

Original Article

JMJD5 is a potential oncogene for colon carcinogenesis

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Abstract: Objective: To observe the effects of Jumonji C domain-containing (JMJD) 5 depletion on colon cancer (CC). Methods: A short-hairpin RNA targeting JMJD5 was transfected into a lentivirus to make Lv-shJMJD5 for infection into the Caco-2 human cell. Besides, a negative control shRNA was constructed. The mRNA and protein levels of JMJD5 were determined by quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting, respectively. Cell proliferation, migration, and invasion were assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), soft agar colony assay and transwell assay, respectively. In addition, immunohistochemical (IHC) staining was performed to investigate the expression of JMJD5 in adjacent normal tissues and tumor tissues from patients with CC. Results: Compared with control group, mRNA and protein levels of JMJD5 was significantly reduced after infection with Lv-shJMJD5 ($P < 0.05$), and Caco-2 cell proliferation, migration, and invasion were all obviously inhibited ($P < 0.05$). The results of IHC showed that JMJD5 was significantly up-regulated compared with normal tissues ($P < 0.01$). Additionally, follow-up data demonstrated that the survival rate of patients with high expression of JMJD5 was obviously lower than that with low expression ($P < 0.01$). Conclusions: JMJD5 depletion could significantly inhibit human CC cell proliferation, migration, and invasion, implying that JMJD5 might be a potential oncogene.

Keywords: Colon cancer, JMJD5, Lv-shJMJD5, proliferation, migration, invasion

Introduction

Colon cancer (CC) is heralded as being the third most common cancer in both men and women worldwide [1]. It is characterized by a long life history, and showed an elevated risk rate in over 50-year-old people [2]. The commonest clinical symptoms of CC include abdominal discomforts, persistent diarrhea or constipation, and hematochezia. This disease still remains a major cause of global mortality, and it has been reported that more than 1.2 million new cases were diagnosed yearly [3]. Despite the advanced development of numerous therapeutic strategies, the progression for CC still remains bleak. The accurate genetic factors responsible for CC progressions remain blurred. There is an increasing amount of evidence demonstrating that the development and progression of CC involves in accumulation of various genetic changes for a long time [4-7]. Hence, more concern is centered on the regulation of gene expression to explore an effective therapy for CC.

Several landmark discoveries have found that histone modifications, especially histone methylation, may quickly become an important epigenetic mark in regulating great critical cellular functions, such as a cancer setting [8]. Histone demethylases indicate the reversibility of chromatin mark and have effects on expression of gene. Jumonji C domain-containing (JMJD) proteins are a new class of histone demethylases [9-12], which is now believed that the family exerts enzymatic activity at the promoters of specific target genes besides histone demethylases [13, 14]. JMJD5 is now renamed KDM8, which belongs to the JMJD family and is an H3K36me2 demethylase. Despite the precise cellular substrates and functions of JMJD5 remain uncertain, it has been shown to positively regulate cyclin A1 but negatively regulate p53 and CDKN1A (p21). Previous studies indicated that JMJD5 was necessary for proliferation of Michigan Cancer Foundation (MCF)-7 breast cancer cells [15]. Besides, JMJD5-/- embryos displays serious retarded growth, leading to embryonic lethality at the midgesta-

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tion stage [8]. In addition, a recent study found that JMJD6, another JMJD family protein, was a potential biomarker for aggressiveness of CC and a potential target intervention for CC [16]. However, the relationship between the functions of JMJD5 and CC remains unclear. In this study, we investigated the functions of JMJD5 in CC.

Materials and methods

Plasmids, antibodies and reagents

Rabbit anti-human JMJD5 antibody, mouse anti-human β -actin antibody and corresponding secondary antibody were all purchased from Abcam (Abcam; Cambridge, UK). Specific shRNA targeting JMJD5 (shJMJD5) was commercially from Santa Cruz (CA, USA). For control, a negative control shRNA (shNC) was also used. Matrigel was purchased from BD Biosciences (San Jose, CA, USA). Cell counting kit-8 (CCK-8) was purchased from Dojindo (Japan).

