Long non-coding RNA MLK7-AS1 promotes proliferation in human colorectal cancer via downregulation of p21 expression

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Abstract. Current studies have highlighted long non-coding RNAs (lncRNAs) as critical regulators in various cancers, including colorectal cancer (CRC). By utilizing publicly available data from The Cancer Genome Atlas dataset, MLK7 antisense RNA 1 (MLK7-AS1) was identified as a novel lncRNA that correlated with CRC progression. The results of reverse transcription-quantitative polymerase chain reaction (RT-qPCR) revealed a significant upregulation of MLK7-AS1 in both CRC tissue samples and cell lines. In addition, a positive correlation was observed between increased MLK7-AS1 expression and several clinicopathological factors in patients with CRC. Importantly, MLK7-AS1 knockdown suppressed CRC cell proliferation and promoted G1/G0 phase arrest and apoptosis *in vitro*, whereas MLK7-AS1 overexpression exhibited opposite effects. Consistently, decreased MLK7-AS1 expression inhibited tumor growth *in vivo*. Furthermore, RT-qPCR and western blot assays revealed that p21 may be a potential downstream target of MLK7-AS1. To the best of the authors' knowledge, this is the first study to report that MLK7-AS1 has potential as a biomarker and may promote proliferation in CRC partially through downregulating p21 expression.

Introduction

Colorectal cancer (CRC) is one of the common malignancies, with an estimation of 693,900 deaths in 2012 (1). Patients with CRC are frequently diagnosed at an advanced stage due to lack of effective diagnostic biomarkers and the prognosis is unsatisfactory even with comprehensive therapies (2-4). Thus, it is necessary to identify novel biomarkers and therapeutic targets for CRC patients. Identification of crucial molecules will help to accelebrate the research on CRC pathogenesis.

With the improvement of high-resolution microarray and RNA sequencing technology, it has been verified that non-coding RNAs (ncRNAs) occupies a higher rate of 98% in transcripts of human genome (5-7). Long non-coding RNAs (lncRNAs), newly identified counterparts of ncRNAs, have been demonstrated to be dysregulated and serve as critical regulators in various tumors (8-10). Current investigations have focused on the pathogenesis of lncRNAs in multiple cancers (11-14). Dysregulation of lncRNAs can alter the process of many biological events, such as cell cycle, apoptosis, invasion, and epigenetic regulation (15,16). Nie *et al* (17), found an obvious upregulation of lncRNA ANRIL in non-small-cell lung cancer (NSCLC) tissue samples. ANRIL acts as an oncogene in CRC partly via decreasing p21 and Kruppel like factor 2 (KLF2) expression (17). In contrast, increased lncRNA-LET inhibits cell proliferation, invasion, and migration in lung cancer (18). Thus, lncRNAs may be oncogenes or tumor suppressors to mediate cancer progression. There is a critical need to investigate correlations between lncRNAs and carcinogenesis, especially CRC. However, only a small portion of lncRNAs have been functionally studied, more lncRNAs should be identified (19).

To detect aberrantly expressed lncRNAs associated with CRC progression, we analyzed The Cancer Genome Atlas (TCGA) colon cancer and normal tissue RNA sequencing data (49 normal and 648 cancer samples), and focused on a remarkably overexpressed lncRNA termed MLK7 antisense RNA 1 (MLK7-AS1). LncRNA MLK7-AS1 is located on human chromosome 2 with a length of 2551 bp. It was firstly reported to be significantly upregulated in gastric cancer (GC) tissues (20). Further, MLK7-AS1 can serve as an independent prognosis indicator in GC patients (20). However, the expression pattern, functional role, and clinical significance of MLK7-AS1 in CRC still remain uncharacterized. In this study, we show the first reporting of expression pattern and funtional role associated with MLK7-AS1 in CRC. MLK7-AS1 is significantly increased in CRC tissues and cells. Further, MLK7-AS1 overexpression exhibits tight associations with clinicopathologic factors in CRC patients. *In vitro* and *in vivo* experiments suggested that decreased MLK7-AS1 expression inhibited cell proliferative capacities, and

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promoted cell cycle arrest and apoptosis in CRC. Consistently, MLK7-AS1 overexpression showed opposite effects.

Cyclin-dependent kinase inhibitors (CKIs) are known to modulate cell cycle progression and function as tumor suppressors (21,22). p21 is an essential member of CKIs family, which includes p15, p16, p27 and p57 (21). Mounting studies reveal that lncRNAs can alter cancer cell phenotypes through silencing tumor suppressors and CKIs are involved in biological functions induced by lncRNAs (10,23,24). Thus, we further investigated the alteration of CKIs family expression levels in DLD-1 and LOVO cells with MLK7-AS1 knockdown. Our findings revealed that decreased MLK7-AS1 expression levels remarkably activated p21 expression at both mRNA and protein levels. Therefore, p21 is partly involved in MLK7-AS1-induced proliferation in CRC. Overall, MLK7-AS1 has potential as a biomarker for CRC patients and promotes CRC cells proliferation partly through suppressing p21 expression.

Materials and methods

Expression profiling data retrieval and analysis of lncRNAs in colorectal cancer. Expression profiling data of CRC and normal tissue samples were downloaded from the TCGA dataset. The expression data are collected from cancer patients before therapeutic intervention (25). The BAM files and normalized probe-level intensity files can be achieved from the Atlas of Non-coding RNAs in Cancer (TANRIC, http://bioinformatics.mdanderson.org/main/TANRIC:Overview) database (25). Gene annotations accords to GENCODE Release 19 annotation for lncRNAs.Reads per kilobase per million mapped reads (RPKM) values were calculated using TCGA RNA-sequencing data in the BAM files.

Tumor tissue samples and matched non-tumor tissue samples. The CRC tissues (n=50) and matched non-tumor tissue samples (n=50) were obtained from CRC patients with surgical resection in Cancer Hospital of China Medical University. The patients who participated in this study do so in the context of informed consents. Median value was selected as cut-off point to better compare correlations between clinical pathological factors and the expression level of certain gene in patients with cancer (10,26,27). In the present study, we divided 50 PC patients into two groups: the high MLK7-AS1 group (n=25, fold change above the median value); and the low MLK7-AS1 group (n=25, fold change below the median value) according to the median value of MLK7-AS1 levels. The study was approved by the Cancer Hospital of China Medical University Ethics Committee. The collected tissue samples were instantly stored in a liquid nitrogen, and then transferred to be kept at -80˚C.

