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Original Article

Long noncoding RNA AFAP1-AS1 facilitates tumor growth through enhancer of zeste homolog 2 in colorectal cancer

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Abstract: Increasing evidences have shown that long noncoding RNAs (lncRNAs) play critical regulatory roles in cancer biology. However, the contributions of lncRNAs to colorectal cancer remain largely unknown. Here, we identify a lncRNA AFAP1-AS1 that facilitates colorectal cancer, where it is upregulated. AFAP1-AS1 expression was associated with colorectal cancer patient survival. AFAP1-AS1 knockdown inhibited cell proliferation, cell cycle, and tumorigenesis in an subcutaneous mouse xenograft model system. Further data demonstrated that AFAP1-AS1 was associated with enhancer of zeste homolog 2 (EZH2) and that this association was required for the repression of EZH2 target genes. Our findings indicate that AFAP1-AS1 is an oncogenic lncRNA that promotes tumor progression and may be a novel prognostic factor in colorectal cancer. Targeting AFAP1-AS1 might be a potential therapeutic strategy for colorectal cancer treatment.

Keywords: lncRNA, AFAP1-AS1, EZH2, colorectal cancer

Introduction

Colorectal cancer is one of the most common human malignancies worldwide, particularly in China, with a significantly increasing incidence [1-3]. Although about 90% of colorectal cancer patients with early stage can be cured due to the recent advances in chemotherapy, radiotherapy, and surgery, the survival of rest of the colorectal cancer patients is closely related to distant metastasis and drug resistance [4, 5]. Therefore, developing molecular target treatment for colorectal cancer will improve the survival rate of patients with colorectal cancer. Increasing evidences showed that lncRNAs play a vital role in colorectal cancer progression [6-9]. However, the precise molecular mechanisms that account for colorectal cancer development and progression remain unclear.

Actin filament associated protein 1 antisense RNA1 (AFAP1-AS1) has been demonstrated to function as an oncogene in multiple cancers. AFAP1-AS1 is a lncRNA derived from the antisense DNA strand in the AFAP1 gene locus, which regulates actin filament integrity and act as an adaptor protein linking Src family members and other signaling proteins associated with actin filaments [10]. AFAP1-AS1 was first shown to contribute to Barrett's esophagus and esophageal adenocarcinoma [11]. Recent AFAP1-AS1 decreases the activity of the Wnt/β-catenin pathway and suppresses the expression of EMT-related genes in tongue squamous cell carcinoma [12]. AFAP1-AS1 regulates cell cycle via inhibition of the RhoA/Rac2 signaling [13]. Moreover, high AFAP1-AS1 levels were associated with malignancy, metastasis and poor prognosis of colorectal cancer, hepatocelluar carcinoma, pancreatic ductal adenocarcinoma, and gall bladder cancer [6-9, 14-16]. However, further explorations are required to elucidate the functions of AFAP1-AS1 in tumor progression, including colorectal cancer.

In this study, we have identified the lncRNA AFAP1-AS1 that are upregulated in colorectal cancer, compared with paired peritumoral tissues. The effects of AFAP1-AS1 were assessed by silencing and overexpressing it in vitro and in vivo.

Materials and methods

Clinical tissue samples

In total, 80 colorectal carcinoma tissues and 10 normal colon tissues were collected from Meizhou Provincial People's Hospital between 2004 and 2007. None of the patients had received chemotherapy and/or radiotherapy before the operation. To use the clinical materials for research, prior patient consent and approval from the Institutional Research Ethics Committee of the Meizhou People's Hospital were obtained. Follow-up information was available for all patients. The use of tissue specimens was carried out in accordance with the approved guidelines of the Meizhou People's Hospital. Written informed consent was obtained from each patient, and all patients granted permission for the data obtained to be used in subsequent studies.

Cell lines

LOVO, SW1116, SW480, HCT116, SW620 and HT29 cells were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. NCM460 was also purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and was cultured in DMEM/F12 (Invitrogen, Carlsbad, CA) supplemented with 5% horse serum, 20 ng/ml epidermal growth factor (EGF), 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μg/ml insulin, and 100 μg/ml penicillin-streptomycin. Cells were cultured in a humidified incubator in a 5% CO_2 atmosphere at 37°C.

ChIP-qPCR

ChIP was performed using the Chromatin Immunoprecipitation Kit (Millipore-Upstate) according to the manufacturer's instructions. Immunoprecipitated DNA was purified after phenol extraction and was used for qPCR. Primers are listed in Table S1.

RNA extraction and quantitative RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen). First-strand cDNA was generated using the PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). Real-time PCR was performed in the StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA). *ACTB* was used as a control. Primers were listed in Table S1.

Cell proliferation and colony formation

Cell proliferation assay was performed using a WST-1 Assay Kit (Roche). Briefly, cells were seeded in each of the triplicate wells of a 96-well plate and incubated at 37°C. Then, cells were split and detected with a WST-1 assay kit.

RNA immunoprecipitation

We performed RNA immunoprecipitation (RIP) experiments using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA), according to the manufacturer's instructions. The EZH2 antibodies used for RIP were ab3748 (Abcam, Hong Kong, China). The coprecipitated RNAs were detected by reverse-transcription polymerase chain reaction (RT-PCR). Total RNAs (input controls) and isotype controls were assayed simultaneously to demonstrate that the detected signals were from RNAs specifically binding to enhancer of zeste homolog 2 (EZH2) (n = 3 for each experiment).

RNA pull-down assay

Briefly, biotin-labeled RNAs were in vitro transcribed with the Biotin RNA Labeling Mix (RocheDiagnostics, Indianapolis, IN) and T7 RNA polymerase (Roche), treated with RNase-free DNase I (Roche), and purified with the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA). Cell nuclear proteins were extracted using the ProteoJETTM Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas, St. Leon-Rot, Germany). One milligram of SW480 cell nuclear extract was then mixed with 50 pmol of biotinylated RNA biotin-labeled RNAs. Sixty microliters of washed streptavidin agarose beads (Invitrogen, Carlsbad, CA) were added to each binding reaction and further incubated at room temperature for 1 hour. Beads were washed briefly five times and boiled in sodium dodecyl

sulfate buffer, and the retrieved protein was detected by the standard western blotting technique.

Construction of vectors

The cDNA encoding AFAP1-AS1 and EZH2 were purchased from Nanjing Yidao biotech Company Limited. AFAP1-AS1 and EZH2 were PCR-amplified by Q5 High-Fidelity DNA Polymerase (BioLabs) and subcloned into the EcoR1 and Xho1 sites of the pcDNA3 vector (Invitrogen), subsequently named pCDNA3-AFAP1-AS1 and pCDNA3-EZH2. pLVX-AFAP1-AS1 and pLVX-EZH2 was generated from pCDNA3-AFAP1-AS1 and pCDNA3-EZH2, respectively. EZH2 shRNA (Cat# MLCC8133 and MLCC8135) were purchased from addgene. shRNAs were designed to target AFAP1-AS1 (shAFAP1-AS1-1 target sequence: 5'-GCTTCCTTCTCTACGTCTTCA-3'; shAFAP1-AS1-2 target sequence: 5'-GCCACTTGCTTGTCTGCATGT-3') following the method described in http://rnaidesigner. invitrogen. com/rnaiexpress.

