Identification of long non-coding RNA 00312 and 00673 in human NSCLC tissues

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Abstract. Non-small cell lung cancer (NSCLC) is a fatal disease to human health. Despite the advanced progresses in cancer therapy during the past decades, NSCLC still remains the leading cause of cancer death worldwide. The long non-coding RNAs (lncRNAs) recently have been considered as key regulators of tumor malignant. Previous studies identified that long non-coding RNAs, linc00312 and linc00673 are markedly associated with lung cancer. However, current understanding of the two lncRNAs involving in NSCLC remains unclear. The aim of the present study was to profile their expression and clinicopathological significance in 76 patients' NSCLC tissues compared to non-tumor tissues using reverse transcription-quantitative polymerase chain reaction. Data have indicated that the linc00312 expression level was significantly decreased in NSCLC tissues (P<0.001), while a higher linc00673 level has been detected in the same tumor tissues (P<0.01). In addition, the low expression of linc00312 was associated with the Tumor-Node-Metastasis stage of NSCLC (P<0.05), whereas the high expression of linc00673 was related with the histological types of NSCLC (P<0.05). In conclusion, lncRNA 00312 and 00673 may serve as potential novel biomarkers for lung cancer early diagnosis, which may play a vital role in treatments of NSCLC.

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Introduction

Lung cancer is one of the most common malignant cancers in China and with the highest mortality and low five-year survival rate of 16.6% (1). Non-small cell lung cancer (NSCLC) which accounts for ~85% of lung cancer includes lung adenocarcinoma, lung squamous and large cell lung cancer (2). The most common types of NSCLC are lung adenocarcinoma (ADC) and lung squamous cell cancer (SCC) (3). The effective treatment for the patients with early-stage NSCLC remains to be complete surgical resection, indicating that the disease is medically operable. The effectiveness of surgery, however, is limited by high rates of distant recurrence because of the presence of metastatic diseases (4). Therefore, it is necessary to exploit the underlying mechanisms during the development of NSCLC and find new biological biomarkers with high sensitivity and specificity to improve the early diagnosis and treatment of NSCLC.

Long non-coding RNAs (lncRNAs) are evolutionarily conserved non-coding RNA that are >200 nucleotides with no protein-coding capacity (5). Accumulating research has demonstrated that lncRNAs participate in multiple cancers through diverse pathogenic processes, such as cell differentiation, proliferation and invasion (6,7). The potential regulation pattern of lncRNAs to modulate the expression of associated genes may be at transcriptional, post-transcriptional and epigenetic levels (8). In previous years, abundant lncRNAs have been reported to have critical roles in the development of NSCLC, including some upregulated lncRNAs like MALAT1 (9), H19 (10), PVT1 (11) and UCA1 (12), and downregulated ones such as GAS5 (13) and MEG3 (14), Spry4-IT1 (15) and BANCR (16). Due to the advances of high throughout technology, such as microarray and RNA-sequencing, numerous lncRNAs have been detected and profiled in lung cancer. It is well established that the expression patterns of lncRNAs are different in lung adenocarcinoma LAD and lung squamous cell carcinoma (LSCC).

The long intergenic non-coding RNA linc00312, also called NAG7, located at Ch3p25.3 and originally identified in nasopharyngeal carcinoma as a novel putative tumor suppressor gene. Previous studies have revealed that linc00312 significantly declined in NPC tissues compared

Table I. Correlation between clinicopathological factors and linc00312 and linc00673 expression levels in non-small cell lung cancer patients.

		Linc00312 expression			Linc00673 expression		
Variable	Cases (n)	Low	High	P-value	Low	High	P-value
Gender				0.111			0.289
Male	57	32	25		31	26	
Female	19	6	13		7	12	
Age (years)				0.819			0.819
≤60	36	17	19		18	18	
>60	40	21	19		21	19	
Smoking				0.217			0.217
Never	24	9	15		11	13	
Yes	52	29	23		27	25	
Tumor size (cm)				0.176			0.419
≤3	18	6	12		7	11	
>3	58	32	26		31	27	
Histological stage				0.023[a]			0.165
I	40	18	22		16	24	
II	12	3	9		8	4	
III	24	17	7		14	10	
T-stage				0.392			0.392
1	18	6	12		7	11	
2	40	21	19		21	19	
3	8	3	5		4	4	
4	10	6	4		6	4	
Regional lymph nodes				0.08			0.674
N0	52	24	28		25	27	
N1	9	3	6		4	5	
N2	15	11	4		9	6	
Histological differentiation				0.685			0.511
Poorly	15	6	9		6	9	
Moderately-poorly	8	6	2		6	2	
Moderately	47	23	24		24	23	
Moderately-well	3	2	1		1	2	
Well	3	1	2		1	2	
Left-right				0.167			0.357
Left	35	14	21		15	20	
Right	41	24	17		23	18	
ADC/SCC				1.000			0.037[a]
ADC	43	21	22		17	26	
SCC	33	17	16		21	12	