Cell culture. Human colonic epithelial cells (HCoEpiC) and CRC cells (SW480, HCT116, LOVO and DLD-1) are all cultured in DMEM medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 mg/ml streptomycin, and 100 U/ml penicillin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C with 5% O_2.

Reverse transcription-quantitative polymerase chain reaction PCR (RT-qPCR) analyses. A Reverse Transcription Kit (Thermo Fisher Scientific Inc.) was used to reverse total RNA into cDNA. Then, cDNA and primers were used to perform qRT-PCR assays on 7500 Real-Time PCR System (Thermo Fisher Scientific Inc.). The specific primers are listed in Table II. The results downloaded from this instrument were then normalized to GAPDH expression. The collected data were analyzed, expressed relative to threshold cycle values, and then switched to fold changes. Each sample was analyzed in triplicate.

Cell transfection. CRC cell lines were transfected with three individual MLK7-AS1 (MLK7-AS1 no. 1, no. 2 and no. 3), scrambled negative control (NC) small interfering RNAs (siRNAs), as well as vectors such as pcDNA-MLK7-AS1, empty vector, and sh-MLK7-AS1-vector. SiRNAs were transfected into cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Plasmid vectors were extracted by DNA Midiprep kit (Qiagen GmbH, Hilden, Germany) and transfected into cells using Fugene (Roche Diagnostics, Basel, Switzerland). The full-length complementary DNA of MLK7-AS1 was synthesized by Realgene (China), and subcloned into the pcDNA3.1(+) vector (Invitrogen; Thermo Fisher Scientific, Inc.). Up to transfection after 48 h, collected cells were used to conduct qRT-PCR and western blot experiments. The sequences for siRNAs and shRNAs are listed in Table III.

Assays of MTT and colony formation. The cells are cultured in 96-well plates. MTT experiments were conducted to test cell viability at 490-nm-wavelength by Microplate Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For assays of colony formation, cells with transfection after 48 h are placed in six-well plates. Up to 14 days, methanol-fixed colonies were stained with crystal violet of 0.1% and then colony numbers in each group can be counted.

EdU assay. 5-ethynyl2-deoxyuridine (EdU) labeling/detection kit (Guangzhou RiboBio Co., Ltd., Guangzhou, China) was used to assess proliferating cells. SW480 and LOVO cells were cultivated in 96-well plates and transfected with siRNAs for 48 h. Then, the cells were cultured in EdU labeling medium and incubated for 2 h at 37˚C under 5% CO_2. After treatment with 4% paraformaldehyde and 0.5% Triton X-100, anti-EdU working solution was ued to stain cells. And DAPI was used to label cell nuclei. The percentage of EdU-positive cells was calculated from five random fields in three wells.

Flow cytometry. DLD1 and LOVO cells with 48 h transfection of si-MLK7-AS1 no. 1, no. 2 and si-NC were stained with FITC-Annexin-V and propidium iodide (PI). Then, flow cytometry (FACScan®; BD Biosciences, Franklin Lakes, NJ, USA) was used to detect alterations of cell cycle. Furthermore, cells were classified into viable cells, dead cells, early apoptotic cells, and apoptotic cells through analysis of flow cytometry.

Western blot analysis and antibodies. RIPA buffer with proteinase inhibitor cocktail (Medchem Express, NJ, USA) was used to lyse cells. The membranes were incubated with antibodies against CDK2, CDK4 and P21. GAPDH was used as a reference control. All the antibodies are obtained from Cell Signaling Technology, Inc., (Danvers, MA, USA).

Table I. Correlation between MLK7-AS1 expression and clinicopathological factors of colorectal cancer patients.

Variables	MLK7-AS1 expression		P-value
	High	Low	
Age (years)			
>60	11	16	0.156
≤60	14	9	
Gender			
Male	17	17	1.000
Female	8	8	
Tumor size			
≤5 cm	9	18	0.011[a]
>5 cm	16	7	
TNM stage			
I/II	4	11	0.031[a]
III/IV	21	14	
Lymph node metastasis			
Positive	21	14	0.031[a]
Negative	4	11	

[a]P<0.05.

Xenograft model in nude mice. Male athymic BALB/c nude mice of 4 weeks old were purchased from Institute of laboratory animal medicine, Chinese Academy of Medical Sciences. The study accords with rules of Cancer Hospital of China Medical University Ethics Committee. A total of 100 μl empty-vector-transfected or sh-MLK7-AS1-transfected DLD-1 cells was respectively injected into a single side of each mouse. Measurement of tumor volume was conducted every four days. Up to twenty-four days after injection, the mice were killed and tumors removed from the mice were kept in 4% paraformaldehyde for further research.

Immunohistochemical (IHC) analysis. The tumor tissues derived from control group and sh-MLK7-AS1 group were immunostained for H&E and Ki67. Anti-Ki67 from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA) was used to present the percentage of positive cells, further revealing the proliferative activities in tumor tissues.

Statistical analysis. All assays were repeated for three times. The data were analyzed with SPSS v17.0 software program (SPSS, Inc., Chicago, IL, USA) and presented as mean ± SD (standard deviation). Student's t test (two tailed) was used to compare data derived from different groups. P<0.05 was considered to indicate a statistically significant difference.

Results

MLK7-AS1 is obviously upregulated in CRC tissue samples, and increased MLK7-AS1 expression is significantly correlated with several clinicopathological factors and poor prognosis in patients with CRC. To investigate aberrantly expressed lncRNAs involved in CRC, we analyzed the data downloaded from the TCGA dataset, and found that lncRNA MLK7-AS1 exhibited obvious upregulation in CRC tissue samples relative to normal tissues (Fig. 1A). Then, qRT-PCR experiments were used to determine the expression levels of MLK7-AS1 in 50 pairs CRC tissue samples and matched non-tumor tissue samples. MLK7-AS1 exhibits significant upregulation in CRC tissues. Fold change >1.5 was recognized to be significant. Then, we divided CRC patients into high (above the median value, n=25) and low (below the median value, n=25) MLK7-AS1 expression groups to better study correlations between MLK7-AS1 expression levels and clinicopathological features (Fig. 1B). As shown in Fig. 1D and Table I, increased MLK7-AS1 expression was obviously linked with tumor size (P=0.011), TNM stage (P=0.031), and lymph node metastasis (P=0.031) in CRC patients.

To detect the correlation between MLK7-AS1 expression and the prognosis of CRC patients, Kaplan-Meier analysis and log-rank test were used to explore the effects of MLK7-AS1 on overall survival of CRC patients (Fig. 1G). The median survival time for cases with high MLK7-AS1 expression was 14 months, whereas it was 25 months for low MLK7-AS1 expression. Furthermore, the overall survival rate over 2 years for the low MLK7-AS1 expression group was 32%, while it was 16% for the high MLK7-AS1 expression group. Our findings show that MLK7-AS1 is an unfavourable prognostic factor for CRC patients.

