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Serum exosomal small nucleolar RNA (snoRNA) signatures as a predictive biomarker for benign and malignant pulmonary nodules

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Abstract
Low-dose CT (LDCT) is increasingly recognized as the preferred method for detecting pulmonary nodules. However, distinguishing whether a nodule is benign or malignant often necessitates repeated scans or invasive tissue sampling procedures. Therefore, there is a pressing need for non-invasive techniques to minimize unnecessary interventions. This study aim to investigate the expression profile of exosomal snoRNA in the serum of patients with benign and malignant pulmonary nodules. We identified a total of 278 snoRNAs in serum exosomes, revealing significant differences in snoRNA levels between patients with malignant and benign nodules. Specifically, the upregulated snoRNAs U78 and U37 were validated through qRT-PCR and were found significantly elevated in the serum of patients with malignant pulmonary nodules, positioning them as promising biomarkers for the early detection of lung cancer. This study underscores the potential of serum exosomal U78 and U37 as critical tools for assessing the risk of pulmonary nodules identified through CT screening.

Keywords Serum, Exosome, snoRNA, Biomarker, Benign pulmonary nodules, Malignant pulmonary nodules

Introduction

Lung cancer ranks among the top tumors worldwide, leading in cancer mortality in both advanced and less developed areas [1, 2]. Often presenting without symptoms and developing slowly, it's commonly diagnosed at late stages, with non-small cell lung cancer (NSCLC) accounting for 85% of these cases [1–3]. Treatments include surgery, radiotherapy, chemotherapy, and immunotherapy, but the outlook for NSCLC patients is bleak, as current treatments don't cure the disease [1–3]. The overall five-year survival rate for lung cancer is 22%, highlighting the critical need for more effective treatments [1, 2, 4]. Stage I NSCLC patients receiving proper treatment have a five-year survival rate between 68% and 92%, but this drops to just 14% for patients with advanced stages of the disease [1, 3–6]. Early detection via low-dose chest CT scans over the past 30 years has led to decreased incidence and improved survival rates for NSCLC patients [1, 6, 7]. The use of low-dose CT scans has increased the detection of lung nodules in routine exams, yet distinguishing benign from malignant nodules is challenging [1, 6, 7]. Decisions on further action typically rely on medical judgment or repeated low-dose CT scans at intervals to monitor nodule growth, raising concerns over radiation risk and psychological stress for patients

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[1, 6, 7]. Therefore, identifying new biomarkers to differentiate benign from malignant lung nodules is critically needed to enable more accurate and timely treatment options.

Small nucleolar RNAs (snoRNAs) are a subclass of small non-coding RNAs (ncRNAs) that interact with associated proteins to form small nucleolar ribonucleoproteins [8, 9]. They play a crucial role in the maturation and modification processes of various RNA types, including ribosomal RNAs (rRNAs), messenger RNAs (mRNAs), and small nuclear RNAs [9]. There are two distinct subtypes of snoRNAs: box H/ACA snoRNAs and box C/D snoRNAs, which are distinguished by their structural differences [9]. The former facilitates pseudouridylation catalysis of rRNAs, while the latter orchestrates 2'-O-ribose methylation [9]. Initially classified as housekeeping genes upon their discovery, snoRNAs have emerged as significant contributors to human disease pathogenesis, especially in malignancies [9]. It is worth noting that SNORD88C has been identified as a tumour promoter [10]. It guides 2'-O-methylation of 28 S rRNA to regulate SCD1 translation, thus suppressing autophagy and promoting the growth and metastasis of non-small cell lung cancer cells [10]. Knockdown of either SNORA65 or SNORA7A/B has been shown to inhibit the growth and colony formation of NSCLC cell lines [11]. Moreover, SNORA71A functions as an oncogene by promoting proliferation, migration, and invasion of lung cancer cells through the MAPK/ERK pathway in NSCLC, indicating its potential as a therapeutic target [12]. In addition, SNORD78 affects cancer stem-like cell activity and triggers epithelial-mesenchymal transition (EMT) in A549 cells [13, 14].

Exosomes, which range in diameter from 40 to 150 nm, play a crucial role in cell-to-cell communication by transporting a diverse array of molecules, including nucleic acids, lipids, and proteins [15]. Their unique bilayer membranes provide protection against RNase degradation in bodily fluids, enhancing the stability of their cargo and suggesting their potential as biomarkers for numerous diseases, particularly tumors [16]. Recent studies suggest that snoRNAs may be among the molecules packaged into exosomes [17–21]. Currently, there is insufficient data to determine whether snoRNAs encapsulated in extracellular vesicles could serve as diagnostic biomarkers for lung cancer.

