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MiR-524 inhibits cell proliferation and induces cell apoptosis in thyroid cancer via targeting SPAG9

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Abstract. – OBJECTIVE: To investigate the effect of miR-524 on the proliferation of thyroid cancer and its underlying mechanism.

PATIENTS AND METHODS: MiR-524 expression levels in thyroid cancer samples and para-cancer tissues were tested by quantitative Real-time polymerase chain reaction (qRT[illegible] Cells proliferative ability and apopto[illegible] we[illegible] evaluated through methyl thiazolyl tetra[illegible]um (MTT) and apoptosis assays, respective[illegible] [illegible]uciferase reporter assay was used to confir[illegible] regulatory mechanism.

RESULTS: MiR-524 expressi[illegible] reduc[illegible] in thyroid cancer specimen [illegible]-regulated miR-524 expression inhi[illegible]d the [illegible]iferative ability and enhanced ce[illegible]optosis [illegible] thyroid cancer cells. SPAG9 was[illegible]get [illegible] 524, and was reverse[illegible]regu[illegible]

CONCLUSIONS: [illegible]R-524 [illegible]es thyroid cancer cell prolif[illegible]ion and indu[illegible]ell apoptosis *via* targe[illegible]G9.

Key Words
miR-52[illegible]yroid cancer, [illegible]tion, Apoptosis.

[illegible]ntroduction

Thyro[illegible]er is [illegible] most common malignant [illegible] of end[illegible]gin, accounting for 94.5% of all [illegible]ocrine tu[illegible] and 2.6% of all malignant tumo[illegible] with the highest incidence of head and neck m[illegible]rs. At the beginning of the last cen[illegible]y, the [illegible]dence of thyroid cancer exhibited a [illegible]al upward trend[1,2]. Papillary thyroid cancer ([illegible] the most common type of thyroid cancer, acco[illegible]ting for about 90% of all pathological types[3]. At present, the treatment of papillary thyroid carci[illegible]a includes surgical treatment, thyroid hormone [illegible]pression thera[illegible] isotope iodine 131 treatment [illegible] adjuvant rad[illegible]erapy. With effective and reaso[illegible] treatment[illegible]apillary thyroid carcinoma gen[illegible]eral[illegible]rognosis; the 5-year survival rate is about [illegible] 10-year survival rate is also above [illegible]%[4]. However, some papillary cancers have the [illegible]f dedifferentiation and eventually devel[illegible] [illegible]orly differentiated thyroid cancer or undifferentiated carcinoma, which results in the decline of survival rate and quality of life[5]. Therefore, it is crucial to look for differentially expressed genes, predictive biomarkers and target molecules for intervention therapy, which could further increase the cure rate and survival rate of patients.

Previous researchers[6-11] have found that miRNAs are implicated in cell proliferation, apoptosis, invasion and angiogenesis in the different diseases. In thyroid cancer, miRNAs are differentially expressed in the serum of patients[12]. MiR-146a polymorphism is significantly associated with PTC risk[13].

Upregulation of microRNA-524-5p enhances the cisplatin sensitivity of gastric cancer cells by modulating proliferation and metastasis *via* targeting SOX9[14]. miRNA-106b-mediated down-regulation of C1orf 24 expression induces apoptosis and suppresses invasion of thyroid cancer[15]. MiR-524-5p may function as a novel tumor suppressor gene, and suppresses the growth and invasive abilities of gastric cancer cells[16]. However, there are no relevant reports on the mechanism of action of miR-524 in PTC. The primary purpose of this study was to investigate the effect of miR-524 on the proliferation of thyroid cancer and its underlying mechanism.

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Patients and Methods

Clinical Specimens

We collected fresh cancer tissues and para-cancer tissues from thyroid cancer patients in our hospital. All specimens were pathologically confirmed and quickly placed in inactivated RNAse cryopreservation tube in liquid nitrogen cryopreservation during 15 minutes, and then into 80°C refrigerator to be reserved. All patients did not receive the treatment of preoperative iodine 131 and thyroid hormone suppression therapy. Patients with other malignant tumors, systemic serious infection, and other serious systemic diseases were excluded. This study was approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. Signed written informed consents were obtained from all participants before the study.

