Original Article

MicroRNA-142-3p Promotes Cellular Invasion of Colorectal Cancer Cells by Activation of RAC1

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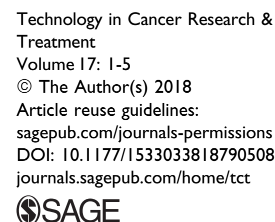

Xiang Gao, MD[1], Wenhuan Xu, MD[1], Tingxun Lu, MD[1], Jialiang Zhou, MD[1], Xiaosong Ge, MD, PhD[1], and Dong Hua, MD, PhD[1]

Abstract

Background: Colorectal cancer has been proved more difficult to treat owing to potently malignant metastasis. The present study was aimed to explore the functional role of miR-142-3p in cell migration and invasion of colorectal cancer cells, as well as its underlying mechanism. **Materials and Methods:** Expressions of miR-142-3p were analyzed in colorectal cancer tissues and cell lines. Ras-related C3 botulinum toxin substrate 1 (RAC1) was predicted as a target of miR-142-3p using software and network resources. SW480 cells were transfected with miR-142-3p expression plasmid and miR-142-3p silencer plasmid, and the expression of RAC1 and the cellular invasion were measured. **Results:** In colorectal cancer cells transfected with miR-142-3p expression plasmid, RAC1 was specifically upregulated and invasiveness of cells was downregulated. Moreover, RAC1 was significantly associated with tumor stage ($P = .029$) and tumor metastasis ($P = .012$). **Conclusion:** miR-142-3p promotes cellular invasion in colorectal cancer cells by activating RAC1. Thereby, miR-142-3p is a potential candidate for molecular targeted therapy of colorectal cancer.

Keywords

microRNA-142-3p, invasion, colorectal cancer, RAC1.

Abbreviations

CRC, colorectal cancer; FBS, fetal bovine serum; UTR, untranslated region, miR, microRNA; OS, overall survival

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Introduction

Colorectal cancer (CRC) is one of the most common malignancies in the world, with approximately 1.36 million new cases diagnosed annually. In men, CRC is the third most common cancer worldwide after lung and prostate cancer and is also the second most common malignancy in women after breast cancer.[1] During the past 2 decades, despite all advances in chemotherapy and cancer control strategies, the survival rates of patients with CRC have not changed, especially in patients with metastatic disease.[2] Therefore, new strategies that have high efficacy and low toxicity and that can overcome drug resistance of colon cancer are required.

MicroRNAs (miRs) are noncoding and small RNAs consisting of approximately 22 nucleotides that modulate gene expression by targeting the 3′-untranslated region (UTR), leading to translation inhibition.[3-5] Increasing evidence suggests that miRs are involved in a wide range of cellular processes including proliferation, apoptosis, and differentiation.[6]

Recent studies have reported that miR-142-3p, which is located at chromosome 17q22, is associated with cellular migration, proliferation, and apoptosis in renal cell carcinoma,[7] hepatocellular carcinoma,[8] and esophageal squamous cell carcinoma.[9] However, the function of miR-142-3p in CRC is largely unknown.

[1] Department of Medical Oncology, Affiliated Hospital of Jiangnan University, Wuxi, China

Corresponding Authors:
Dong Hua, MD, PhD, and Xiaosong Ge, MD, PhD, Department of Medical Oncology, Affiliated Hospital of Jiangnan University, Wuxi Cancer Institute, No. 200 Huihe Road, Wuxi, Jiangsu 214062, China.
Emails: wx89211@163.com; gexiaosong@qq.com

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Previously, our team studied the miR profiles of CRC samples and normal colorectal mucosa tissues using miR gene chip screening. The results indicated that the expression levels of miR-142-3p were significantly increased and were associated with clinicopathological features.[10] Next, we confirmed upregulation of miR-142-3p in CRC samples and cell lines and its involvement in the regulation of cell proliferation.[11] The present study establishes the oncogenic function of miR-142-3p in CRC, demonstrating how it regulates cell migration and influences the overall survival (OS) in patient with CRC.

