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Long non-coding RNA FOXD2-AS1 functions as a tumor promoter in colorectal cancer by regulating EMT and Notch signaling pathway

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Abstract. – OBJECTIVE: A growing number of long noncoding RNAs (lncRNAs) are emerging as new modulators in cancer origination and progression. However, the functions and molecular mechanisms of lncRNAs to colorectal cancer (CRC) are still largely unknown. The aim of this study was to investigate the function and role of lncRNA FOXD2-AS1 (FOXD2-AS1) in human CRC.

PATIENTS AND METHODS: The expression of FOXD2-AS1 was investigated using Real-time reverse transcription-polymerase chain reaction (qRT-PCR) in 45 CRC specimens and matched adjacent normal tissues and CRC cell lines. MTT assays were conducted to explore the impact of FOXD2-AS1 knockdown on the proliferation of human CRC cells. The effects of FOXD2-AS1 on CRC cell migration and invasion were evaluated by cell invasion assays and migration assays. Western blot analysis was used to determine the expression levels of EMT-related and Notch-related proteins.

RESULTS: The results showed that FOXD2-AS1 expression was significantly increased in CRC tissues as well as in CRC cell lines. Moreover, down-regulation of FOXD2-AS1 suppressed cell, proliferation, invasion and migration in vitro. Importantly, we further confirmed that EMT and the Notch signaling pathway were inactivated in CRC cells after FOXD2-AS1 knockdown.

CONCLUSIONS: FOXD2-AS1 promoted the progression of CRC by regulating EMT and Notch signaling pathway. Thus, targeting FOXD2-AS1 may be an effective strategy for CRC treatment.

Key Words
Long noncoding RNA, FOXD2-AS1, Colorectal cancer, EMT, Notch signaling pathway.

Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide, with a high incidence and mortality[1]. In Europe in 2010, CRC was the third most common malignant cancer in both men and women with 250,000 cases of colorectal cancer diagnosed[2]. Although recent advances have been made in diagnostic procedures and therapeutic strategies, the 5-year survival rate for metastatic CRC is only 10-15%[3,4]. Therefore, it is urgent to elucidate the potential mechanism involved in CRC metastasis, which will provide diagnostic and potential targets for therapeutics of CRC patients with metastasis. Recently, more researchers have focused on the effects of lncRNAs on CRC. Recently, non-coding RNAs (ncRNAs) has grasped researchers' attention due to its important in regulating biological progression[5]. NcRNAs are divided into three categories: housekeeping RNAs, small non-coding RNAs, and long non-coding RNAs[6]. Long noncoding RNAs (lncRNAs) are types of transcriptional products of the eukaryotic genome comprising > 200 nt in length[7]. Increasing evidence indicated that lncRNAs may serve as master gene regulators capable of controlling protein-coding and noncoding genes[8]. Furthermore, aberrant lncRNA expression may be a major contributor to tumourigenesis[9,10]. Some well-known lncRNAs have deeply been studied[11,12]. However, the role of most lncRNAs and its potential mechanism was unclear. Long non-coding RNA FOXD2-AS1 (FOXD2-AS1) was a newly identified lnRNA. Previous studies[13,14] indicated that FOXD2-AS1 expression was up-regulated in non-small cell lung cancer and gastric cancer. However, the role of FOXD2-AS1 in progression of CRC remains unknown. It was known to us that EMT and the Notch pathway regulate initiation and progression of various cancers[15,16]. Our present study was designed to detect the expression pattern of FOXD2-AS1 in CRC tissues and to explore whether FOXD2-AS1 could influence EMT and the Notch pathway.

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Patients and Methods

Patients and Tissue Samples

The tissue used in the current study included 45 CRC tissues and paired non-cancerous colorectal tissue samples from patients who underwent surgical treatment at Shanxi Medical University between 2014 and 2016. The patients were confirmed as primary CRC by pathological diagnosis. None of the patients had received chemotherapy or radiotherapy before surgery excision. The Research Ethics Committee of Shanxi Medical University provided Ethical Approval for this study, and all patients provided written informed consent.

Cell Culture and RNA Interference

The normal fetal human colon epithelial cell line CRL-1831 and human CRC cell lines RKO, HCT15, HCT28, HCT116, and SW480, were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Pudong, Shanghai, China). All CRC cell lines were grown in Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA) Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), Sigma-Aldrich (St. Louis, MO, USA). Cultures were incubated at 37°C with 5% CO_2 in a humidified incubator. Small interfering RNAs (siRNAs) and scrambled negative control siRNA (si-NC) were purchased from Invitrogen (Invitrogen, Carlsbad, CA, USA) and were used for FOXD2-AS1 inhibition. The sequences of FOXD2-AS1 was 5'GCTTCCAGGTATGTGGGAA3'. si-FOXD2-AS1 and si-NC were transfected into the cells reaching the exponential growth phase using HiPerFect transfection reagent (Qiagen, Valencia, CA, USA) according to manufacturer's instructions, respectively.

