miR-32-5p Inhibits the Proliferation, Migration and Invasion of Thyroid Cancer Cells by Regulating Twist1

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Research

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Abstract

Background: Thyroid cancer is the most prevalent malignancy and one of the leading causes of cancer-related deaths. Recent studies have revealed that microRNAs (miRNAs) play an important role in tumorigenesis in various cancer types by affecting the expression of its targets. However, the role of miR-32-5p in thyroid cancer remains limited.

Methods: In this study, we attempt to explore the role of miR-32-5p in thyroid cancer and elucidate the underlying mechanism. Expression of miR-32-5p was determined by quantitative reverse transcription PCR. Functional assays were performed by CCK-8 assay, cell colony assay, cell apoptosis assay, cell migration and invasion assays, cell cycle assay and luciferase assay. Protein expression was analyzed by Western blot.

Results: In the present study, the role of miR-32-5p in thyroid cancer was firstly explored. It is found that miR-32-5p was downregulated in thyroid cancer tissues and cells. Overexpression of miR-32-5p inhibited thyroid cancer cells proliferation, migration, invasion and epithelial-mesenchymal transition process; while suppression of miR-32-5p exhibited an opposite effect on thyroid cancer cells. In addition, In addition, a luciferase assay showed Twist1 was identified as a direct target of miR-32-5p in thyroid cancer, and further study showed that restoration of Twist1 attenuated the biological effect of miR-32-5p on thyroid cancer cells.

Conclusion: In conclusion, our results demonstrated miR-32-5p functions as a tumor suppressor by targeting Twist1 in thyroid cancer, providing a novel insight into thyroid cancer therapy.

1. Introduction

Thyroid cancer is a common malignant tumor in the head and neck and the most common malignant tumor in the endocrine system [1]. The treatment of thyroid cancer mainly adopts comprehensive treatment based on surgery, but its limitations still exist. Due to the popularity of early cancer screening, the incidence of thyroid cancer is increasing every year globally. [2]. Although the patients with thyroid cancer present a good prognosis, some patients still exhibit recurrence or distant metastasis [3]. Therefore, exploring the potential molecular mechanisms of thyroid cancer is particularly important for treating patients.

MicroRNA (miRNA), an additional product produced during gene transcription, not only plays an important role in cell morphology, structure, and functional transformation by participating in epigenetic, transcriptional, and post-transcriptional regulation, but also plays an important role as an oncogene or tumor suppressor gene in the occurrence and development of malignant tumors, including thyroid cancer [4-6]. More and more studies have confirmed that miR-32-5p plays a very important role in

the occurrence and development of different malignant tumors in the human body. For example, overexpression miR-32-5p was able to inhibit the triple negative breast cancer cells (TNBC), and further

results elucidated that the LncRNA WEE2 antisense RNA 1 (WEE2-AS1) and transducer of ERBB2, 1(TOB1) were the upstream gene and downstream gene of miR-32-5p, respectively [7]. Additionally, the level of miR-32-5p was downregulated in osteosarcoma (OS) tissues and cells, and Lou et al. found that long noncoding RNA HNF1A antisense RNA 1 (HNF1A-AS1) bound to miR-32-5p to regulate the expression of high-mobility group protein B1 (HMGB1) in OS progression [8]. Moreover, miR-32-5p was found to significantly regulate the radiosensitivity, migration and invasion of colorectal cancer cells via targeting transducer of ERBB2, 1(TOB1) [9]. The application of miR-32-5p in prognosis evaluation of malignant tumors is of great significance. Nevertheless, the relationship between the progression of thyroid cancer and miR-32-5p remains largely unclear. Thus, the expression of miR-32-5p in thyroid cancer and its potential molecular mechanisms were investigated in our study.

2. Materials And Methods

2.1 Tissue samples

Tumor tissues and matched adjacent nontumor tissues were collected from 10 thyroid cancer patients in the Department of General Surgery, Department of pathology, Hubei Provincial Hospital of TCM, (Wuhan, China). All patients received no chemotherapy or radiotherapy before surgery. Tissue samples were frozen immediately in liquid nitrogen following resection and stored at -80°C until RNA extraction.

2.2 Cell Culture

The human thyroid cancer cell lines B-CPAP, TPC-1, KTC-1, HTh-7, C643 and the normal human thyroid cell line HTORI-3 were purchased from Shanghai Cell Bank of the Chinese Academy of Science (Shanghai, China). Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS, Gibco) at 37˚C in a humidified chamber with 5% CO_2 atmosphere.

2.3 Transfection

The mimics-miR-32-5p, inhibitor- miR-32-5p, Twist1 and Twist1 siRNA(si-Twist1), and their respective negative controls were obtained from Shanghai GenePharma Co., Ltd., and separately transfected into thyroid cancer cell lines using Lipofectamine 2000 according to the manufacturer's protocols. After 48 h of transfection, the cells were harvested and used in experiments.

