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MicroRNA-198 inhibits metastasis of thyroid cancer by targeting H3F3A

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Abstract. – OBJECTIVE: This study was designed to investigate the role of microRNA-198 in thyroid cancer (TCa) progression.

PATIENTS AND METHODS: Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out to examine microRNA-198 and H3F3A levels in tumor tissue specimens and paracancerous ones collected from 50 patients with TCa, and the interplay between microRNA-198 or H3F3A and some clinical indicators or prognosis of TCa patients was analyzed as well. MicroRNA-198 and H3F3A overexpression models were constructed using lentivirus in TCa cell lines TPC-1 and BHP2-7, and the impacts of microRNA-198 on TCa cell functions were evaluated by using cell counting kit-8 (CCK-8), plate clone formation, and transwell assays. Finally, recovery investigations were conducted to explore the underlying mechanisms as well as the interaction between microRNA-198 and H3F3A.

RESULTS: QRT-PCR indicated that in tumor tissues of TCa patients, microRNA-198 showed a remarkably lower expression than in adjacent normal tissue samples. Compared with patients with high expression of microRNA-198, those with microRNA-198 low expression had more advanced tumor stage, larger tumor size, higher lymph node metastasis rate, and lower overall survival rate. Meanwhile, the results of research on H3F3A were just opposite to the above observations on microRNA-198. In in vitro cell experiments, overexpression of microRNA-198 significantly weakened the proliferation and migration ability of thyroid tumor cells. Besides, Luciferase reporter gene experiment revealed that H3F3A was a specific target gene for microRNA-198. Moreover, qRT-PCR indicated that H3F3A and microRNA-198 were negatively correlated in thyroid carcinoma tissues. In addition, compared with NC group, overexpression of H3F3A markedly enhanced the migration and proliferative capacity of TCa cells. Lastly, recovery experiment revealed a mutual regulation between microRNA-198 and H3F3A, the two of which may together participate in the malignant progression of TCa.

CONCLUSIONS: MicroRNA-198 is remarkably reduced in TCa and inhibits malignant progression of TCa by regulating H3F3A. Meanwhile, microRNA-198 is remarkably associated with pathological stage, tumor size, lymph node metastasis, and poor prognosis of TCa.

Key Words:
Thyroid cancer, MicroRNA-198, H3F3A, Proliferation.

Introduction

As the most common endocrine organ-source malignant tumor, thyroid carcinoma ranks first among head and neck malignant tumors[1-3], and its incidence is still gradually increasing[1,3]. As the most common type of TCa, papillary thyroid carcinoma (PTC) accounts for about 90% of all pathologic types, and most of PTC patients are adolescent and female, accompanied by cervical lymph node metastasis[1,4,5]. PTC is clinically characterized by slow-growing thyroid masses with multiple foci and a tendency of regional lymph node metastasis[1,4,5]. Currently, the treatment methods include surgical therapy, hormone inhibition treatment, I-131 treatment, and adjuvant radiotherapy[6,8,9]. After effective and reasonable treatment, PTC generally has a good prognosis, with a 5-year survival rate of about 95%, and the 10-year survival rate reaches about 90%[10,11].

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However, some papillary cancers are highly invasive, and some of them tend to dedifferentiate and eventually develop into poorly differentiated or undifferentiated TCa, leading to decreased survival rate and quality of life[10,11]. Therefore, it is of great significance to further improve the survival rate of patients by studying the infiltration and metastasis of thyroid papillary cancer cells and searching for biomarkers for prediction of metastasis and target molecules for intervention of this cancer[12,13].

MicroRNA (MiRNA), or miRNA for short, is a research hotspot in the field of molecular biology and genetics in recent years[14,15]. MiRNA is a newly discovered endogenous non-coding single-stranded small RNA of about 21-25 nucleotides in length. Its main function is to bind to the 3′-untranslated region (3′-UTR) of the miRNA of the downstream target gene and downregulate the expression of proteins, thereby exerting a regulatory effect on cell proliferation, differentiation, invasion, and apoptosis. Besides, it is associated with the occurrence and development of many tumors[15-17]. MiRNAs are differentially expressed between cancer cells and normal cells[17]. Abnormal expression of miRNAs can affect tumor progression by regulating genes involved in cancer-related processes and bypass pathways, suggesting that miRNAs may play a role similar to tumor suppressor genes or proto-oncogenes and regulate a variety of important biological behaviors of tumor cells[17,18]. Of note, microRNA-198 plays an anticancer role in some malignant tumors, but the specific mechanism and its role in TCa remain elusive[19,20].

