

Dietary Fat as a Modulator of Tumor Malignancy in Cholangiocarcinoma: Prognostic Models and Therapeutic Insights

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28

29 **Abstract:** This study aimed to investigate the prognostic significance of dietary
30 fat-related genes in cholangiocarcinoma (CCA). We first retrieved a gene set related to
31 dietary fat from the Comparative Toxicogenomics Database (CTD). Univariate and
32 multivariate Cox regression analyses across multiple cohorts revealed that 21 dietary
33 fat-related genes were significantly associated with patient survival. Based on these
34 findings, we developed a prognostic model using LASSO regression, which
35 demonstrated strong predictive performance with a concordance index (C-index) of
36 0.700. The model included genes such as *CD300A*, *CD44*, *CELSR3*, *CENPA*, *DDIT4*,
37 *DHRS9*, *F3*, *FGFR4*, and *GREM1*. To further assess the clinical relevance of these
38 genes, we constructed a nomogram, which highlighted their potential role in CCA
39 prognosis. Additionally, the model genes showed strong correlations with immune cell
40 infiltration and chemotherapy drug sensitivity, with significant differences observed
41 between high- and low-risk groups. Molecular docking analysis further revealed critical
42 interactions between harmful dietary fats, palmitic acid and elaidic acid, and the model
43 proteins, suggesting their involvement in CCA progression. Collectively, these findings
44 underscore the significant role of dietary fat exposure in CCA prognosis and provide a
45 novel framework for developing personalized therapeutic strategies.

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47 **Keywords:** Dietary fat-related genes; Cholangiocarcinoma; Immune cell
48 infiltration; Molecular docking

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Introduction

Dietary fats are essential macronutrients that contribute to energy supply, membrane structure, and the synthesis of numerous bioactive molecules¹. They are primarily composed of triglycerides and phospholipids, which contain diverse fatty acids (FAs) differing in chain length and saturation degree². Based on the saturation level of their constituent FAs, dietary fats are categorized into saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA)^{3,4}. Industrial processing has additionally introduced trans fatty acids (TFA), a distinct group of unsaturated fats that exhibit pro-inflammatory effects due to their cytotoxic properties⁵. Collectively, dietary fats display heterogeneous health effects, MUFA and PUFA, particularly omega-3 fatty acids, acting beneficially impacts, and SFA and TFA exerting detrimental impacts⁶⁻⁹. Accordingly, the World Health Organization (WHO) recommends limiting total fat intake of adults to less than 30% of total energy, with SFA below 10% and TFA below 1%, while ensuring adequate omega-3 and omega-6 fatty acids intake for cardiovascular health and metabolic balance¹⁰.

Cholangiocarcinoma (CCA) is a highly aggressive malignancy arising from biliary epithelial cells and is characterized by a particularly poor prognosis and an multidrug resistance to pharmacological therapies^{11,12}. Globally, the incidence of CCA has continued to rise over the past decades, and the 5-year survival rate remains below 10%, due in part to the fact that CCA often remains asymptomatic or presents with non-specific symptoms in its early stages, leading most patients to be diagnosed at advanced stage^{13,14}. Despite therapeutic advances, the current treatment landscape remains limited, apart from surgical resection, chemotherapy and radiotherapy are the major therapeutic approaches¹⁵. However, only a minority of patients are eligible for surgery, and nearly two thirds of those who undergo resection experience recurrence^{16,17}. Moreover, existing chemotherapeutic, targeted, and immunotherapeutic regimens provide only modest clinical benefit¹⁸. Multiple risk factors, particularly metabolic and

inflammatory disturbances, contribute to the development of CCA¹⁹. Therefore, there is an urgent need to identify modifiable risk factors and elucidate the molecular determinants that drive its initiation and progression.

Emerging evidence suggests that dietary fat intake, particularly diets enriched in SFA, may influence the development of CCA through metabolic and inflammatory dysregulation. Epidemiological and experimental studies have shown that SFA promote systemic inflammation, insulin resistance, and other metabolic disturbances increasingly linked to elevated CCA risk²⁰⁻²². Mechanistically, excessive dietary fat can alter bile acid composition and enterohepatic circulation, contributing to cholangiocyte injury, oxidative stress, and chronic biliary inflammation, which are pathological processes central to cholangiocarcinogenesis^{23,24}. These effects may be amplified in cholangiocytes, which reside at the interface of bile acid exposure and hepatic metabolic flux and are therefore highly sensitive to metabolic perturbations induced by dietary fat. Despite these insights, the molecular pathways connecting dietary fat exposure to CCA remain largely undefined, and the gene networks through which lipid-related alterations shape tumor initiation, progression, and the immune microenvironment have not been comprehensively characterized.

This study aims to identify dietary fat-related molecular signatures in CCA, elucidate their biological and immunological relevance, and evaluate their potential clinical utility in prognostic assessment and therapeutic stratification. By clarifying how dietary fat-related pathways intersect with cholangiocarcinogenesis, this work seeks to provide new insights into prevention strategies and inform the development of lipid-metabolism-oriented interventions for CCA.

Materials and Methods

Data acquisition and preprocessing

RNA-sequencing data and corresponding clinical pathology information for the

TCGA-CHOL cohort were retrieved from The Cancer Genome Atlas (TCGA) database (<https://portal.gdc.cancer.gov/>). Clinical pathology information and whole-genome expression profiles for the GSE107943 cohort were downloaded from the Gene Expression Omnibus (GEO) database²⁵. In addition, the Fan_match cohort reported in previous studies was obtained for further comparative analysis²⁶. The single-cell RNA-sequencing cohort GSE171899 was also acquired from the GEO database. Information on genes associated with dietary fat exposure and their interaction frequencies were retrieved from the Comparative Toxicogenomics Database (CTD, <http://ctdbase.org/>). All datasets were processed according to their respective platform requirements. The main workflow of this study is shown in Fig. 1.

Functional enrichment and pathway analysis

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were conducted using the “cluster profiler” package, with significance defined as $P < 0.05$. Hallmark gene sets were obtained from the Molecular Signatures Database (MSigDB, <https://www.gsea-msigdb.org/gsea/msigdb>), and pathway activity scores were calculated through Gene Set Variation Analysis (GSVA). Pathways meeting a significance threshold of $P < 0.05$ were retained for further analysis. Gene Set Enrichment Analysis (GSEA) was subsequently performed using the “pheatmap” package to compare functional differences in GO terms between high- and low-risk groups.

Screening and interaction analysis of candidate prognostic genes

Genes associated with dietary fat with more than 3 interactions were evaluated for prognostic relevance using univariate Cox regression analysis within each cohort, applying a significance threshold of $P < 0.1$ to identify genes significantly associated with patient survival. Survival-associated genes shared across the three cohorts were identified through intersection analysis and retained as candidate prognostic genes. Meanwhile, heatmaps were constructed using the “pheatmap” package to visualize the

expression differences of these genes between normal and CCA samples. Protein-protein interaction (PPI) network between these genes was constructed using the STRING database (<https://string-db.org/>) and visualized using the Cytoscape (version 3.10.0).

Prognostic gene signature generated by machine learning

Multiple machine learning algorithms were applied to construct prognostic models using the Fan_match cohort as the training set, and TCGA-CHOL and GSE107943 cohorts as external validation sets. Ten algorithms, including Stepwise Cox (StepCox), generalized boosted regression modeling (GBM), random survival forest (RSF), CoxBoost, elastic network (Enet), partial least squares regression for survival (plsRcox), survival support vector machine (survival-SVM), supervised principal components (SuperPC), Lasso, and Ridge, were combined to generate 116 algorithm combinations. Model construction was performed using leave-one-out cross-validation (LOOCV) based on the expression profiles of prognostic candidate genes. Model performance was quantified using the Harrell's concordance index (C-index) across all cohorts. The algorithm with the highest average C-index was selected as the optimal model to identify model genes. Chromosomal locations of the model genes were obtained from the Ensembl database (<https://asia.ensembl.org/index.html>) and visualized using a circos plot generated with the "RCircos" package.

The risk score calculation formula is as follows:

$$\text{Risk Score} = \sum_{i=1}^9 \beta_i * E_i$$

β_i is the coefficient of the i-th gene derived from LASSO regression analysis (Supplementary Table S1), and E_i is the expression level of the i-th gene in the sample. The index "i" ranges from 1 to 9, corresponding the nine model genes.

Patients in all cohorts were divided into high- and low-risk groups according to the median risk score. Overall survival (OS) was assessed using Kaplan-Meier analysis implemented in the "survival" package. Time-dependent ROC curves were generated using the "pROC" package to evaluate predictive performance at 1-, 3-, and 5-year.

Expression profiling and prognostic analysis of model genes

Differences in model gene expression between CCA and normal tissues were evaluated using the Wilcoxon test. For each gene, the optimal expression cut-off was determined with the “survminer” package, and patients were stratified into high- and low-expression groups accordingly. Kaplan-Meier survival curves were generated to compare OS between groups, and statistical significance was assessed using the Log-rank test.