RNA Extraction and Real-Time Quantitative RT-PCR Assay

Total RNA was isolated from cells or harvested tissues. cDNA synthesis was performed using reverse transcription reagents (Thermo Scientific, Waltham, MA, USA). To measure mRNA expression, Real-time PCR was performed with a miScript SYBR Green PCR Kit (Qiagen, Zeeland, The Netherlands). Relative quantification of lncRNA expression was evaluated using the comparative cycle threshold (CT) method. The expression levels of FOXD2-AS1 were normalized to GADPH. The primers of FOXD2-AS1 and GADPH were purchased from RiboBio (Guangzhou, Guangdong, China). The specific primers are listed as follows: FOXD2-AS1-forward:5'TGGACCTAGCTGCAGCTCCA3', FOXD2-AS1-reverse: 5'AGTTGAAGGTGCACACACTG3'; GAPDH-forward: 5'-GCGAGATCGCACTCATCATCT-3'; GAPDH-reverse: 5'-TCAGTGGTGGACCTGACC-3'.

Cell Proliferation Assay

At 24 h after transfection, cells were seeded into 96-well plates (1×10^3 cells/well). Cell viability was assessed by the CKK-8 assay. The proliferation rate was measured at 0, 24, 48, 72 h after transfection. The optical density was measured at 450 nm on a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Each sample was tested in triplicate and all experiments were performed three times.

Migration and Invasion Assay

Cell migration and invasion were carried out using the BD BioCoat Matrigel Invasion Chamber (BD Bioscience, Franklin Lakes, NJ, USA). 5×10^4 cells were harvested and placed in the upper chamber without serum. The lower chamber contained 10% fetal bovine serum (FBS) was used as a chemo-attractant. After chambers were incubated for 6 h (for migration) or 24 h (for invasion) 37°C in a humidified incubator with 5% CO^2, they were disassembled and the membranes were stained with 2% crystal violet and placed on a glass slide. The invaded and migrated cells were photographed and counted in five randomly selected fields for each well using a light microscope (Olympus, Tokyo, Japan).

Western Blot Analysis

Protein isolation and Western blotting were performed according previous study[17]. The following antibodies were used: Hes-1 (1:300), NICD (1:300), GAPDH (1:500), E-cadherin (1:300), N-cadherin(1:300), vimentin (1:300) (Tianjin Saier Biotech, Tianjin, China). GAPDH was used as control. Protein expression was detected by a chemiluminescence kit (Amersham Biosciences, Little Chalfont, UK).

Statistical Analysis

Statistical analysis was performed using SPSS software 17.0 (SPSS Inc., Chicago, IL, USA). Data were presented as mean ± SEM. Comparisons between group were performed by using paired or non-paired Student's *t*-test. Statistical significance was defined as $p < 0.05$.

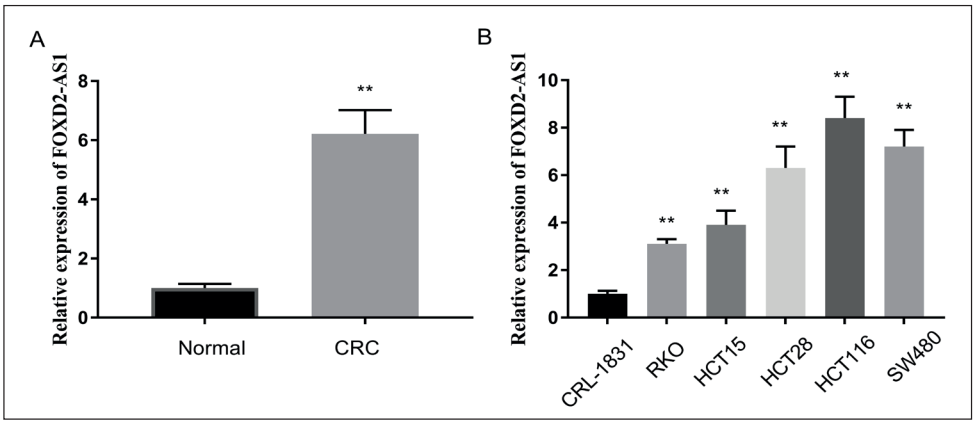

Figure 1. FOXD2-AS expression was upregulated in human CRC tissues and cell lines. **A**, qRT-PCR analysis of FOXD2-AS expression in RKO, HCT15, HCT28, HCT116, and SW480 cells. **B**, qRT-PCR analysis of FOXD2-AS expression in human CRC samples and corresponding normal tissues. *$p < 0.05$, **$p < 0.01$.