Bioinformatics analysis reveals that H3F3A is a specific target gene of microRNA-198. Therefore, in this study, the differences in the expression levels of microRNA-198 and H3F3A in PTC tumor tissues and adjacent tissues were analyzed, and their relationship with the clinicopathological features of PTC was assessed. Meanwhile, the possible mechanism of their involvement in the development of TCa was further explored through the analysis of their correlation.

Patients and Methods

Patients and Thyroid Carcinoma Samples

A total of 50 cases of PTC and their normal adjacent tissues (NCE) with a distance of more than 2 cm from the tumor were collected. The specimens were immediately frozen in liquid nitrogen and then transferred to a refrigerator at –80°C. All patients suffered from PTC, except for malignant tumors in other sites, and did not accept radiotherapy or chemotherapy before surgery. Clinical data were collected for each patient, including age, gender, tumor size, local location of the lesion, and local lymph node metastasis. This investigation was approved by the Ethics Committee of Chinese PLA 988 Hospital. Signed written informed consents were obtained from all participants before the study.

Cell Lines and Reagents

Four PTC cell lines (BHP5-16, TPC-1, BCPAP, K1, and BHP2-7) and a normal human thyroid epithelial cell line (Nthy-ori 3-1) were obtained from the Tumor Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The TCa cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL). All cells were cultured in a 37°C, 5% CO_2 incubator, and were passaged with 1% trypsin + EDTA (ethylenediaminetetraacetic acid) for digestion when growing to 80%-90% confluence.

Transfection

MiR-NC and microRNA-198 mimics were also purchased from Shanghai GenePharma Company (Shanghai, China). Cells were plated in 6-well plates and grew to a cell density of 30%-40%, and then lentiviral transfection was performed. Finally, cells were collected 48 h later for quantitative real-time polymerase chain reaction (qRT-PCR) analysis and cell functional assays.

Cell Counting Kit-8 (CCK-8) Assay

The transfected cells were collected and plated into 96-well plates at 2000 cells per well. After cultured for 24 h, 48 h, 72 h, and 96 h respectively, 10 μl of CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added per well for incubation for 1 h, and then the optical density (OD) value of each well was measured in the microplate reader at 450 nm absorption wavelength.

Colony Formation Assay

After 48 h of transfection, cells were collected, and 200 cells were seeded in each well of a 6-well plate and cultured with complete medium for 2 weeks. The medium was changed after one

week and then twice a week, and it should not be replaced as much as possible in the previous week to avoid cell adhesion. After 2 weeks, the cells were cloned and then fixed in 2 mL of methanol for 20 min. After the methanol was aspirated, the cells were stained with crystal violet for 20 min, photographed, and counted under a light-selective environment.

Transwell Assay

The cells after transfection for 48 h were digested, centrifuged, and resuspended in medium without FBS to adjust the density to 5×10^5 cells/mL. A cell suspension of 200 μL (1×10^5 cells) was added to the upper chamber, and 700 μL of a medium containing 20% FBS was added to the lower chamber. After incubated in a 37°C incubator for 48 h, the chamber was removed, fixed with 4% paraformaldehyde for 30 min, and stained with crystal violet. Subsequently, the inner surface of the basement membrane of the chamber was carefully cleaned to remove the inner layer cells. At last, the perforated cells stained in the outer layer of the basement membrane of the chamber were observed under the microscope.

QRT-PCR

Total RNA was extracted from TCa tissue and cell samples, which was then reversely transcribed into complementary deoxyribose nucleic acid (cDNA). After that, qRT-RCR was carried out using StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The following primers were used: microRNA-198: forward: 5'-CAACGGAAUCCCAAAAGCAGCU-3', reverse: 5'-GGUCCAGAGGGGAGAUAGGUUC-3', U6: forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3', H3F3A: forward: 5'-TCAATGCTGGTAGGTAAGTAAGGA-3', reverse: 5'-GGTTTCTTCACCCCTCCAGT-3', glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward: 5'-CGCTCTCTGCTCCTCCTGTTC-3', reverse: 5'-ATCCGTTGACTCCGACCTTCAC-3'. Three replicate wells were repeated for each sample and the assay was repeated twice.

Western Blot

The transfected TCa cells were lysed using cell lysis buffer, shaken on ice for 30 min, and centrifuged at 14,000 ×g for 15 min at 4°C. Total protein concentration was calculated by bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA). The extracted proteins were separated using a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Immunoblotting was performed according to standard procedures. The primary antibodies against H3F3A and GAPDH, and the secondary antibodies were all provided by Cell Signaling Technology (Danvers, MA, USA).

