

Long noncoding RNA expression profile analysis of colorectal cancer and metastatic lymph node based on microarray data

Peng Yang^{1,*}

Zi-Peng Xu^{1,*}

Tao Chen¹

Zhen-Yu He²

¹The Second Clinical Medical College of Nanjing Medical University,

²Department of General Surgery, The Second Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu Province, People's Republic of China

*These authors contributed equally to this work

Abstract: Long noncoding RNAs (lncRNAs) are emerging as an important part of biological progress in cancers, yet the aberrant lncRNAs implicated in colorectal cancer (CRC) with lymph node metastasis remain unknown. In this study, a total of 390 lncRNA transcripts and 508 mRNA transcripts were dysregulated in tumor tissues compared with paired metastatic lymph nodes. Functional prediction showed that lots of lncRNAs might be involved in biological pathways related to CRC metastasis by *cis*-regulation and *trans*-regulation of coexpressed genes. As a representative, *ENST00000430471* was associated with cell proliferation and invasion of CRC cells. These results provided support for further investigations of the metastatic pathogenesis of CRC.

Keywords: colorectal cancer, lymph node metastasis, long noncoding RNA, microarray, *ENST00000430471*

Introduction

Colorectal cancer (CRC) is one of the most common causes of cancer-related deaths in the world, leading to 600,000 deaths each year worldwide.^{1,2} Clinically, a considerable number of CRC patients with metastasis, such as blood metastasis and lymph node metastasis, fail to respond well with the help of current treatment regimens. Lymph node metastasis is the most common metastatic site, and ~50% of CRC patients with lymph node metastasis experience disease recurrence.^{3,4} Therefore, disclosing the molecular mechanisms underlying metastasis is urgently needed for developing effective therapies and improving patients' prognosis.

Long noncoding RNAs (lncRNAs), unable to be translated into proteins and >200 nt in length, have emerged as an important aspect of biology.^{5,6} Evidences suggest that they are capable of controlling protein-coding and noncoding genes and interacting with known cancer genes.⁷⁻⁹ For instance, HOTAIR, highly expressed in breast tumors, could promote metastasis through reprogramming the chromatin state.¹⁰ Several lncRNAs that play significant roles in tumorigenesis and might be potential biomarkers for CRC diagnostic and prognosis have been proposed in our previous study.¹¹ However, the roles that lncRNAs play in the progress of lymph node metastasis of CRC remain unknown.

To profile the lncRNA expression patterns in tumor tissues of CRC compared with paired metastatic lymph node (MLN), the lncRNA microarray expression profile in three pairs of CRC tumor tissues compared with MLNs was presented in this study. Then, we predicted the potential functions of differentially expressed lncRNAs based

Correspondence: Zhen-Yu He
Department of General Surgery, The Second Affiliated Hospital of Nanjing Medical University, 121 Jiangjiayuan Road, Nanjing 210011, Jiangsu Province, People's Republic of China
Tel/fax +86 25 5850 9900
Email hezhenyu1968@126.com



on their coexpressed protein-coding genes. Next, a novel lncRNA, *ENST00000430471*, that displayed a higher expression level in MLNs compared with tumor tissues was selected for functional analysis and further study.

Patients and methods

Tissue collection

A total of 26 CRC patients who underwent surgical resections at The Second Affiliated Hospital of Nanjing Medical University from 2011 to 2012 were recruited for our study. These patients received neither chemotherapy nor radiotherapy prior to the operation. A written informed consent was obtained from all the patients, and approval was obtained from the ethics committee of The Second Affiliated Hospital of Nanjing Medical University. Two experienced pathologists collected lymph nodes from the CRC patients during the operation and stained them with hematoxylin and eosin. According to the hematoxylin and eosin staining results, the lymph nodes were divided into MLNs and normal lymph nodes. All samples were frozen in liquid nitrogen until further analysis. For microarray analysis, three pairs of samples (three tumor tissues and three MLNs) from the CRC patients were used.

Microarray expression profiling

Three pairs of CRC tumor tissues and MLNs were used to synthesize double-stranded complementary DNA (cDNA), which was labeled and hybridized on the SurePrint G3 Human Gene Expression 8x60K v2 Microarray (Agilent Technologies, Santa Clara, CA, USA). Processed slides were scanned with the Agilent G2505C Microarray Scanner (Agilent Technologies) after hybridization and washing. Raw data were extracted using Feature Extraction (version 10.7.1.1; Agilent Technologies). Then, quantification of normalization and subsequent data processing were performed using the GeneSpring software (version 12.0; Agilent Technologies). After that, raw signals from the microarray were \log_2 transformed and specific expression of mRNAs and lncRNAs were defined when the absolute value of fold change was >2 and P -value was <0.05 . The microarray profiling was conducted by the OE Biotechnology Company (Shanghai, People's Republic of China).

Coexpression network and functional prediction

According to the specific expressed genes, coexpression networks were built to identify the interactions among genes.¹² First, Pearson's correlation coefficient of the dysregulated lncRNA compared with that of each dysregulated mRNA was calculated to find its coexpressed mRNAs. The

absolute value of 0.8 with a correlation P -value <0.05 was considered statistically significant. Then lncRNA gene functions were predicted using the hypergeometric cumulative distribution function based on the coexpression of mRNA using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations. The threshold of statistical significance was set as a P -value <0.05 and false discovery rate <0.01 .

Evidence shows that several lncRNAs can exert their *cis*-regulating functions by recruiting remodeling factors to local chromatin.¹³ We defined *cis*-regulated genes as protein-coding genes coexpressed with one dysregulated lncRNA and within 300 kbp upstream or downstream in genomic distance in the same allele.

The core transcription factors (TFs) are *trans*-regulated by specific lncRNAs to participate in certain biological pathways.^{14,15} Thus, we compared the coexpressed mRNAs of these lncRNAs with the mRNAs that were regulatory targets of certain TFs to predict that these lncRNAs possibly participate in pathways regulated by these TFs. The lncRNA–TF network was constructed using hypergeometric cumulative distribution function of MATLAB 2012b (MathWorks, Natick, MA, USA). The graph of the lncRNA–TF network was drawn using Cytoscape 3.01 (Agilent Technologies and IBS; Agilent Technologies, Santa Clara, CA, USA).

As the lncRNA coexpression genes might participate in lncRNA-mediated gene regulation, we constructed the “TF–lncRNA–genes” network based on the interactions of lncRNAs and target coexpression genes as previously described.¹⁶ The three groups were generated based on the “TF–lncRNA” two-element network with the help of Cytoscape software.

RNA extraction and quantitative real-time polymerase chain reaction analysis

Total RNA was extracted from 26 snap frozen subsets and cultured cells using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. For quantitative real-time polymerase chain reaction (qRT-PCR) analyses, RNA was reverse transcribed to cDNA by using a reverse transcription kit (Takara Biotechnology, Dalian, People's Republic of China). Then, qRT-PCR was performed using SYBR Green (Takara Biotechnology) according to the manufacturer's instructions. The qRT-PCR results were normalized to glyceraldehyde-3-phosphate dehydrogenase.

