MicroRNA-592 promotes cell proliferation, migration and invasion in colorectal cancer by directly targeting SPARC

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Abstract. Colorectal cancer (CRC), one of the most common cancer types, causes a large number of cancer-related mortalities annually worldwide. Dysregulated microRNAs (miRNAs/miR) are closely associated with the malignant progression of CRC. Therefore, the present study aimed to investigate the expression and regulatory role of miR-592 in CRC. It was found that miR-592 expression was significantly elevated in CRC tissues and cell lines, and was associated with the prognosis of patients. Cellular phenotype assays demonstrated that miR-592 could promote CRC cell proliferation, migration and invasion. Bioinformatics analysis demonstrated that miR-592 mainly participated in the positive regulation of transcription, as well as the regulation of cell motility. Moreover, miR-592 targets were enriched in several signaling pathways, such as the ‘mTOR’ and ‘FoxO’ signaling pathways. In addition, secreted protein acidic and rich in cysteine (SPARC) was identified as a target of miR-592 in CRC. The present results suggested that miR-592 acts as an oncogene in CRC, in part, by directly inhibiting SPARC expression. Collectively, the present study provides a novel potential therapeutic strategy for CRC.

Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors, leading to ~700,000 cancer-related mortalities each year worldwide (1). In China, the International Agency for Research on Cancer reported that CRC accounts for 6.3% of all malignancy-related mortalities in 2012 (2). Despite advances in therapeutic methods, almost half of patients with CRC still experience tumor metastasis or recurrence after treatment, which causes a poor overall survival (OS) (3). Therefore, elucidation of the potential mechanisms of CRC proliferation and metastasis is important for improving the treatment and prognosis of patients with CRC.

MicroRNAs (miRNAs/miRs) are a class of small non-coding RNAs (18-25 nucleotides in length) that act as post-transcriptional regulators of gene expression by direct binding to the 3' untranslated regions (3'UTRs) of target mRNAs; this binding induces mRNA degradation and/or translational repression (4). miRNAs participate in a number of biological processes, such as cell cycle progression, proliferation, invasion, migration and cell metabolism (5-8). The regulatory role of miR-592 is context-dependent. For instance, miR-592 functions as a tumor suppressor in breast cancer (9), non-small cell lung cancer (10) and glioma (11), but as an oncogene in gastric cancer (12). Moreover, contrary results have been observed in CRC. Fu et al (13) reported an oncogenic role of miR-592 in CRC by targeting forkhead box O3A (FoxO3A). However, Liu et al (14) revealed that miR-592 can inhibit the proliferation of CRC cells by suppressing cyclin D3 expression.

Therefore, the present study aimed to further verify miR-592 expression and investigate its regulatory mechanism in CRC.

Materials and methods

Clinical samples. A total of 35 paired CRC tissues and adjacent normal tissues (ANTs) were collected from patients who underwent surgical resection at The Affiliated Huai'an No. 1 People's Hospital of Nanjing Medical University (Huai'an, China) between January 2017 and June 2019. The healthy tissue was >2 cm away from the CRC tissue. All samples were confirmed by three pathologists independently and stored at -80˚C until use. The inclusion criteria for the patients to be enrolled in the study were as follows: i) Patients with CRC whose histologic slides were identified by two independent pathologists; and ii) patients with CRC who had not been treated before surgery, such as chemoradiotherapy. Otherwise, patients were excluded. The age of patients with CRC ranged between 43 and 76 years old. Written informed consent was obtained from each participant, and the study was approved by the Ethics Committees of The Affiliated Huai'an No. 1 People's Hospital of Nanjing Medical University.

Cell culture and transfection. The normal colonic epithelial cell (FHC) and CRC cell lines (HCT8, HT29, HCT116, SW480
and SW620) were obtained from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. Cells were cultured in DMEM that was supplemented with 10% FBS (both from Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C. All cells had been authenticated using short tandem repeat profiling. As conducted in previous studies (15,16), the present study performed subsequent experiments using two cancer cells (HT29 and SW480).

