

Original Paper

miR-30a Suppresses Cell Migration and Invasion Through Downregulation of PIK3CD in Colorectal Carcinoma

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Key Words

miR-30a • Colorectal carcinoma • Migration • Invasion • PIK3CD

Abstract

Background/Aims: MicroRNAs (miRNAs) play key roles in tumor metastasis. The aim of this study was to determine the regulation and function of miR-30a in colorectal carcinoma (CRC) metastasis. **Methods:** The expression of miR-30a was detected in CRC cell lines and samples by qRT-PCR. The anti-metastatic effect of miR-30a was determined by both *in vitro* and *in vivo* assays. A luciferase reporter assay was performed to determine target association between miR-30a and phosphoinositide 3-kinase catalytic subunit delta (PIK3CD). **Results:** miR-30a was significantly downregulated in highly metastatic CRC cell lines and metastatic tissues. Overexpression of miR-30a suppressed CRC cell migration and invasion *in vitro* and liver metastasis *in vivo*, whereas miR-30a deletion dramatically promoted cell migration and invasion. Further studies revealed that PIK3CD is a direct target of miR-30a as miR-30a binds directly to the 3'-UTR of PIK3CD, subsequently reducing its expression. Similar to the restoring miR-30a expression, PIK3CD downregulation inhibited cell migration and invasion, whereas PIK3CD overexpression rescued the suppressive effect of miR-30a. Moreover, significant downregulation of miR-30a in metastatic CRC tissues was found to be inversely correlated with PIK3CD expression. Mechanistic studies revealed that miR-30a down-regulated the expression of key components of the Akt/mTOR pathway, whereas PIK3CD overexpression reversed this negative effect. **Conclusion:** Our findings indicate that miR-30a might function as a metastasis suppressor in CRC. miR-30a may be a potential therapeutic target to block CRC metastasis.

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Introduction

Colorectal carcinoma (CRC) is the third leading cause of cancer-related deaths worldwide [1]. Despite achievements in the treatment in the few past decades, CRC remains a major public health concern, resulting in more than 600,000 deaths each year. The major cause of death and relapse from CRC is metastasis, however, promising therapy for CRC metastasis is not available. Given this, understanding the molecular mechanisms that promote metastasis in CRC is of crucial significance to the development of therapeutic strategies for CRC patients.

MicroRNAs (miRNAs) are a recently discovered class of small noncoding RNA molecules that modulate gene expression [2]. Accumulating evidence indicates that aberrant expression of miRNAs correlates with various human cancers, and that miRNAs can act as tumor suppressors or oncogenes [3, 4]. Several studies have also reported that miRNAs are implicated in both the suppression and promotion of tumor metastasis [5]. For example, miR-143 inhibits cell invasion and metastasis in osteosarcoma, colorectal cancer and pancreatic cancer [6-8]; miR-139 targets IGF1R and inhibits *in vitro* and *in vivo* invasion and metastasis in colorectal cancer [9]; and miR-25 promotes cell migration and invasion in esophageal squamous cell carcinoma [10]. Recent studies showed that miR-30a is significantly downregulated in colorectal cancers [11-13]. The involvement of miR-30a in the metastasis of breast cancer and non-small cell lung cancer has been reported [14, 15]; however, its roles and mechanism in CRC metastasis remain largely unknown.

In this study, we revealed significant downregulation of miR-30a in metastatic CRC cell lines and tissues. Overexpression of miR-30a suppressed CRC cell metastasis and invasion *in vitro* and *in vivo*, whereas miR-30a knockdown promoted CRC cell migration and invasion. Furthermore, we identified PIK3CD as a direct and functional target of miR-30a. We also confirmed an inverse correlation between miR-30a and PIK3CD expression in human CRC tissues. Our study reveals a crucial role of miR-30a-dependent PIK3CD modulation in CRC metastasis.

