Downregulation of lncRNA ZFAS1 inhibits the hallmarks of thyroid carcinoma via the regulation of miR-302-3p on cyclin D1

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Abstract. At present, treatment options for thyroid carcinoma remain limited. The present study aimed to investigate the role of ZFAS1 in various major hallmarks of cancer and the underlying mechanisms in thyroid carcinoma cells. The interactions between long non-coding RNAs (lncRNAs), microRNAs (miRs) and target genes were predicted by bioinformatics and confirmed by performing dual-luciferase assays. The mRNA and protein expressions were determined by reverse transcription-quantitative PCR and western blotting. Cell invasion, migration, and viability were evaluated via Transwell, wound-healing and Cell Counting Kit-8 assays, respectively. The results demonstrated that lncRNA ZFAS1 expression was upregulated in thyroid carcinoma tissues, TT and SW579 cells, and was associated with the proliferation of these two cell lines. Notably, downregulation ZFAS1 reduced migration and invasion, and reversed the promotive effects of miR-302a-3p inhibitor on the proliferation, migration and invasion of TT and SW579 cells. Moreover, cyclin D1 (CCND1) is targeted by miR-302a-3p, and was regulated by ZFAS1. In addition, the downregulation of ZFAS1 not only reversed the promotive effects of miR-302a-3p inhibitor on CCND1 expression and the epithelial-mesenchymal transition (EMT) of TT and SW579 cells, but also targeted and increased the expression of miR-302a-3p, and further reduced the expression of CCND1, resulting in suppression of the proliferation, migration, invasion and EMT of thyroid carcinoma cells.

Introduction

Thyroid carcinoma, which is a common malignant tumor of the endocrine system (1), has one of the highest incidence rates for malignant tumors in a number of regions (1,2), such as the United States (3), Korea (4) and southern European countries (5). According to the latest data from the American Cancer Society, there were 52,070 new cases and 2,170 mortalities of thyroid carcinoma in 2019 (3). According to its pathological characteristics, thyroid cancer can be divided into papillary thyroid cancer, follicular thyroid cancer, medullary thyroid carcinoma and undifferentiated thyroid carcinoma (6). At present, the treatments for differentiated thyroid cancer are mainly surgical resection, thyroid-stimulating hormone inhibition treatment, radioactive iodine treatment and molecular-targeted therapy (7). However, due to the strong invasive and migratory nature of thyroid cancer and its high degree of malignancy, recurrence or distant metastasis often occurs in a number of patients after treatment (8). Thus, investigation of the molecular mechanisms involved in the metastasis of thyroid carcinoma, and development of molecular markers and targets of thyroid carcinoma are important for the treatment of the cancer.

Long non-coding (lnc)RNAs are RNAs with >200 nucleotides in length and without protein-coding functions (9). lncRNAs have been reported to regulate numerous biological processes (9); for example, lncRNAs participate in the growth and development of the human body and occurrence of numerous diseases (9), such as cancer (10) and glomerular and tubulointerstitial kidney disease (11). A previous study indicated that the genome is widely transcribed and regulated by lncRNAs, and various lncRNAs serve important roles in different biological processes, such as in chromatin remodeling, transcription, cleavage and translation (12). Gene expression profiling of tumors is indicative of abnormal expressions of lncRNAs in tumors, and functional studies have reported that lncRNAs are involved in general mechanisms underlying tumorigenesis (13,14).

ZFAS1 serves a role in atherosclerosis (15) and a variety of cancer types (16-19), such as ovarian cancer, breast cancer, prostate cancer and hepatocellular carcinoma. ZFAS1 is a candidate biomarker predictive of the prognosis of thyroid carcinoma (20). Han *et al* (20) reported that *Homo sapiens* (hsa)-microRNA (miRNA/miR)-150-5p and hsa-miR-590-3p are competitive endogenous RNAs related to ZFAS1 in thyroid cancer cells. ZFAS1 promotes progression of papillary thyroid carcinoma by sponging miR-590-3p and increasing high-mobility group AT-hook 2 expression (21). lncRNAs

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sponge different miRNAs to regulate the cellular functions (22). Additionally, it has been reported that miR-302a-3p serves a role in a variety of diseases, such as hepatocellular carcinoma (23) and pancreatic ductal adenocarcinoma (24). Long intergenic non-protein coding RNA (LINC)01016 promotes the malignant phenotype of endometrial cancer cells by regulating the miR-302a-3p/miR-3130-3p/nuclear transcription factor Y subunit α/SATB homeobox 1 axis (25). In addition, miR-302a-3p suppresses the progression of hepatocellular carcinoma by inhibiting proliferation and invasion of the tumor cells (23). However, the role of miR-302a-3p in thyroid carcinoma has not been reported.

The present study investigated the role of ZFAS1 in the proliferation, migration, invasion and epithelial-mesenchymal transition (EMT) of thyroid carcinoma cells, and explored the downstream miRNA and target gene via which ZFAS1 exerted its regulatory effects on thyroid carcinoma cells.

Materials and methods

Patients. This study was approved by the Ethics Board of The First Hospital of Qiqihar (approval no. QR20180503112). Samples (n=30) from carcinoma as well as the adjacent tissue (≥5 cm away from the cancer tissue) were extracted from patients (age range, 28-65 years; mean age, 41.2±8.24 years; males, 11; females, 19) diagnosed with thyroid carcinoma in the First Hospital of Qiqihar between June 2018 and April 2019. The inclusion criteria of patients in this study were as follows: Patients who were identified as thyroid carcinoma via pathological examination; and patients who did not receive radiotherapy or chemotherapy before the surgery. Written informed consent was obtained from patients in this study. Based on the median expression value of ZFAS1, the patients were separated into low and high expression groups.

Cell culture. Nthy-ori3-1 (Shanghai YaJi Biological Technology Co., Ltd.; http://www.yajimall.com/) (26), MDA-T68 [American Type Culture Collection (ATCC)] (27), SW579 (ATCC) (28), B-CPAP (The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences) (29) and TPC-1 (The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences) (30) cell lines were cultured in RPMI 1640 (cat. no. 21875091; Thermo Fisher Scientific, Inc.). TT cells (ATCC) (28) were cultured in DMEM (cat no. D0819; Sigma-Aldrich; Merck KGaA). The media all contained 10% FBS (cat. no. F8192; Sigma-Aldrich; Merck KGaA) and incubated with the cells at 37˚C with 5% CO_2.

