

RARRES2 promotes glycolytic reprogramming and malignant phenotypes in clear cell renal cell carcinoma

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1 **RARRES2 promotes glycolytic reprogramming and malignant phenotypes in**
2 **clear cell renal cell carcinoma**

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Abstract

Background: Clear cell renal cell carcinoma (ccRCC) is a metabolically driven malignancy characterized by profound metabolic reprogramming, with enhanced glycolysis representing a key feature. However, the relationship between glycolytic activation and ccRCC progression remains incompletely understood. This study aimed to characterize glycolytic activity in the ccRCC tumor microenvironment and identify a key biomarker associated with glycolytic reprogramming.

Methods: Single-cell RNA-sequencing data from GSE156632 and bulk transcriptomic data from the TCGA-KIRC cohort were analyzed. Single-cell analyses were performed to investigate the association between glycolytic activity and tumor epithelial cells within the tumor microenvironment, as well as related intercellular communication patterns. Bulk transcriptomic analyses were used to evaluate the clinical relevance of glycolysis-related features. Machine-learning algorithms combined with SHAP interpretation were applied to identify candidate biomarkers and construct diagnostic models. In vitro experiments were conducted in ccRCC cells to validate the functional role of RARRES2 in glycolysis and malignant progression. **Results:**

Tumor epithelial cells exhibited significantly elevated glycolysis scores in the ccRCC microenvironment. Highly glycolytic tumor epithelial cells showed enhanced outgoing signaling, particularly through the MIF-CD74/CXCR4 axis, and were associated with poorer prognosis, increased immune infiltration, and activation of hypoxia-related pathways. A stable five-gene signature was identified from 11 candidate genes, and the SVM classifier achieved the best diagnostic performance (AUC = 0.982). SHAP analysis highlighted RARRES2 as a consistent positive contributor. Functional experiments further demonstrated that RARRES2 knockdown suppressed proliferation, colony formation, and migration of ccRCC cells, reduced glucose

consumption, ATP production, and lactate production, and downregulated the mRNA expression of glycolysis-related genes, including HK2, LDHA, PKM2, and GLUT1 ($P < 0.05$). **Conclusions:** Multi-omics and machine-learning analyses identified RARRES2 as a glycolysis-associated biomarker in ccRCC and incorporated it into a diagnostic model. In vitro validation further demonstrated that RARRES2 contributes to glycolytic reprogramming and malignant phenotypes in ccRCC cells, supporting its potential role in the metabolic progression of ccRCC.

Keywords: clear cell renal cell carcinoma (ccRCC); glycolysis; diagnostic model; RARRES2; machine learning

Introduction

Renal cancer is one of the most common malignancies of the urinary system. Epidemiological statistics indicate that the global incidence of renal cancer is increasing, with an estimated 80,450 new cases and 15,160 deaths in the United States in 2026(1-3). Clear cell renal cell carcinoma (ccRCC) is the most prevalent histological subtype (4). Its key biological features include sustained activation of hypoxia signaling driven by loss of the von Hippel-Lindau (VHL) tumor suppressor, profound metabolic reprogramming, and a highly heterogeneous tumor microenvironment (TME) (5, 6). Although immunotherapy and its combinations with targeted therapies have markedly reshaped the clinical treatment landscape of ccRCC, recurrence and acquired resistance remain common(7, 8). Increasing evidence indicates that heterogeneity in therapeutic response in ccRCC is closely linked to metabolism-immunity crosstalk, whereby

lactate accumulation and export resulting from enhanced glycolysis, along with dysregulated lipid metabolism, promote the development of an immunosuppressive microenvironment, thereby impairing effector immune-cell function and limiting the benefit of immunotherapy(9, 10).

Mechanistically, ccRCC is widely regarded as a “metabolism-driven” tumor. Hypoxia adaptation mediated by the von Hippel-Lindau/hypoxia-inducible factor (VHL/HIF) axis coordinately rewires glucose metabolism, lipid metabolism, and redox homeostasis, while continuously remodeling the TME through lactate-driven acidification, nutrient competition, and metabolite signaling. Together, these processes shape the functional states of immune populations such as T cells and myeloid cells, and are associated with patient prognosis and therapeutic sensitivity (11-13). In this context, adipokines with dual properties of immune chemotaxis and metabolic regulation have attracted increasing attention. Chemerin, so known as Retinoic Acid Receptor Responder 2 (RARRES2) is a secreted protein that can regulate immune-cell recruitment and inflammation via the receptors CMKLR1, GPR1, and CCRL2, and it also intersects with lipid metabolism and glucose homeostasis (14). Notably, a recent integrative transcriptomic study incorporated RARRES2 into a CD8⁺ T-cell-related prognostic signature in ccRCC, supporting its potential clinical and immunological relevance in this malignancy(15). In addition, chemerin has been reported to be functionally important in ccRCC biology, particularly in the context of lipid metabolic regulation. As previously reported, RARRES2 can promote invasion and metastasis in gastric cancer while inhibiting melanoma growth. It may influence tumor progression by promoting angiogenesis, recruiting immune cells, and inducing lipid metabolism (16, 17). However, the association of RARRES2 with glycolytic activity and malignant progression in ccRCC remains unclear.

In recent years, on the one hand, single-cell technologies can be used to characterize tumor heterogeneity, reveal the tumor microenvironment, and explore the relationships among different cellular subpopulations in ccRCC. Meanwhile, bulk transcriptomic analysis provides an overall view of the molecular landscape of ccRCC, supporting the identification of subtype-related, prognostic, and functionally relevant genes and pathways (18-20). On the other hand, machine learning (ML) has been widely applied to renal cancer subtyping, diagnosis, and prognostic prediction (21, 22), while SHapley Additive exPlanations (SHAP) quantify feature contributions and reveal potential interactions, improving the transparency and biological interpretability of ML models (23, 24). Therefore, we integrated single-cell and bulk transcriptomic analyses to further elucidate the relationship between glycolysis and ccRCC, while combining machine-learning approaches with SHAP-based interpretation to develop a novel diagnostic model and validate the association of RARRES2 with glycolysis and disease progression.

Materials and Methods

Data sources

This study analyzed data from publicly available databases. Single-cell RNA sequencing data were obtained from the GSE156632 cohort in the GEO database (Gene Expression Omnibus, <https://www.ncbi.nlm.nih.gov/geo/>), which includes 5 normal kidney tissues and 7 ccRCC samples, and were used to characterize the cellular composition and transcriptional features of tumor and normal tissues. Bulk RNA-seq expression data and matched clinical information for the TCGA-KIRC cohort (The Cancer Genome Atlas; <https://portal.gdc.cancer.gov/>) for subtype

identification, prognostic evaluation, and model construction. As all datasets were publicly released and de-identified, ethics approval and informed consent were obtained in the original studies. Accordingly, our analyses complied with the relevant data-use policies and guidelines.

Single-cell transcriptomic analysis

Single-cell RNA sequencing data were analyzed with Seurat (25). During quality control, Cells with 200-6,000 detected genes (nFeature_RNA) and mitochondrial gene content < 20% were retained after quality control, and doublets were removed using DoubletFinder (26). The integrated dataset was then log-normalized with a scaling factor of 10,000, and the 2,000 most variable genes were selected for subsequent analyses. Principal component analysis (PCA) was performed first, followed by Harmony-based correction to reduce batch effects among samples. The corrected data were subsequently subjected to UMAP for visualization and Louvain clustering. Cell types were manually annotated according to canonical marker genes. AUCell was applied to assess quantify single-cell gene-set enrichment based on within-cell gene expression rankings. Within tumor epithelial cells (Epi-Tumor), the median AUCell glycolysis score was used to classify cells into high glycolysis score tumor epithelial cells (High-Epi) and low glycolysis score tumor epithelial cells (Low-Epi).

