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Low expression of microRNA-1266 promotes colorectal cancer progression via targeting FTO

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Abstract. – OBJECTIVE: To explore the role of microRNA-1266 in colorectal cancer (CRC) and its underlying mechanism.

PATIENTS AND METHODS: The expression level of microRNA-1266 in 48 CRC tissues and paracancerous tissues was detected by quantitative Real-time-polymerase chain reaction (qRT-PCR). The relationship between microRNA-1266 expression and basic characteristics of CRC patients was analyzed. The effect of microRNA-1266 on the viability of CRC cells was detected by cell counting kit-8 (CCK-8) assay. Subsequently, a potential target gene for microRNA-1266 was predicted through bioinformatics. Finally, the binding condition between microRNA-1266 and the target gene was verified by RNA binding protein immunoprecipitation (RIP) and luciferase reporter gene assay, respectively.

RESULTS: MicroRNA-1266 was lowly expressed in 48 cases of CRC tissues than that of paracancerous tissues. Clinical data demonstrated that microRNA-1266 expression was correlated to tumor size and TNM of CRC patients. Knockdown of microRNA-1266 promoted proliferation of CRC cells. FTO was predicted to be the target gene for microRNA-1266, which was negatively regulated by microRNA-1266.

CONCLUSIONS: MicroRNA-1266 is lowly expressed in CRC tissues than that of paracancerous tissues. Lowly expressed microRNA-1266 promotes the occurrence and progression of CRC by directly targeting FTO.

Key Words:
Colorectal cancer, MicroRNA-1266, FTO, Cell viability.

Introduction

Colorectal cancer (CRC) is one of the common malignant tumors of the digestive tract. Globally, there are approximately 1 million new cases of CRC each year, and the mortality rate is about 50%. In China, the incidence of CRC has been increased annually with the changes of diet and living habits[1,2]. CRC pathogenesis involves multiple genes and complex biological processes. It is of great significance to explore the mechanism in CRC development, which provides new directions in the early diagnosis and novel treatment for CRC. MicroRNAs are non-coding RNAs with approximately 22 nucleotides in length. MicroRNAs could degrade and downregulate target gene by complementation with the 3' UTR of target gene mRNA, thereby participating in disease development[3]. MicroRNA-1266 is differentially expressed in a variety of tumor cells, affecting tumor growth, invasion and apoptosis[4-6]. However, the specific role of microRNA-1266 in CRC has been rarely reported. In this study, we first detected microRNA-1266 expression in CRC tissues and cell lines. *In vitro* experiments were further carried out to detect the possible roles and mechanisms of microRNA-1266 in regulating CRC. Our study aims to provide new strategies for early diagnosis and treatment for CRC.

Patients and Methods

Sample Collections

CRC tissue and corresponding paracancerous tissues were surgically resected from 48 CRC patients. Samples were immediately stored in liquid nitrogen. All patients completed the informed consent form. The basic characteristics of CRC patients were listed in Table I, including age, gender, tumor diameter, tumor node metastasis (TNM) stage, lymph node metastasis and pathological grade. This study was approved by

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the Ethics Committee of The Affiliated Wujiang Hospital of Nantong University. Signed written informed consents were obtained from all participants before the study.

Cell Culture

Human CRC cell lines (Caco-2, HTC116 and SW620) and human normal colorectal mucosal cell line (FHC) were cultured in RPMI-1640 (Roswell Park Memorial Institute-1640, Gibco, Grand Island, NY, USA) containing 10% FBS (fetal bovine serum), 100 U/mL penicillin and 100 μg/mL streptomycin (HyClone, South Logan, UT, USA). Cells were incubated in a 5% CO_2 incubator at 37°C. Cell passage was performed using trypsin when the cell confluence was up to 85%.

