MicroRNA-592 suppresses the malignant phenotypes of thyroid cancer by regulating lncRNA NEAT1 and downregulating NOVA1

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Received March 10, 2019; Accepted July 5, 2019

DOI: 10.3892/ijmm.2019.4278

Abstract. Numerous studies have demonstrated that various microRNAs (miRs) are aberrantly expressed in thyroid cancer and play critical roles in thyroid cancer malignancy. The aberrant expression of miR-592 has frequently been reported in multiple human cancer types; however, its expression profile and functions in thyroid cancer remain poorly understood. Reverse transcription-quantitative polymerase chain reaction was carried out to determine the expression profile of miR-592 in thyroid cancer tissues and cell lines. The regulatory effects of miR-592 upregulation on thyroid cancer cell proliferation, migration, and invasion *in vitro*, and tumor growth *in vivo* were investigated using a CCK-8 assay, migration and invasion assays, and a xenograft tumor model, respectively. Furthermore, the mechanisms underlying miR-592-mediated suppression of the aggressive phenotypes of thyroid cancer cells were explored in detail. The results indicated that miR-592 was significantly downregulated in thyroid cancer samples, and its downregulation was associated with lymph node metastasis and tumor-node-metastasis stage. Patients with thyroid cancer and low miR-592 expression exhibited shorter overall survival than patients with high miR-592 expression. Overexpression of miR-592 resulted in decreased cell proliferation, migration, and invasion in thyroid cancer. In addition, neuro-oncological ventral antigen 1 (NOVA1) was identified as a novel target gene of miR-592 in thyroid cancer cells. Furthermore, ectopic *NOVA1* expression may effectively abolish the tumor-suppressing effects of miR-592 overexpression in thyroid cancer cells. Notably, the lncRNA *NEAT1* was proposed to function as a sponge of miR-592 in thyroid cancer cells, thereby regulating *NOVA1* expression. Finally, resuming miR-592 expression significantly impaired thyroid cancer tumor growth *in vivo*. The results indicated that the NEAT1/miR-592/NOVA1 pathway may play regulatory roles in thyroid cancer malignancy *in vitro* and *in vivo*. Our findings may provide novel insight into the pathogenesis of thyroid cancer. Therefore, this pathway may be an effective target for treating patients with this disease.

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Key words: thyroid cancer, microRNA-592, neuro-oncological ventral antigen 1, nuclear paraspeckle assembly transcript 1

Introduction

Thyroid cancer is the most commonly diagnosed malignant endocrine tumor worldwide (1). Globally, there are ~300,000 new cases of thyroid cancer and ~40,000 mortalities are reported every year, and these numbers are set to increase (2,3). Thyroid cancer includes four subtypes: Papillary thyroid carcinoma, follicular thyroid carcinoma, poorly differentiated thyroid carcinoma and anaplastic thyroid carcinoma (4). Papillary thyroid carcinoma, the most common form of thyroid cancer, accounts for >80% of thyroid cancer cases (5). Multimodal therapeutic approaches, including surgical resection, radioiodine ablation, and long-term thyrotropin inhibitory therapy, have led to marked reductions in mortality in recent years; however, the development of recurrence and metastasis remains the most common cause of death for patients with thyroid cancer (6). Although numerous genes have been demonstrated to be closely associated with the malignant development of thyroid cancer, the underlying molecular mechanisms have not been fully investigated (7). Therefore, a complete understanding of the molecular events that occur during thyroid cancer formation and progression will aid in the development of effective therapeutic techniques.

MicroRNAs (miRNAs/miRs) are 17-23-nucleotide-long, single-stranded, non-coding RNAs (8). miRNAs post-transcriptionally silence a gene or inhibit protein expression by base-pairing with the 3'-untranslated regions (UTRs) of their target genes (9). Over half of the human miRNAs are located in cancer-related genomic regions or in fragile sites, suggesting that miRNAs may serve critical roles in carcinogenesis and cancer progression (10). The aberrant expression of miRNAs in thyroid cancer has been widely reported in recent years. For example, miR-129 (11) miR-539 (12), and miR-791 (13) are downregulated in thyroid cancer, and inhibit the malignant phenotype. On the contrary, miR-1270 (14), miR-221 (15), miR-222 (16), and miR-625-3p (17) are upregulated and promote thyroid cancer aggressiveness. Hence, further investigation into the detailed functions of cancer-associated miRNAs in thyroid cancer may aid the identification of novel targets for preventing the development of thyroid cancer.

Long noncoding RNA (lncRNA) is a type of noncoding RNA >200 nucleotides in length (18). Several studies have reported the aberrant expression of lncRNAs in thyroid cancer and revealed that the dysregulation of lncRNAs is involved in various biological processes (19-21). At present, the lncRNA-miRNA-mRNA network is considered the most widespread mechanism underlying the action of lncRNAs in tumorigenesis and tumor development (22). Therefore, further investigation into the interaction between lncRNAs and miRNAs may facilitate the identification of effective approaches for managing patients with thyroid cancer.

Downregulation of miR-592 has frequently been observed in multiple human cancer types, including non-small cell lung cancer (23), breast cancer (24), glioma (25,26), hepatocellular carcinoma (27-29), prostate cancer (30), and colorectal cancer (31,32). However, its expression profile and functions in thyroid cancer are yet to be determined. In this study, we examined miR-592 expression in thyroid cancer and assessed its regulatory roles in thyroid cancer progression. In addition, the mechanisms underlying miR-592-mediated restriction of the aggressive phenotypes of thyroid cancer cells were explored in detail.

Materials and methods

Human tissue samples. The Ethics Committee of Jilin Central General Hospital approved the use of human tissue samples. Written informed consent was obtained from all patients prior to surgical resection. Primary thyroid cancer tissues and paired adjacent normal tissues (ANTs) were collected from 51 patients (24 males, 27 females; age range, 46-69 years) with thyroid cancer who underwent surgical resection in the Jilin Central General Hospital. None of these patients had been treated with chemotherapy, radioiodine ablation, or long-term thyrotropin inhibitory therapy before surgery. All patients were graded according to TNM staging system (33), and were divided into either the miR-592-low or miR-592-high expression group based on the median value of miR-592 expression of thyroid cancer tissues. Freshly resected tissues were snap-frozen in liquid nitrogen and stored at -80˚C.

Cell culture. The thyroid anaplastic carcinoma cell line HTH83, papillary thyroid carcinoma cell line TPC-1, thyroid cancer cell line BCPAP, as well as a normal human thyroid cell line (HT-ori3) were purchased from the American Type Culture Collection. All cell lines were maintained in Dulbecco's Modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Sigma-Aldrich; Merck KGaA) and incubated at 37˚C in an incubator supplied with 5% CO_2.

