Effect of miR-205 on proliferation and migration of thyroid cancer cells by targeting CCNB2 and the mechanism

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Abstract. This study explored the target of miR-205 and the effect of miR-205 on the proliferation and migration regulating its target in thyroid cancer cells (TC). Twenty-five pairs of TC and adjacent tissues were collected after surgical resection. Real-time fluorescence quantitative PCR (qRT-PCR) was used to detect the expression of miR-205 in TC tissues and cells (SW579, B-CPAP, TPC-1, WRO). SW579 cells were transfected with miR-205 mimic, and SW579 cells with overexpression of miR-205 were constructed. The effects of miR-205 overexpression on the proliferation and migration of SW579 cells were observed by cell counting kit-8 (CCK-8) and Transwell assays, respectively. Luciferase reporter assay was further used to look for the target of miR-205 and to study the mechanism of miR-205 in the proliferation and migration of TC cells. Compared with normal tissues and cells, the expression of miR-205 was significantly reduced in TC tissues (t=3.47, P=0.031) and cells (t=5.41, P=0.016). Overexpression of miR-205 inhibited the proliferation (t=4.12, P=0.035) and migration (t=4.47, P=0.027) of SW579 cells. Luciferase reporter assays found that CCNB2 was a target gene of miR-205 (t=4.63, P=0.024), qRT-PCR and western blot assays confirmed there was negatively correlation between CCNB2 and miR-205 (t=3.55, P=0.029; t=2.86, P=0.043). CCNB2 overexpression reversed the inhibition of miR-205 on the proliferation (t=3.70, P=0.031) and migration (t=4.12, P=0.022) of SW579 cells. In conclusion, miR-205 inhibits the proliferation and migration of TC cells by targeting CCNB2, which may be a potential target of TC therapy.

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Introduction

Thyroid cancer (TC), as a common malignant thyroid tumor, can be divided into papilla carcinoma, follicular carcinoma, myeloid carcinoma and undifferentiated carcinoma through its pathological classification. According to Chinese scholars, the incidence of TC ranks seventh among female malignant tumors. Most TC patients have no obvious symptoms, the most common manifestation is thyroid nodules, often found in physical examination, and a very small number of patients with cervical lymph node enlargement (1). In recent years, TC treatments have made significant progress, such as resection of thyroid, thyroid stimulating hormone suppression therapy and drug targeting therapy, radiation therapy, have been proven beneficial for patients with TC treatments (2), but its incidence is still rising steadily year by year, as there is no clear molecular explantion for the pathogenesis of TC. Therefore, it is very important for the treatment of TC to explore the pathogenesis of TC from the molecular mechanism perspective.

microRNA (miRNA) is a small molecule intrinsic non-coding RNA. In recent 10 years, research on miRNA in cell differentiation, proliferation and apoptosis has been paid increased attention. miRNA, as a post-transcriptional regulator, is involved in the regulation of gene deletion, mutation or amplification of various mRNAs, which may lead to misexpression, which is related to the occurrence and development of many diseases (3). miRNA, as a tumor inhibitor or promoter, is widely involved in the occurrence and development of many malignant tumors (4). For example, Wang *et al* suggested that miR-205 can be used as a tumor inhibitor in triple-negative breast cancer, which can inhibit the growth, migration and invasion of cancer cells by targeting HMGB1-RAGE pathway (5). Wu *et al* (6) found that miR-501-3p is misexpressed in colorectal cancer. miR-501-3p may be used as a new type of miRNA to promote the development of colorectal cancer by targeting the regulation of APC to promote the development of colorectal cancer. miR-205 is generally used as a tumor inhibitor, has low expression in tumors and affects the occurrence and development of various malignant tumors (7-9). Yang *et al* (10) reported that miR-205 negative regulation of PAR2 promotes invasion and metastasis of colorectal cancer. Pang and Yue (11) found that the

low expression of miR-205 in cervical cancer was related to the degree of tumor differentiation and clinical stage. IGF1R was targeted to inhibit the invasion and metastasis of cervical cancer. Although some progress has been made in tumor research, the role and mechanism of miR-205 in tumors, especially the mechanism of miR-205 in TC, the effect of miR-205 on the proliferation and migration of TC cells has not been reported, and the relationship between miR-205 and TC is still unclear and needs to be further studied.

