Original Research

miR-124-3p alleviated the progress of Colorectal carcinoma

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Abstract

Colorectal cancer is a common malignancy worldwide, and its incidence and mortality rates are increasing at an increasing rate. The common treatment for colorectal cancer is surgery, followed by radiotherapy and chemotherapy, but early and accurate diagnosis is a prerequisite for the treatment of the disease. Tumor signatures of colon cancer are important in the diagnosis of colon cancer. we found miR-124-3p was lower expressed in tissues of colorectal cancer, and it hampered the cell proliferation, apoptosis inhibition, migration, and invasion of colorectal cancer cell lines, and miR-124-3p overexpression hampered the tumor growth in vivo. In conclusion, miR-124-3p served as a tumor suppressor in colorectal cancer by hampered the cell proliferation, apoptosis inhibition, migration, and invasion of colorectal cancer cell lines, and miR-124-3p overexpression hampered the tumor growth in vivo. It can be a potential biomarker for early diagnosis of colorectal cancer to provide innovative ideas and methods for the diagnosis and treatment of colorectal cancer.

Keywords: miR-124-3p; Colorectal carcinoma; microRNA

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Introduction

Colorectal cancer (CRC) is the third most common cancer, with 4%-5% probability of suffering from colorectal cancer, and most lethal malignancies in the world[1, 2]. The known risk factors related with CRC included Age, family history, ethnicity, the gut microbiology, inflammatory bowel disease[3, 4]. For early-stage colon cancer, the treatment modalities mainly include endoscopic and surgical radical resection and post-operative chemotherapy, targeted therapy, and immunotherapy[5]. And for mid- to late-stage tumors, the main modalities are pre-operative neoadjuvant chemotherapy to reduce tumor stage before surgery, palliative resection and radiotherapy, targeted therapy, immunotherapy, Chinese medicine treatment and systemic therapy, etc. [6]. However, studies have shown that a significant number of patients are already at an advanced stage when they present with symptoms and require systemic treatment, and about 20% of colon cancer patients have metastases to the liver, bones, and abdominal implants at the time of diagnosis[7]. The 5-year survival rate is even lower than 20%[8]. The molecular mechanisms of colon cancer development and metastasis are therefore essential to identify new molecular therapeutic targets for colorectal cancer.

MicroRNAs (miRNAs) are small, endogenously expressed non-coding RNAs of approximately 17-25 nucleotides in length[9]. miRNAs regulate gene expression at the post-transcriptional level primarily by pairing with the 3'UTR of the target mRNA. In the last decade, miRNAs have been found to play important roles in different biological processes such as cell differentiation, proliferation, apoptosis, and metabolism[10]. A large number of miRNAs have been found to be located at frequently altered and vulnerable sites and regions in cancer, including amplification, loss of heterozygosity and breakpoint regions[11]. In addition, dysregulation of miRNA processing steps can affect carcinogenesis[12]. Several research indicated that miR-124-3p exerted as a tumor suppressor miRNA is various kind of cancer. miR-124-3p was reported inhibited the migration and invasion of gastric cancer by targeting ITGB3[13]. Zo et al. also reported that miR-124-3p could suppress the proliferation, migration, invasion, and promote apoptosis of bladder cancer cells by targeting DNMT3B[14]. However, the role of miR-124-3p was barely reported. In this manuscript, we discussed the role of tumor suppressor miR-124-3p in colorectal carcinoma.

Materials and Methods

Clinical tissue acquisition

The tumor tissues of colorectal carcinoma and corresponding para-cancerous tissues were harvested from QuJin Affiliated Hospital Medical University. All patients with primary colorectal carcinoma underwent complete resection, but without received neoadjuvant radiotherapy or chemotherapy. All patients were fully informed of the methods of use and data retrieval prior to obtaining specimens and written informed consent was obtained from each patient, this manuscript does not contain any information that might reveal the identification of any relevant patients or violate the rights of an individual.

