Type III TGF-β receptor inhibits cell proliferation and migration in salivary glands adenoid cystic carcinoma by suppressing NF-κB signaling

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Abstract. It is known that the TGF-β superfamily receptors act as master regulators of cancer progression. However, alteration and role of type III TGF-β receptor (TβRIII, or betaglycan) as the most abundant of the TGF-β receptor has not been explored in salivary gland adenoid cystic carcinoma (ACC). Here, we reported that tumor biopsies and matched normal human salivary glands from patients with ACC were examined for the expression of TβRIII. The expression of TβRIII protein is significantly decreased in ACC patients based on immunohistochemistry and western blot analysis. In vitro, a transient overexpression of TβRIII markedly induced apoptosis and cell cycle arrest in the G2/M phase, thereby inhibited cell viability and migration of ACC-M cells. Co-immunoprecipitation revealed that TβRIII, scaffolding protein-arrestin2 (β-arrestin2) and IκBα formed a complex. Transient overexpression of TβRIII decreased p-p65 expression and increased IκBα expression, which was abolished by knockdown of β-arrestin2. The present study defines TβRIII as a biomarker exerting antitumor action on ACC progression. Gene therapy of TβRIII may be a powerful new approach for ACC disease.

Introduction

Salivary gland adenoid cystic carcinoma (ACC) is the most common epithelial malignant neoplasm of salivary glands (1,2), which is characterized by slow but aggressive growth, intensive local invasion, distant metastasis to the lungs at early or late stages, multiple recurrence, and poor long-term survival rates (3,4). The primary treatment of ACC is radical surgery, which can be followed by post-operative radiotherapy. Unfortunately, surgery, chemotherapy, and radiation therapy provide little improvement in survival (5). Therefore, there is a need for better understanding of the biology of ACC and the development of therapeutic approaches based on relevant targets.

Recently, many efforts have been made to examine the antitumor effect of TGF-β superfamily co-receptor, the type III TGF-β receptor (TβRIII, also known as betaglycan) in both in vitro and in vivo cancer models, including breast (6,7), lung (8,9), prostate (10,11), pancreas (12), ovary (13) and oral squamous cell carcinomas (14). Conversely, a report by Gatza et al (15) showed that TβRIII enhances colon cancer cell migration and growth. A recent study by Jovanović et al (16) also demonstrated that TβRIII is a tumor promotor in mesenchymal-stem like triple-negative breast cancer. These findings suggest that TβRIII exerts dual action on cancer progression depending upon the cell type. To date, no studies have investigated the expression and role of TβRIII in progression of ACC disease.

Here, we demonstrated that the expression of TβRIII is reduced in ACC. Transient transfection of TβRIII significantly decreased viability, migration and induced apoptosis in human high metastasis cell lines of ACCs (ACC-M), with significant inhibition of nuclear factor κB (NF-κB) signaling through its interaction with β-arrestin2.
Materials and methods

Tissue samples. Human adenoid cystic carcinoma and their adjacent non-cancer tissues were collected immediately after surgery from the Department of Oral and Maxillofacial Surgery at the Second Affiliated Hospital of Harbin Medical University in China, and informed consent was obtained from all participating patients or their guardians. The study was approved by the Institutional Review Board of Harbin Medical University.

Cell culture. Human high metastasis cell lines of ACCs (ACC-M) were cultured as previously described (17). ACC-M cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 100 U/ml penicillin and streptomycin.

Plasmids and transfections. Cells were transfected with pc-DNA3.1-mTβRIII plasmid (GeneChem Co., Ltd., Shanghai, China) with DNA concentrations of 0.5 and 1 μg/ml. The pc-DNA3.1-plasmid was used as an empty vector. Transient transfections were carried out by Fugene 6 (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s instructions.