2.4 Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA from tissue samples or cultured cells was extracted using the TRIzol reagent according to the manufacturer's protocols. For miR-32-5p, cDNA was reverse transcribed from RNA using the PrimeScript RT reagent kit (Promega Corp.). U6 was used as normalization control gene. For Twist1 detection, cDNA was synthesized by M-MLV reverse transcriptase (Takara, Dalian, China). GAPDH was used as normalization control gene. qRT-PCR was performed by using SYBR Green PCR Master Mix (Invitrogen) in a real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA). Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The primers used for qRT-PCR were as follow: miR-

32-5p forward, 5’-GTCGTATCCAGTGCAGGG TCCGAG-3’, and reverse, 5’-TGCGCTATTGCACAT TACTAAG-3’; U6 forward, 5'-CGCTTCGG CAGCACATATAC-3', and reverse, 5'-AAATATGGAACGCTTCACGA-3'; Twist1 forward, 5'-GG AGTCCGCAGTCTTAC GAG-3', and reverse, 5'-GCTTGAGGGTCTGAATCTTGC-3'; GAPDH forward, 5'-TCAAGAAGGTGGTGAAGCAGG-3', and reverse, 5'-TCAAAGGTGGAGGAGT GGGT-3'.

2.5 Cell Counting Kit-8 (CCK-8) assay

The CCK-8 assay was performed to measure cell proliferation according to the manufacturer's instructions. In brief, after effectively transfection, cultured cells were plated and incubated in 96-well plates at 5×10^4 cells/well. Then, 10 μL CCK-8 solution was added to each well. Proliferation rates were measured with a microplate reader at an absorbance of 450 nm at 24, 48, 72 and 96 h.

2.6 Cell colony assay

Cell samples were seeded in 6-well culture dishes at 200 cells/well for incubation of 2 weeks. After fixed in 4% paraformaldehyde, samples were processed with 0.1% crystal violet, and colony numbers were then counted

2.7 Wound healing assay

Cell samples were seeded in 6-well culture dishes at 5×10^5 cells/well for incubation of 24 h. The cell monolayer was scratched with a 10 μL pipette tip to in a straight line and then washed twice with cold phosphate-buffered saline (PBS). Cells were then cultured with serum-free medium at 37°C for 48 h. Finally, the wounds were observed under a microscope (Carl Zeiss, Germany) and the relative migration ability was calculated.

2.8 Cell apoptosis assay

Cell samples were seeded in 6-well culture dishes at 1×10^6 cells/well for incubation of 24 h. Cells were collected by the pancreatin without EDTA, and cells were stained with AnnexinV-APC and 7-AAD for 5–15 min at room temperature avoiding light, and immediately analyzed on FACSCalibur flow cytometer.

2.9 Cell migration and invasion assays

Cell samples were seeded in 6-well culture dishes at 1×10^6 cells/well for incubation of 24 h. A Transwell chamber with 8-μm pores was used for the migration assay. Complete RPMI-1640 medium containing 10% FBS, was added in the lower layer, and the cell suspension in serum-free media was added in the upper chamber. After 48 h incubation, the cells on the lower surface of the chamber were fixed with 70% ice ethanol solution for 60 min and stained with 0.5% crystal violet for 20 min at room temperature. A total of 10 fields from each chamber were selected randomly for counting and the relative migration ability was calculated. Cell samples were seeded in 6-well culture dishes at 1×10^6 cells/well for incubation of 24 h. Then the cells were plated into the upper layer of the chamber covered with Matrigel, and the same culture method was used to perform cell invasion assays. After staining with 0.5% crystal violet, at least 10 fields from each chamber were selected and the invasive cells were counted and quantified.

2.10 Cell cycle assay

Cell samples were seeded in 6-well culture dishes at 1×10^6 cells/well for incubation of 24 h. All cells were harvested by digesting with trypsin and washed with PBS and then fixed with ice-cold 70% ethanol in PBS for 30 min at -20°C. Fixed cells were washed with PBS, treated with 10 μlL RNase A (1 mg/mL) and resuspended in 10 μl of L 400 μg/mL propidium iodide (PI) for staining. Cell cycle distribution was performed with FACSCalibur flow cytometer.

2.11 Luciferase activity assay

The wild-type (WT) 3'-UTR of Twist1 containing miR-32-5p binding site or the mutant (mut) Twist1 3'-UTR was amplified and then inserted into pUC57 reporter vector. For luciferase activity assay, two thyroid cancer cell lines were cotransfected with mimics-miR-32-5p or the scramble with the WT or mut 3'-UTR of Twist1 reporter vector using Lipofectamine 2000 according to the manufacturer's protocol. Following 48 hours of transfection, cells were harvested and lysed. The luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega) and normalized against renilla luciferase activity.

2.12 Western blot assay

Cell samples were seeded in 6-well culture dishes at 1×10^6 cells/well for incubation of 24 h. The cultured cells were collected, washed twice with cold PBS and lysed with radioimmune precipitation assay buffer containing Protease Inhibitor Cocktail. Total protein concentration was measured using a bicinchoninic acid (BCA) protein kit. Protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were blocked in 5% nonfat milk and then incubated with primary antibodies at 4°C overnight. The following day, membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at 37°C. Finally, the signals were detected using an enhanced chemiluminescent (ECL) Western blot analysis kit.