Cell culture and plasmid transfection

Normal colonic epithelial cell line FHC and colorectal cancer cell lines SW480, SW620, LoVo, HT29, HCT116 and DLD-1 were all purchased form Pricella, China. SW480 and SW620 were cultured in Leibovitz's L-15 (PM151010, Pricella, China) contained 10% fetal bovine serum (FBS) (164210-50, Pricella, China) and 1% Penicillin/Streptomycin (PB180120, Pricella, China) in a humidified 5% CO2 at 37 °C. LoVo was cultured in Ham's F-12K medium contained 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin in a humidified 5% CO_2 at 37 °C. HT29 and HCT116 cultured in McCoy's 5A medium contained 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin in a humidified 5% CO_2 at 37 °C. DLD-1 was cultured in RPMI-1640 medium contained 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin in a humidified 5% CO_2 at 37 °C. miR-124-3p mimics and corresponding negative control was transfected to SW480 and HTC116 via Lipofectamine 2000 agent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol.

qPCR

Total RNA was extracted from cells and tissues with TRIzol reagent, and reversely transcribed by miRNA cDNA synthesis kit. RT-qPCR was carried out by SYBR green Premix Ex Taq II. The relative expression of miR-124-3p was calculated by $2^{-\Delta\Delta Ct}$ with the house keeping gene as U6. The primers were designed and conducted as the sequences showed as followed:

F: GGTAAGGCACGCGGTGAATG

R: GTGCGTGTCGTGGAGTC

CCK8 assay for cell viability

Cells were inoculated into 96-well plates for 48 h. Then cells were incubated with 10 μL CCK-8 reagent for 4 h. Absorbance at 450 nm was measured by an enzyme-labeled meter.

Flow cytometry detection of apoptosis

The transfected cells were rinsed with cold PBS twice, then resuspended with 500 μL 1 × binding buffer (containing 5 μL Annexin-FITC and 5 μL propidium iodide) and incubated for 15 min in darkness, and ratio of apoptotic cells was determined by flow cytometry.

Flow cytometry of the cell cycle

Cells were also trypsinized and harvested. After being washed using PBS, cells were stained with propidium iodide (PI) using Cycletest Plus DNA Reagent Kit (BD Biosciences) following the manufacturer's protocol, and the cell cycle distribution was analyzed by FACSVerse flow cytometer (BD Biosciences). The percentages of cells in G0⁄G1, S, and G2⁄M phases were counted and compared. The experiments were carried out in triplicate.

Plate cloning assay to detect cell clone formation

Cells suspended in medium with 10% FBS and 0.3% noble agar were placed into a 6-well plate with 0.6% noble agar and the medium was replaced every 3 days.14 days after incubation, the cell colonies were fixed by methanol and then stained with 0.1% crystal violet. The number of colonies was counted when the colonies were more than 50 cells.

Transwell invasion assay detects cellular invasion

Cells suspended in serum-free medium were

harvested and 200 μL cell suspension was inoculated into the upper chamber, and the medium included 10% FBS was added in lower chamber. 24 h after incubation, the unpenetrated cells were removed using a cotton swab. Cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for 15 min. Number of cells in 5 random fields on the membrane was counted under the microscope.

Wound-scratch assay for cell migration

A cell free zone was created by scraping with a micropipette tip. The dislodged cells were removed and cells were cultured in serum-free medium. The images were taken at 0 h and 48 h.

Xenomorphic tumor model

Cells at logarithmic growth stage were inoculated subcutaneously on the back of nude mice at 2×10^6/200 μL only and kept for 6 weeks to establish a subcutaneous tumorigenic model. The tumor volume was calculated by measuring the maximum longitude and the transverse longitude perpendicular to the tumor every 5 days, V= (long diameter × short diameter^2)/2, and the tumor growth curve was plotted. 6 weeks later, the mice were executed by cervical dislocation method, and the tumor tissues were peeled off and divided into two, one was fixed with 4% paraformaldehyde for HE staining, and one was immediately frozen in liquid nitrogen and stored at -80 ℃ for qPCR assay.

Statistical method

SPSS 20.0 was employed for data analysis. Measurement data were expressed as mean ± standard deviation (mean ± SD). Comparisons between groups were made using one-way ANOVA, and further two-way comparisons were made using Tukey's test if there were differences. The data were statistically significant when $P<0.05$.

Results

hsa-miR-124-3p was lower expressed in Colorectal carcinoma

The tumor tissues and their corresponding paracancerous tissues were harvested and the expression of hsa-miR-124-3p was detected. The result showed that the expression of hsa-miR-124-3p was significantly lower in tumor tissues then which in paracancerous tissues ($p < 0.05$, Figure 1A). Then, the expression of hsa-miR-124-3p in the normal colonic epithelial cell line FHC, colorectal cancer cell lines SW480, SW620, LoVo, HT29, HCT116 and DLD-1 were detected by qPCR ($p < 0.0001$, Figure 1B). It was found that hsa-miR-124-3p expression levels were significantly lower in all six colorectal cancer cell lines compared to FHC cell line, and SW480 and HCT116 cells, which expressed relatively lower hsa-miR-124-3p, were used in this protocol for the experiments.

