Original Article

Target genes of N6-methyladenosine regulatory protein *ALKBH5* are associated with prognosis of patients with lung adenocarcinoma

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Background: Lung adenocarcinoma (LUAD) exhibits high fatality rates, and effective treatments are lacking. The expression of the N6-methyladenosine (m6A) regulatory protein ALKBH5 is associated with lung cancer. To identify new therapeutic targets for LUAD, we screened target genes of *ALKBH5* and analyzed their potential mechanisms of action.

Methods: LUAD samples from The Cancer Genome Atlas (TCGA) were used to analyze the expression of *ALKBH5* and screen for genes with correlated expression. Intersection of the genes upregulated in cells with *ALKBH5* silencing with the genes significantly associated with *ALKBH5* were defined as *ALKBH5* target genes. STRING was used to evaluate interactions between the target genes, and the relationship between *ALKBH5* target gene expression and LUAD patient prognosis was analyzed using the R package Survminer. Target genes were evaluated by functional enrichment analyses.

Results: *ALKBH5* was highly expressed in LUAD tissues and was significantly associated with a poor prognosis. Fifteen *ALKBH5* target genes were identified, primarily enriched in protein processing in the endoplasmic reticulum, transcriptional coregulator activity, and cell activation involved in the immune response. Upregulation of *ZNF*777, *TCOF1*, *CPLX2*, and *ABL1* was associated with a poor prognosis, whereas upregulation of *ZER1*, *VPS53*, and *RRBP1* was associated with a good prognosis.

Conclusions: This study provides potential therapeutic targets for LUAD and a basis for further studies on the mechanism underlying the effects of ALKBH5.

Keywords: Lung adenocarcinoma (LUAD); N6-methyladenosine (m6A); enrichment analysis; *ALKBH5*; prognosis

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Introduction

Lung cancer has a higher annual fatality rate than pancreatic, prostate, or breast cancer (1). Lung adenocarcinoma (LUAD) is the most common subtype of lung cancer (2), with 5-year survival rate of only 17% (3). Patients with early-stage disease and without metastatic tumors care candidates for standard surgical resection. However, most patients with LUAD are diagnosed at an advanced stage and can only be treated with chemoradiotherapy, which is associated with a high mortality rate (4). Therefore, in-depth studies of the molecular mechanisms underlying LUAD prognosis are of great value for the diagnosis and treatment of this cancer.

N6-methyladenosine (m6A), an adenosine derivative from RNA, plays a major role in regulating gene expression and is the most common mRNA modification in mammals (5). Li *et al.* (6) found that m6A modification contributes to the formation of the tumor microenvironment in patients with LUAD and consequently is associated with its prognosis. The m6A family consists of regulatory proteins that can "write", such as methyltransferase-like protein 3 (METTL3), METTL14, and Wilms' tumor 1-associating protein; "read", including YTH domain-containing family proteins and insulin-like growth factor 2 mRNA-binding protein family; and "erase", including α-ketoglutarate-dependent dioxygenase and α-ketoglutarate-dependent dioxygenase alkB homolog 5 (ALKBH5) (7). ALKBH5 has been identified as the primary demethylase of the m6A family (8). ALKBH5 is a target of hypoxia-inducible factor and is upregulated during hypoxia (9).

Highlight box

Key findings

- ALKBH5 was highly expressed in LUAD tissues and was significantly associated with a poor prognosis. Upregulation of ZNF777, TCOF1, CPLX2, and ABL1 was associated with a poor prognosis, whereas upregulation of ZER1, VPS53, and RRBP1 was associated with a good prognosis.

What is known and what is new?

- The expression of the N6-methyladenosine regulatory protein ALKBH5 is associated with lung cancer. ALKBH5 was highly expressed in LUAD tissues and was significantly associated with a poor prognosis.

What is the implication, and what should change now?

- We screened target genes of ALKBH5 in LUAD, some of which were associated with the prognosis of patients with LUAD. This study provides targets and a basis for the development of novel treatments for LUAD.

