Exosomal miR-224-5p from colorectal cancer cells promotes malignant transformation of human colon normal cells by promoting cell proliferation through downregulation of CMTM4

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Research

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Abstract

Background

Interactions between malignant cells and neighboring normal cells is important for carcinogenesis. In addition, cancer cell-derived exosomes have been shown to promote the malignant transformation of recipient cells, but the mechanisms remain unclear.

Methods

The level of miR-224-5p in colorectal cancer (CRC) cell-derived exosomes was determined by RT-qPCR assay. In addition, PKH26 dye-labeled exosomes were used to assess the efficacy of the transfer of exosomes between SW620 and CCD 841 CoN cells.

Results

In this study, we found that overexpression of miR-224-5p significantly promoted the proliferation, migration and invasion of SW620 cells. In addition, miR-224-5p can be transferred from SW620 cells to CCD 841 CoN cells via exosomes. SW620 cell-derived exosomes overexpressing miR-224-5p markedly promoted proliferation, migration and invasion of CCD 841 CoN cells. Meanwhile, SW620 cell-derived exosomal miR-224-5p notably decreased the expression of CMTM4 in CCD 841 CoN cells. Furthermore, SW620 cell-derived exosomal miR-224-5p significantly inhibited tumor growth in a xenograft model *in vivo*.

Conclusion

These findings suggested that SW620 cell-derived exosomal miR-224-5p could promote malignant transformation and tumorigenesis *in vitro* and *in vivo* via downregulation of CMTM4, suggesting that miR-224-5p might be a potential target for therapies in CRC.

Background

Colorectal cancer (CRC) is one of the most common digestive tract malignancies with high morbidity and mortality [1, 2]. The development and progression of CRC are regulated by a number of factors, such as dietary behaviors, chronic intestinal inflammation, aging, smoking and mutations [3, 4]. It is estimated that around 1 million people will be affected by CRC every year, accompanied by overt metastatic disease [2]. Recently, several methods including radiotherapy, and chemoradiotherapy have been used for the treatment of CRC for years [5–7]. However, the prognosis of patients with CRC is still unsatisfactory [5–7]. Therefore, identifying specific biomarkers is of great importance in order to improve early diagnosis and investigate novel treatment strategies for patients of CRC.

MicroRNAs (miRNAs) have been found to be involved in the development of CRC [8]. Accumulating evidences have shown that miRNAs exert a vital role in regulating CRC progression through acting as tumor suppressors and oncogenes [8, 9]. Exosomes are small (30–100 nm) extracellular vesicles that carry different nucleic acids including miRNAs [10, 11]. In addition, exosomes serve as key mediators in cell-to-cell communication, often to prepare a premetastatic niche, remodel the extracellular environment and escape immune surveillance [11, 12]. Recently, cancer cell-secreted exosomes have been reported to influence tumor microenvironment and promote cancer cell growth, invasion and angiogenesis [13–15]. Meanwhile, cancer cell-secreted miRNAs can be delivered to recipient cells via exosomes, then perform a key regulatory role in migration and invasion of CRC cells [16, 17].

Herein, in this study, we found that CRC cell-derived exosomal miR-224-5p can be transferred to human colon normal cells, promoting the malignant transformation of CCD 841 CoN cells (human colon normal cells) by targeting CMTM4. These findings may provide a novel target for the treatment of CRC.

Materials And Methods

Data collection and differential expression analysis

CRC-related datasets (GSE18392, GSE115513 and GSE126093) were downloaded from the Gene Expression Omnibus (GEO) database. The differentially expressed miRNAs (DEMs) between CRC tissues and adjacent normal tissues were identified using R language. The miRNAs with $P < 0.05$ and $|\log_2(FC)| > 2$ were selected as the significantly DEMs. The overlapping DEMs were identified using a Venn diagram from three datasets (GSE18392, GSE115513 and GSE126093). In addition, TCGA dataset was used to determine the association between the overall survival of patients with CRC and miR-224-5p level.

Cell culture

Human colon normal cell line (CCD 841 CoN), CRC cell lines (HT-29, HCT116, SW620 and SW480) and 293T cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences. Cells were maintained in DMEM medium containing 10% FBS and cultured in a humidified incubator containing 5% CO₂ at 37°C.

Cell transfection

MiRNA negative control (NC), miR-224-5p agomir, miR-224-5p antagomir were obtained from RIBOBIO. The NC, miR-224-5p agomir or miR-224-5p antagomir were transfected into SW620 and HCT116 cells respectively by using lipofectamine 2000.

In addition, the sequence of CMTM4 was amplified by PCR and then subcloned into pcDNA3.1 vector to generate pcDNA3.1-CMTM4 overexpression (CMTM4-OE) plasmids. The pcDNA3.1-NC and CMTM4-OE plasmids were transfected into CCD 841 CoN cells by using Lipofectamine 2000 reagents. Later on, 48 h after transfection, the transfected cells were selected with neomycin (Thermo Fisher Scientific).

