Lentivirus-mediated shRNA interference of trefoil factor 3 blocks cell viability, migration and invasion in the papillary thyroid carcinoma cells

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Trefoil factor 3 (TFF3), a regulatory protein composed of 59 amino acids, has been suggested to be involved in pathogenesis, proliferation, invasion, migration and apoptosis in multiple malignant tumors. However, the roles of TFF3 concerning the viability, migration and invasion in papillary thyroid carcinoma cells have not yet been studied. This study aimed to investigate the effect of TFF3 knockdown on a thyroid papillary carcinoma TPC-1 cell line both in vitro and in vivo. In the present study, lentivirus-mediated short hairpin RNA (shRNA) targeting TFF3 plasmids were first constructed and stable TPC-1 cells were obtained while their TFF3 gene was silenced with either shTFF3-TPC-1, or a scrambled shRNA control. TFF3 expression was detected using quantitative real-time PCR and western blot analyses. The TPC-1 cell viability was measured by CCK-8 assay and colony formation. The cell migration and invasion were assessed by wound scratch assay and transwell filters. AKT phosphorylation, MMP-9, and BCL-2 expression levels were detected by western blot analyses. Our results showed that TFF3 knockdown significantly inhibits TPC-1 cell viability, migration and invasion. AKT phosphorylation, MMP-9, and BCL-2 levels were all remarkably depressed in TFF3 knockdown TPC-1 cells. Using a thyroid papillary carcinoma xenograft mouse model, we further investigated the effects of TFF3 knockdown in vivo. Significantly delayed xenograft emerging, slower growth rate and lower final tumor weights and volumes were observed in the shTFF3 group as compared to the control group. As expected, the expression levels of MMP-9 and BCL-2 in the xenograft are consistent with those of shTFF3-TPC-1 and shTFF3-TPC-1 cells in vitro. Our results suggest that TFF3 plays a vital role in the viability and oncogenesis of TPC-1 cells and may be a potential target for effective treatment of thyroid papillary carcinoma.

Key words: papillary thyroid carcinoma, trefoil factor 3, cell viability, migration, invasion

Papillary thyroid carcinoma (PTC) is the most common type of thyroid carcinoma, accounting for about 80% of thyroid carcinomas, and has a generally favorable prognosis with a mortality rate of less than 10%. PTC-related deaths, in excess of 30,000/year worldwide, are typically preceded by dedifferentiation and resistance to radioactive iodine treatment [1]. PTC is predominantly driven by genetic and epigenetic alterations, including activation of oncogenes and inactivation of tumor suppressor genes. Much still remains unknown about the molecular mechanisms of PTC, even though many genetic and epigenetic alterations have been identified in thyroid carcinoma, including trefoil factor 3 (TFF3), which was thought to be oncogenic in common human solid tumors. Krause et al. previously reported that TFF3 mRNA expression varies significantly between benign and malignant thyroid tissues and proposed that TFF3 might be used as a marker for discriminating between them [2]. We had verified the TFF3 overexpression in PTC tissues and confirmed the functional role of TFF3 in PTC lymph node metastatic progression [3]. To the best of our knowledge, the effect of TFF3 in the development of PTC had not yet been investigated. A better understanding of the biological mechanism involved in PTC is essential to devise better therapeutic strategies for PTC.

TFF3 is a regulatory protein composed of 59 amino acid residues. It plays an essential role in the maintenance and protection of epithelial surface integrity by forming a protective barrier, and acting as a motogen to facilitate cell migration. Recent studies have described the important role of TFF3 in the occurrence, proliferation, invasion, migration and apoptosis of multiple human tumors [4, 5]. Pandey et al. assessed the value of TFF3 expression in predicting metastatic and poor survival outcome of ER+ mammary carcinoma, demonstrating that siRNA inhibition of TFF3 reduced the
invasiveness of ER+ MC cells [6]. Moreover, Vestergaard et al. reported that TFF3 is overexpressed in prostatic carcinoma, and plasma TFF3 levels increase in patients with advanced prostate cancer [7, 8]. TFF3 knockdown had also been shown to significantly enhance the sensitivity of tumor cells to radio- or chemo-therapy, as well as inhibit tumor cell proliferation, invasion and migration [9]. TFF3 has also been described as a potent inhibitor of apoptosis and anoikis [10]. Since the underlying mechanisms and associated molecules remain unclear, this study aimed to explore the effects and mechanism of TFF3 in TPC-1 cells.

