Low Expression of miR-448 Induces EMT and Promotes Invasion by Regulating ROCK2 in Hepatocellular Carcinoma

Huaqiang Zhu  Xu Zhou  Chaoqun Ma  Hong Chang  Hongguang Li  Fangfeng Liu  Jun Lu

Department of Hepatobiliary Surgery, Provincial Hospital Affiliated to Shandong University (East District), Jinan, China

Key Words
miR-448 • ROCK2 • EMT • Invasion • HCC

Abstract
Background/Aims: miR-448 has been reported to exhibit abnormal expression in hepatocellular carcinoma (HCC), however, the essential role of miR-448 in HCC progression is still unclear. Methods: real-time PCR was used to detect the expression of miRNAs and candidate genes in HCC samples (n=117). miR-448 mimics and inhibitor were transfected in human HCC cells. The transwell assay was used to examine the cell invasive ability. The regulation mechanism was confirmed by luciferase reporter assay. The markers of EMT were detected by using Western blot. Results: miR-448 was decreased in HCC samples and associated with HCC development. Inhibition of miR-448 significantly promoted cell invasion, while the effect of miR-448 up-regulation was reverse. miR-448 could regulate ROCK2 in hepatocellular carcinoma. Knockdown of ROCK2 expression partially reversed the effect of miR-448 inhibitor. Abnormal expression of miR-448 could regulate the markers of epithelial-mesenchymal transition (EMT). Conclusions: miR-448 may contribute to the progression of HCC via regulating ROCK2 expression.

H. Zhu and X. Zhou contributed equally to this work.

Copyright © 2015 S. Karger AG, Basel
Introduction

Hepatocellular carcinoma (HCC) is one of the malignant cancers worldwide and the third leading cause of cancer-related death [1]. Despite many strategies have been used to treat HCC, the average 5-year overall survival remains low [2], because of the cancer cells metastasis. However, the complicated molecular and cellular mechanisms of HCC development are not unclear. Therefore, elucidating the potential molecular mechanism of tumor development and identifying the therapeutic targets would improve the therapy of HCC.

MicroRNAs (miRNAs) were identified as an abundant class of small non-coding RNAs that regulated different biological processes of tumors by binding to target gene 3’-untranslated regions (UTRs) [3]. Growing evidence has shown that miRNAs play crucial roles in various physiological processes and are involved in the initiation and progression of HCC [4-8]. A recent study had identified the pathogenesis-related microRNAs in HCC by expression profiling, which showed miR-448 was down-regulated in 40 HCC samples and served as a novel biomarker for HCC [9]. However, the underlying role of miR-448 in the development of HCC is not explored.

Rho-associated coiled-coil-containing protein kinase 2 (ROCK2) belongs to a family of serine/threonine kinases, which are activated via interaction with Rho GTPases [10]. An increasing number of studies reported that ROCK2 is involved in the development of the malignant tumors. Up-regulation of ROCK2 expression is implicated in poor prognosis in different human cancers, including breast cancer and lung cancer [11, 12]. ROCK2 plays a key role in HCC invasion and metastasis [13, 14]. For example, in vitro and in vivo, up-regulation of ROCK2 in HCC is obviously involved in the presence of tumor microsatellite formation, and down-regulation of ROCK2 inhibited HCC migration and invasion [2].

In our study, we demonstrated that miR-448 was down-regulated in HCC samples compared with corresponding adjacent tissues, and the expression analyses exhibited a significantly negative correlation. We first found that miR-448 was related to tumor stage and metastasis of HCC. In addition, we identified that miR-448 could regulate the HCC cells invasion by regulating ROCK2. Moreover, we also revealed that miR-448 could regulate the markers of EMT in HCC cells.

Materials and Methods

Clinical Tissues

A total of 117 HCC samples were collected from the archives of the Provincial Hospital Affiliated to Shandong University (Jinan, China). Total patients had provided the written informed consent. The informed consent approval from the Shandong University Institute Research Ethics Committee was obtained.

