miR-204-5p Inhibits Proliferation and Invasion and Enhances Chemotherapeutic Sensitivity of Colorectal Cancer Cells by Downregulating RAB22A

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Abstract

Purpose: miR-204-5p was found to be downregulated in colorectal cancer tissues in our preliminary microarray analyses. However, the function of miR-204-5p in colorectal cancer remains unknown. We therefore investigated the role, mechanism, and clinical significance of miR-204-5p in colorectal cancer development and progression.

Experimental Design: We measured the expression of miR-204-5p and determined its correlation with patient prognoses. Ectopic expression in colorectal cancer cells, xenografts, and pulmonary metastasis models was used to evaluate the effects of miR-204-5p on proliferation, migration, and chemotherapeutic sensitivity. Luciferase assay and Western blotting were performed to validate the potential targets of miR-204-5p after the preliminary screening by a microarray analysis and computer-aided algorithms.

Results: miR-204-5p is frequently downregulated in colorectal cancer tissues, and survival analysis showed that the downregulation of miR-204-5p in colorectal cancer was associated with poor prognoses. Ectopic miR-204-5p expression repressed colorectal cancer cell growth both in vitro and in vivo. Moreover, restoring miR-204-5p expression inhibited colorectal cancer migration and invasion and promoted tumor sensitivity to chemotherapy. Mechanistic investigations revealed that RAB22A, a member of the RAS oncogene family, is a direct functional target of miR-204-5p in colorectal cancer. Furthermore, RAB22A protein levels in colorectal cancer tissues were frequently increased and negatively associated with miR-204-5p levels and survival time.

Conclusions: Our results demonstrate for the first time that miR-204-5p acts as a tumor suppressor in colorectal cancer through inhibiting RAB22A and reveal RAB22A to be a new oncogene and prognostic factor for colorectal cancer. Clin Cancer Res; 20(23); 6187–99. ©2014 AACR.

Introduction

Colorectal cancer is the third most common cancer and the third leading cause of cancer death in women and men worldwide (1). The development of colorectal cancer involves multiple factors, including cancer inhibitors (tumor suppressors), and cancer inducers (oncogenes). During the past decade, a type of non–protein-coding RNA molecules known as microRNAs (miRNA) has been linked to cancer development by acting as either tumor suppressors or oncogenes (1, 2). These miRNAs are generally endogenous, noncoding RNAs of 19 to 24 nucleotides in length (3). Deregulation of miRNAs has been implicated in the development and progression of nearly all tumor types, including colorectal cancer (2). Generally, miRNAs play critical roles in the negative regulation of gene expression by base pairing to complementary sites in the 3'-untranslated regions (UTR) of their target miRNAs (4).

In colorectal cancer, many miRNAs show aberrant expression patterns (5–7), and some have been shown to be involved in tumorigenesis by targeting tumor-associated genes (7–12). In addition, miRNAs appear to be promising tumor biomarkers (13, 14). Our previous expression profiling data revealed that miR-204-5p (previously known...
Translational Relevance

MicroRNAs (miRNA) expression aberration has been observed in almost all human cancers, thus offering a group of potential diagnostic markers, prognostic factors, and therapeutic targets in tumorigenesis. We now present data showing that miR-204-5p, which is frequently downregulated in colorectal carcinoma tissues, functions as a tumor suppressor in colorectal carcinoma. Ectopic miR-204-5p expression repressed colorectal carcinoma cell growth, migration, and invasion and promoted tumor sensitivity to chemotherapy. RAB22A, a member of the RAS oncogene family, was identified as a direct functional target of miR-204-5p in colorectal carcinoma. Low miR-204-5p and high RAB22A expression are associated with poor prognoses in patients with colorectal carcinoma, suggesting that they could serve as valuable prognostic factors for survival. In addition, therapeutic miR-204-5p overexpression could be useful for the treatment of colorectal carcinoma by targeting RAB22A.

as miR-204 before miRBase release 19.0) was one of the miRNAs that was most significantly downregulated in colorectal cancer tissues compared with adjacent noncancerous tissues (NCT; ref. 7). Several studies have also shown that miR-204 is frequently downregulated in other cancers, suggesting a common role of miR-204 in human tumorigenesis (15–23). However, the role of miR-204-5p in colorectal cancer remains undefined.

