



***E2F1* as a potential prognostic and therapeutic biomarker by affecting tumor development and immune microenvironment in hepatocellular carcinoma**

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Background: *E2F1* plays a crucial role in cell cycle regulation. However, the exact role of *E2F1* in liver hepatocellular carcinoma (LIHC) remains controversial. This study aimed to integrate disparate data by bioinformatics for a deeper insight into the possible roles of *E2F1* in LIHC.

Methods: Differentially overexpressed genes in LIHC were screened by GEO2R. Gene ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway were analyzed by WebGestalt. Then, hub genes were selected via STRING and Cytoscape, followed by validation with Oncomine, GEPIA2, and Human Protein Atlas (HPA). Next, *E2F1* expression was investigated using Oncomine, GEPIA2, TIMER2.0, UALCAN, and HPA. Then, Kaplan-Meier plotter was adopted to investigate survival. After that, *E2F1* promoter methylation, mutations and copy number alterations were analyzed with UALCAN and cBioPortal. Moreover, competing endogenous RNAs (ceRNAs) network were established using ENCORI, miRCancer, and Kaplan-Meier plotter. Additionally, the association between *E2F1* and immune microenvironment was investigated through TISCH and TIMER2.0.

Results: Six hub genes including *E2F1* were identified. *E2F1* was overexpressed in most solid cancers including LIHC. *E2F1* overexpression was correlated with poor prognosis in LIHC. Copy number alterations could positively affect *E2F1* expression. Moreover, ceRNAs network was established with 3 long non-coding RNAs (lncRNAs) named AC025048.4, AC090114.2, and AC092171.5, as well as 4 microRNAs (miRNAs) including miR-150-5p, miR-302c-3p, miR-520d-3p, and miR-330-5p. Single cell sequencing data showed that *E2F1* was mainly expressed in malignant cells and proliferating T cells, and that *E2F1* targets almost exclusively enriched in proliferating T cells. Besides, there existed a positive correlation between *E2F1* and certain immune cells including CD8(+) T cells, CD4(+) T cells, B cells, macrophages, and dendritic cells.

Conclusions: This study elucidated that *E2F1* could affect tumor development and immune microenvironment in LIHC. Thus, *E2F1* might be a potential prognostic biomarker and therapeutic target for LIHC.

Keywords: *E2F1*; hepatocellular carcinoma; prognosis; tumor microenvironment; therapeutic target

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Introduction

Primary liver cancer is perceived as one of the most lethal cancers, which is ranked the seventh and the second of new cases and deaths respectively among all malignancies worldwide (1). Liver hepatocellular carcinoma (LIHC) is the major subtype of primary liver cancer, accounting for approximately 75% (2). In recent years, novel treatment strategies including immune-checkpoint inhibitors, tyrosine kinase inhibitors and monoclonal antibodies have been applied to the treatment for LIHC and have achieved great progress (3); however, the outcome remains poor with a 5-year survival only standing at about 20% in the United States (4).

Since 50–60% LIHC patients need to be exposed to systemic therapies throughout their entire treatment (3), it is particularly necessary to identify more candidate anti-LIHC targets for effective diagnosis, prognosis, and treatment (5). Nevertheless, approximately 25% of LIHC exist with potentially actionable mutations, which are yet to be translated into the clinical practice (6). Therefore, identifying vital biomarkers and exploring their latently clinical significance could facilitate the development of novel precision treatments.

E2F family of transcription factors plays a crucial role in cell differentiation, response to DNA damage, and cell life cycle (7). A variety of *E2F* isoforms have been characterized and can be found in most cell types (8). It has been demonstrated that *E2F* family genes are overexpressed and associated with the development and prognosis in several types of cancers including LIHC (9–13).

Despite that *E2F1* is the classic and the most studied *E2F* member, the role of *E2F1* in LIHC is controversial. Some studies reported that *E2F1* could promote the proliferation of LIHC by activating ERK/mTOR, B-Myb, BRCA1, Stathmin 1 (14–17) or could inhibit c-Myc-driven apoptosis via activating PIK3CA/Akt/mTOR and COX-2 pathway (18). Meanwhile, several studies indicated that *E2F1* exhibited tumor-suppressing activity in LIHC. For instance, TFDP3/*E2F1* pathway could promote LIHC cell apoptosis by upregulating HIF-2 α , and HIF-2 α overexpression was associated with favorable overall survival (OS) of LIHC patients (19). Thus, more studies are needed to further verify the exact role of *E2F1* in LIHC (13).

