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ORIGINAL ARTICLE

Plasma Exosomal CXCL7 is a Potential Biomarker for Lung Adenocarcinoma

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SUMMARY

Background: **Lung cancer is a leading cause of cancer-related death, with lung adenocarcinoma (LUAD) representing the most common subtype. Recently, exosome-based biomarkers have provided new diagnostic approaches for malignancies.**
Methods: **The differential expression profile of plasma exosomal mRNA was established by high-throughput sequencing, and the expression and diagnostic value of plasma exosomal CXCL7 mRNA and protein in LUAD were studied to evaluate their diagnostic value as tumor biomarkers.**
Results: **The expression of plasma exosomal CXCL7 mRNA in patients with LUAD was significantly increased ($p < 0.01$), which had no significant correlation with age, gender, and stage. ROC was used to evaluate the diagnostic value of plasma exosomal CXCL7 mRNA in LUAD patients with AUC = 0.7171. Further analysis signified that the CXCL7 protein of plasma exosomes in LUAD patients was overexpressed, and it was positively correlated with TNM stage and age. The diagnostic value of plasma exosomal CXCL7 in LUAD is better than serum CEA, with an AUC of 0.785, which has higher sensitivity and specificity.**
Conclusions: **This research suggests that plasma exosomal CXCL7 may become an effective biomarker for early diagnosis of LUAD.**
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KEYWORDS

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INTRODUCTION

Lung cancer has long become the major cause of cancer death in the world. Lung cancer is classified into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) because of the significant distinctions in histological types [1,2]. Lung adenocarcinoma (LUAD) is the most common pathological subtype of NSCLC, and it is usually at advanced stages with the poor prognosis when it was diagnosed [3]. At present, low-dose computed tomography (LDCT) is familiarly used clinically to detect tiny lung cancers, while its high false

positive rate may bring about over diagnosis and treatment. In addition, as the supplementary screening method for LDCT, serum markers such as carcinoembryonic antigen (CEA) and cytokeratin fragment antigen 21-1 (CYFRA21-1) still lack sufficient sensitivity and specificity [4]. Therefore, it is necessary to identify rapid and effective indicators for early diagnosis of LUAD.

Exosomes are vesicles with diameter of 30 - 100 nm, which can be secreted by many different types of cells, including immune cells, mesenchymal cells, and cancer cells. They contain proteins, lipids, nucleic acids, and other substances, which can shuttle from donor cells to recipient cells and mediate intercellular communication [5]. Emerging evidence has identified that biomarkers in exosomes serve as diagnostic markers for a variety of cancers, including lung cancer [6,7]. Previous studies have shown that CXCL7 is a member of the CXC chemokine family, which is a platelet-derived growth factor that promotes a variety of cellular processes by binding to its receptor CXCR2 [8,9]. A series of studies indicated that CXCL7 could be the diagnostic marker for lung cancer, colorectal cancer, and hepatoblastoma [10-12]. Furthermore, CXCL7 from serum-purified exosomes can also be used with the diagnosis of oral squamous cell carcinomas [13]. Nevertheless, these studies of CXCL7 were primarily focused on tissue or blood samples, the value of CXCL7 in plasma exosomes as a diagnostic and predictive biomarker for lung cancer has not been explored so far.

In the present study, RNA sequencing and quantitative real-time polymerase chain reaction (qRT-PCR) were used for analysis and discovered that the expression of CXCL7 mRNA was up-regulated significantly. Subsequently, we further expanded the sample size and applied qRT-PCR and enzyme linked immunosorbent assay (ELISA) to detect the expression of CXCL7 mRNA and protein in plasma exosomes. Besides, we also analyzed the relationship between the plasma exosomal CXCL7 expression level and the clinical characteristics of LUAD patients, and used receiver operating characteristic curve (ROC) analysis to evaluate its diagnostic efficacy.

MATERIALS AND METHODS

Clinical information

The 70 pre-treatment plasma samples of patients diagnosed as LUAD by histopathology in Fujian Provincial Hospital between August 2019 and August 2020 were collected as the case group. The case group included 37 males and 33 females, aged from 36 to 82 years with an average of 55.14 years. According to the 2010 Union for International Cancer Control (UICC)/American Joint Committee on Cancer (AJCC) TNM staging standard, the enrolled patients were staged, of which 46 were in stage I-II and 24 were in stage III-IV. In addition, 30 plasma specimens from healthy medical examiners in our hospital who did not have any systemic diseases, lung lesions or other tumors during this time period were collected as the control group. The control group consisted of 14 females and 16 males, aged from 35 to75 years with an average of 42.36 years. The clinical data of all participants were completely collected and organized (Table 1). The Medical Ethics Committee of Fujian Provincial Hospital approved this study.

