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Original Article

Primary microcephaly gene *MCPH1* shows a novel molecular biomarker of human renal carcinoma and is regulated by miR-27a

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Abstract: Microcephalin 1 (MCPH1) gene, initially identified as an hTERT repressor, result in two autosomal recessive disorders: primary microcephaly and premature chromosome condensation syndrome. Recently, several studies have found that MCPH1 has also been shown to be downregulated in several different types of human cancers, suggesting that it could also function as a tumor suppressor gene and a novel molecular biomarker of human cancers. To investigate its potential role in the human renal carcinoma progression, we evaluated the expression of protein MCPH1 in 188 renal cancer and 20 normal renal tissues from 188 patients with renal cancer and 20 healthy persons by immunohistochemistry. Positive MCPH1 staining was found in all normal renal samples and partly in cancerous tissues. But MCPH1-positive cells resulted significantly lower in renal carcinoma tissues compared with normal tissues. We further observed that overexpression of MCPH1 decreased cellular proliferation, cell migration and invasion and induced cell apoptosis, indicating it is tumor suppressor. Using bioinformatics approaches and luciferase reporter assay, we showed that the 3'-UTR of MCPH1 harbors two non-overlapping functional seed regions for miR-27 which negatively regulated its level. The expression level of miR-27a negatively correlated with the MCPH1 protein level in renal cancer. Our study indicates for the first time that, in addition to its role in brain development, MCPH1 also functions as a tumor suppressor gene and is directly regulated by miR-27a.

Keywords: Renal carcinoma, MCPH1, suppressor gene, microRNA, miR-27

Introduction

MCPH1 (microcephalin 1) gene, also known as BRIT1 (BRCT-repeat inhibitor of TERT expression 1), is located at chromosome 8p23.1. Mcph1 mutations in this gene cause an autosomal recessive disorder, primary microcephaly (MCPH), which is characterized by decreased size of cerebral cortex and mental retardation in affected individuals [1-5]. MCPH1 gene mutation also can cause premature chromosome condensation (PCC), then resulting in miscarriage or premature birth with cerebellar malformations fetus. MCPH1 contains 14 coding exons and codes for an 835 amino acids with about 110 kDa of the molecular weight. MCPH1 harbors three BRCT (BRCA1 C-terminal) domains, a NLS (nuclear localization signal) and a CIIBR (condensin II binding region). Bilbao et al. [6] screened the MCPH1 gene for mutations within mononucleotide coding tracts in exons 4, 5 and 8 in 41 MSI (microsatellite instability)-positive and 62 MSI-negative endometrial tumors and found mutations in only five MSI-positive tumors. Most of these mutations were in a heterozygous state [7-9]. Further, MCPH1 was found to be downregulated at the transcript level in 19/30 ovarian cancer specimens and at the protein level in 93/319 breast cancer tissues. Decreased MCPH1 protein levels are associated with triple negative breast cancers and a lower transcript level of MCPH1 correlates with lesser time for metastasis in breast cancer [7, 10-14].

Based on these observations, we hypothesized that MCPH1 may also function as a tumor suppressor gene, in addition to its role in the brain development. The purpose of this study was to

test if MCPH1 also functions as a tumor suppressor gene using different approaches in renal cancer. The results of our study show that MCPH1 has many of these signatures, functions as a tumor suppressor gene and is regulated by miR-27a.

Materials and methods

Immunohistochemistry

The immunohistochemistry for the tissue slides was performed as described previously by Simmons et al. [15] using a rabbit polyclonal anti-MCPH1 antibody (Abcam, Cambridge, MA) at a dilution of 1:100 and a secondary antibody from Bangalore GeneiH, Bangalore, India. The degree of MCPH1 staining in tumors was scored as the percentage of cells stained with DAB (3, 3-diaminobenzidine) in a total of five microscopic fields at a magnification of 40X. A 35% cut-off was used to dichotomize the MCPH1 expression into low and high groups as described by Richardson et al [16].

