



# Transcription factor-target gene regulatory network analysis in human lung adenocarcinoma

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**Background:** Transcription factors (TFs) play a crucial role in the occurrence and progression of lung adenocarcinoma (LUAD), and targeting TFs is an important direction for treating LUAD. However, targeting a single TF often fails to achieve satisfactory therapeutic outcomes. Furthermore, the regulatory TF-target gene networks involved in the development of LUAD is complex and not yet fully understood.

**Methods:** In this study, we performed RNA sequencing (RNA-seq) to analyze the transcriptome profile of human LUAD tissues and matched adjacent nontumor tissues. We selected the differentially expressed TFs, performed enrichment analysis and survival curve analysis, and predicted the regulatory networks of the top differential TFs with their target genes. Finally, alternative splicing analyses were also performed.

**Results:** We found that TFs GRHL3, SIX1, SIX2, SPDEF, and ETV4 were upregulated, while TAL1, EPAS1, SOX17, NR4A1, and EGR3 were significantly downregulated in LUAD tissues compared to normal tissues. We propose a potential GRHL3-CDH15-Wnt- $\beta$ -catenin pro-oncogenic signaling axis and a potential TAL1-ADAMTS1-vascular antioncogenic signaling axis. In addition, we found that alternative splicing of intron retention (IR), approximate IR (XIR), multi-IR (MIR), approximate MIR (XMIR), and approximate alternative exon ends (XAE) showed abnormally increased frequencies in LUAD tissues.

**Conclusions:** These findings revealed a novel TF-target gene regulatory axis related to tumorigenesis and provided potential therapeutic targets and mechanisms for LUAD.

**Keywords:** Lung adenocarcinoma (LUAD); transcription factor (TF); GRHL3; TAL1

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## Introduction

Lung cancer is a common malignant tumor that originates from malignant cells in the lung tissue and is one of the leading causes of death worldwide (1,2). Lung adenocarcinoma (LUAD) is one of the most common subtypes of lung cancer, occurring in the epithelial cells of the LUAD and accounting for approximately 40% of

all lung cancer cases. The etiology and pathogenesis of lung cancer are complex and closely related to genetic, environmental, and lifestyle factors. The pathogenesis of lung cancer involves multiple molecular pathways and signaling transduction pathways. Studies have shown that activation of proliferative signaling pathways, inactivation of tumor-suppressor genes and DNA repair genes, and

epigenetic modifications can lead to the development of lung cancer (3). In addition, inflammation, immune cells, and cytokines in the tumor microenvironment are also closely associated with the occurrence of lung cancer (3,4). Currently, the treatment modalities for lung cancer include surgical resection, radiotherapy, chemotherapy, genetic testing, immunotherapy, and targeted therapy (5-7). Although these treatment modalities have played important roles in lung cancer treatment, there are still challenges and bottlenecks, including tumor drug resistance, difficulties in early diagnosis, individual differences, and genetic heterogeneity.

Transcription factors (TFs) are a class of proteins widely present in organisms and play a regulatory role in gene expression. In the progress and development of LUAD, TFs participate in processes such as cell proliferation, apoptosis, invasion, and metastasis through their regulatory functions. Previous studies have shown that many TFs exhibit specific expression patterns in LUAD. For example, the expression level of FOXP3 is often low in LUAD tissues and is associated with prognosis (8). Other TFs such as p63 and TTF-1 can be used for the diagnosis and differentiation of different types of LUAD (9). The MYC family of TFs is critically involved in the occurrence and development of LUAD (10). Overexpression of c-Myc can promote the proliferation and survival of LUAD cells

and enhance their invasive and metastatic abilities. N-Myc can regulate the expression of various LUAD-related genes, such as *BCL2*, *MCL1*, and *BMI1*, thereby closely influencing the occurrence and development of LUAD. Inactivation of TP53 can lead to abnormal cell cycle and apoptosis inhibition in LUAD, thus promoting malignant transformation and progression. TP53 also regulates the expression of multiple genes related to LUAD, such as *MDM2*, *p21*, and *BAX*, also contributing to the occurrence and development of LUAD (11). Considering the critical role of TFs in the progress of LUAD, targeting TFs has become an important direction in the treatment of LUAD. Currently, many targeted therapy drugs against c-Myc are under development, which inhibit the growth and metastasis of LUAD by suppressing the expression or activity of c-Myc (10). In addition, due to the significant role of TP53 in LUAD, several treatment strategies targeting TP53 are also under investigation. For example, synthetic peptides targeting the core region of TP53 can bind to MDM2, inhibiting MDM2-mediated degradation of TP53 and restoring its function, thus providing a therapeutic approach for LUAD (12).

High-throughput sequencing technology, through comprehensive analysis of all transcripts in LUAD cells or tissues, has assisted in gaining a deeper understanding of the pathogenesis of LUAD and identifying multiple TFs involved in its occurrence and development (4,13,14). A recent transcriptional regulatory network study identified key TFs involved in LUAD development, including SOX10, SPIB, NR4A2, FOXD1, ELF5, HOXA5, KLF5, ESRRB, SREBF1 and REL (14). However, due to the diversity and heterogeneity of LUAD, our knowledge of the TF regulatory network in LUAD remains limited, particularly in terms of downstream target gene regulation networks and signaling pathways. In this study, we continued to use high-throughput sequencing for transcriptomic analysis of normal adjacent tissues and LUAD tissues in humans. We identified differentially expressed TFs in LUAD and discovered several TFs that were significantly upregulated in LUAD tissues, including GRHL3, SIX1, SIX2, SPDEF, and ETV4, while TAL1, EPAS1, SOX17, NR4A1, and EGR3 were significantly downregulated. Subsequently, we constructed a key differential TF-target gene regulatory network by combining differentially expressed genes. Among them, the TFs GRHL3 and TAL1 exhibited the most significant differential expression in LUAD tissues, with GRHL3 being newly discovered as being upregulated in LUAD tissues. Therefore, we further predicted target genes and conducted functional analysis of these potential

### Highlight box

#### Key findings

- This study constructed a regulatory network of key transcription factors (TFs) and target genes involved in lung adenocarcinoma (LUAD). Here, two potential key TFs involved in the regulation of LUAD (GRHL3 and TAL1) were identified, along with five alternative splicing events (XMIR, XIR, XAE, MIR, and IR) that exhibited abnormal occurrence in LUAD.

#### What is known and what is new?

- The regulatory TF-target gene networks involved in the development of LUAD is complex and not yet fully understood.
- Here, we propose the existence of a GRHL3-CDH15-Wnt- $\beta$ -catenin pro-oncogenic signaling axis and a TAL1-ADAMTS1-vascular antioncogenic signaling axis.

#### What is the implication, and what should change now?

- The result indicated a novel TF-target gene regulatory axis related to tumorigenesis and provided potential therapeutic targets and mechanisms for LUAD. Although further experimental validation is warranted, this study provides potential targets and clues for the diagnosis and treatment of LUAD in the future.

**Table 1** The patients' clinical information

Patient No.	Age (years)	Gender	Side	T	N	M
1	73	Male	LL	T1	N0	M0
2	71	Male	RL	T1	N0	M0
3	79	Male	LL	T1	N0	M0
4	68	Male	RL	T1	N0	M0

LL, left lung; RL, right lung.

target genes. The expression of GRHL3 and TAL1 were positively correlated with the survival rate of patients with LUAD. Additionally, to further analyze the transcriptional regulatory mechanisms during the development of LUAD, we examined selective splicing events, an important posttranscriptional regulatory mechanism, and found that intron retention (IR), approximate IR (XIR), multi-IR (MIR), approximate MIR (XMIR), and approximate alternative exon ends (XAE) showed abnormally increased frequencies in LUAD tissues. In summary, for the first time, we constructed a differential TF-target gene regulatory network in LUAD tissues and proposed a potential GRHL3-CDH15-Wnt- $\beta$ -catenin pro-oncogenic signaling axis and a potential TAL1-ADAMTS1-vascular antioncogenic signaling axis, providing novel mechanistic insights into the targeted TF therapy of LUAD. We present this article in accordance with the STREGA reporting checklist (available at <https://jtd.amegroups.com/article/view/10.21037/jtd-23-1688/rc>).

