MicroRNA-204 Inhibits the Growth and Motility of Colorectal Cancer Cells by Downregulation of CXCL8

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Among all of the miRNAs, miR-204 has gained considerable attention in the field of cancer research. This study aimed to reveal the detailed functions and the underlying mechanism of miR-204 in colorectal cancer (CRC) cells. The expressions of miR-204 in CRC tumor tissues and cell lines were monitored. Expressions of miR-204 and CXCL8 in Caco-2 and HT-29 cells were altered by transfection, and then cell viability, apoptosis, migration, invasion, EMT-related protein expression, and PI3K/AKT/mTOR pathway protein expression were assessed. We found that miR-204 was expressed at low levels in CRC tumor tissues and cell lines when compared to their normal controls. miR-204 overexpression reduced the viability, migration, and invasion of Caco-2 and HT-29 cells while significantly inducing apoptosis. miR-204 overexpression upregulated E-cadherin expression and downregulated N-cadherin and vimentin expressions. CXCL8 was a target of miR-204, and miR-204 suppression could not increase cell viability, migration, invasion, and EMT procedure when CXCL8 was silenced. Moreover, miR-204 overexpression decreased the phosphorylated levels of PI3K, AKT, and mTOR. The increased phosphorylations of PI3K, AKT, and mTOR, and the upregulation of CXCL8 induced by miR-204 suppression were all abolished by the addition of LY294002 and AZD8055 (inhibitors of PI3K/AKT and mTOR, respectively). To conclude, we demonstrated a tumor-suppressive miRNA in CRC cell lines, miR-204, which is poorly expressed in CRC tissues and cell lines. miR-204 exerted antigrowth, antimigration, anti-invasion, and anti-EMT activities, which might be via deactivating the PI3K/AKT/mTOR pathway and repressing CXCL8 expression.

Key words: miR-204; Colorectal cancer (CRC); Chemokine C-X-C motif ligand 8 (CXCL8); Epithelial–mesenchymal transition (EMT); PI3K/AKT/mTOR pathway

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide, with a high incidence and mortality in both sexes. The national incidence rate of this cancer has declined during these decades; however, there is still a large population suffering from CRC, and nearly 50,260 deaths were recorded in 2017 in the US. Surgery combined with radio- or chemotherapy is the mostly conventional treatment strategy, but this therapy is highly toxic and had modest results in advanced stage (stage IV) patients. Therefore, new treatment strategies with fewer side effects are urgently required to further improve the survival rate of patients with CRC. In this regard, microRNAs (miRNAs) have gained a lot of attention.

miRNAs are a class of short noncoding RNAs with targeting effects on several mRNAs and thereby can participate in multiple biological processes, including cell proliferation, cell cycle progression, apoptosis, inflammation, differentiation, as well as tumor metastasis. It has been well established that miRNAs are potential biomarkers for CRC, and dysregulation of miRNAs determines CRC cell fate. For instance, low miR-148a and miR-625-3p were associated with a more aggressive phenotype, and both of them might be potential prognostic and predictive biomarkers for CRC. Inhibition of miR-630 reduced the proliferation and induced apoptosis of the CRC cell line HCT116, suggesting tumor-suppressive functions on CRC. In contrast, miR-216a-3p acted as an oncogene, as its overexpression inhibited CRC cell proliferation.

Among all of the miRNAs, miR-204 has gained considerable attention in the field of cancer research.
It has been reported as a tumor suppressor in laryngeal squamous cell carcinoma\textsuperscript{10}, cervical cancer\textsuperscript{11}, hepatocellular carcinoma\textsuperscript{12}, breast cancer\textsuperscript{13}, and prostate cancer\textsuperscript{14}, by modulating tumor cell proliferation, apoptosis, migration, invasion, as well as chemosensitivity. In terms of CRC, miR-204 has been identified as a metastasis-related candidate miRNA, indicating that miR-204 might be a biomarker for CRC liver metastasis\textsuperscript{15}. Besides, a recent study has demonstrated that miR-204 overexpression inhibited the proliferation of two CRC cell lines (HCT116 and SW480 cells) and upregulated 5-fluorouracil (5-FU) chemosensitivity via the phosphatidylinositol 3-kinase (PI3K)/AKT pathway\textsuperscript{16}. In vivo and in vitro investigations showed that reintroduction of miR-204 into CRC cells markedly suppresses cell proliferation and invasion\textsuperscript{17}. It seems that the antitumor functions of miR-204 in CRC cells has been revealed, but the role of miR-204 in CRC cell apoptosis and the epithelial–mesenchymal transition (EMT) process as well as its underlying mechanism remain undefined.

