The tumor suppressive miR-302c-3p inhibits migration and invasion of hepatocellular carcinoma cells by targeting TRAF4

Liu Yang1,#, Yang Guo2,#, Xin Liu3,#, Tongtong Wang4, Xiangmin Tong1, Kefeng Lei5, Jiahui Wang6, Dongsheng Huang1,6, Qiuran Xu1,6

1. Key Laboratory of Tumor Molecular Diagnosis and Individualized Medicine of Zhejiang Province, Zhejiang Provincial People’s Hospital (People’s Hospital of Hangzhou Medical College), Hangzhou, Zhejiang 310014, China
2. Bengbu Medical College, Bengbu, Anhui 233030, China
3. Department of Neurosurgery, Zhejiang Provincial People’s Hospital (People’s Hospital of Hangzhou Medical College), Hangzhou, Zhejiang 310014, China
4. Zhejiang Chinese Medical University, Hangzhou, Zhejiang 310014, China
5. Department of Gynecology, Zhejiang Provincial People’s Hospital (People’s Hospital of Hangzhou Medical College), Hangzhou, Zhejiang 310014, China
6. School of Basic Medical Sciences, Shandong University, Jinan, Shandong 250000, China

#Contributed equally

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Abstract

MicroRNAs (miRNAs) have been recognized as key regulators of tumorigenesis and progression. Serum miR-302c-3p expression is prominently deregulated in HCV-related hepatocellular carcinoma (HCC). However, the expression of miR-302c-3p and its functional role in HBV-related HCC are rarely investigated. In this study, we found that the expression levels of miR-302c-3p were prominently down-regulated in HCC tissues compared to matched tumor-adjacent tissues. Moreover, miR-302c-3p under-expression was detected in HCC cell lines compared to a normal hepatic cell line LO2. Low miR-302c-3p expression was positively correlated with multiple tumor nodes, venous infiltration and advanced TNM tumor stage of HCC patients. Notably, our follow up data and TCGA data demonstrated that low miR-302c-3p expression predicted a poor survival of HCC patients. Functionally, miR-302c-3p overexpression inhibited migration and invasion of MHCC97H cells in vitro. Additionally, miR-302c-3p knockdown showed an opposite effect on these metastatic behaviors of HepG2 cells. MiR-302c-3p negatively regulated tumor necrosis factor receptor associated factor 4 (TRAF4) abundance by directly targeting 3'-UTR of TRAF4 mRNA. The expression of TRAF4 was up-regulated in HCC tissues. The level of TRAF4 mRNA was inversely correlated with miR-302c-3p expression in HCC specimens. Mechanistically, miR-302c-3p restrained AKT-mediated epithelial-mesenchymal transition (EMT) in HCC cells. Importantly, TRAF4 restoration reversed the inhibitory effect of miR-302c-3p on AKT-induced EMT and HCC cell metastasis. MK2206, an AKT inhibitor, inhibited miR-302c-3p knockdown-induced EMT in HepG2 cells. In summary, these results indicate that miR-302c-3p exhibits a tumor suppressive role in HCC by targeting TRAF4. Inhibition of miR-302c-3p/TRAF4 axis may serve as a therapeutic target for HCC.

Key words: miR-302c-3p; TRAF4; hepatocellular carcinoma; EMT, metastasis

Introduction

Hepatocellular carcinoma (HCC), the fifth most common cancer, exhibits extremely high morbidity and mortality rates worldwide [1]. In 2012, more than approximately 700,000 died of HCC, which accounts
for 9.1% in all cancers [2]. Tumor recurrence and metastasis are the main causes for the poor survival rate of patients with advanced stage HCC [3]. Therefore, identification of novel biomarkers for early stage detection and novel potential therapeutic targets for HCC is desirable and urgently needed.

