miR-365 targets ADAM10 and suppresses the cell growth and metastasis of hepatocellular carcinoma

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Abstract. Expression of miR-365 has been reported to be downregulated in hepatocellular carcinoma (HCC). However, the biological function and underlying mechanism of miR-365 in HCC growth and metastasis remain unclear. The aim of the present study was to explore the role of miR-365 in HCC progression. We found that miR-365 expression was downregulated in HCC tissues and cell lines. Further results showed that low expression of miR-365 was significantly associated with tumor-node-metastasis (TNM) stage and lymph node metastasis. Functional assays revealed that overexpression of miR-365 significantly inhibited cell proliferation, colony formation, migration and invasion of HCC cells in vitro, and suppressed tumor growth in vivo. Mechanistic investigations demonstrated that ADAM10 (a disintegrin and metalloproteinase 10) is a target of miR-365 in HCC. In addition, knockdown of ADAM10 in HepG2 cells significantly inhibited cell proliferation, colony formation, migration and invasion, which mimicked the suppressive effects induced by miR-365 overexpression. Restoration of ADAM10 expression partially reversed the suppressive effects mediated by miR-365 overexpression. Taken together, these results indicate that miR-365 functions as a tumor-suppressor in HCC through targeting ADAM10, and may serve as a promising candidate for therapeutic applications in HCC treatment.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common human cancers worldwide, and is ranked as the third leading cause of cancer-related deaths worldwide (1). Although significant progresses in the diagnosis and treatment of HCC have been made in the past decade, the prognosis of HCC patients remains gloomy mainly due to its high rates of recurrence and metastasis (2,3). Therefore, understand the molecular mechanisms which regulate the growth and metastasis of HCC are urgently needed for the development of effective therapeutic strategies.

Recently, molecular targets for treating various human cancers have been widely researched, and microRNAs (miRNAs) have been suggested as promising diagnosis markers and therapeutic targets (4). miRNAs are endogenous small, non-coding regulatory RNA molecules of ~18-25 nucleotides that control the expression of a large number of genes by binding to the 3' untranslated region (3'UTR) of target mRNAs, leading to mRNA cleavage/degradation or translational repression (5,6). Accumulating evidence has demonstrated that miRNAs are involved in tumorigenic progression including cell proliferation, apoptosis, angiogenesis and invasion, and function as tumor-suppressors or oncogenes (7,8). Results from previous investigations have identified a number of oncogenic and tumor-suppressive miRNAs involved in human HCC (9-11), which can serve potentially as effective biomarkers for improving diagnostic and prognostic accuracies, or as targets for novel treatment strategies against malignant HCC.

miR-365, a newly discovery miRNA, has been reported to play crucial roles in tumor progression and development in several types of human cancers (12-20). Recently, a previous study demonstrated that miR-365 expression was downregulated in HCC tissues, and that overexpression of miR-365 suppressed HCC cell proliferation and invasion (21). However, the detailed biological function and underlying molecular mechanism of miR-365 in HCC have not been fully elucidated due to the lack of target gene information. Therefore, the aims of the present study were to investigate the biological function and underlying molecular mechanisms of miR-365 involved in the carcinogenesis of HCC.

Materials and methods

Tissue samples and cell culture. The present study was approved by the Ethics Committee of the First Hospital of Jilin University. Written informed consent was obtained from each patient who participated in the present study. Fresh frozen primary tumor samples and their adjacent non-tumor tissues (>5 cm from the cancer tissue) used in the present study were obtained from 40 patients who underwent resection for HCC.
at the Department of Hepatobiliary and Pancreatic Surgery, The First Hospital of Jilin University (Changchun, China) between July 2011 and September 2015. All tissue samples were immediately frozen in liquid nitrogen after resection, and stored at -80°C until use. The relevant clinical characteristics of the HCC patients were collected and were listed in Table I.

HCC cell lines (SMMC-7721, Hep3B, HepG2 and Huh-7) and a normal hepatic cell line, HL-7702, were obtained from the Institute of Cell Biology of the Chinese Academy of Science (Shanghai, China), and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both from HyClone, Logan, UT, USA), 2 mmol/l glutamine, 100 U/mm penicillin or 100 µg/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37°C.

