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miR-204-5p suppresses cell proliferation by inhibiting IGFBP5 in papillary thyroid carcinoma

Lianyong Liu [1], Jingnan Wang [1], Xiangqi Li, Junhua Ma, Chao Shi, Hongling Zhu, Qian Xi, Jichen Zhang, Xuemei Zhao, Mingjun Gu*

Department of Endocrine, Shanghai Pudong Gongli Hospital, Shanghai 200135, China

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ABSTRACT

microRNAs (miRNAs) are frequently dysregulated in human malignancies. It was recently shown that miR-204-5p is downregulated in papillary thyroid carcinoma (PTC); however, the functional significance of this observation is not known. This study investigated the role of miR-204-5p in PTC. Overexpressing miR-204-5p suppressed PTC cell proliferation and induced cell cycle arrest and apoptosis. The results of a luciferase reporter assay showed that miR-204-5p can directly bind to the 3′ untranslated region (UTR) of insulin-like growth factor-binding protein 5 (IGFBP5) mRNA, and IGFBP5 overexpression partially reversed the growth-inhibitory effects of miR-204-5p. These results indicate that miR-204-5p acts as a tumor suppressor in PTC by regulating IGFBP5 expression and that miR-204-5p can potentially serve as an antitumorigenic agent in the treatment of PTC.

1. Introduction

Papillary thyroid carcinoma (PTC) is the most common histotype of thyroid malignancy, and its incidence has been steadily increasing over the past few decades [1,2]. Although the majority of cases have excellent prognosis and therapeutic response, up to 30% of patients present with locoregional recurrence or distant metastases within 10 years [3,4]. Thus, it is imperative to understand the molecular basis of PTC in order to develop effective diagnostic, prognostic, and therapeutic strategies for its treatment.

microRNAs (miRNAs) are 18–25-nt, non-coding, single-stranded RNAs that regulate gene expression at the post-transcriptional level by binding to the 3′-untranslated region (UTR) of target mRNAs [5,6]. miRNAs regulate a variety of basic physiological processes, including the cell cycle, apoptosis, cell proliferation, migration, invasion, and differentiation [7–10], and miRNA expression or function is dysregulated in various types of malignancies, including PTC [11]. For instance, overexpression of the tumor suppressor miR-146a decreased PTC cell survival and induced PTC cell apoptosis [12], while miR-199a-3p is down-regulated in PTC and its overexpression in PTC cells inhibited migration and proliferation, thereby inducing cell death [1]. On the other hand, miR-222 and miR-146b levels are upregulated in recurrent PTC and serve as circulating biomarkers for this cancer [3]. A recent microarray analysis revealed that miR-204-5p was downregulated in PTC [13]; however, the functional significance of this observation is not known.

In the present study, we validated that miR-204-5p was obviously downregulated in PTC tissues and cell lines. miR-204-5p overexpression suppressed PTC cell growth and induced cell cycle arrest and apoptosis. Insulin-like growth factor-binding protein 5 (IGFBP5) was identified as a direct target of miR-204-5p regulation, and restoring IGFBP5 expression partially reversed the anti-tumorigenic effects of miR-204-5p.

2. Materials and methods

2.1. Cell lines and tissues

Human PTC cell lines (TCP-1 and BCPAP) and HEK293T were cultured in Dulbecco's Modified Eagle's Medium (HyClone/Thermo Scientific, Beijing, China) supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2. Cells were passaged every 2–3 days. PTC and adjacent non-tumorous tissue samples were obtained with informed consent from 16 PTC patients who underwent curative resection at Shanghai Pudong Gongli Hospital. The study protocol

* Corresponding author. Fax: +86 21 58858730.
E-mail address: mjgugonglihos@yeah.net (M. Gu).
[1] These authors contributed equally to this work.

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was approved by the Ethics Committee of Shanghai Pudong Gongli Hospital.

2.2. Vector constructs

The 3′-UTR of IGFBP5 containing two putative miR-204-5p-binding sites was amplified and cloned into the psiCHECK-2 luciferase vector (Promega, Madison, WI, USA). The two sites were mutated using the XL Site-directed Mutagenesis Kit (Qiagen, Hilden, Germany). For IGFBP5 overexpression, the coding sequence of IGFBP5 lacking the 3′-UTR was cloned into the multiple cloning site of the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). All constructs were verified by sequencing.

