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## **miR-1258: A novel microRNA that controls TMPRSS4 expression is associated with malignant progression of papillary thyroid carcinoma**

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***miR-1258*: A novel microRNA that controls *TMPRSS4* expression is associated with malignant progression of papillary thyroid carcinoma**

**Running title:** *MiR-1258/TMPRSS4* axis in PTC

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**Abstract**

**Introduction:** MicroRNA-1258 (*miR-1258*) has been shown to play an anti-cancer role in a variety of cancers, but its relationship with papillary thyroid cancer (PTC) has not been reported. The emphasis of this research was to reveal the biological function of *miR-1258* in PTC and its potential mechanisms.

**Material and methods:** We measured *miR-1258* expression in PTC cells and the transfection efficiency of *miR-1258* mimic and *miR-1258* inhibitor by quantitative real-time PCR (qRT-PCR) assay. Cell Counting Kit-8 assay (CCK8) and Transwell experiments were conducted to examine the influences of altering *miR-1258* expression on the viability, migration, and invasion of PTC cells. Bioinformatics prediction and dual-luciferase experiment were performed to verify the target gene of *miR-1258*. Finally, we carried out a rescue assay to verify whether the regulation of *miR-1258* on the biological behaviour of PTC cells needs to be achieved by regulating *TMPRSS4*.

**Results:** The outcomes revealed that *miR-1258* was lowly expressed in PTC cell lines

and *miR-1258* showed the lowest expression in KTC-1 and the highest expression in B-CPAP among all tested PTC cell lines. Overexpression of *miR-1258* inhibited KTC-1 cell viability and ability to migrate and invade, whereas inhibition of *miR-1258* in B-CPAP cells has the opposite effect. Furthermore, we affirmed that *miR-1258* can directly target *TMPRSS4*, and *miR-1258* can reduce the biological malignant behaviour of PTC cells via regulation of *TMPRSS4*.

**Conclusion:** Taken together, our research raised the possibility that *miR-1258* was an anti-oncogene, which exerts its anti-cancer function by targeting *TMPRSS4*. Hence, it may be possible to treat PTC by targeting the *MIR-1258/TMPRSS4* axis in the future.

**Key words:** *miR-1258*; *TMPRSS4*; target; PTC

## Introduction

Papillary thyroid cancer (PTC), as the most common subtype of thyroid cancer, has the characteristics of high cure rate and long-term survival rate (> 95%) [1–3]. In some patients with poor prognosis, recurrence and metastasis are the direct factors leading to death [4, 5]. Although PTC is more common in female patients, the mortality rate of male patients is higher [6, 7]. To date, most patients with PTC can be cured by surgery, but owing to the increasing incidence of PTC [8] and the high mortality rate of patients with recurrence and metastasis, a useful target of the diagnosis and therapy of PTC still need to be studied and developed.

MicroRNA (miRNA), as an endogenous small RNA in the non-coding region of the genome [9, 10], can regulate more than 30% of human genes and contributes to the regulation of various major diseases in humans [11]. Extensive research has reported the role of miRNAs in PTC; for example, up-regulation of miR-222 expression in PTC was associated with clinical risk stratification [12]. MiR-23a was down-regulated in PTC and participates in the regulation of PTC by targeting CCNG1 [13]. MiR-96-5p induced the invasion and migration of PTC cells by silencing the expression of CCDC67, thus accelerating the development of PTC [14]. In contrast, miR-506 can significantly reduce the migration and invasion ability of PTC cells [15]. MicroRNA-1258 (*miR-1258*), as one of the miRNAs, has been reported to participate in regulating

the process of carcinoma and plays a tumour suppressive role in many cancers, such as NSCLC(17), ovarian cancer [18], osteosarcoma [19], oral squamous cell carcinoma [20], colorectal cancer [21], breast cancer [22], etc. Nevertheless, no previous research has been performed on the relationship between *miR-1258* and PTC.

On the basis of existing literature data, we carried out studies in an effort to investigate the role of *miR-1258* in the viability, migration, and invasion of PTC cells, and to study its potential target genes. It was expected to provide a potentially useful strategy for the diagnosis and therapy of PTC.