Isolation and identification of plasma exosomes

On the basis of instructions furnished by the manufacturer, the total exosomes kit (exoRNeasy SP Maxi Kit-76064, Qiagen, Germany) was used to separate exosomes from plasma. In order to judge whether the exosomes are separated successfully, we not only observed the morphology of exosomes under a transmission electron microscope (TEM), but also detected the expression of exosomes-specific proteins CD9 and CD63 by western blot (WB).

RNA sequencing analysis of plasma exosomes

The high-throughput sequencing analysis of plasma exosomal mRNA was completed by CloudSeq Biotech (Shanghai, China). Clean reads are filtered from raw reads and used for comparison with human reference genome/transcriptome. Under the guidance of the gtf gene annotation file, the expression level of mRNA transcripts was calculated based on the number of fragments per kilobase of transcript per million mapped reads (FPKM), and the differentially expressed mRNA was identified. In addition, the R language clustering analyzer package is used to perform gene ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis on mRNA.

Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR was applied to verify the five genes with the highest differential expression in the sequencing data. Moreover, further expanding the sample size for analyzing the target gene CXCL7. According to the reagent manufacturer's instructions, we used PrimeScript RT Reagent Kit (Takara, Dalian, China) reverse transcription to synthesize template complementary DNA (cDNA). The CXCL7 mRNA expression level was detected by the LightCycler480 fluorescent quantitative PCR system and the TB Green Premix Ex Taq II (Tli RNaseH Plus) kit. With GAPDH as an endogenous control, the relative expression of CXCL7 mRNA was calculated by the 2-ΔΔCt method.

Enzyme-linked immunosorbent assay (ELISA)

We referred to the reagent manufacturer’s protocol and used the human neutrophil chemoattractant protein 2 (NAP-2/CXCL7) enzyme-linked immunoassay kit to quantitatively detect the expression of CXCL7 protein. With the aid of the regression curve, the concentration of CXCL7 protein was calculated based on the absorbance measured by the microplate reader.

Table 1. Clinical characteristics of study subjects.

Characteristics	RT-PCR cohort		ELISA cohort	
	LUAD (n = 70)	Controls (n = 30)	LUAD (n = 64)	Controls (n = 30)
Age, years [a]	55.14	42.36	55.56	41.99
Gender (n)				
Female	33	16	27	17
Male	37	14	37	13
Stage (%)				
Stage I - II	46	15	48	
Stage III - IV	24		16	
CEA (ng/mL) [a]	2.65	1.78	2.75	1.60

[a] Age data are presented as the mean (SD).

Table 2. Correlation between CXCL7 expression and clinicopathologic features in LUAD Patients.

Variable	Patients (n)	log2 (relative expression)	p-value
Age (years)			
> 60	49	2.192 ± 0.2191	0.2602
≤ 60	21	2.683 ± 0.4189	
Gender			
Male	33	2.035 ± 0.206	0.1484
Female	27	2.611 ± 0.3237	
TNM stage			
I - II	46	2.165 ± 0.2318	0.2275
III - IV	24	2.673 ± 0.3685	

Determination of CEA in serum by electrochemiluminescence method

The CEA expression level in the serum was quantitatively analyzed on a Cobas E601 (Roche Diagnostics, Switzerland) by using the original kit reagents and following the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using SPSS Statistics 22.0 (IBM SPSS Statistics for Windows; IBM Corp., Armonk, NY, USA) and GraphPad Prism 5.0 software (GraphPad software, La Jolla, CA, USA). All measurement data are expressed as mean and standard deviation (SD). Chi-squared test was utilized to analyze the correlation between CXCL7 expression and clinicopathological characteristics. The diagnostic value of CXCL7 in LUAD was evaluated by drawing receiver operating characteristic (ROC) curve. $p < 0.05$ was considered statistically significant.

