

# Pan-cancer analysis supports MAPK12 as a potential prognostic and immunotherapeutic target in multiple tumor types, including in THCA

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**Abstract.** P38 mitogen-activated protein kinase (MAPK)12 (also known as P38  $\gamma$ ) is critical in the development and progression of various types of tumors. Despite the extensive literature on the subject, further studies are needed to elucidate its role in cancer progression. Here, a comprehensive bioinformatics analysis of a generalized cancer dataset was performed

to explore the mechanism of MAPK12 regulation in tumorigenesis. Several tumor datasets and online analytical tools, including HPA, SangerBox, UALCAN, GEPIA2, STRING, ImmuCellAI, and MEXPRESS, were used to analyze the expression information on MAPK12 in several types of cancers. Western blotting and reverse transcription-quantitative PCR were used to verify the protein and mRNA expression levels of MAPK12, respectively, in human normal thyroid cells (HTORI-3) and thyroid carcinoma (THCA) cells. Cytotoxicity and EdU assays were used to verify the promoting effect of MAPK12 on cell proliferation in THCA cells. Analysis of several cancers found that MAPK12 was overexpressed in multiple cancer types. Upregulated MAPK12 mRNA expression levels were correlated with a worse prognosis in patients with several types of cancer. Cytotoxicity and EdU experiments showed that MAPK12 knockdown inhibited THCA cell proliferation. Gene Ontology-Biological Process and Kyoto Encyclopedia of Genes and Genomes analyses showed that the enrichment of MAPK12 genes was related to cell proliferation and the tumor immune microenvironment. These results showed that MAPK12 was closely related to the immune checkpoint, microsatellite instability, and tumor mutational burden and affected the sensitivity of the tumor to immunotherapy. This study showed that MAPK12 may be an immunotherapeutic and promising prognostic biomarker in certain types of tumors.

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**Abbreviations:** ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DFS, disease free survival; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck cancer; ICP, immune checkpoint; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MAPK12, P38 mitogen-activated protein kinase 12; MESO, mesothelioma; MSI, microsatellite instability; OS, overall survival; PAAD, pancreatic adenocarcinoma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; RFS, relapse-free survival; STAD, stomach adenocarcinoma; STES, stomach and esophageal carcinoma; THCA, thyroid carcinoma; TMB, tumor mutational burden; UCEC, uterine corpus endometrial carcinoma; UVM, uveal melanoma

**Key words:** MAPK12, pan-cancer, prognosis, immune, thyroid carcinoma

## Introduction

The (MAPK) family is a highly conserved protein family, the members of which participate in several cytokine pathways (1-3). P38 has four isoforms encoded by different genes in mammalian cells: P38  $\alpha$  (MAPK14), P38  $\beta$  (MAPK11), P38  $\gamma$  (MAPK12), and P38  $\delta$  (MAPK13) (3). The MAPK signaling pathway partakes in numerous core biological functions such as in the regulation of cell proliferation, inflammation, survival, innate immunity, and other cellular processes related to cancer progression and development (4,5). Classical MAPKs primarily include P38, JNK1/2/3, and other subtypes, which have been studied in depth (6-8).

Studies suggest that MAPK12 is expressed in multiple tissues and promotes tumorigenesis and tumor progression (9). For example, high MAPK12 expression promoted epithelial-mesenchymal transition (EMT) in breast cancer cells, and the downregulation of MAPK12 inhibited EMT (10,11). MAPK12 overexpression increased the number of cancer stem cells (CSCs), whereas MAPK12 knockdown decreased the proportion of CSCs in breast cancer cells (12). Chen *et al* (13) found that overexpression of MAPK12 enhanced the transformation to a malignant phenotype in renal cell carcinoma (RCC) cells, and MAPK12 may be a novel therapeutic target for the management of RCC.

Here, systematic bioinformatics analysis was used to explore the functions and effects of MAPK12 in a variety of cancers. The significance of abnormal MAPK12 expression in multiple cancer types was comprehensively studied using mRNA expression analysis, patient prognostic indicators, functional analyses of MAPK12-related genes, tumor immunity, and methylation patterns. Additionally, the relationship between MAPK12 expression and thyroid carcinoma (THCA) proliferation was determined using cytotoxicity and EdU assays *in vitro*.

## Materials and methods

**Expression analysis.** The mRNA expression profiles of MAPK12 in various normal tissues were obtained from the Human Protein Atlas (HPA) website (<https://www.proteinatlas.org>). Data sources are Tumor-Node-Metastasis standardized. The 'Gene' module of SangerBox (14), a web-based program (<http://www.sangerbox.com>), was used to examine the mRNA expression levels of MAPK12 in normal and cancer tissues as reported by TCGA (<https://cancergenome.nih.gov/abouttcga/overview>). The GSE33630 (15), GSE27155 (16), and GSE65144 (17) datasets were downloaded to analyze the MAPK12 mRNA expression differences between normal thyroid cells and thyroid carcinoma cells.

**Survival analysis.** The relationship between MAPK12 mRNA expression and overall survival (OS) in each tumor type in the TCGA database was analyzed using the GEPIA2 website (18). Patients were divided into MAPK12 low and MAPK12 high cohorts based on MAPK12 expression levels. Cox regression analysis was used in SangerBox to study the effects of MAPK12 expression on the OS and disease-free survival (DFS) of patients with different types of tumors.

**MAPK12-related gene enrichment analysis.** First, the 'similar Gene' module in GEPIA was used to analyze the top 100 genes that were most commonly associated with MAPK12 pan-cancer, and we screened the top 50 genes. A network map of MAPK12 and the 50 genes was created using STRING (<https://string-db.org/>) (19). Gene Ontology (GO) (20,21) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (22) were used to perform enrichment analysis of these 100 MAPK12-related genes. The TCGA-THCA database was downloaded, and the genes that most significantly correlated with MAPK12 were screened based on thresholds of  $R > 0.35$  and  $P < 0.05$  using R-project (<http://www.R-project.org/>) and R studio (<http://www.rstudio.com/>). GO and KEGG enrichment

analyses were performed for the screened differentially expressed genes using the GeneDenovo tool (<https://www.omicsshare.com/tools/>).

**Immune-related analysis.** The EPIC and QUANTISEQ (<https://www.epicimmuneatlas.org>) datasets from TCGA (<https://icbi.i-med.ac.at/software/quantiseq/doc/index.html>) were used for the immune analysis of all types of infiltrating immune cells. The relationship between the expression levels of MAPK12 mRNA and immune checkpoint (ICP), microsatellite instability (MSI), and tumor mutational burden (TMB) in different cancer types in TCGA were analyzed using the immuno-analysis module of the SangerBox website (<http://vip.sangerbox.com/home.html>). The ImmuCellAI (<http://bioinfo.life.hust.edu.cn/ImmuCellAI#!/>) portal was used to analyze the relationship between MAPK12 expression and immune-related cells in THCA using the dataset from TCGA. The P-values and partial correlation were obtained using the Spearman rank correlation test.

**Methylation analysis.** The MAPK12 promoter methylation level differences in tumor tissues and normal tissues were analyzed using UALCAN (<http://ualcan.path.uab.edu>). The transcripts per kilobase of exon model per million mapped reads (TPM) was used to normalize the methylation expression value of raw data from TCGA. The MEXPRESS website (<https://mexpress.be/>) (23) was used to obtain the DNA promoter methylation patterns of MAPK12 in THCA.

**Cell culture.** The human normal thyroid cell line HTORI-3 and human THCA cell lines (TPC-1, K-1, and HTH-83) were obtained from ATCC. All cell lines were tested for mycoplasma, and STR cell identification was performed. All cell lines used in this study were cultured in DMEM supplemented with 10% FBS (both from Thermo Fisher Scientific, Inc.) and maintained in an incubator at 37°C, supplied with 5% CO<sub>2</sub>, and 95% humidity.