Intercellular communication analysis

The CellChat R package (v1.6.1) was used to infer ligand-receptor-mediated intercellular communication networks in the tumor microenvironment (27). CellChatDB is a curated ligand-receptor interaction database, and CellChatDB.human provides the human-specific interaction repertoire for cell-cell communication analysis. Using the Secreted Signaling subset, we compared

the communication probabilities and interaction strengths of High-Epi and Low-Epi cells with other cell populations. Signaling interactions were then aggregated at the pathway level, and key pathways driving between-group differences were identified through contribution decomposition and centrality analyses.

Enrichment analysis

Gene Set Variation Analysis (GSVA) (28) was performed based on the Hallmark gene sets obtained from MSigDB (29). Glycolysis enrichment scores and Hallmark pathway activity were further quantified using single-sample gene set enrichment analysis (ssGSEA), and differences between subtypes were compared with the Wilcoxon rank-sum test. Functional annotation analyses, including Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG), were carried out using the clusterProfiler package (30) .

Consensus clustering and Survival analysis

Based on the glycolysis-related gene set obtained from MSigDB, the corresponding expression matrix was extracted from the TCGA-KIRC bulk transcriptomic dataset. Consensus clustering was performed with ConsensusClusterPlus using the PAM algorithm with Pearson distance and Ward.D2 linkage, with 1,000 resamplings. The optimal cluster number was determined according to the consensus matrix, cumulative distribution function (CDF) plots, and item consensus level (ICL). We ultimately chose $k = 2$ and assigned patients to clust1 and clust2. Differential expression between clust1 and clust2 was assessed using the limma-voom workflow, with thresholds of $|\log_2FC| > \log_2(1.5)$ and $FDR < 0.05$. Overall survival (OS) differences were analyzed by Kaplan-Meier survival curves and compared using the log-rank test.

Immunological characteristics assessment

The ESTIMATE algorithm was applied to calculate the ImmuneScore, StromalScore, ESTIMATEScore, and tumor purity (31). Immune infiltration was assessed using IOBR by integrating CIBERSORT (32), TIMER(33), and IPS. Immune checkpoint gene expression was compared between subtypes using the Wilcoxon test.

Key gene selection

Three sets of differentially expressed gene (DEG) lists were generated: (i) DEGs between tumor and normal tissues in TCGA-KIRC; (ii) DEGs between glycolysis-related molecular subtypes in TCGA; and (iii) DEGs between glycolysis-stratified tumor epithelial cells in the single-cell dataset. We intersected the three DEG lists to obtain consistently upregulated candidate genes. Feature selection was performed using LASSO (glmnet), SVM-RFE (e1071), and random forest (randomForest). Genes retained by all three methods were defined as the key gene set.

Construction of diagnostic models

Based on the selected key genes, diagnostic classification models were developed in the TCGA-KIRC cohort. The dataset was randomly divided into a training set (70%) and a testing set (30%) by stratified sampling. Multiple machine-learning algorithms (**Table S1**) were implemented within the caret framework using repeated 10-fold cross-validation, and predicted probabilities were obtained for the testing set. Model discrimination was assessed by receiver operating characteristic (ROC) analysis and the corresponding area under the curve (AUC). The model achieving the highest AUC was chosen for subsequent interpretability analysis.

SHAP-based interpretability analysis

To interpret the best-performing model, permutation SHAP (permshap) was used to compute SHAP values for each feature gene, and results were visualized and summarized with shapviz. Gene importance was evaluated using SHAP value distributions across samples and mean absolute SHAP values.

Cell lines and transfection

The human ccRCC cell lines 786-O and 769-P, as well as the normal renal epithelial cell line RPTEC, were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). 786-O and 769-P cells were cultured in RPMI 1640 medium (Pan-Biotech, Aidenbach, Germany) supplemented with 10% fetal bovine serum (FBS; Gibco, Waltham, MA, USA) and 1% penicillin–streptomycin (Gibco, Waltham, MA, USA).

RPTEC cells were cultured in renal epithelial cell basal medium (ATCC, Manassas, VA, USA) supplemented with renal epithelial cell growth kit components according to the manufacturer's instructions. All cells were maintained at 37°C in a humidified incubator with 5% CO₂. Mycoplasma contamination was routinely tested using a MycoStrip® mycoplasma detection kit (InvivoGen, San Diego, CA, USA) according to the manufacturer's instructions, and all cell lines were confirmed to be mycoplasma-free.

For RARRES2 knockdown, a predesigned RARRES2-specific siRNA (IDT; hs.Ri.RARRES2.13.1) and a negative control DsiRNA (IDT; 51-01-14-04) were used. 786-O and 769-P cells (1×10^5 per well) were seeded into 6-well plates and incubated overnight for attachment. Transfection was then performed with siRARRES2 or control siRNA (siNC) using Lipofectamine RNAiMAX (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's

instructions. Cells were harvested 48 h after transfection for subsequent experiments.

Real-time quantitative PCR (RT-qPCR)

Total RNA was isolated with the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), and complementary DNA (cDNA) was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific). Quantitative PCR analysis was then carried out on a QuantStudio 3 Real-Time PCR System (Applied Biosystems) with PowerTrack™ SYBR Master Mix (Applied Biosystems). Relative mRNA expression was normalized against GAPDH and determined using the $2^{-\Delta\Delta C_t}$ method. Primers were obtained from Eurofins, and the primer sequences are listed in **Table S2**.

CCK-8 assay

Cell proliferation was measured using a CCK-8 assay. Briefly, 3×10^3 786-O or 769-P cells per well were seeded into 96-well plates. For five consecutive days, 10 μ L of CCK-8 solution (Dojindo, Kumamoto, Japan) was added to each well at the same time point daily. After 2 h of incubation, absorbance at 450 nm was recorded with a microplate reader (Thermo Fisher Scientific, Germany). OD450 values were used to plot cell proliferation curves.

Scratch assay

Transfected 786-O and 769-P cells were seeded into 6-well plates. After cells reached the appropriate confluence, straight scratches were created using sterile 200- μ L pipette tips. Images were captured at 0, 6, and 12 h after scratching under a ZEISS microscope (Oberkochen, Germany). Wound closure was quantified using ImageJ software (version 1.53; NIH, Bethesda, MD, USA).

211 **Transwell assay**

212 Cell migration were evaluated using Transwell chambers (SARSTEDT, Nümbrecht, Germany).
213 Transfected 786-O and 769-P cells were washed twice with DPBS and resuspended in serum-free
214 medium. A total of 3×10^4 cells in 200 μ L serum-free medium were added to the upper
215 chambers, while 750 μ L complete medium containing 20% FBS was added to the lower chambers
216 as a chemoattractant. After 3 days of incubation, cells were fixed with 4% paraformaldehyde and
217 stained with 0.1% crystal violet. Invaded cells were photographed under a ZEISS microscope.

218 **Colony formation assay**

219 Transfected 786-O and 769-P cells were seeded into 6-well plates at a density of 1,000 cells per
220 well. After incubation for 12 days in 786-O cells or 10 days in 769-P cells, colonies were fixed
221 with 4% paraformaldehyde and stained with 0.1% crystal violet. Colony images were then
222 acquired and recorded.

