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Original Article

Combined identification of long non-coding RNA XIST and HIF1A-AS1 in serum as an effective screening for non-small cell lung cancer

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Abstract: Objective: Long non-coding RNAs (lncRNAs) XIST and HIF1A-AS1 have been shown to play important regulatory roles in cancer biology, and lncRNA-XIST and HIF1A-AS1 are upregulated in several cancers such as glioblastoma, breast cancer and thoracoabdominal aorta aneurysm, however, its value in the diagnosis of non-small cell lung cancer (NSCLC) is unclear. The aim of this study is to evaluate the clinical significance of serum XIST and HIF1A-AS1 as a biomarker in the screening of NSCLC. Methods: Expression levels of lncRNA-XIST and HIF1A-AS1 in tumor tissues and serum from NSCLC patients were evaluated by quantitative real-time PCR, and its association with overall survival of patients was analyzed by statistical analysis. Moreover, the XIST and lncRNA-XIST expression correlation between tumor tissues and plasma was demonstrated by linear regression analysis. Results: The levels of XIST ($P < 0.05$) and HIF1A-AS1 ($P < 0.05$) were significantly increased in tumor tissues or serum from NSCLC patients as compared to those of control group. Correlation of lncRNA-XIST or HIF1A-AS1 expression between tumor tissues and serum from the same individuals was confirmed in NSCLC patients. Moreover, serum levels of XIST and HIF1A-AS1 were significantly decreased after surgical treatment as compared to pre-operative. The ROC curves illustrated strong separation between the NSCLC patients and control group, with an AUC of 0.834 (95% CI: 0.726-0.935; $P < 0.001$) for XIST and 0.876 (95% CI: 0.793-0.965; $P < 0.001$) for HIF1A-AS1, however, the combination of XIST and HIF1A-AS1 yielded an AUC of 0.931 (95% CI: 0.869-0.990; $P < 0.001$), which was significantly improved as compared to XIST or HIF1A-AS1 alone. Conclusion: Our results demonstrated that increased serum XIST and HIF1A-AS1 could be used as a predictive biomarker for NSCLC screening, and that combination of XIST and HIF1A-AS1 had a higher positive diagnostic efficiency of NSCLC than XIST or HIF1A-AS1 alone.

Keywords: Non-small cell lung cancer, long non-coding RNA, XIST, HIF1A-AS1, tumor biomarker

Introduction

Lung cancer is one of the most fatal malignancies in humans, with more than 226,000 new cases at the United States in 2012. Eighty-five percent of lung cancers are non-small-cell lung cancer (NSCLC), which has a 5-year survival of only 10% [1-3]. Although in recent years there are mounting progresses in clinical treatment for NSCLC, the overall survival time of NSCLC patients has not improved dramatically. An important reason for that is the lack of molecular biomarkers. Recent biomarkers such as micro-RNAs [4] and cyclooxygenase [5] are the classic tumor markers commonly used in the management of patients with NSCLC. However, these tumor markers have limited utility in the early detection of NSCLC due to lack of sufficiently high diagnostic sensitivity and specificity. Therefore, the significance of exploration of new biomarkers with high sensitivity and specificity in early detection of NSCLC should be emphasized.

Long non-coding RNAs (lncRNAs) are longer than 200 bases with lack of protein coding capability, which play critical roles in tumor initiation, progression and metastasis by modulating oncogenic and tumor-suppressing pathways [6]. Within 4 years, the number of identified lncRNA genes increase more than 8000 [7]. Although the function of most lncRNAs is still unknown, there are increasing numbers and accumulating evidence involving in many can-

Table 1. Correlation clinicopathological factors and XIST expression levels in NSCLC patients

Variable	Number of patients	XIST-Low	XIST-High	*P* value
Gender				0.001
Male	15	7	8	
Female	17	4	13	
Age (years)				0.787
< 60	12	4	8	
≥ 60	20	7	13	
Tumor size (cm)				
< 3	15	5	10	0.729
≥ 3	17	6	11	
Histological				0.001
I	15	7	8	
II-III	17	4	13	
Lymph nodes metastasis				0.001
Absence (A)	14	6	8	
Presence (P)	18	5	13	

