Clin. Lab. 2019;65:XXX-XXX

ORIGINAL ARTICLE

Expression and Clinical Significance of Long-chain Noncoding RNA LINC00261 in Colon Cancer

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SUMMARY

Background: **To investigate the expression of long non-coding RNA (lncRNA) LINC00261 in colon cancer and its clinical significance.**
Methods: **The Significance Analysis of Microarrays (SAM) software was used to analyze the data of gene microarray GSE33113 which was downloaded from NCBI GEO Data Sets, and differential expression of lncRNA in colon cancer was screened. The expression of LINC00261 in 138 cases of colon cancer and its adjacent normal tissues were detected by in situ hybridization (ISH). Then, the correlation between LINC 00261 expression and clinicopathological characteristics of colon cancer was analyzed, and Cox's proportional hazards regression model was used to assess the value of LINC00261 in predicting the prognosis of colon cancer patients after operation.**
Results: **Analysis of gene microarray GSE33113 data by SAM found 65 differentially expressed lncRNA in colon cancer, including 6 up-regulated lncRNA (fold change > 2) and 59 down-regulated lncRNA (fold change < 0.05). The results of in situ hybridization showed that the negative rates of LINC00261 in colon carcinoma tissues and their adjacent non-cancerous tissues were 63.77% (88/138) and 8.70% (12/115), respectively, with a significant difference between them ($p < 0.01$). LINC00261 was significantly correlated with the clinical staging of patients with colon cancer ($p < 0.05$). Lowered expression of LINC00261 was identified as an independent risk factor affecting postoperative recurrence-free survival time of the colon cancer patients ($p < 0.001$).**
Conclusions: **There was decreased expression of LINC00261 in colon cancer tissues, which was related to clinical stages, lymph node metastasis, and recurrence-free survival time of colon cancer, indicating that LINC00261 might be used as a new molecular biomarker for evaluating the metastasis and recurrence-free survival of colon cancer.**
(Clin. Lab. 2019;65:xx-xx. DOI: 10.7754/Clin.Lab.2019.190402)

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Manuscript accepted June 9, 2019

KEY WORDS

colon carcinoma, long non-coding RNA, LINC00261, in situ hybridization

INTRODUCTION

Colon cancer is a common malignant tumor in the world and also one of the most common tumors in China, with its morbidity and mortality increasing year by year [1]. The occurrence and development of colon cancer is a multi-step, multi-factor process in which long noncoding RNA (lncRNA) plays an important role in addition to genetic changes. IncRNA is a class of RNA

with transcripts of a length greater than 200 nt, which does not encode any protein [2]. Recent studies have found that IncRNA plays an important role in the biological processes such as cell cycle regulation, epigenetic regulation, transcriptional regulation, and post-transcriptional regulation, particularly in the occurrence and development of malignant tumors [3,4]. As more and more tumor-associated IncRNAs are discovered, new technologies are urgently needed to study the biological functions of these lncRNAs at different stages of development and different degrees of differentiation in large-scale and high-throughput formats, thereby screening new tumor molecular markers.

LINC00261 is an intergenic lncRNA located on 20p11.21, and present experimental results show that LINC00261 plays a role as a cancer suppressor gene in a variety of tumors. Yu et al. [5] found that expression level of LINC00261 in gastric cancer tissues was significantly lower than that in para-carcinoma tissues and could inhibit the expression level of Slug protein, thus inhibiting the progress of epithelial cell - mesenchymal transition (EMT) of gastric cancer. Overexpression LINC00261 can promote colon cancer cell apoptosis and increase sensitivity to the chemotherapy drug cisplatin [6]. In human choriocarcinoma [7] and human endometrial cell lines [8], overexpression of LINC00261 can inhibit cell invasion and migration capabilities. Using the bioinformatics approach, this study analyzed the gene chip GSE33113 data on a common platform and found that the expression level of LNC00261 in colon cancer tissue was significantly increased. Using the in situ hybridization (ISH) to detect the in situ expression of the lncRNA in 138 cases of colon cancer and its para-carcinoma tissue, the relationship between the positive expression rate and clinical pathological parameters was analyzed and its potential value evaluated as a new molecular marker of tumor invasion and metastasis.