## **Materials and method**

### ***Data collection***

In this study, we downloaded data from the [The Cancer Genome Atlas](https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) (TCGA) database ([https:// www.cancer.gov/about-nci/organization/ccg/research /structural-genomics/tcga](https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga)) to analyse the difference of the expression of *miR-1258* in PTC cells and normal cells. Similarly, the data of 510 tumour samples and 58 healthy controls were downloaded from the TCGA database and used to analyse differential expression of transmembrane protease serine 4 (*TMPRSS4*).

### ***Cell culture***

Human B-CPAP, BHT101, and KTC-1 PTC cell lines (Shanghai Cell Bank of Chinese Academy of Medical Sciences) and normal thyroid epithelial cell Nthy-ori 3-1 (European Collection of Cell Culture, Salisbury, UK) were routinely cultured in RPMI-1640 medium containing 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37°C with 5% CO<sub>2</sub>.

### ***Cell transfection***

*MiR-1258* mimic/inhibitor and corresponding NC were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China), and pcDNA3.1-*TMPRSS4*, si-*TMPRSS4*, si-con were compounded by Shanghai GenePharma Co., Ltd. (Shanghai, China). The conventionally cultured cells were seeded in a six-well plate before transfection for 24

h. When the cell fusion degree achieved 70–90%, the culture medium was swapped to antibiotic-free and serum-free medium, and then transfected using Lipofectamine 2000 after 2 h. The transfection was carried out according to the instructions of the Lipofectamine 2000 transfection kit. After 48 hours, the transfection was observed, and the cells were collected for further assays.

#### ***Quantitative real-time PCR (qRT-PCR)***

Total RNA was extracted by RNeasy Mini Kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions. After that, M-MLV (Clontech, Palo Alto, USA) and miScript PCR reverse transcription kit (Qiagen, Dusseldorf, Germany) were used for reverse transcription. Then, using the qRT-PCR kit to test the expression level of *miR-1258/TMPRSS4*, *GAPDH* and *U6* were used as the internal reference genes. The qRT-PCR reaction was carried out as follows: 95°C for 5 min, 95°C for 5 s, and 60°C for 30 s; 40 cycles, using the  $2^{-\Delta\Delta C_t}$  method to calculate the relative quantities. The specific primer sequences were:

— *MiR-1258*:

F: 5'-GTTAGGATTAGGTCGTGG-3'

R: 5'-GAACATGTCTGCGTATCTC-3'

— *U6*:

F: 5'-CTCGCTTCGGCAGCACA-3'

R: 5'-AACGCTTCACGAATTTGCGT-3'

— *TMPRSS4*:

F: 5'-GACGAGGAGCACTGTGTCAAGA-3'

R: 5'-GAAACAGGCAGAGAACCAGTTCC-3'

— *GAPDH*:

F: 5'-TAG ATGACACCCGTCCCTGA-3'

R: 5'-ACCTCCACCTGTCCTTAGTG-3'

#### ***Western blotting assay***

Six-well plates with transfected cells were placed on ice, and protein was extracted by

RIPA lysis buffer with protease inhibitor. Then a BCA protein quantitative kit was used to measure the protein content in the samples. About 20 µg of the above protein was conducted to SDS-PAGE electrophoresis, followed by transferral of the separated protein sample to a PVDF membrane. After adding Western Sealing Solution (5% skimmed milk powder) to seal for 1 h, incubation of the membrane overnight at 4°C with primary antibody, followed by rinsing the incubated membrane three times with TBST for five minutes each time was carried out. Then the secondary antibody was added to the membrane and incubated for 1 h at room temperature. After rinsing with TBST for 3 × 5 min, the images were developed with an ECL Western blotting kit (Thermo Fisher Scientific, Inc.). Using Quantity One software to analyse the greyscale value of protein bands, *GAPDH* was used as the internal reference gene.

#### ***Cell counting kit-8 (CCK8) assay***

The transfected cells were prepared into cell suspension and inoculated on a 96-well plate at a density of  $1 \times 10^5$  cells/well, followed by conventional culturing in a 5% CO<sub>2</sub> incubator. OD values at 450 nm wavelength of each well were measured using a microplate reader at 0 h, 24 h, 48 h, and 72 h. Notably, 10 µl of CCK8 solution was added to each well 2 h before detection.