Cells and transfection

Human intestinal epithelial Caco-2 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were cultured in RPMI 1640 medium (GIBCO Invitrogen GmbH, Karlsruhe, Germany) with 10% fetal bovine serum (FBS) (BD Biosciences, San Diego, CA, USA) at 37°C in humidified atmosphere of 5% CO₂. RNA interference technique was employed to knockout the expression of JMJD5 in Caco-2 cells. Transient transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's guidelines. We infected Caco-2 cells with lentivirus stably silencing JMJD5 (Lv-shJMJD5). As a control, we also constructed a negative control lentivirus (Lv-shNC). Green fluorescence (GFP) could be expressed by lentivirus, making it easy to evaluate the infection efficiency of each group.

Real-time reverse transcription PCR

Total cellular RNAs were extracted with the TRIzol reagent (Invitrogen Corp., Carlsbad, CA) and applied to first strand cDNA synthesis using the Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's recommendation. Quantitative RT-PCR was

performed to assess the gene transcripts with an ABI PRISM 7300 sequence detection system (Applied Biosystems, Rotkreuz, Switzerland). The expression of GAPDH was considered as the internal control. GAPDH forward primer, 5-CCCCTCTCCACCTTTGAC-3; GAPDH reverse primer, 5-CATACCAGGAAATGAG CTTGACAA-3; and JMJD5 forward primer, 5-CCGAAGTCAGTTCCTTGTGG-3; JMJD5 reverse primer, 5-GGTCCCCTGTTGT CTGCC-3.

Western blotting

Forty-eight hours after infection, cells were collected for protein extraction. Protein concentration of the supernatant was assessed using the Bio-Rad DC protein Assay kit (Bio-Rad, Ivry sur Seine, France). Total cell lysates were resolved with 10% SDS-PAGE gels, and was then transferred onto nitrocellulose membranes. For Western blotting, membranes were sealed in 5% fresh nonfat dry milk for 2 h. Then the membranes were incubated with mouse anti-human JMJD5 antibody (1:400), mouse anti-human β -actin antibody (1:800) for 1-2 h at room temperature or overnight at 4°C followed by incubation with a secondary antibody.

Cell proliferation assay

Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay. Briefly, Caco-2 cells were rinsed with PBS and were placed in 96-well plate at a final concentration of 2×10^4 per mL in an assay medium. Then the plates were incubated at 37°C for consecutive 5 days with 5% CO₂ incubator. After incubation for further 1, 2, 3, 4, and 5 days, 10 μ l MTT was added to each well, and the plates were incubated at 37°C for another 2 h. The absorbance at 595 nm was determined with a synergy 2 multi-mode microplate reader (Bio Tek Instruments, Winooski, VT, USA). Experiments were carried out 3-5 times.

Soft agar colony formation assay

Caco-2 cells transfected with shRNAs were trypsinized. Then colony formation ability was assessed with soft agar assay. Briefly, 1.5 ml RPMI 1640 medium containing 0.35% agarose were added to each well and added to the top of base agar. The cells were then cultured for 14 days at 37°C under 5% CO₂. Crystal violet