Cell Transfection

One day prior to cell transfection, CRC cells in good growth condition were seeded into 6-well plates at a density of 3×10^5 per well. Cells were transfected with corresponding plasmids when the confluence was up to 50% following the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Culture medium was replaced 4 h later.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (Qrt-PCR)

Total RNA in treated cells was extracted using TRIzol method (Invitrogen, Carlsbad, CA, USA) for reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). RNA concentration was detected using spectrometer and those samples with A260/A280 ratio of 1.8-2.0 were selected for the following qRT-PCR reaction. QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq TM (TaKaRa, Otsu, Shiga, Japan). The relative gene expression was calculated using $2^{-\Delta Ct}$ method. Primers used in the study were as follows: MicroRNA-1266, F: 5'-CAATTATTCAAAAGATGCTTGGG-3', R: 5'-GGTATGGCCCTCCAGGTTAA-3'; FTO, F: 5'-TCAACTGGAAGCACTGTGG-3', R: 5'-AGGCAAGGATGGCAGTCAA-3'; GAPDH, F: 5'-GTTGGAGGTCGGAGTCAACGG-3', R: 5'-GAGGGATCTCGCTCCTGGAGGA-3'.

Cell Counting Kit-8 (CCK-8) Assay

Transfected cells were seeded into 96-well plates at a density of 3×10^3/μL. 10 μL of CCK-8 solution (cell counting kit-8, Dojindo, Kumamoto, Japan) were added in each well after cell culture for 6, 24, 48, 72 and 96 h, respectively. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA). Each group had 5 replicates.

Bioinformatics Prediction

The targeting binding condition of microRNA-1266 and FTO was predicted using online software, including TargetScan, miRanda and PicTar3.

Table I. Correlation of microRNA-1266 expression with clinicopathologic features in patients with colorectal cancer (n = 48).

Clinicopathological features	Number	miR-1266 expression		*p*-value
		Low (n=24)	High (n=24)	
Age (years)				0.7726
≥ 60	25	12	13	
< 60	23	12	11	
Gender				0.2482
Male	24	10	14	
Female	24	14	10	
Tumor size (cm)				0.0002*
≥ 5	23	18	5	
< 5	25	6	19	
TNM stage				0.0014*
I+II	21	5	16	
III+IV	27	19	8	
Lymph node metastasis				0.7628
Absent	31	15	16	
Present	17	9	8	

Luciferase Reporter Gene Assay

The binding site of microRNA-1266 and FTO was predicted for constructing wild-type FTO (FTO wt) and mutant-type FTO (FTO mut). CRC cells were seeded in the 96-well plates and co-transfected with 50 pmol/L microRNA-1266 mimics or negative control and 80 ng FTO wt or FTO mut, respectively. Cells were lysed and incubated for 15 min at room temperature, followed by detection of Firefly-Luc/Renilla Luc (Thermo Fisher Scientific, Waltham, MA, USA).

RNA Binding Protein Immunoprecipitation (RIP)

Cells were washed and cross-linked with 0.01% formaldehyde for 15 min. After centrifugation and cell lysis, cells extracted were incubated with RIP buffer containing protein A/G magnetic beads coated with anti-Ago2 or negative control anti-IgG antibody (Abcam, Cambridge, MA, USA). After overnight incubation at 4°C, cells were incubated with Protein A Agarose for 1 h at 4°C, followed by the isolation of RNA. MicroRNA-1266 level was then detected by qRT-PCR.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 statistical software (IBM, Armonk, NY, USA) were used for data analysis. Measurement data were expressed as mean ± standard deviation ($\bar{x}\pm s$) and compared using the *t*-test. $p<0.05$ considered the difference was statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Results

MicroRNA-1266 was Lowly Expressed in CRC

Clinical data demonstrated that microRNA-1266 expression had no correlation with age, gender and lymph node metastasis of CRC patients. However, microRNA-1266 expression was correlated to tumor size and TNM of CRC patients (Table I). QRT-PCR results showed that microRNA-1266 was lowly expressed in 48 cases of CRC tissues than that of paracancerous tissues (Figure 1A, $p<0.001$). By analyzing the relationship between microRNA-1266 expression and clinical data of CRC patients, we found that the overall survival of CRC patients with higher level of microRNA-1266 was longer than those with lower level (Figure 1B, $p=0.0392$, HR=0.345). Furthermore, microRNA-1266 was lowly expressed in larger CRC tissues (tumor size ≥ 5 cm) compared with those smaller CRC tissues (tumor size < 5 cm) (Figure 1C, $p<0.001$). In addition, microRNA-1266 expression was negatively correlated to TNM stage (Figure 1D, $p<0.001$).