Development and validation of a nomogram for prognostic prediction

Univariate and multivariate Cox regression analyses were performed in the Fan_match cohort to evaluate the correlation between clinical pathological information and OS. Variables with significant prognostic value were incorporated into the construction of a nomogram using the “rms” package. The predictive performance of the nomogram was evaluated through calibration curves generated with the “rms” and “regplot” packages to assess its calibration accuracy.

Immune infiltration analysis

The relative proportions of 22 immune cell types were estimated using the “CIBERSORT” algorithm. Differences in immune cell infiltration between CCA and normal tissues were assessed with the Kruskal-Wallis test. Correlations between model gene expression and immune cell abundance were evaluated using Pearson correlation analysis. A linear regression model was constructed using the “lm” package, and the significance of the linear relationship between gene expression and immune cell abundance was evaluated using Spearman’s rank correlation coefficient. In addition, the expression levels of immune checkpoint-related genes were compared between high- and low-risk groups using the Kruskal-Wallis test.

Chemotherapy drug sensitivity analysis

Chemotherapy drug response data were obtained from the Genomics of Drug

Sensitivity in Cancer (GDSC, <http://www.cancerrxgene.org/>) database. Drug sensitivity values for CCA samples were predicted using the “oncoPredict” package. Correlations between model gene expression and predicted drug sensitivity were assessed using Pearson correlation analysis and visualized as a correlation heatmap. Differences in drug sensitivity between high- and low-risk groups were compared using the Wilcoxon test.

Single-cell data processing and analysis

Single-cell RNA-sequencing data were processed using the Seurat workflow. Quality control was performed to remove low-quality cells and lowly expressed genes, followed by normalization to minimize technical variation. Doublets were identified and excluded using the “DoubletFinder” package. Principal component analysis (PCA) was conducted for initial dimensionality reduction, after which batch effects were corrected using the “Harmony” integration method in combination with the “IntegrateLayers” function of Seurat. Uniform Manifold Approximation and Projection (UMAP) was then applied for further dimensionality reduction, and UMAP plots were generated with “Seurat” to visualize cellular clustering and distribution. For gene expression analysis, expression levels of the model genes were extracted from the integrated object and visualized using Seurat’s “FeaturePlot” function. Module scores for the model gene set were calculated using the “AddModuleScore” function to quantify gene set activity across different cell populations. Finally, intercellular communication networks were inferred using the “CellChat” package based on ligand-receptor interactions to characterize communication patterns among cell types.

Molecular docking analysis

Molecular docking was performed using AutoDock Vina (Version 1.5.7) to evaluate the binding affinity and interaction patterns between dietary fatty acids and their target proteins. The molecular structures of palmitic acid ($C_{16}H_{32}O_2$) and elaidic acid ($C_{18}H_{34}O_2$) were obtained from the PubChem database

(<https://pubchem.ncbi.nlm.nih.gov/>). Three-dimensional structures of the protein targets were retrieved from the PDB (<https://www.rcsb.org/>) or UniProt (<https://www.uniprot.org/>) databases. Unfortunately, the experimentally resolved structure of CELSR3 is currently unavailable. Prior to docking, both ligands and receptors were preprocessed by adding hydrogen atoms, removing water molecules, and converting the files to PDBQT format. The docking grid was positioned at the center of each protein with dimensions of $30 \text{ \AA} \times 30 \text{ \AA} \times 30 \text{ \AA}$ and a grid spacing of $0.05 \text{ \AA}$. Docking simulations were subsequently performed using AutoDock Vina to predict binding modes and calculate binding energies for each ligand-receptor pair.

Statistical analysis

All data processing, statistical analyses, and visualizations were performed in R software (version 4.3.2). Differences between two groups were assessed using the Wilcoxon rank-sum test, and comparisons among multiple groups were evaluated using the Kruskal-Wallis test. Pearson correlation analysis was conducted to determine associations between continuous variables. Univariate and multivariate Cox proportional hazards regression analyses were applied to evaluate the effects of clinical and molecular variables on OS. All statistical tests were two-tailed, and $P < 0.05$ was considered statistically significant.

Results

Identification and functional enrichment of dietary fat-related genes

A total of 7,362 dietary fat-related genes were identified (Supplementary Table S2). Among them, 20 genes exhibited markedly high interaction frequencies with dietary fat exposure, with *TNF* (389 interactions), *IL6* (300 interactions), and *APOE* (293 interactions) ranking among the most strongly associated (Fig. 2A).

GO enrichment analysis showed that these genes were predominantly involved in biological process (BP) such as fatty acid metabolic process, regulation of lipid

localization and regulation of inflammatory response. They were mainly enriched in cellular component (CC) such as the endoplasmic reticulum lumen, lipid droplet, low-density lipoprotein particle, and nuclear envelope. Molecular function (MF) analysis highlighted significant enrichment in cytokine receptor binding, lipoprotein particle binding, and fatty acid binding (Fig. 2B). KEGG pathway analysis further indicated enrichment in signaling pathways including PI3K-Akt, AMPK, p53, and IL-17 (Fig. 2C).

Identification of prognostic genes associated with dietary fat exposure

Univariate Cox regression identified 617 dietary fat-related genes associated with OS in the Fan_match cohort, and 173 and 264 such genes in the TCGA-CHOL and GSE107943 cohorts, respectively (Supplementary Table S3). Intersection analysis across the three datasets revealed 21 consistently prognostic genes, which were retained as candidate genes (Fig. 2D). A forest plot of the Fan_match cohort highlighted 19 risk and 2 protective genes (Fig. 2E), with parallel results for TCGA-CHOL and GSE107943 shown in Supplementary Fig. S1A, B. Expression profiling further demonstrated that 16 candidate genes, including *CBR3*, *CD300A*, and *CD44*, were upregulated in CCA tissues, while *IGFBP3*, *IL1RN*, and *S100A8* were markedly downregulated (Fig. 2F). A similar expression pattern was observed in the GSE107943 cohort (Supplementary Fig. S1C). PPI network analysis revealed extensive interactions among these candidate genes (Fig. 2G), highlighting several central nodes within the network.

Construction and validation of the prognostic model

In the Fan_match cohort, 116 predictive models were constructed using the LOOCV framework. The model incorporating LASSO regularization achieved the highest average concordance index (C-index) of 0.700, and was therefore selected as the optimal algorithm (Fig. 3A). The optimal λ value was determined by minimizing partial likelihood deviance (Fig. 3B), and nine genes with non-zero coefficients were

retained as model genes (Fig. 3C), including *CD300A*, *CD44*, *CELSR3*, *CENPA*, *DDIT4*, *DHRS9*, *F3*, *FGFR4*, and *GREM1*. Their chromosomal distributions are shown in Fig. 3D.

According to the LASSO regression coefficient, risk score of each patient was calculated, and the patients in the Fan_match cohort were stratified into high- and low-risk groups based on the median risk score. GSVA analysis showed that pathways related to immune response, inflammatory signaling, and multiple cancer-associated processes displayed higher activity in the high-risk group, whereas metabolic pathways such as Fatty acid metabolism and Bile acid metabolism exhibited stronger activity in the low-risk group (Fig. 3E). To further characterize functional differences between the two subgroups, GSEA was performed. In the low-risk group, model genes were enriched in pathways such as Adaptive immune response and T cell activation. Conversely, high-risk patients were enriched in pathways including Amino acid metabolic process and Active transmembrane transporter activity (Fig. 3F, G).

Kaplan-Meier survival analyses across the Fan_match, TCGA-CHOL, and GSE107943 cohorts revealed consistently poorer survival outcomes in the high-risk group ($p < 0.05$), although no significant difference was observed in the TCGA-CHOL cohort (Fig. 3H). Time-dependent ROC curves further confirmed the robust predictive performance of the risk score for 1-, 3-, and 5-year survival. The maximum OS for patients in the Fan_match cohort was 4.9 years. (Fig. 3I).

Expression analysis and survival correlation of model genes

To assess the prognostic significance of the model genes, Wilcoxon tests were conducted to compare their expression levels between CCA and normal tissues. In the TCGA-CHOL cohort, the expression of *CD300A*, *CD44*, *CELSR3*, *CENPA*, *DDIT4*, *F3*, *FGFR4*, and *GREM1* was significantly higher in CCA compared to normal samples (Fig. 4A). Similar results were observed in the GSE107943 cohort for *CD44*, *CELSR3*, *CENPA*, *DDIT4*, and *F3* (Supplementary Fig. S2A). Subsequently, patients in the Fan_match cohort were stratified into high and low-expression groups based on the

optimal cut-off values of model gene expression levels. Kaplan-Meier survival analysis revealed that, except for *FGFR4*, high expression of the remaining eight genes was significantly associated with poorer prognosis in CCA patients (Fig. 4B-J). Kaplan-Meier analysis of the Fan_match and GSE107943 cohorts showed similar survival trends (Supplementary Fig. S2B-S).

