MicroRNA-325-3p contributes to colorectal carcinoma by targeting cytokeratin 18

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Abstract. Colorectal carcinoma (CRC) is one of the most common malignant tumors. The present study aimed to investigate a non-invasive molecular marker that can evaluate the diagnosis and potential molecular mechanism of CRC. Microarray assays and reverse transcription-quantitative PCR analysis demonstrated that microRNA (miR)-325-3p expression was significantly increased in both tissues and serum samples of patients with CRC. In addition, miR-325-3p expression in the tissues and serum was significantly associated with differentiation, TNM stage and lymph node metastasis. The results of the dual-luciferase reporter assay and western blot analysis revealed that cytokeratin 18 (CK18) is a target gene of miR-325-3p. Furthermore, treatment with transforming growth factor (TGF)-β increased miR-325-3p expression in a time-dependent manner. Conversely, TGF-β decreased CK18 expression at 48 and 72 h. Western blot analysis demonstrated that TGF-β1 significantly decreased the expression of the epithelial marker, CK18, and increased the expression of the mesenchymal markers, α-SMA and vimentin. Notably, these effects were reversed following inhibition of miR-325-3p expression. Taken together, the results of the present study suggest that miR-325-3p is a key regulator of TGF-β-induced CK18 downregulation. Thus, elevated levels of miR-325-3p is an important factor affecting epithelial-to-mesenchymal transition, and is likely to be a molecular marker in the progression of CRC and act as a potential therapeutic target.

Introduction

Colorectal carcinoma (CRC) is one of the most common malignant tumors, with the incidence of CRC being 15-25/100,000 population in China in 2011 (1,2). Given that there are no specific symptoms in the early stage of CRC, patients are diagnosed when the tumor is in the advanced stage (3). Despite surgical treatment, the 5-year overall survival (OS) rate is still <50% (3). Thus, one of the most effective ways to improve the survival rate is to identify molecular markers that can reflect the invasion, metastasis and prognosis of CRC, and provide targeted adjuvant treatment to patients (4,5).

Recently, it has been demonstrated that small non-coding microRNAs (miRNAs/miRs) play an important role in tumor-related gene regulation (6,7). miRNAs are highly conserved, single-stranded non-coding small RNA molecules (8,9). As endogenous regulators, they regulate the expression of target genes by binding to the 3'-untranslated region (UTR) of target mRNAs at the post-transcriptional level (10). Increasing evidence suggests that abnormal expression of miRNAs is associated with the occurrence, invasion and metastasis of human malignant tumors, indicating that miRNAs are involved in the regulation of tumorigenesis, invasion and metastasis (11-13). For example, miR-92b-3p contributes to CRC invasion by inhibiting FBXW7 (9). In addition, increased levels of miR-15a-5p predict poor disease-free survival and OS of patients with CRC (14).

The present study screened the miRNAs that are dysregulated in the tissues and serum samples of patients with CRC via microarray assay. The present study aimed to investigate miRNAs in CRC and target genes that are associated with CRC.

Materials and methods

Patient samples. A total of 50 patients with CRC (male/female ratio, 24/26; mean age ± SD, 53.6±15.8 years; age range, 34-73 years) and 21 healthy individuals (male/female ratio, 11/10; mean age ± SD, 52.8±17.4 years; age range, 36-72 years) were recruited in the present study between January 2019 and December 2019. Due to financial constraints, the present study could not recruit equal numbers of healthy controls and patients with CRC. Among the patients with CRC, 46 patients received radical surgery, while 4 patients did not receive surgery. CRC tissues and adjacent non-cancer tissues (≥5 cm) were collected. The patient characteristics are present in Table I. All patients had complete clinical and pathological data, and the inclusion criteria were as follows: i) CRC was diagnosed via histopathology at the initial diagnosis and
study were as follows: miR-325-3p-RT, 5'-GTC GTA TCC AGT
internal reference gene U6. The primers used in the present
calculated using the 2^△△Cq method (15) and normalized to the
optical density (OD) 260/OD 280.

