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RAPID COMMUNICATION

# Genetic landscape and clinical significance of cuproptosis-related genes in liver hepatocellular carcinoma

Recently, Peter et al reported a novel form of cell death (cuproptosis) that was different from other known death mechanisms.[1] They found that accumulation of copper ions could induce destabilization of Fe–S cluster proteins and aggregation of mitochondrial lipoylated proteins, ultimately resulting in cuproptosis.[1] Of note, ten genes were identified as cuproptosis-related genes (CRGs) in copper ion-induced cell death, including *PDHB*, *PDHA1*, *DLAT*, *DLD*, *LIPT1*, *LIAS*, *FDX1*, *CDKN2A*, *GLS*, and *MTF1*.[1] Since dysregulation of copper metabolism is involved in many cancers, cuproptosis and CRGs may play vital roles in cancer development and treatment.[1,2] Herein, our pancancer analysis revealed the coordinated upregulation of 9 of 10 CRGs in human liver hepatocellular carcinoma (LIHC) across 33 solid tumors (Fig. S1A; Fig. 1A), suggesting the distinct role of CRGs in LIHC. We then explored the genetic landscape, biological function, and clinical significance of CRGs in LIHC, and validated our bioinformatic findings by *in vitro* experiments and clinical cohorts. The detailed methods were described in the supplementary material.

To identify the prognostic value of CRGs in LIHC, Cox regression analysis, least absolute shrinkage and selection operator regression analysis, and nomogram analyses were conducted. Our data showed the combination of *DLAT*, *CDKN2A*, *LIPT1*, and *GLS* exhibited potent prognostic value in LIHC (Fig. S1B–G; Fig. 1B). To assess the mechanism underlying the upregulation of CRGs in LIHC, the mutation and methylation status of CRGs were analyzed. As shown in Figure S2A and B, the mutation frequency of most CRGs was less than 10%, and genetic alterations were not associated with the prognosis of LIHC (Fig. S3A). Intriguingly, we found that the transcript levels of all CRGs were negatively associated with the DNA methylation of multiple CpG sites (Table S1), and the hypomethylation of *PDHA1*, *DLAT*, *LIPT1*, *GLS*, and *MTF1* was correlated with worse outcomes in LIHC patients (Fig. S3B), suggesting that DNA methylation might be responsible for the upregulation of CRGs in LIHC.

We also explored the coexpression patterns of CRGs in LIHC and identified the top 10 genes with the highest correlation coefficient with each CRG in LIHC (Fig. S4A and Table S2). The protein–protein interaction network of coexpressed genes and CRGs is displayed in Figure 1C. As shown in Figure 1D and Figure S4B, the candidate genes were closely associated with cuproptosis-related processes, such as the citrate cycle (TCA cycle), oxidative phosphorylation, cellular respiration, and mitochondrial respiration.[1]

Emerging evidence has suggested that *FDX1*, *DLAT*, and *LIAS* are the key factors in cuproptosis.[1,2] To elucidate the interaction between these key genes of cuproptosis and immune cell infiltration in LIHC, we further applied a series of genetic-immunologic analyses. As presented in Figure S5A and B, and Table S3–8, the expression of *DLAT*, *LIAS*, and *FDX1* was correlated with the expression of multiple immune checkpoint-associated and immune pathway-associated genes, suggesting their significance in LIHC immunotherapy. Notably, compared with *LIAS* and *FDX1*, only *DLAT* expression was positively correlated with the infiltration of all typical immune cells (Fig. S5C, D; Fig. 1E), suggesting that *DLAT* might play a critical role in the process of genetic–immunological interaction in LIHC. Besides, *DLAT* expression was higher in the malignant cells than that in the immune cells (Fig. 1F). The analysis of TIMER score of immune cells revealed enhanced infiltration of most immune cells in *DLAT*-high LIHC compared with *DLAT*-low LIHC (Fig. 1G). Moreover, in the *DLAT*-high group, high infiltration of $CD8^+$ T cells was associated with better clinical outcomes in LIHC patients, while high infiltration of macrophages was correlated with poor survival in LIHC patients (Fig. 1H). As expected, no significant correlation was observed between immune cell infiltration and LIHC