Construction and evaluation of a nomogram for prognostic prediction in CCA patients

Univariate and multivariate Cox regression analyses were conducted to assess the prognostic value of RiskScore. In the univariate Cox analysis, RiskScore was found to be a significant risk factor for prognostic (HR = 2.970, 95% CI: 2.220-3.960, $P < 0.001$, Fig. 5A). In the multivariate analysis, RiskScore remained an independent prognostic factor even after adjusting for other potential confounding variables (HR = 2.096, 95% CI: 1.325-3.316, $P < 0.01$, Fig. 5B). Based on these results, a prognostic nomogram was constructed using the Fan_match cohort to predict the 1-, 3-, and 4-year OS in CCA patients (Fig. 5C). Calibration curves confirmed the accuracy of the model in predicting the 1-, 3-, and 4-year survival rates (Fig. 5D).

Immune landscape and correlation with model genes in CCA

To assess immune cell infiltration in CCA patients from the Fan_match cohort, the CIBERSORT algorithm was used to calculate the relative abundance of 22 immune cells types. Macrophages M0, Macrophages M2, and T cells CD4 memory resting were the most abundant, while B cells memory, Dendritic cells activated, and Eosinophils showed low relative abundance (Fig. 6A). We further compared immune cell proportions between high- and low-risk groups. B cells memory, T cells CD4 memory activated, Neutrophils, and Macrophages M0 had higher abundance in the high-risk group, while Dendritic cells resting, NK cells activated, Mast cells resting, B cells naive, and T cells CD4 memory resting had higher abundance in the low-risk group ($P < 0.05$) (Fig. 6B). Correlations between the nine model genes and immune cell types are shown

in Fig. 6C. Notably, Activated dendritic cells positively correlated with *F3*, *DHRS9*, and *CD44*, while Type 2 T helper cell and Type 17 T helper cell negatively correlated with *DHRS9*, *DDIT4*, and *CD300A*. Significant correlations were also found between model gene expression and immune cell types such as Activated B cell, Activated CD4 T cell, Activated CD8 T cell, Activated dendritic cell, Macrophage, MDSC, and Neutrophil ($P < 0.05$) (Fig. 6D-O). Immune checkpoint analysis revealed significant differences in the expression of several immune checkpoint genes between the high- and low-risk groups ($P < 0.001$) (Fig. 6P).

Correlation between the dietary fat-related prognostic model and treatment response in CCA

To explore the relationship between the model genes and chemotherapy drug sensitivity, we analyzed the half-maximal inhibitory concentration (IC₅₀) values of various drugs in CCA samples using the GDSC database. The correlations between drug IC₅₀ values and model genes, along with their significance, are shown in Fig. 7A. Drug response prediction was performed using the “oncoPredict” algorithm to identify potential effective drugs for CCA patients. The results indicated that low-risk patients were more sensitive to drugs such as AZD2014_1441, Dactolisib_1057, Ibrutinib_1799, Nutlin-3a(-)_1047, OSI-027_1594, and Rapamycin_1084, while high-risk patients showed higher sensitivity to drugs including 5-Fluorouracil_1073, Cisplatin_1005, Cyclophosphamide_1512, Gemcitabine_1190, Irinotecan_1088, and Trametinib_1372 ($P < 0.05$) (Fig. 7B).

Single-cell analysis of immune cell composition and interactions in CCA

Dimensionality reduction, clustering, and cell annotation were performed on the single-cell data to identify distinct cell clusters. The relative proportions of the eight major cell types in normal and CCA samples were compared (Fig. 8A-C). Differential gene expression analysis revealed no significant differences in the expression of the

351 nine model genes between normal and CCA samples (Fig. 8D). The cell distribution
352 analysis showed differences in the proportions of major cell types among the six tumor
353 samples (Fig. 8E). Further analysis of model gene expression across the eight major
354 cell types indicated that CD44 and DDIT4 were strongly expressed in all cell types (Fig.
355 8F). CD300A was predominantly expressed in NK and Myeloid cells, while GREM1
356 was most highly expressed in T cells. Patients in the single-cell cohort were divided
357 into high- and low-risk groups based on model gene expression levels (Fig. 8G-I).

358 The distribution of the eight major cell types in the high- and low-risk groups is
359 shown in Fig. 8J, K, with notable differences observed. In the low-risk group, B cells
360 were more abundant, while NK cells were more prevalent in the high-risk group. Based
361 on the calculated risk scores, we derived the risk ratios for the six CCA samples in the
362 single-cell cohort and stratified them into high- and low-risk groups (Fig. 8L). The
363 relative proportions and distribution patterns of the eight major cell types in the two
364 groups are shown in Fig. 8M, N. Notably, Fibroblasts were more enriched in the low-
365 risk group, whereas B cells were relatively more abundant in the high-risk group.

366 To explore intercellular interactions within the tumor microenvironment, we
367 examined the signaling pathways promoting communication between immune cell
368 types. High-risk samples exhibited stronger interactions primarily between DC-DC,
369 DC-Myeloid cells, and DC-Fibroblasts (Fig. 8O). In contrast, low-risk samples showed
370 stronger interactions between Endothelial-B cells, Endothelial-Myeloid cells, and
371 Endothelial-Fibroblasts.

372 **Molecular docking**

373 To evaluate the potential interactions between harmful dietary fats and the proteins
374 encoded by the nine model genes, we performed molecular docking using AutoDock
375 Vina (Version 1.5.7). Palmitic acid and elaidic acid, two representative harmful dietary
376 fats, were docked with each model protein, and their binding affinities were calculated
377 to characterize possible interaction patterns. Both fatty acids showed notable binding
378 affinities with seven proteins, including CD300A, CENPA, DDIT4, DHRS9, F3,

FGFR4, and GREM1, and the docking poses revealed clear hydrogen-bond formation and strong electrostatic interactions (Fig. 9A-P). These findings suggest that these fatty acids may directly interact with multiple model proteins and potentially influence their biological activities. In contrast, CD44 exhibited positive binding energies with both palmitic acid and elaidic acid, indicating unfavorable binding and the inability to form a stable ligand-protein complex.

Discussion

CCA is a highly aggressive cancer, and over the past two decades, its incidence has shown a steady increase²⁷. In our daily diet, the average intake of dietary fat is approximately 84 g per day²⁸. However, excessive consumption of saturated fatty acids and trans fats can be highly detrimental to human health, potentially leading to various diseases, including cancer²⁹⁻³¹. A large body of evidence has established a link between dietary fat and CCA; however, the underlying molecular mechanisms and potential drug targets remain poorly understood. Studies suggest that excessive dietary fat can elevate the levels of secondary bile acids in feces, disrupting gut microbiota metabolism and altering the bile acid pool. This, in turn, regulates the Farnesoid X receptor (FXR) signaling pathway, a critical regulator of bile acid-mediated intestinal tumorigenesis³². Additionally, high-fat diet-induced obesity impairs the function of CD8⁺T cells within the tumor microenvironment, thereby accelerating tumor growth³³. Therefore, further investigation into the relationship between dietary fat and CCA, as well as the identification of reliable biomarkers, is crucial for improving the diagnosis, treatment, and prognosis of CCA.

This study developed a risk assessment model based on transcriptomic data from the Fan_match cohort, the TCGA-CHOL cohort, and the GSE107943 cohort. The model consists of nine potential biomarkers and aims to quantify the promotive effects of dietary fat-related genes on CCA risk. CD300A is an inhibitory receptor of the immunoglobulin superfamily, expressed in myeloid and lymphoid immune cells, and

plays a critical role in immune regulation and tumor progression ³⁴. The CD44 gene encodes a non-kinase transmembrane glycoprotein, widely recognized as a molecular marker for cancer stem cells (CSCs) ³⁵. CELSR3 is a seven-pass transmembrane receptor involved in cell-cell interactions and signal transduction. Its downregulation inhibits the proliferation, migration, and invasion of lung adenocarcinoma cells ³⁶. Similarly, CENPA, a member of the mitosis-related protein family, regulates cell division and plays a key role in tumor biology through its interactions with transcription factors ³⁷. DDIT4 is upregulated under stress conditions like hypoxia and DNA damage and regulates tumor cell growth, proliferation, and metabolism by inhibiting the mTORC1 pathway ³⁸. DHRS9, a member of the short-chain dehydrogenase/reductase family, converts retinol to retinal and plays a critical role in cancer development and progression ³⁹. F3 encodes coagulation factor III, which activates tumor cell proliferation and reshapes the tumor microenvironment by activating tumor-autonomous signaling and the extrinsic coagulation pathway ⁴⁰. FGFR4 mediates multiple signaling pathways through its binding with FGF19, promoting cancer development, including hepatocellular carcinoma (HCC), and its high expression is associated with poor prognosis ⁴¹. GREM1 regulates the bone morphogenetic protein (BMP) signaling pathway, and its increased expression promotes cancer cell proliferation and metastasis by altering signal transduction in the tumor microenvironment ⁴².

Based on this risk assessment model, we conducted survival analysis for patients in different risk subgroups. The results revealed a significant correlation between the OS of CCA patients in the Fan_match and GSE107943 cohorts and our developed risk model. This suggests that our prognostic model is reliable and accurate, providing strong support for survival prediction in CCA patients. However, the TCGA-CHOL cohort did not show such a correlation, which may be due to its smaller sample size, resulting in greater variability and affecting the statistical significance of the analysis ⁴³. Nevertheless, the high-risk group in all three cohorts exhibited poorer prognosis.

