Blockage of tropomyosin receptor kinase a (TrkA) enhances chemo-sensitivity in breast cancer cells and inhibits metastasis in vivo

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Abstract: Hyper-activation of the Neurotrophin Receptor Signaling contributes to the development and metastasis of breast cancer. The inhibition of growth factor-dependent growth of breast cancer cell demonstrated a promising way for cancer therapy. In this study, the signaling pathway of tropomyosin receptor kinase A (TrkA) had been investigated for the role it played in the proliferation of chemo-resistance of breast cancer cells. Small interference RNA (siRNA) was used to down-regulate the expression of TrkA in breast cancer cell and tumor xenograft mice model. Our results indicated that siRNA mediated down-regulation of TrkA lead to the proliferation inhibition of cancer cells and arrested cells cycle at G₀/G₁ phase via inactivation of NF-κBp65. Application of TrkA siRNA to cancer cell also increased the chemo-sensitivity to paclitaxel, and further promoted apoptosis in cancer cell through the activation of caspase-3. Moreover, TrkA siRNA increased the efficacy of paclitaxel and decreased the incidence of lung metastasis in tumor xenografted mice. In sum, these results indicate that TrkA signaling plays an important role in breast cancer chemo-resistance and metastasis. It could be a potential pharmacologic target to enhance the effectiveness of chemo-therapy for breast cancer.

Keywords: TrkA, siRNA, breast cancer, metastasis

Introduction

Breast cancer is the second leading cause of death in females despite considerable progresses in diagnostic and therapeutic technologies [1]. Development of a universal therapeutic drug for breast cancer is difficult due to the multifactorial natures of breast carcinogenesis as well as the molecular and cellular diversity of tumor cells. Herceptin, a monoclonal antibody against Receptor tyrosine-protein kinase ErbB2 (ERBB2), is a well-known targeting drug had been used for treating certain type of breast cancer [2]. However, over-expression of ERBB2 was only accounted for 20% of total breast cancer patients, which limited its application. So far, chemotherapy remains the primary treatment for breast cancer. However, the toxicity of chemo-drugs and drug resistance are the two obstacles for an effective chemotherapy of breast cancer. Thus, it is required for an effective means to increase the chemo-sensitivity of breast cancer cells.

Neurotrophin Receptors Signaling has been demonstrated to involve in neuronal cell activities, including neuronal differentiation, survival and growth. There are totally 4 receptors mediating the signal of Neurotrophin, TrkA, TrkB, TrkC and p75 [3]. In recent years, studies demonstrated that Trks also plays important roles in nonneuronal cells [4-6]. Notably, up-regulation of nerve growth factor (NGF) and TrkA are usually observed in breast cancer cells derived from patients with unfavorable prognosis [7]. Furthermore, evidence suggests that activation of TrkA signal by NGF also blocked the cytotoxic effects of chemotherapeutic drugs [4]. Others studies also found that Trks inhibitors process anti-angiogenic activity and reduced the progression and metastasis of tumors [8-11]. Therefore, TrkA overexpression may be responsible for NGF signal mediated protection of cancer cells from chemotherapy-induced cell death. In this study, we focus on investigating the role of TrkA in the development and chemo-resistance of breast cancer.
TrkA as a potential target for breast cancer therapy

**Table 1. Primers used in the study**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TrkA</td>
<td>TTGGCATGAGCAGGATATCTACA</td>
<td>TCTCGGTGTAAGCTTACGTTACA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GGTCCTCTCATGACTCAAGA</td>
<td>AGCCAAATTCTGTTGCTACAT</td>
</tr>
</tbody>
</table>

**Table 2. Volumes of all tumors (cm³)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mice 1</th>
<th>Mice 2</th>
<th>Mice 3</th>
<th>Mice 4</th>
<th>Mice 5</th>
<th>Mice 6</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA + PTX</td>
<td>0.08</td>
<td>0.08</td>
<td>0.09</td>
<td>0.01</td>
<td>0.03</td>
<td>0.06</td>
<td>0.06 ± 0.033*</td>
</tr>
<tr>
<td>Scramble + PTX</td>
<td>0.77</td>
<td>0.68</td>
<td>0.3</td>
<td>0.27</td>
<td>0.64</td>
<td>0.49</td>
<td>0.49 ± 0.23*</td>
</tr>
<tr>
<td>Mock</td>
<td>2.06</td>
<td>1.33</td>
<td>1.12</td>
<td>3.9</td>
<td>1.4</td>
<td>1.05</td>
<td>1.81 ± 1.08</td>
</tr>
<tr>
<td>siRNA</td>
<td>0.78</td>
<td>0.64</td>
<td>0.33</td>
<td>0.26</td>
<td>2.00</td>
<td>0.88</td>
<td>0.815 ± 0.62*</td>
</tr>
</tbody>
</table>

*P<0.05 vs. Other group; **P<0.05 vs. Mock and Scramble group. Mock: untreated MCF-7 cells, Scramble: unrelated siRNA transfected cells, siRNA: TrkA-specific siRNA cells.