## Methods

### *Human LUAD and paracancerous tissue collection*

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Institutional Ethics Committee of Binhai County People's Hospital of Yancheng (No. 2021BHKYLL0023) and informed consent was taken from all the patients. The patients' clinical information, such as age, gender, tumor node metastasis (TNM) classification, and stage, was basically consistent (Table 1). Fresh LUAD tissues and matched adjacent nontumor tissues were collected from four patients with LUAD who underwent surgical resection at Binhai County People's Hospital of Yancheng from February 2021 to October 2022. Prior to surgery, none of the patients had received radiotherapy or chemotherapy, had a history of other

treatments, or had inflammatory diseases. Tissue specimens collected from LUAD patients were immediately frozen and stored at  $-196^{\circ}\text{C}$  in liquid nitrogen for further study.

### *RNA sequencing (RNA-seq) and screening of differentially expressed genes*

Total RNA from LUAD tissues or matched adjacent nontumor tissues was extracted with Trizol reagent (MilliporeSigma, Burlington, MA, USA) according to the manufacturer's instructions. Subsequent RNA-seq was conducted by Shanghai OE Biotech Co., Ltd. (Shanghai, China). The differentially expressed genes were screened using fold change, and P values were calculated using the *t*-test. The threshold set for upregulated and downregulated genes was a fold change  $\geq 2$  and a P value  $\leq 0.05$ .

### *Enrichment analysis*

For enrichment analysis, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<https://www.genome.jp/kegg/>) and Gene Ontology (GO) database (<https://geneontology.org/>) were used to perform pathway and biological function analyses on these differentially expressed messenger RNAs (mRNAs).

### *Construction of a differential TF-target gene regulatory network*

The Gene Transcription Regulation Database (GTRD) (<https://gtrd.biouml.org/>) was used to predict the interaction between differential TFs and target genes and to construct a differential TF-target gene regulatory network. Based on the prediction results of differential genes and differential TFs (fold change  $>5$ ), a Sankey diagram of the relationship of the TF family and the TF with the target genes was drawn using OECloud tools (<https://cloud.oebiotech.com>).

**Table 2** Summary of the quality of RNA-seq

Sample	Raw bases (G)	Valid bases (%)	Q30 (%)	Mapped reads (%)
NM1	7.27	93.28	95.08	97.28
NM2	7.40	92.27	95.09	97.59
NM3	7.20	92.27	95.05	98.19
NM4	7.47	92.27	95.11	98.10
LC1	7.67	92.55	95.12	97.82
LC2	7.59	91.81	94.95	96.92
LC3	7.12	91.65	95.00	97.49
LC4	7.35	90.59	95.01	97.63

RNA-seq, RNA sequencing; Q30, percentage of bases that have a Q-score  $\geq 30$ ; NM, matched adjacent nontumor tissues; LC, LUAD tissues; LUAD, lung adenocarcinoma.

### Survival curve analysis

Using the most authoritative website for survival analysis, the Kaplan-Meier plotter database (<https://kmplot.com/analysis>), we analyzed the impact of the differentially expressed TFs (GRHL3, ETV4, SPDEF, SIX1, SIX2, TAL1) and target gene (*CDH15*) on the survival rate of patients with LUAD.

### Prediction of binding sites of TFs and target genes

To further identify the target gene of *GRHL3* and *TAL1*, the hTFtarget database (<http://bioinfo.life.hust.edu.cn/hTFtarget>) was used to predict the target genes of these two TFs. The overlap of target genes between the hTFtarget database and the GTRD were selected for further analysis. The JASPAR database (<https://jaspar.genereg.net/>) was used to predict the binding motifs of *GRHL3* (or *TAL1*) as well as the binding sites between *GRHL3* (or *TAL1*) and *CDH15* (or *ADAMTS1*).

### Alternative splicing analysis

The alternatively splicing analysis of differentially regulated transcripts isoforms or exons was performed using ASprofile software. The ASprofile software categorizes alternative splicing events into 12 different types: alternative 5' first exon (TSS), alternative 3' last exon (TTS), skipped exon (SKIP), approximate SKIP (XSKIP), multiexon SKIP (MSKIP), approximate MSKIP (XMSKIP), IR, XIR, MIR, XMIR, alternative exon ends (AE), and XAE.

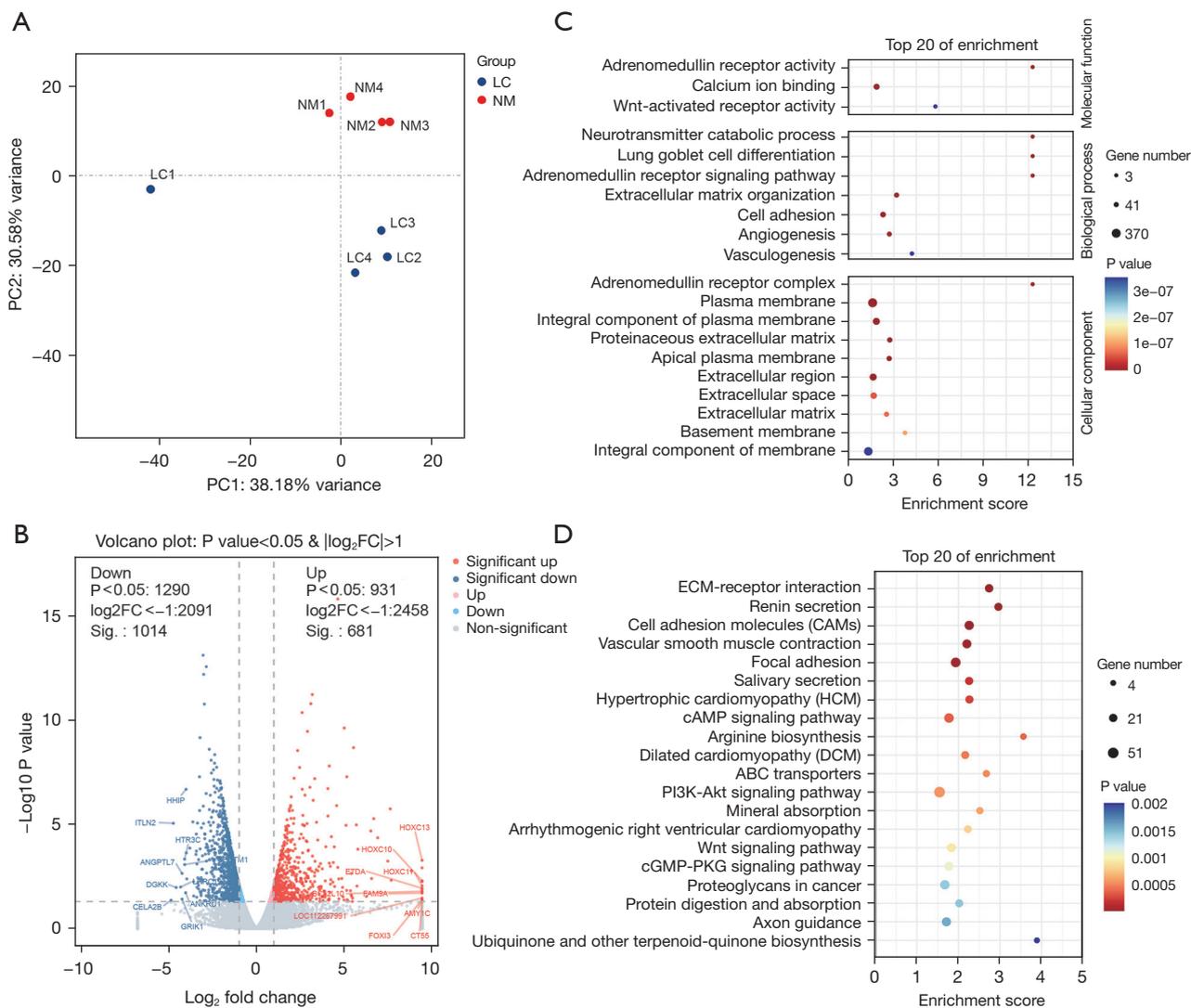
### Statistical analysis

In the RNA-seq data, a P value less than 0.05 and a fold change greater than 2 indicated there to be a differential expression of the same gene between the two samples.

