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CBX1 is a direct target of miR-205-5p and contributes to the progression of pituitary tumor

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MicroRNAs (miRs) are crucial regulators for tumorigenesis through negatively regulating their target genes expression in the manner of 3'-untranslated region (3'-UTR) binding. MiR-205-5p has been reported to function as a tumor suppressor in several cancer types. The aim of this study was to investigate the role of miR-205-5p/chromobox homolog 1 (CBX1) axis in human pituitary tumors. The expression of miR-205-5p was firstly examined by quantitative real-time PCR and the results revealed that miR-205-5p expression was declined in pituitary cell lines compared with normal cell line. Overexpression of miR-205-5p effectively decreased cell proliferation and cell migration. Based on the results of bioinformatic analysis, luciferase reporter assay, and western blot, we identified CBX1 as a direct target of miR-205-5p. Notably, overexpression of CBX1 promoted cell proliferation and migration. The effects of miR-205-5p overexpression on cell proliferation and migration can be reversed by CBX1 overexpression. Based on these findings, we deducted that miR-205-5p inhibits the cell proliferation and migration through directly targeting CBX1.

1. Introduction

Pituitary adenoma is a common neurological tumor with its pathological basis largely unknown (Jie et al. 2016). Most of pituitary adenomas are benign, however, some of them can invade outside to central nervous system (Pichard et al. 2002). Pituitary adenoma has brought greatly physical and mental burden to patients (Tang et al. 2015). Molecular targeted therapy is a novel strategy for tumor treatment and has brought new hope to patients (Gentilin et al. 2013; Shi et al. 2012). Therefore, it is necessary to explore the molecular mechanisms related to the initiation and progression of pituitary adenoma.

MicroRNAs (miRNAs) are a family of small non-coding RNA molecules with the length of 18-25 nucleotides, functioning as crucial roles in gene expression regulation mainly through 3'-untranslated region (3'-UTR) binding (Bartel et al. 2004). miRNAs were reported to play crucial roles in regulating cell behavior but also function as important mediators in the initiation and progression of human cancers (Schickel et al. 2004). Many miRNAs were abnormally expressed in pituitary adenoma (Li et al. 2014), miR-205-5p is generally recognized as a tumor suppressor in a variety of human cancers (Li et al. 2018; Nagai et al. 2018; Yamada et al. 2018). It was found that miR-205-5p expression was downregulated in prostatic carcinoma tissues and cell lines, and the overexpression of miR-205-5p inhibits cell migration and invasion through targeting zinc finger E-box binding homeobox 1 *in vitro* (Li et al. 2018). Another study investigating the function of miR-205-5p in prostate cancer showed that high-mobility group box 3 (HMGB3) was a direct target of miR-205-5p and the miR-205-5p/HMGB3 axis was able to inhibit cancer cell aggressiveness (Yamada et al. 2018). In oral squamous cell carcinoma, miR-205-5p directly targeted tissue inhibitor of metalloproteinases-2 to regulate cancer cell migration and invasion and therefore miR-205-5p was proved to be responsible for cancer development and metastasis (Nagai et al. 2018). However, the role of miR-205-5p in pituitary adenoma remains unknown.

Here, we analyzed the expression status of miR-205-5p in pituitary adenoma cancer cell lines. We also investigated the target of miR-205-5p in pituitary adenoma and confirmed that Chromobox homolog 1 (CBX1) was a direct target of miR-205-5p. Through direct targeting and negative control of CBX1 in pituitary adenoma cell lines, miR-205-5p can inhibit cell proliferation and migration. Our study suggested that miR-205-5p may be a therapeutic target for pituitary adenoma and offered novel insights into the mechanisms regarding the progression of pituitary adenoma.

2. Investigations and results

2.1. miR-205-5p was downregulated in pituitary adenoma cell lines

miR-205-5p expression levels in pituitary adenoma cell lines were examined by qRT-PCR. The results show that miR-205-5p expression was significantly reduced in pituitary adenoma cell lines compared with in HEK-293T cell line, indicating that miR-205-5p may play a crucial role in pituitary adenoma (Fig. 1).

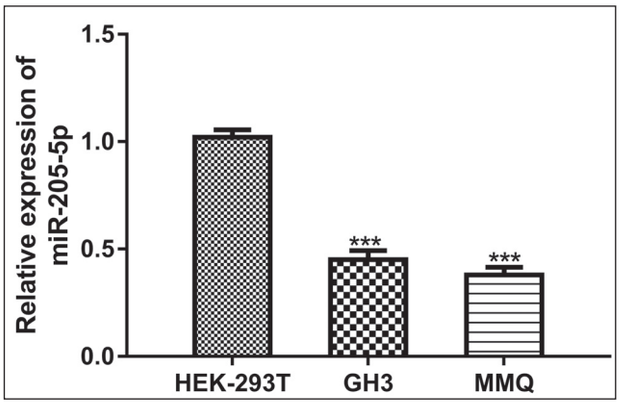

Fig. 1: The miR-205-5p expression levels in pituitary adenoma cell lines (GH3 and MMQ) and human transformed embryonal epithelial cell HEK-293T. (***P<0.001) miR-205-5p: microRNA-205-5p.