Materials and Methods

Bacterial Strain, Plasmids, Tissue Samples, and Cell Lines

Escherichia coli strain DH5α was preserved in the laboratory of Affiliated Hospital of Jiangnan University. Gene expression vector GP/S/EGFP/B/CMV-miR plasmid was purchased from GenePharma (Shanghai, China). Colorectal cancer tissues and adjacent nontumor tissues were collected from 60 patients who underwent CRC radical surgery at the Affiliated Hospital of Jiangnan University in 2010, who did not receive preoperative treatment. Tumor, Node, Metastasis (TNM) classification was defined according to the American Joint Committee on Cancer/Union for International Cancer Control (2010). This study was approved by the Human Ethics Committee, Affiliated Hospital of Jiangnan University, and all patients signed informed consent. The human CRC cell line SW480 was maintained by the laboratory at our hospital. The cells were routinely cultured in Roswell Park Memorial Institute (RPMI)-1640 supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 U/mL) at 37°C in a humidified atmosphere containing 5% (vol/vol) CO_2.

Bioinformatics Analysis

MiR-142-3p sequences were obtained in the miR authority database miRBase (http://www.mirbase.org/). The miR target genes were predicted by miRanda 3.3a, RNAhybrid 2.1, TargetSpy softwares, and the network resources of miRBase targets (http://www.mirbase.org/), TargetScan (http://www.targetscan.org), and PicTar (http://pictar.mdc-berlin.de/). The KEGG pathway database and Expression Analysis Systematic Explorer database were used to further analyze the overlap of these results. The 3′-UTR of target genes were retrieved by UCSC human Gene Sorter (http://genome.ucsc.edu/cgibin/hgNear). All the bioinformatics analysis results are in the supplementary materials.

The Construction of miR-142-3p Expression Plasmid and miR-142-3p Silencer Plasmid

The DNA sequence of miR-142-3p was amplified from complementary DNA isolated from SW480 cells and cloned into the Bbs I and BamH I sites of the GP/S/EGFP/B/CMV-miR plasmid (GenePharma) with the following sequence: hsa-miRAN-142-S: 5′-TGCTGTGTAGTGTTTCCTACTTTATGGAGTTTTGGCCACTGACTGACTCCATAAAAGGAAACACTACA-3′, hsa-miRAN-142-A: 5′-CCTGTGTAGTGTTTCCTT TTATGGAGTCAGTCAGTGGCCAAAACTCCATAAAGTAGGAAACACTACAC-3′. miR-142-3p silencer plasmid was constructed with the following sequences: hsa-miRAN-142-S: 5′-TGCTGTCCATAAAGTAGGAAACACTACAGTTTTGGCCACTGACTGACTCCATAAAAGGAAACACTACA-3′, hsa-miRAN-142-A: 5′-CCT GTCCATAAAGTAGGAAACACTACAGTCAGTCAGTGGCCAAAACTCCATAAAGTAGGAAACACTACAC-3′. Finally, the constructs were verified by sequencing and named pPG/miR/EGFP/Blasticidin-miR-142-3p and pPG/miR/EGFP/Blasticidin-miR-142-3p-silencer, respectively.

Western Blot Analysis

Western blot was performed to detect the relative levels of Ras-related C3 botulinum toxin substrate 1 (RAC1) protein after CRC cell line SW480 was transfected with miR-142-3p expression plasmid, miR-142-3p silencer plasmid, or miR-142-3p-negative control (NC) plasmid. Cell lysates were clarified by centrifuging at 10 000*g*. Immunoblots were probed according to standard protocols with RAC-1 (Santa Cruz Biotechnology, Inc, Santa Cruz, California). The quality of loading and transfer was assessed by immunostaining with β-actin (Santa Cruz Biotechnology Inc). Immunoblots were developed using enhanced chemiluminescent regent (Pierce Biotechnology, Rockford, Illinois, USA), and images were captured by the FUJIFILM LAS-3000 system (Fuji film, Tokyo, Japan).

Migration and Invasion Assay

Cell migration and invasion assays were performed using Transwell chambers. For the migration assay, 5 × 104 cells were added to the upper chamber of 8-μm pore size Transwells (BD Biosciences, Franklin Lakes, New Jersey). For invasion assays, 1×10^5 cells were added to the upper chamber of 8-μm pore size Transwells precoated with Matrigel (BD Biosciences). In these assays, cells were plated in medium without serum and medium containing 10% FBS in the lower chamber, serving as a chemoattractant. After 14 hours of incubation, cells not migrated or invaded through the pores were carefully removed. The filters were then fixed in 90% alcohol, which was followed by crystal violet staining. Five random fields were counted per chamber using an inverted microscope (CKX41; Olympus Corporation, Tokyo, Japan), and each test was performed in triplicate.