2.13 Statistical analysis

All experimental data were presented in the form of mean ± standard deviation from three independent experiments. GraphPad Prism 8 (GraphPad Software, Inc.) was utilized for statistical analysis. Student's t-test was used to compare the statistical difference between two groups, while one-way ANOVA with Dunnett's or Tukey's multiple comparisons test was employed to compare the statistical differences among multiple groups. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1 miR-32-5p was down-regulated in thyroid cancer

Firstly, the relative expression of miR-32-5p in thyroid cancer tissues and normal tissues was measured by qRT-PCR. It was found that the expression of miR-32-5p in thyroid cancer tissues is significantly lower than normal tissues (Fig. 1A). Moreover, the miR-32-5p expression in five thyroid cancer cell lines and normal thyroid cell lines was also examined. Compared to normal thyroid cell lines Htori-3, miR-32-5p was also downregulated in fiver thyroid cancer cell lines, which is consistent with the results of tumor tissue (Fig. 1B). More importantly, among these thyroid cancer cell lines, the TPC-1 and KTC-1 cell lines were contained the most significant upregulation and downregulation of miR-32-5p, respectively, thus they were used for following assays (Fig. 1B). In conclusion, the data indicated that miR-32-5p is downregulated in thyroid cancer tissues and cells lines.

3.2 Overexpression of miR-32-5p repressed cell proliferation, migration and invasion in KTC-1 cells

To elucidate the effect of miR-32-5p in thyroid cancer, KTC-1 cells were transfected with mimics-NC and mimics-miR-32-5p. Compared with mimics-NC group, miR-32-5p expression in KTC-1 cells was increased when cells are transfected with mimics-miR-32-5p. (Fig. 2A). As shown in Fig. 2B, overexpression of miR-32-5p significantly inhibited thyroid cancer cells proliferation (Fig. 2B). The results of cell colony experiments showed the high expression of miR-32-5p obviously inhibited the cell colony (Fig. 2C). Also, the cell apoptosis rate was increased when overexpression of miR-32-5p (Fig. 2D). Moreover, as shown in Fig. 2F-2G, data showed that increased miR-32-5p suppresses cell migration and invasion. Furthermore, we found that miR-32-5p overexpression significantly promoted G1/G0 phase arrest (Fig. 2H). Additionally, the expression of N-cadherin fibronectin, snail and vimentin was decreased while E-cadherin fibronectin expression was increased by miR-32-5p upregulation (Fig. 2I). All in all, these results revealed that overexpression of miR-32-5p inhibits cells proliferation, migration, invasion and cell cycle in KTC-1 cells.

3.3 Knockdown of miR-32-5p promoted cell proliferation, migration, invasion in TPC-1 cells

Then, the expression of miR-32-5p in TPC-1 cells through miR-32-5p inhibitor transfection. As shown in Fig. 3A, miR-32-5p expression was decreased in TPC-1 cells transfected with miR-32-5p inhibitor. Compared with the inhibitor NC, the proliferation of TPC-1 cell was increased in the miR-32-5p inhibitor-treated group (Fig. 3B). As exhibited in Fig. 3C, results showed that the numbers of colonies are increased, while the cell apoptosis was decreased when miR-32-5p inhibition in TPC-1 cells (Fig. 3D). Moreover, our results showed that inhibition of miR-32-5p is able to enhance the migration and invasion ability of TPC-1 cells (Fig. 3E-3G). Furthermore, the S phase was arrest when TPC-1 cells treat with miR-32-5p inhibitor (Fig. 3H). Besides, results indicated that miR-32-5p suppression can remarkably promote EMT process

(Fig. 3I). These findings suggested that the increased proliferation, migration, invasion and cell cycle of TPC-1 cells are associated with miR-32-5p deletion.

3.4 miR-32-5p directly targeted Twist1

Based on miRanda data, we found that Twist1 has miR-32-5p binding sequences in its 3 '-UTR, suggesting that Twist1 may the target of miR-32-5p (Fig. 4A). To confirm the Twist1 as the target of miR-32-5p in our study, the luciferase assay was further studied. As shown in Fig. 4B, the results showed that luciferase activity of WT was repressed by overexpression of miR-32-5p, but not the mut 3 '-UTR of miR-32-5p (Fig. 4B). More importantly, results also revealed that the level of Twist1 is elevated or reduced when inhibition of miR-32-5p in TPC-1 cells or overexpression of miR-32-5p in KTC-1 cells, respectively (Fig. 4C-4D). Together, these results demonstrated that Twist1 is a target of miR-32-5p.

3.5 Restoration of Twist1 attenuated the biological effect of miR-32-5p on thyroid cancer cells

In order to further study the role of Twist1 in thyroid cancer, the KTC-1 and TPC-1 cells were transfected with pcDNA3.1-Twist1 plasmid or siRNA-Twist1, respectively. The results found that Twist1 expression was significantly increased in KTC-1 cells, while there was opposite phenomenon in TPC-1 cells. (Fig. 5A and 5B). Upregulated Twist1 was found to increase cells proliferation, but downregulation of Twist1 partly abolished the effect of inhibitor miR-32-5p on cells proliferation regression (Fig. 5C and 5D). Additionally, the numbers of colonies were increased when overexpression Twist1, while knockdown of Twist1 attenuated the inhibitor miR-32-5p-induced thyroid cancer cell colony (Fig. 5E). Moreover, it is found that restoration of Twist1 increased the proapoptotic effect of mimics-miR-32-5p on thyroid cancer cells (Fig. 5F). it indicated that overexpression of miR-32-5p significantly decreases the migrated and invaded of cell numbers, respectively, while restoration of Twist1 increased the number of migrated and invaded cells on the basis of the up-regulation of miR-32-5p. However, decreased Twist1 shows the opposite effect, implying that Twist1 reverses the ability of miR-32-5p to promote thyroid cancer cell migration and invasion (Fig. 5H-5I). Furthermore, restoration of Twist1 significantly increased the proportion of S phase of mimics-miR-32-5p on thyroid cancer cells, while si-Twist1 decreased t the proportion of S phase on the base of knockdown of miR-32-5p (Fig. 5J). In addition, the results showed that the recovery or knockout of Twist1 could effectively weaken the effect of mimic-miR-32-5p or inhibitor miR-32-5p on the EMT process of thyroid cancer cells (Fig. 5K). These findings suggest that miR-32-5p promotes thyroid cancer cell proliferation by the regulation of Twist1.

