INTRODUCTION

In 2016, estimated 64,300 new cases and 1980 deaths related to thyroid cancer were reported in men and women in the United States [1]. As the most common form, papillary thyroid cancer (PTC) accounts for 85–90% of all thyroid cancers [2]. Other types of thyroid cancers include follicular (FTC) and Hürthle cell (together with PTC are classified as differentiated thyroid cancers), medullary (MTC), and anaplastic (aggressive undifferentiated tumor) [3]. Currently, the preferred treatment for thyroid cancer is surgery, where complete excision is performed in approximately 86% of patients and partial in 12% of patients [4]. In selected patients (with FTC and PTC) radioactive iodine (RAI) remnant ablation is considered, to remove residual thyroid tissue and microscopic disease [5]. Nevertheless, surgical excision of the thyroid gland may damage the parathyroid glands and lead to disorders of calcium metabolism. The surgery may also result in voice changes due to the damage to laryngeal nerves [4]. Moreover, treatment with RAI has been associated with an increased risk of leukemia and fertility disorders in thyroid cancer patients [6].

Genetic and epigenetic alterations of several signaling pathways have been associated with the development of thyroid cancer, including gene mutations, increase in gene copy number and aberrant DNA methylation, thus providing novel prognostic and diagnostic markers as well as therapeutic targets [7,8].

microRNAs (miRNAs) are small noncoding RNAs that regulate gene expression by targeting mRNAs for cleavage or translational repression [9,10], and thus have a crucial role in fundamental biological processes, such as cell proliferation,
differentiation and apoptosis [11]. Previous studies have demonstrated dysregulation of a number of miRNAs in thyroid cancer; moreover, these RNAs showed variable expression patterns (upregulation and downregulation) between different histological types of thyroid tumors and at different stages of tumor progression [12-14]. For example, miR-222 and miR-146b were overexpressed in patients with recurrent PTC compared to the patients without recurrence [15]. The induction of lethal-7 (let-7f) miRNA in a human PTC cell line (TPC-1) that spontaneously harbors the RET/PTC1 rearrangement (which enhances proliferation and dedifferentiation of cells) caused reduced cell proliferation in TPC-1 and induced the expression of markers of thyroid differentiation, indicating a suppressor function of let-7 miRNA in PTC [16]. The involvement of various miRNAs in the development of thyroid tumors and different patterns of their expression related to the individual histological types suggest the use of miRNAs as diagnostic, prognostic, and markers of recurrence in thyroid cancer patients [13].

miR-369-3p has been implicated in the pathogenesis of several diseases. For instance, the expression of miR-369-3p was increased in serum samples and skin tissues of psoriasis patients, and was in positive correlation with disease severity [17]. In cisplatin (DDP)-resistant nonsmall cell lung cancer (NSCLC) tissues, the inhibition of miR-369-3p induced sensitivity of NSCLC cells to and suppressed their invasive capability in the presence of DDP, and conversely, the overexpression of miR-369-3p promoted resistance of NSCLC to DDP and enhanced their invasiveness [18]. However, the role of miR-369-3p in thyroid cancer is still not clear.

Tetraspanins (TSPANs) are cell surface proteins about 204–355 amino acids long, which contain four transmembrane domains, intracellular N- and C-termini, two extracellular domains (also called small and large extracellular loops), and one intracellular loop [19-20]. TSPANs play critical roles in many cellular processes, such as cell-cell adhesion, extracellular matrix-cell interaction, cell growth and survival [21-23]. In *in vitro* studies, downregulation of TSPAN1 inhibited the proliferation and invasion of colon cancer cells [24], overexpression of TSPAN2 in p53-mutated lung cancer cells was associated with cancer invasiveness and metastasis [25], while TSPAN8 contributed to exosome-induced endothelial cell activation in rat adenocarcinoma model [26]. More recently, it was shown that TSPAN13, a largely uncharacterized member of the TSPAN family, was upregulated in human breast cancer tissue and that the inhibition of TSPAN13 expression significantly decreased the growth rate of ZR-75-30 breast cancer cells [27]. Similarly, knockdown of TSPAN13 significantly inhibited colorectal cancer (CRC) cell proliferation and colony formation [28]. Whether TSPAN13 is involved in thyroid cancer initiation and progression is still not elucidated.

