Radiotherapy and Oncology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Radiotherapy and Oncology

journal homepage: www.thegreenjournal.com

Original article

Growth inhibition and chemo-radiosensitization of head and neck squamous cell carcinoma (HNSCC) by survivin-siRNA lentivirus

Zakir Khan [a,b,*], Abdul Arif Khan [c], Godavarthi B.K.S. Prasad [a], Noor Khan [d], Ram Pramod Tiwari [e], Prakash Singh Bisen [a,*]

[a] School of Studies in Biotechnology, Jiwaji University, Gwalior, India; [b] Departments of Biomedical Sciences and Pathology and Laboratory Medicine, Cedars-Sinai Medical Center, Los Angeles, USA; [c] Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia; [d] Division of Plant-Microbe Interactions, National Botanical Research Institute, Lucknow; and [e] Diagnostic Division, Vanguard Diagnostics, New Delhi, India

ARTICLE INFO

Article history:
Received 21 July 2015
Received in revised form 14 December 2015
Accepted 15 December 2015
Available online xxxx

Keywords:
Survivin
HNSCC
Chemo-radioresistance
Lentiviral therapy
Apoptosis

ABSTRACT

Background: Survivin expression is often associated with aggressive tumor behavior and therapy resistance. In this study, we investigated the effect of survivin knockdown by survivin-siRNA lentiviral vector (Svv-Lent) on the response of HNSCC to chemo-radiotherapy, tumor growth and metastasis.
Methods: Four human HNSCC (OSC19, Cal27, Cal33 and FaDu) and one normal HOK cell lines were included in the study, and survivin knockdown was achieved with Svv-Lent treatment. Cell proliferation and apoptosis were measured by MTT and TUNEL assay, respectively. Transwell assays were performed to measure *in vitro* cell migration and matrigel invasion. Xenograft tumors were developed in nude mice by injecting Cal27 cells subcutaneously and following tail-vein injection of lung and liver metastasis.
Results: Knockdown of survivin significantly suppressed HNSCC cell proliferation and induced apoptosis *in vitro*. Survivin inhibition could also significantly reduce *in vitro* cell migration and matrigel invasion that might be due to inactivation of matrix metalloproteinases. *In vivo* studies showed significant repression of Cal27 xenograft tumor growth and tissue metastasis leading to improvement in mice survival in the Svv-Lent treated group compared to controls. Our data indicated that survivin expression in HNSCC cells contributed to chemo-radioresistance, and its down-regulation increased anti-cancer effects of paclitaxel, cisplatin and radiation.
Conclusions: Our findings suggest that sustained survivin expression facilitates HNSCC tumor growth and confers resistance to chemo-radiotherapy. Svv-Lent therapy may be able to enhance the cytotoxic effect of commonly used anticancer drugs such as cisplatin and paclitaxel, and radiotherapy that could provide a promising strategy for the effective control of resistant head and neck cancer.
 Radiotherapy and Oncology xxx (2015) xxx–xxx

Head and neck squamous cell carcinoma (HNSCC) is a sixth most common form of cancer worldwide with more than 90% of head and neck cancer [1,2]. Comprehensive efforts have been made to understand the effective therapy regimens for HNSCC, but long-term survival of HNSCC patients has not improved since the last couple of decades and in most of the cases relapse is mainly due to therapy resistance leading to tumor recurrence [3,4]. The treatment of HNSCC in advance stages requires a combination of radiation, surgery and chemotherapy [5]. Cisplatin (CCDP, cis-diamminedichloroplatinum II) and paclitaxel are two widely used chemotherapeutic drugs for the treatment of solid tumors, mostly in combination with radiotherapy. However, a major obstacle in the use of these drugs for the treatment of advance or recurrent HNSCC tumors is the development of resistance at clinically relevant doses [4,6,7]. Further improvements in HNSCC treatment will depend on understanding the mechanism of resistance and identifying molecular targets for sensitizing cancer cells for conventional therapies.