Experimental design. To investigate the effects of using small interfering (si)RNA to downregulate ZFAS1 expression, TT and SW579 cells were divided into the following groups: i) Control (without transfection); ii) si-Control (transfected with 50 nM si-Control); and iii) si-ZFAS1 (transfected with 50 nM si-ZFAS1). The si-Control (5'-UUCUCCGAACGUGUCACGUTT-3') and si-ZFAS1 (5'-CUAACUGCCUACCUGCAUATT-3') were obtained from Shanghai GenePharma Co., Ltd., and the cells were transfected using Lipofectamine® 3000 (cat. no. L3000015; Thermo Fisher Scientific, Inc.). To specify the linkage between miR-302a-3p and ZFAS1 on the hallmarks of thyroid carcinoma, TT and SW579 cells were divided into the following groups: i) Control (without transfection); ii) inhibitor-negative control (NC; transfected with 50 nM miR-302a-3p inhibitor-NC; iii) inhibitor (transfected with 50 nM miR-302a-3p inhibitor); iv) inhibitor + si-ZFAS1 (transfected with 50 nM miR-302a-3p inhibitor and 50 nM si-ZFAS1); and v) si-ZFAS1 (transfected with 50 nM si-ZFAS1). The miR-302a-3p inhibitor-NC (5'-CAGUACUUUUGUGUAGUACAA-3') and miR-302a-3p inhibitor (5'-UCACCAAAACAUGGAAGCACUUA-3') were obtained from Shanghai GenePharma Co., Ltd, and the cells ($2x10^4$ cells/well; 96-well plate) were transfected using Lipofectamine® 3000 (cat. no. L3000015; Thermo Fisher Scientific, Inc.). The cells were cultured for 24 h prior to subsequent experimentation.

Reverse transcription-quantitative (RT-q)PCR. The total RNAs were extracted from the tissue samples and cells ($1x10^6$ cells) using TRIzol® reagent (cat. no. 15596018; Thermo Fisher Scientific, Inc.). For miRNA analysis, cDNA synthesis was performed on 200 ng of total RNA using a TaqMan™ MicroRNA Reverse Transcription Kit (cat. no. 4366597; Thermo Fisher Scientific, Inc.) according the manufacturer's protocol. Reverse transcription conditions included: 42˚C for 30 min and at 85˚C for 5 min. The qPCR reactions was performed using 2 μl cDNA solution, 5 μl TaqMan 2X Perfect Master Mix (Takara Biotechnology Co., Ltd.), 0.25 μl gene-specific primers and 2.75 μl of nuclease-free water in a final volume of 10 μl with a Bio-Rad IQ5 thermocycler (Bio-Rad Laboratories, Inc.) under the following conditions: Initial denaturation at 95˚C for 3 min, followed by 40 cycles at 95˚C for 30 sec, 62˚C for 30 sec and 72˚C for 25 sec. The U6 gene was used as an internal control. For mRNA analysis, the mRNA templates were reverse transcribed into cDNAs using PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd.). Reverse transcription conditions: At 37˚C for 30 min and at 85˚C for 5 min. According to the protocol of FastStart™ Universal SYBR-Green Master (Rox; cat. no. 4913850001; Roche Diagnostics), 14 μl 2X SYBR-Green master mix, 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), 3 μl cDNA template and 6 μl double distilled H_2O were mixed and reacted in a Bio-Rad IQ5 thermocycler (Bio-Rad Laboratories, Inc.) under the following conditions: 95˚C for 90 sec, 95˚C for 25 sec, 65˚C for 20 sec, 72˚C for 30 sec for 40 cycles. GAPDH was used as an internal control. mRNA expression levels were calculated by the $2^{-\Delta\Delta Cq}$ method (31). The primers used for RT-qPCR were shown in Table I.

Transwell assay. The TT and SW579 cells ($1x10^6$) were collected at the logarithmic growth phase, and pipetted into the upper chamber (containing serum-free medium) of a Transwell insert (8-μm) pre-coated with Matrigel (BD Bioscience; at 37˚C for 4 h). The lower chamber was supplemented with 10% FBS mixed in 400 μl medium. Transwell was incubated at 37˚C with 5% CO_2 for 24 h. Next, cells remaining on the surface of the upper chamber were removed with a cotton swab, the invading cells were fixed with 4% paraformaldehyde for 15 min at room temperature and then stained with 0.2% crystal violet for 10 min at room temperature. The cells in the lower chamber were observed under a light microscope (magnification, x200), and the cells were counted using Image J software (version 1.8.0; National Institutes of Health).

Table I. Primers used in the study.

Gene	Primer sequence (5'→3')
LncRNA ZFAS1	F: CTATTGTCCTGCCCGTTAGAGCTATTGTCCTGCCCGTTAGAG
	R: GTCAGGAGATCGAAGGTTGTAG
miR-302a-3p	AATAAGTGCTTCCATGTTTTGGTGA
Cyclin D1	F: GTCTTCCCGCTGGCCATGAACTAC
	R: GGAAGCGTGTGAGGCGGTAGTAGG
MMP2	F: GGAGGCACGATTGGTCTG
	R: TTGGTTTCCGCATGGTCT
MMP9	F: TGTACCGCTATGGTTACACT
	R: CCTCAAAGGTTTGGAAT
E-cadherin	F: TAACCGATCAGAATGAC
	R: TTTGTCAGGGAGCTCAGGAT
N-cadherin	F: AGTGAGCCTGCAGATTTTAAGGTGGATG
	R: CACTTGCCACTTTTCCTGGGTCTCTT
GAPDH	F: CGCTTCACGAATTTGCGTGTCAT
	R: GAAGATGGTGATGGGATTTC
U6	F: TGCGGGTGCTCGCTTCGGCAGC
	R: CCAGTGCAGGGTCCGAGGT

lncRNA, long non-coding RNA; miR, microRNA; MMP, matrix metallopeptidase; F, forward; R, reverse.

Bioinformatics and dual-luciferase reporter assay. The interactions between ZFAS1 and miR-302a-3p, and cyclin D1 (CCND1) and miR-302a-3p were predicted by Starbase (version 2.0; http://starbase.sysu.edu.cn). The mutants of ZFAS1 and CCND1 were built using a Quick-Change Site-Directed Mutagenesis kit (Agilent Technologies, Inc.). pGL3 plasmid encoding a luciferase reporter gene was purchased from Promega Corporation. Recombinant plasmids containing the wild-type (WT) ZFAS1-3'-untranslataed region (UTR), WT CCND1-3'-UTR or corresponding mutant sequences were constructed. The TT and SW579 cells ($1x10^5$ cells/well) were seeded in a 24-well plate, and the cells were co-transfected with miR-302a-3p mimic (40 nM; 5'-UAAGUGCUUCCAUGU UUUGGUGA-3'; Shanghai GenePharma Co., Ltd.) or miRNA control (40 nM; 5'-UUCUCCGAACGUGUCACGUTT-3'; Shanghai GenePharma Co., Ltd.), recombinant plasmid (20 ng) or corresponding mutants (20 ng) using Lipofectamine 3000. Plasmid pRL-Thymine kinase (TK; Promega Corporation) was used as an internal reference luciferase. The cells were cultured for 48 h prior to the detection of luciferase activity using a Dual-Glo luciferase assay kit (Promega Corporation). The firefly luciferase activity was normalized to *Renilla* luciferase activity.

Wound healing assay. TT and SW579 cells ($1x10^6$) were collected at the logarithmic growth phase. A gap in the middle of the cell layer was created using a sterile 200-μl pipette tip by scratching the monolayer of cells. After washing, the cells were treated with serum-free medium for 48 h and incubated with 5% CO_2 at 37˚C. The images were captured using a light microscope (magnification, x100) and analyzed via ImageJ software 1.8.0 (National Institutes of Health). The mean distance between the upper, middle and bottom edges of the gap were measured and recorded.

