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Role of miR-16-5p in the proliferation and metastasis of hepatocellular carcinoma

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Abstract. – OBJECTIVE: The aim of this study was to investigate the role of miR-16-5p in hepatocellular carcinoma (HCC), and to explore the possible underlying mechanism.

PATIENTS AND METHODS: 100 pairs of cancerous and para-cancerous tissues surgically removed in our hospital were collected. Real Time quantitative-Polymerase Chain Reaction (qRT-PCR) was used to detect the expression level of miR-16-5p in tissues. Bioinformatics and Dual-Luciferase reporter gene assay were used to screen and verify the potential target genes of miR-16-5p, respectively. Human HCC SMMC-7721 cells were used for functional experiments. Cell proliferation was detected by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Cell invasion and migration were evaluated by transwell and scratch wound-healing assay, respectively. The protein expression levels of epithelial-mesenchymal transition (EMT) associated markers were measured by Western blot (WB) assay.

RESULTS: QRT-PCR showed that miR-16-5p expression in HCC tissues was significantly lower than that of adjacent normal liver tissues. At the cellular level, miR-16-5p was lowly expressed in HCC cells (SMMC-7721). Bioinformatics websites (including Targetscan, PicTar, miRanda) predicted that insulin-like growth factor 1 receptor (IGF1R) was a potential target gene of miR-16-5p. Meanwhile, IGF1R was selected for further investigation due to its metastatic function. The results showed that no significant difference was found in the mRNA expression level of IGF1R in HCC tissues. However, the protein level of IGF1R was significantly up-regulated, which was negatively correlated with miR-16-5p. Combined with Dual-Luciferase reporter gene assay, it was confirmed that miR-16-5p could regulate the expression of IGF1R in a targeted manner. Furthermore, down-regulation of IGF1R significantly reduced the inhibitory effect of miR-16-5p on the proliferation and metastasis of SMMC-7721 cells.

CONCLUSIONS: We showed that miR-16-5p suppressed invasion and migration of HCC cells, mechanically by directly targeting and inhibiting IGF1R protein expression. The newly identified miR-16-5p/IGF1R axis might provide new insights into the pathogenesis of HCC and novel potential therapeutic targets for the treatment of HCC.

Key Words:
Hepatocellular carcinoma (HCC), MicroRNA-16-5p (MiR-16-5p), Insulin-like growth factor 1 receptor (IGF1R), Epithelial-mesenchymal transition (EMT).

Introduction

Hepatocellular carcinoma (HCC) is a common malignant tumor in China, whose mortality rate ranks second among all malignant tumors. Approximately 250,000 people die from HCC every year in the world, of which 45% are from China[1]. Despite continuous improvement in the medical level and prognosis of HCC in recent years, the 5-year survival rate of HCC patients is only 30-50% around the world. Meanwhile, the prognosis is far from satisfactory[2]. Currently, radiotherapy and chemotherapy assisted surgical resection is widely used in the treatment of HCC. This method can inhibit tumor growth and metastasis to a certain degree; however, it is far

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from solving the problem of tumor recurrence[3]. In recent years, the research and development of targeted drugs for HCC has gradually become a hotspot. HCC is a multi-factor, multi-stage and multi-gene heterogeneous disease. Moreover, the incidence and development of HCC are accompanied by a complex gene regulatory network. This leads to poor targeting of anti-tumor drugs currently[4]. Therefore, clarifying the underlying molecular mechanism of HCC has important scientific significance and clinical value for exploring new diagnostic and therapeutic targets. Micro-ribonucleic acid (miRNA) family members lin-4[5] and let-7[6] were discovered in nematode in 1993 and 2000, respectively. Due to the ability to regulate gene expression at the post-transcriptional level, miRNAs have attracted increasingly more attention. Meanwhile, they can explain the inconsistency between gene transcriptional level and protein level. With the development of high-throughput gene sequencing technique, more and more miRNAs have been found in animals and plants. At the same time, the functions of miRNAs have been identified. It has been found that miRNAs are involved in the incidence and development of a variety of diseases[7-10]. Furthermore, the research on miRNAs in malignancies has quickly become a hotspot[11-14]. The role of miRNA in cancer has been studied in a large number of works through functional gain and loss. For example, the deleted expression of miR-15a/16-1 cluster in chronic lymphocytic leukemia has been reported previously, thus promoting tumor development[15]. MiR-494 has been found to have a cancer-inhibiting effect on ovarian cancer. Meanwhile, it has the ability in regulating fibroblast growth factor receptor 2 (FGFR2)[16]. These studies provide considerable evidence for extending people's understanding of the pathogenesis of cancer. MicroRNA-16-5p (MiR-16-5p) is an important component of the microRNA regulatory network. Its strong inhibitory effect on the development of breast cancer has been widely reported[17-20]. Moreover, miR-16-5p also plays an important role in other malignant tumors, including gastric cancer[21], osteosarcoma[22] and chordoma[23]. However, no reports have investigated the role of miR-16-5p in HCC. In this work, we first detected the expression of miR-16-5p in HCC, and further studied the role of miR-16-5p in HCC cells. Our study might provide new suggestions and theoretical basis for clinical treatment and prevention of HCC.

