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MicroRNA-375 accelerates the invasion and migration of colorectal cancer through targeting RECK

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Abstract. – OBJECTIVE: This study aims to detect the expression pattern of microRNA-375 in colorectal cancer (CRC), and to examine its specific mechanism in regulating the progression of CRC.

PATIENTS AND METHODS: We detected microRNA-375 expression in 50 pairs of CRC and paracancerous tissues by quantitative real-time polymerase chain reaction (qRT-PCR). The correlation between microRNA-375 expression and pathological indexes of CRC patients was analyzed. Cellular expression of microRNA-... in CRC cell lines was detected as well. Regulatory effect of microRNA-375 on biological behaviors of CRC cells was examined, including proliferative, invasive and migratory abilities. We used bioinformatics method to predict potential target of microRNA-375 and finally explored their interactive function in regulating CRC progression.

RESULTS: MicroRNA-375 expression remained higher in CRC tissues than to paracancerous ones. CRC patients with high level of microRNA-375 tended to have higher rates of lymph node metastasis and distant metastasis compared with those with low level. Transfection of microRNA-375 inhibitor greatly reduced proliferative, invasive and migratory abilities of CRC cells. RECK was predicted to be the target of microRNA-375, which was downregulated in CRC tissues and cells. Besides, RECK expression was negatively regulated by microRNA-375 in CRC. Rescue experiments confirmed that microRNA-375 inhibitor promoted the malignant progression of CRC.

CONCLUSIONS: MicroRNA-375 is upregulated in CRC, correlated to lymph node metastasis and distant metastasis. MicroRNA-375 enhances invasive and migratory abilities of CRC cells via regulating RECK.

Key Words:
MicroRNA-375, RECK, Colorectal cancer, Metastasis.

Introduction

Colorectal cancer (CRC) is a common tumor globally, and its incidence in male and female malignancies accounts for 9.4% and 10.1%, respectively. About 2 million people per year are diagnosed as CRC. More seriously, more than 600,000 patients directly or indirectly die from CRC every year[1,2]. In the developed countries, the incidence of CRC ranks second among tumors, and mortality is as high as 33%[1,3]. With the continuous improvement of economic level and living standards in China, dietary habit changes increase the incidence of CRC at a rate of 4% annually. There are about 130,000 new cases of CRC each year in China, ranks fifth in malignancies. In particular, southeastern coastal zone is a high-incidence area of CRC, where the incidence is about 48/1,000,000. Similar to the developed countries, CRC in China is characterized by younger onset, family aggregation and genetic susceptibility[4,5]. At present, surgery is the major therapeutic approaches for CRC. However, advanced CRC is unable to be operated. Postoperative recurrence or poor response of chemotherapy severely restricts the therapeutic outcome of CRC patients[6-9]. In-depth explorations on CRC pathogenesis contributes to develop effective strategies to improve prognosis of CRC patients.

MicroRNAs (miRNAs) are a class of endogenous, small RNAs of about 16-24 nucleotides in length, which have been well concerned currently. MiRNAs are conserved in both position and sequence[10-12]. They inhibit target gene expressions by binding to 3'UTR of target mRNAs to degrade or silence them, thus participating in cellular behaviors[13,14]. Currently, miRNAs are considered to be important in tumorigenesis and tumor devel-

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opment. A complex network involving multiple miRNAs and their downstream could mediate the malignant progression of tumors[15, 16]. Genome instability is an important feature of tumor cells. MiRNAs are differentially expressed in tumor tissues and corresponding normal tissues with the same differentiation source. About 50% of miRNAs are distributed in chromatin break sites and fragile sites. These sites are in the genome instability region, showing different functions in different types of tumors[17-19]. MiRNA serves as an oncogene or a tumor suppressor in tumor progression[20, 21]. This work firstly analyzed the published microarray data of CRC, and found that microRNA-375 was highly expressed in highly metastatic CRC cell lines.

So far, previous researches on microRNA-375 function in CRC are rarely reported. Moreover, whether microRNA-375 could regulate RECK, thus participating in the occurrence and progression of CRC remains unclear. This study aims to reveal the specific function of microRNA-375/RECK in regulating malignant behaviors of CRC cells.

Patients and methods

Patients and CRC samples

Tumor tissues and paracancerous tissues w harvested from 50 CRC patients 45-76 yea undergoing radical resection patients received any radiotherapy chemot apy before surgery. Pathologi ping criteria for CRC wer in proposed by the U for I onal Cancer Control (UICC). nts and t milies in this study have ly informed s study was approve Eth mmittee of The Second Hospit of Jilin Un y.

