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MiR-610 Restrains the Proliferation and Invasion in Thyroid Cancer Cells by Modulating TAZ Expression

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ABSTRACT In this study, microRNA-610 (miR-610) was used to evaluate the expression and function on the development process of thyroid cancer. The expressions of miR-610 in tissues and cells were quantified by using qRT-PCR. The proliferation and invasion in cells were determined by MTT along with Transwell assay. The relationship between miR-610 and FAZ was measured in the dual luciferase reporter. Besides, TAZ protein expression was evaluated using a Western blot. MiR-610 showed low expressions in the following tissues and cell lines. Functionally, silence of miR-610 enhanced theproliferation and invasion of cells. TAZ was verified to ba a direct target gene of miR-610. Subsequently, the proliferation and invasion of cells were suppressed when TAZ was silenced. Besides, it was demonstrated that the inhibitory effect on cells induced by miR-610 was reversed by TAZ vector. miR-610 was revealed to restrain cells' proliferation and invasion by making TAZ expression restrain in thyroid cancer (TC).

INTRODUCTION

Thyroid cancer, abbreviated TC, is an extremely common malignant tumour in the endocrine system, and its morbidity is increasing in the world (Choudhury et al. 2020). It was known that based on a new report from the International Agency for Research on Cancer (IARC) in 2018, there are 567,233 newly added cases of thyroid cancer, which ranks the ninth among all cancers (Bray et al. 2018). At present, the most important treatment method is radical resection of thyroid cancer. Most patients have a good initial overall survival rate, but the prognosis of some patients is poor (Wang et al. 2018). The cause of TC is not known. Consequently, it is urgent to investigate thoroughly the pathogenesis and progression of TC and to find new therapeutic strategies and effective interventions for the thyroid cancer treatment.

So far, some research experts found that microRNAs (miRNAs) acted an essential part in various cancers (Wang et al. 2019; Ma et al. 2017). MiR-299-3p was discovered to repress cell proliferation and progression, while facilitating cell apoptosis in thyroid cancer (Chen et al. 2019a). Fan and Zhao (2019) verified that miR-451a suppressed the growth, epithelial-mesenchymal transition and induced apoptosis in TC cells. Inversely, miR-652-3p was proved to promote the TC cell cycle and cell proliferation by restraining CDKN1A expression, and provided to be a new therapeutic target for TC therapy (Lei et al. 2016). Recently, miR-610 has been verified to be an aberrant expressed in many human tumours. For instance, miR-610 was found to be low-expressed in lung cancer, and could inhibit lung cancer cells' proliferation and invasion through targeting GJA3 (Jin et al. 2014). Similar function has been identified in colorectal cancer and glioma (Sun et al. 2015; Yan et al. 2016). It must be noted, until now, the role and function of miR-610 has still not been clarified in thyroid cancer. In addition, a previous study has shown that TAZ may function as an oncogene in ovarian cancer (Cao et al. 2019). And also, TAZ was confirmed to be highly expressed and promoting the proliferation, migration and invasion of gastric cancer cells (Zuo et al. 2015). Nevertheless, the correlation between miR-610 and TAZ has not yet been reported in the progression of thyroid cancer.

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Objectives

This study is designed to research the expression of miR-610 and its effects on the thyroid cancer. In addition, the reciprocal action of miR-610 and TAZ in genesis and development of thyroid cancer was also analysed.

METHODOLOGY

Clinical Specimens

Fifty-four thyroid cancer tissues and paracancer tissues were acquired from a hospital called The Third People's Hospital of Qingdao affiliated to Shandong province (Qingdao, China). All the specimens were put into liquid nitrogen, and were next placed in an -80°C refrigerator for the experiments. The experimental patients had not been treated by preoperative radiotherapy, chemotherapy, radiofrequency ablation or other adjuvant therapy. Before the experiment, all the patients signed written informed consents. These experiments were conducted with the approval from the Ethics Committee of the above hospital.

Cell Cultured and Cell Lines

Human TC cell lines (KFC-133, SW579 and TPC-1) along with Nthy-ori3-1 were all from the Chinese Academy of Sciences in Shanghai of China. They were incubated in the DMEM added with foetal bovine serum (10%), penicillin (100 U/mL) and streptomycin (100 U/mL). The culture condition is under 5 percent CO_2 at 37°C. Later, the Lipofectamine 2000™ reagent was applied for transfecting miR-610 mimic, miR-610 inhibitor and the negative controls (GenePharma, Shanghai, China) into cells (Yan et al. 2016). The cells were used for the experiments when they were transfected for 24 hours.

