Pro-carcinogenic actions of miR-155/FOXO3a in colorectal cancer development

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ARTICLE INFO	ABSTRACT
	Colorectal cancer (CRC) ranks third in cancer incidence and second in cancer mortality globally. MicroR-NAs (miRNAs) are promising biomarkers and therapeutic targets for CRC diagnosis and treatment. The miR-155 is reported to induce radiation resistance in CRC. In this study, we aimed to further clarify the role and underlying mechanism of the miR-155 in CRC cell malignancy. We found that miR-155 was significantly up-regulated in CRC tissues. The results of loss-of-function experiments revealed that miR-155 deficiency suppressed the proliferative capacity, invasion, and migration of CRC cells. Moreover, the downstream target genes of miR-155 were screened, and miR-155 was demonstrated to directly bind to FOXO3a in CRC cells to negatively regulate FOXO3a expression. FOXO3a was downregulated in CRC tissues and the expression of FOXO3a and miR-155 was in negative correlation in CRC tissues. FOXO3a overexpression alone was revealed to inhibit CRC cell growth, migration and invasion. Additionally, rescue assays showed that FOXO3a silencing significantly reversed the inhibitory effect of miR-155 deficiency on CRC cell malignant behaviors. In conclusion, miR-155 induces malignant phenotypes of CRC cells including cell proliferation, migration and invasion by targeting FOXO3a, which might provide clues for the targeted therapy of CRC.

Introduction

Colorectal cancer (CRC) is a highly heterogeneous disease with a poor prognosis, with over 1.9 million newly diagnosed cases and 935,000 deaths globally (1). Risk factors such as family history, obesity, dietary habits, lack of physical exercise, drinking and smoking are reported to contribute to CRC (2). For non-metastatic CRC, surgery and chemoradiotherapy are the primary options, but tumor metastasis is less prognostic friendly and about 25% of CRC patients have distant metastases (3), with a 5-year survival rate of only 12.5% (4, 5). Several potential mechanisms are involved in tumor progression, including metastasis and the growth of tumor cells (6, 7). Therefore, it is significant to elucidate the underlying mechanism to develop promising biomarkers and therapeutic targets to improve the diagnosis and treatment outcomes of CRC.

MicroRNAs (miRNAs) are non-coding small RNAs with 21-25 nucleotides without the protein-coding potential. They negatively regulate the target gene expression at the posttranscriptional level by suppressing translation and/or facilitating mRNA degradation (8). They can recognize specific complementary sequences which are mainly located in the 3'UTR of the target mRNA (9, 10). Studies have revealed that miRNAs are powerful regulators of a variety of cellular processes such as proliferation, differentiation, development as well as apoptosis, and are involved in the pathogenesis of diverse diseases, cancer

included (11). Many miRNAs play a key role as oncogenes or tumor suppressors in human cancer (12). According to reports, miR-155 induces tumor growth and invasion in many solid malignancies such as breast cancer (13), gastric cancer (14), liver cancer (15) and oral cancer (16). In addition, miR-155 can promote CRC proliferative capacity, migration, and invasion (17) and even induce radiation resistance of CRC by targeting the FOXO3a gene (18). A study has suggested that p-STAT3 upregulates miR-155-3p expression to subsequently downregulate WDR82 to stimulate CRC progression (19). It is also reported that miR-155 targets the tumor suppressor PTPRJ in CRC cells, and miR-155 overexpression elevates the viability of CRC cells and activates the AKT pathway, which is partially reversed by the PTPRJ overexpression (20). However, the direct effects of miR-155 on CRC malignant phenotypes require to be further elucidated. Therefore, we chose miR-155 as a candidate gene to study its biological function and mechanism in CRC.

Forkhead box O3A (FOXO3a) belongs to the FOXO subfamily of forkhead transcription factors (TFs) that regulates various cellular processes such as proliferation, cell cycle, DNA damage and apoptosis (21). FOXO3a, approximately 71 kDa in size, is conserved in different species. Substantial evidence has demonstrated that FOXO3a is critically implicated in diverse cancer progression. For example, DNMT1-induced downregulation of FOXO3a facilitates breast cancer stem cell properties

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and tumor growth (22). MiR-182-5p promotes hepatocellular carcinoma cell proliferation and migration by targeting FOXO3a to activate the Wnt/β-catenin signaling (23). Additionally, FOXO3a is also reported to be targeted by miR-223 in CRC, and miR-223 suppresses CRC proliferation and induces CRC cell apoptosis by downregulating FOXO3a (24). FOXO3a is downregulated in CRC tissues and miR-592 promotes CRC cell growth and migration partially by targeting FOXO3a (25). However, the role of the miR-155/FOXO3a axis in CRC still requires further exploration.

