Original Research

Long noncoding RNA LRRC75A-AS1 inhibits cell proliferation and migration in colorectal carcinoma

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Impact statement

It is reported that colorectal cancer has seriously threatened human health. The incidence of colorectal cancer in China is increasing year by year. At present, the treatment of cancer is gradually developing towards individualized treatment whose core is targeted therapy, and molecular pathology is the basis of targeted therapy. Previous studies have shown that in addition to protein-coding genes that regulate tumor invasion and metastasis, there are also some non-coding genes involved in tumor encroachment and spread. Our study found that long noncoding RNA LRRC75A-AS1 is closely related to CRC and is related to its proliferation and migration. And it may become a novel molecular marker for clinical diagnosis and treatment.

Abstract

With the continuous improvement of technology in the molecular field, more and more evidence indicated that long noncoding RNAs (lncRNAs) are widely expressed in a broad spectrum of human tumors, playing an important role in the development and progression of tumors. Most studies reported that lncRNAs might serve as reliable biomarkers and effective clinical therapeutic target. Leucine-rich repeat containing 75 A-antisense RNA1 (LRRC75A-AS1) was reported to be relevant to many types of cancers and indicated to do influence on colorectal carcinoma (CRC). This research firstly examined the role of LRRC75A-AS1 in CRC and analyzed its association with the biological behaviors of CRC cells. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) brought to light that LRRC75A-AS1 was remarkably expressed at low levels in CRC tissues. We also found that LRRC75A-AS1 was localized in the cytoplasm. In addition, LRRC75A-AS1 knockdown also notably promoted CRC cell proliferation, metastasis, invasion, and colony formation. To summarize, these experimental results showed that LRRC75A-AS1 might serve as an anti-oncogene for CRC tumorigenesis and advancement, and it may become a novel molecular marker for clinical diagnosis.

Keywords: LRRC75A-AS1, colorectal cancer, long noncoding RNA, migration, proliferation

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Introduction

Colorectal carcinoma (CRC) ranks third in the world's most common malignant tumors.[1] Although mortality has declined for decades due to improved screening with standard treatment strategies and improvements in standardized treatment, relapse and poor prognosis still affect the outcome of CRC treatment.[2] The tumorigenesis of CRC involves multiple oncogenes, anti-oncogenes, and cancer signaling pathways, and there are inextricable links between different signaling pathways, which form a complex signaling network. Therefore, identifying key signal transduction mediators in the development of CRC is an important part of analyzing and developing fresh targets about its targeted treatment. LncRNAs are not only a group of endogenous RNA, which is greater than 200 nucleotides in length[3] but also lack of specific complete open reading frames (ORFs) and the function of encoding protein, and thus lncRNAs are considered as accessory products produced by the transcription of RNA polymerase II and do not have a corresponding biological effect.[4]

However, lncRNAs are abnormally expressed in most tumors, and they play a critically important part in promoting or maintaining tumorigenesis and the development of cancer, having clinical potential as biomarkers and targets for targeted therapies.[5] For instance, it is reported that transcription can be regulated by lncRNAs via local (cis) as well as long-distance (trans) mechanisms. Previous studies show that cis-regulation can affect the expression level of lncRNAs' neighboring genes during the transcription of it. Transduction means that lncRNA may interact with transcription factors (TF) during affecting the expression of TF of the target gene. According to competitive endogenous (Ce) RNA hypothesis, lncRNAs attach to microRNAs (miRNAs) so that they can compete with mRNAs that contain the similar miRNA response elements, thereby affecting miRNA target genes to express.[6] Despite this, many lncRNAs are still unexplored and their abilities to cancer pathogenesis, invasion, metastasis, and other biological behavior, still aren't made out.

The previous study suggested that the lncRNA leucine-rich repeat containing 75 A-antisense RNA1 (LRRC75A-AS1) is located on 17p11.2 and may be related to osteosarcoma, but no further studies have been reported on its functional and structural features.[7] However, information about LRRC75A-AS1 in the development and pathogenesis of different tumors, particularly in CRC, is still limited. Furthermore, the experimental outcomes suggested the gene expression level in human colorectal cancer is lower than that in normal tissues. Further functional experiments indicated that LRRC75A-AS1 plays an important part in regulating cells monoclonal reproduction, adjacent migration, and distant metastasis. Finally, the experimental data suggested LRRC75A-AS1 can be involved in CRC tumorigenesis and development as a non-coding anti-cancer gene.

