



Integrated single-cell and bulk RNA sequencing reveals novel biomarkers of invasive adenocarcinoma subtypes in lung adenocarcinoma

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Background: Lung adenocarcinoma (LUAD) is one of the most common lung cancer subtypes worldwide, and its aggressive subtype invasive adenocarcinoma (IAC) has low survival rates. The precise identification of IAC is vital for the clinical diagnosis and treatment. The purpose of this study is to identify novel biomarkers for LUAD using single-cell and bulk RNA sequencing, so as to provide theoretical basis and practical support for the diagnosis, treatment and prognosis evaluation of lung invasive adenocarcinoma.

Methods: We employed a combination of transcriptomic analysis and single-cell analysis to investigate the molecular characteristics and immune microenvironment of four subtypes of LUAD, including atypical adenomatous hyperplasia (AAH), adenocarcinoma in situ (AIS), minimally invasive adenocarcinoma (MIA), and IAC, with the aim of screening for biomarkers to differentiate pre-invasive lesions from invasive lesions.

Results: Transcriptomic and single-cell analyses revealed that IAC subtypes demonstrated the most substantial molecular differences, particularly in immune cell infiltration and immune-related gene expression. Three genes—*CD27*, *TIGIT*, and *TNFRSF18*—that were significantly upregulated in IAC, predominantly expressed in immune cells and closely linked to immune regulatory pathways. We further analyzed T cell subpopulations in the IAC subtype and explored the expression of transcription factors (TFs) corresponding to these three genes, revealing their critical roles in immune cell function. Additionally, communication between T cells and other cells showed significantly enhanced signaling pathways, particularly those related to immune co-stimulatory molecules and inflammation pathways. Immunohistochemical validation of clinical samples showed that these three genes have high diagnostic value in IAC subtypes. These findings establish a crucial biological foundation for diagnosis, classification, and immunotherapy of LUAD, which contributes to the development of individualized treatment strategies.

Conclusions: This study identifies a three-gene signature (*CD27*, *TIGIT*, and *TNFRSF18*) that not only distinguishes invasive from pre-invasive LUAD with high precision by capturing the immune checkpoint disequilibrium characteristic of IAC, but also provides a clinically actionable biomarker panel for preoperative diagnosis and personalized immunotherapy strategies.

Keywords: Lung cancer; invasive adenocarcinoma of the lung (IAC of the lung); molecular markers; multi-omics

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Introduction

According to the 2022 Global Cancer Burden report by the International Agency for Research on Cancer under the World Health Organization (WHO) in 2024, 2,481,000 new cases of lung cancer were recorded, which comprises 12.4% of all newly diagnosed cancer cases and dominate the most prevalent cancer globally (1). Globally, lung adenocarcinoma (LUAD) is a highly heterogeneous subtype of lung cancer, which accounts for a relatively high proportion (around 35–40%) of lung cancers. And its morbidity and mortality rates continue to rise, posing a great challenge to public health (2). LUAD could be further classified into atypical adenomatous hyperplasia (AAH), adenocarcinoma in situ (AIS), minimally invasive adenocarcinoma (MIA), and invasive adenocarcinoma (IAC) (3,4). AAH, AIS, and MIA are regarded as pre-invasive forms of LUAD, exhibiting a 100% 5-year recurrence-free survival rate (5). However, the IAC subtype is the most aggressive subtype of LUAD, with a significantly reduced 5-year postoperative survival rate of less than 75%, and is highly associated with local infiltration and distant metastasis (6). Pre-invasive lesions can typically be cured through surgical resection, whereas invasive LUAD often necessitates multimodal treatment, including surgery, chemotherapy, radiotherapy, targeted therapy, and immunotherapy (7). Therefore, accurate identification of invasive biomarkers is vital for the rapid initiation of treatment.

Although molecular markers, computed tomography (CT), histopathology, and other diagnostic methods are

popularly used for the differentiation of LUAD subtypes, some limitations exist. With molecular markers, IAC typically presents with EGFR mutation, and elevated programmed death-ligand 1 (*PD-L1*) expression, but AAH is commonly associated with *EGFR* mutation (8). And *PD-L1* expression is influenced by the tumor microenvironment, and its stability may limit its utility in routine diagnosis (9). Besides, other emerging markers, such as microRNAs (miRNAs) and circulating tumor cells, show promise in LUAD diagnosis and prognostic assessment. High expression of miR-17 and FR⁺CTC correlates with the aggressiveness of IAC (10,11). At present, CT and histopathology are commonly used in clinical practice, but they are also plagued by subjective diagnosis. The interpretation of CT and pathological diagnosis also both depend on the doctor's experience, and the judgments vary among the doctors. Although these markers provide valuable diagnostic insights, biological overlap among subtypes results in a lack of sensitivity and specificity, hindering accurate differentiation.

In the exploration of biomarkers for LUAD, bulk RNA sequencing (RNA-seq) remains a fundamental approach for constructing prognostic and diagnostic signatures. It provides strong statistical power in large-scale cohorts such as The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO), and facilitates correlation analyses with clinical outcomes. Using bulk RNA-seq data, Si *et al.* identified a cancer-associated fibroblast related metabolic and immune signature capable of stratifying patient survival (12), while Zhu *et al.* constructed a six-gene prognostic model that was successfully validated across multiple datasets (13). However, bulk RNA-seq cannot distinguish cell type-specific transcriptional signals, making single-cell RNA sequencing (scRNA-seq) an important complementary strategy for uncovering tumor heterogeneity and immune microenvironment characteristics. By integrating bulk and single-cell transcriptomic data, researchers have identified malignancy-associated (14) and immune microenvironment-related long non-coding RNA (lncRNA) signatures (15), which not only improve biological interpretability but also enhance clinical predictive performance. Collectively, these findings indicate that integrated transcriptomic strategies hold significant potential for precision classification and therapeutic guidance in LUAD.

Our study aimed to identify marker genes that distinguish pre-invasive from invasive LUAD. We systematically compared the differences of gene expression

Highlight box

Key findings

- This study identified a three-gene signature (*CD27*, *TIGIT*, *TNFRSF18*) as novel biomarkers for invasive adenocarcinoma (IAC) via integrated single-cell and bulk RNA sequencing.

What is known and what is new?

- Immune checkpoint molecules play a key role in lung adenocarcinoma (LUAD) progression, but specific biomarkers for distinguishing IAC from pre-invasive subtypes remain insufficient.
- This study is the first to integrate single-cell and bulk RNA sequencing to identify *CD27*, *TIGIT*, and *TNFRSF18* as an IAC-specific three-gene signature, providing a theoretical basis and practical support for LUAD diagnosis, treatment, and prognosis evaluation.

What is the implication, and what should change now?