In this study, we confirmed that the miR-204-5p expression is significantly decreased in colorectal cancer using an expanded cohort. This decrease was attributed to the hypermethylation of its promoter. The downregulation of miR-204-5p in colorectal cancer was associated with poor patient prognosis. Functional analyses showed that the reintroduction of miR-204-5p into colorectal cancer cells markedly suppresses cell proliferation and invasion both in vitro and in vivo and increases the sensitivity of colorectal cancer cells to oxaliplatin (OXL), 5-fluorouracil (5-FU), and cisplatin (DDP). Further mechanistic investigations revealed that miR-204-5p inhibits colorectal cancer development and progression by directly targeting RAB22A (a member of the RAS oncogene family), which appears to be a new prognostic factor in colorectal cancer. Collectively, these data highlight the tumor-suppressive role of miR-204-5p and the consequences of its inactivation on the oncogenic activity of RAB22A. These factors might contribute, at least in part, to the poor response of colorectal cancer to conventional chemotherapy.

Materials and Methods

Cell lines and clinical samples

Human colorectal cancer cell lines, including Caco2, DLD1, HCT8, HCT116, HT29, LoVo, SW480, and SW620, were purchased from ATCC. HCT116 and LoVo cells using for functional and mechanism studies in this study were tested and authenticated using short tandem repeat (STR) assay by Genewiz. All of the media (Hyclone) were supplemented with 10% FBS (Gibco). The cells were incubated under the conditions recommended by ATCC as described previously (7).

A total of 272 paired colorectal cancer and NCT tissues were obtained from Fudan University Shanghai Cancer Center (Shanghai, China) and the Affiliated Hospital of Jiangnan University (Jiangsu, China; Supplementary Table S1). All of the samples were gathered according to the Institutional Review Board–approved protocol and the written informed consent from each patient.

DNA and RNA isolation

Genomic DNA was isolated using the General AllgGen Kit (Cwbio) according to the manufacturer’s protocol. Total RNA was extracted using TRizol reagent (Invitrogen). The concentrations of DNA and RNA were determined using a NanoDrop 2000 (Thermo).

Real-time qRT-PCR

cDNA was synthesized using the PrimeScript RT Reagent Kit (TaKaRa). QRT-PCR analyses were conducted to quantitate the relative mRNA expression using SYBR Premix Ex Taq (TaKaRa), with β-actin as an internal control. Stem-loop qRT-PCR assays using TaqMan miRNA probes (Applied Biosystems) were performed to quantify the levels of the mature miRNAs. The reactions were incubated in 96-or 384-well optical plates at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. After the reactions, the cycle threshold (Ct) data were determined using default threshold settings, and the mean Ct was determined from the duplicate PCRs. A comparative ΔCt method was used to compare each condition with the controls, and the values are expressed as 2−ΔCt. The relative levels of miRNAs were normalized to the levels of U6, a ubiquitously expressed small nuclear RNA. The relative DNA copy numbers were determined as described previously (24). All of the primers used are listed in Supplementary Table S2.

Promoter methylation analysis

The gDNA was bisulfite-modified as previously described (25). Bisulfite-treated DNA was amplified with bisulfite sequencing PCR (BSP) primers. The purified BSP products were directly sequenced and the methylation status of each CpG site was determined as described previously (26). To perform methylation-specific PCR (MSP) analysis, bisulfite-treated DNA was subjected to PCR using methylation-specific primers, and the PCR products were analyzed by 2% agarose gel electrophoresis. BSP and MSP primers were designed using MethPrimer (Supplementary Table S2 and Supplementary Fig. S1A; ref. 27). HCT116 and LoVo Cells were treated with 10 μmol/L 5-aza-dC (Sigma), a demethylating agent, for 72 hours, and then subjected to the quantitation of miR-204-5p.
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Plasmid and siRNA
The human pri-miR-204 sequence was amplified from normal human gDNA and cloned into the lentivirus expression vector pWPXL to generate pWPXL-miR-204. The 3′UTRs of potential miR-204-5p target genes were amplified from gDNA using PrimerSTAR Premix (TaKaRa). The amplified 3′UTRs were then cloned into the region directly downstream of a CMV promoter–driven firefly luciferase cassette in a pcDNA3.0 vector (p-Luc). The mutant 3′UTR of RAB22A, which carried the mutated sequence in the complementary site for the seed region of miR-204-5p, was constructed on the basis of the p-Luc-RAB22A 3′UTR-WT plasmid by overlap-extension PCR. The open reading frame (ORF) of RAB22A was amplified and cloned into pWPXL. Duplex siRNAs were purchased from GenePharma.

