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Integrated analysis identifies prognostic biomarkers associated with mitochondrial dysfunction in head and neck squamous cell carcinoma

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Abstract

Objective: Head and neck squamous cell carcinoma (HNSC) is highly heterogeneous and has poor prognosis. Mitochondrial dysfunction contributes to metabolic reprogramming and immune regulation, but its prognostic value in HNSC remains unclear. This study aimed to identify mitochondrial dysfunction-related prognostic genes, construct a risk model, and explore associations with the immune microenvironment, mutations, and immunotherapy response.

Methods: TCGA-HNSC transcriptomic data were integrated with mitochondrial dysfunction-related genes from GeneCards. Prognostic genes were screened by differential expression, log-rank testing, and Cox regression. Patients were divided 7:3 into training and testing cohorts. A model was constructed using univariate Cox, LASSO,

stepwise, and multivariate Cox regression and validated in the training, testing, TCGA-HNSC, and GSE41613 cohorts. CIBERSORT, ssGSEA, ESTIMATE, TIDE, and an independent immunotherapy cohort were used for immune analyses. Somatic mutations and tumor mutational burden were evaluated. PPP1R3A was validated in CAL27 and SCC-25 cells by overexpression, knockdown, qRT-PCR, and Western blotting.

Results: Ninety-eight mitochondrial dysfunction-related prognostic genes were identified, yielding a 10-gene model. The model stratified patients into high- and low-risk groups across internal and external cohorts, with significantly poorer survival in the high-risk group. Risk score was an independent prognostic factor. Low-risk patients showed stronger antitumor immune activity and a higher likelihood of immunotherapy benefit. Risk score was associated with tumor mutational burden and mutation patterns. qRT-PCR and Western blotting confirmed PPP1R3A overexpression and knockdown.

Conclusion: The 10-gene mitochondrial dysfunction-related signature may support prognostic assessment and risk stratification in HNSC and characterize immune and genomic features relevant to immunotherapy response.

Keywords: Head and neck squamous cell carcinoma; mitochondrial dysfunction; tumor immune microenvironment; immunotherapy response

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the most common malignancies of the head and neck, arising mainly from the oral cavity, oropharynx, hypopharynx, and larynx[1,2]. Tobacco use, excessive alcohol consumption, infection with high-risk human papillomavirus (HPV), and genetic and environmental factors are well-established risk factors for HNSCC[3]. Because early symptoms are often nonspecific, a substantial proportion of patients are diagnosed with locally advanced disease or regional lymph node metastasis at initial presentation[4]. Surgery, radiotherapy, and chemotherapy remain the main treatment options for HNSCC, while immune checkpoint inhibitors have increasingly been introduced into clinical practice in recent years. However, treatment response and survival vary considerably among patients, and the prognosis of those with recurrent or metastatic disease remains poor[5]. Identifying reliable molecular biomarkers that better reflect this heterogeneity may therefore improve prognostic assessment, risk stratification, and individualized treatment of HNSCC.

Mitochondria are central to cellular energy metabolism and also participate in redox homeostasis, apoptosis, calcium signaling, and cellular stress responses[6,7]. Growing

evidence suggests that mitochondrial dysfunction is an important metabolic feature of cancer development and progression. It may involve abnormalities in the tricarboxylic acid cycle, impaired electron transport chain activity, mitochondrial DNA mutations, excessive production of reactive oxygen species, and dysregulation of mitochondria-mediated apoptosis[8]. These alterations can affect the energy supply and metabolic adaptability of tumor cells and have been linked to tumor proliferation, invasion, migration, and treatment resistance[9,10]. Thus, changes in mitochondrial function may not only reflect the biological state of tumor cells but may also be associated with disease progression and clinical outcome.

Tumor metabolism is also closely connected to the tumor immune microenvironment[11]. Altered mitochondrial metabolism and redox balance can influence the recruitment, activation, and functional state of immune cells and may contribute to immune escape and differences in treatment response[12]. HNSCC is characterized by a heterogeneous immune microenvironment, and only a subset of patients derives durable benefit from immunotherapy[13,14]. However, whether mitochondrial dysfunction-related molecular features can be used to assess prognosis in HNSCC, or whether they are associated with immune status, genomic alterations, and potential response to immunotherapy, has not been fully explored[15,16]. Most previous studies have focused on individual mitochondrial genes or specific signaling pathways, which may not adequately capture the coordinated effects of multiple mitochondrial abnormalities. A systematic assessment of mitochondrial dysfunction-related genes at the transcriptomic level may therefore provide additional insight into the molecular heterogeneity of HNSCC.

In this study, we systematically analyzed mitochondrial dysfunction-related genes in HNSCC using transcriptomic and clinical data from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) databases. A 10-gene prognostic signature was constructed using a series of survival and regression analyses and was evaluated in internal and independent external cohorts. We further compared immune cell infiltration, immune activity, tumor microenvironment characteristics, somatic mutations, and tumor mutational burden between different risk groups. The association between the risk signature and potential benefit from immunotherapy was assessed using the Tumor Immune Dysfunction and Exclusion (TIDE) algorithm and an independent immunotherapy cohort. We also selected PPP1R3A as a representative gene from the signature and established PPP1R3A overexpression and knockdown models in CAL27 and SCC-25 cells to confirm that its expression could be experimentally manipulated. Through these analyses, we aimed to develop a mitochondrial dysfunction-related molecular signature for prognostic assessment and risk stratification in HNSCC and to explore its potential relevance to immunotherapy response.

2. Materials and Methods

2.1. Collection and processing of publicly available data

RNA-sequencing transcriptomic data (read counts), corresponding clinical information, and somatic mutation data for head and neck squamous cell carcinoma were obtained from The Cancer Genome Atlas Head and Neck Squamous Cell Carcinoma project (TCGA-HNSC) through the NCI Genomic Data Commons (GDC) Data Portal (<https://portal.gdc.cancer.gov/projects/TCGA-HNSC>)[17]. The TCGA-HNSC dataset used in this study comprised 522 HNSC tumor samples and 44 normal controls. Clinical information included survival status, age, sex, and tumor stage. Genes with raw counts < 10 and detected in fewer than 50% of the samples were excluded. Differentially expressed genes (DEGs) between HNSC and adjacent non-tumor tissues were identified using the R package limma, with $|\log_2 \text{fold change (FC)}| \geq 1$ and false discovery rate (FDR) < 0.05 as the cutoff criteria. Volcano plots and heatmaps were generated using the ggplot2 and pheatmap packages, respectively. Raw expression data were subsequently converted to transcripts per million (TPM) values and $\log_2(x + 1)$ -transformed for downstream analyses.

Mitochondrial dysfunction-related genes were retrieved from the GeneCards database using the exact search term “mitochondrial dysfunction” (<https://www.genecards.org/Search/Keyword?queryString=mitochondrial%20dysfunction>)[18]. A total of 8,957 human genes associated with mitochondrial dysfunction were obtained from the search results. The independent external validation dataset GSE41613 was obtained from the NCBI Gene Expression Omnibus (GEO) under accession number GSE41613 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE41613>)[19]. The GSE41613 dataset contained 97 HPV-negative oral squamous cell carcinoma samples profiled using the GPL570 Affymetrix Human Genome U133 Plus 2.0 Array platform. Expression data were $\log_2$ -transformed and normalized before subsequent analyses.

2.2. Identification of prognostic mitochondrial dysfunction-related DEGs

Genes associated with overall survival (OS) in the TCGA-HNSC cohort were initially screened using log-rank tests and Cox regression analyses implemented in the survival and survminer R packages. Prognostic mitochondrial dysfunction-related DEGs were identified by intersecting prognostic genes, HNSCC-associated DEGs, and mitochondrial dysfunction-related genes. The overlapping genes were visualized using a Venn diagram. A total of 98 mitochondrial dysfunction-related prognostic DEGs were retained for subsequent analyses.

2.3. Functional enrichment analysis of mitochondrial dysfunction-related DEGs

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed using the clusterProfiler R package to investigate the biological

functions and pathways associated with the identified genes. Fisher's exact test was used for enrichment analysis, and an FDR-adjusted P value < 0.05 was considered statistically significant.

2.4. Construction of the mitochondrial dysfunction-related prognostic model

Patients with complete survival information in the TCGA-HNSC cohort were randomly divided into training and testing cohorts at a ratio of 7:3 using the caret R package. The prognostic model was developed in the training cohort.

Univariate Cox proportional hazards regression analysis was first performed using the survival package to identify mitochondrial dysfunction-related DEGs associated with OS. Least absolute shrinkage and selection operator (LASSO) Cox regression was subsequently conducted using the glmnet package[20] to reduce dimensionality and minimize model overfitting. Ten-fold cross-validation was used to determine the optimal penalty parameter (λ) according to the minimum criterion. Candidate genes retained by LASSO were further subjected to stepwise regression and multivariate Cox regression to obtain the final prognostic genes.

A mitochondrial dysfunction-related risk score was calculated for each patient according to the expression levels and regression coefficients of the genes included in the final model:

Risk score = $\text{Exp}_1 \times \beta_1 + \text{Exp}_2 \times \beta_2 + \text{Exp}_3 \times \beta_3 + \dots + \text{Exp}_n \times \beta_n$. where Exp represents the expression level of each model gene and β represents its corresponding Cox regression coefficient.

2.5. Evaluation and validation of the prognostic model

Patients were classified into high- and low-risk groups according to the median risk score in the training cohort. Kaplan–Meier survival analysis was performed using the survival and survminer packages to compare OS between the two risk groups.

Time-dependent receiver operating characteristic (ROC) curves were generated using the timeROC package, and the areas under the curve (AUCs) at 1, 3, and 5 years were calculated to assess the predictive performance of the model. The distribution of risk scores, survival status, and expression patterns of the model genes was also examined. Heatmaps showing gene expression patterns in the high- and low-risk groups were generated using the pheatmap package.

The robustness of the prognostic signature was further evaluated in the TCGA-HNSC training cohort, testing cohort, entire TCGA-HNSC cohort, and the independent GSE41613 cohort.

2.6. Association between the risk score and clinical characteristics

Univariate and multivariate Cox regression analyses were performed to determine whether the risk score was independently associated with OS after adjustment for available clinicopathological variables, including age, sex, tumor stage, and TNM classification. Hazard ratios (HRs), 95% confidence intervals (CIs), and P values were calculated for each variable. Associations between the risk groups and clinicopathological characteristics were further assessed using the chi-square test.

2.7. Immune cell infiltration and immune activity analysis

Single-sample gene set enrichment analysis (ssGSEA) was performed using the GSVA R package to estimate immune cell infiltration in individual HNSC samples. Enrichment scores were calculated to reflect the relative abundance of each immune cell type. The activity of 13 immune-related pathways was also quantified using ssGSEA.

CIBERSORT[21] was used to estimate the relative proportions of 22 tumor-infiltrating immune cell populations in each HNSC sample. The LM22 leukocyte signature matrix, comprising 547 marker genes, was used to distinguish 22 immune cell populations, including different subsets of T cells, B cells, natural killer cells, plasma cells, and myeloid cells. Genes with an average expression value of zero were removed before analysis. Differences in the proportions of immune cell populations between the high- and low-risk groups were subsequently compared. Spearman correlation analysis was performed to assess the relationships among the infiltration levels of the 22 immune cell types, the expression of the 10 model genes, and the risk score.

Immune infiltration estimates generated using seven algorithms—TIMER, CIBERSORT, CIBERSORT-ABS, quanTIseq, xCell, MCP-counter, and EPIC—were additionally obtained from TIMER2.0 (<http://timer.cistrome.org/>), with TCGA-HNSC selected as the cancer cohort. Differences in immune cell infiltration between the high- and low-risk groups were visualized using a heatmap. Immune checkpoint gene expression was also compared between the high- and low-risk groups.

2.8. Tumor microenvironment analysis

The ESTIMATE R package was used to characterize the tumor microenvironment (TME) of HNSC based on gene expression profiles. Stromal scores, immune scores, and ESTIMATE scores were calculated for each patient and compared between the high- and low-risk groups. Spearman correlation analysis was further performed to evaluate the associations between the risk score and each TME-related score.

Immune cell infiltration within the HNSC microenvironment was additionally assessed using ssGSEA to provide a complementary assessment of differences in immune composition between the two risk groups.

2.9. Gene set variation analysis and gene set enrichment analysis

Gene set variation analysis (GSVA) was performed to explore differences in biological processes and signaling pathways between the high- and low-risk groups. The GSVA R package was applied using the MSigDB v7.5 curated KEGG pathway gene set `c2.cp.kegg.v7.5.symbols.gmt` (<https://data.broadinstitute.org/gsea-msigdb/msigdb/release/7.5/c2.cp.kegg.v7.5.symbols.gmt>) and Gene Ontology biological-process gene set `c5.go.bp.v7.5.symbols.gmt` (<https://data.broadinstitute.org/gsea-msigdb/msigdb/release/7.5/c5.go.bp.v7.5.symbols.gmt>). Differential pathway activity between the two risk groups was visualized using the pheatmap package.

Gene set enrichment analysis (GSEA) was further performed using the clusterProfiler package to identify biological pathways differentially enriched between the high- and low-risk groups. The MSigDB v7.2 Hallmark gene set `h.all.v7.2.symbols.gmt` (<https://data.broadinstitute.org/gsea-msigdb/msigdb/release/7.2/h.all.v7.2.symbols.gmt>) was used as the reference gene set.

2.10. Assessment of the prognostic model for immunotherapy response

The Tumor Immune Dysfunction and Exclusion (TIDE) algorithm (<http://tide.dfci.harvard.edu/>) was used to estimate the potential response of patients with HNSC to immune checkpoint blockade therapy[22]. TIDE, T-cell dysfunction, and T-cell exclusion scores, as well as predicted immunotherapy response status, were compared between the high- and low-risk groups to evaluate the potential association between the mitochondrial dysfunction-related signature and immunotherapy benefit. To provide an independent assessment in patients treated with immune checkpoint blockade, the IMvigor210 cohort was additionally analyzed. IMvigor210 comprises patients with advanced urothelial carcinoma treated with the anti-PD-L1 antibody atezolizumab. Processed transcriptomic and corresponding clinical/response data were obtained from the IMvigor210CoreBiologies resource (<http://research-pub.gene.com/IMvigor210CoreBiologies/>); the underlying molecular data are archived in the European Genome-Phenome Archive under study accession EGAS00001002556 (<https://ega-archive.org/studies/EGAS00001002556>). Patients were stratified using the established risk-score formula, and survival outcomes and treatment responses were compared between risk groups. Complete response and partial response were considered objective responses, whereas stable disease and progressive disease were considered nonresponses.

