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Integrated machine learning reveals the role of tryptophan metabolism in clear cell renal cell carcinoma and its association with patient prognosis

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Abstract

Background Precision oncology's implementation in clinical practice faces significant constraints due to the inadequacies in tools for detailed patient stratification and personalized treatment methodologies. Dysregulated tryptophan metabolism has emerged as a crucial factor in tumor progression, encompassing immune suppression, proliferation, metastasis, and metabolic reprogramming. However, its precise role in clear cell renal cell carcinoma (ccRCC) remains unclear, and predictive models or signatures based on tryptophan metabolism are conspicuously lacking.

Methods The influence of tryptophan metabolism on tumor cells was explored using single-cell RNA sequencing data. Genes involved in tryptophan metabolism were identified across both single-cell and bulk-cell dimensions through weighted gene co-expression network analysis (WGCNA) and its single-cell data variant (hdWGCNA). Subsequently, a tryptophan metabolism-related signature was developed using an integrated machine-learning approach. This signature was then examined in multi-omics data to assess its associations with patient clinical features, prognosis, cancer malignancy-related pathways, immune microenvironment, genomic characteristics, and responses to immunotherapy and targeted therapy. Finally, the genes within the signature were validated through experiments including qRT-PCR, Western blot, CCK8 assay, and transwell assay.

Results Dysregulated tryptophan metabolism was identified as a potential driver of the malignant transformation of normal epithelial cells. The tryptophan metabolism-related signature (TMRS) demonstrated robust predictive

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capability for overall survival (OS) and progression-free survival (PFS) across multiple datasets. Moreover, a high TMRS risk score correlated with increased tumor malignancy, significant metabolic reprogramming, an inflamed yet dysfunctional immune microenvironment, heightened genomic instability, resistance to immunotherapy, and increased sensitivity to certain targeted therapeutics. Experimental validation revealed differential expression of genes within the signature between RCC and adjacent normal tissues, with reduced expression of DDAH1 linked to enhanced proliferation and metastasis of tumor cells.

Conclusion This study investigated the potential impact of dysregulated tryptophan metabolism on clear cell renal cell carcinoma, leading to the development of a tryptophan metabolism-related signature that may provide insights into patient prognosis, tumor biological status, and personalized treatment strategies. This signature serves as a valuable reference for further exploring the role of tryptophan metabolism in renal cell carcinoma and for the development of clinical applications based on this metabolic pathway.

Introduction

Renal cell carcinoma (RCC) is the most prevalent form of kidney cancer, ranking as the sixth and tenth most common cancer among males and females, respectively [1]. Originating from the renal tubular epithelium, RCC encompasses several subtypes, including clear cell RCC (ccRCC, approximately 70% of cases), papillary RCC (pRCC, 10–15%), chromophobe RCC (5%), and other rare histological variants [2]. Significant advancements in early detection and therapeutic strategies have markedly improved the identification and management of localized RCC, substantially enhancing long-term survival outcomes [3]. Nonetheless, 17% of patients present with distant metastasis at initial diagnosis, and an additional 30% of cases initially confined to the kidney eventually progress to metastatic disease [3, 4]. For these patients, the prognosis is unfortunately poor [3, 4]. Furthermore, substantial intertumoral and intratumoral heterogeneity contributes to markedly varied responses to treatment and diverse clinical trajectories, even among patients at the same disease stage [5–9]. Due to limited means for detailed patient stratification and prognostic assessment, only a minority of patients with ccRCC have truly benefited from precision oncology thus far [10]. Therefore, identifying reliable biomarkers or signatures to accurately predict prognosis and treatment responsiveness in patients with ccRCC remains a crucial pursuit, motivating the present study.

Tryptophan (Trp), an essential amino acid sourced exclusively from the diet, plays a vital role in the human body. Beyond its fundamental role in protein synthesis, Trp serves as a precursor to metabolites that function as signaling molecules in various pathways, orchestrating essential physiological processes [11]. Of the three primary degradation pathways—serotonin, indoleacetate, and kynurenine (Kyn) pathways—the Kyn pathway is the most dominant, processing over 95% of free Trp [11]. Besides their well-known neuroactive functions, Kyn pathway metabolites are associated with immune suppression, cancer cell malignancy, and other metabolic

pathways [11–16], making them a focal point in cancer research.

Alterations in tryptophan metabolism have been observed across various cancers, including colorectal cancer [17, 18], liver cancer [19–21], pancreatic cancer [22, 23], glioma [24, 25], lung cancer [26, 27], ovarian carcinoma [28, 29], breast cancer [30, 31] and RCC [32, 33]. Notably, these metabolic shifts have been linked to patient prognosis, underscoring their potential clinical significance. In RCC, tryptophan metabolism is significantly disrupted, evidenced by elevated levels of downstream metabolites in the kynurenine (Kyn) pathway, such as kynurenine and quinolinate, along with a decrease in tryptophan (Trp) levels [32]. Concurrently, enzymes associated with alternative tryptophan degradation pathways, such as those leading to serotonin and indoleacetate production, are notably downregulated [33–35]. These changes collectively indicate a substantial reconfiguration of tryptophan metabolism in RCC. Despite these observations, the exact implications of abnormal tryptophan metabolic pathways in renal cancer progression remain enigmatic. Their influence on patient prognosis, treatment responsiveness, and overall clinical significance requires further investigation.

This study conducted a comprehensive analysis of single-cell sequencing data and bulk transcriptome data to identify tryptophan metabolism-related genes in ccRCC. Using an integrated machine learning framework, a tryptophan metabolism-related signature (TMRS) was established. Further exploration of multi-omics data revealed promising associations between this signature and ccRCC prognosis, immune microenvironment status, and response to treatment modalities. These findings were validated by clinical samples and experiments, though we acknowledge that further extensive studies and clinical validation are necessary before clinical application. Based on these results, we believe that this study can serve as a reference for exploring the role of tryptophan metabolism in renal cell carcinoma and for the development of clinical applications based on tryptophan metabolism. The workflow of this study is shown in Fig. 1.

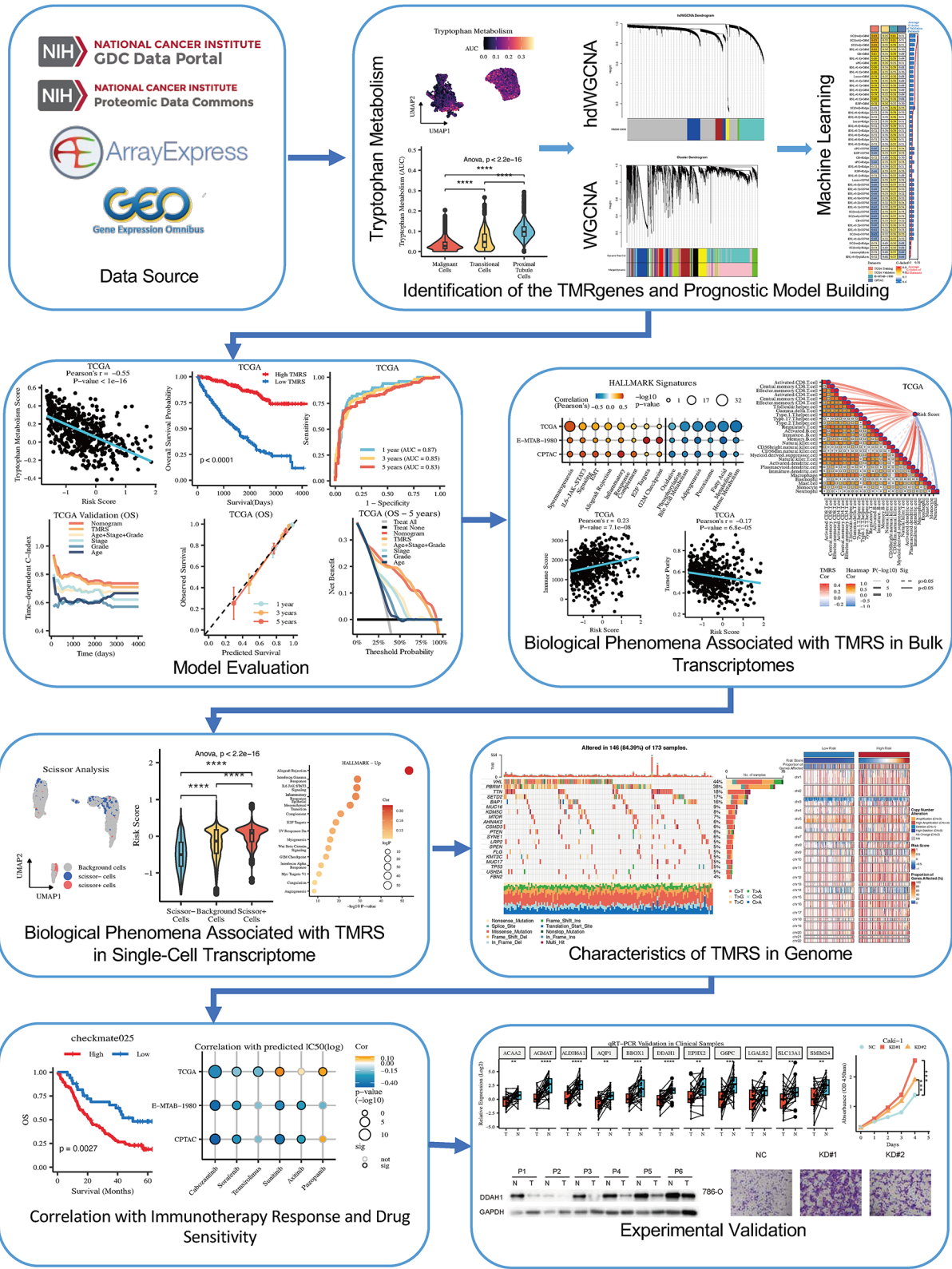


Fig. 1 The Workflow of this study. TMRgenes, tryptophan metabolism-related genes; TMRs, tryptophan metabolism-related signature

Methods

Data acquisition

Six independent public datasets were sourced from the Genomic Data Commons (<https://portal.gdc.cancer.gov/>) [36], Proteomic Data Commons (<https://pdc.cancer.gov/pdc/>), Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>) [37], ArrayExpress database (<https://www.ebi.ac.uk/arrayexpress/>) [38], and supplementary material from a previous publication [39]. The single-cell sequencing dataset GSE156632 was utilized for study and model validation at the single-cell level [40]. Three datasets containing clinicopathological and follow-up data, namely The Cancer Genome Atlas - Kidney Renal Clear Cell Carcinoma (TCGA-KIRC) [41], E-MTAB-1980 [42], and the Clinical Proteomic Tumor Analysis Consortium (CPTAC) ccRCC Discovery Study [43], were employed to construct and validate a prognostic model. The GSE53757 dataset was incorporated into the analysis to evaluate the aberrant expression of genes in TMRS [44]. Transcriptome and survival data from the CheckMate-025 [45], JAVELIN-Renal-101 cohort [46], GSE91061 cohort [47] were leveraged to investigate the association between TMRS and immunotherapy efficacy. Furthermore, proteomic data from the CPTAC ccRCC Discovery Study (PDC000127) and immunohistochemical images from the Human Protein Atlas (<http://www.proteinatlas.org>) [48] were utilized to assess TMRS expression at the protein level. Genetic mutation data in mutation annotation format (MAF) and copy number variation (CNV) data (gene level copy number) from TCGA-KIRC (referred to as TCGA) and the CPTAC ccRCC Discovery Study (referred to as CPTAC) were employed to analyze the relationship between TMRS and genetic characteristics.