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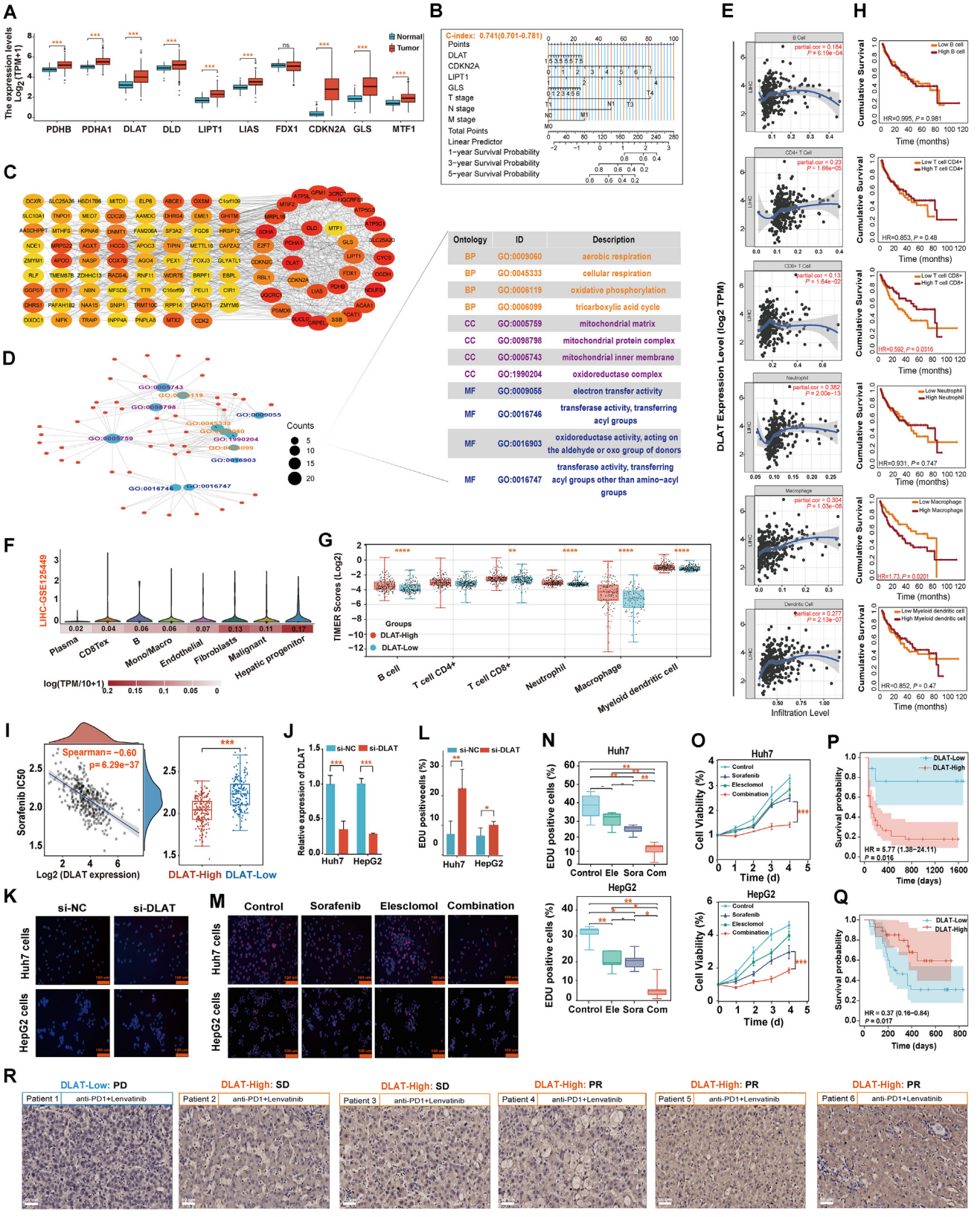


