lncRNA-ENST00000513396 is associated with multiple tumor signaling pathways, promotes the cell proliferation, invasion and migration in colorectal cancer

Jinxin Zhu
Nanjing Medical University
Hui Wang
Nanjing Medical University
Yangbin Du
Nanjing Medical University
Zhenyu He (hezhenyu1968@163.com)
Nanjing Medical University

Research

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Abstract

Purpose

Recently, many studies have revealed that Long noncoding RNAs (lncRNAs) were abundant in kinds of cells and might have multiple functions in a wide range of biological processes, such as proliferation, apoptosis, or cell migration. However, the role of lncRNA in the development of colorectal cancer has not been systematically reported and needs to be studied.

Methods

We applied Agilent microarray in tumoral tissues and paired para-cancer tissues for detecting different expression of lncRNAs and mRNAs. Bioinformatics analysis were used to identify the relationship between lncRNAs and corresponding mRNAs and the potential function of the lncRNAs. lncRNA-ENST00000430471 was chosen for further functional study through constructing overexpression plasmid, cell invasion, and cell migration.

Results

We found that 460 lncRNAs were significantly deregulated in cancer tissues. More importantly, they may participate in a variety of tumor-related signaling pathways through functional enrichment analysis with their co-expressed mRNAs, suggesting that these lncRNAs might play critical roles in tumorigenesis and development. Recently, we found lncRNA-ENST00000513396 was significantly upregulated in cancer tissues compared with para-cancer tissues. The overexpression of ENST00000513396 promotes tumor cell proliferation. Meanwhile ENST00000430471 could promote migration and invasion ability of colorectal cells.

Conclusion

LncRNAs play an important role in the development of colorectal cancer. Differentially expressed lncRNAs are associated with multiple tumor signaling pathways. Overexpression of lncRNA- ENST00000513396 in colorectal cancer cells can promote tumor cell proliferation and increase the ability of cell invasion and migration, which leads to the development of tumor.

Introduction

Colorectal cancer is the third most common malignant tumor in the world, with approximately 1.3 million new cases worldwide each year.[1] In recent years, comprehensive treatment of colorectal cancer has made considerable progress, but its mortality rate is still increasing year by year; about 190, 000 patients per

year died of colorectal cancer.[2] The prognosis of colorectal cancer is closely related to the severity of the disease in diagnosis. Local or distant metastases often indicate a poor prognosis for patients.[3] The occurrence and metastasis of tumor is a complex multi-step biological process. Epithelial-mesenchymal transition (EMT) is considered as a necessary condition for colorectal cancer metastasis, but the specific mechanism behind it is not yet clear.[4] Therefore, in-depth discussion of the mechanism of colorectal cancer development and development, clarifying the key molecules and their roles in the process of colorectal cancer metastasis, is essential for early diagnosis and postoperative recurrence monitoring, which also helps to develop new drugs to block occurrence and development of colorectal cancer. Additionally, it is conducive to improving the survival rate of cancer patients and the quality of life of patients.

Long noncoding RNA (LncRNA) is an RNA molecule with a sequence length greater than 200 nt. It does not encode a protein. The expression is spatiotemporal and tissue specific.[5] It has various biological functions such as regulating cell proliferation, apoptosis, and metastasis.[6–8] LncRNA can be regulated by chromosomal modification, histone modification, DNA methylation and other transcriptional regulation, and plays a regulatory role at the level of transcription and post-transcription.[6, 8–11] For example, LncRNA BCAR4 combined with Smad protein1 in the nucleus, restores the activity of histone acyltransferase p300, acetylates histone in the promoter region of glioma-associated oncogene 2 (GLI2), activates the Hedgehog/GLI2 transcription pathway, and promotes Breast cancer development.[10] This shows that LncRNA plays an important role in the occurrence and development of tumors.

In recent years, high-throughput chip technology has become an important means of basic research. We detected the expression of lncRNA and mRNA in colorectal cancer tissues and adjacent tissues through the LncRNA chip technology. We found that there are 460 differentially expressed lncRNAs in colorectal cancer and adjacent tissues. Through functional enrichment analysis of these differentially expressed lncRNAs, it revealed that these genes are involved in a variety of tumor-related signaling pathways, suggesting that these differentially expressed lncRNAs play an important role in the development and metastasis of colorectal cancer. Through clinical sample testing, we have found that lncRNA-ENST00000513396 is significantly overexpressed in tumor tissues. Overexpression of ENST00000513396 can promote the proliferation of cell and improve the ability of invasion and migration of tumor cells.

This study helped us to explore the expression of lncRNA in colorectal cancer, found that some lncRNA may play an important role in the development and metastasis of colorectal cancer, and took ENST00000513396 as an example to explore its function and mechanism of action in order to find potential molecular markers and drug therapy targets to provide theoretical and experimental basis.

Materials And Methods

Tissue samples

The tumor tissues and corresponding adjacent tissues diagnosed by pathology in this research were all derived from the Second Affiliated Hospital of Nanjing Medical University. All patients signed an informed consent form.

The study protocol was approved by the ethics committee of the Second Affiliated Hospital of Nanjing Medical University.

lncRNA gene chip and Bioinformatics analysis

SurePrint G3 Human Gene Expression 8x60K v2 Microarray (Agilent, USA) was used to examine the expression of lncRNA and mRNA in 3 patients with colorectal cancer tumor tissues and adjacent tissues. Using Agilent Database Processing Software, Feature Extraction software (v11. 0.1. 1) obtained the data and chip map, and reading the value obtained the original data. GeneSpring GX v12. 1 software (Agilent, USA) was used to standardize and subsequently process the gene chip data. After normalizing the raw data, the high-quality screened probes were used for further analysis. Differentially expressed genes with statistical significance were obtained between the two groups of samples and filtrated by volcano graphs. Differentially expressed genes were filtrated by Fold Change. Hierarchical clustering was obtained through R script.

