Original Article

Investigation of the function of the novel tumor marker *BEND5* in lung adenocarcinoma based on data mining and *in vitro* analysis

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Background: BEN domain-containing protein 5 (BEND5) belongs to the BEN family of structural domains, whose members can be found in several animal proteins. The characteristic ability of *BEND5* to inhibit cell proliferation allows it to play a crucial role as a tumor suppressor gene in colorectal cancer. However, the function of *BEND5* in lung adenocarcinoma (LUAD) has not been fully explored.

Methods: The Cancer Genome Atlas (TCGA) database was used to extensively examine *BEND5* dysregulation and its prognostic significance in pan-cancer data. Databases including TCGA, gene expression profiling interactive analysis (GEPIA), and STRING were used to perform analysis of the expression pattern and clinical significance of *BEND5* in patients with LUAD, and the possible regulatory mechanisms responsible for the occurrence and progression of LUAD. To explore the relationship between *BEND5* expression and tumor immunity in LUAD. Finally, transfection experiments using an in vitro model were performed to confirm *BEND5* expression in LUAD cells while investigating its regulatory significance in tumor cell proliferation.

Results: Significantly decreased *BEND5* expression was observed in LUAD and in most other cancers. Further analysis of the Kyoto Encyclopedia of Genes and Genomes database revealed that the genes significantly linked to *BEND5* were mainly enriched in the peroxisome proliferator-activated receptor (PPAR) signaling pathway. Also, *BEND5* was found to be involved in tumor immunity in LUAD via its functional regulation of various tumor cell types, such as B cells and T cells. *In vitro* experimental results showed that *BEND5* overexpression mediated LUAD cell inhibition and decreased the expression of cell cycle-related proteins. Further, *BEND5* activated the PPAR signaling pathway, and knockdown of *PPAR* reversed the effect of *BEND5* overexpression on LUAD cells.

Conclusions: BEND5 expression is low in LUAD and may be associated with poor prognosis, and *BEND5* overexpression inhibits LUAD cells via the PPAR signaling pathway. The dysregulation of *BEND5* in LUAD, its prognostic significance, and its ability to function *in vitro* suggest that *BEND5* could be a deciding factor in the progression of LUAD.

Keywords: BEN domain-containing protein 5 (*BEND5*); lung adenocarcinoma (LUAD); proliferation; PPAR signaling pathway; prognosis

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Introduction

Lung cancer accounts for approximately 2.2 million new diagnoses and 1.79 million deaths every year, making it the most prevalent malignancy worldwide (1). The incidence and mortality rates of lung cancer are constantly rising (2,3). The most prevalent subtype of lung cancer is lung adenocarcinoma (LUAD), which accounts for about 40% of cases (4). Currently, surgical resection is the mainstay treatment for LUAD. However, at the time of presentation, most cases have already progressed to an advanced stage of the disease and are no longer suitable for surgical resection. Furthermore, even if LUAD is detected at an early stage, recurrence and metastasis are extremely common issues (5-7). Despite the significant achievements that have been made in the treatment of LUAD, the 5-year overall survival (OS) rate is still only 15%, which reflects the poor prognosis of the disease (8,9). Therefore, the identification of specific molecular targets holds immense potential for the diagnosis, treatment, and prognosis of LUAD.

BEN domain-containing protein (BEND) encodes a domain protein that plays a pivotal role in the maintenance of DNA chromatin structure. The BEND family has nine members: BEND2, BEND3, BEND4, BEND5, BEND6, BEND7, BEND3P1, BEND3P2, and BEND3P3. BEND is critically important for mediating protein-DNA and protein-protein interactions (PPIs) during chromatin organization and transcription (10,11). *BEND5* is located on chromosome 1p33 (12) and encodes a protein comprising 421 amino acid residues. The involvement of BEND proteins in the regulation of gene expression has been observed during early germ cell development, with *BEND5* overexpression inducing the differentiation of embryonic stem cells into primordial germ progenitor-like cells *in vitro* (13). Deregulation of *BEND5* has been reported in malignant tumors, including colorectal cancer (14) and breast cancer (15). In colorectal cancer, *BEND5* is highly downregulated and exhibits high methylation levels. Apoptosis, autophagy, and differentiation are among the key mechanisms via which *BEND5* causes a reduction in cell proportions in colorectal cancer, and its reduced expression is correlated with patients' clinical status and outcome (14). In breast cancer, Shi *et al.* found that high expression of *BEND5* inhibited tumor cell proliferation and metastasis by interacting with the N-terminal domain (NTD) of RBPJ and further inhibiting Notch signaling (15). The same study also demonstrated that low expression of the *BEND5* gene could predict an advanced clinical stage and poor OS outcome in patients with breast cancer (15). However, to the best of our knowledge, no report has ever thoroughly investigated the role of *BEND5* in LUAD in a comprehensive and integrated manner.

Bioinformatics research has grown with the development of next-generation sequencing (16,17). Public databases such as The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) are ideal for accessing transcriptomic information, which promotes effective ways of identifying gene signatures (18-20). Many studies have attempted to build risk models for biological features or prognostic assessment of malignant tumors that might have a clinical influence (21-23). In the current study, we used the TCGA-LUAD database to assess the expression and prognostic value of *BEND5* in LUAD and performed gene set enrichment analysis (GSEA), Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis, Gene Ontology (GO) enrichment analysis, and immune infiltration analysis. It is hoped our findings will inform a better understanding of the role played by *BEND5* in LUAD.

Highlight box

Key findings

- *BEND5* expression is low in LUAD and may be associated with poor prognosis, and *BEND5* overexpression inhibits LUAD cells via the PPAR signaling pathway.

What is known and what is new?

- *BEND5* belongs to the BEN family of structural domains, whose members can be found in several animal proteins. *BEND5* is dysregulated in colorectal and breast cancer and is strongly associated with tumor progression and prognosis;
- According to bioinformatics, *BEND5* expression was low in LUAD and was related to a poor prognosis. *In vitro* experiments showed that *BEND5* overexpression suppressed LUAD cells and decreased the expression of cell cycle-related proteins. Furthermore, *BEND5* triggered the PPAR signaling pathway, and knocking down PPAR reversed the effects of *BEND5* overexpression on LUAD cells.

What are the implications of the study, and what should change now?

- *BEND5* inhibits the proliferation of lung adenocarcinoma cells via activating PPAR signaling pathways, and it could be used as a prognostic biomarker for patients with the disease.

Methods

BEND5 expression in pan-cancer data

To determine the expression of *BEND5* in pan-cancer, RNA-seq data were downloaded from the Genotype-

Tissue Expression (GTEx) cohort and TCGA database (https://portal.gdc.cancer.gov/) in transcript per million (TPM) format using the University of California Santa Cruz (UCSC) Xena Browser (https://xenabrowser.net) (24,25). The Toil method was then applied for uniform data processing. An evaluation and representation of *BEND5* mRNA expression levels in various cancers were performed with the 'ggplot2' package (version 3.3.3) in R (version 3.6.3). After being normalized to the TPM format, the data from transcript mapping were log2 converted. In total, 10,534 and 15,776 samples were used for paired and unpaired sample analysis, respectively. The expression levels of *BEND5* mRNA were compared between healthy control and tumor groups using the Mann-Whitney U test. Paired sample *t*-tests were conducted to analyze paired sample data that passed the Shapiro-Wilk normality test (P>0.05). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

BEND5 expression in LUAD samples from the TCGA database

For analysis of *BEND5* expression in LUAD samples, level 3 high-throughput sequence (HT-seq) data from patients with LUAD were obtained from the TCGA (https://portal.gdc.cancer.gov/) and UCSC Xena Browser (https://xenabrowser.net) (24,25) databases in fragments per kilobase of TPM mapped reads (FPKM)' format. Samples without clinical data were eliminated. A further analysis was conducted on the TCGA-LUAD dataset, which comprises 594 samples including 535 LUAD tumor and 59 healthy control samples. Log2 transformations were applied following the conversion of FPKM-formatted RNA-sequencing (RNAseq) data to TPM format. The 'ggplot2' package (version 3.3.3) in R (version 3.6.3) was used to evaluate and display *BEND5* mRNA expression levels in LUAD. Both paired and unpaired samples were analyzed by applying the paired *t*-test to paired data that passed the Shapiro-Wilk normality test (P>0.05). The Mann-Whitney U test (Wilcoxon rank sum test) was used to examine unpaired data that failed the normality test (P<0.05).

Analysis of the diagnostic utility of BEND5 mRNA expression in LUAD

The 'pROC' (version 1.17.0.1) and 'ggplot2' (version 3.3.3) packages in R (version 3.6.3) were applied to examine *BEND5* gene expression data using the receiver-operating characteristic (ROC) curve. Clinical status (LUAD tumor versus normal) was selected as an outcome parameter. The X-axis represented the false positive rate (FPR), and the Y-axis represented the true positive rate (TPR). In general, diagnostic tests with an area under the ROC curve (AUC) >0.9 are considered highly accurate; those with an AUC 0.7–0.9 are considered moderately accurate; those with an AUC 0.5–0.7 are considered to have low accuracy; and those with an AUC <0.5 are considered random.

Tumor clinical characteristics of TCGA-LUAD samples

The above-described TCGA-LUAD data served as the foundation for the subsequent analysis. Clinicopathological information, *BEND5* mRNA expression data, and general patient characteristics were extracted from the dataset. Based on *BEND5* expression level, the LUAD samples were classified into a low *BEND5* gene expression group and a high *BEND5* gene expression group. The following categorical variables of cases were examined: T stage, N stage, M stage, pathologic stage, primary therapy outcome, age, sex, race, smoker (yes or no), anatomic neoplasm subdivision (left or right sites and peripheral or central lung), OS events, disease-specific survival (DSS) events, and progression-free interval (PFI) events. The clinical characteristics of cases in the low and high *BEND5* gene expression groups from the TGCA-LUAD dataset are shown in *Table 1*.

When the theoretical frequency of a categorical variable was greater than 5 and the sample size exceeded 40, the chi-squared test was employed, while Fisher's exact test was conducted to determine whether any differences were present between the groups with high and low LUAD expression. When a specific categorical variable did not have a normal distribution (P<0.05), the Wilcoxon rank-sum test was applied. The 'ggplot2' package (version 3.3.3) in R (version 3.6.3) was used for statistical analysis.

Correlation analysis of BEND5 expression level and tumor clinical features

Multiple categories of clinical features were examined to analyze their relationships with *BEND5* mRNA expression level. If data were normally distributed, the 'ggplot2' package (version 3.3.3) in R (version 3.6.3) was applied to perform a one-way analysis of variance (ANOVA; P>0.05, Shapiro-Wilk normality test); otherwise, the Kruskal-Wallis test was performed. Data from 329 LUAD tumor samples

Table 1 Clinical characteristics of patients in the low and high *BEND5* gene expression groups from The Cancer Genome Atlas lung adenocarcinoma cohort

Characteristic	Low *BEND5* expression	High *BEND5* expression	P
N	267	268	
T stage, n (%)			<0.001
T1 (175 cases)	67 (12.6)	108 (20.3)	
T2 (289 cases)	154 (28.9)	135 (25.4)	
T3 (49 cases)	33 (6.2)	16 (3.0)	
T4 (19 cases)	12 (2.3)	7 (1.3)	
N stage, n (%)			0.205
N0 (348 cases)	168 (32.4)	180 (34.7)	
N1 (95 cases)	50 (9.6)	45 (8.7)	
N2 (74 cases)	45 (8.7)	29 (5.6)	
N3 (2 cases)	1 (0.2)	1 (0.2)	
M stage, n (%)			0.884
M0 (361 cases)	189 (49.0)	172 (44.6)	
M1 (25 cases)	14 (3.6)	11 (2.8)	
Pathologic stage, n (%)			0.013
Stage I (294 cases)	129 (24.5)	165 (31.3)	
Stage II (123 cases)	68 (12.9)	55 (10.4)	
Stage III (84 cases)	52 (9.9)	32 (6.1)	
Stage IV (26 cases)	14 (2.7)	12 (2.3)	
Primary therapy outcome, n (%)			0.031
PD (71 cases)	45 (10.1)	26 (5.8)	
SD (37 cases)	20 (4.5)	17 (3.8)	
PR (6 cases)	3 (0.7)	3 (0.7)	
CR (332 cases)	149 (33.4)	183 (41.0)	
Sex, n (%)			<0.001
Female (286 cases)	123 (23)	163 (30.5)	
Male (249 cases)	144 (26.9)	105 (19.6)	
Race, n (%)			0.057
Asian (7 cases)	6 (1.3)	1 (0.2)	
Black or African American (55 cases)	22 (4.7)	33 (7.1)	
White (406 cases)	199 (42.5)	207 (44.2)	
Age, n (%)			0.660
≤65 years (255 cases)	130 (25.2)	125 (24.2)	
>65 years (261 cases)	127 (24.6)	134 (26.0)	

