Original Article
Effect of microRNA338-3p on proliferation of hepatoma cells and its mechanisms

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Abstract: Objective: To clarify the effects of microRNA338-3p on the proliferation of hepatoma cells and its mechanisms. Methods: The microRNA338-3p expressions in the hepatoma cell line HepG2 and hepatic cancer tissue were detected by quantitative PCR (qPCR); the microRNA338-3p analogs were transfected into hepatoma cell line HepG2 with the use of the liposome-mediated transfection; the effects of microRNA338-3p analogs on the proliferation of hepatoma cell line HepG2 were tested by CCK-8; the impacts of the microRNA338-3p analogs on the sphingosine kinase-2 (SphK-2) protein expression in the hepatoma cell line HepG2 were measured by western blot; targeted regulation of SphK-2 expression with microRNA338-3p was detected by the luciferase reporter gene assay. Results: The level of microRNA338-3p in hepatoma cell line HepG2 was strikingly lower than that in the normal hepatic tissue (P<0.001); the level of microRNA338-3p was lower in hepatic cancer tissue than in adjacent cancer tissue (P<0.001); when compared with the control group, the microRNA338-3p analogs substantially inhibited the proliferation of hepatoma cell line HepG2 and downregulated the SphK-2 expression levels (P<0.001); the luciferase reporter gene assay indicated SphK-2 was a target gene regulated by microRNA338-3p. Conclusion: MicroRNA338-3p showed low expression in liver cancer and inhibited the proliferation of hepatoma cells by downregulation of target SphK-2 expression.

Keywords: MicroRNA, miR-338-3p, liver cancer, cell proliferation

Introduction
Liver cancer (LC) is one of the most common malignancies, and the fourth most prevalent and most fatal disease in the world; hepatocellular carcinoma is one of its major pathological subtypes [1-3]. Surgical resection is the choice of treatment for LC. Nevertheless, postoperative recurrence and metastasis, plus poor prognosis bring about great challenges to clinicians and researchers. A good understanding of the mechanisms for the onset and development of LC is beneficial to its treatment. In the development of tumors such as prostate cancer and colon cancer, the metabolites of sphingomyelin (sphingosine, ceramide and phosphate-1) play decisive roles in regulating proliferation and apoptosis of tumor cells, whereas sphingosine kinase-2 (SphK-2) is one of the crucial enzymes maintaining the balance among the metabolites of sphingomyelin [4-7]. The expression of SphK-2 varies greatly in different solid tumors, and SphK-2 can promote tumor growth through the sphingosine phosphate signaling pathway. Nevertheless, little is known with regard to the role of SphK-2 in the onset and development of LC.

Recently, microRNA has become one of the most popular biomolecules in cancer study. Numerous studies have confirmed that microRNA is closely associated with proliferation, metastasis, invasion, differentiation and apoptosis of tumors, and is paramount in the onset and development of LC [8-10]. According to the latest findings of studies, miR-338-3p inhibits the proliferation, invasion and migration of a variety of malignant tumors [11, 12]. However, the role of miR-338-3p in the development of LC and its effects on the protein SphK-2 expression remains unclear. Therefore, the purpose of this study was to investigate the...
effects of miR-338-3p on proliferation of LC cells and the possible mechanisms by detecting the expression of the hepatoma cell line HepG2 and hepatic cancer tissue and transfecting the miR-338-3p analogues into LC cells by means of the liposome-mediated transfection, expecting to provide more experimental evidence for miR-338-3p as a novel therapeutic target for LC.

Materials and methods

Cell line

In this study, the human hepatoma cell line HepG2 was stored and cultured in the DMEM medium supplemented with 10% fetal bovine serum (FBS).

Tissue specimens

All the patients provided written informed consent regarding the clinical specimens collected in this study and an approval was obtained from the Hospital Ethics Committee. The fresh hepatic cancer tissue specimens used in this study were taken from the resected hepatic cancer tissue of the patients who had not undergone radiotherapy or chemotherapy before. All the patients were pathologically confirmed as having LC. A total of 10 specimens were pooled in this study, including hepatic cancer tissue and adjacent cancer tissue. Additionally, 5 specimens of fresh normal hepatic tissue were taken from the resected partial liver of the non-tumor patients with intrahepatic bile duct stones who had received partial hepatectomy. All the specimens were stored at -80°C.