MicroRNAs (miRNAs), as a family of small non-coding RNAs (ncRNAs), are approximately 22 nucleotides in length, which act as post-transcriptional regulators by binding to the 3′-untranslated regions (UTRs) of their target mRNAs [4]. Recent studies have shown the association between miRNAs and HCC progression. For example, miR-187-3p, miR-542-3p, miR-1296 etc. function as tumor suppressors by targeting different genes [5-7], while miR-324-3p, miR-1468 and miR-519a act as oncogenes in HCC [8-10] and miR-367 promotes cell proliferation and metastasis during the progression of human HCC [11]. MiR-302c-3p has been recognized as a cancer-related miRNA in previous studies. MiR-302c-3p is identified a potent estrogen receptor-alpha (ERα) regulating miRNA and inhibits estrogen-induced cell growth of breast cancer [12, 13]. Down-regulation of miR-302c-3p leads to the resistance of tumor cells to natural killer (NK) cells via up-regulation of UL-16 binding protein 2 (ULBP2) and MHC class I chain-related A and B (MICA/B) [14]. Furthermore, miR-302c-3p acts as a tumor suppressor in glioma via inhibiting proliferation and invasion of cancer cells [15]. MiR-302c-3p/interleukin-8 (IL8) axis plays an essential role in receptor for activated C-kinase 1 (RACK1)-mediated metastasis of gastric cancer [16]. Meanwhile, miR-302c-3p suppresses HCC growth via inhibiting endothelial cell-mediated angiogenesis [17]. Recently, serum miR-302c-3p expression is prominently deregulated in hepatitis C virus (HCV)-related HCC [18]. However, the expression of miR-302c-3p and its functional role in hepatitis B virus (HBV)-related HCC are rarely investigated.

In the current study, we were aimed to investigate the expression and clinical significance of miR-302c-3p in HCC, and disclosed the effects of miR-302c-3p on migration and invasion of HCC cells. Down-regulation of miR-302c-3p was observed in HCC tissues. Tumor necrosis factor receptor associated factor 4 (TRAF4) was recognized as a novel target of miR-302c-3p and it possibly mediated the tumor suppressive role of miR-302c-3p in HCC.

**Materials and methods**

**Clinical samples**

Eighty pairs of HCC and adjacent normal tissues were obtained from the Zhejiang Provincial People’s Hospital. All samples were confirmed by experienced pathologists and were conserved in liquid nitrogen for further analysis. All patients did not receive therapies including radiotherapy, chemotherapy and radiofrequency ablation prior to surgery. The study was approved by Ethic Committee of the Zhejiang Provincial People’s Hospital and written informed consent was signed by all patients. The clinicopathologic parameters of patients were shown in Table 1.

**Cell culture, transfection and reagent**

The human HCC cell lines (HepG2, SMMC-7721, Huh7, Hep3B and MHCC97H) and normal hepatic cell line (LO2) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA, USA) added with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and 1% penicillin/streptomycin (HyClone, Logan, UT, USA). All cell lines were incubated in a humidified atmosphere with 5% CO2 at 37°C.

The miR-302c-3p mimics, miR-302c-3p inhibitors and their corresponding negative control were purchased from GeneCopoeia (Guangzhou, China). TRAF4 expression plasmid (pcDNA3.1-TRAF4) was constructed and purchased from Ribobio (Guangzhou, China). Cells transfection was performed by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. An AKT inhibitor, MK-2206 (1μM, Selleck Chemicals, Houston, TX, USA) was used to treat HCC cells for 12h.

**RNA extraction and quantitative real-time PCR (qRT-PCR)**

The total RNA from HCC cells and tissues was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. cDNA was synthesized by TaqMan miRNA reverse transcription (Applied Biosystems, Foster City, CA, USA) and a PrimeScript Reverse Transcriptase kit (Takara, Dalian, China). The relative expression of miR-302c-3p and TRAF4 mRNA were quantified using miRNA-specific TaqMan miRNA Assay Kit (Applied Biosystems) and the SYBR Premix Ex Taq™ Kit (Takara, Shiga, Japan) in the Applied Biosystems 7500 Sequence Detection system. The relative expression of miR-302c-3p and TRAF4 mRNA were normalized by U6 small nuclear RNA and GAPDH, respectively. The primers of miR-302c-3p, U6, TRAF4 and GAPDH were designed and synthesized by Sangon Biotech (Shanghai, China).

**Western blotting**

Western blotting was performed according to the protocol described previously [19]. The protein
concentration was measured using spectrophotometer (BIO-RAD, Hercules, CA), then equal protein was separated by SDS-PAGE and transferred to PVDF membranes. Subsequently, the PVDF membranes were probed with antibody against TRAF4 (Abcam, Cambridge, MA, USA), p-AKT (Ser473, Cell Signaling Technology, Beverly, MA, USA), AKT (Cell Signaling Technology), E-cadherin (Cell Signaling Technology), Vimentin (Cell Signaling Technology), Slug (Cell Signaling Technology) and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA), then probed with HRP-conjugated secondary antibodies (#7074 and #7076, Cell Signaling Technology). The western blot was detected with enhanced chemiluminescence regent (Thermo Scientific, Waltham, MA, USA).

