Original Research

miR-124-3p alleviated the metastasis of Colorectal carcinoma through FOXQ1/ERK pathway

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Abstract

Colorectal carcinoma (CRC) is currently one of the most common malignancies, accounting for approximately 10% of all new cases diagnosed worldwide each year. FOXQ1 regulates metabolic homeostasis, promotes epithelial cell differentiation, inhibits smooth muscle differentiation, mediates skin follicle development, and exerts immune functions against related diseases. However, few research has focus the relationship between miRNAs and FOXQ1 in CRC. In this manuscript, we investigated the mechanism of miR-124-3p on cell proliferation, apoptosis inhabitation, migration, and invasion by targeting FOXQ1/ERK pathway. SW480 was used for research, and hsa-miR-124-3p mimics and pcDNA-FOXQ1 and their negative control were transfected into SW480 cells. Cell viability was detected by CCK-8 and cell colony assay, apoptosis rate and cell cycle were detected by flow cytometry, migration index and invasion number were detected by wound healing assay and Transwell assay. The expression of FOXQ1 in tumor tissues and corresponding para tumor tissues was detected by qPCR and IHC. The expression of FOXQ1 and the activation of ERK/1/2 in SW480 were detected by western blot. The results showed that FOXQ1 was highly expressed in colorectal carcinoma tissues. AS a directly target of hsa-miR-124-3p, hsa-miR-124-3p hampered the cell proliferation, apoptosis inhibition, cell cycle process, cell migration and invasion, and tumor growth through FOXQ1. Also, hsa-miR-124-3p overexpression regulated ERK pathway by targeting FOXQ1. In conclusion, we found that miR-124-3p regulated cell proliferation, apoptosis inhibition, migration, invasion and tumor growth by targeting FOXQ1/ERK pathway. These results may indicate that FOXQ1 can be a potential target of early diagnosis and treatment of colorectal carcinoma.

Keywords: FOXQ1; Colorectal carcinoma; miR-124-3p; microRNA

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Introduction

Clorectal carcinoma (CRC) is currently one of the most common malignancies, accounting for approximately 10% of all new cases diagnosed worldwide each year[1]. Colorectal cancer is associated with a number of factors, with 10-20% of colorectal cancer patients having a family history of the disease Poor lifestyle habits, such as smoking, alcohol abuse and excessive obesity, can also contribute to an increased risk of colorectal cancer[2]. Clinically, patients with colorectal cancer usually present with rectal bleeding, changes in bowel habits, anaemia and abdominal pain. However, for most patients, symptoms usually only appear when the disease has reached an advanced stage[3, 4]. Thanks to effective screening, early intervention and optimal treatment strategies, the mortality rate from colorectal cancer has declined by 35% in recent decades. However, the prognosis for patients with advanced stage colorectal cancer remains poor and the incidence in developing countries and regions increases with the level of regional development increases[5, 6]. Therefore, it is still an urgent to investigate the molecular mechanism of colorectal carcinoma and find out the biomarkers to assist the early diagnose and treatment of colorectal carcinoma clinically.
The Forkhead box (FOX) family is a class of DNA-binding domain transcription factors with a unique helical winged structure, first identified in Drosophila[7-9]. This family of proteins is involved in a variety of important biological processes, such as DNA repair and embryonic development, and the maintenance of metabolic homeostasis in the body[10, 11]. FOXQ1 regulates metabolic homeostasis, promotes epithelial cell differentiation, inhibits smooth muscle differentiation, mediates skin follicle development, and exerts immune functions against related diseases[12]. Research showed that FOXQ1-mediated SIRT1 upregulation augments expression and nuclear translocation of β-catenin and benefits CRC-related intestinal pathological bacterial, thereby enhancing the stemness and radio-resistance of CRC cells[13]. Chen et. al. found that TAMs induce EMT program to enhance CRC migration, invasion, and CTC-mediated metastasis by regulating the JAK2/STAT3/miR-506-3p/FoxQ1 axis[14]. However, few research has focus the relationship between miRNAs and FOXQ1 in CRC.
In this manuscript, we investigated the mechanism of miR-124-3p on cell proliferation, apoptosis inhabitation, migration, and invasion by targeting FOXQ1/ERK pathway. SW480 was used for research, and hsa-miR-124-3p mimics and pcDNA-FOXQ1 and their negative control were transfected into SW480 cells. Cell viability, apoptosis rate, cell cycle, migration index and invasion number were detected.

Materials and methods

Clinical tissue collection

Tumor tissues and corresponding para tumor tissues were collected from QuJin Affiliated Hospital Medical University. All patients were diagnosed as primary colorectal carcinoma underwent complete resection but without 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.

Immunohistochemistry

Paraffin sections were dewaxed in xylene for 15 min and then hydrated in different concentrations of ethanol (100%, 95%, 70%) and rinsed 3 times in PBS (pH 7.4) for 5 min each. Sections were dipped in citrate buffer, heated in the microwave to boiling and then cooled at room temperature for 20-30 min. The sections were stained with primary antibody to FOXQ1 (isotype: IgG, 1:400, ab51340, abcam, UK). Then, slides were incubated with appropriate secondary antibody at 37℃ for 30 min using EnVisionTM+ Dual Link System-HRP (Dako North America, Inc. Carpinteria, CA 93013, USA). DAB (3, 3-diaminobenzidine) and chromogen solution was used for color reaction enhancement. Images were acquired by laser scanning confocal microscopy (LSM710, Carl Zeiss, Germany).

qPCR

Total RNAs were extracted from cells and tissues using TRIzol regent. Reverse transcription was performed using reverse transcriptase kit (Promega, WI, USA) and random primers (Promega) for mRNA. Real-time PCR reaction were performed with SYBR Green qPCR Super Mix-UDG reagent (Invitrogen). GAPDH was used as the normalization control. The relative expression of FOXQ1 was calculated by $2^{-\Delta\Delta Ct}$. The primers were designed and conducted as the sequences showed as followed:

FOXQ1-F 5'-TCTCCATCAAACGTGCCTT-3'

FOXQ1-R 5'-GCAGGCTTCGCAAAGAAACT-3'

Cell culture and transfection

Colorectal cancer cell line SW480 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% CO_2 at 37 °C. miR-124-3p mimics and pcDNA-FOXQ1 and their corresponding negative control was transfected to SW480 via Lipofectamine 2000 agent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol.