Table 1 (*continued*)

Table 1 (*continued*)

Characteristic	Low *BEND5* expression	High *BEND5* expression	P
Residual tumor, n (%)			0.777
R0 (355 cases)	173 (46.5)	182 (48.9)	
R1 (13 cases)	8 (2.2)	5 (1.3)	
R2 (4 cases)	2 (0.5)	2 (0.5)	
Anatomic neoplasm subdivision, n (%)			1.000
Left (205 cases)	103 (19.8)	102 (19.6)	
Right (315 cases)	157 (30.2)	158 (30.4)	
Anatomic neoplasm subdivision 2, n (%)			0.765
Central lung (62 cases)	32 (16.9)	30 (15.9)	
Peripheral lung (127 cases)	70 (37.0)	57 (30.2)	
No. of pack years smoked, n (%)			0.074
<40 years (188 cases)	116 (31.4)	72 (19.5)	
≥40 years (181 cases)	94 (25.5)	87 (23.6)	
Smoker, n (%)			<0.001
No (75 cases)	18 (3.5)	57 (10.9)	
Yes (446 cases)	244 (46.8)	202 (38.8)	
OS event, n (%)			0.022
Alive (343 cases)	158 (29.5)	185 (34.6)	
Dead (192 cases)	109 (20.4)	83 (15.5)	
DSS event, n (%)			0.007
Alive (379 cases)	172 (34.5)	207 (41.5)	
Dead (120 cases)	72 (14.4)	48 (9.6)	
PFI event, n (%)			0.240
Alive (309 cases)	147 (27.5)	162 (30.3)	
Dead (226 cases)	120 (22.4)	106 (19.8)	
Age, median (years) [IQR]	65 [57, 72]	66 [60, 72]	0.299

BEND5, BEN domain-containing protein 5; PR, partial response; CR, complete response; PD, progressive disease; SD, stable disease; OS, overall survival; DSS, disease-specific survival; PFI, progression-free interval; IQR, interquartile range.

were examined. A binary logistic model was also applied to study the relationships between *BEND5* expression and tumor clinical features.

Survival analysis

Gene expression profiling interactive analysis (GEPIA; http://gepia.cancer-pku.cn/index.html) was used to plot survival maps of *BEND5* expression using pan-cancer data. The Kaplan-Meier (KM) plotter (https://kmplot.com/analysis/) was used to evaluate the relationships of *BEND5* gene expression with OS and relapse-free survival in patients with LUAD.

Cox regression with univariate and multivariate survival analysis

To examine the relationship between tumor features and

patient prognosis in LUAD, univariate and multivariate Cox regression models were used. To determine OS outcomes, the Cox regression module and the coxph function of the survival package (version 3.2-10) in R (version 3.6.3) were employed. In addition to the categorical variables mentioned above, *BEND5* gene expression was included as a tumor characteristic feature.

Forest plots

Based on the results of univariate and multivariate Cox regression analyses [hazard ratio (HR), 95% confidence interval (CI), and P value], two forest plots were created using the 'ggplot2' package (version 3.3.3) in R (version 3.6.3). By comparing one level of a binary characteristic with a reference level, the HR reflects the relative mortality risk. In general, an HR value >1 denotes a higher chance of death, whereas an HR value <1 indicates a lower risk.

Nomogram plotting

A LUAD prognosis-related nomogram incorporating clinical characteristics and gene expression was created using the TCGA-LUAD dataset, as well as a predictive nomogram that combined *BEND5* expression levels with clinical variables was also built. In the R program (version 3.6.3), the rms (version 6.2-0) and survival (version 3.2-10) packages were used to create the nomogram, which was then analyzed statistically by Cox regression. The nomograms were created to predict the OS of patients with LUAD.

Calibration plot

The survival (version 3.2-10) and rms (version 6.2-0) packages in R were used to create calibration plots. To assess the performance of the nomogram model in terms of accuracy for predicting prognostic outcomes of LUAD, the fit between the actual fraction survival probability and the nomogram-predicted survival probability was calculated at three time points (1, 3, and 5 years). The nomogram would be considered to show perfect prediction accuracy if the solid lines for the 1-, 3- and 5-year time points fitted well at a 45-degree ideal diagonal line.

Identification of genes associated with BEND5 in LUAD

BEND5 gene correlation analysis was carried out using the 'stat' package (version 3.6.3) in R (version 3.6.3). Solely using protein-coding genes, the Pearson correlation test was applied to determine if the high and low expression groups were linearly related. The Pearson correlation coefficient (Pearson's r, also known as the cor_Pearson value) was evaluated, with a cor_Pearso value of 0.90–1.00, 0.70–0.89, 0.40–0.69, 0.10–0.39, and 0.00–0.10 indicating a very strong, strong, moderate, weak, and negligible correlation, respectively. *BEND5*-correlated genes with P value <0.05 were regarded as significantly correlated genes.

Heat map of the top 20 BEND5-correlated genes

Following their identification, genes that were significantly correlated with *BEND5* in LUAD, were listed in descending order of cor_Pearson value, and the first ten were considered to be the top 10 positively correlated genes. The significantly correlated genes were then listed in ascending order of cor_Pearson value, and the first ten were considered to be the top 10 negatively correlated genes. The expression of these 20 associated genes in LUAD samples was visualized as a heat map using the 'ggplot2' package (version 3.3.3) in R (version 3.6.3).

GSEA

The genes possessing an absolute correlation coefficient >0.1 and a P value <0.05 were selected for GSEA analysis. Significant genes between LUAD and healthy control samples in the TCGA dataset were identified using 'DESeq2' (version 1.26.0) in R (version 3.6.3). To perform GSEA, the $\log_2$[fold change (FC)] values were acquired for genes that were significantly correlated with *BEND5*. The GSEA was performed with the clusterProfiler package (version 3.14.3) in R (version 3.6.3). Further, to find the pathways, the Reactome (REAC) Pathway database, the KEGG pathway database and WikiPathways (WP) database were used. The referenced gene set is located in the MSigDB Collections (https://www.gsea-msigdb.org/gsea/msigdb/collections.jsp#C2) as c2.cp.v7.2.symbols.gmt (Curated). Functional terms were deemed to be significantly enriched on meeting the conditions of P.adjust <0.05, false discovery rate (FDR) or q value <0.25, and |normalized enrichment score (NES)| >1.

Functional enrichment analysis of the top 10 genes significantly associated with BEND5

Significantly correlated genes with the threshold values (|r|

>0.4 and P<0.05) were selected for functional enrichment analysis. To identify highly enriched functional terms for *BEND5*-correlated genes, a functional enrichment analysis was conducted on the top 10 positively and negatively correlated genes. The Benjamini and Hochberg correction was used to calculate adjusted P values for *Homo sapiens*. GO terms—including biological processes (BP), cellular components (CC), and molecular functions (MF)—and KEGG pathways were considered to be significantly enriched for linked genes on meeting the thresholds of P.adjust <0.05 and q value <0.2. Bubble charts to show the outcomes of the enrichment analyses were created using the 'ggplot2' package (version 3.3.3) in R (version 3.6.3).

Cell lines and culture

Five LUAD cell lines (H1299, H460, PC-9, A549, and H1975) and a non-neoplastic lung epithelial cell line (HBE) were obtained from the American Type Culture Collection (ATCC). The H1299 cell line (ATCC number: CRL-5803) is an epithelial-like cell line established from a lymph node metastasis from the lung of a patient who had received prior radiation therapy (URL: https://www.atcc.org/products/crl-5803). The H460 cell line (ATCC number: HTB-177) was isolated from the pleural fluid of a male patient with large cell lung cancer in 1982 (URL: https://www.atcc.org/products/htb-177). The PC-9 (formerly known as PC-14) cell line was derived from a human adenocarcinoma from lung tissue which remains undifferentiated. The A549 cell line (ATCC number: CRM-CCL-185) was initiated through explant culture of lung carcinomatous tissue from a 58-year-old Caucasian male. The H1975 cell line (ATCC number: CRL-5908) is a cell line exhibiting epithelial morphology that was isolated from the lungs of a nonsmoking female with non-small cell lung cancer. The cell lines were grown in RPMI-1640 media (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (Gibco, USA) and incubated at 37 ℃ with 5% CO_2.

Plasmid transfection experiments

Plasmids were purchased from WZ Biosciences (Shandong, China). Following instructions supplied by the manufacturer, the *BEND5* plasmid was transfected utilizing Lipofectamine 3000 (Thermo Fisher Scientific, Inc.). Overexpression of *BEND5* was stimulated in LUAD cells to investigate its effects.

Western blot analysis

Western blot analysis was carried out following a previously published protocol (https://www.abcam.cn/protocols/general-western-blot-protocol#8). Quantification of protein concentration was performed by employing the BCA protein assay method (Beyotime, China) after the protein had been extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Fude Biotechnology, China) supplemented with phosphatase and protease inhibitors (CWBIO, Shanghai, China; Thermo Fisher Scientific). Total protein (30 μg) was separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinyl difluoride membranes (Invitrogen, USA), and then blocked with 5% nonfat dry milk in Tris-buffered saline, pH 7.5. Primary antibodies were incubated with the membranes for an extended period. The primary antibodies used were as follows: PPAR (Abcam, UK), BEND5, GAPDH, CDK2 (Proteintech, USA), CDK4, CCND1, and CCNB1 (Cell Signaling Technology, USA). The membranes were immunoblotted at 4 ℃ overnight. A horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG antibody was used as the secondary antibody (Proteintech, USA). Enhanced chemiluminescence reagents (Fude Biotechnology, China) were used to detect the signals.

Cell Counting Kit-8 (CCK-8) and colonogenic assays

The CCK-8 assay was performed as described in a previously reported protocol (https://www.abcam.com/cell-counting-kit-8-wst-8--cck8-ab228554.html). For this assay, cells (10^3 cells per well) were seeded in 96-well plates. The CCK-8 reagent (Beyotime, China) was added continuously for 5 days at 24-hour intervals. Cell viability was determined by measuring the optical density at a wavelength of 450 nm. For the colonogenic experiment, cells were seeded in 6-well plates with 500 cells/well and cultured for 14 days using 37 ℃ with 5% of CO_2. Afterward, the colonies were cleaned three times with phosphate-buffered saline and stained with crystal violet for 15 minutes.

Analysis of correlations between immune cells and BEND5 expression in LUAD

Using Spearman's statistical technique, the relationship between *BEND5* expression and immune cells in LUAD

tumor tissues was examined. This analysis was carried out using the GSVA package (version 1.34.0) in R (version 3.6.3). The statistical analysis made use of the single-sample GSEA (ssGSEA) method, which is a built-in technique in the GSVA package. The 24 tumor-infiltrating immune cells (TIICs) under study included B cells, CD8 T cells, cytotoxic cells, dendritic cells (DCs), activated DCs, immature DCs, plasmacytoid DCs, macrophages, mast cells, natural killer (NK) cells, NK CD56bright cells, NK CD56dim cells, neutrophils, T cells, T helper cells, T central memory cells, T effector memory cells, T follicular helper cells, gamma delta T cells, Th1 cells, Th17 cells, Th2 cells, and T regulatory cells. The correlation between *BEND5* expression and the 24 TIICs in LUAD samples was visualized using a lollipop plot. Scatter plots were utilized to display the statistically significant relationships between *BEND5* expression and specific types of immune cell in LUAD samples.

