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Combined ARHGEF6 and Tumor Mutation buBden may serve as a potential biomarker for immunotherapy of lung adenocarcinoma

Li Tong [a,b,1], Sichu Wang [a,b,1], Juanjuan Yang [a,1], Qing Zhang [a], Xue Gu [a,c], Taoming Mo [a,c], Yang Luo [a,c], Chenqian Zhang [c], Jianguo Zhang [a], Yifei Liu [a,c,*]

[a] Department of Pathology, Affiliated Hospital of Nantong University, Nantong, China
[b] Dalian Medical University, Dalian, China
[c] Medical School of Nantong University, Nantong, China

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ABSTRACT

ARHGEF6, a member of the Dbl-related guanylate exchanger (GEF) family, is highly expressed in gastric cancer and glioma. However, scientists still do not know whether it plays a pivotal role in the pathogenesis of lung adenocarcinoma (LUAD) . The prognostic significance of ARHGEF6 expression was assessed by TCGA data. This paper focuses on the level of immune infiltration associated with ARHGEF6 and explored the relationship of this gene with the tumor mutational burden (TMB), immune checkpoints, and drug sensitivity. The results showed that the high expression of ARHGEF6 was associated with a good prognosis in LUAD patients, and positively correlated with a variety of immune cells and drugs. Meanwhile, ARHGEF6 was found to be negatively correlated with TMB. In conclusion, the results of this study suggest that ARHGEF6 is a protective gene in LUAD patients. A combination of ARHGEF6 and TMB could be used as a potential biomarker in the screening of immunotherapy regimens, which are provided to patients with LUAD.

1. Introduction

Lung cancer is one of the cancers that pose the greatest threat to human health. Lung adenocarcinoma (LUAD) is a common histological type of lung cancer [1]. Even with the current rise of targeted therapies and immunotherapy, the 5-year survival rate of patients with LUAD remains low [2]. This is mainly because immunotherapy is only effective for a small percentage of patients. Therefore, we need to identify more potential molecular targets for the diagnosis and treatment of LUAD. This would improve the prognosis of patients with LUAD.

The tumor mutational burden (TMB) responds well to immunosuppressive therapy and is a predictive marker for non-small cell lung cancer (NSCLC) [3]. It is defined as the number of somatic mutant loci in the tumor genome and can be used to indicate whether a tumor has the ability to produce neoantigens [4]. When many neoantigens are associated with tumors, the value of TMB is high and immunotherapy NSCLC patients live significantly longer than those with chemotherapy [5].

ARHGEF6 belongs to the following family of proteins: Dbl-related, guanine nucleotide exchange factor (GEF). It catalyzes the exchange of GDP for GTP, and it promotes the activity of Rho-GTPases Rac1 and Cdc42 [6]. It is reported that ARHGEF7 promoted the

* Corresponding author. Department of Pathology, Affiliated Hospital of Nantong University, Nantong, China.
E-mail address: liuyifei@ntu.edu.cn (Y. Liu).
[1] LiTong, Sichu Wang, and Juanjuan Yang contributed equally to this work and share first authorship.

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metastasis of colorectal cancer [7]. In addition, ARHGEF10 exerted a specific inhibitory effect on patients with pancreatic cancer [8]. Interestingly, ARHGEF16 can promote the progression of glioma [9]. However, no previous study has explored the relationship between ARHGEF6 and LUAD. In particular, the relationship between TMB and LUAD remain unclear till date.

The purpose of this study was to determine the expression of ARHGEF6 in LUAD. In addition, this study explored the relationship between ARHGEF6 and TMB-related immunotherapy in LUAD. This makes ARHGEF6 a novel biomarker for therapies related to LUAD.

2. Materials and methods

2.1. Data source and processing

Using The Cancer Genome Atlas (TCGA) (https://portal.gdc.cancer.gov/) database [10], we obtained the transcriptome data of 555 specimens, which included 54 normal and 501 LUAD tissue samples. The main objectives of this study was to explore the differential expression of ARHGEF6 in tumor and non-tumor samples. We excluded the samples with incomplete clinical information. The data from the remaining 461 samples after processing were used to construct survival curves. Determination of whether ARHGEF6 is an important biomarker in the prognosis of LUAD and calculation of the hazard ratio (HR) of ARHGEF6 expression levels on other clinical features of LUAD were performed by logit test and using univariate and multivariate Cox proportional hazards models.

