SPANDIDOS PUBLICATIONS

STAT3 blockade with shRNA enhances radiosensitivity in Hep-2 human laryngeal squamous carcinoma cells

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Abstract. Signal transducer and activator of transcription 3 (STAT3) is an oncogene aberrantly activated in many human tumors. We studied whether radiation combined with STAT3 siRNA enhances radiosensitivity of Hep-2 human laryngeal squamous carcinoma cells (Hep-2 cells). Firstly, STAT3 targeting recombinant plasmid was constructed. Hep-2 cells were transfected with expression vector of STAT3 siRNA using Lipofectamine 2000. Semiquantitive RT-PCR detected effective STAT3 mRNA down-regulation by STAT3 siRNA. Secondly, Hep-2 cells were radiated with different doses of γ-rays after transfection with STAT3 small interference RNA (siRNA). MTT assay showed cell proliferation decreased significantly ($P<0.05$) after STAT3 siRNA transfection combined with radiation. Thirdly, flow cytometry (FCM) demonstrated that cell apoptosis of combined treatment group increased significantly ($P<0.05$) and exhibited time dependency after 6 Gy irradiation ($P<0.05$). Simultaneously, STAT3, p-STAT3, Bcl-2, VEGF, p53 protein levels decreased in Hep-2 cells, with positive correlations between level of p-STAT3 and levels of Bcl-2, VEGF, p53, respectively ($r=0.974$, 0.988, 0.976, all $P<0.01$). Above all, specific siRNA targeting STAT3 gene is able to enhance the radiosensitivity in Hep-2 cells by regulating expression of Bcl-2, VEGF and p53 proteins.

Introduction

Laryngeal carcinoma is a malignant tumor of head and neck cancers that are difficult to cure. Traditionally, patients with laryngeal carcinoma are treated with surgery, radiotherapy alone or adjuvant treatments. Radiation therapy effects are reliable and adverse reactions are clear. But simple radiation therapy only has relatively good efficacy to a section of the radiation-sensitive patients. Meanwhile, the side effects and injury to normal tissue caused by radiotherapy can not be ignored. Therefore, it is urgent to find radiation sensitizers to enhance tumor radiosensitivity and decrease injury in normal tissues. Gene therapy offers hope in this regard, involving regulations of certain target genes such as STAT3.

STAT3 is one member of family of cytoplasmic proteins. In cytoplasm, STAT3 is activated by tyrosine phosphorylation to form dimerization (1), then activated STAT3 translocates to the nucleus to regulate gene expression (2). It has been demonstrated that STAT3 plays a crucial role in a variety of biological processes, including cell motility (3), proliferation (4), differentiation (5,6), cell cycle progression (7,8) and apoptosis (9). In addition to its physiological roles, a number of studies have demonstrated that constitutive activation of STAT3 protein is involved in the genesis and development of primary tumors, including breast (10,11), ovarian (12,13) and prostate cancers (14,15), myelomas (16), leukemias (17), kidney cancers (18) and head and neck cancers (19). Further studies indicate that STAT3 participates in oncogenesis by modulation of p53 expression (20), up-regulation of genes encoding apoptosis inhibitors (Bcl-2, Bcl-xL, survivin, Mcl-1) (21) and induction of angiogenesis by vascular endothelial growth factor (VEGF) (22). It is understood that the therapy of radiation be accompanied by the repair of DNA injury, changes in cell cycle, programmed cell death (PCD) and other mechanisms regulated by oncogenes, all of which eventually affect the radiosensitivity of cells after radiation. Moreover, downstream of the STAT3 pathway has several critical oncogenes related to tumor cell cycle, apoptosis and DNA repair. Accordingly, we speculate STAT3 gene may be involved in cancer resistance to radiotherapy. In fact, one study has indicated that STAT3 (-/-) B-1 cells become susceptible to irradiation (23). The evidence suggests a correlation between STAT3 gene and cellular radiosensitivity. Therefore, it is highly likely that tumor radiosensitivity could be improved by blocking the STAT3 signaling pathway.

Among the different approaches for regulating gene expression in cells, the technique of RNA interference (RNAi) is a powerful method of gene down-regulation. It has been demonstrated that siRNA could effectively inhibit STAT3 gene expression and lead to growth inhibition and induction of apoptosis in Hep-2 cells (24). In our study,

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STAT3 was inhibited by siRNA to investigate whether radiosensitivity could be enhanced in Hep-2 human laryngeal squamous carcinoma cells (Hep-2 cells).