**Transfection.** The MAPK12 small interfering (si)RNAs were purchased from Shanghai GenePharma Co., Ltd. siRNAs were used to knock down the expression of MAPK12 in HTH-83 and K-1 cells. The pcDNA3.1-MAPK12 plasmid was purchased from Genewiz, Inc. and used to overexpress MAPK12. Transient transfection of 3  $\mu$ l si-MAPK12 (20  $\mu$ M) or 2  $\mu$ l MAPK12 plasmid (1,000 ng/ $\mu$ l) was performed in 6-well plates with a cell density of  $2 \times 10^5$  cells using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The empty vector (pcDNA3.1) and si-NC were also used as negative controls. Transfected cells were harvested 48 h after transfection for subsequent analysis and detection. The MAPK12 siRNA sequences were #1: 5'-AAG UAACACGCUUCCAUCCTT'3' and #2: 5'-UACAAAAGG GUCUAUUUCCTT'3'; the si-NC sequence was sense: UUC UCCGAACGUGUCACGUTT and antisense: ACGUGACAC GUUCGGAGAATT.

**Reverse transcription-quantitative (RT-q) PCR.** The total RNA was extracted using TRIzol<sup>®</sup> reagent (Thermo Fisher Scientific, Inc.). The GoScript RT system (Promega Corporation) was used to synthesize cDNA according to

the manufacturer's protocol. The MAPK12 mRNA expression status was detected using GoTaq®qPCR Master Mix (Promega Corporation) on an ABI QuantStudio 3. The PCR system was 20  $\mu$ l in total, including 2X SYBR Green qPCR Master Mix (10  $\mu$ l; Bimake), cDNA (2  $\mu$ l), primer mix (2  $\mu$ l), DNase/RNase-free water (6  $\mu$ l), and the following thermocycling conditions were used: 95°C for 3 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min. The dissolution curve program was: 95°C for 15 sec, 60°C for 1 min, and 95°C for 1 sec. The relative expression levels of the target gene were calculated using the  $2^{-\Delta\Delta C_q}$  method (24). The oligonucleotide primers used for qPCR were: MAPK12 forward, 5'-CCCTGGATGACTTCA CGGAC-3' and reverse, 5'-GCTTCAGGTCCCTCAGCC-3'; GAPDH forward, 5'-GGTGGTCTCCTCTGACTTCAACA-3' and reverse, 5'-GTTGCTGTAGCCAAATTCGTTGT-3'.

**Western blotting.** The plates were carefully washed twice with PBS. Total protein was extracted using RIPA buffer (Beijing Solarbio Science & Technology Co., Ltd.) containing protease inhibitors (Beijing Solarbio Science & Technology Co., Ltd.). The protein concentration was detected using a BCA kit (Beijing Solarbio Science & Technology Co., Ltd.) according to the manufacturer's instructions. The protein lysates (20  $\mu$ g) were loaded on a 10% SDS gel, resolved using SDS-PAGE, and transferred to PVDF membranes (MilliporeSigma). Then membranes were blocked using TBS containing 5% BSA (cat. no. A7906, MilliporeSigma) at room temperature for 2 h followed by incubation with primary antibodies against MAPK12 (1:2,000; cat. no. 9212; Cell Signaling Technology, Inc.) or GAPDH (1:5,000; cat. no. 97166; Cell Signaling Technology, Inc.) overnight at 4°C, followed by incubation with the corresponding HRP-conjugated secondary antibody at 1 h (1:3,000; cat. no. 7074; Cell Signaling Technology, Inc.). The protein bands were visualized and detected using an enhanced chemiluminescence system (Bio-Rad Laboratories, Inc.). GAPDH was used as a loading control.

**Cytotoxicity assay.** A cytotoxicity assay (Dojindo Molecular Technologies, Inc.) was used to detect cell proliferation. A total of  $2 \times 10^3$  cells/well were plated in 96-well plates with 3 replicate wells/group. The treatment group was treated with siMAPK12 knockdown. After 0, 1, 2, 3, 4, and 5 days, 100  $\mu$ l serum-free solution (ApexBio) containing 10% cytotoxicity reagent was added, and cells were further incubated at 37°C for 1 h. The optical density values were obtained at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

**EdU assay.** An EdU assay was used to detect cell proliferation. A total of  $2 \times 10^5$  THCA cells/well (HTH-83, K-1, or TPC1) were plated in 96-well plates. After 24 h, the adherent cells were transfected. After 48 h, 100  $\mu$ l EdU medium containing 10  $\mu$ M (Guangzhou RiboBio Co., Ltd.) was added to each well for 2 h, and the culture medium was washed and fixed with 100  $\mu$ l cell fixator (cat. no. P1110; Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 30 min. The fixed cells were permeabilized with 100  $\mu$ l PBS containing 0.5% Triton X-100, followed by staining using an Apollo staining reaction solution (Guangzhou RiboBio Co., Ltd.) in the dark for 30 min at 37°C. A Hoechst 33342 reaction solution (100  $\mu$ l 1x; Guangzhou RiboBio Co., Ltd.) was used for 10 min at 37°C. The dyed

plate was placed under an inverted fluorescence microscope (x100 magnification) to obtain fluorescence images.

**Statistical analysis.** Statistical analyses were automatically calculated using the aforementioned online tools. Comparisons between two groups were made using an unpaired t-test. Comparisons between multiple groups were made by one-way ANOVA and comparisons between CCK8 groups were made using two-way ANOVA, followed by Bonferroni's post-hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**MAPK12 expression patterns.** The flowchart of this study is shown in Fig. 1. The HPA database revealed that MAPK12 mRNA expression was highest in skeletal muscle, followed by the tongue (nTPM >100; Fig. 2A). MAPK12 mRNA expression levels were detectable but low (nTPM <20) in most other normal human tissues (Fig. 2A). To understand and analyze the differences in mRNA expression levels of MAPK12 in normal tissues compared with the respective tumor tissues, the expression profiles of several cancers were obtained from TCGA and the differences in expression of MAPK12 mRNA in normal tissues and tumor tissues were determined. The mRNA expression levels of MAPK12 in all TCGA tumor datasets are shown in Fig. 2B. MAPK12 mRNA expression levels were higher in several TCGA tumor datasets compared with the corresponding normal tissues. The expression of MAPK12 mRNA was significantly higher in 12 cancers: Cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), esophageal carcinoma (ESCA), head and neck cancer (HNSC), kidney renal clear cell carcinoma (KIRC), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), prostate adenocarcinoma (PRAD), rectum adenocarcinoma (READ), stomach adenocarcinoma (STAD), and THCA. However, MAPK12 mRNA expression was lower in kidney chromophobe (KICH), kidney renal papillary cell carcinoma (KIRP), and uterine corpus endometrial carcinoma (UCEC) (Fig. 2B).

The expression levels of MAPK12 in THCA were further studied to add to the relevance of disciplinary research. First, three GEO datasets were obtained: GSE33630, GSE27155, and GSE65144. MAPK12 expression levels were higher in all three databases compared with the normal tissues (Fig. 2C-E). Three THCA cell lines and a normal thyroid follicular cell line were used to verify the expression of MAPK12 RNA and protein. The expression levels of MAPK12 protein and mRNA were higher in the THCA cell lines compared with the normal thyroid follicle cells (Fig. 2F-G).

In conclusion, these results suggested that MAPK12 expression was upregulated in several tumors, including THCA.

