

miR-205 targets YAP1 and inhibits proliferation and invasion in thyroid cancer cells

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Abstract. MicroRNA-205 (miR-205) has been reported to be downregulated, and serves critical roles in the pathogenesis and progression of several types of cancer, including breast, prostate and lung cancer. However, the underlying mechanism of miR-205 in thyroid cancer remains unclear. In the present study, it was demonstrated that the expression of miR-205 was reduced in thyroid cancer tissues compared with non-cancer tissues. In addition, miR-205-knockdown models in the BHT-101 cell line and ectopic expression models in the 8505-C cell line were used to measure the biological functions of miR-205. The results indicated that miR-205 inhibited certain aspects of thyroid cancer, including cell proliferation, migration and invasion. Furthermore, Yes-associated protein 1 (YAP1) was identified as a target gene of miR-205 and its expression was negatively correlated with that of miR-205 in thyroid cancer tissues. Depletion of YAP1 partially reduced the anti-miR-205-induced cell growth and invasion. The results of the present study suggested that the tumor suppressive functions of miR-205 via targeting YAP1 could be a novel target for the treatment of thyroid cancer.

Introduction

Thyroid cancer is the most common type of tumor of the endocrine system with a rising morbidity rate of >5% annually (1,2). Papillary thyroid carcinoma is the predominant subtype, accounting for ~70% of all thyroid cancer cases and the overall chance of survival is favorable (3). However, patients with poorly differentiated thyroid cancer suffer a poorer prognosis (4). Therefore, urgent investigation into novel agents and treatments for this malignant disease is required. There have been extensive studies on the molecular alterations in thyroid carcinoma and the biomarkers associated with

tumor progression, including telomerase reverse transcriptase, transcription factor SOX2 (SOX2) and zinc finger protein SNAI1 (5-7). Recently comprehensive genetic characterization has been reported, and genome mutations and abnormalities have been demonstrated (8).

MicroRNA (miRs) are comprised of small endogenous noncoding RNAs of 20-30 nucleotides in length (9). miRs may regulate target gene expression by complementary binding to the 3' untranslated region (3'-UTR) of mRNAs, leading to repression of translation and inhibition of protein activity. Recently, studies demonstrated that miRs were aberrantly expressed in tumors, including thyroid cancer, and functioned in pro-oncogenic or tumor suppression roles (10). A number of miRs, including miR-187, let-7, miR-146 and miR-222, have been consistently identified to be deregulated in thyroid cancer (11,12). These results indicate that investigating the association between miRs and thyroid cancer is critical for an improved understanding of tumorigenesis.

miR-205 is reported to be deregulated in several types of tumors, including breast, prostate and lung cancer (13-15). However, its role in thyroid carcinoma remains unknown. In the present study, it was demonstrated that miR-205 was critical in regulating the proliferation, migration and invasion of thyroid carcinoma cells. Additionally, miR-205 was downregulated in thyroid carcinoma tissues and the same observation was demonstrated in other studies. Further study of the mechanism of action revealed that miR-205 inhibited thyroid carcinoma tumorigenesis by targeting YAP1 expression.

Materials and methods

Human tissue specimens. A total of 132 paired thyroid carcinoma and non-tumor tissues were obtained from patients who underwent surgery at the Department of Thyroid Surgery at the Shanxi Provincial People's Hospital (Taiyuan, China) between January 2005 and June 2010. The clinicopathological features are demonstrated in Table I. All tissues samples were immediately frozen in liquid nitrogen and stored at -80°C prior to use. The present study was approved by the Ethics Committee of Shanxi Provincial People's Hospital. Written informed consent was obtained from all patients.

Cell culture and RNA oligonucleotide transfection. The human thyroid cancer cell lines 8505-C (cat. no. SCSP-540),

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BCPAP (cat. no. SCSP-543) and BHT-101 (cat. no. SCSP-544) were obtained from the Cell Bank at the Chinese Academy of Sciences (Shanghai, China), and TPC-1 was from the American Type Culture Collection (Manassas, VA, USA). Human embryonic kidney 293T (HEK293T) cells (ATCC cat. no. CRL-11268) cells were cultured in our laboratory. Cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. For overexpression or knockdown of miR-205, cells were seeded into 6-well plates and transfected with an miR-205 mimic (5'-UCCUUC AUUCCACCGGAGUCUG-3'), normal control (NC) or miR-205 antagonist (anti-miR-205; 5'-CAG ACUCCGGUGGAAUGAA'-3') at 5 µM concentration using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). cells were used 48 h following transfection. For inhibiting endogenous YAP1 expression, small interfering RNA targeting YAP1 (CCGUUCCAGACUACCUU) was purchased from Chang Jing Bio-Tech, Ltd. (Changsha, China) and was transfected at 5 µM concentration to the cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and the cells cultured for the next 48 h.