## Results

### Identification of differentially expressed genes in LUAD

To explore the transcriptomic changes during the development of LUAD, we first collected clinical samples of LUAD tissue and adjacent nontumor tissue and then performed RNA-seq to construct libraries for screening differentially expressed genes in LUAD. We obtained results where the original base number for each sample was greater than 7.1 G, the percentage of effective bases was greater than 91%, and the percentage of bases that have a Q-score  $\geq 30$  (Q30) was greater than 94%. At least 97% of reads matched the human genome, indicating a high reliability of the sequencing (*Table 2*). Principal component analysis (PCA) of the sequencing results revealed that the four normal adjacent tissue samples clustered together, indicating a high similarity between normal samples, while one sample from the four LUAD tissues deviated from the other three samples, which may be related to the heterogeneity of lung cancer (*Figure 1A*). Using a threshold of fold change greater than 2-fold as the cutoff, we identified 1,014 downregulated differentially expressed genes and 681 upregulated differentially expressed genes in LUAD tissue (*Figure 1B*). Among the top 10 upregulated genes identified, *HOXC13*, *HOXC10*, and *HOXC11* have been reported to play a role in promoting LUAD, which indicated the accuracy of our



**Figure 1** Identification and functional analysis of DEGs in human LUAD tissues. (A) PCA of results detected from RNA-seq. (B) Volcano plot of DEGs in LUAD. (C) GO analysis of DEGs in LUAD. (D) KEGG analysis of DEGs in LUAD. PC, principal component; LC, LUAD tissues; NM, matched adjacent nontumor tissues; FC, fold change; Sig., significance; DEGs, differentially expressed genes; PCA, principal component analysis; RNA-seq, RNA sequencing; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

sequencing results (8,15,16). We further conducted molecular functional, cellular process, cellular component, and signaling pathway enrichment analyses on these differentially expressed genes. We found that these genes were mainly enriched in molecular functions such as calcium ion binding and Wnt-activated receptor activity; biological processes such as cell adhesion, angiogenesis, and vasculogenesis; cellular components such as plasma membrane and integral component of the plasma membrane (Figure 1C); and signaling pathways such as cell adhesion molecules, vascular

smooth muscle contraction, and Wnt signaling pathway (Figure 1D). In conclusion, the abovementioned data indicated that abnormal regulation of cell adhesion, the Wnt signaling pathway, and vascular regulation occurs in LUAD, which is closely related to the development of LUAD.

### Differentially expressed TFs in LUAD

To further explore the mechanism for the significant transcriptomic dysregulation in LUAD, we focused on

TFs and selected a total of 50 differentially expressed TFs from the differentially expressed genes, including 34 downregulated and 16 upregulated TFs (Figure 2A,2B). TFs GRHL3, SIX1, SIX2, SPDEF, and ETV4 were the most significantly upregulated, while TAL1, EPAS1, SOX17, NR4A1, and EGR3 were the most significantly downregulated in LUAD tissue. Among them, SIX1 (17), SIX2 (18), ETV4 (19), EPAS1 (20), and NR4A1 (21) have been shown to be closely associated with the proliferation, invasion, and metastasis abilities of lung cancer, further confirming the accuracy of our identified TFs and suggesting the potential value of the remaining unverified TFs in LUAD. The enrichment analysis results showed that these TFs were mainly enriched in cellular components such as chromatin and nucleus; biological processes such as regulation of transcription by RNA polymerase II and cell differentiation; relevant molecular functions such as DNA-binding TF activity; and signaling pathways such as transcriptional misregulation in cancer (Figure 2C,2D). These data indicate the important role of TFs in the transcriptomic dysregulation of LUAD.

#### ***Construction of key differential TF-target gene interaction networks***

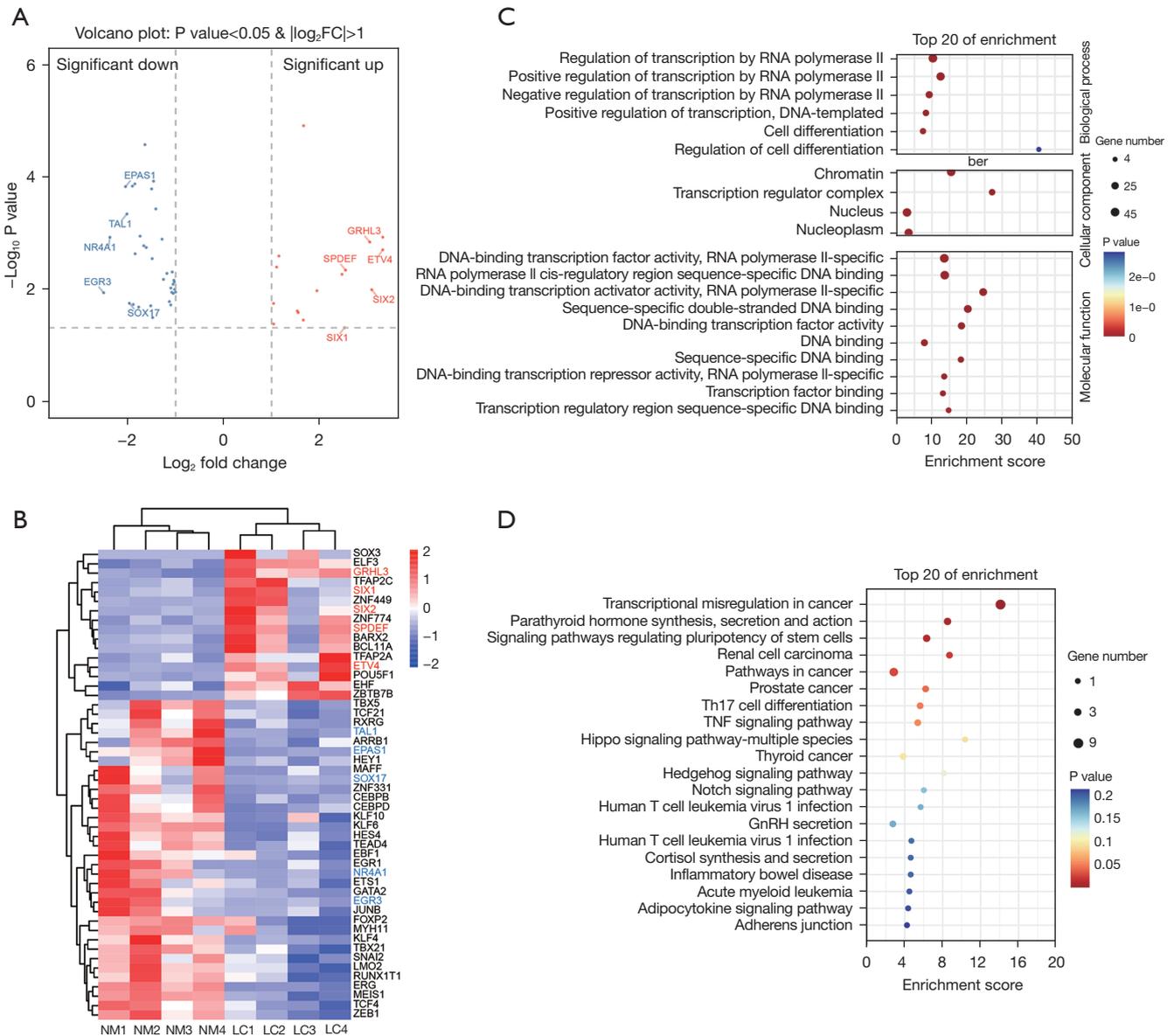
The functional role of TFs primarily relies on their transcriptional regulation of downstream target gene expression. Although previous studies have revealed the expression and function of some TFs in LUAD, no studies to date have systematically analyzed the target genes regulated by these abnormally expressed TFs in LUAD. To further explore the TF regulatory mechanisms in LUAD, we used GTRD to predict the regulatory networks of the top five upregulated and downregulated differential TFs with their target genes in LUAD based on the differentially expressed genes and TFs (with a fold change threshold >5). In this network, among the top five upregulated and downregulated TFs, one TF in each group was not predicted to have consistent expression with its target genes, while the expression trends of the remaining four TFs were consistent with their corresponding target genes (Figure 3A,3B).

