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RS1 gene is a novel prognostic biomarker for lung adenocarcinoma

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Abstract

Background: Although it has a poor prognosis, patients with lung adenocarcinoma (LUAD) have a relatively higher 5-year survival period. Thus, it is necessary to identify effective prognostic markers to evaluate the effect of early treatment. RS1 gene encodes retinoschisin, a key protein in congenital retinoschisis, while few studies have been reported on the association between RS1 and cancer prognosis.

Methods: We performed bioinformatic analyses based on the data obtained from The Cancer Genome Atlas and Gene Expression Omnibus databases to demonstrate the expression level of RS1 was related to the LUAD prognosis and our findings were verified in-vitro and clinical samples. Then, we explored the potential mechanism of how RS1 expression influenced the prognosis of LUAD.

Results: Compared with normal tissues, the RS1 expression was significantly lower in tumor tissues. The Multivariate Cox regression model showed that RS1 could be used as an independent prognostic indicator. Furthermore, we found significant differences in immune cell infiltration between RS1 high and low expression groups, and the proteasome pathway was found enriched in RS1 low expression samples.

Conclusion: In conclusion, our study suggests that RS1 is a novel prognostic biomarker for LUAD. Differences in immune cell infiltration and signaling pathways may contribute to the poor prognosis of LUAD caused by low RS1 expression.

KEYWORDS
bioinformatics, LUAD, prognosis, RS1

INTRODUCTION

The incidence and mortality rate of lung cancer ranks first among malignant tumors. Lung adenocarcinoma (LUAD) is the most common pathological type of lung cancer, accounting for 80%–85% of total lung cancer cases.[1] The prognosis of patients with LUAD is poor, with the 5-year survival rate being less than 30%.[2] However, studies have found that in LUAD patients at TNM stage IA, the 5-year survival rate increased to about 60%.[3,4] Despite the continuous improvement of diagnostic technology in recent years, only 10%–15% of new cases are diagnosed in their early clinical stage.[5] In addition to the limited diagnostic techniques and treatment strategies, recurrence and metastasis contribute to the main causes of death in LUAD.[6] Moreover, the treatment of LUAD is gradually transitioning to molecular targeted therapy.[7] Therefore, identification of molecular markers for early diagnosis and prognosis of LUAD is particularly vital in lowering the risk of metastasis and recurrence and prolonging survival.

Various factors are involved in the prognosis of LUAD, mainly in three aspects: patient differences (gender, age of diagnosis, smoking history and family history of cancer, etc.),[8–10] tumor heterogeneity and differences (cancer stage, degrees of differentiation in tumors, etc.),[11,12] and treatment schemes (surgery, chemotherapy, radiotherapy and targeted therapy, etc.).[13–15] Genetic and molecular factors are increasingly considered to be involved. More recently,

prognostic biomarkers or signatures have been widely studied to predict the risk of LUAD in patients. Salim et al. found that DKK1 might be a potential therapeutic target for non-small cell lung cancer (NSCLC).[16] Shi et al. reported that MAD2L1 is a potential prognostic indicator for LUAD.[17] High RRM2 expression has been indicated to activate Bcl-2 and E-cadherin signalings and serve as a prognostic indicator for LUAD.[18] The facilitation of high-throughput sequencing technology to detect a large number of gene expression changes, combined with bioinformatic methods to analyze tumor-related genes and their regulatory mechanisms, has become an effective and indispensable research method for functional genomics, widely applied in the area of screening potential tumor biomarkers. Retinoschisin 1 (RS1) gene encodes a key extracellular protein in retinal tissue.[19] Numerous evidence indicates that mutation in RS1 would lead to macular degeneration called X-linked retinoschisis (XLRS).[20] Additionally, a recent study has demonstrated that RS1 may form an intercellular adhesive scaffold in the retina.[21] Meanwhile, cell adhesion plays a crucial role in many tumors. In LUAD, Kosibaty et al. have recently demonstrated the crucial role of the focal adhesion cascade.[22] In addition, cell adhesion has been reported to be related to several prognostic markers in LUAD, such as CTHRC1.[23] Thus, the potential of RS1 in LUAD involving cell adhesion or metastasis deserves further exploration. However, as far as we are aware, few studies have focused on the role of RS1 in cancer.

In this study, we herein aimed to evaluate the prognostic value of the RS1 gene based on the LUAD data from the Cancer Genome Atlas (TCGA) database and Gene Expression Omnibus (GEO) databases. Combining bioinformatic mining and experimental validation, our findings are expected to give more insights into the role of RS1 in LUAD.

METHODS

Datasets

The LUAD datasets containing the level 3 RNA-sequencing data of 512 LUAD patients and 56 adjacent lung samples were downloaded from The Cancer Genome Atlas (TCGA, https://tcga-data.nci.nih.gov/tcga/). We divided 499 patients with complete survival information into high and low expression groups according to the median RS1 expression level (see Table 1 for clinical information). In addition, two data sets (GSE32863 and GSE115002) were downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) (clinical information is listed in Table S1). GSE32863[24] contains 58 tumor samples and paired adjacent tissues and was performed on Illumina Human WG-6 V3.0 expression beadchip for mRNA expression data. GSE115002[25] contains 52 tumor samples and paired adjacent tissues and was performed on Agilent-026652 Whole Human Genome Microarray for mRNA expression data.

Analysis of RS1 gene expression level

Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn) is a portal for gene expression analysis based on TCGA and GTEx data. To compare the expression levels of RS1 in different samples and at different cancer stages, we used TCGA-LUAD and GTEx data sets to evaluate the RS1 expression.[26]

Survival analysis

The Survival package and Survivminer package in R were used to estimate the overall survival (OS) of different groups based on the Kaplan–Meier method, with log-rank to test the significant difference in OS between different groups. We used the Kaplan–Meier plotter online database (http://kmplot.com/analysis/) to analyze the survival rate of high and low RS1 expression groups of GSE37745 (the probe ID corresponding to RS1 was 207363_at). To investigate whether RS1 gene expression predicts the survival of patients with LUAD as an independent factor, we applied a multivariate Cox regression model.

