ABSTRACT

Aims: Given their regulatory roles in gene expression, microRNAs play an important role in tumorigenesis. The current study aimed to explore the function and the related mechanisms of miR-616 in hepatocellular carcinoma (HCC).

Methods: The expression of miR-616 was detected using quantitative real-time polymerase chain reaction (qRT-PCR). Chi-square test was applied to estimate the association of miR-616 with clinical characteristics of HCC patients. Cell transfection was performed by Lipofectamine 2000. MTT assay was used to detect cell proliferation, whereas cell motility was estimated using Transwell assay. The protein expression was detected using western blot.

Results: MiR-616 was significantly up-regulated in HCC tissues and cells (p < .05 for all). Moreover, its elevated expression was positively correlated with lymph node metastasis (p = .008) and TNM stage (p = .012). Knockdown of miR-616 resulted in decreased cell proliferation, migration and invasion. Moreover, the inhibition of miR-616 significantly suppressed PI3K/AKT pathway. The bioinformatics analysis and luciferase reporter assay suggested that PTEN was a targeted gene of miR-616. PTEN had the capacity to reverse the oncogenic function of miR-616 in HCC.

Conclusion: MiR-616 activates PI3K/AKT pathway through suppressing PTEN expression, thus promoting the progression of HCC.

Introduction

Hepatocellular carcinoma (HCC) is a highly fatal malignancy around the world, with high prevalence and death rate [1]. A number of risk factors have been confirmed for HCC, including cirrhosis, infections by hepatitis C (HCV) and hepatitis B (HBV) virus, alcohol abuse, liver disease, etc. [2]. In China, HBV infection represents a leading reason for HCC [3]. Recently, there are various treatments for HCC patients, such as surgery, chemotherapy, radiotherapy, immunotherapy, ablation, cell treatments, etc. [4,5]. However, the prognosis of HCC patients still remains unsatisfactory, especially for those diagnosed with advanced stages [6]. It has been reported that the 5-year survival of the HCC patients diagnosed with advanced stages is less than 5% [7]. The dismal clinical outcomes of HCC patients may be attributed to local and systemic metastasis [8]. However, due to the unclear molecular mechanisms underlying HCC, it is difficult to identify the key factors to drive cancer metastasis. Therefore, to explore the molecular aetiology of HCC is of great importance for clinical outcomes of the patients.

MicroRNAs (miRNAs) belong to a family of small non-coding RNAs, and their lengths are about 22 nucleotides [9]. MiRNAs could bind to the 3’end of the untranslated regions of their targeted mRNAs, and result in translational suppression, thus regulating gene expression [10]. miRNAs are involved in various biological processes, and their alterations may lead to uncontrolled cell proliferation and malignant cell motility, ultimately leading to cancer or other diseases [11]. In tumorigenesis, miRNA may act as oncogenes or tumour suppressors [12]. In HCC, growing evidence have demonstrated that the abnormal expression of miRNAs may be responsible for the malignant behaviours of HCC [13,14]. To explore the cancer-related miRNAs may provide a better understanding of HCC aetiology.

MicroRNA-616 (MiR-616) is located on the chromosome region 12q13.3, which is newly confirmed to be a cancer-related miRNA. Its dysregulation has been reported in several cancers, including gastric cancer [15], glioma [16], non-small cell lung cancer [17], etc. The dysregulation of miR-616 may contribute to tumour progression through multiple signalling pathways. However, the functional roles and the associated mechanisms of miR-616 in HCC have been rarely reported.

In this study, we detected the expression of miR-616 in HCC tissues and cell line, as well as its function in HCC progression. In addition, the in vitro experiments were designed to address the molecular mechanisms underlying the function of miR-616 in HCC.
Materials and methods

Patients and tissue collection

In our study, the HCC tissues and adjacent normal tissues were collected from 123 patients who were pathologically diagnosed with HCC in Affiliated Hospital of Nantong University between May 2015 and October 2017. None of the patients had received any preoperative treatments, such as chemotherapy, radiotherapy, immunotherapy, etc. The surgical tissues were immediately frozen in lipid nitrogen and stored in −80 °C. The clinical characteristics of the patients were collected from their medical records. The study was reviewed and approved by the Ethic Committee of the hospital. The written informed consents were obtained from all the participants.

