Comprehensive analysis of the expression and prognosis for RBR E3 ubiquitin ligases in lung adenocarcinoma

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INTRODUCTION

Lung cancer is one of the most prevalent cancers in the world and the main cause of cancer deaths.\(^1\) Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers, including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma.\(^2\) Lung adenocarcinoma is the most common subtype of NSCLC. The main treatments for LUAD include surgical operation, radiotherapy and drug therapy. However, these traditional treatment methods are not effective for terminal patients.\(^3,4\) Further, due to the high incidence of LUAD metastasis and proto-oncogene mutations, there are currently few effective targeted therapeutic methods. Elucidating the mechanisms of...
LUAD oncogenesis and improving innovative approaches for lung cancer treatment are critical to ameliorate patients’ therapeutic outcomes.

Ubiquitination is a kind of protein post-translational modification in which 76 amino acid ubiquitin proteins are coupled to the lysine residues of the target protein. Ubiquitin is widely distributed in eukaryotic cells. Ubiquitin has seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) and an N-terminal methionine (M1) as the ubiquitination position, resulting in different linkage types. Substrate proteins can be attached with a mono- or polyubiquitin. Ubiquitination is controlled by a 3-step cascade reaction including E1-mediated Ub activation by forming a high-energy thioester with ubiquitin, Ub conjugation to E2 and E3 ligase target the substrate protein. In this sequential reaction, E3s are required for recognition of specific substrate proteins instead of E1s and E2s. Many studies have shown that E3 ligase are related to cancers by regulating the stability of tumor promoters or suppressors such as KRAS, p53, NF-kB and PTEN. Ubiquitin ligases (E3) can be divided into three categories: really interesting new genes (RING), homologous to E6AP C-terminus (HECT) and RING-between-RING (RBR). RING E3s can directly transfer ubiquitin from E2s to the substrate protein, while HECT and RBR E3s first receive ubiquitin from E2s, and then transfer ubiquitin to the substrate protein.

In the human genome, RBR E3 ubiquitin ligases can be subclassified into 14 family members including ARIH1, ARIH2, CUL9, ANKIB1, PRKN, RNF144A, RNF144B, RBCK1 (also called HOIL-1), RNF14, RNF19A, RNF19B, RNF31 (also called HOIP), RNF216 (also called TRIAD3), and RNF217. RBR E3 ubiquitin ligases gene mutation and abnormal expression have been identified in many diseases such as infection, inflammation, neurodegenerative diseases and tumorigenesis. PRKN has been reported as a tumor suppressor in many cancers by performing the function of antiapoptosis, anticell proliferation, and anticell metastasis. It has previously been reported that RNF31 (HOIP) is highly expressed in several cancers and can interact with LMP1 and IRF7 to induce cell proliferation and oncogenesis. A study showed that ARIH2 knockout promotes resistance to EGFR tyrosine kinase inhibitors (TKIs) in NSCLC. In human colorectal cancer (CRC), RNF216 promoted cell proliferation and migration by degrading BECN1. A study clarified that RNF217 with mutations of GXXXG motif has been observed in several human cancers. However, little is known about the expression and function of the most members of RBR E3 ubiquitin ligases.

METHODS

TCGA database

The Cancer Genome Atlas has sequencing and clinicopathological data of more than 30 different cancers. By studying, defining, discovering and classifying changes in the human tumor genome, a “whole-genome and multidimensional cancer genome” map has ultimately been created. Gene expression profiles and clinical patients data of LUAD were acquired from the TCGA project (https://cancergenome.nih.gov/). A total of 594 LUAD samples contain 535 tumor tissues and 59 adjacent-normal tissues, and corresponding clinical information (age, gender, TNM stage, OS. time, OS. state) were identified from the TCGA dataset.

