

Original Article

AK001058 promotes the proliferation and migration of colorectal cancer cells by regulating methylation of ADAMTS12

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Abstract: Background: Long noncoding RNA (lncRNA) functions as multiple mechanisms, including DNA methylation in colorectal cancer (CRC). ADAMTS12 was applied as biomarkers in CRC via abnormally DNA methylation. lnc-AK001058 gene, which was reported dysregulated in CRC, is located adjacent to the gene ADAMTS12. However, little is known about the role of AK001058 during the proliferation and migration of CRC. Material and Methods: In present study, quantitative RT-PCR were used to measure AK001058 and ADAMTS12 expression levels, and western blotting assays were performed to measure ADAMTS12 expression in CRC cells. Methylation-specific PCR (MSP) was applied to measure the methylation of the CpG islands of the ADAMTS12 promoter. Cell proliferation, migration, invasion and cycle assays were utilized to analyze the role of AK001058 in CRC. Results: The results indicated that the expression of AK001058 was significantly increased in CRC. Overexpression of AK001058 could suppress the expression of ADAMTS12. AK001058 also significantly promoted cell proliferation, migration and invasion, and prolonged S stage of CRC, while silencing the expression of AK001058 showed contrary effects. Moreover, compared with negative control and AK001058-NC groups, overexpression of AK001058 could increase the DNA methylation level of ADAMTS12 gene promoter in CRC, while si-AK001058 could reverse this effect. Conclusion: In conclusion, AK001058 promotes the proliferation, invasion, migration, and prolonged S stage of CRC by regulating methylation of ADAMTS12. Our research will provide new insights for the biomarker of colorectal cancer diagnose and new clues for clinical treatment.

Keywords: lncRNA-AK001058, ADAMTS12 methylation, colorectal cancer

Introduction

Colorectal cancer (CRC) has become one of the most common malignancies worldwide, which seriously influences people's life quality [1]. The development of CRC consists of cell transformation, angiogenesis, cell migration and invasion [2], which caused approximately 30-40% of the patients dead because of postoperative relapse and metastasis [3]. However, it is still unknown for us to clarify the mechanisms of proliferation, migration and invasion of tumor cells, which should be revealed by novel diagnoses and active intervention.

Long noncoding RNA (lncRNA) was a kind of specific noncoding RNA, which is sized from 200 nt to 100 kb, has been reported critical in many diseases, including tumor formation and progression [4, 5]. lncRNA functions as multi-

ple mechanisms, including regulating the miRNA-sponge action, histone modification and DNA methylation in colon cancer [6, 7]. However, the studies between long noncoding RNA and colorectal cancer remains in its infancy. There is no doubt that exploring the role and mechanism of lncRNA in the pathophysiology of colorectal cancer will deepen the understanding of cancer development.

As mentioned above, DNA methylation is highly associate with CRC, which may mainly involve in the early stage of malignant tumor development [8, 9]. For example, CAHM, a long non-coding RNA gene, adjacent to the gene QKI which is proposed to be a tumor suppressor, hypermethylated in colorectal neoplasia [10]. These changes generally involve in the inflection of CpG islands and shores in the promoter regions of specific genes [11, 12]. The ADAMTS

comprises 19 extracellular metalloproteases, which could be applied as biomarkers in early stages of pancreatic, gastric and colorectal cancers via abnormally methylated [13-15]. ADAMTS12 (A disintegrin and metalloproteinase with thrombospondin motifs 12) was reported regulating cell proliferation, migration, invasion and tumor metastasis [16]. Further, in colon cancer, the ADAMTS12 metalloprotease gene could be silenced in the stroma. Promoter methylation could result in the silence of ADAMTS12 expression in colorectal carcinomas and colon cancer cell lines [17]. These results indicated that lncRNA could regulate the proliferation and migration of colorectal cancer cells by regulating methylation of ADAMTS12.

In previous studies, some researchers screened dysregulated lncRNA in colorectal cancer using microarray (GSE8671, GSE22598, GSE23878, GSE9348 and GSE37364). In these dysregulated lncRNAs, a long coding RNA named AK001058 has been screened and attracted our attention, which has been reported enriched in human gastric cancer tissues and significantly elevated in the plasma of patients with gastric cancer [18]. Moreover, the location of AK001058 gene (position: chr5:33,523,536-33,525,166) is adjacent to ADAMTS12 (position: chr5:33,527,188-33,891,856) in Chromosome 5, indicating AK001058 might inhibit ADAMTS12 expression via methylated way. Our research will provide new insights for the biomarker of colorectal cancer diagnose and new clues for clinical treatment.

