lncRNA EGFEM1P promotes thyroid cancer progression by acting as an miR-369-3p sponge and upregulating TCF4

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Received February 10, 2022; Accepted August 31, 2022

DOI: 10.3892/ol.2022.13576

Abstract. Thyroid cancer is the most commonly diagnosed endocrine cancer, with the incidence of 14.42 per 100,000 person-years in 2010-2013. It is important to conduct an in-depth exploration into the molecular mechanisms of thyroid cancer, providing insights into the improvements of therapy. Long non-coding RNAs (lncRNAs) act as oncogenes or tumor suppressors in thyroid cancer by sponging microRNAs (miRNAs), however, the functions of numerous lncRNAs are still unknown. In the present study, via the comprehensive analysis of microarray data derived from papillary thyroid tumors and the RNA sequencing of thyroid tumors from The Cancer Genome Atlas database, EGF like and EMI domain containing 1 (EGFEM1P) expression levels in papillary thyroid tumors and normal adjacent tissues were explored. Reverse transcription-quantitative PCR was used to detect EGFEM1P, microRNA (miR)-369-3p and T cell factor 4 (TCF4) expression levels. Western blotting was used to detect TCF4 protein and cleaved caspase-3/8 expression levels. Cell proliferative ability and apoptosis were assessed using the Cell Counting Kit-8 assay and flow cytometry, respectively. The interactions between EGFEM1P and miR-369-3p, and miR-369-3p and TCF4, were determined using the dual-luciferase reporter assay. The results demonstrated that EGFEM1P was upregulated in papillary thyroid tumors and thyroid cancer cells compared with normal adjacent tissues and human normal thyroid epithelial Nthy-ori 3-1 cell line. In the examined thyroid cancer cells, EGFEM1P was demonstrated to interact with miR-369-3p and decreased miR-369-3p expression levels. Thereafter, TCF4 was determined to be a target gene of miR-369-3p and EGFEM1P promoted TCF4 expression via regulating miR-369-3p expression levels. At last, it was found that EGFEM1P expression promoted rapid cell proliferation and inhibited cell apoptosis in thyroid cancer cells via acting as a miR-369-3p sponge. In conclusion EGFEM1P promoted thyroid cancer progression via acting as a sponge of the miR-369-3p/TCF4 axis.

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Key words: thyroid carcinoma, cell proliferation, cell apoptosis, EGF-like and EMI domain-containing protein 1, microRNA-369-3p/T cell factor 4 axis

Introduction

Thyroid cancer is the most commonly diagnosed endocrine cancer and mainly originates from follicular epithelial cells (1). The incidence of thyroid cancer increased by 3.6% per year on average during 1974-2013 (from 4.56 per 100,000 person-years in 1974-1977 to 14.42 per 100,000 person-years in 2010-2013 (2). According to histological classification, papillary thyroid carcinoma, which accounts for ~80% of cases, and follicular thyroid carcinoma, which accounts for ~10% of cases, are the two major types of thyroid cancer (3). Treated with specific methods such as surgery (4), radioiodine (5), and thyroid-stimulating hormone suppression (6), numerous patients with solid cancers exerted a relatively optimal improvement in their conditions. However, there is also recurrence and metastasis of tumor, which lead to death in certain cases (7). Thus, it is of great importance to conduct an in-depth exploration into the molecular mechanisms of thyroid cancer, to provide significant insights into the improvements of therapy.

MicroRNAs (miRNAs or miRs), which locate in the non-coding region of the genome and are highly conserved in evolution, are a family of non-coding single-stranded small RNAs with the length of 19-23 nucleotides (nt) and regulate gene expression at translation level (8). miRNAs participate in multiple physiological metabolic processes, including development, cell differentiation and cell apoptosis (9). With the advancement in researches on molecular biology and molecular genetics, miRNAs have been indicated to be related with the development, and metastasis of tumors, including thyroid cancer (10).

Long non-coding RNAs (lncRNAs) are defined as molecules consisting of >200 nt with no protein-coding potential (11). Evidence indicates that lncRNAs have a critical regulatory function in eukaryotic cells (12,13). Moreover, it

has been reported that lncRNAs interact with key proteins and sponge miRNAs to regulate cell proliferation, differentiation, motility and metabolism (14-16). The dysregulation of lncRNAs is involved in the pathogenesis of numerous types of cancer. For example, mini-chromosome maintenance complex component 3 associated protein (MCM3AP)-antisense RNA 1 (AS1) is upregulated in thyroid cancer. This lncRNA promotes thyroid cancer cell proliferation and invasion via acting as an miR-211-5p sponge and elevating secreted protein acidic and cysteine rich (SPARC) protein expression levels (17). Moreover, miR-592 inhibits the progression of thyroid cancer via the regulation of lncRNA nuclear paraspeckle assembly transcript 1 and the downregulation of NOVA alternative splicing regulator 1 (18). Furthermore, the hypoxia-induced lncRNA-AC020978 induces cell proliferation of non-small cell lung cancer via the regulation of the pyruvate kinase M2/hypoxia-inducible factor-1α axis (19). Although the function of several abnormally expressed lncRNAs has been reported, the roles of numerous lncRNAs in cancer remain elusive. In 2018, a novel lncRNA, that is, EGF like and EMI domain containing 1 (EGFEM1P) was found to be associated with thyroid cancer in the study by You *et al* (20); however, since its role in thyroid cancer development has not been discovered, the present study aimed to explore it.

Materials and methods

Tissue samples. From September 2017 to June 2020, thyroid tumor tissues (n=35) and normal adjacent tissues (n=24) were collected from 35 patients (aged from 41 to 72 years old) diagnosed with papillary thyroid cancer during surgery in The Central Hospital of Enshi Tujia and Miao Autonomous Prefecture (Enshi, China). The clinicopathological characteristics of the patients were recorded in Table I. Patients did not receive radiotherapy or other treatments before enrollment in the present study. Written informed consent was obtained from all patients. The present study was approved (approval no. CH ESTJMAP-2017-08) by the Ethics Committee of The Central Hospital of Enshi Tujia and Miao Autonomous Prefecture (Enshi, China). The tissues were stored at -80˚C before being used in the following experiments.