**Plasmids, reagents and transfection.** miR-365 mimic (UAUAUGCCCUAAAAUCCUUAU) and the corresponding miRNA negative control (miR-NC, UUCUCG AACGGUCAGCUUUU) were designed and synthesized by GenePharma Co., Ltd. (Shanghai, China). siRNA against ADAM10 (si-ADAM10) and a corresponding scramble negative control (si-NC) were obtained from our laboratory. A disintegrin and metalloproteinase 10 (ADAM10) overexpression plasmid was purchased from GeneChem (Shanghai, China). Transfections were performed using the Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

**RNA extraction and quantitative real-time RT-PCR.** Total RNA was extracted from the cultured cells and the surgically resected HCC and adjacent non-tumor tissues using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Then, 5 ng of total RNA was reverse transcribed using a reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. The miR-365 levels were determined using miRNA-specific TaqMan MiRNA Assay kit (Thermo Fisher Scientific) on ABI 7900 Sequence Detection System with miR-365 und U6 primers (both from Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. Relative fold-change in expression was normalized to U6 according to the manufacturer's instructions.

**Cell proliferation and colony formation assays.** Cell proliferation assay was performed using the Cell Counting Kit-8 kit (CCK-8; Dojindo, Tokyo, Japan) according to the manufacturer's instructions. In brief, 3x10⁴ transfected cells were seeded into 96-well culture plates and cultured in DMEM supplemented with 10% (v/v) FBS. CCK-8 (10 µl) reagent was added to each well at the indicated time points (24, 48 and 72 h) after seeding and the cells were incubated at 37°C for 2 h. Absorbance was measured for each well at 450 nm using an enzyme immunoassay analyzer (Bio-Rad, Hercules, CA, USA).

<table>
<thead>
<tr>
<th>Variables</th>
<th>No. of cases</th>
<th>Relative miR-365 expression (miR-365/U6)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>&lt;50</td>
<td>19</td>
<td>0.386±0.121</td>
<td></td>
</tr>
<tr>
<td>≥50</td>
<td>21</td>
<td>0.378±0.114</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Male</td>
<td>23</td>
<td>0.376±0.134</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>17</td>
<td>0.390±0.142</td>
<td></td>
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<tr>
<td>TNM stage</td>
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<td></td>
<td>&lt;0.01</td>
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<tr>
<td>I-II</td>
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<td></td>
</tr>
<tr>
<td>III-IV</td>
<td>10</td>
<td>0.124±0.041</td>
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</tr>
<tr>
<td>Tumor size (cm)</td>
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<td>&gt;0.05</td>
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<tr>
<td>&lt;5</td>
<td>27</td>
<td>0.391±0.144</td>
<td></td>
</tr>
<tr>
<td>≥5</td>
<td>13</td>
<td>0.363±0.138</td>
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</tr>
<tr>
<td>Lymph node metastasis</td>
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<tr>
<td>No</td>
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<td>0.456±0.168</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>9</td>
<td>0.127±0.038</td>
<td></td>
</tr>
</tbody>
</table>

For the colony formation assay, transfected cells were resuspended and seeded into 6-well plates at a density of 500 cells/well, and grown in medium containing 10% FBS for 14 days. Colonies were fixed with methanol for 15 min and stained with 0.1% crystal violet for 10 min. Colonies with >50 cells/colony were counted using a light microscope (Olympus, Tokyo, Japan). The percentage of colony formation was calculated by setting the control to 100%.

**Cell migration and invasion assays.** Cell migration and invasion assays were carried out using Transwell invasion chambers (Corning, Tewksbury, MA, USA). For the migration assay, 2x10⁴ transfected cells were placed into the upper chambers (Corning) after resuspending cells in serum-free DMEM. For the invasion assay, 2x10⁴ transfected cells were seeded in the upper chambers which were coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The lower chamber was filled with medium with 10% FBS to attract the cells. After 24 h (migration assay) or 48 h (invasion assay) of incubation, the non-migrated or non-invaded cells on the upper surface of the membrane were removed with a cotton swab, while cells that had migrated to the bottom surface were fixed with 70% ethanol for 30 min and stained with 0.2% crystal violet for 10 min. Images were captured, and the cell numbers were counted for five randomly selected fields under a light microscope (Olympus).

