MicroRNA-135a contributes to the development of portal vein tumor thrombus by promoting metastasis in hepatocellular carcinoma

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Background & Aims: Portal vein tumor thrombus (PVTT) has previously been demonstrated to correlate with poor prognosis of hepatocellular carcinoma. Approximately 50–80% of HCC is accompanied by portal or hepatic vein invasion. The underlying mechanisms of PVTT development remain unclear. This study aimed to elucidate the role of miR-135a in PVTT tumorigenesis.

Methods: In the present study, we investigated the expression of microRNAs and mRNAs in PVTT tissues using advanced microRNA and cDNA microarray techniques. MicroRNA (miR)-135a was noted to be highly over-expressed in PVTT and the cell line CSQT-2 and was selected for further study. We characterized the function of miR-135a in vitro and in vivo. We also analyzed the clinical relevance of miR-135a in relation to the prognosis and survival of HCC patients with PVTT.

Results: Our analyses found that the miRNA and mRNA expression profiles of PVTT were distinct from the parenchyma tumor. Overexpression of miR-135a favors invasive and metastatic behavior in vitro. Furthermore, in a CSQT-2 orthotopic transplantation nude mouse model, blockade of miR-135a significantly reduced PVTT incidence. We also found that miR-135a was transcribed by forkhead box M1 (FOXM1), and metastasis suppressor 1 (MTSS1) was identified as the direct and functional target of miR-135a. Additionally, the cohort analysis revealed the relevance of miR-135a with respect to the prognosis and survival of HCC patients with PVTT.

Conclusions: Our data suggest an important role for miR-135a in promoting PVTT tumorigenesis and indicate the potential application of miR-135a in PVTT therapy.

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Introduction

Hepatocellular carcinoma (HCC) results in hundreds of thousands of deaths worldwide every year, half of which occur in China [1]. Additionally, the mortality of HCC has displayed the fastest rate of increase in the US, while the overall cancer-related mortality has been declining [2].

Portal vein tumor thrombosis (PVTT) in patients with HCC is a major complication and is associated with poor survival [3,4]. If left untreated, a median survival of less than 6 months has been reported [5]. Approximately 50–80% of HCC has been reported to be accompanied by portal or hepatic vein invasion, as demonstrated by magnetic resonance imaging (MRI) and ultrasonography [6]. However, the molecular mechanism of PVTT remains unclear.

PVTT is considered as a special type of HCC metastasis, which is a complex cascade. The alteration of some adhesion molecules involved in HCC metastasis, such as membrane-type 1 matrix metalloproteinase (MT1-MMP) and matrix metalloproteinase-2 (MMP-2), has been described [7]. Our studies and others have shown that altered miRNA levels can also result in aberrant expression of gene products that may contribute to tumor metastasis [8]. With the identification of increasing numbers of miRNAs deregulated in tumors, several miRNAs have been verified to affect HCC metastasis, subtype classification, and prognosis [9,10].

Based on these findings, we designed this study to test whether aberrantly expressed miRNAs contribute to PVTT formation. A miRNA array was used to comparatively analyze the miRNA expression profile of PVTT and the corresponding parenchyma tumor (PT) tissue. Several differentially expressed miRNAs were selected and tested in both tissues and cell lines. On this
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basis, miR-135a was found to be significantly up-regulated and was selected for further study. We characterized the function of miR-135a in vitro and in vivo. Our results show that the up-regulated miR-135a, which is transcribed by forkhead box M1 (FOXM1), promotes the invasion of CSQT-2 cells by repressing the expression of metastasis suppressor 1 (MTSS1). The prognostic value of miR-135a was also evaluated in patients with PVTT.