Materials and methods

Construction of STAT3-targeting siRNA expression vector. Aiming at the sequence of the STAT3 gene cDNA (GeneID: 6774: Accession: NM_003150) provided by GeneBank, two chains of oligonucleotides were synthesized (Gene Pharma Co., Ltd, Shanghai, China) as DNA models [the mRNA coding region of 2144-2162 (25), is underlined]. 5'-CAC CGCAGCAGCTGAACAACATGTTCAAGAGACATGTT GTTCAGCTGCTGCTTTTTTG-3' (sense), 5'-GATCCAAA AAAGCAGCAGCTGAACAACATGTCTCTTGAACATGT TGTTCAGCTGCTGC-3' (antisense). Equal amounts of complementation oligonucleotides were mixed and annealed. Then the new oligonucleotides were linked with linearity plasmid vector (pGPU6/GFP/Neo, Gene Pharma Co., Ltd), which can be digested by restriction enzyme *Bam*HI and *Bbs*I. The connection products were transformed into *E. coli* DH5α competent cells, which were inoculated onto LB plate containing kanamycin (50 μg/μl) and cultured at 37˚C overnight. The plasmid was extracted (Wizard® Plus Megapreps DNA Purification System, Promega, USA) and digested by restriction endonucleases *Bam*HI and *Pst*I. Then the fragment of DNA released was identified by sequencing (Biology Technology Co., Ltd. Shanghai Yingjun, China). Recombinant plasmids were named pGPU6/GFP/Neo-shSTAT3 (pshSTAT3), pGPU6/GFP/Neo-Neg (pshNeg), respectively. Plasmid concentration and purity were determined by ultraviolet spectrophotometry and reserved at -20˚C. The negative control sequence: 5'-CACCGTTCTCC GAACGTGTCACGTCAAGAGATTACGTGACACGTTCG GAGAATTTTTTG-3' (sense), 5'-GATCCAAAAAATTCT CCGAACGTGTCACGTAATCTCTTGACGTGACACGTT CGGAGAAC-3' (antisense) and the target sequence (underline shows the target sequence) has no homology with human or mouse.

Cell line and cell culture. Hep-2 cells (National Cancer Institute, USA) were cultured in complete RPMI-1640 medium (Invitrogen Corporation, USA) containing 10% fetal bovine serum (Hangzhou Sijiqing, China), penicillin 100 IU/ml and streptomycin 100 μg/ml under the conditions of 37˚C, 5% CO_2 and 95% humidity in a carbon dioxide incubator. Logarithmic phase cells were used for the experiment. Twenty-four hours before the transfection, cells were cultured in antibiotics-free medium with serum and were digested by 0.25% trypsin into a single cell suspension to make the cell number 1.0×10^4/well (96-well plates) and 5.0×10^5/well (6-well plates). The cells were distributed into three groups: blank control group (blank), negative control plasmid group (pshNeg) and positive plasmid group (pshSTAT3). The plated cells were cultured for 12 h and the cell confluence reached 80-90% in each well to be used for transfection.

Transfection of plasmids and irradiation of cells. According to Lipofectamine™ 2000 (Invitrogen Corporation) manufacturer's instructions, plasmid and liposome were diluted separately by Opti-MEMI (Invitrogen Corporation). The effective ratio of plasmid: liposome was 1:2.5 (μg: μl, 0.2 μg plasmid/well for 96-well plates and 4 μg plasmid/well for 6-well plates). The diluted plasmid and liposome were mixed and incubated at room temperature for 20 min. The original culture medium in each well was replaced with serum and antibiotics-free medium. Then transfection mixture was added to the medium. Six hours later, the mixture was substituted with complete media. Fluorescence microscopy and flow cytometry were used to confirm transfection efficiency after 24 h followed by irradiation at different time intervals. The delivery of irradiation was conducted under ambient conditions at different radiation dosages. All irradiations were performed at ^{60}Co unit (FCC-7000, Shandong Xinhua Medical Instrument Co., Ltd, China) with the SSD (source skin distance) of 75 cm, radiation area 10x10 cm and dose rate 325 cGy/min. After irradiation, the culture media in each well were replaced with fresh medium immediately for subsequent experiments.

Semi-quantitive RT-PCR analysis. Total RNA was extracted from 1×10^6 cells using the TRIzol reagent (Invitrogen) following the manufacturer's instruction at 48 h post-transfection. The concentration and purity of the RNA samples were determined on an ultraviolet spectrophotometer (752Z, Beijing Optical Instrument Factory, China). RT-PCR was performed by two-step method. cDNA was synthesized according to the protocol of AMV First-Strand cDNA synthesis kit (Protocol-BS251, Shanghai Sangon Biological Engineering Technology & Services Co. Ltd, China). The primers of STAT3 gene were forward: 5'-GTCAGATGCCA AATGC-3', reverse: 5'-CCTGGAGGCTTAGTGC-3'. Amplification of human ß-actin served as an internal standard. The primers of ß-actin were forward: 5'-GCATGGGTGCCC CGACGTTG-3', reverse: 5'-GCTCCGGCCAGAGGCCT CAA-3'. The above oligonucleotide primers were designed and synthesized by Sangon (Shanghai, China). PCR reaction was performed by PCR instrument (UNOII, Biometra, Germany). The reaction conditions were: 95˚C for 5 min, then 30 cycles at 95˚C for 30 sec, 55˚C for 45 sec, 72˚C for 60 sec, followed by a final elongation at 72˚C for 10 min. PCR products were separated in a 2% agarose gel, visualized by ethidium bromide staining and quantified with a gel image analyzer (WD-9413A, Beijing Liuyi Instrument Factory, China). STAT3 mRNA expression was analyzed according to the Optical density (OD) ratio of PCR fragments between STAT3 and ß-actin.