- The three-gene signature paves the way for improved LUAD subtype stratification and personalized immunotherapy.

and immune microenvironmental features of four LUAD subtypes by utilizing bulk RNA-seq and scRNA-seq. By integrating differential expression analysis, functional enrichment analysis, immune microenvironmental characterization, and validation with clinical samples, we identified three novel candidate marker genes—*CD27*, *TIGIT*, and *TNFRSF18*—that effectively distinguish pre-invasive from invasive LUAD and are closely associated with tumor immune dynamics. In the context of LUAD, *CD27* functions as a key co-stimulatory molecule involved in T-cell memory formation and sustained immune responses, and its expression variation may reflect differences in immune effector activity. *TIGIT* is a well-recognized inhibitory immune checkpoint of which its upregulation often indicates T-cell exhaustion and the presence of an immunosuppressive microenvironment. *TNFRSF18*, a member of the tumor necrosis factor (TNF) receptor superfamily, plays an essential role in balancing effector T-cell activation and regulatory T-cell-mediated suppression. This comprehensive multi-omics integration thereby pinpoints key molecular determinants for IAC progression and its immune contexture, providing crucial insights into its molecular underpinnings. We present this article in accordance with the REMARK reporting checklist (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-2025-aw-2503/rc>).

Methods

Patients

This study was a single-center, retrospective analysis conducted at Jinhua People's Hospital. All enrolled cases were pathologically confirmed LUAD patients diagnosed between 2022 and 2023. A total of 12 patients were included, ranging in age from 33 to 62 years, representing four major histological subtypes of LUAD (AIS, MIA, IMA, and IAC), with three cases assigned to each subtype. The cohort consisted of six male and six female patients. All tumor tissues were obtained through video-assisted thoracoscopic surgery (VATS), immediately fixed and processed after resection, and subsequently prepared as pathological sections. All sections were stored at 4 °C to ensure sample integrity and quality. The inclusion criteria for this study were as follows: pathological confirmation of one of the four LUAD subtypes listed above; receipt of thoracoscopic surgical resection at Jinhua People's Hospital; availability of sufficient tumor tissue suitable for pathological sectioning and downstream molecular analyses;

and completeness of basic clinical information. Exclusion criteria included: receipt of neoadjuvant chemotherapy, radiotherapy, targeted therapy, or immunotherapy prior to surgery; insufficient or poor-quality tissue samples that did not meet analytical requirements; and missing essential clinical or pathological data. As the aim of this study was to evaluate the expression patterns of *CD27*, *TIGIT*, and *TNFRSF18* across different LUAD subtypes and to assess their diagnostic performance in distinguishing pre-invasive from invasive lesions, no survival follow-up data were collected, and no survival endpoints or follow-up durations were analyzed. The study was conducted in accordance with the Declaration of Helsinki and its subsequent amendments. The study was approved by the Institutional Review Board of Jinhua People's Hospital (No. IRB-20220012), and all patients provided written informed consent.

Differential expression analysis

DEGs were identified across pathological subtypes (AAH, AIS, MIA, IAC) using the limma package. Gene expression matrices were extracted from the GSE169033 dataset. Following data cleaning and integration with phenotypic information, samples were grouped by pathology type. DEGs were calculated by constructing design matrices using a linear model fitting (lmFit) and comparative analyses and the significance was obtained by Bayesian test (eBayes) statistics. DEGs were defined as those with an absolute log₂ fold change (logFC) >1 or <-1 and an adjusted P value <0.05.

scRNA-seq data processing and integration

For the single-cell dataset GSE189487, initial quality control was performed and low-quality cells were removed based on the following filtering criteria: total number of RNA molecules (nCount_RNA) ≥1,000, number of genes detected (nFeature_RNA) between 200 and 10,000, and percentage of mitochondrial genes (percent.mt) ≤10%. Expression data from all cells were normalized using the NormalizeData function with "LogNormalize" and a scaling factor of 10,000. The top 2,000 highly variable genes were screened based on their mean and dispersion and when scaling these genes, they were The proportion of mitochondrial genes was regressed using the "var.to.regress" option. Subsequently, high variant genes were zero-centred and scaled to unit variance to reduce potential batch effects. To further correct for batch effects, the Harmony method was used to correct for batch origin.

Functional and pathway enrichment analysis

In this study, gene set enrichment analysis (GSEA), a gene functional annotation analysis platform, was used to perform functional enrichment analysis of the input gene list in combination with the Gene Ontology biological process gene set. Firstly, single-cell sequencing expression data were read and gene symbols were mapped to corresponding Entrez IDs. Subsequently, geneList was enriched by GSEA function based on the GO gene set (GOGmt) to obtain significant functional pathways.

Analysis of immune infiltration

TIMER2.0 (<http://timer.cistrome.org/>) is an interactive web-based platform for systematic analysis of immune infiltration in a wide range of malignant tumors. TIMER2.0 employs six advanced algorithms to more rigorously evaluate information on the levels of tumor-infiltrating lymphocytes (TILs) derived from TCGA, thereby obtaining tumor-associated data. We investigated the expression of *CD27*, *TIGIT*, and *TNFRSF18* in LUAD and the relationship with T and B cell expression by gene modules.

Immune cell deconvolution using CIBERSORTx

In this study, we performed a deconvolution analysis of cellular composition using CIBERSORTx. First, we extracted gene expression information as well as sample grouping information from the input data. Subsequently, a signature gene matrix (LM22) containing 22 immune cell types was constructed for deconvolution analysis. For the deconvolution analysis, the raw count matrices were normalized and used as input. Deconvolution was performed using the default parameters of CIBERSORTx, where the number of permutations was set to 1,000. The results of the analysis contained the relative proportions of each immune cell type in each sample.

Immunohistochemistry (IHC) staining

Paraffin-embedded LUAD specimens from Jinhua People's Hospital were used in this study. IHC was used to study the expression of *CD27*, *TIGIT*, and *TNFRSF18* in LUAD subtypes. IHC staining was performed with anti-CD27 antibody (1:200; Daigle Bio, Zhejiang, China, #db13956), anti-TIGIT (1:200; Cell Signaling Technology, Danvers, USA, #99567) and anti-TNFRSF18 antibody (1:200; Daigle Bio, #db15250). The area of positive immunohistochemical

results was quantified using Image J.

Receiver operating characteristic (ROC) curve analysis

The plotting of ROC curves and calculation of AUC values were performed using the Sento Academic online tool (<https://www.xiantao.love/>). The input data consisted of the immunohistochemical positive staining area (%) for *CD27*, *TIGIT*, and *TNFRSF18* across the four histological subtypes (AAH, AIS, MIA, and IAC). ROC curve analysis was used to evaluate the diagnostic performance of each marker in distinguishing among the different subtypes, and the area under the curve (AUC) was used to quantify their predictive accuracy. When dichotomization of marker levels was required, the median value of each marker was used as the cutpoint, rather than any data-driven or pre-specified threshold.

Statistical analyses

Statistical analyses in this study were performed using R (version 4.2.2), GraphPad Prism (version 9), and making the correlation analyses were performed using the Spearman method.