RESULTS

Identification of plasma exosomes

We observed the plasma exosomes isolated from LUAD patients and healthy controls with a transmission microscope and found spherical vesicles with a diameter of 40 - 100 nm, which were consistent with the morphological characteristics of the exosomes (Figure 1A). In addition, WB results exhibited that the specific exosomal marker proteins CD9 and CD63 were highly expressed in plasma exosomes (Figure 1B). Based on the above results, it can be determined that exosomes are effectively extracted from plasma.

Analysis of mRNA expression profile of plasma exosomes

The analysis of mRNA sequencing data demonstrated that compared with healthy controls, plasma exosomes of LUAD patients contained 3,315 differentially expressed mRNAs, of which 994 exosomal mRNAs were up-regulated and 2,321 exosomal mRNAs were down-

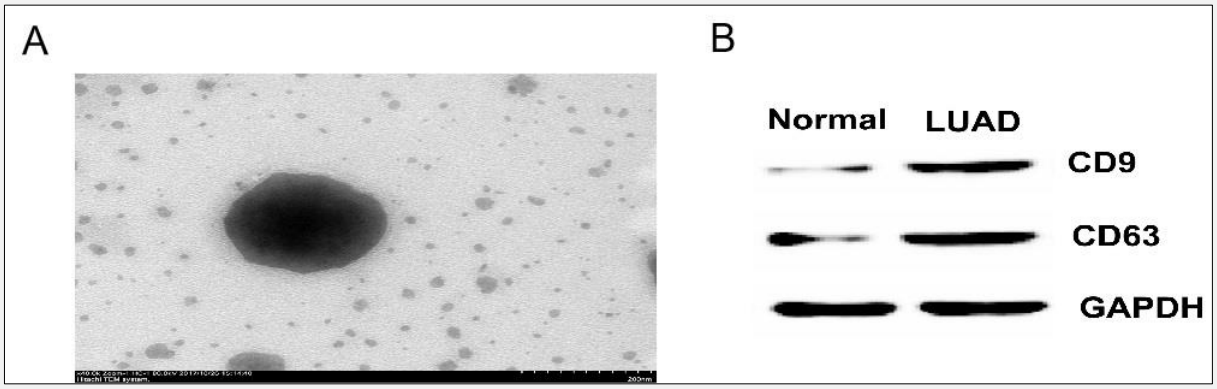

Figure 1. Identification of plasma exosomes.

A. The shape of plasma exosomes under TEM. B. The specific exosomal marker proteins CD9 and CD63 were validated by using WB. TEM - transmission electron microscopy, WB - western blotting.

