Article

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Hypoxia-Induced Downregulation of MiR-199a-3p Promotes Proliferation, Invasion and Epithelial-Mesenchymal Transition in Colorectal Cancer Cells

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Background: Colorectal cancer (CRC) is a prevalent malignancy. Studies reported that microRNAs (miRNAs) are critical for CRC development. Thus, microRNA-199a-3p (miR-199a-3p)'s mechanism of action in CRC cells was investigated in this paper.
Methods: In hypoxic CRC cells, the corresponding gene miR-199a-3p and its target genes were searched for by sequencing, including Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. CRC cell viability was detected via the Cell Counting Kit-8 (CCK-8) assay. CRC cell apoptosis was identified with a flow cytometry assay. Transwell assay was carried out to further assess CRC cells' migrative and invasive abilities. Quantitative reverse transcription PCR (qRT-PCR) was employed to examine miR-199a-3p and fibronectin1 (FN1) mRNA expression. The proteins of FN1, E-cadherin, B-catenin, N-cadherin, and Vimentin were evaluated using western blot. The functional association of miR-199a-3p with its target genes was validated via luciferase reporter assay.
Results: In hypoxia CRC cells, miR-199a-3p was significantly diminished. Overexpressed miR-199a-3p significantly inhibited HCT116 CRC cells' proliferative, migrative, and invasive abilities and promote HCT116 apoptosis. Inhibiting miR-199a-3p reversed its effect. Additionally, overexpressed miR-199a-3p or FN1 knockdown significantly increased epithelial-mesenchymal transition (EMT) marker protein E-cadherin and B-catenin while decreasing EMT marker protein N-cadherin and Vimentin. FN1 overexpression reversed the effect.
Conclusions: In CRC, miR-199a-3p is reduced, and it induces CRC cells' proliferative, migrative, and invasive processes and suppresses their apoptosis via upregulation of FN1. The miR-199a-3p may function as a novel potential target for treating CRC.

Keywords: miR-199a-3p; fibronectin1 (FN1); colorectal cancer; cell apoptosis; epithelial-mesenchymal transition

Introduction

Colorectal cancer (CRC) is a prevalent malignancy of the digestive tract. It ranks second in the global incidence of female malignancies and third in male malignancies. It has the characteristics of easy invasion and migration [1,2]. Diet, obesity, lack of exercise, sedentary, smoking and other adverse risk factors increase the risk of CRC, especially in the western ways of life [3]. Despite a decline in CRC rates in previous years, the decline has been slowing in recent years [4].

Changes in microRNA (miRNA) expression are implicated in the occurrence and progression of human cancers [5]. Due to its ability to targ plentiful molecules, miRNA may have a therapeutic application in cancer treatment [6]. Furthermore, the potential value of miRNAs as CRC markers was explored in recent studies [6,7]. For example, a study demonstrated that microRNA-199a-3p (miR-199a-3p) is associated with gastric cancer cell proliferation, apoptosis and invasion [8]. Other studies reported miR-199a-3p downregulation in esophageal carcinoma cell lines and renal cell carcinoma [9,10]. Besides, inhibiting miR-199a-3p dramatically attenuated the apoptosis induced by breast cancer treatment, while miR-199a-3p overexpression increased its sensitivity to treatment [11]. Under hypoxia, miR-199a-3p is down-regulated, as a miRNA, in ovarian cancer cells. Moreover, overexpressed miR-199a-3p significantly impeded the peritoneal spread in the xenograft model, proving that miR-199a-3p is a promising target for ovarian cancer spread treatment [12]. Although studies suggested that miR-199a-3p was involved in CRC development and progression [13,14], its mechanism of action still needs further exploration.

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Fibronectin (FN) is a high molecular weight glycoprotein, with many biological functions. Fibronectin 1 (FN1) was identified as a novel prognostic biomarker related to pathophysiological changes in sporadic medullary thyroid carcinoma [15]. Besides, a study found that FN1 can inhibit apoptosis in CRC, by interacting with Integrin $\alpha 5$ and promote its viability, migration, and invasion [16]. It has been found that FN1 overexpression can promote the migrative and invasive abilities of nasopharyngeal carcinoma cells and reduce the apoptosis of nasopharyngeal carcinoma cells [17]. In addition, FN1 facilitates cancer cells' invasive and migrative abilities by inhibiting apoptosis and regulating relative proteins on epithelial-mesenchymal transition (EMT) like E-cadherin, N-cadherin and vimentin [18]. A previous study indicated that EMT correlates with the viability of tumor cell [19] and accelerates metastasis not only by enhancing invasion but also by inducing immunosuppression [20]. Thus, FN1 may function as a target for treatment in the future.