Materials and Methods

Cell culture and tumor specimens

The human colorectal cancer (CRC) cell lines, including SW480, CaCo-2, WiDr, HT29, LoVo, HCT116 and SW620, were purchased from American Type Culture Collection. Cells were cultured in RPMI1640 medium (Thermo Scientific HyClone, Beijing, China) supplemented with 10% fetal bovine serum, 100 units/ml of penicillin and 100 µg/ml of streptomycin. The cells were incubated in a 5% CO₂ atmosphere at 37°C. Primary CRC samples and lymph node metastatic tissues were obtained from Renji Hospital, Shanghai Jiaotong University School of Medicine. Studies involving these tissues were approved by the Ethics Review Committee of the Institutional Review Board of the hospital, and written informed consent was obtained from every patient.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesized from 1 µg of total RNA with a reverse reaction kit (Promega, Madison, WI, USA). The expression of PIK3CD and β-actin was quantified by qRT-PCR using SYBR-Green assays (TOYOBO, Tokyo, Japan) as previously reported [16]. To quantify the expression of mature miR-30a, RT-PCR was performed using TaqMan microRNA assays (Applied Biosystems, Foster City, CA, USA) as described previously [17]. U6 snRNA was used as an endogenous control for miRNA detection. The relative expression levels of each gene were calculated and normalized using the 2^{-ΔΔCt} method relative to β-actin or U6 snRNA.

Lentiviral production and infection oligonucleotide transfection

The miR-30a-5p precursor sequence was amplified with the following primers: 5'-TAC GGA TCC CCT TCA TCT TAC TTT TTT CCC CCA A-3' (forward) and 5'-ATC GCT AGC GAA ACT AGA AGC TC GGT GAT GAA

TA-3' (reverse). PIK3CD siRNA was purchased from GeneChem (Shanghai, China). These sequences were separately cloned into the lentivirus-based expression plasmid pLenti6.3 (Invitrogen). Virus packaging and infection were performed according to standard protocols as recommended by the manufacturer. SW620 cells (1×10^5) were infected with 1×10^7 lentivirus transducing units in the presence of 10 $\mu\text{g/ml}$ polybrene (Sigma, St Louis, Missouri, USA). Empty lentiviral vector was used as negative control. The inhibitors anti-miR-30a and anti-control were purchased from Ambion (Austin, TX, USA). SW480 cells were transfected with anti-miR-30a and negative control using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were collected 48 h after transfection.

Plasmid construction

The coding sequences of PIK3CD were cloned into pcDNA3.1 (+) to generate PIK3CD expression vectors. The wild-type PIK3CD 3' UTR was cloned into the pMIR-REPORT luciferase vector (Ambion, Austin, TX, USA). Mutant PIK3CD 3' UTR was generated based on the pMIR-PIK3CD-3' UTR by mutating 3 nt that are recognized by miR-30a. The primers for PIK3CD were: 5'-TGT GGA TCC CTG TCA TCT GGG AAG TAA CAA CGC A-3' (forward) and 5'-AAG TCT AG AGA GCC ACT ACT GCC TGT TGT CTT TG-3' (reverse). The primers for PIK3CD 3' UTR were: 5'-T CTG AGC TCG CTA AAC AGC CAT AAA CGG AAA CGC-3' (forward) and 5'-ACC ACG CGT GCG TA GAT TCT CCT TTA TGG GGC TG-3' (reverse).

Luciferase reporter assays

The reporter plasmid was transiently transfected into SW620 and SW480 cells in the presence of either miR-30a or miR-control. After 48 h, the cells were harvested and lysed, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Renilla-luciferase was used for normalization. The experiments were performed independently in triplicate.

Cell migration and invasion assays

A 24-well transwell plate (Corning, New York, USA) was used to measure each cell line's migratory and invasive ability. For transwell migration assays, 5×10^4 cells suspended in serum-free medium were plated in the upper chamber lined with a non-coated membrane. For invasion assays, chamber inserts were coated with 150 μg of Matrigel (BD Biosciences, Bedford, MD, USA). Then, 5×10^4 cells were plated in the upper chamber. The chambers were then inserted into the wells of a 24-well plate and incubated at 37 °C for 24 h in RPMI-1640 medium with 10% fetal bovine serum before examination. The cells remaining on the upper surface of the membranes were removed, whereas the cells adhering to the lower surface were fixed, stained in a dye solution containing 0.1% crystal violet, air-dried and counted under a microscope (Olympus Corp., Tokyo, Japan) to calculate their relative numbers. Results are presented as an average of triplicate experiments.

