

CD8⁺ T cell-related KCTD5 contributes to malignant progression and unfavorable clinical outcome of patients with triple-negative breast cancer

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Abstract. Triple-negative breast cancer (TNBC) is a highly aggressive and heterogeneous subtype of breast cancer that lacks expression of estrogen receptor, progesterone receptor, and HER2, making it more challenging to treat with targeted therapies. The present study aimed to identify CD8⁺ T cell-associated genes, which could provide insight into the mechanisms underlying TNBC to facilitate developing novel immunotherapies. TNBC datasets were downloaded from public databases including The Cancer Genome Atlas, Molecular Taxonomy of Breast Cancer International Consortium, and Gene Expression Omnibus. Candidate genes were identified integrating weighted gene co-expression network analysis (WGCNA), differential gene expression, protein-protein-interaction network construction and univariate Cox regression analysis. Kaplan-Meier survival, multivariate Cox regression and receiver operating characteristic analysis were performed to evaluate the prognostic value of hub genes. Knockdown experiments, alongside wound healing, Cell Counting Kit-8 and Transwell migration and invasion assays were performed. In total, seven gene modules were associated with CD8⁺ T cells using WGCNA, among which potassium channel tetramerization domain 5 (KCTD5) was significantly upregulated in TNBC samples and was associated with poor prognosis. KCTD5 expression inversely associated with infiltration ratios of 'Macrophages M1', 'Plasma cells', and 'γδ T cells', but positively with 'activated Mast cells', 'Macrophages M0', and

'Macrophages M2'. As an independent prognostic indicator for TNBC, KCTD5 was also associated with drug sensitivity and the expression of programmed cell death protein 1, Cytotoxic T-Lymphocyte-Associated Protein 4 (CTLA4), CD274, Cluster of Differentiation 86 (CD86), Lymphocyte-Activation Gene 3 (LAG3), T Cell Immunoreceptor with Ig and ITIM Domains (TIGIT). Knockdown of KCTD5 significantly inhibited viability, migration and invasion of TNBC cells *in vitro*. KCTD5 was suggested to impact the tumor immune microenvironment by influencing the infiltration of immune cells and may serve as a potential therapeutic target for TNBC.

Introduction

Breast cancer is caused by unregulated cell proliferation. Breast cancer is the most common cancer in women globally (1), however, breast cancer can also occur in men. Triple negative breast cancer (TNBC) constitutes 10-20% of all breast cancer cases. TNBC is distinct from other subtypes of breast cancer and is characterized by the absence of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) (2). Patients with TNBC often exhibit early relapse and distant metastases, including those affecting the liver and central nervous system (3). There are often aggressive clinical features in patients with TNBC, including high tumor grade and mitotic indices with poor prognosis (4). The 5-year survival rate for patients with metastatic TNBC is 30% (5). Traditional treatment methods, including surgery, radiation therapy and chemotherapy are used in TNBC treatment, but the options are limited owing to the absence of molecular targets, in particular HER2 because it is a key target for targeted therapies (6). Currently, TNBC remains a challenge in breast cancer research and clinical practice.

The tumor microenvironment (TME) is composed of immune and stromal cells, blood vessels and extracellular matrix (7). CD8⁺ T cells, a type of cytotoxic T lymphocyte (CTL) are a component in TME that serve a role in immune surveillance against cancer through the recognition of tumor-specific antigens on the surface of cancer cells (8). Higher CD8⁺ T cell scores often indicate better survival in

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TNBC (9). In a number of patients with TNBC, the density of CD8⁺ T cell infiltration is not uniformly distributed. There is a notable pattern where density diminishes from the periphery towards the interior of the tumor cell clusters, with a rise again in the center of tumor (10). This phenomenon is explained by two hypotheses: Physical barrier hypothesis which suggests that the physical structure of TME, such as the extracellular matrix (ECM) fibers, creates a barrier that impedes the migration of CD8⁺ T cells into tumor cell clusters (11) and the biochemical barrier hypothesis which proposes that certain chemical factors or signals within TME may actively repel CD8⁺ T cells, preventing their infiltration into tumor cell clusters (12). CD8⁺ T lymphocytes serve a role in immunotherapy due to their responses to immune checkpoint inhibitors including programmed cell death-1 (PD-1) inhibitors (13). Significantly improved clinical outcomes are observed in patients with TNBC treated with checkpoint inhibitors; this is associated with a tissue-resident memory CD8⁺ T cell gene signature, extracted from tumor-free tissue (14). Exposure to carcinogens like 12-dimethylbenz[a]anthracene (DMBA) triggers an immune response in breast cancer cells, resulting in the production of immune-activating factors such as CCL21. This chemokine enhances the infiltration and activity of CD8⁺ T cells, which in turn, suppress the metastasis of breast cancer by recognizing and eliminating cancer cells that attempt to spread (15).

The aim of the present study was to identify the hub genes associated with CD8⁺ T cells that serve a role in the pathology of TNBC. This knowledge can be applied to the development of targeted immunotherapy approaches with the potential to improve treatment efficacy and patient outcomes, and further contribute to understanding of the immunological dynamics within this aggressive subtype.

Materials and methods

Research objects. mRNA expression data and corresponding clinical information of patients with BC were downloaded from three public databases by searching the key words 'triple negative breast cancer' or 'TNBC'. The inclusion criteria for samples from datasets were as follows: i) Human TNBC samples; and ii) tumor sample size or normal sample size was ≥ 10 . Samples missing survival information were excluded. The detailed information of all datasets were summarized as follows. Samples that tested negative for ER, PR and HER2 were selected from The Cancer Genome Atlas (TCGA; tcga-data.nci.nih.gov/tcga/) resulting 122 TNBC samples. The samples from Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) database were downloaded using cBioPortal tool (cbioportal.org/). Patients with negative 'ER_IHC', 'HISTOLOGICAL_SUBTYPE' of 'Ductal/NST' and 'HER2_SNP6' no 'Gain' were selected from the 'Clinical Data' file. Samples with negative ER, PR and HER2 status and 'BREAST_CANCER_TYPE_DETAILED' of 'Breast Invasive Ductal Carcinoma' were selected from the 'sample' file, resulting in 199 TNBC samples. Furthermore, from the Gene Expression Omnibus (GEO) database (ncbi.nlm.nih.gov/geo/), the following TNBC datasets were also obtained, including inclusion/exclusion criteria cited in the references: GSE76250 (16); GSE38959 (17); GSE21653 (18);

GSE31448 (19); GSE69031 (20); GSE45255 (21); GSE135565 (22) and GSE65194 (23) (Table I).

Tumor IMMune Estimation Resource database (cistrome.shinyapps.io/timer/) was used to assess expression of target gene in numerous types of cancers.

Weighted gene co-expression network analysis (WGCNA). WGCNA was performed using the 'WGCNA' R package (version 1.69) (24) based on gene expression values. The top 25% of genes were selected for WGCNA using variance analysis. Pearson correlation coefficients were calculated and an appropriate soft threshold (β) was applied by criteria of $R^2 \geq 0.9$ to ensure the constructed network met the scale-free network standard. A network was constructed using a one-step method and the adjacency matrix was transformed into a topological overlap matrix. A gene hierarchical clustering tree was generated using hierarchical clustering. Gene and module significance ($P < 0.05$) were calculated to determine the significance of genes and clinical information ($P < 0.05$), respectively. Heatmap was drawn based on 'pheatmap' package (<https://cran.r-project.org/web/packages/pheatmap/>; Version:1.0.12). Finally, key gene modules were identified by calculating correlation between modules and CD8⁺ T cell scores.

Differential gene expression analysis and functional enrichment analysis. The 'limma' package (25) was used to identify differentially expressed genes (DEGs) using cut off values of $\log_2$ fold change absolute > 0.5 and $P < 0.05$. To identify biological processes and pathways enriched with DEGs, enrichment analysis was performed using the 'clusterProfiler' function package (26) in R. Gene Ontology (GO, <http://geneontology.org/docs/ontology-documentation/>) and Kyoto Encyclopedia of Genes and Genomes (KEGG, <https://www.kegg.jp/>) were used as primary sources of functional annotation and GO terms including biological process (BP), molecular function (MF) and cellular component (CC) and KEGG pathways with a statistically significant enrichment cutoff of $P < 0.05$. Gene set enrichment analysis (GSEA) was used to identify pathways differentially regulated between high and low gene expression groups, defined based on the median of the expression of the target gene. Pathways with $P < 0.05$ were considered significantly enriched.

Protein-protein interaction (PPI) network. Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (27) (version 11.0, string-db.org/) was used to analyze and predict functional connections and interactions of proteins. PPI network of candidate genes was constructed and interaction pairs were filtered by default minimum required interaction score > 0.4 . Cytoscape software version 3.7.2 (28) was used to visualize the PPI network and the top 50 genes were selected using the maximum neighborhood component method with the cytoHubba plugin (29) (version 0.1).

Survival analysis. In survival analysis the datasets GSE69031, GSE45255, GSE135565, and GSE65194 were merged after the batch effects had been removed using the ComBat function from the R package 'sva'. Subsequently, R packages 'survival' (CRAN.R-project.org/package=survival) and 'survminer'

Table I. GEO datasets.

