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ORIGINAL ARTICLE

Knockdown of ZEB2-AS1 inhibits cell invasion and induces apoptosis in colorectal cancer

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Summary

Purpose: *To uncover the potential function of long non-coding RNA (lncRNA) ZEB2-AS1 in the progression of colorectal cancer (CRC), and its underlying mechanism.*

Methods: *Relative level of ZEB2-AS1 in CRC tissues and matched normal ones was determined by quantitative real-time polymerase chain reaction (qRT-PCR). Correlation between ZEB2-AS1 level and survival of CRC patients was analyzed by Kaplan-Meier method. Regulatory effects of ZEB2-AS1 on cellular behaviors of CRC cells were evaluated. The interactions between ZEB2-AS1 with LSD1 and EZH2 were explored by RNA immunoprecipitation (RIP) assay. 5-Ethynyl-2'- deoxyuridine (EdU) assay was performed to elucidate the roles of ZEB2-AS1, LSD1 and EZH2 on the proliferative ability of CRC cells. Finally, Spearman's correlation analysis was performed to analyze the relationship between ZEB2-AS1 level and expressions of proliferation- and invasion-related genes.*

Results: *ZEB2-AS1 was upregulated in CRC tissues relative to matched controls. Its level remained higher in CRC patients with ≥6 cm in tumor size, nodal metastasis and stage III-IV. CRC patients with low-level ZEB2-AS1 presented worse survival compared with those with high-level ZEB2-AS1. QRT-PCR data showed higher abundance of ZEB2-AS1 in CRC cell lines than colonic epithelial cell line. Knockdown of ZEB2-AS1 attenuated the proliferative, migratory and invasive abilities, but induced apoptosis of DLD1 and SW620 cells. RIP assay demonstrated the interaction between ZEB2-AS1 and LSD1, EZH2. Moreover, EdU assay revealed that transfection of sh-ZEB2-AS1 attenuated the proliferative ability, which was further reduced after co-transfection of sh-LSD1 or sh-EZH2. Finally, correlation analysis showed that mRNA level of ZEB2-AS1 was positively correlated to those of LSD1, EZH2, MMP9, MMP12 and KRAS, but negatively correlated to KLF2.*

Conclusions: *LncRNA ZEB2-AS1 is upregulated in CRC. It accelerates CRC cells to proliferate via interacting with EZH2 and LSD1, thus promoting the progression of CRC.*

Key words: *colorectal cancer, ZEB2-AS1, EZH2, LSD1, proliferation*

Introduction

Globally, there are nearly 700,000 death cases of colorectal cancer (CRC) each year, which is the fourth lethal cancer following lung cancer, liver cancer and gastric cancer [1]. The 5-year survival of early-stage CRC is 90.1%, which is only 11.7% in CRC patients with distant metastases [2]. With the in-depth researches on the pathogenesis of CRC, targeted therapy has been recently well concerned [3,4]. Nevertheless, its therapeutic efficacy is not satisfactory. To improve the diagnostic and therapeutic efficacies of CRC, it is necessary to clearly uncover the molecular mechanism of CRC development.

Long non-coding RNAs (lncRNAs) are RNAs ranging from 200 nucleotides (nt) to 100,000 nt long, which exert epigenetic, transcriptional and post-transcriptional regulations [5]. It is reported that lncRNA participates in physiological and path-

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ological processes. Besides, lncRNAs are identified to influence the occurrence and progression of various types of human tumors [6,7]. LncRNAs exert a vital role in the progression of CRC [8]. A recent study illustrated that lncRNA ZEB2-AS1 stimulates bladder cancer cells to proliferate through sponging miR-27b [9]. The specific function of lncRNA ZEB2-AS1 in CRC, however, remains unclear.

EZH2 is upregulated in many types of tumors that is capable of regulating tumor cell phenotypes [10]. A relevant study pointed out that Myc binds to EZH2 promoters and directly stimulates its transcription. EZH2 level is positively correlated to Myc level in prostate cancer [11]. In glioblastoma, EZH2 maintains tumor stemness by regulating c-Myc expression [12]. A recent study reported that EZH2 is highly expressed in the colorectum and can promote metastasis of CRC by silencing E-cadherin [13,14].

This study aimed to clarify the potential role of lncRNA ZEB2-AS1 in the progression of CRC, and the potential mechanism.