2.2. Single-cell analysis of ARHGEF6

Using the Tumor Immune Single-Cell Hub (http://tisch.comp-genomics.org/) (TISCH) web tool [11], we performed correlate single cell analysis of ARHGEF6. Briefly, the " NSCLC" cancer type, the "GSE99254" dataset, and the "ARHGEF6" gene name were selected. Then, we quantified and visualized the ARHGEF6 expression levels by constructing a scatter and violin plot.

2.3. Analysis of TIMER and TISIDB databases

Tumor Immune Estimation Resource 2.0 (TIMER2.0) (http://timer.cistrome.org/) is a comprehensive resource for systematical analysis of immune infiltrate [12]. We determined the expression of ARHGEF6 in various types of tumors, which were presented in TIMER2 database. Using the TISIDB web tool (TISIDB (cis.hku.hk)), we evaluated the relationship between ARHGEF6 expression and the abundance of six types of TIICs and with immune checkpoints such as PDCD1, PDCD1LG2, CTLA4, CD274, HAVCR2, LAG3 and TIGIT) [13].

2.4. A correlation exists between the expression of ARHGEF6 and TMB

The TMB scores were determined for all the samples included in this study. The score was based on the somatic mutation data of TCGA database. The correlation between ARHGEF6 expression and TMB was established by using the Spearman's rank correlation coefficient.

2.5. Prediction of multiple therapeutic sensitivities

In this experiment, the concentration required for 50% inhibition is expressed as IC50. We predicted chemotherapeutic responses for each sample. The responses were based on the largest publicly available database on pharmacogenomics [The Genomics of Cancer Drug Sensitivity (GDSC), https://www.cancerrxgene.org/] [14]. The prediction tool chose the R package "pRRophetic".

The Immunophenoscores (IPS) of LUAD was obtained from the Cancer Immunome Atlas (TCIA) database (https://tcia.at/) [15]. Then, we compared the IPS of different immunotherapy methods. Thus, we predicted the sensitivity of immunotherapy methods. The results were calculated by using the R packages "ggpubr" and "limma."

2.6. Clinical samples of lung adenocarcinoma (LUAD)

We obtained 148 pairs of lung adenocarcinoma tissue samples by surgical resection and performed genetic testing on all patients. The clinical information (including gender, age, TNM staging, and chemotherapeutic efficacy) was obtained either by telephonic follow-up or by reviewing the patient's medical records. Genetic testing was performed in 48 of these cases. The patient's TMB value is obtained by genetic testing. There are no accepted criteria to distinguish between high and low TMB. In this study, the mean TMB value of 8.2muts/Mb in lung adenocarcinoma from TCGA (The Cancer Genome Atlas) database was used as a criterion for differentiation. This study was approved by the Medical Ethics Committee of Nantong University Hospital (protocol code 2021-K133 and approved on 1st Jan 2021), and written informed consent was obtained from each patient prior to this study.

2.7. An analysis of Real-time quantitative polymerase chain reaction

Using TRIzol Reagent (Invitrogen), we extracted the RNA from lung tissues. After that, cDNA was synthesized according to the instructions of the Hifair II 1st Strand cDNA Synthesis SuperMix (11120ES60; Yeasen, Shanghai, China). We subjected to qRT-PCR analysis by using Hieff qPCR SYBR Green Master Mix (Low Rox Plus) (11202ES08; Yeasen, Shanghai, China).

2.8. Immunohistochemistry

We first dewaxed the sections with gradient alcohol and xylene, followed by 20 min of antigen repair in an autoclave to block endogenous peroxidase. Finally, we incubated the samples with rabbit anti-human polyclonal *anti*-ARHGEF6 primary antibody (PA5-51965, Invitrogen, Carlsbad, CA, USA). In the medium, we maintained a dilution of 1:200 for 3 h at room temperature. Thereafter, we covered the sections with secondary antibodies. Thereafter, we stained with hematoxylin. The staining intensities and percentages were recorded as 0, 1, 2 and 3, corresponding to weak, low, medium, and strong as well as 0–25%, 26–50%, 51–75% and 76–100%, respectively. The final score is the product of both. A final staining index of ≥ 6 or <6 indicated high or low expression of ARHGEF6, respectively. The sections were scored by two certified pathologists.

