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MicroRNA-449a suppresses hepatocellular carcinoma cell growth via G1 phase arrest and the HGF/MET c-Met pathway

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ABSTRACT

Background: Accumulating evidence demonstrates that microRNAs (miRNAs) play essential roles in tumorigenesis and cancer progression of hepatocellular carcinoma (HCC). Average targets of a miRNA were more than 100. And one miRNA may act in tumor via regulating several targets. The present study aimed to explore more potential targets of miR-449a by proteomics technology and further uncover the role of miR-449a in HCC tumorigenesis.

Methods: Technologies such as iTRAQ-based quantitative proteomic were used to investigate the effect of miR-449a on HCC. The expression of c-Met and miR-449a was detected by qRT-PCR in HCC samples. Gain- and loss-of-function experiments were performed to identify the function and potential target of miR-449a in HCC cells.

Results: In HCC, miR-449a was significantly downregulated, while c-Met was upregulated concurrently. Quantitative proteomics and luciferase reporter assay identified c-Met as a direct target of miR-449a. Moreover, miR-449a inhibited HCC growth not only by targeting CDK6 but also by suppressing c-Met/Ras/Raf/ERK signaling pathway. Furthermore, the inhibition of c-Met expression with a specific siRNA significantly inhibited cells growth and deregulated the ERK pathway in HCC.

Conclusion: The tumor suppressor miR-449a suppresses HCC tumorigenesis by repressing the c-Met/ERK pathway.

Introduction

Hepatocellular carcinoma (HCC) accounts for approximately 695,900 cancer deaths worldwide, and half of them are in China [1]. There have been advances in understanding the genomic landscape of most common forms of human cancer [2], providing an increasingly comprehensive knowledge of HCC [3,4]. However, the molecular mechanism of HCC remains poorly understood. Genome instability and chromosomal aberrations trigger deregulation of gene expression in human tumors, which promotes or "drives" tumorigenesis [5]. To date, understanding the role of individual molecule in HCC is not sufficient to clarify the tumorigenic mechanism or to develop a more effective treatment [6]. Exploring the intrinsic links and interactions between the altered molecules in HCC helps to understand the HCC tumorigenesis.

MicroRNAs (miRNAs) regulate multiple biological processes through inhibiting mRNA translation and promoting mRNA degradation [7]. Accumulated evidences had proved that miRNAs were implicated in variety of human disease, particular in cancers [8]. In a previous study, miR-449a was reported as a tumor suppressor that targeted CDK6 and CDC25A, inhibiting pRb-E2F1 activity and inducing G1 phase cell cycle arrest [9]. E2F3, a regulator of G1/S transition, had also been defined as the direct target of miR-449a [10]. Epigenetic changes may promote cell proliferation in many cancer types, and miR-449a might reverse the changes in epigenetic traits by repressing HDAC-1 expression [11]. Moreover, miR-449a was found suppressing the epithelial-mesenchymal transition of HCC by targeting FOS and MET [12]. Although the previous

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classic approach of molecular cloning has partially revealed the function of miR-449a, individual target mining of the overall profile of miR-449a has not been conducted in HCC. Quantitative proteomics can evaluate the dynamic changes in cells [13], promote the discovery of specific targets of miR-449a, and be used to reveal the exact molecular mechanisms of miR-449a downregulation in HCC.

Here, we searched miR-449a targets by performing an isobaric tag for relative and absolute quantitation technology (iTRAQ) in combination with two-dimensional nano-scale liquid chromatography and tandem mass spectrometry (2D nano-HPLC-MS/MS).

Materials and methods

Tissue specimens, cell lines and experimental animals

Human primary HCC tissues (with adjacent non-tumorous tissues, pathologically diagnosed as HCC), were collected at the Shenzhen Second People's Hospital with written informed consent. Cells were cultured in a humidified atmosphere (5% CO_2, 37 °C), by using Dulbecco's modified Eagle's medium (DMEM), with 10% fetal bovine serum. BALB/c nude mice (female, 4 weeks old) were purchased from the Shanghai Experimental Animal Center of the Chinese Academic of Sciences (Shanghai, China). The study was approved by the Ethical Review Committee and the Experimental Animals Management Committee of Shenzhen University. Animal experiments were performed according to the National Institute Guide for the Care and Use of Laboratory Animal (NIH publication 86-23 revised 1985).

Materials

The hsa-miR-449a mimic (sense: 5′-UGG CAG UGU AUU GUU AGC UGG U-3′; antisense: 5′-CAG CUA ACA AUA CAC UGC AAU U-3′), mimic-NC (negative control, sense: 5′-UUC UCC GAA CGU GUC ACG UTT-3′; antisense: 5′-ACG UGA CAC GUU CGG AGA ATT-3′), miR-449a inhibitors (5′-ACC AGC UAA CAA UAC ACU GCC A-3′, 2′-O-methyl modification), and inhibitor-NC (5′-CAG UAC UUU UGU GUA GUA CAA-3′) were synthesized by Genepharma Inc. (Shanghai, China). iTRAQ Reagents – 4-plex Application Kits were purchased from AB Sciex Pte. Ltd (Foster City, USA).