Dual-Luciferase Reporting Assay

HEK293T cells were seeded in 24-well plates and co-transfected with microRNA-198 mimics/NC and pMIR Luciferase reporter plasmids. The plasmid was introduced into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h, the reporter luciferase activity was normalized to control.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 V5.01 software (La Jolla, CA, USA). Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Data were expressed as mean ± standard deviation. $p<0.05$ was considered statistically significant.

Results

MicroRNA-198 and H3F3A Expression in PTC and NCE Tissues

It was found by qRT-PCR that microRNA-198 showed a remarkable reduction in PTC tissue samples when compared to the NCE (Figure 1A, $p<0.05$). Conversely, PTC tissues contained a significantly higher mRNA expression of H3F3A than NCE (Figure 1B, $p<0.05$).

MicroRNA-198 and H3F3A Expression Was Correlated With TNM stage, Tumor size, and Overall Survival in Thyroid Carcinoma Patients

The correlation between microRNA-198 expression and various clinicopathological factors of PTC patients was analyzed. The results (Table I) showed that microRNA-198 was correlated with TNM stage ($p= 0.013$), tumor size ($p=0.015$), and situation of local lymph node metastasis ($p=0.033$) of patients, but not with

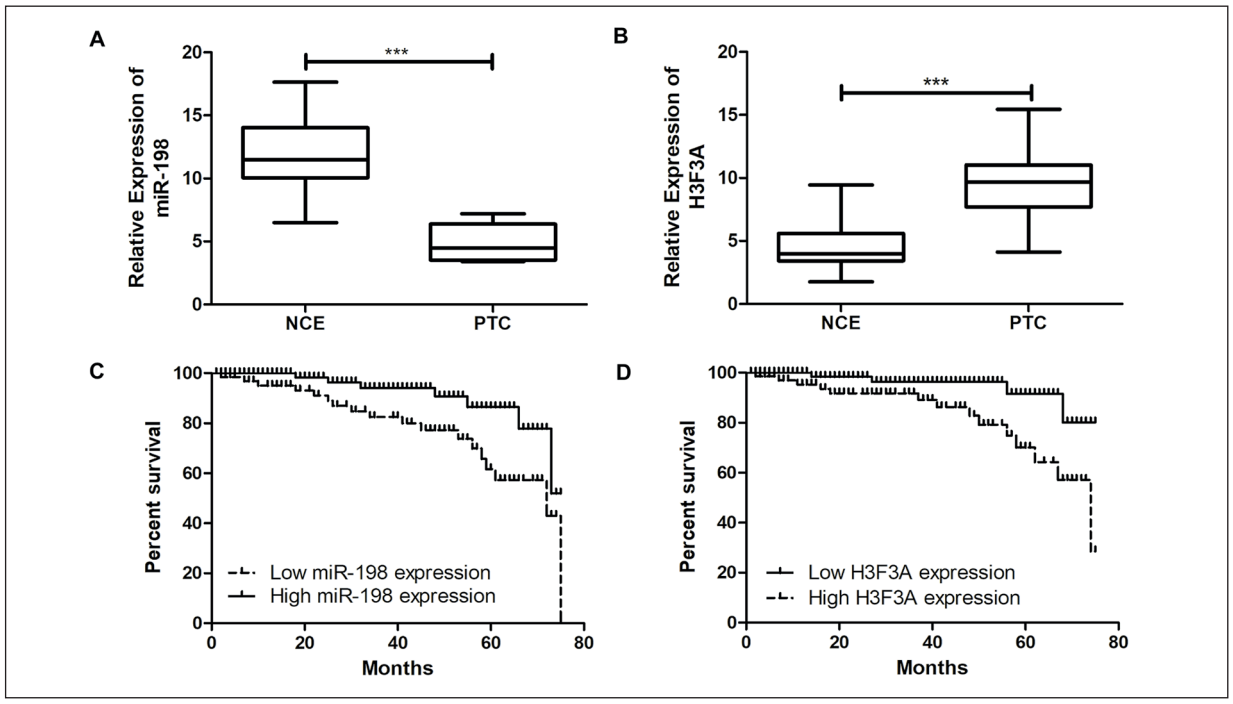

Figure 1. Expression levels of miR-198 and H3F3A in TCa tissues. **A,** QRT-PCR is used to detect the differential expression of miR-198 in tumor tissues and non-tumor tissues adjacent to TCa patients. **B,** QRT-PCR is used to detect the difference of H3F3A expression in tumor tissues and adjacent non-tumor tissues of patients with TCa. **C,** Kaplan Meier survival curve of TCa patients based on miR-198 expression is shown. The prognosis of patients with low miR-198 expression is significantly worse than that of patients in high expression group. **D,** Kaplan Meier survival curve of TCa patients based on H3F3A expression is shown. The prognosis of patients with high H3F3A expression is significantly worse than that of patients in low expression group. Data are mean ± SD, ***p<0.001.