RT-qPCR assay

The TRIpure Total RNA Extraction Reagent (ELK Biotechnology, Hubei, China) was used to extract total RNA from cells. After that, RNA (1 µg) was reversely transcribed into cDNA using EntiLink™ 1st Strand cDNA Synthesis Kit (ELK). Later on, real-time PCR was carried out on a StepOne™ Real-Time PCR System using the EnTurbo™ SYBR Green PCR SuperMix kit (ELK). The primer sequences were as follows: β-actin, Forward: 5'-GTCCACCGCAAATGCTTCTA-3', Reverse: 5'-TGCTGTCACCTTCACCGTTC-3'; CMTM4, Forward: 5'-CTGCCGTGATATTTGGCTTCT-3', Reverse: 5'-CGGATGTAGTCATTGGTGCTCT-3'; U6, Forward: 5'-CTCGCTTCGGCAGCACAT-3', Reverse: 5'-AACGCTTCACGAATTTGCGT-3'; miR-224-5p, Forward: 5'-CAAGTCACTAGTGGTTCCGTTTAG-3', Reverse: 5'-CTCAACTGGTGTCTGGAGTC-3'. The expression of CMTM4 was normalized to β-actin. The expression of miR-224-5p was normalized to U6.

Cell viability assay

The transfected cells were plated onto 96-well plates (about 5,000 per well). At 0, 24, 48 or 72 h post-transfection, CCK-8 reagent (10 µl, MedChem Express) was added into each well, and cells were then incubated for 2 h. After that, the absorbance of each well was detected using a microplate reader at 450 nm.

Immunofluorescence assay

Cells were treated with 4% paraformaldehyde for 20 min and incubated with 5% BSA for 1 h. Then, cells were incubated with primary antibodies anti-Ki67 at 4°C overnight. Later on, cells were incubated with a corresponding secondary antibody for 1 h. Subsequently, images were captured using a fluorescence microscope. Nuclei were stained by DAPI.

Transwell assay

Transwell migration or invasion assays were performed using transwell chambers uncoated or coated with Matrigel. Cells (2×10^4 cells/well) were seed onto the upper chamber of each insert (Corning). In addition, the lower chambers were loaded with DMEM (600 µL) containing 10% FBS. After 24 h of incubation, the cells on the lower surface were stained with 0.1% crystal violet. Subsequently, a fluorescence microscope was used to observe migrated or invasive cells.

Flow cytometry assay

Cell apoptosis was detected using an Annexin-V-FITC apoptosis detection kit. Briefly, 1×10^5 cells were stained with Annexin-V-FITC (5 µl) and PI (5 µl) staining solution for 15 min in darkness. Subsequently, cell apoptosis was analyzed by a flow cytometer.

Dual-luciferase reporter assay

293T cells were co-transfected with pGL6-miR-based luciferase reporter plasmids containing wild-type (WT) or mutant (MT) 3'-UTR of CMTM4, and miR-224-5p agomir using Lipofectamine 2000. Later on, the luciferase activity in cell lysates was measured by the Dual Luciferase Reporter Assay System (Beyotime, Beijing, China).

Exosome isolation and characterization

The conditioned media (CM) of SW620 cells was collected. Later on, exosomes were isolated using the GETTM Exosome Isolation Kit (GeneExosome technologies). Nanoparticle-tracking analysis (Particle Metrix, Meerbusch, Germany) was applied to determine the size of exosomes. Next, a transmission electron microscopy (TEM) was used to visualize the morphology of exosomes as described previously [18].

Exosome labeling and uptake

SW620 cell-derived exosomes were mixed with PKH26 dye for 30 min. After that, PKH26-tagged exosomes were added into CCD 841 CoN cells and incubated for 24 h. Subsequently, CCD 841 CoN cells that uptake the labelled exosomes was observed by a fluorescence microscope. Nuclei were stained by DAPI.

Western blot assay

Proteins were separated by 10% SDS-PAGE and then transferred onto a PVDF membrane. Later on, the membrane was incubated overnight at 4°C with primary antibodies against CMTM4, p-Akt, Akt, p-ERK, ERK, CD63, TSG101, β -actin, and then incubated with the corresponding secondary antibody at room temperature for 1 h. Subsequently, immune complexes were detected using the ECL reagents.

Animal studies

The BALB/c nude mice (4–5 weeks old) were purchased from the Vital River Laboratories (Beijing, China). SW620 cells (1×10^7 cells) were subcutaneously injected into left flank of nude mice. When the tumors reach about 200 mm³, mice were divided randomly into four groups: control, Exo-NC, Exo-miR-224-5p agomir and Exo-miR-224-5p antagomir. Then, mice were intravenously injected with PBS, Exo-NC, Exo-miR-224-5p agomir or Exo-miR-224-5p antagomir twice a week. The tumor size was measured with a vernier caliper every week and the volume was calculated by the following formula $V = \text{length} \times \text{width}^2 \times 0.5$. After 3 weeks of tumor cell implantation, the mice were sacrificed, and the tumors from different groups were removed and weighted. All animal experiments were approved by the Ethics Committee of the Harbin Medical University Cancer Hospital and performed following the procedures of National Institutes of Health guide for the care and use of laboratory animals.