Cell line and cell culture

Human HCC cell line HepG2 (code: SCSP-510) and human immortalized hepatocytes THLE-3 (code: GNHu40) cell lines were purchased from the Cell Bank of the Chinese Academy of Science (CSP600232; Shanghai, China). The cells were cultured using RPMI-1640 medium, with addition of 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Then, the cell medium was incubated at 37 °C with 5% CO2. The cells were harvested at logarithmic growth phase for the subsequent analyses.

RNA extraction and quantitative analysis

Total RNA was extracted from the collected tissue and cell specimens using Trizol reagent (Invitrogen, Thermo Fisher Scientific, Inc.), according to the manufacturer’s instruction. The quality of the extracted RNA samples was estimated using a spectrophotometer, and only the RNA sample with ODA260/A280 ratio of 1.8–2.0 was used for the subsequent experiments. The first-strand cDNA was synthesized using PrimerScript RT reagent kit (Takara, Dalian, China). The quantitative analysis was performed using quantitative real-time polymerase chain reaction (qRT-PCR) which was carried out using SYBR Green PCR master mix (Applied Biosystems, USA) in the 7300 Real-Time PCR System (Applied Biosystems, USA). The specific primer sequences were as follows: U6 forward: 5’-CTTCGCCTCCGACGCACA-3’; reverse: 5’-AAGCGTCTTCA CGAATTTCGTG-3’; miR-616 forward: 5’-TTAGTAAATCCTCC TCTC-3’; reverse: 5’-CAGTCATTCCCTCTGCTTCTT-3’; GAPDH forward: 5’-TGCAACCAACGTCTGAGAC-3’; reverse: 5’-GGCATG GACTGGGTACTGAG-3’; PTEN: forward: 5’-GGATTTCCATCTAGTACTGAC-3’; reverse: 5’-GGTGGTTATGGTCTTCAAAAGG-3’. U6 was employed as an internal control for the detection of miRNA, whereas GAPDH acted as an internal reference for mRNA. The expression levels of the detected genes were calculated using 2−ΔΔCt method. Each test was repeated three times.

Cell transfection

To explore the functional roles of miR-616 in progression of HCC, the miR-616 inhibitor, miR-616 mimic, and the corresponding negative controls were designed and synthesized by HANBIO Company (Shanghai, China). In addition, the RNA interference vector was also designed to target PTEN expression (si-PTEN) in HCC cells. The harvested cells were seeded to 6-well plate in a density of 1 × 10⁵. The designed vectors were transfected to the cells using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Then, the cells were incubated at 37 °C with 5% CO2. Forty-eight hours later, the relative expression of miR-616 was detected in the transfected cell using qRT-PCR to estimate the transfection efficacy.

Cell proliferation

The effect of miR-616 expression on cell proliferation ability was estimated using MTT assay. The transfected cells were cultured in 96-well plate with a density of 2 × 10⁴ cells/well and maintained in an incubator with 5% CO2 at 37 °C. Then, 50 µL MTT (5 mg/mL) was added to cell medium at daily intervals, including 0 h, 24 h, 48 h and 72 h. The cells were incubated with MTT for 4 h, then 150 µL DMSO was added to the medium, and incubated at dark for 10 min. The absorbance at 490 nm was read using a Microplate Reader (TECAN, Salzburg, Austria).

Cell migration and invasion

Transwell assay was performed to estimate cell migration and invasion. Two hundred microlitres RPMI-1640 medium was added to the upper chamber (8.0 µm pore size, Costar, Shanghai, China), whereas 500 L RPMI-1640 medium with 10%FBS was added to the lower chamber. Additionally, for invasion analysis, the upper chamber was coated with Matrigel (Corning Glass Works, Corning, NY, USA). The procedures were according to the manufacture’s introduction. In brief, 200 µL cell suspension solution (5 × 10⁴/mL) was seeded to the upper chamber and then incubated at 37 °C with 5% CO2. Forty-eight hours later, the lower chamber was stained using crystal violet and counted under inverted microscope (IX31; Olympus Corporation, Tokyo, Japan). Five random files were selected for each sample.