Evaluation of IHC staining

The quantification of degree of staining of MCPH1 protein expression was based on previous studies; we used the intensity and extent of staining to evaluate MCPH1 expression. The entire tissue sections were observed under the optical microscope (100×) to assign scores. Each section was examined independently in a blinded fashion by two pathologists (Ning Wang, Hongsheng Lu). Extent of staining was scored as 0 (no staining), 1 (<25% of staining), 2 (26%-50% of staining), 3 (51%-75% staining), or 4 (75%-100% staining), according to the percentages of the positive staining areas in carcinomatous sections and entire section for the normal samples (36). Meanwhile, intensity of staining was scored as 0 (no staining), 1 (light yellow staining), 2 (yellow staining), 3 (brown staining). The sum of the intensity and extent scores was used as the final staining score (0 to 7) of MCPH1. Section having a final staining score (<3) were grouped into low MCPH1 expression and those with scores (≥3) were grouped into high MCPH1 expression.

Cell culture and generation of stable cell lines

Human renal cancer cells (ACHN, 768-0) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented 10% fetal bovine serum (Invitrogen, USA), in a humidified atmosphere of 5 % CO_2 at 37°C. ACHN, 768-o cells were infected with lentivirus Lent-MCPH1 or negative control (Lent-control) at an MOI (multiplicity of infection) of 10, 20 and 40, respectively. Each cell line was divided into the following three experimental groups: (1) Lent-MCPH1 group (Lent-MCPH1-infected cells), (2) Lent-control group (Lent-control-infected cells) and (3) W/O group (noninfected cells).

Cell proliferation assay

Cell proliferation assay was performed by cell Titer 96Aqueous one Solution Cell Proliferation Assay (Promega, USA) according to the manufacturer's protocol.

In vitro migration and invasion assay

For transwell migration assays, 2.5×10^3 to 5×10^3 cells were plated in the top chamber with the non-coated membrane (24-well insert; pore size, 8 mm; BD Biosciences). For invasion assays, 1.25×10^3 cells were plated in the top chamber with Matrigel-coated membrane (24-well insert; pore size, 8 mm; BD Biosciences). In both assays, cells were plated in medium without serum or growth factors, and medium supplemented with growth factors (for HMECs) or serum (for all other cells) was used as a chemoattractant in the lower chamber. The cells were incubated for 24 h and cells that did not migrate or invade through the pores were removed by a cotton swab. Cells on the lower surface of the membrane were stained with the Diff-Quick Staining Set (Dade) and counted.

Immunoblotting

Cells were harvested in RIPA lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.5, 1% NP40, 1% deoxycholate, 0.1% SDS, protease inhibitor cocktail (Roche). Proteins from total cell lysates were resolved by the NuPAGE 4-12% Bis-Tris gradient gel (Invitrogen), transferred to the PVDF membrane, blocked in 5% non-fat milk in PBS/Tween-20, and blotted with the antibodies for anti-MCPH1 (Abcam, UK), anti-Actin (Sigma, USA.

In analysis of MicroRNA targets in MCPH1

The 5.4 kb 3'-UTR of MCPH1 was analyzed for miRNA binding sites using five miRNA predic-

