

MicroRNA-142-3p suppresses cell proliferation, invasion and epithelial-to-mesenchymal transition via RAC1-ERK1/2 signaling in colorectal cancer

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Abstract. Aberrant expression of microRNAs (miRNAs/miRs) is associated with the initiation and progression of colorectal cancer (CRC), but how they regulate colorectal tumorigenesis is still unknown. The present study was designed to investigate the expression profile of miRNAs in human CRC tissues, and to reveal the molecular mechanism of miRNA-142-3p in suppressing colon cancer cell proliferation. The expression of miRNA was examined using an Exiqon miRNA array. Bioinformatics was used to predict the target genes of differentially expressed miRNAs and to analyze their biological function in CRC. The effect of miR-142-3p in colon cancer cells was evaluated *in vitro* using cell proliferation, colony formation and Transwell assays. Dual-luciferase reporter gene assays were performed to investigate the association between miR-142-3p and Rac family small GTPase 1 (RAC1). The effect of miR-142-3p regulation on colon cancer proliferation was assessed through western blotting and quantitative polymerase chain reaction analysis. Compared with their expression in adjacent non-cancer mucosal tissues, 76 miRNAs were upregulated and 102 miRNAs were downregulated in CRC. One of the most significantly and differentially regulated miRNAs was miR-142-3p, which was downregulated in 81.0% (51/63) of primary CRC tissues. After transfection of

miR-142-3p mimics into colon cancer cells, proliferation and colony formation were decreased, and migration and invasion were markedly suppressed. *RAC1* was a possible target of miR-142-3p, which was confirmed by dual-luciferase reporter assay. Transfection of miR-142-3p mimics decreased the levels of RAC1 and suppressed epithelial-to-mesenchymal transition in colon cancer cells. The phosphorylation of extracellular signal-regulated kinase (ERK) was decreased significantly by the inhibition of RAC1 or transfection of miR-142-3p mimics in colon cancer cells. In conclusion, aberrant miRNAs are implicated in CRC. Decreased expression of miR-142-3p may be associated with CRC tumorigenesis via Rac1-ERK signaling.

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide and the second leading cause of cancer-associated death (1). In China, the incidence and mortality rates of CRC have been increasing gradually over the past decade, with 190,000 people dying of CRC every year, despite improved surgical and medical management (2). Numerous studies have revealed that microsatellite instability, CpG island methylation, frequent gene mutations, and the PI3K, WNT, MAPK, p53, transforming growth factor (TGF)- β and DNA mismatch repair pathways are involved in the initiation and progression of CRC (3,4). However, the genetic and genomic changes associated with colorectal tumorigenesis and their significance are still unclear.

MicroRNAs (miRNAs/miRs) are endogenous long non-coding RNAs of 21-25 nucleotides that are generally transcribed from non-coding regions of the genome. miRNAs are processed from a 60-70-nucleotide hairpin precursor. Over the past decade, >2,000 miRNAs have been found in the human genome (5); they participate in several physiological and pathological processes by regulating the expression of >60% of human genes (5). Mature miRNAs are the most important members of the active RNA-induced silencing complex, and play a key role in gene regulation. Mature miRNAs suppress the translation or degradation of their target mRNAs by interacting with the 3'-untranslated region (UTR),

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Abbreviations: CRC, colorectal cancer; MAPK, mitogen-activated protein kinase; TGF, transforming growth factor; miRNA, microRNA; UTR, untranslated region; EMT, epithelial-to-mesenchymal transition; qPCR, quantitative polymerase chain reaction; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; ERK, extracellular signal-regulated kinase; TNM, Tumor-Node-Metastasis; RAC1, Rac family small GTPase 1; CCK-8, Cell Counting Kit-8

Key words: miR-142-3p, CRC, RAC1, ERK1/2, microarray, miRNA

open reading frame (ORF), or 5'-UTR of the mRNA (6,7). miRNAs repress protein production by post-transcriptional suppression of mRNA translation or promotion of mRNA degradation, allowing miRNAs to regulate cell differentiation, proliferation, apoptosis and homeostasis (5,8,9). Dysregulation of miRNAs has been implicated in human diseases and tumorigenesis, including tumor invasion, metastasis, staging and prognosis (10-14). Accumulating evidence has shown that miRNAs function as regulators in CRC (11,12,15).

Since the low expression of miR-143 and miR-145 in CRC was first reported (16), the dysregulation of miRNAs has been found to be a frequent event in CRC, and is considered to be associated with CRC tumorigenesis and progression (11,12,15). Nagy *et al* (17) found that miR-18a, -18b, -431, -503, -1246 and -4417 are upregulated during the adenoma to carcinoma sequence in CRC, whereas miR-133a, -375, -378, -422, and -479 are downregulated during this sequence. It was also shown that the expression profiles of miRNAs in colorectal adenoma and CRC are significantly different (17). Further studies have revealed that aberrant miRNAs, including miR-142-3p, are involved in cell proliferation, cell cycle transition, cell apoptosis, autophagy, epithelial-to-mesenchymal transition (EMT), invasion and metastasis in CRC by regulating the WNT/ β -catenin, epidermal growth factor receptor, TGF- β , Rac family small GTPase 1 (RAC1) and p53 signaling pathways (11,12,15,18). The aforementioned results suggest that some miRNAs may be effective biomarkers for diagnosis and prognosis, and can be used as drug targets for therapy in CRC. However, although the dysregulation of miRNAs plays a key role in CRC progression, the specific mechanism by which miRNAs regulate colon tumorigenesis is still largely unknown.

In the present study, the Exiqon miRNA oligonucleotide microarray was used to assess miRNA expression profiles in CRC, and bioinformatics was employed to analyze the biological function of differentially expressed miRNAs and their role in CRC tumorigenesis and progression. The functions and mechanisms of miRNA-142-3p were assessed in CRC cell lines.

Materials and methods

Tissue specimens. A total of 63 CRC tissues and their corresponding adjacent non-cancer tissues were collected from January 2017 to December 2018 in the Department of Pathology of the First Affiliated Hospital of Hainan Medical University (Haikou, China). All patients (age, 28-86 years; mean age, 58.3 years) included in the study had undergone surgical resection but without previous surgery, radiotherapy or chemotherapy. All cases were diagnosed pathologically by two senior pathologists. Clinicopathological characteristics, such as age, sex, tumor size and tumor TNM stage according to the AJCC 8th Edition (19), were obtained from electronic medical records and are summarized in Table I. To maintain the consistency of the biological information, three pairs of cases with similar clinical data were selected for miRNA microarray analysis (Table SI and Fig. S1). All the patients provided written consent for their specimens to be used in the study. The study was approved by the Ethics Committee of the First Affiliated Hospital of Hainan Medical University.