MTT assay. To prepare for MTT assay, 20 μl MTT (5 mg/ml, Sigma) was added to each well of 96-well plates and the cells were incubated for 4 h at 37˚C. After incubation, the culture media were disposed and 150 μl DMSO was added to develop coloration. The plates were swirled gently with cover in the dark for 2 h at room temperature. To observe the viability of cells, the absorbance values at 490 nm (A490) of each well was measured using Enzyme-linked Immunosorbent Detector (Model 550, Bio-Rad, USA). Cell survival rates were calculated according to the formula: cell survival rate = experimental group (each dose point group) *A* value/control group (0 Gy group) *A* value x 100%, taking the absorbed dose as X-axis and the survival rate as Y-axis to depict curves.

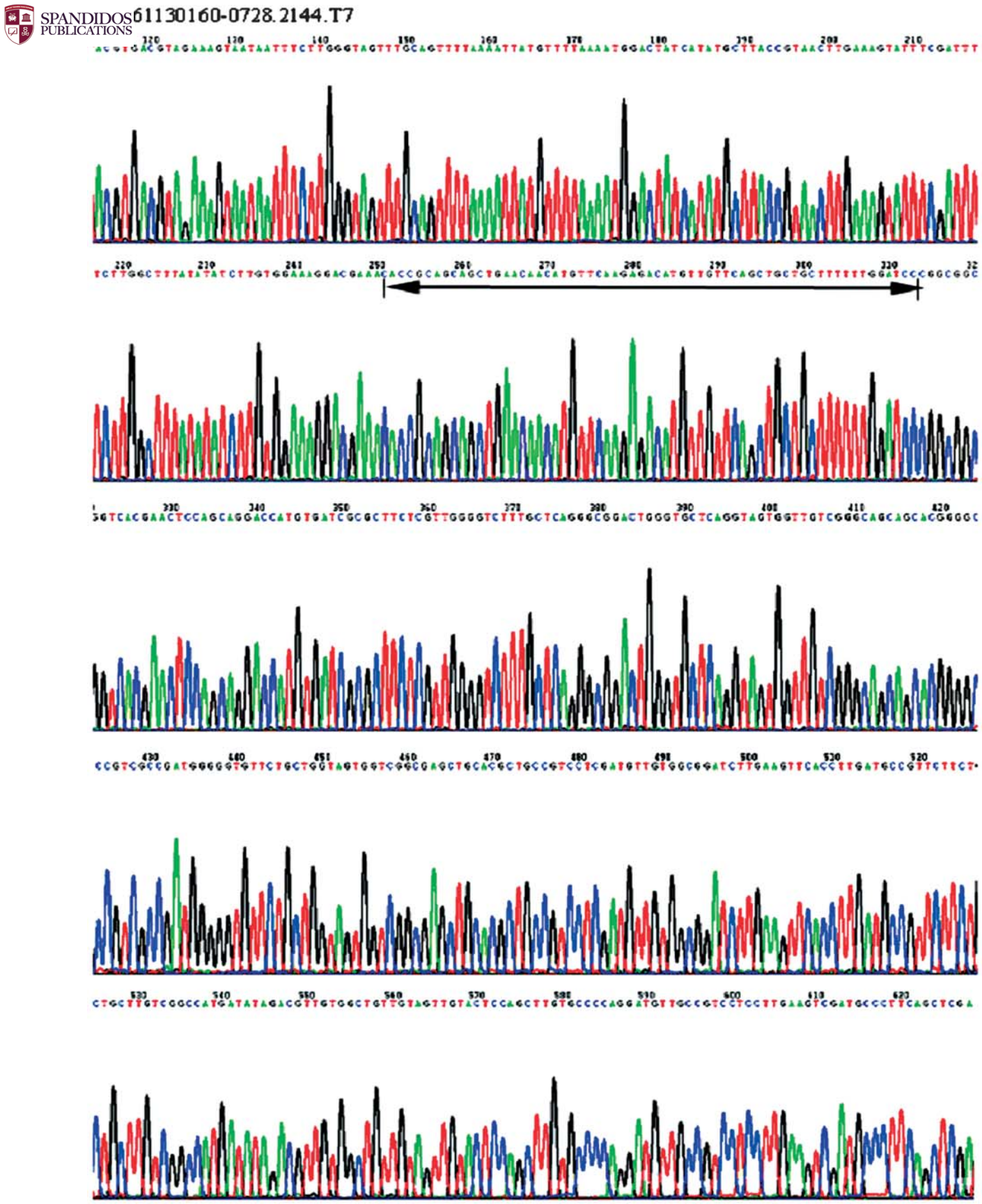

Figure 1. Sequencing of plasmid pGPU6/GFP/Neo-shSTAT3 (pshSTAT3).