Western blot analysis

Cells were lysed in RIPA buffer, agitated for 20 minutes at 4°C, sonicated for 15 seconds using a sonic oscillator and centrifuged at 12,000 rpm for 15 minutes. The total protein concentration was determined using the BCA method. Equal amounts of the total protein (30 μg) were then denatured and loaded on 10% SDS polyacrylamide gels for separation. The proteins were transferred onto polyvinylidene difluoride membranes that were subsequently blocked with 8% non-fat milk in TBST. The membranes were incubated with primary antibodies including EZH2 (1:1000, Abcam), p15 (1:1000, Cell Signaling Technology), p16 (1:1000, Cell Signaling Technology), and p21 (1:1000, Cell Signaling Technology) at 4°C overnight. β-actin (1:5000, Protech) was used as the loading control. After washing, the membranes were incubated with HRP-conjugated goat anti-rabbit, goat anti-mouse (Cell Signalling Technology, dilution 1:5000) secondary anti-bodies for 1 h at room temperature and visualized with an enhanced chemiluminescence detection kit (Millipore). determined using the BCA method.

Xenografts

Female BALB/c-nude mice (4-5 weeks old and weighing 15-18 g) were housed under pathogen-free conditions. SW480 cells (2×10^6) were trypsinized, washed twice with serum-free medium, recon-stituted in serum-free medium DMEM, mixed 1:1 with Matrigel (Becton-Dickinson) and then inoculated subcu-taneously into the right flank of each nude mouse. All experimental procedures were carried out in accordance with the guidelines of and were approved by the Guidance of Institutional Animal Care and Use Committee of the Zhejiang Provincial People's Hospital. Bioluminescence imaging was perfomed using the IVIS Lumina imaging station (Caliper Life Sciences). Mice group allocation, surgery and assessing the outcome of mice were performed independently by different investigators.

Statistical analysis

Statistical analyses were performed in GraphPad Prism version 5.0 for Windows (GraphPad Software Inc., San Diego, CA, USA). The significance of the data from patient specimens was deter-mined by Pearson's correlation coefficient. The significance of the in vitro and in vivo data between experimental groups was determined by Student's test or Mann-Whitney U-test. $P < 0.05$ was statistically significant.

Results

AFAP1-AS1 expression is prognostic for clinical colorectal cancer

To examine the role of AFAP1-AS1 in colorectal cancer, we first determined expression of AFAP1-AS1 in clinical specimens of patients. We downloaded the Cancer Genome Atlas (TCGA) RNA-seq dataset of clinical colorectal cancer samples.

In this dataset, 50 pairs of clinical colorectal cancer tumors and the peritumoral tissues were included. Compared with the paired peritumoral tissues, the expression level of AFAP1-AS1 was significantly elevated in colorectal cancer specimens (**Figure 1A**). Moreover, we examined AFAP1-AS1 expression in one colorectal cancer RNA-seq dataset, GSE50760, and demonstrated that AFAP1-AS1 was expressed at higher levels in colorectal cancer compared with normal colon tissues (**Figure 1B**).

Then, we performed qRT-PCR assays in the total 80 clinical colorectal cancer tissues and 10 normal colon tissues. As shown in **Figure 1C**, AFAP1-AS1 was upregulated in the colorec-

