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Bioinformatics Analysis Confirms FLAD1 as a Prognostic and Immune Biomarker in Hepatocellular Carcinoma

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Abstract Objective: To investigate the functional role and molecular mechanisms of flavin adenine dinucleotide synthetase 1 (FLAD1) in the initiation and progression of hepatocellular carcinoma (HCC). Methods: The gene expression and clinical data of HCC patients were obtained from TCGA and GEO, and the expression of FLAD1 was analyzed using TIMER2 and HPA. Differential analysis was performed using DESeq2 and limma packages, and feature genes were screened by single-factor COX survival analysis and three machine learning algorithms. Genes related to immune regulation were screened by WGCNA and PPI analysis, and co-expression analysis was performed with FLAD1. Drug sensitivity analysis combined with molecular docking revealed the relationship between FLAD1 and commonly used cancer drugs. The impact of FLAD1 on the biological functions of liver cancer cells was evaluated through colony formation assays, Transwell migration and invasion assays, as well as subcutaneous tumor xenograft experiments in mice.Results: The expression of FLAD1 in liver cancer tissues was significantly higher than that in normal tissues, and was associated with poor prognosis. Immunoinfiltration analysis showed that the immunomicroenvironment score of the group with high expression of FLAD1 was significantly lower than that of the group with low expression, suggesting that FLAD1 might inhibit immune response. WGCNA and PPI analysis identified genes closely related to immune infiltration and co-expression with FLAD1.The FLAD1 gene promotes the proliferation, invasion, and migration of liver cancer cells. Conclusion: FLAD1 can be used as a biomarker for poor prognosis of hepatocellular carcinoma, and its mechanism may be related to remodeling immunosuppressive microenvironment, and provide a potential strategy for combined target therapy.

Index Terms HCC, Machine learning, Single-cell sequencing, Molecular docking

I. Introduction

Hepatocellular carcinoma (HCC), the predominant histologic subtype of primary liver cancer, ranks as the sixth most common malignancy globally and the third leading cause of cancer-related mortality worldwide [1]. Despite continuous advancements in diagnostic and therapeutic technologies, approximately 70% of patients are diagnosed at intermediate or advanced stages due to tumor heterogeneity and the lack of early diagnostic biomarkers, resulting in a dismal five-year survival rate below 20% [2]. This critical situation underscores the urgent need to explore novel molecular biomarkers and therapeutic targets.The breakthrough development of multi-omics technologies has established a new paradigm for deciphering tumor molecular mechanisms through integrative analysis of large-scale biomedical databases such as TCGA and ICGC [3]. Notably, metabolic reprogramming, recognized as one of the hallmarks of cancer, has brought increasing attention to the key regulatory molecule FLAD1 (Flavin Adenine Dinucleotide Synthetase 1) [4]. This enzyme catalyzes the final step of FAD biosynthesis and plays pivotal roles in oxidative phosphorylation and DNA damage repair. Emerging evidence has demonstrated that aberrant FLAD1 expression correlates with progression in various malignancies including breast and colorectal cancers [5], [6]. However, its functional role in HCC and the dynamic interplay with the tumor immune microenvironment remain largely unexplored.This study employs a multidimensional bioinformatics strategy to systematically investigate FLAD1-related molecular characteristics in HCC: First, we integrated GEO datasets (GSE14520, GSE10143) with TCGA-LIHC data to comprehensively evaluate FLAD1 transcriptomic features through differential expression analysis. Subsequently, Cox proportional hazards models and Kaplan-Meier survival analysis were employed to assess its prognostic value. To further elucidate FLAD1's immunoregulatory network, we comprehensively analyzed tumor immune infiltration characteristics using CIBERSORT and ESTIMATE algorithms, followed by WGCNA-based co-expression network construction. Additional drug sensitivity analysis and molecular docking studies revealed potential associations between FLAD1 and therapeutic agents. Both in vitro and in vivo experiments with HCC cell lines validated FLAD1's role in promoting tumor cell proliferation, invasion, and migration.As the first study to unravel FLAD1's molecular mechanisms in HCC from an immunometabolic perspective, our findings identify FLAD1 as a

potential diagnostic biomarker for HCC and provide a theoretical foundation for developing personalized therapeutic strategies and targeted drug discovery.

II. Materials and Methods

II. A. Data Acquisition and Databases

Gene expression profiles and clinical data of hepatocellular carcinoma (HCC) patients were obtained from the TCGA database [7] and the GEO datasets GSE14520 and GSE74627 [8], respectively. Single-cell RNA sequencing (scRNA-seq) data were derived from two samples (GSM4955419 and GSM4955421) of the GSE162616 dataset. The transcriptomic expression features of the FLAD1 gene were analyzed across multiple dimensions using the TIMER2 database [9], while protein-level expression of FLAD1 in HCC tissues was validated via immunohistochemistry (IHC) data from the HPA database. Genomic drug sensitivity data were sourced from the GDSC (Genomics of Drug Sensitivity in Cancer) database. Molecular docking was performed using the CB-DOCK2 database.