Results

Bulk RNA-seq reveals inter-subtype heterogeneity in LUAD subtypes

The bulk RNA-seq GSE169033 analysis delineated distinct transcriptomic landscapes across the four LUAD subtypes (AAH, AIS, MIA, IAC) compared to normal tissue. We identified respectively 12 (11 upregulated, 1 downregulated), 53 (28 upregulated, 25 downregulated), 83 (39 upregulated, 44 downregulated), and 72 (33 upregulated, 39 downregulated) significantly differentially expressed genes (DEGs) in AAH, AIS, MIA, and IAC (*Figure 1A*). Further analysis of DEG across LUAD subtypes showed that the number of subtype-specific DEGs increased with malignancy, from 6 in AIS and 22 in MIA to 32 in IAC (*Figure 1B*). Notably, four genes (*CCL19*, *CD24*, *CXCL14*, *THY1*) were consistently up-regulated across all subtypes (*Figure 1C*), with expression levels positively correlating with disease progression (*Figure 1C*). However, no gene was consistently down-regulated across all subtypes (*Figure 1D*). Furthermore, direct comparison between subtypes revealed 115 (72 upregulated and 43 downregulated), 9 (2 upregulated and 7 downregulated), 97 (51 upregulated and 46 downregulated), and 308 (162 upregulated and 146 downregulated) DEGs for AAH, AIS,

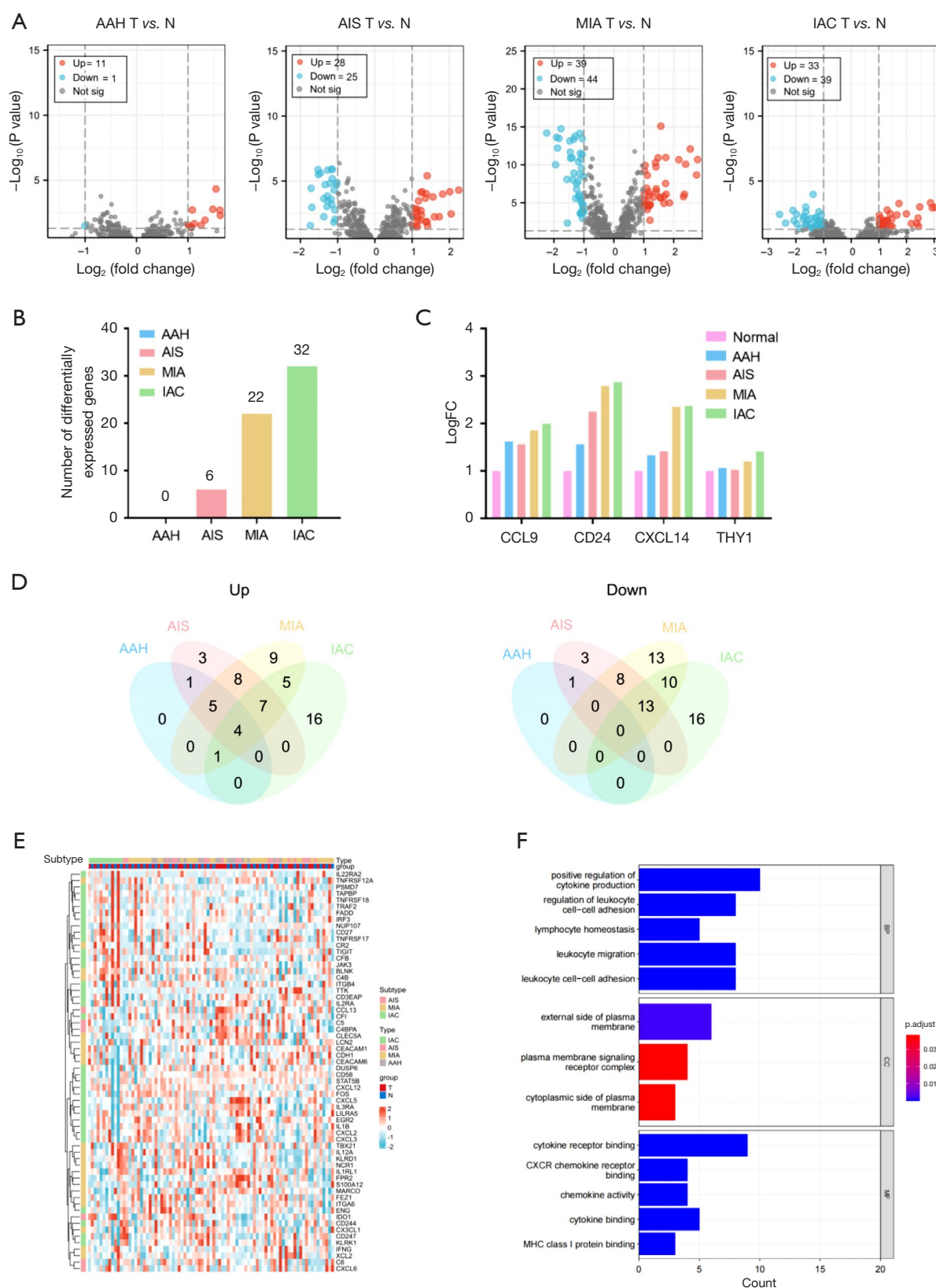


Figure 1 Bulk RNA-seq reveals inter-subtype heterogeneity in LUAD subtypes adenocarcinoma. (A) Volcano plots of DEGs between each

LUAD tumor (T) subtype (AAH, AIS, MIA, IAC) and normal tissues (N). Upregulated, downregulated, and non-significant genes are shown in red, blue, and gray, respectively. Dashed lines indicate the statistical thresholds for significance. (B) Bar graph quantifying the number of subtype-specific DEGs for each LUAD subtype. (C) Expression fold changes (*vs.* normal tissue) of CCL9, CD24, CXCL14 and THY1 across all four subtypes. (D) Venn diagrams showing the overlap of up-regulated and down-regulated DEGs among the four subtypes. (E) The expression of subtype-specific DEGs in normal and LUAD subtype (AAH, AIS, MIA, IAC). Tissue types are indicated by the color bar at the top of the heatmap. (F) GO enriched for DEGs in the IAC subtype. AAH, atypical adenomatous hyperplasia; AIS, adenocarcinoma in situ; BP, biological process; CC, cellular component; DEG, differentially expressed gene; FC, fold change; GO, Gene Ontology; IAC, invasive adenocarcinoma; LUAD, lung adenocarcinoma; MF, molecular function; MIA, minimally invasive adenocarcinoma.

MIA, and IAC, respectively, underscoring significant inter-subtype heterogeneity (Figure S1). Heatmap analysis further revealed unique expression patterns for subtype-specific DEGs, including *CCL13*, *C5*, and *C4BP4* in AIS; *CEACAM6*, *CEACAM1*, and *TRAF2* in MIA; and *IRF3*, *TIGIT*, and *TNFRSF18* in IAC (Figure 1E). Functional enrichment analysis of IAC-specific DEGs highlighted significant alterations in immune-related pathways, including cytokine regulation and leukocyte activation (Figure 1F). These findings suggest significant inter-subtype heterogeneity.

scRNA-seq reveals inter-subtype heterogeneity in LUAD subtypes

Bulk RNA-seq capture the overall characteristics and patterns of gene expression but lack resolution at the single-cell level. To complement bulk RNA-seq data and resolve gene expression at a cellular level, we also analyzed scRNA-seq GSE189487 data on LUAD subtypes (AIS, MIA, IAC). First, uniform manifold approximation and projection (UMAP) clustering identified major cell populations, including endothelial cells, fibroblasts, T-cells, B-cells, plasma cells, neutrophils, and others (Figure 2A). Dot plot analysis illustrated the DEGs and their intensities across these cell types (Figure 2B). Subtype-specific comparative analysis revealed an increasing number of DEGs with disease progression: 27 (14 upregulated and 13 downregulated) in AIS, 55 (28 upregulated and 27 downregulated) in MIA, and 84 (32 upregulated, 52 downregulated) in IAC (Figure 2C). Venn diagram analysis of the three differential gene sets showed that the IAC possessed the highest number of unique DEGs (Figure 2D). The expression patterns of subtype-specific DEGs were further delineated with heatmaps. The results revealed that genes such as *WFDC2*, *TACSTD2*, and *PIGR* were differentially expressed only in AIS; *CD36*, *BTN3A3*, and *ITGA1* were differentially expressed only in MIA; and *CD27*, *TNFRSF18*, and *CXCL2* were differentially expressed exclusively in IAC subtypes