Overexpression of tumor markers is often linked with the tumor resistance, recurrence and metastasis. Survivin is a novel anti-apoptotic protein that belongs to inhibitor of apoptosis (IAP) family [8]. Its overexpression is associated with carcinogenesis, cancer progression and drug resistance in several cancers [9–11], which makes it an ideal target for cancer therapeutics. In earlier studies, we have shown that survivin is overexpressed in premalignant and malignant HNSCC tissues [12]. Our results demonstrated that increased expression of survivin plays a critical role in HNSCC cell survival [8–10,13]. Here we used lentiviral vector (Svv-Lent) for survivin knockdown in HNSCC cells and investigated the role of

* Corresponding authors at: School of Studies in Biotechnology, Jiwaji University, Gwalior, India.
E-mail addresses: zakirq.khan@gmail.com, zakir.khan@cshs.org (Z. Khan), psbisen@gmail.com (P.S. Bisen).

http://dx.doi.org/10.1016/j.radonc.2015.12.007
0167-8140/

survivin in chemo-radioresistance. Overall results suggest that survivin knockdown by Svv-Lent greatly enhanced the therapeutic responses of HNSCC to conventional cancer therapies, and suppressed tumor growth and metastasis.

Materials and methods

Cell lines and culture conditions

Four HNSCC (OSC19, Cal27, Cal33 and FaDu) and one normal human oral keratinocyte (HOK) cell line were used in this study. Cell lines were provided by Dr. Jean-Louis Fischel and Dr. Cedric Boura as a kind gift. Methods essentially followed for culture conditions and maintenance of these cell lines are described elsewhere [7].

Svv-Lent and pcDNA3-survivin vectors

In this study, we used survivin-siRNA oligonucleotides (sense, 5′-CUG-GAC-AGA-GAAAGA-GCC-ATT-3′; antisense, 5′-UGG-CUC-UUUCUCUGU-CCA-GTT-3′ [7,13]. In brief, siRNA sequences were subcloned into the lentiviral siRNA delivery vector FG-12; empty vector was used (Cont-Lent) as a control. Lentiviruses were produced by cotransfection of 293T cells with FG-12 along with packaging vectors pMDLg/pRRE, pRSVRev, and pMD.G, as described earlier [14]. The viral supernatant was collected, titrated for p24, and infection efficiency was monitored [10,14]. For survivin-knockdown, cell lines were transduced with Svv-Lent with 30, 50 and 150 ng p24 titer per well of 96-well, 24-well and 6-well plate, respectively. For overexpressing survivin, HOK cell line was transfected with plasmid containing survivin (pcDNA3-survivin) or an empty plasmid (pcDNA3-neo) as described earlier [7].

Chemotherapeutic drugs and radiation treatments

Cell lines were treated with a range of paclitaxel (0.01–5 μM), cisplatin (1–50 μM) and radiation (3 and 6 Gray unit, Gy) for 24 h to examine the cell death by chemo-radiotherapy *in vitro*. In combination treatment, cell lines were first infected with Svv-Lent/Cont-Lent for survivin knockdown for 24 h followed by treatment with either paclitaxel or cisplatin or radiation for another 24 or 48 h. Low concentrations ($\leqslant IC_{20}$) of cisplatin (3 μM), paclitaxel (0.1 μM) and radiation (6 Gy whole body irradiation) were used in combination treatments. Intratumor injections of Svv-Lent or Cont-Lent were given at days 3, 8, and 15 (1 μg p24 dose per injection) in combination with either cisplatin (1 mg/kg cisplatin, one i.p. dose every 3rd day until 21 day) or radiation (6 Gy at day 7 and 15) for *in vivo* experiments. First treatment started at day 3 when the tumor had grown equally in each group, and tumor size was measured every day until day 30.

Western blotting (WB), real-time PCR (qPCR),

(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide), TUNEL (Tdt-mediated dUTP Nick-End Labeling) and clonogenic assay

Experiments were performed as per methods described earlier [7,10,13,15].

