

# MiR-224-5p targets EGR2 to promote the development of papillary thyroid carcinoma

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**Abstract. – OBJECTIVE:** Various microRNAs (miRNAs) have been reported to be involved in the pathogenesis and development of human cancers, including papillary thyroid carcinoma (PTC). However, the role of miR-224-5p in PTC progression remains unclear. Therefore, the purpose of this study is to illuminate the function of miR-224-5p in PTC.

**PATIENTS AND METHODS:** Expression of miR-224-5p and EGR2 was examined in PTC by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Transwell assay was used to detect cell migration and invasion. Western blot analysis was used to detect epithelial-mesenchymal transition (EMT). The relationship between miR-224-5p and EGR2 was confirmed by Dual-Luciferase assay.

**RESULTS:** Upregulation of miR-224-5p and downregulation of EGR2 expression were detected in PTC tissues and cells. Upregulation of miR-224-5p was found to be associated with TNM stage and lymph node metastasis. Meanwhile, it also predicted poor prognosis in PTC patients. Functionally, upregulation of miR-224-5p promoted cell metastasis and EMT in PTC. In addition, miR-224-5p was detected to directly target EGR2. EGR2 expression was negatively correlated with EGR2 expression in PTC. Of note, overexpression of EGR2 attenuated the carcinogenic effects of miR-224-5p in PTC.

**CONCLUSIONS:** MiR-224-5p promotes cell migration, invasion, and EMT in PTC by targeting EGR2.

*Key Words:*

Papillary thyroid carcinoma, MiR-224-5p, Epithelial-to-mesenchymal transition, Metastasis, Prognosis, EGR2.

thelial cells of malignant tumors<sup>1</sup>. The vast majority of thyroid cancers originate from follicular epithelial cells, including papillary, follicular, undifferentiated, and medullary carcinoma<sup>2</sup>. In particular, papillary thyroid carcinoma (PTC) is a low-grade malignancy that is one of the most common pathological types of thyroid cancer<sup>3</sup>. PTC tumors are generally small and develop slowly, but PTC can be metastasized early<sup>4</sup>. However, PTC patients have better prognosis and lower mortality rate<sup>5</sup>. In addition, most PTC patients are highly differentiated, and the tumor stage is early<sup>6</sup>. Therefore, there is an urgent need to investigate the molecular mechanisms involved in the pathogenesis of PTC.

MicroRNAs (miRNAs) have been shown to regulate tumorigenesis by degrading mRNAs and/or inhibiting translation of some genes<sup>7</sup>. In addition, the abnormal expression of microRNA, as well as its relationship with clinicopathological features have been analyzed in PTC<sup>8</sup>. Dysregulation of many miRNAs is involved in the tumorigenesis and progression of PTC, such as miR-96, miR-144, and miR-363<sup>9,11</sup>. The abnormal expression of miR-224 has been widely identified in human cancers, which are contradictory in different cancers. Upregulation of miR-224 was found in gastric cancer<sup>12</sup>, colorectal cancer<sup>13</sup>, and hepatocellular carcinoma<sup>14</sup>. However, downregulation of miR-224 was detected in breast cancer<sup>15</sup>, prostate cancer<sup>16</sup>, and non-small cell lung cancer<sup>17</sup>. Especially, miR-224 has been reported to function as a potential target for miR-based therapy of cancer<sup>18</sup>. However, the potential role of miR-224 in PTC is still unclear. Therefore, this study was designed to illuminate the function of miR-224-5p in PTC.

Early growth response 2 (EGR2) has been reported to act as a zinc finger transcription

## Introduction

Thyroid cancer is the most common thyroid malignant tumor, originating from thyroid epi-

factor to regulate the development of the hind-brain and nervous system<sup>19</sup>. EGR2-dependent miR-138 is essential for peripheral nerve myelination<sup>20</sup>. Furthermore, EGR2 was found to promote apoptosis in various cancers by directly regulating BNIP3L and BAK expression<sup>21</sup>. In addition, the interaction between miRNAs and EGR2 was also reported. Of note, miR-20a promoted the progression of gastric cancer by targeting EGR2<sup>22</sup>. It has also been reported that EGR2 together with miR-20a suppressed cell proliferation in hepatocellular carcinoma<sup>23</sup>. Despite this, the regulatory mechanism of miR-224-5p/EGR2 in PTC is still unknown.

In this study, the expression level of miR-224-5p and EGR2 was detected in PTC tissues and cells. Meanwhile, the function of miR-224-5p/EGR2 and their relationship were investigated in PTC. We hope that these findings can provide a new approach for the diagnosis and treatment of PTC.

## Patients and Methods

### *Clinical Tissues*

Fifty-seven pairs of PTC surgical specimens and adjacent tissues were obtained from the Affiliated Hospital of Qingdao University. All PTC patients provided written informed consents. These patients did not receive any treatment prior to surgery. Then, these tissues were frozen in liquid nitrogen and stored in a refrigerator at  $-80^{\circ}\text{C}$ . This investigation was approved by the Institutional Ethics Committee of the Affiliated Hospital of Qingdao University.

### *Cell Culture and Transfection*

Human thyroid epithelial cell line Nthyori3-1 and PTC cell lines TPC-1, K1, BCPAP were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). These cells were inoculated in Roswell Park Memorial Institute-1640 (RPMI-1640; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and incubated with 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ .

MiR-224-5p mimic, miR-224-5p inhibitor or EGR2 siRNA were purchased from GenePharma (Shanghai, China). Next, they were transfected into PTC cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), respectively.