Cell Counting Kit-8 (CCK-8) assay. The TT and SW579 cells ($1x10^6$) were transfected with si-ZFAS1 or miR-302a-3p inhibitor, divided into groups (control, si-control, si-ZFAS1, inhibitor-NC, inhibitor, inhibitor + si-ZFAS1) and cultured for 24, 48 and 72 h. The cell viability was detected by a CCK-8 assay (cat. no. 96992-100TESTS-F; Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. The absorbance was determined at 450 nm using a Multiskan microplate reader (Thermo Fisher Scientific, Inc.).

Western blotting. Following cell transfection for 24 h, $1x10^6$ cells were obtained and lysed using RIPA lysate (cat. no. R0278; Sigma-Aldrich; Merck KGaA) with protease inhibitor (cat. no. S8830; Sigma-Aldrich; Merck KGaA) to extract the total protein. The bicinchoninic acid method (cat. no. BCA1; Sigma-Aldrich; Merck KGaA) was used to determine the concentration of total protein. The proteins (25 μg/lane) were separated by 12% SDS-PAGE, and then transferred to a PVDF membrane. The membrane was blocked with 5% non-fat milk for 1 h at room temperature and incubated with anti-cyclin D1 (1:10,000; cat. no. ab134175; 34 kDa), matrix metallopeptidase (MMP)-9 (1 μg/ml; cat. no. ab73734; 78 kDa), MMP2 (1:1,000; cat. no. ab37150; 72 kDa), E-cadherin (1:10,000; cat. no. ab40772; 97 kDa), N-cadherin (1 μg/ml; cat. no. ab18203; 130 kDa); and GAPDH (1:10,000; cat. no. ab181602; all purchased from Abcam) primary antibodies overnight at 4˚C. The membrane was then washed with TBS-Tween 20 (0.1% Tween-20) and incubated with horseradish peroxide-conjugated goat anti-rabbit secondary antibody (1:2,000; cat. no. ab205718; Abcam) for 1 h at room temperature. The proteins blots were developed using SignalFire™ ECL Reagent (cat. no. 6883; Cell Signaling Technology, Inc.) and quantified using ImageJ Software (version 1.46; National Institutes of Health).

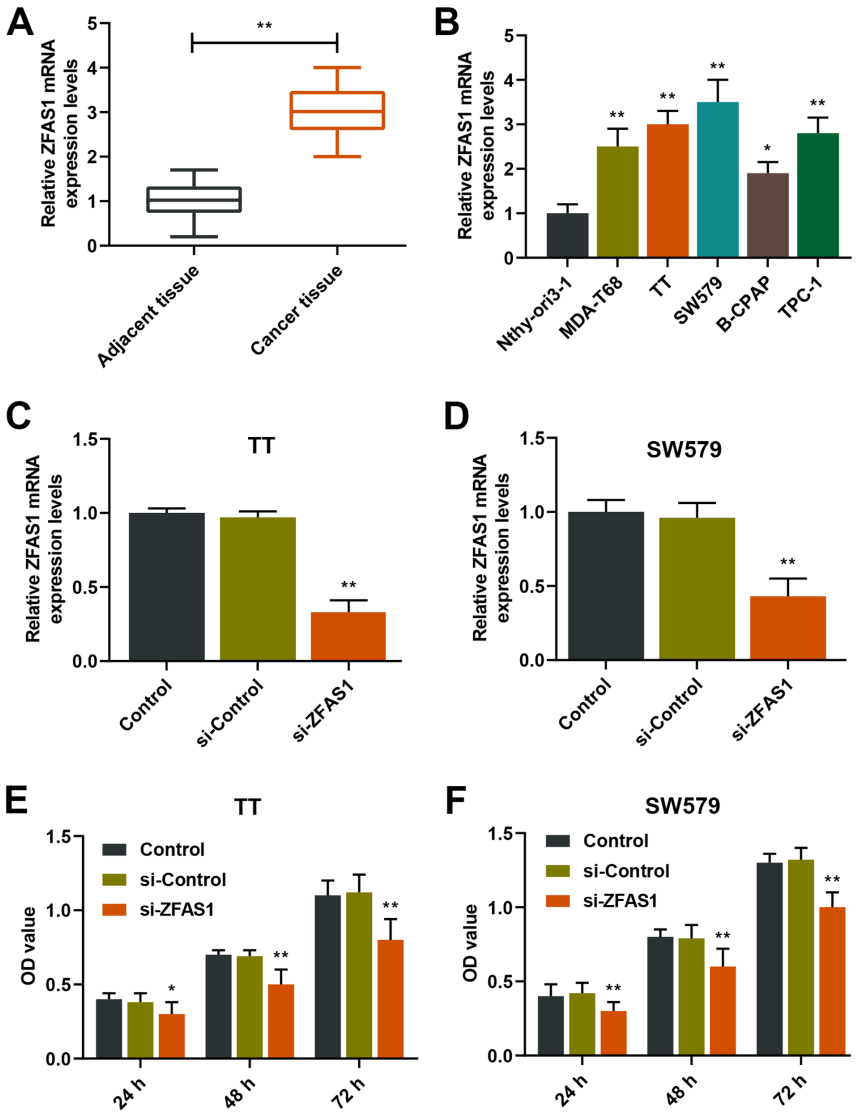

Figure 1. Expression levels of ZFAS1 in thyroid carcinoma tissue and cell lines, as well as the effects of downregulating ZFAS1 expression levels on cell viability. (A) Relative ZFAS1 expression in thyroid carcinoma tissue and (B) cell lines. *P<0.05, **P<0.01 vs. Adjacent tissue or Nthy-ori3-1. Relative (C) ZFAS1 expression in TT and (D) SW579 cells when ZFAS1 expression was silenced. OD value of (E) TT and (F) SW579 cells at 24, 48 and 72 h when ZFAS1 expression was silenced. *P<0.05, **P<0.01 vs. si-Control. OD, optical density; si, small interfering.

Statistical analysis. Data were expressed as the mean ± SD of three independent experiments. The statistical differences between two groups were analyzed by paired and unpaired Student's t-test, whereas differences among multiple groups were analyzed by one- or two-way analysis (for the CCK-8 data) of variance followed by Tukey's post hoc test. Pearson's correlation coefficient test was used for the analysis of correlation, Fisher's exact test and χ^2 test used to analyze associations between categorical variables. All results were analyzed using GraphPad Prism 8.0 (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

ZFAS1 expression levels in tissues and cell lines of thyroid carcinoma are upregulated and positively associated with the proliferation of thyroid carcinoma cells. In order to determine the expression and effects of ZFAS1 in thyroid carcinoma, the expression levels of ZFAS1 in tumor tissues and cell lines were detected. In addition, the cell viabilities of TT and SW579 cells with silencing ZFAS1 were determined. The results demonstrated that ZFAS1 expression levels were upregulated in thyroid carcinoma tissues compared with in adjacent tissues, and were upregulated in MDA-T68, TT, SW579, B-CPAP and TPC-1 cells compared with in Nthy-ori3-1 cells (P<0.01; Fig. 1A and B). In addition, the associations between ZFAS1 expression and clinical characteristics were analyzed, and the results demonstrated that ZFAS1 expression levels were significantly associated with tumor size, lymph node status and tumor stage (Table II). In addition, ZFAS1 expression levels were significantly decreased in the si-ZFAS1 group compared with those of the si-control group in TT and SW579 cells (P<0.01; Fig. 1C and D). Notably, the cell viabilities of TT and SW579 cells were significantly lower in the si-ZFAS1 group compared with those of the si-Control group after 24, 48 and 72 h (P<0.05 or P<0.01; Fig. 1E and F). These results suggested that the expression levels of ZFAS1 were elevated in thyroid carcinoma and associated with the proliferation of thyroid carcinoma.