Cell culture. The human normal thyroid epithelial Nthy-ori 3-1 cell line was purchased from the European Collection of Authenticated Cell Cultures. The two papillary thyroid carcinoma cell lines (TPC-1 and KTC-1) and one follicular thyroid carcinoma cell line (FTC-133) were purchased from the American Type Culture Collection. Cells were maintained in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin solution (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were cultured in a humid incubator with 5% CO_2 at 37˚C.

Bioinformatic analysis. To screen thyroid cancer-related lncRNAs, the gene expression data of papillary thyroid tumor tissues and normal adjacent tissues from the GSE66783 dataset (normal adjacent thyroid tissues, 5; papillary thyroid carcinoma tissues, 5) (21) and The Cancer Genome Atlas (TCGA; solid tumor normal tissues, 54; primary tumors, 478; metastatic tumors, 8. Tissues were not derived from the same patients) (22) were used. The differentially expressed genes from these two datasets were overlapped and EGF-like and EMI domain-containing protein 1 (EGFEM1P) was identified as one of the most significantly upregulated lncRNAs. The patients were divided into two groups according to high and low EGFEM1P expression levels. Furthermore, Kaplan-Meier analysis was performed to investigate the association between EGFEM1P expression levels and the prognosis of patients. The target miRNAs of EGFEM1P were predicted using miRDB (http://mirdb.org/) (23). The target mRNAs of miR-369-3p were also predicted using miRDB (23), TargetScan (http://www.targetscan.org/vert_72/) (24) and miRWalk (http://mirwalk.umm.uni-heidelberg.de/) (25).

Transfection. Control small interfering (si)RNA (5'-UUCUCCGAACGUGUCACGUU-3'), si-EGFEM1P-1 (5'-GCGGCGAGCGCGUUUCCAUGG-3') and siEGFEM1P-2 (5'-GGAGGGCGGCGAGCGCGUUUC-3') were designed and synthesized by Suzhou GenePharma Co., Ltd. miR-negative control (NC) mimic (5'-CUGAACUGCUAGGACGCGUA-3'), miR-NC inhibitor (5'-CUCGAUAGCGCAUGGUCCGAGCUA-3'), miR-369-3p mimic (5'-AAUAAUACAUGGUUGAUCUUU-3') and miR-369-3p inhibitor (5'-AAAGAUCAACCAUGUAUUAUU-3') were synthesized by Guangzhou RiboBio Co., Ltd. siRNA (100 nM), miR-369-3p mimic (40 nM) or miR-369-3p inhibitor (40 nM) or the corresponding controls was transfected into KTC-1 cells using Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 48 h according to the manufacturer's protocol. The transfection efficiencies of the siRNA, miR-369-3p mimic and miR-369-3p inhibitor constructs were determined using reverse transcription-quantitative PCR (RT-qPCR) 48 h after transfection.

Cell proliferation assay. Cell proliferation of KTC-1 cells ($1x10^5$/well) was analyzed using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) assay. In brief, the cell medium was replaced with medium containing 10% CCK-8 solution and cells were incubated for 2 h at 37˚C. The absorbance was detected at 450 nm using a Microplate Reader (Bio-Rad Laboratories, Inc.).

Cell apoptosis assay. The percentage of apoptotic KTC-1 cells was determined using the Dead Cell Apoptosis Kit with an Annexin V FITC and PI kit (Invitrogen; Thermo Fisher Scientific, Inc.). In brief, cells were collected and suspended in Annexin V binding buffer containing PI. Annexin V-FITC was then added and cells were incubated for 15 min at room temperature. Subsequently the cells were subjected to flow cytometric analysis. The apoptotic cells were estimated by a FACSCalibur flow cytometer (BD Biosciences). The data were analyzed using FlowJo software 10.7 (BD Biosciences). Apoptotic cells were identified as being positive for Annexin V and/or PI.

RT-qPCR. Total RNA was extracted from cells and tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Complementary DNA was synthesized using the PrimeScript™ RT Reagent kit (Takara Bio, Inc.) according to

Table I. The clinicopathological characteristics of the patients with papillary thyroid cancer.

Clinicopathological factors	Number of patients (%)
Age, years	
≤55	17 (48.57)
>55	18 (51.43)
Sex	
Male	15 (42.86)
Female	20 (57.14)
TNM staging	
I-II	25 (71.43)
III-IV	10 (28.57)
Tumor size, cm	
≤2	22 (62.86)
>2	13 (37.14)
Lymph node metastasis	
Yes	21 (60.00)
No	14 (40.00)
Distant metastasis	
Yes	11 (31.43)
No	24 (68.57)
Extrathyroidal extension	
Yes	19 (54.29)
No	16 (45.71)

Table II. Primer sequences.

Primer name	Primer sequence (5'-3')
EGFEM1P	F: ATATTGTATTATAATTAGTT
	R: AACTAATTATAATACAATAT
miR-30d-3p	F: CTTTCAGTCAGATGTTTGCTGC
	R: GCAGCAAACATCTGACTGAAAG
miR-30e-3p	F: CTTTCAGTCGGATGTTTACAGC
	R: GCTGTAAACATCCGACTGAAAG
miR-369-3p	F: AATAATACATGGTTGATCTTT
	R: AAAGATCAACCATGTATTATT
TCF4	F: CTTAACAGCTGTATTATCTTAAACCCA
	R: TGGGTTTAAGATAATACAGCTGTTAAG
GAPDH	F: GCACCGTCAAGGCTGAGAAC
	R: TGGTGAAGACGCCAGTGGA
U6	F: GTGCTCGCTTCGGCAGCACAT
	R: AATATGGAACGCTTCACGAAT

EGFEM1P, EGF-like and EMI domain-containing protein 1; miR, microRNA; TCF4, T cell factor 4; F, forward; R, reverse.

the manufacturer's instructions. qPCR was performed using TB Green Advantage qPCR Premix (Takara Bio, Inc.). The thermocycling conditions were as listed: Initial denaturation at 95˚C for 1 min; followed with 42 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 31 sec and elongation at 72˚C for 30 sec, and final extension at 72˚C for 5 min. Expression levels were normalized to GAPDH and U6 for mRNA and miRNA, respectively. Expression levels were analyzed using the $2^{-\Delta\Delta Cq}$ method (26). The primer sequences were listed in Table II.