Luciferase reporter assay

The bioinformatics analysis demonstrated that PTEN might be a potential targeted gene of miR-616 in HCC. In order to verify the results, the luciferase reporter assay was performed. First, the 3’UTR sequence containing the binding site of miR-616 (PTEN-wt), and the fragment with the mutated binding site (PTEN-mt) were amplified using PCR method. Then, the fragments were cloned to the luciferase reporter vector pGL3 (Promega, USA), respectively. Subsequently, the recombinant plasmids (PTEN-wt or PTEN-mt) combined with miR-616 mimic or mimic NC were transfected to HepG2 cells using
Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The cells were incubated at 37°C with 5% CO2 for 48 h. Then, the luciferase activity of the transfected cells was estimated using Dual-Luciferase Reporter Assay System (Promega Corporation).

**Western blot analysis**

The protein analysis was performed using western blot. In brief, the cells were harvested for protein isolation which was performed using RIPA Lysis and Extraction Buffer (Thermo Scientific, Waltham, MA, USA). Then, the obtained proteins were quantified using BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Next, the equal volume of protein was separated using 10% SDS-PAGE. Then, the proteins were transferred to a polyvinylidene fluoride membrane (PVDF) (0.45 μm pore size; EMD Millipore, Billerica, MA, USA) under the treatment with 10 mA for 40 min. Following, 5% skim milk powder was applied to block the membranes at room temperature for 2 h. Next step, the membranes were marked by the specific primary antibody, and the reaction was carried out at 4°C for overnight. Subsequently, the membranes were washed using PBS buffer for three times and then incubated with the secondary anti-rabbit IgG antibody (dilution, 1:2000; cat. No. ab6709; Abcam) for 2 h at room temperature. Finally, the protein bands were analyzed using ECL substrate reagent kit (GE Healthcare) on a Gel Doc XR imaging system (Bio-RAD, USA). The used specific primary antibodies were as follows: anti-PTEN antibody (1:1000, Abcam), anti-p-AKT antibody (1:1000, Abcam), anti-AKT antibody (1:2000, Abcam), PI3K antibody (1:1000, Abcam), p-PI3K antibody (1:1000, Abcam), anti-GAPDH antibody (1:1000, Abcam). The anti-GAPDH antibody served as the loading control. Each test was in triplicate.

**Statistical analysis**

The continuous variables were expressed as mean ± standard deviation (SD) and compared between two groups using student’s t-test. Chi-square test was performed to estimate the association of miR-616 expression with clinical characteristics of HCC patients. All the tests were two-tailed, and the p values less than 0.05 indicated the statistical significance of the results. The statistical analysis was performed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA), and the figures were plotted using GraphPad Prism version 5.0 (GraphPad, San Diego, CA, USA).

**Results**

**Baseline characteristics of the study population**

A total of 123 HCC patients including 78 males (63.41%) and 45 (36.59%) females were included in our research, and their mean age was 61.23 ± 10.25 years. Seventy-eight (63.41%) of them had smoking history, whereas 45 (36.59%) patients had drinking habit. Sixty-eight (55.28%) of the subjects exhibited tumour size less than 3 cm, and lymph node metastasis was observed in 51 (42.46%) patients. According to TNM staging, 69 (56.10%) patients were confirmed with stages I–II, whereas the rest 54 (43.90%) patients were diagnosed with stages III–IV. The detailed clinical information of the included patients is summarized in Table 1.

**Uproadregulation of miR-616 in HCC tissues and cells**

QRT-PCR method was used to investigate the relative expression of miR-616 in HCC tissues and cell line. As displayed in Figure 1, the levels of miR-616 were significantly increased in HCC tissues and cells, compared to the non-cancerous specimens (p < .01 for both).

