Glycolysis-related gene dihydrolipoamide acetyltransferase promotes poor prognosis in hepatocellular carcinoma through the Wnt/β-catenin and PI3K/Akt signaling pathways

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Background: Recent research suggests that dihydrolipoamide acetyltransferase (DLAT), which is a copper-induced cell death-related gene, is involved in multiple biological events in tumors. This study sought to investigate the relationship between DLAT and hepatocellular carcinoma (HCC).

Methods: In the Cancer Genome Atlas (TCGA) database, we first identified the differentially expressed gene (i.e., DLAT), then confirmed DLAT expression, and found a link between it and the prognosis of HCC patients. An internal validation nomogram was built based on a multivariate Cox regression analysis. Data from the Tumor Immune Estimation Resource (TIMER) database was used to examine the association between DLT and immunological cells. A gene set enrichment analysis (GSEA) was conducted to investigate the probable mechanism of action. Finally, *in vitro* cytological research was conducted to further examin the involvement of DLAT in HCC-related unfavorable biological events.

Results: The database screenings showed that DLAT was a differentially expressed molecule; that is, DLAT was more highly expressed in the cancer tissues than normal tissues. TCGA results and Kaplan-Meier-plotter data sets showed that HCC patients with reduced DLAT expression had greater disease-specific survival (DSS), overall survival (OS), and progression-free interval (PFI). The prediction model had a concordance index of 0.659 (0.614–0.704), which indicates high accuracy. According to the TIMER database, tumor cells in the HCC microenvironment may be able to bypass the immune system due to the expression of DLAT. The *in vitro* cytological tests showed that DLAT knockdown significantly decreased the proliferation and invasion of the HCC cells. It also inhibited the activity of the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (Akt) and Wnt/β-catenin signaling pathways.

Conclusions: Decreased DLAT expression significantly prolongs the OS, PFI, and DSS of HCC patients. DLAT may be employed as a new predictive biomarker for HCC, and may be linked to the immune system in HCC patients. The tumor microenvironment (TME) may have a significant effect on the ability of tumor cells to evade the immune system. The PI3K/Akt and Wnt/β-catenin signaling pathways may affect the prognosis of HCC by interfering with DLAT. Given these findings, HCC may be an ideal target for the development of anti-cancer therapies.

Keywords: Dihydrolipoamide acetyltransferase (DLAT); hepatocellular carcinoma (HCC); glycolysis; bioinformatics analysis; prognosis

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Introduction

Liver cancer ranks 6th in morbidity, 3rd in mortality, and 4th in cancer-related fatalities among malignant tumors (1). Hepatocellular carcinoma (HCC) accounts for 75–85% of primary liver cancers (2). In 2020, the incidence of HCC was higher than that of other malignancies, and the World Health Organization (WHO) has estimated that over 1 million individuals will die of HCC by 2030 (3). HCC is highly heterogeneous and has a complicated etiology. The main causes of this neoplasm include metabolic disorders, chronic hepatitis virus infection, smoking, and excessive drinking (4,5). As the early clinical symptoms are not obvious and its pathogenesis is unclear, HCC patients often have advanced stage HCC or distant metastases at the time of diagnosis, and thus a poor prognosis (6). Consequently, the treatment of HCC is challenging.

Currently, the primary methods for treating HCC include surgery, intervention, and targeted therapy. These frequently used therapeutic interventions may increase the overall survival (OS) time of patients, but they have substantial drawbacks and are unable to significantly lower the death and recurrence rates associated with liver cancer (7,8). HCC is now thought to be affected by a number of "tumor suppressor genes", "oncogenes," and different signaling pathways. For example, the low expression of kinesin family member 20A (KIF20A) has been shown to reduce cell proliferation, enhance chemosensitivity, and to be closely connected to HCC (9). HCC patients with a 11beta-hydroxysteroid dehydrogenase 1 (11βHSD1) deficiency have more glycolysis, a higher intrahepatic metastasis risk, and a poor prognosis (10). The Thr-328 phosphorylation of pyruvate kinase M2 (*PKM2*) by heat shock protein 90 (*Hsp90*) promotes cellular glycolysis, and proliferation, and inhibits apoptosis in HCC patients (11). Forkhead box (FOX) G1 facilitates the epithelial to mesenchymal transition in HCC by forming a T-cell factor-4/β-catenin/FOXG1 complex (12). At present, the commonly used diagnostic methods for HCC, such as serum tumor markers, imaging techniques, and other clinical results, are not ideal (13). The early detection of HCC necessitates the screening of indicators linked with the disease.

Dihydrolipoamide acetyltransferase (DLAT) is a mitochondrial protein involved in glucose metabolism (14). DLAT is also the E2 subunit of the pyruvate dehydrogenase complex (PDC) in the catabolic glucose pathway and is an important gene related to glycolysis (15). Previous research has shown that glycolysis in tumors is linked to the tumor microenvironment (TME) (16,17). Glycolysis activity is related to active immune characteristics in cancer, because highly glycolytic tumors present an immune stimulating TME, and even related to immune checkpoints such as PD-L1 expression in tumors (18).

A study has shown that aberrant glycolysis is closely linked to the emergence and growth of cancers. For example, pancreatic cancer cells have been shown to be less sensitive to gemcitabine when glycolysis is present (19). Honokiol has been shown to inhibit hypoxia inducible factor-1alpha (HIF-1α)-mediated glycolysis, which in turn stops breast cancer growth (20). Further, the serine/threonine protein kinase 25 (STK25)-induced inhibition of aerobic glycolysis through the Golgi phosphoprotein 3 (GOLPH3)-mTOR pathway has been shown to inhibit

Highlight box

Key findings

- The reduced expression of DLAT can significantly prolong the prognosis of patients with hepatocellular carcinoma, and may interfere with the DLAT through Wnt/β-Catenin and PI3K/Akt signaling pathways are involved in the different prognosis of hepatocellular carcinoma.

What is known and what is new?

- DLAT is involved in a variety of biological events of tumors, including lung cancer, colorectal cancer, etc.
- We have increased the study of DLAT in liver cancer and carried out cytological verification, which provides a reference for further mechanism research in the future.

What is the implication, and what should change now?

- In the future, we will increase *in vivo* and *in vitro* cytological experiments to further study the mechanism of DLAT in liver cancer.

cell proliferation in colorectal cancer (21). When studying HCC, it is preferable to focus on glycolysis-related genes, as the liver is a key organ for glycolysis.

In this study, we examined the role of DLAT in predicting HCC patients' survival rate by screening The Cancer Genome Atlas (TCGA) database for a differentially expressed gene (i.e., DLAT), which is associated with the prognosis of HCC patients. A bioinformatics analysis and *in vitro* tests were also used to explore the effect of DLAT on the behavior of tumor cells and the underlying mechanisms of influence on tumor cells. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-5272/rc).

Methods

Data analysis using TCGA database

We used TCGA database (https://tcga-data.nci.nih.gov/) to examine the expression of DLAT in HCC.

TCGA database DLAT and RCC clinical parameters

HCC patients' clinical parameters were retrieved from TCGA database. A comparative analysis was then conducted to examine the correlation between DLAT and these clinical parameters and patient prognosis. The log-rank test and the Mantel-Cox test for gene expression in HCC were used to examine the prediction accuracy and risk score of the DLAT gene in the time receiver operating characteristic (ROC) analysis (22-24).

Construction and evaluation of nomograms

A multivariate analysis was used to construct nomograms that projected patients' survival probability at 1, 3, and 5 years based on our findings. The RMS R program was used to generate nomograms that comprised DLAT clinical characteristics and calibration plots. Calibration curves are the most common method used to evaluate model performance. In this study, the calibration curve was assessed graphically by mapping the nomogram predicted probabilities to the observed ratios, with the 45-degree line representing the best predicted value. The bootstrap approach with 1,000 resamples was used to assess the nomograms' discriminative ability, which yielded the concordance index (C-index). Additionally, the C-index was used to assess the prediction accuracy of the nomograms and specific prognostic factors.

