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<td>Oncology Letters</td>
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microRNA-204 suppressed proliferation and motility capacity of human hepatocellular carcinoma via directly targeting ZEB2

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Running title: miR-204 in HCC.

Key words: microRNA-204, ZEB2, proliferation, metastasis, motility, HCC
Abstract

Abnormal expression of microRNA-204 (miR-204) has been found in various kinds of human cancer. However, the expression and functions of miR-204, and the underlying molecular mechanism involved in initiation and progression of hepatocellular carcinoma (HCC) remain to be investigated. The results showed that miR-204 was down-regulated in HCC tissues and cell lines. Notably, ZEB2 was identified as a direct target of miR-204 in HCC. In addition, miR-204 negatively regulated ZEB2 expression in HCC at post-transcriptional level. In functional studies, overexpression of miR-204 inhibited proliferation, migration and invasion of HCC cells. Moreover, knockdown of ZEB2 could mimics the functions of miR-204 in HCC, suggesting ZEB2 was a direct function target of miR-204. In conclusion, these results of the present study indicated that miR-204 suppressed the tumor growth, migration and invasion of HCC by directly targeting ZEB2 and may serve as a novel therapeutic target for HCC.
**Introduction**

Primary human hepatocellular carcinoma (HCC), the most common form of liver cancer, is the fifth most frequently diagnosed malignancies and the third leading cause of cancer-related death around the world(1). According to the statistical analysis, in 2015, an estimated 35,660 new HCC cases and 24,550 cancer deaths due to HCC in the USA(2). Chronic hepatitis B or C viral (HBV and HCV) infection, alcohol abuse, non-alcoholic fatty liver disease, autoimmune mediated hepatitis, primary biliary cirrhosis and exposure to carcinogens are the mainly risk factors of HCC(3). Among these risk factors, infection with HBV is the most prevalent cause of the disease worldwide and is responsible for the increasing incidence of HCC, especially in China(4, 5). HCC patients in China contribute to 55% of all patients with HCC worldwide(6). Currently, liver resection, liver transplantation, radiotherapy, chemotherapy, and targeted therapy are the standard therapeutic treatments for HCC patients with early stage(7, 8). However, the 5-year survival rate remains very low, especially for HCC patients diagnosed at advanced stages, mainly due to lack of effective therapeutic treatments(4, 9). Therefore, it is important to investigate novel therapeutic treatments so as to improve effectiveness of therapy and prognosis for HCC patients.

Micro-ribonucleic acids (miRNAs) belongs to a group of evolutionary, conserved, and non-coding small RNAs with 19 to 25 nucleotides in length(10). In human, 1881 miRNA precursors and 2588 mature miRNA sequences are deposited in miRBase since the first discovery of miRNA lin-4 in Caenorhabditis elegans(11-13). They have been demonstrated to negatively modulate expression of targeted mRNAs in animals, plants and viruses by directly binding to the 3'untranslated region (3'UTR) of targeted mRNAs in base pairing, resulting in mRNAs degradation or translational repression either at the translational or post-transcriptional levels(14, 15). A great deal of evidences have documented that miRNAs involved in a variety of cellular biological processes, such as proliferation, cell cycle, apoptosis, invasion, and migration, metastasis and so on(16-18). Abnormal expression of miRNAs has been found in various kinds of human malignancies, including HCC, suggesting that miRNAs may
contributed to the carcinogenesis and progression of these cancers (19-21). Further studies also indicated that miRNAs could be proposed as potential novel therapeutic targets for cancers (22, 23). Therefore, it is of great significance to explore the abnormal expressed miRNAs and their roles in HCC, which will provide effective and novel therapeutic targets for patients with HCC.

In the present study, we found that miR-204 was significantly down-regulated in HCC tissues and cell lines. Notably, ZEB2 was identified to be the direct target gene of miR-204 in HCC. In functional studies, enforced miR-204 expression suppressed growth, migration and invasion of HCC cells. Collectively, miR-204 involved in the growth and metastasis of HCC via negatively regulation of ZEB2 expression.

Material and Methods

Clinical specimens and cell lines.