2.11. Cell culture and transfection

The human tongue squamous cell carcinoma cell lines CAL 27 (ATCC CRL-2095) and SCC-25 (ATCC CRL-1628) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in the corresponding complete

culture medium at 37 °C in a humidified incubator containing 5% CO₂ and were transfected after reaching an appropriate confluence.

For PPP1R3A overexpression experiments, cells were divided into Control, OE-NC, and OE-PPP1R3A groups. For PPP1R3A knockdown experiments, cells were divided into Control, sh-NC, and sh-PPP1R3A groups. PPP1R3A overexpression or knockdown vectors and their corresponding negative controls were introduced into CAL27 and SCC-25 cells according to the manufacturer's instructions for the transfection reagent.

Cells were collected at the indicated time after transfection. PPP1R3A expression at the mRNA and protein levels was examined by quantitative real-time PCR (qRT-PCR) and Western blotting, respectively, to verify the efficiency of overexpression and knockdown.

2.12. Quantitative real-time PCR

Total RNA was extracted from cultured cells using TRIzol reagent and reverse-transcribed into complementary DNA (cDNA). qRT-PCR was performed under the following cycling conditions: 95 °C for 10 s, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 30 s. α -Tubulin was used as the reference gene, and relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method.

The primer sequences were as follows:

PPP1R3A forward, 5'-TGGTTTCTCCCCTCAACCAAG-3';

PPP1R3A reverse, 5'-TCCGTCCCTAAGTCAAAAGTGG-3';

α -tubulin forward, 5'-TCTCTTACATCGACCGCCTAA-3';

α -tubulin reverse, 5'-CATTGCCAATCTGGACACC-3'.

2.13. Western blotting

CAL27 and SCC-25 cells from each treatment group were collected and lysed in RIPA buffer supplemented with protease inhibitors. Total protein concentrations were determined using a bicinchoninic acid (BCA) assay. Equal amounts of protein were separated by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes.

The membranes were blocked with 5% non-fat milk for 2 h at room temperature and then incubated overnight at 4 °C with primary antibodies against PPP1R3A and α -tubulin. After washing with Tris-buffered saline containing Tween-20 (TBST), the membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature. Protein bands were visualized using enhanced chemiluminescence (ECL).

α -Tubulin was used as the loading control. Band intensities were quantified using ImageJ, and PPP1R3A expression was normalized to α -tubulin. All experiments were independently repeated at least three times.

2.14. Statistical analysis

All experimental assays were independently repeated at least three times. Quantitative data are presented as the mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism version 10.1.2 (GraphPad Software, La Jolla, CA, USA). Differences between two groups were assessed using Student's t-test, whereas comparisons among multiple groups were performed using one-way analysis of variance (ANOVA). A two-sided P value < 0.05 was considered statistically significant.

3. Results

Fig. 1 Workflow of this study

3.1. Identification of prognostic mitochondrial dysfunction-related genes

The overall workflow of this study is shown in Fig. 1. To identify mitochondrial dysfunction-related genes (MDGs) associated with prognosis, we first used the limma R package to perform differential expression analysis between HNSC and normal samples based on TCGA RNA-seq data. A total of 1,140 differentially expressed genes (DEGs) were identified, as shown in the volcano plot (Fig. 2A). We then performed rank-sum tests and univariate Cox regression analyses using the whole-transcriptome data from the TCGA-HNSC cohort to identify genes associated with patient prognosis. The prognostic genes, DEGs, and mitochondrial dysfunction-related genes were subsequently intersected and visualized using a Venn diagram. This analysis identified 98 prognostic mitochondrial dysfunction-related genes in HNSC (Fig. 2B). The single-nucleotide variant (SNV) landscape of these 98 genes is presented in Supplementary Fig. 1D, showing their mutation frequencies in HNSC and further supporting the potential involvement of mitochondrial dysfunction-related genes in HNSC.

Fig. 2 Screening of mitochondrial dysfunction-related prognostic genes and construction of the LASSO–Cox prognostic model in head and neck squamous cell carcinoma. (A) Volcano plot showing differentially expressed genes between HNSC tumor tissues and normal tissues. (B) Intersection analysis of differentially expressed genes, prognosis-related genes, and mitochondrial dysfunction-related genes. (C) Cross-validation curve of the LASSO regression model. (D) LASSO coefficient profiles of the candidate genes. (E) Forest plot of univariate Cox regression analysis for the candidate genes. (F) Heatmap showing the expression patterns of the 10 candidate prognostic genes in normal and tumor tissues

3.2. Functional enrichment analysis of mitochondrial dysfunction-related DEGs

To investigate the potential biological functions of the 98 prognostic mitochondrial dysfunction-related DEGs, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed. GO analysis showed that these genes were significantly enriched in terms related to biological process (BP), cellular component (CC), and molecular function (MF), including response to mechanical stimulus, myosin complex, and actin binding (Supplementary Fig. 1A).

KEGG pathway analysis further identified six significantly enriched pathways (Supplementary Fig. 1B). The enriched pathways were mainly associated with cardiomyopathy-related pathways, motor proteins, and ECM–receptor interaction. In addition, the heatmap showed distinct expression patterns of the 98 mitochondrial dysfunction-related genes between HNSC and normal samples (Supplementary Fig. 1C).

3.3. Construction of the prognostic risk score in the TCGA training cohort

A total of 510 patients with HNSC from the TCGA cohort were randomly divided into a training cohort and a testing cohort at a ratio of 7:3 using the caret R package. To establish an optimal prognostic model, univariate Cox regression analysis for overall survival (OS) was first performed on the 98 candidate genes in the TCGA training cohort. As shown in Fig. 2E, 52 genes were significantly associated with OS. These genes were then subjected to least absolute shrinkage and selection operator (LASSO) Cox regression analysis, and 14 genes were retained based on partial likelihood deviance and the optimal penalty parameter (Fig. 2C, D).

To further reduce the number of genes included in the model, stepwise regression analysis with both forward and backward selection (direction = both) was performed, followed by multivariate Cox regression analysis. Finally, 10 mitochondrial dysfunction-related genes were selected to construct the prognostic model. The risk score for each patient was calculated according to the expression level of each gene and its corresponding regression coefficient as follows: Risk score = $0.1191 \times \text{DHRS2} + 0.1514 \times \text{PLAU} + 0.1997 \times \text{ULBP2} + 0.2240 \times \text{HOXB9} - 0.2272 \times \text{CELSR3} + 0.1151 \times \text{FST} - 0.3519 \times \text{TNFRSF4} + 0.1368 \times \text{RBP4} + 0.1368 \times \text{PPP1R3A} + 0.1279 \times \text{IFI27}$.

We next examined the expression patterns, molecular interactions, and prognostic value of these 10 genes. Eight genes were upregulated in tumor tissues, whereas two genes were downregulated compared with normal tissues ($P < 0.001$; Fig. 2F). The molecular interactions among the 10 genes are shown in Fig. 5C. Kaplan–Meier survival analysis further showed that all 10 genes were significantly associated with OS in patients with HNSC (Supplementary Fig. 2A–J).

Patients in the TCGA training cohort were subsequently classified into high- and low-risk groups according to the median risk score. Kaplan–Meier analysis showed that patients in the high-risk group had significantly poorer OS than those in the low-risk group (Fig. 3A). Risk scores were then calculated for individual patients and ranked in ascending order. As

the risk score increased, survival time tended to decrease and the proportion of deaths increased (Fig. 3D, G). The expression patterns of the 10 model genes also differed between the two risk groups, as shown in the heatmap (Fig. 2F).

Time-dependent receiver operating characteristic analysis showed that the area under the curve (AUC) values for predicting 1-, 3-, and 5-year OS were 0.695, 0.721, and 0.731, respectively (Fig. 3M). These findings indicate that the 10-gene mitochondrial dysfunction-related signature has potential value for prognostic stratification in patients with HNSC.

Fig. 3 Construction and validation of the 10-gene mitochondrial dysfunction-related prognostic model in HNSC. (A–C) Kaplan–Meier survival analysis of the high- and low-risk groups in the TCGA training cohort, testing cohort, and entire cohort. (D–F) Risk score distributions in the TCGA training cohort, testing cohort, and entire cohort. (G–I) Distributions of survival time and survival status in the TCGA training cohort, testing cohort, and entire cohort. (J–L) Heatmaps showing the expression patterns of the 10 prognostic model genes in the TCGA training cohort, testing cohort, and entire cohort. (M–O) Time-dependent ROC curves of the prognostic model in the TCGA training cohort, testing cohort, and entire cohort

3.4. Validation of the prognostic risk score

To further evaluate the stability and effectiveness of the risk score in predicting survival in patients with HNSC, patients in the validation cohorts, including the TCGA testing cohort, the entire TCGA-HNSC cohort, and GSE41613, were also divided into high- and low-risk groups according to the risk scores calculated using the established risk formula, following the same approach used in the TCGA training cohort. To assess differences in survival time and survival status between the two groups, Kaplan–Meier survival curves, risk score distribution plots, heatmaps, and ROC curves were generated for the validation cohorts.

Consistent with the results obtained in the TCGA training cohort, Kaplan–Meier survival analysis and risk score distribution plots showed that patients in the high-risk group had significantly poorer prognosis than those in the low-risk group (Fig. 3A–F; Fig. 4A, B). Similarly, the expression levels of the 10 genes included in the model differed significantly between the high- and low-risk groups (Fig. 3G–I; Fig. 4C).

Time-dependent receiver operating characteristic (ROC) curves at 1, 3, and 5 years were further generated to evaluate the sensitivity and specificity of the prognostic model developed in the TCGA training cohort. In the TCGA testing cohort, the AUC values for predicting 1-, 3-, and 5-year overall survival (OS) were 0.736, 0.731, and 0.651, respectively (Fig. 3N). In the entire TCGA-HNSC cohort, the corresponding AUC values were 0.690, 0.691, and 0.714, respectively (Fig. 3O). In the GSE41613 cohort, the AUC values for predicting 1-, 3-, and 5-year OS were 0.733, 0.754, and 0.702, respectively (Fig. 4D). These results suggest that the 10-gene risk model has good prognostic performance for predicting OS in patients with HNSC.

Fig. 4 Validation of the 10-gene prognostic model in the GSE41613 cohort. (A) Kaplan–Meier survival analysis. (B) Risk score distribution. (C) Survival status distribution. (D) Time-dependent ROC curves for 1-, 3-, and 5-year survival. (E) Heatmap of the 10 model genes in the high- and low-risk groups. (F) Nomogram for predicting 1-, 3-, and 5-year survival. (G) Calibration curves of the nomogram. (H) Cox regression forest plot of the risk score and clinicopathological characteristics

3.5. Association between the risk score and clinicopathological characteristics

Given the prognostic performance of the mitochondrial dysfunction-related risk model, we further examined the association between the risk score and clinicopathological characteristics. Univariate and multivariate Cox regression analyses incorporating age, sex, tumor stage, TNM stage, and risk score showed that the risk score remained an independent predictor of overall survival (OS) in patients with HNSC (Fig. 5A, B).

Patients in the TCGA-HNSC cohort were further stratified according to age, sex, tumor stage, T stage, N stage, and M stage. No significant differences in risk scores were observed among most clinicopathological subgroups (Fig. 5D–J). We then assessed the prognostic value of the 10-gene mitochondrial dysfunction-related signature within different clinical subgroups. Kaplan–Meier analysis showed that high-risk patients had significantly poorer OS than low-risk patients across the age, sex, tumor stage, T-stage, N-stage, and M0 subgroups (Supplementary Fig. 3A–K). No significant survival difference was observed in the M1–X subgroup (Supplementary Fig. 3L, $P = 0.4015$). Overall, these findings suggest that the prognostic signature retains its risk-stratification ability across most clinicopathological subgroups.

Fig. 5 Independent prognostic value and clinicopathological associations of the 10-gene mitochondrial dysfunction-related prognostic model in HNSC. (A) Univariate Cox regression analysis of the risk score and clinicopathological variables. (B) Multivariate Cox regression analysis evaluating the independent prognostic value of the risk score. (C) Correlation chord diagram among the 10 model genes; red indicates positive correlations and green indicates negative correlations. (D–I) Comparison of risk scores across age, sex, clinical stage, T stage, N stage, and M stage subgroups. (J) Comparison of risk scores between the high- and low-risk groups. (K) ROC curves comparing the prognostic performance of the risk score with clinicopathological variables. (L) Distribution of clinicopathological characteristics between the high- and low-risk groups

3.6. Biological differences between the high- and low-risk groups

To explore the biological differences between the high- and low-risk groups, we first performed principal component analysis (PCA) based on RNA-seq data from the TCGA-HNSC cohort. The two risk groups showed partially distinct overall expression patterns

(Fig. 6A). Differential expression analysis using the limma R package identified 810 differentially expressed genes (DEGs), including 603 downregulated and 207 upregulated genes (Fig. 6B).

GO enrichment analysis was then performed to characterize the biological functions of these DEGs (Fig. 6C). GSEA further revealed distinct pathway enrichment patterns between the two risk groups. In the high-risk group, the enriched pathways mainly included arrhythmogenic right ventricular cardiomyopathy, cardiac muscle contraction, dilated cardiomyopathy, focal adhesion, and hypertrophic cardiomyopathy (Fig. 6D). In contrast, the low-risk group was mainly enriched in immune-related pathways, including allograft rejection, asthma, autoimmune thyroid disease, intestinal immune network for IgA production, and primary immunodeficiency (Fig. 6E). These findings indicate distinct biological and immune-related features between the high- and low-risk groups.