Cell Culture

Thyroid cancer cell lines WRO [DMEM (Dulbecco's Modified Eagle Medium)] and TPC1 [RPMI (Roswell Park Memorial Institute)] [illegible]um were supplemented with 10% fetal bo[illegible]rum (FBS; Life Technologies, Grand Isla[illegible]Y, USA). Both of them were maintained in a 5% [illegible] and 37°C humidified incubator.

Cell Transfection

MiR-524 mimics and [illegible]-524 [illegible]ics NC (normal control) were syn[illegible]ized and [illegible]rified by Shanghai Jima Co., Ltd. (S[illegible]hai, [illegible] were seeded into 6-w[illegible] plate[illegible]sity of 2.[illegible] × 10^5/mL. After 48 [illegible]iRNA-524 [illegible] and miR-524 mimics NC [illegible]ransfected in[illegible]RO and TPC1 cell lin[illegible] with [illegible]fectamine 2000 (Invitrogen, Carlsbad, CA, US[illegible]cording to the manufacturer's protocol. The fi[illegible]oncentration was 50 nM.

Qu[illegible]tativ[illegible]eal-Time Polymerase Chai[illegible]on (qR[illegible]CR)

Total [illegible] was e[illegible]acted from the cell lines [illegible]e tissu[illegible]he TRIzol reagent kit (Life Te[illegible]logies, G[illegible]d Island, NY, USA). Then, all [illegible]NA was synthesized into complementary [illegible] Nucleic Acid (cDNA) *via* using [illegible]MuLV reverse transcriptase (Fisher Scientific, [illegible]burgh, PA, USA). Real-time PCR was cond[illegible] using SYBR Green mix (TaKaRa, Dalian, China). The detection system condition was 94°C for 3 min, followed by 35 cycles of 94°C for 30 s and 60°C for 30 s. MiR-524 expression was normalized to U6 RNA. Primer[illegible]es used in this study were as follow[illegible] F: 5'-GCTGTGACCCTACAAA[illegible]GA-3', R: 5'-AGCATCAACTTCAACGCT[illegible]3'; SPAG9, F: 5'-TCCTGAGCTGGATATG[illegible]AAGA-3', R: 5'-GCCTGAGCCAGC[illegible]AAGA[illegible]'; U6, F: 5'-CTCGCTTCGGCA[illegible]ACA-3', R: [illegible]AC GCTTCACGAATTTG[illegible]T-3'.

Methyl Thiazolyl T[illegible]ol[illegible]n (MTT) Assay

Cells (1 × [illegible] cells/well) [illegible] se[illegible]ed into a 96-well plat[illegible] incubated fo[illegible], 4, 5, and 6 days. M[illegible] re[illegible] (20 µL) was added to each well for 4 h incuba[illegible]. Then, dimethyl sulfoxide (DMSO) (150 µL) wa[illegible]ed to each well. The ab[illegible]e at 540 nm wa[illegible]ngth was detected.

[illegible]ll Apoptosis Detection

[illegible]n accordanc[illegible]ith the manufacturer's protoco[illegible]ll apoptos[illegible]as detected by using an Annex[illegible] (Becton, Dickinson and Company, Fr[illegible] Lakes, NJ, USA). Cells (5×105) were re-suspended in the binding buffer (200 µL) [illegible]bated with FITC Annexin V (5 µL) and [illegible]opi[illegible]m iodide solution (1 µL). Finally, cell apoptosis was analyzed by Calibur Flow Cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Luciferase Reporter Assay

With PCR Amplification kit (TaKaRa, Dalian, China), SPAG9 3'UTR including the predicted binding site was amplified, and then cloned into the psiCHECK-2 reporter vector (Promega, Madison, WI, USA). By using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), psiCHECK-2-SPAG9-wild (200 ng) or psiCHECK-2-SPAG9-mutant (200 ng) and miR-524 mimics (100 nmol/L) were co-transfected into WRO cells. The reporter activity was examined by a Dual Luciferase Reporter Assay kit (Promega, Madison, WI, USA).