Methyl Thiazolyl Tetrazolium (MTT) Assay

The thyroid cancer cells (concentration: 5×10^3/well) were put into a 96-well plate. Cells in wells were filled with MTT reagents (20 μL, M1020-500T, Solarbio, Bejing, China), and cultured for 4 hours at 37°C again. DMSO (100 μL) was joined to dilute the crystals sufficiently after the supernatant was removed. Finally, the researchers determined the relative optical density values (OD) at 490 nm through the use of a microplate reader.

Transwell Assay

Firstly, TPC-1 cells were in serum-free medium and then they were dropped into the upper cavity (8 μm). Low cavity was filled with DMEM containing 10 percent foetal bovine serum FBS. Methanol (YZ-1601623, Solebao, Beijing, China) was added into the cells as a fixer after the above cells were incubated for 48 hours. Then, TPC-1 cells were stained by 0.1 percent crystal violet (IC0600, Solibao). At last, the number of invasive cells was counted using the optical microscope under magnification of 200 times.

Quantitative Real-time Quantitative PCR

All RNAs were isolated from tissues and cell lines by the TRIzol® reagent (Takara Bio, Inc.). The expressions of miR-610 and TAZ were clarified using a LightCycle 96 thermocycle. The expressed level of miR-610 was normalised by GAPDH as endogenous controls, while TAZ expression was normalised to β-actin.

MiR-610, forward: 5´-UGAGCUAAAUGUGUGCUGGGA-3´; reverse: 5´-CCAGCACACAUUUAGCUCAUU-3´.

TAZ, forward: 5´-ACCGTGTCCAATCACCAGTC-3´; reverse: 5´-TCCAACGCATCAACTTCAGGT-3´.

GAPDH, forward: 5´-CCTGACCTGCGTGTGGACT-3´; reverse: 5´-GCTGTGGATGGGGAGGTGTC-3´.

β–actin, forward: 5´-CCACTGGCATCGTGATGGA-3´; reverse: 5´-CGCTCGGTGAGGATCTTCAT-3´.

The $2^{-\ddot{A}\ddot{A}Ct}$ method was utilized to assess the above data (Livak and Schmittgen 2001).

Dual Luciferase Reporter

Next, the target genes that bind to miR-610 were predicted by TargetScan version. The dual luciferase reporter assay checked whether there existed a relationship between miR-610 and TAZ. TPC-1 cells were transfected with TAZ-wt or TAZ-mut and miR-NC or miR-610 mimic through

a Lipofectamine 3000 transfection reagent (Invitrogen, USA), and then cells were put into the 48-well plates and the culture conditions were 37°C, 5 percent CO_2 and about 95 percent humidity. After they were cultured for 48 hours, the luciferase activity of TAZ was examined by the kit (Promega Corporation).

Western Blot Assay

The samples were prepared by using the TRIZOL method (Kirkland et al. 2006). Protein concentration was measured with a bicinchoninic acid kit (Shanghai Weimeng Biotechnology Co., Ltd, Shanghai, China). Total protein was separated by the SDS-PAGE (10%, P1203, Solebao, Beijing, China) and transfected immediately onto a polyvinylidene difluoride (PVDF) membrane. Following blocked with a bovine serum albumin (3%) in tris-buffered saline contained Tween-20 solution, this membrane was then incubated via a primary antibody against TAZ (orb39498, 1:1000, Biorbyt, Shanghai, China) and β-actin (orb181785, 1:2000; Biorbyt). It was let go overnight when the temperature was 4°C. Next, it was incubated properly by a goat anti-rabbit IgG (dilution, 1:1,000; Abcam) secondary antibody for 50 minutes. The gel image processing system was implemented to analyse the molecular weight and optical density of the target strip. Finally, according to the standard curve, the protein content was calculated.

Statistical Analysis

The data were analysed based on the GraphPad Prism 6. For the data, the form of mean ± SD was presented by the researchers. Subsequently, the one-way analysis of variance or Student's *t*-test was exerted to analyse the differences. The researchers repeated the experiments at least 3 times. When $p<0.05$, differences were considered as statistically significant.