Materials and Methods

Bioinformatics Analysis

Sequencing analysis was performed on hypoxic and normoxic CRC cells, to detect the miRNA expression in hypoxic CRC cells. Tests for associations were performed on a dataset offered by the Broad Institute Firehose (https://gdac.broadinstitute.org; January 28, 2016), including mature colon adenocarcinoma (COAD) collection from The Cancer Genome Atlas (TCGA) database of 293 colon cancer patients with miRNA expression data. The miR-199a-3p target genes' biological functions were identified via Gene Ontology (GO) analysis. The Kyoto Encyclopedia of Genes and Genomes (KEGG) was used, in order to search for potential biological pathways in which the anticipated target genes miRNAs with diverse expression were engaged. The Cancer Genome Atlas - colon adenocarcinoma (TCGA-COAD) database's expanded version was applied to test whether there were linear correlations between the miR-199a-3p and FN1 expression levels (n = 439, the TCGA public repository, https://cancergenome.nih.gov; March 8, 2016).

Cell Culture and Cell Transfection

HCT116 cell line was obtained from and authenticated by the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). It was cultured in a dulbecco's modified eagle medium (DMEM) medium (Gibco, 11966025, Shanghai, China) containing 10% Fetal bovine serum (FBS) (Gibco, 10099141C, Shanghai, China) at 37 °C and cultured at 5% CO_2 in a humid environment. HCT116 cell line was tested for mycoplasma and only negative lines were included in the study. They were not listed in the The International Cell Line Authentication Committee (ICLAC) and Expert Protien Analysis System (ExPASy) Cellosaurus database of commonly misidentified cell lines.

The miRNA mimics, miRNA inhibitor, and miR-199a-3p's negative control miRNA were obtained from RiboBio Co. Ltd (Guangzhou, China). HCT116 cells were transfected with Lipofectamine 2000 reagent (Invitrogen, 11668019, Shanghai, China).

CCK-8

Cell activity was measured via a Cell Counting Kit-8 (CCK-8) (Sigma, 96992, St. Louis, MO, USA). A total of 96-well plates were used at a concentration of 1×10^3/well, to culture the cells in the complete medium. After culturing for 24 h, the complete medium was changed into one with 10% fatal bovine serun (FBS), followed by the addition of 10 μL cell counting kit-8 (CCK8) solution after 24 h of incubation per well. In order to determine the viability of the cells, additional 4 h incubation was implemented. Every 12 h following transfection, the determination of proliferation rates was performed. With a microtiter plate reader, the determination of the optical density was implemented at 450 nm. Every experiment was conducted at least three times and the average was used to express the findings.

Transwell

HCT116 cells were seeded in each well of a transwell polycarbonate membrane's upper chamber (Costar, 3422, 8-μm pore size, Cambridge, MA, USA), which was coated with Matrigel (BD Biosciences, 356234, San Jose, CA, USA) for cell migration and invasion tests. The lower chamber was added with a cell culture medium with 20% FBS. Next, the upper chamber cells were collected and rinsed and then stained using 0.5% crystal violet after 24 h culture. The stained cell number was measured via the light microscope.

Flow Cytometry

Cell apoptosis was examined using the fluorescein isothiocyanate fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit I (BD Biosciences, 556547, San Jose, CA, USA) under the guidance of the introduction of the manufacturer. First, after treating the cells for 24 h, they were washed with cold Phosphate Buffered Saline (PBS) twice. After that, the cells were suspended (1×10^6 cells/mL) in buffer solution again and 100 μL of solution (1×10^5 cells) were transferred to 5 mL culture tubes and 5 μL FITC and 5 μL PI were added. Then, the cells were vortexed gently and cultured in the dark, at 25 °C for 15 min, with 400 μL buffer in each tube. The CytoFLEX flow cytometer (V5-B5-R3, Beckman Coulter, Brea, CA, USA) was used for analyzing and quantifying cell death, within 1 h.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA reversely transcribed into cDNA was extracted using the Trizol reagent (Invitrogen, 15596018,

Carlsbad, CA, USA), according to the manufacturer's instructions. Ex Taq (TAKARA, RR001, Dalian, China) was applied to perform the Quantitative PCR and the cycle conditions were as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 62 °C for 40 s. Additionally, GAPDH was used as a loading control. The primers were as follows: GAPDH, forward 5′-TGAAGCAGGCATCTGAGGG-3′ and reverse 5′-CGAAGGTGGAAGAGTGGGAG-3′; MiR-199a-3p, forward 5′-TCAAAGCGTGGTTGGTGAGT-3′ and reverse 5′-GGGATCTTGCCACTCCAACT-3′; HCT116 FN1, forward 5′-TCCGGGCCTCAATCCAAATG-3′ and reverse 5′-ACTGTAAGGGCTCTTCGTCG-3′. The mRNA relative expression level was calculated via the $2^{-\Delta\Delta Ct}$ method.

Dual-Luciferase Reporter Assay

MiR-199a-3p and FN1 were combined to the pmiR-RB-REPORT™ vector (Ribobio, Guangzhou, China). Mutant-type (MUT)-MiR-199a-3p and MUT-FN1 were utilized for the control group. The construction of the miR-199a-3p 3′UTR and FN1 3′UTR mutation vectors and the identification of the recombinant plasmid sequences were performed. QuikChange II XL Site-Directed Mutagenesis Kit (Agilent, 200522, SantaClara, CA, USA) was used to carry out the Mutation. For the assay of luciferase, HCT116 cells were seeded in 96-well plates. pMIR-3′-UTR constructs were co-transfected with miR-199a-3p mimics via the Lipofectamine 2000 method. After transfecting the cells for 24 h, the sediment was collected before adding to the non-transparent 96-well plates containing firefly luciferase. The luciferase activities were examined via Dual-Luciferase Reporter Assay Kit (Promega, E1910, Madison, WI, USA).