Fig. 6 Differentially expressed genes and functional enrichment analysis between the high- and low-risk groups. (A) Principal component analysis (PCA) showing the overall expression distribution of samples in the high- and low-risk groups. (B) Volcano plot of differentially expressed genes between the high- and low-risk groups; a total of 810 differentially expressed genes were identified, including 603 downregulated genes and 207 upregulated genes. (C) GO enrichment analysis of the differentially expressed genes, including biological process (BP), cellular component (CC), and molecular function (MF). (D) GSEA results for the high-risk group. (E) GSEA results for the low-risk group

3.7. Genetic and transcriptional alterations of prognostic MDGs in HNSC

Accumulating evidence suggests that genomic alterations play an important role in tumor progression. The overall mutation landscape of HNSC is shown in Supplementary Fig. 4A and B. Missense mutation was the most common variant classification in the TCGA-HNSC cohort, while single-nucleotide polymorphism (SNP) was the predominant variant type. Among the different single-nucleotide variant classes, C>T substitution occurred most frequently.

Among the 508 patients with available mutation data, 484 (95.28%) harbored at least one somatic mutation, with TP53 showing the highest mutation frequency at 69% (Fig. 7A). We then examined the mutation profiles of the 10 prognostic mitochondrial dysfunction-related genes (MDGs). Only 29 patients (5.71%) carried mutations in these model genes, indicating a relatively low overall mutation frequency of the prognostic MDGs in HNSC (Fig. 7B). Co-occurrence and mutual exclusivity among mutations in the prognostic MDGs were further analyzed (Fig. 7D).

Among the 488 patients with complete risk-group information, 246 were assigned to the high-risk group and 242 to the low-risk group. Somatic mutations were detected in 241 patients (97.97%) in the high-risk group and 225 patients (92.98%) in the low-risk group (Fig. 7F, G). The most frequently mutated genes in the high-risk group included TP53, TTN, CDKN2A, FAT1, and NOTCH1, whereas those in the low-risk group mainly included TP53, TTN, CSMD3, PIK3CA, and CDKN2A. Fisher's exact test was

subsequently used to compare mutation frequencies between the two risk groups, and genes with $P < 0.05$ were considered differentially mutated. Their odds ratios, mutation frequencies, and mutation types are shown in Fig. 7C, E, and H, respectively

Fig. 7 Somatic mutation characteristics of prognostic mitochondrial dysfunction-related genes and differential mutation analysis between the high- and low-risk groups in HNSC. (A) Waterfall plot of somatic mutations in the TCGA-HNSC cohort. (B) Somatic mutation profiles of the 10 prognostic MDGs. (C) Forest plot comparing mutation frequencies between the high- and low-risk groups using Fisher's exact test. (D) Co-occurrence and mutual exclusivity analysis of mutations among the prognostic MDGs. (E) Comparison of mutation frequencies and mutation types of differentially mutated genes between the two risk groups. (F) Somatic mutation waterfall plot of the high-risk group. (G) Somatic mutation waterfall plot of the low-risk group. (H) Distribution of differentially mutated genes between the high- and low-risk groups

3.8. Association between immune cell infiltration and the risk score

Immune cell infiltration plays an important role in tumor progression, treatment response, and prognosis. To examine immune heterogeneity between the two risk groups, we used the CIBERSORT algorithm to estimate the relative proportions of 22 immune cell types based on transcriptomic data. Considerable variation in immune cell composition was observed among individual patients, and the high- and low-risk groups showed distinct immune infiltration patterns (Supplementary Fig. 5A). Correlation analysis further revealed both positive and negative associations among the 22 immune cell populations (Supplementary Fig. 5B). Kaplan–Meier analysis showed that the infiltration levels of eosinophils, activated mast cells, regulatory T cells, and $\gamma\delta$ T cells were significantly associated with overall survival in patients with HNSC (all $P < 0.05$; Supplementary Fig. 5C, D).

As shown in Fig. 8A, several immune cell populations differed significantly between the two risk groups. Compared with the high-risk group, the low-risk group had higher levels of naïve B cells, memory B cells, plasma cells, CD8⁺ T cells, activated memory CD4⁺ T cells, follicular helper T cells, regulatory T cells, resting mast cells, and eosinophils. In contrast, resting memory CD4⁺ T cells, resting NK cells, M0 macrophages, M1 macrophages, and activated mast cells were more abundant in the high-risk group (all $P < 0.05$). Correlation analysis between the 10 prognostic mitochondrial dysfunction-related genes and the 22 immune cell types showed varying degrees of positive and negative associations (Fig. 8B).

Analysis using multiple immune infiltration algorithms from the TIMER2.0 platform also showed distinct immune cell infiltration patterns between the high- and low-risk groups (Fig. 8C). We next evaluated 13 immune-related functions using single-sample gene set enrichment analysis. The low-risk group showed higher scores for chemokine receptor

activity, immune checkpoint activity, cytolytic activity, HLA, inflammation-promoting activity, T-cell co-inhibition, T-cell co-stimulation, and type II interferon response. In contrast, parainflammation and type I interferon response scores were higher in the high-risk group ($P < 0.05$; Fig. 8D). No significant differences were observed in APC co-inhibition, APC co-stimulation, or MHC class I scores between the two groups ($P > 0.05$).

Fig. 8 Immune cell infiltration patterns and immune functional differences between the high- and low-risk groups in HNSC. (A) Comparison of the relative proportions of 22 immune cell types between the high- and low-risk groups based on the CIBERSORT algorithm. (B) Correlation heatmap showing the associations between the 10 prognostic mitochondrial dysfunction-related genes and the infiltration levels of 22 immune cell types. (C) Heatmap of immune cell infiltration characteristics in the high- and low-risk groups evaluated by multiple algorithms based on the TIMER2.0 platform. (D) Comparison of 13 immune-related functional scores between the high- and low-risk groups based on single-sample gene set enrichment analysis (ssGSEA)

3.9. Association of the mitochondrial dysfunction-related prognostic model with the tumor immune microenvironment and immunotherapy response

To further examine the relationship between the mitochondrial dysfunction-related prognostic model, the tumor immune microenvironment, and immunotherapy response in HNSC, we performed a series of immune-related analyses in the high- and low-risk groups. ESTIMATE analysis showed significant differences in Stromal Score, Immune Score, and ESTIMATE Score between the two groups, indicating distinct tumor microenvironmental features across risk groups (Fig. 9A). Several immune checkpoint-related genes were also differentially expressed between the high- and low-risk groups, further suggesting an association between the risk score and tumor immune status (Fig. 9B).

The TIDE algorithm was then used to assess potential immune evasion and response to immunotherapy. Significant differences were observed in TIDE, T-cell Dysfunction, and T-cell Exclusion scores between the two risk groups (Fig. 9C). TIDE predicted a higher proportion of immunotherapy responders in the low-risk group than in the high-risk group (36% vs. 27%, $P = 0.028$; Fig. 9D). In addition, responders had significantly lower risk scores than non-responders ($P = 0.006$; Fig. 9E), suggesting that a lower risk score may be associated with a better response to immunotherapy.

The model was further evaluated in the IMvigor immunotherapy cohort. Kaplan–Meier analysis showed that patients in the low-risk group had better survival outcomes than those in the high-risk group ($P = 0.011$; Fig. 9F). Patients who achieved complete or partial response (CR/PR) also had significantly lower risk scores than those with stable or progressive disease (SD/PD) ($P = 0.013$; Fig. 9G). These findings suggest that the risk

model reflects differences in the tumor immune microenvironment and may also have value in predicting immunotherapy response.

Fig. 9 Tumor immune microenvironment characteristics and immunotherapy response between the high- and low-risk groups. (A) Comparison of Stromal Score, Immune Score, and ESTIMATE Score between the high- and low-risk groups. (B) Differential expression of immune checkpoint-related genes. (C) Comparison of TIDE, Dysfunction, and Exclusion scores. (D) Proportions of predicted immunotherapy responders in the high- and low-risk groups based on TIDE. (E) Comparison of risk scores between responders and non-responders. (F) Kaplan–Meier survival analysis of the high- and low-risk groups in the IMvigor immunotherapy cohort. (G) Comparison of risk scores between patients with CR/PR and those with SD/PD

3.10. Rationale for selecting PPP1R3A and in vitro validation

The 10-gene mitochondrial dysfunction-related prognostic model established in this study consisted of DHRS2, PLAU, ULBP2, HOXB9, CELSR3, FST, TNFRSF4, RBP4, PPP1R3A, and IFI27. Among these genes, DHRS2, PLAU, ULBP2, HOXB9, CELSR3, FST, TNFRSF4, and IFI27 showed an overall tendency toward higher expression, whereas RBP4 and PPP1R3A were downregulated. To further support the bioinformatics findings and assess the experimental reproducibility of a low-expression gene included in the model, PPP1R3A was selected for in vitro validation.

PPP1R3A is closely involved in glycogen metabolism and cellular energy regulation, providing a biological link to the metabolic alterations and mitochondrial dysfunction examined in this study. In addition, compared with several other genes in the signature that have been more extensively studied, the role of PPP1R3A in HNSC remains relatively unexplored. Its selection therefore provided both a representative target from the prognostic model and an opportunity for further investigation.

It should be emphasized that PPP1R3A was selected primarily as a representative downregulated gene for experimental validation of the model. This selection does not imply that PPP1R3A carries the greatest prognostic weight or represents the only functionally important gene within the 10-gene signature.

Fig. 10 Validation of PPP1R3A overexpression and knockdown efficiency in CAL27 and SCC-25 cells. (A) qRT-PCR analysis of PPP1R3A mRNA expression after overexpression. (B) qRT-PCR analysis of PPP1R3A mRNA expression after knockdown. (C, D) Western blot analysis of PPP1R3A protein expression after overexpression and knockdown, with α -tubulin as the loading control. (E, F) Quantification of PPP1R3A protein expression after overexpression in CAL27 and SCC-25 cells. (G, H) Quantification of PPP1R3A protein expression after knockdown in CAL27 and SCC-25 cells. Data are presented as mean $\pm$ SD. ns, not significant; $P < 0.01$, $P < 0.001$, $P < 0.0001$

To verify the efficiency of PPP1R3A manipulation, PPP1R3A overexpression and knockdown models were established in CAL27 and SCC-25 cells and evaluated by qRT-PCR and Western blotting. qRT-PCR showed no significant difference in PPP1R3A mRNA expression between the Control and corresponding negative control groups. In contrast, PPP1R3A expression was significantly reduced in the sh-PPP1R3A group and markedly increased in the OE-PPP1R3A group in both cell lines, confirming successful modulation of PPP1R3A at the transcriptional level (Fig. 10A, B).

Western blotting yielded consistent results. PPP1R3A protein expression was markedly increased after overexpression and decreased after knockdown in both CAL27 and SCC-25 cells (Fig. 10C, D). Densitometric analysis showed the same expression pattern, with significantly increased PPP1R3A protein levels following overexpression and significantly reduced levels following knockdown (Fig. 10E–H). These findings confirmed the successful establishment of PPP1R3A overexpression and knockdown models at both the mRNA and protein levels, providing an experimental basis for further investigation of the biological role of PPP1R3A in HNSC.

4. Discussion

Mitochondria are central to energy metabolism, redox homeostasis, apoptosis, and intracellular signaling, and mitochondrial dysfunction is increasingly recognized as an important feature of metabolic reprogramming in cancer[23,23]. In this study, we systematically identified mitochondrial dysfunction-related prognostic genes in HNSC using TCGA and GEO data and established a 10-gene prognostic signature. The model was further evaluated for its associations with the tumor immune microenvironment, immunotherapy response, and somatic mutation characteristics, and PPP1R3A was selected as a representative gene for experimental validation.

The final signature consisted of DHRS2, PLAU, ULBP2, HOXB9, CELSR3, FST, TNFRSF4, RBP4, PPP1R3A, and IFI27. Among these genes, DHRS2, PLAU, ULBP2, HOXB9, CELSR3, FST, TNFRSF4, and IFI27 showed an overall tendency toward higher expression in HNSC, whereas RBP4 and PPP1R3A were downregulated. The model consistently separated patients into groups with distinct survival outcomes in the training cohort, testing cohort, entire TCGA-HNSC cohort, and the external GSE41613 cohort. The AUC values for predicting 1, 3, and 5-year overall survival ranged from approximately 0.651 to 0.754 across the different cohorts, indicating relatively stable prognostic performance. Multivariate Cox regression analysis further showed that the risk score remained independently associated with overall survival after adjustment for conventional clinicopathological variables. These results suggest that the mitochondrial dysfunction-related signature may provide prognostic information complementary to traditional clinical factors[24].

Marked differences in the immune landscape were observed between the two risk groups. The low-risk group showed higher infiltration of several adaptive immune cell populations,

including CD8⁺ T cells, activated memory CD4⁺ T cells, naïve and memory B cells, and plasma cells. In contrast, resting memory CD4⁺ T cells, resting NK cells, M0 macrophages, M1 macrophages, and activated mast cells were more abundant in the high-risk group. Differences were also observed in several immune-related functional scores. In particular, the low-risk group showed higher cytolytic activity, HLA activity, T-cell co-stimulation, and type II interferon response. Together, these findings indicate that the two risk groups have distinct immune microenvironmental states rather than simply differing in the overall amount of immune infiltration[25]. Differential expression of multiple immune checkpoint genes further supports an association between the mitochondrial dysfunction-related signature and tumor immune status[26,27].

The relationship between the risk score and immunotherapy response was further examined using TIDE and an independent immunotherapy cohort. TIDE analysis showed differences in predicted immune dysfunction, exclusion, and treatment response between the two risk groups. The predicted responder rate was higher in the low-risk group than in the high-risk group (36% vs. 27%, $P = 0.028$), and predicted responders had significantly lower risk scores than non-responders ($P = 0.006$). Similar findings were obtained in the independent immunotherapy cohort, in which low-risk patients had better survival outcomes ($P = 0.011$), while patients achieving complete or partial response had lower risk scores than those with stable or progressive disease ($P = 0.013$). These results suggest that the risk signature may contain information relevant to immunotherapy response in addition to its prognostic value. However, these findings should be interpreted cautiously because TIDE is based on computational prediction and the independent immunotherapy analysis does not directly establish a treatment-predictive role for the signature. Further validation in HNSC cohorts receiving immune checkpoint inhibitors will therefore be necessary.

