

Overexpression of Long Noncoding RNA LBX2-AS1 Promotes the Proliferation of Colorectal Cancer

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Abstract

Background: LBX2 antisense RNA I (LBX2-AS1), a long noncoding RNA, has been identified to be closely associated with the progression of various cancers. However, the role of LBX2-AS1 in colorectal cancer (CRC) is still poorly understood. In this study, we aimed to investigate the expression and function of LBX2-AS1 in CRC. **Material and Methods:** Expression data from the Gene Expression Omnibus (GEO) and Gene Expression Profiling Interactive Analysis (GEPIA) databases and results obtained from clinical samples/patients were used to determine the correlation between LBX2-AS1 expression and pathological stages, overall survival (OS). Furthermore, knockdown of LBX2-AS1 in CRC cells using the short interfering RNA (siRNA) technique, and observed its biological functions using western blotting, quantitative reverse transcription-polymerase chain reaction (qRT-PCR), cell counting kit-8 (CCK-8) and flow cytometry assay in the CRC cell line. **Results:** Our study demonstrated that the expression levels of LBX2-AS1 were higher in CRC cell lines than in normal colon mucosal cell lines. Bioinformatics analysis revealed that CRC patients with high LBX2-AS1 expression levels had poor OS. Furthermore, knockdown of LBX2-AS1 in CRC cells could attenuate the proliferative ability of CRC cells *in vitro*, which is associated with decreased expression of cyclin-dependent kinase (CDK) 3, CDK6, and CCND1 and enhanced expression of cyclin-dependent kinase inhibitor 1A. **Conclusions:** LBX2-AS1 plays a crucial role in the tumorigenesis of CRC, providing a potential therapeutic target for CRC patients.

Keywords

long noncoding RNA, LBX2 antisense RNA I, colorectal cancer, prognosis, therapy

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Introduction

Colorectal cancer (CRC), one of the most common and aggressive human malignancies, is the third leading cause of cancer-induced death worldwide.^{1,2} In 2015, approximately 0.8 million new cases and 0.4 million deaths from CRC in developed countries were reported.³ With the improvement of systemic therapy for CRC, in the past decades, substantial progress has been achieved in the treatment of CRC. However, the mortality rate of CRC caused by recurrence has not evidently changed.⁴ The progression and development of CRC may involve a multi-step process that is closely associated with inherited factors and environmental factors, which may result in a series of changes in important molecules, thereby inducing cancer cell apoptosis, proliferation, and differentiation.^{5,6} With the popularity of molecular targeting therapy, some studies have focused on the molecular pathogenesis of CRC by analyzing molecular abnormalities in the tumorigenesis of CRC.^{7,8}

Long noncoding RNAs (lncRNAs), a class of noncoding RNAs, have been recently identified.^{9,10} LncRNAs have a

transcribing length of greater than 200 nt and have an insufficient complete functional open reading frame.¹¹⁻¹³ In recent years, lncRNAs have been gradually gaining significant attention owing to their effects on cell biological behavior, specifically in tumor cells. Accumulating studies have revealed that lncRNAs are aberrantly expressed in several cancers, such as nasopharyngeal carcinoma,¹⁴ breast cancer,¹⁵ non-small cell lung cancer

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(NSCLC),¹⁶ and CRC.¹⁷ These abnormally expressed lncRNAs act as biomarkers for the diagnosis and therapy of cancers.

LBX2 antisense RNA 1 (LBX2-AS1) is a member of the lncRNA. It is located in the chr2p13.1 region and is 1786 bp in length. Emerging evidence indicates that LBX2-AS1 is associated with tumorigenesis in various cancers, including hepatocellular carcinoma (HCC),¹⁸ esophageal squamous cell carcinoma (ESCC),¹⁹ gastric cancer (GC),²⁰ and NSCLC.²¹ However, their specific effects on the tumorigenesis of CRC are still poorly understood. In this study, we found that LBX2-AS1 was overexpressed in CRC cell lines and CRC samples, and CRC patients with high expression levels showed poor overall survival (OS). Furthermore, knockdown of the expression of LBX2-AS1 suppressed the proliferative ability of CRC cells *in vitro*. These results indicated that lncRNA LBX2-AS1 may be a novel target for cervical cancer therapy.

Materials and Methods

Bioinformatics Analysis

Microarray expression data was deposited in the Gene Expression Omnibus (GEO) database: GSE41328 (Affymetrix Human Genome U133 Plus 2.0 platform). The GSE41328 dataset contains 10 pairs of CRC and adjacent non tumor tissues.