MATERIALS AND METHODS

Bioinformatics analysis of gene chip data

The human colon cancer gene chip data GSE33113 was downloaded from the United States National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO), containing 96 colon cancers and 6 para-carcinoma tissues (pathologically unaffected cells). The chip platform is Affymetrix Human Genome U133 Hus 2.0 Array (GPL570). The Significance Analysis of Microarrays (SAM) software was downloaded from the Stanford University website and installed in Microsoft Excel. Organize the gene chip data in the required format in Microsoft Excel, click the SAM plugin in the Excel menu bar, select the two-category unpaired comparison, perform the *t*-test, and output the result graph. Then, the result map is parameterized, the error discovery rate (FDR) is controlled at about 1%, the expression value is changed by > 2, and the gene probe ID number with significant difference in expression is output. The gene probe ID number is re-annotated on online websites such as DAVID, Ensembl, and GeneCards, where the gene name represented by the probe ID number is found. Gene type is long noncoding lncRNA, antisense transcript (antisense), long intergenic noncoding RNA (hncRNA), sense overlapping and processed transcripts can be considered as lncRNA [9] and drawn by r software into a heat map.

Specimen collection

Colon carcinoma tissue and its para-carcinoma tissue (distance from the boundary portion cancerous > 2.0 cm, no cancer cells after pathological diagnosis) which were removed surgically and diagnosed through postoperative histopathological examination, included 138 cases, including 89 males and 49 females, aged 35 - 75 years, mean (56.84 ± 12.54) years, from April 2014 to June 2016, in the First Affiliated Hospital of Nanjing University of Traditional Chinese Medicine, were collected. According to the World Health Organization (WHO) histological criteria, colon cancer in 138 cases was all adenocarcinoma, including 46 poorly differentiated cases, 65 moderately differentiated cases, and 27 highly differentiated cases. The American Joint Committee on Cancer (AJCC) TNM classification of colon cancer was used for clinical staging, including 21 cases of clinical Phase I, 64 cases of clinical Phase II, and 53 cases of clinical Phases III - IV. All patients did not receive anti-tumor treatments such as radiotherapy, chemotherapy, and biological therapy before surgery, and no other colon diseases such as colitis and colon polyps were found in diagnosis.

Disease-free survival was defined as the time from the start of surgery to the time of diagnosed recurrence or the last follow-up time. The median follow-up time was 30 months. The follow-up included: postoperative conditions (progression, relapse, death), imaging examination changes, serum alpha-fetoprotein levels, and liver function. Our study was reviewed and approved by the Institutional Ethics Committee of Nanjing University of Traditional Chinese Medicine. Our study obtained written informed consents from each participant at the time of enrollment for the present study.

Specimens were fixed with 4% paraformaldehyde [prepared with 0.1 mol/L PBS, containing 0.1% diethylpyrocarbonate (DEPC), pH 7.4], and subjected to conventional dehydration, transparency, and paraffin embedding, and cut into 4 μm serial sections.

Main instruments and reagents

The in situ hybridization kit and the DAB chromogenic kit were purchased from Wuhan Boster Company, Harris hematoxylin was purchased from Beijing ZSGB-BIO, DEPC was purchased from Sigma, USA, and the microscope Olympus BX-41 was purchased from Olympus Corporation of Japan. The oligonucleotide probe was designed and synthesized by Wuhan Boster Company.