Statistical Analysis

The results were carried out in triplicate. Graphpad Prism v5.0 (GraphPad Software, La Jolla, CA, USA) was used for analyses. The comparisons were examined using the Student's *t*-test. A p value of <0.05 was regarded as statistically significant.

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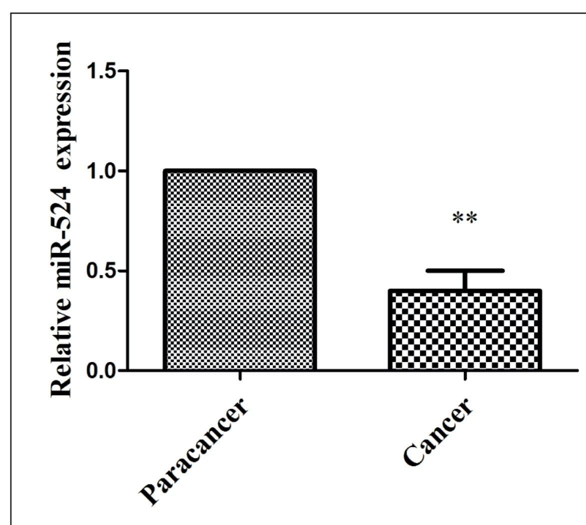

Figure 1. The relative miR-524 expression was detected by qRT-PCR between paracancer tissues and cancer tissues (**p<0.01).

Results

MiR-524 Was Down-Regulation In Thyroid Cancer Samples

To investigate the unknown functions [illegible] 524 in thyroid cancer, we firstly detec[illegible] the expression of miR-524 in cancer tissues a[illegible]ra-cancer tissues *via* using qRT-PCR method. [illegible] analysis exhibited that lower exp[illegible]ion of mi[illegible] 524 was found in cancer tiss[illegible]red wit[illegible] the para-cancer tissues (Fi[illegible]e 1). [illegible] finding demonstrated that dysre[illegible]tion of [illegible]R-524 is implicated in the progress[illegible] thy[illegible]

MiR-524 Overe[illegible]ession Co[illegible] Repress Cell P[illegible]rative Abili[illegible]

Furthermo[illegible] to [illegible]e the influence of miR-524 down-regulation o[illegible] proliferative ability, MTT me[illegible]d was used. I[illegible]ingly, the result of MT[illegible]igure 2A and 2B) s[illegible]ed that the prolifer[illegible]e ability of cells transfected with miR-524 mi[illegible]as d[illegible]eased as compared to the control group, [illegible]uggested [illegible]t miR-524 overexpres[illegible] could [illegible]ss ce[illegible]oliferative ability.

Up[illegible]gulatio[illegible] miR-524 Co[illegible] Promote Cell Apoptosis

[illegible]e futher investigated the changes [illegible] cell apoptosis responding to up-regulation of [illegible]524 through an apoptosis assay. The results o[illegible]optosis assay showed that more cell apoptosis was detected in over-expressing miR-524 group compared with the control group (Figure 2C and 2D). These results indicated that up-regulation of miR-524 could promote cell apoptosis.

SPAG9 Was a Target Gene of miR-524

Various studies[6-9] have report[illegible]hat miRNAs influences the different cancer [illegible]ment by modulating the target gene[illegible]. To fi[illegible] target gene of miR-524, we use[illegible]ree miRN[illegible]in formatics predictive so[illegible]are (miRDB, R[illegible] and TargetScan). Th[illegible]tersecti[illegible] of the three bioinformatics predic[illegible]ft[illegible]e were a[illegible]essed (Figure 3A). A t[illegible] of [illegible]ntial ta[illegible] genes of miR-524 w[illegible] shown in [illegible] 3[illegible]. Additionally, accord[illegible] the research [illegible]arg et al [17], SPAG9 w[illegible]ig[illegible]essed in thy[illegible]d cancer, and knockdown of SPA[illegible]xpression in cell significantly reduced cellula[illegible]th and colony formatio[illegible], we focused o[illegible]AG9.