ALKBH5 demethylase activity can promote the progression of various cancers, including liver cancer and glioblastoma (10,11), but its overexpression has been reported to inhibit cancer cell growth in bladder and pancreatic cancers (12,13). The conflicting roles of ALKBH5 in different cancers warrant further investigation. In LUAD, the upregulation of *METTL3* and downregulation of *ALKBH5* together lead to an increase in m6A levels and are often associated with a poor prognosis. Furthermore, knockdown of *METTL3* and overexpression of *ALKBH5* can inhibit lung tumor formation in a mouse model (14). Therefore, studies of the regulatory mechanisms of ALKBH5 are crucial to understand its influence on LUAD formation, prognosis, and treatment.

A549 cell lines with and without ALKBH5 knockdown were sequenced by methylated RNA immunoprecipitation sequencing (MeRIP-seq). We combined these data with mRNA sequencing data and clinical information for LUAD samples from The Cancer Genome Atlas (TCGA). The goal was to identify target genes of ALKBH5 and their association with LUAD prognosis. The newly identified target genes provide insights into the mechanism of action of ALKBH5 and are candidate targets for the treatment of LUAD. We present this article in accordance with the STREGA reporting checklist (available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-1464/rc).

Methods

Expression of ALKBH5 in LUAD and paracarcinoma tissues

TCGA-LUAD expression and clinical profile data were downloaded using the Xena online platform of the University of Santa Cruz [https://xenabrowser.net/datapages/?cohort=GDC%20TCGA%20Lung%20Adenocarcinoma%20(LUAD)&removeHub=https%3A%2F%2Fxena.treehouse.gi.ucsc.edu%3A443]. The relative expression levels of *ALKBH5* in 50 pairs of LUAD and paracancerous tissues were compared using paired-sample *t*-tests.

In addition, using 478 LUAD samples from TCGA, the relative expression levels of *ALKBH5* were compared among patients at different pathological stages by *t*-tests. The ggpubr R package was used to visualize the results, and the significance threshold was set to $P<0.05$.

Relationship between ALKBH5 expression and LUAD prognosis

To analyze the relationship between the expression

of *ALKBH5* and the prognosis of LUAD, prognostic information and *ALKBH5* expression data for 478 patients with LUAD were obtained from TCGA. The cutoff R package was used to calculate the optimal cutoff value for *ALKBH5* expression based on the smallest P value. The survival package in R (15) was used for survival analysis, and the Survminer R package was used to draw the Kaplan-Meier (KM) survival curve for *ALKBH5*.

Screening of genes related to ALKBH5 expression

Based on the expression profiles for 478 LUAD samples, Pearson correlation coefficients between the expression levels of *ALKBH5* and other genes were calculated. The thresholds for significantly correlated expression were correlation coefficient $|r| > 0.2$ and $P<0.05$.

Genes significantly associated with *ALKBH5* were further evaluated by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses using the clusterProfiler R package (version 4.4.4) (16). Terms with counts greater than or equal to 2 and Bonferroni-corrected $P<0.05$ were considered significantly enriched.

Analyzing ALKBH5 target genes

The LUAD cell line A549 was transfected with three small hairpin RNAs (shRNAs) and the scramble lentiviral vector pLHO.1 for stable *ALKBH5* silencing. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was used to verify the transfection efficiency. RNA was extracted using TRIzol. m6A RNA immunoprecipitation (IP) was performed using the GenSeq™ m6A-MeRIP Kit (GenSeq Inc., Nanjing, China) following the manufacturer's instructions. Both input samples without IP and m6A IP samples were used for RNA-seq library generation using the NEBNext® Ultra II Directional RNA Library Prep Kit (New England Biolabs, Inc., Ipswich, MA, USA). The library quality was evaluated with the BioAnalyzer 2100 system (Agilent Technologies, Inc., Santa Clara, CA, USA). Library sequencing was performed using an Illumina HiSeq instrument to obtain 150-bp paired-end reads. These reads were subjected to quality control with a threshold score of Q30. Subsequently, 3' adaptor trimming and the removal of low-quality reads were performed using cutadapt (v1.9.3). Clean reads from all libraries were aligned to the reference genome (HG38) using Hisat2 (v2.0.4). Methylated sites on RNAs (peaks) were identified using magnetic cell sorting (MACS). Differentially methylated sites were identified using diffReps. Peaks identified by both MACS and diffReps overlapping with exons were identified using homemade scripts.