LINC00261 mRNA in situ hybridization experiment

The kit instructions were followed and a slight improvement was made: Paraffin sections were routinely dewaxed, hydrated, and washed 3 times with PBS/DEPC instead of DEPC water for 5 minutes, and treated with 3% H_2O_2 for 10 minutes at room temperature to inactivate endogenous peroxidase. This was followed with 3 rinses with DEPC water. Then, 30 μL of pepsin freshly diluted with 3% citric acid was added dropwise, digested at 37°C for 20 minutes, and rinsed 3 times with PBS/DEPC for 5 minutes. It was fixed with DEPC treated 1% paraformaldehyde (0.1 mol/L PBS, pH = 7.4) for 10 minutes at room temperature and rinsed 3 times with DEPC water. Then 20 μL of pre-hybrid solution was added dropwise to each section, and pre-hybridization was carried out in a wet box at 37°C. After 2 to 4 hours, 20 μL of probe hybridization solution was added and kept at 37°C overnight (16 to 18 hours). After hybridization, elution was done with a gradient of 2 x SSC, 0.5 x SSC, 0.2 x SSC preheated to 37°C, and the excess probes were washed away. Then, 50 μL of confining liquid was added dropwise to the section, which was blocked at 37°C for 30 minutes, then 50 μL of biotinylated murine anti-human-digoxin antibody (ready to use) was added dropwise, and incubated at 37°C for 120 minutes (60 minutes more than the specification). Rinsing in PBS was done 4 times for 5 minutes, 50 μl streptavidin-biotin complex was added dropwise. Incubation was conducted at 37°C for 30 minutes followed by rinsing 3 times in PBC 5 minutes, Biotinylated peroxidase 50 μL was added dropwise and then incubated at 37°C for 30 minutes, rinsing 3 times in PBC for 5 minutes. DAB coloration was performed for 5 minutes, and counterstaining was done with hematoxylin. After dehydration and transparency, the section was sealed with neutral gum. The hybridization solution was replaced with a probe-free pre-hybrid solution as a negative control. Result judgment: Under the Olympus BX-41 optical microscope, the coloration of LINC00261 in the tissue was observed. As for the judgment standard, the reference [10] is referred to and slightly modified: (1) According to the positive staining intensity, the cells have no staining, 0 point; the cells are light brown, 1 point; cells were stained brown without background coloration, or dark brown with the light brown background, medium positive, 2 points; and the cells were dark brown without background coloration, strong positive, 3 points. (2) Scoring is done according to the number of positive cells, 0 points if there are no positive cells; 1 point when the number of positive cells ≤ 25%; 2 points when the number of positive cells < 25%; and strong positive, 3 points when the number of positive cells is more than 50%. The product of the two is the final score, and each of the sections was randomly observed in 5 high power fields and averaged. Zero to 1 point represents negative, and 1 to 9 points represent positive.

Statistical analysis

SPSS 18.0 software was used. The positive rate of LINC00261 in colon cancer tissues and corresponding para-carcinoma tissues was compared by cross tabulation χ^2 test or Fisher's exact test, and its relationship with clinicopathological parameters was analyzed. The difference between tumor-free survival time of patients with linc00261 high expression and low expression was compared using Kaplan-Meier method, and log-rank method was used for hypothesis testing; COX proportional hazard model was used to evaluate the predictive effect of LINC00261 expression on postoperative tumor-free survival time of patients. With $\alpha = 0.05$ as the test level, a two-sided test was used.

RESULT

Bioinformatics analysis results

The gene chip GSE33113 contains 90 colon cancer tissues and 6 normal colon tissues. Through SAM analysis and probe re-annotation, it was found that there are 65 clearly annotated differentially expressed lncRNAs in colon cancer tissues (fold change > 2 or < 0.5), 6 upregulated, 59 downregulated; for lncRNA with more obvious difference in the expression (fold change > or 3 < 0.33), see Figure 1. In downregulated lncRNA, differential expression of LINC00261 is most significant, so LINC00261 is used for this study for further experimental verification.

In situ hybridization detection results

The detection results of LINC00261 in 138 colon cancers and their corresponding para-carcinoma tissues showed that LINC00261 was negatively expressed in colon cancer tissues and positive in para-carcinoma tissues (Figure 2). The negative expression rates of LINC-00152 in colon cancer tissues and corresponding para-carcinoma tissues were 63.77% (88/138) and 8.70% (12/115), respectively, and the difference was statistically significant ($p < 0.01$).

Relationship between expression of LINC00261 and clinicopathological parameters of colon cancer

According to the results, LINC00261 was significantly correlated with the clinical staging of patients with colon cancer ($p < 0.05$), negative rate of Phases I and II of LINC00261 was significantly lower than that of Phases III - IV ($p < 0.05$, for all), but there was no significant difference when compared with Phases I and II ($p > 0.05$); the negative rate of LINC00261 was higher in colon cancer tissues with lymphatic metastasis, but was not related to patient age, gender, tumor location, tumor size, and tumor cell differentiation ($p > 0.05$, for all). The relevant data is shown in Table 1.

Table 1. The relationship between LINC00261 expression and clinicopathological parameters in colorectal cancer (n, %).