2.2. Cell proliferation and migration were inhibited by miR-205-5p overexpression

In order to examine the role of miR-205-5p in pituitary adenoma cell lines, miR-205-5p mimic and NC-mimic were transfected into the GH3 and MMQ cell lines. qRT-PCR analysis results showed that miR-205-5p mimic transfection significantly enhanced the levels of miR-205-5p in pituitary adenoma cell lines (Fig. 2A). MTT assay showed that compared with the NC-mimic group, miR-205-5p mimic reduced the cell proliferation of pituitary adenoma cell lines (Fig. 2B). A wound-healing assay showed that miR-205-5p mimic transfection significantly reduced cell migration compared with NC-mimic (Fig. 2C).

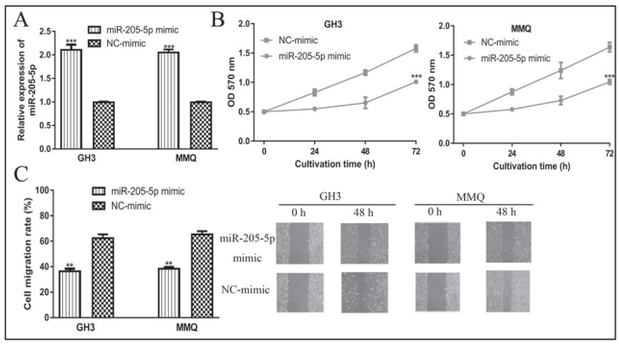

Fig. 2: Overexpression of miR-205-5p inhibits cell proliferation and migration. A, miR-205-5p expression, B, Cell proliferation, and C, Cell migration in pituitary adenoma cell lines (GH3 and MMQ) with miR-205-5p mimic or NC-mimic transfection. (***P<0.001) miR-205-5p: microRNA-205-5p; NC-mimic: negative control for miR-205-5p mimic.

2.3. CBX1 was a direct target of miR-205-5p

The prediction results of TargetScan showed the 3'-UTR of CBX1 contains a binding site for miR-205-5p (Fig. 3A). Luciferase activity reporter assay demonstrated that the transfection of miR-205-5p mimic reduced the luciferase activity of CBX1-wt, while almost no influence on the luciferase activity of CBX1-mut was seen (Fig. 3B). Besides, western blot assay showed that CBX1 protein expression was inhibited by miR-205-5p mimic in pituitary adenoma cell lines (Fig. 3C).

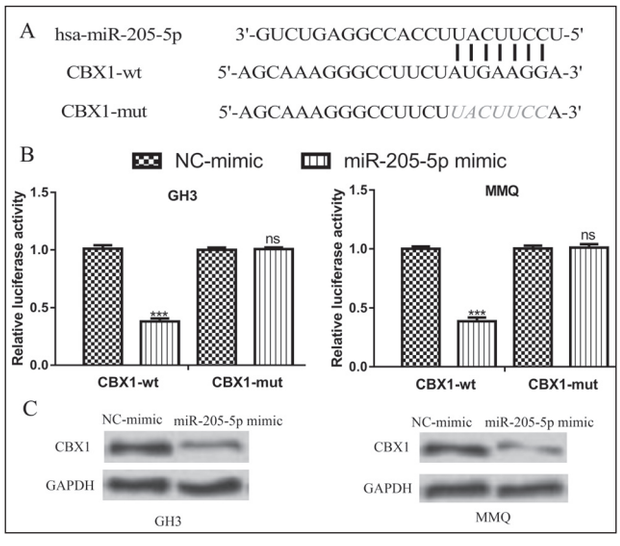

Fig. 3: CBX1 was a target of miR-205-5p. A, Putative binding site between miR-205-5p and the 3'-UTR of CBX1. B, Luciferase activity in cells with miR-205-5p mimic or NC-mimic and CBX1-wt or CBX1-mut transfection. C, CBX1 expression in cells with miR-205-5p mimic or NC-mimic transfection. (ns not significant; ***P<0.001) miR-205-5p: microRNA-205-5p; NC-mimic: negative control for miR-205-5p mimic; CBX1: chromobox homolog 1; wt: wild-type: mut: mutant; UTR: untranslated region.