2.3. Lentivirus production and transduction

A 450-bp fragment containing pre-miR-204-5p was cloned into the pWPXL vector to generate pWPXL-miR-204. The pWPXL or pWPXL-miR-204-5p construct was cotransfected with the packaging plasmids psPAX2 and pMD2G into HEK293T cells using Lipofectamine 2000 (Invitrogen), and viral particles were collected 48 h later. TPC-1 and BCPAP cells were infected with recombinant lentivirus-transducing units with 8 μg/ml polybrene (Sigma, St Louis, MO, USA). Stable transgene expression was confirmed by quantitative real-time (qRT)-PCR.

2.4. qRT-PCR

Total RNA, including miRNAs, was extracted using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The SuperScript III reverse transcription kit (Invitrogen) was used to transcribe RNA into cDNA, and qRT-PCR was performed using SYBR Premix Ex Taq II (Takara Bio Inc., Dalian, China) on an ABI 7900HT system (Applied Biosystems, Carlsbad, CA, USA). The level of target gene expression was normalized to that of the internal control (U6 small nuclear RNA or β-actin) and was determined by the $2^{-\Delta\Delta CT}$ method.

2.5. Cell proliferation and colony formation assays

Cell viability was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as previously described [14]. Briefly, 2×10^3 cells were cultured in 96-well plates and stained at 24, 48, 72, and 96 h with 20 μl MTT (0.5 mg/ml) for 4 h. The supernatant was discarded and 200 μl dimethylsulfoxide were added to each well to dissolve the precipitate. The absorbance was measured at 490 nm. For the colony formation assay, cells were seeded in 6-well plates at 2×10^2/well and grown for 2 weeks. Colonies were washed with phosphate-buffered saline, fixed with methanol, and stained with 0.5% crystal violet. The number of colonies was counted under an IX81 inverted microscope (Olympus, Tokyo, Japan).

2.6. Cell cycle and apoptosis analyses

For cell cycle analysis, 2×10^5 transfected cells were collected, fixed with 70% ethanol and incubated at 4 °C overnight. After washing, cells were treated with RNase A (100 μg/ml) for 30 min, then stained with propidium iodide (PI; 50 μg/ml) for 30 min in the dark. Cell cycle distribution was analyzed using a FACScan flow cytometer (BD Biosciences, Bedford, MD, USA). Apoptosis of cultured cells was evaluated using the Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's protocol.

2.7. Luciferase assay

HEK293T cells were cultured in 96-well plates and co-transfected with 50 ng of the wild-type or mutant IGFBP5 3′-UTR construct, or 50 mM negative control or miR-204-5p mimic. Cells were collected 48 h later, and Renilla luciferase activity was measured with the dual luciferase reporter system (Promega). The assay was performed in duplicate in three independent experiments.

2.8. Western blotting

Cells were lysed and protein concentration was measured using a bicinchoninic acid kit (Tiangen, Beijing, China). Denatured proteins (30 μg) were resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes, which were blocked with 5% non-fat milk in Tris-buffered saline for 1 h at room temperature and incubated with primary antibodies against IGFBP5 (Abcam, Cambridge, MA, USA) and glyceraldehyde-3-phosphate dehydrogenase (Abcam) at 4 °C overnight, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein bands were visualized by chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA).

2.9. Xenograft model

Male BALB/c nude mice (4–6 weeks of age) were housed under standard conditions according to protocols approved by the Animal Experiments Ethics Committee of Second Military Medical University. TCP-1 cells (2×10^6) stably expressing miR-204-5p or the control vector were transplanted subcutaneously into the right flank of each mouse. Tumor growth was assessed every 2 days, and tumor volume was calculated weekly using the formula: $V = (A \times B^2) \times 0.5$, where A and B are the longest and shortest diameters, respectively. After 5 weeks, mice were sacrificed and the tumors were dissected.