We used the Perl script to screen out protein coding genes adjacent to lncRNA (100Kb upstream and downstream) as cis-target genes of lncRNA. We predicted the target gene of lncRNA by calculating the Pearson correlation coefficient. When the absolute value of the Pearson correlation coefficient between the coding gene and lncRNA is bigger than 0.9, we believed that the coding gene is the target gene of lncRNA. The standard enrichment calculation method was used for GO enrichment analysis and KEGG analysis.

By calculating the intersection of the coding gene collection co-expressed with lncRNA and the target gene collection of the transcription factor/chromatin regulatory complex, and using the hypergeometric distribution to calculate the enrichment degree of this intersection, the transcription factors significantly related to lncRNAs were obtained to predict the transcription factor/chromatin regulatory factor that possibly plays a regulatory role with lncRNAs. And a visual network diagram was drawn according to the results of hypergeometric distribution.

Cell culture, plasmid construction and cell transfection

The colorectal cancer cell lines (SW480) used in this experiment are all from the Institute of Biochemistry and Cell Research, Chinese Academy of Sciences (Shanghai). The medium of colorectal cancer cell line SW480 is a mixed medium of DMEM high glucose medium containing 10% fetal bovine serum.

We constructed the LncRNA overexpression plasmid. PCR amplified the G0138630-1 fragment and identify the primer sequence. The upstream primer was synthesized by General Bio: PEGFP-N-5 TGGGAGGTCTATATAAGCAGAG. Downstream primer: PCDNA3. 1R ACAGTGGGAGTGGCACCTTC. Positive clones were screened by bacterial solution PCR method. The obtained positive bacterial solution was

shaken to extract plasmid at 37℃ and sent to General Biological Company for sequencing. The constructed plasmid was transformed into SW480 cells for targeted-gene expression detection.We transfected siRNA into sw480cells by lipo2000 transfection reagent and performed the next QPCR detection. The siRNA is shown in Table 1.

Table 1
The siRNA design for the knockdown of lncRNA (The first is the forward primer and the second is the reverse primer)

Primers	Primer sequences
Control	CAAGATCATGGCAGGCATCC
	TCTCAACAGCTCCAACCTCA
NC	UUACUUUUCCCUCAUUUGGUU
	CCGAAUAAGGGAUAAGAAAGG
SiRNA1	UUUUACUUCCCUUUCAUGGUU
	CCAUGAAAGGGAAGUAAAAGG
SiRNA2	UCAGAUUUCUCAUUGAGUCCA
	GACUCAAUGAGAAAUCUGAAG
SiRNA3	AGAACUUUGGUGUCAUUGGUC
	CCAAUGACACCAAAGUUCUGA

Real-time PCR Assay

Total RNA was extracted from CRC cells using the Trizol method (Synthgene, China). Reverse transcription was performed using reverse transcriptase kit (Synthgene, China) according to the manufacturer's protocol.

Real-time PCR was performed using an SYBR premix Ex Taq kit on 12000 Real-Time PCR System (Applied Biosystems, USA) according to the manufacturer's protocol.
The primer sequences used were obtained from General Bio.

Cell proliferation, invasion, and migration assay

Collected the logarithmic phase cells, treated with MTT(Synthgene, China) and dimethyl sulfoxide, and then measured the absorbance of each well at OD490nm of the enzyme-linked immunoassay detector(hermo scientific, USA).

The transwell inserts were firstly treated with Matrigel (60μl). Then, HCT116 cells transfected with GAPLINC siRNAs or control siRNAs were collected and resuspended in serum-free DMEM medium. Subsequently, the cell suspensions was added into the upper chamber of the inserts, and the complete

medium (containing 20% FBS) were added into the lower chamber of the transwell. Finally, a microscope was applied to take images of the invaded cells after the cells were stained with 0.1% crystal violet.

Cells were seeded and transfected on six-well plates with targeted siRNA or control, then an artificial scratch wound on a confluent monolayer of the cells was created with a 200ul pipette tip. Observe the width of the track in different treatment groups after taking the scratch for 0 hours and 24 hours. Each experiment was repeated three times..

Statistical Analysis

Data were expressed as the mean ± SD of at least three independent experiments. Statistical analysis was carried out using The SPSS 21.0 software package (SPSS, Chicago, IL). Student's t-test or ANOVA was performed to analyze the data. $P < 0.05$ was considered statistically significant.

Results

Differentially expressed LncRNAs in colorectal cancer and adjacent tissues

We randomly selected 3 cases of colorectal cancer tumor tissue and relative para-cancerous tissues, extracted total RNA, and detected the expression profile of lncRNA using the Agilent lncRNA chip. It was found that 460 lncRNAs (change factor ≥ 2, $P \leq 0.05$, calculated by student t test), of which 326 lncRNAs were highly expressed and 134 lncRNAs were lowly expressed (Fig. 1).

Cis-acting target gene prediction means that lncRNA affects its spatially adjacent protein-coding genes through related cis-acting elements, thereby regulating gene expression. The lncRNA target gene is predicted based on the positional relationship between lncRNA and gene. Trans-target gene prediction refers to the case where there is a positive or negative correlation between the expression of LncRNA and some distant genes, and the target gene can be predicted by analyzing the correlation between the expression of lncRNA and protein-coding genes between samples. We used the Perl script to screen out protein coding genes adjacent to lncRNA (100Kb upstream and downstream) as cis-target genes of lncRNA. By calculating the Pearson correlation coefficient between the coding gene and lncRNA to predict the target gene of lncRNA, when the absolute value of the Pearson correlation coefficient between the two is greater than 0.9, we think that the coding gene is the target gene of lncRNA (Table 2 and tableS1).