Reagents and instruments

The hyperglycaemia DMEM solution and FBS were purchased from Gibico in the USA, GAPDH antibodies and rabbit anti-human SphK-2 antibodies from Santa cruz in the USA, the miR-338-3p analogs from Shanghai Zimmer in China, CCK-8 from Sigma in the USA, the transfection reagents Trizol Regent and Lipofectamine TM 2000 from Invitrogen in the USA, an inverted microscope from Niko in Japan, qRT-PCR kits SYBR Green from Roche in the USA, RNaseA from Boehringer Mannheim in the USA, and gel imaging system from Bio-Rad in the USA.

Detection of miR-338-3p expression in hepatoma cell line HepG2 and hepatic cancer tissue by quantitative PCR (qPCR)

Total miRNAs was isolated following the one-step miRNA procedures from the hepatoma cell line HepG2, hepatic cancer tissue, adjacent cancer tissue and normal hepatic tissue, followed by reverse transcription into cDNA with the use of TaqMan MicroRNA reverse transcription kit. miR-338-3p or U6 internal primers were added for amplification. The primers were designed as follows: miR-338-3p forward, 5’-CCTCCTATTTCCAGCATCAGTG-3’; miR-338-3p reverse, 5’-TATGCTTGTTCTCGTCCTGTGTC-3’; internal reference U6 forward, 5’-ATTTGGAACGATACAGAGAATT-3’; internal reference U6 reverse, 5’-GGAACGCTTACGAGAATTG-3’. The reaction system contained the following reagents: 0.4 μL each of forward and reverse primers, 10 μL SYBR Green Realtime PCR Master Mix, 2 μL of cDNA, and 7.2 μL ddH2O. The quantitative PCR was carried out under the following cycling conditions: initial pre-denaturation at 95°C for 3 min, denaturation at 95°C for 12 s and annealing/extension at 60°C for 45 min, with 40 cycles in total. After completion of the PCR, the gene amplification was analyzed on a 7300 ABI Sequence Detection System for the corresponding Ct values. U6 was used as an internal reference to normalize the copy numbers of the PCR template. The gene relative quantification was calculated using the 2-ΔΔCT method.

Detection of hepatoma cell line HepG2 proliferation by CCK-8

The hepatoma cell line HepG2 was inoculated on a 96-well plate. Cells were transfected at 60% confluency with miR-338-3p analog using the Lipo2000 transfection reagent. The cells un-transfected with miR-338-3p were taken as controls. The cells were cultured in an incubator with 5% CO2 at 37°C for days 1, 2, 3, 4 and 5, respectively. After adding CCK-8 solution (10 μL) into each well, the mixture was cultured for additional 4 hours. Absorbance at each well was measured at 450 nm using a microplate reader and the mean value of 5 wells was calculated.

Detection of protein SphK-2 expression by western blot

Cells were transfected at 60% confluency with miR-133a analogs using the Lipo2000 trans-
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WT Sphk-2 3′-UTR 5′  ggACCAGA–CG–UGAUGCUGGa 3′
miR-338-3p  3′ guUUGUUUAGUAGCUACGACCu 5′
Mut Sphk-2 3′-UTR 5′  ggACCAGA–CG–UCUACGAGCa 3′

Figure 1. Binding sequences of Sphk-2 3′-UTR-WT and miR-338-3p, but Sphk-2 3′-UTR-MUT sequence was not bound to miR-338-3p

Effect of sphk-2 overexpression on the proliferation of HepG2

The segments in the encoding region were cloned into the pcDNA3.1 (+) plasmids based on the human sphk-2 gene sequence (Gene ID: 56848), followed by construction of the pcDNA3.1 (+) -Sphk-2 eukaryotic expression vectors. HepG2 was inoculated on a 96-well plate. Cells were transfected at 60% confluency with pcDNA3.1 (+) -Sphk-2 eukaryotic expression vectors using the Lipo2000 transfection reagent. The cells transfected with pcDNA 3.1 (+) empty vector were served as controls. The transfected cells were cultured in an incubator with 5% CO₂ at 37°C for days 1, 2, 3, 4 and 5, respectively. After adding CCK-8 solution (10 μL) into each well, the mixture was cultured for additional 4 hours. Absorbance at each well was measured at 450 nm using a microplate reader and the mean value of 5 wells was calculated.