Methods

Subjects and samples

CRC tissues and matched normal tissues (3 cm away from tumor edge) were harvested from 60 cases of CRC patients undergoing surgical resection in the First Affiliated Hospital of Gannan Medical University from February 2008 to October 2016. Tissue samples were immediately preserved in liquid nitrogen. Enrolled CRC patients were pathologically diagnosed and did not have previous treatment nor medical history of other malignancies. This study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Gannan Medical University and written informed consent was provided from each subject.

Table 1. Primer sequences

Gene	*Primer sequences*
MMP9	F:5'-GAACCAATCTCACCGACAGG-3'
	R:5'-GCCACCCGAGTGTAACCATA-3'
MMP12	F:5'-AGTTTTGATGCTGTCACTACCG-3'
	R:5'-CACTGGTCTTTGGTCTCTCAGAA-3'
KRAS	F:5'-GACTCTGAAGATGTACCTATGGTCCTA -3'
	R:5'-CATCATCAACACCCTGTCTTGTC -3'
KLF2	F:5'-TTCGGTCTCTTCGACGACG-3'
	R:5'-TGCGAACTCTTGGTGTAGGTC-3'
EZH2	F:5'-TGCACATCCTGACTTCTGTG-3'
	R:5'-AAGGGCATTCACCAACTCC-3'
LSD1	F:5'-AGCGCCTACGCTGTCAAAG-3'
	R:5'-CTCAAAGTGGTGCGAAAAACG-3'
ZEB2-AS1	F:5'-CCTGGAAAGGGAAATCCTG-3'
	R:5'-AGGATGAATATAGACAGGCCA-3'
GAPDH	F:5'-CGGAGTCAACGGATTTGGTCGT-3'
	R:5'-GGGAAGGATCTGTCTCTGACC-3'

Cell culture and transfection

CRC cells and human colon epithelial normal cells (NCM460) were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) in a 5% CO2 incubator at 37°C. For cell transfection, cells were seeded in a 6-well plate with 1×10^4 cells per well. 1.5 mL of serum-free medium and 500 µL of Lipofectamine™ 2000 transfection solution (Invitrogen, Carlsbad, CA, USA) were applied in each well. Complete medium was replaced 6 h later.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), chloroform and isopropanol. The extracted RNA was quantified and reversely transcribed into cDNA, followed by PCR using SYBR Green method (TaKaRa, Tokyo, Japan). Primer sequences are listed in Table 1.

Cell Counting Kit (CCK-8) assay

Cells were seeded in 96-well plate with 2×10^3 cells per well. Absorbance (A) at 450 nm was recorded at the appointed time points using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for depicting the viability curve.

Colony formation assay

Cells were seeded in 6-well plate with 2.5×10^3 cells per well and cultured for 2 weeks. Subsequently, cells were subjected to 15-min fixation in 4% paraformaldehyde and 10-min staining in Giemsa solution. After removing the staining solution, colonies were washed, air-dried and observed under a microscope.

Apoptosis determination

Cells were prepared for single-cell suspension. Cells were double-stained with 5 µl of Annexin V/propidium iodide (PI) and 5 µl of fluorescein isothiocyanate (FITC) in the dark for 10 min. Apoptotic rate was finally determined using flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA).

Transwell assay

Cell density was adjusted to 2×10^4/mL. 400 µL of suspension were applied in the upper side of Transwell chamber (Millipore, Billerica, MA, USA) pre-coated with Matrigel (BD bioscience, Franklin Lakes, NJ, USA). In the bottom side, 700 µL of medium containing 10% FBS was applied. After 48 h of incubation, invasive cells were fixed in methanol for 30 min, dyed with 0.1% crystal violet for 10 min and counted using a microscope. Penetrating cells were counted in 6 randomly selected fields per sample. Transwell migration assay was similarly conducted without Matrigel pre-coating.

RNA immunoprecipitation (RIP)

Cells were treated according to the procedures of Millipore Magna RIPTM RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA). Cell lysate was incubated with anti-EZH2, anti-LSD1 or IgG antibody at 4°C for 6 h. A protein-RNA complex was captured and digested with 0.5 mg/ml proteinase K containing 0.1% sodium dodecyl sulphate (SDS) to extract RNA. The magnetic beads were repeatedly washed with RIP washing buffer to remove non-specific adsorption as much as possible. Finally, the extracted RNA was subjected to mRNA level determination using qRT-PCR.