**Materials and methods**

**Cells, chemical and animals**

Human breast cancer cell line MCF-7 (obtained from Bengbu Medical College, Anhui, China) was used in the study. Cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah, USA), 100 μg/ml streptomycin and 100 units/ml penicillin at 37°C in 5% CO₂. Human recombinant NGF-β was purchased from Pepotech INC (London, UK). Female SCID mice (6-8 weeks old) were purchased from Changzhou card Vince experimental animal (Guangzhou, China).

**Construction of siRNA plasmids and transfection of cell**

Plasmid PSilencer 4.1-CMVneo (Ambion, Austin, USA) was used for siRNA expression. The siRNA (Sequence: 5’-GATCCACCTCACCTCGTGAAGAG-GTTCAAGAGACTCTCTACGATGGAGGTAAA-3’ and 5’-AGCTTAAACCTCACCTCGTGAAGAGCTCTCAAGAGGAGGTAA-3’) against mRNA of human TrkA (GenBank Accession No. NM-002529.3) were designed by the RNAi Designer (http://bioinfo.clontech.com/rnaidesigner). The original pSilencer™ 4.1-CMV neo vector containing control siRNA sequence had been used as the scramble siRNA.

The complementary oligonucleotides of siRNA were incubated in annealing buffer at 94°C for 3 min, and then annealed at 37°C for 1 h. The T4 DNA-PNK (Takara, Dalian, China) was used for phosphorylation of annealed oligonucleotides according manufacturer’s instruction. Then the oligonucleotides were ligated into the linearized pSilencer™ 4.1-CMV neo plasmid (BamHI/HindIII) by using T4 DNA ligase (Takara). The ligation product was transformed into competent E.coli DH5α cells as previously described [12]. The positive bacterial clone was verified by DNA sequencing (Shenggong Biotech, Shanghai, China).

MCF-7 Cells were seeded into 6-well plate. 24 hours later, the cells were transfected with 6-8 μg of siRNA siRNA plasmids by using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen, Carlsbad, USA). The knock down efficacy of was confirmed by Western blot and Real-Time PCR. The primers used in Real-Time PCR were listed in Table 1. Target sequence of TrkA siRNA in TrkA mRNA is AAACCTCACCATCGTGAAGAG (nt339-360).

**Western blot analysis**

Western blot assay was conducted as previously described [13]. Briefly, a total of 2 × 10⁶ cells were washed twice with ice-cold PBS and then lysed with 300 μl lysis buffer (50 mM Tris pH8.1, 1% SDS, β-glycerophosphate, sodium orthovanadate, sodium fluoride, EDTA, leupeptin) on ice for 5 min. The lysates were clarified by centrifugation at 15000 × rpm for 15 min at 4°C. A totally 50 ug protein of each sample were resolved in a 10% polyacrylamide gel. The separated proteins were then transferred onto a nitrocellulose membrane and probed with rabbit antibodies against TrkA (Beijing Biosynthesis, Beijing, China), NF-κBp65 (Zhongshan Golden Bridge, Beijing, China), Caspase-3 (Zhongshan Golden Bridge), and GAPDH (Zhongshan Golden Bridge), Specific reaction products were detected using goat anti-rabbit IgG conjugated with horseradish peroxidase (Shanghai Kangcheng, Shanghai, China) and revealed using a chemiluminescence substrate. The chemiluminescence signal was recorded digitally by a ChemiDoc XRS imaging system (Bio-Rad Laboratories, Hercules, CA).

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Real-time PCR

Total RNA was isolated with TRizol reagent (Invitrogen) by following the manufacturer’s instructions. Reverse transcription was performed by using 1 μg of RNA, 0.5 μg of random hexamers, and 200 units of moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, Wisconsin) according to manufacturer’s instructions. Real-time PCRs were performed using a Quantitect SYBRGreen PCR kit (Takara) with 2 μl of 10 × diluted cDNA and 500 nM of primers. The PCR cycles setting was 40 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 30 s. All samples were processed in triplicate. Transcripts of GAPDH were also amplified from the samples and used to normalize the total amount of input RNA (Table 1).

