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Original Article

UBE2S promotes glycolysis in hepatocellular carcinoma by enhancing E3 enzyme-independent polyubiquitination of VHL

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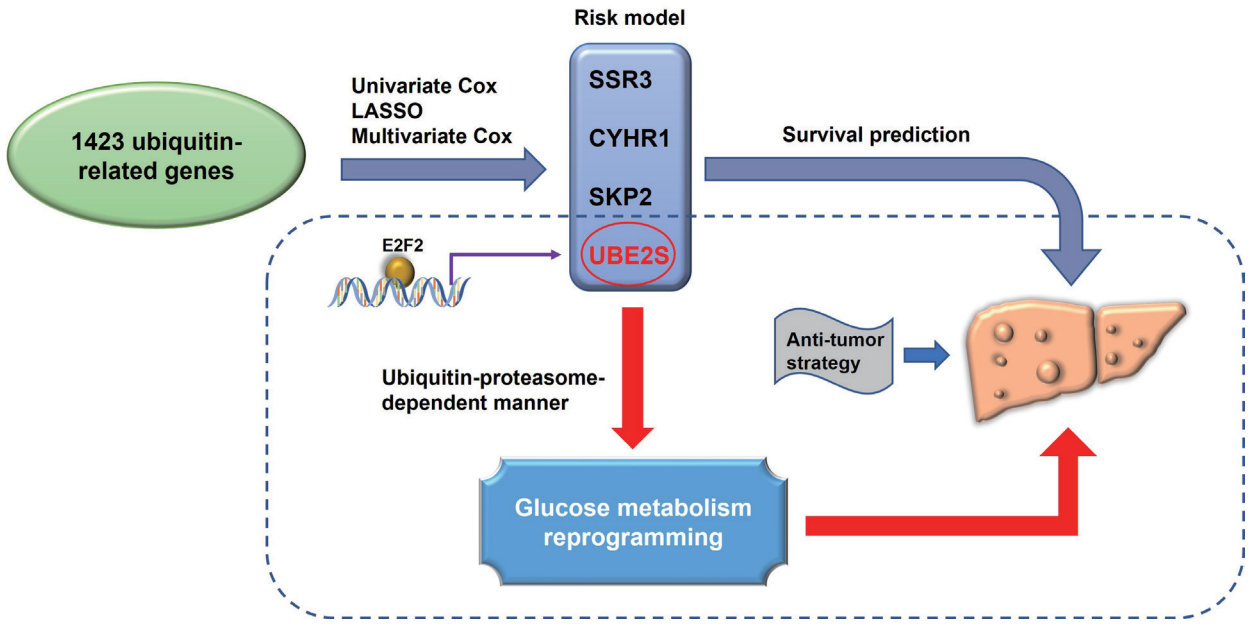
Graphical Abstract



UBE2S is a key biomarker of HCC and promotes glycolysis in a ubiquitin proteasome-dependent manner.

Study Highlights

- A four-gene risk prediction model is constructed to evaluate the prognosis of patients with HCC based on 1423 ubiquitin-related genes, including E1s, E2s, E3s, and DUBs.
- UBE2S, an E2 enzyme, is a key biomarker in HCC among the thousands of ubiquitin-related genes, and E2F2 transcriptionally upregulates UBE2S expression by directly binding to its promoter.
- UBE2S promotes glycolysis of HCC cells by enhancing K11-linkage polyubiquitination at lysine residues 171 and 196 of VHL in an E3 enzyme-independent manner.
- A combined strategy of targeting E2 enzyme and its downstream pathway significantly inhibits HCC growth.

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Background/Aims: Ubiquitination is widely involved in the progression of hepatocellular carcinoma (HCC) by regulating various cellular processes. However, systematic strategies for screening core ubiquitin-related genes, clarifying their functions and mechanisms, and ultimately developing potential therapeutics for patients with HCC are still lacking.

Methods: Cox and LASSO regression analyses were performed to construct a ubiquitin-related gene prediction model for HCC. Loss- and gain-of-function studies, transcriptomic and metabolomics analysis were used to explore the function and mechanism of UBE2S on HCC cell glycolysis and growth.

Results: Based on 1,423 ubiquitin-related genes, a four-gene signature was successfully constructed to evaluate the prognosis of patients with HCC. UBE2S was identified in this signature with the potential to predict the survival of patients with HCC. E2F2 transcriptionally upregulated UBE2S expression by directly binding to its promoter. UBE2S positively regulated glycolysis in a HIF-1α-dependent manner, thus promoting the proliferation of HCC cells. Mechanistically, UBE2S enhanced K11-linkage polyubiquitination at lysine residues 171 and 196 of VHL independent of E3 ligase, thereby indirectly stabilizing HIF-1α protein levels by mediating the degradation of VHL by the proteasome. In particular, the combination of cephalomannine, a small molecule compound that inhibits the expression of UBE2S, and PX-478, an inhibitor of HIF-1α, significantly improved the anti-tumor efficacy.

Conclusions: UBE2S is identified as a key biomarker in HCC among the thousands of ubiquitin-related genes and promotes glycolysis by E3 enzyme-independent ubiquitination, thus serving as a therapeutic target for the treatment of HCC. **(Clin Mol Hepatol 2024;30:771-792)**

Keywords: Ubiquitination; Ube2S protein, human; Hepatocellular carcinoma; Glycolysis; VHL protein, human

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Abbreviations:

BATF3, basic leucine zipper ATF-like transcription factor 3; CYHR1, cysteine and histidine rich 1; DEN, diethylnitrosamine; ECAR, extracellular acidification rate; E2F2, E2F transcription factor 2; FOXD3, forkhead box D3; GLUT1, glucose transporter type 1; HCC, hepatocellular carcinoma; HIF-1α, hypoxia inducible factor-1α; HK2, hexokinase 2; LDHA, lactate dehydrogenase A; PFKL, phosphofructokinase, liver type; POU2F2, POU class 2 homeobox 2; SKP2, S-phase kinase associated protein 2; SSR3, signal sequence receptor subunit 3; UBE2S, ubiquitin conjugating enzyme E2 S; VHL, von Hippel-Lindau tumor suppressor

INTRODUCTION

Ubiquitination is an important type of post-translational modification that is widely involved in the progression of hepatocellular carcinoma (HCC) by regulating various cellular processes such as endoplasmic reticulum homeostasis, genomic integrity, epigenetic markers, cell growth, cell death, autophagy, and metabolic reprogramming.[1-6] Although a broad range of options including liver transplantation, surgical resection, percutaneous ablation, and radiation, as well as systemic therapies are currently available for the treatment of HCC, most patients cannot receive radical treatment strategies due to the advanced stages of the disease at diagnosis.[7] Nonetheless, there is no obvious breakthrough in the identification of biomarkers for the early diagnosis and monitoring of HCC.[8] Hence, identifying potential biomarkers in the process of ubiquitination used to evaluate prognosis or predict treatment response is of great value in improving the clinical translation of patients with HCC.

Ubiquitination is a cascade of enzymatic reactions in which ubiquitin molecules are covalently linked to substrate proteins by ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s).[9] Moreover, ubiquitination is also a highly reversible process which can be antagonized by deubiquitinating enzymes (DUBs).[10] The human genome encodes two E1s, at least 40 E2s, >800 E3s, and more than 100 DUBs.[11-13] Thanks to technological advances in mass spectrometry and proteomics, our understanding of the functions and mechanisms of the molecules involved in the process of ubiquitination has greatly improved.[14] In particular, a number of small molecule inhibitors targeting E1s, E2s, E3s, and DUBs have been developed and their antitumor effects are gradually being tested.[15] However, the current research on ubiquitination mainly focuses on the exploration of certain enzymes, systematic strategies for screening key molecular targets among the thousands of ubiquitin-related genes are lacking. Therefore, approaches to identify core ubiquitin-related molecules with potential clinical value and in-depth studies on their functions and mechanisms are urgently needed.

In this study, we aimed to construct a ubiquitin-related gene risk prediction model to evaluate the prognosis of patients with HCC by identifying 1,423 ubiquitin-related genes. Particularly, our results demonstrate the important clinical value of ubiquitin-conjugating enzyme E2 S (UBE2S) in HCC and identified E2F transcription factor 2 (E2F2), which upregulates UBE2S expression. Furthermore, our study clarifies the regulatory role of UBE2S in glucose metabolism reprogramming through ubiquitination in an E3 enzyme-independent manner and provides a potential strategy for the treatment of patients with HCC.

MATERIALS AND METHODS

Identification and differential expression analysis of ubiquitin-related genes

Ubiquitin-related genes were acquired from the integrated annotations for Ubiquitin and Ubiquitin-like Conjugation Database (iUUCD, http://iuucd.biocuckoo.org/browse.php), the Molecular Signatures Database (MSigDB, https://www.gsea-msigdb.org/gsea/msigdb/) and the human E3 ubiquitin ligase database (https://esbl.nhlbi.nih.gov/Databases/KSBP2/Targets/Lists/E3-ligases/).[16] The identified genes were presented in Supplementary Table 1. RNA sequencing data and corresponding clinical information from 374 liver tumor tissues and 50 adjacent tissues were downloaded from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/). The “limma” package in R software was used to identify differentially expressed genes (DEGs) in tumors and adjacent tissues among the ubiquitin-related genes. The screening criteria were false discovery rate (FDR) <0.05 and |log (fold change)| ≥1.0.

Construction and evaluation of ubiquitin-related gene risk prediction model for HCC

The “caret” package in R software was used to randomly divide 370 HCC samples with complete clinical information into two groups, a training cohort (n=224) and a testing cohort (n=146). In the training cohort, the “survival”, “glmnet”, and “survminer” packages in R software were used to construct a ubiquitin-related gene prognostic signature by successively performing univariate Cox, least absolute shrinkage and selection operator (LASSO), and multivariate Cox regression analyses. The risk score of each patient with HCC in the training and testing cohorts was calculated as

the sum of the expression values of each ubiquitin-related gene in the model multiplied by its regression coefficient. The median was used as the threshold to divide the patients into high- and low-risk groups. The "survival" and "timeROC" packages in R software were used to analyze Kaplan–Meier survival curve and receiver operating characteristic (ROC) curve for high- and low-risk groups of patients with HCC, and the area under the curve (AUC) was used to evaluate the accuracy and specificity of the risk prediction model.

Statistical analysis

All data were analyzed using GraphPad Prism software (version 8.0) or R software (version 4.1.0) and presented as the mean±standard deviation. Cox regression analyses were performed to evaluate the effects of ubiquitin-related genes on the survival of patients with HCC. Student's *t*-tests were used to test the differences between two groups. One-way ANOVA was used to calculate differences among multiple groups. The log-rank test was used to assess the statistical significance of the Kaplan–Meier survival curve. Two-way ANOVA was used to calculate the statistical significance of dynamic curves of tumor volume in mice treated with different compounds. $P<0.05$ was considered statistically significant.

RESULTS

Ubiquitin-related gene risk prediction model for HCC is constructed

Based on the iUUCD (including E1s, E2s, E3s, and DUBs), MSigDB, and human E3 ubiquitin ligase databases,[16] we collected a ubiquitin-related gene set consisting of 1,423 genes (Fig. 1A). By differential expression analysis of this gene set in the TCGA database, a total of 92 DEGs were identified in HCC and adjacent tissues, of which 91 were upregulated and one was downregulated (Fig. 1B). To explore the clinical significance of these DEGs, 370 patients with HCC that had complete clinical information in the TCGA database were randomly divided into training (n=224) and testing (n=146) cohorts. Next, we conducted a univariate Cox regression analysis in the training cohort and found that 28 DEGs were significantly positively correlated with poor prognosis in patients with HCC (Fig. 1C). Furthermore, we performed LASSO regression and multivariate Cox regression analyses and identified four core ubiquitin-related genes (*SSR3, CYHR1, UBE2S,* and *SKP2*) that could be used to construct a risk model for HCC survival prediction (Fig. 1D, E). The regression coefficients of the four genes in the prognostic signature were 0.0248, 0.0783, 0.0613, and 0.0592, respectively. The formula for calculating the risk score for each patient with HCC is the sum of the expression values of these four genes multiplied by their respective regression coefficients. Thus, a risk prediction model for HCC based on ubiquitin-related genes was successfully constructed.