Immune cell infiltration analysis

CIBERSORT,[27] validated by a leukocyte gene signature matrix consisting of 22 human immune subsets and a total of 547 genes, can characterize the composition of immune cells by deconvolution algorithm. We used CIBERSORT to calculate the relative proportion of the 22 immune cells in each LUAD sample of TCGA.

Gene set enrichment analysis (GSEA)

In GSEA software,[28,29] the Molecular Signatures Database (MSigDB, https://www.gsea-msigdb.org/gsea/index.jsp, version 7.2), which contains different categories of gene sets, is provided to perform gene enrichment analysis. We used KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway gene sets to perform the signaling pathways enrichment in the high and low RS1 expression groups (the permutation in a gene set was set to 1000) and detected the top pathways in the two groups.

Cell lines, cells culture, and qRT-PCR

Human normal lung epithelial cell line BEAS-2B and LUAD cell line NCI-H1975 were purchased from Fenghui Biotechnology Co., Ltd. LUAD cell line NCI-H441 was purchased from BeNa Culture Collection (BNCC). BEAS-2B cells were cultured in DMEM medium (Gibco, C11995500BT) with 1% penicillin (HyClone, SV30010) and 10% FBS (Gibco, 10099–141). NCI-H441 and NCI-H1975 cells were cultured

in 1640 medium (Gibco, C11875500BT) with 1% penicillin (HyClone, SV30010) and 10% FBS (Gibco, 10099–141). The cells were cultured at 37°C in an incubator containing 5% CO_2. Then, the total RNA was extracted with TRIZOL reagent (Tiangen, dp424, China) and detected for concentration and purity using ultramicro ultraviolet–visible spectrophotometer Nanodrop 2000 (Thermo). After reverse transcription (RevertAid First Strand cDNA Synthesis Kit; Thermo), PCR was performed with a fluorescent quantitative PCR detector (ABI Veriti; Life Technologies) using TB Green Premix Ex Taq II (Takara, RR820A). GAPDH was used as a reference gene. Three repeat samples were taken

TABLE 1 Relationship between RS1 expression level and clinicopathological variables in LUAD patients

			RS1 expression			
Characteristics	**Groups**	**Total**	**Low**	**High**	X^2	***p*-value**
Age	Median	66	65	66	0.0102	0.9949
	Range	33–88	38–88	33–86		
Gender	Female	269	134	135	0.0552	0.9728
	Male	230	117	113		
TNM stage	I	269	121	148	10.238	0.2487
	II	118	65	53		
	III	80	50	30		
	IV	25	13	12		
	Unknown	7	2	5		
T stage	T1	167	67	100	11.124	0.1948
	T2	268	148	120		
	T3	43	25	18		
	T4	18	10	8		
	TX	3	1	2		
N stage	N0	323	151	172	10.74	0.3781
	N1	94	53	41		
	N2	69	42	27		
	N3	2	2	0		
	NX	10	3	7		
	Unknown	1	0	1		
M Stage	M0	332	175	157	2.5767	0.8598
	M1	24	12	12		
	MX	139	62	77		
	Unknown	4	2	2		
EGFR mutation	Wild	431	220	211	0.7324	0.9473
	Mutation	64	29	35		
	Unknown	4	2	2		
Smoking history	Non-smoker	72	37	35	4.9176	0.5544
	Smoker	118	60	58		
	Reformed smoker	295	143	152		
	Unknown	14	11	3		
Status	Alive	319	144	175	9.4171	0.0090
	Dead	180	107	73		

TABLE 2 Primer sequences for RT-PCR

Genes	**Forward primer (5′-3′)**	**Reverse primer (5′-3′)**	**Product length (bp)**	**Tm (°C) (°C)**
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	227	58
RS1	ACCCAATGCTCTGTGGTCTGC	GGTGTGACCTCCCCTGACTCG	99	62