Cell and reagents

cell li (HT29, HCT-8, HCT-116) and colo a cell (FHC) were provided by Am Type ure Collection (ATCC) assas). Cells were cultured in Du co's M Eagle's Medium (DMEM) (ex for HC 116 cells were cultured in RP- M ell Park Memorial Institute-1640)) taining % fetal bovine serum (FBS) (Gibco, kville, MD, USA), 100 U/mL penicillin-strep- in and maintained in a 5% CO_2 incubator at 37 Cell passage was conducted using trypsin containing ethylene diamine tetraacetic acid (EDTA) until 80-90% of confluence.

Transfection

CRC cells in logarithmical period were seeded into 6-well plates. Until 50-70% of confluence, transfection of microRNA-375 inhibi RECK was conducted using Lipofec ne 20 (Invitrogen, Carlsbad, CA, USA) h medium was replaced 6 hours later. Trans cells were harvested at 48 h.

Cell proliferation ass

Cells were seeded ne 96 plate w 2.0×10^3 cells per well ility determined at the appointed tim oin ell co g kit-8 (CCK-8) kit (ido La ries amoto, Japan). Abso e (OD) at 4 s recorded for plottin feration cur

Wound healing a

ted cells for were digested and a ted to 5.0×10^5/mL. Until 90% of confluence, rtificial wo was created in the confluent monolayer g a 200 μL pipette tip. The i were tak t 0 and 24 h using an inverted mic ctively. Percentage of wound closure alculated from three independent ecords.

ell cell migration and invasion assay

Transfected cells for 48 h were digested and adjusted to 2.0×10^5/mL. 200 μL/well suspension was applied in the upper side of Matrigel-coated Transwell chamber (Millipore, Billerica, MA, USA). In the bottom side, 500 μL of medium containing 10% FBS was applied. After 48 h incubation, invasive cells were fixed in 4% paraformaldehyde, dyed with crystal violet and counted using a microscope. Penetrating cells were counted in 5 randomly selected fields per sample. Transwell migration assay was conducted in the same procedures except for Matrigel pre-coating.

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

Total RNA was extracted from tissues or cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and quantified using UV spectrophotometer. Extracted RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan) and amplified by real-time quantitative PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Relative levels were quantitatively analyzed using the $2^{-\Delta\Delta Ct}$ method. U6

and β-actin were used as internal references. Primer sequences were as follows: MicroRNA-375, forward: 5'-ATCGGATCCAGGGTGGCTGGGAAAGGA-3', reverse: 5'-ATCGAATTCCCCGTATTACGACGCAGAATG-3'; U6, forward: 5'-CGCAAGGATGACACGCAAATTC-3', reverse: 5'-TATATCACTCTTGCTTCA-3'; RECK, forward: 5'-TGGAGTCACTGTACACCCTC-3', reverse: 5'-CGGACATCCGCTAAACAGGT-3'; β-actin, forward: 5›-CCTGGCACCCAGCACAAT-3›, reverse: 5›-GCTGATCCACATCTGCTGGAA-3›.

Western blot

Total protein from cells was extracted using radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China), quantified by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA) and loaded for electrophoresis. After transferring on a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), it was blocked in 5% skim milk for 2 hours, incubated with primary antibodies at 4 °C overnight and secondary antibodies for 2 h. Bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image S[illegible] (NIH, Bethesda, MD, USA).

Statistical analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Measurement data were analyzed the *t*-test and [illegible] data were analyzed by χ^2 test or Fisher's exact test. Kaplan-Meier was introduced for survival analysis. $p<0.05$ was considered statistically significant.

Results

MicroRNA-375 was highly expressed in CRC tissues and cell lines

Compared with paracancerous tissues, microRNA-375 expression remained higher in CRC tissues, displaying a significant difference (Figure 1A). In addition, microRNA-375 was also highly expressed in CRC cell lines relative to colorectal mucosa cell line FHC, especially in HCT-8 and HCT-116 cells (Figure 1B). In the subsequent experiments, HCT-8 and HCT-116 cell lines were selected as *in vitro* objects.