RNA extraction and reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells or human tissues by TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The first-strand cDNA was synthesized using the PrimeScript™ RT reagents kit (Takara Biotechnology Co., Ltd., Dalian, China). qPCR was performed using SYBR-Green method as described previously (16). YAP1 was normalized to the expression of GAPDH and miR-205 was normalized to the small nuclear (sn)RNA U6. The gene expression was quantified using the 2^{-ΔΔC_q} method (17). Primers used were as followed: YAP1 forward, 5'-TAGCCCTGCGTAGCCAGTTA-3' and reverse, 5'-TCATGCTTAGTCCACTGTCTGT-3'; snRNA U6 forward, CTCGCTTCGGCAGCACA and reverse, AACGCTTCACGAATTTGCGT and GAPDH forward, 5'-ACAACCTTGGTATCGTGGAAGG-3' and reverse, 5'-GCCATCAGCCACAGTTTC-3'.

Western blotting. Cells were lysed using the radioimmuno-precipitation assay buffer (Thermo Fisher Scientific, Inc.) containing a protease inhibitor and phosphatase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). Total protein concentration was measured by the bicinchoninic acid assay method. Equal amounts of protein (60 µg) were separated by 10% SDS-PAGE and then transferred to nitro-cellulose membranes. Following blocking with bovine serum albumin (Wuhan Boster Biological Technology, Ltd., Wuhan, China) for 1 h at room temperature, the membranes were incubated with specific primary antibodies at 4°C overnight. Then the membranes were incubated with the secondary antibody (HRP conjugated mouse anti-rabbit antibody; sc-2357; 1:5,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at room temperature for 1 h and visualized by enhanced chemiluminescence method (Pierce; Thermo Fisher Scientific,

Table I. Clinicopathological features of 132 patients with thyroid cancer.

Clinicopathological features	Value
Median age, years (range)	49 (22-78)
Gender, n	
Male	45
Female	87
Age, n	
>35 years-old	41
≤35 years-old	91
T classification, n	
T1-T2	109
T3-T4	23
N classification, n	
N0	52
N1-N2	80
Stage, n	
I-II	89
III-IV	43
Histological type, n	
Papillary thyroid carcinoma	96
Undifferentiated thyroid carcinoma	36
T, tumor; N, node.	

Inc.). GAPDH was used as internal control. The primary antibodies used were as followed: Anti-YAP1 (cat. no. ab52771; 1:1,000), anti-Translin-associated zinc finger protein 1 (TAZ; cat. no. ab110239; 1:1,000) and anti-Bcl2-like protein 1 (Bcl-xl; cat. no. ab32370; 1:1,000; all Abcam, Cambridge, MA, USA) and anti-GAPDH (cat. no. sc-365062; 1:2,000; Santa Cruz Biotechnology, Inc.).

Predicting the miRNA target. For exploring the miRNAs targeting YAP1, Targetscan (http://www.targetscan.org/vert_71/) was used. By typing the gene symbol, the database could give the miRNAs which could potentially bind to the target.

Dual-luciferase reporter assay. Luciferase reporter vectors of wild-type (WT) and mutant (MUT) YAP1 3'-UTR were synthesized. The sequence region was cloned into the pMIR-REPORT miR vector (Ambion; Thermo Fisher Scientific, Inc.) and the open reading frame of firefly luciferase. For the Dual-luciferase assay, 293T cells were seeded in 6-well plate (1×10⁵ cells/well) and transfected with the above vectors and co-transfected with miR-205 or control using Lipofectamine RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.). Following 24 h culturing, cells were harvested and the firefly and *Renilla* luciferase activities were detected by Dual-Glo luciferase assay kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol.