[illegible]R-524 Coul[illegible] Reversely [illegible]ulate SPA[illegible] Through Bounding t[illegible]AG9 3`U[illegible]

[illegible] binding site of miR-524 to SPAG9 [illegible] was shown in Figure 4A. To con[illegible]m it, we planned a luciferase reporter assay. [illegible] displayed that the luciferase activity [illegible]as s[illegible]ficantly lessen in psiCHECK-2-SPAG9-Wild+mimics (Figure 4B), which demonstrated that miR-524 could bound to SPAG9 3`UTR.

Furthermore, we used qRT-PCR method to detect the regulatory correlation between miR-524 and SPAG9. This test displayed that declined SPAG9 was expressed when miR-524 was up-regulation (Figure 4C and 4D). All these results indicated that miR-524 could reversely regulate SPAG9 through bound to SPAG9 3`UTR.

Discussion

MiRNAs, as small single-chain non-coding RNAs with 19-22 nt in length, can regulate the expression of specific mRNAs at the transcriptional level, ultimately resulting in functional changes in cells or tissue. Evidence had indicated that abnormal up-regulation or down-regulation of miRNAs is associated with the development, invasion, and metastasis of various tumors[18,19]. Our work found that there is a significant difference in the expression of miR-524 between thyroid cancer and para-cancerous tissues. Through the previous functional tests, we discovered that miR-524 acts as a tumor suppressor gene in thyroid cancer. Ac-

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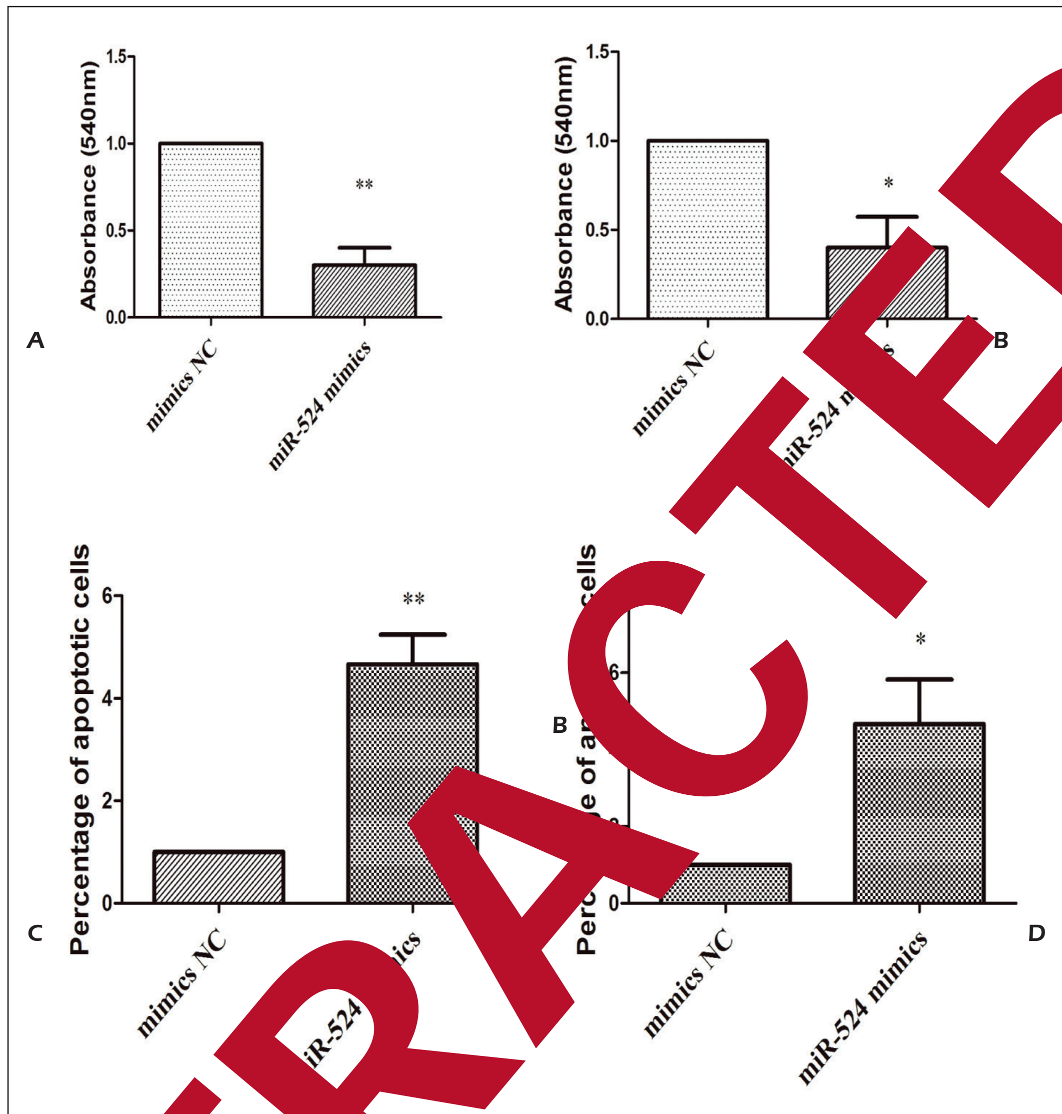