**Figure 1** Genetic landscape and clinical significance of CRGs in LIHC. **(A)** Expression of CRGs in LIHC tissues and adjacent normal liver tissues. **(B)** Logistic and least absolute shrinkage and selection operator regression-based nomogram model for predicting the survival of LIHC patients. **(C)** Gene—gene interaction network for CRGs and their most correlated genes in LIHC (Cytoscape). (**D**) GO

survival in the *DLAT*-low group (Fig. S6). Collectively, these data suggest a close correlation between *DLAT* expression and immune infiltration in LIHC.

Sorafenib is the first approved targeted drug for LIHC at an advanced stage; however, only a small number of patients can benefit from Sorafenib treatment.[3] To explore whether cuproptosis or CRGs affect the therapeutic effect of Sorafenib on LIHC, the Genomics of Drug Sensitivity in Cancer database was used. As shown in Figure 1I, *DLAT* expression was negatively correlated with the half-inhibitory concentration ($IC_{50}$) of Sorafenib in LIHC. To verify the potential association between *DLAT* expression and Sorafenib response in LIHC, we compared the proliferation ability of Huh7 and HepG2 cells transfected with negative control siRNA (si-NC) or DLAT siRNA (si-DLAT ) upon Sorafenib treatment (Fig. 1J). As shown in Figure 1K and L, the inhibition of LIHC cell proliferation by Sorafenib could be dramatically impaired by DLAT knockdown. To assess the effect of cuproptosis on the targeted therapy of LIHC, elesclomol was utilized in this study. As shown in Figure 1M and N, elesclomol administration notably enhanced the suppressive effect of Sorafenib on the proliferation of Huh7 and HepG2 cells. Consistently, cell viability assay also showed that LIHC cell proliferation was dramatically inhibited in the combination group compared with other groups (Fig. 1O), which further confirmed the synergistic effect of cuproptosis and Sorafenib treatment in LIHC cells.

A clinical cohort with 80 LIHC patients was enrolled (cohort-1) to verify the prognostic value of DLAT in LIHC, while a Sorafenib cohort (cohort-2) with 57 LIHC patients was used to compare the therapeutic effect of Sorafenib on LIHC patients with various DLAT levels. DLAT expression in LIHC patients from the above clinical cohorts was examined using the standard immunohistochemistry method. The characteristics of the cohorts are shown in Table S9. Importantly, high expression of DLAT was associated with worse 5-year Disease-Free Survival (DFS) in LIHC patients in cohort-1 (Fig. 1P), while patients with higher DLAT expression had better DFS in the cohort-2 (Fig. 1Q), suggesting DLAT-high patients might be more sensitive to Sorafenib treatment.

Accumulating evidence has demonstrated the synergistic effect of targeted agents and immunotherapy,[4] and the combination of targeted agents and immunotherapy has been accepted as an optimized treatment for advanced LIHC.[5] To analyze the correlation between DLAT expression and patient response to targeted agents and immunotherapy, we enrolled 6 LIHC (progressive disease (PD): 1, stable disease (SD): 2, partial response (PR): 3) patients in advanced stage who underwent Lenvatinib plus PD-1 inhibitor treatment. We found that DLAT-high patients exhibited superior clinical outcomes (SD or PR) to Lenvatinib plus anti-PD1 treatment (Fig. 1R), suggesting DLAT might act as a potential biomarker for LIHC treatment.