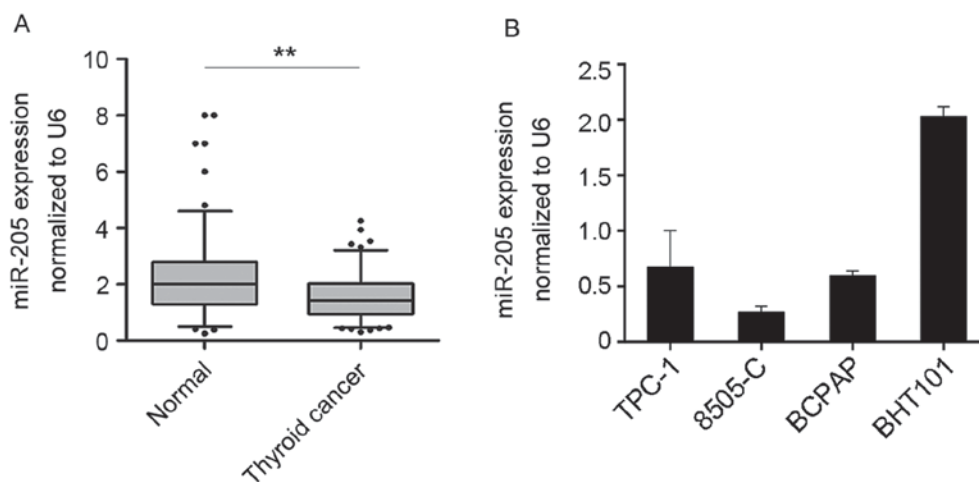


Figure 1. miR-205 is downregulated in human thyroid cancer tissues and cell lines. (A) The expression of miR-205 was detected using the reverse transcriptase-quantitative polymerase chain reaction in thyroid cancer and adjacent non-cancer tissues, and the data were presented graphically using a box and whisker plot. The box represents the upper and lower quartiles and median; the whiskers indicate the 5-95% data points. The external points represent the data out of the 5-95% range. A Mann-Whitney-U test was used to compare the expression of miR-205 in two groups. (B) The expression of miR-205 in thyroid cancer cell lines. ** $P < 0.01$, *** $P < 0.001$. miR, microRNA.

Cell proliferation and colony formation assay. Cell proliferation was measured using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Cells were seeded in 96-well plates at a concentration of 2,000 cells/well. The proliferation rates were recorded at 24, 48, 72 and 96 h at 450 nm following the manufacturer's protocol. For the colony formation assay, 1,000 cells were seeded in 6-wells plates and cultured for 2 weeks. Then cells were fixed with 4% paraformaldehyde and stained with crystal violet at room temperature for 30 min. Colony numbers were calculated under a phase contrast microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Wound healing assay. Cells were cultured in a 6-well plate to form a monolayer. The wounds were scratched using a sterile 200 μ l tip/well and cells were cultured in low serum media (1% fetal bovine serum; FBS). The wound distance was measured at the times of 0 and 72 h. Representative images were taken by phase contrast microphotography.

Matrigel invasion assay. A total of 10^5 cells in serum-free medium were seeded into the upper chamber of a Transwell insert (8- μ m pore size) coated with Matrigel® (BD Biosciences, Franklin Lakes, NJ, USA). Then media containing 15% FBS were added to the lower chamber and following 48 h of culture, the cells remaining on the upper membrane were removed and cells that invaded to the lower membrane were fixed in 4% paraformaldehyde at room temperature for 10 min and stained with 0.1% crystal violet at room temperature for 10 min and imaged. The cell numbers were calculated under an upright metallurgical microscope (Leica Microsystems GmbH) at 100x magnification.

Statistical analysis. *In vitro* results were presented as the mean \pm standard error of the mean from at least three independent experiments. Mann-Whitney-U test, Spearman rank correlation coefficient test, Student's t test, analysis of variance

and multiple comparison between the groups using the S-N-K method were performed using SPSS software (version 21.0; IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-205 is repressed in human thyroid cancer tissues and cell lines. To investigate the expression of miR-205 in human thyroid cancer tissues, the expression profile of miR-205 in 132 paired thyroid cancer and non-tumor tissues was detected by RT-qPCR. The data demonstrated that the expression levels of miR-205 were significantly decreased in tumor tissues compared with non-tumor tissues ($P < 0.01$; Fig. 1A). In addition, a panel of thyroid cancer cell lines was tested, including TPC-1, 8505-C, BCPAP and BHT-101. The results indicated that miR-205 expression was relatively high in BHT101 cells (Fig. 1B). These data suggested that miR-205 may be involved in thyroid cancer development.