Western Blot

The cells were lysed using the Radioimmunoprecipitation (RIPA) buffer (Thermo Fisher, 89901, Waltham, MA, USA). Protein concentration was determined via a Biochanin A (BCA) protein assay kit (Applygen Technologies Inc., P1511-1, Beijing, China) following the introduction of the manufacturer. Electrophoresis was followed. Subsequently, the protein was sealed at room temperature for 2 h in 5% skim milk. After that, it was transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, IPFL00010, Billerica, MA, USA). Next, it was assayed using primary antibodies against FN1 (1:1000, CST, 26836, Danvers, MA, USA), E-cadherin (1:1000, CST, 14472, Danvers, MA, USA), β-catenin (1:1000, CST, 8480, Danvers, MA, USA), N-cadherin (1:1000, CST, 13116, Danvers, MA, USA), Vimentin (1:1000, CST, 5741, Danvers, MA, USA) and GAPDH (1:1000, CST, 5174, Danvers, MA, USA) at 4 °C for 12 h. After that, the corresponding horseradish-peroxidase-conjugated secondary antibodies were grown with the membranes. Protein signals were detected via ECL detection reagents bought from Beyotime (Beyotime, P0018FS, Shanghai, China). The protein band gray value was measured through the Image J software (NIH Image, version 1.52, Bethesda, MD, USA).

Statistical Analysis

Statistical Product Service Solutions (SPSS) 20.0 (Chicago, IL, USA) and GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA) were employed to analyze all data. The distribution of continuous data was checked for normality, using Shapiro-Wilk test. Normally distributed continuous data described as mean $\pm$ standard deviation (SD) and compared using Student's *t*-test, resorting to one-way analysis of variance (ANOVA) for comparisons involving more than two groups. When a statistically significant difference was found between more than two groups, each pair was compared. Continuous data that did not fit a normal distribution were described as the median [inter-quartile range] and compared using Wilkinson signed rank test, resorting to the Mann-Whitney test or non-parametric analysis of the variance (ANOVA) for comparisons involving more than two groups. Categorical data were described as frequency (%) and compared using the chi-squared test, resorting to the Mann-Whitney U test when frequencies <5 were observed in $\geq$20% of cells and Fisher's exact test for comparisons that fitted 2×2 tables. Correlations were assessed using Pearson's correlation test. A p value < 0.05 was considered statistically significant. Each experiment was carried out three times. $p < 0.05$ was considered statistically significant.

Results

MiR-199a-3p Expression in CRC Cells HCT116 was Significantly Down-Regulated under Hypoxia

According to the volcano plot, a total of 162 genes were detected in the hypoxia and non-hypoxia CRC cells by gene sequencing analysis, and 8 genes were down-regulated significantly and 3 genes were up-regulated significantly in the hypoxia group (Fig. 1A). Meanwhile, differentially expressed miR-199a-3p of CRC cells was significantly lowered in the hypoxia group according to the heat map (Fig. 1B). In addition, the miR-199a-3p of HCT116 CRC cells was significantly down-regulated under hypoxia (Fig. 1C). Therefore, miR-199a-3p was chosen as the subsequent study object.

MiR-199a-3p Suppressed CRC Proliferation, Migration and Invasion and Facilitated Apoptosis in Vitro

The miR-199a-3p expression was significantly elevated following transfecting miR-199a-3p mimics into HCT116 CRC cells (Fig. 2A). After transfecting HCT116 by miR-199a-3p inhibitor, miR-199a-3p expression was significantly decreased.

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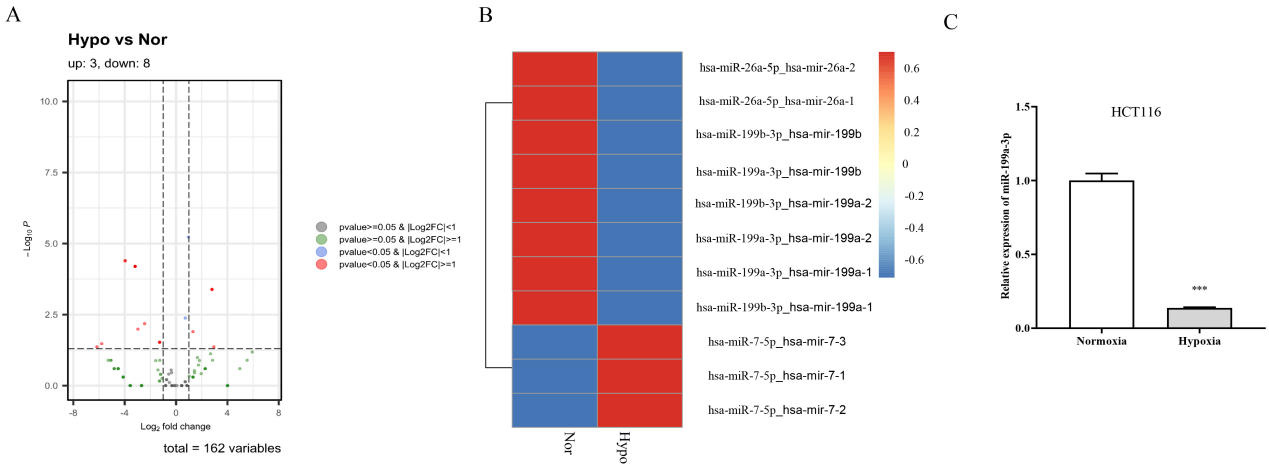

