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Expression of TRIM44 and its correlation with TLR4 in laryngeal squamous cell carcinoma

Jing Bai[1], Xiangyi Liu[2], Shurong Zhang[2], Shoutao Dang[2], Yong Zhang[3], Guojun Zhang[4*]

[1] Department of Clinical Laboratory, Beijing Tiantan Hospital Affiliated to Capital Medical University, NO. 119 South Fourth Ring West Road, Fengtai District, Beijing100050, China
[2] Department of Oncology, Beijing Tongren Hospital Affiliated to Capital Medical University, Beijing 100730, China
[3] Department of Pathology, Beijing Tongren Hospital Affiliated to Capital Medical University, Beijing 100730, China
[4] Department of Clinical Laboratory, Beijing Tiantan Hospital Affiliated to Capital Medical University, Beijing100050, China

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ABSTRACT

Some members of the tripartite motif-containing protein family have been reported as important regulators of carcinogenesis. In the present study, it was investigated whether tripartite motif-containing protein 44 (TRIM44) acts as a pro-oncogene through their over-expression in laryngeal squamous cell carcinoma. Its results showed that TRIM44 was up-regulated in tumor tissues and cell lines of laryngeal squamous cell carcinoma. In vitro, knockdown of TRIM44 significantly inhibited cell growth of laryngeal squamous cell carcinoma. Furthermore, TRIM44 knockdown inhibited tumor growth in nude mice in vivo, further suggesting the oncogenic activity of TRIM44 in laryngeal squamous cell carcinoma. Also, TRIM44 positively correlated with TLR4 at the mRNA and protein levels, and Si-RNA-NF-κB restrained laryngeal squamous cell carcinoma from proliferating. All indicated that TRIM44 might play a key role in tumor invasion through their over-expression and inhibition of TRIM44 is an effective strategy for the treatment of laryngeal squamous cell carcinoma.

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Introduction

With an increasingly high incidence, laryngeal squamous cell carcinoma (LSCC) accounts for approximately 90% of all laryngeal malignancies and is the second most common malignancy of the respiratory system (1-3). Currently, LSCC patients receive the surgical intervention or total laryngectomy before radiotherapy and chemotherapy as the main treatment option (4). However, these treatment modalities are only effective for patients in the early stages, whereas those with advanced LSCC have a low 5-year overall survival rate and are likely to experience LSCC recurrence mainly due to high invasiveness and acquired chemoresistance (5). Therefore, it is important to uncover the pathogenesis of LSCC in order to develop new treatment strategies.

The tripartite motif (TRIM)-containing protein family is a highly conserved group of proteins with three domains, consisting of a RING finger, one or two B-box motifs and a coiled-coil domain (6). They are involved in a variety of cellular processes, such as protein degradation, cell cycle progression, transcriptional regulation, DNA repair and signal transduction (7-9). Compared with most TRIM family members, TRIM44 contains a zinc-finger ubiquitin-specific protease (UBP) domain and acts as a deubiquitinating enzyme (10). Previous studies have linked TRIM44 to a variety of diseases across cancer, developmental disorders, neurodegenerative diseases and viral infections (11-13). Recent studies have further shown that TRIM44 is aberrantly overexpressed and acts as a proto-oncogene in several types of human malignancies, like prostate cancer (14), osteosarcoma (15), breast cancer (16), papillary carcinoma (17), gastric cancer (18), lung cancer (19), testicular germ cell tumors (20), and ovarian cancer (21). In addition, overexpression of TRIM44 is associated with poor prognosis and advanced clinicopathological features in multiple human malignancies (22,23). Although TRIM44 promotes migration and invasion of human cancer cells, its underlying mechanisms remain unclear during metastasis in laryngeal squamous cell carcinoma.

Toll-like receptor 4 (TLR4) is a pattern recognition receptor (PRR) expressed mainly on the cytoplasmic membrane of haematopoietic stem cells such as macrophages, monocytes and dendritic cells (24). TLR4 is activated by specific exogenous substances, including bacterial LPS and endotoxin, as well as by the well-known pathogen-associated molecular pattern (PAMP), which triggers innate immune responses and inflammation (25). Additionally, numerous studies have demonstrated that TLR4 is involved in the regulation of tumorigenesis and tumor progression (26). For example, TLR4 signaling promotes the migration of human melanoma cells (27). Several important inflammatory factors such as tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) have been shown to be frequently released from damaged tissues of LSCC patients. These inflammatory factors enhance the malignant process of LSCC by acting on TLR4 to activate main downstream signaling pathways (28). However,

* Corresponding author. Email: tiantanzgj@163.com

few reports focused on the correlation between TRIM44 and TLR4 in LSCC, so this study aimed to investigate the function of TRIM44 in LSCC and to explore the relevance of TRIM44 and TLR4 in LSCC.