Dataset	TNBC samples	Normal tissue samples	Total breast cancer samples
GSE76250	165	33	-
GSE38959	30	13	-
GSE21653	237	29	-
GSE31448	263	31	-
GSE69031	19	-	130
GSE45255	19	-	139
GSE65194	41	11	153
GSE135565	84	-	-

(<https://cran.r-project.org/web/packages/survminer/>) were used to estimate the overall survival (OS) rates based on the Kaplan-Meier (KM) method. The log-rank test was performed to determine the significance of differences in survival rate. A multivariate Cox regression model was used to determine the independent prognostic factors for TNBC.

Immune landscape analysis. Cell-type Identification By Estimating Relative Subsets Of RNA Transcripts (CIBERSORT) (30) was used to calculate relative proportion of 22 types of immune cell in the TNBC samples from METABRIC database. The CIBERSORT software uses a deconvolution algorithm with 547 barcode genes to characterize the composition of immune cell infiltration based on the gene expression matrix. The sum of all estimated proportions of immune cell types in each sample equaled 1. xCell (xcell.ucsf.edu/, version 3.8) and Tumor-immune system interactions and drug bank database (TISIDB, cis.hku.hk/TISIDB/) tools were also used to calculate the abundance of immune cells.

Cell culture. A human normal breast epithelial cell line MCF10A and two human TNBC cell lines, MDA-MB-231 and MDA-MB-453, were purchased from Procell Life Science & Technology Co., Ltd. The cell lines were cultured following the methods described previously (31).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from MCF10A, MDA-MB-231 and MDA-MB-453 cells by TRNzol Universal Total RNA Extraction Reagent (cat. no. DP424; Tiangen Biotech Co., Ltd.). RT-qPCR was performed as previously described (31). GAPDH served as the internal reference gene. Primer sequences are shown in Table II. RT-qPCR was performed in three biological replicates and three technical replicates.

Western blotting (WB). WB was performed as described in a previous study (32). Primary antibodies against potassium channel tetramerization domain 5 (KCTD5; 1:1,000, cat. no. ab194825; Abcam) and secondary goat anti-rabbit IgG (H+L) antibodies conjugated with horseradish peroxidase (HRP; 1:10,000; cat. no. ZB-2301; OriGene Technologies, Inc.) were used. GAPDH was used as a loading control with primary (1:50,000; cat. no. 60004-1-Ig; Proteintech

Group, Inc.) and secondary HRP-labeled goat anti-mouse IgG (H+L) antibodies (1:10,000; cat. no. ZB-2305; OriGene Technologies, Inc.). Finally, band intensities were analyzed using Image J software (imagej.en.softonic.com/, version 1.6.0). WB experiment was performed in three biological replicates.

Transfection. Short interfering (si)RNAs targeting KCTD5 and negative control (NC) were purchased from Guangzhou RiboBio Co., Ltd. (Table III). In a 6-well plate, 5×10^5 MDA-MB-231 cells were cultured overnight at 37°C. Transfection was performed using Lipofectamine™ 3000 (cat. no. 2343152, Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The cells were incubated for 48 h at 37°C and 5% CO₂. Transfection was performed in three biological replicates.

Wound healing assay. The migratory capacity of the MDA-MB-231 cell line was assessed using a wound healing assay. A total of 5×10^5 Cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM, PM150210, Procell) supplemented with 1% Penicillin/Streptomycin (P/S, 164210, Procell) and 10% Fetal Bovine Serum (FBS, 164210, Procell). Once the cells reached approximately 90% confluence, a standardized wound was created by scratching the cell monolayer with a pipette tip, ensuring that the tip was perpendicular to the ruler behind the culture dish to maintain a consistent angle and avoid tilting.

After scratching, the cells were gently washed three times with Phosphate-Buffered Saline (PBS) to remove detached cells and debris, followed by the addition of serum-free medium to facilitate cell migration without proliferation. The cultures were then placed in a 37°C incubator with 5% CO₂ to maintain optimal growth conditions.

At 0 and 48 h post-wounding, images of the wound area were captured using an inverted fluorescence microscope (IMT-2; Olympus) with a 10x magnification. The wound healing rate was quantified by measuring the wound area at both time points using ImageJ software. The healing ratio was calculated as the difference in wound area between 0 and 48 h divided by the wound area at time zero, expressed as a percentage. Wound healing assay was performed in three biological replicates and three random fields of view were photographed for each sample.

Cell Counting Kit-8 (CCK-8) assay. After 24 h cell culture at 37°C, 100 μ l cell suspension was added to each well of a 96-well plate at a cell density of 5,000 cells/well. The cells were cultured at 37°C and 5% CO₂ for 24 h. A total of 10 μ l CCK-8 solution (C0037, Beyotime Biotech Inc., Shanghai, China) was added to each well and incubated for 1 h before measuring optical density at 450 nm. CCK-8 assay was conducted in three technical replicates.

Transwell assay. For the migration assay, 600 μ l high-glucose DMEM (PM150210, Procell) supplemented with 1% P/S (164210, Procell) and 10% FBS (164210, Procell) was added to the lower chamber. In the upper chamber, 1×10^4 cells/well in 100 μ l cell suspension was added. The Transwell inserts used were 8.0 μ m polycarbonate membranes with a

Table II. Primer sequences for quantitative PCR.

Gene	Sequence, 5'→3'	Length, bp	RefSeq	Product length, bp	Location
KCTD5	F: GGAGCTGCTGGGATTCCTTT	20	NM_018992	168	6th KCTD5 exon 1773-1792
	R: GTCAGTCTGCACAGTACCCC	20			6th KCTD5 exon 1921-1904
GAPDH	F: GAAGGTGAAGGTCGGAGTC	19	NM_001357943.2	172	2nd GAPDH exon 82-100
	R: GAAGATGGTGATGGGATTTTC	20	NM_001289745.3	226	4th GAPDH exon 253-234
					2nd GAPDH exon 174-192
			NM_001289746.2	226	4th GAPDH exon 399-380
					1st GAPDH exon 322-340
			NM_002046.7	226	3rd GAPDH exon 547-528
					2nd GAPDH exon 82-100
					4th GAPDH exon 307-288

F, forward; R, reverse; KCTD5, potassium channel tetramerization domain 5.

disposable cell culture insert (TCS020006, Guangzhou JET Bio-filtration Co., Ltd., Guangzhou, China). The upper chamber was filled with DMEM containing 0.1% FBS, while the lower chamber was filled with DMEM supplemented with 10% FBS. After 48 h at 37°C, cells were fixed with 4% paraformaldehyde for 30 min at room temperature. After removing the non-migratory cells from surface, the cells were stained with 0.1% crystal violet for 1 h at room temperature and then rinsed with PBS (P1020, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Transwell chambers were then air-dried and images were captured using a fluorescence microscope (IMT-2, Olympus).

For the invasion assay, Matrigel (cat. no. 356234; Corning Life Sciences, Inc.) was in moved from -20°C to a 4°C refrigerator overnight. The Matrigel was diluted on ice with DMEM to a concentration of 300 µg/ml and evenly applied to the surface of the Transwell inserts at 37°C for 2 h to allow the Matrigel to solidify. The subsequent protocol was the same to the migration assay. For the Transwell migration and invasion assays, triplicate biological repeats were conducted, and during image acquisition, three random fields of view were photographed for each sample.

Statistical analysis. All statistical analyses were performed in R (version 4.2.1). Data are presented as the mean ± SEM of three independent experimental repeats; all experimental data were analyzed using GraphPad Prism 6 (Dotmatics). Comparisons two groups were performed using unpaired t-test, while comparisons between multiple groups were conducted using ANOVA with Bonferroni's post hoc test. Wilcoxon rank-sum test was used to compare gene expression and immune cell infiltration. The 'cor' function was used to perform Pearson correlation analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Seven CD8⁺ T cell-associated gene modules were screened by WGCNA. WGCNA analysis was conducted on TNBC

Table III. siRNA sequences.

siRNA	Sequence, 5'→3'
siRNA1	T: GAACGAGACAGCAAAACAT
	S: GAACGAGACAGCAAAACAU
	A: AUGUUUUGCUGUCUCGUUC
siRNA2	T: AGCATCGGCTCCTCTTACA
	S: AGCAUCGGCUCUCUUAACA
	A: UGUAAGAGGAGCCGAUGCU
siRNA3	T: GTTGGAGGAAGCAGAATTT
	S: GUUGGAGGAAGCAGAAUUU
	A: AAAUUCUGCUUCCUCCAAC
si-NC	T: ACGGAGGCTAAGCGTCGCAA
	S: ACGGAGGCUAAGCGUCGCAA
	A: UUGCGACGCUUAGCCUCCGU

siRNA, short interfering RNA; T, target; S, sense; A, antisense; NC, negative control.

samples from METABRIC database, using a soft thresholding value of $\beta=4$ (Fig. 1A) to construct a gene network, which yielded 13 gene modules (Fig. 1B). The CIBERSORT was used to estimate infiltration ratios of 22 types of immune cells in the TNBC samples and CD8⁺ T cell scores from each sample were selected as the trait data for WGCNA. The association between gene module and CD8⁺ T cell scores was calculated (Fig. 1C and D) and seven gene modules [yellow (cor=0.26), green (cor=-0.21), magenta (cor=-0.17), turquoise (cor=-0.27), black (cor=-0.16), pink (cor=0.16) and brown (cor=0.15)] were significantly related to CD8⁺ T cell. These modules comprised a total of 2,815 genes, which were considered CD8⁺ T cell-related genes in TNBC. GO and KEGG enrichment analyses were performed on these genes and 36 significantly enriched KEGG pathways, 622 significantly enriched BP, 25 MF and 45 CC terms were identified. The top 10 significantly enriched KEGG pathways