MicroRNA-375 expression was correlated with lymph node and distance metastasis of CRC

According to microRNA-375 expression in collected tumor tissues, CRC patients were divided into high-level and low-level group. Correlation analysis showed that high expression of microRNA-375 was positively correlated to lymph node metastasis and distant metastasis, while it was not correlated to age, gender and clinical grade of CRC (Table I).

Knockdown of microRNA-375 inhibited cell proliferation, migration and invasion

To explore the regulatory effect of microRNA-375 on CRC cells, we first constructed microRNA-375 inhibitor and verified its transfection efficacy by qRT-PCR (Figure 2A). Subsequently, proliferative, invasive and migratory abilities of HCT-8 and HCT-116 cells were evaluated. CCK-

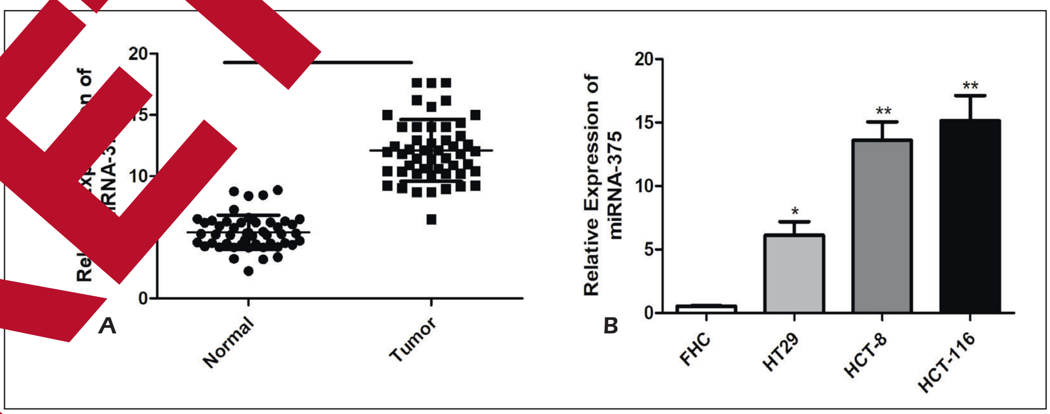


Figure 1. MicroRNA-375 was highly expressed in CRC tissues and cell lines. **A**, MicroRNA-375 expression remained higher in CRC tissues compared with paracancerous tissues detected by qRT-PCR. **B**, MicroRNA-375 was highly expressed in CRC cell lines relative to colorectal mucosa cell line FHC.

Table I. Correlation of miRNA-375 expression with pathological characteristics of colorectal cancer.

Parameters	No. of cases	miRNA-375 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.923
<60	18	11	7	
≥60	32	20	12	
Gender				0.608
Male	24	14	10	
Female	26	17	9	
T stage				
T1-T2	30	18	12	
T3-T4	20	13	7	
Lymph node metastasis				
No	32	24	8	
Yes	18	7	11	
Distance metastasis				0.002
No	40	29	11	
Yes	10	2	8	

8 assay indicated the inhibited viability in CRC cells transfected with microRNA-375 inhibitor (Figure 2B). Compared with those transfected with negative control, CRC cells with microRNA-375 knockdown revealed a lower p[illegible]t of wound closure (Figure 2C). Transwe[illegible] revealed that transfection of microRNA-3[illegible]n-hibitor suppressed migratory and invasive [illegible] in HCT-8 and HCT-116 cells relative to cont[illegible] (Figure 2D).