Cell Culture and Transfection

CRC cell lines (HT29, LoVo, SW620 and HCT116) and the human normal colon mucosal cell line NCM460 were used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, USA). These cell lines (NCM460, LoVo, SW620, HT29) were cultured in RPMI-1640 medium (Invitrogen, USA) with 10% fetal bovine serum (FBS, Invitrogen, USA), and HCT116 cells were maintained in DMEM media (Invitrogen, USA) with 10% FBS. When the cells covered 60% of the bottom of the bottle, 50nM siRNA oligos were transfected by Lipofectamine 3000 (Invitrogen, USA), according to the manufacturer's instructions. The sequences of the *LBX2-AS1* targeting siRNAs were: *LBX2-AS1*-si-1: 5'-AGGAATGTTT GCTGAATTAATGG -3'; *LBX2-AS1*-si-2: 5'-CCCAAGTTA TAAACTATAATGC-3'; Sequences of non-target scramble controls were provided by RiboBio (Guangzhou, China).

Subcellular Fractionation Analysis

The cytoplasmic and nuclear RNAs of CRC cell lines were separated using the PARIS Kit (Invitrogen, USA), and the computing method was according to described previously.²²

Quantitative Real-Time Polymerase Chain Reaction

RNA isolation and amplification were performed as described previously.²³ SYBR_Premix ExTaq II kit (Takara, China) was used to execute the quantitative real-time polymerase chain reaction (qRT-PCR) assay in the CFX96 Real-Time PCR Detection System (Bio-Rad, USA), thereby analyzing the

relative expression levels of target genes. The sequences of qRT-PCR primers: *LBX2-AS1* forward: 5'-AGTTTGTCCCAG GTTTGGCA-3', reverse: 5'-CATGCCAGG GTCCTTGT TCT-3'; Human *CCND1* forward: 5'-TCGTTGCCCTCTGTG CCACA-3', reverse: 5'-GCAGTCCGGGTACACTTGA-3'; Human *CDK3* forward: 5'-CCAGCTCTTTCGTATCTTT CGT-3', reverse: 5'-CCAGCTCTTTCGTATCTTTCGT-3'; Human *CDK6* forward: 5'-CCAGATGGCTCTAACCTC AGT-3', reverse: 5'-AACTTCCACGAAAAAGAGGCTT -3'; Human *CDKN1A* forward: 5'-CGATGGAACCTTCGACTT TGTCA-3', reverse: 5'-GCACAAGGGTACAAGACAGTG -3'; Human *GAPDH* forward 5'-CCACATCGCTCAGACACCAT-3', reverse 5'-TGACAAGCTTCCCCTTCTCA-3'; Human β -*actin* forward 5'-TCACCAACTGGGACGACATG-3', reverse 5'-GT CACCGGAGTCCATCAGAT-3'; Human *U6* (nuclear) forward 5'-CTCGCTTCGGCAGCACA-3', reverse 5'-AACGCTTCACG AATTTGCGT-3'.

Western Blot Assays

Western blot assays were performed as previously described.²³ Primary anti-CCND1, CDK3, CDK6, CDKN1A and GAPDH antibody (Cell Signaling Technology, Danvers, MA, USA) and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) were used.

CCK8 Proliferation Assay

CCK8 proliferation assay was used to detect the cell proliferative ability, the operating steps carried out as described previously.²⁴ Each experiment was repeated for 3 times independently.

Cell Cycle Detected by Flow Cytometry

After transfection with si-NC or si-LBX2-AS1 36 h, approximately 1×10^6 HCT116 cells were collected and fixed with 70% precooled ethanol for flow cytometry assay. DNA content was detected using propidium iodide (PI) (Sigma, San Antonio, USA) staining, and cell cycle distribution were detected by flow cytometry (Beckman Coulter, South Kraemer, USA), according to the methods described in a previous study.²⁵ Each experiment was repeated for 3 times independently.

Statistical Analysis

SPSS 20.0 software was utilized for analysis. The measurement data were expressed by mean \pm standard deviation. The differences among groups were analyzed by the *t* test was adopted for comparison between groups. $p < 0.05$ suggested that the difference was statistically significant.

Results

LBX2-AS1 Overexpression in Colorectal Cancer (CRC)

To analyze the expression of lncRNA LBX2-AS1 in CRC, we first analyzed GEO datasets GSE41328 using Affymetrix