Transfection assay. The agomir-592, agomir-NC, *NEAT1* small interfering RNA [siRNA; si-nuclear paraspeckle assemble transcript 1 (NEAT1)], and nontargeting control siRNA (si-NC) were designed and synthesized by Shanghai GenePharma Co., Ltd. The agomir-592 sequence was 5'-UUG UGUCAAUAUGCGAUGAUGU-3' and the agomir-NC sequence was 5'-UUCUCCGAACGUGUCACGUTT-3'. The si-NEAT1 sequence was 5'-GUGAGAAGUUGCUUAGAA ACUUUCC-3' and the si-NC sequence was 5'-UACUGUCUA GUCGCCGUAC-3'. The coding sequences of neuro-oncological ventral antigen 1 (*NOVA1*) were amplified by GeneRay and cloned into the pcDNA3.1 plasmid (GeneRay; restriction sites, *Hind*III + *Xho*I) to produce the pcDNA3.1-NOVA1 (pc-NOVA1) plasmid. The *NEAT1* overexpression plasmid pcDNA3.1-NEAT1 (pc-NEAT1) was also chemically synthesized by GeneRay. Empty pcDNA3.1 plasmid was utilized as a negative control. Cells were plated in 6-well plates 24 h prior to transfection, and were transfected with agomir-592 (50 nM), agomir-NC (50 nM), siRNA (100 pmol), or plasmid (4 μg) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. After incubation at 37˚C for different periods, transfected cells were harvested and used for subsequent analysis. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR), Cell Counting Kit-8 (CCK-8) assay and xenograft tumor model was carried out at 24 h post-transfection. Migration and invasion assays and western blotting were performed at 48 and 72 h after transfection, respectively.

RT-qPCR. Total RNA was extracted using TRIzol® (Thermo Fisher Scientific, Inc.) from tissues and cultured cells. To quantify miR-592 expression, an miRcute miRNA First-Strand cDNA Synthesis kit (Tiangen Biotech, Co., Ltd.) was used to prepare cDNA from total RNA, according to the manufacturer's protocols. The synthesized cDNA was then subjected to qPCR using a miRcute miRNA qPCR Detection kit SYBR-Green (Tiangen Biotech, Co., Ltd.). U6 small nuclear RNA was used as an internal control for miR-592 expression. The cycling conditions for qPCR were as follows: 15 min at 95˚C, followed by 45 cycles of 94˚C for 20 sec and 60˚C for 34 sec. To measure the mRNA levels of *NOVA1* and *NEAT1*, total RNA was reverse transcribed into cDNA with a PrimeScript® RT reagent kit (Takara Bio, Inc.), according to the manufacturer's protocols. Thereafter, qPCR was performed on an ABI 7500 PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) using a SYBR® Premix Ex Taq™ II kit (Takara Bio, Inc.). The cycling conditions for qPCR were as follows: 5 min at 95˚C, followed by 40 cycles of 95˚C for 30 sec and 65˚C for 45 sec. The relative mRNA expression of *NOVA1* and *NEAT1* was normalized against that of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Relative gene expression was analyzed using the $2^{-\Delta\Delta Cq}$ method (34). The primers were designed as follows: miR-592, 5'-CCATGACATTGT GTCAATATGCGA-3' (forward) and 5'-CGTCATGATGTT GCGTCACC-3' (reverse); U6, 5'-GCTTCGGCAGCACAT ATACTAAAAT-3' (forward) and 5'-CGCTTCACGAATTTG CGTGTCAT-3' (reverse); *NOVA1*, 5'-GGTCTCAGCCAAGCA GCAGCAA-3' (forward) and 5'-TTGCAGCAGTAGCAGCAG CCAG-3' (reverse); *NEAT1*, 5'-TGGCTAGGCTCAGGCTT CAG-3' (forward) and 5'-TCTCCTTGCCAAGCTTCCTTC-3' (reverse); and *GAPDH*, 5'-TGAACGGGAAGCTCCTGG-3' (forward) and 5'-TCCACCCCTGTTGCTGTA-3' (reverse).

Cell Counting Kit-8 (CCK-8) assay. Transfected cells were seeded in 96-well plates at a density of $2x10^3$ cells/well. The CCK-8 assay was conducted to measure cellular proliferation every 24 h for a period of 72 h. Briefly, 10 μl of CCK-8 solution (Dojindo Molecular Technologies, Inc.) was added to each

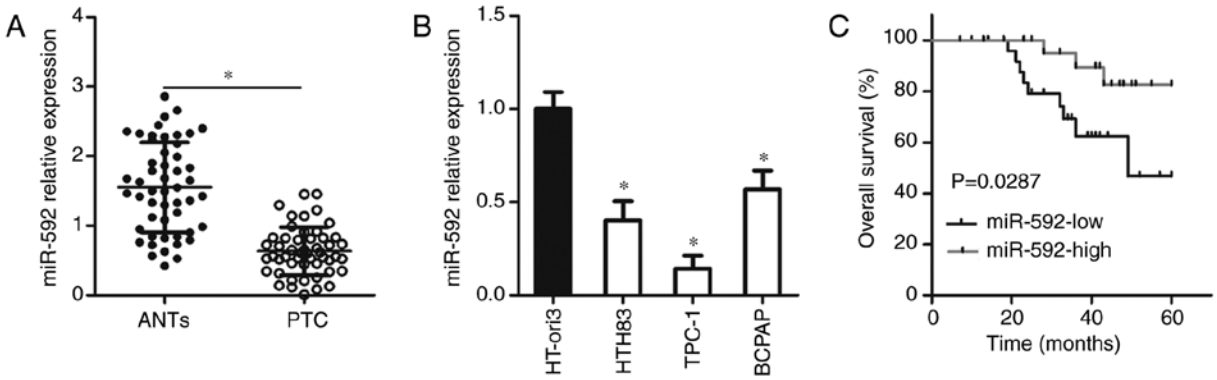

Figure 1. miR-592 expression is decreased in thyroid cancer. (A) Expression level of miR-592 was analyzed in 51 pairs of thyroid cancer tissues and ANTs using RT-qPCR. *P<0.05 vs. ANTs. (B) RT-qPCR was used to measure miR-592 expression in thyroid anaplastic carcinoma cell line HTH83, papillary thyroid carcinoma cell line TPC-1, and thyroid cancer cell line BCPAP. A normal human thyroid cell line (HT-ori3) acted as the control. *P<0.05 vs. HT-ori3. (C) Patients with thyroid cancer with low miR-592 expression exhibited shorter overall survival than patients with high miR-592 expression, as indicated by a log-rank test. P=0.0287. ANTs, adjacent normal tissues; miR, microRNA; PTC, papillary thyroid cancer; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

well prior to incubation at 37˚C for another 2 h. Finally, the absorbance at 450 nm was detected using a microplate reader (BioTek Instruments, Inc.).

Migration and invasion assays. Transfected cells were detached using 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.) and then resuspended in FBS-free DMEM at a concentration of $1x10^5$ cells/ml. For the migration assay, 200 μl cell suspension was added to the upper compartments of Transwell inserts (8 μm pore size, Costar; Corning, Inc.). For the invasion assay, an equivalent number of cells were added to the upper compartments of Transwell inserts that were coated with Matrigel (BD Biosciences). In both assays, the bottom compartments were covered with 600 μl DMEM containing 20% FBS (Gibco; Thermo Fisher Scientific, Inc.). After 24 h of routine culture, non-migrated and non-invaded cells were gently removed with a cotton swab. Cells that migrated/invaded to the lower surface of the Transwell inserts were fixed with 4% paraformaldehyde at room temperature for 30 min and stained with 0.5% crystal violet at room temperature for 30 min. The number of migrated/invaded cells in five randomly selected visual fields was counted under an IX83 inverted microscope (x200 magnification; Olympus Corporation).

