Exosomes-derived miR-548am-5p promotes colorectal cancer progression

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ARTICLE INFO

Original paper

Article history:
Received: June 04, 2023
Accepted: August 14, 2023
Published: November 30, 2023

Keywords:
MiR-548am-5p, exosome, colorectal cancer, RORA, stemness

ABSTRACT

Exosomes are vital modulators in intercellular communication and microRNAs (miRNAs) are enriched within exosomes. MiRNAs are important participants in affecting colorectal cancer (CRC) progression, but the influence and latent mechanism of cancer-secreted exosomal miRNAs in colorectal cancer are not fully understood. miR-548am-5p has been reported to be differentially expressed in colon cancer and is indicated as a biomarker for colon cancer diagnosis at the early stage. In this study, we aimed to explore the role of exosomes-derived miR-548am-5p in CRC development. ISH and FISH were implemented to assess miR-548am-5p expression and location in CRC. CRC cells-secreted exosomes were identified via transmission electron microscopy and western blot. Colony formation, sphere formation and flow cytometry assessed the changes in proliferation, stemness and apoptosis of CRC cells. Bioinformatic analyses and mechanical experiments verified the binding of miR-548am-5p and RAR-related orphan receptor A (RORA). Our study identified miR-548am-5p was highly expressed in CRC tissues and cells. Tumor-derived exosomes expedited CRC cell proliferation and stemness along with secreted miR-548am-5p. Moreover, miR-548am-5p inhibition suppressed CRC cell proliferation and stemness while promoting cell apoptosis. RORA was the target mRNA of miR-548am-5p. Down-regulation of RORA was discovered in CRC and its expression was repressed by CRC cell-derived exosomes. As a result, our work elucidated that tumor-derived exosomal miR-548am-5p promoted CRC cell proliferation and stemness via targeting RORA, providing a valuable sight for CRC therapy.

DOI: http://dx.doi.org/10.14715/cmb/2023.69.12.17

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Introduction

Colorectal cancer (CRC) occupies the third most common diagnosed tumor and is deemed to be the main cause of cancer-linked death (1). In spite of great advances in screening and therapy for CRC, the 5-year survival time for CRC patients is disillusionary, majorly because of its high metastatic character (2). Therefore, exploring the actual pathophysiological mechanism of CRC is imperative, in order to offer a basis for early diagnosis and therapy of CRC.

MicroRNAs (miRNAs) are non-coding RNAs with 21–25 nucleotides. They combine with complementary mRNA sequences to affect gene expression, leading to enhanсe degradation of their target mRNAs (3). MiRNAs have been confirmed to take part in the genesis and progression of plentiful cancers, working as oncogenes or tumor suppressors resting with their target mRNAs (4). In CRC, many miRNAs have been declared to be prognostic biomarkers, containing miR-27a, miR-130a and miR-1180-3p (5, 6). MiR-548am-5p is a novel miRNA and Jixi Liu et al have pointed out the involvement of miR-548am-5p in the pathogenesis of early-stage colon adenocarcinoma (7), but its specific role and potential mechanism in CRC is obscure.

As a peculiar subset of tumor cells existing in tumors, cancer stem cells (CSCs) have the properties of self-renewal, multidirectional differentiation, unlimited proliferation as well as tumor regeneration (8). In recent years, miRNAs have also been discovered to take part in CSC progression or the CSC-like characteristics of tumor cells. For example, miR-375 represses the stemness of gastric cancer cells (9). MiR-3065-3p induces stemness via targeting CRLF1 in CRC (10). MiR-137 targets KLF12 to restrain stemness traits of pancreatic cancer cells (11). However, the influence of miR-548am-5p on CRC cell stemness is unclear.

Exosomes are considered as lipid bilayer microvesicles with a diameter of 30 to 150 nm (12). They are composed of a lipid bilayer and contain multifarious functional biological molecules, such as proteins, miRNAs and DNA (13). In accordance with recent reports, exosomes can induce cancer progression via the mediation of miRNA communication between tumor cells and surrounding cells. For all we know, mesenchymal stem cells-derived exosomal miRNA-142-3p inhibitor hinders tumorigenicity of breast cancer (14). Exosomal miR-205 secreted by ovarian cancer cells promotes tumor metastasis (15). Exosomal miR-106b promotes lung cancer metastasis by targeting PTEN (16). Herein, the relationship between exosome and miR-
miR-548am-5p in CRC cells was also studied. In this study, the GSE156732 database demonstrated the miR-548am-5p up-regulation in CRC tissues. Thus we further investigated the relationship between CRC cell-derived exosomes and miR-548am-5p, as well as probed the function and mechanism of miR-548am-5p in CRC cell proliferation and stemness. The data implicated miR-548am-5p might be a therapy targeting CRC.