Eighty-two HCC tissues and paired adjacent non-tumor liver tissues used in this study were obtained from The First Affiliated Hospital of Xiamen University. None of these HCC patients received chemotherapy, radiotherapy or other treatments before operation. HCC tissues and non-tumor liver tissues were snap-frozen in liquid nitrogen after surgery and stored at -80°C. All patients provided written informed consent, and this study was approved by the Research Ethics Committee of The First Affiliated Hospital of Xiamen University.

The human HCC cell lines (HepG2, PLC-5, SMMC-7721, HuH-7, HLE) and immortalized normal liver epithelial cell line (THLE-3) were both purchased from American Type Culture Collection (ATCC; Manassas, VA, USA), and cultured in Dulbecco's modified eagle's medium (DMEM; Gibco, Grand Island, NY) high glucose medium containing 10% v/v fetal bovine serum (FBS; Gibco, Grand Island, NY) and 100 IU/ml penicillin, and 100 μg/ml streptomycin (Gibco, Grand Island, NY).

Cell transfection.

miR-204 mimics and the corresponding negative control (NC) were purchased from GenePharma (Shanghai, China). The pGL3-ZEB2-3’UTR Wt and pGL3-ZEB2-3’UTR Mut were also obtained from GenePharma. For knockdown
ZEB2 expression, siRNA targeting ZEB2 (ZEB2 siRNA) or NC (NC siRNA) were purchased from Ribobio (Guangzhou, China). Oligonucleotide transfection or co-transfection was conducted with Lipofectamine® 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions.

**Quantitative real-time polymerase chain reaction (qRT-PCR).**

Total RNA was isolated from HCC tissues, adjacent non-tumor liver tissues, HCC cell lines and immortalized normal liver epithelial cells THLE-3 using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), following to the manufacturer's protocol. For miR-204 expression, cDNA was synthesized from total RNA using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, MA, USA), followed by qRT-PCR using specific TaqMan MicroRNA assays (Applied Biosystems, MA, USA). For ZEB2 mRNA expression, cDNA was synthesized using GeneAmp RNA PCR kit (Life Technologies), followed by qRT-PCR using the Power SYBR® Green Master Mix (Life technologies). qRT-PCR reaction was performed in Applied Biosystems 7500 Real-time PCR System (Applied Biosystems, Foster City, CA). U6 and GADPH was used as an internal control for miR-204 and ZEB2 mRNA expression, respectively.

**Dual-luciferase report assay.**

For the report assays, cells were seeded into 24-well plates and were co-transfected with miR-204 mimics, or NC and pGL3-ZEB2 -3’UTR Wt or pGL3-ZEB2-3’UTR Mut. At 48 h after transfection, cells were collected and luciferase activities were detected with Dual-luciferase report assay (Promega, Madison, WI), following to the manufacturer's protocol. The renilla luciferase activities were measured as an internal control for firefly activities.

**Western blot.**

Transfected cells were washed with phosphate-buffered saline (PBS), harvested and homogenized using RIPA Lysis Buffer (Beyotime, Jiangsu, China) supplemented with a Protease Inhibitor (Pierce, WI, USA), following to the manufacturer's protocol. The protein concentration was detected with a BCA assay protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA), and equal amounts of protein were
separated with 10% SDS-PAGE and transferred to PVDF membrane (Invitrogen, Carlsbad, CA, USA). Then, the membranes were blocked with TBST containing 5% skimmed milk for 2 h at room temperature, followed by incubation with primary antibodies for overnight at 4°C. After washing with TBST for three times, the membranes were probed with appropriate horseradish peroxidase-conjugated secondary antibody and visualized with FluorChem imaging system (Alpha Innotech, San Leandro, CA, USA). In the present study, the primary antibodies were mouse anti-human monoclonal ZEB2 (sc-271984; Santa Cruz Biotechnology, CA, USA) and mouse anti-human monoclonal β-actin (sc-47778; Santa Cruz Biotechnology, CA, USA). β-actin was used as an internal control for ZEB2.

**Cell proliferation assay.**

Cell counting kit 8 (CCK8; Dojindo, Kumamoto, Japan) assay was adopted to assess the effect of miR-204 in cell proliferation ability. Cells were seeded into 96-well plates at a density of 3,000 cells/well. After incubation overnight, cells were transfected with miRNAs or siRNAs. Cell proliferation assay was performed every 24 h after transfection until 96 h. In briefly, cells were treated with 10ul CCK8 assay solution for 2 h at 37°C. Absorbance at 450 nm was detected for each well with a microplate reader (Bio-Rad, Richmond, CA, USA). All experiments were performed in 5 replicates.