Single-cell RNA sequencing (scRNA-seq) data processing

Single-cell sequencing data processing was primarily conducted using the R package “Seurat” [49]. Quality control, doublet removal (using the “DoubletFinder” package [50]), normalization, feature selection, scaling, principal component analysis (PCA), and batch effect (using the “Harmony” package [51]), graph-based clustering, and Uniform Manifold Approximation and Projection (UMAP) dimension reduction were conducted as part of the data preprocessing pipeline. Cell type annotation was based on canonical cell markers (Table S1). Additionally, the developmental trajectory of epithelial cells was reconstructed using the Monocle2 algorithm [52]. Details of the scRNA-seq processing are provided in the Supplementary Methods.

Pathway activity analysis and gene enrichment analysis

The activity of specific pathways at the single-cell level was quantified using the R package “AUCell” [53]. At the bulk-cell level, pathway activity was assessed using the

R package “GSVA” [54]. Enrichment scores for “Tryptophan Metabolism” from Kyoto Encyclopedia of Genes and Genomes (KEGG) utilized in the weighted gene co-expression network analysis, were calculated with the single sample Gene Set Enrichment Analysis (ssGSEA) algorithm, while scores for other gene sets, when investigating TMRS mechanisms, were derived using the Gene Set Variation Analysis (GSVA) algorithm. Gene enrichment analyses were conducted with the R package “clusterProfiler” [55]. Gene sets in this study were sourced from the Molecular Signatures Database (MSigDB) [56].

Weighted gene co-expression network analysis (WGCNA) and high-dimensional weighted correlation network analysis (hdWGCNA)

The R packages “WGCNA” and “hdWGCNA” (formerly “scWGCNA”) were used to construct scale-free co-expression networks, with “WGCNA” applied to bulk data [57] and “hdWGCNA” used for single-cell data analysis [58, 59]. In both approaches, the module most strongly correlated with tryptophan metabolism enrichment scores was identified as the tryptophan metabolism-related module. Within this module, genes exhibiting high gene significance (GS) and module membership (MM) values (both within the top 50%) were considered to be associated with tryptophan metabolism. Further details on the WGCNA and hdWGCNA methodologies are provided in the Supplementary Methods.

Integrated machine learning-based development of prognostic tryptophan metabolism-related signature

Genes identified through WGCNA and hdWGCNA were selected as candidate tryptophan metabolism-related genes (TMRgenes) for signature construction. The prognostic value of these TMRgenes was first assessed using univariate Cox regression to filter genes with potential prognostic relevance. In the next step, 246 combinations of 10 machine learning algorithms were applied to construct a prognostic model using the TCGA training set. Finally, the performance of each model was evaluated using Harrell’s concordance index (C-index), and the model with the highest C-index across validation sets was selected as the optimal prognostic signature. Detailed methods are provided in the Supplementary Methods.

Nomogram construction and model performance evaluation

To maximize the predictive power and clinical value of the signature, predictive nomograms for overall survival (OS) and progression-free survival (PFS) were constructed based on the TMRS risk score and other clinical covariates using the “rms” R package. The performance of TMRS and the nomograms was comprehensively evaluated through univariate and multivariate Cox regression,

Kaplan-Meier analysis, receiver operating characteristic (ROC) curves, time-dependent ROC analysis, concordance index (C-index), calibration plots, and decision curve analysis (DCA). The R packages utilized in this process included “survival”, “survminer”, “rms”, “pROC”, “timeROC”, and “dcurves”. Additionally, Single-Cell Identification of Subpopulations with bulk Sample phenotype correlation (SCISSOR) analysis was performed to elucidate the association between TMRS and prognostic phenotypes at the single-cell level, integrating survival data and transcriptomic profiles from the TCGA cohort [60].

Analysis of immune characteristics and prediction of treatment response

The immune cell infiltration profiles across samples were characterized using three algorithms: Estimation of STromal and Immune cells in MAlignant Tumours using Expression data’ (ESTIMATE) [61], cell-type identification by estimating relative subsets of RNA transcripts (CIBERSORT) [62], and ssGSEA. Gene signatures of 28 immune cell types employed in the ssGSEA approach were retrieved from the Tumour Immune System Interaction Database (TISIDB) (<http://cis.hku.hk/TISIDB/index.php>) [63]. The association between TMRS and the cancer-immunity cycle, which delineates the sequential processes of T cell activation, tumor recognition, and cancer cell elimination [64], was also investigated. Activity scores quantifying each step of the cancer-immunity cycle for the TCGA cohort were obtained from the Tracking Tumor Immunophenotype (TIP) database (<http://biocc.hrbmu.edu.cn/TIP/index.jsp>) [65]. Additionally, patients’ predicted responses to immunotherapy and targeted therapy regimens were evaluated using the Tumor Immune Dysfunction and Exclusion (TIDE) algorithm [66] and the “oncoPredict” R package [67].

Genomic characteristics analysis

The somatic genetic mutation landscape across samples was visualized, and co-occurring and mutually exclusive mutation patterns were analyzed using the “maftools” R package [68]. This package also quantified tumor heterogeneity metrics, including mutant-allele tumor heterogeneity (MATH) scores [69] and tumor mutation burden (TMB) estimates. Gene-level copy number variation profiles were illustrated with the “ComplexHeatmap” R package [70].

Clinical samples and ethical approval

All clinical renal cell carcinoma tissue specimens were obtained from patients at Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University. Informed consent was obtained from each participant prior to sample collection. This study adhered to the principles outlined in the Declaration of Helsinki and received approval from

the Ethics Committee of Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University (No.20220317).

Cell lines

The ccRCC cell lines 786-O (Research Resource Identifiers (RRID): CVCL_1051) and Caki-1 (RRID: CVCL_0234) were purchased from the National Collection of Authenticated Cell Cultures (NCACC, Shanghai, China). Detailed cell culture conditions, along with the phenotypic experimental procedures used to assess the proliferation and metastatic capabilities of these cells, are provided in the Supplementary Methods.

Cell transfection

DDAH1-specific short interfering RNAs (siRNAs) were obtained from GenePharma (Shanghai, China), and cell transfection was conducted using RNAiMAX reagent (Invitrogen) following the manufacturer’s guidelines. The sense sequences (5’-3’) of si-DDAH1#1 and si-DDAH1#2 were GUGCAAAGGUUUAUGAGAATT and CAGCUC AAUAUAGUAGAGATT, respectively.

Quantitative real-time PCR (qRT-PCR)

RNA was extracted from renal cell carcinoma tissues and cell lines using the TRIzol reagent (Invitrogen, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed to measure relative gene expression. Detailed experimental processes are provided in the Supplementary Methods. Primer sequences used are listed in Table S2.

Western blot assay

Western blot analysis was conducted to evaluate protein expression levels in cell and tissue samples. Briefly, protein extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes, and probed with specific primary antibodies. Immunoreactive bands were detected using an enhanced chemiluminescence system. Detailed experimental procedures are provided in the Supplementary Methods.

The detection assays of tryptophan and kynurenine

After 48 h incubation, supernatants of cells were collected and the amount of kynurenine and tryptophan in the supernatant was quantified using the tryptophan assay kit (ab211098; abcam, UK) and human kynureninase kit (ab313980; abcam, UK), following manufacturer’s instructions. Then, the kynurenine to tryptophan ratio was calculated.

Statistical analysis

Statistical analyses were performed using R software (version 4.3.1). Continuous variables are expressed as mean ± standard deviation and analyzed *via* the Student’s

t-test or the Wilcoxon rank-sum test, where applicable. For multiple groups of continuous numerical data, Analysis of Variance (ANOVA) is used to determine if there are any significant differences between the means of the groups. Categorical variables were evaluated with the chi-square test or Fisher's exact test. A P-value below 0.05 was deemed statistically significant.

Result

Tryptophan metabolism undergoes abnormal alterations in tumor cells of clear cell renal cell carcinoma

The role of tryptophan metabolism in ccRCC was explored by assessing its activity at the single-cell level (GSE156632). After rigorous quality control and batch effect removal, 45,427 cells from seven tumor samples and five adjacent normal samples from seven patients with ccRCC were analyzed (Figure S1). These cells were categorized into six major cell types: epithelial, endothelial, fibroblast, myeloid, B, and T & NK cells (Figure S2A). The enrichment score of "KEGG Tryptophan Metabolism" was calculated using the AUCell algorithm. Significant differences in tryptophan metabolism scores were observed among various epithelial cells, characterized by a gradual decrease from left to right on the UMAP plot, indicating a correlation between tryptophan metabolism and diverse biological states of epithelial cells (Figure S2B). sub-clustering and annotation of epithelial cells identified primary differences originating from proximal tubule cells (Figure S2C-D). Further analysis classified proximal tubule cells into normal, transitional (expressing markers of both normal proximal tubule and malignant cells), and malignant cells (Fig. 2A). The tryptophan metabolism score exhibited a gradual decline across normal, transitional, and malignant cells, suggesting its involvement in the malignant transformation of proximal tubule cells (Fig. 2A). Pseudotime analysis supported this, demonstrating a steady decrease in tryptophan metabolism score with increasing pseudotime (Fig. 2B). These findings collectively underscore the critical role of abnormal tryptophan metabolism in the pathogenesis of ccRCC. The expression of marker genes used in this step-wise annotation were shown in Figure S3.

