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MicroRNA-433 Represses Proliferation and Invasion of Colon Cancer Cells by Targeting Homeobox A1

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The aberrant expression of miR-433 has been validated in some types of cancers. However, the expression profile and the biological function of miR-433 on colon cancer are still elusive. This study was designed to investigate the function of miR-433 on the proliferation and invasion of colon cancer cells. We detected the expression of miR-433 in colon cancer tissues, adjacent normal tissues, and cell lines. CCK8 and Transwell assays were performed to explore the impact of miR-433 on colon cancer cell proliferation and invasion. The luciferase reporter assay was applied to identify the direct target of miR-433. The results demonstrated that miR-433 was downregulated in colon cancer tissues and cell lines when compared with the control. Overexpression of miR-433 significantly suppressed the ability of colon cancer cell proliferation and invasion, whereas knockdown of miR-433 remarkably enhanced cell proliferation and invasion. Homeobox A1 (HOXA1) was identified as a target of miR-433, and it mediated the functions of miR-433 on colon cancer cells. To conclude, we revealed that miR-433 was downregulated in colon cancer, and it inhibited colon cancer cell proliferation and invasion by directly targeting HOXA1.

Key words: miR-433; Colon cancer; Proliferation; Invasion; Homeobox A1 (HOXA1)

INTRODUCTION

Colorectal cancer is one of the most common cancers worldwide. The mortality of colorectal cancer ranks as second in all types of cancers all over the world[1]. It has greatly impacted people's health and has brought great economic burden for society[2,3]. Although the diagnosis and treatment for colorectal cancer have improved in recent years, it is essential to explore the molecular biological mechanisms and potential therapy target for colorectal cancer[4].

Noncoding RNAs (also called non-protein-coding RNA) are a family of RNAs that is not translated into a protein. The family of noncoding RNAs consists of microRNAs (miRNAs), long noncoding RNAs (lncRNAs), circular RNAs (circRNAs), and other noncoding RNAs[5]. miRNA is one type of noncoding RNA that contains 20–21 nucleotides. miRNAs exert important roles in most physiological and pathological processes through targeting the 3′-untranslated region (3′-UTR) of their downstream molecules, which leads to translational inhibition or degradation of mRNAs[6,7]. The aberrant expression of miRNAs has been proved in many types of cancers. It is also well known that miRNAs play important roles in the processes of tumorigenesis and tumor progression[8]. Research has proved that miRNAs affect the proliferation, invasion, cell cycle, and apoptosis of cancer cells[9–12].

There have been studies indicating the aberrant expression of miR-433 in some types of cancers. miR-433 has been demonstrated to be downregulated in retinoblastoma, ovarian cancer, and oral squamous cell carcinoma[13–15]. However, the expression and function of miR-433 in colon cancer are largely elusive. In this study, we aimed to explore the expression pattern and biological function of miR-433 in colon cancer, hoping to uncover more molecular biological mechanisms of colon cancer.

MATERIALS AND METHODS

Human Tissue Specimens

A total of 20 colon cancer patients were enrolled in this research. None of the patients received antitumor therapy before operation for colon cancer resection. After

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obtaining informed consent of all enrolled patients, colon cancer tissue specimens were collected and kept in liquid nitrogen until use. The research was approved by the ethics committee of TungWah Hospital of Sun-Yat Sen University.

Cell Culture and Transfection

All the cells, including Caco-2, LOVO, HT29, SW480, and SW620, were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultivated in Dulbecco's modified Eagle's medium (Gibco, Carlsbad CA, USA). The medium was supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco). miR-433 mimic, miR-433 inhibitor, and miR-433 control (Ribobio, Guangzhou, China) were used for the overexpression or knockdown of miR-433. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for transfection according to the manufacturer's instructions.

RNA Extraction and Real-Time PCR

The total RNA of tissues or cells was isolated by TRIzol (Invitrogen). Quantitative real-time reverse transcription PCR was performed using SYBR Green Real-Time PCR Master Mixes (Thermo Fisher Scientific, Waltham, MA, USA) by LightCycler 480 real-time PCR platform (Roche, Meylan, France).

