

Identification of Molecular Subtypes and a Novel Prognostic Model of Thyroid Cancer Based on a Pyroptosis and Mitophagy Related Gene Signature

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Background: The global incidence of thyroid carcinoma (THCA) is gradually increasing. Our investigation aims to establish a signature relevant to pyroptosis and mitophagy, which could provide new insights into prognostic biomarkers and therapeutic targets associated with THCA.

Methods: Gene sequencing data and clinical information were obtained from The Cancer Genome Atlas (TCGA) database and Gene Expression Omnibus (GEO) database. A six-gene prognosis signature of pyroptosis- and mitophagy-related differentially expressed genes (PMRDEGs) was constructed using least absolute shrinkage and selection operator (LASSO) regression analysis. The prognostic efficacy of the risk score model in the TCGA-THCA cohort was evaluated using Kaplan-Meier (KM) survival plots and receiver operating characteristic (ROC) curve analysis. Functional enrichment analysis identified the potential for differential expression of biological pathways across various subgroups of risk scores. The features of the tumor microenvironment associated with the risk score were investigated using the CIBERSORT algorithm for immune infiltration analysis. The pyroptosis and mitophagy score was calculated to predict the response to immune-checkpoint inhibitor (ICI) treatment in thyroid cancer. Additionally, real-time quantitative PCR (qPCR) and Western Blot (WB) were utilized to investigate the expression levels of hub genes.

Results: A prognostic signature consisting of six pyroptosis- and mitophagy-related genes (Apolipoprotein E (*APOE*), Ephrin type-A receptor 2 (*EPHA2*), DEAD-box helicase 3 (*DDX3X*), Centrosome protein 55 (*CEP55*), Kinesin family member 23 (*KIF23*), H2B clustered histone 9 (*H2BC9*)) was established, and participants were stratified into high- and low-risk groups based on their risk levels. When comparing the progression-free interval (PFI) of the high-risk group with that of the low-risk group, Kaplan-Meier (KM) analysis revealed a statistically significant reduction in PFI for the high-risk group ($p < 0.05$). Gene Set Variation Analysis (GSVA) illustrated differential expression of 20 pathways between the high- and low-risk groups, including activation of Neurotrophic receptor tyrosine kinase 2 (NTRK2) signals through the Phosphoinositide 3-kinase (PI3K) pathway ($p < 0.001$). Immune infiltration analysis demonstrated significant variations in immune cell distribution between the two investigated groups ($p < 0.05$). According to quantitative real-time PCR (qRT-PCR) findings, mRNA expression levels of *APOE*, *EPHA2*, *CEP55*, and *KIF23* were significantly higher in tumor cells ($p < 0.01$). Additionally, expression levels of the pyroptosis-associated proteins NOD-like receptor thermal protein domain associated protein 3 (NLRP3), Gasdermin-D, and Caspase-1 were significantly downregulated in the si-KIF23 group ($p < 0.05$).

Conclusions: Our study developed a prognostic risk model for thyroid cancer, comprising six pyroptosis- and mitophagy-related genes. This model serves as a valuable marker for prognosis, diagnosis, and predicting the response to immunotherapy in individuals with THCA.

Keywords: pyroptosis; mitophagy; prognostic signature; thyroid carcinoma; bioinformatics

Introduction

Thyroid carcinoma (THCA) is a prevalent endocrine malignancy that has exhibited a significant rise in incidence in the last few years [1], with a total of 586,202 new cases worldwide in 2020 [2]. THCA could be classified into at least four subtypes depending on the histological characteristics: papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), medullary thyroid cancer (MTC), and anaplastic thyroid cancer (ATC). PTC is recognized as the predominant subtype of THCA, constituting over 90% of all thyroid cancers [3]. Even though well-differentiated thyroid tumors, such as PTC, have a decent prognosis, MTC and ATC nevertheless have relatively high mortality rates [4]. There are still some patients who develop local and distant metastases, resulting in a weak prognosis outcome [5]. Moreover, the management of refractory thyroid cancer continues to pose a significant challenge in the field [6]. A study has shown that a prognostic marker consisting of six senescence-related genes identified in The Cancer Genome Atlas (TCGA) dataset was significantly correlated with clinical characteristics, survival outcomes, and immunotherapy response in THCA patients [7]. Characterization of molecular subtypes of thyroid cancer can help in risk stratification management of tumors and precision therapy, and the development of bioinformatics will greatly facilitate this advancement. To improve the identification of high-risk patients at an earlier stage and provide better personalized treatment, it is imperative to conduct additional research on novel biomarkers that could serve as prognostic indicators and therapeutic targets.

Mitophagy, a specialized form of autophagy, plays a crucial role in maintaining mitochondrial homeostasis by regulating the removal of damaged mitochondria [8]. In response to various pathological challenges such as the collapse of mitochondrial transmembrane potential (MMP), oxidative stress, hypoxia, and iron starvation, mitophagy can be strongly induced [9]. Several mechanisms of mitophagy have been identified, typically classified into pathways dependent on PRKN (parkin RBR E3 ubiquitin protein ligase) and those that are PRKN-independent [10]. Increasing evidence suggests that dysregulation of mitophagy contributes to various human pathologies, including heart damage, kidney fibrosis, and neurodegenerative diseases [11–13]. Recent study has highlighted the crucial role of mitophagy in tumorigenesis and progression. Mitophagy deficiency mediated by depletion of ULK1 in breast cancer cells has been shown to promote the onset of breast cancer and bone metastasis [14]. Additionally, mitochondria-eating protein (MIEAP), a key molecule in non-canonical mitophagy, has been demonstrated to inhibit tumor progression in a V-raf murine sarcoma viral oncogene homolog B1 (BRAF)^{V600E+} mouse model of thyroid cancer [15]. However, the precise mechanism of mitophagy in thyroid tumors

remains unclear, and the potential of targeting mitophagy as an anti-tumor therapeutic strategy warrants further investigation.

Pyroptosis represents a form of inflammatory cell death triggered by the activation of Caspase-1/4/5/11, resulting in cell swelling, plasma membrane disruption, chromatin fragmentation, and intracellular secretion of proinflammatory molecules [16]. Pyroptosis has been directly associated with various disorders of the neurological system, autoimmune diseases, infectious diseases, cardiovascular disorders, and malignancies [17–20]. Alantolactone has been found to exert antitumor effects by inducing GSDME-dependent pyroptosis in anaplastic thyroid cancer (ATC) through the reactive oxygen species (ROS) mitochondria-dependent caspase pathway [21]. Pyroptosis exhibits different roles in different tumors, and its relationship with thyroid cancer remains unclear.

Mitophagy and pyroptosis represent two distinct forms of programmed cell death. Studies have indicated that mitophagy serves as a compensatory mechanism for pyroptotic cell death by removing pyroptosis-induced mitochondrial damage through the interaction of NOD-like receptor thermal protein domain associated protein 3 (NLRP3) and CASP1 proteins [22]. Conversely, impaired mitophagy leads to the overexpression of mitochondrial ROS, thereby enhancing hepatocyte pyroptosis [23]. Despite numerous studies confirming the critical roles of mitophagy and pyroptosis in tumor development, the significance of their genetic markers in thyroid cancer remains unclear.

Throughout this investigation, gene sequencing data of THCA were utilized to construct a prognostic model comprising six pyroptosis- and mitophagy-associated genes. Further analyses were conducted on tumor immune infiltration and the response to treatment with immune checkpoint inhibitors (ICIs) associated with the signature in THCA. The aim of our research was to investigate the relationship between the pyroptosis- and mitophagy-related prognostic signature and THCA. Our findings indicate that this signature serves as a valuable marker for the diagnosis, prognosis, and response to immunotherapy in THCA patients.

Materials and Methods

Data Download

The THCA dataset was acquired from The Cancer Genome Atlas (TCGA) through the TCGA biolinks package [24] (<https://portal.gdc.cancer.gov/>). It comprised count sequencing data from 510 THCA samples and 58 paracancerous samples (normal group), totaling 568 samples for subsequent analysis. The baseline characteristics of the THCA patients are summarized in **Supplementary Table 1**. Additionally, count sequencing data were normalized to Fragments Per Kilobase-Million (FPKM) format, while

corresponding clinical information was obtained from the UCSC Xena database [25]. TIDE immune score results for the TCGA-THCA dataset were obtained from the tumor immune dysfunction and exclusion (TIDE) database [26,27] (<http://tide.dfci.harvard.edu>). Furthermore, the immunogenicity score (IPS) for 20 cancers, including THCA, were obtained from The Cancer Immunome Atlas (TCIA) database [28] (<https://tcia.at/home>), which serves as a reliable predictor of CTLA-4 and PD-1 responsiveness. The IPS data for THCA were retrieved from the TCIA database for further analysis using the ggplot2 R package (<https://ggplot2.tidyverse.org>).

The expression profile datasets GSE336308 [29], GSE53157 [30], and GSE35570 [31] were obtained from the Gene Expression Omnibus (GEO) database [32] using the “GEOquery” package [33] (<https://github.com/seandavi/GEOquery>, <http://seandavi.github.io/GEOquery>) for THCA patients. Both GSE33630 and GSE53157 datasets were from Homo sapiens. The GSE33630 dataset comprised 49 thyroid tumor tissues and 45 paracancerous tissue samples (normal group) from THCA patients, totaling 94 samples for analysis. The GSE53157 dataset consisted of seven thyroid tumor tissues from THCA patients, two paraneoplastic tissues, and one normal thyroid tissue sample, with a total of ten samples included in the analysis. In the GSE35570 dataset, there were 65 specimens of thyroid tumor tissues from THCA patients, including 33 samples exposed to radiation (excluded) and 32 unexposed samples, along with 51 normal thyroid tissue samples, totaling 83 samples for analysis. The data platform utilized in this study were GPL570 [HG-U133_Plus_2], which is available from the Affymetrix Human Genome U133 Plus 2.0 Array (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL570>), and it is a microarray platform used for the analysis of gene expression. The TCGA-THCA dataset served as the test set, while the datasets GSE33630 and GSE53157 were merged to form the Merged dataset for validation analysis. Additionally, dataset GSE35570 was utilized as another validation set. **Supplementary Table 2** provides detailed information on the datasets used in this study. All data for this study were obtained from public databases and did not require ethical approval.

Acquisition of Pyroptosis- and Mitophagy-Related Genes and Differential Analysis

To gain insight into the characteristics and trends of cancer samples and paracancer samples in the TCGA-THCA dataset, we initially conducted a principal component analysis (PCA) using the “prcomp” R package (version 3.6.3) (<https://www.r-project.org/>). We standardized the data by setting the parameter scale=TRUE and centered it using center=TRUE. To identify genes for subsequent differential analysis, we searched the GeneCards database [34] (<http://www.genecards.org/>) for “Pyroptosis” and retrieved 429 pyroptosis-related genes (PRGs). Similarly, by search-

ing for “Mitophagy”, we obtained 3486 mitophagy-related genes (MRGs). The intersection of PRGs and MRGs yielded 172 pyroptosis- and mitophagy-related genes (PMRGs). Utilizing the grouping information (thyroid cancer group/paracancer group) within the dataset, we performed differential analysis using the R package limma to identify differentially expressed genes (DEGs). Genes with up-regulated expression were identified based on a $\log_2$ Fold Change (FC) >0.5 and $p.\text{adj} < 0.05$, while those with downregulated expression had a $\log_2\text{FC} < -0.5$ and $p.\text{adj} < 0.05$. Pyroptosis- and mitophagy-related differentially expressed genes (PMRDEGs) have been acquired through the intersection of DEGs with PMRGs.

Prognostic Model Construction

To identify pyroptosis- and mitophagy-related genes associated with THCA survival outcomes, pyroptosis- and mitophagy-related differentially expressed genes (PMRDEGs) in the TCGA-THCA dataset underwent univariate Cox regression analysis to establish prognostic models. The glmnet package [35] was employed to select variables with $p < 0.1$ based on different levels of pyroptosis- and mitophagy-related gene expression within the TCGA-THCA dataset. This selection process utilized least absolute shrinkage and selection operator (LASSO) [36] regression, incorporating a ten-fold cross-validation method and 1000 cycles to mitigate overfitting. LASSO regression is a widely used machine learning technique for constructing diagnostic models, particularly in the context of developing prognostic diagnostic models.

$$\text{Risk Score} = \sum_i \text{Coefficient}(\text{hub gene}_i) \times \text{mRNA Expression}(\text{hub gene}_i)$$

The prognostic significance of pyroptosis- and mitophagy-related genes was further assessed through multivariate Cox regression analysis. Subsequently, the resulting coefficients from this multivariate Cox regression were extracted. The prognostic model’s risk score for pyroptosis- and mitophagy-related differentially expressed genes (PMRDEGs) was then computed based on the expression levels of pyroptosis- and mitophagy-related genes. The median risk score value of all THCA participant samples with available progression-free interval (PFI) clinical information was utilized as the threshold parameter to stratify THCA patient specimens into high- and low-risk groups. Finally, clinical data from the TCGA-THCA dataset were integrated to examine the prognostic impact of the pyroptosis- and mitophagy-related gene prognostic model. The “survival” R package was employed to conduct Kaplan-Meier curve analysis, investigating the variation in PFI between the two groups within the TCGA-THCA dataset.

The “rms” R package was employed to construct nomograms, utilizing the outcomes of the multivariate Cox regression analysis. A nomogram plot provides a graphical representation for calculating an aggregate score to predict the likelihood of an event occurrence. This is achieved by establishing a specific scale to score individual variables within the multivariate regression model [37]. Subsequently, the precision of the nomogram plots generated by the multivariate Cox prognostic model was assessed. This evaluation involved utilizing calibration curves to assess their ability to discriminate between participants. Calibration plots were utilized to evaluate the model’s predictive performance against actual outcomes [38]. These plots primarily evaluate the fitness of the model constructed via the Cox regression technique to real-world situations. Decision Curve Analysis (DCA) [39] is a straightforward approach used to evaluate molecular markers, diagnostic tests, and clinical prediction models. DCA plots were utilized to assess the precision and discriminative ability of the model. The multivariate Cox prognostic model’s predictive effect was further evaluated by generating DCA plots using the “ggDCA” R package (<https://rdocumentation.org/package/ggDCA/versions/1.1>).

GO and KEGG Analysis

Gene Ontology (GO) enrichment analysis [40] is a widely used method for conducting comprehensive functional enrichment investigations, typically across three distinct categories: biological process (BP), molecular function (MF), and cellular component (CC) [41]. Kyoto Encyclopedia of Genes and Genomes (KEGG) is a frequently utilized resource that catalogues data related to biological pathways, genomes, disorders, and drugs [42]. To explore the relationship among biological processes, molecular functions, cellular components, and biological pathways of differentially expressed genes (*DEGs*) in thyroid cancer within the high-risk and low-risk score groups of the TCGA-THCA dataset prognostic model, the “clusterProfiler” [43,44] R package (<https://doi.org/10.1016/j.xinn.2021.100141>) was utilized. This package facilitated GO and KEGG enrichment analysis on the high- and low-risk groups of pyroptosis- and mitophagy-related gene prognostic models within the TCGA-THCA dataset. Statistical significance of entry screening criteria was determined based on a $p_{\text{adj}} < 0.05$ and FDR value (q -value) < 0.05 , with the p -value correction technique being Benjamini-Hochberg (BH).