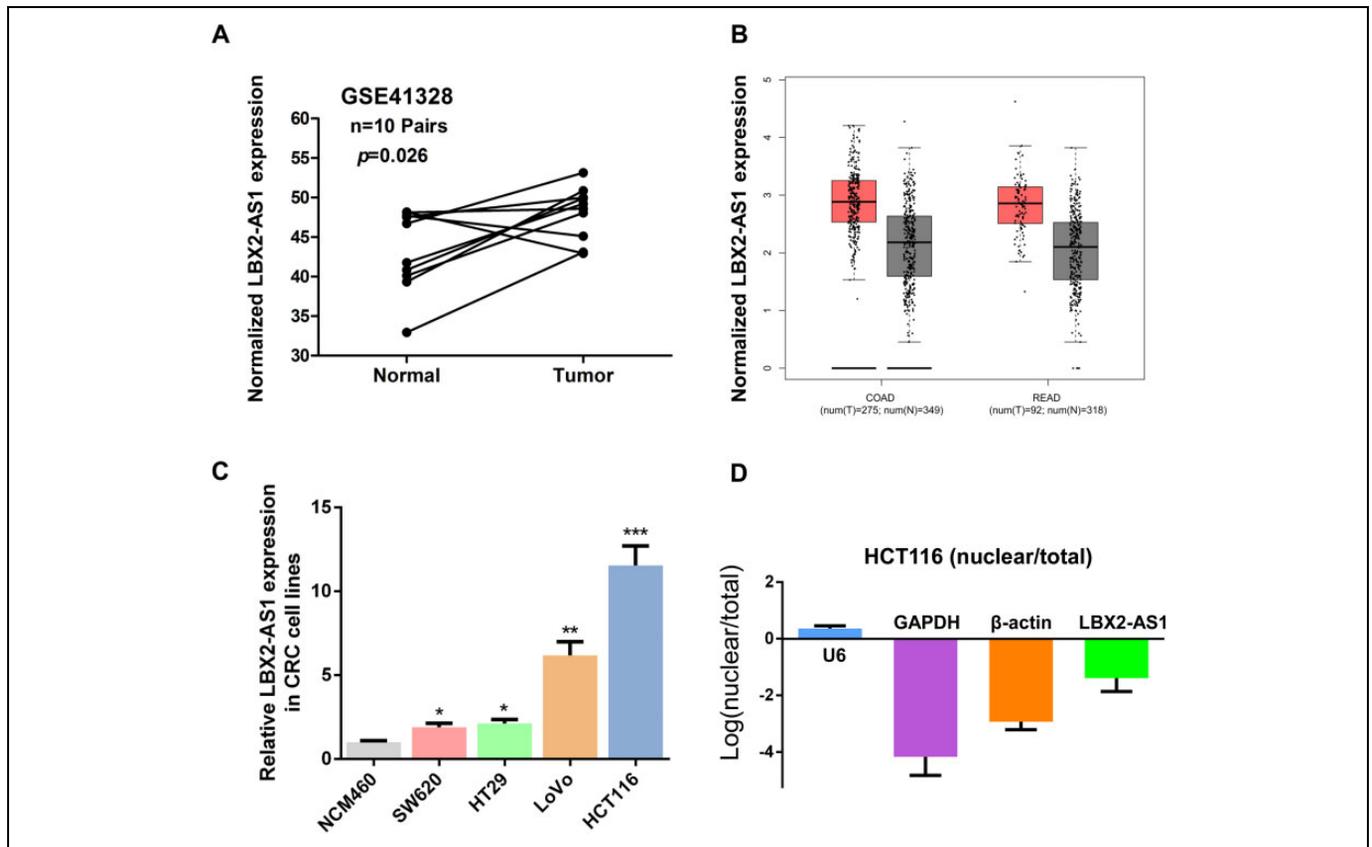


Figure 1. LBX2 antisense RNA 1 (LBX2-AS1) is overexpressed in colorectal cancer (CRC). (A) LBX2-AS1 expression, as measured by Affymetrix microarray, was more upregulated in CRC tissues compared with normal colon mucosal tissues in #GSE41328 (containing 10 pairs of CRC tissues and corresponding normal colorectal tissues) from the Gene Expression Omnibus database. (B) The Gene Expression Profiling Interactive Analysis database was used to analyze the expression of LBX2-AS1 in CRC tissues using the display form of box plots. (C) LBX2-AS1 expression was significantly higher in CRC cell lines (HT29, LoVo, SW620, and HCT116) than in the normal colon mucosal cell line NCM460. (D) Nucleic and cytoplasmic RNA were analyzed using quantitative real-time polymerase chain reaction to detect the expression of LBX2-AS1 in HCT116 cells. U6 was used as a nucleic RNA control; glyceraldehyde 3-phosphate dehydrogenase and β -actin were used as cytoplasmic RNA controls. Data are shown as mean \pm standard error of the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with control