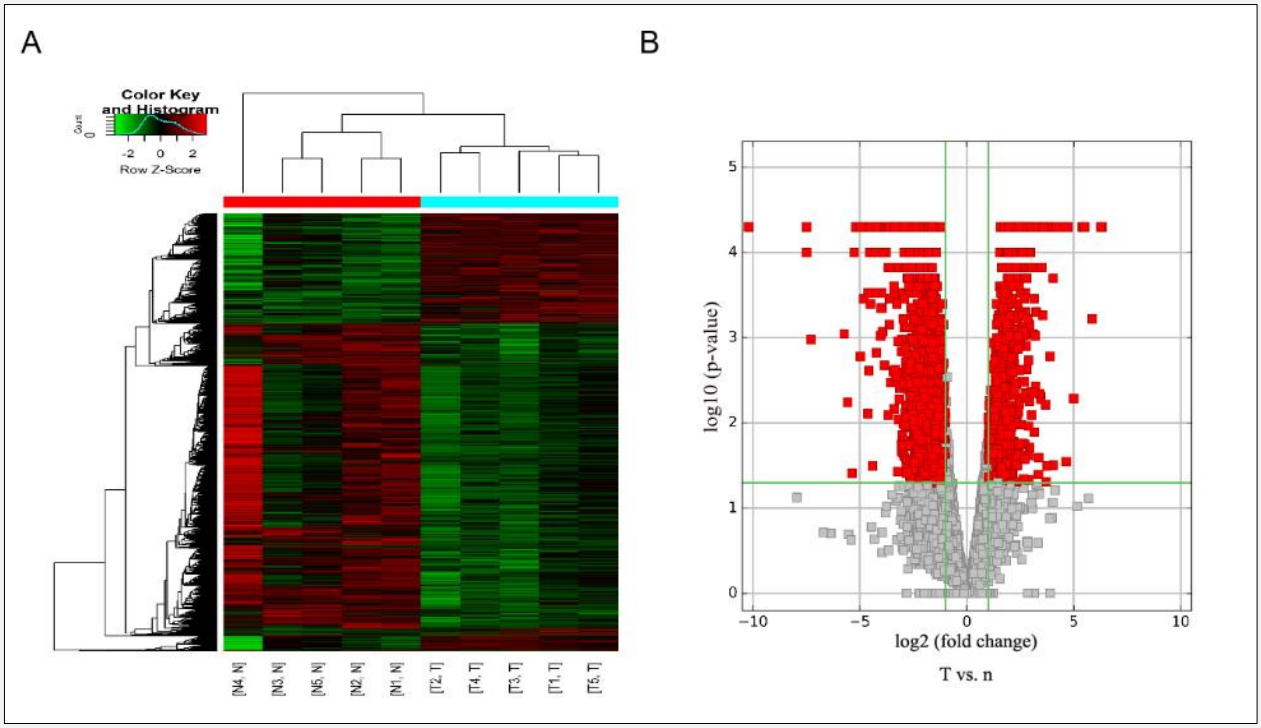

Figure 2. Analysis of differentially expressed mRNAs in plasma exosomes from LUAD patients compared to controls by RNA sequencing.

A. Hierarchical clustering presents a distinct expression profile of mRNAs between the two groups. B. Scatter plots are used to access the changes in mRNAs expression between the two groups. The mRNAs above and below the green line represent more than 2.0-fold changes between two groups.

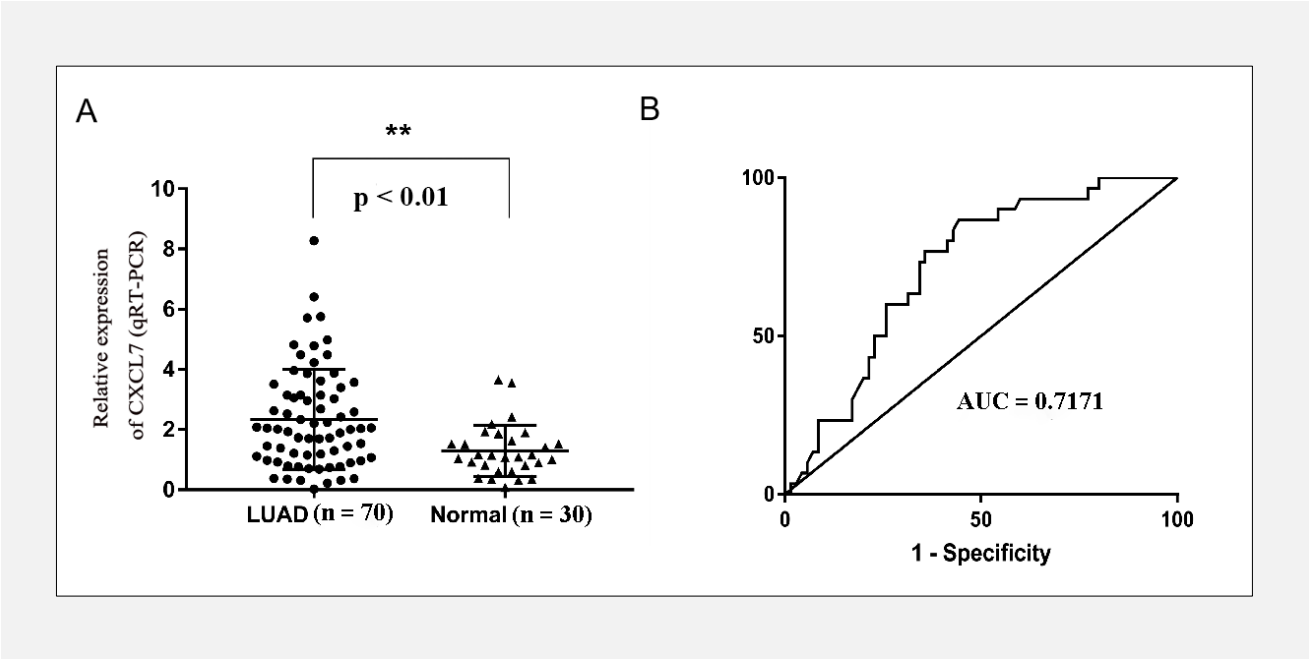

Figure 3. Plasma exosomal CXCL7 mRNA as a novel marker for LUAD.