their gender or age (p>0.05). And the same results were observed in qPCR detection of H3F3A expression in PTC tissue samples. Furthermore, Kaplan-Meier survival curve was plotted to explore the interplay between the expression of microRNA-198, as well as H3F3A and the prognosis of patients with TCa. As a result, it was found that low expression of microRNA-198

Table I. Association of miR-198 and H3F3A expression with clinicopathologic characteristics of thyroid carcinoma.

Parameters	No. of cases	miR-198 expression			H3F3A expression		
		High (%)	Low (%)	*p*-value	High (%)	Low (%)	*p*-value
Age (years)				0.854			0.696
< 40	22	12	10		9	13	
≥ 40	28	16	12		13	15	
Gender				0.696			0.854
Male	26	13	9		10	12	
Female	24	15	13		12	16	
TNM stage				0.013			0.013
I/II	28	20	8		8	20	
III/IV	22	8	14		14	8	
Tumor size				0.015			0.015
< 2 cm	30	21	9		9	21	
≥ 2 cm	20	7	13		13	7	
Lymph node metastasis				0.033			0.049
Negative	31	21	10		17	14	
Positive	19	7	12		5	14	

or high expression of H3F3A was remarkably associated with poor prognosis of TCa, which is, the lower the microRNA-198 expression or the higher the H3F3A level, the worse the prognosis ($p<0.05$, Figure 1C, D).

Up-Regulation of MicroRNA-198 Inhibited PTC Cell Growth and Migration

In *in vitro* cell assays, qRT-PCR detection also revealed a remarkably lower microRNA-198 expression in TCa cell lines than in Nthy-ori 3-1 cells (Figure 2A), suggesting that microRNA-198 may act as a tumor suppressor gene in TCa. To further investigate the cellular functional changes of microRNA-198 in TCa, a microRNA-198 overexpressing lentiviral vector was constructed and transfected into TPC-1 and BHP2-7 cell lines, and the transfection efficiency was verified through qPCR (Figure 2B). After that, CCK-8, plate cloning, and transwell assays were carried out to examine the proliferation and migration ability of TCa cells. The CCK-8 results showed that compared with miR-NC, microRNA-198 mimics remarkably attenuated the proliferative ability of TCa cells (Figure 2C). Similarly, microRNA-198 overexpression