Microarray assay. To compare the miRNA transcriptome
between CRC tissues/serum samples and the respective
controls, seven different samples were taken as one mixture. For each group, three different mixtures were included in each
group for miRNA profiling using an Agilent miRNA array
(Agilent Technologies, Inc.). Each miRNA was detected using
probes (Agilent Technologies, Inc.) and repeated 30 times.
The array also contained 2,164 Agilent control probes. The
miRNA samples were Cy3 labeled using the Agilent miRNA Complete Labeling and Hyb kit (cat. no. 5190-0456; Agilent
Technologies, Inc.), according to the manufacturer's instruc-
tions. The differentially expressed miRNAs were obtained
using a combine threshold of fold change >1.5 and t-test P<0.05
for transcriptome comparisons.

Reverse transcription-quantitative (RT-q)PCR. Total RNA
was isolated from the serum and tissue samples using RNAVzol
(LS or RNAVzol (Vigorous Biotechnology Beijing Co., Ltd.), according to the manufacturer's protocol. The concentration and purity of the RNA samples were determined
by measuring the optical density (OD) 260/OD 280.

Cell culture. The human CRC cell line, HT-29, was purchased
from the American Type Culture Collection and authenticated
via STR profiling. The cells were maintained in Ham's F-12
medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; HyClone; Cytiva), 100 U/ml penicillin (Invitrogen; Thermo Fisher
Scientific, Inc.) and 0.1 mg/ml streptomycin (HyClone; Cytiva),
at 37°C with 5% CO2.

The human CRC cell lines, SW480 and HCT116, the
normal colon cell line, FHC, and 293T cells were purchased
from the Chinese Academy of Sciences Cell Bank of Type
Culture Collection. All cells were maintained in RPMI-1640
medium (HyClone; Cytiva) supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, at 37°C with
5% CO2.

Transient transfection. miR-325-3p mimic (5'-AATACCTCCT
CCAGGAGTTTTT-3') or inhibitor (5'-AAATAACTCTCG
GAGGTAGTTT-3') and the respective negative controls (NCs; NC
mimic, 5'-TTTCTCGAACGTGTCAGT-3'; NC inhibitor,
5'-TTTCTCGAACGTTGTCACGT-3')) (all from Shanghai
GenePharma Co., Ltd.) are chemically modified analogs, which
can be transfected into cells without using a vector. Transfection
was performed using HiPerFect Transfection Reagent (Qiagen
GmbH), as previously described (16). Briefly, HT-29 cells
were seeded in a 6-well plate at a density of 10^5 cells/well.
Subsequently, the cells were transfected with miR-325-3p
mimic, inhibitor or NC for 48 h using HiPerFect Transfection
Reagent according to the manufacturer's instructions. A total of
12 µl HiPerFect Transfection Reagent was mixed with
100 µl cell culture in serum-free DMEM (Invitrogen; Thermo
Fisher Scientific, Inc.). Meanwhile, 10 µl miR-325-3p mimic,
inhibitor or NC was mixed with serum-free DMEM. Then, the
two mixtures were mixed and incubated at room temperature
for 15 min. After that, the mixture was added in the 6-well plate at a final concentration of 20 nM. Following transfection
for 48 h, the cells were collected for subsequent experiments.

Cell viability analysis. To examine cell viability, HT-29 cells
were seeded in 96-well plates at a density of 1.0x10^4 cells/well.
miR-325-3p mimics, inhibitors or NC were transfected into
cells and the viability of transfected cells was measured at 24,
48 and 72 h after seeding of cells. MTT assay was performed
as previously described (17).

Cell migration and invasion. Cell migration assays were
performed using Boyden chambers (8-µm pore filter; Corning
Inc.). For the cell invasion assay, the filter surfaces were
precoated with Matrigel (BD Biosciences) at 37°C for 2 h.
Briefly, transfected HT-29 cells were seeded at a density of
10^5 cells/well in the upper chamber for 24 h in RPMI-1640
medium without FBS. RPMI-1640 medium (600 µl) with 20% FBS was plated in the lower chamber. After 48 h of incubation
at 37˚C, non-migratory and non-invading cells were removed with cotton swabs. The migratory or invasive cells located on the lower side of the chamber were fixed in methanol for 30 min at 37˚C and stained with 0.5% crystal violet for 1 h at 37˚C. Stained cells were counted in 5 random fields using fluorescence microscopy (magnification, x40). All experiments were performed in triplicate.