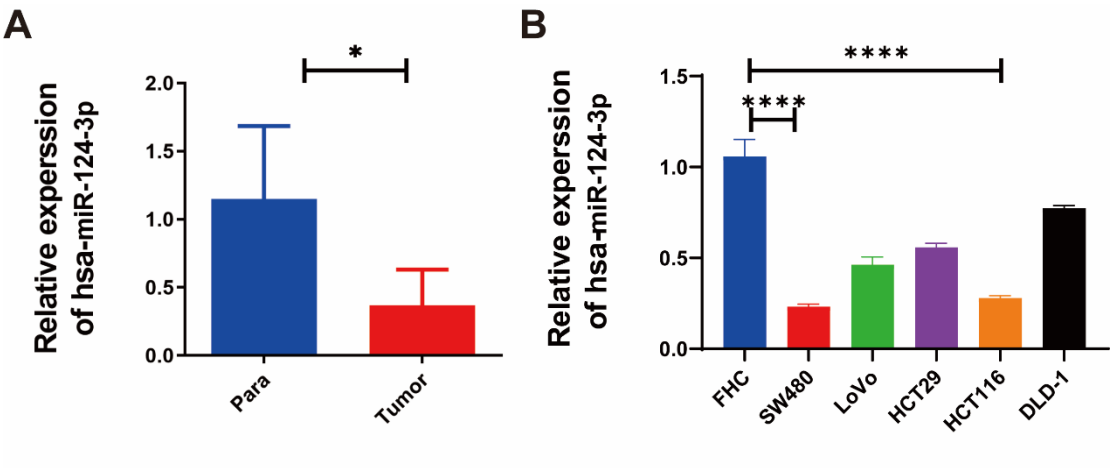

Figure 1. hsa-niR-124-3p was lower expressed in Colorectal carcinoma. (A) the expression of hsa-miR-124-3p in tumor tissues and their corresponding paracancerous tissues. (B) the expression of hsa-miR-124-3p in in the normal colonic epithelial cell line FHC, colorectal cancer cell lines SW480, SW620, LoVo, HT29, HCT116 and DLD-1 were detected by qPCR. Error bars represent SD. Lines stands for the comparison between two groups. *, $p < 0.05$; ****, $p < 0.0001$.

hsa-miR-124-3p overexpression inhibited the cell proliferation of colorectal cancer cell lines

The expression of hsa-miR-124-3p was increased by the transfection of miR-124-3p mimics. The transfection rate was detected by qPCR (Figure 2A-B). After transfection of miR-124-3p mimics, the expression of hsa-miR-124-3p was increased significantly, which indicated the successful transfection of hsa-miR-124-3p mimics. The cell morphology was observed (Figure 2E). The SW480 and HTC116 in the control and negative control group were clearly delineated, mostly triangular and round-like, and in some areas the cells were indistinctly fused, with tumor giant cells, surrounded by more fibroblast-like cells, scattered, or connected to each other. After transfection of miR-124-3p mimics, the cells were rounded in shape, refractive, crumpled or dislodged and float in the medium. Also, the number of floating cells increased, the walled growth became sparse, the cytoplasm became more granular and the cell membrane was blister. Then the cell proliferation was detected by CCK-8 (Figure 2C-D) and cell colony assay (Figure 2F). The result showed that after transfected with hsa-miR-124-3p mimics, cell viability and the number of colonies was decreased compared with control group.