Identification of hub genes related to tryptophan metabolism in both single-cell and bulk RNA sequencing

High-dimensional weighted correlation network analysis (hdWGCNA) was performed on proximal tubule cells to pinpoint genes intricately linked with tryptophan metabolism at the single-cell level. Employing a soft power threshold of 10 ($R^2=0.87$), a scale-free topological network was constructed, unveiling six distinct modules differentiated by colors (Figure S4A-C). The turquoise module's eigengenes displayed the strongest correlation with the tryptophan metabolism enrichment score

(Spearman's $r=0.72$, Fig. 2C). The correlation between gene significance (GS) and module membership (MM) within this module was exceptionally high (0.98), reinforcing the robustness of these findings (Figure S4D). Consequently, 635 genes from the turquoise module, with both GS and MM in the top 50%, were identified as potential hub genes associated with tryptophan metabolism at the single-cell level (Figure S4D and Table S3).

To address the limitations of single-cell analyses, including high false positive rates and the lack of clinical data, weighted correlation network analysis (WGCNA) was also applied to bulk RNA-seq data from TCGA-KIRC. With a soft power threshold set at 6 ($R^2=0.96$), 207 genes within the purple module, which showed the highest correlation with tryptophan metabolism enrichment scores (Spearman's $r=0.74$), were identified as potential hub genes at the bulk RNA level, with both GS and MM in the top 50% (Figure S5 and Table S4). Ultimately, 142 overlapping genes from both hdWGCNA and WGCNA analyses were designated as tryptophan metabolism-related genes (TMRgenes, Fig. 2E). GO enrichment analysis revealed these genes predominantly participate in metabolic processes, including amino acid, aldehyde, and fatty acid metabolism (Fig. 2F). Univariate Cox regression analysis indicated that 35 of these genes were significantly associated with patient prognosis across the TCGA, CPTAC, and E-MTAB-1980 datasets. These genes were selected for further analyses (Fig. 2G and Table S5).

Construction of a consensus prognostic tryptophan metabolism-related signature through integrated machine learning

Following the initial identification of 35 genes, a refined gene signature and a prognostic model were developed through a comprehensive machine-learning strategy to enhance clinical applicability. The TCGA-KIRC dataset was randomly partitioned into a training set and an internal validation set with a 7:3 split (Table S6). Utilizing 246 combinations from 10 distinct machine learning algorithms, gene screening and prognostic model development were conducted within the TCGA training set. Each model's concordance index (C-index) was assessed across all datasets (Table S7). Notably, the combination of backward stepwise Cox regression and generalized boosted regression modeling (GBM) emerged as the superior approach, achieving the highest mean C-index of 0.758 across three validation datasets (Fig. 3A). This highlights its exceptional predictive accuracy for the prognosis of patients with ccRCC, establishing it as the definitive model for the tryptophan metabolism-related signature (TMRS). Specifically, among the initial 35 genes, 11 were selected for the final TMRS based on their minimal Akaike Information Criterion (AIC) values

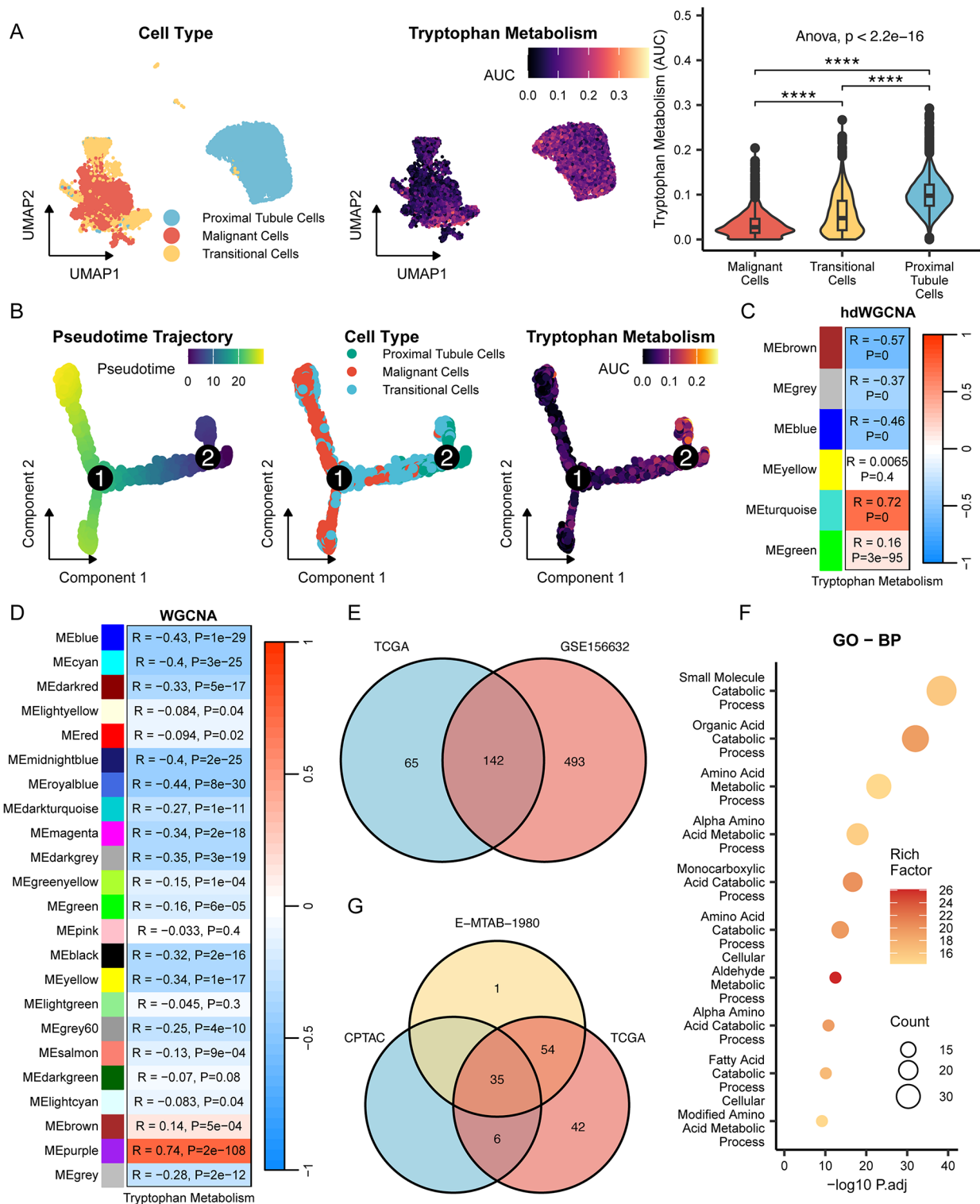


Fig. 2 Identification of tryptophan metabolism-related genes. **(A)** Assessment of tryptophan metabolism activity scores in normal & malignant proximal tubule cells. **(B)** Distribution of tryptophan metabolism activity scores in normal and malignant proximal tubule cells across varying pseudotime stages inferred by Monocle2. **(C-D)** Association between modules and tryptophan metabolism in hdWGCNA at the single-cell level **(C)** and WGCNA at the bulk-transcriptome level **(D)**. **E** The overlapping genes between hdWGCNA and WGCNA results. **F** Gene Ontology (GO) enrichment analysis of the tryptophan metabolism-related genes. **G** Identification of tryptophan metabolism-related genes with robust prognostic value via univariate cox regression screening. UMAP, uniform manifold approximation and projection; AUC, area under the curve; ANOVA, analysis of variance; ME, module eigengene; hdWGCNA, high-dimensional weighted gene co-expression network analysis; WGCNA, weighted gene co-expression network analysis; GO, gene ontology; BP, biological process; Padj, adjusted P value; ****, $p < 0.0001$

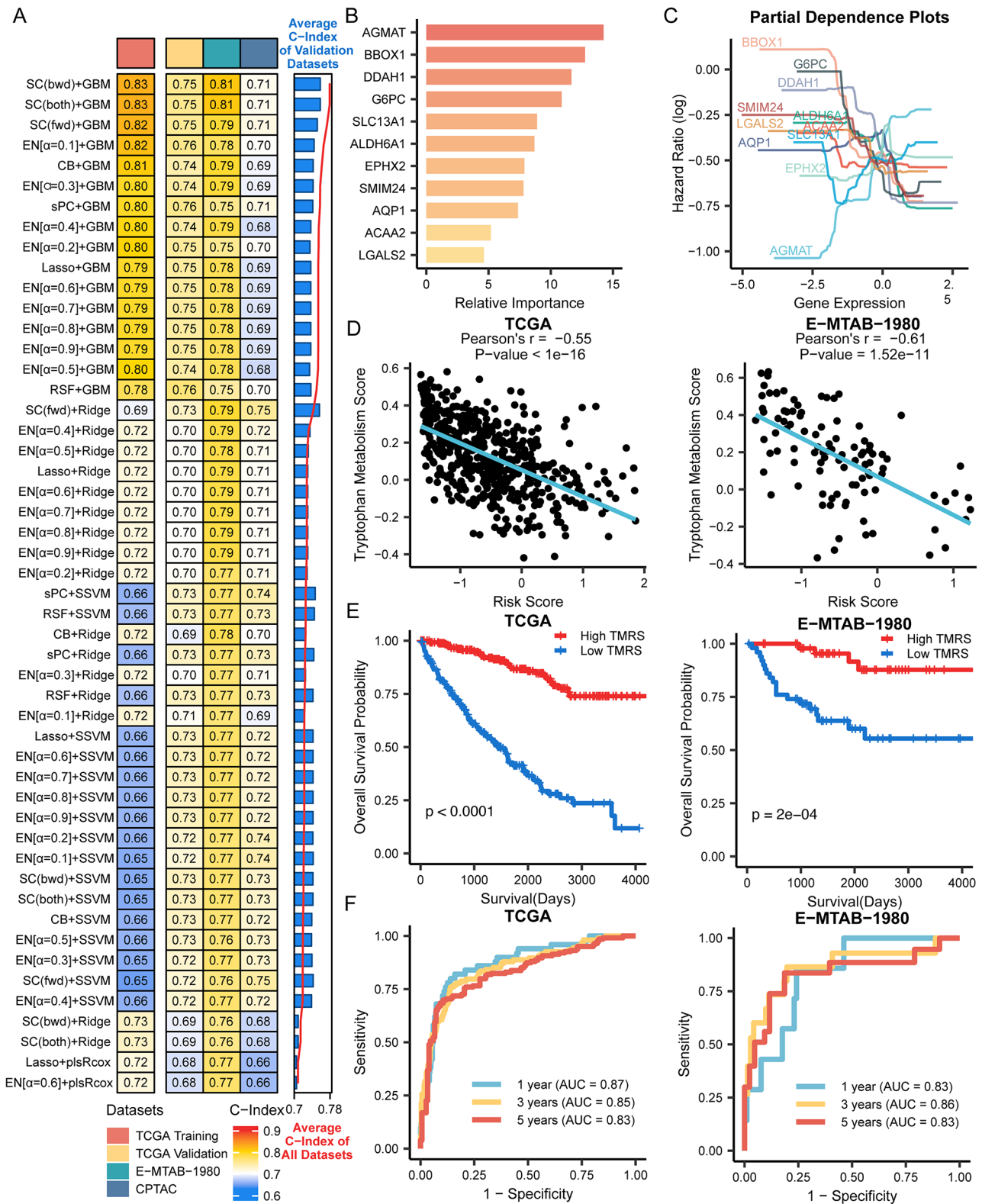


Fig. 3 (See legend on next page.)