RECK was lowly expre[illegible]d in CRC tissues and c[illegible]nes

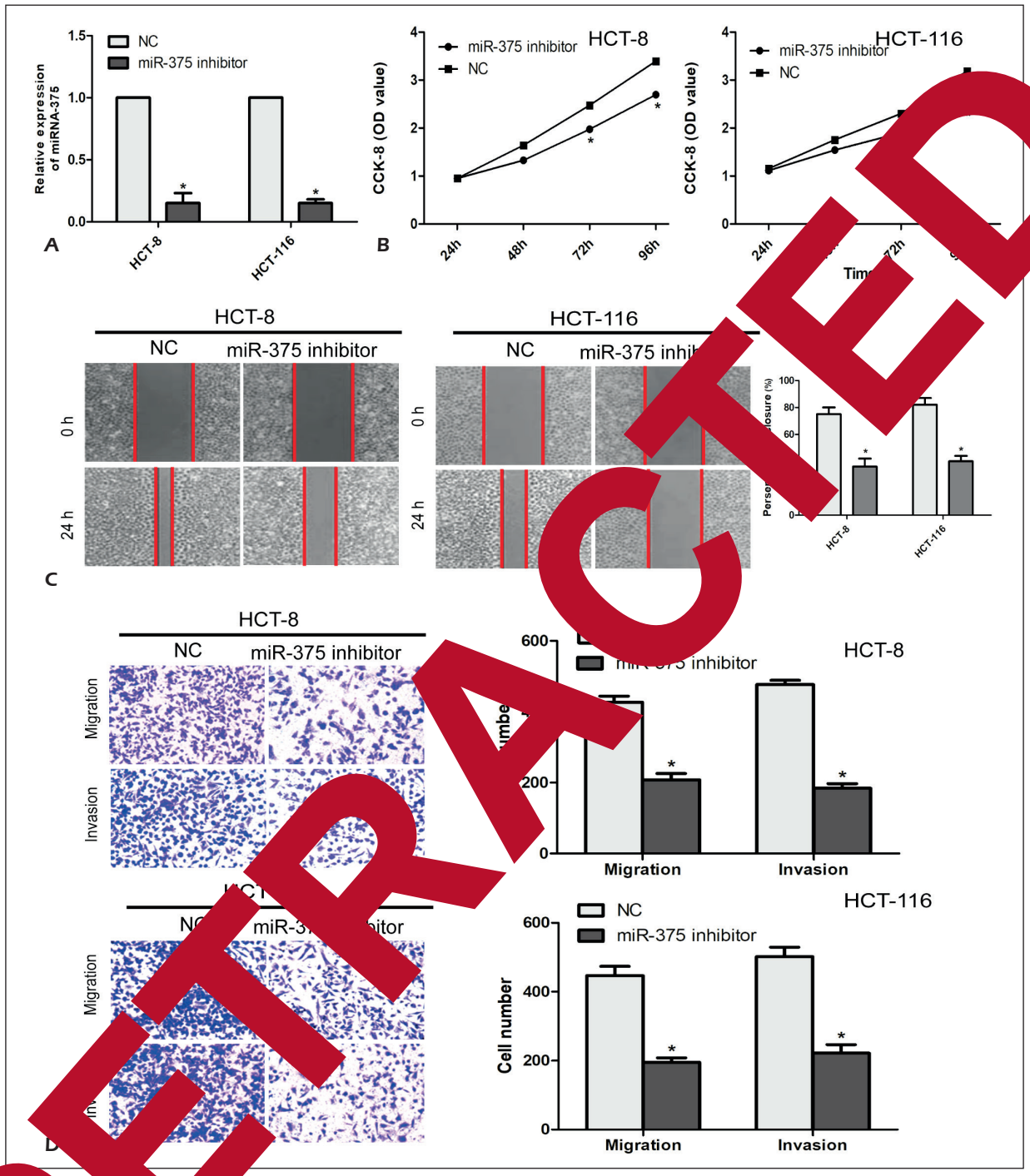
Subsequently, REC[illegible] w[illegible]d to [illegible] target gene of micr[illegible]A-37[illegible]gh bioinformatics (data not s[illegible]n). In this [illegible] we found a lower level [illegible] in CRC tis[illegible] relative to paracancer[illegible] tissu[illegible]igure 3A). Identically, RECK wa[illegible]wly expres[illegible] CRC cells (Figure 3B). By [illegible]ting mRNA lev[illegible] microRNA-375 and R[illegible]K in CR[illegible] tissues, a negative correlation bet[illegible] these[illegible] genes was observed (Figure 3C). [illegible] P[illegible]data sh[illegible]ed that transfection of microR[illegible] inhi[illegible] markedly upregulated [illegible]K exp[illegible]n[illegible]ther confirming their negati[illegible]orrelat[illegible]gure 3D).

M[illegible]75 regulated cellular [illegible]haviors of CRC cells via targeting [illegible]CK

[illegible] explore the mechanism of microRNA-375 in [illegible]ulating malignant progression CRC, cells were transfected with si-NC, si-RECK, si-NC+ microRNA-375 inhibitor or si-RECK+ microR-[illegible]375 inhibito[illegible]spectively. Both mRNA and [illegible]ein levels of [illegible]CK were downregulated by t[illegible]ction of s[illegible]ECK. Knockdown of microRNA[illegible]ed RECK expression, which was fu[illegible]eversed by co-transfection of si-RECK and microRNA-375 inhibitor (Figure 4A, [illegible]nswell assay indicated that RECK knockdow[illegible]nhanced migratory and invasive rates of CRC cells. More importantly, the inhibited migratory and invasive abilities due to knockdown of microRNA-375 were partially reversed by RECK silence (Figure 4C, 4D).