Fig. 1. CRC cell gene expression in normoxic and hypoxic conditions. (A) Volcano plot of CRC cell gene expression in normoxic and hypoxic conditions. (B) Heat map of CRC cell gene expression in normoxic and hypoxic conditions. (C) HCT116's miR-199a-3p expression level in hypoxia condition. Compared to the Normoxic group, $^{***}p < 0.001$. Abbreviation: Hypo, Hypoxia; Nor, Normoxia.

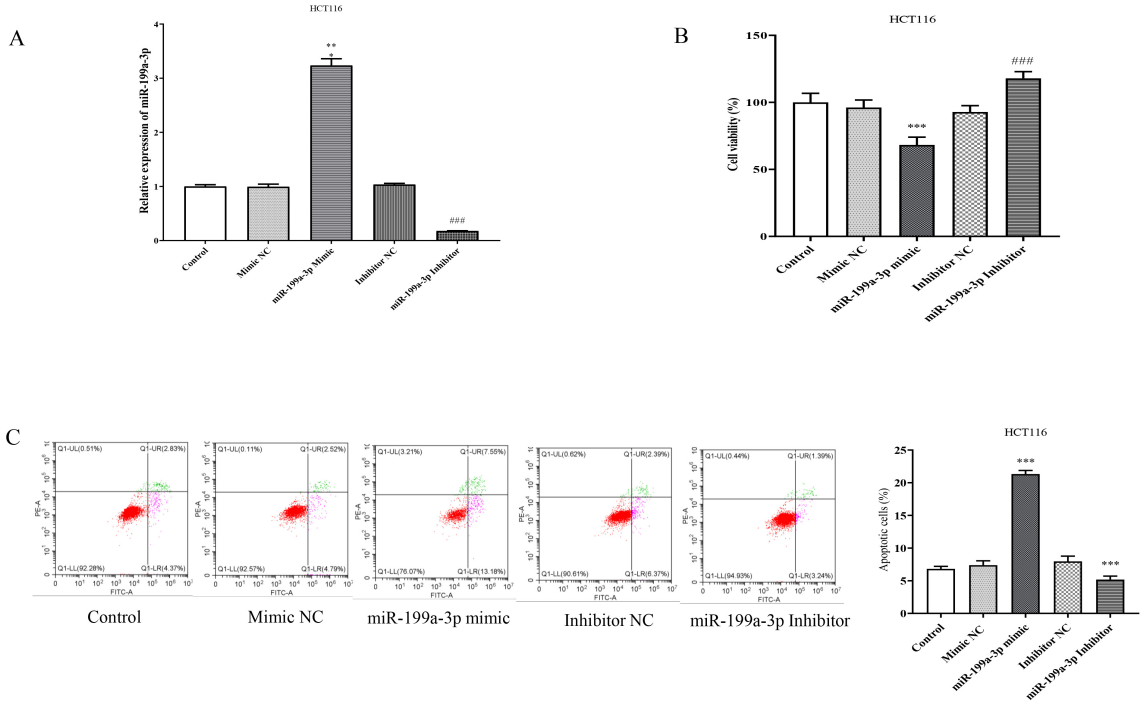

Fig. 2. Effect of miR-199a-3p overexpression or inhibitor on proliferation and apoptosis of CRC cells. (A) MiR-199a-3p expression level of on HCT116, following transfecting miR-199a-3p mimics or inhibitors. (B) CCK-8 assay after treating HCT116 cells using miR-199a-3p mimic and inhibitor. (C) Flow cytometry following treating HCT116 using miR-199a-3p mimic and inhibitor. Compared to the control group, $^{**}p < 0.01$, $^{***}p < 0.001$, in comparison to the inhibitor NC group, $^{###}p < 0.001$.

Overexpressed miR-199a-3p significantly curbed the HCT116 cell proliferation rate (Fig. 2B) and promoted the cell apoptosis (Fig. 2C). Suppressing miR-199a-3p significantly reversed the effect. Overexpressed miR-199a-3p significantly impeded HCT116 cell migration and invasion, which was significantly promoted via suppressing miR-199a-3p (Fig. 3).