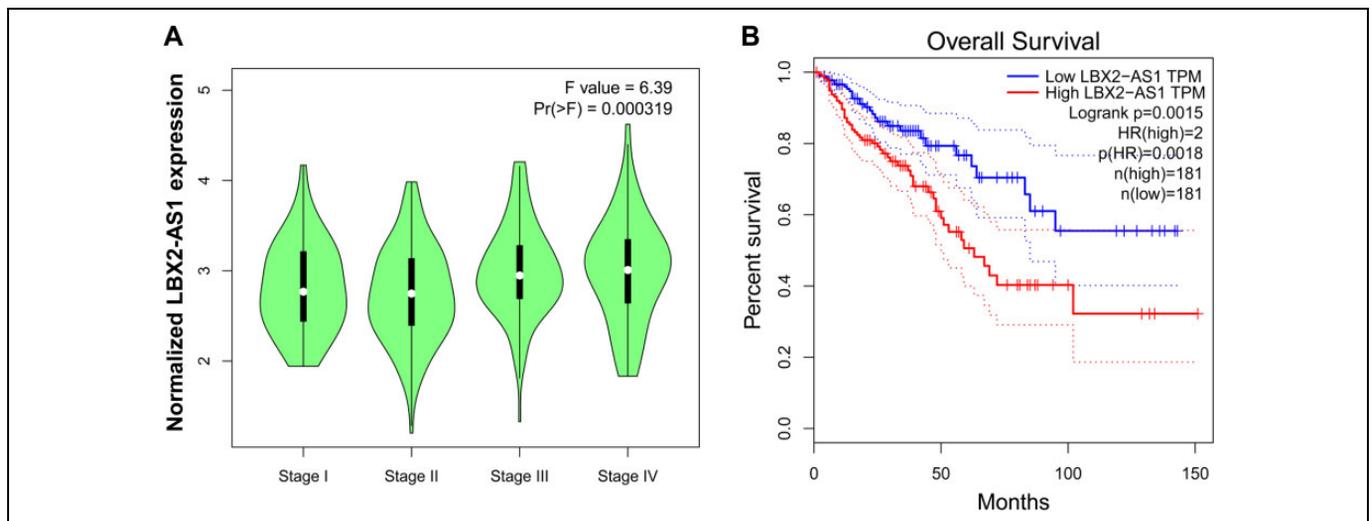


Figure 2. Upregulated LBX2 antisense RNA 1 (LBX2-AS1) is associated with the clinicopathological features of colorectal cancer (CRC). (A) The Gene Expression Profiling Interactive Analysis (GEPIA) database was used to analyze the association between LBX2-AS1 expression and the pathological stages of CRC. (B) The GEPIA database was used to analyze the clinical impact of LBX2-AS1 expression patterns on CRC patients' survival in a CRC specimen expression profile dataset, group 1 = low expression of LBX2-AS1, n = 181; group 2 = high expression of LBX2-AS1, n = 181.

