

Type III TGF- β receptor inhibits cell proliferation and migration in salivary glands adenoid cystic carcinoma by suppressing NF- κ B 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 (T β RIII, 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 T β RIII. The expression of T β RIII protein is significantly decreased in ACC patients based on immunohistochemistry and western blot analysis. *In vitro*, a transient overexpression of T β RIII 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 T β RIII, scaffolding protein-arrestin2 (β -arrestin2) and I κ B α formed a complex. Transient overexpression of T β RIII decreased p-p65 expres-

sion and increased I κ B α expression, which was abolished by knockdown of β -arrestin2. The present study defines T β RIII as a biomarker exerting antitumor action on ACC progression. Gene therapy of T β RIII 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 (T β RIII, 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 T β RIII enhances colon cancer cell migration and growth. A recent study by Jovanović *et al* (16) also demonstrated that T β RIII is a tumor promoter in mesenchymal-stem like triple-negative breast cancer. These findings suggest that T β RIII exerts dual action on cancer progression depending upon the cell type. To date, no studies have investigated the expression and role of T β RIII in progression of ACC disease.

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

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Abbreviations: ACC, adenoid cystic carcinoma; TGF- β , transforming growth factor β ; NF- κ B, nuclear factor κ B; BMPs, bone morphogenetic proteins; GDFs, growth and differentiation factors; T β RIII, the type III TGF- β receptor; ACC-M, high metastasis cell lines of ACCs; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; FITC, fluorescein isothiocyanate; PI, propidium iodide; H&E, hematoxylin and eosin

Key words: adenoid cystic carcinoma, T β RIII, NF- κ B, β -arrestin2, I κ B α

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-mT β RIII plasmid (GeneChem Co., Ltd., Shanghai, China) with DNA concentrations of 0.5 and 1 μ g/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 1×10^4 cells/well and treated as designated. Cells were incubated with 20 μ l 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 μ l 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% (v/v) paraformaldehyde (PFA) containing 2.5% (w/v) glutaraldehyde (Paesel-Lorei) buffered in HMSS (Hank's modified salt solution) at 4°C for 4 h. The cells were further fixed in 1% (w/v) OsO₄ solution buffered by 0.1 M cacodylate (pH 7.2) at 4°C for 2 h. Then, the cells were scraped off from the plastic and dehydrate dinethanol. 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 T β RIII 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 μ g/ml propidium iodide (PI) and immediately subjected to Coulter Epics XL flow cytometer (Beckman Coulter, Miami, FL, USA). The effect of T β RIII 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 Epics XL flow cytometer (Beckman Coulter).

RNA interference. RNA interference was performed with siRNA duplexes (GenePharma, Shanghai) with sequences specifically targeting β -arrestin2 (5'-AAGGACCGCAAAGU GUUUGUG-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 5 μ g of antibody specific to T β RIII 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 T β RIII or β -arrestin2 protein binds to one of them, T β RIII or β -arrestin2 protein can be identified by western blot analysis. Briefly, proteins were resolved by SDS-polyacrylamide gel electrophoresis, and transferred to PVDF membranes (Amersham Biosciences). All the blots 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-T β RIII, anti-p-p65, anti- β -arrestin2, or anti-I κ B α (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 \times 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 1×10^5 cells/6-well plate and allowed to grow overnight. Cells were transiently transfected with pcDNA3.1 expressing empty vector or T β RIII. 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- μ m pore; BD Biosciences), respectively. Briefly, 5×10^4 cells were resuspended in 250 μ l serum-free RPMI-1640 and added into the inserts. A total of 500 μ l 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 $\times 100$ fields.

Immunohistochemistry (IHC). Serial sections (5-6- μ m thick) were made from paraffin-embedded tissue blocks and

Table I. Clinicopathological features of ACC samples.

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

M, male; F, female.