Table 2
LncRNA target gene prediction results based on positional relationship (partial)

#lncRNA	Genes
ABCC6P1	NOMO2
ABCC6P2	NOMO1; NPIPA3; NPIPA2
ABHD11	ABHD11; STX1A; WBSCR22; WBSCR27; VPS37D; CLDN4; CLDN3; DNAJC30
AC000079.1	CDC45; UFD1L; HIRA; C22orf39; MRPL40
AC002117.1	NMT1; HEXIM2; CAKD; ACBD4; PLCD3; FMNL1; SPATA32;HEXIM1
AC005682.8	TOMM7;FAM126A

We also did GO enrichment analysis and KEGG enrichment analysis of differentially expressed lncRNA cis-target gene and trans-target gene (Fig. 2 and Fig. 3). We have found some signal molecules or pathways related to tumors, such as cell adhesion molecules(CAMs), transcriptional misregulation in cancer, and Rap 1 signaling pathway.Based on existing research, lncRNA interacts with transcription factors to affect downstream gene expression. Through association analysis of lncRNA-transcription factor, we found that ACAP1, A4AGLT, and ABCF2 were related to multiple lncRNAs, such as ENST00000513396, while ACAP1, A4AGLT, and ABCF2 were all related to the occurrence and development of tumors, which suggests that the interaction between lncRNA and transcription factors may play an important role in the process of colorectal cancer metastasis (Fig. 4).

Cell biological behaviors in vitro after Overexpression of ENST00000513396

We selected 10 lncRNAs with significantly different expressions and verified them in clinical tissue samples. It was found that the expression difference of lncRNA-ENST00000513396 was the most obvious in tumor tissues, so we chosen ENST00000513396 for the next experiment. We conducted in-depth research through constructing ENST00000513396 to knockout expressed and overexpressed cell lines (Fig. 5).The results are as follows.

Firstly, we observed the effect of overexpression of ENST00000513396 on cell proliferation and other biological functions by MTT method. We found that overexpression of ENST00000513396 can significantly enhance the proliferation ability of colon cancer cell line HCT116, suggesting that overexpression of ENST00000513396 can enhance the proliferation ability of tumor cells *in vitro* (Fig. 6).

Secondly, we analyzed the effect of ENST00000513396 on the migration ability of colon cancer cells using a cell scratch test. The results showed that after overexpression of ENST00000513396, the migration ability of tumor cells was significantly improved (Fig. 7).

And finally, we used Transwell cell technology to analyze the effect of ENST00000513396 on the invasion ability of colon cancer cells. The results showed that after overexpression of ENST00000513396, the invasion ability of colon cancer cells was significantly improved (Fig. 8).

So in summary, the overexpression of ENST00000513396 can obviously promote the proliferation, migration and invasion of colon cancer cells *in vitro*.

Discussion

Colorectal cancer (CRC) is one of the common malignant tumors worldwide. Despite the tremendous achievements made in surgery, chemotherapy, and biotherapy in recent years, about 700, 000 patients still die from colorectal cancer each year, due to the lack of early diagnosis and tumor metastasis and recurrence.[12] Therefore, in-depth research on the mechanism of the occurrence and development of colorectal cancer is expected to make a breakthrough in the early diagnosis, metastasis and targeted therapy of tumors.

Non-coding RNA plays a broad and powerful regulatory role in cells. In recent years, with the progress of research, it has been confirmed that it plays an important role in the process of tumorigenesis and development, and becomes a hot spot in recent years.[13, 14] RNA that exceeds 200nt in length is called long noncoding RNA (lncRNA). LncRNA has obvious spatial and temporal specificity and tissue specificity. Previous reports have shown that lncRNA plays an important role in many physiological processes such as cell proliferation and differentiation.[15–17] In recent years, with the deepening of the research on the mechanism of tumorigenesis and development, the abnormal expression of lncRNA in various tumors has been discovered, indicating that it plays an important role in the process of tumorigenesis and development, and may provide new methods for the diagnosis and treatment of colorectal cancer.[18–20]

In this study, through high-throughput chip technology, combined with bioinformatics analysis methods, to find differentially expressed lncRNA in colorectal cancer and adjacent tissues, we found 460 differentially expressed lncRNA (change factor ≥ 2, $p \leq 0.05$). Through functional enrichment analysis of the co-expressed mRNA, it was found that the co-expressed mRNA function of these differentially expressed lncRNAs was mostly related to tumor-related signaling pathways, indicating that these differentially expressed lncRNAs may play an important role in the occurrence and development of colorectal cancer.

We selected lncRNA-ENST00000513396 with obvious differential expression as the research object. Through functional enrichment of its co-expressed mRNA, it was found to be related to multiple tumors signaling pathways. At the same time, we verified in clinical samples and found that it is the highest expressed in tumor tissues, so we determined ENST00000513396 as the research object to further study its function and mechanism of action. We constructed the overexpression and knockout plasmids of ENST00000513396. *in vitro* experiments, it was found that after overexpression of ENST00000513396,

the proliferation of cells was accelerated, and the proliferation rate of colorectal cancer cells that knocked down the expression of ENST00000513396 decreased, suggesting that overexpression of ENST00000513396 can promote tumor proliferation *in vitro*. Cell scratch experiments and Transwell cell experiments showed that overexpression of ENST00000513396 can improve the ability of tumor cells to migrate and invade, which is closely related to the occurrence and development of tumors. In summary, ENST00000513396 can promote the proliferation of tumor cells and increase the ability of tumor cells to invade and migrate. Therefore, ENST00000513396 may play an important role in the development of colorectal cancer.

Although this subject studied the function of ENST00000513396 *in vitro*, it has not been verified in vivo and the relationship between it and the prognosis of clinical patients has not been studied, which needs further improvement. It is essential to continue to explore the key molecules and signaling pathways that affect the ability of colorectal cancer to proliferate, migrate and invade, and work hard to study new diagnostic and therapeutic methods. Therefore, this will be the direction of our further discussion.

This subject systematically analyzes the abnormal expression of lncRNA in colorectal cancer tissues and adjacent tissues, and takes ENST00000513396 as the research object to elaborate its impact on cell function, providing a theory for its use as a diagnostic marker and therapeutic target for colorectal cancer basis.