GSVA between High- and Low-Risk Groups

To explore the variability between high- and low-risk subgroups within the TCGA-THCA dataset, Gene Set Variation Analysis (GSVA) was conducted on all genes. GSVA is an unsupervised and non-parametric analytical approach and is commonly utilized to assess enrichment of specific biological pathways across multiple specimens [45]. The

reference gene set “h.all.v7.4.symbols.gmt” was obtained from the Molecular Signatures Database (MSigDB, <http://www.gsea-msigdb.org/>) for GSVA of gene expression matrices for high- and low-risk groups of pyroptosis- and mitophagy-related gene prognostic models in the TCGA-THCA dataset. Functional differences in GSVA enrichment analysis results of enriched pathways in the TCGA-THCA dataset for the pyroptosis- and mitophagy-related gene prognosis model among high-expression and low-expression subgroups of samples were assessed, with significance determined by $p_{\text{adj}} < 0.05$.

Calculating Phenotype Scores

To quantify the relative expression abundance of each gene in a single sample of the dataset, pyroptosis and mitophagy scores were computed for every specimen within the TCGA-THCA and Merged dataset, as well as the GSE35570 dataset. This computation utilized the expression matrices of pivotal genes associated with pyroptosis and mitophagy in thyroid cancer, employing the “GSVA” R package (<https://github.com/rcastelo/GSVA>) and utilizing the single-sample Gene Set Enrichment Analysis (ss-GSEA) algorithm.

GSEA in High- and Low-Risk Groups for Pyroptosis- and Mitophagy-Related Genes

The objective of this investigation was to assess the impact of gene expression levels on thyroid cancer incidence in the TCGA-THCA dataset, particularly within subgroups characterized by high- and low-risk scores. The Gene Set Enrichment Analysis (GSEA) method was utilized to examine the distribution pattern of genes within pre-defined gene sets, organized based on their correlation with phenotypes. This method allows for the determination of the genes’ influence on the phenotype [46]. In this study, all genes within the TCGA-THCA dataset were categorized into two groups based on their logarithm of fold change (logFC) values. These groups were distinguished by high and low logFC values. Subsequently, the genes were further divided into high- and low-risk groups according to the ranking of their logFC values within the prognostic model of pyroptosis- and mitophagy-related genes. Next, the clusterProfiler package was employed to perform gene enrichment analysis on each gene within the high- and low-risk groups of the pyroptosis- and mitophagy-related gene prognosis model in the TCGA-THCA dataset. The reference gene set c2.cp.v7.2.symbols.gmt was obtained from the MSigDB database [47].

Immune Infiltration and Immune Therapy Analysis

CIBERSORT [48] was employed to assess the composition and abundance of immune cells in samples across different subgroups (High Risk/Low Risk). The matrix data were integrated into CIBERSORT and merged with the LM22 signature gene matrix to identify data featur-

ing immune cell enrichment scores exceeding 0. Subsequently, the resulting matrix of immune cell infiltration abundance was obtained and visualized. The Spearman algorithm was utilized to determine correlations among immune cells across various subgroups, and the results were visualized using the “ggplot2” R package. Additionally, the TIDE algorithm was utilized to evaluate the potential of pyroptosis and mitophagy scores as predictors of response to Immune Checkpoint Blockade (ICB) therapy in THCA. Furthermore, the immunophenoscore (IPS) associated with THCA from the TCIA database was downloaded to further analyze the predictive capability of the pyroptosis and mitophagy score model for immunotherapy.

Pyroptosis- and Mitophagy-Related Gene Analysis

We plotted receiver operating characteristic (ROC) curves of the screened prognostic pyroptosis- and mitophagy-related genes for further analysis and presentation of the results. The receiver operating characteristic (ROC) curve [49] is a composite measure that represents the continuous variables of specificity and sensitivity. The prognostic pyroptosis- and mitophagy-related gene's ROC curve in THCA patients underwent plotting utilizing the pROC R package, and the area under curve (AUC) values were computed to estimate the expression of pyroptosis- and mitophagy-related gene diagnostic effect on the survival of THCA patients. Typically, the AUC of ROC curve falls within the range of 0.5 and 1. A diagnostic performance is considered better when the AUC is 1. The AUC exhibits a diminished accuracy within the range of 0.5 to 0.7, moderate accuracy within the range of 0.7 to 0.9, and an elevated accuracy for AUC values exceeding 0.9.

Cell Culture

The normal human thyroid cell line Nthy-ori 3-1 (FH1088) and thyroid cancer cell line TPC-1 (FH1039) were obtained from Fu Heng Biology (Shanghai, China). Both cell lines underwent STR identification and mycoplasma detection to ensure their quality. The cells were cultured in two distinct media: DMEM and RPMI 1640, both supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cultures were maintained at a temperature of 37 °C in an environment containing 5% CO₂.

Real-Time Quantitative PCR (qPCR)

Total RNA extraction from Nthy-ori 3-1 and TPC-1 cells was performed using TRIzol (ET101-01-V2, TransGen Biotech, Beijing, China), followed by reverse transcription using EasyScript Onestep gDNA Removal and cDNA Synthesis SuperMix (AE311-02, TransGen Biotech, Beijing, China). Quantitative PCR (qPCR) was carried out using TransStart Green qPCR SuperMix (AQ111-01, TransGen Biotech, Beijing, China). The qPCR conditions were

as follows: pre-denaturation at 94 °C for 30 seconds, followed by 42 cycles each consisting of denaturation at 94 °C for 5 seconds, annealing at 55 °C for 15 seconds, and elongation at 72 °C for 10 seconds. Gene expression levels were measured using a CFX Opus Deepwell Real-Time PCR System (YQ20211009000476, Bio-rad, Hercules, CA, USA). Primer sequences are provided in **Supplementary Table 3**. The expression of relative genes was determined using the $2^{-\Delta\Delta C_t}$ method.

Western Blot Analysis

Cellular total protein was extracted using RIPA lysis buffer (APPLYGEN) supplemented with protease and phosphatase inhibitors (GLP BIO). The protein concentration was determined using the bicinchoninic acid protein assay kit (DQ111-01, TransGen Biotech, Beijing, China). Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was employed to separate the proteins, which were subsequently transferred onto polyvinylidene difluoride membranes (03010040001, Millipore, Burlington, MA, USA). The membrane was then blocked in 5% nonfat dry milk in phosphate-buffered saline with Tween 20 (PBST) for 1 hour, followed by overnight incubation at 4 °C with diluted primary antibodies: rabbit anti-Kinesin family member 23 (KIF23) (ab174304, 1:1000, Abcam, Cambridge, UK), rabbit anti-NLRP3 (WL02635, 1:2000, WANLEBIO, Shenyang, China), rabbit anti-GSDMD (ab219800, 1:2000, Abcam, Cambridge, UK), rabbit anti-Caspase-1 (22915-1-AP, 1:2000, proteintech, Wuhan, China), rabbit anti-Total and Cleaved Caspase-1 (PY10200, 1:1000, abmart, Shanghai, China) and mouse anti-Beta Actin (81115-1-RR, 1:5000, proteintech, Wuhan, China).

After incubation, the membrane was washed with PBST three times for 5 minutes each. Subsequently, it was incubated with horseradish peroxidase-conjugated secondary antibody (BA1038, BA1039, Boster Biological Technology, Wuhan, China) for 2 hours at room temperature to bind with the primary antibodies, followed by three washes in PBST buffer for 5 minutes each. The immunoblots were visualized using enhanced chemiluminescence (ECL, AR1191, TIANGEN Biotech, Beijing, China) reagents. With Beta Actin serving as the internal reference, the expressions were quantified using ImageJ software, version 7.6.5 (National Institutes of Health, Bethesda, MD, USA). Each assay was repeated at least three times to minimize deviations.

Transfections of Small Interfering RNAs

TPC-1 cells were seeded in 6-well plates and cultivated at 37 °C in a 5% CO₂ atmosphere until cell growth covered 60% of the dish area. Predesigned and validated small interfering RNAs (siRNAs) specific for *KIF23* were used. After overnight incubation, *KIF23* siRNA and *negative control* siRNA were separately mixed with TransIn-

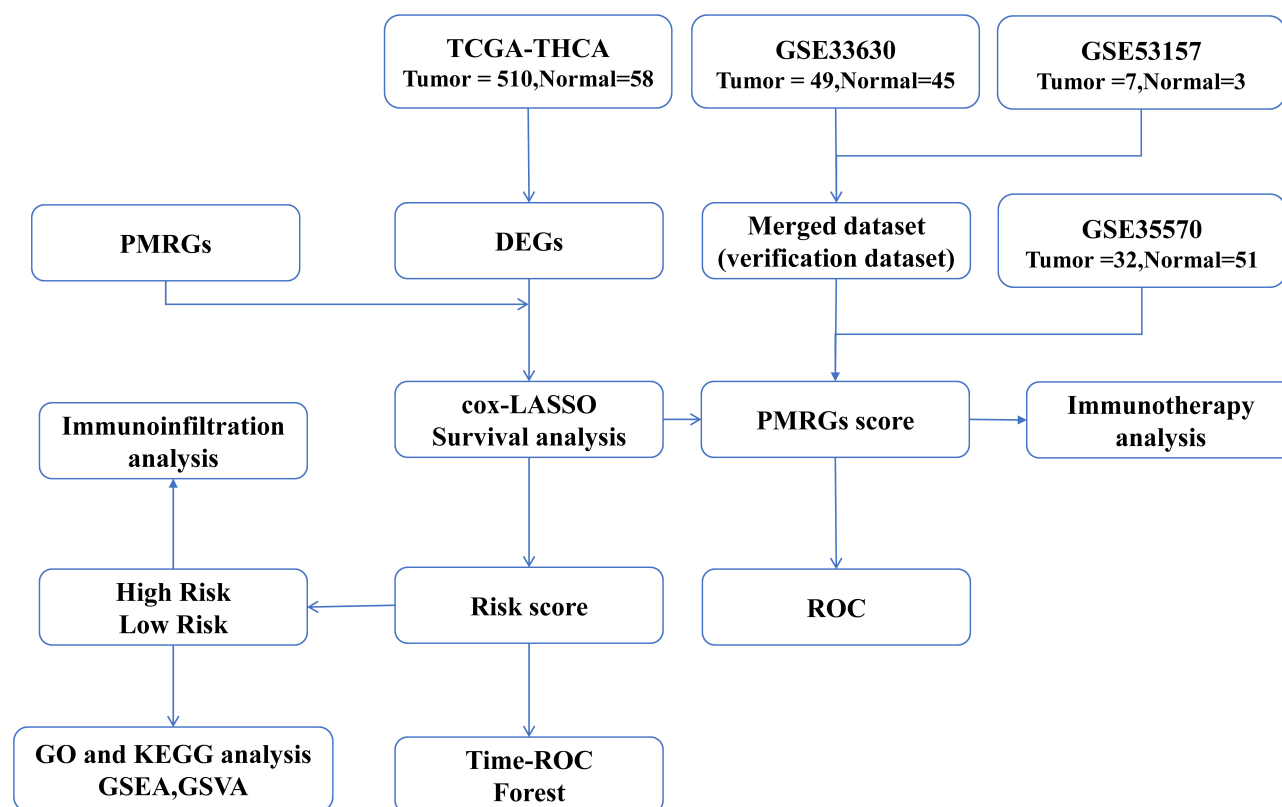


Fig. 1. Flowchart of bioinformatics analysis. TCGA, The Cancer Genome Atlas; ROC, receiver operating characteristic; GSEA, Gene Set Variation Analysis; GSEA, Gene Set Enrichment Analysis; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; LASSO, least absolute shrinkage and selection operator; PMRGs, pyroptosis- and mitophagy-related genes; DEGs, differentially expressed genes; THCA, thyroid carcinoma.

tro EL (FT201-01, TransGen Biotech, Beijing, China). Initially, the siRNA was diluted by adding 250 pmol of siRNA to 180 μ L of Opti-MEM solution and gently stirring. The diluted siRNA was then mixed with an additional 7.5 μ L of TransIntro EL, gently mixed, and allowed to stand at room temperature for 15 to 20 minutes before being introduced to the cell culture medium. The transfection medium was removed after 4 to 6 hours. Cells were then incubated with complete medium for 24 hours before being harvested for subsequent investigations.

The sequences for *KIF23*-siRNA were as follows:

Sense: 5'-GACUAUAUCUAGAUAUGUCU-3'

Antisense: 5'-ACAUGAUCUAGAUAUAGUCUU-3'

The sequences for the *negative control* siRNA were:

Sense: 5'-UUCUCCGAACGUGUCACGUTT-3'

Antisense: 5'-ACGUGACACGUUCGGAGAATT-3'

These siRNAs were synthesized by GENEray (Shanghai, China).

Statistical Analysis

The present study utilized R software (Version 4.1.2) (<https://www.r-project.org/>) for data processing and analysis. The statistical significance of normally distributed vari-

ables in comparing the two groups of continuous variables was estimated using an independent Student's *t*-test. For non-normally distributed variables, the Mann-Whitney U test was employed to analyze variances. Survival analysis was conducted using the survival R package. Kaplan-Meier survival curves were employed to illustrate variances in survival rates, and the log-rank test was used to evaluate the statistical significance of variances in survival times between the two groups of patients. All statistical *p*-values were two-sided, with $p < 0.05$ considered statistically significant. Spearman correlation analysis was utilized to determine the correlation coefficients between various molecules unless otherwise specified.

Results

Analysis Process

The data in this article were analyzed according to the following process (Fig. 1).