A. Relative levels of exosomal CXCL7 mRNA in LUAD patients compared to controls. B. ROC curve analysis for CXCL7 mRNA in LUAD patients versus healthy controls. ** p < 0.01.

regulated (fold change > 2; p < 0.05). The results of hierarchical cluster analysis and volcano map displayed that the differentially expressed exosomal mRNAs were significantly agglomerated and differentiated in LUAD patients, which was completely different from the healthy control group (Figure 2A, 2B). In addition, GO functional analysis illustrated that in LUAD patients, the differentially up-regulated exosomal mRNAs participate in related processes of the immune system, as well as play a role in molecular functions such as ribosome structure, protein binding, and cell adhesion. They also mediate the cellular functions of vesicles, extracellular matrix of exosomes, and extracellular vesicles. Enrichment analysis of the KEGG signaling pathway also showed that the up-regulated differentially expressed exosomal mRNA is involved in the regulation of ribosomes, HIF-1, and cell adhesion molecules and other signaling pathways.

The expression and diagnostic value of plasma exosomal CXCL7 mRNA in LUAD

The consequences of qRT-PCR analysis indicated that the expression of exosomal CXCL7 mRNA in the LUAD group was up-regulated markedly compared with the control group (p < 0.01) (Figure 3A). However, there was no significant correlation between the expression of CXCL7 mRNA in exosomes and clinical characteristics such as age, gender, and TNM stage (p > 0.05) (Table 2). Further ROC curve analysis signified that the area under the curve (AUC) of plasma exosomal CXCL7 mRNA was 0.7171 (Figure 3B).

Expression of plasma exosomal CXCL7 protein in LUAD and its diagnostic value

The ELISA test results evidenced that the expression of plasma exosomal CXCL7 protein in LUAD patients was higher than that in the healthy control group (p < 0.001) (Figure 4A). Correlation analysis of clinical characteristics showed that the expression of plasma exosomal CXCL7 protein was not overtly different in gender (p > 0.05) (Figure 4B), but it was significantly related to age and TNM stage (p < 0.05) (Figure 4C, 4D). The expression of plasma exosomal CXCL7 protein was not only higher in late LUAD than in early stage, but also increased conspicuously with the increase of patients' age. In addition, in the ROC curve analysis, it was also found that the AUC of the plasma exosomal CXCL7 protein was 0.785, with the diagnostic sensitivity and specificity of 76.67% and 71.88%, respectively (Figure 4E). We also tested CEA, a commonly used serum marker for LUAD screening, and the outcomes indicated that its expression in LUAD was increased compared with normal controls, and the AUC was 0.6734 (p < 0.05) (Figure 4F, 4G). By comparison, we discovered that the diagnostic efficiency of plasma exosomal CXCL7 protein for LUAD is better than that of serum CEA.