2.9. Statistical analysis

All the statistical analyses were performed by using the R statistical language (version 4.1.1) and the SPSS software (version 26.0). P-values were obtained by case weighting and after calculating Pearson correlations. Wilcoxon test was used for comparison of two groups, while Kruskal-Wallis test was used for comparison of more than two groups. Survival curves were constructed by the Kaplan-Meier method. Logarithmic tests were performed to determine statistically significant differences. In all the analyses, $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. ARHGEF6 is a prognostic indicator of LUAD

Tumor Immunization Estimation Resource version 2 (TIMER2, http://timer.cistrome.org/) was used to analyze the differential expression of ARHGEF6 in different types of tumor tissues and adjacent normal tissues. As shown in Fig. 1A, the expression of ARHGEF6 gene was higher in tumor tissues of following types: glioblastoma multiforme, renal pheochromocytoma, and renal clear cell tumor. The expression of ARHGEF6 gene was found to be lower in tumor tissues of following types: bladder urothelial carcinoma, breast invasive carcinoma, cervical squamous cell carcinoma and endocervical adenocarcinoma, colon adenocarcinoma, lung

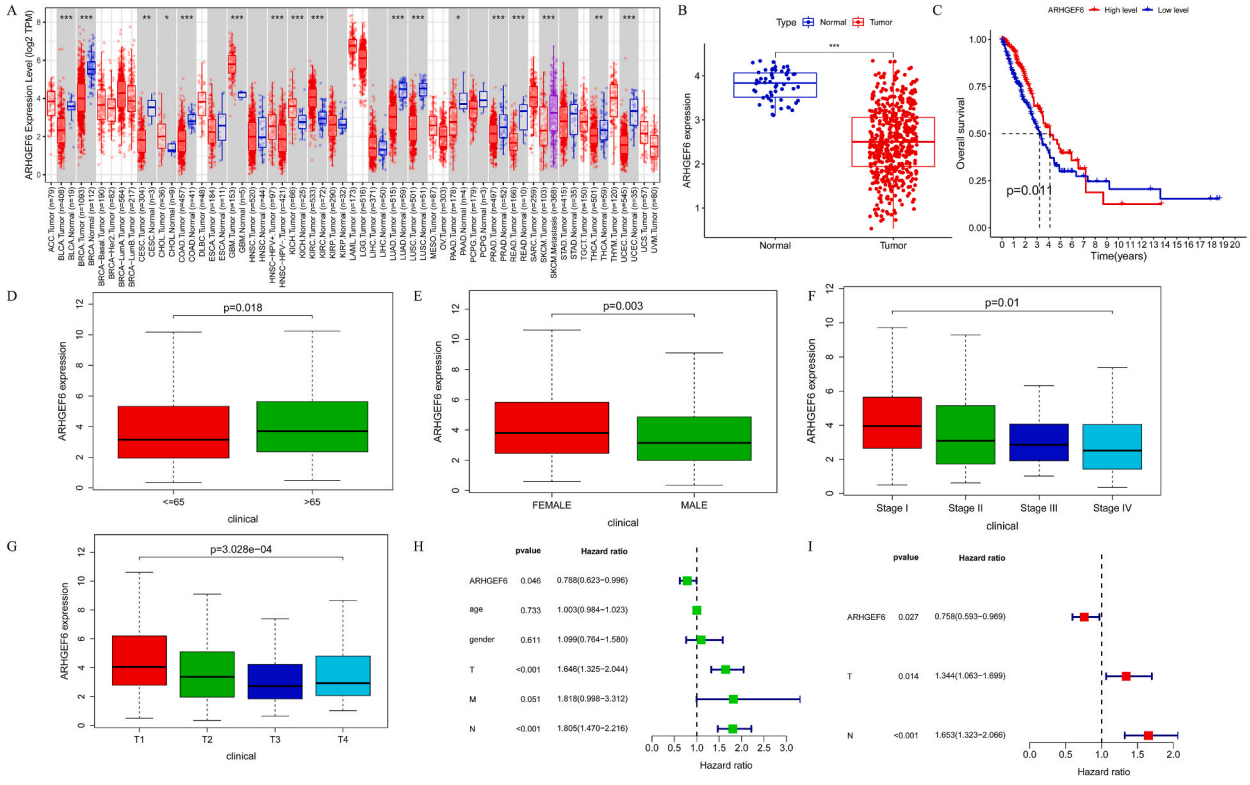

Fig. 1. ARHGEF6 may serve as a new prognostic marker for lung adenocarcinoma (LUAD). (A) ARHGEF6 mRNA levels in different tumor types were determined by TIMER2.0. (B) ARHGEF6 expression in TCGA database paired lung adenocarcinoma data. (C) ARHGEF6 expression was significantly negatively correlated with OS in TCGA. (D–G) Relationship between ARHGEF6 and clinicopathological parameters. (H–I) Univariate and multifactorial analysis of ARHGEF6. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

adenocarcinoma, lung squamous cell carcinoma, and prostate adenocarcinoma (all $P < 0.05$).

We further evaluated the expression of ARHGEF6 gene and the relationship with clinicopathological parameters of LUAD. The results indicate that ARHGEF6 gene was highly expressed in normal tissues ($P < 0.001$) (Fig. 1B). Using Kaplan-Meier analysis, we found that ARHGEF6 gene was highly expressed and showed better prognosis ($P = 0.011$; Fig. 1C) (Supplementary Table 1). Moreover, it was found to be associated with age ($P = 0.018$), gender ($P = 0.003$), tumor stage ($P = 0.01$) and tumor size ($P = 3.028e-04$) (Fig. 