Materials and methods

Cell culture

NCM460, HT-29, SW480 and LoVo cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The NCM460, SW480 and HT-29 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FBS (Life Technologies, Gaithersburg, MD, USA). LoVo were cultured in Ham's F12K medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FBS (Life Technologies, Gaithersburg, MD, USA). Cells were maintained at 37°C in a 5% CO₂/atmosphere saturated with water.

Plasmid construction and transfection

AK001058 plasmid and its negative control (AK001058-NC), small interference RNA (si-RNA) targeting AK001058 (si-AK001058) and its scramble control (si-NC) were obtained from Gene Pharma Co., Ltd. (Shanghai, China). AK001058 sequences were cloned into pcDNA3.1 (Invitrogen) to produce AK001058 overexpression vectors. AK001058, AK001058-NC, si-AK001058 and si-NC were respectively expressed in LoVo cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. Transfection with nothing was used as normal control.

RNA isolation and quantitative RT-PCR

Total RNA from the cultured cells were isolated using TRIzol Reagent (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. Real-Time RT-PCR for AK001058, ADAMTS12 and GAPDH were measured using an Applied Biosystems 7300 Sequence Detection System (Applied Biosystems) and SYBR Green Master Mix (Roche). The primer sequences are as follows: AK001058 forward: 5'-GCTTGCTGTACAAGAGGGGA-3' and reverse: 5'-CTGCGATGGCCCTCTCTATT-3'; ADAMTS12 forward: 5'-CAGAAAGGACATCTTGCTGG-3' and reverse: 5'-TCCTGGCAGAAGGTGCATTC-3'; GAPDH forward: 5'-TGGGTGTGAACCACGAGAA-3' and reverse: 5'-GGCATGGACTGTGGTCATGA-3'; U6 forward: 5'-CTCGCTTCGGCAGCAC-3' and reverse: 5'-AACGCTTCACGAATTTGCGT-3'. The thermocycling conditions: initial denaturation at 95°C for 10 min, followed by 35 cycles of 95°C for 10 s and 60°C for 30 s. GAPDH and U6 were applied to normalize the level of expression of ADAMTS12 and AK001058 respectively using 2^{-ΔΔCt} method as relative quantification.

DNA extraction and methylation-specific PCR (MSP)

An interval of 2.0 kb upstream and 0.5 kb downstream from the first ADAMTS12 ATG codon was analyzed for CpG island identification using the Methyl Primer Express Software v1.0 (<https://productcs.appliedbiosystems.com>). This program was also used to design MSP primers. Cells were collected following 5-aza-2'-deoxycytidine (Aza; Sigma-Aldrich, St. Louis, MO, USA, 10 μM) treatment as described previously [19]. Genomic DNA was

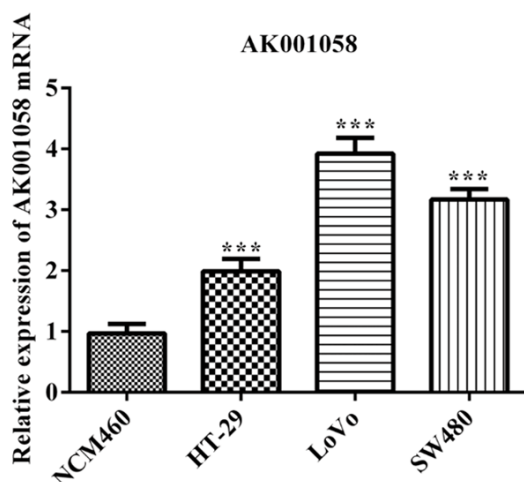


Figure 1. AK001058 expression in colorectal cancer cells. Expression of AK001058 in colorectal cancer cell line HT-29, SW480, LoVo and the normal colonic epithelial cell line NCM-460 was detected using qRT-PCR, *** $P < 0.001$ vs. NCM-460. Data were expressed as the mean \pm SD.

extracted using the QIAmp DNA blood Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Bisulfite modification of DNA and methylation of the CpG islands of the ADAMTS12 promoter were performed as described previously [17]. Primers for the unmethylated reaction were 5'-GAGTTTGGG-AGGAAGATGTATT-3' and 5'-CTAACAATATCCAC-TTTCACAAAA-3', and for the methylated reaction 5'-GAGTTCGGGAGGAAGATGTATC-3' and 5'-ACAATATCCGCTTTCGACG-3'.

Cell proliferation assay

Cell proliferation of LoVo cells were measured by CCK-8 assay (Sigma-Aldric, USA). Cells were seeded in 96-well culture plates (1×10^4 cells/well, 100 μ l each well) and cultured in appropriate medium containing 10% FBS for 24 h. CCK-8 reagents (10 μ l/well) were added to each well and cells were incubated according to the manufacturer's protocol, followed by measuring the absorbance (optical density, OD) at 450 nm on an enzyme immunoassay analyzer (Bio-Rad, USA).