#### ***GRHL3 may act as a potential proto-oncogene by transcriptionally regulating CDH15 expression and subsequently regulating Wnt signaling pathway in LUAD***

To further understand the functions of these dysregulated

TFs in the development of LUAD, we conducted survival analysis using the authoritative Kaplan-Meier plotter database to examine the impact of the top five upregulated differential TFs (GRHL3, ETV4, SPDEF, SIX1, SIX2) on the survival rate of patients with LUAD (Figure 4). We found that the TF GRHL3 had the most significant effect on the survival rate of these patients. Patients with high expression of GRHL3 exhibited a significantly shortened lifespan of up to 24 months compared to those with low expression of GRHL3 (Figure 4A,4F), suggesting that GRHL3 may act as an oncogene and is associated with poor prognosis in LUAD. Among the other 4 TFs, only SIX1 showed a correlation with poor prognosis in LUAD although SIX1, SIX2, and ETV4 have been demonstrated to promote proliferation and migration of non-small LUAD cells (17-19). Patients with a high expression of SIX2 had a lifespan shortened by 14 months compared to those with low expression of SIX2, while SIX1, ETV4, and SPDEF did not have significant association on the survival rate of patients with LUAD (Figure 4B-4F). Therefore, we next focused on investigating the functions and regulatory mechanisms of GRHL3 in LUAD.

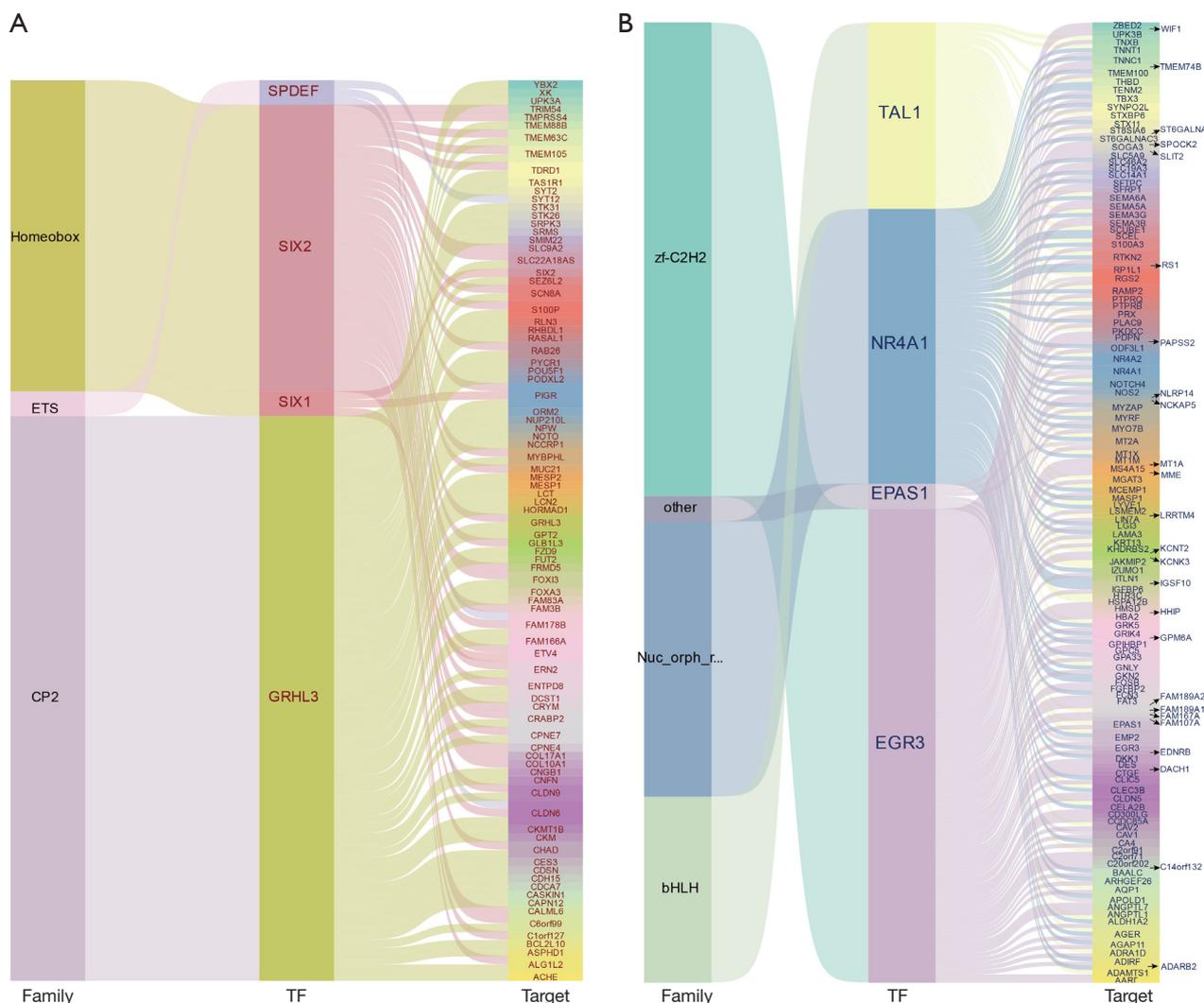
Through enrichment analysis of the predicted target genes of GRHL3 obtained from GTRD, it was found that the target genes of GRHL3 were mainly enriched in the cell adhesion molecules and Wnt signaling pathway (Figure 5A), which are also significantly enriched in the differential genes of LUAD (Figure 1D), suggesting that GRHL3 may play an important role in the transcriptional dysregulation of LUAD. The hTFtarget database contains the most comprehensive information on human TF-target interactions, integrating large-scale human TF-target gene data (22). Therefore, we further predicted the target genes of GRHL3 using the hTFtarget database and performed Venn diagram analysis to identify the intersection between the predicted target genes from GTRD and the predicted target genes from the hTFtarget database, resulting in 22 potential target genes (Figure 5B). Among these 22 potential targets, *CDH15* was enriched in the cell adhesion molecules signaling pathway and exhibited significant upregulation in LUAD (Figure 5C). Survival curve analysis revealed that patients with high expression of *CDH15* had a significantly shortened lifespan of up to 11 months compared to those with low expression of *CDH15* (Figure 5D), further indicating the potential regulatory role of *CDH15* in the development of LUAD. By comparing the conserved binding motifs of GRHL3 (23) with the promoter sequence of *CDH15*, two potential binding sites,



**Figure 2** Identification and functional analysis of differentially expressed TFs in human LUAD tissues. (A) Volcano plot of differentially expressed TFs in LUAD. (B) Clustered heatmap of differentially expressed TFs in LUAD. The top five significantly upregulated TFs are highlighted in red, while the top five significantly downregulated TFs are highlighted in blue. (C) GO analysis of differentially expressed TFs in LUAD. (D) KEGG analysis of differentially expressed TFs in LUAD. FC, fold change; NM, matched adjacent nontumor tissues; LC, LUAD tissues; LUAD, lung adenocarcinoma; TF, transcription factor; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

