MiR-599 serves a suppressive role in anaplastic thyroid cancer by activating the T-cell intracellular antigen

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Abstract. Anaplastic thyroid cancer (ATC) has a mean survival time of 6 months and accounts for 1-2% of all thyroid tumors. Understanding the underlying molecular mechanisms of carcinogenesis and progression in ATC would contribute to the identification of novel therapeutic targets. A previous study revealed that microRNA (miR)-599 was associated with tumor initiation and development in certain types of cancer. However, the specific functions and mechanisms of miR-599 in ATC are poorly understood. The objective of the present study was to identify its expression, function and molecular mechanism in ATC. The expression levels of miR-599 in 10 pairs of surgical specimens and human ATC cell lines were examined by reverse transcription-quantitative polymerase chain reaction. Function assays illustrated that miR-599 overexpression not only suppressed KAT-18 cell viability, proliferation and metastasis in vitro and decreased tumor growth in the tumor xenograft model but also induced cell apoptosis. Furthermore, T-cell intracellular antigen (TIA1), a tumor suppressor, was confirmed as a direct target of miR-599. It was demonstrated that TIA1 silencing rescued the inhibitory effect of migration and invasion induced by the overexpression of miR-599 in KAT-18 cells. In conclusion, the present study revealed that miR-599 inhibited ATC cell growth and metastasis via activation of TIA1. Therefore miR-599 may be a novel molecular therapeutic target for ATC.

Introduction

Thyroid cancer incidence is rapidly increasing in the USA. In 2017, an estimated annual diagnosis rate of 56 870 people and an annual mortality rate of 2 010 thyroid cancer cases were reported (1). Thyroid cancers are typically classified as papillary, follicular and anaplastic carcinomas. Anaplastic thyroid cancer (ATC) accounts for 1 to 2% of all thyroid tumours (2). ATC is characterized by aggressive, local invasion and common distant metastases. Available therapies for ATCs include chemotherapy, radiotherapy and surgery. However, no effective target treatment is available. ATC is still one of most fatal types of cancer, with a mean survival time of 6 months after diagnosis (3). Therefore, improved understanding of the molecular mechanisms underlying ATC carcinogenesis and progression will contribute to find novel diagnosis markers and therapeutic targets.

MicroRNA (miRNA) is a group of small noncoding RNAs that regulate gene expression by translation repression or messenger RNA (mRNA) degradation by binding to the 3'-untranslated region (3'-UTR) of the target Mrna (4). Increasing evidence indicate that miRNAs are involved in the regulation of cell survival, proliferation and migration through mediating the expression of their target genes. Recent studies on microRNA in thyroid tumours have provided new insights for the development of novel biomarkers that can be used to diagnose thyroid cancer and optimize its management (5-8). To date, however, our understanding of how miRNAs affect ATC development and progression remains unclear.

In the present study, we identified T-cell intracellular antigen (TIA1) as a direct target gene of miR-599 in ATC. Moreover, we provided evidence that miR-599 can promote ATC cell proliferation and metastasis in vitro and accelerate tumour growth in vivo by targeting TIA1.

Materials and methods

Cell culture and tissue collection. The ATC cell lines (SW1736 and KAT-18) and human immortalized follicular cell line (Nthy-ori3–1) were obtained from the ATCC (Manassas, VA, USA). The cell lines were authenticated via short-tandem repeat profiling performing by BMR Genomics. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone, Beijing, China) supplemented with 10% foetal bovine serum (FBS) (HyClone, Gaithersburg, Maryland, USA). All the cells were incubated
at 37°C in a humidified atmosphere with 5% CO₂. Human ATC specimens and adjacent normal thyroid tissues (10 pairs) were obtained from patients who underwent surgery according to an approved human protocol at the WeiHaiWei people’s hospital. All of the patient materials were obtained with written informed consent.