Flow cytometry. Cell samples from parallel experiments under the same treatments were taken. A flow cytometer (Epics-XLII type, Beckman Coulter, USA) was used to detect the changes of cell apoptosis and protein expression, using a 150

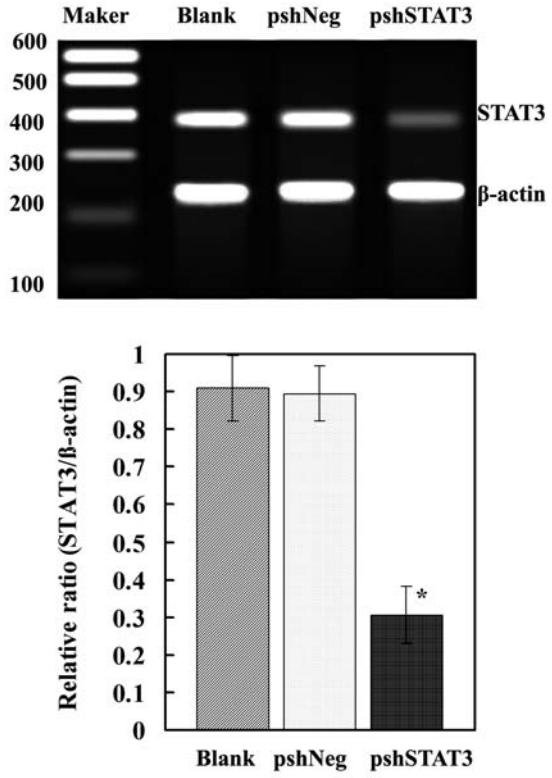

Figure 2. Semi-quantitative RT-PCR analysis of STAT3 mRNA in Hep-2 cells of different groups. Hep-2 cells were transfected with negative plasmid (pshNeg) or the plasmid for the expression of STAT3 siRNA (pshSTAT3) and RT-PCR was performed at 48 h post-transfection. The ratio (STAT3/ß-actin) of PCR products were 0.91±0.09, 0.90±0.07 and 0.31±0.08 in blank control, negative control and pshSTAT3 groups, respectively. The statistical analysis showed that the expression of STAT3 mRNA in pshSTAT3 cells was down-regulated significantly (P<0.05). The transfecion with negative plasmid had no effect on the expression of STAT3 mRNA (P>0.05) (*P<0.05, vs. blank control and negative control groups) (6-well plates, n=6).

mW argon ion laser regulated to provide an excitation wave length at 488 nm. Immunofluorescence data were analyzed by Expo32ADC software. To analyze apoptosis, cells were harvested by trypsinization, washed with PBS, fixed in 75% ethanol and stored at 4˚C. After the removal of ethanol by centrifugation (1,000 rpm, 5 min), cells were stained with a solution (1 ml) containing 50 mg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO, USA), 0.01% Triton X-100 and 0.01 mg/ml sodium citrate at 4˚C for 30 min. To observe the levels of protein expression, the treated cells were harvested, fixed overnight or longer in 70% ethanol and stored at -20˚C prior to analysis by flow cytometry. For each sample, 0.1 ml of each first antibody including STAT3, p-STAT3, Bcl-2, VEGF, p53 (mouse anti-human monoclonal antibody, Santa Cruz Biotechnology, USA) was diluted in 1:100 and added to ~10^5 cells. Following incubation for 30 min at room temperature, the cells were washed once with PBS containing 0.1% Triton X-100, resuspended in 100 μl fluoro-isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:50 dilution, Santa Cruz Biotechnology) and incubated for 30 min at room temperature. The cells were washed in 10 ml PBS and centrifuged. After the removal of supernatants, cells were resuspended in 0.1 ml PBS. Samples were analyzed by flow cytometry. According to Morkve and Laerum (26) and Kelsten *et al* (27), protein fluorescence index (FI) was used to determine the protein relative expression content. The formula is as follows: Protein FI = experimental samples mean channel value divided by normal sample values, mean channel value = l g modex340.

Statistical analysis. Data are expressed as mean ± standard deviation (SD). All statistical analyses were performed by SPSS15.0 software. Factorial analysis was applied to values measured by MTT and apoptosis rate. FI values of STAT3, p-STAT3, Bcl-2 VEGF, p53 proteins were analyzed by one-way ANOVA and Pearson correlation analysis. Paired LSD t-test was used to determine statistical significance of values between different groups and P<0.05 was considered significant.

Results

The construction of STAT3-targeting siRNA recombinant plasmid and its effects on STAT3 mRNA expression. The recombinant plasmid targeting STAT3 could be cut off by *Bam*HI to release a DNA fragment ~5100 bp (data not shown) but not by *Pst*I. And sequencing results showed that shRNA coding sequence was in exact conformity to DNA fragments designed, confirming the correct plasmids (Fig. 1).

Twenty-four hours after transfection, equal amount of green fluorescence could be observed in the cells transfected with recombinant plasmid and negative plasmid under inverse fluorescence microscope, and sustained the same level at 48 h and decreased at 72 h and thereafter (data not shown). Transfection efficiency from flow cytometry was 85.63%, but blank control group showed no green fluorescence. The expression of STAT3 mRNA was significantly down-regulated after transfection with pshSTAT3 (Fig. 2), indicating potent effects of pshSTAT3 in blocking STAT3 mRNA expression. The down-regulation of STAT3 mRNA was also accompanied by simultaneous significant decreased levels of STAT3 protein expression by FI values (data not shown).