Regulation of MLK7-AS1 expression in CRC cell lines. We detected MLK7-AS1 expression levels in CRC cell lines, found that MLK7-AS1 exhibited higher levels in DLD-1 and LOVO cells (Fig. 1C). In attempt to evaluate the role of MLK7-AS1 in CRC cells, MLK7-AS1 expression was decreased by transfection with siRNAs or shRNA vector. And qPCR assays were used to test the interference efficiencies of three siRNAs transfected in CRC cells. Si-MLK7-AS1 no. 1 and si-MLK7-AS1 no. 2 exhibited more efficient silencing abilities than si-MLK7-AS1 no. 3 (Fig. 1E). Thus, we selected si-MLK7-AS1 no. 1 and si-MLK7-AS1 no. 2 for all subsequent assays. Moreover, we also examine the expression levels of MLK7-AS1 in DLD-1 and LOVO cells transfected with pcDNA-MLK7-AS1. Compared with the NC, MLK7-AS1 expression significantly increased in pcDNA-MLK7-AS1-transfected CRC cells (Fig. 1F).

The effects of MLK7-AS1 dysregulation on cell viability and colony-formation ability in CRC cells. MTT experiments were performed to test CRC cells viability, and the results demonstrated that cell viabilities of DLD1 and LOVO cells following transfection with si-MLK7-AS1 no. 1 or si-MLK7-AS1 no. 2 were obviously suppressed compared with control cells (Fig. 2A). Furthermore, MLK7-AS1 knockdown impaired CRC cells clonogenic survival in DLD-1 and LOVO cells (Fig. 2B). Consistently, the results of MLK7-AS1 overexpression showed opposite effects (Fig. 2A and B). These findings indicated the effects of MLK7-AS1 on CRC proliferation.

The function of MLK7-AS1 in cell cycle progression and apoptosis of CRC cell lines. To explore whether MLK7-AS1 is involved in cell cycle regulations, flow cytometry assays were

Table II. Sequences of specific primers for reverse transcription-quantitative polymerase chain reaction.

	Primer sequences (5'-3')	
Gene	Forward	Reverse
MLK7-AS1	CAGCCTCCCGAGTTGAGTAA	CAAATGACACGAGCCTTCCT
GAPDH	GAAGAGAGAGACCCTCACGCTG	ACTGTGAGGAGGGGAGATTCAGT
p15	ACGGAGTCAACCGTTTCGGGAG	GGTCGGGTGAGAGTGGCAGG
p16	ATGGAGCCTTCGGCTGACT	GGCCTCCGACCGTAACTATT
p21	CAGCAGAGGAAGACCATGTG	GGCGTTTGGAGTGGTAGAAA
p27	TGCAACCGACGATTCTTCTACTCAA	CAAGCAGTGATGTATCTGATAAACAAGG
p57	CACGATGGAGCGTCTTGTC	CCTGCTGGAAGTCGTAATCC

Table III. Sequences of siRNAs and shRNAs.

siRNAs	
si-MLK7-AS1 no. 1	GAGAGUACUUUGGUCACCACGGGAA
si-MLK7-AS1 no. 2	CCAAGGGCUCUGUUCAUAAACUGUU
si-MLK7-AS1 no. 3	CCAAGCUACUUGUAAUCCUUCCAAA
shRNAs	
sh-MLK7-AS1 no. 1	CACCGTTCCCGTGGTGACCAAAGTACTCTCCGAAGAGAGTACTTTGGTCACCACGGGAA
sh-MLK7-AS1 no. 2	CACCGAACAGTTTATGAACAGAGCCCTTGGCGAACCAAGGGCTCTGTTCATAAACTG
sh-MLK7-AS1 no. 3	CACCGTTTGGAAGGATTACAAGTAGCTTGGCGAACCAAGCTACTTGTAATCCTTCCAAA

Si, small interfering; sh, short hairpin.

performed in DLD-1 and LOVO cell lines. Compared with control cells, DLD1 and LOVO cells with MLK7-AS1 knockdown showed an obvious G1/G0 phase arrest (Fig. 3A and B). Importantly, flow cytometry experiments were further used to study the effects of MLK7-AS1 overexpression on cell cycle regulation in DLD-1 and LOVO cell lines. Consistently, the results showed that DLD-1 and LOVO cells with MLK7-AS1 overexpression had a significant decrease in G1/G0 phase and an obvious increase in G2/S phase (Fig. 4A and B). Edu staining assays also revealed the proliferation promotion mediated by MLK7-AS1 in CRC (Fig. 5A). As known to all, CKIs exert indispensable roles in cell cycle progression and serve as tumor suppressors in many cancers, including CRC (28) Moreover, western blot assays revealed the significant alteration of cyclin dependent kinase 2 (CDK2) and cyclin dependent kinase 4 (CDK4) in DLD-1 and LOVO cells with MLK7-AS1 knockdown (Fig. 4C).

To comfirm the findings that MLK7-AS1 exerts regulatory roles in cell cycle regulation, we investigated the alteration of CKIs family in DLD-1 and LOVO cells following transfection with si-MLK7-AS1 no. 1 or si-MLK7-AS1 no. 2. Interestingly, qPCR and western blot experiments both demonstrated that p21 was dramatically increased in DLD-1 and LOVO cells with MLK7-AS1 knockdown (Fig. 5B). These results highlighted p21 as a novel target gene of MLK7-AS1. Next, we use flow cytometry to study whether MLK7-AS1 could induce CRC cells apoptosis. The results showed that downregulation of MLK7-AS1 by MLK7-AS1 siRNAs significantly increased apoptotic abilities of CRC cells (Fig. 3C and D). These findings suggest that MLK7-AS1 promotes CRC cells proliferative abilities partly via silencing p21 expression.

MLK7-AS1 downregulation inhibited tumor growth in mice. Sh-MLK7-AS1-transfected or empty-vector-transfected DLD-1 cells are injected into nude mice to establish *in vivo* models (10,29). Up to 24 days after injection, the size of tumor formed from sh-MLK7-AS1-transfected DLD-1 cells was dramatically smaller than the size of tumor in control group (Fig. 6A). Additionally, the tumor volumes and weights were obviously decreased compared with the controls (Fig. 6C and D). The expression of MLK7-AS1 in tumors derived from DLD-1 cells with MLK7-AS1 knockdown exhibited remarkable downregulation, relative to that of control (Fig. 6B). Immunohistochemistry (IHC) assays demonstrated that the tumor tissues formed from DLD1/sh-MLK7-AS1 cells displayed lower Ki-67 expression (Fig. 6E). These results suggested that MLK7-AS1 knockdown could inhibit CRC cells growth *in vivo*.