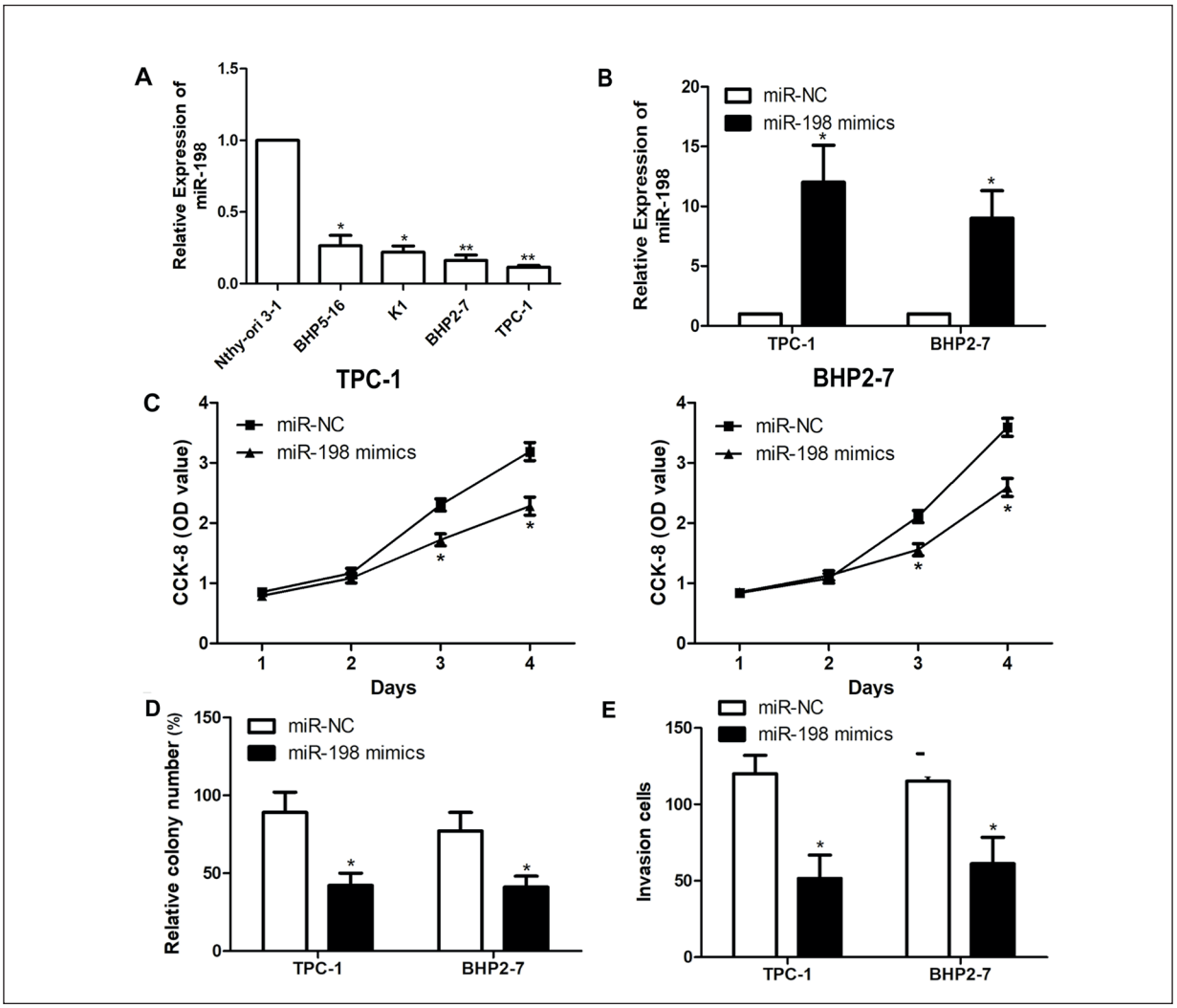

Figure 2. Overexpression of miR-198 inhibits proliferation and migration of TCa cells. **A,** QRT-PCR was used to detect the mRNA expression level of miR-198 in TCa cell lines. **B,** QRT-PCR verifies the transfection efficiency of miR-198 after transfection of the miR-198 overexpression vector in the TPC-1 and BHP2-7 cell lines. **C,** CCK-8 assay detects the effect of overexpressing miR-198 on the proliferation of TCa cells in TPC-1 and BHP2-7 cell lines. **D,** Plate cloning assays are performed to detect the number of TCa positive cells after overexpression of miR-198 in TPC-1 and BHP2-7 cell lines (magnification: 10×). **E,** Transwell assay further confirms the invasion ability of TCa cells after overexpression of miR-198 in the TPC-1 and BHP2-7 cell lines (magnification: 40×). Data are mean ± SD, *$p<0.05$.

group also showed a reduction in positive proliferating cells, measured by plate cloning experiment (Figure 2D). In addition, the transwell assay revealed a significant decrease in TCa cell migration after upregulation of microRNA-198 (Figure 2E).

Up-regulation of H3F3A Promoted PTC Cell Growth and Migration

Compared to Nthy-ori 3-1 cells, H3F3A was found markedly to be highly expressed in TCa cell lines (Figure 3A), suggesting that H3F3A may serve as a cancer-promoting gene in TCa. To figure out the impact of H3F3A on TCa cell functions, a H3F3A overexpression lentiviral vector was transfected in TPC-1 and BHP2-7 cell lines, and qPCR was used to verify the transfection efficiency (Figure 3B). Afterwards, compared to the control group, H3F3A overexpression dramatically enhanced the proliferative capacity of TCa cells, showed by CCK-8 (Figure 3C) and plate cloning assay (Figure 3D). Meanwhile, a significant elevation in migration ability of TCa cells was verified through performing transwell assay (Figure 3E).