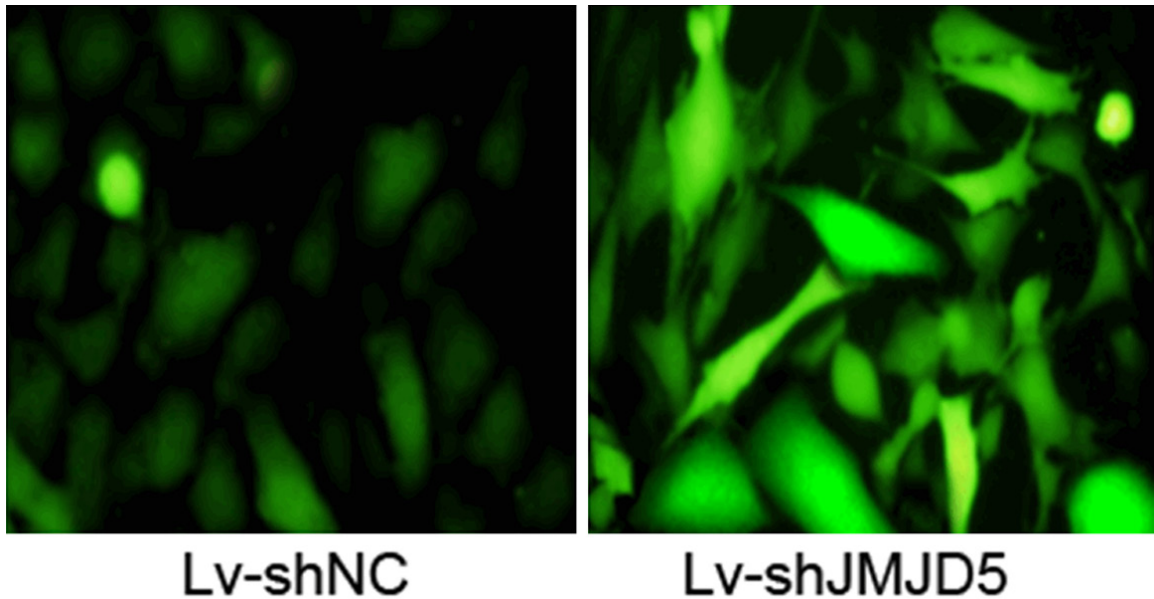


Figure 1. Infection efficiency in both Lv-shNC and Lv-shJMJD5 groups.

was employed to stain the plates for 1 hour, and the colonies were measured and photographed using a microscope (Olympus, Tokyo, Japan).

Transwell migration and cell invasion assay

Transwell chamber assay were coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA, 354230) for invasion. Briefly, cells were collected after infected with shRNAs, and then cells were suspended in serum-free DMEM media at a concentration of 1×10^5 cells/ml. About 100 μ l cell suspensions were placed to the upper chamber of the transwell, and 0.5 ml of media containing 10% FBS were put onto the lower chamber. Then the cells in the top well were removed after incubation at 37°C in 5% CO₂ for 24 h. The membranes were then stained with 0.5% crystal violet reagent and the remaining cells were calculated. Four high powered fields were counted for each membrane.

Patients and specimens

The studies concerning human tumor specimen were approved by the local Ethics Committee and informed consents were obtained from all patients. Between July 2008 and May 2009, totally 130 patients with CC treated at our hospital were enrolled into the retrospective study. Patients were diagnosed as CC according to the clinical pathological find-

ings. According to histological grading, 20 patients were at grade I, 56 were at grade II, and 54 were at grade III. According to the clinical TNM stage, 13 patients were stage I, 67 patients were stage II, 45 patients were stage III, and 5 patients were stage IV. All patients did not receive preoperative special treatment (e.g. radiotherapy, chemotherapy and or other treatment). The colon carcinomas tissues and the matched adjacent normal tissues that were located 5 cm away from the tumor margin were obtained from surgical specimens from all patients. Of the patients, 88 were men and 42 were women. The ages of the patients ranged from 30 to 85 years (mean, 60.4 years). All patients were completed the follow up for survival. By August 2014 (the time of data analysis), 62 patients had died and 68 patients were alive. The median survival time was 53 months.

Tissue microarray (TMA) and immunohistochemical analysis (IHC)

Tissues from both adjacent normal and tumor tissue were fixed in formalin and embedded in paraffin. TMAs were then assembled with a 0.6-mm-diameter punch (Beecher Instruments, Silver Spring, MD, USA). Sections (4 μ m) were constructed by a tape-transfer system (Instrumedics, Hackensack, NJ). The samples were deparaffinized followed by a graded alcohol, antigen retrieval. Then the samples were incubated at 4°C overnight in primary antibody

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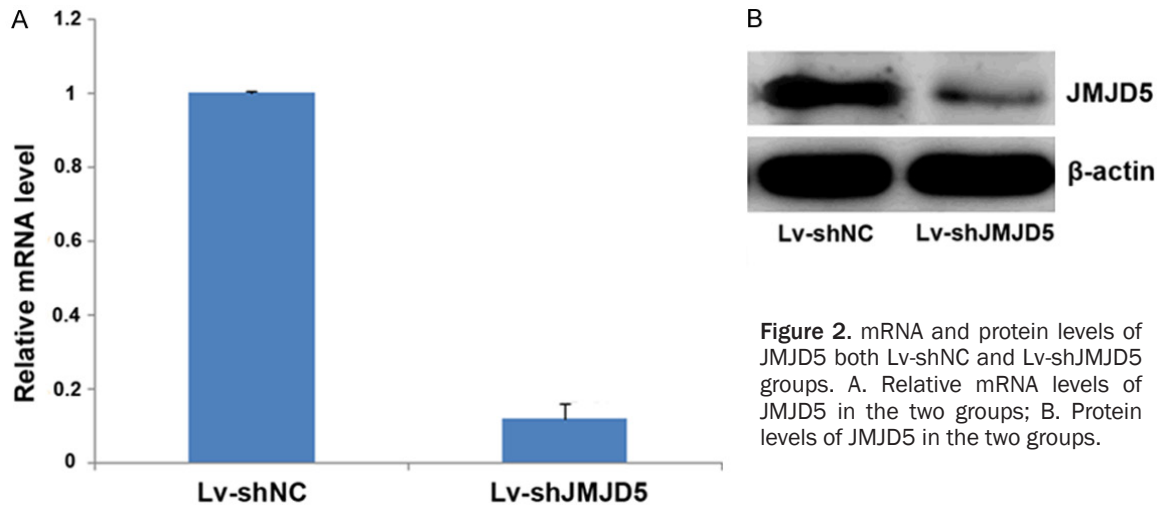