Microarray analysis. CRC and paired non-cancer mucosa tissues from three sets of cases were homogenized using the TissueLyser II (Qiagen GmbH), and total RNA was isolated using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and purified with the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Microarray hybridization and data analysis were performed by KangChen BioTech Co., Ltd., according to the protocols for the miRCURY miRNA Array (v.18.0, Exiqon) which comprised >3,100 probes for capturing miRNAs. Samples were labeled with Hy3 using the miRCURY Array Power Labeling kit (Exiqon). rRNA was removed using the rRNA removal kit, and the remaining mRNA was hybridized to each array in a hybridization oven at 55°C and 20 rpm for 20 h. The array was washed with the Gene Expression Wash Buffer (Exiqon) and scanned using the Axon Genepix 4000B Scanner (Exiqon). Then scanned images were analyzed with the GenePix Pro 6.0 software (Axon Instruments; Molecular Devices, LLC). Background subtraction was performed, and the raw data were normalized using the quantile algorithm. Aberrantly expressed miRNAs were classified as those with a 2-fold change in expression and a $P < 0.05$. The obtained microarray data were deposited in the Gene Expression Omnibus database (accession no. GSE101502).

RNA extraction and quantitative polymerase chain reaction (qPCR). Total RNA and miRNAs were extracted from 63 pairs of CRC and matched non-cancer mucosa tissues using the RNA and miRcute miRNA isolation kits, respectively (cat. nos. DP439 and DP502; Tiangen Biotech Co., Ltd.). Total RNA was reverse transcribed into first-strand cDNA by incubating at 42°C for 15 min using the RNA first-strand cDNA kit (cat. no. KR118; TIANGEN Biotech Co., Ltd.) and the TaqMan MicroRNA Reverse Transcription kit (cat. no. 4366596; Thermo Fisher Scientific, Inc.). The specific stem-looped RT primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.) and are shown in Table II. The PCR primer sequences for the miRNAs, small nuclear RNA U6 (U6), RAC1, and GAPDH were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.) and are listed in Table III. Quantification of miRNAs and RAC1 mRNA was performed using the ABI viia7 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was performed using the FastFire qPCR kit and the miRcute miRNA qPCR Detection kit (cat. nos. FP208 and FP401, TIANGEN Biotech Co., Ltd.), according to the manufacturer's instructions. The initial denaturation step during qPCR was performed at 95°C for 120 sec, followed by 40 amplification cycles at 95°C for 15 sec, and annealing and extension at 60°C for 20 sec. The relative expression levels of RAC1 mRNA and miRNAs were calculated using the $2^{-\Delta\Delta C_q}$ method (20). U6 and GAPDH were used as internal references.

Bioinformatics analysis. CRC-associated miRNAs were identified in the dbDEMC 2.0 (21) and miRCancer (22) databases. These databases were also used to find the tripartite overlapping data of differentially expressed miRNAs and miRNAs associated with CRC using Biovenn (23). The target genes of differentially expressed miRNAs were predicted by the PicTar (24), TargetsScan (25) and miRanda (26) databases. The cut-off value determined using mirSVR was -0.49. The regulatory network of miR-142-3p and its targeted genes

Table I. Association between miR-142-3p expression and pathological characteristics of patients with colon cancer.

Clinicopathological parameter	n	miR-142-3p		χ^2	P-value
		Low expression	High expression		
Age, years				0.226	0.464
≥60	42	8	34		
<60	21	3	18		
Sex				0.017	>0.99
Male	39	7	32		
Female	24	4	20		
Depth of invasion				2.56	0.16
T1+T2	22	7	15		
T3+T4	41	4	37		
Clinical TNM stage				8.53	0.01
I+II	27	9	18		
III+IV	36	2	34		
Tumor grade				0.49	0.31
I+II	41	6	35		
III+IV	22	5	17		
Lymph node metastasis				4.88	0.04
N0	28	8	19		
N1+N2	35	3	33		
Distant metastasis				0.15	0.55
Yes	4	1	3		
No	59	10	49		

miR, microRNA; TNM, Tumor-Node-Metastasis.

Table II. Stem-looped reverse transcription primers of miRNAs.

miRNAs	Primer sequence
miR-15b-5p	5'-GTCGTATCCAGTGC GTGTCGTGGAGTCGGCAATTGCACTGGATACGACTGTA AAA-3'
miR-31-5p	5'-GTCGTATCCAGTGC GTGTCGTGGAGTCGGCAATTGCACTGGATACGACAGCTAT-3'
miR-139-5p	5'-GTCGTATCCAGTGC GTGTCGTGGAGTCGGCAATTGCACTGGATACGACACTGGA-3'
miR-142-3p	5'-GTCGTATCCAGTGC GTGTCGTGGAGTCGGCAATTGCACTGGATACGACTCCATA AAA-3'
miR-196b-3p	5'-GTCGTATCCAGTGC GTGTCGTGGAGTCGGCAATTGCACTGGATACGACGAAGGC-3'
miR-342-5p	5'-GTCGTATCCAGTGC GTGTCGTGGAGTCGGCAATTGCACTGGATACGACTCAATCAC-3'
miR-378a-5p	5'-GTCGTATCCAGTGC GTGTCGTGGAGTCGGCAATTGCACTGGATACGACACACAGG-3'
miR-455-5p	5'-GTCGTATCCAGTGC GTGTCGTGGAGTCGGCAATTGCACTGGATACGACCGATGTA-3'

miR/miRNA, microRNA.

were visualized using Cytoscape (27). Tripartite overlapping genes were identified, and enriched pathways and cellular functions were identified by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses (david.ncifcrf.gov/tools.jsp) using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.8 (28).

Cell culture. The human colon cancer cell lines, SW620 and 293T, were purchased from the China Center for Type Culture

Collection. Cells were incubated in Dulbecco's modified Eagle's supplemented with 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 U/ml streptomycin in a 5% CO₂ incubator at 37°C. When the cells reached 70% confluence at the logarithmic growth phase, they were used for the experimental study.

Dual-luciferase assay. A dual-luciferase assay was performed using a Nano-Glo® Dual-luciferase® Reporter Assay

Table III. Primer sequences for miRNAs, *GAPDH* and *RAC1*.

Gene	Primer sequences	Annealing temperature, °C	Product length, bp
miR-15b-5p	F: 5'-GGGTAGCAGCACATCATGG-3'; R: 5'-GTGCGTGTTCGTGGAGTCG-3'	60	63
miR-31-5p	F: 5'-GGGAGGCAAGATGCTGGC-3'; R: 5'-GTGCGTGTTCGTGGAGTCG-3'	60	65
miR-139-5p	F: 5'-GGGGTCTACAGTGCACGTGT-3'; R: 5'-CAGTGCCTGTTCGTGGAGT-3'	60	65
miR-142-3p	F: 5'-GGGGGTGTAGTGTTCCTA-3'; R: 5'-CAGTGCCTGTTCGTGGAGT-3'	60	68
miR-196b-3p	F: 5'-GGCTCGACAGCACGACACT-3'; R: 5'-GTGCGTGTTCGTGGAGTCG-3'	60	63
miR-342-5p	F: 5'-GGGAGAGGGGTGCTATCTG-3'; R: 5'-GTGCGTGTTCGTGGAGTCG-3'	60	64
miR-378a-5p	F: 5'-GGCTCGTACTCCAGGT-3'; R: 5'-CAGTGCCTGTTCGTGGAGT-3'	60	64
miR-455-5p	F: 5'-GGGCAGTATGTGCCTTTGG-3'; R: 5'-CAGTGCCTGTTCGTGGAGT-3'	60	68
<i>U6</i>	F: 5'-GCTTCGGCAGCACATATACTAAAAT-3'; R: 5'-CGCTTCACGAATTTGCGTGCAT-3'	60	89
<i>RAC1</i>	F: 5'-ATGTCCTGCAAAGTGGTATC-3'; R: 5'-CTCGGATCGCTTCGTCAAACA-3'	60	86
<i>GAPDH</i>	F: 5'-GCCAAAAGGGTCATCATCTC-3'; R: 5'-GTAGAGGCAGGGATGATGTTTC-3'	60	124

miR/miRNA, microRNA; F, forward; R, reverse.