MicroRNA-198 Modulated H3F3A in

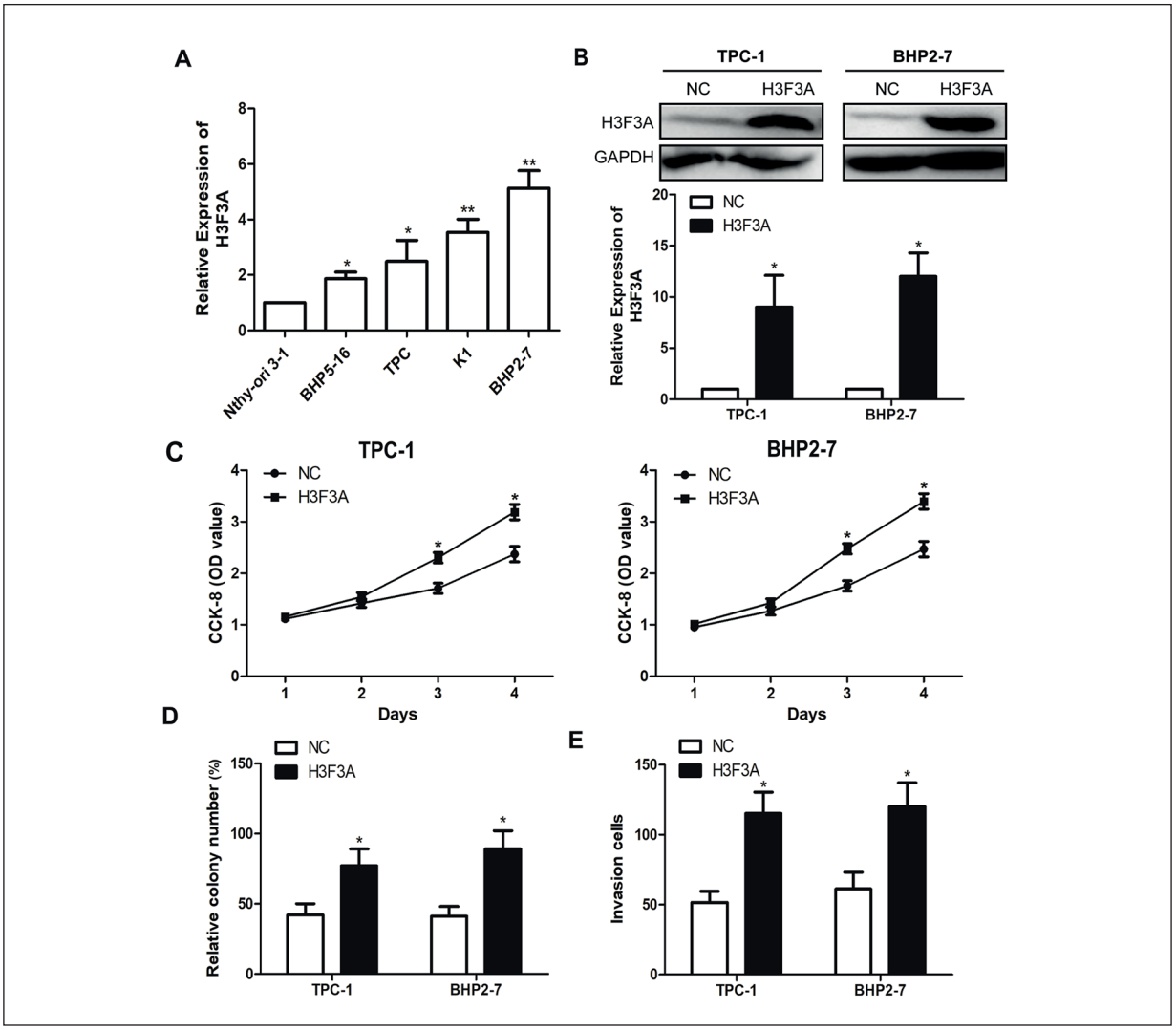

Figure 3. Overexpression of H3F3A inhibits proliferation and migration of TCa cells. **A,** QRT-PCR is used to detect the mRNA expression level of H3F3A in TCa cell lines. B, qRT-PCR verifies the transfection efficiency of H3F3A after transfection of H3F3A overexpression vector in TPC-1 and BHP2-7 cell lines. **C,** CCK-8 assay detects the effect of overexpression of H3F3A on the proliferation of TCa cells in TPC-1 and BHP2-7 cell lines. **D,** Plate cloning assays are performed to detect the number of TCa positive cells after overexpression of H3F3A in TPC-1 and BHP2-7 cell lines. **E,** Transwell assay further reveals the ability of TCa cells to migrate after overexpressing H3F3A in the TPC-1 and BHP2-7 cell lines. Data are mean ± SD, *$p<0.05$.