cers [7-9]. Previous studies have proved that upregulated lncRNAs ANRIL [10], AK001796 [11], BCYRN1 [12] and HNF1A-AS1 [13] are proved to induce cell migration and tumor metastasis of NSCLC. In contrast, an lncRNA named HMlincRNA717 is downregulated and associated with tumor progression in human NSCLC [14]. In accordance to the literature, HIF1A-AS1 is expressed in a number of renal cancers, suggesting that it may have a role in cancer development [15]. Moreover, the expression of HIF1A-AS1 is upregulated in the thoracoabdominal aorta aneurysm, and the interaction between HIF1A-AS1 and apoptotic proteins plays a key role in the proliferation and apoptosis of VSMCs in vitro [16]. However, the underlying signaling mechanisms accounting for HIF1A-AS1 in NSCLC are still not well characterized. The lncRNA X-inactive specific transcript (XIST), a product of the XIST gene, is the master regulator of X inactivation in mammals, and XIST gene is exclusively transcribed from the inactive X chromosome [17]. XIST is found highly expressed in some carcinomas of the glioblastoma [18], breast cancer [19] and ovarian cancer [20], suggesting that XIST may serve as a biomarker for the diagnosis of these cancers [21]. A recent study has shown that the lncRNA-XIST is essential for long term survival of hematopoietic stem cells [22]. Functionally, knockdown of XIST exerted tumor suppressive functions by reducing cell proliferation, migration and invasion as well as inducing apoptosis. The in vivo studies also showed that knockdown of XIST suppressed tumor growth and produced high survival in nude mice [18]. These results indicate that XIST plays an important role in the occurrence and developing progress of malignant tumors. Nevertheless, there is no relevant report about the interaction between XIST and the progression of NSCLC. Thus, the role of XIST in LC and its underlying mechanism remain to be determined.

In this study, we were examined the expression levels of XIST and HIF1A-AS1 in tissues and plasma, and their potential use as tumor markers for NSCLC detection were evaluated. We hypothesized that these NSCLC-related lncRNAs might be released into the circulation during NSCLC initiation and could be utilized to detect and screen NSCLC.

Materials and methods

Patients and specimens

Sixth-fourth NSCLC tissues and matched adjacent non-tumor tissues were collected from Shanghai Chest Hospital and Shanghai First People's Hospital, Shanghai Jiaotong University (Shanghai, China) between Jan 2012 and June 2014. All patients recruited in this study were not subjected to preoperative radiotherapy or chemotherapy and diagnosed with NSCLC based on histopathological evaluation. Clinicopathological characteristics analyses were shown in **Tables 1** and **2**. All collected tissue samples were immediately stored at liquid nitrogen until use. Human samples were obtained with written informed consent from all patients. The study was approved by the Ethics Committee of the Shanghai Chest Hospital and Shanghai First People's Hospital, Shanghai Jiaotong University, China.

Real-time PCR

Total RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reaction mix-

Table 2. Correlation clinicopathological factors and HIF1A-AS1 expression levels in NSCLC patients

Variable	Number of patients	HIF1A-AS1-Low	HIF1A-AS1-High	*P* value
Gender				0.717
Male	15	6	9	
Female	17	7	10	
Age (years)				0.549
< 60	12	5	7	
≥ 60	20	8	12	
Tumor size (cm)				
< 3	15	5	10	0.01
≥ 3	17	8	9	
Histological				0.001
I	15	8	7	
II-III	17	5	12	
Lymph nodes metastasis				0.001
Absence (A)	14	8	6	
Presence (P)	18	5	13	

ture (20 μl) containing 2 μg of total RNA was reversely transcribed to cDNA by using PrimeScript RT-polymerase (Takara, Dalian, China). Quantitative PCR was performed on the cDNA using specific primers (Sangon, Shanghai, China) for XIST. The first strand cDNAs served as the template for the regular polymerase chain reaction (PCR) performed using a DNA Engine (ABI 9700). The cycling conditions were 30 s polymerase activation at 95°C followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. PCR with the following primers: XIST, Forward 5'-CTCTCCATTGGGTTCAC-3' and Reverse 5'-GCGGCAGGTCTTAAGAGATGAG-3'; HIF1A-AS1, Forward 5'-GTCACGATTCGGTACAC-3' and Reverse 5'-CGCGCAGGTCATAAGAGTT-GTG-3'; GAPDH, Forward 5'-ACAGGGGAGGTG-ATAGCATT-3' and Reverse 5'-GACCAAAAGC-CTTCATACATCTC-3'. Glyceraldehyde-phosphate dehydrogenase (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 according to the kit instructions. After completion of the reaction, the amplification curve and melting curve were analyzed. Gene expression values are represented using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All statistical analyses were performed using SPSS version 18.0 software. Data were analyzed using independent two-tailed t test. Categorical data were analyzed using the two-side chi-square test. Overall survival was estimated by using Kaplan-Meier method, and univariate analysis was conducted by log-rank test. The Cox proportional hazards model was used in the multivariate analysis. Values of $P < 0.05$ were considered statistically significant.