Cell cycle assay

48 h after transfection, cell cycle assay was applied to determine cell cycle distribution. Briefly, LOVO cells were collected using 0.25% trypsin, washed, and then fixed with 70% ethanol at 4°C overnight. Then, the cells were stained with RNase A and PI at 4°C for 30 min.

Finally, flow cytometer (FACSort; FACSCanto II, BD Biosciences, Franklin Lakes, NJ, USA) was performed to analyze the cell cycle distribution. Tests were repeated at least for 3 times.

Cell invasion assay

For invasion assay, the invasion of LoVo were measured using Transwell assay as the reference. Cells were seeded in 24-well transwell plates (5×10^4 cells/well) in the upper chambers with Matrigel-coated membrane (BD Bioscience, San Jose, USA) without serum. And the cell culture medium containing 20% FBS was added the bottom of the chambers as a chemoattractant. Cells were incubated through the polyethylene terephthalate membrane for 24 h at 37°C with 5% CO₂. Finally, the invasive cells which passed through the membrane were fixed and then stained with hematine. The number of invaded cells was counted using a light microscope, and the average value was determined through five different random fields.

Cell migration assay

For wound healing assay, LoVo cells were seeded in 6-well plates (5×10^5 cells/well). A pipette tip was used to create a wound after 48 h transfection. The cells were then cultured in serum-free medium. Cell migration was determined by detecting the average distance of growing cells migrated into wound surface under an inverted microscopy at 0 h and 48 h.

Statistical analysis

All results were obtained from at least three separate experiments. The data are expressed as the means \pm SD. SPSS 17.0 was used for the statistical analyses. The statistical comparisons were performed using one-way analysis of variance and Student's t-test. Differences were considered significant at $P < 0.05$. Two-tailed tests were used for the univariate comparisons.

Results

AK001058 was significantly up-regulated in CRC cell lines

The expression levels of AK001058 in human CRC cell lines (HT-29, SW480, LoVo) and the normal human colonic epithelial cells NCM460 were analyzed using qRT-PCR analysis. As shown in **Figure 1**, the expression of AK001058