Thyroid Carcinoma

To further understand the way in which microRNA-198 inhibits the malignant progression of TCa, bioinformatics analysis was performed and it was found that there might exist an interplay between microRNA-198 and H3F3A. Subsequently, Luciferase reporter gene assay demonstrated that H3F3A was a specific target gene for microRNA-198 (Figure 4A). Therefore, it was hypothesized that microRNA-198 might repress malignant progression of TCa *via* modulating H3F3A. Subsequently, Western blotting assay revealed that overexpression of microRNA-198 remarkably suppressed H3F3A protein expression (Figure 4B). Meanwhile, qPCR indicated that overexpression of H3F3A significantly reduces the expression of microRNA-198 (Figure 4C). In addition, TCa tissue validation suggested a significant negative correlation between microRNA-198 and H3F3A (Figure 4D). Subsequently, H3F3A and microRNA-198 were simultaneously overexpressed in TPC-1 and BHP2-7 cell lines to further clarify their interaction in TCa, and qPCR assay detected the transfection efficiency of microRNA-198 (Figure 4E). Consequently, upregulation of H3F3A was found to be capable of counteracting the impact of microRNA-198 overexpression on proliferative and migratory abilities of TCa cells (Figure 4F-4H).

Discussion

PTC is an endocrine tumor with a low degree of malignancy. Surgical resection of the lesion is the basic way to treat this cancer, which can be combined with endocrine suppressive therapy and I-131 treatment programs to achieve a better prognosis[3-5]. In recent years, the incidence of thyroid cancer has gradually increased in China, becoming a solid malignant tumor with the fastest growth rate of mortality and mor-