Lentivirus production and transduction
The pWPXL, pWPXL-miR-204, and pWPXL-RAB22A plasmids were cotransfected into HEK-293T cells along with the packaging plasmid ps-PAX2 and the envelope plasmid pMD2G using Lipofectamine 2000 (Invitrogen). Virus particles were harvested 48 hours after transfection. Then, the particles were individually used to infect HCT116 and LoVo cells. The cells were then harvested 3 days after infection for Western blotting and qRT-PCR validation.

Cell proliferation assay and colony formation assay
For the cell proliferation assay, 1,000 cells were plated in 96-well plates, incubated and detected with the Cell Counting Kit-8 (CCK-8, Dojindo) according to the manufacturer’s instructions. For the colony formation assay, 1,000 cells were placed in each well of a 6-well plate and maintained in media containing 10% FBS for 2 weeks. The colonies were fixed with methanol and stained with 0.1% crystal violet in 20% methanol for 20 minutes. The number of colonies was counted using an inverted microscope.

Cell migration and invasion assay
The migration ability of colorectal cancer cells was tested in a Transwell Boyden Chamber (8-μm pore size, BD Biosciences) as previously described (12). For the cell invasion assay, the polycarbonate membranes of the upper compartment of the chambers were precoated with a matrix gel.

Assessment of chemotherapy sensitivity and apoptosis
LoVo or HCT116 cells stably expressing miR-204 or the control vector were treated with oxaliplatin (range, 0–24 μg/mL), and cell inhibition was then assessed by CCK-8 assay. The half-maximal inhibitory concentration (IC50) was calculated. For the apoptosis analysis, colorectal cancer cells were treated with 5 μg/mL oxaliplatin for 48 hours. The cells were then harvested and subjected to apoptosis analysis using an Annexin V-FITC and propidium iodide labeling kit (Invitrogen).

Tumor formation in nude mouse
LoVo or HCT116 cells stably expressing miR-204 or the blank vector were subcutaneously injected into either flank of the same athymic male BALB/c nude mouse at 5 weeks of age. The mice were sacrificed after a period of 4 to 6 weeks and examined for the growth of subcutaneous tumors. For the in vivo metastasis assays, 2 × 106 LoVo cells stably expressing miR-204 or the control vector were suspended in 100 μL DMEM and were injected into the caudal vein of each nude mouse. The nude mice were maintained under specific pathogen-free conditions in the Experimental Animal Department of Fudan University.

Microarray analysis
Expression profiling was performed using an Agilent human whole genome oligo microarray chip (4 × 44 K; Agilent). A total of 5 × 10^5 HCT116 cells were seeded in 6-cm² tissue culture plates and transfected with the miR-204-5p or the negative control (NC) as described above. After propagation for 48 hours, total RNA was extracted for the expression profiling analyses. The microarray profiling was performed as described in our previous work (12), and the results had been submitted to GEO (GSE59897).

Luciferase reporter assay
HEK-293T or HCT116 cells were cultured in 96-well plates and cotransfected with 50 nmol/L of miR-204-5p mimic (or NC), 50 ng of luciferase reporter, and 10 ng of pRL-CMV Renilla luciferase reporter using Lipofectamine 2000. Forty-eight hours after transfection, the luciferase activities were assayed using a luciferase assay kit (Promega).

Western blotting
The levels of RAB22A were analyzed by Western blotting using a rabbit monoclonal anti-human RAB22A antibody at a dilution of 1:1,000 (Abcam). Normalization was performed by blotting the same samples with an antibody against β-actin (Abcam).

Immunohistochemistry
Tissue microarrays were constructed using paired colorectal cancer and NCT tissues from 2 different colorectal cancer cohorts. Immunohistochemical (IHC) staining was performed on 4-μm sections of paraffin-embedded tissue samples to detect the expression levels of RAB22A protein. In brief, the slides were incubated in RAB22A antibody diluted to 1:200 at 4°C overnight. The subsequent steps were performed using the EnVision FLEX High pH 9.0 Visualization System (DAKO).

Statistical analyses
The results are expressed as the mean ± SEM. The data were subjected to Student t tests, the Mann–Whitney U test, or the Kruskal–Wallis test unless otherwise specified (γ test or Spearman correlation). The survival curves were plotted according to the Kaplan–Meier method, with the log-rank test applied for comparisons. Cox proportional hazards
regression analysis was used to estimate the HRs and the 95% confidence intervals (CI). A value of $P < 0.05$ was considered to be statistically significant. The SPSS 16.0 package (IBM) was used for the statistical analyses.