Analysis of correlations between immune checkpoint genes (ICGs) and BEND5 expression

The 59 ICGs include 23 checkpoint inhibitor genes and 36 checkpoint stimulator genes (26). Using Spearman's statistical approach, the association between the expression of *BEND5* expression and each individual ICG in LUAD was assessed. A correlation coefficient of r>0 and a P value <0.05 indicated a significant positive correlation, while r<0 and P value <0.05 indicated a significant negative correlation. The expression patterns of the 59 ICGs in LUAD tumor samples were visualized as a heat map with the 'ggplot2' package (version 3.3.3) in R (version 3.6.3). Scatter plots were also created to display the correlations between *BEND5* and ICGs that were confirmed to be statistically significant, again using 'ggplot2'.

Statistical analysis

GraphPad Prism (version 8.0) was used for data processing. Unless otherwise stated, the data values are presented as the mean with standard deviation (SD). Student's *t*-test (two-tailed) was employed for comparing independent survey contrasts between two groups when both SDs were the same, and Student's *t*-test with Welch's correlation was used when the SDs of two groups differed. An ANOVA (one way) was applied when variances between multigroup sample statistics were equal, and Welch's ANOVA was adopted if the variances were not equal. Statistically significant differences were indicated by P<0.05, P<0.01, or P<0.001.

Results

Flowchart of the current study

Figure 1 is a flowchart that depicts the current research study design.

BEND5 dysregulation in pan-cancer data and LUAD

Analysis of pan-cancer data from the TCGA database revealed significant downregulation of *BEND5* in multiple cancer types (*Figure 2A*,*2B*). Unpaired sample analyses showed that *BEND5* was dysregulated in multiple cancers, including breast invasive carcinoma (BRCA), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), kidney chromophobe (KICH), LUAD and pancreatic adenocarcinoma (PAAD), among others (*Figure 2A*). Paired sample analyses showed that *BEND5* was dysregulated in several cancers including BRCA, KICH, kidney renal papillary cell carcinoma (KIRP), LUAD, rectum adenocarcinoma (READ) and stomach adenocarcinoma (STAD), among others (*Figure 2B*). Further, in comparison with healthy control samples, LUAD tumor samples showed a significant decrease in *BEND5* expression (*Figure 2C*,*2D*). The ROC curve analysis also showed that *BEND5* expression was useful for differentiating LUAD from healthy control samples (AUC =0.688 >0.5; *Figure 2E*).

Prognostic significance of BEND5 in pan-cancer and LUAD

Data from TCGA were used to determine the prognostic value of *BEND5* expression in pan-cancer (*Figure 3A*,*3B*). Analysis of the association between *BEND5* expression and OS using pan-cancer data showed that elevated *BEND5* mRNA expression levels were linked to a poor prognosis in adrenocortical carcinoma (ACC), KICH, READ and STAD but with a good prognosis in BLCA, BRCA, CESC, lymphoid neoplasm diffuse large B-cell lymphoma (DLBC), KIRP, LUAD, PAAD, PRAD, and uveal melanoma (UVM). In terms of relapse-free survival, *BEND5* overexpression was found to be linked to a short recurrence time in ACC, KICH, READ, and STAD but to a long recurrence time in BLCA, mesothelioma, PAAD, and UVM. The prognostic

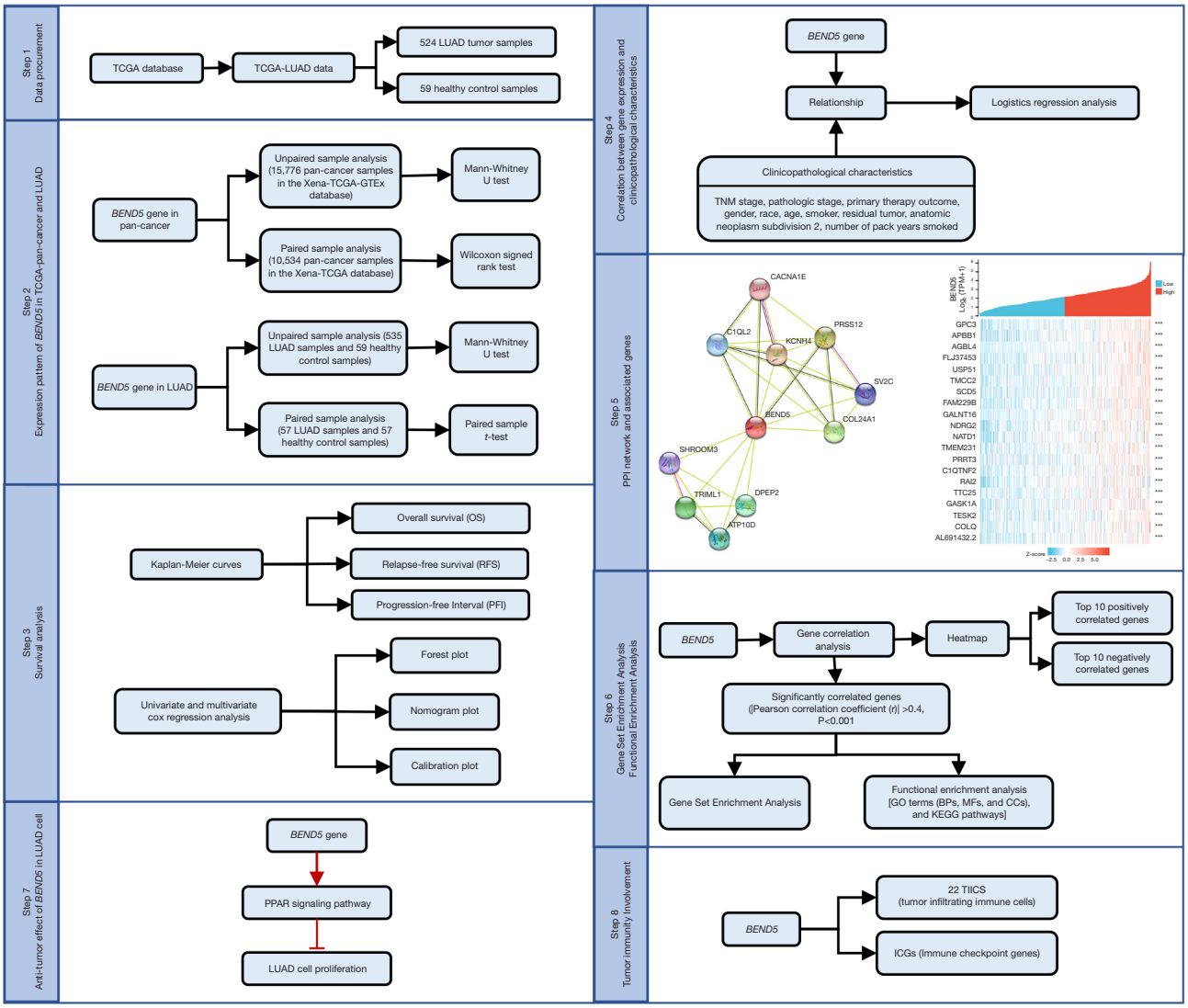

Figure 1 The flowchart of the research design. ***, P<0.001. TCGA, The Cancer Genome Atlas; LUAD, lung adenocarcinoma; *BEND5*, BEN domain-containing protein 5; GTEx, Genotype-Tissue Expression; TPM, transcript per million; PPI, protein-protein interaction; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; MF, molecular function; CC, cellular component.

value of *BEND5* for LUAD was confirmed via the KM method, and the outcome was consistent with earlier findings that *BEND5* expression was significantly related to OS and DSS (*Figure 3C*,*3D*). Cox regression analysis (univariate and multivariate) was applied to determine whether *BEND5* could serve as a biomarker for predicting the prognosis of patients with LUAD independently, and the results revealed that *BEND5* expression could strongly predict OS and relapse-free survival in LUAD (*Figure 3E*,*3F*, *Table 2*).

Correlation between BEND5 expression and clinicopathological characteristics of LUAD

To investigate the putative function of *BEND5* in the pathogenesis of LUAD, its expression levels and their association with tumor clinicopathological characteristics were examined. A comparison of tumor characteristics between patients with low and high *BEND5* gene expression in LUAD is shown in *Table 1*, and a comparison of clinical features between these two groups is shown in *Table 3*.

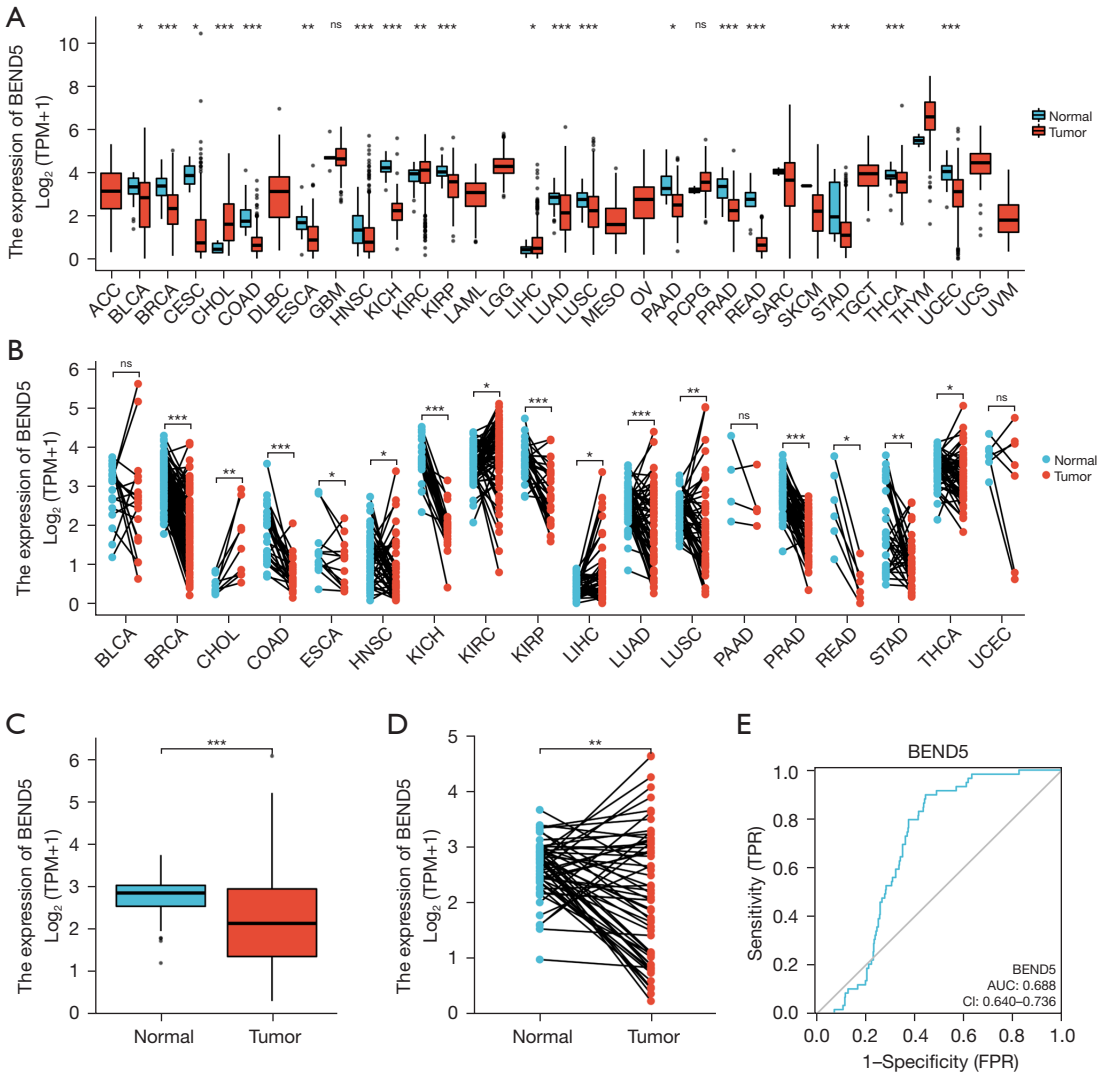

Figure 2 Dysregulation of *BEND5* in pan-cancer and lung adenocarcinoma and the diagnostic value of *BEND5*. (A) Results of unpaired sample analysis of the expression level of BEND5 mRNA in various types of tumors based on data from TCGA. (B) Expression patterns of *BEND5* in pan-cancer data were analyzed using paired sample analysis. (C) Results of unpaired sample analysis of the expression level of *BEND5* mRNA in LUAD and in normal lung tissues from TCGA. (D) Results of paired sample analysis of the expression level of *BEND5* mRNA in LUAD and in normal lung tissues from TCGA. (E) Receiver-operating characteristic curve for the evaluation of the diagnostic value of *BEND5* in discriminating LUAD samples from healthy controls. *, P<0.05; **, P<0.01; ***, P<0.001. *BEND5*, BEN domain-containing protein 5; ns, not significant; TPM, transcript per million; ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, Colon adenocarcinoma; DLBC, diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, Acute myeloid leukemia; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma; AUC, area under the curve; CI, confidence interval; FPR, false positive rate; TPR, true positive rate; TCGA, The Cancer Genome Atlas.