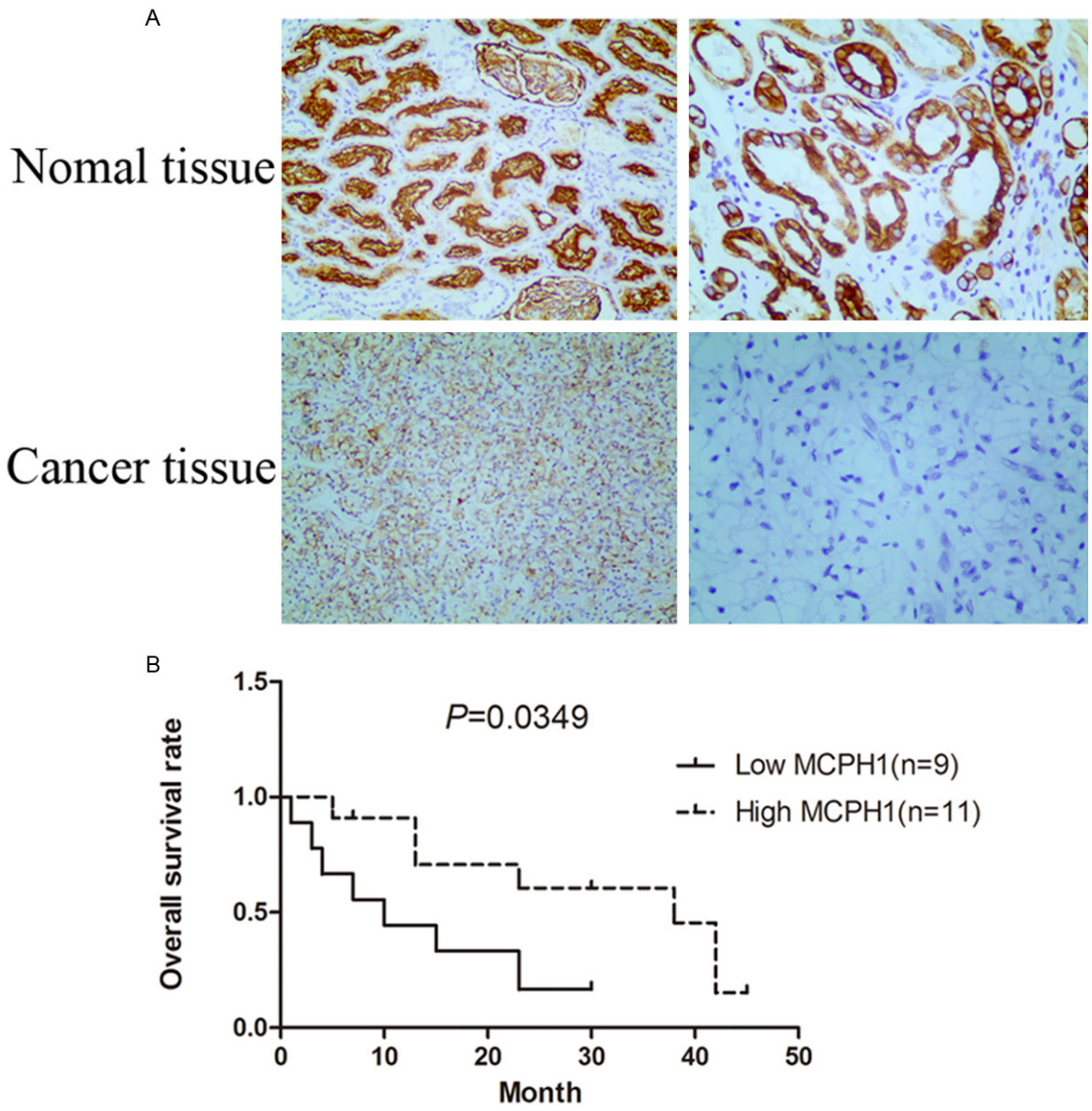

Figure 1. MCPH1 is decreased expression in renal cancer tissues. A. Up. Normal renal tissue showing strong expression of MCPH1. MCPH1 showing low expression in renal cancer tissue and mainly locating in the cytoplasm. Left (×200). Right (×400). B. Kaplan-Meier plots of MCPH1 expression in 20 cases of renal cancer patients. Overall survival rate was performed by log-rank test. $P<0.05$ indicate significant differences between two groups.

tion softwares: MicroCosm (http://www.ebi.ac.uk/enright-srv/microcosm), miRTAR (http://mirtar.mbc.nctu.edu.tw/), microRNA (http://www.microrna.org/microrna/home.do), miRDB (http://mirdb.org/miRDB/) and TargetScan (http://www.targetscan.org/).

miRNA gene cloning and ectopic expression

In order to generate a construct with a full length pre-miR-27a, the human miRNA gene was PCR-amplified from normal genomic DNA and cloned into the MDH1-PGK-GFP 2.0 retroviral vector. The production of amphotropic viruses and infection of target cells were described previously48. The GFP1 cells accounted for over 90% of infected cells, as determined by fluorescence-activated cell sorting (FACS) analysis.