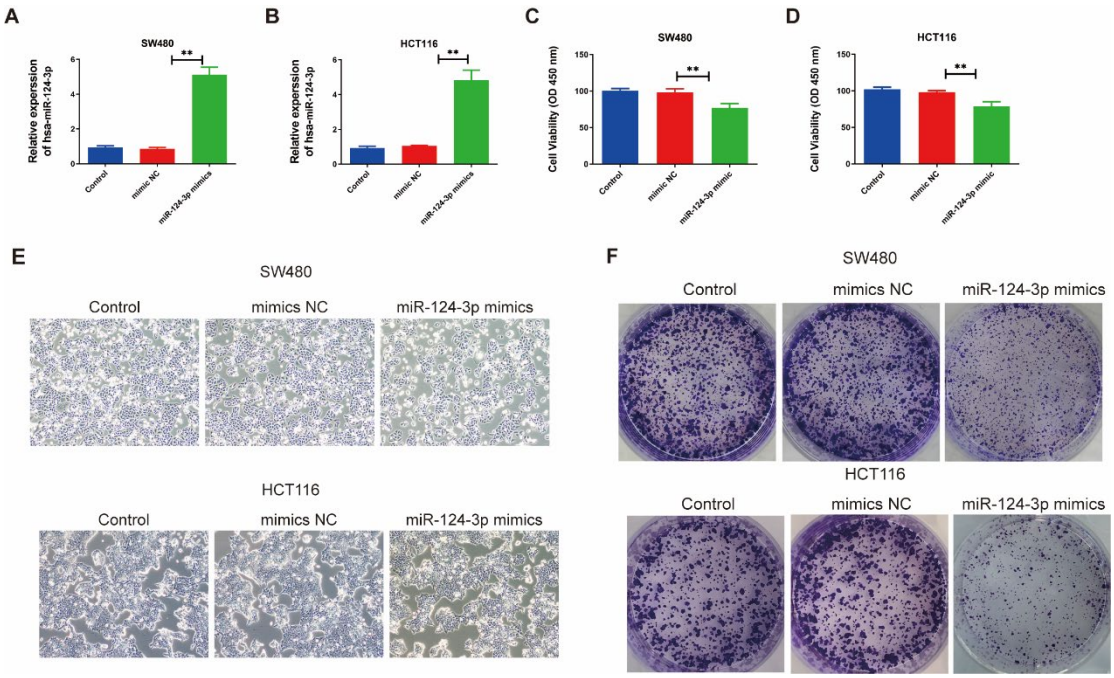

Figure 2. hsa-miR-124-3p overexpression inhibited the cell proliferation of colorectal cancer cell lines. (A-B) The transfection rate was detected by qPCR. (C-D) The cell proliferation was detected by CCK-8. (E)The cell morphology was observed. (F) The cell proliferation was cell colony assay. Error bars represent SD. Lines stands for the comparison between two groups. **, $p < 0.01$.

hsa-miR-124-3p overexpression aggravated the cell apoptosis of colorectal cancer cell lines

The cell apoptosis was detected by flow cytometer (Figure 3). The result showed that after transfected with hsa-miR-124-3p mimics, cell apoptosis rate was increased compared with control group.

