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Original Article

The upregulation of TGM2 is associated with poor prognosis and the shaping of the inflammatory tumor microenvironment in lung squamous cell carcinoma

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Abstract: Tissue transglutaminase (TGM2) is a member of the glutamine transferase superfamily, located within cells and their membranes. When secreted, it catalyzes the cross-linking of extracellular matrix proteins and promotes the formation of extracellular matrix scaffolds. To determine the function of TGM2 in the tumorigenesis of lung squamous cell carcinoma (LUSC), we conducted a comprehensive bioinformatics analysis of TGM2. Our findings indicate that high expression of TGM2 in LUSC was associated with a poorer prognosis. Additionally, we found that high expression of TGM2 is closely related to tumor-promoting inflammation and may increase sensitivity to immunotherapy. We further confirmed the cancer-promoting effect of TGM2 in LUSC through in vitro overexpression and knockdown experiments and showed that TGM2 primarily affects cancer cell proliferation, apoptosis, and invasion. In summary, TGM2 promoted the progression of LUSC, and targeting TGM2 is expected to become a new therapeutic approach for LUSC treatment.

Keywords: TGM2, lung squamous cell carcinoma, inflammatory tumor microenvironment, metastasis

Introduction

Lung cancer is one of the most common cancers worldwide and exhibits the highest mortality rates [1]. Non-small cell lung cancer NSCLC accounts for 85% of all lung cancers, with about 30% of these being lung squamous cell carcinoma (LUSC) [2]. Patients with early-stage lung cancer can achieve radical treatment through surgery [3]. However, most lung cancer patients have already developed metastasis by the time of diagnosis, and their 5-year survival rate is below 20% [4]. Compared to lung adenocarcinoma (LUAD), LUSC exhibits more severe genomic abnormalities, but no effective treatment targets are available [5]. Thus, LUSC is usually associated with a worse prognosis. Although there is significant progress in immunotherapy in recent years, most LUSC patients are insensitive to it. One important reasons for this is that the tumor microenvironment (TME) of LUSC interferes with the immune response [6]. Therefore, there is an urgent need to develop new biomarkers to guide the treatment of LUSC patients.

TGM2 is a multifunctional protein that plays an indispensable role in tumor autophagy, metastasis, drug resistance, and tumor-promoting inflammation [7]. The increase in TGM2 expression is closely related to the drug resistance and metastatic phenotype of most tumors [8-13], mainly due to its inhibitory effect on tumor autophagy [13, 14]. The detachment of epithelial-derived tumors from the primary site and the acquisition of mesenchymal phenotype represent key steps in tumor metastasis. TGM2 is an important promoter in this process, as it can activate FAK, Akt, and NF-κB signals, as well as enhance the EMT process of cancer

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cells, thereby promoting metastasis [15]. After the knockdown of TGM2, the TGF-β loses its ability to induce EMT in breast epithelial cells [16]. Additionally, TGM2 is involved in the regulation of innate and adaptive immune processes, including inhibiting the proliferation and activation of T cells and B cells, and promoting the infiltration of myeloid suppressor cells [7]. In mice with TGM2 deficiency, T cells activation is suppressed, and the proportion of myeloid-derived suppressor cells (MDSC) and tumor-associated macrophage (TAM), which exert immunosuppressive effects, is significantly reduced. This greatly enhances the anti-tumor effect of immune cells in the tumor microenvironment (TME) [17]. Therefore, TGM2 is also considered a novel immune regulatory target. Promoting tumor inflammation is one of the hallmarks of cancer, and it is highly correlated with tumor progression, angiogenesis, and metastasis [18], TGM2 is considered a key factor in this process [19]. Small molecule drugs targeting TGM2 have also shown promise [20]. However, the role of TGM2 in LUSC has not been clearly defined.

In this study, we employed bioinformatics to elucidate the role of TGM2 in LUSC, encompassing its expression pattern, function, and potential mechanisms affecting tumor progression. We further investigated the influence of TGM2 in immunotherapy and chemotherapy, providing rationale for the clinical application of TGM2. Finally, through overexpression and knockdown experiments, we validated its potential function in LUSC. Collectively, our study offers a new research perspective and is anticipated to contribute to the development of new strategies for individualized treatment of LUSC patients.

Materials and methods

Data collection

The expression data of TGM2 in pan-cancers were obtained from the TIMER database [21]. mRNA expression data of TGM2 in LUSC, along with corresponding clinical information, were acquired from TGCA. Additionally, GEPIA was utilized to validate TGM2 expression and to downloaded survival analysis data for LUSC, including overall survival (OS) and disease-free survival [22].

Protein-protein interaction network

TGM2 was input into the STRING database [23], and the top 20 genes based on correlation coefficient were retrieved to construct a protein-protein interaction network (PPI).

GO and KEGG enrichment analysis

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses map genes to different biological functions or pathways, offering a framework for bioinformatics research. The GO/KEGG enrichment analysis in this study was performed using the "clusterProfiler" R package [24].

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) is a computational method used to analyze the overall trend of gene expression data to determine the enrichment of related pathways. It can capture subtle but synergistic functional pathways, particularly gene sets with small differential multiples. Through GSEA software, we analyzed the significant pathways associated with TGM2 from a holistic perspective [25].

Immune microenvironment analysis

Immune-associated molecules were obtained from TISIDB [26], including chemokine, immunostimulator, immunoinhibitor, MHC molecule, and receptor, which reflect the state of the tumor microenvironment. TISIDB database also provides correlation analysis between the expression of a single gene and multiple immune cells. Additionally, we further validated the infiltration degree, immune score, matrix score, and microenvironment score of 64 immune cells using the xCell algorithm [27].

Prediction of immunotherapy response

The characteristics of immune infiltration suggest that the immune phenotype and the mechanism of tumor immune escape are dependent on the tumor genotype. The Immunophenolscore (IPS) serves as a robust predictive indicator for evaluating the efficacy of anti-CTLA-4 and anti-PD-1 immunotherapy [28]. We obtained relevant IPS scores from the TCIA database and analyzed the potential response of LUSC patients to immunotherapy. Additionally, the TISMO database provides sequenc-

ing data from mouse models undergoing various immunotherapies [29]. We downloaded relevant data on immunotherapy in lung cancer mouse models and assessed the predictive efficacy of TGM2 in these models.

Prediction of chemotherapy response

Sensitivity analysis of LUSC to different chemotherapy drugs was conducted utilizing the "pRRophetic" package, which contains drug sensitivity experiments from 727 cell lines [30]. We used 'LUNG' as the reference tissue for analysis.

Reagents

RPMI 1640 medium was purchased from Biosharp (Anhui, China), and fetal bovine serum (FBS) was from Tianhang Company (Zhejiang, China). Penicillin-streptomycin was obtained from HYCLONE (Utah, USA). The Annexin V-FITC/PI Apoptosis Detection Kit was procured from Meilunbio Inc (Dalian, China), and Small interference RNA (siRNA) targeting TGM2 was from GenePharma Biotechnology Company (Suzhou, China). Lipofectamine 3000 reagent was purchased from Thermo Fisher Company (Massachusetts, USA). Trizol reagent and reverse Transcription reagent were obtained from Beyotime (Nanjing, China). SYBR Green Master Mix and ECL exposure solution were purchased from Vazyme Biotech Company (Nanjing, China).

Cell culture and transfection

Human lung squamous cell carcinoma cell lines SK-MES-1 and NCI-H226 were purchased from BOHUI BIOTECHNOLOGY Co. Ltd. (Guangzhou, China) and cultured in RPMI 1640 medium containing 10% FBS and 1% penicillin-streptomycin. TGM2-specific siRNAs were transfected into NCI-H226 and SK-MES-1 cells to silence TGM2. The selected sequences of siRNAs were as follows: siTGM2-2: (5'-GCGUCGUGACCAACUACAA-3', 5'-UUGUAGUUGGUCACGACGC-3') and si-TGM2-3: (5'-GGCUGAAGAUCAGCACUAA-3', 5'-UUAGUGCUGAUCUUCAGCC-3'). To overexpress TGM2, pCDNA3.1-TGM2-puro construct was transfected into NCI-H226 and SK-MES-1 cells by electroporation. The primer sequences used for cloning were as follows: pCDNA3.1-TGM2-F: 5'-tttaaaCTTAAGCTTGGTACCgccaccATGGCCGAGGAGCTGG-3' and pCDNA3.1-TGM2-R: 5'-TTTAAACGGGCCCTCTAGACTCGAGTTAGGCGGGGCCAATGATGACA-3'.