Cell viability assay. Cells were seeded in a 96-well microplate at a density of 1x10^4 cells/well and treated as designated. Cells were incubated with 20 μl of MTT (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) for 4 h at 37°C in the dark. After medium was removed, 150 μl of DMSO was added to the wells. The absorbance was measured using a microplate spectrophotometer (Tecan, Austria) at 490 nm. The cell viability was calculated as a ratio of the mean OD value in treated versus untreated cells.

Assessment of electron microscopy. ACC-M cells were cultured in 60-mm plates, collected in phosphate-buffered saline (PBS) solution and fixed with 2.5% (w/v) glutaraldehyde (Paesel-Lorei) buffered solution and fixed with 2% (v/v) paraformaldehyde (PFA) in 60-mm plates, collected in phosphate-buffered saline (PBS) with 1% (w/v) OsO4 solution buffered with 0.1 M cacodylate (pH 7.2) at 4°C for 4 h. The cells were further fixed in 1% (w/v) OsO4 solution buffered with 0.1 M cacodylate (pH 7.2) at 4°C for 2 h. Then, the cells were scraped off from the plastic and dehydrated in ethanol. Dehydration was completed in propylene oxide. The specimens were embedded in Epon medium and dissected into 60-70-nm sections. Specimens were analyzed and documented with a JEOL 1200 electron microscope (JEOL Ltd., Tokyo, Japan).

Quantification of apoptosis and cell cycle by flow cytometry. Apoptosis in ACC-M cells, transfected with pcDNA3.1 expressing empty vector or TβRIII for 24 h, was quantified using the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Invitrogen, Carlsbad, CA, USA). Briefly, the cells were harvested, washed in cold PBS, and resuspended in Annexin V-labeling solution. After a 15-min incubation at room temperature, cells were then stained with 5 μg/ml propidium iodide (PI) and immediately subjected to Coulter Epics XL flow cytometer (Beckman Coulter, Miami, FL, USA). The effect of TβRIII overexpression on cell cycle distribution was determined by flow cytometry of DNA content from the nuclei of the cells. After treatment, ACC-M cells were harvested, washed with PBS and fixed in 70% ethanol and treated with 80 mg/ml RNase A at 37°C for 30 min. DNA was stained with 50 mg/ml PI and analyzed using a Coulter Epic XL flow cytometer (Beckman Coulter).

RNA interference. RNA interference was performed with siRNA duplexes (GenePharma, Shanghai) with sequences specifically targeting β-arrestin2 (5’-AAGGACCGCAAAAGUGUUUGUG-3’), or control non-specific siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Knockdown of expression of the target was determined by western blot analysis.

Co-immunoprecipitation (Co-IP) and western blot analysis. Total protein samples were extracted from the cultured cells. Approximately 5μg of antibody specific to TβRIII or β-arrestin2 protein was added to cell lysates and then incubated for 12 h at 4°C. The antibody-protein immune complexes were precipitated together with protein A/G PLUS-Agarose (rabbit polyclonal; Santa Cruz Biotechnology), which binds most antibodies, then incubated at 4°C overnight. When TβRIII or β-arrestin2 protein binds to one of them, TβRIII or β-arrestin2 protein can be identified by western blot analysis. Briefly, proteins were resolved by SDS-polyacylamide gel electrophoresis, and transferred to PVDF membranes (Amersham Biosciences). All the bands were blocked with 5% non-fat dry milk powder in Tris-buffered saline with Tween-20 for 2 h and then incubated with either primary rabbit anti-TβRIII, anti-p-p65, anti-β-arrestin2, or anti-β-arrestin2 (1:1,000; Cell Signaling Technology, Beverly, MA, USA). Western blot bands were quantified using Odyssey v1.2 software by measuring the band intensity (area x OD) for each group and normalizing it to GAPDH or β-actin (Zhongshan, Beijing, China) as an internal control.

Scratch wound-healing assay. For the scratch assay, ACC-M cells were seeded at 1x10^5 cells/well plate and treated to grow overnight. Cells were transiently transfected with pcDNA3.1 expressing empty vector or TβRIII. After a 24-h transfection, a scratch wound was applied using a pipette tip, and a baseline image was obtained. Scratch wound closure was monitored over a 24 h period. The healing of the wounds through cell migration was quantified by measuring the wound distance.