Western blot analysis

Cells were lysed with RIPA lysis buffer. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Blots were blocked and then probed with antibodies against PIK3CD (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-Akt (Ser473; 1:400 dilution; Santa Cruz Biotechnology), Akt (1:1000 dilution; Santa Cruz Biotechnology), phospho-mTOR (1:500 dilution; Cell Signaling Technology Inc., Beverly, MA, USA), mTOR (1:1000 dilution; Santa Cruz Biotechnology), β -actin (1:500 dilution; Cell Signaling Technology Inc.). After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies and visualized using super enhanced chemiluminescence detection reagent (Amersham Biosciences, Piscataway, NJ, USA).

Animal studies

Athymic BALB/c nude mice (4–6 weeks old, 18–22 g body weight) were randomly assigned to two groups (SW620-miR-30a or SW620-miR-control, 6 mice per group), and 1×10^6 cells were injected intravenously via tail vein. All mice were euthanized at 6 weeks after the initial injection, and the livers were excised to count the tumor nodules under a stereomicroscope (Olympus). The nude mice were provided by the Experimental Animal Center of Renji Hospital, Shanghai Jiaotong University School of Medicine.

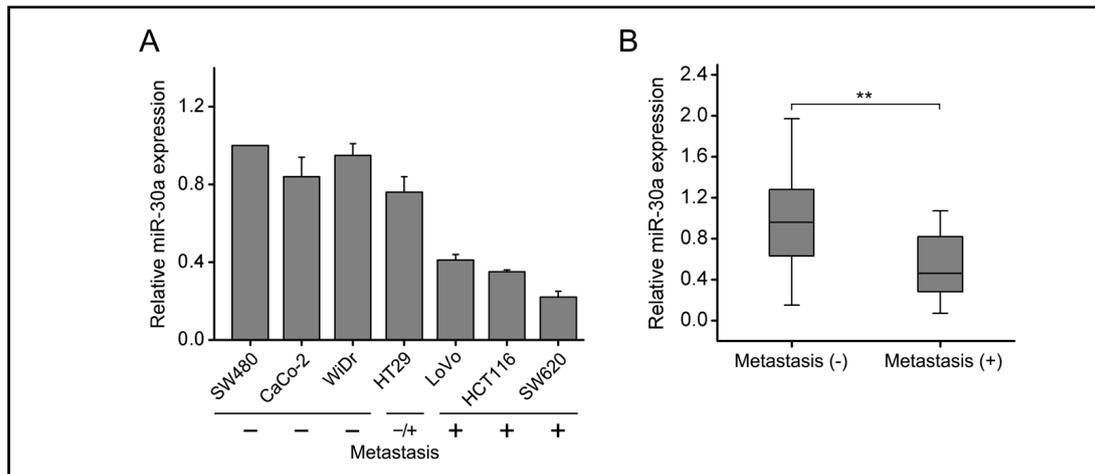


Fig. 1. Downregulation of miR-30a in metastatic CRC cells and lymph node metastatic tissues. (A) qRT-PCR analysis of miR-30a expression in CRC cell lines with differential metastasis potential. (B) miR-30a expression in CRC with or without lymph node metastasis.

All animal studies complied with Shanghai Jiaotong University animal use guidelines and the protocols approved by Shanghai Jiaotong University Animal Care Committee.

Statistical analysis

Statistical analysis was performed using the SPSS13.0 program. Data were expressed as the means \pm SEM of at least three independent experiments. The Student's t-test was used to analyze differences between two groups. The relationship between miR-30a and PIK3CD mRNA expression was explored by Spearman's correlation. Differences were considered statistically significant at $p < 0.05$, * $p < 0.05$ and ** $p < 0.01$.