Evaluation of Immunohistochemical Variables

Immunohistochemical staining results were assessed by 2 pathologists independently with no knowledge of patient characteristics. Discrepancies were resolved by consensus. RAC1 staining was evaluated using the following criteria: positive, dark brown staining in >50% tumor cells completely obscuring cytoplasm and absent, no appreciable staining in tumor cells (Figure 1B).

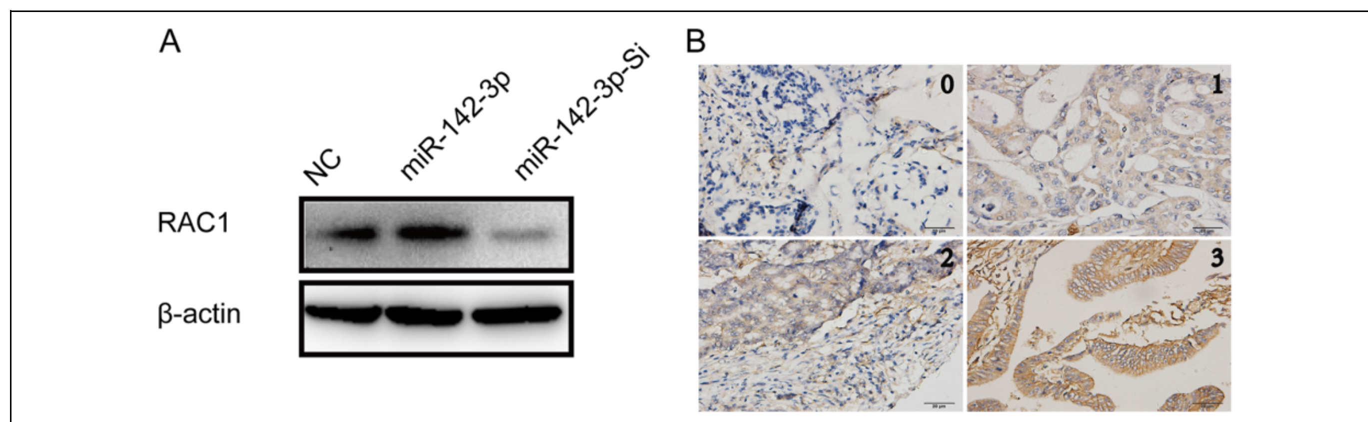

Figure 1. RAC1 expression by Western blot and immunohistochemistry (IHC). A, RAC1 expression in SW480 cells transfected with NC/miR-142-3p/ miR-142-3p silencer was analyzed by Western blot. β-Actin was used as control. B, RAC1 expression in CRC tissue microarrays by IHC. The upper right number represents the IHC score.

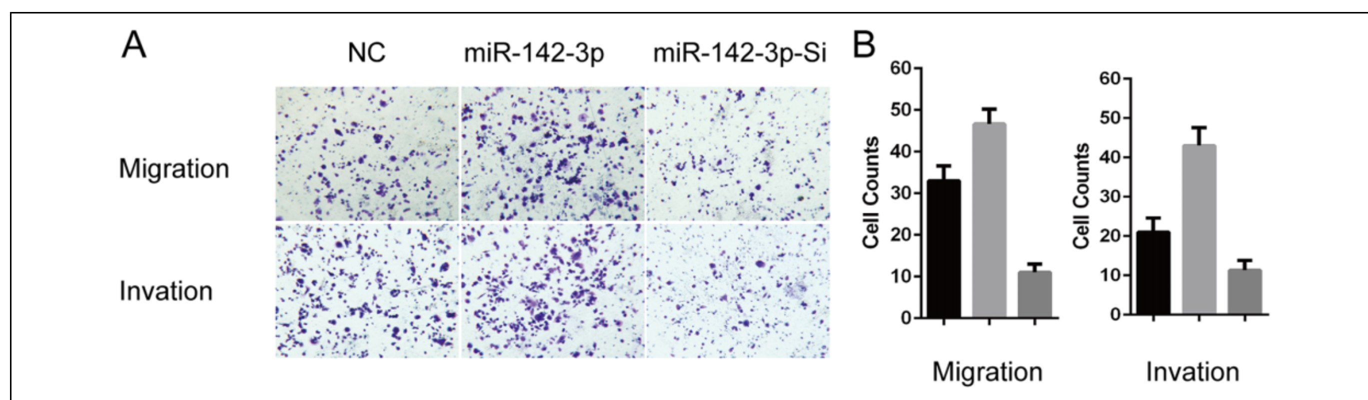

Figure 2. RAC1 affected migration and invasion of colorectal cell line SW480. Migrating and invading cells were stained by crystal violet and photographed (n = 3, $*P < .05$).

