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PLASMA LONG NON-CODING RNA SNHG-18 IS A VALUABLE MARKER FOR THE SCREENING OF NON-SMALL CELL LUNG CANCER

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ABSTRACT

Many research studies have proven that long non-coding RNAs (lncRNAs) may play a critical role in various cancer development and progression. However, little is known the clinical aspect of small nucleolar RNA host gene (SNHG18) in non-small cell lung cancer (NSCLC). This work aimed to investigate the diagnostic impact of SNHG18 in NSCLC plasma and to assess its association with clinical characteristics. Firstly, the expression level of SNHG 18 was detected by quantitative reverse transcription-polymerase chain reaction (qRT -PCR) in 64 pairs of NSCLC tissue. Then, we analyzed the differential expression and diagnostic value of SNHG 18 in 126 NSCLC patient and 87 healthy controls. Results showed that SNHG18 expression was significantly up-regulated in NSCLC plasma compared with healthy control plasma (P=0.0005), and its diagnostic efficacy was satisfactory. The combination of SNHG18 and CEA could provide a more accurate diagnosis than SNHG18 or CEA alone (AUC 0.7217, 95% CI: 0.6279-0.8155, P<0.0001). Additionally, plasma SNHG18 levels in preoperative patients with NSCLC were significantly higher than those in the postoperative group (P=0.0181). In conclusion, our results showed that SNHG 18 might serve as an oncogene and can be considered as a tumor marker for diagnosing and monitoring NSCLC patients.

KEYWORDS: Long non-coding RNAs, SNHG18, plasma.

INTRODUCTION

Lung cancer, the leading cause of cancer-related deaths, represents 27% of cancer-related deaths worldwide.[1] In 2033, lung cancer incidence rate will be increased, and it will be the leading cause of global death. Lung cancer comprises of two common types: small cell lung cancer (SCLC, 15% of cases), and (NSCLC, 85% of cases). And NSCLC includes adenocarcinoma (ADC), squamous cell carcinoma (SCC), large cell carcinoma (LCC) and several other types.[2,3] Despite significant progress in research, screening and treatment options, physicians find out the diagnosis of lung cancer mostly at very late stages. For example, the screening of high-risk heavy smokers by low-dose computed tomography help doctors to diagnose lung cancer at an earlier stage. However, only 2-4% of millions of people were eligible for the screening, even in a developed country like the United States of America in 2010. Chronic treatment followed by a fast onset of chemo resistance is also a problem. Therefore, diagnosis of lung cancer at the very early stage is critical to reduce the morbidity and motility rate. Highly accurate, efficiently sensitive, cost-effective, stable and easily available, non-invasive, prognostic and predictive biomarkers are still needed in urgency.[1,4,5] Studies for circulating protein biomarkers panels,[6] microRNA profiles,[2] long non-coding RNAs profiles,[7] genetic markers[8] have been explored.

Numerous researchers have assessed that miRNAs are stable non-invasive biomarkers for early diagnosis, prognosis and evaluation of lung cancer stages.[9] LncRNAs are functional RNA molecules released as a result of tumor cell secretion processes, and they are packaged in small, membrane-covered vesicles such as exosomes and microparticles, which protect them from degradation. They play critical roles in oncogenic and tumor-suppressing pathophysiological pathways including cell growth, apoptosis, metastasis, and necrosis.[10] Nowadays, many studies highlighted lncRNAs as stable tumor markers of cancer and also for future therapeutic targeted therapy. For example, HOTAIR was highly expressed in small cell lung cancer,