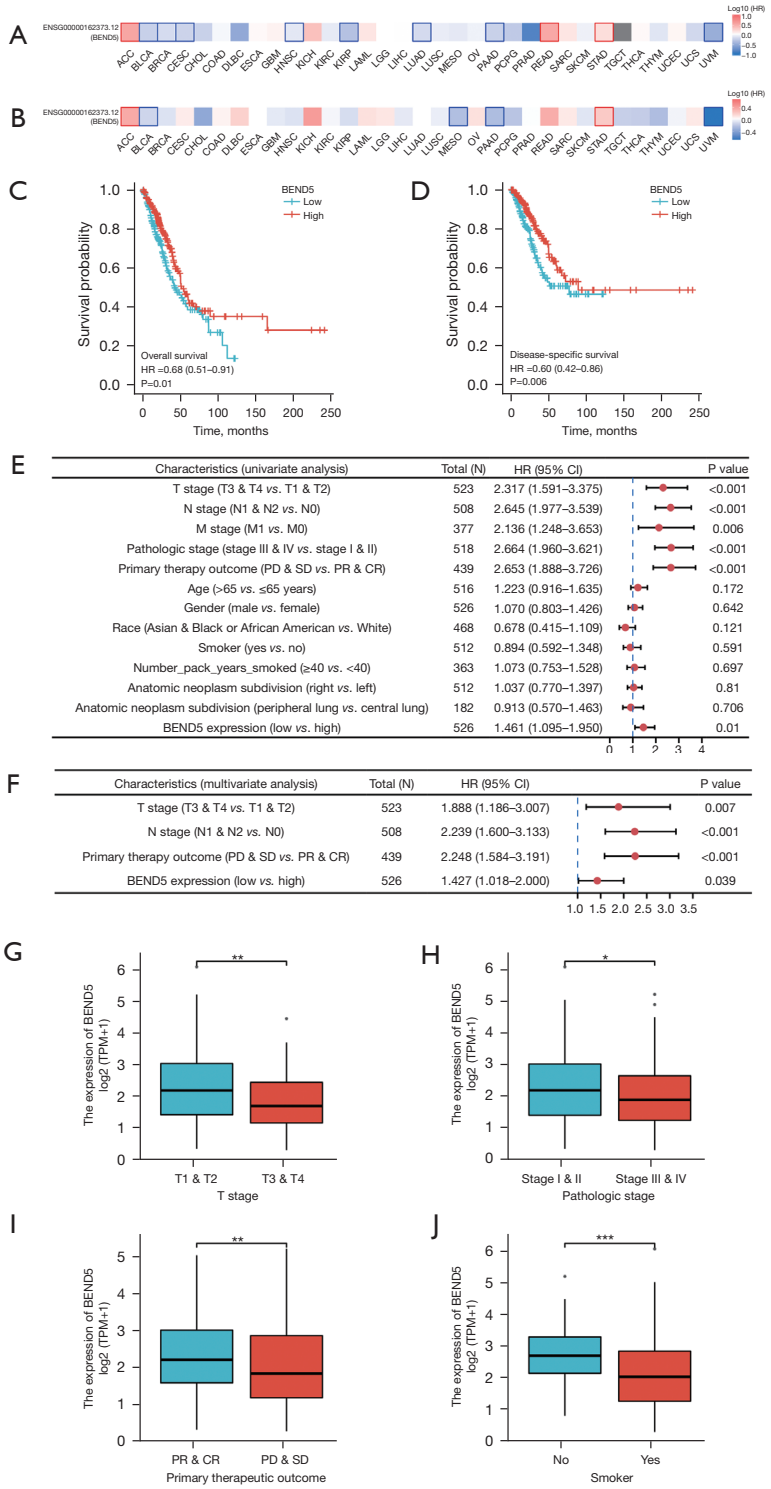

Figure 3 The prognostic value of *BEND5* in pan-cancer and lung adenocarcinoma data from The Cancer Genome Atlas. (A) According to the GEPIA2 database, the *BEND5* gene has a prognostic impact on OS outcomes in numerous cancers. The bounding boxes reflect significantly (P<0.05) unfavorable (red) and favorable (blue) outcomes. (B) HR survival heat map depicting the prognostic impact of the *BEND5* on the outcome of RFS in multiple cancer types. (C) Prognostic relationship of *BEND5* gene expression and OS in patients with LUAD. (D) Prognostic association of *BEND5* expression and DSS in patients with LUAD. (E,F) Forest plots showing the results of

univariate and multivariate regression analysis of the relationships of BEND5 and clinicopathologic characteristics with OS in patients with LUAD. (G-J) The relationships between tumor characteristics (G: T stage; H: pathologic stage; I: primary therapeutic outcome; J: smoker) and *BEND5* expression in LUAD. *, P<0.05; **, P<0.01; ***, P<0.001. OS, overall survival; RFS, recurrence free survival; DSS, disease free survival; *BEND5*, BEN domain-containing protein 5; ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma; HR, hazard ratio; CI, confidence interval; TPM, transcript per million; PR, partial response; CR, complete response; PD, progressive disease; SD, stable disease.

Table 2 Cox regression analysis of the associations of overall survival with clinical characteristics and *BEND5* mRNA expression level in patients with LUAD (27)

Characteristics	Total (N)	Univariate analysis		Multivariate analysis	
		HR (95% CI)	P value	HR (95% CI)	P value
T stage (T3 + T4 *vs.* T1 + T2)	523	2.317 (1.591–3.375)	<0.001	1.888 (1.186–3.007)	0.007
N stage (N1 + T2 *vs.* N0)	508	2.645 (1.977–3.539)	<0.001	2.239 (1.600–3.133)	<0.001
M stage (M1 *vs.* M0)	377	2.136 (1.248–3.653)	0.006	–	–
Pathologic stage (stage III + IV *vs.* stage I + II)	518	2.664 (1.960–3.621)	<0.001	–	–
Primary therapy outcome (PD + SD *vs.* PR + CR)	439	2.653 (1.888–3.726)	<0.001	2.248 (1.584–3.191)	<0.001
Age (>65 *vs.* ≤65 years)	516	1.223 (0.916–1.635)	0.172	–	–
Sex (male *vs.* female)	526	1.070 (0.803–1.426)	0.642	–	–
Race (Asian + Black or African American *vs.* White)	468	0.678 (0.415–1.109)	0.121	–	–
Smoker (yes *vs.* no)	512	0.894 (0.592–1.348)	0.591	–	–
No. of pack years smoked (≥40 *vs.* <40 years)	363	1.073 (0.753–1.528)	0.697	–	–
Atomic neoplasm subdivision (right *vs.* left)	512	1.037 (0.770–1.397)	0.810	–	–
Anatomic neoplasm subdivision 2 (peripheral lung *vs.* central lung)	182	0.913 (0.570–1.463)	0.706	–	–
BEND5 expression (high *vs.* low)	526	1.461 (1.095–1.950)	0.010	1.427 (1.018–2.000)	0.039

BEND5, BEN domain-containing protein 5; LUAD, lung adenocarcinoma; HR, hazard ratio; CI, confidence interval; PR, partial response; CR, complete response; PD, progressive disease; SD, stable disease.

The expression level of *BEND5* mRNA in LUAD was significantly linked to four tumor characteristics including T stage, pathologic stage, primary therapy outcome, and smoker (*Figure 3G-3J*); however, no such correlation was found for other variables such as N stage, M stage, age, race, and the number of pack-years smoked.

Nomogram plot and calibration plot

To assess the survival probability of patients with LUAD at 1, 3, and 5 years, a nomogram plot (*Figure 4A*) was constructed incorporating *BEND5* expression levels along with independent clinical characteristics. The points for genetic score, age, and TNM stage were added to obtain

Table 3 Logistic regression analysis outcomes showing the association of *BEND5* expression with clinical characteristics of patients with lung adenocarcinoma (27)

Characteristics	Total (N)	Odds ratio (95% CI)	P value
T stage (T3 + T4 *vs.* T1 + T2)	532	0.578 (0.339–0.968)	0.039
N stage (N1 + N2 + N3 *vs.* N0)	519	0.773 (0.535–1.116)	0.170
M stage (M1 *vs.* M0)	386	0.873 (0.378–1.970)	0.744
Pathologic stage (stage III + IV *vs.* stage I + II)	527	0.656 (0.427–1.001)	0.052
Primary therapy outcome (PD + SD *vs.* PR + CR)	446	0.526 (0.336–0.816)	0.004
Sex (male *vs.* female)	535	0.622 (0.441–0.875)	0.007
Race (Asian + Black or African American *vs.* White)	468	1.179 (0.690–2.028)	0.548
Age (>65 *vs.* ≤65 years)	516	1.048 (0.742–1.480)	0.792
Smoker (yes *vs.* no)	521	0.286 (0.161–0.489)	<0.001
Residual tumor (R1 + R2 *vs.* R0)	372	0.658 (0.234–1.751)	0.406
Anatomic neoplasm subdivision2 (peripheral lung *vs.* central lung)	189	0.716 (0.388–1.315)	0.282
No. of pack years smoked (≥40 *vs.* <40 years)	369	1.334 (0.884–2.018)	0.171

BEND5, BEN domain-containing protein 5; PR, partial response; CR, complete response; PD, progressive disease; SD, stable disease; CI, confidence interval.

the total points, with a higher number of total points on the nomogram suggesting a worse prognosis. A calibration curve was established to evaluate the nomogram model's prediction accuracy. The nomogram-predicted 1-, 3- and 5-year OS was found to match the actual survival results, as indicated by the calibration curves (*Figure 4B*).

PPI network plotting and heatmap

PPI network analysis, performed using the online STRING database, was used to evaluate the possible relationships of *BEND5* with other genes. The PPI network graph includes BEND5 and 10 proteins to which it was strongly related (*Figure 5A*). The proteins KCNH4, PRSS12, and COL24A1 had the highest combined scores, with corresponding values of 0.647, 0645, and 0.637, respectively.

The heat map expression patterns of genes related to *BEND5* in LUAD samples are depicted in *Figure 5B*. The top 10 genes that were positively associated and the top 10 genes that were negatively associated with *BEND5* were identified. The top 10 genes that showed a positive correlation with *BEND5* were *GPC3*, *APBB1*, *AGBL4*, *FLJ37453*, *USP51*, *TMCC2*, *SCD5*, *FAM229B*, *GALNT16*, and *NDRG2*. The top 10 genes that showed a negative correlation with *BEND5* were *NATD1*, *TMEM231*, *PRRT3*, *C1QTNF2*, *RAI2*, *TTC25*, *GASK1A*, *TESK2*, *COLQ*, and *AL691432.2*.

The functional terms enriched for BEND5-correlated genes

Mountain plots depicting the five functional terms that met the criteria of P.adjust <0.05, q value <0.25, and |NES| >1 are shown in *Figure 6A*. Through GSEA analysis, *BEND5*-associated genes were revealed to be primarily enriched in two functions: M_PHASE (*Figure 6B*) and SIGNALING_BY_NUCLEAR_RECEPTORS (*Figure 6C*).