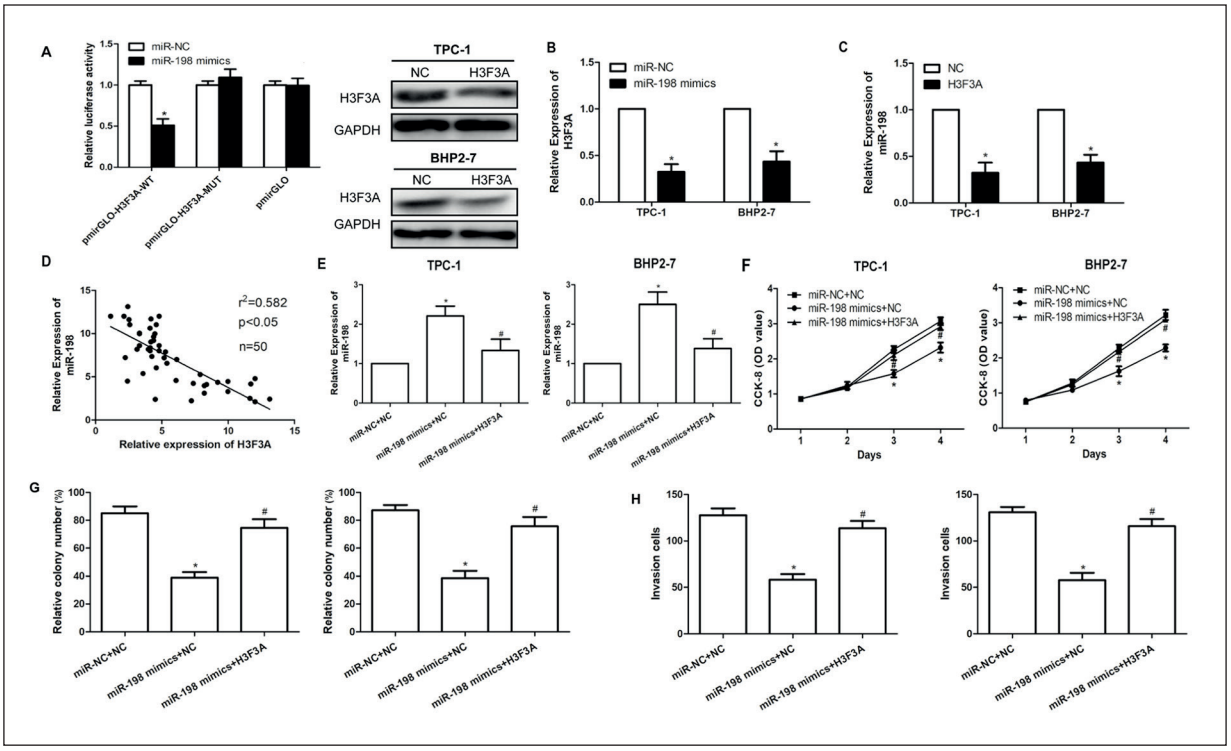

Figure 4. MiR-198 regulates the expression of H3F3A in TCa. **A,** Luciferase reporter gene experiments suggest that miR-198 specifically binds to H3F3A. **B,** Protein expression of H3F3A after overexpression of miR-198 is detected by Western Blotting assay. **C,** The expression level of miR-198 after overexpression of H3F3A is detected by qRT-PCR. **D,** There is a significant negative correlation between miR-198 and H3F3A expression in TCa tissues. **E,** The expression level of miR-198 is detected by qRT-PCR after co-transfection of miR-198 and H3F3A into TCa cell lines. **F,** CCK-8 detects the TCa cell proliferation after co-transfection of miR-198 and H3F3A. **G,** Plate cloning assay is performed to detect TCa positive cells after co-transfection of miR-198 and H3F3A (magnification: 10×). **H,** Transwell experiments further confirm TCa cell migration ability after co-transfection of miR-198 and H3F3A (magnification: 40×). Data are mean ± SD, *#p<0.05.

bidity, which further threatens human health[1,6,7]. With the development of medical standards, the improved early detection rate and the comprehensive treatment provided by multidisciplinary diagnosis and treatment team have advanced the survival time of PTC patients[8-10]. Excessive intake of iodine-containing food, history of radiation exposure, and continuous increase of estrogen levels are all important causes of TCa, but the pathogenesis and mechanism are not yet clear[11-13].

MicroRNAs (miRNAs) regulate gene expression by inhibiting target mRNAs at the post-transcriptional level mainly through complete or incomplete complementary binding with 3'UTR of the target genes[14-17]. About 30% of protein-coding genes in the human body are regulated by miRNAs, which have become a research hotspot in the field of life sciences in recent years[16,17]. MiRNAs interweave into a complex regulatory system by regulating the interaction between target genes and proteins and other biological networks, playing a pivotal role in the occurrence of tumors and widely participating in the modulation of expression of genes involved in the process of tumor invasion and metastasis[15,18]. In addition, microRNA-198 has been found to play a pivotal role in tumorigenesis and development, but no clear association with TCa was identified[19,20]. In this study, it was found that the expression of microRNA-198 in TCa tissues was remarkably lower than that in adjacent tissues, and was positively correlated with TNM stage, tumor size, local lymph node metastasis, and poor prognosis of TCa patients. Therefore, it was suspected that microRNA-198 might act as a tumor-suppressor in TCa. In addition, bioinformatics analysis and Luciferase reporter assay revealed that H3F3A was a specific target gene for microRNA-198, and the results of TCa tissue validation manifested that H3F3A might play a role of promoting cancer in TCa.

To further investigate the molecular mechanism of microRNA-198 in the development of TCa, *in vitro* cell experiments were conducted. In TCa cell lines with microRNA-198 overexpression, CCK-8, plate cloning, and transwell migration experiments revealed that microRNA-198 could inhibit the proliferation and migration of TCa cells. In addition, the molecular mechanism of H3F3A in the progression of TCa was also investigated, and the results indicated that overexpression of H3F3A could accelerate the proliferative rate and metastasis of TCa cells.

At present, functional studies on competitive endogenous RNA suggest that miRNAs can regulate each other through competitive binding of corresponding mRNA response elements, thus effectively controlling the subsequent post-transcriptional regulation[15-17]. The results of this study demonstrated that the expression levels of microRNA-198 and H3F3A were negatively correlated in TCa tissues. In addition, cell recovery experiment revealed that overexpression of H3F3A could counteract the effect of overexpression of microRNA-198 on TCa cell functions. Therefore, the above findings suggest that microRNA-198 can inhibit metastasis and proliferative ability of TCa cells *via* regulating the expression of H3F3A. The novelty of this study was that the anticancer role of microRNA-198 in TCa was reported, and the possible mechanism in the development of TCa was uncovered, which could provide a new therapeutic target for TCa.

Conclusions

Altogether there results demonstrated that microRNA-198 is remarkably decreased in TCa tissues and cell lines, which can inhibit the malignant progression of TCa *via* modulating H3F3A. Meanwhile, microRNA-198 is remarkably correlated with pathological staging, tumor size, lymph node metastasis, and poor prognosis of thyroid carcinoma patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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