System (cat. no. N1630; Promega Corporation) according to the manufacturer's instructions. Briefly, a wild-type sequence of the *RAC1* 3'-UTR (WT-*RAC1*, AAGACA GTATTTTGACAAAATACGAAGTGGAGATTTACTA CATTGTACAAGGAATGAA) and a mutant sequence of the *RAC1* 3'-UTR (MUT-*RAC1*, AAGACAGTATTTTGACAA AATACGAAGTGGAGATTTTGTGATGTTTGTACAAGG AATGAA) were inserted into the luc2 site of the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega Corporation) using *SacI* and *XhoI*. The 293T cells were seeded at 5×10^4 cells per well into 24-well plates, co-transfected with 5 ng reporter vectors and 40 pmol miR-142-3p mimics (UGU AGUGUUUCCUACUUUAUGGA) or miR-142-3p negative control (NC) mimics (UUUGUACUACACAAAAGUACU G; both Guangzhou RiboBio Co., Ltd.) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 12 h, the transfection medium was replaced with a complete culture medium, and cells were further cultured for 48 h. At that point, the cells were lysed, and dual-luciferase activity was measured. All assays were performed in triplicate. *Renilla* luciferase was used for normalization.

Cell viability and colony-formation assays. SW620 cells at the logarithmic growth phase were seeded into 96-well plates or 6-well plates at a density of 1,000 cells/well. The cells were transfected with 40 pmol miR-142-3p mimics or miR-142-3p NC mimics using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) or treated with *RAC1* inhibitor (NSC23766 trihydrochloride; cat. no. ab142161; Abcam). Cell proliferation was assessed using Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc.) according to the manufacturer's instructions. The transfection medium was replaced after 12 h, and cells were cultured for 24, 48, 72 or 96 h. At the appropriate time point, 10 μ l CCK-8 reagent and 90 μ l DMEM were added into each well for 2 h. Absorbance was

measured at a wavelength of 450 nm using a multifunctional enzyme-labeling instrument.

For the colony-formation assay, SW620 cells were cultured until the number of colonies in the control group was >50 , which took ~ 15 days. The cell colonies were fixed in 10% formalin at 25°C for 1 h and stained with 0.1% crystal violet (Beyotime Institute of Biotechnology) at 25°C for 5 min. The number and average size of colonies were counted using an inverted light microscope at x100 magnification (Olympus Corporation). All assays were performed in triplicate.

Cell migration and invasion assays. The cell invasion and migration assays were performed using Transwell chambers (Corning, Inc.), with or without Matrigel (BD Biosciences), respectively. Briefly, 5×10^4 or 1×10^5 transfected cells were suspended in serum-free medium and plated in the upper chamber for the migration and invasion assays, respectively. The medium in the lower chamber was supplemented with 10% FBS. After 16 or 24 h, cells in the lower chamber were fixed in 10% formalin at 25°C for 10 min and stained with 0.1% crystal violet at 25°C for 5 min and counted using an inverted light microscope at x100 magnification (Olympus Corporation).

Western blotting. SW620 cells were lysed in lysis buffer [1% SDS, 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 1 mM EDTA, 1 mM NaF, 10 mM PMSF, 1 mM Na_3VO_4 and protease inhibitor cocktail], denatured, and centrifuged at 10,000 x g and 4°C for 10 min, and the supernatant was collected. The protein concentration was measured using the BCA method (Beyotime Institute of Biotechnology). Protein (30 μ g) was used for sodium dodecyl sulfate polyacrylamide gel electrophoresis using 12% acrylamide gels. The proteins were transferred and fixed on the polyvinylidene fluoride membranes, blocked with 5% skimmed milk supplemented with 95% PBS at

25°C for 2 h, and incubated with the primary antibodies at 4°C overnight. All primary and secondary antibodies were purchased from Abcam. The primary antibodies used were: RAC1 (cat. no. ab155938; 1:200), GAPDH (cat. no. ab9485; 1:2,000), MMP9 (cat. no. ab76003; 1:500), extracellular signal-regulated kinase (ERK)1/2 (cat. no. ab184699; 1:500), pERK1/2 (cat. no. ab201015; 1:500), vimentin (cat. no. ab8978; 1:1,000), E-cadherin (cat. no. ab1416; 1:500) and N-cadherin (cat. no. ab18203; 1:500; all Abcam). After washing the membranes three times, horseradish peroxidase-conjugated secondary antibodies (goat anti-Rabbit, cat. no. ab150077, 1:2,000 and anti-mouse IgG, cat. no. ab150113, 1:2,000) were added, and the membranes were incubated at 25°C for 0.5 h. The membranes were washed and developed using BeyoECL Plus (Beyotime Institute of Biotechnology, Suzhou, China). Bands were imaged using Tanon 4600SF Automatic Chemiluminescence System (Tanon Science and Technology Co., Ltd.) and intensities were quantified using ImageJ v1.8.0 (imagej.nih.gov/ij/).

Statistical analysis. IBM SPSS Statistics for Windows, version 22.0 (IBM Corp.) was used for the statistical analysis. Student's t-test (paired or unpaired depending on the data) was used for the analysis of continuous variables (mean \pm standard deviation). One-way analysis of variance with Tukey's post hoc test was used to evaluate the differences between multiple groups. Associations among pathological features (categorical variables) were evaluated using the χ^2 and Fisher's exact tests. Correlations were assessed using the Pearson correlation coefficient. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Aberrant expression of miRNAs in CRC tissues. A microarray analysis was performed to determine the differentially expressed miRNAs between CRC and paired non-cancer tissue. In total, >2,000 miRNAs were detected, and 178 miRNAs were differentially expressed between cancer and non-cancer mucosal tissues. Of the, 178 miRNAs, 76 were upregulated in CRC tissues and 102 were downregulated (Fig. 1A and B). Unsupervised hierarchical clustering of 3 CRC and paired non-cancer tissue samples analyzed by miRNAs microarray using the 178 differentially expressed miRNAs showed that the expression profile of miRNAs was clearly divided into two groups, corresponding to CRC and paired non-cancer tissues (Fig. 1C). Therefore, the expression of miRNAs in CRC was different from that in non-cancer mucosa tissue.

Analysis and validation of miRNAs dysregulated in CRC. In order to identify the miRNAs that were most closely associated with CRC, the dbDEMC2 and miRcancer databases were utilized to search for the dysregulated miRNAs in CRC. A total of 359 and 371 miRNAs that were dysregulated in CRC were obtained from the dbDEMC2 and miRcancer databases, respectively. The differentially expressed miRNAs identified from the microarray analysis were compared with the miRNAs obtained from the two databases (Fig. 2A and B), and 8 miRNAs found in all databases were identified. The

downregulated miRNAs included miR-139-5p, miR-15b-5p, miR-342-5p, miR-142-3p and miR-378a-5p; and the upregulated miRNAs included miR-196b-5p, miR-455-5p and miR-31-5p. In the present study, qPCR (Fig. 2C) showed that the expression of these miRNAs was consistent with the microarray analysis results (Fig. 2D) ($r=0.903$; $P=0.02$). The expression of miR-142-3p was downregulated 4.3-fold in CRC tissue compared with that in adjacent non-cancer mucosa tissue ($t=12.473$; $P=0.006$).