Discussion

Advances in sequencing technologies facilitate the completion of new massively human sequencing projects, such as the Encyclopedia of DNA Elements (ENCODE) and GENCODE (30,31). LncRNAs are known as newly identified ncRNAs, which occupies a higher rate of 98% in transcripts of human genome (8). Emerging evidence has highlighted lncRNAs as critical regulators in multiple biological processes,

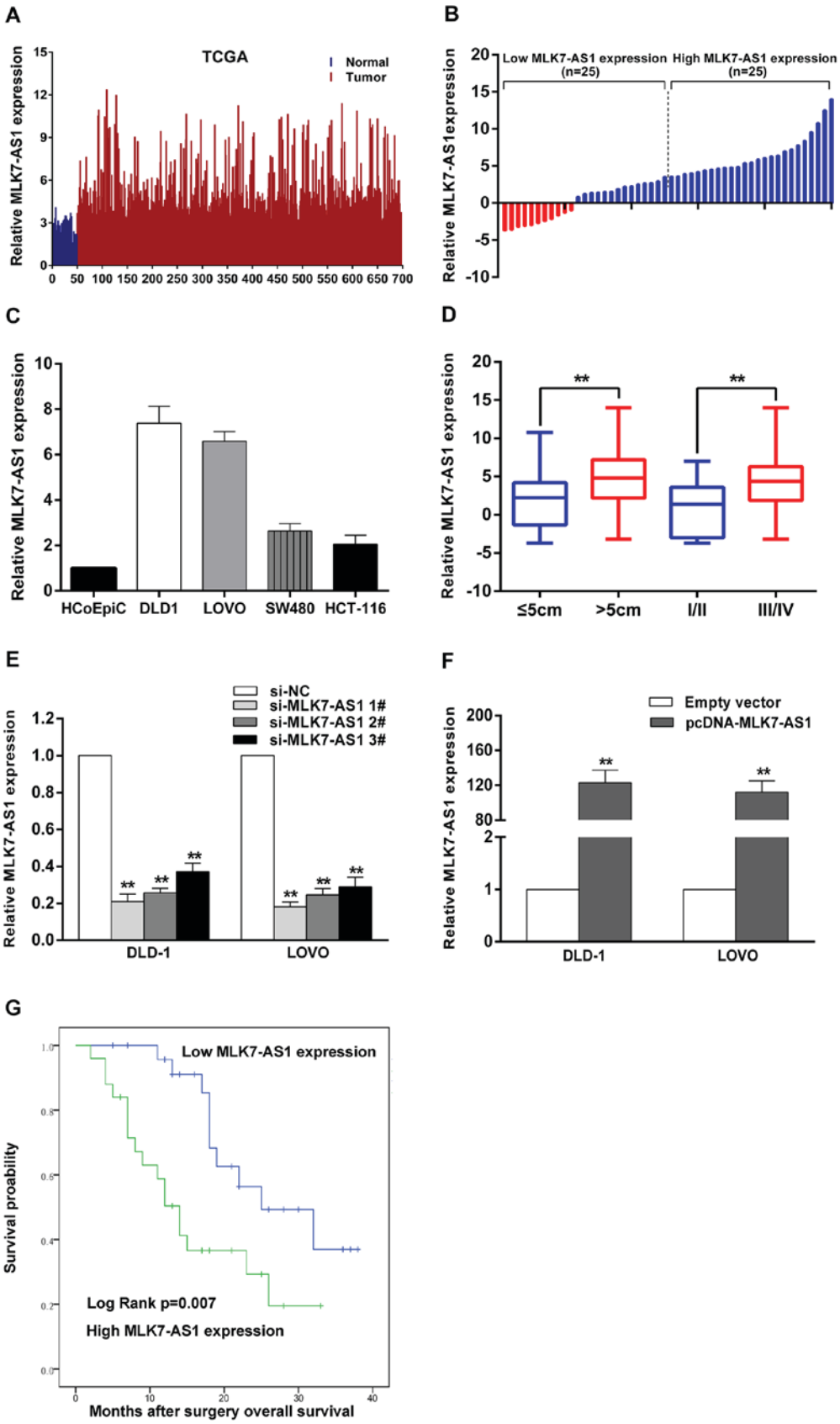

Figure 1. MLK7-AS1 overexpression correlates with clinicopathological factors and overall survival of CRC patients, and its modulation in CRC cell lines. (A) Upregulation of MLKF-AS1 in CRC tissues compared with normal tissues in TCGA dataset. (B) CRC patients were divided into high MLK7-AS1 group and low MLK7-AS1 group according to the median value. (C) The expression levels of MLK7-AS1 are examined in CRC cell lines and HCoEpiC cells using qRT-PCR. (D) Increased MLK7-AS1 expression was significantly associated with tumor size (**P<0.01 vs. tumor size ≤5 cm group) and TNM stage (**P<0.01 vs. I/II group) in CRC patients. (E) The interference efficiencies are tested in DLD-1 and LOVO cell lines using qRT-PCR (**P<0.01 vs. NC group). (F) The relative expressions of MLK7-AS1 between pcDNA-MLK7-AS1-transfected CRC cells and empty-vector-transfected CRC cells are examined using qRT-PCR. (G) Association between MLK7-AS1 expression and patient survival in CRC. MLK7-AS1, MLK7 antisense RNA 1; CRC, colorectal cancer; TCGA, The Cancer Genome Atlas.