The aim of our research was to characterise snoRNAs in exosomes extracted from serum obtained from age-matched individuals with malignant and benign lung nodules. We identified 278 snoRNAs in serum exosomes and found significant variations in snoRNA abundance between individuals with malignant and benign lung nodules. Our study found a significant increase in U78 and U37 in serum exosomes from malignant lung nodules compared to benign ones. This suggests that they could be valuable biomarkers for early lung cancer detection.

Materials and methods

Data set source

The snoRNAs expression data of lung cancer tissues and adjacent normal tissues were downloaded from the SNORic database (http://bioinfo.life.hust.edu.cn/SNORic) [22].

Collection of clinical samples

The study was approved by the Ethics Committee of The Fifth People's Hospital of Wuxi Affiliated to Jiangnan University (2024-016-1). The sample consisted of 73 cases of benign nodules from individuals undergoing annual physical examinations at the Department of Respiratory, The Fifth People's Hospital of Wuxi Affiliated to Jiangnan University. The Department of Oncology, under the jurisdiction of The Fifth People's Hospital of Wuxi Affiliated to Jiangnan University, collected 227 cases of stage I lung adenocarcinoma (malignant lung nodules). An equal number of male and female cases were evenly distributed across the discovery and internal validation cohorts to mitigate gender bias. Participants self-reported their gender and provided informed consent voluntarily without compensation. The subjects with benign nodules had stable evaluations during follow-up CT scans spanning 2–5 years at the time of analysis, except for one case in the external validation set. This case was collected before surgery, and a diagnosis of chronic bronchitis was confirmed through histopathological examination. Cases of lung adenocarcinoma were collected before lung resection surgery and verified through pathological diagnosis. Blood samples were collected after fasting using serum separator tubes without anticoagulants. The samples were allowed to clot at room temperature for 1 h, followed by centrifugation at 1000 x g for 10 min at 4 °C to obtain the serum supernatant. The serum aliquots were frozen at -80 °C until exosomes extraction.

Serum exosomes extraction and identification

Serum exosomes were enriched and purified using the exosomes isolation kit (130-111-572, Miltenyi Biotec) and identified by the electron microscopy (TEM) analysis, Nanoparticle tracking analysis (NTA) and western blotting according to the manufacturer's guidelines and previous report [23]. The CD63 (25682-1-AP) and GM130 (11308-1-AP) antibody used for western blotting were purchase from Proteintech (Wuhan, China).

Exosome small RNA extraction, RNA sequencing and qRT-PCR

Exosome small RNA was extracted and purified using Qiagen miRNeasy Mini Kit (Cat. No. 217004, Qiagen, Hilden, Germany) following the manufacturer's instructions. For RNA sequencing, the Illumina NovaSeq 6000 System (Illumina, San Diego, CA) were performed to sequence the small RNA libraries following the manufacturer's instructions. The sequencing data were analysed using the exceRpt pipeline, as implemented in Genboree [23, 24].

For qRT-PCR, Serum exosomes were enriched and purified using the exosomes isolation kit (130-111-572, Miltenyi Biotec) from 200ul serum. Subsequently, the exosome RNA was extracted and purified using Qiagen miRNeasy Mini Kit (Cat. No. 217004, Qiagen, Hilden, Germany). The extracted RNAs were reverse-transcribed into cDNA using the Mix-X miRNA First Strand Synthesis Kit (TaKaRa Bio, Nojihigashi), following the TB-Green Premix Ex Taq II Reagent (TaKaRa) according to the manufacturer's instruction. U6 was used as an endogenous control as previously reports [25–32]. The qRT-PCR reaction was evaluated by melting curve analysis. Standard curves were used to quantify snoRNA expression and data was normalized to U6. The relative expressions of snoRNAs were evaluated by the absolute quantification of snoRNA after U6 normalization, as described previously [27–32]. Each sample was analyzed in duplicate. The qRT-PCR primers are listed in Table 1.