Dual luciferase assay

The 3'-UTR sequences of FOXQ1 and the binding sites to miR-124-3p were amplified from the genomic DNA and sub-cloned into the psi-CHECK2 (Promega) to construct the wild type or mutate FOXQ1 plasmids. HEK293T were co-transfected with wild type or mutate FOXQ1 plasmids and miR-124-3p mimics and its negative control. Renilla luciferase reporter vector pRL-SV40 (Promega) was referred as an internal transfection control. Luciferase activities were determined using Luciferase Reporter Assay kit (Promega).

RNA fluorescence in situ hybridization assay

Cells were fixed by 4% paraformaldehyde for 30 min and permeabilized with proteinase K (1:200) for 5 min. Then the cells were incubated with the Cy3-labelled FOXQ1 probes and FAM-labelled miR-124-3p (Sandon biotech, China) for 48 h at 37 ◦C using miRNA Hybridization Buffer (FOCOFISH, China). DAPI (Sigma-Aldrich, USA) was used to stain the nuclei before cells were sealed onto the glass slides.

Western blot

Cells were lysed using RIPA buffer, including a proteases inhibitor cocktail inhibitor (Thermo Scientific, USA). The proteins were separated by SDS-PAGE gels and transferred to PVDF membranes (Millipore, USA). The membranes

were incubated with FOXQ1 (sb51340, 1:400, abcam), ERK1/2 (4695, 1:1000, CST), p-ERK1/2 (4370, 1:1000, CST), GAPDH (P30008M, 1:2000, abmart). Diluted secondary antibody (horseradish peroxidase-labeled IgG, 1:25,000) was added at room temperature for 2 h. ECL chemiluminescence reaction solution was added dropwise for visualized development.

Detection of Cell viability

For CCK-8 assay, SW480 cells were incubated with 10 μL CCK-8 work fluid for 1 h and absorbance at 450 nm was measured with a microplate reader (Tecan, Switzerland). For colony formation assay, 500 cells were planted in 6-well plates and cultured for 2 weeks. Cells were the fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. The experiments were carried out in triplicate.

Detection of cell apoptosis and cell cycle

For detection of cell 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. For detection of 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.

Cell migration and invasion

Cells was incubated into 24-well plates, and a zero-cell zone was made by dragging a plastic pipette tip across the cell surface. The remaining cells were washed three times in PBS to remove cellular debris and incubated with serum-free medium. The image was photographed after 24 h, 36 h., 48 h and 60 h respectively. Cell invasion assay was performed using 24-well Transwell (8 μm pore size, Coming, USA) pro-coated with Matrigel (BD biosciences, USA). $1x10^5$ cells were suspended in Leibovitz's L-15 medium containing 1% FBS and added to the upper chamber, Leibovitz's L-15 medium with 10% FBS was placed in the lower chamber. After 48 h, Matrigel and the cells remaining in the upper chamber were removed. Cells on the lower chamber were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. The experiments were carried out in triplicate.

Xenomorphic tumor model

Six-week-old female BALB/c nude mice were subcutaneously injected into flank of mice with SW480 cells or cells transfected with miR-124-3p mimics or its negative control. Thirty days after cell injection, the mice were euthanized and tumor tissues were harvested.

Statistical analysis

The Kolmogorov-Smirnov test for normality of the data was performed by Graph-Pad Prism 8.0 software (GraphPad Software, San Diego, CA). Normally distributed continuous variables were expressed as mean ± standard deviation (mean ± SD) and statistically analysed by Graph-Pad Prism 8.3 software. Comparisons between groups were made using one-way ANOVA, with further two-way comparisons tested by Tukey's if there were differences, and data from non-normal distributions were expressed as medians (interquartile spacing, Q1-Q3) and subjected to Kruskal-Wallis rank sum tests by Graph-Pad Prism 8.3 software, with further two-way comparisons tested by Dunn's test for two-by-two comparisons, with $P<0.05$ being

considered a statistically significant difference.

Results

FOXQ1 was highly expressed in colorectal carcinoma tissues

To detect the expression of FOXQ1 in colorectal carcinoma, we first collected the tumor tissues and corresponding para carcinoma tissues from the patients diagnosed as primary colorectal carcinoma and underwent surgical radical resection. The result of immunohistochemistry showed that FOXQ1 was located in cytoplasm and nucleus and positively expressed in colorectal carcinoma tissues. The result of qPCR also showed that the mRNA expression of FOXQ1 was higher expressed in tumor tissues. Then we verified the FOXQ1 expression in multiple cancer in TIMER (ver. 2.0). The result showed that FOXQ1 was differentially expressed in 18 out of 24 types of cancers, including COAD. Also, the expression of FOXQ1 was found highly expressed in cancer samples from TCGA-COAD database. These results showed that FOXQ1 was highly expressed in colorectal carcinoma tissues.