TUNEL assay

Cell apoptosis in tumor tissues were assessed using an APO-BrdU™ TUNEL Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software (version 7.0, La Jolla, CA, USA). Differences between three or more groups were analyzed by One-way analysis of variance (ANOVA) and Tukey's tests. Data are presented as the mean $\pm$ standard deviation (S.D.). The differences were considered significant at *P < 0.05. All data were repeated in triplicate.

Results

Identification of DEMs in CRC

To identify the DEMs between CRC tissues and adjacent normal tissues, R language was performed to analyze the expression profiles of miRNAs from three CRC-related datasets (GSE18392, GSE115513 and GSE126093). As shown in Fig. 1A, 1B and 1C, a total of 24, 44 and 417 DEMs were identified from the GSE18392, GSE115513 and GSE126093 respectively. In addition, 4 overlapping DEMs were identified in three datasets, including miR-31-5p, miR-135-5p, miR-183-5p and miR-224-5p (Fig. 1D). Zheng et al revealed that miR-224 level was increased in CRC tissues [19]. Meanwhile, in the TCGA dataset, high levels of miR-224-5p in patients with CRC are associated with a shorter overall survival, indicating that increased miR-224-5p levels may predict poor overall survival in patients with CRC (Fig. 1E).

Overexpression of miR-224-5p promoted the proliferation, migration and invasion of CRC cells

To investigate the role of miR-224-5p in CRC cells, RT-qPCR was performed to assess the level of miR-224-5p in CCD 841 CoN cells and CRC cells. As indicated in Fig. 2A, miR-224-5p level was markedly increased in HT-29, HCT116, SW620 and SW480 cells compared with CCD 841 CoN cells. HCT116 and SW620 cell lines exhibited higher levels of miR-224-5p (Fig. 2A). As shown in Fig. 2B and 2C, miR-224-5p agomir notably increased miR-224-5p level in SW620 and HCT116 cells, while miR-224-5p antagomir displayed the opposite results. Moreover, miR-224-5p agomir notably promoted the viability of SW620 and HCT116 cells, while miR-224-5p antagomir obviously suppressed cell viability (Fig. 2D and 2E). Meanwhile, as shown in Fig. 3A, 3B and 3C, miR-224-5p agomir significantly promoted the proliferation, migration and invasion of SW620 cells. In contrast, miR-224-5p antagomir suppressed cell proliferation, migration and invasion, triggered cell apoptosis (Fig. 3A, 3B, 3C, 3D, 3E and 3F). Collectively, overexpression of miR-224-5p could promote the proliferation, migration and invasion of CRC cells.

CMTM4 is a binding target of miR-224-5p

Evidences have shown that miRNAs can negatively regulate protein expression though binding to the 3'-UTR of their target mRNA [20, 21]. To find the target genes of miR-224-5p, five bioinformatics tools (miRWalk, miRanda, miRDB, RNA22 and TargetScan) were used. We found that CMTM4 was one of the best candidates (Fig. 4A). Xue et al indicated that CMTM4 level was frequently downregulated in CRC

tissues [22]. As predicted, a marked decrease in CMTM4 at mRNA level was found in SW620 and HCT116 cells (Fig. 4B). To validate that CMTM4 is a binding target of miR-224-5p, dual-luciferase reporter assay was used. As shown in Fig. 4C, miR-224-5p agomir notably reduced the luciferase activity of CMTM4-WT. Meanwhile, miR-224-5p agomir obviously decreased the expression of CMTM4 at mRNA level in SW620 cells, while miR-224-5p antagomir displayed the opposite results (Fig. 4D). These results indicated that CMTM4 is a direct binding target of miR-224-5p.

MiR-224-5p can be transferred from SW620 cells to CCD 841 CoN cells via exosomes

Evidences have shown that cancer cell-derived exosomes exert a vital role in the process of malignant transformation [23, 24]. In this study, exosomes were isolated from the CM of CCD 841 CoN cells and SW620 cells. TEM and NTA analysis demonstrated that exosomes secreted from CCD 841 CoN and SW620 cells had the characteristic size (40 to 100 nm) and cup-shaped morphology, and expressed the exosomal markers, TSG101 and CD63 (Fig. 5A, 5B and 5C), suggesting that exosomes were isolated from cells. Meanwhile, miR-224-5p level was increased in exosomes derived from SW620 cells compared with that in exosomes derived from CCD 841 CoN cells (Fig. 5D).