### *Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)*

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA containing miRNA. QRT-PCR was performed on ABI 7500 Fast Real-Time PCR system (Applied Biosystems; Foster City, CA, USA) using Fast SYBR-Green master mix (GeneCopoeia; Rockville, MD, USA). U6 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control for miR-224-5p or EGR2. Finally, the expression of miR-224-5p and EGR2 was calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method. The primers sequences used were: miR-224-5p: Forward primer, 5'-CTG GTA GGT AAG TCA CTA-3' Reverse primer, 5'-TCA ACT GGT GTC GTG GAG-3'; U6: Forward primer, 5'-CTG GTA GGG TGC TCG CTT CGG CAG-3' Reverse primer, 5'-CAA CTG GTG TCG TGG AGT CGG C-3'. EGR2, Forward primer 5'-CCT GCG ACC TCG AAA GTA-3' and Reverse primer 5'-TCG TCA CTC CTG GCA AAC-3'. GAPDH: Forward primer, 5'-TGACTTCAACAGCGACAC-CCA-3' and Reverse primer, 5'-GGAGTGTTG-GAGAAGTCATATTAC-3'.

### *Western Blot Analysis*

Protein samples were obtained using radio-immunoprecipitation assay (RIPA; Beyotime, Shanghai, China) lysis buffer. The proteins were then separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred into polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Next, the protein was incubated with 5% skim milk at room temperature for 2 h. The membranes were then incubated with rabbit monoclonal anti-EGR2 (1:1000; Abcam, Cambridge, MA, USA), rabbit monoclonal anti-GAPDH antibody (1:1000; Epitomics, Burlingame, CA, USA) overnight at  $4^{\circ}\text{C}$ . After washing, the protein was incubated with goat polyclonal anti-rabbit IgG secondary antibody (1:1000; Abcam, Cambridge, MA, USA) for 1 h. Finally, protein expression levels were measured by enhanced chemiluminescence (ECL; Pierce, Rockford, IL, USA). In addition, antibodies against Vimentin, E-cadherin, and N-cadherin were obtained from Abcam (Cambridge, MA, USA).

### *Transwell Assays*

Transwell chambers (8  $\mu\text{m}$  pore size; Millipore, Billerica, MA, USA) were used to assess cell migration and invasion in 24-well plates. Serum-free PTC cells were placed in the up-

per chamber uncovered membrane. RPMI-1640 medium with 10% FBS was added in the lower chamber. The cells in the upper chamber coated with membrane were used for invasion assay. These cells were incubated for cell migration and invasion for 24 h. The migrated and invaded cells were stained with 0.1% crystal violet and counted by a microscope (Olympus, Tokyo, Japan).

### Dual-Luciferase Reporter Assay

The 3'-UTR of wild type or mutant EGR2 was inserted into the pmirGLO luciferase vector (Promega, Madison, WI, USA). Then, the above vector and miR-224-5p mimics were transfected into A375 cells for 48 h. Finally, Dual-Luciferase assay system (Promega, Madison, WI, USA) was used to analyze luciferase activity.

### Statistical Analysis

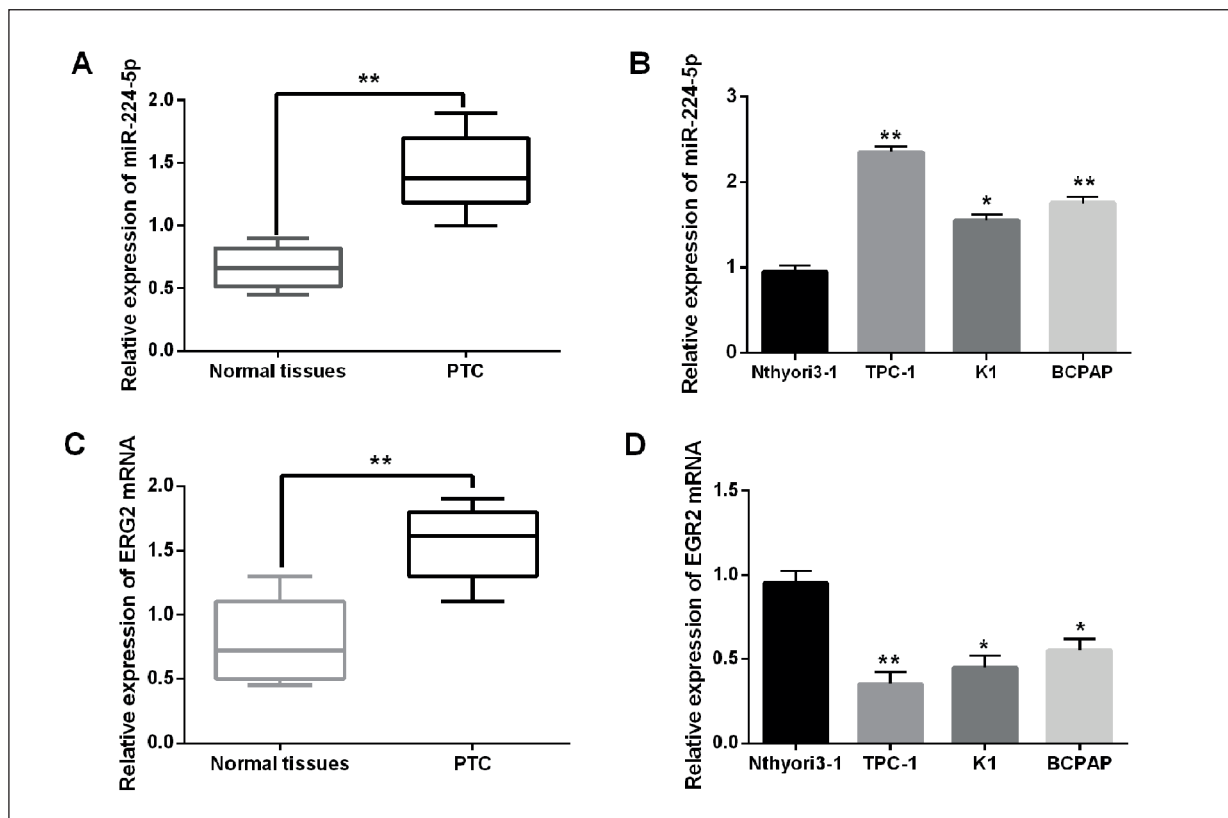
Data were analyzed by SPSS 19.0 (SPSS IBM, Armonk, NY, USA) and GraphPad Prism 6 (La