**Cell transfection.** A lentiviral Phhvl-u6-puro vector was purchased from GenePharma (Shanghai, China). Lentiviruses carrying miR-599 or miR-NC were packaged following the manufacturer's instructions. The sequences were as follows: miR-599 mimic, 5′-CUGUCCACAGUG UGUUUGUAAG-3′; miR-NC 5′-ACUACUGAGUGA CAGUAGA-3′. KAT-18 cells were grown in 6-well plates until they reached 50% confluency. The medium was replaced with 1 ml of fresh culture medium supplemented with 100 µl viral supernatant (1x10⁵ UT/ml) and 8 µg/ml Polybrene for 24 h. The KAT-18 cells were further cultured in medium containing puromycin at 3 µg/ml. Individual puromycin-resistant colonies were isolated during drug screening. Mammalian TIA1 expression plasmids were purchased from Genescript (Nanjing, China). An empty plasmid served as a negative control (control plasmid). SiRNAs designed to specifically silence TIA1 were purchased from GenePharma (Shanghai, China). A scrambled siRNA served as a control. The siRNA sequences were as follows: si-TIA1: TGCAACAACAAATGGCCA GTA. Transient transfection was carried out by using Lipofectamine 2000 Transfection Reagent (Invitrogen, CA, USA) according to the manufacturer's instructions.

**Cell viability assay.** Cell viability was detected via CCK-8 assay (Beyotime Institute of Biotechnology). The cells were trypsinized and seeded at 3,000 cells/well in a 96-well plate. After culturing for indicated time (0, 24, 48 and 72 h), 10 µl of the CCK-8 was added into each well. The resulting mixtures were incubated at 37°C for 3 h. Then, the absorbance of each well was examined by using a Multi-skan MK3 spectrophotometer set at a wavelength of 450 nm.

**Colony formation assay.** KAT-18 cells transfected with miR-599 or miR-NC or siTIA1 were seeded at 400 cells in six-well plates and cultured for approximately 10 d until colony formation was observed. Colonies were fixed with methanol and stained with 0.5% crystal violet (Sigma, USA). The colonies were photographed, and scored.

**Analysis of apoptosis.** The fraction of apoptotic cells was detected via Annexin V and propidium iodide (PI) staining method according to the manufacturer's protocol (KeyGEN BioTHCH, Nanjing, Jiangsu, China). Apoptotic cells were analyzed by using a Flow cytometer (Beckman, CA, USA). The results are presented as the percentage of apoptotic cells relative to the total number of cells.

**Cell migration and cell invasion assays.** Cell migration and invasion ability of KAT-18 were analyzed by polycarbonate membrane transwell inserts (BD Biosciences, Bedford, MA, USA). Briefly, the upper sides of the filters were coated with 50 µl Matrigel solution (matrigel: DMEM=1:8) for invasion. Cells were harvested at 48 h post transfection, and 1x10⁴ cells with 200 µl of serum-free medium were seeded in the upper chamber. The lower chamber was filled with medium supplemented with 5% FBS (invasion) or not (migration). Following incubation for 8 h (migration) or 12 h (invasion) at 37°C with 5% CO₂, cells on the lower filter were fixed with methanol, stained with crystal violet, and then counted under a light microscope (CKX41; Olympus Corporation).

**Real-time quantitative PCR (qPCR).** Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA) was utilized to operate qRT-PCR experiment. The primers used for amplification were: TIA1 forward 5′-TCCGCTCC AAGAGTACATAGG-3′, and reverse 5′-AAAATAGG CATGTGCTGACACCT-3′; miR-599 forward 5′-GGUGUUG UGACUUUAACAC-3′, and reverse 5′-GGUGGUUCUA GUUUAUACAC-3′; β-actin forward 5′-GATCATTGCTCTCG TTTCGCAGC-3′, and reverse 5′-CCAGTGCAGGTCGCC AGGT-3′; β-actin forward 5′-GATCATTGCTCTCG TTTCGCAGC-3′, and reverse 5′-ACTCCCTGTTGCTGATCCAC-3′.

**Western blot assay.** The method used for Western blot analysis has been described in our previous study (9). The primary antibodies were TIA1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), TGF-β1 (Abcam, Cambridge, MA, USA) and β-actin antibody (Beyotime, Nantong, China).

**In vivo tumorigenesis assay.** All experiments involving mice were performed in The Model Animal Research Center of Weifang medical University. Animal experiments were approved by the Animal Management Rule of the Chinese Ministry of Health and were performed in accordance with the approved guidelines and experimental protocols of Weifang medical University (Weifang, China). Female nude mice (4-week-old) were obtained from Jilin Laboratory Animal Center (Changchun, China), and bred in special pathogen-free (SPF) condition. 1x10⁴ KAT-18 cells transfected with lentiviral miR-599 mimic or lentiviral control were suspended in 100 µl serum-free medium and subcutaneously injected into the back of nude mice. Xenograft volume (V) was monitored by measuring the length (L) and width (W) with calipers and was calculated as V=0.5 x L (length) x W² (width). The tumor tissues were dissected, weighted, and stored at -80°C until use.