1D–G). Then, we performed univariate analysis on the data obtained from 331 LUAD specimens of TCGA database. Thus, we identified the three risk factors of LUAD samples, that is, the expression of ARHGEF6, lymph node status, and tumor size (Fig. 1H). The multivariate Cox proportional hazards model established that ARHGEF6 expression, lymph node status, and tumor size were independent prognostic factors of LUAD (Fig. 1I).

3.2. Association of ARHGEF6 with immune infiltration and immunotherapy

In this experiment, we had to determine the predominant cell types associated with the expression ARHGEF6 gene. Fig. 2A illustrates how ARHGEF6 gene is mainly expressed in immune cells, especially in T cell subsets. Using the GSE99254 dataset, we analyzed 12,346 cells of 14 NSCLC patients. We found that ARHGEF6 gene was highly expressed in T-cell subsets of NSCLC microenvironment (Fig. 2B and C).

Using TISIDB databases, we found that the expression of ARHGEF6 gene was positively correlated with the following cell types: B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells (Fig. 2D). Moreover, it showed a positive correlation with the following multiple immune checkpoints (PDCD1, PDCD1LG2, CTLA4, CD274, HAVCR2, LAG3, and TIGIT) (Fig. 2E).

Since TMB is associated with immunotherapy. In this experiment, we analyzed the relationship between ARHGEF6 gene and TMB. As shown in Fig. 3A, ARHGEF6 was found to be negatively correlated with TMB. We also compared the differences in IPS between high and low expression groups of ARHGEF6. IPS, IPS-PD1, IPS-CTLA4, and IPS-PD1 + CTLA4 were used to determine the potential application of ARHGEF6 gene. IPS-PD1, IPS-CTLA4 and IPS -PD1+CTLA4 were significantly different in the high and low expression groups (all $P < 0.05$). However, there was no significant difference in IPS ($P = 0.9$) (Fig. 3B–E).

3.3. Immunohistochemical analysis of tissue chips

We had to validate the expression of ARHGEF6 in LUAD patients and the relationship with clinicopathological parameters and TMB. Therefore, we performed the immunohistochemical staining of tissue microarrays, which contained 148 LUAD samples. The results indicate that the expression of ARHGEF6 gene was low in the tumor tissues (Fig. 4A) (Supplementary Fig. S2). In addition, ARHGEF6 gene expression correlated with tumor size ($P = 0.002$), stage ($P = 0.044$), but not well with other clinicopathological