Luciferase assay

HEK293 cells, seeded into 6-well plates, were cotransfected with 1 mg of the reporter gene

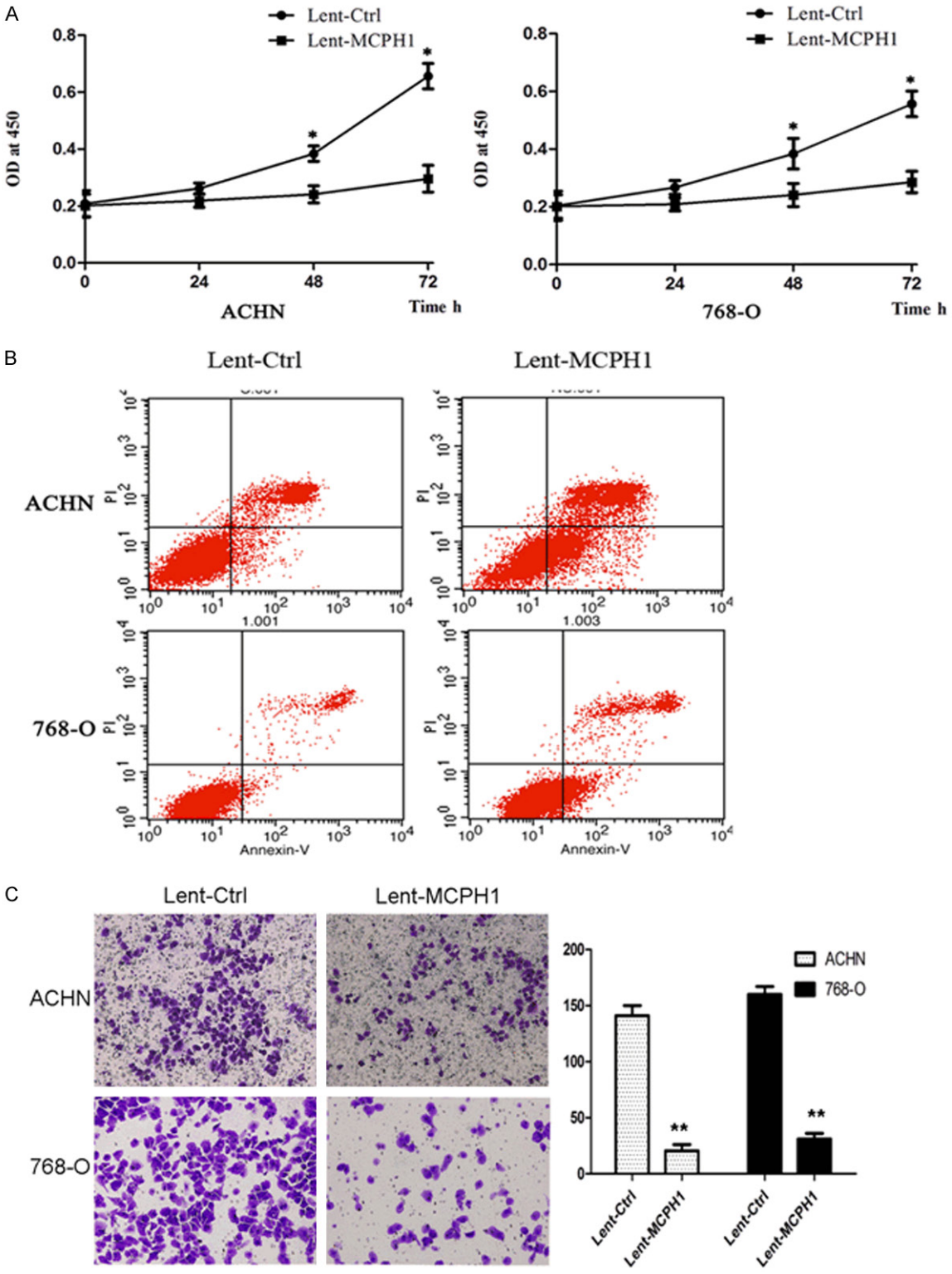

Figure 2. Tumor suppressor activity of MCPH1. A. MCPH1 overexpression inhibits cell growth examined by cell proliferation assay in ACHN and 768-O cells, compared with the Lent-ctrl. The curve represents the OD (optical density) measurement at 450 nm. The values are the mean OD450 nm of three independent experiments. *indicates $P < 0.05$. B. Flow cytometry analysis of apoptosis of renal cancer cells. MCPH1 overexpression induces apoptosis of ACHN and 768-O cells, 72 h after infection. C. Matrigel invasion analysis of cell invasion in renal cells. Note: MCPH1 overexpression reduced invasiveness in ACHN and 768-O cells as compared to Lent-ctrl cells. The quantitative representation of the cell invasion assay data. The values are the mean ± SD of the number of invaded cells counted in four random microscopic fields.