Reduction of ZFAS1 expression levels reduces the migratory and invasive ability of thyroid carcinoma cells. TT and SW579 cells were transfected with si-ZFAS1 to further observe the effects of ZFAS1 on the migratory and invasive ability of thyroid carcinoma cells. The results demonstrated that the migration and invasion rates of TT and SW579 cells were significantly decreased in the si-ZFAS1 group compared with the si-Control group after 24 h (P<0.01; Fig. 2), suggesting that ZFAS1 expression was positively associated with the migratory and invasive ability of thyroid carcinoma cells.

miR-302a-3p is targeted by ZFAS0. Bioinformatics predicted that miR-302a-3p was the target gene of ZFAS1 (Fig. 3A); thus, the relationship between miR-302a-3p and ZFAS1 was further examined. The results demonstrated that the relative luciferase activity was significantly decreased in the ZFAS1-WT + mimic groups in TT and SW579 cells compared with those of the ZFAS1-WT + blank groups (P<0.01; Fig. 3B and C), which indicated that miR-302a-3p was a target of ZFAS1. In addition, the results demonstrated that miR-302a-3p expression levels were significantly decreased in thyroid carcinoma tissues compared with those of adjacent tissues (P<0.01; Fig. 3D), and that there was a negative relationship between the expression levels of ZFAS1 and miR-302a-3p (Fig. 3E). In addition, the expression levels of miR-302a-3p in the inhibitor group were significantly decreased compared with those of the inhibitor-NC group, but were significantly higher in the inhibitor + si-ZFAS1 group compared with those of the inhibitor group in TT and SW579 cells (P<0.01; Fig. 3F and G). In addition, the miR-302a-3p expression levels in the si-ZFAS1 group were significantly increased compared with those in the inhibitor + si-ZFAS1 group (P<0.01; Fig. 3F and G). Taken together, these results suggested that ZFAS1 may target miR-302a-3p to regulate the activity of thyroid carcinoma cells.

Downregulation of ZFAS1 expression levels eliminates the positive effects of miR-302a-3p inhibition on the proliferation, migration and invasion of thyroid carcinoma cells. To investigate the roles of ZFAS1 and miR-302a-3p in the progression of thyroid carcinoma, the changes of the cell viability, migration and invasion in thyroid carcinoma cells treated with or without si-ZFAS1 and miR-302a-3p inhibitor were examined. The results demonstrated that the cell viability, and migration and invasion rates in the inhibitor group of TT and SW579 cells were significantly increased compared with those of the inhibitor-NC group, but they were significantly decreased in the inhibitor + si-ZFAS1 group compared with those of the inhibitor group (P<0.01; Fig. 4). In addition, the cell viability, and migration and invasion rates were significantly decreased in the si-ZFAS1 group compared with the inhibitor + si-ZFAS1 group (P<0.01; Fig. 4). These results suggested that downregulation of ZFAS1 may attenuate the reduced expression of miR-302a-3p in thyroid carcinoma.

miR-302a-3p targets CCND1. The gene via which ZFAS1 and miR-302a-3p exerted their regulatory roles in thyroid carcinoma was determined via bioinformatic analysis. Bioinformatic analysis predicted that CCND1 is targeted by miR-302a-3p in the development of thyroid carcinoma (Fig. 5A). In addition, dual-luciferase reporter assay results demonstrated that the relative luciferase activities of TT and SW579 cell lines were significantly lower in the CCND1-WT + mimic groups compared with those of CCND1-WT + blank groups (P<0.01; Fig. 5B and C).

Table II. Association between ZFAS1 expression and clinical characteristics.

Characteristics	ZFAS1-low cases	ZFAS1-high cases	P-value
Age at diagnosis, years			0.269
≤45	10	7	
>45	5	8	
Sex			0.256
Male	7	4	
Female	8	11	
Location			0.514
Left lobe	7	4	
Right lobe	5	9	
Bilateral	2	1	
Isthmus	1	1	
Focus type			0.121
Unifocal	8	12	
Multifocal	7	3	
Tumor size			0.003[a]
T1-T2	12	4	
T3-T4	3	11	
Lymph node status			0.028[a]
N_0	10	4	
N_1	5	11	
Tumor stage			0.010[a]
I-II	12	5	
III-IV	3	10	

[a]P<0.05.

Downregulation of ZFAS1 expression levels reverses the promotive effects of miR-302a-3p inhibitor on CCND1 expression in thyroid carcinoma cells. Whether CCND1 expression was regulated by ZFAS1 and miR-302a-3p in TT and SW579 cells was explored. The results demonstrated that CCND1 expression levels were significantly increased in TT and SW579 cells in the inhibitor group compared with those of the inhibitor-NC group (P<0.01; Fig. 6). In addition, CCND1 expression levels in the inhibitor + si-ZFAS1 group were significantly reduced compared with those of the inhibitor group, but were significantly increased compared with those in the si-ZFAS1 group (P<0.01; Fig. 6). These results suggested that the expression levels of miR-302a-3p and CCND1 were negatively associated, but that the expression levels of ZFAS1 and CCND1 were positively associated.

Downregulation of ZFAS1 expression levels eliminates the positive effects of miR-302a-3p inhibition on EMT of thyroid