[a]P<0.05. ADC, adenocarcinoma; SCC, squamous cell carcinoma.

with non-cancerous nasopharyngeal epithelium tissues (17,18). However, further study has demonstrated that linc00312 was reduced in NSCLC tissues compared to non-tumorous tissues (19). A recent study (20) has been reported that long intergenic RNA linc00673, which located at Ch17q25.1, could also serve a crucial role in NSCLC progression and may be a potential tumor biomarker for patients NSCLC. In the present study, the expression levels of lncRNA linc00312 and linc00673 in NSCLC patients' tissues were investigated and expect to estimate the association between the expression levels and clinical features.

In the current study, the authors have analyzed the expression levels of linc00312 and linc00673 in 76 tumor tissues from NSCLC patients and paired non-cancerous lung tissues. In addition, the correlation of linc00312 and linc00673 with a variety of clinicopathological parameters were evaluated.

Materials and methods

Tissue samples and cell lines. A total of 76 NSCLC tissues (including 43 LAD and 33 LSCC) and their morphologically normal tissues (located >3 cm away from the tumor) were collected from Zhongnan Hospital of Wuhan University (Wuhan, China) between October 2014 and January 2016. The study was approved by the Medical Ethical Committee of the Zhongnan Hospital of Wuhan University. Patients who underwent surgical resection without any previous anticancer treatment and finally diagnosed as primary NSCLC according to the postoperative histopathological findings were used as experimental subjects. All tissue samples were snap-frozen in liquid nitrogen and stored at -80˚C until total RNA extraction. The use of the tissue samples for the experiment was under the informed consent and agreement of all enrolled patients. All patients were pathologically confirmed by clinical histological features such as histological type and Tumor-Node-Metastasis (TNM). All the patients' clinical and pathological features are presented in Table I.

Four NSCLC cell lines (A549, SPC-A-1, H1299 95-D) and human embryonic lung fibroblasts (HELF) were provided by the School of Public Health of Wuhan University (Wuhan, China). All cell lines were cultured in Dulbecco's modified Eagle's medium (HyClone; GE Healthcare, Chalfont, UK) added with 10% (v/v) fetal bovine serum, 100 IU/ml penicillin and streptomycin (100 U/ml), then incubated at 37˚C in a humidified chamber with 5% (v/v) CO_2. The cell RNA was extracted when all the survival rate of the cells was ~85%, as observed under a light microscope, and experiments were repeated three times.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the tissues and cell lines using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions (1 ml TRIzol/80 mg tissue). The RT-qPCR assay was conducted to detect the level of RNA transcripts. Briefly, reaction mixture (20 μl) containing 1 μg total RNA was reverse transcribed to cDNA by using PrimeScript RT reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd., Dalian, China). RT-qPCR was performed using the SYBR Premix Ex Taq II real-time PCR kit (Takara Biotechnology Co., Ltd.) in a 20 μl reaction volume, which contained 10 μl SYBR Premix Ex Taq II, 0.8 μl of each of forward and reverse primers, 2 μl diluted cDNA template and appropriate amounts of sterile distilled water. Thermocycling conditions were as follows: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles of denaturation at 95˚C for 5 sec, annealing at 63.3˚C for 30 sec and elongation at 72˚C for 30 sec. The sequences of the PCR primers were as follows: Linc00673, forward 5'-CTCGGACCTTAG GGCTGTTG-3', reverse 5'-GGCTGCAATGAACGACAG TG-3'; linc00312, forward 5'-GGTTCTTCTTAATCTGGC TGTTG-3', reverse 5'-TTATTGGCTTGGTTCGCTTGTC-3'; and GAPDH, forward 5'-GGTCTCCTCTGACTTCAACA-3' and reverse 5'-GTGAGGGTCTCTCTCTTCCT-3'. GAPDH as an internal control was used to normalize the data to determine the relative expression of the target genes. The reaction conditions were set based on the kit instructions. Following completion of the reaction, the amplification curve and melting curve were analyzed to measure the specificity of the amplified products method. The relative expression of each lncRNA was calculated using the comparative cycle threshold method ($2^{-\Delta\Delta Cq}$) (21).