Real-time fluorescence quantitative PCR

Total RNA was extracted from tissues using Trizol reagent (TIANGEN, Beijing, China). The relative expression of TGM2 was determined using the 2-ΔΔCt formula, with GAPDH as an internal control. The following primer sequences were used for amplification: TGM2, 5'-TCCACTGGCGTCTTCACC-3' (forward) and 5'-GGCAGAGATGATGACCCTTTT-3' (reverse); GAPDH, 5'-GGCGAACCACCTGAACAAAC-3' (forward) and 5'-GTGTTGTTGGTGATGTGGGC-3' (reverse).

Western blotting

Total proteins from tissue and cells were extracted using RIPA lysis buffer (Beyotime, Nanjing, China), and the protein concentration was determined by BCA protein assay kit (Beyotime, Nanjing, China). Antibodies used in this study were mouse anti-GAPDH (1:1000, Proteintech, Wuhan, China) and rabbit anti-TGM2 (1:1000, Proteintech, Wuhan, China). Standard western blotting protocol was performed, and ECL Chemiluminescence System was employed to assess the binding of antibodies. Data analysis was conducted using the Image J software.

Cell proliferation assay

Cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8). Briefly, a cell suspension was seeded into a 96-well plate at a concentration of 1000 cells per well. The plates were then cultured in a 5% CO_2 atmosphere at 37°C. Prior to measuring the absorbance, 10 µl of CCK-8 solution was added to each well. After a 2-hour incubation, absorbance was measured at 450 nm.

Cell apoptosis assay

Briefly, cells were digested and collected, and then 1× binding buffer was added to the cells to obtain cell suspension at a concentration of 1×10^6. A 100 µL aliquot of cell suspension was added to a new tube along with 5 ul of Annexin VFITC and 5 µL of PI. The mixture was gently mixed and incubated at room temperature in

the dark for 15 minutes. Subsequently, 400 μL of 1× Binding Buffer was added to each tube, and the samples were analyzed using a Beckman Coulter flow cytometer (California, USA).

Wound healing assay

Briefly, transfected cells were culture in a 6-well plate to a confluency of 90%. Then, a scratch was produced with a 10 uL gun tip, followed by rinsing once with PBS. Photographs were taken at 0 and 24 hours to monitor the scratch closure. The magnification of the field of view was 10×.

Transwell invasion assay

An 8-μm pore size transwell apparatus (Corning, NY, USA) coated with matrigel was used to assess cell invasion. Transfected NCI-H226 and SK-MES-1 cells (5×10^4 cells) suspended at 200 μL of FBS-free DMEM were seeded into the upper chamber, while 600 ul RPMI 1640 containing 10% FBS was added to the lower chamber. After incubation at 5% CO_2, 37°C for 24 hours, the cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. For each chamber, five fields of view were randomly selected under a microscope, images were captured, and the number of cells that migrated through the membrane was calculated.

Immunohistochemical (IHC) staining

LUSC and matched normal lung tissue samples were obtained through surgical resection from the Sun Yat-sen University Cancer Center. A total of 5 pairs of matched samples were collected from patients who had not received any treatment prior to surgery and were diagnosed with LUSC through pathological examination post-surgery. This study was approved by the Sun Yat-sen University Cancer Center ethics committee (B2024-064-01). The LUSC tissue sections underwent dewaxing and rehydration procedures, followed by antigen retrieval. Subsequently, endogenous peroxidase activity was inhibited, and the samples were incubated overnight with the TGM2 primary antibody. The secondary antibody was then incubated for 1 hour. Signal visualization was achieved using 3,3'-diaminobenzidine (DAB) reagent, followed by counterstaining with hematoxylin.

Statistical analysis

Results from each experiment, which were independently performed at least three times, were presented as means ± SD. Statistical analysis was performed using GraphPad Prism 8.3.0 software. Image J software was utilized for Image processing and analysis, including cell counting. Experimental data were expressed as X ± S. Each assay was repeated three times, and pairwise comparisons between groups were made using a t-test. Group comparisons were conducted using univariate analysis of the variance (ANOVA), and pairwise comparisons were made using an LSD t-test. Differences between different groups for continuous variables were detected using the Wilcoxon rank-sum test. A p-value < 0.05 was considered significant.

Results

Expression and generation analysis of TGM2 in LUSC

We initially analyzed the mRNA expression of TGM2 across various cancers and observed significant overexpression in multiple cancer types (**Figure 1A**). In lung tissue, while TGM2 exhibited high expression in both LUAD and LUSC, its expression was higher in normal lung tissue. Subsequently, utilizing data from TCGA, we confirmed significant overexpression of TGM2 in normal lung tissue at both overall and paired samples levels (**Figure 1B**, **1C**). However, in LUSC, high expression of TGM2 was significantly associated with worse overall survival (OS) and disease-free survival (DFS) (**Figure 1D**, **1E**), suggesting a potential tumor-promoting ability of TGM2. Then, we collected tumor tissue and normal lung tissue from 5 pairs of matched LUSC patients from our hospital and performed TGM2 immunohistochemical staining. The results confirmed that TGM2 was higher in normal lung tissue (**Figure 1F**).

PPI and enrichment analysis

We constructed a protein-protein interaction network (**Figure 2A**) using the top 20 genes associated with TGM2. The results of enrichment analysis revealed that these genes primarily function in cell-extracellular matrix the interactions, including cell-matrix junctions, cell-matrix adhesion, and cell adhesion molecules (**Figure 2B**, **2C**). To comprehensively