Given that *ALKBH5* is a demethylation gene, the methylation level of genes related to ALKBH5 will increase after knockdown of *ALKBH5* (17). Therefore, genes with elevated methylation levels in *ALKBH5*-knockdown cells compared with normal LUAD cells with $P<0.00001$ and logFC <2 were selected for further analyses. GO and KEGG analyses of these genes were performed using clusterProfiler. Counts greater than or equal to 2 and Bonferroni-corrected $P<0.05$ were set as the significance thresholds for the enrichment analysis.

To further narrow down the number of target genes, the intersection between genes with significantly upregulated methylation levels in *ALKBH5*-knockdown cells and ALKBH5-related genes based on correlated expression patterns were identified as candidate targets of *ALKBH5*. The VennDiagram package in R (version 1.7.3) (18) was used to draw Venn plots. The functions of the target genes were assessed by GO and KEGG analyses using clusterProfiler. The significance threshold for the enrichment analyses was set as previously described.

In addition, interactions between target genes were assessed using the protein-protein interaction (PPI) database STRING (version 11.5) (19). Cytoscape (version 3.9.1) (20) was used to visualize the PPI network with a score of 0.15.

Prognostic analysis of ALKBH5 target genes

Pearson's correlation coefficients between the expression of *ALKBH5* and its target genes were calculated to assess the correlation between the genes. The R package ggplot2 (21) was used to visualize the correlation results. The cutoff package was used to calculate the optimal cutoff value of ALKBH5 target gene expression. The Survminer package was used to draw the KM survival curve for *ALKBH5*. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Statistical analysis

We compared standardized differences for all covariates between LUAD and paracarcinoma tissues. Continuous data are presented as the mean ± standard deviation (SD) and were analyzed with Student's *t*-tests for independent data. Categoric variables are presented as a count and

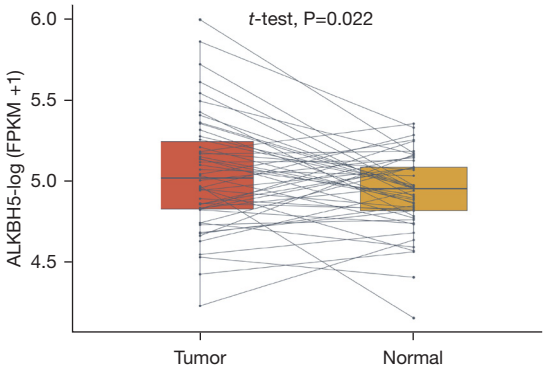

Figure 1 Relative expression of *ALKBH5* in lung adenocarcinoma and paired paracancerous tissues. FPKM, Fragments Per Kilobase of exon model per Million mapped fragments.

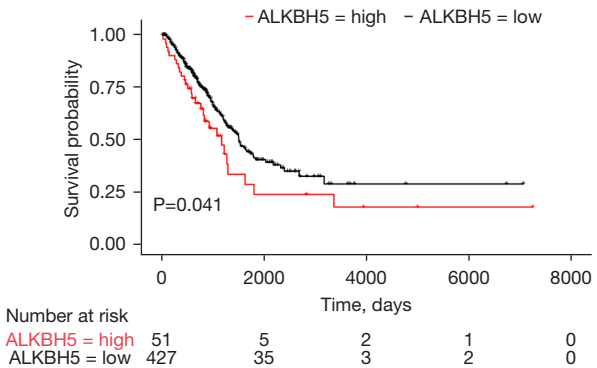

Figure 2 Kaplan-Meier survival curve for *ALKBH5*.

percentage of patients and compared using the χ^2 or Fisher's exact test. Pearson correlation coefficients between the expression levels of different genes were calculated. All P values less than 0.05 were considered statistically significant. All tests were two-sided, with an alpha level of 0.05. For all statistical evaluations, IBM SPSS Statistics 22.0 (IBM Corp, Armonk, NY, USA) and R 2.15.3 (2013; The R Foundation for Statistical Computing, Vienna, Austria) were used.

