**Original Article**

**MicroRNA-485-5p suppresses cell proliferation and invasion in hepatocellular carcinoma by targeting stanniocalcin 2**

Guo-Xiao Guo*, Quan-Ying Li*, Wan-Li Ma, Zhao-Hui Shi, Xue-Qun Ren

Department of General Surgery, Huaihe Hospital of Henan University, Kaifeng, Henan Province, China. *Equal contributors.

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**Abstract:** Increasing evidences indicate that dys-regulation of MicroRNAs contributes to hepatocellular carcinoma. However, the roles of miR-485-5p in HCC are still largely unexplored. In the present study, our quantitative real-time PCR analysis found that miR-485-5p was significantly down-regulated in 50 pairs of human HCC tissues. Moreover, the reduced expression of miR-485-5p was significantly correlated with larger tumor size and more tumor number in patients with HCC. In vitro studies further showed that overexpression of miR-485-5p mimics could inhibit, while its antisense oligos promote cell proliferation and invasion. Results from the dual-luciferase reporter gene assays and western blot further showed that stanniocalcin 2 was a direct target of miR-485-5p. Therefore, our data suggest a novel role for miR-485-5p in the regulation of HCC progression.

**Keywords:** Hepatocellular carcinoma, MicroRNA, miR-485-5p, stanniocalcin 2

**Introduction**

Hepatocellular carcinoma (HCC) has become the third leading cause of cancer mortality worldwide [1, 2]. In China, chronic HBV infection is a critical risk factor for the carcinogenesis and progression of HCC [3, 4]. Thus, the identification of the causes and mechanisms behind the progression of HCC will improve its treatment.

MicroRNAs (miRNAs), a class of small and non-coding RNAs, could inhibit gene expression through binding to the 3'-untranslated region of target mRNAs [5, 6]. It has been shown that many deregulated miRNAs play important roles in the cell proliferation, apoptosis, invasion and metastasis in HCC [7-9]. For instance, miR-101, down-regulated in HCC tissues, promoted apoptosis and suppresses tumorigenicity [10]. Besides, miR-221 silencing was shown to block HCC and promote survival, suggesting that targeting miRNAs might benefit treatment for patients with advanced HCC [11].

Recently, miR-485-5p has been found to be dys-regulated in some types of cancers. For instance, miR-485-5p was down-regulated and correlated significantly with FIGO grade 3 in ovarian epithelial tumors [12]. Besides, Anaya-Ruiz et al. showed that miR-485 may act as a tumor suppressor by inhibiting cell growth and migration in breast carcinoma cells [13]. Moreover, miR-485-5p binding site SNP rs8752 in 15-hydroxyprostaglandin dehydrogenase (HPGD) gene is associated with breast cancer risk [14]. However, the exact function of miR-485-5p in hepatocarcinogenesis has not been revealed yet. In this study, we found that miR-485-5p was down-regulated in HCC tissues and further explored its roles in the tumorigenesis.

**Materials and methods**

50 paired surgically resected HCC tissues and matched adjacent normal liver tissues were collected from patients during operation with informed consent. All tissue samples were flash-frozen in liquid nitrogen immediately after collection and stored at -80°C until use. The study was approved by the hospital institutional review board of Huaihe Hospital of Henan University.
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Human HCC cell lines (HepG2 and Hep3B) were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, St. Louis, Mo., USA) supplemented with 10% fetal bovine serum (EMD Millipore, Wanchai, Hong Kong, China). Cultures were maintained at 37°C in a humidified atmosphere with 5% CO₂.

RNA isolation and real-time PCR

Total RNA from tissues or cell lines was harvested using Trizol according to the manufacturer’s instructions (Invitrogen, Shanghai, China). Expression of mature miRNAs was assayed using Taqman MicroRNA Assay (Applied Biosystems) specific for hsa-miR-485-5p. Small nuclear U6 snRNA was used as an internal control for normalization and quantification of miR-485-5p expression.

BrdU assays

A cell proliferation enzyme-linked immunosorbent assay (BrdU kit; Beyotime) was used to analyze the incorporation of BrdU during DNA synthesis following the manufacturer’s protocols. All experiments were performed in triplicate. Absorbance was measured at 450 nm in the Spectra Max 190 ELISA reader (Molecular Devices, Sunnyvale, CA).

miR-485-5p target predictions

The putative targets of miR-485-5p were predicted using the miRWalk software. The algorithm produced a list of hundreds of target genes for miR-485-5p by searching for the presence of conserved 7-mer and 8-mer sites matching the seed region of miR-485-5p.

Western blot

Cells were harvested and lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 6.8, 485-5p mM 2-ME, 2% w/v SDS, 10% glycerol). After centrifugation at 20000g for 10 min at 4°C, proteins in the supernatants were quantified and separated by 10% SDS PAGE, transferred to NC membrane (Amersham Bioscience, Buckinghamshire, U.K.). After blocking with 10% nonfat milk in PBS, membranes were immunoblotted with antibodies as indicated, followed by HRP-linked secondary antibodies (Cell Signaling). The signals were detected by Super Signal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL) according to manufacturer’s instructions. Anti-Stc2 and GAPDH antibodies were purchased from Abcam (USA). Protein levels were normalized to GAPDH (Santa Cruz, USA).

Luciferase reporter assays

Total cDNA from HepG2 cells was used to amplify the 3’UTR of Stc2 by PCR. The KLF9 3’UTR was cloned into pMir-Report (Ambion), yielding pMir-Report-KLF9. Mutations were introduced in potential miR-485-5p binding sites using the Quik Change site-directed mutagenesis Kit (Stratagene). Cells were transfected with the pMir-Report vectors containing the 3’-UTR variants, and miR-485-5p mimics, negative controls for 36 hours. The pRL-TK vector (Promega, USA) carrying the Renilla luciferase gene was used as an internal control to normalize the transfection efficiency. Luciferase values were determined using the Dual-Luciferase Reporter Assay System (Promega).