Materials and methods

Ethics statement

All experiments in our research were authorized by the Ethics Committee of Southern Medical University (Guangdong, China). And the research protocol is consistent with the principles of the Helsinki declaration and with current laws. All participants gave informed consents.

Cell culture

The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) provided us with the human colorectal cancer cell lines RKO, HT29, Ls-174T, HCT116, LoVo, SW620, and SW480. All these CRC cell lines were preserved in an incubator at 37°C with 5% CO_2 and nourished by RPMI Medium 1640 (Thermo Fisher Scientific, Inc., MA, USA) including 10% fetal bovine serum (GE Healthcare Life Sciences, Logan, UT, USA). Sterility should also be strictly observed during cell culture.

Clinical samples collection

A total of 31 cases of fresh CRC tissue and adjacent normal mucosa tissues were prospectively acquired from surgical patients with primary colorectal cancer at Nanfang Hospital, Southern Medical University (Guangdong, China) from January to June in 2017. All fresh CRC tissue was preserved in a cryopreservation tube in liquid nitrogen, preventing tissue destruction. Among the specimens collected, the average age of the patients was 57.5 years old, including 9 females (29%) and 22 males (71%). According to the size of the tumor, the state of the lymph nodes and a classification system for distant metastases,[8] the patients were classified as following: 4 patients (12.9%) were in the T stage 2, 19 patients (61.3%) were T3 and other 8 patients (25.8%) were T4; There were 19 cases (61.3%) with lymph node metastasis and 12 cases (38.7%) without metastasis. Distant metastasis occurred in four patients (12.9%), while no distant metastasis occurred in the rest patients. The tissue used in the experiments was authorized by the Ethics Committee of Southern Medical University (Guangdong, China). All of these patients did not receive any preoperative chemotherapy or radiotherapy. All these patients have endorsed consent forms before using clinical data for research purposes.

RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT–PCR)

Cells used in the next experiments were washed by PBS for three times, then the RNA of CRC cells was extracted with TRIzol kit (Invitrogen; Thermo Fisher Scientific, Inc., Shanghai, China) and then cDNA was compounded through reverse transcription with the PrimeScript RT kit (Takara Biotechnology Co., Ltd, Dalian, China). The qRT-PCR analysis was carried out using the SYBR Premix Ex Taq kit (Takara Biotechnology Co., Ltd, Dalian, China) in a three-step qRT-PCR technique with the ABI 7500 Real-Time PCR system. The temperature setting of thermal cycling is as following: firstly, 95°C for 10 min, then 40 amplification cycles (95°C for 5 s, 60°C for 20 s, and 72°C for 34 s), then a dissociation stage (95°C for 15 s, 60°C for 1 min, and 95°C for 15 s). In the experiment, GAPDH acted as a blank control group. The result was computed through the relative quantification $2^{-\Delta\Delta Ct}$ method. Primer sequences are shown in Table 1. All sample manipulations are performed on the ice. Every sample was analyzed in triplicate to illustrate technical variability.

Subcellular fractionation location

RNA of nucleus and cytoplasm were isolated and extracted, respectively. CRC cells were washed several

Table 1. Reverse transcription-quantitative polymerase chain reaction used in this study.

Gene	Primer	Sequence(5′-3′)
LRRC75A-AS1	Forward	AGCTCACAGCACACCTGGCTA
	Reverse	AGCTGAGGCAGGAGGACCAT
GAPDH	Forward	ACAGTCAGCCGCATCTTCTT
	Reverse	GACAAGCTTCCCGTTCTCAG
U6	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGCTTCACGAATTTGCGT

LRRC75A-AS1: leucine-rich repeat containing 75A-antisense RNA1

times with ice-cold PBS solution and then transferred to the cell centrifuge tube and centrifuged at $120 \times g$ at 4°C for 5–10 min. The cells obtained were added to the lysis buffer which includes NaCl, $MgCl_2$, Tris-HCl, and 0.5% NP-40, and fully reversed for several minutes. Then centrifuge the mixture at $5000 \times g$ at 4°C for 5 min. The supernatant produced is the cytoplasmic component extract, and the rest was washed twice with pre-cooled PBS for cytoplasmic component extraction. The nuclear and cytoplasmic components were added to TRIzol reagent for RNA extraction, respectively.