This study explored the expression level of miR-205 in TC, and its effect on the proliferation and migration of TC cells and its regulatory mechanism, so as to provide a theoretical basis for explaining the pathogenesis and molecular therapy of TC.

Materials and methods

Tissue specimens. The 25 pairs of TC and paracancer tissues collected in this study were tissue specimens from January 2017 to December 2018, removed after surgical treatment in the department of breast and breast surgery in The Second Affiliated Hospital of Qiqihar Medical University (Qiqihar, China). Among them, 15 were female and 10 were male, with an average age of 42.25±8.73 years. Inclusion criteria: The specimens received surgical treatment in the hospital, and were confirmed to be TC by histopathological examination, and all were primary tumor lesions. Exclusion criteria: Patients who had received preoperative radiation and chemotherapy, patients who had secondary surgery, and patients with other site metastasis. The adjacent tissues were 1-2 cm away from the tumor, and were non-tumor tissues by histopathological examination. Each specimen was stored in liquid nitrogen for 10 min *in vitro* for subsequent analysis. All patients and their families agreed to participate in the experiment and signed the informed consent form. This study was approved by the Ethics Committee of The Second Affiliated Hospital of Qiqihar Medical University.

Cell lines and cell culture. The cell lines (SW579, B-CPAP, TPC-1, WRO, Htori-3) were from the Cell bank of the Chinese Academy of Sciences (Shanghai). The cell lines were cultured with DMEM (Corning, Inc.) culture, and 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) and 1% streptomycin (Corning, Inc.) were added. The cell culture conditions were: CO_2 concentration 5% at 37˚C.

Real-time fluorescence qRT-PCR. Total RNA was extracted by TRIzol kit (Shanghai Pufei). The concentration and quality of RNA were determined by spectrophotometer, and cDNA was obtained by reverse transcription. Specific reaction system (20 μl): The total RNA was 2 μg, 2X miRNA RT Buffer 10 μl, miRNA RT Enzyme Mix 2 μl, and RNase-Free H_2O was added to 20 μl. U6 (Gene) was the internal reference gene, according to the fluorescence quantitative PCR specification (Takara). Establishment of PCR reaction system: SYBR Premix Ex Taq (Takara) 10 μl, forward primer 0.4 μl, reverse primer 0.4 μl, cDNA 2 μl, sterilized distilled water 7.2 μl, reaction conditions: Pre-denaturation at 95˚C for 30 sec, pre-denaturation at 95˚C for 5 sec, at 60˚C for 30 sec, 40 cycles, the relative quantitative analysis of mRNA was carried out using the method of 2-Thiophans (Table I).

Table I. Primer sequences.

Primer		Primer sequences
CCNB2	F:	5'-CAACCCACCAAAACAACA-3'
	R:	5'-AGAGCAAGGCATCAGAAA-3'
miR-205	F:	5'-GCTCCTTCATTCCACCGG-3'
	R:	5'-CAGTGCAGGGTCCGAGGT-3'
U6	F:	5'-CTCGCTTCGGCACAGT-3'
	R:	5'-ACGCTTCACGATTGCT-3'

F, forward; R, reverse.

Cell transfection and group. miR-205 mimic (5'-UCCUUCA UUCCACCGGAGUCUG-3') and CCNB2 overexpression plasmid (5'-CCGUUUCCCAGACUACCUU-3') were from Guangzhou Ruibo Biology Company and inoculated into 6-well culture plate (Corning, Inc.). The cells were transferred to 50-70% of the cells. According to the specification of Lipofectamine® 3000 transfection kit (Invitrogen; Thermo Fisher Scientific, Inc.), the 10 μl transfection reagent was diluted with 250 μl serum-free medium and added to each well of the culture plate for 48 h for subsequent experiments. The TC cell line model of miR-205 overexpression was constructed by transferring miR-205 mimic, into the cell line, that is, miR-205 mimic group, and SW579 cells were transferred into negative virus (5'-TCTCCGAACGTGTCACGT-3'), which was the negative control (NC).