Results

High expression of ALKBH5 in LUAD tissues

The expression levels of *ALKBH5* were compared between LUAD and paired paracancerous tissues. *ALKBH5* expression was higher in LUAD tissues than in paracancerous tissues (P<0.05, *Figure 1*).

ALKBH5 expression levels in patients with LUAD at different pathological stages are presented in Figure S1. No obvious association between *ALKBH5* expression and different tumor node metastasis stages or stages of LUAD was identified.

High expression of ALKBH5 is a poor prognostic factor

According to the optimal cutoff value, patients with LUAD were divided into *ALKBH5* high- and low-expression groups. The KM curve exhibited a significant difference in prognosis between the two groups of patients; in particular, the prognosis of patients in the high *ALKBH5* expression group was worse than that of patients in the low expression group (P<0.05, *Figure 2*).

Correlated expression analysis

Based on Pearson correlation coefficients, we identified 774 genes that were significantly related to *ALKBH5* expression, including 459 and 315 genes with positively and negatively correlated expression, respectively. For functional assessment of these related genes, GO and KEGG enrichment analyses were performed, and only the top 20 terms were retained (*Figure 3*). These terms were all classified within the biological process category in GO analysis. The genes were primarily enriched in proteasomal protein catabolic processes, cellular component disassembly, negative regulation of transport, I-kappaB kinase/nuclear factor kappa B (NF-κB) signaling, and response to molecules of bacterial origin. A KEGG pathway analysis revealed that these genes were primarily involved in herpes simplex virus 1 infection, *Salmonella* infection, lipid and atherosclerosis, and human cytomegalovirus infection.

Fifteen genes were screened as targets of ALKBH5

The expression of *ALKBH5* in A549 cells decreased significantly after shRNA-mediated ALKBH5 silencing (Figure S2). To obtain the *ALKBH5* target genes, we obtained the intersection of genes correlated with *ALKBH5* expression and upregulated genes (365 genes) after *ALKBH5* knockdown in LUAD cells. Ultimately, 15 genes (*ANKRD11*, *LACTB*, *VPS53*, *TCOF1*, *ABL1*, *HSP90B1*, *RRBP1*, *NCOR2*, *TFF3*, *ZER1*, *CPLX2*, *NDST1*, *DHRS7B*, *ZNF777*, and *KATNIP*) were identified as potential candidate target genes of *ALKBH5* (*Figure 4A*). GO and KEGG enrichment analyses were used to functionally characterize these genes (*Figure 4B*). The genes were enriched for 21 GO terms (20 biological