Cell transfection

In order to down-regulate the expression level of LRRC75A-AS1, Genechem (Co., Ltd, Shanghai, China) provided us with a kind of specific short hairpin RNAs (shRNAs) and control shRNAs. The human LRRC75A-AS1 sequence 5′ -GCTCGAGCTTCTGTGGTCT -3′ was transferred into GV248 (GenePharme, Shanghai, China). Manufacturer's instructions were followed during the cell transfection. Virus particles were collected after transfection into the packaging vector GV248, pHelper1.0, and pHelper2.0 into 293 T cells using transfection reagent (GenePharme, Shanghai, China). We picked out SW620 and LoVo cells with good state and planted them into six-well plates, then Lentiviral particles were obtained and transfected into cells. Negative vectors (The GV lentiviral vector series, pHelper 1.0 vector, and 2.0 vector) were used as controls. The transfected CRC cells were collected after culturing for 48 h. To confirm the interference efficiency of LRRC75A-AS1 in the infected cell lines, the relative expression levels of the experimental group and the control group were measured by qRT-PCR. And then infected cell lines were applied for the next experimental operation.

Cell proliferation assay

Referring to the manufacturer's instructions, the cell proliferation rate was evaluated by Counting Kit8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Rockville, MD, USA). After lentivirus transduction was stabilized, SW620 and LoVo cells in the logarithmic phase of growth were transformed into single-cell suspension, and then the suspension was separately transferred to the 96-well plates at the density of 2×10^3 per well, and added CCK-8 reagent to each well (5 mg/mL). Avoiding the light, the reagent was added and left alone for 2 h, and the microplate reader was employed to measure the absorbance at 450 nm. Over the next week, CCK-8 reagent was separately added in every well, respectively, at a given point in time and the absorbance of each well was measured in the same way. Each sample has five duplicate holes, and the average value was caculated by removing the maximum and the minimum.

Colony formation assay

The CRC cells lines with good growth form were selected and placed in the six-well plates and kept the density of cells at 2×10^2 cells per well. Then cells were cultured with 10% FBS in RPMI 1640 for two weeks. After visible cells mass appeared in the plates, we washed the cells twice with ice-cold PBS, fixed with methanol for several minutes, then stained with Giemsa reagent. When the diameter of tumor cell clones was larger than 150 μm, the number of tumor cell clones was measured by Image-Pro Plus v6.0 and the entire orifice plate needed to be calculated and a total of three orifice plates were calculated per sample. Three independent experiments were conducted.

Cell migration assay

The migration capabilities of CRC cells were measured with a wound-healing assay by using 10 μL pipette tube to create a blank scratch area in the orifice and measuring the movement of the cells. The measuring time is 0 h and 48 h, respectively. The migration results of the transfected cells in each group were measured by photographs. The migration was quantified by computing the cell migration area which can be subtracted from the area before migration by the total cell area after migration. Image Pro Plus v6.0 was employed to analyze the area of cell migration. In this experiment, all of the five random fields were surveyed for each board, and three random fields were measured for each sample, and three independent experiments were conducted.

Cell invasion assay

Tumor cells with a concentration of 2×10^5 were added in the upper chamber of the 24-well transwell plate with serum-free medium culturing the cells. After assuring the presence of cells in the upper chamber under a microscope, the lower chamber was added with 10% fetal bovine serum medium as a chemical inducer. Then the bottom of the upper chamber was soaked in the solution of the lower chamber. There was a matrix gel attached below the upper chamber. After the entire orifice plate was incubated in the cell incubator at 37°C for 48 h, tumor cells that did not invade the upper chamber were scraped with a cotton swab. The tumor cells that successfully infiltrated into the lower chamber were fixed with methanol for several minutes, and then the cells were stained with Giemsa for 15 min. Next, the cell numbers were calculated under a microscope. In this experiment, all of the five random areas need to be measured for each plate, and three plates were calculated for every sample, and all independent assays were duplicated at least three times.

Statistical analysis

All data represent the mean ± standard deviation of the indicated number of experiments. Use GraphPad Prism (GraphPad Software, Inc., CA, USA) and SPSS software 20.0 statistical package (SPSS, Inc., Chicago, USA) to conduct statistical analysis. In the statistical analysis, we regard P value < 0.05 as statistically significant. The following statistical tests were used to assess differences among variables: t-test, factorial analysis, χ^2-test, one-way analysis of variance, or Fisher's exact test. All the data used in this paper were significant in the statistic.