**Statistical analysis.** Statistical analyses were performed using SPSS 13.0 software. All experiments were performed in triplicate. Unless otherwise indicated, the data were evaluated as mean ± SD (standard deviation). Differences between two groups were assessed using Student’s t-test (two-tailed). Data of more than two groups were analyzed using one way ANOVA with post hoc test by Tukey’s test. Correlations between TIA1 and miR-599 were analyzed using Spearman rank correlation. P<0.05 was considered to indicate a statistically significant difference.
Results

**MiR-599 is downregulated in ATC tissues and cell lines.** In the present study, we firstly evaluated the expression level of miR-599 in 10 cases of human ATC tissues and their matched adjacent non-tumor tissues via qPCR. As indicated in Fig. 1A, miR-599 was markedly lower in ATC tissues compared to that in adjacent non-tumor tissues. Furthermore, qPCR results showed that miR-599 expression were also downregulated in ATC cell lines (SW1736 and KAT-18) compared to human immortalized follicular cell line Nthy-ori3–1 (Fig. 1B). The lower expression of miR-599 was detected in the KAT-18 cells. Then KAT-18 cell line was selected for further studies because of the lower expression levels of miR-599.

**MiR-599 reduces cell viability, proliferation, induces cell apoptosis in ATC cell line.** To examine the biological role of miR-599 in ATC, KAT-18 cells were transfected with miR-599 mimic or miR-NC. The data showed that miR-599 was significantly increased in cells transfected with miR-599 mimic compared to cells transfected with miR-NC by using qPCR (Fig. 2A). Cell viability of KAT-18 was detected via CCK-8 assay. The results showed that overexpression of miR-599 in KAT-18 cells significantly inhibited cell viability (Fig. 2B). Furthermore, the colony formation assay illustrated that the proliferation of miR-599 overexpression cells was severely inhibited (Fig. 2C and D). In addition, cell apoptosis was investigated in KAT-18 cells transfected with miR-599 mimic or miR-NC. As showed in Fig. 2E and F, overexpression of miR-599 could significantly increase cell apoptosis ratio in KAT-18 cell line.

**TIA1 is a direct target of miR-599.** TargetScan was used to identify the target of miR-599. TIA1 was predicted to be a target gene of miR-599 (Fig. 3A). The WT-TIA1-3’-UTR (Fig. 3A) or MT-TIA1-3’-UTR (Fig. 3A) luciferase reporter vector was generated. The luciferase reporter assay was performed to confirm the relationship of miR-599 and TIA1 in KAT-18 cells. The cells were co-transfected with miR-599 and either wild type or mutated TIA1-3’-UTR reporter. As shown in Fig. 3B, the luciferase activity was markedly enhanced only in KAT-18 cells co-transfected with miR-599 mimics and WT-TIA1-3’-UTR vector, illustrating that miR-599 could directly bind to the 3’-UTR of TIA1 mRNA in ATC cells. Furthermore, we examined the expression of TIA1 in KAT-18. Western blot assay showed that the protein level of TIA1 was markedly enhanced in miR-599-overexpressing KAT-18 cells compared to the control group (Fig. 3C). However, the mRNA expression of KAT-18 was not affected after overexpression of miR-599 (Fig. 3D). Therefore, miR-599 inhibited the expression of its target TIA1 at the post-transcriptional level. Furthermore, we found that the mRNA expression of TIA1 in ATC was significantly downregulated compared with adjacent noncancerous tissues (Fig. 3E) and was positively correlated with miR-599 in ATC tissues (Fig. 3F). To exclude the off-target effect of miR-599 on TIA1, western blot was used to examine the expression level of TGFβ2, which has been identified as a direct target gene of miR-599 (10). The results showed that the expression of TGFβ2 was significantly decreased in KAT-18 transfected with miR-599 mimic (Fig. 3C).