In this study, 25 CRC patients were enrolled, and miR-204 expressions in the CRC tissues and correspondingly nontumor adjacent intestine tissues were detected by performing qRT-PCR. In addition, expressions of miR-204 in normal colonic endothelial cell line and five human CRC cell lines were determined. The expressions of miR-204 in Caco-2 and HT-29 cells were overexpressed or suppressed by miRNA transfection, and then the changes in cell viability, apoptosis, migration, invasion, EMT-related factor expression, and chemokine C-X-C motif ligand 8 (CXCL8) expression were assessed, respectively. Moreover, the effects of miR-204 dysregulation on the activation of the PI3K/AKT/mTOR signaling pathway were explored. The findings of this study further suggest that miR-204 may be a potential therapeutic target for CRC treatment.

**MATERIALS AND METHODS**

**Clinical Specimens**

Twenty-five pairs of CRC tissues and the corresponding nontumor adjacent intestine tissues were obtained from CRC patients who underwent surgical resection between December 2013 and April 2016 at Linyi People’s Hospital. Of the patients recruited, 13 were male and 12 were female, and all were aged between 44 and 67 years (median, 58 years). Of these 25 cases, there were 5 cases in stage I, 12 cases in stage II, 6 cases in stage III, and 2 cases in stage IV. None of the participants received chemo- or radiotherapy before tissue collection. Informed consent was obtained from each patient, and the present study was approved by the Ethics Committee of our institute.

**Cell Culture and Treatment**

Human CRC cell lines Caco-2, HT-29, Colo-320, Colo-205, and DLD-1, and a normal colonic endothelial cell line CCD-18Co were all purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Caco-2, HT-29, and CCD-18Co cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL, Gaithersburg, MD, USA). DLD-1, HT-29, Colo-320, and Colo-205 were grown in Roswell Park Memorial Institute (RPMI; Gibco-BRL) medium. Both of the media were supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco-BRL). The cells were maintained at 37°C in a 5% CO\textsubscript{2} humid atmosphere.

For EMT induction, the cells were treated with transforming growth factor-β1 (TGF-β1) (10 ng/ml) for 24 h following serum starvation for 8 h. LY294002 (20 μM) and AZD8055 (0.8 nM) both purchased from Sigma-Aldrich (St. Louis, MO, USA) were used as inhibitors of the PI3K/AKT and mTOR pathways, respectively.

**miRNA and siRNA Transfection**

miR-204 mimic, miR-204 inhibitor, and their corresponding negative controls (mimic NC and inhibitor NC) were all synthesized by GenePharma Co. (Shanghai, PR, China). The sequences for miRNAs were as follows: miR-204 mimic, UUCCUUUUGUCACUUGCU (sense) and GCAUAGGAUGACAAAGGGAUU (antisense); miR-204 inhibitor, AGGCAUAGGAUGACAAAGGG AA, CXCL8 specific short interfering RNA (siRNA) was purchased from Santa Cruz Biotechnology (sc-39631; Santa Cruz, CA, USA). All cell transfections were performed using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol. After 48 h of transfection, the cells were collected for use in the following analyses.