Jolla, CA, USA). Differences were calculated using Student's *t*-test or ANOVA. Pearson's  $\chi^2$ -tests were used for analysis of the correlation between clinicopathological features and miR-224-5p expression in PTC patients. Kaplan-Meier survival analysis was used for analysis of survival rate and *p*-value was calculated by the log-rank test  $p < 0.05$  was defined as a significant difference.

## Results

### Upregulation of MiR-224-5p and Downregulation of EGR2 Were Identified in PTC

First, the expression level of miR-224-5p was observed in PTC tissues. Upregulation of miR-224-5p was found in PTC tissues compared to normal tissues (Figure 1A). Next, the expression of miR-224-5p was observed in TPC-1, K1, BCPAP, and Nthyori3-1 cell lines. We found that



**Figure 1.** Upregulation of miR-224-5p and downregulation of EGR2 were identified in PTC. **A**, Expressions of miR-224-5p in PTC tissues detected *via* qRT-PCR. **B**, Expression of miR-224-5p in TPC-1, K1, BCPAP cell lines and Nthyori3-1 cells (control). **C**, Expressions of EGR2 in PTC tissues detected *via* qRT-PCR. **D**, EGR2 expression in TPC-1, K1, BCPAP cell lines, and Nthyori3-1 cells (control). \* $p < 0.05$ , \*\* $p < 0.01$ .

**Table I.** Relationship between miR-224-5p expression and their clinic-pathological characteristics of PTC patients.

Characteristics	Cases	miR-224-5p		p-value
		High	Low	
Age (years)				0.186
≥ 60	37	20	17	
< 60	20	12	8	
Gender				0.071
Male	26	16	10	
Female	31	18	13	
Tumor size				0.237
< 5 cm	35	20	15	
≥ 5 cm	22	14	8	
TNM stage				0.007*
I-II	40	24	16	
III-IV	17	10	7	
Lymph node metastasis				0.028*
No	42	27	15	
Yes	15	10	5	

Statistical analyses were performed by the  $\chi^2$ -test. \* $p < 0.05$  was considered significant.

miR-224-5p expression was higher in TPC-1, K1, BCPAP cell lines than that of NHOK cells (Figure 1B). In addition, EGR2 expression was examined in PTC tissues and cells. In contrast to the trend of miR-224-5p, EGR2 expression in PTC tissues and cell lines was reduced (Figure 1C, 1D). These results show that abnormal expression of miR-224-5p and EGR2 may be associated with tumorigenesis of PTC.

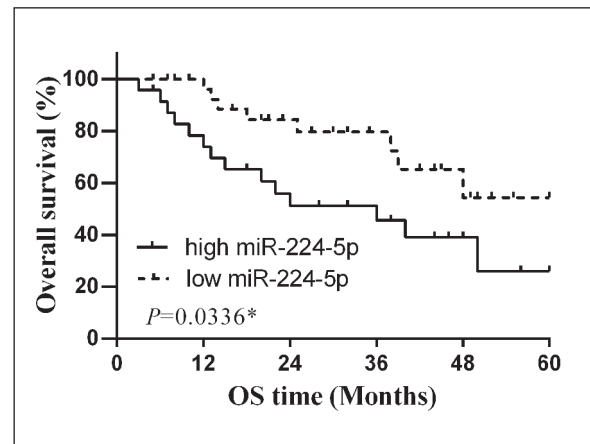
**Upregulation of MiR-224-5p Was Related to Clinic-Pathological Features and Poor Prognosis of the PTC Patients**

The correlation between miR-224-5p expression and clinicopathological features in PTC patients was investigated. High expression of miR-224-5p was found to be associated with TNM stage ( $p=0.007$ , Table I) and lymph node metastasis ( $p=0.028$ , Table I) in PTC patients. Thus, we consider that miR-224-5p may be involved in the development of PTC. Additionally, survival analysis indicated that high expression of miR-224-5p was associated with shorter overall survival in PTC patients ( $p=0.0336$ ; Figure 2). Based on these findings, we consider that upregulation of miR-224-5p predicts adverse clinical outcomes in PTC patients.