Cell Counting Kit 8 (CCK8) and Invasion Assays

The CCK8 assays (Dojindo, Kumamoto, Japan) were used for accessing the ability of cell proliferation. The colon cancer cells were transfected with miRNA, siRNA, or control. After transfection, the cells were plated in 96-well plates. The CCK8 assays were conducted at 24, 48, 72, and 96 h. All the procedures were performed according to the manufacturer's protocol. For the migration assays, the lower chamber of the Transwell inserts (Corning, Corning, NY, USA) was covered by Matrigel matrix (BD, Franklin Lakes, NJ, USA). Then transfected cells with no serum medium were plated into the upper inserts. The lower chambers were surrounded by 10% FBS medium (Gibco). The invaded cells were fixated with methanol (Beyotime, Beijing, China) and stained with 0.1% crystal violet (Beyotime) after 48 h. The invaded cells were counted in 10 randomized fields.

Western Blot

The proteins of cells were extracted using RIPA Mix (Beyotime). The proteins were separated by 10% SDS-PAGE and transferred to the PVDF membrane (Millipore, Billerica, MA, USA). The membrane was soaked with 5% nonfat milk and then incubated with homeobox A1 (HOXA1) or GAPDH antibody (Abcam, Cambridge, MA, USA) at 4°C overnight. The membrane was then incubated with secondary antibody (Biogot Technology, Nanjing, China). The protein bands were visualized via the enhanced chemiluminescence detection system (Millipore).

Luciferase Reporter Assay

The psiCHECK-2 vector (Promega, Madison, WI, USA) was cloned with the sequences of the HOXA1 3′-UTR, which contained the miR-433 predicted target site. The former luciferase reporter vector was then transfected with or without miR-433 mimic. The Dual-Luciferase Reporter Assay System (Promega) was applied to detect luciferase activity. *Renilla* luciferase activity was used as the normalized control.

Statistical Analysis

The results are shown as mean ± SD. All experiments were independently repeated three times. SPSS statistical software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Expression of miR-433 Was Significantly Lower in Colon Cancer

We collected 20 paired colon cancer tissues and adjacent normal tissues. We isolated the total RNA of these tissues and detected the expression pattern of miR-433. The results showed that the expression of miR-433 was significantly decreased in colon cancer tissues when compared with the adjacent normal tissues (Fig. 1A). Furthermore, we extracted the total RNA from five colon cancer cell lines including Caco-2, LOVO, HT29, SW480, and SW620. Quantitative real-time reverse transcription PCR was performed to detect the expression of miR-433 in these colon cancer cell lines. Compared with control cells, the expression of miR-433 was significantly downregulated in colon cancer cell lines (Fig. 1B).

miR-433 Suppressed Colon Cancer Cell Proliferation and Invasion

SW480 cells were selected for the overexpression of miR-433 for its low endogenous profile. For the experimental group, miR-433 mimic was transfected into the cells. For the control group, miR-433 mimic control (mimic NC) was transfected into the cells. The efficiency of transfection was validated by quantitative real-time reverse transcription PCR (Fig. 2A). The results indicated that overexpression of miR-433 significantly suppressed the proliferation and invasion abilities of the cells (Fig. 2C and E).

SW620 cells were selected for the lost function experiment due to their high endogenous profile. The cells of the experimental group were transfected with miR-433