2.10. Statistical analysis

Statistical analyses were performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± SD. The Student's t test was used for between-groups comparisons. The correlation between IGFBP5 and miR-204-5p expression was evaluated by Spearman's correlation test. Results were considered statistically significant at $P < 0.05$.

3. Results

3.1. miR-204-5p expression is downregulated in PTC tissues and cell lines

miR-204-5p expression in 16 sets of PTC and adjacent normal tissue was assessed by qRT-PCR. miR-204-5p expression was downregulated in PTC relative to adjacent non-tumorous tissues (Fig. 1A). Similarly, miR-204-5p levels were reduced in the TPC-1 and BCPAP PTC cell lines as compared to normal tissue (Fig. 1B). These data indicate that miR-204-5p expression is attenuated in PTC.

3.2. miR-204-5p suppresses proliferation and promotes apoptosis in PTC cells

To investigate the biological function of miR-204-5p in PTC, TPC-1 and BCPAP cells were infected with a lentivirus expressing miR-

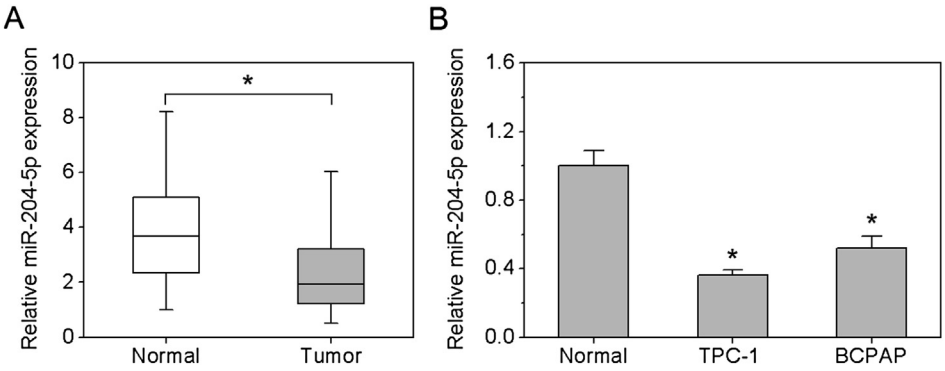

Fig. 1. miR-204-5p expression is downregulated in PTC cell lines and tissue. (A) Quantitative analysis of miR-204-5p levels in PTC and matched, adjacent normal tissues, as determined by qRT-PCR. (B) Relative miR-204-5p expression in the TPC-1 and BCPAP PTC cell lines. $^{*}P < 0.05$.

204-5p. miR-204-5p expression was increased in infected cells (Fig. 2A), which showed a concomitant decrease in proliferation rate relative to control-infected cells, as determined by the MTT assay (Fig. 2B). Similar results were obtained in the colony formation assay (Fig. 2C). miR-204-5p overexpression also decreased the fraction of cells in S-phase as compared to control cells (Fig. 2D), and increased the rate of apoptosis (Fig. 2E). These results suggest that miR-204-5p has a tumor suppressor role in PTC.

3.3. miR-204-5p inhibits PTC cell tumorigenicity in vivo

To investigate the role of miR-204-5p in tumor growth, TPC-1 cells stably expressing miR-204-5p were subcutaneously injected into nude mice. Tumors formed by control cells (miR-neg) grew more rapidly than those formed by miR-204-5p-overexpressing cells (Supplementary Fig. 1A) both in terms of volume (Supplementary Fig. 1B) and weight (Supplementary Fig. 1C). These