**CCK-8 Assay**

The viability of transfected cells was assessed using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Gaithersburg, MD, USA). Caco-2 and HT-29 cells after transfection were seeded in 96-well plates with a density of 5×10\textsuperscript{4} cells/well for adherence. After culturing in the humid incubator for 48 h, the culture medium was removed from each well, 10 μl of CCK-8 solution was added, and the plates were incubated for 4 h at 37°C in humidified 5% CO\textsubscript{2}. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

**Transwell Assay**

Cell migration was determined using a modified Boyden chamber with an 8-μm pore polycarbonate filter...
insert (Costar-Corning, New York, NY, USA). The transfected cells (5 × 10⁴) were suspended in serum-free medium and were added into the upper chamber. The lower chamber was filled with complete medium. After 24 h of incubation at 37°C in humidified 5% CO₂, nontraversed cells in the upper chamber were removed by cotton sticks. Traversed cells were stained with crystal violet (Beyotime, Nantong, P.R. China) for 30 min. Relative cell migration was calculated by counting the stained cells under an inverted fluorescence microscope.

Cell invasion was assessed the same as for cell migration, except that before testing the filter insert was pre-coated with Matrigel.

Apoptosis Assay

Cell apoptosis was performed using an Annexin-V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (Beyotime, Shanghai, P.R. China). The transfected cells were collected and resuspended in 300 μl of binding buffer containing 10 μl of annexin V-FITC and 5 μl of propidium iodide (PI). After incubation in the dark at room temperature for 30 min, 200 μl of ice-cold phosphate-buffered saline (PBS) was added, and the samples (1 × 10⁴ cells/sample) were analyzed under a fluorescence-activated cell sorting (FACS) Calibur flow cytometer (Becton-Dickinson). FITC⁺ and PI⁻ cells were recognized as apoptotic cells.

Dual-Luciferase Reporter Activity Assay

The constructed pMiR-luciferase report vector (Promega, Madison, WI, USA) with the 3’-untranslated region (3’-UTR) of CXCL8 carrying the putative hsa-miR-204-5p binding sites was amplified by PCR and referred to as CXCL8-WT. To mutate the putative binding site of hsa-miR-204-5p in the CXCL8 3’-UTR, the sequence of the putative binding site was replaced and referred to as CXCL8-MUT. Cells were cotransfected with the reporter constructs and miR-204 mimic using Lipofectamine 2000 (Invitrogen). Reporter analyses were performed using the Dual-Luciferase Reporter Assay system (Promega) according to the manufacturer’s information.

qRT-PCR

For miR-204 expression detection, total miRNAs in the transfected cells were extracted by miRNeasy Mini Kit (Qiagen, Shenzhen, P.R. China). Reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), and qRT-PCR was performed by the TaqMan Universal Master Mix II with the TaqMan MicroRNA Assay (Applied Biosystems). U6 was used as an internal control. For the analysis of E-cadherin, N-cadherin, and vimentin expression, total RNA was isolated by the TRIzol reagent–phenol chloroform procedure provided by Invitrogen. Five micrograms of total RNA from each sample was subjected to reverse transcription using the Transcriptor First-Strand cDNA Synthesis Kit according to the manufacturer’s instructions. FastStart Universal SYBR Green Master (ROX) was used in qRT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Data were analyzed with the 2⁻¹⁰Ct method.

Western Blot

Total protein was extracted from the transfected cells using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime). The protein concentration was determined by BCA Protein Assay Kit (Pierce, Appleton, WI, USA), and 0.1 mg of protein samples was resolved over sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. Following block in 5% non-fat milk for 1 h, the membranes were probed by primary antibodies overnight at 4°C for the specific detection of E-cadherin (ab11512), N-cadherin (ab19348), vimentin (ab137321), CXCL8 (ab7747), PI3K (ab86714), phosphorylated (p)-PI3K (ab182651), AKT (ab8805), p-AKT (ab38449), mTOR (ab32028), p-mTOR (ab109268), and GAPDH (ab9484; Abcam, Cambridge, UK). The membranes were then incubated in the secondary antibodies for 1 h at room temperature, protein bands were developed using enhanced chemiluminescence, and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad).