Changes of radiosensitivity by cell survival and apoptosis after pshSTAT3 transfection. As shown in Fig. 3, the viability of transfected Hep-2 cells decreased in an irradiation dose-dependent manner. Under the same absorbed dose, growth inhibition showed differences among the three groups. Cell proliferation of pshSTAT3 group decreased significantly (F=138.612, P=0.000, P<0.01) and the increase of growth inhibition became less at higher radiation dose (10 Gy), but was still statistically significant compared with controls. There was little difference in growth inhibition between pshNeg group and blank control group at the same doses of irradiation (P=0.965, P>0.05) (Fig. 3). At 12, 24 and 48 h after 6 Gy irradiation, cell viability changed in time dependency (F=13.938, P<0.05). Viability of Hep-2 cells transfected with pshSTAT3 decreased significantly compared to that of any other group at the same time point (F=257.399, P=0.000, P<0.01) (Fig. 4).

Apoptosis rate of pshSTAT3 group (25.86%) was significantly increased compared to that of the blank control (2.58%) and pshNeg group (3.12%) after transfection and the difference had statistical significance (P<0.05), but not

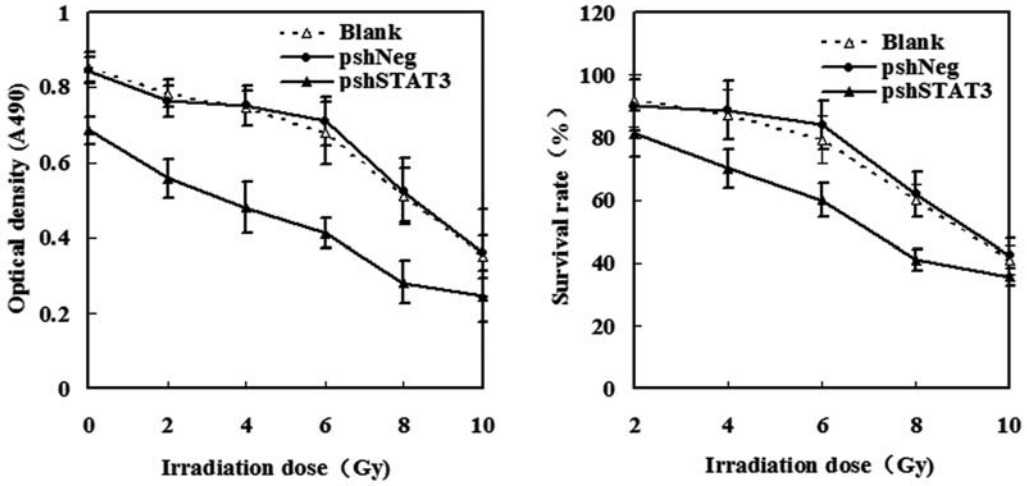

Figure 3. (A) (Left panel) Viabilities of the transfected Hep-2 cells exposed to different doses of irradiation. Hep-2 cells were transfected by pshSTAT3 and pshNeg. Twenty-four hours after transfection, cells were irradiated with single-doses (0, 2, 4, 6, 8 and 10 Gy) of γ-rays. MTT was performed at 48 h after transfection. Absorbance value at A490 was measured to show cell viability (96-well plates, n=6). (B) (Right panel) Survival rate of Hep-2 cells exposed to different doses of irradiation. Twenty-four hours after transfection, cells grown in 96-well plates were irradiated with 0, 2, 4, 6, 8 and 10 Gy of γ-rays under ambient conditions. Twenty-four hours later, cells were harvested, MTT assay performed and cell survival rate calculated (n=6).