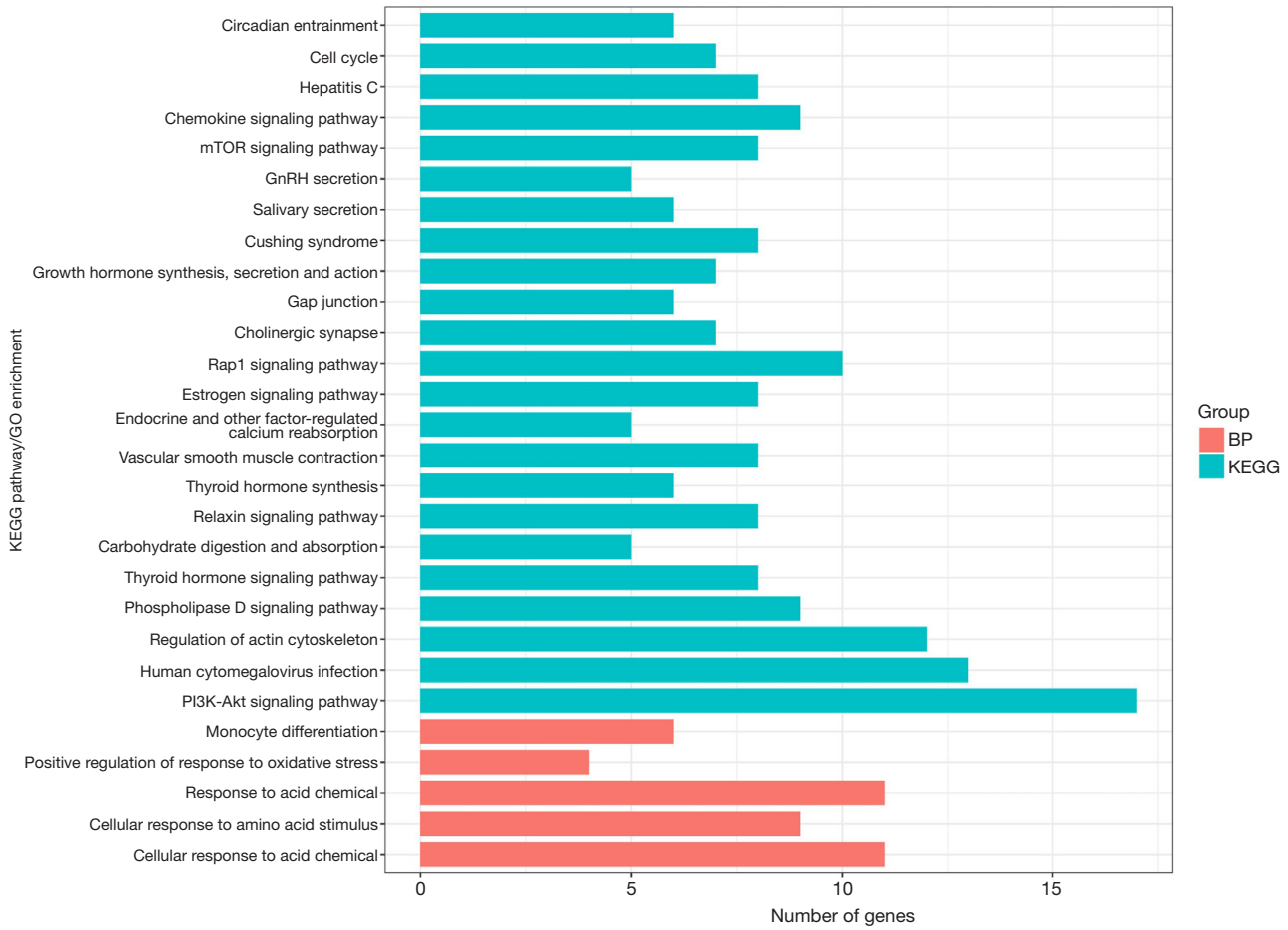

Figure 3 Enrichment analysis of genes associated with *ALKBH5*. BP, biological process; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology.

processes and one molecular function) and one KEGG pathway. These 21 terms included protein processing in the endoplasmic reticulum, transcriptional coregulator activity, regulation of small molecule metabolic processes, and cell activation involved in the immune response. In addition, a PPI network constructed using these 15 targets demonstrated that 10 target genes were included (*Figure 4C*). Nuclear receptor corepressor 2 (*NCOR2*) was a core gene in this network and interacted with ankyrin repeat domain-containing 11 (*ANKRD11*), zinc finger protein 777 (*ZNF777*), and nonreceptor tyrosine kinase (*ABL1*).

Prognostic value of target genes

Except for β-actin (*ACTB*), the target genes were positively correlated with the expression of *ALKBH5* (Figure S3). In addition, we analyzed the relationship between the 15 target genes and the prognosis of patients with LUAD (only significant results are presented in *Figure 5*). Patients with high expression of *ZNF777*, treacle ribosome biogenesis factor 1 (*TCOF1*), complexin 2 (*CPLX2*), or *ABL1* exhibited worse survival probabilities, whereas patients with high expression of zyg-11 related cell cycle regulator (*ZER1*), a subunit of the GARP complex (*VPS53*), or ribosome binding protein 1 (*RRBP1*) had better prognoses.