Bioinformatics is an effective way to integrate and process vast life sciences data (20). Thus, this study adopted bioinformatics to merge disparate data sources to explore the possible roles of *E2F1* in LIHC. Hub genes in LIHC were identified followed by exploring the expression

level and prognostic value of *E2F1*. Moreover, possible regulating mechanisms including promotor methylation, mutation, copy number alterations, and competing endogenous RNAs (ceRNAs) of *E2F1* were investigated. In addition, the association between *E2F1* and tumor immune microenvironment was examined using single cell sequencing data. We present the following article in accordance with the REMARK reporting checklist (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-22-218/rc>).

Methods

Identification of overexpressed genes in LIHC

Two Gene Expression Omnibus (GEO) series, named GSE46408 (21) and GSE54238 (22,23), containing LIHC and normal liver tissue gene expression profiles were selected from GEO (RRID:SCR_004584) (24). Six LIHC tissue samples and 6 paired normal liver parenchyma samples of GSE46408, as well as 13 advanced LIHC tissue samples and 10 normal liver samples of GSE54238 were respectively compared by GEO2R (25) to screen differentially expressed genes (DEGs). Significantly overexpressed DEGs were chosen by the criteria of adjusted P value <0.05 and log₂FC \geq 1.5 (FC: fold change). Some genes displayed multiple log₂FC values in one GEO series due to the use of different probes. Those genes that displayed conflicting log₂FC values, some of which >0 (representing overexpression in LIHC) and the others <0 (indicating under-expression in LIHC), were excluded. The shared overexpressed DEGs in 2 GEO series were chosen for further study.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis

The biological process, cellular component, molecular function annotations of GO, as well as KEGG pathway enrichment were adopted to analyze the functions of the shared overexpressed DEGs using WebGestalt 2019 (RRID:SCR_006786) (26). Parameters are as following: organism: homo sapiens, method: over-representation analysis, reference set: genome protein coding. The results were visualized through R programming.

PPI network construction and hub genes selection

STRING database Version 11.5 (RRID:SCR_005223) (27)

was applied to construct protein-protein interaction (PPI) network of the shared overexpressed DEGs with the following parameters: network type: full STRING network, active interaction sources: all, interaction score: at least 0.700 (high confidence). The STRING analyzing result including nodes (genes) and experimentally determined interaction parameters was input into Cytoscape software (RRID:SCR_003032) (28) with CytoHubba plugin (RRID:SCR_017677). Calculating via betweenness methods, hub genes were selected with the interaction score >160.

Hub genes validation

Next, to verify the expression of selected hub genes, the difference of mRNA expressions of each hub gene in LIHC and normal liver tissue were compared via Oncomine (RRID:SCR_007834) (29) and GEPIA2 (RRID:SCR_018294) (30) respectively. Besides, immunohistochemical staining images in Human Protein Atlas (HPA, RRID:SCR_006710) (31-33) were adopted to illustrate the protein expression of each hub gene in LIHC and normal liver tissue.

Analysis of *E2F1* mRNA and protein expression in various cancers including LIHC

The mRNA expression levels of *E2F1* in various cancers including LIHC and paired normal tissues were compared by Oncomine, GEPIA2, and TIMER2.0 (RRID:SCR_018737) (34). In Oncomine, the thresholds were set as: P value of 1E-4, fold change of 2, gene rank of top 10%, and data type of all. TIMER2.0 used Wilcoxon test to count *E2F1* expression difference between cancer and the corresponding normal tissue. Besides, to verify *E2F1* expression level in LIHC, TCGA data containing *E2F1* mRNA expression level and clinicopathological features of LIHC were synthesized and analyzed through UALCAN web (RRID:SCR_015827) (35), and were graphically illustrated with Graphpad Prism Version 9.2.0 (RRID:SCR_002798). Moreover, the *E2F1* protein expressions in different kinds of cancers including LIHC were traced from HPA, which present the percentage of patients with high or medium immunohistochemical staining using 3 different *E2F1* antibodies (HPA008003, CAB000329, CAB019308) respectively.