In this study, for the first time, we investigated the role of miR-369-3p in PTC and its association with TSPAN13. We showed that miR-369-3p is downregulated and the TSPAN13 gene is upregulated in PTC, FTC, and tall cell (TC) variant of PTC, and that the expressions of miR-369-3p and TSPAN13 are inversely correlated in thyroid cancer tissues. Both low expression of miR-369-3p and high expression of TSPAN13 were associated with a short survival time in thyroid cancer patients. Re-establishment of miR-369-3p expression in PTC cell lines inhibited the proliferation and promoted apoptosis in the cells probably by targeting TSPAN13.

**MATERIALS AND METHODS**

Bioinformatics analysis

To analyze the expression of miR-369-3p and TSPAN13 gene in thyroid cancer, expression profiles of miR-369-3p miRNA-seq and TSPAN13 mRNA were downloaded from the Cancer Genome Atlas database (TCGA dataset, https://gdc-portal.nci.nih.gov/) under the Illumina HiSeq 2000 RNA Sequencing Platform. A total of 572 specimens were available in the database, including 513 thyroid cancer and 59 normal thyroid tissues. According to the histological type, 513 thyroid cancer tissues were classified into PTC (*n = 363*), FTC (*n = 100*), TC variant (*n = 36*), and other unknown types (*n = 14*).

Cell lines and transfection

Human PTC cell lines (TPC-1 and GLAG-66) and human embryonic kidney 293T (HEK293T) cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS), 100 units/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA), and 0.1 mg/ml streptomycin (Enpromise, Hangzhou, China). All cells were maintained in a humidified incubator containing 5% CO₂ at 37°C.

The synthetic miRNA mimics for miR-369-3p, small interfering RNA (siRNA) for TSPAN13 (siTSPAN13) and their corresponding negative controls (NC mimics and siNC) were purchased from Genechem (Shanghai, China). For cell transfection assay, TPC-1 and GLAG-66 cells were seeded in 6-well plates, grown until they reached confluence of 40–50%, and then transfected with miR-369-3p mimics or siTSPAN13, or their corresponding negative controls, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) per the manufacturer’s instructions. After 48 hours of transfection, cells were harvested for functional analysis.

Quantitative reverse transcription PCR (RT-qPCR)

Total RNA was isolated from cells using TRIzol RNA Isolation Reagent (Invitrogen, Carlsbad, CA) according to
the manufacturer’s instructions, and reverse-transcribed by PrimeScript™ RT reagent kit (Takara, Dalian, China). Quantitative PCR was performed using the StepOnePlus Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). U6 small nuclear RNA or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene were used as internal controls. The relative expression of target genes was calculated using the 2^ΔΔCT method. The primer sequences were as follows: miR-369-3p forward 5´-TGACCTAGGGACTCCACCA-3´, reverse 5´-TAGCAATATTGCACAGAAGGC-3´; U6 forward 5´-CTCGTCGTTCCGACCGACA-3´, reverse 5´-AACGGTTTACACACTTCC-3´.

Cell proliferation assay

Cell Counting Kit-8 (CCK-8) assay was performed to determine cell proliferation in TPC-1 and GLAG-66 cells. After 48 hours of transfection, approximately 4 × 10^4 cells were seeded in a 96-well plate. After culturing for 48 hours, CCK-8 solution (Dojindo Laboratories, Japan) was added to each well and incubated for an additional hour. The absorbance of each well was detected in a microplate reader (BioTek, Winooski, VT, USA) at a wavelength of 450 nm.

Colony formation assay

For colony formation assay, after 48 hours of transfection, cells were seeded in another 6-well plate at 500 cells per well. Following approximately 2 weeks of culturing, colony formation was observed. Then, the colonies were fixed with 5% nonfat milk for 1 hour and incubated with biotinylated antibodies against TSPAN13 (13570-1-AP, 1:1000, Proteintech, Chicago, IL, USA) and GAPDH (10497-1-AP, 1:5000, Proteintech) at 4°C overnight. After washing with Tris-buffered saline/Tween 20 (TBST), the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000) for 2 hours at room temperature, followed by washing twice with TBST. The signals were detected with an enhanced chemiluminescence (ECL) western blot detection system (Millipore, Bedford, MA, USA).

Flow cytometry analysis of cell apoptosis

Cell apoptosis assay was performed using the APC Annexin V Apoptosis Detection Kit with 7-AAD (KeyGEN, Nanjing, China), according to the manufacturer’s instructions. Briefly, after 48 hours of transfection, cells were seeded into 6-cm dishes at a density of 50,000 cells per dish. Following another 48 hours of culturing, cells were collected, stained by APC Annexin V/7-AAD and analyzed by flow cytometry (BD Biosciences, USA).