Transwell migration assay. Transwell migration and invasion assay were performed using the 24-well cell culture inserts without Matrigel and Matrigel invasion chambers (8-μm pore; BD Biosciences), respectively. Briefly, 5x10^4 cells were suspended in 250 μl serum-free RPMI-1640 and added into the inserts. A total of 500 μl DMEM with 10% FBS was added to the lower chamber. After allowing cells to migrate for 4 h or invasion for 22 h, cells on the upper surface of the membrane were removed using a cotton swab, and the membranes were fixed with methanol and stained with crystal violet. The number of migrating or invading cells was determined by averaging cell counts from nine randomly selected x100 fields.

Immunohistochemistry (IHC). Serial sections (5-6-μm thick) were made from paraffin-embedded tissue blocks and
mounted on silane-coated glass slides (Matsunami Glass, Osaka, Japan). One section from each tissue block was stained with hematoxylin and eosin (H&E), and the others were used for IHC. IHC staining was performed using the standard streptavidin-biotin-peroxidase complex method. Briefly, paraffin sections of ACC tissues were deparaffinized, blocked with 10% normal goat serum for 10 min, and incubated with anti-TβRIII overnight at 4˚C. The tissue section was then incubated with biotinylated goat anti-rabbit immunoglobulin at a concentration of 1:75 at 37˚C for 30 min. The status of TβRIII expression was assessed by two independent investigators without prior knowledge of the clinicopathological data.

**Statistical analysis.** The results are expressed as the mean ± standard deviation (SD). Statistical significance was evaluated using the unpaired Student's t-test. A p-value <0.05 was considered statistically significant.

**Results**

**Tumor characteristics.** Table I summarizes the clinical attributes of the patients, who provided the 12 ACC tumor samples. All the tumors had arisen sporadically. Of these, 8 occurred in men, and the median age at the time of presentation was 61 years (range, 48-77 years). Tumors arose at the following sites: submandibular gland (7 tumors), parotid gland (3 tumors), and tongue (2 tumors). The tumors were classified as follows based on morphologic subtype: combined tubular and cribriform (8 tumors), cribriform (2 tumors), combined tubular and solid (2 tumors).

**Decreased TβRIII expression in human adenoid cystic carcinoma.** Loss or reduced expression of TβRIII has been demonstrated in multiple human cancers. To confirm the expression of TβRIII in human adenoid cystic carcinoma, we

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**Table I. Clinicopathological features of ACC samples.**

<table>
<thead>
<tr>
<th>Case</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Tumor location</th>
<th>Histological type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>74</td>
<td>Submandibular gland</td>
<td>Tubular + cribriform</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>69</td>
<td>Submandibular gland</td>
<td>Tubular + cribriform</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>57</td>
<td>Parotid gland</td>
<td>Tubular + cribriform</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>77</td>
<td>Submandibular gland</td>
<td>Tubular + cribriform</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>63</td>
<td>Tongue</td>
<td>Cribriform</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>51</td>
<td>Parotid gland</td>
<td>Tubular + solid</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>48</td>
<td>Parotid gland</td>
<td>Tubular + solid</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>66</td>
<td>Tongue</td>
<td>Cribriform</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>54</td>
<td>Submandibular gland</td>
<td>Tubular + cribriform</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>66</td>
<td>Submandibular gland</td>
<td>Tubular + cribriform</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>59</td>
<td>Submandibular gland</td>
<td>Tubular + cribriform</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>49</td>
<td>Submandibular gland</td>
<td>Tubular + cribriform</td>
</tr>
</tbody>
</table>

M, male; F, female.
To validate the positive functional involvement of TβRIII expression in normal salivary glands and negative TβRIII expression in adenoid cystic carcinoma, we transfected ACC-M cells with a plasmid encoding the constitutively active TβRIII. Successful transfection of TβRIII was verified by our data shown in Fig. 2A. Western blot analysis showed that the TβRIII protein level was increased in a concentration-dependent manner in cells treated with 0.5 and 1 µg/ml of TβRIII plasmid DNA (Fig. 2A). Cell viability was determined by MTT assay. Fig. 2B shows that the viability of ACC-M cells transfected with 0.5 or 1 µg/ml TβRIII plasmid was reduced dose-dependently.

