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Effects of miR-223 on colorectal cancer cell proliferation and apoptosis through regulating FoxO3a/BIM

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Abstract. – OBJECTIVE: Colorectal cancer is a common malignant tumor of the digestive tract. It frequently occurs at the junction of the rectum and sigmoid colon. It is characterized by high mortality and poor prognosis. Bcl-2 interacting mediator of cell death (BIM) plays a role in the regulation of cell proliferation and apoptosis, and involves in the pathogenesis of colorectal cancer. The transcription factor forkhead, transcription factor O subfamily 3a (FoxO3a) plays a role in the regulation of BIM expression and is associated to the pathogenesis of colorectal cancer. Bioinformatics analysis suggests that there is a targeted relationship between FoxO3a and microRNA-223 (miR-223). This study aims to investigate effects of miR-223 on the regulation of FoxO3a/BIM signaling pathway and colorectal cancer cell proliferation and apoptosis.

MATERIALS AND METHODS: Colorectal cancer cell line SW620 and normal colorectal epithelial cell line NCM460 were cultured *in vitro*. Dual luciferase reporter assay was used to validate the relationship between miR-223 and FoxO3a. Flow cytometry was adopted to detect apoptosis. EdU staining was applied to test cell proliferation. Western blot was selected to determine FoxO3a and BIM protein expressions.

RESULTS: There was targeted regulatory relationship between miR-223 and FoxO3a. MiRa-223 up-regulated, FoxO3a and BIM expressions reduced, and cell proliferation was enhanced in SW620 cells compared with NCM460 cells. MiR-223 inhibitor or pIRES2-FoxO3a transfection significantly increased FoxO3a and BIM expressions, attenuated cell proliferation, and enhanced cell apoptosis.

CONCLUSIONS: MiR-223 targeted inhibited expression of FoxO3. Down-regulating the expression of miR-223, it increased the expressions of FoxO3a and BIM, weakened SW620 cells proliferation and induced apoptosis.

Key Words

miR-223, FoxO3a, BIM, Colorectal cancer, Proliferation, Apoptosis.

Introduction

Colorectal cancer (CRC) is a common clinical malignant tumor of the digestive tract, accounting for the third leading malignant tumor of the body[1]. CRC is featured as concealed onset, easy to metastasis, and poor prognosis. Therefore, exploring the pathogenesis of CRC is of great significance for early diagnosis, individual treatment, improving the therapeutic effect, and perfecting patient survival and prognosis.

Forkhead transcription factor O subfamily 3a (FoxO3a) is a tumor suppressor gene in FoxO family, which can regulate multiple genes that participate in cell proliferation, cell cycle, and apoptosis, such as Bcl-2 interacting mediator of cell death (BIM), p53 up-regulated modulator of apoptosis (PUMA), cyclin-dependent kinase inhibitor (CKI), p27Kip1, and cyclin-dependent kinase D1 (cyclin D1). It controls various signal pathways and multiple biological processes of tumor cells[2]. Decreased FoxO3a expression or activity is associated with the occurrence, progression, and drug resistance of a variety of tumors, including breast cancer[3], pancreatic cancer[4], liver cancer[5], and bladder cancer[6]. It was showed that[7,8] FoxO3a expression was abnormally reduced in CRC, suggesting its possible tumor suppressor effect. MicroRNAs are a class of non-coding single-stranded RNAs at about 22-25 nucleotides in length. They can negatively regulate over 1/3 of human genes by targeting degradation of mRNA or inhibiting translation of target mRNAs, thus it is involved in the regulation of tissues and organs development, cell proliferation, apoptosis, and differentiation. Their abnormal expression and function in tumorigenesis received more and more attention[9]. MiR-223 is a well-reported miRNA, which is located at q12 of chromosome X. MiR-223 plays

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a dual role in promoting cancer or tumor suppressor genes depending on the target genes. For example, elevated expression of miR-223 is associated with the occurrence of lung cancer[10] and the development and drug resistance of gastric cancer[11,12]. On the contrary, it plays a tumor suppressor role in gallbladder cancer to reduce drug resistance[13]. It was found that abnormal expression of miR-223 in both tumor tissue[14] and peripheral blood[15] of CRC patients suggests its potential role in promoting cancer. Bioinformatics analysis revealed the targeted complementary relationship between miR-223 and the 3'-UTR of FoxO3a. This study further explores whether miR-223 plays a role in the regulation of FoxO3a expression and CRC proliferation and apoptosis.