The risk prediction model is useful to evaluate the survival of patients with HCC

To evaluate the clinical significance of this risk-prediction model, we calculated the risk score of each patient with HCC in the training cohort and divided them into high- and low-risk groups based on the median score (Fig. 1F, G). In particular, the survival status of patients gradually decreased as the risk score increased (Fig. 1H). Moreover, Kaplan–Meier survival analysis showed that the overall survival of patients with HCC in the high-risk group was significantly lower than that of patients in the low-risk group (Fig. 1I). ROC curve analysis showed that the 1-year, 2-year, and 3-year survival AUC values of the risk prediction model were 0.76, 0.72, and 0.68, respectively (Fig. 1J). In addition, we calculated the risk score of patients with HCC in the testing cohort using the same formula and found a clinical significance consistent with that of the training cohort (Supplementary Fig. 1A–E).

To further examine the potential of the risk score derived from the model as an independent prognostic factor in patients with HCC, we performed univariate and multivariate Cox regression analyses on five variables (age, sex, tumor grade, tumor stage, and risk score) for the entire TCGA cohort. We found a significant association between tumor stage and risk score with the overall survival of patients with HCC (Table 1). The results indicate that the risk score can be used as an independent prognostic factor to evaluate the overall survival of patients.

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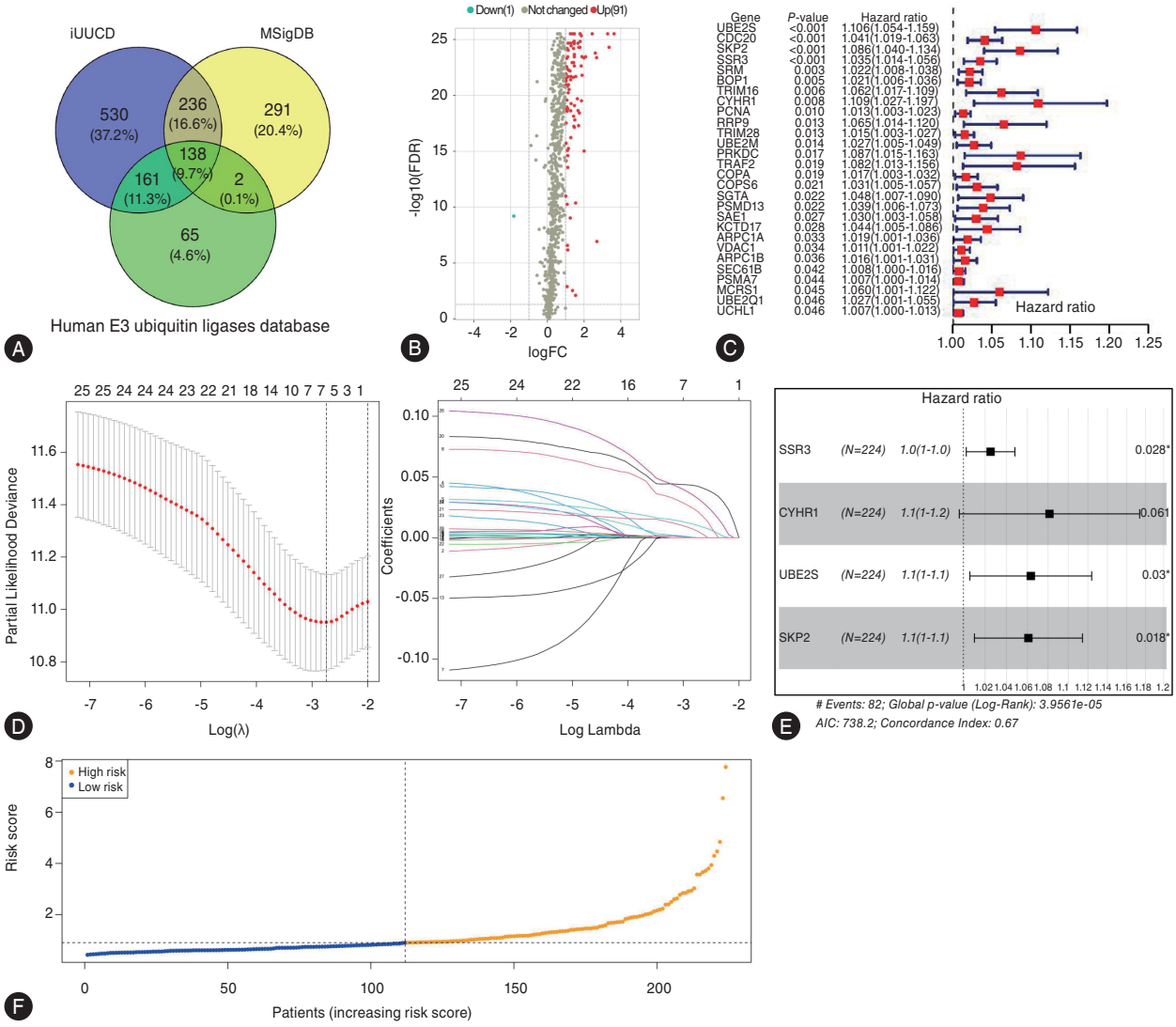
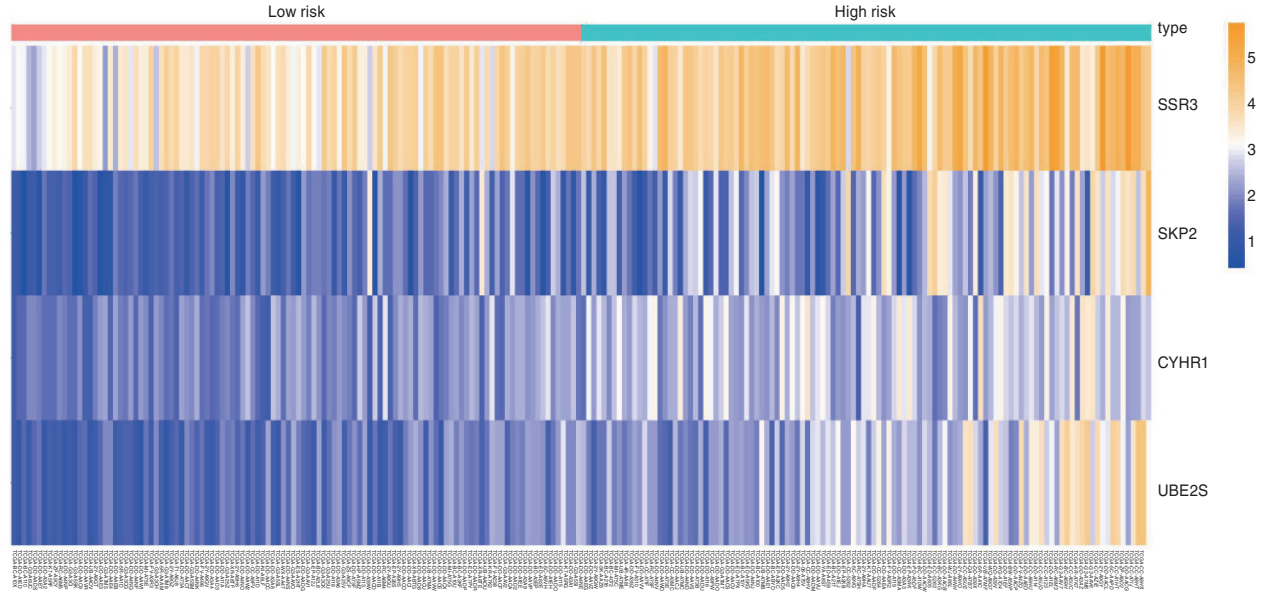
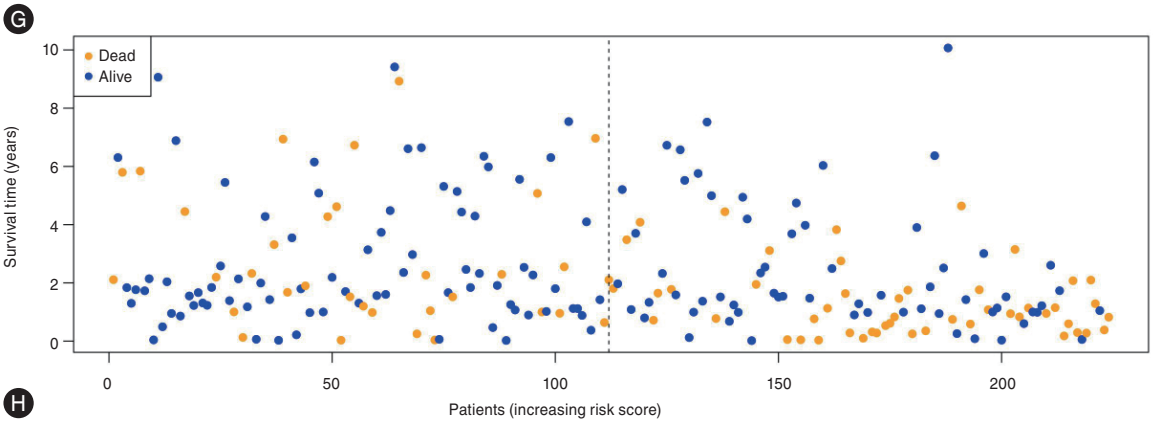


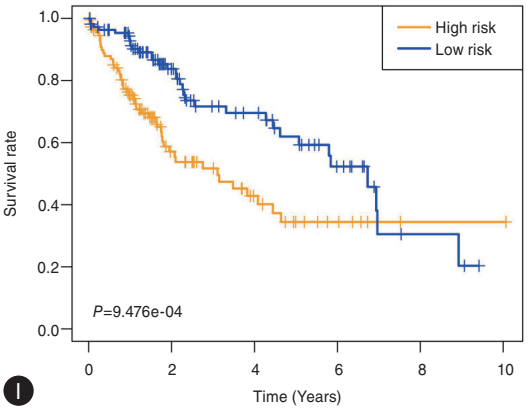
Figure 1. Construction of a prognostic model for patients with HCC based on ubiquitin-related genes and evaluation of its clinical significance in the training cohort. (A) Venn plot of ubiquitin-related genes identified based on iUUCD, MSigDB, and human E3 ubiquitin ligase database. (B) Volcano plot of ubiquitin-related differentially expressed genes in human HCC and adjacent tissues. FDR <0.05, |logFC| ≥1.0. (C) Forest plot of 28 ubiquitin-related genes associated with overall survival of patients with HCC based on univariate Cox regression analysis. (D) Distribution plot of the partial likelihood deviation and coefficient of the LASSO regression. (E) Forest plot of 4 ubiquitin-related genes for the construction of a prognostic model for patients with HCC based on multivariate Cox regression analysis. (F) Visualization of high- and low-risk groups based on risk scores of patients with HCC in the training cohort. (G) Expression profiles of 4 ubiquitin-related genes in high- and low-risk groups in the training cohort. (H) Visualization of survival status and risk score in patients with HCC in the training cohort. (I, J) Kaplan–Meier and ROC analyses of risk score and overall survival in HCC patients within the training cohort. HCC, hepatocellular carcinoma; FDR, false discovery rate; LASSO, least absolute shrinkage and selection operator; ROC, receiver operating characteristic.

UBE2S is identified as a key ubiquitin-related gene using the risk prediction model in HCC

Next, we examined the expression characteristics and clinical significance of each of the four genes in the risk model. The results showed that only CYHR1 and UBE2S, but not SSR3 and SKP2, had significantly higher RNA and protein levels in HCC tissues than in adjacent normal tissues (Supplementary Fig. 2A). In addition, we evaluated the correlation between the expression levels of the four genes in the model and the survival of patients with HCC. We found a significant correlation of only the expression







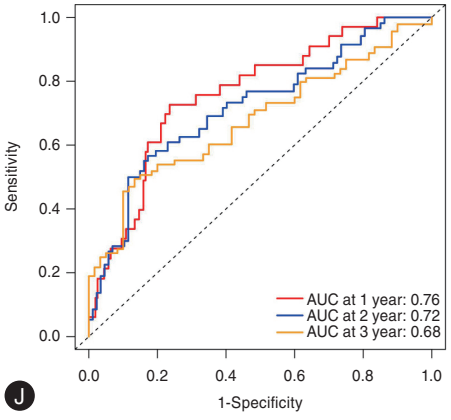


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Figure 1. Continued.