RESULTS

miR-610 Showed a Decreased Expression in Thyroid Cancer

First of all, miR-610 expression level was explored by the method of qRT-PCR in 54 thyroid cancer tissues. miR-610 was found to show a significant lower expression in thyroid cancer tissues than in normal ones ($p<0.01$; Fig. 1A). Meanwhile, miR-610 expression was examined in the human TC lines (KFC-133, TPC-1 and SW579) and Nthy-ori3-1 cells (Fig. 1B). The result showed that a reduced expression of miR-610 in TC cell lines was found compared to the Nthy-ori3-1 cells ($p<0.01$; Fig. 1B). Next, the researchers analysed the correlation relationship of miR-610 expression and clinical characteristics in the TC patients (Table 1). From the Table 1, it was seen that miR-610 expression was re-

Table 1: The Correlation between miR-610 expression and clinical characteristics of TC patients (n=54)

Characteristics	*Number of cases n=54*	*miR-610 expression*		
		Low (n=34)	*High (n=20)*	*p-value*
Age (years)				0.690
>45	36	22	14	
<45	18	12	6	
Sex				0.568
Male	38	23	15	
Female	16	11	5	
Tumor Size (cm)				0.614
>2 cm	30	18	12	
<2 cm	24	16	8	
TNM Stage	26	20	6	0.041*
I-II	28	14	14	
Lymph Node Metastasis				0.005**
No	22	9	13	
Yes	32	25	7	

*$p<0.05$, **$p<0.01$ mean the difference is significant, highly significant, respectively

markably connected with the TNM stage and lymph node metastasis (p=0.041 and p=0.005). Nevertheless, there was no obvious significance between miR-610 expression and ages (p=0.690), sex (p=0.568) as well as tumour size (p=0.614), separately. Furthermore, thyroid cancer patients were grouped into low expression and high expression groups based on the expression of miR-610 (Fig. 1C). The Kaplan survival analysis results demonstrated the survival time in TC patients who had a low expression of miR-610 was remarkably shorter than that in patients with high expression (p<0.05; Fig. 1C). This revealed that miR-610 was an indicator of poor prognosis for TC.

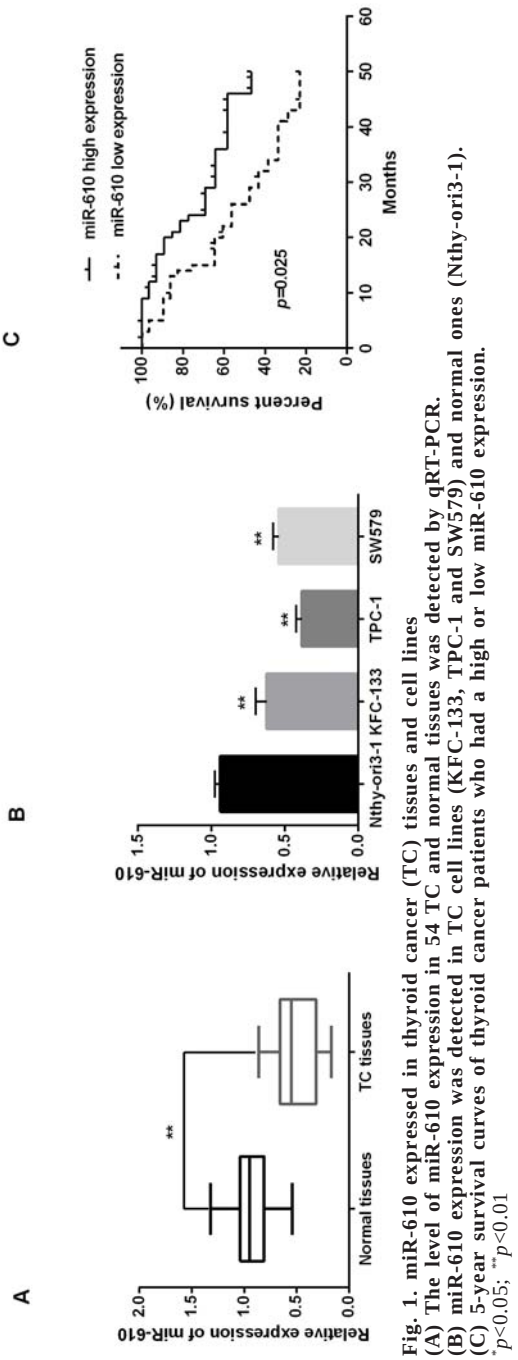

Fig. 1. miR-610 expressed in thyroid cancer (TC) tissues and cell lines
(A) The level of miR-610 expression in 54 TC and normal tissues was detected by qRT-PCR.
(B) miR-610 expression was detected in TC cell lines (KFC-133, TPC-1 and SW579) and normal ones (Nthy-ori3-1).
(C) 5-year survival curves of thyroid cancer patients who had a high or low miR-610 expression.
$^{*}p$<0.05; $^{**}p$<0.01