Statistical Analysis

All data were expressed as the mean±SD from at least three independent experiments. Statistical analyses were performed using the SPSS version 13.0 program (SPSS Inc., Chicago, IL, USA). The p values were calculated using a one-way analysis of variance (ANOVA) with LSD(L) procedure. A value of p<0.05 was considered as a statistical significance.

RESULTS

miR-204 Was Poorly Expressed in CRC Tissues and Cell Lines

We first analyzed the expression changes of miR-204 between CRC tissues and the corresponding non-tumor adjacent intestine tissues, which were obtained from 25 patients with CRC. According to the qRT-PCR analytical results (Fig. 1A), miR-204 expression was much lower in tumor tissues when compared to those in non-tumor adjacent tissues (p<0.001). The expression of miR-204 in a normal colonic endothelial cell line
CCD-18Co and five human CRC cell lines was monitored by qRT-PCR. Results in Figure 1B showed that the RNA expression of miR-204 in Caco-2, HT-29, Colo-320, Colo-205, and DLD-1 cells was much lower than that in CCD-18Co cells \((p<0.01\) or \(p<0.001\)). These data suggested that miR-204 might be implicated in the occurrence and development of CRC.

**Antigrowth, Antimigration, and Anti-invasion Roles of miR-204 in CRC Cell Lines**

To confirm our hypothesis, we altered the expression of miR-204 in two CRC cell lines (Caco-2 and HT-29), and then the effects of miR-204 dysregulation on cell viability, apoptosis, migration, and invasion were detected. The qRT-PCR results displayed in Figure 2A and B show that miR-204 expression was significantly increased by transfection with miR-204 mimic \((p<0.001)\), while expression was reduced by miR-204 inhibitor \((p<0.01)\), indicating that cells overexpressing and suppressing miR-204 were successfully obtained. Data in Figure 2C–F show that miR-204 overexpression significantly decreased cell viability \((p<0.05\) or \(p<0.01\)) and increased apoptosis \((p<0.001)\); in contrast, miR-204 suppression increased cell viability \((p<0.05)\), while suppression has no significant impact on cell apoptosis.

Transwell assay results (Fig. 3A–D) showed that miR-204 overexpression significantly reduced both cell migration and invasion in Caco-2 and HT-29 cells \((p<0.05)\). As expected miR-204 suppression resulted in significant increases in cell migration and invasion \((p<0.05)\). Then Caco-2 and HT-29 cells were treated with TGF-\(\beta\)1 (an EMT inducer), and the expression of EMT-related factors was assessed to reveal whether miR-204 inhibited CRC cell migration and invasion via modulation of EMT. As shown in Figure 4A–D, mRNA and protein level expressions of E-cadherin were upregulated in response to miR-204 overexpression, while N-cadherin and vimentin were downregulated by miR-204 overexpression. miR-204 suppression resulted in a completely opposite result, in that E-cadherin expression was downregulated and N-cadherin and vimentin expressions were upregulated.

**miR-204 Exerted Antitumor Functions by Repressing the Expression of CXCL8**

Given that CXCL8 has been widely recognized as an important multifunctional cytokine to modulate tumor proliferation, invasion, and migration in an autocrine or paracrine manner \(^{18-20}\); herein we explored whether miR-204 has a regulatory role in CXCL8 expression. By performing dual-luciferase activity assay, we found that the luciferase activity was significantly decreased by cotransfection with CXCL8-WT and miR-204 mimic \((p<0.05)\), rather than cotransfection with CXCL8-MUT and miR-204 mimic (Fig. 5A). Western blot analytical results in Figure 5B showed that CXCL8 protein expression was downregulated in miR-204-overexpressing cells and was upregulated in miR-204-suppressing cells, indicating a negative regulation between miR-204 and CXCL8. Caco-2 and HT-29 cells were cotransfected with miR-204 inhibitor and CXCL8 siRNA to simultaneously suppress the expression of miR-204 and CXCL8. As shown in Figure 5C, increases of CXCL8 induced by miR-204 inhibitor were abolished by CXCL8 siRNA. These data suggested that CXCL8 might be a target of miR-204, and miR-204 negatively regulated the expression of CXCL8.