The observed immune differences may also be related to alterations in mitochondrial metabolism[27,28]. Changes in mitochondrial function can influence reactive oxygen species production, cellular energy availability, and metabolite levels, which in turn may affect both tumor cells and surrounding immune cells[23,29–31]. Consistent with this possibility, functional enrichment analyses in the present study revealed distinct biological patterns between the two risk groups, while immune-related pathways were particularly enriched in the low-risk group. These observations suggest a possible link between mitochondrial dysfunction and remodeling of the HNSC immune microenvironment, although the underlying causal relationships remain to be established experimentally.

Differences in somatic mutation patterns were also observed between the two risk groups. TP53 was the most frequently mutated gene in the TCGA-HNSC cohort, while other commonly altered genes, including CDKN2A, NOTCH1, FAT1, PIK3CA, and CSMD3, showed different mutation distributions across risk groups. Only a small proportion of

patients carried mutations in the 10 genes included in the prognostic signature, suggesting that the prognostic information captured by the model is more likely to reflect differences in gene expression and associated biological states than recurrent mutations within the model genes themselves. Taken together, the genomic and transcriptomic findings indicate that the mitochondrial dysfunction-related signature may reflect multiple aspects of HNSC biology, including metabolic state, immune composition, and genomic alterations[26].

Among the 10 genes in the signature, PPP1R3A was selected for further experimental validation. This choice was not based on its having the largest regression coefficient or the greatest contribution to the prognostic score. Rather, PPP1R3A was selected because of its expression pattern, biological function, and relatively limited characterization in HNSC. PPP1R3A and RBP4 were the two genes showing a downregulated pattern in the model, making PPP1R3A a representative low-expression candidate. In addition, PPP1R3A is involved in the regulation of glycogen synthesis and glycogen metabolism, processes closely linked to cellular energy homeostasis and metabolic adaptation[32]. Glycogen can serve as an alternative energy reserve under metabolic stress, including hypoxia and nutrient limitation[33]. It is therefore reasonable to speculate that altered PPP1R3A expression may be associated with metabolic adaptation in HNSC. Nevertheless, the precise relationship among PPP1R3A, glycogen metabolism, mitochondrial function, and HNSC progression remains unclear and requires direct mechanistic investigation.

In CAL27 and SCC-25 cells, qRT-PCR and Western blotting confirmed successful modulation of PPP1R3A at both the mRNA and protein levels. PPP1R3A expression increased after overexpression and decreased following knockdown in both cell lines. These experiments demonstrate that PPP1R3A can be effectively manipulated in HNSC cells and provide a basis for subsequent functional studies. Importantly, the current experiments were designed to verify intervention efficiency rather than establish a tumor-promoting or tumor-suppressive role for PPP1R3A. Future studies should examine cell proliferation, migration, invasion, and other tumor-related phenotypes and incorporate measurements of glycogen content, ATP production, reactive oxygen species, mitochondrial membrane potential, and related metabolic parameters. Such experiments will be needed to determine whether PPP1R3A influences HNSC progression through glycogen metabolism or mitochondrial metabolic reprogramming.

Several limitations of this study should be acknowledged. First, the prognostic signature was developed mainly from retrospective public datasets, and its clinical applicability requires validation in larger, multicenter cohorts. Second, immune infiltration and immunotherapy response were largely evaluated using transcriptome-based computational approaches. Although an independent immunotherapy cohort provided additional support, prospective validation in patients with HNSC receiving immune checkpoint inhibitors is still required. Third, the current experimental validation of

PPP1R3A was limited to confirmation of overexpression and knockdown efficiency. Functional and mechanistic experiments, together with validation in clinical tissues and in vivo models, will be necessary to clarify the role of PPP1R3A and other genes included in the signature. Finally, although the model showed consistent prognostic performance across different cohorts, its predictive ability remains moderate, and further optimization and comparison with established clinical prognostic models will be required before clinical application.

Overall, we developed and validated a 10-gene mitochondrial dysfunction-related prognostic signature for HNSC that was associated with survival, immune microenvironment characteristics, immunotherapy response, and somatic mutation patterns. PPP1R3A was further selected as a representative candidate based on its expression pattern, metabolic relevance, and limited previous investigation in HNSC, and its overexpression and knockdown were successfully established in CAL27 and SCC-25 cells. These findings provide a framework for further investigating the relationship between mitochondrial dysfunction, tumor immunity, and clinical outcome in HNSC.

5. Conclusion

In this study, we developed and validated a 10-gene mitochondrial dysfunction-related prognostic signature for HNSC based on TCGA and GEO datasets. The signature, comprising DHRS2, PLAUI, ULBP2, HOXB9, CELSR3, FST, TNFRSF4, RBP4, PPP1R3A, and IFI27, showed consistent risk-stratification ability, with poorer overall survival observed in the high-risk group. The risk score also retained independent prognostic value. Further analyses revealed distinct differences between the two risk groups in immune cell infiltration, tumor microenvironment characteristics, immune checkpoint expression, immunotherapy response, and somatic mutation patterns, suggesting that the model may provide additional information relevant to immunotherapy benefit. Based on its expression pattern, relative novelty, and biological link to glycogen metabolism, PPP1R3A was selected for in vitro validation. Successful overexpression and knockdown were confirmed in CAL27 and SCC-25 cells by qRT-PCR and Western blotting. Overall, this mitochondrial dysfunction-related signature may provide a useful basis for prognostic assessment and risk stratification in HNSC and support further investigation of PPP1R3A and glycogen metabolism in HNSC biology.

Supplementary Materials

Supplementary Fig. S1 presents the functional enrichment, expression patterns, and mutation characteristics of the 98 prognostic mitochondrial dysfunction-related genes (MDGs). Supplementary Fig. S2 shows Kaplan–Meier survival analyses of the 10 genes included in the prognostic signature. Supplementary Fig. S3 presents subgroup survival analyses of the prognostic model according to different clinicopathological

characteristics. Supplementary Fig. S4 summarizes the somatic mutation landscape and single-nucleotide variant characteristics of the TCGA-HNSC cohort. Supplementary Fig. S5 shows immune cell infiltration patterns and their associations with overall survival in patients with HNSC.

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Author contributions

Zhenyu Chen/ Jiangpeng Pu: Study design, data analysis, manuscript writing.

Jingjie Zhang/ Junlong Xue: Literature review, manuscript preparation.

Shaoqin Tu/ Haitao Wang: Supervision, study conceptualization, manuscript revision.

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Ethics

This study involved secondary analyses of de-identified data obtained from established repositories and previously published datasets, including TCGA-HNSC, GSE41613, and the IMvigor210 immunotherapy cohort, together with in vitro experiments using established human cancer cell lines. No new human participants, human tissue specimens, or experimental animals were recruited or collected for the present study. Ethical approval and informed consent for the original human studies were obtained by the original investigators as applicable. Therefore, no additional institutional ethics approval or informed consent was required for this secondary analysis and in vitro study.

Data availability

The datasets and public resources analyzed in this study are available from their respective repositories. TCGA-HNSC transcriptomic, clinical, and somatic mutation data are available through the NCI Genomic Data Commons under project identifier TCGA-HNSC (<https://portal.gdc.cancer.gov/projects/TCGA-HNSC>). The independent external validation dataset is available from NCBI GEO under accession GSE41613 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE41613>). Mitochondrial

dysfunction-related genes were retrieved from GeneCards using the exact query term “mitochondrial dysfunction” (<https://www.genecards.org/Search/Keyword?queryString=mitochondrial%20dysfunction>). Gene sets used for GSEA and GSEA were obtained from MSigDB: c2.cp.kegg.v7.5.symbols.gmt (<https://data.broadinstitute.org/gsea-msigdb/msigdb/release/7.5/c2.cp.kegg.v7.5.symbols.gmt>), c5.go.bp.v7.5.symbols.gmt (<https://data.broadinstitute.org/gsea-msigdb/msigdb/release/7.5/c5.go.bp.v7.5.symbols.gmt>), and h.all.v7.2.symbols.gmt (<https://data.broadinstitute.org/gsea-msigdb/msigdb/release/7.2/h.all.v7.2.symbols.gmt>). Immunotherapy-response estimates were generated using TIDE (<http://tide.dfci.harvard.edu/>), and complementary immune-infiltration estimates were obtained from TIMER2.0 (<http://timer.cistrome.org/>) using the TCGA-HNSC cohort. The independent IMvigor210 immunotherapy dataset is available through the IMvigor210CoreBiologies resource (<http://research-pub.gene.com/IMvigor210CoreBiologies/>), and the corresponding molecular data are archived in the European Genome-Phenome Archive under study accession EGAS00001002556 (<https://ega-archive.org/studies/EGAS00001002556>). Data generated from the in vitro experiments are included in the article and Supplementary Materials where applicable; additional raw experimental data are available from the corresponding authors upon reasonable request.

Competing interests

The authors declare no competing interests.

Declarations

Consent to Participate declaration: not applicable.

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Data Sources

TCGA-HNSC cohort
522 tumor tissues vs.
44 normal tissues

Differentially expressed genes
(DEGs)

HNSC patients with survival information
(n = 510)
Training cohort: n = 357
Testing cohort: n = 153
(7:3 split)

Log-rank analysis

Cox regression analysis

GSE41613
External validation cohort

GeneCards
Mitochondrial dysfunction-related genes
(MDGs)
n = 8,957

MDGs

98 prognostic mitochondrial dysfunction-related DEGs
(98 mdg-DEGs)

Univariate Cox regression analysis
52 candidate genes

LASSO regression analysis
14 genes

Stepwise regression analysis (direction = both)
and multivariate Cox regression analysis

10-gene MDG prognostic signature

Prognostic model construction

Risk score calculation, and patients were divided into
high- and low-risk groups according to the median risk score

Validation Cohorts

Training cohort

Testing cohort

Entire TCGA-HNSC cohort

GSE41613 external cohort

Downstream Analyses

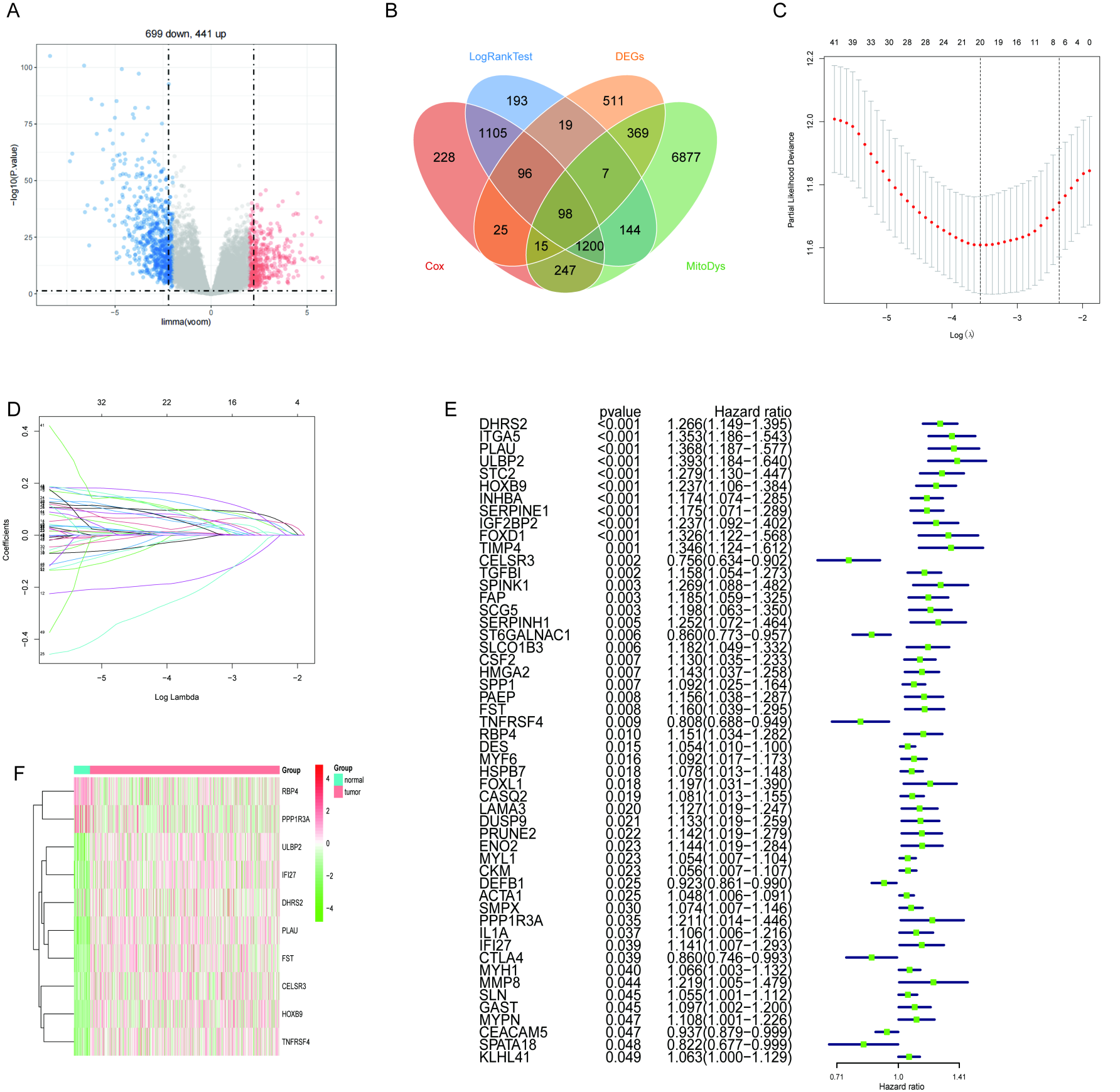
Association between
risk score and
clinicopathological
features

