Biomedicine & Pharmacotherapy 105 (2018) 670–676

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

MicroRNA-302c represses epithelial–mesenchymal transition and metastasis by targeting transcription factor AP-4 in colorectal cancer

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ARTICLE INFO

Keywords:
miR-302c
Transcription factor AP4
Colorectal cancer
Epithelial-mesenchymal transition, tumor metastasis

ABSTRACT

MicroRNAs (miRNAs) contribute to tumorigenesis and progression via acting as tumor suppressors or oncogenes in human cancer. Aberrant expression of miR-302c has been reported in various types of cancer except colorectal cancer (CRC). Thus, our study was aimed to verify the expression of miR-302c and its functional role in CRC. We found a significant reduced expression of miR-302c in CRC tissues compared to tumor-adjacent tissues. Low miR-302c level was remarkably correlated with deeper tumor invasion, lymph node metastasis and advanced TNM stage. Importantly, low miR-302c expression was identified as an independent indicator for poor prognosis of CRC patients. Overexpression of miR-302c repressed migration and invasion capacities of SW620 and SW480 cells *in vitro*. Mechanistically, miR-302c inversely regulated transcription factor AP4 (TFAP4) abundance in both SW620 and SW480 cells, and it negatively correlated with TFAP4 mRNA expression in CRC samples. Herein, TFAP4, a regulator of epithelial-mesenchymal transition (EMT), was recognized as a direct target gene of miR-302c in CRC. Otherwise, miR-302c overexpression increased E-cadherin expression and reduced the levels of Vimentin and SNAI1, suggesting an inhibitory effect of miR-302c on EMT of CRC cells. Notably, our findings established that the EMT and metastasis of Caco-2 cells were enhanced by miR-302c knockdown, and subsequently reversed by TFAP4 silencing. Collectively, these data indicate that miR-302c represses EMT and CRC metastasis possibly by targeting TFAP4, and it may serve as a potential prognostic factor and therapeutic target for CRC.

1. Introduction

Colorectal cancer (CRC) is one of the leading cause for cancer-related deaths and ranks the third commonest cancer worldwide [1]. Surgery remains the most common curative therapy for CRC patients [2]. In spite of great advancement in the therapy strategies for CRC such as systemic chemotherapy, radiotherapy, immunotherapy and targeted therapy, the post-surgical outcome of CRC patients has been limitedly improved due to the high frequency of tumor recurrence and metastasis [3]. In this regard, it is still urgently needed to uncover the molecular pathways underlying CRC progression and identify the novel biomarkers for predicting the post-surgical prognosis of CRC patients.

MicroRNAs (miRNAs), a family of endogenous small non-coding RNAs (~22 nucleotides in length), negatively regulate the expression of target genes by inducing translational inhibition and/or messenger RNAs (mRNAs) degradation, via incompletely base-pairing to a complementary sequence in the 3′-untranslated region (3′-UTR) [4,5]. Accumulating evidence reveal that aberrant expression of miRNAs is a frequent event in cancer initiation and metastasis, and they also act as promising prognostic biomarkers and therapeutic targets for cancer diagnosis and treatment [6–9]. A lot of studies indicate that miRNAs play critical roles in CRC progression [10,11]. Recently, miR-302c has been recognized as a cancer-related miRNA in several studies. For instance, miR-302c is identified as a potent estrogen receptor-alpha (ERα) regulating miRNA and inhibits estrogen-induced cell growth of breast cancer [12,13]. Knockdown of miR-302c results in the resistance of

Abbreviations: miRNAs, microRNAs; CRC, colorectal cancer; TFAP4, transcription factor AP4; EMT, epithelial-mesenchymal transition; mRNAs, messenger RNAs; 3'-UTR, 3'-untranslated region; ERα, estrogen receptor-alpha; NK, natural killer; ULBP2U, L-16 binding protein 2; MICA/B, MHC class I chain-related A and B; IL8, interleukin-8; RACK1, receptor for activated C-kinase 1; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; siRNA, small interfering RNA; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; RCC, renal cell carcinoma; bHLH, basic helix-loop-helix; USP22, ubiquitin specific peptidase 22

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https://doi.org/10.1016/j.biopha.2018.06.025
Received 14 March 2018; Received in revised form 1 June 2018; Accepted 5 June 2018

cancer cells to natural killer (NK) cells via up-regulating UL-16 binding protein 2 (ULBP2) and MHC class I chain-related A and B (MICA/B) expression [14]. Moreover, miR-302c functions as a tumor suppressor via repressing proliferation and invasion of glioma cells [15]. Otherwise, miR-302c/interleukin-8 (IL8) axis plays an important role in receptor for activated C-kinase 1 (RACK1)-mediated metastasis of gastric cancer [16]. In addition, miR-302c inhibits tumor growth via suppressing endothelial cell-mediated angiogenesis in hepatocellular carcinoma (HCC) [17]. Moreover, circulating miR-302c expression is markedly deregulated in hepatitis C virus (HCV)-related HCC [18]. However, the expression of miR-302c and its functional role in CRC are rarely investigated. Thus, it is worth to investigate the clinical significance of miR-302c and its role in CRC.