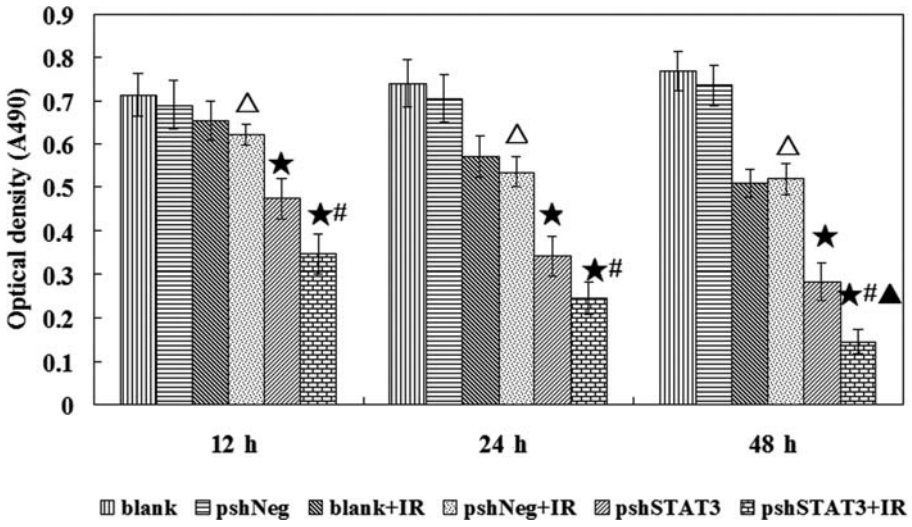

Figure 4. Viabilities of Hep-2 cells at different times after exposure to irradiation. Twenty-four hours after transfection, cells were exposed to 6 Gy γ-rays. Cell viabilities were detected by MTT assay at 12, 24 and 48 h after irradiation (96-wells, IR=irradiation, n=6) (✶ vs. groups untransfected with pshSTAT3, *P<0.05; △ vs. blank+IR, △P>0.05; # vs. pshSTAT3 or blank+IR, #P<0.05; ▲ vs. 12 and 24 h pshSTAT3+IR, ▲P<0.05).

between blank control and pshNeg group (P=0.671, P>0.05). At the same absorbed irradiation doses (2-10 Gy), the apoptosis rate of pshSTAT3 group increased significantly in comparison with that of the other two groups (P<0.01). Moreover, apoptosis rates in each separate group increased in a radiation dose-dependent (2-8 Gy) manner. Although the apoptosis rate still increased at 10 Gy, it showed no significant difference compared to that of 8 Gy (P=0.182, P>0.05) (Fig. 5). Besides, cells apoptosis increased time-dependently after 6 Gy irradiation. The apoptosis rate of Hep-2 cells transfected with pshSTAT3 increased significantly compared to that of any other group at 12, 24 and 48 h after irradiation (F=257.399, P=0.000, P<0.01) (Fig. 6).

Expression of important proteins in STAT3 signaling pathway by FI after pshSTAT3 transfection. In order to observe simultaneous changes of several important proteins in the STAT3 pathway, cells from parallel experiments were subjected to flow cytometry to detect the FI value, a factor indicating protein expression. Forty-eight hours after irradiation, STAT3, p-STAT3, Bcl-2, VEGF and p53 protein expression were measured (Table I). FI values of the pshSTAT3 plus 6 Gy irradiation group were lower than that of the other two groups, the differences were statistically significant (P<0.05). There were positive correlations between p-STAT3 expression and Bcl-2, VEGF and p53 expression (r=0.974, 0.988, 0.976, all P<0.01) by the Pearson correlation test analysis as demonstrated in Fig. 7.

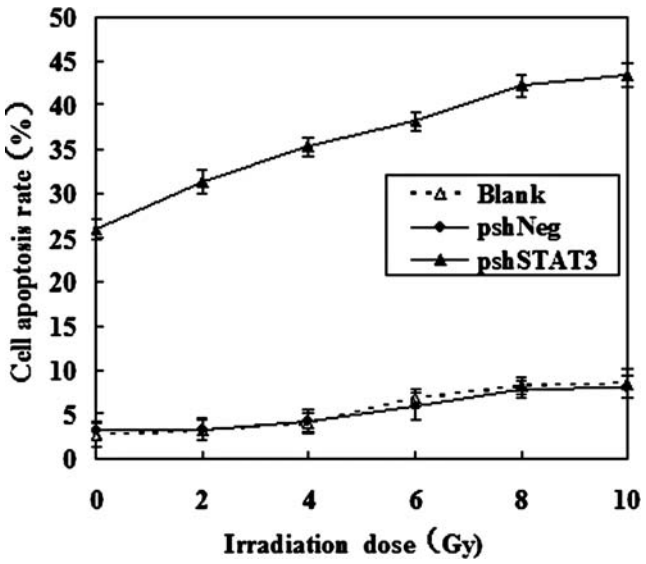

Figure 5. Apoptosis rate of Hep-2 cells exposed to different doses of γ-rays. Twenty-four hours after transfection, cells were exposed to different doses of irradiation. Forty-eight hours post-radiation, cells were analyzed by flow cytometry. (Blank, blank control group cells; pshNeg, cells transfected with negative plasmid; pshSTAT3, cells transfected with STAT3 recombinant plasmid) (n=6).

Discussion

The treatment of head and neck squamous cell carcinomas such as laryngeal cancer generally comprises operation and/or radiotherapy to radicate both primary and metastatic lesions. However, most of the times, limited tumor-controlling efficacy is achieved by radiation alone (28,29) due to tumor inherent radio-resistance. In this sense, developing new strategies to improve the sensitivity of cancers to radiotherapy is urgently needed. In recent years, potential targets for therapeutic intervention in therapy-resistance of head and neck squamous cell carcinoma have been extensively studied, one of which is STAT3.