4. Discussion And Conclusion

Abnormal regulation of miRNA plays an important role in tumorigenesis and development [5, 6]. During the occurrence and development of different types of human cancers, miRNAs consider an oncogene or tumor suppressor gene in the occurrence and development, respectively [5, 6]. The application of miRNAs in the diagnostic classification and prognostic evaluation of thyroid cancer has been a research hotpot in

recent years. For example, Hou et al. found that the proliferation and migration of dedifferentiated thyroid cancer cells are inhibited when miR-146b-3p deletion, and the results of further study showed that regulation the expression and localization of sodium/iodine cotransporter related with targeting MUC20 [10]. Moreover, miR-153-3p was found to inhibit the cell proliferation and glycolysis by inhibiting the expression of E3F3, and it may consider as a potential biomarker for thyroid cancer diagnosis [11]. Additionally, the results from Wu et al. demonstrated that the The miR-199a-3p/ DNMT3A pathway is involved in the aggression of papillary thyroid carcinoma and directly targets RAP2A [12]. MiR-32-5p has been extensively studied in different human diseases, including cancer, metabolic syndrome, and neuropathic pain [13–15]. However, the relationship between miR-32-5p and thyroid cancer has not been studied. Therefore, it is of great significance to study the occurrence and development of miR-32-5p mediated thyroid cancer. In our study, miR-32-5p was down-regulated in both thyroid cancer tissues and cancer cells, and further study showed that it is positively correlated with cell proliferation, migration and invasion. Each miRNA can regulate its downstream target, and then control to promote or inhibit the proliferation of tumor cells [16]. In previous study, Twist1 is a pro-malignant transcription factor, which plays an important role in the invasion and metastasis of various malignant tumors [17]. It was reported that the induction of Twist1can can regulate tumor metastasis in hepatocellular carcinoma [18]. Furthermore, Twist1 was regulated by several miRNAs in cancers. Yin et al. found that miR-361-5p can inhibit tumorigenesis and EMT in hepatocellular carcinoma by targeting Twist1[19]. In addition, miR-186 also targets Twist1 to mediate proliferation, migration, and EMT inhibition in breast cancer cells [20]. In our research, we found that Twist1 is a direct target of miR-32-5p. Moreover, rescue experiments showed that overexpression or knockdown of Twist1 can decrease the role of mimics or inhibitor miR-32-5p on thyroid cancer cells proliferation and EMT process. All results indicate that miR-32-5p may play an anti-thyroid cancer effect by targeting Twist1.

In conclusion, this study is the first to show that miR-32-5p is downregulated in thyroid cancer tissues and cell lines and miR-32-5p inhibited tumorigenesis and the EMT of thyroid cancer by targeting Twist1. These findings suggest that miR-32-5p may serve as a novel biomarker in thyroid cancer diagnosis or have potential clinical values in thyroid cancer treatment.

Declarations

Acknowledgements

Not applicable

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Authors' contributions

Qing Liu and Jidong Chen contributions to design of this study. Li Ouyang, Chi Zhou, Yu Wang and Chunxue He carried out the study and analyzed the data. All the authors drafted the manuscript; Chunxue

He and Silei Li revised it critically for important intellectual content. All the authors read and approved the final manuscript.

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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.

Ethics approval and consent to participate

This study protocol has been approved by the Department of General Surgery, Department of pathology, Hubei Provincial Hospital of TCM, (Wuhan, China)

Consent for publication

The recruited personnel have signed written informed consent.

Competing interests

The authors declare that they have no competing interests.