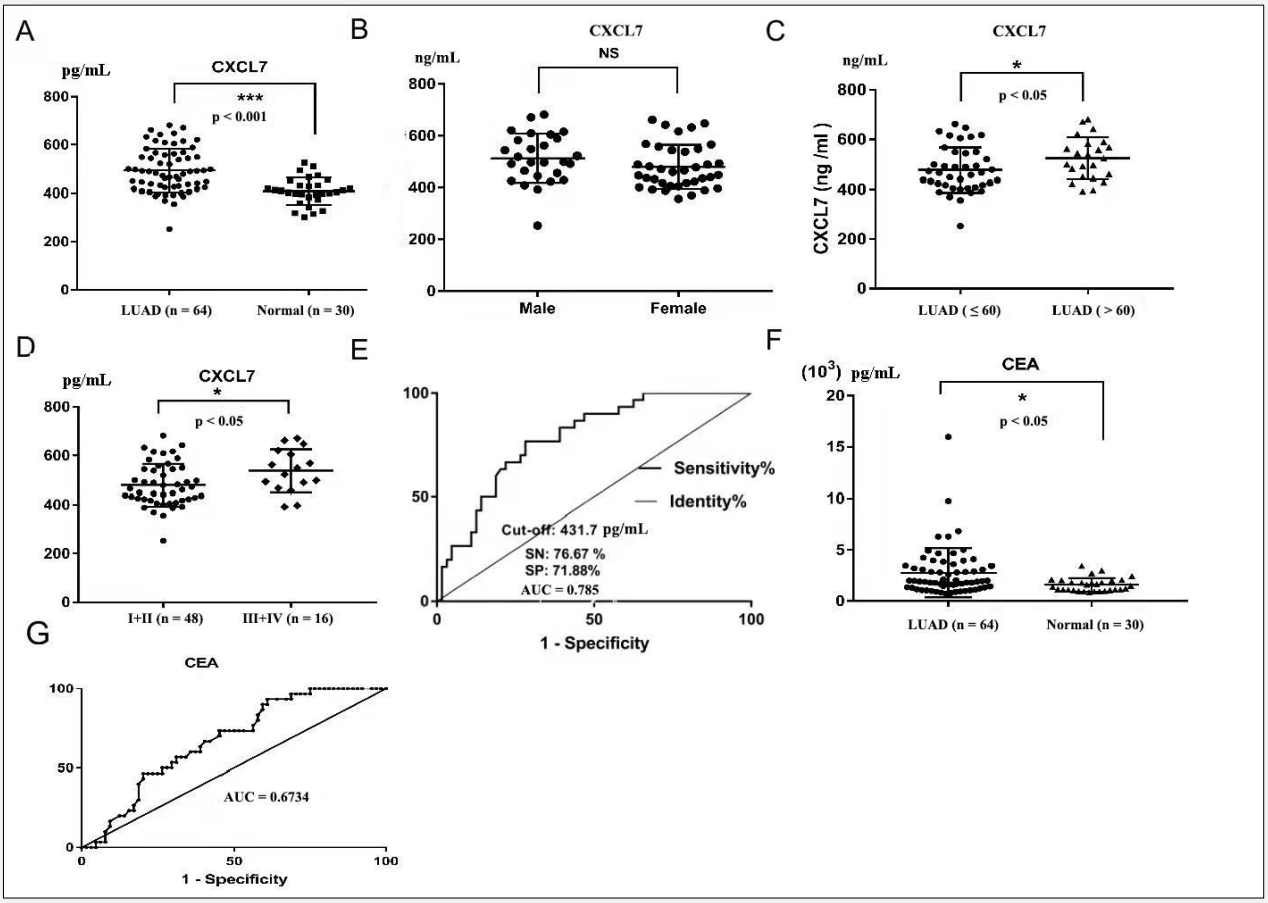

Figure 4. Plasma exosomal CXCL7 as a novel marker for LUAD.

A. Relative levels of exosomal CXCL7 in LUAD patients compared to controls. B, C, D. Correlation between Plasma exosomal CXCL7 Levels and clinical characteristics in LUAD patients. E. ROC analysis to discriminate LUAD patients from healthy controls according to plasma exosomal CXCL7 Levels. F. Relative levels of serum CEA in LUAD patients compared to controls. G. ROC analysis to discriminate LUAD patients from healthy controls according to serum CEA.
*** p < 0.05, ** p < 0.01, *** p < 0.001.**

DISCUSSION

Exosomes are vesicles with a diameter of 40-100 nm formed by budding endosomes, which are released into the extracellular environment after fusion with the plasma membrane. [14,15]. Exosomal membranes can protect the various functional proteins, DNA, and RNA carried within the exosome from degradation by circulating enzymes [16,17]. However, a major defect of serum markers commonly used in lung cancer diagnosis is that they are easily damaged by the external environment. The emergence of exosomes can make various biomarkers stable in the circulation [18]. Tumor cells also can secrete a large number of exosomes, which promote tumor progression by participating in tumor angiogenesis, cell proliferation, and metastasis [19-21]. Exosome-derived miR-1246 contributes to proliferation and migration of metastatic breast cancer cell by regulating the down-regulation the expression of Cyclin-G2 (CCNG2) [22]. Hsa-miR-199a-3p in exosomes secreted by neuroblastoma cells promotes cell proliferation via inhibiting its target gene NEDD4 [23]. It can be seen that exosomes are expected to become a non-invasive marker for early cancer screening and diagnosis [24]. Tao et al. hold that exosomal lncRNAs TBILA and AGAP2-AS1 have promising diagnostic capabilities for NSCLC patients [4]. Previous studies of our group have also affirmed that microRNAs and circRNAs in exoso-

mes are potential biomarkers of lung cancer, which laid a reliable theoretical and experimental foundation for this research [3,25].