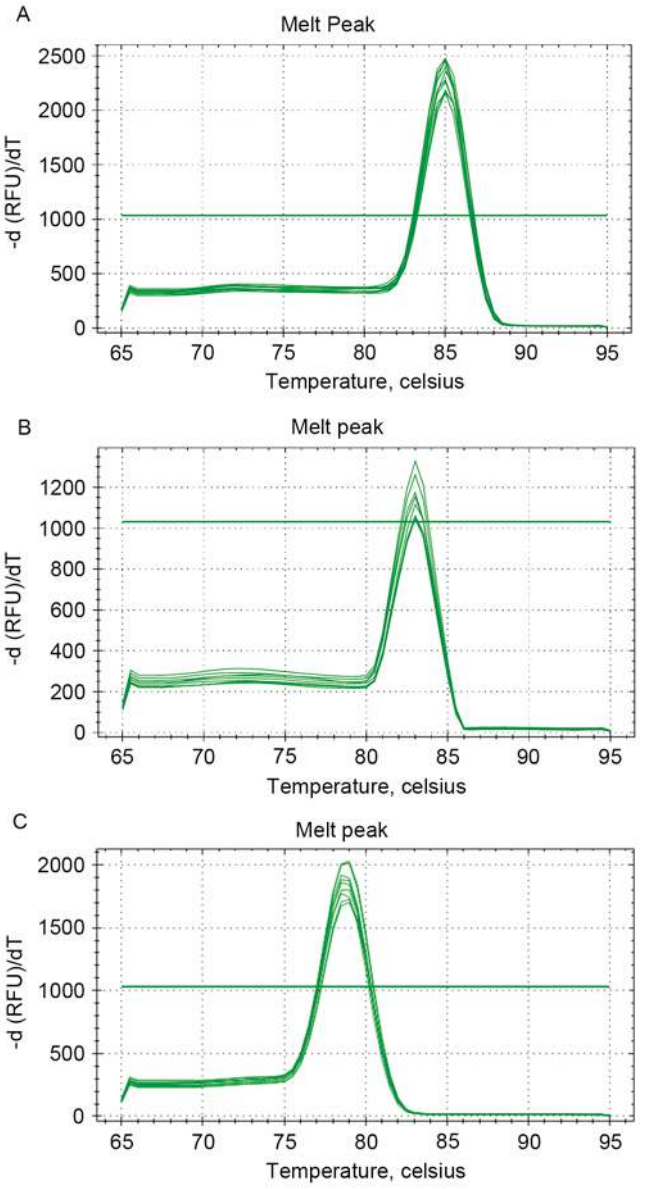

Figure 1. The figures of melt peak in melting curve analysis: (A) Endogenous control, GAPDH, (B) linc00673, (C) linc00312. LincRNA, long intergenic non-coding RNA.

Statistical analysis. All statistical analyses were performed using the SPSS software (version, 17.0; SPSS, Inc., Chicago, IL, USA). Paired-sample Student's t-test and Wilcoxon test were applied to compare the expression levels of linc00312 and linc00673 between NSCLC tissues and normal tissues. The association between the expression levels and clinicopathological factors was analyzed by the two-sided chi-square test. A receiver operating characteristic (ROC) curve was applied to evaluate the diagnostic value. Data are presented as mean ± standard error of the mean. $P<0.05$ was considered to indicate a statistically significant difference.