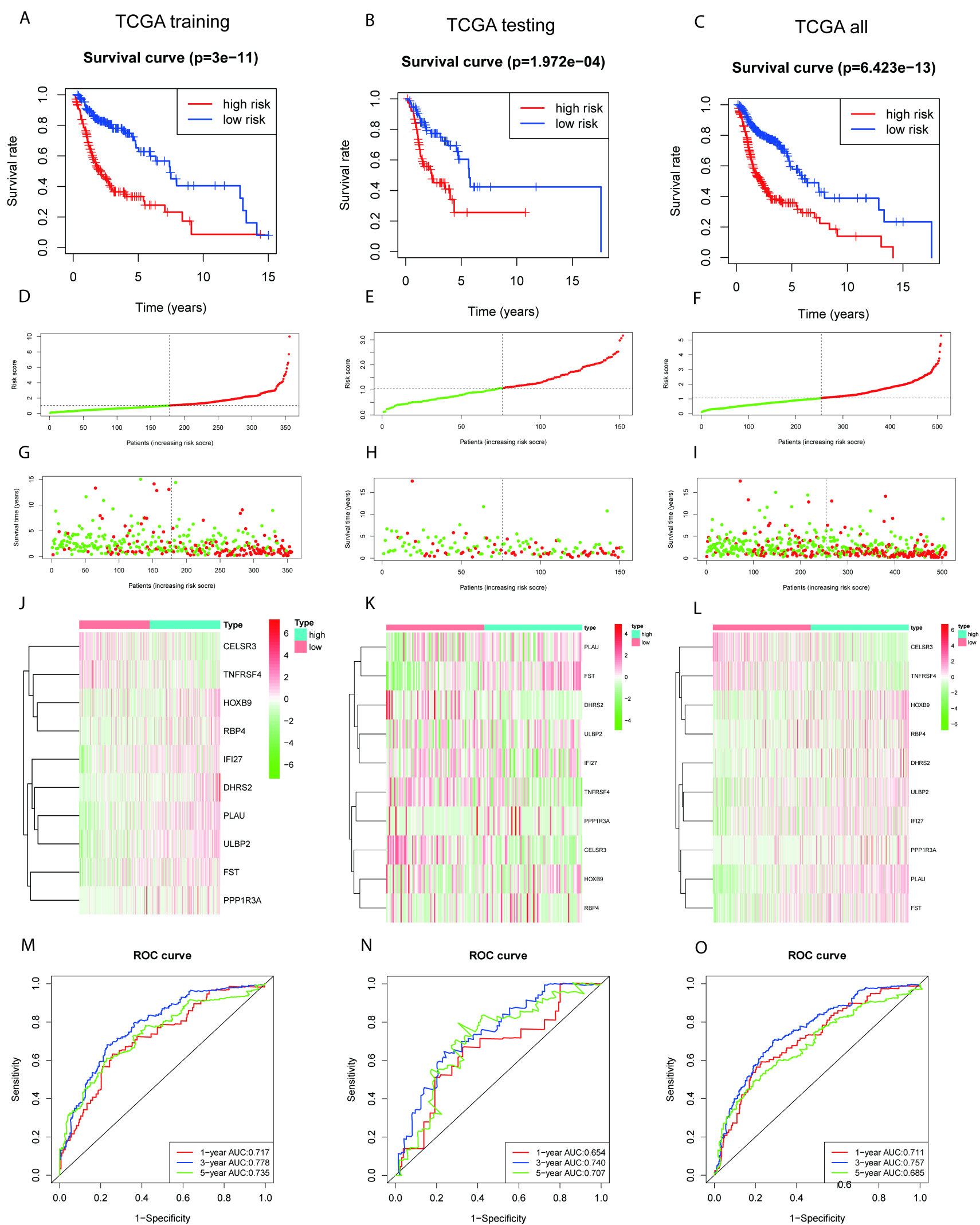
Biological differences between
high- and low-risk groups

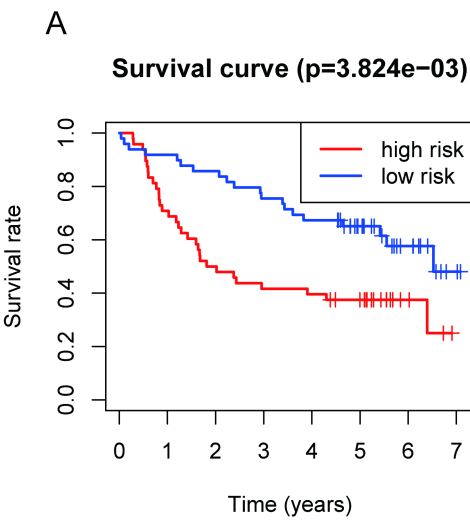
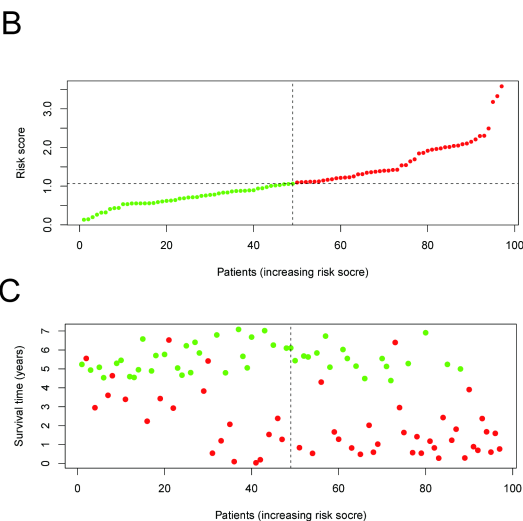
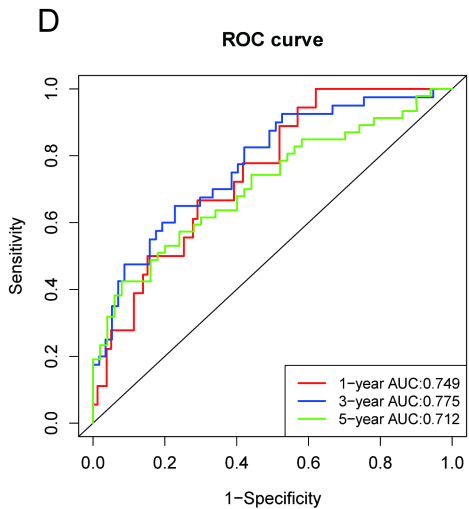
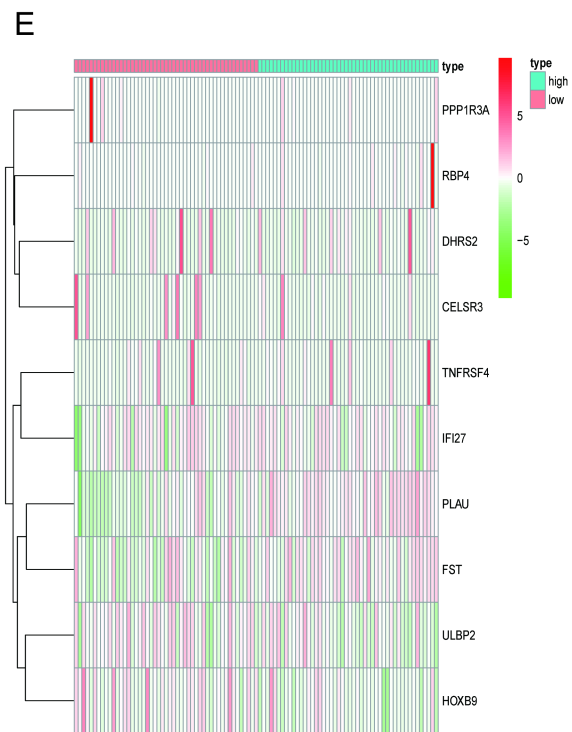
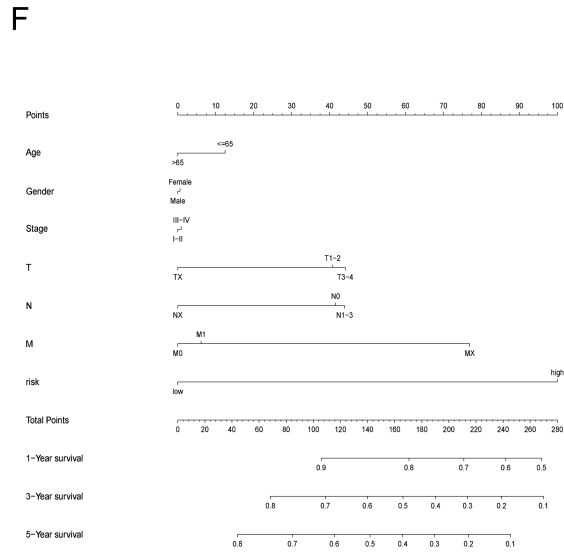
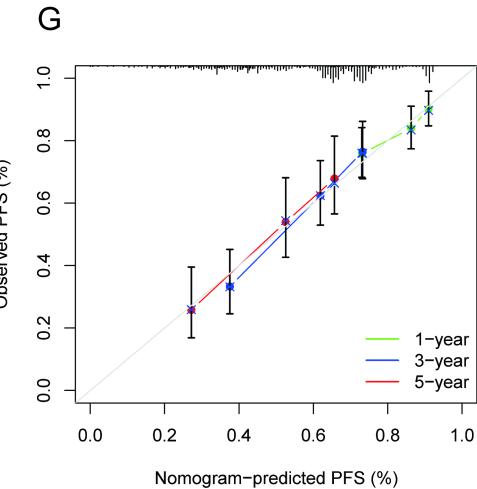
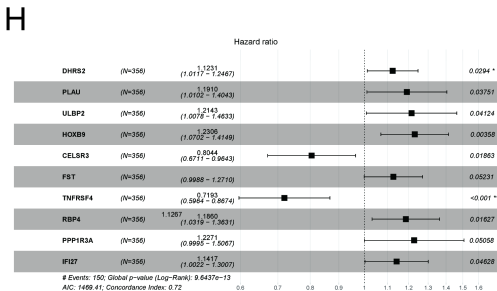
Mutation landscape of 10 MDGs
in HNSC

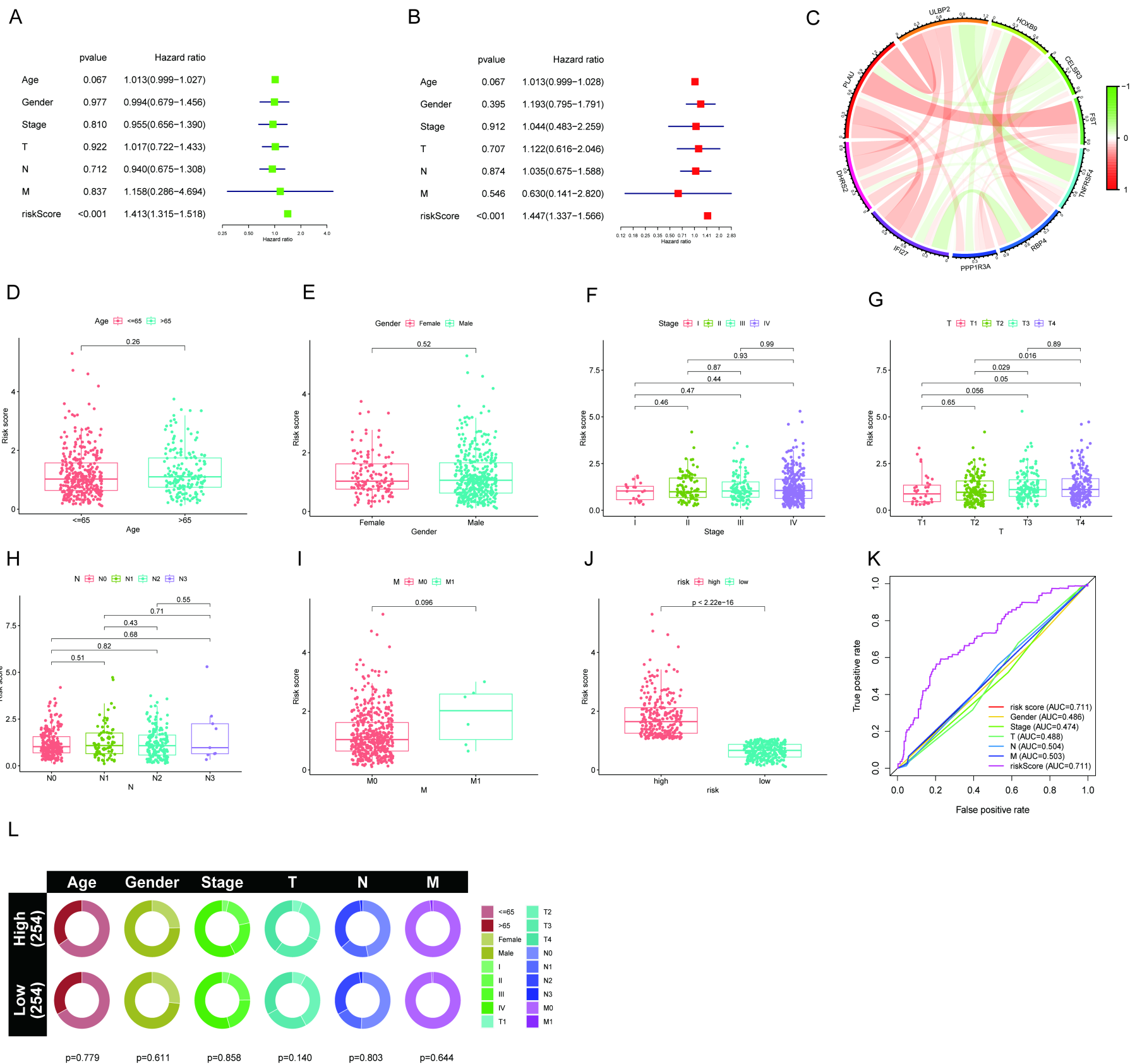
Predictive performance evaluation
(ROC curves)