Cell proliferation assay and cell cycle analysis

Cells (2 × 10^4/well) were seeded in 96 well plates with antibiotic-free medium for overnight. Then NGF was added at a final concentration of 100 ng/ml. NGF-induced proliferation was determined 24 h, 48 h, 72 h and 96 h after treatment. By using MTS Cell Proliferation Colorimetric Assay kit (Biovision, Milpitas, CA, USA) according manufacturer’s instruction.

For the cell cycle analysis, cells were collected and fixed with ethanol. The fixed cell was incubated with 500 μl of PBS containing 100 units/ml RNaseA at 37°C for 2 h. Then the cells were stained with propidium iodide (50 μg/ml) for 30 min on ice in dark, followed by Flow Cytometry (FCM) assessment.

Synergistic effect of paclitaxel and TrkA siRNA on tumor xenograft

Six week old SCID mice were housed and maintained in laminar airflow cabinets under specific pathogen-free condition. Each mice was injected with 3 × 10^6 cells to form a tumor xenograft. Mice were randomized into four groups (n = 6): 1) TrkA siRNA + Paclitaxel (PTX); 2) Scramble + PTX; 3) Mock 4) TrkA siRNA. Mock and TrkA siRNA was received intraperitoneal (i.p.) injection of 100 μl NS (0.9% sodium chloride solution), TrkA siRNA + PTX and Scramble siRNA + PTX were received intraperitoneal (i.p.) injection of paclitaxel (15 mg/kg) in 100 μl PBS twice a week. Four weeks later, mice were euthanized by cervical dislocation in deep CO2 and the tumor volume was calculated by using the formula: volume = length × width^2 × 0.5. Pulmonary metastasis of lung was determined by hematoxylin-eosin staining.

Statistical analysis

Statistical analysis was performed by using a SPSS package (version 13.0). The significant differences in cellular mRNA, cell viability, cell proliferation, caspase 3 levels and cell cycle between the groups of siRNA transfected cells with or without adding PTX were assessed by Student’s t test. A two-tailed P value of less than 0.05 was considered significant.

Results

TrkA-specific siRNA suppressed TrkA expression

By using Psilencer™ 4.1-CMV neo plasmid as a delivery vector (si-vector), we generated a
siRNA plasmid targeting TrkA. TrkA-specific siRNA vector was then transfected into breast cancer cell lines and the down-regulation of TrkA was verified by real-time PCR and western blot. As shown in Figure 1A and 1B, mRNA and protein levels of TrkA were decreased significantly compared with Scramble or Mock (P<0.05). No significant difference was detected between Scramble or Mock. The results demonstrated that TrkA-siRNA was effective in inhibiting the expression of TrkA.

**Down-regulation of TrkA expression inhibited NGF-induced proliferation and NF-κBp65 activity in breast cancer MCF-7 cell lines**

Cell proliferation assay showed that TrkA siRNA transfection suppressed NGF stimulated proliferation of MCF-7 in a time-dependent manner. Scramble siRNA had no suppression of proliferation. Furthermore, TrkA siRNA arrested cell cycle of MCF-7 at G0/G1 phase which was significantly higher than that of Mock and Scramble. Furthermore, expression of NF-κBp65 was detected predominantly in Scramble induced by NGF (Figure 2C), but not in TrkA siRNA group (with or without NGF). The results indicated that TrkA siRNA treatment could inhibit NF-κBp65 activity and proliferation of cancer cell.

**Down-regulation of TrkA promoted paclitaxel-induced apoptosis**

Paclitaxel is widely used for the therapy of patients with breast cancer. Since paclitaxel alone has been reported to cause severe mitotic arrest and apoptosis [2], we designed a sequential treatment which involved pretreatment with TrkA siRNA for 24 h or 48 h, and then followed by additional paclitaxel (8 μM) treatment for 24 h or 48 h respectively. As shown in Figure 3A, paclitaxel inhibited the cell proliferation in a time-dependent manner. Comparing to the group applying Scramble siRNA, pre-treatment of TrkA specific siRNA significantly inhibited the cell proliferation. However, no inhibition of cell proliferation was observed in the Scramble group.

The morphology of cells also confirmed the result. Cells in Mock of were flat, irregular and adherent to the well, while cells in Scramble + PTX and siRNA + PTX group were round and aggregated, especially in the siRNA + PTX group that cells almost detached from the well. Cells in Scramble were similar to these in the Mock (Figure 3B).

Furthermore, paclitaxel induced caspase-3 activation was increased markedly after transfection of TrkA siRNA, but it was unaffected after transfection of Scramble (Figure 3C). Previous study had shown that paclitaxel induced apoptosis of tumor cells through caspase activation [1]. These results demonstrated that inactivation of TrkA can sensitize human breast cancer cells to paclitaxel induced apoptosis by caspase-mediated pathway.