**Cell migration and invasion assays.**

Cell migration and invasion assays were performed using transwell chamber (8 μm pore size; Corning, Cambridge, MA, USA) coated without Matrigel (BD Biosciences, San Jose, CA) or with Matrigel, respectively. For migration and invasion assays, 1x10^5 transfected cells in 300 μL FBS-free culture medium were transferred to the top of the transwell chambers. The lower trasnwell chambers were added with 500 μL culture medium containing 20% FBS. After incubation for 24 h, cells migrating or invading to the low surface membranes of the trasnwell chambers were fixed with methanol and stained with 0.5% crystal violet. After washing with PBS for three times, cells were counted under a inverted microscope (Olympus Corporation, Tokyo, Japan).
Statistical analysis.

Data were expressed as mean ± standard deviation (SD), and compared using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). P < 0.05 was considered to be statistically significant.

Results

miR-204 was down-regulated in HCC tissues and cell lines

To explore the expression levels of miR-204 in HCC, qRT-PCR was adopted to measure differential expression levels of miR-204 in HCC tissues and paired adjacent non-tumor liver tissues. The results showed that miR-204 expression levels were decreased in HCC tissues compared with that in adjacent non-tumor liver tissues (Fig. 1a).

In addition, we detected the expression levels of miR-204 in five HCC cell lines (HepG2, PLC-5, SMMC-7721, HuH-7, HLE) and immortalized normal liver epithelial cell line (THLE-3). The results showed that, compared with THLE-3, the five HCC cell lines had lower expression levels of miR-204 (Fig. 1b). In these five HCC cell lines, HepG2 and HuH-7, had the lower miR-204 expression levels, were selected for further functional studies. These findings indicated that miR-204 may be involved in carcinogenesis and progression of HCC.

miR-204 directly targeted the 3’UTR of ZEB2 in HCC.

We used TargetScan (http://www.targetscan.org/) to predict miR-204 potential target genes. The analysis showed that ZEB2 contained a miR-204 seed match at position at 218–225 of ZEB2 3’UTR (Fig.2a). To confirm this predication, Dual-luciferase reporter assays were performed. As shown in Fig.2b, ectopic of miR-204 expression in HepG2 and HuH-7 cells significantly suppressed the luciferase activities of pGL3-ZEB2-3’UTR Wt, but had no effect on the luciferase activities of pGL3- ZEB2-3’UTR Mut. Above all, miR-204 directly targeted the 3’UTR of ZEB2 in HCC.

miR-204 negatively regulated ZEB2 expression at post-transcriptional level.

Next, we performed qRT-PCR and western blot to elucidate whether miR-204 could regulate ZEB2 expression. The qRT-PCR results indicated that high expression
levels of miR-204 decreased ZEB2 mRNA expression levels in HepG2 and HuH-7 cells (Fig.3a). Meanwhile, western blot showed that over-expression of miR-204 could also reduce ZEB2 protein expression levels in HepG2 and HuH-7 cells (Fig.3b). Collectively, miR-204 negatively modulated ZEB2 expression levels in HCC through mRNA cleavage mechanism at the post-transcriptional level

**miR-204 suppressed cell proliferation, migration and invasion of HCC cells.**

To explored the cellular functions of miR-204 in HCC, miR-204 mimics or NC was introduced into HepG2 and HuH-7 cells. qRT-PCR was performed to assess the transfection efficiency. The results showed that miR-204 expression levels were remarkably increased in miR-204 mimics-transfected HepG2 and HuH-7 cells, compared with the NC groups (Fig.4a).

Next, cell proliferation assay, cell migration and invasion assays were conducted to investigate the cellular functions of miR-204 in growth, migration and invasion of HCC. The results showed that high expression levels of miR-204 resulted into a significant decrease in cell proliferation of HepG2 and HuH-7 cells (Fig.4b). The migration and invasion assays showed that enforced miR-204 expression suppressed migration and invasion abilities of HepG2 and HuH-7 cells (Fig.4c). Thus, restoration of miR-204 expression suppressed growth and motility in HCC.