Results

LncRNA LRRC75A-AS1 is down-expressed in CRC tissues

In order to understand the role of LRRC75A-AS1 in CRC tumorigenesis, the qRT-PCR analysis was used to research the expression level of LRRC75A-AS1 in CRC tissue and adjacent normal tissue. Compared to the adjacent normal mucosa tissue, expression of LRRC75A-AS1 was lower in 22 of 31 (70.97%) CRC specimens ($P < 0.05$) (Figure 1(a)). The results revealed that the expression level of LRRC75A-AS1 in tumor tissues was lower than that in the corresponding adjacent normal mucosa tissues (Figure 1(b)), suggesting that lncRNA LRRC75A-AS1 may play a significant part in the pathogenesis and development of CRC.

LRRC75A-AS1 expression level in CRC cell lines was detected, and LRRC75A-AS1 is located in the cytoplasm

In order to further study LRRC75A-AS1 proliferation and migration, the expression level of the LRRC75A-AS1 in seven cell lines was detected and several cell lines with higher expression level were screened out. The expression level of the LRRC75A-AS1 in seven CRC cell lines (HT29, Ls-174T, RKO, HCT116, SW620, LoVo, and SW480) was analyzed by qRT-PCR. And the results suggested that the expression levels of LRRC75A-AS1 were highest in SW620, RKO, and LoVo cell lines (Figure 1(c)). For ascertaining the subcellular localization of LRRC75A-AS1, nuclear/cytoplasmic isolation of SW620 and RKO cells was carried out, and then RNA was extracted from the treated cell