**Synergistic anti-cancer effects of TrkA siRNA and paclitaxel**

To evaluate the synergistic anti-cancer activity of TrkA siRNA and paclitaxel in vivo, human breast cancer xenograft mouse model was used in the study. Compared to Mock, tumor growth was suppressed in the TrkA siRNA + PTX and Scramble + PTX group (Figure 4A). Tumor mass of TrkA siRNA was also smaller than that of the Mock (Table 2). The smallest tumor mass of TrkA siRNA + PTX group indicated synergistic anti-cancer effect of TrkA siRNA and paclitaxel (Figure 4A). Furthermore, down-regulation of TrkA by siRNA inhibited the lung metastasis. There were 5 mice had unequal cancer focus of lung metastasis in Mock. However, no mice had lung metastasis in TrkA siRNA + PTX group (Figure 4B), 2 mice had lung metastasis in Scramble siRNA + PTX group and 3 mice had lung metastasis in TrkA siRNA group. The volumes of all tumors were listed in Table 2.

**Discussion**

Aberrant expression of NGF and TrkA receptor has been reported to be associated with the development and progression of varieties of human cancers [11]. Up-regulation of TrkA and its phosphorylated form had been identified in human breast cancer biopsies [11]. TrkA was activated following binding of NGF, which has been shown to be involved in tumorigenesis of both neuronal and non-neuronal cells [2]. In this study, our result demonstrated that siRNA targeting TrkA inhibited NGF-induced proliferation and arrested cell cycle at G0/G1 phase, which is consisted with previous finding that targeting NGF and their receptors led to inhibition of cell survival, proliferation and invasion of breast cancer cell [14].
Figure 2. TrkA siRNA inhibits NGF-induced proliferation and NF-κB p65 activity. A. Cell viabilities in different groups; B. Cell cycle distributions in three groups; C. Flow Cytometry picture for cell cycle analysis; D. Activities of NF-κBp65 after treatment. *P<0.05.
The Trks receptor tyrosine kinases pathways were found to be constitutively activated by NGF, as revealed by the high levels of pAkt, pERK and pp38 [11]. NGF activation of the TrkA
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Signal transduction block the cytotoxic effects of chemotherapeutic drugs [4]. Furthermore, TrkA is involved in promoting cell proliferation [15], and activation of NF-κB pathway can further increase its anti-apoptotic ability [16]. In this study, NF-κBp65 activation induced by NGF can be inhibited by TrkA siRNA. Application of TrkA siRNA promoted apoptosis of cancer cell as well, which indicate the important role of TrkA in cancer cell survival. Taken together, our data suggests the TrkA siRNA may induce apoptosis through suppressing activation of NF-κB.

Chemotherapy drug paclitaxel is a first line anticancer drug for breast cancer. It had been reported that paclitaxel can induce apoptosis in tumor cells through the activation of caspase [16, 17]. However, side effects and drug resistance resulted from administration of paclitaxel are the main causes led to failure of chemotherapy. Therefore, a lot of effort had been paid for improving the chemo-sensitivity of cancer cell. In our study, caspase-3 activation induced by paclitaxel was significantly enhanced by the transfection of TrkA-specific siRNA, which suggested that inactivation of TrkA can sensitize cancer cells to chemotherapy. Our data also suggested TrkA overexpression and its related NGF signal pathway may be responsible for protecting cancer cells from chemotherapy-induced cell death. A previous study demonstrated similar result Trk tyrosine kinase inhibitor (CEP-701) reduced the tumor growth volume up to 50-70% in xenografts athymic nude mice [10]. More importantly, our data also indicated that TrkA siRNA could increase the efficacy of paclitaxel mediated inhibition of tumor growth and lung metastasis. However, mechanism underlying synergistic effects of TrkA inhibition and chemotherapy need further investigation. A possible explanation is that blocking of TrkA pathway can facilitate the binding of paclitaxel to tubulin, which increase the sensitivity of cancer cell to paclitaxel [16].

Metastasis is a complicate process which could be affected by multiple factors. The acquisition of anoikis resistance can increase the metastatic capacity of cancer cells. It had been reported that overexpression of TrkA rendered breast cancer cells more resistant to anoikis [16]. Notably, the inhibition of TrkA pathway also inhibited metastasis, which may imply a new target for cancer therapy.

In sum, our data showed that down-regulation of TrkA inhibited breast cancer cells proliferation and enhanced chemo-sensitivity of cancer cells via promoting caspase-3 activity. It also decreased tumor growth and lung metastasis in vivo. These findings indicated that administration of pharmacologic inhibitors of TrkA together with chemo-drugs may potentiate the chemotherapy efficiency during the treatment of breast cancer.

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Disclosure of conflict of interest

None.

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