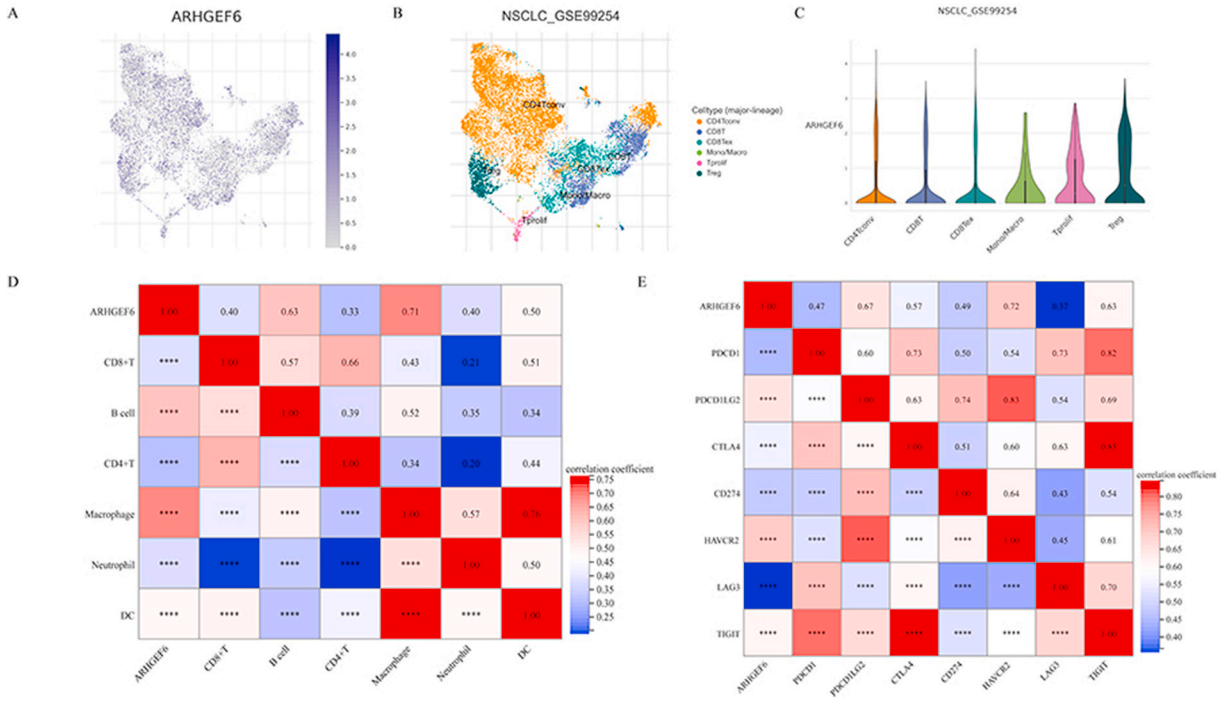

Fig. 2. ARHGEF6 has the potential to predict immunotherapy. (A–C) Single-cell analysis of ARHGEF6. (D) Correlation between ARHGEF6 expression and 6 types of immune cells in the TISIDB database. (E) Correlation between ARHGEF6 expression and 7 types of immune checkpoints in the TISIDB database.