**MAPK12 expression is associated with prognosis across several types of cancer, including THCA.** First, the correlation between MAPK12 mRNA levels and prognosis using patient survival-related information obtained from TCGA was determined, and the OS curves were plotted. The results showed that higher levels of MAPK12 mRNA in multiple cancer types



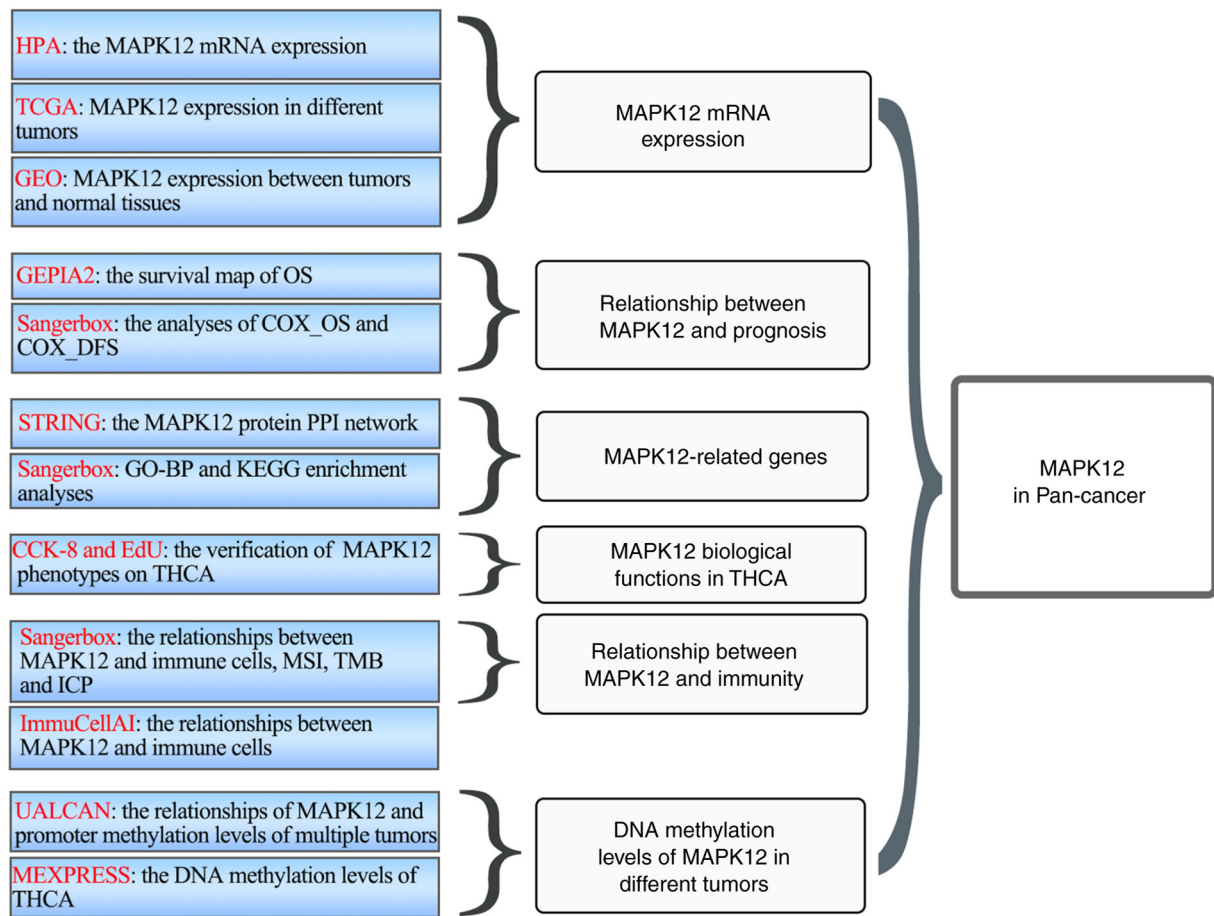


Figure 1. Flow chart of the bioinformatics analyses.

were associated with a poorer prognosis and shorter survival in patients with multiple tumors, including bladder urothelial carcinoma (BLCA), LIHC, mesothelioma (MESO), THCA, and uveal melanoma (UVM) (Fig. 3). Cox regression analysis was used to further analyze the relationship between MAPK12 mRNA levels with OS and DFS in tumor patients. The results showed that high mRNA levels of MAPK12 were associated with a shorter OS in the pan-kidney cohort (KIPAN), LAML, HNSC, LIHC, lung adenocarcinoma (LUAD), BLCA, LAML, COADREAD, COAD, ACC, MESO, THCA, and UVM, and a shorter DFS in KIPAN, STES, HNSC, BRCA, KIRP, BLCA, ACC, COAD, COADREAD, UVM, MESO, and THCA (Fig. S1A and B).

Overall, the analyses suggested that high levels of MAPK12 mRNA were associated with a poorer prognosis pan-cancer, including in THCA.

**Enrichment analysis of MAPK12-related genes.** To further examine the molecular biological mechanism of MAPK12 function in tumors, MAPK12 expression-related proteins were identified, a protein-protein interaction network was constructed, and functional enrichment analysis of the MAPK12 expression-related genes obtained above was performed. First, the interaction of the top 50 proteins associated with MAPK12 in the form of a network diagram was shown using STRING (Fig. 4A). Second, the top 100 genes with a significant correlation with the MAPK12 gene in the

generalized carcinoma dataset from TCGA were determined using GEPIA2. Third, GO-biological process (BP) and KEGG functional enrichment analyses were performed using the top 100 positively related genes (Fig. 4B and C). The results showed that the top 100 genes were enriched in cell proliferation-related pathways and immune-related pathways, including ‘cell cycle process’ and ‘activation of immune response’.

The THCA dataset from TCGA was downloaded and analyzed regarding MAPK12-related genes based on thresholds of  $R > 0.35$  and  $P < 0.05$ . The related genes were analyzed using GO-BP and KEGG functional enrichment. The results were similar to that of the pan-cancer analysis. MAPK12 THCA was also enriched in cell proliferation, and in immune-related functions and pathways (Fig. 4D and E).

Based on these results, it was speculated that MAPK12 promoted the development of tumors pan-cancer, particularly in THCA, by influencing cell proliferation and the tumor immune microenvironment (TIM). Therefore, the underlying mechanism was further examined.

**MAPK12 expression is associated with THCA cell proliferation *in vitro*.** Although the functional enrichment analysis results confirmed that MAPK12 promoted cancer progression in THCA, experimental verification was required to confirm the bioinformatics results. Three classical THCA cell lines, TPC-1, HTH-83, and K-1, were selected for the cell proliferation experiments. MAPK12 expression in HTH-83 and