(Figure S2A). Additionally, the composition ratios of cell types across subtypes also showed significant differences (Figure 2E). With advancing malignancy, the proportion of T cells, B cells, and neutrophils gradually increased. Integrated analysis of bulk and single-cell data localized the expression of common deregulated genes (*CCL19*, *CXCL14*, *THY1*) predominantly to fibroblasts (Figure S2B). In the IAC subtype, the proportion of naïve T cells was significantly reduced, while cytotoxic and exhausted CD8⁺ T cells were notably increased, indicating a shift from naïve to effector/exhausted T cell states during tumor progression. Additionally, activated B cells were elevated, accompanied by a decrease in naïve B cells, suggesting a transition from resting to activated B cell states in IAC (Figure S3A). In the IAC (Figure 2F,2G) subtype, communication between T cells and tumor cells with other cells shows significantly enhanced major signaling pathways compared to the AIS (Figure S3B) and MIA (Figure S3C) subtypes. T cell communication mainly involves immune co-stimulatory molecules and inflammation-related pathways, while tumor cells are associated with immune regulatory molecules, chemotactic factors, and pro-inflammatory pathways.

Combined bulk RNA-seq and scRNA-seq reveals CD27, TIGIT, and TNFRSF18 as biomarkers of IAC

Bulk RNA-seq offer a broad perspective on gene expression in cellular populations, whereas scRNA-seq reveals cellular heterogeneity. Integrating both approaches enables a more systematic and accurate understanding of gene expression patterns, facilitating the discovery of novel cell subpopulations, biomarkers, and functional-phenotypic correlations. By combining bulk RNA-seq and scRNA-seq data and visualizing the overlap via Venn plots, we identified three differential genes—*CD27*, *TIGIT*, and *TNFRSF18* that were significantly upregulated in IAC subtype (Figure 3A). UMAP visualization revealed that these genes were predominantly expressed within immune cells, showing

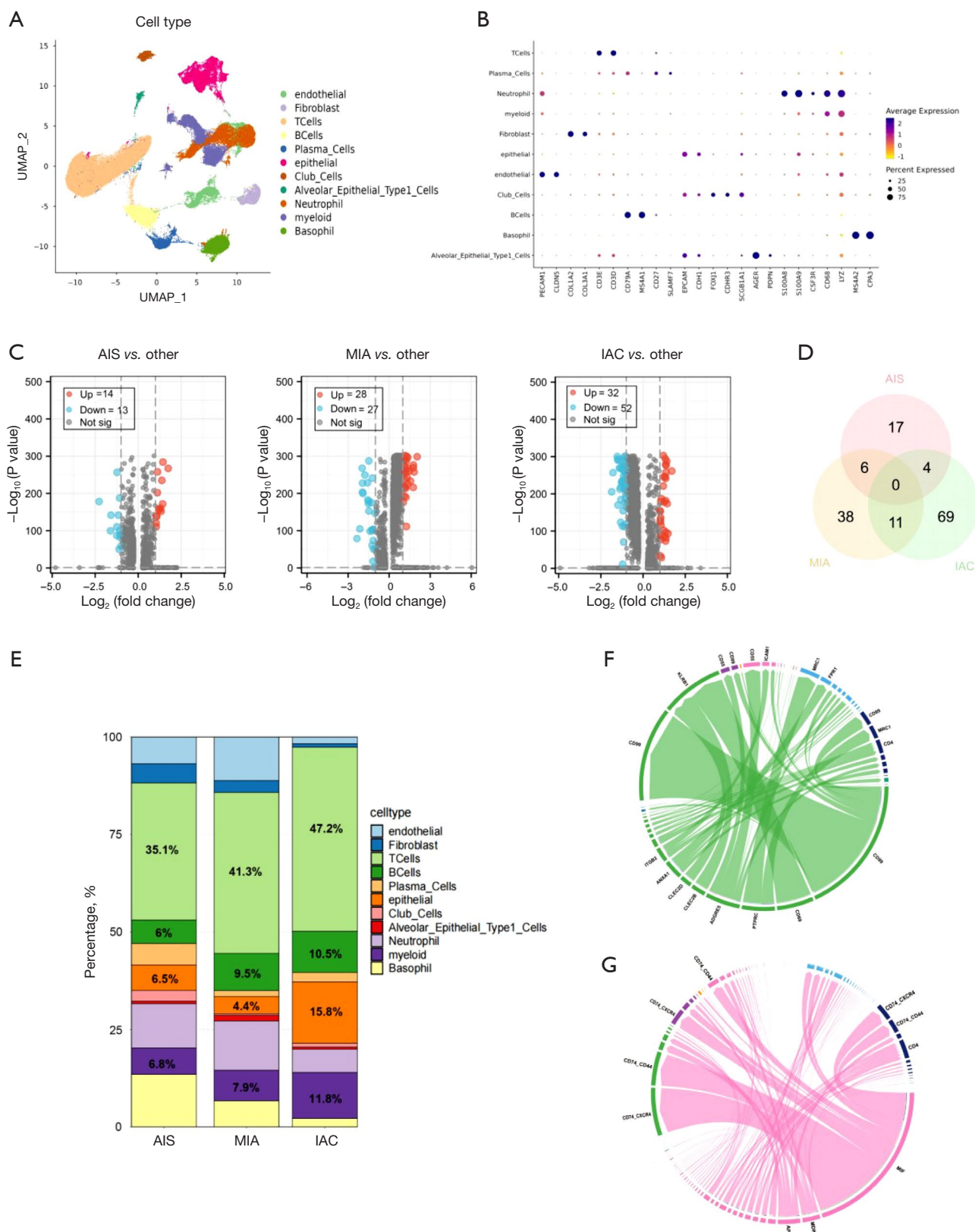
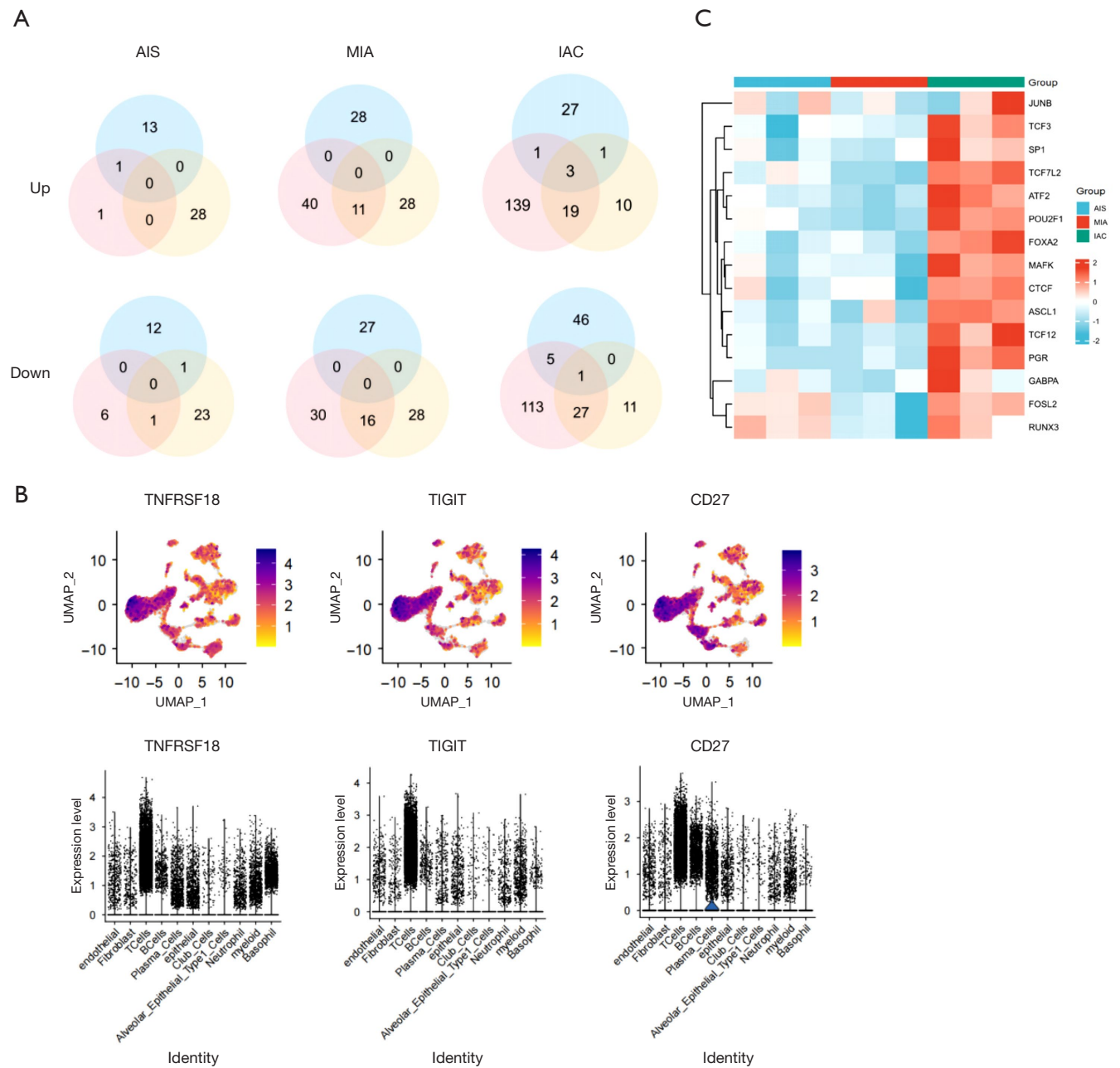


