Research Article

Colorectal cancer-derived exosomal miR-106b-3p promotes metastasis by down-regulating DLC-1 expression

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Cancer-derived exosomal miRNAs play an important role in the development of metastasis, but the effects and underlying mechanisms remain unclear. In the present study, we investigated the miRNA expression profiles of 5 paired serum exosomal samples from metastatic colorectal cancer (mCRC) and non-mCRC patients via RNA sequencing. After we evaluated the differentially expressed miRNAs in 80 CRC patients, miR-106b-3p was selected as a metastasis-associated miRNA of CRC. We showed that the expression level of serum exosomal miR-106b-3p was significantly higher in CRC patients with metastasis than those without metastasis. Additionally, high serum exosomal miR-106b-3p expression in patients was correlated with a poor prognosis. Coculture of low-metastatic CRC cells with high-metastatic CRC cell-derived exosomes promoted cell migration, invasion, and epithelial-to-mesenchymal transition (EMT), which was caused by the transport and transduction of miR-106b-3p in vitro. Moreover, exosomal miR-106b-3p promoted lung metastasis of CRC cells in vivo. In addition, we demonstrated that miR-106b-3p regulated metastasis by targeting deleted in liver cancer-1 (DLC-1). A negative correlation was also identified between miR-106b-3p and DLC-1 expression in human CRC tumour tissues and in mouse lung metastatic lesions. Collectively, our study indicated that metastasis-associated miR-106b-3p from serum exosomes could be used as a potential prognostic biomarker and therapeutic target for CRC patients.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer, causing approximately 600,000 deaths each year, most of which are due to metastasis [1]. Approximately 45–60% of CRC patients experience metastasis and frequent tumour recurrence after surgical resection [2]. In addition, metastasis of colorectal cancer usually results in a poor prognosis, and current interventions are insufficient. Therefore, identification of an effective early diagnostic method and elucidation of the potential mechanism of CRC metastasis are important for improving the current treatment strategies.

MicroRNAs (miRNAs) are small non-coding RNAs that act as post-transcriptional regulators of gene expression by inhibiting the translation of mRNA [3]. According to previous studies, miRNA plays a major role in various biological processes, such as cell proliferation, invasion and migration [4,5]. Alterations of miRNAs in circulating, inflammatory cell populations or pathological samples of malignancies have also been documented [6,7]. MiRNAs can be encapsulated by exosomes and then transported and transduced to target cells to facilitate cell-to-cell communication [8]. Exosomes are nanoscale membrane vesicles...
with a diameter of 30–150 nm, which contain various nucleic acids [9]. Exosomes can affect target cells through gene regulation, which is mediated by the transfer of miRNAs [10,11]. Exosomes are secreted into the circulation from various cell types and are transported to target cells throughout the body. Advanced studies have demonstrated that the exosome-derived miRNAs may be shipped to the premetastatic niche in various tumour types [12] and play a key role in the functions of recipient cells [10,11]. Cancer-derived exosomes have also been reported as biomarkers for monitoring disease progression [13,14]. Furthermore, quantitative detection of serum exosomal miRNA levels could facilitate the diagnosis of metastasis and predict the prognosis of patients [15,16]. The biological functions as well as the diagnostic and therapeutic implications of exosomes in CRC have been reviewed previously [17]. Previous studies have focused on the communication of CRC cells with non-neoplastic cells through exosomes. Whether exosomes from highly invasive CRC cells can promote the invasiveness of surrounding cancer cells remains to be further investigated.

Herein, after serum exosomal RNA sequencing, we demonstrated that miR-106b-3p was highly expressed in metastatic CRC (mCRC) patients and closely correlated with a poor prognosis. In addition, we identified that exosomes from CRC cells with high invasive potential can deliver miR-106b-3p to CRC cells with low invasive potential, thus enhancing CRC cell migration and invasion by targeting deleted in liver cancer-1 (DLC-1). Moreover, exosomal miR-106b-3p promoted lung metastasis of CRC cells in vivo. Our findings indicated that exosomal miR-106b-3p may serve as a prognostic biomarker and novel therapeutic target for CRC patients.

Materials and methods

Human tissue

A total of 80 CRC patients (including 40 mCRC patients and 40 non-mCRC patients) and 20 healthy people were enrolled in the present study from November 2015 to December 2016 at the Tumour Hospital of Harbin Medical University (Heilongjiang, China). Clinical data for the 80 included patients are shown in Supplementary Table S1. All patients with primary tumours underwent curative surgical resection, and no patient received any preoperative treatment. Serum samples were collected before the operation and 1 week after complete resection. All patients and healthy blood donors provided written consent, and the Ethics Committee of Harbin Medical University (Harbin, China) approved the present study. The RNA sequences of serum exosomes from 10 patients (including 5 mCRC patients and 5 non-mCRC patients) were determined by Novogene (Beijing, China). The fresh CRC biopsy specimens with adjacent mucosa samples were collected from Harbin Medical University Cancer Hospital. Tissue pieces were frozen immediately in liquid nitrogen and stored at −80°C after surgery.