Results

Downregulation of miR-204-5p predicts poor survival in colorectal cancer

miR-204-5p emerged as one of the most prominently downregulated miRNAs in colorectal cancer in our previous microarray data (7). To validate the result, we examined the miR-204-5p levels in an expanded colorectal cancer cohort using qRT-PCR. Consistent with the microarray data, miR-204-5p expression was frequently downregulated in colorectal cancer tissues compared with the corresponding NCTs ($P < 0.0001$, Fig. 1A and B). These cases were then divided into 3 groups (high: the 25% highest, low: the 25% lowest, or not significantly changed) for survival analysis based on their miR-204-5p levels. We found that miR-204-5p expression was significantly associated with overall survival, and patients with low miR-204-5p expression had shorter overall survival than patients with high miR-204-5p expression ($P = 0.017$, Fig. 1C). After adjusting for age, gender, tumor size, stage, and grading, multivariate analyses confirmed that miR-204-5p expression was an independent prognostic factor for colorectal cancer survival (HR, 0.303;
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95% CI, 0.147–0.622; \( P = 0.001 \)). No significant association was found between miR-204-5p expression in colorectal cancer and tumor size, location, stage, and grading.

To further evaluate the impact of miR-204-5p on prognosis, we analyzed the miR-204-5p expression data from The Cancer Genome Atlas (TCGA) database. A total of 159 colorectal cancer samples were selected for further analysis. A significant positive correlation was found between miR-204-5p expression in colorectal cancer and tumor size, location, stage, and grading.

To analyze the miR-204-5p expression levels and survival time, \( P = 0.007 \), Fig. 1D). After adjusting for age, gender, T stage, N stage, TNM stage, and vascular/lymphovascular invasion, Cox multivariate analyses showed that miR-204-5p expression was an independent prognostic factor for colorectal cancer survival. Tumors with high miR-204-5p expression levels (\( > \) median) exhibited a decreased risk of death (HR, 0.182; 95% CI, 0.067–0.499, \( P = 0.001 \)).

**Downregulation of miR-204-5p caused by promoter hypermethylation in colorectal cancer cells**

The coding sequence of miR-204 is located within an intronic region of the TRPM3 gene. TRPM3 and miR-204 share the same transcriptional regulatory motif and are derived from a single transcription unit (28, 29). Previous study reported that the promoter CpG islands of TRPM3 were hypermethylated in glioma (21). We next sought to investigate whether the promoter hypermethylation also occurred in colorectal cancer and whether it was responsible for the downregulation of miR-204-5p. The BSP results showed that most of the 8 colorectal cancer cell lines were hypermethylated in the TRPM3 promoter (Supplementary Fig. S1B). The expression of miR-204-5p was linearly correlated with that of TRPM3 in these colorectal cancer cell lines (Supplementary Fig. S1C–S1E). We then examined promoter methylation of TRPM3 in colorectal cancer tissues. The results showed that the methylation ratio of TRPM3 in colorectal cancer tissues (23 of 28, 82.1%) was significantly higher than that in NCTs (14 of 28, 50.0%; \( P = 0.011 \), and TRPM3 was unmethylated in 5 normal colonic epithelia samples. To further examine the effect of DNA methylation on miR-204 expression, HCT116 and LoVo cells were treated with the DNA methyltransferase inhibitor 5-aza-dC, and the expression of miR-204-5p in the treated cells was significantly restored (Supplementary Fig. S1F).

Together, these data demonstrate that promoter hypermethylation is an important mechanism for silencing miR-204 expression in colorectal cancer.

Copy number variants (CNV) also cause aberrant expression of genes, including miRNAs (12). We analyzed the CNV of miR-204 in 48 paired colorectal cancer s and NCTs and observed no significant changes between them (data not shown).