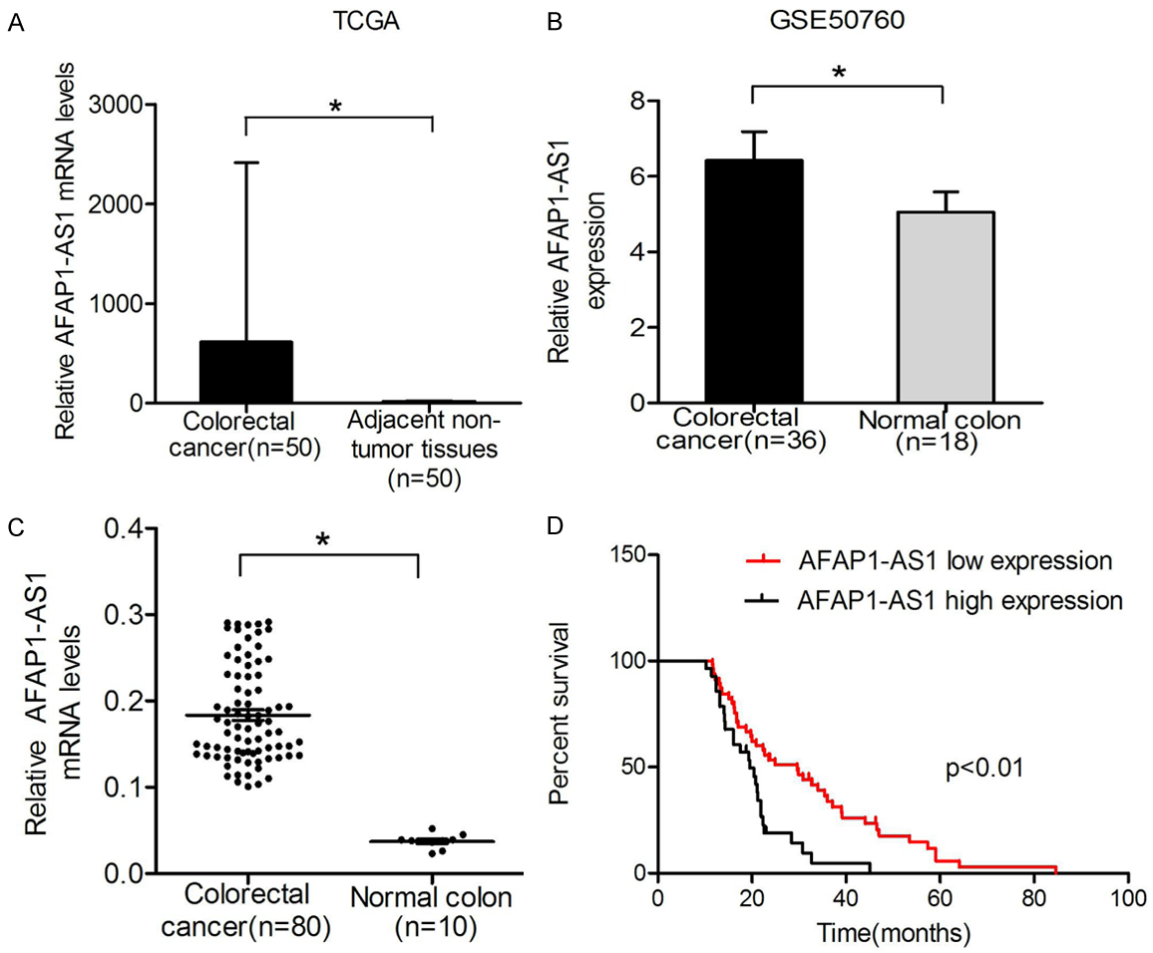

Figure 1. AFAP1-AS1 expression is prognostic for clinical colorectal cancer. A. Expression levels of AFAP1-AS1 mRNA are significantly higher in colorectal cancer samples compared with adjacent non-tumor tissues. Expression data of AFAP1-AS1 mRNA were downloaded from the TCGA dataset. B. Expression levels of AFAP1-AS1 mRNA are significantly higher in colorectal cancer samples compared with normal colon tissues. Expression data of AFAP1-AS1 mRNA were downloaded from the GSE50760 dataset. C. qRT-PCR analysis of AFAP1-AS1 in clinical colorectal cancer tissues and normal colon tissues specimens. D. Kaplan-Meier analysisof patients with high AFAP1-AS1 mRNA-expressing colorectal cancer versus low AFAP1-AS1 mRNA-expressing colorectal cancer. Statistical analysis was performed by log-rank test in a GraphPad Prism version 5.0 for Windows. Error bars ± SD. *, $P < 0.05$. Data are representative from two independent experiments.

tal cancer tissues, suggesting that the levels of AFAP1-AS1 expression are upregulated in tumor tissues.

Finally, we examined the relationship of AFAP1-AS1 expression and colorectal cancer patient survival by Kaplan-Meier survival analysis. As shown in **Figure 1D**, Kaplan-Meier survival analysis revealed a statistically significant worse prognosis for colorectal cancer patients with high AFAP1-AS1 mRNA levels compared with those with low. Taken together, these observations strongly indicate that upregulation of AFAP1-AS1 was closely associated with progression and poor prognosis in colorectal cancer patients.

Knockdown of AFAP1-AS1 contributes to cell-cycle arrest

To demonstrate the role of AFAP1-AS1 in colorectal cancer cell-cycle, we first determined LOVO, SW1116, SW480, HCT116, SW620, HT29 colorectal cancer cells and NCM460 colonic epithelial cells. AFAP1-AS1 expression was much higher in colorectal cancer cells than in NCM460 cells (**Figure 2A**), suggesting that higher AFAP1-AS1 expression may be associated with colorectal cancer phenotype. Next, we used lentivirus-mediated short hairpin RNAs (shRNA) of AFAP1-AS1 or a control shRNA to deplete AFAP1-AS1 in SW480 and HCT116

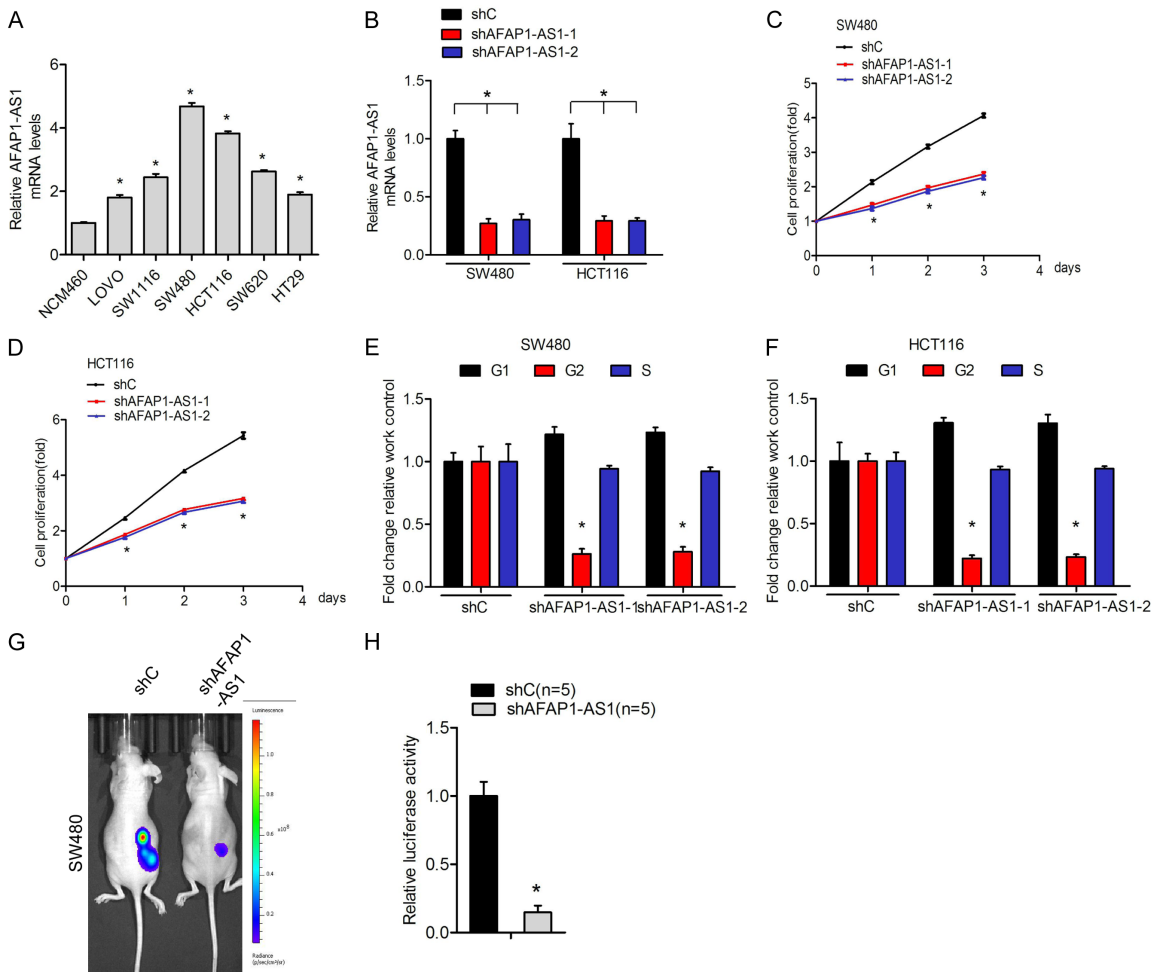

Figure 2. Knockdown of AFAP1-AS1 contributes to cell-cycle arrest. (A) qRT-PCR analysis of AFAP1-AS1 mRNA expression in colorectal cancer cells and normal colonic epithelial cells. Actin was used as a control. (B) qRT-PCR analysis of AFAP1-AS1 knockdown in SW480 and HCT116 cells. (C, D) Effects of AFAP1-AS1 knockdown on colorectal cancer cell proliferation. (E, F) Effects of AFAP1-AS1 knockdown on colorectal cancer cell cycle. (G) Representative bioluminescence images of AFAP1-AS1 knockdown-inhibited SW480 subcutaneous tumour generation. Mice were imaged at 3-4 weeks after implantation. Data were from two independent experiments with 5 mice per group with similar results. (H) Quantification of the bioluminescence activity in (G). Error bars ± SD. *, $P < 0.05$. **, $P < 0.01$. Data are representative from two independent experiments.

colorectal cancer cells (**Figure 2B**). As shown in **Figure 2C-F**, knockdown of endogenous SOX2 significantly inhibited cell proliferation, and a significant decrease in the percentages of cells in the G2 phases in both colorectal cancer cells. These results suggest that AFAP1-AS1 may contribute to cell-cycle arrest.

