PIWI-interacting RNAs are aberrantly expressed and may serve as novel biomarkers for diagnosis of lung adenocarcinoma

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Funding information
Natural Science Foundation of Shandong Province, Grant/Award Number: ZR2020MH008; Young Taishan Scholar Program of Shandong Province

Abstract
Background: Lung adenocarcinoma (LUAD) is the main subtype of primary lung cancer and is a leading cause of cancer-related death worldwide. PIWI-interacting RNAs (piRNAs) are a type of small non-coding RNAs that may play crucial roles in cancer progression and serve as biomarkers for tumor detection. This study aimed to explore the expression profiles and diagnostic values of piRNAs in LUAD.

Methods: Small RNA sequencing was performed to investigate tissue piRNA profiles of LUAD. The expression of selected upregulated piRNAs were detected in tissues and serum exosome samples by quantitative real-time polymerase chain reaction (qRT-PCR). Serum exosomes were identified by transmission electron microscope, nanoparticle tracking analysis, and western blot analysis. Receiver operating characteristic (ROC) curve was adopted to quantify the diagnostic potentials of piRNAs in LUAD. Finally, a piRNA panel was developed by multivariate logistic regression model.

Results: We identified that 76 piRNAs were overexpressed and 9 piRNAs were underexpressed in LUAD tissues compared with adjacent non-tumor tissues. Among the top 10 overexpressed piRNAs, 4 piRNAs (piR-hsa-26925, piR-hsa-5444, piR-hsa-30636, and piR-hsa-8757) were verified by qRT-PCR to be significantly upregulated in LUAD tissues. Moreover, piR-hsa-26925 and piR-hsa-5444 had a significantly higher level in serum exosome samples of LUAD patients than those of healthy controls. We finally established a 2-piRNA panel composed of piR-hsa-26925 and piR-hsa-5444, which showed higher diagnostic performance for LUAD with an AUC of 0.833.

Conclusions: Our finding revealed the abnormally expressed piRNAs in LUAD, and serum exosomal piR-hsa-26925 and piR-hsa-5444 could serve as potential biomarkers for LUAD diagnosis.

KEYWORDS
biomarker, diagnosis, lung adenocarcinoma, PIWI-interacting RNAs, serum exosome

INTRODUCTION
As one of the most common malignant tumors worldwide, lung cancer is the leading cause of cancer-related morbidity in 2020, seriously threatening human health. According to the morphological characteristics of tumor cells, lung cancer is typically divided into two main types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC is the deadliest malignancy worldwide, among which lung adenocarcinoma (LUAD) is the most prevalent histopathologic type, with increasing morbidity year by year. The tumorigenesis of LUAD is a complex process involving multiple stages and factors. Despite recent advances in medical technology, the 5-year survival rate of...
LUAD remains low, mainly because LUAD lacks noticeable symptoms in the early stage, making it difficult to detect. Therefore, it is urgently necessary to identify potential hallmarks correlated with effective diagnosis of LUAD.

PIWI-interacting RNAs (piRNAs) are a type of small non-coding RNAs with a length of ~30 nucleotides (nt) and more than 30,000 members were described in the human genome. Investigation into the role of piRNAs was initially limited to germ cells and gonadal cells during early embryonic development. Recent reports have shown that piRNAs are expressed in a tissue-specific manner in a variety of human tissues, and regulate key signaling pathways at the transcriptional or post-transcriptional level. An increasing number of studies have indicated that the abnormal expressions of piRNAs and PIWI proteins in various cancers may serve as new biomarkers and therapeutic targets for tumor diagnosis and treatment. For example, Mai et al. suggested that piRNA-54265 can be used as a biomarker for the early detection and clinical monitoring of colorectal cancer. Tan et al. revealed that piRNA-36712 is a novel tumor suppressor and may serve as a prognostic predictor of breast cancer. Furthermore, recent studies have revealed that piRNAs can be stably present in body fluids and detected in exosomes of cancer cells. However, the potential clinical significance of serum exosomal piRNAs as biomarkers for the diagnosis of LUAD remains elusive.

To fill this important gap in knowledge, in the present study, we systematically analyzed the expression profiles of piRNAs in five pairs of LUAD tissues and adjacent normal tissues by small RNA sequencing (SRS). Following rigorous bioinformatic analysis and prioritization of aberrantly piRNAs, the top 10 upregulated piRNAs were validated in 14 LUAD tissues and paired normal tissues, four (piR-hsa-26925, piR-hsa-5444, piR-hsa-30636, and piR-hsa-8757) of which were significantly upregulated in LUAD tissues. We thereafter, conducted a systematic analysis on the serum exosomal levels of the above-mentioned four piRNAs and established a panel comprised of 2 piRNAs (piR-hsa-26925 and piR-hsa-5444) for the diagnosis of LUAD. In summary, all these findings suggested that serum exosomal piRNAs could be used as potential biomarkers for the diagnosis of LUAD.