II. B. Feature genes of HCC were screened using machine learning algorithms

We constructed an integrated feature selection framework incorporating four classical machine learning algorithms:(1) Random Forest (RF) Algorithm: Utilizing bootstrap aggregation (bagging) to generate an ensemble of decision trees, we performed gene selection based on Gini importance scores [10].(2) Gradient Boosting Machine (GBM): Employing a stage-wise additive modeling approach, this algorithm sequentially constructed weak learners to minimize residual errors, enabling high-precision feature selection [11].(3) eXtreme Gradient Boosting (XGBoost): Building upon GBM's foundation, this implementation incorporated distributed computing efficiency, L1/L2 regularization, and second-order gradient optimization to enhance feature selection robustness [12].

II. C. Expression Level Analysis

We performed comprehensive expression analysis of FLAD1 using three independent datasets: TCGA, GSE14520, and GSE74627 to ensure robust cross-platform validation of its differential expression in hepatocellular carcinoma (HCC) patients.Subsequently, we expanded our investigation to pan-cancer analysis through the TIMER2 database to characterize FLAD1 expression patterns across multiple cancertypes.Finally, protein-level validation was conducted using immunohistochemistry data from the Human Protein Atlas (HPA) database to confirm FLAD1 expression at the translational level.

II. D. Survival Analysis

Kaplan-Meier survival curves for FLAD1 were generated separately in both TCGA and GSE14520 datasets.

II. E. GO and KEGG pathway enrichment analyses

Using the clusterProfiler package, we performed comprehensive functional module annotation. This included three-dimensional analysis of Gene Ontology (GO) databases (molecular functions, biological processes, and cellular components) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment, implemented through a hypergeometric distribution algorithm.

II. F. Immune infiltration analysis

We employed the ESTIMATE algorithm to quantify the tumor microenvironment composition, where the StromalScore represents the stromal component proportion, the ImmuneScore reflects the degree of immune cell infiltration, and the ESTIMATEScore indicates the overall tumor heterogeneity level. Subsequently, we utilized the CIBERSORT deconvolution algorithm [13] with the LM22 signature matrix and 1,000 permutation iterations to analyze the relative abundance changes of 22 immune cell subsets.

II. G. Drug sensitivity analysis

Drug sensitivity prediction analysis was performed using the oncoPredict R package, leveraging pharmacogenomic data from the GDSC (Genomics of Drug Sensitivity in Cancer) database. By integrating tumor sample data from hepatocellular carcinoma (HCC) patients, we systematically evaluated the differential drug sensitivity of FLAD1 expression subgroups to clinically relevant targeted therapies and chemotherapeutic agents. The half-maximal inhibitory concentration (IC_{50}) was calculated for each patient to identify potential effective treatment regimens.

II. H. Molecular docking

Molecular docking was performed via CB-Dock2 to examine FLAD1's interactions with three differentially expressed drugs, generating three sets of docking configurations.

II. I. Colony formation assay

or the colony formation assay, Huh7 cells with stable FLAD1 knockdown and negative controls were plated in 6-well plates (1,500 cells/well, triplicates) and cultured for 14 days. After visible colony formation, cells were PBS-washed, fixed with 4% paraformaldehyde (30 min), stained with 0.1% crystal violet (30 min), thoroughly rinsed, and imaged for analysis.

II. J. Transwell assay

FLAD1-knockdown and control Huh7 cells in logarithmic phase were trypsinized, counted, and seeded (5×10^4 cells/insert) into Matrigel-coated upper chambers (serum-free DMEM), with 20% FBS-DMEM as chemoattractant in lower chambers. After 48 h, non-migrated cells were removed, and migrated cells were fixed (4% PFA, 30 min), stained (0.1% crystal violet, 30 min), PBS-washed, and imaged for ImageJ quantification.

II. K.Subcutaneous Xenograft Tumor Assay in NOG Mice

Female NOG mice aged 6-8 weeks were selected and randomly assigned into experimental groups. The axillary hair was shaved, and Huh7 cells (3×10^6 cells/mouse) with FLAD1 knockdown or control treatment were subcutaneously inoculated into the axillary region using a 1-mL syringe. Prior to inoculation, the cells were digested, centrifuged, and resuspended in an appropriate medium.Tumor formation was monitored starting one week post-inoculation. The number of tumor-bearing mice was recorded, and tumor volume was measured every two days for four weeks. After the observation period, the mice were euthanized in accordance with institutional guidelines, and the tumors were dissected and photographed for further analysis.