Figure 2. MiR-524 [illegible] expression inhi[illegible]l proliferation and promoted cell apoptosis. MTT assessment of up-regulated miR-524 on cell pr[illegible], absorbance ([illegible]) was obtained in WRO (**A**) and TPC1 (**B**); cell apoptosis assay was performed in WRO [illegible] and [illegible]**D**) (*p<0.05, **p<0.01).

cording [illegible] the mechanism of [illegible]RNA on downstrea[illegible]arget ge[illegible]es, it can inhibit the translational exp[illegible]n of [illegible]get gene mRNA and reduce the protei[illegible]t throug[illegible]omplete or incomplete [illegible]ompleme[illegible]y wit[illegible]e target gene. Therefore, [illegible]rget ge[illegible]R-524 should meet the follow[illegible] conditio[illegible]irstly, the target genes are oncog[illegible]s, which are involved in promoting the deve[illegible] progression of tumors. Secondly, [illegible]re is complementation of miRNAs with their [illegible]t sites. Thirdly, there are conserved types o[illegible]NA target sites between different species. Four[illegible]ly, there is the thermal stability between miRNA and mRNA double-stranded. Fifthly, the miRNA target site should not have complex secondary structure. We chose three miRNAs bioinformatics predictive software (miRDB, RNA22, and TargetScan) together to predict the target genes of miR-524 according to the above conditions. The intersection of the three bioinformatics predictive software was assessed. Then, to verify whether SPAG9 was a downstream gene of miR-524, we detected SPAG9 mRNA expression *via* qRT-PCR. We found that miR-524 could up-regulate the expression of SPAG9. Additonally, miR-524 was further verified to act specifically on SPAG9 3'UTR by dual luciferase reporter gene system.