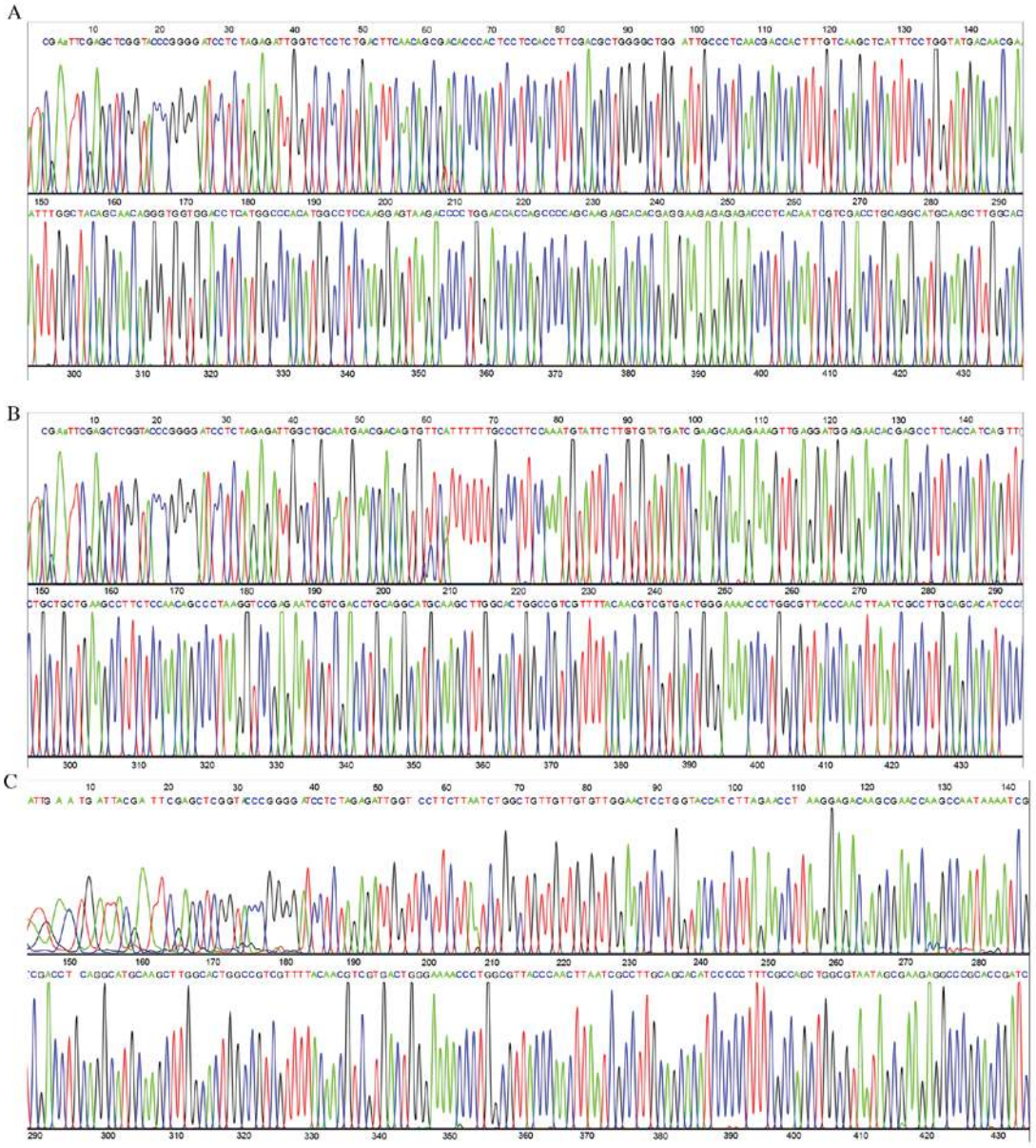

Figure 2. Sequencing results of reverse transcription-quantitative polymerase chain reaction products: (A) Endogenous control, GAPDH, (B) linc00673, (C) linc00312. LincRNA, long intergenic non-coding RNA.

Results

The validation of linc00312 and linc673 expression in NSCLC. Cancer-related lncRNAs were searched in the LncRNA Disease database (http://www.cuilab.cn/lncrnadisease) with 'NSCLC' as the search term. Based on the previous findings, the authors focused on the two novel lncRNAs (linc00312 and lin00673) and further detected their expression in NSCLC cell lines and tissues. To confirm that linc00312 and linc00673 could be detected by RT-qPCR, the melting curve analysis (Fig. 1) and their PCR product sequence results were available as expected, which were completely consistent with the entries from the NCBI database (Fig. 2).

The expression of linc00312 and linc00673 in NSCLC tissues. Long intergenic non-coding RNA 673 and 312 expression was detected in 76 paired NSCLC samples and corresponding normal tissues by RT-qPCR. The results revealed that the linc00312 was distinctly decreased (P<0.001), while linc00673 was significantly increased in NSCLC tissues compared with normal tissues (P<0.01; Fig. 3). The waterfall plot demonstrated that linc00312 was reduced by at least three-fold in 59.2% (46/76) of the NSCLC patients. In addition, linc00673 was overexpressed by at least two-fold in 50% (38/76) of NSCLC patients (Fig. 4). Further statistics analysis indicated that linc00312 was downregulated in both LAD and LSCC tissues compared with the paired normal tissue (P<0.001). However, linc00673 was extremely upregulated only in LAD patients' tissues (P<0.01) but not in LSCC cancer tissues (P>0.05) (Fig. 5). To assess the correlation the expression of linc00673 and linc00312 with clinicopathological data, their expression levels in tumor tissues were categorized as low or high

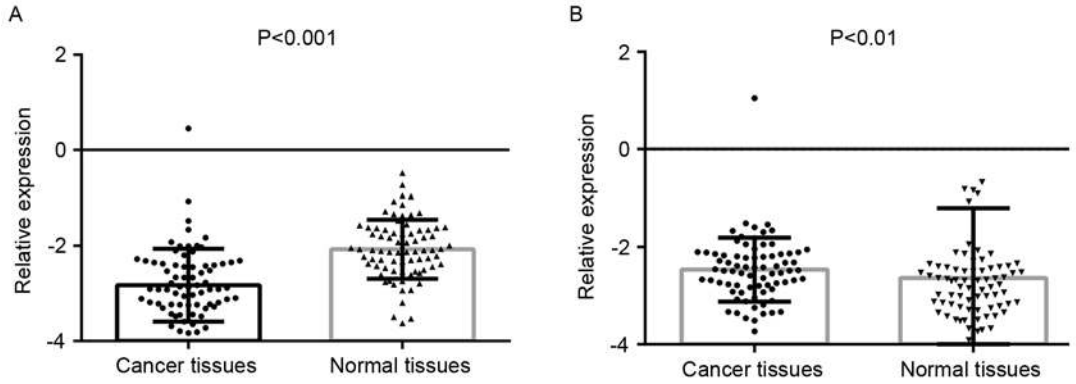

Figure 3. (A) linc00312 was downregulated and (B) linc00673 was upregulated in NSCLC tissues. Long non-coding RNAs expression were examined by reverse transcription-quantitative polymerase chain reaction and normalized to GAPDH expression in 76 pairs of NSCLC tissues, compared with normal tissues. The expression levels were calculated using the lg[2(-ΔCT)] method. Values were expressed as mean ± standard error of the mean. P<0.05 vs. non-tumorous group. NSCLC, non-small cell lung cancer; lincRNA, long intergenic non-coding RNA.