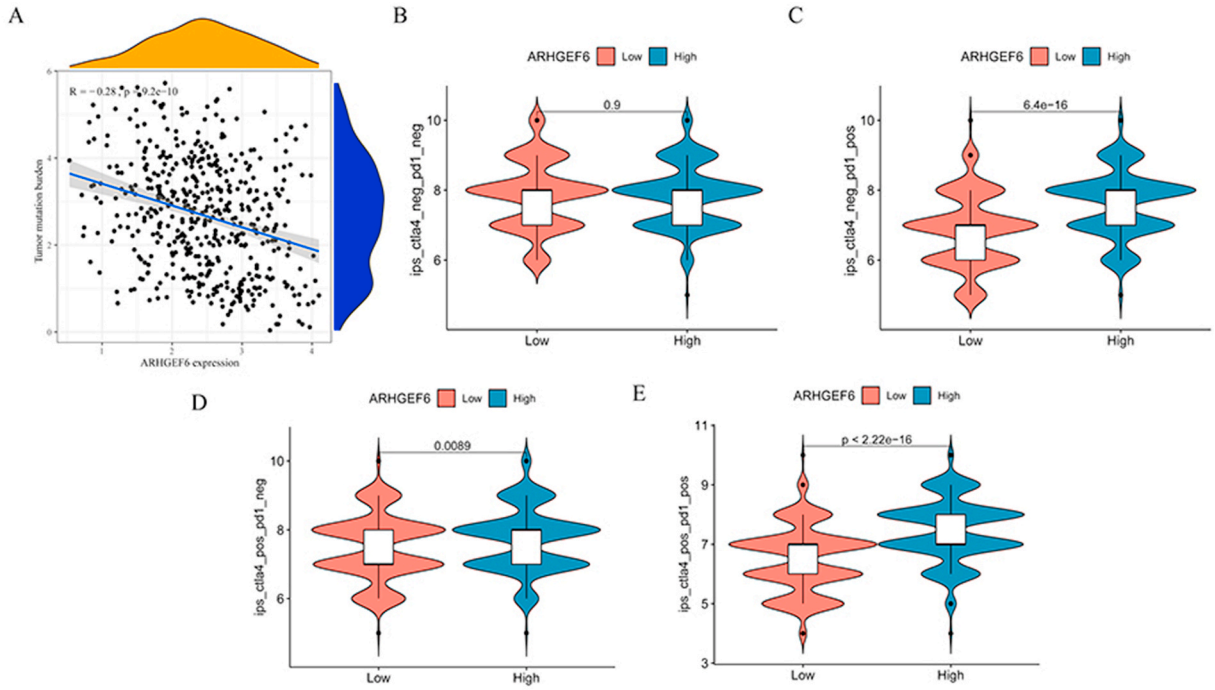

Fig. 3. Analysis of ARHGEF6 immunotherapy. (A) Correlation between ARHGEF6 expression and Tumor Mutuation Burden (TMB). (B–E) Correlation between ARHGEF6 expression and IPS.

parameters (Table 1). In 48 microarrays after genetic testing, we found that ARHGEF6 expression was higher in the tumor tissues of the TMB low expression group (P = 0.014). (Table 2) (Fig. 4B).

3.4. Drug Sensitivity Analysis of ARHGEF6

We had to determine whether ARHGEF6 gene was suitable for use in individualized treatment of LUAD patients. Therefore, we investigated the relationship between ARHGEF6 gene and the IC50 of commonly used investigational drugs of LUAD. These drugs include camptothecin, cisplatin, crizotinib, dabrafenib, docetaxel, erlotinib, gemcitabine, and paclitaxel. The LUAD patients with a high expression of ARHGEF6 were found to be more sensitive to most drugs. This trend was not observed in patients with a low-expression group of ARHGEF6. However, the high-expressing group may not benefit much from erlotinib treatment. The two

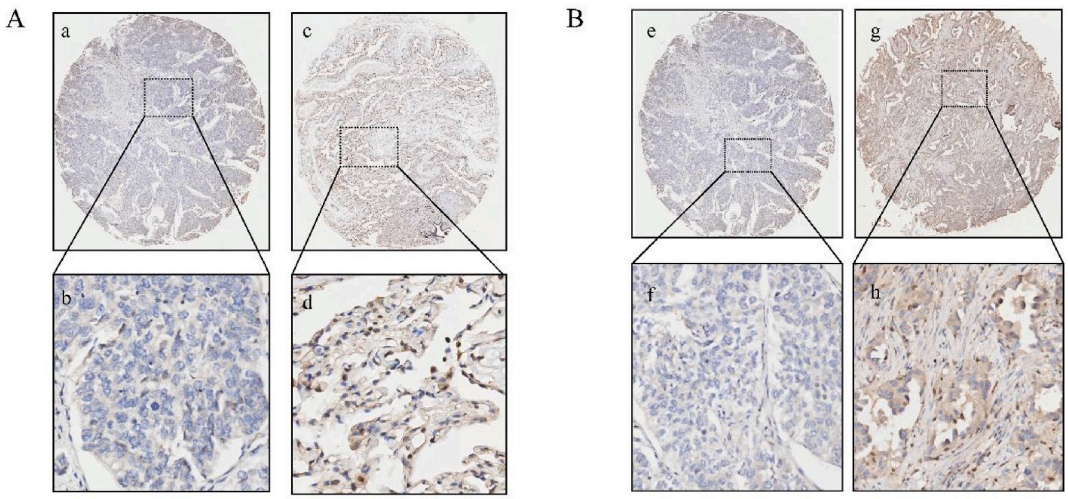

Fig. 4. The results of immunohistochemical staining in LUAD. (A) ARHGEF6 expression in LUAD tissue (a,b) and normal lung tissue (c,d). (B) ARHGEF6 expression in high TMB tissue (e,f) and low TMB issue (g,h). (a,c,e,g) Magnification, ×2.5; (b,d,f,h) Magnification, ×10.