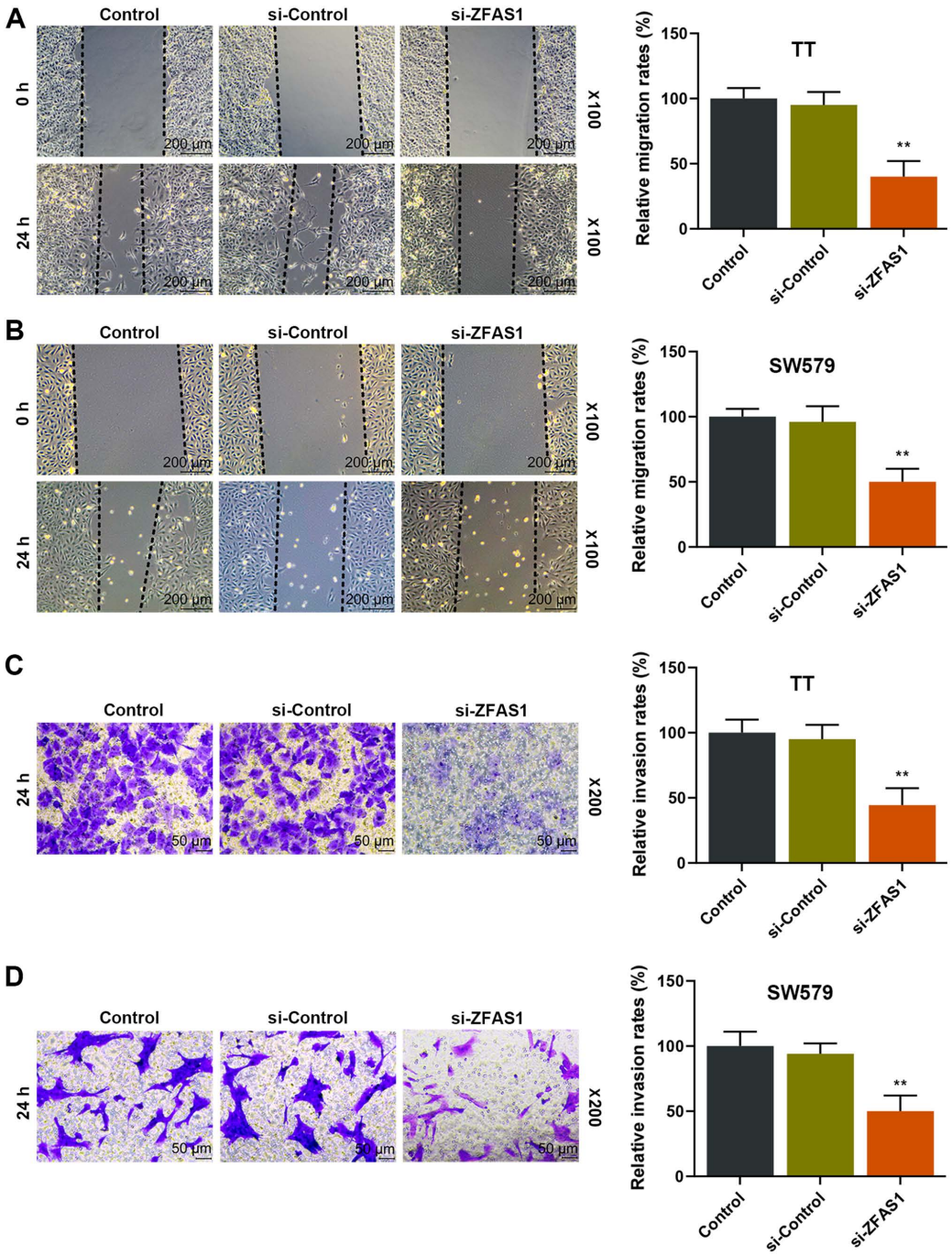

Figure 2. Effects of downregulating ZFAS1 expression levels on cell migration and invasion. Relative migration rates of (A) TT and (B) SW579 cells when ZFAS1 was silenced. Scale bar, 200 μm; magnification, x100. Relative invasion rates of (C) TT and (D) SW579 cells when ZFAS1 was silenced. Scale, 50 μm; magnification, x200. **P<0.01 vs. si-Control. si, small interfering.

carcinoma cells. The expression levels of MMP2, MMP9, E-cadherin and N-cadherin were measured to detect the EMT of TT and SW579 cells with or without silencing the expression levels of miR-302a-3p and ZFAS1. The results demonstrated that the expression levels of MMP2, MMP9 and N-cadherin were significantly increased in the inhibitor group compared with those of the inhibitor-NC group (P<0.01; Fig. 7). In addition, the expression levels of MMP2, MMP9 and N-cadherin were significantly decreased in the inhibitor + si-ZFAS1 group compared with the inhibitor group, but were significantly increased compared with in the si-ZFAS1 group (P<0.01; Fig. 7). However, the changes in the expression levels of E-cadherin were the opposite to those observed MMP2, MMP9 and N-cadherin (P<0.01; Fig. 7). Thus, the results indicated that EMT was affected by miR-302a-3p and was regulated by ZFAS1.

Discussion

The present study revealed that ZFAS1 expression was increased in thyroid carcinoma tissues and cell lines, and that downregulation of ZFAS1 expression decreased the proliferation, migration, invasion and EMT of the tumor cells. These properties, however, were promoted by the inhibition