Cell culture

Three human CRC cell lines SW480, HCT116, and SW620 were obtained from the Cell Bank of the Chinese Academy

of Medical Sciences (Shanghai, People's Republic of China). Cells were cultured in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific) in an atmosphere of 5% CO₂ at 37°C. All the media were supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Thermo Fisher Scientific).

Plasmid DNA transfection

According to the full-length *ENST00000430471* sequence in Ensembl, the *ENST00000430471* sequence was synthesized and subcloned into a pCDNA3.1 vector (Thermo Fisher Scientific). The empty vector was used as the control. The pCDNA-*ENST00000430471* and empty vector were transfected into HCT116 cells seeded at six-well plates using Lipofectamine 2000 (Thermo Fisher Scientific), according to the manufacturer's suggested protocol. The expression level of *ENST00000430471* was detected by qRT-PCR.

Cell proliferation assay

The viability of HCT116 cells was assessed using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) following the manufacturer's instructions. CCK-8 solution was used to measure cell viability at 24 hours, 48 hours, and 72 hours after transfection. The absorbance value of each well was measured at 450 nm. For the colony formation assay, a total of 500 cells were seeded in six-well plates to allow colony formation for 2 weeks. The colonies were fixed with methanol and stained with Giemsa, and the number of colonies was counted after 20 minutes.

Flow-cytometric analysis

For the cell cycle analysis, transfected cells were fixed in 75% ethanol overnight. The cells were stained for 30 minutes with propidium iodide (50 µg/mL; Sigma-Aldrich Co., St Louis, MO, USA) and 0.25 mg/mL of RNase A (Sigma-Aldrich Co.). Next, the cells were analyzed by flow cytometry (FACScan; BD Biosciences, San Jose, CA, USA) using CellQuest software (BD Biosciences). The percentage of the cells in G0–G1, S, and G2–M phases was counted and compared.

For the cell apoptosis assay, the cells were treated with fluorescein isothiocyanate-Annexin V and propidium iodide in the dark, according to the manufacturer's instructions. Then the cells were detected by flow cytometry with the help of CellQuest software. Cells were discriminated into dead cells, viable cells, early apoptotic cells, and late apoptotic cells. Next, the percentage of early apoptotic cells and late apoptotic cells was compared with empty vector from each experiment. Each assay was repeated in triplicate.

Cell migration and invasion assays

In migration assays, 3×10⁴ cells at 48 hours after transfection were seeded in the upper chamber of the wells in a 200 µL serum-free medium (8 µm pore size; EMD Millipore, Billerica, MA, USA); for the invasion assays, 1×10⁵ cells in serum-free medium were seeded in the upper chamber coated with Matrigel (BD Biosciences). The lower chambers were filled with 800 µL of 20% medium containing 20% fetal bovine serum. Following the incubation for 24 hours, cells on the filter surface were fixed with methanol, stained with 0.1% crystal violet, and photographed with a phase-contrast inverted microscope. Experiments were independently repeated three times.

Statistical analysis

The statistical significance of differences between groups was estimated by Student's *t*-test on SPSS software (version 18.0; SPSS Inc., Chicago, IL, USA). A *P*-value of <0.05 was chosen for statistical significance. The results are reported as mean ± SD. All experiments were performed at least three times.

Results

lncRNA and mRNA expression profiles

Differentially expressed lncRNAs and mRNAs (fold change ≥2, *P*≤0.05) were observed in Figure 1. A total of 53 lncRNAs exhibited upregulated expression levels and 337 lncRNAs exhibited downregulated expression levels, whereas 102 mRNAs exhibited upregulated expression levels and 406 mRNAs exhibited downregulated expression levels in the tumor tissues compared with MLNs (Table 1). Among the dysregulated lncRNA transcripts, the most upregulated lncRNA was *uc010fsr.1* (up 46.06), whereas *ENST00000430471* (down 9.50) was the most downregulated lncRNA. These results suggested that these lncRNAs and mRNAs might also have common functions in facilitating the transfers of CRC tumor cells from the primary tumor to lymph nodes. Table 1 lists the top 20 dysregulated lncRNAs from our microarray. A novel lncRNA, *ENST00000430471*, that displayed the lowest expression in tumor tissues compared with MLNs was selected for further study.

Coexpression profiles and the lncRNA function prediction

One lncRNA can be coexpressed with hundreds of mRNAs. For instance, *uc010fsr.1* was coexpressed with 666 mRNA transcripts and *ENST00000430471* with 3,749 mRNA transcripts. A heat map was built to show the relationships between every differentially expressed lncRNAs

