

Pan-cancer prognostic value of TGFBR1: Bioinformatics and experimental validation with a focus on gastric cancer

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Abstract. TGF- β receptor 1 (TGFBR1) is a key component of TGF- β signaling and has been implicated in tumor progression. However, its expression patterns, prognostic relevance and associated molecular contexts vary among malignancies and remain incompletely characterized within a consistent pan-cancer framework. In the present study, TGFBR1 expression, clinicopathological associations, survival outcomes, immune and stromal infiltration, genomic features, co-expression profiles and transcriptional programs were systematically evaluated using The Cancer Genome Atlas (TCGA), TCGA-Genotype-Tissue Expression and complementary public datasets. Multivariable Cox regression was performed with adjustment for age, sex and pathological stage and the independent GSE15459 gastric cancer (GC) cohort was used for external prognostic validation and gene-set enrichment analysis. TGFBR1

exhibited cancer type-dependent expression alterations rather than uniform upregulation across malignancies. Higher TGFBR1 expression remained associated with poorer overall survival (OS) in mesothelioma (MESO), adrenocortical carcinoma (ACC) and kidney renal papillary cell carcinoma and with poorer disease-specific survival in ACC, esophageal carcinoma, MESO, lung squamous cell carcinoma, stomach adenocarcinoma and bladder urothelial carcinoma after multivariable adjustment. In GSE15459, elevated TGFBR1 expression was associated with shorter OS and remained significant after adjustment for age, sex, pathological stage and Lauren classification. TGFBR1 expression was also associated with distinct immune, stromal, genomic and transcriptional contexts across cancer types. In GC, enrichment analyses revealed an association between higher TGFBR1 expression and apoptosis- and stress-response-associated programs, epithelial cell proliferation, focal-adhesion assembly and actin-cytoskeleton organization. *In vitro*, TGFBR1 knockdown reduced the proliferation and migration of GC cells, altered epithelial-mesenchymal transition-associated protein expression and promoted apoptosis. Collectively, these findings define the cancer-type-specific clinical and biological relevance of TGFBR1 and support its prognostic importance in selected malignancies, particularly GC, while further mechanistic and prospective validation remains necessary.

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Abbreviations: TGFBR1, TGF- β receptor 1; EMT, epithelial-mesenchymal transition; TIME, tumor immune microenvironment; OS, overall survival; DSS, disease-specific survival; DFI, disease-free interval; PFI, progression-free interval; DFS, disease-free survival; TMB, tumor mutational burden; MATH, mutant-allele tumor heterogeneity; MSI, microsatellite instability; HRD, homologous recombination deficiency; LOH, loss-of heterozygosity; CAF, cancer-associated fibroblast

Key words: pan-cancer, TGF- β receptor 1, prognosis, tumor microenvironment, gastric cancer

Introduction

At present, research has primarily focused on studying the mechanisms of tumorigenesis by identifying typical biomarkers across diverse human cancers (1). Pan-cancer analysis refers has gained recognition for identifying tumor biomarkers and signaling pathways, further enhancing the understanding of tumor occurrence and progression at the molecular level and aiding clinicians in improving patient outcomes (2-4). Thus, screening and identifying biomarkers with diagnostic or therapeutic value using pan-cancer analysis not only has the potential to markedly improve traditional cancer therapies but also presents novel avenues for personalized treatment strategies, ultimately contributing to more effective and targeted cancer management.

TGF- β receptor 1 (TGFBR1), also known as ALK5, is an important molecule in TGF- β signaling pathway (5). TGFBR1, which is composed of signal peptide, hydrophilic extracellular region, transmembrane domain and intracellular region, can phosphorylate serine or threonine in downstream signaling proteins (6,7). The extracellular region includes a number of cysteines and has sites for glycosylation, while the intracellular region close to the membrane features glycine-rich and serine-rich segments that are associated with self-phosphorylation (8). In addition, serine/threonine rich fragments exist in the intracellular region of TGF- β receptor 2, which phosphorylates TGFBR1 during signal transduction, activates the kinase region of TGFBR1, further phosphorylates downstream substrates and transmits TGF- β signals into the cell, thereby regulating numerous physiological and pathological processes, such as cell cycle phases, immunosuppression and epithelial-mesenchymal cell transformation (EMT) (9-11). Alterations in TGFBR1 expression, activity and genetic background have been reported in numerous malignancies, including breast, colorectal and gastric cancer (GC), amongst others (12-18). Pharmacological inhibitors of TGFBR1, including LY2157299 (galunisertib) and TEW-7197 (vactosertib), have also been investigated in preclinical and clinical studies, with evidence suggesting that TGFBR1 inhibition may suppress the tumor-promoting effects of TGF- β signaling (19,20).

A previous pan-cancer study characterized genomic alterations affecting mediators and regulators of TGF- β superfamily signaling across The Cancer Genome Atlas (TCGA) cancer types (21). In GC, He *et al* (22) reported elevated TGFBR1 expression and associations with clinicopathological characteristics, prognosis and immune-cell infiltration using TCGA, Gene Expression Profiling Interactive Analysis (GEPIA) and Human Protein Atlas (HPA) data. More recently, functional studies have shown that pharmacological or genetic inhibition of TGFBR1 could suppress GC cell proliferation, migration, invasion or treatment resistance through TGF- β -associated signaling contexts (22,23). These findings establish the biological relevance of TGFBR1 but also indicate that its role in GC is not entirely unexplored. Despite this, a number of clinically relevant aspects of TGFBR1 remain insufficiently characterized. In particular, to the best of our knowledge, the extent to which its expression and prognostic relevance vary across cancer types has not been systematically compared using a consistent analytical framework. It also remains necessary to determine whether the observed survival associations persist after adjustment for key clinicopathological variables and can be reproduced in an independent GC cohort. In addition, the immune, stromal, genomic and transcriptional features associated with TGFBR1 may differ notably among malignancies and therefore require cancer-type-specific interpretation.

Accordingly, the present study conducted an integrated analysis of TGFBR1 across TCGA and TCGA-Genotype-Tissue Expression (GTEx) cancer cohorts. The present study systematically evaluated its cancer-type-specific expression patterns, clinicopathological associations, multiple survival endpoints, immune and stromal characteristics, genomic features, co-expression profiles and functional transcriptional programs. Multivariable Cox regression was performed to assess the prognostic relevance of TGFBR1 after adjustment

for age, sex and pathological stage, with the independent GSE15459 GC cohort being used for external prognostic validation and transcriptomic enrichment analysis. Functional experiments in GC cell lines were further performed to examine the effects of TGFBR1 knockdown on proliferation, migration, EMT-associated protein expression and apoptosis. Through the integration of pan-cancer comparisons, adjusted survival analyses, independent cohort validation and cellular experiments, the present study aimed to refine the clinical and biological importance of TGFBR1 across malignancies, with a particular emphasis on GC (Fig. 1).

Materials and methods

Analysis of Gene Expression. TCGA (portal.gdc.cancer.gov/) database contains a wide range of information on numerous types of human cancers. The uniformly standardized pan-cancer dataset was obtained from the UCSC Xena platform (xenabrowser.net/), including TCGA tumor and GTEx normal tissue samples. Given that some tumors in TCGA lack sufficient normal tissue, the GTEx data (commonfund.nih.gov/) were additionally incorporated for normal tissue comparison. Subsequently, samples were filtered from 'Solid Tissue Normal', 'Primary Blood Derived Cancer-Peripheral Blood' and 'Primary Tumor' and a $\log_2(x+1)$ transformation was applied to each expression value. After excluding cancer types with <3 samples in a single cancer type, 34 cancer types were obtained. Comparisons of TGFBR1 expression between two clinicopathological groups, including sex, were performed using the two-sided Wilcoxon rank-sum test. Comparisons involving >2 groups, including pathological stage and tumor grade, were performed using the Kruskal-Wallis test. P-values were adjusted across cancer types for each clinicopathological variable using the Benjamini-Hochberg method and an adjusted $P < 0.05$ was considered to indicate a statistically significant difference. The abbreviations for each tumor are summarized in Table SI.

Analysis of TGFBR1 subcellular localization. The subcellular localization of TGFBR1 was assessed based on immunofluorescence images obtained from the Human Protein Atlas (HPA) database (proteintlas.org/search/TGFBR1). The HPA subcellular localization dataset provides antibody-based immunofluorescence profiling of human proteins in cultured cells. TGFBR1 localization information and representative immunofluorescence images were retrieved from the HPA database and used for descriptive analysis. The immunofluorescence data available in the HPA database were generated according to the standardized experimental and imaging protocols established by the Human Protein Atlas project.

Evaluation of the prognostic importance of TGFBR1 expression. Clinical and survival data were obtained from TCGA Pan-Cancer Clinical Data Resource, and relevant prognostic analyses were conducted based on overall survival (OS), disease-specific survival (DSS), disease-free interval (DFI) and progression-free interval (PFI). Patients with available TGFBR1 expression data and corresponding clinical follow-up information were included in the prognostic analyses. Patients with missing survival information or follow-up duration

