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Knockdown of Stat3 expression using RNAi inhibits growth of laryngeal tumors *in vivo*

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Key words

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Abstract

Aim: To study the effect of pSilencer1.0-U6-siRNA-stat3 on the growth of human laryngeal tumors in nude mice. **Methods:** Hep2 cells were transplanted into nude mice, then at the time of tumor formation, growth rates were observed. After the tumor formed, pSilencer1.0-U6-siRNA-*stat3* was injected. Tumor volumes were calculated, and growth curves were plotted. Representative histological sections were taken from mice bearing transplantation tumors in both treated and control groups, and *stat3*, *pTyr-stat3*, *Bcl-2*, *cyclin D1*, and *survivin* expression were detected by Western blotting. survivin mRNA levels were detected by Northern blotting, hematoxylin and eosin staining and terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end-labeling (TUNEL) assay to confirm the apoptosis of tumors. **Results:** In nude mice, pSilencer1.0-U6-siRNA-*stat3* significantly suppressed the growth of tumors compared with controls ($P<0.01$). It suppressed stat3 expression, and downregulated *BcL2*, *cyclin D1*, and *survivin* expression within the tumor. This significantly induced apoptosis of the tumors. **Conclusion:** pSilencer1.0-U6-siRNA-*stat3* was able to inhibit the growth of transplanted human laryngeal tumors in nude mice and induce apoptosis.

Introduction

Signal transducers and activators of transcription (STATs) were originally identified as key components of the cytokine signaling pathways that regulate gene expression[1,2]. Recent studies suggest that they have potential as novel molecular targets for control of the development and survival of laryngeal carcinomas. In mammals, there are 7 members in the *stat* family. Constitutive activation of one *stat* family member, *stat3*, has been shown to play a key role in promoting proliferation, differentiation, anti-apoptosis and cell cycle progression. Constitutive activation of *stat*3 occurs in a variety of tumor cell lines[3–7], thus suggesting that *stat3* is an important molecular target for tumor therapy. Constitutive *stat3* signaling represents one of the key molecular events in the multistep process leading to carcinogenesis.

Several recent reports demonstrate that blockade of *stat3* expression in human cancer cells suppresses proliferation *in vitro* and tumorigenicity *in viv*o. Attempts to block *stat3* expression have been made using tyrosine kinase inhibitors[8,9], antisense oligonucleotides[5], decoy oligonucleotides[10], dominant-negative *stat3* protein[11,12] and RNA interference (RNAi)[13,14]. *In vitro* studies have shown that inhibition of *stat3* activity in human tumor cells induced apoptosis and/or growth arrest. In human head and neck squamous carcinoma cells, blocking of *stat3* signaling by decoy oligonucleotides or antisense oligonucleotides abrogates transforming growth factor and suppresses the oncogenic growth of these cells[15,16].

In the RNAi approach, sequence-specific post-transcriptional gene silencing is achieved by small interfering RNA (siRNA): short double-stranded RNA molecules in which the antisense strand is complementary to the target mRNA of a given gene[17,18]. RNAi technology is currently being used not only as a powerful tool for analyzing gene function, but also for developing highly specific therapeutics. Our previ-

ous studies have demonstrated that blockade of *stat3* expression by siRNA in Hep2 human laryngeal tumor cells suppresses proliferation and induces apoptosis *in vitro*[19]. However, it has not been determined whether blocking *stat3* signaling with siRNA is sufficient to inhibit tumor growth *in vivo*.

In the present study, we used a DNA-vector-based *stat3*-specific RNAi approach to block *stat3* signaling and to evaluate the biological consequences of *stat3* downmodulation on tumor growth in a mouse model. The results indicate that blockade of *stat3* expression using a specific RNAi approach can significantly reduce laryngeal tumor growth and induce apoptosis *in vivo*.