Patients and Specimens

All the medical charts of colorectal carcinoma between the period January 2003 and December 2008 were reviewed in the database of Affiliated Hospital of Jiangnan University. Patients were excluded if they did not receive the majority of their treatment at Affiliated Hospital of Jiangnan University or if they had more than 1 primary cancer. A total of 192 cases obtained the clinical outcome, with the last update in December 2014. Information on potential prognostic factors such as age, gender, tumor stage, lymph node metastasis, and metastasis was captured.

Statistical Analysis

The follow-up period was defined as the time from surgery to the last observation for censored cases or death for complete observations. Overall survival probability was derived from the Kaplan-Meier estimate, and the differences between survival curves were compared by means of the log-rank test. RAC1-positive tumors were compared to RAC1-negative tumors for the same clusters, using the 2-sided Person χ^2 test. Statistics was analyzed using Stata/SE version 10.0 (Stata Corporation, College Station, Texas). All P values are 2 sided, and $P < .05$ was considered significant.

Results

RAC1 is a Target of miR-142-3p

To identify the miR-142-3p targets, we predicted its potential target genes using miR and a 3.3a, RNA hybrid 2.1; Target Spy; TargetScan, miRBase, and PicTar algorithms. The genes predicted by all the algorithms were chosen as the candidate target genes of miR-142-3p. Among them, RAC1 was found to have a putative miR-142-3p binding site within its 3′-UTR.

miR-142-3p Promotes Metastasis by Targeting RAC1

To examine the role of miR-142-3p, SW480 cells were transfected with miR-142-3p expression plasmid and miR-142-3p silencer plasmid, respectively. RAC1 protein levels were increased in miR-142-3p-transfected SW480 cells but

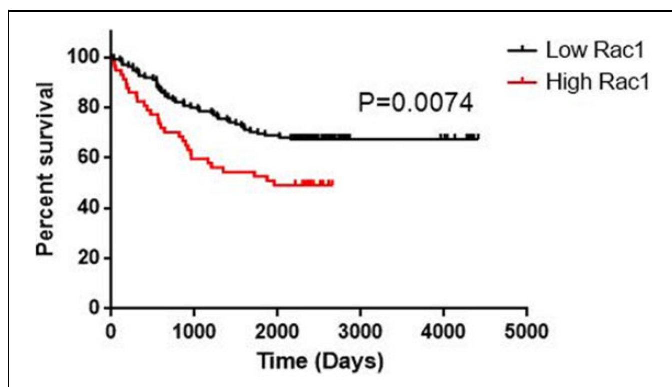

Figure 3. Kaplan-Meier analysis of survival for patients with low versus high levels of RAC1.

Table 1. Correlation Between Ras-related C3 botulinum toxin substrate 1 (RAC1) Expression and Clinicopathological Characteristics of Colorectal Cancer.

		RAC1 Expression		
Characteristics	Cases	Low (135)	High (57)	*P* Value
Gender				.611
Male	99	68	31	
Female	93	67	26	
Age (mean = 62)				.394
<62	100	54	46	
≥60	97	46	51	
Clinical stage				.029
I	34	25	91	
II	67	46	21	
III	81	61	20	
IV	10	3	7	
T				.932
T1-2	47	33	14	
T3	101	72	29	
T4	44	30	14	
N				.712
N0	102	71	31	
N1	53	36	17	
N2	37	28	9	
M				.012
M0	182	132	50	
M1	10	3	7	

decreased in miR-142-3p silencer-transfected SW480 cells compared with NC-transfected SW480 cells (Figure 1A). Further results indicated that cell migration and invasion was promoted in SW480 cells transfected with miR-142-3p ($P < .05$), while those cells transfected with miR-142-3p-silencer had significant cell migration and invasion inhibition compared with that of the cells transfected with NC and blank controls ($P < .05$; Figure 2).

Prognostic Influence of RAC1 Expression

Immunohistochemical staining results showed that RAC1 was overexpressed in CRC tumor tissues (data not shown).

Table 2. Univariate Analysis for Overall Survival (Cox Proportional Hazards Regression Model).