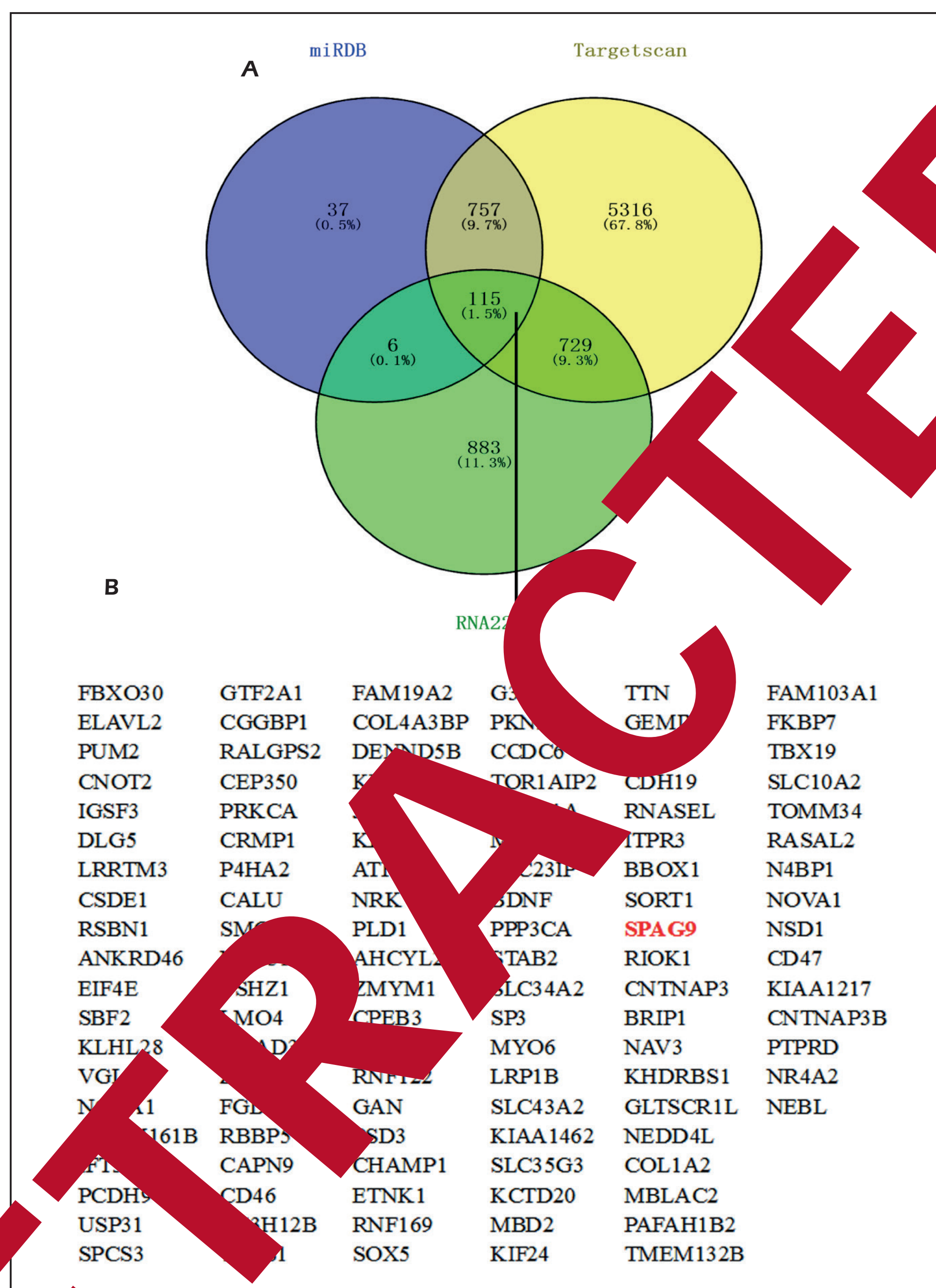

Fig... SPA... was a target gene of miR-524. **A**, The intersection of the three bioinformatics predictive software (miRDB, RNA22 ...tScan) wa...essed; **B**, 115 potential target genes of miR-524 were shown.

...vious stu... have found that SPAG9 expre...n levels are overexpressed in prostate can-ce... cancer[22], hepatocellular cancer[23,24], ...eosarcoma[25,26], breast cancer[27], and endometri...ncer[28]. Abnormal expression of SPAG9 contri... to the molecular biology processes.

To sum up, we first found the lower expression of miR-524 in thyroid cancer tissues, and the function of miR-524 tumor as suppressor gene through *in vitro* functional experiments. In addition, we further verified that SPAG9 is a downstream gene of miR-524. Further studies are needed to explore the role of SPAG9 downstream genes in thyroid cancer, so as to discover a complete regulatory pathway, and then improve the regulatory network in thyroid cancer.