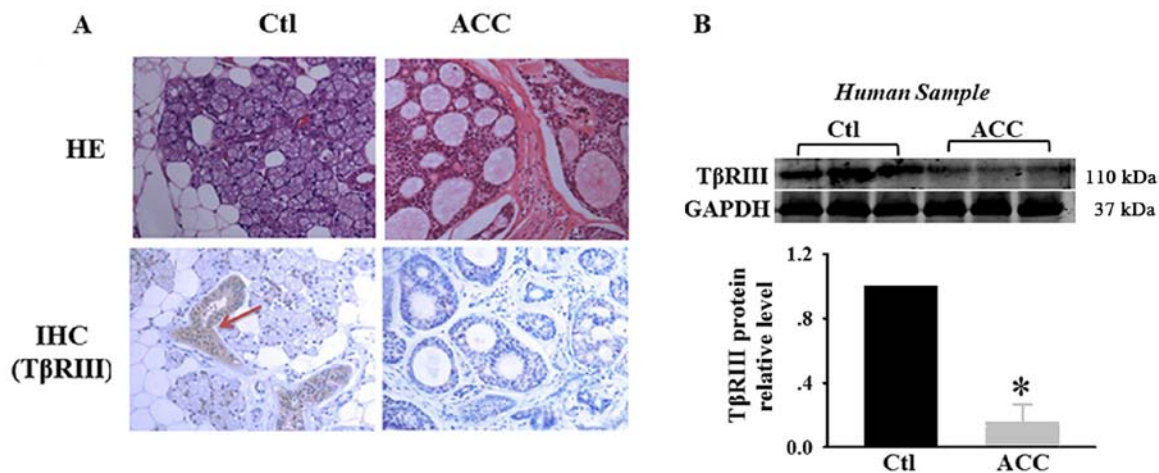


Figure 1. Expression of TβRIII in adenoid cystic carcinoma patient specimens. (A) H&E stain and IHC analysis for TβRIII expression in matched normal human salivary glands and adenoid cystic carcinoma specimens (magnification, x400). (B) Expression of TβRIII in specimens was evaluated by immunoblot analysis with antibodies against TβRIII. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. These changes were quantitated using densitometry (B). Data presented as mean ± standard deviation from three independent experiments. *P<0.05.

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

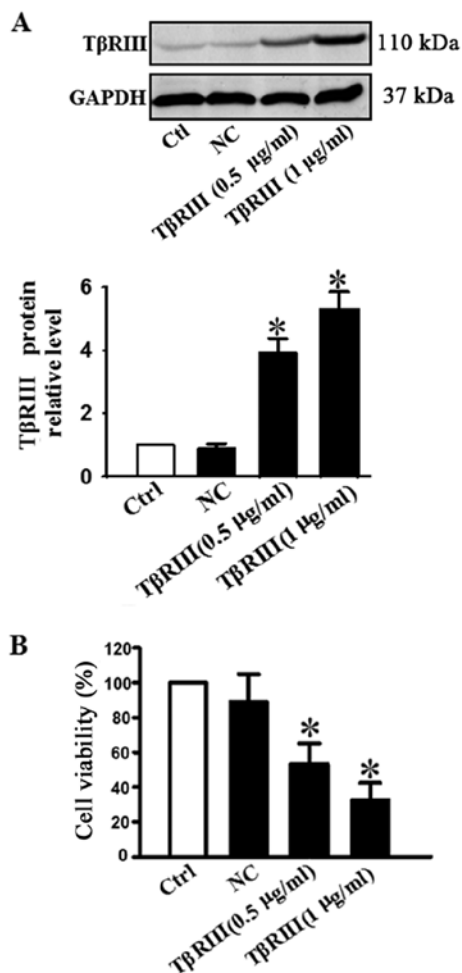


Figure 2. Overexpression of T β RIII suppresses the viability of ACC-M cells. ACC-M cells were transfected with plasmid encoding T β RIII at concentrations of 0.5 and 1 μ g/ml. NC represents empty vectors (1 μ g/ml of pc-DNA3.1 plasmid) transfected into ACC-M cells, which serves as an NC. (A) T β RIII expression determined by western blot analysis, and the averaged band densities from three independent experiments are shown. (B) Relative cell viability determined by MTT assay. Data are shown as the rate (%) of growth control (viability of control) cultured in growth medium without transfection. Data are presented as mean \pm standard deviation from three independent experiments. *P<0.05.

performed IHC analysis for T β RIII expression in matched normal human salivary glands and adenoid cystic carcinoma specimens. Representative samples are shown in Fig. 1A. H&E staining showed that adenoid cystic carcinoma exhibited cribriform architecture with pseudocystic spaces filled with eosinophilic coagulum. IHC showed moderate to strong T β RIII expression in normal salivary glands and negative T β RIII expression in ACC specimens. We also examined the expression of T β RIII in matched normal human salivary glands and adenoid cystic carcinoma specimens. Lower levels of T β RIII protein were observed in human adenoid cystic carcinoma specimens (Fig. 1B). These results suggest that T β RIII expression is significantly decreased in adenoid cystic carcinoma, with loss of T β RIII expression correlating with adenoid cystic carcinoma progression.