according to the median level. As presented in Table I, the low expression level of linc00312 was associated with the histologic grade of NSCLC patients (P<0.05), and the high level of linc00673 was related with ADC compared to SCC (P<0.05). No other significant differences were observed between the expression of the two lncRNAs and the patients' clinical characteristics including age, gender, smoking status, tumor size, advanced T-stage and regional lymph node metastasis.

The expression of linc00312 and linc00673 in NSCLC cell lines. Long intergenic non-coding RNA 673 and 312 expression was detected in four NSCLC cell lines (A549, SPC-A-1, H1299 and 95-D) and HELF by RT-qPCR. The results demonstrated a lower expression of linc00312 in H1299, SPC-A-1, 95-D and A549 cell lines, compared with that in HELF cells (Fig. 6A). Among the six NSCLC cell lines, linc00312 decreased the most in A549 and H1299 cell lines. Moreover, it was identified that linc00673 expressed higher in the four NSCLC cell lines compared with normal lung cell line HELF (Fig. 6B).

ROC curve analysis of linc00312 and linc00673 in tumor tissues. To investigate the diagnostic contribution of the two lncRNA in NSCLC, ROC curve and the area under the ROC curves (AUC) were performed on data from all subjects, including 76 NSCLC patients (Fig. 7). The ROC curves illustrated strong separation between the tumor tissues and control group, with an AUC of 0.803 (95% CI: 0.732-0.873; P<0.0001) for linc00312 and 0.653 (95% CI: 0.5663-0.7395; P<0.001) for linc00673 respectively (Fig. 7A and B). However, ROC curves was further performed to assess the significance of linc00312 and linc00673 in the subtypes of NSCLC including ADC and SCC compared with their paired non-tumorous groups. The ROC curves indicated that there was strong separation between LAD and paired normal tissues, with an AUC of 0.717 (95% CI: 0738-0.917; P<0.001) for linc673 (Fig. 7E and F), while the AUC of LSCC for linc00673 was 0.5886 (95% CI: 0.4534-0.238; P=0.203). In addition, there was no difference between the LAD and LSCC group, with an AUC of 0.828 (95% CI: 0738-0.917; P<0.0001) and 0.7959 (95% CI: 0.687-0.905; P<0.0001) for linc00312, respectively (Fig. 7C and D). Therefore, linc00312 can provided the highly diagnostic power for the detection of NSCLC, while linc00673 may be more specific for LAD.

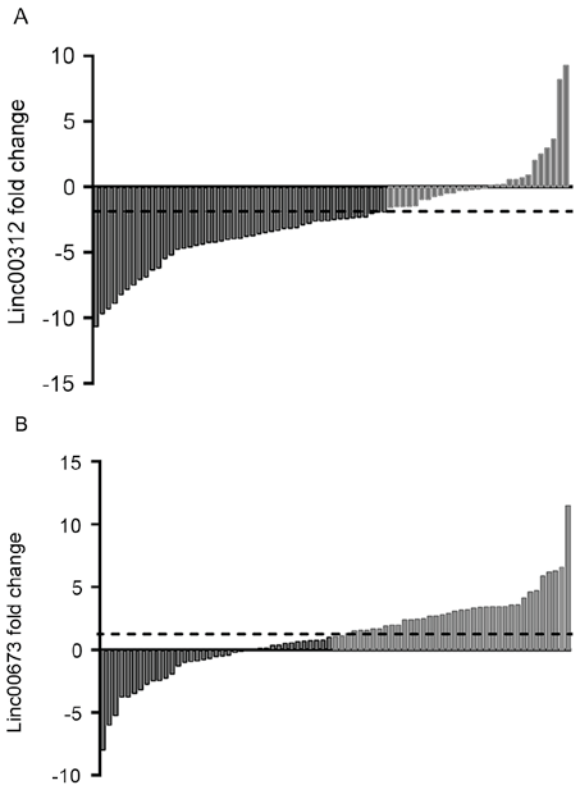

Figure 4. (A) Waterfall plot demonstrated that linc00312 was downregulated in 59.2% (46/76) of human NSCLC samples by at least threefold; (B) and linc00673 was upregulated in 50% (38/76) of human NSCLC samples by at least two-fold. NT, non-tumorous tissues of NSCLC; NSCLC, non-small cell lung cancer; linc, long intergenic non-coding RNA.