miR-205 inhibits proliferation and invasion of thyroid cancer cells. To investigate the biological functions of miR-205 in thyroid cancer, the expression of miR-205 was modulated using exogenous transfecting miR mimics (Fig. 2A) or inhibitors (Fig. 2B). The effect of miR-205 on cell growth using CCK-8 assays was then examined. As exhibited in Fig. 2C, the miR-205 overexpression group demonstrated significantly decreased proliferation compared with the control group at 96 h. Similarly, when miR-205 expression was knocked down, cell growth was increased (Fig. 2D). Also, the colony formation assay confirmed the observations (Fig. 2E and F) that miR-205 expression suppressed cell viability. Wound healing and Transwell assays were performed to evaluate the impact of miR-205 on the migratory and invasive capability of thyroid cancer cells. As illustrated in Fig. 2G and H, compared with the control group, overexpression of miR-205 decreased migratory and invasive. However, knocking down miR-205 expression

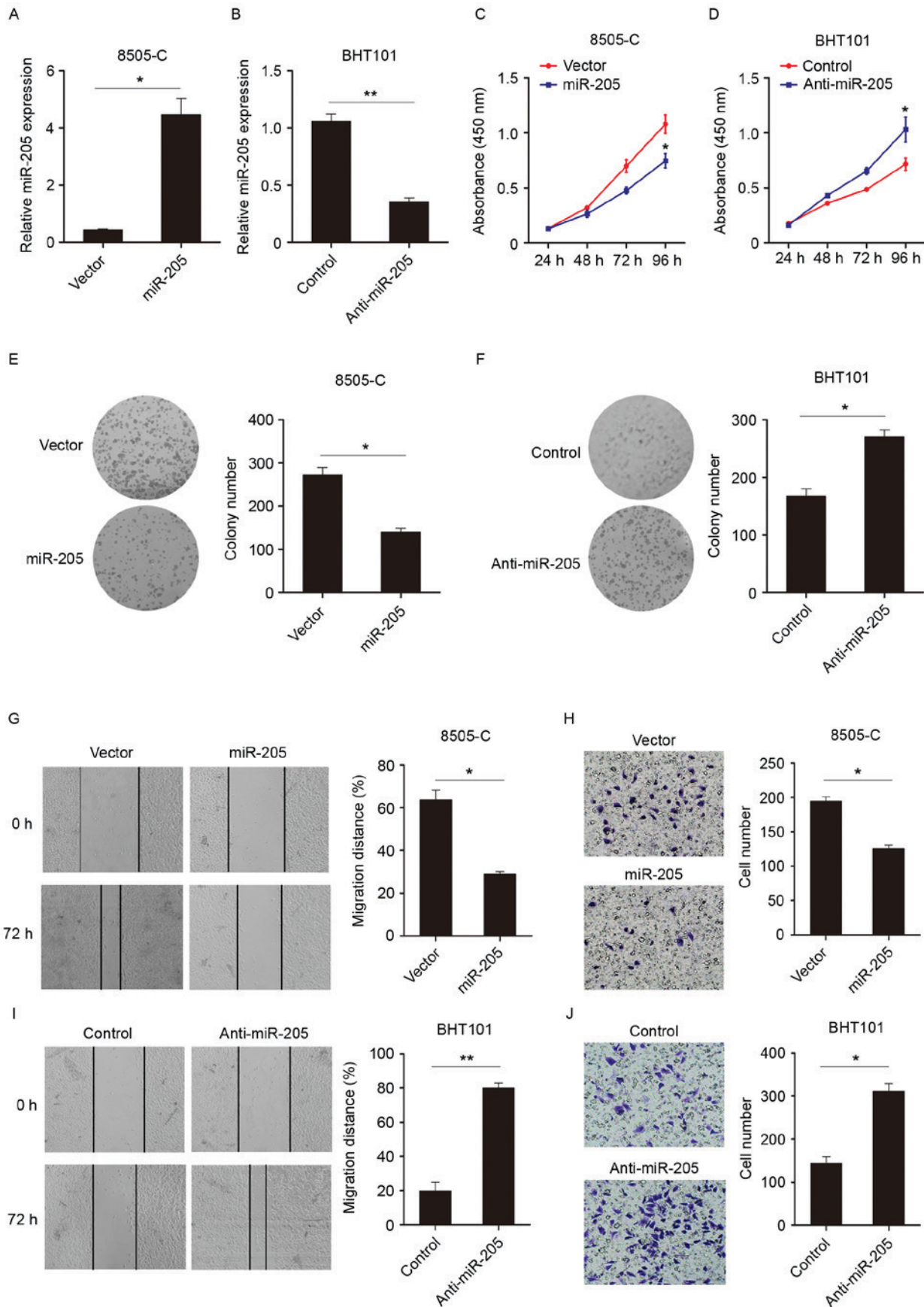


Figure 2. miR-205 suppresses growth, migration and invasion of thyroid cancer cells. The reverse transcriptase-quantitative polymerase chain reaction was performed to detect the (A) ectopic expression of miR-205 in 8505-C cells and (B) knockdown of miR-205 in BHT-101 cells. Cell proliferation assay in (C) 8505-C cells treated with the vector alone and miR-205, and (D) BHT101 cells treated with the control and anti-miR-205. Colony formation assay images and quantification of (E) 8505-C cells treated with the vector alone and miR-205, and (F) BHT101 cells treated with the control and anti-miR-205. miR-205 inhibited cell (G) migration and (H) invasion by wound healing assay and Transwell assay. Knocking down of miR-205 promoted (I) cell migration and (J) invasion. *P<0.05, **P<0.01. miR, microRNA.