Analysis of the correlation between DLAT and immune cells in the Tumor Immune Estimation Resource (TIMER) database and gene set enrichment analysis (GSEA) enrichment analysis

Bar graphs were created using the TIMER database (https://cistrome.shinyapps.io/timer/) to show the relationship between DLAT expression and other immune cells in HCC. Functional analysis was performed online using Metascape (https://metascape.org/gp/index.html"\l"/main/step1). Differential genes were added to Metascape for functional analysis. Genes with different functions were added to Metascape.

Tissues and cell lines

SMMC-7721 and HepG2 cell lines from the Shanghai Chinese Academy of Sciences Cell Bank (Shanghai, China) were used in this study. The HCC tissues and the corresponding adjacent tissues of 25 patients from the Tumor Hospital Affiliated to Nantong University (Nantong, China) were collected. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of the Tumor Hospital Affiliated to Nantong University (No. 2020-050), and informed consent was obtained from all the patients.

Reverse transcription-quantitative PCR was used to identify DLAT messenger ribonucleic acid (mRNA)

First, we pulverized 100 mg of tumor and paracrine tissue from the patient samples, and then froze the powder. Next, 1 mL of Trizol lysate wad added in accordance with the manufacturer's instructions to obtain the total ribonucleic acid (RNA). The RNA was reverse transcribed to obtain the complementary deoxyribonucleic acid (cDNA). A fluorescent quantitative polymerase chain reaction (PCR) instrument was used to conduct the real-time quantitative PCR. The relative expression of the mRNA for the target molecule was determined using the $2^{-\Delta\Delta Ct}$ method.

Validation of in vitro cytological experiments

CCK-detection of cell viability

Cell proliferation was quantified by Cell Counting Kit-8

(CCK-8) assays in accordance with the manufacturer's instructions. Briefly, seed 1,500 cells/96-well plate. The next day, CCK-8 solution was added to every well. Absorbance (OD at 450 nm) was assessed in a microplate reader (BioRad) after 4 h at 37 ℃ in a 5%-carbon dioxide (CO_2) incubator. The experiments were conducted a total of 3 times, and the average of the 3 results was taken.

Transwell invasion assays

For the cell invasion tests, Matrigel was diluted with cold serum-free Dulbecco's Modified Eagle Medium (DMEM) at a ratio of 1:8 at 4 ℃ (BD Biosciences, San Jose, USA). And carefully use a 50 µL coated polycarbonate filter (8 µm; Corning, NY, USA). The cells were incubated overnight at 37 ℃. The top chamber was then seeded with 5×10^5 cells in 200 µL of serum-free DMEM. The lower chamber was then seeded with 500 µL of DMEM and 10% fetal bovine serum (FBS). An atmosphere of 5% CO_2 was used to grow the cells at 37 ℃. After 24 h, the upper chamber was fixed with paraformaldehyde and stained with 0.5% crystal violet. The surface cells were counted using a microscope to eliminate the non-invading cells.

Western blot detection

A cold radio immunoprecipitation assay buffer containing protease inhibitors was used to lyse the cells after they had been washed twice with phosphate buffered solution at 4 ℃. The protein concentrations of the samples were determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was used to denature the total protein and transfer it to nitrocellulose membranes. Tween-20 [Tris-Buffered Saline with Tween (TBST)] was added to tris-buffered saline (TBS) containing 5% non-fat milk for 1 hour at room temperature to block the membranes. The membrane was then incubated with the primary antibody overnight at 4 ℃. The membranes were treated with a secondary antibody (anti-rabbit immunoglobulin G) for 1 hour at room temperature before being washed 3 times with TBST to identify the target protein. The target protein was then detected by ECL reagent (EMD Millipore, MA, USA).

Statistical analysis

Significance of continuous parameters expressed as mean ± standard deviation was determined by student *t*-test. GEOquery (25) (version 2.54.1) and Limma (26) (version 3.42.2) in R software (version 3.6.3) were used to download the data and conduct the difference analysis, respectively. The DLAT expression levels of the normal and malignant tissues were compared using the Wilcoxon test, while the Kruskal-Wallis test was used to examine the link between DLAT level and patient stage. Kaplan-Meier curves were used to measure the survival outcomes, and Spearman's correlation coefficient was used to examine the correlations. A P value <0.05 was considered statistically significant.

Results

DLAT is elevated in HCC tissue

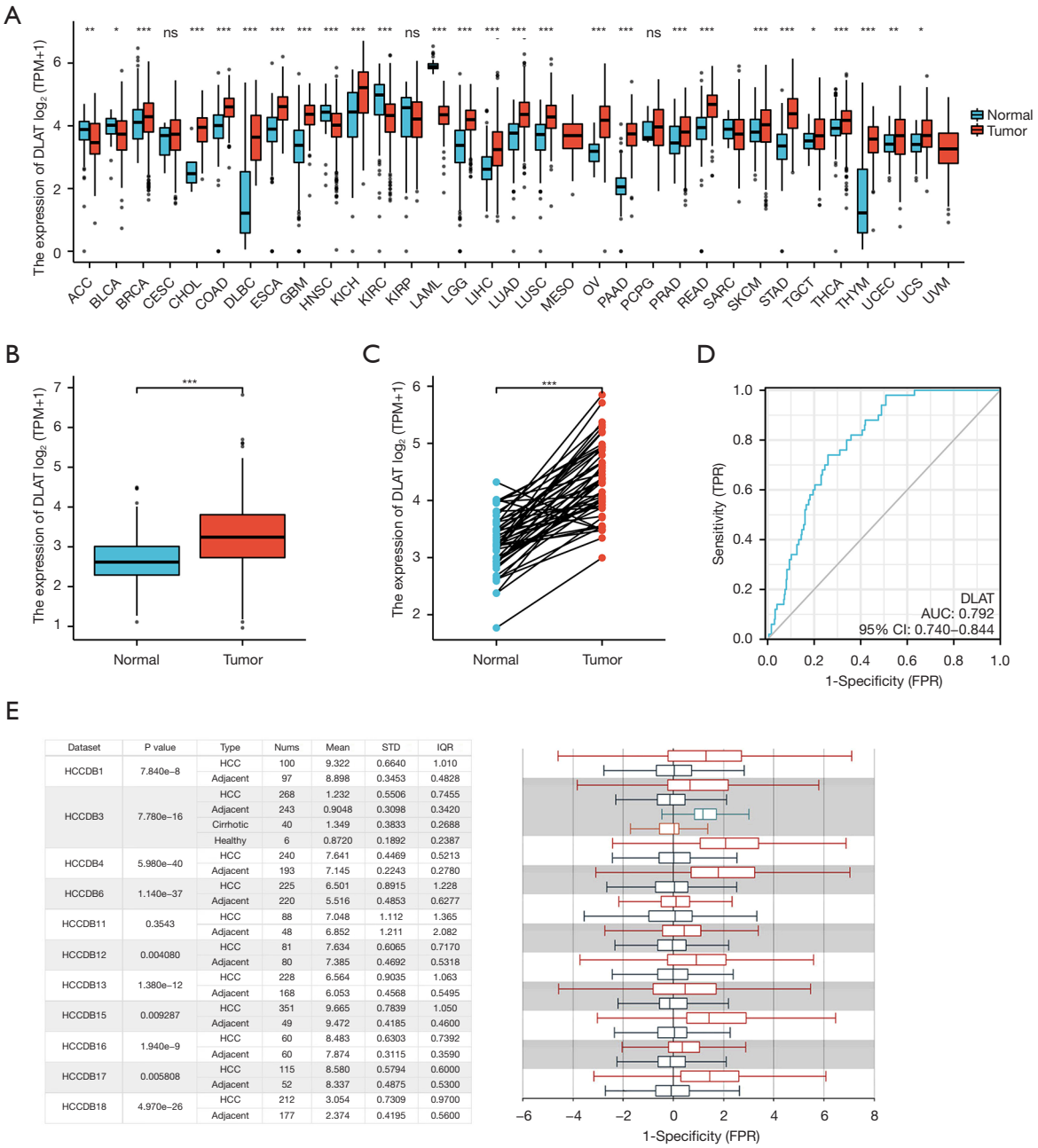
The initial pan-cancer investigation of TCGA database showed that DLAT was differentially expressed in the tumor tissues (see *Figure 1A*). Further research revealed that DLAT expression was increased in the HCC tumor tissues (see *Figure 1B*,*1C*). Constructing the ROC curve found that the area under the curve (AUC) was 0.792, so DLAT had a good prediction effect (see *Figure 1D*). The DLAT transcript levels were considerably greater in the HCC tissues than the surrounding healthy tissues (see *Figure 1E*).