MiR-610 Overexpression Restrains the Proliferation and Invasion of TPC-1 Cells

The miR-610 mimic or inhibitor was transfected into TPC-1 cells iso as to verify the effect of miR-610 on thyroid cancer progression (Fig. 2A). The data indicated that the level of miR-610 expression was ascended by the miR-610 mimic, and reduced by the miR-610 inhibitor (p<0.01; Fig. 2A). Then, the roles and effects of miR-610 on cell viability were investigated through the MTT assay and Transwell assay in TPC-1 cells (Fig. 2B, 2C). These data revealed that up-regulation of miR-610 expression restrained TPC-1 cells' proliferation , while miR-610 knockout promoted it (p<0.01; Fig. 2B). Similarly, miR-610 mimic inhibited TPC-1 cells' invasion, while miR-610 inhibitor could promote cell invasion (p<0.01; Fig.2C). The studies illustrated that overexpression of miR-610 acted a strong inhibitory effect on the progression of TC cells.

TAZ Was a Target Gene of miR-610 in TC

Targetscan Version 7.2 predicts that Mir-610 has binding sites with TAZ (Fig. 3A), and dual-luciferase reporter assay confirmed the hypothesis (Fig. 3B). These results revealed that miR-610 mimic notably lessened the luciferase activity of TAZ-wt, but had no effect for TAZ-mut (p<0.01; Fig. 3B). Meanwhile, the researchers examined that the expression levels of TAZ mRNA and protein were regulated by miR-610 mimic or inhibitor (Fig. 3C, 3D). The results displayed that miR-610 mimic

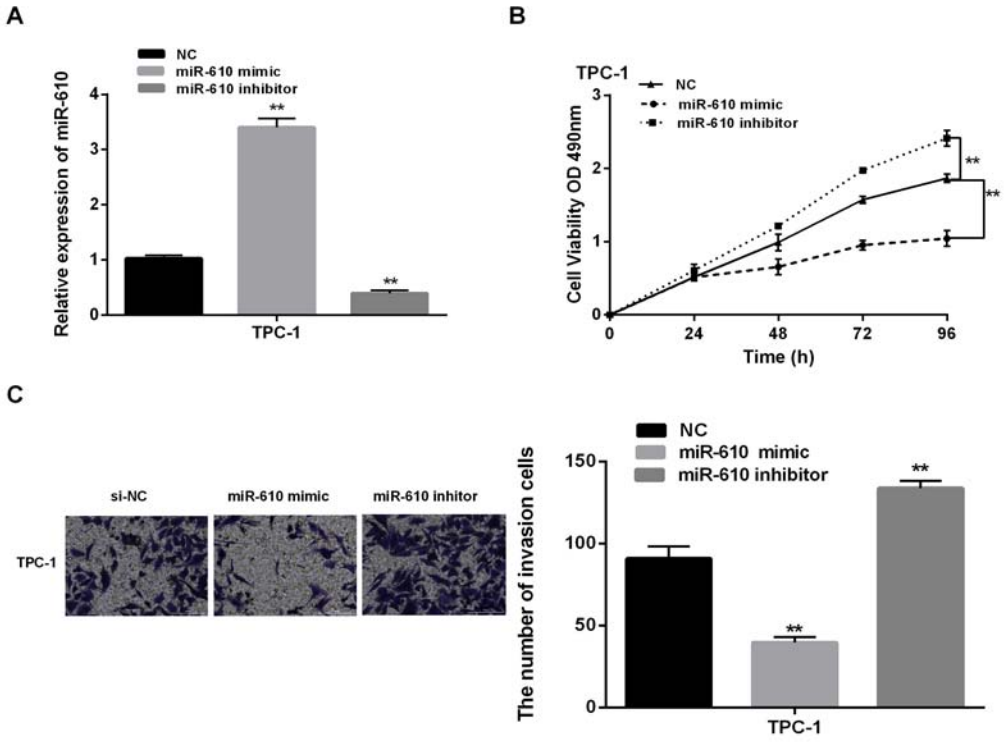

Fig. 2. Aberrant expression of miR-610 effected the proliferation and invasion of TPC-1 cells
(A) The relative expression of miR-610 was explored when transfected by miR-610 mimic or inhibitor.
(B) Cell proliferation of miR-610 mimic and inhibitor was assessed in TPC-1 cells.
(C) The invasion of miR-610 mimic and inhibitor was determined in TPC-1 cells (scale bar=100ìm).
$^{**}p<0.01$

significantly reduced the expressions of TAZ mRNA and protein, while miR-610 inhibitor enhanced that ($p<0.01$) in TPC-1 cells. Moreover, the Pearson's correlation analysis data displayed that miR-610 expression and TAZ presented a negative correlation in thyroid cancer tissues ($R^2=0.6138$; $p<0.01$; Fig. 3E). The above results mean that TAZ was a target gene of miR-610 in TC.