Cell counting kit-8 (CCK-8). The logarithmic cells were digested by trypsin to make cell suspension. Cells (2,000) were inoculated into a 96-well plate, one group for every 5 compound wells. CCK-8 solution (Corning, Inc.) Ten microliters was added to each well and incubated for 1 h. The absorbance of 450 nm was measured by enzyme labeling instrument (Tecan Infinite) on the 1st day, 2nd day, 3rd day, 4th day and 5th day, respectively.

Transwell transfer experiment. A 24-well plate was placed in a Transwell chamber (Corning, Inc.), and 100 μl of serum-free medium was added to the upper layer to prepare a serum-free cell suspension (concentration of 2 to 105/ml) and a 600 μl of 30% FBS-containing medium was added to the lower layer and incubated for 24 h at 37˚C in an incubator. The chamber was carefully removed, 4% paraformaldehyde was fixed at room temperature for 30 min, and stained with 0.1% crystal purple (Shanghai Health and Industry Co., Ltd.) at room temperature for 10 min. The solution was cleaned and placed under a microscope (Olympus) after drying.

Double luciferase test. Target prediction database TargetScan (http://www. Targetscan.org), DIANA (http://athena-innovation.gr) and MiRDB (http://mirdb.org) were used to predict the target gene of miR-205. Double luciferase kit (Promega) was used to detect wild-type and mutant CCNB2, which were constructed by Shanghai Jikai Gene Co., Ltd. The logarithmic SW579 cells were inoculated into a 96-well plate. Twenty-four hours later, both miR-205 mimic group and NC group

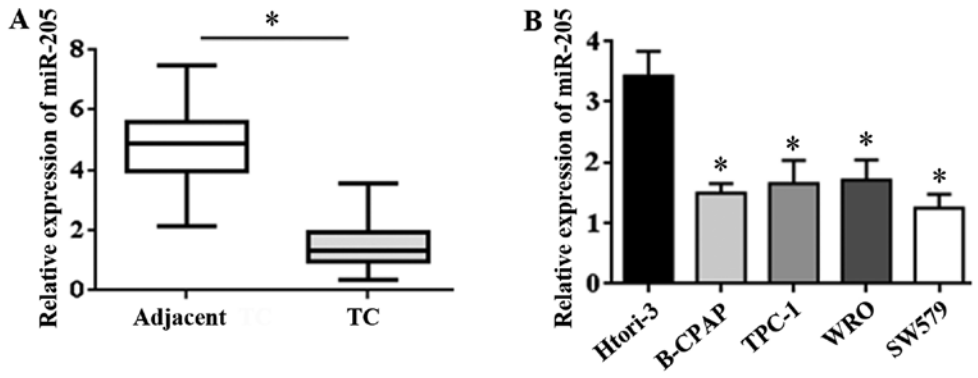

Figure 1. Expression of miR-205 in TC tissues and cells. (A) Expression of miR-205 in TC was detected by qRT-PCR, where TC is thyroid carcinoma and adjacent is paracancerous tissue. (B) qRT-PCR was used to detect the expression of miR-205 in TC cells, and (B) qRT-PCR was used to detect the expression of miR-205 in TC cells. TC, thyroid cancer. *P<0.05.

were transfected with wild-type and mutant CCNB23'UTR plasmid, and the fluorescence intensity was detected by fluorescence detector (Glomax20/20; Promega). Fluorescein detection reagent II (100 μl) was placed in 1.5 ml centrifuge tube. A bioluminescence detector (GloMax) was used to read the luciferase activity Firely luciferase (FLUC) of firefly by pre-reading for 2 sec, detecting 10 sec per well, adding 20 μl cell lysate, and fully mixing it with 20 μl cell lysate. Then 1X Stop&Glo preparation 100 μl was added, fully mixed, to read the luciferase activity *Renilla* luciferase (RLUC) of sea kidney on the luminous instrument. The relative fluorescence intensity was calculated by the ratio of RLUC/FLUC.