Construction and activity detection of luciferase reporter gene

According to SphK-2 sequencing and Target Scan prediction, 3′UTR sequence in the DNA sequence amplified and constructed by PCR was cloned into pGL-Basic reporter gene plasmid (pGL-UTR), sequenced, and constructed into luciferase reporter vector pGL3-WT-Sph2-3′UTR and pGL3-MUT-SphK2-3′UTR. Sequencing was completed by commissioned Takara Bio (Dalian; Figure 1). Lipo2000 liposomes mediated the two plasmids and chemically-synthesized miR-338-3p analogs, respectively, and 70% of pooled HepG cells were transfected by nonsense control sequence. At 48h, the activity of luciferase was detected using dual luciferase assay kits (Promega, US).

Statistical analysis

The statistical data analyses were conducted by the SPSS software, version 21.0. Mea-
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### Results

**miR-338-3p expression level in hepatic cancer cell line**

The qPCR detection indicated that miR-338-3p level in hepatoma cell line HepG2 was 0.764 ± 0.204, which was significantly lower than that (5.493 ± 0.378) in the normal hepatic tissue. The expression levels were remarkably different between the two groups (P<0.001, Figure 2).

**miR-338-3p expression in hepatic cancer tissue**

The miR-338-3p expression levels in 10 hepatic cancer tissue specimens (adjacent cancer tissues and hepatic cancer tissue) from the patients were detected by qPCR. The miR-338-3p level in hepatic cancer tissue (1.697 ± 0.615) was substantially lower than that (5.486 ± 0.782) of the adjacent cancer tissue and that (5.493 ± 0.378) in the normal hepatic tissue (P<0.001); conversely, no significant differences in miR-338-3p levels were observed between adjacent cancer tissue and normal hepatic tissue (P=0.658), as reported in Figure 3.

**Effects of miR-338-3p overexpression on HepG2 cell proliferation**

The results of cell proliferation CCK-8 assay revealed that overexpression of miR-338-3p, as compared with controls, significantly suppressed the proliferation of HepG2 cells at 1 d, 2 d, 3 d, 4 d, and 5 d, respectively (all P<0.001, Table 1).

**Effect of miR-338-3p overexpression on Sph-2 expression in hepatic cell line HepG2**

A remarkably lower level of Sph-2 protein in hepatoma cell line HepG2 was noted in the miR-338-3p mimics group than in the control group (P<0.001, Figure 4).

**Effect of Sphk-2 overexpression on the HepG2 cell proliferation**

Sphk-2 overexpression, as compared with the controls, significantly promoted the proliferation of HepG2 cells, cells at 1 d, 2 d, 3 d, 4 d, and 5 d, respectively (all P<0.001, Figure 5).

### Table 1. Effects of miR-338-3p overexpression on proliferation of hepatoma cell line HepG2

<table>
<thead>
<tr>
<th>Group</th>
<th>1 d</th>
<th>2 d</th>
<th>3 d</th>
<th>4 d</th>
<th>5 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMG</td>
<td>0.296 ± 0.184*</td>
<td>0.419 ± 0.168*</td>
<td>0.574 ± 0.212*</td>
<td>0.689 ± 0.197*</td>
<td>0.793 ± 0.224*</td>
</tr>
<tr>
<td>CG</td>
<td>0.365 ± 0.192</td>
<td>0.536 ± 0.175</td>
<td>0.866 ± 0.271</td>
<td>1.159 ± 0.284</td>
<td>1.476 ± 0.271</td>
</tr>
</tbody>
</table>

Note: MMG denotes miR-338-3p mimics group, CG Control group; compared with the control group, *P<0.001.
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SphK-2 target regulated by miR-338-3p detected by the luciferase reporter gene

For the SphK-2 mutant type, there were merely small differences in the luciferase activity between the miR-338-3p mimics group and the control group (P>0.05); however, for the SphK-2 wild type, much lower activity of luciferase was seen in the miR-338-3p mimics group (P<0.001), as shown in Figure 6.