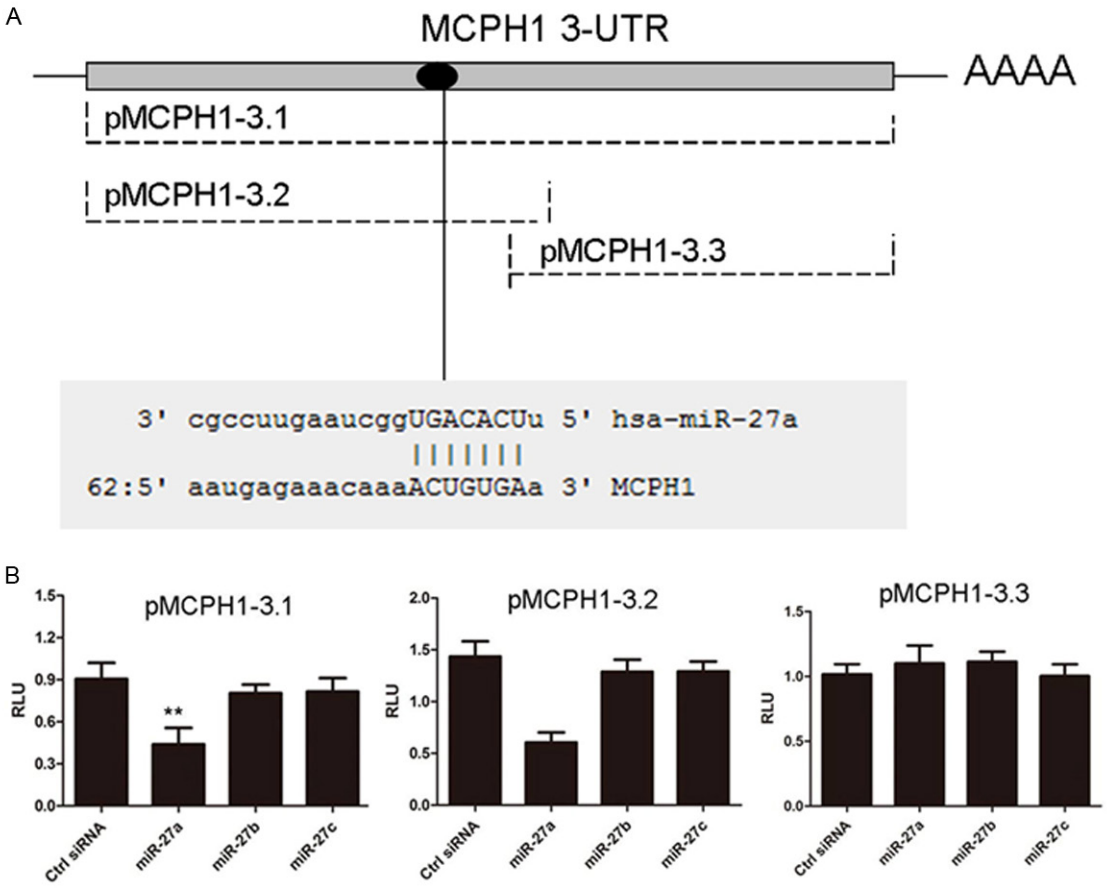

Figure 3. Binding of miR-27 family members to the 3'-UTR of MCPH1. A. Scheme of the MCPH1 3'-UTR containing luciferase constructs (pMCPH1-3.1, pMCPH1-3.2 and pMCPH1-3.3). The gray horizontal bar represents the MCPH1 3'-UTR, dashed lines indicate different 3'-UTR fragments fused to Renilla luciferase in psiCHECK2. The black dot indicates the location of the predicted miR-27a binding site. B. Plasmid reporter gene constructs were cotransfected with the indicated miRNA mimics or control siRNA into HEK293 cells. After 48 hours, lysates from transfectants were analyzed for luciferase activity miR-27a and miR-27b as well as miR-27c. Data of normalized Renilla luciferase activity (RLU) represent means (+SEM) of n=3 experiments **, $P<0.01$.

constructs (pMCPH1-3.1, pMCPH1-3.2, pMCPH1-3.3, pMCPH1-3.1 Mut) and 2.5 nmol/L of miScript miRNA mimics. After 48 hours, cells were lysed and analyzed for Renilla and Firefly luciferase activity using the Dual-Glo luciferase assay system (Promega). Each transfectant was assayed in triplicates. Activity of Renilla luciferase was normalized to Firefly luciferase.

Quantitative real-time RT-PCR

Total miRNA and mRNA were isolated from tumor cells using the TRIzol reagent (Invitrogen), according to the protocol of the manufacturer. Reverse transcription of miRNA was carried out with the Taqman MicroRNA Assay kit, mRNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was carried out using specific TaqMan Gene Expression assays or miRNA assays in combination with the StepOnePlus Real-Time PCR system (Applied Biosystems). Relative RNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method after normalizing expression levels of MPH1 mRNA to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Mrna and miRNA to RNU6B.

Results

Decreased expression of MCPH1 in renal cancer tissues

To determine the expression of MCPH1 in renal tissues, we examined the expression MCPH1 protein in 180 paraffin-embedded renal cancer