Discussion

With the living standard improvement and dietary structure change, the incidence of CRC in China has been on the rise for the past 30 years. CRC currently ranks the third in the incidence of malignancies in China, which is elevated to the second in some developed cities[1-3]. Female morbidity of CRC is rising faster than males; disease onset is becoming younger; and the incidence of right sided colon carcinoma is elevating relative to other locations[3,4]. Pathogenesis of CRC involves dietary habits, psychological factors, family inheritance and infection, among which genetic factors and dietary habits are particularly prominent[5,6]. In addition, many CRC patients lose the surgical opportunity due to local infiltration or distant metastasis. Postoperative recurrent rate remains high in advanced CRC patients. Therefore, searching for



2. Ki microRNA-375 inhibited cell proliferation, migration and invasion. **A**, Transfection efficacy of microR 375 inhi in HCT-8 and HCT-116 cells detected by qRT-PCR. **B**, CCK-8 assay indicated the inhibited viability in HC nd HCT-116 cells transfected with microRNA-375 inhibitor. **C**, Wound healing assay indicated the decreased wound cl and HCT-116 cells transfected with microRNA-375 inhibitor. **D**, Transwell assay indicated the inhibited ation and vasion in HCT-8 and HCT-116 cells transfected with microRNA-375 inhibitor.