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Fig. 3 Development and validation of a consensus tryptophan metabolism-related signature (TMRS). **(A)** The top 50 machine learning combinations by average concordance index (C-index) in the validation set, selected from 246 possible combinations. **(B)** The relative importance of variables within the TMRS prognostic model. **(C)** Partial dependence plots demonstrating the impact of specific variables on the TMRS prognostic model's predictions. **(D)** The correlation between TMRS risk scores and tryptophan metabolism enrichment scores in TCGA and E-MTAB-1980 datasets. **(E)** Kaplan-Meier survival analyses revealing a significant correlation between TMRS risk scores and overall survival in patients with ccRCC, using the median value as the cut-off point, across the TCGA and E-MTAB-1980 datasets. **(F)** Time-dependent ROC curves assessing the TMRS prognostic model's ability to predict 1, 3, and 5-year overall survival rates in the TCGA and E-MTAB-1980 datasets. SC, stepwise Cox regression; bwd, backward; GBM, generalized boosted regression modeling; fwd, forward; EN, elastic net; CB, CoxBoost; sPC, supervised principal components; Lasso, least absolute shrinkage and selection operator; RSF, random survival forest; SSVM, survival support vector machine; plsRcox, partial least squares regression for Cox; C-Index, concordance index; TMRS, tryptophan metabolism-related signature; AUC, area under the curve

obtained through backward stepwise Cox regression. These genes were then incorporated into the GBM. With the interaction depth set at 3 and the shrinkage parameter at 0.001, the optimal number of boosting iterations for the GBM model was determined to be 2721, as established *via* 10-fold cross-validation. The genes ultimately included in the model are as follows: AGMAT, BBOX1, DDAH1, G6PC, SLC13A1, ALDH6A1, EPHX2, SMIM24, AQP1, ACAA2, LGALS2. The relative importance and partial dependence plots for each variable in the final model are presented in Fig. 3B and C, offering insights into the contribution and influence of each gene within the model.

To ensure the reliability of the genes included in the predictive model, separate analyses were conducted. These 11 genes exhibited reduced expression in tumor samples compared to adjacent normal tissues, both at the transcriptional level across three datasets (Figure S6A-C) and at the protein level in proteome data from CPTAC (Figure S6D) as well as in immunohistochemical images from the HPA (Figure S7). Most of these genes also demonstrated significant associations with disease stage (Figure S8) and tumor histological grade (Figure S9), and showed notable independent prognostic value in multivariate Cox regression analyses (Figure S10-S12). These findings support the validity and reliability of the predictive model based on these 11 genes.

Assessment of the TMRS model's performance

To validate the TMRS's capacity to accurately reflect tryptophan metabolism status in ccRCC, TMRS risk scores were compared with canonical single-sample Gene Set Enrichment Analysis (ssGSEA) enrichment scores for the "KEGG_Tryptophan_Metabolism" signature. The analysis revealed a pronounced negative correlation between TMRS risk scores and ssGSEA enrichment scores across all datasets (Fig. 3D and Figure S13A-C). Subsequently, the TMRS's predictive power concerning patient prognosis was assessed. Figure 3E and Figure S13D-F showed that patients in the high-risk group exhibited significantly poorer overall survival (OS) across all datasets. Additionally, high-risk groups were associated with less favorable progression-free survival (PFS) as well, as illustrated in Figure S13G, H. The precision of the TMRS model was

further corroborated through receiver operating characteristic (ROC) curve analysis, which demonstrated 1-, 3-, and 5-year area under the curves (AUCs) of 0.87, 0.85, and 0.83 in TCGA dataset; 0.83, 0.86, and 0.83 in the E-MTAB-1980 dataset; 0.79, 0.67, and 0.59 in the CPTAC dataset; 0.91, 0.87, and 0.86 in the TCGA-training set; and 0.81, 0.81, and 0.77 in the TCGA-validation set (Fig. 3F and Figure S13J-L). The relatively low 5-year survival AUC in the CPTAC cohort may be attributed to the cohort's shorter follow-up duration. Among the 98 patients with available survival data in the CPTAC dataset, only nine (9.2%) were monitored for over five years, compared to 152 out of 531 (28.6%) in the TCGA dataset and 43 out of 101 (42.6%) in the E-MTAB-1980 dataset. Besides, the AUC value of TMRS for predicting PFS is also quite high, exceeding 0.75 in all datasets (Figure S13M-O).

The relationship between TMRS and clinical characteristics, commonly used in clinical settings to predict patient outcomes, was also explored. As depicted in Fig. 4A-B and Figure S14A, TMRS risk scores were notably higher in patients with advanced disease stages and grades within the TCGA dataset. This pattern was similarly evident in the E-MTAB-1980 and CPTAC datasets (Fig. 4A, B, Figure S14B, and Figure S15A-B). Multivariate Cox regression was performed to examine whether TMRS had independent prognostic value. As shown in Fig. 4C and Figure S15D, after adjusting for age, histological grade, and disease stage, patients with higher TMRS scores still had significantly worse overall survival across all three databases. This independent prognostic value of TMRS scores was further validated by subgroup analysis. In patients with different stages and grades, TMRS demonstrated good predictive ability for overall survival, especially in patients with early-stage ccRCC (Figure S15E-F). The diagnostic efficacy of TMRS was also examined. ROC curve analysis demonstrated the outstanding accuracy of TMRS in distinguishing between normal and malignant renal tissues (Figure S15G).

To enhance the clinical applicability of the TMRS, two nomograms were created to predict patients' OS and PFS, integrating the TMRS with clinical characteristics. The OS nomogram, depicted in Fig. 4D, E and Figure S16A, B, demonstrates exceptional accuracy in predicting

patients' survival outcomes, with Harrell's C-index and time-dependent C-index scores approaching 0.8 across all datasets. Calibration curve analysis confirms the nomogram's precision, aligning predicted probabilities with observed events, ensuring reliable prognostic assessments (Fig. 4F and Figure S16C). Decision curve analysis (DCA) further establishes the OS nomogram's clinical value, showcasing its potential to guide treatment decisions and effectively improve patient care (Fig. 4G and Figure S16D, F). The PFS model evaluation yielded similar outcomes (Figure S17), collectively underscoring the nomograms' predictive precision, dependability, and practical utility in clinical settings.

Exploring the underlying mechanisms of TMRS on patient outcomes

Subsequent investigations elucidated the molecular mechanisms by which TMRS influences patient prognosis. The UMAP visualization in Fig. 5A and Figure S18A reveals significant variations in patient distribution across TMRS risk score groups, highlighting distinct biological states. Gene set variation analysis on Hallmark signatures, presented in Fig. 5B, identified pathways significantly associated with TMRS across all datasets, including those related to metabolism, malignancy, and immune response. Specifically, metabolic pathways such as fatty acid metabolism and oxidative phosphorylation exhibited a negative correlation with TMRS scores, whereas malignancy-related pathways (e.g., epithelial-mesenchymal transition, G2M checkpoints, E2F Targets) and immune-related pathways showed a positive correlation. These findings suggest that ccRCC with high TMRS scores may display more aberrant metabolic pathways, increased malignancy, and enhanced immune responses. Given TMRS's foundation in tryptophan metabolism and the observed poorer prognosis in patients with high scores, its association with abnormal metabolism and increased malignancy is expected. However, the correlation with an enhanced immune response warrants further investigation.

Further analysis of the relationship between TMRS and immune status in ccRCC was conducted. ESTIMATE analysis demonstrated a consistent positive correlation between TMRS scores and immune scores across three databases, and a negative correlation with tumor purity (Fig. 5C, D and Figure S18B, C), suggesting that higher TMRS scores correlate with greater immune cell infiltration in ccRCC. The infiltration levels of specific immune cell types were then evaluated. The ssGSEA algorithm indicated a higher abundance of most immune cells in patients with elevated TMRS scores, encompassing both traditional "anti-cancer" and "pro-cancer" immune cells (Fig. 5E and Figure S18D, E). The CIBERSORT algorithm showed slight variations, with significant increases in

regulatory T cells and M0 macrophages across all datasets, and significant decreases in M1 macrophages, monocytes, and resting mast cells in two of the three datasets (Fig. 5F and Figure S19A, B). These results imply that the tumor immune microenvironment in patients with elevated TMRS risk scores is characterized by extensive immune cell infiltration but may also be functionally impaired. This observation aligns with previous studies indicating that a highly inflamed microenvironment in ccRCC correlates with adverse outcomes [71–73]. Further analysis of the cancer-immunity cycle supports this notion, revealing that while most steps in the immune cycle positively correlate with TMRS scores, the critical final step, "killing of cancer cells," trends towards a negative correlation (Figure S19C).

High TMRS was associated with poor prognosis and higher malignancy at the single-cell scale

Given the inherent limitations of bulk RNA sequencing, which provides an averaged expression profile across all cells, findings were corroborated using single-cell RNA sequencing data. As illustrated in Fig. 6A, a progressive increase in TMRS risk scores from normal cells to transitional cells, and finally to malignant cells, underscores its link to malignancy. Subsequent analysis focused on malignant cells for a detailed examination (Fig. 6B, C). To determine the prognostic significance of TMRS, the Scissor (Single-Cell Identification of Subpopulations with bulk Sample phenotype correlation) analysis was employed. Utilizing bulk RNA-seq data and survival information from the TCGA dataset, the Scissor algorithm categorized malignant cells into those associated with worse survival (Scissor+), better survival (Scissor-), and non-specific background cells (Fig. 6D). Notably, both the TMRS risk score and the proportion of high-risk cells incrementally increased from Scissor- to background, and then to Scissor+ cells, emphasizing a strong correlation between elevated TMRS and adverse prognosis (Fig. 6E, F).

