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TRB3 overexpression due to endoplasmic reticulum stress inhibits AKT kinase activation of tongue squamous cell carcinoma

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SUMMARY

Our investigation aims to evaluate the significance of TRB3, an endoplasmic reticulum stress (ERS)-inducible gene, and explore its relationship with AKT in oral tongue squamous cell carcinoma (OTSCC). Expression of TRB3 and phosphorylated AKT (p-AKT) in OTSCC tissues and adjacent normal tissues were assessed by RT-PCR, Western blot and immunohistochemistry assay. Correlation of TRB3 and AKT was validated by TRB3 adenovirus plasmid (Ad-TRB3) transfection and short hairpin RNA (shRNA) inhibition. The mRNA expression of TRB3 was significantly higher than adjacent noncancerous tissues by RT-PCR in 15 of 18 specimens of OTSCC (83.3%, $P < 0.01$). Both of TRB3 and AKT were highly expressed in 13 of 18 (72.2%) specimens of OTSCC comparing with adjacent noncancerous tissues by Western blot assay ($P < 0.05$). TRB3 was significantly elevated in 49.2% (63/128) of pathologically confirmed specimens and 13.3% (4/30) of adjacent noncancerous specimens by immunohistochemical analysis ($P < 0.01$). TRB3 overexpression was closely correlated with tumor pathological T stage, lymph node metastasis and tumor recurrence. In addition, both mRNA and protein expression of TRB3 was increased under thapsigargin (TG) or tunicmycin (TU)-induced ERS in Tca8113 and CAL-27 cells. Moreover, expression of p-AKT protein decreased when Ad-TRB3 was transected with OTSCC Tca8113 cells. However, expression of p-AKT protein increased when TRB3 was inhibited by TRB3 shRNA inhibition. TRB3 expression was closely correlated with OTSCC prognosis. Under ERS, TRB3 was up-regulated, resulting in inhibiting the activation of AKT in OTSCC.

Introduction

Oral tongue squamous cell carcinoma (OTSCC) is the most common kind of oral cancer and has increased by 1.9% of annual percent change between 1975 and 2007, especially among young adults aged from 20 to 44 years.[1,2] Even with combined treatment of surgery, chemotherapy and radiation, the five-year survival rate of OTSCC is only 50–60%.[2,3] Therefore, exploring a new biomarker for OTSCC prognosis and treatment is necessary. TRB3, a mammalian homolog of Drosophila tribbles, is identified in 1999.[4], has been found overexpressed in primary lung carcinoma, colorectal carcinoma, breast carcinoma, uterus and ovary tumor with the positive rate ranged from 60% to 90.9%.[5–8] For colorectal carcinoma, patients with high TRB3 expression are statistically susceptible to a recurrence of the disease, and show poorer overall survival than those with low expression.[7] However, the expression of TRB3 in OTSCC remains unknown.

OTSCC is a kind of solid tumor. With growing of tumor, nutrient deprivation, hypoxia and ischemia, cancer cells become more and more serious, leading to metabolism stress of tumor cells.[8] Protein is the basal form of life and endoplasmic reticulum is the major place of protein synthesis, modification and transportation. So endoplasmic reticulum stress (ERS) is an important form of cells' response to environment stress and becomes common in solid tumor.[9] TRB3 is not only overexpressed in tumor, but also plays an important role in ERS. Under low environment stress, ERS stimulates the unfolded protein response (UPR) to protect the cells. However, a severe or prolonged stress up-regulates TRB3 and induces cell apoptosis.[10] TRB protein family has no ATP combining

Abbreviations: ERS, endoplasmic reticulum stress; OTSCC, oral tongue squamous cell carcinoma; Ad-TRB3, TRB3 adenovirus plasmid; shRNA, short hairpin RNA; HNSCC, head and neck squamous cell carcinoma; UPR, unfolded protein response; p-AKT, phosphorylated AKT; TG, thapsigargin; TU, tunicmycin; CHOP, C/EBP homologous protein; ATF4, activating transcription factor 4.

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site, and therefore requires a connection with other kinase binding areas to take effects.[4] AKT, a serine/threonine protein kinase, regulates a variety of cellular processes including survival, proliferation, protein translation and metabolism.[11] ERS has been found to mediate cellular processes of cancer via AKT signaling pathway.[12–14] In addition, AKT signaling pathway is closely correlated with carcinogenesis and the development of head and neck squamous cell carcinoma.[15] Moreover, TRB3 inhibits AKT activation through combining with its threonine or serine activated site,[16,17] resulting in activating the downstream signal to induce cell apoptosis.

Here, we showed that both of TRB3 and phosphorylated AKT (p-AKT) were overexpressed in OTSCC. Additionally, TRB3 overexpression was closely correlated with tumor pathological T stage, lymph node metastasis and tumor recurrence. More importantly, we found that overexpression of TRB3 reduced the phosphorylation of Akt while suppression of TRB3 by shRNA induced Akt phosphorylation in Tca8113 cells.