Patients and Methods

Tissue Samples and Cell Lines

From July 2015 to September 2017, 100 patients pathologically diagnosed with HCC in our hospital were enrolled. Meanwhile, 100 pairs of carcinoma tissues and para-carcinoma tissues surgically resected were collected. Signed informed consent was obtained from each subject before the study. No patient received radiotherapy, chemotherapy and other adjuvant therapies before the operation. This study was approved by the Ethics Committee of Shanghai Pudong New Area People's Hospital. Human HCC cell line SMMC-7721 and normal liver cell line HL-7702 were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). After resuscitation using the conventional method, SMMC7721 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) in a 5% CO_2, 37°C incubator.

Determination of Target Gene

Bioinformatics prediction software [miRBase (http://www.mirbase.org/), TargetScan (http://www.targetscan.org/) and PicTar (http://pictar.mdc-berlin.de/)] predicted that insulin-like growth factor 1 receptor (IGF1R) was a downstream target gene of miR-16-5p. Meanwhile, it had a binding site at the 3'-untranslated region (UTR). Dual-Luciferase reporter gene assay: construction of the Luciferase reporter plasmid for 3'- UTR of IGF1R: the 3'-UTR fragment of IGF1R containing a seed sequence of miR-16-5p was cloned from genomic DNA. Subsequently, it was inserted into the downstream of firefly Luciferase reporter gene plasmid PGL-3, namely wild-type (WT) gene (IGF1R-3'-UTR-WT). At the same time, the 3'-UTR site-directed mutation was performed for a seed sequence of miR-16-5p using the QuickChange™ site-directed mutation kit (StrataGene, La Jolla, CA, USA), namely mutant-type (MT) gene (IGF1R-3'-UTR-MT). SMMC-7721 cells were first inoculated into 48-well plates. Subsequently, the cells were incubated with miR-16-5p mimics or NC for 6 h. Next, they were transfected with the 3'-UTR firefly Luciferase reporter plasmid and internal control plasmid pRLCMV containing Renilla Luciferase reporter gene for 48 h. The firefly Luciferase signal was measured. Finally, the binding effect of miR-16-5p to IGF1R mRNA 3'-UTR was eva-

luated by the ratio of firefly Luciferase activity to Renilla Luciferase activity.

Cell Transfection

When the density of the cells was about 80%, they were digested with 0.25% trypsin, passaged and inoculated into 6-well plates (1×10^5/well). When the cell density was about 70-80%, the original medium was replaced by serum-free medium. SMMC7721 cells were transfected with miR-16-5p mimics, NC and LV-IGF1R according to the instructions of Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA). After 48 h, the expression level of miR-16-5p in SMMC7721 cells was detected *via* Real Time quantitative-Polymerase Chain Reaction (qRT-PCR).

QRT-PCR Analysis

The expression levels of miR-16-5p and IGF1R in tissues or cells were detected *via* qRT-PCR. Total RNA was extracted from tissues or cells in strict accordance with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The purity and concentration of extracted RNA were detected using an ultraviolet spectrophotometer. 10 ng total RNA was taken from each specimen, and complementary deoxyribonucleic acid (cDNA) was synthesized according to the instructions of One Step PrimeScript cDNA Synthesis Kit (TaKaRa, Otsu, Shiga, Japan). Subsequently, 2 μL cDNA was taken for qRT-PCR according to the instructions of One Step SYBR PrimeScript™ RT-PCR Kit (TaKaRa, Otsu, Shiga, Japan). U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal references for miR-16-5p and IGF1R, respectively. The relative expression level of the gene was calculated by the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were as follows: miR-16-5p, F: 5'-CTTAAGAACCCTCCTTACTC-3', R: 5'-AAGCTACCCTAGGGGAAGGA-3'; IGF1R, F: 5'-CCAATGTAAACATCCTCGACTG-3', R: 5'-GGTTGTGTCGTGGAGTCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot (WB) Analysis

48 h after transfection, the cells in each group were collected, and 200 μL protein lysis buffer was added for total protein extraction. The protein concentration was detected using bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). The amount of extracted protein in each group was 20 μg. The protein samples were mixed evenly with loading buffer and were boiled in boiling water for 10 min for protein denaturation. After separating by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under a constant voltage, the proteins on the gel were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) using the wet transfer method. After sealing with 5% bovine serum albumin and vibrated on a shaking table for 2 h, the PVDF membranes were washed with Tris-Buffered Saline and Tween-20 (TBST) 3 times. Then the membranes were incubated with primary antibodies (1:1000) of β-actin and IGF1R at 4°C overnight. After washing again with TBST 3 times, the membranes were incubated with secondary antibody (1:5000) at room temperature for 1 h. After that, the membranes were washed with TBST 3 times. Immunoreactive bands were developed by the electrochemiluminescence (ECL) developer (Thermo Fisher Scientific, Waltham, MA, USA), followed by observation and photography using the gel imaging device. Finally, the protein expression level was expressed as the ratio of optical density target protein/optical density GAPDH.