Small RNA sequencing

The RNA in serum exosomes was sequenced by Novogene (Beijing, China). Briefly, 3 μg of RNA was used as the input material of a small RNA library for each sample. After the 3′ ligation reaction, the SR RT primer was hybridized with the excess part of the 3′ SR adaptor, and the single-stranded DNA adaptor was transformed into the double-stranded DNA molecule. Next, the 5′ ends adapter was ligated to the 5′ ends of the miRNAs. Then, first-strand cDNA was synthesized by M-MuLV Reverse Transcriptase (RNase H-), and the PCR products were purified on an 8% polyacrylamide gel (100 V, 80 min). Finally, library quality was assessed on the Agilent Bioanalyzer 2100 system using DNA High Sensitivity Chips. After cluster generation, the library preparations were sequenced on an Illumina Hiseq 2500/2000 platform, and 50-bp single-end reads were generated. After quality control, we chose a certain length range from clean reads for all downstream analyses. Differential expression analysis of two groups was performed using the DESeq R package (1.8.3). The P-values were adjusted using the Benjamini and Hochberg method. A corrected P-value of 0.05 and fold change > 1.5 were set as the threshold for significantly differential expression by default.

Predicting miR-106b-3p targets

The public database miRWalk 2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/miRretsys-self.html) was used to search for potential target genes of miR-106b-3p. To predict the binding site of miR-106b-3p in the 3′-UTR of DLC-1, three publicly available databases, RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid?id=rnahybrid_view_submission), RegRNA (http://regrna2.mbc.nctu.edu.tw/detection.html) and RNA22 version 2.0 (https://cm.jefferson.edu/rna22/Interactive/) were used (limit score ≥120, energy ≤ -15 in RegRNA).
Cell culture

Human CRC cell lines (HCT116, SW480, SNU-C1, SW1116, LoVo and KM12SM) and a human colorectal epithelial cell line (NCM460) were cultured in the Myocardial Ischemia Laboratory, affiliated with Harbin Medical University. The SW480, SNU-C1, SW1116, KM12SM and HCT116 cell lines were maintained in RPMI-1640 containing 10% foetal bovine serum (FBS; ExCell Bio, FSP500, South America) and 1% penicillin/streptomycin. LoVo cells were cultured in F12-K medium containing 10% FBS (ExCell Bio, FSP500, South America). All the cultures were incubated at 37°C with 5% CO₂.

Exosome isolation and characterization

Exosomes were isolated from 20 ml of CRC cell supernatant and 1 ml of sera from patients or healthy donors. Cells were maintained in medium containing 10% exosome-depleted FBS (Vivacell, Shanghai, China). Briefly, cell supernatants (collected after 48 h) or sera were differentially centrifuged at 300 × g and 3000 × g for 10 min to remove cellular debris. Then, the supernatants were filtered through a 0.22-μm filter (Millipore) and centrifuged at 120,000 × g for 1 h at 4°C. Next, the exosomes were resuspended in phosphate-buffered saline (PBS), and the exosome number was measured with a BCA protein kit (KeyGen Biotech).

Exosomes were irradiated with a laser, and their Brownian motion was recorded. A 10-s sample video was analysed with nanoparticle tracking analysis (NTA) software (version 2.3, Nanosight). Exosomes were observed by transmission electron microscopy (TEM) (H-7650, HITACHI, Japan) as described previously [18].

Exosome labelling and electron microscopy

Exosomes were stained with PKH67 membrane dye (Sigma). After culture with labelled exosomes for 3 h, cells were fixed and stained with Hoechst. Cellular uptake of exosomes was detected by using an electron microscope (JEM-1220, JEOL, Ltd., Japan). For the in vitro experiments, 1 × 10⁵ receptor cells were cocultured with 50 μg of exosomes.

Animal studies

Five-week-old female athymic BALB/c-nu/nu mice (purchased from Shanghai SLAC Laboratory Animal Co., Ltd) were raised in specific pathogen-free animal facilities, and all animal experiments were conducted in Second Affiliated Hospital of the Harbin Medical University Laboratory. All animals received humane care and were provided free access to water and food. The Hospital Scientific Affairs Committee on Animal Research and Ethics approved the study protocol (SYDW2019-208). Mice were anesthetized with 1–3% isoflurane [19] and killed by CO₂ inhalation. All animal experiments were undertaken in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

For the tail vein metastasis assay, exosomes from KM12SM CM (KM12SM exosomes) were first purified. Then, 1 × 10⁶ stable luciferase-transfected SW480 cells (stimulated SW480 cells) were cocultured with 200 μl of KM12SM exosomes, and another group of luciferase-transfected SW480 cells (non-stimulated SW480 cells) were added with an equal amount of PBS. After 24 h, the stimulated SW480 cells and non-stimulated SW480 cells were injected via the tail vein (n = 5). On day 45 after SW480 injection, mice were anaesthetized and injected (i.p.) with luciferin at 150 mg/kg in a volume of 100 μl. At 10–15 min after luciferin injection, mice were imaged to observe luciferase expression using an IVIS-200 Imaging System (Xenogen Corporation, U.S.A.).