**Dual-luciferase reporter assay.** Based on the TargetScan database (http://www.targetscan.org/mamm_31/), a conserved binding site was identified in the 3'-UTR of cytokeratin 18 (CK18). The 3'-UTR of CK18 containing the predicted binding site was cloned into the pmirGLO luciferase reporter vector (Promega Corporation). Subsequently, the plasmid and/or miR-325-3p mimic was transfected into HT-29 cells using Vigofect transfection reagent (Vigorous Biotechnology Beijing Co., Ltd.), according to the manufacturer's instructions. Briefly, 10 µl Vigofect transfection reagent was mixed with 100 µl cell culture in serum-free DMEM. Meanwhile, 10 µl miR-325-3p mimic or NC and pmirGLO-CK18-3'-UTR plasmid was mixed with the aforementioned mixture. Then, the two mixtures were mixed and incubated at room temperature for 10 min. Subsequently, the mixture was added in the 6-well plate at a final concentration of 20 nM. Following transfection for 48 h, the luciferase activity was detected using a Dual Luciferase Reporter Assay System (Promega Corporation). Firefly luciferase activity was normalized to Renilla luciferase activity.

**Western blotting.** Total protein isolated from CRC samples or HT-29 cells was extracted using RIPA buffer (Beijing Solarbio Science & Technology Co., Ltd.). A bicinchoninic protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used to determine the protein concentration. A total of 20 µg protein/ lane was separated by 10% SDS-PAGE and subsequently transferred onto PVDF membranes (EMD Millipore). Membranes were blocked with 5% skimmed milk at room temperature for 2 h. The membranes were incubated with primary antibodies against: CK18 (1:1,000; cat. no. ab133263; Abcam), α-SMA (1:1,000; cat. no. 19245; Cell Signaling Technology, Inc.), vimentin (1:1,000; cat. no. 5741; Cell Signaling Technology, Inc.) and GAPDH (1:1,000; cat. no. 5174; Cell Signaling Technology, Inc.) overnight at 4˚C. Membranes were washed with PBST three times, and subsequently incubated with HRP-conjugated anti-rabbit secondary antibodies (1:5,000; cat. no. ZB-2301; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) for 2 h at room temperature. Protein bands were visualized using the ECL Plus detection system (EMD Millipore), according to the manufacturer's instructions. GAPDH was used as the internal control.

**TGF-β treatment.** HT-29 cells were treated with 10 ng/µl TGF-β (Sigma-Aldrich; Merck KGaA) for 24, 48 and 72 h at 37˚C. To determine the effect of miR-325-3p on epithelial-to-mesenchymal transition (EMT), HT-29 cells were treated with or without TGF-β for 24 h at 37˚C. Subsequently, the cells were transfected with or without miR-325-3p inhibitors for 48 h, as aforementioned. The cells were collected for further analysis.

**Statistical analysis.** Statistical analysis was performed using SPPS 20.0 software (IBM Corp.). Data are presented as the mean ± standard deviation. Two-tailed unpaired Student's t-test or paired Student's t-test (for tumor vs. adjacent non-cancer tissues) was used to compare differences between two groups. One-way ANOVA followed by Tukey's post hoc test were used to compare difference between multiple groups. A receiver operating characteristic (ROC) curve was used to analyze the area under the curve (AUC) in the diagnosis of patients with CRC. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Microarray assay.** Tissue and serum samples were collected from patients with CRC and healthy individuals. A microarray
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A microarray assay was performed to identify the differentially expressed miRNAs in the tissue and serum samples of patients with CRC and healthy individuals. miR-325-3p expression was significantly increased in both the (A) tissues and (B) serum samples of patients with CRC compared with the healthy individuals. miRNA/miR, microRNA; CRC, colorectal carcinoma.

Figure 1. A microarray assay was performed to identify the differentially expressed miRNAs in the tissue and serum samples of patients with CRC and healthy individuals. miR-325-3p expression was significantly increased in both the (A) tissues and (B) serum samples of patients with CRC compared with the healthy individuals. miRNA/miR, microRNA; CRC, colorectal carcinoma.

miR-325-3p expression is elevated in patients with CRC. Based on the microarray data, RT-qPCR analysis was performed to detect miR-325-3p expression in patients with CRC. The results demonstrated that miR-325-3p expression was significantly upregulated in the tissues and serum samples of patients with CRC compared with the healthy individuals (Fig. 1A and B).