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Table 1. Univariate and multivariate Cox regression analysis of risk score and overall survival in patients with HCC

Clinicopathological characteristics	Univariate analyses			Multivariate analyses		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age	1.011	0.996–1.025	0.150	1.009	0.995–1.023	0.221
Gender	0.776	0.532–1.133	0.189	0.829	0.557–1.234	0.356
Grade	1.130	0.878–1.453	0.343	1.135	0.869–1.483	0.351
Stage	1.658	1.352–2.032	<0.001*	1.572	1.268–1.949	<0.001*
Risk score	1.225	1.129–1.330	<0.001*	1.156	1.056–1.265	0.002*

HCC, hepatocellular carcinoma; HR, hazard ratio; CI, confidence interval.
*indicates statistically significant.

level of *UBE2S* with the overall survival and disease-free survival of patients with HCC, wherein the survival of patients with high expression of *UBE2S* was significantly reduced compared to that of the low expression group (Supplementary Fig. 2B). Therefore, in subsequent experiments, we focused on exploring the biological functions and mechanisms of UBE2S in HCC.

E2F2 binds directly to the UBE2S promoter and activates UBE2S transcription

Since the expression level of UBE2S in HCC tissues is significantly higher than that in normal tissues (Supplementary Fig. 2A), we therefore explored the factors regulating UBE2S expression in HCC. Based on JASPAR database analysis, we identified four transcription factors, BATF3, FOXD3, E2F2, and POU2F2, which might bind to the promoter region of UBE2S (Fig. 2A). Further, we found that only the expression level of E2F2, but not the other three transcription factors, in HCC tissues was significantly higher than that in normal tissues (Supplementary Fig. 3A). In particular, the correlation coefficient between the RNA expression levels of UBE2S and E2F2 in HCC tissues was significantly higher than that of the other three transcription factors (Supplementary Fig. 3B). Moreover, we found a significant positive correlation between the protein expression levels of E2F2 and UBE2S by immunohistochemistry in a microarray containing 90 HCC tissue samples (Fig. 2B). qPCR and Western blotting demonstrated that E2F2 overexpression significantly upregulated the RNA and protein levels of UBE2S (Fig. 2C, D). Furthermore, we constructed a luciferase reporter containing the UBE2S promoter to explore whether the transcription of UBE2S is regulated by E2F2. As shown in Figure 2E, E2F2 overexpression increased UBE2S promoter activity. Next, luciferase gene reporters containing truncated domains of the UBE2S promoter were constructed. When the UBE2S promoter domain was truncated to –250 bp/+199 bp, the increase in UBE2S promoter activity mediated by E2F2 overexpression was abolished, indicating that E2F2 could bind to the domain (–500 bp/–250 bp) of the UBE2S promoter (Fig. 2F). To further confirm the binding sites of E2F2 in the UBE2S promoter, we predicted four potential binding sites using the JASPAR database and constructed luciferase gene reporters containing the UBE2S promoter with E2F2 binding site mutations. Mutations in the first binding site of E2F2 did not impede the increase in UBE2S promoter activity mediated by E2F2 overexpression, whereas the other three mutations significantly alleviated the increase in activity, suggesting that E2F2 could bind to binding sites 2, 3, and 4 of the UBE2S promoter (Fig. 2G). In addition, the binding of E2F2 to the UBE2S promoter was confirmed by chromatin immunoprecipitation (ChIP) and qPCR assays (Fig. 2H). Taken together, these results indicate that E2F2 directly binds to the UBE2S promoter and transcriptionally upregulates UBE2S expression.

UBE2S promotes the proliferation of HCC cells by positively regulating glycolysis

To systematically elucidate the downstream functions of UBE2S, we constructed Huh-7 cells with UBE2S-knockout using CRISPR-Cas9 and performed RNA sequencing (Supplementary Fig. 4A). A total of 2,458 genes were upregulated, and 2,558 genes were down-regulated after the knockout of UBE2S (Fig. 3A). Further, KEGG analysis revealed a significant relationship between the DEGs and the metabolic pathways (Fig. 3B). Next, a non-target metabolo-

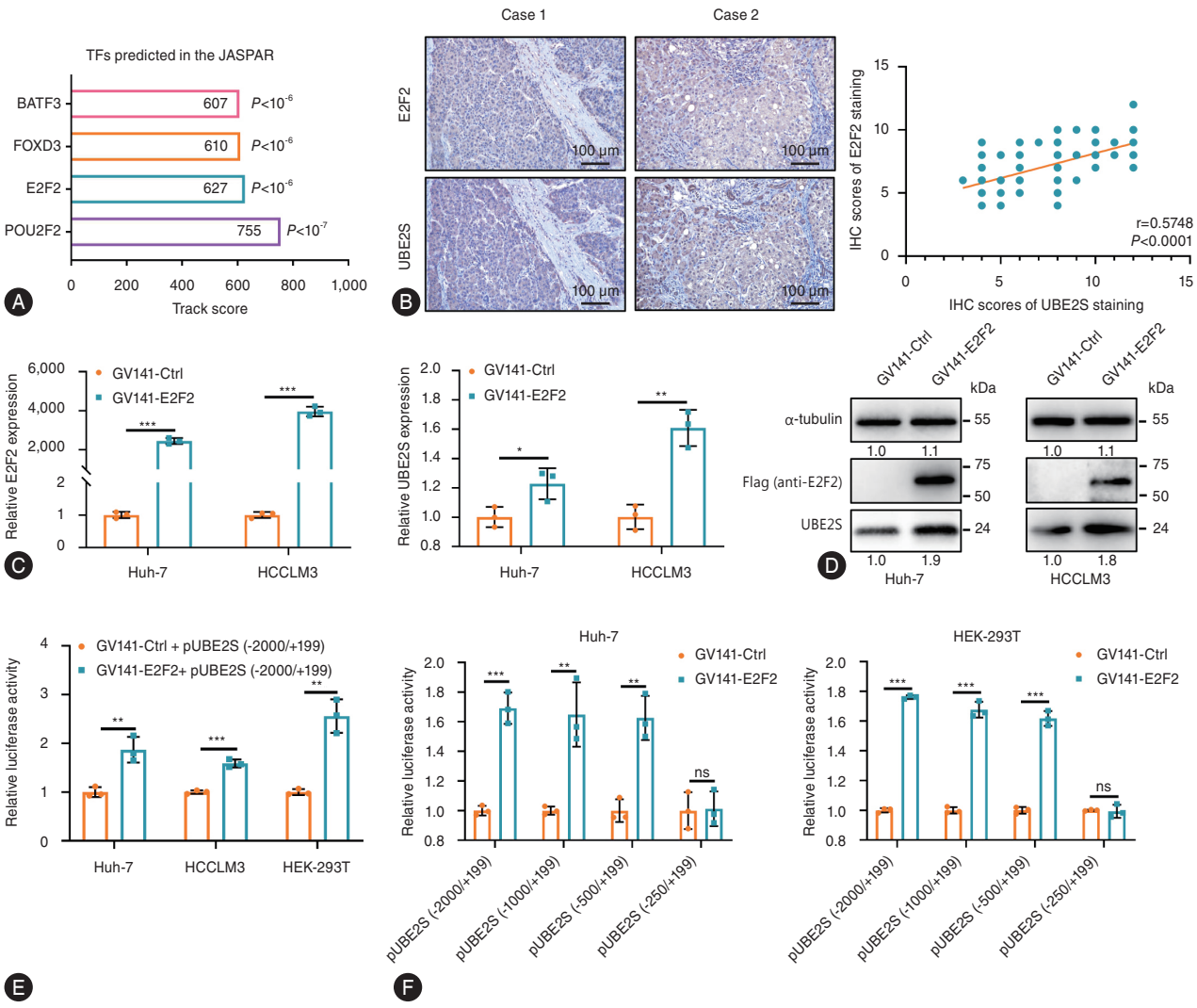

Figure 2. E2F2 binds directly to the UBE2S promoter and transcriptionally upregulates UBE2S expression. (A) Four upstream transcription factors of UBE2S were identified using the JASPAR database. (B) Representative immunohistochemical images of E2F2 and UBE2S expression in 90 HCC tissue samples and correlation analysis of quantitative scores. (C, D) qPCR and Western blot analyses of UBE2S expression in Huh-7 and HCCLM3 cells overexpressing E2F2. Data were analyzed by Student's *t*-test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. (E) The effects of E2F2 on the activity of the UBE2S promoter (–2,000/+199) were determined using a dual-luciferase reporter assay. Data were analyzed by Student's *t*-test. $^{**}P<0.01$, $^{***}P<0.001$. (F) The effects of E2F2 on the activity of different truncated UBE2S promoters in Huh-7 and HEK-293T cells were determined using a dual-luciferase reporter assay. Data were analyzed by Student's *t*-test. $^{**}P<0.01$, $^{***}P<0.001$. (G) Binding sites and mutation strategies of E2F2 in the UBE2S promoter (–500/–250) in the JASPAR database. The effects of E2F2 on the activity of different UBE2S promoter mutants in Huh-7 and HEK-293T cells were determined using a dual-luciferase reporter assay. Data in different groups were analyzed by one-way ANOVA, comparing each MUT group to the WT. Data in each group were analyzed by Student's *t*-test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. (H) The sequence of the UBE2S promoter in ChIP samples from Huh-7 or HCCLM3 cells transfected with the GV141-E2F2 plasmid was detected by qPCR. IgG was used as the negative control. Histone H3 was used as the positive control. Data were analyzed by Student's *t*-test. $^{***}P<0.001$. E2F2, E2F transcription factor 2; UBE2S, ubiquitin conjugating enzyme E2 S; HCC, hepatocellular carcinoma; ns, no statistical difference.

mic analysis was performed to identify specific metabolic pathways regulated by UBE2S (Supplementary Fig. 4B). Differential abundance score analysis revealed a downregulation of all identified metabolites in five pathways, namely glycerolipid metabolism, pentose and glucuronate interconversion, glycolysis/gluconeogenesis, pentose phosphate pathway, and phenylalanine, tyrosine and tryptophan biosynthesis, in Huh-7 cells with UBE2S knock-

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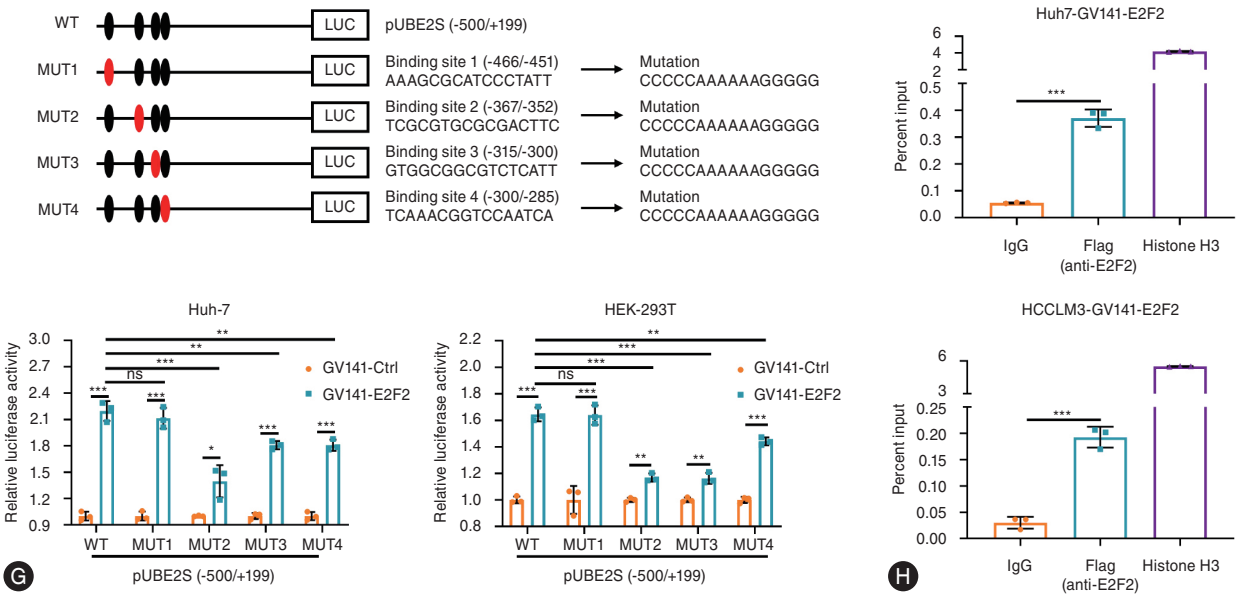

Figure 2. Continued.

down (Fig. 3C). Previous studies have shown that glycolysis interacts with several metabolic pathways such as the pentose phosphate pathway, lipid metabolism, and amino acid metabolism and thus plays a central role in the metabolic network.[17] Therefore, we focused on the regulatory role of UBE2S in glycolysis in subsequent experiments.