Exploring the relationship between *E2F1* expression and survival in LIHC

The prognostic value of *E2F1* in LIHC was probed using Kaplan-Meier plotter (RRID:SCR_018753) (36), which integrated gene expression and clinical data simultaneously from TCGA database (RRID:SCR_003193), GEO database, and European Genome-Phenome Archive (EGA, RRID:SCR_004944) database. The OS, disease-specific survival (DSS), progression-free survival (PFS), and relapse-free survival (RFS) in each clinicopathological characteristic cohort were analyzed according to *E2F1* expression level by the following parameters: cut-off: median, RNAseq ID: 1869, and default settings for all other parameters.

Moreover, the clinical data and *E2F1* expression data were retrieved from TCGA database. Multivariate Cox regression analysis was carried out by SPSS Version 26 (RRID:SCR_019096) to analyze whether *E2F1* expression level could serve as an independent prognostic factor for LIHC. High and low *E2F1* expression subgroups were categorized based on the median expression level of *E2F1*.

Investigating *E2F1* promotor methylation level in LIHC

E2F1 promotor methylation levels in LIHC and normal liver tissue were investigated from TCGA database via UALCAN web. Results were graphically presented through Graphpad Prism Version 9.2.0. In addition, 373 LIHC samples containing data of mRNA Expression z-Scores (RNA Seq V2 RSEM) and Methylation (HM450) beta-value simultaneously were obtained from TCGA Firehose Legacy Cohort via cBioPortal (RRID:SCR_014555) (37,38). Methylation (HM450) beta-value was normalized into 0 to 1. Then simple linear regression was performed by SPSS Version 26 to calculate the correlation between promotor methylation and mRNA expression and visualized by Graphpad Prism Version 9.2.0.

Analysis of mutations and copy number alterations (CNAs) of *E2F1* in LIHC

916 LIHC samples of 914 patients in 3 studies [TCGA, Firehose Legacy; AMC, Hepatology 2014 (39); INSERM, Nat Genet 2015 (40)] were adopted to analyze the frequency and types of *E2F1* mutation via cBioPortal. Following that, 673 samples of 671 patients in 2 LIHC studies (TCGA,

Firehose Legacy; AMC, Hepatology 2014) were used to acquire the frequency of copy-number alterations. Meanwhile, *E2F1* mRNA expression levels in different copy number groups of the TCGA Firehose Legacy Study were compared with One-way ANOVA using SPSS Version 26. Moreover, 349 samples of the TCGA Firehose Legacy Study containing mRNA expression data and copy-number alterations data simultaneously were retrieved from cBioPortal, the correlation of the relative linear copy-number values and the mRNA expression z-scores (RNA Seq V2 RSEM) was analyzed by simple linear regression via SPSS Version 26 and exhibited via Graphpad Prism Version 9.2.0.

Exploring possible ceRNAs of *E2F1*

According to the mechanism of ceRNAs (41), it is assumed that long non-coding RNAs (lncRNAs) as ceRNAs (ce-lncRNAs) could negatively affect the expression of certain microRNAs (miRNAs) which then negatively affect *E2F1* levels. Thus, ce-lncRNA levels should be raised and certain miRNA levels should be lowered. ENCORI (RRID:SCR_016303) (42) was employed to explore possible lncRNAs as ceRNAs (ce-lncRNAs) with the following parameters: genome of human, assembly of hg19, miRNA number ≥ 2 , P value ≤ 0.01 , FDR ≤ 0.01 , and with at least one cancer type data. Following that, only those ce-lncRNAs showing positive coefficient with *E2F1* expression level (P value ≤ 0.01 and FDR ≤ 0.01) in LIHC were supposed to be ce-lncRNAs. Next, miRNAs which could be regulated by the aforementioned ce-lncRNAs were retrieved from ENCORI. The expression of those miRNAs was investigated through miRCancer database (<http://mircancer.ecu.edu/>) (43), only those downregulated miRNAs in LIHC were selected for further study. Then, the relationship between those miRNAs' expression level and OS was investigated using Kaplan-Meier plotter, those miRNAs showing favorable OS for LIHC patients were included into ceRNAs network.