**miR-204-5p inhibits colorectal cancer cell proliferation in vitro and in vivo**

Cell proliferation assays revealed that miR-204-5p overexpression significantly reduced the growth rates of HCT116 and LoVo cells (\( P < 0.01 \), Fig. 2A), whereas silencing miR-204-5p expression significantly promoted the growth of colorectal cancer cells (\( P < 0.01 \), Fig. 2B). Colony formation assays confirmed the proliferation-repressing function of miR-204 in colorectal cancer cells (\( P < 0.01 \), Fig. 2C). A tumor formation assay in a nude mouse model was performed to evaluate the in vivo function of miR-204 and revealed that miR-204 overexpression significantly repressed the tumorigenesis of colorectal cancer cells compared with the vector control (\( P < 0.01 \), Fig. 2D).

miR-204-5p enhances oxaliplatin-induced cytotoxicity in vitro and in vivo

To determine whether miR-204-5p could modulate the metastasis ability of colorectal cancer, we first examined the effect of miR-204-5p on colorectal cancer cell migration and invasion using a Transwell assay. As shown in Fig. 3A, miR-204-5p–transfected cells exhibited considerably slower migration and invasion compared with the control. We then assessed the impact of miR-204-5p on in vivo metastasis using a mouse model of pulmonary metastasis. Overexpression of miR-204-5p significantly reduced the number of lung metastasis sites (Fig. 3B), further validating that the invasive behavior of colorectal cancer could be suppressed by miR-204-5p.

**miR-204-5p increases sensitivity of colorectal cancer cells to chemotherapy**

Because low miR-204-5p expression was significantly associated with poor patient survival and increased cell growth, we assessed the potential effect of miR-204-5p on the sensitivity of colorectal cancer cells to oxaliplatin, one of the most commonly used drugs in colorectal cancer treatment. The results revealed that the IC_{50} of miR-204-5p–overexpressed colorectal cancer cells was significantly lower than that of the control (LoVo: 2.961 vs. 1.501 \( \mu \)g/mL; HCT116: 2.749 vs. 1.012 \( \mu \)g/mL; \( P < 0.01 \), Fig. 3C), suggesting that miR-204-5p strongly increases the sensitivity of colorectal cancer cells to oxaliplatin. To further confirm that miR-204-5p enhances oxaliplatin-induced cytotoxicity, we examined the apoptosis of oxaliplatin-treated colorectal cancer cells. Following treatment with 5 \( \mu \)g/mL of oxaliplatin for 48 hours, significantly increased apoptosis ratios were observed in miR-204-5p–overexpressed HCT116 and LoVo cells, relative to the controls (\( P < 0.01 \), Fig. 3D).

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To investigate whether miR-204-5p overexpression could affect apoptosis in colorectal cancer cells exposed to chemotherapy agents other than oxaliplatin, we exposed colorectal cancer cells to 5-FU and DDP, which are also commonly used in colorectal cancer treatment. Following treatment with 5-FU or DDP, significantly increased apoptosis was observed in miR-204-overexpressing colorectal cancer cells (Supplementary Fig. S3). Taken together, these observations suggest that miR-204-5p, through enhanced apoptosis, significantly increases the sensitivity of colorectal cancer cells to chemotherapy.

Identification of RAB22A as a target of miR-204-5p

Genomic-wide expression profiling was first performed in miR-204-5p- or NC-transfected HCT116 cells using a microarray (GSE59897). A total of 1,367 downregulated transcripts (>2-fold change) were identified in miR-204-5p–transfected cells compared with the control (Supplementary Table S3). Using 2 computer-aided algorithms, TargetScan and PicTar, we identified 123 genes that are potentially regulated by miR-204-5p. By comparing all of the downregulated genes with the candidate genes predicted by the programs, 15 downregulated genes were selected (Fig. 4A). Because it is generally accepted that miRNAs exert their function by inhibiting the expression of their target genes, miR-204-5p may execute its tumor-suppressive function by downregulating targets that normally have tumor-promoting functions. On the basis of this rationale, 4 candidate genes (BCL2, CCPO1, KLF12, and RAB22A) were selected from the 15 genes. Luciferase reporter assays were