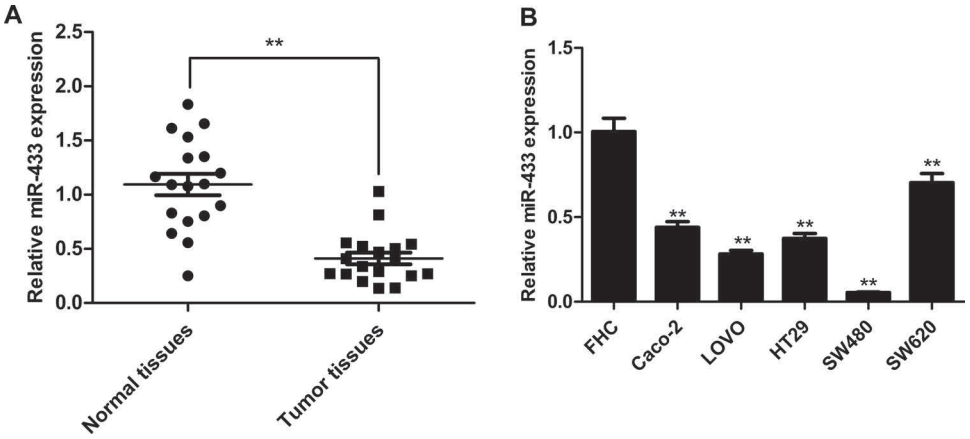

Figure 1. miR-433 was notably downregulated in colon cancer tissues and cell lines. (A) Compared with adjacent normal tissues, the expression of miR-433 was remarkably decreased in colon cancer tissues. $^{**}p<0.01$. (B) Compared with control cells, the expression of miR-433 was remarkably decreased in colon cancer cell lines. $^{**}p<0.01$ versus FHC.

inhibitor and the control group with miR-433 inhibitor control (inhibitor NC). The efficiency of transfection was also validated (Fig. 2B). The functional experiments showed that knockdown of miR-433 significantly enhanced the proliferation and invasion abilities of the cells (Fig. 2D and F).

HOXA1 Was a Direct Target of miR-433

miRNAs usually exert their function via binding to the 3′-UTR of the target gene. The targets of miRNA were usually predicted by a computer algorithm. TargetScan 7.1 was used for the prediction of the target of miR-433. We found that the 3′-UTR of HOXA1 contains a putative 7-bp binding site for miR-433 (Fig. 3A). We conducted dual-luciferase reporter vectors including HOXA1 wild-type (wt) 3′-UTR and HOXA1 mutant-type (mt) 3′-UTR vector. The dual-luciferase reporter assay showed that overexpression of miR-433 significantly decreased the relative luciferase activity of pmirGLO-HOXA1 wt 3′-UTR in SW480 cells, but there was no difference in the pmirGLO-HOXA1 mt 3′-UTR cells (Fig. 3B).

Furthermore, we evaluated the mRNA and protein levels of HOXA1 in colon cancer cells after overexpression or knockdown of miR-433. The results demonstrated that the mRNA and protein levels of HOXA1 were significantly reduced by miR-433 mimic in SW480 cells (Fig. 3C and D). In LS174t cells, the expression levels of HOXA1 mRNA and protein were significantly increased by miR-433 inhibitor (Fig. 3E and F). These results indicated that miR-433 directly targeted HOXA1.

Effects of miR-433 on Colon Cancer Cells Could Be Attenuated by HOXA1

It is still unknown whether the effects of miR-433 on colon cancer cells could be affected by HOXA1. We synthesized siRNA aimed at knocking down the expression of HOXA1. We detected the efficiency of si-HOXA1 in mRNA levels (Fig. 4A). The efficiency of si-HOXA1 in HOXA1 protein was also validated, and the siRNA was selected for follow-up experiments (Fig. 4B and C). We transfected miRNA control and siRNA control, miRNA-433 inhibitor and siRNA control, miRNA-433 inhibitor and si-HOXA1 into the LS174t cells, respectively, and then detected the mRNA expression of HOXA1 (Fig. 4D). We conducted CCK8 assays and Transwell invasion assays in the cells of the three groups. The experiments demonstrated that knockdown of HOXA1 could abolish the effects of miR-433 on cell proliferation and invasion (Fig. 4E and F). These results indicated that HOXA1 could mediate the effects of miR-433 on colon cancer cells.