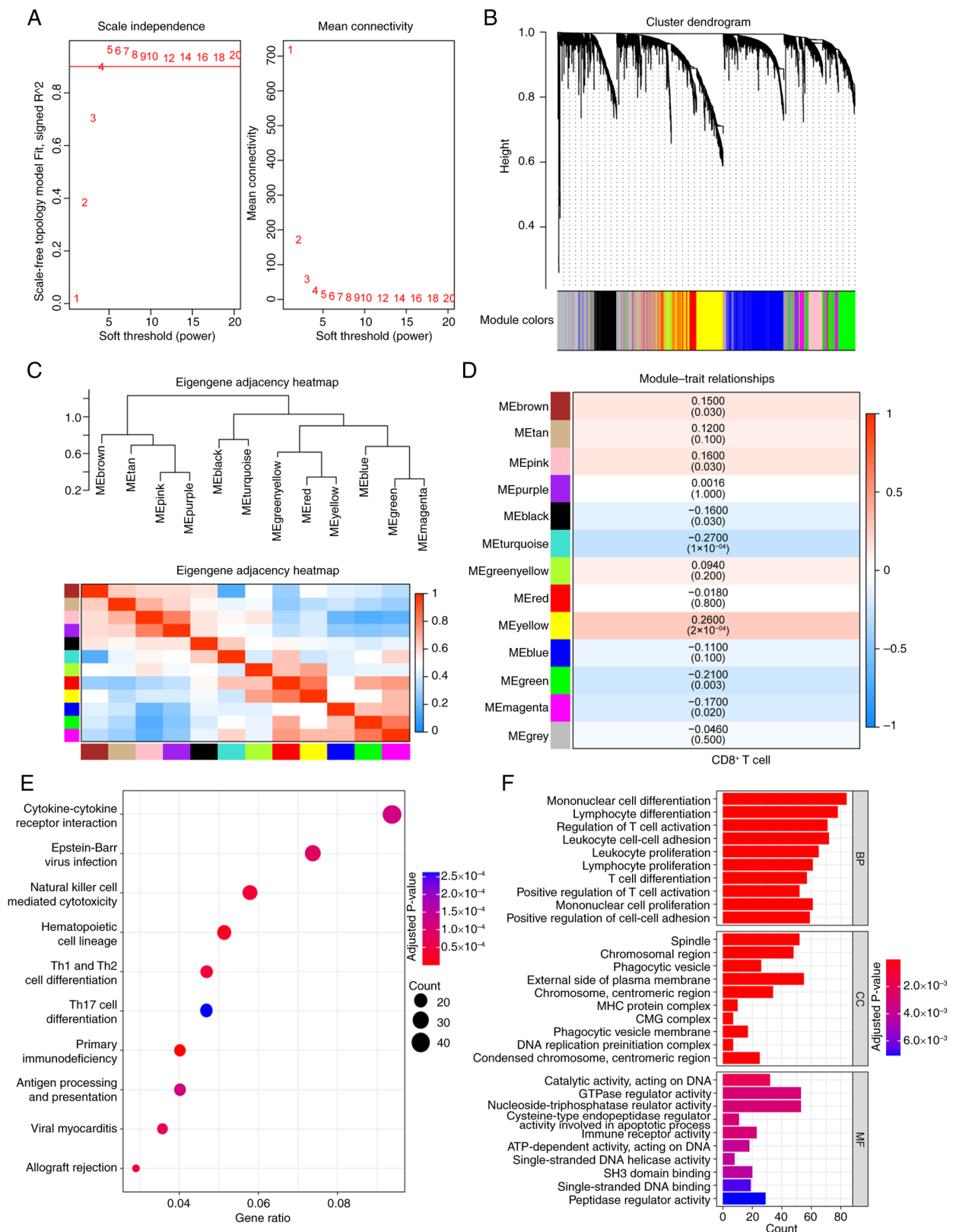


Figure 1. Weighted gene co-expression network analysis and functional enrichment analysis on triple negative breast cancer samples from Molecular Taxonomy of Breast Cancer International Consortium database. (A) Determination of soft threshold. (B) Clustering of gene modules. (C) Heatmap of eigengene adjacency. (D) Association between gene modules and CD8⁺ T cell levels. Top 10 significantly enriched (E) Kyoto Encyclopedia of Genes and Genomes pathways and (F) Gene Ontology terms. BP, biological process; MF, molecular function; CC, cellular component; ME, module eigengene.

and the top 10 significantly enriched GO terms are shown in Fig. 1E and F, respectively. The detailed results are presented in Table SI.

CD8⁺ T cell-associated *KCTD5* is significantly upregulated in TNBC and associated with poor prognosis. Differential gene expression analysis was performed using the GSE76250

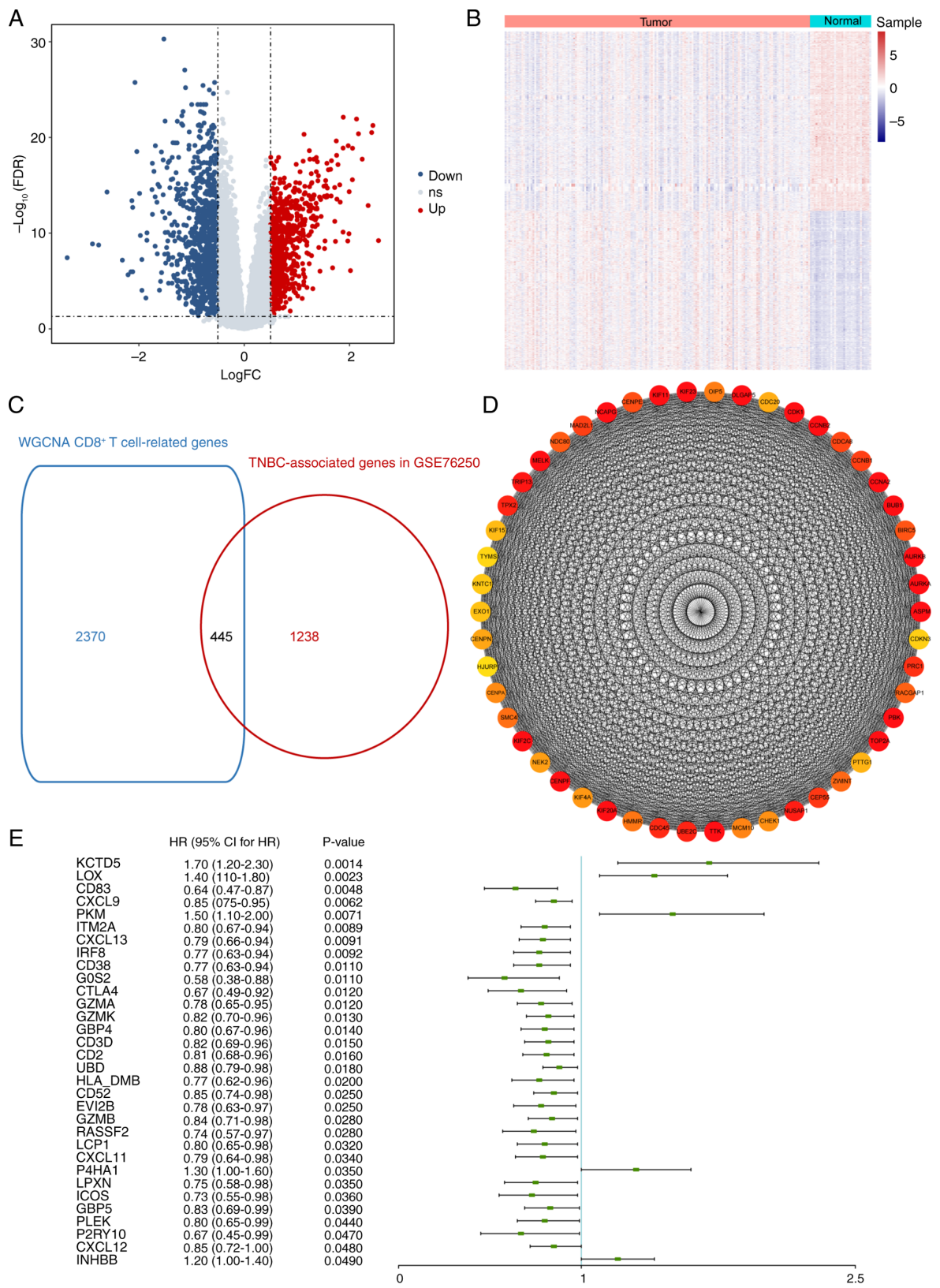


Figure 2. Selection of target gene KCTD5. (A) Volcano map and (B) heatmap of differential gene expression analysis between TNBC and normal samples using GSE76250 dataset. (C) Intersection of differentially expressed genes. (D) Protein-protein interaction network of candidate genes. (E) Univariate Cox regression analysis of candidate genes. FDR, false discovery rate; ns, not significant; TNBC, triple negative breast cancer; WGCNA, weighted gene co-expression network analysis; HR, hazard ratio.

dataset. Compared with normal samples, 1,683 DEGs were identified in TNBC samples including 791 up- and 892 down-regulated genes (Fig. 2A and B). A cross analysis between the

mentioned 1,683 DEGs and 2,815 CD8⁺ T cell-associated genes from the WGCNA was conducted, resulting in 445 CD8⁺ T cell-associated genes with aberrant expression in TNBC

(Fig. 2C; Table SII). PPI network from the 445 candidate genes was constructed and interaction pairs were filtered based on STRING database. PPI network was visualized and the top 50 genes were selected (Fig. 2D).