#### ***Transwell assay***

40µl of Matrigel Matrix glue was added to the upper chamber of the Transwell chamber and incubated overnight at 37°C to make the Matrigel gelatinous. The migration assay did not require gluing treatment. After digestion, serum-free culture medium was used to prepare the cell suspension. The cell suspension was added to the upper chamber at the density of  $1 \times 10^5$  cells, and 500 µl of complete culture medium was added to the lower chamber. Then the chamber was cultured at 37°C for 24 h in a 5% CO<sub>2</sub> incubator. Afterwards, the chamber was removed and the remaining cells were wiped from the upper chamber with a cotton swab. The cells in the lower chamber were fixed with 4% paraformaldehyde for 15 minutes and stained with 0.1% crystal violet for three minutes. Finally, five visual fields were randomly selected under an optical microscope to

observe and count the cells.

### ***Bioinformatics prediction***

The target gene of *miR-1258* was predicted by the target gene prediction and analysis websites miRbase (<http://www.mirbase.org/>) and TargetScan (<http://www.targetscan.org/>).

### ***Dual-luciferase reporter assay***

In order to verify whether *miR-1258* can directly target *TMPRSS4*, dual-luciferase reporter assay was performed. The wild type and mutant type 3'UTR of *TMPRSS4* were cloned into pmirGLO luciferase vector to construct a luciferase expression plasmid *TMPRSS4*-WT and *TMPRSS4*-MUT, respectively, and they were co-transfected into HEK-293T cells with *miR-1258* mimic or *miR-1258* mimic NC. Lipofectamine 2000 was used for transfection according to the instructions. Luciferase activity was detected after 24 h.

### ***Statistical analysis***

The experimental data were analysed by SPSS22.0 statistical analysis software. T-test was used to compare the differences between the two groups, and one-way ANOVA analysis and post-test of Tukey were used to compare the differences between three or more groups. Statistical results were considered to be significantly different when  $p < 0.05$ .

## **Results**

### ***MiR-1258 was lowly expressed in PTC cell lines***

Firstly, the bioinformatics analysis results in Figure 1A showed that *miR-1258* expression was markedly lower in PTC tumour tissues than in normal tissues ( $p < 0.0001$ ). Subsequently, we confirmed this difference in PTC cell lines by qRT-PCR assay. Figure 1B indicated that *miR-1258* expression was markedly lower in PTC cell lines B-CPAP, BHT101, and KTC-1 than that in normal healthy cell Nthy-ori 3-1 ( $p <$

0.01). Interestingly, we also found that *miR-1258* showed unequal expression in different PTC cell lines, with the lowest expression in KTC-1 and the highest expression in B-CPAP.

#### ***The effect of miR-1258 inhibitor/mimic transfection on the expression of miR-1258 in PTC cells***

To detect the function of *miR-1258* inhibitor/mimic, we selected KTC-1 for over-expression assay and B-CPAP for knockdown assay. KTC-1 was transfected with *miR-1258* mimic and *miR-1258* mimic NC, simultaneously B-CPAP was transfected with *miR-1258* inhibitor and *miR-1258* inhibitor NC. Cells that were treated with transfection reagent were considered as controls. Then the expression of *miR-1258* in KTC-1/ B-CPAP cells after transfection was detected by qRT-PCR. Fig.1C-D shows that *miR-1258* expression was markedly increased in KTC-1 cells transfected with *miR-1258* mimic compared with that in KTC-1 cells transfected with *miR-1258* mimic NC and in controls ( $p < 0.01$ ), while the expression of *miR-1258* was obviously reduced in CPAP cells transfected with *miR-1258* inhibitor compared to that in B-CPAP cells transfected with *miR-1258* inhibitor NC and in controls ( $p < 0.01$ ). These results illustrated a successful transfection and a high transfection efficiency.