Conclusion

In this study, lncRNA chips were used to analyze colorectal cancer tissues and adjacent tissues, and 460 differentially expressed lncRNAs were screened. Combined with bioinformatics analysis, these differentially expressed lncRNAs were found to be related to multiple tumor signaling pathways.

LncRNA-ENST00000513396 is highly expressed in colorectal cancer tissues. The results of *in vitro* experiments show that ENST00000513396 high expression promotes tumor cell proliferation, and can increase the ability of tumor cell migration and invasion.

These fundings contribute to a better understanding of the potential mechanism of the progression of CRC, which could be a novel diagnostic and therapeutic target for CRC.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the The Second Affiliated Hospital of Nanjing Medical University ([2018]KY No.25). Informed consent was obtained from all participants included in the study and experimental procedures were performed according to the guidelines of the non-profit, statecontrolled HTCR (Human Tissue and Cell Research) foundation.

Consent for publication

Not applicable.

Availability of data and materials

Datasets are available on request from the corresponding author on reasonable request. The raw data and all related documents supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

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Author Contributions

All authors contributed toward data analysis, drafting, and revising the paper and agree to be accountable for all aspects of the work.

Competing interests

The authors report no conflicts of interest in this work.

Author details

Jinxin zhu. E-mail: 737419628@qq.com

Hui Wang. E-mail:359718219@qq.com

Yangbin Du. E-mail:1760661092@qq.com

Zhenyu He. E-mail:hezhenyu1968@163.com

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Figures

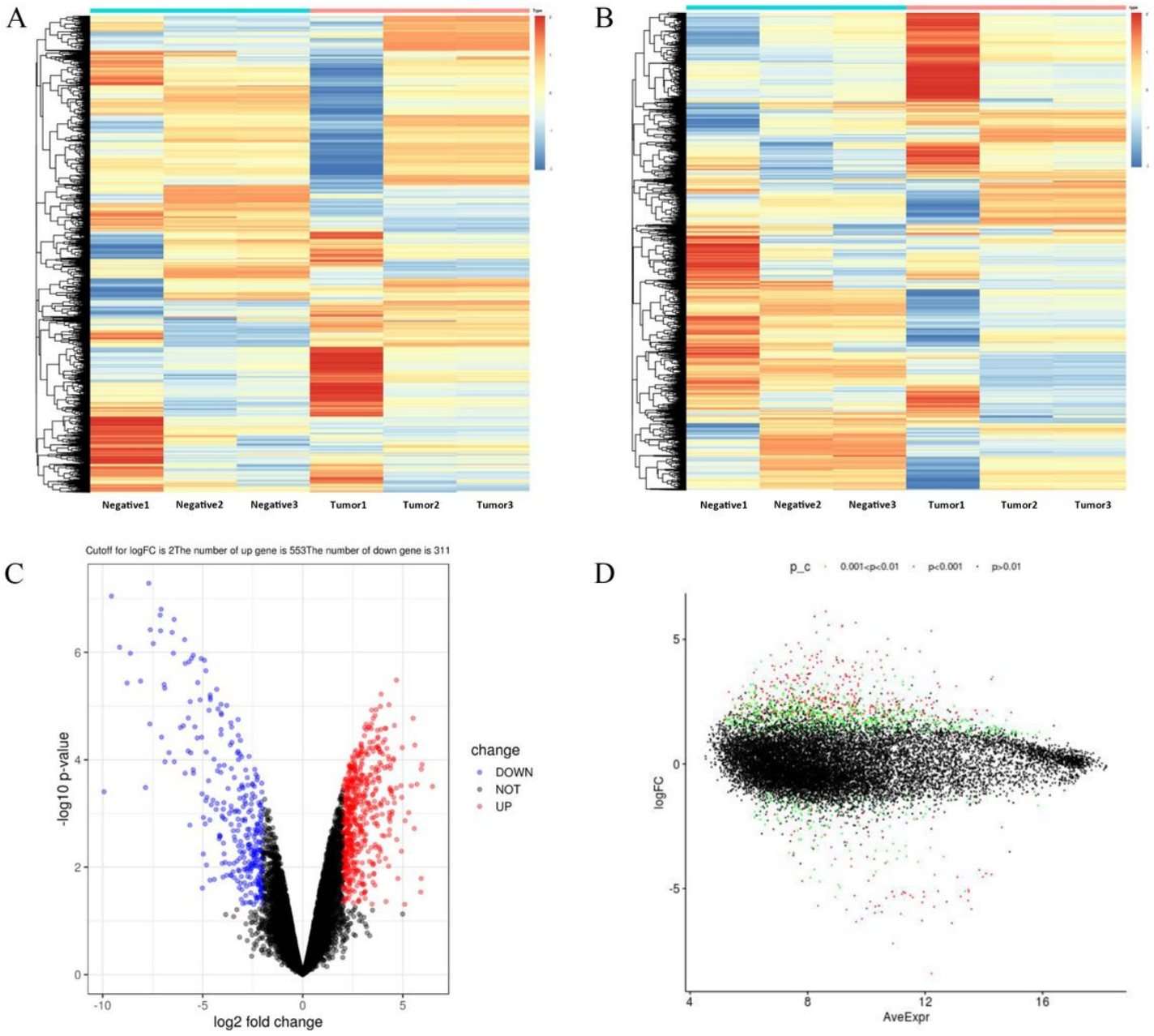

Figure 1

Differentially expressed LncRNA in colorectal cancer and adjacent tissues. A: Hierarchical cluster analysis of differentially expressed mRNA. B: Differential expression lncRNA hierarchical cluster analysis. C: Volcano graph of differentially expressed lncRNA. The abscissa is log2FC and the ordinate is log10

(corrected p-value). The red dots are up-regulated genes. The blue dots are down-regulated genes. The gray dots are not significant genes. D: MA map of differentially expressed lncRNA. The X-axis is the average value of all samples for comparison after normalization. The Y-axis is log2 (FoldChange). Significant (according to differential screening conditions) differential genes are marked in red.