RNA extraction and quantitative RT-PCR

Total RNA was extracted and reverse transcribed to cDNA using a miRcute miRNA Isolation Kit and a miRcute Plus miRNA First-Strand cDNA Synthesis Kit (TIANGEN BIOTECH, China) according to standard protocols. PCR was performed on a Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, U.S.A.). The relative expression level of miR-106b-3p was normalized to that of U6, and the expression of DLC-1 mRNA was normalized to that of GAPDH. MiRNAs in exosomes were normalized using cel-miR-39 (Applied Biosystem, CA, U.S.A.) as an external control. The following primer sequences were used:

Luciferase activity and RIP assays

The 3’-UTR fragment of the DLC-1 gene was amplified and transfected into vectors. For luciferase analysis, HEK293T cells were cultured with miR-106b-3p mimic or inhibitor at 40 nM in a 24-well plate. Renilla luciferase vectors were transfected to determine the transfection efficiency. Cells were collected after 48 h of transfection and tested by relative light units as described by the manufacturer.
RIP assays were conducted with a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) in SW480 and KM12SM cell lines following the manufacturer’s instructions. Ago2 antibodies and IgG were used in the assays (ab5072, rabbit polyclonal antibody, Cambridge, MA, U.S.A.).

**Cell transfection**

CRC cells were transfected with Lipofectamine 3000 (Invitrogen) and Opti-MEM (Gibco, U.S.A.). The miR-106b-3p mimic, inhibitor and negative control (NC) were used at a dose of 100 pmol for miRNA up- and down-regulation. After transfection for 24 or 48 h, the cells were harvested and total RNA was isolated. Oligonucleotides were annealed and bound to shRNA vectors (GeneChem, Shanghai, China). The ectopic expression of the DLC-1 plasmid was amplified and subcloned into the XhoI and KpnI sites of pcDNA3.1 (GeneChem, Shanghai, China). Plasmid vectors were transfected with Lipofectamine 3000 (Invitrogen), and blank pcDNA3.1 vector was used as a control. KM12SM cells were transfected with the miR-106b-3p inhibitor or NC, and exosomes were then isolated from RPMI-1640 medium supplemented with exosome-free FBS (Vivacell, Shanghai, China).

**Cell proliferation, migration and invasion assays**

For the CCK-8 assay, cell proliferation was determined following the manufacturer’s directions for the CCK-8 assay (Dojindo, Japan) every 24 h.

For the cell invasion assay, $1 \times 10^5$ cells were cultured with serum-free medium in transwell chambers (Corning, 3422) coated with Matrigel (BD Biosciences, U.S.A.). Medium supplemented with 10% FBS was maintained in the lower chamber. After 6 h, the cells on the bottom were treated with paraformaldehyde, stained with a Crystal Violet solution and counted.

For the wound healing assay, the cell migration was calculated by measuring the wound area at 0 and 48 h, and the cells were observed and photographed.

**Western blot analysis**

Western blots were carried out following standard protocols. The primary antibodies used were as follows: anti-human E-cadherin (Wanleibio, China, WL00941, 1:500 dilution), N-cadherin (Wanleibio, China, WL01047, 1:500 dilution), vimentin (Wanleibio, China, WL01960, 1:500 dilution), apolipoprotein A1 (ApoA1; ab52945, Abcam, U.S.A., 1:1000 dilution), SW480 cell lysate (ab3957, Abcam, U.S.A.), TSG101 (Abcam, ab125011, 1:1000 dilution), CD63 (ab217345, Abcam, U.S.A., 1:1000 dilution), Alix (ab186429, Abcam, U.S.A., 1:1000 dilution), Calnexin (ab22595, Abcam, U.S.A., 1:1000 dilution), β-actin (Proteintech, 20536, 1:2000 dilution) and DLC-1 (Bioss, bs-20131R, 1:500 dilution). After incubation with the secondary antibody (Invitrogen, U.S.A.), the protein bands were visualized with Super chemiluminescent reagent (HaiGene, Harbin, China) using a Bio-Rad ChemiDoc XRS system (Bio-Rad, CA, U.S.A.).

**Immunohistochemistry (IHC)**

IHC performance and analysis were carried out as previously described [20] using a 1:50 antibody dilution for DLC-1 (Bioss, China, bs-20131R). Subsequently, a secondary antibody (ZSGB-BIO, Beijing, China) was added and incubated for 20 min at 4°C.

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Statistical analysis
Each experiment was repeated at least three times, and statistical analyses were performed using SPSS18.0 software. The data are described as the mean ± standard deviation (SD). A two-tailed Student’s t test was used to compare two independent groups. Multiple independent groups were compared by one-way analysis of variance (ANOVA). Spearman’s correlation was used to study the relationship between DLC-1 expression and miR-106b-3p, and the relationship between the invasive potential of different CRC cell lines and cellular miR-106b-3p expression. Survival curves were calculated by the log-rank test. A probability level of 0.05 indicated statistical significance.