#### ***The influence of altering the miR-1258 expression level on the biological behaviour of PTC cells***

To verify whether *miR-1258* plays a biological function in PTC cells, CCK8 and Transwell experiments were set up to detect cell viability, migration, and invasion. It can be seen from Figure 2A that the cell viability of KTC-1 cells transfected with *miR-1258* mimic declined more than that in the control group ( $p < 0.01$ ), whereas it can be seen from Fig. 2D that the cell viability showed a marked increase after inhibition of *miR-1258* expression in B-CPAP cells ( $p < 0.01$ ). The results obtained from the Transwell assay indicated that after overexpression of *miR-1258* in KTC-1 cells, the number of migrating and invading cells through the Transwell membrane was markedly reduced compared to that in the control group (Fig. 2 BC,  $p < 0.01$ ), whereas



suppression of *miR-1258* in B-CPAP cells showed the opposite effect (Fig. 2EF,  $p < 0.01$ ). Overall, these results demonstrate that overexpression of *miR-1258* can inhibit the cell viability, migration, and invasion in PTC, and thus *miR-1258* may be a tumour suppressor gene in PTC.

#### ***MiR-1258 directly targets TMPRSS4***

To explore the mechanism of regulation of PTC by *miR-1258* we used a target gene prediction website to predict and analyse the target of *miR-1258*. Bioinformatics analysis indicated that *TMPRSS4* was more highly expressed in PTC cells than in normal cells (Fig. 3A,  $p < 0.0001$ ), and it may be a potential target gene for *miR-1258*, which has predicted target sites for *miR-1258* in the 3'-UTR region (Fig. 3B). To validate further whether *TMPRSS4* is a direct target gene of *miR-1258*, *TMPRSS4*-WT or *TMPRSS4*-MUT and *miR-1258* mimic or *miR-1258* mimic NC were co-transfected into HEK 293T cells to determine the cell luciferase activity. From Figure 3C we can see that *miR-1258* mimic significantly reduced the luciferase activity of wild-type compared to the negative control group ( $p < 0.01$ ), whereas the luciferase activity of the mutant did not change markedly compared with the control group.

Subsequently, Western blot assay was carried out to test the influence of overexpression/suppression of *miR-1258* on *TMPRSS4* protein levels. The results from Figure 3DE revealed that the level of *TMPRSS4* protein in KCT-1 cells transfected with *miR-1258* mimic decreased significantly compared to that in the control group ( $p < 0.01$ ), while the level of *TMPRSS4* was markedly higher in KCT-1 cells co-transfected with mimic and pcDNA3.1-*TMPRSS4* compared to that in the *miR-1258* mimic group ( $p < 0.01$ ). Simultaneously, it can be concluded from Figure 3EF that inhibition of *miR-1258* expression in B-CPAP cells can significantly increase the level of *TMPRSS4* protein compared to the control group ( $p < 0.01$ ), whereas *TMPRSS4* protein level in B-CPAP cells co-transfected with *miR-1258* inhibitor and si-*TMPRSS4* was reduced significantly compared to those in the inhibitor group. Overall, these results illustrate that *miR-1258* can directly target the *TMPRSS4* gene and negatively regulate its expression.

### ***Regulation of the miR-1258/TMPRSS4 axis on the phenotype of PTC***

To confirm whether *miR-1258* regulates the biological behaviour of PTC cells by regulating *TMPRSS4*, a rescue experiment was performed. Firstly, KTC-1 cells were transfected with *miR-1258* mimic or *miR-1258* mimic plus pcDNA3.1-*TMPRSS4*, and B-CPAP cells were transfected with *miR-1258* inhibitor or *miR-1258* inhibitor plus si-*TMPRSS4*, and negative control groups were set respectively. The viability, migration, and invasion of cells under different conditions were examined by CCK8 and Transwell experiments. As shown in Figure 4A–C, the cell viability, migration, and invasion were reduced significantly more in KTC-1 cells transfected with *miR-1258* mimic than that in the controls ( $p < 0.01$ ), whereas after overexpression of *TMPRSS4* in the *miR-1258* mimic group, the cell viability and the number of migrating and invading cells were markedly elevated compared to those in the mimic group ( $p < 0.01$ ). At the same time, down-regulation of *miR-1258* expression in B-CPAP cells significantly increased cell viability, migration, and invasive ability, while knockdown of *TMPRSS4* in the inhibitor group showed the opposite effect. This suggests that overexpression of *TMPRSS4* can reverse the anti-cancer properties of *miR-1258*. Thence, *miR-1258* can reduce the viability, migration, and invasion of PTC cells via regulating *TMPRSS4*.