Materials and Methods

Tissue sample
CRC tissues and adjacent normal tissues (n=18) were collected from the Affiliated Hospital of Xuzhou Medical University. None of the patients in this study received chemotherapy or radiation therapy before the operation. All CRC patients were confirmed through histopathological analysis of surgically resected tissues. This study was approved by the ethical committee of the Affiliated Hospital of Xuzhou Medical University, and all patients received informed consent before enrollment. All samples were preserved at -80 °C before use.

In situ hybridization (ISH) assay
In brief, slices were washed and then hatched with anti-miR-548am-5p oligodeoxynucleotide probes (RiboBio, Guangzhou, China) in a hybridization solution including 1% blocking solution overnight. Afterwards, slices were washed and be stained by DAPI, followed by observation using a fluorescent microscope.

Cell culture and transfection
CRC cells (LoVo, HCT116, SW480 and RKO) were bought from Procell (Wuhan, China), and severely maintained in Ham’s F-12K medium, McCoy’s 5A medium, Leibovitz’s L-15 medium and MEM. Normal human colonic epithelial cells HCoEpiC were offered by Green Flag Biotechnology Development Co., LTD (Shanghai, China) and maintained in DMEM. Cell culture mediums were treated with 10% fetal bovine serum (FBS), 100 U/mL streptomycin and 100 μg/mL penicillin (Invitrogen, Carlsbad, CA, USA) at 37 °C. For cell transfection, miR-548am-5p inhibition and NC inhibitor, miR-548am-5p mimics and NC mimics, along with sh-RORA and sh-NC were all synthesized by GenePharma (Shanghai, China). Lipofectamine 3000 was used for cell transfection.

RT-qPCR
Total RNA was extracted from CRC cells and tissues using TRIzol (Invitrogen, Carlsbad, CA, USA). The cDNA was reversely transcribed. SYBR Green master mix (Applied Biosystems, USA) was used for qRT-PCR assay. The RNA levels were normalized to GAPDH or U6. The 2−ΔΔCt method was implemented for the calculation of gene expression level.

RNA fluorescence in situ hybridization (FISH)
This assay was performed using a FISH Kit (RiboBio, China) in the light of the protocol. MiR-548am-5p probe was designed by RiboBio (China). Cells were fixed and permeabilized, respectively. After that, miR-548am-5p probe was added to the hybridization solution for incubation overnight. Cells were redyed with DAPI and imaged using a fluorescent microscope.

Exosome extraction
Using ultracentrifugation or an exosome isolation kit, exosomes were purified from CRC cells or serum from CRC patients. After culturing for 48 h, exosomes from the conditioned medium were collected, followed by centrifugation at 4,000 × g for 10 min, and then at 17,000 × g for 1 h. The supernatant was further ultracentrifuged at 200,000 × g for 1 h and ultracentrifuged at 200,000 × g for 60 min. Finally, the exosomes were prepared for further analysis.

Western blot
Cell lysates or exosomes were extracted. 12% SDS-PAGE was implemented for protein separation. Then proteins were shifted to PVDF membranes. Followed by sealing with 5% defatted milk, the primary antibody TSG101 (Abcam, ab125011, 1:2000), CD9 (Abcam, ab236630; 1:1000), ALIX (Abcam, ab275377, 1:1000), GAPDH (Abcam, ab9485, 1:2500), NANOG (Abcam, ab109250, 1:2000), SOX2 (Abcam, ab92494, 1:2000), OCT4 (Abcam, ab181557, 1:1000) and RORA (Abcam, ab256799, 1:1000) were all cultivated with the membranes overnight at 4 °C. Subsequently, a secondary antibody was cultivated with the membranes and the bands were visualized with enhanced chemiluminescence using an ECL kit (Beyotime, Shanghai, China). The relative densities of protein bands were analyzed by ImageJ (v1.8.0; National Institutes of Health).