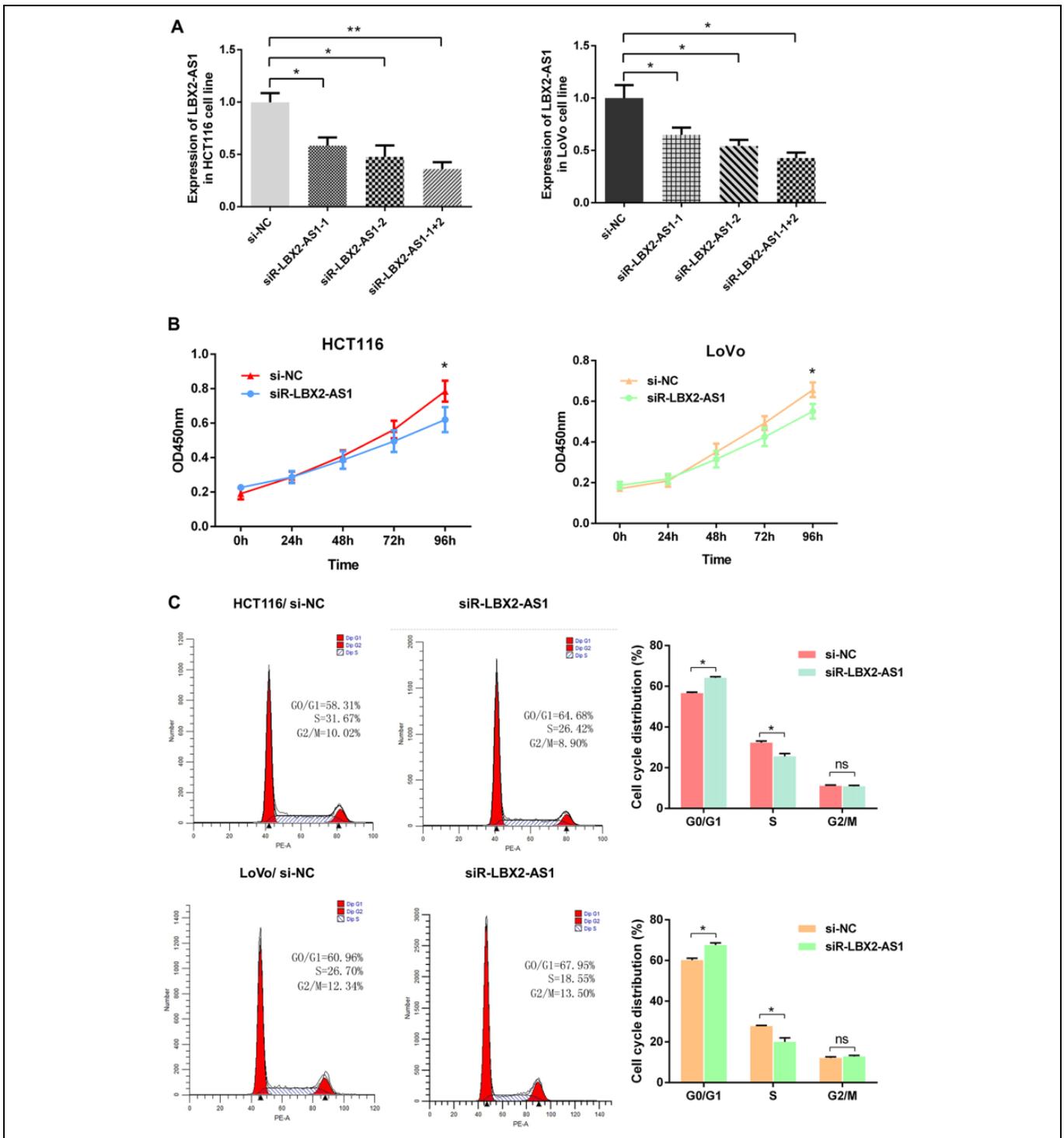


Figure 3. LBX2 antisense RNA 1 (LBX2-AS1) knockdown suppressed the proliferative ability of colorectal cancer cells. (A) The interference efficiency of si-LBX2-AS1 was verified in HCT116 and LoVo cells. HCT116 and LoVo cells were transfected with either si-NC or si-LBX2-AS1 (1#, 2#, 1+2#) for 48 h, and subsequently, LBX2-AS1 expression was analyzed by quantitative real-time polymerase chain reaction. (B-C) Cell Counting Kit-8 proliferation assay and flow cytometry assay were used to detect the cell proliferative ability after transfection with si-NC or si-LBX2-AS1 for 48 h in HCT116 and LoVo cells. Data are shown as mean \pm standard error of the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with control.

HG_U133 Plus 2 arrays to identify dysregulated lncRNAs in CRC. We found that LBX2-AS1 was significantly upregulated in GSE41328 ($p < 0.001$, Figure 1A). Meanwhile, LBX2-AS1

was significantly upregulated in Colon adenoma (COAD) and Rectum adenoma (READ) tissues of The Cancer Genome Atlas (TCGA) database by analyzing the Gene Expression Profiling