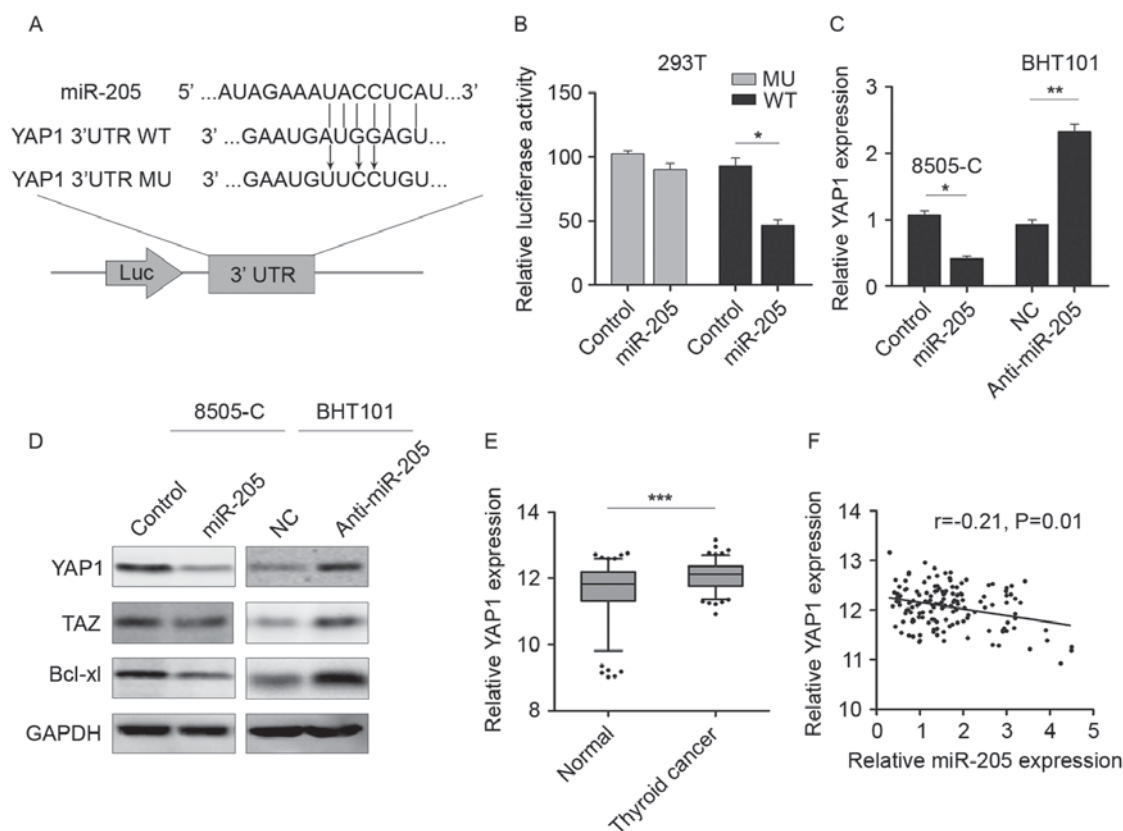


Figure 3. YAP1 is a direct target of miR-205. (A) Schematic of the binding sites of miR-205 in the 3'UTR of YAP1. (B) Luciferase reporter assay in 293T cells cotransfected with miR-205 and luciferase vector. (C) RT-qPCR was performed to detect the YAP1 expression following miR-205 overexpression or knockdown. (D) Western blotting was performed to detect the YAP1, TAZ and Bcl-x1 expression following miR-205 overexpression or knockdown. (E) RT-qPCR was performed to detect YAP1 expression in thyroid cancer tissues. The data were presented graphically using a box and whiskers plot. The box represents the upper and lower quartiles and the median; the whiskers indicate the 5-95% data points. The external points represent the data out of the 5-95% range. Mann-Whitney-U test was used to compare the expression of YAP1 in two groups. (F) Spearman's rank correlation coefficient analysis was performed to measure the correlation between the YAP1 and miR-205 in thyroid cancer tissues. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. YAP1, Yes associated protein 1; Taz, anti-Translin-associated zinc finger protein 1; miR, microRNA; Bcl-x1, Bcl2-like protein 1; NC, normal control; RT-qPCR, reverse transcriptase-quantitative polymerase chain reaction; UTR, untranslated region; Luc, luciferase.

promoted cell migration and invasion (Fig. 2I and J). These data suggested that miR-205 could suppress cell proliferation, migration and invasion.

YAP1 is a direct target of miR-205. The potential targets of miR-205 were further investigated by predicting the binding site using TargetScan and combined with the protein expression in GEO database (18). As demonstrated in Fig. 3A, miR-205 could bind to the 3'-UTR of YAP1. Therefore, luciferase reporter vectors containing WT and MU 3'-UTR were constructed and transfected into 293T cells together with miR-205 or NC. Following 48 