MicroRNA-592 targets IGF-1R to suppress cellular proliferation, migration and invasion in hepatocellular carcinoma

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Abstract. MicroRNAs (miRs) can function as tumor suppressors or oncogenes in different types of human malignancy, and may provide an effective therapy for cancer. The expression and functions of miR-592 have previously been studied in relation to cancer. However, the expression and potential functions of miR-592 in hepatocellular carcinoma (HCC) are still unknown. Using quantitative polymerase chain reaction, MTT assays, cellular migration and invasion assays, bioinformatics software, western blot analysis and dual-luciferase report assays, the present study explored the expression and roles of miR-592 in HCC. It was identified that miR-592 was significantly downregulated in HCC tissues and cell lines. The statistical analysis revealed that low expression of miR-592 was evidently associated with tumor node metastasis stage and lymph node metastasis. Additionally, the present study provided the first evidence that miR-592 was likely to directly target the insulin-like growth factor 1 receptor in vitro. The present results indicated that miR-592 could be investigated as an efficacious therapeutic target for HCC in the future.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common types of malignancy and is the third most common cause of cancer-related mortality worldwide (1,2). HCC can be divided into four histological groups based on tumor differentiation: Well differentiated; moderately differentiated; poorly differentiated; and undifferentiated (3). There are various factors which contribute to the initial development and progression of HCC, including infection with the hepatitis B or C virus, chronic inflammation, alcoholic liver disease and obesity (4). Although advances have been made in the treatment of patients with HCC, including hepatectomy and liver transplantation, the prognosis for patients with HCC remains unsatisfactory due to the rapid progression of HCC (5,6). The 5-year survival rate is <5% for patients with HCC with intra-hepatic or extra-hepatic metastasis (7). Therefore, it is important to additionally investigate the molecular mechanisms underlying the tumorigenesis of HCC in order to develop effective new therapeutic targets and prognostic markers.

Numerous studies have demonstrated that microRNAs (miRs) are dysregulated in HCC (8-10). miRs are a group of highly conserved, single-strand, non-protein-coding small RNAs that are ~22-25 nucleotides in length. The present study negatively regulated target mRNA expression at the post-transcriptional level by binding to the 3'untranslated regions (3'UTR) of mRNA in a base-pairing manner, which resulted in the cleavage of target mRNA or translation repression (11,12). miRs are involved in variety of physiological and pathological processes, including differentiation, proliferation, angiogenesis, apoptosis, cell cycles and metastasis (13-15). Current studies have acknowledged that more than half of miRs are located in cancer-associated genomic regions which suggests that dysregulation of miRs may perform important functions in carcinogenesis and the progression of cancer (16). Growing evidence has revealed that miRNAs may function as either tumor suppressors or oncogenes in different types of human malignancy, as a result of a change in the expression level of their target mRNAs (17-19). Therefore, miRNAs could be investigated for their potential role in the diagnosis, therapy, prognosis and monitoring of cancer.

The present study explored the expression and functions of miR-592 in HCC. The molecular mechanism of miR-592 action on HCC cells was also studied. The present results revealed that miR-592 was evidently downregulated in HCC tissues and human HCC HepG2 and SMMC-7721 cell lines. The low expression of miR-592 was significantly associated with tumor node metastasis (TNM) stage and lymph node metastasis of HCC patients. In addition, ectopic expression of miR-592 decreased HCC cellular proliferation, migration and invasion. Notably, insulin-like growth factor 1 receptor (IGF-1R) was identified as a novel miR-592 target. These findings collectively suggested that miR-592 acted as a tumor suppressor via the blocking of IGF-1R expression and...
may be investigated as an efficacious therapeutic target for HCC.

**Materials and methods**

**HCC clinical specimens and ethics statement.** For the use of tissue samples, written informed consent was obtained from all patients involved in the present study. The present study was approved by the Medical Ethics Committee of The Second Hospital of Hebei Medical University (Shijiazhuang, China). A total of 42 pairs of HCC tissues and corresponding adjacent non-tumor tissues were collected from patients with HCC (24 males, 18 females) who had undergone surgery treatment at The Second Hospital of Hebei Medical University. None of these patients received chemotherapy or radiotherapy prior to surgery. Tissues were put into liquid nitrogen immediately following surgery and then stored at -80°C until use.