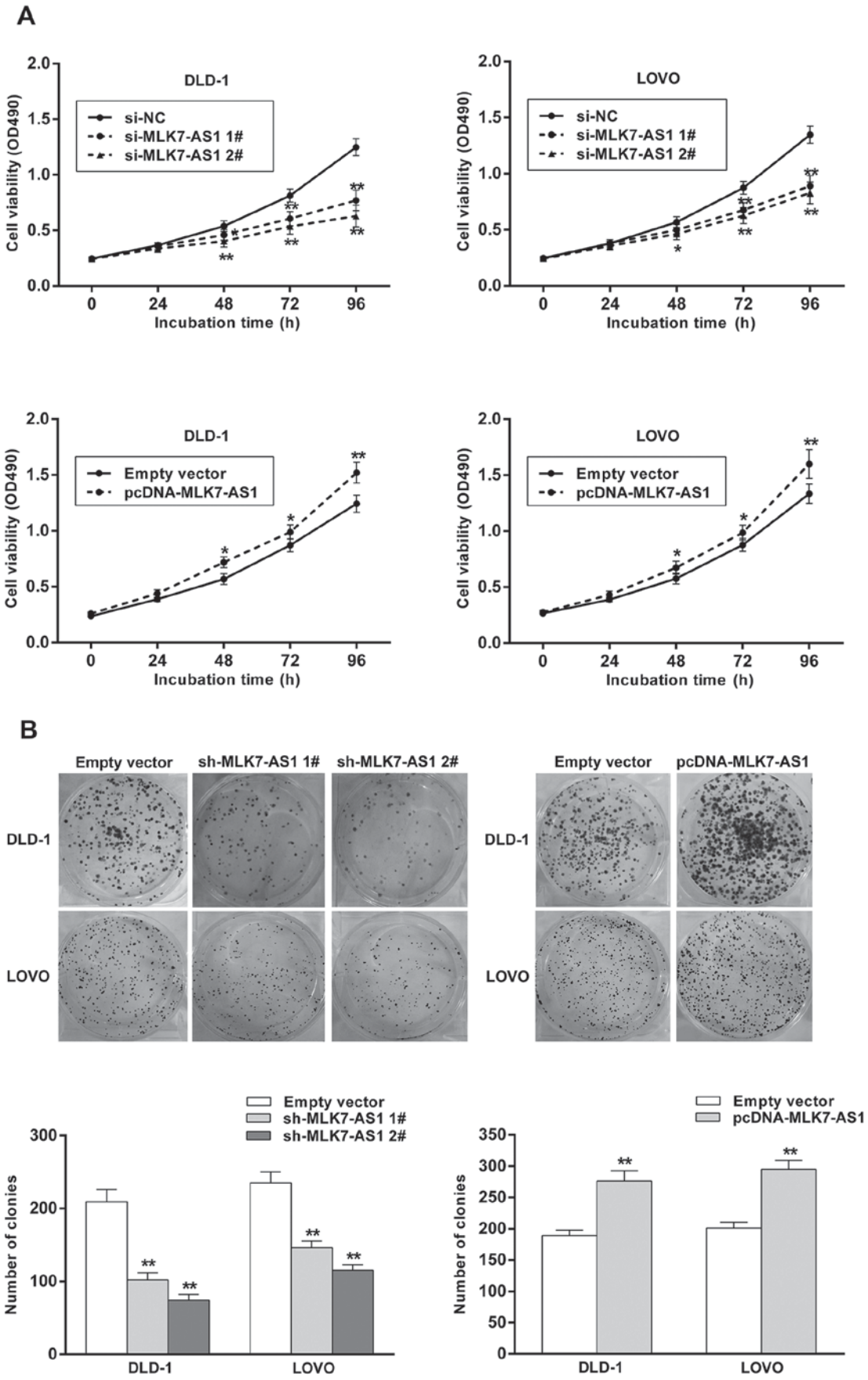

Figure 2. The effects of MLK7-AS1 on CRC proliferation. (A) The cell viability was measured in DLD-1 and LOVO cell lines with MLK7-AS1 knockdown or MLK7-AS1 overexpression, compared with respective controls (*P<0.05, **P<0.01 vs. NC group). (B) MLK7-AS1 knockdown impaired colony-formation abilities of CRC cells, whereas MLK7-AS1 overexpression increased colony-formation abilities of CRC cells (**P<0.01 vs. Empty vector group). MLK7-AS1, MLK7 antisense RNA 1; CRC, colorectal cancer.