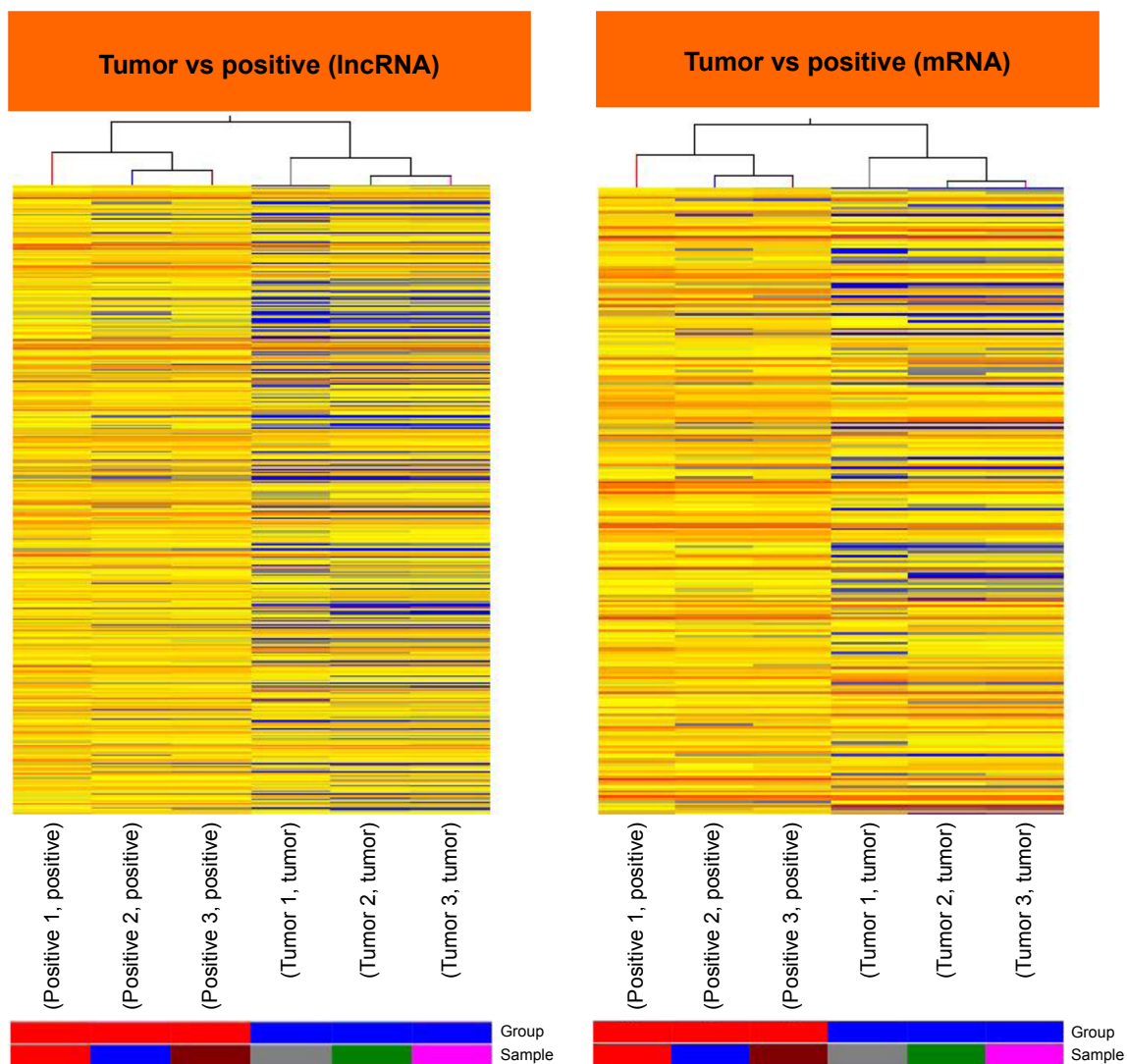


Figure 1 Hierarchical clustering for differentially expressed lncRNAs and mRNAs in tumor vs MLN (positive).

Notes: “Red” indicates high relative expression, and “blue” indicates low relative expression. 1–3 represent patients 1–3, whereas tumor represents tumor tissue and positive represents MLN.

Abbreviations: lncRNA, long noncoding RNA; MLN, metastatic lymph node.

and its coexpression mRNAs using the unsupervised hierarchical clustering analysis. We exhibited the map of *ENST00000430471* with its coexpression mRNAs in Figure S1.

The functions of differentially expressed lncRNAs were predicted by the GO and KEGG pathway annotations of their coexpressed mRNAs. According to the *P*-value and enrichment, we counted and summarized the top 200 and 500 credible annotations for coexpressed and aberrant lncRNA genes, respectively. In the GO pathway analyses, the most frequently predicted functions of aberrant lncRNAs were “transforming growth factor beta (TGF- β)-activated receptor activity”, “DNA binding TF activity”, “transmembrane signaling receptor activity”, “protein binding”, and “DNA binding”

(Figure 2A and B), while the most common pathways involved in the KEGG pathway were “transcriptional misregulation in cancer”, “osteoclast differentiation”, “mitogen-activated protein kinase (MAPK) signaling pathway”, and “nuclear factor-kappa B signaling pathway” as shown in Figure 2C and D. We listed the representative KEGG terms of the top 20 dysregulated lncRNAs in Table 1.

The lncRNA *ENST00000430471* was also annotated using GO and KEGG pathway analyses. According to the aforementioned selection criteria, the top 20 enrichment KEGG terms are listed in Table 2, indicating that *ENST00000430471* was associated with “adrenergic signaling in cardiomyocytes”, “MAPK signaling pathway”, “regulation of actin cytoskeleton”, and “pathways in cancer”. The results of the pathway

Table 1 Top 20 dysregulated lncRNAs (tumor vs positive)

lncRNA	P-value	FC	Regulation	KEGG term	TFs
<i>uc010fsr.1</i>	0.009893517	46.06492737	Up	NF-kappa B signaling pathway	BATF, SPI1, NFKB1
<i>ENST00000434499</i>	0.000675539	37.12658809	Up	Osteoclast differentiation	TCF12, EBF1, PRDM1, USF2
<i>ENST00000430471</i>	0.033238088	9.501604567	Down	MAPK signaling pathway	USF2, GATA2, RAD21, SPI1
<i>uc001pjf.3</i>	0.007060995	9.270161245	Down	MAPK signaling pathway	TCF12, EBF1, USF2, POU2F2
<i>uc002btm.2</i>	0.012791482	7.898165872	Down	Transcriptional misregulation in cancer	TCF12, GATA1, PRDM1
<i>AK024164</i>	0.028566538	7.558821916	Down	Focal adhesion	TCF12, GATA1, RAD21
<i>ENST00000233836</i>	0.0109523	7.164441114	Down	MAPK signaling pathway	TCF12, E2F1, EBF1, USF2
<i>uc001dbm.2</i>	0.000997457	6.862444929	Down	TGF- β signaling pathway	EBF1, SPI1, BATF, NFKB1
<i>BC032569</i>	0.00420414	6.739981509	Down	NF-kappa B signaling pathway	EBF1, TCF12, SPI1, BATF
<i>ENST00000429048</i>	0.034423918	6.48362627	Down	PI3K-Akt signaling pathway	TCF12, RAD21, SMC3, JUN
<i>AK022228</i>	0.02360072	5.887638121	Down	Pathways in cancer	SPI1, USF2, USF1, TCF12
<i>BX483760</i>	0.001321745	5.700537409	Down	NF-kappa B signaling pathway	SPI1, BATF, EBF1, NFKB1
<i>NR_029467</i>	0.003048984	5.54162953	Down	NF-kappa B signaling pathway	SPI1, TCF12, BATF, NFKB1
<i>CN273898</i>	0.002144023	5.338684774	Down	TGF- β signaling pathway	TCF12, PRDM1, FAM48A
<i>NR_001558</i>	0.012046172	5.1594478	Up	NF-kappa B signaling pathway	TCF12, ESRR, GATA2
<i>AK126261</i>	0.006970672	5.148190123	Down	NF-kappa B signaling pathway	BATF, EBF1, SPI1, RFX5
<i>nc-HOXB6-181+</i>	0.003952154	5.090352861	Up	NF-kappa B signaling pathway	EBF1, TCF12, BATF, TAF1
<i>HIT000389365</i>	0.012777917	5.030105062	Down	MAPK signaling pathway	TCF12, USF2, GATA2
<i>ENST00000508517</i>	0.048584219	4.901906275	Down	Transcriptional misregulation in cancer	SPI1, JUN, RAD21, SMC3
<i>AX747038</i>	0.044646349	4.83483839	Down	Alzheimer's disease	USF1, EBF1, SPI1, ZBTB7A

Abbreviations: lncRNA, long noncoding RNA; FC, fold change; KEGG, Kyoto Encyclopedia of Genes and Genomes; TF, transcription factor; NF, nuclear factor; MAPK, mitogen-activated protein kinase; TGF, transforming growth factor; PI3K, phosphoinositide 3-kinase.

analyses consistently showed that *ENST00000430471* is associated with the pathogenesis of CRC.

Cis-regulation of lncRNA

According to the aforementioned criteria, a total of 104 lncRNA transcripts with their predicted *cis*-regulating protein-coding genes were found through accurate genomic mapping. The “*cis*” analyses of some representative lncRNAs are shown in Table 3.