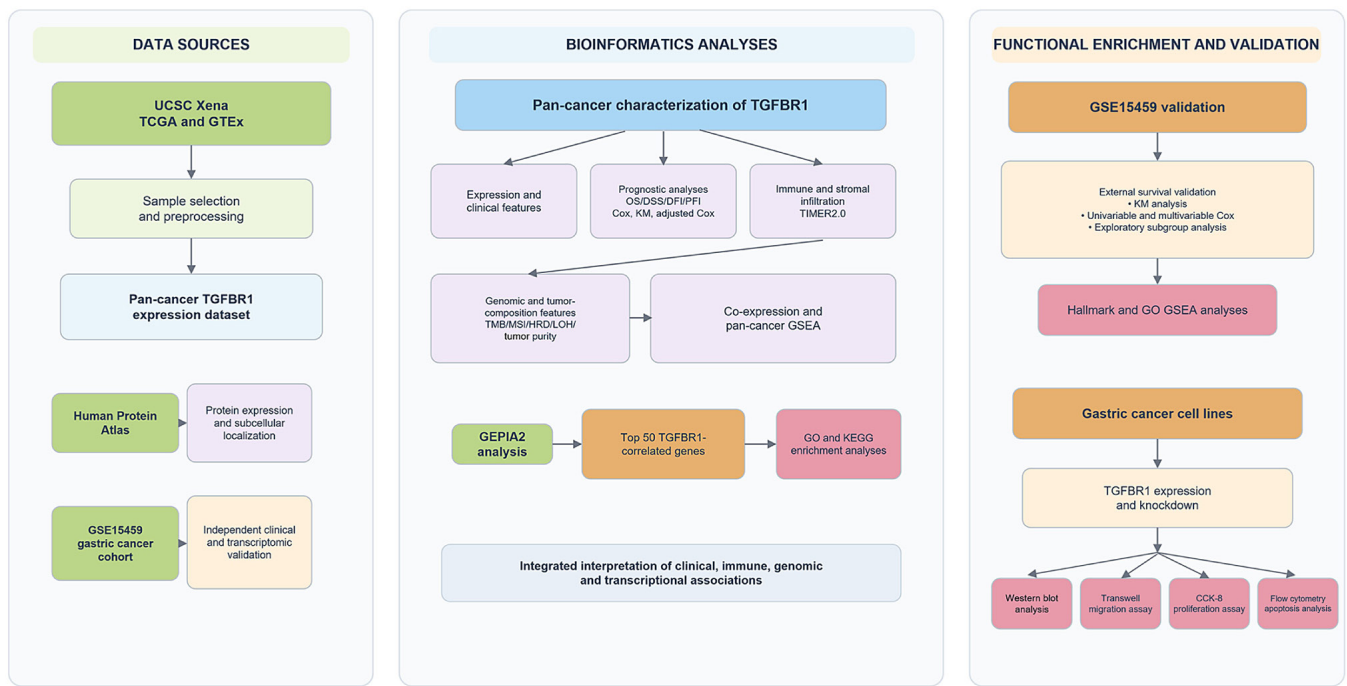


Figure 1. Technical flow chart of the present study. OS, overall survival; TMB, tumor mutational burden; GO, Gene Ontology; GSEA, Gene Set Enrichment Analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; CCK-8, Cell Counting Kit-8; TCGA, The Cancer Genome Atlas; UCSC, University of California, Santa Cruz Genome Browser; GTEx, Genotype-Tissue Expression; GEPIA2, Gene Expression Profiling Interactive Analysis 2; TIMER2.0, Tumor Immune Estimation Resource version 2.0; TGFBR1, TGF- β receptor 1; DSS, disease-specific survival; DFI, disease-free interval; PFI, progression-free interval; MSI, microsatellite instability; HRD, homologous recombination repair deficiency; LOH, loss-of-heterozygosity.

<30 days were excluded. OS, DSS, DFI and PFI were assessed using univariable Cox regression and Kaplan-Meier analysis. TGFBR1 expression was standardized as a z-score for Cox analysis, while patients were divided into high- and low-expression groups according to the median expression level for Kaplan-Meier analysis. Multivariable Cox regression for OS and DSS was further performed with adjustment for age, sex and pathological stage. The proportional hazards assumption was evaluated using Schoenfeld residuals and P-values across cancer types were adjusted using the Benjamini-Hochberg method. The prognostic value of TGFBR1 in GC was externally validated in the independent GSE15459 cohort obtained from the Gene Expression Omnibus (GEO) database (ncbi.nlm.nih.gov/geo/). GSE15459 contains gene expression profiles of primary gastric tumors generated using the Affymetrix Human Genome U133 Plus 2.0 Array reported by Ooi *et al* (24). The TGFBR1 probe with the largest interquartile range was selected, followed by Kaplan-Meier, univariable Cox, multivariable Cox adjusted for age, sex and stage and exploratory subgroup analyses according to age, sex, stage and Lauren classification. GEPIA2 (<http://gepia2.cancer-pku.cn/>), an online platform based on TCGA and GTEx datasets, was additionally used as a complementary TCGA-based analysis of OS and DFS. All analyses were performed using the 'survival' R package (version 3.8-3) and 'survminer' R package (version 0.5.1) (25,26).

TGFBR1 expression and immune infiltration analysis. Tumor Immune Estimation Resource 2.0 (TIMER2.0) (<https://compbio.cn/timer2/>) is a platform for immune infiltration analysis based on TCGA data. It uses high-throughput

sequencing technology (including RNA-sequencing expression profiling) to assess immune cell infiltration in tumor tissue, primarily providing insight into B cells, CD4⁺ T cells, CD8⁺ T cells, T regulatory cells (Tregs), neutrophils, macrophages and dendritic cells, amongst others. In the present study, the TIMER2.0 technique was used to evaluate P-values and correlation results in tumor samples, with the aim of exploring the potential association between TGFBR1 expression and immune infiltration levels. Given that tumor purity may confound the association between gene expression and immune infiltration, purity-adjusted partial Spearman's correlation was used where applicable. For Estimating the Proportions of Immune and Cancer cells (epic.gfellerlab.org/) and quanTiseq (<http://icb-research.it/quantiseq/>), which estimate cell fractions (CC) relative to total cells, correlations were calculated without additional adjustment for tumor purity in accordance with the TIMER2.0 recommendations (27). Spearman's correlation coefficients and corresponding P-values were summarized using heatmaps and a two-sided nominal $P < 0.05$ was considered to indicate a statistically significant difference.

Database analysis of TGFBR1 gene expression in tumor immune subtypes and molecular subtypes. From the UCSC database, the uniformly standardized pan-cancer dataset was downloaded: TCGA Pan-Cancer ($n=10,535$; $G=60,499$). Subsequently, the expression data of the TGFBR1 gene in each sample was extracted. Subsequently, the samples derived from 'Primary Blood Cancer-Peripheral Blood' and 'Primary Tumor' were screened. The tumor mutation burden (TMB) and mutant-allele tumor heterogeneity (MATH) was

calculated for each tumor to assess the genetic landscape and heterogeneity within the tumors, respectively, using the TMB function and the infer-heterogeneity function of the 'maftools' R package (version 2.8.05; Posit Software, PBC) (28). We obtained microsatellite instability (MSI), neoantigen, ploidy, homology-dependent recombination repair deficiency (HRD), loss-of-heterozygosity (LOH) scores for each tumor from previous studies (29,30). Samples with expression levels of 0 were filtered out and each expressed value was subsequently transformed using the $\log_2(x+1)$ method. In addition, cancer types that had <3 samples were excluded from the present analysis. Within each cancer type, Pearson correlation analysis was used to evaluate the associations between TGFBR1 expression and the genomic or tumor-related features. Correlation coefficients and corresponding two-sided P-values were summarized graphically and $P < 0.05$ was considered to indicate a statistically significant difference. These analyses were considered exploratory.

Association between TGFBR1 expression and genes related to EMT, cell proliferation, apoptosis, autophagy, drug resistance and immune checkpoint. Co-expression of TGFBR1 and genes associated with EMT, cell proliferation and apoptosis autophagy, drug resistance and immune checkpoint were analyzed using the 'limma' R package (version 3.64.3) (31). Within each cancer type, Pearson correlation coefficients and corresponding two-sided P-values were calculated between TGFBR1 expression and the expression of each selected gene. The results were visualized as heatmaps using R software (Post Software, PBC). A nominal $P < 0.05$ was considered to indicate a statistically significant difference. Given the numerous genes and cancer types examined, these analyses were considered exploratory.

Functional enrichment analysis of TGFBR1-correlated genes. A list of the top 50 genes with expression patterns most similar to TGFBR1 were identified using the 'Similar Genes Detection' module of GEPIA2. Genes were ranked according to their Pearson correlation coefficients with TGFBR1 expression across the selected TCGA datasets. The resulting TGFBR1-correlated genes were subsequently submitted to the Database for Annotation, Visualization and Integrated Discovery (david.ncifcrf.gov/) for Gene Ontology (GO; geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses (32).

Gene-set enrichment analysis (GSEA). GSEA is a computational approach used to assess whether a predefined collection of genes demonstrates significant, consistent differences in expression between two biological states or phenotypes. In the present study, the 'clusterProfiler' R package (version 4.12.2; Posit Software, PBC) was used for GSEA, with the aim of clarifying the biological role of TGFBR1 in tumor development (33). After excluding normal samples, tumor samples were divided into TGFBR1-high and TGFBR1-low groups according to the median expression level. Genes were ranked by the $\log_2$ fold change between the two groups. The Molecular Signatures Database (MSigDB; version 7.1; gsea-msigdb.org/gsea/msigdb/) C2 KEGG pathway collection and C5 ontology gene-set collection were analyzed

separately (34). Gene sets with a nominal $P < 0.05$ were retained for exploratory interpretation. To further evaluate the transcriptional programs associated with TGFBR1 in an independent GC cohort, additional GSEA was performed using the GSE15459 dataset. The eight samples marked as excluded in the Gene Expression Omnibus (GEO; ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE15459) sample annotations were removed, leaving 192 gastric adenocarcinoma samples for analysis. The processed microarray expression values were $\log_2$ -transformed. Among the three GPL570 probe sets annotated to TGFBR1, 'probe set 236561_at', which had the largest interquartile range, was selected and consistently used for survival analysis and GSEA. Samples were divided into TGFBR1-high and -low groups according to the median expression of TGFBR1.

GPL570 probe sets were mapped to gene symbols using the 'hgu133plus2.db' annotation R package (version 3.13.0; Bioconductor) (35). When multiple probe sets corresponded to the same gene, the probe set with the largest interquartile range was retained. Differential expression between the TGFBR1-high and TGFBR1-low groups was evaluated using the 'limma' R package and genes were ranked according to the moderated t-statistic for the high-vs.-low contrast. GSEA was then performed using the 'clusterProfiler' R package with the MSigDB 'Hallmark' and 'C5' GO gene-set collections. Gene sets containing 10-500 genes were analyzed. Normalized enrichment scores, nominal P-values and Benjamini-Hochberg-adjusted P-values were calculated. An adjusted $P < 0.05$ was considered to indicate a statistically significant difference.

Cell lines and cell cultures. In the present study, the human gastric mucosal epithelial cell line (GES-1), alongside GC cell lines, including MGC803, SNU719, YCCEL1, AGS and AGS-EBV, were utilized. All cell lines were cultured in RPMI-1640 medium enriched with 10% FBS (Gibco, Thermo Fisher Scientific, Inc.) and 2% penicillin-streptomycin and were kept in a humidified incubator at 37°C with 5% CO₂.