**TβRIII overexpression induces apoptosis in ACC-M cells.** TGF-β signaling pathway induces programmed cell death in a variety of cell types. To determine the effects of TβRIII overexpression on ACC-M cell apoptosis, we used electron microscopy and flow cytometry to confirm the apoptotic changes. Under an electron microscope the cells with TβRIII overexpression exhibited robust changes in microstructure, including cell surface microvilli reduction, nuclear chromatin condensation, margination, and membrane blistering (Fig. 3A). We transfected ACC-M cells with a plasmid encoding TβRIII or NC plasmid, and apoptosis was assessed by flow cytometry in live cells stained with Annexin V-FITC/PI. As shown in Fig. 3B, TβRIII overexpression induced apoptosis in ACC-M cells in a dose-dependent manner.

**TβRIII affects cell cycle progression in ACC-M cells.** To define the mechanisms by which TβRIII regulated viability of ACC-M cells further, we examined the effect of increasing TβRIII expression on the cell cycle progression in ACC-M cells in vitro. Cell cycle analysis revealed that TβRIII overexpression resulted in a remarkable G2/M arrest in ACC-M cells (Fig. 4).

**TβRIII delays and decreases ACC-M cells migration in vitro.** We further examined whether TβRIII overexpression affected cell migration ability in ACC-M cells. Wound healing and Transwell assays were evaluated. As shown in Fig. 5, both wound healing and Transwell assays showed that TβRIII overexpression significantly inhibited the cellular transmigration ability compared with controls. These results strongly indicated that TβRIII may also regulate the cell migration ability of adenoid cystic carcinoma.

**TβRIII negatively regulates NF-κB signaling through interacting with β-arrestin2 in ACC-M cells.** NF-κB is a dimeric transcription factor that regulates genes involved in cell survival and proliferation (18). Increased NF-κB activity has been demonstrated in diverse human malignancies, including ACC-M, which is believed to enhance tumor cell survival by accelerating mitosis and inhibiting apoptosis (19). TGF-β has been reported to activate (20) or inhibit (21) NF-κB signaling through mechanisms yet to be fully defined. You et al (22) reported that TβRIII was involved in NF-κB regulation via its interaction with β-arrestin2 in MCF10A breast epithelial and MDA-MB-231 breast cancer cells. We next investigated the effect of TβRIII overexpression on NF-κB activity in ACC-M. Our results indicated that transiently increasing TβRIII expression decreased p-p65 expression (Fig. 6A) and increased IκBα expression (Fig. 6B). These results suggest that inhibition of NF-κB signaling represents a potential mechanism.
for TβRIII-mediated inhibition of cell apoptosis, migration, and mitotic arrest in ACC-M. Our results also confirmed that TβRIII interacted with β-arrestin2, and β-arrestin2 interacted with IκBα (Fig. 6C). TβRIII overexpression decreased p-p65 expression, and co-transfection of TβRIII and siRNA of β-arrestin2 resulted in an increase in phosphorylation of p65 (Fig. 6E). These results suggest that TβRIII through its interaction with β-arrestin2, negatively regulates NF-κB signaling in ACC-M.