and it mediated chemo resistance of SCLC by regulating HOXA1 methylation for new adjuvant therapies.[11,12] LncRNAs like HOTAIR, HOTTIP, ANRIL, MG3, TUG1, MALAT-1, AK001796, LUADT1, HNF-1A-AS1, PVT1 were confirmed as diagnostic, prognostic and predictive biomarkers.[13] Long non-coding RNA H19 has been identified as a stable biomarker in plasma for gastric cancer diagnosis.[14] Zheng et al. showed that SNHG18 was up-regulated in glioma tissue and it strengthened the glioma cell radio resistance through inhibition of Sema5A.[15] But no one has studied the clinical role of SNHG18 in NSCLC. Therefore, after analyzing in 64 pairs of tissue, SNHG18 expression was compared in plasma samples from 126 patients and 87 controls. In this study, we demonstrated that plasma SNHG18 levels are useful to detect NSCLC and monitor tumor dynamics after tumor resection. Then we analyzed the correlation between SNHG18 and clinicopathological characteristics. Afterwards, we evaluated the diagnostic efficiency of circulating SNHG18 for patients with NSCLC.

MATERIALS AND METHODS

Study population

Tissue samples collection

64 specimens (50 males and 14 females, mean age: 61±4) of NSCLC tissues and adjacent normal tissues (3cm from a cancerous tumor) were collected from NSCLC patients whom had not previously undergone radiotherapy or chemotherapy treatment in Zhongnan Hospital of Wuhan University from October 2014 to January 2016. All patients were pathologically classified using the tumor-node-metastasis staging system, and histological grade was evaluated by the National Comprehensive Cancer Network clinical practice guidelines. The tissue samples were immediately snap frozen in liquid nitrogen and stored at - 80 ◦C until total RNA extraction.

Plasma samples collection

A total of 213 plasma samples were collected from 87 healthy controls (50 men and 37 women, mean age:56±9) and 126 NSCLC patients (97 men and 29 women, mean age:61±2) before surgery, radiation and chemotherapy from Zhongnan hospital of Wuhan University between November 2016 and August 2017. Moreover, 23 paired postoperative samples were also collected. The recruitment of subjects to the control group was conducted in the Medical Examination Centre of the Zhongnan Hospital without cancer and other health problem.

All plasma samples collected, were kept at room temperature no more than 4 hours, using ethylene diamine tetra acetic acid (EDTA) as an anticoagulant. Furthermore, procedure was followed by two-step centrifugation protocol (2000 rpm at 4˚C for 5 min, 12,000 rpm at 4˚C for 5 min) and stored in the ultra-low freezer (−80◦C) before performing experiments.

Ethical Approval

All patients' specimens collected had signed their written informed consent before biological examination or surgery was performed, and Wuhan University approved all aspects of the study. The study was recognized by the Ethics Committee of Zhongnan Hospital of Wuhan University (Wuhan, China).

Clinical data collection

The stage of NSCLC patients was determined according to the eighth edition of tumor node metastasis classification for lung cancer (UICC). The clinical characteristics of the patients including tumor markers (CEA, CA125, NSE, SCC, CA199), age, sex, smoking history, tumor size, lymph node metastasis, histological grade were collected from Laboratory Information System of Zhongnan hospital for data analysis. Age, gender, CEA, CA125 and CA199 of healthy controls were also collected.

RNA Extraction & Quantitative Reverse Transcription Polymerase Chain Reaction

Tissue RNA was extracted using TRIzol reagent (Invitrogen, CA, USA), and plasma RNA was extracted using blood/liquid sample Total RNA Rapid Extraction kit (spin-column) (Bioteke, Beijing, China). We used NanoDrop ND2000 (Thermo, CA, USA) to quantify the concentration and purity of extracted RNA. Briefly, total RNA was reversely transcribed to cDNA, followed the protocol of 42˚C for 2 min, then 37˚C for 15 min and 85˚C for 5 secs by using Prime Script RT reagent Kit with gDNA Eraser (Takara, Dalian, China). Quantitative real-time PCR (qRT-PCR) was performed using the SYBR®Premix Ex Taq™ II real-time PCR kit (Takara) on Bio-Rad CFX PCR machine. With total 20-µl reaction volume, containing SYBR-Green master PCR mix (10 µl), forward primer (0.8 µl) and reverse primers (0.8 µl), diluted cDNA template (2 µl), and appropriate amounts of sterile distilled water. The cycling conditions were initial denaturation at 95˚C for 5 min; 40 cycles of denaturation at 95˚C for 30 secs, annealing at 61.4˚C for 30 secs, and elongation at 72˚C for 30 sec. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous control for data normalization to determine the relative expression of the target gene. The sequences of PCR primers were synthesized by TsingKe, Wuhan, China as follow: SNHG-18, forward:5'- GACCTGGACCTCACCTAA -3', reverse: 5'- GCTGCTTCCTTGAACTTG--3'; GAPDH: forward: 5'-GCT CTC TGC TCC TCC TGT TC-3', reverse:5'-ACGACCAAATCCGTTGACTC-3'. All reaction conditions were carried out according to the manufacturer's instructions and then analyzed the amplification curve and melting curve to calculate the specificity of the amplified PCR products. The relative expression was calculated using the comparative cycle threshold (Ct) method $-\log 2^{\Delta Ct}$.