Materials and methods

Tissue specimens and patients

For reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis, we collected 18 paired OTSCC and adjacent noncancerous tongue tissues (at a distance greater than 2 cm from tumor) from patients who underwent half-tongue resection between May and July 2010. In addition, 128 paraffin-embedded samples of OTSCC and 30 specimens of adjacent noncancerous tongue tissues were collected between January 2000 and December 2002 for immunohistochemical testing. All tissue samples were collected before chemotherapy or radiotherapy and were histologically and clinically diagnosed by the cancer center at Sun Yat-sen University in Guangzhou, China. The tumor stage of each patient was classified according to the 2002 AJCC staging system.[18] The median follow-up time of patients with immunohistochemical testing was 76 months for patients still alive at the time of analysis, and ranged from 8 to 128 months. This study was approved by the Institutional Review Boards. The samples were collected after receiving informed consent.

RT-PCR

The mRNA of OTSCC tissues was purified using TRIzol Reagent (Invitrogen, USA), and 1 μg of each sample was reversely transcripted using a TIAN Script Kit (Invitrogen, USA). The TRB3 sense primer was 5′-AGCAGAAATGCATCGAACAA-3′, and the antisense primer was 5′-CCTAACCAGATGAAGTTGCTGA-3′. PCR reactions were performed on a PTC-200 PCR system (Bio-Rad, USA) using the following cyclical procedure: 10 min at 94 °C, followed by 33 cycles of 30 s at 94 °C, 30 s at 54 °C, 30 s at 72 °C, and a final cycle at 72 °C for 10 min. For OTSCC cells, real-time PCR and data collection were performed with an ABI PRISM 7900 HT sequence detection system. The GAPDH housekeeping gene was used as an internal control to normalize expression levels of TRB3.

Western blot analysis

Cells were lysed with 1× sodium dodecyl sulfate (SDS) sample buffer (62.5 mmol/l Tris–HCl, 2% SDS, 10% glycerol and 5% 2-mercaptoethanol). The protein concentration was determined using a Bio-Rad protein assay. Total of 30 μg protein was separated electrophoretically in 15% SDS–polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membrane and incubated sequentially with primary and peroxidase-conjugated secondary antibodies, including rabbit monoclonal anti-TRB3 (1:5000, EPITOMICS, USA), rabbit polyclonal anti-p-AKT (Ser-473) and AKT (both of 1:1000, Invitrogen, USA) and anti-rabbit (1:2000, Santa Cruz, USA). After washing, the bound antibody complex was detected using an ECL chemi-luminescence reagent and XAR film (Eastman Kodak Company, USA). The Western blot bands were scanned and were analyzed by the Quantity One program (Bio-Rad, USA).

Immunohistochemistry assay

In brief, paraffin-embedded tongue tissue specimens were cut into 4-μm sections and baked at 65 °C for 30 min. The sections were washed with xylene and rehydrated. TRB3 sections were submerged for 2 min into an EDTA buffer at 95 °C and 90 kpa for antigenic retrieval. The p-AKT sections were submerged into EDTA and microwaved for 3 min for antigenic retrieval. Both types of section were treated with 3% hydrogen peroxide in methanol, followed by incubation with 1% rabbit serum albumin. The specimens were incubated overnight at 4 °C with anti-TRB3 (1:200, EPITOMICS, USA) and rabbit polyclonal anti-p-AKT (Ser-473, 1:100, Invitrogen, USA). For negative control, the primary antibody was replaced with the non-immune rabbit IgG of the same isotype. The degree of immuno-staining of sections was reviewed and scored by two independent pathologists. The proportion of cells expressing TRB3 and p-AKT were scored as follows: 0 (no expression), 1 (0–25%), 2 (26–50%), 3 (51–75%) or 4 (76–100%). The intensity of cell staining was scored as either 0 (no expression), 1 (yellow) or 2 (brown). The total score for proportion and intensity was calculated and divided into low expression (0–2) or high expression (3–6) for both TRB3 and p-AKT.

Cell lines and cell culture

OTSCC line Tca8113 was obtained from the Committee of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). CAL-27 cell line was obtained from American Type Culture Collection. All cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, USA), penicillin (100 U/ml), and streptomycin (100 U/ml) at 37 °C in a humidified 5% CO_2 incubator. All cells were plated one day prior to ERS inducer treatment.

Plasmids and transfection

For TRB3 expression plasmid, the full-length coding region of TRB3 cDNA was amplified by RT-PCR, and the digested and purified PCR products was directly cloned into a pEGF vector (Invitrogen, USA) to generate pEGF-C3-TRB3. For TRB3 knockdown plasmid, the annealed TRB3 shRNA oligonucleotides were cloned into pSUPER-retro-puro vector (Invitrogen, USA) to generate pSUPER-shTRB3. All plasmids were verified by sequencing. Stable transfected cell lines (Tca8113 scramble, Tca8113 shTRB3#1 and Tca8113 shTRB3#2) were created by retrovirus infection and chosen by antibiotic selection according to plasmid instruction.

Statistical analysis

All statistical analyses were carried out using SPSS 16.0 statistical software package. The $\chi 2$ test for proportion was used to analyze the relationship between TRB3 and p-AKT expression, the clinical and pathological characteristics. Survival curves were plotted using the Kaplan–Meier method and compared by log-rank test. The significance of variables for survival was analyzed by the Cox proportional hazards model in multivariate analysis. $P < 0.05$ was considered statistically significant.