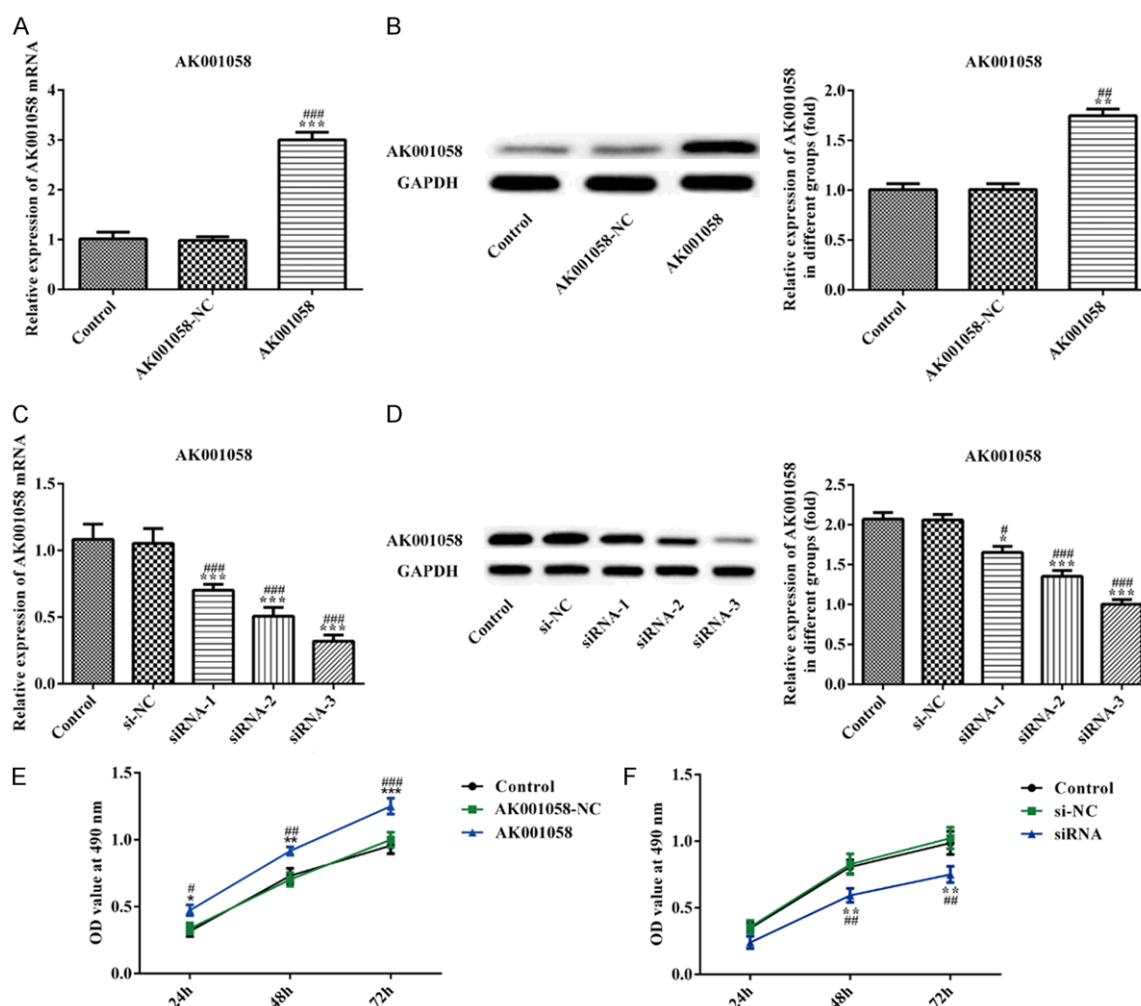


Figure 2. Effect of AK001058 on colorectal cancer cell viability. The mRNA (A) and protein level (B) of AK001058 was increased by transfecting AK001058 plasmid. The mRNA (C) and protein level (D) of AK001058 was decreased by interfering AK001058. 48 h after transfecting AK001058 (E) or siRNA (F), cell viability was detected using CCK-8 assay in LoVo cells. *, **, ***P < 0.05, 0.01, 0.001 vs. Control; #, ##, ###P < 0.05, 0.01, 0.001 vs. NC. Data were expressed as the mean \pm SD. NC: negative control of AK001058 or siRNA.

in HT-29, SW480 were significantly increased compared with NCM460, and LoVo cells had the highest expression of AK001058. These results indicated that AK001058 was significantly up-regulated in CRC cell lines. And we used LoVo cells to perform further analysis.

AK001058 promoted cell proliferation, migration, invasion, and inhibited G1 phase arrest in LoVo cells

To investigate the role of AK001058 in CRC cells, AK001058 was upregulated or downregulated in CRC cell lines (LoVo) by transfection with the AK001058 plasmid or specific siRNAs for AK001058 (siRNA-1, siRNA-2, siRNA-3), respectively. At 48 h after transfection, transfection efficiency was determined using RT-

qPCR. As depicted in **Figure 2**, the transfected AK001058 significantly increased AK001058 expression in LoVo cells, while the siRNAs especially siRNA-3 significantly decreased AK001058 expression. And we used siRNA-3 to perform further analysis.

Furthermore, cell viability was analyzed with a CCK-8 assay. Cell migration, invasion and cell cycle were analyzed with Transwell assay, wound healing assay and flow cytometry, respectively. In the CCK-8 assays, the results indicated that the AK001058 significantly promoted LoVo cell viability at 48 and 72 h, and the effect could be reversed by siRNA (**Figure 2**). Similarly, cell migration and invasion could be significantly regulated by AK001058 and siRNA (**Figure 3**). In flow cytometry, compared