Furthermore, the expression levels of the model genes were significantly associated with the survival status of CCA patients, highlighting their potential as prognostic biomarkers. However, our findings suggest that patients with low expression of FGFR4 had a worse prognosis, which contradicts the commonly observed high expression of FGFR4 in tumor samples. A possible explanation is that FGFR4 may exert dual roles through different mechanisms in certain tumors. Low expression of FGFR4 could be linked to changes in the tumor microenvironment, immune evasion, or metabolic reprogramming, all of which may influence prognosis⁴⁴. Conversely, high expression of FGFR4, under specific conditions, may contribute to increased resistance to treatment⁴⁵. Therefore, the role of FGFR4 is likely complex and context-dependent, varying across tumor stages and microenvironments. Further in-depth studies are needed to explore its clinical applications as a therapeutic target or biomarker.

Tumor cells can evade immune surveillance by disrupting normal host immune functions⁴⁶. In this study, we observed a significant decrease in the expression of anti-tumor immune cells, such as Dendritic cells resting, NK cells activated, and T cells CD4 memory resting, in high-risk patients. On the other hand, the relative proportions of immune-suppressive cells, such as Neutrophils and Macrophages M0, were significantly increased in the high-risk group. These results suggest that immune evasion mechanisms, such as the accumulation of immunosuppressive cells, may have been enhanced in the tumor microenvironment. Furthermore, the expression levels of the model genes showed significant correlations with various key immune cell types, including Activated B cells, Activated CD4⁺T cells, and Neutrophils. This finding suggests that the model genes identified in this study may play an important role in regulating immune cell functions and their interactions with tumor immunity.

This study explored the differences in cell types and changes in the immune microenvironment between CCA and normal samples. This was achieved using dimensionality reduction, clustering, and cell annotation analysis of single-cell data. CD44 and DDIT4 were strongly expressed across all cell types, suggesting their

potential involvement in shaping the immune microenvironment of CCA. CD300A was primarily expressed in NK and Myeloid cells, indicating its role in immune response regulation and possibly contributing to tumor immune evasion^{47,48}. GREM1 showed higher expression in T cells, which is likely associated with T cell-mediated immune regulation, potentially influencing tumor immunity. These findings provide new insights into understanding the immune mechanisms in CCA. Furthermore, intercellular interaction analysis revealed that in the high-risk group, DC exhibited stronger interactions with Myeloid cells and Fibroblasts, which may facilitate tumor immune evasion by promoting immune suppression or altering the tumor microenvironment⁴⁹. In contrast, the low-risk group showed stronger interactions between Endothelial cells, B cells, Myeloid cells, and Fibroblasts, which may promote angiogenesis and facilitate the coordination of immune responses, thus supporting a more favorable tumor immune environment⁵⁰.

Palmitic acid, a type of SFA, is commonly present in many foods. However, excessive intake of palmitic acid may increase the risk of obesity and inflammation^{51,52}, insulin resistance⁵³, and cardiovascular disease⁵⁴, and may also promote tumor metastasis^{55,56}. Trans fatty acids are also known to be harmful to human health⁵⁷. These fatty acids primarily include ruminant trans fats and industrially produced partially hydrogenated vegetable oils⁵⁸, which primarily consist of elaidic acid, trans-linoleic acid, and trans-alpha-linolenic acid⁵⁹. Therefore, we predicted the interactions and potential binding sites between the harmful dietary fats, palmitic acid and elaidic acid, and the proteins encoded by our model genes using molecular docking. The results revealed that these harmful dietary fats form stable binding interactions with the proteins, suggesting that these proteins play a crucial role in the biological functions of these dietary fats, particularly in regulating fatty acid-related signaling and metabolic processes.

This study has several limitations. First, we defined the model genes as “dietary fat-related” according to chemical-gene interaction data in the CTD database, selecting

genes with a higher number of interactions with dietary fat-associated exposures, rather than on the basis of individual dietary records or lipid profiles. This indirect strategy may introduce selection bias and cannot reflect patient-level exposure heterogeneity. Second, the immune landscape and cell-cell communication patterns were inferred mainly from computational deconvolution and a relatively small set of single-cell datasets, so these findings should be corroborated by experimental techniques such as multiplex immunostaining, flow cytometry, or spatial transcriptomics. Third, the interactions between palmitic/elaidic acid and the proteins encoded by the model genes were only supported by molecular docking, and their functional significance needs to be tested in follow-up mechanistic studies in vitro and in vivo.

Conclusion

In summary, this study successfully developed a prognostic model for CCA based on dietary fat-related genes using machine learning algorithms. Our analysis revealed the significant impact of these genes on risk stratification, survival prognosis, immune cell infiltration, and drug sensitivity in CCA patients. These findings not only improve prognostic assessment but also suggest that these genes could serve as biomarkers for personalized treatment strategies, potentially guiding drug selection and optimizing therapeutic approaches. The model offers promising clinical applications for improving patient outcomes and enabling more effective treatment strategies in CCA.

Data Availability

All datasets used in this study are publicly available in the Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>), The Cancer Genome Atlas (TCGA, <https://portal.gdc.cancer.gov/>), and the previous studies (<https://doi.org/10.1016/j.ccell.2021.12.006>).

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Author contributions

Yuan L, Zhu YL: Conceptualization, data curation, formal analysis, writing – original draft preparation. Ma ZL, Fan N: Investigation, Methodology, validation, writing – review & editing. Wang GS: Supervision, project administration, resources, writing – review & editing. All authors read and approved the final manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

This study did not involve human or animal subjects; therefore, ethics committee approval is not applicable.

Consent for publication

All authors consent to the publication of this manuscript.

Figure Legends

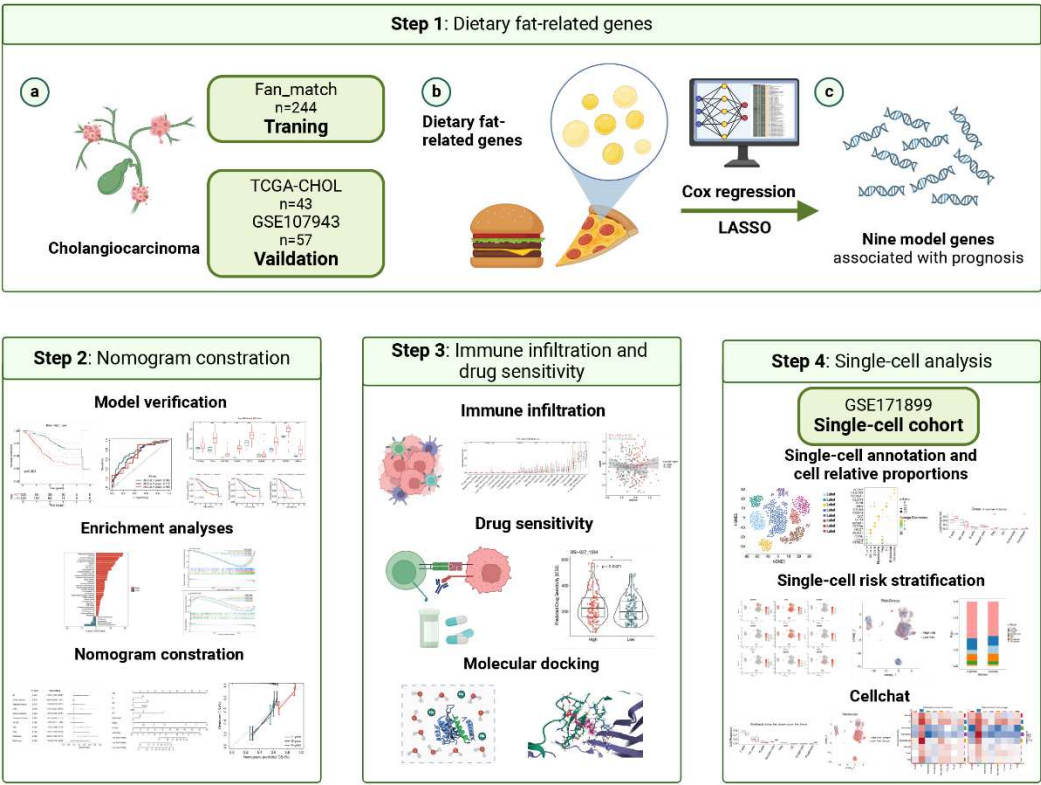


Fig. 1. Graphical abstract of this study. Created with BioRender (<https://www.biorender.com/>).