Material and methods

Plasmid construction pSilencer1.0-U6 (Ambion, Austin, TX, USA) was used for DNA vector-based siRNA synthesis under the control of the U6 promoter *in vivo*. The vector was constructed by first synthesizing the double-stranded DNA template encoding the siRNA oligonucleotides (GenBank accession number for human *stat3*: NM003150), which contained a sense strand of 19 nucleotides followed by a short space (TTCAAGAGA), then the reverse complement of the sense strand, followed by five thymidines as a RNA polymerase III transcriptional stop signal. The sequences were: forward 5'-GCAGCAGCTGAACAAC ATGTTCAAGAGA-CATGTTGTTCAGCTGCTGCTTTTTT-3' and reverse 5'-AATTAAAAAAGCAGCAGCTGAACAACATGTCTCTTGAA-CATGTTGTTCAGCT GCTGCGGCC-3' (located in the SH2 domain). The oligonucleotides were annealed in a buffer [100 mmol/L potassium acetate, 30 mmol/L *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-KOH (pH 7.4), and magnesium acetate 2 mmol/L] and the mixture was incubated at 90 ºC for 3 min and then at 37 ºC for 1 h. The double-stranded oligonucleotides were cloned into a *ApaI*-*Eco*RI site in the pSilencer 1.0-U6 vector (Ambion), in which short hairpin RNAs (shRNA) were expressed under the control of the U6 promoter. A negative control scrambled siRNA (Ambion), which had no significant homology to mouse or human gene sequences, was designed to detect any nonspecific effects.

Cell culture and establishment of animal model Hep2 cells (2×10^6/150 μL) were inoculated subcutaneously into the right flanks of nude mice, and establishment of palpable tumors was confirmed. The tumor volume ($m_1^2\times m_2\times0.5236$, where m_1 represents the short axis, and m_2 the longer axis) was measured every 2–3 d. When tumors reached an average volume of ~50.69±11.25 mm^3, 3 experimental groups (5 mice per group) were tested: (1) mock transfection (phosphate-buffered saline [PBS] buffer alone); (2) scrambled siRNA control (20 μg/mouse); and (3) pSilencer1.0-U6-STAT3-3 siRNA (20 μg/mouse). The samples were diluted in 50 μL of PBS buffer, and injected percutaneously into the tumor by using a syringe with a 27-gauge needle. Immediately after injection, tumors were pulsed with an electroporation generator (ECM 830, BTX Holliston, MA, USA). Pulses were delivered at a frequency of 1/s 150 V/cm for a duration of 50 ms. This process was repeated on day 20. Mice were killed on d 27, the tumors treated with either scrambled siRNA or STAT3 siRNA were excised for hematoxylin and eosin (HE) staining, and terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) and fluorescence-activated cell sorting (FACS) assays.

HE staining and TUNEL assays Serial sections of tumor tissue excised from animals were fixed in formalin, stained with HE, and processed for routine histological examination. The TUNEL assay was performed by using the *in situ* Cell Death Detection Kit (Roche), which relies on fluorescent labeling of DNA strand breaks. Three-micrometer sections from paraffin-embedded tissues were dewaxed and hydrated according to the standard protocol. After incubation with proteinase K (200 μg/mL) for 30 min at 21 °C, the TUNEL reaction mix containing BrdUTP, terminal deoxynucleotidyl transferase, and reaction buffer was added to the slides, and they were incubated in a humidified chamber for 60 s at 37 °C, followed by washing and incubation with a fluorescein isothiocyanate-labeled anti-BrdU monoclonal antibody for 30 min at room temperature. The reaction was visualized by fluorescence microscopy. TUNEL-positive cells exhibited green fluorescence.

Western blot analysis Anti-stat3, anti-phospho-Tyr705-stat3 (p-stat3), anti-cyclin D1, anti-survivin and anti-β-actin antibodies were obtained from Santa Cruz Biotech. Anti-Bcl-2 antibody was obtained from Dako Biotech. For Western blot analyses, 100 mg tumor tissue as described earlier was lysed with lysis buffer [5 mmol/L ethylenediamine tetraacetic acid (EDTA), 300 mmol/L NaCl, 0.1% Igepal, 0.5 mmol/L NaF, 0.5 mmol/L Na_3VO_4, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 10 μg/mL each of aprotinin, pepstatin, and leupeptin; Sigma]. After centrifugation at 15 000×*g* for 30 min, the supernatant was analyzed for protein content using Bradford reagent (Bio-Rad, USA). For the analysis of *stat3*, 50 μg of total protein was electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, transferred onto a PVDF membrane (Millipore, Bedford, MA, USA), and incubated with anti-STAT3 or anti-p-*stat3* antibody as indicated earlier. For *Bcl-2*, 50 μg