Dual luciferase reporter assay

According the basic mechanism by which miRNAs regulate mRNA expression, the potential binding sites of miR-369-3p in TSPAN13 mRNA were predicted using online bioinformatics databases including TargetScan, miRanda, and miRWalk. The predicted wild-type and mutated miR-369-3p binding sites in the 3´ untranslated region (UTR) of TSPAN13 (TSPAN13 WT and TSPAN13 MUT, respectively) were amplified from human complementary DNA (cDNA) and inserted into the psicHECK-2 dual-luciferase expression vector (Promega, Madison, WI, USA). For reporter assay, HEK293T cells were cultured in 96-well plates and co-transfected with psicHECK-TSPAN13 WT/MUT and miR-369-3p or NC mimics using Lipofectamine 2000. Luciferase activity was measured with the Dual Luciferase Reporter Assay Kit (Promega, Madison, USA) at 48 hours after transfection.

Western blot analysis

Total protein was extracted from the cells using ice-cold RIPA lysis buffer (Beyotime, Shanghai, China). The protein concentration was quantified using a bicinchoninic acid (BCA) assay kit (Beyotime, Shanghai, China). Approximately 30 μg of protein were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Bio-Rad, Hercules, CA, USA) and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% nonfat milk for 1 hour and then incubated with primary antibodies against TSPAN13 (13570-1-AP, 1:3000, Proteintech, Chicago, IL, USA) and GAPDH (10497-1-AP, 1:5000, Proteintech) at 4°C overnight. After washing with Tris-buffered saline/Tween 20 (TBST), the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000) for 2 hours at room temperature, followed by washing twice with TBST. The signals were detected with an enhanced chemiluminescence (ECL) western blot detection system (Millipore, Bedford, MA, USA).

Statistical analysis

Statistical analyses were performed with GraphPad Prism version 6.0 (GraphPad, San Diego, CA, USA). All quantitative data are expressed as mean ± standard deviation (SD) from at least three independent experiments. Differences between NC mimics and miR-369-3p mimics or between siNC and siTSPAN13 were determined by Student’s t-test. For the bioinformatics analysis, the expression of miR-369-3p and TSPAN13 in normal tissues was compared with PTC, FTC and TC variant using the t-test. A correlation between miR-369-3p and TSPAN13 expression in thyroid cancer types was calculated using the Pearson correlation coefficient (PCC). In addition, 513 thyroid cancer tissues were classified into groups with high and low expression according to the median expression of miR-369-3p and TSPAN13, and the overall survival (OS) of patients in relation to the expression levels was analyzed by
the Kaplan–Meier method with the log-rank test. One-way ANOVA was used to compare miR-369-3p and TSPAN13 expression between normal tissue, PTC, FTC and TC groups. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Comparison of TCGA-derived miR-369-3p and TSPAN13 expression profiles between normal and thyroid cancer tissues

To explore the potential role of miR-369-3p and TSPAN13 in thyroid cancer, their miRNA-seq and mRNA expression profiles, respectively, were retrieved from the TCGA database and the differential expression was compared between normal and tumor tissues. As shown in Figure 1A, the expression of miR-369-3p was significantly downregulated in thyroid cancer tissues, including PTC and FTC, compared with normal thyroid tissues ($p < 0.001$). On the contrary, TSPAN13 mRNA expression was remarkably upregulated in PTC, FTC and TC variant tissues, in comparison with normal tissues (Figure 1B, $p < 0.001$). In addition, the PCC indicated a negative correlation between miR-369-3p and TSPAN13 expression in all thyroid cancer tissues (Figure 1C, $p < 0.001$, PCC = -0.2413). These results suggest that miR-369-3p and TSPAN13 play an important role in the development and progression of thyroid cancer.

Survival analysis of thyroid cancer patients based on TCGA-derived expression profiles of miR-369-3p and TSPAN13

We determined the prognostic value of miR-369-3p and TSPAN13 in thyroid cancer by analyzing the relationship between miR-369-3p or TSPAN13 and clinical follow-up information in thyroid cancer patients. We found that lower expression levels of miR-369-3p were associated with lower patient survival rates ($p = 0.01754$), and similarly OS was shorter in thyroid cancer patients with higher TSPAN13 expression (Figure 2, $p = 0.03205$). These findings indicate that miR-369-3p and TSPAN13 may be used as prognostic markers in patients with thyroid cancer.