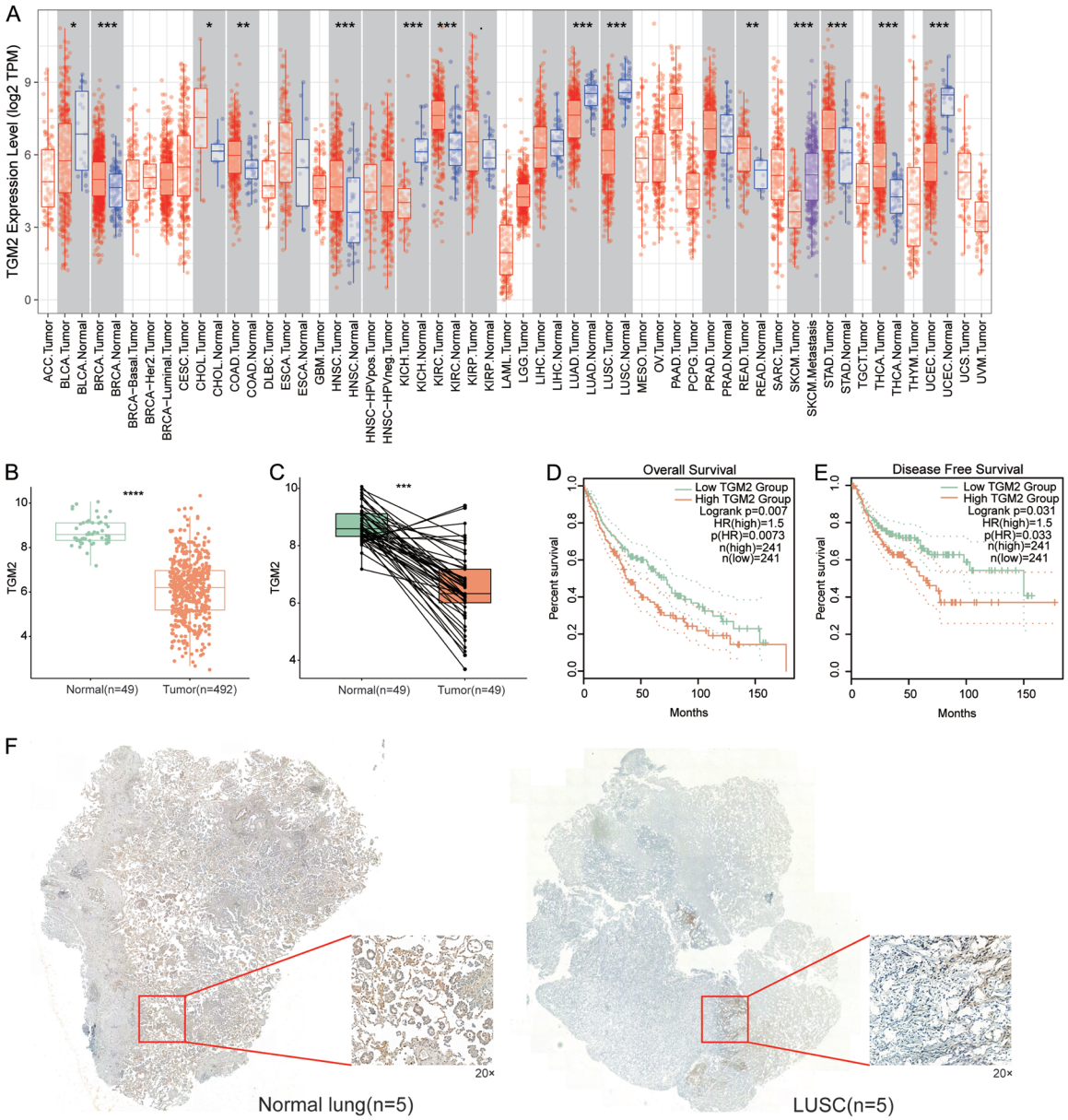
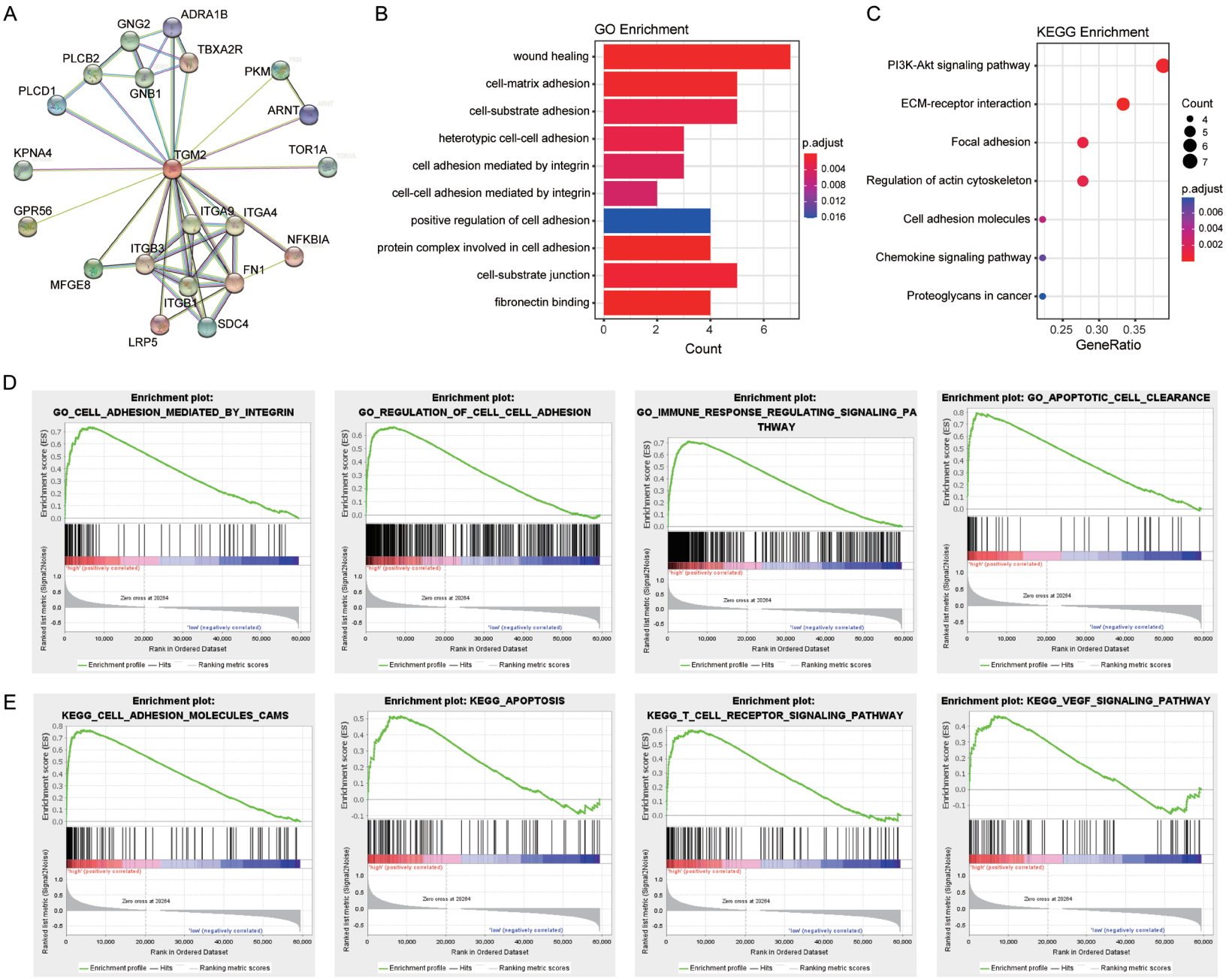


Figure 1. Expression information of TGM2 in Databases. (A) Expression of TGM2 in pan-cancer from the TIMER database. (B) mRNA levels of TGM2 in 492 tumor and 49 normal samples. (C) mRNA level of TGM2 in 49 matched pairs of samples. Survival analysis of TGM2 in (D) overall survival (OS) and (E) disease-free survival (DFS) from the GEPIA database. (F) Immunohistochemical (IHC) staining of 5 matched pairs of LUSC and normal lung tissue. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

explore the function of TGM2, we divided the TCGA-LUSC samples into four equal fractions based on TGM2 expression and performed Gene Set Enrichment Analysis (GSEA) using the top and bottom 25% of samples. The pathways significantly enriched in the high TGM2 expression group included regulation of cell adhesion, cell apoptosis, activation of immune-related pathways, and the VEGF pathway (**Figure 2D**, **2E**), thereby highlighting the function of TGM2 in cell-extracellular matrix the interactions.

Construction and validation of the nomogram

To determine whether TGM2 is an independent risk factor affecting the prognosis of LUSC, we conducted Cox regression analysis on multiple clinically relevant factors. The results of univariate and multivariate Cox regression analysis revealed that TGM2 was indeed an independent risk factor for the prognosis of LUSC (HR=1.19, 95% CI: 1.076-1.317; HR=1.266, 95% CI: 1.128-1.420, **Figure 3A**, **3B**). A nomo-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ADRA1B
GNG2
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ITGA9
ITGA4
NFKBIA
ITGB3
FN1
MFGE8
ITGB1
SDC4
LRP5
B
GO Enrichment
wound healing
cell-matrix adhesion
cell-substrate adhesion
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cell-cell adhesion mediated by integrin
positive regulation of cell adhesion
protein complex involved in cell adhesion
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PI3K-Akt signaling pathway
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Regulation of actin cytoskeleton
Cell adhesion molecules
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Enrichment plot:
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GO_IMMUNE_RESPONSE_REGULATING_SIGNALING_PATHWAY
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Enrichment plot: KEGG_APOPTOSIS
Enrichment plot:
KEGG_T_CELL_RECEPTOR_SIGNALING_PATHWAY
Enrichment plot: KEGG_VEGF_SIGNALING_PATHWAY
Enrichment score (ES)
Ranked list metric (Signal2Noise)
'high' (positively correlated)
'low' (negatively correlated)
Zero cross at 20264
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

Figure 2. Exploration of TGM2 function. (A) Construction of protein-protein network. Gene Ontology (GO) (B) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (C) enrichment analysis. GO (D) and KEGG (E) enrichment analysis from Gene Set Enrichment Analysis (GSEA) of TGM2 high expression group.