Univariate Cox regression analysis was performed on 445 candidate genes and the hazard ratio was calculated. Among all the candidate genes identified as significant variables ($P < 0.05$), KCTD5 demonstrated the highest significance and was selected as the target gene for subsequent assessment (Fig. 2E). KCTD5 was categorized in the yellow module (Table SI), which was positively correlated with CD8⁺ T cell levels.

Expression of hub gene KCTD5 in TNBC in both public datasets and TNBC cell lines was assessed. In the GSE38959 dataset, KCTD5 mRNA expression was significantly increased in the TNBC compared with the normal samples (Fig. 3A). Tumor IMMune Estimation Resource database (cistrome.shinyapps.io/timer/) was used to assess expression of KCTD5 in numerous types of cancers. KCTD5 was significantly upregulated in Bladder Urothelial Carcinoma (BLCA), Breast Invasive Carcinoma (BRCA), Cervical Squamous Cell Carcinoma (CESC), Cholangiocarcinoma (CHOL), Esophageal Carcinoma (ESCA), Glioblastoma Multiforme (GBM), Head and Neck Squamous Cell Carcinoma (HNSC), Kidney Renal Clear Cell Carcinoma (KIRC), Kidney Renal Papillary Cell Carcinoma (KIRP), Liver Hepatocellular Carcinoma (LIHC), Lung Adenocarcinoma (LUAD), Lung Squamous Cell Carcinoma (LUSC), Prostate Adenocarcinoma (PRAD), Stomach Adenocarcinoma (STAD), and Uterine Corpus Endometrial Carcinoma (UCEC) compared with normal samples (Fig. 3B).

TNBC samples in the METABRIC database were divided into high KCTD5 expression group (HEKG) and low KCTD5 expression group (LEKG) by the median KCTD5 expression value (6.067). KM survival analysis demonstrated that patients in HEKG had a significantly shorter overall survival (OS) compared with LEKG (Fig. 3C). Expression of KCTD5 was validated in TNBC cell lines MDA-MB-231 and MDA-MB-453 by RT-qPCR and WB with the control of human normal breast epithelial cell line MCF10A. mRNA expression levels of KCTD5 were significantly higher in MDA-MB-231 compared with normal cells (Fig. 3D). Likewise, both TNBC cell lines demonstrated significantly higher KCTD5 protein levels compared with the normal cell line (Fig. 3E).

KCTD5 is associated with malignant progression of TNBC. TNBC samples from TCGA were grouped into HEKG and LEKG according to the median expression of KCTD5. The DEGs between HEKG and LEKG were assessed and upregulated genes were subjected to KEGG and GO enrichment analyses. There were 18 significantly enriched KEGG pathways, 602 significantly enriched BP, 97 MF and 144 CC entries, with the top ten enriched KEGG pathways and top ten enriched GO pathways shown in Fig. 4A and B, respectively. GSEA was performed between HEKG and LEKGs. The results demonstrated that there were 136 pathways significantly enriched in HEKG compared with LEKG (Table SIII), including IL-17 signaling pathway, MAPK signaling pathway, NF- κ B signaling pathway, PI3K-Akt signaling pathway, Wnt signaling pathway, etc. which were associated with

proliferation, survival, invasion, and metastasis of cancer cells (Fig. 4C). The enrichment of functions, including those associated with cell cycle and tumor-associated pathways, suggested the role of KCTD5 in the malignant progression of TNBC, influencing key processes such as tumor occurrence and invasion.

Expression of KCTD5 in a number of breast cancer subtypes with differential malignancy was assessed using the GSE21653 and GSE31488 datasets. The results demonstrated that in both datasets, expression of KCTD5 was significantly higher in breast cancer subtypes than normal samples, and the expression median gradually increased with the increase of malignant degree of subtypes, where the most malignant subtype was TNBC (Fig. 4D and E). Expression of marker of proliferation Ki-67 (MKI67) was assessed in HEKG and LEKGs using TCGA datasets, which demonstrated that expression of MKI67 was significantly higher in HEKG compared with the LEKG (Fig. 4F). Therefore, KCTD5 may promote tumor cell proliferation and the malignant progression of TNBC.

Immune landscape of samples with high KCTD5 expression.

As KCTD5 was identified as a potential driver of malignant progression in TNBC, the impact of KCTD5 on the immune landscape and TME in TNBC was assessed. The immune response and TME are vital for cancer progression and patient response to therapy (33). Using TCGA database, the correlation between KCTD5 and abundance of numerous infiltrating immune cells was analyzed based on two databases, xCell and TISIDB. In xCell, KCTD5 demonstrated a significant positive association with 'Smooth.muscle', 'Th1.cells', megakaryocyte-erythroid progenitor cells 'MEP' and 'pro.B.cells' and a significant negative association with common myeloid progenitor 'CMP', megakaryocytes, regulatory T cells 'Tregs', conventional dendritic cells 'cDC', 'monocytes', 'astrocytes', 'endothelial.cells', lymphatic endothelial cells 'ly.Endothelial.cells', hematopoietic stem cells 'HSC' and 'adipocytes' (Fig. 5A). In TISIDB tool analysis, KCTD5 demonstrated a significant negative association with 'eosinophil', $\gamma\delta$ T cells 'Tgd', 'mast' cells, 'neutrophil' and T helper 2 'Th2' cells (Fig. 5B).

The infiltration of 22 types of immune cell between HEKG and LEKG groups was evaluated. The results demonstrated a significantly higher infiltration of 'activated dendritic cells', 'macrophages M0', 'macrophages M2', 'activated mast cells', 'monocytes', and 'neutrophils' in HEKG compared with the LEKG, and significantly lower infiltration of 'macrophages M1', 'plasma cells', and 'Tgd cells' in HEKG compared with the LEKG (Fig. 5C). Further analysis of Pearson correlation was conducted and six of cell types displayed significant correlation with KCTD5 expression level. KCTD5 expression was significantly negatively correlated with 'plasma cells', 'macrophages M1' and 'Tgd cells', and significantly positively associated with 'activated mast cells', 'macrophages M0' and 'macrophages M2' (Fig. 5D).

These 22 immune cells were divided into six categories: Lymphocytes, macrophages, monocytes, neutrophils, dendritic cells and mast cells. It was demonstrated that the infiltration rates of macrophages, monocytes and neutrophils were significantly higher in the HEKG compared with LEKG, while