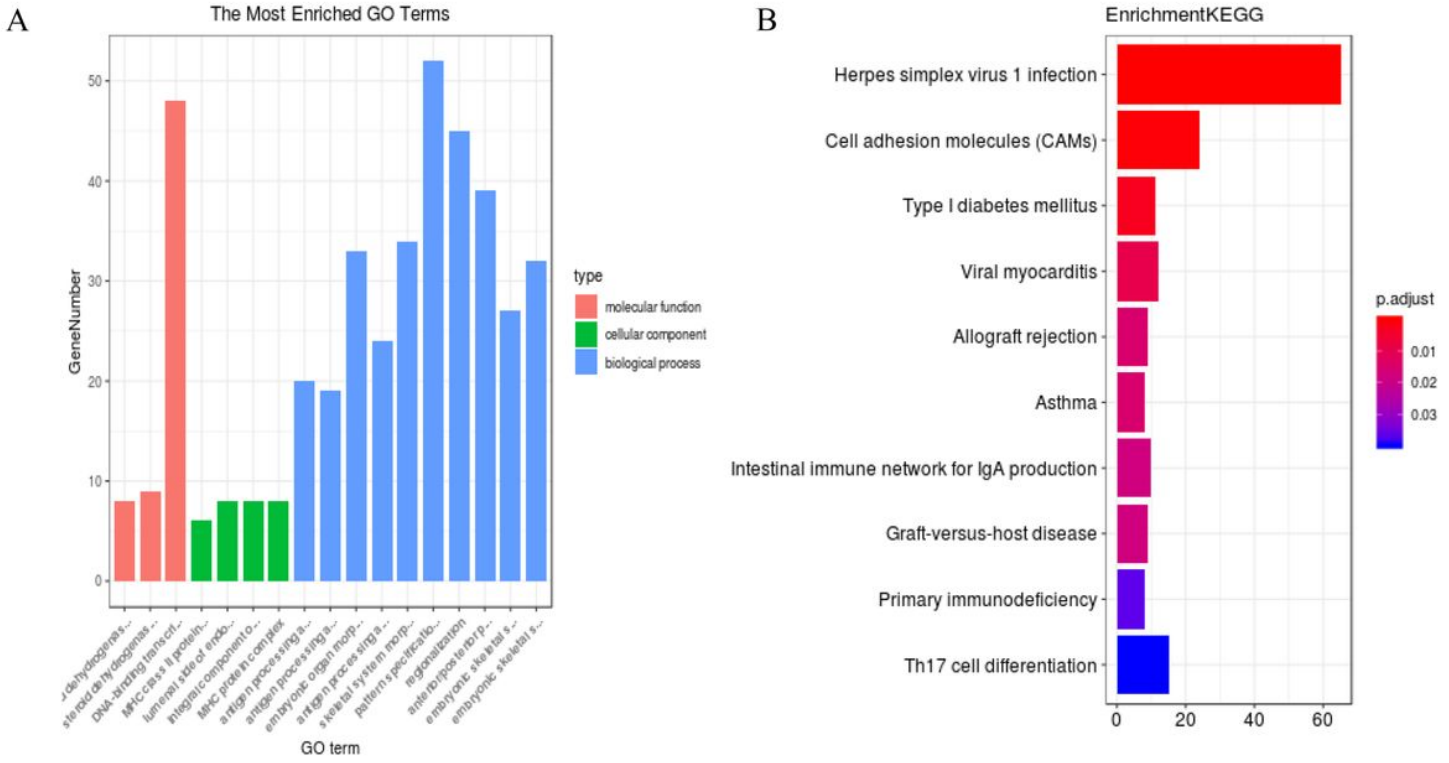

Figure 2

Enrichment analysis of lncRNA cis-target genes with differential expression. A is GO enrichment analysis (abscissa is count: the number of genes enriched to the entry; ordinate Terms: the entry enriched by GO, including GO description, the red color means the smaller the p-value, the more obvious the enrichment. Horizontal The axis is GeneRatio, which represents the ratio of the number of differential genes under the GO term to the total number of differential genes, and the ordinate is the enrichment entries. B is the KEGG enrichment analysis (the abscissa is the count, and the ordinate is the number of enrichment).