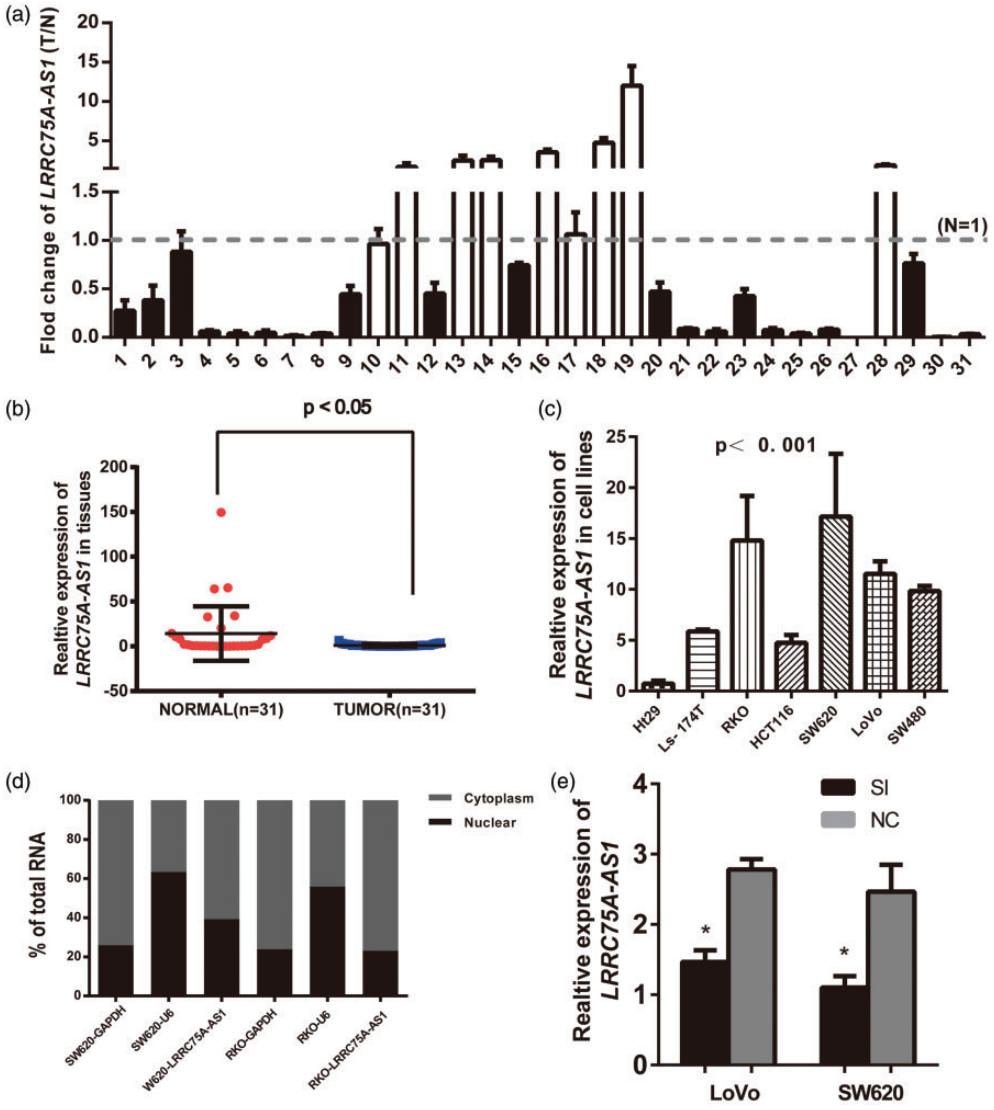

Figure 1. LRRC75A-AS1 expression is down-regulated in CRC tissue and cells. (a) The qRT-PCR analysis of LRRC75A-AS1 expression in 31 pairs of CRC tissue and adjacent normal mucosa tissue. LRRC75A-AS1 expression was normalized to GAPDH, and the results are shown as the fold change in tumor tissues relative to the matched adjacent normal mucosa tissues. And error bars show the means ± standard deviation of three independent experiments. Blank bars represent T/N>1. (b) Relative expression of LRRC75A-AS1 in the 31 paired human CRC tissue and paired adjacent normal mucosa tissue. (c) The qRT-PCR analysis of LRRC75A-AS1 expression in seven CRC cell lines. And error bars indicate the means ± SD of three independent experiments. (d) LRRC75A-AS1 cell localization, as identified using the qRT-PCR analysis in fractionated SW620 and RKO cells. GAPDH and U6 were used as cytosolic and nuclear fractionation indicators, respectively. (e) Relative expression of LRRC75A-AS1 was detected in SW620 and LoVo cells by qRT-PCR following transfection with either a specific siRNA (SI) or a negative control siRNA (NC). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (A color version of this figure is available in the online journal.)

materials for reverse transcription, followed by qRT-PCR. In this experiment, primer GAPDH and primer U6 RNAs were employed to act as the differential controls of the separation indexes of cytoplasmic and nuclear extracts. These results showed that the expression of the LRRC75A-AS1 in the nucleus of these two cell lines was significantly lower than the cytoplasm (Figure 1(d)), indicating that LRRC75A-AS1 was chiefly located in the cytoplasm of CRC cells.

Downregulating of LRRC75A-AS1 promotes CRC cell proliferation and clonal group formation in vitro

This research used specific siRNA to knockdown the expression of LRRC75A-AS1 in CRC cells by viral vector for further studying the effect of LRRC75A-AS1 on the biological behavior of CRC cells. We selected the highest expression of three cell lines SW620, RKO, and LoVo for LRRC75A-AS1 knockdown by RNA interference, and SW620 and LoVo with better transfection effect were selected for the next experiments. The qRT-PCR analysis revealed that after transfection with specific siRNA, the expression of LRRC75A-AS1 was down-regulated by 48.19% in LoVo cells and 55.47% in SW620 cells in comparison with the control group (Figure 1(e)). The cell proliferation curve measured by CCK-8 kit assay indicated that LoVo and SW620 cells proliferation was increased after LRRC75A-AS1 was down-regulated (Figure 2(a) and (b)). Moreover, the results of colony formation test made clear