Discussion

The early diagnosis of NSCLC is generally poor conducted due to the lack of significantly biomarkers. Recently, micro-RNAs (22) and cyclooxygenase (23) have drawn considerable attention compared with the previous classic tumor

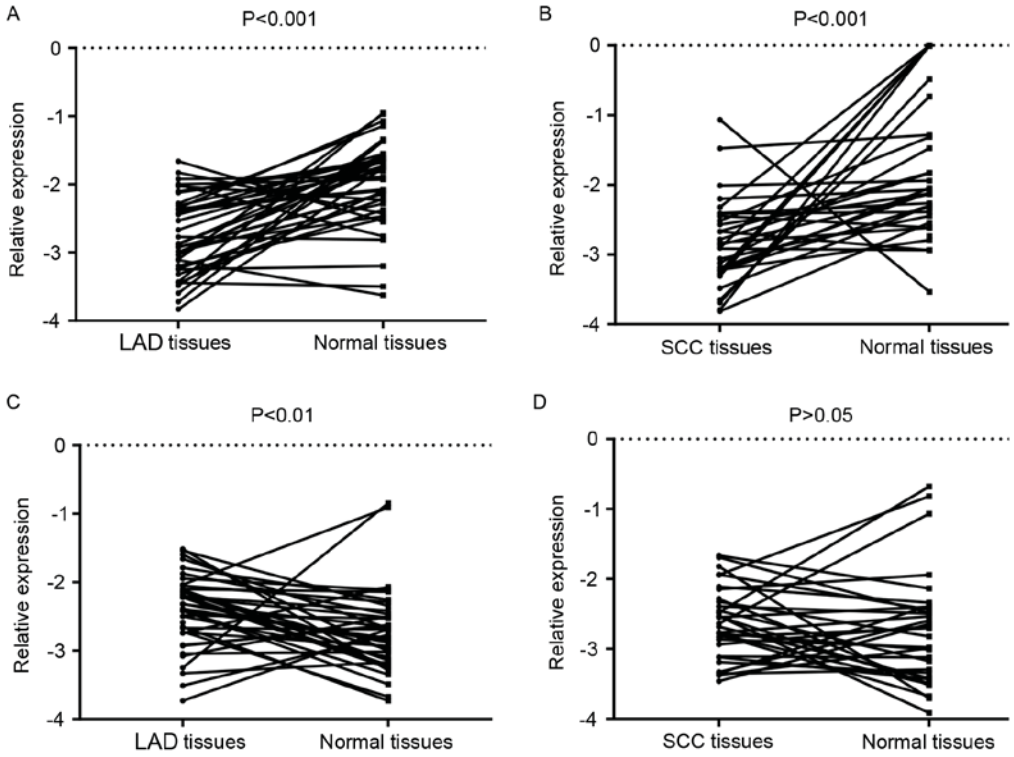

Figure 5. linc00312 decreased both in (A) LAD and (B) LSCC cancer tissues. Linc00673 increased in (C) LAD cancer patients, but not in (D) LSCC cancer tissues. The lncRNA expression was examined by reverse transcription-quantitative polymerase chain reaction, and normalized to GAPDH expression in 43 pairs of LAD tissues and 33 pairs of LSCC tissues, compared with normal tissues. The expression levels were calculated using the lg[2(-ΔCT)] method. LincRNA, long intergenic non-coding RNA; LAD, lung adenocarcinoma; LSCC, lung squamous cell carcinoma.

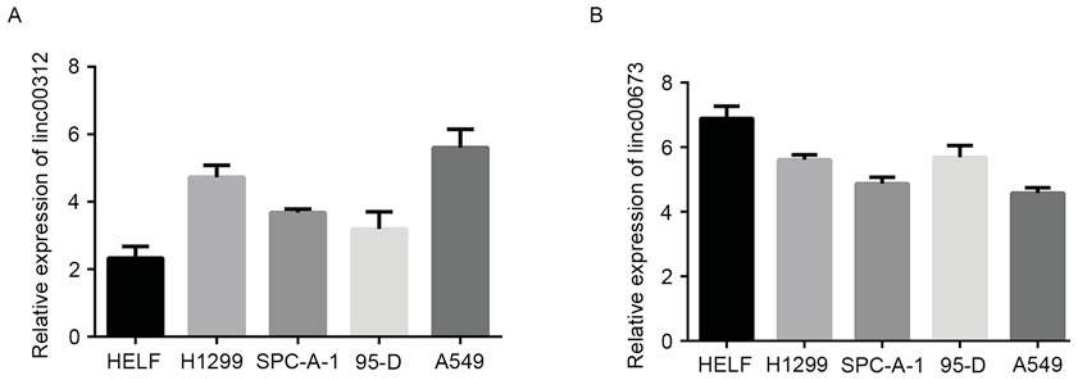

Figure 6. The expression levels of (A) linc00312 and (B) linc00673 in four non-small cell lung cancer cell lines and the normal HELF cell line. Assays were performed in triplicate. Data are presented as mean ± standard error of the mean. LincRNA, long intergenic non-coding RNA.

markers commonly used in NSCLC treatment. However, the lack of diagnostic sensitivity and specificity appear to be obstacle barrier for their clinical amplification in the early detection of NSCLC.