Cell culture, cell transfection and RNA interference

Five HCC cell lines (HepG2, Hep3B, Huh-7, QSG-7701 and SMMC-7721) and a normal cell line (LO2) were cultured in DMEM Medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS (Life Technologies, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C. We conducted the transfections by using a Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA). Cell lines were transfected with miR-448 mimics or inhibitor (GenePharma, Shanghai, China). Short interfering RNA targeting ROCK2 (Santa Cruz Biotechnology, Santa Cruz, USA) were transfected into cells in 6-well plates using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions.

RNA isolation and real-time PCR

Isolation of total RNA from cells was performed using the miRVana miRNA Isolation Kit (Ambion, Austin, TX, USA). According to the manufacturer’s instructions (Ambion, Austin, TX, USA), miR-448 was investigated using the miRVana real-time PCR miRNA Detection Kit and real-time PCR Primer Sets. The primers for real-time PCR were shown as below. miR-448: Forward primer: TTATGGCGATGTGTTCCCTATG.
Reverse primer: ATGATGCGACGGGCTACATACCT; ROCK2: Forward primer: TCAGAGGTCTACAGATGAAGGC, Reverse primer: CAGGGGCTATTGGCAAGAG. U6 small nuclear RNA was used for normalization. Real-time PCR assay was performed on ABI PRISM7500 system (Applied Biosystems, California, USA).

Western blotting assay
Total protein obtained from cultured cells was examined using a BCA Protein Assay Kit (Beyotime, Jiangsu, China). The blots were blocked in BSA (5% w/v in PBS + 0.1% Tween 20) at room temperature. The antibodies which are against ROCK2, Vimentin, and GAPDH were used according to the manufacturer’s instructions, and were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies against N-cadherin and E-cadherin were obtained in our previous study, which were purchased from Abcam (Cambridge, MA, USA). After using the secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, USA) at 1:2,000 (v/v) dilutions in PBS + 0.1% Tween 20 for 1 h, the signals were shown using ECL kit (Thermo Scientific Pierce, Thermo Fisher Scientific, Rockford, USA).

Cell proliferation assays
We performed CCK-8 assays to investigate cells proliferation. According to the instructions, Cell Counting Kit-8 (CCK-8) (Dojindo, Tokyo, Japan) reagent was added at 0, 24, 48, and 72 h respectively after seeding 4 × 10⁴ cells per well in a 96-well plate, and incubated at 37°C for 2 h. The OD (optical density) value was detected by using a microplate reader (Bio-Rad, Richmond, CA, USA).

Apoptosis assay
Cells apoptosis was measured in cells with an Annexin V assay kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) were added after treating cell according to the instructions. Then 300 µL of 1× binding buffer was added. The flow cytometry with a FACS Calibur (BD Bioscience, San Jose, CA, USA) was performed to evaluate the result.

Transwell invasion assay
Cell invasion assays were performed with BioCoat Matrigel (BD Biosciences, San Jose, CA) and 8-µm pore size invasion chambers (Millipore, Eschborn, Germany) according to the manufacturer’s instructions [15]. After treatment, cells were harvested. Then, cells (1×10⁴) were seeded into the upper chamber of a 24-well plate with serum-free medium. After 24 h incubation in 37°C and 5% CO₂, cells that remained on top of the filter were stained with crystal violet. The images were acquired by using NIS Elements image analysis software (Nikon, Tokyo, Japan). For the membrane images, we measure the migrated cells using image analysis software ImagePro Plus 6.0 (Media Cybernetics, Bethesda, USA).

Luciferase reporter assay
Luciferase reporter assay was performed according to the manufacturer’s protocol. 48 hours after transfection, luciferase activity was evaluated in Victor 1420 Multilabel Counter (Wallac, Finland) using Luciferase Assay System (Promega, USA).