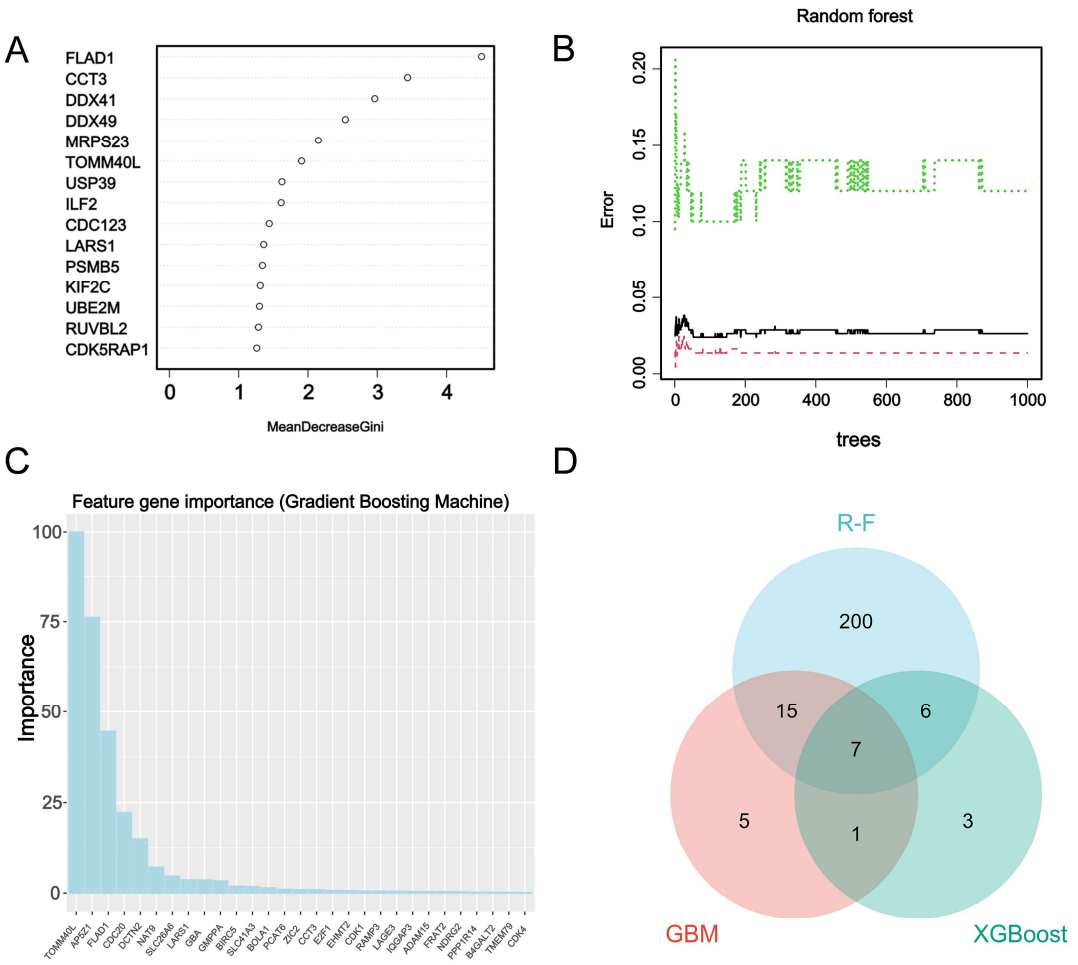

Figure 1. Machine learning-based screening of disease signature genes. A. Random Forest analysis.B. Gradient Boosting Machine (GBM) analysis. C. XGBoost analysis.D. Venn diagram showing overlapping candidate genes

II. L. Statistical Analysis

To ensure data reliability, each experimental group contained more than three replicates (n > 3). All statistical analyses were performed using GraphPad Prism software, with quantitative data expressed as mean ± standard deviation (Mean ± SD).

III. Results

III. A. Identification and Characterization of Hepatocellular Carcinoma-Related Genes

We performed univariate Cox regression analysis on TCGA samples for batch survival analysis, with screening thresholds set at HR>1 and P<0.05, identifying 1,451 genes significantly associated with hepatocellular carcinoma (HCC) patient prognosis. By intersecting these prognostic genes with differentially expressed genes, we obtained 618 prognosis-related differentially expressed genes.For these candidate genes, we employed multiple machine learning approaches including Random Forest, GBM (Gradient Boosting Machine), and XGBoost for further screening (Figure 1 A-C). The selection criteria for both GBM and XGBoost were set at importance scores >0. Finally, we identified consensus genes by intersecting the results from all machine learning methods (Figure 1 D).