Results
Exosomal miR-106b-3p is abundant in mCRC patients
To investigate the differential expression of miRNAs in exosomes during CRC metastasis, serum exosomes were extracted from CRC patients with or without metastasis and identified. First, exosomes were isolated by differential ultracentrifugation. Then, TEM was used to verify the exosomal cup-shaped membranes (Figure 1A). Nanosight particle tracking revealed that the sizes of these exosomes were approximately 100 nm (Figure 1B). In addition, Western blot analysis confirmed the expression of specific exosomal markers such as CD63, Alix and TSG101. In contrast, exosomes isolated from CRC patients were negative for non-EV markers, such as Calnexin, and for serum contaminants, such as ApoA1 (Figure 1C). Next, exosomal RNA sequencing (RNA-seq) was conducted, and the miRNA profiles were compared and are shown in Figure 1D and Supplementary Table S1. As shown in the volcano plot, miRNAs that reached a significance level of \( P < 0.05 \) and had a fold-change \( > 1.5 \) were analysed further. Based on the above results, we selected five miRNAs that were significantly up-regulated and expressed in serum exosomes from CRC patients with metastasis (miR-1307-3p, miR-106b-3p, miR-6842-3p, miR-151a-3p and miR-744-5p) (Figure 1E). RT-PCR analyses confirmed that three of these miRNAs (miR-106b-3p, miR-6842-3p and miR-151a-3p) showed higher levels in serum exosomes from mCRC patients than those from patients without metastasis (10 non-mCRC patients and 10 mCRC patients) (Figure 1F). In addition, qPCR analyses in 20 paired fresh CRC tissues and matched adjacent normal mucosa showed that miR-106b-3p, miR-6842-3p and miR-151a-3p were substantially up-regulated in CRC tissues. (Figure 1G and Supplementary Figure 1A–D). Most importantly, miR-106b-3p was closely associated with metastasis (Figure 1H and Supplementary Figure 1E and F). Therefore, miR-106b-3p was selected for further study.

Serum exosomal miR-106b-3p is correlated with poor prognosis
Serum exosomes were extracted from healthy donors, mCRC patients and non-mCRC patients to determine whether exosomal miR-106b-3p was correlated with metastasis. Compared with that in exosomes from healthy controls, miR-106b-3p in circulating exosomes was increased in CRC patients, and higher levels of miR-106b-3p were found in the metastasis group than in the non-metastasis group \((n = 100)\) (Figure 2A). In addition, miR-106b-3p was highly expressed in CRC tissues compared with the adjacent mucosa according to qRT-PCR (Figure 2B), and a positive correlation between miR-106b-3p expression in tumour tissues and in circulating exosomes was observed \((n = 20)\) (Figure 2C). We also examined the level of serum exosomal miR-106b-3p from CRC patients before and after the operation. In sum, exosomal miR-106b-3p expression was decreased significantly in 95% \((19/20)\) of patients after CRC tissue removal (Figure 2D). The above results proved that miR-106b-3p up-regulation in CRC tissues may contribute to the elevated miR-106b-3p expression in serum exosomes. Next, we detected miR-106b-3p expression in six human CRC cell lines (HCT116, SW480, SNU-C1, SW1116, LoVo and KM12SM) and in a colorectal epithelial cell line (NCM460). Compared with that in NCM460 cells, the expression level of miR-106b-3p was significantly higher in CRC cells (Figure 2E) and in exosomes from CRC cell supernatants (Figure 2F). Next, patients were categorized into low and high expression groups according to their serum exosomal miR-106b-3p levels. The relationship between miR-106b-3p expression and the clinicopathological characteristics of 80 CRC patients was evaluated. Patients with high serum exosomal miR-106b-3p expression had an advanced TNM stage and larger tumour volume \((P < 0.05)\) (Supplementary Table S2), indicating that the up-regulated expression of exosomal miR-106b-3p was involved in CRC progression. We also discovered that patients with low serum exosomal miR-106b-3p expression tended to have a good prognosis (Figure 2G,H). Collectively, our clinical data showed that a high level of miR-106b-3p in serum exosomes was correlated with poor prognosis.

MiR-106b-3p promotes CRC cell invasiveness via EMT initiation by targeting DLC-1
Matrigel invasion assays were performed to measure the invasive ability of different CRC cells (Figure 3A). According
Figure 1. Exosomal miR-106b-3p is abundant in mCRC patients

(A–C) Exosomes released by mCRC patients and non-mCRC patients were detected by electron microscopy, NTA and Western blot analysis. Exosomes were positive for EV-specific protein markers (Alix, CD63, TSG101). Calnexin and ApoA1 could not be detected in any of the exosome samples. SW480 cell lysates were used as a control for Alix, TSG101 and Calnexin. Recombinant ApoA1 and SW480 cell lysate served as a control for ApoA1; scale bar, 100 nm. (D) Exosomal RNA sequences from CRC patients with and without lung metastasis are presented in the heatmap. (E) The expression profile changes in exosomal miRNAs in the volcano plot indicate up- and down-regulated miRNAs in mCRC samples compared with non-mCRC samples. (F) RT-PCR analysis of five miRNA (miR-106b-3p, miR-6842-3p, miR-151a-3p, miR-1307-3p and miR-744-5p) levels in 20 cases of serum exosomes from non-mCRC or mCRC patients (10 cases without metastasis, 10 cases with metastasis). (G) RT-PCR analysis of miR-106b-3p expression in 20 paired fresh CRC tissues and matched adjacent normal mucosa samples. The expression of miR-106b-3p in normal mucosa was normalized to 1. (H) RT-PCR analysis of miR-106b-3p expression in 20 cases of non-mCRC or mCRC tissues. The experiments were independently repeated three times, and the data are presented as the mean ± SD; *P < 0.05, **P < 0.01.
Figure 2. Serum exosomal miR-106b-3p correlates with metastatic progression in CRC