Materials and methods

Samples

Human liver PT tissues and PVTT tissues were obtained from HCC patients with PVTT in the Eastern Hepatobiliary Surgery Hospital after informed consent. The follow-up procedures have been described in our previous study [3]. Overall survival (OS) and disease-free survival (DFS) were defined as previously described [11]. Female athymic nude mice (4–6 weeks old) were purchased from the Transgenic Animal Research Center, Second Military Medical University. The mice were maintained in a pathogen-free facility and used in accordance with the institutional guidelines for animal care. CSQT-2 originated from a PVTT and was established in our lab [12]. Hep3B, HepG2, and SK-HEP-1 cell lines were obtained from the American Tissue Culture Collection (ATCC), and HuH7 was obtained from the Japanese Collection of Research Bioresources (JCRB). Cells were cultured as previously described [9].

Microarray

miRNA microarray and cDNA microarray analyses were supplied by KangChen Corp. Details regarding the arrays are listed in Supplementary Materials and Methods (Microarray).

Western blot and immunohistochemistry

Western blot and immunohistochemistry were performed as described in Supplementary Materials and Methods. The following antibodies were used: FOXM1 rabbit Ab (1:300; C-20, Santa Cruz Biotech. Inc.), MTSS1 mouse mAb (1:300; SS-3, Santa Cruz Biotech. Inc.), and β-actin (Cell Signaling).

In situ hybridization

Probes of pri-miR-135a with a digoxin label were synthesized for in situ hybridization (ISH). ISH was performed on sections of tumor tissues as previously reported [13].

Statistical analysis

The Student’s t-test was used to compare two groups unless otherwise indicated (χ² test). Categorical data were analyzed using Fisher’s exact test, and quantitative variables were analyzed using the t-test or Pearson’s correlation test. The Cox regression model was used to perform multivariate analysis. Receiver operating characteristic (ROC) curve analysis was used to determine the predictive value of the parameters. p < 0.05 was considered statistically significant.

Results

miRNAs are differentially expressed in PVTT compared to PT

We first screened for specific candidate miRNA molecules involved in PVTT carcinogenesis. For this purpose, cancerous tissues were collected from five HCC patients with PVTT (Supplementary Table 2). miRNA expression profiles were evaluated using microarray hybridization studies. A series of miRNAs was aberrantly expressed in PVTT tissues compared to PT nodules (Supplementary Fig. 1A). The miRNAs with the greatest fold changes are listed in Table 1. These selected miRNAs were further confirmed by real-time PCR (Supplementary Fig. 2). The real-time PCR data agreed with the microarray data (correlation coefficient = 0.981), and the two methodologies demonstrated high internal consistency. Bootstrapping hierarchical clustering analysis of the miRNAs listed in Table 1 generated a map with a clear distinction between the two types of samples (PVTT tissues and PT tissues) (Supplementary Fig. 1B). These data indicated that the significantly altered expression of these miRNAs might be involved in the pathogenesis or phenotypic behavior of PVTT.

miR-135a promotes cell invasion and metastasis in vitro

We next assessed the contribution of aberrantly expressed miRNAs to PVTT carcinogenesis. Because miR-135a showed the greatest increase, this miRNA was selected as the representative molecule for further study. First, we analyzed miR-135a expression in four HCC cell lines (HepG2, Hep3B, SK-HEP-1 and HuH7) and one PVTT-derived cell line (CSQT-2). As shown in Fig. 1A, although miR-135a was readily detectable in all of the HCC cell lines, its expression was significantly up-regulated from approximately twofold (compared to SK-HEP-1) to eightfold (compared to Hep3B) in CSQT-2 cells. Next, we determined whether the miR-135a up-regulation would affect the cancer biology. Hep3B cells, which express the lowest level of miR-135a, were selected for the gain-of-function analysis. The loss-of-function analysis was performed in CSQT-2 cells. For each condition, miR-135a expression was assessed using real-time PCR after transfection of mimics or inhibitors to ensure effective miRNA modulation (Supplementary Fig. 3A). Then, we analyzed the proliferation and induced apoptosis potential of the transfected cells (Hep3B transfected with miR-135a or its mock, miR-mock; CSQT-2 transfected with miR-135a antisense, AS or its mock, AS-mock). The CCK-8 assay demonstrated that miR-135a had no significant effect on cell proliferation (Fig. 1B). The modulation of miR-135a expression also had no significant effect on the rate of apoptosis (from 3% to 6%) (Supplementary Fig. 3B). Because the apoptosis rates in each group were all at very low levels, an apoptosis inducer (Ap) was thus employed in the culture system to confirm the results. As shown in Fig. 1C, aberrant miR-135a expression indeed had no significant effect on apoptosis in vitro. Therefore, we next evaluated the capacity for invasion. The gain-of-function analysis showed that when compared to the blank or miR-mock group, Hep3B cell invasion was significantly increased after transfection with miR-135a mimics. The invasion of CSQT-2 cells was markedly inhibited by transfection with miR-135a AS. Invasion was
not significantly altered in cells that were co-transfected with AS-mock (Fig. 1D). This result strongly indicates a functional role for miR-135a in mediating cell invasion.