III. B. Expression Profiling and Prognostic Analysis

To comprehensively elucidate the clinical relevance of FLAD1 in hepatocellular carcinoma (HCC), we conducted an integrated multi-platform analysis utilizing diverse omics databases. Pan-cancer analysis through TIMER2 demonstrated significantly elevated FLAD1 mRNA expression in HCC tissues compared to normal controls (P<0.001, Figure 2 A). This finding was consistently validated across four independent datasets: GSE14520 (Figure 2 B), GSE76427 (Figure 2 C), and GEPIA2 (Figure 2 D), confirming FLAD1's stable overexpression pattern in HCC.Notably, diagnostic performance evaluation in the TCGA cohort revealed FLAD1's exceptional discriminative capacity with an AUC of 0.991 (Figure 2 E), underscoring its potential as a diagnostic biomarker. Subsequent survival analysis established a significant association between FLAD1 expression levels and patient outcomes. Both TCGA (Figure F) and GEPIA2 (Figure G) datasets demonstrated markedly superior overall survival in the FLAD1-low expression group compared to FLAD1-high expressors (log-rank P<0.05).Protein-level validation via immunohistochemistry in the Human Protein Atlas (HPA) database confirmed FLAD1's specific overexpression in HCC tissues (Figures H-I), with predominant cytoplasmic staining patterns in malignant cells.

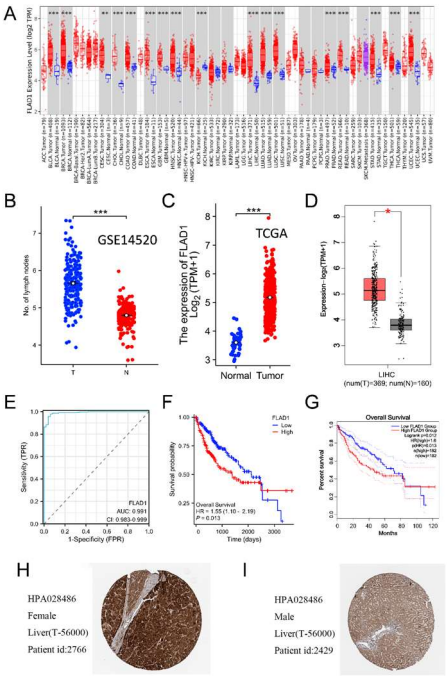

Figure 2： Pan-Cancer and Hepatocellular Carcinoma-Specific Analysis of FLAD1 Expression and Prognostic Significance. A.FLAD1 Expression Across Cancer Types in the TIMER2 Database B-D. FLAD1 is significantly overexpressed in hepatocellular carcinoma (HCC) compared to normal liver tissues across multiple genomic datasets including GSE14520, GSE76427, and GEPIA2. E.ROC curve of FLAD1 in the TCGA dataset. F-G. Lower FLAD1 expression correlates with better prognosis in TCGA and GEPIA2 databases.H-I.IHC staining results from the HPA database

III. C. GO, KEGG, and GSEA enrichment analysis

Differential analysis was conducted on samples with high FLAD1 expression and those with low FLAD1 expression, and differentially expressed genes were selected for enrichment analysis. GO enrichment analysis indicated that FLAD1 participates in the progression of liver cancer through copper ion detoxification (FAD as a cofactor of copper metabolism enzymes), inorganic compound detoxification (affecting the detoxification function of P450 enzymes), and hormone signal regulation (interfering with steroid metabolism) (Figure 3 A, Table 1). KEGG analysis suggested that it was significantly associated with drug metabolism pathways (abnormal P450-mediated chemotherapy drug metabolism) (Figure 3 B, Table 1). GSEA further revealed that high expression of FLAD1 was accompanied by inhibition of immune-related pathways (complement activation, stress response) and imbalance of metal ion (zinc/copper) homeostasis, jointly leading to accumulation of oxidative stress and microenvironment remodeling (Figure 3 C-D, Table 1). The above results systematically explained the molecular mechanism by which FLAD1 drives the development of hepatocellular carcinoma through metabolic reprogramming and immune regulatory networks.