Materials and Methods

Main Reagents and Instruments

Human normal colorectal epithelial NCM460 and HEK293T cells were purchased from Shanghai Suer Biological Technology Co., Ltd. (Shanghai, China). CRC SW620 cells were obtained from Jiangsu Feiya Biological Technology Co., Ltd. (Nanjing, China). Roswell Park Memorial Institute-1640 (RPMI-1640) medium, penicillin, streptomycin, option minimum Eagle's medium (Opti-MEM), and fetal bovine serum (FBS) were purchased from Gibco BRL. Co. Ltd. (Grand Island, NJ, USA). TRIzol and Lipofectamine RNAiMAX were purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA). PrimeScript™ RT reagent kit was purchased from TaKaRa (Dalian, China). Annexin V/PI apoptosis detection kit was purchased from Beyotime Biotechnology (Shanghai, China). EdU staining cell proliferation detection kit was derived from Molecular Probes (Eugene, OR, USA). Luciferase activity detection kit was purchased from Promega (Madison, MI, USA). Luciferase reporter plasmid pLUC was obtained from BioVector (Beijing, China). Rabbit anti-human polyclonal antibodies FoxO3a and BIM were bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-human polyclonal antibody β-actin was got from Gene Tex (Irvine, CA, USA). Horseradish peroxidase (HRP) conjugated secondary antibody was purchased from Bio-Rad Laboratories (Hercules, CA, USA). MiR-223 mimic, miR-223 inhibitor, and miR-NC were provided by Ribobio (Guangzhou, China). Biosafety cabinet and cell incubator were supplied by Thermo Scientific Pierce (Rockford, IL, USA). Polyacrylamide gel electrophoresis (PAGE) electrophoresis apparatus and Real-time PCR amplifier were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Western blot transfer box was got from Tanon (Shanghai, China). Flow cytometry was purchased from Beckman Coulter Inc. (Brea, CA, USA). Inverted microscope was provided by Nikon (Mode: TS100, Tokyo, Japan).

Cell Culture

NCM460 and SW620 cells were cultured in RPMI 1640 medium containing 10% FBS and 1% penicillin-streptomycin. The cells were cultured at 37°C and 5% CO_2, and passaged at 1:4.

Dual-Luciferase Reporter Gene Assay

HEK293T cells were lysed by TRIzol to extract mRNA. The PCR products containing the full length of FoxO3a gene 3'-UTR segment or mutant segment were cloned to pLUC. After transforming to DH5α competent cells, the positive clone was named as pLUC-FoxO3a-wt or pLUC-FoxO3a-mut and co-transfected to HEK293T cells using Lipofectamine 2000 together with miR-223 mimic (miR-223 inhibitor or miR-NC) and pRL-null Renilla luciferase. The luciferase activity was detected according to the Dual-Glo Luciferase Assay System manual after cultured for 48 h. The primer sequences were listed in Table I.

Table I. qRT-PCR primer pairs.

Primers	Forward	Reverse
FoxO3a-wt	5′-CTAGTGTGGGCAAAGCAGACCCTC AAACTGACACAAGACCTACAGAGAAAAC-3′	5′- AGCTTGGTTTTCTCTGTAGGTCTTG TGTCAGTTTGAGGGTCTGCTTTGCCCACA-3′
FoxO3a-mut	5′- CTAGTGTGGGCAAAGCAGACCCT CACAAGACCTACAGAGAAAACCA-3′	5′-AGCTTGGTTTTCTCTGTAGGTCTTG TGAGGGTCTGCTTTGCCCACA-3′

FoxO3a Overexpression Plasmid Construction

The coding sequence (CDS) region of FoxO3a gene was amplified and connected to pIRES2 plasmid after Xho I and BamH I digestion. Next, it was transformed to component cell JM109 and the plasmid was extracted. The plasmid with correct target segment was named as pIRES2-FoxO3a.