Figure 2 scRNA-seq reveals inter-subtype heterogeneity in LUAD subtypes. (A) UMAP visualization characterizing the major cell types

identified in the LUAD ecosystem. Each dot represents a single cell, and cell types are color-coded. (B) Dot plot depicting cell-type-specific marker genes, where dot size represents the fraction of expressing cells and color intensity indicates the average expression level. (C) Volcano plots of DEGs between each LUAD subtype (AIS, MIA, IAC). Red dots represent upregulated genes, blue dots represent downregulated genes, and gray dots represent genes with no significant changes. The selection criteria are indicated by dashed lines. (D) Venn diagram showing the overlap of DEGs among AIS, MIA and IAC. (E) Bar chart quantifying the proportion abundance of each major cell type across LUAD subtypes. (F,G) Chord diagrams illustrate cell-cell communication networks involving T cells (F) and tumor cells (G) with other cell types in the IAC subtype, where edges represent ligand-receptor interactions and edge width indicates the strength of these interactions. AIS, adenocarcinoma in situ; DEG, differentially expressed gene; IAC, invasive adenocarcinoma; LUAD, lung adenocarcinoma; MIA, minimally invasive adenocarcinoma; UMAP, uniform manifold approximation and projection.



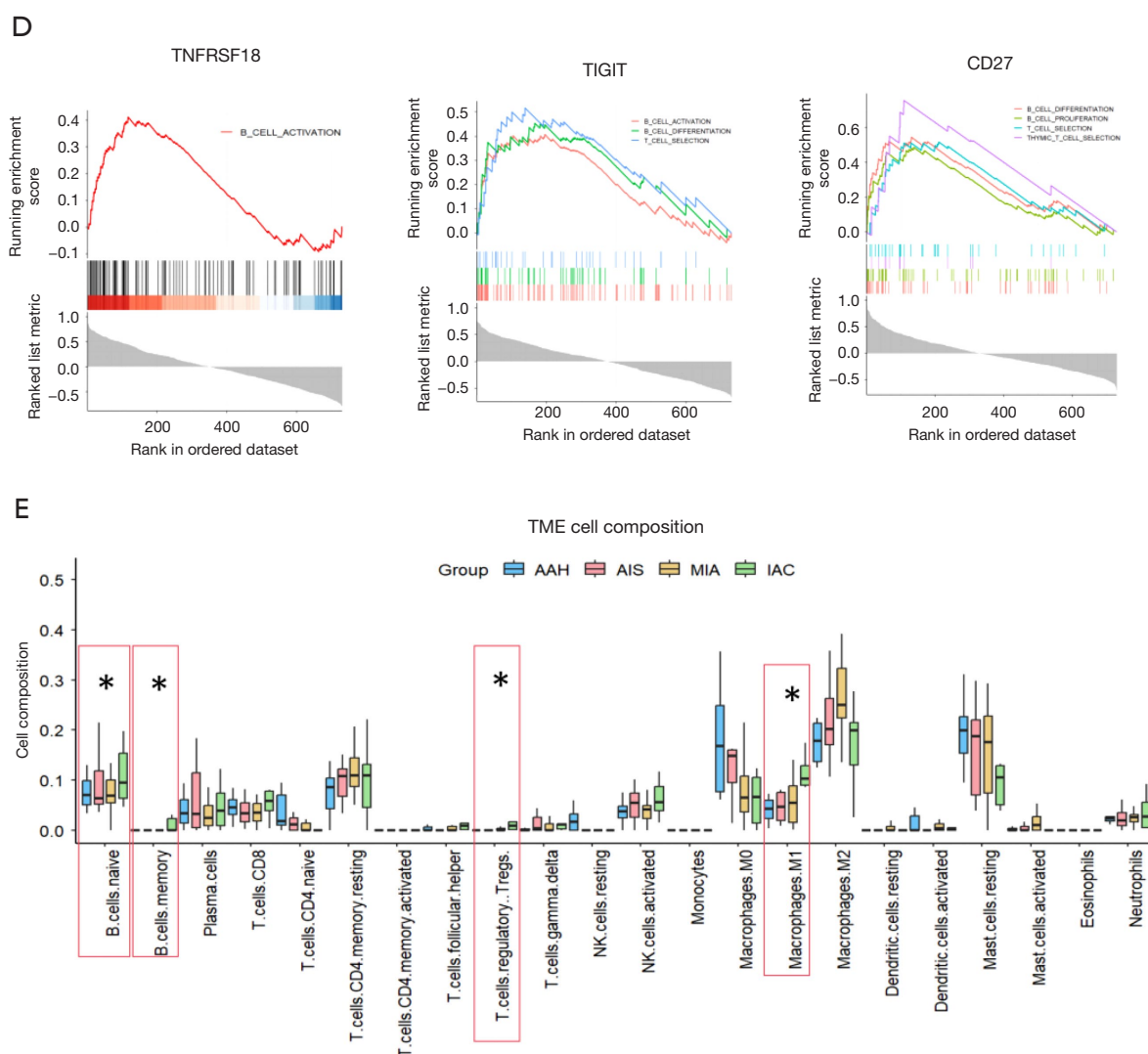


Figure 3 Combined bulk RNA-seq and scRNA-seq reveals CD27, TIGIT, and TNFRSF18 as biomarkers of IAC. (A) Venn diagram illustrating the overlap of subtype-specific DEGs identified through three analytical approaches: single-cell sequencing (blue), transcriptomic comparison of each subtype versus normal tissue (yellow), and inter-subtype transcriptomic comparison (red). (B) Heatmap and violin plot showing the expression levels of three genes across different cell types. (C) Heatmap showing the expression of transcription factors associated with CD27, TIGIT, and TNFRSF18 in the IAC subtype, compared to AIS and MIA subtypes. The colors represent the expression levels, with red indicating upregulation and blue indicating downregulation. (D) GSEA of CD27, TIGIT, and TNFRSF18. (E) Quantification of immune cell composition in the tumor microenvironment of different subtypes using CIBERSORT analysis, presented as a bar chart. The x-axis represents immune cell types, and the y-axis represents the proportion of immune cells. Different colors represent different LUAD subtypes. *, $P < 0.05$. AAH, atypical adenomatous hyperplasia; AIS, adenocarcinoma in situ; DEG, differentially expressed gene; GSEA, gene set enrichment analysis; IAC, invasive adenocarcinoma; LUAD, lung adenocarcinoma; MIA, minimally invasive adenocarcinoma; NK, natural killer; TME, tumor microenvironment.

notable clustering in T-cell subpopulations (Figure 3B). Dot plots of gene expression confirmed markedly elevated levels of all three genes in IAC compared to other subtypes, with *TNFRSF18* showing the most pronounced upregulation in IAC (Figure S4). To investigate the upstream transcriptional regulation of *CD27*, *TIGIT*, and *TNFRSF18*, we first curated a list of putative transcription factors (TFs) targeting these three genes from multiple public regulatory databases, including hTFtarget and JASPAR. We then intersected this predicted TF pool with our single-cell transcriptomic data and retained only those TFs that were significantly upregulated in IAC and positively correlated with the expression of *CD27*, *TIGIT*, or *TNFRSF18* in the T-cell compartment. We identified 15 IAC-upregulated TFs—including AP-1 complex members (*JunB*, *FOSL2*, *ATF2*) and checkpoint regulators (*SP1*, *RUNX3*)—that directly or indirectly regulate *CD27*, *TIGIT*, and *TNFRSF18*, thereby providing a transcriptional regulatory rationale for their coordinated upregulation in invasive disease (Figure 3C). To