Statistics

Subjects' age was analyzed by unpaired two-sample t-test and one-way ANOVA. QRT-PCR data were analyzed by two-sample t-test and one-way ANOVA followed by

Table 1 Sequence information of the primers for qRT-PCR

U78	**RT**	**GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTCTTC**
	F	TGAGCATGTAGACAAAGGTAACACT
	R	AGTGCAGGGTCCGAGGTATT
U28	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGCCAT
	F	CAAAAGTTAATAGCATGTTAGAGTTCTG
	R	AGTGCAGGGTCCGAGGTATT
U75	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCCTC
	F	AGAAGGGATTTCTGAAATTCTATTCT
	R	AGTGCAGGGTCCGAGGTATT
U83A	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCTGTT
	F	ATTCTCTACTGCCTTCCTTCTGAG
	R	AGTGCAGGGTCCGAGGTATT
U74	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCACCA
	F	TGAATGCCAACCGCTCTGA
	R	AGTGCAGGGTCCGAGGTATT
HBII-180 A	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCAGGG
	F	CCGGGGGTCTGAGGGG
	R	AGTGCAGGGTCCGAGGTATT
U37	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCTCAG
	F	GTCTACTGAAGAAAGCCTGCGT
	R	AGTGCAGGGTCCGAGGTATT
U45C	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGGTCT
	F	GATTATTACTACTTTAGCTCTAGAATTACTCTG
	R	AGTGCAGGGTCCGAGGTATT
U36B	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTCAG
	F	AAGAGTAAAACCGAGCTTTTTAACA
	R	AGTGCAGGGTCCGAGGTATT
U58C	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGGTTG
	F	GGAATAACTATGAAAGAAAACTATTCTGAG
	R	AGTGCAGGGTCCGAGGTATT
U18B	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGTCAG
	F	TGAAACACATGATATTTGTGGAAATT
	R	AGTGCAGGGTCCGAGGTATT
U6	RT	CGCTTCACGAATTTGCGTGTCAT
	F	GCTTCGGCAGCACATATACTAAAAT
	R	CGCTTCACGAATTTGCGTGTCAT

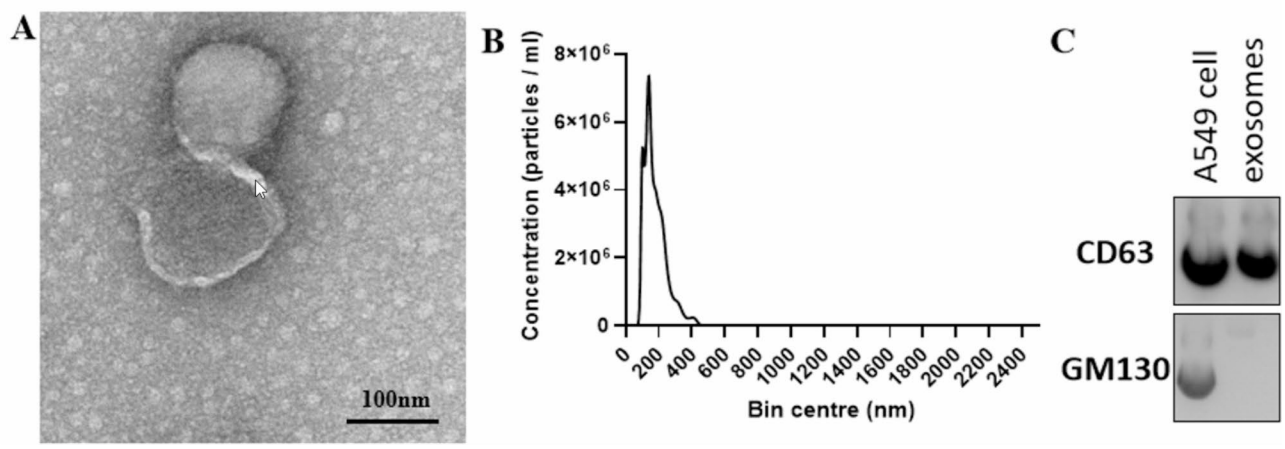

Fig. 1 Identification of serum exosomes isolated by the exosomes isolation kit. **(A)** Structural characteristics of exosomes under TEM. **(B)** Exosome diameter was detected by NTA. **(C)** Exosome specific protein CD63 and Golgi protein GM130 was detected by Western blotting

Table 2 Characteristics of benign and malignant lung nodules

Features	Benign PNs ($n=73$)	Malignant PNs ($n=227$)	p
Sex			0.914
Male	50	157	
Female	23	70	
Age	50.35 ± 12.66	52.58 ± 13.55	0.845
Smoking			0.698
Yes	58	185	
No	15	42	
Histologic diagnosis			
Lung adenocarcinoma		227	
Granuloma	15		
Infammation	18		
Hamartoma	11		
Dysplasia	15		
Other	14		

Tukey's post hoc multiple comparisons test. SnoRNAs were considered significantly differentially enriched when $p<0.05$. All t-tests or ANOVAs were performed using GraphPad Prism, v8.4.2.