Using gene knockdown techniques, here we investigated the effect of TFF3 down regulation on TPC-1 cell viability, migration and invasion. The potential mechanisms mediating such effects were also explored.

Materials and methods

Cell lines. The human TPC-1, normal thyroid follicular epithelial cells Nthy-ori 3-1 and 293T cells were cultured in RPMI 1640 medium, F12K, and Dulbecco’s modified Eagle’s medium respectively, supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) and incubated at 37 °C with 5% CO₂.

Construction of TFF3 small hairpin (sh)RNA lentivirus vectors, virus packaging and infection of target cell line. TFF3 small hairpin RNA (shTFF3), the highest silencing efficiency targeting site 258 of human TFF3 (NM_003226), was designed to knockdown TFF3 expression [11]. In parallel, a scramble shRNA with no homology to any known human genes was used as a control (shCon). The stem-loop-oligos were designed and cloned into the lentiviral expression vector pLVX-shRNA2-Puro (Biowit Technologies, China), using the restriction sites EcoRI and BamHI (Takara Biotechnology, Dalian, China). The above plasmids, Lv-shTFF3 and Lv-shCon, were transformed into competent cells (Escherichia coli strain DH5α; Biowit Technologies, China), and extracted with a plasmid purification kit. Successful ligation was determined by polymerase chain reaction (PCR) and sequencing analyses. The recombinant expressing shTFF3 or control shRNA (shCon) vectors were next transfected into 293T cells. The culture supernatants expressing shTFF3 or control shRNA (shCon) vectors were then harvested and passaged 2:1, and cells were screened every 2 to 3 days with 5 μg/ml puromycin complete medium for 1 week, then screened for another week with 2 μg/ml puromycin complete medium to obtain a stable transfection in shTFF3-TPC-1 and shCon-TPC-1 cell lines. The efficiency of knocking down TFF3 was subsequently evaluated by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and western blot analysis.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR). Total RNA extraction, cDNA synthesis, and qRT-PCR were performed as previously described [12]. Briefly, total RNA was extracted according to the manufacturer’s instructions. Two micrograms of total RNA were then used to prepare the cDNA. A reverse transcriptase kit (Takara Dalian, China) was used for cDNA synthesis. The transcripts were quantified using an ABI 7500 Real-Time PCR system and SYBR® Premix Ex Taq™ II (Qiagen, UK), according to the manufacturer’s instructions. The primers used in each reaction were as follows: TFF3 forward: 5’-AATGACACCTTCTGAGGCACCT-3’ and reverse: 5’-CGTTAAGACATCAGGCTCCAGAT-3’; and β-actin forward: 5’-CCTGGGACATGGACTCTGTG-3’ and reverse: 5’-AGGGCGCCGACTGTCATAC-3’.

Western blot analysis. A total of 2×10⁵ cells (blank group TPC-1, shTFF3-TPC-1 and shCon-TPC-1) were seeded in each well of a 6-well plate and incubated for 72 h. The cells were then washed with ice-cold PBS and lysed in a modified RIPA buffer containing 1 mM DTT, 1 mM PMSF, and a complete protease inhibitor cocktail for 30 min. Protein was extracted using Mammalian Protein Extraction Reagent (Pierce Inc., Rockford, IL) and its concentration was determined by BCA (Pierce) assay. Supernatants were mixed with an equal volume of 2× loading buffer and boiled for 10 min. Total proteins (40 μg) were separated by 12% SDS-PAGE gel, and transferred to a polyvinylidine difluoride membranes (Millipore, Billerica, MA, USA). The membrane was blocked with 5% milk and incubated at room temperature for 1 h, then incubated with the following primary antibodies at 4°C overnight: TFF3 (sc-81467), Akt (sc-1619), p-Akt (sc-16646-R), MMP-9 (sc-393859) (Santa Cruz, CA, USA). All antibodies used in this study were diluted at a 1:300 ratios. The membranes were washed three times with Tris-buffered saline plus and incubated with a secondary antibody horseradish peroxidase(HRP)- conjugated goat anti-rabbit or mouse IgG f:6,000 (MultiSciences) for 1 h at room temperature, and the proteins were visualized using enhanced chemiluminescent reagents (32106, Thermo Scientific).