Western blot

Total protein was extracted from cells or tissues using radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) and loaded for electrophoresis. After electrophoresis, polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), were blocked in 5% skim milk for 2 h, incubated with primary antibodies at 4°C overnight and with secondary antibodies for 2 h. Bands were exposed by electrochemiluminescence (ECL) (Pierce, Rockford, IL, USA) and analyzed by Image Software.

5-Ethynyl-2'- deoxyuridine (EdU) assay

Cells were seeded in 24-well plates with 4×10^4 cells per well. Cells were labeled with 50 μmol/L EdU at 37°C for 2 h and subjected to 30-min fixation in 4% paraformaldehyde and 20-min incubation in phosphate buffered saline (PBS) containing 0.5% Triton-100. After washing with PBS containing 3% bovine serum albumin (BSA), 100 μl of dying solution was applied per well for 1 h incubation in the dark and cells were counter-stained with 4',6-diamidino-2-phenylindole (DAPI) for 30 min. The ratio of EdU-positive cells was calculated.

Statistics

SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Intergroup differences were analyzed by the t-test. Spearman's correlation analysis was conducted for evaluating the expression relationship between two genes. Kaplan-Meier method followed by log rank test were performed for analyzing the survival rate. P<0.05 was considered statistically significant.

Results

Upregulated ZEB2-AS1 in CRC and its negative correlation to disease progression

QRT-PCR data showed that ZEB2-AS1 was upregulated in CRC tissues relative to matched normal tissues (Figure 1A). In particular, ZEB2-AS1 level was higher in CRC tissues with ≥ 6 cm in size than those < 6 cm (Figure 1B). Based on the lymph node metastasis condition, CRC patients in this category had higher level of ZEB2-AS1 relative to those patients without nodal metastasis (Figure 1C). CRC