DISCUSSION

The aberrant expression pattern of miR-433 had been illustrated in many types of cancers. It had been demonstrated that the expression of miR-433 was significantly lower in retinoblastoma, ovarian cancer, and oral squamous cell carcinoma. Li et al. found that miR-433 was downregulated in retinoblastoma tissues and negatively regulated retinoblastoma cell proliferation, migration, and invasion[13]. It was also found that miR-433 was markedly downregulated in ovarian cancer tissues compared with matched normal ovary tissues. miR-433 significantly suppressed the migration and invasion of ovarian cancer cells and negatively regulated Notch1[14]. The expression level of miR-433 was frequently downregulated in oral squamous cell carcinoma when compared with adjacent normal tissues. Overexpression of

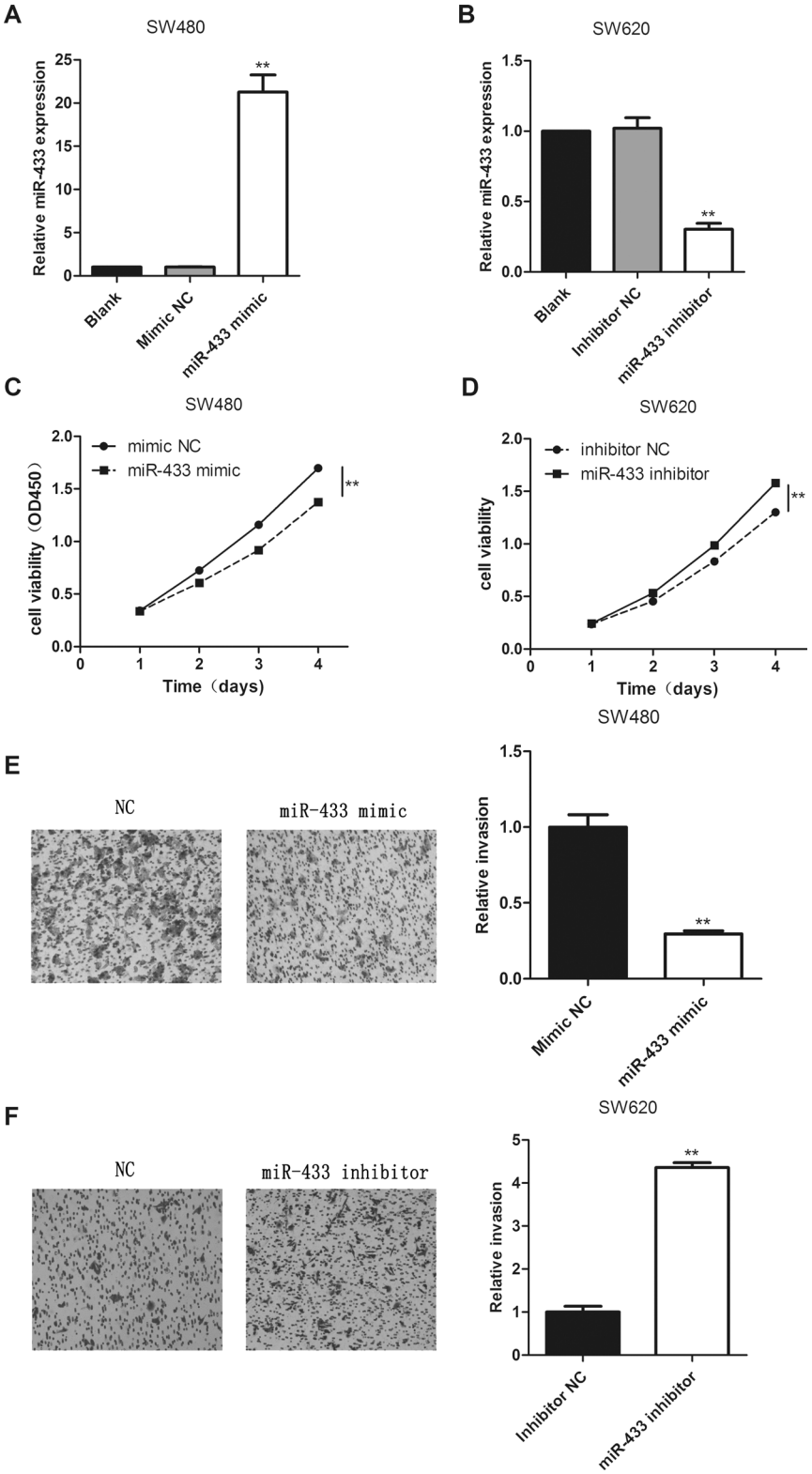

Figure 2. The function of miR-433 on colon cancer cells. (A) The miR-433 mimic significantly increased the expression of miR-433 in SW480 cells. (B) The miR-433 inhibitor significantly inhibited the expression of miR-433 in LS174t cells. (C) Overexpression of miR-433 significantly suppressed the cell proliferation. (D) Knockdown of miR-433 significantly enhanced the cell proliferation. (E) Overexpression of miR-433 significantly suppressed the cell invasion. (F) Knockdown of miR-433 significantly enhanced the cell invasion. $^{**}p<0.01$ versus mimic NC or inhibitor NC.