223 **Assessment of Cellular Glucose Metabolism**

224 Glycolysis-related metabolic activity was assessed by measuring glucose consumption, lactate
225 production and ATP levels. Lactate concentrations in culture supernatants were determined using
226 the L-Lactate Assay Kit (Abcam, Cambridge, UK) according to the manufacturer's protocol, and
227 absorbance was detected with a microplate reader. Glucose consumption was indirectly evaluated
228 by quantifying residual glucose in the culture supernatants using a Glucose Colorimetric Detection
229 Kit (Invitrogen, Frederick, MD, USA), followed by absorbance measurement with a microplate
230 reader. Intracellular ATP levels were measured with the ATP Determination Kit (Invitrogen,
231 Eugene, USA), and bioluminescence signals were acquired using a Tecan Spark plate reader

(Tecan, Männedorf, Switzerland). All assays were performed in at least three independent experiments.

Statistical analysis

All statistical analyses were performed using R (version 4.3.1) and GraphPad Prism 8.0. Student's t test and one-way analysis of variance (ANOVA) were used for statistical comparisons. All in vitro experiments were independently repeated at least three times ($n \geq 3$), and data are presented as mean $\pm$ standard deviation (SD). A two-sided $P < 0.05$ was considered statistically significant.

Results

Single-cell analysis reveals glycolytic predominance in ccRCC tumor epithelial cells

The overall analytical workflow is shown in **Figure 1**. Single-cell RNA-seq analysis revealed substantial cellular heterogeneity in ccRCC, and eight major cell types were identified through unsupervised clustering and cell-type annotation (**Figure 2A**), including normal epithelial cells (Epi-Normal), transdifferentiated epithelial cells (Epi-Changed), Epi-Tumor, myeloid cells, T cells, B cells, endothelial cells, and fibroblasts. Representative marker-gene expression patterns across these populations (**Figures 2B-C**) showed clear, cell-type-specific transcriptional features, supporting the accuracy of the annotations. Then, we compared cellular composition between normal and tumor tissues and found increased proportions of endothelial cells, fibroblasts, and myeloid cells in ccRCC tumors, whereas the proportion of Epi-Changed cells was decreased (**Figures 2D-E and Figure S1A**). Meanwhile, correlation analysis indicated that epithelial cells

from different tissue origins still exhibited a degree of transcriptional similarity (**Figure S2B**).

GSVA revealed that Epi-Tumor cells were significantly enriched in metabolism- and proliferation-related programs, including glycolysis, hypoxia, and reactive oxygen species pathways (**Figure 2F**). Correspondingly, glycolytic pathway analysis showed that Epi-Tumor cells had the highest glycolysis scores among all major cell types (**Figure 2G**). When stratified by tissue origin, epithelial cells in tumor tissues exhibited significantly higher glycolysis scores than those in normal tissues. In contrast, glycolysis scores were significantly lower in myeloid cells, T cells, endothelial cells, and fibroblasts in tumor tissues (**Figure 2H**; $p < 0.05$).

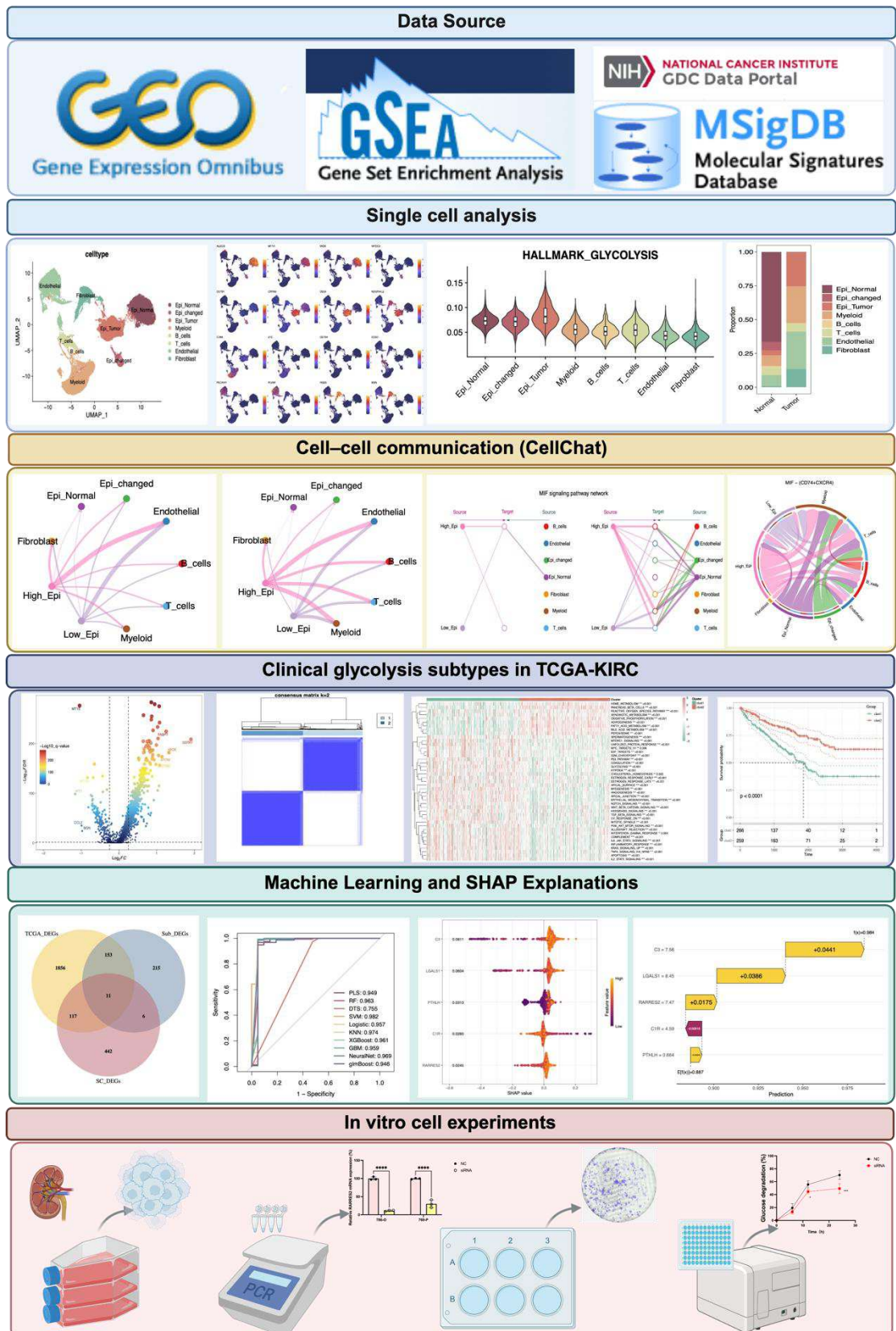


Figure 1. Study Workflow. Data were obtained from GEO (scRNA-seq), TCGA-KIRC (bulk transcriptomics),

and MSigDB gene sets. Single-cell analyses identified major cell populations and quantified glycolysis activity,

264 followed by CellChat-based inference of ligand-receptor communication between High-Epi and Low-Epi epithelial
265 subsets (highlighting MIF-CD74+CXCR4/CD44 signaling). TCGA-KIRC consensus clustering defined glycolysis-
266 associated clinical subtypes with distinct transcriptomic features and survival outcomes. Machine-learning models
267 with SHAP interpretation prioritized key genes, and in vitro assays in 786-O and 769-P cells validated the role of
268 RARRES2 in malignant phenotypes and glycolysis-related metabolism.