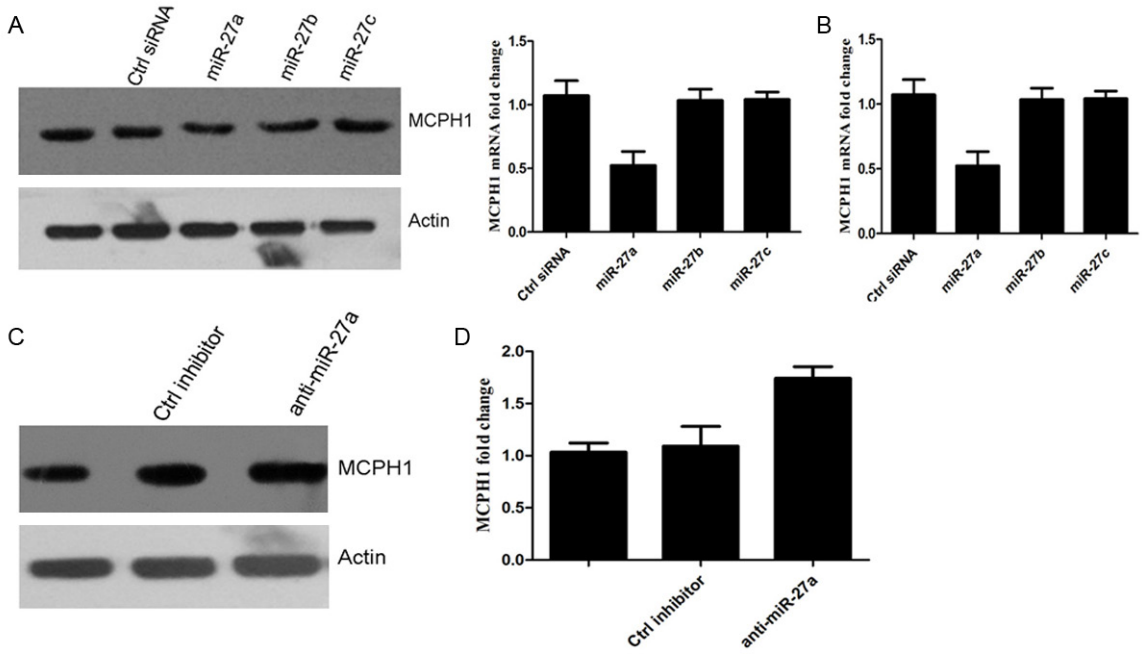

Figure 4. miR-27a controls MCPH1 expression in renal cells. A. Left, transfection of miR-27 mimics downregulates ULBP2 expression in melanoma cells. ACHN cells were transfected with miRNA mimics or control siRNA. After 48 hours, lysates of cells were analyzed for MCPH1 expression by Western blot. Actin served as loading control and for normalization. A. Right, fold change in MCPH1expression was calculated from n=3 experiments. B. The influence of miR-27 mimic transfection on MCPH1 mRNA levels was determined by qRT-PCR. Expression of MCPH1 mRNA was normalized to endogenous GAPDH mRNA. Mean expression levels (+SEM) of n=3 experiments are presented relative to control siRNA-transfected cells. C. Endogenous miR-27a reduces MCPH1 expression in renal cells. ACHN cells were transfected with either anti–miR-27a or control anti-miRNA. After 48 hours, cells were lysed and MCPH1 expression levels were determined by Western blot. Actin served as loading control and for normalization. D. The fold change in MCPH1 expression was calculated from n=3 experiments. *, $P<0.05$.

samples and 20 normal samples by immunohistochemical analysis. As shown in **Figure 1**, MCPH1 showed low expression in renal cancer samples (**Figure 1A**). MCPH1 protein was predominantly found to be strongly stained in normal sections (**Figure 1A**). And MCPH1 was mainly expressed in the cytoplasm of cancer cells, normal renal tissue showed strong nuclear expression of MCPH1 protein, which was consistent with recent research. These data suggest that MCPH1 show lower expression in samples compared to normal samples, which indicates MCPH1, is related to the differentiation and metastasis of renal cancer. Decreased MPCH1 expression also found to correlate with overall survival of patients (**Figure 1B**).