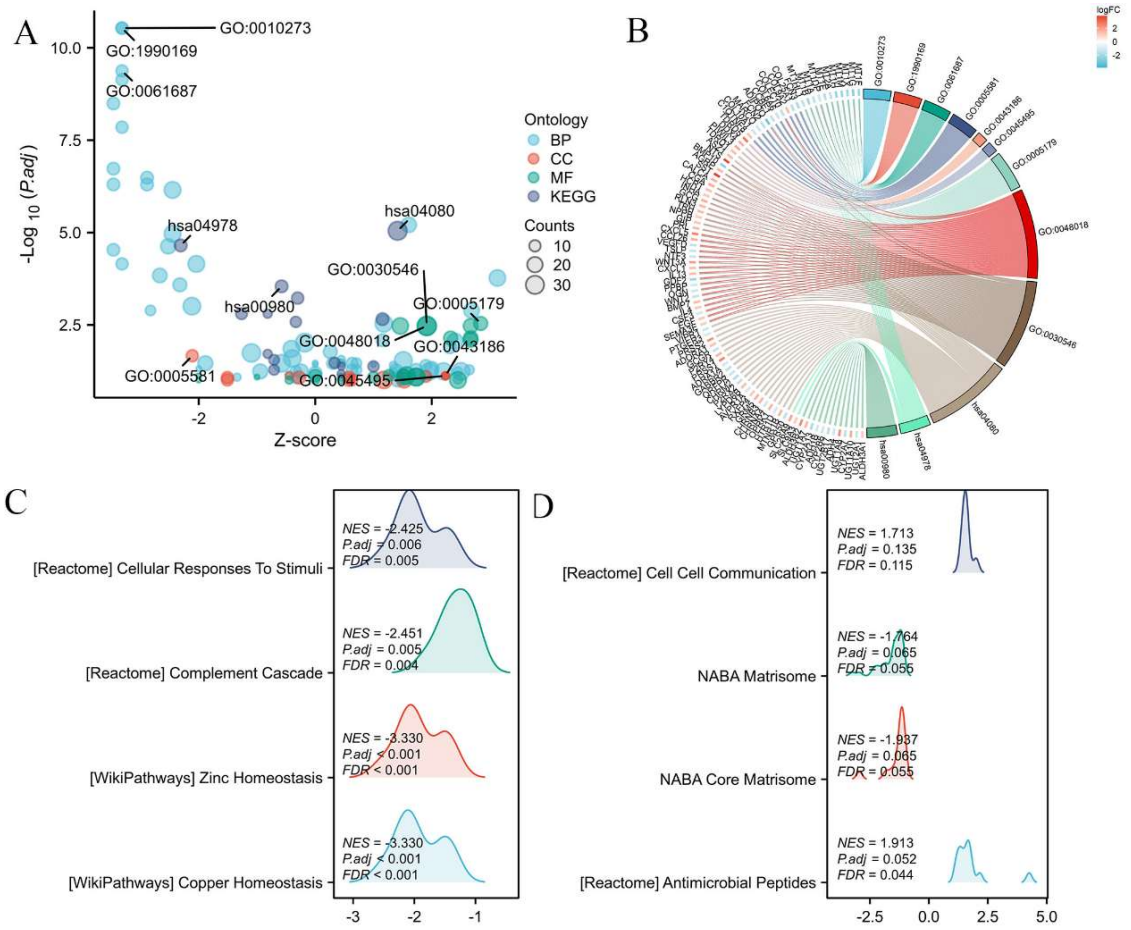

Figure 3: Enrichment analyses.A-B. GO and KEGG enrichment analyses;C-D. GSEA (Gene Set Enrichment Analysis)

Table 1: Description

ONTOLOGY	ID	Description
BP	GO:0010273	detoxification of copper ion
BP	GO:1990169	stress response to copper ion
BP	GO:0061687	detoxification of inorganic compound
CC	GO:0005581	collagen trimer
CC	GO:0043186	P granule
CC	GO:0045495	pole plasm
MF	GO:0005179	hormone activity

MF	GO:0048018	receptor ligand activity
MF	GO:0030546	signaling receptor activator activity
KEGG	hsa04080	Neuroactive ligand-receptor interaction
KEGG	hsa04978	Mineral absorption
KEGG	hsa00980	Metabolism of xenobiotics by cytochrome P450

III. D. High expression of FLAD1 inhibits cellular immune responses, thereby reducing the prognosis of patients.

Violin plot analysis revealed that the stromal score (StromalScore), immune score (ImmuneScore), and comprehensive assessment score (ESTIMATEScore) in the low-expression group of FLAD1 were significantly higher than those in the high-expression group, suggesting that high expression of FLAD1 might affect the clinical prognosis of patients by suppressing the cellular immune response in the tumor microenvironment (Figure 4 A). Immune cell infiltration analysis based on the CIBERSORT algorithm indicated significant differences in the distribution of immune cell subtypes among different groups. The proportions of three immune cell subtypes showed significant statistical differences between the tumor group (red) and the normal tissue group (blue) (Figures 4 B-C). Further correlation analysis revealed that the expression level of FLAD1 was significantly associated with 13 immune cell subtypes, including 5 positive regulatory ($r > 0$) and 8 negative regulatory ($r < 0$) relationships (Figure 4 D).