Cell Grouping and Transfection

SW620 cells were cultured *in vitro* and divided into four groups, including miR-NC, miR-223 inhibitor, pIRES2-Blank, and pIRES2-FoxO3a groups. The above-mentioned were incubated with Lipofectamine 2000 at room temperature for 30 min. Then, they were added to the cells and incubated for 6 h. After changing the medium and incubation for 72 h, the cells were collected for the following experiments.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted using Total RNA Kit and adopted for PCR reaction by PrimeScript™ RT reagent kit. The reverse transcription system was as follows: ligdT Primer (50 μM) 0.5 μl, Random 6 mers (100 μM) 0.5 μl, PrimeScript RT Enzyme Mix 0.5 μl, RNA 0.5 μl, 5×PrimeScript Buffer 2 μl, RNase Free dH_2O up to 10 μl. The PCR reaction system was as follows: SYBR Fast qPCR Mix 10 μl, Forward Primer (10 μM) 0.8 μl, Reverse Primer (10 μM) 0.8 μl, complementary DNA (cDNA) 2 μl, RNase Free dH_2O 6.4 μl. The PCR reaction was composed of 95°C pre-denaturation for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 15 s. Real-time PCR was performed on Bio-Rad (Hercules, CA, USA) CFX96 Real-time PCR Detection System to test the relative expression. The primers were listed in Table I.

Western Blot

Total protein was extracted by sodium dodecyl sulfate (SDS) lysis from cells. A total of 40 μg protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at 50 V for 3.5 h and transferred to membrane at 300 mA for 1.5 h. Next, the membrane was blocked by 5% skim milk at room temperature for 60 min and incubated in primary antibody at 4°C overnight (FoxO3a, BIM, and β-actin at 1:2000, 1:2000, and 1:10000, respectively). Next, the membrane was incubated in HRP conjugated secondary antibody (1:15000) for 60 min after washed by phosphate buffered solution and Tween-20 (PBS-T) for three times. At last, the protein expression was detected by enhanced chemiluminescence (ECL, Amersham Biosciences, Little Chalfont, Buckinghamshire, England).

Cell Proliferation Assay

The cells were added with 10 μM EdU solution for 2 h. After incubated for 48 h, the cells were digested and collected. After washed by PBS, fixed, and penetrated, the cells were incubated in reaction liquid at room temperature avoid of light for 30 min. The cells were washed and tested by Beckman Coulter FC 500 MCL flow cytometry (Brea CA, USA).

Cell Apoptosis Detection

The cells were digested and resuspended in binding buffer. Next, the cells were incubated in 5 μl Annexin V-FITC and 5 μl propidium iodide (PI) avoid of light for 10 min. At last, the cells were tested on Beckman FC 500 MCL flow cytometry to evaluate cell apoptosis.

Statistical Analysis

All data analyses were performed on SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The measurement data were depicted as mean ± standard deviation and compared by *t*-test. $p<0.05$ was considered statistically significant.

Results

MiR-101 Targeted Inhibited MEK1 Expression

MicroRNA.org online prediction showed the targeted binding site between miR-223 and 3'-UTR of FoxO3a mRNA (Figure 1A). Dual luciferase assay revealed that miR-223 mimic transfection significantly declined, while miR-223 inhibitor transfection markedly elevated the relative luciferase activity of HEK293T cells. Meanwhile, miR-223 inhibitor presented no statistical impact on the relative luciferase activity in HEK293T cells transfected by pLUC-FoxO3a-mut, indicating the regulatory relationship between miR-101 and MEK1 mRNA (Figure 1B). qRT-PCR detection demonstrated that FoxO3a mRNA expression markedly declined in miR-223 mimic transfection group, whereas apparently elevated in miR-223 inhibitor transfection group compared with miR-NC (Figure 1C).