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Figures

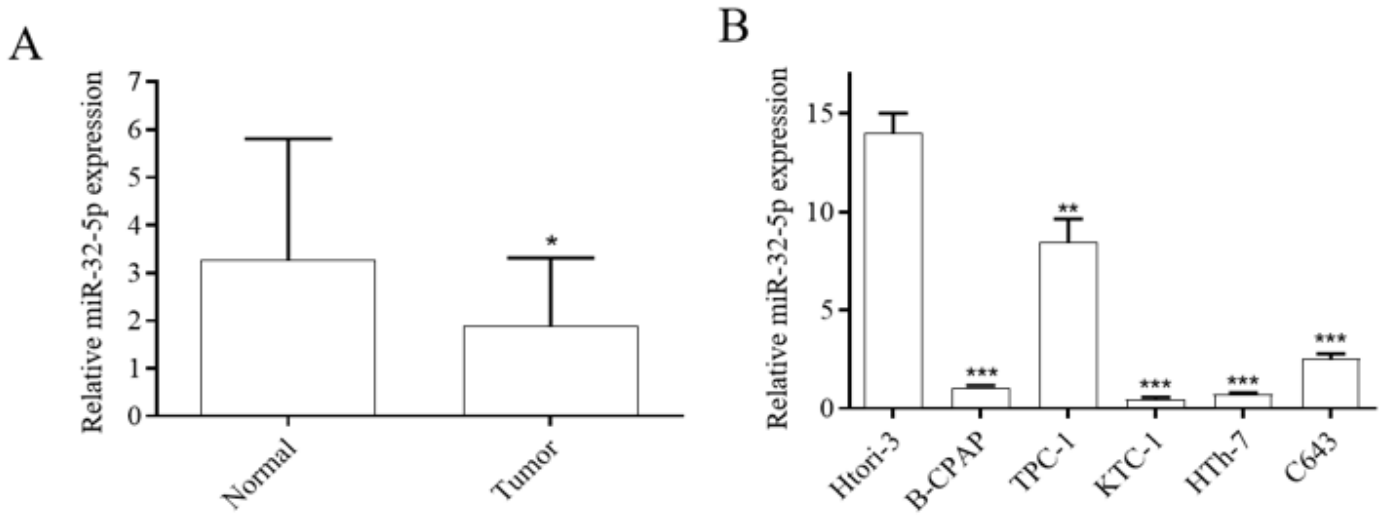

Figure 1

miR-32-5p was downregulated in thyroid cancer tissues and cell lines. (A) The expression of miR-32-5p in thyroid cancer tissues and normal thyroid tissues. (B) The expression of miR-32-5p in five thyroid cancer cell lines and normal human thyroid cell line Htori-3. Data were expressed as mean ± SD. ***P < 0.001, **P < 0.01.