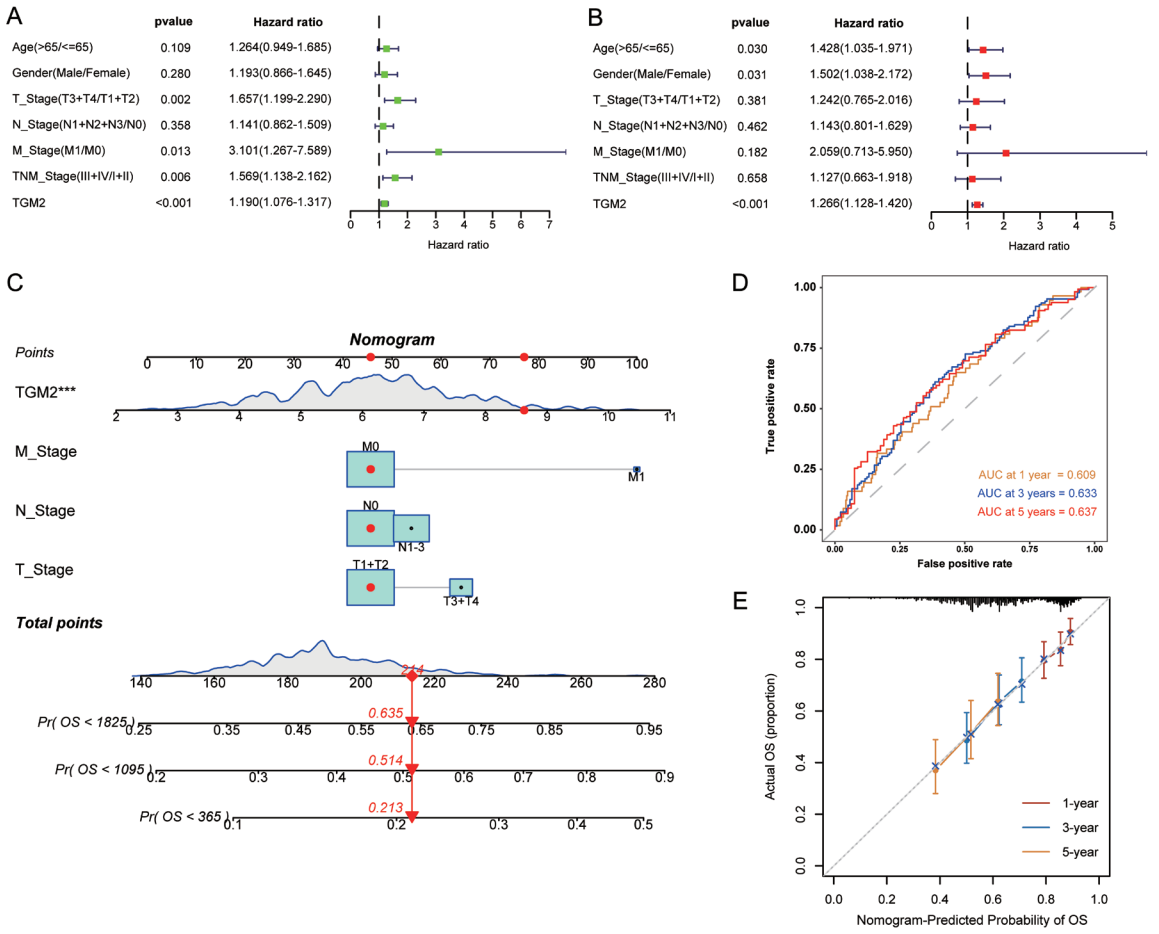

Figure 3. Construction of the nomogram to predict the prognosis of LUSC. (A) Univariate and (B) multivariate Cox regression analysis of TGM2 and other clinical information. (C) Nomogram for predicting 1-, 3-, and 5-year survival probability. (D) Time-dependent receiver operating characteristic (ROC) analysis of the nomogram. (E) Calibration curves for evaluating accuracy. ***P < 0.001.

gram was then constructed based on the expression level of TGM2 and clinical information (**Figure 3C**), with the receiver operating characteristic curve area for predicting 1-, 3-, and 5-year OS rates being 0.609, 0.633, and 0.715, respectively (**Figure 3D**). The calibration curve indicated a high consistency between the predicted and actual results of the nomogram (**Figure 3E**).

Analysis of immune-associated molecules

Given that TGM2 expression is associated with the activation of immune related pathways, we analyzed the expression differences of various immune molecules across different TGM2 expression groups (**Figure 4A-E**). The majority of immune related molecules exhibited higher levels in the high TGM2 expression group, suggesting a significant correlation between high TGM2 expression and elevated immunogenicity.

TGM2 is associated with tumor-promoting inflammation

We further analyzed the association between TGM2 and important immune cells using the data from the TISIDB database (**Figure 5A**). The infiltration levels of nearly all types of immune

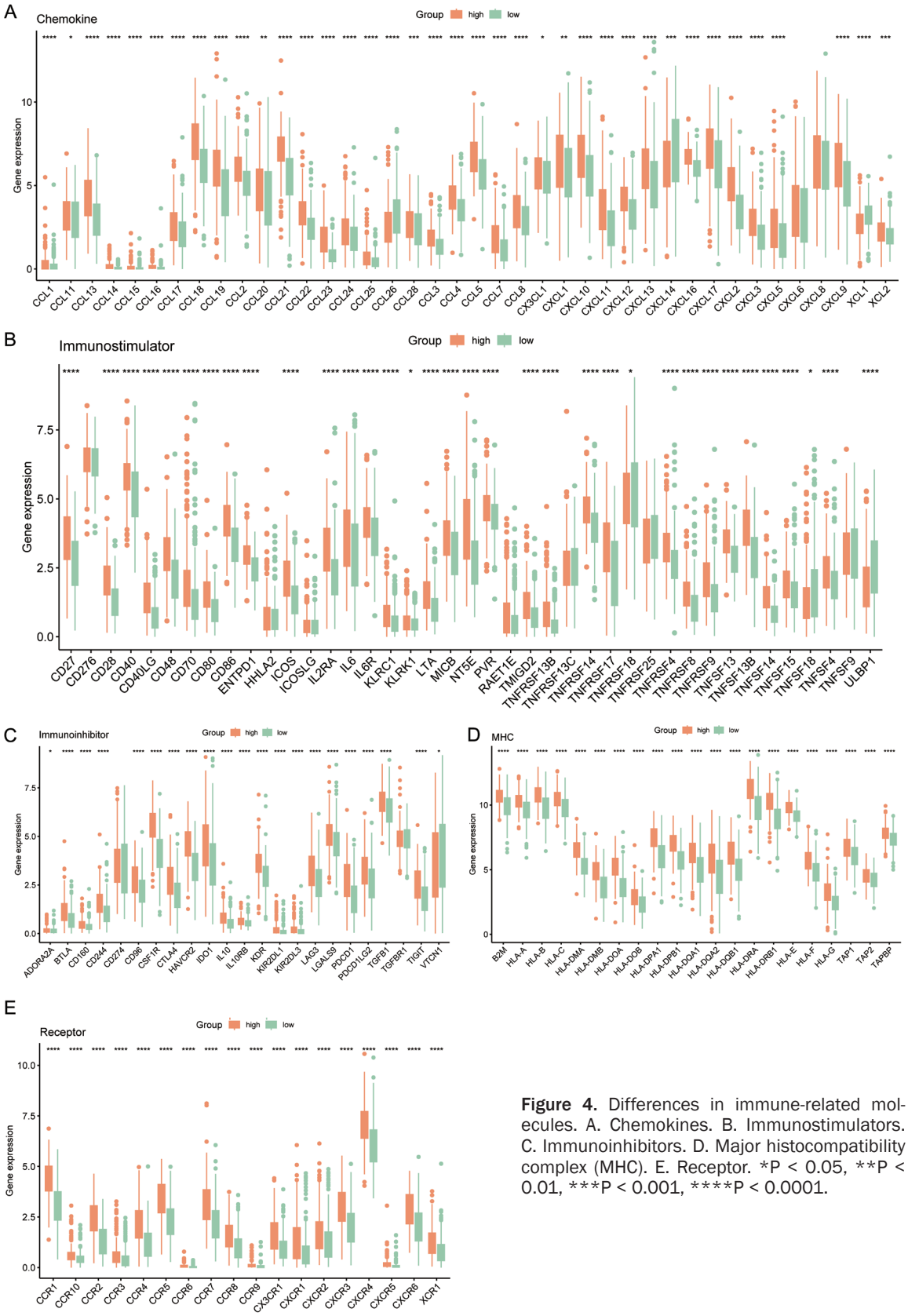

Figure 4. Differences in immune-related molecules. A. Chemokines. B. Immunostimulators. C. Immunoinhibitors. D. Major histocompatibility complex (MHC). E. Receptor. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