Subsequently, we analyzed the pathways associated with TMRS at the single-cell level. As shown in Fig. 6G, HALLMARK signatures positively correlated with TMRS are primarily associated with immunity (e.g., allograft rejection, interferon gamma response, and inflammatory response) and tumor malignancy (e.g., epithelial-mesenchymal transition, E2F targets, and G2M checkpoint). In contrast, pathways negatively correlated with TMRS are mainly involved in metabolic processes (Fig. 6H). Similar findings were observed in the KEGG pathway analysis (Figure S20). These results are in line with our observations in bulk RNA sequencing and are consistent with the significantly poorer prognosis seen in patients with high TMRS.

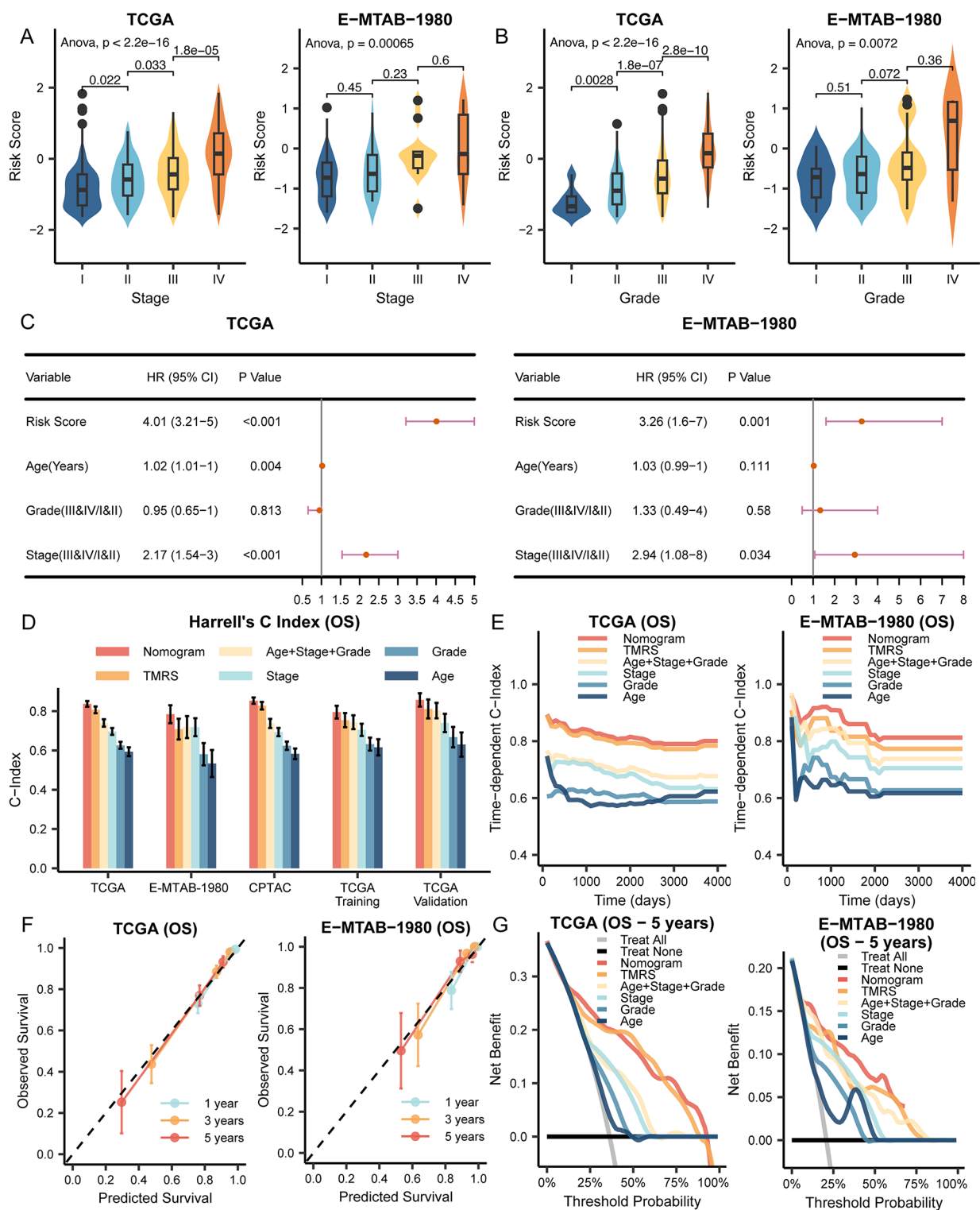


Fig. 4 Association of TMRS with clinical characteristics and development of a tryptophan metabolism-related nomogram. **A–B.** Correlation between TMRS risk score and disease stage (**A**) and histological grade (**B**) of ccRCC in the TCGA and E-MTAB-1980 datasets. **C.** Multivariate Cox regression analyses evaluating the prognostic significance of TMRS alongside clinical characteristics for overall survival (OS) in the TCGA and E-MTAB-1980 datasets. **D.** Harrell's concordance index comparison of nomogram, TMRS, and other clinical parameters for predicting OS across various datasets. **E.** Time-dependent concordance index of the nomogram for predicting OS within the TCGA and E-MTAB-1980 datasets. **F.** Calibration curve of the nomogram for 1-year, 3-year, and 5-year OS predictions within the TCGA and E-MTAB-1980 datasets. **G.** Decision curve analysis demonstrating the net benefit of utilizing the nomogram, TMRS, and other clinical features for predicting 5-years OS within the TCGA and E-MTAB-1980 datasets. Anova, analysis of variance; HR, hazard ratio; CI, confidence interval; OS, overall survival; TMRS, tryptophan metabolism-related signature

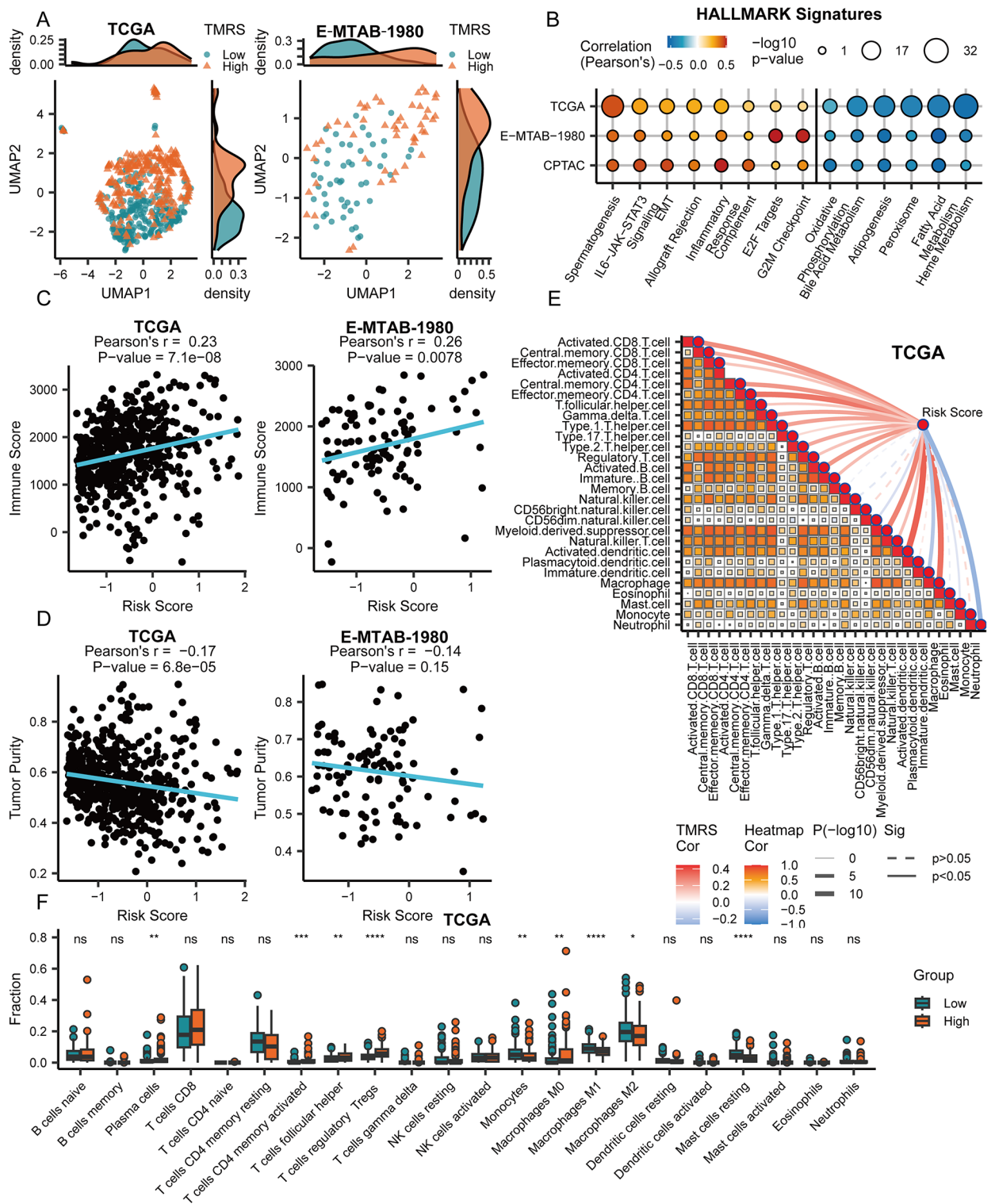


Fig. 5 Biological status and immune landscape of patients with different TMRS risk scores. **(A)** UMAP plots revealing distinct biological status in patients stratified by TMRS risk scores in TCGA and E-MTAB-1980 datasets. **(B)** Hallmark signatures consistently correlated with TMRS across TCGA, E-MTAB-1980, and CPTAC Datasets. **(C-D)** ESTIMATE algorithm indicated elevated immune infiltration **(C)** and reduced tumor purity **(D)** in patients with high TMRS risk scores in TCGA and E-MTAB-1980 datasets. **(E-F)** Association between TMRS risk scores and abundance of various immune cells in the tumor microenvironment assessed by ssGSEA **(E)** and CIBERSORT algorithms **(F)** in TCGA dataset. UMAP, uniform manifold approximation and projection; TMRS, tryptophan metabolism-related signature; EMT, epithelial-mesenchymal transition; Cor, Pearson's correlation; Sig, significance; ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$

