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miR-23c suppresses tumor growth of human hepatocellular carcinoma by attenuating ERBB2IP

Lei Zhang[a], Yufeng Wang[b], Liang Wang[b], Guozhi Yin[b], Weimin Li[c], Yao Xian[c], Wei Yang[b,*], Qingguang Liu[b,*]

[a] Department of Geriatric Surgery, The First Affiliated Hospital of Xi'an Jiaotong University, 277 Yanta West Road, Xi'an, Shaanxi Province 710061, China
[b] Department of Hepatobiliary Surgery, The First Affiliated Hospital of Xi'an Jiaotong University, 277 Yanta West Road, Xi'an, Shaanxi Province 710061, China
[c] Department of Nutrition, The First Affiliated Hospital of Xi'an Jiaotong University, 277 Yanta West Road, Xi'an, Shaanxi Province 710061, China

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ABSTRACT

MicroRNAs (miRNAs) regulate a variety of development and physiologic processes, and play prominent roles in the initiation and progression of human cancers including hepatocellular carcinoma (HCC). MiR-23c is recently emerging as a cancer-associated miRNA, while its expression status and functional role in HCC are unrevealed yet. Here, we found that miR-23c underexpression was associated with the tumorigenesis of HCC based on TCGA data. qRT-PCR analysis revealed that miR-23c expression was reduced in HCC tissues and cell lines. Clinical analysis indicated that low miR-23c expression was correlated with large tumor size, high tumor grade, advanced tumor stage and poor survival of HCC patients. Our *in vitro* experiments found that overexpression of miR-23c inhibited cell proliferation and induced apoptosis of HCC cells. While miR-23c knockdown led to HCC cell growth arrest and apoptosis. Additionally, miR-23c overexpression repressed tumor growth of HCC *in vivo*. Mechanistically, erbb2 interacting protein (ERBB2IP) was identified as a direct target of miR-23c in HCC cells. miR-23c suppressed ERBB2IP expression in HCC cells and inversely correlated with ERBB2IP mRNA expression in HCC tissues. Notably, ERBB2IP silencing restrained HCC cell proliferation and induced apoptosis. ERBB2IP restoration reversed the inhibitory effects of miR-23c on HCC cell growth. In conclusion, our observations suggested that miR-23c inhibited cell proliferation and accelerated apoptosis by attenuating ERBB2IP. Targeting miR-23c might open a new avenue for HCC treatment.

1. Introduction

Hepatocellular carcinoma (HCC) treatment is a knot that still cannot be solved by the surgeons and physicians worldwide. Surgical resection is the only and best option for early stage HCC [1]. However, most of HCC patients are often diagnosed at advanced stage accompanied with intrahepatic and distant metastases [1]. Even though patients with advanced HCC were treated with surgical resection, transarterial chemoembolization (TACE) or sorafinib, the survival is still dismal due to postoperative recurrence and metastasis [2–4]. Thus, it is imperative to explore the molecular mechanism underlying the initiation and progression of HCC.

MicroRNAs (miRNAs) are an abundant class of small non-coding RNA molecules (20–24 nt)), which are expressed in various tissues and cell types, and they are involved in post-transcriptional modulation of gene expression by targeting mRNA for degradation and translational inhibition [5]. Increasing evidence support that miRNAs play critical roles in distinct and diverse biological procedures, including cell proliferation, differentiation, apoptosis, cell cycle progression and stem cell division [6]. Importantly, miRNAs have been shown to be aberrantly expressed in many human diseases including cancer [7]. The specific miRNAs that function as oncogenes or tumor suppressers, contribute to the initiation and progression of human cancer [8]. Our research team have reported several hepatocarcinogenesis-associated miRNAs,