Small interfering RNA (siRNA) transfection. siRNA sequences targeting TGFBR1 and the corresponding negative control (NC) were designed and synthesized by Shanghai GenePharma Co., Ltd. A total of 50 nM siRNA (sequence 1: 5'-GGGUCUGUGACUACAACAUTT-3' and 5'-AUGUUGUAGUCACAGACCCTT-3'; sequence 2: 5'-CUCGAUGAUUCCAUAUAUATT-3' and 5'-UAUUUAUGGAAUCAUCGAGTT-3') or NC; sequence: 5'-UUCUCCGAACGUGUCACGUTT-3' and 5'-ACGUGACACGUUCGGAGAATT-3') oligonucleotides was transfected with the Lipofectamine™ 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Briefly, siRNA-Lipofectamine complexes were incubated with cells at 37°C for 6 h, after which the medium was replaced with fresh complete medium. Functional assays and protein/RNA analyses were performed 48 h after transfection.

Western blotting. Western blotting was employed to assess protein expression in the YCCEL1 and AGS gastric cancer cells following si-TGFBR1 transfection. Gastric cancer cells were collected and washed twice with cold PBS. The cell precipitation was mixed with a RIPA buffer mixture

(consisting of RIPA:PMSF:phosphatase inhibitor at a 100:1:1 ratio) which was added to the cells and lysed on ice for 20 min. Subsequently, the protein content was measured using a BCA assay. Equal amounts of protein (25 μ g per lane) were separated using 10 or 15% SDS-PAGE and then transferred to a PVDF membrane (MilliporeSigma). Following the transfer, the PVDF membranes were blocked with 5% skim milk at room temperature for 2 h and subsequently incubated with primary antibodies at 4°C overnight. The next day, after washing the membrane three times with TBST (Tween 20), the bands were then incubated with HRP-conjugated goat anti-rabbit (Beyotime Biotechnology; cat. no. A0208; 1:2,500) or HRP-conjugated goat anti-mouse IgG (H+L) (both Beyotime Biotechnology; cat. no. A0216; 1:3,000) for 2 h at room temperature. Proteins of interest were detected using an ECL detection system. The specific antibodies used were included: Anti-TGFBR1 (Santa Cruz Biotechnology, Inc.; cat. no. sc-515875; 1:1,000), anti-N-cadherin (PTM BIO LLC; cat. no. PTM-5221; 1:2,000), anti-E-cadherin (PTM BIO LLC; cat. no. PTM-6222; 1:2,000), anti-Snail (Santa Cruz Biotechnology, Inc.; cat. no. sc-271977; 1:500), anti- β -catenin (Abmart Pharmaceutical Technology Co., Ltd.; cat. no. PK02151; 1:1,000), anti-Vimentin (Abmart Pharmaceutical Technology Co., Ltd.; cat. no. T55134; 1:1,000), anti-zinc finger E-box binding homeobox 1 (ZEB1; Proteintech Group, Inc.; cat. no. 21544-1-AP; 1:2,000), anti-GAPDH (Abcam; cat. no. ab8245; 1:3,000), anti-Bax (Cell Signaling Technology, Inc.; cat. no. 5023; 1:1,000) and anti-Bcl2 (Cell Signaling Technology, Inc.; cat. no. 4223; 1:1,000).

CCK-8 proliferation assay (CCK-8). CCK-8 (Boster Biological Technology) was performed to assess the ability of YCCEL1 and AGS cells to proliferate. Cells (24 h after transfection with different siRNAs) were seeded into 96-well plates at 4×10^3 cells/100 μ l/well. After 0, 24, 48 and 72 h of incubation, the CCK-8 solution (10 μ l) was added to each well. The absorbance was measured at 450 nm after incubation for 2 h using a Soft-Max device (Bio-Tek ELx 808; Agilent Technologies).

Transwell migration assay. YCCEL1 and AGS gastric cancer cells were used to evaluate migratory ability using Transwell chambers with 8- μ m pores (Corning, Inc.). Briefly, cells (5×10^4 /well) were counted and re-suspended in serum-free DMEM (200 μ l) and inoculated in the upper chamber. The lower chamber was filled with medium supplemented with 20% FBS. Following incubation at 37°C for 48 h, non-migrated cells were removed, and migrated cells on the lower surface of the membrane were fixed with 4% paraformaldehyde at room temperature for 30 min and stained with 0.1% crystal violet solution at room temperature for 30 min. Migrated cells were observed using an inverted light microscope (Olympus Corporation) and ImageJ software (National Institutes of Health) was used for cell counting.

Apoptosis assay. An Annexin V-cyanocyanin/PI apoptosis detection kit (Tianjin SM Biotech Co, Ltd.) was used to detect apoptosis. AGS and YCCEL1 gastric cancer cells (1×10^6) were seeded into six-well plates and, after attachment, transfected with siTGFBR1-2 or siNC as described above. A total of 48 h later, cells were resuspended with binding buffer (200 μ l) containing Annexin V-APC and PI and incubated at room

temperature in the dark for 15 min. The stained cells were analyzed using a BD FACSCanto II flow cytometer (BD Biosciences), and data were processed using FlowJo software (version 10.8.1; FlowJo LLC). All experiments were conducted in triplicate.

Statistical analysis. Experimental data are presented as the mean $\pm$ SD. For *in vitro* experiments involving three groups (NC, siTGFBR1-1 and siTGFBR1-2), including western blot densitometry and Transwell migration assays, statistical differences were evaluated using one-way ANOVA, followed by Dunnett's multiple comparisons tests, with the NC group serving as the control. CCK-8 proliferation data were analyzed using two-way ANOVA followed by Tukey's multiple comparisons test to compare each TGFBR1-knockdown group with the NC group at each time point. For experiments involving only two groups, including the flow-cytometric apoptosis assay, a two-tailed unpaired Student's t-test was used. Data analyses were performed using R software (version 4.4.1; Posit Software, PBC) and GraphPad Prism (version 8.0.2; Dotmatics), with $P < 0.05$ being considered to indicate a statistically significant difference.

Results

TGFBR1 exhibits cancer-type-dependent expression patterns. TCGA and TCGA-GTEX data obtained from UCSC were analyzed to investigate TGFBR1 expression across cancer types. Findings revealed distinct TGFBR1 expression patterns among different tumor types. TCGA dataset showed that TGFBR1 expression was significantly elevated in 12 cancer types, including glioblastoma multiforme (GBM), glioma (GBMLGG), brain lower grade glioma (LGG), colon adenocarcinoma (COAD), colon adenocarcinoma/rectum adenocarcinoma esophageal carcinoma (COADREAD), stomach and esophageal carcinoma (STES), stomach adenocarcinoma (STAD), head and neck squamous cell carcinoma (HNSC), liver hepatocellular carcinoma (LIHC), thyroid carcinoma (THCA), pheochromocytoma and paraganglioma (PCPG) and cholangiocarcinoma (CHOL). Conversely, significantly lower expression was observed in 9 tumors, including lung adenocarcinoma (LUAD), BRCA, kidney renal papillary cell carcinoma (KIRP), pan-kidney cohort (KIPAN), prostate adenocarcinoma (PRAD), uterine corpus endometrial carcinoma (UCEC), lung squamous cell carcinoma (LUSC), bladder urothelial carcinoma (BLCA) and kidney chromophobe (KICH). No significant differences were observed in cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), esophageal carcinoma (ESCA), kidney renal clear cell carcinoma (KIRC), rectum adenocarcinoma (READ) and pancreatic adenocarcinoma (PAAD) (Fig. 2A).

Given that certain tumors lacked normal tissue samples in the TCGA dataset, TCGA-GTEX data was concurrently analyzed. This analysis revealed that TGFBR1 was upregulated in 18 tumors, including GBM, GBMLGG and LGG, while being downregulated in 10 tumors, such as UCEC, LUAD, KIRP and COAD. No significant expression changes were detected between normal and tumor tissues in READ, testicular germ cell tumors (TGCT), acute lymphoblastic leukemia and kidney chromophobe (KICH) (Fig. 2B). The