Xenograft tumor model. TPC-1 cells transfected with agomir-592 or agomir-NC were harvested after 24 h of incubation and subcutaneously injected into the flanks of 4-6-week-old BALB/c nude mice (n=4 for each group) purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All nude mice were maintained under specific pathogen-free conditions (25˚C; 50% humidity; 10-h light/14-h dark cycle; free access to food and water). After 14 days following inoculation, tumor size was measured every 2 days for 14 days using a caliper, and tumor volumes were determined based on the following formula: Tumor volume = length x $(width)^2/2$. All nude mice were sacrificed 4 weeks post-injection, and tumor weights were measured. The tumor xenografts were resected and stored for western blotting and RT-qPCR analysis. Animal experiments were approved by the Animal Care and Use Committee of Jilin Central General Hospital and were performed in accordance with the Animal Protection Law of the People's Republic of China-2009 for experimental animals.

Bioinformatics analysis. miRDB (last modified: January 22, 2019; http://mirdb.org/) and TargetScan (Release 7.2: March 2018; http://www.targetscan.org) were utilized for predicting the potential target gene of miR-592. The binding site between miR-592 and *NEAT1* was predicted using DIANA tools -LncBase Experimental v2 (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2%2findex-experimental).

Luciferase reporter assay. The wild-type (wt) *NEAT1* containing the predicted miR-592 binding site and mutant (mut) *NEAT1* were amplified by Shanghai GenePharma Co., Ltd. and inserted into the pmiR-REPORT reporter plasmid (Promega Corporation) to generate NEAT1-wt and NEAT1-mut, respectively. The NOVA1-wt and NOVA1-mut reporter plasmids were synthesized in a similar manner. Cells were plated in 24-well plates with a density of $1.0x10^5$ cells per well and transfected with a synthetic luciferase reporter plasmid, along with agomir-592 or agomir-NC, using Lipofectamine® 2000. Then, 48 h after transfection, luciferase activity was quantified using a Dual Luciferase Assay (Promega Corporation). Firefly luciferase activity was normalized to that of *Renilla* luciferase activity.

Western blot analysis. Total protein was isolated from tissue specimens, cells, or tumor xenografts was conducted using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology. The total protein concentration was measured with a BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Equal amounts of protein (30 μg) were loaded, separated by electrophoresis using 10% SDS-PAGE, and then transferred onto polyvinylidene fluoride membranes (Beyotime Institute of Biotechnology). Prior to overnight incubation at 4˚C with primary antibodies against NOVA1 (ab183024; 1:1,000 dilution; Abcam) or GAPDH (ab128915; 1:1,000 dilution; Abcam),

the membranes were blocked at room temperature with 5% skim milk diluted in Tris-buffered saline buffer containing 0.1% Tween-20 (TBST) for 2 h. Thereafter, the membranes were incubated with a goat anti-rabbit horseradish peroxidase-linked IgG secondary antibody (ab97051; 1:5,000 dilution; Abcam) at room temperature for 1 h. Finally, protein signals were visualized using an Enhanced Chemiluminescence Detection System (Pierce; Thermo Fisher Scientific, Inc.). Quantity One software version 4.62 (Bio-Rad Laboratories, Inc.) was utilized for quantify protein expression.

Statistical analysis. All results are presented the mean ± standard deviation from at least three separate experiments, and data processing was performed with SPSS 21.0 statistical software (IBM Corp.). The χ^2 test was used to examine the association between miR-592 and clinicopathological parameters in patients with thyroid cancer. One-way analysis of variance followed by the Bonferroni post-hoc test was used to analyze the data among multiple groups; the difference between two groups was assessed using a two-sided Student's t-test. The correlation between genes was examined via Spearman's correlation analysis. A log-rank test was applied to determine the overall survival rates, following Kaplan-Meier analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-592 is downregulated in thyroid cancer, and its downregulation is inversely associated with the prognosis of patients with thyroid cancer. To explore the roles of miR-592 during the development of thyroid cancer, we first measured miR-592 expression in primary thyroid cancer tissues and ANTs obtained from 51 patients with thyroid cancer. The expression of miR-592 was significantly downregulated in thyroid cancer tissues compared with that in ANTs, as demonstrated by RT-qPCR (P<0.05; Fig. 1A). miR-592 expression in three thyroid cancer cell lines and a normal human thyroid cell line (HT-ori3) was also detected via RT-qPCR. The results indicated that the miR-592 levels were significantly downregulated in thyroid cancer cell lines than in HT-ori3 cells (P<0.05; Fig. 1B).

All patients were divided into either the miR-592-low or miR-592-high expression group based on the median value of miR-592 expression in thyroid cancer tissues. To examine the clinical value of miR-592 in thyroid cancer, we investigated the association between miR-592 expression and clinicopathological parameters in patients with thyroid cancer. As presented in Table I, decreased miR-592 expression was associated with a higher incidence of increased lymph node metastasis (P=0.002) and advanced tumor-node-metastasis (TNM) stage (P=0.009). In addition, expression levels of miR-592 exhibited an inverse association with overall survival in patients with thyroid cancer (P=0.0287; Fig. 1C). Thus, miR-592 was determined to be downregulated in thyroid cancer, and its decreased expression could predict poor prognosis for patients with thyroid cancer.

Restoring miR-592 expression suppresses the proliferation, migration, and invasion of thyroid cancer cells. To restore miR-592 expression, the HTH83 and TPC-1 cell lines, which had the lowest miR-592 expression among the three thyroid cancer cell lines examined, were transfected with agomir-592. A significant increase in miR-592 expression in HTH83 and TPC-1 cells after agomir-592 transfection was confirmed via RT-qPCR analysis (P<0.05; Fig. 2A). Subsequently, the influence of miR-592 upregulation on thyroid cancer cell proliferation, migration, and invasion was determined by CCK-8, and migration and invasion assays, respectively. The proliferation (P<0.05; Fig. 2B), migration (P<0.05; Fig. 2C), and invasion (Fig. 2D, P<0.05) of HTH83 and TPC-1 cells were significantly suppressed by miR-592 restoration. These results suggested that the upregulation of miR-592 can impair the malignancy of thyroid cancer cells *in vitro*.

NOVA1 is a novel target of miR-592 in thyroid cancer cells. To identify the molecular mechanism of miR-592 in regulating the malignant phenotypes of thyroid cancer cells, *NOVA1* was validated as a putative target of miR-592 using bioinformatics analysis (Fig. 3A). To determine whether miR-592 could recognize and interact with the 3'-UTR of *NOVA1*, a luciferase reporter assay was conducted in HTH83 and TPC-1 cells after co-transfection with agomir-592 or agomir-NC, and NOVA1-wt or NOVA1-mut. Restoring miR-592 expression significantly reduced the luciferase activity of the NOVA1-wt (P<0.05; Fig. 3B), but not the NOVA1-mut in HTH83 and TPC-1 cells. In addition, RT-qPCR and western blot analysis were conducted to determine whether endogenous *NOVA1* expression could be directly regulated by miR-592 in thyroid cancer cells. The results indicated that expression levels of *NOVA1* mRNA

Table I. Association between the clinicopathological features and miR-592 expression in patients with papillary thyroid cancer.

	miR-592 expression		
Features	Low	High	P-value
Age (years)			0.579
<60	15	12	
≥60	11	13	
Sex			0.095
Male	9	15	
Female	17	10	
Tumor size (cm)			0.264
<1 cm	12	16	
≥1 cm	14	9	
Lymph node metastasis			0.002[a]
Negative	13	23	
Positive	13	2	
TNM stage			0.009[a]
I-II	15	23	
III-IV	11	2	

[a]P<0.05. miR, microRNA; TNM, tumor-node-metastasis.