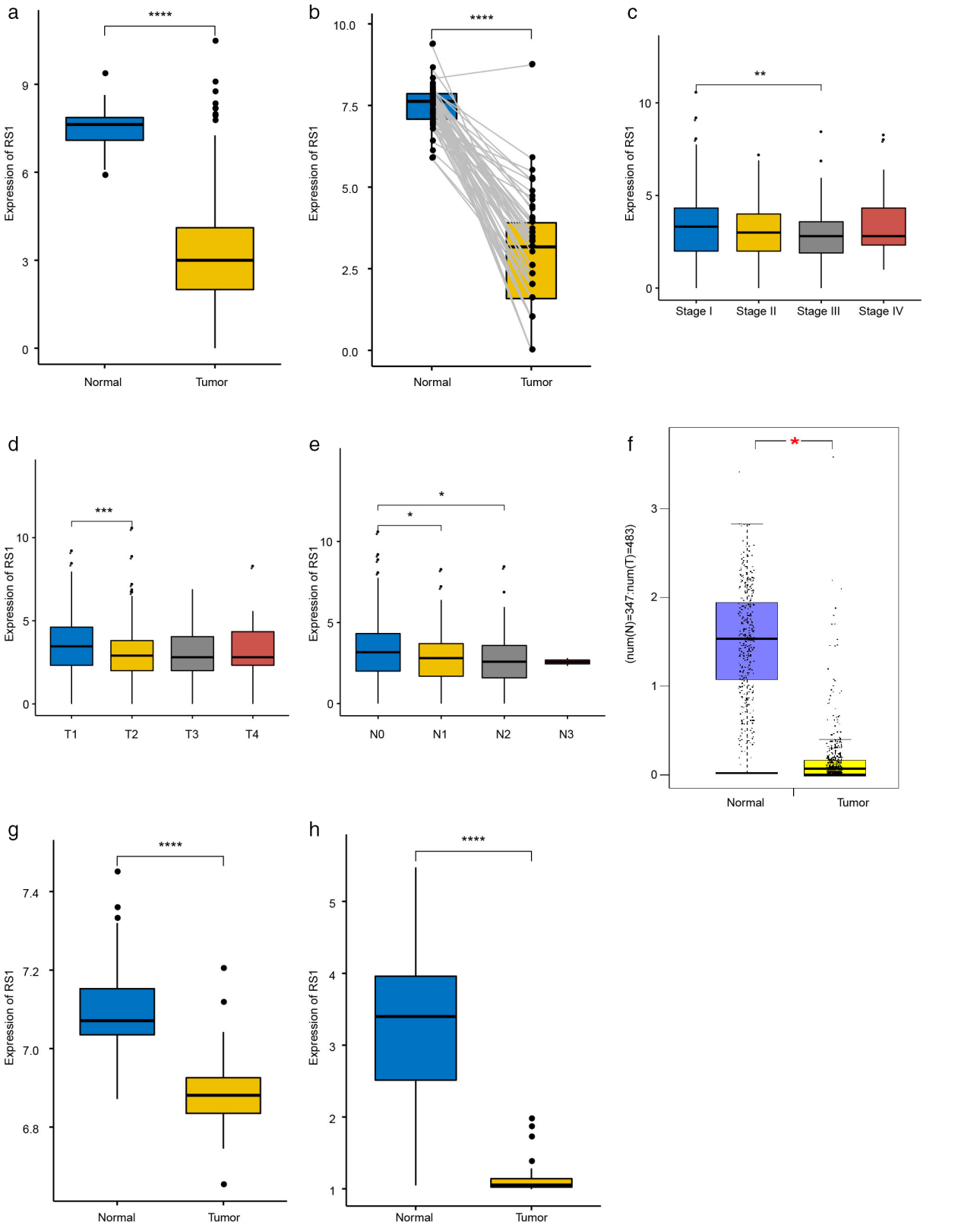

FIGURE 1 Expression levels of RS1 gene in lung adenocarcinoma (LUAD). (a) Expression difference of RS1 gene between tumor tissues and adjacent tissues of LUAD samples in the TCGA database. The longitudinal axis represents the relative expression level of mRNA. (b) Expression difference of RS1 gene between 56 LUAD tissues and paired adjacent tissues in the TCGA database. (c) RS1 expression levels at different TNM stages. (d) RS1 expression levels at different T stages. (e) RS1 expression levels at different N stages. (f) Expression difference of RS1 gene between tumor and adjacent tissues of LUAD samples in GEPIA database. (g) Different expression levels between tumor and paired adjacent tissues in the GSE32863 dataset. (h) Different expression levels between tumor tissues and paired adjacent tissues in GSE115002 dataset. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

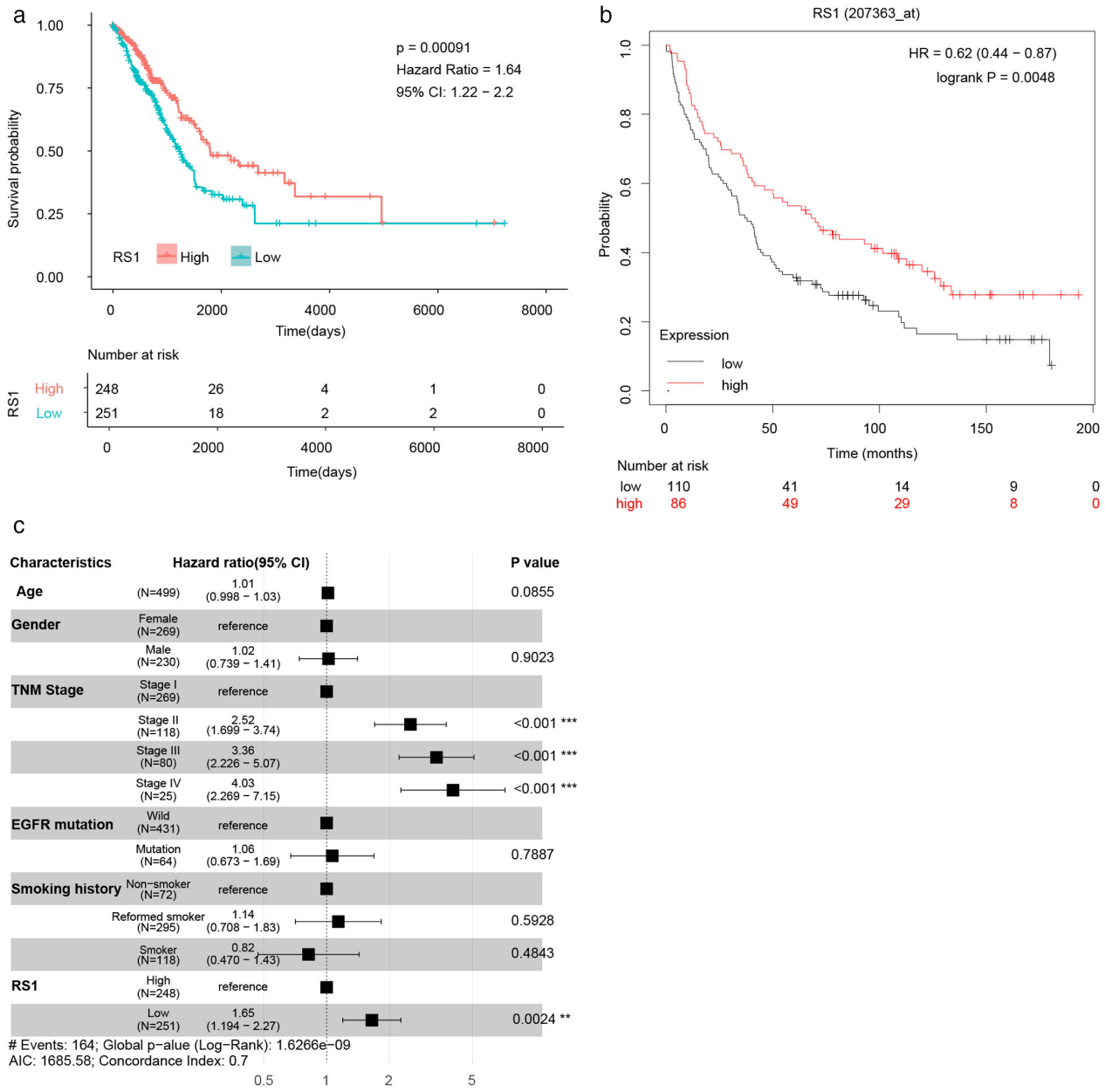

FIGURE 2 RS1 gene is an independent prognostic indicator for lung adenocarcinoma (LUAD). (a) Kaplan–Meier survival analysis for TCGA dataset showed poor overall survival (OS) in samples with low expression of RS1. (b) Kaplan–Meier survival analysis for GSE37745 dataset. (c) A forest map of six factors included in multivariate Cox regression analysis. Compared with reference, HR >1 represents a higher risk of death, and HR <1 represents a lower risk of death.

for each sample (see Table 2 for primer sequences). The mRNA expression level was calculated by $2^{-\triangle\triangle CT}$.