of total protein was resolved on a 12% SDS-PAGE gel, transferred onto PVDF membranes and then probed with anti-Bcl-2 antibody. For cyclin D1, 50 μg of total protein was resolved on a 10% SDS-PAGE gel, transferred onto PVDF membranes and then probed with anti-cyclin D1 antibody. For survivin, 50 μg of total protein was resolved on a 12% SDS-PAGE gel, transferred onto PVDF membranes and then probed with anti-survivin antibody. The immunoblots were visualized by using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, USA). The optical density of each band in these western blots was measured by using densitometry and the results are given as the relative expression of tumors versus normal tissue.

Northern blot analysis Total RNA was extracted from tissue with the Trizol reagent (Invitrogen) following the manufacturer's instructions. For Northern blot analysis, 20 μg of total RNA was electrophoresed on a 1.2% agarose-formaldehyde gel, and blotted onto Hybond-N^+ membranes (Amersham Pharmacia Biotech). Hybridization was performed using the express Hyb buffer (BD Clontech) with ^{32}P-labeled cDNA of *survivin* and *acti*n as probes. Blots were exposed to Kodak MS film and then quantitated using a Molecular Dynamics PhosphorImager.

Statistical analysis A χ^2-analysis was performed to evaluate the significance of differences between the experimental groups. For a single comparison of two groups, Student's *t*-test was used. Two-way ANOVA using the Student-Newman-Keuls method was used for comparisons of tumor size in mice after different treatments. For all analyses, the level of significance was set at $P<0.05$. All statistical calculations were performed using the SigmaStat statistical software package (SPSS, Chicago, IL). Data are presented as the Mean±SD.

Results

Antitumor activity of *stat3* siRNA In order to evaluate the effects of the *stat3* siRNA vector on laryngeal tumor growth *in vivo*, we examined its antitumor efficacy using a nude mouse model. Mice were subcutaneously inoculated with 2×10^6 Hep2 cells into their right flank. By d 13, palpable tumors had developed at the sites of injection (mean volume 50.69±11.25 mm^3, n=5). The mice were divided into 3 groups of 5 mice each and injected intratumorally with TE Tris EDTA buffer, scrambled-siRNA control or pSilencer1.0-U6-STAT3 siRNA. This process was repeated on d 20. Animals were killed on d 27 and tumor sizes were determined. The mean tumor volume in mice treated with buffer alone was 918.12±89.03 mm^3 on d 27. The mean tumor volume in mice treated with scrambled siRNA control was 896.42±92.23 mm^3, and that in the group treated with *stat3*-siRNA was 306.24±28.13 mm^3. The difference in tumor size between the mice treated with buffer and those treated with siRNA did not achieve statistical significance ($P>0.05$), whereas the group treated with *stat3* siRNA showed markedly suppressed tumor growth compared with the controls (Figure 1A, 1B; $P<0.01$). To determine the mechanism of tumor growth inhibition *in vivo*, Hep2 tumors treated with either scrambled siRNA control or *stat3* siRNA were excised for HE staining and analyzed by using TUNEL assay. The results from both experiments showed that *stat3* siRNA-treated tumors had undergone massive apoptosis compared with the controls (Figure 1C–1H). These data suggest that *stat3* siRNA injection into the tumor can exert significant antitumor effects.

Reduction of *stat3* expression siRNA-specific to the *stat3* gene can significantly suppress stat3 protein expression and inhibit the growth of cells *in vitro*[19]. To further study the molecular mechanism of growth arrest of the tumor *in vivo*, stat3 and p-stat3 expression in the tumors were analyzed by Western blotting, and the results indicate that stat3 and p-stat3 expression are markedly reduced in tumors treated with siRNA stat3, whereas scrambled and buffer groups had high levels of stat3 and p-stat3 expression ($P<0.01$; Figure 2A, 2B).