Wound scratch assay. Cell migration was monitored by a wound scratch assay. Briefly, the TPC-1, shTFF3-TPC-1 and shCon-TPC-1 cells were seeded in a 12-well plate and grown to confluence. A scratch wound was created using a sterile 10μl pipette tip, and incubated in medium with 0.5% FBS.
Phase contrast images were captured at 0, 6 and 12 h after the scratch using an inverted microscope (Nikon, Japan).

**Growth curve assay.** The three groups of cells were seeded in a 96-well plate (2,000 cells/well), and the number of cells was measured daily using a Cell Counting Kit-8 (CCK-8) assay (CA1210, Solarbio) for 5 days. 10 μl of reagent WST-8 was added to each well and incubated for 2 h at 37°C. The absorbance was measured at 450 nm with an ultraviolet spectrometer (Beckman Coulter, Brea, CA, USA). The experiments were performed in five parallel wells and repeated in triplicate from day 1 to day 5.

**Colony formation assay.** The procedures of colony formation assays were performed as described previously [13]. Briefly, TPC-1, shTFF3-TPC-1 and shCon-TPC-1 cells were seeded in 6-well plates at a density of 100 cells per well and continually cultured at 37°C with 5% CO₂ for 14 days. The colonies were then fixed and manually counted.

**Transwell invasion assay.** Double-chamber migration system was used for the transwell invasion assay. Briefly, TPC-1, shTFF3-TPC-1 and shCon-TPC-1 (1×10⁵) cells were seeded in serum-free medium in the upper chamber containing a thin layer of matrigel basement membrane matrix while 15% FBS medium was added to the lower chamber. After 24 h, cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for 20 min. Then the crystal violet was dissolved in DMSO, and the OD value was calculated using (length × width²)/2. After xenograft establishment mean tumor weight and volume immediately following sacrifice 28 days after tumor inoculation, and the volume of each tumor was calculated using (length × width²)/2. After xenograft primary tumors were harvested, one section of the tissue was fixed in formalin and embedded in paraffin for HE and immunohistochemical staining, and the other sections were frozen in liquid nitrogen.

**Statistical analysis.** Data were statistically analyzed using SPSS 13.0 (SPSS, USA). The data were presented as the mean ± SD of triplicate independent experiments. One-way analysis of variance (ANOVA) followed by LSD multiple comparison tests were used to evaluate the significance of differences among TPC-1, shTFF3 and shCon. All shTFF3 group compared with shCon group by a two-tailed Student’s t-test.

**Results**

**TFF3 expression in TPC-1 and normal thyroid follicular epithelial cells.** qRT-PCR and western blot and results showed that normal thyroid follicular epithelial cells Nthy-ori 3-1 express lower levels of TFF3 mRNA and protein expression compared to TPC-1 (p<0.05, Figure 1A, B).

**shTFF3 strongly suppressed TFF3 expression in TPC-1 cells.** Stable transfection was achieved in the cell lines, as shown in Figure 1C. The transduction of shTFF3 led to a considerable decrease in the TFF3 protein and mRNA expression compared with that in untreated TPC-1 cells or shCon controls (p<0.01, Figure 1D, E), whereas the expression of TFF3 mRNA and protein was similar in the two control groups.

