Research Article

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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.

Keywords: miR-135a; Portal vein tumor thrombus; Hepatocellular carcinoma; Metastasis; CSQT-2.

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Abbreviations: HCC, hepatocellular carcinoma; PVTT, portal vein tumor thrombus; miR-135a, microRNA-135a; FOXM1, forkhead box M1; MTSS1, metastasis suppressor 1; PT, parenchyma tumor nodules tissue; UTR, untranslated region; siRNA, small interfering RNA; AS, antisense oligonucleotides; Ap, apoptosis inducer; ChIP, chromatin immunoprecipitation; TF, transcription factor.

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).

Chromatin immunoprecipitation (ChIP) assay

ChIP was performed in CSQT-2 cells using the ChIP assay kit (Agilent) as previously described [9]. The FOXM1 rabbit Ab (C-20, Santa Cruz Biotech. Inc.) was used. Primers specific to the human *miR-135a* promoter are listed in Supplementary Table 1.

Athymic nude mouse surgery, necropsy, and histopathology

An orthotopic transplantation nude mouse model was established as previously described [9]. The experiments were performed as described in the Supplementary Materials and Methods (athymic nude mouse surgery, necropsy, and histopathology).

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 (χ^2 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.

For the miR-135a density *in situ* hybridization, a minimum *p* value was sought, and the fiftieth percentile value was defined as the cut-off value for the high and low density, in this study [14].

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