Expression of Pyroptosis- and Mitophagy-Related Genes in THCA

We initiated the analysis by performing a principal component analysis on the TCGA-THCA data using the

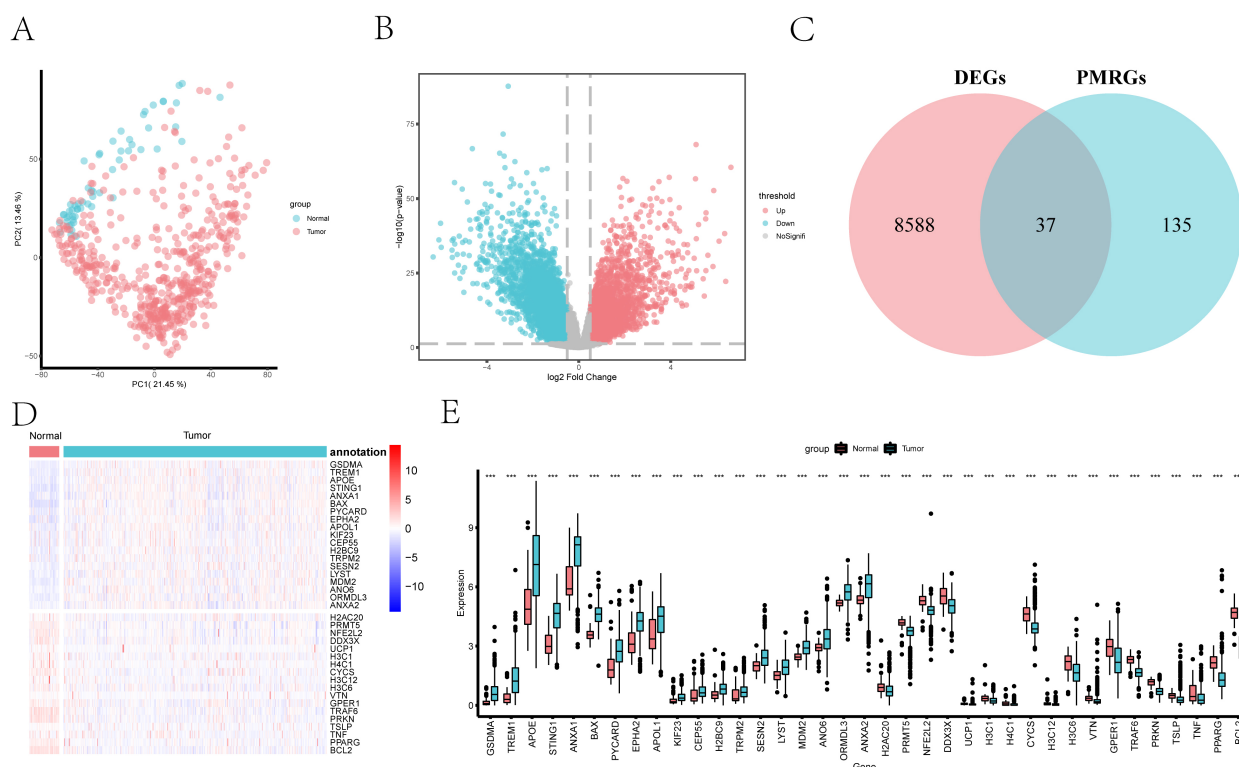


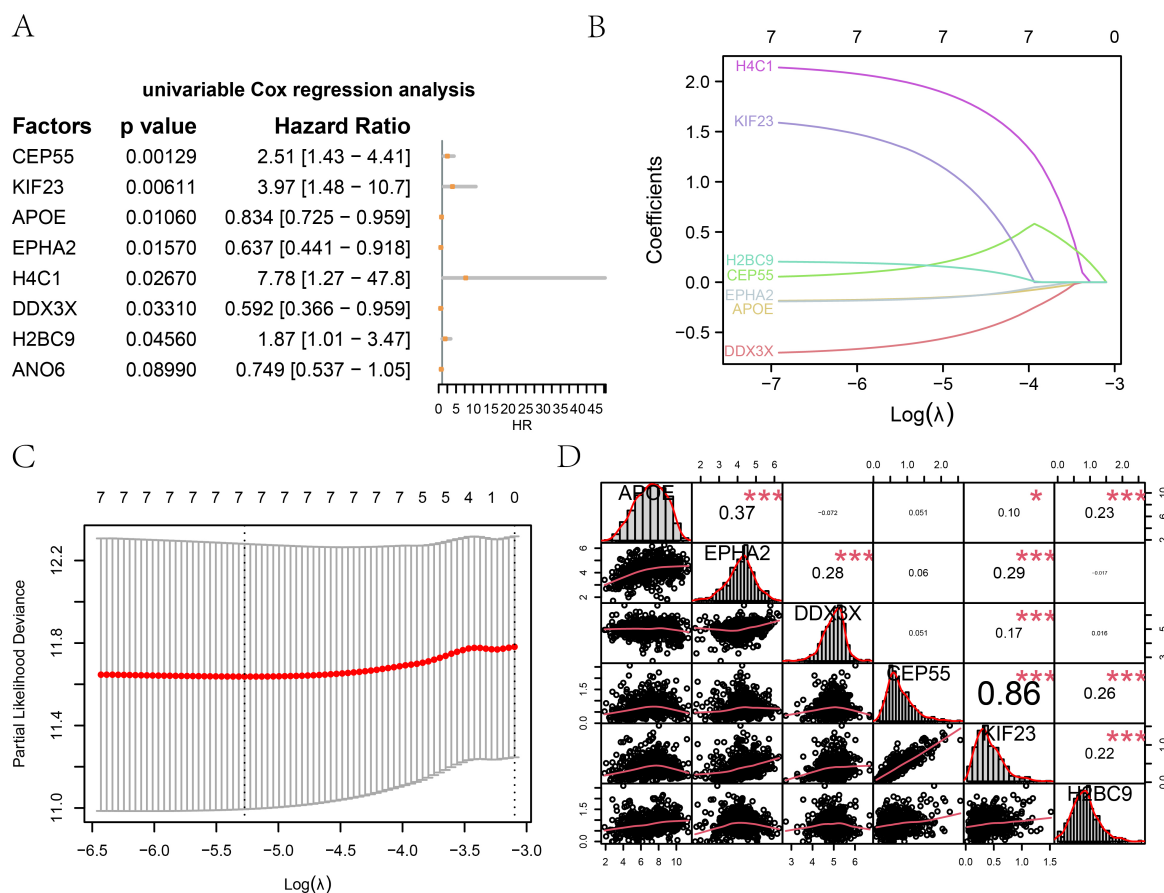
Fig. 2. Expression of genes correlated with pyroptosis and mitophagy in thyroid cancer and paraneoplastic tissues. (A) PCA analysis, red indicates tumor tissue, and blue indicates paraneoplastic tissue (normal). (B) Volcano map of the DEGs, red nodes indicate up-regulated DEGs, blue nodes indicate down-regulated DEGs and gray nodes indicate genes that showed no significant DEGs. (C) Venn diagram of thyroid cancer group and para cancer group DEGs and PMRGs. (D) Heat map of the DEGs, vertical coordinates indicate genes, horizontal coordinates indicate sample grouping, red indicates paraneoplastic tissue (normal), and blue indicates tumor tissue. (E) Comparison of differentially expressed PMRGs groupings, red indicates normal group, and blue indicates tumor group. *** $p < 0.001$. DEGs, differentially expressed genes; PMRGs, pyroptosis- and mitophagy-related genes; *APOE*, Apolipoprotein E; *EPHA2*, Ephrin type-A receptor 2; *DDX3X*, DEAD-box helicase 3; *CEP55*, Centrosome protein 55; *KIF23*, Kinesin family member 23; *H2BC9*, H2B clustered histone 9.

'prcomp' function of R (Fig. 2A). The results revealed that most of the thyroid cancer samples were better differentiated from the para-cancer samples. Subsequently, we systematically analyzed the differential gene expression between the normal and thyroid cancer groups. Employing thresholds of $|\log_2FC| > 0.5$ and adjusted p -value (p_{adj}) < 0.05 , we identified the DEGs between the normal and thyroid cancer groups (Fig. 2B). Next, we intersected the DEGs with pyroptosis- and mitophagy-related genes (PMRGs) (Fig. 2C) to obtain 37 PMRDEGs, including genes such as *GSDMA*, *TREMI*, Apolipoprotein E (*APOE*), *STING1*, *ANXA1*, *BAX*, *PYCARD*, Ephrin type-A receptor 2 (*EPHA2*), *APOL1*, *KIF23*, Centrosome protein 55 (*CEP55*), H2B clustered histone 9 (*H2BC9*), and others. Among these, 19 genes were overexpressed, and 18 were underexpressed. A heatmap was generated to visualize the expression patterns of these genes (Fig. 2D). Furthermore, we analyzed the variations in the expression levels of the 37 PMRDEGs between paraneoplastic and thyroid cancer tissues within the TCGA-THCA dataset using the Wilcoxon

rank sum test. The outcomes of the expression difference analysis were presented through group comparison plots (Fig. 2E), which demonstrated that all 37 genes exhibited statistically significant differences between the thyroid cancer and normal groups.

Screening Prognostic Genes

Univariate Cox regression analysis was utilized for screening candidate genes (*APOE*, *EPHA2*, *H4C1*, DEAD-box helicase 3 (*DDX3X*), *CEP55*, *KIF23*, *H2BC9*, *ANO6*) (Fig. 3A, **Supplementary Table 4**) with a threshold of $p < 0.1$. These candidate prognostic genes underwent additional screening through LASSO regression analysis. Finally, seven prognostic genes (*APOE*, *EPHA2*, *H4C1*, *DDX3X*, *CEP55*, *KIF23*, *H2BC9*) were selected with the best parameters. Among them, there were more samples with zero values of expression in the *H4C1* gene, so *APOE*, *EPHA2*, *DDX3X*, *CEP55*, *KIF23*, and *H2BC9* were selected as prognostic genes for the subsequent analysis. Fig. 3B shows the Lambda selection for LASSO-Cox re-



gression analysis. Fig. 3C displays the coefficient selection, and Fig. 3D demonstrates the correlation analysis results of the six prognostic genes, with a significant positive connection observed between *CEP55* and *KIF23* ($R = 0.86$, $p < 0.01$).

Construction of Prognostic Model

We combined the gene expression values of six prognostic genes (*APOE*, *EPHA2*, *DDX3X*, *CEP55*, *KIF23*, *H2BC9*) with the progression-free interval (PFI) of THCA individuals from the TCGA-THCA dataset based on the median gene expression grouping for prognostic Kaplan-Meier (KM) curve analysis (Fig. 4A–F). The median subgroups of *APOE*, *CEP55*, *EPHA2*, and *DDX3X* gene expressions exhibited statistically significant differences in PFI of THCA individuals in the TCGA-THCA dataset ($p < 0.05$). The study involved the development of a prognostic model through the application of multivariate Cox analysis on these six genes (Supplementary Table 5). The model utilized multivariate Cox coefficients in conjunction with gene expression data to calculate risk scores.

As a result, we visualized the grouping of the samples within the constructed prognostic model with risk factor plots (Fig. 4G). These plots depict the risk grouping and survival outcome of the dataset samples, alongside a heatmap illustrating the expression patterns of prognosis-related genes. The findings indicated that a greater number of specimens within the high-risk group experienced worsening thyroid cancer. Additionally, genes such as *CEP55*, *KIF23*, and *H2BC9* were expressed to a greater extent in the high-risk group.

Prognostic Analysis of the Prognostic Model

The findings demonstrate that the prognostic model risk score exhibited a degree of precision in anticipating the progression-free interval (PFI) of THCA individuals in the TCGA-THCA dataset ($AUC1 = 0.764$, $AUC3 = 0.693$, $AUC5 = 0.727$) (Fig. 5A). Additionally, prognostic Kaplan-Meier curve analysis was conducted by integrating the prognostic model risk scores with PFI of THCA individuals within the TCGA-THCA dataset, based on the median risk score subgroup (Fig. 5B). The results revealed a statistically significant variance in PFI of THCA individuals be-

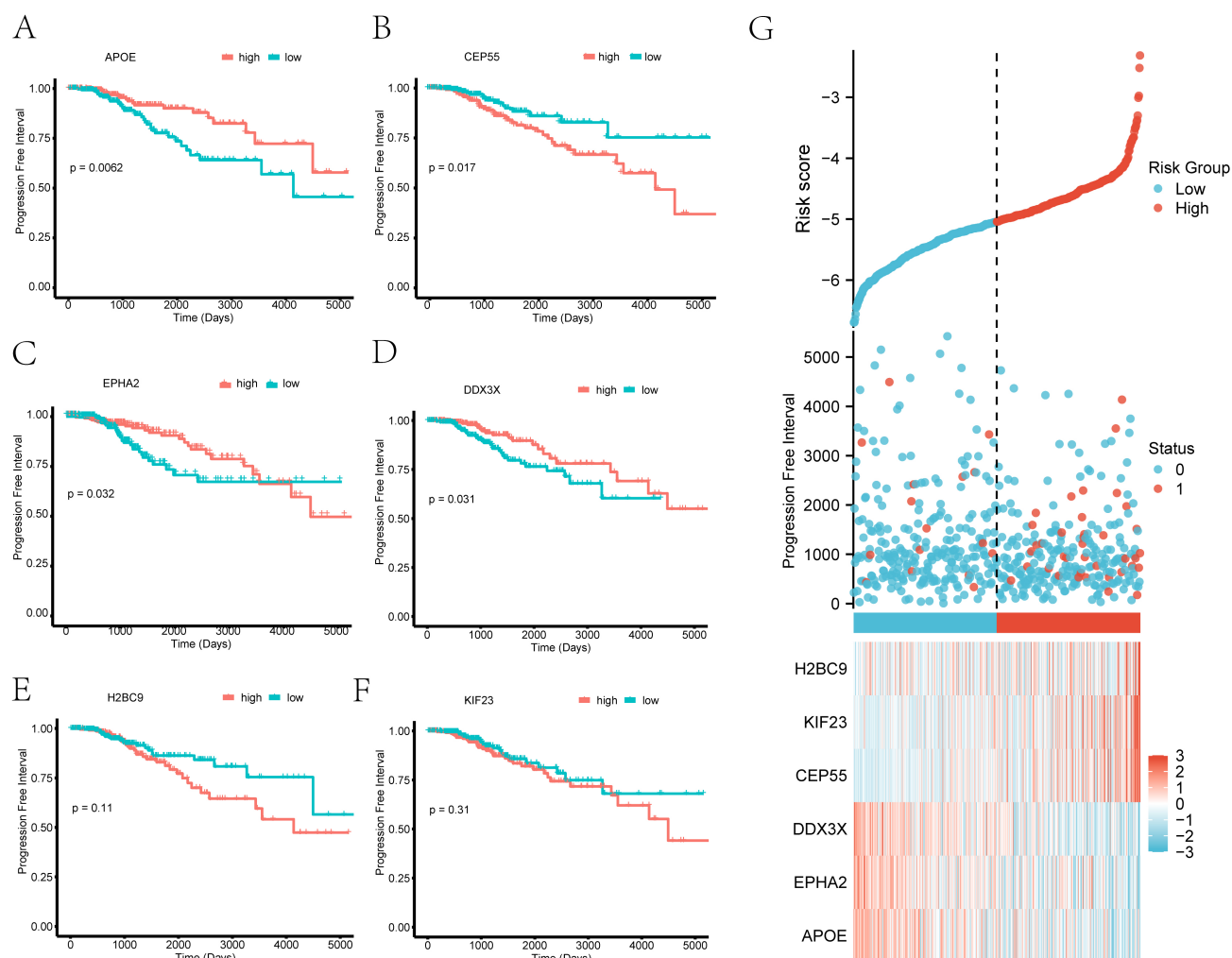


Fig. 4. Expression analysis of prognostic genes and prognostic analysis. (A–F) KM curves of median expression of *APOE* (A), *CEP55* (B), *EPHA2* (C), *DDX3X* (D), *H2BC9* (E), *KIF23* (F) in TCGA-THCA dataset. (G) Risk factor plots of prognostic models. The risk factor plots consist of (1) Risk grouping: The dataset samples were grouped by median risk score predicted by the prognostic model. (2) Dot plots were used to display the survival period and outcome of clinical samples in the TCGA-THCA dataset. (3) Heat map of the prognosis-associated genes from our constructed prognostic model (*APOE*, *EPHA2*, *DDX3X*, *CEP55*, *KIF23*, *H2BC9*). KM, Kaplan-Meier; TCGA, the Cancer Genome Atlas; THCA, thyroid carcinoma.

tween the two investigated groups ($p < 0.05$). Moreover, we combined clinical data such as risk scores, patient age, and patient gender factors to plot Decision Curve Analysis (DCA) curves. It was observed that the model curves were farther away from the two reference lines, indicating better model accuracy (Fig. 5C). For a deeper understanding of the potential significance of the risk score for clinical purposes, multivariate Cox regression analysis was conducted, incorporating risk score, patient age, and patient gender factors. A forest plot was obtained according to Fig. 5D. The risk score was positioned to the right of the null line, with HR = 2.4, CI = 1.66–3.5, and $p < 0.001$, suggesting a significant association between the risk score and prognostic clinical information.