Results

Identification of tumor tissues-enriched lncRNA implicated in NSCLC patients

To identify lncRNAs that were potentially involved in the progression of NSCLC, we searched for lncRNAs that were enriched in tumor tissues. Firstly, the lncRNA expression profiles and hierarchical cluster analysis were performed in 3 NSCLC tissues and paired corresponding nontumourous tissues, and we identified over 30 lncRNAs that were enriched in tumor tissues of NSCLC patients. Nineteen lncRNAs were found to be significantly down-regulated and twelve lncRNAs to be significantly up-regulated in the NSCLC tissues by microarray assay, and we finally focused on XIST and HIF1A-AS1 in our study (**Figure 1**).

XIST and HIF1A-AS1 were detectable in tumor tissues and serum

Real-time PCR analysis was performed to determine the expression level of XIST and HIF1A-AS1 in 32 pairs of NSCLC tumor tissues and corresponding nontumourous specimens. We found that the expression of XIST and HIF1A-AS1 in tumor tissues was conspicuously higher than that of the adjacent nontumourous tissues ($P < 0.05$, **Figure 2A** and **2B**). To explore whether these NSCLC-related lncRNAs could reach the circulation at levels sufficient to be detectable, real-time PCR was used to examine

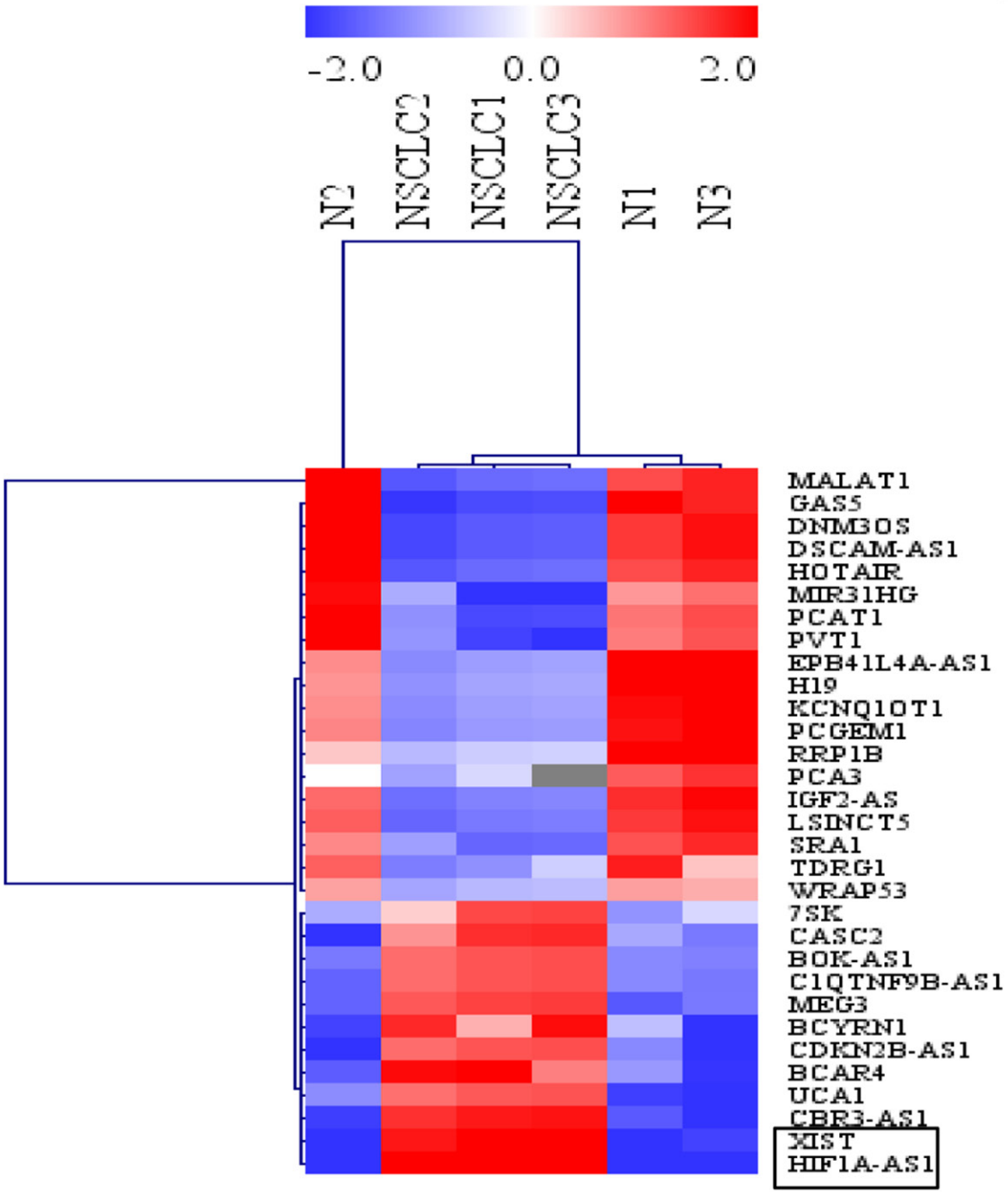

Figure 1. Identification of tumor tissues-enriched lncRNA implicated in NSCLC patients. The figure is drawn by MeV software (version 4.2.6). Differentially expressed LncRNAs chosen from lncRNA and disease database. Correlation similarity matrix and average linkage algorithms are used in the cluster analysis. Each row represents an individual LncRNA, and each column represents a sample. The dendrogram at the left side and the top displays similarity of expression among LncRNAs and samples individually. The color legend at the top represents the level of mRNA expression, with red indicating high expression levels and blue indicating low expression levels.

expression of XIST and HIF1A-AS1 in 64 serum samples (32 NSCLC patients and 32 normal controls). As shown in **Figure 2C** and **2D**, the levels of XIST ($P < 0.05$) and HIF1A-AS1 ($P < 0.05$) were significantly increased in NSCLC patients as compared to those of control group.

Correlation of lncRNAs expression between tumor tissues and serum in NSCLC patients

To test whether there was a relationship between tumor tissues and serum lncRNAs level, XIST and HIF1A-AS1 were measured in