We further explored whether miR-224-5p can be transferred from SW620 cells to CCD 841 CoN cells via exosomes. First, exosomes were isolated from SW620 cells that were transfected with NC, miR-224-5p agomir or miR-224-5p antagomir (Exo-NC, Exo-miR-224-5p agomir and Exo-miR-224-5p antagomir). As revealed in Fig. 5E, these exosomes expressed the exosomal markers, TSG101 and CD63. Next, to determine whether CCD 841 CoN cells could take up SW620 cell-derived exosomes, CCD 841 CoN cells were co-cultured with PKH26-label exosomes. As revealed in Fig. 5F, PKH26 fluorescence dye was observed in CCD 841 CoN cells. In addition, miR-224-5p level was upregulated in CCD 841 CoN cells incubated with Exo-miR-224-5p agomir, whereas CCD 841 CoN cells co-cultured with Exo-miR-224-5p antagomir displayed the opposite results (Fig. 5G). Collectively, oligonucleotide sequences (miR-224-5p agomir or miR-224-5p antagomir) could be transferred from SW620 cells to CCD 841 CoN cells via exosomes.

Intercellular transfer of miR-224-5p agomir by exosomes promoted malignant transformation of CCD 841 CoN cells via downregulation of CMTM4

Next, we investigated the role of Exo-miR-224-5p agomir or Exo-miR-224-5p antagomir in recipient cells. As revealed in Fig. 6A, CMTM4 level was markedly upregulated in CCD 841 CoN cells after transfection with CMTM4-OE. In addition, Exo-miR-224-5p agomir significantly promoted the viability, proliferation, migration and invasion of CCD 841 CoN cells compared with Exo-NC group; however, these Exo-miR-224-5p agomir-induced changes were inhibited when CCD 841 CoN cells were transfected with CMTM4-OE plasmids (Fig. 6B-6H). In contrast, Exo-miR-224-5p antagomir notably suppressed the viability, proliferation, migration and invasion, and triggered the apoptosis of CCD 841 CoN cells compared with Exo-NC group (Fig. 6B-6J). Moreover, Exo-miR-224-5p agomir markedly downregulated the expression of CMTM4 and upregulated p-Akt and p-ERK protein expressions in CCD 841 CoN cells; however, these phenomenon were reversed when CCD 841 CoN cells were transfected with CMTM4-OE plasmids (Fig. 7A,

7B, 7C and 7D). These data suggested that exosomal miR-224-5p is involved in the malignant transformation of CCD 841 CoN cells.

Exo-miR-224-5p antagomir suppressed the growth of CRC cells *in vivo*

We further investigated the role of exosomal miR-224-5p on tumor growth *in vivo*. As shown in Fig. 8A, 8B and 8C, the tumor volume and tumor weight were significantly increased in the Exo-miR-224-5p agomir group, while the opposite results were observed in the Exo-miR-224-5p antagomir group. In addition, the level of miR-224-5p was upregulated in tumor tissues of tumor-bearing mice that were received Exo-miR-224-5p agomir; whereas mice that received Exo-miR-224-5p antagomir displayed the opposite results (Fig. 8D). Moreover, TUNEL assay indicated that Exo-miR-224-5p antagomir obviously induced cell apoptosis in tumor tissues (Fig. 8E and 8F). Meanwhile, the expression of CMTM4 was decreased and p-Akt and p-ERK protein expressions was increased in tumor tissues of mice that received Exo-miR-224-5p agomir compared with Exo-NC group; whereas mice that treated with Exo-miR-224-5p antagomir displayed the opposite results (Fig. 8G, 8H, 8I and 8J). Collectively, Exo-miR-224-5p antagomir could suppress the growth of CRC cells *in vivo* via upregulation of CMTM4.

Discussion

The development of human cancers is a complex process [25]. Tumor cells are the major driving force behind the development and progression of human cancers [26]. García-Olmo found that interactions between tumor cells and neighboring normal cells in the tumor microenvironment might be essential for tumor progression [27]. Local microenvironments have been found to exert a vital role in mediating intercellular communication between malignant cells and nonmalignant cells [28, 29]. In addition, cancer cell-derived exosomes can act as communicative vectors, participating in remodeling the tumor microenvironment [30, 31]. Importantly, cancer cell-derived exosomes have been shown to promote the malignant transformation of recipient cells, increasing cell migratory and invasive abilities [32]. Meanwhile, cancer cell-derived exosomes can carry different miRNAs, and these exosomal miRNAs can modulate the function of recipient cells via activation or inactivation of multiple cancer related pathways through transferring into recipient cells and mediating protein expression [33, 34]. Thus, the discovery of novel circulating biomarkers of CRC may help to improve diagnosis or treatment of CRC.