(A) The expression levels of miR-106b-3p in serum exosomes from healthy controls or CRC patients (20 healthy donors, 40 non-mCRC patients, and 40 mCRC patients) *P < 0.05 relative to the control. (B) RT-PCR analysis of miR-106b-3p expression in adjacent mucosa and CRC tissues, n = 20, Student’s t test. (C) Spearman’s correlation analysis between the miR-106b-3p levels in CRC tissues and the miR-106b-3p levels in circulating exosomes. Pearson’s correlation coefficient (r) and the P value are shown, n = 20. The P value was derived from Spearman’s test. (D) Fold change in miR-106b-3p in serum exosomes from 20 patients after surgery. The expression of miR-106b-3p at the postoperative stage was normalized to 1. (E) Expression levels of miR-106b-3p in six CRC cell lines (HCT116, SW480, SNU-C1, SW1116, LoVo and KM12SM) and a human colorectal epithelial cell line (NCM460). (F) qRT-PCR detected the levels of miR-106b-3p in exosomes from the supernatants of different cell lines. (G and H) High levels of circulating exosomal miR-106b-3p predicted poor prognosis in CRC. The expression level of exosomal miR-106b-3p in serum was detected by qPCR. Each experiment was performed in triplicate and data were presented as mean ± SD; *P < 0.05, **P < 0.01, ***P < 0.001.

we measured the protein levels of EMT biomarkers, including N-cadherin, vimentin and E-cadherin via Western blot assays. The data demonstrated that the expression levels of E-cadherin, N-cadherin and vimentin were significantly changed after miR-106b-3p overexpression (Figure 3H). In addition, miR-106b-3p-induced EMT was abolished by treatment with the miR-106b-3p inhibitor. These results suggested that EMT-related gene expression was regulated by miR-106b-3p.

Subsequently, we used the public database miRWalk 2.0 to search for potential target genes of miR-106b-3p. DLC-1 was identified as one of the top candidates and has been shown to inhibit metastasis and EMT [26]. In three publicly available databases, RNAhybrid, RegRNA and RNA22 version 2.0, the binding sites of miR-106b-3p in the 3’-UTR of DLC-1 were predicted, and these binding sites were consistent (Figure 4A and Supplementary Figure S2). In addition, EMT plays an important role in the effect of exosomes on tumour metastasis [27]. Therefore, we studied the regulatory effect of miR-106b-3p on DLC-1. RT-PCR analysis showed that miR-106b-3p knockdown robustly increased the level of DLC-1 mRNA, while miR-106b-3p overexpression significantly decreased DLC-1 expression in KM12SM cells (Figure 4B). To investigate the binding ability between miR-106b-3p and the 3’-UTR of DLC-1, a luciferase assay was conducted.
Figure 3. MiR-106b-3p enhances EMT progression and metastatic ability in CRC cells

(A) The invasion ability of different cell lines was determined by transwell assays. (B) qRT-PCR analysis of the relative miR-106b-3p content in SW480 and KM12SM cells after treatment with miR-106b-3p mimic or inhibitor. (C) The transwell assay results demonstrated the effect of miR-106b-3p on cell invasion. (D) Quantitative analysis of (C). (E) Wound healing assays verified that miR-106b-3p promoted cell migration. (F) Quantitative analysis of (E). (G) The CCK-8 assay results demonstrated the viability of SW480 cells (treated with mimic NC, miR-106b-3p mimic) and KM12SM cells (treated with inhibitor NC and miR-106b-3p inhibitor). Two-way ANOVA. (H) The protein levels of vimentin, N-cadherin and E-cadherin were determined by Western blot after cells were transfected with different reagents for 48 h. Results are shown as mean ± SD. All experiments were carried out three times; *P < 0.05, **P < 0.01, ***P < 0.001.

performed. Luciferase reporter vectors carrying wild-type or mutant sequences of the DLC-1 mRNA 3′-untranslated region (UTR) were constructed and cotransfected with the miR-106b-3p mimic or inhibitor. The results showed that treatment with the miR-106b-3p mimic caused a substantial decrease in luciferase activity, while treatment with the miR-106b-3p inhibitor enhanced luciferase activity. Additionally, the mutant sequence of the 3′-UTR of DLC-1 did not impair the luciferase activity response to the miR-106b-3p mimic or inhibitor (Figure 4C). Furthermore, an RNA immunoprecipitation (RIP) assay revealed the specificity of the interaction between miR-106b-3p and DLC-1 in both SW480 and KM12SM cells (Figure 4D). In total, our results demonstrate that DLC-1 is a direct target gene of miR-106b-3p.