Results

Expression of FOXD2-AS1 in CRC Tissues and Cell Lines

In order to study the potential mechanism of FOXD2-AS1 within CRC, we performed RT-PCR assay to determine the expression levels of FOXD2-AS1 in both CRC tissues and cell lines. As shown in Figure 1A, mean FOXD2-AS1 levels were significantly higher in CRC tissues compared to normal colorectal tissues ($p < 0.05$). In addition, we observed that FOXD2-AS1 level was increased in CRC cells (RKO, HCT15, HCT28, HCT116, and SW480) compared with that in CRL-1831 cells (all $p < 0.01$, Figure 1B).

Knockdown of FOXD2-AS1 Inhibits CRC Growth

To explore the potential role of FOXD2-AS1 in CRC, we performed loss-of-function studies in HCT116 and SW480. As shown in the Figure 2A, FOXD2-AS1 was significantly down-regulated following transfection with si-FOXD2-AS1. Knockdown of FOXD2-AS1 resulted in a significant decrease in cell viability, as detected by CKKK-8 assays (Figure 2B and 2C). These results demonstrated that FOXD2-AS1 significantly promoted CRC cell proliferation.

Depletion of FOXD2-AS1 Inhibited Migration and Invasion of CRC Cells

To explore whether FOXD2-AS1 was related to tumor metastasis, we detected the role of FOXD2-AS1 on cell migration and invasion. HCT116 and SW480 cells were transfected with si-FOXD2-AS1 or si-NC, after 24 h, as shown in Figure 3A. The results of transwell showed that depletion of FOXD2-AS1 expression appeared to inhibit cell migration in both HCT116 and SW480 cells ($p < 0.01$, respectively). At the same time, depletion of FOXD2-AS1 also could inhibit cell invasion in both HCT116 and SW480 cells (Figure 3B, $p < 0.01$, respectively).

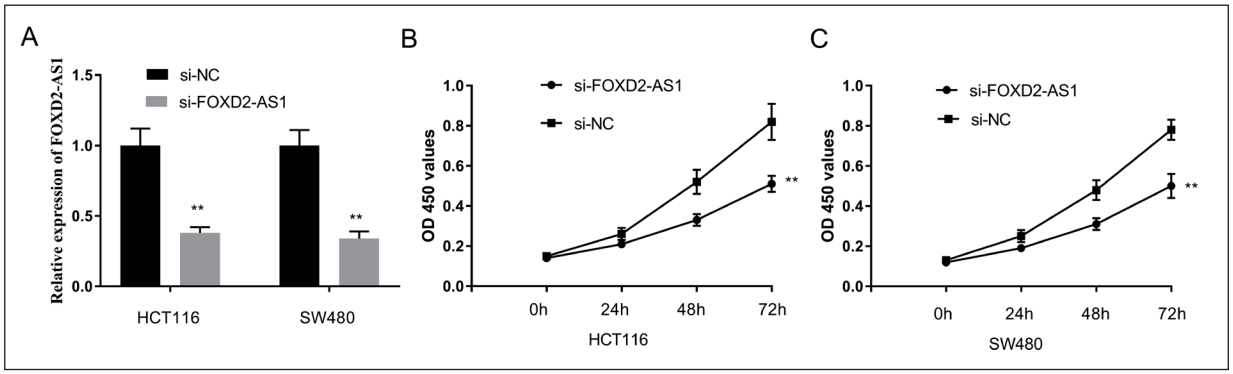

Figure 2. Knockdown of FOXD2-AS suppressed the proliferation of HCT116 and SW480 cells. **A**, Validation of FOXD2-AS expression levels after transfection by PCR analysis. (**B**-**C**) Proliferation of HCT116 and SW480 cells with aberrant expression of FOXD2-AS was evaluated by CKK-8 assay. *$p < 0.05$, **$p < 0.01$

