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miR-181a-5p is downregulated in hepatocellular carcinoma and suppresses motility, invasion and branching-morphogenesis by directly targeting c-Met

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ABSTRACT

c-Met receptor tyrosine kinase has been regarded as a promising therapeutic target for hepatocellular carcinoma (HCC). Recently, microRNAs (miRNAs) have been shown as a novel mechanism to control c-Met expression in cancer. In this study, we investigate the potential contribution of miR-181a-5p dysregulation to the biology of c-Met overexpression in HCC. Herein, we found an inverse expression pattern between miR-181a-5p and c-Met expression in normal, cirrhotic and HCC liver tissues. Luciferase assay confirmed that miR-181a-5p binding to the 3′-UTR of c-Met downregulated the expression of c-Met in HCC cells. Overexpression of miR-181a-5p suppressed both HGF-independent and -dependent activation of c-Met and consequently diminished branching-morphogenesis and invasion. Combined treatment with miR-181a-5p and c-Met inhibitor led to a further inhibition of c-Met-driven cellular activities. Knockdown of miR-181a-5p promoted HGF-independent/-dependent signaling of c-Met and accelerated migration, invasion and branching-morphogenesis. In conclusion, our results demonstrated for the first time that c-Met is a functional target gene of miR-181a-5p and the loss of miR-181a-5p expression led to the activation of c-Met-mediated oncogenic signaling in hepatocarcinogenesis. These findings display a novel molecular mechanism of c-Met regulation in HCC and strategies to increase miR-181a5p level might be an alternative approach for the enhancement of the inhibitory effects of c-Met inhibitors.

1. Introduction

HCC is one of the most common and aggressive cancers worldwide [1,2]. Although many advances have been made in the diagnosis and management of HCC, the prognosis of patients with HCC remains poor due to metastasis, recurrence and development of resistance to conventional chemotherapy and radiotherapy [1–3]. Intense efforts have been made over the past decade to understand molecular mechanisms of HCC, and HGF/c-Met signaling pathway has become one of the most promising targets for the molecular therapy of HCC [4–6].

Classically, upon binding to HGF, c-Met becomes activated and drives complex biological activities including proliferation, survival, migration, invasion, and morphogenesis [4–8]. Abnormalities in HGF/c-Met signaling were reported to be linked to an unfavorable clinicopathological status, including high proliferation index, low degree differentiation, vascular invasion and metastasis in several cancer types [4–6]. We and others have identified ligand independent kinase activity for c-Met in HCC, occurring by several mechanisms including gene amplification, activating point mutations, receptor crosstalk, receptor overexpression, and non-coding RNAs [7,8]. Recent studies have shown that miRNAs regulating c-Met expression are down-regulated in HCC and directly contributes to cell migration and invasion of HCC cells [7,9–11].

miRNAs are evolutionary endogenous regulatory small non-coding RNAs that play critical roles in post-transcriptional gene expression through base pairing with the 3′untranslated region (3′UTR) of target mRNAs [12,13]. miRNAs regulate gene expression by directly degrading messenger RNA (mRNA) or repression translation [12,13]. It is now known that one miRNA can simultaneously control the expression of hundreds of different genes and over 60% of human protein-coding genes are predicted to contain miRNA-binding sites within 3′UTRs [12]. These properties of miRNAs make them a powerful regulator in human physiology and pathology, including cancer [14,15]. Therefore, miRNAs represent a novel and attractive therapeutic strategy for human diseases.

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Several studies have explored that dysregulated miRNAs possess important roles in HCC progression and directly contribute to proliferation, apoptosis and metastasis of HCC [15–20]. Recently, we have identified HCC Epithelial-to-Mesenchymal-Transition (EMT)-associated miRNAs. Among these miRNAs, miR-181a-5p has been computationally predicted to target c-Met. In this study, we investigated the potential role of miR181a5p in the regulation of c-Met expression and c-Met induced motility, invasion and branching-morphogenesis in HCC.

2. Material and methods

2.1. Cell culture

Human HCC cell lines Mahlavu (MV), and SNU-449, were cultivated as described [8]. Hepatocyte growth factor/scatter factor (HGF) was from R&D Systems (MN, US). HGF (40 ng/mL) was used at specific time points after overnight starvation in DMEM with 2% FBS. For the inhibition of c-Met, SU11274 (Calbiochem 448101, US) was added to the culture medium in the indicated doses and times. DMSO (Applichem, US) was used as solvent for SU11274.

2.2. The prediction of candidate miRNAs targeting c-Met

To investigate the target genes and the conserved sites bound by the seed region of miR-181a-5p, the miRDB, and microRNA.org-Targets and Expression, algorithms were used.