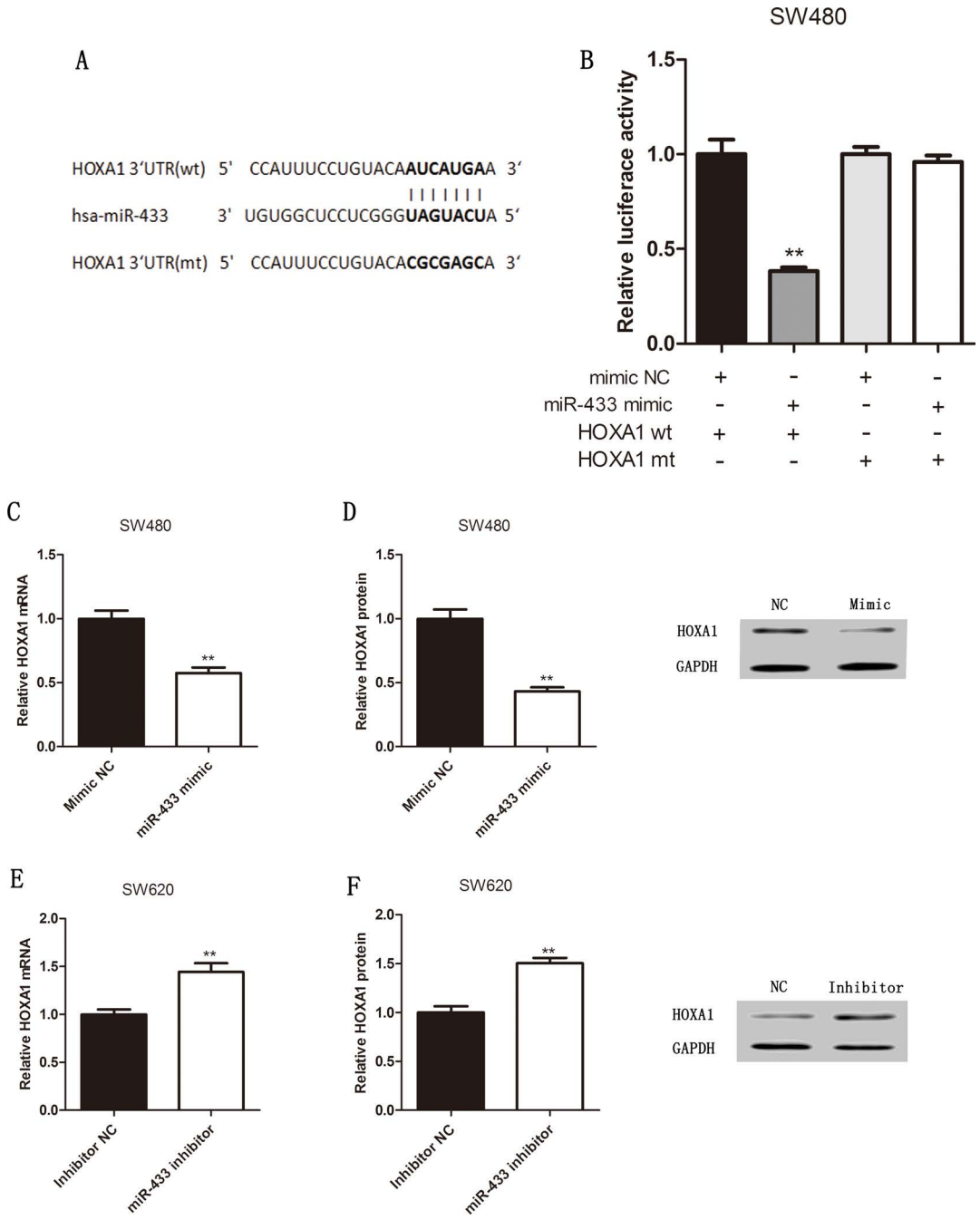

Figure 3. Homeobox A1 (HOXA1) was newly identified as a target of miR-433. (A) The predicted binding sites between miR-433 and HOXA1. (B) Luciferase assay demonstrated that miR-433 directly targeted HOXA1. (C) Overexpression of miR-433 notably decreased the mRNA expression of HOXA1 in SW480 cells. (D) Overexpression of miR-433 notably decreased the protein level of HOXA1 in SW480 cells. (E) Knockdown of miR-433 notably increased the mRNA expression of HOXA1 in LS174t cells. (F) Knockdown of miR-433 notably increased the protein level of HOXA1 in LS174t cells. $^{**}p<0.01$ versus mimic NC or inhibitor NC.

miR-433 dramatically inhibited cell growth, invasion, and migration[15]. Accumulating evidence has suggested the tumor suppressor role of miR-433. Consistent with previous studies, our study indicated that miR-433 was downregulated in colon cancer tissues and colon cancer cell lines. Gain-of-function or loss-of-function experiments suggested that miRNA-433 inhibited the proliferation and invasion of colon cancer cells. These results, combined with former research, indicated the tumor suppressor role of miR-433.