Results

Isolation and identification of serum exosomes

The microvesicles derived from serum of the benign nodules and lung adenocarcinoma patients were characterized by NTA, TEM, and Western blot analysis after isolation. TEM revealed that exosomes had a spherical morphology with an average size distribution of 100 nm (Fig. 1A). NTA further confirmed the average size distribution with 100 nm (Fig. 1B). The enrichment of exosomes was further confirmed by Western blot, which showed the presence of the exosome marker CD63 and the absence of GM130 (a Golgi protein) (Fig. 1C).

Identification and differential analysis of snoRNAs in serum exosomes of malignant and benign lung nodules

Exosomes were isolated from the serum of randomly selected 7 patients with malignant lung nodules and 7 patients with benign lung nodules (Table 2). The RNA-seq sequencing data were analyzed using the exceRpt pipeline for the snoRNA expression profile [23, 24]. The expression patterns of exosomal snoRNAs in the serum of malignant and benign lung nodules were found to differ significantly, as confirmed by the heatmap (Fig. 2A). Principal component analysis (PCA) was also used to determine the expression of exosomal snoRNAs in the serum, and showed the snoRNAs in the serum exosomes could distinguish between benign and malignant lung nodules with complete accuracy (Fig. 2B). The volcano plot was used to visualize the differentially expressed exosomal snoRNAs between benign and malignant lung nodules (Fig. 2C). The analysis using exceRpt revealed 255 snoRNAs across the 14 samples. To further screen the biomarkers for the malignant lung nodules, we selected genes with an average count greater than 100, a fold change greater than 2, and a p-value less than 0.05 for further study. We identified 11 snoRNAs that passed this cut-off (Table 3). All 11 snoRNAs were box C/D snoRNAs (Table 2).

Verification of differentially enriched snoRNAs in serum exosomes of an independent group of malignant and benign lung nodules

To improve the reliability of the sequencing data, we conducted a qRT-PCR assay to identify the 11 significantly overexpressed snoRNAs in 20 patients with malignant lung nodules and 20 patients with benign lung nodules in the training stage (Table 2). As illustrated in Fig. 3A, exosomal U78 and U37 exhibited significant overexpression in NSCLC, while the other 9 evaluated snoRNAs did not show any significant differences (Fig. 3A). To further

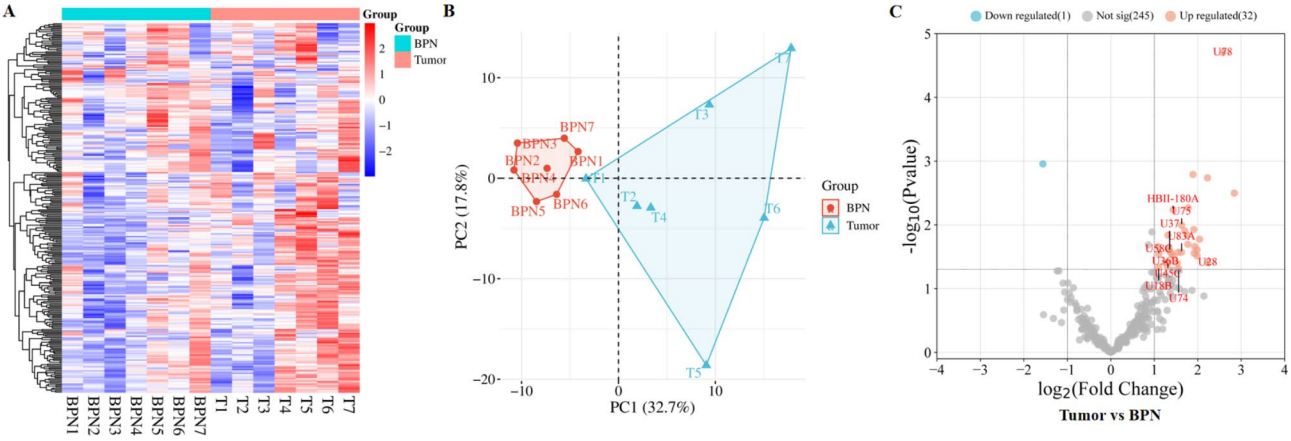

Fig. 2 Serum exosomal snoRNA expression profile in benign and malignant lung nodules. **(A)** The heat map of exosomal snoRNA expression profile in benign (BPN) and malignant lung nodules (T). **(B)** The PCA analysis of exosomal snoRNA expression profile in benign (BPN) and malignant lung nodules (T). **(C)** The volcano map of exosomal snoRNA expression profile in benign (BPN) and malignant lung nodules (Tumor)