To probe the effects of AFAP1-AS1 on cancer cell dynamics in vivo, AFAP1-AS1-down-regulated (firefly luciferase-labeled SW480 shRNA-1 cells), or respective control cells separately implanted intracranially to generate subcutaneous xenografts in immunocompromised mice. Our results showed that the growth of tumors from AFAP1-AS1-down-regulated xenografts was significantly inhibited, compared with that of tumors formed from control xenografts (**Figure 2G** and **2H**). These data support that AFAP1-AS1 is critical for cell cycle.

Association of AFAP1-AS1 and polycomb repressive complex 2

Recent studies have reported that lncRNAs recruit polycomb-group proteins to target genes [17, 18]. Twenty percent of all human lncRNAs have been shown to physically associate with Polycomb Repressive Complex 2 (PRC2 complex) [19], suggesting that lncRNAs may have a

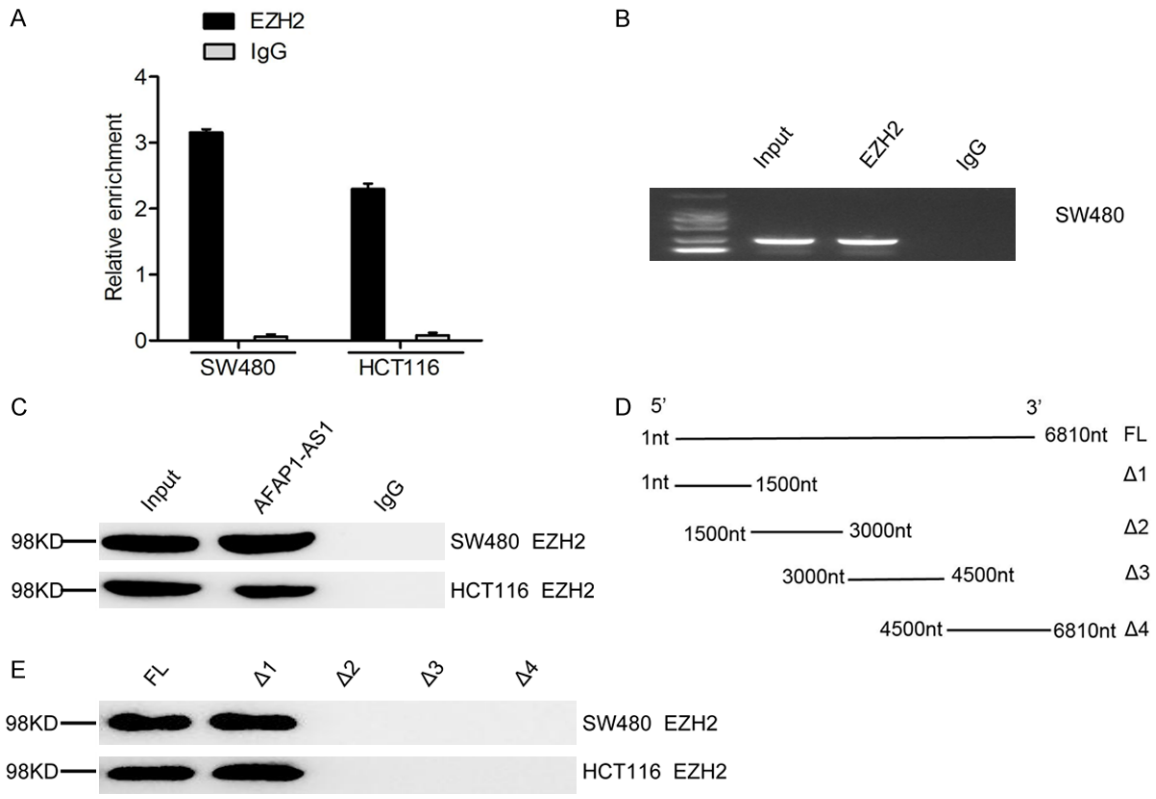

Figure 3. Association of AFAP1-AS1 and Polycomb Repressive Complex 2. (A) RIP enrichment was determined as RNA associated with EZH2 IP relative to an input control. (B) RIP experiments were performed using the EZH2 antibody to immunoprecipitate (IP). (C) Biotinylated AFAP1-AS1 was incubated with nuclear extracts (SW480 and HCT116 cells), targeted with streptavidin beads, and washed, and associated proteins were resolved in a gel. Western blotting analysis of the specific association of EZH2 and AFAP1-AS1 (n = 3). (D, E) RNAs corresponding to different fragments of AFAP1-AS1 were treated as in (C), and associated EZH2 was detected by western blotting (n = 3). Error bars ± SD. *, $P < 0.05$. **, $P < 0.01$. Data are representative from two independent experiments.

general role in recruiting polycomb-group proteins to their target genes. Thus, we hypothesized that AFAP1-AS1 might affect gene expression in such a manner. To test this, we first performed RNA Immunoprecipitation (RIP) PCR assays with an antibody against enhancer of zeste homolog 2 (EZH2; an important subunit of the PRC2 complex) from nuclear extracts of SW480 and HCT116 cells. We demonstrated that AFAP1-AS1 specifically bound to endogenous EZH2 protein (**Figure 3A** and **3B**). We next performed RNA pulldown (**Figure 3C**), to validate the association between AFAP1-AS1 and EZH2, and performed deletion-mapping experiments (**Figure 3D** and **3E**) to determine whether EZH2 would associate within a specific region of AFAP1-AS1. These analyses identified a 1500-nt region at the 5' end of AFAP1-AS1 required for the association with EZH2 (**Figure 3E**). Together, the RIP, RNA pull-down, and deletion mapping results demonstrate a specific association between EZH2 and AFAP1-AS1.

AFAP1-AS1 induces alterations in cell-cycle-related genes require EZH2

To determine the functions relevance of the association between AFAP1-AS1 and EZH2, we first overexpressed EZH2 in SW480 and HCT116 cells (**Figure 4A** and **4B**). These data presented the effectiveness of EZH2 overexpression. We next performed EZH2 overexpression in AFAP1-AS1-knockdown cells. Previous evidences showed that the cell-cycle regulation genes, such as p15 [20], p16 [21, 22], p21 [23], are direct target of PRC2. Analysis with RT-PCR and western blotting confirmed that these cell-cycle regulation genes are induced by AFAP1-AS1 depletion (**Figure 4C-E**). Furthermore, the stimulation of genes by AFAP1-AS1 knockdown was restored by EZH2 overexpression (**Figure 4C-E**). These data suggest that AFAP1-AS1 induced alterations in cell-cycle-related genes require EZH2.