Overexpression of miR-369-3p inhibited cell proliferation and induced apoptosis in PTC cell lines

The gain-of-function assay was performed in PTC cell lines, TPC-1 and GLAG-66, by transfection with miR-369-3p or NC mimics. As determined by qRT-PCR, miR-369-3p was overexpressed in both cell lines (Figure 3A, $p < 0.001$). The CCK-8 assay indicated that the cell proliferation ability was significantly reduced in TPC-1 and GLAG-66 cells transfected with miR-369-3p mimics compared to negative control (Figure 3B, $p < 0.001$). Consistently with CCK-8 results, the colony formation assay showed that the overexpression of miR-369-3p significantly inhibited colony formation ability of PTC cells, i.e., there was a reduction in the number of cell colonies in both cell lines (Figure 3C, $p < 0.001$). Further analysis by flow cytometry revealed that miR-369-3p overexpression promoted cell apoptosis in PTC cell lines, including early apoptosis (Annexin V+/7-AAD-) and late apoptosis [Annexin V+/7-AAD+] (Figure 3D, $p < 0.001$), which was consistent with miR-369-3p-induced suppression of cell proliferation in PTC cell lines.

miR-369-3p regulates the expression of TSPAN13 by directly targeting its 3′ UTR in PTC cells

The binding sites of miR-369-3p in TSPAN13 mRNA were predicted using online bioinformatics databases. As shown in Figure 4A, we found a broadly conserved binding site between the 3′ UTR of TSPAN13 and miR-369-3p. The dual-luciferase reporter assay was used to investigate whether...
miR-369-3p directly targets TSPAN13. Our results revealed that TSPAN13 expression was significantly decreased when wild-type TSPAN13 3’ UTR was co-transfected with miR-369-3p mimics, compared to co-transfection with NC mimics (Figure 4B, \( p < 0.001 \)). However, this effect was not observed, when the mutant TSPAN13 3’ UTR construct was used for co-transfection. Next, we analyzed the effect of miR-369-3p on the expression of TSPAN13 in PTC cells. As shown in Figure 4C, overexpression of miR-369-3p downregulated the expression of TSPAN13 at both mRNA and protein levels, in
TPC-1 and GLAG-66 cells (Figure 4C-E, \( p < 0.001 \)). The finding above suggests that miR-369-3p negatively regulates the expression of TSPAN13 by directly binding to its 3' UTR.

**Knockdown of TSPAN13 phenocopied the effect of miR-369-3p in PTC cells**

The correlation between miR-369-3p and TSPAN13 expression has been verified in thyroid cancer tissues retrieved from the TCGA database. Next, we performed loss-of-function assays to confirm whether TSPAN13 is a downstream effector of miR-369-3p. As shown in Figure 5A, the expression of TSPAN13 protein was remarkably downregulated in TPC-1 cells after transfection with siTSPAN13. Furthermore, we used CCK-8, colony formation and flow cytometry assays to evaluate the effects of knockdown of TSPAN13 on cell proliferation and apoptosis in PTC. Consistently with the effects of miR-369-3p overexpression, the knockdown of TSPAN13 significantly suppressed cell proliferation (Figure 5B and C, \( p < 0.01, p < 0.001 \)) and promoted apoptosis in TPC-1 cells (Figure 5D, \( p < 0.001 \)).

**DISCUSSION**

miRNAs have been associated with the development and progression of tumor in all types of analyzed human cancers, which is characterized by abnormal expression of miRNA transcripts in tumor compared to corresponding normal tissues [29,30]. In this study, we showed that miR-369-3p and TSPAN13 expression levels are inversely correlated in thyroid cancer tissues, and downregulation of miR-369-3p and upregulation of TSPAN13 were predictive of lower survival rates in patients with thyroid cancer. Moreover, our *in vitro* experiments demonstrated that TSPAN13 is a direct target of miR-369-3p, and that the siRNA-mediated silencing of TSPAN13 phenocopies the effect of miR-369-3p mimics in PTC cells, including decreased proliferation and accelerated apoptosis of cells. Overall, our results indicate that the downregulation of miR-369-3p and consequent upregulation of its target TSPAN13 are involved in pathophysiology of PTC.