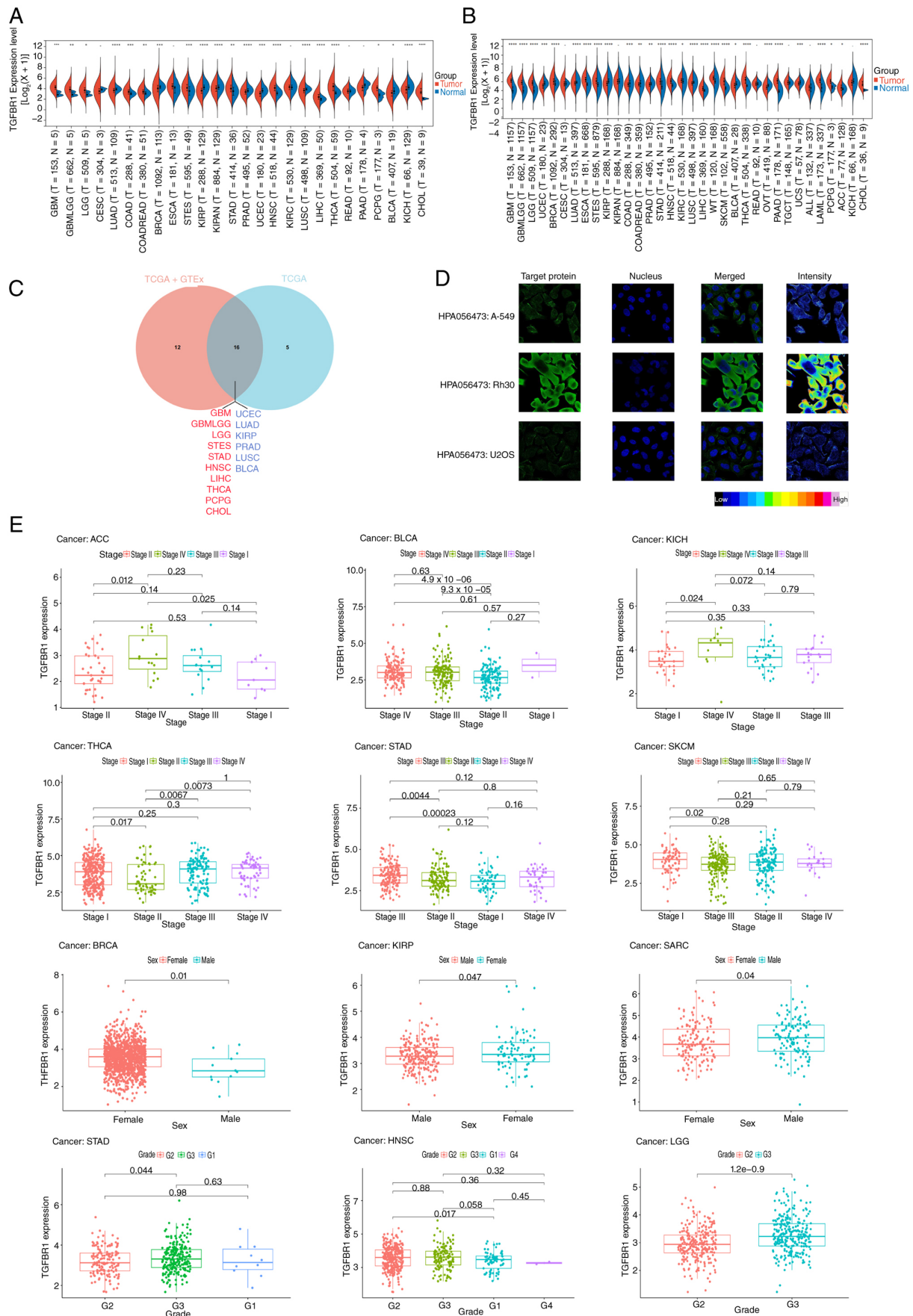


Figure 2. TGFBR1 expression in the pan-cancer. (A) TGFBR1 expression in the 26 tumors derived from TCGA database. (B) TGFBR1 expression in the 34 tumors derived from the TCGA-GTEx samples. (C) A 3D Venn diagram of the intersection of TCGA database and TCGA-GTEx database algorithm. (D) Immunofluorescence staining was performed to determine the subcellular localization of TGFBR1 using data from the HPA database. (E) Association between TGFBR1 expression in tumors and clinical features. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. TGFBR1, TGF- β receptor 1; TCGA, The Cancer Genome Atlas; GTEx, Genotype-Tissue Expression; HPA, Human Protein Atlas.

intersection of the two analyses was taken, revealing consistent TGFBR1 upregulation in 10 cancer types (GBM, GBMLGG, LGG, STES, STAD, HNSC, LIHC, THCA, PCPG and CHOL) and significant downregulation in six tumors (LUAD, KIRP, PRAD, UCEC, LUSC and BLCA; Fig. 2C). In addition, based on the HPA database, immunofluorescent localization of TGFBR1 in A-549, Rh30 and U-2 osteosarcoma cells demonstrated that TGFBR1 predominantly localizes in the cytoplasm of these cells (Fig. 2D). Collectively, these findings indicate that TGFBR1 expression alterations are cancer-type dependent rather than uniformly increased across malignancies.

TGFBR1 expression varies across selected clinicopathological subgroups. To investigate the association between TGFBR1 and clinical indicators, TGFBR1 expression levels were assessed across different clinical stages, sexes and grades for each type of cancer. Significant differences in the expression of TGFBR1 in different clinical stage samples were observed in the following four tumors: STAD, ovarian serous cystadenocarcinoma (OV), BLCA and adrenocortical carcinoma (ACC). Furthermore, in BRCA, TGFBR1 expression levels were higher in female compared with male patients. However, female patients with KIRP and sarcoma (SARC) exhibited lower TGFBR1 expression levels compared with male patients. Subsequently, the effect of TGFBR1 expression on tumor malignancy was examined and significant variation was observed in its increased expression among STAD, HNSC and LGG. TGFBR1 expression levels did not differ significantly across pathological stages in BRCA, COAD or ESCA, amongst others (Figs. 2E and S1-S3).

Prognostic importance of TGFBR1 expression across cancers and external validation in GC. Prognostic importance of TGFBR1 expression was initially evaluated across TCGA cancer cohorts using univariable Cox regression and Kaplan-Meier survival analyses. Higher TGFBR1 expression was associated with shorter OS in numerous cancer types, including LGG, ACC, mesothelioma (MESO), KIRP, STAD and BLCA (Figs. 3A and B and S4). Similarly, elevated TGFBR1 expression was associated with poorer DSS in LGG, ACC, KIRP, STAD, BLCA, THCA, and MESO (Figs. 3C and D and S5). Complementary analysis using GEPIA2 indicated an association between higher TGFBR1 expression and unfavorable OS, whereas no significant association was observed for DFS (Fig. 3E and G). In addition, higher TGFBR1 expression was associated with shorter DFI in ACC, HNSC, LIHC and PAAD and with shorter PFI in ACC, BLCA, GBM and LUAD (Figs. 3F and H; S6 and S7). To assess whether these prognostic associations were independent of key clinicopathological variables, multivariable Cox regression analyses were performed with adjustment for age, sex and pathological stage. TGFBR1 expression remained significantly associated with poorer OS in MESO, ACC and KIRP (Fig. 4A). For DSS, significant associations were observed in ACC, ESCA, MESO, LUSC, STAD and BLCA after multivariable adjustment (Fig. 4B). These findings indicate that the prognostic relevance of TGFBR1 varies among cancer types and may be influenced by clinicopathological characteristics in certain malignancies.

The prognostic value of TGFBR1 in GC was further evaluated in the independent GSE15459 cohort. Kaplan-Meier

analysis showed that patients with high TGFBR1 expression exhibited significantly shorter OS compared with those with low expression (Fig. 4C). Each one-standard-deviation increase in TGFBR1 expression was associated with an increased risk of mortality in the univariable Cox model (HR=1.27; 95% CI: 1.03-1.57). This association remained significant after adjustment for age, sex and pathological stage (HR=1.38; 95% CI: 1.10-1.74). The sensitivity model additionally adjusted for Lauren classification yielded a comparable result (HR=1.42; 95% CI: 1.10-1.82). Exploratory subgroup analyses exhibited significant associations between higher TGFBR1 expression and poorer OS among patients aged ≥ 65 years, male patients, patients with stage III-IV disease and patients with intestinal-type GC (Fig. 4E). However, because formal interaction tests were not performed, these subgroup findings should not be interpreted as evidence of significant differences in the prognostic effect of TGFBR1 between subgroups. Collectively, the multivariable TCGA analyses and independent GSE15459 validation support the prognostic relevance of TGFBR1 in selected malignancies, particularly GC.

TGFBR1 expression is associated with distinct immune and stromal contexts across cancers. The tumor immune microenvironment (TIME) serves an important role in influencing tumor progression. To explore the association between TGFBR1 expression and immune cells within this microenvironment, analysis using the TIMER2 database was performed to evaluate the association between TGFBR1 and immune cell presence. The association between TGFBR1 expression and immune cell populations such as CD4⁺ T cells, CD8⁺ T cells, Tregs, B cells, neutrophil, monocyte, myeloid derived suppressor cells, natural killer cell, mast cell, cancer associated fibroblast (CAF), common lymphoid progenitor, common myeloid progenitor and endothelial cells are represented as heatmaps (Fig. 5A). TGFBR1 expression exhibited positive associations with CAF estimates in numerous cancer types, whereas its associations with lymphoid and myeloid cell populations varied among tumors and analytical methods. In CESC, an inverse correlation between TGFBR1 expression and T cells CD8⁺, B cell infiltration was observed. Furthermore, there was a positive correlation between CAF infiltration and TGFBR1 expression in other cancers except for uveal melanoma (UVM), TGCT, skin cutaneous melanoma (SKCM)-metastasis and lymphoid neoplasm diffuse large B-cell lymphoma (DLBC). Furthermore, it was found that TGFBR1 expression was positively correlated with neutrophils in PRAD. Overall, the present findings suggest that TGFBR1 expression is associated with cancer-type-specific immune and stromal characteristics rather than a uniform pan-cancer immune pattern.

TGFBR1 expression is associated with cancer-specific genomic and tumor-composition features. To characterize the genomic and tumor-composition features associated with TGFBR1 expression, the TMB, tumor purity, MSI, neoantigen, MATH, ploidy, HRD and LOH were separately calculated for each cancer sample. The direction and magnitude of these associations varied markedly across cancer types. As shown in Fig. 5B, a significant correlation between high TGFBR1 expression and TMB in four tumors was found, including a strong positive association in ACC and PCPG. In KIRP and