Exploring the association between *E2F1* and tumor immune microenvironment via single cell sequencing data

Four human LIHC datasets (LIHC_GSE125449_aPDL1aCTLA4, LIHC_GSE140228_10X, LIHC_GSE140228_Smartseq2, and LIHC_GSE98638) were retrieved from TISCH database (<http://tisch.comp-genomics.org/>) (44). *E2F1* expression levels in different

major-lineage cell clusters of each dataset were investigated. Moreover, the hallmark of up-regulated genes in different cell clusters, as well as the enrichment of E2F targets were also explored via TISCH database. Furthermore, the correlations between *E2F1* expression and the infiltrations of different types of immune cells in LIHC were analyzed by TIMER2.0 database.

The brief study process was illustrated by flowchart (see Figure 1).

Statistical analysis

All statistical analysis were performed by SPSS Version 26. Multivariate Cox regression analysis was carried out to identify independent prognostic factors for LIHC. Simple linear regression was performed to calculate the correlation between promotor methylation and mRNA expression of *E2F1*, as well as relative linear copy-number values and mRNA expression of *E2F1*. Besides, *E2F1* mRNA expression levels in different copy number groups were compared with One-way ANOVA. P value < 0.05 is considered statistically significant, the confidence interval was set at 95%.

Ethical statement

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Results

Overexpressed genes in LIHC

A total of 664 and 289 overexpressed DEGs were identified from GSE46408 and GSE54238 respectively. Among those, 100 overexpressed DEGs shared in these 2 GEO series were selected for further investigation (see Figure 2A).

Gene ontology and KEGG pathway enrichment

Analyzed by WebGestalt, the biological process annotation of the 100 overexpressed DEGs mainly enriched in DNA replication, mitotic cell cycle phase transition, cell cycle phase transition, mitotic cell cycle process, mitotic cell cycle, regulation of cell cycle process, cell cycle process, DNA metabolic process, regulation of cell cycle, and cell cycle (see Figure 2B). Cellular component mainly focused on mitotic spindle, spindle pole, spindle, chromosomal region, nuclear