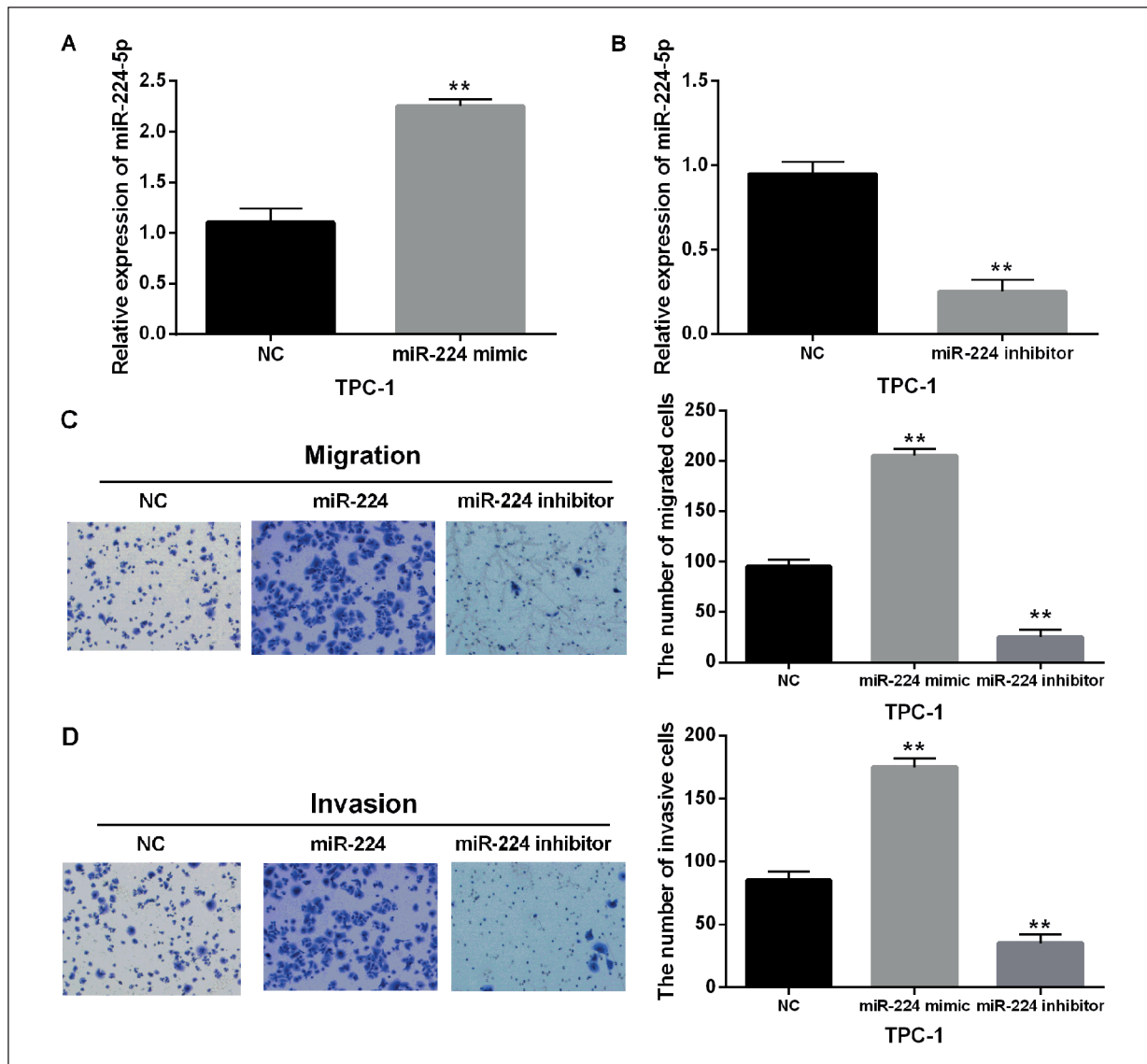
**Upregulation of MiR-224-5p Contributed to Cell Migration and Invasion in PTC**

To investigate the specific function of miR-224-5p in PTC, miR-224-5p mimics or inhibi-

tor was transfected into TPC-1 cells. We found that miR-224-5p mimics significantly increased its expression (Figure 3A), while miR-224-5p inhibitor decreased its expression in TPC-1 cells (Figure 3B). Furthermore, transwell assay showed that upregulation of miR-224-5p promoted cell migration and invasion in TPC-1 cells (Figure 3C, 3D). Meanwhile, downregulation of miR-224-5p inhibited TPC-1 cell migration and invasion (Figure 3C, 3D). Therefore, it is confirmed that upregulation of miR-224-5p contributes to cell metastasis in PTC.



**Figure 2.** MiR-224-5p could predict the prognosis of PTC patients. High miR-224-5p expression patients showed shorter OS. \* $p < 0.05$ .



**Figure 3.** The effect of miR-224-5p on cell migration and invasion was detected in PTC. **A-B**, Expression of miR-224-5p was examined in TPC-1 cells contained miR-224-5p mimics or inhibitor *via* qRT-PCR. **C-D**, Cell migration and invasion were measured in cells with miR-224-5p mimics or inhibitor *via* transwell analysis (magnification 200 $\times$ ). \*\* $p < 0.01$ .