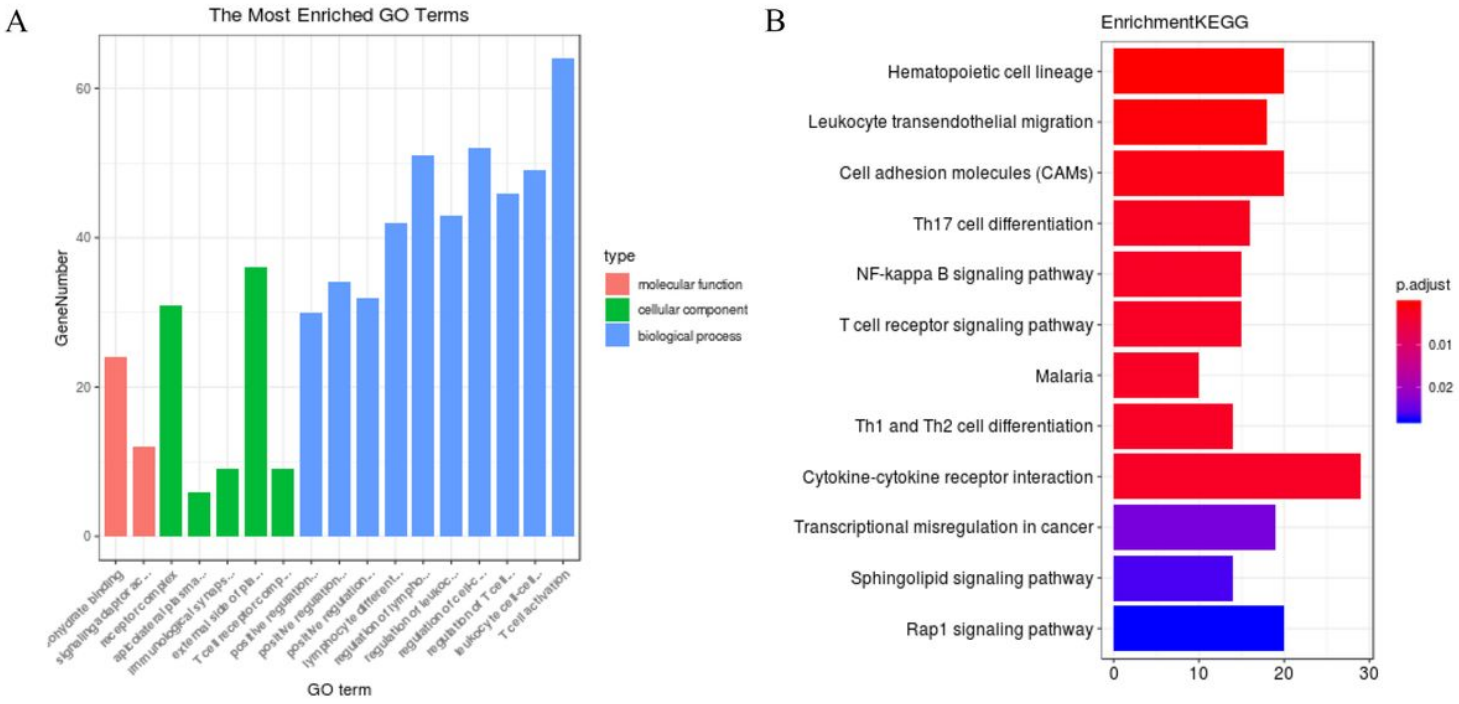

Figure 3

Enrichment analysis of lncRNA trans-target genes with differential expression. A is GO enrichment analysis (abscissa is count: the number of genes enriched to the entry; ordinate Terms: entries enriched by GO, including GO description, the red color means the smaller the p-value, the more obvious the enrichment). B is KEGG enrichment analysis (abscissa is count, ordinate is enrichment number).

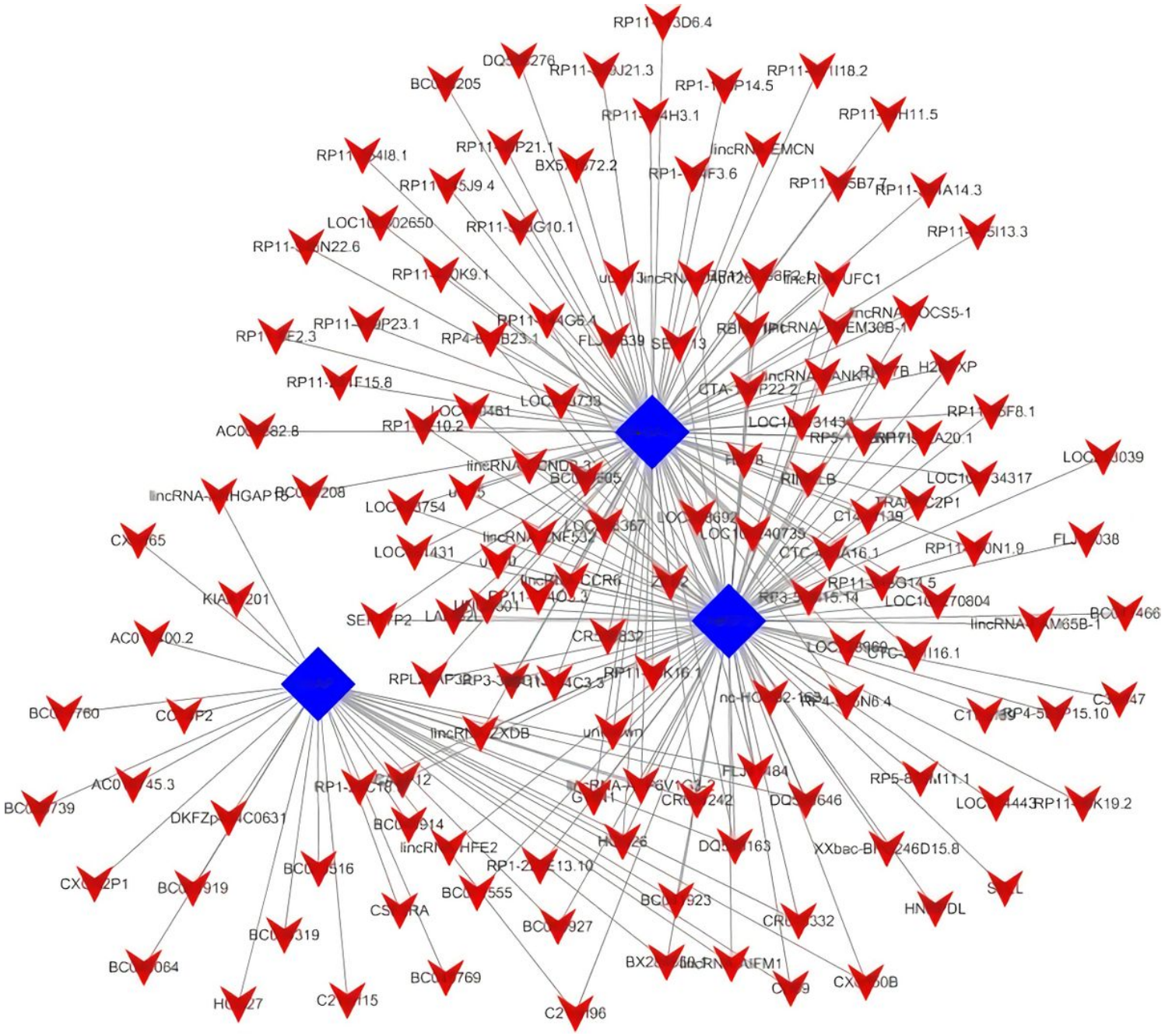

Figure 4

lncRNA-transcription factor regulatory network. Note:Red represents lncRNA and blue represents transcription factor.