**Association of miR-616 with clinical characteristics of HCC patients**

The included patients were divided into high expression group (n = 55) and low expression group (n = 55) according to their mean expression levels of miR-616 in HCC tissues. Chi-square test was performed to estimate the relationship between miR-616 expression and the clinical characteristics of HCC patients. The results demonstrated that the expression of miR-616 was positively correlated with lymph node metastasis (p = .008) and TNM stage (p = .012). Meanwhile, miR-616 expression had no association with patients’ age, gender, smoking, drinking, or tumour size (p > .05 for all) (Table 1).

**Knockdown of miR-616 could suppress HCC cell proliferation, migration and invasion**

MiR-616 inhibitor was designed to suppress miR-616 expression in HCC cells. QRT-PCR detection suggested that the transfection of miR-616 could significantly suppress the expression of miR-616 in HCC cells (p < .001) (Figure 2).
The biological behaviours of the transfected cells were investigated to explore the functional roles of miR-616 in the progression of HCC. MTT analysis demonstrated that knock-down of miR-616 could obviously suppress HCC cell proliferation ability (Figure 3(A)), migration (Figure 3(B)) and invasion (Figure 3(C)) ($p < .05$). MiR-616 could promote the malignant behaviours of HCC cells in vitro.

**MiR-616 might target PTEN in HCC**

According to the bioinformatics analysis (TargetScan 6.2 and miRanda), PTEN was confirmed as a potential target of miR-616. The 3’UTR of PTEN gene contained the complementary sequence of miR-616 (Figure 4(A)). In order to verify the prediction, the luciferase reporter assay was performed. The HepG2 cells were co-transfected by miR-616 mimic and PTEN-wt vector or miR-616 mimic and PTEN-mt vector. The cells co-transfected by mimic NC and PTEN-wt vector or mimic NC and PTEN-mt vector were employed as internal control. Analysis results demonstrated that the over-expression of miR-616 lead to obviously decreased luciferase activity in the cells with PTEN-wt ($p < .01$), whereas the up-regulation of miR-616 had no significant effects on luciferase activity of the cells with PTEN-mt ($p > 0.05$) (Figure 4(B)).

QRT-PCR was used to investigate the expression of PTEN mRNA in HCC. The results demonstrated that the levels of PTEN were significantly decreased in HCC cells, compared to the normal hepatic cells ($p < .01$) (Figure 5(A)). Furthermore, the knockdown of miR-616 lead to up-regulation of PTEN in HCC cells ($p < .01$) (Figure 5(B)). All the data revealed that miR-616 could regulate the expression of PTEN through binding to its 3’UTR.

**MiR-616 could regulate the activity of PI3K/AKT signalling pathway in HCC**

PTEN was confirmed as a regulator of PI3K/AKT signalling pathway [18]. Given the targeted relationship between PTEN...
and miR-616, we hypothesized that miR-616 might influence the activity of PI3K/AKT signalling pathway. Western blot analysis demonstrated that the protein levels of p-PI3K and p-AKT were significantly decreased in the HCC cells transfected using miR-616 inhibitor (p < .05) (Figure 6). The knockdown of miR-616 lead to inactivation of PI3K/AKT signal pathway.

**MiR-616 activated PI3K/AKT pathway through targeting PTEN, thus contributing to malignant progression of HCC**

HepG2 cells were co-transfected by miR-616 inhibitor and si-PTEN vectors to investigate the molecular mechanisms underlying the oncogenic function of miR-616 in HCC. The HCC cells transfected by miR-616 inhibitor served as control. Western blot analysis found that the inhibition of PTEN could significantly enhance the expression of p-PI3K and p-AKT (p < .05), revealing the activation of PI3K/AKT signalling pathway (Figure 7). Furthermore, the transfection of si-PTEN obviously promoted cell proliferation (Figure 8(A)), migration (Figure 8(B)) and invasion (Figure 8(C)) (p < .05 for all). All the results demonstrated that PTEN could reverse the function of miR-616 in HCC. MiR-616 activated PI3K/AKT signalling pathway through targeting PTEN, thus contributing to malignant progression of HCC.