MicroRNA-1266 Expressions in CRC Cell Lines

MicroRNA-1266 expressions in human CRC cell lines (Caco-2, HTC116 and SW620) and human normal colorectal mucosal cell line (FHC) were detected by qRT-PCR. The data indicated that SW620 cells expressed the highest and HTC116 cells expressed the lowest level of microRNA-1266 (Figure 2A). Subsequently, microRNA-1266 mimic and inhibitor were constructed. Transfection efficacies of microRNA-1266 mimic and inhibitor in SW620 and HTC116 cells were verified by qRT-PCR, respectively (Figure 2C and 2D).

MicroRNA-1266 Inhibited Proliferation of CRC Cells

CCK-8 results suggested that microRNA-1266 knockdown increased viability of SW620 cells (Figure 2E, $p<0.001$). On the contrary, the overexpression of microRNA-1266 decreased viability of HTC116 cells (Figure 2F, $p<0.001$). The above results indicated that microRNA-1266 serves as a tumor-suppressor gene in CRC.

MicroRNA-1266 Directly Regulated FTO

QRT-PCR results showed that FTO expression in 48 CRC tissues was remarkably higher than that of paracancerous tissues (Figure 2B, $p<0.001$). The correlation analysis revealed that microRNA-1266 expression was negatively correlated to FTO in CRC (Figure 3B, $p=0.0006$, $R^2=0.2264$). Further bioinformatics prediction analysis demonstrated that FTO is a potential target gene for microRNA-1266.

RIP was performed to confirm whether FTO interacts with microRNA-1266. It is well known that Ago2 is present in the core of the RNA-induced silencing complex (RISC) and miRNAs silence the target gene by interacting with RISC[7]. Compared with the control group, microRNA-1266 was mainly enriched in the Ago2 co-precipitation group (Figure 3C and 3D, $p<0.001$). Subsequently, we predicted the binding site of microRNA-1266 and FTO through TargetScan and constructed the FTO wt and FTO mut sequences (Figure 3A). Dual-luciferase reporter assay results showed that the fluorescence value of cells co-transfected with