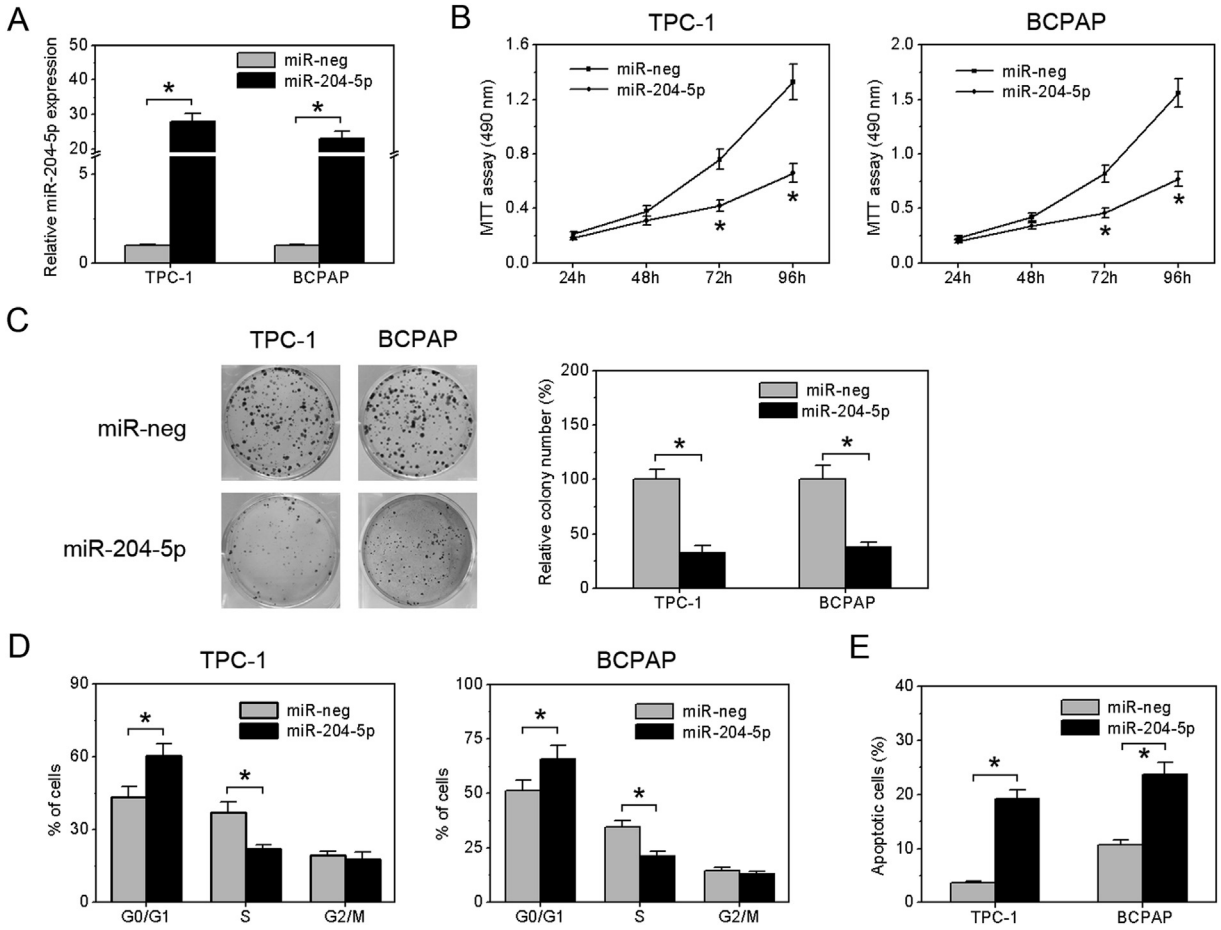

Fig. 2. miR-204-5p inhibits PTC cell proliferation. (A) TPC-1 and BCPAP cells were infected with lentivirus encoding miR-204-5p and expression was detected by qRT-PCR. Cell proliferation was assessed by (B) MTT and (C) colony formation assays. Representative micrographs and quantitative analysis of colony formation are shown. (D) Flow cytometry analysis of cell cycle. (E) The percentage of cells undergoing apoptosis was measured by annexin V/PI staining. Data represent mean ± SD from three independent experiments. $^{*}P < 0.05$.

results validate the inhibitory effects of miR-204-5p on tumor growth in a mouse model of PTC.