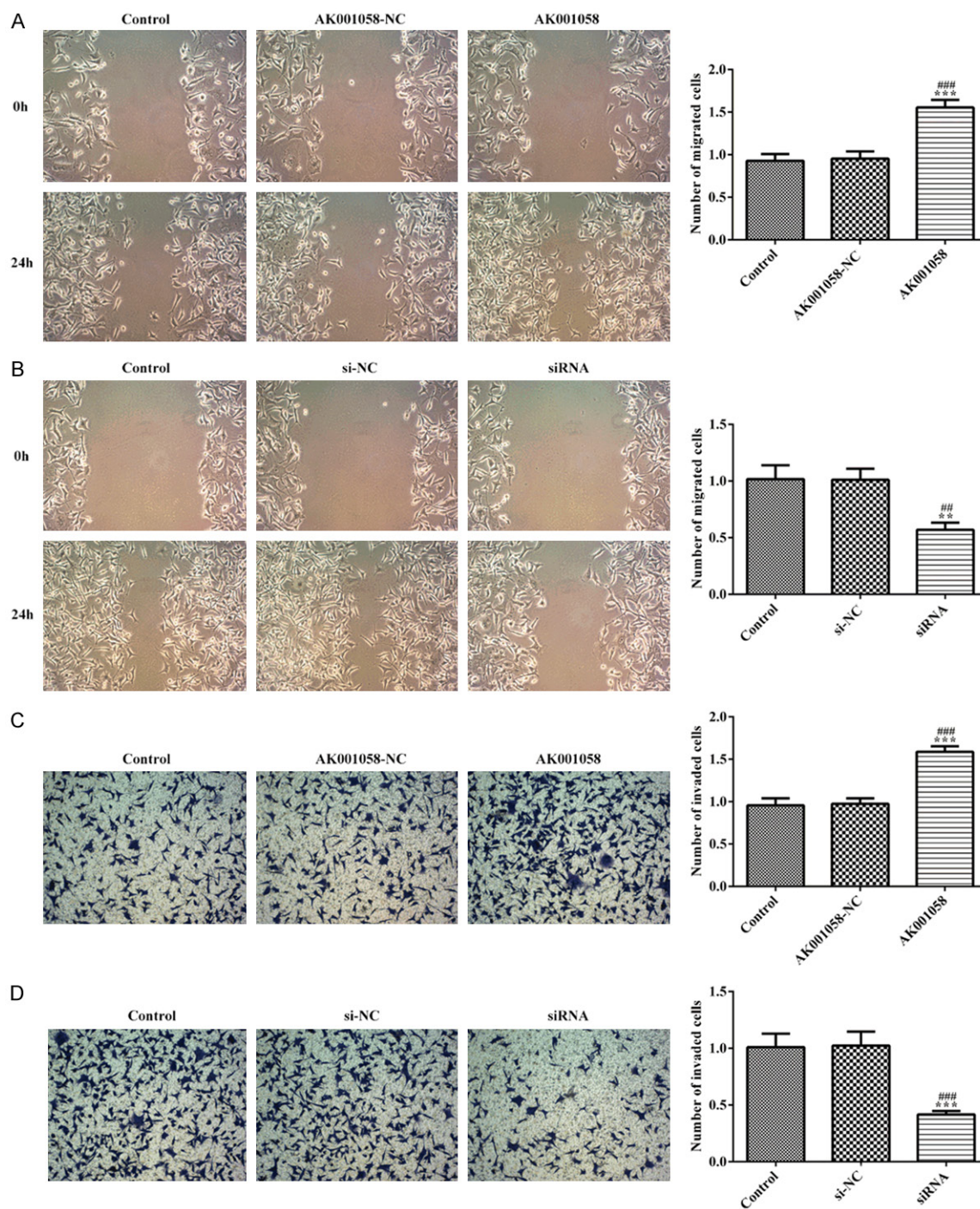


Figure 3. Effect of AK001058 on cell migration and invasion of colorectal cancer. 48 h after cell transfection, cell migration and invasion of LoVo were analyzed in wound healing assay (A and B) and Transwell assay (C and D). *, **, ***P < 0.05, 0.01, 0.001 vs. Control; #, ##, ###P < 0.05, 0.01, 0.001 vs. NC. Data were expressed as the mean \pm SD. NC: negative control of AK001058 or siRNA.

with the control and NC group, the AK001058 inhibited G1 phase arrest. Additionally, there were significant increase of S-phase cells in the AK001058 group of LoVo cells, while siRNA indicated the opposite effects (Figure 4).

AK001058 regulated ADAMTS12 expression via methylation in CRC

In this study, we showed the regulatory mechanism of AK001058 in LoVo cells. We found that