Bioinformatics analysis of miR-142-3p. The expression level of miR-142-3p was further examined by qPCR in 63 cases of CRC and matched non-cancer tissues. The results showed that miR-142-3p was downregulated in 81.0% of primary CRC tissues compared with that in matched non-cancer colorectal mucosa tissues ($t=2.595$; $P=0.012$; Fig. 3A). Additionally, miR-142-3p expression was associated with lymph node metastasis and Tumor-Node-Metastasis (TNM) stage (Table I). In order to understand the biological roles of miR-142-3p, the PicTar, TargetsScan and miRanda databases were used to predict its possible targets. A total of 56 potential targets were identified after examining genes that were present in all three databases (Fig. 3B). Next, those genes were imported into DAVID to perform GO and KEGG pathway enrichment analyses. The results revealed that these targets were associated with 'cell adhesion', 'cell autophagy', 'small GTPase-mediated signal transduction', 'cell proliferation' and 'regulation of transcription', among others, and the involved pathways included 'pathways in cancer', 'regulation of actin cytoskeleton', the 'Ras signaling pathway', 'colorectal cancer' and other associated signaling pathways (Fig. 3C).

miR-142-3p has the function of regulating RAC1 expression. RAC1 had the highest score as a predicted target of miR-142-3p and had a putative binding site within its 3'-UTR (Fig. 4A). In order to investigate whether miR-142-3p regulates the expression of RAC1, a dual-luciferase assay was performed to determine whether miR-142-3p can bind to the 3'-UTR of RAC1 and repress RAC1 expression. The results showed that luciferase activity in 293T cells co-transfected with miR-142-3p mimics and RAC1-WT was decreased by >40% compared with that in control cells co-transfected with miR-142-3p NC mimics and RAC1-WT ($t=35.64$; $P=0.001$). However, 293T cells co-transfected with miR-142-3p mimics and the mutant 3'-UTR of RAC1 had no significant decrease in luciferase activity ($t=3.64$; $P=0.68$; Fig. 4B). In order to further verify that RAC1 is a target of miR-142-3p, SW620 cells were transfected with miR-142-3p mimics (KD cells), which increased miR-142-3p expression by 732.1% ($t=11.47$; $P=0.008$; Fig. 4C), while decreasing RAC1 expression by 63.8%, compared with the SW620 cells transfected with miR-142-3p NC mimics (NC cells) ($t=23.99$; $P=0.002$; Fig. 4D). The aforementioned results suggest that RAC1 may be a target of miR-142-3p.

Increased miR-142-3p inhibits the viability, invasion and migration of SW620 cells. In order to analyze the role of miR-142-3p in CRC, miR-142-3p mimics were transfected into SW620 cells and cell viability was assessed by the CCK-8

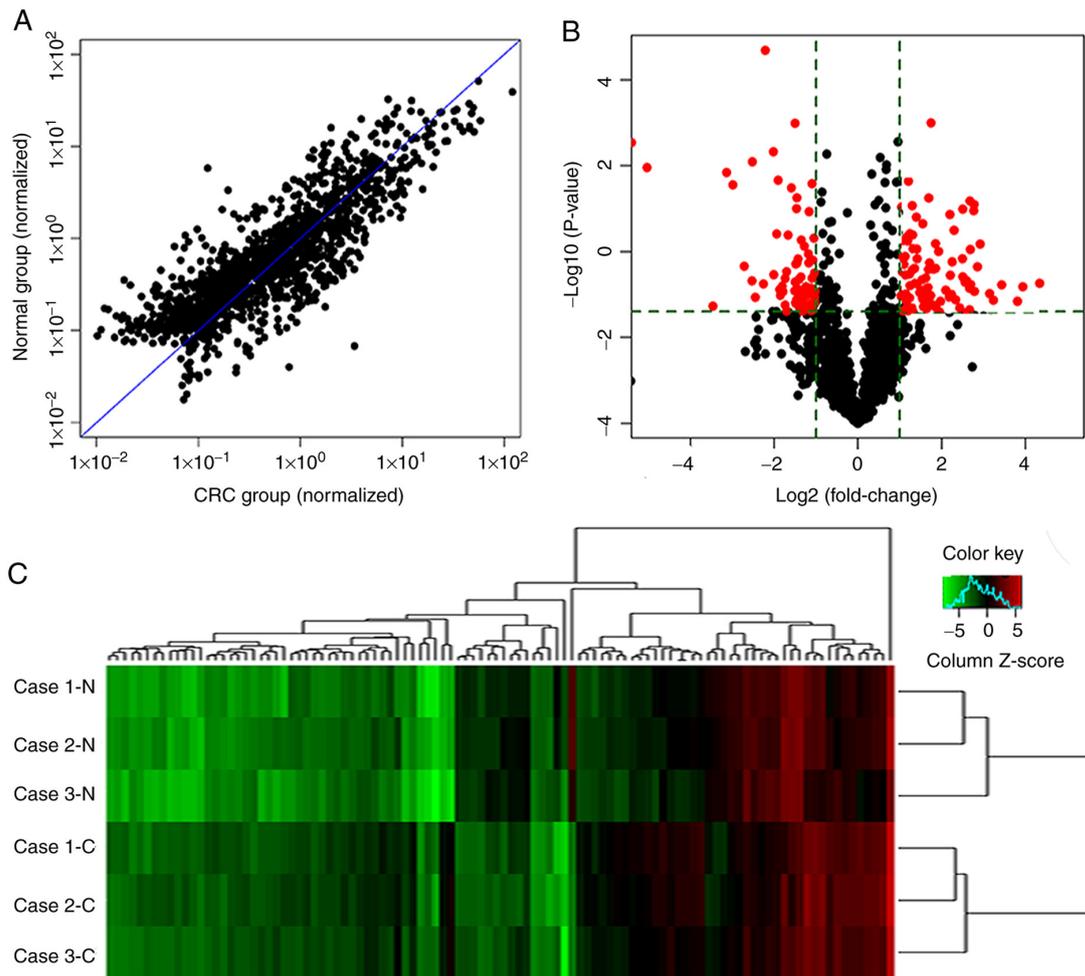


Figure 1. miRNA expression profiles in CRC. (A) A scatter plot was used to assess the variation in miRNA expression between CRC and paired normal tissues. The green lines indicate two-fold changes. (B) Volcano plot assessing P-values and fold-change values of miRNAs. The vertical lines correspond to two-fold upregulation and downregulation, and the horizontal line represents a P-value of 0.05. Red points represent differentially expressed miRNAs with statistical significance. (C) Cluster heat map showing the differentially expressed miRNAs with over two-fold change. Red indicates upregulation, and green indicates downregulation. miRNA, microRNA; CRC, colorectal cancer; N, adjacent non-cancer tissue; C, CRC tissue.