Correlation between DLAT and HCC prognosis in KM-plotter database

When then examined the clinical data from the Kaplan-Meier-plotter database for HCC, and found that patients with low DLAT expression levels had a better prognosis in terms of OS, disease-specific survival (DSS), and progression-free interval (PFI) than those with high DLAT expression levels (see *Figure 2A*). Further subgroup stratified analysis showed that among different types of HCC patients with alpha-fetoprotein (AFP) ≤400 ng/mL, G1, G2, G3, M0, N0, Stage I, T1, T2, Child-Pugh grade A and vascular invasion, HCC patients with high DLAT expression had worse prognosis (see *Figure 2B*).

Nomogram construction

A nomogram with a C-index of 0.659 (0.614–0.704) was developed using the findings of the multivariate analysis to estimate the survival probability of HCC patients at 1, 3, and 5 years (see *Figure 3A*). The bias correction line in the calibration plot is close to the ideal curve (i.e., the 45-degree line), indicating some agreement between predicted and



Dataset	P value	Type	Nums	Mean	STD	IQR
HCCDB1	7.840e−8	HCC	100	9.322	0.6640	1.010
		Adjacent	97	8.898	0.3453	0.4828
HCCDB3	7.780e−16	HCC	268	1.232	0.5506	0.7455
		Adjacent	243	0.9048	0.3098	0.3420
		Cirrhotic	40	1.349	0.3833	0.2688
		Healthy	6	0.8720	0.1892	0.2387
HCCDB4	5.980e−40	HCC	240	7.641	0.4469	0.5213
		Adjacent	193	7.145	0.2243	0.2780
HCCDB6	1.140e−37	HCC	225	6.501	0.8915	1.228
		Adjacent	220	5.516	0.4853	0.6277
HCCDB11	0.3543	HCC	88	7.048	1.112	1.365
		Adjacent	48	6.852	1.211	2.082
HCCDB12	0.004080	HCC	81	7.634	0.6065	0.7170
		Adjacent	80	7.385	0.4692	0.5318
HCCDB13	1.380e−12	HCC	228	6.564	0.9035	1.063
		Adjacent	168	6.053	0.4568	0.5495
HCCDB15	0.009287	HCC	351	9.665	0.7839	1.050
		Adjacent	49	9.472	0.4185	0.4600
HCCDB16	1.940e−9	HCC	60	8.483	0.6303	0.7392
		Adjacent	60	7.874	0.3115	0.3590
HCCDB17	0.005808	HCC	115	8.580	0.5794	0.6000
		Adjacent	52	8.337	0.4875	0.5300
HCCDB18	4.970e−26	HCC	212	3.054	0.7309	0.9700
		Adjacent	177	2.374	0.4195	0.5600

Figure 1 HCC has increased DLAT expression. (A) Pan-cancer DLAT expression; (B) unpaired HCC tissues (n=374) and paracancerous tissues (n=50) in TCGA database both have high levels of DLAT expression; (C) paired HCC tissues (n=50) and paracancerous tissues (n=50) in TCGA database both have high levels of DLAT expression; and (D) the ROC analysis of DLAT revealed that (E) the HCC tissues in the HCCDB had considerably higher mRNA levels than the nearby normal tissues. *P<0.05, **P<0.01, ***P<0.001 indicate that the difference is statistically significant. DLAT, dihydrolipoamide acetyltransferase; TPM, transcripts per million; ns, no significant; TPR, true positive rate; FPR, false positive rate; AUC, area under the curve; CI, confidence interval; STD, standard deviation; IQR, interquartile range; HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; ROC, receiver operating characteristic; HCCDB, Hepatocellular Carcinoma Database; mRNA, messenger ribonucleic acid.

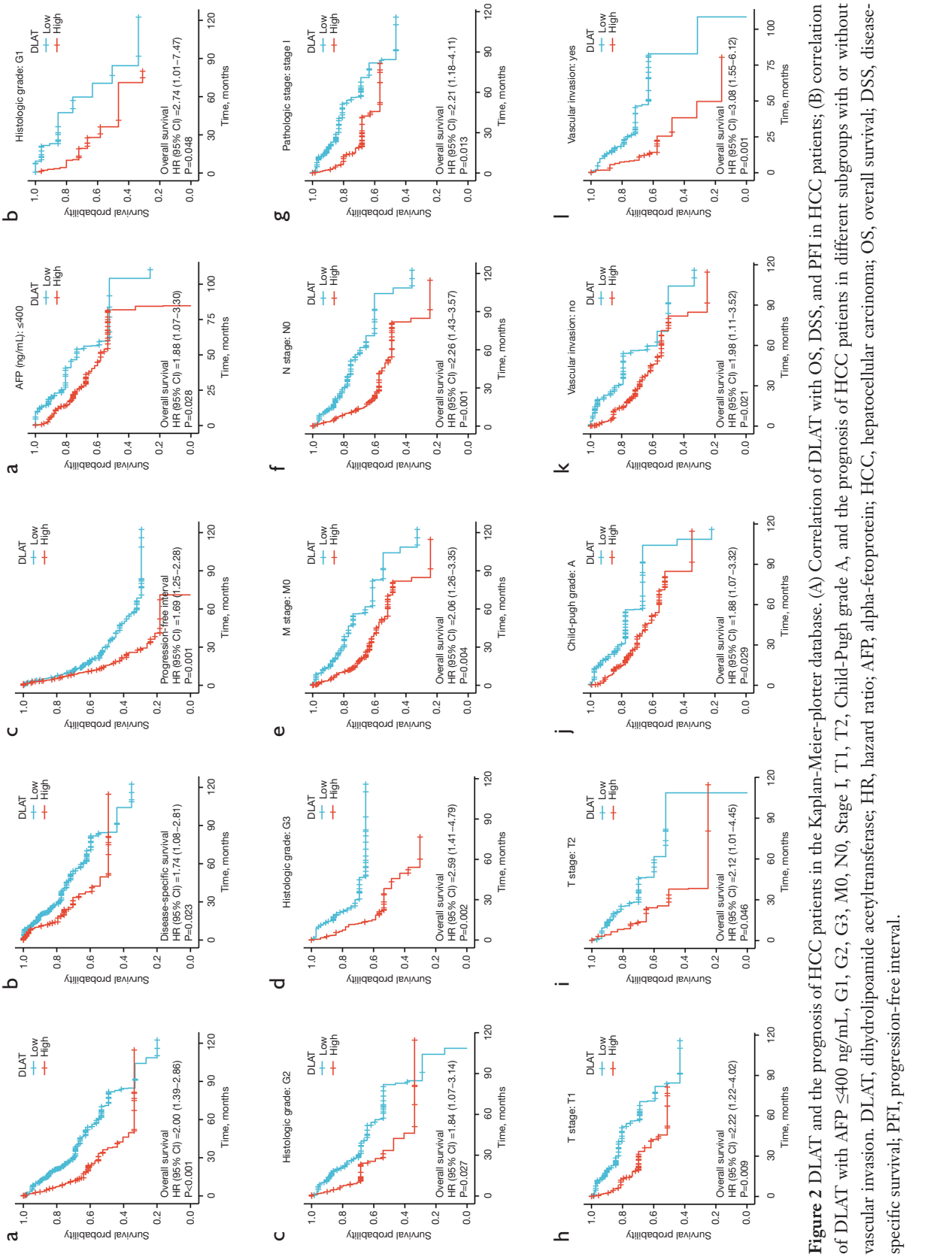


Figure 2 DLAT and the prognosis of HCC patients in the Kaplan-Meier-plotter database. (A) Correlation of DLAT with OS, DSS, and PFI in HCC patients; (B) correlation of DLAT with AFP ≤400 ng/mL, G1, G2, G3, M0, N0, Stage I, T1, T2, Child-Pugh grade A, and the prognosis of HCC patients in different subgroups with or without vascular invasion. DLAT, dihydrolipoamide acetyltransferase; HR, hazard ratio; AFP, alpha-fetoprotein; HCC, hepatocellular carcinoma; OS, overall survival; DSS, disease-specific survival; PFI, progression-free interval.