We selected risk score, patient age, and patient gender factors for nomogram analysis (Fig. 6A) to assess the

prognostic model capacity. Additionally, prognostic calibration analysis was conducted on nomogram plots for 1-, 3-, and 5-year intervals, and calibration curves were plotted (Fig. 6B–D). The results indicate a ranking of predicted effects: 5-year > 3-year > 1-year.

GSVA of High- and Low-Risk Groups

We conducted Gene Set Variation Analysis (GSVA) on all genes belonging to the high-risk and low-risk subgroups of the TCGA-THCA dataset to examine the variation among subgroups (Fig. 7A, **Supplementary Table 6**). Pathways meeting the criterion of $p_{\text{adj}} < 0.05$ in the GSVA findings were considered statistically significant. We selected the top 10 results of $|\log_{2}FC|$ for display, which included pathways such as thyroid cancer poor survival downregulated, escaped from X inactivation, skin

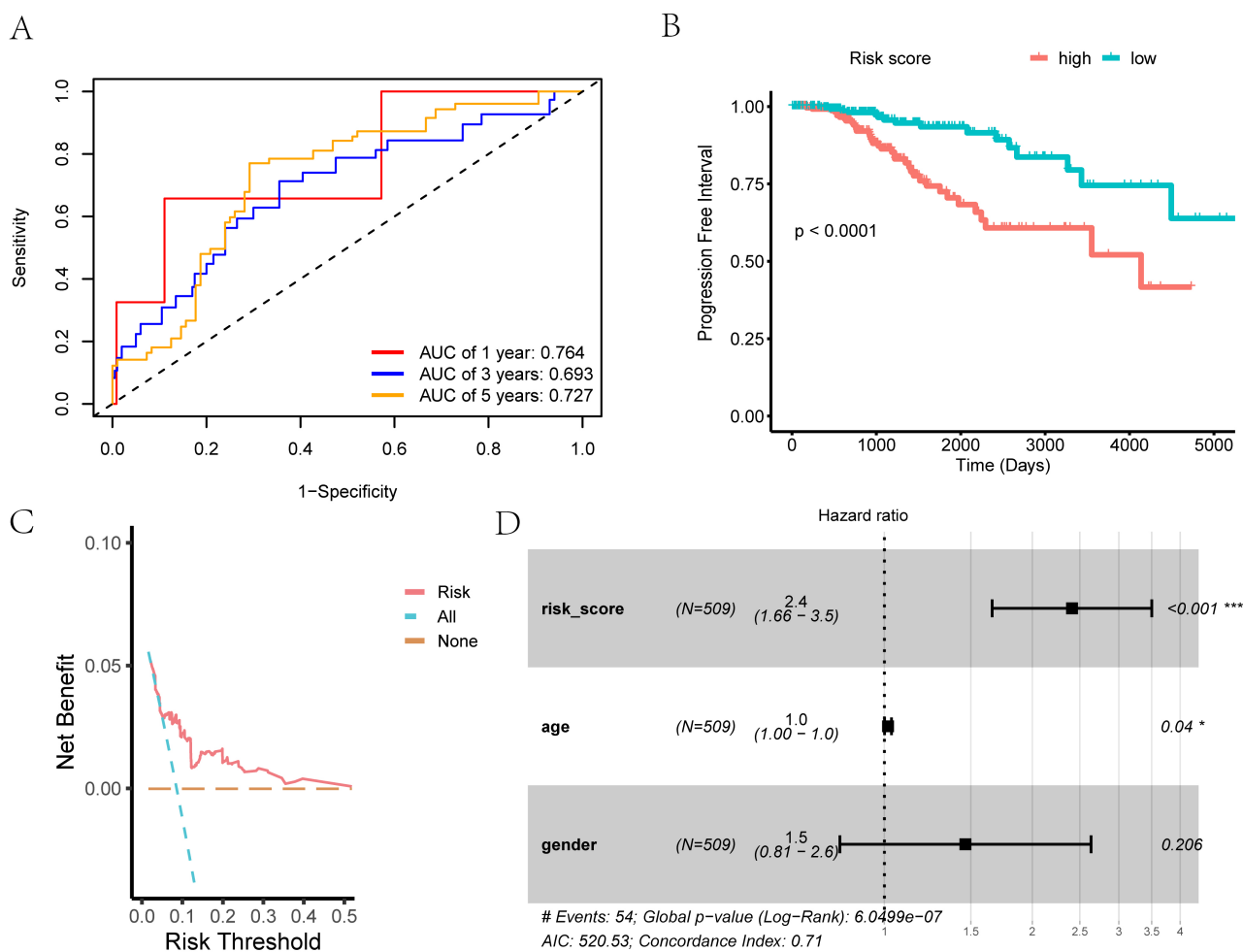


Fig. 5. Prognostic analysis of the prognostic model. (A) Time-dependent ROC curve of the risk score in TCGA-THCA dataset for the prognostic model. (B) KM curve of the risk score in TCGA-THCA dataset. (C) DCA curve with threshold probability in horizontal coordinate and net benefit in vertical coordinate. (D) Forest plot of risk score, age, and gender, showing HR and p -values. * $p < 0.05$, *** $p < 0.001$. ROC, receiver operating characteristic; DCA, Decision Curve Analysis.

wound response, apoptosis CDKN2A downregulated, activated Neurotrophic receptor tyrosine kinase 2 (NTRK2) signals through Phosphoinositide 3-kinase (PI3K), circadian pathway, loss of function of SMAD2/3 in cancer, amplification hot spot 5, regulation of FOXO transcriptional activity by acetylation, and trafficking of myristoylated proteins to the cilium. These pathways were found to be overexpressed within the low-risk group. Conversely, pathways such as AURKB targets, silenced by methylation downregulated, multiple myeloma pathway upregulated, AURKA targets, response to sulindac downregulated, aging middle downregulated, E2F4 targets, thyroid cancer poor survival upregulated, response to nutlin 3a downregulated, and gender effect upregulated were found to be overexpressed within the high-risk group. Furthermore, we evaluated the extent of group variations among the 20 pathways in various subgroups of the TCGA-THCA dataset using the Mann-Whitney U test. The findings were subsequently presented through a comparison of group plots

(Fig. 7B), which demonstrated that the differential expression levels of the 20 pathways between distinct subgroups of the TCGA-THCA dataset were statistically very significant ($p < 0.001$).

GO and KEGG Analysis of Subgroups

We conducted Gene Ontology (GO) gene function enrichment analysis on genes with differential expression (Supplementary Table 7). The results revealed that genes displaying differential expression in thyroid cancer were predominantly enriched in multiple biological process (BP), including cell junction assembly, modulation of chemical synaptic transmission, calcium-independent cell-cell adhesion through plasma membrane cell-adhesion molecules, cell-cell adhesion through plasma-membrane adhesion molecules. They were also enriched in cellular component (CC), such as ion channel complex, sodium channel complex, cell-cell junction, voltage-gated sodium channel complex. Additionally, they exhibited enrichment

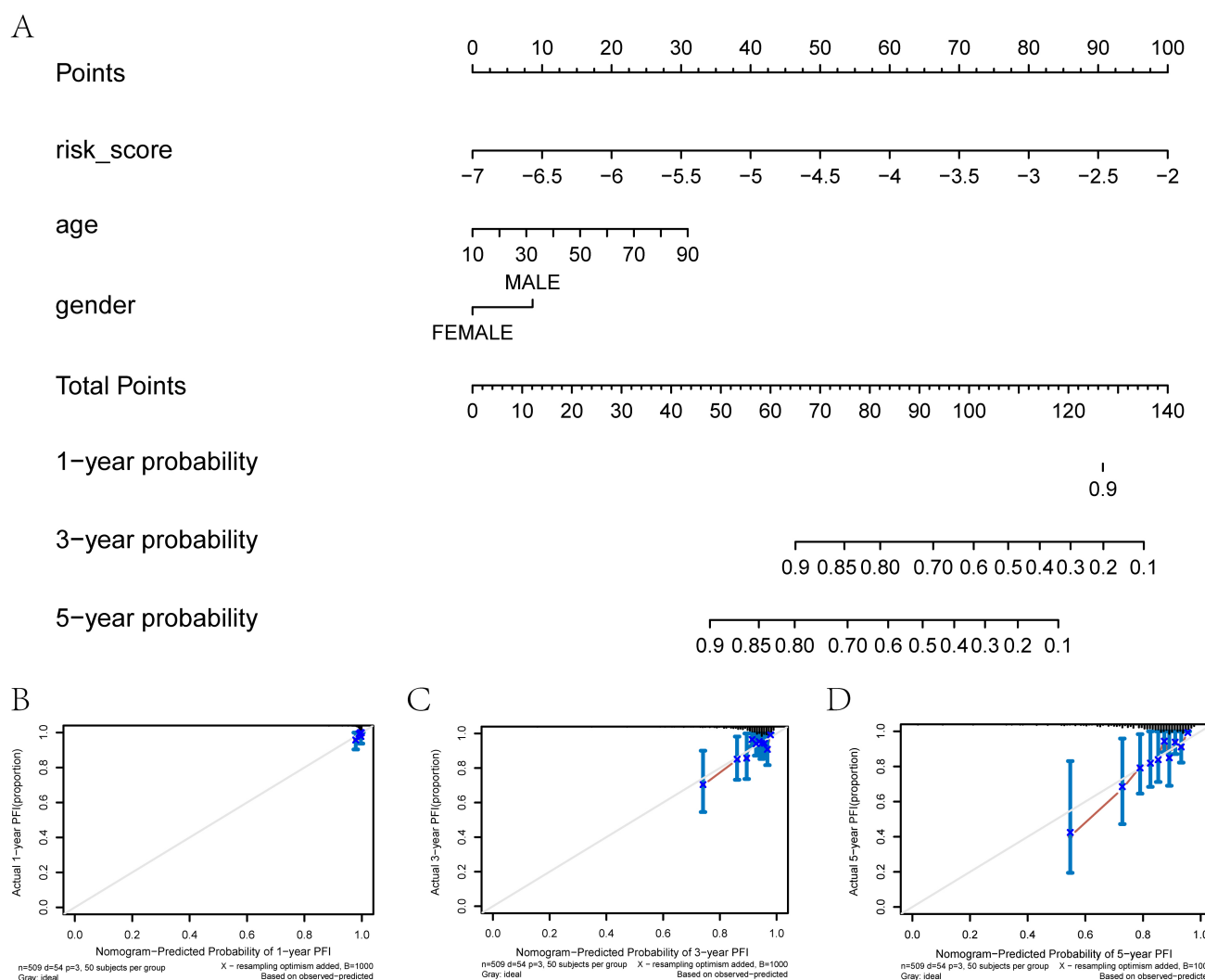


Fig. 6. Nomogram and calibration analysis of the prognostic model. (A) Columnar line plot constructed by risk score combined with clinicopathological characteristics that predicts the 1-, 3-, and 5-year PFI status of individuals according to the risk score, gender, and age. (B–D) Calibration curves of the 1 (B), 3 (C), and 5 (D) year columnar line plots, the horizontal coordinates are the PFI status obtained from the columnar line plot predictions. The vertical coordinate is the actual observed PFI state. PFI, progression-free interval.

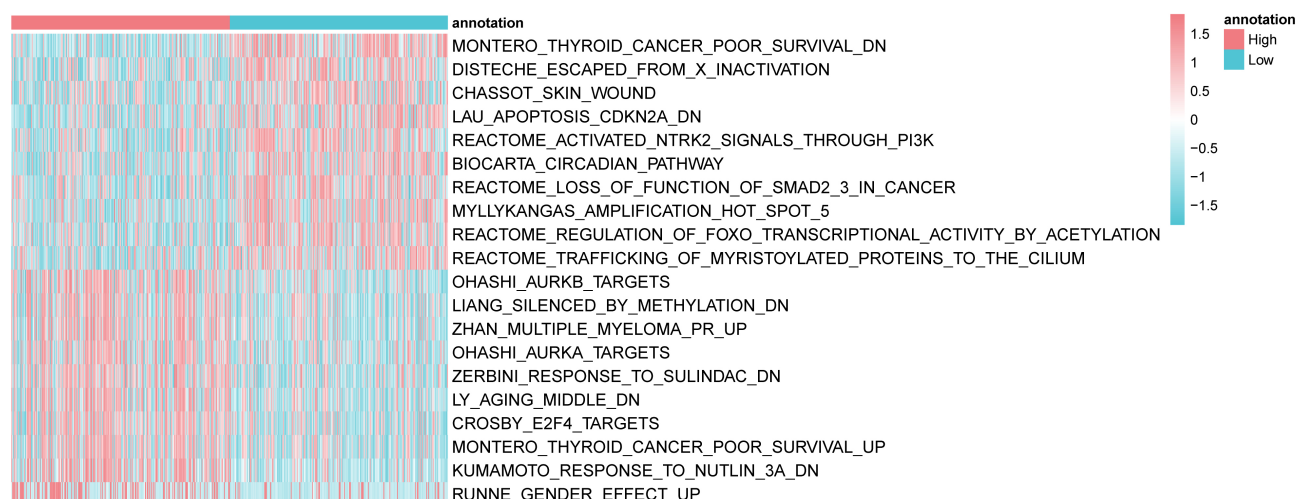
in molecular function (MF), such as voltage-gated sodium channels, serine-type endopeptidase, serine-type peptidase, and serine hydrolase activities. Furthermore, we performed KEGG pathway enrichment analysis for DEGs. The results indicated that DEGs in thyroid cancer were primarily enriched in Neuroactive ligand-receptor interaction, Cell adhesion molecules, and other KEGG pathways. We presented the findings of our study through bar graphs depicting the outcomes of KEGG pathway and GO functional enrichment analyses (Fig. 8A). Additionally, we illustrated the BP pathway results (Fig. 8B), CC pathway results (Fig. 8C), and MF pathway results (Fig. 8D) of GO gene functional enrichment analysis as a circular network diagram. Subsequently, a KEGG enrichment analysis of differential genes was conducted in both investigated groups, using joint logFC by providing the logFC values of DEGs in the TCGA-THCA differential analysis results and

calculating the corresponding Z-score for each gene based on enrichment analysis. The outcomes of the joint logFC KEGG enrichment analysis were presented as a chord diagram (Fig. 8E).