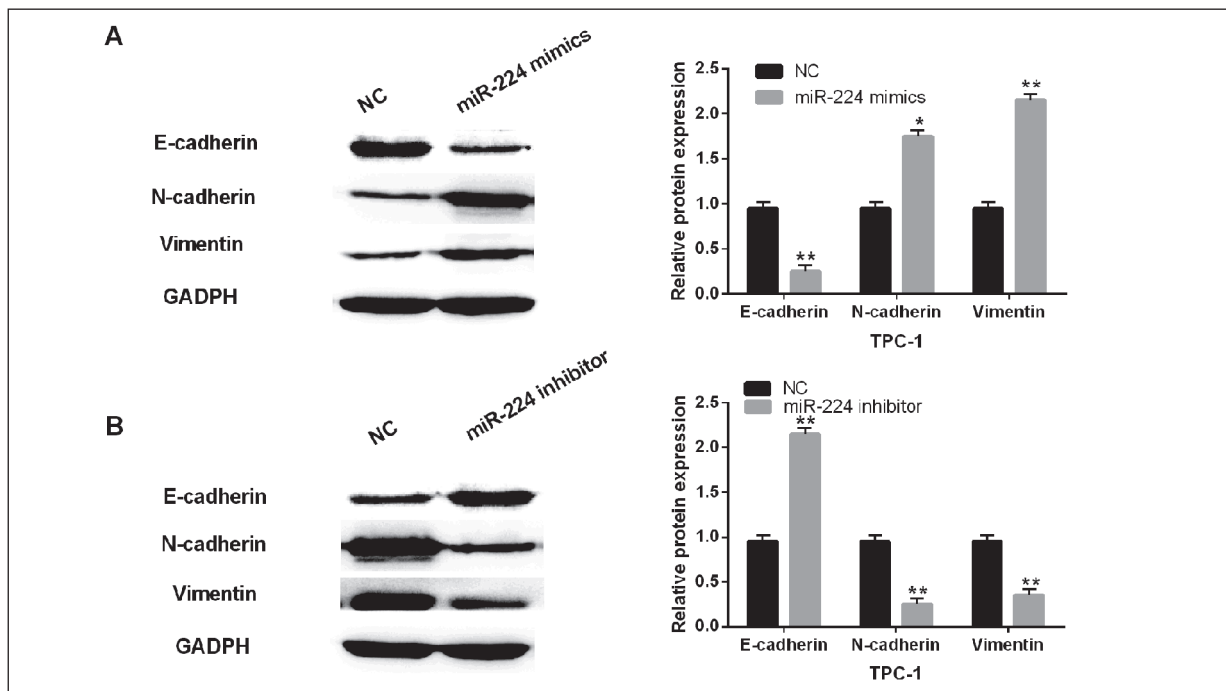
### ***Downregulation of MiR-224-5p Was Found to Prevent EMT in PTC***

The expression levels of EMT markers were detected to further explore the effect of miR-224-5p on cell metastasis in TPC-1 cells. Western blot analysis showed that overexpression of miR-224-5p inhibited the expression of E-cadherin and promoted the expression of N-cadherin and Vimentin (Figure 4A). In contrast, downregulation of miR-224-5p promoted E-cadherin expression and suppressed N-cadherin and Vi-

mentin expression (Figure 4B). Briefly, upregulation of miR-224-5p promotes EMT to regulate cell metastasis in PTC.

### ***MiR-224-5p Directly Targeted EGR2 in PTC Cells***

TargetScan ([http://www.targetscan.org/vert\\_71/](http://www.targetscan.org/vert_71/)) was used to predict the target genes of miR-224-5p. Then, we selected EGR2 as a candidate target for miR-224-5p (Figure 5A). A luciferase reporter assay was then performed to confirm the



**Figure 4.** Overexpression of miR-224-5p promoted EMT in PTC cells. **A-B**, Expression of E-cadherin, N-cadherin, and Vimentin in TPC-1 cells contained miR-224-5p mimics or inhibitors. \* $p < 0.05$ , \*\* $p < 0.01$ .

binding sites between EGR2 and miR-224-5p. We found that miR-224-5p mimics significantly inhibited the luciferase activity of wild-type EGR2 (EGR2-Wt), but had little effect on luciferase activity of mutant-type EGR2 (EGR2-Mut, Figure 5B). In addition, EGR2 expression was found to be inversely correlated with miR-224-5p expression in PTC tissues ( $R^2=0.4044$ ,  $p < 0.01$ , Figure 5C). Furthermore, overexpression of miR-224-5p significantly inhibited the mRNA and protein expression of EGR2, while downregulation of miR-224-5p promoted EGR2 expression in TPC-1 cells (Figure 5D, 5E). Collectively, miR-224-5p directly targets EGR2 and is inversely correlated with EGR2 expression in PTC.

#### **The Inhibitory Effect of EGR2 Was Examined in PTC**

To explore the function of EGR2 in PTC, EGR2 siRNA was transfected into TPC-1 cells. We found that EGR2 siRNA reduced the expression of EGR2 in TPC-1 cells (Figure 6A). Furthermore, EGR2 siRNA was found to inhibit E-cadherin expression and promote N-cadherin and Vimentin expression (Figure 6B). In addition, knockdown of EGR2 was found to promote cell migration and invasion in PTC cells (Figure