Western blot analysis. KTC-1 cells were lysed by radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology), then the bicinchoninic acid assay (Beyotime Institute of Biotechnology) was conducted to assess protein concentration. Protein lysates (20 μg/lane) were separated by 10% SDS-PAGE, then electrophoretically transferred onto PVDF membranes, which were blocked by 5% BSA (Beijing Solarbio Science & Technology Co., Ltd.) in Tris-buffered saline (TBS)-0.5% Tween-20 at room temperature for 1 h. Subsequently, proteins were incubated sequentially with antibodies from Cell Signaling Technology, Inc. against GAPDH (cat. no. 5174; dilution 1:1,000), cleaved caspase-3 (cat. no. 9664; dilution 1:1,000), caspase-3 (cat. no. sc-7272; dilution 1:1,000), cleaved caspase-8 (cat. no. 9496; dilution 1:1,000), caspase-8 (cat. no. sc-56070; dilution 1:1,000), cleaved PARP (cat. no. 5625; dilution 1:1,000), PARP (cat. no. sc-136208; dilution 1:1,000), BAX (cat. no. 2772; dilution 1:1,000), and TCF4 (cat. no. 2569; dilution 1:1,000) for 24 h at 4˚C, followed by anti-rabbit (cat. no. 7074; dilution 1:2,000) horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at room temperature. Enhanced chemiluminescence detection system for HRP (EMD Millipore) was used. Proteins were analyzed with a LAS-4000 luminescence image analyzer (FUJIFILM Wako Pure Chemical Corporation). The band intensities were analyzed using Image Lab™ (version 4.0; Bio-Rad Laboratories, Inc.).

Dual-luciferase reporter assay. EGFEM1P wild-type (WT), EGFEM1P truncate (1-3,000 nt), EGFEM1P mutant (Mut), TCF4 3'untranslated region (UTR) WT and TCF4 3'UTR Mut were inserted into the pmirGLO plasmid (Promega Corporation). pmirGLO-EGFEM1P, the truncated form, or Mut form were co-transfected with miR-NC mimic or miR-369-3p mimic into KTC-1 cells using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). To confirm the interaction between TCF4 and miR-369-3p, TCF4 3'UTR WT or TCF4 3'UTR Mut were co-transfected with miR-NC mimic or miR-369-3p mimic into TPC-1 and KTC-1 cells using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). After incubation at 37˚C for 48 h, the luciferase activity of each group was determined using the Dual-Luciferase Reporter Assay System (Promega Corporation) according to the manufacturer's protocol. The method of normalization was comparison with *Renilla* luciferase activity.

Statistical analysis. All data were analyzed using GraphPad Prism 5.0 software (GraphPad Software, Inc.) and are presented as the mean ± SD. All experiments were repeated three times. Student's t-test (paired for tissues, unpaired for cell lines) was used for statistical comparisons between two groups. More than two groups (including differentially expressed genes from TCGA) were statistically compared using one-way ANOVA followed by Tukey's post hoc test.