T β RIII overexpression suppresses the viability of ACC-M cells. To validate the positive functional involvement of T β RIII in

adenoid cystic carcinoma, we transfected ACC-M cells with a plasmid encoding the constitutively active T β RIII. Successful transfection of T β RIII was verified by our data shown in Fig. 2A. Western blot analysis showed that the T β RIII protein level was increased in a concentration-dependent manner in cells treated with 0.5 and 1 μ g/ml of T β RIII 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 μ g/ml T β RIII plasmid was reduced dose-dependently.

T β RIII 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 T β RIII 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 T β RIII 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 T β RIII or NC plasmid, and apoptosis was assessed by flow cytometry in live cells stained with Annexin V-FITC/PI. As shown in Fig. 3B, T β RIII overexpression induced apoptosis in ACC-M cells in a dose-dependent manner.

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

T β RIII delays and decreases ACC-M cells migration in vitro. We further examined whether T β RIII 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 T β RIII overexpression significantly inhibited the cellular transmigration ability compared with controls. These results strongly indicated that T β RIII may also regulate the cell migration ability of adenoid cystic carcinoma.

T β RIII negatively regulates NF- κ B signaling through interacting with β -arrestin2 in ACC-M cells. NF- κ B is a dimeric transcription factor that regulates genes involved in cell survival and proliferation (18). Increased NF- κ B 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- κ B signaling through mechanisms yet to be fully defined. You *et al* (22) reported that T β RIII was involved in NF- κ B regulation via its interaction with β -arrestin2 in MCF10A breast epithelial and MDA-MB-231 breast cancer cells. We next investigated the effect of T β RIII overexpression on NF- κ B activity in ACC-M. Our results indicated that transiently increasing T β RIII expression decreased p-p65 expression (Fig. 6A) and increased I κ B α expression (Fig. 6B). These results suggest that inhibition of NF- κ B signaling represents a potential mechanism

