MicroRNA-221 promotes colorectal cancer cell invasion and metastasis by targeting RECK

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Abstract

MicroRNAs (miRNAs) have recently emerged as regulators of metastasis. We provide insight into the behavior of miR-221 in colorectal cancer (CRC) metastasis by showing that miR-221 is significantly upregulated in metastatic CRC cell lines and tissues. MiR-221 overexpression enhances, whereas miR-221 depletion reduces CRC cell migration and invasion in vitro and metastasis in vivo. We identify RECK as a direct target of miR-221, reveal its expression to be inversely correlated with miR-221 in CRC samples and show that its re-introduction reverses miR-221-induced CRC invasiveness. Collectively, miR-221 is an oncogenic miRNA which may regulate CRC migration and invasion through targeting RECK.

1. Introduction

Colorectal cancer (CRC) causes more than 600,000 deaths annually, with more than 1 million cases diagnosed each year [1]. As for all cancers, the overwhelming cause of death from CRC is metastasis, a complex series of multiple sequential steps during which primary cancer cells acquire an invasive phenotype enabling them to translocate from the primary tumor to a distant organ and form a secondary tumor [2]. Understanding the molecular mechanisms underlying CRC metastasis is therefore essential for developing therapies for CRC patients.

MicroRNAs (miRNAs) are emerging as regulators of metastasis by acting on multiple signaling pathways [3]. MiRNAs are a class of small cellular RNAs that regulate post-transcriptionally protein-coding genes by pairing with their 3′ untranslated regions (UTRs), thereby inhibiting translation or causing target degradation [4]. Imperfect target binding allows miRNAs to posttranscriptionally regulate entire sets of genes, making miRNAs attractive candidate upstream regulators of metastatic progression, both as promoters and suppressors [5]. In particular, CRC invasion and metastasis are stimulated by miR-21 and miR-103/107 by downregulating tumor suppressors PDCD4, and DAPK and KLF4, respectively, but inhibited by miR-137 and miR-30a by downregulating oncogenes FMNL2 and PIK3CD, respectively [6–9].

MiR-221 has been reported to be deregulated in a variety of tumor types. It is encoded in tandem from a gene cluster on the X chromosome and has been shown to target critical cancer-related pathways in various cellular contexts, including CRC. It has been well studied in its oncogenic role as a suppressor of key cell cycle inhibitors p27(Kip1) and CDKN1C/p57 [10–12]. MiR-221 is also overexpressed in human CRC tissues where it has been shown to promote cell proliferation in vitro by inhibiting cell cycle inhibitor CDKN1C/p57 in CRC cells [12,13]. However, its role in metastatic CRC processes remains unexplored.

Reversion-inducing cysteine-rich protein with Kazal motifs (RECK) is a tumor suppressor that inhibits matrix metalloproteinases (MMPs) −2, −9 and −14 and is involved in breaking down the extracellular matrix [14,15]. RECK downregulation has been described in a number of cancers [16–18], and restored expression of RECK decreased tumor angiogenesis, invasion and metastasis in vitro and in vivo [19]. Studies on RECK regulation may therefore reveal further insights into the mechanisms of metastasis.

In this study, we explored the possibility that miR-221 may be involved in CRC cell invasion and metastasis and partially elucidated the molecular mechanism underlying its effects. We showed that miR-221 promoted metastasis in vitro and in vivo and identified RECK as a direct and functional target for miR-221 in cell invasion, thus providing a candidate target for CRC treatment.
2. Materials and methods

2.1. Cell lines and clinical specimens

Human CRC cell lines SW480, HCT116, HT29, LoVo and SW620 were obtained from the Cell Bank of Type Culture Collection (Shanghai, China). All cell lines were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics. Ten matched normal colon tissue, primary CRC and lymph node metastatic samples were collected directly after surgical resection at Renji Hospital (Shanghai Jiaotong University School of Medicine, Shanghai, China). All samples were immediately frozen in liquid nitrogen and stored at -80°C until use. Approval for this study was obtained from the Ethics Review Committee of the Institutional Review Board of Renji hospital and informed consent was obtained from each patient.