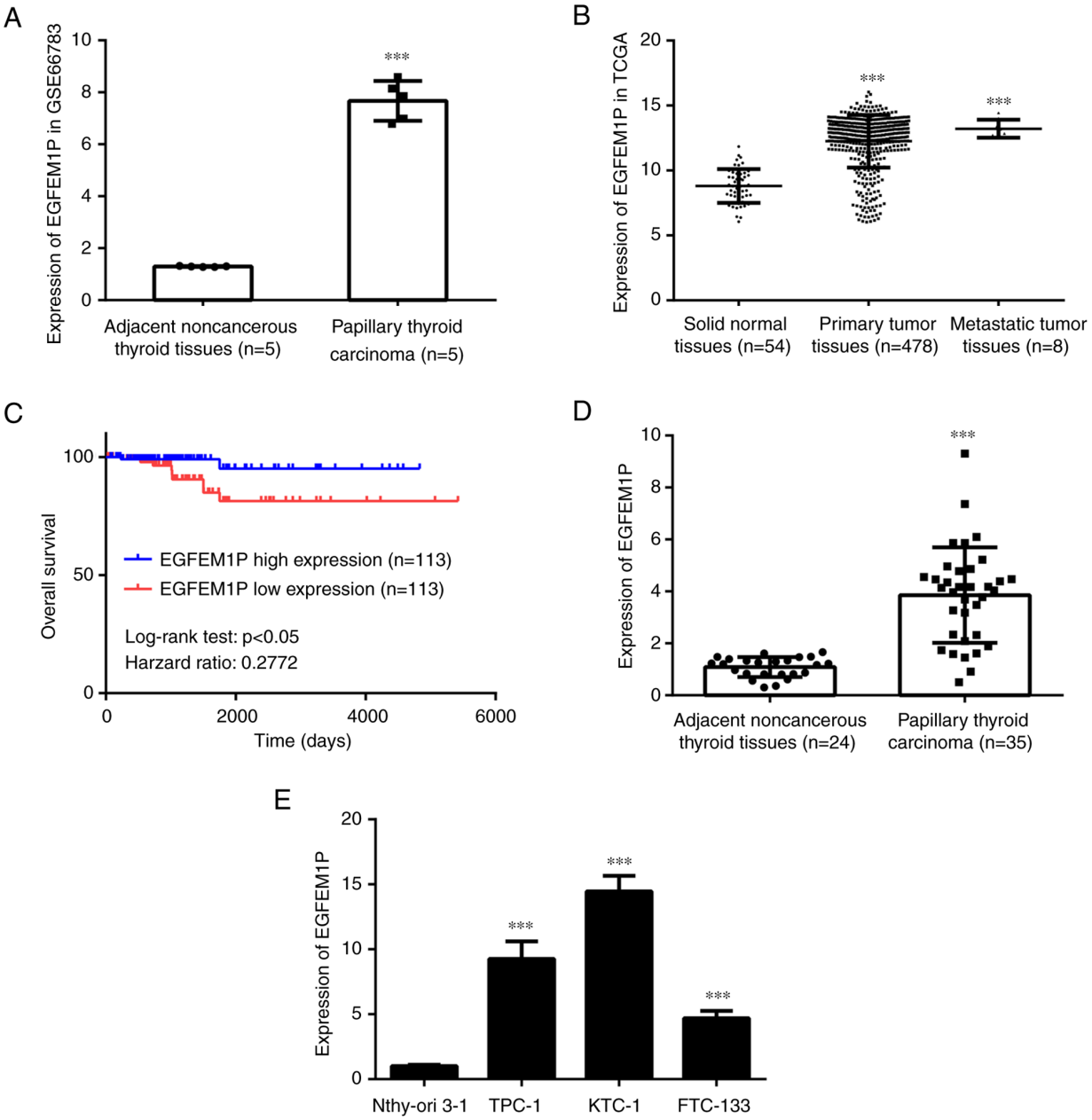

Figure 1. EGFEM1P expression is upregulated in thyroid cancer tissues and cell lines. (A and B) Data retrieved from GSE66783 and TCGA database demonstrated that EGFEM1P expression levels were significantly higher in papillary thyroid cancer tissues compared with adjacent normal thyroid tissues. (C) Kaplan-Meier analysis demonstrated a positive association between EGFEM1P overexpression and the poor prognosis of patients with thyroid cancer. (D) In the collected samples EGFEM1P expression levels were significantly increased in papillary thyroid cancer tissues compared with normal adjacent thyroid tissues. (E) EGFEM1P expression levels were significantly higher in TPC-1, KTC-1 and FTC-133 cells compared with Nthy-ori 3-1 cells. ***P<0.001 vs. normal adjacent thyroid tissues, solid normal tissues and Nthy-ori 3-1 cells. EGFEM1P, EGF-like and EMI domain-containing protein 1; TCGA, The Cancer Genome Atlas.

P<0.05 was considered to indicate a statistically significant difference.

Results

EGFEM1P is upregulated in thyroid cancer tissues and cell lines. According to the data from GSE66783, EGFEM1P was significantly increased in papillary thyroid cancer tissues compared with normal adjacent thyroid tissues (Fig. 1A). Furthermore, the data from the TCGA database demonstrated that EGFEM1P was also significantly increased in primary tumor tissues and metastatic tumor tissues compared with normal adjacent tissues (Fig. 1B). Kaplan-Meier analysis demonstrated a positive association between EGFEM1P overexpression and the poor prognosis of patients with thyroid cancer (Fig. 1C). In the collected samples from the present study, EGFEM1P expression levels were significantly increased in papillary thyroid cancer tissues compared with the normal adjacent thyroid tissues (Fig. 1D). Moreover, compared with Nthy-ori 3-1 cells, EGFEM1P expression levels were significantly increased in TPC-1, KTC-1 and FTC-133 cells (Fig. 1E). As KTC-1 cells exhibited relatively higher EGFEM1P expression levels, this cell line was used in the subsequent experiments.