Currently, miR-224-5p has been identified to be dysregulated in human cancers, including CRC [19, 35]. In this study, we found that miR-224-5p level was significantly upregulated in CRC cells, and downregulation of miR-224-5p could induce cell apoptosis and inhibit cell migration and invasion. Consistent with our present results, zheng et al showed that miR-224 overexpression promoted CRC cell proliferation and migration via targeting BTRC [19]. In addition, liang et al showed that downregulation of miR-224 could suppress the proliferation and trigger the apoptosis of adriamycin-resistant CRC cells [36]. Our data found that miR-224-5p may act as an oncogene for CRC.

For the first time, we found that exosomal miR-224-5p is secreted by SW620 cells that could be internalized by normal colon cell line CCD 841 CoN, suggesting that SW620-secreted miR-224-5p can be

delivered into CCD 841 CoN cells via exosomes. A series of functional experiments indicated that exosomal miR-224-5p derived from CRC cells could promote CCD 841 CoN cell proliferation, migration and invasion. Consistent with our present results, wei et al showed that miR-15b-3p can be transferred from GC cells to normal GES-1 gastric epithelium cells via exosomes, and then promoted GES-1 cell malignant transformation [37]. In addition, evidence has shown that inactivation of tumor-suppressor and activation of oncogene are considered as the key causes driving the progressive transformation of normal cells to malignant cells [26]. In the present study, we found that CMTM4 was a binding target of miR-224-5p. It has been shown that CMTM4 is a tumor suppressor in human cancers, including CRC [22, 38]. Our data showed that exosomal miR-224-5p promoted the proliferation, migration and invasion of CCD 841 CoN cells via downregulation of CMTM4. These results suggested that SW620 cell-derived exosomal miR-224-5p could promote the malignant transformation of CCD 841 CoN cells via inactivation of a tumor suppressor CMTM4.

Conclusion

Collectively, our data showed that exosomes secreted from SW620 cells can deliver miR-224-5p into CCD 841 CoN cells, promoting malignant transformation of CCD 841 CoN cells via downregulation of CMTM4, suggesting that miR-224-5p might be a potential target for therapies in CRC.

Abbreviations

ANOVA, analysis of variance; CCK-8, Cell Counting kit-8; CM, conditioned media; CRC, colorectal cancer; DEMs, differentially expressed miRNAs; GEO, Gene Expression Omnibus; miRNAs, microRNAs; NC, negative control; NTA, Nanoparticle-tracking analysis; S.D., standard deviation; TEM, transmission electron microscopy.

Declarations

Acknowledgements

Not applicable.

Consent for publication

All authors reached an agreement to publish the study in this journal.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Feng Wu made major contributions to the conception, design and manuscript drafting of this study. Jiani Yang, Guoyin Shang, Zhijia Zhang, Sijia Niu, Yang Liu, Jing Jing and Hongru Liu were responsible for data acquisition, data analysis, data interpretation and manuscript revision. Yu Fang made substantial contributions to conception and design of the study and revised the manuscript critically for important intellectual content. All authors agreed to be accountable for all aspects of the work. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Ethics Committee of the Harbin Medical University Cancer Hospital and performed following the procedures of National Institutes of Health guide for the care and use of laboratory animals.

Conflicts of Interest

The authors declare no potential conflicts of interest.

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References

1. Wang Q, Jia S, Jiao Y, Xu L, Wang D, Chen X, Hu X, Liang H, Wen N, Zhang S, et al: **SSRP1 influences colorectal cancer cell growth and apoptosis via the AKT pathway.** *Int J Med Sci*2019, **16**:1573-1582.
2. Jiang L, Zhao XH, Mao YL, Wang JF, Zheng HJ, You QS: **Long non-coding RNA RP11-468E2.5 curtails colorectal cancer cell proliferation and stimulates apoptosis via the JAK/STAT signaling pathway by targeting STAT5 and STAT6.** *J Exp Clin Cancer Res*2019, **38**:465.
3. Li SQ, Su N, Gong P, Zhang HB, Liu J, Wang D, Sun YP, Zhang Y, Qian F, Zhao B, et al: **The Expression of Formyl Peptide Receptor 1 is Correlated with Tumor Invasion of Human Colorectal Cancer.** *Sci Rep*2017, **7**:5918.
4. Wu H, Liu J, Yin Y, Zhang D, Xia P, Zhu G: **Therapeutic Opportunities in Colorectal Cancer: Focus on Melatonin Antioncogenic Action.** *Biomed Res Int*2019, **2019**:9740568.
5. Dellas K, Reese T, Richter M, Arnold D, Dunst J: **Concurrent chemoradiation of metastases with capecitabine and oxaliplatin and 3D-CRT in patients with oligometastatic colorectal cancer: results of a phase I study.** *Radiat Oncol*2012, **7**:83.
6. Wang X, Liu J, Jiang L, Wei X, Niu C, Wang R, Zhang J, Meng D, Yao K: **Bach1 Induces Endothelial Cell Apoptosis and Cell-Cycle Arrest through ROS Generation.** *Oxid Med Cell Longev*2016, **2016**:6234043.