**miR-135a is directly regulated by the transcription factor FOXM1**

We next determined how the transcription of miR-135a was controlled under our experimental conditions. To identify candidate transcription factors (TFs), we used a NimbleGen cDNA microarray platform, which has already been validated by numerous studies [15,16], to evaluate the mRNA expression profiles of CSQT-2 and Hep3B cells. The result from hierarchical clustering based on experimental conditions shows a distinguishable gene expression pattern between the samples (Supplementary Fig. 4), suggesting that these two cell lines differ with respect to biological origin and behavior. For the subsequent analysis, the most significantly up-regulated TF mRNAs and most down-regulated mRNAs in CSQT-2 cells were selected and are listed in Supplementary Table 3. To determine whether TFs could regulate miR-135a expression, miR-135a in CSQT-2 cells was quantified using real-time PCR. The expression of miR-135a, infected into Hep3B cells, and overexpression of FOXM1 was performed into Hep3B cells. The construct pcDNA-foxm1 was transiently transfected into Hep3B cells. In contrast, no significant change occurred in the expression of both miR-302d and miR-214 (Supplementary Fig. 5A). Subsequently, we tested whether miR-135a expression could be reduced or inhibited by siRNA against FOXM1. For this purpose, we designed four different siRNA sequences (siRNA 1–4) targeting human FOXM1 mRNA different sites and scrambled sequences using Ambion’s recommended procedure (Supplementary Table 1). FOXM1 was immunostained and detected by flow cytometry to screen for the most effective siRNA (Supplementary Fig. 5B). The most effective siRNA, siRNA2, and its scrambled control were used in the ensuing vitro analyses. Forty-eight hours after transfection of the siRNA2 into CSQT-2 cells, the reduction in FOXM1 protein expression, miR-302d and miR-214 was then investigated by real-time PCR. To determine whether FOXM1 specifically regulates miR-135a expression, miR-302d and miR-214 were selected as controls based on the miRNA microarray results, which indicated that miR-302d was up-regulated and miR-214 was down-regulated (Table 1). As shown in Fig. 2B (right panel), miR-135a was reduced by approximately 40% compared to the mock-transfected cells. In contrast, no significant change occurred in the expression of both miR-302d and miR-214 (Supplementary Fig. 5C). To further confirm regulation of miR-135a expression by FOXM1, gain-of-function analysis was then performed using Hep3B cells. The construct pcDNA-foxm1 was transiently transfected into Hep3B cells, and overexpression of FOXM1 was detected by Western blot (Fig. 2B). The expression of miR-135a, miR-302d and miR-214 was then investigated by real-time PCR. When FOXM1 was overexpressed in Hep3B cells by transfection with pcDNA-foxm1 (foxm1), miR-135a was markedly up-regulated compared to cells transfected with pcDNA empty vector (con) (Fig. 2B). The two control miRNAs (miR-302d and