The miR-592 inhibitor and negative control (NC) were designed by Shanghai GenePharma Co., Ltd., and their corresponding core sequences were as follows: miR-592 inhibitor, 5'-ACATCATCAGCTATTGACACAA-3' and NC, 5'-TTCTCC GAACTGTCTACGTTTCTC-3'. The small interfering (si)RNA of secreted protein acidic and rich in cysteine (SPARC, siSPARC) was generated from Guangzhou Ribobio Co., Ltd., and the corresponding sequences were 5'-AAAAACAGACCAUU CGACUCUUCC-3' (siSPARC) and 5'-GCACACAGCUCAAU CCUAUC-3' (siNC). Both miR-NC and siNC were non-targeting. The transfection was conducted at a concentration of 100 nM using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 6 h according to the manufacturer's protocol. After transfection for 48 h, subsequent experiments were conducted.

**RNA extraction and reverse transcription-quantitative (RT-q) PCR.** Total RNA was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instruction. RT-qPCR of miR-592 was performed using a Hairpin-it™ miRNA Normalization RT-PCR Quantitation kit (Shanghai GenePharma Co., Ltd.), and U6 snRNA was regarded as the internal control. The following thermocycling conditions of miRNA RT-qPCR were as follows: Initial denaturation at 95°C for 3 min, 40 cycles of denaturation at 95°C for 15 sec, annealing and elongation at 62°C for 34 sec. The RT-qPCR primer sequences were as follows: miR-592 forward, 5'-ACGTTGTGTCATATATGGCTGA-3' and reverse, 5'-GTG CAGGGTGCCAGGTT-3'; and U6 forward, 5'-CTCGTTTGGG CAGGCC-3' and reverse, 5'-AACGCTTCACGAATTTG CAGGGTCCGAGGT-3'. The small interfering (si)RNA of secreted protein acidic and rich in cysteine (SPARC, siSPARC) was generated from Guangzhou Ribobio Co., Ltd., and their corresponding core sequences were 5'-AACAAGACCUU CGACUCUUCC-3' (siSPARC) and 5'-GCACACAGCUCAAU CCUAUC-3' (siNC). Both miR-NC and siNC were non-targeting. The transfection was conducted at a concentration of 100 nM using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 6 h according to the manufacturer's protocol. After transfection for 48 h, subsequent experiments were conducted.

**Cell Counting Kit (CCK)-8 assay.** The proliferative ability of cells was measured using the CCK-8 assay (Nanjing KeyGen Biotech Co., Ltd.), according to the manufacturer's instructions. Briefly, HT29 and SW480 cells were plated into a 96-well plate at a density of 1.0x10⁴ cells/well and incubated at 37°C for 24, 48 and 72 h. Then, the absorbance was measured at 450 nm on a microplate reader (Infinite M200 PRO; Tecan Group, Ltd.).

**Migration assay.** HT29 and SW480 cells were seeded into 6-well plates in DMEM without FBS and grown until 90% confluency for 24 h. Then, the cells were scratched using 200-µl pipette tips and washed twice with PBS. Next, cells were cultured in an incubator containing 5% CO₂ at 37°C. Images were captured after 0 and 24 h under an Olympus FSX100 light microscope.
(Olympus Corporation) at x200 magnification. Images were subsequently analyzed by ImageJ software (v1.53; National Institutes of Health).

**Transwell invasion assay.** The top chambers of Transwell chambers (8-µm pore size; BD Biosciences) were coated with Matrigel mix (BD Biosciences) at 37°C for 2 h and 1.5x10^5 cells were seeded on the upper chamber containing 100 µl DMEM without FBS. Then, 500 µl DMEM with 10% FBS was added to the bottom chamber. After being cultured at 37°C for 24 h, cells that passed through the Matrigel were fixed with 100% methanol at room temperature for 20 min and stained with crystal violet (0.1%) at room temperature for 30 min. Finally, cells were counted under an Olympus FSX100 light microscope (Olympus Corporation) at x200 magnification.

**Immunohistochemistry analysis.** The tissue samples were fixed with 4% paraformaldehyde at room temperature for 24 h and embedded in paraffin. Then, slices (5 µm thickness) were washed twice with phosphate buffered saline at room temperature for 5 min each time and incubated with 10% goat serum (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 30 min. Immunohistochemistry analysis was performed on paraffin-embedded sections using a primary antibody against SPARC (1:1,000; cat. no. HPA003020; Sigma-Aldrich; Merck KGaA) overnight at 4°C. After being washed three times, sections were incubated with a horseradish peroxidase-conjugated IgG (1:1,000; cat. no. ab7090; Abcam) at room temperature for 1 h. In total, three high power fields (magnification, x400) were randomly selected from CRC tissues using an Olympus FSX100 light microscope (Olympus Corporation). For histological scoring, the degree of positivity was initially classified according to scoring both the proportion of positively stained tumor cells and the staining intensities. Scores representing the proportion of positively stained tumor cells were graded as follows: i) 0, ≤10%; ii) 1, 11-25%; iii) 2, 26-50%; iv) 3, 51-75%; and v) 4, >75%. The intensity of staining was determined as follows: i) 0, no staining; ii) 1, weak staining (light yellow); iii) 2, moderate staining (yellow brown); and iv) 3, strong staining (brown). The reactivity degree was assessed by ≥2 pathologists independently.