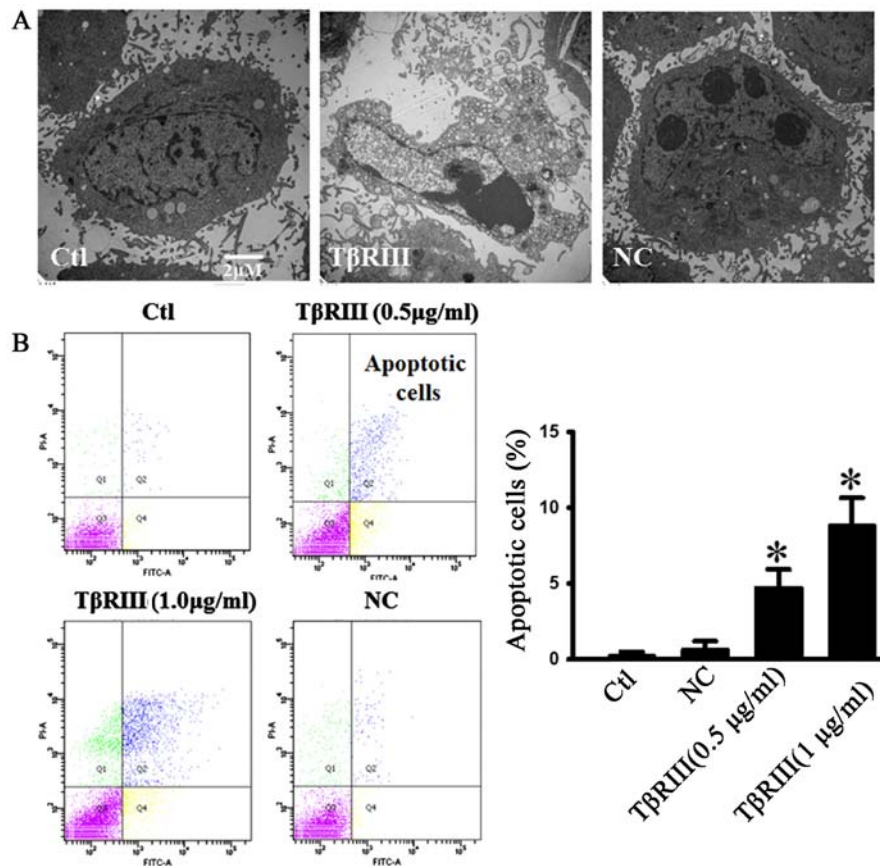


Figure 3. TβRIII overexpression induces apoptosis in ACC-M cells. (A) Transmission electron microscopy to estimate micromorphological changes (magnification, x8,000). (B) Apoptosis was assessed by Annexin V-FITC/propidium iodide staining of live cells. Data are presented as mean ± standard deviation from three independent experiments. *P<0.05.

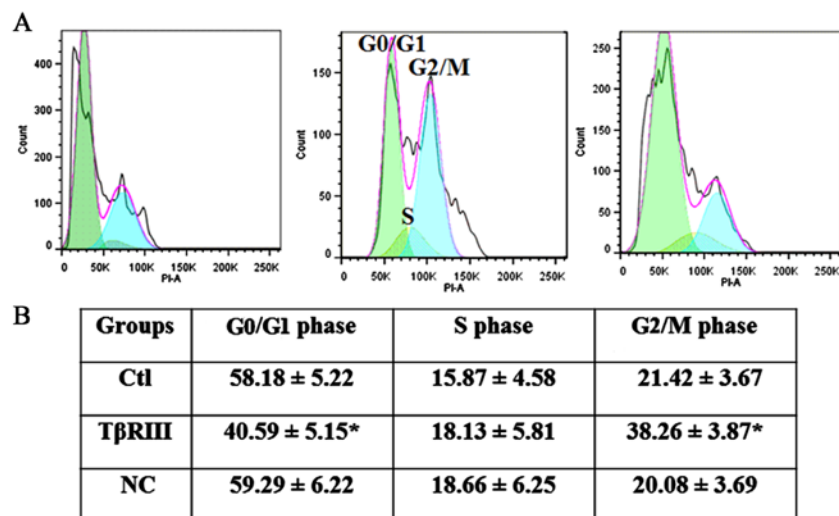


Figure 4. TβRIII overexpression induces G2/M arrest in ACC-M cells. (A) Cell cycle distribution at 24 h after TβRIII overexpression. (B) Cell cycle analysis revealed that TβRIII overexpression resulted in a remarkable G2/M arrest in ACC-M cells.

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.