Discussion

In recent years, with the in-depth research in the role of miRNAs in tumors, increasing evidence demonstrates that miRNA is associated with the onset and development of tumors [13, 14]. Multiple studies have shown that miR-338-3p expresses lowly in many tumors, and miR-338-3p suppresses the growth of colon cancer cells by downregulating SMO protein expression [15]. In the case of prostatic carcinoma, miR-338-3p inhibits the migration and invasion of cancer cells via the CXCR4 axis [16]. Nevertheless, little is reported regarding the mechanisms of miR-338-3p in the development of LC. In this study, in order to clarify the effect of miR-338-3p on the proliferation of hepatoma cells, the expression levels of miR-338-3p in hepatoma cell line HepG2 and hepatic cancer tissues were detected by qPCR, and the effects of miR-338-3p over expression on proliferation of hepatic cancer cell lines were investigated with the use of the cell proliferation CCK-8 assay. The results indicated that the miR-338-3p level in the hepatoma cell line HepG2 was remarkably lower than that in normal hepatic tissue, and the miR-338-3p level was also lower in the hepatic cancer tissue than in the adjacent cancer tissue; miR-338-3p overexpression inhibited the proliferation of the hepatoma cell line HepG2. This suggests that miR-338-3p negatively regulates the proliferation of hepatic cancer cells. Zhang et al. reported downregulation of miR-338-3p expression levels in hepatoma cells was associated with significantly higher levels of β-catenin, MACC1 protein and vascular endothelial growth factor (VEGF), which further promoted tumor angiogenesis [17]. According to Xu et al., miR-338-3p significantly reduced the expression level of hypoxia-induced factor 1α and improved the sensitivity of LC cells to chemotherapeutic agents [18]. The results of this study further indicate that miR-338-3p is essential to the onset and development of LC. However, few reports are involved in the downregulated genes of miR-338-3p.

SphK is a key enzyme for synthesis of 1-sphingosine phosphate in cells, and 1-sphingosine phosphate is a crucial signal molecule for cellular survival and proliferation. Li et al. argued that 1-sphingosine phosphate was a potent regulator during the proliferation of gastric cancer cells [19]. The expression of SphK mRNA significantly increased in methanogenic-induced colon cancer in mice, and SphK/1-sphingosine signaling pathway was closely related to the growth of colon cancer [20]. Additionally, it is reported that the elevated VEGF levels are closely correlated with the clinicopathological features of LC, which contributes to the recurrence, metastasis and invasion of the disease [21]. Purdie et al. held that in bladder cancer cells and endothelial cells, VEGF activated SphK signaling pathway via PKC, indicating that SphK/1-sphingosine signal is involved in transduction of the VEGF signal pathway [22]. Further studies have demonstrated that in LC cells, SphK/1-sphingosine signal pathway is one of the important pathways to regulate the VEGF signaling pathway [23, 24]. Of note, Sphk plays a key role in the pathogenesis of liver cancer.

In this study, to clarify the effect of miR-338-3p expression on the SphK expression in LC, we mediated the transfection of miR-338-3p analogs with the use of liposome and found that lower expression of Sphk-2 in inhibition of the LC cell proliferation by miR-338-3p, while overexpression of Sphk-2 significantly promoted the
proliferation of HepG2 cells; the luciferase reporter gene assay further confirmed regulation of target SphK-2 expression by miR-338-3p.

In conclusion, overexpression of miR-338-3p significantly inhibited the proliferation of LC cells, and was associated with regulation of target SphK-2 expression. Additional studies are required to explore the associated signaling pathways of target genes regulation by miR-338-3p, hence bringing new insights into the management of LC.

Disclosure of conflict of interest

None.

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