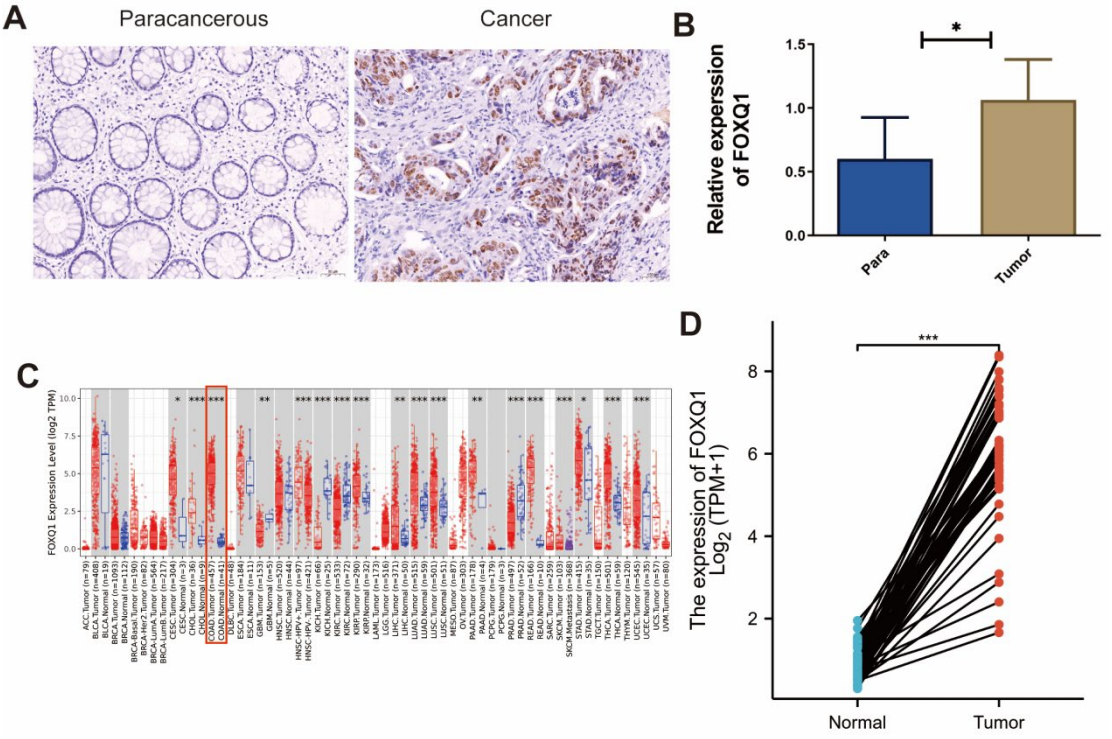

Figure 1 FOXQ1 was highly expressed in colorectal carcinoma tissues. (A) Immunohistochemistry was used to detect the expression of FOXQ1; (B) The mRNA expression of FOXQ1 was used to detect the mRNA expression of FOXQ1; (C) the expression of FOXQ1 in multiple cancers was detected by TIMER (ver. 2.0); (D) the expression of FOXQ1 in paired para-cancer and cancer samples from TCGA database. Error bars represent SD. Lines stands for the comparison between two groups. *, $p < 0.05$; ***, $p < 0.001$.

FOXQ1 was a target of hsa-miR-124-3p

In previous study, we found hsa-miR-124-3p was lower expressed in colorectal carcinoma and exerted as a tumor suppressor. Therefore, the targeted site of hsa-miR-124-3p with FOXQ1 were predicted via ENCORI (https://starbase.sysu.edu.cn/ago ClipRNA.

php?source=mRNA) and dual luciferase assay was conducted to verify the bind of miR-124-3p with FOXQ1. FOXQ1 had an 8-base complementary pairing with miR-124-3p as a potential binding site. Therefore, WT-FOXQ1 and MUT-FOXQ1 plasmids were constructed (Figure 2A). There was no clear distinction in relative luciferase activity after co-transfection of MUT-FOXQ1 plasmid with miR-NC and miR-124-3p mimic, respectively, while compared to NC, the relative luciferase activity was decreased after co- transfection of miR-124-3p mimic with WT-FOXQ1 (Figure 2B). Also, the result of FISH assay showed was FOXQ1 and miR-124-3p were both located in cytoplasm and nucleus (Figure 2C), also the overexpression of miR-124-3p decreased the expression of FOXQ1. These results showed that FOXQ1 directly bind with FOXQ1.

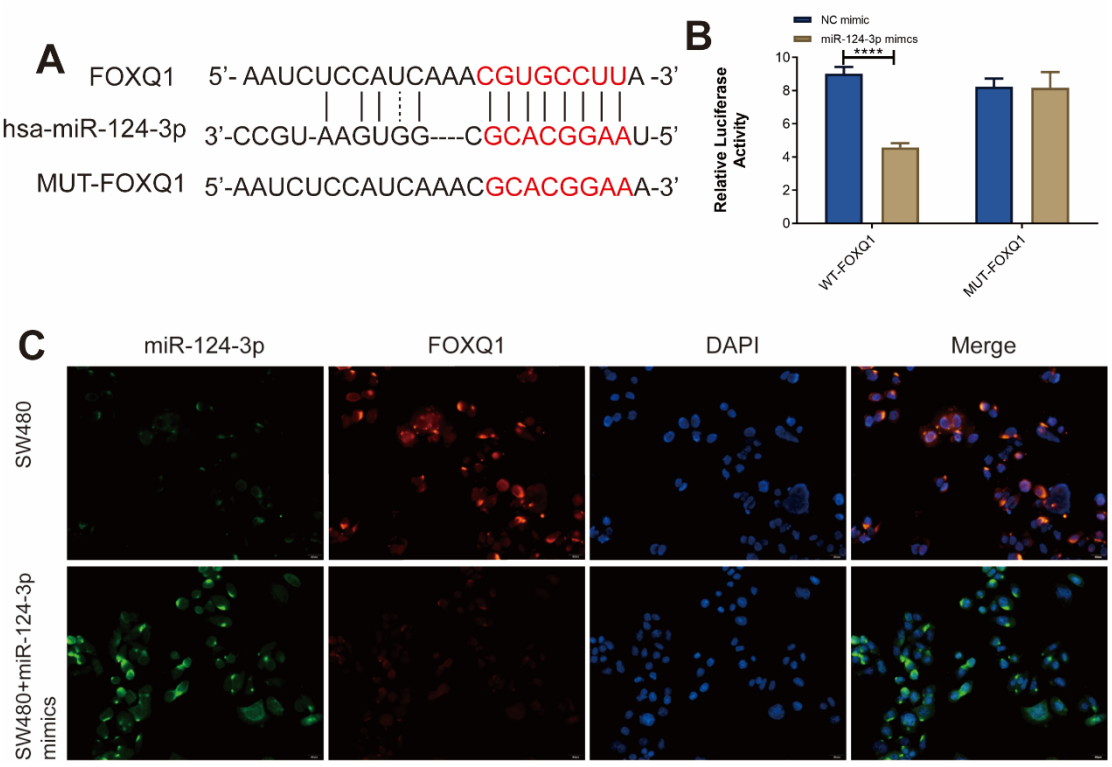

Figure 2 FOXQ1 was a target of hsa-miR-124-3p. (A) Database prediction of hsa-miR-124-3p targeting sites with FOXQ1; (B) validation of the targeting relationship by dual luciferase. (C)Fish assay was used to locate the subcellular location of FOXQ1 and miR-124-3p. ****, $p < 0.0001$.

hsa-miR-124-3p hampered the cell proliferation of CRC cell line through FOXQ1

To further investigate the mechanism of hsa-miR-124-3p on cell proliferation of CRC cell line, the miR-124-3p mimics and pcDNA-FOXQ1 and corresponding negative control was transfected into SW480. When miR-124-3p was transfected into SW480, the mRNA and protein expression of FOXQ1 was decreased, while when miR-124-3p mimics and pcDNA-FOXQ1 was co-transfected into SW480, the mRNA and protein expression of FOXQ1 was reversely increased. These indicated the successful transfection of miR-124-3p mimics and pcDNA-FOXQ1, also, miR-124-3p can negatively regulate the expression of FOXQ1 (Figure 3).