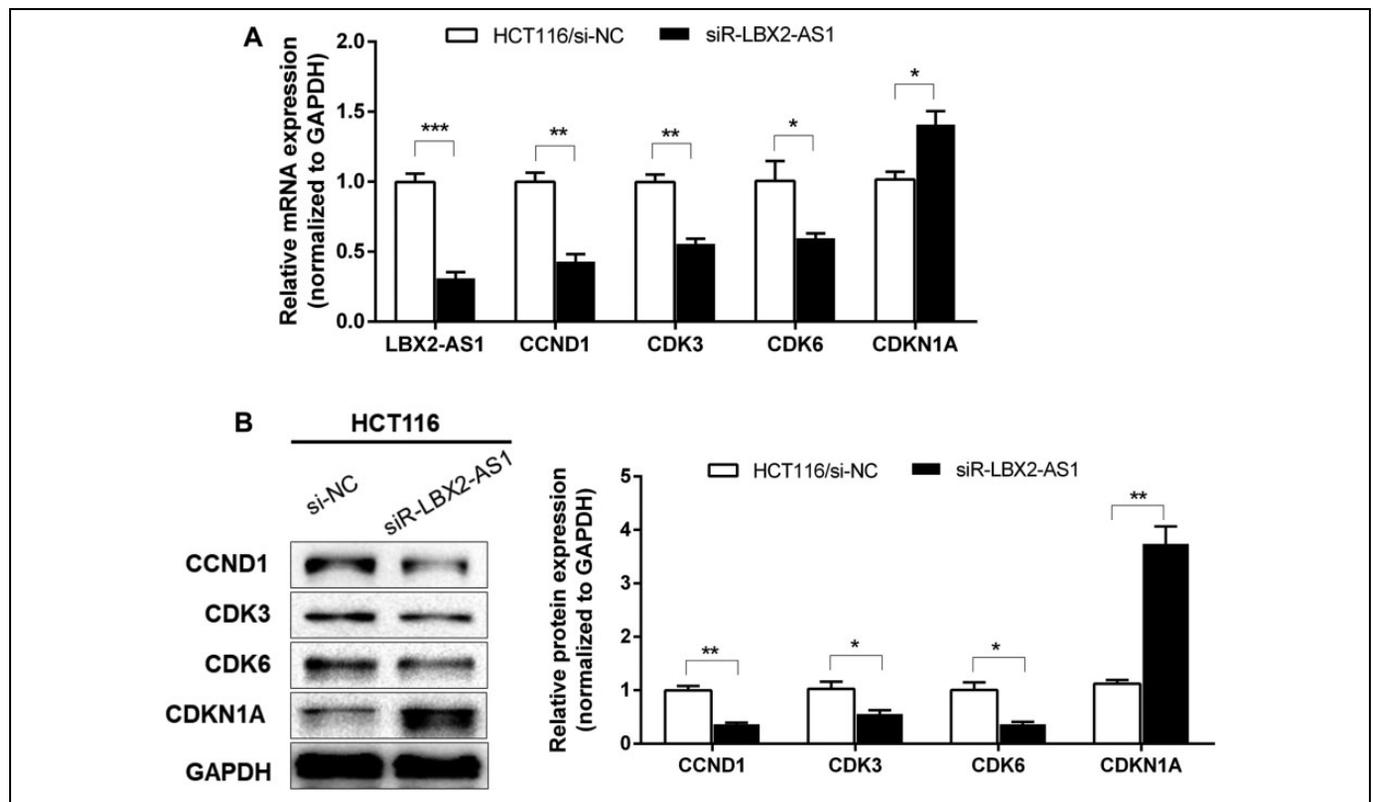


Figure 4. LBX2 antisense RNA 1 (LBX2-AS1) knockdown inhibited the expression of the proliferation-associated markers in colorectal cancer (CRC) cells. After transfection with si-NC or si-LBX2-AS1 for 48 h in HCT116 cells, mRNA expression of CCND1, cyclin-dependent kinase 3 (CDK3), CDK6, and cyclin-dependent kinase inhibitor 1A (CDKN1A) in CRC cells were analyzed by quantitative real-time polymerase chain reaction (A); meanwhile, the protein expressions of CCND1, CDK3, CDK6, and CDKN1A in CRC cells were analyzed by western blotting and densitometry (B). Data are shown as mean \pm standard error of the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with control.

Interactive Analysis (GEPIA) public platform²⁶ (Figure 1B). Subsequently, we detected the expression of LBX2-AS1 in CRC cell lines by qRT-PCR and found that *LBX2-AS1* expression was higher in the CRC cell lines (HT29, LoVo, SW620, and HCT116) than in the normal colon mucosal cell line NCM460 (all $p < 0.05$, Figure 1C). Additionally, *LBX2-AS1* expression was the highest in HCT116 cells. Moreover, we analyzed the cellular localization of LBX2-AS1 in HCT116 cells and found that the cytoplasm proportion of LBX2-AS1 was higher than the nuclear proportion of LBX2-AS1, indicating that the subcellular localization of LBX2-AS1 in the CRC cells was mainly located at the cytoplasm (Figure 1D).

Upregulated LBX2 Antisense RNA 1 (LBX2-AS1) Is Associated With the Clinicopathological Features of CRC

Poor prognosis of CRC patients. Next, we assessed the association between LBX2-AS1 expression and the pathological stages of CRC by analyzing the GEPIA public platform. We found that the higher expression of LBX2-AS1 was significantly associated with the pathological stages of CRC (Figure 2A). To assess the prognostic value of LBX2-AS1 expression in CRC patients, we examined the association between LBX2-AS1 expression levels and OS using Kaplan-Meier analysis with the

log-rank test by analyzing the GEPIA public platform. The results revealed that patients with high LBX2-AS1 expression levels had lower OS ($p = 0.0018$, Figure 2B). Taken together, these results indicate that high LBX2-AS1 expression is an independent risk factor for CRC patients.

LBX2-AS1 knockdown suppresses the proliferative ability of CRC cells. To verify LBX2-AS1 function in colon cancer cells, we first measured the efficiency of the short interfering RNA (siRNA) siRNA-LBX2-AS1 (si-LBX2-AS1). The si-LBX2-AS1-1+2 group showed the higher interfering efficiency in HCT116 and LoVo cells than the si-LBX2-AS1-1 and si-LBX2-AS1-2 groups (Figure 3A). Therefore, we selected si-LBX2-AS1-1+2 to knockdown LBX2-AS1 expression in CRC cells and subsequently assessed the function of LBX2-AS1 in CRC cells. We investigated the effect of LBX2-AS1 knockdown on the cell proliferative ability of CRC cells. The cell growth rates were determined by performing the CCK-8 proliferation assay. Knockdown of LBX2-AS1 expression significantly inhibited HCT116 and LoVo cell proliferation, relative to control cells ($p < 0.001$, Figure 3B). Meanwhile, flow cytometry analysis demonstrated that knockdown of LBX2-AS1 expression had lower percentage of cells in the S phase and higher percentage of cells in the G1 phase in CRC cells than