Western blot. Logarithmic cells were collected so that the lysate was in contact with the cells. The total cell protein was extracted. After the protein concentration was detected by BCA method (Biyuntian), the gel was made. The protein was separated by SDS-PAGE electrophoresis at 4˚C and 300 mA constant current for 90 min. the protein was transferred to PVDF membrane (Millipore) by wet transfer method. PVDF membrane was left at room temperature with 5% skim milk for 1 h. Then the diluted first antibody (mouse anti-human CCNB2 monoclonal antibody, Abcam, ab18250, dilution ratio 1200; mouse anti-human GAPDH, SC-32233; Santa Cruz Biotechnology, Inc.) was incubated for 1 h, and the second antibody (sheep anti-rabbit IgG; Santa Cruz Biotechnology, Inc., sc-2004, dilution ratio 1-2,000) was incubated. The chemiluminescence solution containing ECL substrate (Thermo Fisher Scientific, Inc.) was added to PVDF film and exposed to GelPro Analyzer (Media Cybernetics, Silver Spring) for band analysis.

Statistical analysis. All the data were analyzed by SPSS 24 (IBM, Corp.) and GraphPad 5.0 (GraphPad Software, Inc.). The data were obtained from more than three independent experiments and expressed as mean ± standard deviation. t-test was used to compare the measurement data, and the Chi-square test is used to compare the counting data. P<0.05 was considered as statistically significant.

Results

Expression of miR-205 in TC tissues and cells. The expression of miR-205 in the tissue was detected by qRT-PCR. The results showed that the expression of miR-205 in the cancer tissues was significantly lower than that in the adjacent tissues (t=3.47, P=0.031) (Fig. 1A). The expression of miR-205 in 4 TC cell lines (SW579, B-CPAP, TPC-1, WRO) and 1 human normal thyroid cell line Htori-3 was detected by qRT-PCR assay. The results showed that the expression level of miR-205 in TC cell line was lower than that in the normal cell line (t=5.41, P=0.016) (Fig. 1B). The above results show that miR-205 is significantly downregulated in TC tissue and cells.

Overexpression of miR-205 affects the proliferation and migration of SW579 cells. Results of qRT-PCR test showed that expression of miR-205 in miR-205 mimic group was significantly higher than that in NC group (t=3.92, P=0.035) (Fig. 2A). It can be concluded that the SW579 cell model with overexpression of miR-205 was successfully constructed.

The results of CCK-8 assay showed that in SW579 cells, compared with NC group, the proliferation ability of miR-205 mimic group was significantly weakened (t=4.12, P=0.035) (Fig. 2B). The results of Transwell migration test showed that the cell mobility in miR-205 mimic group was significantly lower than that in NC group (t=4.47, P=0.027) (Fig. 2C and D). These results showed that overexpression of miR-205 inhibited the proliferation and migration of SW579 cells.

Target relationship between CCNB2 and miR-205. miR-205 can be combined with CCNB23'UTR through target prediction database TargetScan, DIANA and MiRDB, prediction miR-205. Double luciferase assay was used to further verify that the wild-type and mutant sequences of CCNB2 combined with miR-205 as shown in Fig. 3A. Overexpression of miR-205 significantly decreased the fluorescence activity of wild-type 3'UTR containing CCNB2, but had no significant effect on the fluorescence activity of 3'UTR containing CCNB2 mutant (t=4.63, P=0.024) (Fig. 3B). The results of western blot and qRT-PCR experiments show that after upregulation of miR-205 in SW579 cells, CCNB2 expression level decreased significantly compared with NC group (t=3.55, P=0.029; T=2.86, P=0.043) (Fig. 3C-E). Expression of miR205 and CCNB2 may be negatively regulated. Therefore, this result confirms that CCNB2 is the downstream target of miR-205.