TAZ Knockdown Inhibited Cell Progression in Thyroid Cancer

Then, the expression of TAZ was explored in the TC tissues (Fig. 4A) and cell lines (Fig. 4B) by using of qRT-PCR. The expression of TAZ was found to be up-regulated ($p<0.01$). To illustrate the role of TAZ in the TC progression, functional experiments were performed. As shown in Figure 4C, TAZ siRNA significantly inhibited TPC-1 cells' proliferation in contrast to the control group ($p<0.01$). Likewise, the capacity of cell invasion was significantly decreased when TPC-1 cells those transfected with TAZ siRNA ($p<0.01$; Fig. 4D). All the findings implied that knockdown of TAZ suppressed TC cells proliferation and invasion.

MiR-610 Inhibited theProgression via Regulating TAZ in Thyroid Cancer Cells

To investigate whether miR-610 regulates the progression of TC by targeting TAZ, miR-610 mimic together with TAZ vector were co-transfected into TPC-1 cells (Fig. 5A). Based on RT-PCR results, the expression of TAZ was showed to decrease induced by miR-610 mimic, and this effect

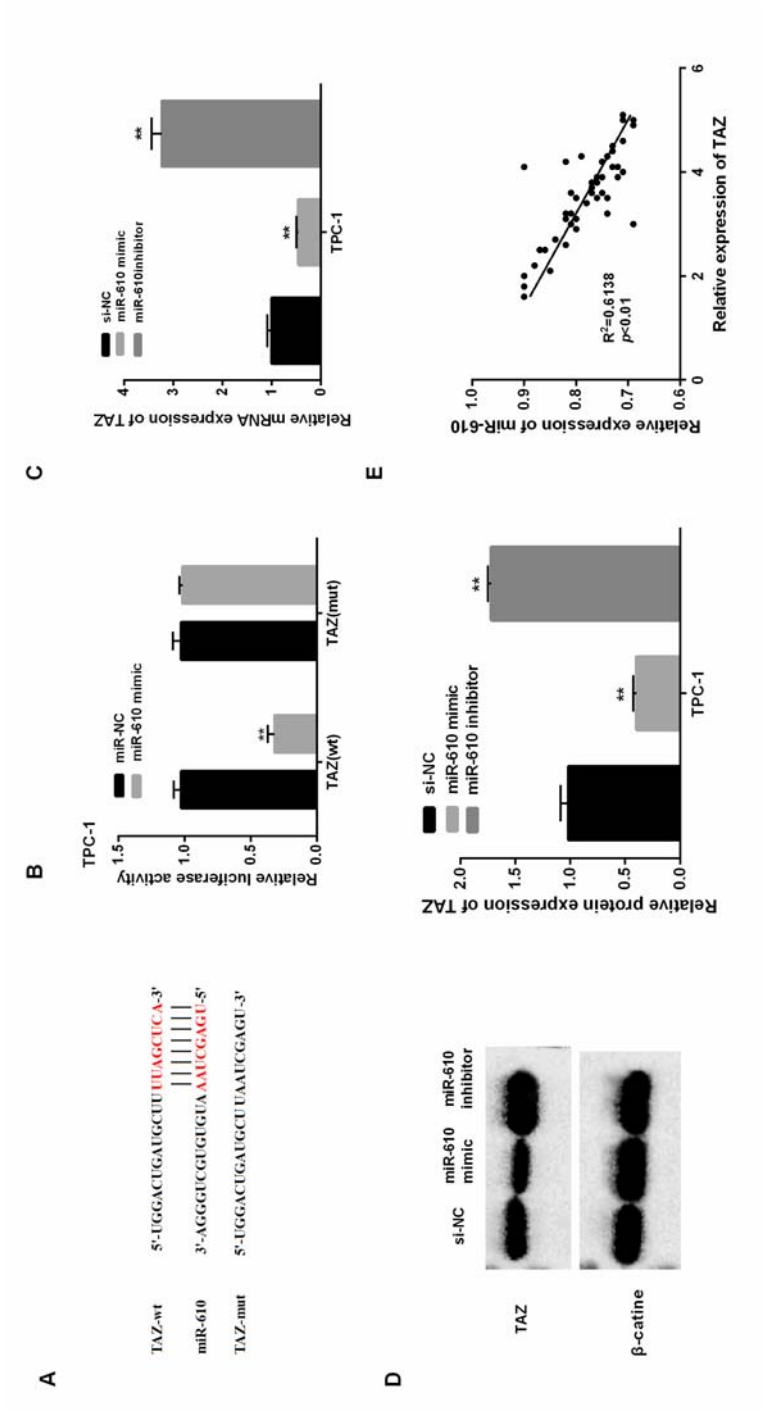

Fig. 3. TAZ was a target of miR-610 in thyroid cancer
(A) MiR-610 was predicted to have binding sites with TAZ by TargetScan.
(B) The interaction of miR-610 with TAZ was verified by the Dual luciferase assay.
(C) The level of TAZ mRNA expression was measured by qRT-PCR when miR-610 mimic or inhibitor transfected into TPC-1 cells.
(D) The level of TAZ protein was quantified by Western blot with miR-610 mimic or inhibitor transfection in TPC-1 cells. (E) TAZ was negatively correlated with miR-610 expression in thyroid cancer tissues.

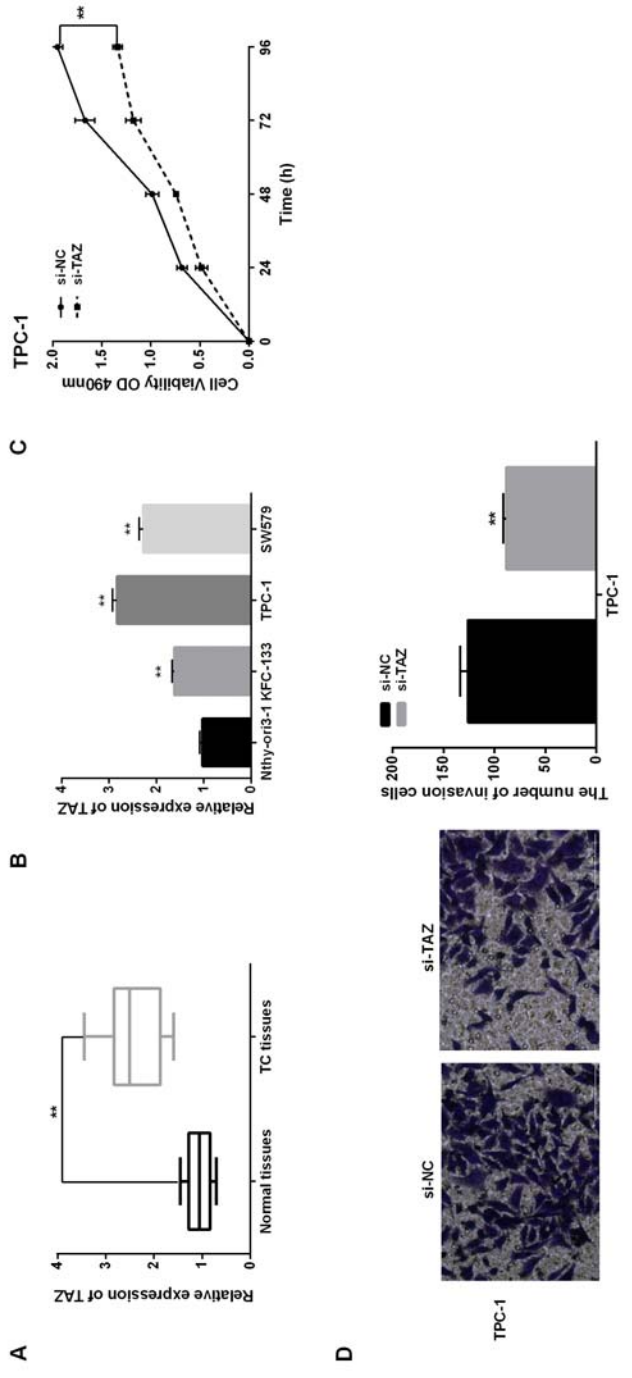

Fig. 4. *TAZ* knockout acted an inhibitory effect on the proliferation and invasion in TC cells
(A) The relative expression of TAZ was detected by the way of qRT-PCR in thyroid cancer tissues.
(B) The relative expression of TAZ in TC cell lines.
(C) MTT assay was exerted to indicate that TAZ knock-out decreased the proliferation in TPC-1 cells.
(D) TAZ knockdown inhibited the invasion in TPC-1 cells from the Transwell assay results (scale bar=100 μm).
$^{**}p<0.01$