Discussion

A large number of patients with LUAD develop drug resistance, and a considerable proportion of patients die from the disease despite rapid therapeutic development (22). Studies have demonstrated that the genesis and development of tumors are regulated by m6A modifications (23). Here,

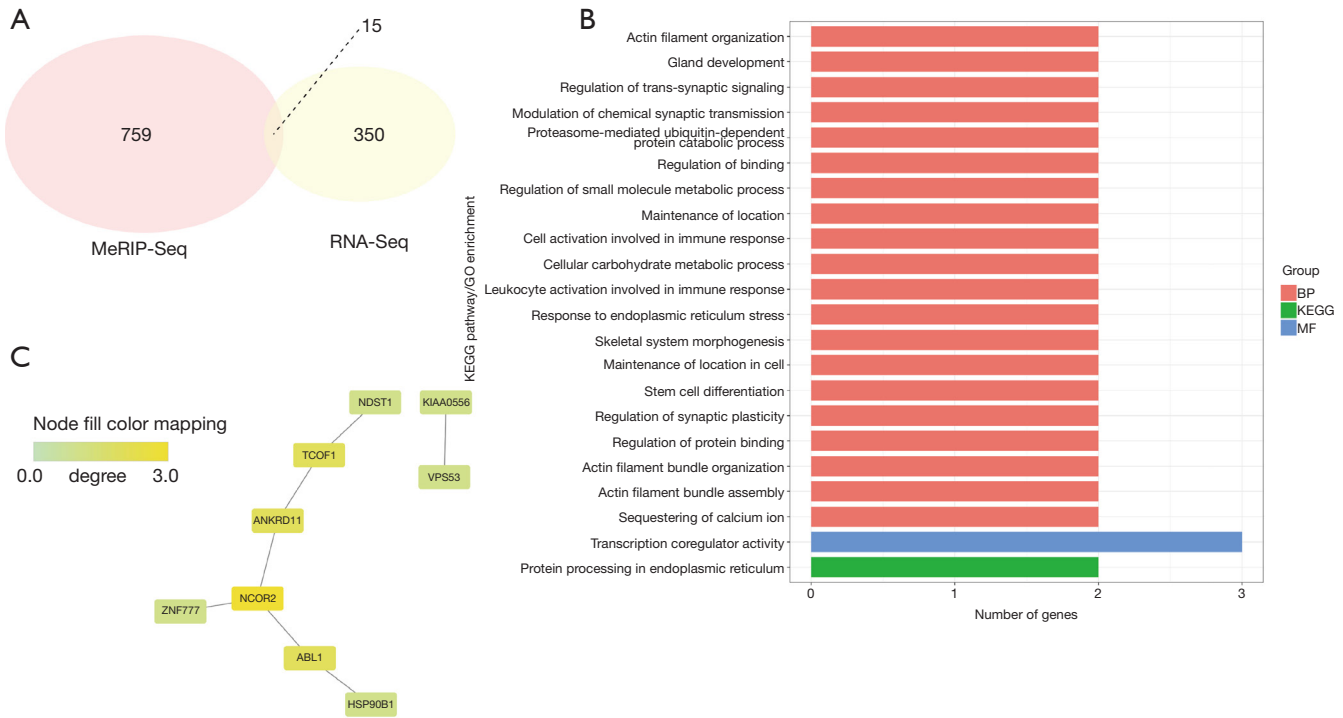

Figure 4 Screening of *ALKBH5* target genes. (A) Intersection of genes associated with *ALKBH5* found in TCGA database and upregulated genes in cells with *ALKBH5* interference compared with levels in normal LUAD cells from a MeRIP-Seq dataset. (B) Enrichment analysis of the target genes of *ALKBH5*. (C) Protein-protein interaction analysis of the *ALKBH5* target genes. MeRIP-Seq, methylated RNA immunoprecipitation sequencing; BP, biological process; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; TCGA, The Cancer Genome Atlas; LUAD, lung adenocarcinoma.

we found that *ALKBH5* is differentially expressed between LUAD and paracancerous tissues and is associated with poor prognosis. Furthermore, we synthesized TCGA-LUAD expression profile data and MeRIP-seq data for A549 cell lines with and without ALKBH5 silencing and identified 15 target genes.