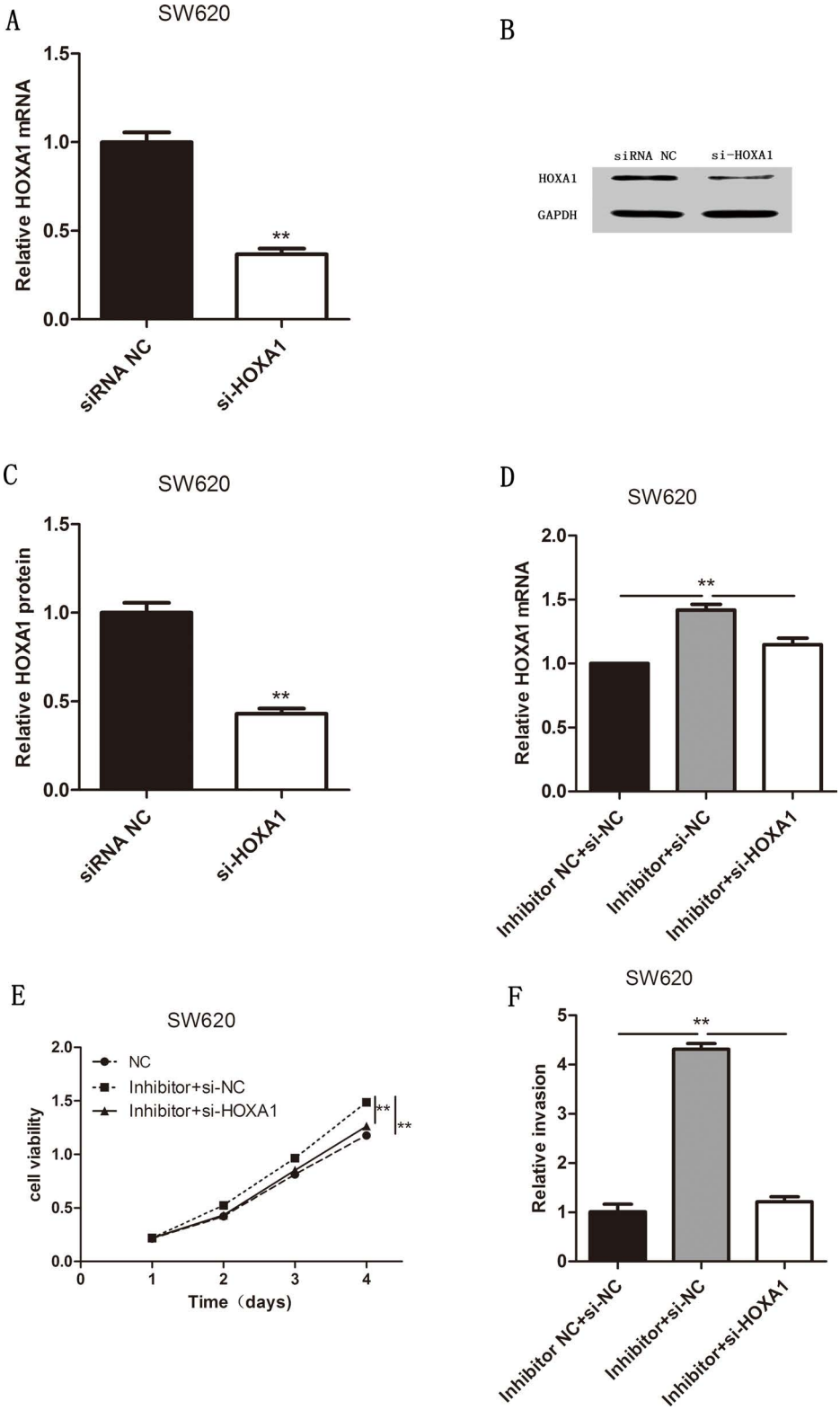

Figure 4. The effects of miR-433 on colon cancer were mediated by HOXA1. (A) The efficiency of siRNA was measured at the mRNA level. (B) The Western blot results of siRNA in silencing HOXA1 protein. (C) siRNA significantly reduced the expression of HOXA1 protein. (D) The mRNA expression of HOXA1 was detected after transfection. (E) The effects of miR-433 inhibitor on proliferation in LS174t cells could be rescued by siRNA2-HOXA1. (F) The effects of miR-433 inhibitor on invasion in LS174t cells could be rescued by siRNA2-HOXA1. $^{**}p<0.01$.

It is well known that miRNAs play their regulatory role via targeting the 3′-UTR site of genes. Some targets of miR-433 had been explored and validated. Xue et al. reported that miR-433 directly targeted p21-activated kinase (PAK4), and the effects of miR-433 on hepatocellular carcinoma could be partially rescued by forced expression of PAK4. Furthermore, the miR-433/PAK4 axis could affect the PI3K/AKT signaling in HepG2 cells[16]. Another research identified cAMP response element-binding protein (CREB1) as a direct target of miR-433, and CREB1 mediated the effects of miR-433 on hepatocellular carcinoma cells[17]. The other targets of miR-433 included histone deacetylase 6 (HDAC6), Notch1, paired box 6 (PAX6), and growth factor receptor bound protein 2 (GRB2)[18].

HOXA1 is one of the members of the homeodomain-containing transcription factor family, which includes HOXA1, HOXA7, HOXB8, HOXB9, HOXC4, HOXC5, HOXC6, HOXC9, and HOXD3[19]. It is widely accepted that HOXA1 affects many physiological processes in cells, including growth, differentiation, development, and organogenesis[20]. It also has been illustrated that the aberrant expression of HOXA1 could influence the growth, apoptosis, and oncogenic transformation of cancer cells[21–24]. Furthermore, high expression of HOXA1 was capable of initiating oncogenic transformation in normal human mammary epithelial cells[25]. The expression of HOXA1 was also found to be associated with clinical features in small cell lung cancer[26]. Herein we newly identified HOXA1 as a direct target of miR-433. In addition, we found that the function of miR-433 on colon cancer was mediated by HOXA1. Our exploration of HOXA1 helped to reveal its crucial role in cancer cells.

In conclusion, our study found that miR-433 was frequently downregulated in colon cancer tissues and cell lines. Overexpression of miR-433 significantly inhibited the proliferation and invasion of colon cancer. We also newly identified HOXA1 as a direct target of miR-433. The effects of miR-433 on colon cancer cells were mediated via HOXA1.

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