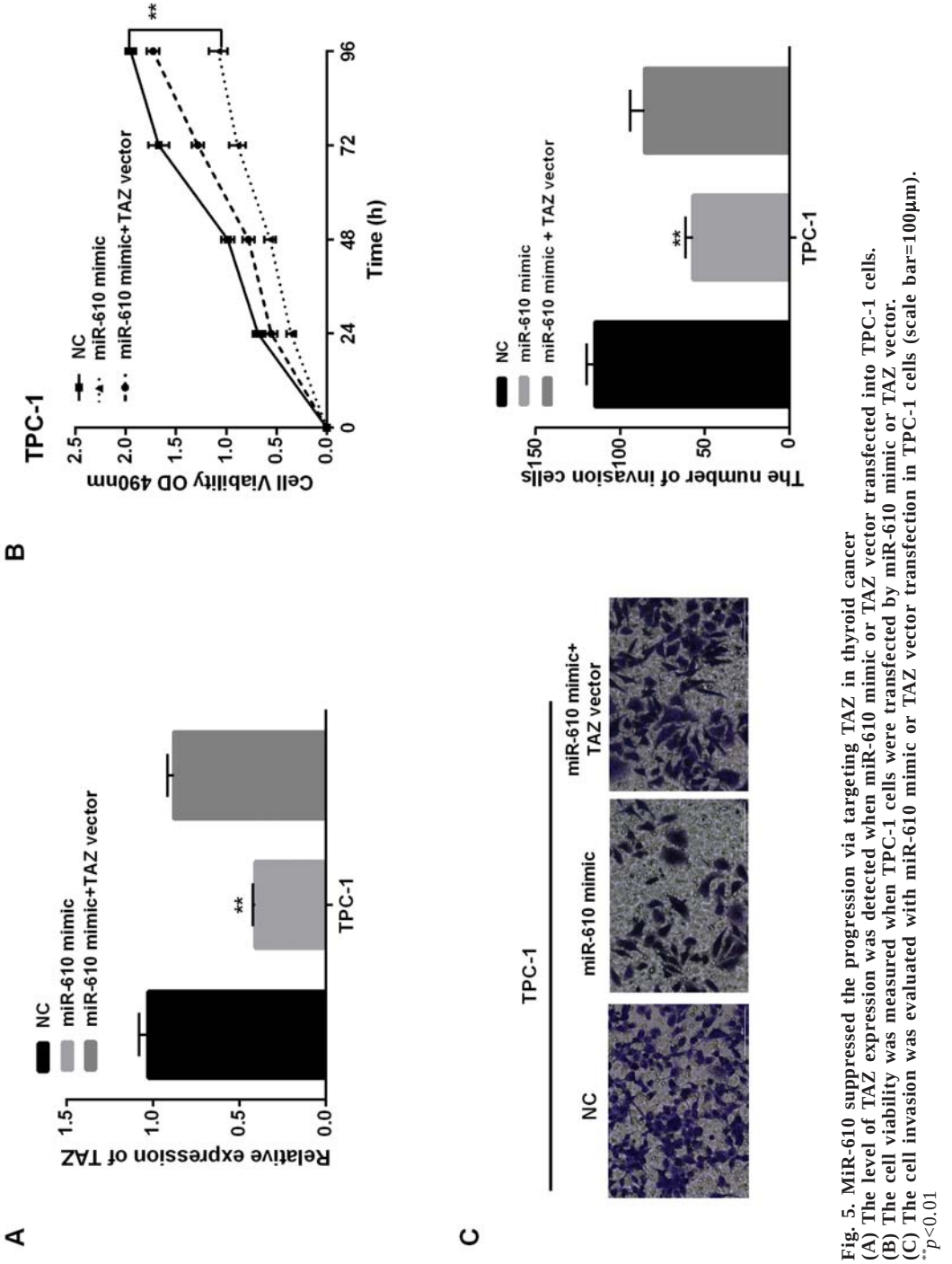

Fig. 5. MiR-610 suppressed the progression via targeting TAZ in thyroid cancer
(A) The level of TAZ expression was detected when miR-610 mimic or TAZ vector transfected into TPC-1 cells.
(B) The cell viability was measured when TPC-1 cells were transfected by miR-610 mimic or TAZ vector.
(C) The cell invasion was evaluated with miR-610 mimic or TAZ vector transfection in TPC-1 cells (scale bar=100μm).
**$p<0.01$

was repaired by TAZ vector in the TPC-1 cells ($p<0.01$; Fig.5A). Moreover, MTT assay (Fig. 5B) and Transwell assay (Fig. 5C) were performed to verify their functional interaction. As seen in Figure 5B, miR-610 mimic inhibited TPC-1 cell proliferation, while TAZ vector restored the suppression effect ($p<0.01$; Fig. 5B). Transwell assay data showed the downtrend in miR-610 mimic induced cell invasion was inverted by TAZ vector ($p<0.01$; Fig. 5C). In conclusion, the researchers consider that miR-610 can regulate assuredly the proliferation and invasion in thyroid cancer cells by way of modulating TAZ. There is an opposite effect between miR-610 and TAZ for thyroid cancer cells.