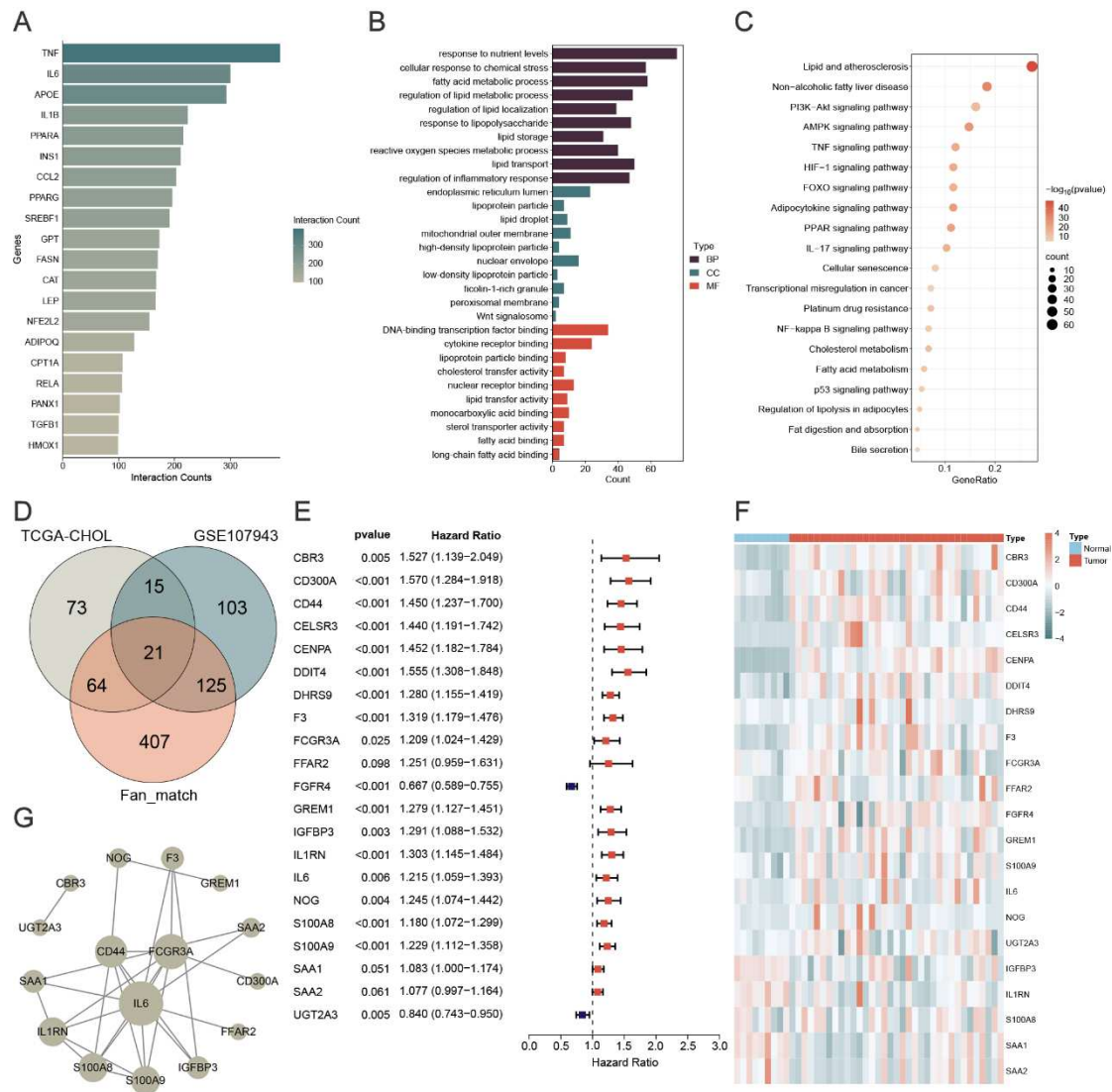


Fig. 2. Identification of dietary fat-related prognostic genes. (A) Top 20 genes ranked by dietary fat-interaction counts. (B) GO enrichment analysis of dietary fat-related genes with interaction counts of 20 and above. (C) KEGG enrichment analysis of dietary fat-related genes with interaction counts of 20 and above. (D) Venn diagram showing survival-related genes shared across the Fan_match, TCGA-CHOL, and GSE107943 cohorts. (E) Univariate Cox regression results for the 21 prognostic candidate genes in the Fan_match cohort. (F) Expression heatmap of the 21 genes in CCA and normal tissues in the TCGA-CHOL cohort. (G) Protein-protein interaction (PPI) network of the 21 prognostic candidate genes.



assessing the predictive performance of the model for 1-, 3-, and 5-year survival in the three cohorts.

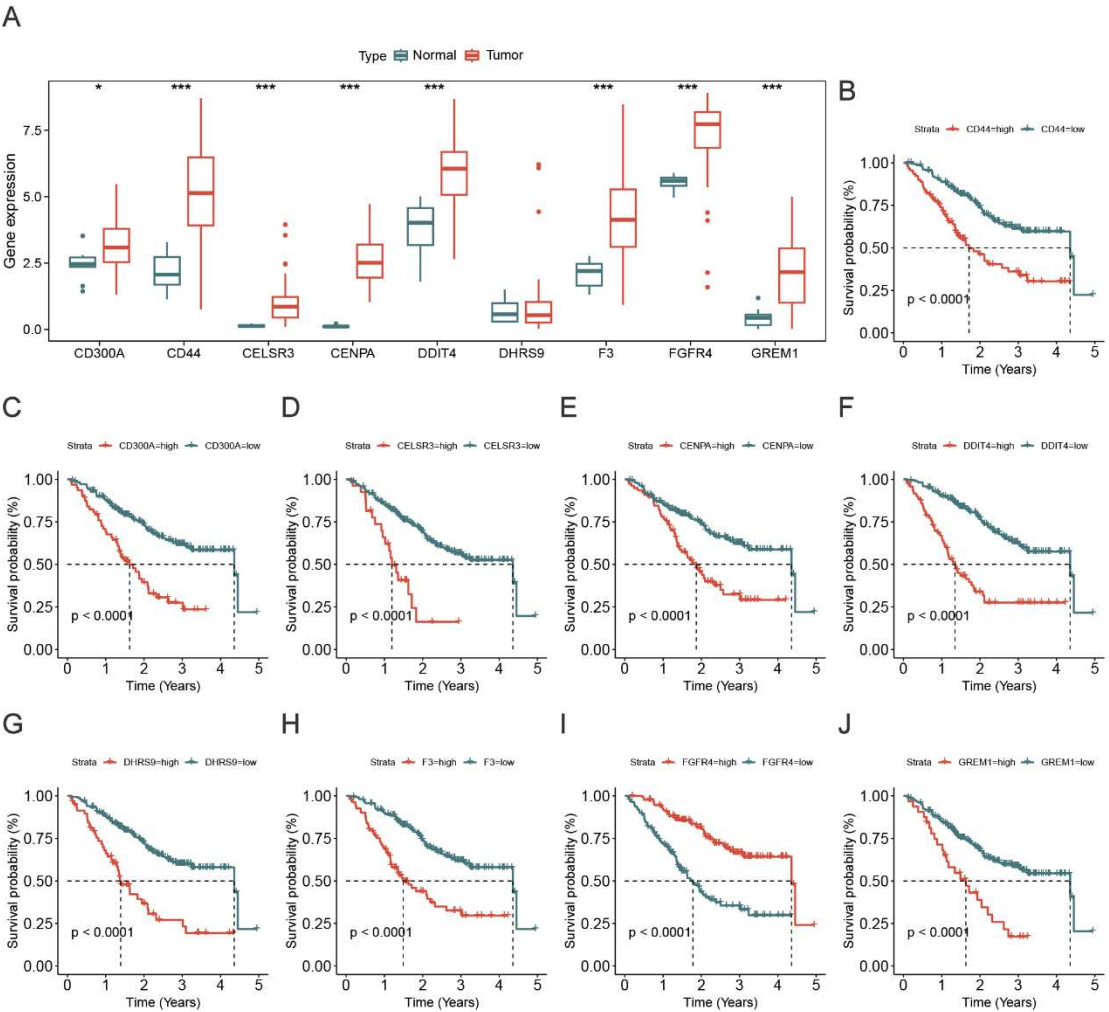


Fig. 4. Expression and survival analysis of dietary fat-related model genes in CCA. (A) Boxplot showing the differential expression of model genes between CCA and normal tissues in the TCGA-CHOL cohort. (B-J) Kaplan-Meier survival curves comparing prognostic differences between high- and low-expression groups for the nine model genes in the Fan_match cohort. *: $P < 0.05$; ***: $P < 0.001$.