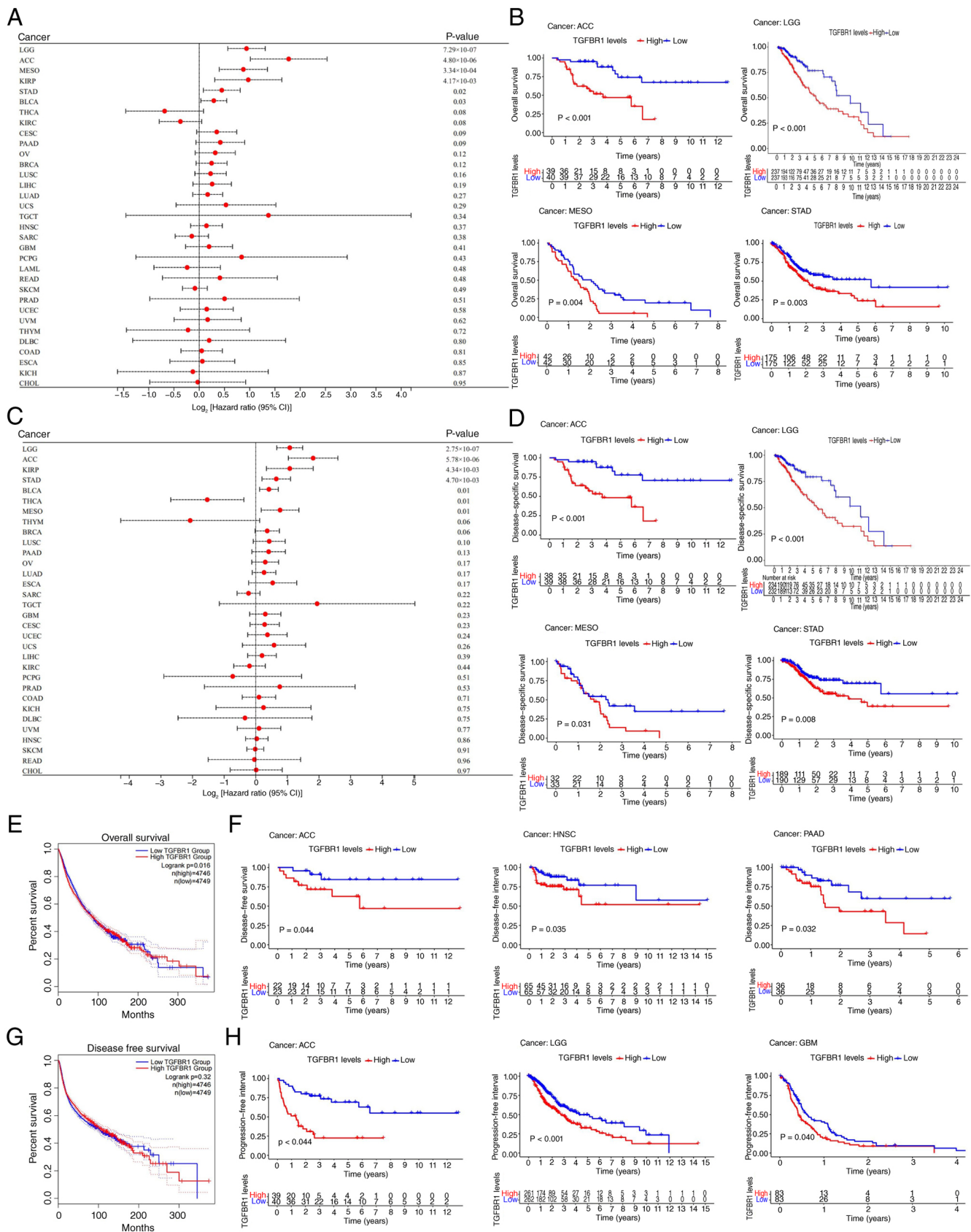


Figure 3. Univariate Cox regression analysis and Kaplan-Meier survival curves were conducted to evaluate the association of TGFBR1 expression with patient outcomes. (A) Univariable Cox regression analysis of the association between TGFBR1 expression and overall survival across cancer types. (B) Kaplan-Meier overall survival curves according to TGFBR1 expression in ACC, LGG, MESO and STAD. (C) Univariable Cox regression analysis of the association between TGFBR1 expression and disease-specific survival across cancer types. (D) Kaplan-Meier disease-specific survival curves according to TGFBR1 expression in ACC, LGG, MESO and STAD. (E) Overall survival analysis according to TGFBR1 expression using GEPIA2. (F) Kaplan-Meier disease-free interval curves according to TGFBR1 expression in ACC, HNSC and PAAD. (G) Disease-free survival analysis according to TGFBR1 expression using GEPIA2. (H) Kaplan-Meier progression-free interval curves according to TGFBR1 expression in ACC, LGG and GBM. TGFBR1, TGF- β receptor 1; OS, overall survival; DFI, disease-free interval; PFI, progression-free interval; DSS, disease-specific survival; DFS, disease-free survival.

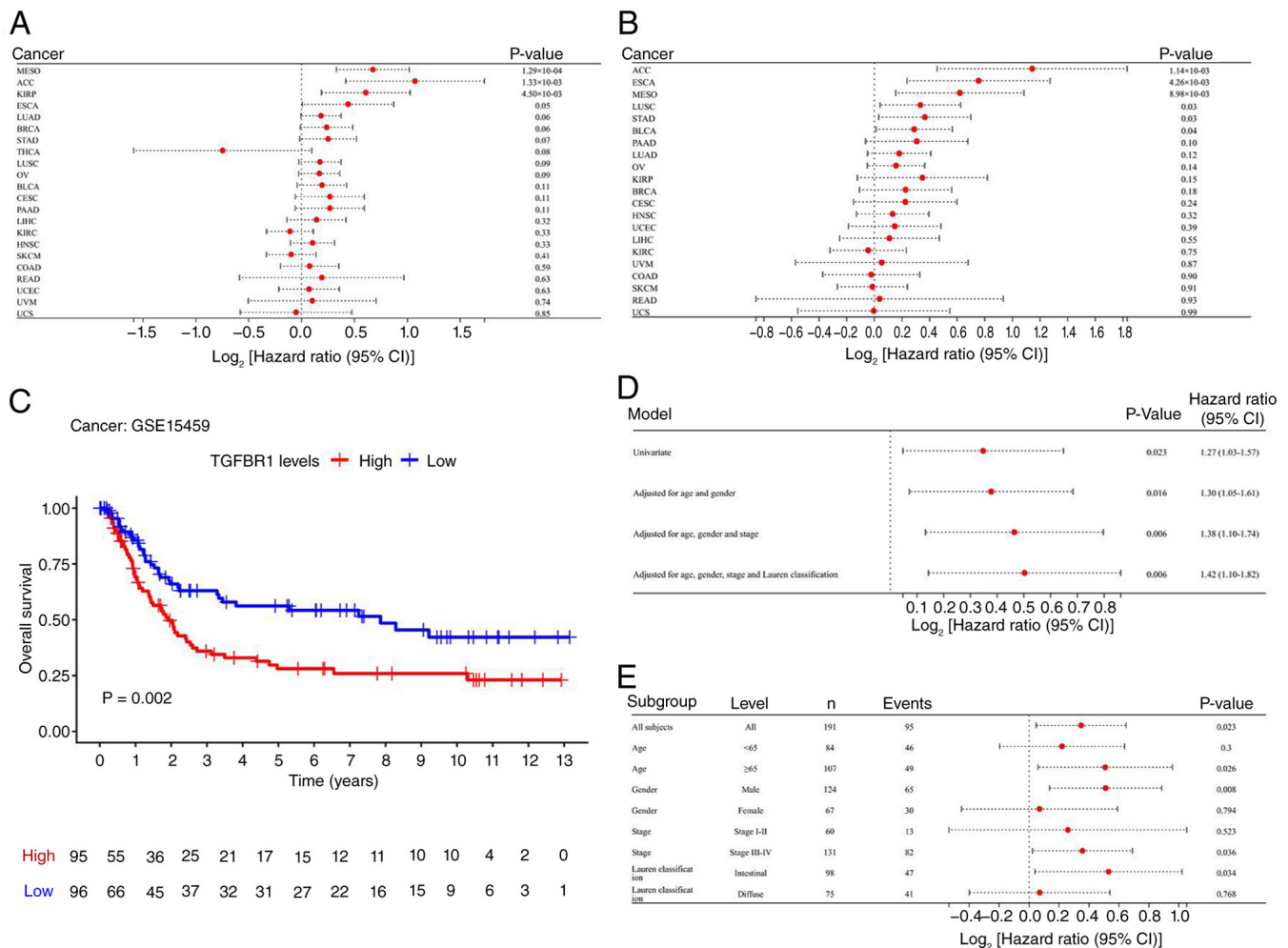


Figure 4. Multivariable Cox regression and external validation of the prognostic value of TGFBR1. (A) Multivariable Cox analysis of TGFBR1 expression and overall survival across TCGA cancer types, adjusted for age, sex and pathological stage. (B) Multivariable Cox analysis of TGFBR1 expression and disease-specific survival across selected TCGA cancer types, adjusted for age, sex and pathological stage. (C) Kaplan-Meier analysis of OS in the independent GSE15459 gastric cancer cohort using the median TGFBR1 expression as the cut-off. (D) Univariable and sequentially adjusted multivariable Cox regression analyses in GSE15459. (E) Exploratory subgroup Cox analyses according to age, sex, stage and Lauren classification. TGFBR1, TGF- β receptor 1; OS, overall survival.

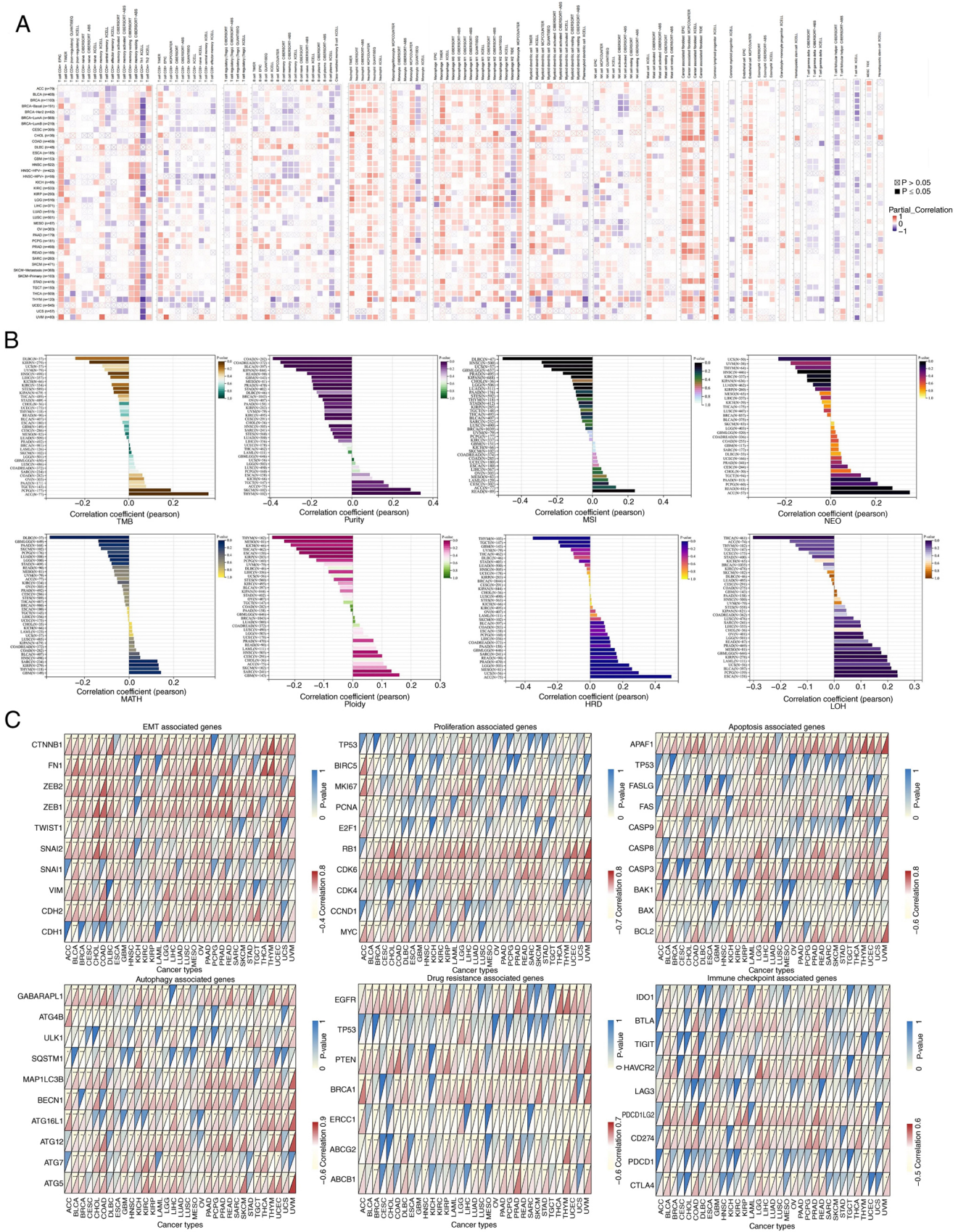
HNSC, high expression of TGFBR1 exhibited a negative correlation with TMB. TGFBR1 expression was significantly associated with purity in 18 tumors, such as thymoma (THYM), SKCM, GBM, CESC, LUAD, COAD, COADREAD, BRCA, STES, KIRP, KIPAN, STAD, PRAD, HNSC, KIRC, READ, OV, BLCA and included a significant positive correlation in THYM and SKCM tumors. Furthermore, the MSI scores for each tumor were obtained and the MSI and gene expression data of the samples were integrated.