**Immunofluorescence analysis.** For the immunofluorescence assay, cells were fixed in 4% paraformaldehyde at room temperature for 20 min, permeabilized using 0.5% Triton X-100 at room temperature for 20 min, blocked with 5% goat serum at room temperature for 2 h, and incubated with a primary antibody against SPARC (1:1,000; cat. no. HPA003020; Sigma-Aldrich; Merck KGaA) overnight at 4°C. After being washed three times, cells were incubated with a secondary antibody (1:500; cat. no. ab150081; Abcam) at room temperature for 1 h. Coverslips were counterstained with DAPI at room temperature for 5 min and imaged with a confocal laser scanning microscope (Olympus FV1000; Olympus Corporation) at x400 magnification.

**Bioinformatics analysis.** The differentially expressed miRNAs and mRNAs in CRC deposited in the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) were analyzed using the online tool GEO2R (https://www.ncbi.nlm.nih.gov/geo2r/). Three GEO datasets for miRNA expression in CRC (GSE128446, GSE126093 and GSE125961) (18) were found using the key words ‘microRNA’ and ‘colorectal cancer’. The putative targets of miR-592 were identified using the TargetScan (v7.2) database (http://www.targetscan.org/vert_72/) and the miRDB database (http://mirdb.org). Gene Ontology (GO) (19) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (18) analyses were conducted on the Database for Annotation, Visualization and Integrated Discovery website (https://david.ncifcrf.gov). The protein-protein interaction (PPI) network was constructed using Search Tool for the Retrieval of Interacting Genes/Proteins (https://string-db.org) and Cytoscape (v3.8.2; https://cytoscape.org). Analysis of functional modules and hub genes were explored using the MCODE and Cytohubba tools in Cytoscape. The expression and location of SPARC protein in CRC cells (RH-30) revealed by immunohistochemical staining and immunofluorescence were further examined using The Human Protein Atlas (https://www.proteinatlas.org) database (20).

**Results**

miR-592 expression is significantly elevated in CRC and is associated with the prognosis of patients. The GEO database was first used to investigate the expression level of miR-592 in CRC. In total, three GEO datasets for miRNA expression in CRC (GSE128446, GSE126093, GSE125961) (18) were found using the key words ‘microRNA’ and ‘colorectal cancer’. The characteristics of these GEO datasets are listed in Table I. As presented in Fig. 1A-C, miR-592 expression was significantly upregulated in CRC tissues compared with that in healthy tissues. Moreover, RT-qPCR analysis demonstrated that miR-592 expression was significantly elevated in CRC tissues and cell lines compared with normal tissues and FHC cells, respectively (Fig. 1D and E). The expression of miR-592 in lymphatic metastatic nodules was higher compared with that in primary tumor sites (Fig. 1D).

To assess the association between miR-592 expression and the clinicopathologic characteristics of patients with CRC, patients were categorized into the miR-592 high group (n=18) and low group (n=17) according to the median value of miR-592 expression. As presented in Table II, miR-592 expression was significantly associated with TNM stage (P=0.018) and lymphatic metastasis (P=0.041). Furthermore, Kaplan-Meier survival analysis indicated that patients with CRC with higher miR-592 expression may have shorter OS times (hazard ratio =2.3; P=0.047) (Fig. 1F).
miR-592 knockdown suppresses the proliferation, migration and metastasis of CRC cells. To examine the biological role of miR-490-3p in CRC, HT29 and SW480 cells were transfected with miR-592 inhibitor and NC. RT-qPCR analysis was used to confirm the transfection efficiency (Fig. 2A). The CCK-8 and EdU incorporation assays demonstrated that miR-592 knockdown significantly inhibited the proliferative ability of CRC cells (Fig. 2B and C). In addition, the wound healing assay identified that, compared with NC transfection, miR-592 inhibitor transfection significantly decreased cell migration (Fig. 2D). The Transwell invasion assay also demonstrated that the invasive ability of CRC cells transfected with inhibitor was significantly decreased compared with that of cells transfected with NC (Fig. 2E).