Oncomine

Oncomine is a large tumor gene chip database, which can be used to discover new biomarkers or new therapeutic targets. Genes with differential expression analysis and gene coexpression analysis can be performed to determine the genes that are differentially expressed in a certain cancer. It was used to investigate the mRNA expression levels of RBR E3 ubiquitin ligases in different clinical cancer samples and corresponding normal candidates.

cBioPortal

Cbioportal for cancer genomics is an open online resource for the interactive exploration of a series of cancer genomics datasets. The TCGA-LUAD dataset were chosen for further analysis of RBR E3 ubiquitin ligases (ARIH2, RNF144B, RNF216, and RNF217). The data of “OncoPrint”, “Cancer Types Summary” were finally obtained (http://www.cbioportal.org/).

String database

The interaction proteins network of RBR E3 ubiquitin ligases was built using a string database (https://string-db.org/), an online database of predicted functional relationships between proteins. “Homo sapiens” was selected and interactions (medium confidence) with a combined score greater than 0.4 were considered significant.
LinkedOmics

LinkedOmics is a publicly available portal that includes multi-omic data from all TCGA cancer types. Gene expression correlations were calculated by Pearson’s test and the threshold was set at \( p \)-value < 0.05. Gene set (GO analysis) enrichment analysis was performed with 500 times simulation and the minimum number of genes was 3.

Functional enrichment analysis

A total of 36 genes that interacted with RBR E3 ubiquitin ligases (ARIH2, RNF144B, RNF216, and RNF217) gene-association networks was made by GeneMANIA. Funrich (version 3.1.3) was used to perform functional enrichment analysis. The chosen analysis terms include “Protein domain”. DAVID 6.8 bioinformatic resources was used for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotations. R package: clusterProfiler was used for gene ontology (GO) functional enrichment analysis and visualization. Bubble charts were plotted using the ggplot2 package of R software. FDR <0.05 was considered a threshold (http://genemania.org/).

Cell culture and qRT-PCR

Three NSCLC cell lines (A549, H1650 and H460) and HBE (purchased from Cell Bank of Chinese Academy of Sciences)
| Characteristics | Total(N) | HR(95% CI) | P value |
|-----------------|---------|------------|---------|
| Histogram       |         |            |         |
| Lung adenocarcinoma | 719    | 0.49(0.39-0.63) | 1.3e-08 *** |
| Gender          |         |            |         |
| Female          | 317     | 0.41(0.27-0.62) | 1.7e-05 *** |
| Male            | 344     | 0.59(0.42-0.83) | 0.0025 ** |
| Smoking history |         |            |         |
| smoker          | 246     | 0.44(0.27-0.73) | 9e-04 *** |
| non-smoker      | 143     | 0.47(0.21-1.08) | 0.0706 |
| Stage           |         |            |         |
| Stage 1         | 370     | 0.20(0.12-0.33) | 1.3e-12 *** |
| Stage 2         | 136     | 0.72(0.44-1.17) | 0.1837 |
| Stage 3         | 24      | 1.29(0.47-3.6)  | 0.6303 |
| AJCC stage T    |         |            |         |
| 1               | 123     | 0.76(0.42-1.39) | 0.3758 |
| 2               | 105     | 1.37(0.78-2.4)  | 0.2749 |
| AJCC stage N    |         |            |         |
| 0               | 184     | 0.68(0.42-1.1)  | 0.1123 |
| 1               | 44      | 1.15(0.53-2.51) | 0.727 |
| AJCC stage M    |         |            |         |
| 0               | 231     | 0.80(0.54-1.19) | 0.2762 |
| Surgery success |         |            |         |
| only surgical margins negative | 204 | 0.58(0.28-1.23) | 0.1549 |