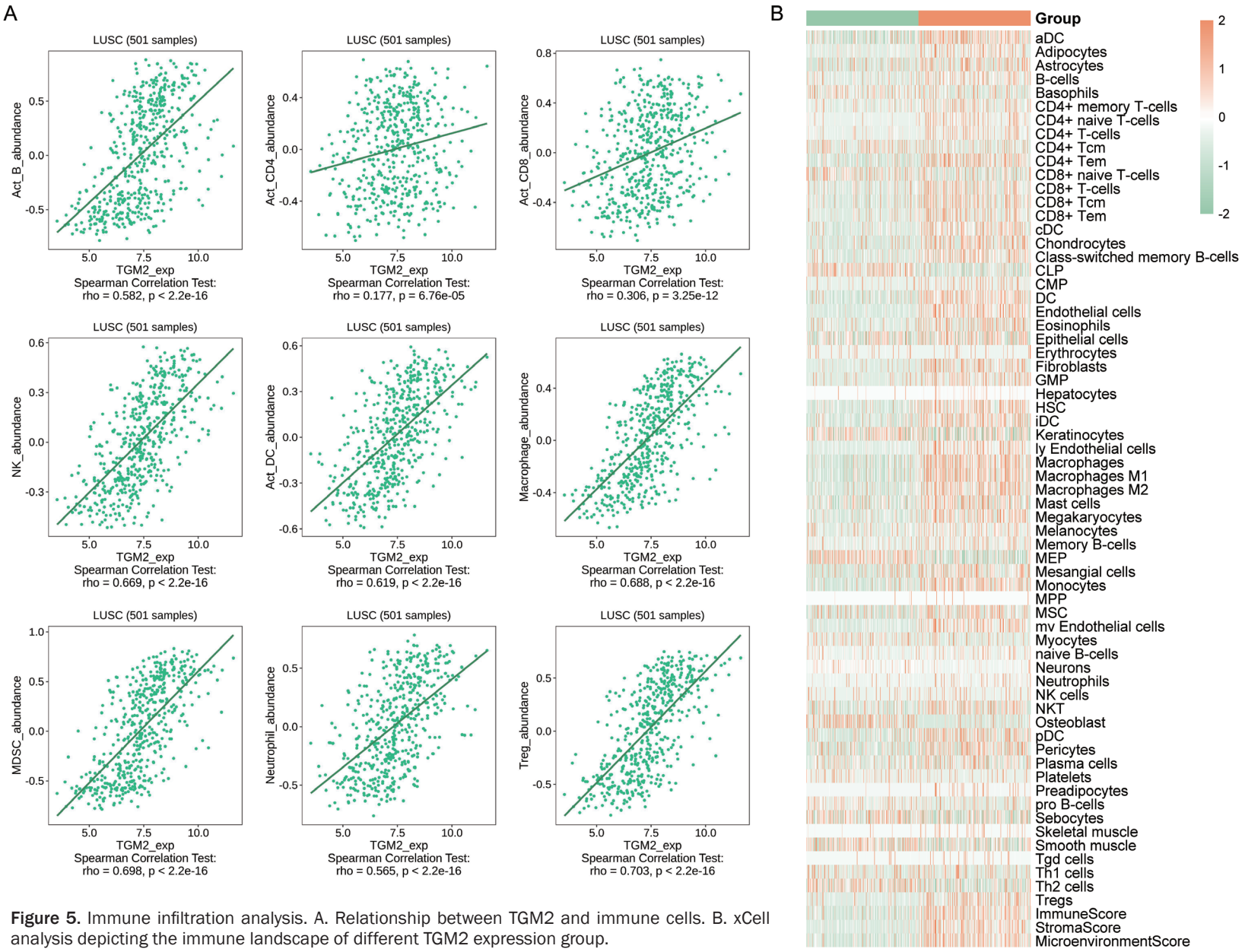

Figure 5. Immune infiltration analysis. A. Relationship between TGM2 and immune cells. B. xCell analysis depicting the immune landscape of different TGM2 expression group.

cells exhibited a significant positive correlation with TGM2 expression. Subsequently, we analyzed the differences of 64 immune cell types across different TGM2 expression groups using the xCell algorithm. Consistently, the majority of immune cells were closely associated with high TGM2 expression (**Figure 5B**). These findings suggest that TGM2 may contribute to the shaping of the inflammatory tumor microenvironment in tumors.

Prediction of treatment response

The Immune Prognosis Score (IPS) reflects the immunogenicity of tumors, and higher scores indicating a greater likelihood of response to immunotherapy. We then analyzed the IPS scores based on TGM2 grouping and found no significant difference between the two groups only when CTLA-4 and PD1 were both negative. However, when either CTLA-4 or PD1 was positive, the TGM2 high expression group demonstrated a higher response to immunotherapy (**Figure 6A-D**). Subsequently, we assessed the potential effects of TGM2 using the TISMO database in a mouse immunotherapy model. In the lung cancer mouse model, non-responders exhibted relatively higher TGM2 expression (**Figure 6E**). In in vitro lung cancer models, TGM2 was significantly overexpressed in samples that responded to IFNb and IFNg. Although the expression of TGM2 increased in samples that responded to TNFa, the difference was no statistically significant (**Figure 6F**). Since chemotherapy remains an indispensable first-line treatment for advanced lung cancer, we evaluated the sensitivity of LUSC to different chemotherapy drugs and observed that samples with low TGM2 expression were more sensitive to various drugs (**Figure 6G**).

TGM2 promotes cell proliferation and inhibits apoptosis

To experimentally determine the role of TGM2 in cell proliferation, we manipulated the expression level of TGM2 by either using small interfering RNA (siRNA) to knock down TGM2 expression or overexpressing TGM2 in NCI-H226 and SK-MES-1 cells. Altered TGM2 expression was confirmed by qRT-PCR and western blotting (**Figure 7A**, **7B**). Results from CCK-8 assay indicated that the proliferation capacity of NCI-H226 and SK-MES-1 cells decreased after TGM2 knockdown, while overexpression of TGM2 promoted cell proliferation (**Figure 7C**, **7D**). Furthermore, knockdown of TGM2 significantly decreased the apoptosis rate of NCI-H226 and SK-MES-1 cells (**Figure 7E**, **7F**), whereas overexpression of TGM2 significantly enhanced apoptosis, indicating a pro-apoptotic effect (**Figure 7G**, **7H**).

TGM2 promotes cell migration and invasion

Given the important role of TGM2 in tumor extracellular matrix remodeling, we investigated the effect of TMG2 on the mobility of cancer cells. Wound healing assays revealed a decreased migration of NCI-H226 and SK-MES-1 cells after TMG2 knockdown (**Figure 8A**, **8B**), and, consistently, Transwell assays further confirmed a significantly decreased invasion of NCI-H226 and SK-MES-1 cells after TGM2 knockdown (**Figure 8C**, **8D**).

Discussion

Previous studies have found a close relationship between EMT and the occurrence and development of promoting tumor inflammation, involving angiogenesis, tumor metastasis, and drug resistance [31]. These processes are closely regulated by key factors such as TGM2 [32]. In the early stages of tumor inflammation, TGM2 activates the NF-κB signaling pathway to enhance the aggregation of inflammatory cells, which is beneficial for wound healing. However, tumors are often referred to as “non-healing wounds”, and under the stimulation of long-term chronic inflammation, they gain stronger invasiveness and drug resistance [33]. The use of anti-inflammatory drugs to prevent cancer progression has been supported by multiple studies [34, 35]. Therefore, a better understanding of the relationship between tumor-promoting inflammation and tumor progression is beneficial for improving the prognosis of cancer patients.

In this study, we comprehensively analyzed the molecular characteristics of TGM2 in LUSC using bioinformatics methods. We found that the expression of TGM2 in normal tissues was significantly higher than in LUSC tissues. Additionally, LUSC patients with high expression of TGM2 were significantly associated with a worse prognosis. Previous studies have con-