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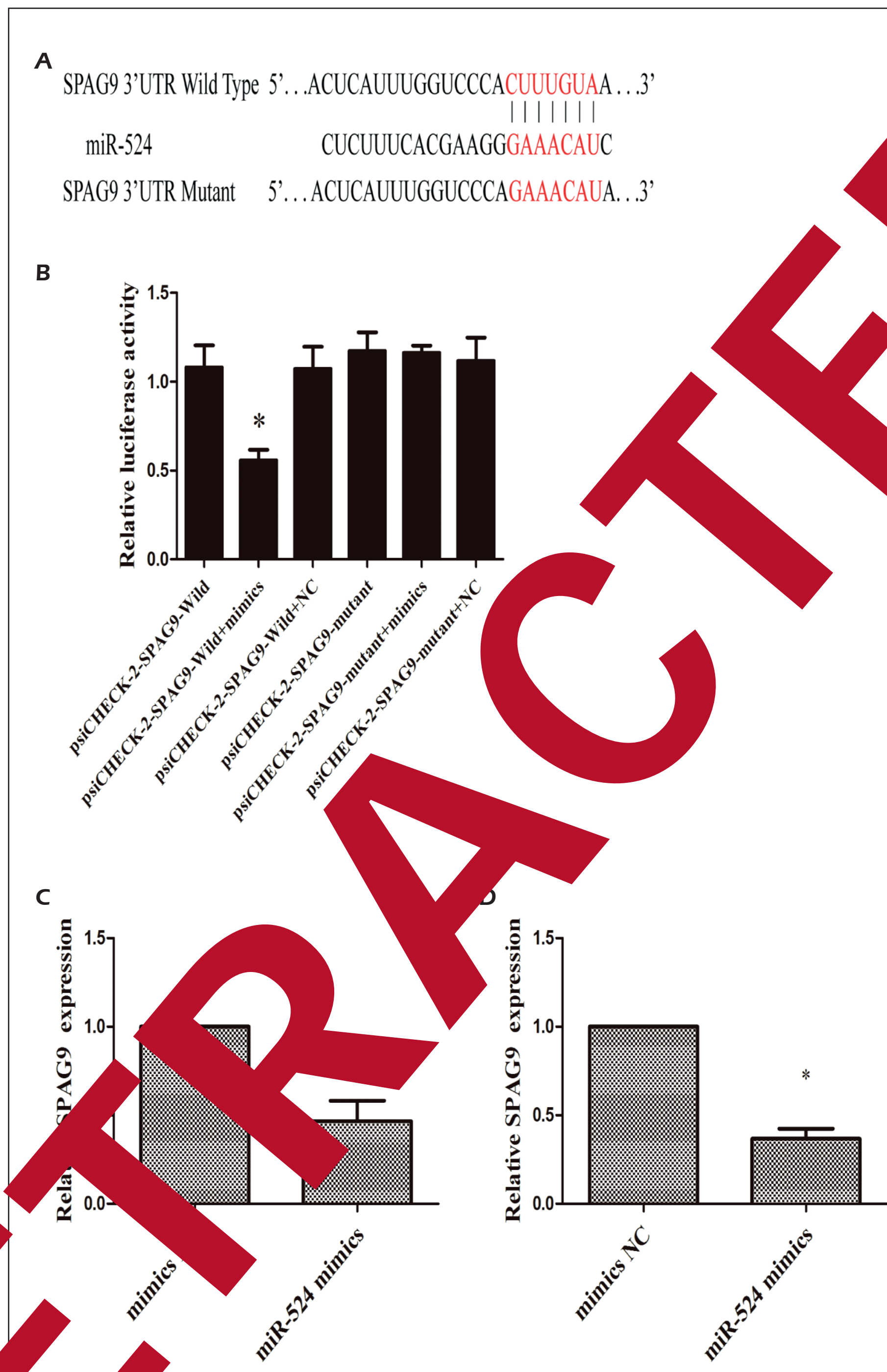

[illegible]re 4. M[illegible] inhi[illegible]d SPAG9 expression via targeting 3`UTR of SPAG9. **A**, The potential binding site was displayed [illegible]pe and m[illegible]; The luciferase activity was assessed; qRT-PCR analysis showed the relative SPAG9 expression in WR[illegible]) and TPC[illegible]) (*p<0.05).

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Conclusions

[illegible] first identified that miR-524 represses thyr[illegible]d cancer cell proliferation and induces cell apoptosis *via* targeting SPAG9, which may provide a new diagnostic criterion and potential therapeutic target in treatment for PTC.

Conflict of interest

The authors declared no conflict of interest.

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