	N	LINC00261		p
		Negative	Positive	
Year old				0.784
< 55	43	26 (60.47)	17 (39.53)	
≥ 55	95	62 (65.26)	33 (34.74)	
Gender				0.998
Male	89	56 (62.92)	33 (37.08)	
Female	49	31 (63.27)	18 (36.73)	
Location				0.284
Left half colon	78	53 (67.95)	25 (32.05)	
Right half colon	60	35 (58.33)	25 (41.67)	
Tumor diameter (cm)				0.259
< 5	56	32 (57.14)	24 (42.86)	
≥ 5	82	56 (68.29)	26 (31.71)	
Differentiated degree				0.341
High	27	20 (74.07)	7 (25.93)	
Middle	65	37 (56.92)	28 (43.08)	
Low	46	30 (65.22)	16 (34.78)	
TNM				0.000 a
I phase	21	6 (28.57)	15 (71.43)	0.189 b
II phase	64	29 (45.31)	35 (54.69)	0.001 c
III - IV phase	53	41 (77.36)	12 (22.64)	0.000 d
Lymphatic metastasis				
Yes	64	52 (81.25)	12 (18.75)	0.000
No	74	36 (48.65)	38 (51.35)	

a - General comparison of clinical stages, b - Comparison between phase I and phase II, c - Comparison between phase II and phase III - IV, d - Comparison between phase I and phase III - IV.

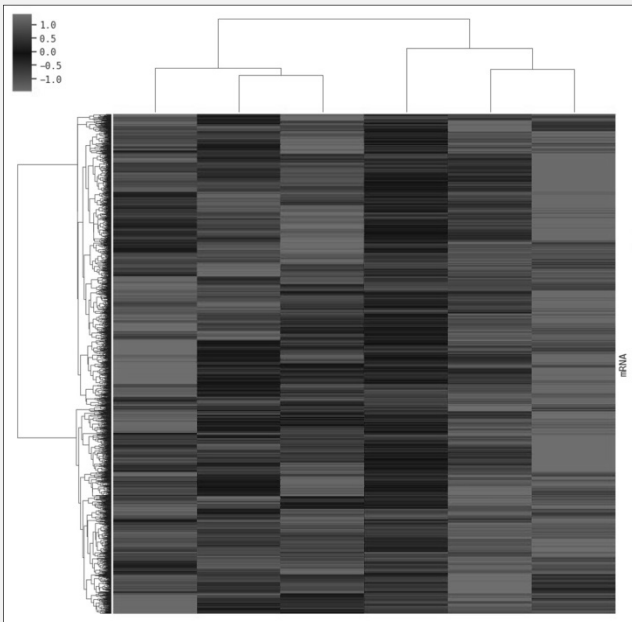
Relationship between expression of LINC00261 in hepatocellular carcinoma and postoperative tumor-free survival

Kaplan-Meier survival analysis showed that the tumor-free survival time of the low-expression group of LINC-00261 was significantly lower than that of the high-expression group, and the median tumor-free survival time was 7.66 and 70.37 months, respectively ($p < 0.001$, Figure 3).

DISCUSSION

With the rapid development of gene chips and high-throughput sequencing technologies, the successive discovery of lncRNAs has brought human genomics research into a new situation. However, how to find new tumor-associated lncRNAs is still the bottleneck of research. The NCBI database is rich in resources, many gene chip data are available for download, and the probes on the gene chips can specifically match lncRNAs. Thus, many researchers use bioinformatics methods to analyze these data to find disease-related lncRNAs [11,12]. In this study, SAM software and DAVID, Ensembl, GeneCards, and other online websites were used to analyze gene chip data to obtain differential expression profiles of colon cancer lncRNAs to lay the foundation for the next experiment. In the screened differentially expressed lncRNAs, studies have confirmed that metastasis associated lung adenocarcinoma transcript (MALAT1) and colorectal neoplasia differentially expressed (CRNDE) are associated with colon cancer [13,14], while LINC00261 has the most significant differential expression. No relevant experimental studies were found, so this study selected this lncRNA for in situ hybridization experiments.