explore the biological roles of these genes, we performed GSEA. The results indicated that high expression of *CD27*, *TIGIT*, and *TNFRSF18* was significantly associated with T cell activation and immunoregulatory pathways, underscoring their importance in shaping the immune microenvironment of IAC (Figure 3D). We further quantified immune cell composition in the tumor microenvironment across subtypes using CIBERSORT. The analysis demonstrated significantly elevated immune infiltration in IAC, including higher proportions of B cells and regulatory T cells (Tregs) (Figure 3E). To validate the immunological significance of *CD27*, *TIGIT*, and *TNFRSF18*, we analyzed the correlation between these genes and tumor immune infiltration levels using the TIMER2.0 database. The results showed that high expression of these genes was significantly correlated with the infiltration levels of T cells (CD8⁺ T cells, Tregs) and B cells (Figure S5). To sum up, we demonstrated that the integration of bulk RNA-seq and scRNA-seq data identified *CD27*, *TIGIT*, and *TNFRSF18* as biomarkers of IAC and revealed their pivotal role in shaping an immunosuppressive microenvironment.

Clinical validation of *CD27*, *TIGIT*, and *TNFRSF18* as a biomarker for IAC

To validate the expression patterns of *CD27*, *TIGIT*, and *TNFRSF18* across different LUAD subtypes and evaluate their diagnostic potential, we performed IHC on patient

tissue sections and evaluated their utility as IAC biomarkers through quantitative statistics, correlation analysis, and ROC curve analysis. We performed IHC on a cohort of LUAD patient tissues encompassing AAH, AIS, MIA, and IAC subtypes. IHC analysis revealed markedly stronger staining intensity for all three proteins in IAC compared to the pre-invasive subtypes (Figure 4A). Quantitative assessment further confirmed that the positive staining areas of *CD27*, *TIGIT*, and *TNFRSF18* were significantly larger in IAC samples ($P < 0.001$; Figure 4B). Moreover, correlation analysis demonstrated a strong positive relationship between mRNA expression levels and protein levels of *CD27*, *TIGIT*, and *TNFRSF18* (mRNA: $R = 0.79$ for *CD27*, 0.80 for *TIGIT*, and 0.90 for *TNFRSF18*; all $P < 0.001$; Figure 4C). We next constructed a logistic regression model based on the IHC staining areas incorporating the three markers: $\text{score} = 1.0204 + 0.0142 \times \text{TNFRSF18} + 0.0057 \times \text{TIGIT} + 0.0128 \times \text{CD27}$ to distinguish IAC from non-IAC cases. ROC curve analysis demonstrated outstanding diagnostic performance, with individual AUC values of 0.920 (*CD27*), 0.872 (*TIGIT*), and 0.930 (*TNFRSF18*). Notably, the combination of all three genes achieved an exceptional AUC of 0.988 , underscoring their collective power as a diagnostic biomarker for IAC (Figure 4D). High expression of *CD27*, *TIGIT*, and *TNFRSF18* was associated with improved overall survival (Figure S6A). *CD27* expression was significantly higher in immune-active samples, correlating with immune activation, and was higher in responders to anti-PD-1/PD-L1 therapy, suggesting better survival outcomes (Figure S6B). Similarly, *TIGIT* expression correlated with immune activation and treatment response (Figure S6C). Further drug association analysis revealed that high *CD27* expression was linked to synergistic effects with multiple chemotherapy and targeted drugs (such as docetaxel, erlotinib, gefitinib, and bleomycin), while being associated with resistance to certain drugs (such as BMS-754807, AZD8911, and afatinib) (Figure S6D). Similarly, high *TIGIT* expression was correlated with synergistic effects with several drugs (such as SB505124, trichostatin A, vismodegib, and temozolomide), but showed resistance patterns with certain drugs (such as BMS-754807, linsitinib, and AZD6094) (Figure S6D).