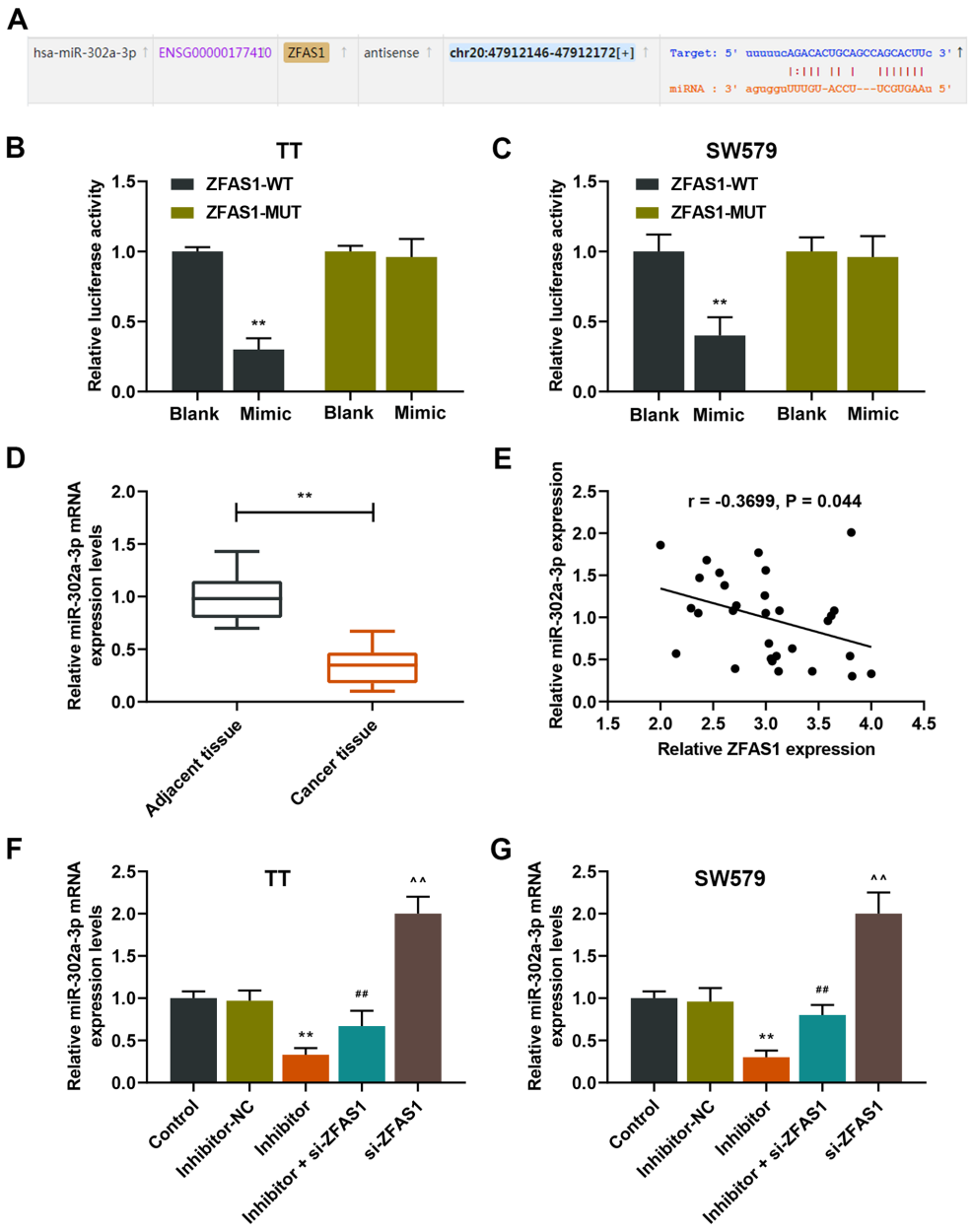

Figure 3. Negative correlation between the expression levels of ZFAS1 and miR-302a-3p. (A) Complementary sequences of ZFAS1 and miR-302a-3p. Relative luciferase activity in (B) TT and (C) SW579 cells treated with miR-302a-3p mimic. **P<0.01 vs. Blank. (D) miR-302a-3p expression levels in adjacent or cancer tissue. **P<0.01. (E) Correlation between miR-302a-3p and ZFAS1 expression. Relative miR-302a-3p expression levels in Control, inhibitor-NC, inhibitor, inhibitor + si-ZFAS1 and si-ZFAS1 groups in (F) TT and (G) SW579 cells. **P<0.01 vs. inhibitor-NC; $^{\#\#}$P<0.01 vs. inhibitor; $^{\wedge\wedge}$P<0.01 vs. inhibitor + si-ZFAS1. NC, negative control; si, small interfering; WT, wild-type; MUT, mutant; miR, microRNA.

of miR-302a-3p expression, potentially by effects on CCND1 expression. These novel findings of a possible regulatory pathway may contribute to the development of interventions for thyroid carcinoma.

The results of the present study demonstrated that ZFAS1 expression levels were increased in thyroid carcinoma tissues and cell lines, and the proliferation, migration and invasion of TT and SW579 cells were reduced after silencing ZFAS1 expression levels. Dong *et al* (32) reported that ZFAS1 overexpression facilitates the development of clear cell renal cell carcinoma. Additionally, Xie *et al* (33) demonstrated that ZFAS1 promotes the metastasis of colorectal cancer by sponging miR-484. Thus, ZFAS1 is potentially a regulator in the progression of thyroid carcinoma.

The MMPs are a family of endogenous proteolytic enzymes with cofactors as metal ions (34). Hydrolyzed proteins require Zn^{2+} and Ca^{2+} to fulfill their functions (34). During the hydrolysis process, the MMP family serves an important role in hydrolyzing most of the extracellular matrix (ECM) components (35). The degradation of ECMs by MMPs affects a number of pathologically related physiological processes, such as the development of cancer, arthritis, genetic diseases, chronic renal failure and cardiovascular diseases (36,37). In cancer-related studies, abnormally expressed MMPs mainly affect tumor cell invasion and migration (37-39). MMP2 and MMP9 are two major members in MMPs and are widely used as the biomarkers of EMT in cancer research (40). EMT refers to the process

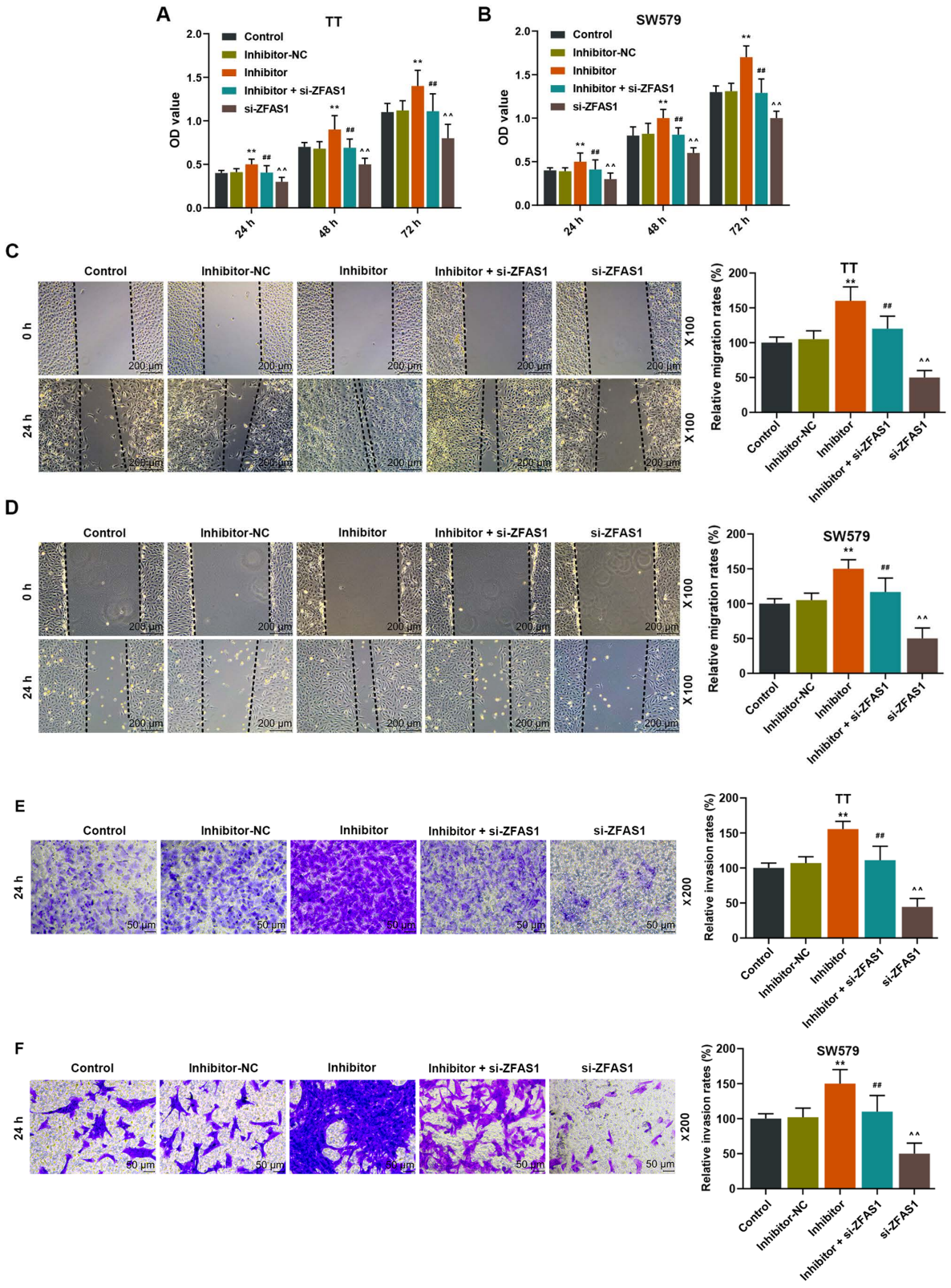

Figure 4. Effects of downregulating ZFAS1 on the cell viability, migration and invasion of TT and SW579 cells treated with miR-302a-3p inhibitor. OD values in Control, inhibitor-NC, inhibitor, inhibitor + si-ZFAS1 and si-ZFAS1 groups in (A) TT and (B) SW579 cells. Relative migration rates of (C) TT and (D) SW579 cells in each group. Scale, 200 μm; magnification, x100. Relative invasion rates of (E) TT and (F) SW579 cells in each group. Scale, 50 μm; magnification, x200. **P<0.01 vs. inhibitor-NC; $^{\#\#}$P<0.01 vs. inhibitor; $^{\wedge\wedge}$P<0.01 vs. inhibitor + si-ZFAS1. OD, optical density; NC, negative control; si, small interfering; miR, microRNA.