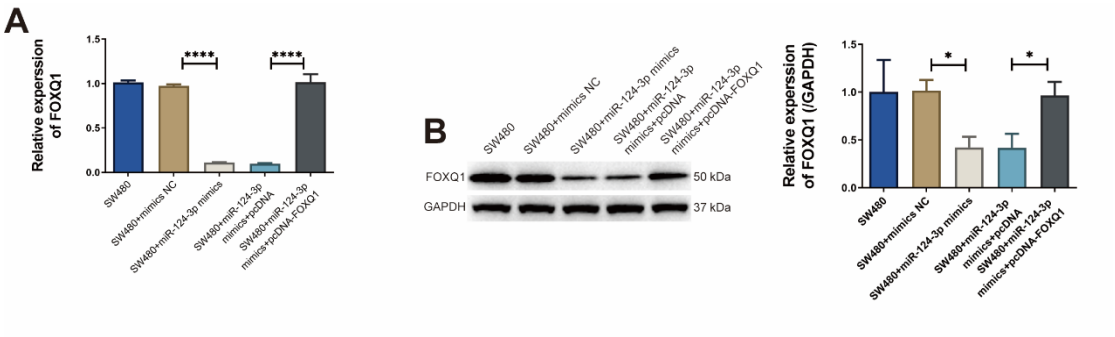

Figure 3 The transfection rate was detected by qPCR(A) and western blot (B). Error bars represent SD. Lines stands for the comparison between two groups. *, $p < 0.05$, ****, $p < 0.0001$.

The result of CCK8 showed that when miR-124-3p was overexpressed in SW480, the cell viability was decreased, while the transfection of pcDNA-FOXQ1 reversely increased the result. Also, the number of cell colonies was decreased when miR-124-3p was overexpressed, while the transfection of pcDNA-FOXQ1 reversely increased the result (Figure 4 A-C). These results showed that hsa-miR-124-3p hampered the cell proliferation of CRC cell line through FOXQ1.

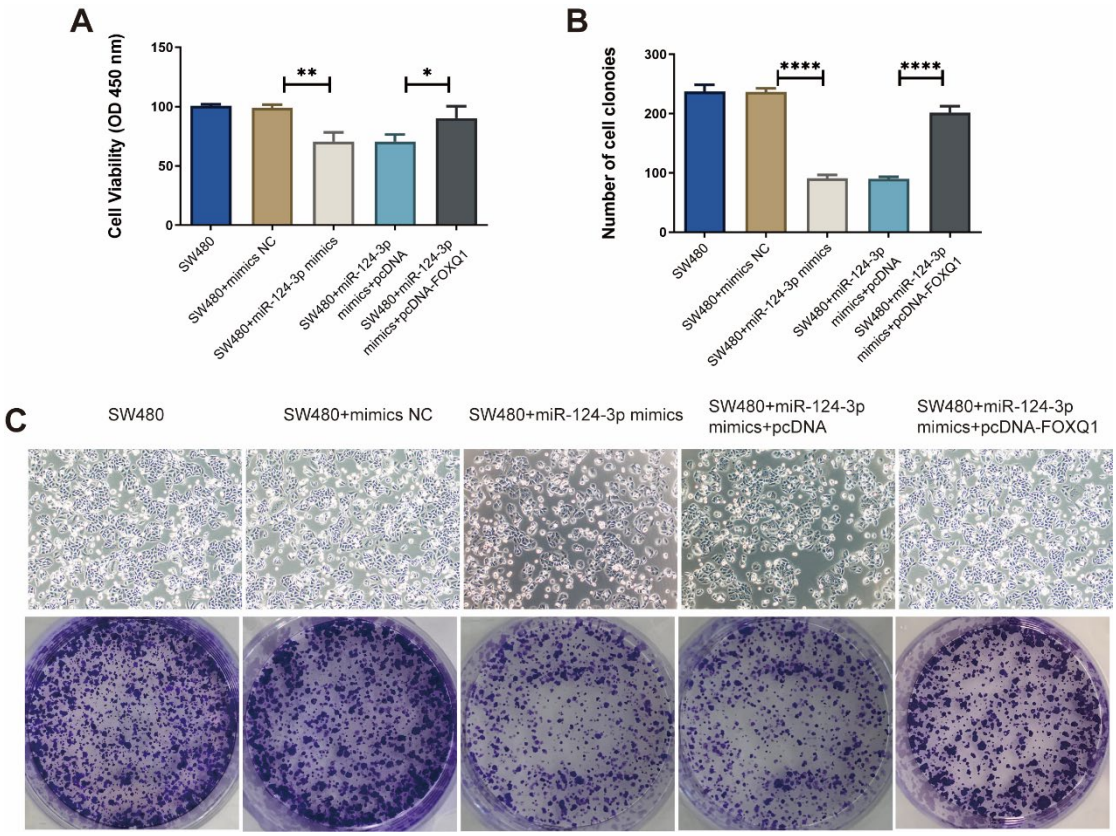

Figure 4 hsa-miR-124-3p hampered the cell proliferation of CRC cell line through FOXQ1. (A) The cell proliferation was detected by CCK-8. (B-C) The cell proliferation was cell colony assay. Error bars represent SD. Lines stands for the comparison between two groups. *, $p < 0.05$, **, $p < 0.01$, ****, $p < 0.0001$.

hsa-miR-124-3p hampered the cell apoptosis inhibition of CRC cell line through FOXQ1

The cell apoptosis was detected by flow cytometer (Figure 5). The result showed that after transfected with hsa-miR-124-3p mimics, cell apoptosis rate was increased compared with control group, while the transfection of pcDNA-FOXQ1 reversely decreased the result (Figure 5). These results showed that hsa-miR-124-3p hampered the cell apoptosis inhibition of CRC cell line through FOXQ1.

