Gene therapy in a mouse tumor model of breast cancer by siRNA-mediated down-regulation of STAT3

Research Article

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Keywords: Breast cancer, STAT3, RNA interference, gene therapy

Received: 31 January 2012; Revised: 5 March 2012
Accepted: 7 March 2012; electronically published: 10 March 2012

Summary

Breast carcinoma is one of the most common forms of cancer, with a high prevalence and mortality rate worldwide. Signal transducer and activator of transcription 3 (STAT3) plays a key role in tumor cell survival and proliferation, angiogenesis, apoptosis. It is aberrantly activated in several types of cancers, including breast cancer. We assessed the therapeutic effects using a DNA vector-based STAT3-specific small interfering RNA (pSi-STAT3) on a murine breast cancer model. We observed the tumor growth in every group and further discussed the mechanism underlying. STAT3 was significantly down-regulated at both the mRNA and protein levels in the pSi-STAT3 group. The growth of the tumors was significantly reduced in the pSi-STAT3-treated mice. Flow cytometry revealed that the number of early apoptotic cells was significantly elevated in the pSi-STAT3 group. Moreover, in the pSi-STAT3 group, the mRNA expression of the STAT3 downstream genes Bcl2 and c-Myc was also significantly inhibited, and immunohistochemistry revealed that the expression of STAT3, HIF1 and PCNA protein were reduced in the tumor tissues. Our results suggested that STAT3-specific siRNA significantly suppressed tumor growth in breast cancer-bearing mice. It might be a useful therapeutic strategy in malignancies.
I. Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in females worldwide, with a high prevalence and mortality rate worldwide. About half the breast cancer cases and 60% of the deaths are estimated to occur in economically developing countries. In China, it is ranked first place in the world for mortality rate. It is estimated that 1.38 million females are diagnosed annually with breast carcinoma, with 0.46 million deaths occurring every year (Jemal et al., 2011). The incidence and mortality rate of breast cancer are increasing by approximately 0.2–0.3% each year, and the disease is affecting increasing numbers of younger women. Despite intensive research, the current therapy for breast cancer has a high post-operative recurrence and metastasis rate (Autier et al., 2010; Mettlin, 1999). Further studies on the mechanisms and treatment of breast cancer are required, therefore, new therapies were imperative.

The signal transducer and activator of transcription 3 (STAT3) protein is constitutively activated in many human cancers, where it functions as a critical mediator of oncogenic signaling through transcriptional activation of genes encoding apoptosis inhibitors (e.g., Bcl2, Mcl1, and survivin), cell-cycle regulators (e.g., Cyclin D1 and c-Myc), and inducers of angiogenesis (e.g., vascular endothelial growth factor). Therefore, STAT3 is a research hotspot for cancer gene therapy (Bromberg et al., 1999; Darnell, 1997; Yuan et al., 2005). In this study, we used tumor-bearing mice as a model of breast cancer to assess the anti-tumor effects of a small interfering RNA (siRNA)-STAT3 plasmid, and explored the mechanism. We believe this will provide the basis for a new gene therapy strategy for cancer.

II. Material and Methods

A. Cell lines and plasmids

The 4T1 cell line was purchased from the Institute of Biochemistry and Cell Biology (SIB, CAS, Shanghai, China) and was cultured in Iscove's modified Dulbecco's medium (GIBCO/Invitrogen Corp., Carlsbad, CA, USA) containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO₂. Cells were digested with 0.25% trypsin. C57BL/6 male mice, 10 weeks old, body weight 20–24g, were purchased from the Institute of Zoology, Chinese Academy of Sciences (Beijing, China). All animal experiments were conducted in accordance with the regulations of the Jilin University Experimental Animal Research Committee. The pSi-Scramble and pSi-STAT3 plasmids were donated from the Prostate Disease Research Center of Jilin University (Changchun, Jilin, China).