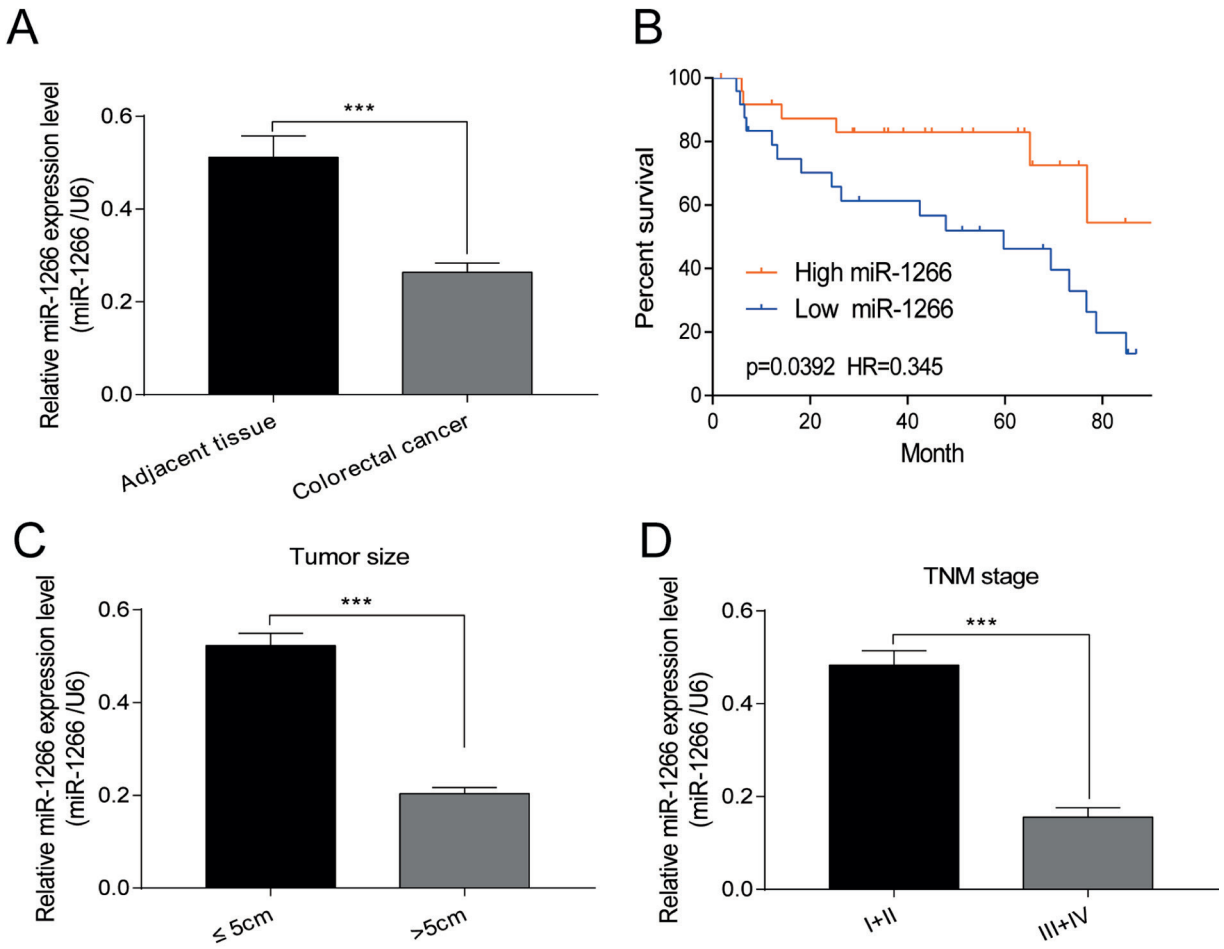

Figure 1. MicroRNA-1266 was lowly expressed in CRC. ***A,*** MicroRNA-1266 was lowly expressed in 48 cases of CRC tissues than that of paracancerous tissues. ***B,*** Overall survival of CRC patients with higher level of microRNA-1266 was longer than those with lower level. ***C,*** MicroRNA-1266 was lowly expressed in larger CRC tissues (tumor size ≥ 5 cm) compared with those smaller CRC tissues (tumor size < 5 cm). ***D,*** MicroRNA-1266 expression was negatively correlated to TNM stage.

FTO wt and microRNA-1266 mimics were remarkably decreased (Figure 3E and 3F, $p<0.001$). The above results indicated that microRNA-1266 directly regulates FTO.

Discussion

It has been reported[8] that more than 30% of protein-coding genes can be directly regulated by miRNAs. Accumulating evidence has shown that miRNAs are involved in tumor growth, differentiation, invasion, metastasis, and angiogenesis[3]. Recent studies[9-12] have found some certain microRNAs are differentially expressed in tumor tissues, which could be served as diagnostic markers in malignancies. MicroRNA-1266 is involved in tumorigenesis and progression. However, microRNA-1266 exerts different roles in tumors as an oncogene or tumor-suppressor gene. In this study, the expression level of microRNA-1266 in CRC tissues was lower than that in adjacent tissues. Besides, microRNA-1266 expression was closely related to tumor size and TNM stage of CRC, indicating its clinical value in CRC. Subsequent function experiments indicated that overexpression of microRNA-1266 reduces viability of CRC cells. Hence, we considered that microRNA-1266 may be a tumor-suppressor gene in CRC, which could inhibit the proliferation of CRC cells.

Through bioinformatics prediction, FTO was found to be a potential target gene for microRNA-1266. FTO was the first candidate gene found to be associated with obesity in the general population through genome-wide association analysis