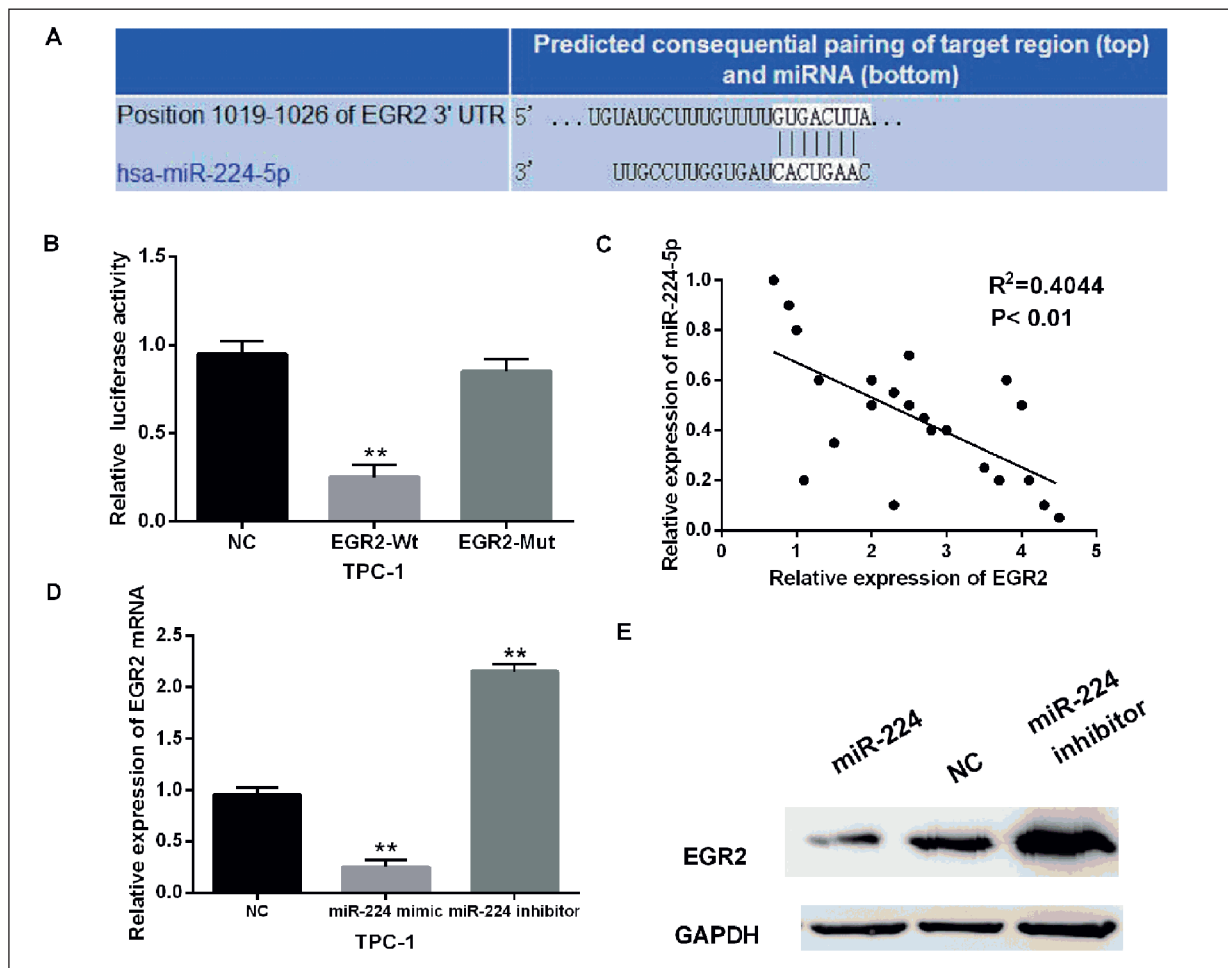
6C, 6D). Taken together, knockdown of EGR2 promoted cell metastasis in PTC.

#### **Overexpression of EGR2 Weakened the Carcinogenesis of MiR-224-5p in PTC**

EGR2 vector and miR-224-5p mimics were transfected into TPC-1 cells to investigate their interaction in PTC. The rescue experiments suggested that the decreased expression of EGR2 induced by miR-224-5p mimics was recovered by EGR2 vector (Figure 7A, 7B). Functionally, upregulation of EGR2 vector also weakened the promoting effect of miR-224-5p on cell migration and invasion in TPC-1 cells (Figure 7C, 7D). Therefore, we consider that overexpression of EGR2 attenuates the carcinogenic effects of miR-224-5p in PTC.

### **Discussion**

Recently, many studies have demonstrated that miRNAs are involved in the development of PTC. For example, miR-613 has been reported to inhibit cell growth, migration, and invasion in PTC by regulating SphK2 expression<sup>24</sup>. It was also found that miR-146b-5p promoted cell me-