In this study, we intended to investigate the effects and underlying mechanisms of the miR-155 in CRC. We hypothesized that miR-155 promotes CRC cell malignant phenotypes by targeting FOXO3a. The findings of our work might deepen the understanding of the role of miR-155 in colorectal cancer and provide novel therapeutic targets for CRC patients.

Materials and Methods

Patient and tissue samples.

The surgical specimens of CRC tissues and adjacent tissues (3 cm from the tumor) were obtained from 50 CRC patients treated in the Affiliated Hospital of Sijing Hospital from January 2017 to February 2019. All tissues underwent pathological confirmation and staging identification based on the National Comprehensive Cancer Network Guidelines. After the operation, the samples were quickly frozen in liquid nitrogen and preserved at -80˚C. All CRC patients experienced pathological diagnosis and received no preoperative treatment. All patients provided their complete clinical, pathological and follow-up data, as well as written informed consent. The ethics committee of the Affiliated Hospital of Hebei University of Engineering approved the plan.

Cell culture and transfection

Human CRC cell lines DLD1 and SW480 and normal colorectal epithelial cell line NCM460 (ATCC, Manassas, VA, USA) were incubated in RPMI-1640 medium containing 10% heat-inactivated FBS (Gibco; Thermo Fisher Scientific) at 37˚C with 5% CO_2. GenePharma (Shanghai, China) produced miR-155 mimic/inhibitor and their negative controls (NCs). Cells (5×10^4) were seeded into the 6-well plates and transfected with vectors or plasmids the following day using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific). The final concentration of oligonucleotide was 20 nmol/l. RT-qPCR was used to evaluate the transfection efficiency after 48 h.

Dual luciferase reporter gene experiment

FOXO3a wild-type 3'-UTR carrying the miR-155 binding site (wt-FOXO3a) was inserted into the pMirGLO reporter vector (Promega). This 3'-UTR was mutated with QuickChange II site-directed mutagenesis kit (Agilent Technologies, USA) to generate mut-FOXO3a. HEK-293T cells were co-transfected with wt-FOXO3a or mut-FOXO3a and miR-155 mimic. The luciferase activity was evaluated in the luciferase reporter system (Promega) following the manufacturer's protocol.

MTT

DLD1 and SW480 cells were cultured continuously for 1, 2, 3, and 4 days in 96-well plates and further incubated with MTT (5 mg/mL) for 4 h. After removal of the cell supernatant, 150 μl DMSO was added in each well, and the optical density values were measured at 450 nm.

Scratch healing experiment

DLD1 and SW480 cells in the logarithmic growth phase were plated in a 24-well plate at 2.5×10^5 cells/well for 24 h, rinsed with PBS twice, and wounded by a 200 μl pipette tip vertically, about 3 to 4 lines per well. Then, each well was rinsed with PBS 3 times and added with 1 mL serum-free culture medium, and observed and and photographed under an inverted microscope after 24 h.

Transwell invasion experiment

A Transwell chamber (Corting, NY, USA) precoated with Matrigel (200 mg/ml, BD, SanJose, USA) was used for cell invasion assays. DLD1 and SW480 cells were added to the upper chamber filled with serum-free medium, and the lower chamber was filled with DMEM (500 μl) containing 10% FBS. Cells were cultured for 24 h, fixed with paraformaldehyde, stained with crystal violet solution, and imaged with a phase contrast microscope (Olympus, Tokyo, Japan).