Transmission electron microscopy (TEM)
Exosomes were fixed with 4% paraformaldehyde and then spotted onto 300-mesh carbon grids. The grids were dyed using uranyl acetate in water and imaged by TEM (JEOL, Tokyo, Japan).

Colony formation
Transfected cells (600 cells/well) planted into 6-well plates were cultivated for 10 days. Then, cells were fixation and staining. The number of colonies was counted using a microscope.

Sphere formation
Transfected cells were seeded in 6-well plates and treated with Sigma (Shanghai, China) offered B27 (1:50), EGF (20 ng/ml) and bFGF (20 ng/ml) for sphere formation. The number of spheres was counted on a microscope.

Flow cytometry
On the basis of the instructions, cell apoptosis was assessed using the Annexin V-FITC/PI detection kit purchased from Nanjing KeyGen Biotech Co., Ltd. Briefly, transfected cells were hatched with 5 μl Annexin V-FITC along with 5 μl propidium iodide at 4°C. Apoptotic cells were assessed via flow cytometry using a FACScan flow cytometer (BD Biosciences).

In vivo experiment
Male BALB/c nude mice (6-week-old) were stochastically divided into two groups (5 mice/group). MiR-548am-5p stably silenced CRC cells (Antagomir miR-548am-5p) and the negative controls (Antagomir NC) were subcutaneously injected into the mice, followed by measuring the volume of tumors. Four weeks later, the mice were euthanatized, and tumor tissues were obtained for subsequent analysis. The animal experiments were as required by ins-
tutional guidelines and approved by the animal ethical committee of the Affiliated Hospital of Xuzhou Medical University.

**Immunohistochemistry (IHC)**

Sections (4-µm thickness) cut from paraffin tissue were dewaxed with xylene, soaked with 0.01 mol/L sodium citrate and heated in the microwave. Then the sections were hatched with primary antibodies against Ki-67 (Abcam, ab92742, 1:500), NANOG (Abcam, ab109250, 1:200), OCT4 (Abcam, ab181557, 1:1000), SOX2 (Abcam, ab92494, 1:100) and RORA (Abcam, ab256799, 1:800). Next, the sections were hatched with HRP conjugated secondary antibody. The reaction was developed by diaminobenzidine and redyed with hematoxylin and observed via a microscope (Olympus, Tokyo, Japan).

**RNA pull-down assay**

Cells were lysed and treated with a biotinylated RORA probe (RIBOBIO, China), followed by an addition of Dynabeads M-280 streptavidin (Invitrogen, USA). After elution, the enrichment of miR-548am-5p was analyzed by RT-qPCR.

**A dual luciferase reporter assay**

The RORA 3’UTR containing miR-548am-5p binding sequence was cloned into the pmirGLO reporter vector (Promega, Madison, WI, USA) to get the pmirGLO-RORA 3’UTR plasmid. Then, cells were co-transfected with miR-548am-5p mimics or NC mimics and the pmirGLO-RORA 3’UTR plasmid. Luciferase activity was measured using a dual-luciferase reporter assay System (Promega) and normalized to Renilla luciferase activity.

**Statistical analysis**

Data from three independent experiments were presented as the mean ± standard deviation (SD). Student’s t-test or one-way ANOVA was performed to compare groups. Spearman’s correlation analysis was implemented to assess the correlation between miR-548am-5p and RORA. All statistical analyses were computed via GraphPad Prism 8 software. The results were significant when the p-value was less than 0.05.

**Results**

**Up-regulation of miR-548am-5p in CRC**

On the grounds of the GSE156732 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE156732) database, we discovered miR-548am-5p expression was significantly increased in CRC tissues (Figure 1A). To prove this result, we assessed miR-548am-5p expression in tumor and adjacent non-tumor tissues from CRC patients using ISH and RT-qPCR. As revealed in Figure 1B-1C, miR-548am-5p was obviously up-regulated in the tumor tissues relative to the adjacent non-tumor tissues. Consistently, compared to normal human colonic epithelial cells HCoEpiC, miR-548am-5p was distinctly up-regulated in CRC cells, especially in LoVo and HCT116 cells (Figure 1D). Additionally, the FISH assay showed that miR-548am-5p was distinctly up-regulated in CRC cells and exerted the regulatory function through the post-transcriptional mechanism.