Effects of downregulation of *stat3* expression Recent studies[20–27] indicate that a constitutively active stat3 induces the expression of anti-apoptotic genes such as *Bcl*-2, *cyclin D1*, and *survivin*. In order to determine if *stat3* downregulation results in the suppression of these genes, Western blot and Northern blot analyses were performed on the extracts from tumors transfected with stat3 siRNA. Western blotting showed that the intracellular Bcl-2, cyclin D1, and survivin levels were significantly decreased in *stat3* siRNA-transfected tumors compared with controls (Figure 2C–E). Northern blot analysis showed that intracellular survivin mRNA was significantly decreased in the tumors (Figure 3). Thus, we concluded that *stat*3 siRNA treatment downregulated the expression of Bcl-2, cyclin D1, and survivin.

Discussion

Laryngeal carcinoma, especially late-stage laryngeal carcinoma, is associated with high morbidity and poor long-term survival because of the absence of effective treatment methods. Current therapies for advanced laryngeal cancer are only marginally effective. Thus, better understanding of the molecular mechanisms underlying proliferation, differentiation and survival of laryngeal carcinoma is critical for the development of optimal therapeutic methodologies.

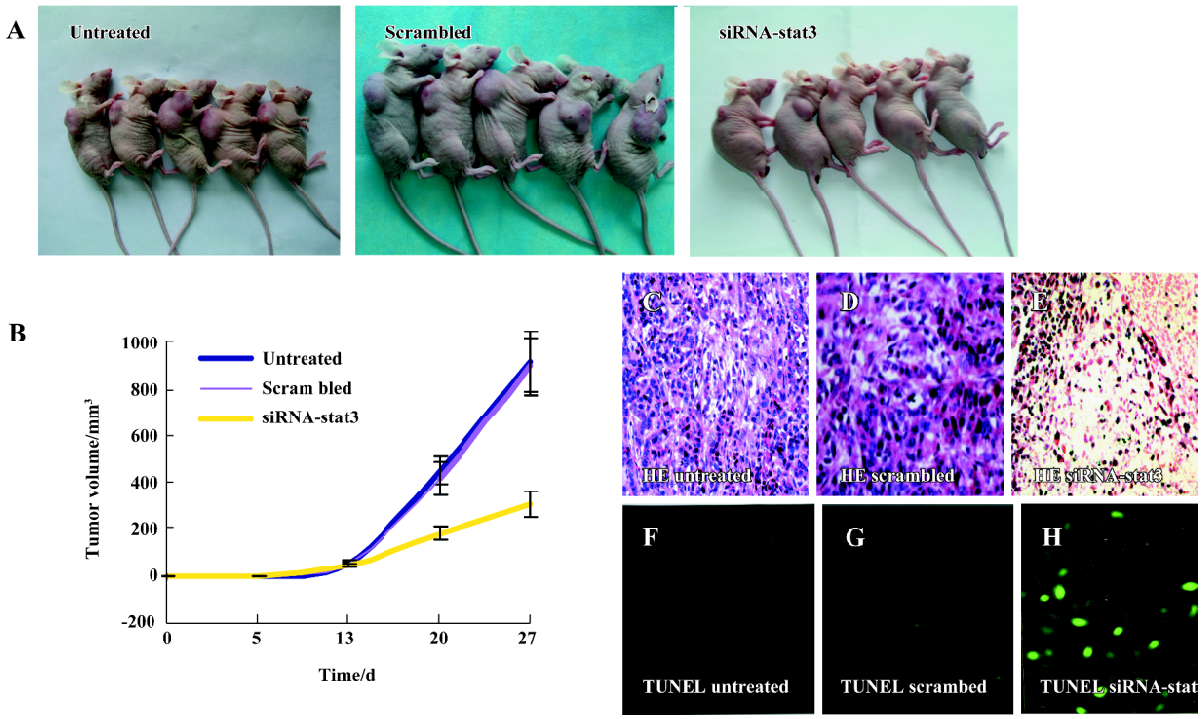

Figure 1. Intratumoral electroinjection of STAT3 siRNA resulted in significant inhibition of tumor growth and induced apoptosis in tumor cells *in vivo*. (A) Mice treated with a scrambled vector control had visible tumors, whereas mice treated with 20 μg of the STAT3 siRNA vector had reduced tumor volumes. (B) Growth curves of Hep2 tumors treated with STAT3 siRNA. Mice were inoculated subcutaneously with Hep2 cells, and by d 13, the mean volume of the palpable tumors reached ~50.69±11.25 mm^3 (n=5) at the sites of injection. At this time, the mice were divided into 3 groups and injected intratumorally with buffer, STAT3 siRNA or a scrambled vector control. This injection was repeated on d 20, and the tumor sizes were determined on days 0, 5, 13, 20, and 27. Mean±SEM. n=5. ^cP<0.01. (C–E) HE staining at 60× magnification, and (F–H) TUNEL assay at 400×magnification of *in vivo* Hep2 tumors treated with buffer (C,F), scrambled siRNA (D,G), or STAT3 siRNA (E,H). TUNEL-positive cells are green.