RT-qPCR

According to the human miR-155 and FOXO3a mRNA sequences in GeneBank, primers were designed using Primer 5.0 software and synthesized by Sangon (Shanghai, China). TRIzol was used to extract total cellular RNA and a UV spectrophotometer was used to determine the purity and concentration of RNA. Then cDNA was produced by reverse transcription. This chain was used as a template to amplify miR-155 and FOXO3a mRNA fragments using an ABI7300 fluorescent quantitative PCR instrument, with GAPDH as the internal reference for FOXO3a mRNA, and U6 as the internal reference for miR-155. The relative expression of target genes was calculated with the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Values are expressed as the mean ± standard deviation. GraphPad *P* value calculator was used to compare the two groups and calculate the *P* value using the t-test. If necessary, a one-way analysis of variance was performed on multiple groups, and then the post-group test was used for paired comparison. *p*-value ≤0.05 indicated that the difference was considered statistically significant.

Results

MiR-155 is up-regulated in CRC

MiR-155 levels in 50 pairs of clinical CRC and adjacent normal tissues were detected. We found miR-155 was up-regulated in CRC tissues compared with the adjacent normal samples (Figure 1A) and higher miR-155 levels were related to the late stage of CRC (Figure 1B). MiR-155 was also revealed to be highly expressed in CRC cell lines and was more upregulated in human CRC DLD1 and SW480 cells (Figure 1C). Then miR-155 mimic and inhibitor were transfected into SW480 and DLD1, respectively, leading to miR-155 overexpression and inhibition correspondingly (Figures 1D and 1E). MTT experiment showed that miR-155 overexpression enhanced the prolif-
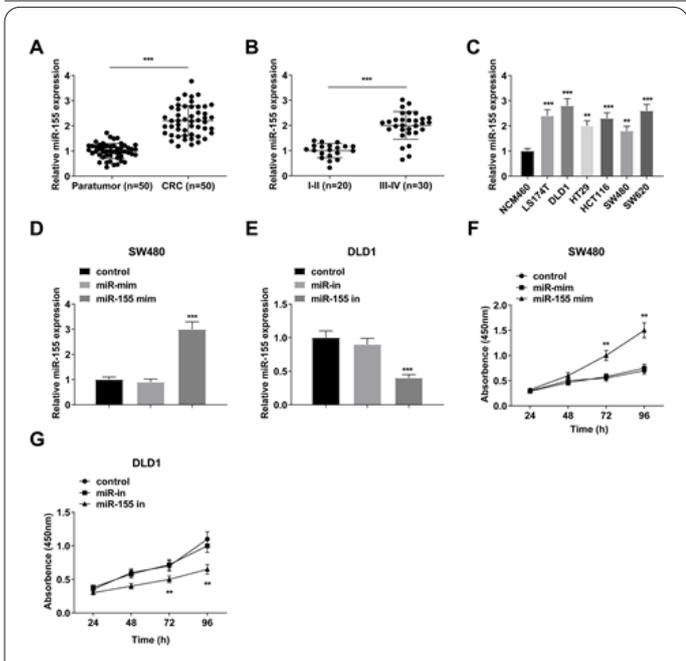
Figure 1. MiR-155 is up-regulated in CRC tissues and cells and promotes the proliferation of CRC cells. A: qRT-PCR was used to detect the expression of miR-155 in CRC tissue (n=50) and adjacent normal tissues (n=50). B: qRT-PCR was used to detect the expression of miR-155 in the tumor tissues of CRC patients at different stages. C: qRT-PCR was used to detect the expression of miR-155 in human colorectal epithelial cell lines NCM460 and CRC cell lines. D: After transfection of the miR-155 mimic, qRT-PCR was used to detect the expression of miR-155 in CRC cells. E: After transfection of the miR-155 inhibitor, qRT-PCR was used to detect the expression of miR-155 in the cells. F-G: After transfection of miR-155 mimic and inhibitor, the proliferation level of CRC cells was detected by the MTT method. **$P<0.01$, ***$P<0.001$ (compared with miR-mim or miR-in group).

Figure 2. Low expression of miR-155 inhibits the migration and invasion of DLD1 cells. A: After transfection with miR-155 inhibitor, the migration of DLD1 cells was detected by the scratch healing test. B: After the miR-155 inhibitor was transfected, the transwell test was used to detect the invasion of DLD1 cells. *** $P<0.001$ (compared with the miR-in group).