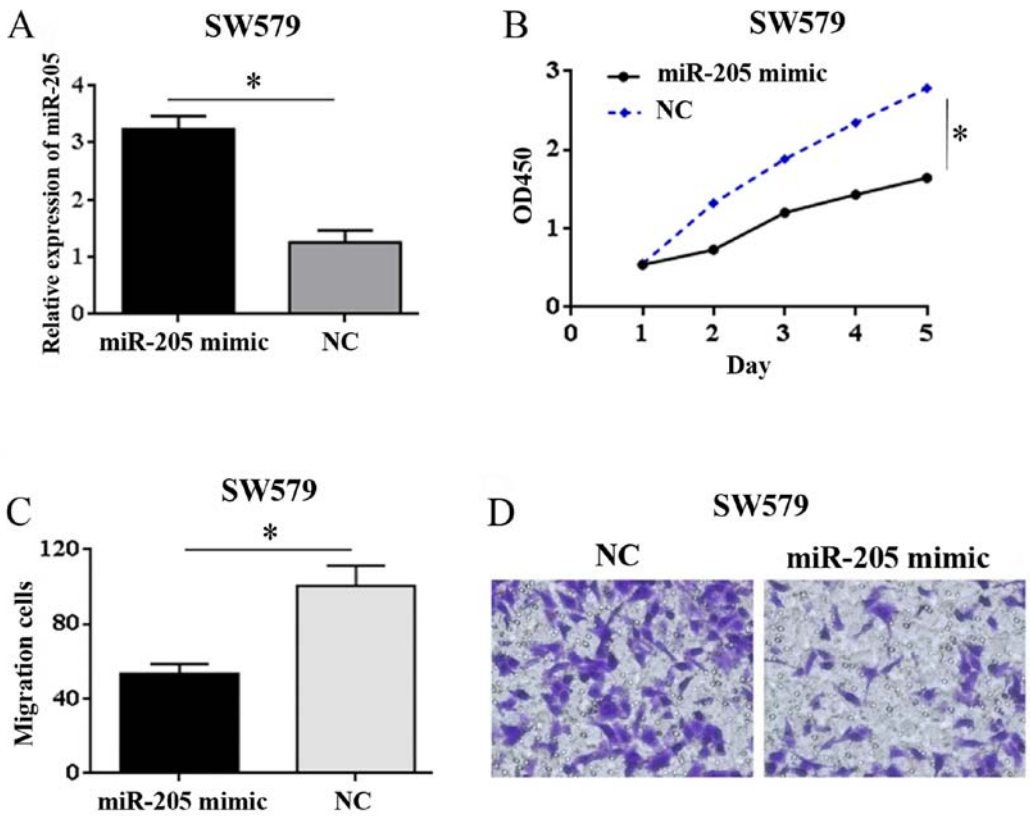

Figure 2. Effect of overexpression of miR-205 on proliferation and migration of SW579 cells. (A) qRT-PCR assay was used to detect the expression of miR-205 in each group. NC, negative control group, *P<0.05. (B) CCK-8 experiment observed the effect of miR-205 overexpression on the proliferation of SW579 cells. NC, negative control group, *P<0.05. (C and D) Transwell experiment observed the effect of miR-205 overexpression on the migration of SW579 cells. NC, negative control group, (D) is a morphological graph, and *P<0.05. TC, thyroid cancer; CCK-8, cell counting kit-8.

Effect of upregulation of CCNB2 on proliferation and migration of SW579 cells regulated by miR-205. In order to further explore whether miR-205 regulates the proliferation and migration of TC cells by acting on its downstream target CCNB2, this study continued to transfect SW579 cells with CCNB2 overexpressed plasmid, and used CCK-8 and Transwell experiments to observe whether the upregulation of CCNB2 could reverse the proliferation and migration of TC cells caused by miR-205. CCK-8 experimental results showed that in SW579 cells, the proliferation capacity of miR-205 mimic+CCNB2 group was significantly higher than that of miR-205 mimic group (t=3.70, P=0.031), while there was no significant difference compared with NC group (t=1.54, P=0.122) (Fig. 4A). The results of Transwell assay showed that the cell migration ability of miR-205 mimic CCNB2 group was significantly higher than that of miR-205 mimic group (t=4.12, P=0.022), but there was no significant difference between miR-205 mimic CCNB2 group and NC group (t=0.39, P=0.765) (Fig. 4B and C). These results suggest that upregulation of CCNB2 can reverse the inhibitory effect of miR-205 on the proliferation and migration of TC cells.