In situ hybridization, as an experiment for visually detecting target DNA or RNA in tissue cells, has high sensitivity and specificity and can be used as a semi-quantitative detection method. LINC00261 belongs to interge-



Probe ID	GenBank	Gene name	Fold	*P*	Chromosome position
225799_at	BF209337	LINC00152	4.5168	0.0029	2p11.2
2245677_x_at	BG534952	MALAT1	3.2587	0.0079	11q13.1
238021_s_at	AA954994	CRNDE	20.7984	0.0165	16q12.2
1564323_at	AK025042	LOC101928405	0.0987	0	3q26.1
1569040_s_at	AA632139	ANKRD36BP2	0.1759	0	2p11.2
1556364_at	AK057923	ADAMTS9-AS2	0.1088	0	3p14.1
228601_at	AW340112	HOXD-AS1	0.2698	0	2q31.2
236719_at	AI042187	ID2-AS1	0.2877	0	2p25.1
233249_at	AU155297	LOC100507073	0.1798	0	2p14
229385_s_at	AI743780	TINCR	0.3541	0	19p13.3
1562678_at	BC037883	HTR4-1T1	0.2874	0	5q32
219791_s_at	NM_024748	HAND2-AS1	0.0599	0	4q34.1
233298_at	AK026494	MDNL1-AS1	0.2157	0	3q25.1
227183_at	AI417267	MIR143HG	0.2744	0	5q32
1553420_at	NM_153031	SATB2-AS1	0.2154	0	2q33.1
230595_at	BF677651	PGM5-AS1	0.0484	0	9q21.11
219791_s_at	NM_024748	HAND2-AS1	0.0784	0	4q34.1
236444_x_at	HE785577	C5orf66-AS1	0.1972	0	5q31.2
1559884_at	BC038540	CDKN2B-AS1	0.1274	0	9q21.3
230432_at	AI733124	LOC100422737	0.3244	0	6q21
1554733_at	AK097110	LOC728175	0.3541	0	4q35.1
235217_at	AW148862	LINC00261	0.001	0.0001	20p11.21
236575_at	AI672383	ARHGDF26-AAS1	0.3097	0.0054	3q25.2

Figure 1. Abnormal lncRNA expression in colon cancer.