Figure 3. High expression of miR-155 promotes the migration and invasion of SW480 cells. A: After transfection of the miR-155 mimic, a scratch healing test was used to detect the migration of SW480 cells. B: After the miR-155 mimic was transfected, the transwell experiment was used to detect the invasion of SW480 cells. *** $P<0.001$ (compared with the miR-mim group).

erative capacity of CRC cells (Figure 1F), while miR-155 inhibition limited CRC cells to proliferate (Figure 1G).

Depleting miR-155 inhibits CRC cell migration and invasion

Scratch-healing assays were performed to explore the effects of miR-155 deficiency on CRC cell migration ability. The wound closure rate of DLD1 cells after miR-155 inhibition was significantly reduced (Figure 2A). The transwell invasion experiments were used to investigate the influence of miR-155 on the invasion ability of CRC cells. Depleting miR-155 significantly dampened DLD1 cell invasion (Figure 2B). In SW480 cells, the wound closure rate of SW480 cells after miR-155 upregulation was elevated (Figure 3A) and the cell invasion ability was enhanced (Figure 3B).

MiR-155 directly binds to the 3'-UTR of FOXO3a

TargetScan (http://www.targetscan.org/vert_72/) was used to screen the target gene of miR-155. We found that FOXO3a contains a conserved binding site for miR-155. The wt-FOXO3a or mut-FOXO3a 3'UTR was inserted into the pMirGLO reporter vector (Figure 4A). After transfection, luciferase activity assay showed that miR-155 overexpression reduced the luciferase activity of wt-FOXO3a and showed no significant impact on mut-FOXO3a (Figure 4B). According to the RT-qPCR an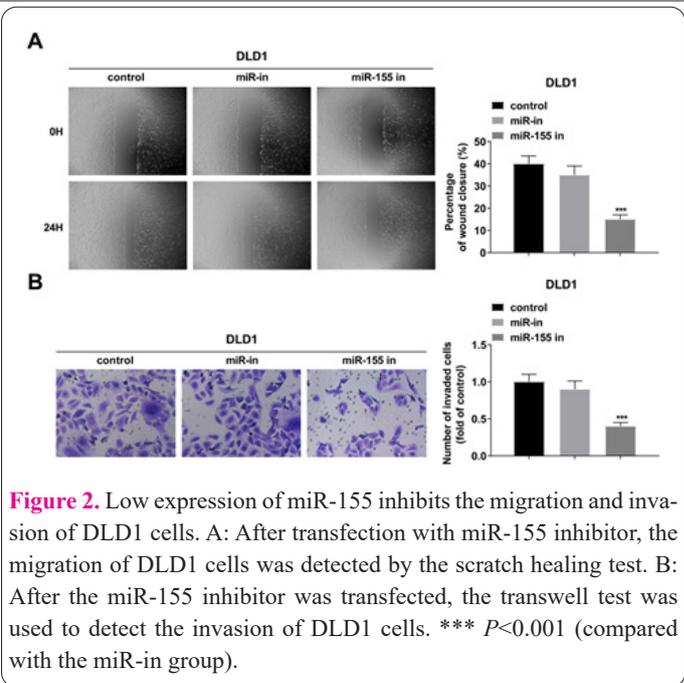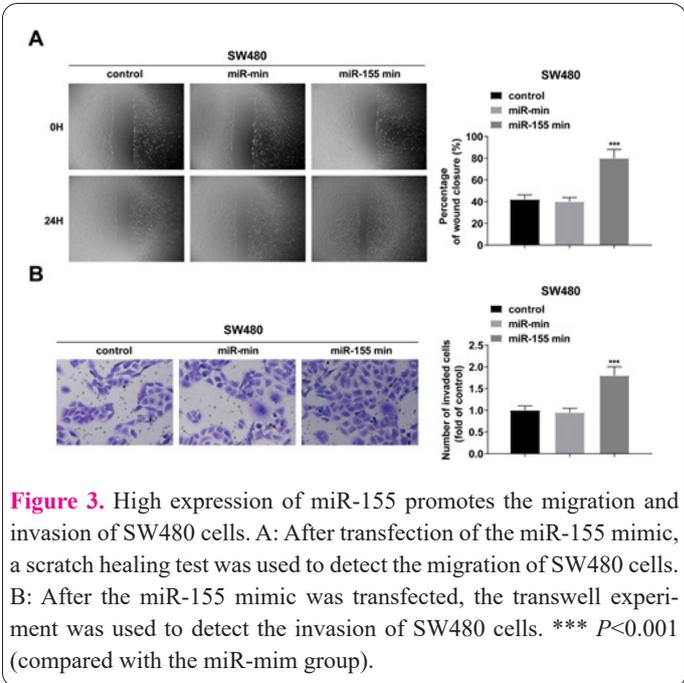alysis, the expression of FOXO3a in CRC cells (DLD1 and SW480) was lower than that in human colorectal epithelial cell line NCM460 (Figure 4C), and FOXO3a was also lowly expressed in CRC tissues compared with adjacent normal tissues (Figure 4C, D). MiR-155 expression was negatively correlated with FOXO3a expression in CRC patient tissues (Figure 4E). RT-qPCR experiments and Western blotting data manifested that FOXO3a expression was down-regulated after transfection of miR-155 mimic and upregulated after transfection of miR-155 inhibitor into CRC cells (Figure 4F, G).