We screened 774 genes significantly associated with *ALKBH5* expression and investigated their functions in GO and KEGG enrichment analyses. NF-κB is a regulator of inflammation and cell survival and contributes to tumor growth, proliferation, and metastasis (24,25). Abnormal activation of the NF-κB signaling pathway is related to the invasion and migration of LUAD, whereas its downregulation induces apoptosis in non-small cell lung cancer cells (26). In this study, *ALKBH5*-related genes were enriched in the NF-κB signaling pathway.

ALKBH5-related genes were also enriched in proteasomal protein catabolic processes. The proteasome plays an important role in protein homeostasis, critical for maintaining cellular stress responses (27), and dysfunctional complexes may contribute to cancer development. Proteasome complex-related genes that promote the cell cycle are highly expressed in lung squamous cell carcinomas (28). These genes have been identified as targets of anticancer drugs (29). Zhang *et al.* (30) reported that proteasome subunits are highly correlated with proteasome 26S subunit (*PSMC6*) expression and that PSMC6 may promote the progression of LUAD by activating the Wingless-type (WNT) signaling pathway.

The 15 target genes identified in this study were primarily involved in the regulation of metabolic processes of small molecules and cell activation involved in the immune response. The PPI network revealed that *NCOR2* may be an important gene that interacts with *ZNF777*, *ABL1*, and *ANKRD11*. NCOR2 modifies chromatin structures and inhibits the basal transcriptional activity of target genes; its abnormal expression is associated with various cancers (31). Downregulation of *NCOR2* is associated with poor prognosis in breast cancer and LUAD, as *NCOR2* can enhance the invasive ability of breast cancer cells and may contribute to cisplatin resistance in LUAD