Cell Proliferation

SMMC7721 cells in logarithmic growth phase were first inoculated into 96-well plates at a density of 5.0×10^3 cells/well. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Sigma-Aldrich, St. Louis, MO, USA) was performed at 24, 48, 72, 96 and 120 h after transfection. Briefly, 4 μL MTT solution was added in each well, followed by incubation for another 4 h. After the addition of 150 μL dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) in each well, the absorbance at the wavelength of 490 nm was measured by a microplate reader.

Transwell Assay

50-100 μL Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) diluent (1:20) was first evenly coated onto the basement membrane covering the bottom of chamber and air dried at 4°C. Then 100 μL Roswell Park Memorial Institute-1640 (RPMI-1640) culture medium (HyClone, South Logan, UT, USA) was added into each transwell chamber to hydrate the basement membrane, followed by standing at 37°C for 1 h. After transfection, the cells in each group were collected, washed with serum-free medium, digested with

0.25% trypsin and centrifuged at 1200 rpm for 5 min. Then the cells were re-suspended with RPMI-1640 medium containing 1% FBS to make a final concentration of 1×10^5 cells/mL. The liquid in the upper transwell chamber was discarded and 900 µL RPMI-1640 medium containing 10% FBS was added into the lower chamber. Meanwhile, 200 µL cell suspension were added into the upper chamber (about 1×10^5 cells/chamber). After routine culture for 24-48 h, the transwell chamber was removed, washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 30 min. After the bottom membrane of the chamber was air dried, the transwell chamber was stained with crystal violet for 20 min. The cells were observed and photographed under an optical microscope. 5 visual fields were randomly selected for counting in each group.

Wound Healing Assay

HCC cells were first inoculated into 6-well plates. When the fusion degree of cells grew to 90%, 200 µL sterile pipette tip was used to sterilize the vertical scratch. The initial scratch distance was measured under a microscope. After incubation for 48 h, the scratch distance was measured and the cell mobility was calculated.

Statistical Analysis

Statistical analysis was performed with Student's *t*-test or *F*-test. All *p*-values were two-sided, and $p<0.05$ were considered statistically significant. Prism 6.02 software (La Jolla, CA, USA) was used for all statistical analyses. All data were presented as means ± standard deviations.

Results

Abnormal Expression of MiR-16-5p in HCC Cases and Cells

The basis of our study was the discovery of aberrantly expressed miRNAs in clinical samples of HCC. We found that miR-16-5p was lowly expressed in 100 HCC tissue samples. The expression of miR-16-5p in normal liver tissues was approximately three times higher than that of the HCC tissues (Figure 1A). The above results aroused our interest. To further explain, we detected the expression of miR-16-5p in HCC cells and normal liver cells by qRT-PCR. As expected, the expression of miR-16-5p in HCC cells was also significantly lower than that of normal liver cells (Figure 1B).

IGF1R Was a Potential Target Gene of MiR-16-5p

The possible targets of miR-16-5p were predicted by miRBase, TargetScan and PicTar online websites. It was found that IGF1R was a potential recognition and binding target of miR-16-5p (Figure 2A). In view of this, we used qRT-PCR