FOXO3a inhibits the malignant phenotypes of CRC cells

FOXO3a is a tumor suppressor gene in human CRC (26). RT-qPCR (Figure 5A) and Western blotting (Figure 5B) demonstrated that FOXO3a in CRC cells transfected with FOXO3a overexpression plasmid were significantly increased. We observed that up-regulating FOXO3a inhibited the proliferative capacity of DLD1 cells (Figure 5C), as well as the migration (Figure 5D) and invasion (Figure
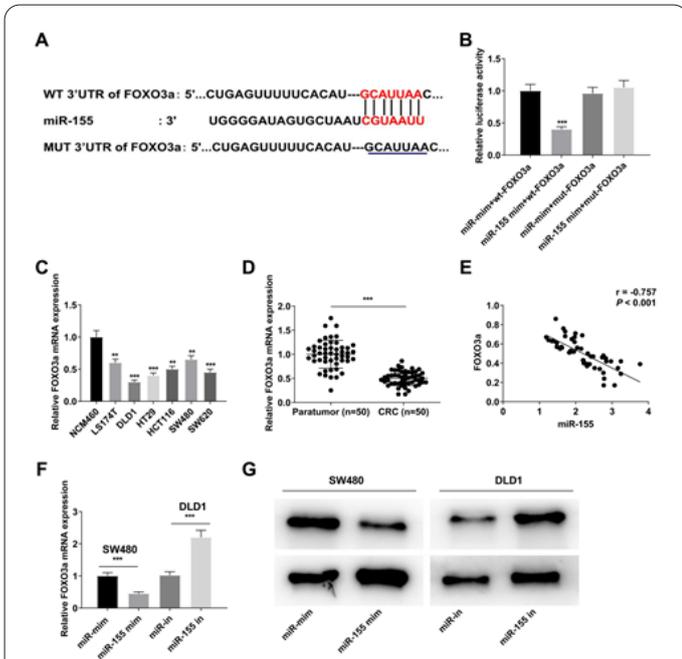
Figure 4. MiR-155 targets FOXO3a in CRC cells. A: The bioinformatics database shows that miR-155 has binding sites for FOXO3a. B: The dual luciferase reporter gene experiment shows that miR-155 has a binding relationship with FOXO3a. C: qRT-PCR was used to detect the level of FOXO3a mRNA in human colorectal epithelial cells and CRC cells. D: qRT-PCR was used to detect the expression of FOXO3a mRNA in CRC tissues and adjacent normal tissues. E: Pearson correlation analysis was used to analyze the relationship between the expression of miR-155 and FOXO3a in CRC tissues. F-G: After transfection of miR-155 mimic and inhibitor, qRT-PCR and western blot were used to detect the mRNA and protein expression of FOXO3a in CRC cells. *** $P<0.001$.

Figure 5. High expression of FOXO3a inhibits the proliferation, migration and invasion of CRC cells. A: After overexpression of FOXO3a, qRT-PCR was used to detect the expression of FOXO3a mRNA in DLD1 cells. B: After overexpressing FOXO3a, a western blot was used to detect FOXO3a expression in DLD1 cells. C: After overexpression of FOXO3a, the proliferation level of DLD1 cells was detected by the MTT assays. D: After FOXO3a overexpression, the scratch healing test was used to detect the migration level of DLD1 cells. E: After overexpression of FOXO3a, the Transwell assay was used to detect the invasion level of DLD1 cells. ** $P<0.01$, ***$P<0.001$. (Compared with the pLV-vector group).