5'-AAGCCGCT-3' and 5'-GAACAGGTG-3', were identified in the CDH15 promoter (Figure 5E), further suggesting that CDH15 is a potential target gene of GRHL3. CDH15 is an M-cadherin, and cadherins have been found to positively regulate myogenic differentiation induced

by the canonical Wnt signaling pathway by regulating the phosphorylation of GSK-3β and β-catenin (24). The abovementioned results indicate that GRHL3 may regulate the Wnt signaling pathway by modulating the expression of the cell adhesion molecule CDH15, thereby



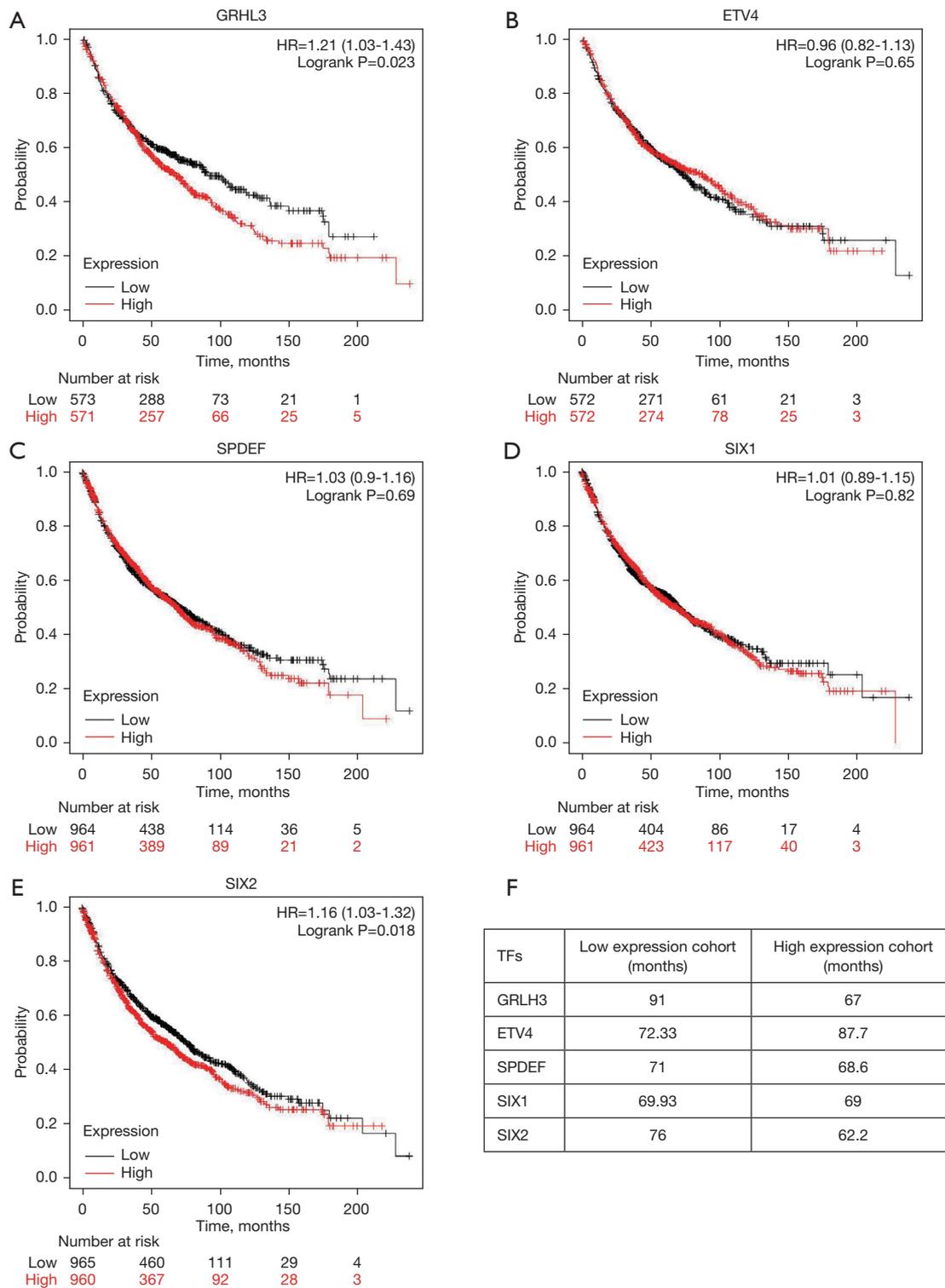
**Figure 3** Differential TF-target gene regulatory network. (A) Sankey diagram of the regulatory network of the top five upregulated TFs and target genes. (B) Sankey diagram of the regulatory network of the top five downregulated TFs and target genes. TF, transcription factor.

promoting the development of LUAD (Figure 5E).

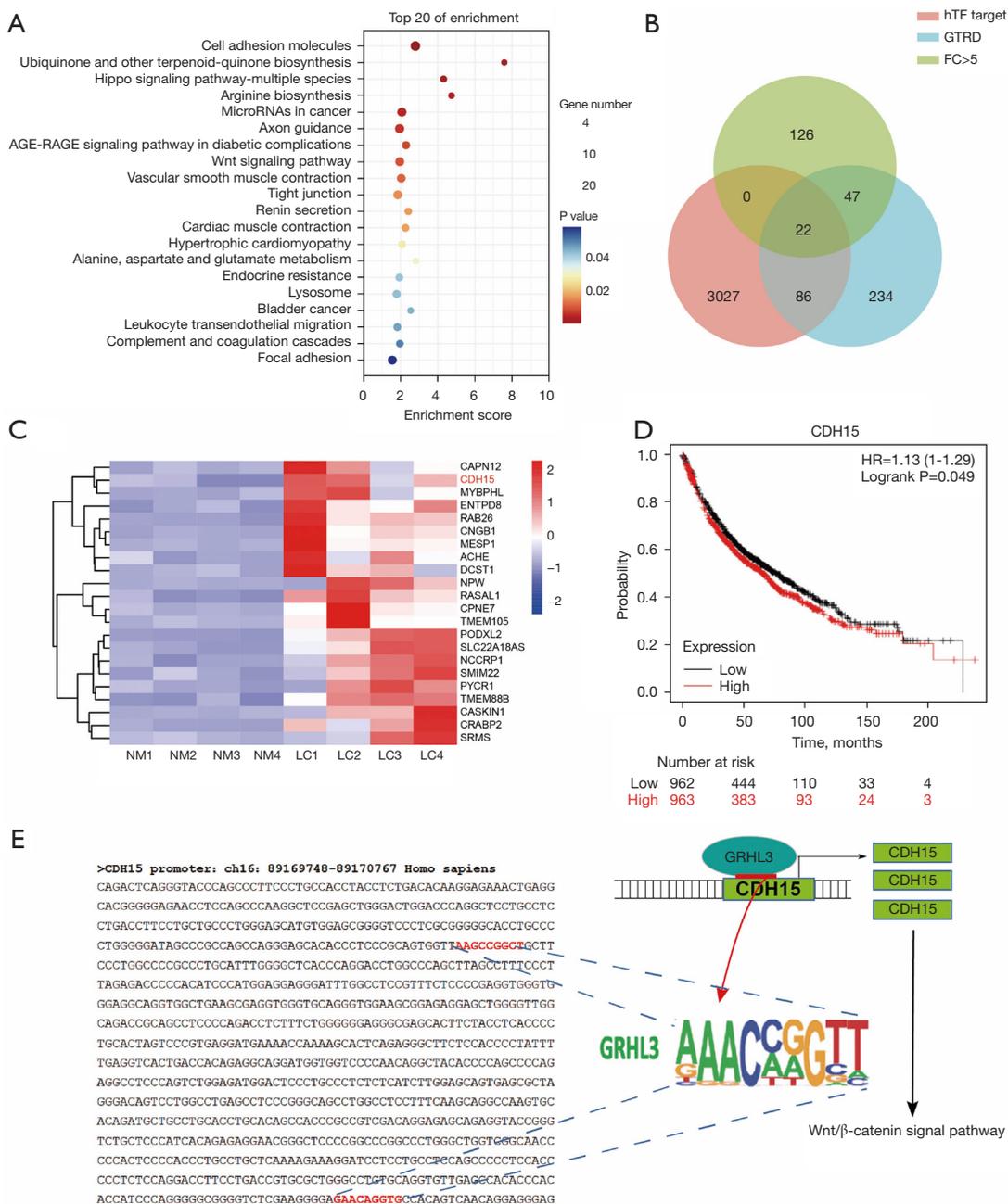
**TAL1 may function as a potential tumor-suppressor gene by regulating angiogenesis through transcriptional regulation of ADAMTS1 expression in LUAD**