Functional analyses results showed that miR-204 suppression could not increase cell viability when CXCL8 was knocked down, and CXCL8 silencing induced significant increases in apoptotic cell rate \((p<0.01\) or \(p<0.001)\) (Fig. 6A–D). Results in Figure 7A–D show that miR-204 suppression could not increase the relative migration and invasion when CXCL8 was knocked down. CXCL8 silencing attenuated or abolished the miR-204
suppression-induced downregulation of E-cadherin and upregulation of N-cadherin and vimentin (Fig. 8A–D).

**miR-204 Repressed CXCL8 Expression via the PI3K/AKT/mTOR Signaling Pathway**

To further reveal the underlying mechanism by which miR-204 regulated CXCL8 expression, we focused on the PI3K/AKT/mTOR signaling pathway since this signaling pathway has a regulatory role in CXCL8 expression21. Western blot analytical results (Fig. 9) showed that miR-204 overexpression decreased the phosphorylated levels of PI3K, AKT, and mTOR, while miR-204 suppression increased the phosphorylated levels of these three proteins. By addition of LY294002 (an inhibitor of PI3K/AKT) and AZD8055 (an inhibitor of mTOR), the increased phosphorylation of PI3K, AKT, and mTOR induced by miR-204 suppression were all abolished. More importantly, miR-204 suppression-induced increases in CXCL8 were also abolished by the addition of LY294002 and AZD8055.

**DISCUSSION**

miRNAs have been the first noncoding genes involved in cancer; recent studies have linked them with cancer initiation, progression, diagnosis, and prognosis22,23. However, the functions and molecular mechanisms of most
Figure 3. miR-204 overexpression inhibits the migration and invasion of CRC cells. Relative migration of Caco-2 (A) and HT-29 (B) cells transfected with miR-204 mimic, mimic NC, miR-204 inhibitor, or inhibitor NC. (C, D) Relative invasion of the transfected cell lines. *p<0.05.

Figure 4. miR-204 overexpression inhibits the epithelial–mesenchymal transition (EMT) process of CRC cells. (A, B) mRNA expression of EMT-related factors. (C, D) Protein expression of EMT-related factors. *p<0.05.
Figure 5. Chemokine C-X-C motif ligand 8 (CXCL8) is a target of miR-204. (A) Targeting effect of miR-204 on the 3'-untranslated region (3'-UTR) of CXCL8 was measured by dual-luciferase reporter activity assay. (B) Protein expression of CXCL8 in cell lines transfected with miR-204 mimic, mimic NC, miR-204 inhibitor, or inhibitor NC. (C) Protein expression of CXCL8 in cell lines transfected with inhibitor NC, miR-204 inhibitor, and miR-204 inhibitor plus CXCL8 short interfering RNA (siRNA). *p<0.05.

Figure 6. miR-204 exerted antigrowth function by repressing CXCL8 expression. (A, B) Viability and (C, D) apoptotic cell rate of cells after transfection with inhibitor NC, miR-204 inhibitor, and miR-204 inhibitor plus CXCL8 siRNA. *p<0.05, **p<0.01, ***p<0.001.
of the miRNAs remain unknown. Herein we found that miR-204 was expressed at lower levels in CRC tumor tissues and cell lines, and miR-204 overexpression exerted antitumor activities, as evidenced by the decrease in cell viability, migration, and invasion, and the increase in apoptosis, as well as the dysregulation of EMT-related factors. CXCL8 was a downstream gene of miR-204. In addition, miR-204 repressed CXCL8 expression possibly via deactivation of the PI3K/AKT/mTOR pathway.