during which epithelial cells change their protein expression levels and transform into mesenchymal cells under the effects of external factors (41). The cadherin family, a class of Ca^{2+}-dependent transmembrane glycoproteins, serves an important role in tissue morphogenesis and coordination of cell movement (42). E-cadherin and N-cadherin are two representatives of the cadherin family and are associated with EMT (43,44). Thus, increases in MMP2, MMP9, N-cadherin

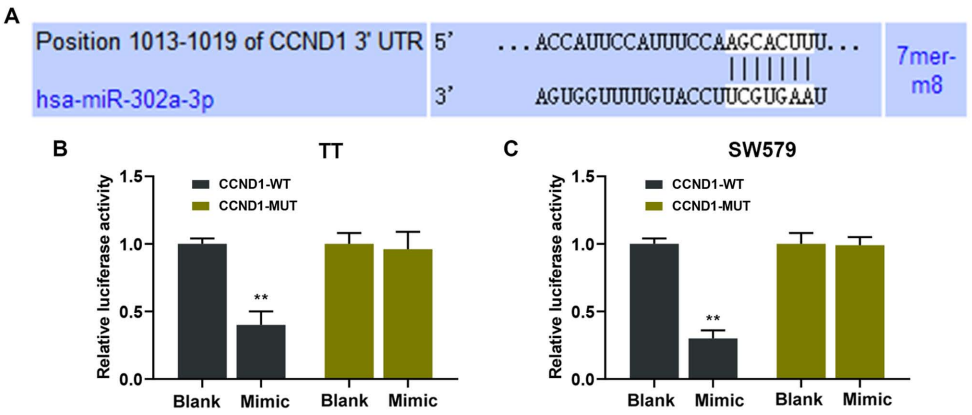

Figure 5. CCND1 is the target of miR-302a-3p. (A) Complementary sequences of CCND1 and miR-302a-3p. Relative luciferase activity in (B) TT and (C) SW579 cells treated with miR-302a-3p mimic. **P<0.01 vs. Blank. CCND1, cyclin D; miR, microRNA; wt, wild-type; mut, mutant.

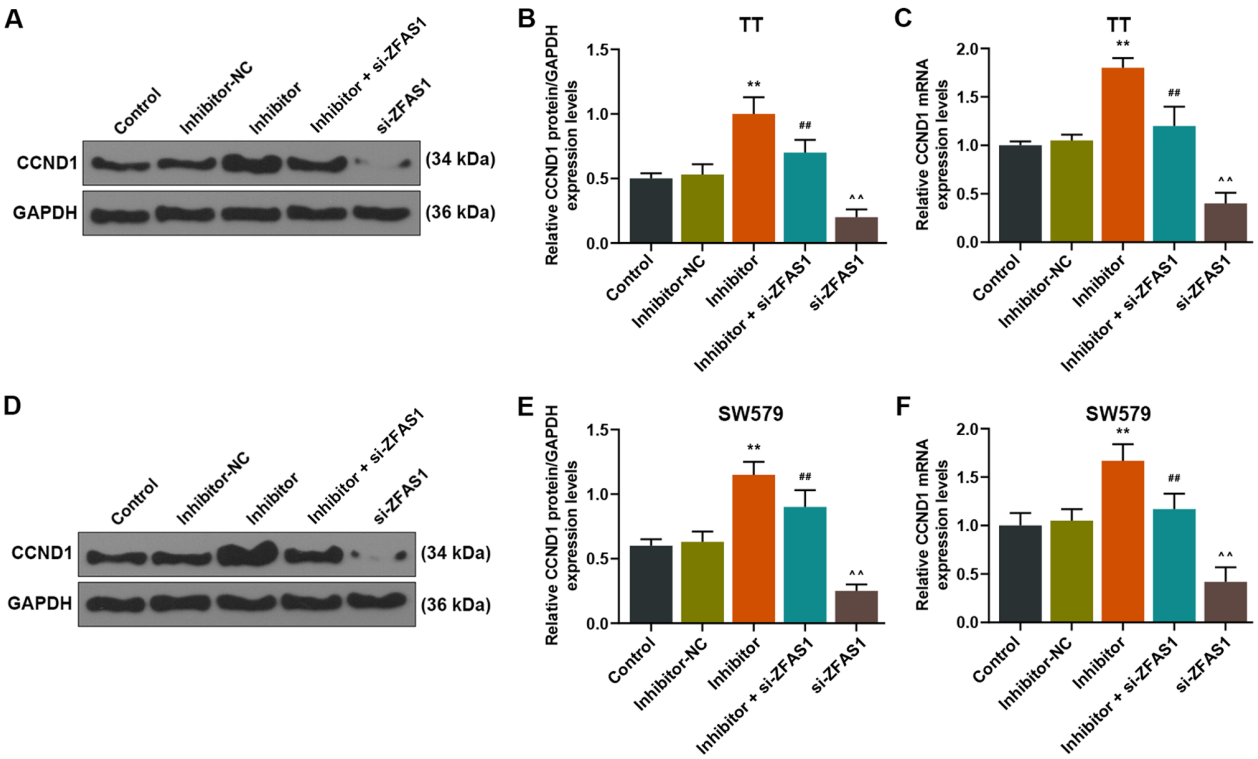

Figure 6. Effects of downregulating ZFAS1 on the expression of CCND1 in TT and SW579 cells treated with miR-302a-3p inhibitor. (A) Protein blots of CCND1 expression in Control, inhibitor-NC, inhibitor, inhibitor + si-ZFAS1 and si-ZFAS1 groups in TT cells. Relative CCND1 (B) protein and (C) mRNA levels in each group of TT cells. (D) Protein blots of CCND1 expression in each group of SW579 cells. Relative CCND1 (E) protein and (F) mRNA levels in each group of SW579 cells. **P<0.01 vs. inhibitor-NC; ##P<0.01 vs. inhibitor; ^^P<0.01 vs. inhibitor + si-ZFAS1. CCND1, cyclin D1; miR, microRNA; NC, negative control; si, small interfering.

expression levels, and decreased E-cadherin expression are indicative of EMT process.

The results of the present study indicated that miR-302a-3p expression was downregulated in thyroid carcinoma tissue and targeted by ZFAS1. In addition, inhibition of miR-302a-3p expression increased the proliferation, migration, invasion and EMT of thyroid carcinoma cells, and such effects were partially reversed by silencing ZFAS1. Zhang *et al* (45) demonstrated that inhibiting miR-302a-3p expression targets the suppressor of the cytokine signalling 5/STAT3 signaling axis and further promotes the metastasis of pancreatic cancer. Additionally, Pan *et al* (25) reported that miR-302a-3p overexpression is involved in the regulation of endometrial cancer, and it inhibits growth of endometrial cancer cells and is sponged by LINC01016. In addition, Ye *et al* (23) observed that the upregulation of miR-302a-3p suppresses the metastatic potential of hepatocellular carcinoma. Thus, miR-302a-3p expression serves a protective role in thyroid carcinoma. Taken together, it is hypothesized that the loss of miR-302a-3p expression contributes to the promotion of thyroid carcinoma, and this may be partially reversed by the downregulation of ZFAS1 expression.