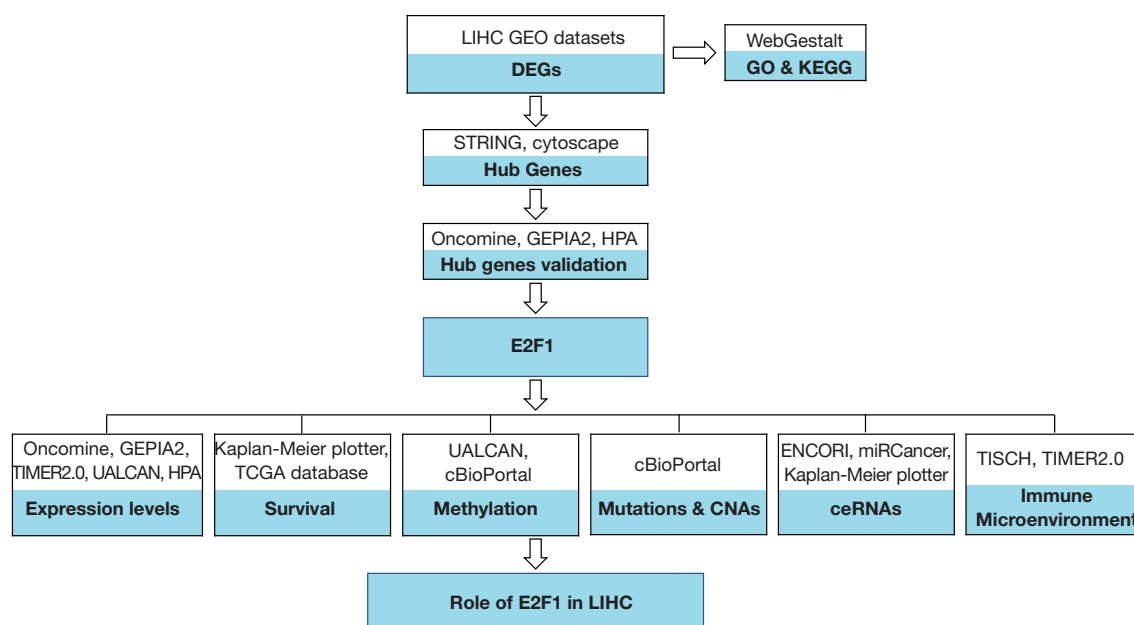


Figure 1 Flowchart of brief study process. DEGs, differentially expressed genes; LIHC, liver hepatocellular carcinoma; GEO, Gene Expression Omnibus; KEGG, Kyoto Encyclopedia of Genes and Genomes; HPA, Human Protein Atlas; CNAs, copy number alterations.

chromosome part, nuclear chromosome, chromosomal part, chromosome, microtubule cytoskeleton, and cytoskeletal part (see *Figure 2C*). Molecular function was mainly related to RNA-DNA hybrid ribonuclease activity, DNA helicase activity, helicase activity, catalytic activity, acting on DNA, kinase binding, protein kinase binding, ATP binding, purine ribonucleoside triphosphate binding, purine ribonucleotide binding, and purine nucleotide binding (see *Figure 2D*). KEGG pathway mainly concentrated on DNA replication, cell cycle, and oocyte meiosis (see *Figure 2E*).

PPI network construction and hub genes selection

Sixty-four largest connected nodes (genes) and 375 edges were constructed through STRING and illustrated by Cytoscape. The other 36 nodes (genes) belonging to separate interaction cluster were not included. Moreover, by using CytoHubba plugin, 6 hub genes named *CCNB1*, *RFC4*, *E2F1*, *DSN1*, *TYMS*, and *AURKA* were identified (see *Figure 2F*).

Hub genes validation

Four datasets containing the expression levels of numerous genes including 6 hub genes were selected and analyzed in

Oncomine. Each hub gene was significantly overexpressed in LIHC in at least 2 out of 4 datasets and no hub gene was downregulated in these 4 datasets (see *Figure 3A*). Analysis by GEPIA2 also demonstrated that the mRNA expression level of each hub gene in LIHC was statistically higher than that in normal liver tissue (see *Figure 3B*). The immunohistochemical staining images showed that the protein expression of each hub gene exhibited higher level in LIHC samples than that in normal liver tissue (see *Figure 3C*).

***E2F1* mRNA and protein expression in various cancers including LIHC**

To deepen the understanding, this study selected one hub gene named *E2F1* for further investigation. Firstly, the mRNA expression of *E2F1* in various cancers and paired normal tissues were investigated. Analysis via Oncomine revealed that *E2F1* was overexpressed in most solid tumors, such as liver cancer, lung cancer, breast cancer, colorectal cancer, kidney cancer, ovarian cancer, etc., except for brain and central nervous system cancer (see *Figure 4A*). Exploration by GEPIA2 found that *E2F1* was significantly overexpressed in 23 out 31 kinds of solid tumors including LIHC, and no type of cancer exhibited downregulation of *E2F1* (see *Figure 4B*). These findings were verified by