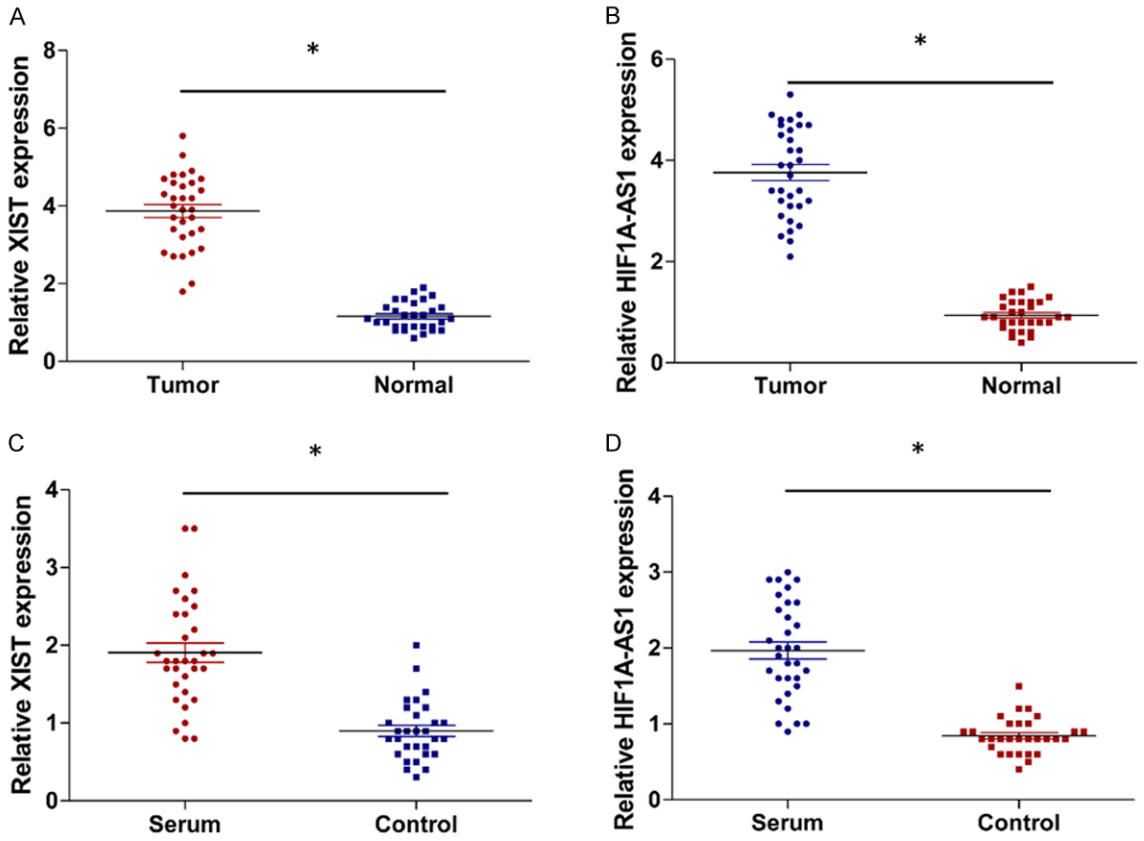

Figure 2. XIST and HIF1A-AS1 were detectable in tumor tissues and serum. XIST (A) and HIF1A-AS1 (B) expression was examined by real-time PCR and normalized to GAPDH expression in 32 pairs of NSCLC tissues compared with adjacent nontumourous tissues. The serum levels of XIST (C) and HIF1A-AS1 (D) was examined by real-time PCR. Values were expressed as mean ± SEM, $^{*}P < 0.05$ versus nontumourous group or normal control group.

tumor tissues and serum from the same individuals. As shown in **Figure 3A** and **3B**, measurements obtained from tumor tissues and serum were strongly correlated for XIST (r = 0.826, **Figure 3A**) and HIF1A-AS1 (r = 0.806, **Figure 3B**). The results suggested that serum samples were acceptable for evaluation of NSCLC-related biomarkers.

The expression levels of XIST and HIF1A-AS1 in pre-operative and post-operative serum samples

Since circulating lncRNAs were primarily released or leaked from tumor cells, they would revert to normal after the tumor has been resected [6]. In our study, the XIST and HIF1A-AS1 were carried out to investigate the differences in NSCLC-related lncRNAs in serum pro-operative and 14 days post-operative. As expected, serum levels of XIST and HIF1A-AS1 were significantly decreased after surgical treatment as compared to pre-operative (**Figure 4A** and **4B**).

Evaluation of XIST and HIF1A-AS1 in serum as predictive NSCLC-related biomarkers

To investigate the characteristics of XIST and HIF1A-AS1 as potential biomarkers for NSCLC, receiver operating characteristics (ROC) curves and the area under the ROC curves (AUC) were performed on data from all subjects, including 32 NSCLC patients and 30 healthy donors. The ROC curves illustrated strong separation between the NSCLC patients and control group, with an AUC of 0.834 (95% CI: 0.726-0.935; $P < 0.001$) for XIST and 0.876 (95% CI: 0.793-0.965; $P < 0.001$) for HIF1A-AS1 (**Figure 5A** and **5B**). Intriguingly, there is increasing evidence showing that combination several tumor markers could improve diagnostic accuracy [6]. In the present study, we determined whether the combination of UCA and HIF1A-AS1 could