7. Villanova L, Barbini C, Piccolo C, Boe A, De Maria R, Fiori ME: **miR-1285-3p Controls Colorectal Cancer Proliferation and Escape from Apoptosis through DAPK2.** *Int J Mol Sci*2020, **21**.
8. To KK, Tong CW, Wu M, Cho WC: **MicroRNAs in the prognosis and therapy of colorectal cancer: From bench to bedside.** *World J Gastroenterol*2018, **24**:2949-2973.
9. Slattery ML, Herrick JS, Mullany LE, Samowitz WS, Sevens JR, Sakoda L, Wolff RK: **The co-regulatory networks of tumor suppressor genes, oncogenes, and miRNAs in colorectal cancer.** *Genes Chromosomes Cancer*2017, **56**:769-787.
10. Zeng Z, Li Y, Pan Y, Lan X, Song F, Sun J, Zhou K, Liu X, Ren X, Wang F, et al: **Cancer-derived exosomal miR-25-3p promotes pre-metastatic niche formation by inducing vascular permeability and angiogenesis.** *Nat Commun*2018, **9**:5395.
11. Elsherbini A, Bieberich E: **Ceramide and Exosomes: A Novel Target in Cancer Biology and Therapy.** *Adv Cancer Res*2018, **140**:121-154.
12. Becker A, Thakur BK, Weiss JM, Kim HS, Peinado H, Lyden D: **Extracellular Vesicles in Cancer: Cell-to-Cell Mediators of Metastasis.** *Cancer Cell*2016, **30**:836-848.
13. Yan W, Wu X, Zhou W, Fong MY, Cao M, Liu J, Liu X, Chen CH, Fadare O, Pizzo DP, et al: **Cancer-cell-secreted exosomal miR-105 promotes tumour growth through the MYC-dependent metabolic reprogramming of stromal cells.** *Nat Cell Biol*2018, **20**:597-609.
14. Bigagli E, Luceri C, Guasti D, Cinci L: **Exosomes secreted from human colon cancer cells influence the adhesion of neighboring metastatic cells: Role of microRNA-210.** *Cancer Biol Ther*2016, **17**:1062-1069.
15. Chen X, Zhang S, Du K, Zheng N, Liu Y, Chen H, Xie G, Ma Y, Zhou Y, Zheng Y, et al: **Gastric Cancer-Secreted Exosomal X26nt Increases Angiogenesis and Vascular Permeability by Targeting VE-cadherin.** *Cancer Sci*2020.
16. Zhou W, Fong MY, Min Y, Somlo G, Liu L, Palomares MR, Yu Y, Chow A, O'Connor ST, Chin AR, et al: **Cancer-secreted miR-105 destroys vascular endothelial barriers to promote metastasis.** *Cancer Cell*2014, **25**:501-515.
17. Liu T, Zhang X, Du L, Wang Y, Liu X, Tian H, Wang L, Li P, Zhao Y, Duan W, et al: **Exosome-transmitted miR-128-3p increase chemosensitivity of oxaliplatin-resistant colorectal cancer.** *Mol Cancer*2019, **18**:43.
18. Che Y, Shi X, Shi Y, Jiang X, Ai Q, Shi Y, Gong F, Jiang W: **Exosomes Derived from miR-143-Overexpressing MSCs Inhibit Cell Migration and Invasion in Human Prostate Cancer by Downregulating TFF3.** *Mol Ther Nucleic Acids*2019, **18**:232-244.
19. Zheng Q, Yu JJ, Li C, Li J, Wang J, Wang S: **miR-224 targets BTRC and promotes cell migration and invasion in colorectal cancer.** *3 Biotech*2020, **10**:485.