### **Discussion**

In this paper, we found for the first time that *miR-1258* was lowly expressed in PTC cell lines. Subsequently, a series of experimental results showed that suppression of *miR-1258* in PTC can improve cell viability, migration, and invasion, while overexpression of *miR-1258* showed the opposite effect. Finally, we confirmed that *miR-1258* could directly target *TMPRSS4*, and it promoted the biological behaviour of PTC cells via regulation of *TMPRSS4*.

To date, a number of studies have revealed that *miR-1258* has an imbalanced expression in various cancers. For example, down-regulated *miR-1258* expression in osteosarcoma was related to unfavourable prognosis [19]. *MiR-1258*, as an anti-cancer gene, also plays a role in inhibiting the process of oral squamous cell carcinoma [20]. The anti-

cancer effect of *miR-1258* in colorectal cancer was achieved by negative regulation of E2F8 [21]. *MiR-1258* was a diagnostic and therapeutic marker of hepatocellular carcinoma [23], and also can be used as a downstream functional target to regulate the process of hepatocellular carcinoma [24]. Recently, Filippova EA et al. found that methylation of *miR-1258* can be used as a predictor of ovarian cancer metastasis [18]. This paper first identified the low expression of *MIR-1258* in PTC, and up-regulation of *miR-1258* markedly reduced the viability, migration, and invasion of PTC cells, while suppression of *miR-1258* showed the opposite effect. Taken together, we concluded that *miR-1258* may have the tumour suppressor function of regulating the biological behaviour of PTC cells.

In this paper, we identified that *TMPRSS4* might be a target gene of *miR-1258* by using a target gene prediction website. Type II transmembrane serine proteases (TTSP) have been found to be dysfunctional in a variety of cancers, which can activate signalling pathways leading to cancer [25]. *TMPRSS4*, as a new member of TTSP, has been found to be highly expressed in many cancers, such as pancreatic cancer [26], gastric cancer [27], lung adenocarcinoma [28], hepatocellular carcinoma [29], and breast cancer [30]. In addition, Mahati et al. pointed out that up-regulation of *TMPRSS4* expression can promote the occurrence and development of various biological behaviours of cancer cells [29]. Based on the bioinformatics analysis and experimental results, we verified that *TMPRSS4* was the direct target gene of *miR-1258*, and up-regulation of *TMPRSS4* expression reduced the influence of *miR-1258* to inhibit the viability, migration, and invasion in PTC cells, while knockdown of *TMPRSS4* has the opposite effect. Hence, it is possible that *miR-1258* plays an anti-cancer role by regulating *TMPRSS4* in PTC, but the underlying mechanisms require further study.

From this paper, we concluded that *miR-1258* was lowly expressed in PTC, and overexpression of *miR-1258* can inhibit cell viability, migration, and invasion, which raises the standpoint that *miR-1258* is an anti-oncogene. Furthermore, we found that *miR-1258* may play an anti-cancer role in PTC by regulating *TMPRSS4*, suggesting that the *miR-1258/TMPRSS4* axis may be a potential biomarker and therapeutic target for

the diagnosis and therapy of PTC. In the next step, we will further validate our experimental data through in vivo experiments.