Statistical analysis

Data are expressed as the mean ± SEM from at least three separate experiments. Differences between groups were analyzed using Student’s t-test analysis. A value of $P < 0.05$ was considered statistically significant.

Results

Down-regulation of miR-485-5p expression in lung carcinoma tissues

The expression levels of miR-485-5p were detected in 50 cases of HCC tissues and adjacent noncancerous liver tissues using a quanti-
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As a result, we found that its expression was significantly reduced in HCC tissues in comparison with the adjacent normal tissues (Figure 1). Furthermore, the low miR-485-5p expression group showed a higher incidence of increased tumor size and tumor number. However, no significant differences were observed with respect to sex, age, AFP, TNM stage or metastasis in HCC (Figure 2). Therefore, these results suggest the importance of miR-485-5p down-regulation in HCC progression.

Overexpression of miR-485-5p inhibits HCC cell proliferation

We then investigated the effects of miR-485-5p on cell proliferation and invasion in HepG2 and Hep3B cells. As shown in the Figure 3A and 3B, miR-485-5p was significantly up-regulated following transfection with miR-485-5p mimics. Abilities of cell proliferation and invasion were significantly enhanced by miR-485-5p overexpression (Figure 3C-F). On the other hand, transfection with miR-485-5p inhibitor led to a significant reduction in cell proliferation and invasion in both cells (Figure 4A-F), indicating that miR-485-5p functions as a potential tumor suppressor in HCC carcinogenesis.

Stc2 is a direct target of miR-485-5p

To fully understand the mechanisms by which miR-485-5p executed its function, we adopted the bioinformatic algorithms (miRWalk software) for target gene prediction [15]. Among these candidates, Stc2, an important oncogene, was identified as one of the potential targets of miR-485-5p and selected for further analysis (Figure 5A). To test that Stc2 may be a direct target of miR-485-5p, the reporter plasmid harboring the wild-type or mutant 3'-UTR region of Stc2 was constructed. As shown in Figure 5B, overexpression of miR-485-5p mimics led to a reduction of luciferase activity when the reporter construct contained the Stc2 3'-UTR (Figure 5B). However, mutation of the miR-485-5p binding site from the Stc2 3'-UTR abolished this effect of miR-485-5p (Figure 5B). Then, western blot analysis was conducted to measure the effect of miR-485-5p on endogenous Stc2 expression. Our results showed that Stc2 protein levels were substantially down-regulated by miR-485-5p (Figure 6A, 6B). In contrast, inhibition of miR-485-5p led to an increased protein level of Stc2 in HCC cells (Figure 6C, 6D). Therefore, down-regulated miR-485-5p in HCC promoted the expression of Stc2, which in turn accelerates tumorigenesis.

Discussion

The roles of miRNAs in the development of HCC have attracted recent attention by a variety of studies [9, 16]. The current work showed that...
Figure 3. Overexpression of miR-485-5p mimics inhibits HCC cell proliferation. (A, B) Expression levels of miR-485-5p were determined in HepG2 (A) and Hep3B (B) cells after transfection with miR-485-5p mimics or negative control (NC). ***P < 0.001 compared with NC. (C-F) The cell proliferative potential (BrdU) and invasion abilities were determined in HepG2 and Hep3B cells transfected with miR-485-5p mimics or negative control (NC). *P < 0.05, **P < 0.01 compared with NC.
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Figure 4. miR-485-5p inhibitors promote the proliferation of HCC cells. (A, B) Expression levels of miR-485-5p were determined in HepG2 (A) and Hep3B (B) cells after transfection with miR-485-5p antisense or negative control (NC). ***P < 0.001 compared with NC. (C-F) The cell proliferative potential (BrdU) and invasion abilities were determined in HepG2 and Hep3B cells transfected with miR-485-5p antisense or negative control (NC). *P < 0.05, **P < 0.01 compared with NC.
miR-485-5p expression is significantly reduced in human HCC, compared with matching adjacent nontumoral tissue. Besides, ectopic overexpression of miR-367 mimics inhibited, whereas its antisense promoted cell proliferation and invasion in HCC cells. In addition, luciferase reporter assays and western blot analysis found that miR-485-5p could interact with 3'-UTR of Stc2 gene, to inhibit its protein expression.

Stanniocalcin (STC) belongs to a family of secreted glycoprotein hormones that was first studied in the corpuscles of Stannius, an endocrine gland of fish [17, 18]. Initially, Stc2 was identified as a negative regulator of postnatal growth, as evidenced by mice with Stc2 deletion [19]. Subsequent studies showed that Stc2 is up-regulated in many types of human cancers, including gastric, breast, prostate and ovarian cancer [20-23]. Besides, Stc2 is shown to be up-regulated by hypoxia-mediated HIF1 activation and promote cell proliferation [24]. Importantly, Stc2 is also up-regulated in hepatocellular carcinoma, which predicts poor prognosis of hepatocellular carcinoma, and pro-
motes cell proliferation and migration in vitro [25, 26]. However, the molecular determinants for the aberrant expression of Stc2 remains poorly understood. Therefore, our results provide an alternative mechanism for Stc2 in HCC.

Interestingly, a recent study found that Stc2 could be suppressed by miR-206 in gastric cancer [27]. Taken together, these results indicate that certain miRNAs might be applied as potential prognostic biomarker and new therapeutic target for cancer therapy.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Guo-Xiao Guo, Department of General Surgery, HuaIhe Hospital of Henan University, 8 Baobei Road, Kaifeng 475000, Henan Province, China. E-mail: guoxiaoguohn@126.com

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