**METHODS**

**Sample collection**

A total of 19 pairs of LUAD tissues and matched adjacent non-tumor tissues were obtained from patients who underwent surgery at Qilu Hospital of Shandong University from December 2020 to May 2021. None of the patients received chemotherapy or radiotherapy before surgery, and all LUAD tissues and paired non-tumor tissues were confirmed by experienced pathologists (Dr. Chengjun Zhou and Dr. Binxin Guan). These tissues were immediately frozen in liquid nitrogen and then stored at −80°C. Among these samples, five pairs of samples were used for SRS and 14 pairs were used for quantitative real-time polymerase chain reaction (qRT-PCR) verification. Serum samples from 70 patients with LUAD and 57 healthy controls were also obtained at Qilu Hospital of Shandong University and frozen at −80°C. The study protocol was approved by the Institutional Review Board of Qilu Hospital of Shandong University, and written informed consents from all participants were obtained before the initiation of the research.

**SRS**

Five pairs of LUAD tissues and the corresponding adjacent non-tumor tissues were used for SRS. Total RNA was isolated using miRNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. The concentration and quality of RNA were determined by the Qubit 2.0 fluorometer (Life Technologies), and samples with an RNA integrity number (RIN) above 7.0 were used for subsequent sequencing.

Paired-end libraries were synthesized using the QIAseq miRNA Library Kit (Qiagen) following TruSeq RNA Sample Preparation Guide. The products were then purified and enriched with PCR to establish the final complementary DNA (cDNA) library. The purified libraries were quantified by Qubit 2.0 fluorometer (Life Technologies) and validated by Agilent 2100 bioanalyzer (Agilent Technologies), and samples with an RNA integrity number (RIN) above 7.0 were used for subsequent sequencing.

For data analysis, reads of each sequencing sample were mapped to the piRbank using CLC genomics workbench 5.5 to calculate the expression level of each piRNA (count number). The abundance of piRNA was estimated by the transcripts per million (TPM). The differentially expressed piRNAs were selected using the criteria of |log2(fold change)| > 1 and p value < 0.05 by edgeR software.

**Prediction of piRNA target genes**

Possible target genes of the known piRNAs were predicted using the miRanda software. The interaction between piRNAs and the potential target genes were visualized using Cytoscape 3.6.0. To clarify the biological functions of the genes and the involved signaling pathways, Gene Ontology (GO) analysis for biological processes, cellular components, and molecular function was performed. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (http://www.genome.ad.jp/kegg) was conducted via “enrichR” package.
Exosome extraction and verification

Exosomes were isolated from serum using ExoQuick Exosome Precipitation Solution (System Biosciences) according to the manufacturer’s protocols. Subsequently, morphology observation of exosomes was conducted using a transmission electron microscope (TEM). The concentration, size distribution, and zeta potential of isolated exosomes were detected by nanoparticle tracking analysis (NTA) using a ZetaView (Particle Metrix). TSG101 and CD9 proteins were used as markers to identify exosomes by western blot analysis.

RNA extraction

Total RNA was extracted from tissues using miRNeasy Mini Kit and from serum exosomes using miRNeasy Micro Kit (50) according to the manufacturer’s instructions. The quality and concentration of the purified total RNA were detected using a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific).

qRT-PCR

Total RNA was reversely transcribed to synthesize cDNA using PrimeScript RT reagent kit (Takara) on a T100 Thermal Cycler (Bio-Rad). qRT-PCR was carried out using SYBR Green Premix Ex Taq reagent kit (Takara) on a CFX96 Real-Time System (Bio-Rad) in a 10-μL reaction system consisting of 1 μL cDNA, 5 μL SYBR Green PCR Master Mix, 0.3 μL miScript Universal Primer (RiboBio), 0.3 μL specific primers for each piRNA (RiboBio), and 3.4 μL RNase-free water. Briefly, after an initial denaturation step at 95°C for 10 minutes, the amplifications were carried out with 40 cycles at a melting temperature of 95°C for 2 seconds, an annealing temperature of 60°C for 20 seconds, and an extension temperature of 70°C for 10 seconds. The threshold cycle (Ct) was defined as the fractional cycle number, at which the fluorescence passed the fixed threshold. The relative expression level of each piRNA was determined by the 2−ΔΔCt method and normalized against U6.