After calculating the Pearson correlation in each tumor, a high expression of TGFBR1 was found to be significantly associated with 10 tumors types, including READ. Expression of TGFBR1 was significantly correlated with neoantigen (NEO) in four types of tumors: READ, ACC, KIPAN and HNSC. MATH represents the deviation of the distribution of MAF values for tumor-specific variable sites, indicating the extent to which MAF differs from the overall MAF distribution within this sample. Therefore, the association between TGFBR1 and MATH was analyzed. Results revealed a significant correlation across four tumor types, with a notable positive correlation observed in KIRP tumors. This indicated that

increased expression of TGFBR1 was associated with greater tumor heterogeneity. TGFBR1 expression was nominally associated with tumor ploidy in six cancer types (Fig. 5B), showing a positive correlation in SARC and negative correlations in ESCA, KIRP, THYM, THCA and MESO.

Genomic instability is a potential factor in tumorigenesis and DNA damage is one of the primary causes of genomic instability (36). HRD produces specific, quantifiable and stable genomic alterations. It was observed that TGFBR1 was significantly associated with HRD in 12 tumors, with significant positive associations in 9 tumors: GBMLGG, LGG, COADREAD, SARC, PRAD, LIHC, MESO, uterine carcinosarcoma and ACC, with significant negative associations reported in 3 tumors, namely GBM, THYM and TGCT.

HRD status is a key indicator of the treatment choice and prognosis of numerous tumor types, representing new possibilities for tumor-targeted therapy and immunotherapy (36,37). LOH is a chromosomal event capable of causing the loss of the entire gene and its nearby chromosomal regions (38,39). Pearson correlation analysis showed that TGFBR1 expression was nominally associated with genome-wide LOH scores in



10 cancer types, including a positive correlation in THCA and negative correlations in GBMLGG, LGG, acute myeloid leukemia, ESCA, KIRP, PRAD, OV, PCPG and BLCA. These findings indicate cancer-type-specific associations between TGFBR1 expression and overall LOH burden. However, the LOH score used in the present analysis represents genome-wide LOH rather than LOH specifically occurring at the TGFBR1 locus. Therefore, these results indicate associations between TGFBR1 expression and overall genomic LOH burden but do not demonstrate loss of heterozygosity or functional loss of TGFBR1 itself.

TGFBR1 exhibits cancer-type-specific co-expression patterns with tumor-related genes. To determine whether TGFBR1 expression was associated with common or cancer-type-specific functional gene-expression patterns, its correlations with selected genes involved in EMT, cell proliferation, apoptosis, autophagy, drug resistance and immune checkpoints was examined across cancer types. The correlation patterns varied notably among malignancies, indicating that the transcriptional context associated with TGFBR1 was cancer-type dependent rather than uniform across cancers (Fig. 5C). In LGG, TGFBR1 expression showed coordinated positive associations with multiple EMT-associated genes, including VIM, CDH2 and SNAI1, as well as proliferation-associated genes, including MKI67 and PCNA, and immune checkpoint-related genes, including PDCD1LG2 and CD274. Positive correlations between TGFBR1 expression and autophagy-related genes, including ATG5 and BECN1, were more evident in UVM. In STAD, TGFBR1 expression was associated with apoptosis-related genes, including BAX, BCL2 and CASP3. In addition, TGFBR1 expression showed correlations with drug resistance/treatment response-related genes, including EGFR, PTEN and BRCA1, across several cancer types. Collectively, these findings indicate that the co-expression patterns of TGFBR1 with tumor-associated genes vary across cancer types, further supporting the context-dependent nature of TGFBR1-associated molecular features.

Functional enrichment reveals TGFBR1-associated stress-response, proliferation, adhesion and cytoskeletal programs. To characterize the biological processes associated with TGFBR1 at the pathway level, the top 50 TGFBR1-correlated genes identified using GEPIA2 were subjected to GO and KEGG enrichment analyses. KEGG pathway analysis indicated that TGFBR1 was associated with the 'regulation of the actin cytoskeleton', 'PI3K/Akt signaling pathway', 'protein processing in endoplasmic reticulum', 'axon guidance', 'human cytomegalovirus infection' and 'sphingolipid signaling pathway', amongst others (Fig. 6A).

In addition, it was found that in the present CC analysis, that TGFBR1-associated genes were enriched in the 'cytosol', 'early endosome', 'membrane coat', 'coated membrane', 'vesicle coat', 'endosome', 'COPII vesicle coat', 'coated vesicle', 'nucleoplasm' and 'pseudopodia'. MF analysis showed that the role of TGFBR1 in tumor pathogenesis was associated with 'enzyme binding', 'kinase binding', 'clathrin heavy chain binding' and 'protein kinase binding'. BP analysis showed that TGFBR1-associated genes were primarily involved in processes including 'cargo loading into vesicle',

'vesicle-mediated transport', 'cytosolic transport' and 'regulation of vesicle-mediated transport' (Fig. 6B). Representative pan-cancer GSEA further demonstrated that the transcriptional programs associated with TGFBR1 varied markedly among cancer types. KEGG- and GO-based analyses revealed cancer-type-dependent enrichment of immune-associated and cellular regulatory pathways. Representative enriched KEGG terms included 'KEGG_CYTOSOLIC_DNA_SENSING_PATHWAY', 'KEGG_RIG_LIKE_RECEPTOR_SIGNALING_PATHWAY', 'KEGG_TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY', and 'KEGG_PROTEIN_PROCESSING_IN_ENDOPLASMIC_RETICULUM'. GO-based analysis further identified variable enrichment of biological processes, including 'GO_CELLULAR_RESPONSE_TO_INTERFERON_GAMMA', 'GO_IMMUNE_RESPONSE', and 'GO_MRNA_PROCESSING' across different cancer types. These findings indicate that TGFBR1-associated transcriptional programs are context-dependent rather than uniformly enriched across malignancies (Fig. 6C and D).

GC is among the most prevalent cancers globally, particularly in Asia (40). Given the particular focus on GC, the present study further evaluated TGFBR1-associated transcriptional programs in the independent GSE15459 cohort. Hallmark analysis identified significant associations between TGFBR1 expression and the following transcriptional programs: 'HALLMARK_APOPTOSIS', 'HALLMARK_HYPOXIA', 'HALLMARK_P53_PATHWAY', 'HALLMARK_TNFA_SIGNALING_VIA_NFKB' and 'HALLMARK_UNFOLDED_PROTEIN_RESPONSE' (Fig. 6E). The 'HALLMARK_APOPTOSIS', 'HALLMARK_HYPOXIA' and 'HALLMARK_TNFA_SIGNALING_VIA_NFKB' were enriched in the TGFBR1-high group, whereas 'HALLMARK_P53_PATHWAY' and 'HALLMARK_UNFOLDED_PROTEIN_RESPONSE' were enriched in the TGFBR1-low group (Fig. 6E). The different enrichment directions indicated that the associations between TGFBR1 expression and cellular stress or apoptosis-associated programs were heterogeneous rather than uniformly directed. GO-based GSEA further showed that 'GOBP_ACTIN_CYTOSKELETON_REORGANIZATION', 'GOBP_FOCAL_ADHESION_ASSEMBLY', 'GOBP_POSITIVE_REGULATION_OF_FOCAL_ADHESION_ASSEMBLY' and 'GOBP_POSITIVE_REGULATION_OF_EPITHELIAL_CELL_PROLIFERATION' were significantly enriched in the TGFBR1-high group (Fig. 6F). Collectively, these findings demonstrate that TGFBR1 expression was associated with heterogeneous apoptosis- and stress-response-associated transcriptional programs, together with epithelial proliferation, cell adhesion and cytoskeletal-remodeling programs in GC. These transcriptional associations provided a rationale for the subsequent functional evaluation of migration, proliferation and apoptosis in GC cells.

TGFBR1 knockdown suppresses malignant phenotypes in GC cells. GC is one of the three leading causes of cancer-associated mortality, with >1 million new cases occurring each year (41). Based on aforementioned available bioinformatics data analysis, the present study chose to determine the expression of TGFBR1 in tumor cells and its impact on tumor prognostic value through further experimental studies in GC. Western blotting experimental results showed that TGFBR1