Abbreviations: HCC, hepatocellular carcinoma; TACE, transarterial chemoembolization; miRNA, microRNAs; LIHC, liver hepatocellular carcinoma; ELAVL1, ELAV like RNA binding protein 1; GSK3β, glycogen synthase kinase 3 beta; ECM, extracellular matrix; CRC, colorectal cancer; ERBB2IP, Erbb2 interacting protein; TGF-β, transforming growth factor beta; ERα, estrogen receptor-α; QSER1, glutamine and serine rich 1; FNIP1, folliculin interacting protein 1; BTLA, B and T lymphocyte associated; TNFIP3, tumor necrosis factor alpha-induced protein 3; BRCA, breast invasive carcinoma; CHOL, cholangiocarcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; PRAD, prostate adenocarcinoma; STAD, stomach adenocarcinoma

* Corresponding authors.
E-mail addresses: dr_bobyang@163.com (W. Yang), liuqingguang@vip.sina.com (Q. Liu).

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including miR-1468 [9], miR-876-5p [10], miR-542-3p [11], miR-367 [12], miR-1296 [13], miR-519a [14] and miR-187-3p [15]. Analysis of TCGA data from ONCOMIR (http://www.oncomir.org) reveals that there are 398 miRNAs associated with tumorigenesis of liver hepatocellular carcinoma (LIHC). Among these miRNAs, miR-23c is poorly characterized in HCC. miR-23c is found to repress renal tubular epithelial pyroptosis by targeting ELAV like RNA binding protein 1 (ELAVL1) in diabetic nephropathy [16]. Moreover, miR-23c directly targets glycogen synthase kinase 3 beta (GSK3β) and affects nucleus pulposus cell proliferation and extracellular matrix (ECM) synthesis *via* Wnt signaling in intervertebral disc degeneration [17]. The first evidence for the involvement of miR-23c in human cancer originates from the higher expression of miR-23c in colorectal cancer (CRC) tissues with recurrence [18]. Furthermore, steviol suppresses gastrointestinal cancer cell proliferation partially by regulating miR-23c [19]. However, the expression and functional role of miR-23c are rarely known in HCC.

Erbb2 interacting protein (ERBB2IP), also known as Erbin, is a leucine-rich repeat and PDZ domain protein [20]. Increasing studies indicate that ERBB2IP regulates cancer cell apoptosis, proliferation and inflammatory response [21–24]. The function of ERBB2IP depends on cell types and contexts. ERBB2IP acts as a tumor suppressor *via* inhibiting oncogenic pathways including ERK [25] and transforming growth factor beta (TGF-β) [26]. In contrast, ERBB2IP contributes to the tumorigenesis of breast cancer [27], CRC [23] and HCC [28]. ERBB2IP expression is elevated and functions as an oncogene by destabilizing estrogen receptor-α (ERα) in HCC [28]. However, the mechanism underlying aberrant overexpression of ERBB2IP in HCC is still unclear.

At present, we confirmed the prognostic significance of miR-23c in HCC. Then we investigated the role of miR-23c in HCC progression through functional experiments *in vitro* and xenograft tumor model *in vivo*. miR-23c regulation of ERBB2IP was demonstrated by bioinformatics and luciferase reporter assays.

2. Materials and methods

2.1. Clinical samples and cell lines

All 80 pairs of HCC and tumor-adjacent tissues were obtained from the First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China). All patients signed written informed consent before enrolling in this study. The collected specimens were pathologically confirmed and initially snap frozen in liquid nitrogen, then stored at −80 °C until use. All patients did not receive immunotherapy, radiotherapy or chemotherapy prior to operation. This study was reviewed and approved by the Ethic Committee of 1st Affiliated Hospital of Xi'an Jiaotong University in accordance with the guidelines outlined in the Declaration of Helsinki. The clinicopathological features of HCC patients were summarized in Table 1.

Human immortalized normal hepatocyte cell line (LO2) and HCC cell lines (Huh7, SMMC-7721, Bel-7402, MHCC97H) were previously maintained in our lab. HCC cells were grown in Dulbecco's modified Eagle medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Sigma-Aldrich, Inc., St-Louis, MO, USA) in the incubator at 37 °C at 5% CO_2.