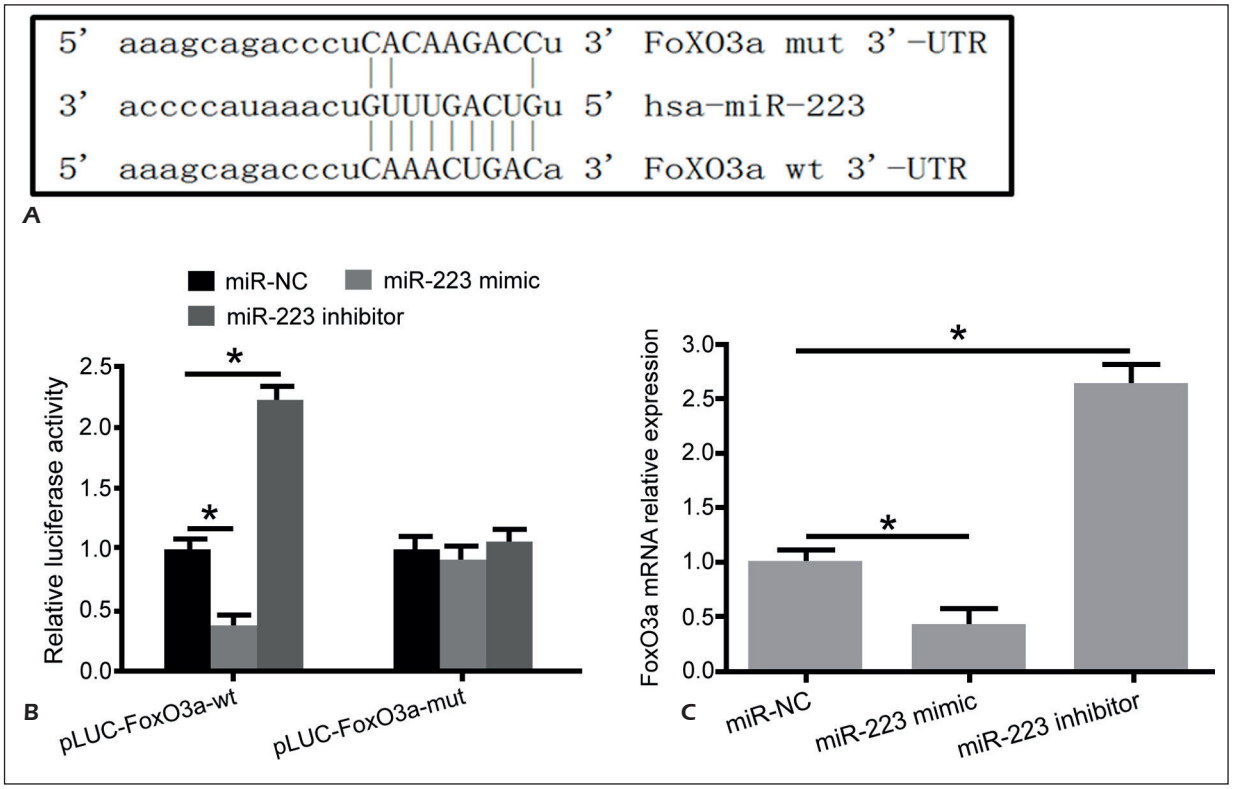

Figure 1. MiR-223 targeted regulated FoxO3a expression. **A**, The binding site between miR-223 the 3'-UTR of FoxO3a mRNA. **B**, Dual luciferase assay. C, qRT-PCR detection of FoxO3a mRNA expression.

MiR-223 and FoxO3a Expression Changes in SW620 Cells

Flow cytometry exhibited that the EdU positive rate in SW620 cells was significantly higher than that in NCM460 cells, suggesting its stronger proliferative ability (Figure 2A). qRT-PCR demonstrated that miR-223 significantly upregulated, while FoxO3a and BIM mRNA expressions markedly reduced in SW620 cells compared with NCM460 cells (Figure 2B). Western blot showed that FoxO3a and BIM protein levels apparently decreased in SW620 cells compared with NCM460 cells (Figure 2C).

Down-Regulation of miR-223 Attenuated SW620 Cell Proliferation and Induced Cell Apoptosis

FoxO3a and BIM expressions significantly enhanced in SW620 cells transfected by miR-223 inhibitor compared with miR-NC group (Figure 3A, B). Flow cytometry presented that cell proliferation significantly attenuated (Figure 3C), whereas cell apoptosis markedly enhanced (Figure 3D) in SW620 cells transfected by miR-223 inhibitor compared with miR-NC. FoxO3a and BIM expressions significantly enhanced in SW620 cells transfected by pIRES2-FoxO3a compared with pIRES2-blank group (Figure 3A, B). Flow cytometry presented that cell proliferation significantly attenuated (Figure 3C), whereas cell apoptosis markedly enhanced (Figure 3D) in SW620 cells transfected by pIRES2-FoxO3a compared with pIRES2-blank.

Discussion

In clinical, 40-50 age group show a high CRC incidence. Moreover, the incidence of male is 2 to 3 times compared with female. Following the improvement of living standard and the changes of diet structure in our country, the incidence of CRC shows an increasing and younger trend year by year, which causes a serious threat to health and quality of life[16]. The early symptom of CRC is not obvious, easily leading to miss diagnosis. The majority of patients with symptoms are in advanced stage, resulting in difficult treatment, bad effect, and poor prognosis.