Statistical analysis

All statistical analyses were performed using SPSS

software package version 23.0 (SPSS, Chicago, Illinois, USA). All figures were drawn by Graph Pad Prism 5.0 software (Graph Pad Software, La Jolla, California). The paired-sample t-test was used to compare differences in SNHG18 expression in paired plasma samples before and after surgery. The independent two-tailed t-test was used to compare differences in plasma lncRNA concentrations between the cancer group and healthy group. Two-sided Chi-square test was used to assess the association between the expression level and clinicopathological factors. Receiver–operating characteristic (ROC) curves and the area under the curve (AUC) were applied to evaluate the feasibility of using plasma lncRNA concentrations as a diagnostic tool for identifying NSCLC. P value < 0.05 was considered to be statistically significant.

RESULT

Validity of Detection Method

We evaluated the repeatability and precision of the qRT-PCR results by using intra-assay and inter-assay coefficient of variation (CV) of Ct value. The intra-assay CV and inter-assay CV of SNHG18 and GAPDH were all <5% (Table I).

Tables and Graphs

Table I: Intra-assay CV and inter-assay CV of SNHG18 and GAPDH in two groups.

CV	Group	SNHG18	GAPDH
Intra-assay	NSCLC	1.40	1.18
CV%	Healthy control	1.24	1.60
Inter-assay	NSCLC	2.2	1.02
CV%	Healthy control	0.82	1.2

SNHG18 expression level in NSCLC tissues

At first, to validate the expression pattern of SNHG18 in NSCLC, we used qRT-PCR to detect the expression levels of SNHG18 in 64 paired NSCLC tissues and corresponding adjacent normal tissues. The result showed SNHG18 was significantly higher in NSCLC tissues than in the corresponding adjacent normal tissues (P =0.0042) (Figure A). The correlation of SNHG18 expression level in tissues with clinicopathological features was assessed as shown in the Table(II). The expression level of SNHG18 was associated with histological grades of NSCLC patients (P<0.05). However, there was no significant difference between the gene expression level and the clinical characteristics of the patients including age, gender, smoking status, TNM stage, and regional lymph node metastasis (P>0.05).