**Tumor cell-derived exosomes motivate CRC cell proliferation and stemness along with secrets miR-548am-5p**

Former studies have declared that tumor-derived exosomes are connected with tumor progression (17). Herein, we separated tumor cell-derived exosomes from the supernatant of two selected CRC cells. As shown in Figure 2A, the presence of exosome-linked markers containing TSG101, CD9 and ALIX were characterized by western blot. TEM also showed the exosomes were rounded particles with a diameter of 30-150 nm (Figure 2B). We then tested whether exosomes impact CRC cell proliferation and stemness. We extracted exosomes from the CRC tissues of clinical patients and co-cultured them with CRC cells. At the same time, the untreated CRC cells were added with exosome inhibitor GW4869. Colony formation assays displayed that cell proliferation was enhanced by exosome introduction while inhibited by GW4869 treatment (Figure 2C). Sphere formation assay uncovered exosomes increased the relative sphere size of CRC cells while GW4869 caused the opposite results (Figure 2D). More importantly, we discovered miR-548am-5p expres-
The xenograft model was built in vivo via subcutaneous injection of miR-548am-5p-silencing CRC cells in mice. As indicated in Figure 4A, miR-548am-5p expression was diminished in tumors transfected with antagonir miR-548am-5p. Besides, we observed that miR-548am-5p knockdown resulted in a smaller tumor volume than the control group (Figure 4B). More, IHC results unmasked that miR-548am-5p repression weakened the expression of Ki-67, NANOG, OCT4 and SOX2 (Figure 4C). These data suggested that miR-548am-5p could facilitate CRC cell proliferation, and stemness and inhibit cell apoptosis.

Down-regulation of RORA in CRC and its expression is repressed by CRC cell-derived exosomes

Through the miRDB (http://mirdb.org/) website, we predicted a set of target mRNAs of miR-548am-5p. We selected FAM135A, NFAT5 and RORA for further screening due to their target score being 100. On the basis of RT-qPCR analysis, we discovered that only RORA (RAR-related orphan receptor A) presented low expression in colon adenocarcinoma (COAD) and rectal adenocarcinoma (READ) tumor tissues (Figure 5B). Besides, Spearman’s correlation analysis testified that RORA expression was negatively correlated with miR-548am-5p expression (Figure 5C, R²=0.3723, P=0.0072). Moreover, RORA expression was also down-regulated in CRC cells, particularly in LoVo and HCT116 cells (Figure 5D). Notably, RORA expression was inhibited in two CRC cells treated with exosomes, whereas was enhanced in those upon GW4869 treatment (Figure 5E). In short, we concluded that RORA was downregulated in CRC cells and its expression could be inhibited by CRC cell-derived exosomes.

Binding of RORA 3'UTR with miR-548am-5p

Furthermore, we proved that RORA mRNA and protein levels were promoted in CRC cells transfected with miR-548am-5p inhibitor (Figure 6A-6B). IHC staining further validated this outcome in vivo (Figure 6C). In addition, the binding sequences of miR-548am-5p and RORA 3'UTR were obtained from the TargetScan database (https://www.targetscan.org/vert_71/) and manifested in Figure 6D. RNA pull-down assay validated the effectiveness of their binding, as shown that miR-548am-5p was highly enriched in biotin-labeled RORA 3’UTR probes (Figure 6E). A dual luciferase reporter assay exhibited that miR-548am-5p mimics brought out the diminished luciferase activity of pmirGLO-RORA 3’UTR, which compared to the NC mimics groups (Figure 6F). Thus, we proved that RORA was targeted and negatively regulated by miR-548am-5p in CRC.
MiR-548am-5p affects CRC cell proliferation, apoptosis and stemness via targeting RORA

To determine whether miR-548am-5p promotes CRC cell proliferation and stemness via targeting RORA, we stably silenced RORA expression and found that the enhanced RORA expression in CRC cells introduced with miR-548am-5p inhibitor was offset upon co-transfection of sh-RORA (Figure 7A). Colony formation assays suggested that RORA silence reversed the inhibited cell proliferation in miR-548am-5p inhibitor-transfected CRC cells (Figure 7B). Flow cytometry analysis certified that simultaneous RORA decrease could counteract the increased cell apoptosis induced by miR-548am-5p inhibition (Figure 7C-7D). Moreover, the suppressive sphere formation ability as well as the lessened protein levels of stemness-linked markers triggered by miR-548am-5p down-regulation was neutralized when RORA was down-regulated together (Figure 7E-7F). These results demonstrated that miR-548am-5p could regulate CRC cell proliferation, apoptosis and stemness via targeting RORA.