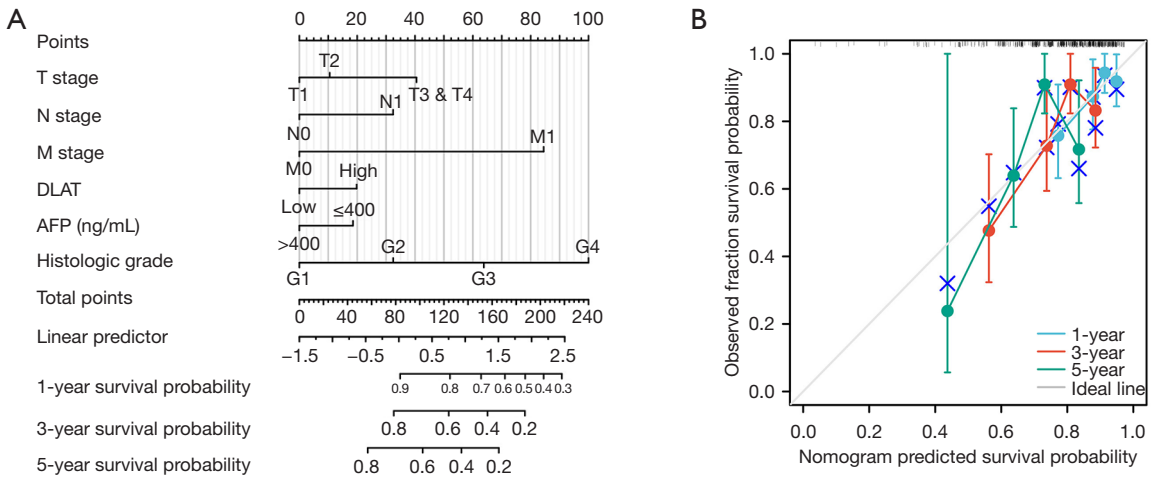


Figure 3 A calibration plot and nomogram. (A) A nomogram for HCC patients that predicts the survival probability of HCC patients at 1, 3, and 5 years; (B) a calibration plot of that nomogram. DLAT, dihydrolipoamide acetyltransferase; AFP, alpha-fetoprotein; HCC, hepatocellular carcinoma.

observed values (*Figure 3B*). Therefore, it also shows that the prediction model has certain prediction accuracy.

DLAT-interacting genes and proteins

GeneMania was used to create a gene-gene interaction network of altered neighboring genes (see *Figure 4A*). A protein-protein interaction (PPI) network was generated via the functional protein association networks (STRING) database for DLAT (see *Figure 4B*).

Screening of co-expressed genes of DLAT

Data mining from the TCGA database was used to identify positively or negatively correlated genes co-expressed with DLAT. The graph shows the top 50 genes positively and negatively associated with DLAT in HCC (*Figure 5A,5B*).

DLAT and immune cell correlation in TIMER

We created bar graphs from the TIMER database to visualize the link between DLAT expression and tumor purity in HCC and other immune cells. A positive correlation between DLAT expression and B cells, cluster of differentiation (CD)4$^+$ T cells, CD8$^+$ T cells, neutrophils, macrophages, and dendritic cells was found (see *Figure 6A*). We also found that DLAT affected the cellular environment of the TME. DLAT was shown to be related to higher numbers of T helper cells, Central Memory T cell (Tcm), T helper 2 (Th2) cells, macrophages, activated dendritic cells (aDC), and immature dendritic cell (iDC) infiltration. Specifically, we found that the T, B, and Th17 cell infiltration levels were negatively linked to the DLAT levels (see *Figure 6B*). Further research revealed a statistically significant correlation between DLAT expression and the immunological checkpoint markers of CD274 and CTLA-4, but not programmed cell death protein 1 (PDCD1) (see *Figure 6C*). Based on these findings, we can speculate that the expression of DLAT may be associated with tumor immune infiltration in HCC.

Correlation between DLAT and molecular targeted therapy drug target molecules in the TCGA database

Currently, there are targeted therapy options available for advanced HCC patients; for example, the latest National Comprehensive Cancer Network guidelines for HCC recommend the detection of relevant target molecules and the selection of related drugs. For treatment-naïve patients with advanced liver cancer, the optimal regimens recommended by the guidelines are sorafenib and lenvatinib, which are standard 1st-line treatments. Among them, the recommendation degree of sorafenib is grade 1, and the recommendation degree of lenvatinib is grade 2A. The 2 most recommended treatment regimens for advanced liver cancer patients with drug-resistant recurrence are regorafenib, cabozantinib, and

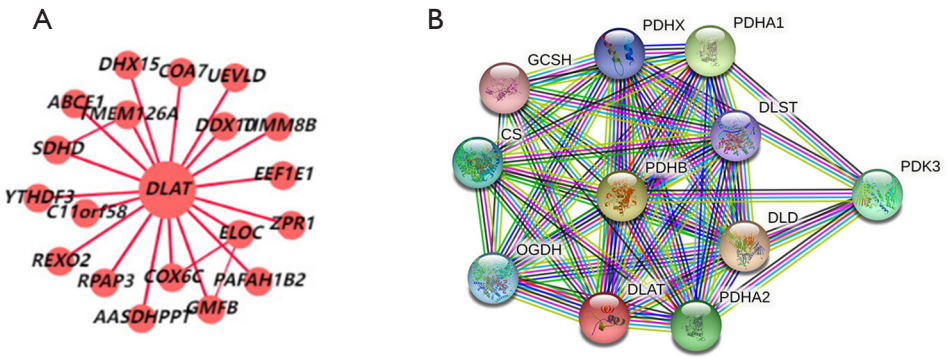


Figure 4 GeneMania was used to create the gene-gene interaction network of DLAT, and STRING was used to create the PPI network. DLAT, dihydrolipoamide acetyltransferase; STRING, functional protein association networks; PPI, protein-protein interaction.