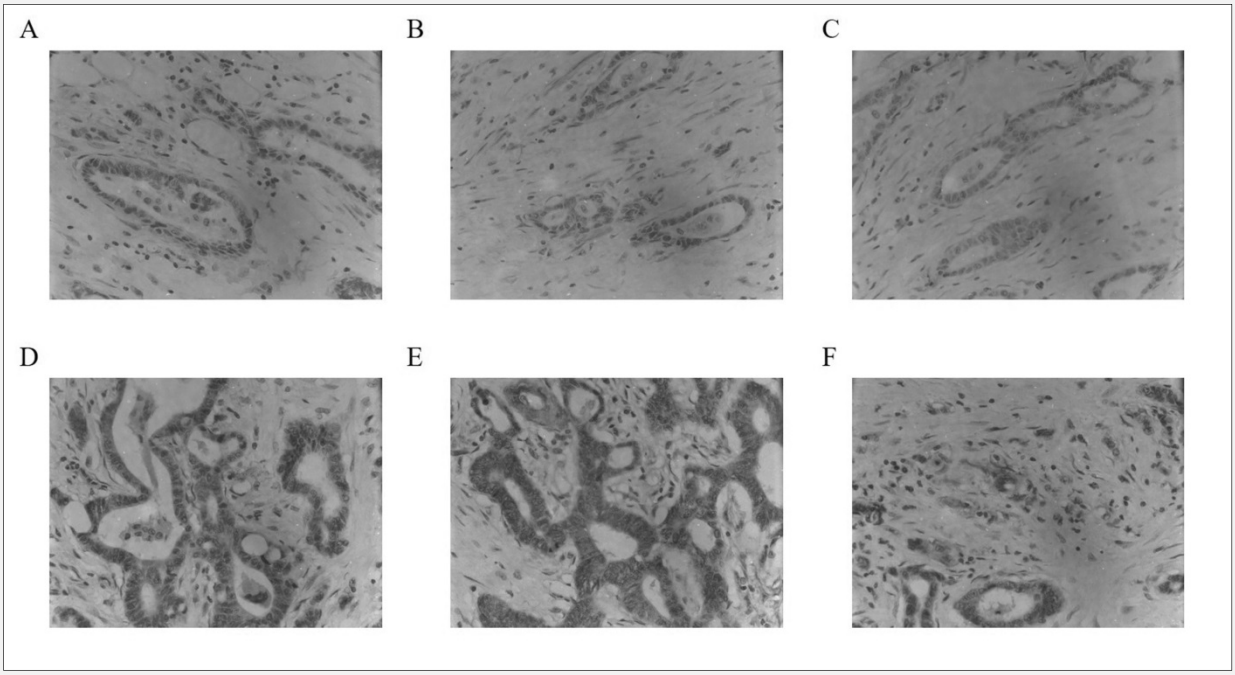


Figure 2. LINC00261 expression in colon cancer and adjacent normal tissues by ISH (400 x) A - D: Colon cancer tissues, LINC00261 expression as follows: negative, weak positive, moderately positive, and false positive; E, F: paracancerous tissue, LINC00261 expression as: positive and strong positive.

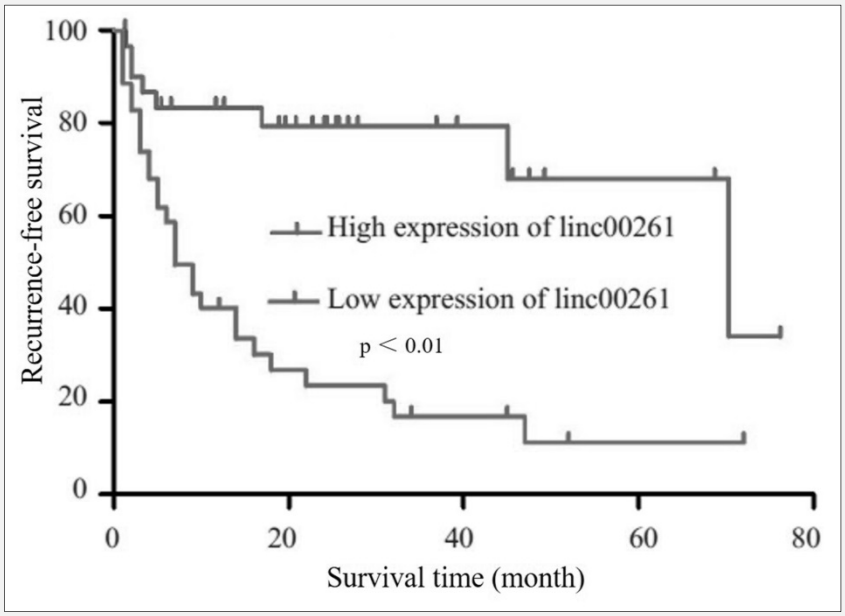


Figure 3. Kaplan-Meier recurrence-free survival curves of patients with high and low expressions of LINC00261.

nic lncRNA, and the gene is located at ch10p11.21. Oligonucleotide probes designed in this experiment are combined with mRNA specificity transcribed in tissue cells to detect the transcriptional level and position of target mRNA. The results showed that the expression level of LINC00261 in colon cancer tissues was significantly lower than that in para-carcinoma tissues, which was consistent with the results of gene chip. The low expression of LINC00261 may result in the inhibition of the synthesis of related proteins, leading to changes in the biological function of the cells, and ultimately causing cancer of the cells. Related basic studies showed that high expression of LINC00261 can effectively inhibit the biological activity of various tumors [15-18]. In this study, the correlation between the expression of LINC00261 and the clinicopathological parameters of patients with colon cancer was analyzed. It was found that the negative rate of LINC00261 was significantly correlated with the clinical staging and lymph node metastasis of patients, and these clinical parameters were the manifestations of malignant biological behavior of tumors. Lymph node metastasis is an important indicator of unfavorable prognosis in patients, suggesting that abnormal expression of LINC00261 may indicate unfavorable prognosis of patients. From this we conclude that LINC00261 can be used as a potential biomarker for predicting unfavorable prognosis in patients. However, this study only explored the expression of LINC00261 in colon cancer, and the specific mechanism of action needs to be explored in the next experiment.

CONCLUSION

In our present study, we found that lncRNA LINC00261 might be an anti-tumor factor in colon cancer. LncRNA LINC00261 expression was closely correlated with recurrence-free survival in colon cancer patients. Meanwhile, lncRNA LINC00261 was also correlated with colon cancer TNM stage and lymphatic metastasis. We will research the relative mechanisms in our future study.

Declaration of Interest:
The authors declare that they have no conflicts of interest.