The cyclin family are a class of proteins widely existing in eukaryotic cells (46); they function periodically in the cell cycle and act on cyclin-dependent kinases (CDKs) to regulate

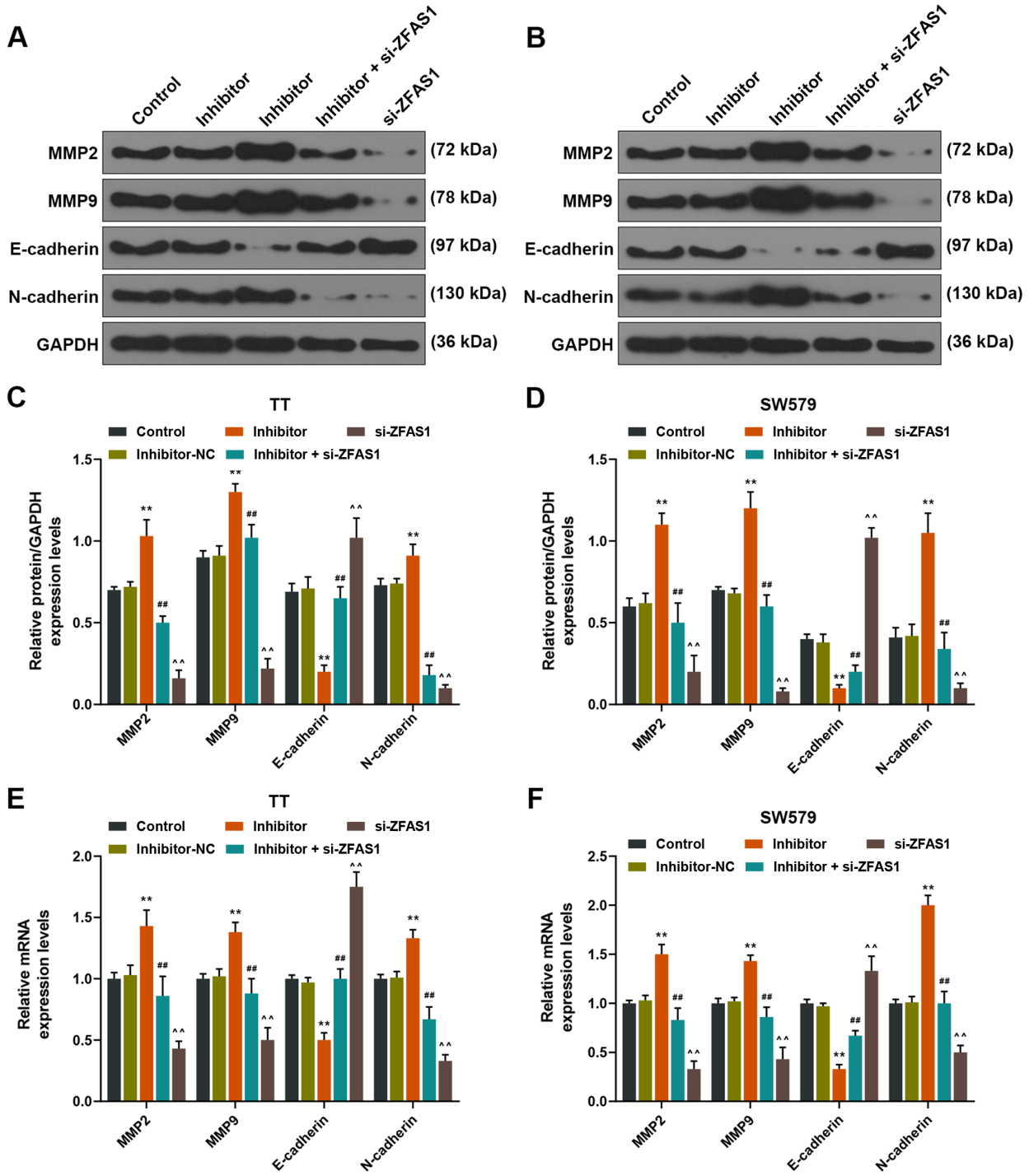

Figure 7. Effects of downregulating ZFAS1 expression on the epithelial-mesenchymal transition capability of TT and SW579 cells treated with miR-302a-3p inhibitor. Protein blots of MMP2, MMP9, E-cadherin and N-cadherin in Control, inhibitor-NC, inhibitor, inhibitor + si-ZFAS1 and si-ZFAS1 groups of (A) TT and (B) SW579 cells. Relative protein expressions of MMP2, MMP9, E-cadherin and N-cadherin in each group of (C) TT and (D) SW579 cells. Relative mRNA expressions of MMP2, MMP9, E-cadherin and N-cadherin in each group of (E) TT and (F) SW579 cells. **P<0.01 vs. inhibitor-NC; $^{\#\#}$P<0.01 vs. inhibitor; $^{\wedge\wedge}$P<0.01 vs. inhibitor + si-ZFAS1. miR, microRNA; NC, negative control; si, small interfering; MMP, matrix metallopeptidase.

cell cycle progression (46). Among them, CCND1 is a highly conserved cell cycle family protein (47). CCND1 binds to CDKs, such as CDK4 or CDK6, to form complexes and act as their regulatory subunit, promoting cell cycle progression from G1 to S phase and completing the regulation of cell cycle (47). The overexpression of CCND1 occurs in different tumors, such as in breast cancer and gastric cancer, and promotes cell invasion and migration, leading to poor prognosis (48-50). Guo *et al* (51) observed that the lncRNA NR2F1-AS1 sponged miRNA-338-3p to upregulate CCND1 expression and promote thyroid carcinoma progression. In addition, Jeon *et al* (52) proposed that the CCND1 splice variant may serve as a biomarker for the diagnostic and prediction of thyroid carcinoma. These findings confirmed that the overexpression of CCND1 serves a key role in the promotion of thyroid carcinoma. The results of the present study demonstrated that

CCND1 was a target of miR-302a-3p, and that the inhibition of miR-302a-3p expression increased CCND1 expression levels, which were reversed by the downregulation of ZFAS1 expression. Thus, it is hypothesized that CCND1 may be the target gene through which ZFAS1 and miR-302a-3p exert their regulatory functions in thyroid carcinoma. However, the present study also has some limitations. For example, downregulated lncRNA ZFAS1 was only demonstrated to inhibit the proliferation, migration and invasion of thyroid cancer cells *in vitro* by regulating miR-302a-3p/CCND1. These result needs to be further confirmed in *in vivo* experiments. In addition, the effect of ZFAS1 expression on thyroid cancer also needs to be further studied.

The results of the present study demonstrated that the downregulation of ZFAS1 targeted and increased the expression of miR-302a-3p, which further suppressed the expression of CCND1, resulting in the inhibition of the proliferation, migration, invasion and EMT of thyroid carcinoma. These findings contribute to the development of drug for the treatment of thyroid carcinoma, however, the specific regulatory network should be further specified.

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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

WC designed and conceived the study, and wrote the manuscript. LZ, HL, YL, QZ, DX and WF acquired, analyzed and interpreted the data. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the ethics board of The First Hospital of Qiqihar (approval no. QR20180503112). Written informed consent was obtained from patients in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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