Figure 2. miR-204-5p inhibits colorectal cancer cell proliferation in vitro and in vivo. A and B, miR-204-5p overexpression repressed the proliferation of LoVo and HCT116 cells (A), whereas knockdown of miR-204-5p enhanced the cell growth rate of LoVo and HCT116 cells (B). The CCK-8 assay was used to determine the cell growth rate (\( P < 0.05; \quad P < 0.01 \)). C, miR-204-overexpressed LoVo and HCT116 cells exhibited decreased colony formation rates compared with the control cells. D, the effect of miR-204 on tumor formation in a nude mouse xenograft model. LoVo (2 \( \times 10^6 \)) and HCT116 (1 \( \times 10^6 \)) cells stably expressing miR-204 or the control were injected subcutaneously into the right flank of each nude mouse. The growth and tumor weight of the miR-204 group were significantly decreased compared with the control. NC, negative control.
then conducted to determine the influence of miR-204-5p on the expression of these 4 genes. The results revealed that miR-204-5p could inhibit the expression of the reporter gene in recombinant plasmids containing the 3’UTRs of RAB22A and BCL2, particularly RAB22A (Fig. 4A). To test whether RAB22A is a direct target of miR-204-5p, the wild-type and mutant RAB22A 3’UTRs were independently cloned into p-Luc (Fig. 4B). As shown in Fig. 4C, the mutant RAB22A 3’UTR was completely refractory to the miR-204-5p-mediated luciferase reporter repression in both HEK-293T and HCT116 cells. In concordance with these results, RAB22A protein expression was significantly decreased in miR-204-5p–overexpressed colorectal cancer cells and enhanced in miR-204-5p–depleted cells (Fig. 4D). In addition, although about 20% decreased luciferase activity was observed in the recombinant plasmid containing the BCL2 3’UTR (Fig. 4A), we did not observe significant down-regulation of BCL2 protein in miR-204-5p–overexpressed colorectal cancer cells (data not shown).

**RAB22A expression is increased in colorectal cancer and inversely correlated with miR-204-5p expression and survival**

To further study the relationship between miR-204-5p and RAB22A in human colorectal cancer, we assessed the RAB22A protein expression in 140 paired colorectal cancer and NCT samples using HIC (Supplementary Fig. S4A). As indicated in Fig. 5A and B, 93 of 140 (66.4%) tumors showed increased RAB22A expression compared with paired NCTs. The RAB22A protein levels in the colorectal cancer tissues were inversely correlated with the miR-204-5p levels (P < 0.001, Fig. 5C), suggesting that miR-204-5p acts as a regulator of RAB22A expression in clinical colorectal cancer tumors.

Survival analyses revealed that increased RAB22A protein levels (score 2 or 3) were associated with shorter survival time (P = 0.019, Fig. 5D). After adjusting for age, gender, nodal status, tumor differentiation, tumor size, and tumor stage, multivariate analyses showed that RAB22A expression was an independent risk factor for survival. Tumors with higher RAB22A expression presented a higher risk of death (HR, 4.229; 95% CI, 2.018–8.861, P < 0.0001). To further validate these results, the impact of RAB22A on prognosis was evaluated in an independent test cohort. RAB22A detection was available in 132 tumors and 106 NCTs, and more than half of tumors (58.5%) showed increased RAB22A expression compared with paired NCTs (Supplementary Fig. S4B). The negative correlation between RAB22A expression and survival time was confirmed using both univariate (P = 0.002, Fig. 5D) and multivariate analyses (HR, 2.695; 95% CI, 1.258–5.777, P = 0.011). Collectively, RAB22A appears to be a novel prognostic factor for colorectal cancer.

**Role of miR-204-5p in tumorigenesis via directly targeting RAB22A**

To determine the functional significance of RAB22A in the miR-204-5p–induced phenotype, we performed a series of restoration assays using HCT116 and LoVo cells. We used siRNA to knockdown RAB22A expression, and a construct containing the RAB22A ORF to increase RAB22A expression (Supplementary Fig. S5A–S5C). As for the cell proliferation assay, siRNA-mediated RAB22A silencing could phenocopy the proliferation-repressing effect of miR-204-5p, whereas anti-miR-204-5p could not restore cell proliferation in RAB22A-depleted colorectal cancer cells (Fig. 6A). Furthermore, we revealed that RAB22A ORF overexpression could significantly abrogate the inhibitory effect of miR-204-5p on cell proliferation (Fig. 6B). Likewise, RAB22A overexpression could restore miR-204-5p–induced apoptosis and migration-repression in colorectal cancer cells (Fig. 6C and D). Taken together, these results imply that miR-204-5p exerts tumor-suppressive function in colorectal cancer via directly targeting RAB22A.

**Discussion**

As important gene regulators, miRNAs are predicted to regulate more than 60% of human protein-coding genes (30). According to the latest version of miRBase (Release 20: June 2013), 2,578 human mature miRNAs have been annotated; however, the functions and molecular mechanisms of most of these miRNAs remain unknown.