Dysregulation of miR-369-3p has been reported in many diseases, including cancer. For example, the expression of miR-369-3p in DDP-resistant NSLC tissues was higher compared...
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to the normal tissues. In the same study, the inhibition of miR-369-3p sensitized lung cancer cells to DDP and attenuated their invasion capability by targeting human solute carrier 35F5 (SLC35F5) [18], Pan et al. [31] found that in Hirschsprung disease (HSCR) tissues miR-369-3p was significantly upregulated compared to adjacent normal tissues. Moreover, dysregulation of miR-369-3p (and its another predicted target, transcription factor SOX4) significantly suppressed cell proliferation and migration in human neuroblastoma (SH-SY5Y) and HEK293T cell lines [31]. Similar studies on the role of miR-369-3p in thyroid cancer are currently lacking. In the present study we showed for the first time that miR-369-3p is significantly underexpressed in PTC, FTC and TC variant tissues. Moreover, the downregulation of miR-369-3p was associated with poor survival rate in thyroid cancer patients, indicating that miR-369-3p may be used as a prognostic biomarker in thyroid cancer.

TSPANs are a large family of four-pass transmembrane proteins that are ubiquitously expressed in mammals, and emerging evidence supports their crucial role in human cancer [32]. Since TSPANs were first identified in human leukocytes, numerous studies reported that their expression correlates with tumor type, stage, and clinical outcome of patients [33]. TSPANs are expressed in almost all cell and tissue types [33] and are known to modulate major cellular processes such as cell growth, mortality, proliferation, survival, and adhesion; with this regard, they may have a tumor suppressor or promoter role in cancer [34]. Arencibia et al. [35] showed that TSPAN13 is overexpressed in prostate cancer tissues compared with adjacent normal tissues and that there was a direct correlation between TSPAN13 expression and presence of prostatic intraepithelial neoplasia (PIN) in tumor tissue, suggesting an important role of this protein in the development of prostate cancer [35]. Estrogen receptor (ER)-positive breast cancer cells in which TSPAN13 expression was inhibited showed a decreased growth rate and cell cycle arrest at G0/G1 phase; also, the knockdown of TSPAN13 in these cells affected the expression of downstream signaling molecules including downregulation of B-cell lymphoma 2 (Bcl-2), phospho (p)-Akt, p-mammalian target of rapamycin (mTOR) and upregulation of cleaved caspase-3 [27]. Similarly, a role of TSPAN13 as an oncogene was suggested in CRC, where the knockdown of TSPAN13 in CRC cell lines increased the expression of p27, cleaved caspase-3 and cleaved poly (ADP-ribose) polymerase (PARP) and decreased cyclin-dependent kinase 4 (CDK4) expression [28]. Our results showed increased expression of TSPAN13 in thyroid cancer tissues compared with normal tissues. Using bioinformatics analysis

**FIGURE 5. TSPAN13 knockdown suppressed proliferation and promoted apoptosis of papillary thyroid cancer (PTC) cells.** (A) Expression of tetraspanin-13 (TSPAN13) in TPC-1 cells was measured by western blot after transfection with small interfering (siRNA) against TSPAN13 (siTSPAN13). (B) The CCK-8 assay showed that the proliferation ability of TPC-1 cells was significantly decreased after TSPAN13 knockdown. (C) The colony formation assay showed that the rates of colony formation in TPC-1 cells were decreased after transfection with siTSPAN13. (D) Flow cytometry was used to analyze cell apoptosis in TPC-1 cells after transfection with siTSPAN13. The data shown are the means ± SD of three independent experiments. **p < 0.01, ***p < 0.001 vs. negative control siRNA.
and luciferase reporter assay, we also validated a putative binding site for miR-369-3p in the 3′ UTR of TSPAN13 mRNA. Moreover, the siRNA-mediated inhibition of TSPAN13 phenocopied the suppressive effect of miR-369-3p on the proliferation of PTC cells. Taken together, these results suggest a negative role of miR-369-3p in thyroid cancer development, and the underlying mechanism possibly involves targeting the TSPAN13 mRNA.

CONCLUSION

To the best of our knowledge, this study is the first to demonstrate that miR-369-3p is downregulated in human thyroid cancer tissues. Re-establishment of miR-369-3p by mimics transfection suppressed cell proliferation, colony formation and induced apoptosis in PTC cell lines. Our results support the potential role of miR-369-3p and TSPAN13 as biomarkers for diagnosis and prognosis in thyroid cancer. Understanding the molecular mechanisms underlying thyroid cancer tumorigenesis and metastasis is of key importance for developing new anticancer therapeutic strategies.

DECLARATION OF INTERESTS

The authors declare no conflict of interests.

REFERENCES