Statistical analysis

Statistical analysis was performed with GraphPad Prism version 5.0 and MedCalc version 19.6. Wilcoxon matched-pairs tests were used to evaluate the group differences of tissue samples. For serum exosome samples, the expressions of piRNAs between different groups were analyzed by Mann–Whitney tests. The expression levels of key piRNAs were used to construct a multivariate model to distinguish LUAD from healthy controls based on the multivariate logistic regression analysis. The receiver operating characteristic (ROC) curve was adopted to assess the diagnostic potential of piRNAs, and the area under the ROC curve (AUC), sensitivity, and specificity were calculated. Only a two-sided p value <0.05 was considered statistically significant.

RESULTS

Genome-wide screening to identify dysregulated piRNAs in LUAD

To identify the most differentially expressed piRNAs, five pairs of LUAD tissues and corresponding adjacent non-tumor tissues were analyzed by SRS. In general, the classes of small RNAs can be identified by the peak of length distribution, which ranges from 18 to 35 nt. MicroRNAs (miRNAs) are mainly distributed in length of 21 or 22 nt, whereas piRNAs are concentrated in 26–32 nt.18 Consistent with this, Figure 1(a) shows that in the LUAD and paired adjacent non-tumor tissue libraries, small RNAs were clustered in two ranges by length, 19–24 and 32–34 nt. More SRS data is available in Table S1. Further analysis of the sequencing data showed that 1474 piRNAs were expressed in the tissue samples. A total of 85 differentially expressed piRNAs were identified by the criteria of \( \log_2 \) (fold change) >1 and \( p \) value <0.05, among which 76 were highly expressed and 9 were lowly expressed in LUAD tissues compared with adjacent non-tumor tissues (Figure 1(b) and (c)). These data indicated that the above-mentioned abnormally expressed piRNAs might play a crucial role in the progression of LUAD.

Selection of four differently expressed piRNAs in LUAD tissues

Considering that the multi-biomarker combination has better diagnostic performance than single candidates,19 this study aims to identify and validate a panel of differentially expressed piRNAs that can distinguish individuals with LUAD from healthy. The top 10 piRNAs were chosen from significantly upregulated piRNAs in LUAD tissues (Table 1, Figure 2(a)). The differential expressions of these piRNAs were verified by qRT-PCR in tumor tissues and paired adjacent non-tumor tissue samples from 14 patients with LUAD. The results showed that the expressions of four piRNAs (piR-hsa-26925, piR-hsa-5444, piR-hsa-30636, and piR-hsa-8757) were significantly upregulated in LUAD tissues compared with non-tumor tissues (Figure 2(b)–(e), \( p < 0.05 \)), which was consistent with the results of SRS. There was no significant difference in the expressions of the other six piRNAs between LUAD tissues and non-tumor tissues (Figure S1(a)–(f), \( p > 0.05 \)).

To predict the possible functions of the four candidates, gene-set enrichment analysis was performed, showing 283 target mRNAs in the interaction network (Figure S2(a)). GO analysis was also performed, and the results revealed significant enrichment of multiple target genes in vesicle-mediated transport, intracellular signal transduction, cell...
junction, and purine nucleotide-binding (Figure S2(b)). KEGG pathway analysis showed that differential genes were enriched in the Wnt signaling pathway, pathways in cancer, and NSCLC (Figure S2(c)). These data indicated that the candidate piRNAs investigated in our study might regulate several downstream genes involved in the progression of LUAD.

Characterization of serum exosomes

Exosomes were isolated from the serum of LUAD patients and healthy controls. The TEM results showed that the exosomes obtained from serum were 40–150 nm in size with saucer-shaped morphology (Figure 3(a)). The exosomes were also confirmed by NTA, with data showing that the average diameter of isolated exosomes was ~100 nm (Figure 3(b)). Western blot analysis further indicated the presence of exosomal markers TSG101 and CD9 (Figure 3(c)). Taken together, these results suggested that exosomes were successfully isolated from serum samples.

