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Original Paper

MiR-30a-5p Suppresses Tumor Metastasis of Human Colorectal Cancer by Targeting ITGB3

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Key Words
MiRNA-30a-5p • Colorectal cancer • Integrin β3 • Metastasis

Abstract
Aims: MicroRNAs (miRNAs) are dysregulated in a wide range of malignant diseases, confirming their crucial role in tumor metastasis. MiRNA-30a-5p, a member of the miR-30 family, has been implicated in many types of cancers, including colorectal cancer, a leading cause of death worldwide. ***Methods:*** qRT-PCR, Western blot, Transwell assay,luciferase reporter assay were performed in the present study. ***Results:*** In this study, miR-30a-5p was found to be significantly downregulated in human colorectal cancer tissue specimens and cell lines compared with non-cancerous tissues and cells. The overexpression of miR-30a-5p inhibited the migratory and invasive abilities of colorectal cancer cells and suppressed the epithelial–mesenchymal transition, a crucial process in metastasis. Bioinformatic algorithms and luciferase reporter assays revealed that integrin β3 (ITGB3) is a direct target of miR-30a-5p. Importantly, overexpression of ITGB3 in colorectal cancer cells rescued these cells from miR-30a-5p-mediated suppression of metastasis and restored the epithelial–mesenchymal transition. ***Conclusion:*** Taken together, our study provides the first evidence that miR-30a-5p suppresses colon cancer metastasis through the inhibition of ITGB3. Thus, targeting miR-30a-5p might serve as a promising therapeutic strategy for the treatment of colorectal cancer.

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Introduction

MiRNAs are evolutionarily conserved, endogenous, non-coding small RNAs (18–25 nucleotides in length) that negatively regulate gene expression by binding to specific target mRNAs, leading to either cleavage or translational inhibition [1]. It is now believed that each

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miRNA is capable of modulating the expression of hundreds of target genes, thereby affecting a diverse range of biological processes [2]. Over the last decade, miRNAs have been recognized as critical regulators in the development and progression of cancer via the modulation of cellular pathways, such as those involved in proliferation [3], differentiation [4, 5], apoptosis [6, 7] and invasion [8-10]. In a number of human cancers, including colon, breast, lung and stomach cancer, as well as leukemia and lymphoma, miRNA expression profiles have been reported to differ significantly between carcinoma cells and corresponding non-carcinoma cells [3, 4, 11]. The dysregulation of miRNA expression in a wide range of malignant diseases has confirmed the crucial role of miRNAs in tumor metastasis [12], however, in many cases it remains to be determined which step(s) in the multistep metastatic process these miRNAs regulate.

In the case of colorectal cancer (CRC), one of the leading causes of death worldwide [13], global miRNA expression analyses have identified multiple dysregulated miRNAs including miRNA-21 [14], miRNA-124 [15], miRNA-625 [16], miRNA-339-5p [17], miRNA-27b [18], although their functional roles are yet to be confirmed experimentally in the majority of cases. It was confirmed that a series of miRNAs, including miR-140-5p [19], miR-149 [20], miR-301α [21], play importat roles in the development of colorectal cancer. In addition, miRNA 30a(miR-30a-5p), a member of the miR-30 family, has been repeatedly reported to be downregulated in a number of cancers including CRC [22-24]. Studies have shown that miRNA-30a can act as a tumor suppressor, inducing growth inhibition and suppression of cell migration and invasion in CRC, by targeting denticleless protein homolog, PIK3CD and insulin receptor substrate 2 [25-27]. miRNAs have also been reported to modulate the expression of integrins, transmembrane receptors involved in signal transduction, and dysfunction in miRNA-regulated integrin signaling has been linked to tumor metastasis and apoptosis in a number of cancers including CRC [28].

The conversion of an epithelial cell to a mesenchymal cell, known as epithelial–mesenchymal transition (EMT) is critical to embryonic morphogenesis. It is also now well-established that EMT plays a specific role in the migration of cells from a primary tumor into the circulation, the process of metastasis, which is often a crucial prognostic factor [29-31]. The loss of epithelial functions and the acquisition of mesenchymal functions during EMT is accompanied by increased migratory and invasive properties. TGF-β and RTK/Ras signaling, autocrine factors and Wnt-, Notch-, Hedgehog- and NF-κB-dependent pathways have been reported to contribute to EMT. Repression of E-cadherin by transcriptional regulators such as Snai1 or Twist is known to be a critical step driving EMT [32]. In fact, reduced E-cadherin protein expression (an epithelial-specific marker) is used as an indicator of EMT, as is increased expression of vimentin (a mesenchymal-specific marker) [33]. A recent report by Liu et al. [10] identified Snai1 as a direct target of miRNA-30a and demonstrated miRNA-30a as a novel regulator of EMT. Similarly, another study reported that miRNA-29b suppresses CRC metastasis by regulating EMT via its target Tiam1 [34].