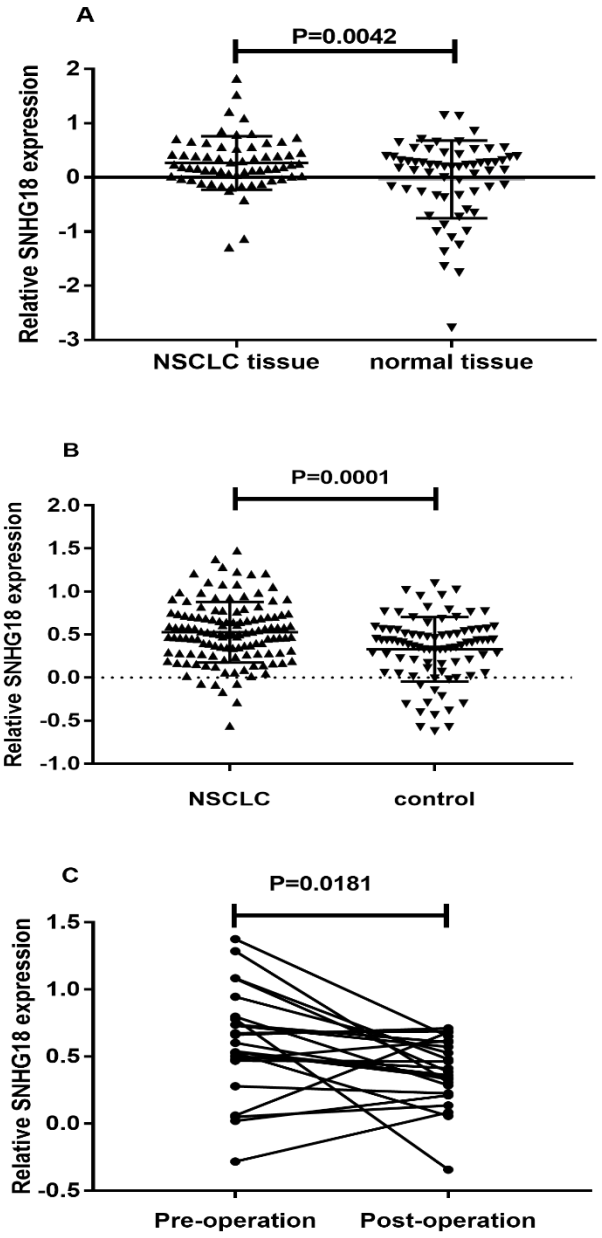

Figure (A): The SNHG18 expression levels were significantly upregulated in NSCLC tissue samples compared with normal tissue (n=64, P=0.0042).
Figure (B): The expression levels of plasma SNHG18 in NSCLC patients (n=126) and healthy controls (n=87). The SNHG18 expression levels were significantly upregulated in NSCLC patients compared with healthy control (P=0.0001).
Figure (C): The SNHG18 expression levels were significantly upregulated in pre-operative plasma samples compared with post-operation (n=23, P=0.0181).

Table II: Correlation clinicopathological factors and SNHG18 level -log2$^{\Delta Ct}$ in NSCLC patients' tissue samples. The low and high groups of SNHG18 expression were divided by median relative expression level. *Data missing in some groups.

Characteristics	Number*	SNHG18-low	SNHG18-high	P-value (Chi-square test)
Gender				
Male	50	25	25	0.765
Female	14	6	8	
Age (years)				
≤60	35	20	15	0.128
>60	28	10	18	
Smoking				
Never	16	7	9	0.771
Ever	40	20	20	
Tumour size (cm)				
≤3	10	2	8	0.145
>3	33	17	16	
Histological grade				
Stage I, II	29	9	20	**0.019***
Stage III, IV	30	19	11	
Lymph node metastasis				
Positive	21	9	12	0.401
Negative	38	19	19	
Histological classification				
ADC	26	9	17	0.068
SCC	30	18	12	