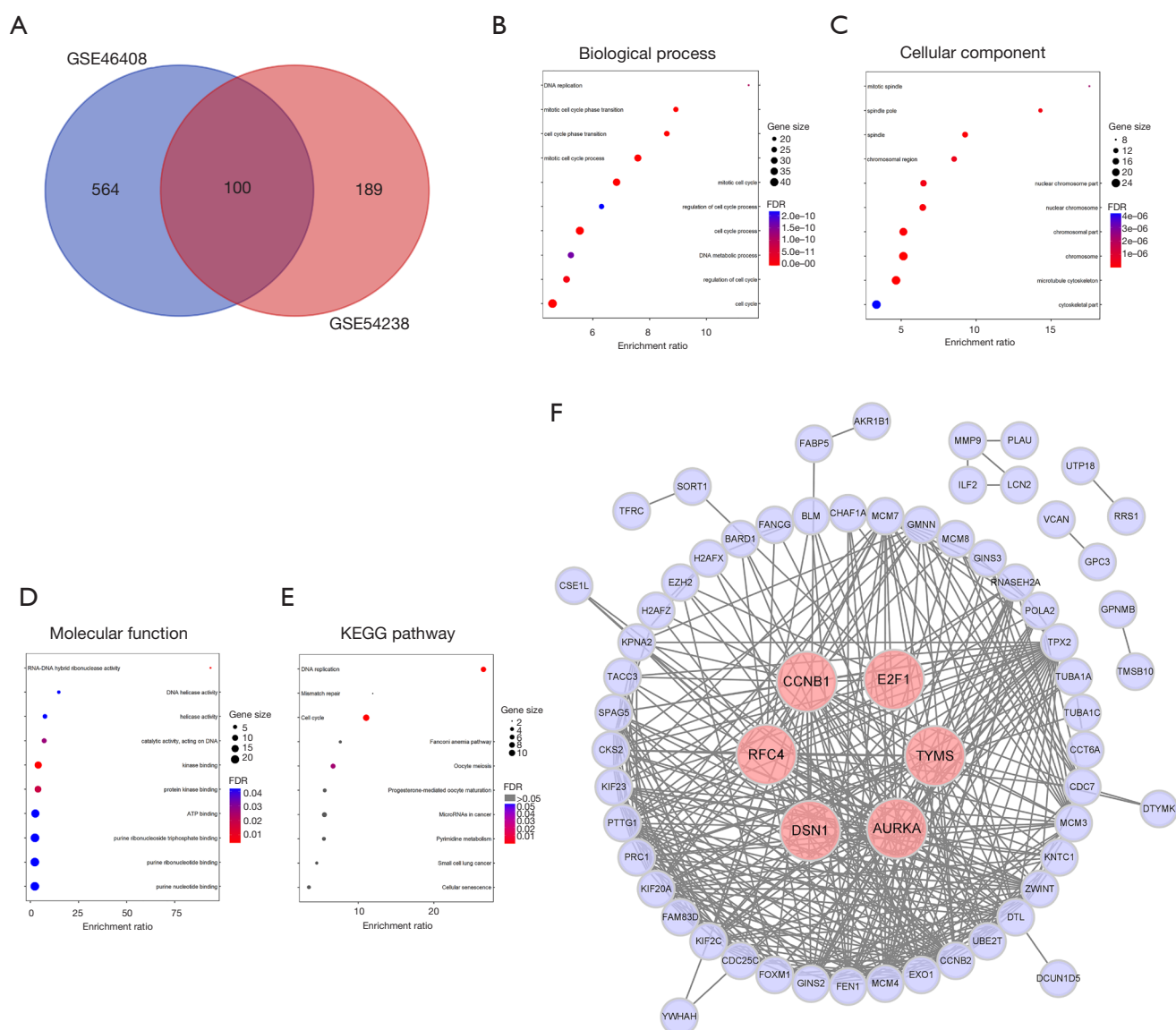


Figure 2 Hub genes selection in LIHC. (A) Venn diagram showed 100 differentially overexpressed genes identified from 2 GEO series; (B) biological processes enrichment of differentially overexpressed genes; (C) cellular component enrichment of differentially overexpressed genes; (D) molecular function enrichment of differentially overexpressed genes; (E) KEGG pathway enrichment of differentially overexpressed genes; (F) PPI network and hub genes. LIHC, liver hepatocellular carcinoma; GEO, Gene Expression Omnibus; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction.

investigating TIMER2.0, which showed that *E2F1* was statistically overexpressed in 20 kinds of cancers containing LIHC (see *Figure 4C*).