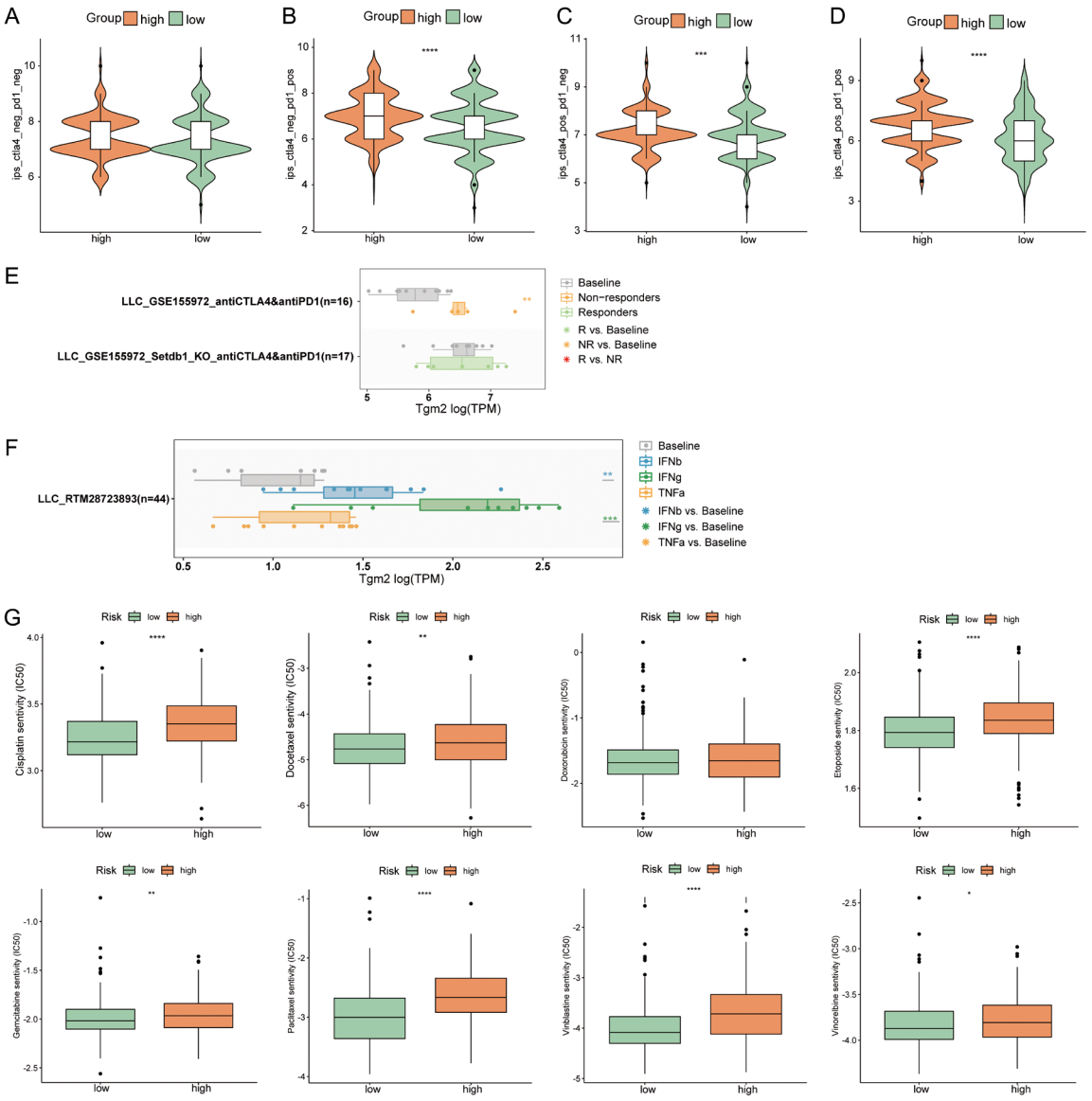

Figure 6. Prediction of immunotherapy and chemotherapy. Predicting the efficacy of immunotherapy based on the expression grouping of CTLA-4 and PD-1, including CTLA-4neg_PD1neg (A), CTLA-4neg_PD1pos (B), CTLA-4pos_PD-1neg (C), and CTLA-4pos_PD1pos (D). (E) Expression of TGM2 in the mouse immunotherapy model for lung cancer. (F) Expression of TGM2 in the lung cancer cell immunotherapy model. (G) Chemotherapy sensitivity analysis, including cisplatin, docetaxel, doxorubicin, gemcitabine, paclitaxel, vinblastine, and vinorelbine. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. pos: positive; neg: negative.

firmed the role of TGM2 in promoting tumor progression in various cancers [12, 17, 19, 36-40]. To explore the potential function of TGM2 in LUSC, we used GSEA to analyze the pathways associated with high TGM2 expression in LUSC, including immune response, cell adhesion, extracellular matrix production, and cell apoptosis. All these characteristics, without exception, indicate that TGM2 plays a pro-cancer role in LUSC. Chhabra et al. believe that TGM2 can both promote apoptosis and protect cells from apoptotic damage. These two distinct effects mainly depend on the type of cell, which explains why TGM2 is highly expressed in normal lung tissue [41]. Subsequently, we conducted cytological experiments to knock down or overexpress TGM2 to validate its role in LUSC. Decreased expression of TGM2 suppressed the malignant phenotype of LUSC, including proliferation, apoptosis, and invasion,

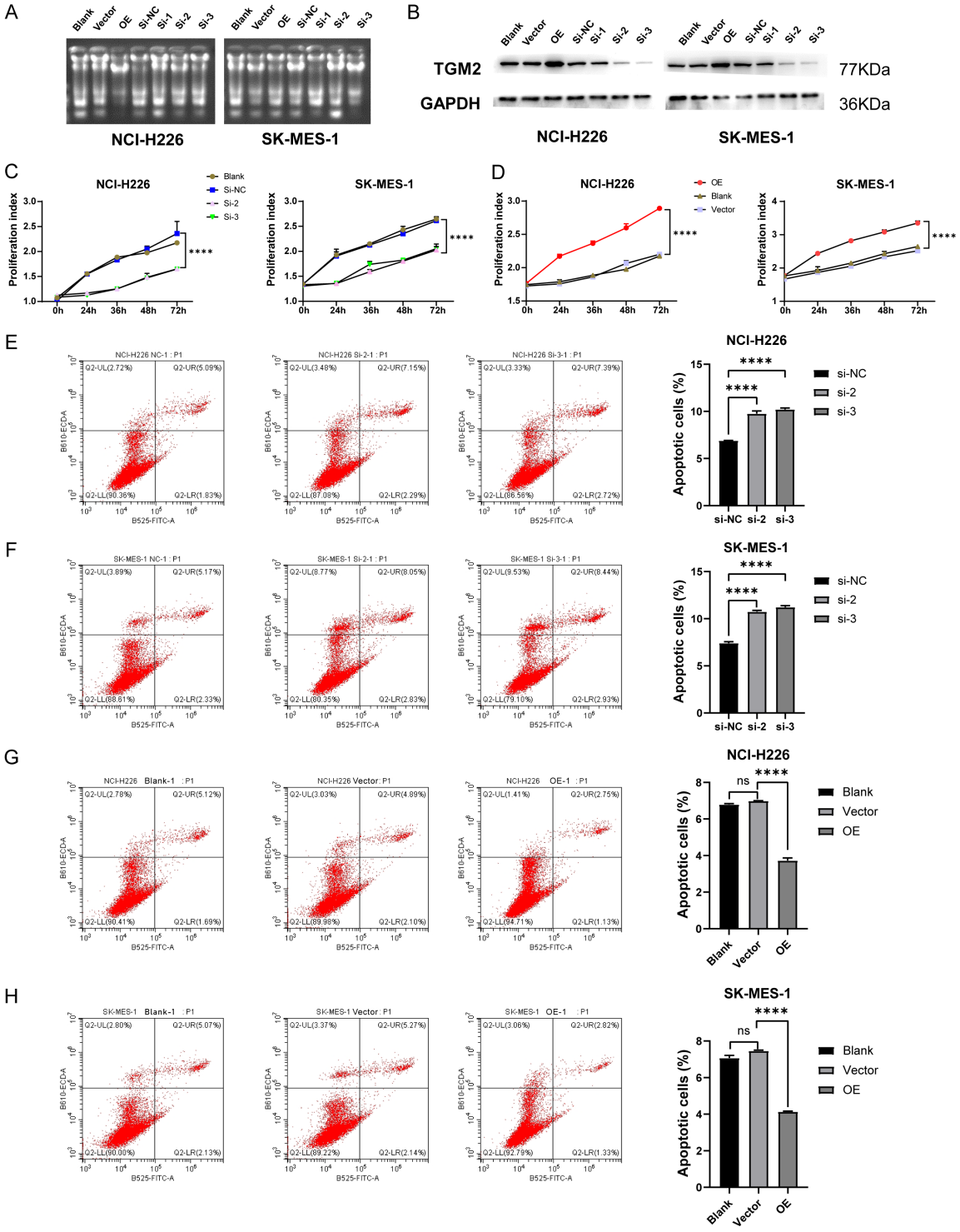

Figure 7. TGM2 promotes cell proliferation and inhibits apoptosis. qRT-PCR (A) and Western blotting (B) detecting the expression level of TGM2 in NCI-H226 and SK-MES-1 cells. (C, D) TGM2 promotes proliferation of NCI-H226 and SK-MES-1 cells. (E, F) Knockdown of TGM2 promotes cell apoptosis rate. (G, H) Overexpression of TGM2 inhibits cell apoptosis rate. ****P < 0.0001.

while overexpression of TGM2 promoted these features.

In inflammation resulting from tissue damage, inflammatory cells appeared briefly to combat