Luciferase reporter assay

The sequence of TRAF4 3’-UTR containing the putative miR-302c-3p binding region was amplified from human genomic DNA. Then the sequence was cloned into pGL3 luciferase reporter vector (Promega, Madison, WI, USA). The potential miR-302c-3p binding sites were mutated by the Quick-change site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). The wild type (wt) TRAF4 3’-UTR vector or mutant (mt) TRAF4 3’-UTR vector and miR-302c-3p mimics or miR-302c-3p inhibitors were co-transfected into HepG2 cells by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) under luminometer (Berthold Detection System, Pforzheim, Germany), and luciferase activity was normalized to Renilla activity.

Cell migration and invasion assay

1 × 10^4 indicated HCC cells were seeded into the upper chamber coated with or without Matrigel (BD Bioscience, San Jose, CA, USA) and added DMEM without FBS. Then, the chamber was put into the cell culture plate containing DMEM supplemented with 10% FBS and incubated at 37°C for 24 hours. Subsequently, the cells inside the upper chamber were carefully removed with cotton swabs. Migrated and invaded cells were fixed with 1% paraformaldehyde for 10 min and subsequently stained by hematoxylin for 5 min. The migratory and invasive cells were finally examined and counted under microscope.

Statistical analysis

All data were shown as mean ± standard deviation (SD) and analyzed by using GraphPad Prism software version 5.0 (San Diego, CA, USA). Statistical analysis was calculated by Chi-squared test, Student’s t-test, ANOVA, Pearson correlation analysis, Kaplan-Meier method and Log-rank test. P-value < 0.05 was considered as statistical significance. Each experiment was repeated three times.

Results

The expression of miR-302c-3p in HCC specimens

First, the expression of miR-302c-3p between HCC and tumor-adjacent tissues was determined by qRT-PCR. Our data showed that the expression of miR-302c-3p in HCC was obviously lower than that in tumor-adjacent tissues (P<0.0001, Figure 1A). Furthermore, the levels of miR-302c-3p in different HCC cell lines were detected. In accordance, low miR-302c-3p level was observed in all HCC cell lines compared to normal hepatic cell line LO2 (P<0.05, respectively, Figure 1B). These data reveal an under-expression of miR-302c in HCC.

Figure 1. miR-302c-3p expression is down-regulated in HCC. (A) The expression of miR-302c-3p in HCC tissues was significantly lower than that in matched tumor-adjacent tissues. n=80, P<0.0001 by Student’s t-test. (B) The expression differences of miR-302c-3p between HCC cell lines (HepG2, SMMC-7721, MHCC97H, Huh7 and Hep3B) and normal hepatic cell line LO2 (P<0.05, respectively, Figure 1B). These data reveal an under-expression of miR-302c in HCC.
Figure 2. The prognostic significance of miR-302c-3p in HCC patients. (A) Different subgroups (low/high miR-302c-3p expression) were divided according to the median of miR-302c-3p in our cohort. HCC patients with low miR-302c-3p level had a significant shorter overall survival compared to those with high miR-302c-3p level. P<0.05 by Log-rank test. (B) TCGA data showed that low miR-302c-3p level predicted an obvious poor prognosis of HCC patients. P<0.05 by Log-rank test.

Table 1. Clinicopathological correlation analysis of miR-302c-3p expression in hepatocellular carcinoma

<table>
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<th>miR-302c-3p expression</th>
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<td></td>
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<td>High level (n=40)</td>
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HBV, hepatitis B virus; AFP, alpha-fetoprotein; TNM, tumor-node-metastasis.

The clinical significance of miR-302c-3p in HCC patients

Different subgroups (low/high miR-302c-3p expression) were divided according to the median level of miR-302c-3p in the cohort. Clinicopathological correlation analysis revealed that low miR-302c-3p level was correlated with multiple tumor nodes, venous infiltration and advanced TNM tumor stage (P < 0.05, respectively, Table 1). Notably, Kaplan-Meier plots indicated that HCC patients with low miR-302c-3p level had a significant shorter 3-year overall survival compared to cases with high miR-302c-3p expression (P=0.0393, Figure 2A). Meanwhile, The Cancer Genome Atlas (TCGA) data also showed that low miR-302c-3p level indicated a poor prognosis of HCC patients (P=0.0011, Figure 2B). These data suggest that miR-302c-3p is a potential prognostic biomarker for HCC patients.