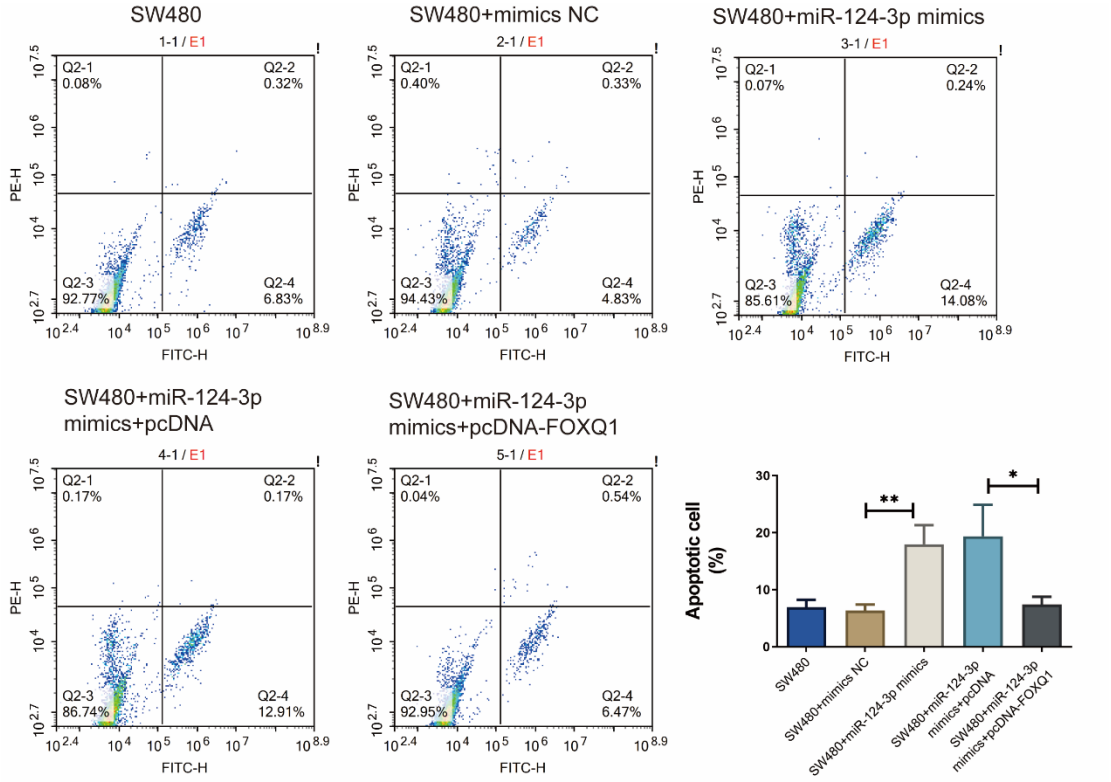

Figure 5. hsa-miR-124-3p hampered the cell apoptosis inhibition of CRC cell line through FOXQ1. Error bars represent SD. Lines stands for the comparison between two groups. *, $p < 0.05$; **, $p < 0.01$.

hsa-miR-124-3p overexpression hampered the cell cycle of colorectal cancer cell line by targeting FOXQ1

The ratio of accumulated cells in each phase was detected by flow cytometer (Figure 6). 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, while the transfection of pcDNA-FOXQ1 reversed the result (Figure 6). These results showed that hsa-miR-124-3p hampered the cell the cell cycle of CRC cell line through FOXQ1.

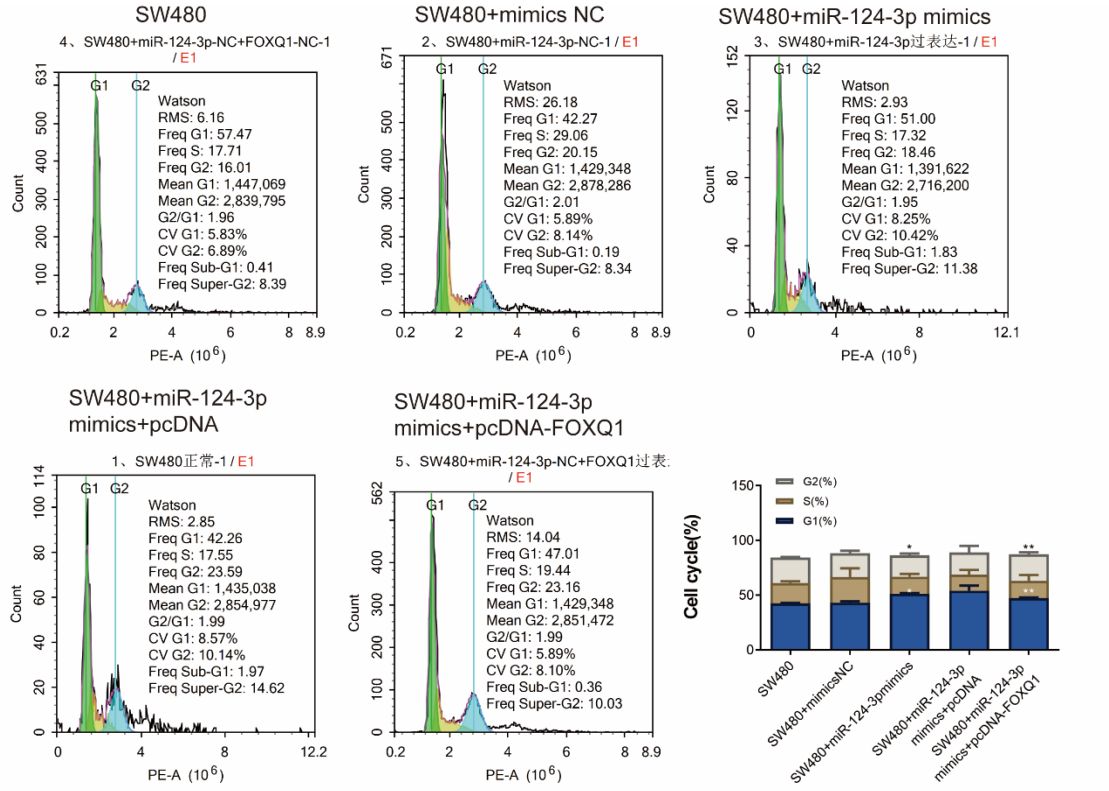

Figure 6 hsa-miR-124-3p overexpression hampered the cell cycle of colorectal cancer cell line by targeting FOXQ1. Error bars represent SD. Lines stands for the comparison between two groups. *, $p < 0.05$; **, $p < 0.01$.

hsa-miR-124-3p overexpression hampered the cell migration and invasion of colorectal cancer cell line by targeting FOXQ1

The cell migration was detected by wound healing assay (Figure 7). The result showed that after transfected with hsa-miR-124-3p mimics, migration index was decreased compared with control group, while the transfection of pcDNA-FOXQ1 reversed the result. Also, cell invasion was detected by Transwell chambers (Figure 8). The result showed that after transfected with hsa-miR-124-3p mimics, the number of invasion cells was decreased compared with control group, while the transfection of pcDNA-FOXQ1 reversed the result.