Discussion

Combined transcriptome and single-cell transcriptome sequencing offer significant advantages in identifying key biomarkers for LUAD subtypes. Although traditional

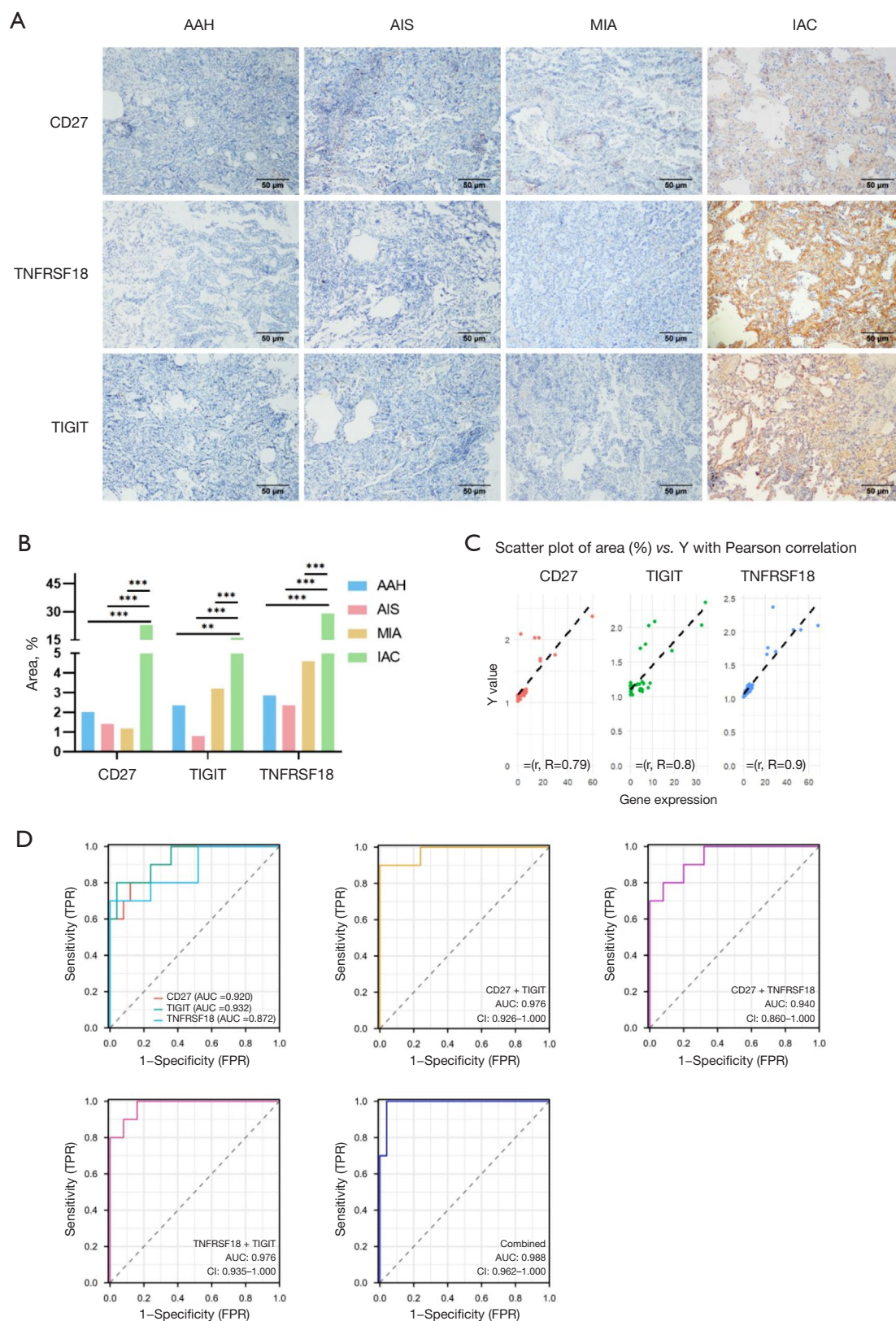


Figure 4 Clinical validation of CD27, TIGIT, and TNFRSF18 as a biomarker for IAC. (A) IHC images showing the protein expression

of CD27, TIGIT, and TNFRSF18 across LUAD subtypes (AAH, AIS, MIA, IAC). Scale bars represent 50 μm . (B) Quantification of the IHC positive staining area for each marker. **, $P < 0.01$; ***, $P < 0.001$. (C) Correlation analysis between mRNA expression levels and IHC staining area ($R = 0.79, 0.80, \text{ and } 0.90$, respectively, $P < 0.001$). (D) ROC curves evaluating the diagnostic efficacy of individual and combined markers for distinguishing IAC from non-IAC (AAH, AIS, MIA) lesions. The x-axis represents 1-Specificity (FPR), and the y-axis represents Sensitivity (TPR). AAH, atypical adenomatous hyperplasia; AIS, adenocarcinoma in situ; AUC, area under the curve; CI, confidence interval; FPR, false positive rate; IAC, invasive adenocarcinoma; IHC, Immunohistochemistry; LUAD, lung adenocarcinoma; MIA, minimally invasive adenocarcinoma; ROC, receiver operating characteristic; TPR, true positive rate.

techniques, such as gene mutation analysis, have had some success in marker screening, they are often restricted to specific biological features, which limits their ability to comprehensively reveal the tumor's molecular mechanisms and the dynamics of the microenvironment. For instance, gene mutation analysis confirmed the high-frequency occurrence of ALK rearrangements and KRAS mutations in IAC, indicating a significant correlation with tumor aggressiveness (16). However, these methods are primarily based on population average responses and fail to capture intercellular heterogeneity. Single-cell sequencing alone cannot provide a global gene expression signature or systematic differential analysis, which limits a comprehensive understanding of molecular mechanisms across cell types and reduces the reliability and generalizability of marker screening. Therefore, this study integrated transcriptome and single-cell sequencing results to construct a multi-level molecular signature profile, aiming to systematically address tumor heterogeneity, microenvironment characteristics, and their interaction mechanisms, ultimately providing new biomarkers and potential targets for accurate diagnosis and treatment.

In this study, based on the transcriptome and single-cell transcriptome analyses, we revealed that IAC exhibited the most DEGs and the largest increase in immune cell proportions, suggesting significant alterations in both immune cell composition and gene expression patterns in the tumor microenvironment with increased malignancy of LUAD. The highest number of DEGs reflected dramatic changes in the expression of genes associated with the biological behaviors of tumor cells, including up-regulation of genes related to malignant features such as cell proliferation (*FOS*, *STAT5B*, *IL1B*), invasion, and metastasis (*ENG*, *CXCL12*), and down-regulation of genes related to the function of normal cells. Meanwhile, the proportion of immune cells, such as T cells, B cells, and plasma cells, increased the most, indicating that the tumor microenvironment shifted from an immune surveillance state to an inflammatory state characterized by immune

escape or pro-tumor growth (17). These combined results suggest that the interaction between the immune system and tumor cells has been altered. Tumor cells may recruit and reprogram immune cells through the release of specific signals, such as the CXCL12/CXCR4 axis (18), which attracts Tregs and myeloid-derived suppressor cells (MDSCs); CCL5/CCR5 (19), which mediates Treg recruitment; and the immunosuppressive cytokine IL-10 (20), which suppresses dendritic cell function and promotes Treg polarization. Collectively, these signals shift immune cells from an anti-tumor to a pro-tumor state, thereby driving the malignant progression of LUAD. We also found significant upregulation of genes, such as *CD27*, *TIGIT*, and *TNFRSF18* in IAC. They were highly enriched in immune-regulation-related pathways, which suggests that these genes may play a crucial role in regulating the tumor microenvironment in IAC. These complex intercellular communication mechanisms may be further amplified by the synergistic effects of *CD27*, *TIGIT*, and *TNFRSF18*, driving immune escape and invasive features of IAC.