5E) abilities of DLD1 cells.

FOXO3a overexpression reverses the effect of miR-155 on CRC

To explore whether miR-155 regulated CRC cell malignancy in a FOXO3a-dependent manner, miR-155 inhibitor and sh-FOXO3a were co-transfected into DLD1 cells with cells transfected with miR-NC used as controls. RT-qPCR (Figure 6A) and Western blotting analysis (Figure 6B) suggested that co-transfection with miR-155 inhibitor and sh-FOXO3a down-regulated FOXO3a in DLD1 cells compared with the miR-155 in+ sh-NC group. The experimental results of MTT (Figure 6C), wound healing (Figure 6D) and Transwell invasion (Figure 6E) showed that FOXO3a silencing reversed the suppressive effect of downregulating miR-155 on the malignant phenotypes of CRC cells.

Discussion

CRC is a global threat to health that causes massive deaths worldwide (27). According to reports, among all patients diagnosed with CRC in the United States from 2001 to 2007, the 5-year survival rate of patients with carcinoma in situ was 90.1%, that of patients with regional lymph node metastasis was 69.2%, and that of patients with distant metastasis was 11.7%. Due to the low rate of endoscopy in patients with gastrointestinal diseases in my country, many patients are diagnosed at the advanced stage of CRC (28). At this stage, CRC treatment is still based on
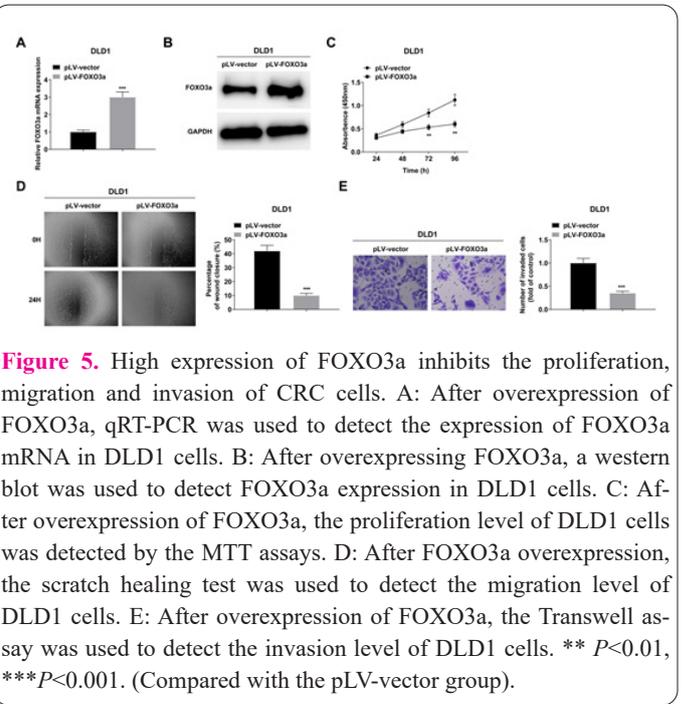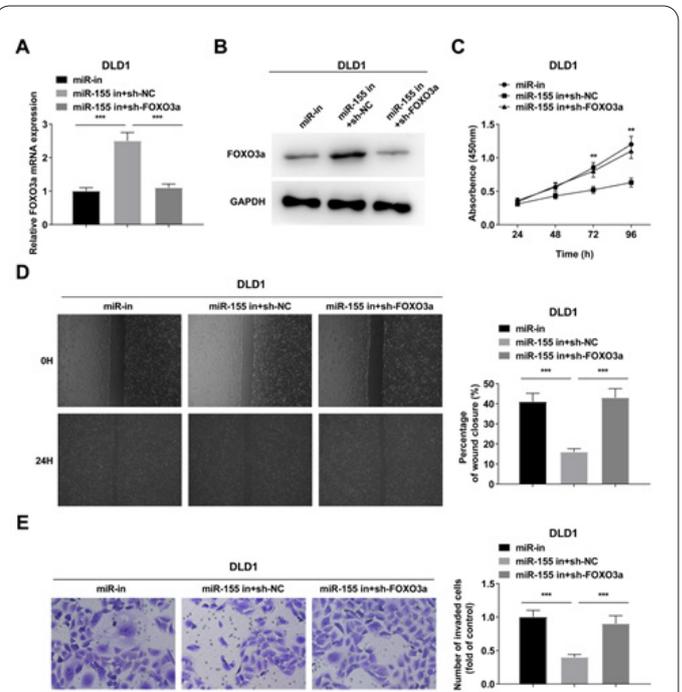