Trans-regulation of lncRNA

Because many lncRNAs were involved, we generated a core network map using the top 100 lncRNA–TF pairs in Figure 3. The map displayed that the TF TCF12 modulated the expression of 33 lncRNAs, whereas the TF SPI1 modulated the expression of 21 lncRNAs and the TF EBF1 the expression of 16 lncRNAs. As shown in Table 1, the relative TFs of the top 20 dysregulated lncRNAs were provided. Then, in order to determine the “TF–lncRNA–genes” relationship, we selected the top 1,500 target genes into the “TF–lncRNA” network based on the results of lncRNA coexpression analysis (Figure S2). In short, valuable information about TFs, lncRNAs, and target genes were provided in these maps.

ENST00000430471 is upregulated in CRC

To validate the differential expression of *ENST00000430471*, we performed qRT-PCR assay in 26 CRC tumor tissues,

paired normal tissues, and corresponding MLNs (Figure 4A). The qRT-PCR data showed that *ENST00000430471* was significantly upregulated in MLN tissues with an average increasing fold of 3.57 and 6.89 ($P < 0.01$), compared with paired tumor tissues and normal tissues. These results indicated that *ENST00000430471* might be significantly related to the progress of lymph node metastasis of CRC.

ENST00000430471 promotes proliferation in HCT116 cells

To investigate the functional role of lncRNA *ENST00000430471* in CRC cells, first, *ENST00000430471* expression was detected by qRT-PCR in three human CRC cell lines (Figure 4B). Notably, SW480 cells expressed relatively lower levels of *ENST00000430471* compared with HCT116 and SW620 cells. Then we put our efforts to discover how the CRC cell behavior changes when upregulating the expression of the lncRNA *ENST00000430471*. After transfection, the results of CCK-8 assays revealed that the growth of HCT116 cells with pCDNA–*ENST00000430471* was promoted compared with control cells ($P < 0.05$; Figure 5A). The colony formation assays were performed to find that the ability of colony formation of pCDNA–*ENST00000430471* cells was significantly stronger than the negative groups ($P < 0.05$; Figure 5B). Taken together, *ENST00000430471* was involved in CRC cell proliferation.

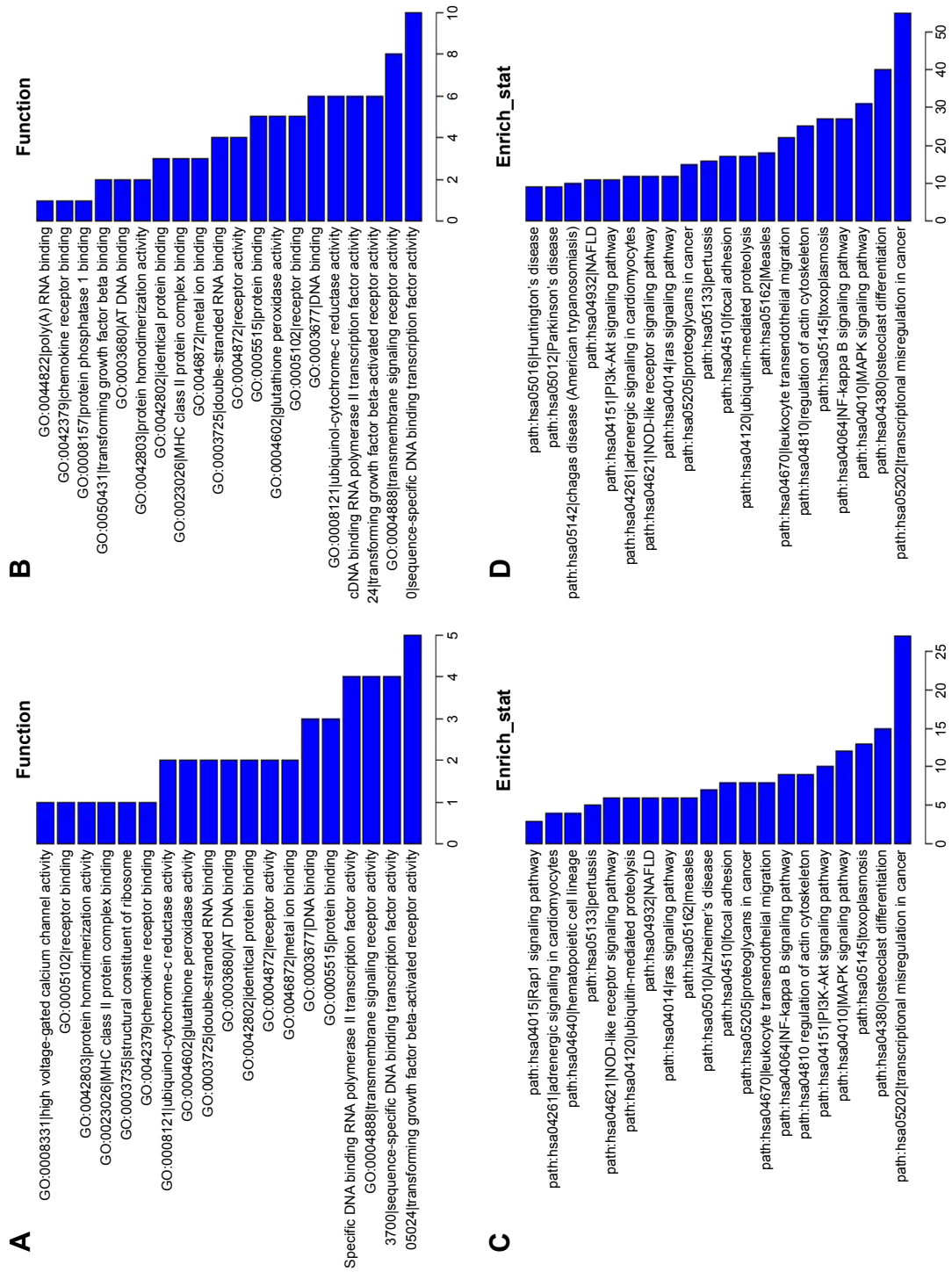


Figure 2 The top 200 and top 500 GO and KEGG annotations for the different lncRNA coexpression genes between the two groups of tumor tissues and MLNs. **Notes:** The top 200 (A) and top 500 (B) GO annotations for the different lncRNA coexpression genes between the two groups. The x-axis shows the number of lncRNAs annotated, and the y-axis shows the GO annotations. The top 200 (C) and top 500 (D) KEGG terms for the different lncRNA coexpression genes. The x-axis shows the number of lncRNAs annotated, and the y-axis shows the KEGG terms. **Abbreviations:** GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; lncRNA, long noncoding RNA; MLN, metastatic lymph node; MHC, major histocompatibility complex; AT, autosomal; NOD, nucleotide oligomerization domain; NAFLD, nonalcoholic fatty liver disease; NF, nuclear factor; PI3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase.