2.4. Cell proliferation and migration were affected by miR-205-5p via targeting CBX1

In order to further confirm the relationship between miR-205-5p and CBX1, CBX1-construct and miR-205-5p mimic were transfected into pituitary adenoma cell lines. We found that CBX1-construct transfection enhanced the protein expression of CBX1, whereas little change for CBX1 expression was found in cells transfected with miR-205-5p mimic and CBX1-construct compared with NC-vector (Fig. 4A). Importantly, CBX1 remarkably weakened the inhibitory effects of miR-205-5p on cell proliferation and migration in pituitary adenoma cell lines (Figs. 4B and 4C).

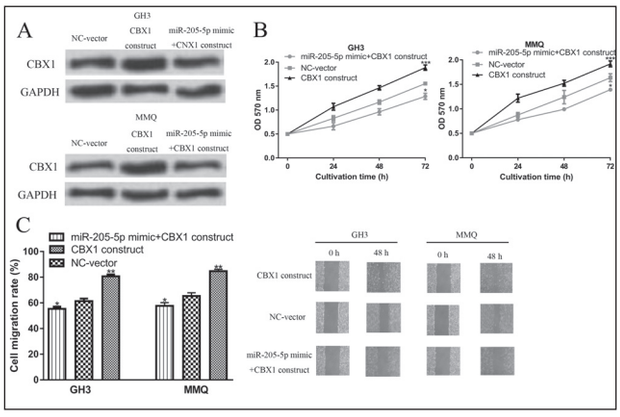

Fig. 4: Overexpression of CBX1 reversed the suppressive effects of miR-205-5p on cell proliferation and migration. A, CBX1 expression, B, Cell proliferation, and C, Cell migration in pituitary adenoma cell lines (GH3 and MMQ) with miR-205-5p mimic and CBX1-construct, CBX1-construct or NC-vector transfection. (*P<0.05; ***P<0.001) miR-205-5p: microRNA-205-5p; CBX1: chromobox homolog 1; NC-vector: negative control vector.

3. Discussion

The importance of miRNAs in pituitary adenoma has been recognized in recent years. He et al. (2017a) revealed that activated leukocyte antigen molecule (ALCAM) was a direct target of both miR-148b-3p and miR-152 in pituitary adenoma, and the overexpression of miR-148b-3p, miR-152 could inhibit proliferation and invasion, and promote apoptosis of pituitary adenoma cells. Liao et al. (2013) showed that miR-200c inhibition leads to the upregulation of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and downexpression of phosphorylated Akt (p-Akt) and as a results pituitary tumor formation was inhibited. Moreover, Wang et al. (2016) found miR-133 can inhibit pituitary adenoma cell migration and invasion by directly targeting forkhead box C1. Furthermore, miR-106b was demonstrated to function as an oncogene and regulate pituitary tumor cell proliferation and invasion *in vitro* by targeting PTEN through PI3K/AKT signaling pathway (Zhou et al. 2016). In this study, we found miR-205-5p expression was downregulated in pituitary adenoma cell lines, suggested miR-205-5p may function as a tumor suppressor in pituitary adenoma. Furthermore, we investigated the biological roles of miR-205-5p in pituitary adenoma and our *in vitro* analysis results showed that miR-205-5p overexpression inhibits cell proliferation and migration.

One gene can be targeted by multiple miRNAs and one miRNA may affect multiple target genes (He et al. 2017a; Liao et al. 2013; Wang et al. 2016; Zhou et al. 2016). In this work, the TargetScan algorithm was conducted to predict the targets of miR-205-5p and the analysis results showed that CBX1 contains a binding site for miR-205-5p in its 3'-UTR. CBX1 was selected for further investigations as it was found as an oncogene in several human cancers (Liang et al. 2017; Yang et al. 2018). For example, Liang et al. (2017) found high CBX1 expression correlated with worsened relapse-free survival of breast cancer patients. Yang et al. (2018) showed that CBX1 overexpression was a predictor of unfavorable overall and disease-free survival in hepatocellular carcinoma and the upregulation of CBX1 promoted cell proliferation and

migration. Here, luciferase activity reporter assay and western blot assay were conducted and validated CBX1 as a direct target of miR-205-5p in pituitary adenoma. Rescue experiments showed that the overexpression of CBX1 reversed the suppression effects of miR-205-5p on cell proliferation and migration. Previous studies showed that miR-205-5p expression could be regulated by long non-coding RNAs, implicating a more complex regulatory network regarding miR-205-5p (Chen et al. 2018; He et al. 2017b). In this work, we investigated the downstream target of miR-205-5p but did not explore its upstream regulator. Therefore, further studies are needed to fully elucidate the role of miR-205-5p in pituitary adenoma.