**FIGURE 2** The prognostic value of the clinicopathological factors for the four potential biomarkers. (a–d) Effect of the clinicopathological factors of ARIH2, RNF144B, RNF216, RNF217 on OS using the Kaplan–Meier plotter. * p < 0.05; ** p < 0.01; ***p < 0.001
were cultured in RPMI-1640 medium (HyClone) with 10% fetal bovine serum at 37°C in a 5% CO2 incubator. Total RNA was extracted by TRIzol reagent (Thermo Fisher Scientific) according to the protocol and reverse transcribed to cDNA using random primer amplification. Primers used for the qRT-PCR analysis were performed as follows: GAPDH (Forward: ACAACTTTGGTATCGTGGAAGG; Reverse: GCCATCACGCCACAGTTTC); ARIH2 (Forward: TCCC GAGGAGTACCAGTTC; Reverse: GCAGTTGAGCA- GAATTGGACTT); RNF144B (Forward: CTGGTAGGCTC ...

FIGURE 3. Relationship between four potential biomarkers mRNA expression levels and clinicopathological characteristics in LUAD. (a) The relationship between mRNA expression levels of these four RBR E3 ubiquitin ligases and patients’ pathological stage. (b–d) The relationship between mRNA expression levels of these four RBR E3 ubiquitin ligases and patients’ TNM stage. *p < 0.05; **p < 0.01; ***p < 0.001.
CACTATCTCG; Reverse: GGGCAAGTGATGGGAGACC; RNF216 (Forward: TAGGCTTCTTTACAGAAAGCC; Reverse: GAGGAACGACCTGGTTGTTAT); RNF217 (Forward: TTAACGCATGAAGACTCCATAA; Reverse: CCAGACGAAATGGCAGGTAGG). The mRNA levels were normalized to GADPH using the $\Delta \Delta Ct$ method.\textsuperscript{31}

Immune infiltration

R package GSVA (ssGSEA)\textsuperscript{32} was used to estimate the correlation between gene expression of potential biomarkers and the abundances of 24 immune cell types.\textsuperscript{33} Spearman’s correlation analysis was used to analyze the gene expression of potential biomarkers and the expression of immune biomarkers. $p < 0.05$ was considered statistically significant.

Statistical analysis

Statistical analysis and mapping were performed using R software (version 3.6.3). Expression of RBR E3 ubiquitin ligases was observed using Wilcoxon rank sum test in unpaired samples. Kaplan–Meier curve and log-rank tests were used to determine the prognosis of LUAD patients by Kaplan–Meier Plotter. Moreover, log regression analysis was performed for potential genes. The forest plot was used to show the total number, $p$-value, HR and 95% CI of each variable through R package ggplot2. A two-sided $p$-value <0.05 was set to be the statistical signature. The public databases used in this study are described above.

RESULTS

Defining differentially expressed RBR family of E3 ubiquitin ligases in LUAD

We investigated the online database The Cancer Genome Atlas (TCGA) to explore the expression profiles of RBR E3 ubiquitin ligases genes in LUAD. A total of 535 tumor samples were used for further analysis (Table S1). Correlation analysis demonstrated that each member of the RBR E3 ubiquitin ligases genes was moderately to highly correlated in LUAD (Figure 1a). As shown in Figure 1b–d, the expression level of ARIH2, PRKN, RNF144B, RNF217 were decreased in tumor tissues, whereas CUL9, ANKIB1, RNF31, and RNF216 were significantly elevated in LUAD. Then, we further analyzed these eight differentially expressed RBR E3 ubiquitin ligases. Data from Oncomine revealed that the mRNA level of ARIH2, RNF216, RNF144B, and RNF217 was differentially expressed in lung cancer (Figure S1). However, there was no evidence of abnormal expression of other RBR E3 ubiquitin ligases family members in lung cancer compared with adjacent-normal tissues.