Figure 2. mRNA and protein levels of JMJD5 both Lv-shNC and Lv-shJMJD5 groups. A. Relative mRNA levels of JMJD5 in the two groups; B. Protein levels of JMJD5 in the two groups.

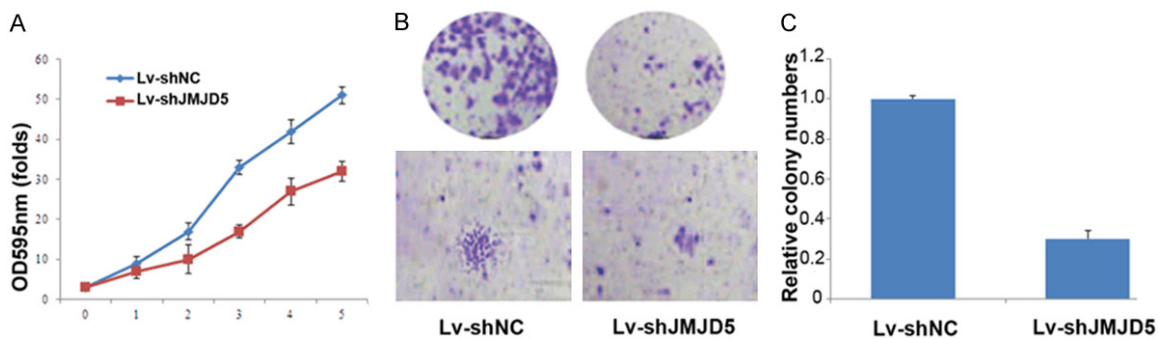


Figure 3. Cell proliferation, migration and invasion are inhibited after JMJD5 depletion in Caco-2 cells. A. The cell numbers at monitored point (on consecutive day 1, 2, 3, 4 and 5); B. Colony formation in the two groups; C. Relative colony numbers in the two groups.

solution of anti-JMJD5 (1:200), and incubated with secondary antibodies for 1 h at room temperature. 3,3'-diaminobenzidine (DAB) were then added to the tissues. The immunostaining results were assessed on the following scale: 0, negative; 1, weak; 2, moderate; 3, strong. The mean percentage of positive tumor cells was quantified (at least five areas, and at 400 \times magnification) and assigned one of the following five categories: 0, <5%; 1, 5%-25%; 2, 25%-50%; 3, 50%-75%; 4, >75%. The final score was calculated by multiplying the intensity and the quantity scores.

Statistical analysis

The data were showed as mean \pm standard deviation (SD). All the statistical analysis employed the SPSS software version 18.0 (SPSS, Chicago, IL). Paired-samples t test was performed to compare the differences between

cancer and adjacent normal tissue. The chi-square test was used to examine the various clinicopathological characteristics of JMJD5 expression. Univariate survival analysis was assessed using Kaplan-Meier method, and log-rank test was used to analyze the difference between the survival curves. $P < 0.05$ was considered statistically significant.