**Cell culture and cell transfection.** The human HCC HepG2 and SMMC-7721 cell lines and immortalized normal liver epithelial THLE-3 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). All cell lines were maintained under the conditions stated by the supplier.

**RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from tissues and cells, according to the manufacturer's protocol. The concentration and purity of total RNA was measured by ND-2000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.) with a 260/280 ratio greater than 1.8. RNA was treated with DNA-free (Ambion; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA (1 µg) was reverse transcribed into cDNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.). cDNA was then synthesized using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) with 1 µg RNA. Subsequent to RT, qPCR was performed using TaqMan MicroRNA assay kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) with a 260/280 ratio greater than 1.8. For U6 forward, 5'-CCATCGATGATATGTATGCTTCC-3' and reverse, 5'-ACTTCGACTGAGCTCC-3'. For U6 forward, 5'-CGTATACGAGCTCC-3' and reverse, 5'-AAGCCTACTTCGATGACGGTCA-3'. Relative expression of miR-592 was analyzed using the 2^(-ΔΔCq) method (20).

**MTT assay.** Cellular viability was assessed using an MTT assay (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). Following a 24-h transfection, transfected cells (miR-592 and NC) were seeded in 96-well plates at a density of 2,500 cells/well. Subsequent to being incubated at 37°C for 24, 48, 72 and 96 h, 5 µl MTT solution (5 mg/ml) was added into each well and incubated for 4 h at 37°C. Cell culture medium was removed carefully and replaced with 200 µl dimethyl sulfoxide (Sigma-Aldrich; Merck Millipore). Absorbance at 490 nm was detected using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All experiments (5 replicates in each) were repeated ≥3 times.

**Cellular migration and invasion assay.** The cellular migration assay was assessed using Transwell chambers with a pore size of 8 µm (Corning Incorporated, Cambridge, MA, USA). Following transfection for 48 h, 5x10⁴ transfected cells (miR-592 and NC) in 300 µl medium without FBS were seeded into the upper chamber of the Transwell, while 500 µl medium supplemented with 20% FBS was placed into the lower chamber. For the Matrigel invasion assay, the Transwell chamber was coated with Matrigel (BD Biosciences, San Jose, CA, USA). A total of 5x10⁴ transfected cells (miR-592 and NC) in 300 µl medium without FBS were seeded into the upper chamber of the Transwell, while 500 µl medium supplemented with 20% FBS was placed into the lower chamber. Cells were incubated at 37°C for a further 24 h for the migration assay and 48 h for the invasion assay. In the two assays, the cells were fixed with 100% methanol for 5 min (Beyotime Institute of Biotechnology, Haimen, China) and stained with 0.5% crystal violet (Beyotime Institute of Biotechnology) for 5 min. Following this, cells remaining on the upper surface of the membranes were removed carefully using cotton swabs. The migrated and invaded cells were then counted in five randomly selected fields with an inverted microscope (Olympus Corporation, Tokyo, Japan). Each experiment was repeated ≥3 times.

**Bioinformatics analysis.** The potential target genes of miR-592 were generated using publicly available databases: miRanda (Memorial Sloan-Kettering Cancer Center, New York, NY, USA) and TargetScan (Whitehead Institute for Biomedical Research, Cambridge, MA, USA).