Next, we conducted a series of in vitro experiments to determine whether DLC-1 is a mediator during the process of metastasis induced by miR-106b-3p in CRC cells. DLC-1 mRNA and protein levels were decreased after transfection with a short hairpin RNA (shRNA) targeting DLC-1 (DLC-1-shRNA) (Figure 4E,F). Concomitant treatment
Figure 4. MiR-106b-3p promotes EMT by targeting DLC-1

(A) Wild-type and mutant binding site between miR-106b-3p and DLC-1. (B) DLC-1 mRNA levels were analysed in the different KM12SM cell groups by qRT-PCR. (C) Relative luciferase activity of DLC-1 in the presence of the indicated treatments. (D) The interaction between miR-106b-3p and DLC-1 was verified through RIP assays in SW480 (left) and KM12SM (right) cells. The experiments were performed in triplicate. (E and F) qRT-PCR and immunoblot assays of DLC-1 expression in KM12SM cells treated with DLC-1-shRNA or control shRNA. (G) Transwell assay results. (H) Quantitative analysis of (G). (I) Wound healing assay results. (J) Quantitative analysis of (I). (K) The protein levels of DLC-1, vimentin, N-cadherin and E-cadherin were determined by Western blot after transfection with miR-106b-3p inhibitor or DLC-1-shRNA1/2. Results are shown as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001. All of the experiments were performed in triplicate.

with an inhibitor of miR-106b-3p and DLC-1-shRNA significantly restored the miR-106b-3p inhibitor-repressed invasion and migration of CRC cells (Figure 4G–J). Furthermore, the reintroduction of DLC-1-shRNA reversed the miR-106b-3p inhibitor-repressed expression of EMT-related proteins (Figure 4K). These results indicate that miR-106b-3p induces EMT through DLC-1.

Exosomal miR-106b-3p promotes invasiveness and EMT in CRC cells in vitro

To investigate whether exosomes mediated the transfer of miR-106b-3p, we purified exosomes from the conditioned medium (CM) of SW480 (low invasive potential) and KM12SM (high invasive potential) cells by differential centrifugation (Figure 5A). Exosomes were identified by TEM (Figure 5B) and Western blot analysis (Figure 5C). To demonstrate that exosomes can participate in intercellular communication, we cocultured SW480 cells with exosomes (KM12SM exosomes), inhibitor miR-106b-3p exosomes, NC exosomes or PBS (Figure 5D). The inhibitor miR-106b-3p exosomes were obtained after transfecting KM12SM cells with the miR-106b-3p inhibitor. Then, exosomes were extracted from CM; qRT-PCR confirmed that exosomal miR-106b-3p expression predominantly changed upon transfection (Figure 5E). Exosomes stained with PKH67 efficiently entered recipient cells and colocalized in the cytoplasm, as detected by confocal microscopy (Figure 5F). Notably, after SW480 cells were cocultured with KM12SM exosomes for 24 h, the expression of mature miR-106b-3p (Figure 5G), not pri/pre-miR-106b-3p (Figure 5H), was...
Figure 5. MiR-106b-3p can be delivered into SW480 cells via exosomes

(A) Flow chart of exosome extraction. (B and C) Exosomes isolated from the conditioned medium (CM) of low-metastatic (SW480) and high metastatic (KM12SM) potential cells were detected by electron microscopy and Western blot analysis. Exosomal markers CD63, TSG101 and Alix were detected in exosomes isolated from the cell supernatant. In addition, Calnexin and ApoA1 were absent in our isolated exosome samples. SW480 cell lysates were used as controls for Alix, TSG101 and Calnexin. Recombinant ApoA1 and SW480 cell lysates served as a control for ApoA1; scale bar, 100 nm. (D) Schematic diagram of the experiments. A total of 50 μg of exosomes were isolated from 1 × 10⁷ KM12SM cells and cultured with 1 × 10⁵ SW480 cells. (E) Expression of miR-106b-3p in KM12SM cell exosomes, inhibitor miR-106b-3p exosomes and NC exosomes. (F) Confocal microscopic image of fluorescent-labelled KM12SM exosomes after uptake by SW480 cells; scale bars, 50 μm. (G and H) Levels of mature and pri/pre-miR-106b-3p in exosome-treated SW480 cells. Results are presented as mean ± SD. **P < 0.01, ***P < 0.001. All of the experiments were performed in triplicate.

remarkably increased in SW480 cells. The above results indicated that high miR-106b-3p expression in the SW480 cells was caused by exosome transfer rather than miR-106b-3p transcription.

To verify whether exosomal miR-106b-3p promotes CRC cell invasiveness in vitro, SW480 cells were treated with different exosomes or PBS for 24 h. Transwell (Figure 6A, B) and wound healing (Figure 6C, D) assays showed that SW480 cells incubated with KM12SM exosomes exhibited enhanced cell invasive ability. In contrast, inhibitor miR-106b-3p exosomes impaired the invasive capacity of SW480 cells. Next, a Western blot assay was used to verify whether exosomal miR-106b-3p could initiate EMT progression. The data showed that vimentin and N-cadherin up-regulation and E-cadherin down-regulation were observed in SW480 cells after incubation with KM12SM exosomes. These results were reversed when SW480 cells were cocultured with inhibitor miR-106b-3p exosomes (Figure 6E).