Functional enrichment analysis revealed that differentially expressed genes in LUAD were significantly enriched in the Wnt signaling pathway and vascular regulation pathway (Figure 1C,1D), both of which play crucial roles in the development and progression of LUAD. Through the analysis of significantly upregulated TFs in LUAD, we identified GRHL3 as a potential key TF involved

in regulating the Wnt signaling pathway in LUAD (Figure 5). Next, we analyzed the top five downregulated TFs in LUAD (TAL1, EPAS1, SOX17, NR4A1, and EGR3) and found that TAL1 has been reported to be critically involved in angiogenesis and vascular system development (25). TAL1 regulates the expression of VE-cadherin in endothelial cells, contributing to the differentiation and development of endothelial cells and the hematopoietic lineage (26). Therefore, we hypothesized that TAL1 may be a key TF involved in regulating the vascular system in LUAD and subsequently focused on investigating the function and mechanisms of TAL1 in LUAD. Survival curve analysis revealed a positive correlation between TAL1



**Figure 4** Survival curve analysis of the top five upregulated differential TFs in human LUAD tissues. (A-E) Kaplan-Meier plotter depicting the association between the expression of GRHL3 (A), ETV4 (B), SPDEF (C), SIX1 (D), and SIX2 (E) and the survival rate of patients with LUAD. (F) Statistics analysis showing the relationship between the expression of the top five TFs and the survival time of patients with LUAD. HR, hazard ratio; TF, transcription factor; LUAD, lung adenocarcinoma.



**Figure 5** GRHL3 may regulate the progress of LUAD by transcriptionally controlling the expression of *CDH15* and regulating the Wnt signaling pathway. (A) KEGG analysis of target genes of GRHL3 predicted by the GTRD. (B) Venn diagram analysis of the intersection target genes of GRHL3 predicted by GTRD and hTFtarget database and the DEGs with more than a 5-fold change in LUAD. (C) Clustered heatmap of the 22 intersecting differentially expressed target genes of GRHL3. (D) The Kaplan-Meier plotter mapped the relationship between *CDH15* expression and survival of patients with LUAD. (E) Schematic diagram illustrating the hypothesis of GRHL3 binding to the *CDH15* promoter, transcriptionally regulating *CDH15* expression, and modulating the Wnt signaling pathway. The red color in the left part marks the sequence of sites where the *CDH15* promoter may bind to GRHL3. The red arrow on the right is the conserved binding motif of GRHL3. GTRD, Gene Transcription Regulation Database; FC, fold change; NM, matched adjacent nontumor tissues; LC, LUAD tissues; LUAD, lung adenocarcinoma; HR, hazard ratio; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes.

expression and the survival rate of patients with LUAD. Patients with high TAL1 expression had a significantly extended lifespan of up to 17 months compared to those with low TAL1 expression (Figure 6A), suggesting that TAL1 may function as a tumor suppressor. Through enrichment analysis, we found that the predicted target genes of TAL1 in the GTRD were significantly enriched in the vascular smooth muscle contraction pathway (Figure 6B), which was also significantly enriched in the differentially expressed genes of LUAD (Figure 1D), further highlighting the crucial role of TAL1 in the regulation of the vascular system in LUAD. Furthermore, we used the hTFtarget database to accurately predict the target genes of TAL1 and identified 23 potential target genes through Venn diagram analysis of the predicted target genes from the GTRD and the hTFtarget database (Figure 6C). Among these 23 potential targets, *ADAMTS1* was significantly upregulated in LUAD tissues (Figure 6D) and has been reported to be closely associated with angiogenesis and vascular function (27), exhibiting inhibitory effects on some tumors (28). Additionally, through JASPAR database analysis, we identified three potential binding sites of TAL1 in the *ADAMTS1* promoter sequence: 5'-TGAACATATCAT-3', 5'-CAACTTTCTGGT-3', and 5'-CAGCCCTCTGCT-3' (Figure 6E). This further suggested that *ADAMTS1* as a potential target gene of TAL1. Based on the aforementioned analysis results, we hypothesize that TAL1 may regulate the vascular system of LUAD cells through the transcriptional regulation of *ADAMTS1*, thereby inhibiting the development and progression of LUAD (Figure 6E).

#### ***Global identification of dysregulation alternative splicing in LUAD***

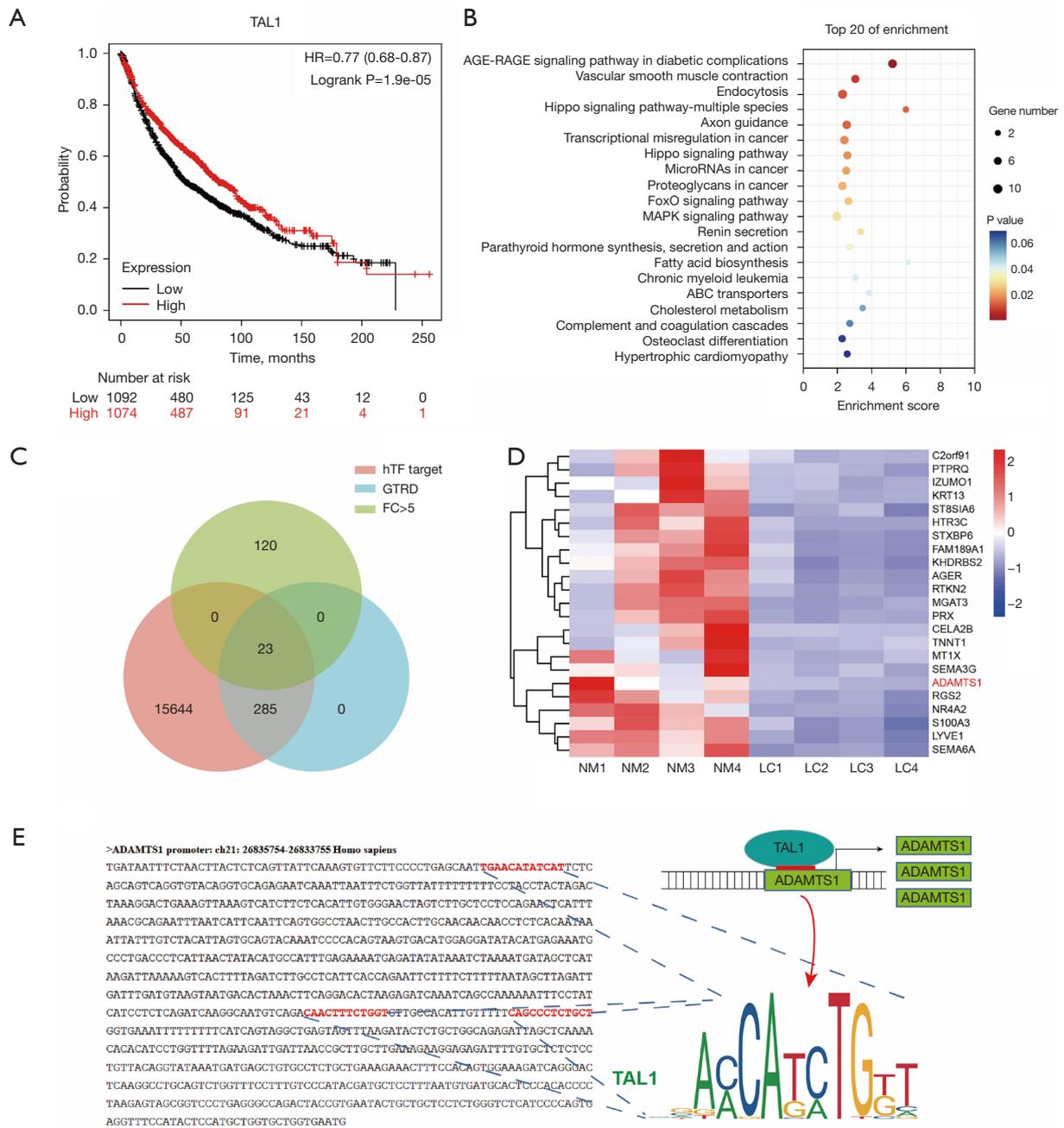
Alternative splicing is an important posttranscriptional gene regulation mechanism that is closely associated with tumorigenesis. Studies have shown that various abnormal splicing events occur during the development of lung cancer, leading to altered expression and functional changes in tumor-related genes (29-31). For example, selective splicing alterations in genes such as *BCL2L1*, *MDM2*, *MDM4*, *NUMB*, and *MET* can affect the apoptosis, proliferation, and cell adhesion of lung cancer cells (30). Furthermore, a recent study has identified 3688 abnormal alternative splicing events in LUAD. Seven types of alternative splicing events were identified, with SKIP being most significant type in LUAD (30). In this study, we used