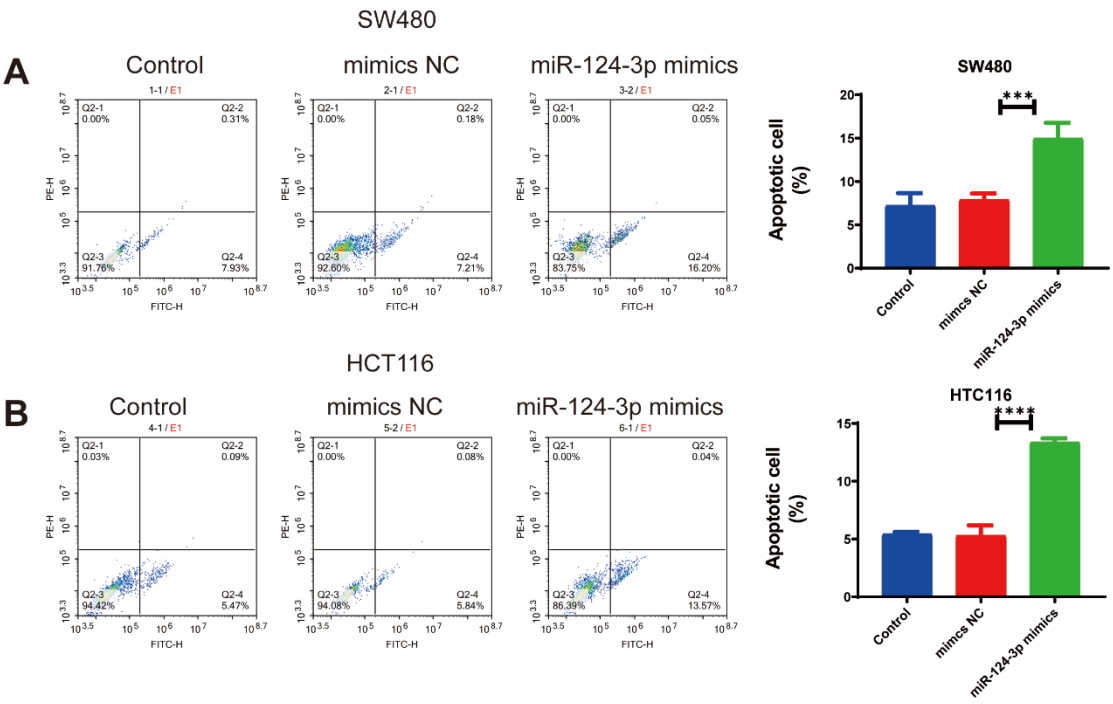

Figure 3. hsa-miR-124-3p overexpression aggravated the cell apoptosis of colorectal cancer cell lines. Error bars represent SD. Lines stands for the comparison between two groups. ***, $p < 0.001$; ****, $p < 0.0001$.

hsa-miR-124-3p overexpression hampered the cell cycle of colorectal cancer cell lines

The ratio of accumulated cells in each phase was detected by flow cytometer (Figure 4). The result showed that the ratio of colorectal cancer cells in G0/G1 phase was increased while the ratio of cells in G2/M phase was decreased after transfected with hsa-miR-124-3p mimics.

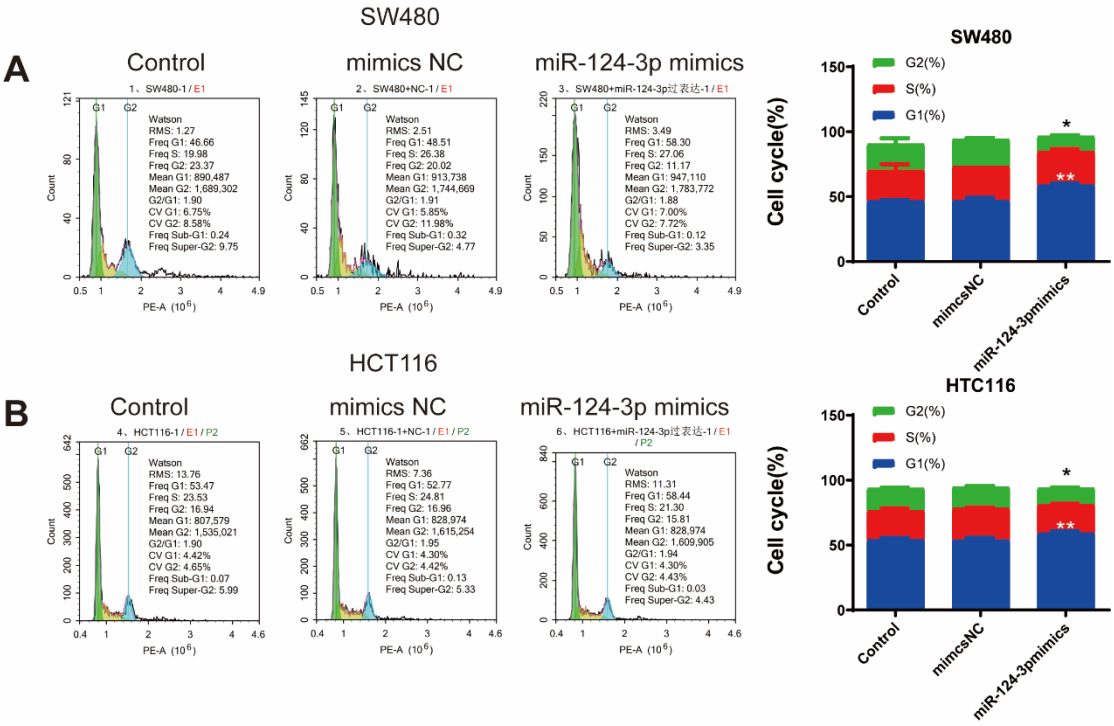

Figure 4. hsa-miR-124-3p overexpression hampered the cell cycle of colorectal cancer cell lines. Error bars represent SD. Lines stands for the comparison between two groups. *, $p < 0.05$; **, $p < 0.01$.

hsa-miR-124-3p overexpression hampered the cell migration and invasion of colorectal cancer cell lines

The cell migration was detected by wound healing assay (Figure 5). The result showed that after transfected with hsa-miR-124-3p mimics, migration index was decreased compared with control group. Also, cell invasion was detected by Transwell chambers (Figure 6). The result showed that after transfected with hsa-miR-124-3p mimics, the number of invasion cells was decreased compared with control group.