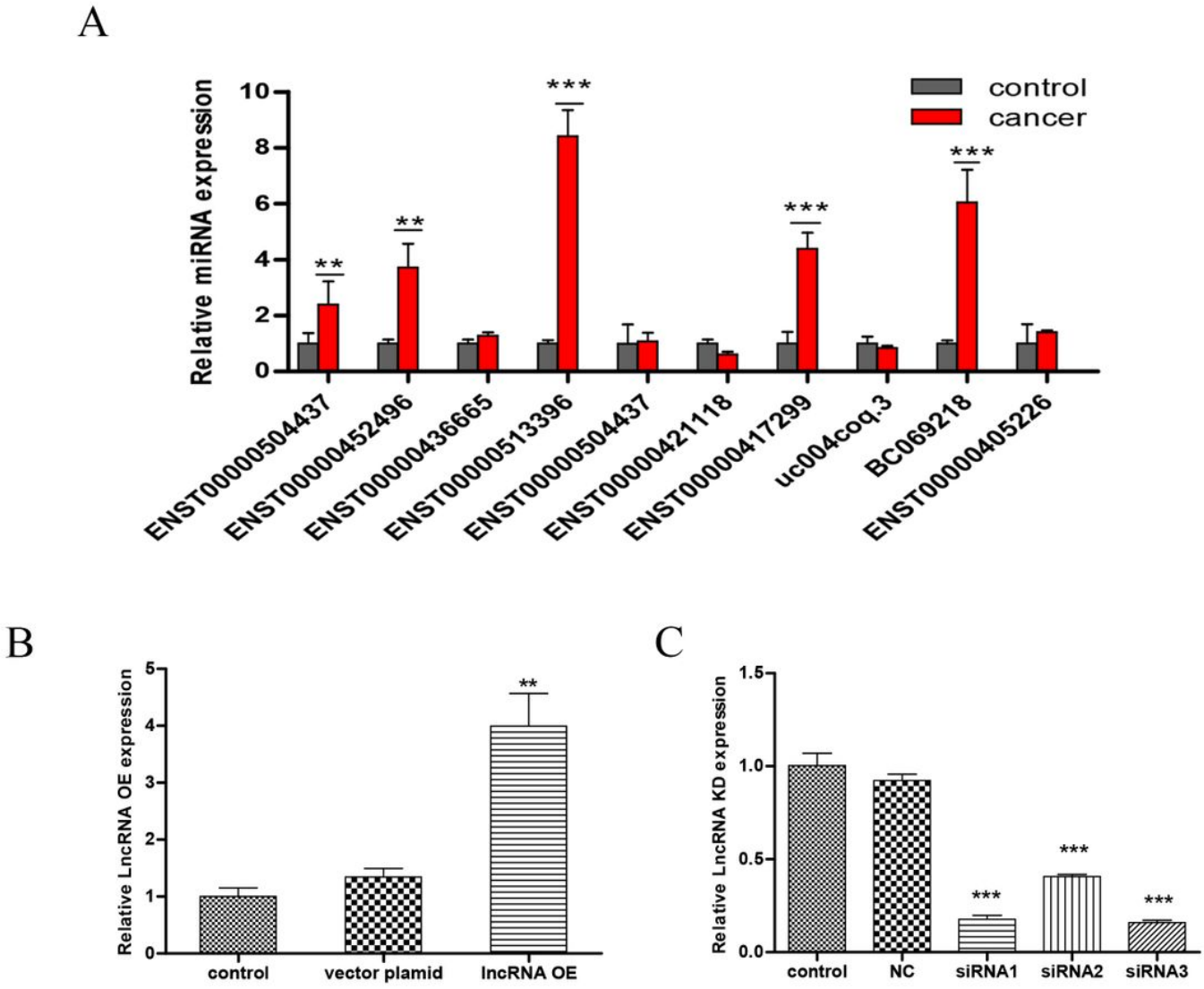

Figure 5

ENST00000513396 was verified in clinical specimens and cell lines and constructed overexpression plasmids. A: The expression of lncRNA in clinical samples, of which ENST00000513396 has the most obvious difference in clinical specimens. B: Expression of ENST00000513396 after SW480 cell line was transfected with empty and overexpression plasmids. C: Expression of ENST00000513396 after SW480 cell line was transfected with empty and knockout expression plasmids, respectively.