Discussion

TC is a common malignant tumor in clinic. Although most of TC are differentiated tumors and the malignant degree is low, the incidence of TC has shown an upward trend in recent years. Therefore, it is of great significance to explore the molecules that can affect the occurrence of TC. miRNA is a kind of non-coding RNA, non-coding protein, which is often used as upstream regulatory molecule to affect the biological process of many tumors and plays an important role in the occurrence and development of tumors (12-14). In TC, many studies have also reported that miRNA is involved in the regulation of its biological process. Jiao *et al* (15) found that ZEB1 overexpression reverses the inhibitory effect of miR-873 overexpression on the proliferation and invasion of TC cells, and miR-873 may play a tumor inhibitory role in the development of TC by inhibiting ZEB1. Guo and Zhang (16) suggest that miR-30a plays a role in inhibiting tumor growth in TC by directly targeting the E2F7 gene. miR-30a may be a new therapeutic target for TC. miR-205 is misexpressed in many tumors, Dai *et al* (17) reported that miR-205 is often underexpressed in glioma tissues. miR-205 can inhibit the growth, invasion and reverse the EMT process of glioma by downregulating its target gene HOXD9. In pancreatic cancer, the expression of miR-205 is also downregulated. miR-205 can inhibit the proliferation and migration of tumor cells by targeting RUNX2 in pancreatic cancer (18). Lu *et al* (19) confirmed that the expression of miR-205 in HCC cells decreased, which could inhibit the migration and invasion of HCC cells. miR-205 may become a therapeutic target for HCC. However, the expression and role of miR-205 in TC has not been reported. In this study, by detecting the expression of miR-205 in TC and paracancerous tissues, we found that the expression of miR-205 in cancer tissues was lower. Moreover, expression of miR-205 in cancer