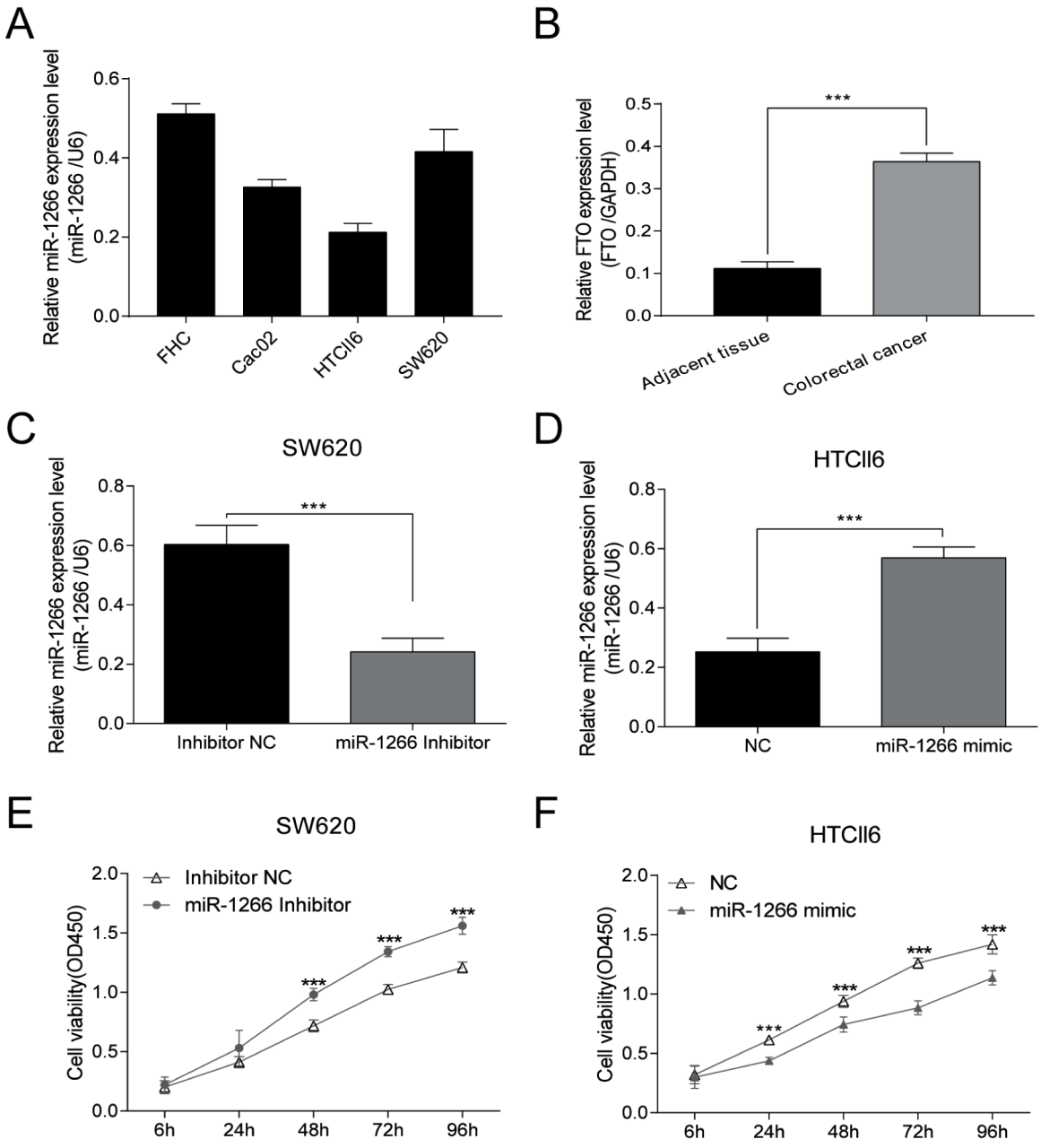

Figure 2. MicroRNA-1266 inhibited proliferation of CRC cells. ***A,*** SW620 cells expressed the highest and HTC116 cells expressed the lowest level of microRNA-1266. ***B,*** FTO expression in 48 CRC tissues was remarkably higher than that of paracancerous tissues. ***C, D,*** Transfection efficacies of microRNA-1266 mimic and inhibitor in SW620 and HTC116 cells were verified by qRT-PCR. ***E,*** MicroRNA-1266 knockdown increased viability of SW620 cells. ***F,*** Overexpression of microRNA-1266 decreased viability of HTC116 cells.

(17434869)[13]. Further researches confirmed that obesity and FTO are not only related to metabolic syndrome, but also related to tumor development[14,15]. The correlation analysis in 48 CRC tissues found that microRNA-1266 expression was negatively correlated to FTO. RIP experiments and dual-luciferase reporter assay further revealed that microRNA-1266 can directly bind to FTO. In summary, we found that microRNA-1266 was lowly expressed in CRC tissues compared with that of paracancerous tissues. MicroRNA-1266 expression was negatively correlated with FTO.