The top 10 genes that were positively and negatively related to *BEND5* were found to be significantly enriched in several BP including the site of polarized growth, growth cone, collagen trimer, synaptic cleft, MKS complex, glucose import, regulation of glucose import, positive regulation of glucose transmembrane transport, and positive regulation of glucose import (*Figure 6D*). Enriched KEGG pathways for the positively and negatively associated genes included the PPAR signaling pathway, fatty acid metabolism, other types of O-glycan biosynthesis, mucin type O-glycan biosynthesis, and biosynthesis of unsaturated fatty acids (*Figure 6E*).

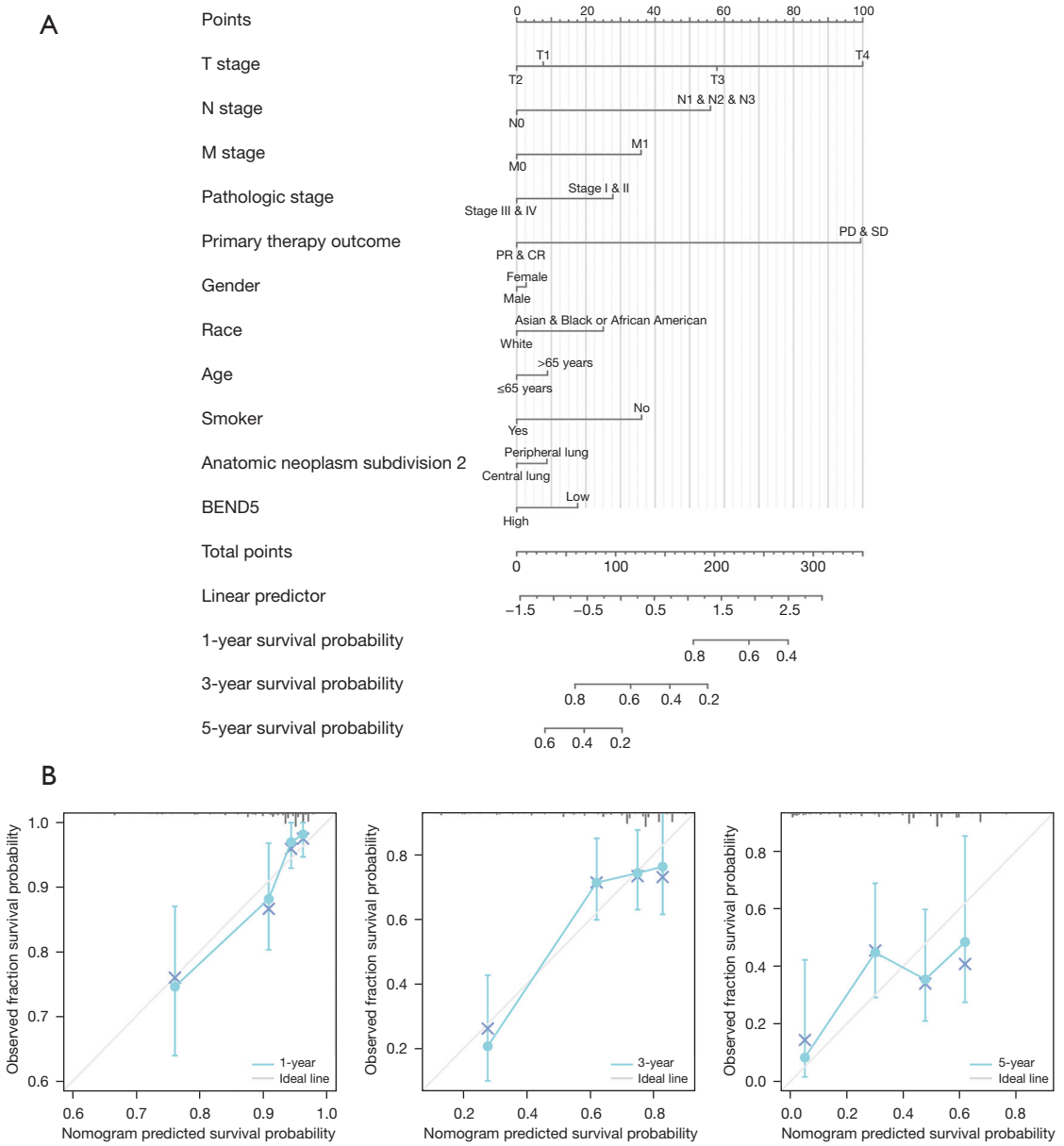

Figure 4 Nomogram and calibration plot. (A) Nomogram predicting the 1-, 3- and 5-year OS probability of patients with LUAD. (B) Calibration curve for 1-, 3- and 5-year OS predicted by the nomogram. *BEND5*, BEN domain-containing protein 5; PR, partial response; CR, complete response; PD, progressive disease; SD, stable disease; LUAD, lung adenocarcinoma; OS, overall survival.

Increased BEND5 expression inhibits LUAD cell proliferation

Western blot was applied for the assessment of BEND5 expression levels in five LUAD cell lines and a human bronchial epithelial cell line. As shown in Figure S1A, results revealed that *BEND5* had low levels of protein expression in the five different LUAD cell lines, with the A549 and PC-9 cell lines exhibiting the lowest expression levels. This finding prompted us to use these two cell lines for our subsequent research. Western blot analysis revealed that *BEND5* plasmids significantly increased *BEND5* expression in A549 and PC-9 cells (Figure S1B).

To gain further insight into the intrinsic mechanism of *BEND5* in the pathogenesis of LUAD, the PPAR signaling pathway was investigated and was found to be activated

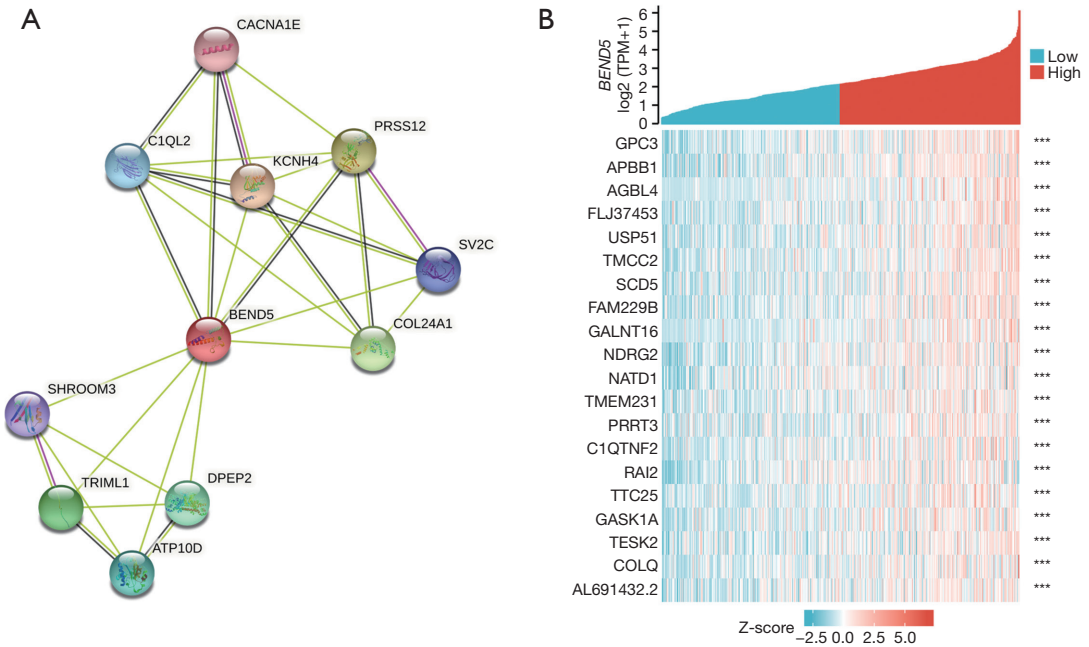

Figure 5 Genes correlated with *BEND5* expression in lung adenocarcinoma. (A) Protein-protein interaction network of genes co-expressed with *BEND5* in LUAD constructed from the STRING database. (B) Heat map indicating the expression patterns of the top 10 positively and negatively associated genes for *BEND5* in LUAD samples. ***, P<0.001. *BEND5*, BEN domain-containing protein 5; TPM, transcript per million; LUAD, lung adenocarcinoma.

by *BEND5* overexpression (*Figure 7A*). Since the PPAR signaling pathway is associated with cell proliferation, *in vitro* functional assays were performed. Overexpression of *BEND5* significantly inhibited A549 and PC-9 cell proliferation, as demonstrated by colonogenic assay and CCK-8 assay results (*Figure 7B,7C*). Western blot showed that BEND5 overexpression inhibited the cyclin-dependent kinase CDK2/4 and the cyclin proteins CCND1 and CCNB1 (*Figure 7D*).

PPAR inhibition restores cell proliferation in BEND5-overexpressing LUAD cells

Previous research shows that overexpression of *BEND5* activates the PPAR signaling pathway. However, the contribution of PPAR in the anti-cell proliferation function of *BEND5* in LUAD has not yet been clarified. To address this, we conducted colonogenic assay and CCK-8 assays on A549 and PC-9 cells, and the results showed that *BEND5* overexpression with *PPAR* inhibition increased the proliferation ability of LUAD cells (*Figure 8A,8B*). Results of Western blot further showed that CDK2/4, CCND1, and CCNB1 were restored in *BEND5*-overexpressing cells with *PPAR* inhibition (*Figure 8C*).

Analysis of the relationship between BEND5 expression and immune infiltration in LUAD

Because the functions of *BEND5* in the regulation of the immune and tumor microenvironments of LUAD are unknown, the next part of our study investigated the relationship between *BEND5* expression and immune scores. The expression of *BEND5* was correlated significantly with ESTIMATE and stromal scores in patients with LUAD; however, the immune score did not show a significant correlation (Figure S1C).

Investigating the relationship between BEND5 expression and immune cells in LUAD

BEND5 expression was found to be significantly positively associated with several TIICs, including B cells, DCs, eosinophils, immature DCs, macrophages, mast cells, NK CD56bright cells, NK cells, plasmacytoid DCs, T cells, T follicular helper cells, and T regulatory cells. *BEND5* expression was significantly negatively correlated with