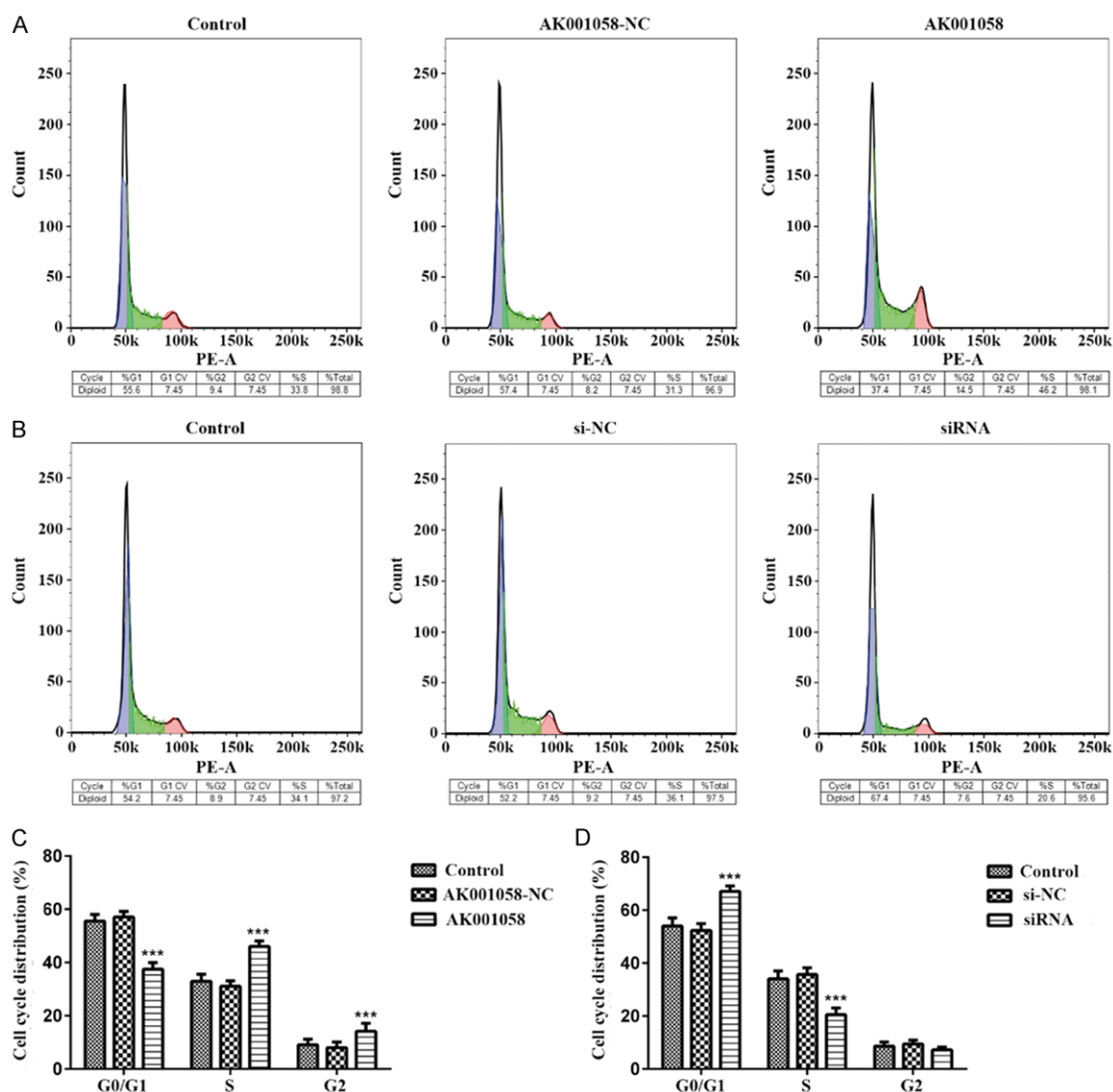


Figure 4. Effect of AK001058 on colorectal cancer cell cycle distribution. 48 h after transferring AK001058 (A and C) or siRNA (B and D), cell cycle distribution of LoVo cells were analyzed using FCM. *, **, ***P < 0.05, 0.01, 0.001 vs. Control. Data were expressed as the mean \pm SD.

overexpression of AK001058 significantly inhibited the expression of ADAMTS12 in LoVo cells, while siRNA enhanced the ADAMTS12 expression (Figure 5). This result indicated that AK001058 promoted cell proliferation, migration, invasion and cell cycle by regulating ADAMTS12 expression. MSP amplification of a 138 bp promoter within the ADAMTS12 CpG Island was performed on LoVo cells, which enhanced or interfered the expression of AK001058. Compared with the control group and NC group, ADAMTS12 gene promoter was found barely methylated in AK001058 group and more methylated in siRNA group (Figure 6).

The above results implied that ADAMTS12 expression could be regulated by AK001058 via methylation pathway.

Discussion

In the present study, it was demonstrated that AK001058 was significantly up-regulated in CRC cell lines. AK001058 promoted cell proliferation, migration, invasion, and inhibited G1 phase arrest by hypermethylating ADAMTS12 promoter in the CRC cell lines. Therefore, AK001058 might be the biomarker of colorectal cancer diagnose and new clues for clinical treatment.