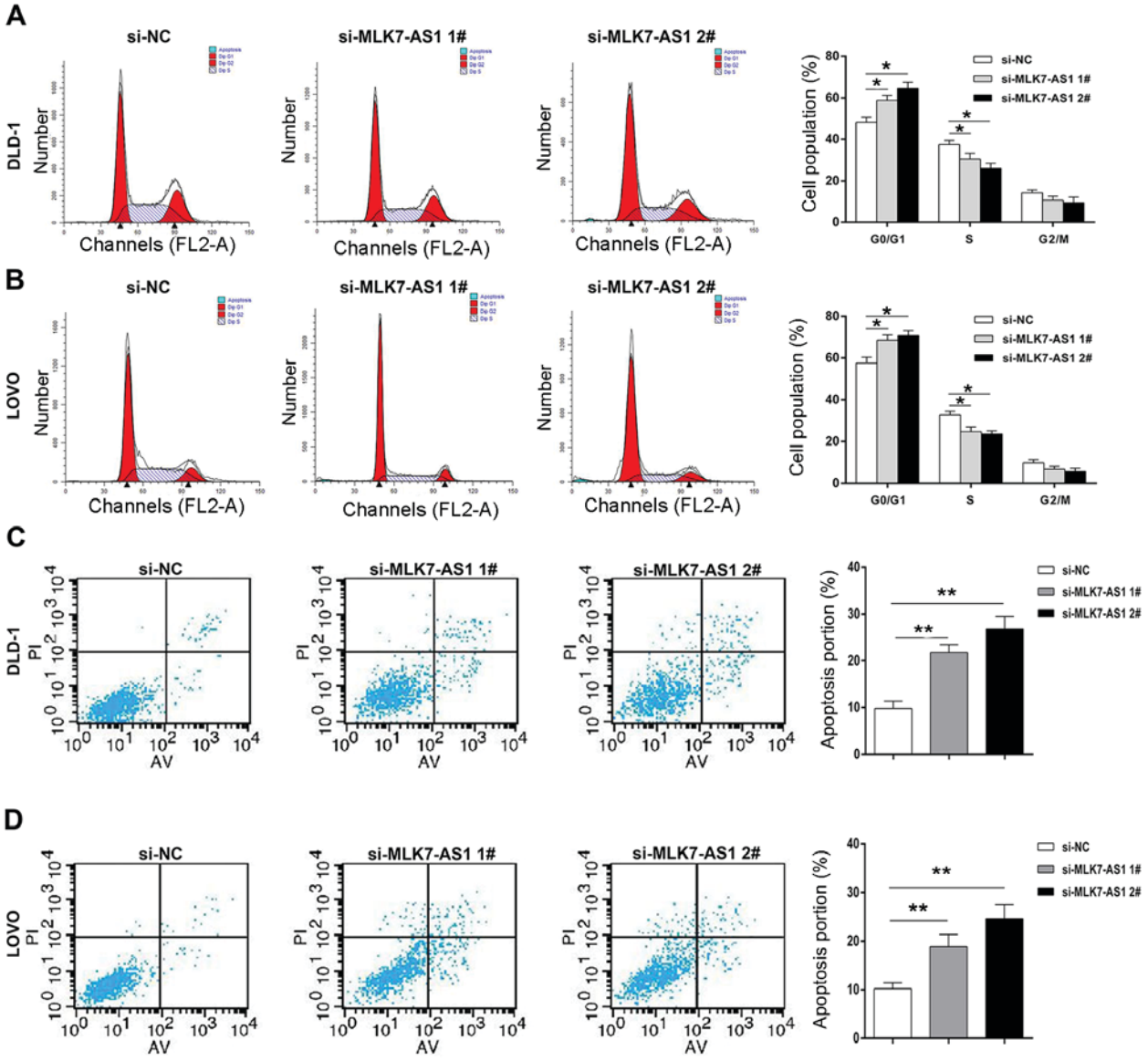

Figure 3. The function of MLK7-AS1 in cell cycle progression and apoptosis of CRC cell lines. (A and B) Compared with control cells, decreased MLK7-AS1 expression promotes G1/G0 phase arrest in CRC cells transfected with si-MLK7-AS1 no. 1 or si-MLK7-AS1 no. 2. (C and D) MLK7-AS1 knockdown increased CRC cells apoptosis in DLD1 and LOVO cell lines (*P<0.05, **P<0.01 vs. NC group). MLK7-AS1, MLK7 antisense RNA 1; CRC, colorectal cancer.

and the dysregulation of lncRNAs has been found to exert important roles in various cancers (8,16,17,23). For example, lncRNA HOXA11-AS is found to be significantly upregulated in GC tissues and alters GC cells phenotypes by modulating cell cycle, apoptosis, and invasion (32). Importantly, lncRNAs exhibit different cell phenotypes in various cancer, and its expression patterns are tissue-specific (32). However, only a few of lncRNAs have been well-studied in cancer progression (33,34). Thus, more lncRNAs should be investigated in tumors, especially CRC.

Here, we utilize publicly available data from TCGA dataset and focus on the overexpressed lncRNAs. LncRNA MLK7-AS1 exhibited obvious upregulation in expression data of CRC and was screened out as a potential oncogene in CRC progression. Then, qRT-PCR experiments were performed to validate the expression levels of MLK7-AS1 in a cohort of 50 paired CRC tissue samples and matched non-tumor samples. Overexpression of MLK7-AS1 is significantly correlated with tumor size, TNM stage, and lymph node metastasis in CRC patients. Functional studies revealed that MLK7-AS1 knockdown promoted CRC cells apoptotic abilities, suppressed proliferation *in vitro*, and contributed to tumor growth inhibition *in vivo*. Consistently, upregulation of MLK7-AS1 showed opposite effects. These findings indicated that MLK7-AS1 may be an oncogene in CRC progression, suggesting its utilities as a potential biomarker and a therapeutic target.

Mounting investigations have focused on the antisense transcripts and its corresponding protein-coding genes. For example, lncRNA KRT7-AS promotes GC cell progression by increasing KRT7 expression (35). LncRNA FEZF1-AS1 facilitates cell proliferation and migration in CRC through mediating FEZF1 expression (36). These studies suggested a

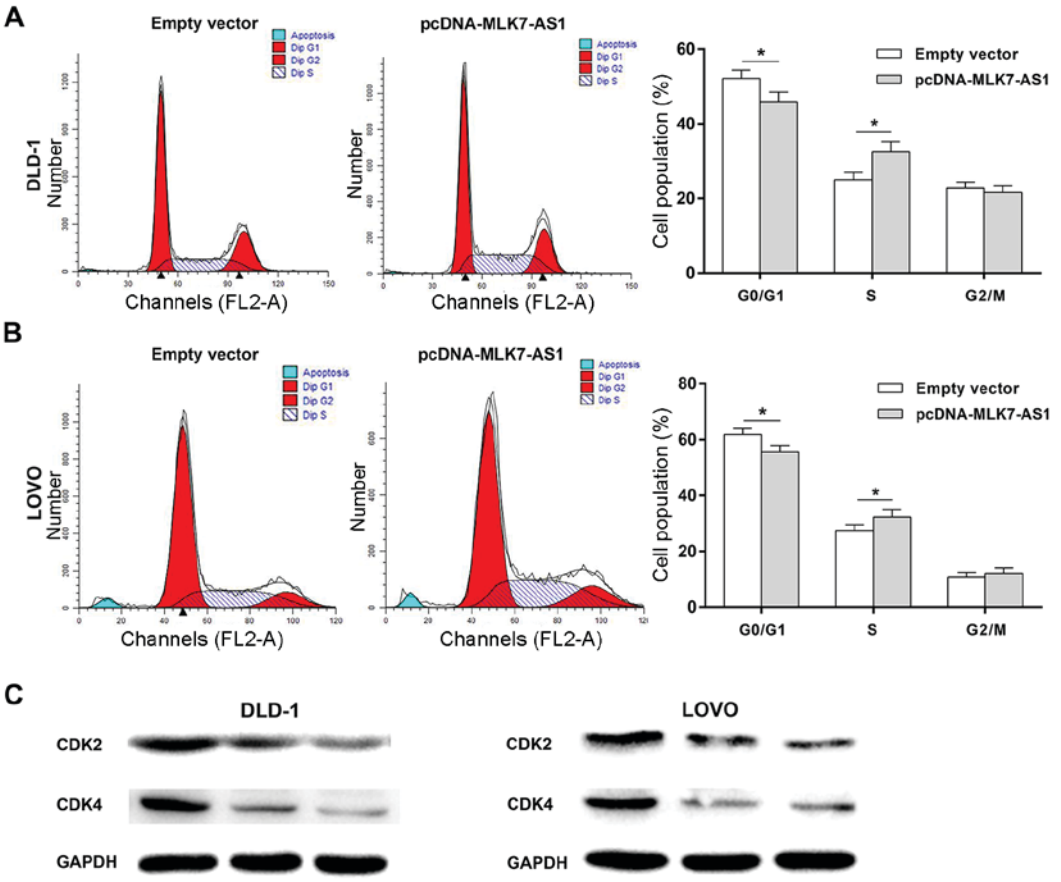

Figure 4. MLK7-AS1 overexpression modulated CRC cells cycle regulation, and MLK7-AS1 knockdown altered the protein levels of cell-cycle-related genes in CRC cells. (A and B) DLD-1 and LOVO cells with MLK7-AS1 overexpression had a significant decrease in G1/G0 phase and an obvious increase in G2/S phase. (C) The protein levels of CDK2 and CDK4 exhibited obvious alteration in DLD-1 and LOVO cell lines with MLK7-AS1 knockdown (*P<0.05 vs. Empty vector group). MLK7-AS1, MLK7 antisense RNA 1; CRC, colorectal cancer.