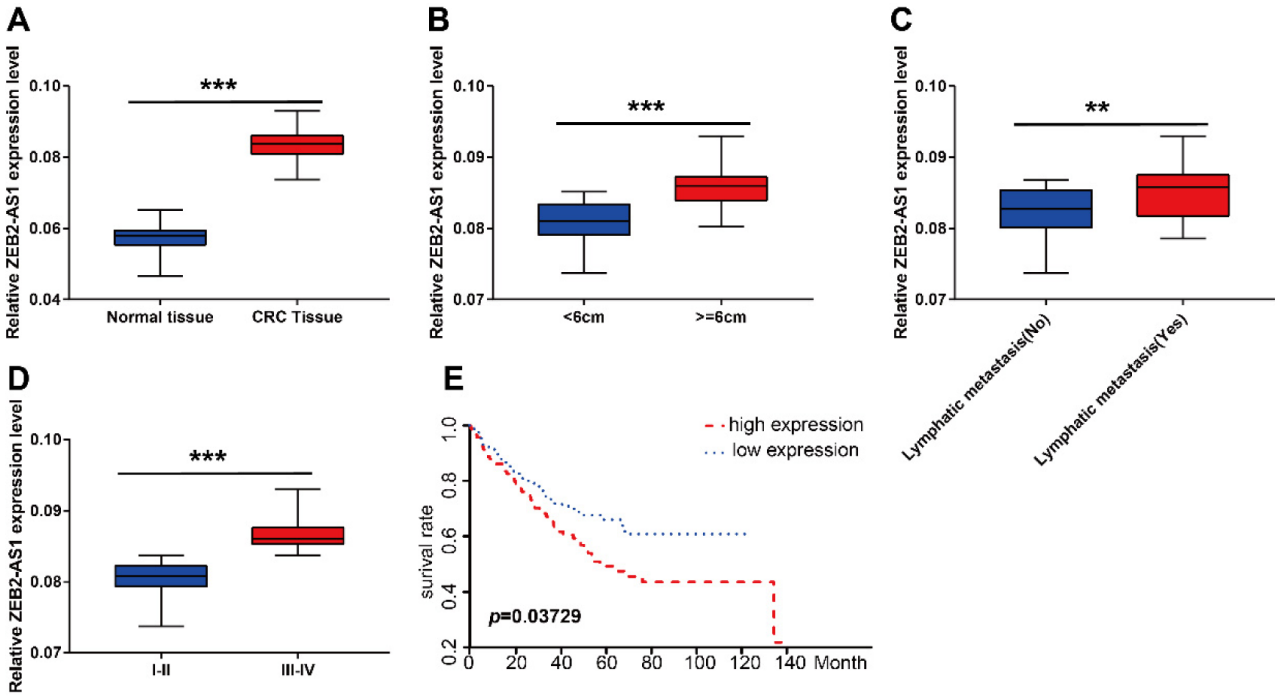

Figure 1. Upregulated ZEB2-AS1 in CRC and its negative correlation to disease progression. **A:** Relative level of ZEB2-AS1 in CRC tissues and matched normal tissues. **B:** Relative level of ZEB2-AS1 in CRC tissues with ≥ 6 cm in size and those < 6 cm. **C:** Relative level of ZEB2-AS1 in CRC patients either with lymph node metastasis or not. **D:** Relative level of ZEB2-AS1 in CRC patients in stage III-IV and stage I-II. **E:** Kaplan-Meier curve introduced for survival rate in CRC patients with high expression and low expression of ZEB2-AS1 (**p<0.01, ***p<0.001).

patients in stage III-IV had higher level of ZEB2-AS1 compared with those in stage I-II (Figure 1D). Survival analysis revealed a worse prognosis in CRC patients with high expression of ZEB2-AS1 (Figure 1E). The above data demonstrated that ZEB2-AS1 was closely correlated to progression of CRC.

ZEB2-AS1 accelerated the proliferative ability and inhibited apoptosis of CRC cells

ZEB2-AS1 was highly expressed in CRC cell lines relative to NCM460 cells (Figure 2A). To elucidate the biological function of ZEB2-AS1 in CRC, we first constructed sh-ZEB2-AS1 1#, sh-ZEB2-AS1