SNHG18 expression level of plasma in NLCLC patients

We determined plasma SNHG18 levels using plasma from 126 NSCLC patients and 87 healthy controls by qRT-PCR assays. Our results showed that SNHG18 expression was significantly higher in NSCLC patients than that in controls (Figure B, P =0.0001). Representation of the data using a ROC plot showed excellent separation between the two groups, with an AUC of 0.6405 (95% CI;0.5657–0.7154, P=0.0005) (Fig; D). The sensitivity and specificity were 63.49% and 62.07%, respectively. We also analyzed the association between the SNHG18 level and clinical factors by two-sided Chi-square test as shown in (Table III). The SNHG18 expression levels had a statistical relationship with drinking (P<0.05). However, no other significant difference was found between the expression level and the clinical characteristics of the patients including age, gender, smoking status, tumor size, histological subtype, TNM stage, and regional lymph node metastasis (P>0.05). To understand the potential value of SNHG18 as a clinical biomarker for diagnosing NSCLC, we compared the diagnostic value of SNHG18 to CEA, which is one of common NSCLC biomarkers. The results showed that plasma SNHG18 (AUC=0.6405 ,95% CI;0.5657–0.7154, P=0.0005) had a higher diagnostic efficiency than CEA (AUC=0.6064, 95%CI: 0.5046-0.7082, P=0.044). Moreover, the combination of SNHG18 and CEA could provide a stronger diagnostic efficiency than SNHG18 or CEA alone (AUC=0.7217, 95%CI: 0.6279-0.8155, P<0.0001) (Table V). These results showed that SNHG18 could serve as a potential biomarker for diagnosing NSCLC. Then, we analyzed the connections among SNHG18 and traditional tumor markers (including CEA, NSE, SCC, CA199 and CA125) (Table IV). There was a significant association between SNHG18 and CA125, but not with other markers.

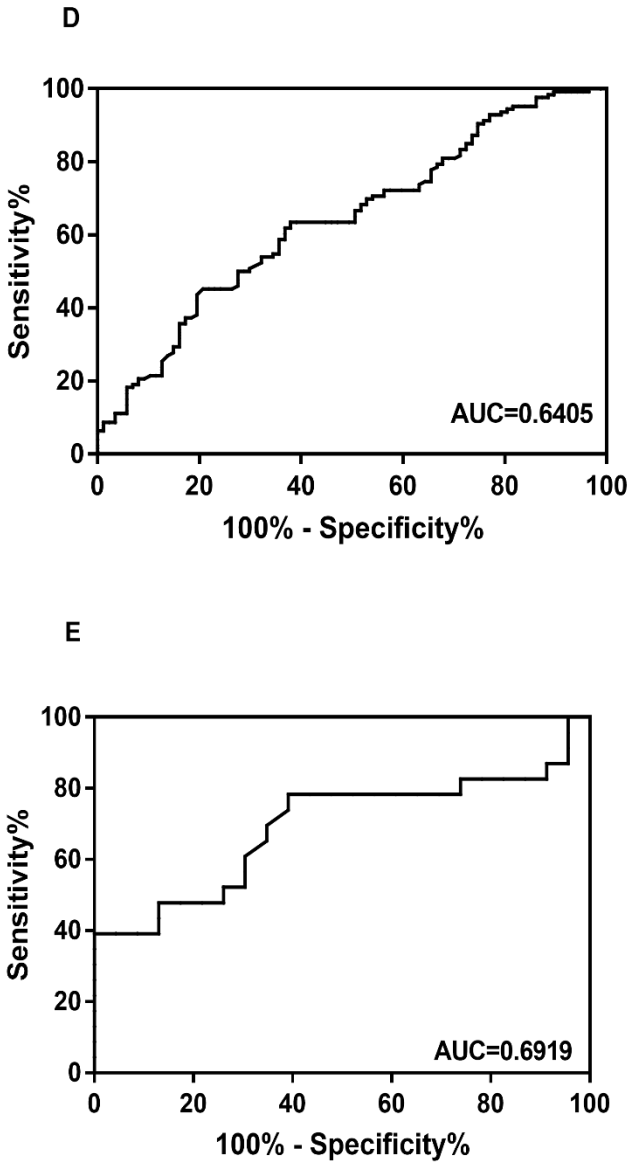

Figure (D): The ROC curve of SNHG18 expression levels in plasma for NSCLC (AUC=0.6405, 95% CI, 0.5657-0.7154, P=0.0005).
Figure (E): The ROC curve of SNHG18 expression levels in plasma (pre-operation vs post-operation) for NSCLC (AUC=0.6919, 95% CI, 0.5321-0.8517).

Table III: Correlation between clinicopathological factors and plasma SNHG18 level -log2$^{\Delta Ct}$ in NSCLC patients. The low and high groups of SNHG18 expression were divided by median relative expression level. *Data missing in some groups.