Furthermore, through analyzing 371 LIHC tissues and 50 normal tissues, UALCAN showed that *E2F1* mRNA was overexpressed in LIHC regardless of race, gender, age, stage (except for stage 4), grade, and TP53 mutation status

(see *Figure 4D*). Besides, stage 2 and 3, grade 3, as well as TP53 mutant subgroups exhibited higher *E2F1* expression level compared to stage 1, grade 1 and 2, TP53 non-mutant subgroups respectively (see *Figure 4D*). In addition, HPA database showed that most kinds of cancers including liver cancer displayed moderate to strong immunoreactivity to *E2F1* irrespective of different antibodies (see *Figure 4E*). To

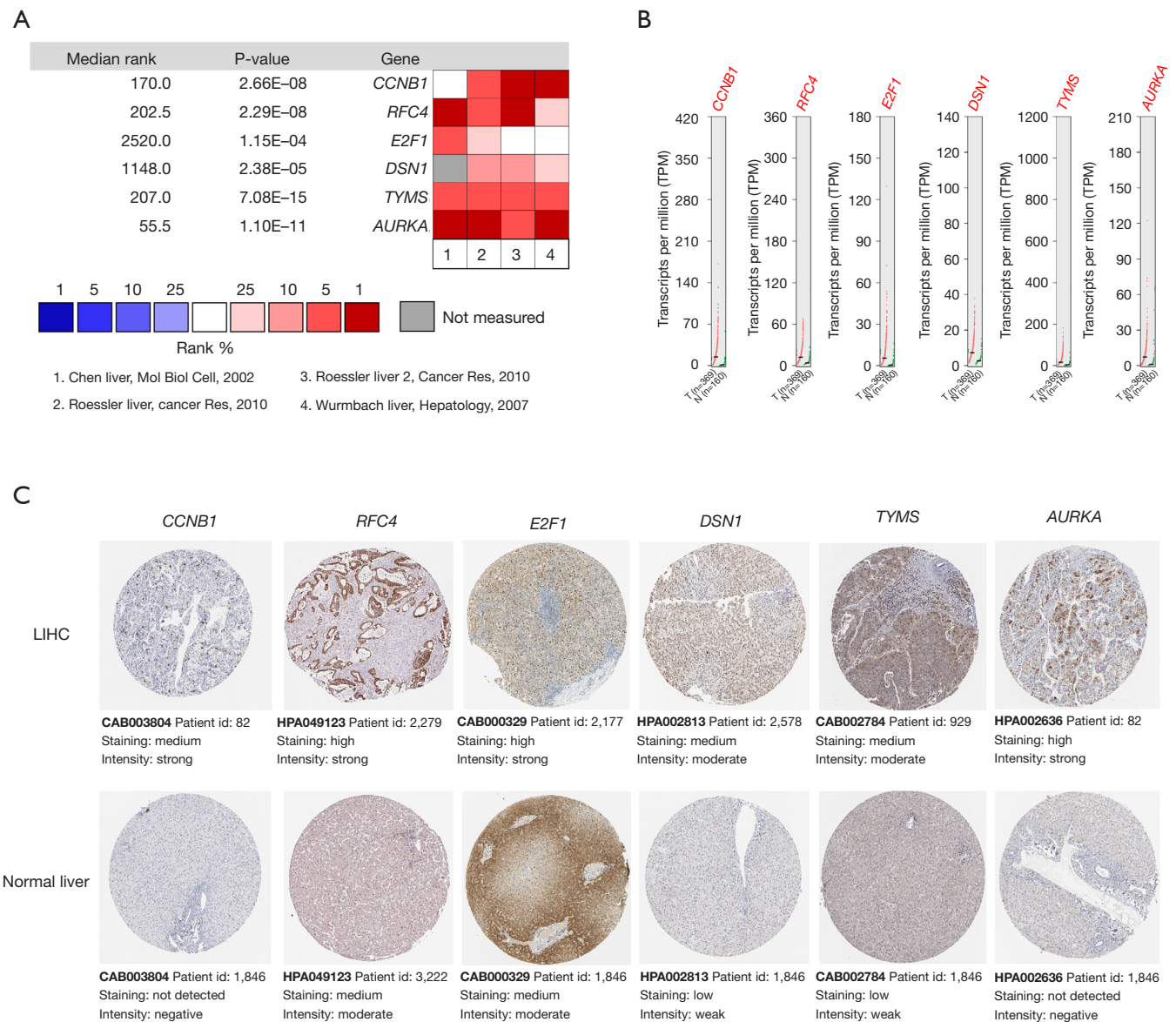


Figure 3 Hub genes validation in LIHC. (A) The expression of 6 hub genes in 4 LIHC datasets via Oncomine; (B) the expression of 6 hub genes in LIHC via GEPIA; (C) the immunohistochemistry images of 6 hub genes via HPA. LIHC, liver hepatocellular carcinoma; HPA, Human Protein Atlas.