3.4. miR-204-5p suppresses IGFBP5 expression by direct binding to the 3′-UTR

Putative targets of miR-204-5p regulation were identified using three bioinformatic algorithms (miRanda, Pictar, and targetscan). Two highly conserved miR-204-5p-binding sites were predicted in the IGFBP5 3′-UTR (Fig. 3A), suggesting that it is a potential target of miR-204-5p. To determine whether there is direct binding, a luciferase reporter system was established with wild-type or one of two mutant IGFBP5 mRNA 3′-UTR fragments. miR-204-5p transfection decreased relative luciferase activity in HEK293T cells expressing the wild-type IGFBP5 3′-UTR, but the activity was largely unaltered in cells transfected with the 3′-UTR with mutated miR-204-5p-binding sites (Fig. 3B). Additionally, overexpressing miR-204-5p decreased IGFBP5 mRNA and protein levels, whereas inhibiting miR-204-5p had the opposite effect (Fig. 3C and D). IGFBP5 expression was also downregulated in PTC as compared to adjacent non-tumorous tissue (Fig. 3E); indeed, miR-204-5p and IGFBP5 expression were inversely correlated (Fig. 3F). Taken together, these data demonstrate that IGFBP5 is directly and negatively regulated by miR-204-5p.

3.5. IGFBP5 overexpression reverses the effects of miR-204-5p

It was hypothesized that if IGFBP5 is a direct target of negative regulation by miR-204-5p in PTC, IGFBP5 overexpression should reverse the phenotypes caused by miR-204-5p overexpression in PTC cells. To test this hypothesis, an IGFBP5-expressing vector lacking the 3′-UTR was used in a rescue experiment. The restoration of IGFBP5 expression was confirmed by western blot analysis (Fig. 4A). Decreases in cell viability (Fig. 4B) and colony formation rate (Fig. 4C), and the increase in the rate of apoptosis (Fig. 4D) induced by miR-204-5p were partly abrogated by IGFBP5 overexpression, confirming that IGFBP5 is a target of miR-204-5p regulation.

4. Discussion

miRNAs play critical roles in the initiation, promotion, and progression of human cancers by regulating target gene expression [15,16]; as such, they can be functionally distinguished as tumor