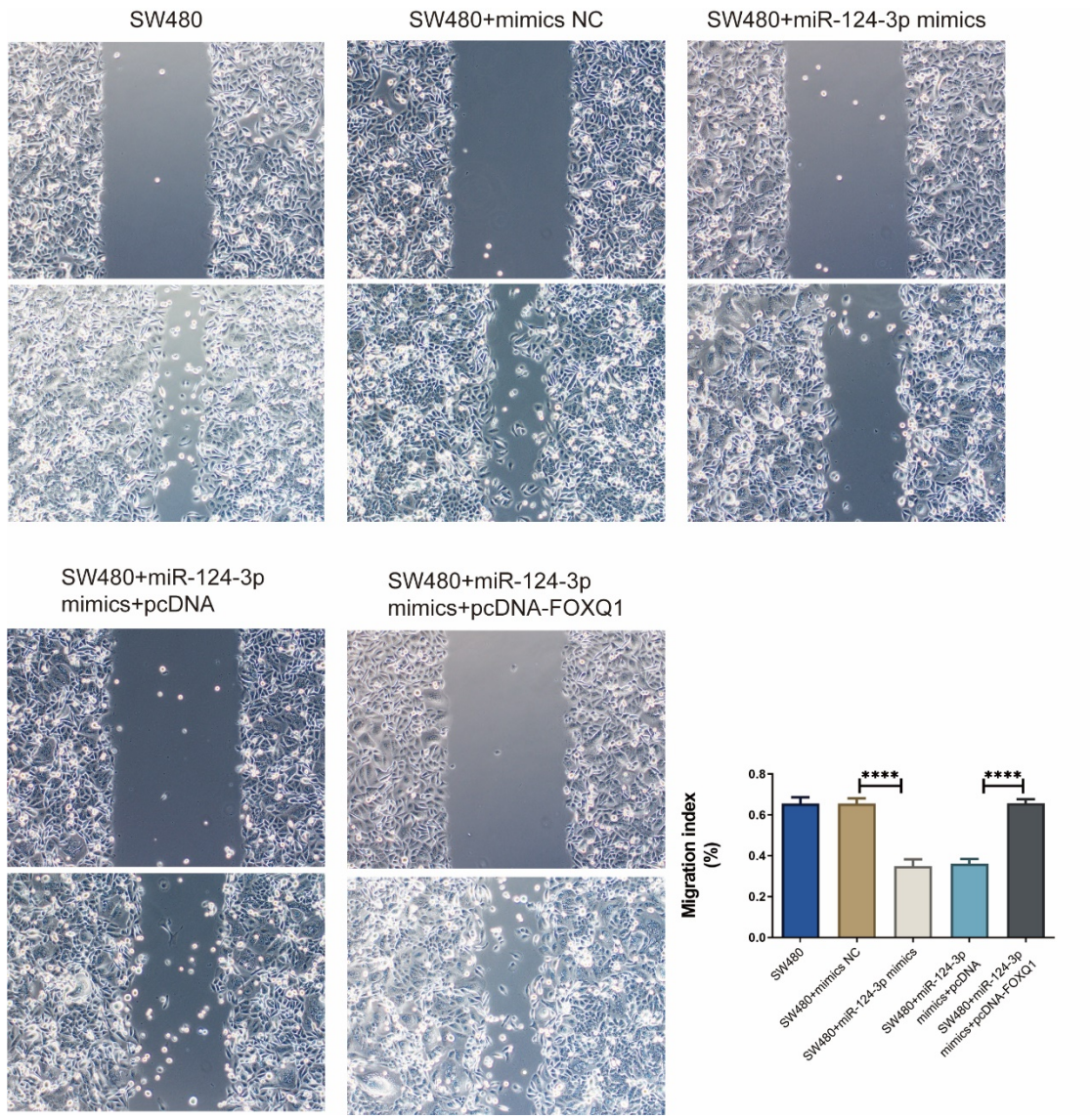

Figure 7. The cell migration was detected by wound healing assay. Error bars represent SD. Lines stands for the comparison between two groups. ****, $p < 0.0001$.

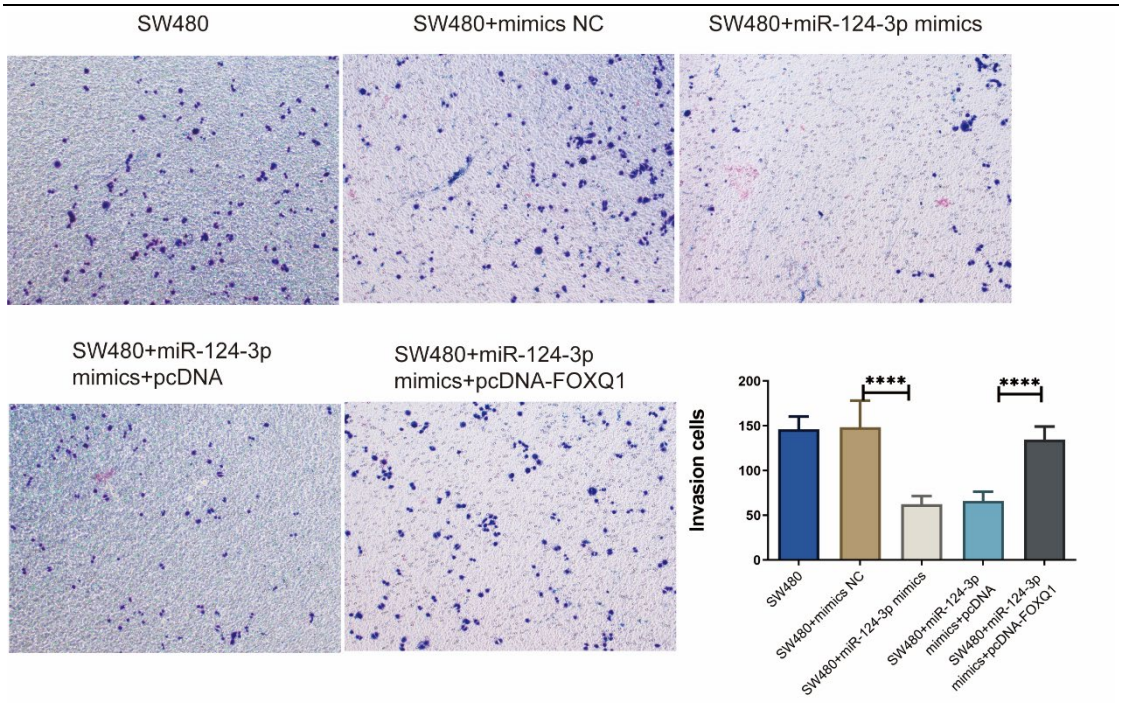

Figure 8. The cell invasion was detected by Transwell chambers. Error bars represent SD. Lines stands for the comparison between two groups. ***, $p < 0.001$; ****, $p < 0.0001$

hsa-miR-124-3p overexpression regulated ERK pathway by targeting FOXQ1

To further detect the mechanism of FOXQ1 on malignant phenotype of colorectal cancer cell line, we detected the expression of downstream EKR pathway (figure 9). qPCR result showed that the mRNA expression of ERK 1/2 was no obvious distinct. While the result of western blot showed that the phosphorylation of ERK 1/2 was decreased when miR-124-3p was increased, while the transfection of pcDNA-FOXQ1 reversed the result. These results showed that hsa-miR-124-3p overexpression regulated ERK pathway by targeting FOXQ1.