Elevated *stat3* activities have been detected in the primary tissues and cell lines of laryngeal tumors. *Stat3* activates several genes whose products promote cell cycle progression, for example *cyclin D1*, and *c-Myc*[20–22], and prevent apoptosis, for example *Bcl-2* and *Bcl-XL*[23–27]. Our previous studies showed that *stat3* plays a key role in promoting laryngeal tumor proliferation *in vitro*. In the present study, we further demonstrated that STAT3 played a key role in promoting laryngeal tumor proliferation *in vivo*.

Western blot analysis with anti-STAT3 or anti-phospho-STAT3 antibodies showed that *stat3* siRNAs suppress *stat3* expression in laryngeal tumors *in vivo*. The expression of p-*stat3* in laryngeal tumors treated with *stat3* siRNAs declined approximately 90%, indicating a good silencing efficiency (Figure 2B). More importantly, direct inhibition of *stat3* signaling was accompanied by growth inhibition and induction of apoptosis in laryngeal tumors. We observed that Bcl-2, cyclin D1, and survivin expression were greatly diminished in tumors transfected with *stat3* siRNA (Figures 2B, 2C, 3). Additionally, massive apoptosis of the tumor cells were detected by TUNEL and HE assays. The results of our study are consistent with those of two recent reports, in which *stat3* siRNA was also used for the study of astrocytomas and human prostate cancer[13,28]. The results of all 3 studies support the hypothesis that *stat3* participates in oncogenesis is by inhibiting apoptosis through the induction of anti-apoptotic genes. Konnikova *et al* reported that *stat3* was required for the survival of the anti-apoptotic genes survivin and Bcl-xL (a member of the Bcl-2 family of proteins) in astrocytoma cells[14]. Likewise, Lee *et al* also showed that inhibition of *stat3* gene expression by siRNA induces apoptosis in human prostate cancer[13]. Moreover, emerging evidence suggests that constitutive activation of *stat3* appears to be ubiquitous in tumors, which renders tumor cells resistant to apoptotic death by unbalancing the expression levels of anti-apoptotic and apoptotic genes[13,28].

Chemical synthesis of siRNAs is not cost-effective for large-scale screening projects, and simple synthetic siRNAs