B. Murine breast tumor model

C57BL/6 mice were inoculated subcutaneously with 10⁶ 4T1 breast cancer cells. When the tumor grew to approximately 1 cm diameter, about three weeks later, the mouse was sacrificed and the tumor was removed. The tumor was dissected from each mouse, placed in cold 0.9% sterile saline, and then cut into blocks with a diameter of 1.2 ± 0.2 mm. The mice were injected intraperitoneally with 0.1 ml of 1% pentobarbital sodium as an anesthetic. A tumor block was then embedded under the skin on each mouse’s back, and the wound sutured with 8-0 non-invasive surgical sutures. When the mice recovered, they were returned to their cages.

C. Therapeutic treatment of breast cancer mice

Ten days after embedding the tumor block, when the tumor reached 100–450 mm³, the 30 mice were randomly assigned into three groups (n = 10 each): the pSi-STAT3 group; the pSi-Scramble group; and the Mock group. The mice in each group were injected with each plasmid (pSi-STAT3, pSi-Scramble), diluted in 50 μl PBS per mouse respectively, while Mock group was injected with 100ul PBS into tumors, respectively, at two tumor locations. Immediately after injection, tumors were pulsed with an electroporation generator (ECM 830, BTX). Mice were treated once a week. Mice were sacrificed on day 40, and tumor sizes were determined. In the Mock group, an equal volume of PBS was injected into the tumor. Tumor size was measured with a caliper every 2 days; tumor volumes were determined using the formula: tumor volume = length × width² × 0.52.

D. Semi-quantitative revers transcription PCR (RT-PCR)

When the tumors in the Mock group grew sufficiently large, all the mice were sacrificed and the tumors dissected out. Total cellular RNA was extracted from implanted tumor tissues using Trizol reagent (Gibco BRL) according to the manufacturer’s instructions. Subsequently, the isolated total messenger RNA (mRNA) was converted into cDNA using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). Primers for RT-PCR were designed using Premier 5.0 software according to the GenBank nucleotide sequences for murine STAT3, Bcl2, c-Myc, and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) (Table 1). RT-PCR was performed by annealing at 55°C, with 30 cycles. Products were run on 1% agarose gels and photographed using a GIS Gelatum imaging system (Tanon, Shanghai, China).

E. Western blotting

Western blotting was performed according to “molecular biology cloning” described methods. Fifty micrograms of protein per lane were resolved by 10% SDS-polyacrylamide gel electrophoresis, and then electro-transferred onto polyvinylidene fluoride membrane. Blots were probed with primary antibodies (1:500) and horseradish peroxidase (HRP)-conjugated secondary antibodies (1:500). The sections were then visualized after 3,3'-diaminobenzidine (DAB) staining, and the optical density of the bands was quantitated using a GIS Gelatum imaging system.
F. Pathological examination

The harvested tumors were fixed in 4% formaldehyde, then stained with hematoxylin and eosin (HE) and labeled with monoclonal antibodies against STAT3, hypoxia inducible factor 1 (HIF1) and proliferating cell nuclear antigen (PCNA). Antibody staining was also performed on 4-μm histological sections of formalin-fixed, paraffin-embedded tumor and adjacent normal samples. Serial 4-μm sections were mounted on pretreated glass slides, deparaffinized, rehydrated, and microwaved for 15 min at high power in 10 mmol/L citrate buffer (pH 6.0) to unmask the epitopes. Endogenous peroxidase was quenched using 3% H2O2 for 10 min; slides were then washed in PBS pH 7.5, and incubated with 5% bovine serum albumin for 20 min. Sections were incubated overnight at 4°C with a 1:100 dilution of primary antibodies. After washing, the sections were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. After washing, tissues were stained for 5 min with DAB and counterstained with HE, dehydrated, and coverslipped. Each experiment was performed in duplicate.

G. Analysis of apoptosis

Tumors were harvested and homogenized to a cell suspension. Cells were collected and washed twice with PBS, then stained with an Annexin V–fluorescein isothiocyanate (FITC) propidium iodide (PI) Detection Kit (Keygene Biology Institute, Nanjing, China), following the manufacturer’s instructions. Annexin V has a high affinity for phosphatidylserine, which is exposed on the cell surface in apoptotic cells. Early apoptotic cells that would bind Annexin V–FITC showed green staining in the plasma membrane, whereas late apoptotic or necrotic cells that have lost membrane integrity show red PI staining throughout the nucleus and a halo of green staining (FITC) on the cell surface.