**Western blot analysis.** Western blot analysis was performed according to the standard protocol. Following 72-h transfection, transfected cells (miR-592 and NC) were washed and harvested using radioimmunoprecipitation assay lysis buffer (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, along with a protease inhibitor (Pierce; Thermo Fisher Scientific, Inc.). Total protein concentration was measured using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein (20 µg) were then separated by 10% SDS-PAGE (Beyotime Institute of Biotechnology) and transferred to polyvinylidene difluoride membranes (Merck Millipore). Following a blocking incubation with 5% non-fat milk at
room temperature for 2 h, the membranes were incubated at 4°C overnight with primary anti-IGF-1R (dilution, 1:1,000; cat no. ab131476; Abcam, Cambridge, MA, USA) and anti-GAPDH (dilution, 1:1,000; cat no. ab201822; Abcam), followed by incubation at room temperature for 1 h with the goat anti-rabbit horseradish peroxidase conjugated secondary antibody (1:3,000 dilution; cat no. ab97051; Abcam). The proteins were detected with an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.) and visualized using the FluorChem imaging system (Alpha Innotech, San Leandro, CA, USA). GAPDH was used as an internal control.

**Dual-luciferase report assay.** The dual-luciferase report assay was performed to determine whether miR-592 directly targeted the 3'UTR of IGF-1R. Cells were seeded in 24-well plates at a density of 50-60% confluence, and were co-transfected with 100 ng PGL3-IGF-1R-3'UTR wild type (Wt) or PGL3-IGF-1R-3'UTR mutant (Mut), and 100 nM miR-592 mimic or NC, using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Following a 48-h transfection, the firefly luciferase activity was detected using the dual-luciferase reporter assay (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. The Renilla luciferase activity was used as an internal control. Each experiment (3 replicates in each) was repeated >3 times.

**Statistical analysis.** Data are presented as the mean ± standard deviation. A two-tailed Student's t-test or ANOVA was used to compare differences between groups using SPSS 19.0 (IBM SPSS, Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

**Results**

miR-592 is downregulated in HCC tissues and cell lines. The present study detected the expression of miR-592 in HCC tissue samples and the corresponding adjacent non-tumor tissues using RT-qPCR. It was observed that miR-592 was significantly downregulated in HCC tissues compared with in corresponding adjacent non-tumor tissues (Fig. 1A and B; P=0.019).

The expression of miR-592 in HCC HepG2 and SMMC-7721 cell lines and immortalized normal liver epithelial THLE-3 cells was also measured. As presented in Fig. 1C, miR-592 expression levels were also decreased in HepG2 (P=0.011) and SMMC-7721 (P=0.015) cells compared with THLE-3. Therefore, miR-592 may perform important functions in HCC carcinogenesis and progression.

**The association of miR-592 expression with clinicopathological factors in patients with HCC.** Statistical analysis was also performed to assess the association of miR-592 expression with clinicopathological factors in patients with HCC. As presented in Table I, low miR-592 expression was significantly associated with TNM stage (P=0.010) and lymph node metastasis (P=0.001). However, no correlation was observed between miR-592 expression and other clinicopathological factors, including age (P=0.750), gender (P=0.754) and differentiation (P=0.330).

miR-592 is upregulated in HCC HepG2 and SMMC-7721 cells following transfection with miR-592 mimics. To clarify the functions of miR-592 on HCC, miR-592 mimics were transfected into HepG2 and SMMC-7721 cells. The present study analyzed the expression of miR-592 subsequent to a 48-h transfection using RT-qPCR. As demonstrated in Fig. 2, miR-592 was significantly upregulated in HCC HepG2 (P<0.001) and SMMC-7721 (P<0.001) cells transfected with miR-592 mimics, compared with cells transfected with NC.
miR-592 decreases the proliferation of HepG2 and SMMC-7721 cells. To explore the functional roles of miR-592, the present study additionally investigated the function of miR-592 on HCC cellular proliferation by using an MTT assay. As presented in Fig. 3, miR-592 significantly decreased cellular proliferation at 72 h and 96 h in HCC HepG2 (P=0.030) and SMMC-7721 (P=0.023) cells. These results suggest that the aberrant expression of miR-592 could regulate HCC cell growth.

miR-592 decreases migration and invasion in HCC HepG2 and SMMC-7721 cells. A cellular migration and invasion assay was performed to assess the role of miR-592 on HCC cellular motility. As demonstrated in Fig. 4A, miR-592 decreased HCC HepG2 (P=0.034) and SMMC-7721 (P=0.027) cellular migration ability. miR-592 also inhibited the HCC HepG2 (P=0.021) and SMMC-7721 (P=0.015) cellular invasion ability, compared with cells transfected with NC (Fig. 4B). These results indicate that the abnormal expression of miR-592 may be capable of regulating HCC metastasis.