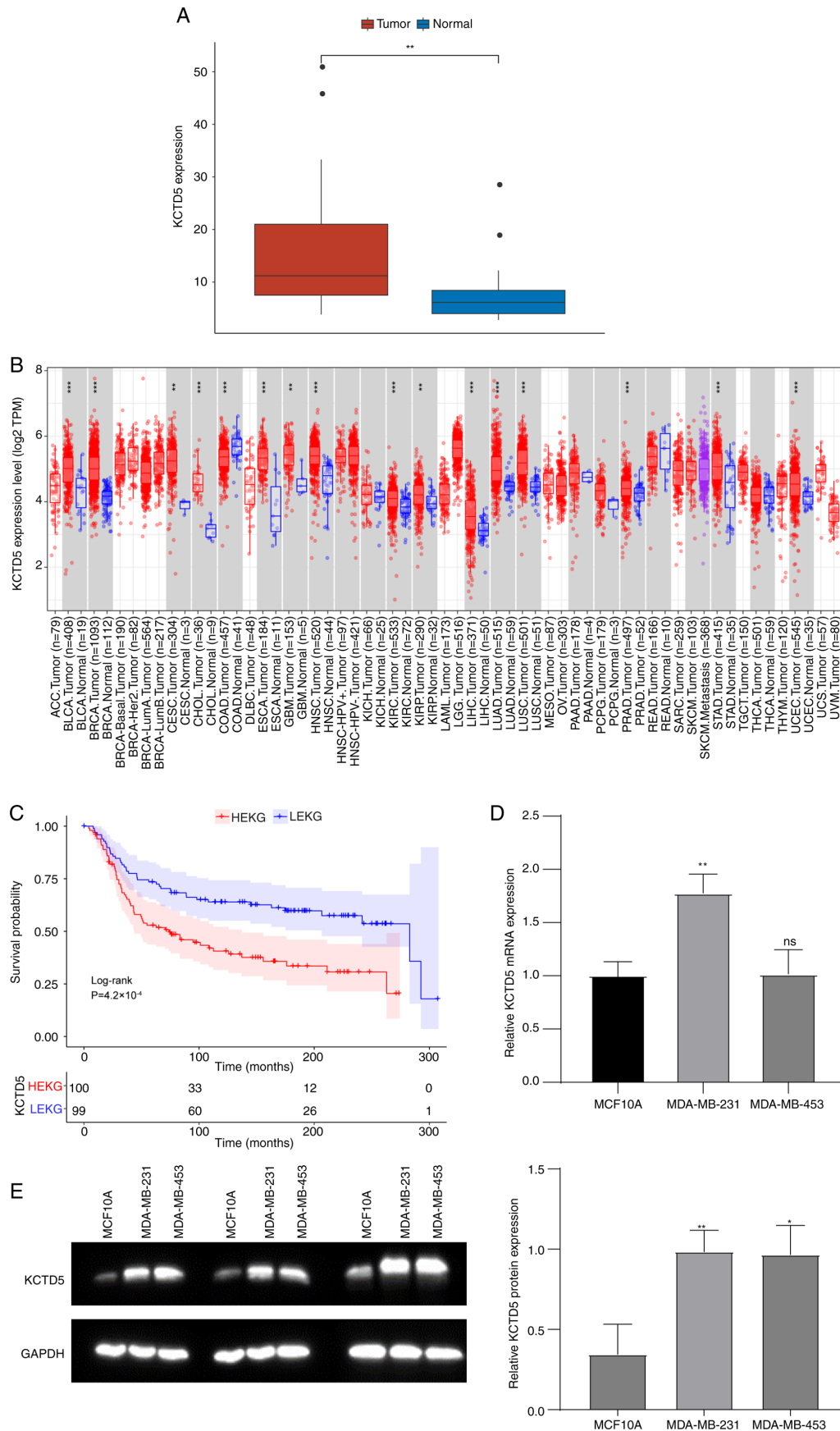


Figure 3. KCTD5 expression and prognosis in TNBC samples. (A) Expression of KCTD5 in TNBC and normal samples of GSE38959 dataset. (B) KCTD5 expression in cancers based on Tumor IMmune Estimation Resource database. (C) Kaplan-Meier OS curve. (D) Comparison of KCTD5 expression levels between TNBC and normal cell line determined by qPCR using a human normal breast epithelial cell line MCF10A and two human TNBC cell lines, MDA-MB-231 and MDA-MB-453. (E) Comparison of KCTD5 protein levels between TNBC and normal cell line detected by western blot based on normal cell line MCF10A and two human TNBC cell lines, MDA-MB-231 and MDA-MB-453. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. normal samples. TPM, transcripts per million; KCTD5, potassium channel tetramerization domain 5; TNBC, triple negative breast cancer.

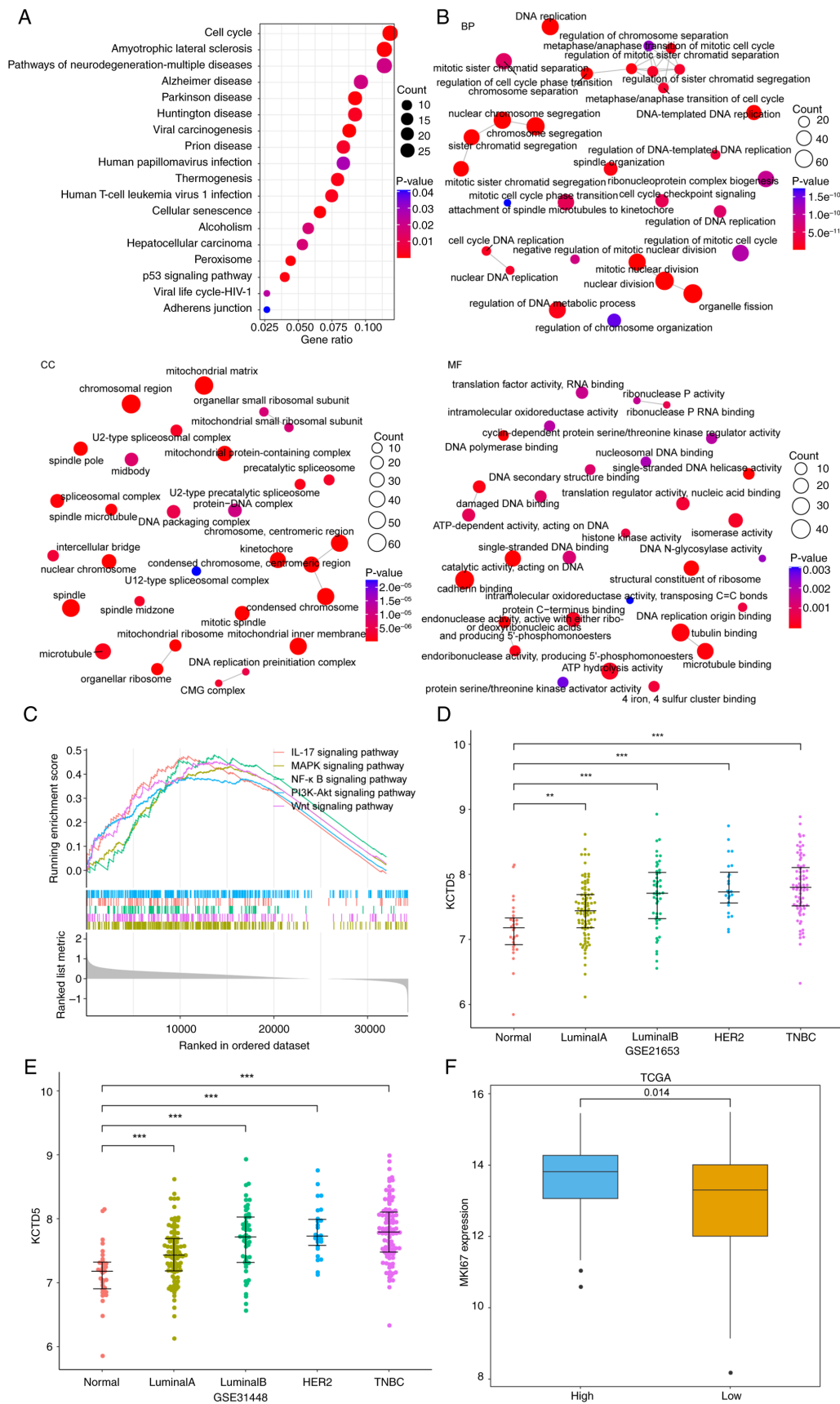


Figure 4. KCTD5 is associated with malignant progression in TNBC. Top 10 (A) KEGG pathways and (B) GO functional enrichment analysis network of upregulated differentially expressed genes between HEKG and LEKG in TCGA-TNBC. (C) Gene set enrichment analysis of HEKG and LEKG in TCGA-TNBC. The expression of KCTD5 in numerous breast cancer subtypes was using (D) GSE21653 and (E) GSE31488 datasets, and the comparisons were made between different subtypes and normal samples. (F) Expression of proliferation marker gene MKI67 in HEKG and LEKG of TCGA-TNBC dataset. ** $P < 0.01$ and *** $P < 0.001$ vs. normal samples. BP, biological process; MF, molecular function; CC, cellular component; TCGA, The Cancer Genome Atlas; TNBC, triple negative breast cancer; HEKG, high KCTD5 expression group; LEKG, low KCTD5 expression group; KCTD5, potassium channel tetramerization domain 5; MKI67, marker of proliferation Ki-67; HER, human epidermal growth factor receptor 2.

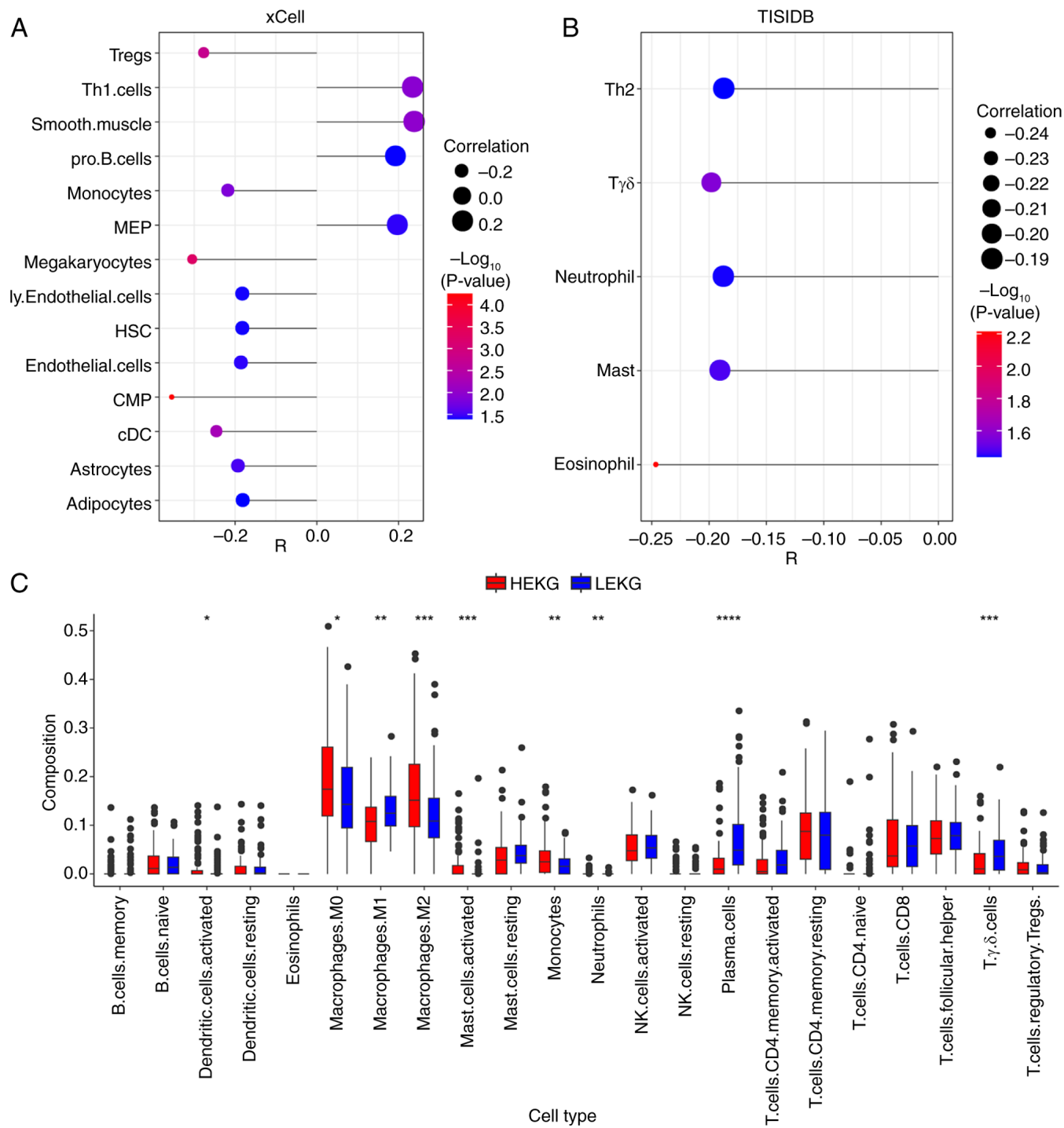


Figure 5. Continued.