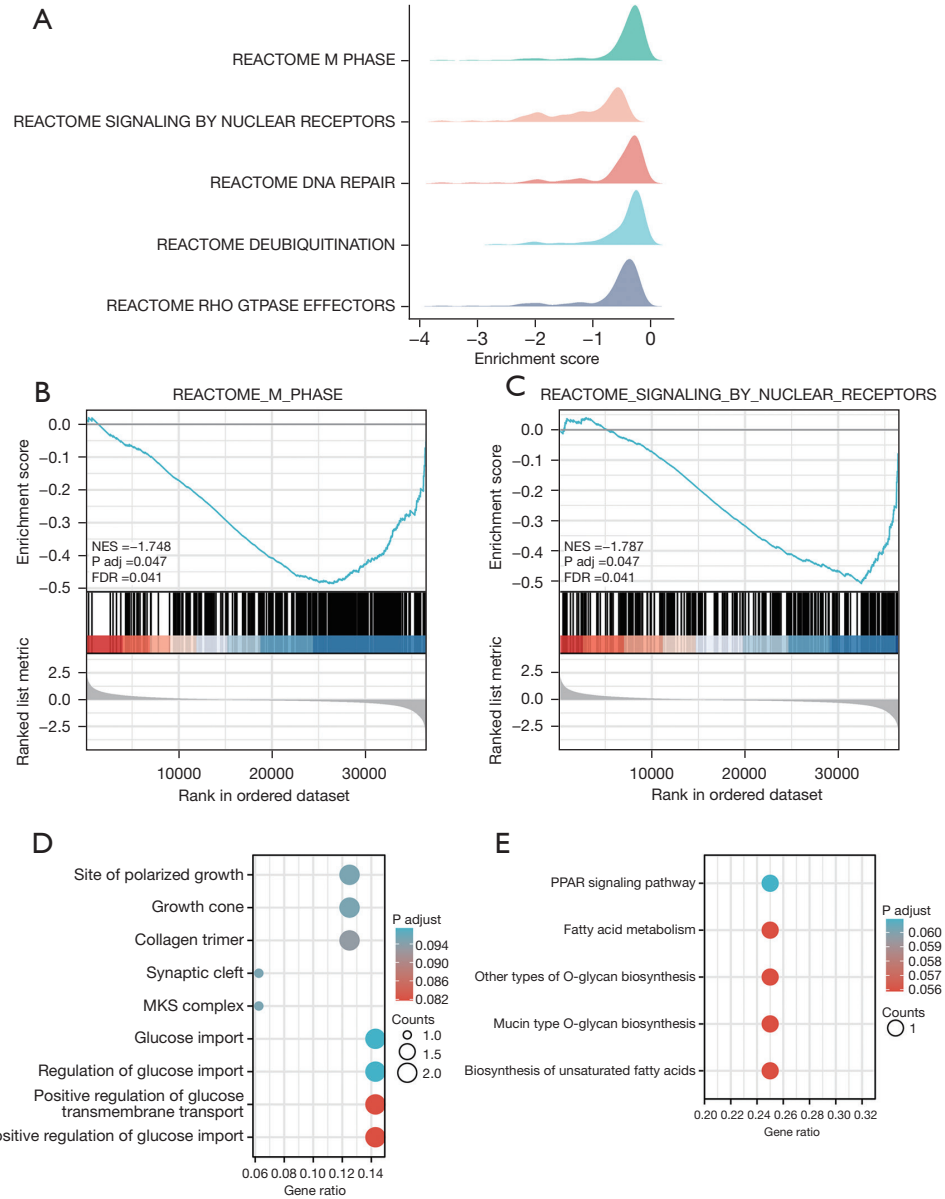

Figure 6 Results of gene set enrichment analysis and functional enrichment analysis. (A) GSEA results are represented by mountain and enrichment plots. Functional terms that fulfilled the criteria of P.adjust <0.05, q value <0.25, and |NES| >1 are displayed in the mountain plot. (B,C) Results of GSEA analysis showing that *BEND5*-associated genes are primarily enriched in two functions: M_PHASE and SIGNALING_BY_NUCLEAR_RECEPTORS. (D) Functional enrichment analysis was performed to obtain the significantly enriched gene ontology terms, including biological processes, cellular components, and molecular functions, for genes significantly related to *BEND5*. (E) Functional enrichment analysis was performed to obtain the significantly enriched Kyoto Encyclopedia of Genes and Genomes pathways for genes significantly related to *BEND5*. *BEND5*, BEN domain-containing protein 5; NES, normalized enrichment score; FDR, false discovery rate; PPAR, peroxisome proliferator-activated receptor; GSEA, gene set enrichment analysis.

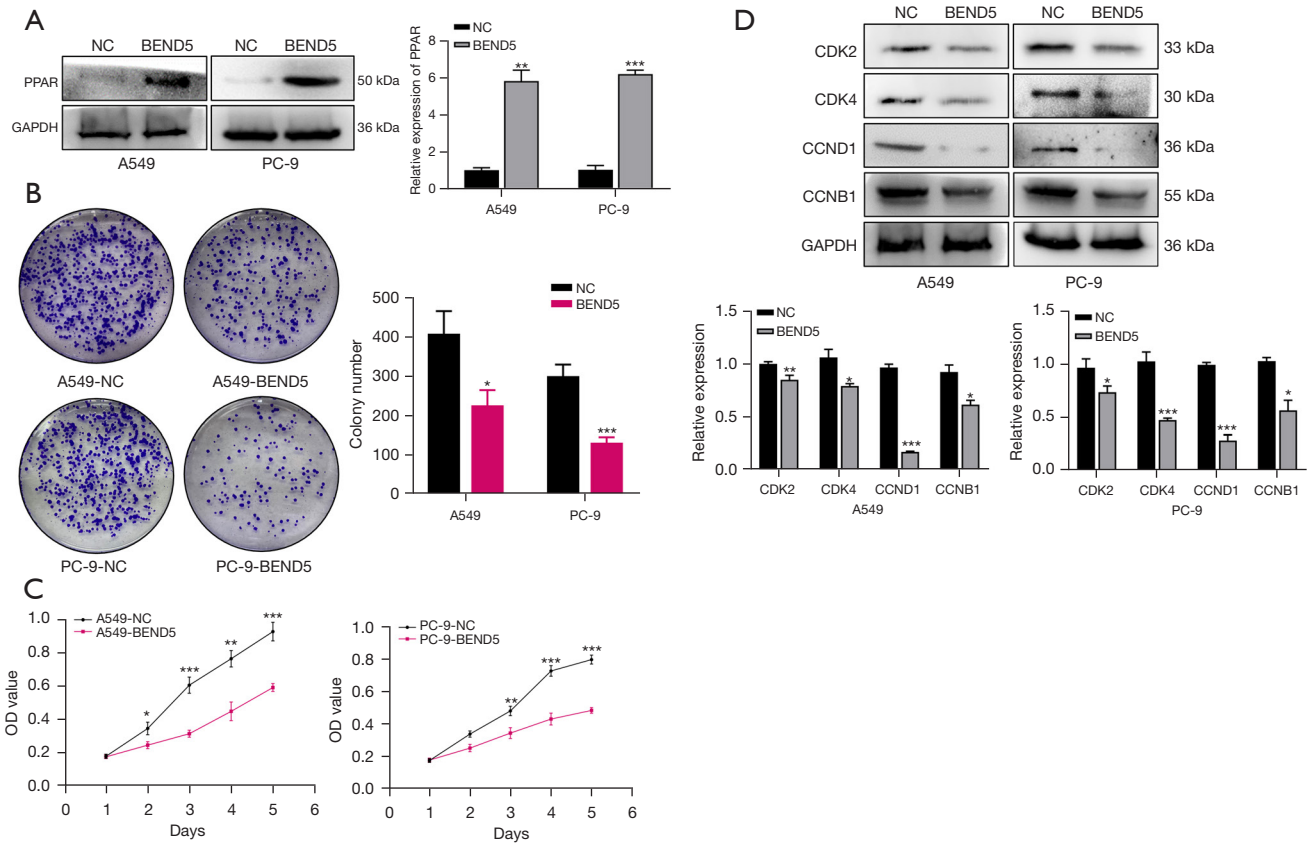

Figure 7 *BEND5* inhibits the proliferation of lung adenocarcinoma cells. (A) Western blot analysis results showing the expression level of the PPAR signaling pathway-related proteins after *BEND5* overexpression in LUAD cells. (B) Colonogenic assay results showing that overexpression of *BEND5* inhibited the proliferation of LUAD cells (crystal violet staining). (C) Cell Counting Kit-8 assay results showing that overexpression of *BEND5* inhibited LUAD cell proliferation. (D) Western blot results showing the expression levels of proliferation signal-related proteins after *BEND5* overexpression in LUAD cells. *, P<0.05; **, P<0.01; ***, P<0.001. *BEND5*, BEN domain-containing protein 5; NC, negative control; PPAR, peroxisome proliferator-activated receptor; OD, optical density; LUAD, lung adenocarcinoma.

several others cell types, including NK CD56dim, gamma delta T cells, and Th2 cells (Figure S2A, S2B).

Investigating the relationships between BEND5 expression and 59 ICGs in LUAD

The correlations of *BEND5* expression with 23 checkpoint inhibitor genes are shown in Figure S3A. Only 14 of the 23 ICGs showed a statistically significant correlation with *BEND5* expression, and the other ICGs had no significant correlation. *BEND5* expression was positively correlated with BTLA, CTLA4, SLAMF7, IDO1, ARG1, IL13, IL4, ADORA2A, VEGFB, VTCN1, EDNRB, and IL12A, and negatively correlated with CD274 and VEGFA (Figure S3B).

The correlations of *BEND5* expression with 36 checkpoint stimulator genes are shown in Figure S4A. Only 16 of the 36 ICGs showed a statistically significant correlation with *BEND5* expression, and the other ICGs had no significant correlation. *BEND5* expression was positively correlated with CX3CL1, TNFRSF14, BTN3A1, CD40LG, IL2, CD27, CD28, TNFRSF4, CD40, ENTPD1, SELP, ITGB2, TNF, and CD70, and negatively correlated with IFNG and TNFSF9 (Figure S4B).

Discussion

LUAD is a common respiratory cancer whose morbidity and mortality rates are constantly increasing. As LUAD can develop resistance to radiation and chemotherapy, there is an unmet need for new treatment approaches. To improve

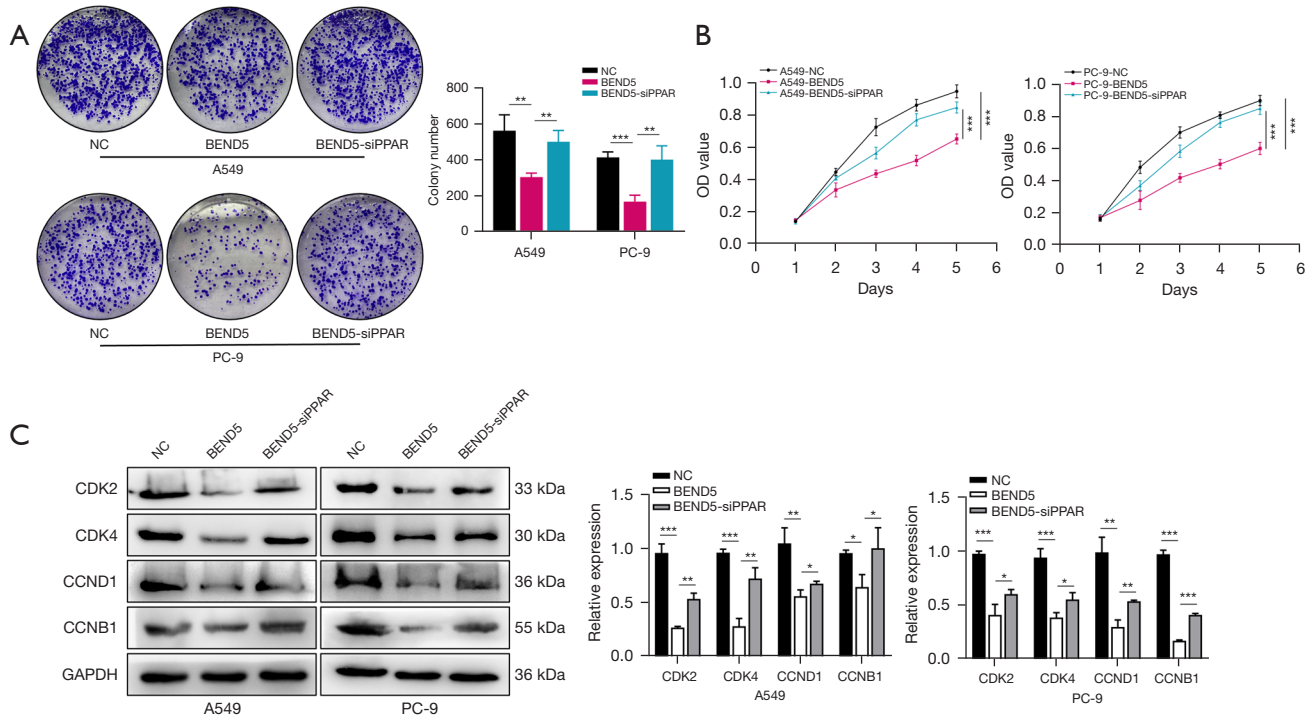

Figure 8 *BEND5* inhibits the proliferation of lung adenocarcinoma cells by activating the PPAR signaling pathway. (A) Colonogenic assay results showing that inhibition of the PPAR signaling pathway restored the proliferative capacity of *BEND5*-overexpressing LUAD cells (crystal violet staining). (B) CCK-8 assay results showing that inhibition of the PPAR signaling pathway restored the proliferative capacity of *BEND5*-overexpressing LUAD cells. (C) Western blot results showing that inhibition of the PPAR signaling pathway in *BEND5*-overexpressing LUAD cells restored the expression levels of proliferation signal-related proteins. *, P<0.05; **, P<0.01; ***, P<0.001. *BEND5*, BEN domain-containing protein 5; NC, negative control; PPAR, peroxisome proliferator-activated receptor; OD, optical density; LUAD, lung adenocarcinoma; si, small interfering; CCK-8, Cell Counting Kit-8.

the early diagnosis and survival rates of patients with LUAD, specific major molecular pathways and multiple sensitive and reliable biomarkers need to be identified. The relationship between *BEND5* and tumor development has been shown in earlier studies (14,15). However, an in-depth description of the role of *BEND5* in LUAD is lacking. In the current study, we developed a prognostic model based on *BEND5* gene expression in LUAD. Enrichment analysis showed that *BEND5* was involved in processes such as cell proliferation and immune infiltration of tumor cells. This is the first investigation to study the effects of *BEND5* in LUAD *in vitro*, and our findings may contribute to the development of future investigations.