There are ~15,000 lncRNAs in the human transcriptome (24). LncRNAs, which have a similar structure to mRNA, are transcribed by polymerase II. Usually, they have fixed polyA tail and promoter, following alternative splicing. Though they contain a lower number of exons than mRNAs and characterized by different, characteristic expression patterns in cancers and other diseases (25-27). On average, lncRNAs, which were considered as 'transcription noise' and 'junk', now has been reported to serve important biological functions in various of diseases and cancers such as cardiovascular disease (28), prostate cancer (29), thyroid cancer (30), hepatocellular carcinoma (31) and gastric cancer (32). So far, quite a few lncRNAs have been better characterized the possible mechanisms involving in chromatin remodeling through multiple chromatin-based mechanisms and RNA cross-talking (5,33,34) and some of which could be prognostic biomarkers and therapeutic targets (35,36). For instance, MALAT-1 generally can be cleaved to small cytoplasmic RNA and large MALAT-1 fragments by RNaseP and RNaseZ, and the latter has the function of epigenetic repression of target genes in cancers through alternative splicing (37); HOTAIR can silence some tumor suppressor genes through H3K27

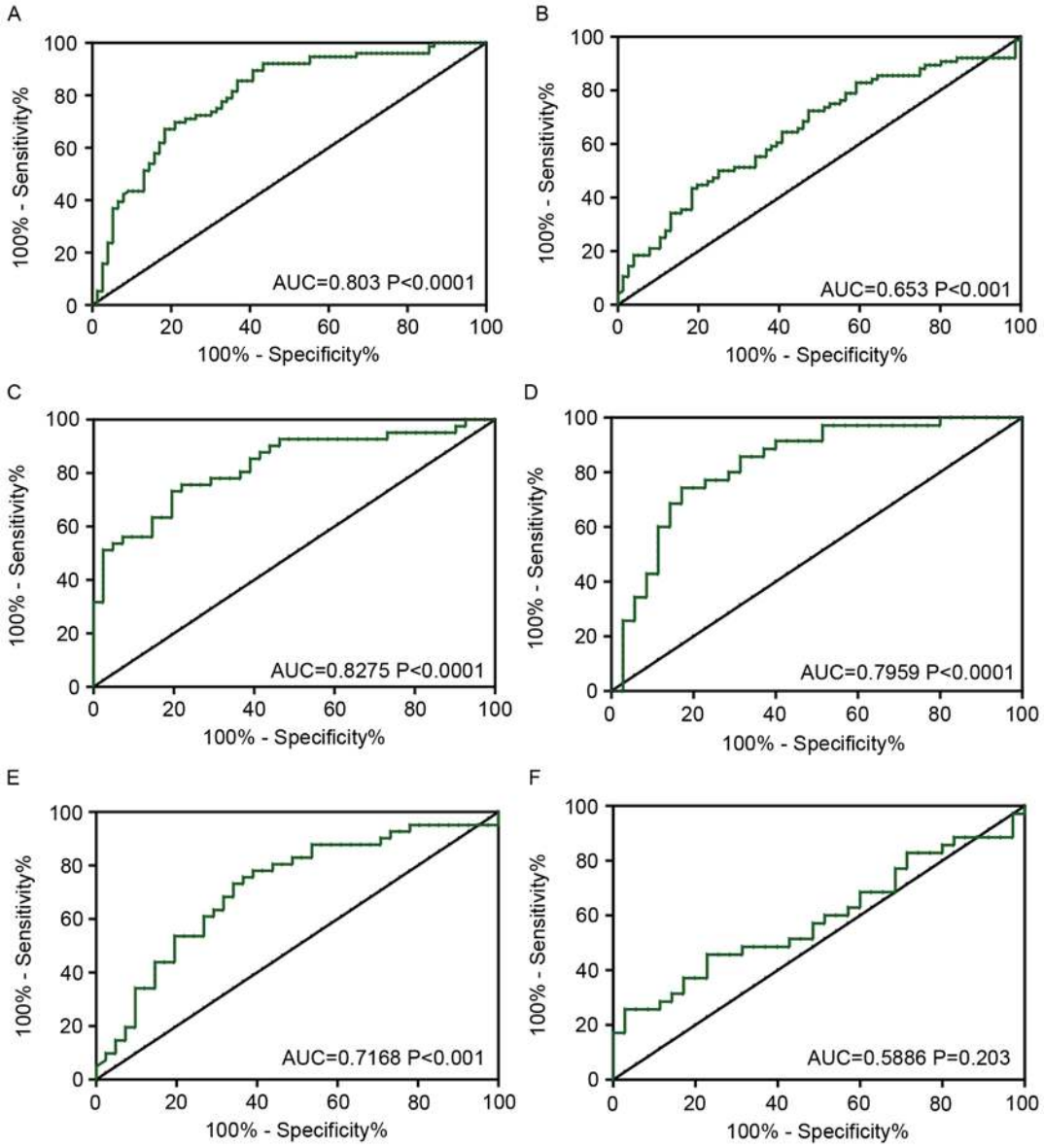

Figure 7. Receiver operator characteristic curve analysis of linc00312 and linc00673 for non-small cell lung cancer. (A) The AUC was 0.803 (95% CI: 0.732-0.873; P<0.0001) for linc00312; (B) AUC was 0.653 (95% CI: 0.5663-0.7395; P<0.001) for linc00673; (C) AUC of linc00312 was 0.8275 for LAD (95% CI: 0738-0.917; P<0.0001); (D) AUC of linc00312 was 0.7959 for LSCC (95% CI: 0.687-0.905; P<0.001); (E) AUC of linc00673 was 0.7168 for LAD (95% CI: 0738-0.917; P<0.001); (F) AUC of linc00673 was 0.5886 for LSCC (95% CI: 0.4534-0.238; P=0.203). AUC, area under curve; lincRNA, long intergenic non-coding RNA; LSCC, lung squamous cell carcinoma; LAD, lung adenocarcinoma.

and H3K4 methylation with the combination of PCR2 and LSD (38). H19 involves in the development of lung cancer by imprint loss and regulation expression of c-myc (39). Those underlying mechanisms of lncRNA have inspired us to identify more specific and valuable lncRNAs. To date, some known lncRNAs have been confirmed to be dysregulated and play functional roles in NSCLC, such as MALAT1, HOTAIR, H19, MEG3, GAS5, ANRIL and SOX2OT (34,40,41), but also many novel lncRNAs. For example, long non-coding RNA 01133 could repress KLF2, P21 and E-cadherin transcription through binding with EZH2, LSD1 and its over-expression is associated with patients' poor prognosis and short survival time in NSCLC (42). Linc00857 may mediate tumor progression through the way of regulating cell cycle in NSCLC (43).