**Discussion**

HCC is difficult to cure, even after radical resection, the recurrent rate within 5 years may be up to 70% [19]. In order to explore the key factors drive the disease progression, more and more researches have been constructed to explore the molecular mechanisms of HCC. It is generally considered that HCC is regulated by the interactions of environmental and genetic factors [20]. With the developments of molecular techniques and next generation sequencing, the function of genetic factors in aetiology of HCC attracts more and more attention.
attentions. MiRNAs are a group of endogenous RNAs, and they have no protein-encoding ability, but they could regulate gene expression via binding to the 3' UTR of their targeted genes [21]. MiRNAs are considered as promising candidates for diagnostic and prognostic biomarkers, as well as therapeutic targets in the management of malignancy [22]. To explore the functional roles and the related molecular mechanisms of miRNAs in tumour progression may provide a new insight into the pathogenesis. In the present study, we investigated the role of miR-616 in HCC, as well as its related molecular mechanisms.

In our study, we found that the expression of miR-616 was significantly increased in HCC tissues and cell lines. Moreover, its up-regulation predicted positive lymph node metastasis and advanced TNM stages for the patients. The knockdown of miR-616 in HCC cells could result in decreased cell proliferation, migration and invasion. Based on the data, we calculated that miR-616 might be an oncogene in progression of HCC. The over-expression of miR-616 might promote malignant disease progression through enhancing cell proliferation and motility. The conclusion was in accordance with the published article. The study designed by Zhang et al. suggested that the expression of miR-616 exhibited increased trend in HCC, moreover, its up-regulation predicted aggressive disease progression and poor prognosis for the patients. MiR-616 might be a promoter in the malignant progression of cancer [23]. In addition, the oncogenic function of miR-616 had also been reported in other types of cancer, like gastric cancer, glioma, non-small cell lung cancer [15–17]. The dysregulated miR-616 in tumorigenesis might be a potential therapeutic target.

Despite without protein encoding ability, miRNAs could regulate the expression of the genes which are involved in cell growth, motility and differentiation, thus taking part in various biological processes [24,25]. For example, miR-506 suppressed invasiveness and metastasis of HCC through inhibiting the expression of IL8 [26]. Pang et al. reported that miR-214-5p was involved in the progression of HCC through targeting KLFS. KLFS might be a potential target of miR-214-5p [27]. Fu et al. demonstrated that miR-142-3p regulated HCC progression through controlling the expression of HMGB1 gene [28]. In our study, the bioinformatics analysis and luciferase reporter assay demonstrated that PTEN might be a potential targeted gene of miR-616 in HCC. MiR-616 could regulate the expression of PTEN through binding to its 3' UTR. The conclusion was consistent with the study of
Zhang et al. [23]. In addition, Wu et al. also demonstrated that miR-616 targeted PTEN in gastric cancer [15]. However, miR-616 bound to TFPI-2 in prostate cancer [29]. MiR-616 might be involved in tumorigenesis through multiple targeted genes.

In our study, we also found that miR-616 could activate PI3K/AKT signalling pathway through suppressing PTEN expression. PTEN was considered as a negative switch of PI3K/AKT pathway [18]. MiR-616 suppressed the expression of PTEN, thus activated PI3K/AKT signalling pathway and promoted malignant cancer progression. Despite the encouraging results, several limitations in the current study should be stated. First, the sample size was relatively small. The clinical significance of miR-616 for HCC needed further verification. Second, the oncogenic function of miR-616 in normal hepatic cells had not been explored. In our study, we only confirmed that the inhibition of miR-616 in HCC cells could suppress the oncogenic process, but whether the normal hepatic cells with over-expression of miR-616 would exhibit oncogenic behaviours remained unclear. In addition, the in vivo experiments were not designed to verify our results. Therefore, further well-designed researches are in urgent need to verify and improve our results.

In conclusion, miR-616 as an oncogene promotes the malignant HCC progression via enhancing cell proliferation, migration and invasion. In HCC, miR-616 may activate PI3K/AKT pathway through suppressing PTEN expression, thus promoting cancer progression.

Disclosure statement

No potential conflict of interest was reported by the authors.

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