**TIA1 overexpression inhibits ATC cell viability, proliferation, migration and invasion in vitro.**

To further determine whether TIA1 plays a critical role in cell proliferation, we performed in vitro gain-of-function analyses by overexpressing TIA1 with a plasmid in KAT-18 cells. The western blot results showed that TIA1 was significantly overexpressed in KAT-18 cells (Fig. 4A). Cell viability of KAT-18 was detected via CCK-8 assay. The results showed that overexpression of TIA1 in KAT-18 cells significantly inhibited cell viability (Fig. 4B). Furthermore, the colony formation assay illustrated that the proliferation of TIA1 overexpression cells was severely inhibited (Fig. 4C and D). In addition, the transwell migration and invasion assay showed that the TIA1 silencing significantly promoted the migration and invasion of KAT-18 cell (Fig. 5A-C).

**MiR-599 inhibits cell migration and invasion via TIA1 in KAT-18 cell line.**

Figure 1. miR-599 is downregulated in ATC tissues and cell lines. (A) Relative miR-599 expression levels were analyzed by reverse transcription-quantitative polymerase chain reaction in 10 pairs of ATC specimens and adjacent normal tissues. (B) Levels of miR-599 were significantly lower in the SW1736 and KAT-18 cell lines compared with the human immortalized follicular cell line Nthy-ori3–1. U6 RNA levels served as an internal control. *P<0.05. ATC, anaplastic thyroid cancer; miR, microRNA.
The migration and invasion of cell plays an important role in tumor metastasis. Thus, the effect of miR-599 overexpression on the migration and invasion of ATC cells was examined in vitro. The ability of migration and invasion was examined via transwell migration and invasion assay. The data illustrated that miR-599 mimic markedly inhibited the cell migration and invasion (Fig. 5A-C).

To discover the functional correlation of TIA1 targeting by miR-599 in ATC cell, we assessed whether TIA1 silencing reversed miR-599 biologic function of cell migration and invasion. KAT-18 cells were co-transfected with miR-599 mimic or miR-NC and siTIA1 or sicontrol. The western blot results illustrated that the protein level of TIA1 was markedly downregulated in miR-599 TIA1 mimic combination with siTIA1 compared to miR-599 combination with sicontrol (Fig. 5D). Furthermore, the results showed that siTIA1 could partially abrogated effect of miR-599 mimic on cell migration and invasion (Fig. 5A-C), suggesting that miR-599 suppressed human ATC cell migration and invasion via the activation of TIA1.

**MiR-599 promotes ATC growth in vivo by targeting TIA1.** At last, we showed the function of miR-599 in the growth of ATC xenograft. Nude mice were subcutaneously implanted with KAT-18 cells transfected with lentiviral miR-599 and lentiviral control. On day 30 after implantation, all mice were sacrificed and obtained the tumor xenograft (Fig. 6A). Moreover, the growth rate (Fig. 6B) and weight (Fig. 6C) of the tumor xenograft were significantly decreased in the miR-599-overexpressing group when compared to the control group. qPCR analysis of miR-599 expression levels in the miR-599 mimics tumor xenograft mice and control tumor xenograft mice (Fig. 6D). The western blot results showed that the protein level of TIA1 was higher in the tumor transfected miR-599 mimic (Fig. 6E).
Figure 3. TIA1 is a direct target of miR-599. (A) Sequence alignment of miR-599 with 3'-UTR of TIA1 as predicted by TargetScan. (B) Luciferase assay results for KAT-18 cells revealed that miR-599 upregulation enhanced luciferase activity. (C) TIA1 and TGFβ2 protein expression in KAT-18 cell lines was examined by western blot analysis. β-actin served as the loading control. (D) TIA1 mRNA expression in KAT-18 cell lines transfected with the miR-NC or miR-599 mimic was examined by reverse transcription-quantitative polymerase chain reaction. (E) TIA1 mRNA level was determined in ATC tissues and adjacent normal tissues. β-actin served as the loading control. (F) Spearman's correlation analysis examined the correlation between the protein expression levels of TIA1 and miR-599 in ATC. *P<0.05. miR, microRNA; NC, negative control; UTR, untranslated region; TGF, transforming growth factor; TIA1, T-cell intracellular antigen; Mut, mutant; Wt, wild type.