**Bioinformatic prediction and dual luciferase reporter assay.** TargetScan (www.targetscan.org) was used to predict the putative target genes of miR-365. The wild-type 3'UTR segment
of human ADAM10 containing the predicted target sites of miR-365 was amplified by PCR and inserted into the downstream of the pGL3/Luciferase vector (Ambion, Austin, TX, USA) at the NheI and XhoI sites, named as Wt-ADAM10-3'UTR. The mutant type 3'-UTR of ADAM10 was generated using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA), and inserted into the downstream of the pGL3/Luciferase vector (Ambion) at the NheI and XhoI sites, and referred to as Mut-ADAM10-3'UTR. All constructs were verified by sequencing. For the luciferase reporter assay, HepG2 cells were cultured to 60-70% confluency in a 24-well plate, and co-transfected with 100 ng of the Wt-ADAM10-3'UTR or Mut-ADAM10-3'UTR plasmid, plus 50 nM of the miR-365 mimic or miR-NC using Lipofectamine 2000. After 48 h of transfection, the Dual-Luciferase reporter assay system (Promega Corporation, Madison, WI, USA) was used to determine the luciferase activity. Firefly luciferase activity was normalized against Renilla luciferase activity.

Western blotting. Cultured cells or tissues were solubilized in cold radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific) to extract protein, which was quantified using a BCA assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Equal amounts of proteins (20 µg) were separated on 10% sodium dodecylsulfate-polyacrylamide gels (SDS-PAGE; Pierce) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific). The membrane was incubated with mouse anti-human ADAM10 monoclonal (1:1,000) and mouse anti-human β-actin monoclonal antibodies (1:5,000) (both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4°C overnight. The membrane was incubated with the peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (1:5,000; Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. The protein band was visualized using a chemiluminescent detection system (ECL; Thermo Scientific, Rockford, IL, USA) and exposed to X-ray film (Thermo Fisher Scientific).

In vivo nude mouse tumorigenesis assay. This experiment was approved by the Animal Ethics Committee of Jilin University (Changchun, China). All animal experiment were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and local institutional ethical guidelines for animal experiments. HepG2 cells (2x10⁶) with stable expression of miR-365 or miR-NC were suspended in 100 µl of serum-free DMEM, and subcutaneously injected into the side of the posterior flank of each BALB/c-a-nu (nu/nu) mouse (Experimental Animal Center of Jilin University, Changchun, China). The tumor volume (V) was monitored every 7 days by measuring the length (L) and width (W) of each tumor using a digital caliper and was calculated with the following formula: \( V (\text{mm}^3) = \frac{1}{2} \times L \times W^2 \). Five weeks after the implantation, the mice were sacrificed and the xenograft tumors were excised.

Statistical analysis. All data are shown as mean ± standard deviation (SD) from at least triplicate experiments. Statistical analysis was performed using one-way ANOVA or a two-tailed Student's t-test. The relationship between miR-365 expression and HCC clinical features was analyzed using Chi-square test. The relationship between ADAM10 and miR-365 expressions was analyzed using Pearson's correlation. The SPSS version 19.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. P-value >0.05 was considered statistically significant for all tests.

Results

miR-365 is downregulated in HCC tissues and cell lines, and is associated with malignant progression. To reveal the role of miR-365 in HCC, we first examined the expression of miR-365 in HCC and adjacent non-tumor liver tissues by qRT-PCR. As shown in Fig. 1A, the miR-365 expression levels were significantly decreased in the HCC tissues compared to the levels noted in the adjacent non-tumor liver tissues. Furthermore, our data showed that lower levels of miR-365 were significantly associated with the malignant progression of HCC, including lymph nodal metastasis and tumor-node-metastasis (TNM) clinical stage (Table I). However, no association was found between miR-365 expression and age, gender or tumor size (Table I). We also found that the expression levels of miR-365 were decreased in four HCC cell lines (SMMC-7721, Hep3B, HepG2 and Huh-7) and a normal hepatic cell line (HL-7702) were determined by qRT-PCR. U6 was used as a loading control; *P<0.05, **P<0.01.
Hep3B, HepG2 and Huh-7) compared to the levels noted in the normal hepatic cell line, HL-7702 (Fig. 1B). As HepG2 cells showed the most significant decrease in miR-365 expression, we used this cell line in the following experiments (Fig. 1B).