**shTFF3 suppressed the viability, migration and invasion of TPC-1 cells.** The effects of TFF3 on the growth of TPC-1 cells were evaluated by a continuous 5-day CCK-8 assay. The shTFF3 group exhibited a remarkable inhibition of cell viability from day 2 to day 5, as compared with untreated TPC-1 cells or shCon control group (p<0.05, Figure 2A). These data indicate that TFF3 knockdown significantly decreased the viability of TPC-1 cells.

Cell migration and invasion were evaluated in the TFF3-knockdown TPC-1 cells. In addition, a wound healing assay (Figure 2B, C) and transwell invasion assay (Figure 2D) demonstrate that TFF3 knockdown inhibited cell migration and invasion (p<0.05).

**shTFF3 inhibits tumor growth in vitro and in vivo.** Furthermore, we assayed colony formation to determine the effect of TFF3 on TPC-1 cell tumorigenesis in vitro. The results showed that TFF3 knockdown in TPC-1 cells caused a substantial reduction in colony formation compared with shCon-TPC-1 cells. There were clear differences in the colony numbers between shTFF3-TPC-1 and shCon-TPC-1 (p<0.01, Figure 3A).

To confirm the tumorigenic effect of TFF3 in vivo, a xenograft model was used to compare the tumorigenesis of shTFF3-TPC-1 and shCon-TPC-1 cells. The antitumor efficacy of TFF3 knockdown was determined by considering mean tumor weight and volume immediately following sacrifice 28 days after inoculation (Figure 3B, C). The final tumor weights showed significant difference (Figure 3Ba) and volumes showed a significant decrease in the shTFF3 group as compared to shCon group (p<0.01, Figure 3Bb, Cc).

HE and immunohistochemical staining of the xenografts show (Figure 3D a–c) characteristics of cancer cells, such as pathological multiple nuclei and nuclear division phase, and with little connective tissue among the cancer cells. The TFF3 immunoreactive positive signal was located in the cytoplasm. The AOD value of TFF3 was significantly lower in shTFF3 cells than in shCon cells (Figure 3Dd, p<0.01). Thus, these data validate our in vitro findings and show that inhibition of TFF3 with lv-shTFF3 strongly inhibits thyroid tumor growth and viability in vivo, and indicate that TFF3 is critical for TPC-1 viability and tumorigenicity.

**shTFF3 inhibited PI3K/AKT, BCL-2, and MMP-9 expression.** The infection of lv-shTFF3 showed no impact on the expression of total AKT in TPC-1 cells and xenografts. To investigate whether the regulation of the PI3K/AKT survival pathway is involved in the shTFF3 induced inhibition of cell viability and invasion on TPC-1 cells and xenografts, the
Figure 1. TFF3 expression in Nthy-ori 3-1 and TPC-1 cells. (A) Relative expression of TFF3 mRNA by qRT-PCR analysis. (B) Relative expression of TFF3 protein by western blot analysis. The TFF3 mRNA and protein levels were lower than that of TPC-1 cells. *p<0.05. Inhibition of the TFF3 expression in TPC-1 cells by Lv-shTFF3. (C) Representative micrographs of shTFF3-TPC-1 and shCon-TPC-1 cell. Green fluorescent expressed in stable transfection TPC-1 cells with Lv-shTFF3 and Lv-shCon. (Left) Light microscopy; (Right) fluorescence microscopy; scale bar=100μm. (D) qRT-PCR analysis revealed that in the shTFF3 groups, the mRNA levels of TFF3 decreased significantly compared to shCon-TPC-1 and TPC-1. **p<0.01 vs. shCon and TPC-1. (E) Western blot demonstrated that the protein levels of TFF3 in shTFF3-TPC-1 cells were significantly decreased compared to shCon-TPC-1 and TPC-1 cells. **p<0.01 vs. shCon and TPC-1.
expression of BCL-2 and MMP-9 proteins was measured by western blot. The results indicate that the downregulation of TFF3 reduced the levels of p-AKT compared with shCon and TPC-1 (p<0.05) that further led to a decrease in BCL-2 and MMP-9 expression in both in vitro and in vivo xenografts (p<0.05, Figure 4A, B). Taken together, such data suggest that TFF3 effect on viability, migration and invasion of TPC-1 cells may involve the PI3K/AKT pathway.