In summary, we found that most CRGs were upregulated in LIHC and that the overexpression of CRGs was associated with DNA hypomethylation and poor patient survival. In addition, the CRGs and their coexpressed genes were enriched in the processes of the TCA cycle, oxidative phosphorylation, and cellular and mitochondrial respiration. Importantly, the expression of CRGs, in particular *DLAT*, was closely correlated with the immunological status and DLAT-high patients could benefit more from the treatment of targeted agents and immunotherapy.

## Ethics declaration

All participants were enrolled from the Eastern Hepatobiliary Surgery Hospital (EHBH), and the sample

enrichment analyses predicted the roles of target genes in three aspects, including biological process, cellular components, and molecular functions. **(E)** Correlation between *DLAT* expression and the immune cell infiltration of different immune cells in LIHC. **(F)** The violin diagram displays the distribution and average expression of *DLAT* in different cell types from the LIHC_GSE125449_aPDL1CTLA4 dataset. **(G)** The box plot shows immune cells with significantly different infiltration levels between *DLAT*-high and *DLAT*-low subtypes. **(H)** Kaplan–Meier curves for the corresponding immune infiltrates in *DLAT*-high group. The hazard ratio and the log-rank *P*-value for the KM curve are presented. **(I)** Correlation between the Sorafenib $IC_{50}$ score and *DLAT* expression. The density curve on the right represents the trend in the distribution of the $IC_{50}$ score, and the upper-density curve represents the trend in the distribution of the gene expression. The abscissa represents different groups of samples, and the ordinate represents the distribution of the $IC_{50}$ score. **(J)** DLAT silencing effect in Huh7 and HepG2 cells. **(K)** The results of the EdU assay for Huh7 and HepG2 cells transfected with negative control (si-NC) and si-DLAT exposed to Sorafenib. The indicators of proliferation and nuclei are EdU (red) and Hoechst (blue) fluorescence (magnification, $\times$100). Representative images, scale = 100 $\mu$m. **(L)** EdU-positive Huh7 and HepG2 cells in si-NC and si-DLAT groups. The proliferation rate of each cell line was compared between groups. **(M)** The results of the EdU assay for Huh7 and HepG2 cell proliferation in the control, elesclomol, Sorafenib, and combination groups. The indicators of proliferation and nuclei are EdU (red) and Hoechst (blue) fluorescence (magnification, $\times$100). Representative images, scale = 100 $\mu$m. **(N)** EdU-positive Huh7 and HepG2 cells in the control, elesclomol, Sorafenib, and combination groups. The proliferation rate of each cell line was compared between groups. **(O)** Viability of Huh7 and HepG2 cells in each stimulation group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars indicate $\pm$ SD. **(P)** Kaplan–Meier curves of 5-year DFS of LIHC between DLAT-high and DLAT-low groups from clinical cohort 1. **(Q)** Kaplan–Meier curves of DFS of LIHC between DLAT-high and DLAT-low groups from the Sorafenib cohort (clinical cohort 2). **(R)** IHC staining of DLAT in LIHC from Lenvatinib plus PD-1 inhibitor-administrated patients with a different response, including progressive disease (PD), stable disease (SD), and partial response (PR). CRGs: cuproptosis-related genes; LIHC: liver hepatocellular carcinoma; $IC_{50}$: half-inhibitory concentration; si-NC: negative control siRNA; si-DLAT: DLAT siRNA; Disease-Free Survival.

collection procedure was approved by the ethics committee of EHBH.

## Author contributions

All authors searched the literature, designed the study, interpreted the findings and revised the manuscript. Dingtao Hu, Yichuan Wang, and Xu Shen carried out data management and statistical analysis and drafted the manuscript. Dingtao Hu, Yichuan Wang, Xu Shen, Tiantian Mao, Xijun Liang, Tengjiao Wang, and Weifeng Shen helped with cohort identification and data management. Jin Ding and Yugang Zhuang contributed to the critical revision of the manuscript.

## Conflict of interests

The authors declare that they have no conflict of interests.

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## Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2023.03.010.

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