**miR-365 inhibits HCC growth in vitro and in vivo.** To further study the role of miR-365 in HCC growth, we established stable cells to restore the expression of miR-365 in HepG2 cells, and the overexpression levels of miR-365 were confirmed by qRT-PCR (Fig. 2A). We then investigated the effect of miR-365 on HCC cell proliferation. CCK-8 assay showed that miR-365 overexpression significantly suppressed cell proliferation of HepG2 cells (Fig. 2B). To assess the long-term effect of miR-365 on cell growth, a colony formation assay was performed. We found that miR-365 overexpression markedly attenuated colony formation of the HepG2 cells (Fig. 2C). Moreover, the role of miR-365 in tumor growth was determined in vivo. The stable cells either overexpressing miR-365 or overexpressing miR-NC were subcutaneously injected into the flank of nude mice. Our data indicated that miR-365 significantly inhibited tumor growth in vivo (Fig. 2D and E). These data suggest that miR-365 suppresses HCC growth in vitro and in vivo.

**miR-365 suppresses HCC cell migration and invasion in vitro.** To investigate the biological effect of miR-365 on migration and invasion in HCC, Transwell assays were performed. Consistent with the clinical features, miR-365 overexpression markedly decreased the migration and invasion capacities of the HepG2 cells, when compared to these capacities observed in the miR-NC group (Fig. 3A and B). Accordingly, miR-365 inhibits the migration and invasion of HCC cells.

**ADAM10 is a target of miR-365 in HCC cells.** To explore the direct target of miR-365, we identified its potential targets using publicly available algorithm TargetScan. ADAM10 was selected as a direct target gene of miR-365, since it has been reported to be involved in tumor progression in various types of cancer including HCC (22). To validate whether ADAM10 is a bona fide target of miR-365 in HCC, we generated luciferase vectors containing Wt or Mut of ADAM10 3’-UTR (Fig. 4A). Luciferase reporter assay was further performed in the HepG2 cells. As indicated in Fig. 4B, the luciferase activity was significantly decreased in the HepG2 cells co-transfected with the Wt ADAM10 3’UTR and miR-365 mimic (Fig. 4B), whereas the luciferase activity was unchanged in the HepG2 cell line transfected with miR-365 mimic or miR-NC.
cells cotransfected with the Mut ADAM10 3'UTR vector and miR-365 mimic (Fig. 4B), suggesting that miR-365 can directly bind to the 3'UTR of ADAM10. Since miR-365 can bind to the 3'UTR of ADAM10, we examined whether miR-365 could decrease ADAM10 expression in HepG2 cells. As expected, overexpression of miR-365 significantly decreased ADAM10 expression at both the mRNA and protein level (Fig. 4C and D).
ADAM10 is highly upregulated in HCC tissues and is inversely correlated with miR-365 levels. To further confirm the relationship between miR-365 and ADAM10 in HCC tissues, we first examined the mRNA levels of ADAM10 in HCC tissues and adjacent non-tumor liver tissues. The result of qRT-PCR indicated that the mRNA levels of ADAM10 were significantly upregulated in HCC tissues compared to these levels found in the adjacent non-tumor liver tissues (Fig. 5A). Furthermore, we found that the mRNA levels of ADAM10 were inversely correlated with the miR-365 levels in HCC tissues by Pearson’s correlation analysis (Fig. 5B; $r=-0.533$; $P=0.0003$).

Downregulation of ADAM10 has effects similar to those of miR-365 overexpression in the HCC cells. To investigate the biological roles of ADAM10 in HCC, we performed RNA interference-based silencing of ADAM10 in the HepG2 cells, and the knockdown efficiency of ADAM10 was verified by qRT-PCR and western blotting (Fig. 6A and B). After transfection with si-ADAM10, cell proliferation, colony formation, migration and invasion were determined. As presented in Fig. 6C-F, downregulation of ADAM10 by si-ADAM10 significantly inhibited cell proliferation, colony formation, migration and invasion of HepG2 cells, which mimicked the effect of miR-365 overexpression in HepG2 cells.
Overexpression of ADAM10 rescues the effects of miR-365 overexpression in HCC. To investigate the functional relevance of ADAM10 targeting by miR-365, we assessed whether overexpression of ADAM10 could rescue the suppressive effects of miR-365 on HCC cells. To this end, miR-365-overexpressing HepG2 cells were further transfected with ADAM10 overexpression plasmid to restore its expression. After transfection, qRT-PCR and western blotting were conducted to examine ADAM10 expression. As shown in Fig. 7A and B, overexpression of the ADAM10 plasmid restored ADAM10 expression at the mRNA (Fig. 7A) and protein levels (Fig. 7B). In addition, our results also showed that overexpression of ADAM10 reversed the inhibitory effects of miR-365 on cell proliferation, colony formation, migration and invasion in the HepG2 cells (Fig. 7C-F). These data indicate that miR-365 exerts an inhibitory effect on HCC growth and metastasis partially by repressing ADAM10.