Bioinformatics Analysis of miR-199a-3p Target Genes

The miR-199a-3p target genes and their biological functions investigation using the bioinformatics method is shown in Fig. 4A. The KEGG analysis showed that PI3K/AKT signaling pathway, cancer pathways, and actin cytoskeleton regulation had the strongest correlation with

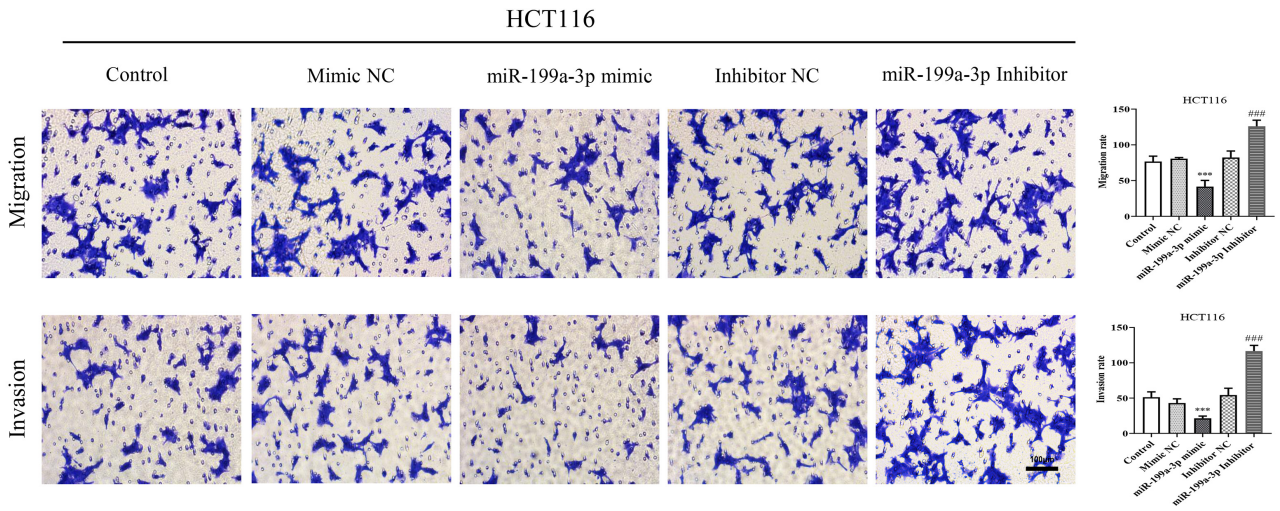

Fig. 3. Effect of miR-199a-3p overexpression or inhibitor on CRC cell migration and invasion. HCT116 cell migration and invasion were measured after treating the cells using miR-199a-3p mimic and inhibitor. Compared to the Control group, $^{***}p < 0.001$, in comparison to the inhibitor NC group, $^{###}p < 0.001$. Scale bar = 100 μm.

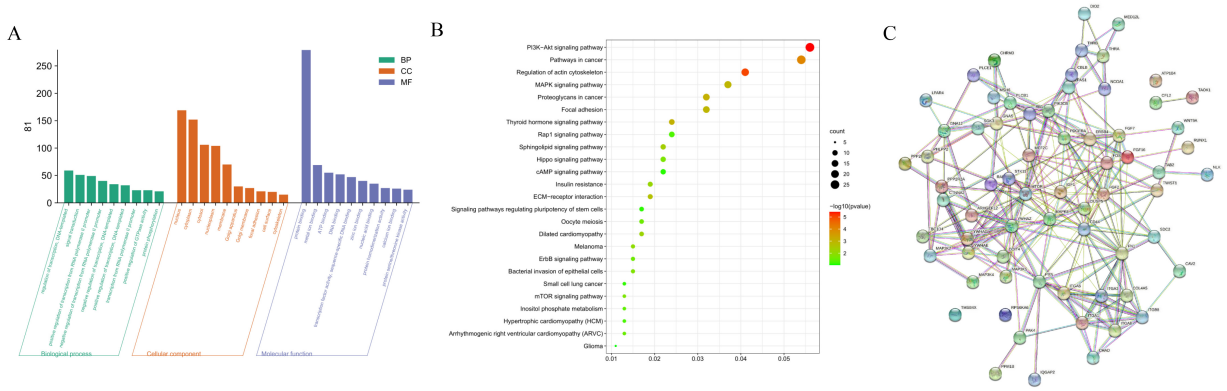

Fig. 4. GO analysis, KEGG analysis and PPI network analysis. (A) GO analysis utilization to determine miR-199a-3p target genes' biological functions. (B) The relationship between enrichment pathways and target genes, as shown by KEGG analysis. (C) Protein-protein interactions of target genes, as shown by PPI network analysis.

miR-199a-3p target genes (Fig. 4B). Protein-Protein Interaction (PPI) network analysis of protein-protein interactions of the first eight KEGG pathway target genes and a PPI network was constructed (Fig. 4C). As shown in Table 1, MTOR and FN1 were the most suitable target genes. MTOR was widely studied in CRC, while there were relatively few studies related to FN1. Therefore, FN1 was selected for verification.