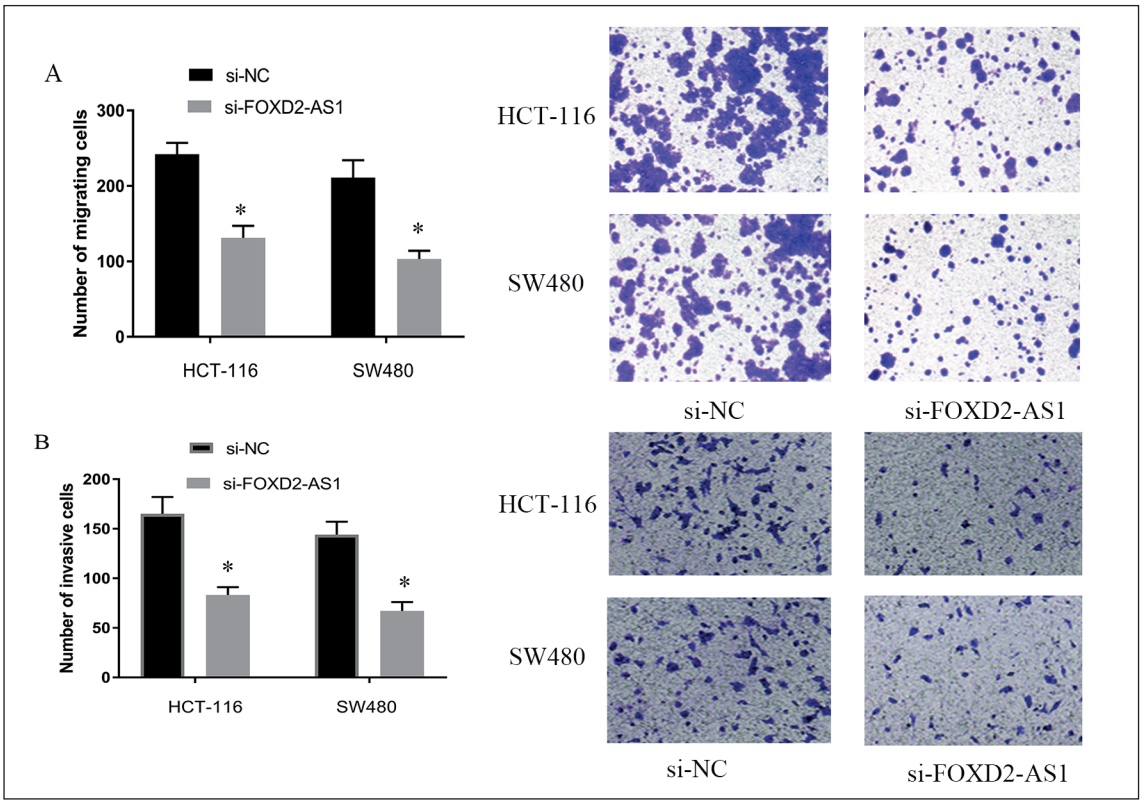

Figure 3. Knockdown of FOXD2-AS inhibited migration and invasion in CRC cells. **A**, The transwell invasion assay determined cell migration in HCT116 cells transfected with anti-FOXD2-AS or miR-NC. **B**, The transwell invasion assay determined cell migration in SW480 cells transfected with si-FOXD2-AS or miR-NC. All experiments were repeated independent three times. *$p < 0.05$, **$p < 0.01$.

FOXD2-AS1 Exerted its Effect by Regulating EMT and Notch Signaling Pathways

It was known that EMT and Notch signaling pathways were closely related to cancer cell metastasis ability; we examined related markers of both signaling pathways in si-FOXD2-AS1 transfected and control CRC cells. As shown in Figure 4A, knockdown of FOXD2-AS1 increased the epithelial marker E-cadherin protein expression and decreased N-cadherin and Snail protein expression. Also, we observed that knockdown of FOXD2-AS1 decreased Hes-1 and NICD protein expression (Figure 4B). These results revealed that FOXD2-AS1 might promote CRC development via the EMT and Notch signaling pathways.