Discussion

Accumulating evidence indicates that miRNAs play important roles in tumorigenesis and tumor progression of hepatocellular carcinoma (HCC) (9-11). However, the molecular mechanisms by which miRNAs regulate the biological behavior of HCC cells remain largely unknown. In the present study, we showed that miR-365 was significantly downregulated in HCC tissues and cell lines, and that lower miR-365 levels were strongly associated with higher malignant potential of HCC, which was consistent with previous results (21). We also demonstrated that ectopic expression of miR-365 significantly suppressed cell proliferation, clone formation, migration and invasion abilities in the HCC cells. In addition, a disintegrin and metalloproteinase 10 (ADAM10) was identified as a functional target of miR-365 in HCC. Overexpression of ADAM10 reversed the inhibitory effects of miR-365 on cell proliferation, colony formation, migration and invasion in the HCC cells. These results suggest that miR-365 may play a fundamental role in the growth and metastasis of HCC. These data suggest that miR-365 may act as a novel therapeutic agent for HCC.

Recently, miR-365 has been reported to act as a tumor suppressor in several types of cancer including lung cancer (12,13), melanoma (14), osteosarcoma (15) and colon cancer (16). However, miR-365 was also found to be upregulated and function as an oncogene in gastric cancer (17), cutaneous squamous cell carcinoma (18,19) and pancreatic cancer (20). These controversial findings suggest that dysregulation of miR-365 in various cancers may be dependent on the cellular microenvironment and specific tumor type. Recently it was reported that miR-365 suppresses the proliferation and invasion of HCC cells (21). However, due to the lack of target gene information concerning miR-365, the biological function of miR-365 in HCC as well as the molecular mechanisms by which miR-365 exerts its functions have not been fully understood. In the present study, we found that miR-365 was downregulated in HCC tissues and cell lines, and reduced miR-365 levels were significantly correlated with TNM stage and lymph node metastasis, which was consistent with a previous study (21). In addition, we found that overexpression of miR-365 significantly suppressed HCC cell proliferation, colony formation, migration and invasion in vitro, and suppressed tumor growth.
in vivo. These finding together with previous research suggest that miR-365 may function as a tumor-suppressor in HCC.

ADAM10, a member of the ADAM family, has been reported to play important roles in cancer initiation and development (23,24). Our previous study showed that ADAM10 expression was significantly upregulated in HCC tissues, and was significantly associated with tumor grade, tumor size and lymph node metastasis (25). Recently, several studies have shown that downregulation of ADAM10 inhibited HCC cell migration and invasion (22,26), and increased sorafenib sensitivity in HCC cells (27). These results suggest that ADAM10 functions as an oncogene in HCC. In addition, ADAM10 has been reported to be regulated in HCC cells by several miRNAs including miR-449a (28), miR-655-3p (29) and miR-122 (30).

In the present study, we identified ADAM10 as a direct target of miR-365 in HCC cells by bioinformatic prediction, luciferase reporter assay, qRT-PCR and western blot analysis. We found that ADAM10 expression was upregulated in HCC tissues, and its mRNA expression level was negatively correlated with the expression level of miR-365 in the HCC tissues. Additionally, further study indicated that knockdown of ADAM10 could phenocopy the effects of miR-365 overexpression in HCC cells, and the overexpression of ADAM10 partially restored the inhibitory effect of miR-365 in the HCC cells. These results suggest that miR-365 inhibits cell growth and invasion in HCC, at least in part, by targeting ADAM10.

In conclusion, the present study showed that miR-365 was significantly decreased in HCC cell lines and tissues, and its expression was negatively correlated with TNM stage and lymph node metastasis, and that miR-365 significantly suppressed HCC cell proliferation, colony formation, and migration and invasion by directly binding to ADAM10 and downregulating its expression. Taken together, our findings suggest that miR-365 may function as a tumor suppressor by targeting ADAM10. Further research is needed to ascertain whether miR-365 is a potential target for the development of an antitumor treatment strategy for HCC.

References