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### Table 1. miRNAs aberrant expression in PVTT.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Means ± SD</th>
<th>p value</th>
<th>Chromosome location</th>
<th>Potential targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased expression &gt;1.5-fold</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>miR-135a</td>
<td>5.94 ± 0.10</td>
<td>&lt;0.05</td>
<td>3p21.1, 12q23.1</td>
<td>MTSS1, GAS7</td>
</tr>
<tr>
<td>miR-302d</td>
<td>2.56 ± 0.34</td>
<td>&lt;0.01</td>
<td>4q25</td>
<td>RASSF2, RGL1</td>
</tr>
<tr>
<td>miR-517b</td>
<td>2.35 ± 0.51</td>
<td>&lt;0.05</td>
<td>19q13.42</td>
<td>WNT4, TSC1</td>
</tr>
<tr>
<td>miR-34a</td>
<td>2.06 ± 0.33</td>
<td>&lt;0.05</td>
<td>1p36.22</td>
<td>FKBP1B, FOXP1</td>
</tr>
<tr>
<td>miR-424</td>
<td>1.92 ± 0.07</td>
<td>&lt;0.01</td>
<td>Xq26.3</td>
<td>MYB, PAPPA</td>
</tr>
<tr>
<td>miR-130a</td>
<td>1.84 ± 0.06</td>
<td>&lt;0.01</td>
<td>11q12.1</td>
<td>DDX6, GJA1</td>
</tr>
<tr>
<td>miR-195</td>
<td>1.66 ± 0.26</td>
<td>&lt;0.05</td>
<td>17p13.1</td>
<td>CD28, KIF23</td>
</tr>
<tr>
<td>miR-624</td>
<td>1.63 ± 0.34</td>
<td>&lt;0.05</td>
<td>14q12</td>
<td>NBEA, NFIB</td>
</tr>
<tr>
<td>miR-150</td>
<td>1.53 ± 0.11</td>
<td>&lt;0.05</td>
<td>19q13.33</td>
<td>RC3H1, PIK3R1</td>
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<tr>
<td>miR-199b</td>
<td>1.52 ± 0.09</td>
<td>&lt;0.05</td>
<td>9q34.11</td>
<td>GARNL1, SULF1</td>
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<tr>
<td>Decreased expression &lt;0.6-fold</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>miR-214</td>
<td>0.37 ± 0.07</td>
<td>&lt;0.01</td>
<td>1q24.3</td>
<td>RC3H1, ZFAND3</td>
</tr>
<tr>
<td>miR-654</td>
<td>0.35 ± 0.14</td>
<td>&lt;0.05</td>
<td>14q32.31</td>
<td>MTSS1, KIF21B</td>
</tr>
<tr>
<td>miR-675</td>
<td>0.33 ± 0.10</td>
<td>&lt;0.01</td>
<td>11p15.5</td>
<td>MARK4</td>
</tr>
<tr>
<td>miR-503</td>
<td>0.32 ± 0.19</td>
<td>&lt;0.05</td>
<td>Xq26.3</td>
<td>ZNF423, TNC6B</td>
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<tr>
<td>miR-433</td>
<td>0.17 ± 0.18</td>
<td>&lt;0.05</td>
<td>14q32.2</td>
<td>NAV1, SORB31</td>
</tr>
</tbody>
</table>
miR-135a regulates the expression of MTSS1

We next examined the mechanisms by which miR-135a induces tumor invasion and metastasis by combining a computational method (TargetScan Release 5.1, April 2009; http://www.targetscan.org/) with cDNA microarray information to identify miR-135a targets in humans. Among the 50 candidate genes (Supplementary Table 4), MTSS1 was one of the highest scoring candidates. In addition, MTSS1 has been found to be related to HCC metastasis [17,18] and has an inverse correlation with patient prognosis and survival [19]. Most importantly, MTSS1 expression was significantly down-regulated in CSQT-2 cells (Supplementary Table 3). This finding was first confirmed by immunostaining HCC patients’ tissues. Compared to negative control, (NC), MTSS1 staining was primarily found in the cytoplasm of tumor cells (Fig. 3A). MTSS1 expression in PT tissues was stronger than in other hepatocarcinoma cell lines. (B) miR-135a has no effect on cell proliferation as assessed by the CCK-8 assay. (C) miR-135a is not involved in cell apoptosis as analyzed by flow cytometry. (D) Transwell invasion assay revealed that miR-135a promotes cell invasion. Data are shown as the means ± SD based on at least three independent experiments. *p < 0.05; **p < 0.01. AS, antisense oligonucleotides; Ap, apoptosis inducer.