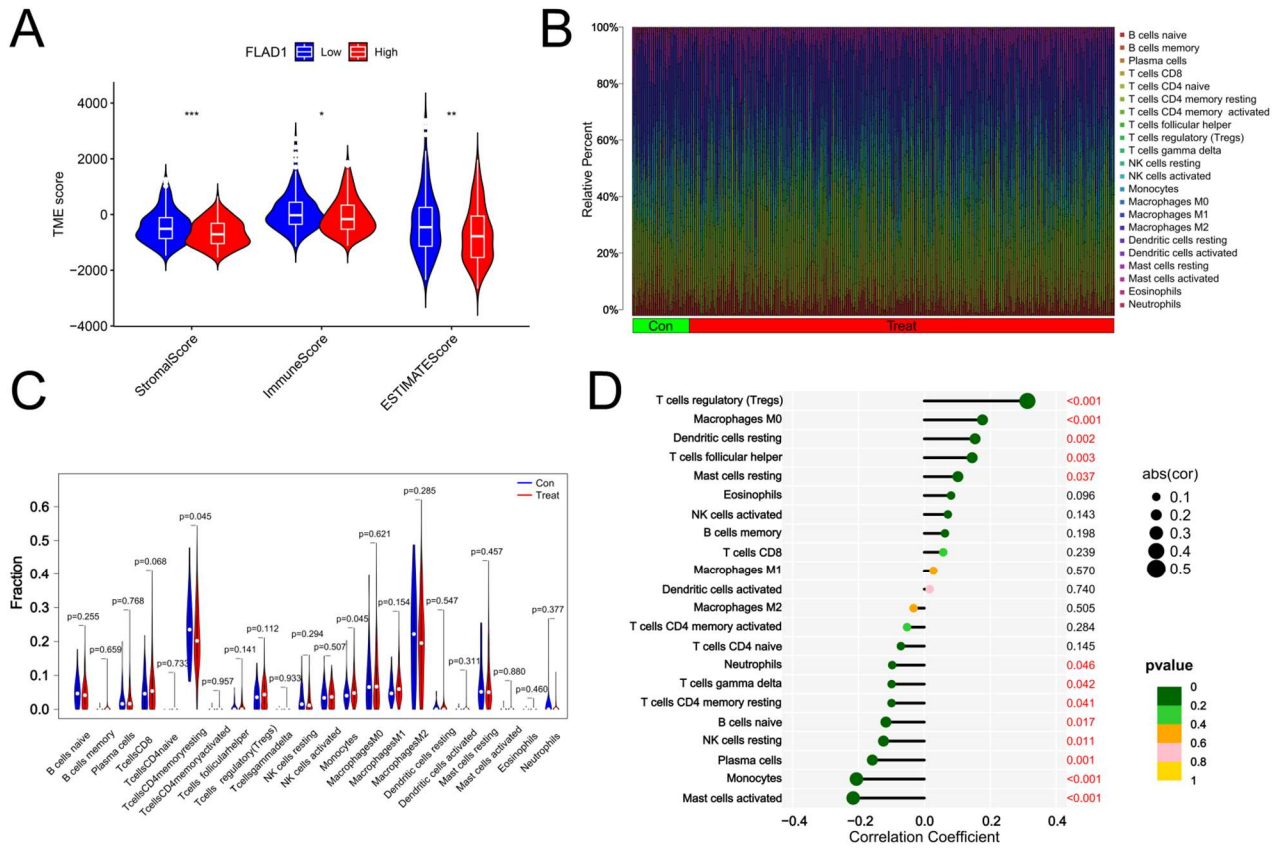

Figure 4: Immune infiltration analysis.A. Score difference plot.B. CIBERSORT bar chart.C. Immune cell difference violin plot.D. FLAD1 and immune cell correlation lollipop chart

III. E. WGCNA analysis identifies immune infiltration-related genes

In the Weighted Gene Co-expression Network Analysis (WGCNA), we screened and selected the genes most strongly correlated with immune infiltration and incorporated them into the STRING database for further analysis. Subsequently, Cytoscape software was used for additional filtering (Figure 5 A), where the top five genes with the highest Degree values (SALL4, CDX2, CT45A1, ISL1, and PITX2) were selected for co-expression analysis with FLAD1. We observed that FLAD1 exhibited co-expression with all five genes, among which SALL4, CDX2, and

CT45A1 showed statistically significant correlations (Figure 5 B). This finding further supports the association between FLAD1 and immune infiltration.