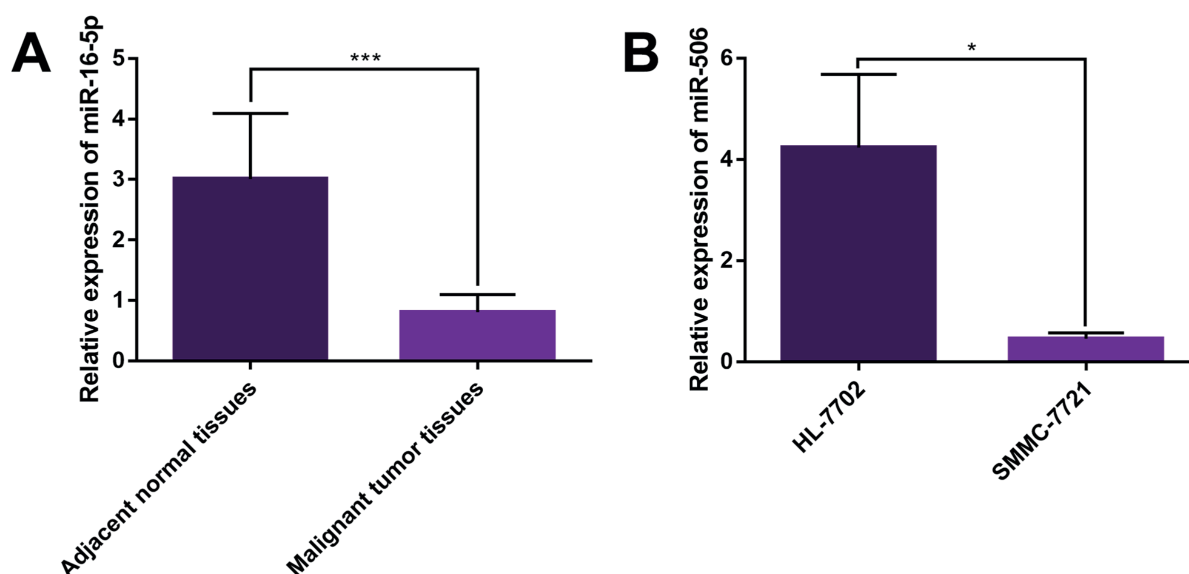

Figure 1. The expressions of miR-16-5p in HCC tissue samples and cells. ***A,*** Difference in the expression of miR-16-5p between HCC tissues and adjacent normal tissues (***$p<0.001$). ***B,*** The expression of miR-16-5p in HCC cell lines (SMMC7721) and normal liver cell lines (HL-7702) (*$p<0.05$).

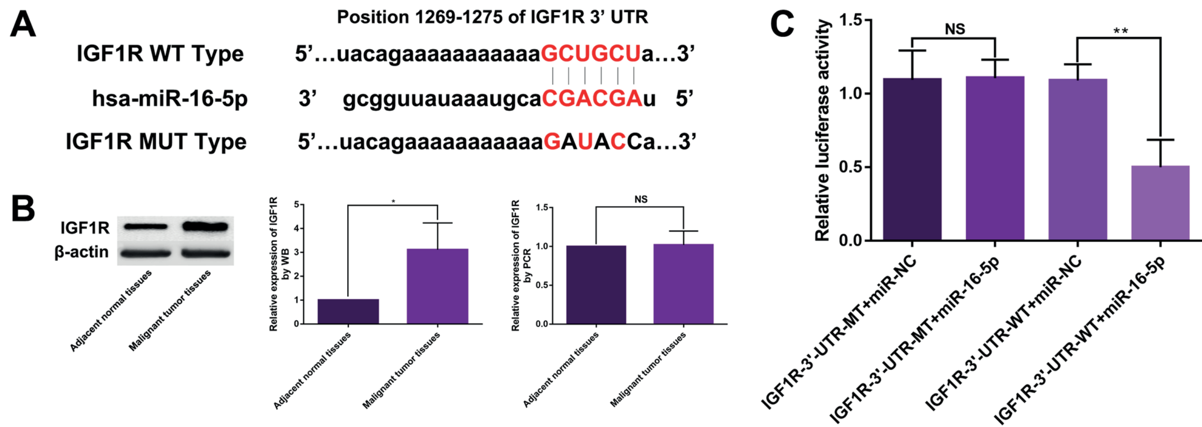

Figure 2. IGF1R was a direct and functional target of miR-16-5p. ***A,*** Diagram of putative miR-16-5p binding sites of IGF1R. ***B,*** The mRNA and protein expressions of IGF1R in HCC tissues and adjacent normal tissues were detected by WB and qRT-PCR assay ($^{*}p<0.05$). ***C,*** Relative activities of Luciferase reporters ($^{**}p<0.01$).

and WB assay to detect the mRNA and protein expressions of IGF1R in HCC tissues and adjacent normal tissues, respectively. Interestingly, significant differences were found in the protein expression level, whereas no significant difference was found in mRNA expression level (Figure 2B). This powerful evidence indicated that the regulation of IGF1R expression was mediated by post-transcriptional regulation. Dual-Luciferase reporter gene assay demonstrated that miR-16-5p remarkably inhibited the Luciferase activity in cells transfected with IGF1R-3'-UTR-WT. However, it had no significant effect on the Luciferase activity in cells transfected with IGF1R-3'-UTR-MT (Figure 2C). The results indicated that IGF1R was negatively regulated by miR-16-5p in HCC, which functioned as a target of miRNA-16-5p.

The Role of MiR-16-5p/IGF1R in HCC Cell Function

Three different groups were established in cell function experiments, including miR-NC group (negative control), miR-16-5p mimics group (SMMC7721 cells transfected with miR-16-5p mimics) and miR-16-5p mimics+IGF1R group (SMMC7721 cells transfected with miR-16-5p mimics and LV-IGF1R). Consistent with clinical trial results, the protein expression of IGF1R was significantly reduced in HCC cells overexpressing miR-16-5p (Figure 3A). This also confirmed the negative regulation of miR-16-5p on IGF1R at the cellular level. MTT results indicated that the transfection of miR-16-5p mimics could significantly inhibit the proliferation of HCC cells. However, the proliferative capacity of HCC has remarkably increased again after up-regulating the expression of IGF1R (Figure 3B). The impacts of miR-16-5p on the migration and invasion of SMMC7721 cells were examined by wound-healing and transwell assay, respectively. As shown in Figure 4A, miR-16-5p mimics transfection significantly reduced the invasion and migration abilities of HCC cells. Subsequently, we detected changes in the expression of epithelial-mesenchymal tran-