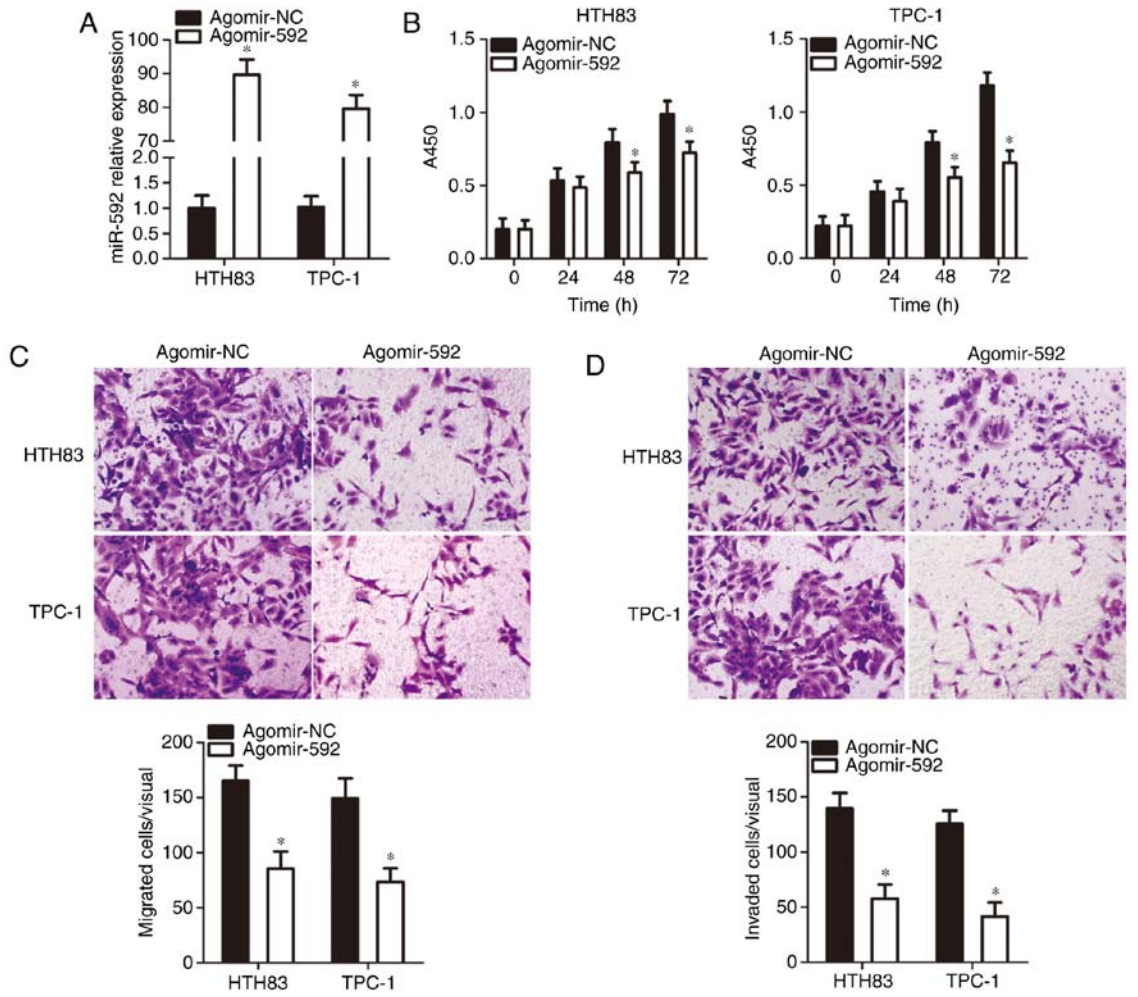

Figure 2. Ectopic miR-592 expression restricts the proliferation, migration, and invasion of thyroid cancer cells. (A) Agomir-592 or agomir-NC was introduced into HTH83 and TPC-1 cells. Transfected cells were collected after 48 h of culture and subjected to reverse transcription-quantitative polymerase chain reaction analysis for determining transfection efficiency. *P<0.05 vs. agomir-NC. (B) The effect of miR-592 upregulation on HTH83 and TPC-1 cell proliferation using a Cell Counting Kit-8 assay. *P<0.05 vs. agomir-NC. (C and D) The migration and invasion of HTH83 and TPC-1 cells were suppressed by transfection with agomir-592, as indicated by migration and invasion assays (x200 magnification). *P<0.05 vs. agomir-NC. miR, microRNA; NC, negative control.

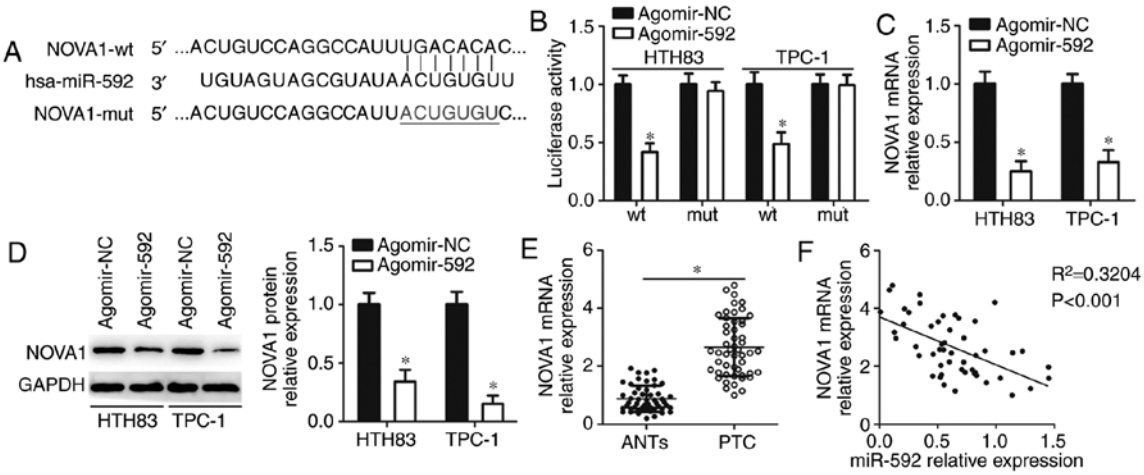

Figure 3. *NOVA1* is a novel direct target of miR-592 in thyroid cancer cells. (A) Sequence of the wt and mut miR-592 binding sequences within the 3'-untranslated region of the *NOVA1* gene. (B) HTH83 and TPC-1 cells were co-transfected with agomir-592 or agomir-NC and NOVA1-wt or NOVA1-mut reporter plasmids. Luciferase activities were measured 48 h after transfection. *P<0.05 vs. agomir-NC. (C and D) The mRNA and protein expression levels of *NOVA1* in miR-592-overexpressing HTH83 and TPC-1 cells were analyzed by RT-qPCR and western blot analysis, respectively. *P<0.05 vs. agomir-NC. (E) RT-qPCR analysis revealed the increased expression level of *NOVA1* in thyroid cancer tissues compared with that in ANTs. *P<0.05 vs. ANTs. (F) Spearman's correlation analysis was used to determine the correlation between miR-592 and *NOVA1* mRNA expression in thyroid cancer tissues. R^2=0.3204, P<0.0001. ANTs, adjacent normal tissues; hsa, *homo sapiens*; NOVA1, neuro-oncological ventral antigen 1; miR, microRNA; Mut, mutant; NC, negative control; PTC, papillary thyroid cancer; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; wt, wild-type.