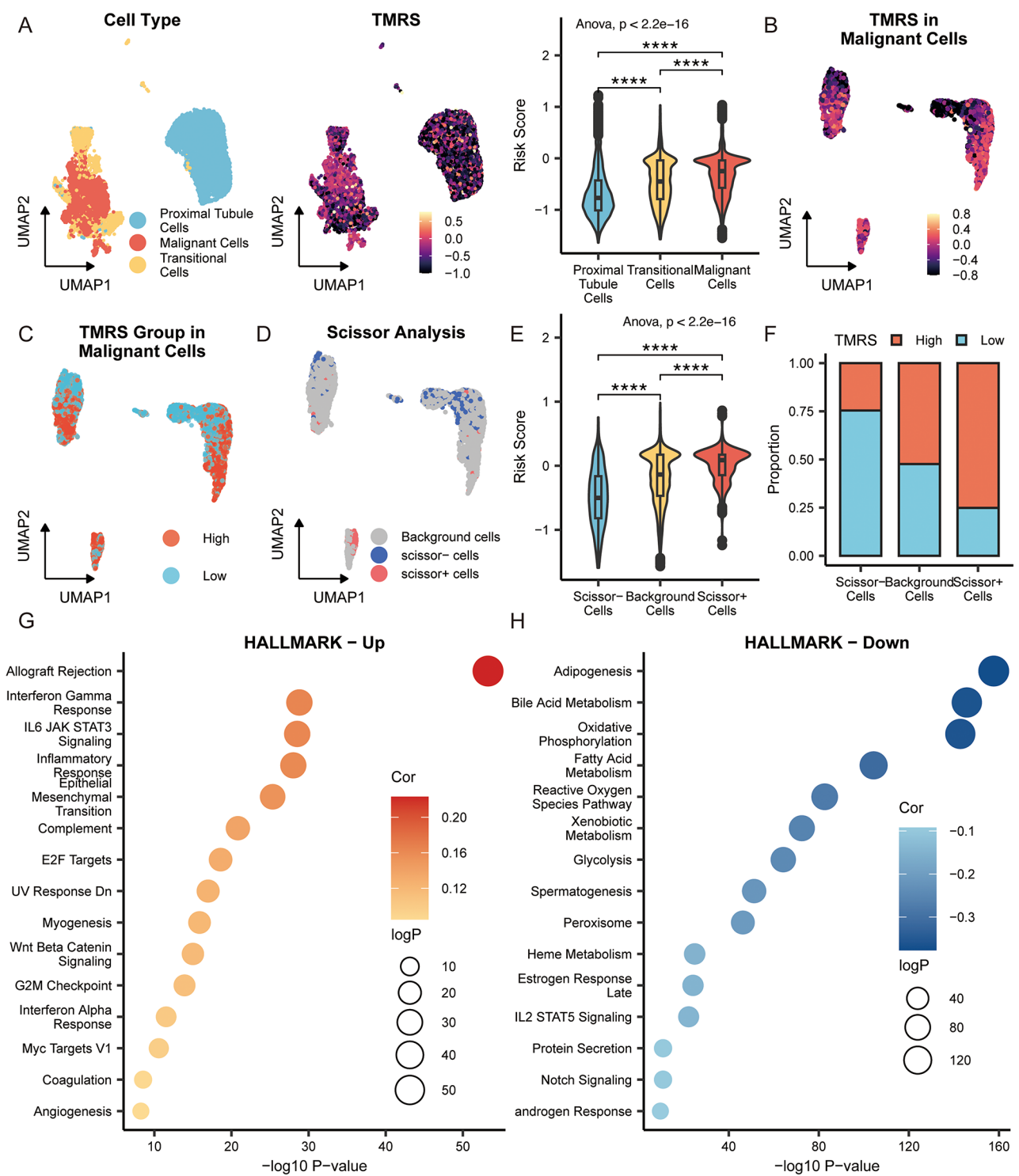


Fig. 6 Correlation of TMRS with survival phenotype and biological status at single cell scale. **A**. UMAP plot depicting the TMRS risk score of normal and malignant proximal tubule cells. **B-D**. UMAP plots showcasing the TMRS risk score distribution (**B**), TMRS group classifications (**C**), and survival phenotypes identified by the Scissor algorithm (**D**) in malignant cells. **E-F**. Scissor+ malignant cells exhibit notably elevated TMRS (**E**) and a predominance of cells within high-risk categories (**F**). **G-H**. Bubble plots showing the top 15 Hallmark gene signatures positively (**G**) and negatively (**H**) associated with the TMRS risk score. The activity score of signatures were calculated by AUCell algorithm. UMAP, uniform manifold approximation and projection; TMRS, tryptophan metabolism-related signature; Scissor, single-cell identification of subpopulations with bulk sample phenotype correlation; EMT, epithelial–mesenchymal transition; Cor, Pearson’s correlation; Anova, analysis of variance; UV, ultraviolet radiation; Dn, down; Cor, Spearman’s correlation; ****, $p < 0.0001$

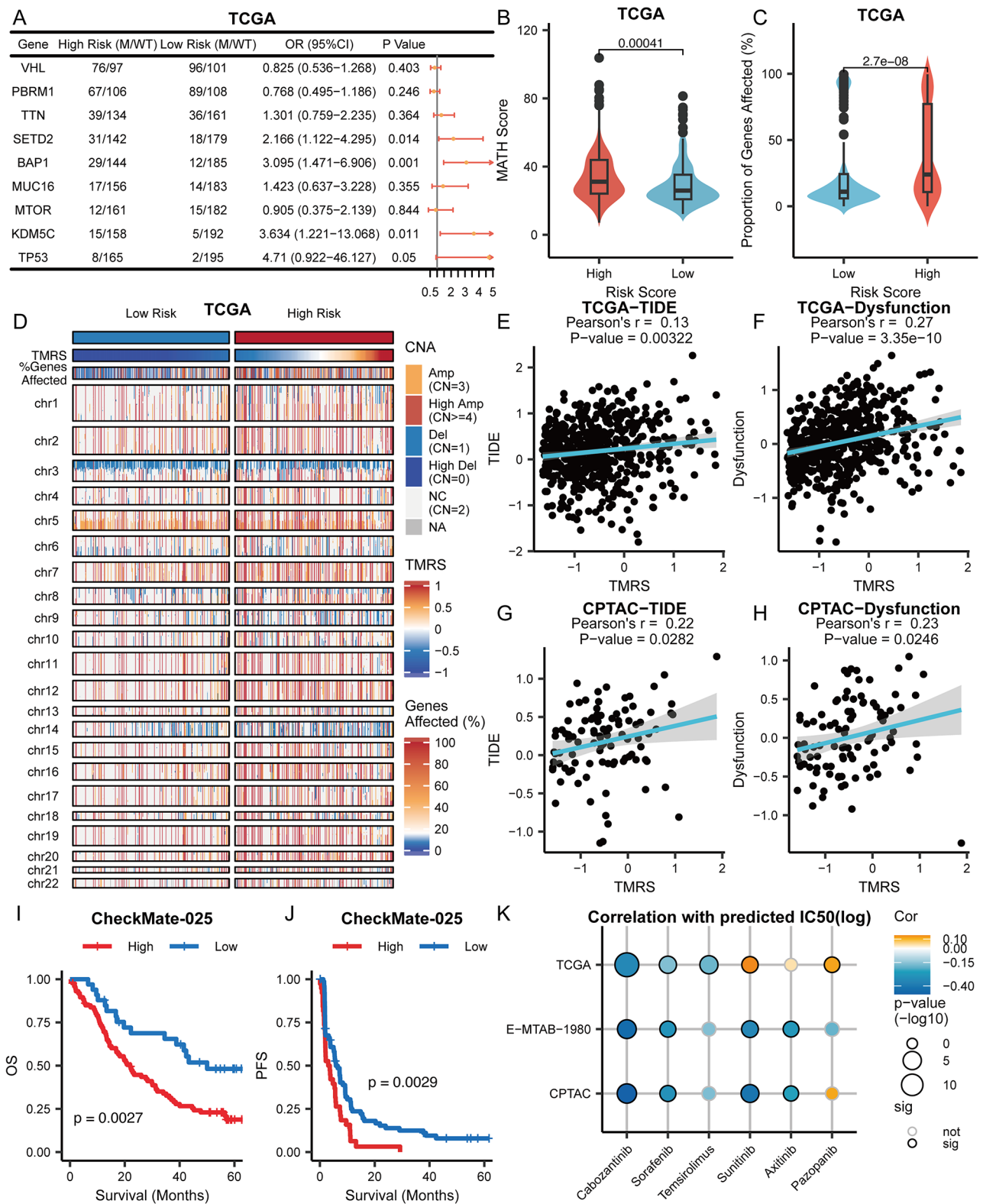


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Fig. 7 Association of TMRS with genomic characteristics and sensitivity to immunotherapy and targeted therapy. **A.** Examination of gene mutation frequency disparities between high and low TMRS risk score groups in TCGA dataset through Fisher's exact test. **B-C.** Patients with a high TMRS risk score exhibited significantly higher mutant allele tumor heterogeneity (MATH) scores (**B**) and a greater proportion of genes affected by copy number alterations (CNA) (**C**) in TCGA dataset. **D.** Depiction of the gene level copy number alteration landscape across patients with varying TMRS risk scores in the TCGA dataset. The horizontal axis of the heatmap represents individual patients, while the vertical axis displays genes ordered by their chromosomal positions. **E-H.** Tumor immune dysfunction and exclusion (TIDE) analysis indicating a positive association between TMRS scores and T cell dysfunction scores in TCGA (**E, F**) and CPTAC (**G, H**) datasets. **I-J.** Kaplan-Meier survival analyses illustrate the association between TMRS and overall survival (OS) (**I**) as well as progression-free survival (PFS) (**J**) in patients who underwent nivolumab treatment in the CheckMate-025 clinical trial. The cut-off value was determined by `surv_cutpoint` function. **K.** Association between TMRS and patient sensitivity to targeted therapeutics commonly utilized in the clinical management of ccRCC, as predicted by OncoPredict algorithm. M, mutated; WT, wild type; OR, odds ratio; CI, confidence interval; MATH, mutant allele tumor heterogeneity; TMRS, tryptophan metabolism-related signature; CNA, copy number alteration; Amp, amplification; CN, copy number; Del, deletion; NC, no change; NA, not available; TIDE, Tumor immune dysfunction and exclusion; OS, overall survival; PFS, progression-free survival; IC₅₀, half-maximal inhibitory concentration; Cor, Pearson's correlation; sig, significance