Prognostic value of RBR E3 ubiquitin ligases in LUAD

To investigate the prognostic value of eight RBR E3 ubiquitin ligases family genes with differential expression in LUAD, we analyzed the correlation between their expression with patient survival by Kaplan–Meier Plotter databases.
FIGURE 5  Genetic mutations and their association with LUAD prognosis of ARIH2, RNF144B, RNF216, and RNF217 genes. (a) OncoPrint of c-BioPortal showed the mutation types and proportions of these four genes, respectively from TCGA samples. (b) Cancer types summary of c-BioPortal specifically showed the types of mutations and their proportions contained in each cancer. (c) Overall survival and disease-free survival in cases with/without these four genes alterations.
Combining differentially expressed genes from the TCGA and Oncomine databases with prognostic curve results from Kaplan–Meier Plotter, we identified four RBR E3 ubiquitin ligases with the highest prognostic value. Among them, ARIH2, RNF144B, RNF216, and RNF217 predicted better prognosis in LUAD patients, whereas RNF216 predicted poor prognosis of patients, not only overall survival, but also first progression survival. Next, clinicopathological parameters of ARIH2, RNF144B, RNF216, and RNF217 on OS analyzed by Kaplan–Meier Plotter showed that the four genes were significantly associated with overall survival in clinical stage I patients (Figure 2). We further explored the relationship of the four RBR E3 ubiquitin ligases with clinicopathological parameters in LUAD. As shown in Figure 3a–d, the expression of ARIH2 was significantly associated with pathological stage II and nodal metastasis status, compared with adjacent normal
tissues. The expression of RNF144B, RNF216 and RNF217 were significantly associated with pathological stages, tumor grades, nodal metastasis and distant metastasis status. Then, qPCR was used to verify expressions of ARIH2, RNF144B, RNF216, and RNF217 in a panel of cell lines, as shown in Figure 4. ARIH2, RNF144B, RNF216, and RNF217 were highly expressed in normal HBE cells, and RNF216 was higher in LUAD cells. Therefore, levels of ARIH2, RNF144B, RNF216, and RNF217
could be used as valuable biomarkers of prognosis prediction in LUAD patients.

**Genetic alteration and coexpression of RBR E3 ubiquitin ligases in LUAD**

Furthermore, the genetic mutations of ARIH2, RNF144B, RNF216, and RNF217 in LUAD were investigated with the c-BioPortal online tool. We determined that DNA copy number amplifications and mRNA upregulation were common mutations in the four genes. Moreover, RNF216 exhibited the highest mutation rate among them (Figure 5a,b). The correlation between RBR E3 ubiquitin ligases mutations and prognosis were further researched. Survival plots showed that genetic alteration was not significantly correlated with patient OS or DFS in LUAD (Figure 5c). Linkedomics database was applied to identify the coexpressed genes in TCGA to better understand the function of ARIH2, RNF144B, RNF216, and RNF217. The volcano plot shows that genes in red dots were positively linked to them while genes in blue dots were negatively correlated within LAUD (p-value < 0.01) (Figure 6a–d). The top 50 positively and negatively correlated genes are listed in Figure 6e–h. GSEA enrichments were employed in the four altered EBR E3 ubiquitin ligases in the TCGA-LUAD cohort. ARIH2 was closely correlated with transition metal ion transport, transition metal ion homeostasis and transforming growth factor