References:

1. Siegel RL, Miller KD, Fedewa SA, et al. Colorectal cancer statistics, 2017. CA Cancer J Clin. 2017;67:177-93 (PMID: 28248415).

2. Maruyama R, Suzuki H. Long noncoding RNA involvement in cancer. BMB Rep. 2012;45:604-11 (PMID: 23186998).

3. Yang G, Lu X, Yuan L. LncRNA: a link between RNA and cancer. Biochim Biophys Acta. 2014;1839:1097-109 (PMID: 25159663).

4. Spizzo R, Almeida MI, Colombatti A, Calin GA. Long non-coding RNAs and cancer: a new frontier of translational research? Oncogene. 2012;31:4577-87 (PMID: 22266873).

5. Yu Y, Li L, Zheng Z, Chen S, Chen E, Hu Y. Long non-coding RNA linc00261 suppresses gastric cancer progression via promoting Slug degradation. J Cell Mol Med. 2017;21:955-67 (PMID: 27878953).

6. Wang ZK, Yang L, Wu LL, et al. Long non-coding RNA LINC-00261 sensitizes human colon cancer cells to cisplatin therapy. Braz J Med Biol Res. 2017;51:e6793 (PMID: 29267503).

7. Wang Y, Xue K, Guan Y, et al. Long noncoding RNA LINC-00261 suppresses cell proliferation and invasion and promotes cell apoptosis in human choriocarcinoma. Oncol Res. 2017;25: 733-42 (PMID: 27983929).

8. Sha L, Huang L, Luo X, et al. Long non-coding RNA LINC-00261 inhibits cell growth and migration in endometriosis. J Obstet Gynaecol Res. 2017;43:1563-9 (PMID: 28707780).

9. Derrien T, Johnson R, Bussotti G, et al. The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. Genome Res. 2012;22:1775-89 (PMID: 22955988).

10. Zhang W, Huang C, Gong Z, et al. Expression of LINC00312, a long intergenic non-coding RNA, is negatively correlated with tumor size but positively correlated with lymph node metastasis in nasopharyngeal carcinoma. J Mol Histol. 2013;44:545-54 (PMID: 23529758).

11. Kim K, Jutooru I, Chadalapaka G, et al. HOTAIR is a negative prognostic factor and exhibits pro-oncogenic activity in pancreatic cancer. Oncogene. 2013;32:1616-25 (PMID: 22614017).

12. Yang YR, Zang SZ, Zhong CL, Li YX, Zhao SS, Feng XJ. Increased expression of the lncRNA PVT1 promotes tumorigenesis in non-small cell lung cancer. Int J Clin Exp Pathol. 2014;7:6929-35 (PMID: 25400777).

13. Zheng HT, Shi DB, Wang YW, et al. High expression of lncRNA MALAT1 suggests a biomarker of poor prognosis in colorectal cancer. Int J Clin Exp Pathol. 2014;7:3174-81 (PMID: 25031737).

14. Graham LD, Pedersen SK, Brown GS, et al. Colorectal Neoplasia Differentially Expressed (CRNDE), a Novel Gene with Elevated Expression in Colorectal Adenomas and Adenocarcinomas. Genes Cancer. 2011;2:829-40 (PMID: 22393467).

15. Zhang B, Li C, Sun Z. Long non-coding RNA LINC00346, LINC00578, LINC00673, LINC00671, LINC00261, and SNHG9 are novel prognostic markers for pancreatic cancer. Am J Transl Res. 2018;10:2648-58 (PMID: 30210701).

16. Fan Y, Wang YF, Su HF, et al. Retraction Note to: Decreased expression of the long noncoding RNA LINC00261 indicate poor prognosis in gastric cancer and suppress gastric cancer metastasis by affecting the epithelial-mesenchymal transition. J Hematol Oncol. 2018;11:2 (PMID: 29301540).

17. Liu Y, Xiao N, Xu SF. Decreased expression of long non-coding RNA LINC00261 is a prognostic marker for patients with non-small cell lung cancer: a preliminary study. Eur Rev Med Pharmacol Sci. 2017;21:5691-5 (PMID: 29272004).

18. Wang ZK, Yang L, Wu LL, et al. Long non-coding RNA LINC-00261 sensitizes human colon cancer cells to cisplatin therapy. Braz J Med Biol Res. 2017;51:e6793 (PMID: 29267503).