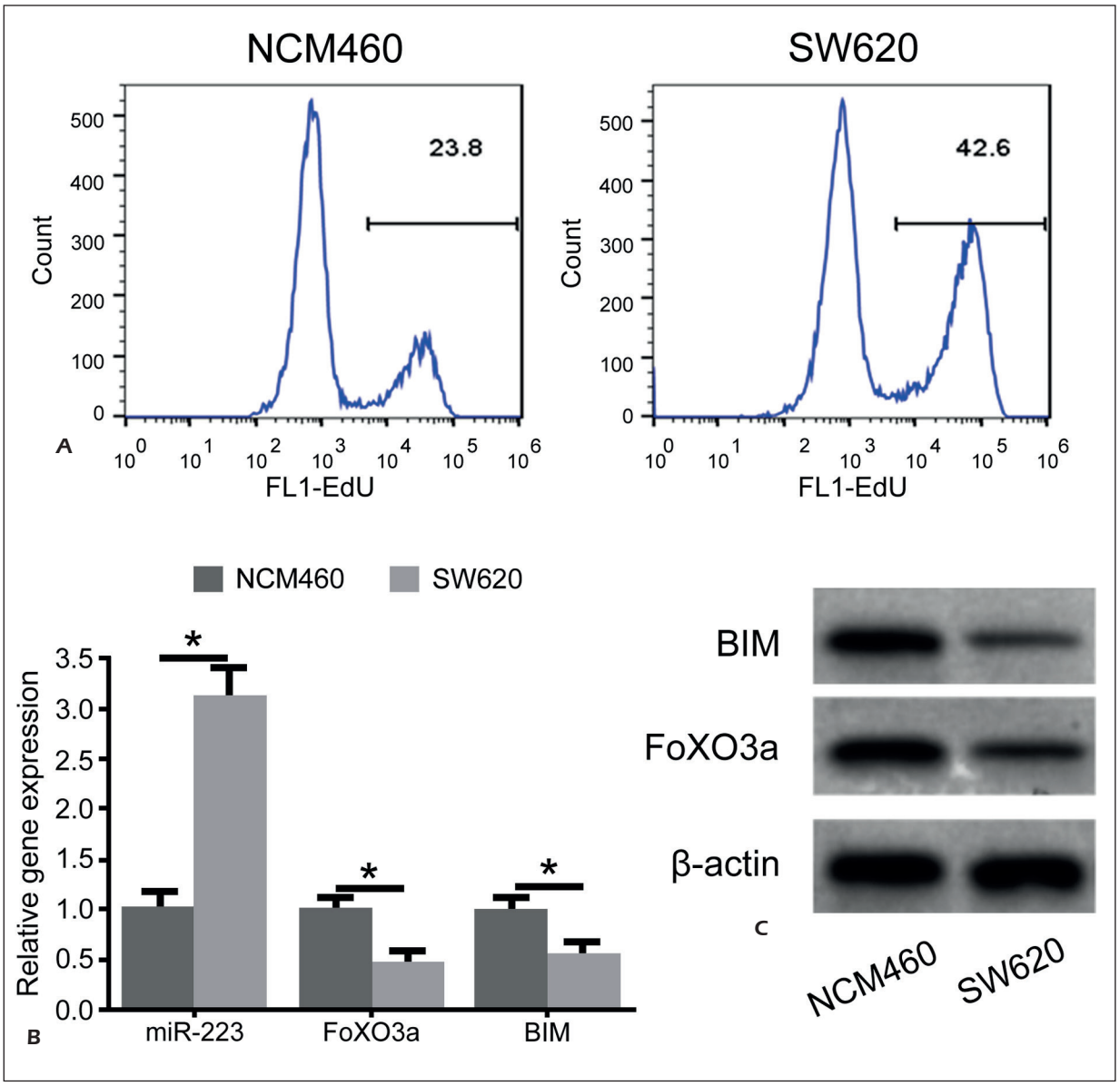

Figure 2. MiR-223 and FoxO3a abnormal expressions in SW620 cells. **A**, EdU staining detection of cell proliferation. **B**, qRT-PCR detection of gene expression. **C**, Western blot detection of protein expression.