Characteristics	Number*	SNHG18-low	SNHG18-high	P-value (Chi-square test)
Gender				
Male	97	47	50	0.503
Female	29	16	13	
Age (years)				
≤60	64	23	41	0.076
>60	62	32	30	
Smoking				
Never	34	19	15	0.418
Ever	86	41	45	
Drinking				
Never	54	19	35	**0.004**
Ever	34	23	11	
Tumour size (cm)				
≤3	25	14	11	0.721
>3	11	5	6	
Histological grade				
Stage I, II	31	15	16	0.174
Stage III, IV	40	13	27	
Lymph node metastasis				
Positive	21	6	15	0.363
Negative	25	11	14	
Histological classification				
ADC	68	36	32	0.183
SCC	38	15	23	
Ki67 proliferation index (%)				
≤60	24	7	17	0.080
>60	24	14	10	

Table IV: Relationship of plasma SNHG18 levels -log2$^{\Delta Ct}$ among plasma tumor markers of NSCLC patients. The low and high groups of SNHG18 expression were divided by median relative expression level *Data missing in some groups.

Factor	Number*	SNHG18-low	SNHG18-high	P-value (Chi-square test)
CEA(ng/ml)				
≤5.0	87	46	41	0.480
>5.0	37	17	20	
NSE(μg/l)				
≤18.3	77	38	39	0.293
>18.3	17	11	6	
SCC(ng/ml)				
≤1.5	16	10	6	0.611
>1.5	5	2	3	
CA125(U/ml)				
≤5.0	38	35	3	**<0.001**
>5.0	42	24	18	
CA199(U/ml)				
≤37	81	38	43	0.197
>37	10	7	3	

Table V: Analysis of ROC curves within groups.

Group	AUC	95%CI	P-value	%Se	% Sp
CEA	0.6064	0.5046-0.7082	0.0444	46.7	75
SNHG18	0.6405	0.5657-0.7154	0.0005	63.5	62.1
CEA+SNHG18	0.7217	0.6279-0.8155	<0.0001	96.7	45

AUC, area under the receiver operating characteristic curves; Se, sensitivity; Sp, specificity

Evaluation of the use of SNHG18 for monitoring surgical resection in NSCLC patients

The expression level of SNHG18 was analyzed in 23 paired pre- and postoperative plasma samples from NSCLC patients who underwent surgery. As shown in Figure C, the expression level of SNHG18 was found to be significantly reduced in postoperative samples (P= 0.0181) indicating that circulating SNHG18 in plasma significantly decreased following surgery. Further analysis of the ROC curve for post-operation was shown in Fig. C, with an area under the curve (AUC) of 0.6919 (95% CI, 0.5321- 0.8517, P=0.0258) Figure. E. Taken together, circulating SNHG18 can be used for monitoring surgical treatment in NSCLC patients and has a potential as a combined biomarker for NSCLC screening. It provides a useful auxiliary diagnostic method to screen for a large number of candidate patients and increase our understanding of NSCLC. Therefore, circulating SNHG18 levels may reflect tumor dynamics of NSCLC patients.