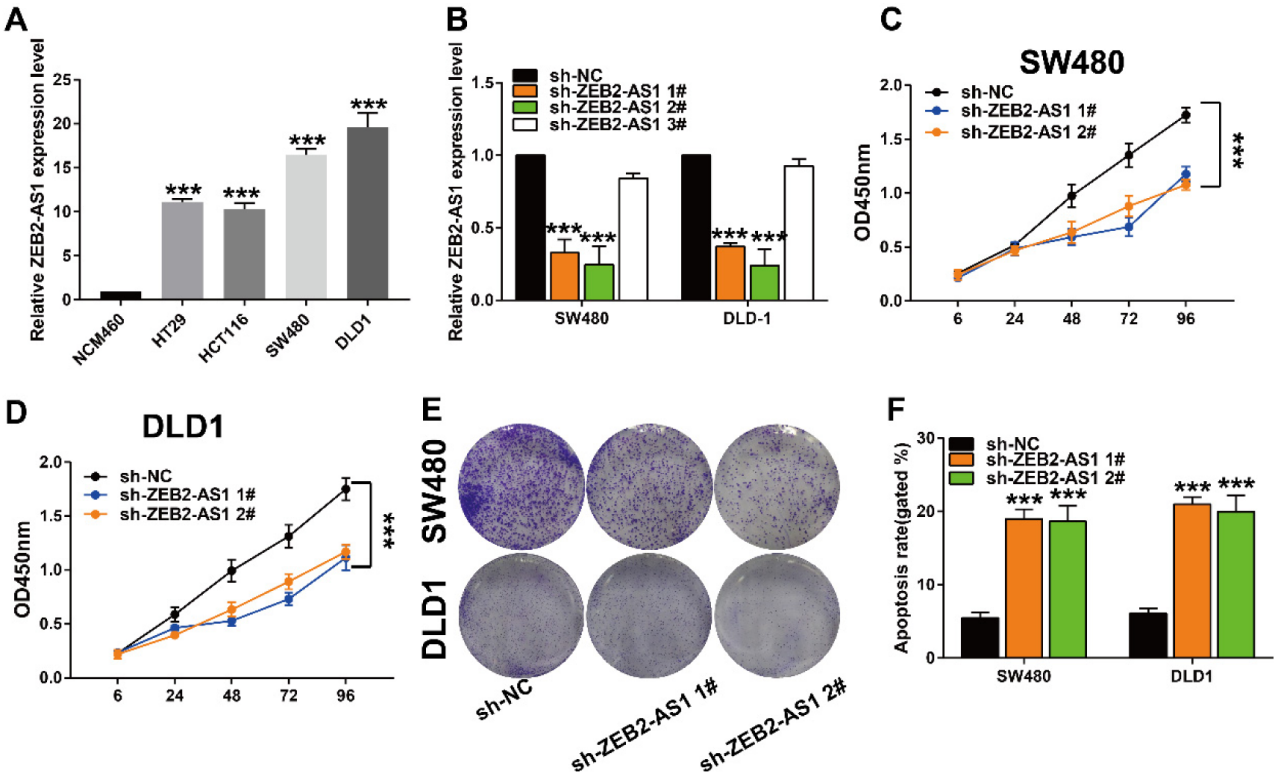

Figure 2. ZEB2-AS1 accelerated the proliferative ability and inhibited apoptosis of CRC cells. **A:** Relative level of ZEB2-AS1 in CRC cell lines (HT29, HTC116, SW480 and DLD1) and human-epithelial normal cells (NCM460). **B:** Transfection efficacy of sh-ZEB2-AS1 1#, sh-ZEB2-AS1 2# and sh-ZEB2-AS1 3# in SW480 and DLD1 cells. **C:** CCK-8 showed the viability in SW480 cells transfected with sh-NC, sh-ZEB2-AS1 1# or sh-ZEB2-AS1 2#. **D:** CCK-8 showed the viability in DLD1 cells transfected with sh-NC, sh-ZEB2-AS1 1# or sh-ZEB2-AS1 2#. **E:** Colony formation assay showed the colonies in SW480 and DLD1 cells transfected with sh-NC, sh-ZEB2-AS1 1# or sh-ZEB2-AS1 2#. **F:** Apoptotic rate in SW480 and DLD1 cells transfected with sh-NC, sh-ZEB2-AS1 1# or sh-ZEB2-AS1 2# (***p<0.001).

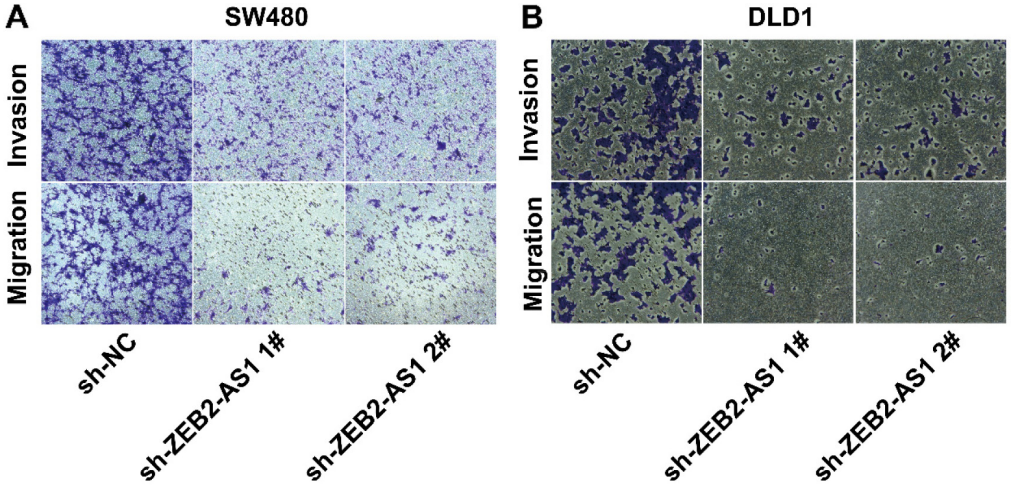

Figure 3. ZEB2-AS1 accelerated CRC cells to invade and migrate. **A:** Transwell assay showed invasion and migration in SW480 cells transfected with sh-NC, sh-ZEB2-AS1 1# or sh-ZEB2-AS1 2#. **B:** Transwell assay showed invasion and migration in DLD1 cells transfected with sh-NC, sh-ZEB2-AS1 1# or sh-ZEB2-AS1 2#.