Discussion

Previous researches[18] have indicated that lncRNAs served as oncogenes or tumor suppressor in various tumors including CRC. For instance, Wang et al[19] reported that lncRNA AFAP1-AS1 is significantly up-regulated in CRC, which depletion resulted in the inhibition of CRC cell proliferation and colony formation. Kong et al[20] found that lncRNA LINC01133 knockdown promoted EMT and metastasis in CRC cells by interacting with SRSF6. Another study by Han et al[21] revealed that up-regulation of lncRNA RNA H19 was associated with a poor prognosis of CRC and promoted tumor growth by recruiting and binding to eIF4A3. Those results highlighted the role and function of lncRNAs in development and progression of CRC. Recently, Li et al[13] reported that up-regulated expression of FOXD2-AS1 was observed in gastric cancer. They further performed Kaplan-Meier method and confirmed that FOXD2-AS1 predicted poor prognosis of gastric cancer. Rong et al[14] found that FOXD2-AS1 is significantly up-regulated in non-small cell lung cancer tissues. Furthermore, they provided evidence that FOXD2-AS1 served as a tumor promoter in non-small cell lung cancer by modulating Wnt/b-catenin signaling. These findings revealed a tumor-promoting role in both

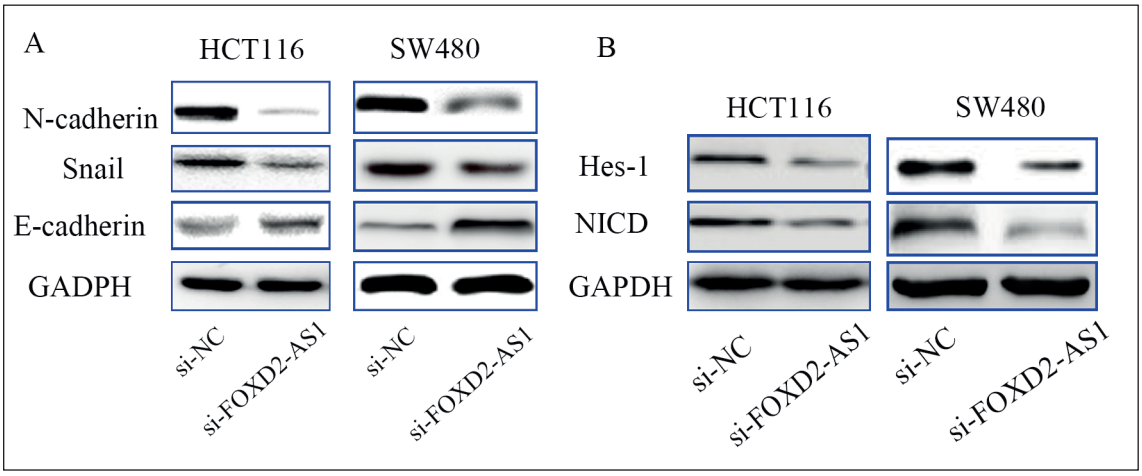

Figure 4. Effect of FOXD2-AS on the EMT and Notch pathway. **A**, Western blot analysis of the phenotypic markers, including E-cadherin, N-cadherin and Vimentin in HCT116 and SW480 cells. **B**, Notch-related proteins (Hes-1 and NICD) were detected by Western blot after HCT116 and SW480 cells transfected with si-FOXD2-AS or si-NC. *$p < 0.05$, **$p < 0.01$.

gastric cancer and non-small cell lung cancer. However, to our best knowledge, it was unknown whether FOXD2-AS1 also plays an important role in CRC. In the present work, we firstly detected the expression levels of FOXD2-AS1 in CRC tissues and matched normal tissues by PCR. Our results showed that FOXD2-AS1 expression was significantly un-regulated in CRC tissues compared with that in normal tissues. Then, we performed *in vitro* assay in CRC cell lines. We observed that knockdown of FOXD2-AS1 significantly suppressed CRC cell proliferation, migration and invasion. Based on above results, FOXD2-AS1 may serve as an oncogene in CRC. In order to explore underlying molecular mechanism of FOXD2-AS1 promoting CRC progress, our attention focused on EMT and Notch signaling pathways. EMT is a physiological mechanism which is present during development, and its presence was observed in human colon during colorectal carcinogenesis and tumor invasion[22,23]. It has been confirmed that many genes could regulate EMT signaling pathways[24,25]. Notch signaling pathways play key roles not only in maintaining the growth and proliferation of CRC, but also in metastasis of CRC[26,27]. Importantly, Notch is actively involved in a process known as EMT. In the current study, we confirmed that FOXD2-AS1 inhibition significantly influenced the expression levels of EMT-related proteins and Notch signal-related proteins. These findings suggested that FOXD2-AS1 might promote CRC development via regulated EMT and Notch signaling pathways.

Conclusions

This is the first report showing that FOXD2-AS1 serves as a tumor promoter by regulating EMT and Notch signaling pathways in CRC. Thus, FOXD2-AS1 appears to be a critical player in CRC, so it may become one of the most important lncRNAs for future investigations in CRC.

Conflict of Interests:

The Authors declare that there are no conflicts of interest.

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