Western blot

Protein was extracted from cells and detected for purity and concentration using the BCA test kit (P0011; Beyotime Biotechnology). Western blot procedure was consistent with that carried out in a previous study.[30] In the Western blot, the primary antibody was RS1 polyclonal antibody (1:1000, H00006247-B01P; Novus), and the secondary antibody was IgG-HRP (1:3000, bs-0295G-HRP; Bioss). GAPDH (1:2000, bs-2188R; Bioss) was used as a reference protein. Gelpro32 software was used to analyze the gray value of the strips.

Tissue microarray and immunohistochemical analysis

LUAD tissue microarray (HLugA150CS03; Outdo Biotech), which consists of 75 LUAD tumor tissues and paired adjacent tissues, was used for clinical research (see Table S2 for

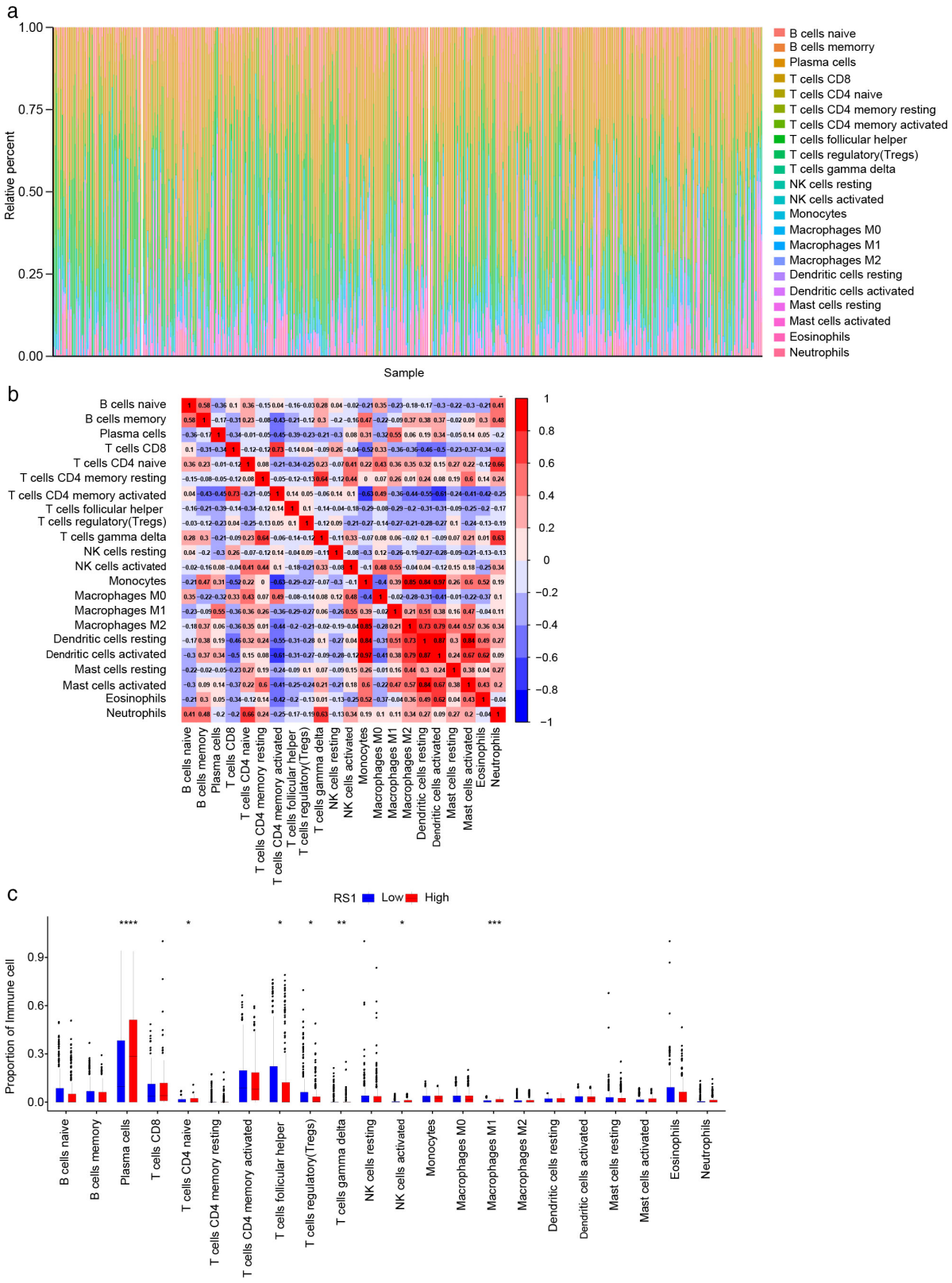

FIGURE 3 Immune infiltration of lung adenocarcinoma (LUAD) patients in the high and low RS1 expression groups. (a) Infiltration rate of 22 immune cells in all patients. (b) A correlation matrix of 22 immune infiltration proportions. Red represents positive correlation and blue represents negative correlation, with darker colors meaning stronger correlation. (c) The infiltration difference of immune cells in the RS1 high and low expression groups. The vertical axis represents the relative infiltration proportions of immune cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