Effect of AK001058 on colorectal cancer through methylation of ADAMTS12

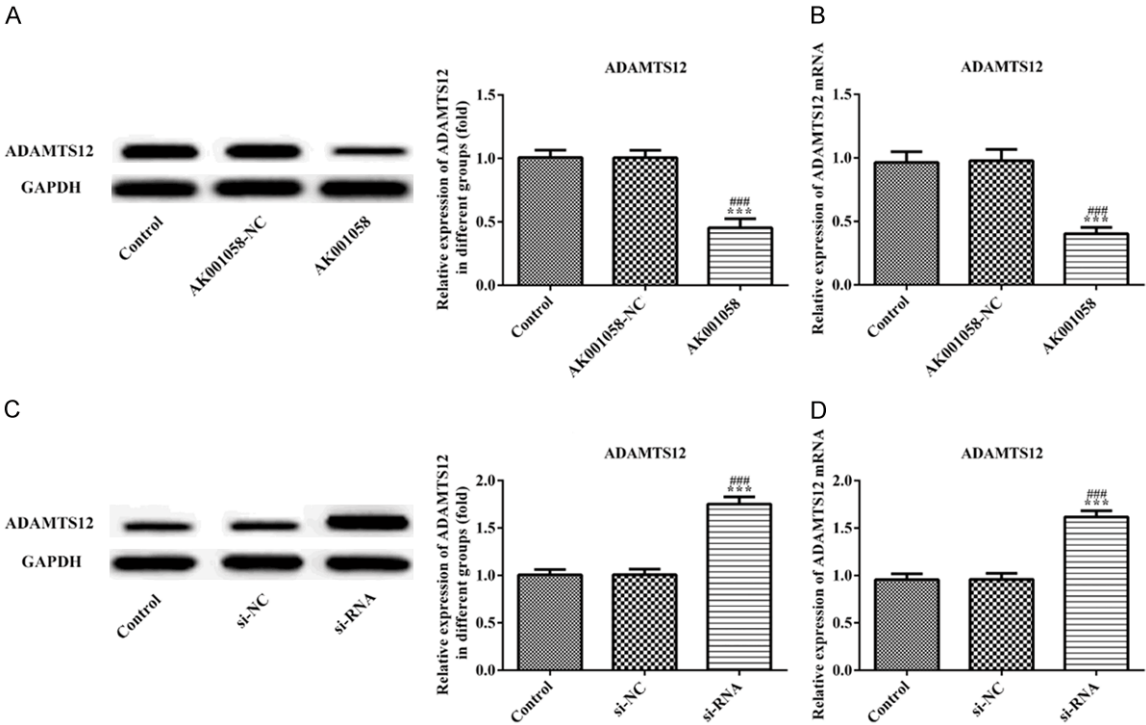


Figure 5. Effect of AK001058 on ADAMTS12 expression in colorectal cancer cells. 48 h after transferring AK001058 (A and B) or siRNA (C and D), ADAMTS12 mRNA and protein level in LoVo cells. *** $P < 0.001$ vs. Control. ### $P < 0.001$ vs. NC. Data were expressed as the mean \pm SD. NC: negative control of AK001058 or siRNA.

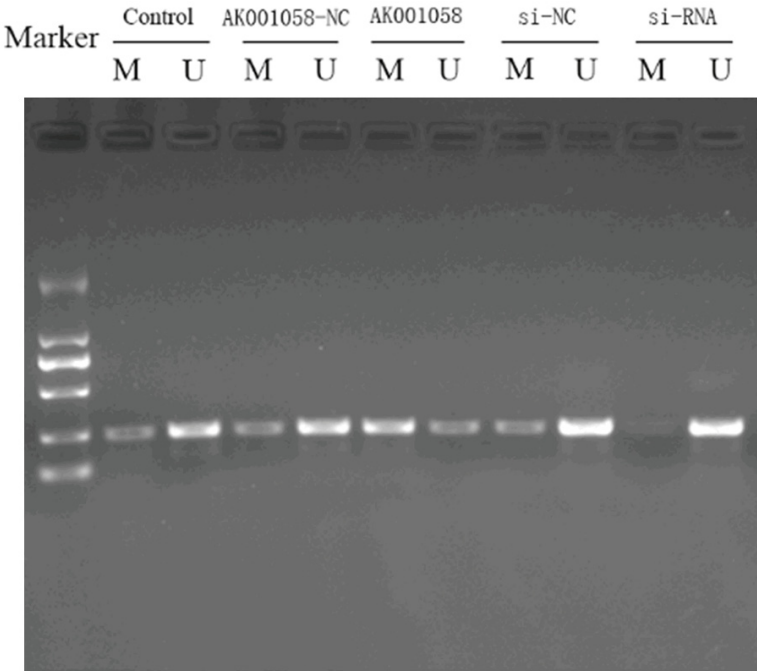


Figure 6. Methylation status of ADAMTS12 after transferring AK001058 or siRNA in LoVo cells. PCR-methylation analysis was carried out using the specific primers for either methylated (M) or modified unmethylated DNA (U).