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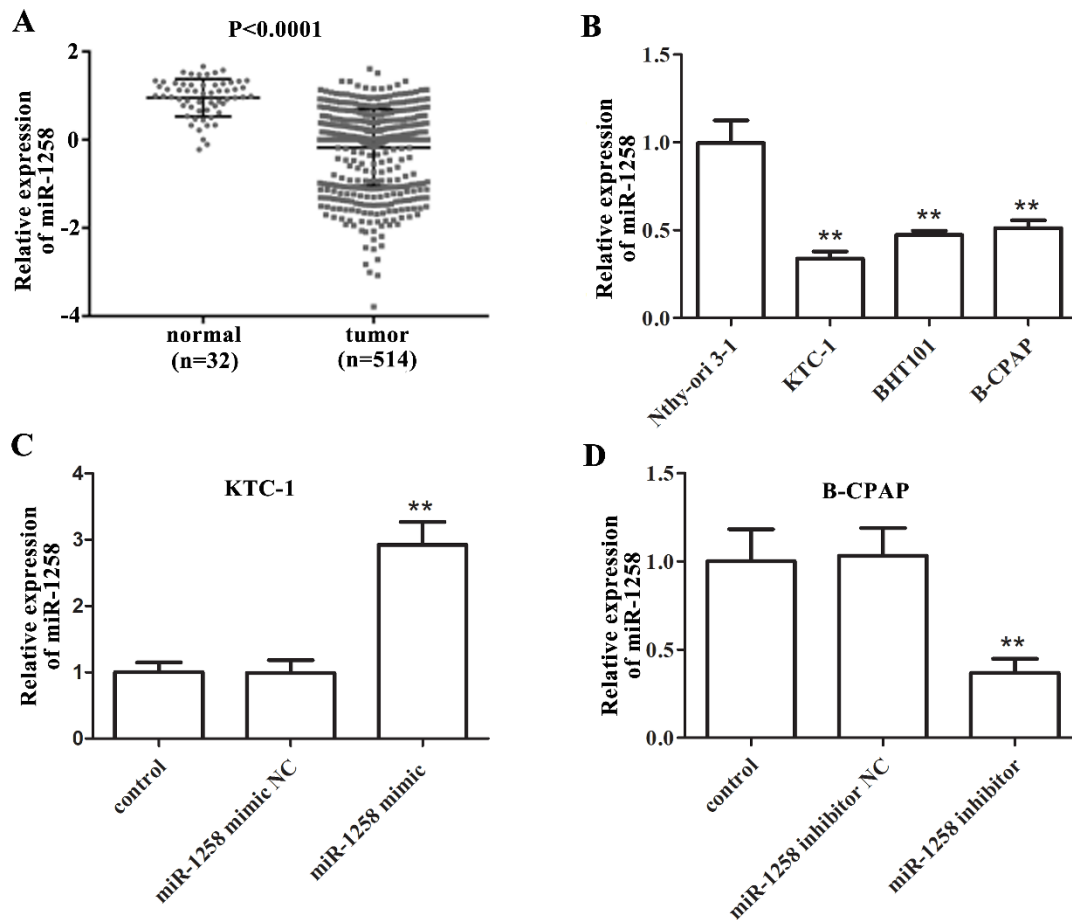
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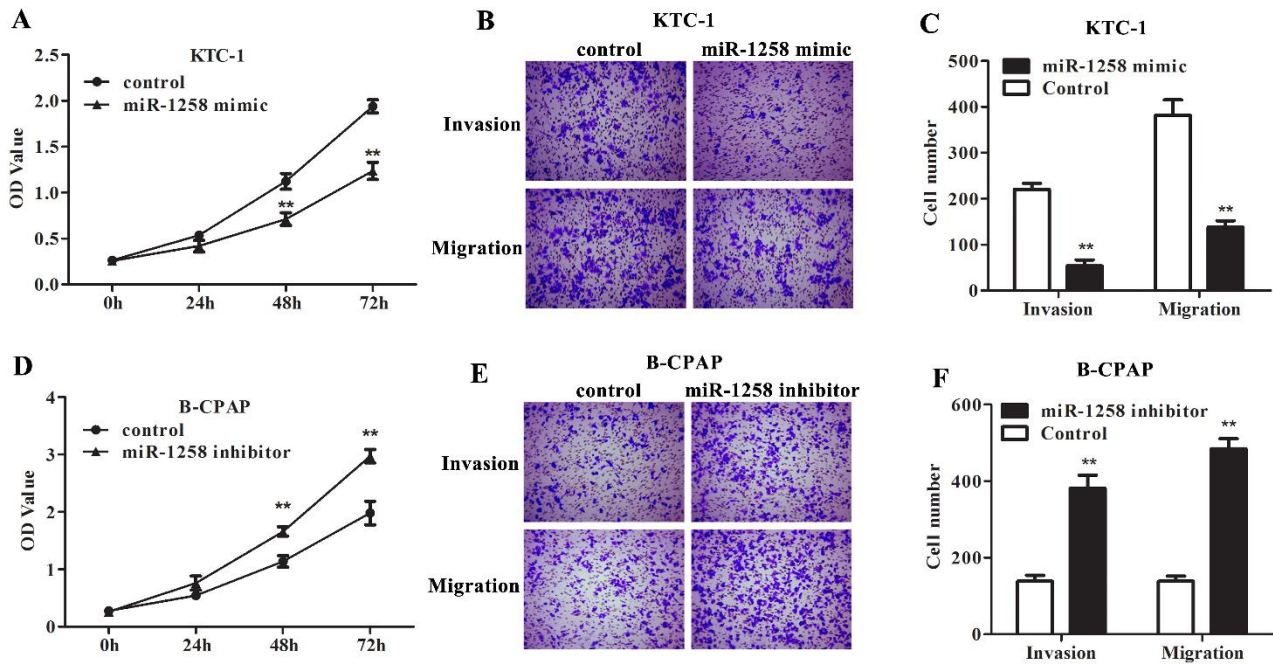
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**Figure 1.** miRNA expression level of *miR-1258* in papillary thyroid cancer (PTC). **A.** *MiR-1258* was lowly expressed in the tumour group (n = 514) and the normal group (n = 59) on the basis of data from TCGA (p < 0.0001). **B.** Through quantitative real-time PCR (qRT-PCR) experiment, the expression of *miR-1258* was markedly reduced in PTC cell lines B-CPAP, BHT101, KTC-1 than that in normal healthy cell Nthy-ori 3-1 (\*\*p < 0.01). **C.** The expression of *miR-1258* in KTC-1 cells transfected with *miR-1258* mimic showed an obvious increase compared with that in KTC-1 cells transfected with mimic NC and that in controls (\*\*p < 0.01). **D.** The expression of *miR-1258* in B-CPAP cells transfected with *miR-1258* inhibitor was significantly decreased compared with that in B-CPAP cells transfected with *miR-1258* inhibitor NC and in controls (\*\*p < 0.01)

