNA or control siRNA. At the indicated time points, the cells were counted using MTT assay in triplicate samples. The results represent the mean ± SD. The asterisks indicate significant differences between siRNA survivin and untreated (*p < 0.05, Student’s t-test). **B,** The effect of survivin siRNA on the clonogenic survival of KB cells, the cells were trypsinized and plated for clonogenic survival assay at 120 h. Colonies were stained with crystal violet and counted after 14 days. Each experiment was repeated at least three times; the error bars represent ± SD. The significance of the difference between siRNA survivin and untreated (*p < 0.05, Student’s t-test).
assays of KB cells demonstrated an increased apoptosis in cells treated with combined survivin siRNA plus IR as compared to IR or siRNA alone (Figure 4C).

**siRNA Survivin Potentiates the Inhibition of Tumor Growth by Irradiation in vivo**

We have shown that siRNA survivin enhances cell growth inhibition by irradiation in KB cells in vitro. To investigate whether siRNA survivin could potentiate the inhibition of tumor growth by irradiation in vivo, tumor-bearing mice were irradiated with 6 Gy of IR. Figure 5A shows that siRNA survivin combined with irradiation caused a significant inhibition of tumor growth compared with siRNA alone in KB xenografts. On day 26, the tumor-inhibition rates of KB-siRNA survivin group, IR group and KB-siRNA survivin+IR group were 21.7, 23 and 62.4%, respectively (p < 0.05). The results reveal that siRNA survivin enhanced the cell growth inhibition by irradiation in KB xenografts.

**siRNA Survivin Enhances the Apoptosis Induction By Irradiation in vivo**

To investigate the effect of siRNA survivin on apoptosis induction by irradiation in KB xenografts, we measured apoptotic cells by TUNEL assay. We found that a much higher apoptosis index was observed in the siRNA survivin +IR group compared with siRNA control group or siRNA survivin group in KB xenografts (Figure 5B).

**Discussion**

Survivin is a bifunctional protein that acts as a suppressor of apoptosis and plays a central role in cell division. The protein is strongly expressed in the most common human neoplasms, has prognostic relevance for some of them and appears to be involved in tumor cell resistance to anticancer agents and ionizing radiation. On the basis of these findings, survivin has been proposed as an attractive target for new anticancer interventions. Several preclinical studies have demonstrated that down-regulation of survivin expression or function, accomplished by means of various strategies, reduced tumor growth potential, increased the apoptotic rate and sensitized tumor cells to chemotherapeutic drugs and radiation in different human tumor models.

**Figure 3.** Effect of survivin siRNA on KB cell apoptosis. KB cells were transfected with 2 ug survivin siRNA or control siRNA for 5 days. Annexin V assays was used for apoptosis detect. The significance of the difference between siRNA survivin and untreated (*p < 0.05, Student’s t-test).
There is evidence that survivin plays an important role in the drug resistant phenotype of human cancer cells. Giodini et al. first reported that infection of HeLa cells with an adenoviral vector expressing survivin suppressed apoptosis induced by taxol. Successively, we found that stable transfection of OAW42 and IGROV-1 human ovarian cancer cell lines with survivin cDNA was
able to protect the cells from the cytotoxic effects induced by taxol and taxotere, with IC50 values for the survivin-transfectant cell populations 4- to 6-fold those of control cells33. Regarding the role of survivin in determining the response of human tumor cells to radiation, Asanuma et al34 first reported that survivin acts as a constitutive radioreistance factor in pancreatic cancer cells. Specifically, in a panel of established cell lines, they found an inverse relationship between survivin

Figure 5. Combined treatment with siRNA survivin and irradiation on tumor cell growth and apoptosis in vivo. A, Tumor-bearing mice were irradiated with 6 Gy of IR. Tumors were assessed for growth by measuring the volume of xenografts. B, Tumor-bearing mice were irradiated with 6 Gy of IR and apoptosis was determined by terminal dUTP nick end labeling (TUNEL) staining. Data are expressed as percentage of apoptosis-positive cells examined with TUNEL. The error bars represent ± SD. *p < 0.05, Student’s t-test.
mRNA expression and in vitro sensitivity to X-irradiation. Moreover, they demonstrated that survivin mRNA expression was increased by sublethal doses of X-irradiation, which would suggest that the protein also acts as an inducible radioresistance factor. Rodel et al\(^5\) also showed an inverse correlation between survivin expression and apoptotic response to irradiation in a panel of colorectal carcinoma cell lines. It is worthy of note that survivin can contribute to radiation resistance also by promoting the survival of tumor vascular endothelial cells.

In the previous report, little has been mentioned about the role of survivin in regard to radioresistance in OSCC. The current study was designed to examine whether survivin is functionally associated with radiosensitivity of OSCC in vitro and in vivo. So far, limited information is available on the role of survivin in radiation response of OSCC cells.

In our study, the downregulation of survivin alone was efficient in induction of apoptosis and inhibits proliferation in KB cells. Interestingly, the combination of survivin siRNA plus IR induced significantly higher apoptosis as compared to siRNA or IR-alone in both oral cell lines. To address the fact that the induction of apoptosis may necessarily lead to long-term response to radiotherapy, we performed the clonogenic assay which demonstrated that combination of IR and survivin downregulation synergistically reduced clonogenic survival as compared to each treatment alone. Our studies demonstrate that survivin downregulation potentially enhanced radiosensitivity of KB cells in vitro.

More recent research shows that survivin expression levels are correlated with sensitivity of cancer cells to radiotherapy and chemotherapy, and survivin inhibitor could enhance the efficacy of conventional cancer treatment such as radiotherapy in vivo. In this investigation, combined treatment with siRNA survivin and irradiation not only enhanced the cell growth inhibition and apoptosis induction, but also increased the tumor-inhibition ratio (%). The present study is the first to confirm that silencing of survivin enhanced sensitivity of human KB cells to radiotherapy in vivo. Therefore, inhibition of survivin protein by survivin-specific siRNA may be a strategy to overcome radioresistance and then improve its therapeutic efficacy for OSCC. Furthermore, the clinical use of survivin siRNA in combination with radiotherapy is unexplored to date and yet important to investigate in human OSCC patients.

**Conclusions**

Survivin expression may be associated with radioresistance in OSCC cells, survivin siRNA may enhance the radiosensitivity of oral cancer cells, and survivin may be an effective radiotherapeutic target of oral cancer and a marker for radiation sensitivity based on in vitro studies.

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**Conflict of Interest**

The Authors declare that there are no conflicts of interest.

**References**