		Cases	OS (days)	*P* Value
Stage	I	34	3604	<.0001
	II	67	3556	
	III	81	2585	
	IV	10	591	
pT	T1 + 2	47	3703	.001
	T3	101	3006	
	T4	44	2200	
pN	N0	102	3569	<.0001
	N1	53	2528	
	N2	37	2229	
pM	M0	182	3162	<.0001
	M1	10	591	
RAC1	Low	135	3259	.007
	High	57	1657	

Abbreviation: OS, overall survival.

Table 3. Multivariate Analysis for Overall Survival (Cox Proportional Hazards Regression Model).

	HR (95% CI)	*P* Value
Stage		
I vs II vs III vs IV	0.999 (0.525-1.899)	.997
N		
N0 vs N1 vs N2	1.690 (1.056-2.705)	.029
T		
T1 + 2 vs T3 vs T4	1.594 (1.104-2.300)	.013
M		
M0 vs M1	3.866 (1.268-11.784)	.017
RAC1		
Low vs High	1.925 (1.178-3.145)	.009

Abbreviations: CI, confidence interval; HR, hazards ratio.

Survival analysis showed that increased RAC1 expression was associated with a shorter OS ($P < .0001$, Figure 3). Meanwhile, RAC1 was significantly associated with tumor stage ($P = .029$) and tumor metastasis ($P = .012$), but not associated with gender, age, tumor invasion status, and nodal status, as seen in Table 1. Moreover, univariate analysis of OS with the Cox proportional hazards model revealed that pT stage, lymph node metastasis (pN), metastasis (pM), and RAC1 were all significant variables (Table 2). Multivariate analysis revealed that high RAC1 was an independent risk factor for OS in patients with CRC (Table 3, hazards ratio = 1.925, 95% confidence interval = 1.178-3.145, $P = .009$).

Discussion

MiR-142-3p has been reported as an oncogenic miRNA in human T-cell acute lymphoblastic leukemia by targeting the glucocorticoid receptor and the cyclic adenosine monophosphate/protein kinase A pathways.[12] Overexpression of miR-142-3p also regulated the properties of breast cancer stem cells, at least in part by activating the WNT signaling pathway and

miR-150 expression.[13] However, relatively little is known about the role of miR-142-3p in CRC.

In this study, the impacts of miR-142-3p on cellular invasion and migration were analyzed by using Transwell chambers. The results demonstrated that downregulation of miR-142-3p significantly suppressed cell migration and invasion, suggesting that miR-142-3p may function as an oncogene during CRC tumorigenesis.

Using miRanda 3.3a, RNAhybrid 2.1, TargetSpy, and the TargetScan, miRBase, and PicTar algorithms, we predicted RAC1 was a potential target of miR-142-3p. RAC1 belongs to Rho family of GTPases, which include Cdc42 and RhoA,[14] and is implicated in many cellular functions, such as cell trafficking, actin polymerization, gene transcription, and cell proliferation. RAC1 has been shown to act as an effecter of tumor progression via NF-κB signaling pathway.[15] In addition, GTP-RAC1 can directly bind to Bcl-2 to elicit antiapoptotic cell responses.[16] Moreover, GTP-RAC1 regulates tumor angiogenesis by increasing vascular endothelial growth factor1/2 production.[17]

Our findings show that patients with high RAC1 expression had a shorter OS than those with low Rac1 expression. RAC1 expression was elevated in patients with higher tumor stage and metastasis. These results indicated the important role of RAC1 in tumor proliferation and invasion. However, RAC1 protein levels were increased in miR-142-3p-transfected SW480 cells but decreased in miR-142-3p silencer-transfected SW480 cells. One of the reasons may be that RAC1 was regulated indirectly by miR-142-3p.

Conclusion

In summary, our study identified the role of miR-142-3p and its target gene RAC1 in CRC and may help understand the potential molecular mechanisms of CRC development. MiR-142-3p may be a candidate target for molecular therapy of CRC.

Authors' Note

Xiang Gao and Wenhuan Xu designed and carried out experiments, analyzed, and interpreted part of the data. Xiaosong Ge, Tingxun Lu, and Jialiang Zhou drafted the manuscript. Xiaosong Ge and Dong Hua analyzed and interpreted part of the data. All authors read and approved the final manuscript. This study was approved by the Human Ethics Committee, Affiliated Hospital of Jiangnan University, and all patients signed an informed consent.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

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