Cluster analysis showed that the levels of glucose-6-phosphate (G-6-P), fructose-1,6-diphosphate (F-1,6-BP), dihydroxyacetone phosphate (DHAP), 3-phosphoglycerate (3-PG), 2-phosphoglycerate (2-PG), and phosphoenolpyruvate (PEP) in the glycolysis pathway were significantly downregulated following UBE2S knockdown (Fig. 3D, E). Further, we sequentially treated HCC cells with glucose, oligomycin (an inhibitor of ATP synthase), and 2-deoxy-D-glucose (2-DG, an inhibitor of glycolysis) and performed the Seahorse XF glycolysis stress test. The dynamic curve of extracellular acidification rate (ECAR) is shown in Figure 3F and Supplementary Figure 4C. UBE2S knockdown suppressed the glycolysis and glycolytic capacity (Fig. 3F), whereas UBE2S overexpression enhanced these abilities (Supplementary Fig. 4C). Moreover, glucose uptake and lactate production were reduced in Huh-7 and HCCLM3 cells by UBE2S silencing (Fig. 3G, H) and enhanced in MHCC-97H cells following UBE2S overexpression (Supplementary Fig. 4D, E). Crucially, CCK8 assays revealed that the cell proliferation enhanced by UEB2S overexpression in different HCC cell lines was significantly inhibited by 2-DG (Fig. 3I). Taken together, these results suggest that UBE2S promotes the proliferation of HCC cells by positively regulating glycolysis

UBE2S enhances HIF-1α-dependent glycolysis in HCC cells

To elucidate the molecular mechanism by which UBE2S promotes glycolysis in HCC cells, we focused on changes in the metabolic enzymes involved in this process. The cluster heat map analysis revealed significant down-regulation of the RNA levels of a series of enzymes involved in glycolysis, such as *GLUT1*, *HK2*, *PFKL*, and *LDHA*, in Huh-7 cells with UBE2S knockout (Fig. 4A). These results were further verified using qPCR and Western blotting (Fig. 4B, C, Supplementary Fig. 5A). To investigate the mechanisms underlying the UBE2S regulation of these glycolytic enzymes, we performed gene set enrichment analysis (GSEA) and found the inhibition of several hypoxia-related signaling pathways in Huh-7 cells with UBE2S knockout (Fig. 4D). Moreover, KEGG pathway analysis also revealed a significant relationship between the DEGs and the HIF-1 signaling pathway (Fig. 3B). Furthermore, we found that UBE2S silencing clearly decreased the protein level of HIF-1α in Huh-7 cells, whereas UBE2S overexpression remark-

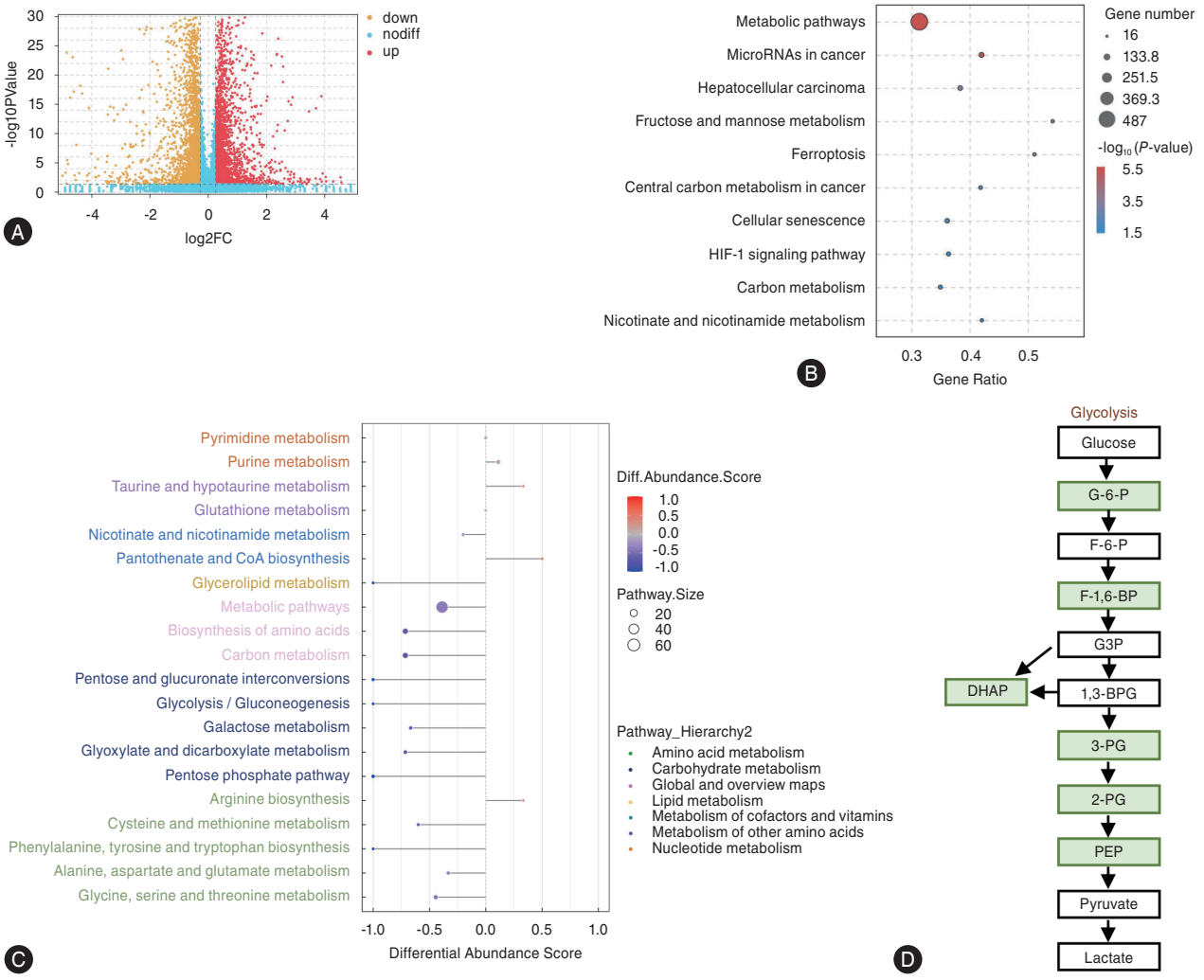

Figure 3. UBE2S positively regulates glycolysis of HCC cells. (A, B) Volcano plot and KEGG enrichment analysis of differential genes in Huh-7 cells with UBE2S knockout and wild-type cells. (C) Abundance scores of differential metabolic pathways in Huh-7 cells with UBE2S knockdown and control cells. (D) Pattern diagram of glycolysis. The green box indicates metabolites that are significantly down-regulated, while the white box indicates metabolites that are undetected or not significantly different in the metabolomics data after UBE2S knockdown in Huh-7 cells. (E) Cluster heat plot of differential metabolites in Huh-7 cells with UBE2S knockdown and control cells. Variable importance for the projection (VIP) >1, $P<0.05$. (F) Dynamic curves of extracellular acidification rate and glycolysis ability in Huh-7 and HCCLM3 cells with UBE2S knockdown were detected by seahorse XF glycolysis stress test. Data were analyzed by Student's *t*-test. $^{*}P<0.05$, $^{***}P<0.001$. (G, H) Glucose uptake and lactate production in Huh-7 and HCCLM3 cells with UBE2S knockdown. Data were analyzed by Student's *t*-test. $^{**}P<0.01$, $^{***}P<0.001$. (I) CCK-8 assay to detect the changes in cell proliferation of HCCLM3 or MHCC-97H cells with UBE2S overexpression after being treated with the glycolysis inhibitor, 2-DG. The concentration of 2-DG is 1.5 mM in HCCLM3 cells or 2.5 mM in MHCC-97H cells. Data were analyzed by one-way ANOVA. $^{***}P<0.001$. UBE2S, ubiquitin conjugating enzyme E2 S; HCC, hepatocellular carcinoma.

ably enhanced HIF-1α protein expression under both normal and hypoxic conditions (Fig. 4E). Since HIF-1α is a key transcription factor regulating cellular glycolysis,[18] these results suggest that UBE2S may promote the glycolysis of HCC cells by upregulating the expression of HIF-1α-mediated metabolic enzymes.

To further clarify whether the promotion of glycolysis by UBE2S is dependent on HIF-1α, the Seahorse XF glycolysis stress test was performed. Although UBE2S overexpression in HCCLM3 and MHCC-97H cells increased glycolysis by converting glucose into pyruvate, these functions were significantly inhibited after HIF-1α knockdown (Fig.

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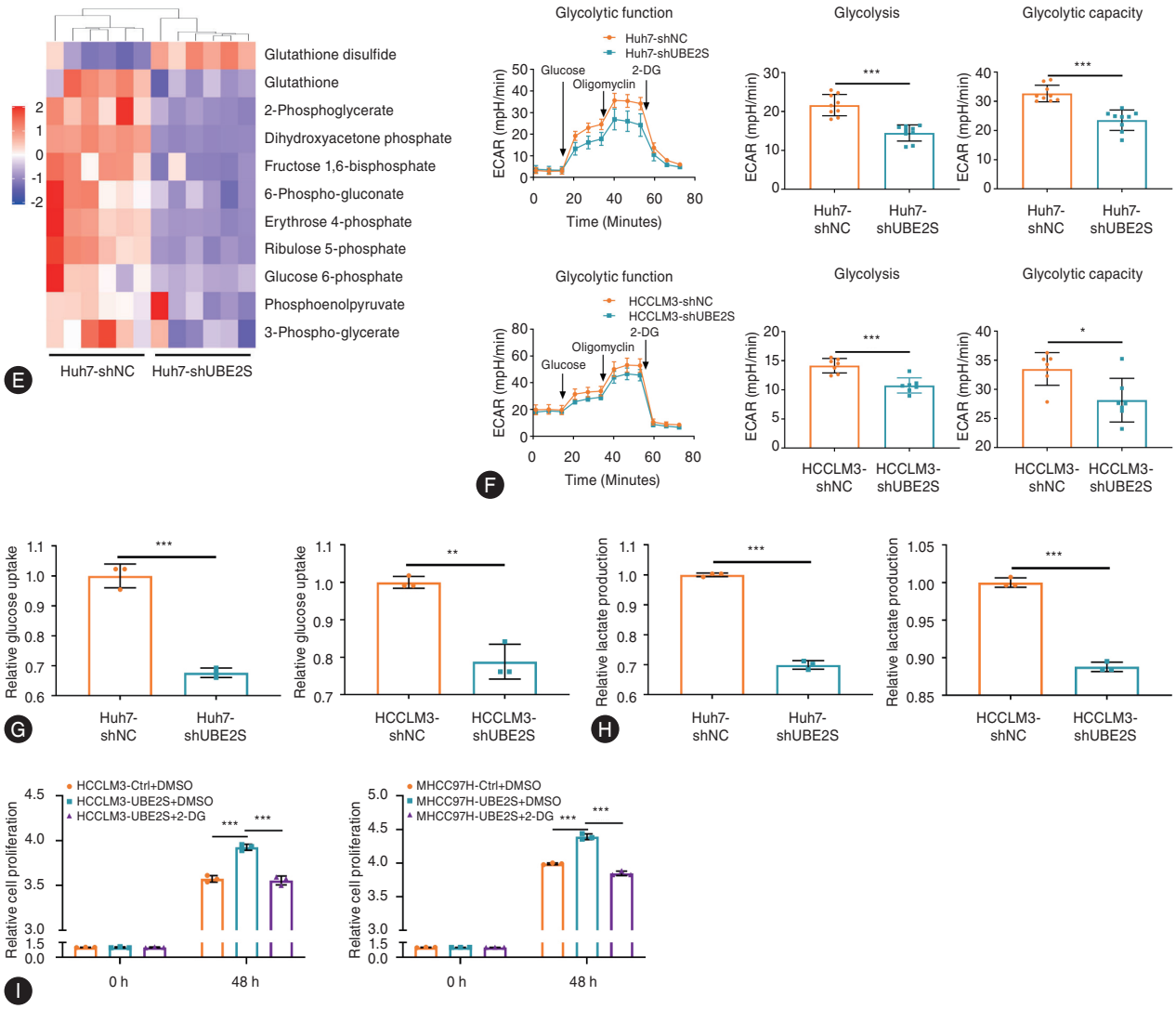