BEND5 belongs to the BEN structural domain family, whose members are present in a variety of animal proteins. Despite the fact that the mammalian genome encodes many BEN domain proteins, little research has been done on the BEN domains (10). The first member of this family to be identified was BANP (also known as BEND1 or SMAR1) (28). The tumor suppressor *SMAR1* has been reported to act as a transcriptional regulator of the cyclin D1 protein and inhibits its expression (29). In addition, SMAR1 is a protein that interacts with p53, which inhibits tumor progression *in vivo* as well as delays the cell cycle (30). Another BEN family member, BEND3, is associated with several human malignancies, such as prostate cancer (31), breast cancer (32), and ovarian cancer (33). Also, BEND3 acts as a transcriptional repressor and is involved in transcriptional repression processes through interaction with histone deacetylases and Sall4 (34). The first study of *BEND5* was reported in colorectal cancer and showed the contribution of *BEND5* hypermethylation to the proliferation of cells, marking its possibility as a prognostic marker (14). *BEND5* has also been identified as a tumor suppressor gene associated with growth and metastasis in breast cancer (15). Our bioinformatics analysis showed that

BEND5 is expressed at low levels in many cancer types, including colorectal and breast cancer, which is consistent with the findings reported in previous studies. The results from our study based on TCGA data showed that *BEND5* was associated with OS in 14 out of 33 cancers, and with relapse-free survival in 10 out of 33 cancers, indicating that *BEND5* could be used as a molecular marker for cancer prognosis. The KEGG enrichment analysis demonstrated the involvement of *BEND5* in the PPAR signaling pathway, fatty acid metabolism, mucin-type O-glycan biosynthesis, and other processes.

PPARs belong to a class of ligand-dependent transcriptional regulators that are involved in developmental and metabolic processes (35-37). Three isoforms of PPARs have been identified in different species: α, β/δ, and γ. The most common isoform is PPARβ/δ, which is expressed in all tissues and is involved in functions such as cell proliferation and differentiation (38), lipid and glucose metabolism (39), and inhibition of inflammatory processes (40-42). Several studies have suggested the significance of *PPAR* in tumorigenesis and progression (43,44). For example, studies have indicated a correlation between *PPAR* proliferation and the rapid recurrence of non-small cell lung cancer (35,36,44), while others have suggested that PPARβ/δ has a protective effect against lung cancer (40). Therefore, we considered that *BEND5* may be involved in the proliferation of LUAD cells through the PPAR signaling pathway. *In vitro* functional assays validated this conjecture, showing that overexpression of *BEND5* inhibited the proliferative capacity of LUAD cells, whereas knockdown of *PPAR* reversed these effects.

The tumor immune microenvironment is crucial to tumor development. Immunotherapy has become an extremely popular treatment for cancers and is also helpful in LUAD. Although a handful of studies have reported the role of *BEND5* in some cancers (14,15), the immunomodulatory function of *BEND5* in LUAD is not well understood at present. Therefore, our main focus was to evaluate the relationship between the level of *BEND5* expression, immune score, and immune cell profile of LUAD. We found that in LUAD, *BEND5* expression was positively correlated with several immune infiltrating cells, including B cells, NK cells, and T cells. In the tumor microenvironment, TILs are heterogeneous immune cell populations that produce different immune responses under the influence of different cellular activation mechanisms and cytokines. The synergistic role of immune infiltrating cells is crucial in the anti-tumor response. Moreover, *BEND5* expression was found to be associated with 15 non-cancerous cell types as well as 23 checkpoint inhibitor genes and 36 checkpoint stimulator genes, suggesting that it plays a key role in the regulation of the tumor immune microenvironment. Overall, these findings add to our knowledge of the role of *BEND5* in tumor immunology and its putative application as a LUAD biomarker.

To evaluate the present study thoroughly, it is important to recognize both its strengths and limitations. The main strength of this study is that it explored the significance of the *BEND5* gene in LUAD through a series of bioinformatic analyses. The study explored the correlation of *BEND5* expression patterns with clinical variables as well as the prognostic value of *BEND5* and its significantly associated genes, KEGG and signaling pathways, and involvement in tumor immunity in LUAD. Furthermore, the role of *BEND5* in LUAD proliferation was also validated by *in vitro* experiments. Our study's main limitation is the absence of *in vivo* tests to confirm the involvement of *BEND5* in LUAD proliferation. Also, it remains to be seen whether *BEND5* regulates LUAD proliferation through multiple signaling pathways.

To the best of our knowledge, our study has revealed, for the first time, the role of *BEND5* in the progression and pathogenesis of LUAD, and has identified its potential clinical value. The potential clinical utility of the current study's primary findings should be underlined. The survival analysis results indicate the potential utility of molecular characterization of patients who are candidates for treatment with BNED5 medicines as a precision medicine methodology. BEND5 agonists have the potential to be a novel therapy strategy for LUAD. Considering the significance of *BEND5* to LUAD prognosis, *BEND5* may be a meaningful prognostic biomarker. Therefore, it is necessary to explore the role of *BEND5* in LUAD and the molecular mechanisms involved more extensively in future.

Conclusions

Using integrated bioinformatics analyses, our study demonstrated that *BEND5* was downregulated in LUAD and could potentially serve as a prognostic biomarker for the disease. *BEND5* inhibited the proliferation of LUAD cell lines by activating the PPAR signaling pathway. Our results provide evidence for prospective research of the associations of LUAD with the *BEND5* gene; however, more investigations are warranted to comprehensively illuminate the role of *BEND5* in LUAD.

Acknowledgments

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Footnote

Data Sharing Statement: Available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-314/dss

Peer Review File: Available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-314/prf

Conflicts of Interest: Both authors have completed the ICMJE uniform disclosure form (available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-314/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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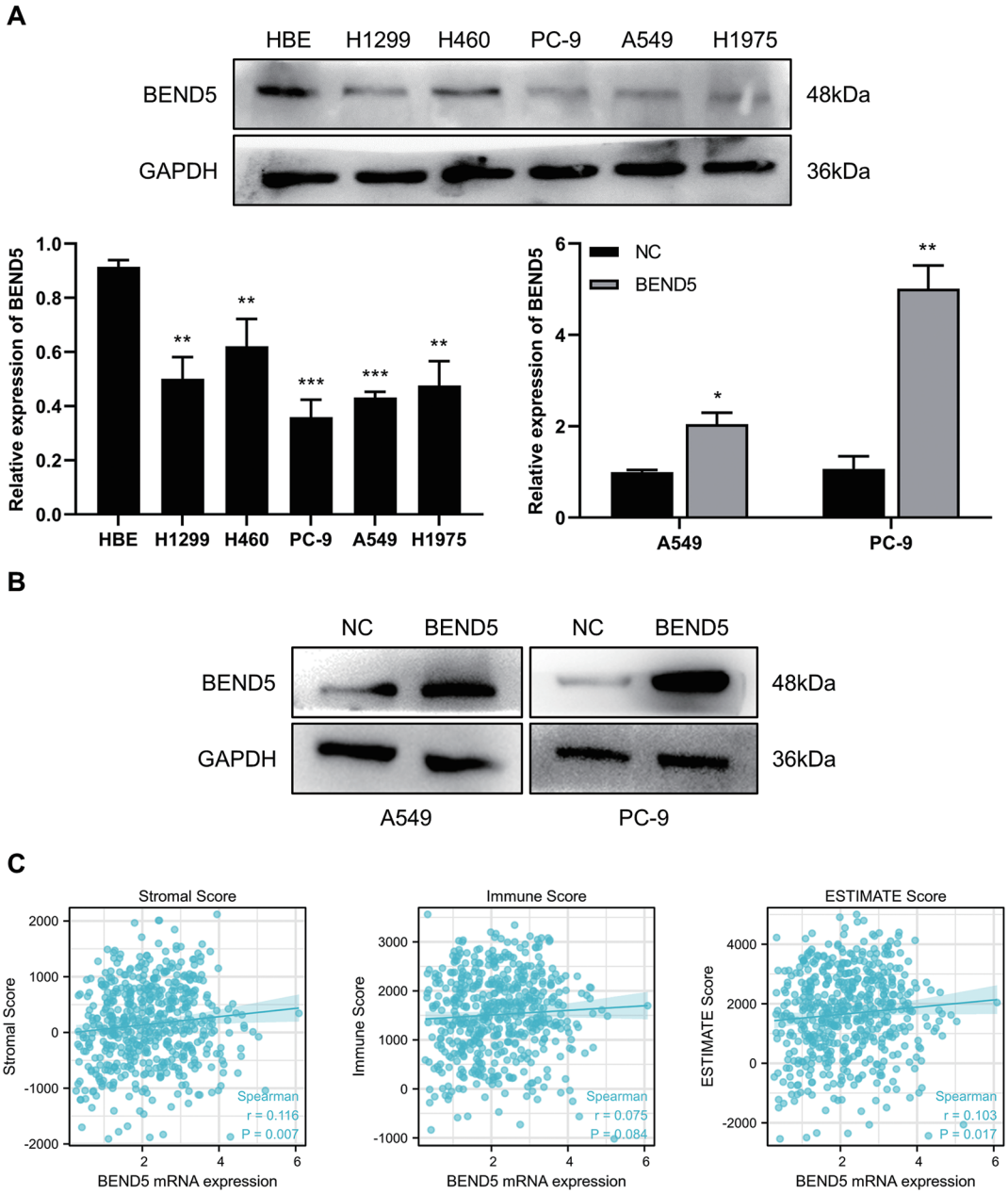

Figure S1 Expression of *BEND5* in lung adenocarcinoma cells. (A) *BEND5* protein expression in five individual LUAD cell lines and a human bronchial epithelial cell line. (B) Plasmid transfection increased the level of *BEND5* protein expression. (C) Immune score, stromal score, and ESTIMATE score are correlated with *BEND5* expression in patients with LUAD. *, P<0.05; **, P<0.01; ***, P<0.001. *BEND5*, BEN domain-containing protein 5; NC, negative control; LUAD, lung adenocarcinoma.