Association of TMRS with genomic characteristics and patients' responsiveness to immunotherapy and targeted treatments

The genomic profiles of patients with varying TMRS risk scores were also scrutinized. Due to the absence of genomic data in the E-MTAB-1980 dataset, the analysis focused on the TCGA and CPTAC datasets. As depicted in Fig. 7A and Figure S21A, B, within the TCGA dataset, patients in the high-risk group exhibited significantly higher mutation frequencies in three critical tumor suppressor genes: SETD2 (17% versus 9%) [74], BAP1 (16% versus 6%) [75], and KDM5C (8% versus 2.6%) [76]. A similar trend was observed in the CPTAC dataset, although only SETD2 reached statistical significance (Figure S21C-E). Additionally, in the TCGA dataset, patients with a high TMRS risk score showed a higher incidence of co-occurring mutations, while this trend was not evident in the CPTAC dataset (Figure S22). Two prominent prognostic genomic mutation biomarkers, intra-tumor heterogeneity (ITH), assessed *via* the mutant allele tumor heterogeneity (MATH) algorithm, and tumor mutation burden (TMB), were also evaluated. Figure 7B and Figure S23A-C demonstrate that, across both TCGA and CPTAC datasets, MATH scores were significantly elevated in the high-risk group, whereas TMB levels did not display a significant difference. Furthermore, the analysis of the copy number alteration landscape revealed that, within the TCGA dataset, the high-risk group exhibited a higher number of copy number alterations (Fig. 7C, D). This phenomenon was not observed in the CPTAC dataset (Figure S23 D, E). Collectively, these findings indicate that the genomes of patients with ccRCC exhibiting high TMRS scores are characteristically more unstable, suggesting a potential for higher malignancy and a poorer prognosis.

Considering the association between TMRS, tumor immunity, and genomic instability, and to enhance the clinical applicability of TMRS, the connection between TMRS and sensitivity to immunotherapy and targeted therapy was explored. Tumor immune dysfunction and exclusion (TIDE) analysis revealed that TMRS risk score was positively correlated with TIDE score and T cell

dysfunction score, but not with T cell exclusion score in both TCGA and CPTAC datasets (Fig. 7E-H and Figure S23F, G). These findings suggest that patients with higher TMRS scores may exhibit a diminished response to immunotherapy, characterized by infiltrating immune cells with functional abnormalities. This observation aligns with the results of the immune cell infiltration analysis. The research findings were further validated through a clinical trial on renal cell carcinoma using the PD-1 inhibitor nivolumab (CheckMate-025). As shown in Fig. 7I-J, patients with a high TMRS risk score demonstrated significantly reduced overall survival and progression-free survival. Similar phenomenon was found in other immunotherapy cohorts, including JAVELIN-Renal-101 cohort (avelumab+axitinib, renal cell carcinoma, Figure S23H), GSE91061 cohort (nivolumab, melanoma, Figure S23I). Additionally, the OncoPredict algorithm was used to investigate the association between TMRS scores and responsiveness to six targeted therapies commonly used in treating renal clear cell carcinoma. As depicted in Fig. 7K, the analyses revealed that the predicted half-maximal inhibitory concentration (IC₅₀) values for cabozantinib and sorafenib were significantly lower in patients with high TMRS risk scores across all three datasets, suggesting that these drugs might be more effective in patients with higher TMRS scores, potentially leading to better treatment outcomes. In summary, these results suggest that the TMRS score may assist in determining whether patients with ccRCC should undergo these treatment measures.

Experimental validation of aberrant expression of genes in TMRS and the role of DDAH1 in tumor malignancy

The findings were validated through experimental analysis of clinical samples and ccRCC cell lines. qRT-PCR results indicated significant downregulation of all 11 TMRS genes in tumor samples compared to adjacent non-tumorous tissues (Fig. 8A), consistent with earlier database observations. The TMRS scores and prognosis information of these patients were also calculated and collected to validate the association between TMRS and prognosis in the clinical cohort. KM analysis indicated

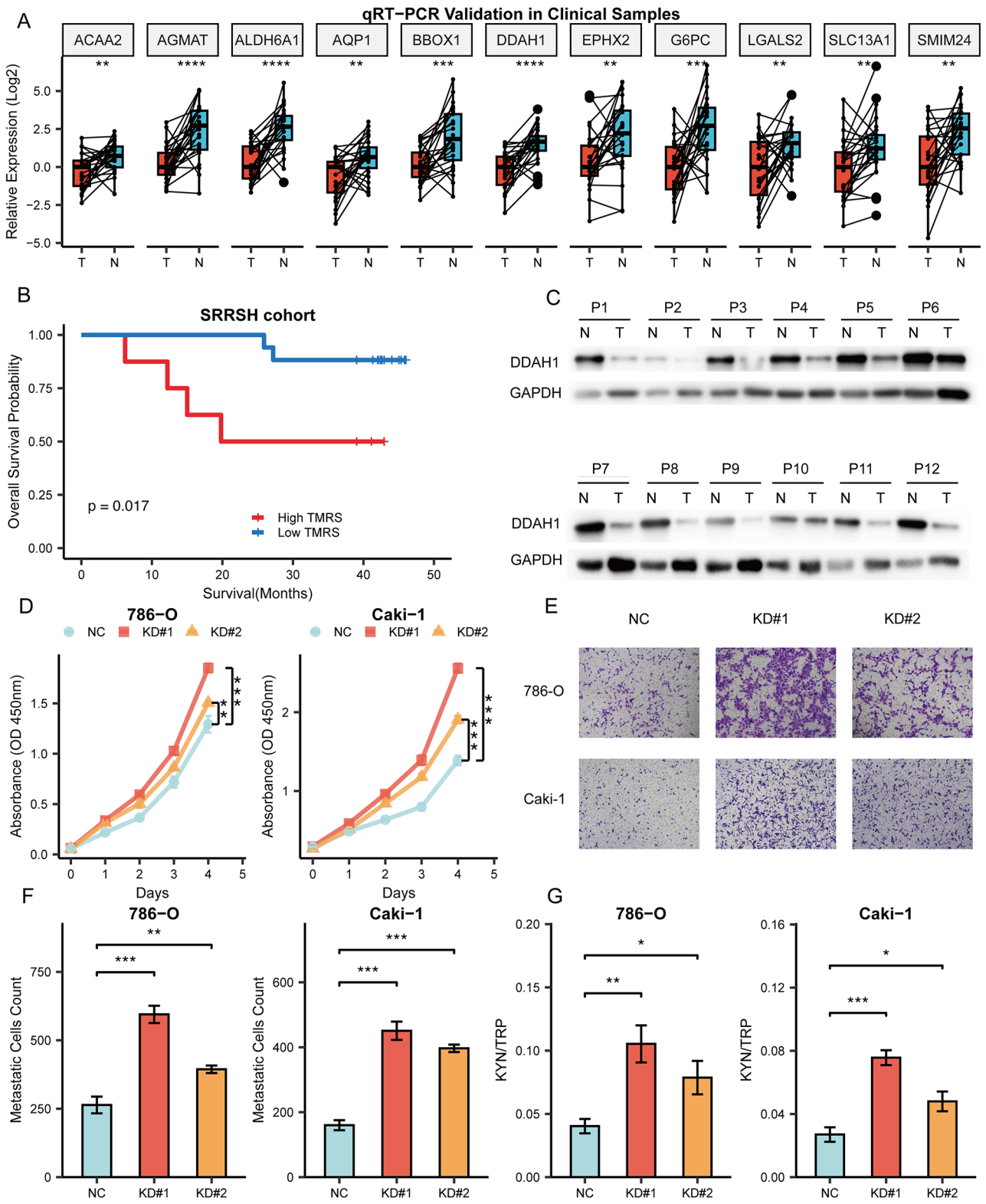


Fig. 8 (See legend on next page.)

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Fig. 8 Experimental validation of TMRS genes expression and DDAH1's association with ccRCC malignancy **(A)** mRNA expression of 11 TMRS genes in 25 pairs of clinical ccRCC samples and matched adjacent normal tissues, assessed by qRT-PCR. **(B)** The Kaplan-Meier plot of patients with ccRCC in SRRSH cohort. The cutoff value was determined by `surv_cutpoint` function. **(C)** Protein expression levels of DDAH1 in 12 pairs of ccRCC and matched adjacent normal tissues from patients, evaluated through Western blot analysis. **(D)** Proliferative capacities of 786-O and Caki-1 cells transfected with DDAH1 siRNAs compared to siRNA control, determined by CCK-8 assays. **E-F.** Migration capabilities of 786-O and Caki-1 cells post-transfection with DDAH1 siRNAs or siRNA control, assessed using transwell assays. **G.** The kynurenine-to-tryptophan ratio in the cell supernatants of 786-O and Caki-1 cells treated with DDAH1 siRNAs or control siRNA. qRT-PCR, quantitative reverse transcription polymerase chain reaction; T, tumor; N, normal; SRRSH, Sir Run Run Shaw Hospital; NC, negative control; KD, knockdown; OD, optical density; P, patient; KYN, kynurenine; TRP, tryptophan; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$

that patients with high TMRS scores had significantly worse prognoses (Fig. 8B). Among these genes, DDAH1 was selected for further exploration due to its relatively unexplored role in ccRCC. Further analysis revealed that DDAH1 was also downregulated at the protein level in tumor samples (Fig. 8C), corroborating findings from the CPTAC database. To investigate DDAH1's involvement in ccRCC progression, two small-interfering RNAs (siRNAs) specifically targeting DDAH1 were employed. Their knockdown efficacy was confirmed *via* qRT-PCR (Figure S24A) and Western blot analysis (Figure S24B). Subsequent Cell Counting Kit-8 (CCK-8) assays indicated that silencing DDAH1 significantly enhanced the proliferation rates of 786-O and Caki-1 cell lines (Fig. 8D). Additionally, transwell assays demonstrated increased cell migration capability upon DDAH1 knockdown (Fig. 8E, F). Finally, we validated the association between DDAH1 and tryptophan metabolism by detecting the ratio of kynurenine to tryptophan, which is a marker of kynurenine pathway. As shown in Fig. 8G, the ratio of kynurenine to tryptophan significantly increased after DDAH1 knockdown, indicating a significant correlation between the expression level of DDAH1 and tryptophan metabolism (Fig. 8G). Collectively, these findings suggest that reduced DDAH1 expression may contribute to the enhanced malignancy of ccRCC cells.