h culture, the luciferase activity was significantly reduced in cells transfected with YAP1 WT 3'-UTR and miR-205, rather than with MU 3'-UTR (Fig. 3B). Then the expression of YAP1 was determined by RT-qPCR and western blotting. As demonstrated in Fig. 3C and D, the expression of YAP1 mRNA and protein was decreased in miR-205 mimic-transfected cells, as well as TAZ and Bcl-x1, which are considered to be downstream genes of YAP1 (19,20). Inhibition of miR-205 could restore YAP1 expression and activate the serine/threonine-protein kinase 4 (hippo) signaling pathway. The YAP1 level in thyroid cancer tissue was measured and the results demonstrated that YAP1 was

significantly upregulated in tumor tissues compared with the non-tumor tissues (Fig. 3E). As YAP1 was a potential target of miR-205, the correlation between YAP1 and miR-205 was also examined in thyroid cancer tissues. An inverse correlation between the expression of YAP1 and miR-205 ($r = -0.21$; $P = 0.01$; Fig. 3F) was demonstrated. These data revealed that YAP1 was a potential target of miR-205 in thyroid cancer.

YAP1 is involved in the inhibitory effects of miR-205. To further investigate the role of YAP1 in miR-205 mediated tumor suppression, RNA interference was used to knockdown YAP1 expression. As demonstrated in Fig. 4A, knockdown of miR-205 increased YAP1 and activated hippo signaling pathway to induce TAZ expression. When YAP1 was subsequently knocked down, the expression of TAZ was reduced. This result indicated that miR-205 suppressed the hippo signaling pathway by targeting YAP1. Further experiments demonstrated that knocking down YAP1 reversed the anti-miR-205-dependent effect on cell growth and invasion in thyroid cancer cells (Fig. 4B-D). These data revealed that downregulation of YAP1 could attenuate the protumorigenic effects of miR-205 inhibitors, suggesting that YAP1 is involved in miR-205-mediated tumor suppression.