Results

miR-30a is downregulated in metastatic CRC cells and lymph node metastatic tissues

To gain an insight into the roles of miR-30a in CRC metastasis, the expression levels of miR-30a were first examined in a panel of CRC cell lines showing differential metastasis potential by qRT-PCR. The results showed that inverse correlations between the levels of miR-30a and the metastasis potential of these cells (Fig. 1A). Furthermore, tissues from lymph node metastases showed lower levels of miR-30a than primary CRC tissues, indicating a potential metastasis-suppressive function of miR-30a in CRC (Fig. 1B). Taken together, these results suggest that downregulation of miR-30a is correlated with increased CRC metastasis.

Overexpression of miR-30a suppresses cell migration and invasion in CRC cells

To determine whether miR-30a can affect CRC cell migration and invasion, we stably overexpressed miR-30a in SW620 cells. These cells (with and without miR-30a overexpression) were then used to determine their migratory and invasive ability. As shown in Fig. 2A, overexpression of miR-30a significantly reduced the migration of SW620 cells in Transwell assays without Matrigel and suppressed the invasion of SW620 cells in Transwell assays with Matrigel. In contrast, the migration and invasion of SW480 cells were promoted when endogenous miR-30a was knocked down with antisense oligonucleotides (Fig. 2B). These results demonstrate that miR-30a can suppress CRC cell migration and invasion *in vitro*. To further investigate whether overexpression of miR-30a would act the same *in vivo*, miR-30a-overexpressed and scramble control SW620 cells were injected into nude mice