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4F). Moreover, the levels of key enzymes involved in glycolysis, such as GLUT1, HK2, PFKL, and LDHA, were significantly increased when UBE2S was upregulated in HCCLM3 and MHCC-97H cells, which was decreased with HIF-1α deletion (Fig. 4G). In addition, the increase of glucose uptake and lactate production caused by UBE2S overexpression in HCCLM3 and MHCC-97H cells was also significantly inhibited by PX-478, a HIF-1α inhibitor (Supplementary Fig. 5B, C). Furthermore, CCK8 assays revealed that cell proliferation enhanced by UBE2S overexpression could be significantly inhibited in different HCC cell lines after HIF-1α knockdown or PX-478 treatment (Fig. 4H). Taken together, these results suggest that UBE2S promotes HCC cell proliferation by upregulating HIF-1α-pathway dependent glycolysis.

UBE2S stabilizes HIF-1α by degradation of VHL via E3 enzyme-independent polyubiquitination

Given that UBE2S knockout did not alter the RNA level of HIF-1α (Supplementary Fig. 6A), we therefore focused on exploring the regulatory role of UBE2S on HIF-1α at the protein level. Von Hippel-Lindau tumor suppressor (VHL) is a classical ubiquitin ligase that mediates the ubiquitination of HIF-1α, which can destroy the protein stability of HIF-1α by causing its degradation.[19] Interestingly, we found that

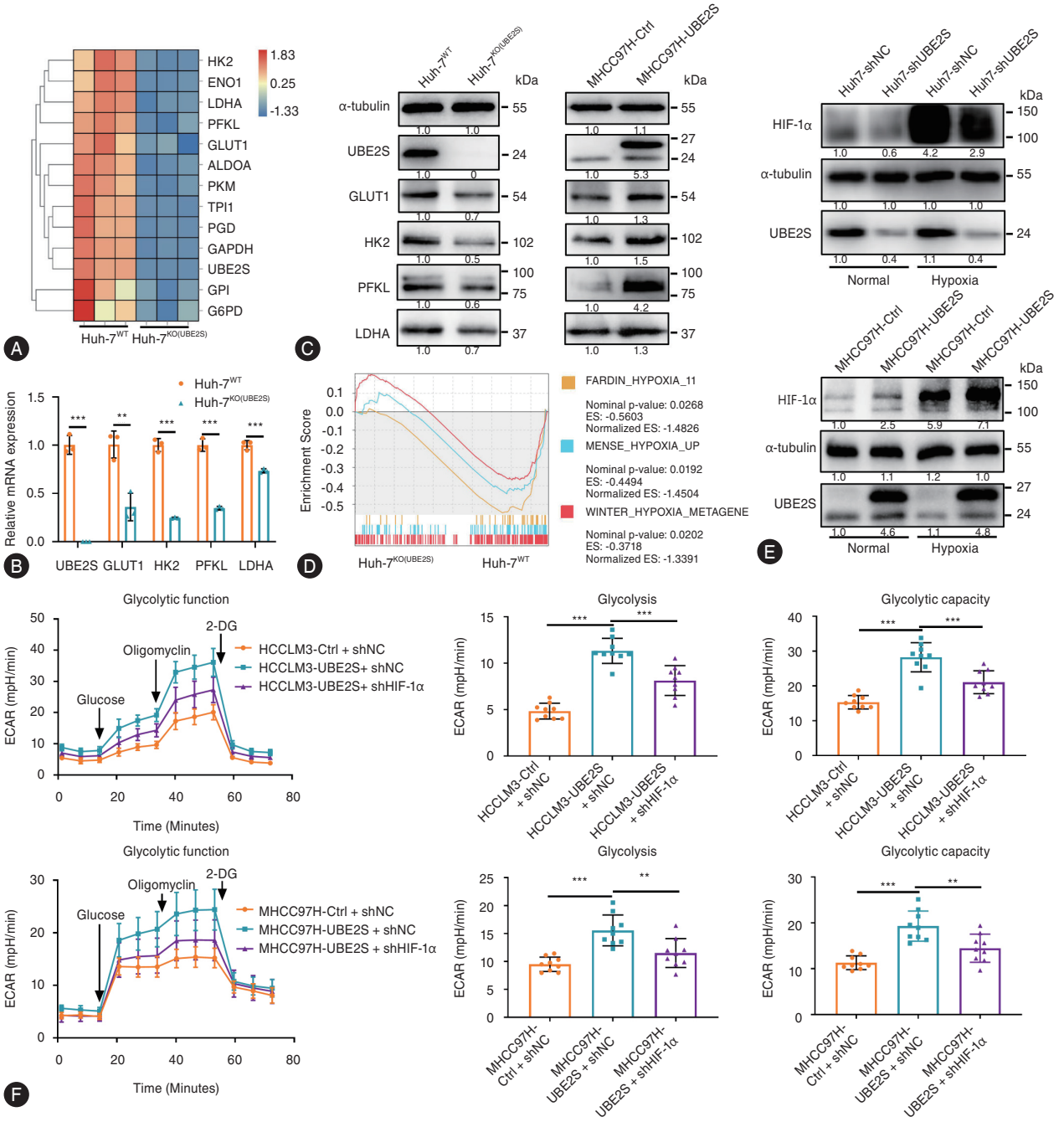

Figure 4. UBE2S promotes glycolysis of HCC cells by enhancing the expression of HIF-1α-regulated metabolic enzymes. (A) Cluster heat plot of differential genes in transcriptome sequencing data from Huh-7 cells with UBE2S knockout and wild-type cells. (B) RNA expression of key metabolic enzymes in glycolysis determined by qPCR assays after UBE2S knockout in Huh-7 cells. (C) The protein expression of key metabolic enzymes was assessed by Western blotting in Huh-7 cells with UBE2S knockout or MHCC-97H cells with UBE2S overexpression. (D) GSEA enrichment analysis of transcriptome sequencing data. (E) The expression of HIF-1α protein was assessed by Western blotting in Huh-7 cells with UBE2S knockdown or MHCC-97H cells with UBE2S overexpression under normal or hypoxic conditions. (F) Dynamic curves of extracellular acidification rate and glycolysis ability in HCCLM3 or MHCC-97H cells with UBE2S overexpression and HIF-1α knockdown were assessed by seahorse XF glycolysis stress test. Data were analyzed by one-way ANOVA. $^{**}P<0.01$, $^{***}P<0.001$. (G) The protein expression of key metabolic enzymes was assessed by Western blot assays in HCCLM3 or MHCC-97H cells with UBE2S overexpression and HIF-1α knockdown. (H) The cell proliferation rate was detected by CCK-8 assays in HCCLM3 or MHCC-97H cells with UBE2S overexpression and HIF-1α knockdown or PX-478 treatment. The concentration of PX-478 is 10 μM in HCCLM3 cells or 5 μM in MHCC-97H cells. Data were analyzed by one-way ANOVA. $^{**}P<0.01$, $^{***}P<0.001$. UBE2S, ubiquitin conjugating enzyme E2 S; HCC, hepatocellular carcinoma; HIF-1α, hypoxia inducible factor-1α; GSEA, gene set enrichment analysis.

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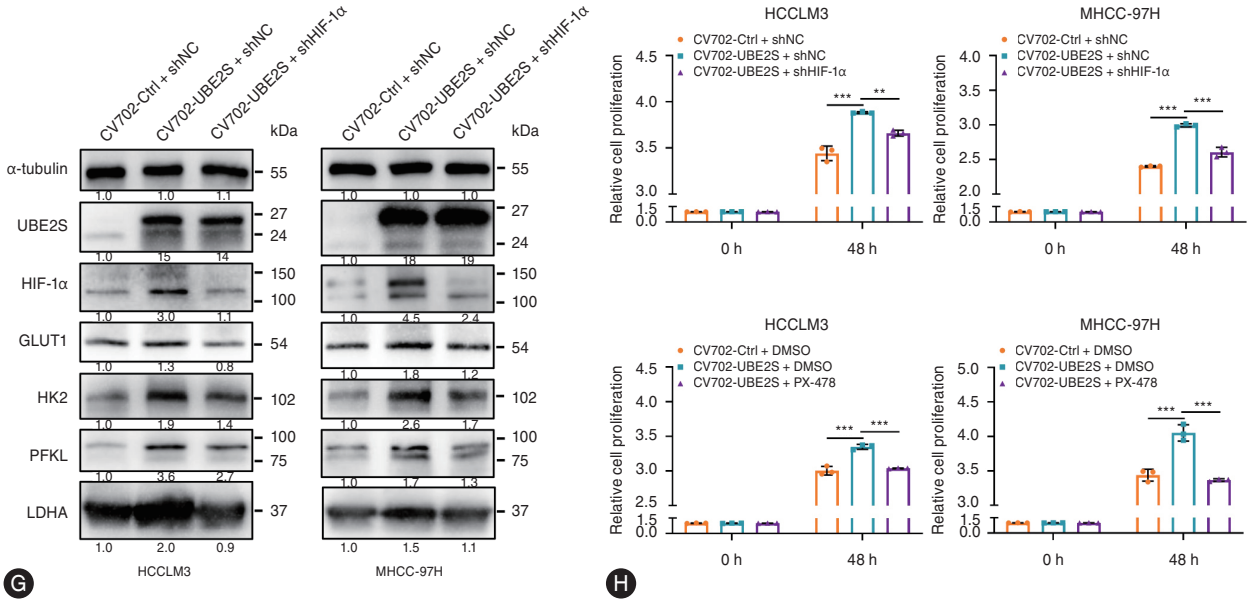

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UBE2S overexpression in different HCC cell lines significantly reduced the expression of VHL and increased the stability of the HIF-1α protein (Supplementary Fig. 6B). Crucially, rescue experiments showed that VHL overexpression could significantly inhibit the increased protein stability of HIF-1α caused by UBE2S overexpression (Supplementary Fig. 6C). These results suggest that UBE2S improves the protein stability of HIF-1α in a VHL-dependent manner. Next, we found that the negative regulatory effect of UBE2S on VHL was significantly inhibited when the cells were treated with MG132, the proteasome inhibitor (Supplementary Fig. 6B). In addition, we also constructed a plasmid encoding catalytically inactive UBE2S in which cysteine was mutated to alanine at site 95.[20] Mutant UBE2S alleviated the inhibitory effect of wild-type UBE2S on VHL protein expression and could not upregulate the protein expression level of HIF-1α (Fig. 5A, Supplementary Fig. 6D). In particular, UBE2S overexpression had no effect on the RNA level of VHL (Supplementary Fig. 6E), indicating that UBE2S negatively regulates VHL in a ubiquitin-proteasome-dependent manner.