**Discussion**

In the present study, we report the expression status of TβRIII in salivary gland adenoid cystic carcinoma for the first time.
We define an important role for TβRIII expression in salivary gland adenoid cystic carcinoma as an inhibitor of cell growth and migration through its inhibitory effects on NF-κB signaling via its interaction with β-arrestin2.
Here, we demonstrated that the TβRIII expression was markedly repressed in adenoid cystic carcinoma specimens compared with matched normal human salivary glands, suggesting that a low TβRIII expression level is associated with salivary gland adenoid cystic carcinoma progression. Loss of TβRIII expression has also been described in breast, lung, prostate, pancreas, ovary, and oral squamous cell carcinomas as mentioned above. Turley et al (29) reported that the restoration of TβRIII expression in prostate cancer cells inhibits migration and invasion. Zheng et al (23) reported that transient overexpression of TβRIII induces apoptosis in human nasopharyngeal carcinoma CNE-2Z cells. Forced overexpression of TβRIII upregulated pro-apoptoticBad, Bcl-2, and XIAP protein. These results support that the TβRIII regulated multiple targets involved in cell proliferation by mediating the TGF-β superfamily ligand independent signaling. Furthermore, some studies reported that TβRIII had direct effects on regulating tumor migration, invasion, and proliferation without any cytokines and ligands (23,24). These data indicated that gene therapy of TβRIII may be a powerful new approach for cancer.

How does decreased TβRIII expression promote salivary gland adenoid cystic carcinoma progression? The great potential for hematogenous metastasis at an early stage is one of the unique characteristics of ACCs. Zhang and Peng (19) reported that the high expression levels of NF-kB were significantly correlated with ACC metastasis. Our results showed that the reason of high levels of NF-kB in ACC may be correlated with reduced expression of TβRIII. Regulation of TβRIII expression occurs at multiple levels. At the transcriptional level, TβRIII expression is negatively regulated by TGF-β through inhibiting of the promoter in multiple cell types. In some tumors, especially late-stage tumors, a large amount of TGF-β was secreted, and inhibited the expression of TβRIII (24).

Zanotto-Filho et al (25) demonstrated that the pharmacological NF-kB inhibitors BAY117082 and MG132 induce cell arrest and apoptosis in leukemia cells through ROS-mitochondria pathway activation. Other groups also confirmed the result that inhibition of NF-kB pathways induces G2/M arrest and apoptosis in different cancer cells, including prostate cancer (26), glioblastoma (27) and melanoma (28). We studied the effects of TβRIII overexpression on cell viability, apoptosis, cell cycle, and in vitro cell migration in highly metastatic cell lines of human ACC-M. We demonstrated that TβRIII inhibited ACC-M cell growth and migration through its inhibitory effects on NF-kB signaling. As NF-kB activation should be accompanied by proteasome-mediated degradation of IkBα, we also investigated IkBα protein expression in response to TβRIII stimulation. Our results indicated that transiently increasing TβRIII expression decreased p-p65 expression and increased IkBα expression in ACC-M.

We also showed a novel interaction of TβRIII with the scaffolding protein, β-arrestin2, which results in TβRIII internalization and downregulation of TGF-β signaling. β-arrestin2 also scaffolds interacting receptors with the IκBα (Fig. 6C). The results are consistent with other groups. TβRIII has been shown to interact with β-arrestin2 through its cytoplasmic domain (29). Gao et al (30) have shown that β-arrestin2 directly interacts with IκBα and prevents phosphorylation and degradation of IκBα. Similar results have also been obtained from Witherow et al (31). You et al (22) demonstrated that TβRIII, through its interaction with β-arrestin2, negatively regulates NF-kB signaling in breast cancer.

In summary, we demonstrated that the TβRIII expression was markedly repressed in adenoid cystic carcinoma. Transient TβRIII overexpression induced apoptosis and G2/M arrest, inhibited cell growth and migration in ACC-M cells. We established the TβRIII/β-arrestin2 as an important negative regulator of NF-kB signaling in ACC-M and a potential mechanism for TβRIII-mediated inhibition of ACC-M migration and ACC progression. The present study defines TβRIII as a biomarker exerting antitumor action on ACC progression, thus, gene therapy of TβRIII may be a new approach for ACC disease.

Acknowledgements

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References