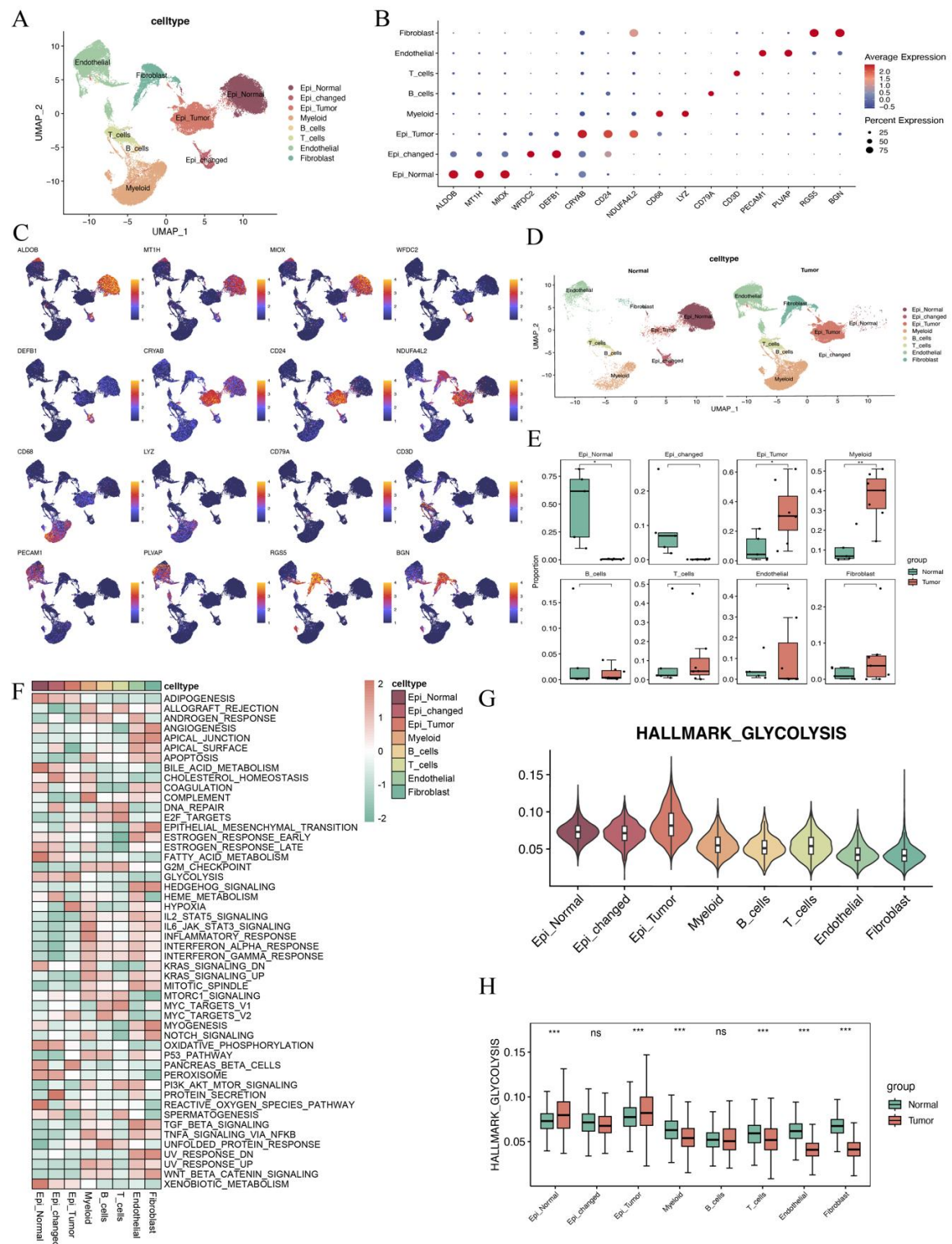


Figure 2. Single-cell annotation and pathway activity profiling across major cell populations.(A) UMAP

visualization of cell clustering and annotation across major cell populations and epithelial subtypes.(B) Dot plot

summarizing canonical marker gene expression across cell populations/subtypes.(C) Feature plots showing the

spatial localization of representative marker genes in UMAP space to support cell-type assignments.(D) UMAPs split by sample origin (Normal and Tumor) to visualize group-specific cellular distributions.(E) Cell-type/subtype composition summarized as proportions by sample origin.(F) Heatmap summarizing Hallmark pathway activity scores across major cell populations and epithelial subtypes.(G) Distribution of HALLMARK_GLYCOLYSIS activity scores across cell populations/subtypes (violin plot). (H) Comparison of HALLMARK_GLYCOLYSIS activity scores between Normal and Tumor within each cell population/subtype (box plots with statistical annotation).

Highly glycolytic tumor epithelial cells displayed distinct transcriptional and pathway activation features

To systematically characterize the molecular features of tumor epithelial cells across distinct glycolytic states, we performed differential expression analysis between High-Epi and Low-Epi. Compared with Low-Epi, multiple genes were significantly upregulated in High-Epi cells, including FABP7, MIOX, CTSD, and GSTA1 (**Figure 3A**). KEGG analysis showed that the differentially expressed genes were significantly enriched in oxidative phosphorylation (**Figure 3B**). GO analysis further highlighted pathways related to energy generation, cofactor metabolism, and oxidoreductase activity (**Figure 3C-E**). Consistently, Hallmark pathway scoring revealed that High-Epi cells were significantly enriched in hypoxia, oxidative phosphorylation, reactive oxygen species pathway, fatty acid metabolism, and peroxisome (**Figure 3F**). Together, these findings suggest that distinct glycolytic states in tumor epithelial cells are associated with broader metabolic reprogramming.

Hallmark pathway activity scores across grouped cell populations.

Distinct intercellular communication patterns between highly and lowly glycolytic tumor epithelial cells

To compare intercellular communication differences between tumor epithelial cells with distinct glycolytic states, we analyzed ligand-receptor-mediated communication networks of High-Epi and Low-Epi tumor epithelial cells. At the global network level, High-Epi cells formed a denser communication network as signal senders and established broader interactions with multiple cell types (**Figures 4A-B**). Comparison of communication strength showed that High-Epi cells exhibited higher interaction strength than Low-Epi cells in both incoming and outgoing directions, with a more pronounced increase in outgoing signaling (**Figure 4C**). To identify pathways underlying these differences, we next compared outgoing signals from the two epithelial subsets at the pathway level. Among these pathways, the MIF signaling axis (MIF-CD74+CXCR4/CD44) was the most prominently activated in High-Epi cells (**Figures 4D-E**). Consistent with this, High-Epi cells showed stronger outgoing communication links with multiple cell types as the signal source (**Figure 4F**). In addition, contribution analysis of receptor combinations within the MIF pathway revealed that the CD74+CXCR4 receptor pair was dominant (**Figure S1C**). Within this pathway, High-Epi cells further displayed stronger signal output capacity (**Figure 4G**).

(CD74+CXCR4) axis across cell populations.

The high-glycolysis group exhibited poorer prognosis and distinct immune microenvironment characteristics

To evaluate the clinical relevance of glycolysis in ccRCC, we performed consensus clustering of patients in the TCGA-KIRC cohort based on the expression patterns of glycolysis-related genes, stably identifying two molecular subtypes (Cluster 1 and Cluster 2) (**Figure 5A**). Cluster 1 exhibited significantly higher glycolytic score (**Figure 5B**; $p < 0.001$) and markedly poorer overall survival than Cluster 2 (**Figure 5C**; $p < 0.0001$). Differential expression patterns of glycolysis-related genes between the two clusters are shown in **Figure S1D**, further supporting the molecular heterogeneity associated with distinct glycolytic states. GO and KEGG analyses of the differentially expressed genes indicated that the differentially expressed genes were mainly enriched in extracellular matrix-related processes and canonical cancer-associated signaling pathways such as PI3K-Akt pathways (**Figures 5D-E**). GSVA further showed that Cluster 1 had significantly increased activities of hypoxia response, IL6-JAK-STAT3 signaling, and PI3K-Akt-mTOR signaling, whereas oxidative phosphorylation and the reactive oxygen species (ROS) pathway were reduced (**Figure 5F**). Notably, bulk transcriptomic subtypes reflect not only epithelial-intrinsic programs but also variations in stromal and immune cell composition, which may influence the apparent oxidative phosphorylation and ROS signatures observed at the cohort level.