and colony-formation assays. After transfection, the proliferation of SW620 cells was inhibited in a time-dependent manner ($t=12.32$; $P=0.001$), whereas no significant difference was detected between NC cells and control cells ($t=1.325$; $P=0.277$). Cell proliferation decreased by 26.2% 4 days after transfection of miR-142-3p mimics, compared with NC cells ($t=4.94$; $P=0.039$; Fig. 5A). In addition, following miR-142-3p transfection, the colony-forming capacity of SW620 cells was significantly decreased by 37.4% ($t=32.04$; $P=0.001$; Fig. 5B and C). Transwell assays were used to assess the effect of miR-142-3p on invasion and migration in SW620 cells. Compared with NC cells, cell invasion was decreased by 68.7% ($t=10.39$; $P=0.009$) after cells were transfected with miR-142-3p mimics (Fig. 5D and F), and migration was decreased by 58.2% ($t=6.351$; $P=0.028$; Fig. 5E and G). When SW620 cells were treated with the RAC1 inhibitor NSC23766, similar results were found. Compared with the control cells, the NSC cells showed a significant decrease in proliferation, cloning, invasion and migration abilities by 29.8% ($t=8.893$; $P=0.003$), 67.3% ($t=7.336$; $P=0.018$), 62.1% ($t=13.423$; $P=0.006$) and 42.5% ($t=13.510$; $P=0.006$), respectively (Fig. 5). These results suggest that miR-142-3p may

play a key role in blocking the viability, invasion and migration of SW620 cells, which may be associated with aberrant expression of RAC1.

miR-142-3p inhibits EMT by targeting RAC1 in SW620 cells. In order to investigate whether miR-142-3p affects EMT in SW620 cells, western blotting was used to detect the expression of EMT-associated markers and the phosphorylation of ERK1/2. The expression of RAC1, vimentin, MMP9, N-cadherin and phosphorylated (p)-ERK was decreased, but that of E-cadherin was increased in SW620 cells transfected with miR-142-3p mimics, compared with NC cells (Fig. 6). Similar results were observed when cells were treated with the RAC1 inhibitor NSC23766 (Fig. 6). Compared with the control cells, the expression of RAC1 did not change in NSC cells (Fig. 6). The aforementioned results indicate that miR-142-3p may also play an important role in blocking EMT in CRC cells by regulating RAC1/ERK1/2 signaling. Increasing miR-142-3p inhibited EMT of colon cancer cells by decreasing RAC1 expression; this was consistent with the effect of NSC23766 suppressing RAC1-GTP activation.

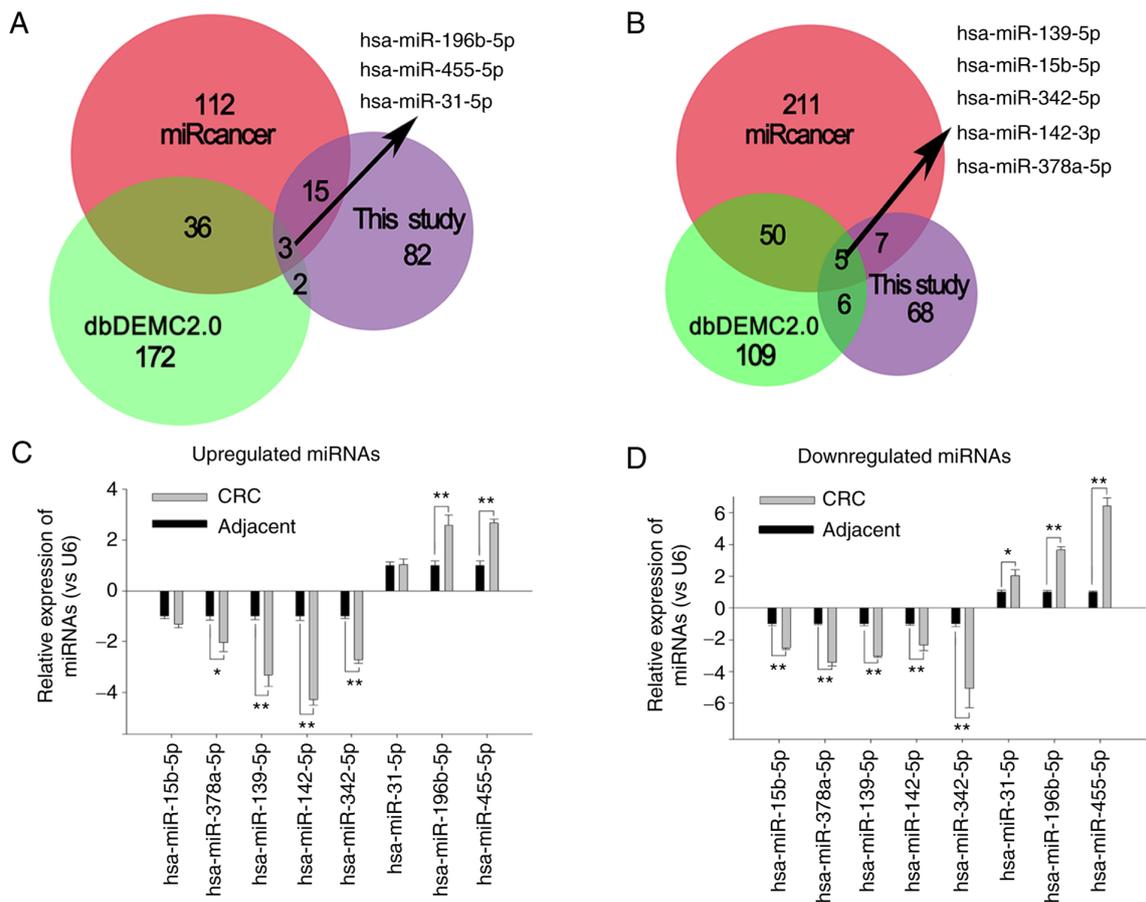


Figure 2. Analysis and validation of miRNAs associated with CRC. Overlap of (A) upregulated and (B) downregulated miRNAs from the present study and the miRcancer and dbDEMC2 databases. (C) The expression analysis of miRNAs in CRC and its adjacent healthy tissues by quantitative PCR. (D) The expression analysis of miRNAs in CRC and its adjacent healthy tissues by microarray analysis. *P<0.05; **P<0.01. dbDEMC2, a database of differentially expressed miRNAs in human cancer (version 2.0); Adjacent, adjacent healthy tissues; miRNA/miR, microRNA; CRC, colorectal cancer.

Discussion

The miRNA microarray is one of the most common methods of screening disease-associated miRNAs, and several studies using this technology have revealed aberrant miRNA expression in human malignant tumors (10,29), including CRC (17). Volinia *et al* (29) used miRNA microarrays to study the expression patterns of miRNAs in 540 tumor tissues and found that miRNAs are differentially expressed in non-cancer and cancer tissues. Further, these miRNAs are strongly tissue-specific. Zhang *et al* (30) found that 35 miRNAs demonstrated aberrant expression in colon cancer tissues using miRNA microarrays. In the present study, the Exiqon miRNA microarray was used to assess differentially expressed miRNAs in paired CRC and non-cancer tissues. Compared with paired non-cancer tissues, 76 miRNAs were upregulated and 102 were downregulated in CRC samples, and qPCR verified the microarray results.