Discussion

RCC, often termed a “metabolic disease” [34, 77], involves complex alterations across multiple metabolic pathways. This condition extends beyond the disruption of well-known glucose and fatty acid metabolism, encompassing significant abnormalities in amino acid metabolic pathways, particularly tryptophan metabolism [34, 77]. Abnormalities in tryptophan metabolism have been linked to tumor growth [13], metastasis [78], metabolic reprogramming [14], and an immunosuppressive tumor microenvironment [11]. However, its specific role in ccRCC and its relationship with patient outcomes remain unclear. This study conducted a comprehensive multi-omics integrated analysis of tryptophan metabolism in ccRCC, culminating in the establishment of a robust tryptophan metabolism-related signature (TMRS).

A thorough investigation into the molecular mechanisms underlying TMRS was conducted, examining its

effects at both single-cell and bulk-cell levels. Findings indicate associations of TMRS with pathways related to immunity, metabolism, and cellular proliferation and metastasis, aligning with prior research on tryptophan metabolism. Specifically, in terms of immunity, pathway enrichment and various immune cell infiltration analyses using different algorithms suggest that individuals with higher TMRS scores may exhibit extensive immune infiltration coupled with abnormal immune cell functions. Previous research has categorized ccRCC as an inflamed tumor marked by pronounced immune infiltration [71]. Unlike most other cancer types, in renal cancer, a high level of immune infiltration may predict a worse prognosis [71, 79]. This effect is particularly pronounced in immune-regulated tumors, which not only have high immune cell infiltration but also exhibit dysfunctional immune cell activity, resulting in the most deleterious prognostic outcomes [79]. Our findings align with these observations, indicating the reliability of our model.

Regarding its association with tumor metabolism, previous studies have reported relatively little on this. Newman et al. reported that abnormal tryptophan metabolism can facilitate tumor purine synthesis through the supply of one-carbon units [14]. Furthermore, considering that many metabolic products of the tryptophan metabolism pathway play crucial roles in other aspects of tumor metabolism—such as reactive oxygen species production, one-carbon metabolism, NAD(P)⁺ synthesis, alanine synthesis, and the integration of carbons into the tricarboxylic acid cycle through α -ketoadipate—it is understandable that it significantly associates with other metabolic pathways as part of the metabolic reprogramming of tumor cells [14]. However, this area warrants further research.

Finally, the relationship between TMRS and the processes of tumor cell proliferation and metastasis has been widely reported. For instance, Optiz et al. highlighted that kynurenine can enhance tumor metastasis through the activation of the AhR pathway [78]. Similarly, Liu et al. found that aberrant tryptophan metabolism supports tumor growth by hindering ferroptotic cell death [13].

The relationship between TMRS and the genomic features of ccRCC was thoroughly investigated. Findings reveal that patients with high TMRS scores are more likely to exhibit a higher mutation frequency in specific

tumor suppressor genes, such as BAP1 [75] and SETD2 [74], and display a greater extent of copy number variation according to the TCGA database. Furthermore, the MATH algorithm suggests that elevated TMRS scores could be associated with increased intra-tumor heterogeneity, a significant contributor to unfavorable prognosis and rapid development of treatment resistance [80]. Overall, patients with high TMRS risk scores might possess more unstable genomes, correlating with their poorer prognostic outcomes.

Additionally, given the association between TMRS, the immune microenvironment, and genomic characteristics, its relation to immunotherapy and targeted drug sensitivity was explored to maximize its clinical utility. Both TIDE analysis and data from the CheckMate-025 trial suggested that high TMRS scores might correlate with poorer immunotherapy responses. Some studies have reported a potential link between dysregulated tryptophan metabolism and immunotherapy resistance [81, 82]. However, due to the complexity of immunotherapy and the failure of indoleamine 2, 3 dioxygenase (IDO) inhibition to improve progression-free survival over anti-PD-1 monotherapy [83], this association warrants further validation. Similarly, limited evidence exists regarding the relationship between tryptophan metabolism and targeted therapy efficacy. Although the OncoPredict algorithm predicted increased sensitivity to cabozantinib, sorafenib in patients with high TMRS across all three datasets, this finding requires additional studies to confirm its clinical relevance.

To ensure the reliability of the model, the genes within it were tested across multiple dimensions. Their aberrant expression at the mRNA level was validated in three databases (TCGA, CPTAC, GSE53757), and their abnormal expression at the protein level was verified through CPTAC proteomic data and immunohistochemistry results from the HPA database. Moreover, considering the potential limitations of bioinformatics methods, aberrant expression at the mRNA level was validated in 25 pairs of clinical samples using qRT-PCR. Additionally, the abnormal protein expression of DDAH1, a gene not thoroughly studied in ccRCC, was validated through Western blot in 12 pairs of ccRCC samples. Furthermore, the function of DDAH1 in promoting ccRCC proliferation and metastasis was confirmed in two cell lines. Knocking down DDAH1 significantly enhanced the proliferation and metastasis capabilities of the ccRCC cell lines, consistent with the model.

To explore the potential mechanism underlying these findings, we measured the kynurenine-to-tryptophan ratio in the supernatant of DDAH1-knockdown cells. The results showed a significant increase in the ratio, suggesting that DDAH1 knockdown may activate the kynurenine pathway, which is aberrantly activated in RCC.

This observation aligns with our current understanding of DDAH1's function and the kynurenine pathway of tryptophan metabolism. DDAH1 primarily catalyzes the degradation of ADMA, an endogenous inhibitor of nitric oxide synthase (NOS) [84, 85]. As a result, DDAH1 knockdown leads to increased ADMA levels, which inhibits NOS activity and reduces nitric oxide (NO) production. Since NO is a known inhibitor of indoleamine 2, 3 dioxygenase (IDO) [86, 87], the key enzyme responsible for converting tryptophan to kynurenine, DDAH1 knockdown would lower NO levels, thereby diminishing its inhibition of IDO activity. This, in turn, could activate IDO and trigger the kynurenine pathway.

While this study has produced promising results, several limitations accompany our findings. Firstly, given that the role and clinical significance of tryptophan metabolism-related genes in renal cancer are relatively underexplored, this study was intended as a comprehensive exploration of this area. Our goal was to provide a reference for ourselves and other researchers for more specific future studies on these genes. Consequently, we explored TMRS from various perspectives across multiple databases to achieve a more holistic understanding, rather than focusing on a single aspect. However, this broad approach may have resulted in a lack of depth in our exploration of individual directions. Therefore, we hope to conduct more in-depth studies on these specific aspects in our future research. For example, while cell phenotype experiments (CCK8 and Transwell) and measurements of the kynurenine-to-tryptophan ratio following DDAH1 knockdown provide valuable insights, they may not be sufficient to definitively establish the relationship between DDAH1 and tumor malignancy, as well as its role in tryptophan metabolism. However, given that thoroughly determining the function of a single gene involves extensive and time-consuming experiments, which could potentially shift the focus of our manuscript, we intend to pursue this as a separate research topic in the future. Secondly, despite evaluating and validating TMRS in the training set, internal validation set, and external validation set, as well as at both bulk-cell and single-cell levels, the evidence remains insufficient, especially its prognostic capabilities. Rigorous large-scale prospective clinical studies are necessary for further verification. Thirdly, in our pursuit of comprehensive model evaluation, certain content areas, such as the association between TMRS and immunotherapy/targeted therapy sensitivity, were inadequately explored and warrant dedicated clinical studies. Due to the limited number of patients who subsequently received immunotherapy, we lack data on the efficacy of immunotherapy in these patients, making it impossible to directly validate the association between TMRS and immunotherapy effectiveness within our own cohort. Conducting a

new prospective cohort study would require significant time; therefore, we hope to further explore this in future research. Fourthly, we observed inconsistent findings among different datasets. For instance, patients with high TMRS in TCGA exhibited more copy number variations, whereas this phenomenon was absent in CPTAC. These inter-dataset inconsistencies require additional investigation. Finally, although we employed various analytical methods across multiple datasets to explore the potential biological functions of TMRS and conducted experimental validations to minimize algorithmic and dataset biases, these studies remain correlational. For example, due to constraints in research scope and current experimental conditions—such as limited funding and a lack of relevant immune therapy animal models—we are currently unable to directly validate the relationship between TMRS and tumor immunotherapy through experimental means. Therefore, more in-depth in vivo and in vitro experiments are needed in the future to elucidate the biological functions of these genes.

Conclusion

This study comprehensively investigated the role of tryptophan metabolism in ccRCC, establishing a tryptophan metabolism-related signature (TMRS) that accurately predicts prognosis in patients with ccRCC and offers guidance for personalized treatment strategies.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13062-024-00576-w>.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Author contributions

Fan Li: Conceptualization, Methodology, Software, Formal analysis, Writing – Original Draft. Haiyi Hu: Data curation, Formal analysis, Writing – original draft. Liyang Li: Writing – Original Draft, Data curation, Methodology. Lifeng Ding: Methodology, Validation. Zeyi Lu: Investigation, Validation. Xudong Mao: Data curation. Ruyue Wang: Investigation, Validation. Wenqin Luo: Data curation, Methodology. Yudong Lin: Investigation, Validation. Yang Li: Visualization. Xianjong Chen: Visualization. Ziwei Zhu: Visualization. Yi Lu: Visualization. Chenghao Zhou: Data curation. Mingchao Wang: Funding acquisition. Liquan Xia: Funding acquisition, Project administration, Supervision, Writing – review & editing. Gonghui Li: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. Lei Gao: Conceptualization, Project administration, Supervision, Writing – review & editing. All authors reviewed the manuscript.

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The funder played no role in study design, data collection, analysis and interpretation of data, or the writing of this manuscript.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

All clinical renal cell carcinoma tissue specimens were obtained from patients at Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University. Informed consent was obtained from each participant prior to sample collection. This study adhered to the principles outlined in the Declaration of Helsinki and received approval from the Ethics Committee of Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University (No.20220317).

Competing interests

The authors declare no competing interests.

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