In this study, we attempted to disclose the potential of miR-302c to function as a prognostic biomarker for CRC patients after surgery and provide a better understanding of its biological function as well as underlying mechanism in CRC progression.

2. Materials and methods

2.1. Patients and samples

Ninety CRC and matched noncancerous tissues (> 5 cm away from tumor edge) were collected from patients, who were underwent surgery in the Second Affiliated Hospital of Xi'an Jiaotong University. Ethical approval was obtained from the Research Ethics Committee of the Second Affiliated Hospital of Xi'an Jiaotong University. All patients signed informed consent forms. None of them received immunotherapy, radiotherapy or chemotherapy prior to operation. The median follow-up time was 23 months (from 6 to 142 months). All samples were pathologically confirmed and conserved in liquid nitrogen for further analysis. The clinicopathologic characteristics of CRC patients were shown in Table 1.

2.2. Cell culture and transfection

Human CRC cell lines including Caco-2, SW620, and SW480 were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). All cells were cultured in the standard growth medium at 37 °C with 5% CO2 in a humidified incubator as previously descried [19]. Precursor miR-302c and inhibitors against miR-302c were purchased from GeneCopoeia (Guangzhou, China). Small interfering RNA (siRNA) used for TFAP4 silencing was purchased from GenePharma (Shanghai, China). Lipofectamine 3000 (Invitrogen, USA) was employed for cell transfection. The specific TFAP4 siRNA targeting sequence: 5′-GUG AUA GGA GGG CUC UGU AG-3′.

Table 1
Clinical association analysis of miR-302c expression in colorectal cancer.

Clinical features		n = 90	miR-302c expression		P
			Low (n = 45)	High (n = 45)	
Age (y)	≤60	52	27	25	0.670
	> 60	38	18	20	
Sex	Male	48	25	23	0.673
	Female	42	20	22	
Tumor grade	G1/G2	70	32	38	0.128
	G3/G4	20	13	7	
Size (cm)	< 5	28	11	17	0.172
	≥5	62	34	28	
Tumor invasion	T1/T2	11	2	9	0.024*
	T3/T4	79	43	36	
Lymph node metastases	Absent	66	28	38	0.017*
	Present	24	17	7	
Distant metastasis	Absent	85	41	44	0.361
	Present	5	4	1	
TNM stage	I/II	64	27	37	0.020*
	III/IV	26	18	8	

* Statistically significant. TNM, tumor-node-metastasis.

2.3. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from transfected CRC tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol, and was reverse transcribed into cDNA using a PrimeScript RT reagent kit (Takara, Dalian, China) and TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Amplification of miR-302c and TFAP4 mRNA was performed in the Applied Biosystems 7500 Sequence Detection system using a miRNA-specific TaqMan miRNA Assay Kit (Applied Biosystems) and a SYBR Premix Ex Taq™ Kit (Takara, Shiga, Japan). The primers for qRT-PCR were listed in Table 2. The relative expression of miR-302c and TFAP4 mRNA were normalized by U6 small nuclear RNA and GAPDH, respectively.

2.4. Western blotting analysis

The protocol of Western blotting was described previously [19]. Briefly, primary antibodies against TFAP4 (Sigma-Aldrich, St-Louis, MO, USA), Vimentin (Epitomics, Burlingame, CA, USA), E-cadherin (Cell Signaling Technology, Beverly, MA, USA), SNAI1 (Cell Signaling Technology) and GAPDH (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were used for membrane incubation at 4 °C overnight. The following day, the membranes were incubated with HRP-labeled secondary antibodies (Cwbiotech, Shanghai, China) for 2 h. The blots were detected using enhanced chemiluminescence reagents (Thermo Fisher Scientific, Waltham, MA, USA).

2.5. Cell migration and invasion assay

The CRC cell migration ability was determined using wound healing assay. CRC cells were transfected with corresponding vectors (miR-302c mimics, miR-302c inhibitors or TFAP siRNA) and seeded in a 6-well plate. When cells reached 90% confluence, a 100 μL pipette tip was used to make a scratch in the middle of the confluent cultures. The area of scratch was recorded using a phase-contrast microscope before and 24 h after incubation. The transwell chamber assay was performed according to standard methods described before [11].

2.6. Luciferase reporter assay

3′UTR of TFAP4 containing the putative miR-302c binding sites was amplified from human genomic DNA. Then wile type (wt) 3′UTR of TFAP4 was cloned into pGL3 luciferase reporter vector (Promega, Madison, WI, USA). Mutant (mt) 3′UTR of TFAP4 was generated using a Quick-change site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). The wt or mt 3′UTR of TFAP4 vector and miR-302c mimics or inhibitors were co-transfected into SW620 cells. Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) under luminometer (Berthold Detection System, Pforzheim, Germany) was used for measuring the luciferase activity, which was normalized to Renilla activity.

2.7. Immunohistochemical staining

The protocol of immunohistochemistry was described previously [19]. Briefly, sections were incubated with primary antibodies against TFAP4 (Sigma-Aldrich), Vimentin (Epitomics) and E-cadherin (Cell Signaling Technology) at 4 °C overnight and were subsequently incubated with HRP-conjugated secondary antibodies (Beijing Golden