Analysis of putative targets of miR-592. To identify the targets of miR-592, the current study first overlapped predicted results searched in the TargetScan and miRDB algorithms, and 323 putative targets were found (Fig. 3A). GO analysis of these targets indicated that miR-592 participated in various biological processes, such as ‘positive regulation of transcription’, ‘extracellular matrix organization’ and ‘regulation of cell motility’ (Fig. 3B). KEGG analysis identified that the targets of miR-592 were significantly associated with several signaling pathways, including the ‘ErbB signaling pathway’, ‘mTOR signaling pathway’ and ‘FoxO signaling pathway’ (Fig. 3C). In addition, a PPI network of miR-592 targets was constructed, among which five functional modules were discovered using the MCODE tool (Fig. 3D). Cytohubba analysis revealed seven hub genes among the PPI network (Fig. 3E).

It has been reported that one miRNA may regulate the expression of multiple genes and vice versa (21). Considering the upregulation of miR-592 in CRC, it was suggested that these targets with downregulated expression in CRC may be regulated by miR-592. It was found that SPARC, one of the hub genes, was significantly downregulated in CRC tissues compared with that in ANTs based on GSE126092 analysis (Fig. 3G). However, the expression of the other six hub genes was either upregulated or undifferentiated in CRC tissues compared with ANTs (data not shown). Additionally, SPARC belonged to a functional module in the PPI network (Fig. 3F). Immunohistochemistry analysis demonstrated that SPARC was barely expressed in CRC cells but was highly enriched in the tumor microenvironment (Fig. 3H). In addition, immunofluorescence analysis identified that SPARC was mainly located in the vesicles of RH-30 tumor cells (Fig. 3I).

miR, microRNA.

SPARC is a direct target of miR-592 in CRC. To identify SPARC as the direct target of miR-592 in CRC, its expression was firstly evaluated. RT-qPCR analysis demonstrated that SPARC expression was significantly downregulated in CRC tissues and cell lines compared with ANTs and FHC cells, respectively (Fig. 4A and B). Furthermore, a weak negative correlation was identified between miR-592 expression and
SPARC expression in CRC tissues (Fig. 4C). When the miR-592 inhibitor was transfected into HT29 and SW480 cells, the expression of SPARC at both the transcriptional and protein levels was significantly upregulated in CRC cells (Fig. 4D and E). Moreover, the direct regulatory role of miR-592 on SPARC expression was assessed using a dual-luciferase assay, in which Renilla luciferase was used for normalization. Interestingly, the SPARC 3'UTR sequence had two binding sites of miR-592 with a high conserved score. Therefore, several luciferase reporter plasmids were constructed with Wt and Mut1-3 SPARC 3'UTRs (Fig. 4F). As presented in Fig. 4G, after co-transfecting with miR-592 inhibitor, the luciferase activity was significantly elevated in luciferase reporter groups with Wt and Mut1-2 SPARC 3'UTRs, but not in the luciferase reporter group with Mut3 SPARC 3'UTRs, which suggested that miR-592 can directly bind to two sites in SPARC 3'UTRs.

Silencing SPARC significantly reverses the effect of miR-592 knockdown on CRC cells. To investigate the regulatory role of SPARC in CRC, SPARC expression was inhibited by transfecting cells with siSPARC. Western blotting results demonstrated that siSPARC effectively suppressed SPARC expression (Fig. 5A). The cellular phenotypes indicated that SPARC knockdown significantly promoted the proliferation, migration and invasion of CRC cells (Fig. 5B-E).

Subsequently, CRC cells were co-transfected with siSPARC and miR-592 inhibitor to investigate whether the oncogenic role of miR-592 was directly mediated by SPARC inhibition (Fig. 5A). The CCK-8 and EdU incorporation assays suggested that SPARC knockdown significantly reversed the effect of miR-592 knockdown on cell proliferation (Fig. 5B and C). Consistently, the migration and Transwell invasion assays identified that both the migratory and invasive abilities, which were suppressed by miR-592 knockdown, were restored after SPARC knockdown in HT29 and SW480 cells (Fig. 5D and E).