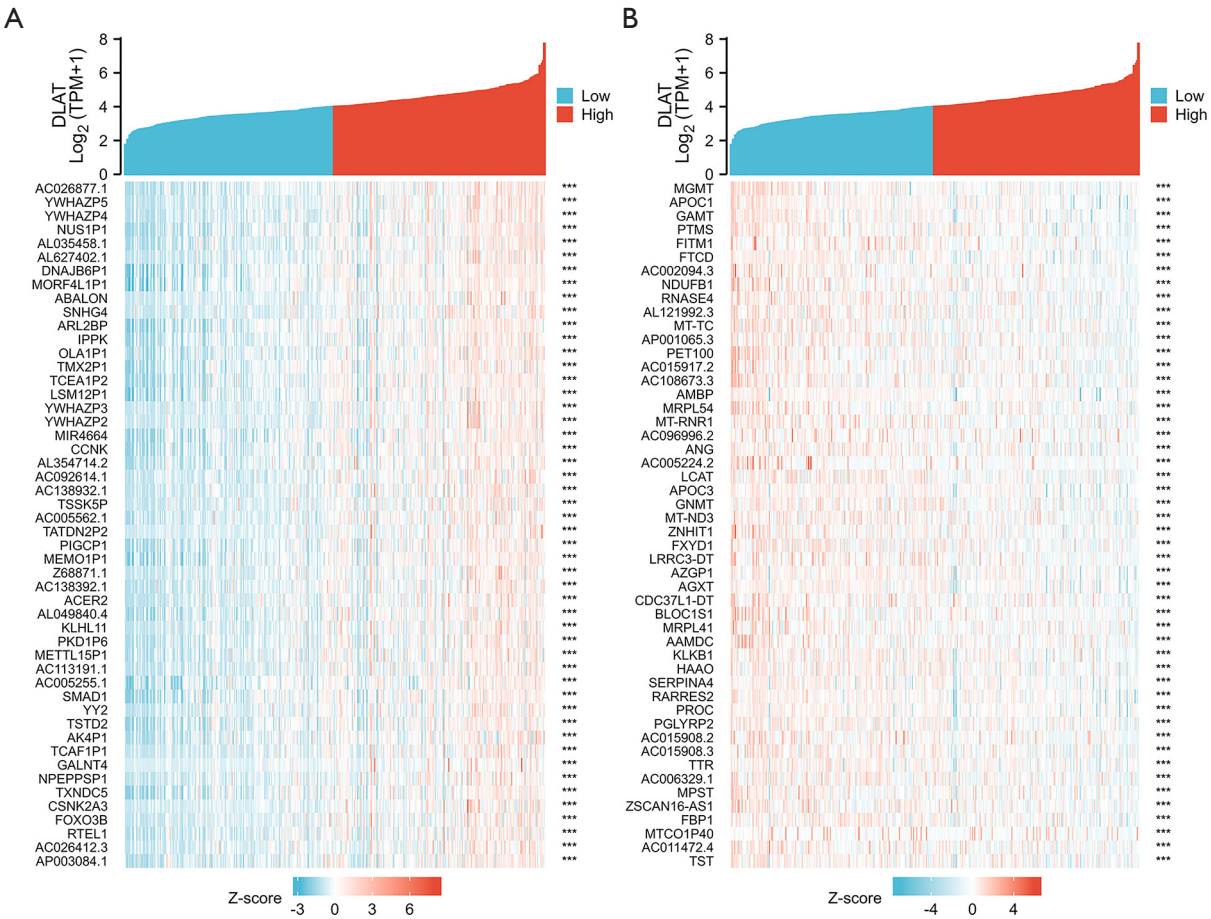


Figure 5 The DLAT screening of co-expressed genes. Heatmaps of the top 50 genes favorably and negatively linked with DLAT in human colorectal cancer in (A) and (B), respectively. ***P<0.001 indicate that the difference is statistically significant. DLAT, dihydrolipoamide acetyltransferase; TPM, transcripts per million.

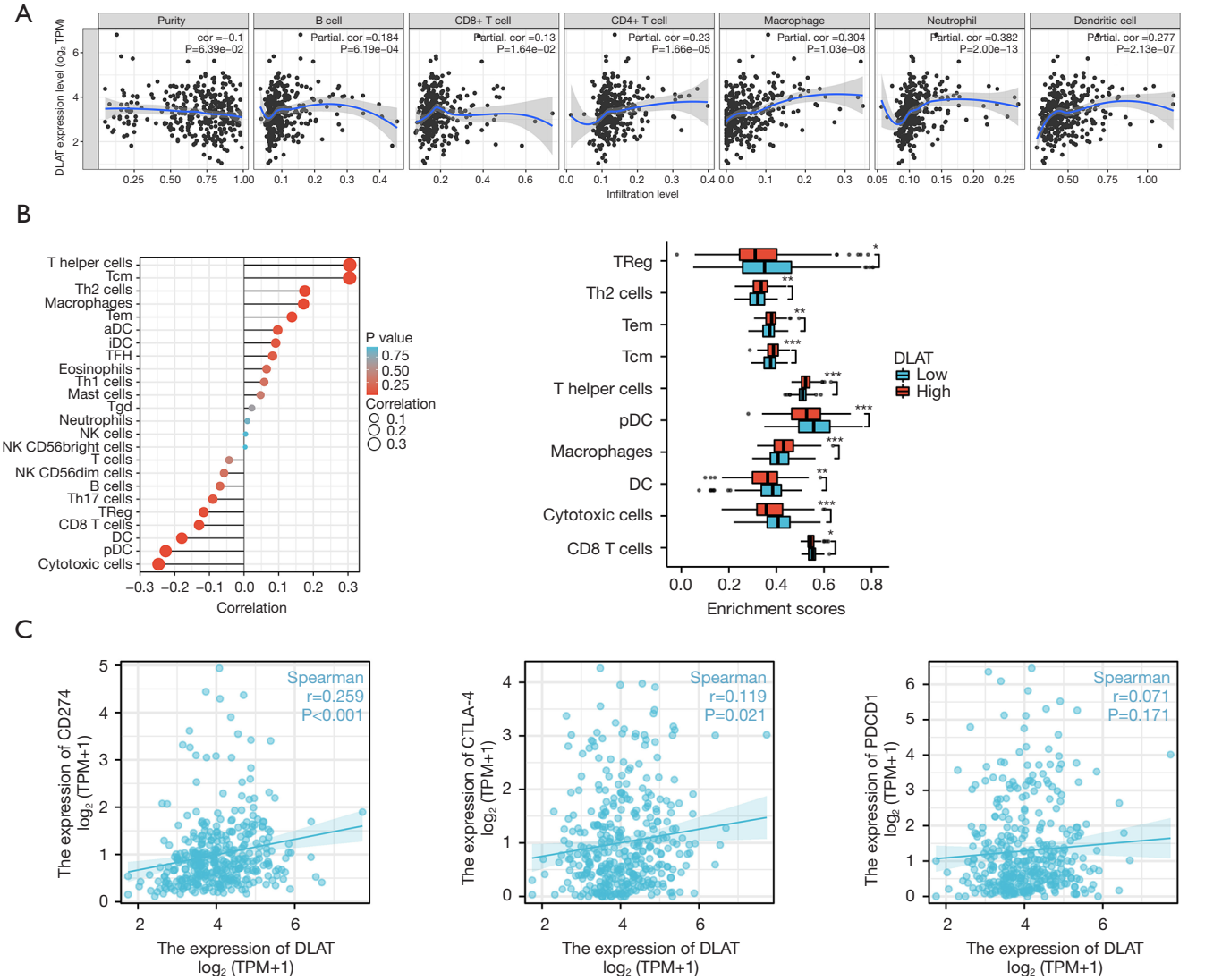


Figure 6 Correlation between DLAT expression and immune infiltration level. (A) The relationship between DLAT expression and the levels of immunological infiltration; (B) the expression of DLAT was closely linked to immune cell infiltration in HCC, and (C) CTLA-4, CD274, and PDCD1 sex scatterplots. *P<0.05, **P<0.01, ***P<0.001 indicate that the difference is statistically significant. DLAT, dihydrolipoamide acetyltransferase; TPM, transcripts per million; CD, cluster of differentiation; Tcm, Central Memory T cell; Th2, T helper 2; aDC, activated dendritic cells; iDC, immature dendritic cell; PDC, pyruvate dehydrogenase complex; PDCD1, programmed cell death protein 1; HCC, hepatocellular carcinoma.

ramucirumab, each of which has a level 1 recommendation. Thus, we continued to study DLAT and the HCC-related therapeutic targets. We found that DLAT was correlated with KIT, PDGFRA, PDGFRB, RAF1, CLDN18, EGFR, VEGFA, VEGFC, VEGFD, FGFR1, FGFR3, and MET (P<0.001) (see *Figure* 7). This also provides some reference opinions for the research and development of targeted drugs in the future, and also shows the potential significance of our research on DLAT.