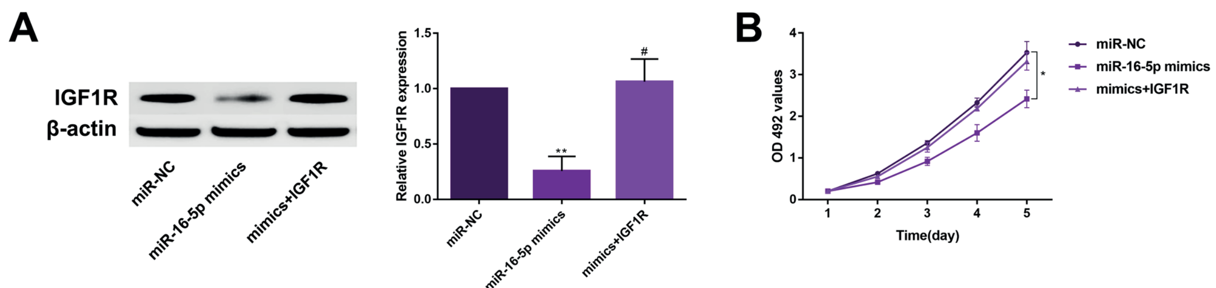

Figure 3. ***A,*** MiR-16-5p decreased the protein expression level of IGF1R (**$p<0.01$ *vs.* NC group; #$p<0.05$ *vs.* miR-16-5p Mimics group). ***B,*** MiR-16-5p blocked the proliferation of HCC cells (*$p<0.05$).

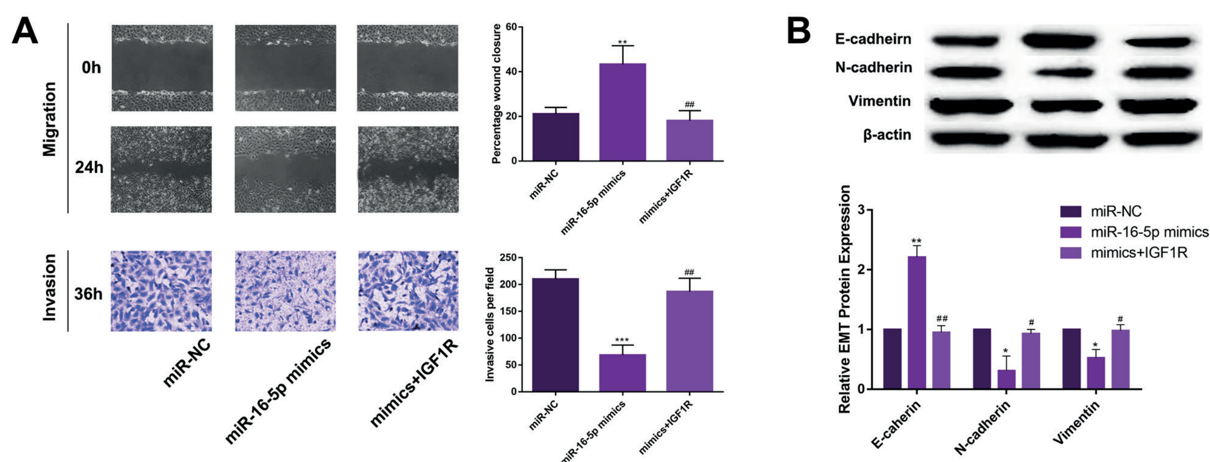

Figure 4. MiR-16-5p/IGF1R axis inhibited the invasion and migration of HCC cells. ***A,*** The migration and invasion of HCC cells were detected by wound-healing assay and transwell assay, respectively (**$p<0.01$, ***$p<0.001$ *vs.* NC group; ##$p<0.01$ *vs.* miR-16-5p Mimics group)**.** ***B,*** The epithelial-mesenchymal transition (EMT) of HCC cells. The expression levels of EMT markers after transfection were detected by WB assay (*$p<0.05$, **$p<0.01$ *vs.* NC group; #$p<0.05$, ##$p<0.01$ *vs.* miR-16-5p Mimics group).

sition (EMT)-related proteins. The results found that EMT was also inhibited in miR-16-5p mimics group. However, after the expression of IGF1R was restored, the metastatic ability of HCC cells also correspondingly recovered (Figure 4B).