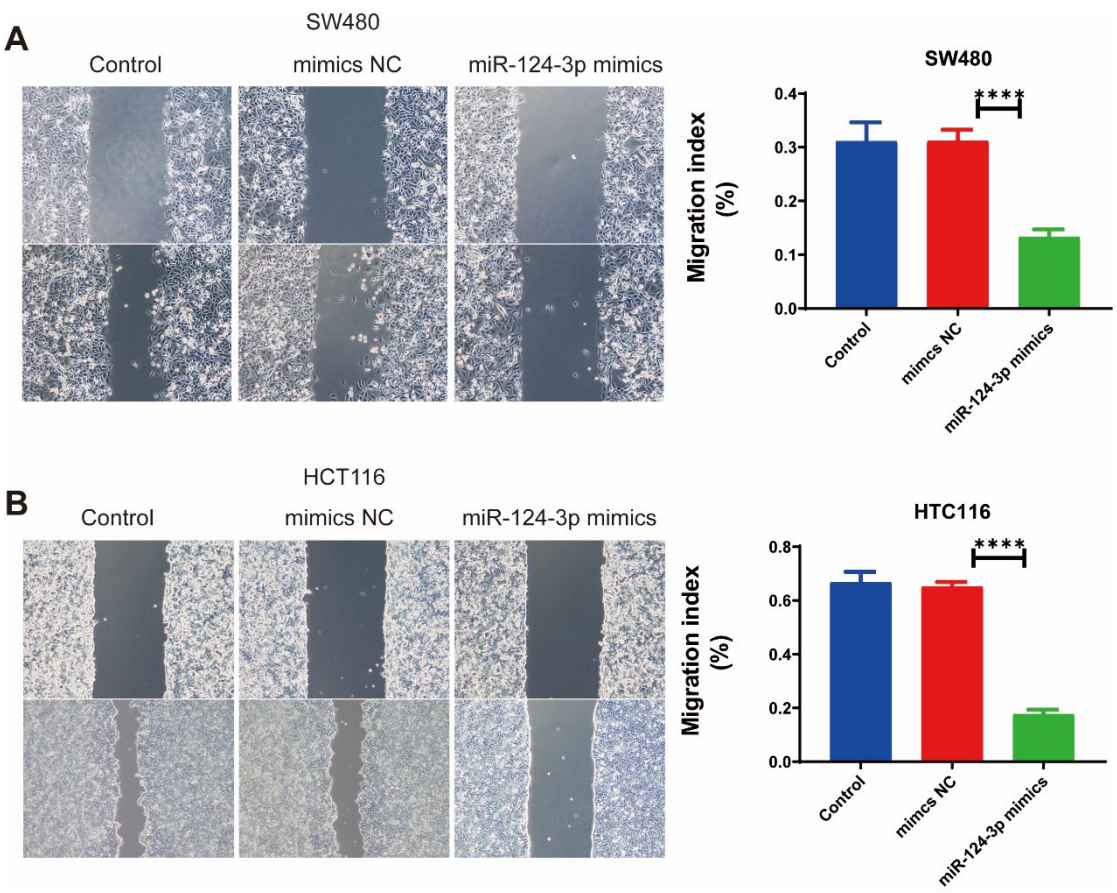

Figure 5. The cell migration was detected by wound healing assay. Error bars represent SD. Lines stands for the comparison between two groups. ****, $p < 0.0001$.