novel mechanism that sense gene regulation can be controlled by antisense transcripts through forming duplex. LncRNA MLK7-AS1 is located on the antisense chain of the gene coding MLK7 protein. MLK7 has been demonstrated to be significantly upregulated in CRC tissues (37,38). CRC RNA-sequencing data showed that MLK7 increased by about threefold in CRC tissue samples (8). Both MLK7 and MLK7-AS1 exhibited obvious upregulation in CRC tissues. However, the regulatory relationship between MLK7-AS1 and MLK7 remains undefined. The potential mechanism between MLK7-AS1 and MLK7 deserves to be focused in the future research.

Recently, it has been revealed that lncRNAs can increase or inhibit the expression levels of target genes to exert effects in cancer progression (24). CKIs are known to be critical tumor suppressors in various cancers and play key roles in cell cycle regulation (10,22,28) Importantly, CKIs are involved in the biological role of lncRNAs (28,39,40). The abnormal methylation in promoter regions of CKIs leads to gene expression inhibition, thus contributing to alteration of cell cycle (41,42). For example, linc00668-mediated cell proliferation in GC is partly via silencing the expressions of CKIs at epigenetical levels (24). Thus, we further examined the expression levels of CKIs in CRC cells after MLK7-AS1 knockdown. Interestingly, p21 expression was obviously upregulated at both mRNA and protein levels. Current studies have highlighted p21 as a critical tumor suppressor in many cancers, including CRC (21,43). For example, BRAF activated non-coding RNA (BANCR) could promote CRC proliferation by silencing p21 (44). The significant increase of p21 can partly explain the finding that decreased MLK7-AS1 contributes to significant increase in G1/G0 phase, and activates apoptosis in CRC cells. Thus, MLK7-AS1 could promote CRC cell proliferation and induce apoptosis partly via silencing p21 expression. SH2 Domain-Containing Inositol-5'-Phosphatase 1 (SHIP1) has been reported to be involved in pathways associated with p21 (45). Additionally, dysregulated SHIP1 exerts regulatory effects on cell viability and cell death in many diseases, including CRC (46-48). It is also of great significance to investigate the relationship between MLK7-AS1 and other potential target gene, including SHIP1.

P21, also known as cyclin dependent kinase inhibitor 1A (CDKN1A), is a crucial component of CKIs family, and has been elucidated to mediate tumor-suppressive activities in many cancers (21,49,50). Current studies have showed the effects of lncRNAs on cell cycle regulation (51). Identifications of lncRNAs and associated regulatory target genes in CRC are important, and will help to explore CRC pathogenesis. Our findings represent first reporting of remarkable upregulation of MLK7-AS1 in CRC tissue samples and cell lines. MLK7-AS1 inhibition can decrease cell proliferative abilities and promote