Discussion

Dysregulated miR-592 has been reported to serve a vital role in the malignant progression of multiple cancer types, such as hepatocellular carcinoma and prostate cancer (22-24). Liu et al (25) revealed that miR-592 expression was upregulated in clinical CRC tissues. Consistently, the present study demonstrated that miR-592 expression was significantly upregulated in CRC tissues and cell lines compared with ANTs and FHC cells, respectively. Furthermore, patients with CRC with higher miR-592 expression had a poorer prognosis compared with those with low expression. Fu et al (13) reported that miR-592 can promote the proliferation and metastasis of CRC cells, in part, by targeting FoxO3A. In the present study, SPARC was identified as another
target of miR-592 in CRC. Moreover, it was found that miR-592 acted as an oncogene by directly suppressing SPARC expression in CRC. Liu et al (25) also discovered that serum miR-592 expression was elevated in patients with CRC compared with healthy individuals and was significantly decreased after radical surgery. Therefore, it was hypothesized that CRC cells may secrete intracellular miR-592 into a circulating system, which offers a novel potential diagnostic biomarker of CRC. However,
a contrary report regarding the low expression of miR-592 in CRC was previously published (14), and it was suggested that this phenomenon may be attributed to the heterogeneity of CRC tissues and the small sample size used in this previous study. The promoter of SPARC has been reported to be epigenetically hypermethylated, which results in decreased SPARC expression in CRC (26). The present study demonstrated that SPARC expression was also controlled by miRNA at the
PAN et al.: ONCOGENIC ROLE OF miR-592 IN CRC

Post-transcription level. Furthermore, the immunohistochemistry analysis identified that SPARC was highly enriched in the tumor microenvironment. In line with the current findings, SPARC was previously found to be upregulated in the tumor stroma, and fibroblast-derived SPARC can promote the invasiveness of CRC cells (27). Interestingly, the immunofluorescence analysis indicated that SPARC was mainly located in the vesicles of RH-30 tumor cells. Emerging evidence has revealed that cancer cells can release cellular RNAs into peripheral blood through vesicles, such as exosomes (28,29). Therefore, it was hypothesized that SPARC may also be located in the vesicles of CRC cells and that the downregulation of SPARC in CRC cells may also be attributed to their enrichment in secretory vehicles.

There are several limitations in the present study. First, the number of clinical samples was relatively small. Second, the current study did not further investigate the expression or regulatory role of miR-592 and SPARC in the tumor microenvironment. Third, the association between SPARC and secretory vehicles in CRC will be examined in future studies.

In conclusion, the present findings demonstrated that miR-592 expression was significantly upregulated in CRC, and that miR-592 overexpression contributed to the promotion of cell proliferation, migration and metastasis by directly suppressing SPARC expression, which provides a novel potential therapeutic target for patients with CRC.

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Figure 5. Knockdown of SPARC significantly reverses the effect of miR-592 knockdown on CRC cells. (A) Transfection effect of siSPARC and miR-592 inhibitor on SPARC expression in CRC cells. (B) Cell Counting Kit-8 and (C) incorporation assays demonstrated that siSPARC significantly promoted CRC cell proliferation, but co-transfecting with the miR-592 inhibitor reversed such effects. (D) Wound healing assay results indicated that siSPARC significantly promoted CRC cell migration, but co-transfecting with miR-592 inhibitor reversed these effects. (E) Transwell invasion assay results identified that siSPARC significantly promoted CRC cell invasion, but co-transfecting with miR-592 inhibitor reversed such effects. Scale bar, 100 µm. **P<0.01, ***P<0.001. NC, negative control; CRC, colorectal cancer; si, small interfering RNA; miR, microRNA; OD, optical density; SPARC, secreted protein acidic and rich in cysteine; EdU, 5-Ethynyl-2'-deoxyuridine
Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The GEO datasets analyzed during the current study are available in the GEO repository, (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE128446; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126093;https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE125961).

Authors’ contributions

CG and ZP designed the present study and drafted the initial manuscript. RX and WS acquired the clinical samples and performed RT-qPCR. Both RX and ZP performed the statistical analysis. CG and WS assessed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Research and Ethical Committee at The Affiliated Hua’an No. 1 People’s Hospital of Nanjing Medical University, and written informed consent was provided by all patients before the start of the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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