the infiltration rate of lymphocytes was significantly higher in LEKG compared with the HEKG. There was no significant infiltration difference in dendritic cells or neutrophils (Fig. 5E). Moreover, single-sample GSEA was performed on TNBC samples from the METABRIC database to assess activity of immune cells indicated by enrichment score. Immune cells were divided into two groups: Innate and adaptive immunity. There was a significant increase in the enrichment score for adaptive immunity in LEKG compared with the HEKG, while no significant difference was demonstrated in innate immunity between groups (Fig. 5F). The adaptive immunity group

was further divided into T cell response and other. LEKG had a significantly higher enrichment score for T cell and other response compared with HEKG (Fig. 5G). *TNBC samples with high expression of KCTD5 exhibit worse clinical outcomes.* Finally, survival rate of patients was assessed. GSE69031, GSE45255, GSE135565 and GSE65194 datasets were merged after batch effect removal and 163 TNBC samples were selected based on complete survival information. KM survival analysis was performed on HEKG and LEKG samples. The OS of patients in HEKG was significantly

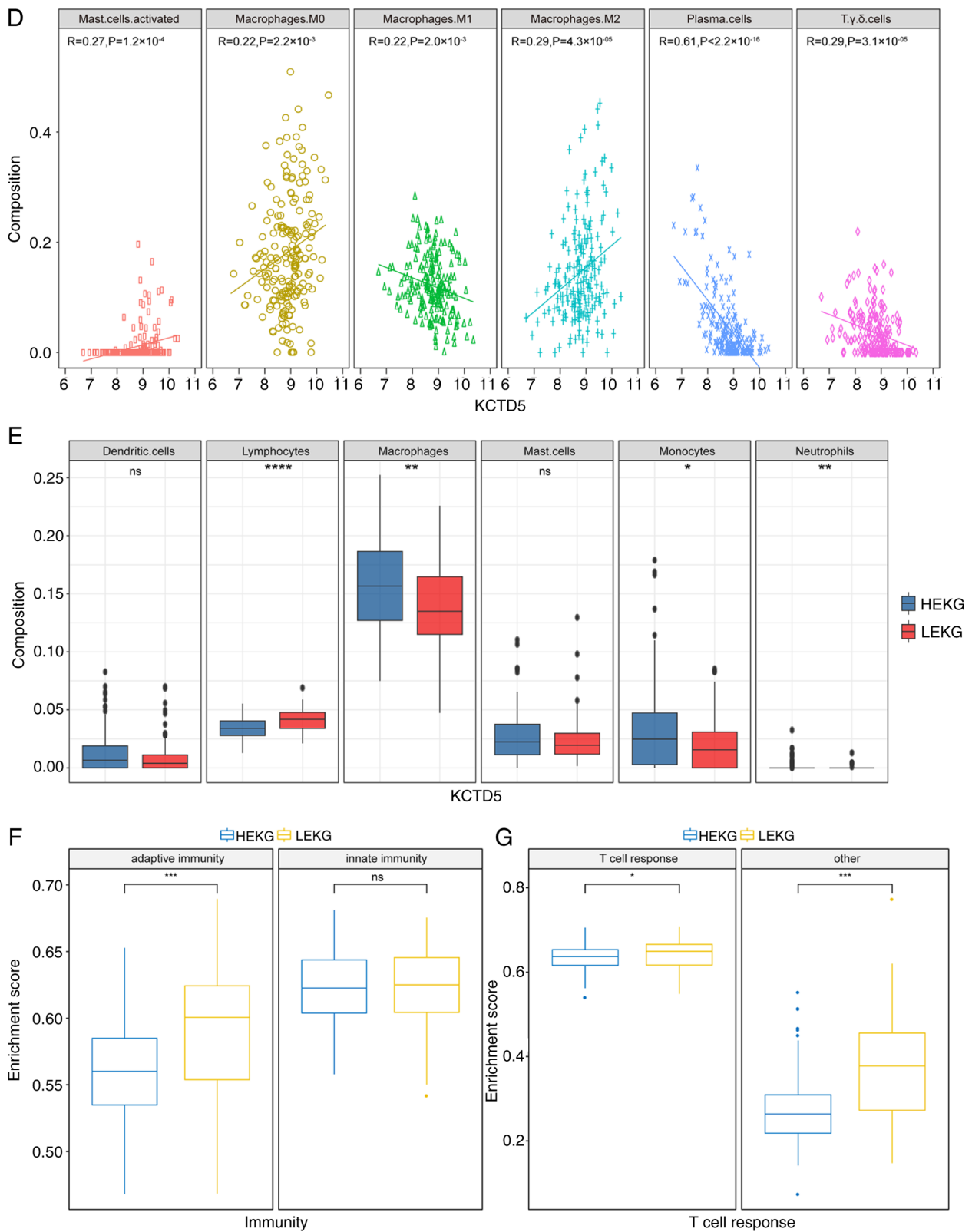


Figure 5. Analysis of immune landscape. Correlation between KCTD5 expression and abundance of infiltrating immune cells in (A) x Cell and (B) TISIDB databases. (C) Comparisons of infiltration of 22 types of immune cell between HEKG and LEKG. (D) Correlation of KCTD5 expression and 6 types of immune cell. (E) Comparison of infiltration of immune cells in HEKG and LEKG. (F) Innate immunity, adaptive immunity and (G) T cell response comparisons between HEKG and LEKG. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ vs. LEKG. ns, not significant. HEKG, high KCTD5 expression group; LEKG, low KCTD5 expression group; KCTD5, potassium channel tetramerization domain 5; TISIDB, Tumor-immune system interactions and drug bank database.

shorter compared with LEKG (Fig. 6A). GSE21653 dataset demonstrated that patients in the HEKG had a significantly shorter disease-free survival (DFS) compared with LEKG

(Fig. 6B). METABRIC database demonstrated that HEKG had a significantly shorter relapse-free survival (RFS) compared with the LEKG (Fig. 6C).