Diagnostic value of miR-325-3p in patients with CRC. The ROC curve analysis was performed to determine the diagnostic value of tissue and serum miR-325-3p levels in patients with CRC. As presented in Fig. 3A, tissue miR-325-3p expression could be used to differentiate patients with CRC from healthy individuals, with an AUC value of 0.876. When the cut-off value was 3.89, the sensitivity was 95.4% and the specificity was 93.6%. Furthermore, the AUC value of serum miR-325-3p expression was 0.795 in screening patients with CRC from healthy individuals. P<0.001). miR, microRNA; CRC, colorectal carcinoma.

Figure 2. Tissue and serum miR-325-3p levels in patients with CRC and healthy individuals. miR-325-3p expression was significantly upregulated in the (A) tissues and (B) serum samples of patients with CRC compared with the healthy individuals. **P<0.001. miR, microRNA; CRC, colorectal carcinoma.

Patients with CRC have higher serum miR-325-3p expression. The association between miR-325-3p expression and the clinicopathological characteristics of patients with CRC was assessed. As presented in Fig. 4A and D, tissue and serum miR-325-3p levels were significantly upregulated in patients...
with well differentiated CRC than those with poorly differentiated CRC. In addition, tissue and serum miR-325-3p levels were significantly upregulated in patients at TNM stages III and IV compared with those at stages I and II (Fig. 4B and E). Furthermore, tissue and serum miR-325-3p levels were significantly upregulated in patients with lymph node metastasis (Fig. 4C and F).

miR-325-3p promotes the migration and invasion of CRC cells. miR-325-3p expression was detected in the CRC cell lines, SW480, HCT116 and HT-29, as well as the normal colon cell line, FHC. The results demonstrated that miR-325-3p was highly expressed in HT-29 cells (Fig. 5A), thus, this cell line was used for subsequent experimentation. HT-29 cells were transfected with miR-325-3p mimics or inhibitors and RT-qPCR analysis was performed to assess transfection efficiency. The results demonstrated that transfection with miR-325-3p mimic significantly enhanced miR-325-3p expression, the effects of which were reversed following transfection with miR-325-3p inhibitor (Fig. 5B). The effect of miR-325-3p on the viability, migration and invasion of CRC cells was subsequently assessed. As presented in Fig. 5C, overexpression of miR-325-3p increased the cell viability in a time-dependent manner. In addition, the cell migratory and invasive abilities were significantly enhanced following transfection with miR-325-3p mimic (Fig. 5D). Conversely, miR-325-3p knockdown decreased HT-29 cell viability at 24, 48 and 72 h (Fig. 5E). Furthermore, the migratory and invasive abilities of HT-29 cells decreased following transfection with miR-325-3p inhibitors (Fig. 5F). Collectively, these results suggest that miR-325-3p acts as an oncogene in CRC.

CK18 is a target gene of miR-325-3p. Notably, a conserved binding of miR-325-3p was identified in the 3'-UTR of CK18 (Fig. 6A). The results of the dual-luciferase reporter assay demonstrated that miR-325-3p significantly suppressed the relative luciferase activity of the pmirGLO-CK18-3'-UTR compared with that of the blank pmirGLO plasmid (Fig. 6B). Western blot analysis demonstrated that overexpression of miR-325-3p significantly suppressed CK18 expression; however, miR-325-3p knockdown significantly elevated CK18 expression (Fig. 6C). Taken together, these results suggest that CK18 is a target gene of miR-325-3p.

miR-325-3p knockdown partially reverses transforming growth factor (TGF)-β-induced CK18 downregulation. The results demonstrated that treatment with TGF-β upregulated miR-325-3p expression in a time-dependent manner (Fig. 7A). Conversely, TGF-β significantly decreased CK18 expression at 48 and 72 h (Fig. 7B). Western blot analysis demonstrated that TGF-β significantly decreased the expression of the epithelial marker, CK18, and increased the expression levels of the mesenchymal markers, α-SMA and vimentin (Fig. 7C). Notably, these effects were reversed following inhibition of miR-325-3p (Fig. 7C). Collectively, these results suggest that miR-325-3p is a key regulator in TGF-β-induced CK18 downregulation.