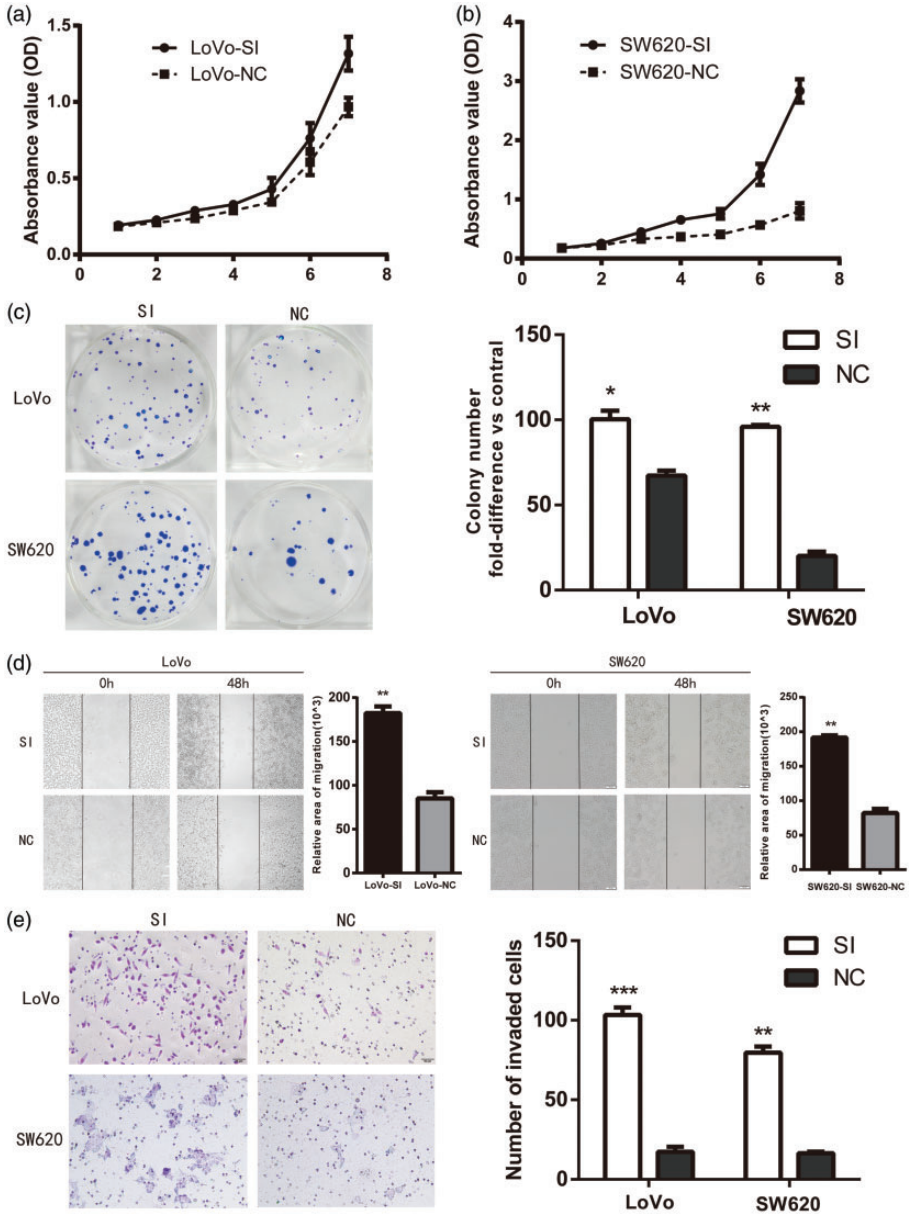

Figure 2. LRRC75A-AS1 knockdown promotes CRC cell proliferation, migration, invasion, and colony formation *in vitro*. LoVo and SW620 cells were transfected with either a LRRC75A-AS1 specific siRNA or a negative control siRNA. (a) Cell proliferation was evaluated by CCK−8 assay in LoVo cells. (b) Cell proliferation was evaluated by CCK-8 assay in SW620 cells. (c) Colony-forming assays were performed to determine the growth of CRC cells. (d) Migration ability was tested by a scratch-wound healing assay (magnification, ×100). (e) Invasion ability was tested in Matrigel-coated transwell invasion chambers (magnification, ×200). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (A color version of this figure is available in the online journal.)

that the decreased expression of LRRC75A-AS1 led to the enhanced population formation ability of LoVo and SW620 cells (Figure 2(c)).

LRRC75A-AS1 inhibits the capacity of CRC cells to migrate and invade in vitro

Specific siRNA was employed to degrade the transcription of LRRC75A-AS1 in CRC cells by a viral vector for further investigating the effect of LRRC75A-AS1 on cell migration and invasion. The wound-healing experiment was employed to measure the migration capacity of LoVo and SW620 cell lines, and its results showed that the knockout of LRRC75A-AS1 obviously promoted the migration of LoVo and SW620 cells in comparison with the control group (Figure 2(d)). In addition, cell invasion experiment showed that the knockdown of LRRC75A-AS1 significantly increased the invasiveness of LoVo cells by 83.23% and SW620 cells by 79.50% when contrasting with the negative control induction scheme (Figure 2(e)). In conclusion, the down-regulated expression of LRRC75A-AS1 can significantly enhance the invasiveness and metastasis of CRC cells *in vitro*.