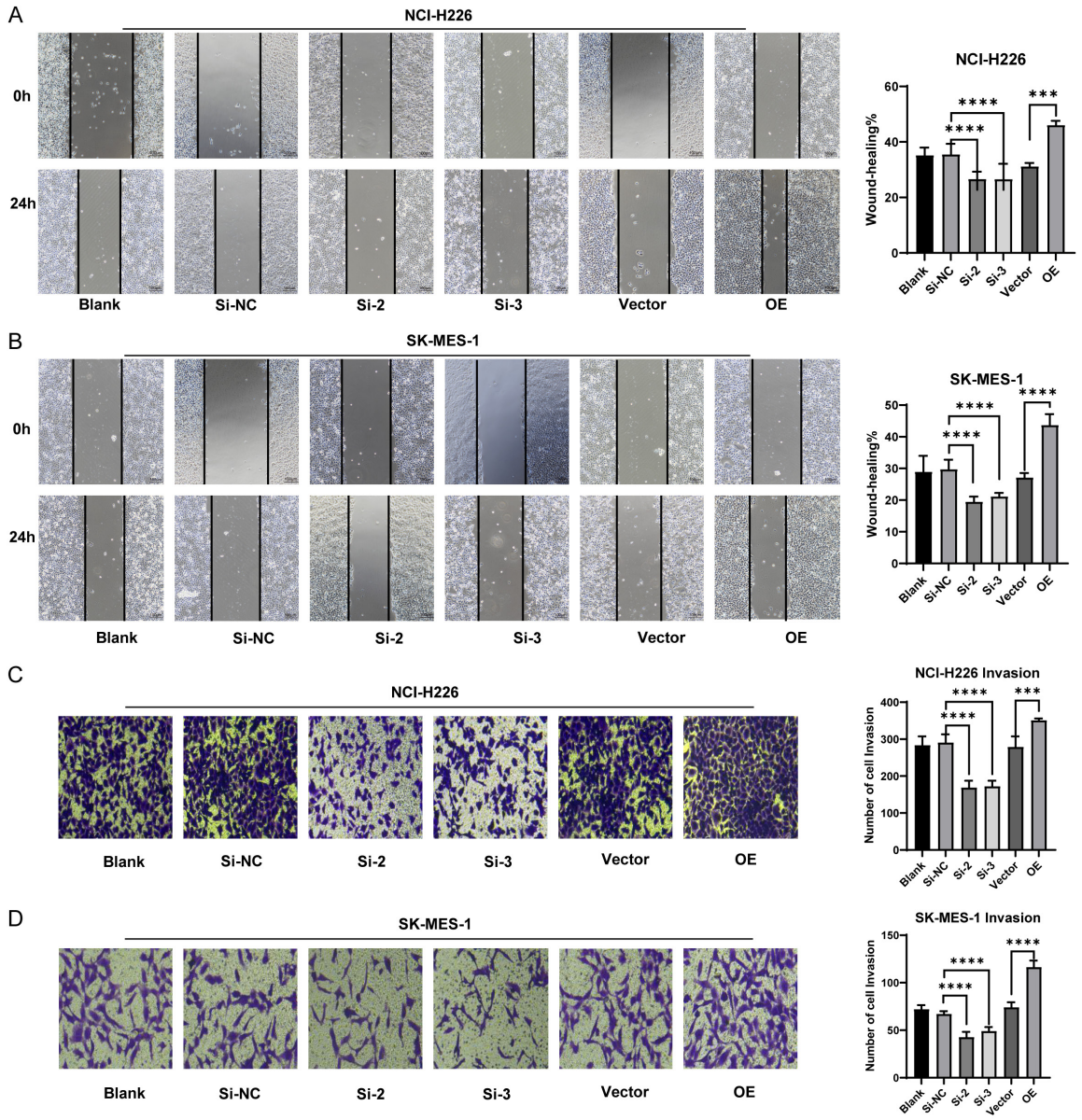

Figure 8. TGM2 promotes cell healing and invasion. TGM2 promotes the healing ability of NCI-H226 (A) and SK-MES-1 (B) cells. TGM2 promotes invasion of NCI-H226 (C) and SK-MES-1 (D) cells. ***P < 0.001, ****P < 0.0001.

the inflammation before disappearing. However, tumors sustain persistent chronic inflammation, wherein the prolonged presence of inflammatory cells will promotes tumor progression through angiogenesis, extracellular matrix remodeling, and immune escape [42]. This inflammatory state, distinct from that induced by tissue damage, is termed tumor-promoting inflammation [18], consistently linked to poorer patient outcomes [43]. Previous studies have reported that TGM2 could exacerbate tumor-promoting inflammation in gastric cancer, significantly correlating with the recruitment of macrophages, neutrophils, blood vessels, and lymphatics, thereby contributing to a worsened prognosis [19]. The results of GSEA analysis and immune infiltration analysis in the TGM2-high group indicated that these patients were in a state of tumor-promoting inflammation, leading to suppression of the anti-tumor immune response and, consequently, a worse prognosis. However, high TGM2 expression also upregulated PD-L1 [44], enhancing the efficacy of immune checkpoint inhibitors (ICIs).

Similar results were obtained from immunotherapy prediction using IPS. Therefore, TGM2 expression level can be used to identify patients suitable for immune checkpoint inhibitor therapy.

Increased TGM2 levels are closely related to the enhanced drug resistance in various cancers [45], as TGM2 can induce EMT. In pancreatic cancer, high TGM2 expression has been associated with strong resistance to gemcitabine treatment and greater metastatic potential [46]. We conducted sensitivity analyses on multiple chemotherapy drugs and obtained similar results. High TGM2 expression significantly decreased the sensitivity to various chemotherapy drugs. This demonstrates an effective combination of bioinformatics analysis and experimental validation, indicating that we can first improve the efficiency of experiments before conducting experimental validation.

TGM2 has strong potential as a therapeutic target. Mehta et al. developed DOPC liposomes as carriers to transport TGM2 siRNA into nude mice, downregulating TGM2 expression. This approach inhibited tumor growth and metastasis in mice and increased the effectiveness of chemotherapy [47]. Since metastasis is the primary cause of death in the majority of malignant tumors, inhibiting metastasis-related molecules has gradually become a new cancer treatment strategy [48].

However, there are some limitations to this study. Firstly, further animal experiments are needed to confirm the role of TGM2. Secondly, additional clinical trials are necessary to explore its sensitivity to drug therapy.

Conclusion

TGM2 is involved in tumor-promoting inflammation in LUSC and affects tumor metastasis and chemotherapy resistance. Targeting TGM2 is expected to improve the prognosis of LUSC patients.

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We ensured that each patient and their guardian signed and submitted a written informed consent form to respect and protect their rights.

Disclosure of conflict of interest

None.

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References

[1] Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A and Bray F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2021; 71: 209-249.

[2] Zappa C and Mousa SA. Non-small cell lung cancer: current treatment and future advances. Transl Lung Cancer Res 2016; 5: 288-300.

[3] Howington JA, Blum MG, Chang AC, Balekian AA and Murthy SC. Treatment of stage I and II non-small cell lung cancer: diagnosis and management of lung cancer, 3rd ed: American College of Chest Physicians evidence-based clinical practice guidelines. Chest 2013; 143 Suppl: e278S-e313S.

[4] Siegel RL, Miller KD and Jemal A. Cancer statistics, 2020. CA Cancer J Clin 2020; 70: 7-30.

[5] Perez-Moreno P, Brambilla E, Thomas R and Soria JC. Squamous cell carcinoma of the lung: molecular subtypes and therapeutic opportunities. Clin Cancer Res 2012; 18: 2443-2451.

[6] Jhunjhunwala S, Hammer C and Delamarre L. Antigen presentation in cancer: insights into tumour immunogenicity and immune evasion. Nat Rev Cancer 2021; 21: 298-312.

[7] Zaltron E, Vianello F, Ruzza A, Palazzo A, Brillo V, Celotti I, Scavezzon M, Rossin F, Leanza L and Severin F. The role of transglutaminase 2 in cancer: an update. Int J Mol Sci 2024; 25: 2797.