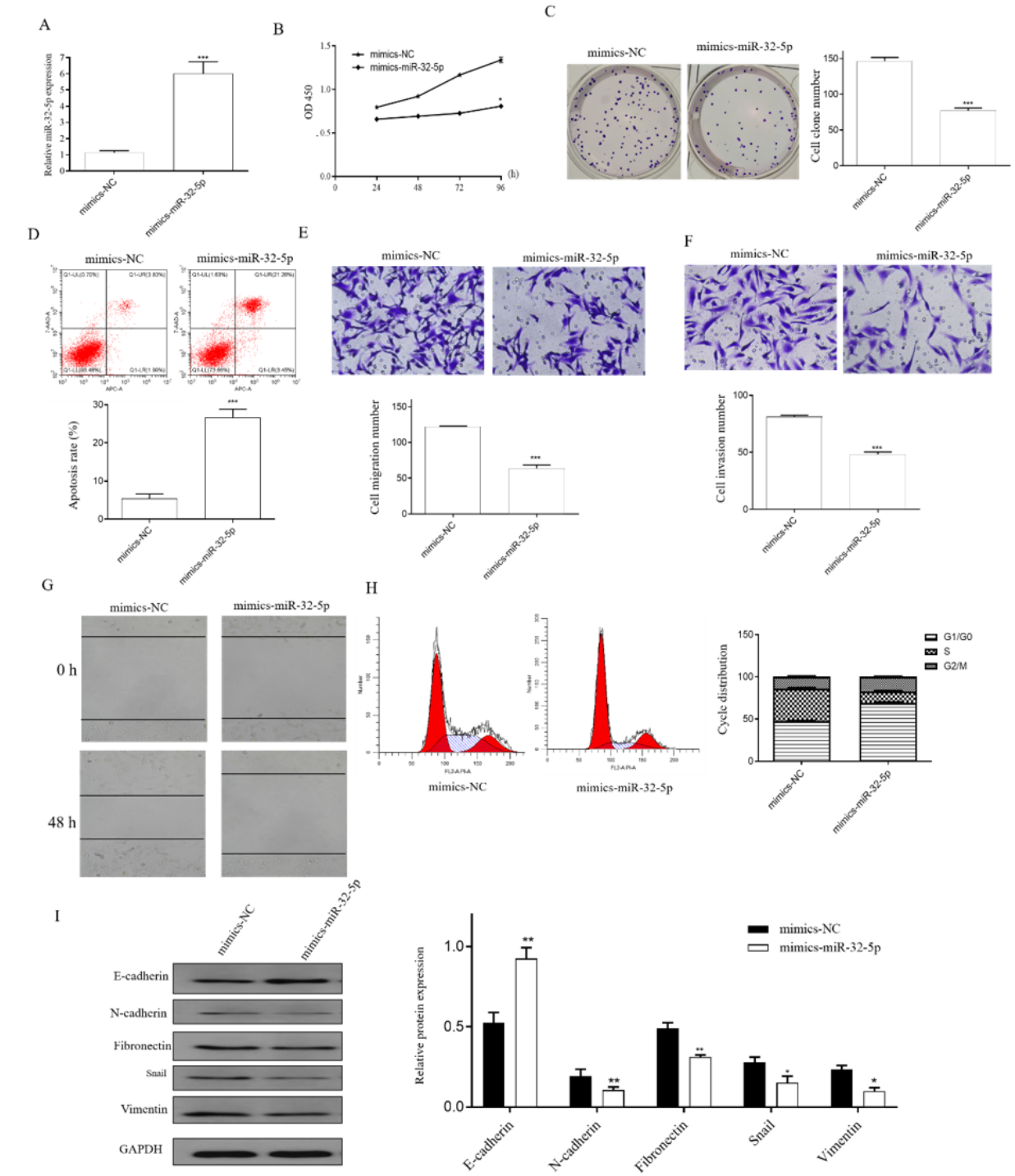

Figure 2

Overexpression of miR-32-5p inhibited cells proliferation, migration, invasion and cell cycle in KTC-1 cells. (A) The relative expression of miR-32-5p in transfected cells was measured by RT-qPCR. (B) Cell proliferation was determined by CCK-8 assay. (C) The number of colony cells was determined by cell colony experiment. (D) Cell apoptosis was determined by cell flow cytometry. (E and G) Cell migration was evaluated by Transwell migration and wound healing assay. (F) Cell invasion was evaluated by

invasion assay. (H) Cell cycle was determined by cell flow cytometry. (I) The expression of E-cadherin, N-cadherin fibronectin, snail and vimentin was determined by Western blot analysis. Data were expressed as mean ± SD. ***P < 0.001, **P < 0.01, *P < 0.05.

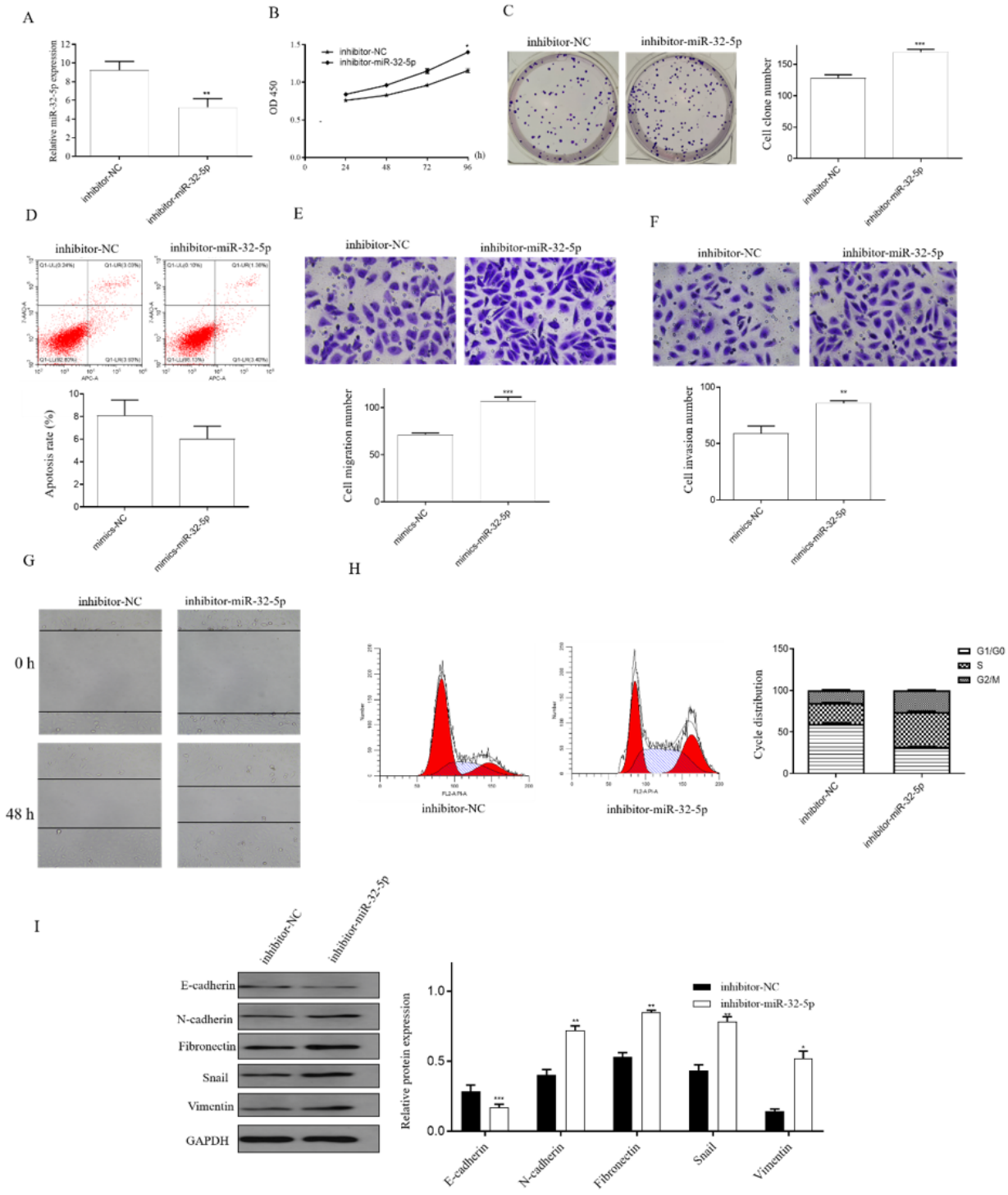

Figure 3

Inhibition of miR-32-5p inhibited cells proliferation, migration, invasion and cell cycle in TPC-1 cells. (A) The relative expression of miR-32-5p in transfected cells was measured by RT-qPCR. (B) Cell proliferation

was determined by CCK-8 assay. (C) Cell colony was determined by cell colony experiment. (D) Cell apoptosis was determined by cell flow cytometry. (E and G) Cell migration was evaluated by Transwell migration and wound healing assay. (F) Cell invasion was evaluated by invasion assay. (H) Cell cycle was determined by cell flow cytometry. (I) The expression of E-cadherin, N-cadherin fibronectin, snail and vimentin was determined by Western blot analysis. Data were expressed as mean ± SD. ***P < 0.001, **P < 0.01, *P < 0.05.