TABLE 3 GSEA enriched KEGG pathways in high and low expression of RS1

NAME	SIZE	NES	NOM *p*-value
High expression of RS1			
KEGG_CARDIAC_MUSCLE_CONTRACTION	73	1.81	0.0000
KEGG_VASCULAR_SMOOTH_MUSCLE_CONTRACTION	114	1.77	0.0061
KEGG_RENIN_ANGIOTENSIN_SYSTEM	17	1.71	0.0061
KEGG_HYPERTROPHIC_CARDIOMYOPATHY_HCM	83	1.69	0.0126
KEGG_GNRH_SIGNALING_PATHWAY	101	1.68	0.0140
KEGG_DILATED_CARDIOMYOPATHY	90	1.64	0.0168
KEGG_PPAR_SIGNALING_PATHWAY	69	1.64	0.0120
KEGG_PROXIMAL_TUBULE_BICARBONATE_RECLAMATION	23	1.63	0.0183
KEGG_GLYCEROPHOSPHOLIPID_METABOLISM	77	1.62	0.0097
KEGG_ARACHIDONIC_ACID_METABOLISM	58	1.61	0.0157
KEGG_ARRHYTHMOGENIC_RIGHT_VENTRICULAR_CARDIOMYOPATHY_ARVC	74	1.55	0.0401
KEGG_ALPHA_LINOLENIC_ACID_METABOLISM	19	1.55	0.0157
KEGG_ETHER_LIPID_METABOLISM	33	1.53	0.0388
KEGG_LINOLEIC_ACID_METABOLISM	29	1.51	0.0244
KEGG_PRIMARY_BILE_ACID_BIOSYNTHESIS	16	1.50	0.0259
KEGG_LONG_TERM_DEPRESSION	70	1.50	0.0441
KEGG_CALCIUM_SIGNALING_PATHWAY	177	1.49	0.0348
KEGG_BETA_ALANINE_METABOLISM	22	1.48	0.0447
KEGG_PURINE_METABOLISM	155	1.48	0.0104
KEGG_BASAL_CELL_CARCINOMA	55	1.46	0.0420
Low expression of RS1			
KEGG_PROTEASOME	44	−1.66	0.0353

clinical information). The microarray was incubated with primary antibody RS1 polyclonal antibody at 4°C overnight and then incubated with secondary antibody IgG-HRP (1:150, SE131; Solarbio) at room temperature for 35 min. Diaminobenzidine (DAB) histochemistry kit (Dako, K5007) was used for color development. After being counterstained with hematoxylin, the microarray was dehydrated and examined with a microscope for image acquisition and analysis.

Statistical analysis

A chi-square test was conducted to determine the significant difference of clinical information between different groups, regarding all LUAD samples in Table 1. A Shapiro–Wilk test was used to determine whether the data was in accordance with normal distribution, and *t*-test was used for the data with normal distribution. As for the data with non-normal distribution, the Wilcoxon signed-rank test was performed to determine the significance of difference (including RS1 expression difference, immune cell infiltration difference). $p < 0.05$ was the threshold for statistical significance. All statistical analyses were performed on R software v3.5.2.

RESULTS

Low expression of RS1 gene was related to the occurrence and development of LUAD

Analyses for LUAD samples in the TCGA database showed that the expression level of the RS1 gene was significantly lower in tumor tissues (Figure 1a). In addition, RS1 expression in tumor samples significantly decreased compared with in normal adjacent tissues (Figure 1b). Moreover, RS1 expression level was closely related to the TNM stages; RS1 expression level at stage III was significantly lower than that at stage I (Figure 1c), and RS1 expression level in T2 stage samples was significantly lower than that in T1 stage samples (Figure 1d). The RS1 expression level was lower at N1 and N2 stage than at N0 stage (Figure 1e). Moreover, based on the GEPIA database, there were also significantly lower RS1 expression level in LUAD compared with normal samples (Figure 1f). In addition, in both GSE32863 (Figure 1g) and GSE115002 datasets (Figure 1h), the expression levels of RS1 gene in cancer samples were found to be significantly lower than those in paired adjacent samples. These results suggested that the downregulation of RS1 gene may play a regulatory role in the occurrence and development of LUAD.