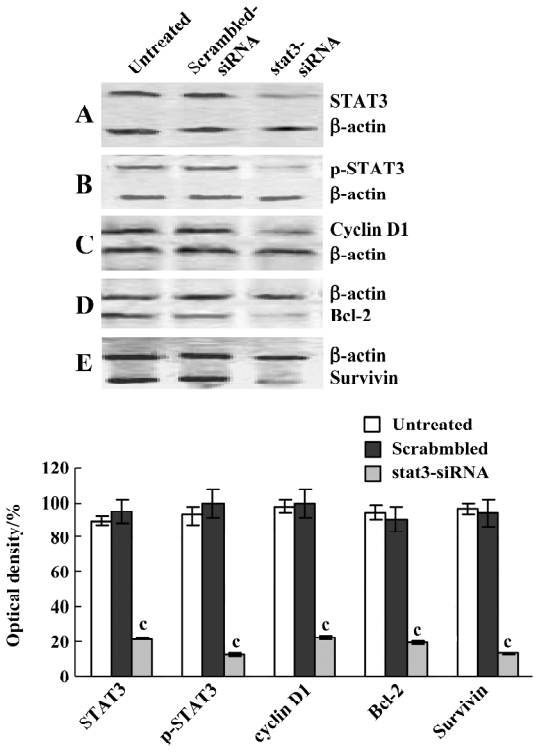

Figure 2. Western blot analyses for STAT3, p-STAT3, Bcl-2, cyclin D1 and survivin expression in Hep2 tumors transfected with STAT3 siRNAs. (A) STAT3 protein level in Hep2 tumors transfected with STAT3 siRNAs and scrambled siRNA revealed by Western blotting. (B) p-STAT3 protein level in Hep2 tumors after transfection with STAT3-siRNAs revealed by western blot analysis. (C) Cyclin D1 protein level in Hep2 tumors after transfection with STAT3-siRNAs revealed by western blot analysis. (D) Bcl-2 protein level in Hep2 tumors after transfection with STAT3-siRNAs revealed by Western blot analysis. (E) Survivin protein level in Hep2 tumors after transfection with STAT3-siRNAs revealed by western blot analysis. (F) Amounts of STAT3, p-STAT3, cyclin D1, Bcl-2 and survivin protein in Hep2 tumors transfected with STAT3 siRNAs, Mean±SEM in 3 separate experiments, $^cP<0.01$ *vs* untreated.

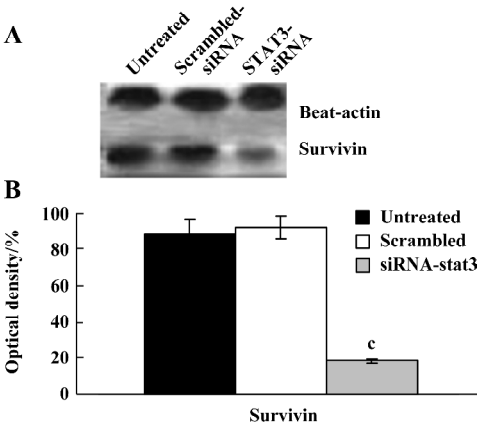

Figure 3. Northern blot analysis of survivin mRNA in Hep2 tumors. (A) Hep2 tumors were transfected with either 20 μg of pSilencer1.0-U6 vector STAT3 siRNAs or scrambled siRNA. Twenty micrograms of total RNA extract was used for Northern blot analysis. (B) STAT3 mRNA expression as mean±SEM of 3 separate experiments, normalized to the expression level of β-actin. $^bP<0.05$ *vs* buffer and scrambled siRNA.

are unstable in mammalian cells, especially for use in *in vivo* studies. Fortunately, this problem has been addressed by using plasmid expression vectors as a delivery tool[11,29,30]. These vector systems produce stable amounts of siRNA by utilizing the cellular machinery[31]. Mammalian expression vectors synthesizing siRNA-like transcripts are able to cause gene knockdown[32]. In order to evaluate the effects of stat3 siRNA on *in vivo* laryngeal tumor growth, we examined the antitumor efficacy of STAT3 siRNA in a nude mouse tumor model. We discovered that inhibition of *stat3* by administration of appropriate vector-based siRNAs into the tumor was an effective and feasible approach for laryngeal cancer therapy. In this study, we used DNA injection as a tool for suppressing *stat3* and tumor growth. Further efforts to evaluate the therapeutic value of this promising approach should be followed.

In conclusion, the data presented here show that the blockade of *stat3* signaling using the RNAi approach significantly suppressed *stat3* expression *in vivo*, suggesting that *stat3* signaling is a potential molecular target for laryngeal cancer therapy. Plasmid-based siRNA therapy for tumor suppression may offer an effective and inexpensive approach for the treatment of laryngeal tumors.

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