Results

Specific depletion of JMJD5 in CC cells

As shown in **Figure 1**, most Caco-2 cells were infected with corresponding lentivirus in both Lv-shNC and Lv-shJMJD5 groups. Infection efficiency in both groups was about 90% as demonstrated by the GFP signals. In addition, mRNA level of JMJD5 significantly decreased when the cells were infected with Lv-shJMJD5 (1.000 ± 0.003 versus 0.119 ± 0.037 ; $P < 0.05$, **Figure**

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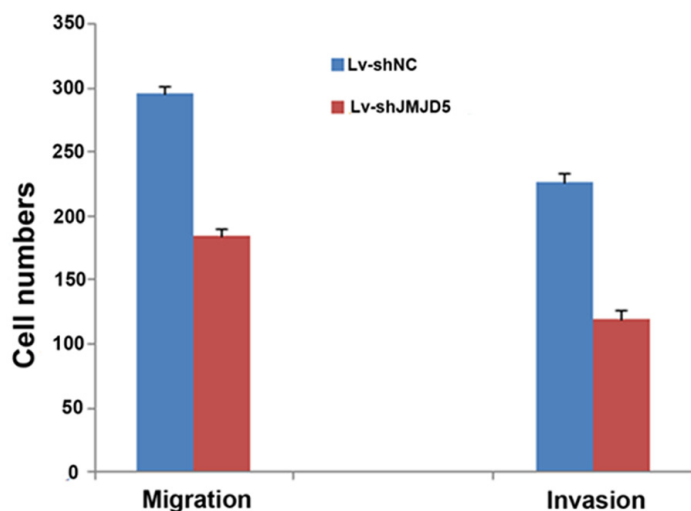


Figure 4. Migration assay and invasion assay in both Lv-shNC and Lv-shJMJD5 groups.

2A). Concomitantly, compared with Lv-shNC-infected Caco-2 cells group, the expression levels of JMJD5 revealed by western blotting analysis were also obviously lowered in Lv-shJMJD5-infected cells (**Figure 2B**).

Depletion of JMJD5 inhibits cell growth in Caco-2 cells

Cells infected with Lv-shNC had still retained a high proliferative rate, while cells infected with Lv-shJMJD5 were greatly impeded from duplication (**Figure 3A**). In parallel, colony formation assay showed that the cells infected with Lv-shJMJD5 exhibited lower colony formation ability (**Figure 3B**). Quantification of the colonies showed that Lv-shJMJD5 could statistically inhibit cells colony formation compared with Lv-shNC (1.000 ± 0.015 versus 0.301 ± 0.042 ; $P < 0.05$, **Figure 3C**).

Depletion of JMJD5 inhibits cell migration and invasion in Caco-2 Cells

The results of migration assay showed that average 296 migrated cells in Lv-shNC group were observed to transmigrate to lower chamber while average 184 cells did that in Lv-shJMJD5 group. There were significant differences between the two groups (296 ± 4.8 versus 184 ± 5.6 , $P < 0.05$, **Figure 4**). The results of invasion assay showed that cells transmigrated to lower chamber were also significantly reduced after infection with Lv-shJMJD5 (226 ± 7.6 versus 119 ± 6.8 , $P < 0.05$, **Figure 4**).

Expression level of JMJD5 in CC patients

To explore the role of JMJD5 in CC, we collected 130 CC samples with paired adjacent normal tissues and performed IHC. The results showed that JMJD5 protein was significantly up-regulated in CC compared with adjacent normal tissues (paired-samples t test, $P < 0.01$) (**Figure 5A**). Additionally, the results of follow-up data demonstrated that the survival rate of patients with high expression of JMJD5 was obviously lower than that with low expression of JMJD5 (log-rank test, $P < 0.01$) (**Figure 5B**).

Discussion

In the present study, the effects of JMJD5 on CC tissue/cell were investigated. We found that JMJD5 was significantly up-regulated in CC tissue compared with normal tissues. After infection with Lv-shJMJD5, the mRNA and protein levels of JMJD5 were significantly reduced compared with control group, and Caco-2 cell proliferation, migration, and invasion were all obviously inhibited. We concluded that JMJD5 depletion could obviously inhibit human CC cell proliferation, migration, and invasion, suggesting that JMJD5 might be involved in the progression of CC.