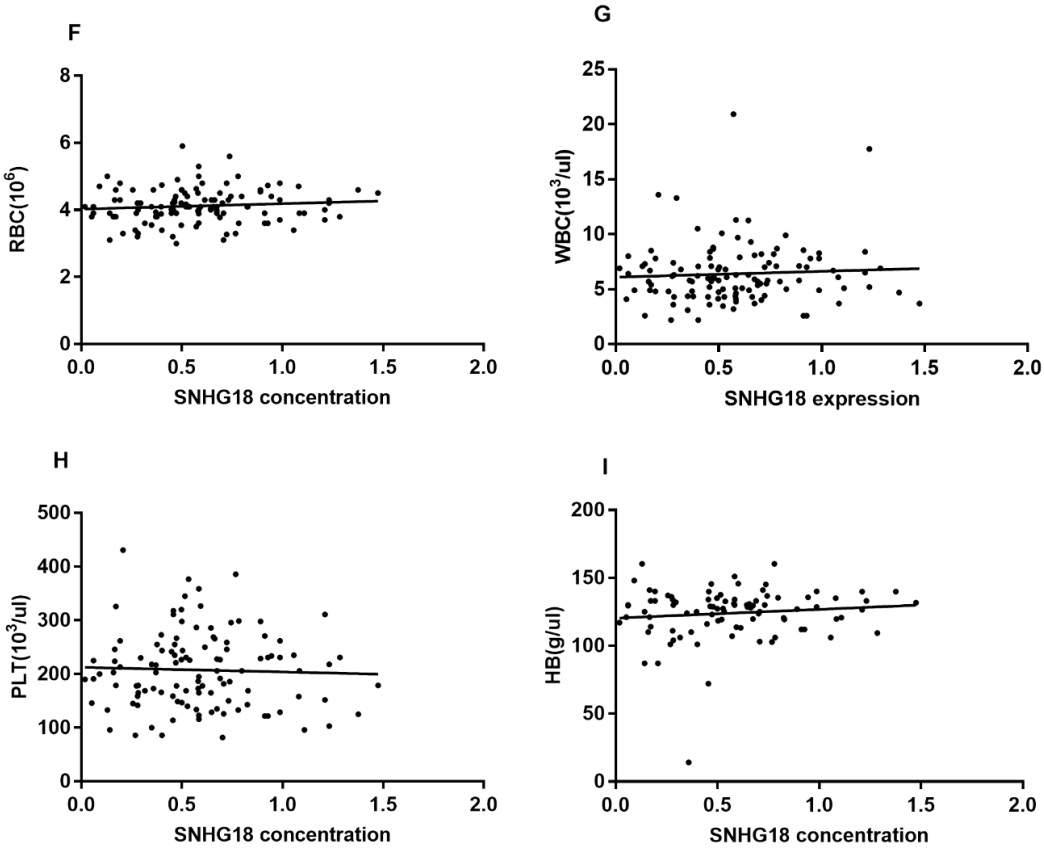

Figure F, G, H, I: Correlation between plasma SNHG18 concentrations and the haematocytes of peripheral blood in NSCLC patients. There was no significant correlation between SNHG18 concentrations and any type of peripheral haematocytes in plasma (P>0.05)

DISCUSSION

Many studies have centered on the role of biomarkers in early diagnosis, predicting prognosis and the therapeutic monitoring of numerous diseases. Although various biomarkers are now in clinical use, there are cases in which the disease of the patients relapsed where biomarkers showing no significant changes.[16] Tumor markers like cytokeratin 19-fragments (CYFRA 21-1), CEA, plasma kallikrein B1 (KLKB1) and squamous cancer cell antigen (SCCA) for NSCLC and progastrin-releasing peptide (ProGRP), neuron-specific enolase (NSE) for SCLC are most widely used for today lung cancer management. But these markers also have the limitation in specificity and sensitivity. For example, CEA was used as a specific marker for lung cancer, but later it was also a marker for gastric cancer.[17] CYFRA 21-1, a useful marker for epithelial malignancies including NSCLC, was also significantly elevated in

patients with obstructive respiratory diseases or inflammatory pulmonary diseases compared with healthy controls. Moreover, combined use of more than one biomarker was recommended for a more accurate diagnosis and prognosis. And combined use of biomarkers gives us greater sensitivity and greater diagnostic accuracy in lung cancer patients, but less in specificity compared with each individually.[18]