2.2. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using Quizol reagent (Qiagen, Valencia, CA). Mature miR-211 and U6 levels were quantified with TaqMan miRNA assays (Applied Biosystems, Foster City, CA). RECK and β-actin mRNA levels were determined by qRT-PCR using the SYBR Green Master Mix on the HT 7500 System (Applied Biosystems). The PCR primers for RECK were 5'-AGCAACC-GAGCCCGTATGT-3' and 5'-CCGAGTAGGCAGCACACA-3'. The relative expression levels of each gene were calculated and normalized using the 2-ΔΔCt method relative to U6 or β-actin. All of the reactions were run in triplicate.

2.3. Lentivirus infection and oligonucleotide transfection

Pre-miR-221 and RECK encoding sequences were subcloned into the pCDH-CMV-MCS-EF1-coGFP constructs (System Biosciences, Mountain View, CA). RECK siRNA sequences were subcloned into the pLKO.1 vector (Addgene, Cambridge, MA). Lentiviral vectors were prepared in accordance with standard protocols. SW480 cells were infected with the recombinant lentivirus-transducing units plus 8 µg/ml polybrene (Sigma, St Louis, MO). The miR-221 oligonucleotide inhibitor (anti-miR-221) and its negative control (anti-miR-ctrl) were synthesized by Ribobio (Guangzhou, China). SW620 cells were transfected with oligonucleotides using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Cells were collected 48 h after transfection.

2.4. Vector construction and luciferase reporter assay

The full-length RECK 3'UTR was amplified from normal human cDNA and cloned into the NotI/XhoI sites downstream of the stop codon of Renilla luciferase in the pGL-control vector (Promega, Madison, WI). The corresponding mutant constructs were created by mutating the seed regions of the miR-221-binding sites. For the luciferase reporter assay, stably miR-221 or miR-ctrl expressing HEK293T or SW480 cells were co-transfected with 200 ng of wild-type 3' UTR or mut 3' UTR and 10 ng pRL-SV40 Renilla luciferase construct. After 48 h, the cells were harvested and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). The Renilla luciferase activities were used as an internal control. The experiments were performed independently in triplicate.

2.5. Wound healing and invasion assays

Artificial wounds were created by using a sterile 200 µl tip to scrape SW480 and SW620 monolayers that had been serum starved for 24 h after reaching near confluence in 6-well plates. Cells were washed with serum-free medium to remove floating cells and debris. Representative images (40×) of cells migrating into the wounds were captured at 0 h and 48 h using an inverted microscope. Cell invasion was determined using 24-well transwell chambers coated with Matrigel (BD Biosciences, Bedford, MA). Cells (1 × 10⁵) in serum-free medium were placed into the upper chamber. The chambers were then inserted into the wells of a plate incubated for 24 h in DMEM with 10% fetal bovine serum. Cells which had invaded to the lower surface were fixed, stained and counted using an inverted microscope (100×). All experiments were performed in triplicate.

2.6. Western blotting

Cells were lysed with RIPA lysis buffer containing protease inhibitor. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Blots were blocked and then probed with antibodies against RECK (Cell Signaling Technology, Beverly, MA) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies and visualized using super enhanced chemiluminescence detection reagent (Amersham Biosciences, Piscataway, NJ).

2.7. In vivo models

All mouse experiments were conducted with approval from Shanghai Jiaotong University Animal Care Committee. SW480/miR-221 or SW620/anti-miR-221 cells were resuspended (1 × 10⁶ cells/0.1 ml PBS) and injected into the tail vain of 6-week old BALB/c nude mice (six in each group). The mice were housed and maintained under specific pathogen-free conditions. Liver metastasis was examined 8 weeks after implantation.

2.8. Statistical analysis

Data were expressed as the mean ± S.D. from at least 3 independent experiments. The difference between groups was analyzed using Student’s t-test when comparing only two groups or one-way analysis of variance when comparing more than two groups. The association between miR-221 and RECK expression was evaluated using Spearman’s correlation analysis. P values <0.05 were considered to be significant.

3. Results

3.1. miR-221 is upregulated in metastatic CRC cell lines and clinical specimens

To probe miR-221’s involvement in CRC metastasis, we measured miR-221 transcript levels in a panel of CRC cell lines of different metastatic potential. qRT-PCR analysis showed that miR-221 levels were higher in metastatic CRC cells (SW620 and LoVo) than in nonmetastatic ones (SW480, HCT116 and HT29) (Fig. 1A). We then explored the clinical relevance of this finding by comparing miR-221 expression in normal colon tissues with primary CRC...
and lymph node metastatic tissues. In keeping with previous studies [12,13], miR-221 expression was significantly higher in primary CRC tissues compared to normal colon tissues. We extended our investigations to lymph node metastatic tissues and found that its miR-221 expression was in turn significantly higher than that of primary CRC tissues (Fig. 1B). These findings suggest that upregulation of miR-221 might play a role in CRC metastasis.