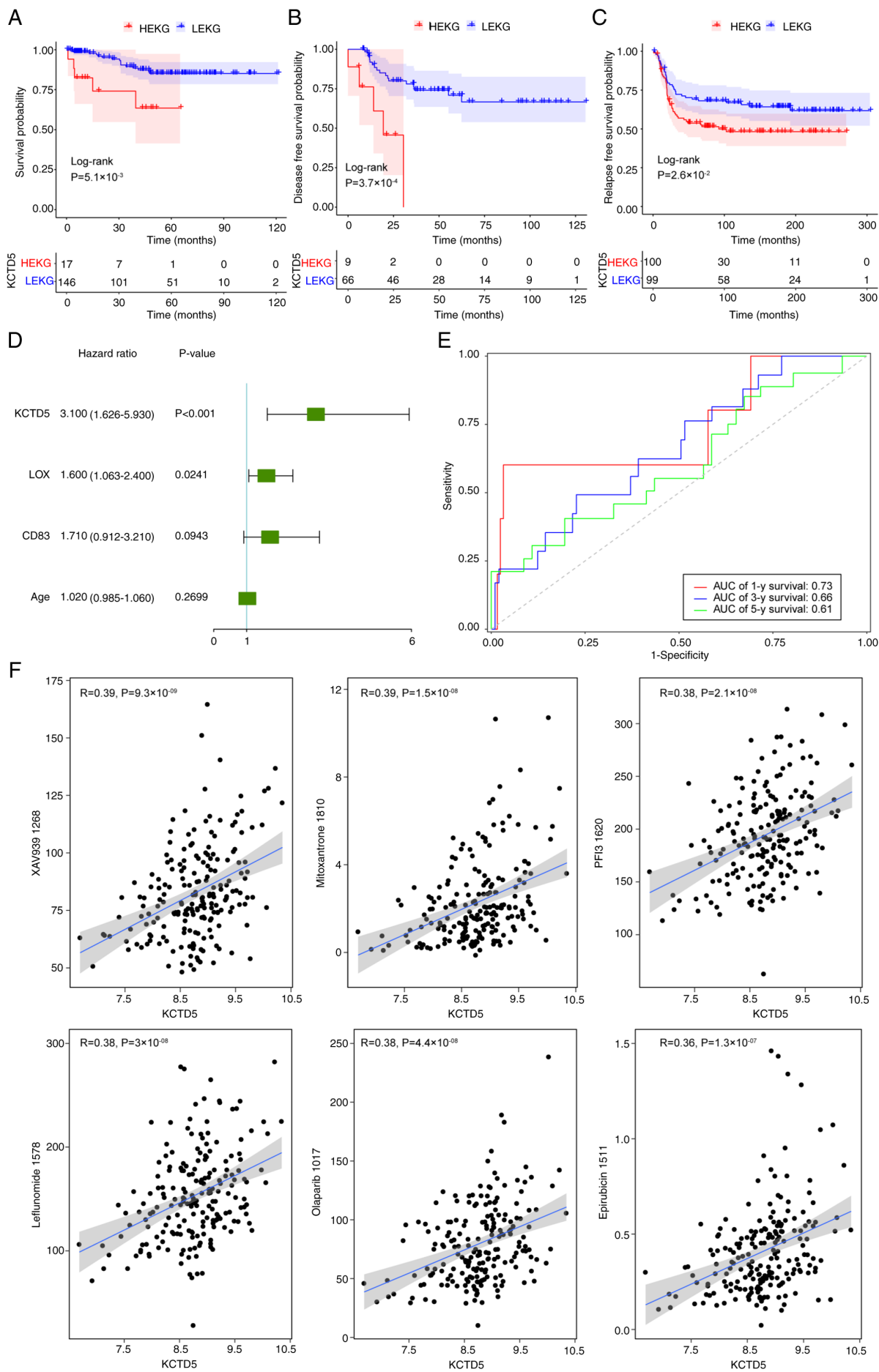


Figure 6. Continued.

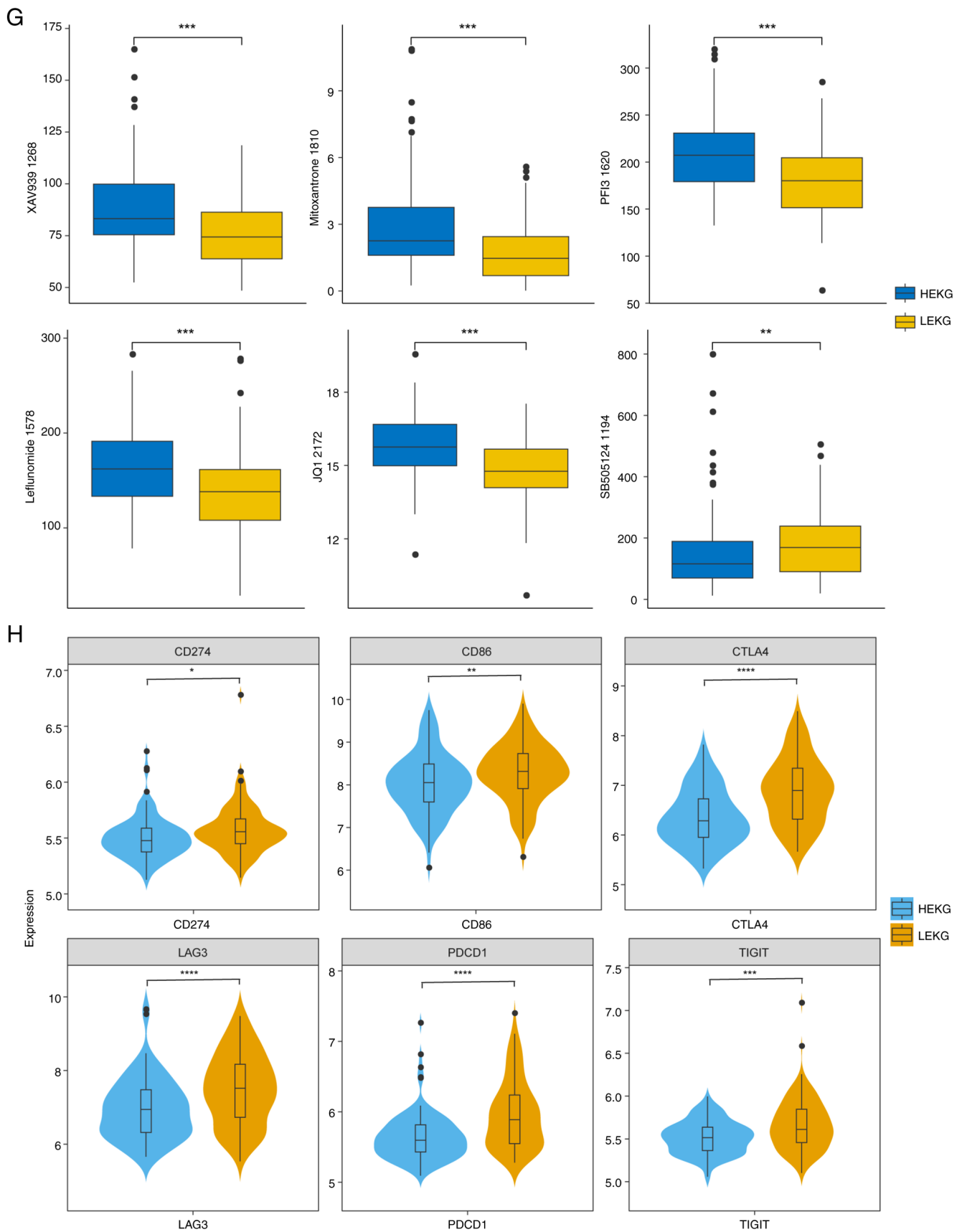


Figure 6. Survival analysis and clinical outcomes of TNBC samples. (A) KM curve of merged GSE69031, GSE45255, GSE135565 and GSE65194. (B) KM disease-free survival curve based on the GSE21653 dataset. (C) KM relapse-free survival curve based on METABRIC database. (D) Multivariate Cox regression analysis of TNBC samples in the merged dataset. (E) Receiver operating characteristics analysis of KCTD5 expression based on the merged dataset. (F) Correlation between KCTD5 expression and drugs using METABRIC dataset. (G) IC_{50} in HEKG and LEKG in TNBC samples. (H) Expression of immune checkpoint genes in HEKG and LEKG based on METABRIC data. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ vs. LEKG. TNBC, triple negative breast cancer; KM, Kaplan-Meier; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium; HEKG, high KCTD5 expression group; LEKG, low KCTD5 expression group; AUC, area under curve; y, year.

As patients with high KCTD5 expression demonstrated a shorter OS, DFS and RFS in different datasets, it was determined whether KCTD5 was an independent indicator for poor prognosis. In the merged dataset, lysyl Oxidase (LOX), CD83 and age were included in a multivariate Cox regression analysis, which demonstrated that KCTD5 and LOX could serve as independent prognostic factors for patients with TNBC (Fig. 6D). Time-dependent receiver operating characteristic analysis demonstrated that the area under curve values for 1, 3 and 5-year survival were 0.73, 0.66 and 0.61, respectively, suggesting that KCTD5 could effectively predict prognosis of patients with TNBC (Fig. 6E).

To identify potential targets for drug development and select more effective drugs for individual patients, drug sensitivity was predicted using METABRIC dataset. The correlation between KCTD5 and drug half-maximal inhibitory concentration (IC_{50}) was analyzed, demonstrating that KCTD5 was significantly negatively correlated with BI.2536_1086, SB505124_1194 and Acetalax_1804 (Table SIV). KCTD5 was also significantly positively correlated with 160 other drugs including XAV939_1268, Mitoxantrone_1810, PF13_1620, Leflunomide_1578, Olaparib_1017, and Epirubicin_1511 (Fig. 6F; Table SIV). For drugs that demonstrated a significant correlation, IC_{50} between HEKG and LEKG in TNBC samples was compared. IC_{50} values of XAV939_1268, Mitoxantrone_1810, PF13_1620, Leflunomide_1578 and JQ1_2172 were significantly higher in HEKG compared with LEKG, while SB505124_1194 was significantly higher in LEKG compared with HEKG (Fig. 6G).

Furthermore, to identify potential targets for immunotherapy and personalized treatment, correlation between KCTD5 and immune checkpoints proteins was assessed. The differential expression of immune checkpoint genes programmed cell death protein 1 (PDCD1), Cytotoxic T-Lymphocyte-Associated Protein 4 (CTLA4), CD274, Cluster of Differentiation 86 (CD86), Lymphocyte-Activation Gene 3 (LAG3), T Cell Immunoreceptor with Ig and ITIM Domains (TIGIT) was assessed based on METABRIC data. Expression levels of these genes were significantly lower in HEKG compared with LEKG (Fig. 6H).

KCTD5 knockdown inhibits viability, migration and invasion of TNBC cells. To assess the effect of KCTD5 downregulation on TNBC cells, a KCTD5 knockdown expression model was constructed in MDA-MB-231 cells. RT-qPCR and WB validated the decreased KCTD5 expression after transfection with siRNA targeting KCTD5. The mRNA and protein levels of KCTD5 in siRNA1, siRNA2 and siRNA3 groups was significantly reduced compared with the si-NC (Fig. 7A and B). In subsequent experiments, si-RNA3 was selected as si-KCTD5. CCK-8 assay demonstrated that KCTD5 knockdown significantly decreased the viability of MDA-MB-231 cells compared with the si-NC group (Fig. 7C). Wound healing assay demonstrated that KCTD5 knockdown significantly inhibited migration ability of MDA-MB-231 cells compared with si-NC group (Fig. 7D). Transwell assays demonstrated that the migration and invasion abilities of MDA-MB-231 cells were significantly decreased after KCTD5 knockdown compared with the si-NC group (Fig. 7E).