H. Statistical analyses

Statistical analysis was performed using ANOVA and Student’s t-test (SPSS10.0 statistical software). A p-value < 0.05 was considered statistically significant.

III. Results

A. Treatment with pSi-STAT3 heavily inhibited the expression of STAT3

Compared with the Mock group and pSi-Scramble group, the expression of both the STAT3 gene and protein in the pGC-Si-STAT3 treatment group (p < 0.01) (Fig. 1A–D).

B. Growth of breast cancer tumor xenografts were significantly inhibited by the pSi-STAT3 plasmid treatment

In the pSi-STAT3 group, both tumor weight and volume were significantly lower than the other two control groups (p < 0.01), but there was no statistically significant difference in the average body weight (p > 0.05) (Fig. 2 and Table 2).

C. Treatment with pGC-pSi-STAT3 significantly induced tumor cell apoptosis in breast cancer xenografts

As shown in Fig. 3A and B, Annexin V–FITC staining showed that 39.68% of cells were in early apoptosis after pSi-STAT3 treatment. This was significantly higher than that in the pSi-Scramble group (10.64%) and the Mock group (4.5%) (p < 0.01).

D. Expression of STAT3 downstream genes was decreased by pSi-STAT3 treatment

Both the mRNA and protein expression of the STAT3 downstream targets c-Myc (an oncogene) and Bcl2 (an anti-apoptosis gene) was significantly decreased in the pGC-Si-STAT3 group versus the pSi-Scramble and Mock untreated group (p < 0.05) (Fig. 4A–D).

E. pSi-STAT3-treated cells displayed morphology characteristic of apoptosis

In the Mock untreated group, the tumor cells were visible with H&E staining as large, polygonal, differentiated cells, with deeply stained nuclei and abundant cytoplasm. In contrast, the pSi-STAT3 group contained many tumor cells exhibiting blue-black condensed and/or fragmented nuclei, indicating apoptosis (Fig. 5A).

F. Treatment of pSi-STAT3 decreased the expression of STAT3, HIF1, and PCNA

Immunohistochemistry was performed on the tumor cells to investigate the expression of STAT3, HIF1, and PCNA. The levels of all three proteins were notably decreased in the pSi-STAT3-treated tumor cells compared with the Mock untreated cells and pSi-Scramble group (Fig. 5B–D).
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Figure 1. Treatment of the pSi-STAT3 plasmid inhibited the growth of breast cancer in vivo. A, Relative tumor sizes of breast tumor xenografts removed from C57B/L mice in each group as indicated. B, Statistical analysis of Average Tumor weight in every group.

Figure 2. pSi-STAT3-treated tumor showed low level of the STAT3 expression. A, The level of expression of STAT3 in every group by RT-PCR and Western blot. C, The expression of STAT3 protein level in three group. B, D, The histogram of relative.
Figure 3. The apoptotic effect of using the pSi-STAT3 plasmid. A, Apoptosis analysis using flow cytometry. B, The average percentage of apoptotic cells calculated for data A.

Figure 4. The expression of STAT3 target genes Bcl-2 and c-Myc detection. A,C, RT-PCR and Western blot detection of STAT3 downstream targets c-Myc and Bcl2. B, D, The histogram of relative gene expression for A and C.
Gene therapy for breast cancer by siRNA-STAT3

Figure 5. HE staining and immunohistochemical detect in tumor tissue. A, HE staining (×200) B, C, D, Immunohistochemistry analysis the expression of STAT3, HIF-1 and PCNA in tumor tissue in each group (×200).

<table>
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<tr>
<th>Gene name</th>
<th>Primer</th>
<th>PCR product (bp)</th>
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<tr>
<td>STAT3</td>
<td>Sense</td>
<td>TTGCCAGTTGTGGTGATC</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>AGACCCAGAAGGAGAAGC</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Sense</td>
<td>ACTTGACAGAAGATCATGCC</td>
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<tr>
<td></td>
<td>Antisense</td>
<td>GGTTATCATAACCTGTTCTC</td>
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<tr>
<td>c-Myc</td>
<td>Sense</td>
<td>AGTTGGACAGTGGCAGGG</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>ACAGGATGTAGGCGGTGG</td>
</tr>
<tr>
<td>β-actin</td>
<td>Sense</td>
<td>CTGGGACGACATGGAGAAA</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>AAGGAAGGCTGGGAAGAGTG</td>
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Table 1 The amplification of caspase-3, caspase-12, β-actin gene PCR primers
IV. Discussion

Gene therapy represents a new and promising therapeutic opportunity against breast cancer. Some research achievements have already been translated into clinical practice, with significant therapeutic effects obtained (Geyer et al., 2009; Sims et al., 2006).