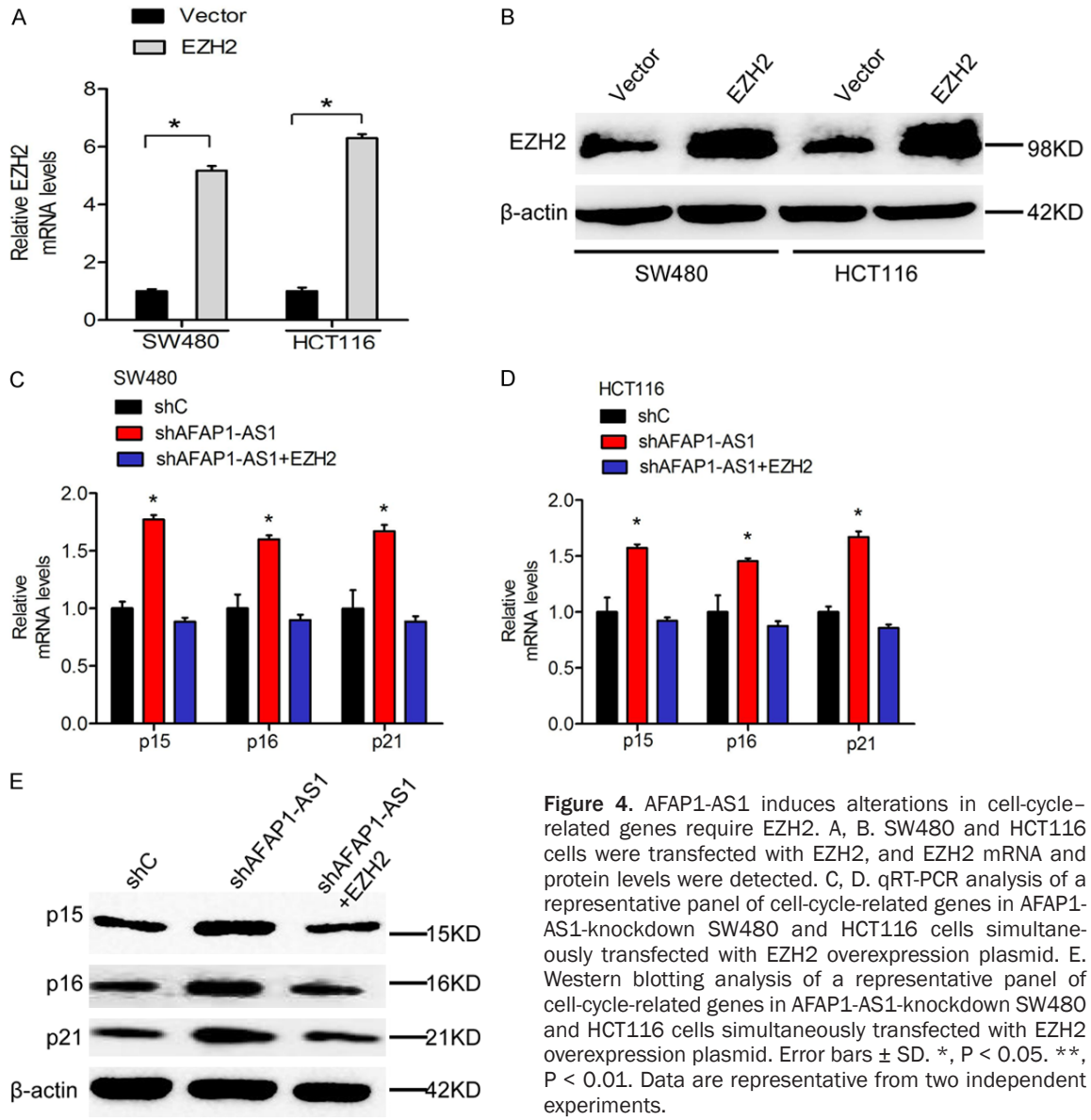

Figure 4. AFAP1-AS1 induces alterations in cell-cycle–related genes require EZH2. A, B. SW480 and HCT116 cells were transfected with EZH2, and EZH2 mRNA and protein levels were detected. C, D. qRT-PCR analysis of a representative panel of cell-cycle-related genes in AFAP1-AS1-knockdown SW480 and HCT116 cells simultaneously transfected with EZH2 overexpression plasmid. E. Western blotting analysis of a representative panel of cell-cycle-related genes in AFAP1-AS1-knockdown SW480 and HCT116 cells simultaneously transfected with EZH2 overexpression plasmid. Error bars ± SD. *, $P < 0.05$. **, $P < 0.01$. Data are representative from two independent experiments.

AFAP1-AS1 is involved in transcriptional repression through enrichment of EZH2 to target gene promoters

To address whether AFAP1-AS1 is involved in transcriptional repression through enrichment of EZH2 to target gene promoters, we first performed in silico analysis of the putative transcription factors binding to the promoter of p15, p16, and p21 (http://jaspar.genereg.net/). Six putative EZH2 binding sites were found in the promoter of these genes (**Figure 5A**). We next conducted ChIP analysis in SW480 cells. ChIP analysis demonstrated that AFAP1-AS1 depletion decreased the binding of EZH2 and H3K27me3 levels across the p15, p16, and p21 promoters, which could be rescued by EZH2 overexpression (**Figure 5B** and **5C**). Since HMT inhibitor 3-deazaneplanocin A (DZNep) had been reported to inhibit H3K27me3 deposition [24], we assessed whether AFAP1-AS1 is involved in transcriptional repression through histone methylation modifcation. As shown in **Figure 5D**, AFAP1-AS1 overexpression increased the binding of H3K27me3 levels across the p15, p16, and p21 promoters, which could be rescued by DZNep. Taken together, these data demonstrated that AFAP1-AS1 is involved in transcriptional repression through enrichment of EZH2 to target gene promoters in a H3K27me3 modification.