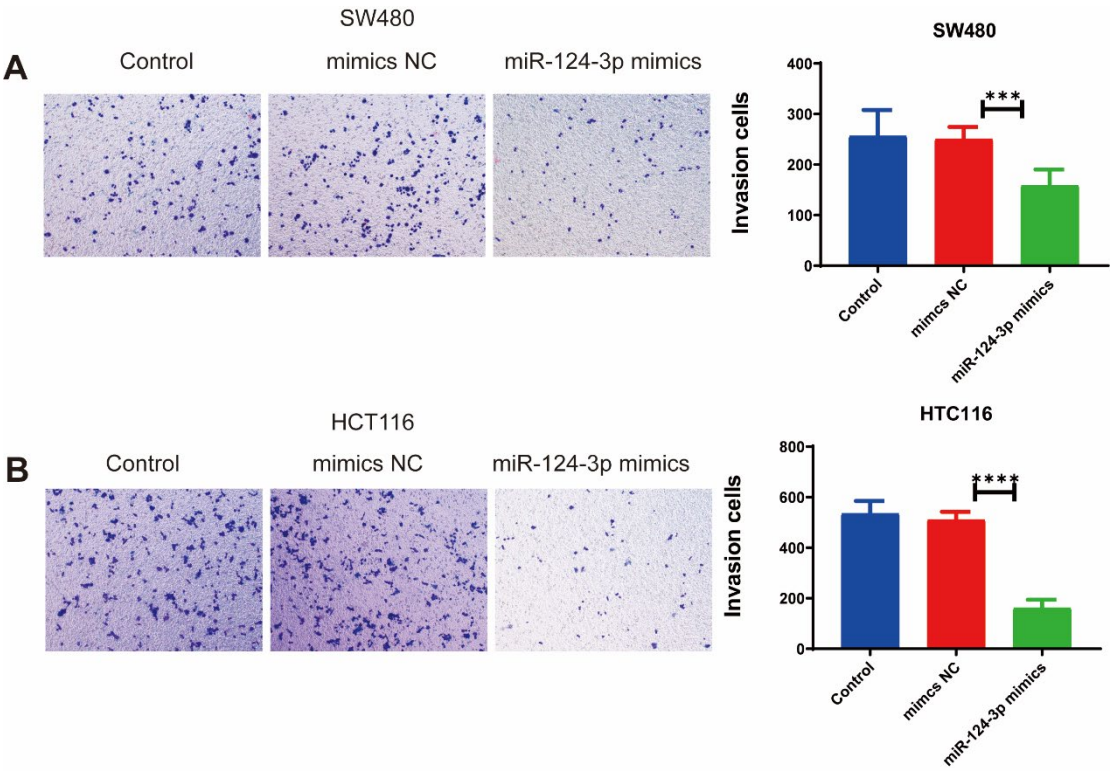

Figure 6. The cell invasion was detected by Transwell chambers. Error bars represent SD. Lines stands for the comparison between two groups. ***, $p < 0.001$; ****, $p < 0.0001$.

Figure 6. The cell invasion was detected by Transwell chambers. Error bars represent SD. Lines stands for the comparison between two groups. ***, p < 0.001; ****, p < 0.0001.

hsa-miR-124-3p overexpression hampered the tumor growth *in vivo*

To verify the inhibition of miR-124-3p on tumor growth, the SW480 and SW480 transfected with miR-124-3p mimics was injected subcutaneously on the back of nude mice. The expression of miR-124-3p was increased when SW480 transfected with miR-124-3p mimics was injected (Figure 7A). The tumor volume was measured each 6 days, the results showed that when miR-124-3p was overexpressed in tumor tissues, the tumor grew slower and the tumor volume was decreased (Figure 7B). Further observation by HE staining showed that the tumor tissue in the control group had small necrotic areas with vigorous tumor cell growth, obvious nuclear heterogeneity, and abundant interstitial blood vessels, whereas the tumor sections in the experimental group had significantly larger necrotic areas, few interstitial blood vessels and visible fibrosis formation, and the tumor cell growth was significantly inhibited (Figure 7C). These results indicated that hsa-miR-124-3p overexpression hampered the tumor growth in vivo.