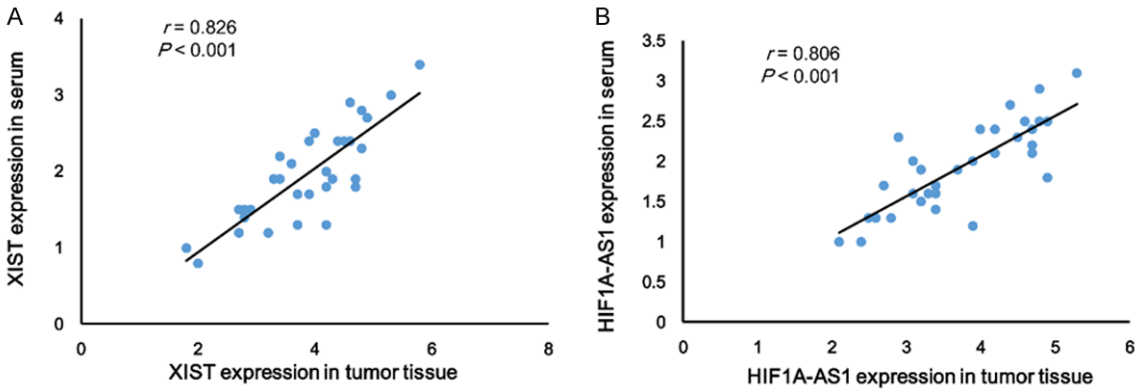

Figure 3. Correlation of lncRNAs expression between tumor tissues and serum in NSCLC patients. Linear correlation plot of XIST (A) and HIF1A-AS1 (B). There was a high correlation comparing the indicated lncRNAs levels between tumor tissues and serum.

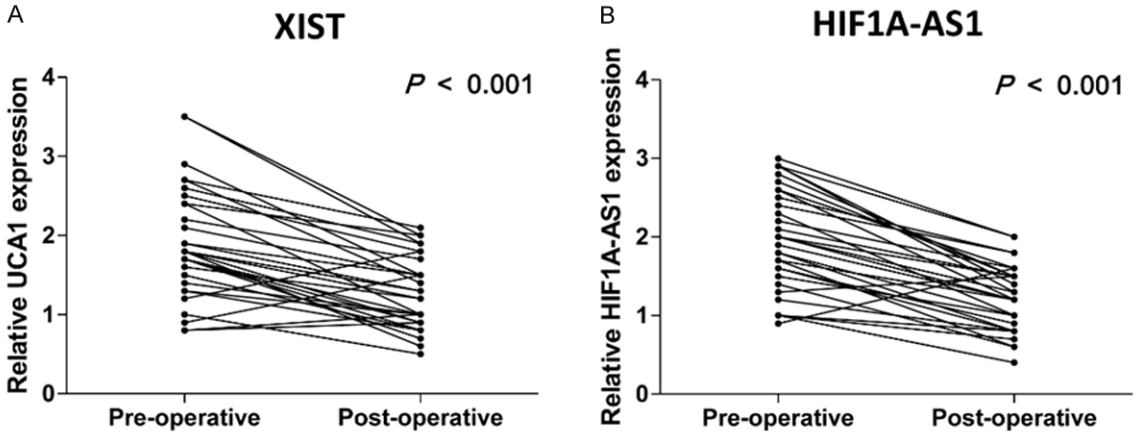

Figure 4. The expression levels of XIST and HIF1A-AS1 in pre-operative and post-operative serum samples. NSCLC-related lncRNA XIST (A) and HIF1A-AS1 (B) expressions were examined by real-time PCR in post-operative samples as compared to pre-operative samples.

provide a more effective screening for NSCLC. The results indicated that combination of XIST and HIF1A-AS1 yielded an AUC of 0.931 (95% CI: 0.869-0.990; $P < 0.001$), which was significantly improved as compared to XIST (AUC = 0.834) or HIF1A-AS1 (AUC = 0.876) alone (**Figure 5C**).

Discussion

So far, numerous studies have indicated that lncRNAs play an important role in tumor occurrence, invasion and metastasis by regulating gene expression as well as signaling pathways [23]. Currently, an increasing number of lncRNAs have been identified to be correlated with cancers. For example, lncRNA-PVT1 is increased in gastric cancer and promotes cancer cell proliferation by modulating the P15 and P16 signal pathway [24]. Metastasis associated in lung adenocarcinoma transcript 1 (MALAT1) is overexpressed in many cancers, especially in colorectal cancer. Further study demonstrates that MALAT1 promotes cancer cell proliferation, migration and invasion via PRKA kinase anchor protein 9 (AKAP-9) [25]. Moreover, MALAT1 promotes tumor growth and metastasis in colorectal cancer through binding to SFPQ and releasing oncogene PTBP2 from SFPQ/PTBP2 complex [26]. Therefore, these aberrantly expressed lncRNAs are expected to become molecular markers for cancer diagnosis and prognosis.