Table 1
Relationship between ARHGEF6 expression and pathological parameters of LUAD patients.

Clinicopathological parameters	All cases	ARHGEF6 expression		P-value
		Low	High	
Age				0.681
>65	42	21	21	
≤65	106	57	49	
Gender				0.821
Male	81	42	39	
Female	67	36	31	
Stage				0.044
I	107	51	56	
II	32	20	12	
III	6	5	1	
IV	3	2	1	
T classification				0.002
T1	104	46	58	
T2	35	24	11	
T3	7	7	0	
T4	2	1	1	
N classification				0.187
N0	108	54	54	
N1	38	22	16	
N2	2	2	0	
M classification				0.501
M0	78	77	1	
M1	70	68	2	

groups did not show any significant difference to docetaxel and gemcitabine treatment. (Fig. 5A–H).

4. Discussion

Using the data of previous transcriptome sequencing, we screened the LUAD samples for ARHGEF6 molecule. ARHGEF6 is the first disease gene identified to cause non-syndromic mental retardation [16]. Protein levels of this molecule have been shown to be significantly upregulated in AGS gastric cancer cells and in tissues of GBM patients [17,18]. However, scientists have yet to elucidate its role in the pathogenesis of LUAD. Here, we verified the expression level of ARHGEF6 in LUAD samples by immunohistochemistry and qPCR: ARHGEF6 was highly expressed in normal tissues (Supplementary Fig. S1). Meanwhile, results from the TISIDB database showed a positive correlation between this molecule and a variety of immune cells and immune checkpoints. This suggests that ARHGEF6 is not only an indicator of lung prognosis in lung adenocarcinoma, but may also be relevant to immunotherapy.

Subsequently, we performed immunohistochemical staining of selected clinical samples. The results established the relationship between ARHGEF6 and TMB. Moreover, previous studies have shown that TMB is a biomarker in immunotherapy and that there is a correlation between the efficacy of immunotherapy and the number of mutations [19]. This implies that the high TMB group benefitted more from immunotherapy. In addition, we explored the relationship between ARHGEF6 and immunotherapy, wherein IPS-PD1 had the best effect.

In previous studies, the levels of PD1+$CD8^+$ T cells and PDL1+$CD8^+$ T cells were associated with immunotherapy, which was provided to NSCLC patients [20,21]. A high expression of PD-L1 would make tumor cells more sensitive to PD-1/PD-L1 inhibitors [22]. Therefore, patients with high TMB and high PD1 levels had the longest median progression-free survival (FPS) [23]. However, TMB did not correlate well with the expression of PD1 [24]. In our late follow-up results, most of the high TMB groups had relatively low PD1/PDL1 expression. The results were similar in the low-TMB group. As a result, these patients did not receive immunotherapy. Additional patients opted for targeted therapy and conventional chemotherapy. Based on our follow-up of 16 patients with complete data, six patients in the low ARHGEF6 expression group had a poor prognosis compared to three patients in the high expression group.

In this study, we combined an online database with immunohistochemical results to validate the prognostic role of ARHGEF6 in lung adenocarcinoma and the negative correlation with TMB. According to the follow-up data, patients in both the high and low TMB groups did not benefit from immunotherapy in the treatment of lung adenocarcinoma. We speculate that one reason for this may be related to the amount of ARHGEF6 expression in the patients. However, this speculation has not been tested by us in clinical data. In this regard, this study suggests that ARHGEF6 and TMB could be combined to better determine the subsequent treatment options for patients: for patients with high ARHGEF6 and high TMB, immunotherapy could be tried; for the other groups, chemotherapy or targeted therapy regimens would be appropriate.