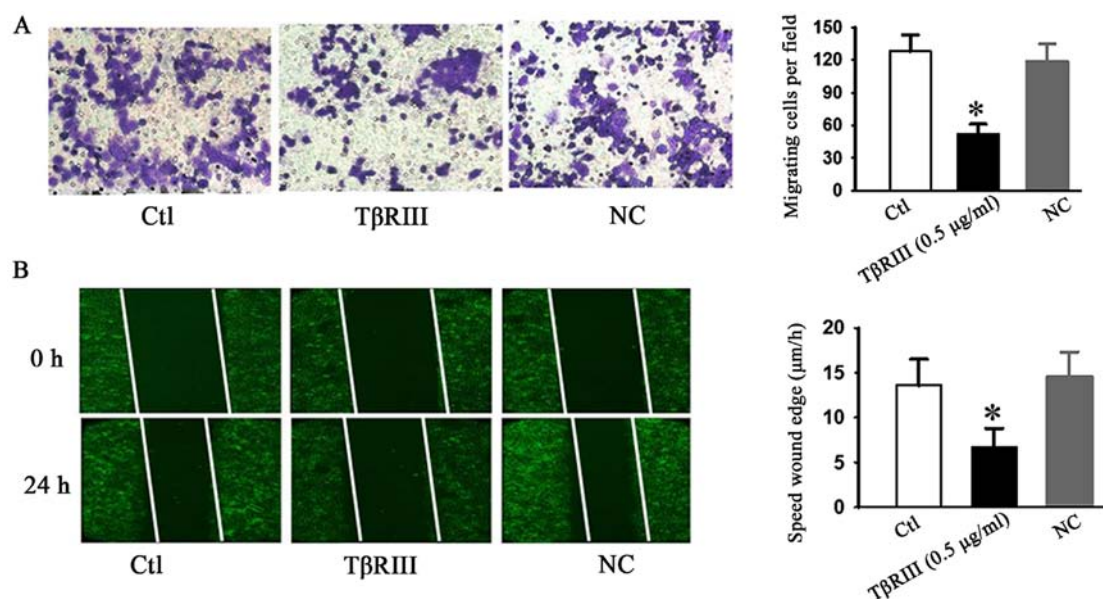


Figure 5. T β RIII overexpression inhibits migration in ACC-M cells. ACC-M cells were transiently transfected with the NC plasmid and T β RIII plasmid, respectively. Following plasmid transfection, cells were subjected to Transwell migration (A) and wound-healing assay (B), respectively. Data are presented as mean \pm standard deviation from three independent experiments. * $P < 0.01$.