Discussion

In the present study, KCTD5 was identified as a CD8⁺ T cell-associated hub gene in TNBC. KCTD5 was significantly upregulated in TNBC and was an indicator of poor prognosis. KCTD5 promoted malignant progression of TNBC such as invasion and cell viability and affected the TME and expression of a number of immune checkpoint genes. These checkpoints serve a role in regulating the immune response and maintaining self-tolerance (34). KCTD5 knockdown inhibited the viability, migration and invasion abilities of a TNBC cell line. The correlation between drug sensitivity and KCTD5 expression suggested that KCTD5 expression could be a useful predictor of drug sensitivity in TNBC, identifying drugs that might be more effective in patients with high or low KCTD5 expression. Moreover, KCTD5 was suggested to be an independent indicator of prognosis of patients with TNBC. Of 26 members of the KCTD protein family, only KCTD5 has a resolved three-dimensional structure (35), underscoring its potential as a subject for detailed biological and medicinal research in TNBC. KCTD5 serves a role in anti-proliferative response (36). Here, expression of the proliferation marker gene MKI67 was significantly increased in HEKG compared with the LEKG. Previous studies have reported that KCTD5 is overexpressed in all types of breast cancer (37), is involved in expression of transient receptor potential melastatin 4 channels and Ca^{2+} sensitivity (38) and serves as a negative regulator of cell migration, which impacts the likelihood of metastasis (39). KCTD5 has been reported to interact with E3 ubiquitin ligase (40), a ubiquitin ligase that promotes degradation of cyclin E (41), which is essential for the G1/S transition of the cell cycle (42).

To the best of our knowledge, there is limited research assessing the association between KCTD5 and CD8⁺ T cells (43). Here, adaptive immunity was significantly more activated in LEKG than HEKG and low expression of KCTD5 was suggested to induce a relatively stronger T cell response compared to high expression of KCTD5. A previous study reported that the expression of KCTD5 is upregulated in peripheral blood lymphocytes stimulated by T cell receptor (40). In the present study, KCTD5 expression was positively associated with CD8⁺ T cells, which was consistent with the positive correlation reported between KCTD5 expression and CD8⁺ T cells in lung adenocarcinoma (43). This positive correlation seems contradictory as KCTD5 is associated with a poor prognosis but CD8⁺ T cells represent antitumor activity, which is an indicator of favorable prognosis. One explanation is the non-uniform distribution of CD8⁺ T cells within tumor tissue, which can lead to variability in results due to random nature of sampling. The TME is complicated, involving numerous regulators. The infiltration of immune cells varies according to diseases and different stages. Although KCTD5 is associated with poor prognosis of TNBC and promotes migration, it is a negative regulator of metastasis in melanoma mediated by Rac1 and Ca^{2+} signaling pathway (39). CD8⁺ T cells inhibit metastasis; metastasis poses a challenge in TNBC treatment due to the aggressive nature of the disease, the lack of targetable receptors, and the potential for immune evasion and therapy resistance.

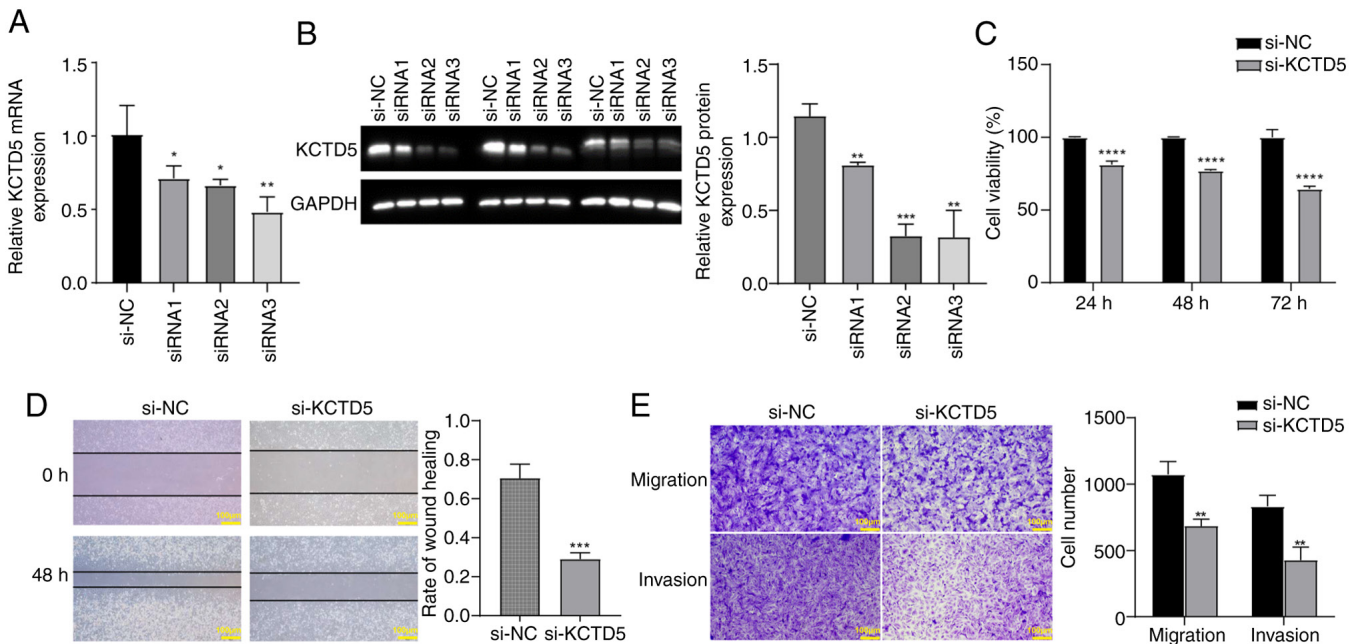


Figure 7. Knockdown experiments of KCTD5 in MDA-MB-231. Validation of expression of KCTD5 by (A) reverse transcription-quantitative PCR and (B) western blotting. (C) Cell viability differences between si-NC and si-KCTD5 was compared. (D) Wound healing, and (E) Transwell migration and invasion assays of MDA-MB-231 cells. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ and **** $P<0.0001$ vs. si-NC. si, short interfering; NC, negative control; KCTD5, potassium channel tetramerization domain 5.

KCTD5 expression was significantly negatively associated with macrophages M1, plasmacells and Tgd cells and significantly positively associated with activated mast cells, macrophages M0 and macrophages M2. When KCTD5 is upregulated in TNBC, macrophages are prone to polarize to M2 subtype, which is an anti-inflammatory subtype (44). Oncogene multiple copies in T cell malignancy 1 (MCT1) exerts a similar effect on polarization of macrophages in TNBC, namely activation of MCT-1, which enriches tumor-promoting M2 macrophages in the TME (45). It is hypothesized that silencing KCTD5 may inhibit M2 macrophages recruitment, in a similar manner to CCL2, a potential therapeutic target gene of TNBC (46).

Furthermore, knockdown of KCTD5 has been reported to suppress proliferation, migration and invasion of melanoma cells (39). KCTD5 may serve as a modulator of cell migration by influencing cell motility and focal adhesion dynamics, potentially via Rac1 activity and Ca^{2+} signaling pathways, which has been reported by previous studies (39,47,48). KCTD5 is a cancer marker associated with programmed cell death (49). KCTD5 knockdown increases the apoptosis of human lung cancer cell line A549 (49). These findings suggest the potential value in downregulating KCTD5 in TNBC treatment.

There was no significant difference in CD8^+ T cell infiltration between HEKG and LEKG. This may be attributed to KCTD5 influencing CD8^+ T cell behavior in an indirect manner, potentially via its effects on the cytokine environment or by impacting other immune cells that regulate CD8^+ T cell activity, rather than by directly recruiting or expanding CD8^+ T cells. Given the complexity of the immune system, the association between KCTD5 expression and CD8^+ T cell infiltration may be influenced by a multitude of interrelated factors. These include the complex regulatory mechanisms, such as intricate immune cell interactions, the cytokine signaling networks that mediate

communication among immune cells, the epigenetic changes that can affect gene expression, and the post-translational modifications that can modulate protein activity. Moreover, heterogeneity of tumors may lead to variability in CD8^+ T cell infiltration that the present analysis could not fully assess owing to differences in TME between patients. Further studies such as *in vitro* co-culture experiment, immune cell isolation and transfer experiments, immunofluorescence and histological analysis are required to elucidate the precise effects of KCTD5 on CD8^+ T cells in TNBC.

In conclusion, the present study identified CD8^+ T cell-associated hub gene KCTD5, which was upregulated in TNBC and associated with poor prognosis. Viability, migration and invasion of TNBC cells was inhibited by KCTD5 knockdown. Furthermore, KCTD5 could influence the TME in TNBC. These findings suggested that KCTD5 may serve a role in the pathogenesis of TNBC and may be a potential therapeutic target for TNBC. Further studies are required to understand the molecular mechanisms underlying the functions of KCTD5 in TNBC and to effective therapeutic strategies targeting this protein.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JL and JY conceived the study and analyzed data. JL wrote the manuscript. JY edited the manuscript. JL and JY confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

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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