Figure 6. MiR-155 affects the progress of CRC by regulating FOXO3a. A: After co-transfection of miR-155 inhibitor and sh-FOXO3a, qRT-PCR was used to detect the expression of FOXO3a mRNA in DLD1 cells. B: After co-transfection of miR-155 inhibitor and sh-FOXO3a, a western blot was used to detect the expression of FOXO3a in DLD1 cells. C: After co-transfection of miR-155 inhibitor and sh-FOXO3a, the proliferation of DLD1 cells was detected by the MTT assays. D: After co-transfection of miR-155 inhibitor and sh-FOXO3a, the scratch healing test was used to detect the migration of DLD1 cells. E: After co-transfection of miR-155 inhibitor and sh-FOXO3a, the transwell experiment was used to detect the invasion of DLD1 cells. ** $P<0.01$, ***$P<0.001$.

surgery, supplemented by chemotherapy and radiotherapy. The biological behavior of tumors is increasingly recognized to affect the clinical treatment plan and the judgment of the prognosis (29). Therefore, it is of great significance to identify biomarkers with prognosis predictive values and explore their mechanisms. In this study, we revealed

the upregulation of miR-155 in CRC, and that induction of miR-155 encouraged CRC cell proliferative capacity, migration and invasion while suppressing miR-155 had the opposite effect. In terms of mechanism, miR-155 acted on CRC cells by targeting FOXO3a.

miRNAs are important regulators of cell proliferation, apoptosis, and differentiation (30). The role of miRNA in human disease originally emerged from high-throughput and functional studies of cancer cells. The differences in miRNA expression reflect a single developmental lineage and transformation mechanisms. Despite the overall dysregulation of miRNAs, most miRNAs are suppressed in cancer tissues compared with normal tissues, indicating a general loss of tumor cell differentiation (31, 32). Therefore, miRNAs abnormal expression in tumors and their mechanism are closely related to disease development. This study noted the overexpressed miR-155 in CRC, and up-regulating miR-155 can promote the proliferative capacity, migration and invasion of CRC cells.

So far, more than 1400 miRNAs have been found in the human body, and these genes account for approximately 1%-3% of the human genome. 30-60% of protein-coding genes are regulated by miRNA (33), and the interaction between miRNAs and their target proteins has attracted great attention in tumor development (34-36). FOXO3a is involved in tumorigenesis and tumor suppression (37), and its location and phosphorylation status are prognostic factors for ovarian cancer, prostate cancer, and bladder cancer (38-42). FOXO3a may be a direct target of miR-155 (43), which is consistent with the finding of our study. FOXO3a reversed the effects of miR-155 on CRC cells.

However, this study still has some shortcomings. Other downstream targets of miR-155 need to be further screened and verified. FOXO3a is reported to be involved in the inactivation of several oncogenic pathways (25), and the effects of the miR-155/FOXO3a on downstream pathways in CRC requires further analysis, as well as the influence of miR-155 on CRC cell apoptosis. It is of great significance to further investigate the effect of miR-155 on CRC, and its value in nude mouse tumors needs to be further explored. In summary, this study confirmed that miR-155 promotes CRC cell proliferative capacity, migration and invasion via targeted negative regulation of FOXO3a.

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Interest conflict
The authors declare that they have no conflict of interest.

Consent for publications
The author read and proved the final manuscript for publication.

Availability of data and material
All data generated during this study are included in this published article

Authors' Contribution
All authors had equal roles in study design, work, statistical analysis and manuscript writing.

Ethics approval and consent to participate
The present study was approved by the Ethics Committee of Maternity and Sijing Hospital and written informed consent was provided by all patients prior to the study start. All procedures were performed in accordance with the ethical standards of the Institutional Review Board and The Declaration of Helsinki, and its later amendments or comparable ethical standards.

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