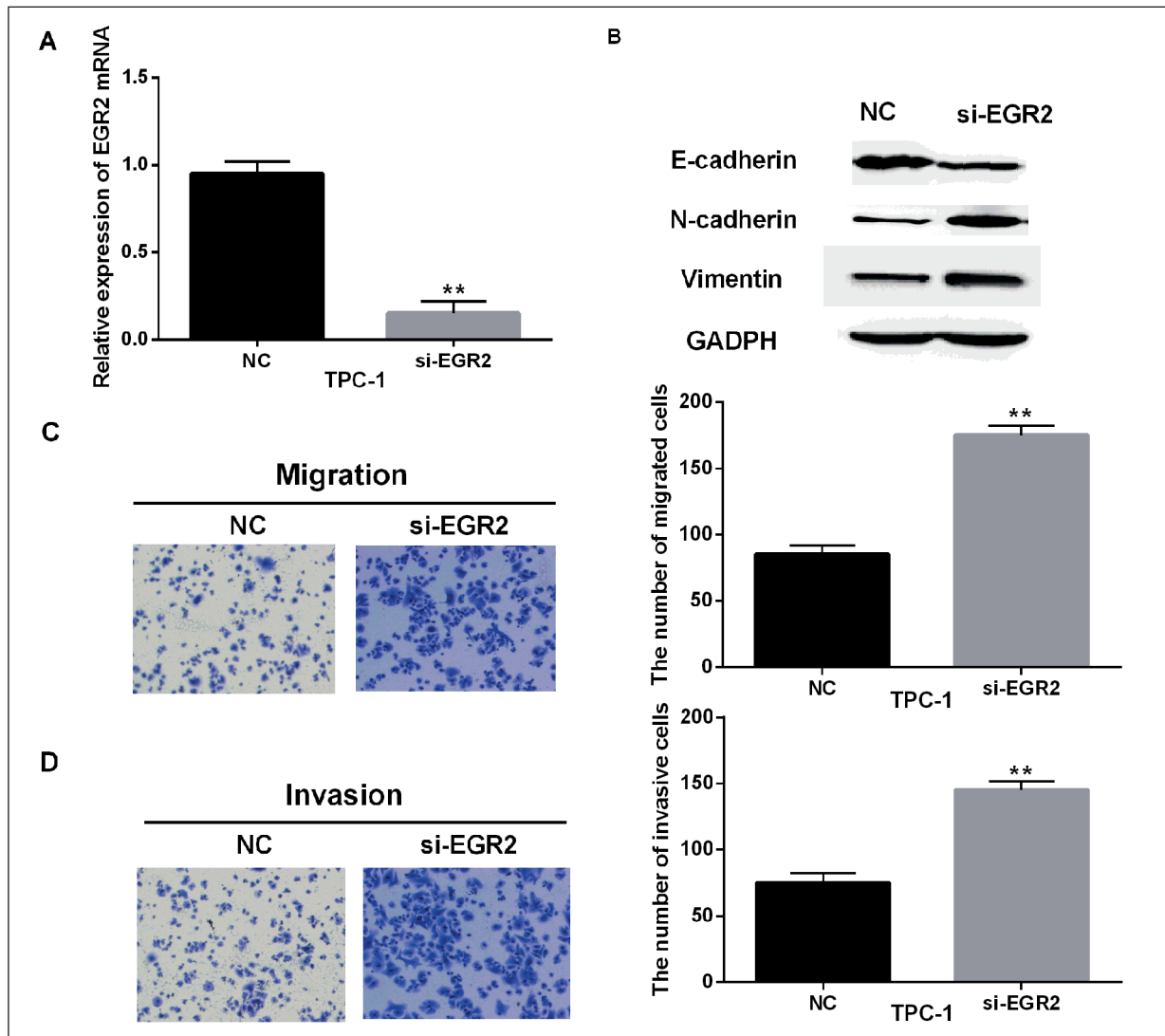


**Figure 5.** MiR-224-5p directly targeted EGR2 in PTC cells. **A**, Binding sites of miR-224-5p on the 3'-UTR of EGR2. **B**, Luciferase reporter assay. **C**, Correlation between miR-224-5p and EGR2. **D-E**, mRNA and protein expression of EGR2 was analyzed in cells containing miR-224-5p mimics or inhibitor  $**p<0.01$ .

tastasis and EMT in PTC by suppressing ZNRF3 expression<sup>25</sup>. In addition, miR-204-5p inhibited cell proliferation in PTC by downregulation of IGFBP5<sup>26</sup>. Overexpression of miR-101 promoted mitochondrial apoptosis by modulating MCL-1 and c-met in PTC<sup>27</sup>. In this study, downregulation of miR-224-5p was detected in PTC tissues and cells. Furthermore, upregulation of miR-224-5p was closely associated with TNM stage and lymph node metastasis and predicted poor prognosis in PTC patients. The results have not been found in previous studies. Functionally, miR-224-5p promoted cell migration and invasion as well as EMT in PTC.

The function of miR-224 depends on the type of human cancers. In fact, MiR-224 has been identified as a biomarker for detecting hepatocellular carcinoma at an early stage<sup>28</sup>. MiR-224

was downregulated in colorectal cancer and inhibited cell migration by targeting Cdc42<sup>29</sup>. The inhibitory effect of miR-224 on cell metastasis was also found in prostate cancer<sup>30</sup>. However, miR-224 was upregulated in non-small cell lung cancer and promoted tumor progression<sup>31</sup>. Additionally, miR-224 also served as an oncogene in hepatocellular carcinoma through affecting AKT expression<sup>32</sup>. These results are consistent with our findings. Different from previous studies, we found that miR-224-5p promotes cell metastasis by activating EMT in PTC. EMT is a critical factor for the advanced-stage cancer cells to gain invasive characteristics. The activation of EMT is characterized by the reduced expression of epithelial marker such as E-cadherin and the increased expression of mesenchymal cell-surface markers such as N-cadherin and Vimentin<sup>33</sup>.