FN1 is the miR-199a-3p Target

Luciferase activity detection revealed that miR-199a-3p significantly lessened luciferase activity, by combining with the 3′UTR position of FN1 gene. However, there was no significant change in luciferase activity after mutating the 3′UTR binding site of FN1 gene (Fig. 5A). Furthermore, miR-199a-3p overexpression significantly diminished FN1 protein and FN1 mRNA expression in HCT116 CRC cells (Fig. 5B,C), while inhibition of miR-199a-3p had the contrary effect.

FN1 Overexpression Promoted the Proliferation, Migration and Invasion of miR-199a-3p Mediated HCT116, and Inhibited Its Apoptosis

FN1 overexpression can compensate for the proliferation inhibition of HCT116 caused by miR-199a-3p overexpression (Fig. 6A). Besides, FN1 overexpression can compensate for the increased apoptosis of HCT116 cells resulted from miR-199a-3p overexpression (Fig. 6B). In addition, FN1 overexpression can compensate for the inhibition of HCT116 migration and invasion owing to miR-199a-3p overexpression (Fig. 6C). The above experiments proved that miR-199a-3p regulates the CRC cell proliferation, migration, invasion, and apoptosis of via targeting FN1.

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Table 1. List of central protein-coding genes identified in the target genes of the first eight significantly different KEGG pathways.

Gene symbol	Node degree	Gene name
MTOR	22	mechanistic target of rapamycin
FN1	19	fibronectin 1
CD44	18	CD44 molecule (Indian blood group)
IGF1	18	insulin like growth factor 1
YWHAZ	17	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta
FGF2	15	fibroblast growth factor 2
FOS	15	Fos proto-oncogene, AP-1 transcription factor subunit
MAPK8	15	mitogen-activated protein kinase 8
PIK3CB	14	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta
ERBB4	13	erb-b2 receptor tyrosine kinase 4
ITGA6	13	integrin subunit alpha 6
PXN	13	Paxillin
STK11	12	serine/threonine kinase 11
YWHAE	12	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon
PDGFRA	11	platelet derived growth factor receptor alpha
YWHAG	11	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma
ITGA1	10	integrin subunit alpha 1
ITGA3	10	integrin subunit alpha 3

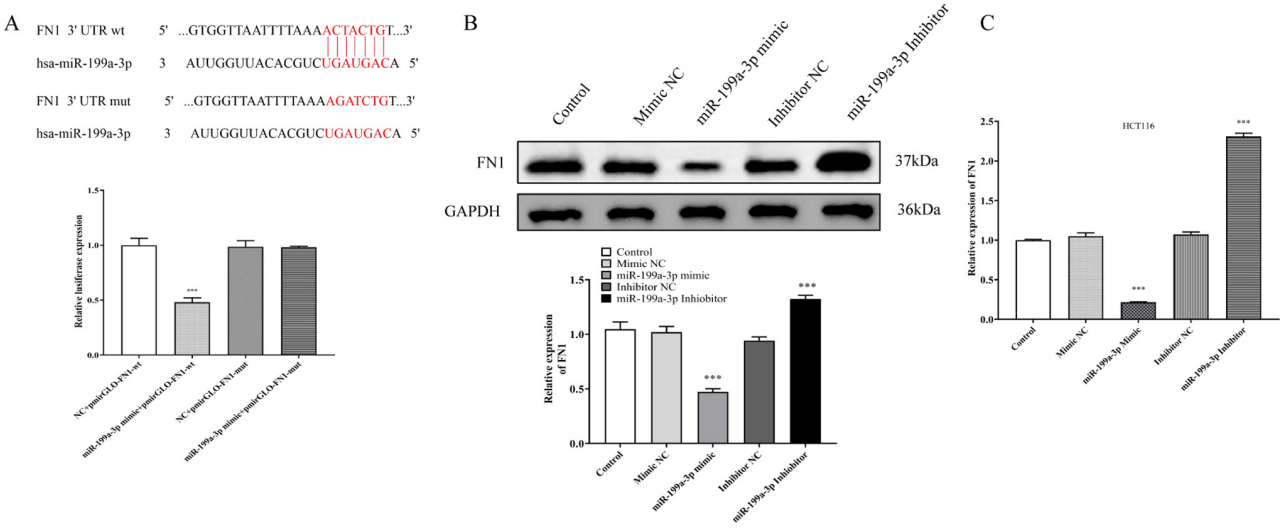

Fig. 5. Effect of miR-199a-3p overexpression or inhibitor on protein and mRNA content of FN1. (A) Effects of different binding sites of FN1 gene on luciferase activity. (B) The protein level of FN1 on HCT116 cells treated with miR-199a-3p mimic and inhibitor was measurement with western blot. (C) qRT-PCR utilization to determine the FN1 mRNA on HCT116 cells treated with miR-199a-3p mimic and inhibitor. In comparison to the Control group, $^{***}p < 0.001$.

MiR-199a-3p and FN1 Interactions Regulate Epithelial-Mesenchymal Transition (EMT) in CRC Cells

MiR-199a-3p overexpression significantly raised the EMT marker protein E-cadherin and B-catenin and diminished N-cadherin and Vimentin on HCT116. The opposite result was obtained in miR-199a-3p inhibitor group (Fig. 7A). Moreover, FN1 knockdown also significantly increased the EMT marker protein E-cadherin and B-catenin and lessened N-cadherin and Vimentin on HCT116 (Fig. 7B). On the other hand, FN1 overexpression significantly reversed miR-199a-3p overexpression's impact (Fig. 7C).