Generally, activation of STAT3 is a transient process in normal cells, only lasting for several minutes to several hours. However, in abnormal conditions, STAT3 has been shown to be constitutively activated and involve in the pathogenesis of cancers (30). Moreover, STAT3 may mediate chemo-resistance in certain malignant tumors such as multiple myeloma (31) pancreatic cancer (32), and breast cancer (33). It has been reported that STAT3 inhibition with a STAT3 antisense oligonucleotide enhances radiation-induced apoptosis in prostate cancer cells (34). However, little is known about radio-sensitivity in head and neck carcinoma when blocking the STAT3 pathway. Therefore, we designed the experiments to test the hypothesis that blocking STAT3 pathway increases the radiosensitivity in Hep-2 cells. Although several other inhibition strategies including phosphotyrosyl peptides (35), transcription factor decoy (36), short peptides (37), antisense oligonucleotides, dominant-negative mutant (38) have been employed to the direct blocking of targets in STAT3 signaling to investigate human cancers, siRNA (39) has become a powerful tool for this purpose, characterized by its high efficiency, specificity and low toxicity. We successfully constructed STAT3-targeting recombinant plasmid that is very effective in blocking STAT3 mRNA expression. When cells were transected with the pshSTAT3 plasmid, the inhibition of STAT3 mRNA became obvious at 24 h after transfection.

In the subsequent experiments, we demonstrated that sensitivity of Hep-2 cells to irradiation could be affected by inhibiting STAT3 protein expression with siRNA. Recombinant plasmid with short hairpin STAT3 could reduce cell survival and enhance apoptosis with simultaneous down-regulations of STAT3, p-STAT3, Bcl-2,VEGF and p53 protein expression after irradiation. This observation is in

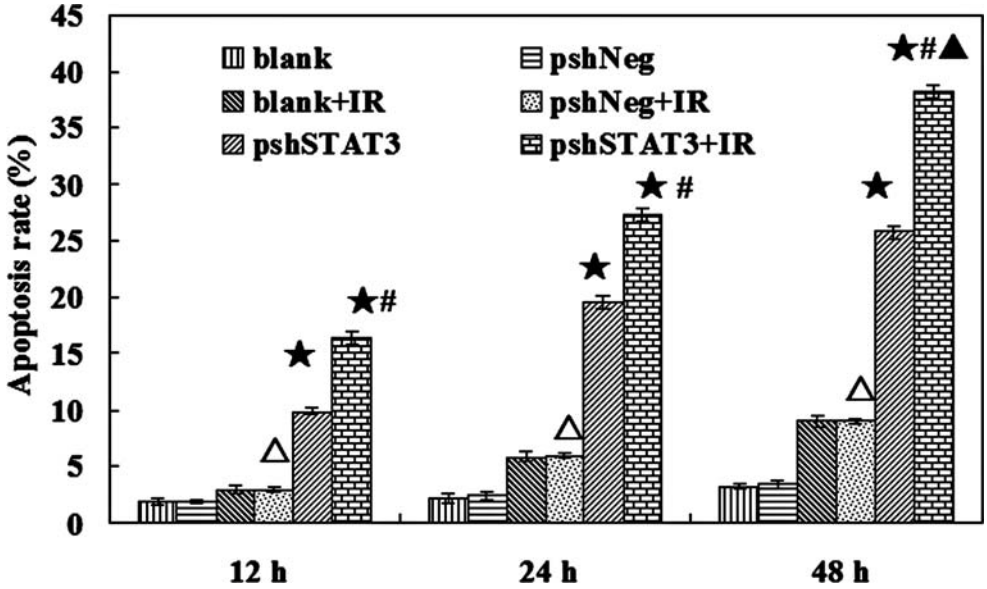

Figure 6. Apoptosis rates of treated cells at 12, 24 and 48 h post-irradiation. (✶ vs. groups untransfected with pshSTAT3, *P<0.05; △vs. blank+IR, △P>0.05; # vs. pshSTAT3 or blank+IR, #P<0.05; ▲ vs. 12 and 24 h pshSTAT3+IR, ▲P<0.05). Twenty-four hours after transfection, cells were exposed to 6 Gy irradiation. Cell apoptosis rate was detected by flow cytometry at 12, 24 and 48 h after irradiation (IR, irradiation, n=6).

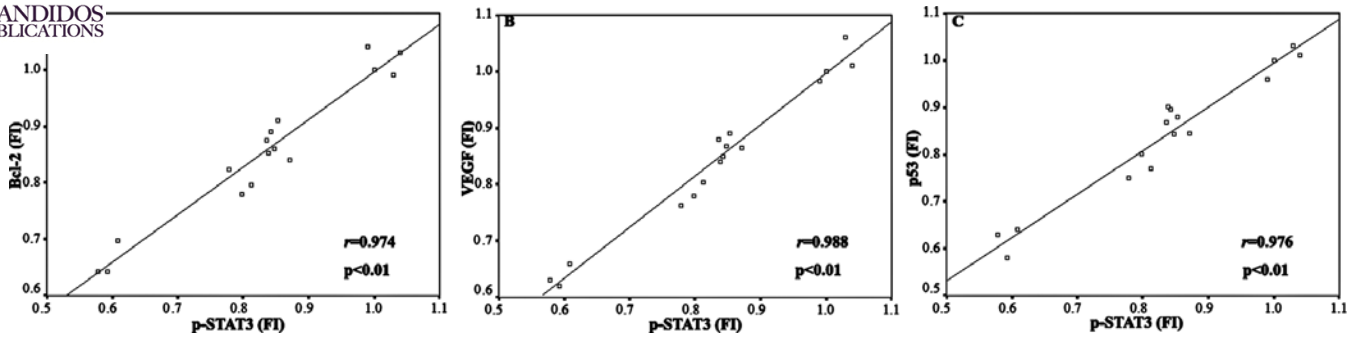

Figure 7. Scatter plotting for the Pearson correlation analyses of protein expression between STAT3 and several independent proteins.