[20]. The number of patients suffering from CRC in China is increasing annually, and CRC occurs more frequently in younger patients [21]. Therefore, the diagnosis and treatment of CRC are crucial [22]. LncRNA serves significant function, including structural or trafficking roles, cell differentiation, and apoptosis [23]. LncRNA also has a broad range of mechanisms, including regulating the neighboring gene, miRNA-sponge action, and coding small peptide to suppress the colon cancer [24]. LncRNA GAPLINC was reported to promote CRC cell invasion via binding to PSF/NONO and activating SNAI2 expression [25]. Up-regulation of BC029135 suppresses CRC invasion and inactivates Wnt/ β -catenin signaling [26].

In previous study, the expression levels of nine cancer-associated lncRNAs were dysregulated in two gastric tissue micro-

CRC is one of the malignant tumor types of the digestive system that endangers human health

arrays, and their relative expression levels were detected by qRT-PCR. AK001058 was found enriched in human gastric cancer tissues and significantly elevated in the plasma of patients with gastric cancer [18]. In present study, we also found that AK001058 was significantly up-regulated in CRC cell lines. AK001058 significantly promoted cell proliferation, migration and invasion, and prolonged S stage of CRC, while silencing the expression of AK001058 showed contrary effects.

ADAMTS12 is a complex metalloproteinase that contains multiple TSP-1 (Thrombospondin-1) repeats in its C-terminal region [27]. Functional studies have demonstrated the participation of ADAMTS in a variety of processes, such as collagen maturation, organogenesis, proteoglycan degradation, inhibition of angiogenesis, reproduction and inflammation [28]. ADAMTS12 also promotes the invasive ability of trophoblastic cells and acts as an early marker of male gonad differentiation in chicken [29]. In addition, ADAMTS12 expression has been mainly detected in some human fetal tissues as well as in a variety of carcinomas and cancer cell lines. Previous study has suggested that ADAMTS12 and fibulin-2 could be a good prognosis marker in breast cancer diagnosis [30]. The ADAMTS12 metalloproteinase also exhibited antitumorigenic properties through modulation of the Ras-dependent ERK signal pathway [16]. Studies have shown that ADAMTS12 expression is deficient in colon cancer cells and in tissues [31]. In our study, we found that overexpression of AK001058 significantly inhibited the expression of ADAMTS12 in CRC cells, while siRNA enhanced the ADAMTS12 expression. The result indicated that AK001058 promotes the proliferation and migration of colorectal cancer cells by regulating ADAMTS12.

At present, epigenetic silencing and genetic inactivation in ADAMTS family members has been frequently reported. ADAMTS15 is genetically silenced in human colorectal cancer [32]. ADAMTS1 and ADAMTS9 have been found to be epigenetically silenced in diverse malignant tumors [33, 34]. The most widely studied epigenetic alteration in cancer is aberrant DNA methylation. Many laboratories further identified panels of genes methylated with high frequency in CRC, such as ALX4, BMP3, CACNA1G, CDKNA2, CDH1, Corf50, IGF2, NEUROG1,

RUNX3, SEPT9, SFRP2, SOCS1, THBD, TMEFF2, VHL, and VIM [35]. ADAMTS12 has been identified as potential tumor suppressor in colorectal cancer. The ADAMTS12 gene was subjected to a generalized methylation process in colorectal tumors and in a variety of tumor cells from different origins, a mechanism of growing relevance in the inactivation of tumor-suppressor genes [17]. In present study, ADAMTS12 gene promoter was found aberrantly methylated after the interference of AK001058 in CRC. This result was consistent with previous studies, and revealed that ADAMTS12 could be regulated via methylation in CRC. More importantly, a recent ENCODE survey revealed that the expression of almost 3% of lncRNAs shows high positive correlation with that of a neighboring mRNA [36]. As reported previously, the CAHM gene is located on chromosome 6, hg19 chr6:163,834,097-163,834,982. It lacks introns, encodes lncRNA and is located adjacent to the gene QKI, which encodes an RNA binding protein. CAHM transcripts might also be RNA binding partners for the Qki protein and may perhaps be involved in directing its specificity. Methylation would appear to extend across the CpG island and encompass both the CAHM and QKI transcription start site (TSS) [10]. In our research, the location of AK001058 gene (position: chr5:33,523,536-33,525,166) is adjacent to ADAMTS12 (position: chr5:33,527,188-33,891,856) in Chromosome 5, indicating AK001058 might inhibit ADAMTS12 expression via methylated way. In-depth mechanism should be investigated in further studies.

In summary, AK001058 was significantly up-regulated in CRC, which promoted cell proliferation, migration, invasion, and inhibited G1 phase arrest. AK001058 could inhibited the expression of ADAMTS12, which might be regulated via methylated way. AK001058 and ADAMTS12 hypermethylation provided biomarker worthy of future study and with promise to enhance the early detection and inform treatment of CRC.

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Disclosure of conflict of interest

None.

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