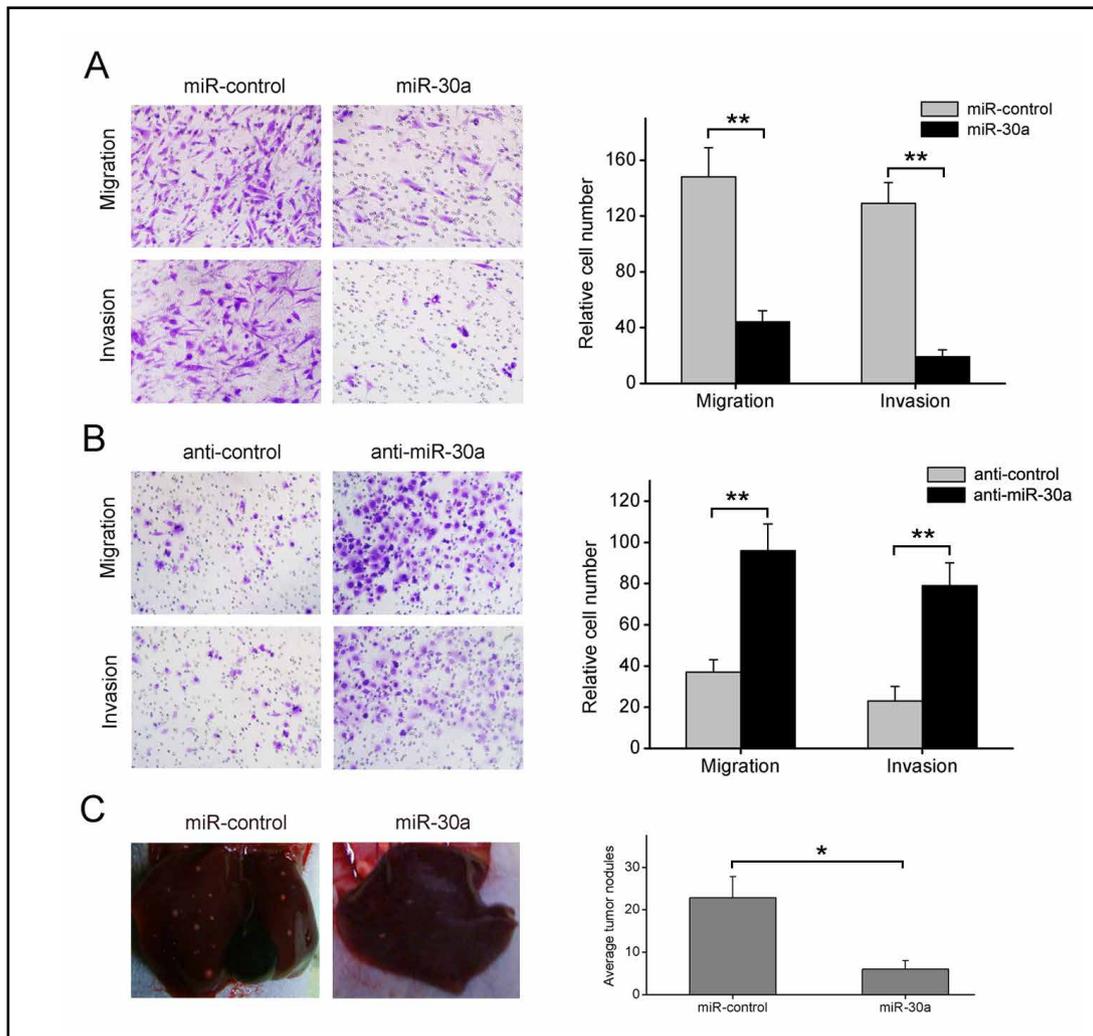


Fig. 2. miR-30a suppresses CRC cell invasion and metastasis *in vitro* and *in vivo*. (A) Overexpression of miR-30a significantly impeded cell migration and invasion in SW620 cells, in comparison with scramble controls. (B) Inhibition of miR-30a expression by transfecting miR-30a inhibitors promoted cell migration and invasion of SW480 cells, compared with scramble controls. (C) miR-30a affects liver metastasis as revealed by the experimental metastasis animal model. Representative images of livers from mice injected with SW620-miR-30a or SW620-miR-control are shown on the left. The number of macroscopic nodules were counted and analysed.

by intravenous tail injections. We found that the mean number of metastatic nodules on the surface of the liver was significantly repressed in SW620-miR-30a groups compared to negative controls (Fig. 2C). Taken together, these results indicate that stable overexpression of miR-30a is able to suppress CRC invasion both *in vitro* and *in vivo*.

PIK3CD is a direct target of miR-30a

To explore the mechanism by which miR-30a inhibits CRC metastasis and invasion, we next aimed to investigate the potential gene targets of miR-30a using target prediction programs, PicTar and TargetScan. Our analysis revealed that PIK3CD was a potential target of miR-30a. The 3'-UTR of PIK3CD mRNA contains a complementary site for the seed region of miR-30a (Fig. 3A). To verify whether or not PIK3CD is a direct target of miR-30a, a human