MCPH1 reduces cell proliferation

In order to determine whether MCPH1 reduces cell proliferation, equal number of cells were plated into 6-well plate and infected with Lent-MCPH1 or Lent-control. We generated MCPH1 over-expressing stable clones in renal cells by G418 selection. To understand the effect of MCPH1 on cell proliferation, we monitored cell growth by cell proliferation assay; 24 h after infection, the cell number of ACHN with MCPH1 overexpression slightly increased; from 48 h on, 768-0 lines with MCPH1 overexpression showed slower growth than that of control, and at 96 h, the difference was much more significant (**Figure 2A**). Thus, these results showed that MCPH1 may function as a negative regulator of cell proliferation.

MCPH1 induces apoptosis

We were further interested in understanding the mechanism of tumor suppression by MCPH1. To quantify the apoptotic cells with MCPH1 overexpression, ACHN and 768-0 cells were stained with Annexin-V/PI and analyzed by flow cytometry, which showed significantly increased apoptotic cell fractions in ACHN and 768-0 MCPH1 overexpression cells,

whereas the apoptotic rate was not significantly different between the two groups of control cells in each cell line (**Figure 2B**), suggesting that MCPH1 induces cell death.

MCPH1 inhibits cell migration and invasion

We performed the cell invasion assay to see the effect of MCPH1 overexpression on the invasive ability of ACHN cells. The results showed a significant decrease in number of cells that had invaded through the matrigel matrix in MCPH1 overexpressing of ACHN and 768-0 (**Figure 2C**).