Recently, the medical community has paid significant attention on the role of epigenetics deregulation in development of human cancer [17-19]. Compared with DNA mutations, epigenetic deregulation, primarily involved with cell structure and function changes that mediated by histone post-translational modifications (PTMs), are biochemically reversible [20]. Besides, drug therapies targeting epigenetic lesions might be more individualized and have underlying less toxicity to sufferers. Histone tail methylation/demethylation is one of the chief PTMs, and plays a crucial role in regulating the gene expression. Aberrant methylation has been well established in oncogenesis, resulting in genomic instability, abnormal imprinting, and silencing genes [21, 22]. Dysregulation of these processes is associated with cancer progression and development [23, 24]. JMJD5 (KDM8)

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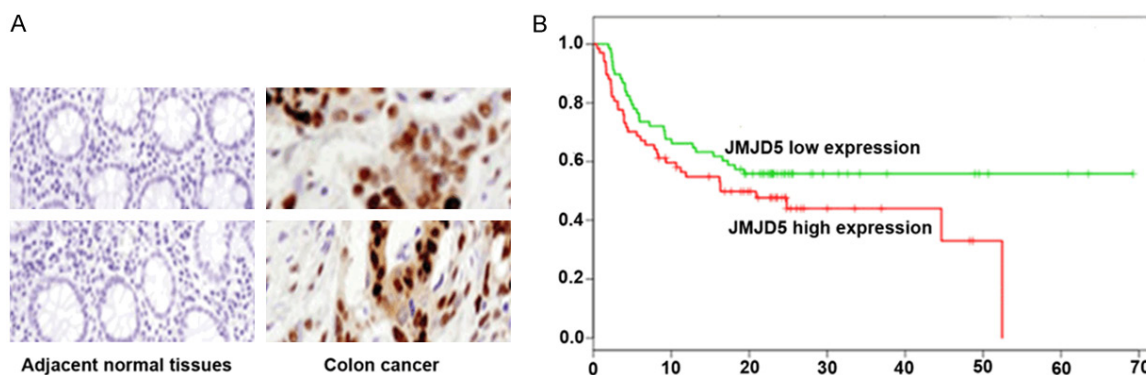


Figure 5. Expression level of JMJD5 in CC patients. A. IHC staining of JMJD5 in paired samples of CC (magnification, $\times 100$); B. The survival rate of CC patients by follow-up data; IHC, Immunohistochemical staining; CC, colon cancer.

acts on H3K36me2 demethylation, which specifically removes methyl moieties from dimethylated lysine 36 on histone H3 [25]. It has been reported that JMJD5 appears to be crucial during embryonic development, osteoclastogenesis and cell proliferation by repressing of p53 expression, implying its oncogenic activity [26]. In addition, JMJD5 could directly interact with pyruvate kinase M2 (PKM2) to modulate metabolic factors associated with tumorigenesis [27]. It has been reported that JMJD5 was increased in leukaemia and breast cancer [25, 28, 29]. While knockdown JMJD5 could inhibit breast cancer cell metastasis [30].

Wang *et al.* [16] found that JMJD6 was significantly up-regulated in CC tissue, and JMJD6 depletion could stimulate cell apoptosis and suppress the proliferation of p53-dependent colon cell and tumor formation. Considering the results, he concluded that JMJD6 was a potential biomarker for CC aggressiveness and a potential target for CC intervention by negative regulation of p53. Similarly, in our study we aimed to elucidate the mechanism JMJD5 depletion on CC progression and aggression, as well as tumor metastasis. A significantly reduced mRNA and protein levels of JMJD5 were found in JMJD5 knockdown cells, suggesting the lentivirus stably silencing JMJD5 was successfully constructed into Caco-2 cells. Moreover, cell proliferation, migration, and invasion after infection with non-specific shRNA were assessed by MTT, soft agar colony assay and transwell assay. We found that cells infected with non-specific shRNA could retain high proliferative rate, and exhibited lower colony formation ability, indicating JMJD5 knockdown could inhibit cell growth in Caco-2 cells. In addition,

our IHC staining showed that compared with normal tissues, JMJD5 protein was significantly up-regulated in CC, and the follow-up data showed that the survival rate of patients with high expression of JMJD5 was significantly lower than that with low expression of JMJD5. Taken together, these results indicated a strong relationship between JMJD5 expression and the aggressive clinical behaviors of CC, reflecting a potential functional contribution of JMJD5 in CC and offering new insights into molecular basis for treatment of JMJD5.

In conclusion, human CC cell proliferation, migration, and invasion could be significantly inhibited by depleting JMJD5. JMJD5 might be a potential oncogene for CC.

Disclosure of conflict of interest

None.

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