Here, we investigated further the role of miRNA-30a-5p in CRC progression and the mechanisms underlying the function of miR-30a-5p in CRC metastasis. We identified ITGB3 as a direct target of miR-30a-5p and revealed the relationship between miR-30a-5p, ITGB3 and the EMT.

Materials and Methods

Clinical specimens

Fresh primary CRC specimens (with and without metastases) and noncancerous colorectal tissue were provided by the Tumor Tissue Bank of the Central Hospital of Xingxiang (Xinxiang, Henan, China). In each case, a diagnosis of primary CRC had been made, and the patient had undergone elective surgery for CRC in the Centrol Hospital of Xingxiang between 2007 and 2010. The pathological diagnosis was made in the Department of Pathology of the Centrol Hospital of Xingxiang.

Cell lines and culture conditions

Human colon cancer cell lines (HT29, LoVo, SW116 and SW480), and HEK293T cells, were cultured in RPMI1640 medium (Thermo Scientific HyClone, Beijing, China) supplemented with 10% fetal bovine serum, and the normal human colon epithelial cell line NCM460 was cultured in M3 base culture media (Incell Corporation, San Antonio, TX, USA) as previously described [35]. Both media contained 10% fetal bovine serum, 100 U/ml penicillin G and 100 µg/ml streptomycin (Gibco Laboratories). All cells were cultured at 37°C in a humidified incubator containing 5% CO_2.

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 AGAAGCTCGGTGATGAATA-3´ (reverse). These sequences were 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. Lovo and SW480 cells ($1x10^5$) were infected with $1x10^7$ lentivirus transducing units in the presence of 10 µg/ml polybrene (Sigma, St Louis, Missouri, USA). Empty lentiviral vector was used as negative control. Cells were collected 48 h after transfection.

qRT-PCR analysis

Total RNA was extracted with Trizol reagents (Life Technologies, CA, USA) according to the manufacturer's instructions. Then, 1 µg of RNA was reverse transcribed into cDNA using a reverse reaction kit (Promega, Madison, WI, USA). qRT-PCR was then performed using specific primers and the 7500 real-time PCR system (Applied Biosystems, Mannheim, Germany). U6 was used as the endogenous control for miRNA expression analysis. Relative quantification analysis was performed using the comparative threshold cycle (CT) method (2-ΔΔCT) [36].

Transwell assay

Migration and invasion of LoVo and SW480 cells were assessed using Transwell plates (Millipore). Cells were plated on uncoated upper chambers (24-well inserts; pore size, 8 mm; BD Bioscience, Bedford, MA, USA) for Transwell migration assays. Cells were plated on Matrigel-coated upper chambers (24-well inserts; pore size, 8 mm; BD Bioscience) for invasion assays. Briefly, fresh media containing 5% fetal bovine serum (FBS) were placed in the lower wells. Cells were incubated for 24 h in medium containing 1% FBS, trypsinized, and suspended at a final concentration of $1×10^6$ cells/mL in medium containing 1% FBS. Then, 200 mL of the cell suspension was loaded into each of the upper wells, and the chamber was incubated at 37°C for 24 h. Non-migrating cells on the upper surface of the filter were removed by wiping with a cotton swab. Chemotaxis was quantified by counting the cells that migrated to the lower side of the filter for eight random fields in each assay with an optical inverted microscope (×200, Nikon, Tokyo, Japan).

Western blot analysis

Equal amounts of cell lysate were separated by 10% SDS-PAGE, and electrophoretically transferred to PVDF membrane. The membrane was blocked and probed with mouse anti-humanITGB3 monoclonal antibody (Abcam, Cambridge, UK), followed by horseradish peroxidase-labeled goat-anti-mouse IgG (Abcam). Chemiluminescence was used to analyze protein levels and β-actin was used as a protein loading control.

Bioinformatic analysis

Potential miRNA targets were predicted and analyzed using three publicly available algorithms: PicTar, TargetScan and miRanda [37]. The number of false-positive results was decreased by accepting only putative target genes that were predicted by at least two programs.