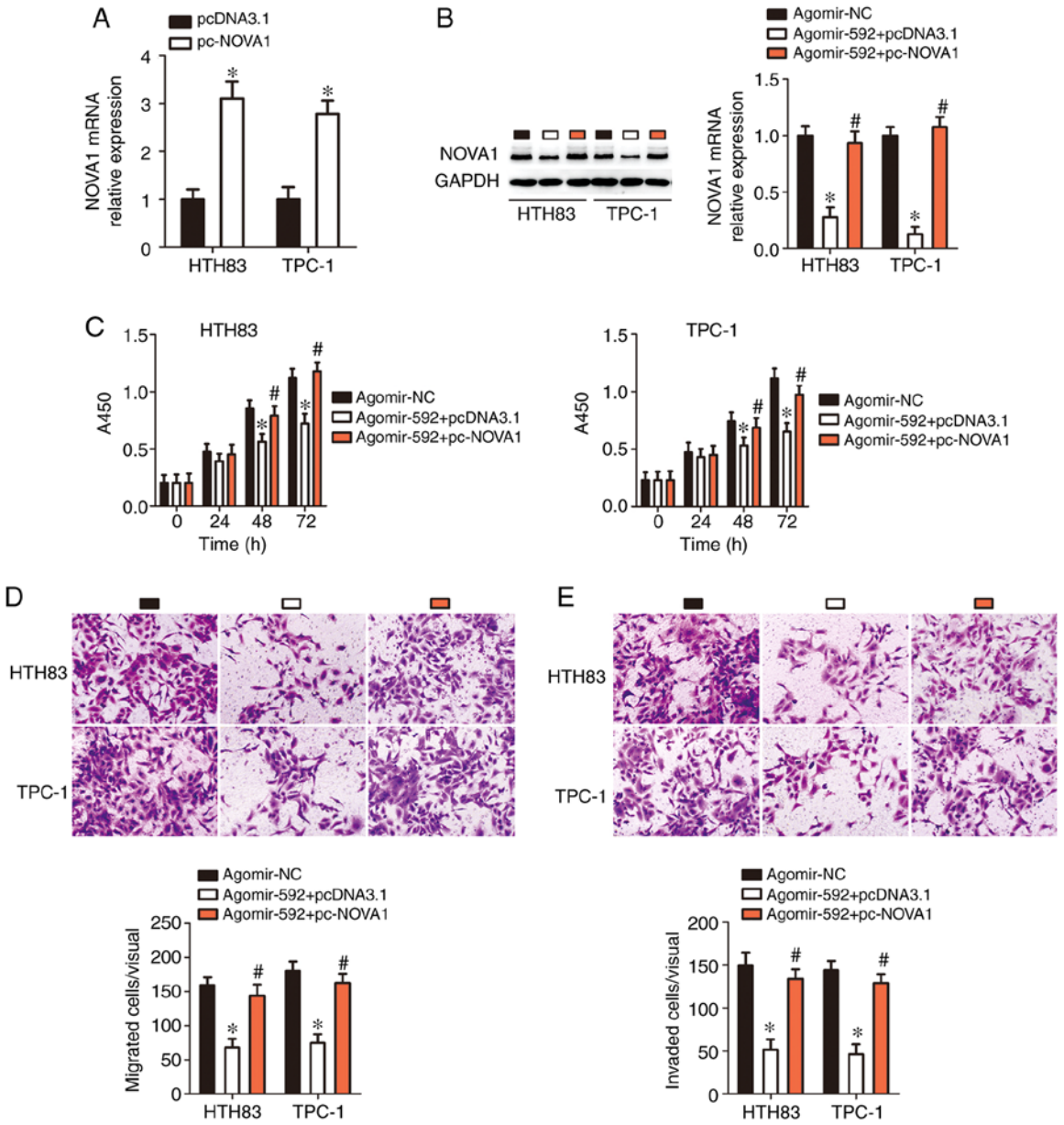

Figure 4. Restoring *NOVA1* expression abolishes the tumor-suppressing effects of microRNA-592 on thyroid cancer cell proliferation, migration, and invasion *in vitro*. (A) pc-NOVA1 or pcDNA3.1 was introduced into HTH83 and TPC-1 cells. After transfection, reverse transcription-quantitative polymerase chain reaction analysis was used for determination of *NOVA1* expression. *P<0.05 vs. pcDNA3.1. (B) pc-NOVA1 or pcDNA3.1 in combination with agomir-592 was transfected into HTH83 and TPC-1 cells. Decreased *NOVA1* expression, induced by miR-592 overexpression, was successfully restored in HTH83 and TPC-1 cells after co-transfection with pc-NOVA1. *P<0.05 vs. agomir-NC. #P<0.05 vs. agomir-NC. (C-E) The proliferation, migration (x200 magnification), and invasion (x200 magnification) of HTH83 and TPC-1 cells treated as aforementioned were assessed using Cell Counting Kit-8, and migration and invasion assays, respectively. *P<0.05 vs. agomir-NC. #P<0.05 vs. agomir-592 + pcDNA3.1. NOVA1, neuro-oncological ventral antigen 1; NC, negative control.

(P<0.05; Fig. 3C) and protein (P<0.05; Fig. 3D) were significantly downregulated in HTH83 and TPC-1 cells transfected with agomir-592 compared with the control. Furthermore, the expression of *NOVA1* mRNA in thyroid cancer tissues and ANTs was detected using RT-qPCR, and the results showed that *NOVA1* mRNA was markedly upregulated in thyroid cancer tissues in comparison with that in ANTs (P<0.05; Fig. 3E). Spearman's correlation analysis also demonstrated that the expression of miR-592 was inversely correlated with *NOVA1* mRNA expression in the same thyroid cancer tissues (R^2=0.3204, P<0.0001; Fig. 3F). These results suggest that *NOVA1* is a novel direct target of miR-592 in thyroid cancer cells.

Ectopic NOVA1 expression mitigates the effects of miR-592 upregulation in thyroid cancer cells. Rescue experiments were further used to determine whether the tumor-suppressing effects of miR-592 in thyroid cancer cells were achieved through decreasing *NOVA1* expression. Agomir-592 was co-transfected with pc-NOVA1 or pcDNA3.1 into HTH83 and TPC-1 cells. First, RT-qPCR analysis indicated that the transfection of pc-NOVA1 significantly increased NOVA1 expression in HTH83 and TPC-1 cells compared with the control (P<0.05; Fig. 4A). Downregulation of *NOVA1* caused by miR-592 overexpression was recovered in HTH83 and TPC-1 cells through co-transfection with pc-NOVA1 (P<0.05; Fig. 4B). Of note, the suppressive effects of miR-592 overexpression on HTH83 and