miR-592 decreases IGF-1R protein expression in HCC HepG2 and SMMC-7721 cells. As miR-592 contributed to HCC carcinogenesis and progression, the present study aimed to identify the potential mechanism by which miR-592 could regulate HCC cellular growth, migration and invasion. To investigate the target mRNA of miR-592, the bioinformatics software miRanda and TargetScan were used. As presented in Fig. 5A, the bioinformatics software predicated that IGF-1R mRNA contained a miR-592 seed match at position 1,745-1,752 of the IGF-1R 3’UTR. To explore the effect of miR-592 on its target IGF-1R protein expression level, western blot analysis was performed. As presented in Fig. 5B, IGF-1R was significantly downregulated in HCC HepG2 (P=0.018) and SMMC-7721 (P=0.013) cells transfected with miR-592.

Table I. Association of miR-592 expression with the clinicopathological features of patients with hepatocellular carcinoma.

| Clinical features                          | Case number | miR-592 expression | P-value |
|-------------------------------------------|-------------|--------------------|---------|
|                                          |             | Low    | High   |         |
| Age                                       |             |        |        |         |
| <50 years                                 | 19          | 13     | 6      | 0.750   |
| ≥50 years                                 | 23          | 14     | 9      |         |
| Gender                                    |             |        |        |         |
| Male                                      | 24          | 16     | 8      | 0.754   |
| Female                                    | 18          | 11     | 7      |         |
| Tumor node metastasis stage               |             |        |        |         |
| I-II                                      | 25          | 12     | 13     | 0.010   |
| III-IV                                    | 17          | 15     | 2      |         |
| Lymph node metastasis                     |             |        |        |         |
| Negative                                  | 22          | 9      | 13     | 0.001*  |
| Positive                                  | 20          | 18     | 2      |         |
| Differentiated                            |             |        |        |         |
| Well and moderate                         | 26          | 15     | 11     | 0.330   |
| Poor                                      | 16          | 12     | 4      |         |

*P<0.05. miR, microRNA.
PGL3-IGF-1R-3’UTR Mut. Notably, IGF-1R was a direct target of miR-592 in vitro.

**Discussion**

The deregulation of miRNA expression in tumor sample tissues vs. normal tissues is a common event and may be essential for tumorigenesis and development (21). Therefore, miRNAs may be investigated as a novel candidate and screening tool that may be applied to clinical diagnosis, therapy and prognosis in various types of cancer. In 2011, Oberg et al (22) reported that miR-592 expression in proficient DNA mismatch repair (MMR) and deficient MMR-derived colorectal cancer was different using miRNA profiles. In colorectal cancer, miR-592 expression levels were significantly upregulated in serum and primary tumor tissues (21). Following surgery, miR-592 was downregulated in serum compared with the preoperative level (21). In 2014, Kim et al (23) demonstrated that miR-592 could be a novel biomarker between primary lung adenocarcinoma and colorectal cancer metastases in the lung using microRNA microarrays. In addition, miR-592 has been observed to be involved in other diseases (24). For example, in neuronal ischemic injury, miR-592 was downregulated in cerebral ischemia and the upregulation of miR-592 inhibited pro-apoptotic signaling and cell death in neurons (24). However, there are no studies regarding the expression of miR-592 in human HCC. The present study identified that miR-592 was significantly downregulated in HCC tissues and cell lines. The present study expands the knowledge on the expression of miR-592 in cancer.