Luciferase reporter assay

HEK293T cells were seeded in a 96-well plate at 50–60% confluence. After 24 h, cells were transfected with 120 ng of miRNA-30a-5p expression vector, miRNA-30a-5p inhibitor, control vector, or negative control. Cells were co-transfected with 30 ng of the wild-type or mutant 3'-UTR of ITGB3 mRNA. Transfections were performed using 0.45 μL of Fugene (Promega, Madison, WI, USA). Cells were collected 48 h after transfection, and Renilla luciferase activity was measured using a dual-luciferase reporter system (Promega). Luciferase reporter assays were performed in duplicate and repeated in three independent experiments. Luciferase activity was detected using an Orion II microplate luminometer (Berthold Technologies, Germany).

Liver metastasis model

Mice were injected with SW480 colon cancer cells in the spleen to produce liver metastasis. The mice were anesthetized with 2.5% pentobarbital sodium by peritoneal injection. After sterilization of the skin in the area of surgery, an abdominal incision paralleling the left subcostal margin was made. Tumor cells (1×10^7) in 100 μl phosphate-buffered saline solution were injected into the spleen. Mice were euthanized 5 weeks after tumor cell implantation. Liver metastases were examined and counted under a dissecting microscope equipped with bright field imaging.

Statistical analysis

Statistical analysis was performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Data are expressed as the mean ± SEM. The difference between two independent groups was assessed using a two-tail Student's t test. A p value <0.05 was considered statistically significant. All experiments were performed in triplicate.

Results

MiR-30a-5p is downregulated in CRC tissue and cell lines

We compared miR-30a-5p expression in 40 CRC tissues with metastases (mCRC) and 27 CRC tissues without metastases (nmCRC) and 40 normal non-cancerous matched tissues (Normal) by qRT-PCR. The average relative expression level of miR-30a-5p was significantly lower in CRC specimens compared with non-cancerous tissues (Fig. 1A, P<0.01). Decreased expression of miR-30a-5p was also observed in all four CRC cell lines tested (HT29, LoVo, SW116 and SW480) compared with the normal human colon epithelial cell line NCM460 or

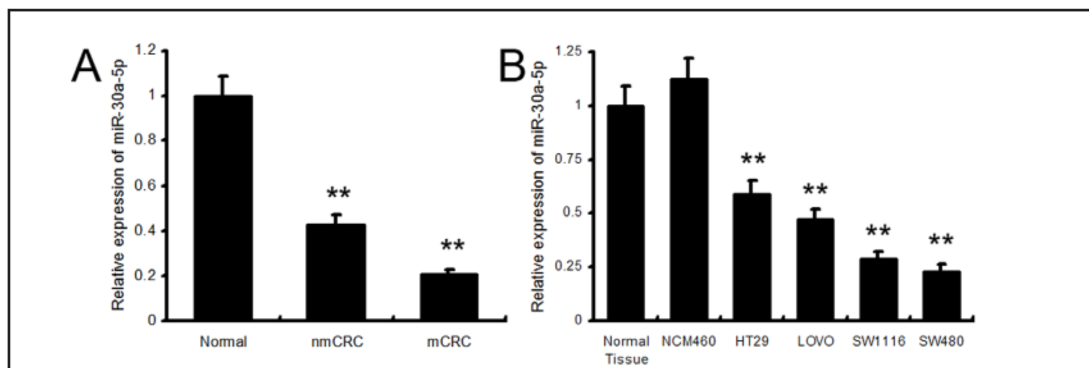

Fig. 1. Downregulated expression of miR-30a-5p in CRC tissues and cell lines. (A) miR-30a-5p expression in CRC tissues with or without metastases relative to normal matched tissues, as determined by qRT-PCR. nm-CRC denotes CRC tissues without metastases; mCRC denotes CRC tissues with metastases. (B) The relative expression of miR-30a-5p in four CRC cell lines (HT29, LoVo, SW1116, SW480) compared with the normal human colon epithelial cell line NCM460 or the mean rate of expression of miR-30a-5p in 24 non-cancerous tissue samples (Normal tissue), as determined by qRT-PCR. **P<0.01.