Table 3 The upregulated snoRNA in the exosomes of benign and malignant lung nodules

snoRNA	Type	log2FoldChange	*p*-value
U78	C/D	2.575730946	1.98611E-05
U28	C/D	2.228789614	0.039170736
U75	C/D	1.628626085	0.010160565
U83A	C/D	1.627131485	0.026931507
U74	C/D	1.558597992	0.049100033
HBII-180 A	C/D	1.43368837	0.005811596
U37	C/D	1.355009405	0.025671157
U45C	C/D	1.313701209	0.038808089
U36B	C/D	1.255498475	0.038693444
U58C	C/D	1.103087207	0.028983979
U18B	C/D	1.100406986	0.046330309

investigate the variance in expression levels of U78 and U37 in lung cancer, we downloaded the data of snoRNA expression in the lung tumor tissue and adjacent tissue from SNORic database [22]. The analysis indicates a significant increase in U78 and U37 within tumour tissue (Fig. 3B), suggesting that U78 and U37 should be further investigated as potential candidates.

The validity of the results of the training stage was verified in an independent group of 200 patients with malignant lung nodules and 46 patients with benign lung nodules using the qRT-PCR assay (Table 2). As depicted in Fig. 4A and B, there was a significant upregulation in both U78 and U37 for patients with malignant lung nodules compared to those with benign lung nodules. To identify predictors for malignant lung nodules, Receiver Operating Characteristic (ROC) curves were calculated for U78 and U37. The AUC values for U78 and U37 were 0.9316 and 0.8434, respectively (Fig. 4C). The sensitivity and specificity were 94.93% and 84.78% for U78, and 92.01% and 76.09% for U37. The combined AUC value calculated for both U78 and U37 was 0.9678 with 100% sensitivity and 91.3% specificity (Fig. 4C).

Changes of U78 and U37 in the exosomes before and after surgery

To confirm the potential downregulation of U78 and U37 in exosomes after tumour removal, we analysed exosomal snoRNAs from the serum of 40 LUAD patients before and one week after surgery. Our findings showed a significant reduction in both U78 and U37 levels in serum exosomes seven days post-surgery compared to pre-surgery levels (Fig. 5A-B). In addition, we examined the correlation between these two snoRNA levels and disease progression-free survival (PFS). Our analysis revealed a strong association between higher snoRNA levels and shorter disease progression-free survival in patients (Fig. 6A-B). These results suggest that U78 and U37 within exosomes could be promising prognostic biomarkers for LUAD.

Discussion

Each year, over one million people worldwide receive a diagnosis of pulmonary nodules, a significant proportion of which are malignant [33]. It is crucial to promptly identify the nature of these nodules to select the most appropriate treatment strategies, ensure timely intervention in malignant cases, and avoid unnecessary treatment for benign nodules [33]. Although current methods for detecting pulmonary nodule tissue biopsies are effective, they have limitations such as invasiveness, false negatives, and the risk of tumor spread [33]. Therefore, there is a pressing need for a detection method that is safer, simpler, and more reliable. Recent advancements in testing techniques have uncovered valuable biomarkers that aid in the diagnosis of patients with pulmonary nodules. Research has shown that combining VEGF-C expression in plasma with CT or PET scans can enhance the diagnostic process for pulmonary nodules [33]. Additionally, utilizing a combination of multiple biomarkers such as