By microarray and RNA-sequencing, high through technology have employed to detect huge number of lung cancer-associated lncRNAs (44), especially LAD and LSCC. In the present study, the authors mainly focused on two intergenic lncRNA 00312 and 00673 and aimed to establish their regulation pattern in NSCLC. Linc00312 locates in the genomic 3p25.3 whose length is 2,903 nt and includes only one exon (18). Linc00312 was originally regarded as mRNA, namely NAG7. A previous study demonstrated that as a protein-coding RNA, it can play critical roles in the interaction of estrogen receptor alpha (ERa) and other molecules including JNK2, c-Fos and MMP-1 (17). However, the HUGO Gene Nomenclature Committee identified that it belongs to the group of long non-coding RNA according to the definition of lncRNAs (18). Although literature has suggested that

linc00312 could promote nasopharyngeal carcinoma invasion through the JNK2/AP-1/MMP1 signaling pathway (17), its mechanism allowing it to participate in the development and progression of NSCLC remains unclear. No study has been available regarding the relationship between the expression level of linc00312 and the clinicopathological parameters of NSCLC. The present study identified that linc00312 was significantly downregulated in NSCLC patients' tissues compared with normal tissues, including LAD and LSCC. Further statistical analysis revealed that the low expression level of linc00312 was correlated with TNM stage of NSCLC, suggesting that linc00312 could be a potential candidate lncRNA to NSCLC as a suppressor gene. Another lncRNA linc00673 locates at Ch17q25.1 with 2275 bp and consists of four exons and two introns. According to Shi *et al* (20), linc00673 may participate in the development and progression of NSCLC as an oncogene through silencing NCALD transcription by binding with histone demethylase lysine specific demethylase 1. Furthermore, the authors proved that linc00673 expressed higher in LAD but not in LSCC tissues. In addition, the ROC curve illustrated that linc00673 could be a LAD specific lncRNA as an oncogene. However, no significant relationship was achieved among the expression of linc00312 and linc00673 with patients' other clinical features.

In conclusion, lncRNA 00312 may serve an important role in the tumorigenesis and deterioration of human NSCLC. Moreover, linc00673 is upregulated in LAD but not in LSCC tissues, implying that it can be a potential candidate tissue specific lncRNA for NSCLC.

Acknowledgments

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