The function of miR-592 in cancer has been previously studied. In colorectal cancer, a high expression level of miR-592 was associated with the tumor size, TNM stage, distance metastasis and preoperative carcinoembryonic antigen level (21). Survival analysis also demonstrated that high miR-592 expression in patients with colorectal cancer resulted...
in a significantly shorter overall survival rate, compared with patients with low expression levels of miR-592 (21). In addition, upregulation of miR-592 enhanced cellular growth, wound healing and cellular invasion in vitro (21). According to these studies, the downregulation of miR-592 could be a potential target for the therapy of patients with colorectal cancer. However, to the best of our knowledge there have been no previous studies regarding the roles of miR-592 in HCC. The present study identified that low expression of miR-592 was associated with TNM stage and lymph node metastasis. In addition, miR-592 inhibited cellular proliferation, migration and invasion ability. These above conflicting studies suggested that the roles of miR-592 in cancers are tissue-type dependent. The present study expands upon the functions of miR-592 in cancer.

Identification of miR-592 target genes is important for understanding the associated roles in HCC carcinogenesis and development. It is also important for developing novel targeted therapies for patients with HCC. In the present study, an important molecular link between miR-592 and IGF-1R was observed. First, bioinformatics software predicted that IGF-1R was a direct target of miR-592. Secondly, western blot analysis revealed that miR-592 inhibited the expression levels of IGF-1R at the protein level in HCC cells. Finally, the dual-luciferase reporter assay also demonstrated that miR-592 directly targeted the IGF-1R 3'-UTR. These findings suggest that miR-592 has a tumor suppressor role in the initiation and development of HCC by directly targeting IGF-1R.

IGF-1R is a transmembrane tyrosine kinase receptor of the insulin receptor family that contains two extracellular α subunits with the ligand-binding site and two transmembrane β subunits with intracellular tyrosine kinase activity (25). Increasing studies have demonstrated that IGF-1R performs vital functions in biological processes, including malignant transformation, growth, apoptosis, cellular development, migration, invasion and distant metastasis (26-28). IGF-1R has been observed to be upregulated in numerous types of human cancer, including HCC, osteosarcoma, non-small cell lung and prostate cancer (29-31). In response to various stimulatory signals, IGF-1R activates the phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (mTOR) and the Ras/Raf/mitogen-activated protein kinase signaling pathway (32). In HCC, the IGF-1R/mTOR signal pathway has been revealed to be frequently dysregulated (33,34). Several agents targeting IGF-1R have been developed or are in development, and some of them currently being clinically used for the treatment of cancer (35). Therefore, regarding cancer-related functions, IGF-1R must be considered as a potential target for inhibition in HCC. The present study revealed that miR-592 decreased HCC cellular proliferation, migration and invasion by directly targeting IGF-1R. It is also suggested that miR-592 could be investigated as a target for the therapy of HCC.

IGF-1R has been identified to be regulated by multiple miRs in various types of cancer (36-38). For example, in HCC miR-133a decreased cellular proliferation, colony formation, migration, invasion and enhanced cell cycle arrest at the G0/G1 stage, and enhanced cell apoptosis by directly targeting IGF-1R (36). In addition, miR-122 suppressed HCC cellular growth and tumorigenesis by regulating IGF-1R directly (37). In addition, miR-99a and miR-378 inhibited HCC cellular proliferation by blocking IGF-1R (9,39). In non-small cell lung cancer, miR-139-5p, miR-99a, miR-195, miR-133a and miR-140 function as tumor suppressors by directly downregulating IGF-1R (40-44). In glioma, miR-323-5p suppresses cellular proliferation and migration via the blockade of IGF-1R (38). In colorectal cancer, miR-143 decreases cellular proliferation and angiogenesis and increases chemosensitivity to oxaliplatin by targeting IGF-1R (45). Notably, miRs may act as regulators of IGF-1R. In the present study, the overexpression of miR-592 in HCC cell lines inhibited cellular proliferation, migration and invasion by blocking IGF-1R. Therefore, miRs could be investigated for their importance in the targeted therapy of HCC.

To the best of our knowledge, this is the first study to demonstrate that miR-592 is significantly downregulated in HCC and associated with TNM stage and lymph node metastasis. The present study also observed that miR-592 contributes to cellular proliferation, migration and invasion by directly targeting IGF-1R in HCC. The identification of the candidate target gene of miR-592 may provide an understanding of potential carcinogenic mechanisms in HCC. These findings have therapeutic implications and may be exploited for further treatment of HCC.

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