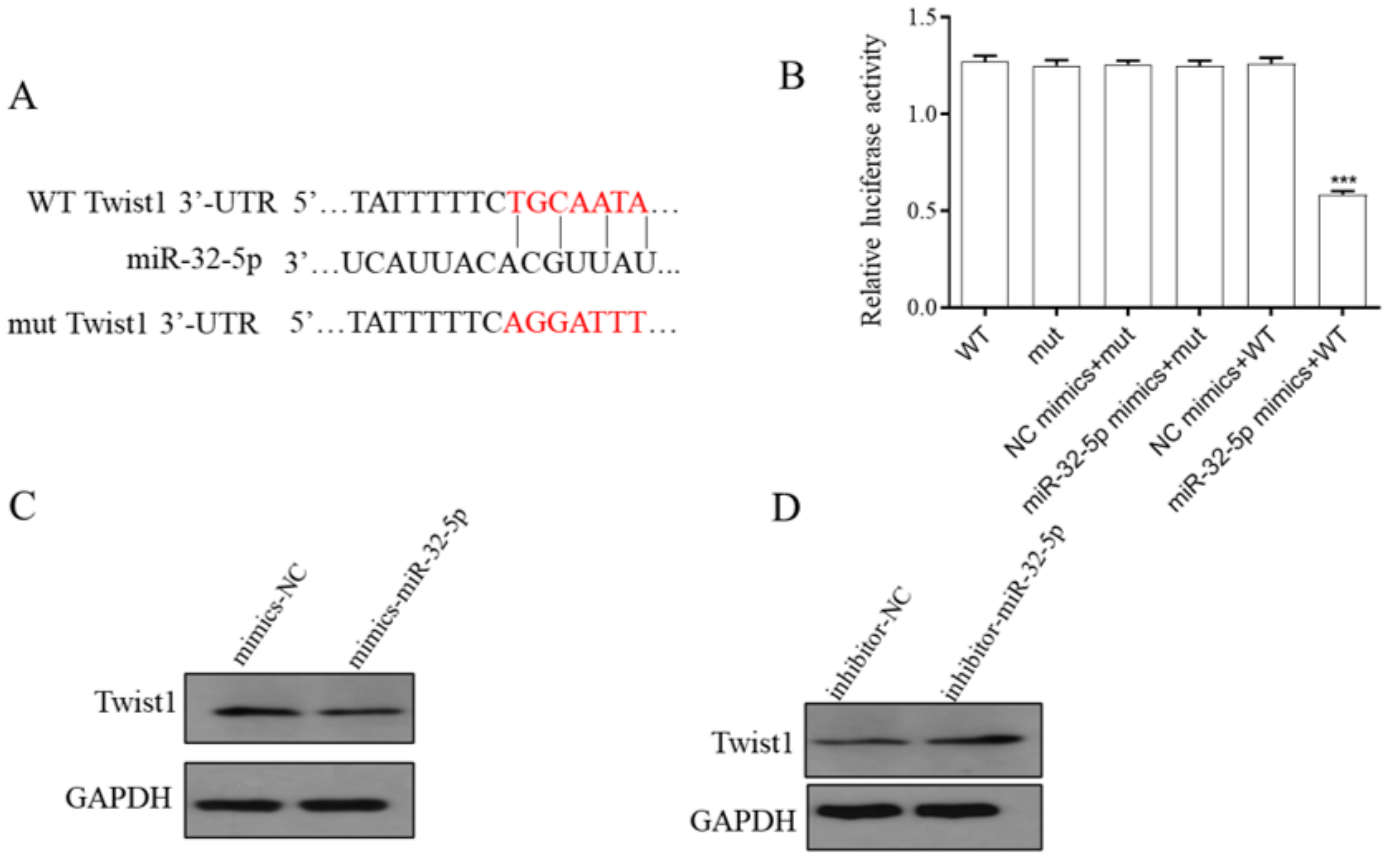

Figure 4

Twist1 was a direct target of miR-32-5p in thyroid cancer. (A) The predicted sequences of miR-32-5p target sequence in Twist1 3'-UTR. (B) Luciferase activity was measured after co-transfection with luciferase reporter plasmids (Twist1 3'-UTR WT/mut), and miR-32-5p mimics or NC mimics in KTC-1 cells. The sequences of WT and mut plasmids are shown in (A). The expression of Twist1 in KTC-1 cells and TPC-1 cells following miR-32-5p overexpression (C) and miR-32-5p suppression (D) ***P < 0.001. mut, mutant; UTR, untranslated region; WT, wild type. Data were expressed as mean ± SD. ***P < 0.001.