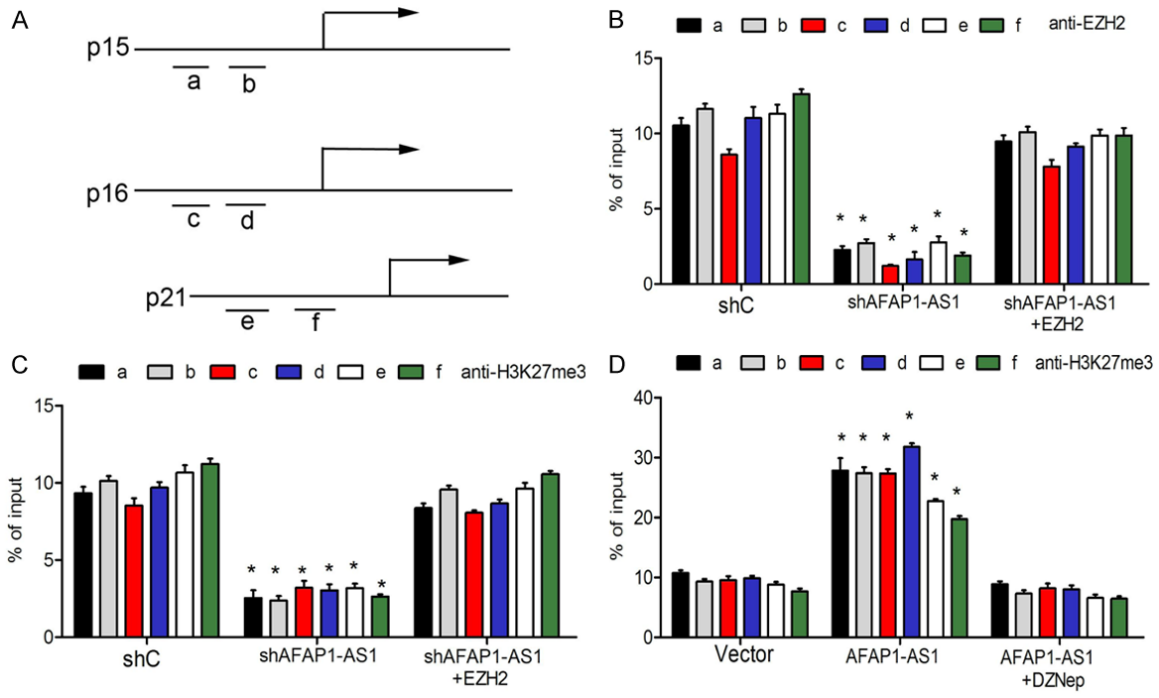

Figure 5. AFAP1-AS1 is involved in transcriptional repression through enrichment of EZH2 to target gene promoters. A-C. ChIP analyses of AFAP1-AS1-knockdown but EZH2-overexpressing SW480 cells were conducted on p15 (primer set a-b), p16 (primer set c-d), p21 (primer set e-f) promoter regions using the indicated antibodies. Enrichment was determined relative to input controls. D. ChIP analyses of AFAP1-AS1-overexpressing but DZNep treated SW480 cells were conducted on p15 (primer set a-b), p16 (primer set c-d), p21 (primer set e-f) promoter regions using the indicated antibodies. Enrichment was determined relative to input controls. Error bars ± SD. *, $P < 0.05$. **, $P < 0.01$. Data are representative from two independent experiments.

AFAP1-AS1 is involved in cell-cycle arrest through EZH2 expression

To further ascertain whether AFAP1-AS1 drive colorectal cancer cell-cycle arrest via EZH2 expression, we first examined the proliferation and cell cycle of colorectal cancer cells depleting AFAP1-AS1 but overexpression in EZH2. Knockdown of AFAP1-AS1 in SW480 and HCT-116 cells caused them to exhibit less cell proliferation (**Figure 6A**), and more cell-cycle arrest (**Figure 6B** and **6C**). Overexpression of EZH2 rescued these effects (**Figure 6A-C**). Moreover, overexpressing AFAP1-AS1 increased more cell proliferation and less more cell-cycle arrest (**Figure 6D**), which could be restored by DZNep (**Figure 6E**). These data suggest that AFAP1-AS1 is involved in cell-cycle arrest through EZH2 expression.

Discussion

In this study, we have identified lncRNA AFAP1-AS1 that is overexpressed in human colorectal cancer, compared to paired peritumoral tissues. We described AFAP1-AS1 plays a key role in cell cycle and cell proliferation regulation. AFAP1-AS1 association with EZH2, leading to the expression of EZH2-regulated target genes, resulted in enhanced colorectal cancer cell cycle.

For colorectal cancer, upregulated expression of both mRNA and microRNAs have been shown to have considerable potential in predicting the prognosis of colorectal patients [25]. Here, we showed that expression of AFAP1-AS1 was upregulate colorectal cancer samples. Moreover, evaluation of colorectal cancer patient samples revealed a negative correlation between AFAP1-AS1 expression and survival in colorectal cancer patients. Previous reports showed that AFAP1-AS1 highly upegulated in colorectal cancer and could also be used as prognostic biomarkers of colorectal cancer [6-9]. Increasing evidences indicate that AFAP1-AS1 is critical for the development of the malignant phenotype of colorectal cancer [7, 8]. Recent depletion of AFAP1-AS1 was revealed to markedly inhibited cell proliferation, cell apop-