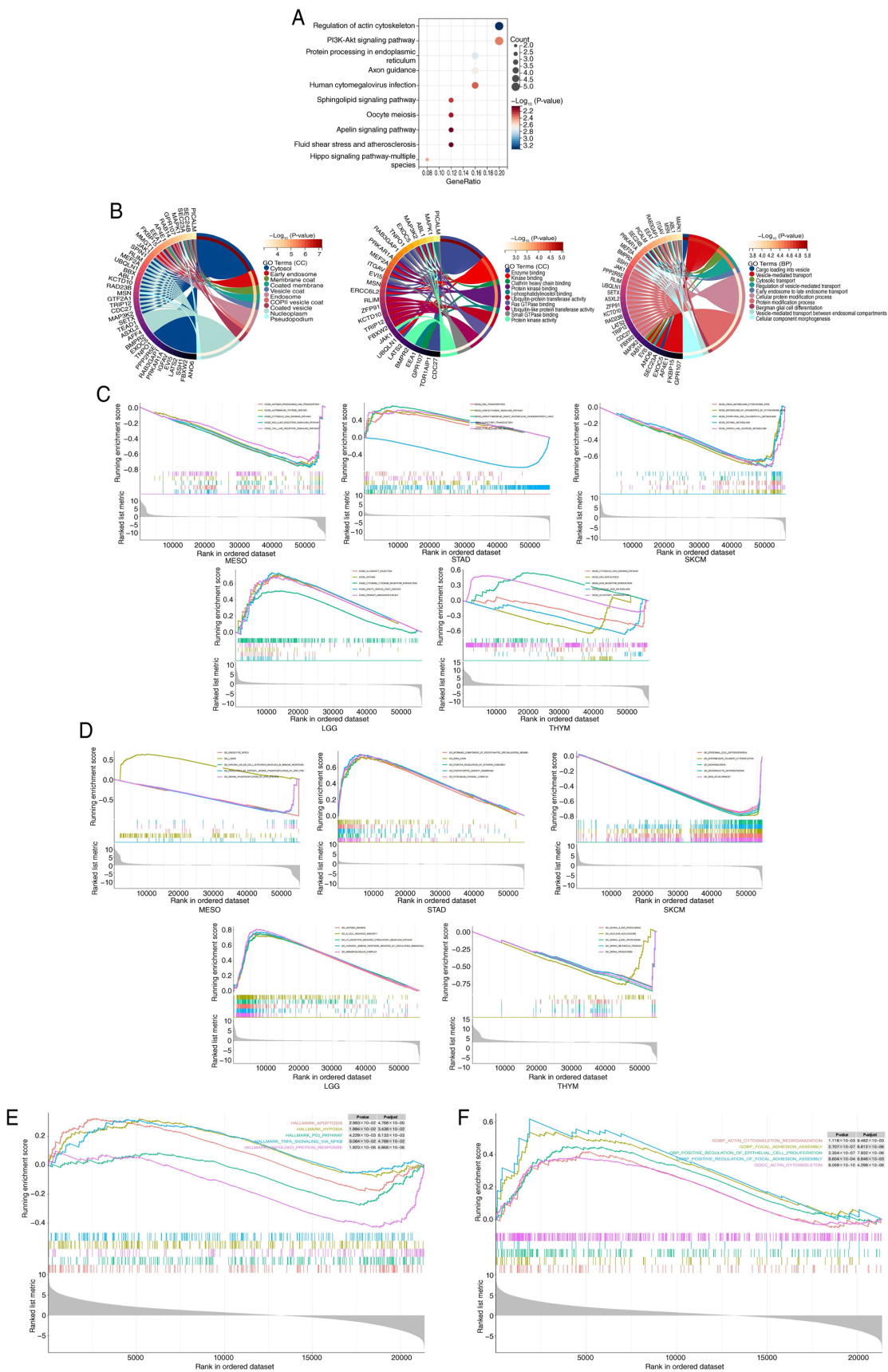


Figure 6. Functional enrichment analysis of TGFBR1-associated genes and experimental validation in GC cells. (A) KEGG pathway analysis of the 50 TGFBR1-associated genes. (B) Analysis of GO functional enrichment (CC, MF and BP). (C) KEGG-based GSEA of TGFBR1-associated pathways in MESO, STAD, SKCM, LGG and THYM. (D) GO-based GSEA of TGFBR1-associated biological processes in MESO, STAD, SKCM, LGG and THYM. (E) Hallmark GSEA of the independent GSE15459 GC cohort. (F) GO-based GSEA of the GSE15459 cohort. TGFBR1, TGF- β receptor 1; GC, gastric cancer; GO, Gene Ontology; GSEA, Gene Set Enrichment Analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; CC, cellular component; MF, molecular functions; BP, biological processes; adjust., adjusted; MESO, mesothelioma; STAD, stomach adenocarcinoma; SKCM, skin cutaneous melanoma; LGG, brain lower grade glioma; THYM, thymoma.

was significantly upregulated in GC cell lines compared with GES-1, the human normal gastric mucosal cells (Fig. 7A). This was consistent with the data analysis results. To reduce the possibility of sequence-specific effects, two independent TGFBR1-targeting siRNAs were used. Both siTGFBR1-1 and siTGFBR1-2 significantly reduced TGFBR1 protein expression in YCCEL1 and AGS cells compared with the NC group, demonstrating effective TGFBR1 knockdown (Fig. 7B and C). Transwell assays showed that TGFBR1 knockdown significantly reduced the migration of both YCCEL1 and AGS cell lines, with consistent effects observed using the two independent siRNAs (Fig. 7D and E). Western blotting further showed that TGFBR1 knockdown was accompanied by significantly reduced expression of the mesenchymal-associated proteins N-cadherin, Vimentin, Snail, β -catenin and ZEB1, together with significantly increased E-cadherin expression in both YCCEL1 and AGS cell lines (Fig. 7F and G), consistent with suppression of an EMT-associated phenotype. This suggests that TGFBR1 may promote GC cell migration. CCK-8 assays further demonstrated that knockdown of TGFBR1 significantly reduced the proliferative capacity of YCCEL1 and AGS cells, particularly at the later time points following transfection (Fig. 7H and I). Flow-cytometric analysis using Annexin V-APC/PI staining showed that TGFBR1 knockdown significantly increased apoptosis in both cell lines compared with the NC group (Fig. 7J and L). Consistently, western blotting analysis demonstrated significantly increased Bax expression and significantly decreased Bcl2 expression following TGFBR1 knockdown and similar expression changes were observed with both independent siRNAs (Fig. 7K and M). These results indicate that TGFBR1 knockdown suppressed proliferation and migration, altered EMT-associated protein expression and promoted apoptosis in GC cell lines.

Discussion

In metastatic human melanoma cells, TGFBR1 could promote tumor angiogenesis by upregulating MMP-9 (17). TGFBR1-associated signaling has also been implicated in GC cell proliferation, migration, invasion and EMT (22), as well as in the proliferation and migration of papillary thyroid cancer cells (42). In addition, genetic variation in TGFBR1 has been associated with the prognosis of patients with GC (15). Previous studies have further characterized genomic alterations affecting mediators of TGF- β superfamily signaling across cancer types and reported elevated TGFBR1 expression, prognostic relevance and immune associations in STAD (21,43). These findings demonstrate that TGFBR1 is an established cancer-associated molecule. However, its cancer-type-specific expression patterns, adjusted prognostic relevance and associated immune, genomic and transcriptional contexts have not been fully evaluated within a consistent pan-cancer analytical framework.

In the present study, an integrated pan-cancer analysis of TGFBR1 was performed from the perspective of gene expression, prognosis, the TIME, genomic features and transcriptional programs. Significant differences in TGFBR1 expression between tumor and normal tissues were observed in the TCGA and TCGA-GTEx datasets. TGFBR1 expression was found to be upregulated in numerous cancer types,

including GBMLGG, GC and thyroid cancer, but was reduced in other malignancies. These findings indicate that alterations in TGFBR1 expression are cancer-type dependent rather than uniformly increased across tumors. Associations between TGFBR1 expression and clinicopathological variables were also observed in selected malignancies. For example, TGFBR1 expression differed across pathological stages in STAD, OV, BLCA and ACC and across tumor grades in STAD, HNSC and LGG. Sex-associated differences were identified in BRCA, KIRP and SARC. These results suggest that the clinicopathological relevance of TGFBR1 may vary among tumor types; however, this did not establish TGFBR1 as a universal diagnostic marker.

The prognostic value of TGFBR1 in numerous cancer types has not been comprehensively studied yet. In the present study, analysis based on the TCGA database clarified the multifaceted prognostic impact of TGFBR1 upregulation on OS. Upregulated TGFBR1 had an impact on the overall prognosis such as OS. In particular, in cancers such as ACC, LGG, MESO and STAD, elevated TGFBR1 expression was associated with poor OS and DSS. For ACC, HNSC and PAAD, elevated TGFBR1 expression was associated with poor DFI. Cancers such as ACC, HNSC and PAAD were associated with poor PFI. In addition, dysregulated TGFBR1-mediated TGF- β signaling contributes to tumor progression by regulating cancer cell proliferation, epithelial-mesenchymal transition and metastatic dissemination (44,45). When reviewing the relevant databases, a lack of studies exploring the prognostic impact of TGFBR1 on cancers such as ACC and BLCA was noted. Therefore, the present study was expanded. Results highlighted the association between TGFBR1 expression and the prognosis of these unexplored malignant tumors. Future research should aim to pay particular attention the underlying mechanisms behind this association to enhance the understanding of tumor evolution.

Notably, the prognostic relevance of TGFBR1 in GC was further evaluated in the independent GSE15459 cohort. Higher TGFBR1 expression was associated with shorter OS and this association remained significant after adjustment for age, sex and pathological stage. A sensitivity model additionally adjusted for Lauren classification yielded a comparable result. These findings extend previous TCGA-STAD-based observations (43) by demonstrating that the prognostic association of TGFBR1 can be reproduced in an independent GC cohort. Despite this, the present subgroup analyses were exploratory and the absence of formal interaction tests prevents conclusions regarding differences in the prognostic effect of TGFBR1 between patient subgroups.

With the advent of immunotherapy, the tumor microenvironment has gained increasing attention (46,47). The tumor microenvironment is composed of tumor cells, immune cells, stromal cells, cytokines, chemokines and extracellular-matrix components and is associated with tumor growth, metastasis, immune escape and treatment response (48-50). To characterize the immune and stromal contexts associated with TGFBR1 expression across cancers, the present study analyzed its association with immune and stromal cell populations. In the present study, TGFBR1 expression was found to be positively associated with CAF estimates in a number of cancer types, whereas its associations with lymphoid and