2.2. Cell transfection

Lentiviral vector-mediated miR-23c (miR-23c, HLMIR1272) and negative control (NCLMIR001) were purchased from Sigma. miR-23c inhibitors (anti-miR-23c, HmiR-AN1916-AM03) and negative control (CmiR-AN0001-AM03) were obtained from GeneCopoeia, Inc (Guangzhou, China). Lentivirus infection of HCC cells was performed in the presence of Polybrene (8 ng/ml). ERBB2IP expression plasmid (pcDNA3.1-ERBB2IP), small interfering RNA (siRNA) targeting ERBB2IP and non-targeting (NT) siRNA were purchased from GenePharma (Shanghai, China). These oligonucleotides and vectors were transfected into HCC cells using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

Table 1
Correlation between the clinicopathologic characteristics and miR-23c expression in hepatocellular carcinoma.

Characteristics		n = 80	miR-23c		*P*
			Low expression (n = 40)	High expression (n = 40)	
Age (y)	< 60	45	20	25	0.260
	≥60	35	20	15	
Sex	Male	63	29	34	0.172
	Female	17	11	6	
HBsAg	Negative	13	5	8	0.363
	Positive	67	35	32	
Serum AFP level (ng/mL)	< 20	36	14	22	0.072
	≥20	44	26	18	
Tumor size (cm)	< 5	47	18	29	0.012*
	≥5	33	22	11	
Liver cirrhosis	Absent	22	9	13	0.317
	Present	58	31	27	
Microvascular invasion	Absent	35	14	21	0.115
	Present	45	26	19	
Edmondson-Steiner grade	I + II	46	18	28	0.024*
	III + IV	34	22	12	
TNM tumor stage	I + II	64	28	36	0.025*
	III + IV	16	12	4	

HBV, hepatitis B virus; AFP, alpha-fetoprotein; TNM, tumor-node-metastasis.
* Statistically significant.

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs were extracted from tissues and cultured cells with Trizol Reagent (Thermo Fisher Scientific). Total RNA was reverse transcribed into cDNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo-Fisher Scientific). qRT-PCR analyses were performed using SYBR® Premix Ex Taq™ II (Takara, Dalian, China) and an All-in-One miRNA qPCR Detection kit (GeneCopoeia, Rockville, MD, USA) on an ABI PRISM 7300 Sequence Detection system (Applied Biosystems, Foster City, CA, USA). The primer sequences were listed as follows: miR-23c forward, 5′-CCA GAA GGA CGT AGA AG-3′and reverse, 5′-CTT CAC TGT GAT GGG CTC-3′; U6 forward, 5′-GCT TCG GCA GCA CAT ATA CTA AAA T-3′ and reverse, 5′-CGC TTC ACG AAT TTG CGT GTC AT-3′; ERBB2IP forward, 5′- CTC TGT GGG GAC TTC AAC G-3′ and reverse, 5′-TGG GTG TCA GCT TGG TGT T-3′; GAPDH forward, 5′-CCA TGT TCG TCA TGG GTG TG-3′ and reverse, 5′-GGT GCT AAG CAG TTG GTG GTG-3′. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative gene expression normalized by GAPDH and U6.

2.4. Western blot

Total proteins from cultured cells were lysed using RIPA buffer (Beyotime, Shanghai, China) and were quantified with a BCA protein assay kit (BIO-RAD, Hercules, CA, USA). Proteins were fully electrophoresed on 10% SDS polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with primary antibodies at 4 °C overnight. The rabbit-anti-human ERBB2IP primary antibody was purchased from Bethyl Laboratories (A303-763A, Montgomery, TX, USA). The mouse-anti-human GAPDH primary antibody was obtained from Santa Cruz Biotechnology (sc-47724, Santa Cruz, Dallas, TX, USA). Then, the