Gene Set Enrichment Analysis of Subgroups

We conducted Gene Set Enrichment Analysis (GSEA) to explore gene expression patterns, biological processes, cellular components, and molecular functions across various subgroups within the TCGA-THCA dataset. The results demonstrated significant enrichment of PMRGs within the TCGA-THCA dataset in several pathways (Fig. 9A–G, **Supplementary Table 8**). These pathways included the [WikiPathways] Hippo Signaling Regulation Pathways (Fig. 9B), [WikiPathways] Wnt Signaling Pathway and Pluripotency (Fig. 9C), [Reactome] PI3K Akt Signaling in Cancer (Fig. 9D), [WikiPathways] Mapk Signal-

A



B

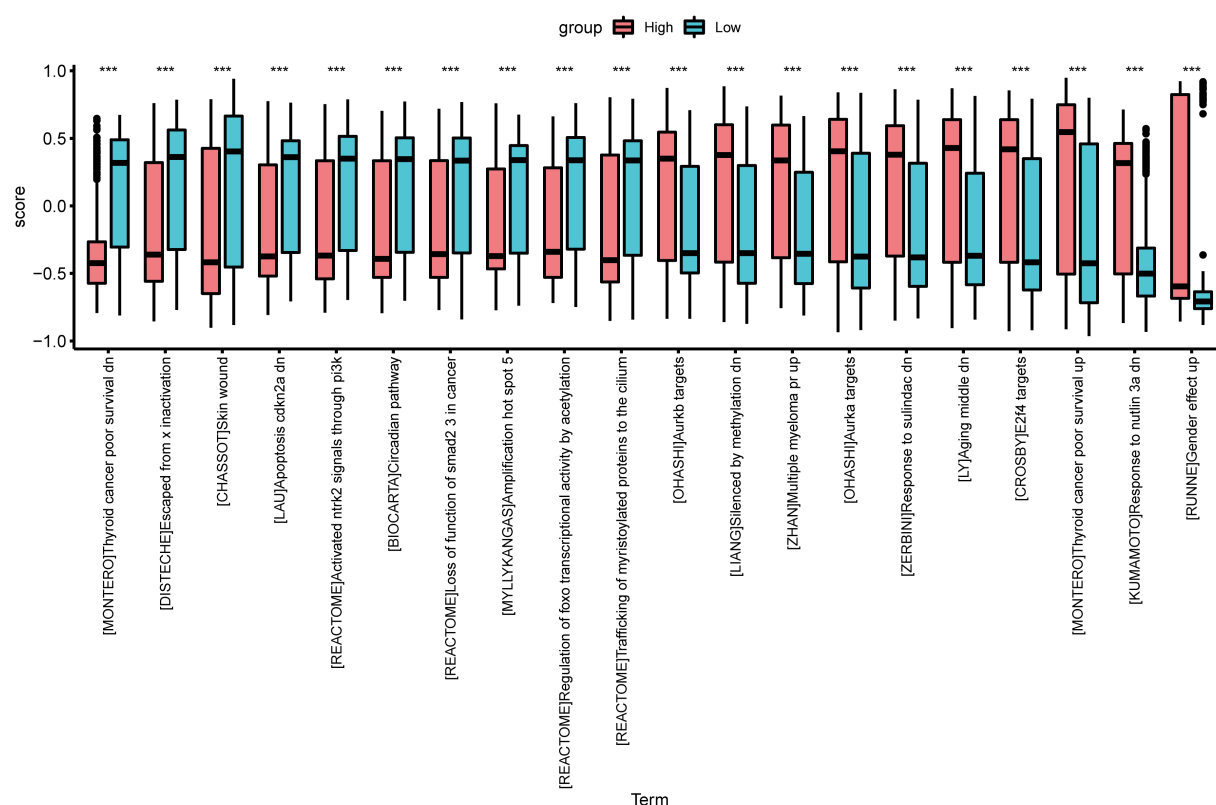


Fig. 7. GSVA for risk subgroups. (A) Complex heat map of the outcomes of GSVA of genes between risk subgroups for the pyroptosis and mitophagy-related gene prognosis model in TCGA-THCA dataset. (B) Grouped comparison plot display. *** $p < 0.001$. GSVA, Gene Set Variation Analysis.

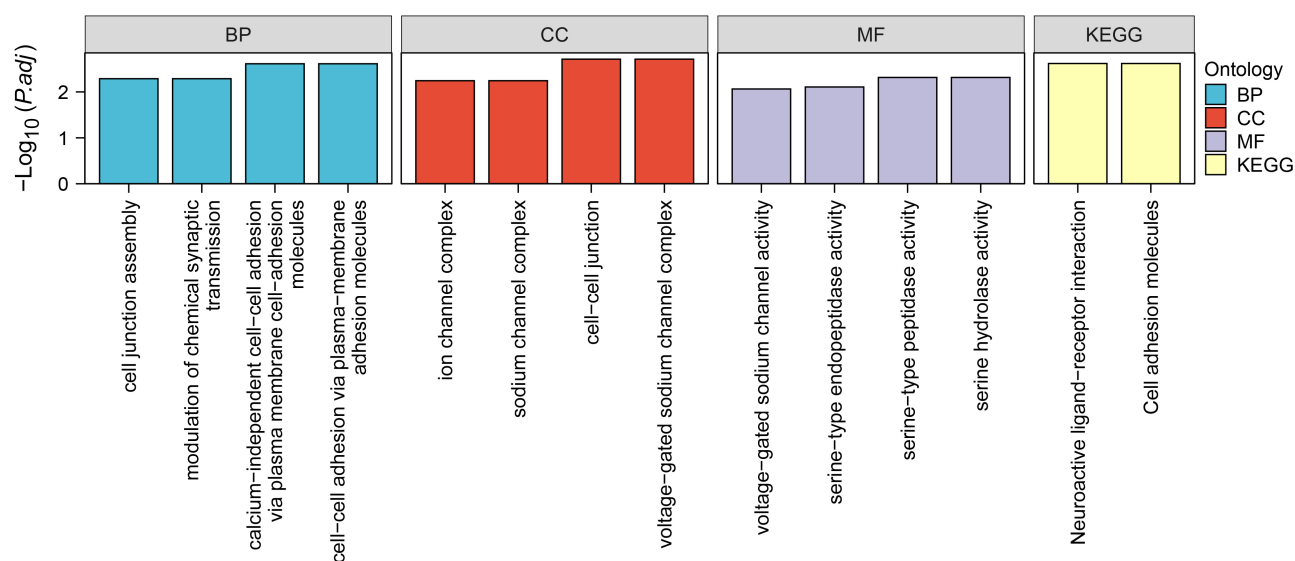
ing Pathway (Fig. 9E), [WikiPathways] Tgfbeta Signaling Pathway (Fig. 9F), and [WikiPathways] Ras Signaling (Fig. 9G).

Immune Infiltration Analysis

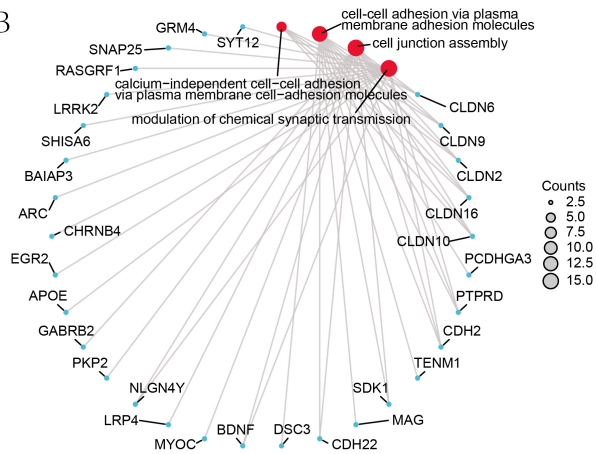
The current study utilized the CIBERSORT algorithm to assess the relative abundance of 22 distinct immune

cell categories in various samples from subgroups (High Risk/Low Risk). The aim was to investigate potential variations in immune infiltration. The findings were depicted using a barplot (Fig. 10A), followed by a comparison of the relative abundance of each of the 22 immune cell types (Fig. 10B). The results indicated that several immune cell types, including naive B cells, Plasma cells, T

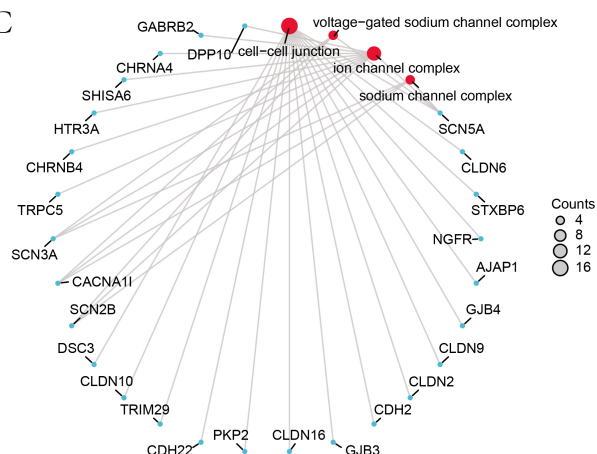
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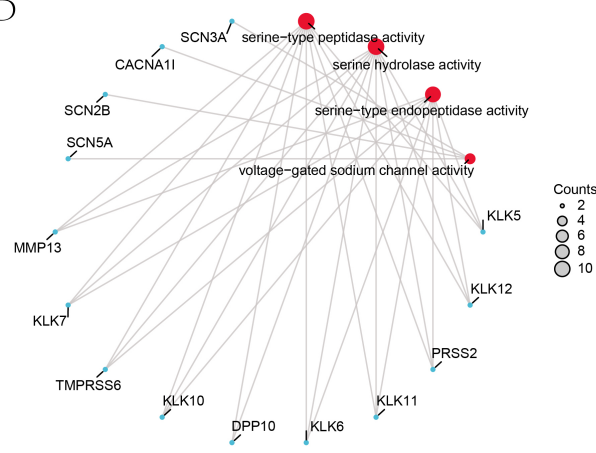
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E

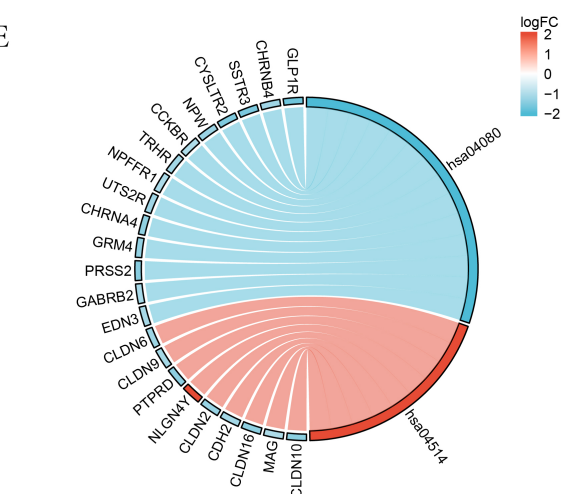
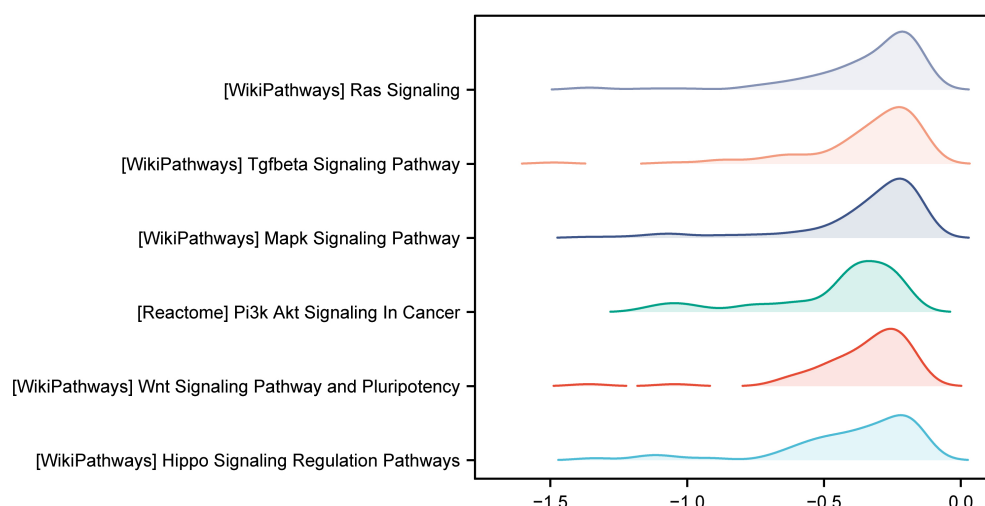
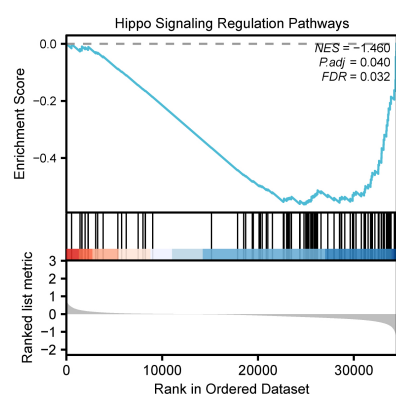


Fig. 8. Functional enrichment analysis (GO) and pathway enrichment (KEGG) analysis for high risk and low risk groups. (A) Histogram display of GO functional enrichment analysis and KEGG pathway enrichment analysis outcomes for differentially expressed genes. **(B)** Loop network diagram display of GO functional enrichment analysis BP pathway outcomes for differentially expressed genes. **(C)** Loop network diagram display of CC pathway results. **(D)** Loop network diagram display of MF pathway results. **(E)** String diagram of KEGG enrichment analysis. BP, biological process; CC, cellular component; MF, molecular function.