MiR-302c-3p inhibits migration and invasion of HCC cells

Since our clinical data revealed the correlation between miR-302c-3p expression and metastatic features of HCC, the regulatory effect of miR-302c-3p on migration and invasion of HCC cells was determined. MHCC97H cells with low miR-302c-3p level and HepG2 cells with high miR-302c-3p level were used for gain- and loss-of-function experiments, respectively. Further experiments were performed in MHCC97H cells after miR-302c-3p overexpression (P < 0.05, Figure 3A). Transwell assays showed that miR-302c-3p overexpression remarkably decreased the migration and invasion capacities of MHCC97H cells (P < 0.05, respectively, Figure 3B). Furthermore, miR-302c-3p knockdown prominently facilitated migration and invasion of HepG2 cell (P < 0.05, respectively, Figure 3C and 3D). Thus, miR-302c-3p functions as a tumor suppressor by suppressing migration and invasion of HCC cells.

miR-302c-3p regulates TRAF4 abundance by directly targeting its 3’UTR in HCC cells

Next, the candidate target genes of miR-302c-3p were predicted using TargetScan (http://www.targetscan.org) and miRanda (microRNA.org). We found that the 3’-UTR of TRAF4 mRNA contained the complementary sequence of miR-302c-3p according to public available database analysis (Figure 4A). Thus, TRAF4 was selected for further experimental validation. Subsequently, we found that miR-302c-3p restoration significantly reduced while miR-302c-3p silencing increased the levels of TRAF4 mRNA and protein in HepG2 cells (P<0.05, respectively, Figure 4B). Then, plasmids with wt 3’-UTR of TRAF4 or mt 3’-UTR of TRAF4 were transfected into HepG2 cells. Notably, miR-302c-3p restoration prominently reduced while miR-302c-3p silencing increased the fluorescence intensity of cells transfected with wt
3'-UTR of TRAF4 (P<0.05, respectively, Figure 4C). While, the fluorescence intensity had no significant variation after being transfected with miR-302c-3p mimics or inhibitors in the mutant group (Figure 4C). Our data further confirmed the overexpression of TRAF4 mRNA in HCC tissues compared to tumor-adjacent tissues (P<0.0001, Figure 4D). Notably, an inverse correlation between TRAF4 mRNA and miR-302c-3p expression was detected in HCC tissues (r=-0.562, P<0.0001, Figure 4E). Furthermore, six HCC tissues with different expression levels of miR-302c-3p were subjected to immunoblotting for TRAF4 expression. Quantitative data revealed that TRAF4 protein expression in HCC tissues with low miR-302c-3p level was prominently higher than that in HCC tissues with high miR-302c-3p level (P<0.05, Figure 4F). Taken together, we firstly disclose miR-302c-3p regulation of TRAF4 in HCC tissues.

miR-302c-3p inhibits AKT-mediated EMT in HCC cells

Since previous study reports that TRAF4 contributes to HCC cell metastasis via PI3K/AKT-mediated epithelial-mesenchymal transition (EMT) [20]. As an upstream regulator of TRAF4, the regulatory effect of miR-302c-3p on AKT-mediated EMT in HCC cells was subsequently determined. As expected, miR-302c-3p overexpression reduced the expression of p-AKT and mesenchymal markers (Vimentin and Slug), and increased the level of epithelial marker (E-cadherin) in MHCC97H cells (P<0.05, respectively, Figure 5). Furthermore, miR-302c-3p knockdown promoted AKT signaling activation and EMT progression in HepG2 cells (P<0.05, respectively, Figure 5). Thus, miR-302c-3p is a regulator of EMT process in HCC cells.

TRAF4 mediates the role of miR-302c-3p in HCC cells

To clarify whether TRAF4 was a downstream effector of miR-302c-3p, the rescue experiments were performed in HCC cells. TRAF4 expression was restored in miR-302c-3p overexpressing MHCC97H cells (P<0.05, Figure 6A). TRAF4 restoration increased the levels of p-AKT, Vimentin and Slug, and reduced E-cadherin expression in MHCC97H cells with miR-302c-3p overexpression (P<0.05, respectively.
Functional experiments revealed that TRAF4 restoration reversed the inhibitory effect of miR-302c-3p on migration and invasion of MHCC97H cells (P<0.05, respectively, Figure 6B). An AKT inhibitor, MK2206, was used to block AKT activation in HepG2 cells with miR-302c-3p knockdown (P<0.05, respectively, Figure 7). Furthermore, inactivation of AKT increased E-cadherin expression and reduced the levels of Vimentin and Slug in miR-302c-3p silencing HepG2 cells (P<0.05, respectively, Figure 7). These results suggest that TRAF4 is a downstream functional effector of miR-302c-3p in HCC.
Deregulated expression of miR-302c-3p has been found in various human cancers including breast cancer [13], glioma [15, 21] and gastric cancer [22]. Meanwhile, aberrant expression of miR-302c-3p is associated with prognostic features of human cancer. For instance, low miR-302c-3p expression is positively
miR-302c-3p reduced the levels of p-AKT, Vimentin pathway and EMT progression in HCC cells.