STAT3 initiates a signaling cascade comprising EGFR, IL6/JAK, Sac, and other tyrosine kinases, and is persistently over-activated in a variety of cancers, including head-neck squamous cell carcinoma, breast, pancreatic, colorectal, and ovarian cancers, melanoma, leukemia and multiple myeloma (Yu et al., 2007). After excessive activation, STAT3 induces abnormally high expression of genes that play a key role in cell proliferation, differentiation, and apoptosis inhibition, thereby promoting tumor growth and metastasis. The expression level of STAT3 is related to tumor growth rate, metastasis, and prognosis (Bollrath et al., 2009; Porta et al., 2009). Therefore, blocking the STAT3 signal transduction pathway in tumors may be a useful avenue to explore tumor gene therapy (Gao et al., 2005a; Gao et al., 2006; Gao et al., 2005b).

In this study, we used a breast cancer mouse model to study the potential therapeutic effect of the siRNA targeting the STAT3 gene. The siRNA plasmid was locally injected into tumors and electrotansfect into tumor cells. There was no sign of infection or discomfort of mice in any group, and this method is safe, non-toxic, and without side effects. The siRNA correctly and efficiently targeted the STAT3 gene and down-regulated its expression, as shown by semi-quantitative RT-PCR and western blotting.

Treatment of pSi-STAT3 significantly reduced tumor volume compared with mice in the Mock group. Next, we detected the apoptosis rate in every group. Flow cytometry experiment showed that pSi-STAT3 treatment caused more apoptosis cells: there were more early apoptotic cells in the pSi-STAT3 group (39.68%). We conclude that the pSi-STAT3 treatment can inhibit tumor growth by increasing apoptosis.

Moreover, to explore the molecular mechanism of apoptosis and proliferation, we demonstrated that pSi-STAT3 treatment reduced expression of the STAT3 downstream genes (Bcl2 and c-Myc), which are the apoptosis and proliferation factors. PCNA is a 36-kDa nuclear protein whose expression is tightly regulated throughout the cell cycle. It is used as a marker of cellular proliferation because it is an auxiliary protein of DNA polymerase δ and contributes to DNA replication (Cazzalini et al., 2010; Shen et al., 2011). The down-regulation of PCNA we observed in the pSi-STAT3 group indicates cell proliferation inhibition. In summary, down-regulation of STAT3 in a mouse breast cancer model resulted in tumor growth reduced, apoptosis increased and cellular proliferation factors decreased. We suggest that siRNA-mediated silencing of STAT3 is worthy of further investigation and may ultimately be useful for clinical application.

References


Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>B W (g)</th>
<th>Tumor weight (g)</th>
<th>Tumor volume (mm³)</th>
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<tbody>
<tr>
<td>Mock</td>
<td>27.68±4.52</td>
<td>2.42±1.32</td>
<td>2264.09±1745.17</td>
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<tr>
<td>pSi-Scramble</td>
<td>26.52±2.44</td>
<td>2.35±0.44</td>
<td>2151.98±347.01</td>
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<tr>
<td>pSi-STAT3</td>
<td>24.54±2.23</td>
<td>1.13±0.49*</td>
<td>1001.99±370.16*#</td>
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*P < 0.05 vs. Mock group, #P < 0.05 vs. pSi-Scramble group.

Acknowledgments

This work was funded by the National Natural Science Foundation of China (No. 30801354 and 30970791). The Ph.D. Programs Foundation of Ministry of Education of China (grant no. 200801831077) and the Jilin Provincial Science & Technology Department (grant no. 20080154).

The authors declare that they have no competing interests.
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