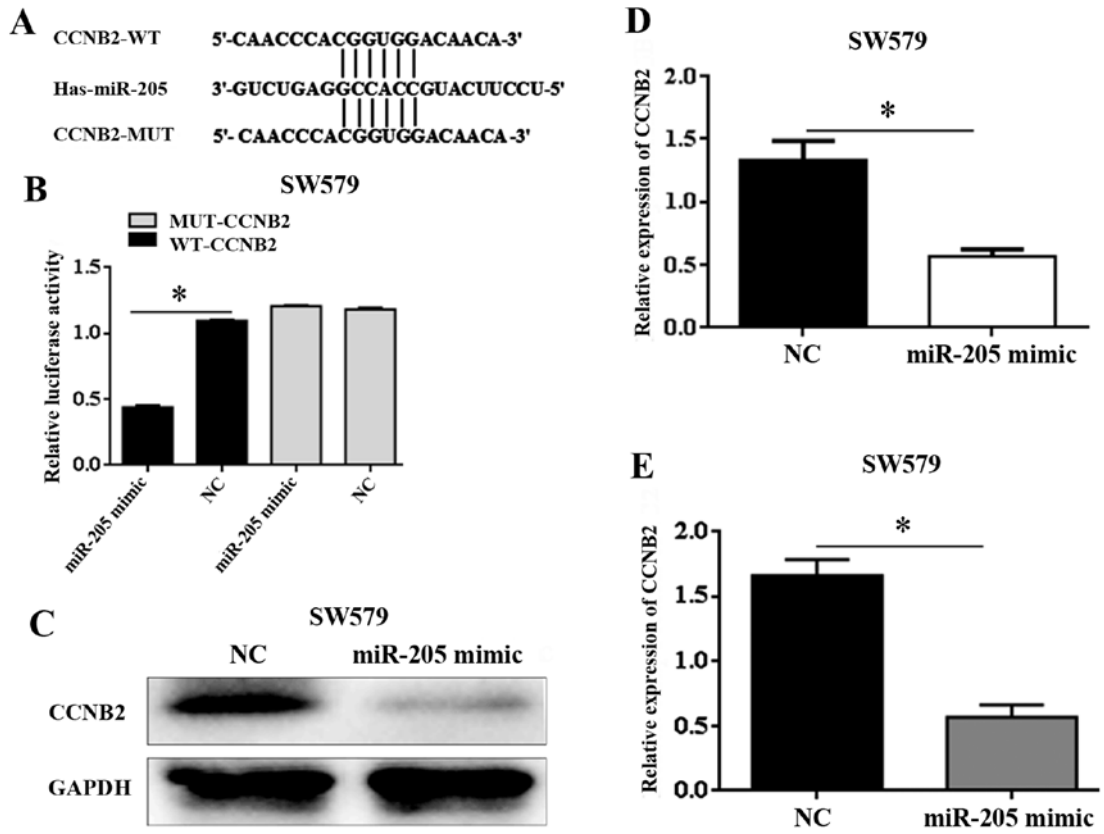

Figure 3. Target regulation relationship between miR-205 and CCNB2. (A) The binding site of CCNB2 to the miR-205. (B) The effect of miR-205 overexpression on the fluorescence activity of CCNB2 wild-type (WT-CCNB2) and mutant (MUT-CCNB2) 3'UTR was observed in the dual-luciferin reporter enzyme experiment. NC, negative control group, *P<0.05. (C and D) Western blot experiment verified the effect of upregulated miR-205 on the expression level of CCNB2, where (D) is the statistical analysis figure of protein band gray scale, NC, negative control group, and *P<0.05. (E) qRT-PCR experiment verified the effect of upregulation of miR-205 on the expression level of CCNB2. NC, negative control group, *P<0.05.

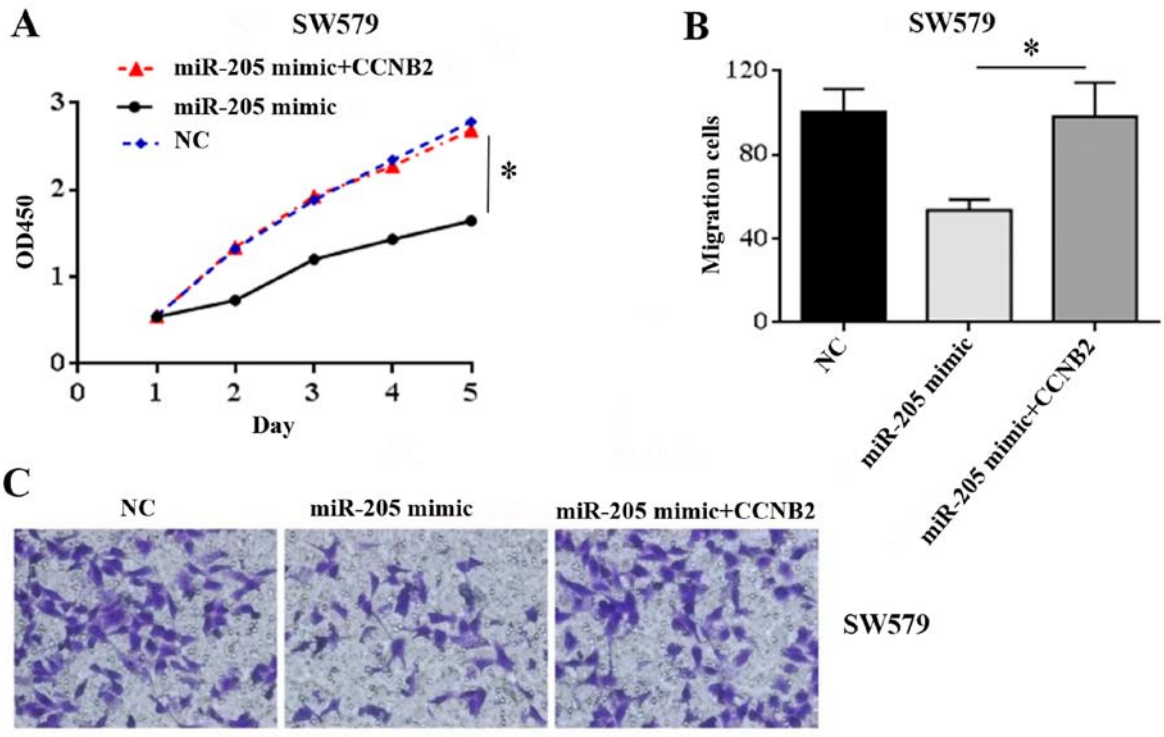

Figure 4. Effect of upregulation of CCNB2 on proliferation and migration of SW579 cells regulated by miR-205. (A) CCK-8 experiment was conducted to observe the effect of upregulated CCNB2 on miR-205 regulating the proliferation of SW579 cells, *P<0.05. (B and C) The effect of upregulation of CCNB2 on the migration of SW579 cells induced by miR-205 was observed by Transwell assay. NC, negative control group, where (C) is a morphological map, *P<0.05. CCK-8, cell counting kit-8.