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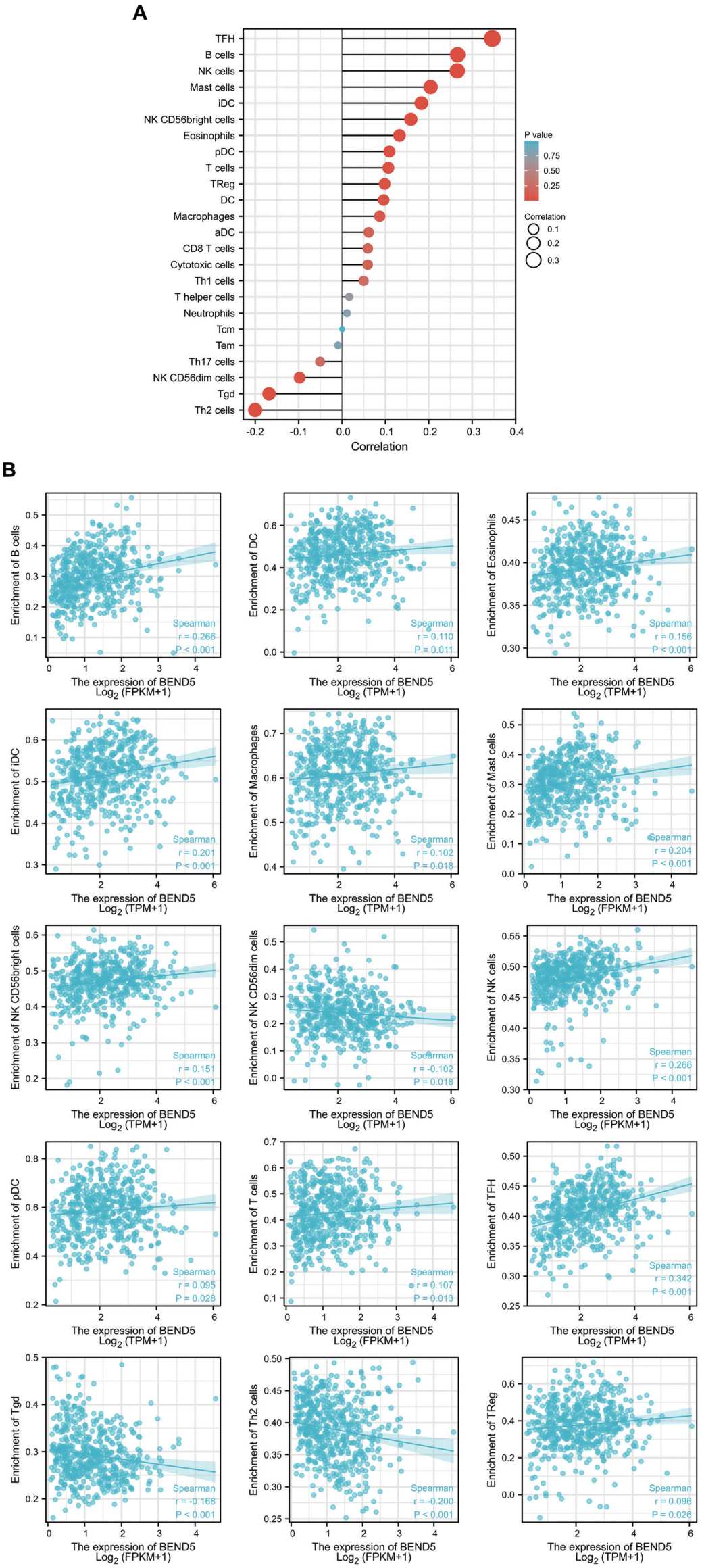

Figure S2 The correlations between *BEND5* expression and TIICs. (A) The correlations between *BEND5* expression and 24 TIICs in LUAD are depicted in a lollipop plot. (B) Significant positive correlations between *BEND5* expression and 12 TIIC types (B cells, DCs, eosinophils, iDCs, macrophages, mast cells, NK cells, NK CD56bright cells, pDCs, T cells, TFH cells and Tregs) and significant negative correlations between *BEND5* expression and 3 TIIC types (NKs CD56dim cells, Tgd and Th2 cells), are shown. TFH, T follicular helper; NK, natural killer; iDCs, immature dendritic cells; pDCs, plasmacytoid dendritic cells; Tregs, T regulatory cells; DCs, dendritic cells; aDCs, activated dendritic cells; TIICs, tumor-infiltrating immune cells; Tcm, T central memory; Tem, T effector memory; Tgd, T gamma delta; *BEND5*, BEN domain-containing protein 5; FPKM, fragments per kilobase of transcript per million mapped reads; TPM, transcript per million; LUAD, lung adenocarcinoma.

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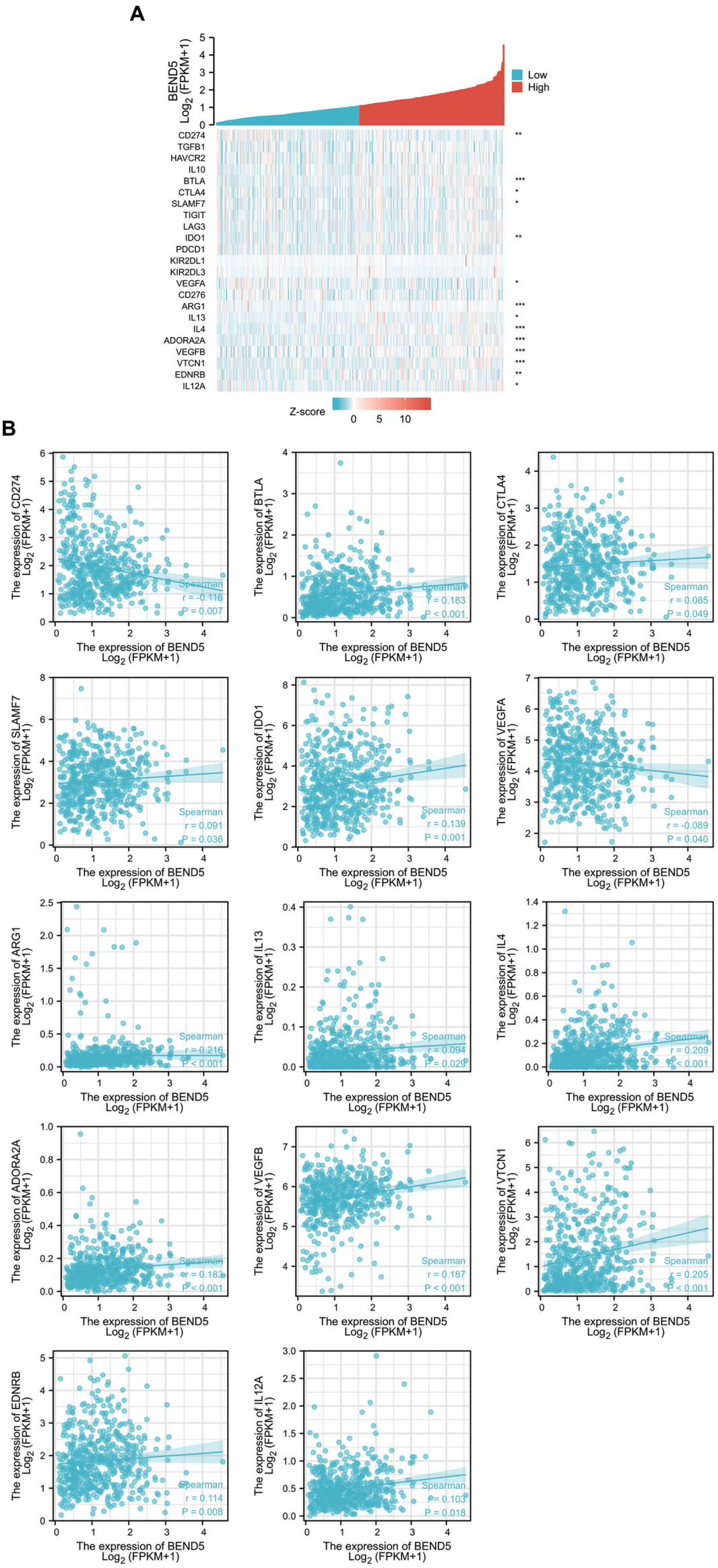

Figure S3 The correlations between *BEND5* expression and 23 checkpoint inhibitor genes in lung adenocarcinoma tumor samples. (A) Heat map indicating the expression patterns of 23 checkpoint inhibitor genes in LUAD. (B) Scatter plots showing positive correlations between *BEND5* expression and BTLA, CTLA4, SLAMF7, IDO1, ARG1, IL13, IL4, ADORA2A, VEGFB, VTCN1, EDNRB, and IL12A, and negative correlations between *BEND5* expression and CD274 and VEGFA. *, P<0.05; **, P<0.01; ***, P<0.001. *BEND5*, BEN domain-containing protein 5; FPKM, fragments per kilobase of transcript per million mapped reads; LUAD, lung adenocarcinoma.

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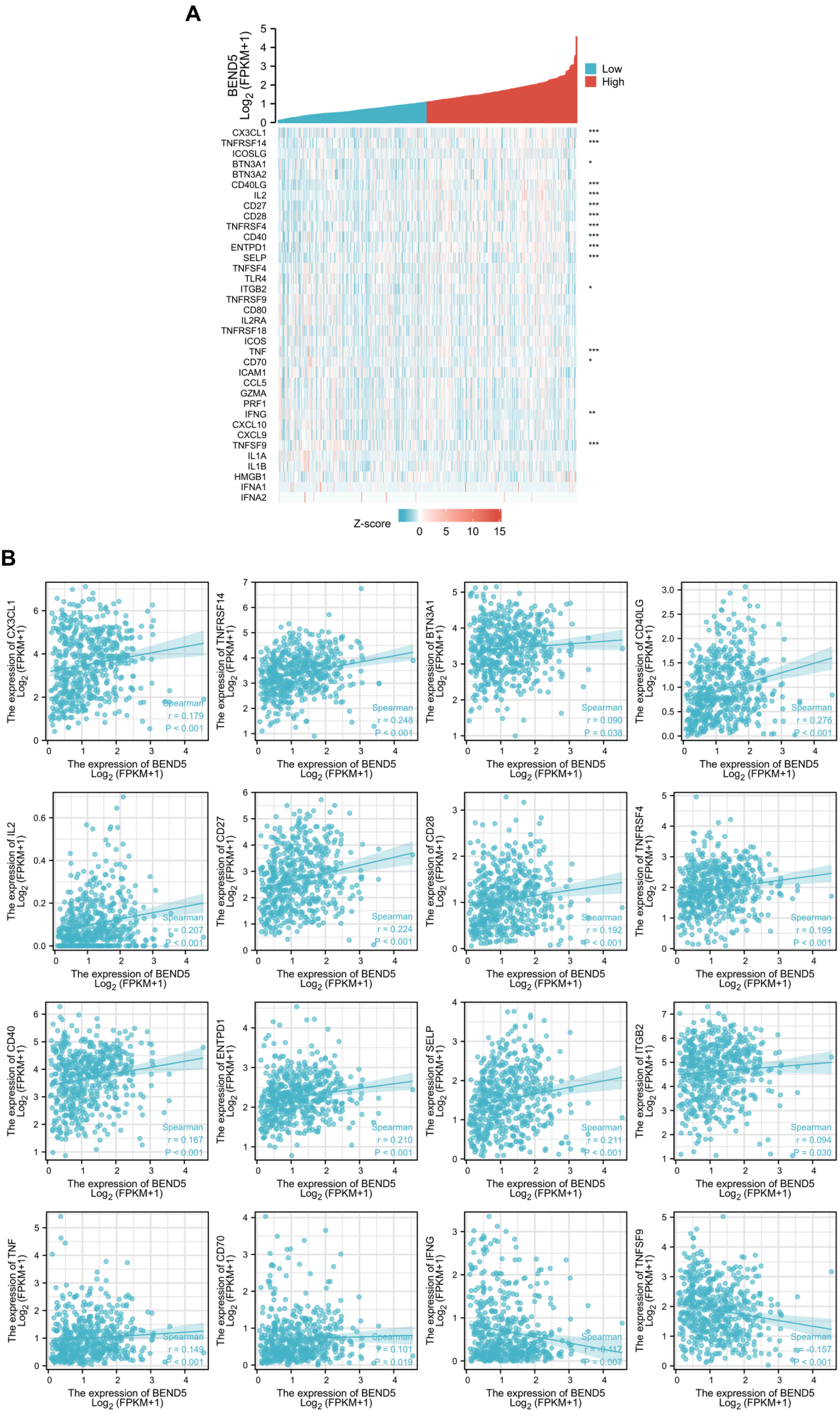

Figure S4 The correlations between *BEND5* expression and 36 checkpoint stimulator genes in lung adenocarcinoma tumor samples. (A) Heat map indicating the expression patterns of 36 checkpoint stimulator genes in LUAD. (B) Scatter plots showing positive correlations between *BEND5* expression and CX3CL1, TNFRSF14, BTN3A1, CD40LG, IL2, CD27, CD28, TNFRSF4, CD40, ENTPD1, SELP, ITGB2, TNF, and CD70, and negative correlations between *BEND5* expression and IFNG and TNFSF9. *, P<0.05; **, P<0.01; ***, P<0.001. *BEND5*, BEN domain-containing protein 5; FPKM, fragments per kilobase of transcript per million mapped reads; LUAD, lung adenocarcinoma.

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