In vitro cell migration and invasion assay

Tissue culture transwell insert with 8 μm pore size (BD Falcon) was used [15]. For invasion assay, the upper side of the insert was coated with Matrigel (40 μg matrigel/insert in 50 μl PBS) (BD Biosciences). After 4 h drying at 37 °C, chambers were placed into 24-well dishes containing 750 μL of RPMI medium supplemented with 10% fetal bovine serum (FBS). HNSCC cells (5×10^4) were then added to the upper well of each chamber in 500 μL of serum-free RPMI medium. Similar conditions were used for migration assay, except non-coating inserts were used (no matrigel). FBS in the lower chamber worked as a chemo-attractant. After 24 h, cells on the upper side of the filter were scraped off with a cotton swab, and cells adhering to the underside of the membrane were fixed in 4% formaldehyde, stained with crystal violet and cells were counted in four random fields per experiment with the help of a bright field microscope. The experiments were performed three times employing duplicate samples.

Tumor xenograft mouse model

Tumor cells treated with Svv-Lent/Cont-Lent were inoculated into mice by subcutaneous (s.c.) injection of 0.1 mL aliquots of sterile saline containing 1×10^6 Cal27 cells into the flank of 6 week old athymic nu/nu (nude) mice using 26-gauge needles. The tumor size was measured with calipers and the tumor volume was calculated according to the formula (Length × Width^2)/2 [16]. Mice were left for a long period of time for determining the survival rate. All procedures were approved by the ethics committee constituted for the purpose at the Jiwaji University, Gwalior.

In vivo tail vein metastatic assays

Six-week old female nude mice received injections of Cal27 cell line (3×10^6 cells) with Svv-Lent/Cont-Lent treatment in 0.1 mL of PBS via the tail vein (6 mice for each group). After killing mice at 8 weeks post-injection, lung or liver tumor nodules were counted. Microscopic examination of metastases was performed on the cross-sections of formalin-fixed, paraffin-embedded lung tissues stained with hematoxylin and eosin (H&E).

Statistical analysis

All experiments were performed at least in triplicate and values are presented as means and standard error. Graphpad software (Prism Inc.) was used for preparing graphs. For statistical analysis, a non-parametric *t*-test, Kruskal–Wallis, and a one-way ANOVA were used. A two-way ANOVA was used for group analysis. *p*-Value <0.05 was considered significant $^{*} \leqslant 0.05$, $^{**} \leqslant 0.005$, $^{***} \leqslant 0.0005$.

Results

Survivin is an important factor for HNSCC cell survival

Panel of cell lines used were checked for survivin expression by WB analysis and found that the normal HOK cell line was negative. Lower (OSC19) to higher expression (Cal27 and FaDu) of survivin was detected in HNSCC cell lines (Fig. 1a). Svv-Lent treatment was given to knockdown survivin in order to examine its role in HNSCC cell proliferation. The efficacy and specificity of Svv-Lent derived survivin knockdown was verified by qPCR and WB (Fig. 1b and c). Survivin knockdown induced cell death significantly in HNSCC cell lines, on the contrary, there was no effect of Svv-Lent treatment in the normal HOK cell line (Supplementary Fig. 1a).

Svv-Lent/Cont-Lent treated cells were labeled with propidium iodide to measure DNA content and with TUNEL to measure apoptosis in order to understand the effect of survivin knockdown on cell cycle distribution and apoptosis. As is evident with the results (Supplementary Fig. 1b) that survivin inhibition increased G0/G1 cell population and decreased cells in S/G2/M phages, suggesting a hypodiploid condition in which cells has less chromosome number than multiple of haploid number indicating apoptosis.

Please cite this article in press as: Khan Z et al. Growth inhibition and chemo-radiosensitization of head and neck squamous cell carcinoma (HNSCC) by survivin-siRNA lentivirus. Radiother Oncol (2015), http://dx.doi.org/10.1016/j.radonc.2015.12.007