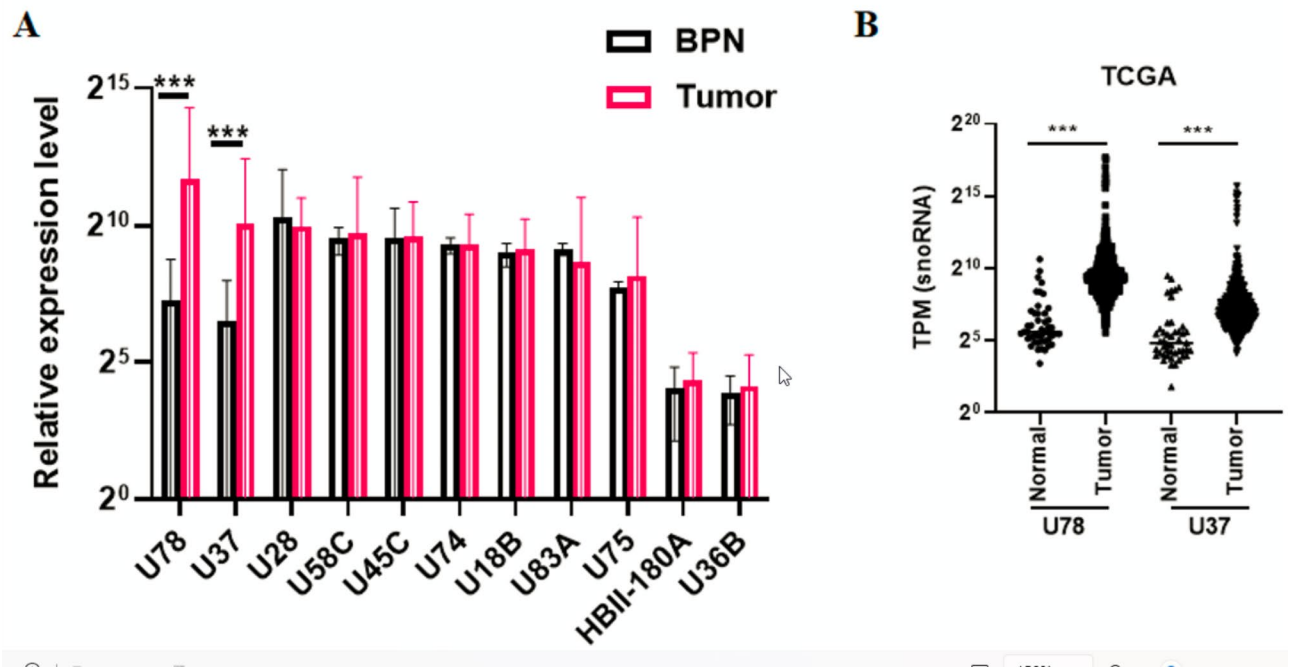

Fig. 3 The eleven upregulated exosomal snoRNA expression profile in benign (BPN) and malignant lung nodules (Tumor) (**A**) and the expression level of U78 and U37 in the lung tumor tissue and adjacent tissue from SNORic database (**B**). The mean differences between diagnostic groups were analyzed by Student's t test. ***$P < 0.001$

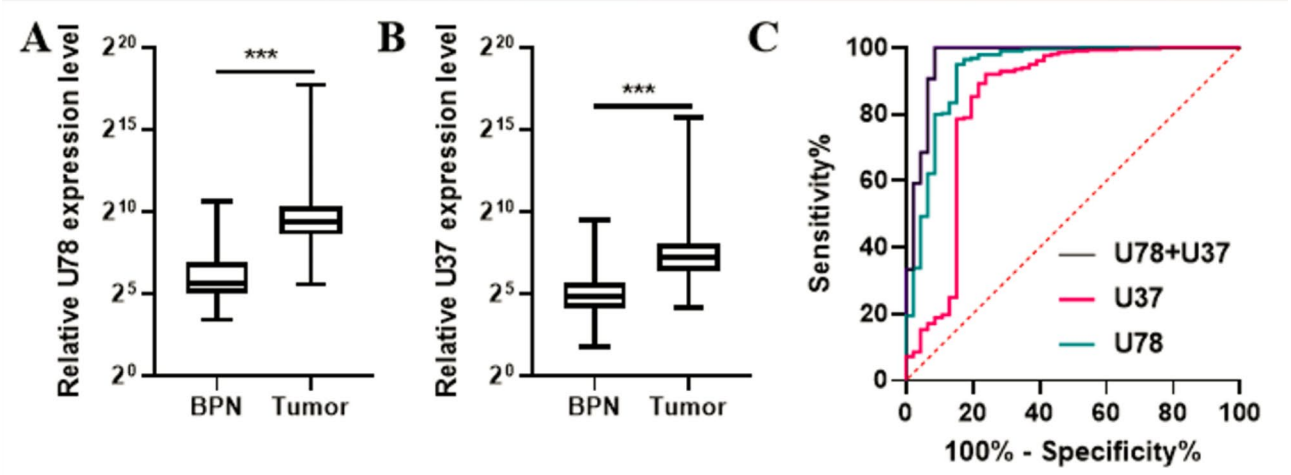

Fig. 4 The expression level and ROC curve analysis of exosomal U78 and U37. **A-B)** The expression level of exosomal U78 and U37. **C)** The ROC curve analysis for exosomal U78, U37 and the combination of U78 and U37. The mean differences between diagnostic groups were analyzed by Student's t test. ***$P < 0.001$

Neuron-specific enolase (NSE), cytokeratin fragment antigen 21−1 (CYFRA21-1), and carcinoembryonic antigen (CEA) can aid in distinguishing between benign and malignant pulmonary nodules [33]. Although these serological markers have been used clinically, their specificity is still not optimal, and their role in early identification of pulmonary nodules is still a subject of debate [33].