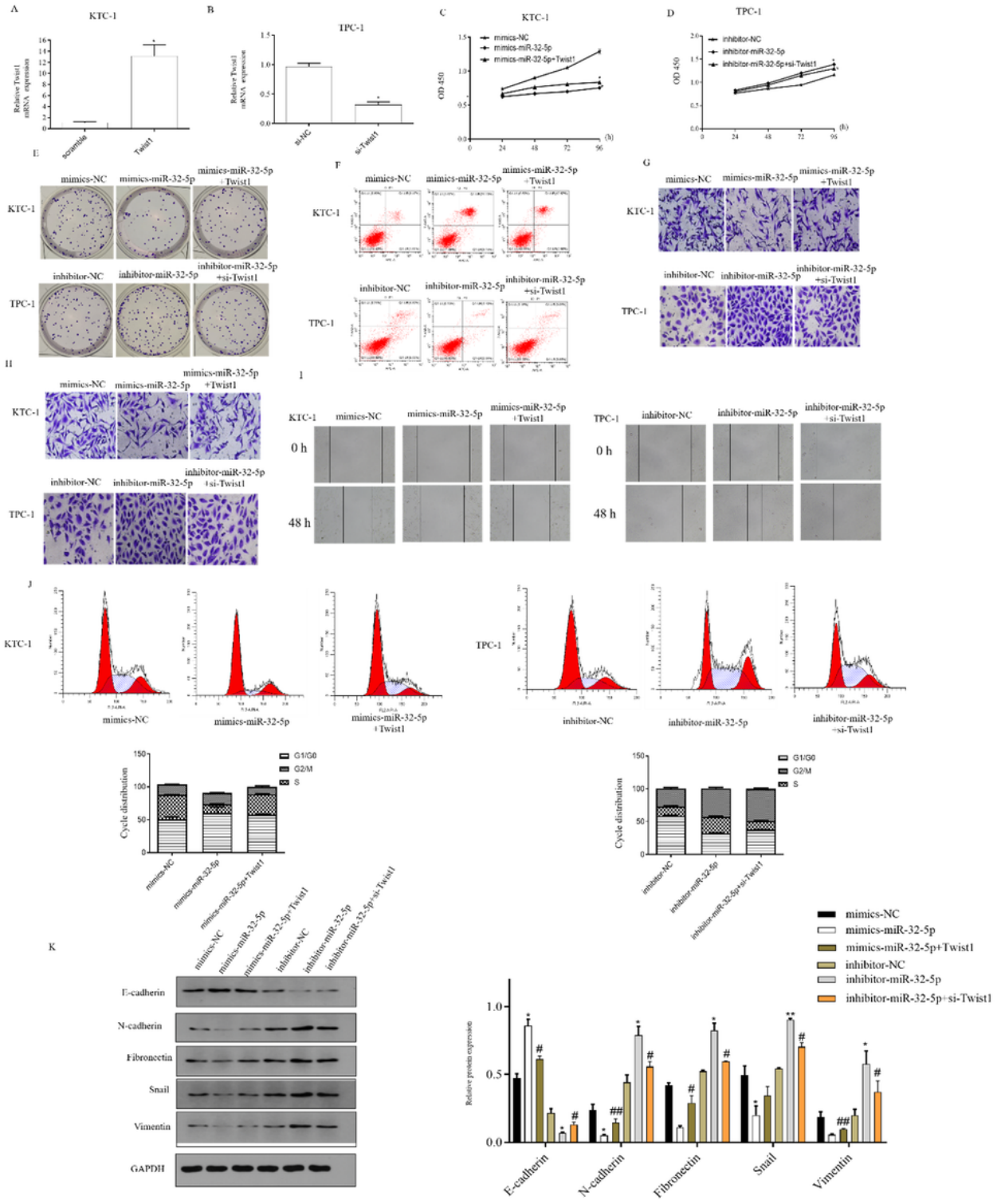

Figure 5

Restoration of Twist1 attenuated the biological effect of miR-32-5p on thyroid cancer cells. The relative mRNA level of Twist1 in KTC-1 cells following Twist1 overexpression (A) and in TPC-1 cells following Twist1 knockdown (B) were determined. Cell proliferation of KTC-1 cells with Twist1 restoration following miR-32-5p overexpression (C) and of TPC-1 cells with Twist1 knockdown following miR-32-5p suppression (D). (E) Cell colony in KTC-1 cells with Twist1 restoration or TPC-1 cells with Twist1

knockdown following miR-32-5p overexpression, respectively. (F) Cell apoptosis in KTC-1 cells with Twist1 restoration or TPC-1 cells with Twist1 knockdown following miR-32-5p overexpression, respectively. (G and I) Cell migration in KTC-1 cells with Twist1 restoration or TPC-1 cells with Twist1 knockdown following miR-32-5p overexpression, respectively. (H) Cell invasion in KTC-1 cells with Twist1 restoration or TPC-1 cells with Twist1 knockdown following miR-32-5p overexpression, respectively. (J) Cell cycle in KTC-1 cells with Twist1 restoration or TPC-1 cells with Twist1 knockdown following miR-32-5p overexpression, respectively. (K) Protein expression in KTC-1 cells with Twist1 restoration or TPC-1 cells with Twist1 knockdown following miR-32-5p overexpression, respectively. Data were expressed as mean ± SD. *P < 0.05.