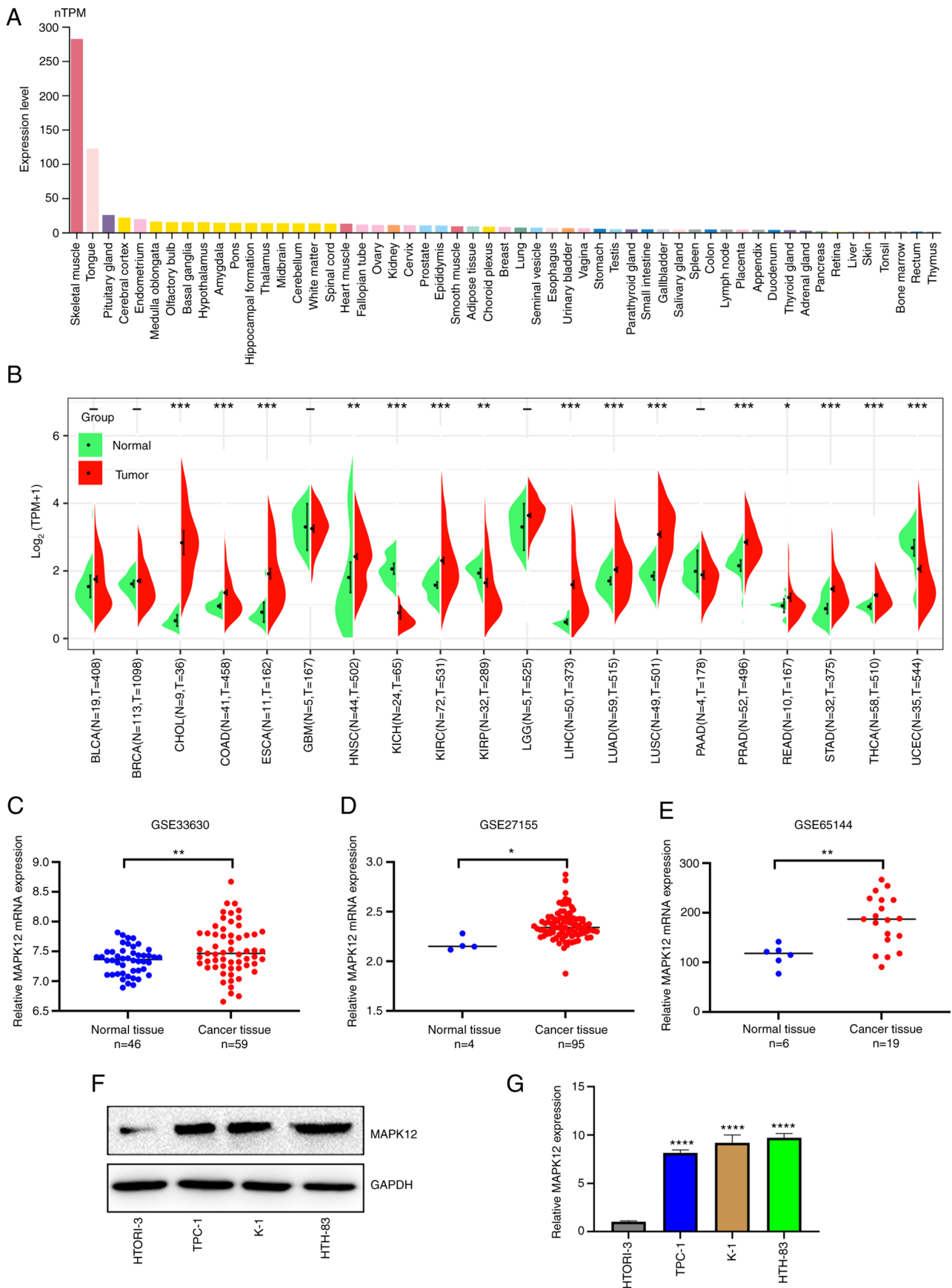


Figure 2. MAPK12 mRNA expression levels in normal and cancer tissues. (A) The mRNA expression levels of MAPK12 in several normal tissues based on the Human Protein Atlas. (B) Based on TCGA data, the mRNA expression levels of MAPK12 in different cancer tissues were analyzed using GEPIA2. (C-E) Based on the GEO data, the mRNA expression levels of MAPK12 mRNA in different cancer tissues were analyzed. Based on (F) western blotting and (G) RT-qPCR analysis, the protein and mRNA expression levels of MAPK12 in a normal thyroid cell line and human thyroid carcinoma cell lines were analyzed. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001. TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; MAPK12, P38 mitogen-activated protein kinase 12.

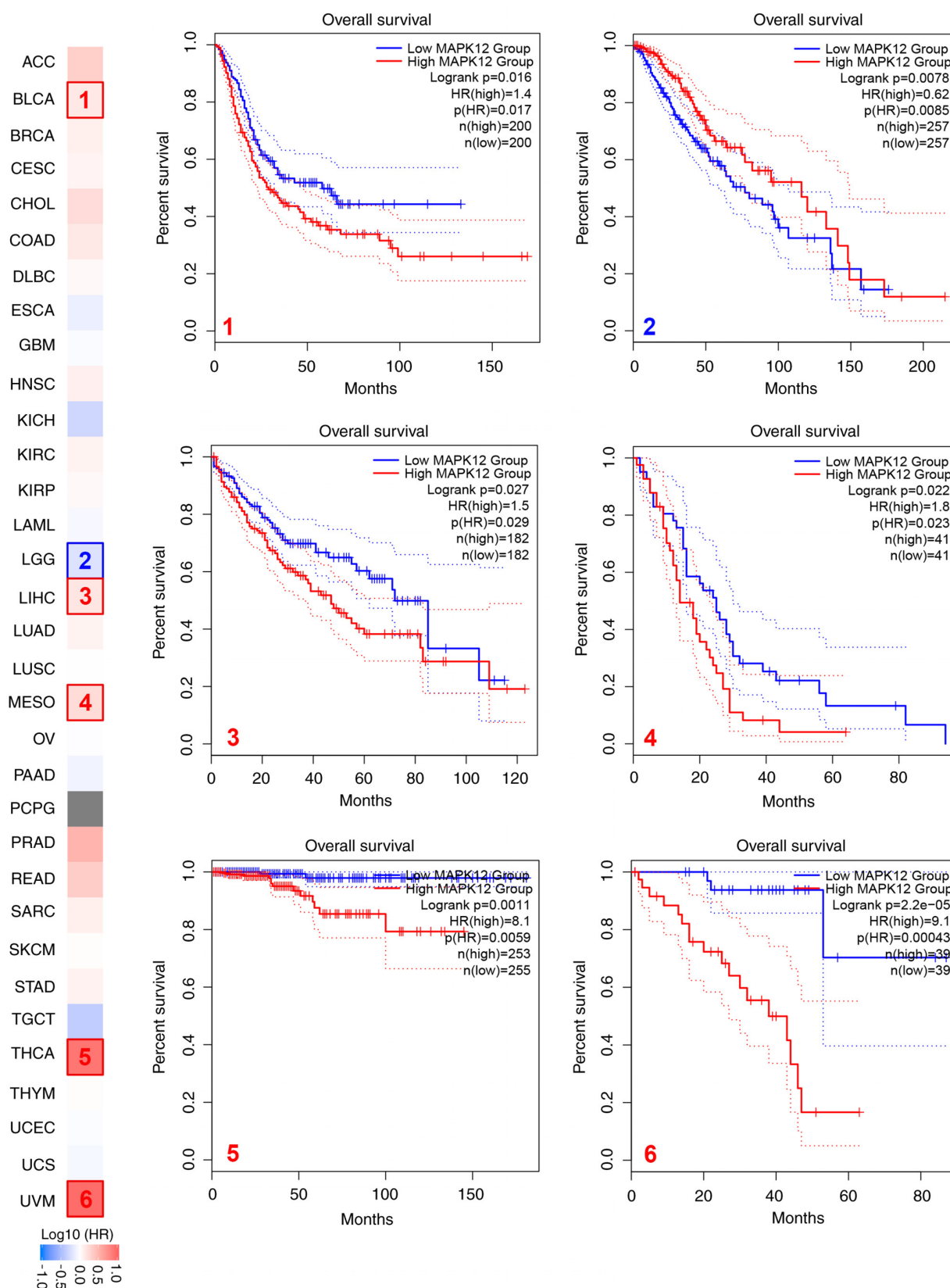


Figure 3. Correlation analysis between MAPK12 expression and survival prognosis in various types of cancer based on data obtained from TCGA. Overall survival between high and low MAPK12 expression groups in different types of cancer was compared using a log-rank test. A survival map with positive results is shown on the left. TCGA, The Cancer Genome Atlas; MAPK12, P38 mitogen-activated protein kinase 12.