To sum up, our results revealed previously unrecognized roles between miR-205-5p and CBX1 in pituitary adenoma. Our results suggested that miR-205-5p may be a potential therapeutic target for pituitary adenoma.

4. Experimental

4.1. Cell culture and cell transfection

Pituitary adenoma cell lines GH3 and MMQ, and the human transformed embryonal epithelial cell HEK-293T were purchased from Gaining Biological (Shanghai, China). Tumor cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10 % fetal bovine serum (FBS, Thermo Fisher Scientific, Inc.). HEK-293T cells were cultured in DMEM containing 10 % FBS and l-glutamine (Thermo Fisher Scientific, Inc.). All cell lines were cultured in a 37 °C humidified incubator containing 5 % CO_2. miR-205-5p mimic and the corresponding negative control (NC-mimic) were purchased from GenePharma (Shanghai, China). Open reading frame expression construct for CBX1 and NC-vector were purchased from GenScript (Nanjing, China). For cell transfection, cells (2×10^5) were plated in a 6-well plate and allowed to adhere overnight. Then, the synthetic miRNAs or expression vectors were transfected to the cells using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

4.2. RNA isolation and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated using Trizol regent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthetized from 1 µg of total RNA per sample using PrimeScript RT Reagent Kit (TaKaRa, Dalian, China). qRT-PCR was performed in ABI7500 PCR instrument (Applied Biosystems, Foster City, CA, USA) using SYBR Green Mix (TaKaRa). qRT-PCR procedure was as follows: denaturation at 94 °C for 5 min; 40 cycles of 94 °C, 58 °C, and 72 °C for 1 min each; and extension step at 72 °C for 7 min using the following primers: miR-205-5p: forward, 5'-TCCTTCATTCCACCGGAGTCTG-3' and reverse, 5'-GCGAGCACAGAATTAATACGAC-3'; U6 snRNA forward, 5'-ATTGGAACGATACAGAGAAGATT-3' and reverse, 5'-GGAACGCTTCACGAATTTG-3'. Relative expression levels of miR-205-5p were analyzed with U6 snRNA as internal control using the $2^{-\Delta\Delta Cq}$ method (Livak et al. 2001).

4.3. Protein isolation and western blot

Total protein sample was extracted using RIPA lysis buffer supplemented with protease inhibitor (Beyotime, Haimen, Jiangsu, China). Protein samples were separated through 10% SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). Subsequently, the membranes were incubated for overnight at 4 °C with anti-CBX1 (1:1,000; ab10811; Abcam, Cambridge, MA, USA), anti-GAPDH (1:1,000; ab181603; Abcam). After washed with TBST, the membranes were incubated with horseradish peroxidase conjugated goat anti-rabbit secondary antibody (1:3,000; ab6721; Abcam). Then, protein expression levels were measured by BeyoECL plus kit (Beyotime).

4.4. Cell proliferation assay

Cells (5×10^3) to be investigated were seeded to 96-well plate. After incubation at 37 °C for 0, 24, 48, or 72 h, 10 µl MTT solution (Beyotime) was added into the medium and further incubated at 37 °C for 4 h. Then, 50 µl of dimethyl sulfoxide (Beyotime) was added and incubated for additional 20 min. Optical density (OD) at 570 nm was measured using ELx800 Absorbance microplate reader (BioTek, Winooski, VT, USA).

4.5. Cell migration assay

Cells were seeded to 24-well plate and cultured to 90 % confluence. Wounds at cell surface were creased with a plastic scriber. Cells were washed to remove debris and then cultured at the abovementioned condition for 48 h. Then, cells were observed and photographed under a microscope.

4.6. Luciferase reporter assay

TargetScan (http://www.targetscan.org/vert_72) was used to analyze the targets of miR-205-5p. CBX1 was predicted as a target of miR-205-5p. Then the wild-type and mutated 3'-UTR sequences of CBX1 were inserted into pmirGLO vector (Promega, Madison, WI, USA) and named as CBX1-wt or CBX1-mut. Then, CBX1-wt or CBX1-mut and miR-205-5p mimic or NC-mimic were transfected into cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Dual-Luciferase Reporter Assay system (Promega) was used to measure luciferase activity after 48 h of transfection.

4.7. Statistical analysis

Data was analyzed at GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA) and presented as mean±SD. Differences between groups were examined by analysis of variance (ANOVA) with Tukey post hoc test (for three or above groups) and Student's t-test (for two groups). $P<0.05$ was regarded as statistical significance.

Conflicts of interest: There is no conflict of interests.

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