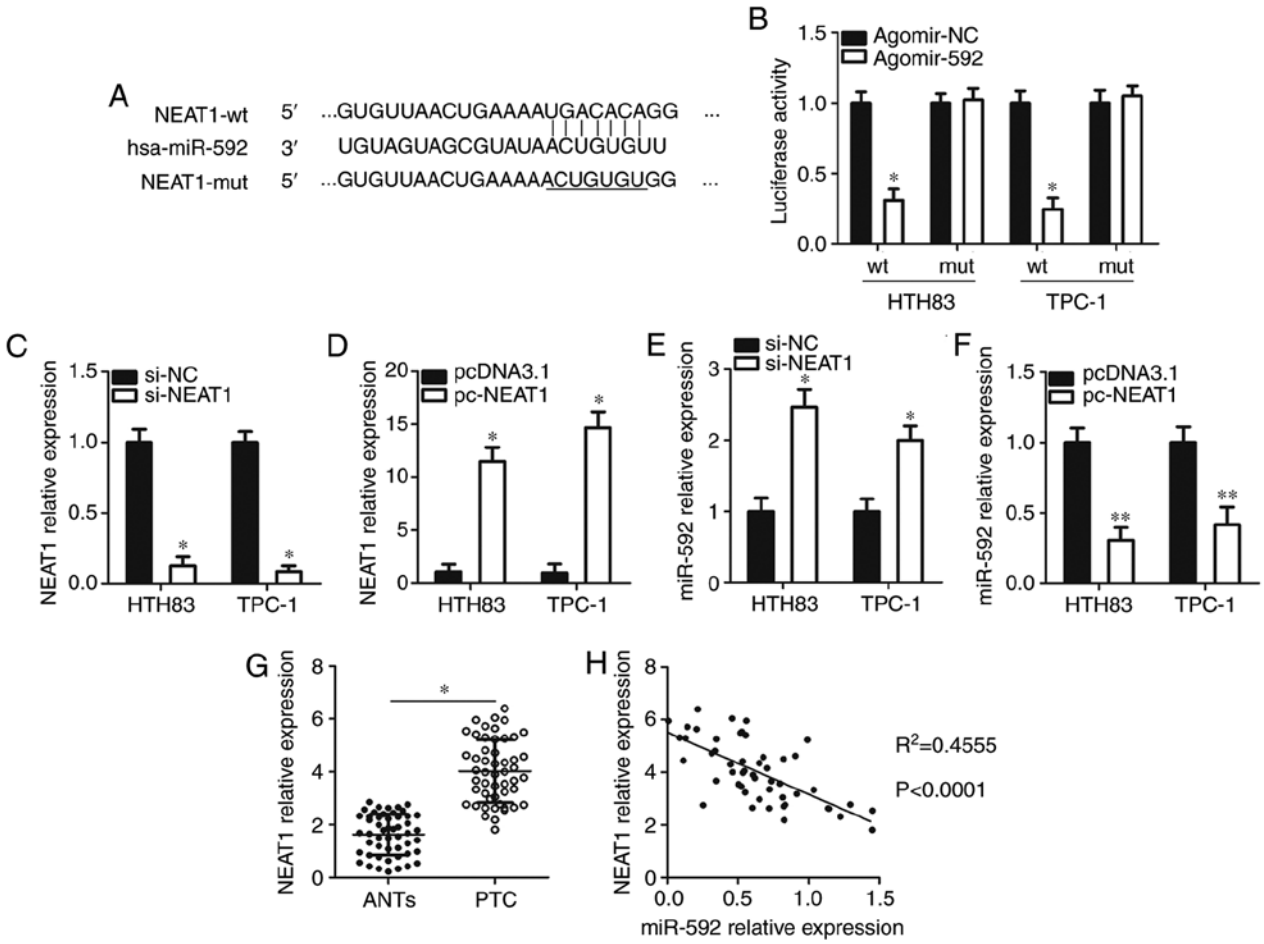

Figure 5. miR-592 is sponged by *NEAT1* in thyroid cancer cells. (A) The wt and mut miR-592 binding sites in *NEAT1*, as predicted by bioinformatics analysis. (B) Luciferase reporter assays were carried out using HTH83 and TPC-1 cells that were co-transfected with agomir-592 or agomir-NC, and NEAT1-wt or NEAT1-mut reporter plasmids. *P<0.05 vs. agomir-NC. (C) *NEAT1* expression in HTH83 and TPC-1 cells after transfection with si-NEAT1 or si-NC was examined using RT-qPCR. *P<0.05 vs. si-NC. (D) HTH83 and TPC-1 cells were transfected with pcDNA3.1 or pc-NEAT1. After transfection 48 h, RT-qPCR was conducted to measure *NEAT1* expression. *P<0.05 vs. pcDNA3.1. (E and F) Expression of miR-592 in *NEAT1*-silenced or *NEAT1*-overexpressing HTH83 and TPC-1 cells was analyzed using RT-qPCR. *P<0.05 vs. si-NC. **P<0.05 vs. pcDNA3.1. (G) The expression levels of *NEAT1* in 51 pairs of thyroid cancer tissues and ANTs was examined through RT-qPCR. *P<0.05 vs. ANTs. (H) The correlation between the expression of *NEAT1* and miR-592 in thyroid cancer tissues was explored via Spearman's correlation analysis. R^2=0.4555, P<0.0001. ANTs, adjacent normal tissues; hsa, *homo sapiens*; miR, microRNA; NEAT1, nuclear paraspeckle assemble transcript 1; mut, mutant; NC, negative control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; si, small interfering RNA; wt, wild-type.

TPC-1 cell proliferation (P<0.05; Fig. 4C), migration (P<0.05; Fig. 4D), and invasion (P<0.05; Fig. 4E) were nearly abolished by restoring *NOVA1* expression. Therefore, these results indicated that *NOVA1* is a functionally relevant target of miR-592 in thyroid cancer cells.

NEAT1 acts as a competing endogenous RNA (ceRNA) for miR-592 in thyroid cancer cells. lncRNAs may act as molecular sponges for miRNAs and play an important role in regulating miRNAs (22). Bioinformatics analysis revealed a putative binding site for miR-592 in the lncRNA *NEAT1* (Fig. 5A), which has been well studied and confirmed to play an oncogenic role in thyroid cancer progression (20,35-37). A luciferase reporter assay was used to confirm this prediction. The results showed that compared with the agomir-NC group, recovery of miR-592 expression significantly decreased the luciferase activity of NEAT1-wt compared with the control (P<0.05; Fig. 5B); however, the luciferase activity of the NEAT1-mut was markedly unchanged after agomir-592 transfection. In addition, silencing and increasing *NEAT1* expression via the transfection of si-NEAT1 (P<0.05; Fig. 5C) and pc-NEAT1 (P<0.05; Fig. 5D), significantly increased (P<0.05; Fig. 5E) and decreased (P<0.05; Fig. 5F) miR-592 expression in HTH83 and TPC-1 cells, respectively compared with the controls. Furthermore, the expression levels of *NEAT1* in thyroid cancer tissues was were upregulated than that in ANTs (P<0.05; Fig. 5G). An inverse correlation was identified between the expression levels of *NEAT1* and miR-592 in thyroid cancer tissues (R^2=0.4555, P<0.0001; Fig. 5H). Collectively, these results suggest that miR-592 may be sponged by *NEAT1* in thyroid cancer cells.