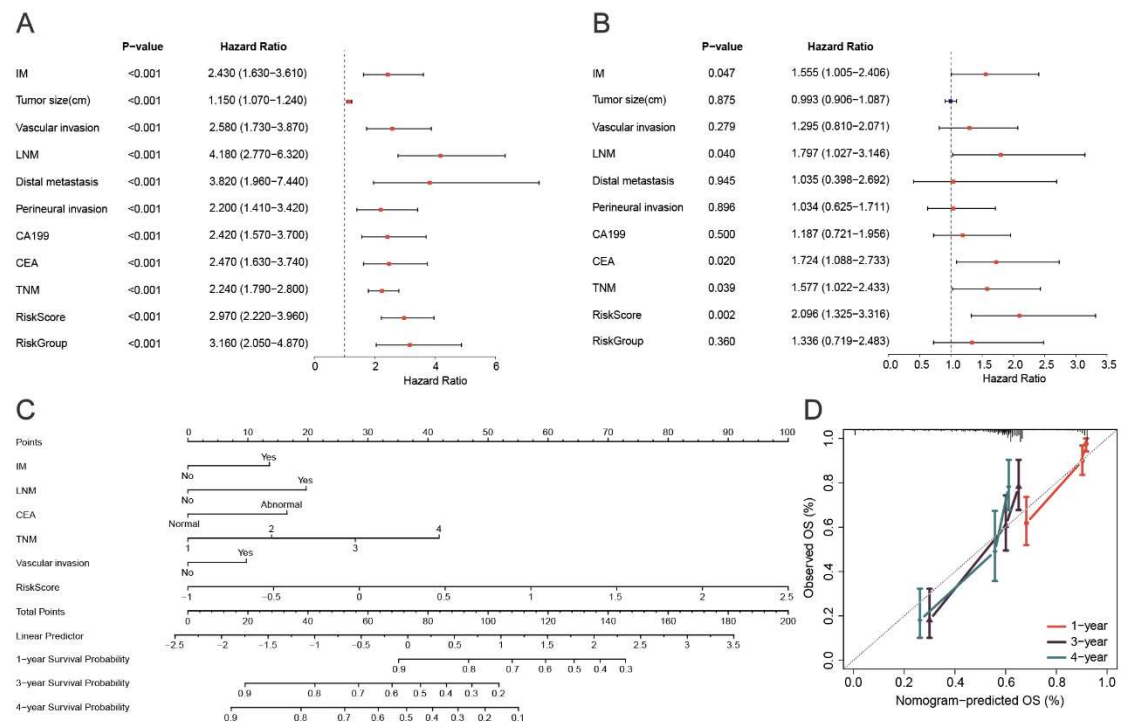


Fig. 5. Development and evaluation of the nomogram survival model. (A) Univariate Cox analysis of risk scores and clinicopathological features in the Fan_match cohort. (B) Multivariate Cox analysis of risk scores and clinicopathological characteristics in the Fan_match cohort. (C) Nomogram for predicting OS in the Fan_match cohort. (D) Calibration curves for the nomogram predicting 1-, 3-, and 4-year OS in Fan_match cohort. Note: “IM” refers to Intrahepatic Metastasis, and “LNLM” refers to Regional Lymph Node Metastasis.

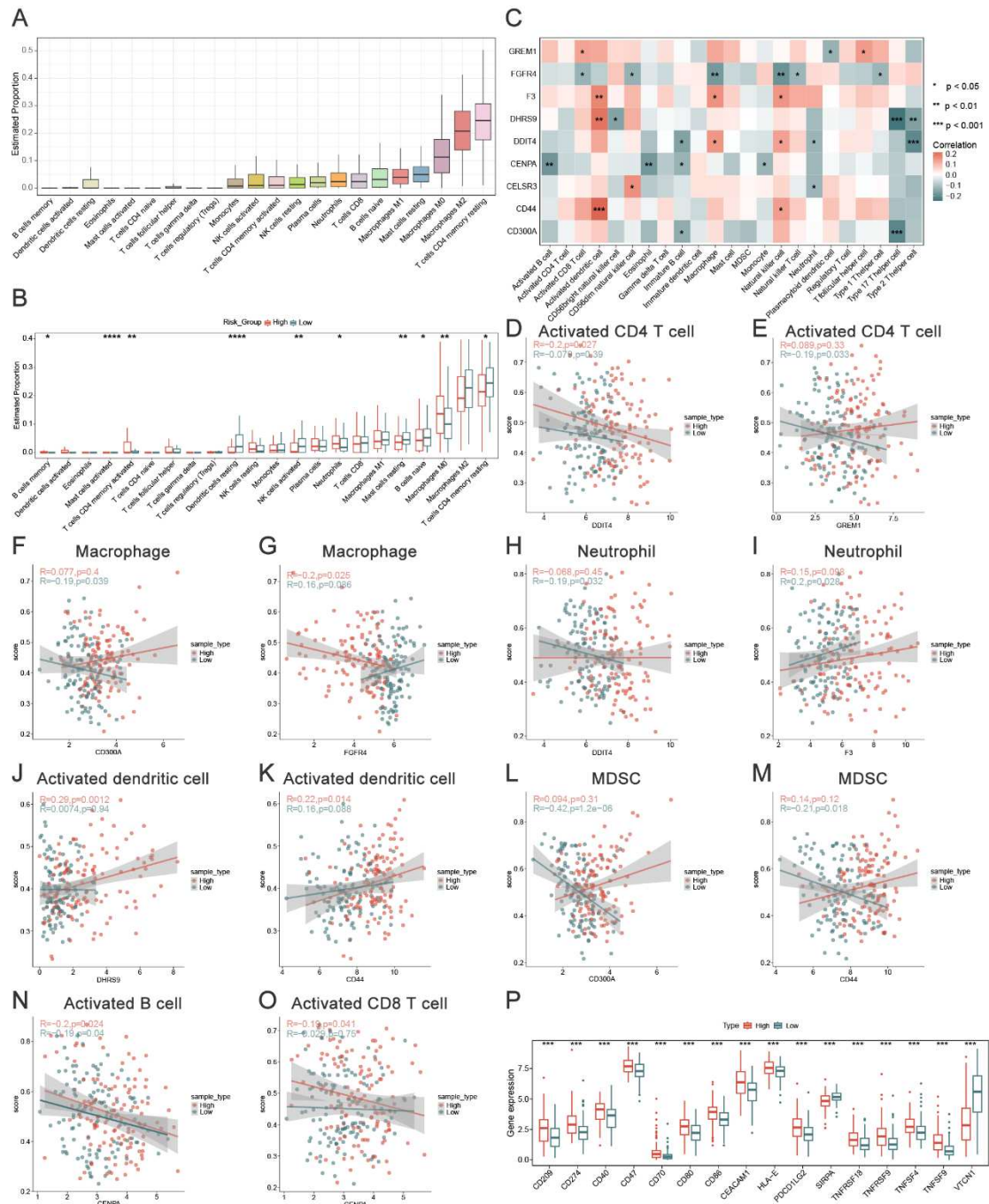


Fig. 6. Immunological features of the two risk groups. (A) Estimated proportions of 22 immune cell subsets based on CIBERSORT analysis. (B) Boxplot showing differences in the infiltration fractions of 22 immune cell subsets between high- and low-risk groups. (C) Heatmap illustrating the relationship between model genes and 23 immune cell types. (D-O) Correlation between the expression levels of model genes and the 23 immune cell types. (P) Boxplot comparing the expression of 16 immune checkpoint-related genes between the high- and low-risk groups. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

0.01; ***: $P < 0.001$; ****: $P < 0.0001$.