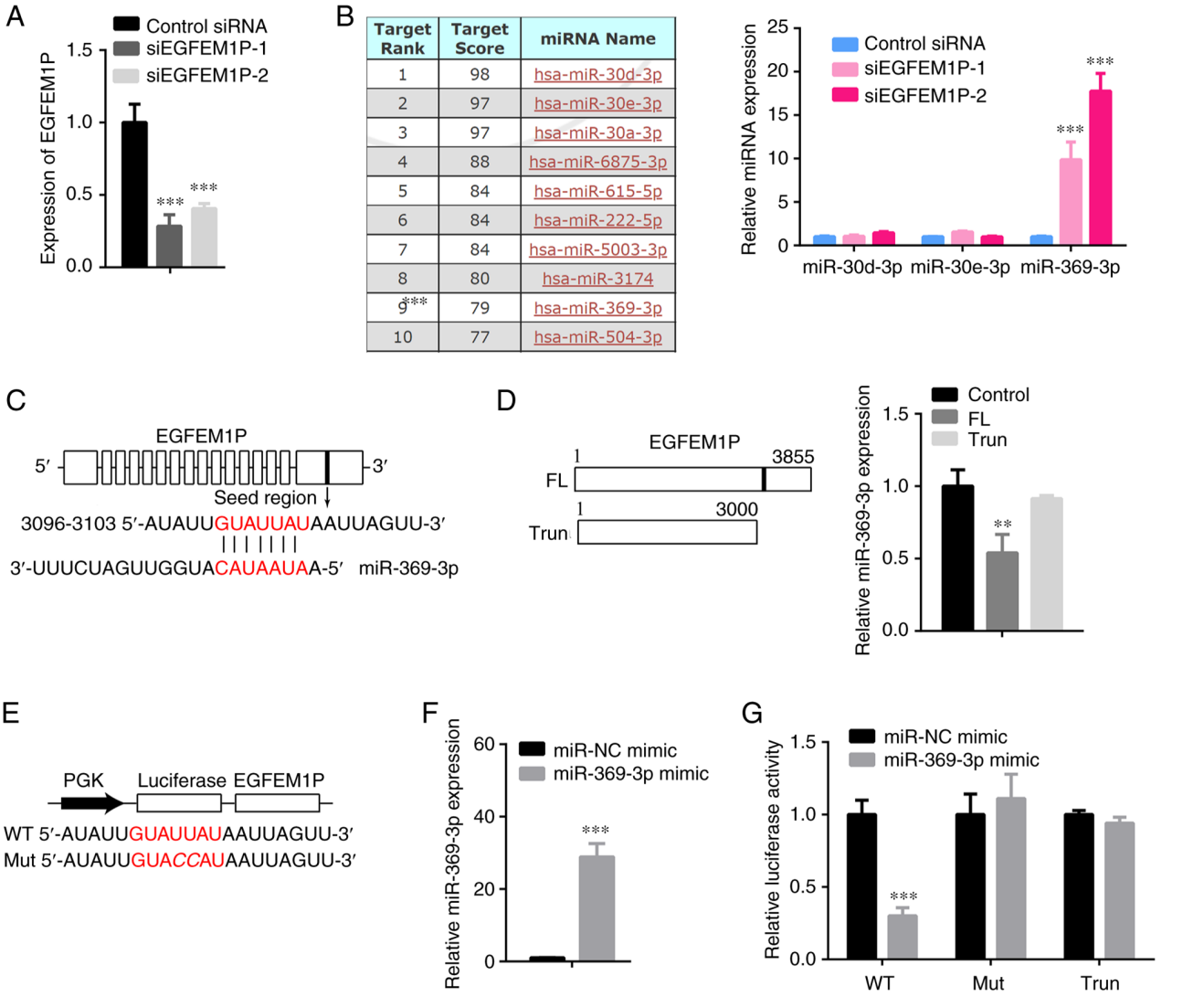

Figure 2. miR-369-3p is sponged by EGFEM1P. (A) siRNAs were used to knockdown EGFEM1P expression levels. (B) Several target miRNAs of EGFEM1P were identified. Compared with control siRNA, miR-369-3p expression levels were reduced in siEGFEM1P-1 and siEGFEM1P-2 transfected KTC-1 cells. (C) Complementary sites between EGFEM1P and miR-369-3p. (D) EGFEM1P inhibited miR-369-3p expression levels in KTC-1 cells. (E) WT and Mut sequences of EGFEM1P complementary sites for miR-369-3p. (F) miR-369-3p expression levels were higher in miR-369-3p mimic than in miR-NC mimic transfected KTC-1 cells. (G) KTC-1 cell luciferase activity when transfected with WT-EGFEM1P was lower in miR-369-3p mimic than in miR-NC mimic co-transfected cells. **P<0.01 vs. control; ***P<0.001 vs. control siRNA and miR-NC mimic. EGFEM1P, EGF-like and EMI domain-containing protein 1; miR, microRNA; siRNA/si, small interfering RNA; WT, wild-type; Mut, mutant; NC, negative control.

miR-369-3p is sponged by EGFEM1P. Subsequently, the miRNA targets of EGFEM1P were explored. Prior to this investigation siRNAs were used to knockdown EGFEM1P expression levels. Both siEGFEM1P-1 and siEGFEM1P-2 were determined to effectively reduce EGFEM1P expression levels (Fig. 2A). However, siEGFEM1P-1 was demonstrated to be more efficient and was therefore used for the subsequent experiments.

Several target miRNAs of EGFEM1P were identified, including miR-30d-3p, miR-30e-3p, miR-30a-3p, miR-6875-3p, miR-615-5p, miR-222-5p, miR-5003-3p, miR-3174, miR-369-3p and miR-504-3p. Moreover, a number of miRNAs were randomly selected to examine their interaction with EGFEM1P. The results demonstrated that compared with control siRNA, siEGFEM1P-1 and siEGFEM1P-2 significantly increased miR-369-3p expression levels but not those of other miRNAs in KTC-1 cells (Fig. 2B). The complementary sites between EGFEM1P and miR-369-3p are presented in Fig. 2C. Furthermore, EGFEM1P significantly decreased the expression levels of miR-369-3p in KTC-1 cells (Fig. 2D). The WT and Mut sequences of EGFEM1P complementary sites for miR-369-3p are presented in Fig. 2E. Compared with miR-NC mimic, miR-369-3p was demonstrated to be increased by miR-369-3p mimic transfection in KTC-1 cells (Fig. 2F). Subsequently, miR-369-3p mimic was demonstrated to reduce luciferase activity in KTC-1 cells transfected with WT-EGFEM1P compared with cells transfected with the miR-NC mimic (Fig. 2G).

TCF4 is targeted by miR-369-3p. The target genes of miR-369-3p were predicted using miRDB (23), TargetScan (24) and miRWalk (25) and are presented in Fig. 3A. These results demonstrated that among the potential target genes identified 36 mRNAs were overlapped. Of these overlapping mRNAs, which were complementary to miR-369-3p, the TCF4 3'UTR