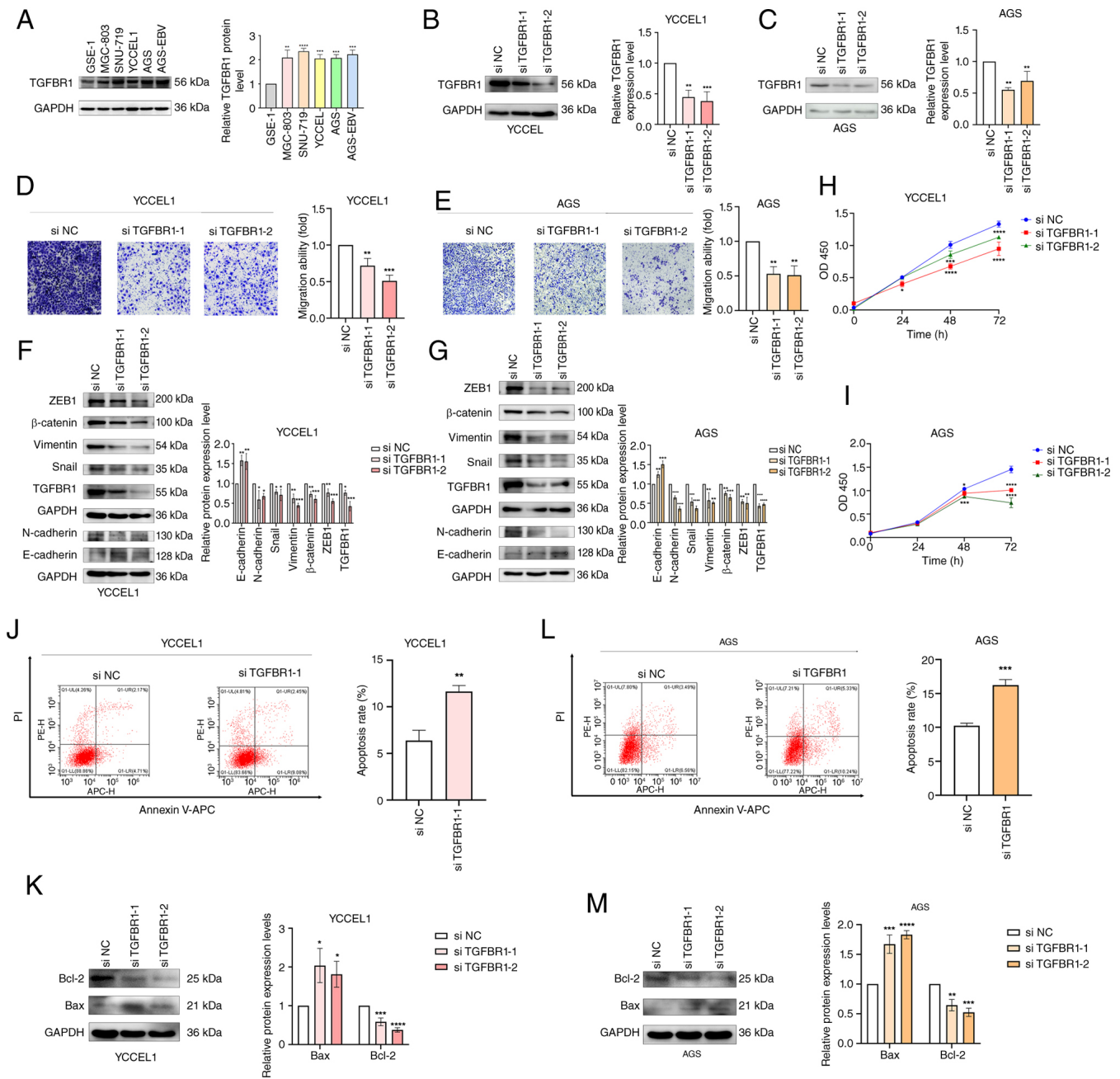


Figure 7. Effects of TGFBR1 knockdown on proliferation, migration, EMT-associated protein expression and apoptosis in GC cells. (A) Expression of TGFBR1 in human normal gastric mucosal cells and GC cells. (B) Knockdown efficiency of TGFBR1 in YCCEL1 cells. (C) AGS cells. (D) Transwell migration assay of YCCEL1 and (E) Transwell migration assay of AGS cells following TGFBR1 knockdown. (F) Western blotting analysis of EMT-associated proteins in YCCEL1 cells following TGFBR1 knockdown. (G) Western blotting analysis of EMT-associated proteins in AGS cells following TGFBR1 knockdown. (H) CCK-8 assay of YCCEL1 cell proliferation following TGFBR1 knockdown. (I) CCK-8 assay of AGS cell proliferation following TGFBR1 knockdown. (J) Flow cytometric analysis of apoptosis in YCCEL1 cells following TGFBR1 knockdown. (K) Western blotting analysis of Bax and Bcl-2 expression in YCCEL1 cells following TGFBR1 knockdown. (L) Flow-cytometric analysis of apoptosis in AGS cells following TGFBR1 knockdown. (M) Western blotting analysis of Bax and Bcl-2 expression in AGS cells following TGFBR1 knockdown. Data are presented as the mean $\pm$ SD. Comparisons among NC, si TGFBR1-1 and si TGFBR1-2 groups were performed using one-way ANOVA followed by Dunnett's multiple comparisons tests. CCK-8 data were analyzed using two-way ANOVA followed by Tukey's multiple comparisons tests. Flow-cytometric apoptosis data involving two groups were analyzed using a two-tailed unpaired Student's t-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. TGFBR1, TGF- β receptor 1; EMT, epithelial-mesenchymal transition; GC, gastric cancer; si, small interfering; CCK-8, Cell Counting Kit-8; NC, negative control; OD, optical density.

myeloid cell populations varied between malignancies and analytical methods. These findings were broadly consistent with the established involvement of TGF- β signaling in immune regulation, fibroblast activation and stromal remodeling (51,52). However, given that immune and stromal infiltration was inferred from bulk transcriptomic data, these

associations remain descriptive and do not demonstrate direct regulation of specific immune-cell populations by TGFBR1. In addition, no immune-checkpoint inhibitor-treated cohort was analyzed; therefore, the present findings could not establish whether TGFBR1 expression predicts immunotherapy response.

The present study also examined the associations of TGFBR1 expression with TMB, tumor purity, MSI, neoantigen burden, MATH, ploidy, HRD and LOH to characterize its genomic and tumor-composition contexts. The direction and magnitude of these correlations varied across cancer types, indicating heterogeneous rather than uniform associations. These analyses were exploratory and did not demonstrate that TGFBR1 directly regulates genomic instability or treatment response. In addition, the LOH score represents genome-wide LOH burden rather than allelic loss specifically at the TGFBR1 locus.

Previous studies have demonstrated that TGFBR1 regulates a number of physiological and pathological processes, including cell cycle progression and epithelial-to-stromal cell transformation (9-11). TGFBR1-associated signaling has also been investigated in relation to proliferative activity and treatment response in GBM models (53). In the present co-expression analysis, TGFBR1 expression was associated with selected genes related to EMT, proliferation, apoptosis, autophagy, drug resistance and immune checkpoints. Coordinated associations with numerous EMT-, proliferation- and immune checkpoint-related genes were observed in LGG, whereas associations with selected apoptosis-related genes were detected in STAD. TGFBR1 was also found to be correlated with genes such as EGFR, PTEN and BRCA1 in a number of cancer types. Functional enrichment analysis showed that TGFBR1-correlated genes were associated with cancer-related pathways, including 'Regulation of actin cytoskeleton', 'PI3K-Akt signaling pathway', 'Protein processing in endoplasmic reticulum', and 'Axon guidance'. GO-based enrichment analysis further indicated involvement of cellular organization and vesicle-associated processes, including 'vesicle-mediated transport', 'cellular protein modification process', and 'protein folding'.

Pan-cancer GSEA further revealed heterogeneous enrichment patterns across malignancies, indicating that TGFBR1-associated transcriptional programs are context dependent. In the independent GSE15459 GC cohort, hallmark GSEA identified enrichment of several stress-response programs, including 'HALLMARK_APOPTOSIS', 'HALLMARK_HYPOXIA', 'HALLMARK_TNFA_SIGNALING_VIA_NFKB', 'HALLMARK_P53_PATHWAY', and 'HALLMARK_UNFOLDED_PROTEIN_RESPONSE'. 'HALLMARK_APOPTOSIS', 'HALLMARK_HYPOXIA', and 'HALLMARK_TNFA_SIGNALING_VIA_NFKB' were enriched in the TGFBR1-high group, whereas 'HALLMARK_P53_PATHWAY' and 'HALLMARK_UNFOLDED_PROTEIN_RESPONSE' were enriched in the TGFBR1-low group. These findings suggest heterogeneous stress-response states rather than uniform regulation of a single pathway. Notably, enrichment of an apoptosis-associated gene set reflects coordinated transcriptional activity and should not be equated with direct measurement of apoptosis.

GO-based GSEA further exhibited enrichment of cell structural and proliferation-related programs, including 'GO_REGULATION_OF_ACTIN_CYTOSKELETON', 'GO_FOCAL_ADHESION_ASSEMBLY', and 'GO_POSITIVE_REGULATION_OF_EPITHELIAL_CELL_PROLIFERATION' in the TGFBR1-high group. Consistent with these associations, TGFBR1 knockdown reduced

proliferation and migration, altered EMT-associated protein expression and promoted apoptosis in YCCEL1 and AGS cells. These findings were consistent with previous studies showing that genetic or pharmacological inhibition of TGFBR1-associated signaling suppresses GC cell proliferation, migration, invasion or treatment resistance (22,23). The use of independent siRNAs further confirmed that TGFBR1 silencing reduced gastric cancer cell proliferation and migration, altered EMT-associated protein expression, and promoted apoptosis in YCCEL1 and AGS cells, supporting the reproducibility of the functional effects observed after TGFBR1 knockdown. Despite this, the present results did not establish a complete downstream mechanism and rescue experiments, pathway perturbation and *in vivo* studies are still required.

A number of limitations should be acknowledged. The majority of the present bioinformatics analyses were retrospective and based on bulk transcriptomic datasets and differences in sample composition, clinical annotation and treatment history may have influenced the present findings. In addition, despite age, sex and pathological stage being included in the multivariable models, grade and treatment information were not consistently available. Furthermore, external validation was limited to one GC cohort and the immune, genomic, co-expression and enrichment analyses were exploratory. Lastly, the present functional experiments were restricted to GC cell lines and lacked rescue experiments and *in vivo* validation.

In conclusion, the present study extends previous TGFBR1-associated research by defining its cancer-type-specific expression and prognostic patterns within a consistent pan-cancer framework. Multivariable analyses and independent validation in GSE15459 support its prognostic relevance in selected malignancies, particularly GC. The present immune, genomic, transcriptional and cell findings provide additional evidence for the context-dependent biological relevance of TGFBR1, while further mechanistic and prospective studies remain necessary.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Authors' contributions

XZ performed the bioinformatics and statistical analyses, prepared the figures and wrote the original draft of the

manuscript. LS performed the cell-based experiments, interpreted the experimental results, prepared the corresponding figures, and substantially revised and edited the manuscript. ZG contributed to data analysis, figure preparation and interpretation of the results. ML and YZ assisted with the cell-based experiments. XL and BT conceived and supervised the study, and reviewed and edited the manuscript. XL and BT confirm the authenticity of all the raw data. All authors read and approved the final manuscript. XZ and LS contributed equally to this work.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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