20. Mohamed ZI, Tee SF, Chow TJ, Loh SY, Yong HS, Bakar AKA, Tang PY: **Functional characterization of two variants in the 3'-untranslated region (UTR) of transcription factor 4 gene and their association with schizophrenia in sib-pairs from multiplex families.** *Asian J Psychiatr*2019, **40**:76-81.
21. Li F, Zhao H, Su M, Xie W, Fang Y, Du Y, Yu Z, Hou L, Tan W: **HnRNP-F regulates EMT in bladder cancer by mediating the stabilization of Snail1 mRNA by binding to its 3' UTR.** *EBioMedicine*2019, **45**:208-219.
22. Xue H, Li T, Wang P, Mo X, Zhang H, Ding S, Ma D, Lv W, Zhang J, Han W: **CMTM4 inhibits cell proliferation and migration via AKT, ERK1/2, and STAT3 pathway in colorectal cancer.** *Acta Biochim Biophys Sin (Shanghai)*2019, **51**:915-924.
23. Uziel O, Gutkin A, Beery E, Lahav M: **[EXOSOMES AS MEDIATORS OF INTERCELLULAR COMMUNICATION: THE CANCER AND TELOMERASE CONNECTION].** *Harefuah*2017, **156**:710-714.
24. Matsumoto Y, Kano M, Murakami K, Toyozumi T, Suito H, Takahashi M, Sekino N, Shiraishi T, Kamata T, Ryuzaki T, et al: **Tumor-derived exosomes influence the cell cycle and cell migration of human esophageal cancer cell lines.** *Cancer Sci*2020, **111**:4348-4358.
25. He M, Rosen J, Mangiameli D, Libutti SK: **Cancer development and progression.** *Adv Exp Med Biol*2007, **593**:117-133.
26. Yuan Y, Jiang YC, Sun CK, Chen QM: **Role of the tumor microenvironment in tumor progression and the clinical applications (Review).** *Oncol Rep*2016, **35**:2499-2515.
27. García-Olmo DC, Picazo MG, García-Olmo D: **Transformation of non-tumor host cells during tumor progression: theories and evidence.** *Expert Opin Biol Ther*2012, **12 Suppl 1**:S199-207.
28. Lu P, Weaver VM, Werb Z: **The extracellular matrix: a dynamic niche in cancer progression.** *J Cell Biol*2012, **196**:395-406.
29. Turley SJ, Cremasco V, Astarita JL: **Immunological hallmarks of stromal cells in the tumour microenvironment.** *Nat Rev Immunol*2015, **15**:669-682.
30. Kalluri R: **The biology and function of exosomes in cancer.** *J Clin Invest*2016, **126**:1208-1215.
31. Kahlert C, Kalluri R: **Exosomes in tumor microenvironment influence cancer progression and metastasis.** *J Mol Med (Berl)*2013, **91**:431-437.
32. Qu JL, Qu XJ, Zhao MF, Teng YE, Zhang Y, Hou KZ, Jiang YH, Yang XH, Liu YP: **Gastric cancer exosomes promote tumour cell proliferation through PI3K/Akt and MAPK/ERK activation.** *Dig Liver Dis*2009, **41**:875-880.
33. Jin X, Chen Y, Chen H, Fei S, Chen D, Cai X, Liu L, Lin B, Su H, Zhao L, et al: **Evaluation of Tumor-Derived Exosomal miRNA as Potential Diagnostic Biomarkers for Early-Stage Non-Small Cell Lung Cancer**

Using Next-Generation Sequencing. *Clin Cancer Res*2017, **23**:5311-5319.

34. Shang A, Gu C, Wang W, Wang X, Sun J, Zeng B, Chen C, Chang W, Ping Y, Ji P, et al: **Exosomal circPACRGL promotes progression of colorectal cancer via the miR-142-3p/miR-506-3p- TGF- β 1 axis.** *Mol Cancer*2020, **19**:117.
35. Gan BL, Zhang LJ, Gao L, Ma FC, He RQ, Chen G, Ma J, Zhong JC, Hu XH: **Downregulation of miR-224-5p in prostate cancer and its relevant molecular mechanism via TCGA, GEO database and insilico analyses.** *Oncol Rep*2018, **40**:3171-3188.
36. Liang CQ, Fu YM, Liu ZY, Xing BR, Jin Y, Huang JL: **The effect of miR-224 down-regulation on SW80 cell proliferation and apoptosis and weakening of ADM drug resistance.** *Eur Rev Med Pharmacol Sci*2017, **21**:5008-5016.
37. Wei S, Peng L, Yang J, Sang H, Jin D, Li X, Chen M, Zhang W, Dang Y, Zhang G: **Exosomal transfer of miR-15b-3p enhances tumorigenesis and malignant transformation through the DYNLT1/Caspase-3/Caspase-9 signaling pathway in gastric cancer.** *J Exp Clin Cancer Res*2020, **39**:32.
38. Li T, Cheng Y, Wang P, Wang W, Hu F, Mo X, Lv H, Xu T, Han W: **CMTM4 is frequently downregulated and functions as a tumour suppressor in clear cell renal cell carcinoma.** *J Exp Clin Cancer Res*2015, **34**:122.

Figures

Figure 1

Identification of DEMs in CRC. (A, B, C) The volcano plot shows the DEMs between CRC tissues and adjacent normal tissues in the GSE18392, GSE115513 and GSE126093 datasets. (D) Venn diagram of overlapping DEMs from intersection of GSE18392, GSE115513 and GSE126093 datasets. (E) The correlation between the level of miR-224-5p and overall survival rate in patients with CRC in the TCGA dataset.

Figure 2

Overexpression of miR-224-5p increased the viability of CRC cells. (A) MiR-224-5p levels in CRC cells (HT-29, HCT116, SW620 and SW480), and CCD 841 CoN cells were detected by RT-qPCR. **P < 0.01 vs. CCD 841 CoN group. (B, C) SW620 and HCT116 cells were transfected with NC, miR-224-5p agomir or miR-224-5p antagonist. RT-qPCR was applied to detect the level of miR-224-5p in SW620 and HCT116 cells. (D, E) Cell viability was determined by CCK-8 assay. **P < 0.01 vs. NC group.