Materials and Methods

Cells and tissues

Two LSCC cell lines (AMC-HN-8 and HEP-2) and the normal human bronchial epithelial cell (NHBEC) line were purchased from Bena Culture Collection. All the cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum. Twenty pairs of primary LSCC paraneoplastic and carcinoma tissues were pooled from Beijing Tiantan Hospital, Affiliated to Capital Medical University. All patients were pathologically confirmed, and fresh tissue was collected from them immediately after surgery and frozen in liquid nitrogen. Tissue collection was approved by the ethical review committee at Beijing Tiantan Hospital, Affiliated to Capital Medical University.

qRT-PCR

Total RNA was extracted from LSCC cells using Trizol reagent before cDNA was reverse transcribed with a PrimeScript kit according to the manufacturer's instructions. Next, qRT-PCR was performed using SYBR Green Mix. The primers were listed as follows: TRIM44, forward 5′-GGCTTGATTTGAGTACCTATT-3′, and reverse 5′-AGTCCACCTGAGTCTTTGC-3′; GAPDH, forward 5'-CACCCACTCCTCCACCTTTG-3', and reverse 5'-CCACCACCCTGTTGCTGTAG-3'.

Western blotting

LSCC cells were lysed with sodium dodecyl sulfate (SDS) and prepared for Western blotting. The primary antibodies used in this study consisted of anti-TRIM44 antibody, anti-GAPDH antibody, anti-TLR4 antibody, anti-AKT antibody, and anti-AKT antibody.

CCK-8

LSCC cells were infected with shNC, shTRIM44-derived lentivirus for 0, 1, 3, 5 and 7 days and then cell growth was assessed according to the manufacturer's instructions.

Plasmids and transfections

The pcDNA3.1 empty vector (EV) was used to construct a TRIM44 overexpression plasmid (TRIM44-OE). EV and TRIM44-OE plasmid were purchased from Genewiz (Suzhou, China). Lentiviral-delivered shRNA was provided by GenePharm (Suzhou, China). The sequence of shTRIM44 used was as listed below: shTRIM44, 5'-GCCTTTGAAGAATTAAGAAGC-3'.

In vivo testing

First, 5×10^6 HEP-2 cells infected with shNC or shTRIM44 were inoculated subcutaneously on the right side of 6-week-old nude mice (n=5 per group) provided by the Capital Medical University Model Animal Institute to assess theirs in vivo growth capacity. The tumor was measured with a vernier caliper in the nude mice every two days to calculate tumor volume using the formulation $V=(0.5\times length\times width)^2$ and then work out tumor growth curves. At the end of the experiment, tumors were excised for further study.

Statistical analysis

Data were expressed as mean ± standard deviation and analyzed using ANOVA and t-test. During the experiment, images were drawn using Graphpad Prism 8. When $p<0.05$, the analysis results were considered statistically significant.

Results

TRIM44 up-regulation in LSCC

TRIM44 expression in HNSC was predicted using the public tumor database GEPIA. As shown in Figure 1A, the database revealed that TRIM44 was significantly up-regulated in HNSC tumor tissues. To further confirm the expression, 20 pairs of paraneoplastic and carcinoma tissues were collected for qRT-PCR analysis. As illustrated in Figure 1B, TRIM44 was significantly up-regulated in LSCC tumor tissues compared to paraneoplastic tissues. Also, qRT-PCR and protein blotting results showed that TRIM44 was up-regulated in the two LSCC cell lines compared to normal human cell lines (Figures 1C and 1D). These findings suggest that TRIM44 is up-regulated in LSCC.