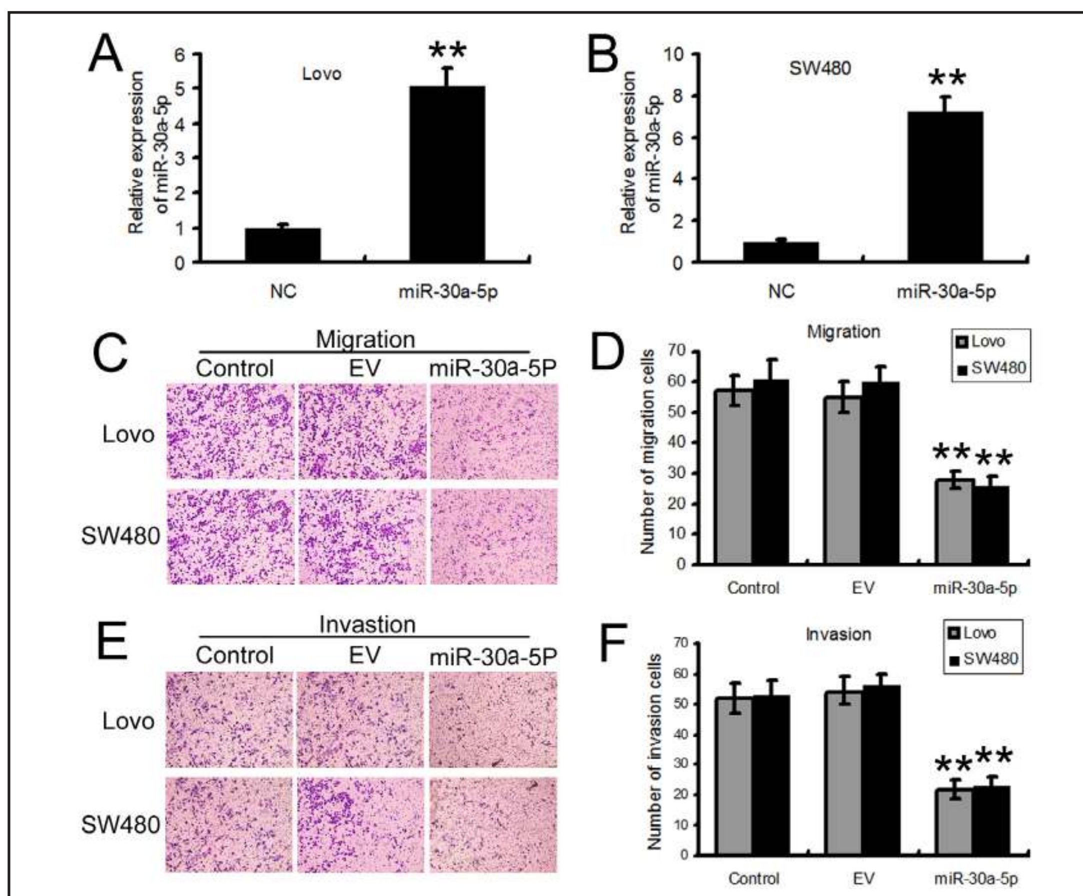

Fig. 2. Ectopic expression of miR-30a-5p inhibited aggressive phenotypes in CRC cells. Relative expression levels of miR-30a-5p in LoVo (A) and SW480 (B) CRC cells transfected with miR-30a-5p compared with normal control (NC) cells, as determined by qRT-PCR. The number of migratory (C) and invasive (D) cells, as determined by Transwell assays at 48 h post-transfection, for Lovo or SW480 CRC cells transfected with miR-30a-5p compared with empty lentivector treatd (EV) or normal control (NC) cells, magnification ×200. Quantification of the migratory (E) and invasive (F) cell numbers determined by the Transwell assay. **P<0.01.

the normal non-cancerous tissue specimens (Fig. 1B, P<0.01). These findings indicated that miR-30a-5p is downregulated in CRC tissue and CRC cell lines.

Exogenous miR-30a-5p suppresses CRC cell metastasis

We transfected the CRC cell lines LoVo and SW480 (which have a lower level of endogenous expression of miR-30a-5p) with a miR-30a-5p mimic and confirmed miR-30a-5p overexpression by qRT-PCR (Fig. 2A and B, P<0.01). At 48 h post-transfection, the effects of miR-30a-5p on cell migration and invasion were assessed by Transwell assays. We found that the number of migratory (Fig. 2C and E) and invading (Fig. 2D and F) cells were decreased for both miR-30a-5p-transfected CRC cell lines compared with control cells and EV (empty lentivector treatd)cells (P<0.01 for both). These findings indicate that ectopic expression of miR-30a-5p inhibited aggressive phenotypes in CRC cells.

Exogenous miR-30a-5p effects EMT in CRC cells

To investigate the effect of exogenous miR-30a-5p on EMT, western blot analysis was performed on the LoVo and SW480 CRC cell lines transfected with miR-30a-5p compared with the EV and control cells. We found that exogenous miR-30a-5p upregulated E-cadherin expression in both CRC cell lines (P<0.01; Fig. 3A and B) and downregulated vimentin