Plasmid constructions

The hsa-miR-449a, containing 100 bp of the sequence flanking the miRNA stem loop (both upstream and downstream), was cloned into the BamH1 and Nhe1 sites of the pEP-miR Cloning and Expression Vector (Cell Biolabs, USA). Reporter constructs containing the wild-type or mutant 3′-UTR sequence of c-Met were cloned into the psiCHECK™-2 Vector (Promega, Madison, WI, USA). A MET lentiviral shRNA was purchased from GeneChem Inc. (Shanghai, China) and the sense sequence was as follows: 5′-ACG ATG AAT ACA TTG AAA T-3′. Correct vector construction was verified by direct sequencing.

miR-449a transfections and RNA interference experiments

Cells were transfected by using Lipofectamine 2000 (Invitrogen, USA), at a final concentration of 100 nmol/L. The miR-449a-carrying plasmid, the MET shRNA lentiviral and control constructs were transfected into Bel-7402 cells. All of the experiments were performed according to the manufacturer's protocol. Cells were isolated after exposure to 3 μg/mL puromycin (Sigma-Aldrich) for 4 weeks to obtain stable transfected cell clones.

iTRAQ-based quantitative proteomic analysis of targets of miR-449a

Sample preparation and protein digestion

Cells were harvested after exposure to exogenous mature miR-449a or an inhibitor for 72 h. Cells were resuspended in SDT buffer (4% SDS, 1 mmol/L DTT, and 150 mmol/L TrisHCl pH 8.0) and homogenized separately by sonication; supernatants were collected by centrifugation. Bradford assay was used to determine the protein concentration. An aliquot of up to 200 μg of protein from each extraction was added into 200 μL of UA buffer (8 mol/L Urea, 150 mmol/L TrisHCl, pH 8.0), transferred into 10 kD ultrafiltration centrifuge tubes, and followed by centrifugation at 4 °C and 14,000 g for 15 min. The samples were alkylated in 50 mmol/L IAA (iodoacetamide) in the dark (20 min, room temperature). After centrifugation, the samples were digested by 2 μg (0.05 μg/μL in 40 μL dissolution buffer) of modified sequencing grade trypsin (Promega, USA) by incubation at 37°C for 18 h.

Peptide labeling with iTRAQ reagents

For the 4-plex iTRAQ experiments, two biological replicates were prepared. The digested peptides from quiescent and activated samples by miR-449a were labeled using chemicals from the iTRAQ reagent kit (Applied Biosystems; Foster City, CA) (Tag 115, 117 for quiescent samples by miR-449a inhibitors, and Tag 114, 116 for activated samples by miR-449a mimics). Each sample was stopped with 0.1% TFA after iTRAQ labeling, and then were mixed.

Sample fractionation using strong cation exchange chromatography

For strong cation exchange chromatography, peptides were fractionated on a Polysulfoethyl Column (Polysulfoethyl 4.6 mm × 100 mm × 5 μm, 200 Å, PolyLC Inc., Maryland, USA). After lyophilized, dissolved and loaded in buffer A (10 mmol/L KH_2PO_4 pH 3.0, and 25% ACN), peptides were then eluted by buffer B (10 mmol/L KH_2PO_4 pH 3.0, 500 mmol/L KCl, and 25% ACN), at a flow rate of 1000 μL/min. A total of 30 fractions were collected into 1.5 mL tubes at suitable intervals and were pooled. Ten pooled samples were dried by vacuum centrifugation before subsequent nano-LC fractionation.

Nano-HPLC-MS/MS

We resuspended sample fractions in loading buffer and separated them in an Easy nLC system (Proxeon Biosystems, now Thermo Fisher Scientific) equipped with a Q-Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Then, samples were injected into an autosampler and loaded onto a trap column (Thermo Scientific EASY- C18 peptide traps 2 cm × 100 μm × 5 μm). Peptides enrichment and desalting lasted for 5 min. We eluted peptides over an analytical column [Thermo Scientific EASY C18 column (75 μm × 100 mm × 3 μm)] at 400 nL/min. Buffer A contained 0.1% formic acid in 99.9% H_2O. Buffer B contained 84.0% ACN and 0.1% formic acid in H_2O. The gradient consisted of 0–35% buffer B over 100 min, which was followed by washing with 35%−100% buffer B for 8 min and re-equilibration with 100% buffer B for 12 min. An LTQ-Orbitrap MS was operated in positive ionization mode. The mass range was to 1800 *m/z* (resolution: 70,000, the automatic gain control: 300,000 ions). The values were excluded for 40 s. MS spectra were acquired from peptides with + 2 to + 5 charge states and were then subjected to a second round of MS. In the octopole collision cell, the ten most intense ions were shattered by HCD with maximum accumulation times of 60 ms. The fragment ion spectra was obtained in the Orbitrap analyzer (resolution: 17,500, at *m/z* 200). Fragmentation was performed under 27 eV (underfill ratio: 0.1%).

Data analysis

Raw data were transferred into mgf and processed in the Mascot 2.2.2 search engine (Matrix Science, UK). Data generated from

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