K-1 cell lines was knocked down using siRNA. The MAPK12 expression levels in the TPC-1 cell line were upregulated using a pcDNA.31-MAPK12 plasmid. The efficiencies of MAPK12

knockdown or overexpression were confirmed using RT-qPCR and western blot analyses (Figs. 5A, B, and S2A). Cytotoxicity and EdU assays were performed, and the results showed that

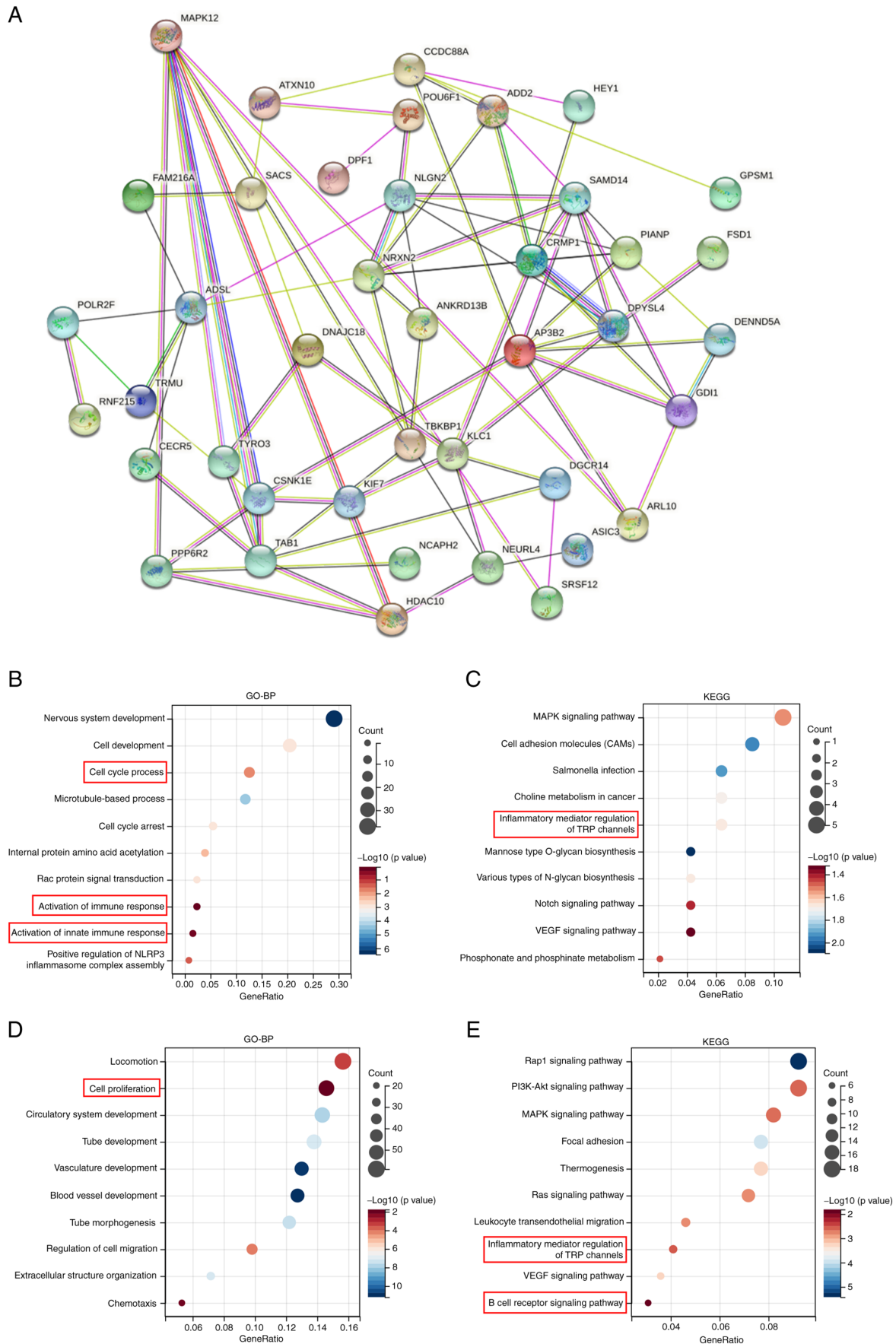


Figure 4. MAPK12-related genes enrichment analysis. (A) MAPK12-binding proteins were obtained using STRING. The significantly enriched (B) GO annotations and (C) KEGG pathways of the top 100 genes positively associated with MAPK12 expression in all TCGA tumor datasets are shown. The significantly enriched (D) GO annotations and (E) KEGG pathways of the top 100 genes positively associated with MAPK12 expression in the THCA dataset from TCGA are shown. TCGA, The Cancer Genome Atlas; MAPK12, P38 mitogen-activated protein kinase 12; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; THCA, thyroid carcinoma.



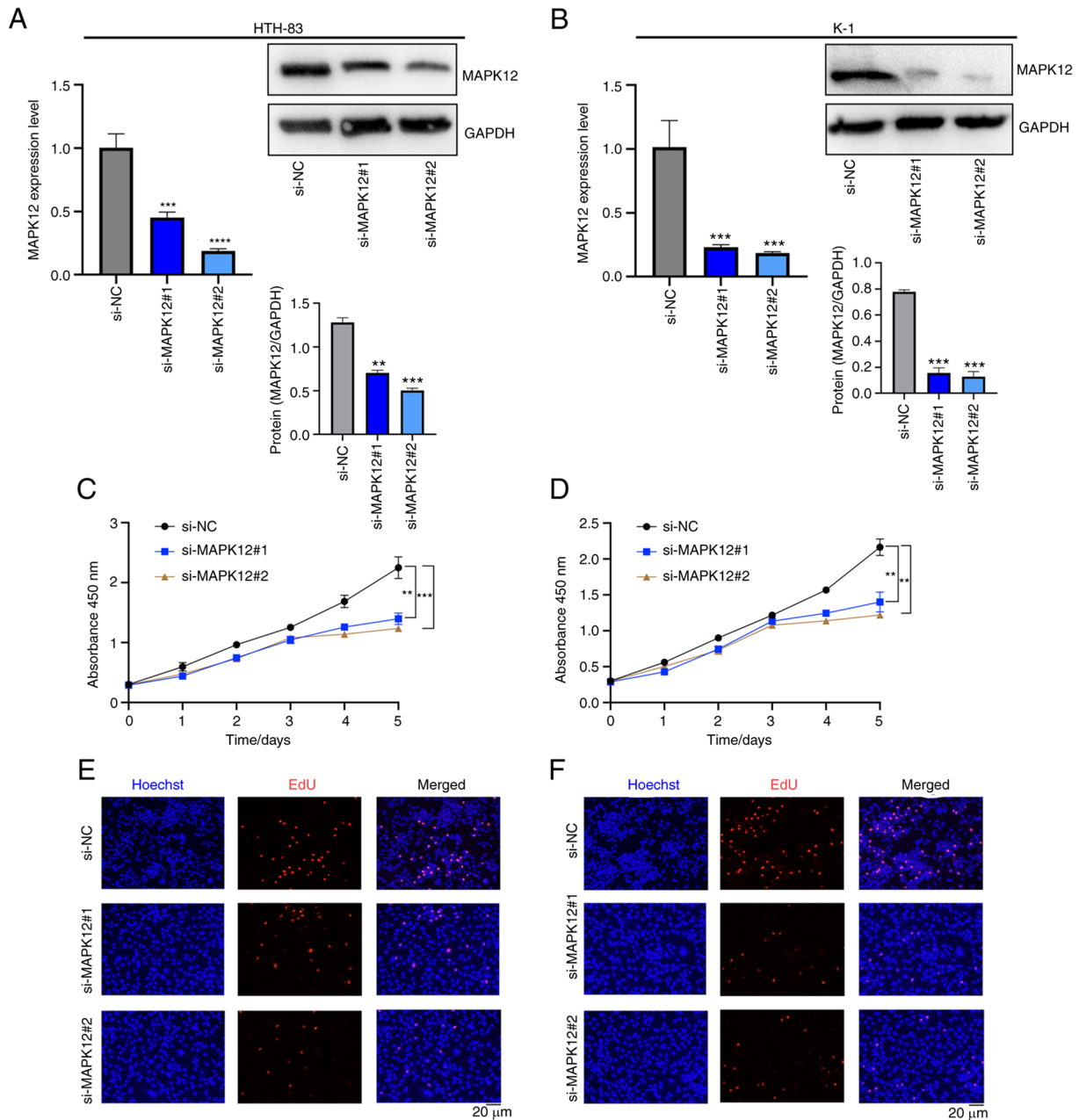


Figure 5. Experimental verification of MAPK12 expression and phenotype in THCA cell lines. The relative expression of MAPK12 at the mRNA and protein level following siRNA-mediated knockdown of MAPK12 in (A) HTH-83 and (B) K-1 cells. Cytotoxicity assays were used to measure cell viability and proliferation following knockdown of MAPK12 in (C) HTH-83 and (D) K-1 cells. EdU assays were used to measure cell viability and proliferation following knockdown of MAPK12 in (E) HTH-83 and (F) K-1 cells.  $^{*}P<0.01$ ,  $^{**}P<0.001$ ,  $^{***}P<0.0001$ . MAPK12, P38 mitogen-activated protein kinase 12; siRNA, small interfering RNA; NC, negative control.