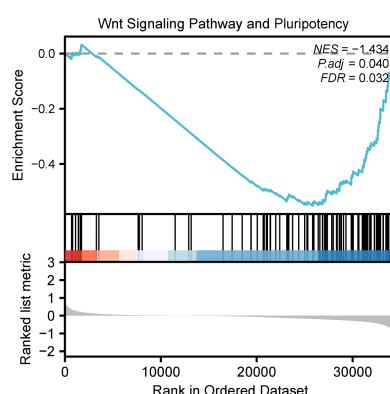
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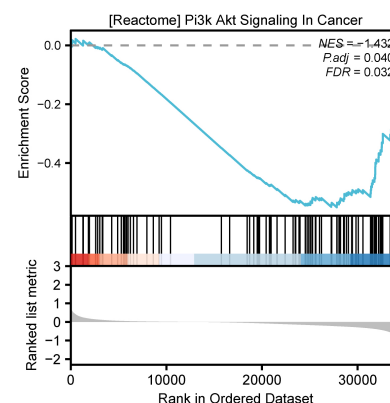
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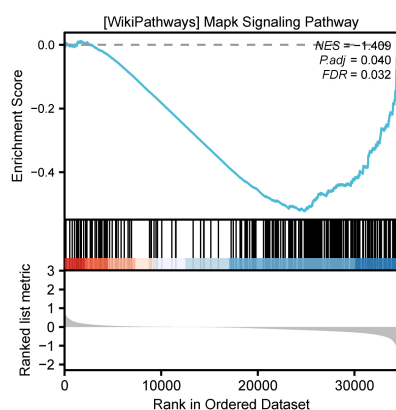
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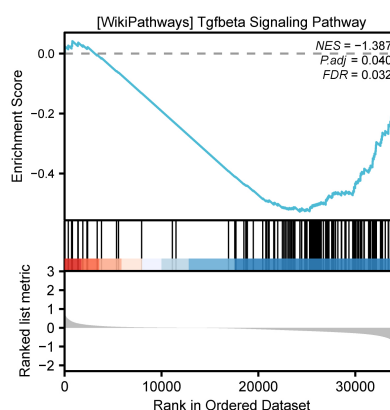
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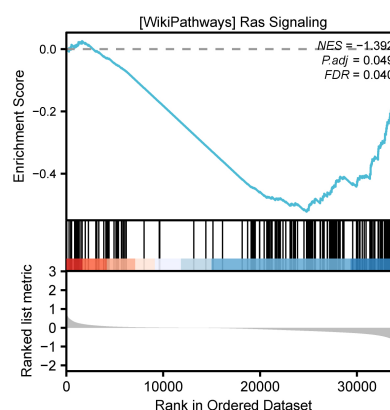


Fig. 9. GSEA analysis of risk subgroups. (A) GSEA enrichment analysis of the main six biological features of genes between high- and low-risk score subgroups in the TCGA-THCA dataset. (B–G) The genes in the TCGA-THCA data set showed significant enrichment in [WikiPathways] Hippo Signaling Regulation Pathways (B), [WikiPathways] Wnt Signaling Pathway and Pluripotency (C), [Reactome] Phosphoinositide 3-kinase (PI3K) Akt Signaling in Cancer (D), [WikiPathways] Mapk Signaling Pathway (E), [WikiPathways] Tgfbeta Signaling Pathway (F) and [WikiPathways] Ras Signaling (G) pathways.

cells CD8, T cells CD4 memory activated, T cells follicular helper, T cells regulatory (Tregs), NK cells activated, Monocytes, Macrophages M0, Macrophages M2, Dendritic cells resting, Dendritic cells activated, Mast cells resting,

Eosinophils, and Neutrophils, exhibited significant distinctions among the different subgroups ($p < 0.05$). Furthermore, we conducted correlation analyses of these differential immune cells separately for the high- and low-risk

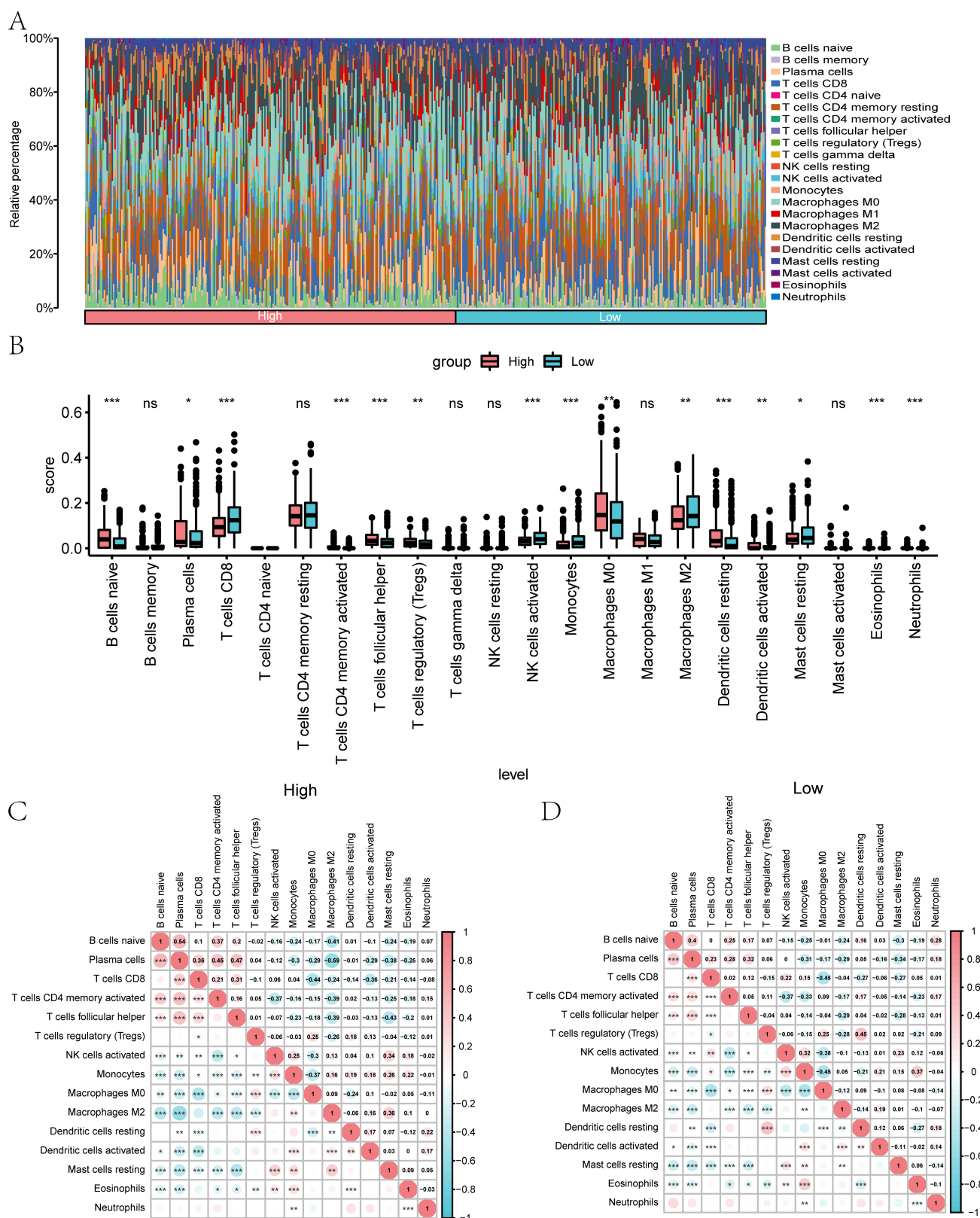


Fig. 10. Immune infiltration analysis. (A) Barplot of immune infiltration analysis results for different subgroups in the disease dataset. (B) Boxplot of immune infiltration abundance of 22 immune cells in different subgroups. (C,D) Correlation of the abundance of immune infiltration in the high- and low-risk subgroups. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

subgroups. In the high-risk group (Fig. 10C), Plasma cells showed significant positive correlations with naive B cells ($R = 0.54$, $p < 0.01$), T cells follicular helper ($R = 0.47$, $p < 0.01$), and T cells CD4 memory stimulated ($R = 0.45$, $p < 0.01$). Conversely, Plasma cells exhibited significant negative correlations with Macrophages M2 ($R = -0.59$, $p < 0.01$), while Macrophages M0 showed negative correlations with T cells CD8 ($r = -0.44$, $p < 0.01$), Mast cells resting with T cells follicular helper ($R = -0.43$, $p < 0.01$), and Macrophages M2 with naive B cells ($R = -0.41$, $p < 0.01$). Moreover, in the low-risk group (Fig. 10D), Dendritic cells resting exhibited a significant positive association with T cells regulatory (Tregs) ($R = 0.45$, $p < 0.01$), whereas Macrophages M0 showed negative correlations with Monocytes ($R = -0.45$, $p < 0.01$) and T cells CD8 ($R = -0.45$, $p < 0.01$).

GSE33630 and GSE53157 Data Set Pre-Processing

The R package sva was employed to mitigate the influence of batch effects on the combined GEO datasets derived from the processing of GSE33630 and GSE53157 datasets (Merged Datasets). Distribution box plots and PCA plots were utilized to compare the datasets before and after the removal of batch effects (Fig. 11A–D). The results of the distribution box plots and PCA plots indicate that the sample's batch effect in the merged datasets was largely eliminated after the batch removal process.

Immune Therapy Analysis

The pyroptosis and mitophagy scores for each specimen within the TCGA-THCA dataset were computed using the “GSVA” R package based on the expression matrix of pyroptosis- and mitophagy-related prognostic genes (*APOE*, *EPHA2*, *DDX3X*, *CEP55*, *KIF23*) associated with thyroid cancer, utilizing the ssGSEA algorithm. As the *H2BC9* was not present in the GSE dataset, the expression matrices of *APOE*, *EPHA2*, *DDX3X*, *CEP55*, and *KIF23* were selected to calculate the pyroptosis and mitophagy scores of the merged dataset and the GSE35570 dataset. The effectiveness of immune-checkpoint blockade (ICB) therapy was assessed using the TIDE algorithm (Fig. 12A). The results indicated significant differences in pyroptosis and mitophagy scores between the TIDE-predicted ICB treatment response and non-response groups, with lower scores observed in the response group. Immune checkpoints serve as intrinsic regulatory mechanisms of the immune system, promoting self-tolerance and preventing inadvertent harm during physiological immune reactions.

We generated box plots to illustrate the differences between different Immune Phenotype Scores (IPS) for high and low groups regarding pyroptosis and mitophagy scores in TCGA-THCA patients using the ggplot function of R (Fig. 12B). The findings reveal significant variations among the high and low groups of pyroptosis and mitophagy scores in IPS-PD1/PD-L1/PD-L2 and IPS-PD1/PD-L1/PD-L2 +

CTLA4. These results suggest that phenotypic scores could serve as predictive indicators of prognosis for immunotherapy.

To investigate the connection between immune checkpoints and phenotypic scores, we plotted box plots to visualize variations within immune checkpoint genes among high- and low-score groups of pyroptosis and mitophagy in the TCGA-THCA dataset using the ggplot function of R (Fig. 12C). The box plots revealed significant differences in the expression of *VTCN1*, *CD28*, *CD209*, *CD276*, *CD160*, *ICOSLG*, *PVR*, *TNFSF4*, *HLA-DOB*, and *BTLA* across groups with high and low scores. Similarly, box plots were generated to visualize the variations in immune checkpoint genes in the pyroptosis and mitophagy high- and low-scoring groups within the merged dataset and the GSE35570 dataset (Fig. 12D,E). The results indicated significant variation in *CD28*, *CD209*, *CD276*, *ICOSLG*, *PVR*, *TNFSF4*, and *HLA-DOB* within the high- and low-scoring subgroups in the Merged dataset. Furthermore, *VTCN1*, *CD28*, *CD209*, *CD276*, *ICOSLG*, *TNFSF4*, *HLA-DOB*, and *BTLA* exhibited significant variation within the pyroptosis and mitophagy high- and low-scoring groups in the GSE35570 dataset.

Diagnostic Analysis of Pyroptosis and Mitophagy Scoring Models

The ROC curves of five genes (*APOE*, *EPHA2*, *DDX3X*, *CEP55*, *KIF23*) were plotted in the TCGA-THCA dataset (Fig. 13A), the Merged dataset (Fig. 13B), and the GSE35570 dataset (Fig. 13C). In the TCGA-THCA dataset, *CEP55* (AUC = 0.677) and *KIF23* (AUC = 0.698) exhibited low accuracy in diagnosing thyroid carcinogenesis, while *APOE* (AUC = 0.779), *EPHA2* (AUC = 0.778), and *DDX3X* (AUC = 0.76) showed some diagnostic accuracy. In the Merged dataset, *DDX3X* (AUC = 0.744), *CEP55* (AUC = 0.75), *KIF23* (AUC = 0.792), and *EPHA2* (AUC = 0.861) demonstrated some diagnostic accuracy for thyroid cancer development, with *APOE* (AUC = 0.916) showing high diagnostic accuracy. In the GSE35570 dataset, *DDX3X* (AUC = 0.714), *CEP55* (AUC = 0.832), *KIF23* (AUC = 0.741), and *EPHA2* (AUC = 0.889) exhibited some diagnostic accuracy for thyroid cancer occurrence, while *APOE* (AUC = 0.963) demonstrated high diagnostic accuracy.

We also generated ROC curves for pyroptosis and mitophagy scores in the TCGA-THCA dataset, the Merged dataset, and the GSE35570 dataset for both cancer and para-cancer subgroups. The findings revealed that the pyroptosis and mitophagy score demonstrated some precision for tumor diagnosis in the TCGA-THCA dataset (AUC = 0.830) (Fig. 13D) and in the Merged dataset (AUC = 0.876) (Fig. 13E). Additionally, the pyroptosis and mitophagy score exhibited an elevated level of precision in the tumor diagnosis process in the GSE35570 dataset (AUC = 0.917) (Fig. 13F).