We further compared immune microenvironment characteristics between the two subtypes.

345 ESTIMATE analysis showed that the ImmuneScore, StromalScore, and ESTIMATEScore were all
346 significantly higher in Cluster 1 than in Cluster 2 (**Figure 5G**). Consistently, immune infiltration
347 analyses indicated enhanced immune-cell infiltration in Cluster 1. CIBERSORT showed increased
348 M0 macrophage infiltration, while TIMER revealed significantly higher infiltration of CD4⁺ T
349 cells, macrophages, and neutrophils. IPS analysis further demonstrated a significantly elevated
350 effector immune-cell score (**Figure 5H**). In addition, immune checkpoint expression analysis
351 demonstrated that CD40LG, IL23A, TNFRSF8, TNFSF14, and PVR were expressed at
352 significantly higher levels in Cluster 1 than in Cluster 2 (**Figure 5I**).

associated differentially expressed genes (DEGs). (E) Circular plot summarizing KEGG pathway enrichment of subtype-associated DEGs. (F) Heatmap of Hallmark pathway activity scores across samples, annotated by subtype. (G) Violin plots comparing ESTIMATE-derived metrics (ImmuneScore, StromalScore, ESTIMATEScore, and TumorPurity) between subtypes. (H) Heatmap summarizing differences in immune infiltration/immune-related signatures estimated by multiple algorithms across subtypes. (I) Box plots comparing the expression of selected immune checkpoint and immunoregulatory genes between subtypes.

Machine learning identifies key genes and builds a high-performing diagnostic model

By intersecting marker genes identified from single-cell RNA-seq data, genes associated with glycolysis-based subtypes in TCGA-KIRC, and TCGA-KIRC differentially expressed genes, we identified 11 significantly upregulated candidate genes closely linked to glycolysis-related subtypes and tumor epithelial cell characteristics (**Figure 6A**). These genes were expressed at significantly higher levels in tumor tissues than in normal tissues (**Figure 6B**), including LGALS1, PTHLH, C3, LOX, TIMP1, and RARRES2, among others. To identify robust features across multiple methods, we applied three machine-learning algorithms for feature selection among the 11 candidate genes, including LASSO regression (**Figures 6C-D**), support vector machine (**Figure 6E**), and random forest (**Figure 6F**). We then took the intersection of features selected by the three algorithms and identified five key genes consistently recognized across models (**Figure 6G**). Furthermore, we constructed 10 machine-learning diagnostic models using these five key genes and systematically evaluated their classification performance. All models achieved $AUC > 0.70$, with the SVM model showing the highest AUC (0.982), indicating the best diagnostic performance among the tested models (**Figure 6H**). These results further support the potential of these key genes to distinguish glycolysis-related molecular subtypes

380 in ccRCC.

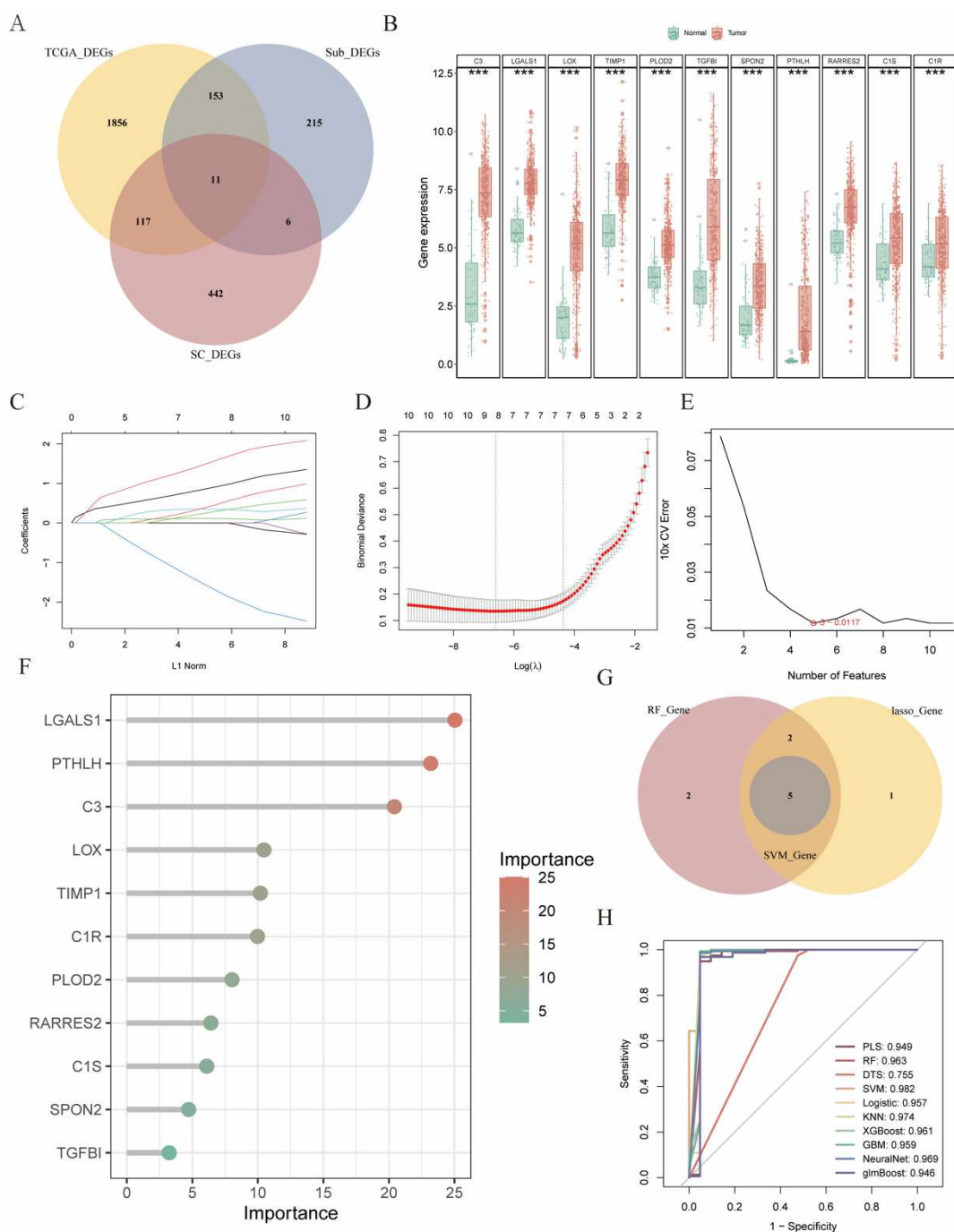


Figure 6. Integrated feature selection and machine-learning model development for a diagnostic signature

(A) Venn diagram showing the overlap among DEGs identified from bulk TCGA analysis, DEGs between glycolytic subgroups, and single-cell-derived DEGs. (B) Box plots comparing the expression of the overlapping candidate genes between Normal and Tumor samples. (C) LASSO coefficient profiles across the regularization

path, showing how candidate gene coefficients change with increasing penalty. (D) Ten-fold cross-validation curve for LASSO used to identify the optimal regularization parameter (λ). (E) Cross-validation error curve used to determine the optimal number of selected features. (F) Random forest variable-importance ranking of candidate genes. (G) Venn diagram summarizing the intersection of features selected by LASSO, SVM-based selection, and random forest to define the final key-gene set. (H) ROC curves comparing the performance of multiple machine-learning classifiers, with AUC values reported for model evaluation.