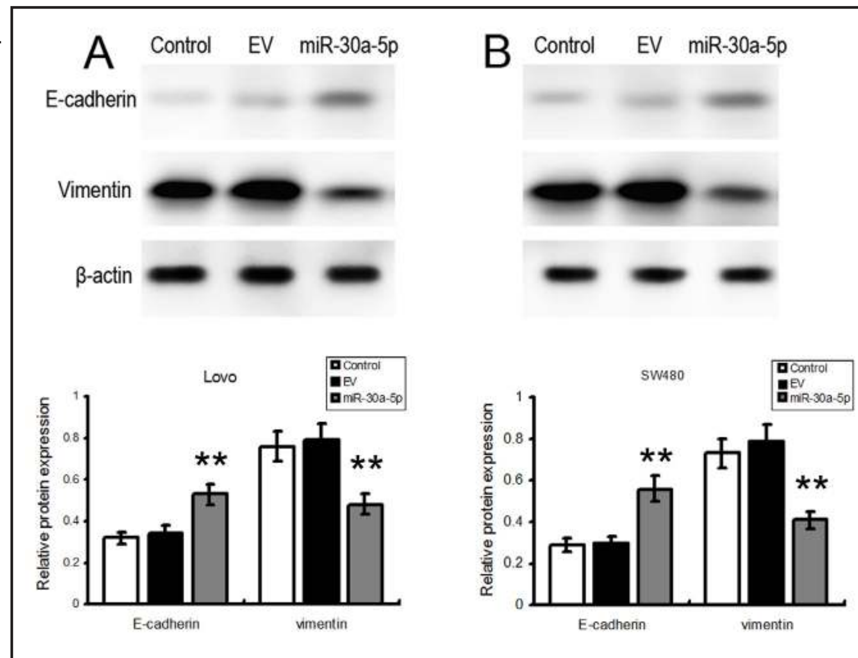

Fig. 3. Effect of ectopic expression of miR-30a-5p on expression of the epithelial-mesenchymal transition (EMT) markers Expression of the EMT markers, E-cadherin and vimentin, was analyzed by western lotting in the (A) LoVo and (B) SW480 CRC cell lines transfected with miR-30a-5p compared with EV or normal control (NC) cells. β-actin was included as a loading control. **P<0.01.

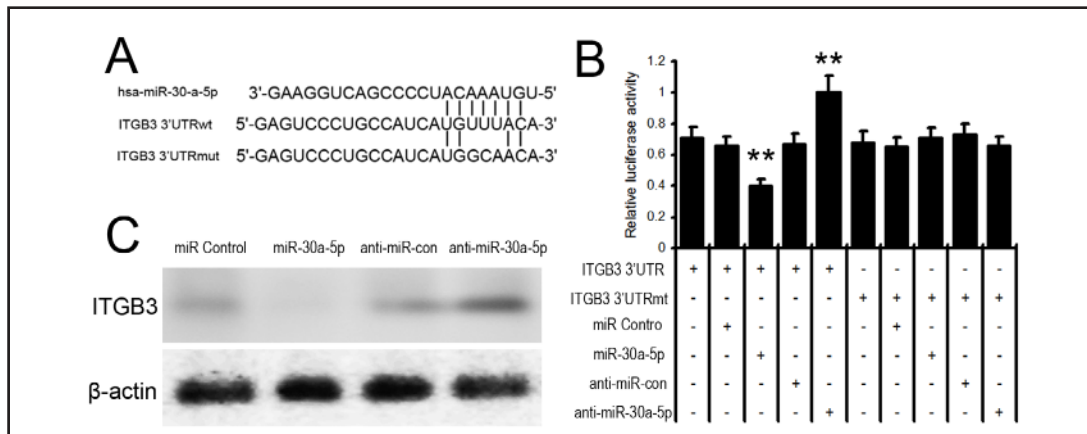

Fig. 4. ITGB3 is a target of miR-30a-5p. (A) The sequence of miR-30a-5p and the 3'UTR of ITGB3 used in the reporter assay. The wild-type and mutant sequences of the ITGB3 3'UTR are shown. The putative binding site between miR-30a-5p and the ITGB3 3'UTR is indicated. (B) The relative luciferase activities observed in reporter assays of HEK-293T cells transfected with reporter vectors containing either the wild-type or mutated 3'UTR of ITGB3, and a miR-30a-5p mimic, anti-miR-30a-5p or their nonspecific controls. (C) Protein levels of ITGB3 in SW480 cells transfected with miR-30a-5p mimic, anti-miR-30a-5p or their nonspecific controls. **P<0.01.

expression (P<0.01; Fig. 3A and B). E-cadherin and vimentin are markers of EMT. Reduced expression of the epithelial-specific marker E-cadherin, accompanied by increased expression of the mesenchymal-specific marker vimentin, is indicative of EMT (Zeisberg and Neilson, 2009). Our findings therefore indicate that exogenous miR-30a-5p inhibits EMT in CRC cells, which is consistent with the suppression of invasive and migratory properties in these cells shown in Fig. 2.