Discussion

With the development of the economy, the incidence rate of CRC continues to increase and has become one of the most notable threats to human health (9). Despite advancements in endoscopic diagnosis and surgical techniques for CRC, the 5-year survival rate was slightly >10% in patients with stage IV disease (18,19). The differential expression of several miRNAs in tumor tissues and normal tissues suggests that miRNAs may be used as molecular markers for tumor diagnosis (7,8). As non-invasive markers, circulating miRNAs are expected to be sensitive and act as specific markers for tumor diagnosis and prognosis evaluation (20).

The present study screened miRNAs that are differentially expressed in the tissues and serum of patients with CRC. The results demonstrated that miR-325-3p tissue and serum levels were significantly upregulated in patients with CRC. AUC: area under the curve; CRC, colorectal carcinoma.
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CRC compared with healthy individuals. In addition, ROC curve analyses demonstrated that both tissue and serum miR-325-3p levels may be used as indicators for the diagnosis of CRC, respectively. Furthermore, high miR-325-3p expression was significantly associated with tumor differentiation, TNM stage and lymph node metastasis. Taken together, these results suggest that miR-325-3p expression can be used as an indicator to monitor the progression of patients with CRC.

EMT is an important mechanism that affects the invasion and metastasis of CRC (21). Several growth factors, such as TGF-β and epidermal growth factor, mediate EMT (22,23). Notably, the results of the present study demonstrated that TGF-β induced miR-325-3p expression. In addition, CK18 was identified as a target gene of miR-325-3p, suggesting that miR-325-3p is associated with the degree of EMT in CRC. EMT is widely involved in the invasion and metastasis of colon cancer due to the loss of epithelial characteristics into mesenchymal
The present study demonstrated that high miR-325-3p expression significantly decreased the expression of the epithelial marker, CK18, but increased the expression levels of the mesenchymal markers, vimentin and α-SMA. It is suggested that HT-29 cells gradually lose the characteristics of epithelial cells and attain the characteristics of mesenchymal cells (24,25). The results of the present study demonstrated that high miR-325-3p expression significantly decreased the expression of the epithelial marker, CK18, but increased the expression levels of the mesenchymal markers, vimentin and α-SMA. It is suggested that HT-29 cells gradually lose the characteristics of epithelial cells and attain the characteristics of mesenchymal
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Figure 6. CK18 is a target gene of miR-325-3p. (A) Based on the TargetScan database, a conserved binding site of miR-325-3p was identified in the 3’-UTR of CK18. (B) The results of the dual-luciferase reporter assay demonstrated that miR-325-3p significantly suppressed the relative luciferase activity of pmirGLO-CK18-3’-UTR compared with that of the blank pmirGLO plasmid. (C) Western blot analysis demonstrated that overexpression of miR-325-3p significantly suppressed CK18 expression; however, miR-325-3p knockdown significantly elevated CK18 expression. *P<0.05; **P<0.01; ***P<0.001 vs. pmirGLO or NC mimics/inhibitors. CK18, cytokeratin 18; miR, microRNA; UTR, untranslated region; NC, negative control.

Figure 7. Inhibition of miR-325-3p partially reverses TGF-β-induced CK18 downregulation. (A) TGF-β treatment upregulated miR-325-3p expression in a time-dependent manner. (B) TGF-β treatment decreased CK18 expression at 48 and 72 h. (C) TGF-β1 significantly decreased the expression of the epithelial marker, CK18, and increased the expression levels of the mesenchymal markers, α-SMA and vimentin, the effects of which were reversed following inhibition of miR-325-3p. *P<0.05; **P<0.01; ***P<0.001 vs. Con. miR, microRNA; TGF, transforming growth factor; CK18, cytokeratin 18; Con, control.
The authors declare that they have no competing interests.

Competing interests
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Authors’ contributions
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Availability of data and materials
The present study was approved by the Research Ethics Committee of Hongqi Hospital Affiliated to Mudanjiang Medical University (Mudanjiang, China; approval no. MDJHQ-20180925) and written informed consent was provided by all participants prior to the study start.

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The present study was not without limitations. First, the sample size was relatively small. Secondly, due to time constraints, the present study was unable to investigate the predictive value of serum miR-325-3p in patients with CRC.

In conclusion, elevated miR-325-3p expression is an important factor that affects EMT, and is likely to act as a molecular marker and potential therapeutic target in the progression of CRC. However, further studies are required to validate the results presented here.

Patient consent for publication
No applicable.

Ethics approval and consent to participate
The authors declare that they have no competing interests.

Competing interests
Not applicable.

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