2# and sh-ZEB2-AS1 3#. Transfection of sh-ZEB2-AS1 1# or sh-ZEB2-AS1 2# greatly downregulated ZEB2-AS1 level in SW480 and DLD1 cells, showing their sufficient transfection efficacy (Figure 2B). Cell viability in SW480 and DLD1 cells markedly decreased after transfection of sh-ZEB2-AS1 1# or sh-ZEB2-AS1 2# (Figure 2C, 2D). The number of colonies was reduced in CRC cells transfected with sh-ZEB2-AS1 1# or sh-ZEB2-AS1 2# (Figure 2E). On the contrary, the apoptotic rate was elevated by transfection of sh-ZEB2-AS1 1# or sh-ZEB2-AS1 2# in CRC cells (Figure 2F). It was concluded that ZEB2-AS1 knockdown attenuated the proliferative ability and induced apoptosis in CRC cells.

ZEB2-AS1 accelerated CRC cells to invade and migrate

To further explore the influence of ZEB2-AS1 on the progression of CRC, Transwell assay was conducted to uncover the invasive and migratory abilities of CRC cells. After transfection of sh-ZEB2-AS1 1# or sh-ZEB2-AS1 2#, the invasive and migratory abilities of SW480 and DLD1 cells were attenuated (Figure 3A, 3B). Therefore, knockdown of ZEB2-AS1 inhibited CRC cells to invade and migrate.

ZEB2-AS1 accelerated CRC cell proliferation via interacting with EZH2 and LSD1

ZEB2-AS1 abundance in the immuno-precipitates EZH2 and LSD1 was much higher than that in IgG as RIP assay revealed (Figure 4A). To elucidate the interaction between ZEB2-AS1 with EZH2 and LSD1 in the progression of CRC, we constructed sh-EZH2 and sh-LSD1. Transfection of sh-EZH2 downregulated the protein level of EZH2 in SW480 cells (Figure 4B). Besides, transfection of sh-LSD1 suf-