2.3. miRNA transfection

MV and SNU-449 cells were transiently transfected with miR-181a-5p-mimics (Ambion, MC10421, US, 100 nM,) or miR-181a-5p-inhibitor (Ambion, MH10421, 100 nM) or respective negative-controls pre-miR miRNA precursor (Ambion, AM:17010, 100 nM, control), or anti-miR miRNA (Ambion, AM:17010, 100 nM, inhibitor control) using "Fugene HD-Promega (US)" following the manufacturer's protocol. After transfection for 24 h, the cells were harvested for further experiments.

2.4. Plasmid constructs and luciferase activity assay

Luciferase vector pEX-MT01 with wild type c-Met miTarget microRNA 3′UTR target clones (product ID HmiT011181-MT01) and mutant with c-Met 3′-UTR with deletions of 7 bp from the site of perfect complementarity of miR-181a-5p (product ID CS-HmiT011181-MT01-01) were purchased from GeneCopoeia (US). Cells were co-transfected with luciferase constructs (100 ng/ml) and miR-181a-5p-mimics or mimic-control or miR-181a-5p-inhibitor or inhibitor-control using Fugene. After 48 h, renilla luciferase activity was measured using a dual-luciferase reporter system (Promega, WI, USA) (For detail see Supplementary Material). The renilla luciferase activities were normalized with the internal firefly luciferase activity.

2.5. Real-time RT-PCR

The total RNA including miRNA was extracted from cell lines using a mirVANA miRNA isolation kit (ambion/RNA by life technologies, USA) according to the manufacturer's instructions. RNA concentration was detected by Nanodrop (Thermo, US). The expression of mature miR-181a-5p was quantified using TaqMan microRNA Assay Kit (Applied Biosystems, US) with specific primers for miR-181a-5p (Applied Biosystems, 000480). miR-181a-5p expression was normalized to RNU6B (Applied Biosystems, 001093) using the $2^{-\Delta Ct}$ method.

2.6. Western blotting

Total protein was prepared by using modified RIPA buffer as described previously [8]. Antibodies against phospho-Met (Y-1234/1235) (cell signaling (cs)-3129, US), phospho-p44/42 ERK1/2 (p-MAPK) (Thr202/Tyr204) (cs-9101), p44/42 ERK1 (MAPK) (C-16) (santa cruz (sc) 93, USA), Calnexin (sc-11397) as described [10]. Equal loading and transfer were confirmed by repeat probing for calnexin (house-keeping gene). Band intensities were quantified as pixels by using ImageJ software (NIH).

2.7. Motility and invasion assay

In vitro motility and invasion assays were performed as described previously [8]. Briefly, cells were transiently transfected with miRNA-mimic or mimic-control or inhibitor or inhibitor-control in 2%DMEM. For c-Met inhibition, cells were pretreated with SU11274 overnight and were placed into upper chambers. DMEM with 2% FBS with/without HGF and/or SU11274 was added to the lower chambers. After 24 h incubation at 37 °C, the medium was removed; cells were fixed and stained with Diff Quick (Siemens Healthcare Diagnostics, UK). Cells that had traversed through the membrane were counted using a bright-field inverted microscope. Total cell numbers were counted for each chamber. Experiments were performed in at least triplicates and repeated at least three times.

2.8. Branching-morphogenesis assays

miRNA, miRNA control treated cells were embedded in three-dimensional collagen I gels (BD 354236, US) that contain SU11274 and/or HGF as previously described [21]. For the quantitation of the morphogenic response, the entire area per experimental condition in each of the 4 independent cultures were photographed using 20X phase contrast objective using Olympus (US) CKX41. All colonies were analyzed and scored on the ability to form branching tubules. Data were expressed as mean ± standard error (SE) for at least four independent experiments.

2.9. Immunofluorescence staining and miRNA LNA in situ hybridization of formalin-fixed, paraffin-embedded tissue section

Human liver tissue microarray (TMA) samples containing 12 normal and 19 cirrhotic liver tissues, and 48 HCC tissues were purchased from US Biomax (catalog no: BC03117 and T031a) to detect miR-181a-5p and c-Met expression levels in HCC, cirrhotic and normal liver tissues by in situ hybridization (ISH) and immunofluorescence (IF), respectively. IF with c-Met was performed on the TMA section after deparaffinization followed by antigen retrieval using proteinase K. After the secondary fluorochrome-conjugated antibodies (Alexa Fluor 488, invitrogen A-21206) treatment, slides were mounted with fluorescence mounting medium (Dako, S3023, Denmark). miR-181a-5p detection was performed on the TMA cores by ISH as described in the Supplementary Material section. DAPI (Invitrogen, US) was used to stain the cell nuclei. The entire TMA section was analyzed by acquisition of c-Met, miR-181a-5p, U6 and DAPI signals by Immunofluorescence Microscopy using Olympus BX50 fluorescence microscope. A score for both miR-181a-5p and c-Met protein expression was given according to the percentage of positively stained cores. Images were generated using Adobe Photoshop (US) and ImageJ software (NIH, US).