Discussion

Tumor is a kind of heterogeneous disease involving multiple gene mutations and stages. The molecular mechanism is very complex and remains unclear. A large number of non-coding genes in the human genome, and non-coding RNAs transcribed by them were initially considered to have no biological functions. In recent years, it has been gradually demonstrated that non-coding RNAs play important roles in regulating the occurrence and development of malignant tumors. Therefore, deeply studying the molecular function and mechanism is helpful to further understand the complexity of gene regulation in the tumorigenic mechanism. It also has some certain important scientific values in searching for effective molecular diagnosis, treatment and prognosis. MiRNA is an important member of non-coding RNAs. Previous studies have indicated that miRNAs can regulate the occurrence and development of tumor by negative regulation of cancer-related gene expression. Meanwhile, it is also considered as a potential molecular target for the diagnosis and treatment of tumor[24-26]. Therefore, further exploring the function and mechanism of differentially-expressed miRNAs in HCC samples is of great scientific significance in clarifying molecular mechanism of the HCC development and investigating new therapeutic targets. In recent years, it has been found that miR-16-5p plays an important role in regulating the malignant biological phenotypes of various diseases. It is also considered as a potential diagnostic and therapeutic target[17,27-29]. However, the role of miR-16-5p in HCC has not been reported. In this study, the expression of miR-16-5p in 100 pairs of HCC and para-carcinoma tissues was detected. QRT-PCR results found that miR-16-5p expression was significantly inhibited in HCC tissues. MiRNA regulatory networks are extremely complex. The expression of multiple genes can be regulated by a single miRNA. Simultaneously, different miRNAs can also co-regulate a single target gene. These complicated regulatory networks are involved in the regulation of various biological behaviors of tumor cells[11,30]. Therefore, searching for specific regulatory targets in diseases has become a key part of research on miRNA. In our study, bioinformatics predicted that IGF1R gene had a targeted sequence matching miR-16-5p in the segment 1269-1275. IGF1R is a transmembrane protein, as well as a tyrosine kinase receptor. IGF1R is activated by two ligands, namely IGF1 and IGF2. IGF1R is lowly or not expressed in normal tissues, whereas is highly expressed in tumor tissues. Besides, the abnormal expression of IGF1R may activate the activity of multiple downstream signal transduction pathways, such

as phosphatidylinositol 3-hydroxy kinase (PI3K)/protein kinase B (Akt), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK), EP2/EP4 and RAS/RAF/ERK signal transduction pathways[31-33]. This may eventually regulate the proliferation and apoptosis of various malignant tumor cells, promote EMT and participate in cell migration and invasion[34-37]. However, a few researches have focused on the regulatory effects of IGF1R on the migration and invasion of HCC cells. Currently, no significant difference was found in the mRNA level of IGF1R in HCC. However, the protein level of IGF1R in HCC was significantly upregulated. This suggested that IGF1R up-regulation in HCC was mediated by post-transcriptional regulation. Meanwhile, IGF1R had a significant negative correlation with miR-16-5p. Combined with the Luciferase reporter assay, it was confirmed that miR-16-5p could regulate the expression of IGF1R in a targeted manner. These results all demonstrated that miR-16-5p was a major factor for post-transcriptional regulation of IGF1R. The epithelial-mesenchymal transition (EMT) is a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal stem cells; these are multipotent stromal cells that can differentiate into a variety of cell types. EMT is essential for numerous developmental processes including mesoderm formation and neural tube formation. EMT has also been shown to occur in wound healing, organ fibrosis and in the initiation of metastasis in cancer progression. Invasion and migration are important factors affecting the prognosis of tumor patients[38,39]. EMT is an important mechanism of tumor cell invasion and migration; it especially plays a vital role in the initiation of metastasis[40,41]. In EMT, the morphology and phenotype of epithelial cells are transformed into mesenchymal cells. Their epithelial marker E-cadherin expression is lowered, and mesenchyme markers N-cadherin and Vimentin are increased. In this study, we used a series of functional experiments to evaluate the invasion and migration abilities of HCC cells. The results showed that after the up-regulation of IGF1R in HCC cells, the invasion and migration of HCC cells were significantly inhibited. Furthermore, even EMT suppression by miR-16-5p was remarkably weakened. Nowadays, the function of miRNAs as well as their target genes has great potential to become new targets and directions for the treatment of HCC. Our findings may also provide a new target for HCC treatment.

Conclusions

We showed that miR-16-5p suppressed the invasion and migration of HCC cells, mechanically by directly targeting and inhibiting IGF1R protein expression. The newly identified miR-16-5p/IGF1R axis provided new insights into the pathogenesis of HCC, and might become a novel potential therapeutic target for the treatment of HCC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References