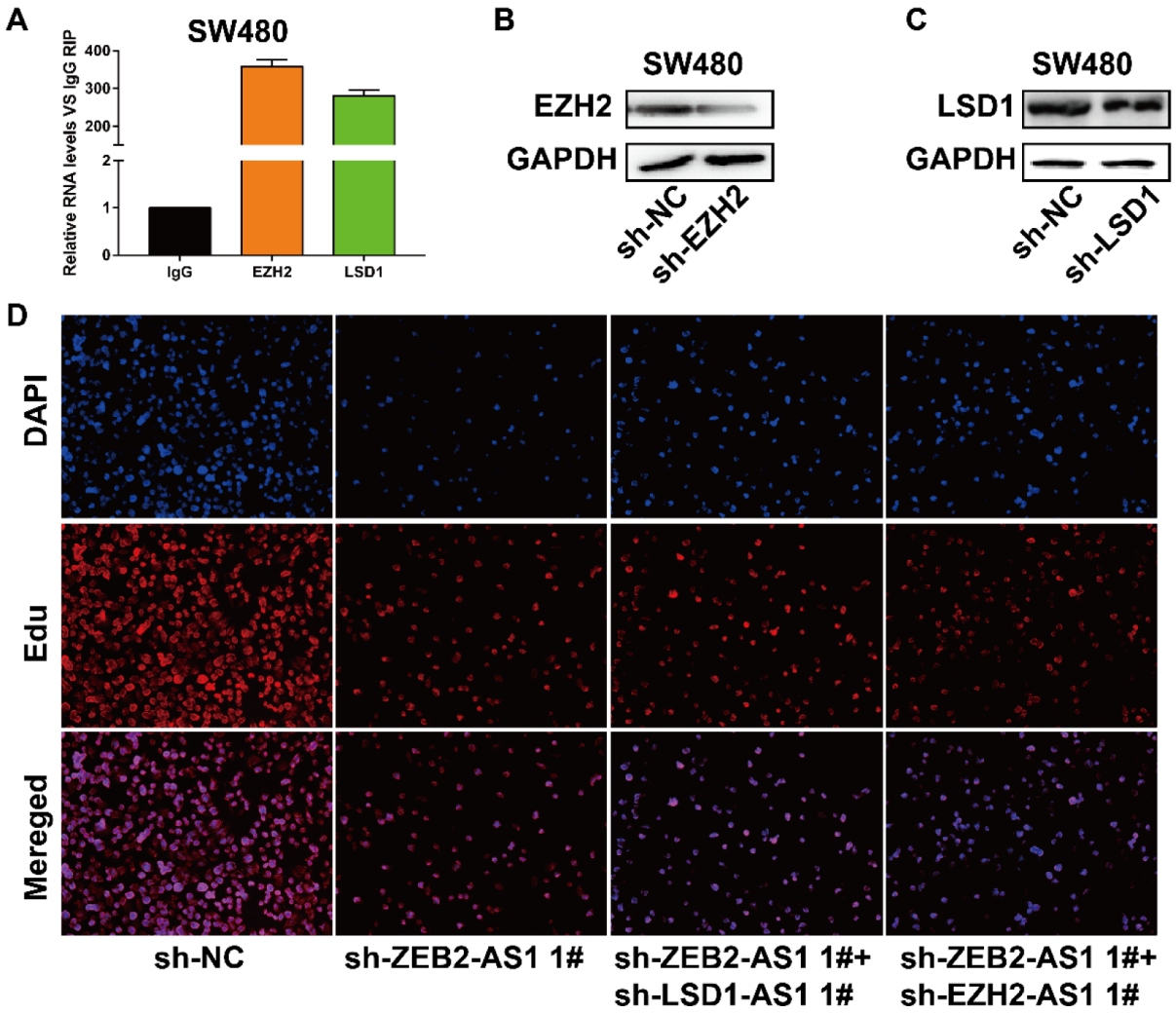

Figure 4. ZEB2-AS1 accelerated CRC cell proliferation via interacting with EZH2 and LSD1. **A:** RIP assay showed ZEB2-AS1 abundance in the immuno-precipitates EZH2, LSD1 and IgG. **B:** Transfection efficacy of sh-EZH2 in SW480 cells. **C:** Transfection efficacy of sh-LSD1 in SW480 cells. **D:** EdU assay showed DAPI-labeled (blue), EdU-labeled (red) and merged images of SW480 cells transfected with sh-NC, sh-ZEB2-AS1 1#, sh-ZEB2-AS1 1#+sh-LSD1 or sh-ZEB2-AS1 1#+sh-EZH2.