In subsequent studies, we will continue to expand the sample size as much as possible and will try to obtain more accurate genetic information by RNA-seq testing. Patients will also be followed up on their treatment, including targeted therapies as well as immunotherapy. Gene expression and TMB values of ARHGEF6 will be collected from patients with significant and poor immunotherapy results, thus validating our hypothesis. This will help us to find a new biomarker for immunotherapy of lung adenocarcinoma.

Table 2
Relationship between ARHGEF6 expression and TMB.

	All cases	ARHGEF6 expression		p-value
		Low	High	
TMB				0.014
Low	39	13	26	
High	9	7	2	

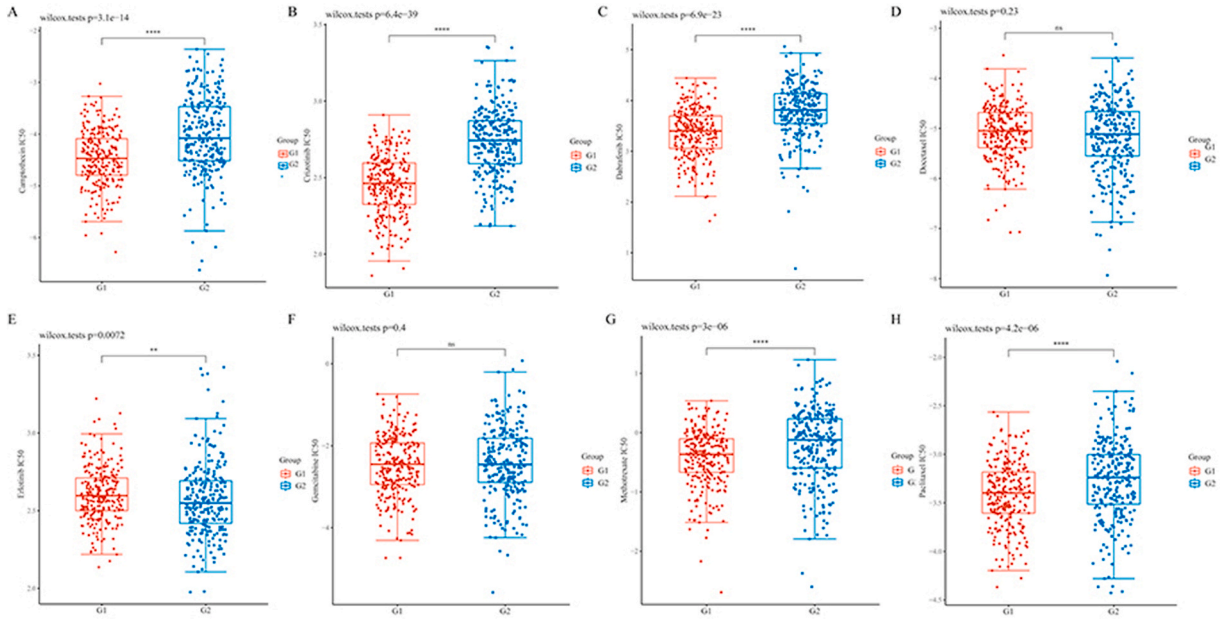

Fig. 5. Drug Sensitivity Analysis of ARHGEF6. (A–H) Correlation of ARHGEF with eight drugs, including camptothecin, cisplatin, crizotinib, dabrafenib, docetaxel, erlotinib, gemcitabine, and paclitaxel. (*P < 0.05, **P < 0.01, ***P < 0.001, G1:low-expression; G2:high-expression).

5. Conclusion

ARHGEF6 was identified as an anti-oncogene in LUAD and its low expression was associated with poor prognosis. ARHGEF6 was negatively correlated with TMB and positively correlated with immune infiltration. Combining TMB and ARHGEF6 may be a new option for immunotherapy of LUAD.

Author contribution statement

Li Tong: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Sichu Wang; Xue Gu; Taoming Mo; Yang Luo; Chenqian Zhang: Analyzed and interpreted the data.
Juanjuan Yang: Performed the experiments.
Qing Zhang: Contributed reagents, materials, analysis tools or data.
Jianguo Zhang; Yifei Liu: Conceived and designed the experiments.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18501.

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