the ASprofile software to categorize variable splicing types into 12 classes: TSS, TTS, SKIP, XSKIP, MSKIP, XMSKIP, IR, XIR, MIR, XMIR, AE, and XAE (Figure 7A). Then, we compared of alternative splicing between NM and LC, and found that the XMIR, XIR, XAE, MIR, and IR events were significantly upregulated in LUAD tissues compared to normal tissues (Figure 7B). These findings deepen our understanding of certain splicing events in LUAD and provide new targets and strategies for further study of the diagnosis and treatment of this disease.

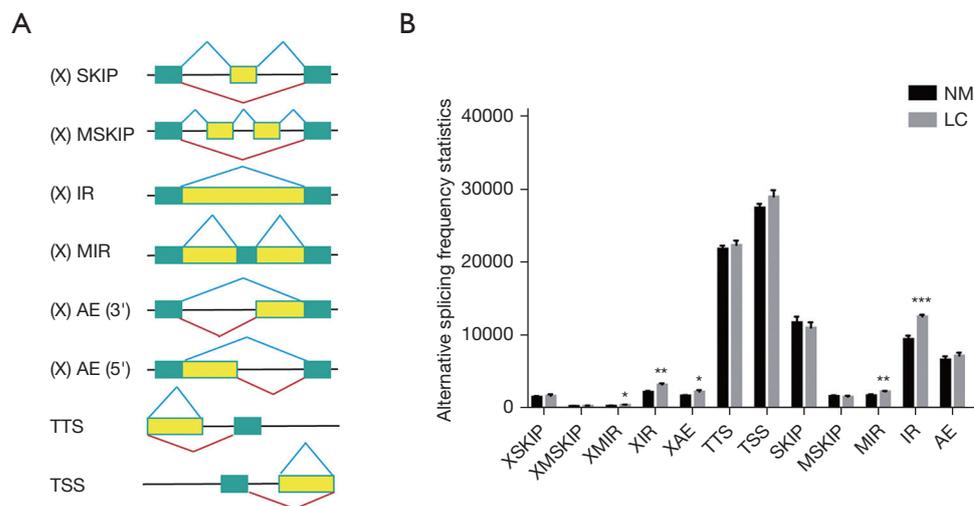
#### **Discussion**

As a malignant tumor, LUAD's occurrence and development are regulated by various complex molecular mechanisms. TFs play a crucial role in LUAD by regulating multiple key genes and signaling pathways, influencing the occurrence, development, and transformation of LUAD. However, the regulatory mechanisms of TFs in LUAD are highly complex. Despite previous studies identifying several important TFs in LUAD, many key TFs remain unexplored, and no research has been conducted on constructing TF-target gene regulatory networks based on the differentially expressed genes in LUAD. Therefore, in this study, we further identified key TFs in LUAD through high-throughput sequencing and, for the first time, constructed a key TF-target gene regulatory network based on the differentially expressed genes in LUAD. Interestingly, during the exploration of target genes regulated by key TFs, we discovered a potential oncogenic TF (GRHL3) that may regulate the Wnt signaling pathway, as well as a potential tumor-suppressive TF (TAL1) that may regulate signaling pathways associated with angiogenesis.

In this study, we first detected a large number of different expressed genes in human LUAD tissues through high-throughput sequencing, and these genes were significantly enriched in the Wnt and angiogenesis-related signaling pathways. In LUAD, the Wnt signaling pathway is frequently aberrantly activated, leading to the enhanced proliferation and invasive capabilities of LUAD cells, and is involved in the epithelial-mesenchymal transition (EMT) process and the maintenance and proliferation of LUAD stem cells (32,33). Angiogenesis also plays a critical role in various aspects of LUAD (34-36). First, angiogenesis provides the needed oxygen and nutrients to tumors, promoting the proliferation and growth of tumor cells. Second, the newly formed blood vessels provide the channels required for invasion and metastasis of LUAD cells. Moreover, angiogenesis



**Figure 6** TAL1 may regulate the progress of LUAD by transcriptionally controlling the expression of *ADAMTS1* and regulating angiogenesis. (A) The Kaplan-Meier plot depicting the association between the expression of *TAL1* and the survival rate of patients with LUAD. (B) KEGG analysis of target genes of *TAL1* predicted by the GTRD. (C) Venn diagram analysis of the intersection target genes of *TAL1* predicted by GTRD and the hTFtarget database and the differentially expressed genes with more than a 5-fold change in LUAD. (D) Clustered heatmap of the 23 intersecting differentially expressed target genes of *TAL1*. (E) Hypothesis diagram of *TAL1* binding the *ADAMTS1* promoter, transcriptional regulation of *ADAMTS1* expression, and regulation of angiogenesis. The red color on the left indicates the potential binding site sequence of the *ADAMTS1* promoter with GRHL3. The red arrow on the right represents the conserved binding motif of *TAL1*. HR, hazard ratio; GTRD, Gene Transcription Regulation Database; FC, fold change; NM, matched adjacent nontumor tissues; LC, LUAD tissues; LUAD, lung adenocarcinoma; KEGG, Kyoto Encyclopedia of Genes and Genomes.



**Figure 7** Global identification of alternative splicing in human LUAD. (A) Schematic diagram of the twelve alternative splicing forms. (B) Statistics analysis of the frequency of alternative splicing in LC and NM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . SKIP, skipped exon; XSKIP, approximate SKIP; MSKIP, multiexon SKIP; XMSKIP, approximate MSKIP; IR, intron retention; XIR, approximate IR; MIR, multi-IR; XMIR, approximate MIR; AE, alternative exon ends; XAE, approximate alternative exon ends; TTS, alternative 3' last exon; TSS, alternative 5' first exon; NM, matched adjacent nontumor tissues; LC, LUAD tissues; LUAD, lung adenocarcinoma.

contributes to therapeutic resistance in LUAD, as the newly formed blood vessels can reduce the effectiveness of drugs by providing alternative routes and lowering drug concentration (35). Therefore, the Wnt signaling pathway and angiogenesis are considered two important therapeutic targets in LUAD.