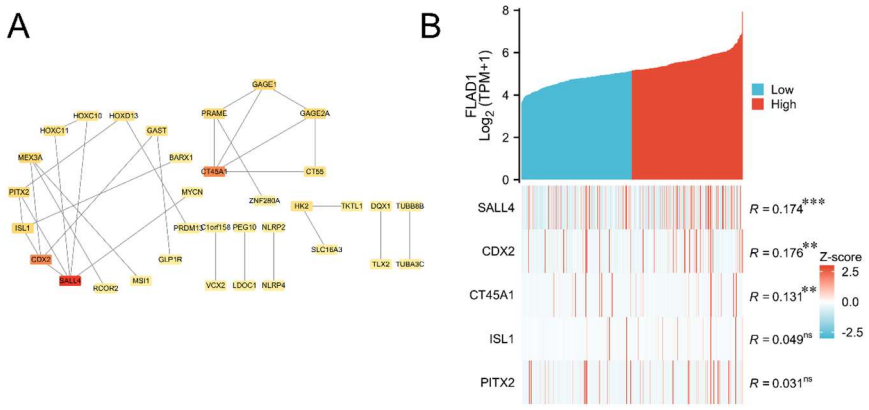

Figure 5: Protein-protein interaction network and co-expression heatmap

III. F. Drug sensitivity analysis

The IC50 values of 5-Fluorouracil, Alpelisib, and SCH772984 were compared between low and high FLAD1 expression groups. Red indicates low FLAD1 expression, while green represents high FLAD1 expression. The results demonstrated significant differences in drug responses between the two groups for all three compounds (Figures A-C, ***P<0.001), suggesting that FLAD1 expression levels may influence their therapeutic efficacy.Molecular docking analysis was performed using the CB-DOCK2 database, which generated three docking results. SCH772984 exhibited the lowest Vinascore, indicating the most favorable binding energy and highest stability with FLAD1. This was followed by Alpelisib (Vinascore = -8.5) and 5-Fluorouracil (Vinascore = -5.1) (Figures 6 A-C).

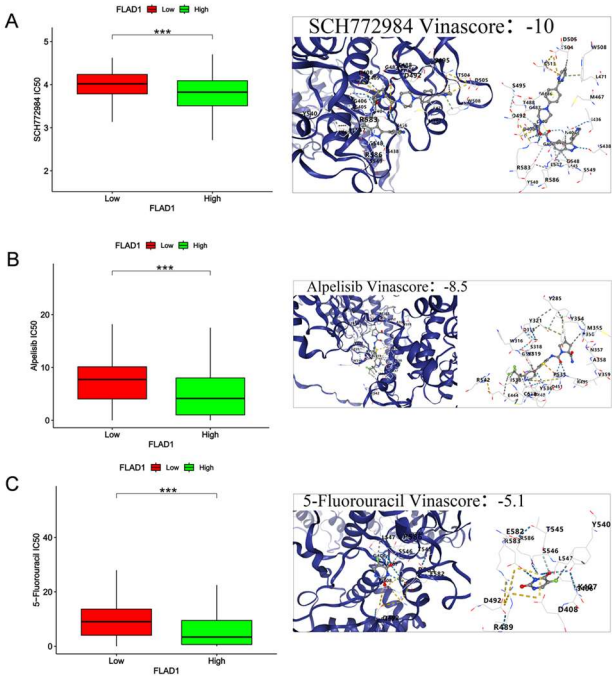

Figure 6: Drug sensitivity analysis and molecular docking results of 5-Fluorouracil, Alpelisib, and SCH772984

III. G. FLAD1 knockdown suppresses in vitro proliferation and metastasis of HCC cells

To further investigate the role of FLAD1 in HCC, we examined phenotypic alterations in HCC cells following FLAD1 knockdown. Western blot analysis confirmed the efficiency of FLAD1 knockdown (Figure 7 A). Colony formation and CCK-8 assays demonstrated that FLAD1 depletion significantly impaired the clonogenic capacity of HCC cells (Figures 7 B-D). Furthermore, transwell migration assays revealed that FLAD1 knockdown markedly reduced the migratory ability of HCC cells (Figures 7 E-F). In vivo subcutaneous xenograft experiments showed that FLAD1 silencing suppressed tumor formation capacity in melanoma models. These findings collectively indicate that FLAD1 plays a crucial role in regulating HCC cell proliferation, invasion, and migration.