We and other groups have shown that miR-204 is downregulated in multiple human cancers (7, 15–23). CpG methylation represents an important mechanism responsible for the inactivation of genes, including miRNAs. miR-204 is an intrinsic miRNA located in the TRPM3 gene. We observed that the promoter region of TRPM3/miR-204 is frequently hypermethylated in colorectal cancer cell lines and colorectal cancer tissues. Aberrant promoter methylation of TRPM3/miR-204 was also observed in NCT tissues but not in normal colonic epithelia, suggesting that TRPM3/miR-204 methylation is likely to be an early event in colorectal cancer tumorigenesis. Expression of miR-204-5p is in proportion to that of TRPM3 in colorectal cancer cells, suggesting that miR-204 and TRPM3 share common regulatory mechanisms, consistent with previous reports (21, 28). In addition, CNV also results in aberrant expression of genes, including miRNAs (12). Imam and colleagues reported that the 9q21.12 chromosomal region containing miR-204 is frequently lost in ovarian cancers, breast cancers, and pediatric renal tumors (31). We observed no significant changes in the copy numbers of the miR-204 gene between the colorectal cancer and NCT samples, suggesting that CNV may not be a key mechanism leading to the downregulation of miR-204. These findings must be confirmed in large colorectal cancer cohorts.

miR-204 has been reported to function as a tumor suppressor in a variety of human cancers through different mechanisms (15–23), suggesting its extensive function in tumorigenesis. For example, miR-204 inhibited tumor growth in renal clear cell carcinoma (17) and pancreatic cancer (32) and suppressed invasion in endometrial cancer (23), glioma (21), gastric cancer (16), intrahepatic...
cholangiocarcinoma (16), and head and neck tumor (18). In addition, miR-204 could affect chemoresistance in neuroblastoma and gastric cancer cells by targeting BCL2 (22, 33). Our data showed that miR-204-5p inhibited cell proliferation and metastasis of colorectal cancer both in vitro and in vivo. In addition, miR-204-5p enhanced apoptosis in colorectal cancer and sensitized colorectal cancer cells to multiple drugs. These data indicate a key role for miR-204-5p in colorectal cancer tumorigenesis and its potential application for colorectal cancer treatment. Previous reports have shown the involvement of miR-204-5p in epithelial-to-mesenchymal transition (EMT) in gastric cancer and intrahepatic cholangiocarcinoma cells (16). Interestingly, we observed that the overexpression of miR-204-5p inhibited EMT in LoVo cells but not in HCT116 cells (data not shown), suggesting that the involvement of miR-204-5p in EMT of colorectal cancer may not be a general phenomenon.

In the subsequent mechanistic study, we demonstrated that miR-204-5p directly targets RAB22A to inhibit proliferation and invasion and enhance apoptosis in colorectal cancer. In miR-204-5p–transfected cells, ectopic RAB22A expression can rescue the proliferation ability attenuated by miR-204-5p, whereas RNAi-mediated knockdown of RAB22A phenocopied the proliferation-promoting effect of miR-204-5p. Li and colleagues performed preliminary luciferase reporter assay to validate the targeting of miR-204-5p to the 3′ UTRs of RAB22A under stress conditions in colorectal cancer. A, initial screening of miR-204-5p target genes using a microarray assay, bioinformatics predictions, and the luciferase reporter assay. Four downregulated genes (BCL2, CCPG1, KLF12, and RAB22A) were selected from the 15 genes in the initial screening based on the functional analysis of these genes, and their 3′ UTRs were assessed using the luciferase reporter assay. Luciferase activity was determined 48 hours after transfection and normalized to Renilla luciferase activity. B, the putative miR-204-5p binding sequence in the RAB22A 3′ UTR. A mutation was generated in the site complementary to the miR-204-5p seed region of the RAB22A 3′ UTR, as indicated. C, analyses of the luciferase activity of the luciferase reporter plasmids containing either wild-type (WT) or mutant-type (MT) RAB22A 3′ UTRs in HEK-293T and HCT116 cells. D, the protein levels of RAB22A were determined by Western blotting in LoVo and HCT116 cells transfected with miR-204-5p mimic, miR-204-5p inhibitor, or the corresponding negative control (NC). β-Actin served as an internal control.