Bioinformatics Analysis
We used the bioinformatic method to predict the potential targeting genes of miR-448. Strikingly, the three websites including microRNA.org (http://www.microrna.org/microrna/), miRDB (http://mirdb.org/cgp-bin/), and TargetScan (http://www.targetscan.org/) database showed that ROCK2 was a candidate gene due to 3’-UTR of ROCK2 binds to miR-448 with the high score.

Statistical analysis
miR-448 and ROCK2 expression levels in the clinical samples was investigated by using chi-square test. All the histogram was evaluated by performing GraphPad Prism, version 4.0 (GraphPad Software, San Diego California, USA). Statistical analyses were performed using Stata. 11.0. P<0.05 indicated statistically significant.
Results

*miR-448 and ROCK2 expression were negatively correlated in HCC tissues*

The expression levels of miR-448 and ROCK2 were detected in 117 HCC samples and the corresponding adjacent tissues by real-time PCR. Strikingly, the results showed that miR-448 expression was lower in HCC tissues than that in corresponding adjacent tissues (p<0.05), whereas ROCK2 expression in tumor tissues was higher than that in corresponding adjacent tissues (p<0.05) (Fig. 1A and B). The scatter plots revealed that miR-448 and ROCK2 expression levels were negatively correlated in HCC tissues (Fig. 1C). All HCC tissues were divided into two groups using median mRNA level of miR-448 (*median=0.3032*) and ROCK2 (*median=3.0074*) as threshold. The correlation between miR-448 expression and clinicopathological characteristics was shown as in Table 1. There was significantly correlation with tumor stage (p=0.007) and metastasis (p=0.000). Thus, low expression level of miR-448 might contribute to the metastasis of HCC.

Table 1. Expression levels of miR-448 and ROCK2 in HCC and corresponding adjacent tissues. *median = 0.3032, *median = 3.0074, * indicates p<0.05

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All Patients</th>
<th>miR-448 low expression</th>
<th>miR-448 high expression</th>
<th>ROCK2 low expression</th>
<th>ROCK2 high expression</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>median=0.3032</td>
<td>median=3.0074</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>58</td>
<td>59</td>
<td>58</td>
<td>28</td>
<td>28</td>
<td>0.642</td>
</tr>
<tr>
<td>≥60</td>
<td>60</td>
<td>39</td>
<td>31</td>
<td>28</td>
<td>29</td>
<td>0.644</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>58</td>
<td>29</td>
<td>29</td>
<td>30</td>
<td>30</td>
<td>0.927</td>
</tr>
<tr>
<td>Female</td>
<td>59</td>
<td>30</td>
<td>29</td>
<td>30</td>
<td>29</td>
<td>0.783</td>
</tr>
<tr>
<td>AFP (µg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;200</td>
<td>60</td>
<td>31</td>
<td>29</td>
<td>30</td>
<td>29</td>
<td>0.519</td>
</tr>
<tr>
<td>≥200</td>
<td>57</td>
<td>27</td>
<td>29</td>
<td>27</td>
<td>28</td>
<td>0.164</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤5</td>
<td>56</td>
<td>23</td>
<td>33</td>
<td>31</td>
<td>32</td>
<td>0.007*</td>
</tr>
<tr>
<td>&gt;5</td>
<td>61</td>
<td>36</td>
<td>25</td>
<td>24</td>
<td>27</td>
<td>0.000*</td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I, II</td>
<td>50</td>
<td>18</td>
<td>32</td>
<td>41</td>
<td>19</td>
<td>0.000*</td>
</tr>
<tr>
<td>III, IV</td>
<td>67</td>
<td>41</td>
<td>17</td>
<td>50</td>
<td>34</td>
<td>0.000*</td>
</tr>
<tr>
<td>Metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>73</td>
<td>22</td>
<td>51</td>
<td>48</td>
<td>37</td>
<td>0.000*</td>
</tr>
<tr>
<td>Yes</td>
<td>44</td>
<td>10</td>
<td>34</td>
<td>17</td>
<td>17</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