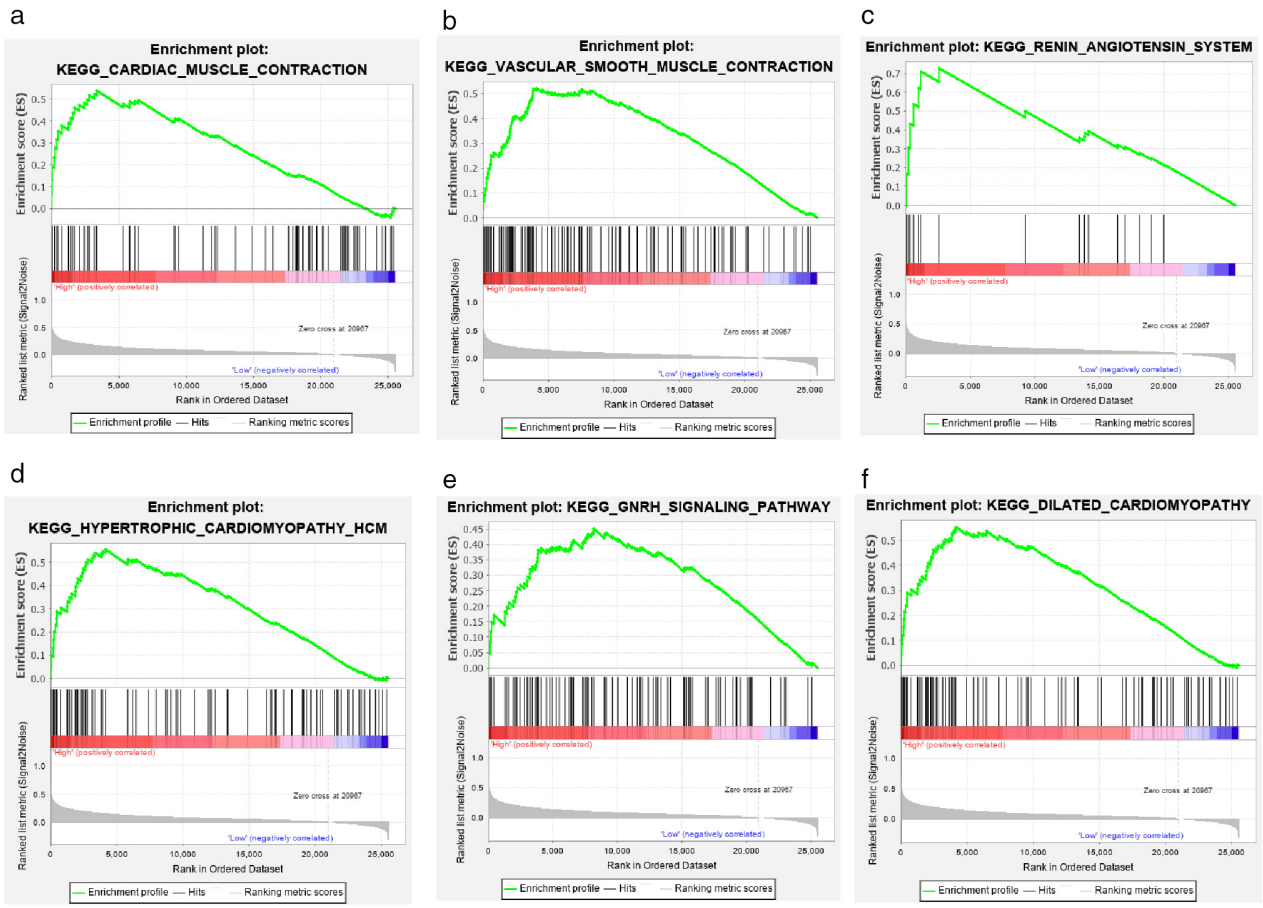

FIGURE 4 Gene set enrichment analysis (GSEA) showed the top six pathways enriched in the high RS1 expression group. (a) Cardiac muscle contraction pathway, (b) vascular smooth muscle contraction pathway, (c) renin angiotensin system pathway, (d) hypertrophic cardiomyopathy (HCM), (e) GnRH signaling pathway and (f) differentiated cardiomyopathy pathway

Low expression of RS1 can be used as an independent prognostic indicator for LUAD

The LUAD samples were then divided into high RS1 and low RS1 expression groups based on the median RS1 expression. First, using LUAD data in TCGA, Kaplan–Meier survival analysis indicated that compared with highly RS1 expressed LUAD samples, LUAD patients with lower RS1 expression had poorer OS (HR = 1.64, 95% CI: 1.22–2.2, $p < 0.05$) (Figure 2a). Additionally, lower RS1 expression patients exhibited relatively worse OS both in LUAD samples at earlier stages (stages I and II) (HR = 1.05, 95% CI: 0.97–1.2, $p = 0.074$) (Figure S1a) and advanced stages (stages III and IV) (HR = 1.32, 95% CI: 1.17–1.75, $p < 0.01$) (Figure S1b). In *EGFR* mutated LUAD samples, there was no significant difference in OS of patients with high and low RS1 expression (Figure S1c). However, in wild-type *EGFR* LUAD samples, lower RS1 expression patients had significantly poorer OS (HR = 1.61, 95% CI: 1.18–1.98, $p = 0.00097$, Figure S1d). In GSE37745, the consistent tendency was also observed, and LUAD patients with lower RS1 expression had worse prognosis compared with high RS1 expression patients ($p < 0.05$) (Figure 2b).

To investigate whether the expression level of RS1 gene can be used as an independent prognostic indicator, various factors such as age, gender, TNM stage, *EGFR* mutation, smoking history, and RS1 expression were included in the multivariate Cox regression analysis based on data in TCGA. Consistent with the above results, RS1 expression level was significantly correlated with OS. Samples with lower RS1 expression had a higher risk of death and poorer prognosis (HR = 1.65, 95% CI: 1.194–2.27, $p < 0.05$) (Figure 2c). These suggested that low RS1 expression level was related to poor prognosis in LUAD and could be an independent prognostic indicator.

RS1 gene is related to immune cell infiltration in LUAD

We applied CIBERSORT to study the immune infiltration of 22 immune cells in LUAD patients in TCGA. The proportions of the 22 immune cells vary in different patients (Figure 3a), indicating differences in proportions could be internal characteristics of individuals receiving immunotherapy. Weak correlation among different proportions in