Validation of exosomal piRNAs in serum samples

To support the translation of our findings from LUAD tissues into clinical practice, we extracted exosomal piRNAs in serum samples from 70 LUAD patients and 57 healthy controls, and explored the potential clinical value of exosomal piRNAs for LUAD diagnosis. The differential expressions of four candidate piRNAs in serum exosomes from patients with LUAD and

| piRNA_ID    | log2FC   | p value   | Regulation |
|-------------|----------|-----------|------------|
| piR-hsa-1008| 4.636    | 0.000157  | UP         |
| piR-hsa-26925| 3.680    | 0.009539  | UP         |
| piR-hsa-28231| 3.676    | 0.004282  | UP         |
| piR-hsa-11256| 3.411    | 0.007259  | UP         |
| piR-hsa-5444| 3.388    | 0.010094  | UP         |
| piR-hsa-30636| 3.356    | 0.010719  | UP         |
| piR-hsa-24143| 3.313    | 0.030070  | UP         |
| piR-hsa-6842| 3.072    | 0.045004  | UP         |
| piR-hsa-8757| 3.069    | 6.35E-05  | UP         |

FIGURE 1 The analysis of dysregulated piRNAs in LUAD patients. (a) Bar graph of small RNA transcripts identified from LUAD patient’s tissue samples. (b,c) The heatmap (b) and volcano plots (c) of 85 differently expressed piRNAs in LUAD patients. T, LUAD tissues; N, paired adjacent non-tumor tissues.
healthy controls were evaluated by qRT-PCR. Data analysis showed that piR-hsa-26925 and piR-hsa-5444 were significantly upregulated in LUAD patients compared to healthy controls (Figure 4(a) and (b), \( p < 0.001 \)), whereas there was no significant difference in piR-hsa-30636 and piR-hsa-8757 (Figure S3(a) and (b), \( p > 0.05 \)). The corresponding AUC values of ROC curves were 0.751 and 0.713, respectively. The sensitivity and specificity were shown in Figure 4(c) and Figure 4(d), respectively. We thereafter, explored the correlation between serum exosomal piR-hsa-26925 and piR-hsa-5444 expression and the clinicopathological characteristics of LUAD patients. The results of Table S2 showed that there was not any association between the expression of these piRNAs and patients’ age, sex, tumor size, (tumor, nodes, and metastases [TNM] stage), or lymph node metastasis (\( p > 0.05 \)).

To further improve the diagnostic accuracy, we established a multivariate logistic regression model based on the expressions of piR-hsa-26925 and piR-hsa-5444. The predictive probability of LUAD from the 2-piRNA panel was calculated using the following formula: Logit
The performance of the 2-piRNA panel in the diagnosis of LUAD was evaluated, and the results of ROC curve showed that the AUC of this panel was 0.833 (95% confidence interval [CI] = 0.756–0.893, sensitivity = 87.1% and specificity = 75.4%, Figure 4(e)). Compared with individual piRNAs, the 2-piRNA panel showed higher diagnostic performance, suggesting that it was a potential diagnostic biomarker for LUAD.

Stability of identified piRNAs in serum exosomes

We next attempted to investigate the stability of the identified piRNAs in serum exosomes, given that this is another essential prerequisite for ideal biomarkers application. Four exosome samples were subjected to harsh conditions, including incubation at room temperature for 0, 4, 8, 12, and 24 hour (Figure 5(a) and (d)), incubation at −80°C for

![LUAD patient and Healthy control TEM images](image)

![LUAD patient and Healthy control NTA analysis](image)

![LUAD patient and Healthy control Western blot](image)

**FIGURE 3** Characterization of serum exosomes. (a) TEM images of exosomes isolated and detected from serum samples (scale bar, 200 nm). (b) the NTA showed the size distribution and the number of serum exosomes. (c) Western blot analysis of exosomal markers, including TSG101 and CD9
0, 1, 2, and 3 months (Figure 5(b) and (e)), and multiple freeze–thaw cycles (0, 2, 4 and 8) (Figure 5(c) and (f)). Total RNA was extracted, and the Ct values of piRNAs were detected by qRT-PCR. Data showed that there was no obvious change in expressions of the exosomal piRNAs under the above-mentioned conditions. Collectively, the results...
indicated that exosomal piRNAs were sufficiently stable in serum exosomes to serve as promising biomarkers for the diagnosis of LUAD.

DISCUSSION

Regardless of advances in the treatment of LUAD, the outcome of these patients remains poor.20,21 The main reason lies in the lack of effective screening methods for LUAD, leading to the majority of patients diagnosed at an advanced stage. Serological biomarkers are considered to be one of the most promising diagnostic methods for tumors. However, markers for LUAD, such as CEA, is of limited use because of the lack of sufficient diagnostic sensitivity and specificity.22 Hence, it is urgently necessary to identify new biomarkers for the diagnosis of LUAD.