Prediction of GSEA-based signaling pathways

We performed a Kyoto Encyclopedia of Genes and Genomes (KEGG) functional analysis online using Metascape. We found that DLAT may affect the biological events of HCC by participating in the WNT pathway, copper homeostasis,

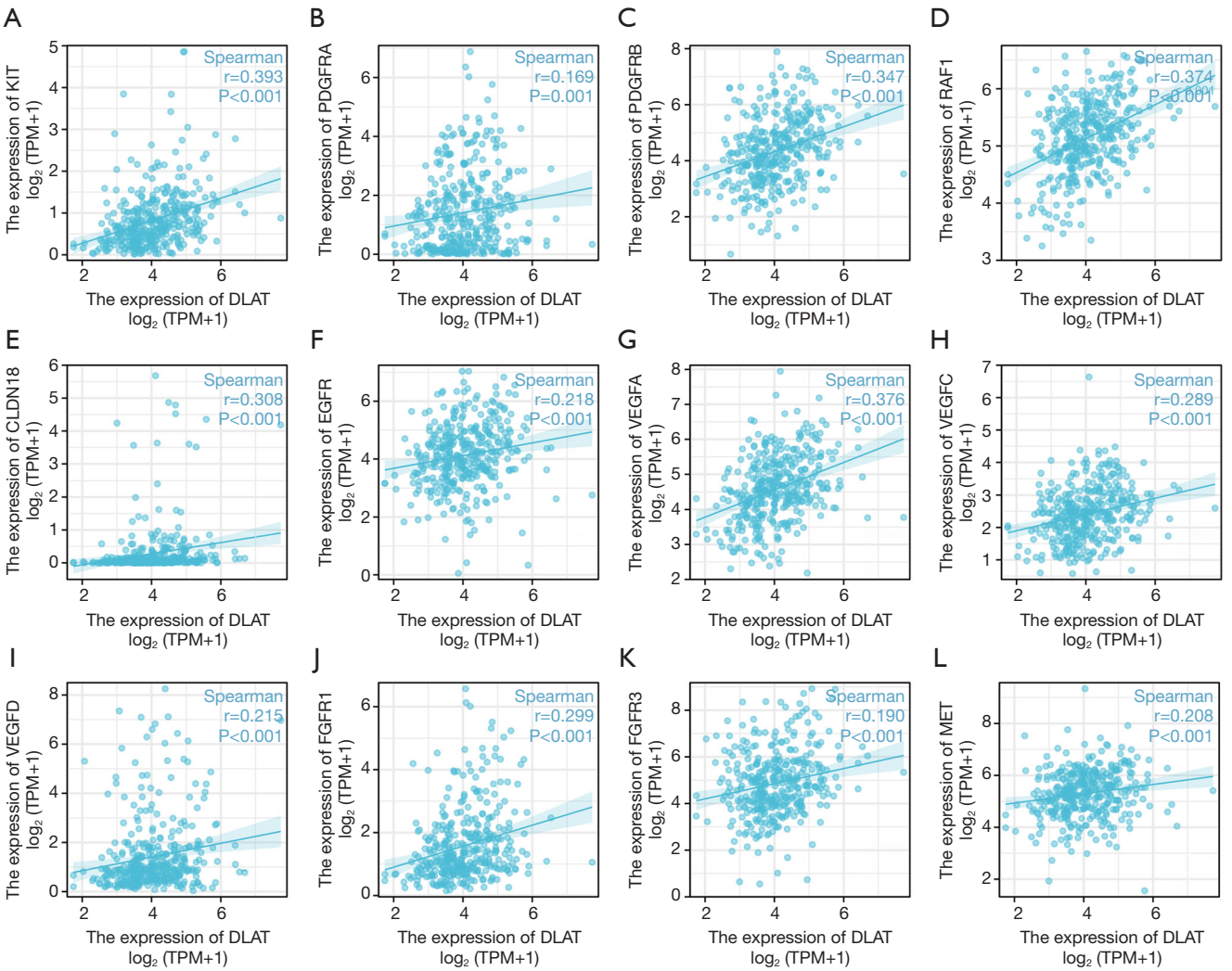


Figure 7 Correlation between DLAT and molecular-targeted drug therapy molecules in TCGA database. TPM, transcripts per million; DLAT, dihydrolipoamide acetyltransferase; TCGA, The Cancer Genome Atlas.

pyruvate metabolism, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (Akt) pathway, NOTCH pathway and angiogenesis, resulting in different prognosis of HCC. Based on the results of these predictions, we hypothesized that the glycolysis-related gene DLAT promotes poor HCC prognosis through the WNT pathway, which also provided a reference for the further *in vitro* experimental research we conducted (see *Figure 8*).

DLAT is highly expressed in liver cancer tissue

First of all, for the results of further data analysis, we collected 25 cases of liver cancer tissues and corresponding adjacent tissues. The results of the real-time quantitative PCR showed that the expression of DLAT in liver cancer was lower than that in adjacent tissues (see *Figure 9A*). Thus, a slow disease vector (short hairpin DLAT, shDLAT) and blank control (scramble) targeting DLAT were designed and transfected into SMMC-7721 and HepG2 liver cancer cells. The expression of DLAT in the SMMC-7721 and HepG2 liver cancer cells was significantly knocked down by adding the slow disease vector (shDLAT) to DLAT (see *Figure 9B*).

DLAT knockdown inhibits liver cancer cell growth and invasion

CCK-8 was used to examine the effects of DLAT

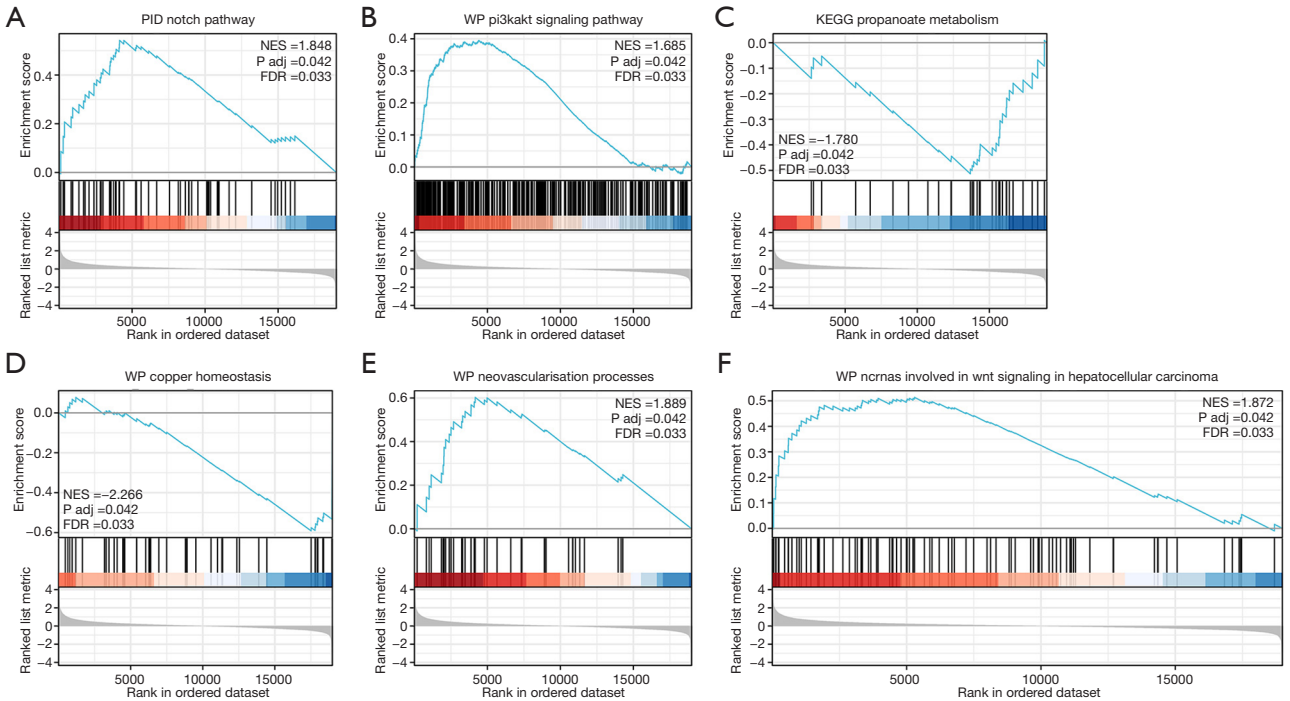


Figure 8 Six statistically significant probable relevant paths. The biological process gene set from MSigDB known as Gene Ear Biology was used. There were 1,600 different combinations of a random sample. NOM-p stands for nominal P value, FDR-q for false discovery rate, and NES for normalized enrichment score.