Some studies demonstrated that the cell-free nucleic acids, such as DNA and micro-RNA, are

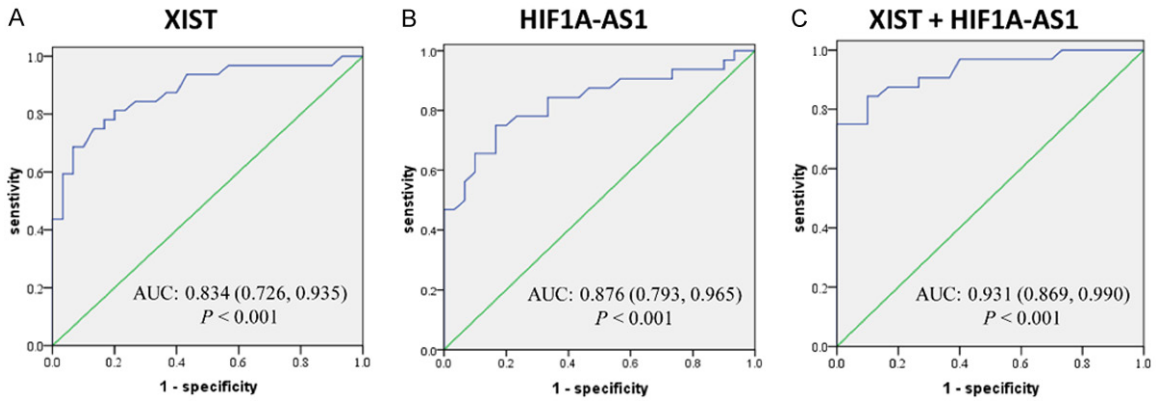

Figure 5. The ROC curve of XIST and HIF1A-AS1 expression levels in serum for NSCLC diagnosis. ROC-XIST (A) and ROC-HIF1A-AS1 (B) for detecting NSCLC from normal controls. ROC corves of a combination of XIST and HIF1A-AS1 to discriminate NSCLC from normal controls (C).

detectable in plasma and serum of cancer patients and therefore may be utilized as a tool for cancer diagnosis [27, 28]. Recent study has shown that plasma lncRNA-POU3F3 can serve as a potential biomarker for diagnosis of esophageal squamous cell carcinoma (ESCC) for early tumor screening, and the combination of lncRNA-POU3F3 and lncRNA-SCCA is more efficient for ESCC detection [6]. Moreover, lncRNA-LINC00152 can be detected in plasma, and one of the possible mechanisms of its stable existence in blood is protected by exosomes. It has the possibility to be applied in gastric cancer diagnosis as a novel blood-based biomarker [23]. Our study detected XIST and HIF1A-AS1 levels in tumor tissues and serum from NSCLC patients. We found that XIST and HIF1A-AS1 levels were significantly upregulated in tumor tissues or serum as compared to control group respectively. We used the ROC curve to analyze the diagnostic value of serum XIST and HIF1A-AS1. The results showed that the individual AUC of XIST and HIF1A-AS1 for the diagnosis of NSCLC were about 0.834 and 0.876, respectively. Intriguingly, the combination of UCA and HIF1A-AS1 could provide a more effective screening for NSCLC. The results indicated that combination of XIST and HIF1A-AS1 yielded an AUC of 0.931, which was significantly improved as compared to XIST (AUC = 0.834) or HIF1A-AS1 (AUC = 0.876) alone.

As we know, early discovery, early diagnosis, and early treatment could greatly increase the survival rate of cancer patients. Biomarkers in body fluid have a potential capacity to detect cancers in early stage [23]. Measurements obtained from tumor tissues and serum were strongly correlated for XIST and HIF1A-AS1. The results suggested that serum samples were acceptable for evaluation of NSCLC-related biomarkers. XIST as a candidate lncRNA has been shown to play an important modulatory role in the development and progression of cancer, and as a biomarker has been applied to screen hepatocellular carcinoma [29] and bladder carcinoma [30]. Moreover, it has only been confirmed that as a newly discovered lncRNA, the expression of HIF1A-AS1 is upregulated in the thoracoabdominal aorta aneurysm (TAA), and the interaction between HIF1a-AS1 and apoptotic proteins plays a key role in the proliferation and apoptosis of vascular smooth muscle cells (VSMCs) in vitro, which may contribute to the pathogenesis of TAA [16]. In our work, the results indicated that combination of XIST and HIF1A-AS1 could significantly improve the diagnostic efficiency of NSCLC.

In the present study, our results demonstrated that increased serum XIST and HIF1A-AS1 could be used as a predictive biomarker for NSCLC screening, and that combination of XIST and HIF1A-AS1 had a higher positive diagnostic rate of NSCLC than XIST or HIF1A-AS1 alone.

Disclosure of conflict of interest

None.

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