With the advanced gene sequencing technologies, non-coding RNAs have emerged as novel genomic regulators in molecular biology. Numerous studies have shown that non-coding RNAs, especially small RNAs, are involved in the pathogenesis of many diseases and emerge as new tumor markers for clinical diagnosis. In terms of small RNAs, previous studies mainly focused on miRNAs, some of which have been validated in lung cancer, including hsa-miR-205-5p, hsa-miR-564, hsa-miR-1260b, and hsa-miR-337.23–26 Moreover, Da et al. have confirmed that miR-1205 can promote the growth of LUAD cells and act as a potential non-invasive biomarker and therapeutic target for LUAD.27 These studies further suggest that small RNAs can participate in the development of LUAD and have potential as biomarkers.

It is worth noting that piRNAs have emerged as another novel class of small non-coding RNAs with more than 30,000 members in the human genome, which is much greater than that of miRNAs.9,10 Emerging studies suggest that piRNAs and PIWI proteins are also involved in tumorigenesis.13 For instance, piR-021285 is associated with the methylation of cancer-related genes in breast cancer,28 and PIWIL1-interacting RNA piR-021285 is associated with the methylation of cancer-related genes, providing clues for investigating their functions and mechanisms involved in LUAD.

Numerous studies have shown that exosomes from cancer cells may exhibit the characteristics of the original tumor, and the nucleic acids and proteins contained in tumor-specific exosomes may serve as more stable tumor diagnostic biomarkers because of lipid bilayer protection compared with cell-free RNA.33–36 Previous studies have shown that the expression levels of RNAs in exosomes remain unchanged even on ribonuclease (RNase) A treatment or incubation with strong acid–base treatment.37,38 A great deal of attention has been paid to the key roles of lung cancer cell-derived exosomes in the development of lung cancer and early detection/diagnosis. The exosome proteomics of NSCLC has identified enriched proteins, such as EGFR, GRB2, and Src, for the early detection of lung cancer.39 Another report has shown that serum exosomal miR-1290 can be a potential diagnostic and prognostic biomarker for LUAD.40 However, the presence of piRNAs in serum exosomes as diagnostic markers for LUAD has not been studied. In the present study, we extracted piRNAs in serum exosomes from 70 patients with LUAD and 57 healthy controls. The expressions of four candidate piRNAs (piR-hsa-26925, piR-hsa-5444, piR-hsa-30636, and piR-hsa-8757) were assessed by qRT-PCR. The results showed that the expression of piR-hsa-26925 and piR-hsa-5444 were significantly higher in patients with LUAD than in healthy controls. The corresponding AUC of ROC curves was 0.751 and 0.713, respectively. To further investigate their combined diagnostic value, we established a multivariate logistic regression model based on the exosomal piR-hsa-26925 and piR-hsa-5444. Compared with single piRNAs, the predictive capability of the 2-piRNA panel was higher with an AUC of 0.833 (95% CI = 0.756–0.893, sensitivity = 87.1% and specificity = 75.4%). In addition, a previous study has shown that the expression of piRNA in serum samples is stable.16 Consistent with this study, we found that repeated freeze–thawing, long-term incubation at room temperature or −80°C did not affect the expressions of piR-hsa-26925 and piR-hsa-5444 in serum exosomes of patients with LUAD. These data supported the potential of serum exosomal piRNAs as promising diagnostic markers for LUAD.

Collectively, our study revealed the abnormally expressed piRNAs in LUAD, and serum exosomal piR-hsa-26925 and piR-hsa-5444 could serve as potential biomarkers for the diagnosis of LUAD. However, there are some limitations in this study. First, the number of subjects included in the study was limited. Second, it was uncertain whether serum exosomal piR-hsa-26925 and piR-hsa-5444 were only specific for LUAD. Therefore, the potential applications of our findings should be validated in larger independent samples.
ACKNOWLEDGMENTS

This research was supported by grant from the Natural Science Foundation of Shandong Province, China (ZR2020MH008) and Young Taishan Scholar Program of Shandong Province.

CONFLICT OF INTEREST

No conflict of interest has been declared by the authors.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Li J, Wang N, Zhang F, Jin S, Dong Y, Dong X, et al. PIWI-interacting RNAs are aberrantly expressed and may serve as novel biomarkers for diagnosis of lung adenocarcinoma. Thorac Cancer. 2021;12:2468–77. https://doi.org/10.1111/1759-7714.14094