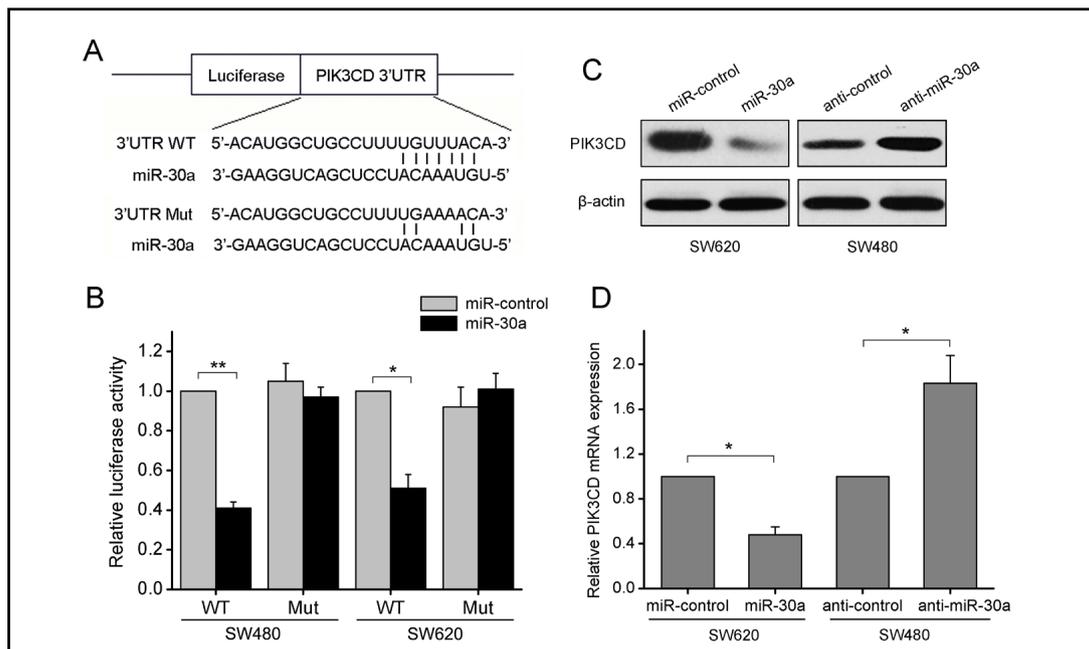


Fig. 3. PIK3CD is a direct target of miR-30a. (A) Putative miR-30a-binding site in the PIK3CD 3'UTR. A mutation was introduced into the PIK3CD 3'UTR sequence by altering 3 nt that are recognized by miR-30a. Wild-type (WT) or mutant PIK3CD 3'UTR (Mut) was subcloned into a dual-luciferase reporter vector. (B) SW620 and SW480 cells were transfected with the luciferase reporter vector containing WT PIK3CD 3'-UTR or Mut PIK3CD 3'-UTR and infected by miR-control or miR-30a. Firefly luciferase activity was determined 48 h posttransfection and normalized to Renilla luciferase activity. (C) PIK3CD protein expression levels in CRC cells infected with miR-control or miR-30a were determined by Western blotting. (D) PIK3CD mRNA expression levels in CRC cells infected with miR-control or miR-30a were determined by qRT-PCR.

PIK3CD 3'UTR fragment containing the wild-type or mutant miR-30a-binding site was inserted downstream of the luciferase open reading frame. These reporter constructs were used to cotransfect SW480 and SW620 cells, which were infected by miR-control-lentivirus or miR-30a-lentivirus. As shown in Fig. 3B, the relative luciferase activity of the reporter containing wild-type PIK3CD 3'-UTR was markedly decreased upon miR-30a cotransfection, whereas the luciferase activity of the reporter containing the mutant binding site was unaffected (Fig. 3B). Furthermore, miR-30a overexpression decreased the levels of PIK3CD mRNA and protein, as determined by qRT-PCR and Western blotting (Figs. 3C and D). These results strongly suggest that PIK3CD is a direct target of miR-30a in CRC cells.

PIK3CD is involved in miR-30a-inhibited CRC cell migration and invasion

To further determine whether miR-30a regulated CRC cell migration and invasion through PIK3CD, we first transfected SW620 cells with siPIK3CD or the negative control. As shown in Fig. 4A, PIK3CD knockdown significantly inhibited SW620 cell migration and invasion. In contrast, PIK3CD overexpression in SW480 cells promoted cell migration and invasion (Fig. 4B). Subsequently, the effect of PIK3CD (without 3'-UTR) were examined in the miR-30a-overexpressed SW480 cells. The Transwell assays indicated that ectopically expressing PIK3CD significantly promoted the migration and invasion of miR-30a-overexpressed SW480 cells (Fig. 4C). To further verify whether miR-30a-induced modulation of PIK3CD is of clinical relevance, we analyzed the correlation between miR-30a and PIK3CD expression in 18 CRC samples with lymph node metastases. As shown in Fig. 4D, when PIK3CD mRNA levels were plotted against miR-30a expression, a significant inverse correlation was observed ($r = -0.782$; $p < 0.001$).