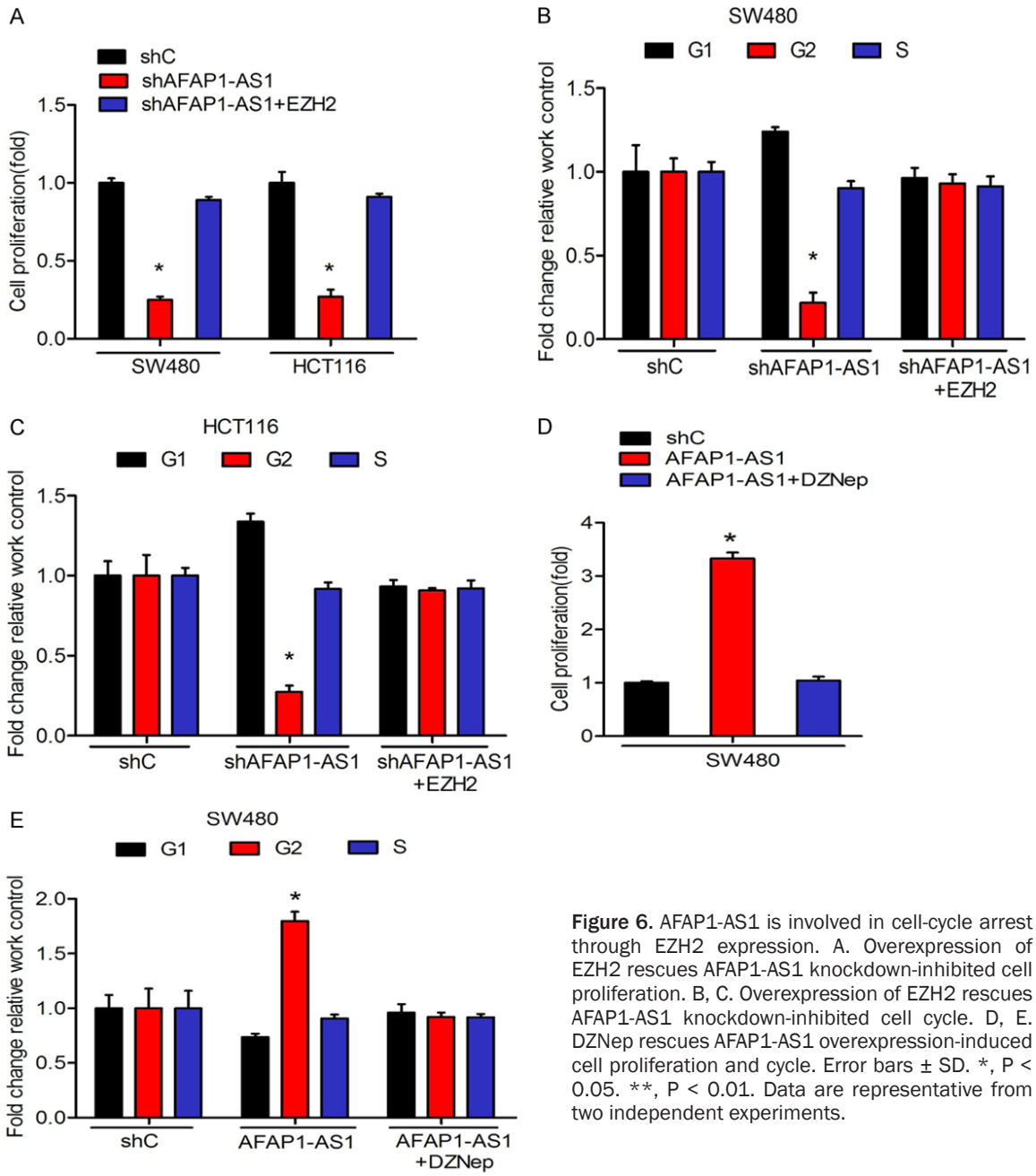

Figure 6. AFAP1-AS1 is involved in cell-cycle arrest through EZH2 expression. A. Overexpression of EZH2 rescues AFAP1-AS1 knockdown-inhibited cell proliferation. B, C. Overexpression of EZH2 rescues AFAP1-AS1 knockdown-inhibited cell cycle. D, E. DZNep rescues AFAP1-AS1 overexpression-induced cell proliferation and cycle. Error bars ± SD. *, $P < 0.05$. **, $P < 0.01$. Data are representative from two independent experiments.

tosis and cell cycle in breast cancer [7, 8]. Here, we show that AFAP1-AS1 promotes colorectal cancer cell cycle. Expression of AFAP1-AS1 was upregulated in colorectal cancer samples. Moreover, evaluation of colorectal cancer patient samples revealed a negative correlation between AFAP1-AS1 expression and survival in colorectal cancer patients. Knockdown of AFAP1-AS1 byshRNAs inhibited colorectal cancer cell proliferation and cell cycle in vitro and vivo. These results show that AFAP1-AS1 is critical for nasopharyngeal carcinoma. Detecting the expression level of AFAP1-AS1, in combination with protein-coding genes or miRNAs, may be valuable to predict the prognosis of colorectal cancer patients more accurately. We will next determine whether upregulation of AFAP1-AS1 and protein-coding genes or miRNAs also correlates with the survival of colorectal cancer patients and whether detecting these genes is more precise in identifying the prognosis of colorectal cancer patients.

Increasing evidences showed that the overexpression of EZH2 correlates with the malignant progression of colorectal cancer and the maliganent biological features of colorectal cancer [26, 27]. We showed that short-hairpin RNA(shRNA)-mediated depletion of AFAP1-AS1 leads to up-regulation of genes that are normally silenced by PRC2 (**Figure 4C-E**). Recent studies have reported that many lncRNAs binding to polycomb-group proteins regulates target gene promoters, thus contributes to biological behaviors [17, 18]. Twenty percent of lncRNAs expressed in various cell types are bound by PRC2, and that additional lncRNAs are bound by other chromatin-modifying complexes [19], suggesting that many lncRNAs likely function through their interaction with PRC2 to their target genes. The binding of AFAP1-AS1 with EZH2 could provide a regulation mechanism of PRC2 complex in colorectal cancer. It is particularly interesting that EZH2 binds AFAP1-AS1 at the site of 1-1500nt. The association of AFAP1-AS1 with EZH2 and our depletion data suggest a role of AFAP1-AS1 in the transcriptional control of gene expression. More important, our data indicate that the association of AFAP1-AS1 with EZH2 are likely to have an effect on colorectal cancer cell cycle. Overall, we provide a model where some lncRNAs binding with chromatin-modifying complexes to regulate target gene expression in colorectal cancer.

Unfortunately, because there is no mouse homolog of AFAP1-AS1, we have no in vivo models available to study this mechanism in more detail. However, the association of AFAP1-AS1 with the EZH2 and our knock-down data suggest a role of AFAP1-AS1 in the transcriptional control of gene expression. Although these data demonstrated that the observed evidences are likely to have a biological effect on cell cycle and proliferation, this possibility needs to be confirmed by specific hypothesis-driven studies. Understanding the precise molecular mechanisms by which AFAP1-AS1 function in colorectal will be critical for exploring these potential new strategies for early diagnosis and therapy of colorectal cancer.

Disclosure of conflict of interest

None.

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References

[1] Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA Cancer J Clin 2014; 64: 9-29.

[2] Siegel R, Naishadham D and Jemal A. Cancer statistics, 2013. CA Cancer J Clin 2013; 63: 11-30.

[3] Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ and He J. Cancer statistics in China, 2015. CA Cancer J Clin 2016; 66: 115-132.

[4] Al Bandar MH and Kim NK. Current status and future perspectives on treatment of liver metastasis in colorectal cancer (Review). Oncol Rep 2017; 37: 2553-2564.

[5] Tauriello DV, Calon A, Lonardo E and Batlle E. Determinants of metastatic competency in colorectal cancer. Mol Oncol 2017; 11: 97-119.

[6] Luo HL, Huang MD, Guo JN, Fan RH, Xia XT, He JD, Chen XF. AFAP1-AS1 is upregulated and promotes esophageal squamous cell carcinoma cell proliferation and inhibits cell apoptosis. Cancer Med 2016; 5: 2879-2885.

[7] Han X, Wang L, Ning Y, Li S and Wang Z. Long non-coding RNA AFAP1-AS1 facilitates tumor growth and promotes metastasis in colorectal cancer. Biol Res 2016; 49: 36.

[8] Wang F, Ni H, Sun F, Li M and Chen L. Overexpression of lncRNA AFAP1-AS1 correlates with poor prognosis and promotes tumorigenesis in colorectal cancer. Biomed Pharmacother 2016; 81: 152-159.

[9] Zhang F, Li J, Xiao H, Zou Y, Liu Y, Huang W. AFAP1-AS1: a novel oncogenic long non-coding RNA in human cancers. Cell Prolif 2018; 51.