Table 2 Top 20 enrichment KEGG terms of *ENST00000430471*

Term	P-value	Pathway
path:hsa04261	0.000115429	Adrenergic signaling in cardiomyocytes
path:hsa04010	0.000166191	MAPK signaling pathway
path:hsa04810	0.000170216	Regulation of actin cytoskeleton
path:hsa05200	0.000227779	Pathways in cancer
path:hsa04380	0.000230427	Osteoclast differentiation
path:hsa05202	0.000369415	Transcriptional misregulation in cancer
path:hsa04510	0.000687107	Focal adhesion
path:hsa05205	0.000850753	Proteoglycans in cancer
path:hsa05162	0.001568171	Measles
path:hsa04024	0.002840745	cAMP signaling pathway
path:hsa05414	0.003834431	Dilated cardiomyopathy
path:hsa04750	0.003843316	Inflammatory mediator regulation of TRP channels
path:hsa04014	0.004130068	Ras signaling pathway
path:hsa05222	0.004139032	Small cell lung cancer
path:hsa04120	0.004468168	Ubiquitin-mediated proteolysis
path:hsa05410	0.005281147	HCM
path:hsa05133	0.00614867	Pertussis
path:hsa04921	0.006379835	Oxytocin signaling pathway
path:hsa04145	0.006998006	Phagosome
path:hsa05134	0.008675511	Legionellosis

Abbreviations: KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinase; cAMP, cyclic adenosine 3',5'-monophosphate; TRP, transient receptor potential; HCM, hypertrophic cardiomyopathy.

Table 3 Representative lncRNAs and their *cis*-regulated genes

lncRNA	PCC	mRNA
<i>uc001dbm.2</i>	0.983455768	ROR1
<i>ROR1</i>	0.922404707	ZNF671
<i>ROR1</i>	0.843639152	ZNF814
<i>ROR1</i>	0.822512303	ZNF418
<i>ENST00000429048</i>	-0.864003773	ARMCX3
<i>ENST00000429048</i>	-0.816859073	DRP2
<i>AK022228</i>	0.916156658	ZNF80
<i>AK126261</i>	-0.99043371	TBC1D8
<i>HIT000389365</i>	0.849861722	STAU2
<i>HIT000389365</i>	-0.818489648	STAU2
<i>CN413083</i>	0.951947071	FCER1G
<i>uc002odt.1</i>	0.97754608	CLIP3
<i>AF308155</i>	0.97778953	RELB
<i>BC031073</i>	-0.863771219	EFCAB4B
<i>uc003xjb.2</i>	0.819161974	NRG1
<i>AK024382</i>	0.833577816	RHCE
<i>AK128058</i>	-0.835535492	SEC11C
<i>nc-HOXD1-48-</i>	-0.957251502	HOXD9
<i>nc-HOXD1-48-</i>	-0.948817424	HOXD9
<i>nc-HOXD1-48-</i>	-0.889280685	HOXD11
<i>nc-HOXD1-48-</i>	-0.884935986	HOXD11
<i>nc-HOXD1-48-</i>	-0.874534069	MTX2
<i>nc-HOXD1-48-</i>	-0.815439428	HOXD10
<i>NR_024344</i>	0.880490052	THYN1
<i>NR_024344</i>	0.879098428	IGSF9B
<i>AK021444</i>	0.828168114	POSTN
<i>AK021606</i>	0.864459707	TGFBR2
<i>AK024173</i>	0.897980516	ZMYND17
<i>AI655567</i>	-0.877234886	SIRPB1

Abbreviations: lncRNA, long noncoding RNA; PCC, Pearson's correlation coefficient.

ENST00000430471 promotes S-phase arrest and inhibits apoptosis

To determine whether the effects of *ENST00000430471* on the proliferation of CRC cells were mediated by changing the cell cycle progression, we followed cell cycle progression in HCT116 cells with flow cytometry. After treatment with pCDNA-*ENST00000430471* or empty vector for 48 hours, the results demonstrated that pCDNA-*ENST00000430471* led to a significant accumulation of cells at the S-phase ($P < 0.05$; Figure 5C). Next, we investigated the effects of overexpression of *ENST00000430471* on apoptosis. As shown, the percentages of apoptotic cells were significantly decreased in the pCDNA-*ENST00000430471* group compared to the control group ($P < 0.05$; Figure 5D). These results suggest that *ENST00000430471* treatment could induce S-phase arrest and diminish CRC cell apoptosis.

Effect of *ENST00000430471* on migration and invasion

In order to examine whether *ENST00000430471* has a role in regulating CRC cell migration and invasion, we evaluated HCT116 cell invasion through Matrigel and migration through transwell. The results showed that upregulation of *ENST00000430471* significantly promoted the migration of HCT116 cells compared with that of the control. Similarly, invasion of HCT116 cells was increased following overexpression of *ENST00000430471* (Figure 5E). These data indicate that *ENST00000430471* could promote migration and invasion of colon cancer cells.

Discussion

Despite recent studies have shown the critical roles of lncRNA on tumorigenesis in different kinds of cancers, few lncRNAs have been characterized in lymph node metastasis of CRC.¹⁷⁻¹⁹ In this study, we first assessed genome-wide lncRNA microarray expression patterns in CRC tumor tissues compared with paired MLNs and explored their possible functions. We found that 390 lncRNA and 508 mRNA transcripts are dysregulated. A novel lncRNA *ENST00000430471*, which was upregulated in MLNs, was chosen for further study.

At present, only a small part of known lncRNAs have functional annotations, so we predict the lncRNA functions based on coexpression gene GO and KEGG pathway annotations in this study. As shown in Figure 2, the top predicted pathways of these lncRNAs were TGF- β -activated receptor activity, transcriptional misregulation in cancer, DNA binding TF activity, and MAPK signaling pathway,