Exosomal miR-106b-3p promotes CRC cells lung metastasis in vivo

We next evaluated whether consistent results would be found in vivo by investigating whether exosomal miR-106b-3p promotes CRC metastasis in mouse lungs. Mice were divided into two groups: one group was injected with exosome-stimulated CRC cells in the tail vein, and the other group was injected with an equal amount of non-stimulated CRC cells. A simplified description of the experiment is shown in Figure 7A. Six weeks after SW480 cell implantation, the mice were intraperitoneally injected with luciferin and imaged to observe the formation of lung metastases (Figure 7B). We found that mice pretreated with stimulated SW480 cells had more lung metastatic sites than mice pretreated with non-stimulated SW480 cells (Figure 7C, D). Moreover, a qRT-PCR assay revealed high
miR-106b-3p expression in the lung metastatic tissues from mice pretreated with stimulated SW480 cells (Figure 7E). These findings implied that KM12SM exosomes could transport and transduce miR-106b-3p to SW480 cells and promote the invasive ability of SW480 cells, thus increasing the number of lung metastases in mice.

Next, we analysed the correlation between miR-106b-3p and DLC-1 expression in tumour tissues. Immunohistochemical analysis showed that DLC-1 expression was lower in lung metastatic lesions from mice pretreated with stimulated SW480 cells than in those from mice treated with non-stimulated SW480 cells (Figure 7F). Likewise, DLC-1 expression in the primary tumours of mCRC patients was significantly lower than that in the CRC patients (Figure 7G). Furthermore, a negative correlation was observed between DLC-1 and miR-106b-3p expression in CRC
Figure 7. **In vivo** verification of exosomal miR-106b-3p-activated metastasis and exosomal miR-106b-3p-suppressed DLC-1 expression

(A) Flow chart depicting the **in vivo** experimental design. Equal amounts of SW480 cells were injected through the tail vein.

(B) At 45 days after tumour cell injection, the mice were injected intraperitoneally with luciferin and imaged using a Xenogen IVIS imaging system; $n = 5$ mice.

(C) The lung metastases in each group are indicated by arrowheads. Lung sections were stained with haematoxylin and eosin (HE); scale bars, 0.5 cm (top panels), 100 μm (middle panels), 50 μm (bottom panels).

(D) Number of lung metastatic nodules.

(E) qRT-PCR assay of miR-106b-3p expression in lung metastatic tissues from mice pretreated with stimulated SW480 cells and non-stimulated SW480 cells.

(F) Immunohistochemistry (IHC) analysis of DLC-1 expression in different mouse groups; scale bars, 100 μm (top panels), 50 μm (bottom panels).

(G) IHC analysis of DLC-1 expression in different human tissues; scale bars, 100 μm (top panels), 50 μm (bottom panels).

(H) Spearman’s correlation analysis showed that miR-106b-3p negatively correlated with DLC-1 in CRC. (I) CRC cells with high invasive potential can confer this capability to CRC cells with low invasive potential via exosomal miR-106b-3p transfer, resulting in DLC-1 inhibition and EMT promotion. The results are presented as the mean ± SD. **$P < 0.01$, ***$P < 0.001$. All experiments were performed in triplicate.
tumour tissues (Figure 7H). These results indicated that miR-106b-3p promotes CRC cells metastasis by inhibiting DLC-1 (Figure 7I).

Discussion

Studies have shown that up to 25% of CRC patients have distant metastasis at diagnosis and that the median overall survival (OS) of patients with mCRC is 28 months [28]. Although surgery combined with chemoradiotherapy can prolong the lifespan and alleviate the pain associated with the disease, mCRC patient prognosis remains poor. Therefore, mechanisms linking metastasis initiation and CRC occurrence need to be determined for effective selection of therapeutic targets for CRC patients.

In the present study, we extracted serum exosomal miRNAs from CRC patients with or without metastasis and evaluated the potential metastasis-associated miRNAs in CRC. We first showed that the expression of miR-106b-3p in serum exosomes was increased in mCRC patients and significantly decreased after the complete resection of CRC tissues, indicating that exosomal miR-106b-3p originated from CRC. In addition, high levels of miR-106b-3p in serum exosomes were associated with a poor prognosis. Previous studies showed that miR-106b-3p can be a promising non-invasive biomarker for cancer metastases [29,30]. For example, Zhao et al. demonstrated the use of plasma miR-106b-3p panels to diagnose pancreatic cancer and to screen for distant metastasis [31]. Since miR-106b-3p has been confirmed to exert carcinogenic effects in various cancers, we investigated whether miR-106b-3p could act as an oncogene in CRC. Functional experiments demonstrated that the invasive ability of CRC cells positively correlated with the content of miR-106b-3p in the cells, and increasing the levels of intracellular miR-106b-3p expression enhanced the invasive ability of the cells. Opposing results were observed when miR-106b-3p was silenced. These findings indicated that metastasis-associated miR-106b-3p from serum exosomes is a novel oncogene in CRC and could be used as a potential prognostic biomarker and therapeutic target for CRC patients. Meanwhile, other overexpressed miRNAs have been detected in serum exosomes from mCRC patients, and the relevance of these alterations to colorectal cancer metastasis needs to be studied further.