TRIM44 knockdown inhibits cell growth of LSCC

Subsequently, the TRIM44 gene was knocked down in AMC-HN-8 and HEP-2 cells to investigate its function in LSCC cells. A CCK-8 assay revealed that TRIM44 knockdown significantly inhibited LSCC cell growth (Figures 2A and 2B). Western blotting further showed that TRIM44 knockdown significantly downregulated the expression of its substrate TLR4 and suppressed the activation of its downstream signals, including p-AKT and NF-κ

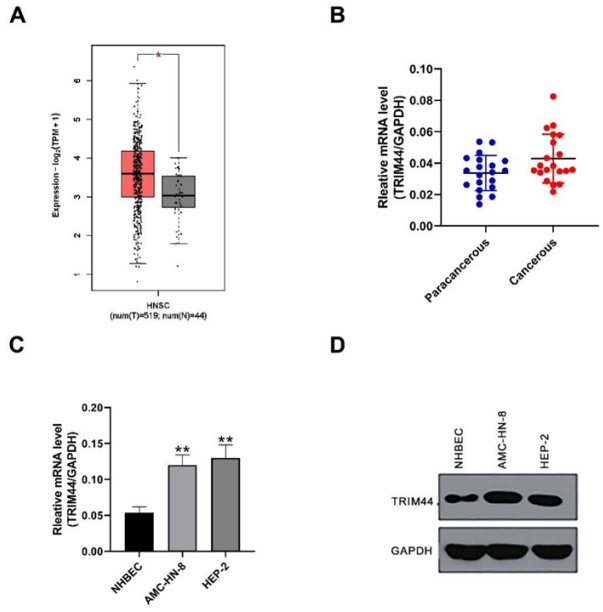

Figure 1. TRIM44 was significantly up-regulated in LSCC. A. Analysis of TRIM44 expression in HNSC using the GEPIA database. B. qRT-PCR for TRIM44 in primary LSCC paraneoplastic and carcinoma tissues. C & D. qRT-PCR and immunoblotting using LSCC cell lines (AMC-HN-8, Hep-2) and NHBEC. *p<0.05; **p<0.01.

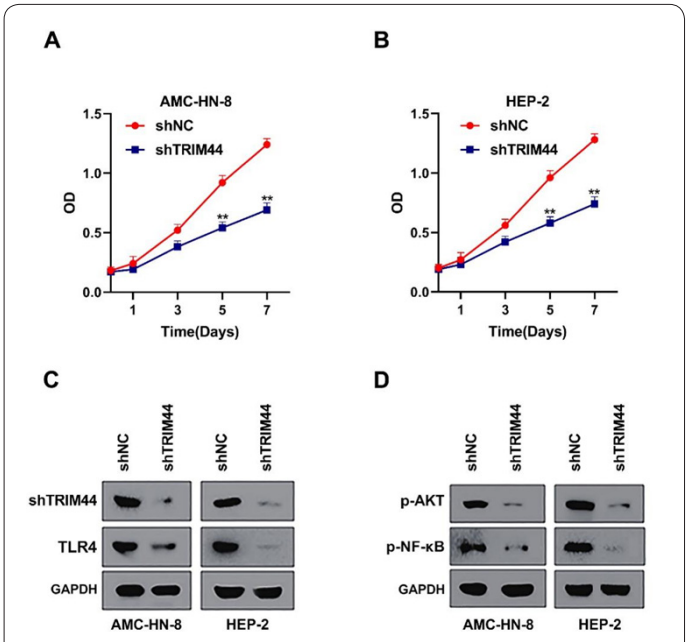

Figure 2. Knockdown of TRIM44 suppresses LSCC cell growth by inhibiting AKT and NF-κB activation. A & B. AMC-HN-8 (A) and HEP-2 (B) cells infected with shNC or shTRIM44 were cultured for 0, 1, 3, 5, 7 days to assess cell viability by CCK-8 assay. C & D. AMC-HN-8 and HEP-2 cells stained with shNC and shTRIM44 were cultured for 3 days before the expressions of TRIM44, TLR4 (C), p-AKT and p-NF-κB (D) were detected by western blotting. **p < 0.01.