SHAP interpretability quantifies gene contributions and reveals potential interactions

To quantify the relative contribution of each key gene to model predictions, we performed a SHAP-based interpretability analysis of the SVM model. RARRES2 exhibited a positive tendency in SHAP value distribution, suggesting a positive contribution to model prediction and potential clinical relevance for ccRCC diagnosis (**Figure 7A**). Consistently, a SHAP force plot for a representative sample indicated that LGALS1, C3, PTHLH, and RARRES2 were the main positive drivers of the prediction, whereas C1R made a weaker and predominantly negative contribution (**Figure 7B**). Moreover, PTHLH exhibited a rapid increase in contribution followed by a plateau, suggesting a saturating effect that was more evident under higher RARRES2 expression. By contrast, C1R showed an overall negative trend, with higher expression associated with lower model output, and this effect was modulated by C3 levels. RARRES2 displayed a non-monotonic pattern, with the most negative SHAP values at intermediate expression levels, while higher PTHLH expression shifted its contribution in a more positive direction, indicating conditional dependence among these features (**Figure 7C**).

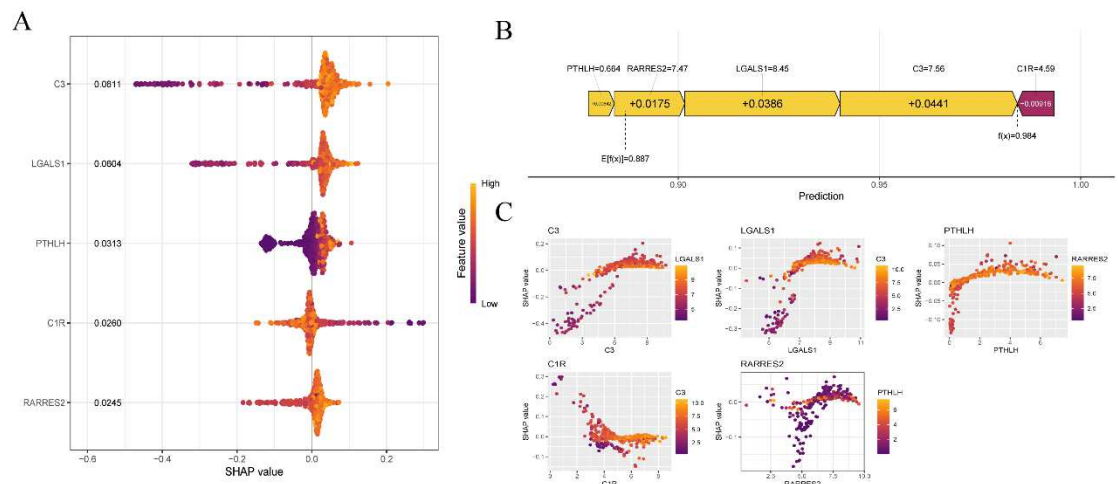


Figure 7. SHAP-based interpretation of the diagnostic model (A) SHAP value distributions for the five key genes (C3, LGALS1, PTHLH, C1R, and RARRES2), summarizing their contributions to model output. (B) Representative SHAP force plot showing the direction and magnitude of feature effects at the single-sample level. (C) SHAP dependence plots highlighting nonlinear feature effects and interactions.

RARRES2 knockdown suppresses malignant phenotypes and impairs glucose metabolism

As shown in **Figure 8A**, RARRES2 expression was significantly upregulated in 786-O and 769-P cells with RPTEC. To further investigate the functional role of RARRES2 in ccRCC, we performed siRNA-mediated knockdown in 786-O and 769-P cells. qRT-PCR analysis demonstrated that, compared with the negative control (NC) group, siRNA treatment significantly reduced RARRES2 mRNA levels in both cell lines (**Figure 8B**; $p < 0.01$), confirming efficient knockdown. We then assessed the effects of RARRES2 knockdown on malignant phenotypes of ccRCC cells. CCK-8 assays demonstrated that, at multiple time points, the siRNA group exhibited significantly reduced proliferative capacity relative to controls (**Figures 8C-D**; $p < 0.05$). In wound-healing assays, RARRES2 knockdown markedly impaired the migratory ability of 786-O

and 769-P cells, with substantially reduced wound closure within 6h and 12 h compared with the NC group (**Figure 8E**; $p < 0.05$). Colony formation assays further showed that RARRES2 knockdown significantly decreased clonogenic capacity in both cell lines, as evidenced by a marked reduction in colony numbers (**Figure 8F**; $p < 0.05$). In addition, Transwell assays indicated that siRNA treatment significantly reduced the number of cells migrating through the membrane (**Figure 8G**; $p < 0.01$), supporting a pro-migratory role of RARRES2 in ccRCC cells.

Given these phenotypic changes and the potential link between RARRES2 and tumor metabolism, we next examined its impact on glucose metabolic programs. qRT-PCR showed that RARRES2 knockdown significantly downregulated the expression of glycolysis-related genes HK2, LDHA, PKM2, and GLUT1 (**Figure 8H**; $p < 0.05$). In the glucose consumption assay, the siRNA group showed significantly reduced glucose consumption compared with the NC group at 6, 12, and 24 h, suggesting that RARRES2 knockdown suppresses glycolytic activity (**Figure 8I**). Consistently, RARRES2 knockdown significantly decreased ATP production (**Figure 8J**; $p < 0.05$) and lactate production (**Figure 8K**) in both cell lines. A schematic model of the proposed mechanism by which RARRES2 regulates glycolysis to promote ccRCC progression is presented in **Figure 9**. Based on our findings, RARRES2 may enhance ccRCC progression by sustaining glycolytic activity, thereby promoting tumor cell proliferation and migration.