FoxO is a class of evolutionarily highly conserved transcription factors that are widely involved in the regulation of many biological processes, such as embryonic development, cell proliferation, cell cycle, and apoptosis. FoxO transcription factor family contains four members, including FoxO1, FoxO3a, FoxO4, and FoxO6, of which FoxO3a is the most studied core member[5,6]. It was showed that[7,8] FoxO3a expression was abnormally reduced in CRC, suggesting its possible tumor suppressor effect. It was found that abnormal expression of miR-223 in both the tumor tissue[14] and peripheral blood[15] of CRC patients suggests its potential role in promoting cancer. This study explores whether miR-223 plays a role in the regulation of FoxO3a expression and CRC proliferation and apoptosis.

Dual luciferase reporter assay demonstrated that the relative luciferase activity in miR-223 mimic transfected HEK293T cells was significantly lower than that in miR-NC group, while miR-223 mimic transfection exhibited no statistical impact on the relative luciferase activity in HEK293T cells transfected by pLUC-FoxO3a-mut. Compared with normal colorectal epithe-

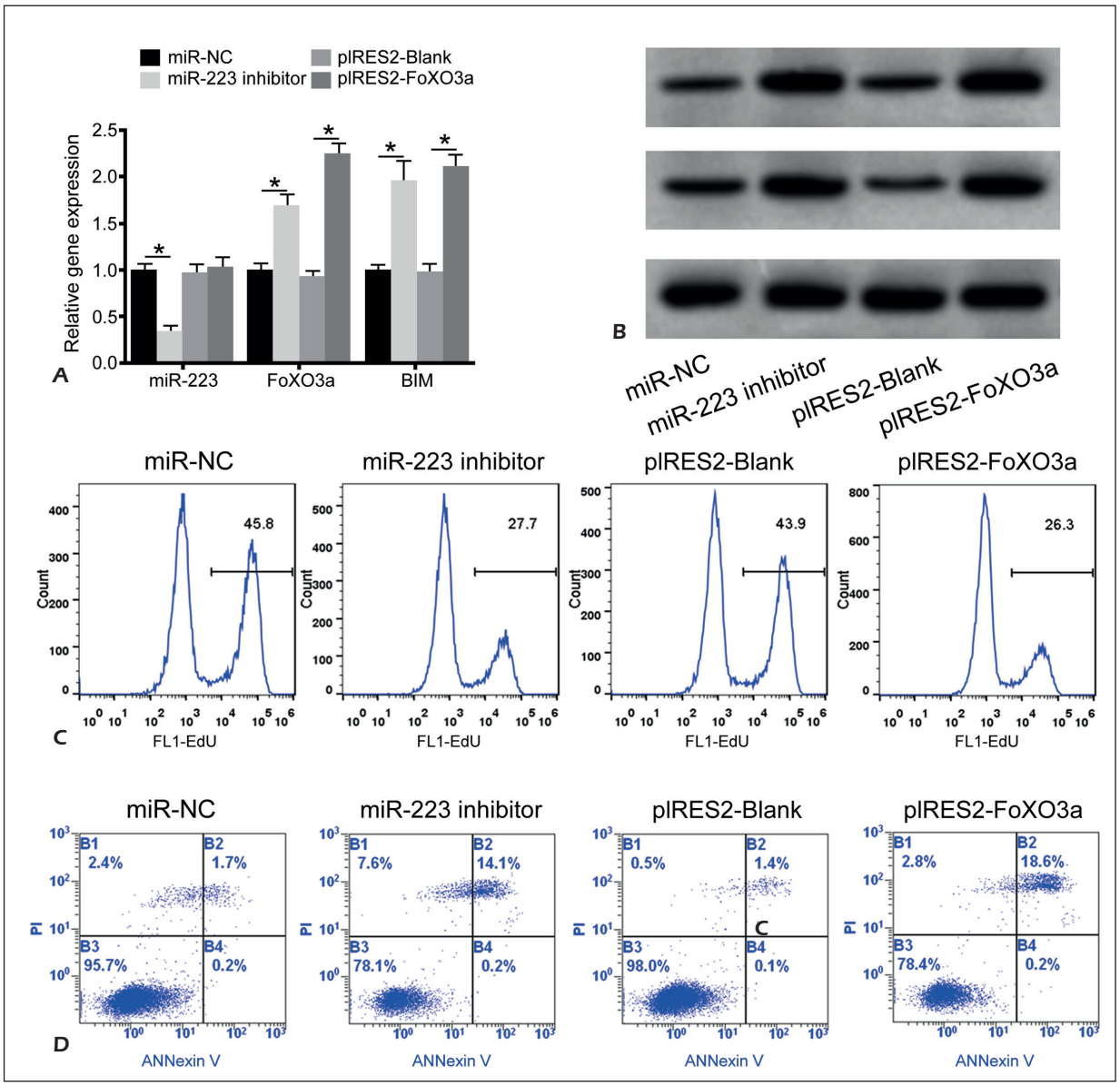

Figure 3. Down-regulation of miR-223 attenuated SW620 cell proliferation and induced cell apoptosis. **A**, qRT-PCR detection of gene expression. **B**, Western blot detection of protein expression. **C**, EdU staining detection of cell proliferation. **D**, Flow cytometry detection of cell apoptosis.

lial cells, miR-223 was significantly increased, whereas FoxO3a was markedly decreased in CRC cells. In the study of the relationship between miR-223 and CRC, Fu et al[17] found that the expression of miR-223 in colorectal cancer tissues was apparently higher than that in normal colorectal tissues. Chang et al[18] reported that miR-223 in plasma of CRC patients was significantly higher than in the control group plasma for about 2.51 times. Li et al[19] demonstrated that compared with adjacent non-cancerous tissue, the expression of miR-223 in the tumor tissue of CRC patients was significantly increased, and its expression was related to TNM stage, lymph node metastasis, and distant metastasis. Patients with higher miR-223 showed worse survival and prognosis than those with lower expression of miR-223, which was similar to this study. All of the above studies revealed the possible role of miR-223 in promoting CRC only from the correlation phenomenon. However, the specific mechanism of miR-223 in affecting the biological effects of CRC and its target genes were still unclear.