Immune infiltration and
tumor microenvironment
analysis

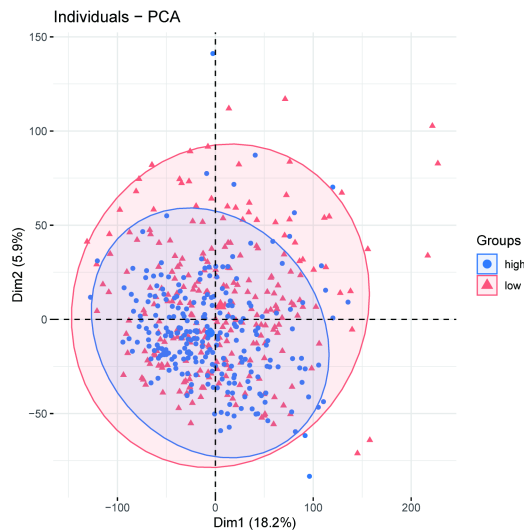




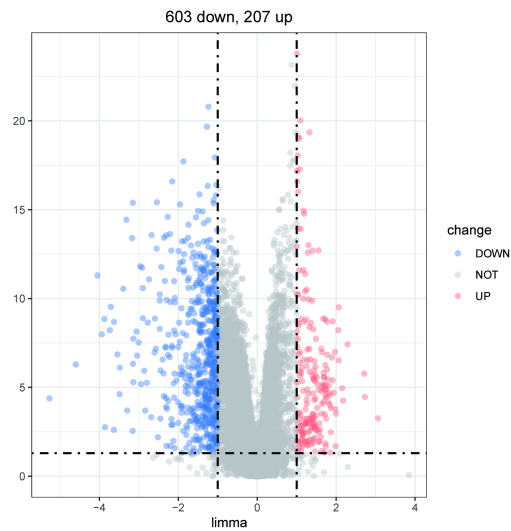




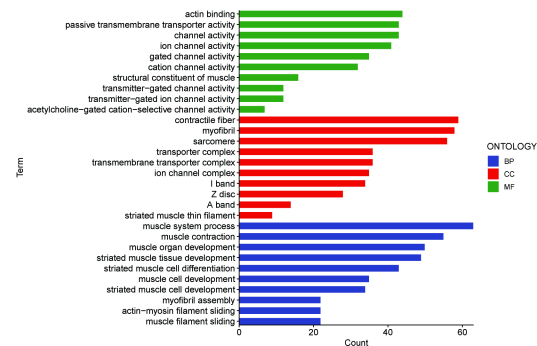
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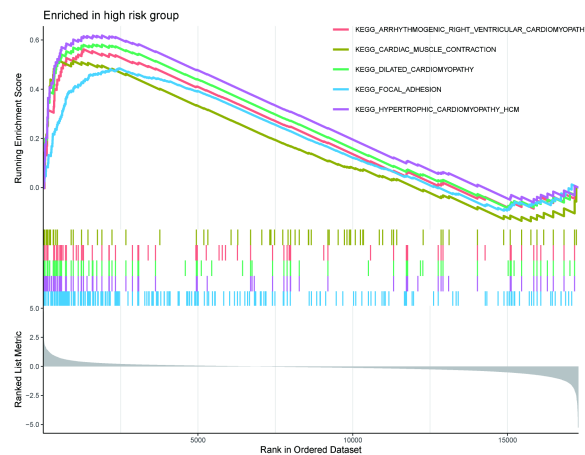
B



C

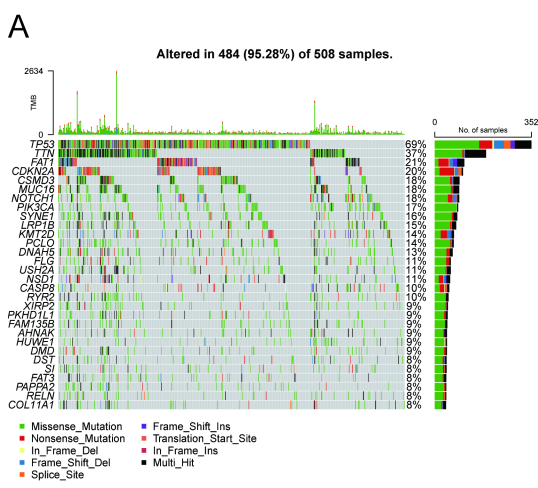
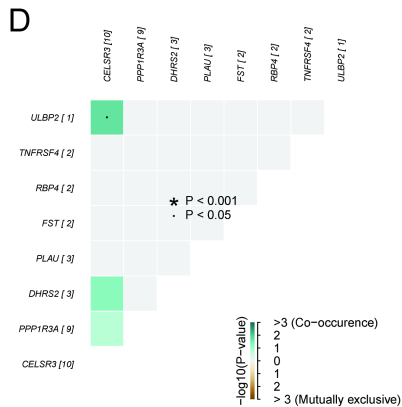
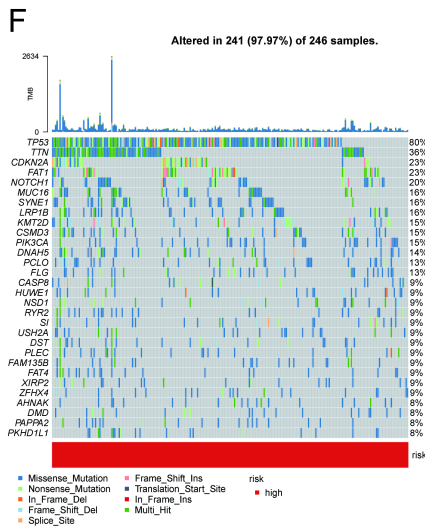
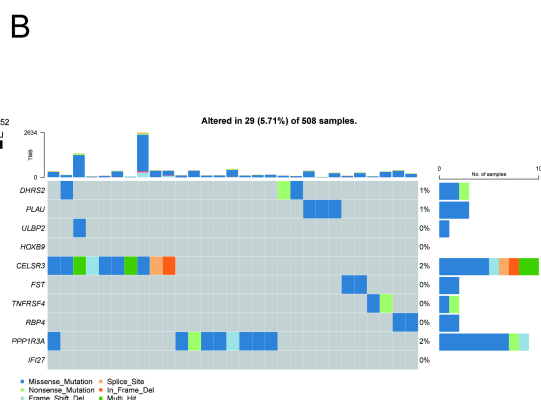
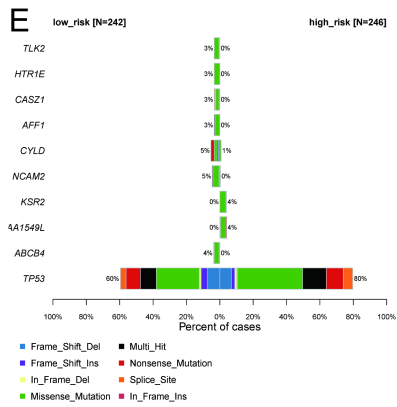
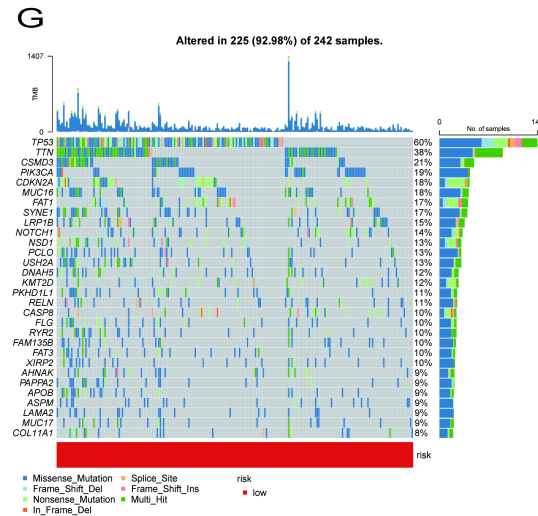
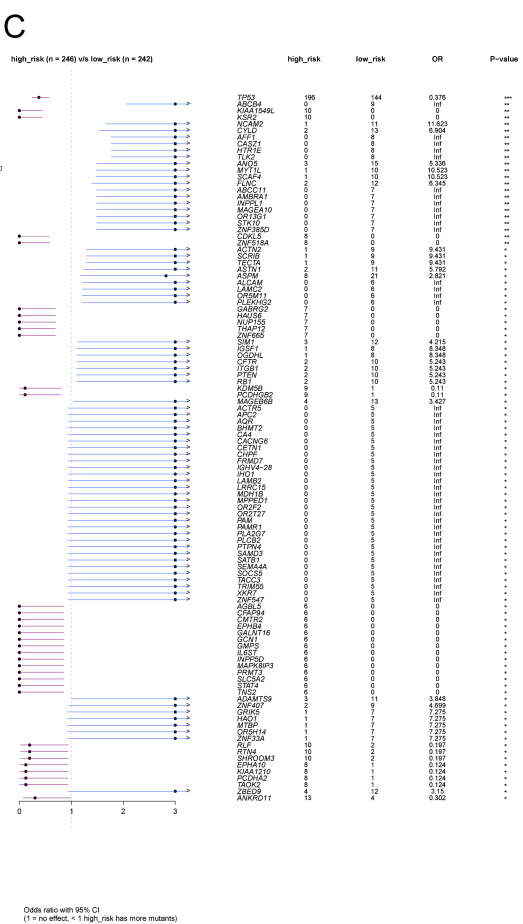


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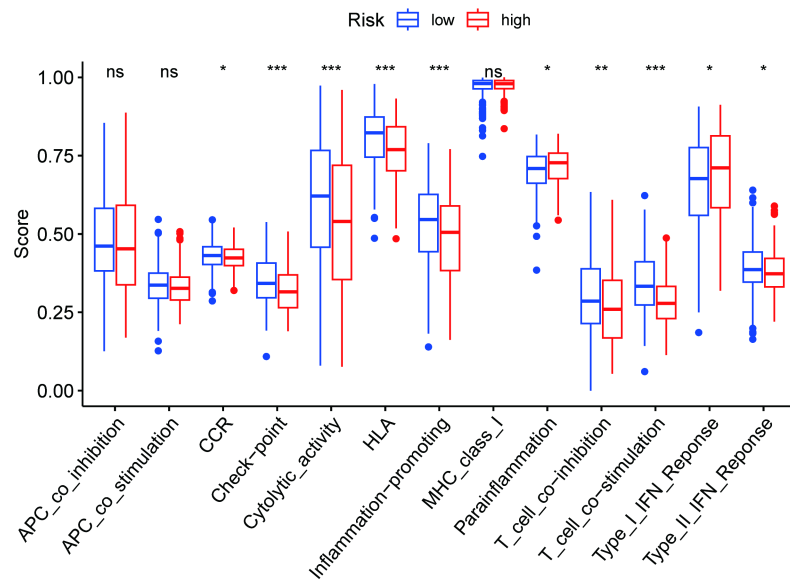


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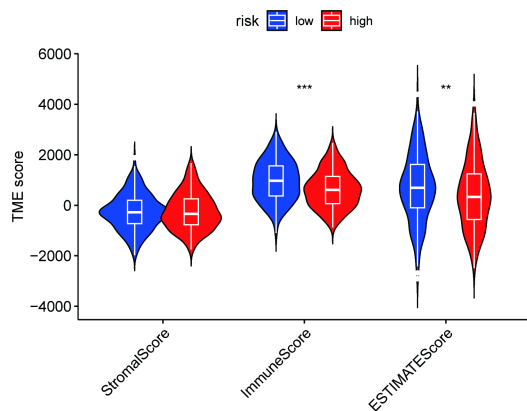