Discussion

In this study, we explored the functions of TFF3 in human papillary thyroid carcinoma TPC-1 cells. Western blot and qRT-PCR analysis showed that TFF3 is expressed in normal and malignant thyroid cells with a higher expression in TPC-1 cells. We infected TPC-1 cells with Lv-shTFF3 and Lv-shCon to knockdown TFF3 expression and acquired stable infection of shTFF3-TPC-1 and shCon-TPC-1 for the first time. Next, the effect of TFF3 knockdown on TPC-1 cells was explored. It has been shown in previous studies that TFF3 affects the proliferation, apoptosis, migration and invasion of normal cells and various cancer cell lines. Sun et al. demonstrated that TFF3 promotes proliferation of gastric mucosal epithelial cells by activating the PI3K/Akt pathway [15]. Forced expression of TFF3 in mammary carcinoma and prostate cancer cells significantly increased cell proliferation [9, 16]. Ahmed et al. thought that although TFF3 protein expression is higher in normal breast and well-differentiated breast cancer, TFF3 has a more sinister role in breast cancer invasion and metastasis [17]. May's group confirmed that TFF3 is an independent biomarker of endocrine response in metastatic breast cancer. TFF3 stimulates migration and invasion of breast cancer cells and TFF3 protein is regulated by oestrogen and inhibited by antioestrogens [18]. A functional role for TFF3 in tumor angiogenesis has been determined in MCF7 cell, which may co-coordinate with the growth promoting and metastatic actions of TFF3 in mammary carcinoma to enhance tumor progression [19]. However, the expression and role of TFF3 in human PTC cell lines has not been investigated to date before this study.

Our study demonstrates that TFF3 knockdown markedly inhibits papillary thyroid carcinoma cell viability and migration in vitro. Colony formation assay showed that TFF3 knockdown in TPC-1 cells causes a substantial reduction in colony formation. Together, these results are consistent with previously published reports suggestive of the vital role which TFF3 plays in the development and progression of some solid malignant tumors such as breast, prostate, gastric and colon cancer [9, 16, 20, 21]. Upregulation of TFF3 confers a metastatic phenotype and correlates with a lower survival
Figure 3. The growth-suppressive effect of TFF3 knockdown on TPC-1 cells in vitro and in vivo. (A) TFF3 knockdown significantly reduced colony formation in TPC-1 cells by colony formation assay, **p<0.01. (B) The tumor volume curve of xenografted TPC-1 in group shCon and shTFF3. Xenografts were visible on the seventh day after the shCon-TPC-1 cells were implanted into nude mice and grew quickly, but on the tenth day after implantation in the shTFF3 group. The initial emergence and the growth rate of the shTFF3 group obviously lagged behind the shCon group. The volumes were smaller and the fluorescence was weaker in the shTFF3 group compared to the shCon group. (a) Histogram of comparison of xenografted weight between shCon and shTFF3, **p<0.01. (b) Significantly different volumes between shTFF3 group and shCon group from the 19th day to 28th day (n=12, *p<0.05,**p<0.01 vs. shCon). (C) (a) Photos captured by camera. (b) Bioluminescence images in group shTFF3 and shCon. (c) Tumor samples removed from xenografted model after the nude mice were sacrificed. (D) (a) Morphology structure of implanted tumor by HE staining. (b) and (c) TFF3 protein expression of implanted tumor in shCon and shTFF3 groups by IHC (scale bar=50μm). (d) Histogram of AOD (Average Optical density) value in different groups,**p<0.01.
rate in gastric, breast and rectal cancer [20, 22, 23]. In these cancers, TFF3 has been shown to promote tumor cell migration, invasion, proliferation, and angiogenesis [16, 24]. Our results show that knockdown of TFF3 in TPC-1 cells inhibits cell viability and migration, which is consistent with previous studies [6].