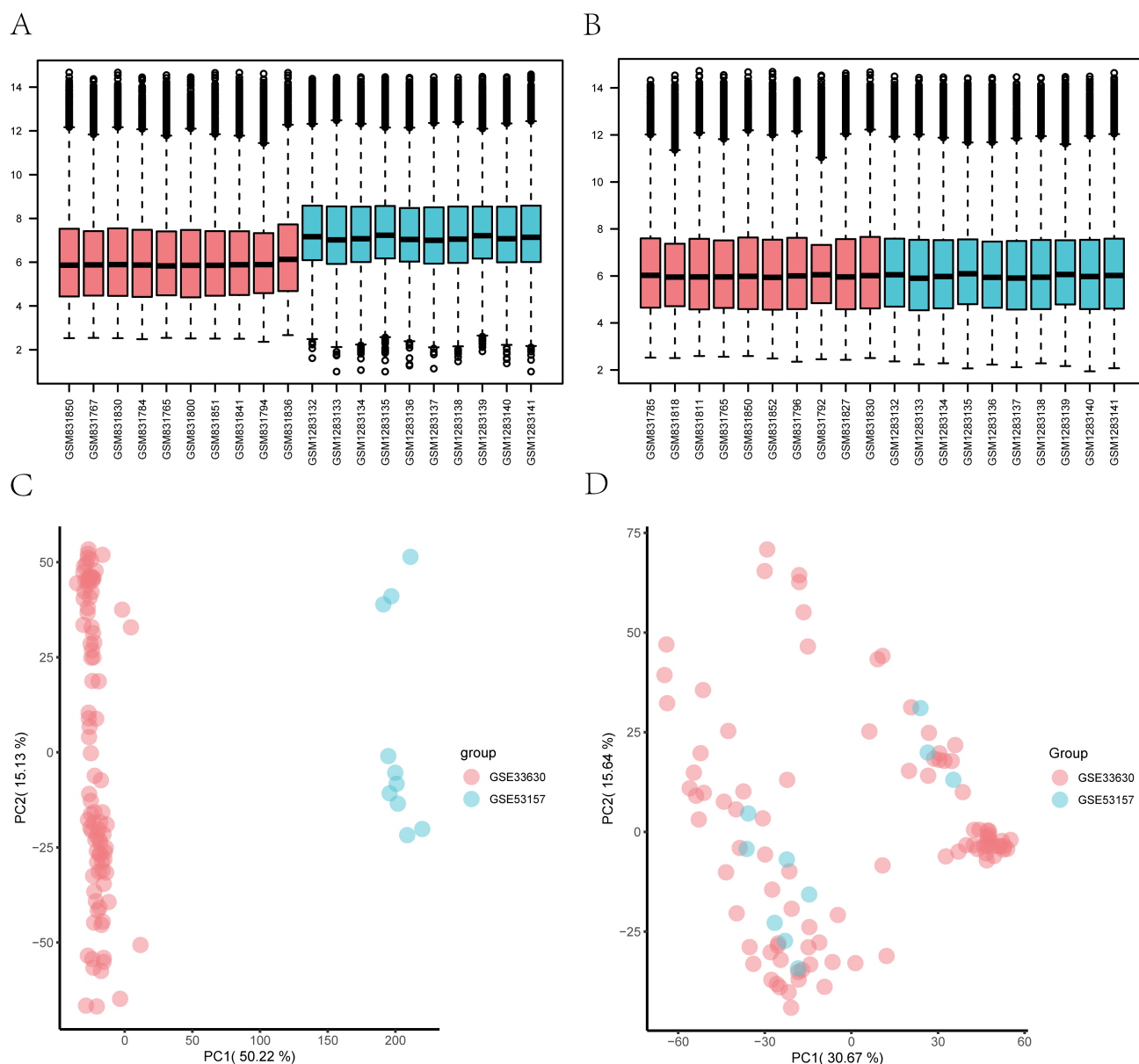


Fig. 11. Dataset pre-processing. (A,B) Distribution box plots of the combined Gene Expression Omnibus (GEO) datasets (Merged Datasets) before and after de-batching. (C,D) PCA plots of the combined GEO datasets (Merged Datasets) before and after de-batching. Blue is dataset GSE53157 and red is dataset GSE33630.

The Expression Levels of Prognostic Genes

The prognostic gene expression levels (*APOE*, *EPHA2*, *CEP55*, *KIF23*) were validated in thyroid cancer cell lines (TPC-1) and normal human thyroid cells (Nthy-ori 3-1) using quantitative real-time PCR (qRT-PCR) analysis. Consistent with the outcomes of bioinformatic analysis, the qRT-PCR findings indicated a statistically significant elevation in the mRNA expression of *APOE*, *EPHA2*, *CEP55*, and *KIF23* in TPC-1 cells ($p < 0.01$) (Fig. 14).

KIF23 Silencing Inhibited Pyroptosis-Associated Proteins in TPC-1

To investigate the role of *KIF23* in thyroid cancer cells, the protein level of *KIF23* was assessed via western blotting in thyroid cancer cell lines (TPC-1). The results demonstrated that the protein level of *KIF23* was up-regulated in the TPC-1 cell lines compared to Nthy-ori 3-1 (Fig. 15A). Subsequently, si-*KIF23* was transfected into TPC-1 cells to suppress *KIF23* expression (Fig. 15B). Furthermore, the results of western blotting revealed that the expression levels of the pyroptosis-related proteins NLRP3, GSDMD, Caspase-1, and Cleaved Caspase-1 were significantly downregulated in the si-*KIF23* group compared to

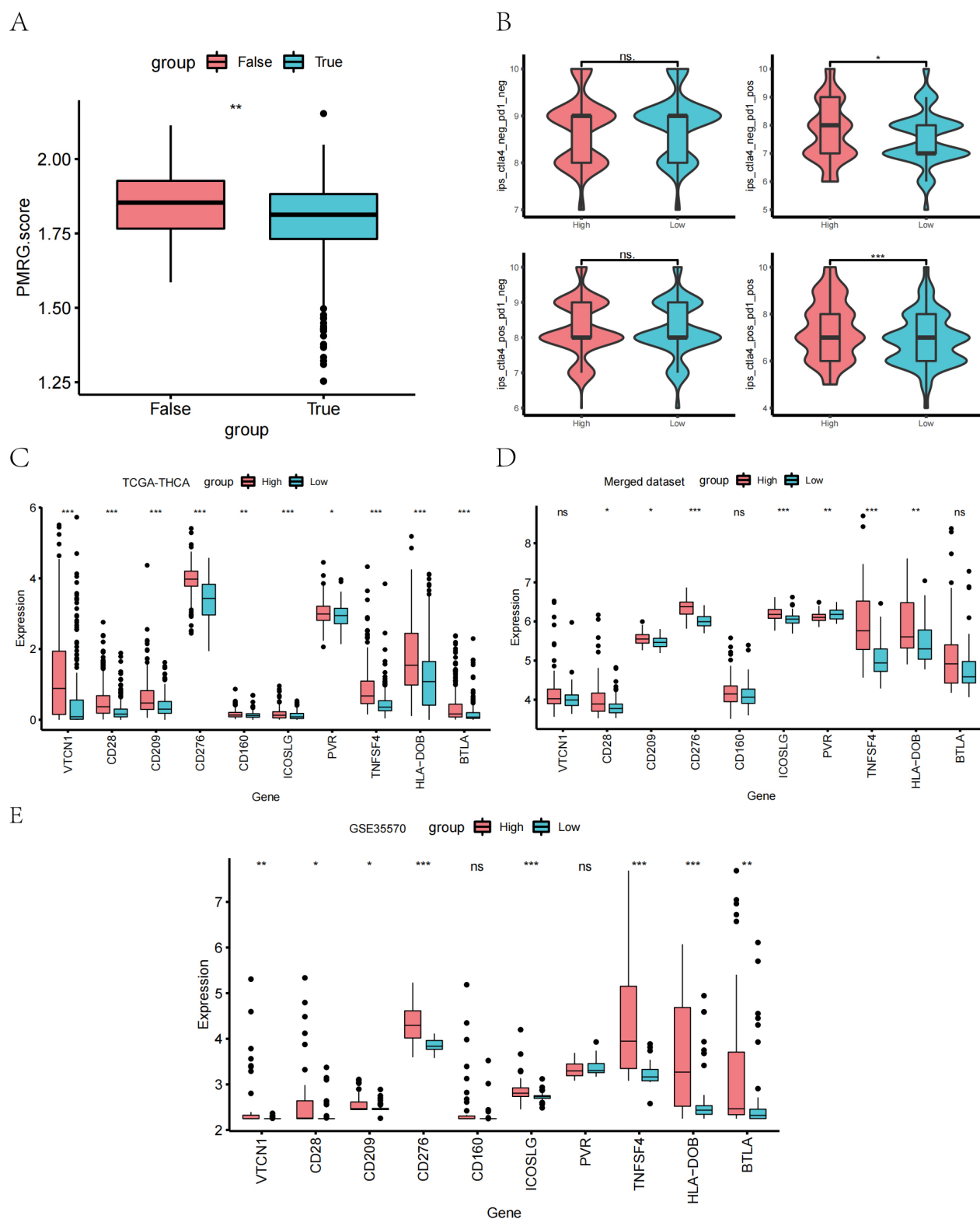


Fig. 12. Immunotherapy analysis. (A) Grouped comparison plots of phenotype scores in the TIDE predicted immunotherapy response and non-response groups. (B) Boxplot of variations in IPS between high and low pyroptosis and mitophagy scoring groups, horizontal coordinates are high and low pyroptosis and mitophagy scoring groups, vertical coordinates are IPS values. (C) Grouped comparison plots of immune checkpoint genes between high- and low-score groups of pyroptosis and mitophagy in the TCGA-THCA dataset. (D,E) Grouped comparison plots of immune checkpoint genes among high and low score groups of pyroptosis and mitophagy in the Merged dataset and GSE35570 dataset. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. TIDE, tumor immune dysfunction and exclusion; IPS, immunogenicity score.

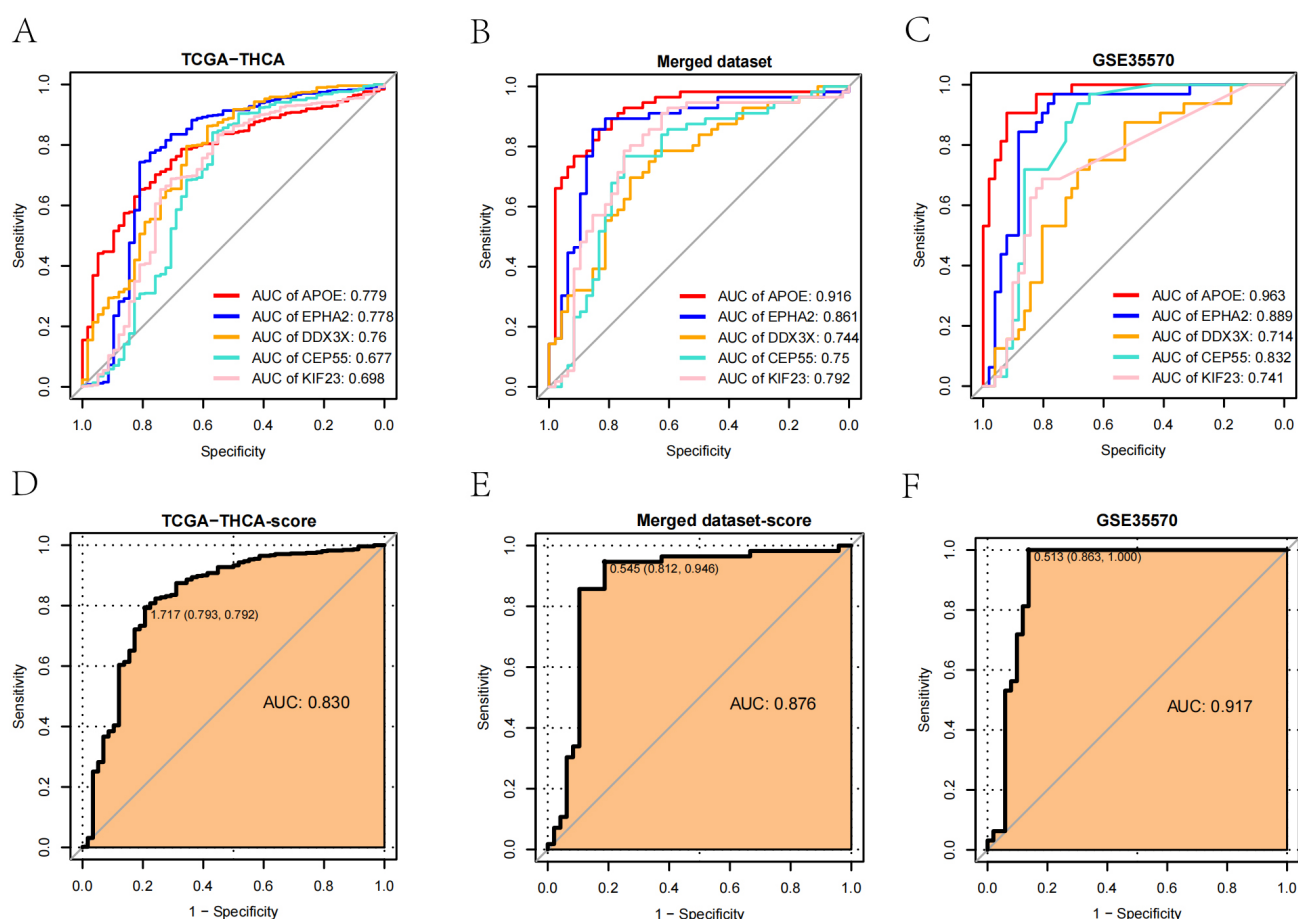


Fig. 13. ROC curves for diagnostic analysis. (A–C) ROC curves of *APOE*, *EPHA2*, *DDX3X*, *CEP55*, and *KIF23* genes in TCGA-THCA dataset (A), Merged dataset (B), and GSE35570 dataset (C). (D–F) ROC curves of pyroptosis and mitophagy scores in the TCGA-THCA dataset (D), the merged dataset (E) and the GSE35570 dataset (F).

those in the si-NC and control groups (Fig. 15C). These data suggest that KIF23 might trigger pyroptosis in thyroid cancer cells.

Discussion

Thyroid cancer has witnessed a rise in prevalence over recent decades, currently ranking as the fourth most common cancer worldwide [50]. Douglas Hanahan and Robert A. Weinberg [51] identified ten hallmarks of cancer that contribute to cancer progression and metastasis. In response to emerging challenges, they proposed four novel hallmarks of cancer in 2022, with resistance to cell death being a key trait [52]. Among various forms of cell death, pyroptosis and mitophagy play significant roles. The role of mitophagy in tumorigenesis is intricate, with increasing evidence suggesting that inhibition of mitophagy promotes tumor progression. For example, dysfunction of BRCA1 impedes stress-induced mitophagy, leading to enhanced tumor proliferation and metastasis [53]. Currently, the relationship between pyroptosis and tumors is garnering more attention. Evidence indicates that this relationship varies

across diseases due to differing genetic backgrounds. Study has shown that PD-L1-mediated expression of GSDMC shifts from TNF α -induced apoptosis of cancer cells to pyroptosis, promoting tumor necrosis [54].