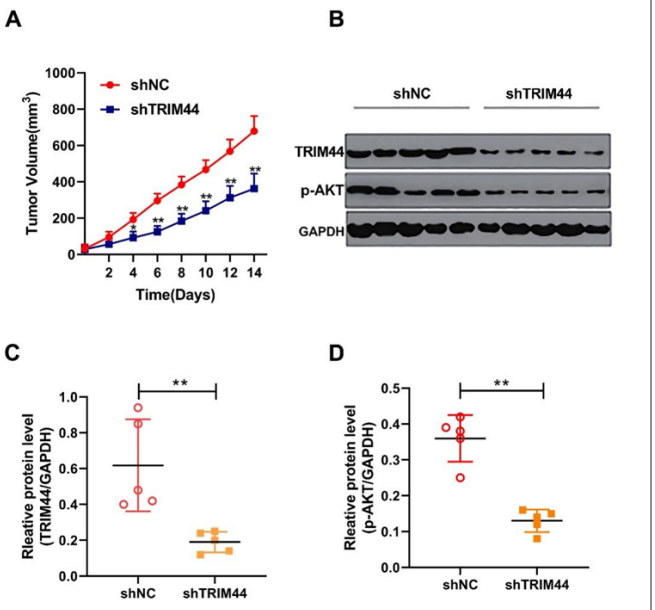

Figure 3. Knockdown of TRIM44 inhibits tumor growth in nude mice. A & B. shNC or shTRIM44-infected cells were inoculated subcutaneously into nude mice to assess their in vivo growth capacity and to calculate tumor growth curves (A). Tumors were prepared for protein blotting to assess TRIM44 and p-AKT expression (B), and then to measure optical density (C and D). *p < 0.05; ** p < 0.01.

b signaling, which was strongly associated with mediating tumor cell survival and growth (Figure 2C and 2D).

TRIM44 knockout inhibits tumor growth in nude mice

To further confirm how TRIM44 regulates LSCC cell growth in vivo, a xenograft model was established in this study. Tumor growth curves in Figure 3A showed that TRIM44 knockdown significantly inhibited tumor growth in nude mice. At the end of the animal experiment, tumors were excised and lysed for Western blotting. As shown in Figure 3B to Figure 3D, protein levels of TRIM44 and phosphorylated AKT were significantly reduced in the shTRIM44 group compared to the control group, indicating that TRIM44 exerts an oncogenic effect by regulating TLR4 signaling in LSCC.

Si-RNA-NF-κB inhibits the proliferation of AMC-HN-8 and HEP-2 cells

To further confirm how TRIM44 exerts oncogenic effects by modulating AKT /NF-κB downstream signaling of TLR4 in LSCC, Si-RNA-NF-κB was transfected into AMC-HN-8 and HEP-2 cells. The results showed that TRIM44, which is up-regulated in AMC-HN-8 and HEP-2 cells, was inhibited by transfection of Si-RNA-NF-κB (Figure 4A and 4B, $P < 0.01$). In addition, cell proliferation verification was performed. Cell proliferation was significantly reduced after transfection of Si-RNA-NF-κB plasmids in AMC-HN-8 and HEP-2 cells cells (Figure 4C and 4D, $P < 0.01$). This suggested that TRIM44, in combination with TLR4, regulates the downstream AKT /NF-κB pathway to play an oncogenic role.

TRIM44 is positively correlated with TLR4 at the mRNA and protein levels

To further investigate the correlation between TRIM44 and TLR4 in LSCC, the relative integral optical density

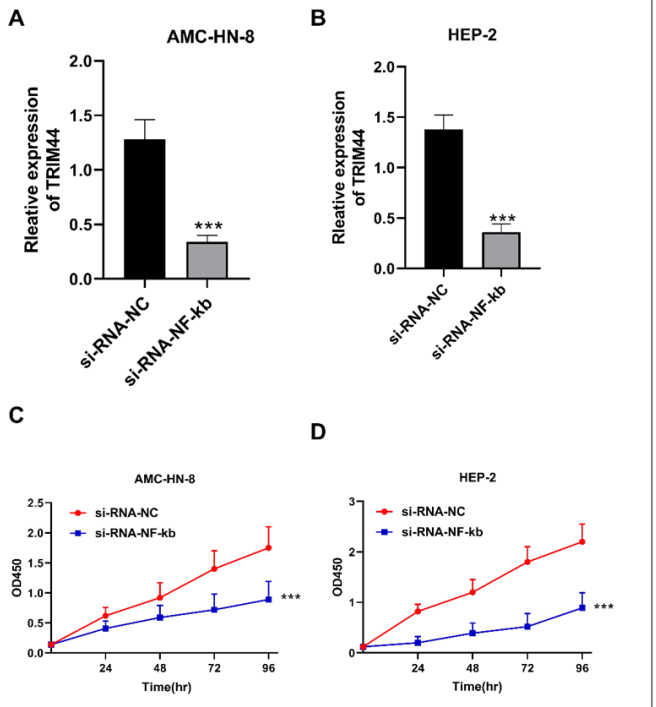

Figure 4. Inhibition of NF-κB expression suppresses TRIM44 expression. (A & B) Si-RNA-NF-κB inhibited TRIM44 expression in AMC-HN-8 (A) and HEP-2 (B) cells; (C & D) Si-RNA-NF-κB inhibited the proliferative capacity of AMC-HN-8 (C) and HEP-2 (D) cells. *** p < 0.001.