Numerous recent reports have identified a dysregulation of miRNAs in CRC (11,15,16,18). Through analyzing data from miR2Disease (21) and miRcancer (22), it was found that there are >300 differentially expressed miRNAs in CRC. These miRNAs are involved in cell proliferation, invasion, metastasis (31), drug resistance (32) and other biological functions (11,15,16,18). Therefore, these miRNAs can be used as markers for diagnosis, prognosis, recurrence and drug efficacy

in CRC (11,15,16,18). The present study identified 8 shared miRNAs from microarray analysis, using the dbDEMC2 and miRcancer databases; of these miRNAs, miR-142-3p was the most significantly downregulated. Furthermore, GO and KEGG pathway enrichment analysis showed that 56 predicted targets of miR-142-3p were associated with 'cell adhesion', 'cell autophagy', 'small GTPase-mediated signal transduction', 'cell proliferation', 'regulation of transcription' and other functional regulation, and the involved pathways included 'pathways in cancer', 'regulation of actin cytoskeleton', the 'Ras-signaling pathway', 'colorectal cancer' and other associated signaling pathways. Therefore, miR-142-3p may play a key role in the tumorigenesis and development of CRC.

Several studies have confirmed that downregulation of miR-142-3p is important in the development and progression of various tumors (33), such as lung (34), breast (35) and gastric (36) cancer. The inhibition of miR-142-3p can promote the growth, invasion and metastasis of tumor cells, whereas increased expression of miR-142-3p inhibits cell migration and proliferation (33-36). Recent studies revealed that miR-142-3p was downregulated in CRC, which is associated with CRC development and progression, and that miR-142-3p could be used as a marker for the diagnosis of the disease (37-39). It was also found that miR-142-3p was significantly downregulated in 51 CRC tissues compared with that in matched non-cancer

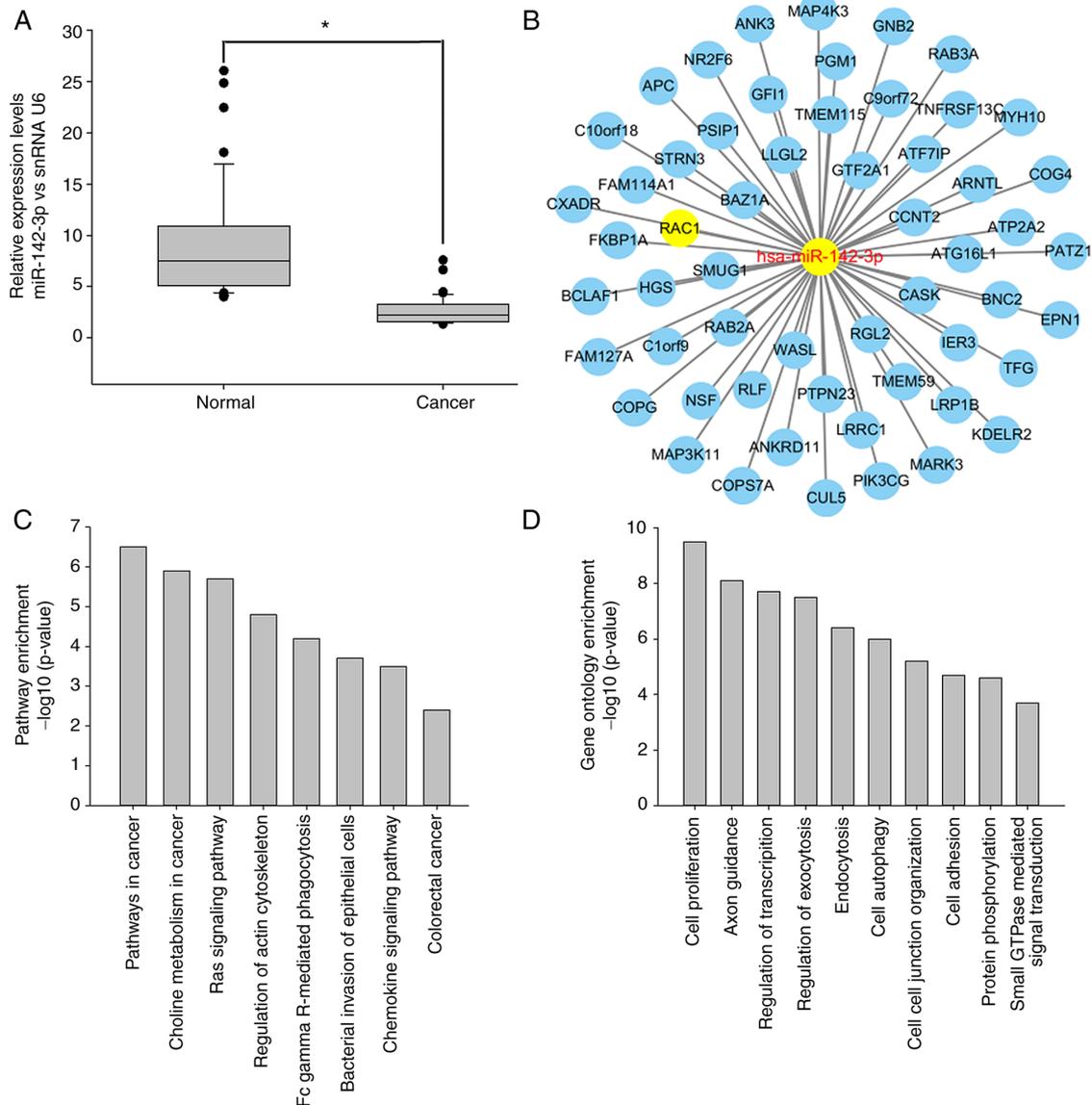


Figure 3. qPCR analysis of miR-142-3p in CRC and bioinformatics analysis of predicted miR-142-3p targets. (A) qPCR analysis of miR-142-3p in 63 CRC cases. (B) The regulatory network of miR-142-3p and its target genes. (C and D) Top 10 functional terms and top 8 signaling pathways associated with miR-142-3p targets according to their P-values in (C) Kyoto Encyclopedia of Genes and Genomes and (D) Gene Ontology analysis. *P<0.05. qPCR, quantitative PCR; miR, microRNA; CRC, colorectal cancer; RAC1, Rac family small GTPase 1.

mucosa tissues, and that miR-142-3p expression was associated with lymph node metastasis and TNM stage. Transfection of miR-142-3p into SW620 colon cancer cells decreased their proliferation and colony-forming capacity, and decreased invasion and migration. The aforementioned data suggest that miR-142-3p may play a key role in regulating the behavior of CRC cells.

Some studies have reported that overexpression of miR-142-3p leads to downregulation of RAC1 in multiple cancer types, and that RAC1 is a target of miR-142-3p (40-42). miR-142-3p downregulates RAC1 by binding to its 3'-UTR (40-43). In the present study, the level of RAC1 was decreased in SW620 cells after transfection with miR-142-3p mimics. Moreover, when the 3'-UTR of wild-type RAC1 was fused to a luciferase construct, there was a significant decrease in luciferase activity after transfection of miR-142-3p mimics; however, this effect dwindled

when the luciferase construct was fused to a mutant RAC1 sequence. The aforementioned results indicated that miR-142-3p can regulate the expression of RAC1 to regulate tumorigenesis.