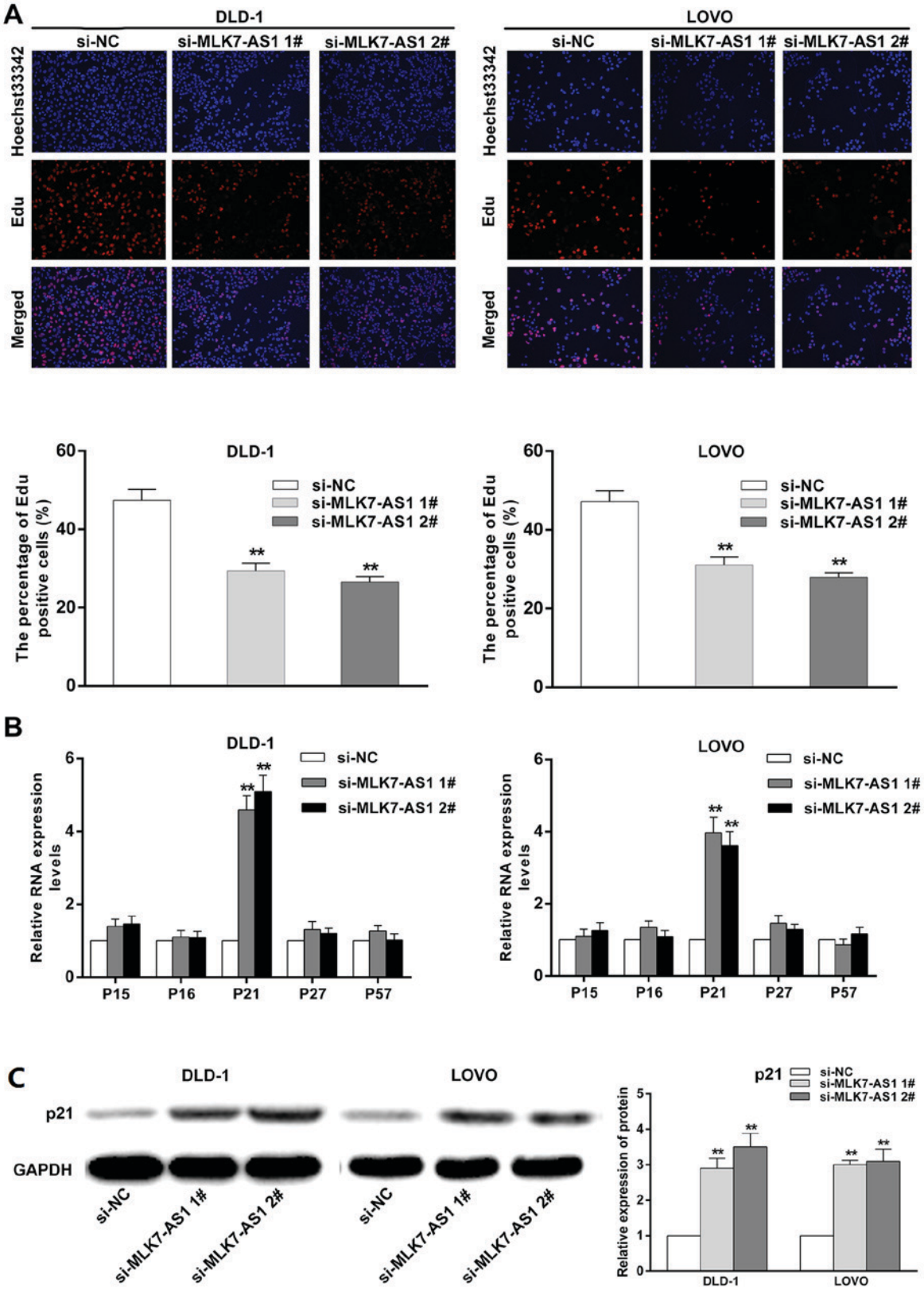

Figure 5. The oncogenic role mediated by MLK7-AS1 is partially through regulating p21 expression. (A) Edu assays were performed to evaluate the effects of MLK7-AS1 inhibition on CRC cells proliferation. Cell nuclei staining with DAPI presents color blue. The Click-it reaction revealed Edu staining (red). (B and C) p21 is the most significantly upregulated gene in DLD-1 and LOVO cells transfected with si-MLK7-AS1 no. 1 or si-MLK7-AS1 no. 2 at both mRNA and protein levels (**P<0.01 vs. NC group). MLK7-AS1, MLK7 antisense RNA 1; CRC, colorectal cancer.

apoptosis in CRC. Importantly, p21 acts as a novel downstream target of MLK7-AS1. The oncogenic role of MLK7-AS1 in CRC progression is partly via silencing p21 expression. Additionally, overexpressed MLK7-AS1 expression may be an

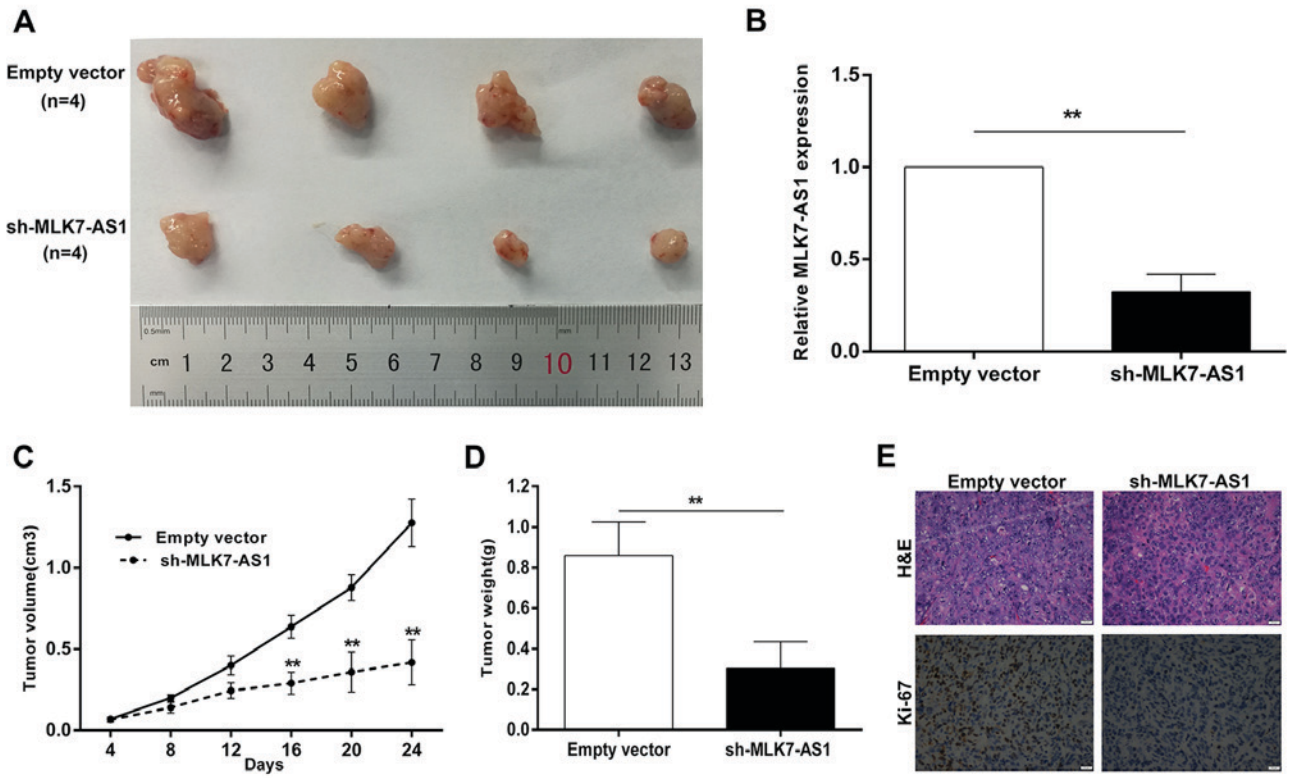

Figure 6. MLK7-AS1 downregulation inhibited tumor growth in mice. (A) The size of tumor formed from sh-MLK7-AS1-transfected DLD-1 cells is obviously smaller than that formed from empty-vector-transfected DLD-1 cells. (B) The relative expression of MLK7-AS1 in the tumors formed from sh-MLK7-AS1 group compared with control group. (C and D) The tumor volumes and tumor weights were obviously decreased compared with the controls (**P<0.01 vs. Empty vector group). (E) The H&E staining images of tumors in control group and sh-MLK7-AS1 group. The tumor tissues formed from DLD1/sh-MLK7-AS1 cells displayed lower Ki-67 staining than those derived from the control cells. MLK7-AS1, MLK7 antisense RNA 1.

unfavourable prognostic factor in CRC patients. Although the functional role of MLK7-AS1 has been investigated, mechanistic investigations between MLK7-AS1 and p21 are required to further the understanding of regulatory mechanism. More numbers of mice can be used to study the effects of MLK7-AS1 dysregulation on tumor growth *in vivo* in the future. Our results support the idea that lncRNA MLK7-AS1 promotes the proliferation in human CRC partly via downregulating p21 expression and suggest that lncRNA MLK7-AS1 may be a potential therapeutic target for CRC patients. Theses findings will shed new light on CRC pathogenesis and promote the development of lncRNAs-directed diagnosis and treatments.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

XZ, RZ, JBL and XFY made substantial contributions to conception and design. RZ, KJ and WYL were responsible for the analysis and interpretation of data. RZ, XL, JFZ and SW were involved the experimental design, drafting the manuscript and revising it critically for important intellectual content. XZ agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The study was approved by the Cancer Hospital of China Medical University Ethics Committee. Informed consent was obtained from participants.

Consent for publication

Informed consent was obtained from participants.

Competing interests

The authors declare that they have no competing interests.

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