**Figure 1.**

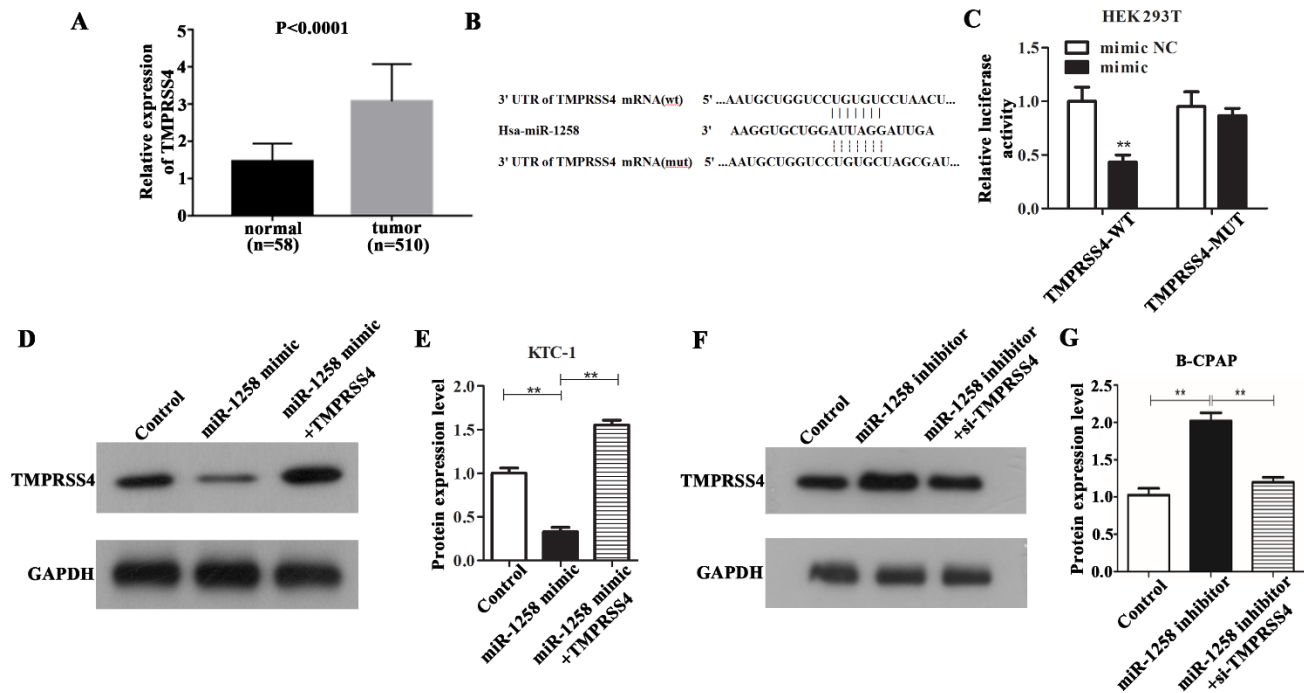


**Figure 2.** Influence of altering the expression of *miR-1258* on the cell biological behaviour in KTC-1 and B-CPAP. **A.** Cell Counting Kit-8 (CCK8) assay results indicated that up-regulation of *miR-1258* expression markedly declined the viability of KTC-1 cells compared to that in controls (\*\*  $p < 0.01$ ). **B.** Down-regulation of *miR-1258* expression markedly enhanced the viability of B-CPAP cells compared to that in controls (\*\*  $p < 0.01$ ). **C, D.** Transwell assay results illustrated that up-regulation of *miR-1258* obviously reduced the number of migrating and invading cells in KTC-1 compared to that in controls (\*\*  $p < 0.01$ ). **E, F.** Inhibition of *miR-1258* expression can improve the cell invasion and metastasis of B-CPAP cells compared to that in controls (\*\*  $p < 0.01$ )



**Figure 3.** *MiR-1258* directly targets *TMPRSS4*. **A.** Relative expression level of *TMPRSS4* in normal cells (n = 58) and tumour cells (n = 510) was analysed on the basis of data from TCGA ( $p < 0.0001$ ). **B.** Alignment of *miR-1258* with *TMPRSS4* sequences. **C.** The fluorescence intensity was markedly decreased in HEK 293T cells co-transfected with *TMPRSS4*-WT and *miR-1258* mimic ( $p < 0.01$ ), while the fluorescence intensity of *TMPRSS4*-MUT and *miR-1258* mimic co-transfected cells was almost unchanged. **D, E.** Overexpression of *miR-1258* markedly decreased the level of *TMPRSS4* protein compared with controls in KTC-1 cells (\*\*  $p < 0.01$ ), while the *TMPRSS4* protein level was markedly increased in pcDNA 3.1-HAO2 and *miR-1258* mimic co-transfected cells compared to the *miR-1293* mimic group (\*\*  $p < 0.01$ ). **F, G.** Inhibition of *miR-1258* in B-CPAP cell can elevate the expression of *TMPRSS4* (\*\*  $p < 0.01$ ), whereas knockdown of *TMPRSS4* in the *miR-1258* inhibitor group, *TMPRSS4* protein level was markedly lower compared with that in the *miR-1258* inhibitor group (\*\*  $p < 0.01$ )





**Figure 4.** The effect of the *miR-1258/TMPRSS4* axis on the function of PTC. **A–C.** Overexpression of *miR-1258* significantly reduced the viability, invasion, and metastasis of KTC-1 cells compared to that in the control group (\*\* $p < 0.01$ , ### $p < 0.01$ ), while the biological behaviour of KTC-1 cells co-transfected with pcDNA3.1-*TMPRSS4* and *miR-1258* mimic was markedly elevated compared to that in the *miR-1293* mimic group. (\*\* $p < 0.01$ , ### $p < 0.01$ ). **D–F.** Suppression of *miR-1293* markedly induced the viability, invasion, and metastasis of B-CPAP cells compared to those in the control group (\*\* $p < 0.01$ ), while the biological behaviour of B-CPAP cells co-transfected with si-*TMPRSS4* and *miR-1293* inhibitors was significant reduced compared to that in the *miR-1293* inhibitor group. (\*\* $p < 0.01$ )