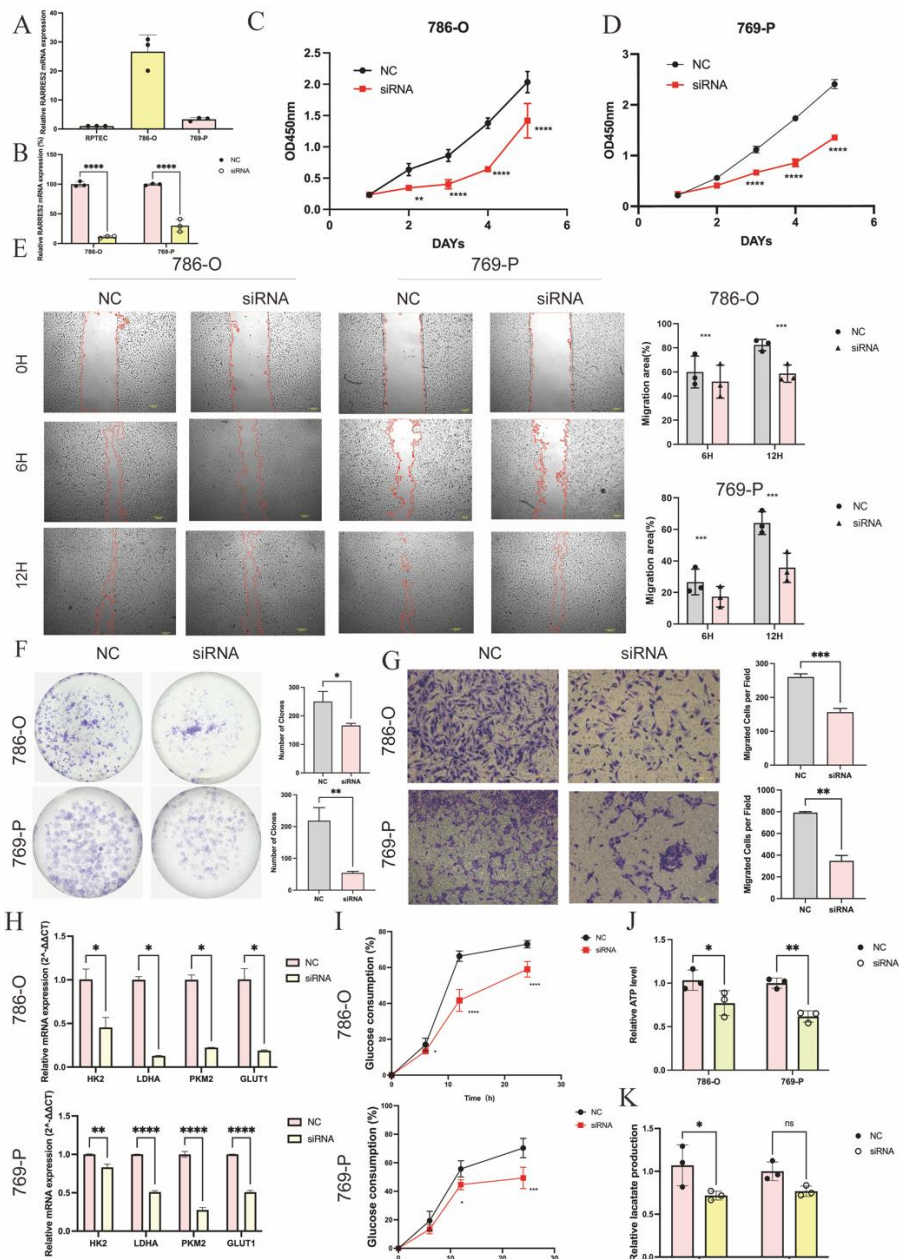


Figure 8. Knockdown of RARRES2 suppresses malignant phenotypes and glucose metabolism in ccRCC cells in vitro (A) Relative mRNA expression of RARRES2 in 786-O and 769-P cells compared with RPTEC. (B) qRT-PCR validation of RARRES2 knockdown in 786-O and 769-P cells after siRNA transfection (B-C) CCK-8 proliferation assays in 786-O (C) and 769-P (D) cells, showing reduced growth upon RARRES2 silencing at the indicated time points. (E) Wound-healing assays with representative images (0 h, 6h and 24 h) and quantification, indicating decreased migration after RARRES2 knockdown. (F) Colony formation assays showing reduced

clonogenic capacity following RARRES2 knockdown. (G) Transwell invasion assays demonstrating fewer invaded cells after RARRES2 knockdown. (H) Knockdown of RARRES2 reduces the expression of key glycolytic genes (HK2, LDHA, PKM2, GLUT1) in ccRCC cells. (I) Glucose consumption assay showing reduced glucose utilization after RARRES2 knockdown. (J) Cellular ATP levels are reduced in both ccRCC cell lines following RARRES2 knockdown. (K) Lactate production is decreased in 786-O and 769-P cells upon RARRES2 knockdown.

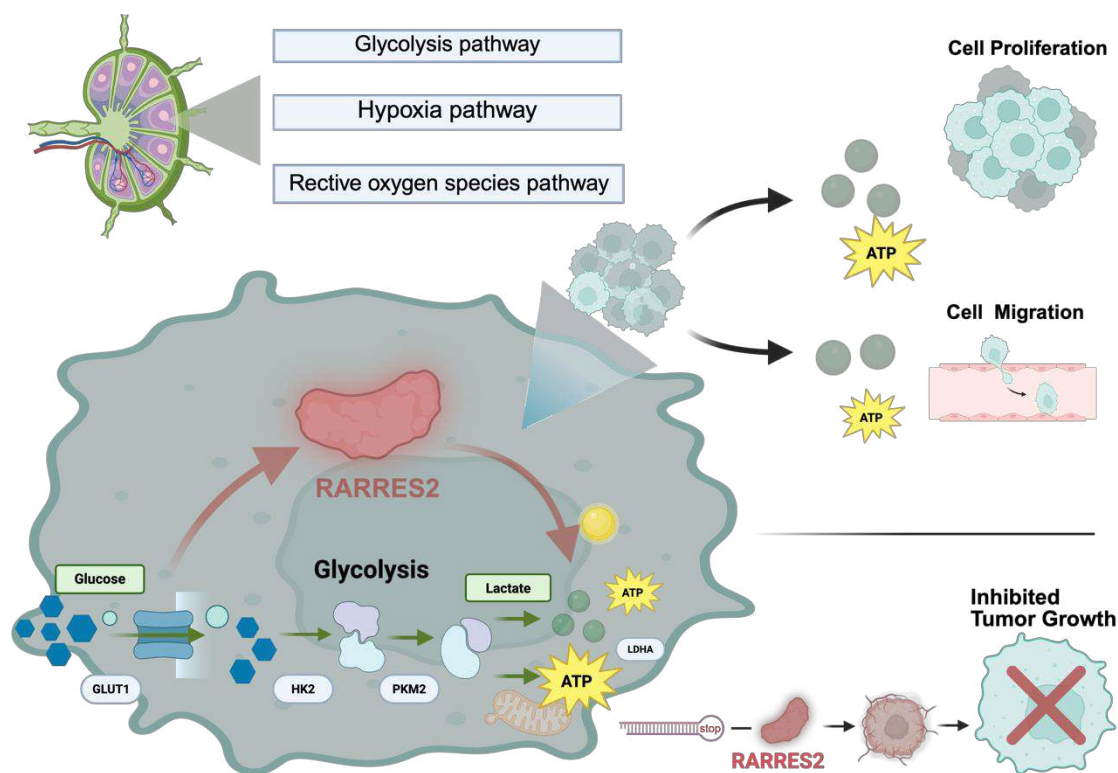


Figure 9. Proposed model of RARRES2-driven glycolysis in clear-cell renal cell carcinoma (ccRCC)

RARRES2 is depicted as a key regulator of the glycolysis program in renal tumor epithelial cells, associated with hypoxia/ROS-related signaling and enhanced glucose uptake, lactate production, and ATP generation, thereby supporting tumor cell growth and migration. RARRES2 inhibition is schematically shown to attenuate glycolysis and suppress malignant behaviors.

Discussion

ccRCC has long been considered a tumor driven primarily by metabolic reprogramming (34). Among these, enhanced glycolysis represents one of the most characteristic metabolic features (6, 35) and is tightly linked to immune microenvironment shaping. To investigate the relationship between ccRCC and glycolysis, we integrated single-cell transcriptomic data with TCGA datasets and found that glycolysis was predominantly enriched in tumor epithelial cells. Moreover, highly glycolytic tumor epithelial cells were associated with poorer prognosis and higher immune scores. Using machine-learning approaches combined with SHAP interpretation, we developed a new diagnostic model with strong performance, in which RARRES2 emerged as the most prominent feature. Further in vitro validation demonstrated that RARRES2 knockdown suppressed ccRCC cell proliferation and migration, accompanied by reduced expression of glycolysis-related genes, reduced glucose consumption, and decreased ATP and lactate production.