**Knockdown of ZEB2 inhibited proliferation, migration and invasion of HCC cells.**

To determine whether the cellular functions of miR-204 in HCC was mediated by ZEB2, HepG2 and HuH-7 cells were transfected with ZEB2 siRNA or NC siRNA. Western blot confirmed that the ZEB2 siRNA significantly decreased expression levels of ZEB2 in HepG2 and HuH-7 cells (Fig.5a).

The effects of ZEB2 siRNA on proliferation, migration and invasion of HCC cells were also assessed using cell proliferation assay, cell migration and invasion assay. Consistently, ZEB2 siRNA suppressed proliferation, migration and invasion of HepG2 and HuH-7 cells (Fig.5b and Fig.5c). These results indicated that miR-204 inhibited growth, migration and invasion of HCC by knockdown of ZEB2 expression.

**Discussion**
HCC is one of the most common malignant around the world and the second most common cause of cancer-related deaths in China(24). Therefore, understanding the molecular mechanisms underlying carcinogenesis and progression of HCC is essential in developing novel therapeutic treatments for HCC patients to improve survival. A large number of evidences demonstrated that abnormal expression of miRNAs may play important functions in HCC initiation and development, suggesting that miRNA could be investigate as a new direction in targeted therapy for HCC(25-27).

In this study, we initially showed that miR-204 was significantly down-regulated in HCC tissues and cell lines, indicating the abnormal expression of miR-204 may contribute to HCC initiation and development. Secondly, TargetScan was used to predicate the potential target genes of miR-204. The results showed that ZEB2 contained a miR-204 seed match at position at 3'UTR of ZEB2. Dual-luciferase reporter assays indicated that miR-204 directly targeted to 3'UTR of ZEB2. qRT-PCR and western blot showed that ZEB2 mRNA and protein expression levels were decreased in HCC cells transfected with miR-204 mimics. These results indicated that miR-204 negatively regulated ZEB2 expression at the post transcription level via directly targeting the 3'UTR of ZEB2. Thirdly, we calculated the cell growth and metastasis through the cell proliferation assay, cell migration and invasion assay to explore the association between miR-204 and the growth and metastasis capacity of HCC cells. The cell growth viability and metastasis of HCC cells transfected with the miR-204 mimics was obviously decreased compared with that in NC groups. Finally, the functions of ZEB2 in HCC was also measured. The results showed that underexpression of ZEB2 could mimics the functions of miR-204 in HCC, suggesting that ZEB2 was a functional target of miR-204 in HCC.

miR-204 has been found to be down-regulated in many human tumors, such as thyroid cancer(28), renal cell carcinoma(29), breast cancer(30), glioma(31), acute myeloid leukemia(32), osteosarcoma(33) and ovarian cancer(34). In functional studies, miR-204 was demonstrated as a tumor suppressor in these cancers. For example, in thyroid cancer, miR-204 suppressed cell proliferation by targeting HMGA2 and
IGFBP5(28, 35). Wu et al. reported that over-expression of miR-204 targeted SOX4 to inhibit growth, migration and invasion of renal cell carcinoma(29). In breast cancer, low expression levels of miR-204 were found to be correlated with TNM stage, metastasis and chemotherapeutic resistance of breast cancer patients. In addition, patients with low miR-204 expression had poorer overall survival time and disease free survival time than those with high miR-204 expression(36). miR-204 improved breast cancer apoptosis via targeting JAK2 through the STAT3/Bcl2 pathway(30). Xia et al. found that upregulation of miR-204 significantly decreased glioma cell growth, migration and invasion in vitro, and suppressed tumorigenesis and increased overall host survival in vivo through regulation of RAB22A(31). Mao and his colleagues reported that miR-204 targeted ezrin to decrease migration and invasion abilities of glioma(37). These findings suggested that miR-204 played important functions, and could be investigated as potential targeted therapeutic treatments for these cancers.