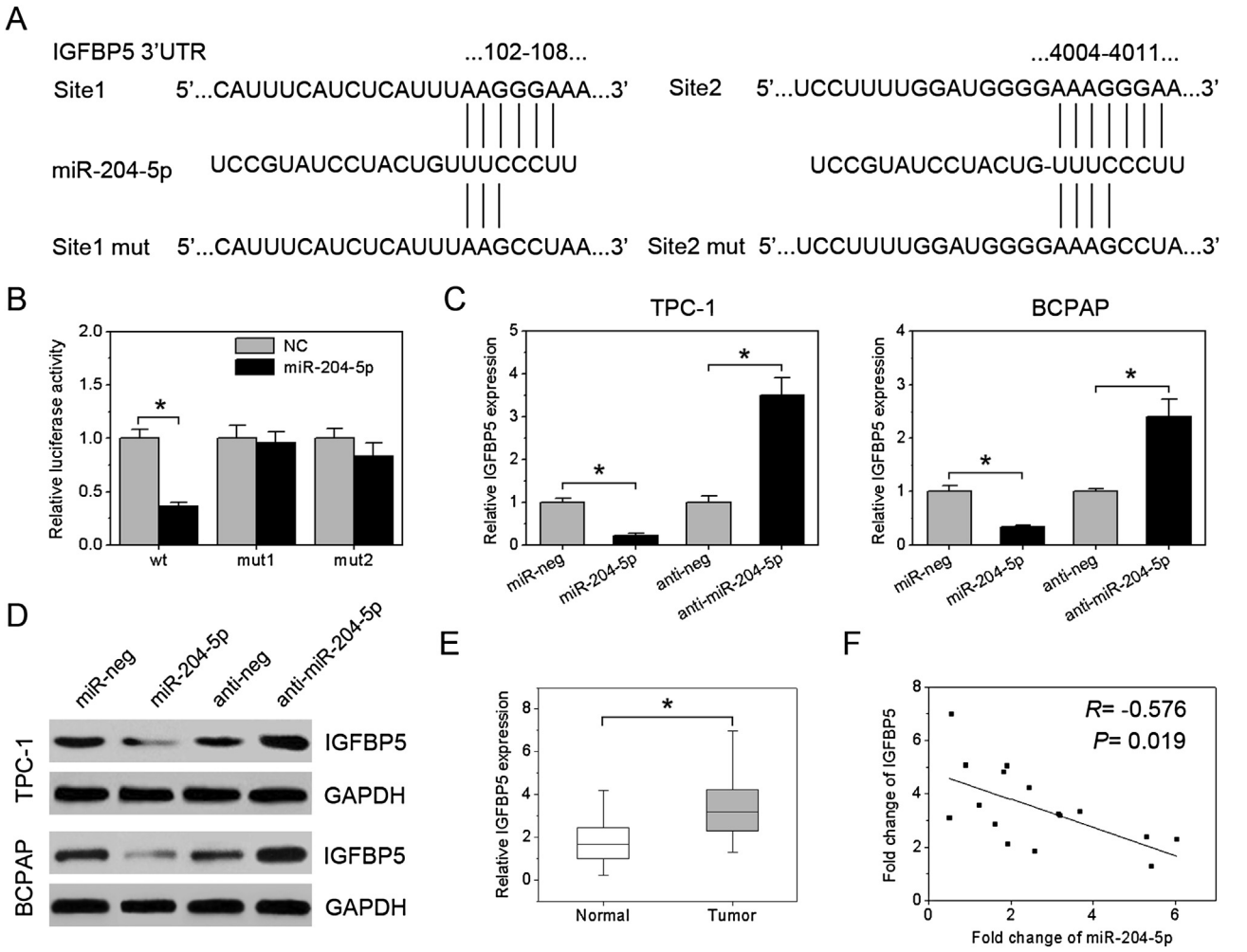

Fig. 3. miR-204-5p suppresses IGFBP5 expression via direct binding to the 3′-UTR. (A) Sequence alignment of two putative miR-204-5p-binding sites in IGFBP5 3′-UTR and corresponding mutant binding sites. (B) HEK293T cells were co-transfected with wild-type or mutant reporter plasmids along with miR-204-5p mimic or negative control (NC), and relative luciferase activity was measured. (C, D) After TPC-1 and BCPAP cells were transfected with miR-204-5p or anti-miR-204-5p, IGFBP5 mRNA and protein expression was assessed by qRT-PCR and western blotting, respectively. (E) IGFBP5 expression in PTC and matched non-tumorous tissues. (F) Correlation analysis between miR-204-5p and IGFBP5 expression in PTC tissues. $^{*}P < 0.05$.