Existing studies have identified that β -1,4-galactosyltransferase 1 (*B4GALT1*), *ALK* and *ROS1* gene fusions, *EGFR* mutations, and *TP53* mutations are relevant to the diagnosis of IAC subtypes, but the utility of these biomarkers for IAC subtyping is constrained by practical and biological factors (21-25). *B4GALT1* shows promise for the early diagnosis of LUAD. However, its variable expression and functional role exhibit considerable heterogeneity among patients, hindering its reliability as a universal biomarker (26). *ALK* and *ROS1* gene fusions, while important, require sophisticated detection methods [e.g., fluorescence in situ hybridization/next-generation sequencing (FISH/NGS)] that are expensive and technically demanding, restricting their accessibility (27). Although *EGFR* mutations are frequent in adenocarcinoma, their heterogeneous prevalence and dynamic nature during IAC progression and treatment affect their consistency as biomarkers (28,29). *TP53* mutations, however, lack the subtype specificity needed for differential diagnosis

(30,31). Our finding that a combination of *TIGIT*, *CD27*, and *TNFRSF18* provides superior diagnostic performance underscores the limitation of relying on single-gene alterations and highlights the necessity of a multi-dimensional immune context-based approach for IAC subtyping. Unlike *B4GALT1* (heterogeneous expression), *EGFR/TP53* (lack of specificity/dynamics), or *ALK/ROS1* (technical barriers), this panel captures complementary aspects of T-cell function and co-stimulation within the tumor immune microenvironment. This allows it to reflect the biological characteristics of IAC subtypes more stably and comprehensively from multiple immune angles, rather than being dependent on a single, potentially variable genetic event. Therefore, this combinatorial strategy effectively addresses the major challenges of heterogeneity, dynamic change, and technical accessibility. While further validation in larger cohorts is needed, this approach presents a promising direction for developing more reliable and accessible diagnostic tools in precision oncology.

Our result revealed significant upregulation of *CD27*, *TIGIT*, and *TNFRSF18* in IAC subtypes of LUAD. These molecules were predominantly expressed in immune cells (particularly T cells), indicating their close involvement in local immune regulation. Some studies indicate that *CD27* promotes T cell proliferation and effector function by binding to its ligand *CD70*. *CD27* agonistic antibodies demonstrate significant anti-tumor activity in breast and colon cancer models and enhance efficacy when combined with *PD-1/PD-L1* inhibitors (32,33). *TIGIT* inhibits T cell and natural killer (NK) cell activity by binding to *CD155* (34,35). Blocking *TIGIT* restores effector T-cell and NK-cell functions in non-small cell lung cancer, colorectal, pancreatic and triple-negative breast cancers and synergistically enhances anti-tumor effects with dual blockade of *PD-1* (36-39). *TNFRSF18* activation signaling suppresses the immunosuppressive effects of regulatory T-cells and enhances the anti-tumor function of effector T-cells. *GITR* agonists have been shown in studies of melanoma and renal carcinoma to optimize the tumor immune microenvironment (40,41). *CD27* and *TNFRSF18* as co-stimulatory molecules that enhance T-cell activation and effector function, whereas *TIGIT* acts as an inhibitory checkpoint associated with T-cell exhaustion and immunosuppression (42-45). Our integrated transcriptomic analysis reveals that these molecules are specifically and coordinately upregulated in IAC, suggesting a balanced yet dysregulated immune landscape where both activating and suppressive signals are heightened (46-50). Although

CD27, *TIGIT*, and *TNFRSF18* are recognized players in tumor immunology, our study provides a novel contribution by elucidating their collective and synergistic evolution throughout the invasive spectrum of LUAD. Leveraging the integration of single-cell and bulk transcriptomics, we established a multi-gene panel capable of precisely distinguishing IAC from pre-invasive lesions. This feature not only enables high-precision discrimination between IAC and precursor lesions (AUC =0.988) but also mechanistically characterizes the imbalance of co-stimulatory and inhibitory signals within the immune microenvironment, explaining the more aggressive biological behavior of IAC. This biomarker combination offers a novel tool for the preoperative precise diagnosis of IAC and provides candidate predictive markers for individualized immunotherapy strategies targeting specific immune checkpoints, such as *TIGIT*.

In our drug association analysis, high *CD27* and *TNFRSF18* expression was correlated with sensitivity to multiple chemotherapeutic and targeted agents but also showed resistance associations with certain compounds, suggesting that co-stimulatory signaling may broadly influence drug response patterns. *TIGIT* expression demonstrated both sensitivity and resistance correlations, in line with its inhibitory role and potential modulation of immune suppression. *CD27*, a *TNFRSF* co-stimulatory receptor, enhances T-cell activation and effector functions, and preclinical studies have shown that agonistic targeting of *CD27* can promote antitumor immunity, although monotherapy clinical responses remain limited, indicating potential resistance mechanisms that may require combination strategies with existing immunotherapies (51). *TNFRSF18* is another co-stimulatory molecule under investigation; agonists targeting *GITR* can augment effector T-cell activity and attenuate regulatory T-cell suppression and have shown synergistic effects when combined with *PD-1* blockade in preclinical models (52). In contrast, *TIGIT* is a co-inhibitory checkpoint that mediates T-cell exhaustion and immune evasion across cancer types; inhibition of *TIGIT* has been explored in combination with *PD-1/PD-L1* inhibitors to overcome adaptive resistance, although some clinical settings have shown limited efficacy or failures, emphasizing the complexity of checkpoint co-regulation and resistance development (45). These findings highlight that rationally combining co-stimulatory activation and inhibitory checkpoint blockade may enhance synergistic antitumor effects while mitigating resistance.

Although these immunomodulatory molecules have

shown efficacy in various tumors, the mechanisms in LUAD remain unclear. Future studies should expand the sample size and incorporate functional validation experiments to explore the specific mechanisms of *CD27*, *TIGIT*, and *TNFRSF18* in regulating the immune microenvironment of LUAD. Additionally, the potential value of these genes in LUAD treatment can be assessed through gene editing or drug intervention. Recent studies have shown that immune checkpoint inhibitors targeting *TIGIT* show promising clinical results in non-small cell lung cancer treatment (53). Furthermore, the spatial heterogeneity of the tumor immune microenvironment across different subtypes of LUAD can be further explored by combining technologies such as spatial transcriptomics.

Conclusions

This study identifies a three-gene signature (*CD27*, *TIGIT*, and *TNFRSF18*) that can accurately distinguish invasive from pre-invasive LUAD by capturing the immune checkpoint disequilibrium of IAC and provides a clinically actionable biomarker panel for preoperative diagnosis and personalized immunotherapy.

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Footnote

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