Fig. 1. miR-448 was reduced and ROCK2 was increased in HCC patients. A: Real-time PCR assay were used to detect miR-448 mRNA expression in HCC tissues (T) and corresponding adjacent tissues (P) (n=117, p<0.05). B: The mRNA levels of ROCK2 relative to GAPDH in human HCC tissues (T) and corresponding adjacent tissues (P) were measured by using real-time PCR assay. C: The scatter plots revealed that miR-448 and ROCK2 expression levels were negatively correlated in HCC tissues (r=0.742, p<0.0001). Data are represented as mean±SEM. * indicates p<0.05. Two biological replicates for each sample were used for real-time PCR analysis and three technical replicates were analyzed for each biological replicate.
Fig. 2. miR-448 regulated cell lines invasion. A: The expression levels of miR-448 in five HCC cell lines (HepG2, Hep3B, Huh-7, QSG-7701 and SMCC-7721) and a normal cell line (LO2) relative to U6 were measured by real-time PCR. Two biological replicates for each sample were used for real-time PCR analysis and three technical replicates were analyzed for each biological replicate. Data are represented as mean±SEM. * indicates p<0.05. B: miR-448 expression level in cell lines transfected with miR-448 mimics, miR-448 inhibitor, control for miR-mimics (NC) and control for miR-448 inhibitor (inhibitor NC). The result was validated by real-time PCR. Two biological replicates for each sample were used for real-time PCR analysis and three technical replicates were analyzed for each biological replicate. Data are represented as mean±SEM. * indicates p<0.05. C: Transwell assay was performed as described in Materials and Methods. HepG2 and Hep3B cells were transfected with NC, miR-448 mimics, inhibitor NC and miR-448 inhibitor for 24h. As shown, the representative images of invasive cells at the bottom of the membrane stained with crystal violet were visualized. For the membrane images, we measure the migrated cells using image analysis software ImagePro Plus 6.0 (Media Cybernetics, Bethesda, USA). The numbers of invading cells are shown in the lower panel (n=3 per condition). All data represent mean ± SEM. *p<0.05 by independent Student’s t-test.
Aberrant expression level of miR-448 was correlated with cell invasion

A recent study reported that miR-448 was reduced in HCC by using microarray [9], however, its roles in the development of HCC have not been elucidated. Here, to explore the potential role of miR-448 expression in HCC tumorigenesis, we detected the expression of miR-448 in five HCC cell lines (HepG2, Hep3B, Huh-7, QSG-7701 and SMMC-7721) and a normal cell line (LO2) by using real-time PCR. As shown in Fig. 2A, the expression of miR-448 was reduced in all five cell lines, compared with the normal cell line. And the expression of miR-448 in HepG2 and Hep3B was between those in Huh-7 and in QSG-7701 (SMMC-7721). Based on this expression pattern, we chose the HepG2 and Hep3B HCC cell lines to investigate the effect of miR-448. Cell lines were transfected with miR-448 negative control (NC), miR-448 mimics, miR-448 inhibitor negative control (inhibitor NC) and miR-448 inhibitor respectively. The transfection efficiency was validated by real-time PCR (Fig. 2B). Furthermore, using a Matrigel-coated transwell chamber, we found that overexpression of miR-448 suppressed cells invasion, whereas inhibition of miR-448 promoted the invasion ability of cells (Fig. 2C). This finding revealed that miR-448 might play an important role in regulate HCC metastasis.
miR-448 had no effect on cell proliferation and apoptosis