Exosomes are the smallest type of extracellular vesicles, ranging from 50 to 100 nm in diameter [15]. They are composed of a phospholipid bilayer and are secreted by various cell types, including cancer cells, into bodily fluids such as blood, plasma, urine, cerebrospinal fluid (CSF), amniotic fluid, and saliva [15, 16]. Exosomal vesicles transport a variety of molecules, such as DNA, mRNA, non-coding RNA, proteins, and lipids [15]. They serve as stable molecular signatures of their cell of origin, making them suitable candidates for cancer biomarkers. Exosomal vesicles play pivotal roles in intracellular

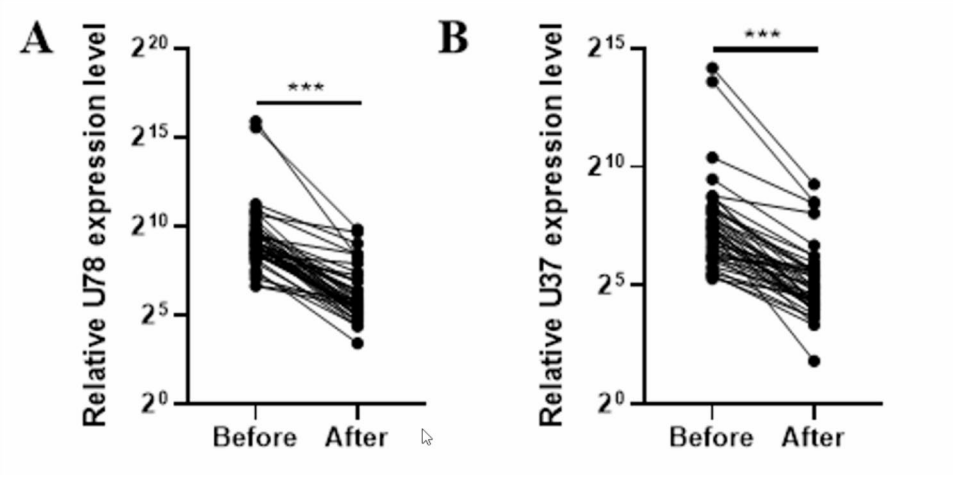

Fig. 5 The expression level of exosomal U78 and U37 in the serum of lung cancer before and one week after surgery