In CRC, many miRNAs show aberrant expression patterns; thus, miRNAs appear to be promising tumor biomarkers in CRC. Previous expression profiling data revealed that miR-204 was one of the miRNAs that was most significantly downregulated in CRC tissues compared with adjacent noncancerous tissues. This was also confirmed in our study, suggesting that miR-204 might be implicated in the tumorigenesis of CRC.

miR-204 has been recognized as a tumor suppressor in a variety of human cancers including CRC. For instance, a previous study has demonstrated that miR-204 inhibited cell proliferation and metastasis, and miR-204 enhanced apoptosis of CRC cells. Another investigation revealed that miR-204 inhibited CRC cell line proliferation by directly targeting high mobility group protein A2 (HMGA2). Consistent with these previous studies, our findings also suggested that miR-204 possessed antitumor activities in CRC cells, as Caco-2 and HT-29 cell viability, migration, and invasion were reduced, while apoptotic cell rates were increased by miR-204 overexpression. More interestingly, previous studies have shown that miR-204 is also involved in EMT in multiple types of cancers, like gastric cancer, oral squamous cell carcinomas, and esophageal cancer. miR-204 overexpression was observed to be related with EMT in the CRC cell line LoVo, rather than HCT116, suggesting that miR-204 expression might also be involved in EMT inhibition in CRC cells. In the current study, we found that miR-204 overexpression increased the expression of E-cadherin (an EMT negatively correlative marker) and decreased the expressions of N-cadherin and vimentin (mesenchymal markers). These data suggested that miR-204-suppressed CRC cell migration and invasion might be through regulating EMT-related proteins.

As important gene regulators, miRNAs are predicted to regulate more than 60% of human protein-coding genes. A growing number of miRNAs have been shown to be...
involved in tumorigenesis by modulating these protein-coding genes. CXCL8 [interleukin-8 (IL-8)] is one of the first and most intensively studied chemokines acting as a proinflammatory chemokine. In addition to its role in inflammation, CXCL8 is also involved in multiple cancer progressions. Elevated expression of CXCL8 has been detected in CRC cancer cells, and CXCL8 exerted regulatory functions in modulating different metastatic phenotypes associated with progression and metastasis.

Our data indicated that CXCL8 might be a target of miR-204, and miR-204 negatively regulated the protein expression of CXCL8. Functional investigations suggested that silencing CXCL8 could rescue miR-204 suppression-induced increases in cell viability, migration, and invasion, implying that miR-204 functioned as a tumor suppressor possibly via repressing the expression of CXCL8.

There are several signaling pathways that regulate CXCL8 production, such as NF-κB, MAPK, and PI3K/AKT signaling pathways. On the other hand, CXCL8 also can induce the phosphorylation of PI3K and AKT. Herein we focused on the PI3K/AKT/mTOR signaling pathway in order to reveal the underlying mechanism of how miR-204 repressed CXCL8 expression. Western blotting results indicated that miR-204-repressed CXCL8 expression might be via deactivation of the PI3K/AKT/mTOR signaling pathway.

Figure 8. miR-204 exerted anti-EMT function by repressing CXCL8 expression. (A, B) mRNA level expressions of EMT-related factors. (C, D) Protein level expressions of EMT-related factors. *p<0.05, **p<0.01, ***p<0.001.

Figure 9. miR-204 repressed CXCL8 expression via the PI3K/AKT/mTOR signaling pathway. Caco-2 and HT-29 cells were transfected with mimic NC, miR-204 mimic, inhibitor NC, and miR-204 inhibitor, and then treated with LY294002 (an inhibitor of PI3K/AKT) and AZD8055 (an inhibitor of mTOR). The protein expressions of phosphorylated (p)-PI3K, PI3K, p-AKT, AKT, p-mTOR, mTOR, and CXCL8.
mTOR signaling pathways. Other mechanisms by which miR-204 suppressed CXCL8 expression should be investigated in future work.

In conclusion, we demonstrated a tumor-suppressive miRNA in CRC cell lines, miR-204, which is poorly expressed in CRC tissues. miR-204 exerted antigrowth, antimigration, anti-invasion, and anti-EMT activities probably via deactivation of the PI3K/AKT/mTOR pathway and repressing CXCL8 expression.

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