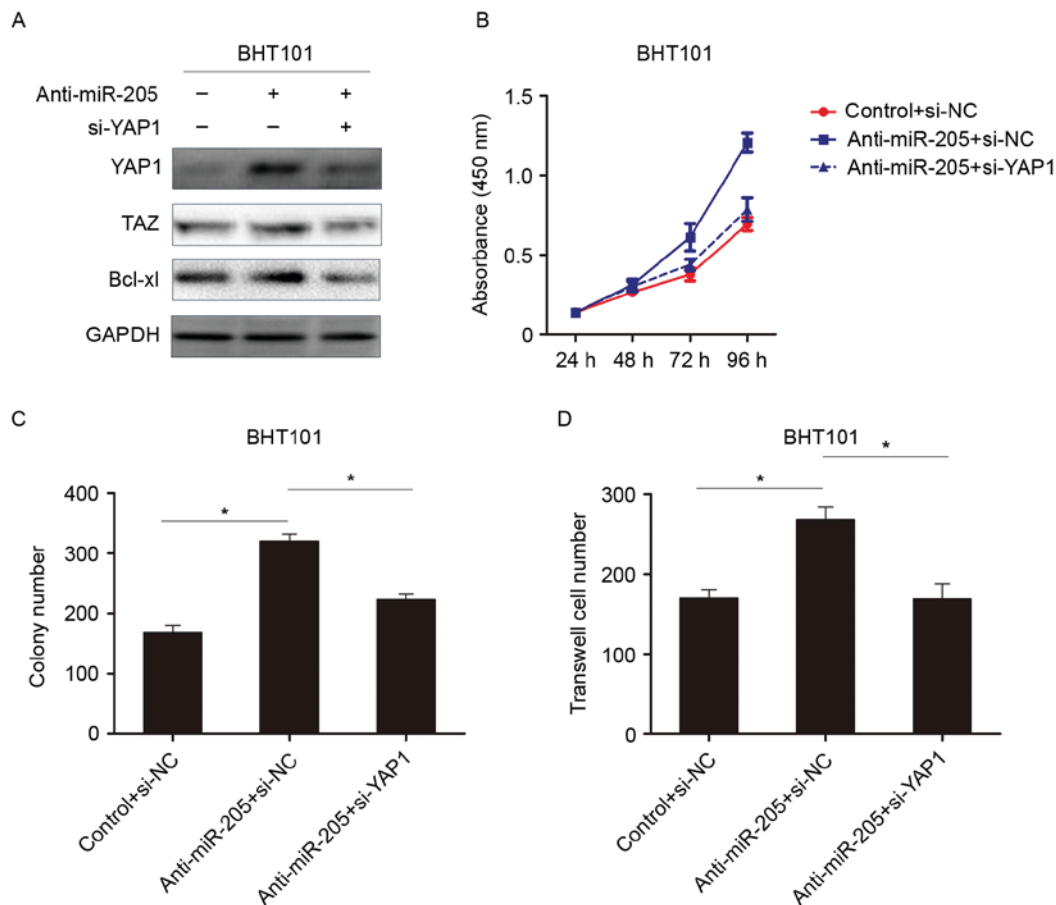


Figure 4. YAP1 is involved in suppressing the function of miR-205. (A) Western blot analysis was performed to detect the indicated protein expression when cells were treated with an miR-205 inhibitor, or YAP1-siRNA and an miR-205 inhibitor. (B) Analysis of the absorbance at 450 nm of cells transfected with anti-miR-205, anti-miR-205 and siYAP1, or NC. (C) Colony assay and (D) invasion assay following treatment with anti-miR-205, anti-miR-205 and siYAP1, or NC. * $P < 0.05$. si, small interfering; YAP1, Yes associated protein 1; Taz, anti-Translin-associated zinc finger protein 1; miR, microRNA; Bcl-xl, Bcl2-like protein 1; NC, normal control.

Discussion

Previous studies have indicated that the deregulated miRs are associated with tumor initiation and development. miR-205 has been identified to be downregulated miR in several types of cancers, including breast, bladder cancer and head and neck squamous cell carcinoma. miR-205 targets phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN, and inhibits renal cell carcinoma progression (21). However, miR-205 was demonstrated to target tumor protein p53-inducible nuclear protein 1, resulting in the tumorigenesis of nasopharyngeal carcinoma. These results indicated that the function of miR-205 was tumor dependent. miR-205 could be an accurate marker for squamous lung cancer (22). Low expression of miR-205 could be a prognostic factor for head and neck squamous cell carcinoma (23). However, the expression and roles of miR-205 in thyroid carcinoma remain unclear.