Further, we performed coimmunoprecipitation and immunofluorescence assays and found an interaction between endogenous UBE2S and VHL in several HCC cell lines (Fig. 5B, C, Supplementary Fig. 7A, B). Consistently, surface plasmon resonance (SPR) demonstrated that UBE2S (1-156 aa, the conserved "core" ubiquitin conjugating domain[21,22]) showed rapid association with the sensor chip-immobilized VHL, producing a concentration-dependent resonance signal, with a calculated dissociation constant (K_D) of 3.56×10^{-5} M (Fig. 5D). These results indicate that UBE2S binds to VHL in a direct manner. In addition, we co-transfected VHL plasmid with the HA tag and ubiquitin plasmid with the Myc-Tag into Huh-7 or HCCLM3 cells and found that UBE2S overexpression significantly enhanced VHL ubiquitination (Fig. 5E). Screening of the mutated forms of ubiquitin for potential lysine ubiquitination types revealed that K11O (Ub with an intact Lys11 residue alone), but not K6O, K27O, K29O, K33O, K48O, or K63O, could be linked to VHL by UBE2S (Fig. 5F). Moreover, UBE2S failed to link K11R (the Ub with only the Lys11 residue was mutated) to VHL in HCCLM3 cells (Fig. 5G). Since substrate proteins are labeled with ubiquitin molecules at lysine residues,[23] three lysine sites of VHL were identified and were mutated separately (namely K159R, K171R, and K196R) to determine the potential UBE2S-catalyzed lysine sites. The IP assay demonstrated that mutations in K171R and K196R abolished UBE2S-mediated ubiquitination (Fig. 5H), indicating that the K171 and K196 sites were specifically responsible for the UBE2S-catalyzed ubiquitination of VHL. Next, we wanted to test whether UBE2S directly ubiquitinates VHL in an E3 enzyme-independent manner. In vitro

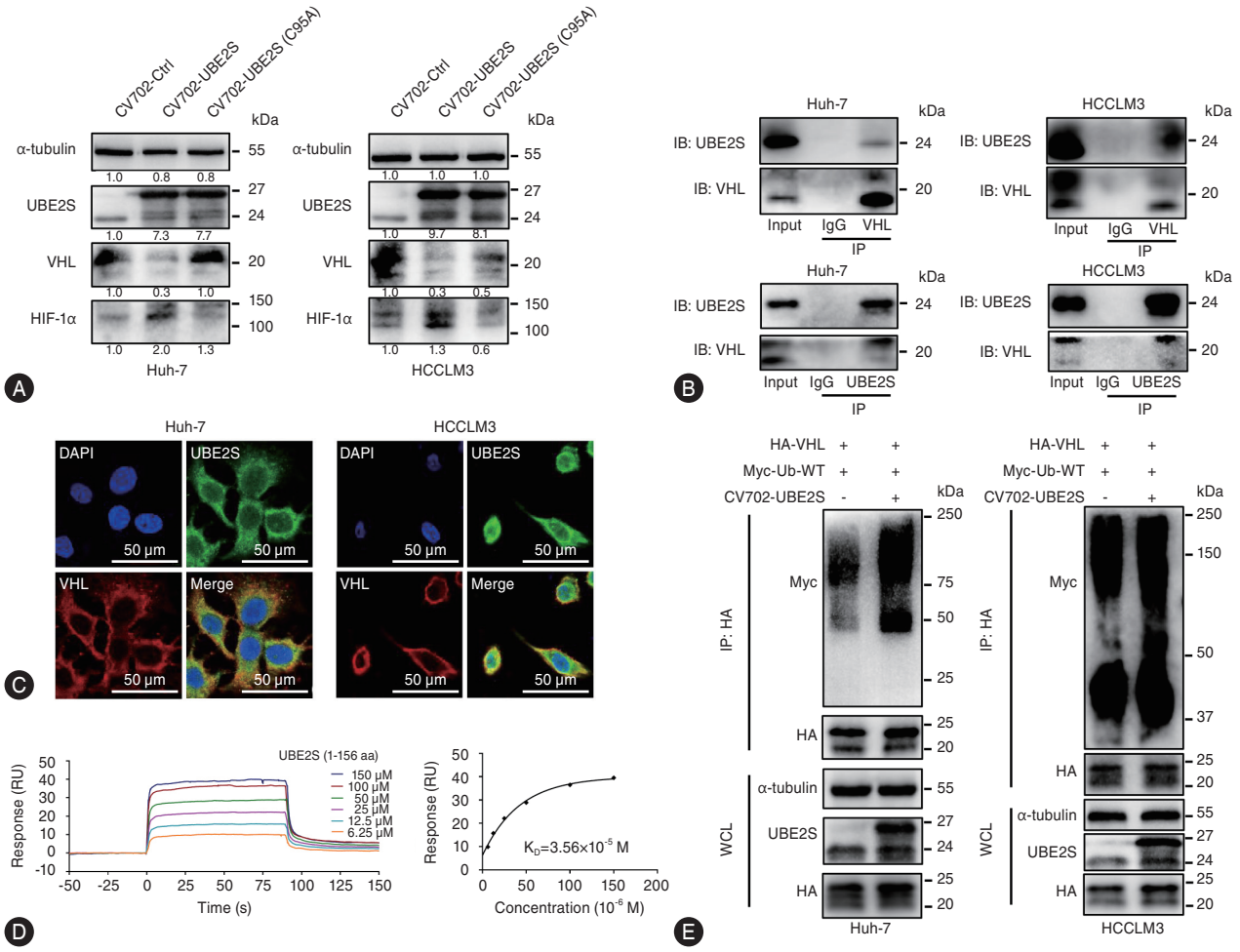

Figure 5. UBE2S destabilizes VHL in a ubiquitin-proteasome-dependent manner. (A) The expression of VHL and HIF-1α was assessed by Western blotting in Huh-7 cells or HCCLM3 cells with wild or mutant UBE2S overexpression. (B) The interactions between endogenous UBE2S and VHL in Huh-7 and HCCLM3 cells were detected by co-immunoprecipitation combined with Western blotting. (C) The co-localization between UBE2S and VHL in Huh-7 and HCCLM3 cells was detected by immunofluorescence. (D) Biophysical analysis of the interaction between VHL and UBE2S (1–156 aa) was detected by SPR. The indicated concentrations of UBE2S (1–156 aa) were injected over immobilized VHL. (E) The effects of UBE2S on polyubiquitination of VHL in Huh-7 and HCCLM3 cells were detected by immunoprecipitation combined with Western blotting. (F) The effects of UBE2S on seven types of polyubiquitination modification of VHL in HCCLM3 cells were detected by immunoprecipitation combined with Western blotting. (G) The effects of UBE2S on K11-linkage polyubiquitination of VHL in HCCLM3 cells were detected by immunoprecipitation combined with Western blotting. (H) The effects of UBE2S on ubiquitination of VHL at different lysine sites were detected by immunoprecipitation combined with Western blotting. (I) In vitro ubiquitination assay. Recombinant Ub and E1 (UBE1) were coincubated with UBE2S protein in the presence or absence of VHL protein at 37°C for 2 hours. The reaction mixture was analyzed by immunoblotting with an anti-Ub antibody (left) or anti-VHL antibody (right). UBE2S, ubiquitin conjugating enzyme E2 S; VHL, von Hippel-Lindau tumor suppressor.

ubiquitination assay showed that coincubation of Ub, E1, UBE2S, and VHL in the absence of additional E3 enzymes leads to the appearance of high-molecular-mass bands corresponding to ubiquitinated VHL (Fig. 5I). Taken together, these results indicate that UBE2S enhances the ubiquitination of VHL and promotes its degradation in an E3 enzyme-independent manner, thus improving the protein stability of HIF-1α.

UBE2S deletion inhibits HCC by regulating glycolysis *in vivo*

To further explore the effect of UBE2S on the promotion of HCC *in vivo*, hepatocyte-specific UBE2S knockout (*UBE2S*$^{-/-}$) mice were established by the crossing of UBE2S$^{loxp/loxp}$ mice with the Alb-Cre mice (Supplementary Fig. 8A). The mice were then treated with diethylnitrosa-

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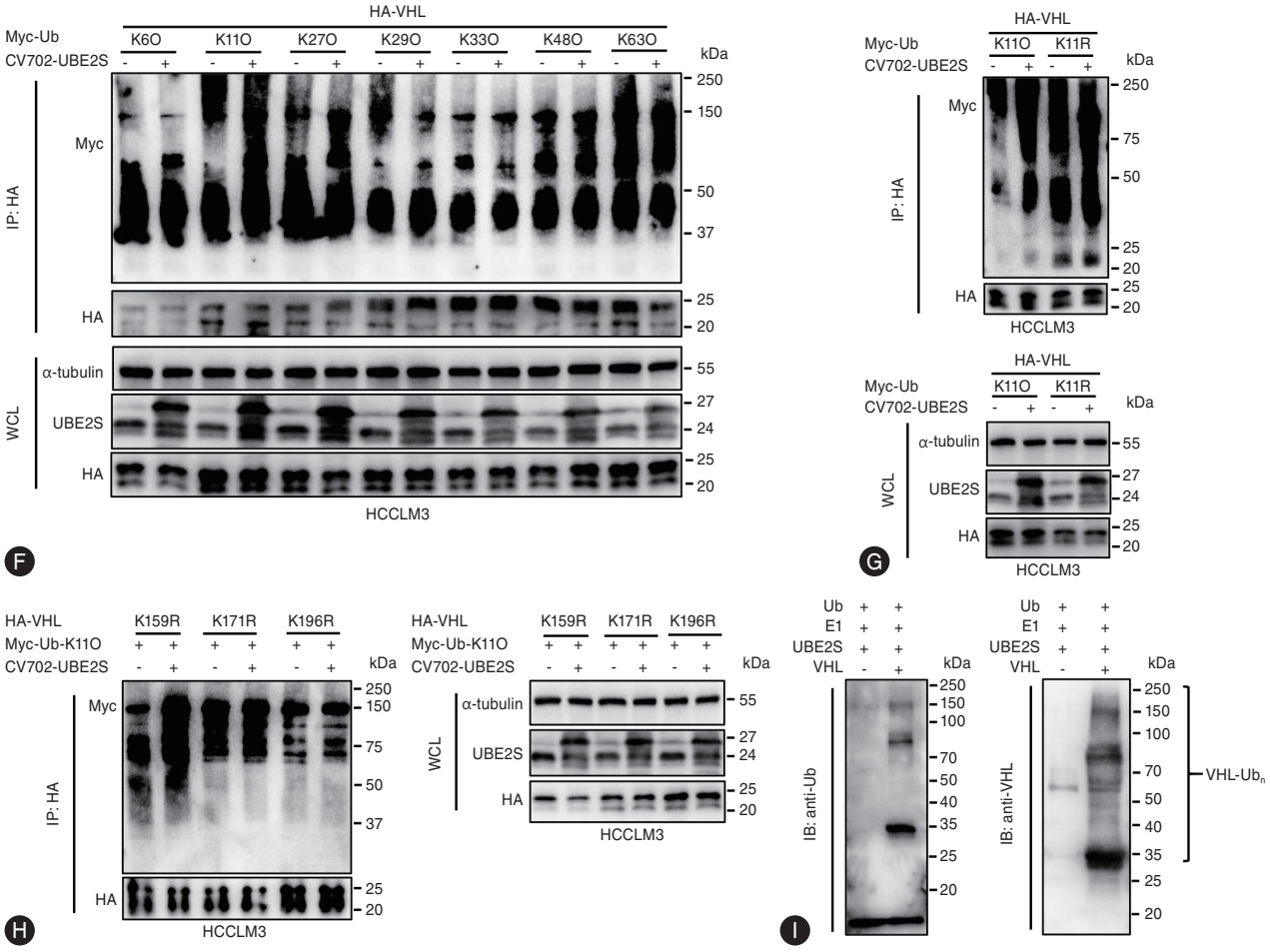