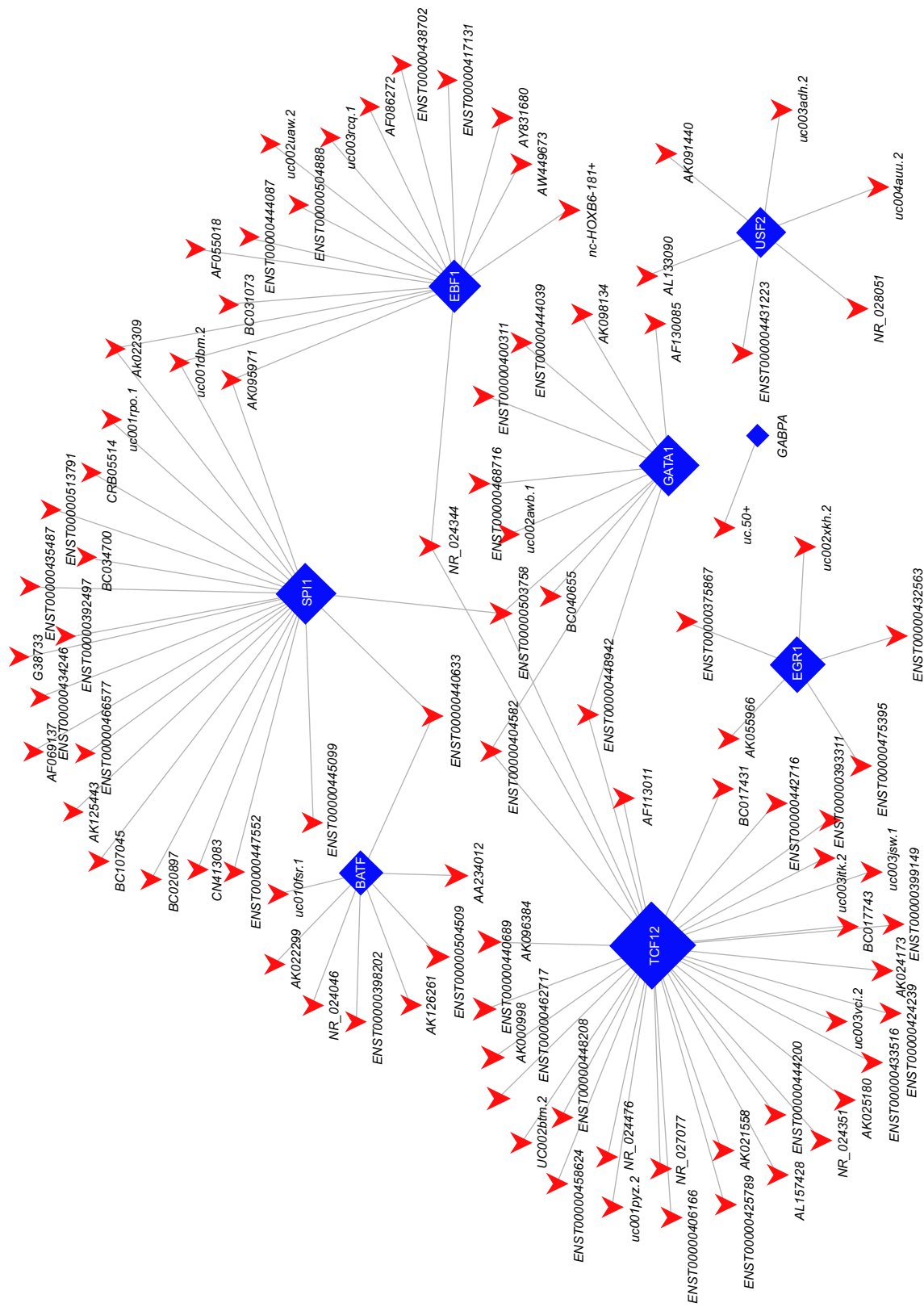


Figure 3 The lncRNA-TF network consisting of the top 100 lncRNA-TF pairs.
Notes: The blue solid squares represent TFs, and the red arrowheads represent lncRNAs; the edges between them mean that the lncRNAs are potentially regulated by the TFs.
Abbreviations: lncRNA, long noncoding RNA; TF, transcription factor.

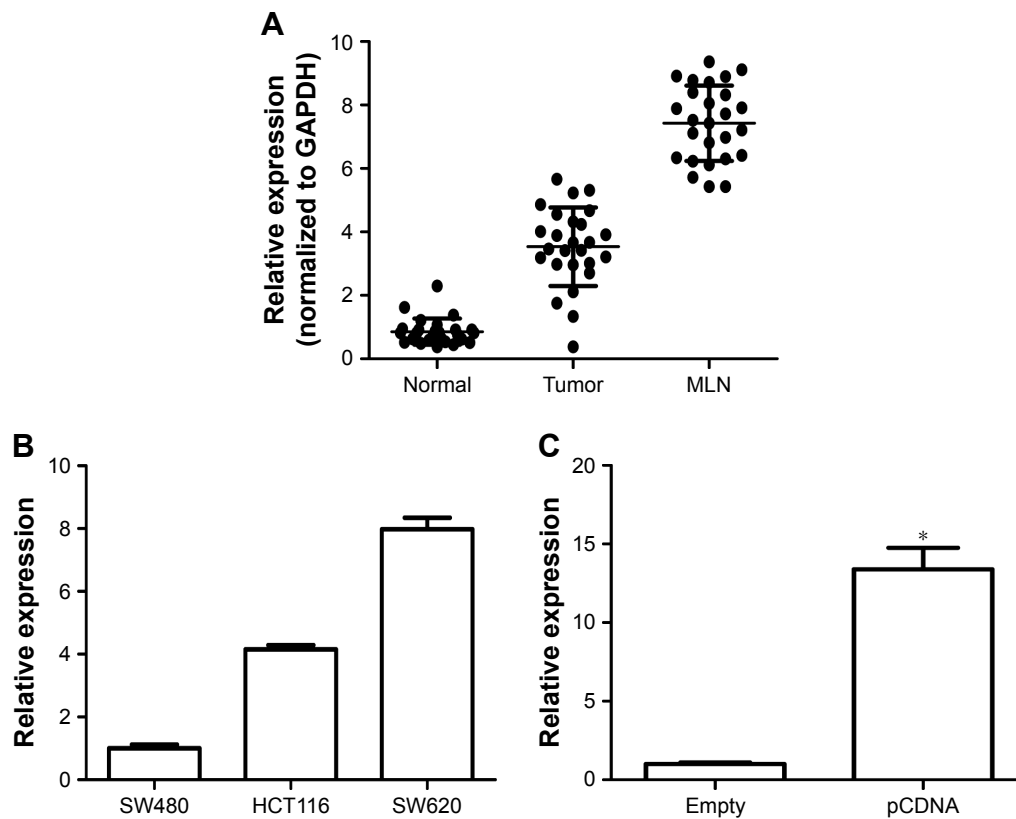


Figure 4 qRT-PCR verification of the expression of *ENST00000430471*.

Notes: (A) The relative expression level of *ENST00000430471* in CRC tumor tissues, paired normal tissues, and corresponding MLNs. (B) The relative expression level of *ENST00000430471* in colorectal cancer cell lines. (C) Transfected with pCDNA-*ENST00000430471* and empty vector. * $P < 0.05$.

Abbreviations: qRT-PCR, quantitative real-time polymerase chain reaction; CRC, colorectal cancer; MLN, metastatic lymph node; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

which correspond well with the pigenetic regulation role of lncRNAs.²⁰ For example, reports have showed that MAPK signaling pathway and TGF- β signaling pathway are related to cell proliferation, invasion, metastasis signaling pathways.^{21,22} *ENST00000430471* was also associated with MAPK signaling pathway and pathways in cancer, which displayed its potential pathogenesis of CRC.