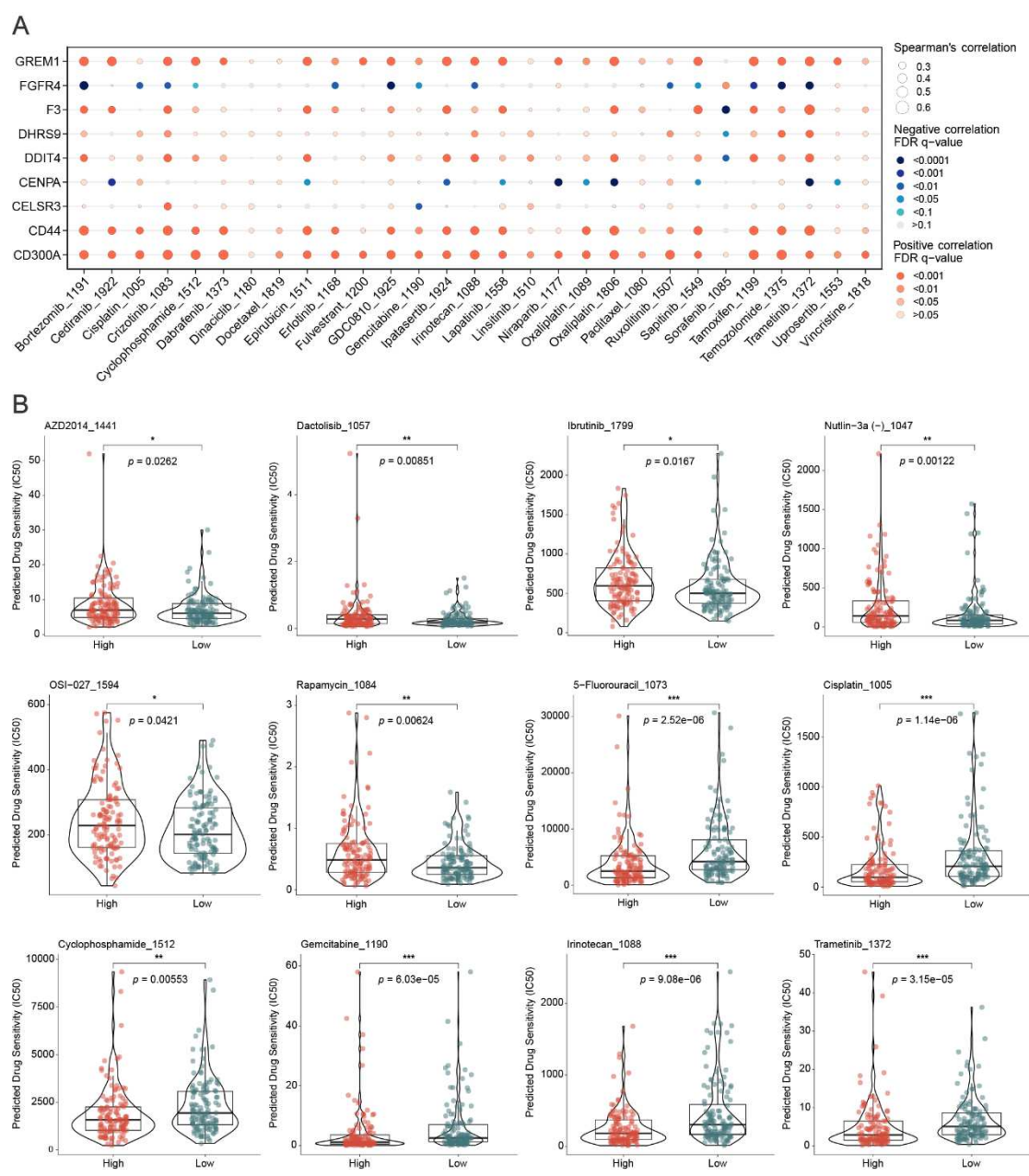


Fig. 7. Correlation between model genes and chemotherapy drug sensitivity. (A) Bubble chart depicting the correlations between chemotherapy drugs and model genes, with statistical significance. (B) Violin plot showing differences in IC50 values of chemotherapy drugs between high- and low-risk groups. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

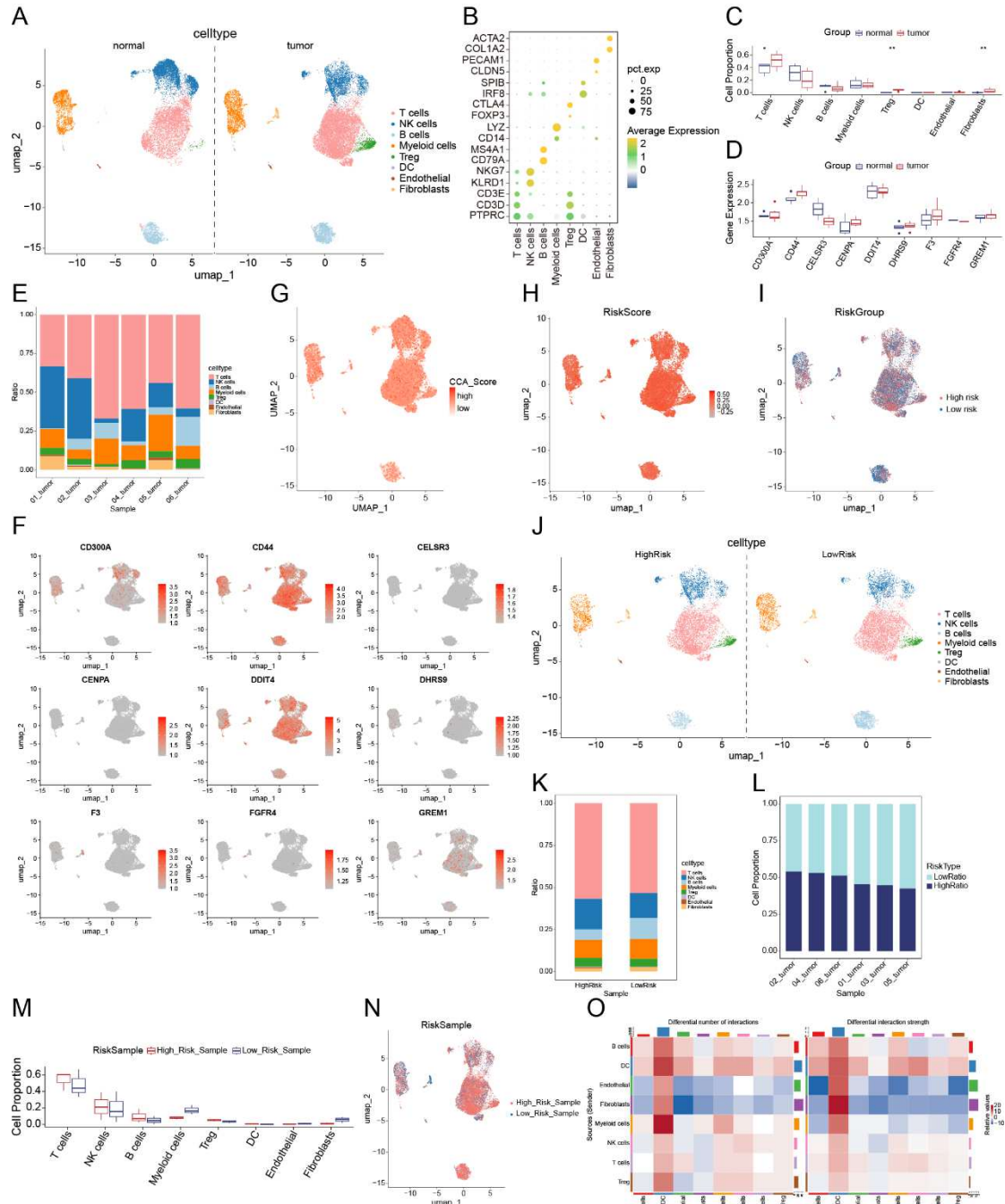


Fig. 8. Single-cell transcriptomic features of normal and CCA tissues based on model genes. (A) UMAP plot of the eight major cell types in CCA and normal tissues. (B) Bubble chart showing the expression of signature genes in each cell type. (C) Boxplot comparing the relative proportion of immune cells between CCA and normal tissues. (D) Gene expression differences of the nine model genes between CCA and normal tissues. (E) Bar chart showing immune cell distribution in six CCA samples from the single-cell cohort. (F) UMAP plot showing the distribution of the nine model

779 genes in single cells. (G) Model gene set scores in major cell types. (H) Risk scores
780 based on LASSO coefficients across major cell types. (I) Patients stratified into high-
781 and low-risk groups based on the median risk score. (J) Differences in the proportions
782 of eight major cell types between high- and low-risk patients. (K) Distribution of the
783 eight major cell types in high- and low-risk patients. (L) Risk proportion in six CCA
784 samples from the single-cell cohort. (M) Comparison of immune cell proportions
785 between two groups based on risk proportion. (N) Distribution of high- and low-risk
786 patients in the single-cell cohort. (O) Cell-to-cell communication strength and
787 frequency based on CellChat between the eight major cell types. *: $P < 0.05$; **: $P <$
788 0.01 ; ***: $P < 0.001$.