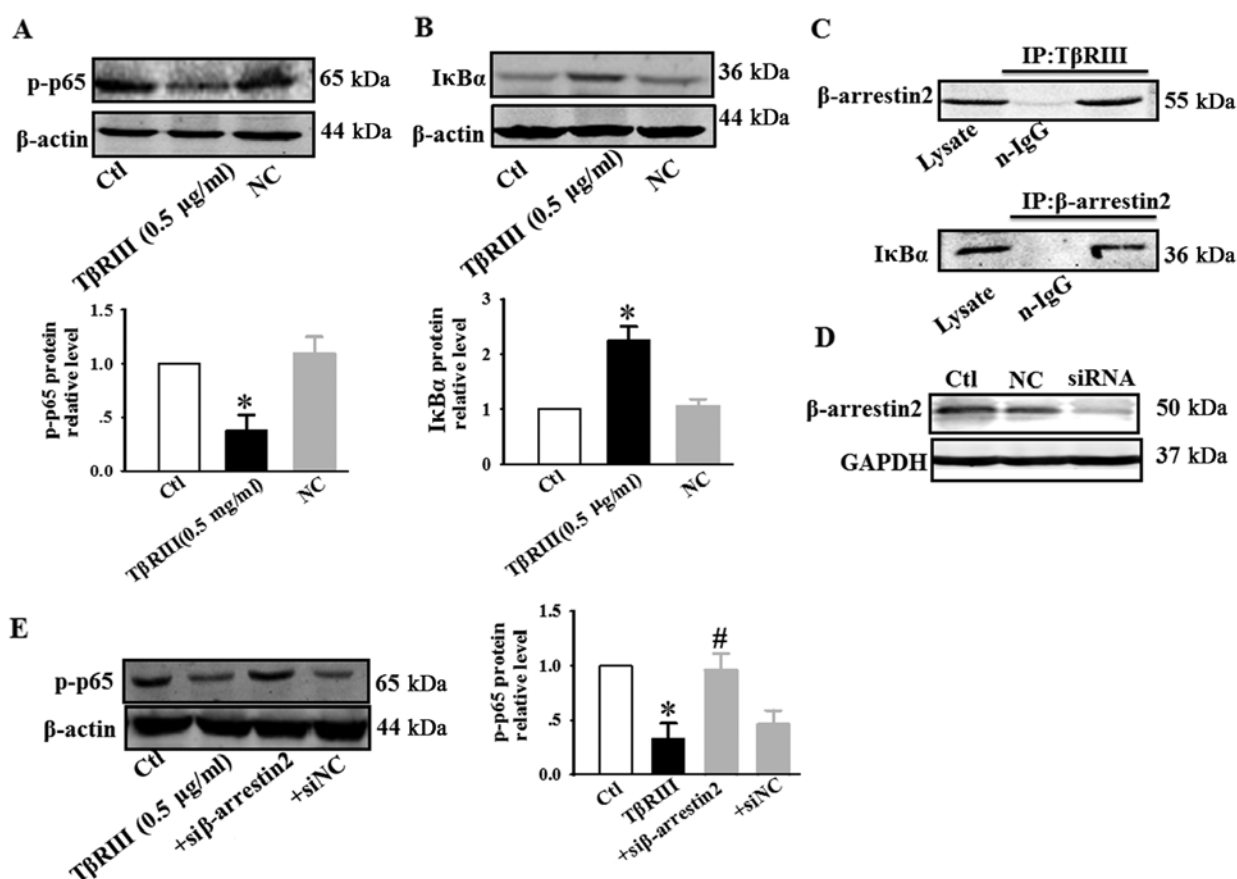


Figure 6. T β RIII negatively regulates NF- κ B signaling through interacting with β -arrestin2 in ACC-M cells. (A) Transiently increasing T β RIII expression decreased p-p65 expression. (B) Transiently increasing T β RIII expression increased I κ B α expression. (C) T β RIII interacted with β -arrestin2, and β -arrestin2 interacted with I κ B α . (D) ACC-M cells were transiently transfected with siRNA for β -arrestin2. (E) T β RIII overexpression decreased p-p65 expression, and co-transfection of T β RIII and siRNA of β -arrestin2 resulted in an increase in phosphorylation of p65. Data are presented as mean \pm standard deviation from three independent experiments. * $P < 0.05$ vs. control, # $P < 0.05$ vs. T β RIII.

We define an important role for T β RIII expression in salivary gland adenoid cystic carcinoma as an inhibitor of cell

growth and migration through its inhibitory effects on NF- κ B signaling via its interaction with β -arrestin2.

Here, we demonstrated that the T β RIII expression was markedly repressed in adenoid cystic carcinoma specimens compared with matched normal human salivary glands, suggesting that a low T β RIII expression level is associated with salivary gland adenoid cystic carcinoma progression. Loss of T β RIII expression has also been described in breast, lung, prostate, pancreas, ovary, and oral squamous cell carcinomas as mentioned above. Turley *et al* (10) reported that the restoration of T β RIII expression in prostate cancer cells inhibits migration and invasion. Zheng *et al* (23) reported that transient overexpression of T β RIII induces apoptosis in human nasopharyngeal carcinoma CNE-2Z cells. Forced overexpression of T β RIII upregulated pro-apoptotic Bad and Bax protein, and downregulated anti-apoptotic p-Bad, Bcl-2, and XIAP protein. These results support that the T β RIII regulated multiple targets involved in cell proliferation by mediating the TGF- β superfamily ligand independent signaling. Furthermore, some studies reported that T β RIII had direct effects on regulating tumor migration, invasion, and proliferation without any cytokines and ligands (23,24). These data indicated that gene therapy of T β RIII may be a powerful new approach for cancer.

How does decreased T β RIII 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- κ B were significantly correlated with ACC metastasis. Our results showed that the reason of high levels of NF- κ B in ACC may be correlated with reduced expression of T β RIII. Regulation of T β RIII expression occurs at multiple levels. At the transcriptional level, T β RIII expression is negatively regulated by TGF- β 1 through inhibition of the promoter in multiple cell types. In some tumors, especially late-stage tumors, a large amount of TGF- β 1 was secreted, and inhibited the expression of T β RIII (24).

Zanotto-Filho *et al* (25) demonstrated that the pharmacological NF- κ B 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- κ B 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 T β RIII overexpression on cell viability, apoptosis, cell cycle, and *in vitro* cell migration in highly metastatic cell lines of human ACC-M. We demonstrated that T β RIII inhibited ACC-M cell growth and migration through its inhibitory effects on NF- κ B signaling. As NF- κ B activation should be accompanied by proteasome-mediated degradation of I κ B α , we also investigated I κ B α protein expression in response to T β RIII stimulation. Our results indicated that transiently increasing T β RIII expression decreased p-p65 expression and increased I κ B α expression in ACC-M.

We also showed a novel interaction of T β RIII with the scaffolding protein, β -arrestin2, which results in T β RIII internalization and downregulation of TGF- β signaling. β -arrestin2 also scaffolds interacting receptors with the I κ B α (Fig. 6C). The results are consistent with other groups. T β RIII 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 I κ B α and prevents phosphorylation and degradation of I κ B α . Similar results have also been obtained

from Witherow *et al* (31). You *et al* (22) demonstrated that T β RIII, through its interaction with β -arrestin2, negatively regulates NF- κ B signaling in breast cancer.

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

Acknowledgements

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