### 3.2. miR-221 modulates CRC cell migration and invasion in vitro

Given its increased expression in metastatic CRC cells, we next investigated whether miR-221 could regulate CRC cell migration and invasion in vitro. To do this, we first established miR-221 stably expressing SW480 cells by lentivirus infection. The wound healing assay demonstrated that miR-221 overexpression significantly increased the mobility of SW480 cells (Fig. 2A). Conversely, knockdown of miR-221 in SW620 cells decreased wound healing (Fig. 2B). Similarly, in the transwell invasion assay, the invasion ability of SW480 cells increased upon miR-221 overexpression while the invasion ability of SW620 cells decreased upon miR-221 knockdown (Fig. 2C and D). These observations suggest that miR-221 significantly promotes in vitro migration and invasion of CRC cells.

### 3.3. miR-221 promotes liver metastasis in vivo

To further explore the role of miR-221 on tumor metastasis in vivo, SW480 cells stably expressing miR-221 were transplanted into nude mice through the lateral tail vein. At 8 weeks after implantation, we found that miR-221 overexpression significantly increased the number of metastatic nodules in the liver, while miR-221 knockdown decreased the number of liver metastases in mice treated with SW620 injection (Fig. 3A and B). These findings add further evidence to miR-221’s role as a metastasis promoter in CRC.

### 3.4. RECK is a direct target of miR-221

To characterize the mechanism by which miR-221 promotes tumor metastasis, we searched for potential target genes of miR-221 using two publicly available databases, TargetScan and miRanda. We were particularly interested in MMP inhibitor and tumor suppressor RECK because in addition to its anti-invasive properties [15], its mRNA and protein expression are negatively correlated with survival in colorectal cancer [20]. Our analysis of the 3’ UTR sequence of RECK identified a possible binding site for miR-221 (Fig. 4A). To test the function of this potential binding site, we inserted wild-type or mutant 3’ UTR sequences immediately downstream of the luciferase reporter gene and co-expressed these with either miR-221 or miR-ctrl in both HEK293 and SW480 cells. As shown in Fig. 4B, miR-221 overexpression caused a clear decrease in relative luciferase activity, whereas activity did not drop at all in the mutant 3’ UTR reporter, indicating that functionality depends on the intact seed sequence. Furthermore, qRT-PCR and western blotting analyses showed that miR-221 overexpression significantly reduced the levels of RECK mRNA and protein in SW480 cells, while miR-221 knockdown increased RECK levels (Fig. 4C and D). Together, these results strongly support a direct suppression of RECK by miR-221 by means of mRNA degradation as well as translational repression.

### 3.5. RECK mediates miR-221-induced invasiveness in CRC cells

To explore the interaction between miR-221 and RECK during metastasis, we performed gain-of-function and loss-of-function studies. As has been shown previously for RECK expression in transformed NIH/3T3 fibroblasts [15], ectopic RECK expression in metastatic CRC cell line SW620 reduced its invasive behavior (Fig. 5A). Conversely, a reduction of RECK protein levels, either by siRNA inhibition (Fig. 5B) or miR-221 expression (Fig. 5C) increased CRC cell line SW480 invasiveness. However, cell invasiveness returned to control levels when RECK is replenished (Fig. 5C), suggesting that miR-221 induced invasiveness by inhibiting tumor suppressor RECK.

### 3.6. Inverse correlation between miR-221 and RECK in CRC tissues

Finally, we measured the levels of RECK mRNA in primary CRC and matched lymph node metastatic tissues from CRC patients. As shown in Fig. 6A, RECK levels were lower in metastatic tissues than in primary CRC tissues. We then correlated RECK with miR-221 expression in the same CRC specimens. A statistically significant inverse correlation was observed between mRNA levels of RECK and miR-221 (R = -0.875, P < 0.001). This provides further evidence of a potential suppression of RECK protein levels by miR-221 in metastatic CRC tissues.