Figure 4. TIA1 overexpression inhibits ATC cell viability and proliferation in vitro. (A) Western blot assay examined the expression of TIA1 in the KAT-18 cell line transfected with TIA1 vector or a control vector. (B) Cell viability and (C and D) proliferation were determined by a CCK-8 and colony formation assay. *P<0.05. ATC, anaplastic thyroid cancer; miR, microRNA.
Discussion

A comprehensive description of the molecular mechanisms underlying ATC initiation and progression will facilitate the novel biomarker identification for early ATC diagnosis and therapy, thereby improving the outcome of patients with ATC. Over the past decade, miRNAs have emerged as a new class of gene regulators involved in a variety of cancers (11). The present study demonstrated for the first time that miR-599 inhibits the tumour suppressor gene TIA1 to promote cancer proliferation in ATC. This study also firstly showed TIA1's function in ATC. Thus, miR-599/TIA1 axis can be used for early diagnosis and it can also be used as a potential target to treat ATC.

Previous studies have suggested the role of miR-599 as a potential anti-onco-miRNA in various types of cancers. A previous study has shown that miR-599 is frequently down-regulated in human gastric cancer, and low miR-599 expression is associated with lymph node metastasis. In addition, miR-599 overexpression suppresses gastric cancer cell migration and invasion and the epithelial–mesenchymal transition process.
dramatically (11). Furthermore, miR-599 may act as a tumour suppressor, playing an important role in regulating lung cancer, glioma, hepatocellular carcinoma and breast cancer metastasis (12-15). However, the function and regulatory mechanisms of miR-599 in ATC remains obscure. Here, miR-599 expression was significantly downregulated in human ATC tissues and cell lines compared with the adjacent normal tissues and cell lines. Moreover, miR-599 overexpression significantly inhibited the ATC cell viability, colony formation, migration and invasion in vitro. MiR-599 overexpression also suppressed ATC tumour growth in vivo. These data suggested that miR-599 may function as a tumour suppressor in ATC.

One of our new findings in the current study indicated that TIA1 is the target gene of miR-599 during the ATC progression inhibition. TIA1 is a RNA binding protein, which is linked to multiple biologic processes associated with RNA metabolism both in the nucleus and in the cytoplasm (16). A recent study has shown that TIA1 serves as a tumour suppressor gene. In addition, TIA1 regulates or interacts with many types of mRNA involved in cancer cell proliferation, apoptosis, angiogenesis, invasiveness and metastasis in many types of cancer (17-20). However, a subsequent study has shown that TIA1 may be a novel oncogenic function of TIA1 in oesophageal squamous cell carcinoma (21). Therefore, whether or not TIA1 is a tumour promoter or suppressor remains controversial. These contradictory results reveal that the function of TIA1 in different tissues depends on the cancer type and tumorigenesis mechanism. Therefore, future research must demonstrate the conclusive function of TIA1 in different types of cancer. The present study demonstrated that TIA1 may be a tumour-suppressor gene, which inhibits ATC cell viability, proliferation and metastasis. Furthermore, because of the myriad of TIA1 tumour-suppressor functions, we elucidated the mechanisms underlying TIA1 regulation during tumorigenesis in ATC. In this study, we firstly demonstrated that TIA1 was regulated by miR-599 in ATC. Furthermore, we demonstrated that TIA1 is a direct target gene of miR-599 via luciferase assay in ATC. However, miRNAs have unique ability to regulate many protein-coding genes. A single miRNA can target a number of genes, thereby regulating numerous biological processes. Therefore, future studies need to identify the new molecular pathways regulated by the tumour-suppressive miR-599.

In conclusion, miR-599 is significantly downregulated in the ATC clinical specimens. MiR-599 also functions as a tumour suppressor in ATC through the regulation of TIA1 expression. Identification of the tumour-suppressive and miRNA-mediated cancer pathways in human ATC can provide new information on the potential therapeutic targets to treat ATC.
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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

JWB and WBC conceived and designed the experiments. WBC wrote and revised the manuscript. YLZ and JTQ conducted all experiments. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of Weihaiwei People's Hospital (Weihai, China) and written informed consent was obtained from all patients prior to their inclusion within the study.

Patient consent for publication

All patients provided written informed consent for the publication of their data and any accompanying images.

Competing interests

The authors declare that they have no competing interests.

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