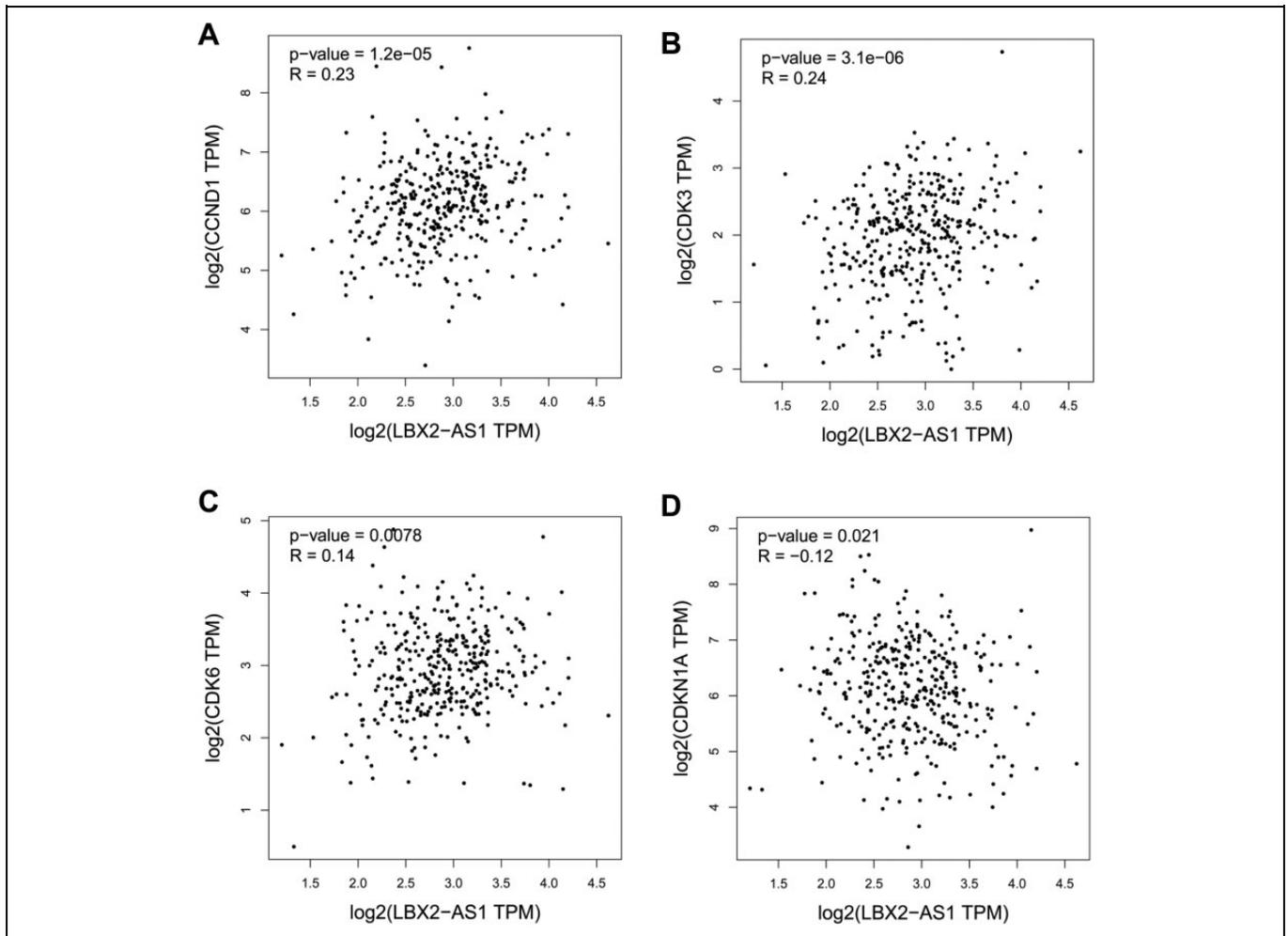


Figure 5. Scatter plots showing the statistical association between LBX2 antisense RNA 1 (LBX2-AS1) expression and proliferation marker expression. The Gene Expression Profiling Interactive Analysis database was used to analyze the association between LBX2-AS1 expression and the expression of CCND1, cyclin-dependent kinase 3 (CDK3), CDK6, and cyclin-dependent kinase inhibitor 1A in colorectal cancer.

control cells (all $p < 0.05$, Figure 3C). These results indicated that LBX2-AS1 knockdown suppresses CRC cell proliferation.