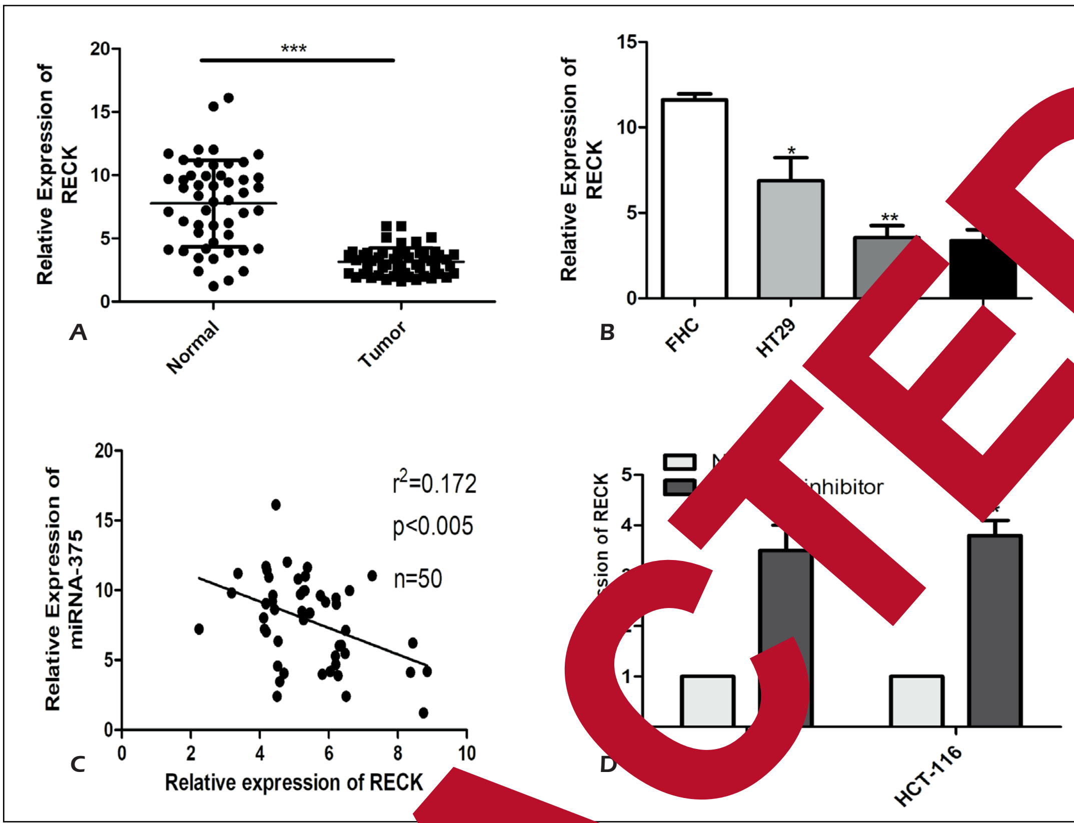
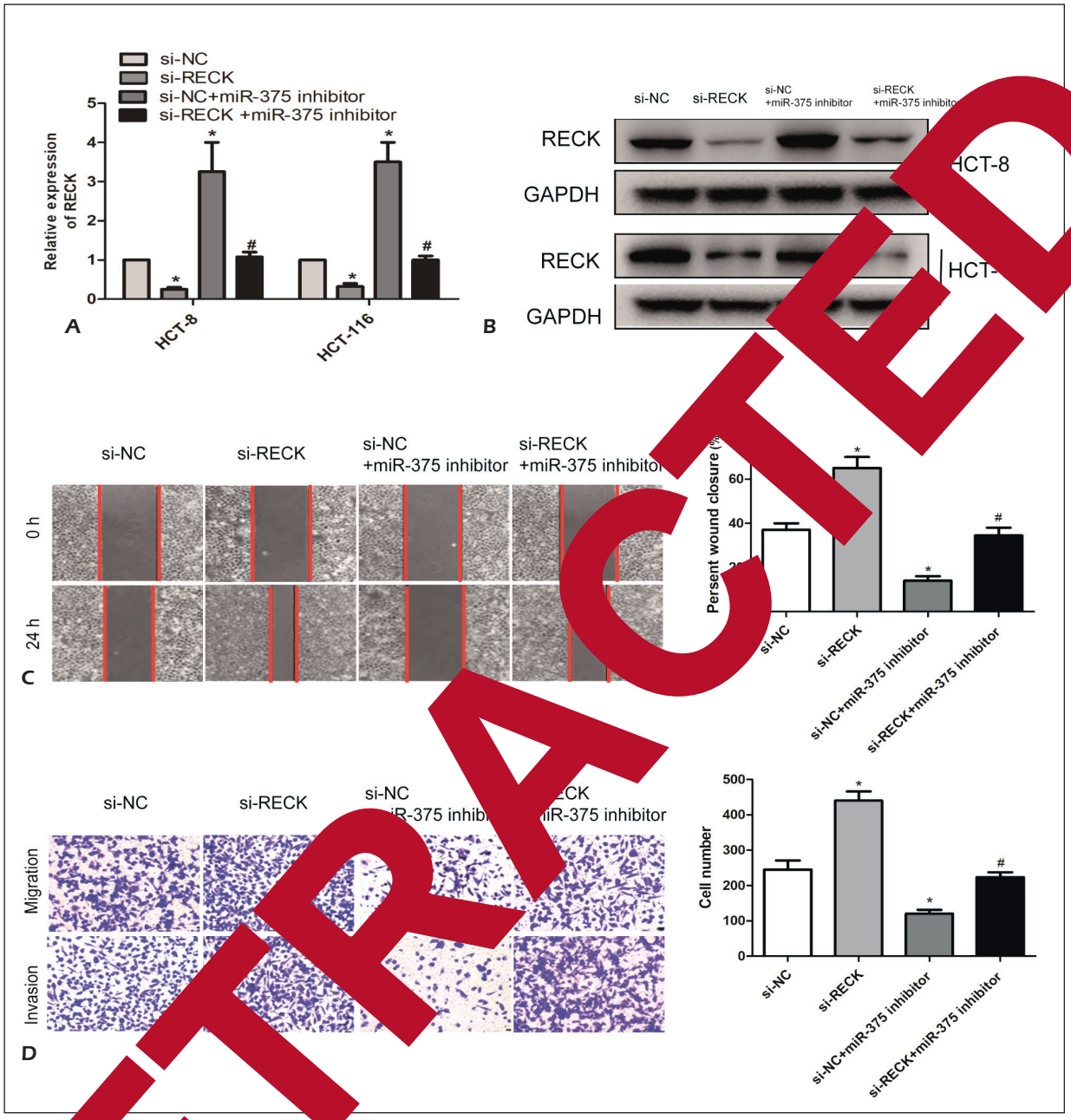


Figure 3. RECK was lowly expressed in CRC tiss[illegible]nd cel[illegible]ECK expression remained lower in CRC tissues compared with paracancerous tissues detected by qR[illegible]. **B**, [illegible]CK [illegible] lowly expressed in CRC cell lines relative to colorectal mucosa cell line. **C**, A negative correlation betw[illegible]K and miR-375 in CRC (r^2=0.172, p<0.005, n=50). **D**, QRT-PCR data showed that transfection of mi[illegible] inhibitor u[illegible]lated RECK expression.