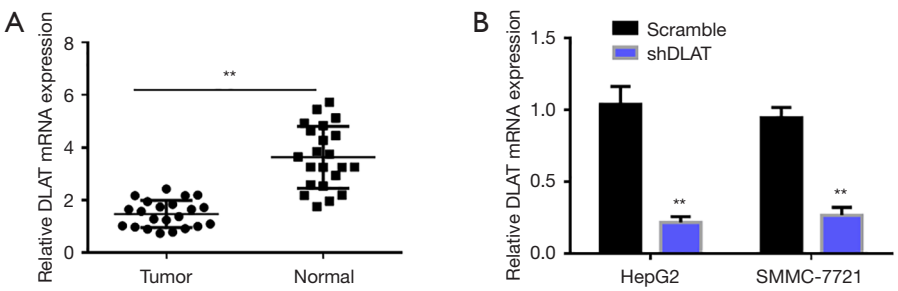


Figure 9 Liver cancer tissues have elevated DLAT expression. (A) 25 liver cancer tissues were examined using fluorescence quantitative PCR to identify the expression of DLAT; (B) a DLAT knockdown liver cancer cell line was created. **P<0.01. DLAT, dihydrolipoamide acetyltransferase; shDLAT, short hairpin DLAT; PCR, polymerase chain reaction.

knockdown on the growth of SMMC-7721 and HepG2 liver cancer cells. ShDLAT significantly decreased the viability of the SMMC-7721 and HepG2 liver cancer cells compared to the scramble control group and the ability of the SMMC-7721 cells to proliferate (see *Figure 10A,10B*). The Transwell tests revealed that DLAT knockdown (shDLAT) significantly reduced the proliferation potential of the SMMC-7721 and HepG2 liver cancer cells compared to the scramble control group (see *Figure 10C,10D*).

Based on our findings, DLAT knockdown has a significant effect on liver cancer cell proliferation and invasion. Further, according to the KEGG function analysis, DLAT may be involved in the WNT and PI3K/AKT pathways. The Western blot detection of SMMC-7721 cells showed that transfecting the liver cancer cells with the DLAT-targeting slow disease vector (shDLAT) greatly reduced the expression of DLAT and the critical Wnt signaling protein β-catenin (see *Figure 11A,11B*). As a result

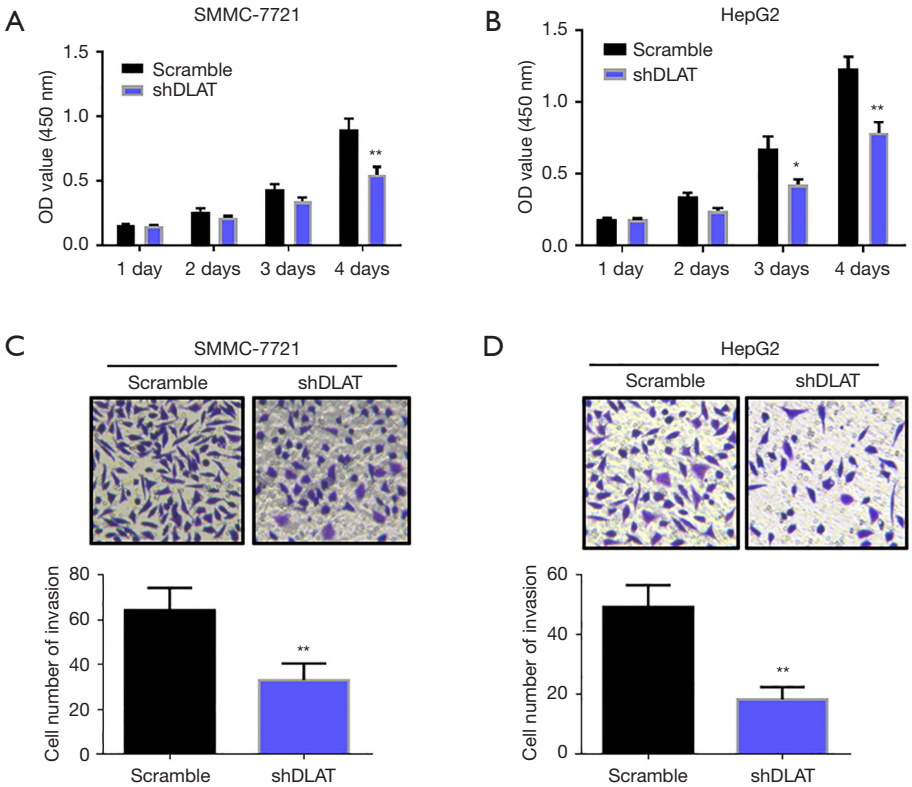


Figure 10 The proliferation and invasion of hepatoma cells were significantly reduced when DLAT was knocked down. (A,B) The Transwell and CCK-8 results both showed that DLAT knockdown affected the capacity of SMMC-7721 and HepG2 liver cancer cells to proliferate. (C,D) The Transwell results also showed that DLAT knockdown affected the ability of SMMC-7721 and HepG2 liver cancer cells to proliferate (crystal violet staining, 200×). *P<0.05, **P<0.01. OD, optical density; shDLAT, short hairpin DLAT; DLAT, dihydrolipoamide acetyltransferase.

of the DLAT knockdown, phosphorylated phosphoinositol 3 kinase (p-PI3K) and Phosphorylated protein kinase B (p-Akt) expression were significantly suppressed; however, the expression of total PI3K (T-PI3K) and T-Akt (T-Akt) was unaffected (see *Figure 11A*,*11C*). When DLAT was knocked down in the liver cancer cells, the activity of the Wnt/β-catenin and PI3K/Akt signaling pathways was significantly reduced.

Discussion

HCC is one of the leading causes of cancer-related mortality worldwide, but its underlying processes are complicated (27). Currently, despite advances in systemic therapy for HCC, patient survival remains low due to late diagnosis and a wide variety of underlying liver disease (28). Following the use of multi-omics technologies, biomarkers have become useful diagnostic, prognostic, and therapeutic tools (29).

In this study, we combined bioinformatics analysis and *in vitro* experiments to explore the role of DLAT in the occurrence and development of HCC and the possible mechanisms through which it acts. Based on data from TCGA database, we found that HCC patients with reduced DLAT expression had superior OS, DSS, and PFS. We also constructed a nomogram by comparing the correlation between DLAT and clinical parameters of HCC patients, so that the prediction effect has more reference value for clinical use.