Table I. Fluorescence index (FI) in Hep-2 cells of different groups at 48 h post-irradiation (n=6, $\bar{x} \pm s$).

Group	STAT3	p-STAT3	Bcl-2	VEGF	p53
pshNeg	1.04±0.03	1.02±0.03	1.02±0.03	1.02±0.04	1.00±0.04
IR	0.87±0.02	0.85±0.02	0.87±0.03	0.86±0.02	0.87±0.03
pshNeg+IR	0.86±0.04	0.85±0.01	0.87±0.03	0.87±0.03	0.88±0.03
pshSTAT3	0.80±0.01	0.80±0.01	0.80±0.02	0.78±0.02	0.77±0.03
pshSTAT3+IR	**0.60±0.02**[a-c]	**0.60±0.02**[a-c]	**0.66±0.03**[a-c]	**0.64±0.02**[a-c]	**0.62±0.03**[a-c]

[a]P<0.05 vs. control and pshNeg group, [b]P<0.05 vs. IR and pshNeg+IR group; [c]P<0.05 vs. pshSTAT3 group and FI of each protein control was 1.00. IR, irradiation.

agreement with a previous study (40), in which the inhibition of STAT3 and Survivin activity by dominant-negative mutant affected survival and angiogenesis of the breast cancer cell line MDA-MB-231 following irradiation. It has been reported that following 3 Gy irradiation, the level of phospho-Stat3 Tyr705 was reduced in human umbilical vein endothelial cells (HUVECs), whereas it remained unchanged in irradiated breast cancer cells. By contrast, our results indicate that the expression of phospho-Stat3 Tyr705 decreased after 6 Gy irradiation in Hep-2 cells. We suppose that the difference may be due to the irradiation dose, detection time after irradiation and inherent radio-sensitivity in different tumor cells.

The radio-sensitivity of tumors depends on several factors, including overall clonogenic burden, type of irradiation rays, surrounding and intrinsic radio-sensitivity (41) regardless of oxygenation status, repopulation capacity and DNA repair (42). Apoptosis may also affect the tumor response to radiation by influencing the overall clonogenic burden through promotion of cell loss during treatment (43,44). In tumor cells with apoptotic propensity, cell may be more sensitive to radiation. Our results indicate that the radio-sensitivity of Hep-2 cells was enhanced after exposure to STAT3 siRNA. At the same irradiation dose, the apoptosis rate of combined group was significantly higher than that of radiation or RNAi alone. Apoptosis rate in each group gradually increased in a dose-dependent manner, but there is no statistical significance between 8 and 10 Gy points. Collectively, these data demonstrate that STAT3 siRNA combined with radiation could promote cell apoptosis at low irradiation doses. This finding suggests that decreasing of cell survival may not be entirely attributed to apoptosis at high-dose radiation because of the direct injury to DNA, resulting in increased cell necrosis and apoptosis. In view of the most significant change of cell viability at 6 Gy, we chose 6 Gy as irradiation dose for further investigations. After 6 Gy irradiation, cell growth inhibition and apoptosis in pshSTAT3 group increased in a time-dependent manner, culminating at 48 h after irradiation. This demonstrates that Hep-2 cells could be inhibited more easily by 6 Gy irradiation combined with short hairpin STAT3.

Furthermore, we determined whether other downstream apoptosis-related factors in the STAT3 pathway are affected by siRNA combined with radiation. In our study, p-STAT3, Bcl-2, VEGF and p53 protein expression were down-regulated at 48 h after radiation with simultaneous changes on cell survival and apoptosis. Our observation is partly consistent with the results of two other studies, in which prostate tumor cells were more sensitive to irradiation after treatment with small molecule medicine (45) and antisense oligonucleotide targeting STAT3 (35). Moreover, we found there was a positive correlation between p-STAT3 expression and expression of Bcl-2, VEGF and p53 proteins, which further states that the combination of pshSTAT3 and irradiation induce cell apoptosis

and inhibit proliferation by down-regulating Bcl-2 and mutant p53 protein expression. In addition, p-STAT3 and STAT3 expression in Hep-2 cells reduced after irradiation compared with no radiation group, indicating that radiotherapy may interfere with the upstream STAT3 pathway, for which the specific mechanisms need to be further studied.

In conclusion, our results demonstrate that radiosensitivity of Hep-2 cells can be enhanced through inhibition of STAT3 by plasmid with siRNA targeting STAT3. This effect is probably mediated by down-regulation of downstream signaling proteins including Bcl-2, VEGF and p53. STAT3 is a potential target of radiosensitization for head and neck squamous carcinomas.

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