In the present study, the expression of miR-205 was observed to decrease in thyroid tumor samples compared with the non-tumor tissues, suggesting that miR-205 may be a potential tumor suppressor in thyroid cancer. In terms of the mechanism of action, miR-205 could be epigenetically regulated by hypermethylation of its promoter (24). The

mechanism of downregulation of miR-205 in thyroid cancer requires further investigation. miR-205 expression in thyroid cancer cells was examined and it was demonstrated that miR-205 was highly expressed in BHT101 cells but exhibited low expression in 8505-C cells. Based on the miR-205 expression level identified, miR-205 was increased or inhibited to assess the biological function in the present study. It was demonstrated that miR-205 suppressed proliferation, migration and invasion of thyroid cancer cells, which may explain miR-205 downregulation in cancer tissues. Furthermore, YAP1 was identified as a direct target of miR-205. To investigate the role of YAP1 further, a luciferase reporter assay was performed, and the results indicated that miR-205 could bind to the 3'-UTR of YAP1 and decrease the YAP1 mRNA level. In addition, it was demonstrated that the levels of miR-205 and YAP1 were inversely correlated in thyroid cancer samples.

While miR-205 inhibited cell migration and invasion, it has been demonstrated to regulate epithelial to mesenchymal transition by targeting zinc finger E-box-binding homeobox 1 and Smad-interacting protein 1, together with the miR-200 family (25). Also, protein kinase C ϵ , receptor tyrosine-protein kinase erbB-3, vascular endothelial growth factor A and Bcl-2-like protein 2 were reported to be the targets of miR-205

in prostate and breast cancer (26-29). These data imply the complex regulatory networks that miRs regulate have multiple targets and a target could be regulated by various miRs. The previous studies indicated that miR-205 level exhibited prognostic value in non-small cell lung cancer, and endometrial and breast cancer (22,30,31). Furthermore, Zhang *et al* (24) demonstrated that hepatitis B virus X protein induced hypermethylation of the miR-205 promoter and suppressed its expression. Also, it is reported that miR-205 could be regulated by long non-coding RNA HOTAIR in bladder cancer cells (32). The regulation of miR-205 in thyroid cancer requires further investigation.

YAP1 is located on chromosome 11q13, coding a downstream nuclear effector of the hippo signaling pathway. As consequence, YAP1 could regulate octamer binding protein 4 activity and SOX2 expression, facilitating a self-renewing ability (33). Also YAP1 could confer resistance to chemotherapy in esophageal cancer (34). YAP1 frequently functions as an oncogene in numerous types of cancer, including hepatocellular carcinoma, breast cancer and oral squamous cell carcinoma. However, its role in thyroid cancer remained unclear. Hudson *et al* (35) has reported the inhibition of YAP1 may be important for medullary thyroid carcinoma development. However Garcia-Rendueles *et al* (36) demonstrated that YAP1 dependent transactivation of RAS promoted poorly differentiated thyroid cancer progression. YAP1-transcriptional enhancer factor TEF-1 could be activated by the hippo signaling pathway and enhance transcription of target genes. In the present study, YAP1 expression was also detected and the downstream genes of hippo signaling pathways, including TAZ and Bcl-xl. The results demonstrated that miR-205 could regulate hippo signaling pathway by targeting YAP1 and knockdown of YAP1 abrogated the pro-tumorigenesis roles of anti-miR-205, which meant YAP1 could promote tumor cell growth and invasion in the present study. Previous studies have demonstrated that several miRs could regulate YAP1, including miR-132 and miR-16-1 (37,38), indicating the key role of the miR-regulatory network in tumor initiation and progression.

In conclusion, it was demonstrated that miR-205 was downregulated in thyroid cancer tissues. Through knockdown and overexpression experiments, it was revealed that miR-205 inhibited tumor cell proliferation and invasion. In addition, miR-205 expression was inversely correlated with YAP1 in thyroid cancer samples. miR-205 suppressed the hippo signaling pathway through targeting YAP1, and inhibited thyroid cancer cell growth and invasion. The results of the present study suggested that overexpression of miR-205 could be a potential therapeutic target for thyroid cancer.

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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

DL contributed significantly to the design of the study and writing and analyzing the data and the manuscript. QW and NL helped with collecting and arranging the patients' information. SZ helped to perform the statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Shanxi Provincial People's Hospital. Written informed consent was obtained from all patients.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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