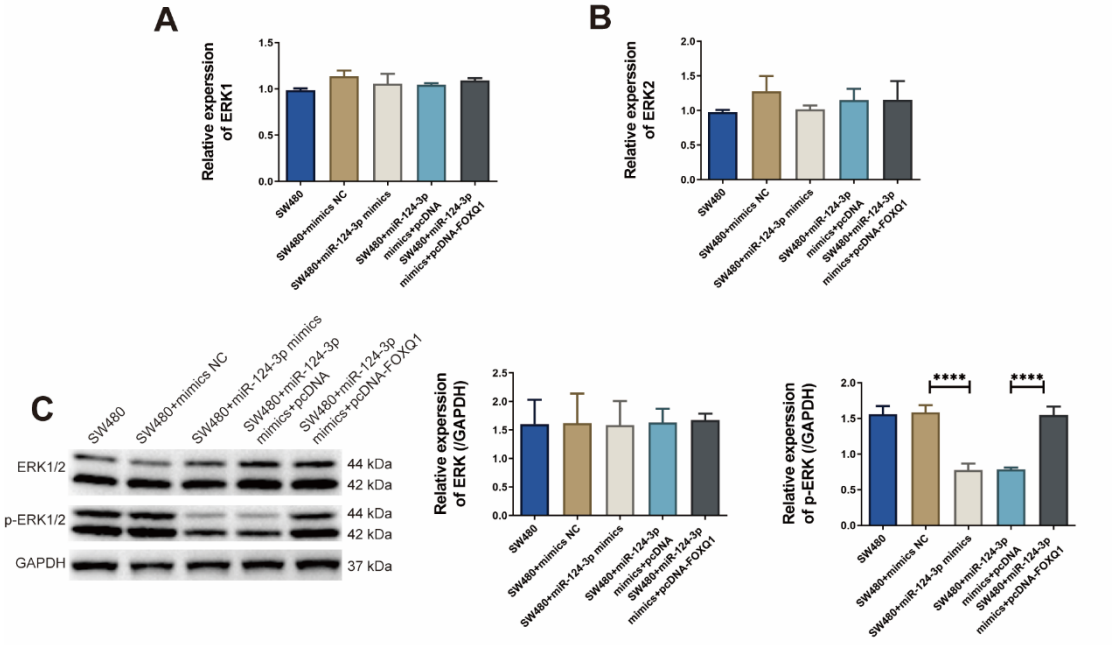

Figure 9 hsa-miR-124-3p overexpression regulated ERK pathway by targeting FOXQ1. (A) The mRNA expression of EKR1/2 was detected by qPCR. (B) The protein expression of ERK1/2 and its phosphorylation was detected by western blot. Error bars represent SD. Lines stands for the comparison between two groups. ****, $p < 0.0001$.

hsa-miR-124-3p overexpression hampered the tumor growth through FOXQ1/ERK *in vivo*

Then, we verified the mechanism in vivo. SW480 was transfected with miR-124-3p mimics and injected subcutaneously on the back of nude mice. HE staining showed that small necrotic areas with vigorous tumor cell growth, obvious nuclear heterogeneity, and abundant interstitial blood vessels in control group, while in miR-124-3p mimics group, cells were irregular arrangement, few interstitial blood vessels and visible fibrosis formation was also observed. The IHC result showed that FOXQ1 was located in cytoplasm and nucleus, and the overexpression of miR-124-3p resulted in the deprivation of FOXQ1 (figure 10).

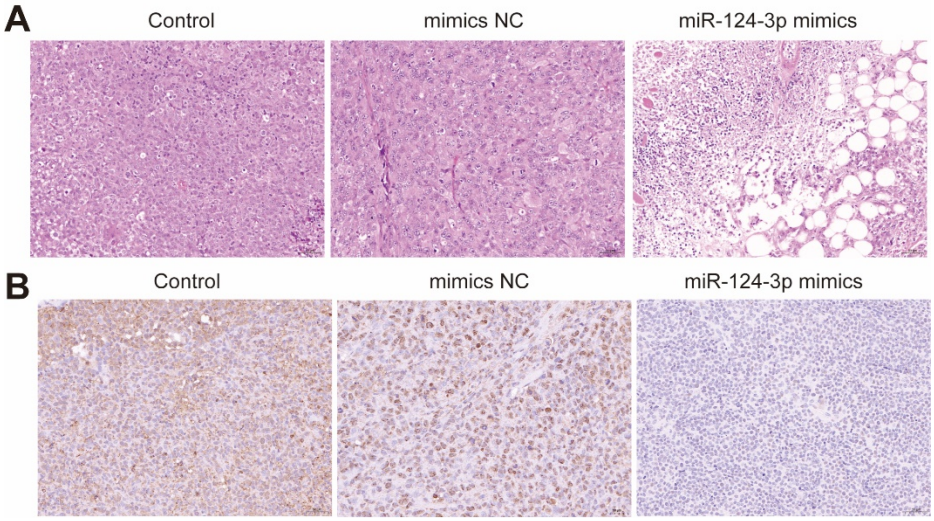

Figure 10. HE staining was used to observe histopathological changes in the tumor (A, *20), IHC was used to detect the FOXQ1 expression and subcellular location in tumor tissues (B, *20).

Also, the mRNA expression of FOXQ1 and ERK 1/2 was detected by qPCR. The result showed that the mRNA expression of FOXQ1 was decreased with the overexpression of FOXQ1, while the mRNA expression of ERK1/2 showed no distinct difference. The protein expression of FOXQ1 and ERK1/2 and their phosphorylation were detected by western blot. The result showed the protein expression of FOXQ1 and ERK1/2 phosphorylation were decreased when miR-124-3p was overexpressed (Figure 11). These results showed hsa-miR-124-3p overexpression hampered the tumor growth through FOXQ1/ERK *in vivo*.