In addition, to explore whether miR-448 could regulate HCC cell lines proliferation and apoptosis, we performed CCK-8 and apoptosis assays. The CCK-8 assay test showed no distinct differences on proliferation after manipulation of miR-448 in cells at 24-hour, 48-
Fig. 5. Knockdown of ROCK2 abrogated the effect of miR-448 inhibition. A: Both HepG2 and Hep3B cells were co-transfected with miR-448 inhibitor and siRNA/ROCK2. The transfection cells of miR-448 inhibitor and the co-transfection cells of miR-448 inhibitor and siRNA/control were used as the control groups. The transfection efficiency was verified by using western blot assay. The blots are representative of three independent experiments and quantification of independent experiments in lower panel. All data represent mean ± SEM. *p<0.05 by Student’s t-test. B: According to the Materials and Methods, transwell assay was performed as shown. The representative images of invasive cells at the bottom of the membrane stained with crystal violet were visualized. For the membrane images, we measure the migrated cells using image analysis software ImagePro Plus 6.0 (Media Cybernetics, Bethesda, USA). The numbers of invading cells are
showed in the lower panel (n=3 per condition). All data represent mean ± SEM. *p<0.05 by independent Student’s t-test. C: E-cadherin, N-cadherin and Vimentin protein expression levels in HepG2 cells transfected with NC, miR-448 mimics, inhibitor NC, and miR-448 inhibitor were detected by using western blotting assay. GAPDH level served as a loading control. The blots are representative of three independent experiments and quantification of independent experiments in right panel. All data represent mean ± SEM. *p<0.05 by Student’s t-test.

hour, and 72-hour time points (Fig. 3A). And there was no obvious difference in the apoptosis assay (Fig. 3B). These findings indicated that miR-448 had no effect on cell proliferation and apoptosis in vitro.

miR-448 could regulate ROCK2 in HCC cells

As described in the Methods, the target genes of miR-448 were predicted by online miRNA target prediction algorithms. We found that ROCK2, which had been reported to promote the invasion and metastasis of hepatocellular carcinoma [16] has the binding site. Then, to identify whether miR-448 could bind to ROCK2 mRNA, we cloned the 3’-UTR fragment involving in the predicted site into pGL3 luciferase reporter vector, named pGL3-ROCK2. We also cloned the 3’-UTR fragment containing mutant sequence as a control group, named pGL3-ROCK2-MUT in the predicted target site. Interestingly, the luciferase reporter showed that the luciferase activity reduced in HepG2 cells with miR-448 mimics and pGL3-ROCK2 vectors. However, miR-448 mimics did not have any effect on luciferase activity when target cells were transfected with pGL3-ROCK2-MUT vector (Fig. 4A). To further identify the regulatory effect of miR-448 on ROCK2, we performed real-time PCR and western blot assays to detect the mRNA and protein expression levels of ROCK2 responses to the changes of miR-448 expression. The assays showed a negative regulatory effect of miR-448 on ROCK2 both in mRNA and protein expression levels (Fig. 4B). Overexpression of miR-448 could reduce ROCK2 expression; meanwhile, inhibition of miR-448 could increase the expression of ROCK2. In sum, these results implied that miR-448 could regulate ROCK2 expression by binding its 3’-UTR. These data suggested that miR-448 regulated ROCK2 in HCC cells.

Knockdown of ROCK2 partly reversed the effect of miR-448 inhibition

In addition, as shown in the previous results, ROCK2 was up-regulated in cells which were transfected with miR-448 inhibitor. Therefore, we silenced the expression of ROCK2 by RNA interference (data not shown). Cells were co-transfected with miR-448 inhibitor and siRNA/ROCK2. The transfection cells of miR-448 inhibitor and the co-transfection cells of miR-448 inhibitor and siRNA/control were the control groups. The transfection efficiency was confirmed by using western blot assay (Fig. 5A). ROCK2 expression was obviously lower in cells both co-transfected with miR-448 inhibitor and siRNA/ROCK2 than that in the control groups. Moreover, the transwell invasion assay revealed that the invasive ability indeed reversed to some extent when ROCK2 down-regulation in cells transfected with miR-448 inhibitor compared to the control groups (Fig. 5B). These results indicated that the functional effect of miR-448 on HCC cell lines depends on ROCK2.