*GRHL3* is a gene that encodes a protein belonging to the grainyhead family. It plays a crucial role in regulating biological processes such as epithelial cell differentiation, proliferation, and migration (37). Aberrant expression of *GRHL3* in tumors is closely associated with the occurrence, development, and prognosis of various cancers. For example, *GRHL3* can suppress squamous cell carcinoma by regulating the activation of PTEN/PI3K/Akt pathway (38). It exerts anticancer effects in esophageal squamous cell carcinoma by regulating HOPX expression and the Wnt signaling pathway (23). In addition, it promotes tumor growth and metastasis in colorectal cancer through the MEK pathway (39). Thus, related studies have largely shown the tumor-suppressive role of *GRHL3*, with a few cases demonstrating its oncogenic potential. This dual role of *GRHL3* in tumors may be attributed to its transcriptional regulation of different target genes. For instance, *GRHL3* can enhance the expression of the tumor suppressors *PTEN* or *HOPX*, thereby inhibiting the progression of esophageal squamous cell carcinoma (23,38). However, to date, no

studies have revealed the specific regulatory target genes responsible for the oncogenic function of *GRHL3*, and there is limited research on the function of *GRHL3* in lung cancer. In this study, we observed upregulated expression of *GRHL3* in LUAD and found a negative correlation between *GRHL3* expression and the survival rate of patients with lung cancer. Through target gene prediction and analysis using multiple databases, we identified multiple binding sites of *GRHL3* in the promoter region of *CDH15*, suggesting *CDH15* as a potential target gene of *GRHL3*. *CDH15*, also known as M-cadherin, belongs to the cadherin superfamily of calcium-dependent cell adhesion proteins and figures prominently in regulating the arrangement and fusion of myocytes (24). Existing research indicates that *CDH15* may be associated with the occurrence, progression, and prognosis of certain tumors. For example, high methylation of *CDH15* is correlated with poor survival in patients with liver cancer (40). Overall, *CDH15* is considered to be a factor that promotes tumor cell invasion and metastasis. A study has suggested that the M-cadherin-mediated signaling pathway attenuates the phosphorylation of  $\beta$ -catenin (Ser31/37/Thr41) by GSK-3 $\beta$  (24). Therefore, we hypothesize that *GRHL3* exerts its oncogenic function by transcriptionally regulating *CDH15* expression and modulating the Wnt/ $\beta$ -catenin signaling pathway.

*TAL1* is a gene that regulates hematopoietic development

by controlling the transcription of multiple genes, including *GATA1*, *GATA2*, and *LMO2* (41). Abnormalities in the *TAL1* gene are common in T-cell acute lymphocytic leukemia (T-ALL), a malignant tumor occurring in children and adults (25). In patients with T-ALL, the *TAL1* gene often undergoes chromosomal translocations, rearrangements, and mutations, leading to overexpression of the TAL1 protein (25). This disruption may interfere with normal hematopoietic differentiation processes and promote abnormal proliferation and survival of leukemia cells. However, there is limited research on the role of *TAL1* in other malignancies, particularly in LUAD. In this study, we observed the significant downregulation of TAL1 expression in LUAD and found a positive correlation between TAL1 expression and the survival rate of patients with LUAD. Further analysis revealed that the potential target genes of TAL1 in LUAD were significantly enriched in the pathways related to vascular smooth muscle contraction and vascular regulation, which is consistent with the known functions of TAL1. Additionally, we identified *ADAMTS1* as a candidate target gene of TAL1 in LUAD. *ADAMTS1* showed a consistent expression pattern with TAL1 in LUAD tissues, and its promoter region contains multiple binding sites for TAL1. *ADAMTS1* is involved not only in vascular development and angiogenesis but also in vascular growth and repair processes through its regulation of endothelial cell proliferation and migration (27). Furthermore, *ADAMTS1* has been found to regulate vascular constriction and dilation by affecting the function of endothelial and smooth muscle cells, thus impacting vascular contractility and hemodynamics (27). These findings suggest that *ADAMTS1* may be a target gene of TAL1 in LUAD. Therefore, we hypothesize that TAL1 may exert its anticancer effects by transcriptionally regulating the expression of *ADAMTS1*, thereby modulating vascular formation or contraction in LUAD.

Alternative splicing is an important gene expression regulatory mechanism that allows a single gene to produce multiple distinct transcripts and protein isoforms. Alternative splicing plays a crucial regulatory role in regulating the Wnt signaling pathway.  $\beta$ -catenin is a core regulatory factor in the Wnt signaling pathway, involved in gene transcription and cell fate determination. Alternative splicing can affect the expression levels and functions of  $\beta$ -catenin. For example, certain tumors exhibit alternatively spliced variants of  $\beta$ -catenin that may display enhanced transcriptional activity and tumor-promoting effects (42). Additionally, alternative splicing is critically involved in angiogenesis, affecting

the expression and regulation of multiple genes involved in vascular formation and function (43). For instance, the precursor mRNA of key regulators in angiogenesis, such as members of the VEGF family including VEGF-A, VEGF-B, VEGF-C, and VEGF-D, undergoes alternative splicing to generate multiple transcript variants encoding proteins with distinct functions (44). These alternative splicing variants can regulate the stability, activity, and receptor-binding capacity of VEGF family members, thereby influencing endothelial cell proliferation, migration, and vascular formation (44). To further identify the transcriptional regulatory mechanisms in LUAD, we performed transcriptome sequencing and analyzed changes in alternative splicing events in LUAD. We found that XMIR, XIR, XAE, MIR, and IR events were significantly upregulated in LUAD tissues compared to normal tissues, suggesting that these five alternative splicing events exert meaningful regulatory roles in the Wnt and vascular-related pathways during the development and progression of LUAD.

In addition, abnormal regulation of cell proliferation, immune evasion, and EMT are also associated with the occurrence and development of LUAD as a malignant tumour (5,45). The major pathways enriched in TFs include not only transcriptional dysregulation in cancer, but also immune response-related pathways such as the TNF signaling pathway. The tumor immune microenvironment is the main source of heterogeneity in lung cancer, affecting disease progression and response to treatment. Heterogeneity and immune editing within genomic tumors are common early phenomena that may occur before invasion. Multi-region whole exome sequencing analysis revealed that mutations in EGFR, ERBB2, NRAS, and BRAF are early clonal genomic events in LUAD carcinogenesis, while TP53 and genes related to cell migration, gap junctions, and metastasis may be late events associated with sub-clonal diversity and tumor progression (46). A latest study utilized a highly reusable imaging quality control cytometer (IMC) to analyze the spatial resolution features related to lung tumour immune microenvironment and clinical outcomes in LUAD patients (47). The results showed that AI based systems can extract features from raw IMC images to predict various LUAD clinical outcomes. We will continue to investigate TFs and their regulatory networks associated with the immune microenvironment in LUAD patients.

## Conclusions

In summary, we used transcriptomic sequencing to

construct a regulatory network of key TFs and target genes involved in LUAD. Two potential key TFs involved in the regulation of LUAD (GRHL3 and TAL1) were identified, along with five alternative splicing events (XMIR, XIR, XAE, MIR, and IR) that exhibited abnormal occurrence in LUAD. We propose the existence of a GRHL3-CDH15-Wnt- $\beta$ -catenin pro-oncogenic signaling axis and a TAL1-ADAMTS1-vascular antioncogenic signaling axis. Although further experimental validation is required to verify these complex transcriptional networks and pro- and antioncogenic signaling axes, this study provides potential targets and clues for the diagnosis and treatment of LUAD in the future.

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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-23-1688/rc>

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*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). The study was approved by the Institutional Ethics Committee of Binhai County People's Hospital of Yancheng (No. 2021BHKYLL0023) and informed consent was taken from all the patients.

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