specific tumor hallmark [illegible] great s[illegible]ce[7]. Advances made in mo[illegible]cul[illegible]lo[illegible]nd g[illegible]ic research of tum[illegible]ontri[illegible] develop effective interventi[illegible]or tumor d[illegible]ment and metastasis. Tar[illegible]y could lar[illegible]mprove the survival a[illegible]fe q[illegible]of CRC patients rather than tr[illegible]tional treat[illegible] such as surgery, radiothe[illegible] and chemother[illegible].

Mi[illegible]s are [illegible]on-coding, s[illegible]all RNAs compos[illegible] 18-25 [illegible]leotides. As a single-stranded RN[illegible] [illegible]inds to [illegible] 3' untranslated region (UTR) [illegible]t gene[illegible]s downregulating protein [illegible]ssion[illegible]he[illegible]plasm and inhibiting mRNA [illegible]nslatio[illegible] miRNAs induce the degradatio[illegible] mRNA, t[illegible]ereby reducing the possibility of m[illegible]tion into proteins. Incomplemen[illegible] pairing between miRNA and target mRNA [illegible]tially blocks the process of protein translation [illegible]osome using RNA[11-14]. One miRNA has hundre[illegible] of target genes controlling a large number of protein-encoding genomes. Several miRNAs have been identified in regulating the metastasis of CRC[15, 17]. This study focused on the regulatory effects of microRNA-375 on CRC cells. First of all, microRNA-375 was highly expressed in CRC tissues relative to paracancerous tissues. Besides, microRNA-375 expression was positively correlated to lymph node metastasis and distant metastasis of CRC. We thereafter speculated that microRNA-375 may be an oncogene in CRC. Identically, microRNA-375 was highly expressed in CRC cell lines, especially in HCT-8 and HCT-116 cells.

Since microRNA-375 was differentially expressed in CRC tissues and normal colon tissues, its complex regulatory network was the focus to be further elucidated. MiRNAs exert crucial functions in cell differentiation, proliferation, apoptosis, etc. Specifically expressed miRNAs may be utilized as prognostic hallmarks. In this paper, we observed that microRNA-375 could accelerate CRC cells to migrate and invade. However, its molecular mechanism in regulating cellular behaviors of CRC cells is unclear. Through bioinformatics method,



Figu Micro 375 regulated cellular behaviors of CRC cells via targeting RECK. HCT-8 and HCT-116 cells were tra d with , si-RECK, si-NC+miR-375 inhibitor or si-RECK+miR-375 inhibitor, respectively. **A**, The mRNA levels of RE cells. protein levels of RECK in treated cells. **C**, Wound healing assay showed percent of wound closure i roup. **D** swell assay showed migration and invasion in each group.

R sidered to be the target gene of roRNA-375.

RECK is a tumor-suppressor gene and an tor of matrix metalloproteinase, which inhib the release of MMP-2, MMP-9 and MTl-MMP. Positive expression of RECK is relatively low in several malignancies, while MMP-2 and MMP-9 are highly expressed. RECK weakens the role of MMPs in degrading basement membrane and promoting neovascularization of tumors, thus inhibiting the invasion and metastasis of malignant tumors. Meanwhile, it inhibits type IV collagenase at post-transcriptional level[22,23]. It is reported that downregulated RECK in breast

cancer is closely related to disease prognosis. RECK expression is negatively correlate to the malignant level of tumors. It is believed that RECK is not only a tumor suppressor, but also a prognostic marker of malignancies.

To verify whether microRNA-375 exerted its function in CRC by targeting RECK, a series of rescue experiments were conducted. Transfection of microRNA-375 inhibitor markedly upregulated the mRNA level of RECK in CRC cells. Subsequently, it is observed that the regulatory effects of microRNA-375 on wound closure, migratory and invasive abilities were partially reversed by RECK. We therefore validated the function of microRNA-375/RECK axis in regulating CRC progression.

Conclusions

In summary, MicroRNA-375 is upregulated in CRC, and correlated to lymph node metastasis and distant metastasis. MicroRNA-375 enhances invasive and migratory abilities of CRC cells *via* regulating RECK.

Conflict of Interests

The authors declared no conflict of interest.

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