Next, we analysed the mechanism by which miR-106b-3p mediates CRC cell metastasis. RIP and luciferase reporter assays confirmed that DLC-1 is a direct target gene of miR-106b-3p. DLC-1 belongs to the Rho GTPase-activated protein family, which is expressed at low levels in a series of cancers [32]. As expected, DLC-1 expression in adjacent mucosa was significantly higher than that in primary tumours of CRC patients. Moreover, there is compelling evidence that DLC-1 inhibits tumour metastasis. Du et al. demonstrated that miR-382-5p/DLC-1 regulated the migration and invasion of liver cancer [33]. Therefore, we investigated whether DLC-1 regulates colorectal cancer metastasis. The results of functional assays verified that DLC-1 knockdown in CRC promoted invasion, migration and the expression of EMT-related proteins. In the course of the present study, we also observed a negative correlation between miR-106b-3p and DLC-1 expression in CRC tumour tissues and in mouse lung metastatic lesions. Therefore, miR-106b-3p promotes CRC metastasis and induces EMT by directly targeting DLC-1.

Emerging evidence suggests that exosomes regulate malignancies by transferring multiple classes of cargo molecules, including DNA, RNA and miRNAs, to recipient cells to mediate intercellular communication [34]. The exosome-derived miRNAs participate in tumour formation, proliferation and metastasis by regulating oncogenes or tumour suppressor genes in the target cells [35]. The miRNAs transferred by exosomes can ultimately change the gene expression and functions of recipient cells. However, the communication of CRC cells with different metastatic potential via exosomes is obscured. In the present study, KM12SM-derived exosomes (with high invasive potential) could deliver miR-106b-3p to SW480 cells (with low invasive potential), thus promoting cell migration, invasion and EMT in vitro and increasing the number of lung metastases in vivo; however, the proliferation of SW480 cells was not affected. Based on these results, we proved that exosomal miR-106b-3p has a major regulatory effect on the metastatic potential of CRC cells but not on tumourigenicity.

In the present study, we further provided evidence that the number of lung metastatic foci in mice increased after stimulated SW480 cells was injected into the tail veins, indicating that increased numbers of CRC cells implanted into the lungs. In this process, cancer cells first convert from an epithelial to a mesenchymal-like cell state (EMT) that allows them to leave the primary tumour and migrate to distant sites. When EMT occurs in cancer cells, it can enhance cell migration and local tissue invasion. Whether mesenchymal-to-epithelial transformation (MET) occurs after cancer cells invade the lung tissue microenvironment and the specific mechanism of tumourigenicity in the lungs need to be further discussed in future studies.
Conclusions

MiRNAs encapsulated by exosomes could facilitate intercellular communication, in which exosomes delivered miRNAs into recipient cell, either near or far from the cells of exosomes origin. We provided valuable information on the role of exosomes in cancer metastasis for future studies. Our study first verified that the level of serum exosomal miR-106b-3p is increased in mCRC and is significantly correlated with poor prognosis. Moreover, exosomes from high-metastatic CRC cells can deliver miR-106b-3p to surrounding cells, thus promoting their metastatic ability and inhibiting DLC-1 in recipient cells in vitro. Furthermore, exosomal miR-106b-3p promotes lung metastasis of CRC cells in vivo. Therefore, the present study reveals new functions of exosomal miR-106b-3p in CRC metastasis and indicates that exosomal miR-106b-3p is a potential prognostic biomarker and a valuable therapeutic target for CRC.

Clinical perspectives

- The effects and mechanisms of exosomes in CRC metastasis are poorly understood.
- The present study described here illustrates the functional role of exosomal miR-106b-3p, which was correlated with poor prognosis and critically regulated metastasis by inhibiting DLC-1 in colorectal cancer.
- Our findings suggest that exosomal miR-106b-3p may promote CRC metastasis and may be used as a potential prognostic biomarker and therapeutic target for CRC patients.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

Conceptualization: He Liu and Binbin Cui; Data curation: Ping Sun; Formal analysis: Kaiming Leng and Yi Xu; Funding acquisition: Binbin Cui; Investigation: Kefei Yao and Juge Bai; Methodology: Yanlong Liu and Binbin Cui; Project administration: Binbin Cui; Resources: Peng Han; Software: Ping Sun and Liqi Mei; Supervision: Binbin Cui; Validation: Bomiao Zhang and Chunbo Li; Visualization: He Liu; Writing – original draft: He Liu; Writing – review and editing: He Liu and Binbin Cui.

Ethics Approval and Consent to Participate

The research protocol was reviewed and approved by the Ethics Review Committees of Harbin Medical University, and written informed consent was obtained from each patient included in the study. All procedures involving animals were performed according to the NIH guidelines for the Care and Use of Laboratory Animals.

Consent for Publication

We received consent for publication from the individual patients who participated in the present study. The consent forms will be provided upon request.

Data Availability

The data in the present study are available from the corresponding authors upon reasonable request.

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Abbreviations

CRC, colorectal cancer; DLC-1, deleted in liver cancer-1; EMT, epithelial–mesenchymal transition; IHC, immunohistochemistry; mCRC, metastatic colorectal cancer; miRNA, microRNA.

References