miR-448 could regulate the markers of EMT

Moreover, we performed western blot assay to explore whether miR-448 could regulate the molecular changes of EMT in HCC cell lines, the expression of mesenchymal markers, including N-cadherin and Vimentin and the epithelial marker, E-cadherin. Thus, HepG2 cells were transfected with NC, miR-448 mimics, inhibitor NC and miR-448 inhibitor. In the protein expression level, up-regulated miR-448 expression promoted E-cadherin expression and decreased N-cadherin and Vimentin expression. Meanwhile, down-regulated miR-448 expression decreased E-cadherin expression and promoted N-cadherin and Vimentin expression (Fig. 5C). This result suggested that miR-448 could regulate the molecular changes of EMT in HCC.
Discussion

HCC is a prevalent malignancy in the world. Cancer metastases are a major factor in the mortality of HCC patients. Aberrant expression of miRNAs plays a critical role in the different cancers development and progression through modulating oncogenic and tumor suppressor pathways [17-19]. Specifically for HCC, it is already reported that miRNAs are implicated in the cells apoptosis, prognosis, proliferation and invasion [20, 21]. For example, miR-486, which is frequently downregulated in HCC, inhibits HCC progression by targeting p85α and PI3K-AKT activation [22].

In the present study, we focused on the mechanism of miR-448 in HCC invasion. The expression level of miR-448 was reduced in HCC. Meanwhile, ROCK2 expression was increased in HCC. To better understand the mechanisms of miR-448 in HCC cell invasion, we identified that miR-448 regulated ROCK2 in HCC using a bioinformatics method. Recently, some studies reported that ROCK2 was frequently overexpressed in human HCC and was closely associated with tumor microsatellite formation, a pathologic feature of intrahepatic metastasis. And ROCK2 promoted HCC migration and invasion both in vitro and in vivo. Using transwell invasion assay, we found that inhibition of miR-448 promoted HCC cells invasion, whereas up-regulation of miR-448 inhibited HCC cells invasion. Furthermore, the CCK-8 and apoptosis assays showed that miR-448 could not regulate the proliferation and apoptosis in HCC cells in vitro.

Growing evidence showed that EMT is involved in migration, tumor invasion and dissemination [23-25]. Decreased E-cadherin and elevated Vimentin and N-cadherin expression is one hallmark of EMT [26]. An increasing studies established functional associations between microRNAs and key effectors of EMT occurring in the context of carcinogenesis and embryonic development, such as miR-200 [27] and miR-10b [28]. In our study, high expression of miR-448 resulted in increased E-cadherin expression and decreased N-cadherin and Vimentin expression. Meanwhile, knockdown miR-448 expression caused decreased E-cadherin expression and increased N-cadherin and Vimentin expression.

Taken together, we identified a critical oncomiR, miR-448, that is frequently low-expressed in HCC. Meanwhile, we found the inverse expression levels of miR-448 and ROCK2, both of which were related to tumor stage and metastasis. Mechanistically, we suggested that miR-448 inhibited HCC cells invasion by suppressing ROCK2 by binding of its 3'-UTR. As the limit on the number of HCC samples, more elaborate studies will be necessary for further exploration of the underlying role of miR-448 in the development of HCC. In future, miR-448 may be a therapeutic target in HCC.

Abbreviations

HCC (hepatocellular carcinoma); UTR (untranslated region); siRNA (small interfering RNA); EMT (epithelial-mesenchymal transition); CCK-8 (Cell Counting Kit-8).

Disclosure Statement

None declared.

Acknowledgments

This study was supported by Natural Science Foundation of Shandong Province in China (Y2008C22 and ZR2014HM099), Excellent Youth Scientist Foundation of Shandong Province in China (2007BS03038), and others of Shandong Province (2014WS0093, 2014GGB14041, 2014WS0095 and 2014WS0096).
References