| GO             | Category                  | Description                                                                 | p-value     | Count |
|----------------|---------------------------|-----------------------------------------------------------------------------|-------------|-------|
| GO:0032480     | GO biological processes   | Negative regulation of type I interferon production                         | 3.32857E-12 | 7     |
| GO:0043161     | GO biological processes   | Proteasome-mediated ubiquitin-dependent protein catabolic process            | 1.55751E-11 | 12    |
| GO:0010498     | GO biological processes   | Proteasomal protein catabolic process                                        | 6.99509E-11 | 12    |
| GO:1903052     | GO biological processes   | Positive regulation of proteolysis involved in cellular protein catabolic process | 7.59907E-11 | 8     |
| GO:0032479     | GO biological processes   | Regulation of type I interferon production                                  | 1.1264E-10  | 8     |
| GO:0032606     | GO biological processes   | Type I interferon production                                                 | 1.27871E-10 | 8     |
| GO:1903364     | GO biological processes   | Positive regulation of cellular protein catabolic process                    | 2.62631E-10 | 8     |
| GO:0045732     | GO biological processes   | Positive regulation of protein catabolic process                             | 2.71839E-10 | 9     |
| GO:0032648     | GO biological processes   | Regulation of interferon-beta production                                     | 4.57194E-10 | 6     |
| GO:0032608     | GO biological processes   | Interferon-beta production                                                   | 5.90346E-10 | 6     |
| GO:2000060     | GO biological processes   | Positive regulation of ubiquitin-dependent protein catabolic process         | 6.41312E-10 | 7     |
| GO:1901800     | GO biological processes   | Positive regulation of proteasomal protein catabolic process                 | 1.21734E-09 | 7     |
| GO:0000209     | GO biological processes   | Protein polyubiquitination                                                   | 7.06565E-09 | 9     |
| GO:1903050     | GO biological processes   | Regulation of proteolysis involved in cellular protein catabolic process     | 7.58462E-09 | 8     |
| GO:0032436     | GO biological processes   | Positive regulation of proteasomal ubiquitin-dependent protein catabolic process | 1.15354E-08 | 6     |
| GO:2000058     | GO biological processes   | Regulation of ubiquitin-dependent protein catabolic process                  | 1.50784E-08 | 7     |
| GO:1903362     | GO biological processes   | Regulation of cellular protein catabolic process                             | 2.32282E-08 | 8     |
| GO:0045862     | GO biological processes   | Positive regulation of proteolysis                                          | 2.45604E-08 | 9     |
| GO:0042176     | GO biological processes   | Regulation of protein catabolic process                                       | 4.19396E-08 | 9     |
| GO:0061136     | GO biological processes   | Regulation of proteasomal protein catabolic process                          | 6.22337E-08 | 7     |
| GO:0016579     | GO biological processes   | Protein deubiquitination                                                     | 6.65789E-08 | 8     |
| GO:0070646     | GO biological processes   | Protein modification by small protein removal                                | 1.0164E-07  | 8     |
| GO:0009896     | GO biological processes   | Positive regulation of catabolic process                                     | 1.06644E-07 | 9     |
| GO:0000151     | GO cellular components    | Ubiquitin ligase complex                                                     | 2.67001E-08 | 8     |
| GO:0004842     | GO molecular functions    | Ubiquitin-protein transferase activity                                       | 3.61547E-19 | 17    |
| GO:0019787     | GO molecular functions    | Ubiquitin-like protein transferase activity                                  | 1.05714E-18 | 17    |
| GO:0061630     | GO molecular functions    | Ubiquitin protein ligase activity                                            | 5.77594E-13 | 11    |
| GO:0061659     | GO molecular functions    | Ubiquitin-like protein ligase activity                                       | 8.94046E-13 | 11    |
| GO:0031625     | GO molecular functions    | Ubiquitin protein ligase binding                                             | 2.82069E-10 | 10    |
| GO:0044389     | GO molecular functions    | Ubiquitin-like protein ligase binding                                        | 5.06901E-10 | 10    |
beta production (Figure 7a). Meanwhile, RNF144B was associated with vasculogenesis, tRNA metabolic process and telomere organization (Figure 7b), RNF216 and RNF217 were correlated with translational initiation and translational elongation RNA catabolic process (Figure 7c, d). Notably, correlated genes of ARIH2, RNF144B, RNF216,
and RNF217 were all associated with NADH dehydrogenase complex assembly (Figure 7e–h). Dehydrogenase complex, involved in various redox reactions in cells, provides energy for cancer cell growth and infinite proliferation.