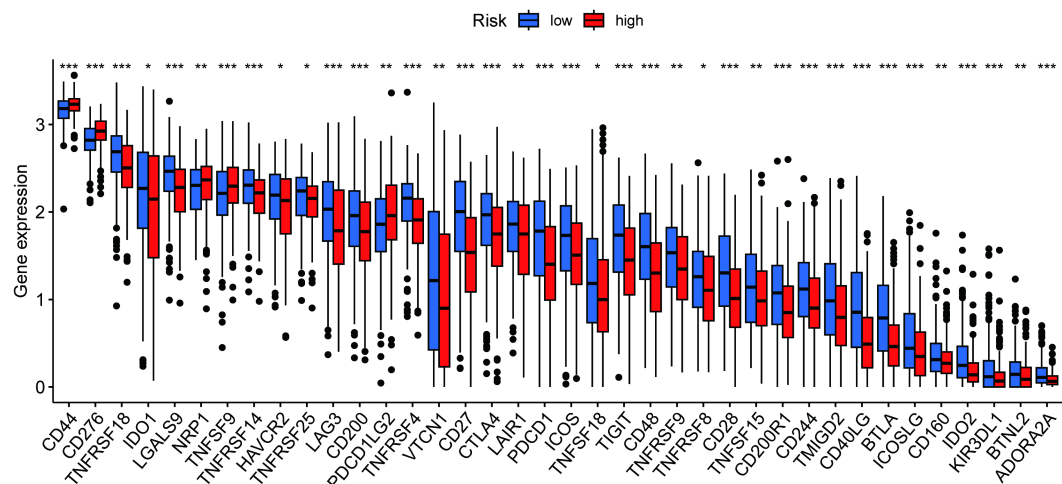
Method



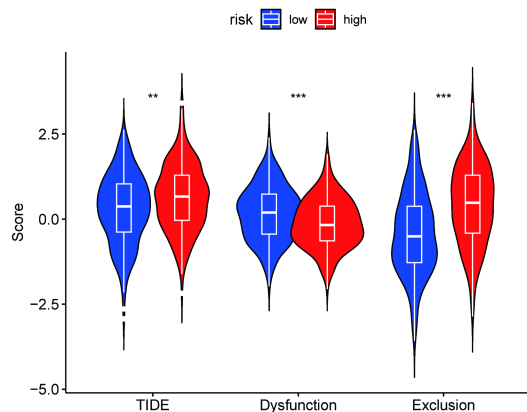
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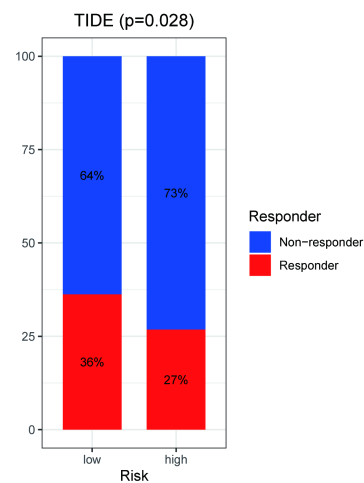
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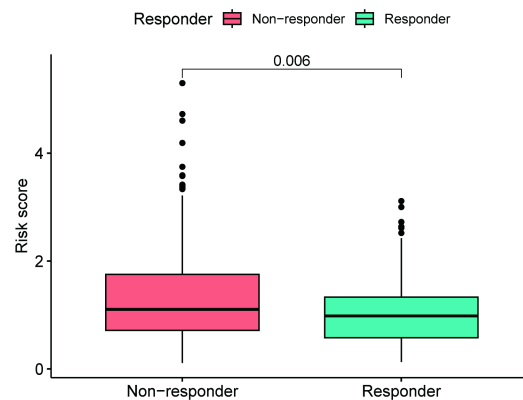
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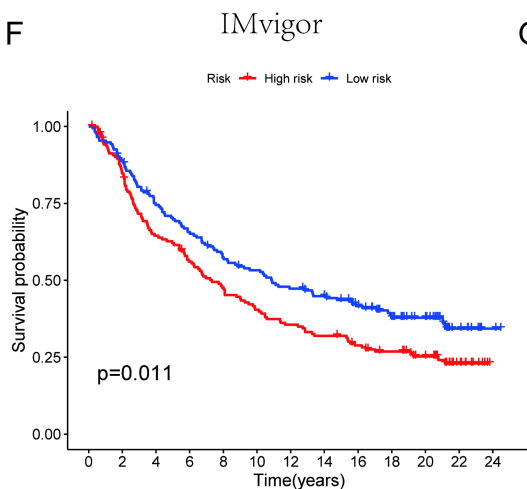
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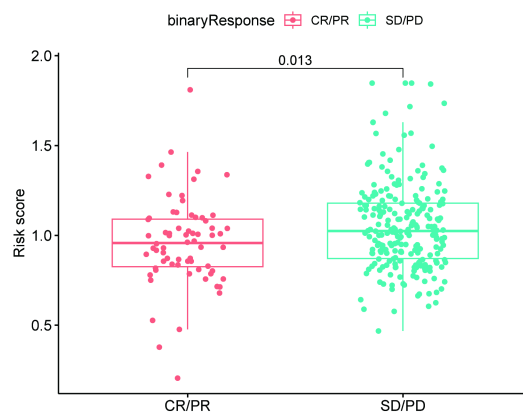
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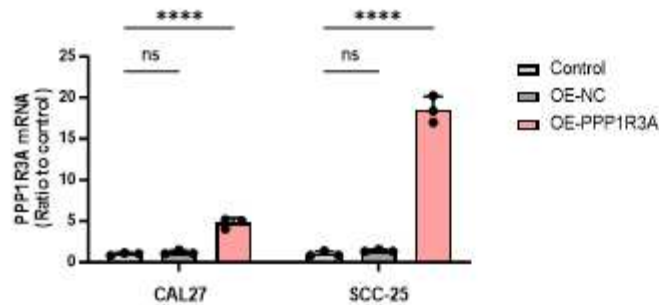
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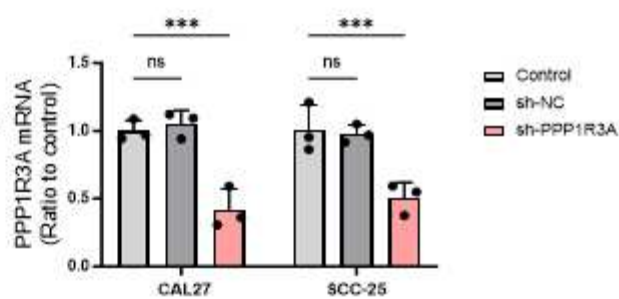
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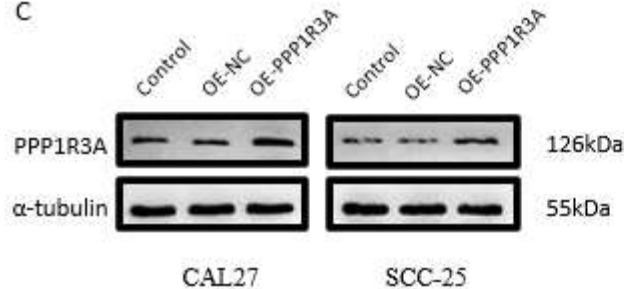
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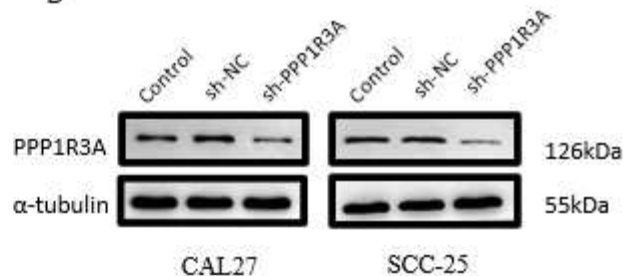
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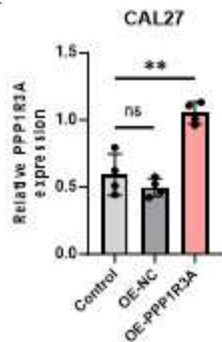
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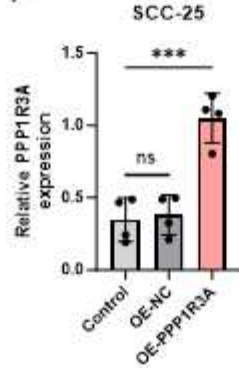
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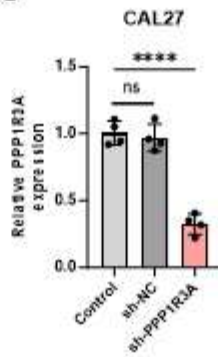
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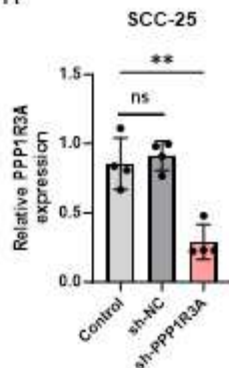
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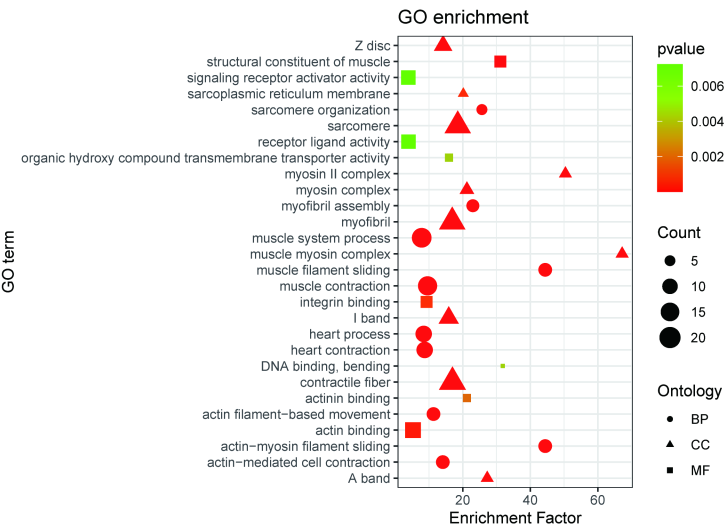
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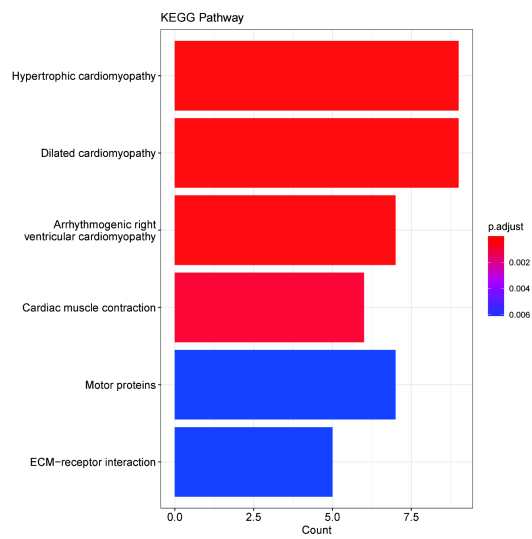
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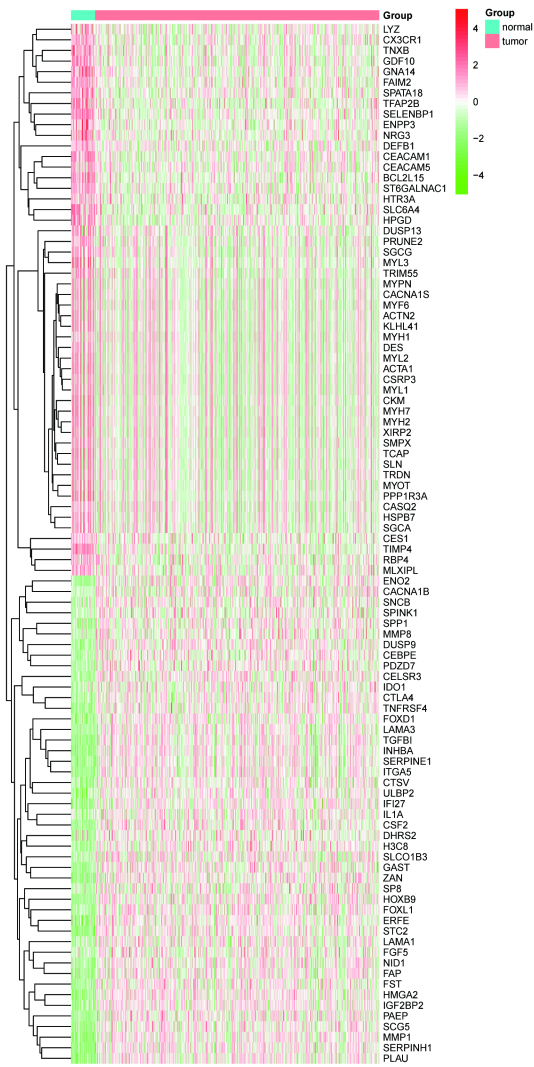
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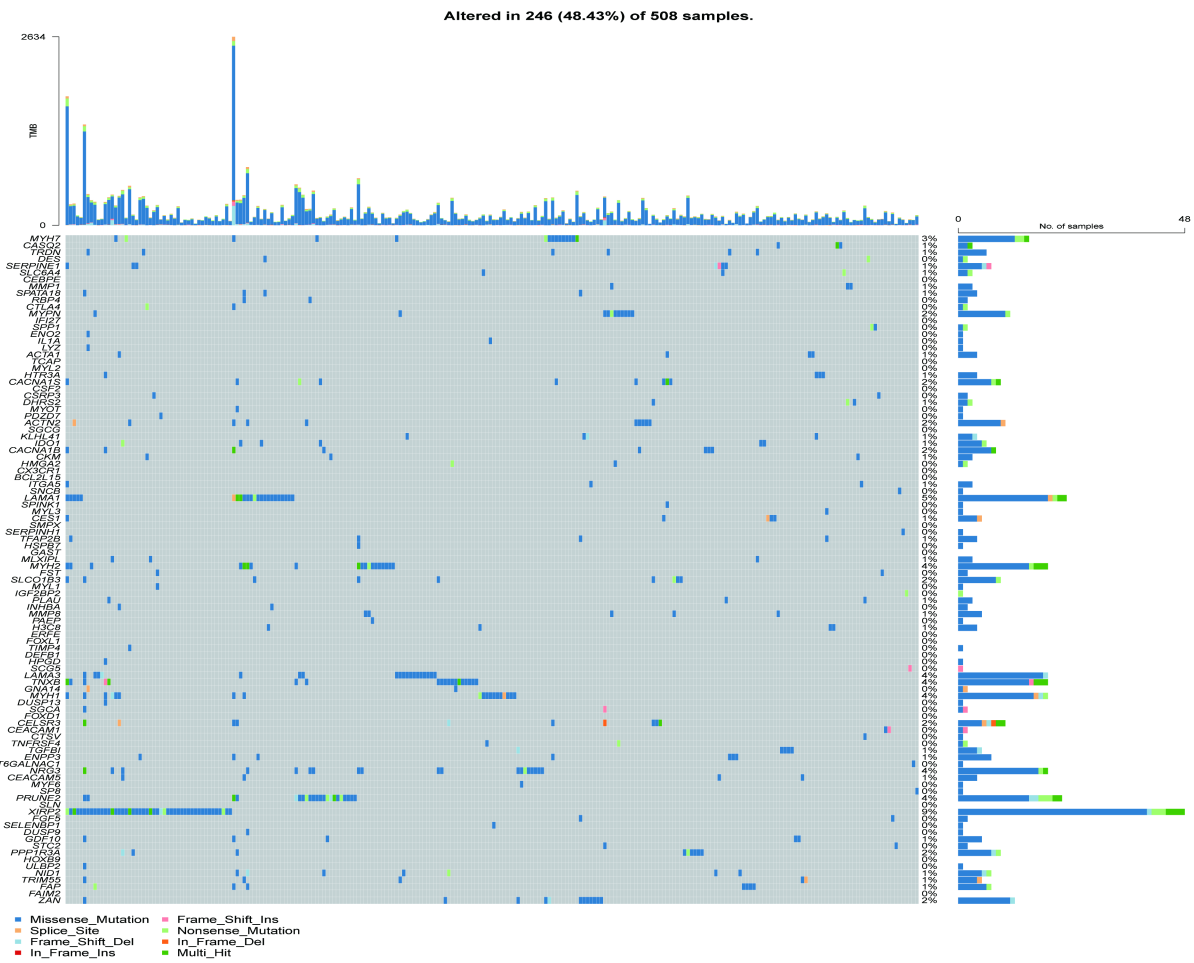
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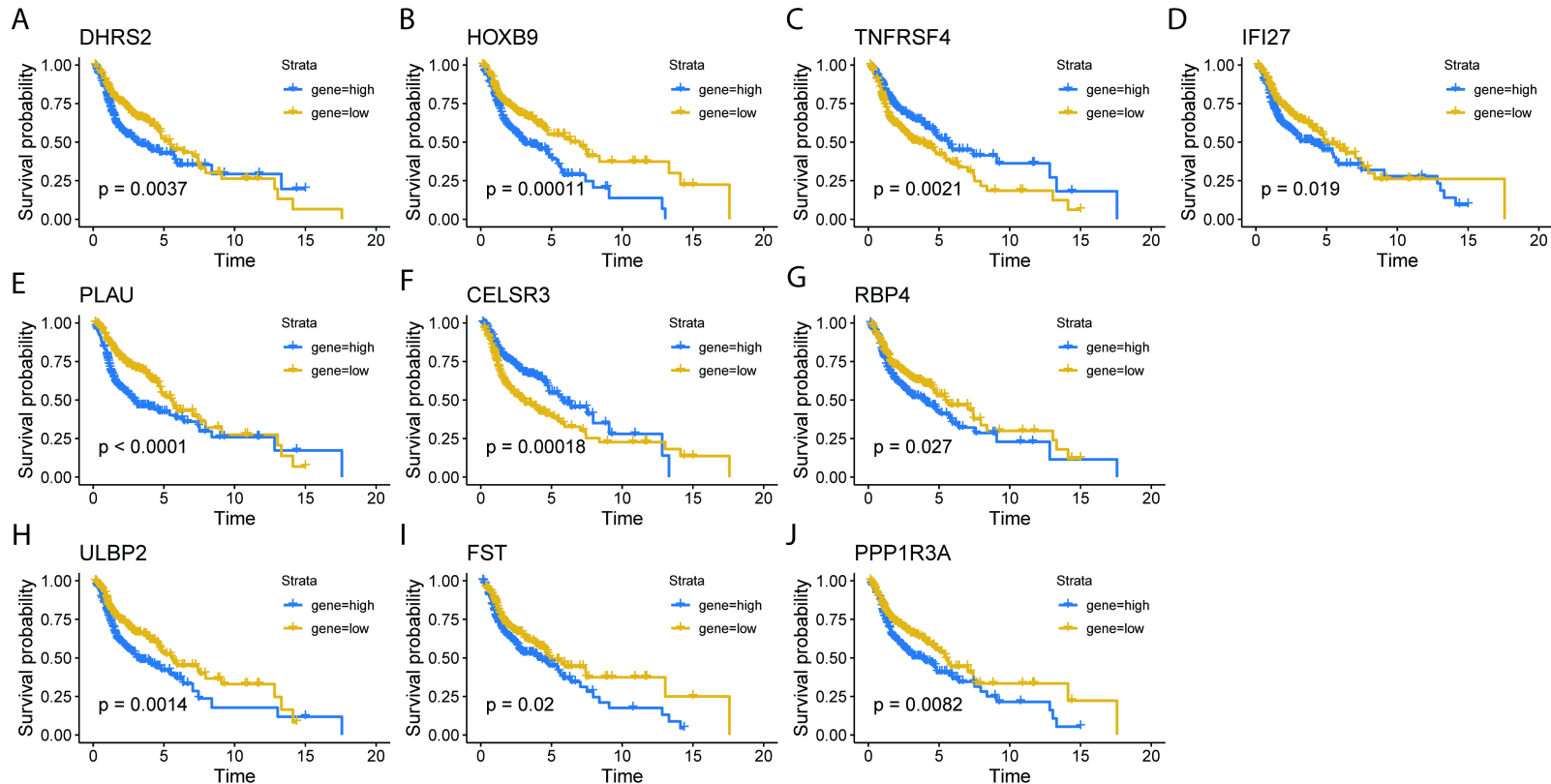