In our study, *cis*-regulation and *trans*-regulation mechanisms were used to get additional information of dysregulated lncRNAs. In all, 104 lncRNAs were predicted to *cis*-regulate their nearby protein-coding genes, and the outstanding lncRNAs are listed in Table 3. The TF-lncRNA and TF-lncRNA-gene networks were constructed with the help of the “trans” analysis. The core TF-lncRNA-gene network (Figure S1) showed that TFs, including TCF12, SPI1, and EBF1, regulated lncRNA expression in CRC. TCF12 is reported to be associated with the occurrence of CRC metastasis by suppressing the expression of E-cadherin.²³ The most relevant with *ENST00000430471*, USF2, is vital for the transcriptional activation of aspartyl (asparaginyl) β -hydroxylase and its truncated homologue humbug.²⁴

Interestingly, humbug over expression is positively associated with tumor grade and inversely with survival in stage II colon cancers.²⁵ Therefore, information of the *cis* and *trans* analyses promoted to interpret lncRNA functions and the pathogenesis of CRC.

A novel lncRNA, *ENST00000430471*, the most upregulated lncRNA in MLNs compared with tumor tissues caught our attention among most of the uncharacterized lncRNAs. In our functional study, *ENST00000430471* overexpression in HCT116 cells promoted cell proliferation and increased colony formation. The results of flow cytometry, *ENST00000430471* overexpression, led to a significant S-phase arrest and a related decrease in apoptosis, revealing that *ENST00000430471* might impact the proliferation of CRC by influencing cell cycle progression and apoptosis. Additionally, transwell assay demonstrated that *ENST00000430471* promoted migration and invasion ability of colorectal cell, suggesting that *ENST00000430471* may be involved in the metastasis of CRC. The effects of *ENST00000430471* on CRC cell laid a good foundation for the next research of CRC with regulation mechanism.

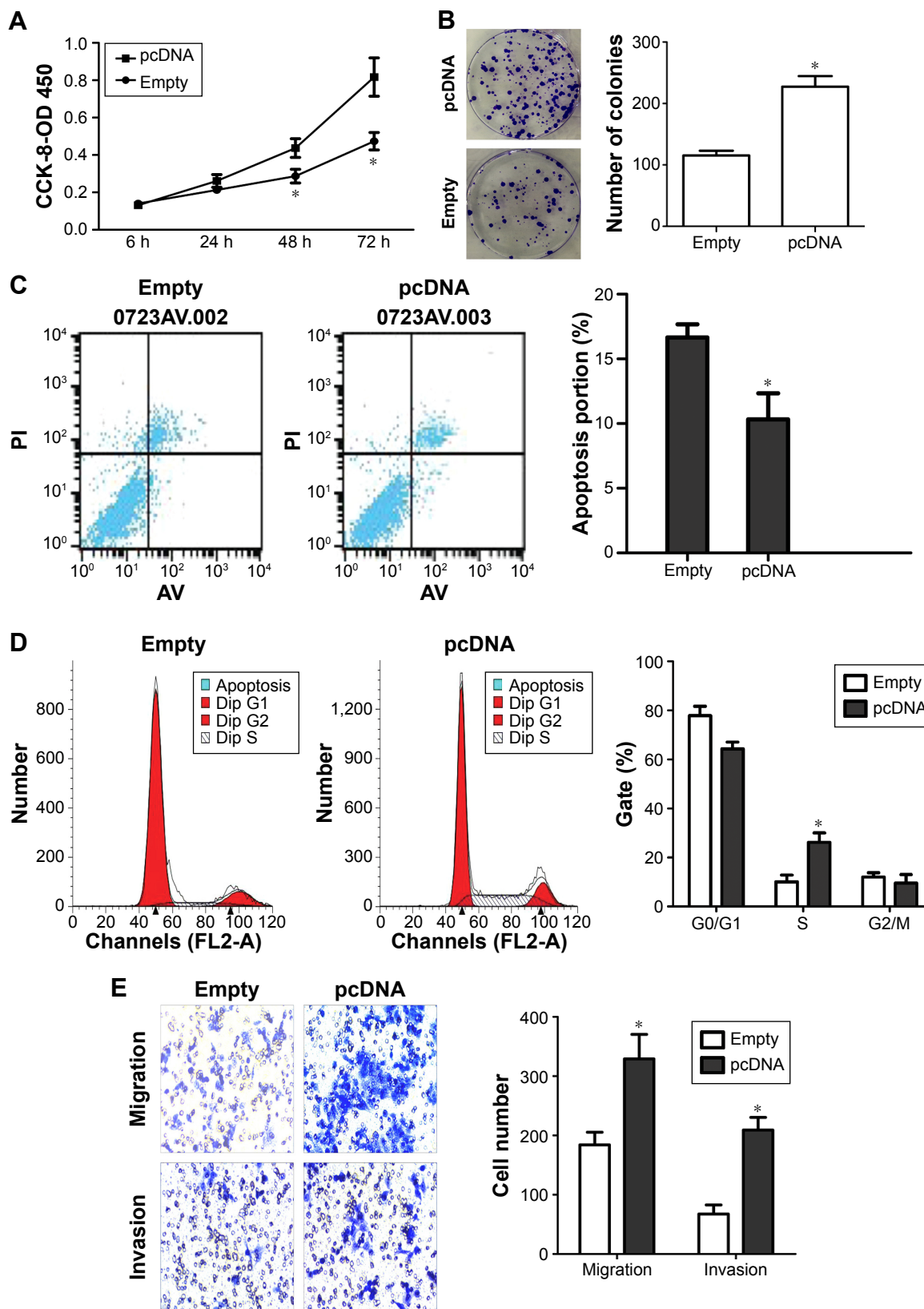


Figure 5 Effects of ENST00000430471 on the CRC cell in vitro.

Notes: (A) CCK-8 assay was performed to determine the proliferation of HCT116 cells. (B) Colony-forming growth assays were performed to determine the proliferation of HCT116 cells. The colonies were counted and captured. (C) The bar chart represents the percentage of cells in G0/G1, S, and G2/M phases. (D) The percentage of apoptotic cells was determined by flow-cytometric analysis. Data represent the mean \pm SD from three independent experiments. (E) Transwell assay showed that ENST00000430471 significantly promoted cell migration and invasion ability. * $P < 0.05$.

Abbreviations: CRC, colorectal cancer; CCK-8, Cell Counting Kit-8; OD, optical density; h, hours; PI, propidium iodide.

Tumor cells usually transfer from the primary tumor to the lymph nodes. To determine whether certain lncRNA would be involved in the lymph node metastasis of CRC, we identified lncRNA-*ENST00000430471* that was most upregulated in MLNs than the tumor tissues. Then we found that *ENST00000430471* played a partial role in the progress of metastasis of HCT116 cells. However, more studies are needed to expand the sample size for clinical research and determine whether *ENST00000430471* can serve as a new diagnostic biomarker and therapeutic target for lymph node metastasis of CRC.

Conclusion

We identified a group of aberrant lncRNAs in tumor tissues and MLNs from three CRC patients. The functional and biological processes of many lncRNAs in the pathogenesis of CRC were determined by *cis*-regulating and *trans*-regulating based on the coexpression genes. Finally, we concluded that *ENST00000430471* might be a novel prognostic marker in CRC.