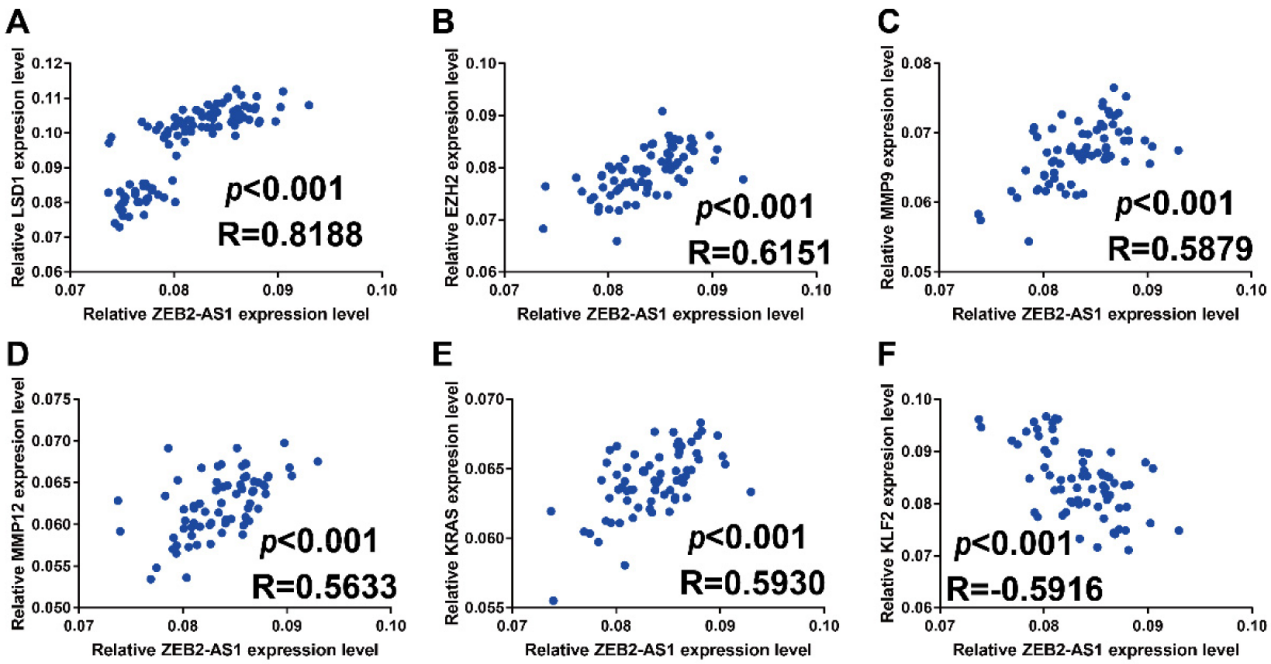

Figure 5. Correlation between ZEB2-AS1 with proliferation- and invasion-related molecules. **A:** Correlation between ZEB2-AS1 and LSD1. **B:** Correlation between ZEB2-AS1 and EZH2. **C:** Correlation between ZEB2-AS1 and MMP9. **D:** Correlation between ZEB2-AS1 and MMP12. **E:** Correlation between ZEB2-AS1 and KRAS. **F:** Correlation between ZEB2-AS1 and KLF2.

ficiently downregulated LSD1 level in SW480 cells (Figure 4C). Moreover, transfection of sh-ZEB2-AS1 1# attenuated EdU-labeled fluorescence in SW480 cells, which was further reduced after transfection of sh-EZH2 or sh-LSD1 (Figure 4D). Collectively, ZEB2-AS1 could bind to EZH2 and LSD1 to accelerate the proliferative ability of CRC cells.

Correlation between ZEB2-AS1 with proliferation- and invasion-related molecules

To clarify the relationship between ZEB2-AS1 and the progression of CRC, we analyzed the correlation between ZEB2-AS1 with proliferation- and invasion-related molecules. What we saw was that ZEB2-AS1 level was positively correlated to those of LSD1, EZH2, MMP9, MMP12 and KRAS (Figure 5A-5E), but negatively correlated to KLF2 (Figure 5F).

Discussion

CRC is a major public health problem worldwide that leads to death [15]. CRC is a heterogeneous disease involving oncogene activation and tumor-suppressor gene inactivation. Genetic mutations and epigenetic changes are responsible for tumorigenesis, and the latter includes DNA methylation, histone modifications and non-coding RNA changes [16,17]. This study investigated the association between ZEB2-AS1 level and survival in Chinese patients with CRC. It was illustrated that CRC patients with high-level ZEB2-AS1 presented worse survival, suggesting the involvement of ZEB2-AS1 in the progression of CRC.

LncRNAs participate in many biological processes by interacting with various macromolecules, such as DNAs, chromatins, proteins and RNAs [18,19]. LncRNAs are reported to be associated with overall survival of cancer patients and may serve as prognostic biomarkers for CRC [20]. LncRNA ZEB2-AS1 promotes the occurrence and progression of bladder cancer by downregulating miR-27b [9]. This study found that ZEB2-AS1 was upregulated in CRC tissues and associated with poor prognosis of CRC patients. ZEB2-AS1 accelerated CRC cells to proliferate by interacting with EZH2 and LSD1.

EZH2 is the catalytic subunit of PRC2, which catalyzes the trimethylation of H3K27me3 and silences its target genes [21-23]. LSD1 removes methyl demethylase from H3K4me1/2 and H3K9me1/2. It is abnormally expressed in many types of cancers, which blocks differentiation and promotes proliferation, metastasis and invasiveness of tumor cells [24]. Recently, HOXA11-AS is reported to simultaneously bind to several RNA binding proteins (PRC2, LSD1 and DNMT1) to promote the proliferative and invasive abilities of gastric cancer [25]. This study found that ZEB2-AS1 was positively correlated with the expressions of invasion-related LSD1 [24], EZH2 [26], MMP9 [27], MMP12 [28] and KRAS [29]. ZEB2-AS1 level

was negatively correlated with the expression of tumor-suppressor gene KLF2 [30]. It is suggested that ZEB2-AS1 could be used as a marker for predicting the progression of CRC.

Conclusions

LncRNA ZEB2-AS1 is upregulated in CRC. It accelerates CRC cells to proliferate *via* interacting with EZH2 and LSD1, thus promoting the progression of CRC. LncRNA ZEB2-AS1 could be utilized as a potential prognostic and therapeutic target for CRC.

Conflict of interests

The authors declare no conflict of interests.

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