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mine (DEN)/CCl_4 to induce liver tumors (Fig. 6A). The number of liver tumors, the maximum tumor diameter, and the ratio of liver weight to body weight were significantly reduced in *UBE2S*$^{-/-}$ mice compared with that of *UBE2S*$^{+/+}$ mice in the same litter (Fig. 6B, C). However, there was no significant difference in the body weight between the two groups of mice (Fig. 6C). Moreover, the serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in *UBE2S*$^{-/-}$ mice were significantly lower than those in *UBE2S*$^{+/+}$ mice, indicating attenuated liver damage by *UBE2S* knockout (Fig. 6D). Kaplan–Meier survival analysis also showed significantly prolonged overall survival of *UBE2S*$^{-/-}$ mice compared with *UBE2S*$^{+/+}$ mice (Fig. 6E). Further, hematoxylin-eosin (HE) staining was used to confirm the morphology of tumor cells (Fig. 6F). Immunohistochemical assays showed that the expression levels of UBE2S, PCNA, and HIF-1α in the tumor tissues of *UBE2S*$^{-/-}$ mice were lower than those of the control group, whereas the expression of VHL was significantly increased (Fig. 6F). The differences in expression of UBE2S, HIF-1α, and VHL in tumor tissue were also confirmed in subsequent Western blot assays (Fig. 6G). In addition, the protein and RNA levels of HIF-1α-regulated metabolic enzymes in tumor tissues of *UBE2S*$^{-/-}$ mice were also downregulated compared with those of *UBE2S*$^{+/+}$ mice (Fig. 6G, H). Since the RNA level of HIF-1α and VHL in tumor tissues between the two groups of mice showed no significant difference (Supplementary Fig. 8B), these *in vivo* results confirmed that the upregulation of glycolytic enzymes induced by UBE2S is dependent on its post-transcriptional regulation of VHL/HIF-1α signaling pathway. Particularly, central carbon-targeted metabolomic analysis was performed to detect differences in metabolites within tumor tissues between the two groups of mice. The results demonstrated

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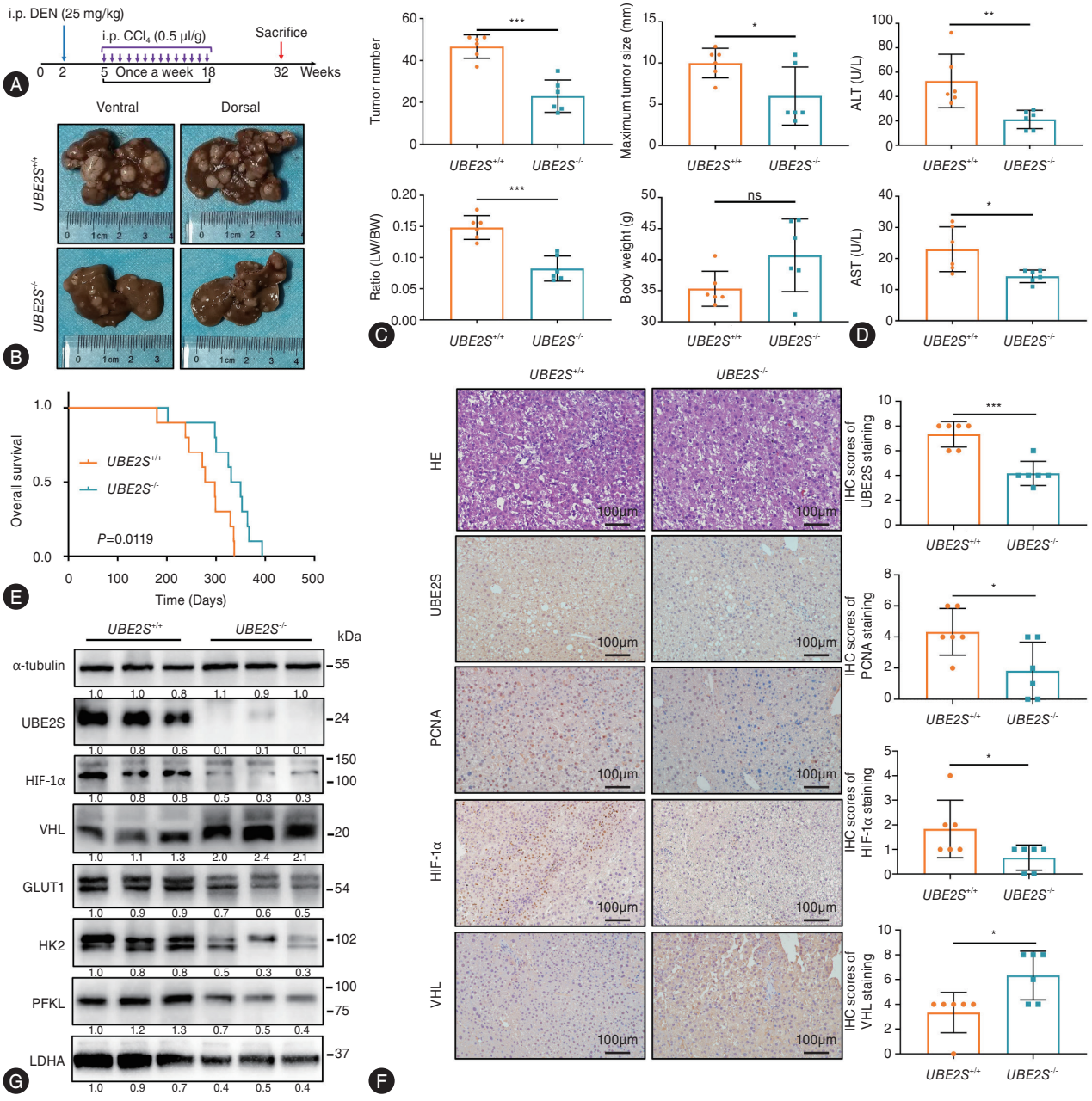

Figure 6. UBE2S promotes hepatocarcinogenesis and glycolysis of HCC *in vivo*. (A) Flowchart showing the construction of DEN/CCl_4-induced mouse HCC models. (B) Representative images of liver tumors in *UBE2S*$^{+/+}$ mice and *UBE2S*$^{-/-}$ mice after 32 weeks of DEN/CCl_4 induction. (C) The number of liver tumors, maximum tumor diameter, the ratio of liver weight to body weight, and the body weight of *UBE2S*$^{+/+}$ mice and *UBE2S*$^{-/-}$ mice on week 32 after DEN/CCl_4 induction. Data were analyzed by Student's *t*-test. $^{*}P<0.05$, $^{***}P<0.001$. (D) The levels of ALT and AST in the serum of *UBE2S*$^{+/+}$ mice and *UBE2S*$^{-/-}$ mice on week 32 after DEN/CCl_4 induction. Data were analyzed by Student's *t*-test. $^{*}P<0.05$, $^{**}P<0.01$. (E) Kaplan–Meier curve analysis of overall survival in *UBE2S*$^{+/+}$ mice and *UBE2S*$^{-/-}$ mice after DEN/CCl_4 induction. (F) Representative images and quantitative statistics of HE or immunohistochemical staining of tumor tissues in *UBE2S*$^{+/+}$ mice and *UBE2S*$^{-/-}$ mice on week 32 after DEN/CCl_4 induction. Data were analyzed by Student's *t*-test. $^{*}P<0.05$, $^{***}P<0.001$. (G, H) The expression of UBE2S and its downstream molecules was assessed by Western blotting or qPCR assays in tumor tissues of *UBE2S*$^{+/+}$ mice and *UBE2S*$^{-/-}$ mice on week 32 after DEN/CCl_4 induction. Data were analyzed by Student's *t*-test. $^{*}P<0.05$, $^{***}P<0.001$. (I) Quantitative statistical analysis of metabolites in tumor tissues of *UBE2S*$^{+/+}$ mice and *UBE2S*$^{-/-}$ mice on week 32 after DEN/CCl_4 induction using UPLC-QQQ-MS. Data were analyzed by Student's *t*-test. $^{*}P<0.05$, $^{**}P<0.01$. UBE2S, ubiquitin conjugating enzyme E2 S; HCC, hepatocellular carcinoma; DEN, diethylnitrosamine; ns, no statistical difference; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

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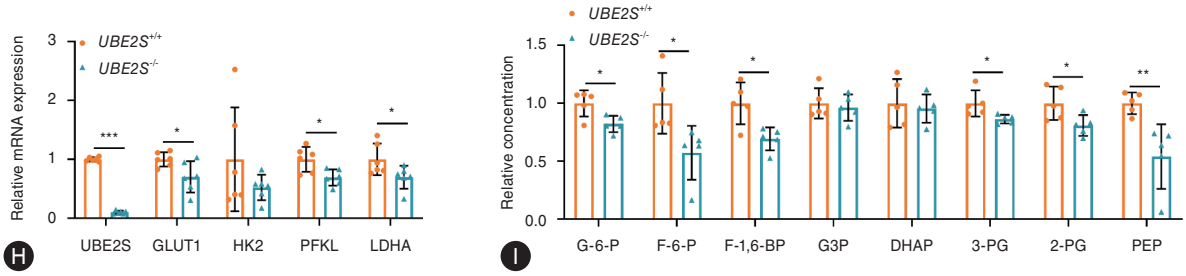

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that G-6-P, F-6-P, F-1,6-BP, 3-PG, 2-PG, and PEP in tumor tissues of *UBE2S*$^{-/-}$ mice were significantly down-regulated compared with those of *UBE2S*$^{+/+}$ mice (Fig. 6I). Taken together, these results suggest that UBE2S acts as an oncogenic gene that promotes HCC progression by regulating glycolysis *in vivo*.

Combined targeting of UBE2S and HIF-1α retards HCC growth

Our previous studies have shown that cephalomannine inhibits the expression of UBE2S.[24] To further evaluate its pharmacodynamic effects, we performed cytotoxicity assays and found that cephalomannine significantly inhibited cell viability in Huh-7 and HCCLM3 cell lines, with IC50 values of 0.11 and 0.06 μM, respectively (Fig. 7A, Supplementary Fig. 9A). Although the viability of different HCC cells gradually decreased with increasing concentration of cephalomannine, it eventually stabilized, indicating that the use of cephalomannine as a therapeutic agent for HCC needs to be improved. Since UBE2S promotes the proliferation of HCC cells by regulating HIF-1α-dependent glycolysis, the effect of cephalomannine combined with PX-478 in the treatment of HCC was further evaluated. Cytotoxicity assays revealed that PX-478 significantly inhibited cell viability in Huh-7 and HCCLM3 cells, with IC50 values of 46.51 and 21.85 μM, respectively (Fig. 7B, Supplementary Fig. 9B). Moreover, compared with single-compound therapy, the combination of the two compounds significantly increased the inhibitory effect on the viability of different HCC cells *in vitro* (Fig. 7C, Supplementary Fig. 9C). In addition, we constructed subcutaneous inoculation models in nude mice using Huh-7 cells that stably expressed firefly luciferase and found that luciferase intensity, tumor size, and tumor weight in the treatment group were significantly reduced compared to those in the negative control group (Fig. 7D–I). In particular, the above indices in the two-compound combination treatment group were further decreased compared to those in the single-compound treatment group (Fig. 7D–I). Taken together, these results indicate that the combination of cephalomannine and PX-478 significantly improves anti-tumor effects, thus providing a potential therapeutic strategy for patients with HCC.

DISCUSSION

HCC presents a significant challenge to global health, characterized by high morbidity, high mortality, and poor prognosis.[25] Although substantial advances have been made in locoregional and systemic therapy, most patients with HCC still have poor outcomes and die from the disease.[7] Therefore, more effective therapies and biomarkers with better predictive power are still necessary for precision medicine in HCC.[7] In view of the above problems, our study has obtained several novel achievements. First, we successfully construct a four-gene risk prediction model, thereby elucidating the clinical importance of ubiquitin-related genes in evaluating the prognosis of patients with HCC. Second, our study identifies UBE2S, an E2 enzyme, as a key biomarker in HCC among the thousands of ubiquitin-related genes and clarifies upstream transcriptional mechanisms that regulate UBE2S expression. Third, this study finds that UBE2S promotes glycolysis of HCC cells by enhancing K11-linkage polyubiquitination at lysine residues 171 and 196 of VHL in an E3 enzyme-independent manner. Fourth, this study proposes a combined strategy to inhibit HCC growth by targeting UBE2S and its downstream pathway.