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MiR-135a reduction inhibits tumor invasion and metastasis in vivo

Next, we determined whether blocking miR-135a could reduce the metastatic activity of CSQT-2 and thus prevent tumors from metastasizing in vivo. To accomplish this goal, CSQT-2 cells were used in an orthotopic transplantation nude mouse model. Subcortaneous tumor pieces were implanted in the livers of the nude mice using a previously described method [9]. pLKO-anti-mock and pLKO-anti-miR-135a were then administered to these nude mice. Sixty days later, all of the mice were sacrificed. First, miR-135a expression levels in the livers were measured by real-time PCR. As shown in Fig. 4A, miR-135a was expressed at high levels in the livers of both mice injected with the anti-mock treatment and untreated mice (blank). In contrast, miR-135a was dramatically reduced in the livers of mice treated with pLKO-anti-miR-135a. In parallel, the level of MTSS1 in the livers of mice in the anti-mock group was very low, whereas, MTSS1 expression was markedly up-regulated when the miR-135a level was reduced by pLKO-anti-miR-135a injection (Fig. 4B). These results show that the high levels of miR-135a were successfully reduced by the administration of pLKO-anti-miR-135a. Local and distant metastasis was then investigated in vivo by hematoxylin–eosin staining (HE); intrahepatic metastasis was observed and no lung metastasis was found (data not shown). Because the CSQT-2 cell line was derived from PVTT and was prone to metastasis, in vivo experiments; analysis is shown. Data are shown as means ± SD based on three independent experiments; *p < 0.01. (C) Real-time PCR analysis of MTSS1 in CSQT-2 cells after transfection of miR-135a and miR-mock. (D) Western blot of MTSS1 after transfection of miR-135a and miR-mock. Western blots were analyzed by Quantity_one_V4 software, and the statistical analysis is shown. Data are shown as means ± SD based on three independent experiments; *p < 0.05 (This figure appears in colour on the web).

Collectively, these results demonstrate that the repression of miR-135a levels by in vivo pLKO-anti-miR-135a administration can effectively inhibit metastasis by tumors that overexpress miR-135a.
Correlation between miR-135a expression profiles and prognosis of patients with PVTT

Next, we determined whether miR-135a expression correlates with prognosis. MiR-135a expression was detected by ISH of the PT and PVTT samples from 50 patients. Compared to samples incubated without the miR-135a probe (negative control, NC), the positive staining was located in the cytoplasm of tumor cells (Fig. 5A and B, indicated by arrows). Compared to PT tissues, PVTT tissues expressed miR-135a at a higher level. Patients were then classified into a high group (n = 27) and a low group (n = 23) according to the miR-135a intensity in their PVTT by the fiftieth percentile value of density as the cut-off, as described in “Materials and methods”. The statistical analysis showed higher miR-135a expression in PVTT compared to PT in both the high and low groups (Fig. 5C). Moreover, real-time PCR was performed to further quantify miR-135a expression in tissues and confirmed the ISH results (Fig. 5D). The correlation between the miR-135a expression level and prognosis was then analyzed. As shown in Supplementary Table 5, miR-135a expression correlated with HBsAg (p = 0.039), tumor encapsulation (p = 0.007) and tumor size (p = 0.007). The median miR-135a intensity value of the initial set of PVTT samples from these 50 patients was used as the cut-off in the Kaplan–Meier survival analysis. Here, differences in both OS (p = 0.02) and DFS (p = 0.011) were statistically significant between the two groups (Fig. 5E and F). The overall 1-year and 2-year survival rates for the high expression group were 22.2% and 0% and for the low expression group were 43.47% and 8.67%, respectively. The 1-year DFS rate was 0% for the high expression group and 17.39% for the low expression group.

The univariate Cox analysis of all 50 investigated patients with regard to miR-135a intensity and clinicopathological factors...
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Supplementary data
Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2011.08.008.

References
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