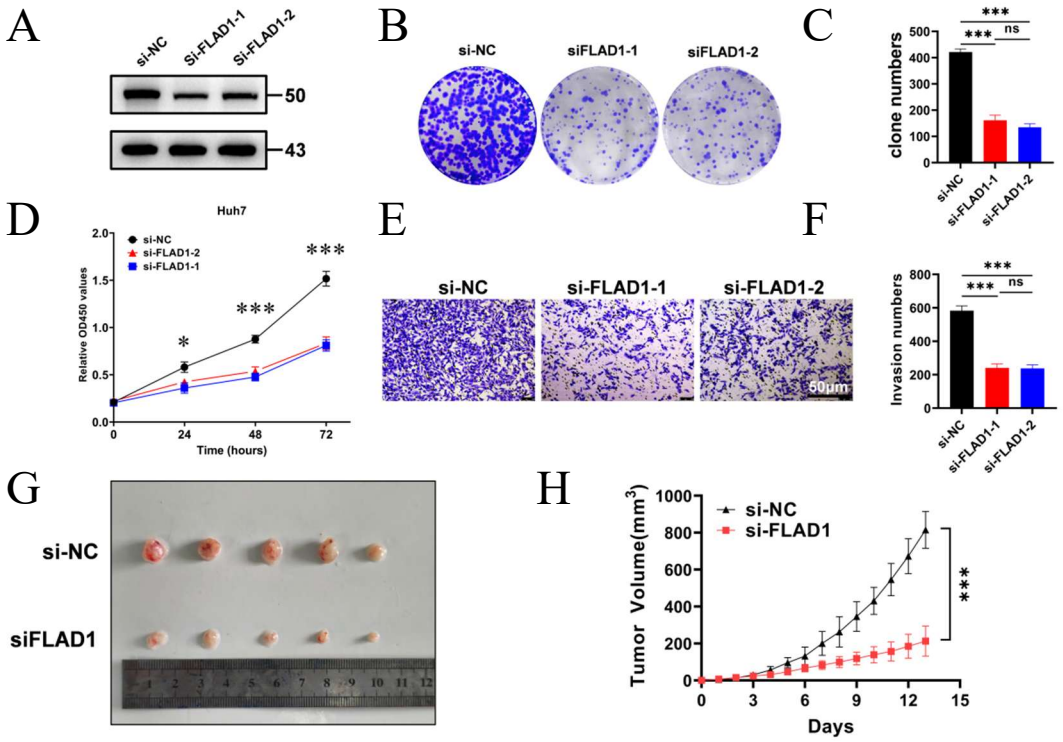

Figure 7: FLAD1 knockdown inhibits HCC cell proliferation and metastasis in vitro.A. Knockdown efficiency of small interfering RNA (siRNA);B-C. Colony formation assay; D. CCK-8 assay;E-F. Transwell invasion assay;G-H. Subcutaneous tumor xenograft model in mice

IV. Disscussion

FLAD1 (FAD Synthetase 1) is a key enzyme involved in the synthesis of flavin adenine dinucleotide (FAD), which plays an essential role in cellular metabolism and redox reactions [14]. FLAD1 may influence cancer cell energy metabolism and oxidative stress balance by modulating FAD levels, thereby contributing to carcinogenesis [15]. Dysfunction of FLAD1 could lead to reduced activity of DNA repair enzymes, increased genomic instability, and subsequent tumor progression. Additionally, FLAD1 may promote tumor development by regulating non-coding RNA expression and modulating the immune microenvironment [16]. However, the specific role of FLAD1 in hepatocellular carcinoma (HCC) pathogenesis remains unclear.

In this study, we employed comprehensive bioinformatics approaches to systematically investigate FLAD1's functional role in HCC, revealing a strong association between FLAD1 overexpression and poor patient prognosis. We found that FLAD1 expression was significantly elevated in HCC tissues compared to normal controls, and its high expression correlated markedly with reduced survival rates. Through GO, KEGG, and GSEA enrichment analyses, we demonstrated that FAD generated by FLAD1 serves as a crucial cofactor for multiple enzymes, participating in key biological processes including copper ion detoxification, inorganic compound detoxification, hormone activity, and receptor-ligand interactions. FLAD1 overexpression may influence HCC initiation, progression, and metastasis through these pathways.Notably, FLAD1 overexpression was associated with significant dysregulation of zinc and copper homeostasis in HCC cells, which is critical for maintaining intracellular metal ion balance. Immune infiltration analysis uncovered FLAD1's role in shaping the tumor immune microenvironment. The

high FLAD1 expression group exhibited significantly lower StromalScore, ImmuneScore, and ESTIMATEScore compared to the low-expression group, suggesting that FLAD1 may suppress immune cell infiltration and impair host immune responses. Furthermore, FLAD1 showed significant correlations with 13 immune cell types (5 positive and 8 negative correlations), highlighting its complex immunomodulatory functions.

Using WGCNA, we identified the blue module genes strongly associated with Treg cells. Protein-protein interaction network and gene co-expression analyses revealed that FLAD1 co-expressed with Treg-related genes including SALL4, CDX2, and CT45A1, providing novel insights into FLAD1's potential mechanisms in HCC immune regulation. Complementary in vitro and in vivo experiments demonstrated FLAD1's functional role in promoting HCC proliferation, invasion, and migration.

In conclusion, our study elucidates FLAD1's critical oncogenic functions and its potential as a prognostic biomarker in HCC, offering new diagnostic and therapeutic strategies for this malignancy. These findings position FLAD1 as a promising molecular target for HCC intervention.

Funding

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