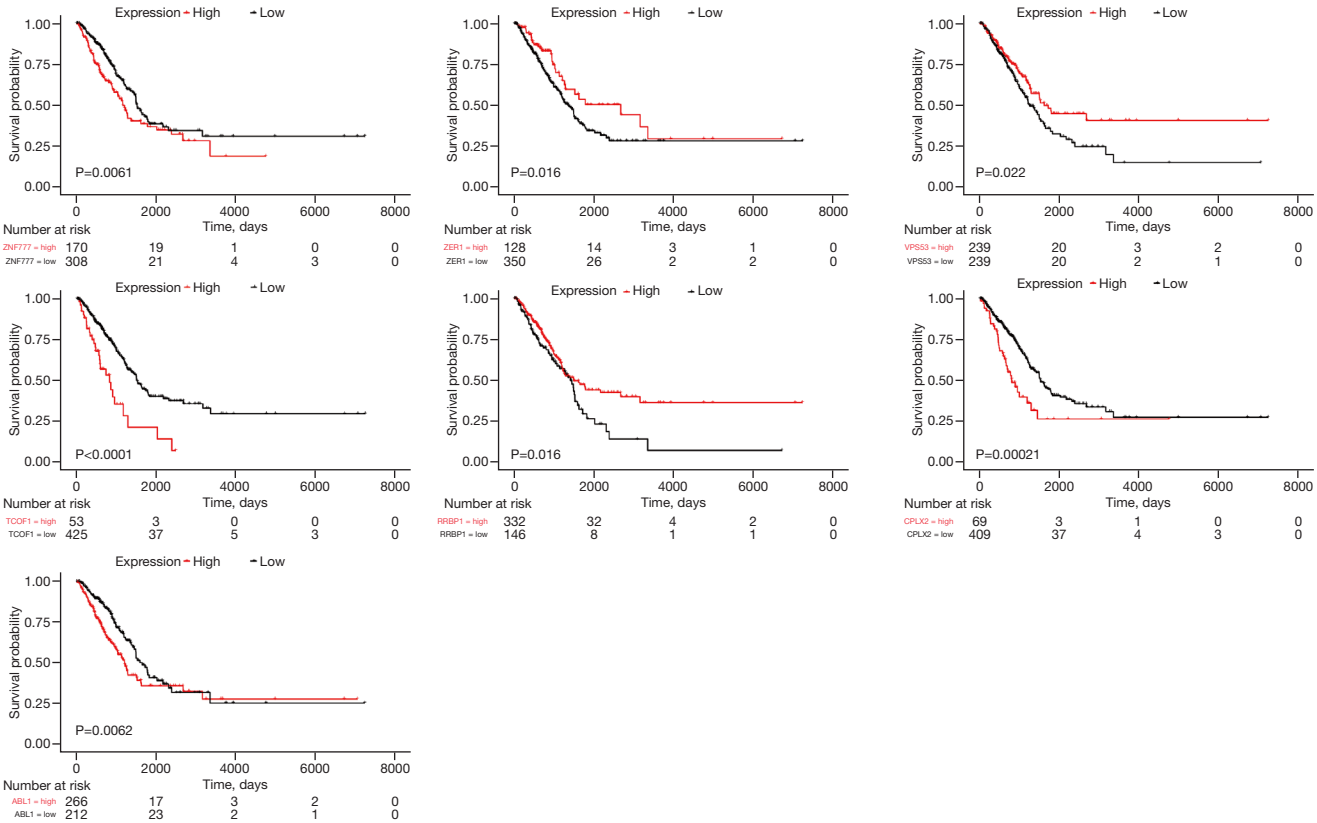

Figure 5 Kaplan-Meier survival curve for *ALKBH5* target genes (only significant results are shown).

cells (32,33). Gong *et al.* (34) reported that chromatin assembly factor 1 subunit B (*CHAF1B*) can induce cisplatin resistance by increasing the ubiquitination and degradation of NCOR2. In a prognostic analysis, patients with LUAD with high expression of *ZNF777*, *TCOF1*, *CPLX2*, or *ABL1* exhibited worse survival probabilities, whereas those with high expression of *ZER1*, *VPS53*, or *RRBP1* had a better prognosis. Several of these genes implicated in prognosis have been associated with cancer. For instance, the downregulation of family with sequence similarity 129 (*FAM129A*) mediated by ZNF777 inhibits colorectal cancer cell proliferation (35). TCOF1 may affect the prognosis of multiple cancer types by participating in the cell cycle and cellular senescence pathways (36). *RRBP1* is highly expressed in lung cancer tissues; knockout of *RRBP1* promotes the endoplasmic reticulum stress response and reduces tumorigenicity (37).

Not all of the target genes were associated with prognosis in LUAD. There are several explanations for this observation. For example, not all targets may contribute to LUAD. Alternatively, these targets may not be involved in LUAD independently but may exert functions via interactions with other genes. Previous studies have demonstrated that not all modified sites are equally important, and only some play a key role in biological processes (10). More research is needed to determine the specific mechanism of action of *ALKBH5* and its target genes. Although the identification of 15 target genes that are strongly associated with LUAD provides a good basis for further analyses, this study had some limitations. First, we only screened genes related to the expression of *ALKBH5*, and the interactions between these genes require further experimental verification. Second, given the heterogeneity of tumors, the amount of retrospective data included in this study was still insufficient. Therefore, it is necessary to expand the sample size in future studies. Finally, more prospective studies are imperative to validate these results.

Conclusions

In conclusion, we screened target genes of ALKBH5 in LUAD, some of which were associated with the prognosis

of patients with LUAD. This study provides targets and a basis for the development of novel treatments for LUAD.

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Footnote

Reporting Checklist: The authors have completed the STREGA reporting checklist. Available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-1464/rc

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-1464/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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