ITGB3 is a target for repression by miR-30a-5p

MiRNAs act by binding to the 3'UTR of target genes sharing sequence homology within the seed sequence. To explore miR-30a-5p-regulated target gene(s) and pathway(s), we used three publicly-available miRNA target prediction tools: Targetscan, ncRNA and mirecords.

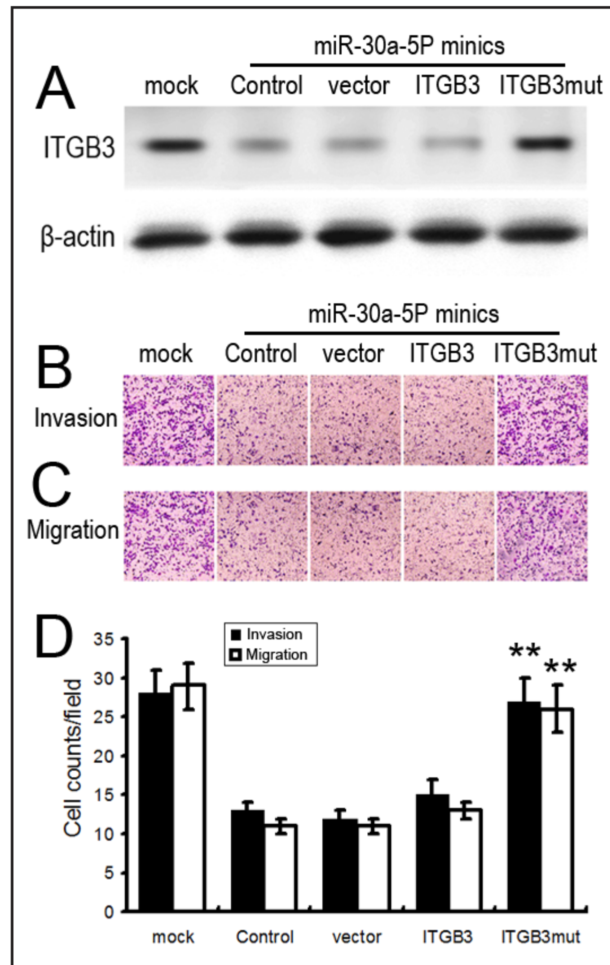

Fig. 5. ITGB3 rescues the suppressive effect of miR-30a-5p on CRC cell metastasis. (A) ITGB3 expression was determined by western blot analysis in cells transfected with miR-30a-5p mimics alone or in combination with either pcDNA 3.1+ (vector) or pcDNA 3.1+ containing a wild-type (ITGB3) or mutant ITGB3 (ITGB3 mut) expression cassette of the miR-30a-5p response element. β-actin was included as an internal control. (B,C) Invasion and migration of the same set of CRC cells were assessed using Transwell assays. (D) Quantification of invasive and migratory CRC cells. **P<0.01 vs. control.

To increase the stringency of the target prediction protocol, we searched for mRNAs simultaneously predicted by all three target-prediction programs and selected ITGB3 as a potential target. To assess whether miR-30a-5p could directly alter the expression of ITGB3, a putative binding site for miR-30a-5p in the 3'UTR of ITGB3 was identified (Fig. 4A) and this mRNA fragment (wt 3'UTR), along with a mutated derivative of this sequence (mut 3'UTR), were cloned into a luciferase reporter vector. HEK-293T cells were then transfected with the reporter vectors containing either the wt or mut 3'UTR of ITGB3 and a miR-30a-5p mimic. As shown in Fig. 4B, luciferase expression was decreased when the wt 3'UTR and miR-30a-5p mimic were cotransfected (P<0.01), while cotransfection with the mut 3'UTR had no effect on luciferase activity. Moreover, cotransfection of HEK-293T cells with the wt 3'UTR and anti-miR-30a-5p reversed the effect seen with the miR-30a-5p mimic (P<0.01).

Collectively, these results indicated that ITGB3 is a direct target of miR-30a-5p. We next elucidated whether the growth-suppressive effect of miR-30a-5p was mediated by repression of ITGB3. We assessed whether expression of ITGB3 changed in response to transfection with the miR-30a-5p mimic or anti-miR-30a-5p in SW480 cells by western blot analysis. Compared with the nonspecific controls, expression of ITGB3 was significantly decreased by miR-30a-5p transfection and increased by anti-miR-30a-5p transfection (Fig. 4C), indicating that miR-30a-5p decreases ITGB3 expression.