Ectopic NEAT1 expression reverses the miR-592-mediated inhibitory effects in thyroid cancer cells. Since we proposed that *NEAT1* acts as a ceRNA for miR-592 in thyroid cancer cells, we then determined whether the effects of miR-592 on thyroid cancer cell proliferation, migration, and invasion were modulated by *NEAT1*. Thus, pc-NEAT1 or pcDNA3.1

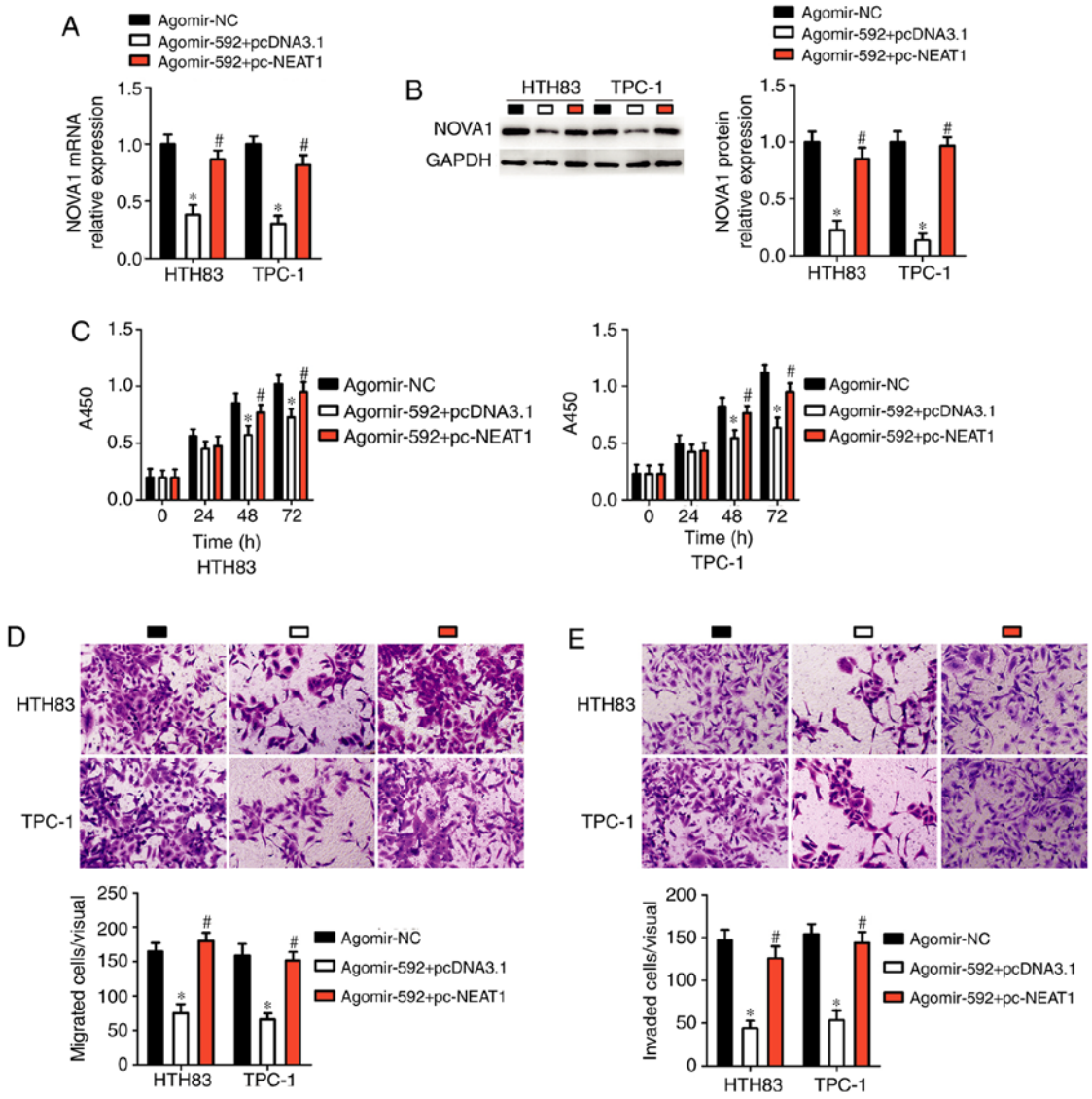

Figure 6. Restoring *NEAT1* expression rescues the effects of microRNA-592 upregulation on thyroid cancer cells. HTH83 and TPC-1 cells were co-transfected with agomir-592 and pc-NEAT1 or pcDNA3.1. (A and B) Following transfection, RT-qPCR and western blot analysis were used to detect *NOVA1* mRNA and protein expression, respectively. *P<0.05 vs. agomir-NC. #P<0.05 vs. agomir-592 + pcDNA3.1. (C-E) Cell Counting Kit-8, and migration and invasion assays (x200 magnification) were conducted to detect the proliferation, migration, and invasion in the aforementioned cells. *P<0.05 vs. agomir-NC. #P<0.05 vs. agomir-592 + pcDNA3.1. NOVA1, neuro-oncological ventral antigen 1; NC, negative control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

was co-transfected with agomir-592 into HTH83 and TPC-1 cells. The mRNA (P<0.05; Fig. 6A) and protein (P<0.05; Fig. 6B) levels of NOVA1 in HTH83 and TPC-1 cells, which were decreased by agomir-592 transfection, were restored by co-transfection with the *NEAT1* overexpression plasmid pc-NEAT1. Functionally, the miR-592-mediated decreases in HTH83 and TPC-1 cell proliferation (P<0.05; Fig. 6C), migration (P<0.05; Fig. 6D), and invasion (P<0.05; Fig. 6E) were substantially reversed after co-transfection with pc-NEAT1. Thus, these results indicated that miR-592 acts as the downstream RNA of *NEAT1* in thyroid cancer cells.

miR-592 impairs tumor growth in vivo. Finally, a xenograft tumor model was established to assess the effect of miR-592 on tumor growth *in vivo*. The tumor xenografts derived from agomir-592-transfected TPC-1 cells exhibited significantly slower growth patterns than those obtained from the agomir-NC-transfected TPC-1 cells (P<0.05; Fig. 7A and B). At the end point of the experiment, the tumor xenografts were resected and weighed. Xenograft tumors in the agomir-592 group weighed significantly less than tumors in the agomir-NC group (P<0.05; Fig. 7C). Furthermore, the expression of miR-592 was significantly upregulated (P<0.05; Fig. 7D), whereas NOVA1 protein expression was decreased (P<0.05; Fig. 7E), in the tumor xenografts derived from agomir-592-transfected TPC-1 cells, compared with the control. These results suggest that miR-592 overexpression impairs the tumor growth of thyroid cancer cells *in vivo*.

Discussion

Numerous studies have demonstrated that various miRNAs are aberrantly expressed in thyroid cancer and play indispensable roles in the malignancy of thyroid cancer (38-40).