Discussion

Numerous reports have identified the dysregulation of miRNAs frequently identified in CRC tissues and cells, and these miRNAs can act as predictive biomarkers in CRC via their tumor-promoting - or - suppressing roles. According to the literature proposed by Longchang Huang et al, miR-4319 prevents CRC progression by controlling ABTB1 (18). Inversely, Min Pan et al have pointed out that miR-106b-5p accelerates CRC cell migration and invasion via binding to FAT4 (19). A former study has indicated that miR-548am-5p is differentially expressed in the pathogenesis of early-stage COAD, but the definite expression pattern is unclear. Through the GSE156732 database, we analyzed miR-548am-5p was up-regulated in CRC cells, and this result was further proved byISH staining and RT-qPCR analysis. Collectively, our results suggested that miR-548am-5p might be a significant contributor to CRC progression.

Recently, miRNAs in exosomes have attracted much attention. Accumulating documents have unveiled that the release of exosomal miRNA derived from cancer cells can be consumed by the surrounding tumor cells, leading to the promotion of cancer progression (20). It has been reported that tumor-secreted exosomal miR-934 stimulates macrophage M2 polarization to enhance liver metastasis of CRC (21). Besides, Chaogang Yang et al have put forward exosomal miR-106b-5p secreted from tumor cells to expedite CRC metastasis (22). More, exosomes isolated from tumor cells packaging miR-34a inhibit tumor progression of CRC (23). In accordance with the above reports, this work also characterized CRC-derived exosomes by western blot and TEM. Subsequently, we proved that CRC cell-secreted exosomes facilitated CRC cell proliferation and stemness. Of note, we confirmed that miR-548am-5p was up-regulated in CRC-derived exosomes. In addition, we attested miR-548am-5p silence hampered...
CRC cell proliferation and stemness in vitro and in vivo. In a word, CRC cell-secreted exosomal miR-548am-5p enhanced CRC cell proliferation and stemness.

RORA is known as a nuclear receptor belonging to the ROR sub-family (24). RORA has been widely documented to function as a tumor suppressor in cancer progression. Overexpression of RORA reverses the promotion influence of miR-652 on endometrial cancer metastasis (25). RORA is up-regulated in gastric cancer cells and suppresses tumor progression (26). Moreover, Anastasia E. Kotorou and his colleague have presented that RORA is down-regulated in CRC (27). Congruously, our work also discovered the low expression of RORA in CRC tissues and cells. More importantly, its expression was negatively correlated with miR-548am-5p and could be repressed by CRC cell-derived exosomes. Further analyses confirmed that RORA was the target mRNA of miR-548am-5p and was controlled by miR-548am-5p. Finally, combined with the rescue assays, we concluded that miR-548am-5p functioned as an oncogene in CRC and affected cell proliferation, apoptosis and stemness via targeting RORA.

There are several limitations to this work. It has been registered that RORA is implicated in the modulation of various signaling pathways, such as the NF-κB signaling pathway (28), Wnt/β-Catenin signaling pathway (29) and STAT3 signaling pathway (30). However, the relationship between RORA and downstream signaling pathways in CRC is blurry. More, the upstream gene of miR-548am-5p as well as the up-regulation mechanism of miR-548am-5p in CRC is undiscovered. Therefore, further relevant studies will be carried out.

In conclusion, our data first demonstrated that tumor cell-derived exosomal miR-548am-5p promoted CRC cell proliferation and stemness via targeting RORA, which might provide a hopeful strategy for CRC treatment.

Availability statement
Original data of this study can be obtained from the corresponding author under reasonable request.

Conflicts of interest
There are no conflicts of interest existing in this study.

Fundings
This work was supported by the National Natural Science Foundation of China (Grant Nos. 82000808 [SL, Z]), the Fundamental Research Funds for the Central Universities (JUSRP12048) and the Natural Science Research Project of Universities in Jiangsu Province (20KJB330002).

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