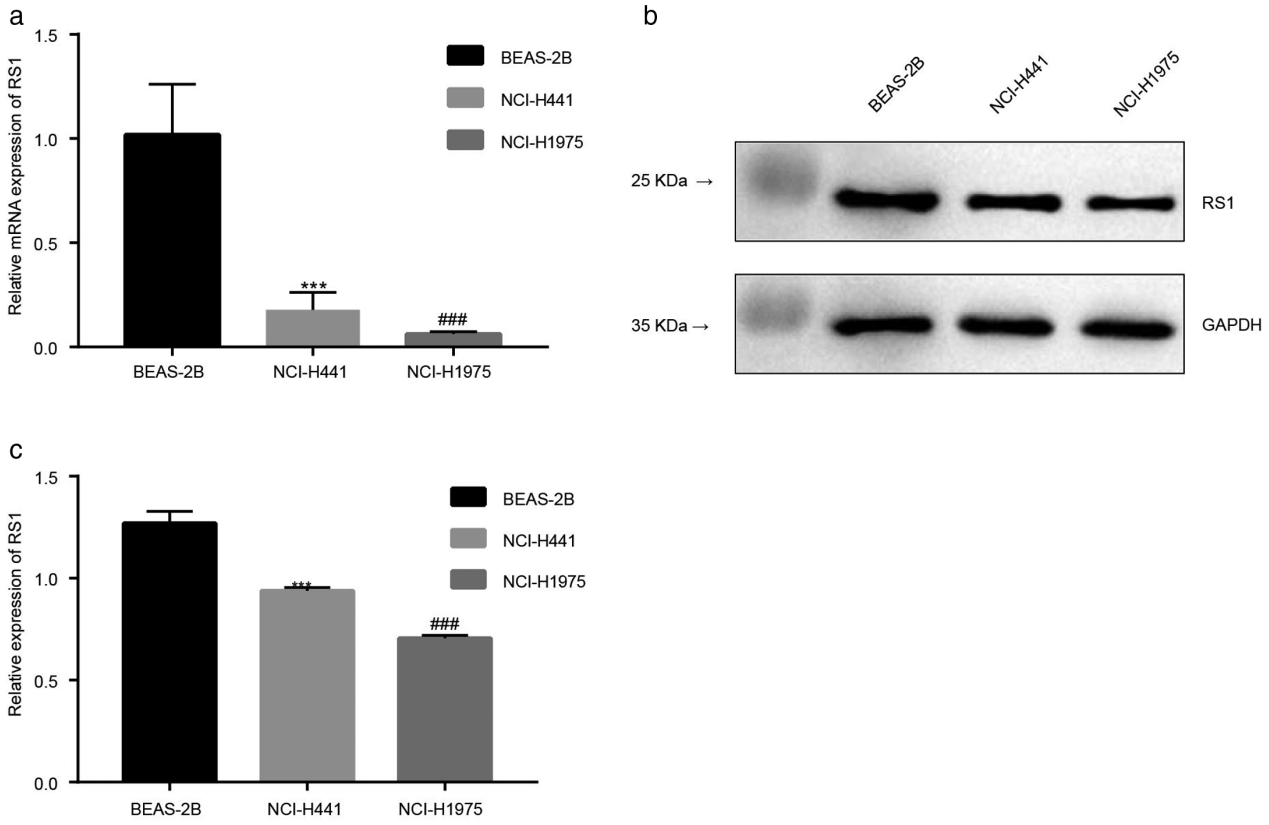

FIGURE 5 RS1 expression in cell lines. (a) RS1 mRNA was downregulated in lung adenocarcinoma (LUAD) cells. $^{***}p < 0.001$ versus NCI-H441; $^{###}p < 0.001$ versus NCI-H1975. (b) Expression of RS1 protein in LUAD cells. (c) Comparison of gray values in WB. $^{***}p < 0.001$ versus NCI-H441; $^{###}p < 0.001$ versus NCI-H1975.

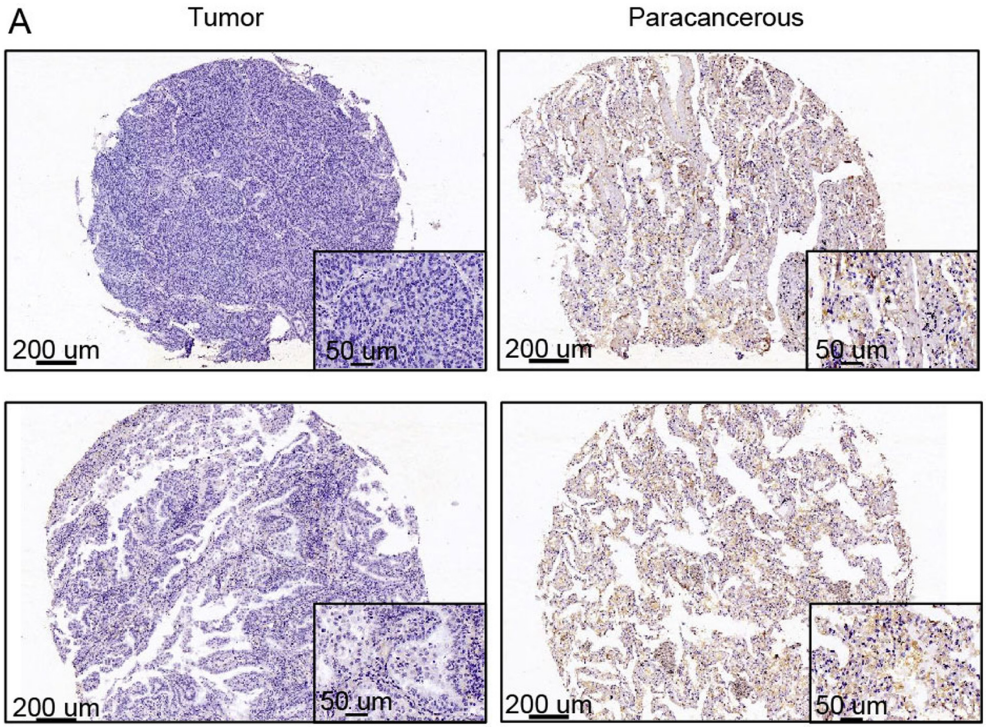

FIGURE 6 Representative images of RS1 protein expressed in clinical samples

different types of immune cells suggested heterogeneity of immune infiltration in LUAD patients (Figure 3b). In addition, we found significant differences in the degree of immune infiltration between the high and low RS1 expression groups. Large proportions of plasma cells, T cells CD4 naive, T cells gamma delta, NK cells activated and macrophages M1 were identified in the high RS1 expression group, while small proportions of T cells follicular helper and T cells regulatory (Tregs) were identified in the low RS1 expression group (Figure 3c). These differences might contribute to different prognoses in patients between the high and low RS1 expression groups.

RS1-related signaling pathways in LUAD

To explore the potential molecular function of RS1 in LUAD, we performed gene set enrichment analysis (GSEA) between samples with high and low RS1 expression, using data in TCGA. A large number of genes were enriched in RS1 high expression samples, with corresponding 20 KEGG pathways were significantly enriched, while one KEGG pathway was enriched in RS1 low expression samples ($p < 0.05$) (Table 3). The top six KEGG pathways; cardiac muscle contraction, vascular smooth muscle contraction, renin-angiotensin system, hypertrophic cardiomyopathy HCM, GnRH signaling pathway, and differentiated cardiomyopathy are shown in Figures 4a–f.

RS1 gene is downregulated in LUAD cells and clinical samples

To verify the expression level of RS1 gene in vitro, we conducted experiments at both cellular and clinical levels. Consistent with the conclusions, the mRNA level was significantly lower in NCI-H441 and NCI-H1975 cells than that in BEAS-2B cells (Figure 5a), with the expression levels of RS1 protein alike (Figure 5b,c). Similarly, immunohistochemistry analysis showed that the expression level of RS1 protein in tumor tissues was lower than that in normal tissues (Figure 6).