### 4. Discussion

It is becoming increasingly evident that miRNA deregulation contributes to invasion and metastasis [21,22]. Specifically for CRC, it is already known that miRNAs regulate diverse cellular behaviors controlling invasion and metastasis. Known oncogenic miRNA targets operate at the levels of transcription (miR-103/107 target KLF4), translation (miR-21 target PDCD4) and cell
motility (miR-103/107 target DAPK) [6,7]. Known tumor suppressor miRNA targets operate at the levels of cell adhesion and motility (miR-137 target FMNL2) and cell signaling (miR-30a target PIK3CD and miR-139 target IGF-1R) [8,9,23]. This study adds miR-221 to the growing group of CRC metastasis-promoting miRNAs and provides evidence for its targeting of MMP inhibitor RECK to promote cell invasion.

miR-221 expression is upregulated in CRC, but prior to this study, apart from its ability to suppress cell proliferation inhibitor CDKN1C/p57 in vitro, the functional relevance of its upregulation in CRC was largely unexplored [12,13]. miR-221 has been extensively studied for its role in regulating key cell cycle inhibitors p27(Kip1) and CDKNC/p57 and is upregulated in a variety of tumors, including hepatocellular carcinoma, bladder cancer and pancreatic cancer [10–12,24–26]. Its invasion promoting effects have been documented for various cancers, including breast cancer and glioma [27,28]. In this study we confirm previous reports that miR-221 is also upregulated in CRC samples and reveal even higher expression levels in metastatic CRC specimens [12,13]. This expression trend is also apparent in the cell line SW620, the metastatic derivative SW480, implying an association between CRC metastasis and increased miR-221 levels. Indeed, Sun and co-workers already observed that miR-221 expression in CRC tumors is higher in advanced TNM stages III and IV and in tumors invading adjacent...
tissue, implying an involvement in tumor invasiveness [12]. We subsequently showed that ectopic miR-221 expression significantly promoted CRC cell invasion and metastasis in vitro and in vivo. Conversely, knockdown of miR-221 reduced cell invasiveness. Interestingly, the passenger strand of miR-221 (miR-221\(^\ast\)) was downregulated as opposed to upregulated in CRC and its overexpression suppressed CRC growth and metastasis in mice [29]. Because miR-221 and its passenger strand are transcribed as one entity, a downregulation of miR-221\(^\ast\) in CRC might reflect a post-transcriptional event specific to the passenger strand’s nucleotide sequence.

To understand the biological role of miR-221 in CRC invasiveness and metastasis, we searched for relevant downstream targets. In a number of cancers, miRNAs regulate cell invasion by targeting MMP suppressor RECK [30–32]. It has been previously reported that RECK expression is low in CRC and is a prognostic marker of low survival in CRC [20]. In this study, we extend these observations to show that RECK expression in malignant CRC cell line SW620 suppresses invasive activity, as has been shown previously in transformed NIH/3T3 fibroblasts [15]. We demonstrated direct binding of miR-221 to the RECK 3’ UTR and showed that miR-221 overexpression diminished but miR-221 knockdown increased RECK mRNA and protein levels in CRC cells. We next established a functional connection between miR-221 and RECK in CRC metastasis by showing that ectopically expressed miR-221’s ability to induce invasiveness is compromised by ectopic RECK expression, indicating that RECK suppression is required for miR-221’s invasive effects. The inverse correlation of miR-221 expression and RECK protein expression in primary CRC tumors provides further evidence for miR-221’s function in metastasis as an inhibitor of tumor suppressor RECK.

To our knowledge, the present study identifies for the first time RECK as a target of miR-221 and demonstrates that miR-221
promotes metastasis in CRC, possibly as a result of targeting RECK. As an MMP inhibitor, RECK is implicated in the control of tissue degradation and invasive growth, processes central to the epithelial-mesenchymal transition in tumor progression. This work adds miR-221 to the growing group of CRC metastasis-promoting miRNAs and provides insight into the molecular mechanisms of its metastatic function. Targeting the miR-221 interaction with RECK would be a helpful therapeutic approach to blocking CRC metastasis.

References


Fig. 6. Inverse correlation between miR-221 and RECK in CRC tissues. (A) Expression of RECK in primary CRC and its matched lymph node metastatic tissues by qRT-PCR (n = 10). (B) Inverse correlation between miR-221 and RECK in CRC tissues determined by Spearman’s correlation analysis (R = −0.875, P < 0.001).