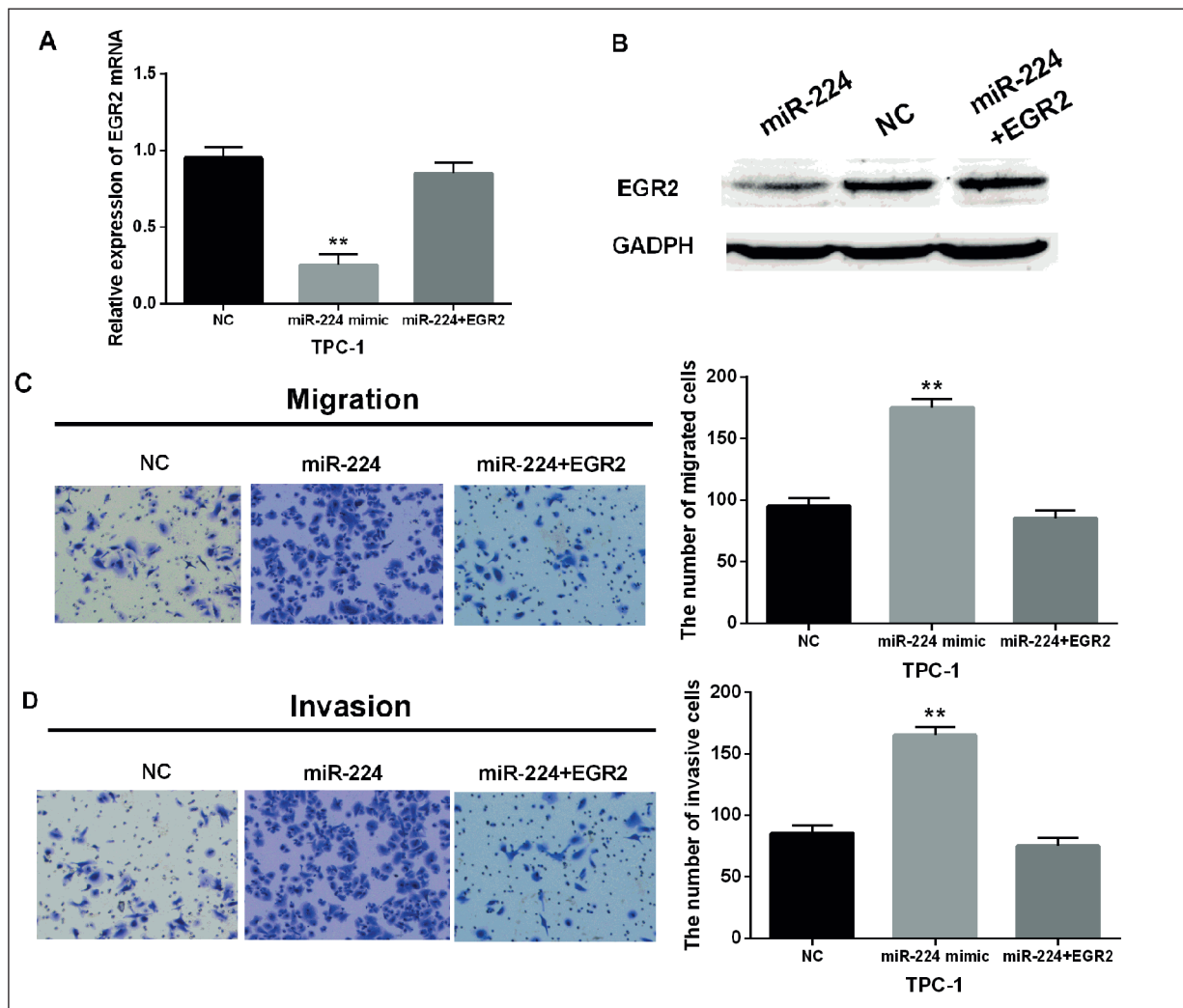


**Figure 6.** The inhibitory action of EGR2 was examined in PTC. **A**, Expression of EGR2 was measured in cells containing EGR2 siRNA. **B**, Western blot analysis of E-cadherin, N-cadherin, and Vimentin in TPC-1 cells contained si-EGR2. **C-D**, Cell migration and invasion analysis of TPC-1 cells with si-EGR2 was detected by transwell assay (magnification 200×) \*\*  $p < 0.01$ .

Here, miR-224-5p also regulated the expression of E-cadherin, N-cadherin, and Vimentin to activate EMT in PTC.

Additionally, it has been reported that miR-224 targets ERG2, which contributes to the progression of meningioma<sup>34</sup>. In the current study, ERG2 was also confirmed to be a direct target of miR-224-5p in PTC. Furthermore, downregulation of EGR2 was found in PTC. Notably, the inhibitory effect of EGR2 on cell migration, invasion, and EMT was also examined in PTC. Besides that, overexpression of EGR2 weakened the carcinogenesis of miR-224-5p in PTC. EGR2

is a member of EGR family that is involved in the regulation of cell proliferation, differentiation, and growth<sup>35</sup>. Furthermore, EGR2 has been reported to regulate tumorigenesis by interacting with several miRNAs, such as miR-150<sup>36</sup> and miR-330<sup>37</sup>. Here, we also found that EGR2 expression can be negatively regulated by miR-224-5p in PTC. In addition, miR-17-5p has been reported to promote gastric cancer cell migration and invasion by directly targeting EGR2<sup>38</sup>, which is consistent with our findings. Here, it was also found that EGR2 inhibited EMT in PTC, which has not been reported previously. All these results



**Figure 7.** Overexpression of EGR2 weakened the carcinogenesis of miR-224-5p in PTC. **A-B**, mRNA and protein expressions of EGR2 were measured in cells containing miR-224-5p mimics and EGR2 vector. **C-D**, Cell migration and invasion in cells containing miR-224-5p mimics and EGR2 vector was measured by transwell assay (magnification 200 $\times$ ). \*\*  $p < 0.01$ .

imply that EGR2 is involved in PTC progression by inhibiting cell metastasis.

#### Conflict of Interest

The Authors declare that they have no conflict of interests.

#### Conclusions

We showed that miR-224-5p was upregulated in PTC and associated with worse clinical outcomes in PTC patients. Functionally, miR-224-5p promoted cell migration and invasion by targeting EGR2. Meanwhile, miR-224-5p/EGR2 axis is involved in PTC by regulating EMT. These findings suggest that miR-224-5p may be a novel diagnostic and prognostic biomarker for human PTC.

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