ITGB3 rescues the suppressive effect of miR-30a-5p on CRC cell metastasis

To identify whether miR-30a-5p inhibits the invasiveness of CRC cells by targeting ITGB3, we performed a "rescue" experiment by co-transfecting SW480 CRC cells with miR-30a-5p mimics and a pcDNA3.1 vector that expresses ITGB3 with a mutated miR-30a seed sequence

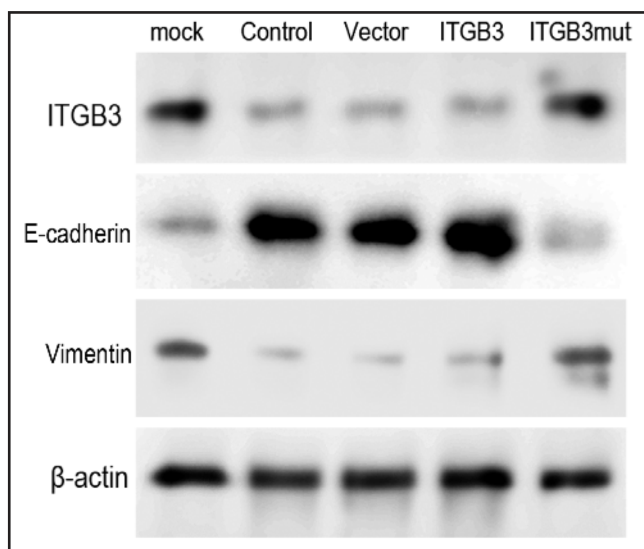

Fig. 6. ITGB3 restores the expression of vimentin and inhibits the expression of E-cadherin. ITGB3, vimentin and E-cadherin expression were determined by western blot analysis in cells transfected with miR-30a-5p mimics alone or in combination with either pcDNA 3.1+ (vector) or pcDNA 3.1+ containing a wild-type (ITGB3) or mutant ITGB3 (ITGB3 mut) expression cassette of the miR-30a-5p response element. β-actin was included as an internal control.

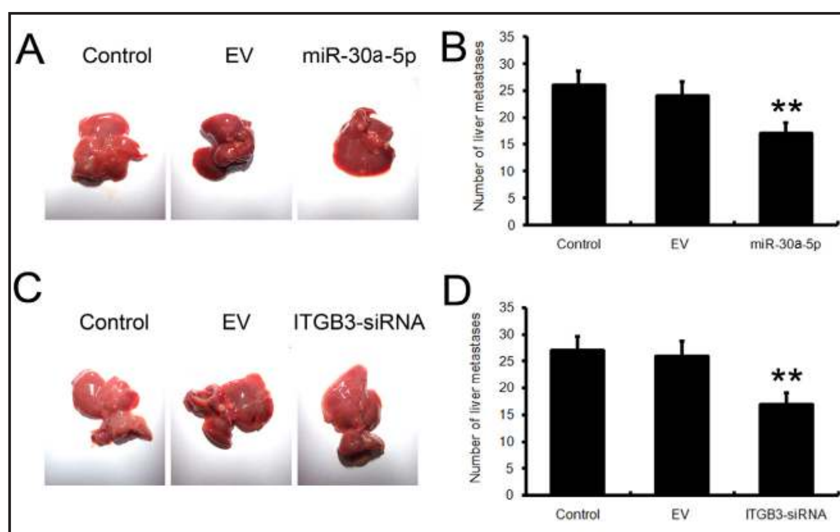

Fig. 7. Effect of Overexpression of miR-30a-5P or knockdown of ITGB3 on colon cancer cell metastasis in a mice model. SW480 cells and SW480 transfected with miR-30a-5P mimics or ITGB3-siRNA were injected into spleen of mice. After 5 weeks, the livers were resected and the number of metastatic lesions were measured. (A&C) Representative images of liver tumors are shown. (B&D) Quantitative evaluation of visible liver metastases on the surface of whole livers. Data represent the mean ± SD. **P < 0.01, n = 5.

(ITGB3 mut) in the 3'-UTR. Western blot analysis demonstrated that co-transfection of CRC cells with miR-30a-5p mimics and ITGB3 mut restored ITGB3 expression, whereas co-transfecting wild-type pcDNA3.1-ITGB3 could not restore ITGB3 expression after silencing by miR-30a-5p mimics (Fig. 5A).

Next, invasion and migration of CRC cells were assessed using Transwell assays. “Rescuing” ITGB3 expression in the presence of miRNA mimics restored the invasive and migratory capacities of SW480 cells (P<0.01; Fig. 5, B–D). Collectively, these data suggested that miR-30a-5p regulates the invasiveness of CRC cells by targeting ITGB3.