1) Siddique O, Yoo ER, Perumpail RB, Perumpail BJ, Liu A, Cholankeril G, Ahmed A. The importance of a multidisciplinary approach to hepatocellular carcinoma. J Multidiscip Healthc 2017; 10: 95-100.
2) Zucman-Rossi J, Villanueva A, Nault JC, Llovet JM. Genetic landscape and biomarkers of hepatocellular carcinoma. Gastroenterology 2015; 149: 1226-1239.
3) Lai KKY, Kweon SM, Chi F, Hwang E, Kabe Y, Higashiyama R, Qin L, Yan R, Wu RP, Lai K, Fujii N, French S, Xu J, Wang JY, Murali R, Mishra L, Lee JS, Ntambi JM, Tsukamoto H. Stearoyl-CoA desaturase promotes liver fibrosis and tumor development in mice via a Wnt positive-signaling loop by stabilization of low-density lipoprotein-receptor-related proteins 5 and 6. Gastroenterology 2017; 152: 1477-1491.
4) Finkin S, Yuan D, Stein I, Taniguchi K, Weber A, Unger K, Browning JL, Goossens N, Nakagawa S, Gunasekaran G, Schwartz ME, Kobayashi M, Kumada H, Berger M, Pappo O, Rajewsky K, Hoshida Y, Karin M, Heikenwalder M, Ben-Neriah Y, Pikarsky E. Ectopic lymphoid structures function as microniches for tumor progenitor cells in hepatocellular carcinoma. Nat Immunol 2015; 16: 1235-1244.
5) Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 1993; 75: 843-854.
6) Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 2000; 403: 901-906.
7) Zampetaki A, Kiechl S, Drozdov I, Willeit P, Mayr U, Prokopi M, Mayr A, Weger S, Oberhollenzer F, Bonora E, Shah A, Willeit J, Mayr M. Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. Circ Res 2010; 107: 810-817.
8) Ikeda S, Kong SW, Lu J, Bisping E, Zhang H, Allen PD, Golub TR, Pieske B, Pu WT. Altered microRNA

expression in human heart disease. Physiol Genomics 2007; 31: 367-373.

9) van Wijnen AJ, van de Peppel J, van Leeuwen JP, Lian JB, Stein GS, Westendorf JJ, Oursler MJ, Im HJ, Taipaleenmaki H, Hesse E, Riester S, Kakar S. MicroRNA functions in osteogenesis and dysfunctions in osteoporosis. Curr Osteoporos Rep 2013; 11: 72-82.

10) Yao YF, Qu MW, Li GC, Zhang FB, Rui HC. Circulating exosomal miRNAs as diagnostic biomarkers in Parkinson's disease. Eur Rev Med Pharmacol Sci 2018; 22: 5278-5283.

11) Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006; 6: 857-866.

12) Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, Magri E, Pedriali M, Fabbri M, Campiglio M, Menard S, Palazzo JP, Rosenberg A, Musiani P, Volinia S, Nenci I, Calin GA, Querzoli P, Negrini M, Croce CM. MicroRNA gene expression deregulation in human breast cancer. Cancer Res 2005; 65: 7065-7070.

13) Hu Z, Chen X, Zhao Y, Tian T, Jin G, Shu Y, Chen Y, Xu L, Zen K, Zhang C, Shen H. Serum microRNA signatures identified in a genome-wide serum microRNA expression profiling predict survival of non-small-cell lung cancer. J Clin Oncol 2010; 28: 1721-1726.

14) Kurozumi A, Goto Y, Matsushita R, Fukumoto I, Kato M, Nishikawa R, Sakamoto S, Enokida H, Nakagawa M, Ichikawa T, Seki N. Tumor-suppressive microRNA-223 inhibits cancer cell migration and invasion by targeting ITGA3/ITGB1 signaling in prostate cancer. Cancer Sci 2016; 107: 84-94.

15) Veronese A, Pepe F, Chiacchia J, Pagotto S, Lanuti P, Veschi S, Di Marco M, D'Argenio A, Innocenti I, Vannata B, Autore F, Marchisio M, Wernicke D, Verginelli F, Leone G, Rassenti LZ, Kipps TJ, Mariani-Costantini R, Laurenti L, Croce CM, Visone R. Allele-specific loss and transcription of the miR-15a/16-1 cluster in chronic lymphocytic leukemia. Leukemia 2015; 29: 86-95.

16) Zhao X, Zhou Y, Chen YU, Yu F. miR-494 inhibits ovarian cancer cell proliferation and promotes apoptosis by targeting FGFR2. Oncol Lett 2016; 11: 4245-4251.

17) Rinnerthaler G, Hackl H, Gampenrieder SP, Hamacher F, Hufnagl C, Hauser-Kronberger C, Zehentmayr F, Fastner G, Sedlmayer F, Mlineritsch B, Greil R. M iR-16-5p is a stably-expressed housekeeping microRNA in breast cancer tissues from primary tumors and from metastatic sites. Int J Mol Sci 2016; 17: 10.3390/ijms17020156.

18) Qu Y, Liu H, Lv X, Liu Y, Wang X, Zhang M, Zhang X, Li Y, Lou Q, Li S, Li H. MicroRNA-16-5p overexpression suppresses proliferation and invasion as well as triggers apoptosis by targeting VEGFA expression in breast carcinoma. Oncotarget 2017; 8: 72400-72410.