Identification of miR-204 target mRNAs is essential for investigating its functions in carcinogenesis and progression of HCC. It is also important for developing new targeted therapies. In the present study, ZEB2 was identified as a functional target gene of miR-204 in HCC. ZEB2, a member of the zinc finger family, has been found to be up-regulated in many human cancers, such as breast cancer, gastric cancer, glioma, ovarian cancer, and non-small cell lung carcinoma(38-44). In HCC, ZEB2 was also up-regulated and expression levels of ZEB2 in nuclear were associated with vasculogenic mimicry and metastasis. HCC Patients with ZEB2 nuclear expression had a shorter survival period than those without expression. In further studies, over-expression of ZEB2 induced motility, invasiveness and vasculogenic mimicry of HCC cells. In contrast, knockdown of ZEB2 suppressed HCC cell motility, invasiveness and vasculogenic mimicry formation(45). This was in accordance with our findings in this study.

ZEB2 has been found to be regulated by multiple miRNAs in a great deal of human cancers. For example, in gastric cancer, miR-141 targeted ZEB2 to inhibit cancer cell migration(46). Zhou et al. reported that miR-153 suppressed cell growth and invasion of ovarian cancer through directly targeting ZEB2(47). In renal cell
carcinoma, miR-205 inhibited cancer cell growth, migration, invasion, and induced apoptosis via blockade of ZEB2(48). All these studies indicated that miRNAs may act as a regulation of ZEB2 in human cancers. In the present study, upregulation of miR-204 in HCC cell lines, interestingly, we showed that miR-204 inhibited cell growth, migration and invasion by negatively regulation of ZEB2. It could be investigated as a targeted therapy of HCC.

In conclusion, the expression of miR-204 was down-regulated in HCC tissues and cell lines. In vitro functional studies showed that miR-204 acted as a tumor suppressor by inhibiting proliferation, migration and invasion of HCC. Its multiple tumor suppressor functions are mediated by ZEB2. Collectively, the down-regulation of miR-204 may play an important role in tumor growth and metastasis, and may be a potential therapeutic target for HCC.
Disclosure

The authors declare that they have no conflict of interest related to the publication of this manuscript.

Acknowledgement

This work was supported by National Natural Science Foundation of China (81200578, 81301923) and National Natural Science Foundation of Fujian (2011D012, 2015J01561).
References


Legends

Fig.1 miR-204 was down-regulated in HCC tissues and cell lines. a Expression levels of miR-204 in HCC tissues were significantly lower than that in paired adjacent non-tumor liver tissues. b The results showed that, compared with THLE-3, the five HCC cell lines had lower expression levels of miR-204.

*P<0.05 compared with their respective controls.

Fig.2 miR-204 directly targeted the 3’UTR of ZEB2 in HCC. a The predicted binding sites of miR-204 on 3’UTR of ZEB2 and the mutant ZEB2 3’UTR sequence at binding site. b HepG2 and HuH-7 cells were co-transfected with pGL3-ZEB2 -3’UTR Wt or pGL3-ZEB2 -3’UTR Mut as well as miR-204 mimics or NC. miR-204 significantly decreased the luciferase activities of pGL3-ZEB2 -3’UTR Wt, but had no
effect on the luciferase activities of pGL3- ZEB2-3'UTR Mut.

*P<0.05 compared with their respective controls.

**Fig.3** miR-204 negatively regulated ZEB2 expression at post-transcriptional level. a qRT-PCR analysis showed that ZEB2 mRNA were obviously down-regulated in miR-204 mimics-transfected HepG2 and HuH-7 cells. b Western blot indicated that miR-204 decreased ZEB2 protein expression in HepG2 and HuH-7 cells.

*P<0.05 compared with their respective controls.

**Fig.4** miR-204 suppressed cell proliferation, migration and invasion of HCC cells. a The relative levels of miR-204 expressed in HepG2 and HuH-7 cells after transfection with miR-204 mimics or NC. b Cell proliferation assays showed that over-expression of miR-204 suppressed cell proliferation in HepG2 and HuH-7 cells. c The migration and invasion assays indicated that enforced miR-204 expression suppressed migration and invasion abilities of HepG2 and HuH-7 cells.

*P<0.05 compared with their respective controls.

**Fig.5** Knockdown of ZEB2 inhibited proliferation, migration and invasion of HCC cells. a ZEB2 expression levels in HepG2 and HuH-7 cells by western blot after transfection with the ZEB2 siRNA or NC siRNA. b ZEB2 siRNA inhibited proliferation of HepG2 and HuH-7 cells. c ZEB2 siRNA suppressed migration and invasion capacity of HepG2 and HuH-7 cells.

*P<0.05 compared with their respective controls.