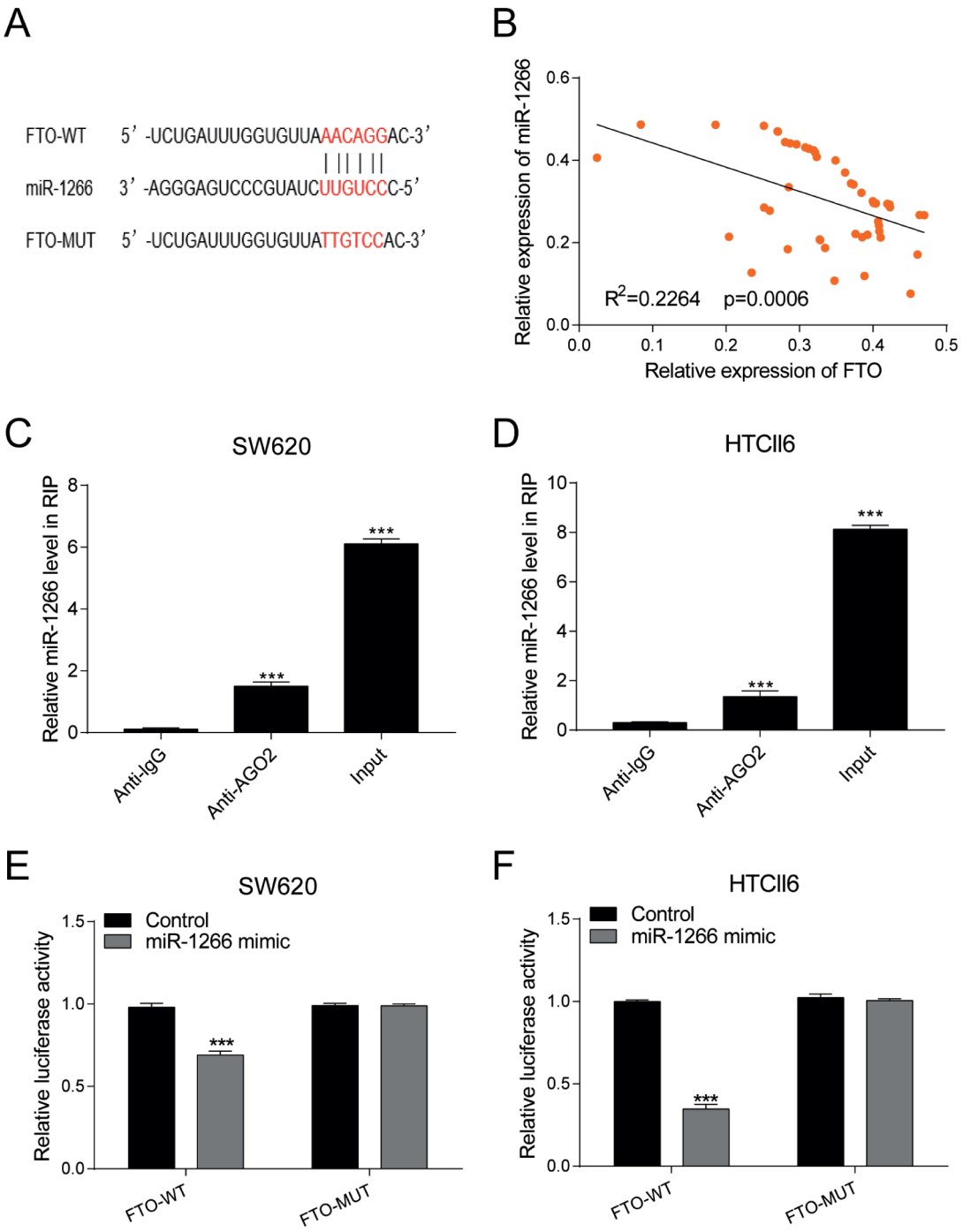

Figure 3. MicroRNA-1266 directly regulated FTO. ***A,*** The binding site of microRNA-1266 and FTO. ***B,*** MicroRNA-1266 expression was negatively correlated to FTO in CRC. ***C, D,*** MicroRNA-1266 was mainly enriched in the Ago2 co-precipitation group. ***E, F,*** Fluorescence value of cells co-transfected with FTO wt and microRNA-1266 mimics was remarkably decreased.

Knockdown of microRNA-1266 remarkably promoted viability of CRC cells. Therefore, microRNA-1266 may be a tumor-suppressor gene in CRC, which affects the progression of CRC *via* targeting FTO.

Conclusions

We found that microRNA-1266 was lowly expressed in CRC tissues than that of paracancerous tissues. Lowly expressed microRNA-1266

promoted the occurrence and progression of CRC by directly targeting FTO.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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