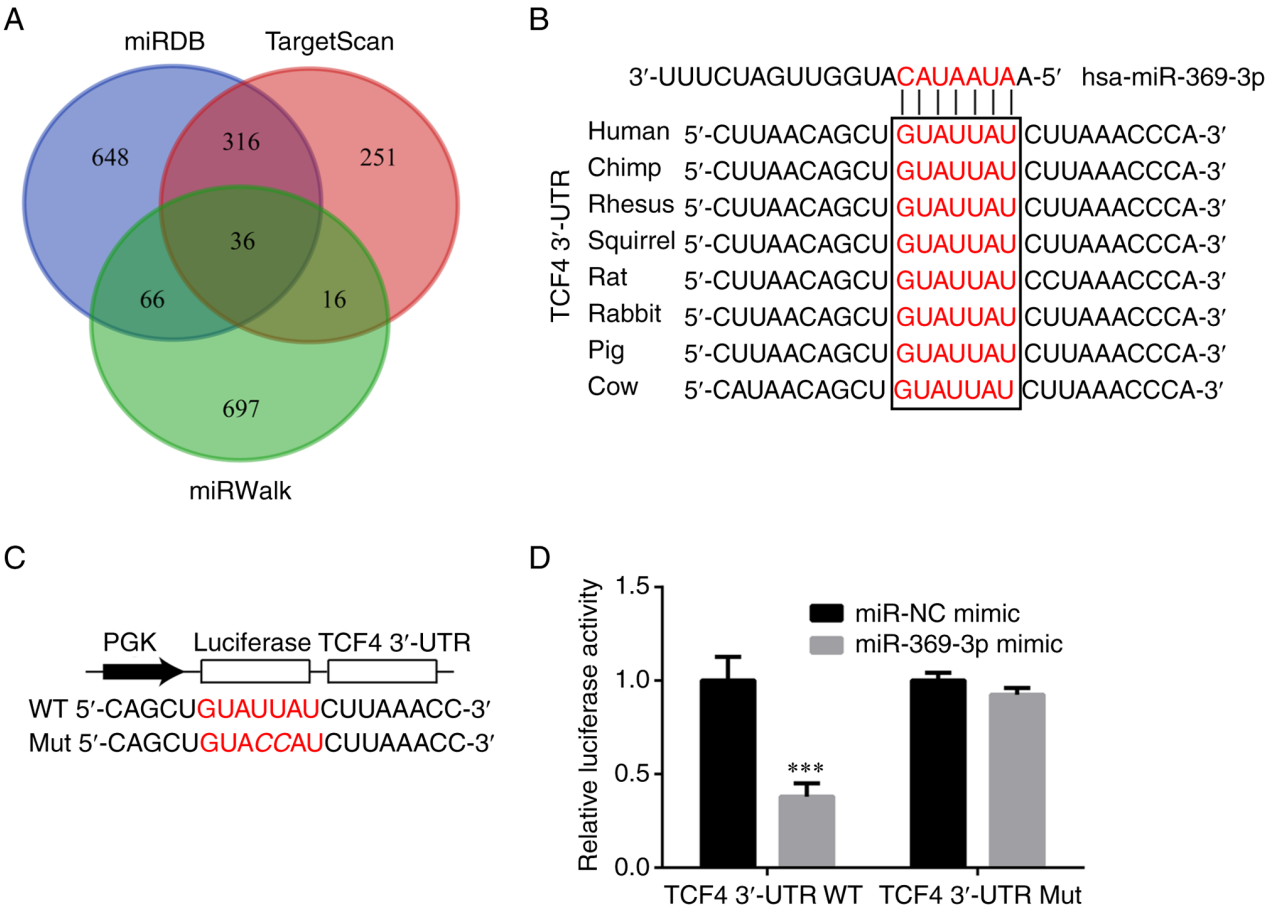

Figure 3. TCF4 is targeted by miR-369-3p. (A) Target genes of miR-369-3p. (B) TCF4 3'UTR was conserved among numerous species. (C) Luciferase reporter constructs containing WT and Mut TCF4 3'UTRs were constructed. (D) KTC-1 cells transfected with WT-TCF4 exhibited lower luciferase activity in miR-369-3p mimic compared with miR-NC mimic co-transfected cells. ***P<0.001 vs. miR-NC mimic. TCF4, T cell factor 4; miR, microRNA; UTR, untranslated region; WT, wild-type; Mut, mutant; NC, negative control.

was conserved among numerous species (Fig. 3B) and therefore became a focus of the present study. Subsequently, luciferase vectors were constructed containing WT- and Mut-TCF4 3'UTR (Fig. 3C). The results demonstrated that compared with miR-NC mimic, miR-369-3p mimic reduced luciferase activity in KTC-1 cells, which were transfected with WT-TCF4 (Fig. 3D).

EGFEM1P induces TCF4 expression via sponging miR-369-3p. Subsequently the effects of EGFEM1P on TCF4 expression levels were assessed. The results demonstrated that in KTC-1 cells, miR-369-3p was decreased by miR-369-3p inhibitor compared with miR-NC inhibitor (Fig. 4A). Moreover, in KTC-1 cells, TCF4 mRNA and protein expression levels were observed to be decreased by siEGFEM1P-1 compared with the control siRNA + miR-NC inhibitor group. This effect was rescued via co-transfection with siEGFEM1P-1 + miR-369-3p inhibitor (Fig. 4B and C).

EGFEM1P induces cell proliferation and reduces cell apoptosis via sponging miR-369-3p. The relationship between EGFEM1P and miR-369-3p in cell proliferation and cell apoptosis was investigated. In KTC-1 cells, compared with the control siRNA + miR-NC inhibitor group, siEGFEM1P-1 significantly suppressed cell proliferation (Fig. 5A); however, siEGFEM1P-1 significantly promoted apoptosis and BAX, cleaved caspase-3/caspase-3, cleaved caspase-8/ caspase-8, cleaved caspase-PARP/PARP ratio (Fig. 5B and C). These aforementioned effects were abolished by the co-transfection of siEGFEM1P-1 + miR-369-3p inhibitor.

Discussion

lncRNAs serve an important regulatory role in eukaryotic cells (12,13). Dysregulation of lncRNAs is involved in the pathogenesis of thyroid cancer. For example, MCM3AP-AS1 promotes cell proliferation and invasion of PTC cells by sponging the miR-211-5p/SPARC axis (17). Furthermore, lncRNA solute carrier family 26 member 4-AS1 inhibits metastasis in thyroid cancer by destabilizing DEAD-box helicase 5 (4). Moreover, lncRNA metastasis-associated lung adenocarcinoma transcript 1 promotes the cell proliferation, migration and invasion of thyroid cancer cells via the miR-204/insulin-like growth factor 2 mRNA binding protein 2/m6A-MYC signaling pathway (27). However, even though the functions of several abnormally expressed lncRNAs have been reported, the roles of numerous abnormally expressed lncRNAs in cancer remain to be elucidated.

In the present study the comprehensive analysis of microarray and RNA sequencing data of thyroid tumors