LBX2-AS1 knockdown inhibits the expression of proliferation-associated markers in CRC cells. To further study the molecular mechanism of LBX2-AS1 in CRC cells, qRT-PCR and western blotting were used to assess the mRNA and protein levels of the proliferation-associated markers CCND1, cyclin-dependent kinase (CDK) 3, CDK6, and cyclin-dependent kinase inhibitor 1A (CDKN1A) in HCT116 cells. Knockdown of LBX2-AS1 expression significantly decreased the mRNA and protein expression levels of CCND1, CDK3, and CDK6 and increased the mRNA and protein expression levels of cell cycle cyclin-dependent kinase inhibitor CDKN1A (all $p < 0.05$, Figure 4A-B). Furthermore, GEPIA database analysis showed that LBX2-AS1 expression was positively associated with the expression of CCND1, CDK3, and CDK6 and negatively associated with the expression of CDKN1A in CRC (all $p < 0.05$, Figure 5). This indicates that *LBX2-AS1* contributes to the regulation of proliferated marker expression in CRC cells.

Discussion

CRC is one of the most common gastrointestinal tumors worldwide,²⁷ specifically in developed countries. CRC is the fifth major cause of cancer-associated deaths in China and has a substantial effect on human health.²⁸ Despite significant advancements in the diagnosis and treatment of CRC, the mortality and morbidity rates of CRC remain high, possibly due to postsurgical recurrence of primary tumors.²⁹ Therefore, determining novel therapeutic targets to better monitor recurrence, metastasis, and other diseases is significantly required.

Increasing studies have indicated that lncRNAs play a significant role in various biological processes by regulating the mechanisms of cellular processes, including cell apoptosis, proliferation, and differentiation.^{30,31} Recently, several studies have demonstrated that disruption or disability expression of lncRNAs is closely associated with the progression of malignant tumors and is involved in cancer cell proliferation, inflammation, epithelial-mesenchymal transition (EMT), and drug resistance.^{11,32} lncRNAs have the following characteristics:

they can be easily extracted, are detected with higher sensitivity and specificity, and exist constantly in the blood and tissue.³³ lncRNAs are considered potential novel biomarkers for cancer diagnosis, recurrence, and prognosis. In CRC, some lncRNAs have been shown to be differentially expressed, indicating poor prognosis, including LINC00467,³⁴ LINC01638,³⁵ CCAT1,³⁶ LINC00152,³⁷ and GAS5.³⁸

With the development of high-throughput sequencing, a series of public databases (e.g. TCGA, GEO) have been widely used to predict and identify valuable lncRNAs.¹⁷ In this study, we analyzed the dysregulated lncRNAs in CRC using the GEO dataset GSE41328 using the Affymetrix Human Genome U133 Plus 2.0 platform. We found that lncRNA LBX2-AS1 was significantly overexpressed in CRC datasets. In previous studies, LBX2-AS1 was overexpressed in various human cancers, and patients with higher expression of LBX2-AS1 had unfavorable OS, including patients diagnosed with HCC,¹⁸ GC,²⁰ and NSCLC.²¹ However, the expression and function of LBX2-AS1 in CRC have not been reported. In our study, we demonstrated that LBX2-AS1 expression in CRC tissues was significantly higher than that in matched adjacent normal tissues. Meanwhile, the overexpression of LBX2-AS1 was significantly associated with shorter OS of CRC patients, which may be potentially developed as a novel biomarker for CRC diagnosis and prognosis.

Regarding the effect of LBX2-AS1 in cancers, convincing evidence has shown that it plays critical roles in regulating the development and progression of cancer. For example, Yang et al. demonstrated that LBX2-AS1 was overexpressed in ESCC, and it could interact with the RNA-binding protein heterogeneous nuclear ribonucleoprotein C to regulate the expression of ZEB1 and ZEB2, thereby promoting cell migration and EMT of ESCC cells.¹⁹ Moreover, Wang et al. showed that LBX2-AS1 could sponge with miR-384 to promote the expression of insulin receptor substrate 1, thereby accelerating cell proliferation, migration, and invasion of HCC cells and inducing apoptosis *in vitro*.¹⁸ However, the effect of LBX2-AS1 on CRC proliferation has been rarely understood. Our results demonstrated that knockdown of LBX2-AS1 expression suppressed cell proliferation in HCT116 cell lines, which was associated with decreased CCND1, CDK3, and CDK6 expression, but enhanced CDKN1A expression. This indicates that LBX2-AS1 regulates the expression of proliferation markers in CRC cell lines.

Conclusions

In summary, our study indicated that the expression of LBX2-AS1 is upregulated in CRC tissues and CRC cell lines, and these patients with high LBX2-AS1 expression showed poor OS. Additionally, knockdown of LBX2-AS1 expression significantly suppressed the proliferative ability of CRC cells. Therefore, LBX2-AS1 may play a crucial role in regulating the tumorigenesis of CRC and provide a prospect for the development of novel CRC therapies after further investigation.

Authors' Contributions

QL, HX, SW and ZZ performed all the experiments, analyzed all the data and were a major contributor in writing the manuscript. QL, HX, SW and ZZ also contributed to performing the experimental tasks and aided in the drafting of the manuscript. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Authors' Note

Qing Li and Hui Xie are also affiliated to the Key Laboratory of Medical Imaging and Artificial Intelligence of Hunan Province.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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