ITGB3 rescues the suppressive effect of miR-30a-5p on EMT

Finally, we investigated the effect of ITGB3 on EMT. Western blot analysis of ITGB3, vimentin and E-cadherin expression in cells co-transfected with miR-30a-5p mimics and pcDNA3.1-ITGB3 harboring the wild-type or mutant miR-30a-5p response element, revealed that ITGB3 restored the expression of vimentin and inhibited the expression of E-cadherin (Fig. 6), thereby rescuing the suppressive effect of miR-30a-5p on EMT.

Overexpression of miR-30a-5P or knockdown of ITGB3 inhibits hepatic metastasis of colon cancer cell in vivo

SW480 cells ($1×10^7$) were injected into the spleen of 30 mice to establish liver metastasis [38] and sacrificed 5 weeks after tumor cell injection for analysis. At 5 weeks after injection, the number of metastases in the livers of mice injected with SW480 cells transfected with miR-30a-5p was significantly lower than that of control and EV SW480 injected mice (Fig. 7A&B). Similar to this,the number of metastases in the livers of mice injected with ITGB3-siRNA SW480 cells was significantly lower than that of control and EV SW480 injected mice (Fig. 7C&D). These results indicated that miR-30a-5p suppresses metastasis of colon cancer cell and ITGB3 has the opposite effect.

Discussion

Although altered miRNA expression profiles have been observed in many types of malignancies [39, 40], the molecular mechanisms by which miRNAs modulate cancer cells largely remain to be determined. Here, we investigated the potential role of miRNA-30a-5p in CRC progression. We demonstrated that miR-30a-5p was significantly downregulated in both human colon cancer tissues and cell lines, and found that downregulation of miR-30a-5p contributed to cancer cell migration and invasion, which was associated with EMT. Furthermore, we showed that ectopic expression of miR-30a-5p in CRC cells inhibited cell migration and invasion, preventing EMT. We investigated the mechanisms underlying the function of miR-30a-5p in colon cancer and using target prediction software and a dual-luciferase reporter assay, identified ITGB3 as a direct target of miR-30a-5p. We then showed the involvement of ITGB3 in the functional consequences of miR-30a-5p suppression. Our findings indicate the important role of miR-30a-5p in the fundamental processes of CRC metastasis (i.e. migration, invasion and EMT). Local invasion and distant metastasis are strongly correlated with poor prognosis in CRC patients and determining the mechanisms that govern metastasis is therefore crucial in developing treatments to improve patient survival rates. Both miR-30a-5p and its physiological target, ITGB3, may therefore be promising candidates for the development of novel therapeutic strategies for CRC.

The aberrant expression and distribution of integrin family members, including ITGB3, has previously been shown to promote tumor metastasis [41-43]. Integrins are transmembrane receptors that mediate cell-to-cell and cell-extracellular matrix attachments and facilitate signal transduction via intracellular signaling pathways such as PI3K and MAPK. Integrin heterodimers (comprising α and β subunits) regulate cell proliferation, survival and migration, predominantly via focal adhesion kinase and Src kinase family members [44, 45], with small GTPases acting as intermediates in this process. In one study, an ITGB3 antagonist led to decreased colon cancer metastasis in mice [46]. The binding of integrins to ligands on the extracellular matrix has been shown to transmit both mechanical and chemical signals to induce cell cytoskeleton remodeling during adhesion and migration. In another study, reactive oxygen species were shown to markedly upregulate the expression of ITGB3, promoting a more aggressive phenotype in a colorectal cancer cell line with increased migratory and invasive capacities [47]. MiRNAs have been linked with the dysregulation of integrin signaling in a number of studies, correlating miRNA-regulated integrin signaling with tumor metastasis and apoptosis in CRC [28], as well as breast, hepatocellular and salivary adenoid cystic carcinoma [7, 48, 49]. Our findings provide further evidence of the targeting of integrins by miRNAs.

In summary, we investigated the potential role of miR-30a-5p in tumor progression and its underlying mechanisms. Our findings indicate that downregulation of miR-30a-5p plays an important role in the development of CRC and that this miRNA is a promising therapeutic target. However, many aspects of miR-30a-5p function remain to be elucidated, including the complex effects on cellular behavior mediated by known targets (denticleless protein homolog, PIK3CD, insulin receptor substrate 2 and ITGB3) and potentially other yet to be determined target proteins.

Ethical approval

This study was approved by the Ethics Committee of the Central Hospital of Xingxiang (Xinxiang, China) and all aspects of the study comply with the Declaration of Helsinki.

Disclosure Statement

The authors have no conflicts of interest.

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