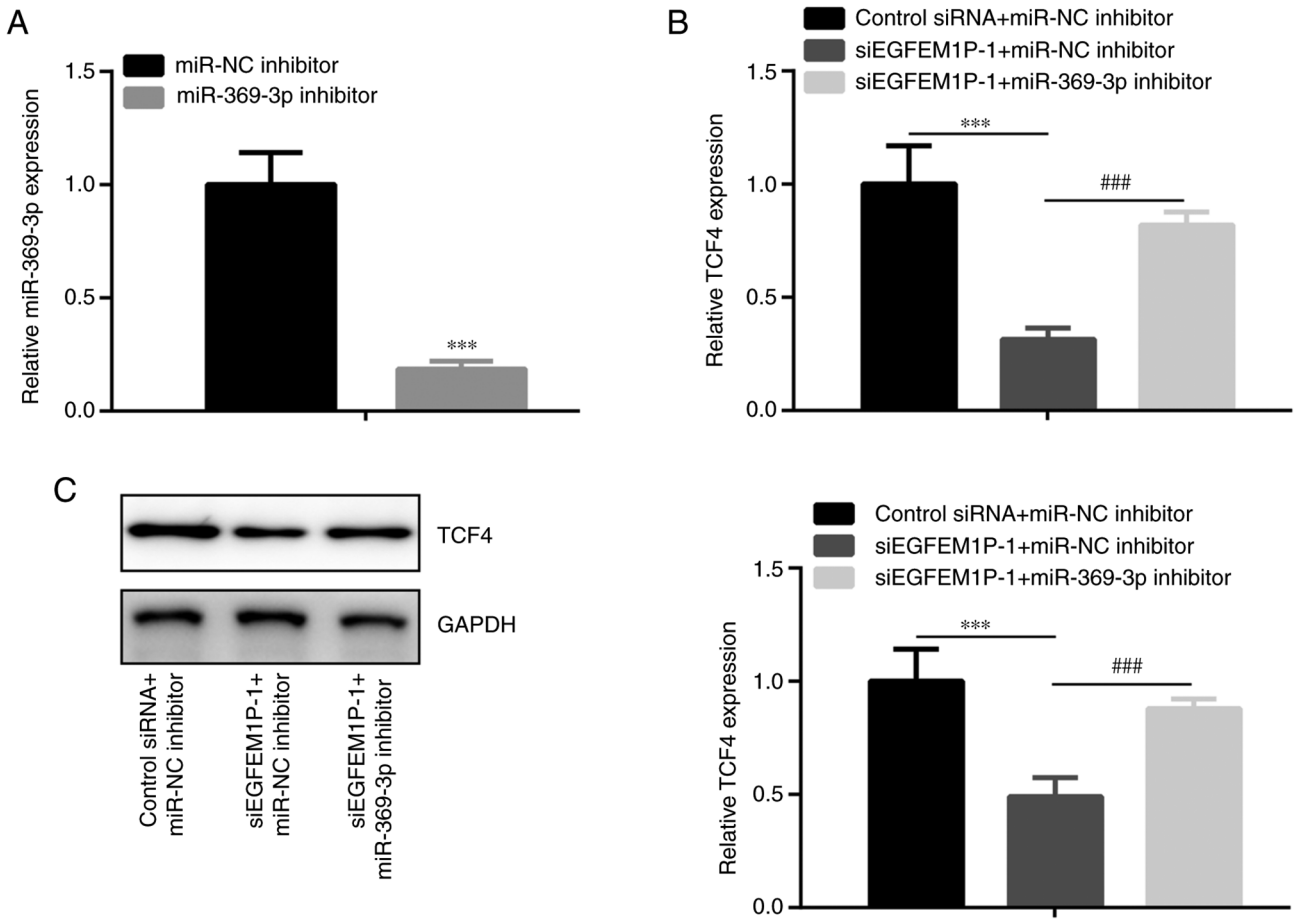

Figure 4. EGFEM1P induces TCF4 expression via sponging miR-369-3p. (A) Lower miR-369-3p expression levels were exhibited in miR-369-3p inhibitor-transfected KTC-1 cells compared with miR-NC inhibitor-transfected cells. (B and C) In KTC-1 cells lower mRNA and protein expression levels of TCF4 were exhibited in the siEGFEM1P-1 transfected group compared with the control siRNA + miR-NC inhibitor group. These effects were reversed by co-transfection with siEGFEM1P-1 + miR-369-3p inhibitor. ***P<0.001 vs. control siRNA + miR-NC inhibitor; $^{###}$P<0.001 vs. siEGFEM1P-1 + miR-NC inhibitor. EGFEM1P, EGF-like and EMI domain-containing protein 1; TCF4, T cell factor 4; miR, microRNA; NC, negative control; siRNA/si, small interfering RNA.

identified EGFEM1P as one of the most significantly upregulated lncRNAs in papillary thyroid tumors. Kaplan-Meier analysis demonstrated that there was a positive association between EGFEM1P overexpression and a poor prognosis in patients with thyroid cancer. RT-qPCR was performed, which confirmed the upregulation of EGFEM1P in papillary thyroid tumors as well as papillary thyroid carcinoma and follicular thyroid carcinoma cell lines. The findings were in consistent with a previous study in 2018, which identified EGFEM1P as an upregulated lncRNA in papillary thyroid cancer tumors, and was positively associated with the TNM staging and lymphatic metastasis of thyroid cancer (20). However, EGFEM1P was previously observed to be a downregulated in and act as a tumor suppressor in human lung adenocarcinoma. This difference between this previous study and the present study could be attributed to the different characteristics of various types of cancer (28). Subsequently, in the present study the miRNA targets for EGFEM1P in thyroid cancer were explored. Current study further investigated the role of EGFEM1P in the progression of thyroid cancer.

Using miRDB (23), several target miRNAs of EGFEM1P were predicted, including miR-30d-3p, miR-30e-3p, miR-30a-3p, miR-6875-3p, miR-615-5p, miR-222-5p, miR-5003-3p, miR-3174, miR-369-3p and miR-504-3p. The results demonstrated that EGFEM1P interacted with miR-369-3p and decreased miR-369-3p expression levels, which demonstrated the potential tumor suppressive role of miR-369-3p in thyroid cancer. This result was consistent with previous studies, which also reported the tumor suppressive role of miR-369-3p in several types of cancer. For example, miR-369-3p inhibits the cell viability and motility of hepatocellular carcinoma by binding to paired box 6 (29), miR-369-3p overexpression inhibits cell proliferation and migration of endometrioid adenocarcinoma (30), miR-369-3p was reduced in the inflamed intestinal regions of patients with inflammatory bowel disease, overexpression of miR-369-3p alleviated LPS-induced inflammation (31), miR-369-3p serves as a tumor suppressor in gastric cancer (32). Moreover, the overexpression of miR-369-3p inhibits cell proliferation and induces cell apoptosis of papillary thyroid cancer cells (33).

MiRDB (23), TargetScan (24) and miRWalk (25) were used to predict the target genes of miR-369-3p, demonstrating that 36 mRNAs were overlapped, of which, the TCF4 3'UTR was conserved among numerous species and therefore became a focus of the present study. Subsequently it was verified in the