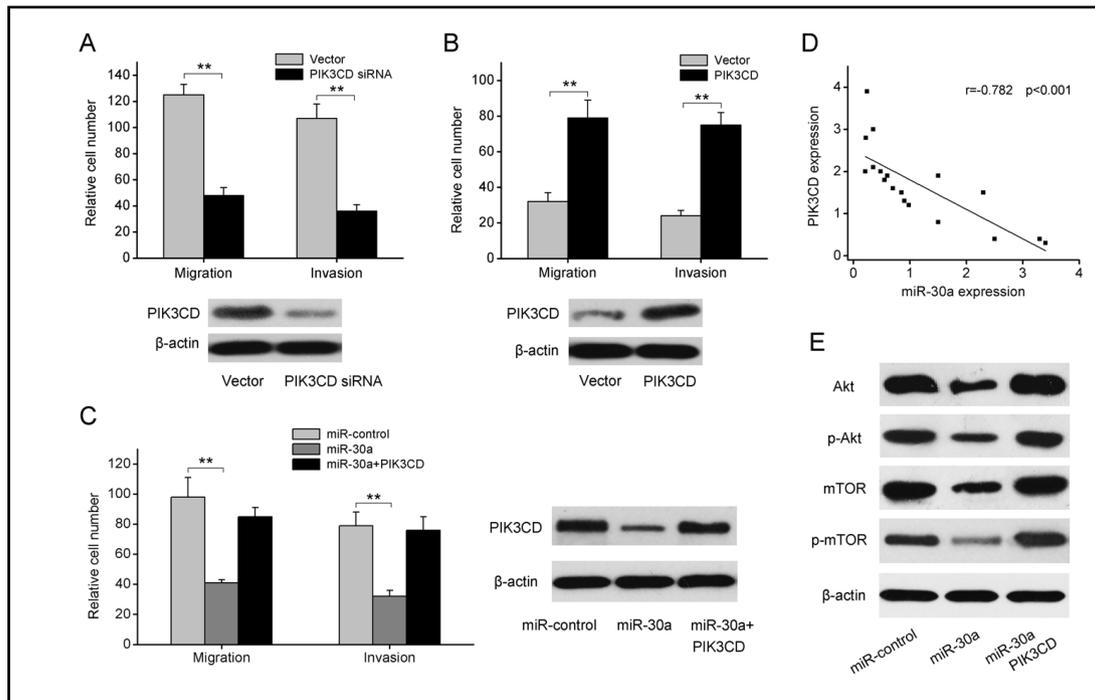


Fig. 4. miR-30a suppresses CRC cell migration and invasion by directly targeting PIK3CD and subsequently affecting PI3K/Akt/mTOR pathway. (A) SW620 cells were infected with PIK3CD siRNA or negative control. Cell migration and invasion were measured by Transwell assays. (B) SW480 cells were transfected with PIK3CD plasmid or vector control, and Transwell migration and invasion assays were then performed. (C) SW620 cells were infected with miR-30a or miR-control, followed by transfection with PIK3CD plasmid, and Transwell migration and invasion assays were then performed. (D) Correlation of miR-30a expression and PIK3CD expression in 18 CRC samples with lymph node metastases. The expression of miR-30a was examined using qRT-PCR analysis and normalized by U6 expression. The expression of PIK3CD was quantified by qRT-PCR and normalized by β -actin expression. (E) Western blotting analysis of the protein levels of Akt and mTOR genes in SW480 cells infected with miR-30a or miR-control or miR-30a infection, in combination with PIK3CD transfection.

miR-30a regulates the Akt/mTOR pathway

Next, we investigated the effect of miR-30a down-regulation of PIK3CD expression on the Akt/mTOR pathway. Analysis of Akt, phospho-Akt (p-Akt), mTOR, and phospho-mTOR (p-mTOR) by Western blotting showed that the levels of these proteins were significantly decreased in miR-30a-overexpressed cells compared to control cells, and this down-regulation was reversed by PIK3CD overexpression (Fig. 4E). These results indicate that miR-30a plays an important role in the regulation of the Akt/mTOR pathway.