Further data analysis results of the TIMER database and the correlation with immune checkpoint-related molecules CTLA-4, CD274, and PDCD1 all support that the expression of DLAT may be significantly related to immune infiltration, and indicate that DLAT plays an important role in HCC tumor microbiome. The environment may play an important role in promoting immune escape of tumor cells. The ability of tumor cells to evade the immune

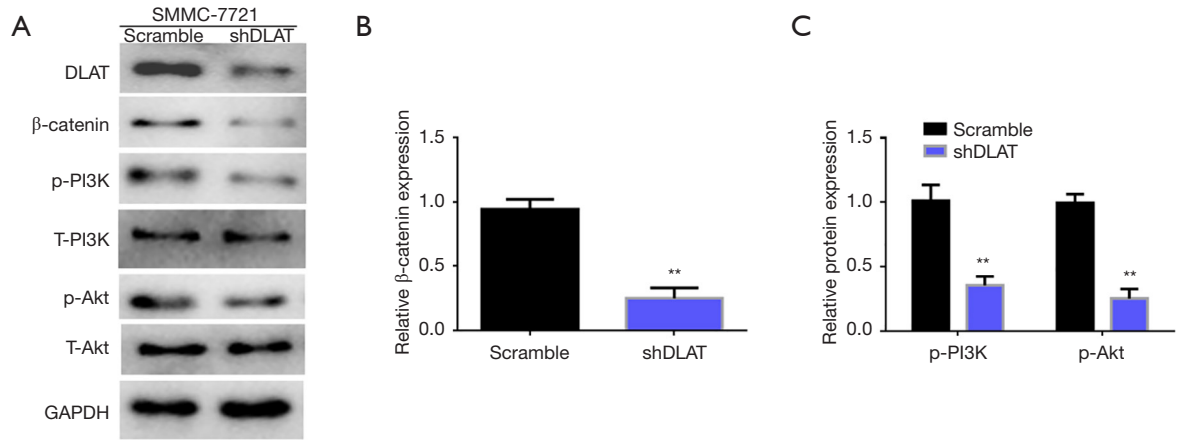


Figure 11 The knockdown of DLAT significantly inhibited the Wnt/β-catenin and PI3K/Akt signaling pathways in the liver cancer cells. (A) Western blot detection showed the effect of DLAT knockdown on the expression of β-catenin, PI3K, and Akt; (B) grayscale analysis and statistics of catenin protein bands on the expression of β-catenin; (C) grayscale analysis and statistics of PI3K and Akt protein bands. **P<0.01. DLAT, dihydrolipoamide acetyltransferase; p-PI3K, phosphorylated phosphoinositol 3 kinase; T-PI3K, total phosphatidylinositol-4,5-bisphosphate 3-kinas; p-Akt, Phosphorylated protein kinase B; T-Akt, total protein kinase B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; shDLAT, short hairpin DLAT; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinas; Akt, protein kinase B.

system may be affected by their immediate surroundings. We also analyzed the correlation between DLAT and the target molecules related to the current HCC-targeted drug therapy, and found that DLAT is related to *KIT*, *PDGFRA*, *PDGFRB*, *RAF1*, *CLDN18*, *EGFR*, *VEGFA*, *VEGFC*, *VEGFD*, *FGFR1*, *FGFR3*, and MET. These results can provide better reference for the selection of targeted drugs from the side, and the current clinical guideline research results also support the clinical value of our research from the side.

Based on the above two results, we found that DLAT is not only associated with immune infiltration in HCC, but may also be associated with targeted drug selection and efficacy in HCC. At present, multiple regimens are used in clinical practice to treat advanced HCC and improve the prognosis of patients, such as immune combined chemotherapy, double immune therapy, and immune combined targeted therapy. There are also the IMbrave150 and GO30140 trials in relevant global clinical trials. For example, 2 large clinical trials (i.e., the IMbrave150 and GO30140 trials) determined that atezolizumab + bevacizumab (T+A) combination therapy should be used as the 1st-line standard regimen for unresectable HCC (30,31). Nivolumab + ipilimumab (Nivo + Ipi) can be safely used as a new adjuvant therapy for HCC without delaying hepatectomy (LR). In terms of radiological and pathological remission, Nivo + Ipi has good anti-tumor efficacy (NCT03682276) (32). The NCT03006926 study compared the efficacy of lenvatinib + pembrolizumab (L + P) in treating unresectable hepatocellular carcinoma (uHCC) patients. This single-arm study showed that changes in serum biomarkers were associated with angiogenesis, fibroblast growth factor (FGF), and interferon cell (IFNc) signaling, which indicated the targeted involvement of lenvatinib + pembrolizumab (L + P) for the treatment of uHCC population (33). Thus, recurrent or metastatic HCC may benefit from immunotherapy in combination with targeted therapy. Additionally, the DLAT molecule studied in this project was not only related to the immune infiltration of HCC, but also to the target molecule, which also reflects the clinical value of our research.

Next, a GSEA was conducted to identify signaling pathways. We found that DLAT may affect the WNT, copper homeostasis, pyruvate metabolism, pPI3K/AKT, and NOTCH pathways, and angiogenesis. All of these mechanisms are linked to HCC prognosis in distinct ways. Wnt2b/β-catenin/c-Myc signaling and glycolysis reprogramming have been found to change the transition of HCC from epithelial to mesenchymal (34). HCC metastasis is inhibited by the Warburg effect, which is inhibited by the PPAR coactivator-1 through the PPAR-dependent WNT/β-catenin/pyruvate dehydrogenase kinase isoenzyme 1 axis (35). In addition to the molecules that have been identified as glycolysis-related molecules, our research molecule is also a copper death-related

molecule (36). Redox scavenger regulation by coumarin-bis (2-pyridylmethyl) amine hybrid molecules was shown to cause DNA damage and cell death in diethylnitrosamine-induced HCCs (37).

Glycolysis refers to the process of decomposing glucose or glycogen into pyruvate, adenosine triphosphate (ATP), and nicotinamide adenine dinucleotide (NADH)+H+. A minimal quantity of ATP is produced as a result of this activity. Glycolysis, gluconeogenesis, the tricarboxylic acid cycle, and amino acid metabolism are all connected to glycolysis as major metabolic nodes. Cheng *et al.* showed that the long non-coding RNA RP11-241J12.3 targeting pyruvate carboxylase promotes invasiveness of HCC by disrupting pyruvate metabolism and DNA mismatch repair system (38). Through the activation of PI3K/Akt/mTOR, XPA suppressed the growth of cancer cells inside the liver by promoting autophagy and cell death (39). Additionally, a study has found that inhibiting RFX6 through the Notch pathway inhibits the invasive ability of tumor cells and affects tumor immunity in HCC (40). Our biological information analysis indicate that DLAT may be involved in the WNT and other pathways that contribute to the poor prognosis of HCC patients.

The cytology part of the article describes the molecular phenotypic function, which is a potential proof of its inhibitory effect on hepatoma cells. We also confirmed that DLAT is highly expressed in liver cancer tissues by real-time PCR. Additionally, we designed a slow disease vector (shDLAT) and a blank control (scramble) targeting DLAT, and used the designed lentivirus to transfect HepG2 and SMMC-7721 cells of liver cancer. Hepatoma cell proliferation and invasion, and the PI3K/Akt or Wnt/β-catenin signaling pathways in hepatoma cells were significantly inhibited by the knockdown of DLAT. Further, these results support previous research findings on liver cancer, indicating that our hypotheses may be correct.

Conclusions

DLAT is critical to HCC progression and that it might be a biomarker for this disease. The prognosis of HCC may be affected by signaling pathways, such as the PI3K/Akt or Wnt/β-catenin signaling pathways. Anti-cancer therapies for HCC could be developed based on these findings.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-5272/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-5272/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-5272/coif). RDP receives consulting fees from AMCA; honoraria for lectures from Servier, bayer, Merck, Lilly, MSD and BMS; support for attending meetings from AstraZeneca and participates on advisory boards from AstraZeneca and Servier. SRM is a consultant for ConMed. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of the Tumor Hospital Affiliated to Nantong University (No. 2020-050), and informed consent was obtained from all the patients.

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