The Warburg effect, characterized by increased aerobic glycolysis, is widely recognized as a metabolic hallmark of diverse cancers (36) and is particularly pronounced in VHL-deficient ccRCC as a consequence of HIF activation(37). VHL loss-induced pseudohypoxia and sustained HIF activation have been shown to promote tumor growth and progression through multiple downstream pathways, with enhanced glycolysis and repression of fatty acid metabolism representing key features of this reprogramming program (38, 39). Our findings are consistent with those of Yang G et al (40) showing that ccRCC exhibits substantial metabolic heterogeneity across different cell types and spatial contexts. In contrast, by stratifying tumor epithelial cells according to glycolysis scores, we found that genes such as FABP7, MIOX, CTSD, and GSTA1 were significantly upregulated in highly glycolytic tumor epithelial cells, and GO enrichment

pointed to redox enzyme activity-related molecular functions. Recent evidence indicates that MIOX exhibits relative specificity and independent prognostic relevance in ccRCC(41), while FABP7, as a fatty acid-binding protein, participates in long-chain fatty acid handling and lipid metabolic regulation(42). Our results further suggest that, in ccRCC, the MIF-CD74+CXCR4 axis may be preferentially activated in highly glycolytic tumor epithelial cells, which is particularly relevant because MIF has been reported to drive tumor pathogenesis by promoting proliferation, angiogenesis, immune evasion, and metastasis (43). These findings raise the possibility that activation of this signaling axis may contribute to the aggressive behavior of highly glycolytic tumor cells.

Our findings showed that patients with high glycolytic status had worse prognosis, which is consistent with prior studies suggesting that glycolytic reprogramming, together with alterations in the immune microenvironment, may jointly promote aggressive tumor phenotypes and unfavorable outcomes (44, 45). Interestingly, despite their poorer prognosis, patients with high glycolytic status also displayed higher immune score, implying that increased immune infiltration in this context may not necessarily reflect effective antitumor immunity. This may be reasonably explained by the progressive decline in immune function during ccRCC progression (46). In addition, lactate production and the acidic tumor microenvironment generated by highly glycolytic tumor cells may further impair T-cell effector functions and promote myeloid immunosuppressive phenotypes, reinforcing the formation and maintenance of an immunosuppressive tumor microenvironment (47, 48).

Chemerin (RARRES2) is a secreted protein with dual immuno-metabolic properties that can regulate the tumor microenvironment via autocrine signaling and influence biological processes

such as tumor cell migration and invasion. Its role can be context dependent, exhibiting either tumor-suppressive or tumor-promoting effects across cancer types (17, 49-51). In ccRCC, mechanistic studies have suggested that RARRES2 is closely involved in metabolic remodeling: it can inhibit fatty-acid oxidation, maintain lipid homeostasis, and modulate susceptibility to ferroptosis, thereby supporting tumor growth and stress tolerance (52, 53). Similarly, we conducted in vitro experiments to further validate this association. In 786-O and 769-P cells, RARRES2 knockdown significantly suppressed cell proliferation and migration and reduced clonogenic capacity. Notably, downregulation of RARRES2 was accompanied by marked impairment of glucose metabolism.

GLUT1, HK2, PKM2, and LDHA represent core components of the glycolytic machinery, spanning sequential steps from glucose uptake to lactate production (54-60). Specifically, GLUT1 facilitates transmembrane glucose import, HK2 catalyzes the first committed step of glucose metabolism, PKM2 governs the terminal glycolytic conversion to pyruvate and is functionally linked to aerobic glycolysis, whereas LDHA drives pyruvate-to-lactate conversion while regenerating NAD⁺, thereby sustaining glycolytic flux. In our study, RARRES2 knockdown consistently reduced the mRNA expression of all four genes. Given that these molecules collectively cover the major functional axis of glycolysis, their coordinated downregulation strongly supports the notion that RARRES2 is closely involved in the maintenance of the glycolytic program in ccRCC. Concomitantly, RARRES2 knockdown led to a marked reduction in glucose consumption, ATP production, and lactate production. In ccRCC, these metabolic readouts are widely used to characterize glycolytic activity, and their simultaneous decline is generally indicative of attenuated aerobic glycolysis and impaired tumor energy metabolism. Therefore,

RARRES2 may serve not only as a key diagnostic gene for ccRCC but also as a molecule tightly coupled to glycolysis-associated metabolic reprogramming, thereby contributing to tumor progression (61, 62).

Overall, through multi-omics analyses centered on glycolytic reprogramming, we characterized the metabolic advantage of ccRCC tumor epithelial cells and developed a high-performing diagnostic model. In vitro assays further demonstrated that RARRES2 contributes to ccRCC cell proliferation, migration, and glycolytic metabolism, supporting its potential role as a link between tumor metabolic adaptation and microenvironment remodeling. Nevertheless, several limitations should be acknowledged. First, the diagnostic model and molecular stratification were primarily derived from retrospective analyses of public datasets and have not yet been validated in large independent clinical cohorts or prospective settings, which may limit their immediate generalizability and clinical applicability. Second, although the biological effects of RARRES2 were consistently validated in ccRCC cells, the present study did not fully elucidate the precise molecular mechanisms underlying RARRES2-mediated glycolytic regulation. In addition, the functional validation mainly relied on the current RNA interference strategy, and further confirmation using complementary approaches will be needed to strengthen causal inference. Future studies should therefore focus on validating the glycolysis-associated signature and diagnostic model in independent clinical cohorts, while further dissecting the mechanistic basis by which RARRES2 regulates glycolytic programs and interacts with microenvironment remodeling.

Conclusion

In summary, our study reveals a biologically meaningful link between glycolysis and

547 ccRCC, establishes a novel glycolysis-associated diagnostic model, and further validates the
548 functional relevance of RARRES2 in ccRCC. Collectively, these findings advance the current
549 understanding of metabolic reprogramming in ccRCC and provide a foundation for future
550 mechanistic investigation and clinical translation.

551 **List of abbreviations**

552 AUC: Area under the curve

553 ATP: Adenosine triphosphate

554 C1R: Complement C1r

555 C3: Complement C3

556 CCRL2: C-C motif chemokine receptor-like 2

557 CMKLR1: Chemerin chemokine-like receptor 1

558 GEO: Gene Expression Omnibus

559 GO: Gene Ontology

560 GPR1: G protein-coupled receptor 1

561 GSVA: Gene set variation analysis

562 HIF: Hypoxia-inducible factor

563 KEGG: Kyoto Encyclopedia of Genes and Genomes

564 LGALS1: Galectin 1

565 MIF-CD74/CXCR4: Macrophage migration inhibitory factor–CD74/CXCR4 signaling axis

566 ML: Machine learning

567 PCA: Principal component analysis

568 PTHLH: Parathyroid hormone-like hormone

569 RARRES2: Retinoic acid receptor responder 2

570 ROC: Receiver operating characteristic

571 SHAP: Shapley additive explanations

572 ssGSEA: Single-sample gene set enrichment analysis

573 SVM: Support vector machine

574 TCGA-KIRC: The Cancer Genome Atlas Kidney Renal Clear Cell Carcinoma

575 TME: Tumor microenvironment

576 UMAP: Uniform manifold approximation and projection

577 VHL: Von Hippel-Lindau

578

579 **Declarations**

580 **Ethics approval and consent to participate:** Not applicable.

581 **Consent for publication:** Not applicable.

582 **Availability of data and materials:** The datasets analyzed in this study are publicly

available. TCGA-KIRC RNA-sequencing, clinical, and mutation data were obtained from The Cancer Genome Atlas (TCGA), and the single-cell RNA-sequencing dataset was obtained from the Gene Expression Omnibus (GEO) under accession number GSE156632. The R scripts used for data analysis are available from the corresponding author upon reasonable request.

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Author contributions: L.C., A.S., and I.G.H.S.-W. conceived and designed the research.

L.C., Y.C., Y.H.L., Y.T.L., J.G., X.L., and X.R. performed the research and acquired the data.

L.C., Y.C., Y.H.L., X.R., and S.M. analyzed and interpreted the data. L.C. drafted the

manuscript, and all authors were involved in revising the manuscript. All authors approved

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