Discussion

The aim of this study was to investigate the regulatory role of miR-199a-3p on CRC and to investigate its mechanism. The study showed that miR-199a-3p was downregulated in hypoxia-induced CRC cells, and that miR-

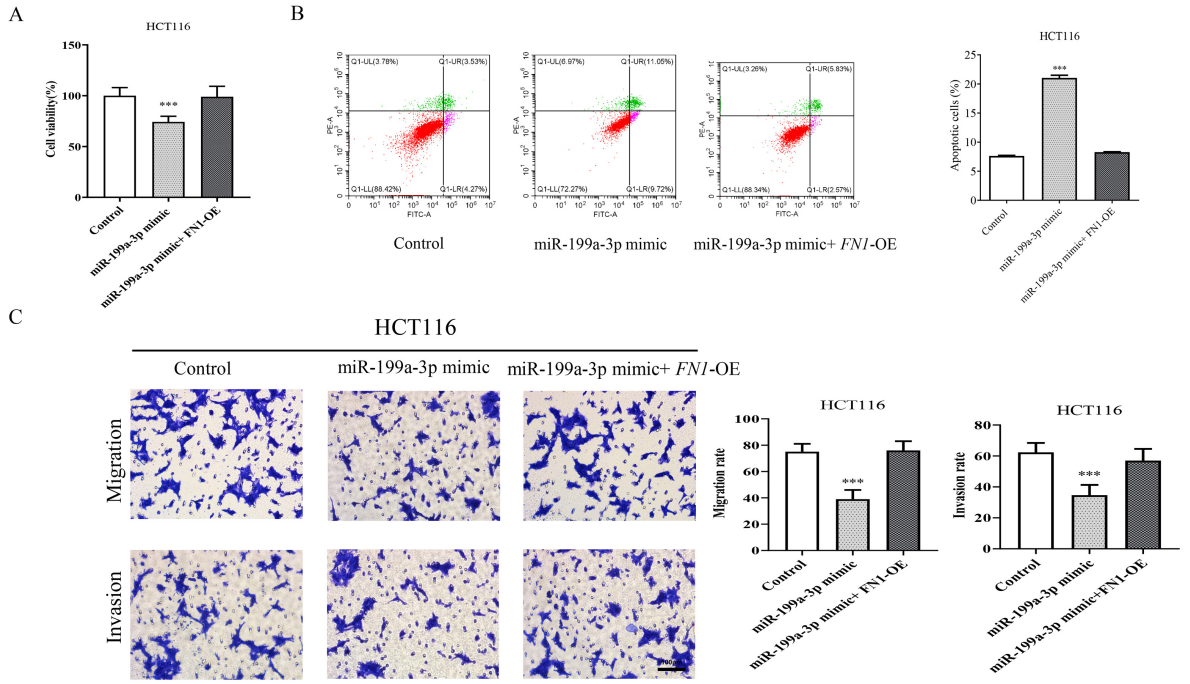

Fig. 6. Effect of FN1 overexpression on CRC cell proliferation, apoptosis, migration and invasion of CRC cells. (A) The CCK-8 assay carried out to detect the HCT116 cell viability after treating the cells with miR-199a-3p mimic and miR-199a-3p mimic + FN1 overexpression. (B) The apoptosis of HCT116 cells treated with miR-199a-3p mimic and miR-199a-3p mimic + FN1 overexpression measurement using the flow cytometry. (C) The transwell assay conducted to examine the migration and invasion following treating HCT116 cells with miR-199a-3p mimic and miR-199a-3p mimic + FN1 overexpression. Compared to the Control group, $^{***}p < 0.001$. Scale bar = 100 μm.