MicroRNAs (miRNAs) are one type of the emerging biomarkers with high specificity and sensitivity, and many types of research have reported the expression of these miRNAs can be functional for the identification, prediction and feedback to the manipulation of lung cancer. Investigations have shown that miR-21, miR-155, miR-17- 92, miR-221/22, miR-205 were up-regulated and miR-106, miR-34, miR-200, miR -548, miR-29 and Let 7 were downregulated in lung cancer.[19] Yu et al. (2017) showed that miR-92a-2 served as a predictive marker for the diagnosis and prognosis of SCLC.[20] Advanced studies proposed circulating tumor cells (CTC) as a valuable marker for early-stage lung cancer, while CT scans limited clinical information to detect tiny nodules and the malignancy stages of lymph nodes. One study showed that patients even without lesion in CT scan but with positive CTCs present risk for lung cancer. They suggested that CTC may even provide prognostic information. However, the major challenge is that CTC demand a very high purification step and its amount is quite low in samples.[21]

Nowadays, lncRNAs are being targeted as a potential biomarker for various cancer types. And many research studies had proven that lncRNAs might bring a new era to cancer diagnosis and aid treatment management. LncRNAs were shown to be valuable in the management of NSCLC. LncRNA TUG1 was involved in cell growth and chemo resistance of SCLC by regulating LIMK2b via EZH2b. Moreover, it was downregulated in NSCLC and could regulate CELF1 on binding to PRC2.[22,23] Li et al. (2017) showed that plasma HOTAIR had high diagnostic accuracy for NSCLC and was significantly lower in postoperative samples than in preoperative samples.[24]

However, nobody has analyzed the clinical importance of SNHG18 in NSCLC. Our study is the first one to investigate SNHG18 expression in plasma and assess its diagnostic value for NSCLC. First, the repeatability and precision of the qRT-PCR method was confirmed by the intra-assay CV and inter-assay CV. Then, we used qRT-PCR to measure the levels of SNHG18 in tissue and plasma of NSCLC patients and healthy controls. To validate the importance of SNHG18 in NSCLC, its expression levels were detected in 64 pairs of NSCLC tissue samples (including 26 ADC and 30 SCC) and corresponding normal tissue by qRT-PCR. The results showed that SNHG18 was significantly increased in NSCLC tissues (P=0.0042) (Figure A).

Then, the levels of SNHG18 in NSCLC patients and healthy controls were detected by qRT-PCR. We found that plasma SNHG18 levels were significantly higher in NSCLC patients than healthy controls and its levels was correlated with drinking habit. We further investigated the diagnostic value of plasma SNHG18.ROC curves showed that SNHG18 had a significant diagnostic power for diagnosis of NSCLC patients (AUC=0.6405, 95% CI;0.5657–0.7154, P=0.0005), which was higher than CEA. Furthermore, the combination of SNHG18 and CEA could provide a more robust diagnostic efficiency than SNHG18 or CEA alone. These results suggested that circulating SNHG18 may serve as the diagnostic biomarker for NSCLC patients. Lastly, we analyzed whether plasma SNHG18 levels could reflect NSCLC tumor dynamics or not. The levels of SNHG18 were analyzed in 23 paired preoperative and postoperative plasma samples. Plasma SNHG18 levels were significantly decreased after surgery. It is possible that plasma lncRNAs were mainly derived from tumor cells, and their level would probably back to normal after removal of the tumor. These results indicated that plasma SNHG18 levels might reflect tumor dynamics of NSCLC patients. Gopal reported that RNA species including lncRNA, messenger RNA, microRNA, DNA, proteins, and lipids are delivered by extracellular vesicles (EVs), especially exosomes and shed micro vesicles, which act as an important delivery medium in the tumor microenvironment between cancer and resident stromal cells to induce tumorigenesis and metastasis.[25] Cell-free and circulating miRNAs, originated from plasma, either from blood cells or endothelial cells, are used for detection of various cancers.[26] Here, we also assessed that whether plasma SNHG18 concentrations and haematocytes of peripheral blood have correlation or not in NSCLC plasma. And there was no common association between plasma SNHG18 concentrations and any types of peripheral haematocyte (P>0.05) (Fig;F,G,H,I).

In conclusion, our study showed that circulating SNHG18 levels were significantly upregulated in tissue and plasma of NSCLC patients suggesting that SNHG18 could be a plasma-based diagnostic biomarker for screening of NSCLC patients, and prediction of prognosis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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