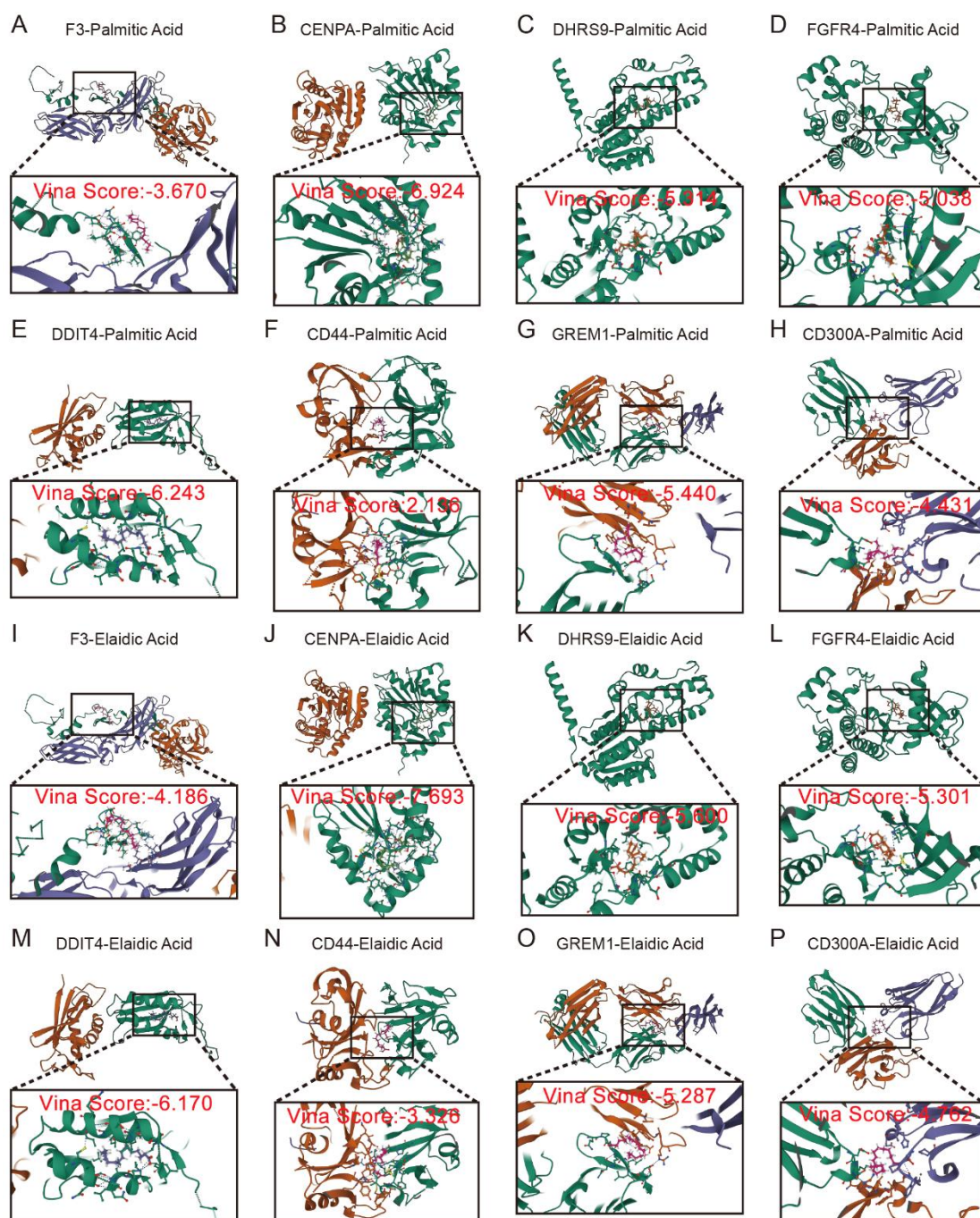
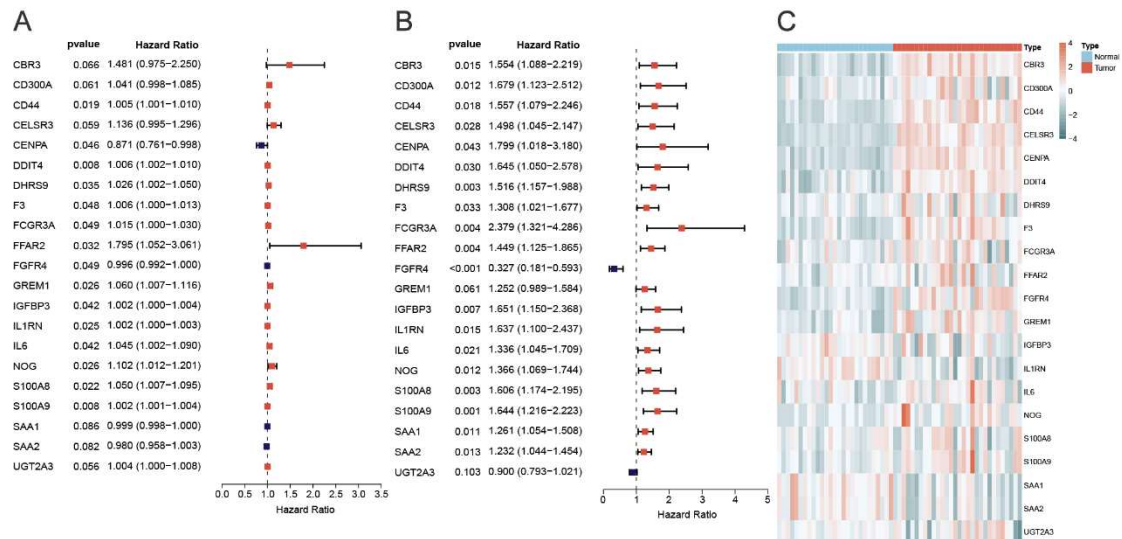
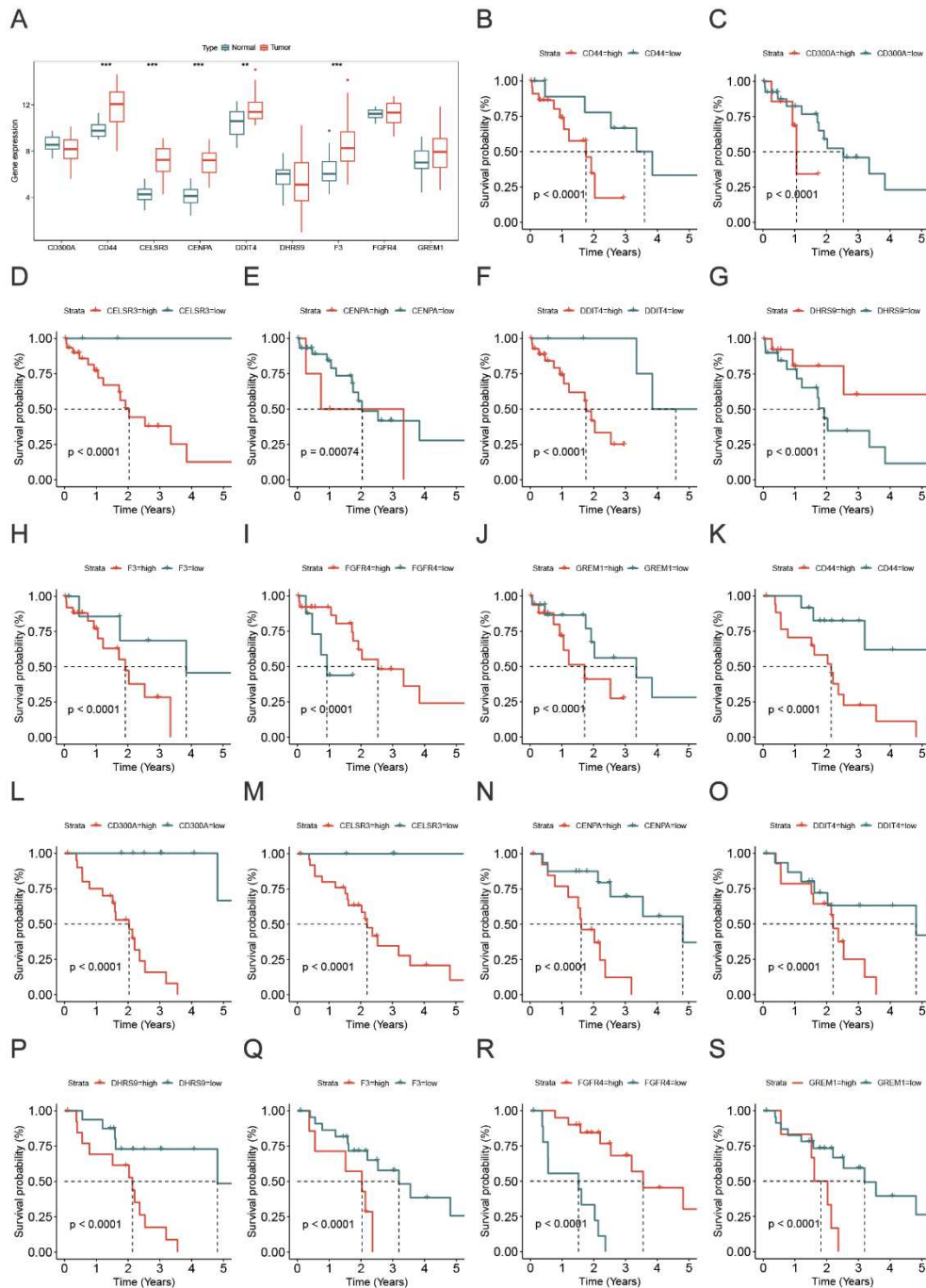


Fig. 9. Molecular docking results of dietary fats with the lowest binding energy in each model gene-encoded protein. (A-P) Docking interactions between model genes and fatty acids (Palmitic Acid and Elaidic Acid). White represents hydrogen, green represents carbon, red represents oxygen, and blue represents nitrogen.

Supplementary materials



Supplementary Fig. S1. Univariate Cox analysis and heatmap of dietary fat-related prognostic genes. (A) Univariate Cox analysis of 21 candidate genes in the TCGA-CHOL cohort. (B) Univariate Cox analysis of 21 candidate genes in the GSE107943 cohort. (C) Heatmap showing the expression of 21 candidate genes in CCA and normal tissues in the GSE107943 cohort.



Supplementary Fig. S2. Expression and survival analysis of dietary fat-related model genes in CCA. (A) Boxplot showing differential expression of model genes between CCA and normal tissues in the GSE107943 cohort. (B-J) Kaplan-Meier survival analysis comparing prognostic differences based on high and low expression levels of nine model genes in the TCGA-CHOL cohort. (K-S) Kaplan-Meier survival analysis comparing prognostic differences based on high and low expression levels of nine model genes in the GSE107943 cohort. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

810 **Supplementary Table S1.** LASSO coefficients for the nine model genes.
811
812 **Supplementary Table S2.** Dietary fat-related genes and their interaction counts.
813
814 **Supplementary Table S3.** Univariate Cox analysis of dietary fat-related genes
815 associated with survival.
816

Supplementary Files

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