[8] Cellura D, Pickard K, Quaratino S, Parker H, Strefford JC, Thomas GJ, Mitter R, Mirnezami AH and Peake NJ. miR-19-mediated inhibition of transglutaminase-2 leads to enhanced invasion and metastasis in colorectal cancer. Mol Cancer Res 2015; 13: 1095-1105.
[9] Mehta K, Fok J, Miller FR, Koul D and Sahin AA. Prognostic significance of tissue transglutaminase in drug resistant and metastatic breast cancer. Clin Cancer Res 2004; 10: 8068-8076.
[10] Verma A, Wang H, Manavathi B, Fok JY, Mann AP, Kumar R and Mehta K. Increased expression of tissue transglutaminase in pancreatic ductal adenocarcinoma and its implications in drug resistance and metastasis. Cancer Res 2006; 66: 10525-10533.
[11] Yuan L, Choi K, Khosla C, Zheng X, Higashikubo R, Chicoine MR and Rich KM. Tissue transglutaminase 2 inhibition promotes cell death and chemosensitivity in glioblastomas. Mol Cancer Ther 2005; 4: 1293-1302.
[12] Zhang L, Li Q, Yang J, Xu P, Xuan Z, Xu J and Xu Z. Cytosolic TGM2 promotes malignant progression in gastric cancer by suppressing the TRIM21-mediated ubiquitination/degradation of STAT1 in a GTP binding-dependent modality. Cancer Commun (Lond) 2023; 43: 123-149.
[13] Dalby KN, Tekedereli I, Lopez-Berestein G and Ozpolat B. Targeting the prodeath and prosurvival functions of autophagy as novel therapeutic strategies in cancer. Autophagy 2010; 6: 322-329.
[14] Zhang H, Chen Z, Miranda RN, Medeiros LJ and McCarty N. TG2 and NF-kappaB signaling coordinates the survival of mantle cell lymphoma cells via IL6-mediated autophagy. Cancer Res 2016; 76: 6410-6423.
[15] Eckert RL, Kaartinen MT, Nurminskaya M, Belkin AM, Colak G, Johnson GV and Mehta K. Transglutaminase regulation of cell function. Physiol Rev 2014; 94: 383-417.
[16] Agnihotri N, Kumar S and Mehta K. Tissue transglutaminase as a central mediator in inflammation-induced progression of breast cancer. Breast Cancer Res 2013; 15: 202.
[17] Sima LE, Chen S, Cardenas H, Zhao G, Wang Y, Ivan C, Huang H, Zhang B and Matei D. Loss of host tissue transglutaminase boosts antitumor T cell immunity by altering STAT1/STAT3 phosphorylation in ovarian cancer. J Immunother Cancer 2021; 9: e002682.
[18] Greten FR and Grivennikov SI. Inflammation and cancer: triggers, mechanisms, and consequences. Immunity 2019; 51: 27-41.
[19] Cho SY, Oh Y, Jeong EM, Park S, Lee D, Wang X, Zeng Q, Qin H, Hu F, Gong H, Liu X, Zhang G, Na D, Lee J, Chae J, Suh YS, Kong SH, Lee HJ, Kim JI, Park H, Zhang C, Yang HK and Lee C. Amplification of transglutaminase 2 enhances tumor-promoting inflammation in gastric cancers. Exp Mol Med 2020; 52: 854-864.
[20] Yin J, Oh YT, Kim JY, Kim SS, Choi E, Kim TH, Hong JH, Chang N, Cho HJ, Sa JK, Kim JC, Kwon HJ, Park S, Lin W, Nakano I, Gwak HS, Yoo H, Lee SH, Lee J, Kim JH, Kim SY, Nam DH, Park MJ and Park JB. Transglutaminase 2 inhibition reverses mesenchymal transdifferentiation of glioma stem cells by regulating C/EBPbeta signaling. Cancer Res 2017; 77: 4973-4984.
[21] Li T, Fan J, Wang B, Traugh N, Chen Q, Liu JS, Li B and Liu XS. TIMER: a web server for comprehensive analysis of tumor-infiltrating immune cells. Cancer Res 2017; 77: e108-e110.
[22] Tang Z, Kang B, Li C, Chen T and Zhang Z. GEPIA2: an enhanced web server for large-scale expression profiling and interactive analysis. Nucleic Acids Res 2019; 47: W556-W560.
[23] von Mering C, Huynen M, Jaeggi D, Schmidt S, Bork P and Snel B. STRING: a database of predicted functional associations between proteins. Nucleic Acids Res 2003; 31: 258-261.
[24] Yu G, Wang LG, Han Y and He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS 2012; 16: 284-287.
[25] Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES and Mesirov JP. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005; 102: 15545-15550.
[26] Ru B, Wong CN, Tong Y, Zhong JY, Zhong SSW, Wu WC, Chu KC, Wong CY, Lau CY, Chen I, Chan NW and Zhang J. TISIDB: an integrated repository portal for tumor-immune system interactions. Bioinformatics 2019; 35: 4200-4202.
[27] Aran D, Hu Z and Butte AJ. xCell: digitally portraying the tissue cellular heterogeneity landscape. Genome Biol 2017; 18: 220.
[28] Charoentong P, Finotello F, Angelova M, Mayer C, Efremova M, Rieder D, Hackl H and Trajanoski Z. Pan-cancer immunogenomic analyses reveal genotype-immunophenotype relationships and predictors of response to checkpoint blockade. Cell Rep 2017; 18: 248-262.
[29] Zeng Z, Wong CJ, Yang L, Ouardaoui N, Li D, Zhang W, Gu S, Zhang Y, Liu Y, Wang X, Fu J, Zhou L, Zhang B, Kim S, Yates KB, Brown M, Freeman GJ, Uppaluri R, Manguso R and Liu XS. TISMO: syngeneic mouse tumor database to model tumor immunity and immunotherapy response. Nucleic Acids Res 2022; 50: D1391-D1397.
[30] Geeleher P, Cox N and Huang RS. pRRophetic: an R package for prediction of clinical chemo-

therapeutic response from tumor gene expression levels. PLoS One 2014; 9: e107468.
[31] Hanahan D and Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011; 144: 646-674.
[32] Su T, Qin XY and Furutani Y. Transglutaminase 2 as a marker for inflammation and therapeutic target in sepsis. Int J Mol Sci 2021; 22: 1897.
[33] Zhao H, Wu L, Yan G, Chen Y, Zhou M, Wu Y and Li Y. Inflammation and tumor progression: signaling pathways and targeted intervention. Signal Transduct Target Ther 2021; 6: 263.
[34] Cuzick J, Otto F, Baron JA, Brown PH, Burn J, Greenwald P, Jankowski J, La Vecchia C, Meyskens F, Senn HJ and Thun M. Aspirin and non-steroidal anti-inflammatory drugs for cancer prevention: an international consensus statement. Lancet Oncol 2009; 10: 501-507.
[35] Rothwell PM, Fowkes FG, Belch JF, Ogawa H, Warlow CP and Meade TW. Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials. Lancet 2011; 377: 31-41.
[36] Zhang S, Yao HF, Li H, Su T, Jiang SH, Wang H, Zhang ZG, Dong FY, Yang Q and Yang XM. Transglutaminases are oncogenic biomarkers in human cancers and therapeutic targeting of TGM2 blocks chemoresistance and macrophage infiltration in pancreatic cancer. Cell Oncol (Dordr) 2023; 46: 1473-1492.
[37] Malkomes P, Lunger I, Oppermann E, Abou-El-Ardat K, Oellerich T, Gunther S, Canbulat C, Bothur S, Schnutgen F, Yu W, Wingert S, Haetscher N, Catapano C, Dietz MS, Heilemann M, Kvasnicka HM, Holzer K, Serve H, Bechstein WO and Rieger MA. Transglutaminase 2 promotes tumorigenicity of colon cancer cells by inactivation of the tumor suppressor p53. Oncogene 2021; 40: 4352-4367.
[38] Li C, Cai J, Ge F and Wang G. TGM2 knockdown reverses cisplatin chemoresistance in osteosarcoma. Int J Mol Med 2018; 42: 1799-1808.
[39] Leicht DT, Kausar T, Wang Z, Ferrer-Torres D, Wang TD, Thomas DG, Lin J, Chang AC, Lin L and Beer DG. TGM2: a cell surface marker in esophageal adenocarcinomas. J Thorac Oncol 2014; 9: 872-881.
[40] Lee J, Condello S, Yakubov B, Emerson R, Caperell-Grant A, Hitomi K, Xie J and Matei D. Tissue transglutaminase mediated tumor-stroma interaction promotes pancreatic cancer progression. Clin Cancer Res 2015; 21: 4482-4493.
[41] Chhabra A, Verma A and Mehta K. Tissue transglutaminase promotes or suppresses tumors depending on cell context. Anticancer Res 2009; 29: 1909-1919.
[42] Grivennikov SI, Greten FR and Karin M. Immunity, inflammation, and cancer. Cell 2010; 140: 883-899.
[43] Crusz SM and Balkwill FR. Inflammation and cancer: advances and new agents. Nat Rev Clin Oncol 2015; 12: 584-596.
[44] Liu J, Liu Q, Zhang X, Cui M, Li T, Zhang Y and Liao Q. Immune subtyping for pancreatic cancer with implication in clinical outcomes and improving immunotherapy. Cancer Cell Int 2021; 21: 137.
[45] Mehta K, Kumar A and Kim HI. Transglutaminase 2: a multi-tasking protein in the complex circuitry of inflammation and cancer. Biochem Pharmacol 2010; 80: 1921-1929.
[46] Zhang S, Yao HF, Li H, Su T, Jiang SH, Wang H, Zhang ZG, Dong FY, Yang Q and Yang XM. Transglutaminases are oncogenic biomarkers in human cancers and therapeutic targeting of TGM2 blocks chemoresistance and macrophage infiltration in pancreatic cancer. Cell Oncol (Dordr) 2023; 46: 1473-1492.
[47] Verma A, Guha S, Diagaradjane P, Kunnumakkara AB, Sanguino AM, Lopez-Berestein G, Sood AK, Aggarwal BB, Krishnan S, Gelovani JG and Mehta K. Therapeutic significance of elevated tissue transglutaminase expression in pancreatic cancer. Clin Cancer Res 2008; 14: 2476-2483.
[48] Suhail Y, Cain MP, Vanaja K, Kurywchak PA, Levchenko A, Kalluri R and Kshitiz. Systems biology of cancer metastasis. Cell Syst 2019; 9: 109-127.