Discussion

The incidence of colorectal carcinoma (CRC) is very high worldwide, yet the mechanisms by which it developed remain unclear.[9] Mammalian genomes are known to encode a lot of lncRNAs.[10] In the past few years, a large number of experimental results have shown that lncRNAs may play an important role in pathophysiology process of many diseases, especially the development of tumors. For example, lncRNA PVT1-214 stabilizes Lin28 and interacts with miR-128 to promote proliferation and invasion of CRC[11]; lncRNA CASC9 can regulate TGF-β signaling in CRC by interacting with CPSF3.[12] These results indicated that lncRNAs is a kind of important marker in the diagnosing and treating of tumors.[13] In the previous study, the lncRNA expression profile of colorectal cancer cell lines from the same parent with different metastatic potential was analyzed, and a series of differentially expressed lncRNAs were obtained. LncRNA FEZF1 antisense RNA1 (FEZF1-AS1) is one of them and the research founded that it was obviously upregulated in primary human CRC and associated with tumor metastasis and poor prognosis in CRC patients.[10] We selected four candidate lncRNAs with different expression levels from the RNA sequencing data and the difference of expression in LRRC75A-AS1 is the most obvious. As a result of this, we chose and paid close attention to this novel lncRNA LRRC75A-AS1 whose function had not been studied in CRC before. So, we explored the expression level and the function of it in CRC.

A recent study performed by Jeong *et al.*[14] found that LRRC75A-AS1 can regulate the vascular calcification negatively, and might be a possible target in the treatment of vascular calcification. Moreover, the chromosome region 17p11.2p12 was founded amplified continually in the high-grade osteosarcomas of human, which implies that there will be one or more oncogenes. In previous studies, nine candidate oncogenes including LRRC75A-AS1 were identified in this region, which suggests that LRRC75A-AS1 may be related to osteosarcoma.[7] LRRC75A-AS1 was also found to be abnormally expressed in the process of normal villous maturation and some cancers, such as breast cancer, and gastric cancer.[15–17] These studies suggest that LRRC75A-AS1 may be related to cancer. Therefore, we are curious about its role in CRC.

The qRT-PCR analysis was employed to analyze the clinical tissue from patients with CRC for exploring the relationship of LRRC75A-AS1 in CRC. We found that LRRC75A-AS1 expression levels were remarkably lower in tumor tissue compared with adjacent normal mucosa tissue. Therefore, our study hinted that LRRC75A-AS1 was likely to be of great importance in the progression and development of cancer. It has not been studied in tumors, so its role and function in tumors are still unclear. Our study first studied its role in colorectal cancer, and the results showed that it played a role as an anti-cancer gene in colorectal cancer, which laid a foundation for the study of this gene in tumors.

Then, the expression level of LRRC75A-AS1 in seven CRC cell lines was examined and the result found that LRRC75A-AS1 is mainly located in the cytoplasm. For testing its function in the cell behaviors of CRC, specific siRNA was used to knockdown LRRC75A-AS1 expression level in LoVo and SW620 cell lines. The result shows that LRRC75A-AS1 knockdown can facilitate CRC cell proliferation, migration, and invasion significantly *in vitro*. All these results indicate that LRRC75A-AS1 might be an anti-oncogene and can suppress the development and progression of CRC.

LncRNAs play an important role in human cancer because of impacting cellular functions by various mechanisms. In this study, LRRC75A-AS1 was first indicated to be an anti-oncogene in CRC. So lncRNAs might restrain the tumorigenesis of CRC. However, current experiments are only being carried out at the cellular level, so more relevant experiments should be practiced on animals to explore its role *in vivo*. Further studies are needed to illuminate the molecular mechanisms that LRRC75A-AS1 might suppress the development and progression in CRC, as well as its application target in clinical practice.

Authors' contributions: JZ led study design and prepared the manuscript; JC conducted most of the experiments; JL prepared the reagents and chemicals; ZY performed the PCR analyses; SD and YH collected and embedded the clinical specimens; YZ performed statistical analysis JZ performed data analysis and interpretation.

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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