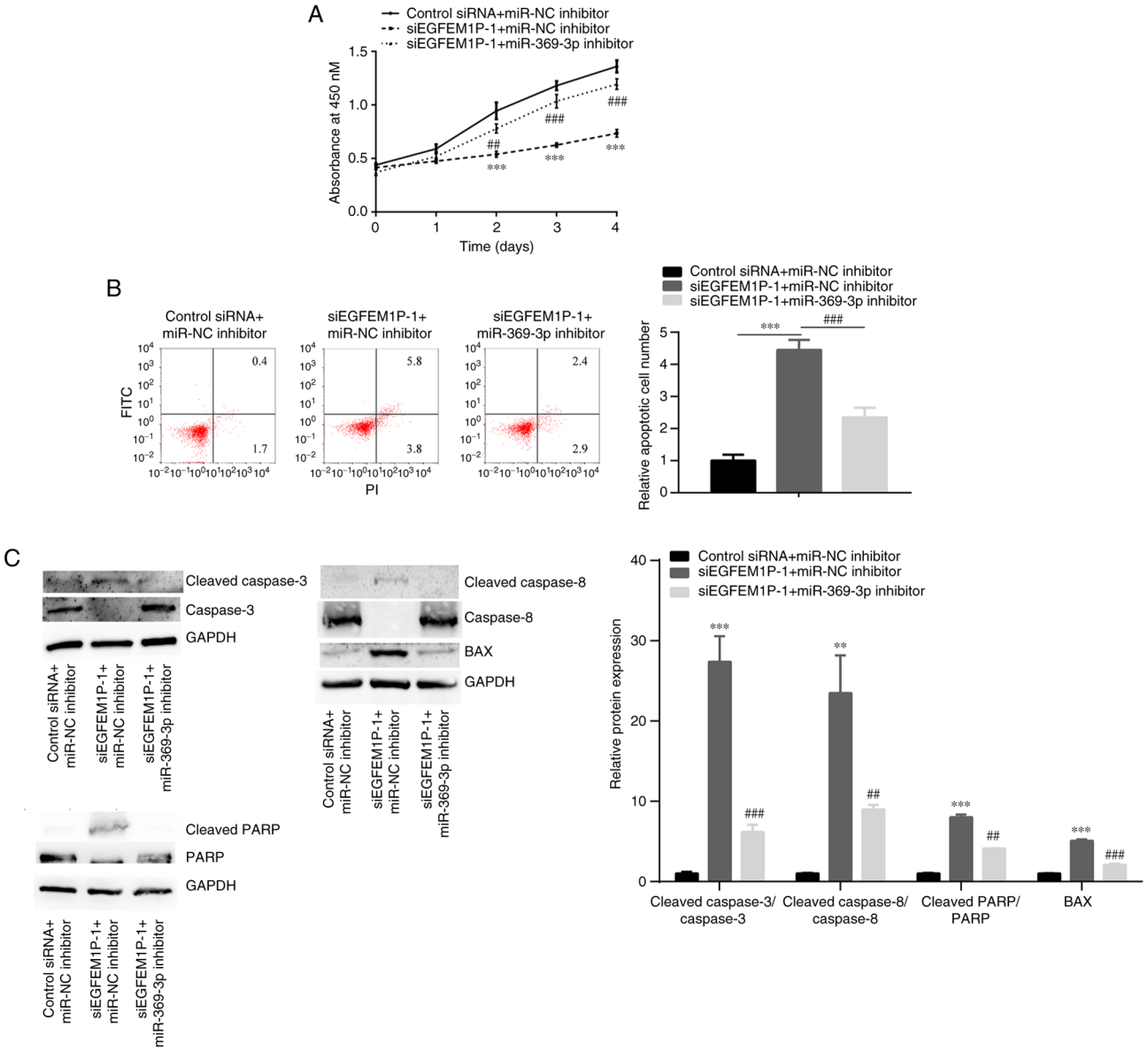

Figure 5. EGFEM1P induces cell proliferation and reduces cell apoptosis via acting as a miR-369-3p sponge. (A and B) KTC-1 cells exhibited reduced cell proliferation and higher cell apoptosis levels in siEGFEM1P-1 transfected cells compared with the control group (control siRNA + miR-NC inhibitor), which was abolished by co-transfection with siEGFEM1P-1 + miR-369-3p inhibitor. (C) In KTC-1 cells higher protein expression levels of BAX, and higher ratio of cleaved caspase-3/caspase-3, cleaved caspase-8/caspase-8, cleaved PARP/PARP were exhibited in the siEGFEM1P-1 transfected group compared with the control siRNA + miR-NC inhibitor group. These effects were reversed by co-transfection with siEGFEM1P-1 + miR-369-3p inhibitor. **P<0.01, ***P<0.001 vs. control siRNA + miR-NC inhibitor; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. siEGFEM1P-1 + miR-NC inhibitor. EGFEM1P, EGF-like and EMI domain-containing protein 1; siRNA/si, small interfering RNA; miR, microRNA; NC, negative control.

present study that TCF4 acted as a target gene of miR-369-3p. The results also demonstrated that EGFEM1P supported TCF4 expression via the regulation of miR-369-3p expression levels. TCF4 is a crucial member of the TCF-4/lymphoid enhancer factor gene family (34) and acts as an oncogene in several types of cancer. For example, TCF4 inhibition represses cell proliferation and invasion but promotes cell cycle arrest and cell apoptosis in glioma (35). Moreover, in thyroid cancer, TCF4 binds to the promoter of lncRNA HLA complex P5 (HCP5) and activates HCP5 expression to facilitate cancer cell proliferation (36). Although TCF4 overexpression has previously been reported in thyroid cancer (34), the underlying mechanism that regulates its expression is unknown. In the present study it was identified that this regulation may be potentially associated with the EGFEM1P/miR-369-3p/TCF4 axis in thyroid cancer.

Apoptosis is involved in the physiological maintenance of normal cell homeostasis, and pathological lesions which accompany numerous diseases (37). The caspase activation is a major leading cause to apoptosis (38), including effector (caspase-3) and initiator (caspase-8) (39,40). poly ADP-ribose polymerase (PARP) is a DNA-binding enzyme involved in apoptosis (41,42). Bax is a direct target in p53-mediated apoptosis (43). Furthermore, the present study demonstrated that EGFEM1P served a critical role in promoting rapid cell proliferation, and inhibiting apoptosis. In addition, detection of apoptosis-related proteins, BAX and cleaved caspase-3/8/PARP was consistent with the apoptotic rate.

In conclusion, the results of the present study indicated that EGFEM1P may be important in promoting thyroid cancer progression via acting as a miR-369-3p sponge.

However, there are several limitations to the present study: i) Immunohistochemistry for TCF4 in thyroid cancer patient samples and normal tissues was not conducted, which is performed in the related ongoing study; ii) The animal study was not conducted, which is performed in the related ongoing study; and iii) The expression profiles of the remaining miRNAs (eg miR-30a-3p) which were predicted to be targeted by EGFEM1P were not detected, which will be performed in the future study.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

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

Authors' contributions

SY and LL conducted the experiments and analyzed the data. ZC conceived the study, analyzed the data and drafted the manuscript. All authors read and approved the final manuscript. SY and ZC confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The study was approved (approval no. CHESTJMAP-2 017-08) by the Ethics Committee of The Central Hospital of Enshi Tujia and Miao Autonomous Prefecture (Enshi, China). Signed written informed consent was obtained from all patients.

Patient consent for publication

Signed written informed consent was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

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