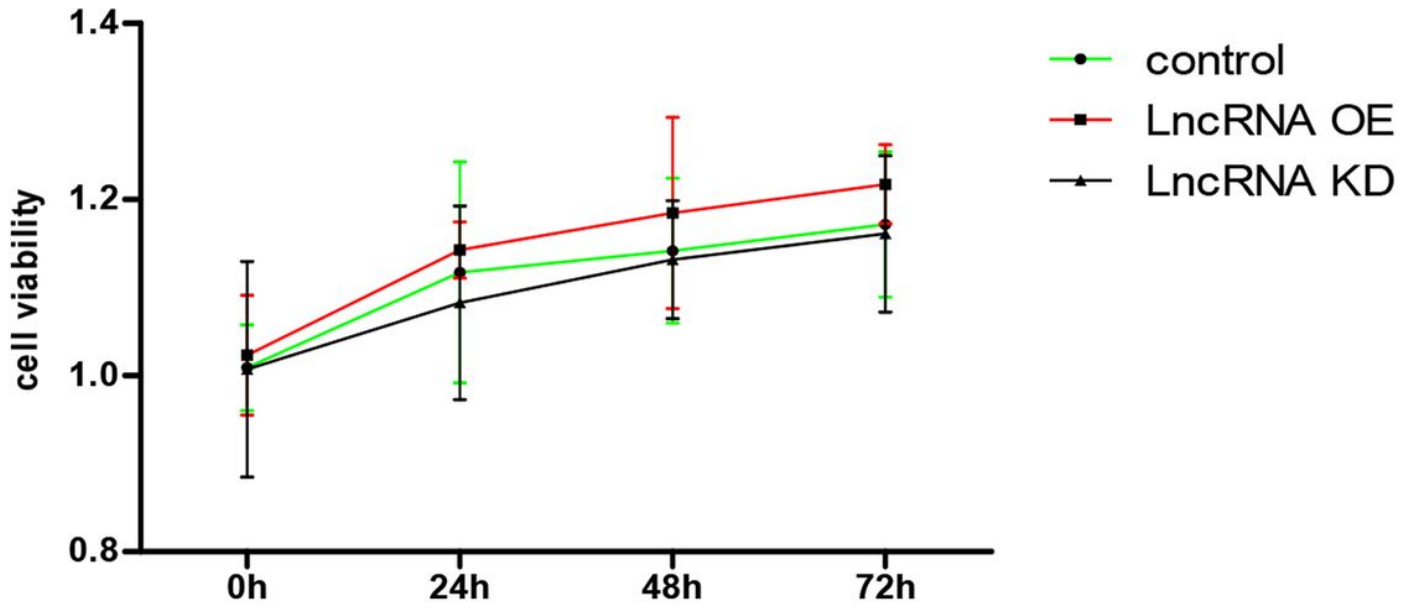

Figure 6

The effect of overexpression of ENST00000430471 on tumor proliferation in vitro. MTT test results of the proliferation ability of tumor cell line SW480 after overexpression of ENST00000513396; the proliferation ability of tumor cell line SW480 was weakened after ENST00000513396 was knocked out. *P<0.05, calculated by student t test.

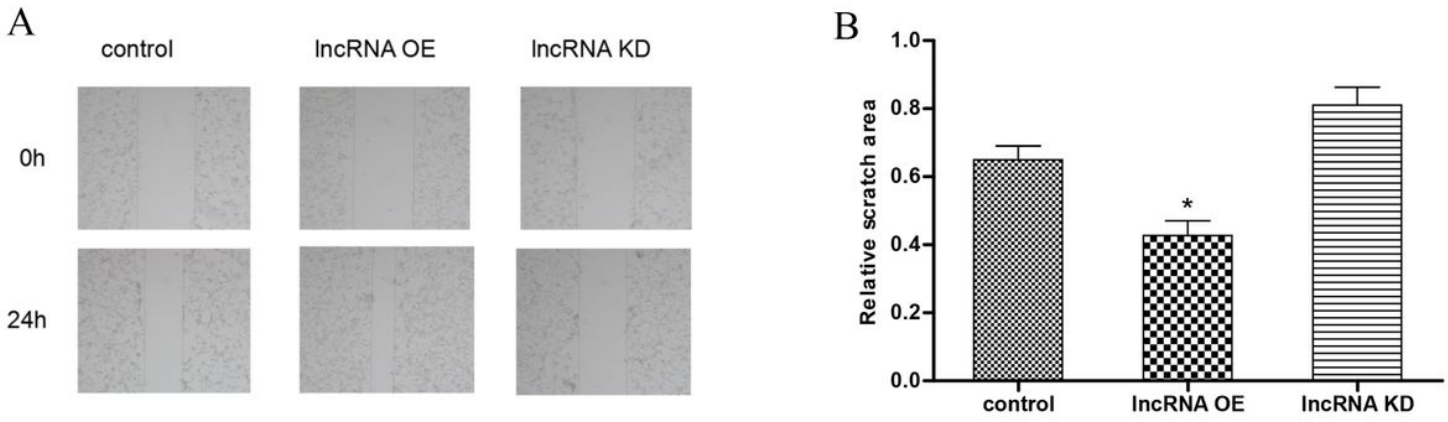

Figure 7

Effect of overexpression of ENST00000513396 on colon cancer cell migration ability. A: Test result of scratch test of tumor cell line SW480 migration ability after overexpression of ENST00000513396; B: area of scratch area. *P<0.05, calculated by student t test.

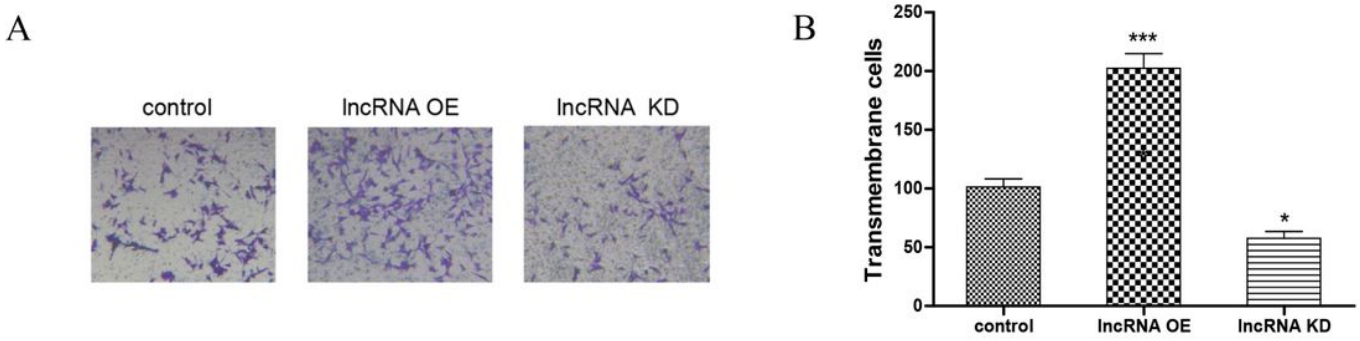

Figure 8

The effect of overexpression of ENST00000513396 on the invasion and migration ability of colon cancer cells. A: Transwell chamber test results of tumor cell line SW480 invasion ability after overexpression of ENST00000513396; B: Data statistics of invading tumor cells. *P<0.05, ***P<0.001, calculated by student t test.

Supplementary Files

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- TableS1.docx