Figure 4. Screening for candidate target genes of miR-204-5p in colorectal cancer. A, initial screening of miR-204-5p target genes using a microarray assay, bioinformatics predictions, and the luciferase reporter assay. Four downregulated genes (BCL2, CCPG1, KLF12, and RAB22A) were selected from the 15 genes in the initial screening based on the functional analysis of these genes, and their 3′ UTRs were assessed using the luciferase reporter assay. Luciferase activity was determined 48 hours after transfection and normalized to Renilla luciferase activity. B, the putative miR-204-5p binding sequence in the RAB22A 3′ UTR. A mutation was generated in the site complementary to the miR-204-5p seed region of the RAB22A 3′ UTR, as indicated. C, analyses of the luciferase activity of the luciferase reporter plasmids containing either wild-type (WT) or mutant-type (MT) RAB22A 3′ UTRs in HEK-293T and HCT116 cells. D, the protein levels of RAB22A were determined by Western blotting in LoVo and HCT116 cells transfected with miR-204-5p mimic, miR-204-5p inhibitor, or the corresponding negative control (NC). β-Actin served as an internal control.
Figure 5. RAB22A protein levels were overexpressed in colorectal cancer and inversely correlated with miR-204-5p levels. A, IHC staining of RAB22A in 140 tumor tissues and adjacent NCTs. Brown cytoplasmic RAB22A staining was observed in colorectal cancer cells but was nearly absent in normal colonic epithelia. B, RAB22A protein expression was frequently increased in the tumor tissues compared with the matched NCTs (66.4%). C, the expression levels of RAB22A were negatively correlated with the miR-204-5p expression levels in the colorectal cancer tissues ($P = 0.0005$). The median value of miR-204-5p levels in 140 colorectal cancer tissues was used to divide these cases into 2 groups with low or high miR-204-5p levels, respectively. D, survival analysis based on the expression levels of RAB22A in 2 different CRC cohorts. The groups were ranked according to the RAB22A staining intensity. The percentage of overall survival in low RAB22A expression (scored 0 or 1) was significantly higher than that of patients with high RAB22A expression (scored 2 or 3; $P = 0.019$ for the training set, $P = 0.002$ for the testing set).
human trabecular meshwork cells without further study (34).

RAB proteins, including more than 60 members in mammals, constitute a Ras superfamily of GTPases (35). They are usually activated by binding GTP in the transport vesicles and then hydrolysed to generate GDP-bound RABs after membrane fusion (36). Recent data suggest an emerging role for RAB GTPases in human cancer (37). RAB22A is a less studied member of the RAB family that plays a role in the endocytic pathway (38). Although RAB22A has recently been reported to be upregulated in hepatocellular carcinoma, cholangiohepatoma (39), and melanoma (40), little is known about its role in human tumorigenesis. Here, we revealed that increased RAB22A expression in colorectal cancer predict poor prognosis and showed that RAB22A overexpression could significantly increase cell growth, migration, and resistance to chemotherapy-induced apoptosis. These data suggest, for the first time, that RAB22A is a potential new oncogene and prognostic factor for colorectal cancer. The detailed role and mechanism by which RAB22A promotes colorectal cancer development and progression should be investigated in future work.

Resistance to conventional chemotherapy remains a significant obstacle in the successful treatment of colorectal cancer. Most chemotherapeutic agents, including oxaliplatin, 5-FU, DDP, and adriamycin, ultimately kill tumor cells by inducing apoptosis, regardless of distinct antitumor mechanisms (41). miR-204-5p could promote the
sensitivity of colorectal cancer cells to the most commonly used drugs for colorectal cancer by targeting RAB22A, suggesting a central role for miR-204-5p/RAB22A signaling in apoptosis regulation. This pathway could be a promising new target for preventive and therapeutic strategies of colorectal cancer.

In conclusion, we have identified an important tumor-suppressive miRNA, miR-204-5p, that is frequently down-regulated in human colorectal cancer. miR-204-5p plays key roles in colorectal cancer development and progression, by repressing colorectal cancer cell growth, migration, and invasion as well as by promoting colorectal cancer cell sensitivity to chemotherapeutic drugs through directly targeting RAB22A. We also demonstrated that miR-204-5p and RAB22A are potential prognostic factors for colorectal cancer. Thus, our study demonstrates the importance of miR-204-5p/RAB22A signaling in colorectal cancer tumorigenesis and suggests that targeting the signaling may represent a new therapeutic approach for human colorectal cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


miR-204-5p Inhibits Proliferation and Invasion and Enhances Chemotherapeutic Sensitivity of Colorectal Cancer Cells by Downregulating RAB22A

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