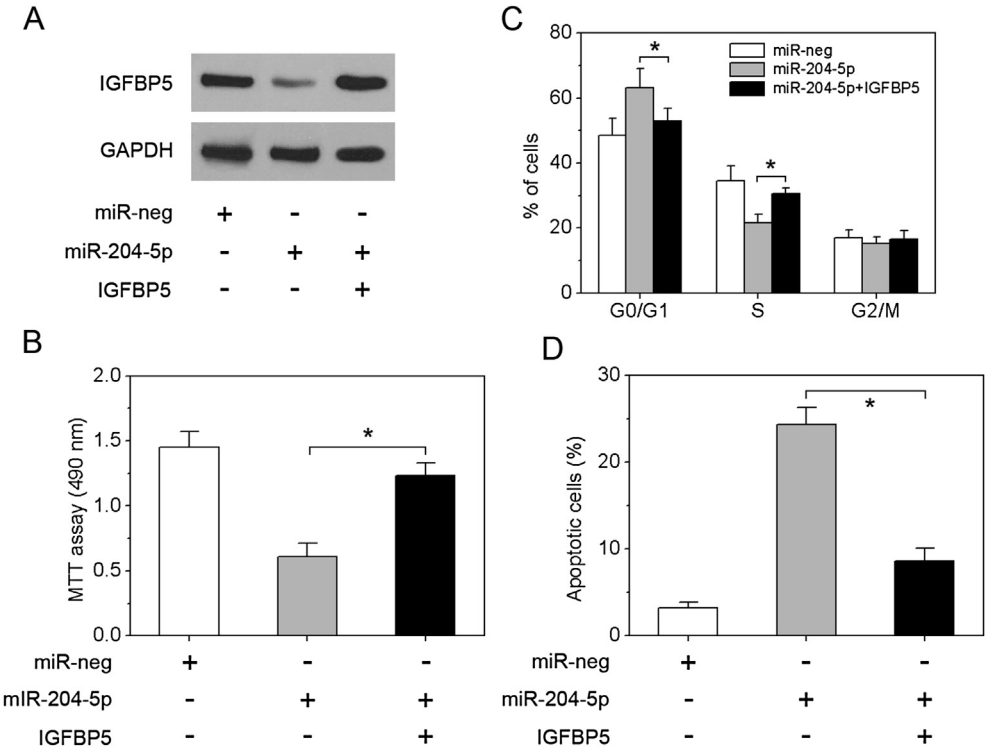

Fig. 4. IGFBP5 overexpression reverses the effects of miR-204-5p. TPC-1 cells were transfected with miR-204-5p or a control miRNA (miR-neg) along with IGFBP5-expressing plasmid lacking the 3′-UTR. (A) IGFBP5 protein level was assessed by western blotting. (B) Cell proliferation was analyzed by MTT assay. (C) A cell cycle analysis was performed by flow cytometry. (D) Apoptosis was measured by annexin V/PI staining. *$P < 0.05$.

suppressors or oncogenes [17]. As a tumor suppressor, miR-204-5p is downregulated in minimal deviation adenocarcinoma [18], clear cell renal cell carcinoma [19], gastric cancer arising from *Helicobacter pylori* infection [20], and melanoma [21]. A recent study also found that miR-204-5p was frequently downregulated in colorectal cancer and associated with poor prognosis, while ectopic miR-204-5p expression inhibited colorectal cancer cell proliferation, migration, and invasion and induced apoptosis and chemotherapeutic sensitivity [22]. A low level of miR-204-5p expression was associated with advanced stage, lymph node metastasis, and low survival rate in endometrial carcinoma; miR-204-5p inhibited the clonogenic growth, migration, and invasion of endometrial carcinoma cells as well as the growth of tumor xenografts [23]. However, little is known about the function and mechanism of miR-204-5p in PTC.

In the current study, we showed that miR-204-5p was prominently decreased in PTC tissues and cell lines. Functional analyses indicated that miR-204-5p could inhibit cell viability and colony formation efficiency, block cell cycle progression and enhance apoptosis *in vitro* and suppress tumorigenicity in vivo. Further investigations revealed that miR-204-5p repressed IGFBP5 by binding to its 3′UTR.

IGFBP5 is the most evolutionarily conserved member of the IGFBP family of secreted proteins [24] and plays a critical role in carcinogenesis involving both IGF-dependent and -independent pathways [25]. The expression and function of IGFBP is specific to cancer type; for instance, protein expression is upregulated in intrahepatic cholangiocarcinoma [26], pancreatic adenocarcinoma [27], and breast cancer [28], but is downregulated in metastatic osteosarcoma [29], head and neck squamous cell carcinoma [30], and cervical carcinoma [31]. In osteosarcoma, a high level of IGFBP5 was associated with an inhibition of cell proliferation, migration, and invasion, and induced apoptosis and cell cycle arrest *in vitro* and in vivo [29]. Similarly, IGFBP5 induced G2/M cell cycle arrest and apoptosis in breast cancer cells and inhibited tumor formation and growth in vivo [32,33]. However, in neuroblastoma cells, IGFBP5 knockdown inhibited cell growth and differentiation, and induced apoptosis [34]. A recent study reported that IGFBP5 was overexpressed in thyroid tumors [35]. Consistent with this report, the present study showed that IGFBP5 expression was elevated in PTC. Overexpressing IGFBP5 partially abrogated miR-204-5p-induced effects on tumorigenesis, and the negative correlation found between IGFBP5 and miR-204-5p expression confirmed a regulatory relationship between these two factors.

In conclusion, miR-204-5p functions as a tumor suppressor that modulates PTC cell growth in part by regulating the expression of the downstream target IGFBP5. These findings suggest that miR-204-5p may be a useful diagnostic marker and potential therapeutic agent for the treatment of PTC.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.037.

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