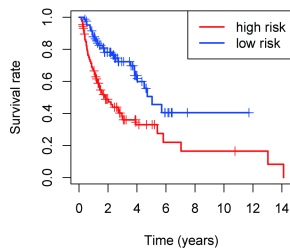
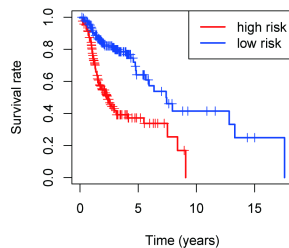
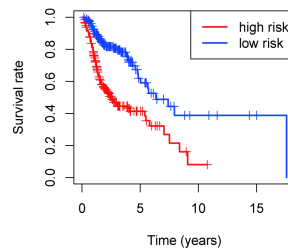
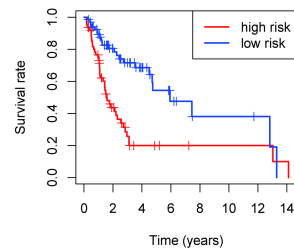
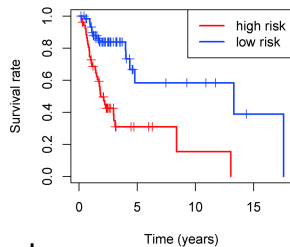
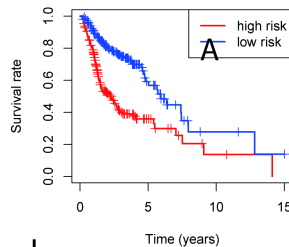
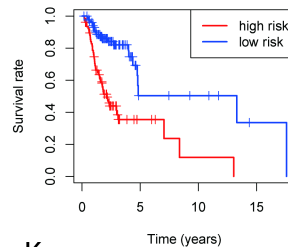
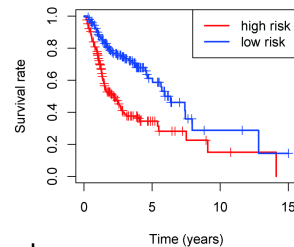
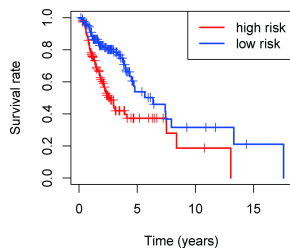
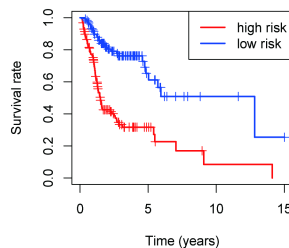
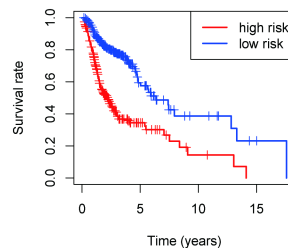
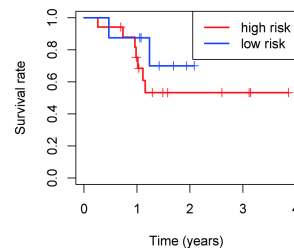
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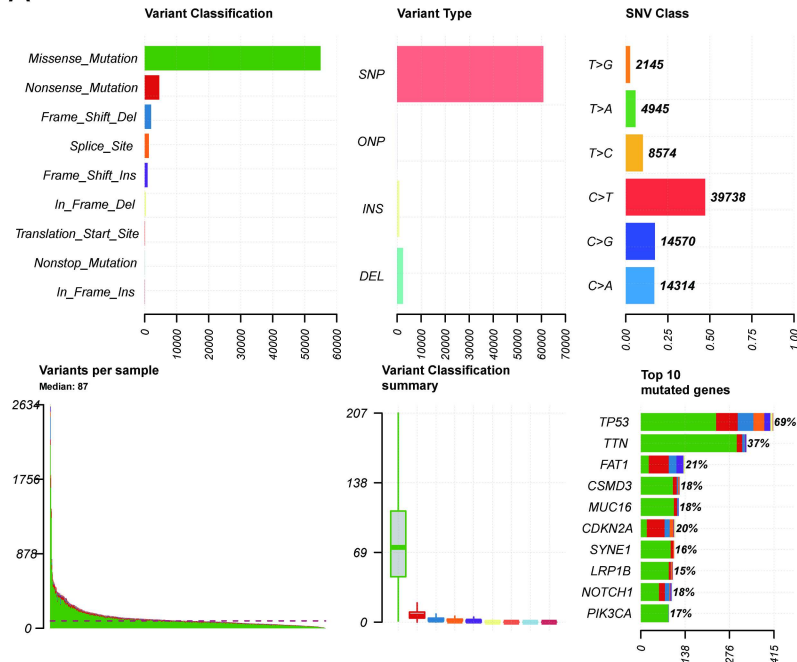
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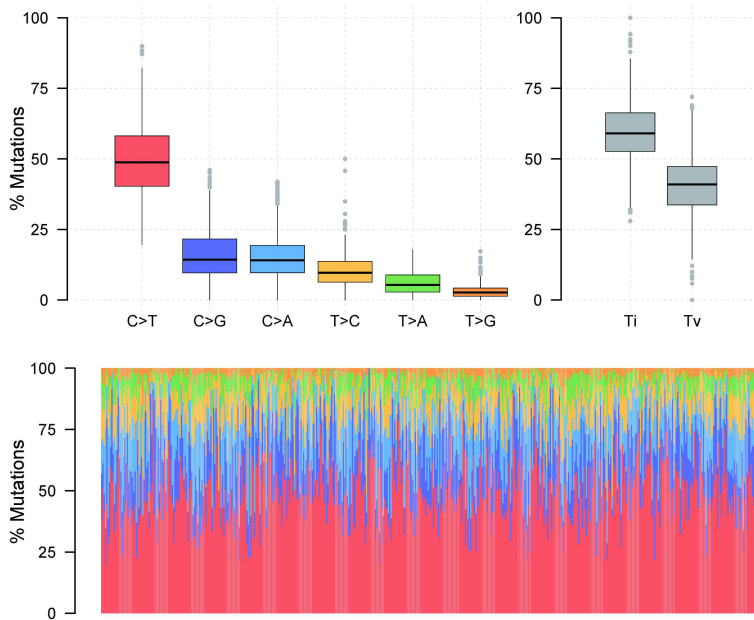


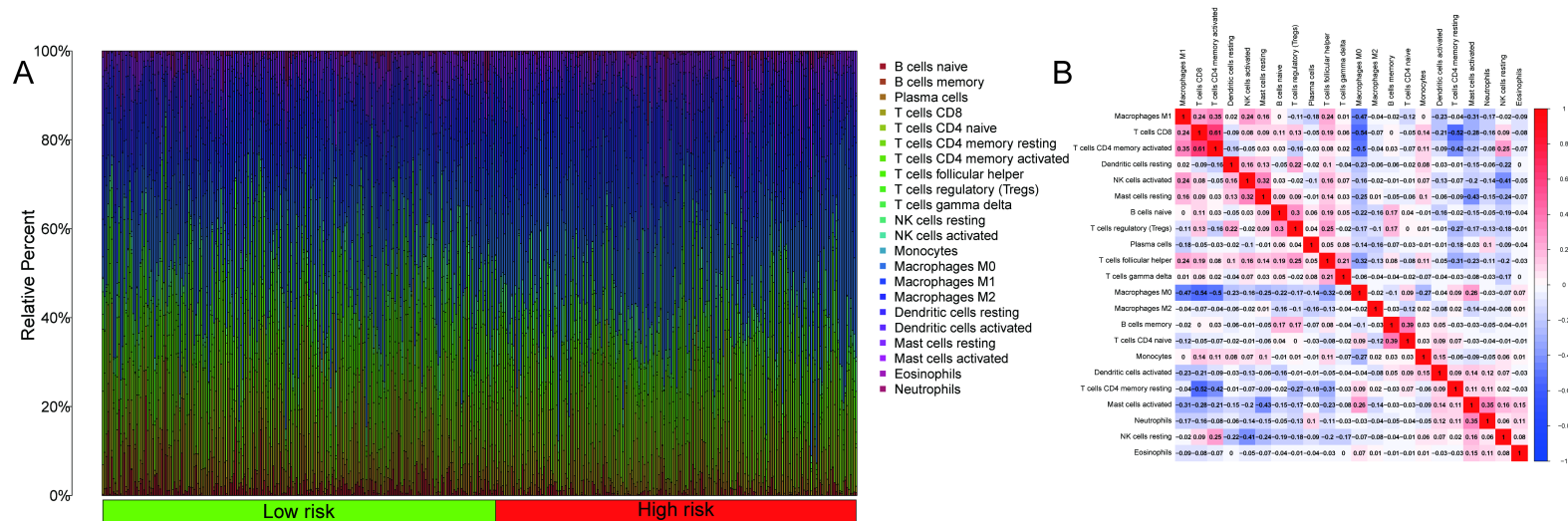
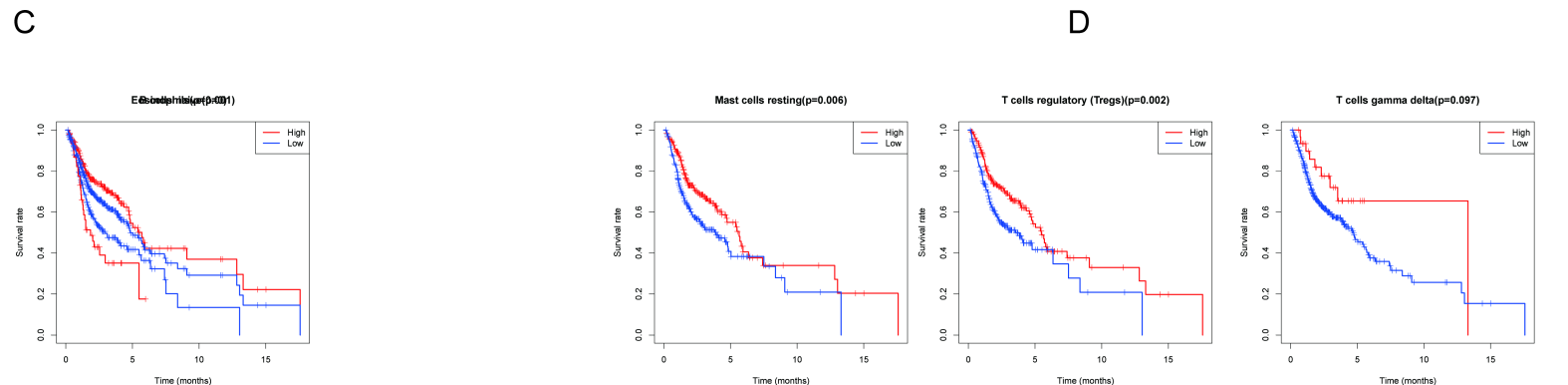
A**Age > 65 (p=8.731e-05)****B****Age <= 65 (p=1.491e-09)****C****Male (p=6.592e-09)****D****Female (p=4.157e-05)****E****Stage I-II (p=5.828e-06)****F****Stage III-IV (p=1.427e-08)****G****T stage 1-2 (p=1.565e-07)****H****T stage 3-4 (p=7.268e-07)****I****N stage 0 (p=1.009e-04)****J****N stage 1-3 (p=1.308e-10)****K****M stage 0 (p=5.651e-13)****L****M stage 1-X (p=4.015e-01)**

A



B





Supplementary Files

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- [Supplementarymaterial.docx](#)
- [Wb.pptx](#)