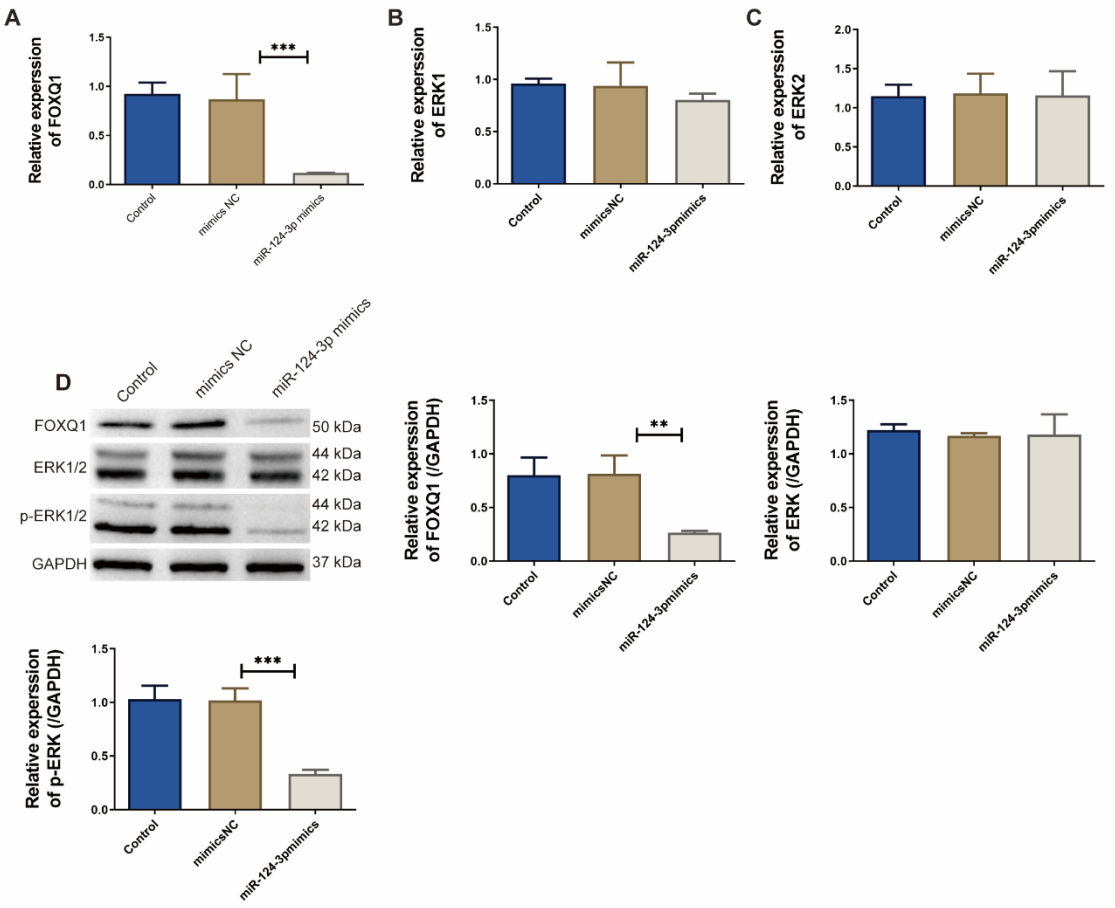

Figure 11 hsa-miR-124-3p overexpression hampered the tumor growth through FOXQ1/ERK in vivo. (A) The mRNA expression of EKR1/2 was detected by qPCR. (B) The protein expression of ERK1/2 and its phosphorylation was detected by western blot. Error bars represent SD. Lines stands for the comparison between two groups. **, p<0.01; ***, p < 0.001.

Discussion

Colorectal cancer is one of the most lethal malignancies in the world[15]. Despite the advances in diagnosis and treatment in recent years, the overall prognosis of colon cancer patients remains poor, one of the main reasons being the lack of effective prognostic biomarkers[16]. Therefore, it is urgent and necessary to explore valuable prognostic markers and therapeutic targets for colon cancer. New predictive factors, molecular imaging and even commercial genomic tests are increasingly helpful in genomic testing of tumors and assisting in the selection of targeted therapies[17]. Patients with advanced CRC require adjuvant targeted therapy with anti-EGFR antibodies and are free of KRAS, BRAF, NRAS and PIK3CA gene mutations[18]. It is reasonable to type the UGT1A1 allele before starting treatment with irinotecan to avoid serious adverse effects[19]. Identification of these biomarkers could provide a non-invasive and cost-effective diagnosis. miRNAs are a class of small noncoding RNAs that can affect a variety of cellular and molecular targets[20]. Our previous research found that miR-124-3p promote cell proliferation, apoptosis inhibition, migration, invasion and tumor growth. In this manuscript, we found that miR-124-3p regulated cell proliferation, apoptosis inhibition, migration, invasion and tumor growth by targeting FOXQ1/ERK pathway.

The Forkhead (FOX) gene family is a large

and versatile family of transcription factors that share a highly conserved forkhead structural domain[21]. Currently, more than 100 FOX family members have been identified in different genera, belonging to 19 subfamilies, based on homology of the DNA structural domain[22]. More than 50 FOX proteins have been shown to exist in the human genome[23]. FOX family members are involved in a wide range of biological activities, including cell cycle regulation, sugar and lipid metabolism, embryonic development and immune regulation[24]. Moreover, they are mutated or aberrantly expressed in a variety of tumors[25, 26]. FOXQ1 gene is located on chromosome 6 (6p25.3), is 2319bp long and contains only one exon. It encodes a 403 amino acid FOXQ1 protein[27]. In this manuscript, we found FOXQ1 was highly expressed in CRC tissues. Which is same as the result found by Wu et al.[28], who found FOXQ1 was highly expressed in CRC patients and involved in CTNNB1 related interaction of the canonical Wnt signaling pathway. Jin et al. found that RP9P promotes colorectal cancer progression by regulating the miR-133a-3p/FOXQ1 axis[29]. Liu et al. found that FOXQ1 promotes cancer metastasis by PI3K/AKT signaling regulation in colorectal carcinoma[30]. Similar, we found FOXQ1 was a directly target of miR-124-3p, and miR-124-3p regulated cell proliferation, apoptosis inhibition, migration, invasion and tumor growth by targeting FOXQ1. Also, FOXQ1 involved in the activation of ERK1/2, which indicated that miR-124-3p can negatively regulate the expression of FOXQ1, which activated the MAPK/ERK pathway, and therefore regulated cell proliferation, apoptosis inhibition, migration, invasion and tumor growth.

In conclusion, we found that miR-124-3p regulated cell proliferation, apoptosis inhibition, migration, invasion and tumor growth by targeting FOXQ1/ERK pathway. These results may indicate that FOXQ1 can be a potential target of early diagnosis and treatment of colorectal carcinoma

Funding

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