In present study, by analyzing the sequencing data of plasma exosomal mRNAs in LUAD patients, it was discovered that the differentially expressed exosomal mRNAs were significantly enriched in tumor-related ribosomes and cell adhesion pathways. Exosomal mRNA has attracted much attention from the scientific community for many years. In addition to being enriched in tumor-related pathways, the level of exosomal mRNA has also increased in a variety of cancers [26, 27]. Our further verification illustrated that among the many differentially expressed exosomal mRNAs, exosomal CXCL7 mRNA may be the best potential marker in LUAD. Practically, CXCL7 is a platelet-derived growth factor in the CXC chemokine family, which is produced and stored in platelets, white blood cells, and tumor cells [9,28,29]. Recent studies have found that serum CXCL7 may be a diagnostic biomarker for obstructive colorectal cancer [30]. Plasma CTAPIII/ CXCL7 levels have latent value in the early diagnosis of non-small cell lung cancer [10]. Nevertheless, there is no research report on CXCL7 derived from plasma exosomes in LUAD. In this study, the presence of CXCL7 mRNA in plasma exosomes of LUAD patients was detected for the first time, and the level of CXCL7 mRNA was significantly higher than that of the control group. Moreover, ROC curve analysis showed that the AUC of plasma exosomal CXCL7 mRNA was 0.7171, which has a good diagnostic value in LUAD.

Although the diagnostic efficacy of CXCL7 mRNA has been established, genes are often transcribed into mRNA and then translated into proteins to perform their corresponding functions. Due to the regulation of many factors during the transcription and translation process, the mRNA expression level cannot fully represent the expression of the protein [31]. Therefore, we further performed an ELISA test, and the results showed that the expression of CXCL7 protein in plasma exosomes of LUAD patients was also significantly up-regulated. Similar results have been supported by the work of other scholars. The increased levels of CXCL7 mRNA and protein in malignant breast cells can help improve the aggressiveness of tumors [32]. Besides, CXCL7 protein is significantly up-regulated in hepatoblastoma and is closely related to clinical staging [12]. We also found in the analysis of the correlation between CXCL7 expression and the clinical characteristics of LUAD, that the overexpression of CXCL7 protein in plasma was positively correlated with TNM stage and age. Furthermore, ROC curve analysis evidenced that the AUC of plasma exosomal CXCL7 protein was 0.785, which was accompanied by higher diagnostic sensitivity and specificity, reaching 76.67% and 71.88%, respectively. In recent years, a variety of exosomal markers have clearly demonstrated their diagnostic effect in LUAD. The AUC of exosomes piR-hsa-26925 and piR-hsa-5444 in LUAD patients are only 0.751 and 0.713, respectively [33]. After comparing the results, it can be affirmed that plasma exosomal CXCL7 has better diagnostic efficiency for LUAD. The analysis of traditional lung cancer diagnostic marker CEA indicated that its AUC was 0.6734, which is far from the diagnostic effect of plasma exosomal CXCL7. In addition, related studies on the diagnostic role of CEA in lung cancer have shown that the higher AUC is only 0.737, which is slightly lower than that of plasma exosomal CXCL7 [34]. Collectively, these findings indicate that plasma exosomal CXCL7 is likely to become a potential biomarker for the diagnosis of LUAD.

However, our study still has a certain degree of limitations. Due to the lack of current samples, we also need to expand the sample size to verify whether plasma exosomal CXCL7 can be a specific diagnostic marker for LUAD. Moreover, it is already clear that CXCL7 can become a key marker for tumor treatment and prognosis [35-37]. There can be no doubt that it is necessary for us to continue to investigate and elaborate the therapeutic and prognostic role of plasma exosomal CXCL7 in LUAD in future studies.

In short, the present study has confirmed the expression of plasma exosomal CXCL7 in patients with LUAD and its clinical implications. Plasma exosomal CXCL7 will be expected to become a novel biomarker for early diagnosis of LUAD. Since the sample size in our study is too small, the results will be biased, so we need more samples and clinical trial data to verify the current research results. Moreover, whether it has value in treatment and prognosis is also worth exploring.

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Declaration of Interest:

The authors declare that they have no conflict of interest.

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