miR-27a bind to the 3'-UTR of MCPH1 mRNA

To address the major mechanisms for the downregulation of MCPH1, we screened the 3'-UTR of MCPH1 mRNA for conserved miRNA-binding sites (**Figure 3A**). Since miRNAs have recently been shown to post-transcriptionally regulate genes, we sought to determine if MCPH1 is regulated by miRNAs. The TargetScan database predicted binding of miR-27 family members, miR-27a and miR-27b as potential miRNAs which could target and regulate MCPH1. To evaluate the role of miR-34 in the regulation of ULBP2 expression, we first generated reporter gene constructs, fusing the MCPH1 3'-UTR downstream to a luciferase reporter gene, yielding the construct pMCPH1-3.1 (**Figure 3B**). Furthermore, 2 deletion variants of pMCPH1-3.1 were generated, one encompassing the proximal half of the 3'-UTR (pMCPH1-3.2; **Figure 3B**) also including the predicted miR-27a binding site, and a second overlapping construct containing the distal half without the miR-27a binding site (pMCPH1-3.3; **Figure 3B**).

miR-27a control MCPH1 expression

Focusing on the regulation of MCPH1 by miR-27a, we first transfected specific miRNA mimics into ACHN cells. Transfection efficiency was verified by qRT-PCR. Western blot analysis of total cell lysates from transfectants showed that both miRNAs, in contrast to control siRNA, downregulated MCPH1 protein expression (**Figure 4A**). This downregulation was associated with a reduction in MCPH1 mRNA levels, suggesting that transfected miR-27a and miR-27c mimics induced degradation of the specific mRNA, though an impact on translation could not be excluded (**Figure 4B**). The strongest inhibitory effect on target expression was exerted by miR-27c, as already observed in reporter gene assays.

Next, we asked for the impact of endogenous miR-27a on the expression level of the specific MCPH1 in renal cancer cells. To decrease endogenous miR-27a levels, ACHN cells were transfected with a specific miR-27a inhibitor (anti–miR-27a) in comparison with a control inhibitor. As shown in **Figure 4C** and **4D**, downregulation of cellular miR-27a in ACHN cells increased the expression of the MCPH1 protein.

Discussion

MCPH1 is identified as transcriptional repressor of hTERT, the catalytic subunit of human telomerase, implicating a potential role in cellular immortalization and tumorigenesis. It harbors three BRCA1-carboxyl terminals (BRCT) domains including the single BRCT domain in the N-terminal and two tandem BRCT domains in the C-terminal. Genomic aberrations at the MCPH1 locus have been showed previously in several cancers, such as breast cancer and ovarian cancer [10, 17-20]. Compared with normal tissues, MCPH1 downregulation in breast cancer and ovarian cancer, these findings indicated that MCPH1 plays a role in the progression of cancerization.

In this study, we first evaluated the expression of MCPH1 in renal tissues of patients with renal cancer and found the expression of MCPH1 was significantly downregulated in renal cancers. In addition, we investigated the biological roles of MCPH1 in renal cancer cell lines and its possible mechanism. Our results showed that MCPH1 overexpression reduced renal cancer cells proliferation, cell migration and invasion, and induced cell apoptosis, suggesting that it indeed functions as a tumor suppressor gene, in addition to its role in brain development.

Studies have shown the involvement of microRNAs in posttranscriptional regulation of genes in cancer [21-23]. We have shown for the first time that MCPH1, being a tumor suppressor gene, is regulated by miR-27a in renal cancer and uses both of its target seed regions in its 3'-UTR. Our study has shown that the overexpression of MCPH1 exhibits anti-tumorigenic effects, and therefore it is alluring to propose that the restoration of MCPH1 could be a thera-

peutic strategy to treat renal cancer. The design of anti-mir oligos for miR-27a, which can upregulate MCPH1, can also be used for therapy. However, the precision of these assumptions require further in depth and extensive pre-clinical validations.

In summary, the results of the present study have suggested that the primary microcephaly gene MCPH1 shows several hallmarks of tumor suppress gene and functions as a tumor suppressor in renal cancer, in addition to its role in brain development. We have for the first time shown that miR-27a targets MCPH1 and regulates its expression level. It is interesting to note that none of the other eight MCPH genes have been shown to be regulated by miRNAs yet. Our study will be useful in designing novel therapeutic methods for the treatment of renal cancer.

Disclosure of conflict of interest

No potential conflicts of interest were disclosed.

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