knockdown of MAPK12 led to a significant decrease in cell proliferation, whereas overexpression of MAPK12 resulted in an increase in cell proliferation (Figs. 5C-F, S2B and C). Together, it was experimentally demonstrated that MAPK12 played a role in tumor occurrence and development by regulating cell proliferation.

*MAPK12 expression is associated with the TIM pan-cancer, including in THCA.* The enrichment analysis of MAPK12-related genes also suggested that MAPK12 influenced the development of tumors by influencing the TIM. Therefore, the impact of MAPK12 on TIM pan-cancer and in THCA was assessed.

First, the correlation between MAPK12 mRNA levels and the abundance of tumor immune cells that had invaded diffuse carcinoma tissues was determined. Tumor-infiltrating lymphocytes (TILs) are an important component of the TIM and are generally associated with the development of tumors. TILs are key predictors of metastatic lymph node status and prognosis in patients with cancer. First, the relationship between TIL abundance and MAPK12 mRNA levels was determined using the EPIC and QUANTISEQ datasets. MAPK12 mRNA levels showed significant correlations with multiple TIIs/TILs in THCA (Fig. 6A and B). MAPK12 expression levels also showed a positive correlation with various TIIs in the TCGA-THCA dataset (Fig. S3). Macrophages are a significant constituent of

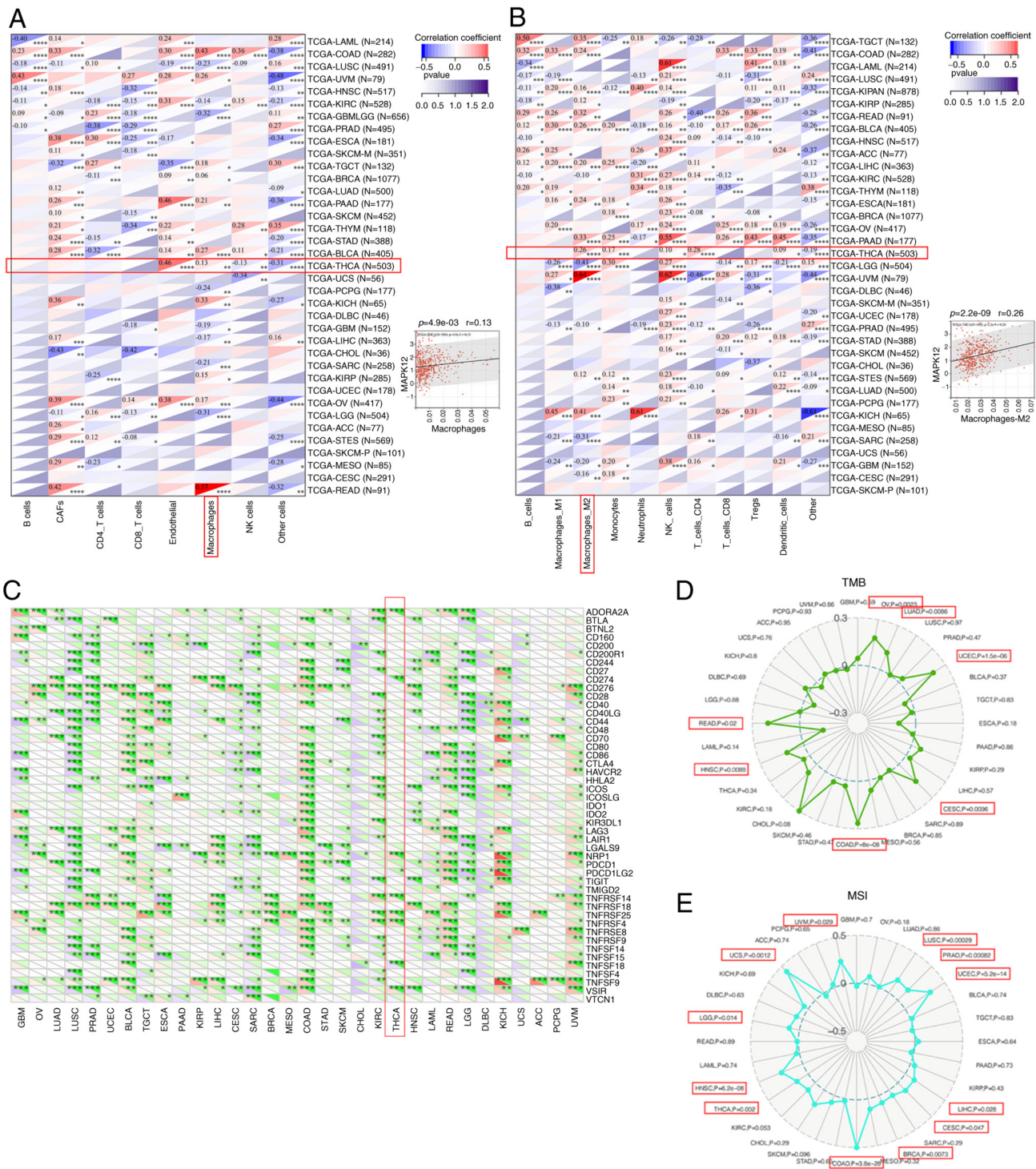


Figure 6. Correlation analysis between MAPK12 expression and immune infiltration levels. (A and B) The relationship between MAPK12 expression and immune cell infiltration level was analyzed in various types of cancer using Sangerbox. (C) The relationship between MAPK12 expression and the expression levels of ICP-related genes in several types of cancer. (D and E) The relationship between MAPK12 expression with TMB and MSI in several types of cancer. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ . MAPK12, P38 mitogen-activated protein kinase 12; ICP, immune checkpoint; TMB, tumor mutational burden; MSI, microsatellite instability.

the innate immune system and play an indispensable role in activating the body's first-line defense against infection and cancer (25). Therefore, the infiltration levels of macrophages were assessed. A positive correlation was obtained in the EPIC database between macrophages and MAPK12 mRNA expression in THCA. Macrophages polarize to antitumor M1 and protumor M2 macrophages. Therefore, the QUANTISEQ

database was used to further analyze the levels of invading M1 and M2 macrophages (26) and found that the M2 invasion levels were positively correlated with the MAPK12 mRNA expression levels in THCA. Therefore, it was hypothesized that THCA tumor cells secreted chemokines to increase the infiltration of tumor-promoting M2 macrophages to increase the degree of tumor malignancy.