We further showed that miR-223 inhibitor or pIRES2-FoxO3a transfection significantly up-regulated FoxO3a expression, increased BIM expression, weakened cell proliferation, and enhanced cell apoptosis in colorectal cancer SW620 cells. Sun et al[20] observed that over-expression of miR-223 promoted the proliferation of colorectal cancer Caco-2 cells and enhanced the tumorigenicity and growth ability of colorectal cancer HT-29 cells in nude mice. Zhang et al[14] revealed that the proliferation, migration, and invasion of Colo320 and LoVo colorectal cancer cells were significantly reduced after transfection with anti-miR-223 to inhibit the expression of miR-223. Therefore, this study found that down-regulation of miR-223 inhibited colorectal cancer cell proliferation, induced apoptosis, and reduced the malignant biological characteristics, which was in accordance with Zhang et al[14]. However, Zhang et al[14] failed to elucidate the specific mechanism of miR-223 in reducing the malignant biological characteristics of colorectal cancer cells. We found that FoxO3a is the target gene of miR-223. MiR-223 affects CRC proliferation and apoptosis through targeted regulating FoxO3a, which is a further integration to Zhang et al[14] study. Fu et al[7] showed that the expression of FoxO3a in colorectal cancer was significantly decreased, indicating that FoxO3a may be a tumor suppressor in the pathogenesis of colorectal cancer. He et al[8] found that the expression of FoxO3a in colorectal cancer tissues was markedly declined. The proportion of G2-M phase of colorectal cancer cells SW480 was apparently increased and the cell cycle progression was accelerated after siRNA was used to disturb the expression of FoxO3a. Marzi et al[21] reported that cetuximab treatment induced CRC cell apoptosis and inhibited cell proliferation through enhancing the expression of FoxO3a and its downstream target genes BIM and p27. In addition, patients with higher FoxO3a expression exhibited better sensitive and outcome to cetuximab than those with lower FoxO3a expression. These studies confirmed *in vitro* or *in vivo* that FoxO3a is a tumor suppressor factor of CRC, which was similar to our results. This investigation combined the expression characteristics of miR-223 and FoxO3a in CRC, revealing the role of miR-223 in regulating FoxO3a expression and affecting the proliferation and apoptosis of CRC cells. However, the influence of miR-223 on FoxO3a expression and the biological process of colorectal cancer *in vivo* are unclear and remain to be further studied.

Conclusions

We showed that miR-223 targeted suppressed the expression of FoxO3. Down-regulating the expression of miR-223, it increased the expressions of FoxO3a and BIM, weakened SW620 cells proliferation and induced apoptosis.

Conflict of interest

The Authors declare that they have no conflict of interests.

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