showed an opposite role in regulating AKT signaling as a direct negative regulator of TRAF4, miR-302c-3p cancer, colon cancer and HCC [20, 29-31]. Recently, in numerous cancers including breast cancer, lung breast cancer and its overexpression has been verified to exhibit its functional role by inhibiting various downstream target genes in human cancer. Subsequently, TRAF4 was identified as a candidate target gene of miR-302c-3p. Public available database and luciferase reporter assay further confirmed that TRAF4 was a direct target of miR-302c-3p in HCC cells (Supplementary Figure 1). Thus, it is worth to disclose the mechanism underlying the regulatory effect of miR-302c-3p in HCC growth. miRNAs exhibit its functional role by inhibiting various downstream target genes in human cancer. miR-302c-3p also suppressed proliferation of HCC cells. Interestingly, we found that miR-302c-3p suppressed proliferation of HCC cells (Supplementary Figure 1). Thus, it is worth to disclose the mechanism underlying the regulatory effect of miR-302c-3p in HCC growth. miRNAs exhibit its functional role by inhibiting various downstream target genes in human cancer. Subsequently, TRAF4 was identified as a candidate target gene of miR-302c-3p. Public available database and luciferase reporter assay further confirmed that TRAF4 was a direct target of miR-302c-3p in HCC cells. TRAF4, a member of TRAFs family, belongs to signal adaptor proteins in the cytoplasm [26]. Some studies reveal the vital role of TRAF4 in embryogenesis and central nervous system myelin homeostasis [27, 28]. TRAF4 is firstly identified in breast cancer and its overexpression has been verified in numerous cancers including breast cancer, lung cancer, colon cancer and HCC [20, 29-31]. Recently, several studies have reported that TRAT4 plays a crucial role in tumorigenesis and progression via regulating multiple signaling pathways, such as PI3K/AKT pathway, transforming growth factor-β (TGF-β) pathway and Wnt/β-catenin[20, 32-34]. As a direct negative regulator of TRAF4, miR-302c-3p showed an opposite role in regulating AKT signaling pathway and EMT progression in HCC cells. miR-302c-3p reduced the levels of p-AKT, Vimentin and Slug, and increased E-cadherin expression. Notably, TRAF4 restoration reversed the tumor suppressive role of miR-302c-3p in HCC cells. Inactivation of AKT by MK2206 repressed miR-302c-3p knockdown-induced EMT of HCC cells. These data indicate that miR-302c-3p inhibits the metastasis of HCC cells by suppressing TRAF4.

In conclusion, we demonstrate that miR-302c-3p acts as a potential prognostic indicator and plays a tumor suppressive role in HCC. MiR-302c-3p inhibits the phosphorylation of AKT, EMT progression, migration and invasion in HCC cells (Supplementary Figure 2). TRAF4 restoration abrogates the tumor suppressive effect of miR-302c-3p in HCC cells. Thus, these findings suggest a potential therapeutic value of miR-302c-3p/TRAF4 axis for HCC.

**Abbreviations**

HCC: hepatocellular carcinoma; miRNAs: microRNAs; ncRNAs: non-coding RNAs; 3'-UTRs: 3'-untranslated regions; ERα: estrogen receptor-alpha; NK: natural killer; ULBP2: UL-16 binding protein 2; MICA/B: MHC class I chain-related A and B; IL8: interleukin-8; RACK1: receptor for activated C-kinase 1; HCV: hepatitis C virus; HBV: hepatitis B virus; TRAF4: tumor necrosis factor receptor associated factor 4; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; TCGA: The Cancer Genome Atlas; EMT: epithelial-mesenchymal transition; KPS: Karnofsky performance status; TGF-β: transforming growth factor-β; AFP: alpha-fetoprotein; TNM: tumor-node-metastasis.

**Supplementary Material**

Supplementary figures.

http://www.jcancer.org/v09p2693s1.pdf

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**Competing Interests**

The authors have declared that no competing interest exists.

**References**