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Author contributions

P Yang and Z-Y He designed the research. Z-P Xu and T Chen performed the research and analyzed the data. P Yang and Z-P Xu wrote the paper. All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

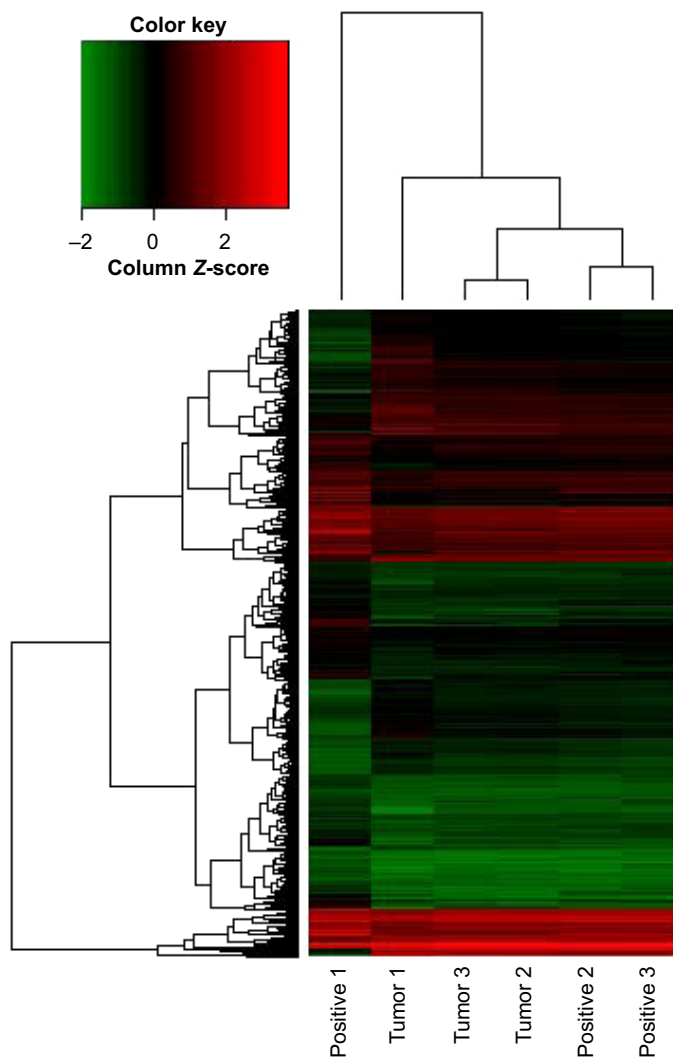


Figure S1 A heat map showing differentially expressed lncRNAs from tumor tissues compared with MLNs.

Notes: Each row represents one lncRNA, and each column represents one sample. The relative lncRNA expression is depicted according to the color scale. Red indicates upregulation and green indicates downregulation.

Abbreviations: lncRNA, long noncoding RNA; MLN, metastatic lymph node.

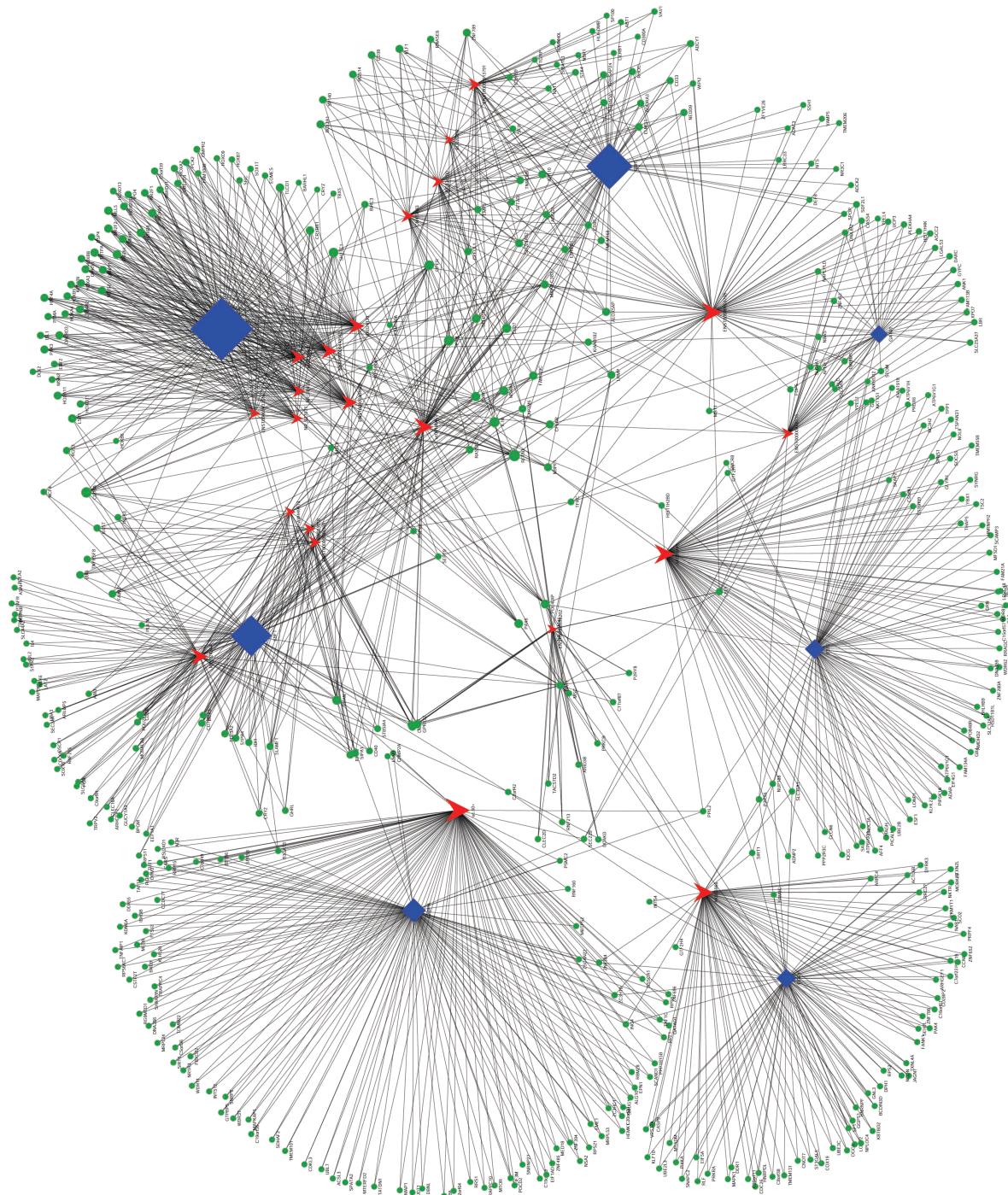


Figure S2 The lncRNA-TF-genes network map consisting of the top 1,500 relevant genes (green dots) based on the lncRNA-TF network.

Note: The blue solid squares represent TFs, and the red arrowheads represent lncRNAs.

Abbreviations: lncRNA, long noncoding RNA; TF, transcription factor.

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