19) Li Z, Peng Z, Gu S, Zheng J, Feng D, Qin Q, He J. Global analysis of miRNA-mRNA interaction network in breast cancer with brain metastasis. Anticancer Res 2017; 37: 4455-4468.

20) Alarmo EL, Havunen R, Hayrynen S, Penkki S, Ketolainen J, Nykter M, Kallioniemi A. Bone morphogenetic protein 4 regulates microRNA expression in breast cancer cell lines in diverse fashion. Genes Chromosomes Cancer 2016; 55: 227-236.

21) Zhu C, Huang Q, Zhu H. Melatonin inhibits the proliferation of gastric cancer cells through regulating the miR-16-5p-Smad3 pathway. DNA Cell Biol 2018; 37: 244-252.

22) Sang S, Zhang Z, Qin S, Li C, Dong Y. MicroRNA-16-5p inhibits osteoclastogenesis in giant cell tumor of bone. Biomed Res Int 2017; 2017: 3173547.

23) Zhang H, Yang K, Ren T, Huang Y, Tang X, Guo W. MiR-16-5p inhibits chordoma cell proliferation, invasion and metastasis by targeting Smad3. Cell Death Dis 2018; 9: 680.

24) Winter J, Diederichs S. MicroRNA biogenesis and cancer. Methods Mol Biol 2011; 676: 3-22.

25) Sassen S, Miska EA, Caldas C. MicroRNA: implications for cancer. Virchows Arch 2008; 452: 1-10.

26) Iorio MV, Croce CM. microRNA involvement in human cancer. Carcinogenesis 2012; 33: 1126-1133.

27) Zhang J, Song Y, Zhang C, Zhi X, Fu H, Ma Y, Chen Y, Pan F, Wang K, Ni J, Jin W, He X, Su H, Cui D. Circulating miR-16-5p and miR-19b-3p as two novel potential biomarkers to indicate progression of gastric cancer. Theranostics 2015; 5: 733-745.

28) Li L, Jia J, Liu X, Yang S, Ye S, Yang W, Zhang Y. MicroRNA-16-5p controls development of osteoarthritis by targeting SMAD3 in chondrocytes. Curr Pharm Des 2015; 21: 5160-5167.

29) Zheng C, Zheng Z, Sun J, Zhang Y, Wei C, Ke X, Liu Y, Deng L, Wang H. MiR-16-5p mediates a positive feedback loop in EV71-induced apoptosis and suppresses virus replication. Sci Rep 2017; 7: 16422.

30) Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005; 120: 15-20.

31) Riedemann J, Macaulay VM. IGF1R signalling and its inhibition. Endocr Relat Cancer 2006; 13 Suppl 1: S33-S43.

32) Zhou F, Nie L, Feng D, Guo S, Luo R. MicroRNA-379 acts as a tumor suppressor in non-small cell lung cancer by targeting the IGF1R-mediated AKT and ERK pathways. Oncol Rep 2017; 38: 1857-1866.

33) Han J, Zhao F, Zhang J, Zhu H, Ma H, Li X, Peng L, Sun J, Chen Z. miR-223 reverses the resistance of EGFR-TKIs through IGF1R/PI3K/Akt signaling pathway. Int J Oncol 2016; 48: 1855-1867.

34) Fagan DH, Yee D. Crosstalk between IGF1R and estrogen receptor signaling in breast cancer. J Mammary Gland Biol Neoplasia 2008; 13: 423-429.

35) Jiang L, Liu X, Chen Z, Jin Y, Heidbreder CE, Kolokythas A, Wang A, Dai Y, Zhou X. MicroRNA-7 targets IGF1R (insulin-like growth factor 1 receptor) in tongue squamous cell carcinoma cells. Biochem J 2010; 432: 199-205.

36) Dziadziuszko R, Merrick DT, Witta SE, Mendoza AD, Szostakiewicz B, Szymanowska A, Rzyman W, Dziadziuszko K, Jassem J, Bunn PA, Varella-Garcia M, Hirsch FR. Insulin-like growth factor receptor 1 (IGF1R) gene copy number is associated with survival in operable non-small-cell lung cancer: a comparison between IGF1R fluorescent in situ hybridization, protein expression, and mRNA expression. J Clin Oncol 2010; 28: 2174-2180.
37) Li J, Li D, Zhang W. Tumor suppressor role of miR-217 in human epithelial ovarian cancer by targeting IGF1R. Oncol Rep 2016; 35: 1671-1679.
38) Friedl P, Wolf K. Tumour-cell invasion and migration: diversity and escape mechanisms. Nat Rev Cancer 2003; 3: 362-374.
39) Hood JD, Cheresh DA. Role of integrins in cell invasion and migration. Nat Rev Cancer 2002; 2: 91-100.
40) Singh A, Settleman J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. Oncogene 2010; 29: 4741-4751.
41) Yilmaz M, Christofori G. EMT, the cytoskeleton, and cancer cell invasion. Cancer Metastasis Rev 2009; 28: 15-33.