RAC1 is an important member of the Rho GTPase family, which regulates various cell biological processes, including cell survival, proliferation, adhesion, EMT, metastasis and invasion. The Rho GTPase family is also involved in PAK, nuclear factor- κ B, MAPK, STAT3 and WNT/ β -catenin signaling (44-46). RAC1-GTP is the active conformation of RAC1, and RAC1 hyperactivation is found in several human cancer types (46,47). It has been reported that eliminating RAC1 GTPase activity can disrupt EMT in CRC cells and significantly inhibit the growth, invasion and metastasis of colon cancer cells (48). Meanwhile, one study showed that upregulated miR-142-3p leads to an increase in RAC1 expression (49), which has a major conflict with the function of most

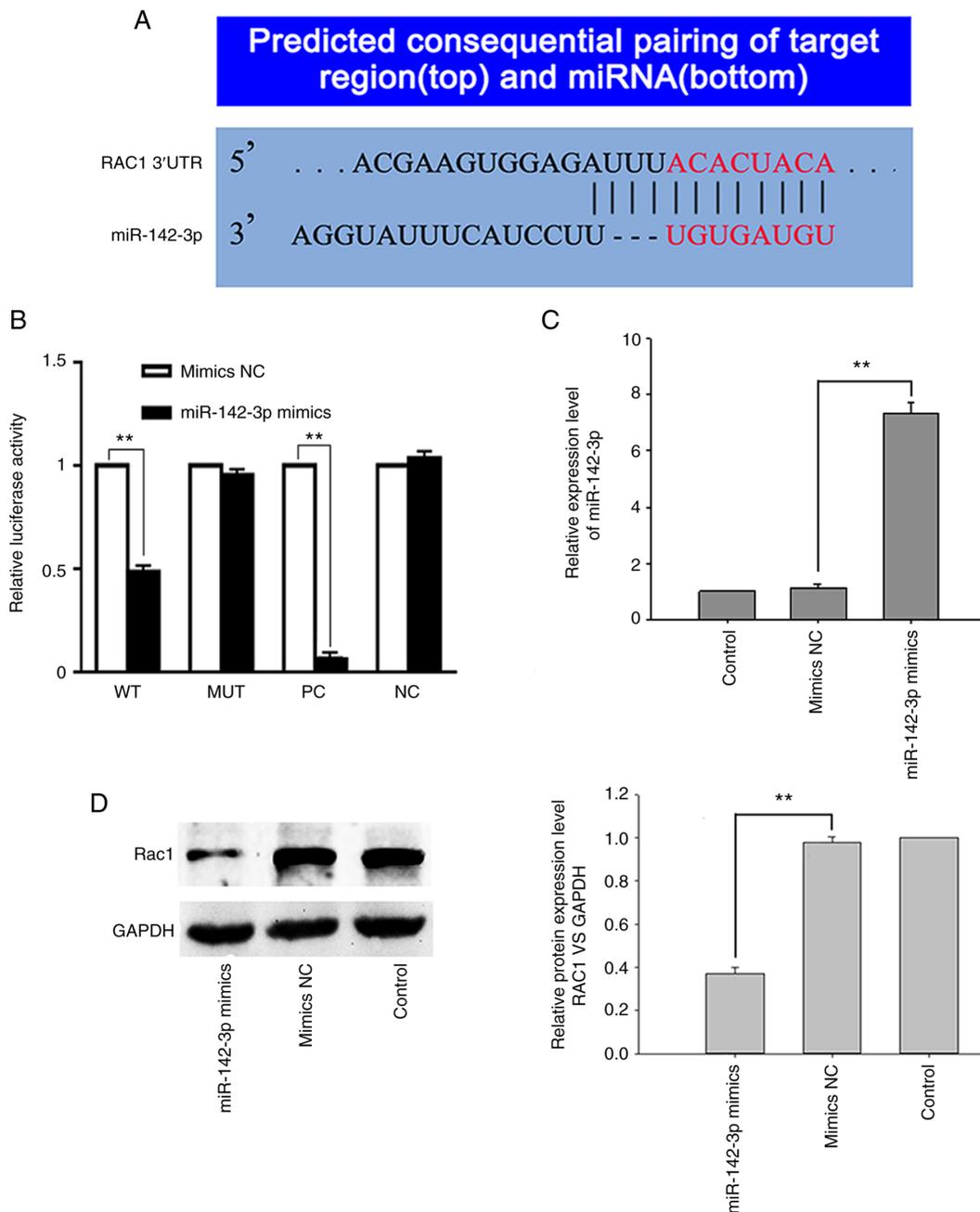


Figure 4. miR-142-3p regulates RAC1 expression. (A) Binding sites of miR-142-3p in the 3'-UTR of *RAC1* (red text). (B) Luciferase activity after co-transfection of miR-142-3p mimics and WT-RAC1 or MUT-RAC1. (C) Expression of miR-142-3p by quantitative PCR after transfection with miR-142-3p. (D) Expression of RAC1 determined by western blotting after transfection with miR-142-3p. * $P < 0.05$; ** $P < 0.01$. miR, microRNA; WT (wild-type SW620 cells transfected with WT-RAC1); MUT, mutant (SW620 cells transfected with MUT-RAC1); PC, positive control cells; NC, negative control cells; RAC1, RAC family small GTPase 1; UTR, untranslated region.

miRNAs. miRNA interacts with the 5'-UTR, ORF or 3'-UTR of its target mRNA to suppress its translation or induce its degradation, but usually does not upregulate its expression; there are only a few cases where miRNAs upregulate protein translation, primarily in mitochondria (6-8,50, 51). In addition, these views are inconsistent with the study results of other authors (40-43), including that of the present study. The present study results showed that RAC1 was significantly downregulated after transfection with miR-142-3p mimics, and EMT, proliferation, invasion and migration were also inhibited. Further studies revealed that the expression of p-ERK decreased after transfection with miR-142-3p mimics

or inhibition of RAC1 activity. Meanwhile the present results are consistent with those of other studies (40-43); if increasing the expression of RAC1 protein in CRC cells can restore the phenotypic changes caused by miR-142-3p upregulation, the result would be more reliable. Further exploration of the effect of changes in RAC1 expression on miR-142-3p function will be helpful to elucidate the molecular mechanism of CRC metastasis.