DISCUSSION

In recent years, more and more scholars have proved that the expression of miRNAs leading to deregulation of tumour suppressors or oncogenes, including thyroid cancer (Chou et al. 2017; Perdas et al. 2016). In thyroid cancer, these miRNAs were found to regulate the cell migration and invasion, including miR-let-7a (Perdas et al. 2016), miR-146b (Chou et al. 2017), miR-125b (Wang et al. 2018) etc. Human miR-610 (MI0003623) was located at the short arm of chromosome 11, p14.1, and its precursor length was 96bp (Mo et al. 2016). Studies have shown that miR-610 can target multiple genes, and plays a key part in tumour growth, invasion and metastasis (Zeng et al. 2014). It is known that miR-610 is confirmed to remarkably restrain cell progression via regulating AGK expression in the oral squamous cell carcinoma (Yao et al. 2019). Similarly, miR-610 inhibited the tumorigenicity via targeting LRP6 and TBL1X in the hepatocellular carcinoma (Zeng et al. 2014). In the research, the researchers revealed that the expression of miR-610 showed a significant down-regulation in both TC tissues and cell lines. And these functional assays displayed that its up-regulation could inhibit TPC-1 cells' proliferation and invasion, consistent with previous findings. The researchers also investigated the relationship of miR-610 expression coordinated with clinico-pathological characteristics. It was displayed that low miR-610 expression was remarkably correlated to TNM stage and lymph node metastasis. This study means miR-610 plays an inhibitory effect in the development of thyroid cancer.

MiRNAs usually affect various life activities of cells through targeted regulation of functional genes (Chen et al. 2019b). In osteosarcoma, miR-610 inhibited the proliferation, cell cycle and the invasion by the way of repressing TWIST1 (Jin et al. 2017). It was also found that miR-610 suppressed the progression of melanoma via targeting LRP6 (Zhang et al. 2017). TAZ, a kind of mitochondria protein, is acquired as one crucial downstream target of the Hippo signalling pathway (Nguyen and Yi 2019). Previous scholars have reported that TAZ was proved to be a target of different miRNAs in the development of many various cancers, and plays an important effect on the proliferation and metastasis of some cancer cells (Hu et al. 2020; Tang et al. 2019). YAP/TAZ was activated and promoted tumour growth and metastasia in thyroid cancer (Bhandari et al. 2019). Yang and other colleagues published that miR-125 inhibited the proliferation and invasion of colorectal cancer and acted by targeting TAZ (Yang et al. 2019). Futhermore, Hu et al. also found the potential of miR-1297/TAZ pathway involved in the triple negative breast cancer (Hu et al. 2020). In this research, it was the first time to study the apparently relations between miR-610 and TAZ on the progression in thyroid cancer. These researchers predicted miR-610 had binding bites with TAZ by TargetScan. Then, the data demonstrated that TAZ was high expressed in TC tissues and cells. Moreover, TAZ silencing restrained the proliferation and invasion of thyroid cancer cell. This study results demonstrated that there was a negatively regulated relevance between miR-610 expression and TAZ in TC. Finally, these experiment results and data also proved that cell proliferation and invasion that reduced by miR-610 could be restored by transfected with TAZ-vector in thyroid cancer. To sum up, this study indicated that TAZ functions as an oncogene in thyroid cancer, which is consistent with its role proved in the colorectal cancer and gastric cancer (Yang et al. 2019; Zuo et al. 2015).

CONCLUSION

As shown above, our experiments and results are the first to reveal that miR-610 restrains TC cells' the proliferation and invasion by means

of modulating TAZ. The results demonstrated that there was a negatively regulated relevance between miR-610 expression and TAZ in thyroid cancer. At the same time, it also discovers that miR-610 may take the important role as a suppressor in thyroid cancer, which indicates thatit may be a novel potential therapeutic target in thyroid cancer patients.

RECOMMENDATIONS

This study demonstrates that miR-610 and TAZ might be new biomarkers for thyroid cancer treatment. However, the efficacy of most of the targeted drugs has not reached the clinical expectation, and further investigation is needed. Highly targeted and effective therapeutic drugs need to be identified to improve the overall prognosis of TC patients in large-scale clinical observation and basic research in the near future, which may lead to better outcomes in patients with thyroid cancer.

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