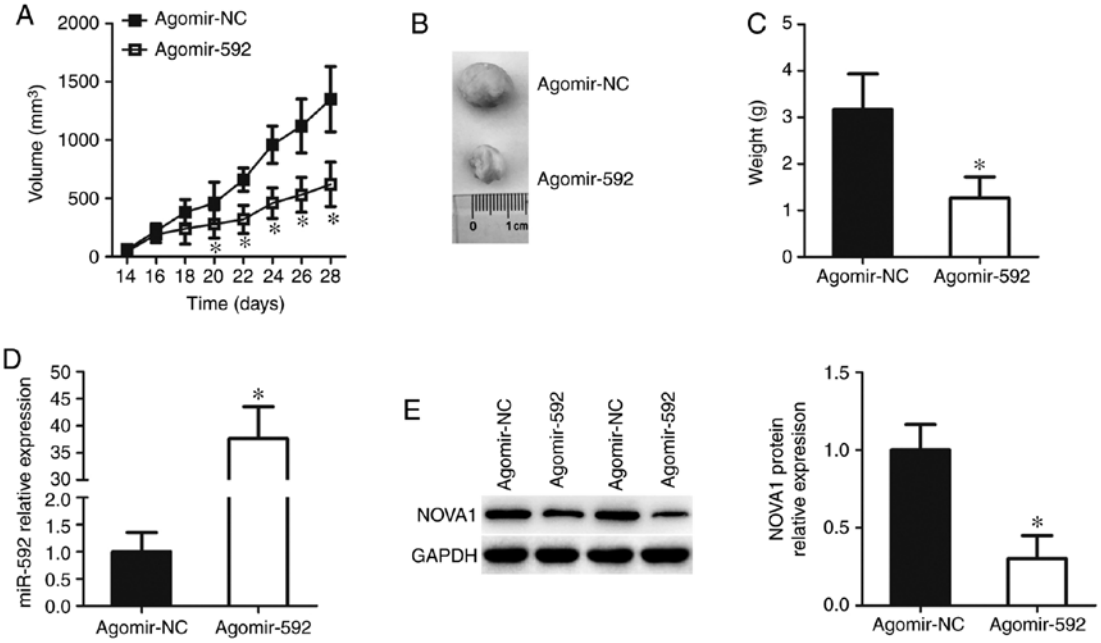

Figure 7. miR-592 inhibits tumor growth *in vivo*. (A) The volume of tumor xenografts was recorded every 2 days for 2 weeks following inoculation. *P<0.05 vs. agomir-NC. (B) Representative images of xenografts derived from the agomir-592 or agomir-NC-transfected TPC-1 cells. (C) Four weeks after injection, the tumor xenografts were resected and weighed. *P<0.05 vs. agomir-NC. (D) Reverse transcription-quantitative polymerase chain reaction analysis was performed to determine the expression of miR-592 in tumor xenografts. *P<0.05 vs. agomir-NC. (E) Total protein was extracted and then subjected to western blotting to measure *NOVA1* protein expression. *P<0.05 vs. agomir-NC. miR, microRNA; NOVA1, neuro-oncological ventral antigen 1; NC, negative control.

Almost all aggressive characteristics of thyroid cancer have been demonstrated to be modulated by miRNAs via regulating the expression of their target genes (41). In the past few decades, multiple miRNAs have been extensively studied in thyroid cancer (13,14,42); however, many miRNAs remain to be investigated. Further exploration of the specific functions of miRNAs in the development of thyroid cancer may aid in identifying potential targets for anticancer therapy. In this study, for the first time, we determined the expression profile of miR-592 in thyroid cancer and examined the prognostic significance of miR-592 in patients with thyroid cancer. Notably, the biological roles of miR-592 with regards to the malignant phenotypes of thyroid cancer and the related molecular mechanisms were explored in detail.

miR-592 is downregulated in non-small cell lung cancer, and its downregulation is significantly associated with TNM stage and lymph node metastasis (23). The expression of miR-592 is decreased in breast cancer and is associated with malignant clinicopathological characteristics (24). miR-592 expression was also reported to be reduced in glioma (25,26) and hepatocellular carcinoma (27-29). On the contrary, miR-592 is upregulated in prostate (30) and colorectal (31,32) cancers. These inconsistent findings led us to explore the expression profile of miR-592 in thyroid cancer. In the present study, we found that miR-592 was downregulated in thyroid cancer tissues and cell lines. Low miR-592 expression was associated with lymph node metastasis and TNM stage. Patients with thyroid cancer samples exhibiting low miR-592 expression exhibited poorer overall survival than patients with high miR-592 expression. These findings suggest that miR-592 could be developed as a novel prognostic marker for patients with thyroid cancer.

miR-592 exerts an inhibitory role in breast cancer through regulating cell growth and metastasis *in vitro*, as well as tumor growth *in vivo* (24). miR-592 was also identified as a tumor-suppressing miRNA in non-small cell lung cancer (23), glioma (25,26), and hepatocellular carcinoma (27-29), serving dispensable roles in regulating a wide range of biological behaviors. On the contrary, miR-592 was found to play tumor-promoting roles in prostate (30) and colorectal (31,32) cancers. These observations indicate that the functional roles of miR-592 in cancer genesis and development exhibit tissue specificity. However, the detailed roles of miR-592 in regulating the malignant development of thyroid cancer had not been studied yet. In the present study, ectopic miR-592 expression suppressed the proliferation, migration, and invasion of thyroid cancer cells *in vitro*. In addition, miR-592 overexpression suppressed the tumor growth of thyroid cancer cells *in vivo*. Accordingly, miR-592 might be an effective target for treating patients with thyroid cancer.

Understanding the molecular mechanisms underlying the tumor-suppressing roles of miR-592 in thyroid cancer is important for identifying effective therapeutic techniques. In the present study, *NOVA1* was validated as a direct target of miR-592 in thyroid cancer cells, and *NEAT1* was proposed to function as a ceRNA to modulate *NOVA1* expression by sponging miR-592. Multiple studies reported that *NEAT1* is expressed at high levels and exerts oncogenic roles in a variety of human cancers, including cervical cancer (43), nasopharyngeal carcinoma (44), breast cancer (45), non-small cell lung cancer (46), colorectal cancer (47,48), and oral squamous cell carcinoma (49). *NEAT1* is also upregulated in thyroid cancer (35-37), and upregulation of *NEAT1* is positively correlated with TNM stage and tumor size (35). *NEAT1* is closely involved in the aggressiveness of thyroid cancer by promoting cell proliferation, migration, invasion, and radioactive iodine resistance *in vitro*; inhibiting cell survival and apoptosis

in vitro; and impairing tumor growth *in vivo* (20,35-37). *NOVA1*, a member of the NOVA family of neuron-specific RNA-binding proteins, is aberrantly downregulated in multiple human cancers and affects a variety of pathophysiological processes associated with carcinogenesis and cancer progression (50-52). The expression and roles of NEAT1 and roles have been studied in other human cancers (43-49). Herein, we identified a novel NEAT1-miR-592-NOVA1 pathway, and this pathway could serve crucial roles in the aggressiveness of thyroid cancer. Our results suggest that silencing *NEAT1* expression and restoring miR-592 expression, thereby decreasing *NOVA1* expression, represents a promising therapeutic approach for preventing thyroid cancer.

This study could not use the TCGA database to validate the relevance of the NEAT1/miR-592/NOVA1 pathway in thyroid cancer, which poses as a limitation of our study, and we aim to resolve this issue in our future studies.

In conclusion, miR-592 was downregulated in thyroid cancer tissues and cell lines. Low miR-592 expression was determined to be associated with the poor prognosis of patients with thyroid cancer. Silencing *NEAT1* expression was proposed to decrease the expression of *NOVA1* by sponging miR-592, thereby suppressing the progression of thyroid cancer.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

This study was designed by LW and YL. YL performed RT-qPCR and the luciferase reporter assays. Migration and invasion assays, and the xenograft tumor model were conducted by TH, JZ, and MZ. PS carried out western blot analysis and the CCK-8 assay. LW and YL wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Ethics Committee of Jilin Central General Hospital approved the use of human tissue samples. Written informed consent was obtained from all patients before they underwent surgical resection.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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