In our study, we selected pyroptosis- and mitophagy-related genes *APOE*, *EPHA2*, *DDX3X*, *CEP55*, *KIF23*, and *H2BC9* as prognostic genes for THCA using the TCGA databases. Subsequently, we developed a prognostic model utilizing these six genes, enabling the prediction of prognosis for thyroid cancer patients and serving as a fundamental framework for potential tumor treatment strategies. We found that the progression-free interval (PFI) of the high-risk group exhibited a significant reduction compared to that of the low-risk group, and we identified 20 signaling pathways with differential expressions between the subgroups. Immune infiltration analysis and immune therapy analysis indicated significant variation in many immune cells and IPS scores across the subgroups. Additionally, qRT-PCR findings demonstrated a statistically significant increase in the expression levels of mRNAs of *APOE*, *EPHA2*, *CEP55*, and *KIF23* in tumor cells, consistent with the results of bioinformatic analysis. These results sug-

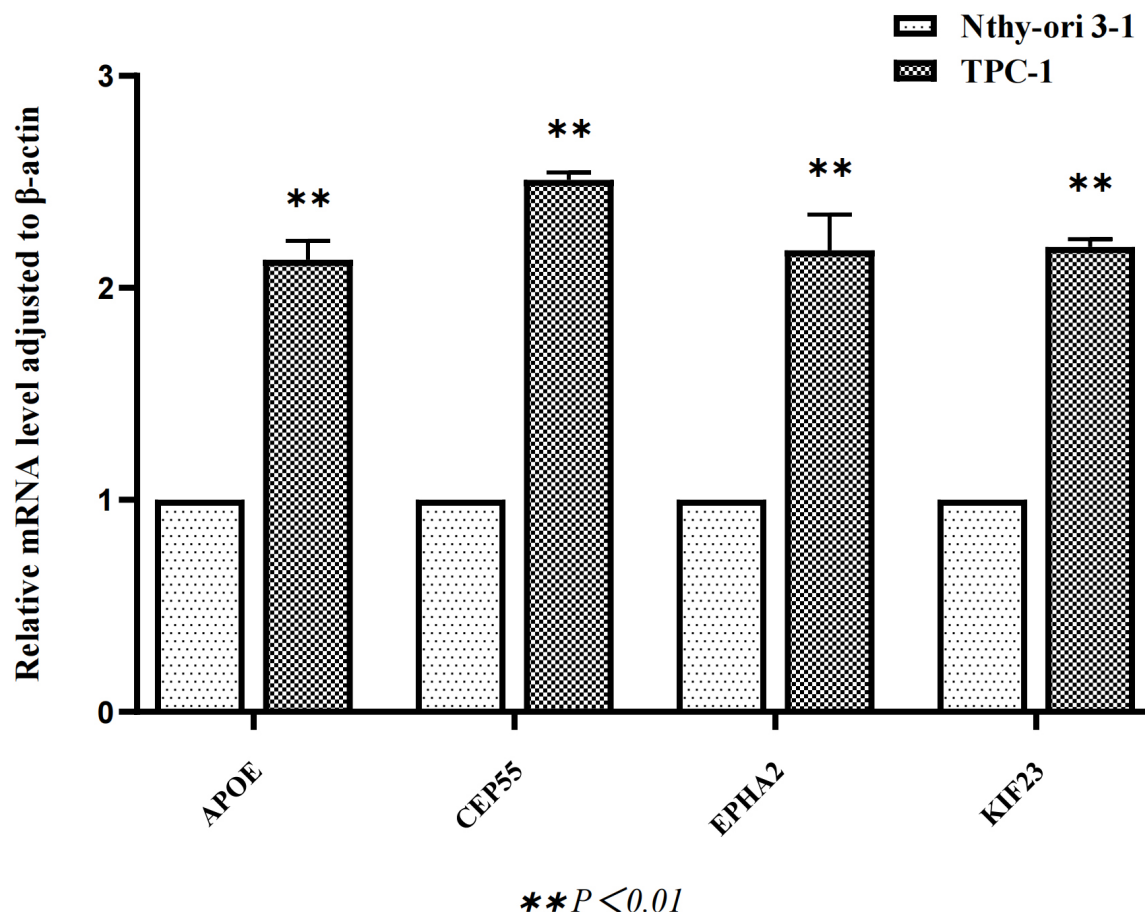


Fig. 14. The mRNA expression levels of *APOE*, *EPHA2*, *CEP55* and *KIF23*. The mRNA expression levels of *APOE*, *EPHA2*, *CEP55* and *KIF23* in thyroid cancer cell lines (TPC-1) and in normal human thyroid cells (Nthy-ori 3-1) were measured using quantitative real-time PCR (qRT-PCR) analysis. ** $p < 0.01$.

gest that the pyroptosis- and mitophagy-related signature is an effective predictor of prognosis and immunotherapy response in thyroid cancer.

Apolipoprotein E (APOE) plays a crucial role in transporting lipids to various human tissues and organs by binding lipids to form lipoproteins. It consists of three primary alleles, each containing 299 amino acids: APOE-2, APOE-3, and APOE-4. While earlier research suggested that APOE primarily contributed to cholesterol metabolism and redistribution, recent study indicates that its up-regulated expression affects the biological function of several malignancies [55]. In THCA tissues, *APOE* expression is up-regulated and positively correlates with larger tumor volume, while its knockdown significantly suppresses glycolysis and the proliferative capacity of PTC cells [56]. In our study, we observed up-regulation of *APOE* in thyroid cancer tissues as a differentially expressed gene in the TCGA-THCA dataset. Furthermore, qRT-PCR analysis confirmed a statistically significant elevation in the mRNA expression of *APOE* in thyroid cancer cells. ROC curve analysis demonstrated that *APOE* exhibited diagnostic accuracy for thyroid cancer occurrence in the TCGA-THCA dataset,

with even higher diagnostic accuracy in the Merged dataset and the GSE35570 dataset. These findings align with previous studies.

DDX3X may serve multiple functions in cancer progression. The University of California Santa Cruz initiated a comprehensive program analyzing the combination of multicancer transcriptomic and matched clinical data. These transcriptome data were sourced from the public TCGA database. The findings unveiled differential expression levels of *DDX3X* across various cancers. Specifically, *DDX3X* demonstrated relatively elevated expression levels in invasive breast carcinoma, stomach adenocarcinoma, and esophageal carcinoma, while exhibiting low expression levels in thymoma, skin cutaneous melanoma, and kidney papillary cell carcinoma [57]. However, the role of *DDX3X* in THCA remains unelucidated. In our investigation, we uncovered that *DDX3X* was downregulated in thyroid cancer tissues within the TCGA-THCA dataset. Moreover, ROC analysis indicated that *DDX3X* exhibited diagnostic accuracy for thyroid cancer occurrence in the TCGA-THCA dataset, the Merged dataset, and in the GSE35570 dataset.

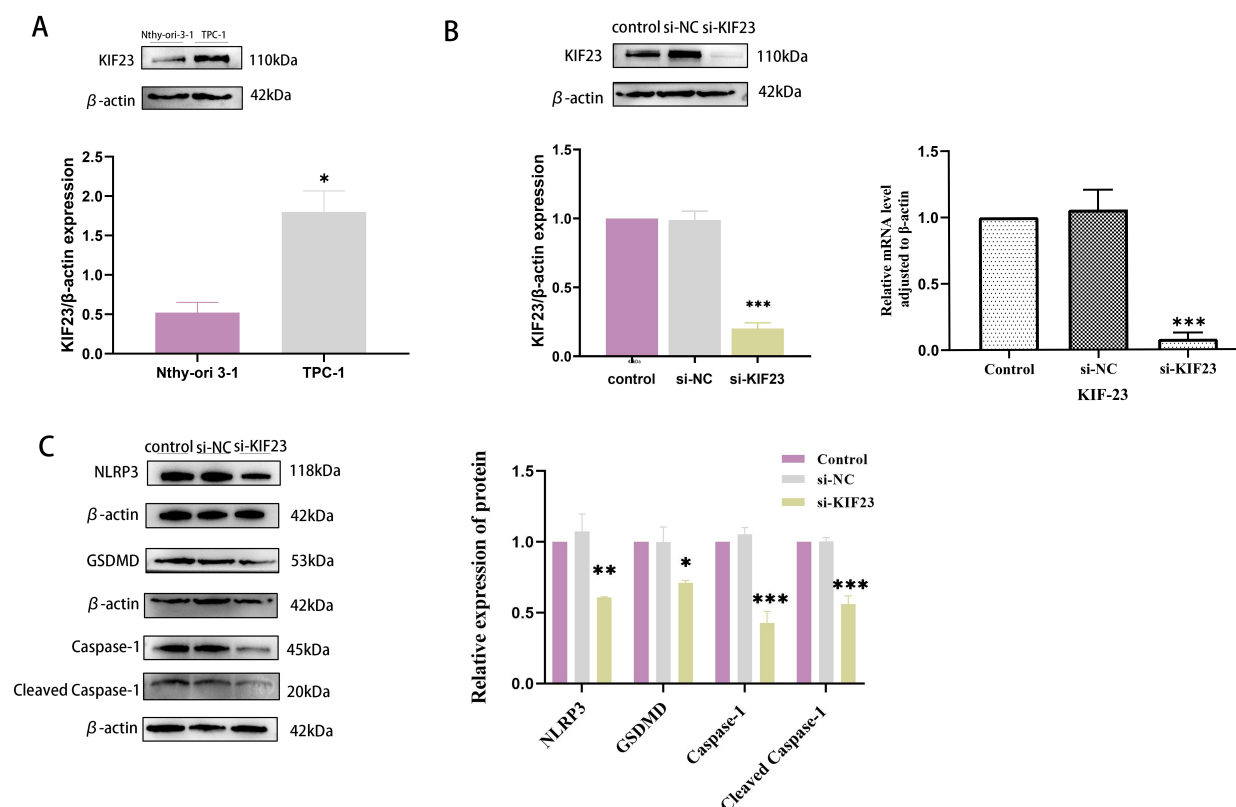


Fig. 15. KIF23 silencing inhibited pyroptosis-associated proteins in TPC-1. (A) KIF23 protein level was upregulated in the TPC-1 cell lines compared to the Nthy-ori 3-1. (B) The expression of KIF23 were downregulated in the si-KIF23 group. (C) The expression levels of the pyroptosis-related protein NOD-like receptor thermal protein domain associated protein 3 (NLRP3), GSDMD, Caspase-1 and Cleaved Caspase-1 were notably downregulated in the si-KIF23 group compared to those in the control and si-NC groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Previous research has indicated that *KIF23* plays crucial roles in the regulation of cytokinesis. Abnormal function of *KIF23* leads to mitotic blockade and the production of binucleated or multinucleated cells, contributing to tumor progression [58]. *KIF23* has been shown to be expressed at elevated levels in various tumors and correlates with tumor progression, such as colorectal cancer [59], breast cancer [60] and bladder cancer [61]. Our current research found elevated expression of *KIF23* in thyroid cancer cells, and *KIF23* silencing inhibited pyroptosis-related proteins in TPC-1 cells. These results suggest that *KIF23* might promote the progression of THCA by enhancing pyroptosis. A recent study also indicates that the desuccinylation of *KIF23* is involved in promoting proliferation and migration of anaplastic thyroid cancer cells [62], and our study also suggests a potential role for *KIF23* in the development of thyroid tumors.

According to the GSEA findings, these pyroptosis- and mitophagy-related genes may potentially participate in various signaling pathways, including Hippo Signaling Regulation, Wnt Signaling Pathway, Pluripotency, PI3K/AKT Signaling in Cancer, Mapk Signaling Pathway, Tgfbeta Signaling Pathway, and Ras Signaling Pathway.

Inactivation of Hippo signaling has been demonstrated to be associated with an unfavorable prognosis in THCA individuals [63]. A study revealed that KDM1A promotes thyroid tumor growth and sustains stemness through the Wnt/ β -catenin signaling pathway [64]. An *in vivo* experiment demonstrated that TR β mutations activate the PI3K/AKT signaling pathway, leading to primary thyroid cancer and metastatic lesions in a mouse model [65]. Some data suggest that thyroid carcinoma cells could be targeted by vitamin C, which suppresses MAPK/ERK and PI3K/AKT pathways through ROS-dependent mechanisms [66]. Furthermore, GSVA was performed on all genes within the high- and low-risk groups. The results showed significant differences in the expression of various pathways between the subgroups, indicating that the risk subgroups of the prognostic model differ in their biological functions. This aspect merits further investigation regarding the underlying mechanisms.

The CIBERSORT algorithm was employed to assess the abundance of 22 different immune cell types within the immune microenvironment of THCA patients, aiming to explore the relationship between the risk score and the immune microenvironment. The findings revealed significant

distinctions in many immune cells, including CD4 memory T cells, CD8 T cells, regulatory T cells (Tregs), follicular helper T cells, Macrophages M0, and Macrophages M2, between the high and low-risk groups. Various types of tumor-infiltrating lymphocytes play distinct roles in thyroid cancer [67]. Elevated levels of circulating myeloid-derived suppressor cells were observed in individuals with thyroid cancer, correlating with the aggressiveness of differentiated thyroid cancer [68]. Moreover, increased scores of macrophages, mast cells, and dendritic cells were linked to low thyroid differentiation scores or BRAF^{V600E} mutations, while higher expressions of CTLA-4 and PD-L1 were noted in BRAF^{V600E} and dedifferentiated thyroid cancers [69]. IPS plays a crucial role in determining the response to immune checkpoint inhibitor (ICIs) therapy. Our study indicated that the IPS-PD1/PD-L1/PD-L2 and IPS-PD1/PD-L1/PD-L2 + CTLA4 scores were higher in the group with high pyroptosis and mitophagy scores. These findings suggest that individuals with THCA who exhibit high pyroptosis and mitophagy scores might display increased sensitivity to immunotherapy, indicating that the pyroptosis and mitophagy score could potentially identify THCA patients who may benefit from PD-1/PD-L1/PD-L2 + CTLA4 inhibitor therapy.

Undoubtedly, our study has certain limitations. Firstly, the majority of our current findings are derived from public databases, highlighting the need for further validation through *in vivo* and *in vitro* studies. Secondly, while we confirmed the differential expression of prognostic genes among cell lines using real-time PCR, data from human tissue specimens are still needed to bolster confidence in our findings. Thirdly, the validity of predictive models necessitates extensive evidence-based validation from multiple centers.

Conclusions

In summary, we identified six genes related to pyroptosis and mitophagy (*APOE*, *EPHA2*, *DDX3X*, *CEP55*, *KIF23*, *H2BC9*) through bioinformatics analysis to construct a prognostic model for individuals with THCA. Our findings indicate that the high-risk group in the prognostic model had significantly lower PFI compared to the low-risk group. Additionally, the IPS-PD1/PD-L1/PD-L2 and IPS-PD1/PD-L1/PD-L2 + CTLA4 scores were higher in the group with elevated pyroptosis and mitophagy scores. This study establishes a novel marker of pyroptosis- and mitophagy-related genes in THCA, which may serve as a prospective biomarker for prognosis and the management of ICIs therapy in thyroid cancer.

Availability of Data and Materials

Our study used public online database. The data can be accessed by following websites: <https://portal.gdc.cancer.gov/>, <http://genome.ucsc.edu>, <http://tide.dfci.harvard.edu>,

<https://tcia.at/home>, <http://www.genecards.org/> and <https://www.gsea-msigdb.org/>. Users can download relevant data for free for research and publish relevant articles.

Author Contributions

YL and WC performed the research and wrote the manuscript. JPZ and JW took part in design of the study. YH and XDH participated in the statistics of the article data. JXX processed data and designed the study. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.23812/j.biol.regul.homeost.agents.20243806.399>.

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