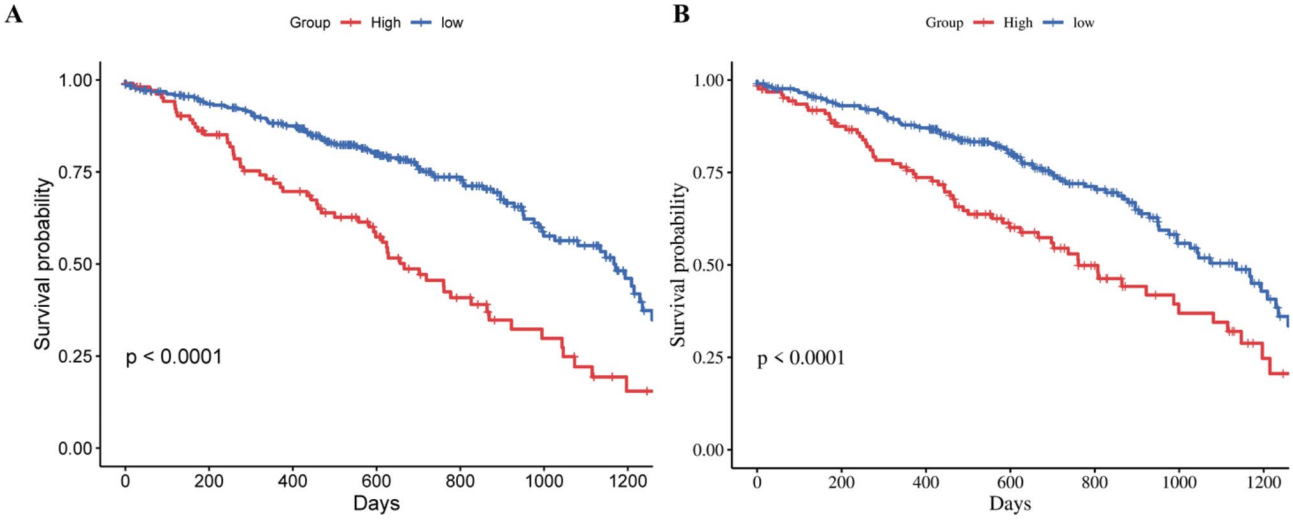

Fig. 6 The correlation between the expression level of exosomal U78 (**A**) and U37 (**B**) and disease progression-free survival

communication, cell signaling, immune response modulation, cancer development, and organ-specific metastasis [15]. Exosomal microRNAs and proteins are potential biomarkers for distinguishing between benign and malignant pulmonary nodules [34–36]. Recent studies have identified FGB and FGG as potential novel biomarkers through significant differences in plasma exosomal proteomics [36]. Additionally, the serum exosomal miR-21/Let-7a ratio shows promise in distinguishing non-small cell lung cancer from benign pulmonary diseases [34]. However, research on whether small nucleolar RNAs (snoRNAs) in extracellular vesicles can differentiate between benign and malignant pulmonary nodules is limited. This study investigates the variance of snoRNA content in serum exosomes between benign and malignant lung nodules using RNA sequencing and quantitative PCR for the first time. The study found significant differences in the levels of snoRNAs, specifically U78 and U37, in exosomes. This suggests that these snoRNAs could be useful biomarkers for distinguishing between benign and malignant lung nodules.

Numerous studies have demonstrated the association between snoRNA dysregulation and cancer progression, including lung cancer [8, 37]. In this investigation, we observed a significant elevation of U78 and U37 in exosomes present in the serum of patients with malignant pulmonary nodules. To further investigate the variance in expression levels of U78 and U37 between tumor tissue and adjacent tissue, we analyzed the SNORic database. The analysis, shown in Fig. 3B, indicates a significant increase in U78 and U37 within tumour tissue, suggesting their potential importance in the initiation and progression of lung cancer. Di Zheng et al. also confirmed the upregulation of SNORD78 in cancer tissues compared to adjacent normal tissues, highlighting its role as an oncogene that promotes cancer cell proliferation,

invasion, and self-renewal [13]. Tumour cells communicate with their microenvironment, including immune cells and fibroblasts, through exosome-mediated RNA secretion [15]. Several studies have revealed small nucleolar RNAs could be secreted via exosomes and direct the 2'-O-methylation in the target cells [8, 18]. Therefore, we hypothesise that U78 and U37 within exosomes may contribute to the progression of malignant pulmonary nodules. However, further investigations are required to validate this hypothesis.

This study has several limitations that require consideration. Firstly, the sample size is relatively small due to various reasons. To confirm whether U78 and U37 in exosomes can serve as biomarkers for distinguishing benign and malignant pulmonary nodules, a larger sample size is necessary for further validation. Secondly, prior investigations have explored the variance between snoRNA in plasma and normal lung cancer [10–14, 27, 31, 38–41]. Wang et al. reported a significant increase in plasma levels of SNARD42B and SNARD111 in patients with non-small cell lung cancer and early-stage non-small cell cancer [27]. This contradicts the differential expression of snoRNAs in serum exosomes as identified in our study. The authors noted that SNORD42B and SNORD111 are present not only within exosomes but also freely in plasma, indicating diversity in snoRNA distribution within serum or plasma [27]. Further investigation is required to determine whether U78 and U37 exhibit distribution patterns similar to those of SNORD42B and SNORD111.

The study demonstrates that snoRNAs, a major class of ncRNAs, specifically U78 and U37, are enriched in exosomes isolated from serum of malignant lung nodules compared to benign lung nodules. Although, the outcomes of much larger independent validation studies are difficult to predict, the results presented here point, at least, serum exosomal snoRNA U78 and U37 could serve as a predictive biomarker for malignant lung nodules.

Author contributions

YZ and FZ designed the experiments. FC and QY performed the experiments and analyzed the results. YZ and FZ wrote the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

The studies involving animal experiments were reviewed and approved by the Ethics Committee of Soochow University.

Declaration of AI-assisted technologies in the writing process

During the preparation of this work the authors used chatGPT in order to improve language and readability. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Competing interests

The authors declare no competing interests.

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