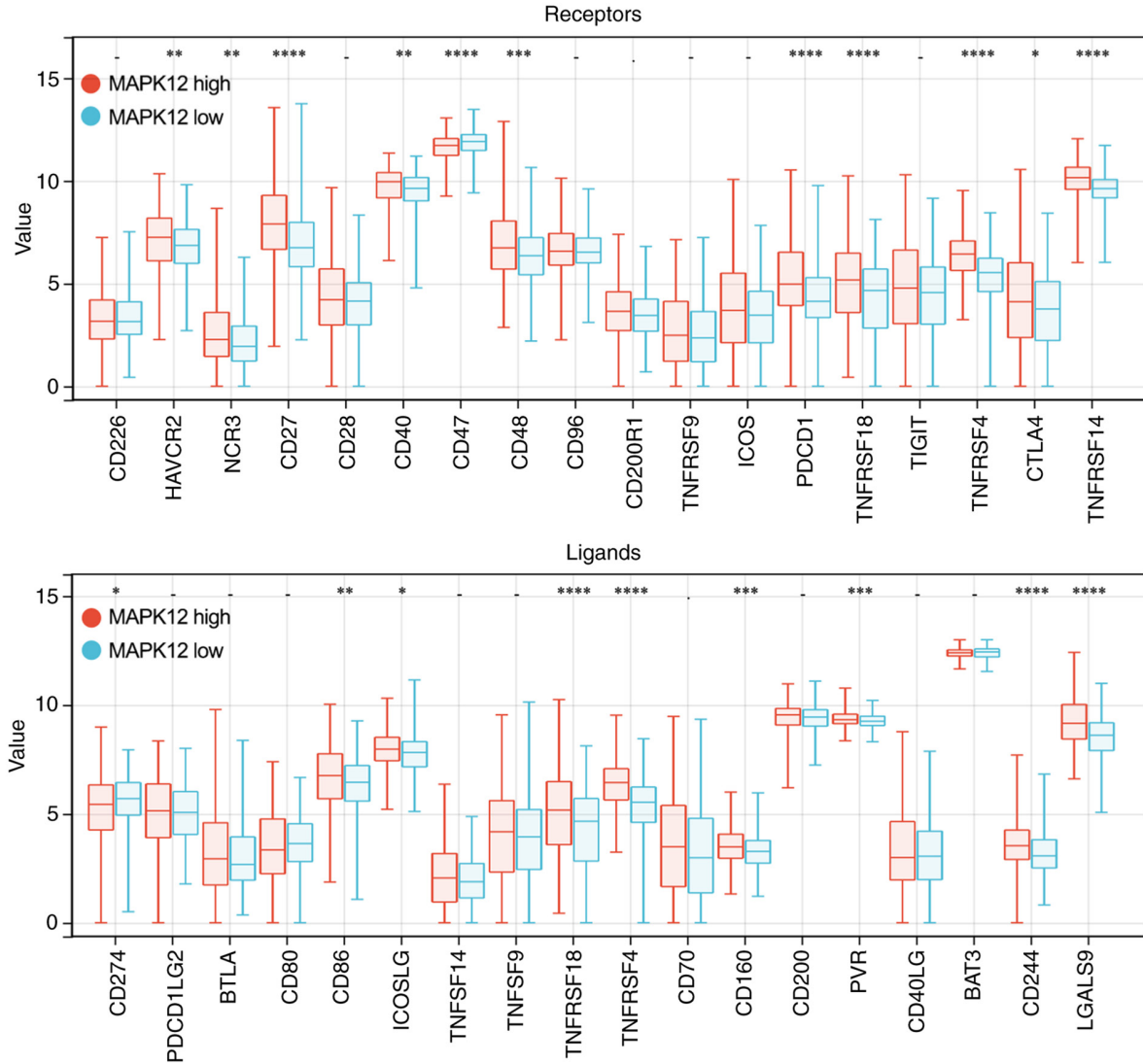


Figure 7. The correlation between MAPK12 expression and ICPs. The relationship between MAPK12 expression and ICP receptors and ligands in THCA. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001. MAPK12, P38 mitogen-activated protein kinase 12; ICP, immune checkpoint; THCA, thyroid carcinoma.

We analyzed whether MAPK12 affected the sensitivity of cancer patients to immunotherapy. ICP proteins are often regarded as promising therapeutic targets in the field of cancer immunotherapy (27-29). Therefore, the relationship between ICP gene expression levels and MAPK12 in various cancer types were determined. MAPK12 mRNA levels were positively correlated with multiple ICP genes in THCA (Fig. 6C). Moreover, MAPK12 expression in THCA showed a positive association with several ICP receptors including HAVCR2, NCR3, CD27, CD40, CD47, CD48, PDCD1, TNFRSF18, TNFRSF4, CTLA4, and TNFRSF14, as well as ICP ligands such as CD274, CD86, ICOSLG, CD160, PVR, CD244, and LGALS9 (Fig. 7). TMB and MSI are also important indicators of whether patients with cancer will benefit from immunotherapy (30,31). The relationship between the expression levels of MAPK12 with TMB and MSI in the TCGA dataset was studied using SangerBox. The results suggested that MAPK12 affected the sensitivity of THCA cells to immune checkpoint inhibitor therapy (Fig. 6D and E). Therefore, it was hypothesized that MAPK12 altered the TIM in THCA tissues by regulating the expression levels of ICP receptors and ligands.

These results suggest that MAPK12 mediates the activation of ICP genes and is thus an ideal target for immunotherapy in THCA patients.

In conclusion, MAPK12 may affect the TIM by modulating the infiltration of immune cells within tumors and the sensitivity of multiple tumors to immunotherapy. Therefore, MAPK12 may serve as an immunotherapeutic target.

*Determination of the MAPK12 methylation levels pan-cancer.* To further study the mechanism of abnormal MAPK12 expression, we also analyzed the DNA methylation patterns of the MAPK12 gene promoter. DNA methylation generally leads to increased expression levels, and upregulation of oncogenes promotes tumor development (32).

The UALCAN online tool was used to explore methylation levels in the MAPK12 promoter region. MAPK12 promoter methylation levels were lower in BLCA, CESC, ESCA, READ, KIRC, KIRP, LUSC, TGCT, THCA, and UCEC (Fig. 8A). These results suggest that methylation of the MAPK12 promoter may lead to its upregulation in a variety of cancer tissues. Next, the MEXPRESS methylation