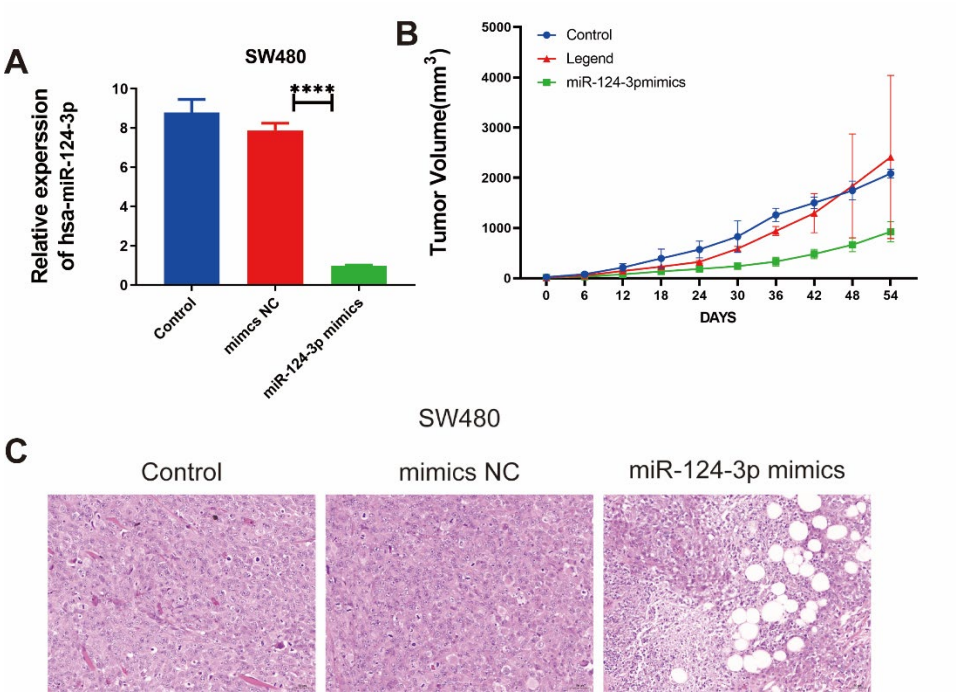

Figure 7. hsa-miR-124-3p overexpression hampered the tumor growth in vivo. (A) The expression of miR-124-3p in tumor tissues was detected by qPCR. (B) The tumor volume was measured each 6 days. (C) HE staining was used to observe histopathological changes in the tumor. Error bars represent SD. Lines stands for the comparison between two groups. ****, $p < 0.0001$.

Discussion

Colorectal cancer is a common malignancy worldwide, and its incidence and mortality rates are increasing at an increasing rate[15]. The common treatment for colorectal cancer is surgery, followed by radiotherapy and chemotherapy, but early and accurate diagnosis is a prerequisite for the treatment of the disease[16]. Tumor signatures of colon cancer are important in the diagnosis of colon cancer. Tumor signatures are substances that are produced on the surface of tumor cells or cell membranes, or by the immune response to tumors, and are secreted or released into the body fluids or tissues[17]. Tumor signatures have important reference value in the early diagnosis, formulation of treatment plan, prognosis assessment and recurrence monitoring of tumor patients[18]. Therefore, finding effective tumor signatures is an important way to improve the survival rate of colorectal cancer patients.

Initially, miRNA is transcribed into long primary miRNAs (pri-miRNAs) by RNA polymerase II (Pol II) or III (Pol III) in nucleus[19]. A pri-miRNA typically consists of one to six hairpins, each containing approximately 70 nt of nucleotides[20]. The double-stranded hairpins are then cleaved and released from the pri-miRNA by a microprocessor complex consisting of DGCR8 and DroshaA[21]. The product, called precursor miRNA (pre-miRNA), is exported from the nucleus to the cytoplasm and is modified by another RNase III endonuclease, Dicer, into a 22-nt miRNA double strand[22]. Finally, one strand of the pre-miRNA is

degraded while the other becomes a mature functional miRNA. These mature miRNAs may bind to the RNA-induced silencing complex (RISC) and cleave the target mRNA or inhibit protein translation[23]. The deprivation of miR-124-3p was found promoted the cell proliferation and metastasis by targeting MGAT5 in breast cancer[24]. miR-124-3p was also reported as a potential marker and suppressed the tumor growth in gastric cancer[25]. The combination of downregulation of ABCC4 with overexpression of miR-124-3p significantly increased sensitivity to adriamycin in breast cancer[26]. Similar, we found miR-124-3p was lower expressed in tissues of colorectal cancer, and it hampered the cell proliferation, apoptosis inhibition, migration, and invasion of colorectal cancer cell lines, and miR-124-3p overexpression hampered the tumor growth in vivo.

In conclusion, miR-124-3p served as a tumor suppressor in colorectal cancer by hampered the cell proliferation, apoptosis inhibition, migration, and invasion of colorectal cancer cell lines, and miR-124-3p overexpression hampered the tumor growth in vivo. It can be a potential biomarker for early diagnosis of colorectal cancer to provide innovative ideas and methods for the diagnosis and treatment of colorectal cancer.

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