Discussion

Metastasis is responsible for most cancer mortality. Therefore, it is essential to identify metastasis-associated molecules and to better understand the mechanism behind cancer metastasis. Recently, mounting evidence indicates that miRNAs are involved in advanced stages of cancer progression and that they can function as activators or suppressors of metastasis, including miR-31, miR-34a and miR-146a [18-20]. However, the functional role of miRNAs in CRC metastasis is still largely unknown. In this study, we investigated the biological role of miR-30a in human CRC metastasis. We found that miR-30a was downregulated in metastatic CRC cells compared with non-metastatic cells. Furthermore, miR-30a expression

was lower in invasive CRC patients with lymph node metastases compared with those of lymph-node-negative patients, suggesting that miR-30a might be involved in both oncogenic transformation and tumor metastasis. Indeed, our results confirm that miR-30a could significantly suppress CRC cell invasion and metastasis both *in vitro* and *in vivo*.

Generally, miRNAs exert their function by regulating expression of their downstream target gene(s). miR-30a has been reported to negatively regulate Beclin 1 mRNA in tumor cells resulting in decreased autophagic activity [21, 22]. Furthermore, the role of miR-30a in inhibition of cellular invasion was recently reported in various cancers, including non-small cell lung cancer, ewing sarcoma and breast cancer [15, 23, 14]. In particular, Baraniskin and colleagues found that miR-30a-5p targets DTL and suppresses tumor growth in colon carcinoma [24]. However, few studies have been performed to determine which genes miR-30a targets to modulate the behavior of CRC metastasis. In this study, we focused on PIK3CD because of its important role as a regulator of cell growth, invasion and migration [25-27]. We found that miR-30a directly bound to the 3'UTR of PIK3CD, which contains a miR-30a-binding site, by dual-luciferase reporter assay. Moreover, miR-30a overexpression significantly down-regulated PIK3CD expression at both mRNA and protein levels. These results suggest that PIK3CD may be a target of miR-30a in CRC cells.

Recently, several miRNAs have been reported to directly target or regulate PIK3CD, including miR-7, miR-384-5p and miR-125b [16, 28, 29]. These studies revealed that multiple miRNAs probably result in the loss of PIK3CD expression in specific types of cancers, and subsequently inhibit the tumorigenesis; however, only miR-7 has been reported to reverse the metastasis of HCC by targeting PIK3CD [16]. In this study, we provide the first evidence that PIK3CD was frequently overexpressed in lymph node metastatic CRC tissues, knockdown of PIK3CD greatly repressed invasion and migration, suggesting a role of PIK3CD in the progression of CRC. Furthermore, we identified PIK3CD as a direct and functional target of miR-30a. miR-30a-induced inhibition of migration and invasion could be rescued by overexpression of PIK3CD. We also showed for the first time that the expression of PIK3CD is inversely correlated with miR-30a levels in metastatic CRC tissues. These results confirm that miR-30a functions as an anti-metastatic miRNA in CRC cells by targeting PIK3CD.

Given that PIK3CD is an important component of the PI3K/Akt pathway, which is frequently activated in human cancers [16, 30, 31], we therefore investigated the effects of miR-30a on the expression of PI3K/Akt/mTOR pathway molecules. We found that overexpression of miR-30a down-regulated the expression of Akt and mTOR and its phosphorylated forms, whereas PIK3CD overexpression reversed this suppressive effect. These results further verify that the inhibitory properties of miR-30a against CRC metastasis is mediated, at least in part, by the inhibition of PI3K/Akt/mTOR signaling pathway. However, our results failed to illustrate the mechanism of PIK3CD through PI3K/Akt/mTOR pathway to reduce CRC metastasis. It is probable that cell apoptosis plays an important role in this process [16]. Further studies of the mechanisms underlying this process are required.

In summary, our study provided the first evidence of the biological role of miR-30a in human CRC metastasis, and identified PIK3CD as a target potentially involved in miR-30a-mediated suppression of CRC cell migration and invasion via the PI3K/Akt/mTOR pathway. These findings may provide the basis for the future use of miR-30a in the treatment of CRC.

Disclosure

All authors are in agreement with the content of this manuscript. The authors declare no conflict of interest.

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