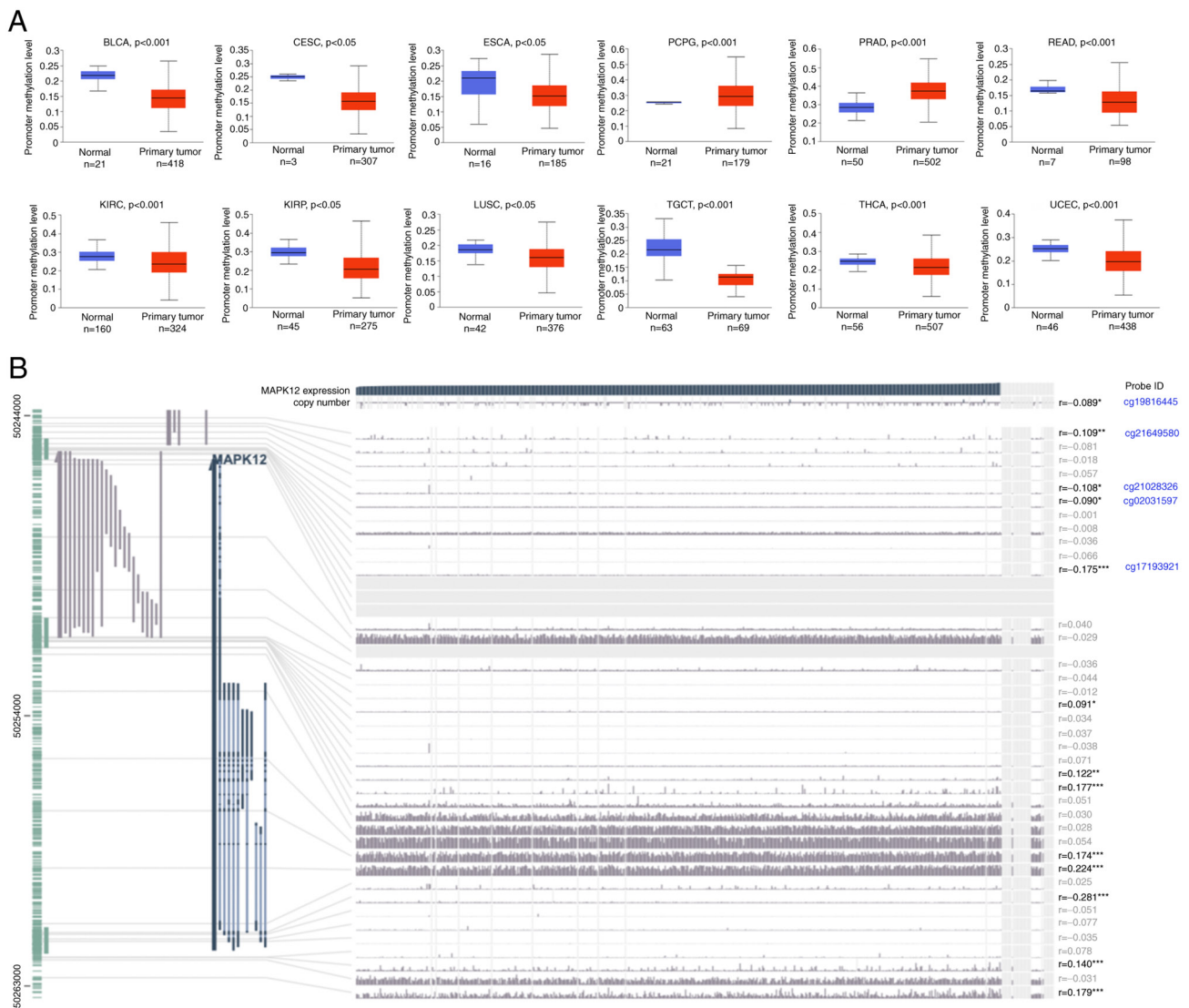


Figure 8. Association between DNA methylation and the mRNA expression levels of MAPK12. (A) Comparison of MAPK12 gene promoter methylation levels between tumor tissues and normal tissues. (B) The correlation between the expression and DNA methylation levels of the MAPK12 gene in THCA based on MEXPRESS. MAPK12, P38 mitogen-activated protein kinase 12; THCA, thyroid carcinoma.

analytical tool was used to analyze THCA promoter levels and the results showed that MAPK12 mRNA expression levels were negatively correlated with MAPK12 methylation levels in THCA. The mRNA levels of MAPK12 were negatively correlated with the MAPK12 methylation levels at probe ID: cg19816445 ( $r=-0.089$ ,  $P<0.05$ ), probe ID: cg21649580 ( $r=-0.109$ ,  $P<0.001$ ), probe ID: cg21028326 ( $r=-0.108$ ,  $P<0.05$ ), probe ID: cg02031597 ( $r=-0.090$ ,  $P<0.05$ ), and probe ID: cg17193921 ( $r=-0.175$ ,  $P<0.0001$ ) in THCA (Fig. 8B).

Taken together, these results suggest that the carcinogenicity of high MAPK12 expression in multiple types of cancer was due to hypomethylation of its promoter, particularly in THCA.

## Discussion

There are four subtypes of p38 MAPK encoded by different genes in mammalian cells: P38 $\alpha$  (MAPK14), P38 $\beta$  (MAPK11), P38 $\gamma$  (MAPK12), and P38 $\delta$  (MAPK13) (33). P38 MAPKs exhibit different expression patterns in different tissues. P38 $\alpha$

was detected in all cells and tissues, and P38 $\beta$  was specifically overexpressed in brain tissue, thymus tissue, and spleen tissues. P38 $\beta$  is expressed at low levels in several tissues, such as the adrenal gland, and it is not expressed in skeletal muscle. In contrast, P38 $\gamma$  is highly expressed in skeletal muscle, whilst being expressed at very low levels in other tissues (34-37). All P38 MAPKs are serine/threonine kinases that are activated by a variety of inflammatory factors in a variety of conditions.

MAPK12, also known as P38 $\gamma$ , ERK6, and SAPK3, regulates some of the processes of malignant transformation in several human cancer cell lines, such as proliferation, cell cycle progression, and apoptosis (38,39). Several researchers found that MAPK12 promoted the development and progression of various types of cancer (40-42). However, the role of MAPK12 in THCA metastasis is not known. Therefore, data from TCGA was used to analyze the functional role of MAPK12 in various tumors, particularly THCA. The analysis performed in this study included the expression of MAPK12 at the RNA level and the effect of differential expression on

prognosis, functional enrichment analysis of MAPK12-related genes, and further analysis of its effect on tumor cell growth and proliferation, and the TIM.

The present study found that MAPK12 was overexpressed in several tumors. The mRNA and protein expression levels of MAPK12 were higher in THCA cell lines compared with normal thyroid follicles. Higher mRNA levels of MAPK12 were associated with a worse OS in KIPAN, LAML, HNSC, LIHC, LUAD, BLCA, LAML, COADREAD, COAD, ACC, MESO, THCA, and UVM, and a shorter DFS in KIPAN, STES, HNSC, BRCA, KIRP, BLCA, ACC, COAD, COADREAD, UVM, MESO, and THCA. GO-BP and KEGG enrichment analyses were performed using the MAPK12-related genes following analysis of the THCA data from TCGA, and the results showed that MAPK12-related genes were enriched in cell proliferation and tumor immune-related functions and pathways. MAPK12 is highly expressed in HNSC, and it promotes the proliferation of ESCC cells and prevents their apoptosis *in vitro* (24). Hou *et al* (25) found that MAPK12 expression was significantly elevated in human colorectal cancer tissues relative to the corresponding normal epithelial tissues, and it promoted the growth, proliferation, and migration of CRC cells whilst inhibiting cellular apoptosis via the direct phosphorylation of PTPH1. Xu *et al* (26) showed that MAPK12 was positively correlated with the grade of glioma and may be a tumorigenic factor that promotes the growth and progression of glioma. Based on these results, it was hypothesized that MAPK12 promoted the development of tumors pan-cancer, particularly in THCA, by affecting cell proliferation and antitumor immunity.

The association between MAPK12 mRNA levels and immune cell infiltration based on the GO and KEGG results of MAPK12-related genes was assessed. As an important component of the TIM, tumor-infiltrating immune cells are generally associated with the occurrence, progression, treatment, and/or metastasis of tumors (43). The MAPK12 mRNA levels were significantly correlated with multiple TIIs/TILs in THCA. A positive correlation was observed between macrophage numbers and MAPK12 mRNA expression levels in THCA in the EPIC database. As macrophages can polarize to antitumor M1 and protumor M2 macrophages (27), the levels of invading M1 and M2 macrophages were analyzed and the results showed that the level of invading M2 macrophages was positively correlated with the expression levels of MAPK12 mRNA in THCA. Therefore, it was hypothesized that tumor cells secreted chemokines to increase the infiltration of tumor M2 macrophages in THCA, which increased the degree of tumor malignancy. Whether MAPK12 affected the sensitivity of cancer patients to immunotherapy was next assessed. ICP, MSI, and TMB analyses showed that MAPK12 may be an ideal target for the treatment of THCA patients, especially in immunotherapy.

In conclusion, the results of the present study suggest that MAPK12 may be a promising prognostic marker and a potential factor for predicting sensitivity to immunotherapy in patients with malignant tumors, particularly THCA.

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## Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

## Authors' contributions

CF and YG conceived and designed the study. JW and ZS performed the experiments. LR, BZ and YZ analyzed the results. TL and XY wrote the manuscript and performed some of the experiments. CF and YG 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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