DISCUSSION

LUAD, which originates from bronchial mucosa epithelial cells, shows few obvious clinical symptoms in its early stage. However, although it grows slowly, blood metastasis can occur in the early stages.[31,32] With the development of large-scale sequencing programs and microarray technology, gene expression profiles are widely acknowledged to play a significant role in tumor diagnosis and prognosis. Therefore, it is critical to investigate the genes associated with the occurrence, invasion, and metastasis of LUAD, which can provide theoretical foundations and identify molecular indicators for early diagnosis and prognostic evaluation, as well as provide specific targeted therapy for patients with LUAD. In the current study, based on gene expression profiling analysis of TCGA and GEO databases, we demonstrated that the expression level of RS1 gene was related to the prognosis of LUAD and low expression of RS1 could be used as an independent prognostic indicator for LUAD. We also found significant differences in immune cell infiltration between high and low RS1 expression LUAD samples. Further enrichment analyses indicated that low expression of RS1 was associated with the proteasome signaling pathway. Our findings provide new insights into the underlying mechanism of RS1 gene in influencing tumor microenvironment and potential application of RS1 in LUAD prognosis.

RS1 (also known as XLRS1, X-linked retinoschisis 1) is the first pathogenic gene identified in congenital retinoschisis, a rare retinal degenerative disease.[33] Congenital retinoschisis mostly occurs in young men, mainly manifested as the splitting between the retinal nerve fiber layer and ganglion cell layer. RS1 gene encodes retinoschisin 1, which is expressed in photoreceptor cells and bipolar cells and participates in the regulation of cell adhesion and signaling pathway, playing an essential role in maintaining the structure and function of the retina.[34] RS1 protein contains two conserved domains, the N-terminal region of the signal peptide, which is involved in the protein secretion from endoplasmic reticulum to cytoplasm, and the highly conserved discoidin domain, which is deemed to mediate cell–cell interaction and is the key part in maintaining retinal integrity and establishing synaptic connections.[35] Over 200 RS1 gene mutations related to retinoschisis have been reported (http://www.hgmd.cf.ac.uk/ac/), some of which can lead to visual impairment by affecting RS1 secretion or protein-octamer formation, resulting in retinoschisis complicated with vitreous hemorrhage, retinal detachment, and glaucoma.[36] In our study, it is somewhat surprising that the expression level of RS1 was found to be related to the prognosis of LUAD. Compared with normal tissues, a lower expression level of RS1 was found in LUAD tumor tissues according to our analyses based on the public database, which was consistent with our validation in LUAD cells and tissue microarrays. In reviewing the literature, however, no relevant research was found on the association between RS1 and LUAD, or other cancers. Moreover, LUAD patients with lower RS1 expression levels had poorer prognosis, and the multivariate Cox regression analysis showed that in addition to the TNM stage, the well-acknowledged scoring system, RS1 could also be used as an independent prognostic indicator. A possible explanation for our findings is the highly conserved discoidin domain of RS1 is related to cell–cell interaction; the reduction of protein secretion might impair cell–cell communication and adhesion, resulting in metastasis of tumor cells. Low expression of RS1 might be involved in the regulation of progression or metastasis of LUAD, and thereby further exerts negative effects of the prognosis of LUAD patients. However, the more detailed underlying relationship between RS1 and LUAD cannot be concluded with our current evidence, and deserves further investigation.

To further explore how the expression level of RS1 influences LUAD prognosis, we performed immune cell infiltration and RS1-related enriched signaling pathways analysis. Increasing evidence has shown the role of tumor immune microenvironment (TME) in tumorigenesis and development. The synergy between tumor cells and their surrounding cells leads to various malignant phenotypes such as immortalized proliferation, antiapoptosis and escaping immune surveillance.[37] Resorting to CIBERSORT to analyze immune cell infiltration enabled us to evaluate the prognostic outcomes of LUAD patients with different RS1 expression levels. A high proportion of T cell follicular helper (Tfh) and T regulatory (Tregs) cells were found in the low RS1 expression group. Tfh and Tregs belong to $CD4^+$T subsets and stimulate the proliferation and differentiation of B cells through a series of cytokines.[38,39] Bcl-6, one of the major cytokines of Tfh, was found in peripheral T-cell lymphomas and primary cutaneous $CD4^+$ small/medium-sized pleomorphic T-cell lymphoma, while six cases of cutaneous follicular B lymphoma managed to obtain good outcomes by performing Tfh lymphoma therapeutic approaching.[40–42] Tfh infiltration was also found in solid tumors such as colon and liver cancer.[43]

Whether Tfh is involved in the development of LUAD mainly via cell infiltration was not clarified in our study, although the probable contribution of Tfh infiltration to poor prognosis could not be neglected. Tregs is thought to weaken the immune effect of T cells, so as to enable tumor growth and inhibit immune surveillance. It has been reported that Tregs can secrete TGF-β in NSCLC, which may be related to tumor progression.[44] Peterson et al. found that the tumor recurrence rate increased with a growing proportion of Tregs in the total tumor infiltrating lymphocytes, suggesting the association between Tregs and the recurrence after surgical resection at NSCLC stage I.[45] In addition, we also demonstrated proteasome pathway was enriched in low RS1 expression samples. The proteasome is designed to degrade enzymes related to cell growth, signal transduction, gene transcription and apoptosis, thus involves in activation or inhibition of certain cellular processes. Hydrolyzation of inhibitor of nuclear factor-κB (IκB) by proteasome will release NF-κB to the nucleus then activate antiapoptotic genes such as Bcl-2, leading to the occurrence of tumors. Studies also have shown increased activity of proteasome degrading IκB in tumor cells and a high level of NF-κB.[46,47] In conclusion, our findings in immune cell infiltration and enriched signaling pathway may partly explain the correlation between low RS1 expression and poor LUAD prognosis.

Although visible progress has been made in molecular targeted therapy for NSCLC, it is still confined to a certain group of patients due to the limited number of molecular targets available in clinic. Screening for new gene targets and prognostic markers is of positive significance for the sustainable control of cancer progression. Here, we identified RS1 as an independent prognostic indicator of LUAD and explored the possible mechanism of association between RS1 expression level and LUAD recurrence. In the future, we will focus more on the prognostic research based on the classification of LUAD (adenocarcinoma in situ, microinvasive carcinoma and invasive adenocarcinoma), and expand the number of clinical samples, especially patients at the early stage, to curb tumor metastasis and recurrence and improve the therapeutic effectiveness at the early stage.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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