[10] Baisden JM, Qian Y, Zot HM and Flynn DC. The actin filament-associated protein AFAP-110 is an adaptor protein that modulates changes in actin filament integrity. Oncogene 2001; 20: 6435-6447.

[11] Wu W, Bhagat TD, Yang X, Song JH, Cheng Y, Agarwal R, Abraham JM, Ibrahim S, Bartenstein M and Hussain Z. Hypomethylation of noncoding DNA regions and overexpression of the long noncoding RNA, AFAP1-AS1, in Barrett's esophagus and esophageal adenocarcinoma. Gastroenterology 2013; 144: 956-966.

[12] Wang ZY, Hu M, Dai MH, Xiong J, Zhang S, Wu HJ, Zhang SS and Gong ZJ. Upregulation of the long non-coding RNA AFAP1-AS1 affects the proliferation, invasion and survival of tongue

squamous cell carcinoma via the Wnt/beta-catenin signaling pathway. Mol Cancer 2018; 17: 3.
[13] Zhang JY, Weng MZ, Song FB, Xu YG, Liu Q, Wu JY, Qin J, Jin T and Xu JM. Long noncoding RNA AFAP1-AS1 indicates a poor prognosis of hepatocellular carcinoma and promotes cell proliferation and invasion via upregulation of the RhoA/Rac2 signaling. Int J Oncol 2016; 48: 1590-1598.
[14] Ma F, Wang SH, Cai Q, Zhang MD, Yang Y and Ding J. Overexpression of LncRNA AFAP1-AS1 predicts poor prognosis and promotes cells proliferation and invasion in gallbladder cancer. Biomed Pharmacother 2016; 84: 1249-1255.
[15] Ye Y, Chen J, Zhou Y, Fu Z, Zhou Q, Wang Y, Gao W, Zheng S, Zhao X, Chen T and Chen R. High expression of AFAP1-AS1 is associated with poor survival and short-term recurrence in pancreatic ductal adenocarcinoma. J Transl Med 2015; 13: 137.
[16] Lu X, Zhou C, Li R, Liang Z, Zhai W, Zhao L and Zhang S. Critical role for the long non-coding RNA AFAP1-AS1 in the proliferation and metastasis of hepatocellular carcinoma. Tumour Biol 2016; 37: 9699-9707.
[17] Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, Tsai MC, Hung T, Argani P, Rinn JL, Wang Y, Brzoska P, Kong B, Li R, West RB, van de Vijver MJ, Sukumar S and Chang HY. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature 2010; 464: 1071-1076.
[18] Tsai MC, Manor O, Wan Y, Mosammaparast N, Wang JK, Lan F, Shi Y, Segal E and Chang HY. Long noncoding RNA as modular scaffold of histone modification complexes. Science 2010; 329: 689-693.
[19] Khalil AM, Guttman M, Huarte M, Garber M, Raj A, Rivea Morales D, Thomas K, Presser A, Bernstein BE, van Oudenaarden A, Regev A, Lander ES and Rinn JL. Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. Proc Natl Acad Sci U S A 2009; 106: 11667-11672.
[20] Paul TA, Bies J, Small D and Wolff L. Signatures of polycomb repression and reduced H3K4 trimethylation are associated with p15INK4b DNA methylation in AML. Blood 2010; 115: 3098.
[21] Aoki R, Chiba T, Miyagi S, Negishi M, Konuma T, Taniguchi H, Ogawa M, Yokosuka O and Iwama A. The polycomb group gene product Ezh2 regulates proliferation and differentiation of murine hepatic stem/progenitor cells. J Hepatol 2010; 52: 854-863.
[22] Chen H, Gu X, Su IH, Bottino R, Contreras JL, Tarakhovsky A and Kim SK. Polycomb protein Ezh2 regulates pancreatic beta-cell Ink4a/Arf expression and regeneration in diabetes mellitus. Genes Dev 2009; 23: 975-985.
[23] Fan T, Jiang S, Chung N, Alikhan A, Ni C, Lee CC and Hornyak TJ. EZH2-dependent suppression of a cellular senescence phenotype in melanoma cells by inhibition of p21/CDKN1A expression. Mol Cancer Res 2011; 9: 418-429.
[24] Zheng S, Xiao L, Liu Y, Wang Y, Cheng L, Zhang J, Yan N and Chen D. DZNep inhibits H3K27-me3 deposition and delays retinal degeneration in the rd1 mice. Cell Death Dis 2018; 9: 310.
[25] Xu P, Wang J, Sun B and Xiao Z. Integrated analysis of miRNA and mRNA expression data identifies multiple miRNAs regulatory networks for the tumorigenesis of colorectal cancer. Gene 2018; 659: 44-51.
[26] Chen Z, Yang P, Li W, He F, Wei J, Zhang T, Zhong J, Chen H and Cao J. Expression of EZH2 is associated with poor outcome in colorectal cancer. Oncol Lett 2018; 15: 2953-2961.
[27] Tao J, Shi L, Huang L, Shi H, Chen H, Wang Y and Wang T. EZH2 is involved in silencing of WNT5A during epithelial-mesenchymal transition of colon cancer cell line. J Cancer Res Clin Oncol 2017; 143: 2211-2219.

Table S1. Oligonucleotide Sequences used in this study

Gene	Sequence
p15	5'CGAAACACAGAGAAGCGGA3'
	5'GCAGACATTGGAGTGAACG3'
p16	5'GGCTTCCTGGACACGCT3'
	5'ATCTAAGTTTCCCGAGGTTTCT3'
p21	5'TGATTAGCAGCGGAACAAG3'
	5'AACAGTCCAGGCCAGTATG3'
ACTB	5'CATGTACGTTGCTATCCAGGC3'
	5'CTCCTTAATGTCACGCACGAT3'
EZH2	5'GAAGCAGGGACTGAAACGG3'
	5'ATTGAGGCTTCAGCACCACT3'
AFAP1-AS1	5'CGTTCACTTCAATAGCCGC3'
	5'GGAGAAGGGATCGTCCCAT3'
p15 (a)	5'GCCCAGTCCTCCTTCCTT3'
	5'CCCTGTCCCTCAAATCCTC3'
p15 (b)	5'ACCCCTTGCCTTCATTTGG3'
	5'AGCCCCTTCCCTCCCTTCT3'
p16 (c)	5'GGCATCAGCAAAGTCTGAGC3'
	5'CTGGGAGACAAGAGCGAAAC3'
p16 (d)	5'AGGGGAAGGAGAGAGCAGTC3'
	5'GGGTGTTTGGTGTCATAGGG3'
p21 (e)	5'AAAGCTGACTGCCCCTATT3'
	5'GAAAGCCCAAGCCTGAAGA3'
p21 (f)	5'GCTTCAAGGCAGTGGGAGA3'
	5'CCAGGATTGTGGCTAAACC3'