In conclusion, the expression of miR-142-3p was decreased in CRC; this may be associated with lymph node metastasis and TNM stage. Transfection of colon cancer cells with miR-142-3p mimics inhibited the expression of RAC1 and

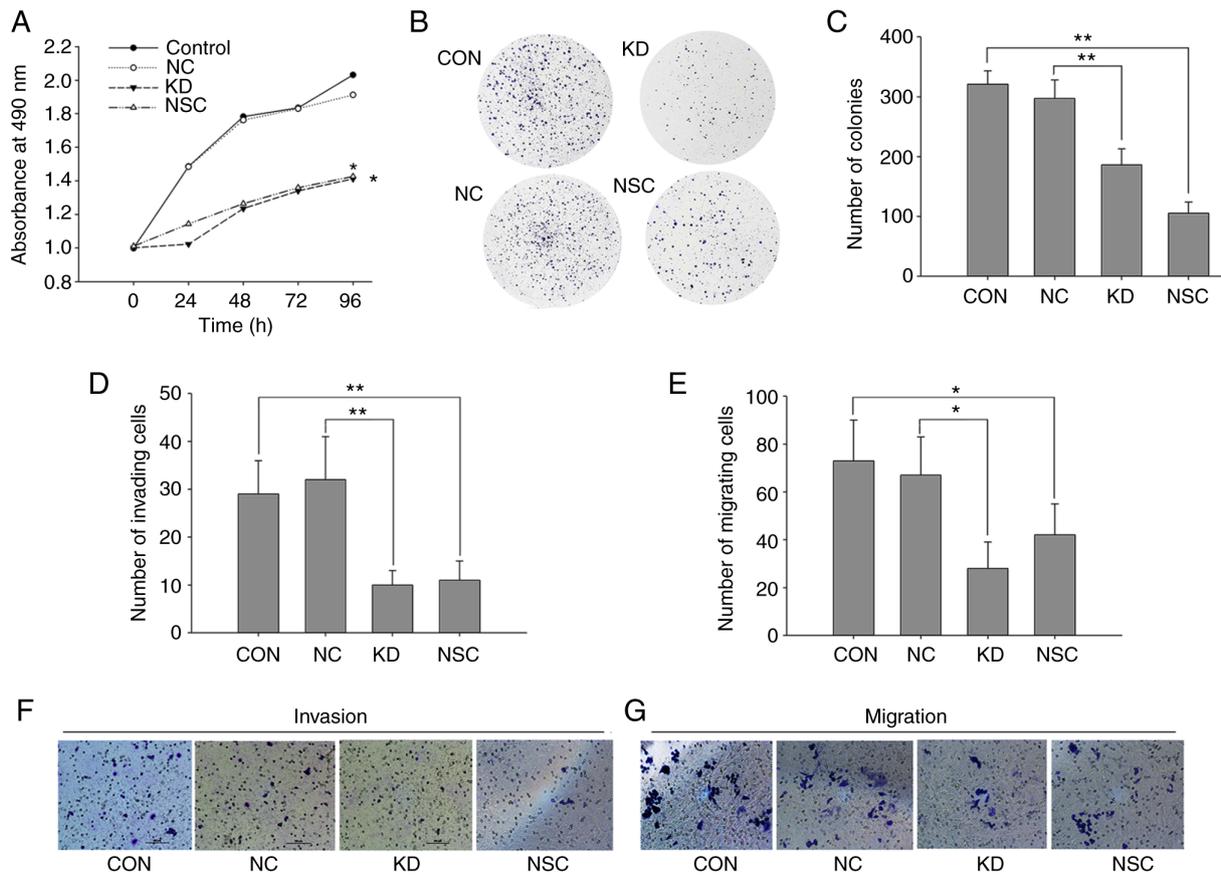


Figure 5. Effect of miR-142-3p on the viability of colon cancer SW620 cells. (A) Cell Counting Kit-8 assays were used to measure cell proliferation in SW620 cells transfected with miR-142-3p mimics or RAC1 inhibitor. (B) Representative images of colony-formation assays of SW620 cells (magnification, $\times 40$). (C) Average number of colonies in SW620 cells transfected with miR-142-3p mimics or RAC1 inhibitor. (D and F) Transwell and (E and G) Matrigel invasion assay of SW620 cells transfected with miR-142-3p mimics or RAC1 inhibitor (magnification, $\times 100$). * $P < 0.05$; ** $P < 0.01$. miR, microRNA; RAC1, RAC family small GTPase 1; KD, miR-142-3p mimic cells; NC, miR-142-3p mimic negative control cells; CON, control cells without any treatment; NSC, RAC1 inhibitor NSC23766.

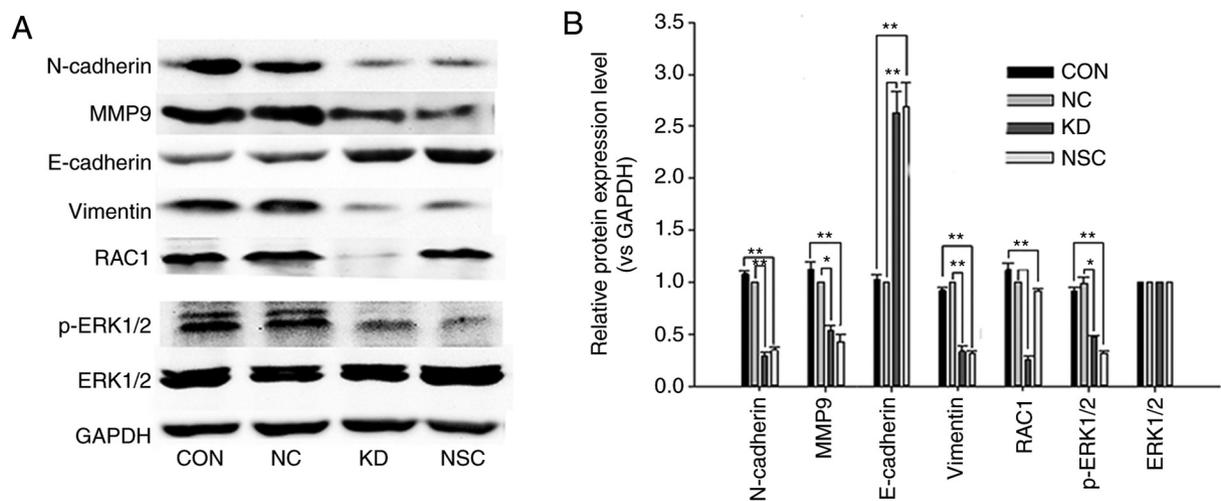


Figure 6. miR-142-3p blocks EMT by regulating RAC1/ERK1/2 signaling in colon cancer SW620 cells. (A) Representative results of western blot analysis of EMT markers and signaling proteins after transfection with miR-142-3p mimics and treatment with RAC1 inhibitor. (B) Relative expression of EMT markers and signaling protein after transfection of miR-142-3p mimics and treatment with RAC1 inhibitor. * $P < 0.05$; ** $P < 0.01$. miR, microRNA; EMT, epithelial-to-mesenchymal transition; RAC1, RAC family small GTPase 1; KD, miR-142-3p mimic cells; NC, miR-142-3p mimic negative control cells; CON, control cells without any treatment; NSC, RAC1 inhibitor NSC23766; MMP, matrix metalloproteinase; p-, phosphorylated-.

decreased the proliferation, invasion and migration of cancer cells; these effects may be associated with the inhibition of

ERK1/2 signaling. The results in the present study suggest that miR-142-3p may be a potential target for the treatment of CRC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article. The obtained microarray data were deposited in the Gene Expression Omnibus database (accession no. GSE101502).

Authors' contributions

NX analyzed and interpreted the data regarding the miRNA microarray and CRC tissues. QM and YZ performed the bioinformatics analysis and qPCR assay, ZL and YL performed the statistical analysis. FX and SL collected the tissue specimens. YH performed the experimental design, cell function experiments, and was a major contributor in writing the manuscript. NX and YH confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the First Affiliated Hospital of Hainan Medical University (approval no. HYFYKJ-2018-32). All the patients provided written consent for their specimens to be used in this study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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