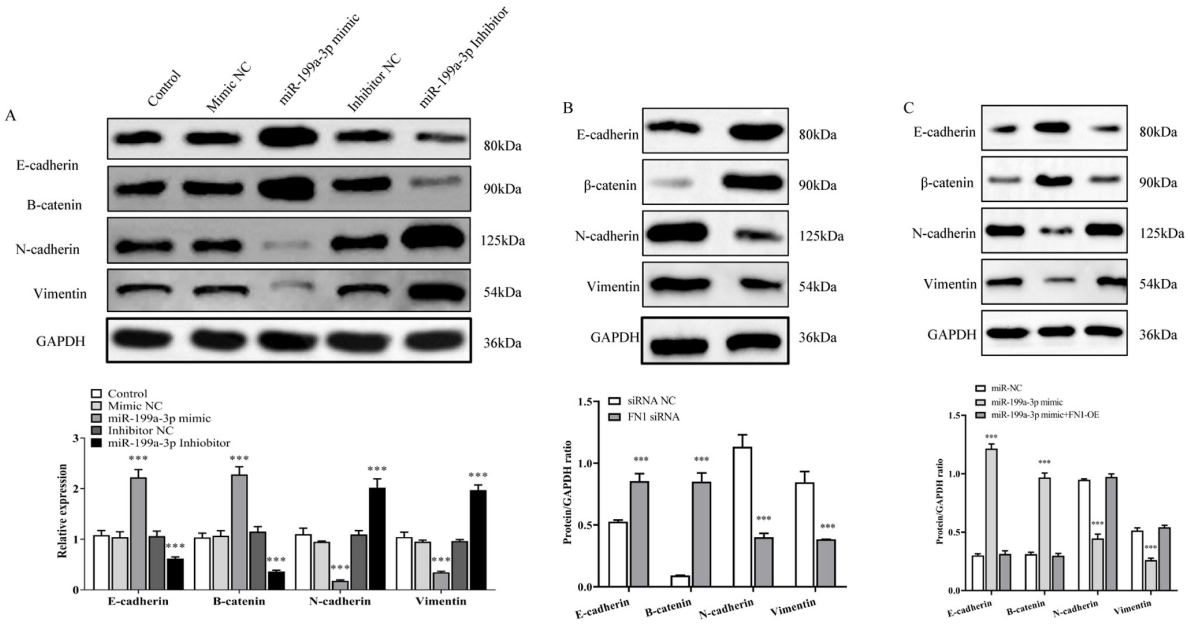

Fig. 7. Effect of miR-199a-3p overexpression and FN1 knockdown or overexpression on EMT protein expression. (A) E-cadherin, B-catenin, N-cadherin, and Vimentin expression on EMT of HCT116 cells by miR-199a-3p overexpression, as measured with western blotting. (B) E-cadherin, B-catenin, N-cadherin and Vimentin expression on EMT of HCT116 cells by FN1 knockdown, as measured with western blot. (C) E-cadherin, B-catenin, N-cadherin and Vimentin expression on EMT of HCT116 cells by FN1 overexpression, as determined with western blot. Compared to the Control group, $^{***}p < 0.001$.

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199a-3p affected CRC cells' proliferation invasion migration and apoptosis through negative regulation of FN1. In addition, it was found that miR-199a-3p and FN1 affected CRC progression through regulation of EMT.

The study showed that the CRC cells' proliferative, migrative and invasive were significantly reduced by overexpressed miR-199a-3p and were significantly promoted via inhibiting miR-199a-3p. The apoptosis of CRC cells was significantly promoted by overexpressed miR-199a-3p and significantly inhibited by suppressing miR-199a-3p. This is similar to the report of a previous study which reported that miR-199a-3p inhibited the progression of ovarian cancer [12]. However, another study illuminated that miR-199a-3p was significantly up-regulated in the cell lines and tissues of gastric cancer, elevated cell proliferation and inhibited apoptosis, by targeting ZHX1 *in vitro* and *in vivo* [8]. This suggests that CRC have a lot to do with miR-199a-3p's target gene or pathway.

This study showed the relationship in HCT116 CRC cells between FN1 and EMT marker proteins. EMT is an vital biological process for malignant tumor cells to acquire the invasive and migrative abilities, and its protein expression is its main feature [21]. FN1 knockdown significantly elevated the EMT marker protein E-cadherin and B-catenin and cut N-cadherin and Vimentin in HCT116. On the other hand, FN1 overexpression had the reversed effect, which in line with the former studies exploring FN1 in ovarian cancer [22].

This study proved that miR-199a-3p could regulate CRC cells' proliferative, apoptotic, migrative and invasive abilities. However, the mechanism requires further clarification. This study observed that miR-199a-3p overexpression decreased FN1-related proteins and mRNA contents in HCT116, while miR-199a-3p inhibitor elevated the contents in HCT116, illustrating the moderating relationship between FN1 and miR-199a-3p for the first time. The study functionally characterized the relationship between these two factors, by using several cell models that manipulate FN1 and miR-199a-3p expression. The study showed that overexpressed FN1 compensated for the miR-199a-3p inhibition on CRC development, suggesting the regulatory effect of miR-199a-3p on FN1's biological function. This study not only identifies the relationship between FN1 and miR-199a-3p for the first time, but also adds to the experimental basis for FN1 as a marker protein for EMT in CRC cells. However, this study still needs to be refined, for example, the results need to be validated *in vivo*.

Conclusions

The aim of this study was to investigate the effect of miR-199a-3p on the CRC phenotype and the mechanism of its association. The study showed that miR-199a-3p can effectively regulate CRC cells' proliferative, apoptotic, migrative and invasive processes. In addition, the study showed that FN1 was a miR-199a-3p target gene and that interactions between miR-199a-3p and FN1 regulated CRC cells, which illustrated that miR-199a-3p and FN1 may be the new targets for treating CRC.

Availability of Data and Materials

The current study contains available datasets which are available from the corresponding author upon reasonable request.

Author Contributions

LL—conceived and designed the study, wrote the manuscript and performed the experiment; BC—performed the experiment, analyzed the data and collected the data and technical information; YL—reviewed and helped revise the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of Qilu Hospital of Shandong University.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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