**PPI network construction of ARIH2, RNF144B, RNF216, and RNF217 and enrichment analysis**

In the meantime, we identified the interacting genes corresponding to ARIH2, RNF144B, RNF216, and RNF217 using STRING. The top 10 genes were used to construct their own protein–protein interaction (PPI) networks (Figure S3a–d). After deleting the duplicated genes, comprehensive PPI network of ARIH2, RNF144B, RNF216, and RNF217 was made (Figure S4). Next, GO functional enrichment analysis of ARIH2, RNF144B, RNF216, and RNF217 and their interactors were performed. Notably, these four RBR E3 ubiquitin ligases and their interactors were most correlated with ubiquitin-protein transferase activity (Table 1). In the aspect of biological process, four RBR E3 ubiquitin ligases were involved in negative regulation of type I interferon production, proteasome-mediated ubiquitin-dependent protein catabolic process and proteasomal protein catabolic process (Figure 8a). RING and IBR domains were the predominant protein domains of the four biomarkers and their interaction genes (Figure 8b). Further, KEGG analysis was conducted by DAVID (Table 2), which suggested that genes were mainly responsible for the cytosolic DNA-sensing pathway, Herpes simplex infection, Hepatitis c and NF-kappa B signaling pathway (Figure 8c).

**Immune infiltration analysis of four potential biomarkers**

Immune cells play a vital role in the immune microenvironment and can affect the prognosis of various cancers. It is unclear whether the four RBR E3 ligases can impact the recruitment of immune cells. We evaluated the correlation between the immune cell infiltration and the four RBR E3 ligases expression by R package GSVA. The scores of 24 immune cell types were calculated based on the TCGA-LUAD database. The results showed that the expression of ARIH2 was associated with Tcm, T helper cells and Tem. The expression of RNF217 was associated with Tcm and T helper cells. In addition, RNF144B was positively associated with Mast cells, Macrophages, iDC, NK cells, eosinopils, neutrophils, Tcm and negatively associated with Th2 cells. Unexpectedly, the correlation between RNF216 and immune infiltration was not strong (Figure S5).

**DISCUSSION**

RBR E3 ubiquitin ligases play pivotal roles in many different diseases, such as neurodegenerative diseases, infection and inflammation, especially in cancer. For instance, RBCK1 and RNF31 are reported to be highly expressed in breast cancers than adjacent nontumor tissue. Elodie Villa et al. demonstrated that ARIH1 functions in resistance to cisplatin in LUAD cells. Knockout of ARIH2 conferred resistance to EGFR inhibition in human NSCLC. In colon cancer, RNF14 participates in promoting cell cycle progression and proliferation. RNF216 promoted colorectal cancer (CRC) cell proliferation, migration and was associated with progression of CRC.

One important finding of our research is that with the online tools, we found ARIH2, PRKN, RNF144B, RNF217 were downexpressed in LUAD, whereas CUL9, ANKIB1, RNF31 and RNF216 were highly expressed. Our results are consistent with previous studies, that RNF216 promotes proteasomal degradation of BECN1 and positively regulates cell proliferation and migration in colorectal cancer. In particular, the RBR E3 ligase RNF216 specifically mediates K63-type ubiquitination modifications. In LPS-mediated inflammatory responses, ubiquitin E3 ligase RNF144B suppresses TBK1 phosphorylation by K63-linked polyubiquitination. Moreover, RNF144B is a potential endometrial cancer biomarker and GSK3β can protect it from proteasomal degradation by phosphorylation. Ferroptosis is essential to regulate tumor growth and ferroportin (FPN) is essential for maintaining systemic iron homeostasis. RNF217 can ubiquitinate and subsequently cause