(IOD) values of TRIM44 and TLR4 were assessed as Spearman's correlation coefficient. As shown in Figure 5A and Figure 5B, TRIM44 was positively correlated with TLR4 at the mRNA and protein levels.

Discussion

TRIM44 has recently been identified as a novel onco-

gene that is overexpressed in a variety of human cancers. Li pang (29) et al. showed that high TRIM44 expression in endometrial cancer was associated with a poor prognosis in patients. According to Kawabata (30) et al., when acting as a regulator of NF-κB signaling, TRIM44 was a poor prognostic factor in breast cancer patients. Linchun Wen (31) et al. demonstrated that sesamin exerts anti-tumor activity in esophageal squamous cell carcinoma by inhibiting TRIM44 and NF-κB signaling (31). TRIM44 overexpression has been reported to induce cell carcinogenesis through direct binding and stabilization of TLR4 (32). Furthermore, NF-κB signaling is known as a downstream target of AKT and TLR4 (33). Therefore, it was hypothesized that TRIM44 expression is involved in the development of LSCC through activation of TLR4/NF-κB signaling.

The expression level of TRIM44 in HNSCs was first predicted using the GEPIA database. Then, qRT-PCR and western blotting were performed after 20 pairs of cancer tissues and paracancerous tissues, as well as tow cancer cell lines and a normal cell line, were collected. The results indicated that TRIM44 was significantly up-regulated in LSCC. Meanwhile, the knockdown of TRIM44 can significantly inhibit the cell growth and tumor growth of LSCC. These findings suggest that targeting TRIM44 may be a potential strategy for the treatment of LSCC.

TLR4 is a member of the TLR family that is expressed in various human cancer cell lines, such as sLNCaP, DU145, PC3, PANC-1 and BxPC-3 (34,35). Numerous studies have shown that TLR4 is involved in mediating inflammation and immunity (36) and in regulating tumorigenesis and tumor progression (37). For example, TLR4 may promote the immune escape of human lung cancer cells and support ovarian cancer progression (38,39). Knockdown of TLR4 has been reported to inhibit the growth of human NSCLC cancer cells (40). Furthermore, TLR4 is highly expressed in LSCC tissues and is involved in the regulation of cell adhesion and metastasis (41,42). However, its effects on LSCC cell proliferation and invasion have been less studied.

To further clarify the regulatory mechanism of TRIM44 in LSCC and its relevance to TLR4, Si-RNA-NF-κB was transfected into AMC-HN-8 and HEP-2 cells. The results showed that TRIM44 expression and cell proliferation were inhibited in LSCC cells. Also, correlation analysis on the IOD values of TRIM44 and TLR4 using Spearman's correlation coefficient revealed a positive correlation at the mRNA and protein levels. These findings suggest that TLR4 and NF-κ b signaling are involved in the regulation of TRIM44 in LSCC cells. This is consistent with previous reports on the introduction of TLR to mediate NFκB activation and indirect regulation of gene transcription processes associated with apoptosis, cell cycle, proliferation, pro-inflammatory and angiogenic cytokine secretion, and growth factors (43-45).

To put together, the findings suggest that TRIM44 is highly expressed in LSCC cells and is involved in the development of LSCC through the activation of TLR4/NF-κB signaling.

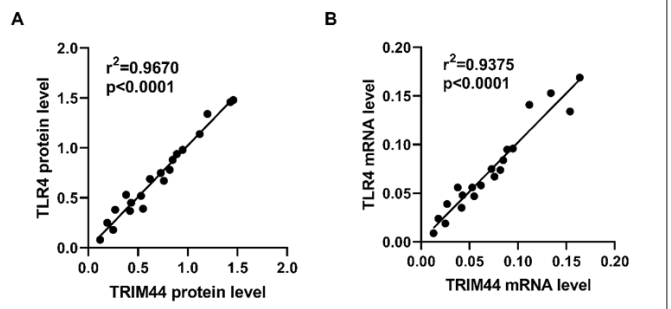

Figure 5. Correlation analysis on the IOD values of TRIM44 and TLR4 using Spearman's correlation coefficient. TRIM44 was positively correlated with TLR4 at the mRNA and protein levels.

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