Figure 3

Overexpression of miR-224-5p promoted the proliferation, migration and invasion of CRC cells. SW620 cells were transfected with NC, miR-224-5p agomir or miR-224-5p antagonist. (A, B) Cell proliferation detected using Ki67 immunofluorescence assay. (C) Transwell migration assay was applied to assess cell migration. (D) Transwell invasion assay was conducted to determine cell migration. (E, F) Cell apoptosis was determined using flow cytometry assay with Annexin V and PI double staining. **P < 0.01 vs. NC group.

Figure 4

CMTM4 is a direct binding target of miR-224-5p. (A) Schematic diagram of binding sites between miR-224-5p and CMTM4, and the mutation of binding sites in CMTM4. (B) The levels of CMTM4 in CCD 841 CoN, SW620 and HCT116 cells were assessed by RT-qPCR. **P < 0.01 vs. CCD 841 CoN group. (C) Luciferase reporter assay in SW620 cells co-transfected with WT or MT CMTM4 3'UTR reporter gene, and NC or miR-224-5p. (D) RT-qPCR analysis of CMTM4 level in SW620 cells transfected with NC, miR-224-5p agomir or miR-224-5p antagomir. **P < 0.01 vs. NC group.

Figure 5

MiR-224-5p can be transferred from SW620 cells to CCD 841 CoN cells via exosomes. (A) TEM of CCD 841 CoN cell and SW620 cell CM secreted exosomes. (B) NTA was used to determine exosome number and size distribution. (C) Western blot analysis of exosomal proteins TSG101 and CD63 in CCD 841 CoN cells, CCD 841 CoN cell-derived exosomes, and SW620 cells, SW620 cell-derived exosomes. (D) RT-qPCR analysis of miR-224-5p level in CCD 841 CoN cell-derived exosomes and SW620 cell-derived exosomes. (E) SW620 were transfected with NC, miR-224-5p agomir or miR-224-5p antagonist for 48 h. Western blot analysis of TSG101 and CD63 levels in exosomes isolated from the transfected SW620 cells. (F) CCD 841 CoN cells were co-cultured with SW620 cell-derived exosomes for 48 h. The uptake of exosomes (Red color) into CCD 841 CoN cells was observed by confocal microscopy. (G) RT-qPCR analysis of miR-224-5p

level in exosomes isolated from the transfected SW620 cells. **P < 0.01 vs. the CCD 841 CoN group or the Exo-NC group.

Figure 6

Intercellular transfer of miR-224-5p agomir by exosomes promoted malignant transformation of CCD 841 CoN cells via downregulation of CMTM4. (A) RT-qPCR analysis of CMTM4 expression in CCD 841 CoN cells transfected with CMTM4-OE. **P < 0.01 vs. NC group. (B) SW620 cells were transfected with NC, miR-224-5p agomir or miR-224-5p antagonist for 48 h. CCD 841 CoN cells were co-cultured with exosomes isolated from the transfected SW620 cells in the presence or absence of CMTM4-OE. Cell viability was determined by CCK-8 assay. (C, D) Cell proliferation detected using Ki67 immunofluorescence assay. (E, F) Transwell migration assay was applied to assess cell migration. (G, H) Transwell invasion assay was conducted to determine cell migration. (I, J) Cell apoptosis was determined using flow cytometry assay with Annexin V and PI double staining. **P < 0.01 vs. the Exo-NC group; ##P < 0.01 vs. the Exo-miR-224-5p agomir group.

Figure 7

Intercellular transfer of miR-224-5p agomir by exosomes promoted malignant transformation of CCD 841 CoN cells via CMTM4/Akt/ERK pathway. SW620 cells were transfected with NC, miR-224-5p agomir or miR-224-5p antagomir for 48 h. CCD 841 CoN cells were co-cultured with exosomes isolated from the transfected SW620 cells in the presence or absence of CMTM4-OE. (A, B, C, D) CMTM4, p-Akt and p-ERK expressions in CCD 841 CoN cells were detected with western blotting. **P < 0.01 vs. the Exo-NC group; ##P < 0.01 vs. the Exo-miR-224-5p agomir group.

Figure 8

Exo-miR-224-5p antagonist suppressed the growth of CRC cells in vivo. (A) The tumor volume was measured every week. (B, C) Tumors were removed and weighted. (D) RT-qPCR was used to determine the level of miR-224-5p in tumor tissues. (E, F) Cell apoptosis in tumor tissues was analyzed using TUNEL assay. (G, H, I, J) CMTM4, p-Akt and p-ERK expressions in tumor tissues were detected with western blotting. **P < 0.01 vs. the Exo-NC group.