Since ubiquitination plays an important role in the pro-

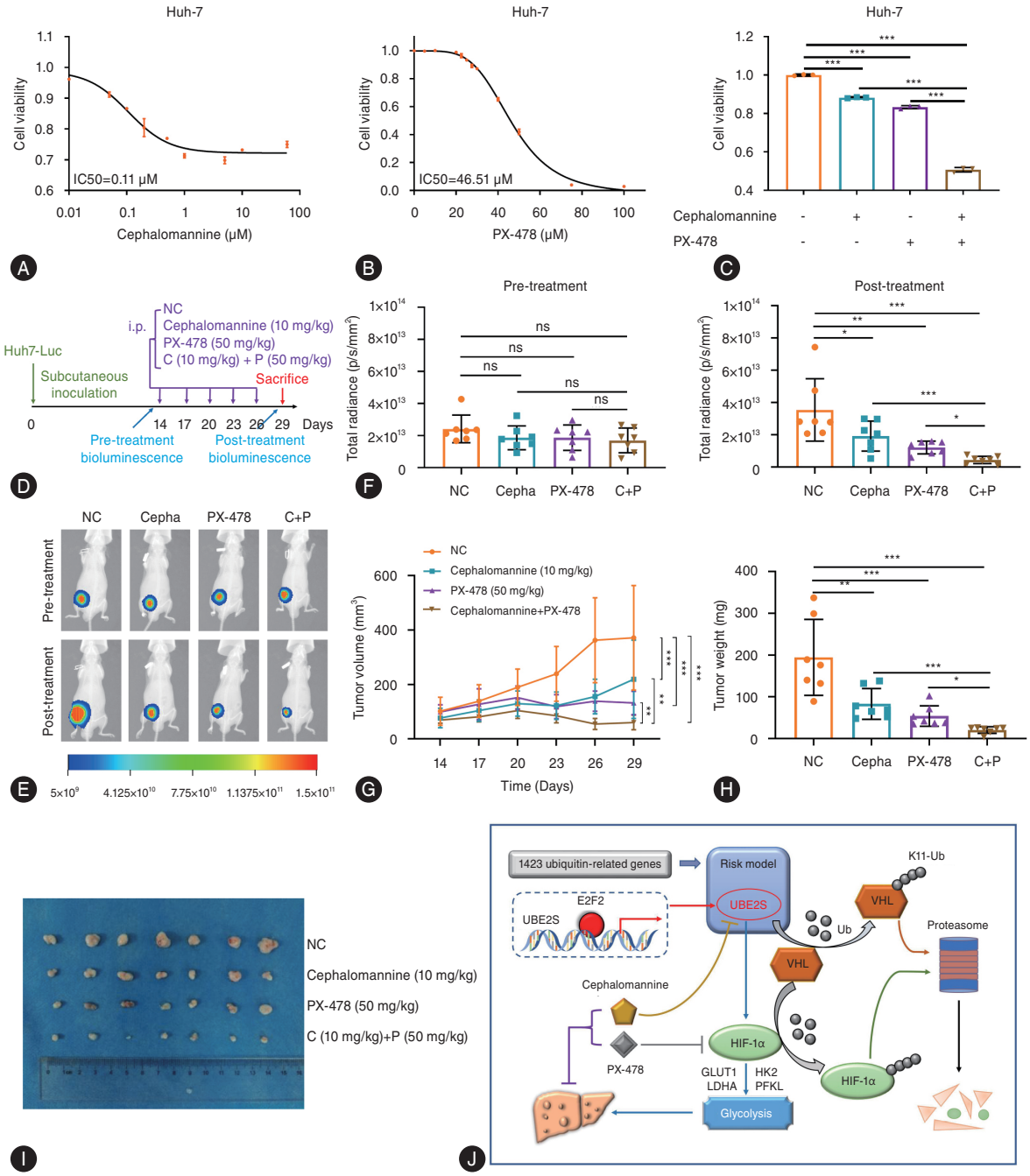

Figure 7. The combination of cephalomannine and PX-478 significantly inhibited the growth of HCC. (A, B) The inhibitory effect of cephalomannine or PX-478 on the viability of Huh-7 cells was detected by cytotoxicity assays. (C) The inhibitory effect of combined treatment of cephalomannine (0.1 μM) and PX-478 (30 μM) on the viability of Huh-7 cells was detected by cytotoxicity assays. Data were analyzed by one-way ANOVA. $^{***}P<0.001$. (D) Flow chart of a subcutaneous tumor model in nude mice injected intraperitoneally with different compounds. (E) Representative fluorescent images of subcutaneous tumors in nude mice before and after treatment with different compounds. (F) Statistical diagram of total radiance in subcutaneous tumors of nude mice before and after treatment with different compounds. Data were analyzed by one-way ANOVA. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. (G) Dynamic curves of tumor volume in mice treated with different compounds. Data were analyzed by two-way ANOVA. $^{**}P<0.01$, $^{***}P<0.001$. (H, I) Tumor weight and tumor tissues of nude mice after treatment with different compounds. Cepha indicates Cephalomannine. C + P indicates Cephalomannine + PX-478. Data were analyzed by one-way ANOVA. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. (J) Schematic of the molecular network. HCC, hepatocellular carcinoma; ns, no statistical difference.

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gression of HCC, systematic screening of ubiquitin-related biomarkers for guiding treatment decisions is of great significance. This study collected a total of 1,423 ubiquitin-related genes, including E1s, E2s, E3s, and DUBs, from three databases, and successfully constructed a four-gene signature that could be used to evaluate the prognosis of patients with HCC. Previous studies had shown that all four genes in this signature had tumor-promoting effects, however, compared with SSR3 and CYHR1, UBE2S and SKP2, which are core members of the E2s and E3s, respectively, played a bigger role in the regulation of ubiquitination modification.[26-29] In particular, only UBE2S had significantly higher RNA and protein levels in HCC tissues than in normal tissues, and was closely related to overall survival and disease-free survival of HCC patients. These results indicate the potential of UBE2S in evaluating survival and postoperative recurrence in patients with HCC, thus leading us to list UBE2S as a key molecule among thousands of ubiquitin-related genes for detailed research.

Our previous studies identified two novel somatic mutations of UBE2S by whole-exome sequencing of HCC tissues and adjacent tissues, and demonstrated that UBE2S overexpression showed several aggressive phenotypes, including cell cycle transition, invasion, and migration.[24] However, the upstream molecules and mechanisms that regulate UBE2S expression in HCC have rarely been reported. In this study, E2F2 was identified as a transcription factor for UBE2S. As an important member of the E2F transcription factor family, E2F2 is widely involved in various cellular processes, such as cell cycle, apoptosis, invasion, and metastasis, through its transcriptional regulation.[30-33] Our study not only verified the positive correlation between the expression levels of E2F2 and UBE2S by immunohistochemistry, but also confirmed that E2F2 could transcriptionally upregulate UBE2S expression by directly binding to the UBE2S promoter. In particular, previous studies have shown that the expression of E2F2 in HCC tissues is significantly higher than that in adjacent tissues and is closely related to poor patient prognosis.[34,35] Considering that these findings are similar to both the expression characteristics and clinical prognostic relevance of UBE2S, these results reinforce our belief that UBE2S can be transcriptionally regulated by E2F2 in HCC.

Ubiquitination is an enzymatic reaction regulated by a cascade of E1s, E2s, and E3s.[9] Previous studies have shown that E2s mainly regulate the type of ubiquitin chain to which the substrate protein is labeled, whereas E3s determine the specificity of the substrate protein recognized.[36,37] Unusually, this study found that UBE2S catalyzes K11-linkage ubiquitination on the K171 and K196 sites of VHL in an E3 enzyme-independent manner, thereby indirectly stabilizing HIF-1α protein levels by mediating the degradation of VHL by the proteasome. Accumulated HIF-1α could upregulate the expression of downstream genes such as *GLUT1*, *HK2*, *PFKL,* and *LDHA*, thereby positively regulating glycolysis and ultimately promoting the proliferation of HCC cells. Taken together, this study not only displays for the first time the regulatory role of UBE2S in metabolic reprogramming, but also reveals an E3 enzyme-independent mechanism for polyubiquitination. Notably, Sun and Fang[38] found that another UBE2E family of E2 enzymes could directly catalyze the monoubiquitination of SETDB1 independent of E3 ligase, while the conjugated ubiquitin was essential for SETDB1's enzymatic activity and function. These results enrich our understanding of the novel mode in which E2s catalyze substrate proteins to be labeled with ubiquitin chains of different lengths in an E3s-independent manner, leading to protein degradation or dysfunction.

Compared with traditional tumor treatment strategies, small-molecule drugs possess advantages such as better oral bioavailability, higher tissue permeability, and a more reasonable half-life; therefore, they can be employed as therapeutics in the field of tumor therapy.[39] Previous studies have identified a range of small compounds that target E3s for cancer treatment, owing to E3s' ability to select, bind and recruit target substrates for ubiquitination.[37] Considering the possibility of E3s-independent ubiquitination, this study highlights the potential of E2s as intervention targets for anti-tumor therapy. Cephalomannine is a small-molecule compound identified in our previous studies that can inhibit the expression of UBE2S.[24] PX-478 is a selective HIF-1α inhibitor that reduces hypoxia-inducible and constitutive HIF-1α protein levels and trans-acting activity in a VHL-independent manner.[40] PX-478 was tested in a phase I clinical trial against advanced solid tumors and lymphomas (NCT00522652), and demonstrated promising results as evidenced by stable disease in approximately 35% of patients.[41,42] Since UBE2S promotes the proliferation of HCC cells by positively regulating HIF-1α-dependent

glycolysis, we evaluated the therapeutic effect of cephalomannine combined with PX-478 on HCC. Our experiments not only confirmed the anti-HCC effect of PX-478 for the first time in nude mice tumor-bearing models, but also demonstrated that the combined strategy of targeting E2 enzyme and its downstream pathway could significantly improve the anti-tumor effect. Recently, Wu et al.[43] found that UBE2S could promote malignant properties via VHL/HIF-1α and VHL/JAK2/STAT3 signaling pathways, and decrease sensitivity to sorafenib in HCC. These results are not only consistent with our conclusions, but also suggest that in addition to HIF-1α inhibitor, the combination of JAK/STAT signaling pathway inhibitors or sorafenib with cephalomannine may also be potential strategies for anti-HCC therapy.

In conclusion, our study constructs a risk prediction model to evaluate the prognosis of patients with HCC and identifies UBE2S as having an important clinical predictive potential among 1,423 ubiquitin-related genes. Moreover, the upstream transcription factor E2F2 regulates UBE2S expression. In particular, our study reveals that UBE2S facilitates ubiquitin-mediated degradation of VHL in an E3 enzyme-independent manner, which subsequently promotes HIF-1α-dependent glycolysis. We finally provide a potential anti-tumor strategy for patients with HCC by targeting UBE2S and HIF-1α (Fig. 7J).

Authors' contribution

Huijie Bian, Zhinan Chen, and Ding Wei designed and supervised the study. Renyu Zhang, Can Li, and Shuai Zhang performed most of the experiments and wrote the manuscript. Minling Kong, Meng Lu, and Man Liu participated in discussions of the research ideas. Zekun Liu, Yule Yong, and Dong Wu conducted the bioinformatics analyses. Yixiao Guo, Ying Sun, and Jianjun Lv assisted molecular biology experiments. Cong Zhang, Tianjiao Zhang, and Haijiao Yang provided technical support for the animal experiments. All the authors had read and approved the final version of the manuscript.

Acknowledgements

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Conflicts of Interest

The authors have no conflicts to disclose.

SUPPLEMENTARY MATERIAL

Supplementary material is available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

Data Availability: RNA-sequencing data has been uploaded to GEO (GSE267607). All the data supporting the findings of this study are available from the corresponding author on reasonable request.

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