| Term       | Category           | Description                          | Count | %       | p-value     |
|------------|--------------------|--------------------------------------|-------|---------|-------------|
| hsa04622   | KEGG pathway       | RIG-I-like receptor signaling pathway | 6     | 15.38461538 | 3.78084E-07 |
| hsa05169   | KEGG pathway       | Epstein–Barr virus infection         | 5     | 12.82051282 | 0.000145329 |
| hsa05164   | KEGG pathway       | Influenza A                          | 5     | 12.82051282 | 0.00056724  |
| hsa04064   | KEGG pathway       | NF-kappa B signaling pathway         | 4     | 10.25641026 | 0.00097147  |
| hsa05160   | KEGG pathway       | Hepatitis C                          | 4     | 10.25641026 | 0.003290683 |
| hsa05168   | KEGG pathway       | Herpes simplex infection             | 4     | 10.25641026 | 0.00833343  |
| hsa04623   | KEGG pathway       | Cytosolic DNA-sensing pathway         | 3     | 7.692307692 | 0.00940191  |
| hsa05162   | KEGG pathway       | Measles                              | 3     | 7.692307692 | 0.037297812 |
| hsa05161   | KEGG pathway       | Hepatitis B                          | 3     | 7.692307692 | 0.043649238 |
In addition, overexpression of ARIH2 has been found to interact with NLRP3 by NACHT domain and mediate the ubiquitination of NLRP3. In summary, the four RBR E3 ubiquitin ligases perform different biological functions through different substrate proteins in a context-dependent manner. Zeng et al. found knocking out ARIH2 participated in resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) in human non-small cell lung cancer (NSCLC). Another interesting finding of this research was that their expressions were associated with clinicopathological parameters in LUAD, such as pathological stage, tumor grade and nodal metastasis status. More importantly, early lung cancer does not yield significant clinical manifestations. Some early symptoms such as fatigue, shortness of breath, or upper back and chest pain were likely to be overlooked. However, early detection and intervention are associated with better survival. For lung adenocarcinoma, early diagnosis dramatically increased the 5-year survival rate and reduced the costs of management of the disease. Here, we demonstrated that the four RBR E3 ubiquitin ligases ARIH2, RNF144B, RNF216, and RNF217 were correlated with the prognosis of LUAD patients, particularly for early-stage LUAD, meaning they could be used as sensitive biomarkers to predict the progression and prognosis of LUAD patients, especially for early ones.

As RBR E3 ubiquitin ligases family belongs to E3 ligases, PPI network reveals that ARIH2, RNF144B, RNF216, and RNF217 mainly involved in ubiquitin specific protease activity and play key roles in protein metabolism. ARIH2 is a posttranslational negative regulator of NLRP3. Its overexpression promoted NLRP3 ubiquitination in macrophages. PARC-mediated ubiquitination of Cytochrome c has been reported to prevent apoptosis in response to stress. It has also been previously reported that RNF144B plays an essential role in p53 protein ubiquitination in poorly differentiated gastric cancer cells. We further identified coexpressed genes in TCGA and used GSEA enrichment analysis to reveal that they were closely correlated with transition metal ion transport, transition metal ion homeostasis, vasculoelasticity, tRNA metabolic process, telomere organization, translational initiation, translational elongation and RNA catabolic process. It has also been reported that RNF217 regulates iron homeostasis through its E3 ubiquitin ligase activity by modulating ferroportin degradation. Interestingly, the correlated genes of ARIH2, RNF144B, RNF216, and RNF217 were all associated with NADH dehydrogenase complex assembly. Based on these findings, we hypothesized that the four RBR E3 ligases may play roles in oxidative stress or ferroptosis. Moreover, this study also clarified the correlation between the four potential biomarkers and immune cells infiltration.

In summary, we have confirmed the aberrant expression of RBR E3 ubiquitin ligases in LUAD, and further validated the prognostic significance of ARIH2, RNF144B, RNF216, and RNF217 and their association with immune infiltration in LUAD. We speculated that these differentially expressed genes may be promising molecular targets for early diagnosis and targeted therapy of LUAD. It is undeniable that our research had some limitations. First, our results were based on database analysis, and qPCR verification has only been confirmed in cell lines, and it would be better to verify the findings of this study by carrying out further experiments.

**CONFLICT OF INTEREST**

No authors report any conflict of interest.

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