cells was also confirmed in 4 TC cell lines (SW579, B-CPAP, TPC-1, WRO) and the normal thyroid cell line Htori-3, which suggested that miR-205 has low expression in TC tumors. It may be working as a tumor suppressor gene. In order to further study the effect of miR-205 on the proliferation and migration of TC cells, a TC cell line model overexpressing miR-205 was constructed in SW579 cells by miR-205 mimic to verify the effect of miR-205 on the biological function of SW579 cells. The results of CCK-8 and Transwell migration also confirmed that overexpression of miR-205 inhibited the proliferation and migration of SW579 cells. These results suggest that miR-205 may be involved in the regulation of cell proliferation and migration of TC as a tumor inhibitor in TC. This is also consistent with the results of Dai *et al* (17) and Lu *et al* (19), which fully indicates that miR-205 may inhibit the proliferation and migration of TC cells. Previous studies have shown that miR-205 is usually an upstream regulator. The studies of Dai *et al* (17) and Lu *et al* (19) also confirmed that miR-205 plays a role in tumor by regulating its target genes. However, the mechanism of miR-205 inhibiting the proliferation and migration of TC cells is not clear.

Therefore, this study continued to verify the target gene of miR-205 through database exploration and double fluorescein reporter enzyme assay, and reveal the mechanism of miR-205 regulating the biological function of TC cells. CCNB2 is an important member of the cell cycle family regulatory network. It can prevent damaged cells from entering mitotic phase and maintain the correct replication of genetic material and genome stability (20,21). The normal growth and development of cells need the organic cooperation of cell cycle family members, orderly regulation, and abnormal expression of CCNB2, may appear as cell cycle regulation disorder, also inducing cell malignant transformation. In recent years, CCNB2 abnormal expression in many malignant tumors has been used as downstream molecules, which affects the development process of tumors, such as non-small cell lung cancer, bladder cancer and gastric cancer (22-24). Shubbar *et al* (25) found that ccnb2 overexpression affects the prognosis of breast cancer patients. During follow-up, ccnb2 overexpression was found to be associated with poor prognosis. Li *et al* reported that CCNB2 is also a risk factor for the prognosis of HCC patients. CCNB2 can promote the proliferation and migration of HCC cells (26). In conclusion, CCNB2, as a member of the cell cycle family, plays an important role in the orderly regulation of the cell cycle. Previous studies have confirmed that CCNB2 may play a carcinogenic role in many malignant tumors. In this study, it was found by public database prediction analysis that miR-205 binds to CCNB2 3'UTR. Therefore, the relationship between the expression of miR-205 and CCNB2 in TC cells and targeted regulation were also explored. In this study, the results of double luciferase assay confirmed that CCNB2 may be a target gene of miR-205. QRT-PCR and western blot experiments also found that overexpression of miR-205 could inhibit the expression of CCNB2, and the expression of miR-205 was negatively correlated with the expression of CCNB2, which may be a negative regulatory relationship. Furthermore, it was found that the proliferation and migration ability of SW579 cells in miR-205 mimic CCNB2 group was significantly higher than that in miR-205 mimic group, but there was no significant difference compared with NC group, which indicated that the upregulation of CCNB2 expression could counteract the inhibitory effect of miR-205 mimic on the proliferation and migration of TC cells. Based on the above results, it can be suggested that miR-205 may play a role in the proliferation and migration of TC cells by targeting the regulation of CCNB2.

In conclusion, this study found that miR-205 was differentially expressed in TC tissues and cells, and miR-205 inhibits the proliferation and migration of TC cells. Further study on the mechanism showed that miR-205 regulated the proliferation and migration of TC cells by inhibiting expression of CCNB2, which provided a theoretical basis for exploring the pathogenesis of TC and a new target for the treatment of TC.

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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

XW wrote the manuscript. XW, YL, HZ, KJ, CZ and HL conceived and designed the study. XW, YL, HZ, KJ, CZ, QM, ZW and CF were responsible for the collection and analysis. XW, YL, HZ, KJ, CZ, HL and QM interpreted the data and drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of The Second Affiliated Hospital of Qiqihar Medical University (Qiqihar, China). All patients and their families agreed to participate in the experiment and signed the informed consent form.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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