Our results show that the level of pAKT and MMP-9 was downregulated in shTFF3-TPC-1 cells and the cells showed decreased viability, migration and invasion. pAkt is an essential component of the PI3K/Akt signaling pathway which is an intracellular signal transducer and plays a key role in regulating cell proliferation, migration and apoptosis [25]. Our results indicate that the PI3K/Akt signaling pathway may be involved in the TFF3-mediated regulation of cell viability, migration and invasion in TPC-1 cells in vitro. Despite the absence of a specific receptor of TFF3, published studies show that TFF3 can affect the ERK1/2 and PI3K signal pathways through EGFR to activate several downstream effector pathways [26, 27]. The defined mechanism of TFF3 effects on TPC-1 cells remains to be clarified.

It is well known that MMP-9 is a major protease that degrades type IV collagen and environmental extracellular matrix (ECM), regulating cell behaviors related to migration and invasion [12]. Zheng et al. showed that the expression of MMP-9, which facilitates cell migration in the gastric cancer cell line SGC7901, was enhanced by TFF3 [28]. Chan et al. found that TFF3 promoted invasiveness of Rat-2 fibroblast cells by upregulating MMP-9 mRNA expression and suggested that TFF3 possessed malignant potential through the promotion of cell invasiveness and alteration of invasion-related genes [29]. Our results are consistent with the studies on glioblastoma multiform cells [30] and prostate cancer cells [31]. Yu et al. reported that MMP-9 may be activated by 5-aza C and promote migration and invasion of human fibrosarcoma HT1080 cells. The activity of MMP-9 is associated with the PI3k/Akt signaling pathways [32], therefore we deduced that the decreased viability, migration and invasion of shTFF3-TPC-1 cells may be related to the deregulation of pAkt and MMP-9.

We also observed that BCL-2 expression decreases in TPC-1 with TFF3 knockdown. Extensive studies have established the role of the BCL-2 family of pro-survival proteins in cell survival, proliferation, and chemoresistance in various tumors. BCL-2 is a protein that can promote cell survival and inhibit cell apoptosis by blocking the transmission of proapoptotic signals. The PI3K/Akt pathway has been implicated as an important signaling pathway for survival in a wide variety of cell types [33]. AKT phosphorylation at the Ser473 site is required for cell survival [34]. Gao et al. reported that knockdown of TFF3 expression facilitated human pituitary adenoma HP75 cell apoptosis by depressing BCL-2 and caspase-3 precursor proteins and elevating Bax and caspase-3 cleaved fragments [35]. Bratton suggested that AKT can regulate BCL-2 expression, promoting MCF-7 cell survival via differential activation of ERα and ERβ as well as regulation of GRIP1 [36]. We reasoned that the deregulation of pAkt and BCL-2 in shTFF3-TPC-1 cells may be related to viability.

We used a subcutaneous PTC xenograft model to validate the hypothesis that TFF3 silencing would inhibit papillary thyroid carcinoma cell growth and tumorigenesis. The stable
silencing of TFF3 in TPC-1 cell xenografts in nude mice was established. In vivo results demonstrate that the knockdown of TFF3 in TPC-1 cells not only delays xenograft formation but also the growth rate at later stage. The volumes of the xenografts from the TFF3 knockdown group were also smaller as compared to the control group. The final tumor weights showed a significant decrease in the shTFF3 group compared with the shCon group. These results indicate that TFF3 is critical for papillary thyroid carcinoma cell growth and tumorigenicity in vivo.

In conclusion, our work provides novel information on the function of TFF3 in the papillary thyroid carcinoma TPC-1 cell line, however, additional mechanisms of TFF3 function on other papillary thyroid carcinoma cell lines such as BCPAP, K1 etc., require further exploration.

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Reference

TFF3 EFFECTS ON PAPILLARY THYROID CARCINOMA CELLS