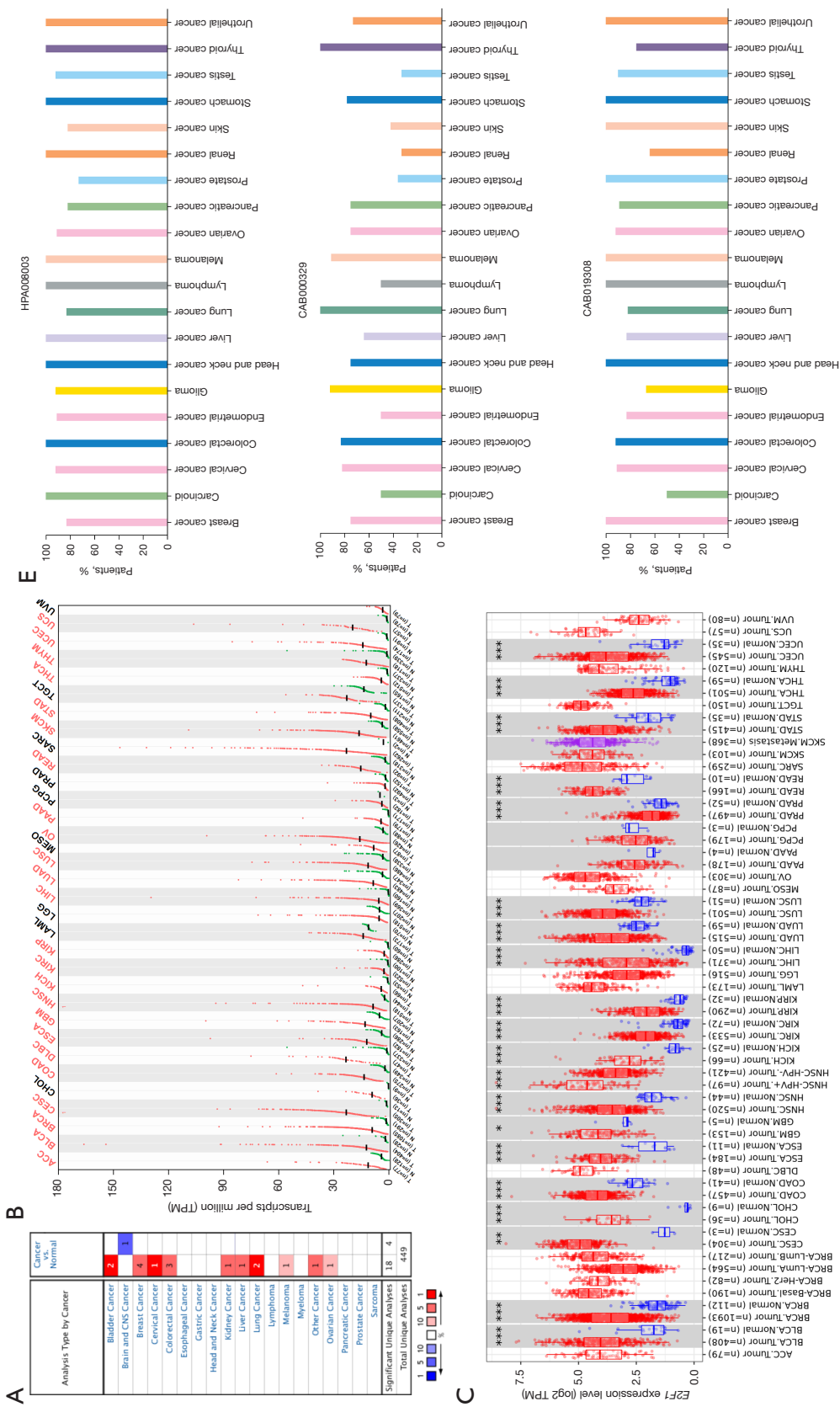
sum up, those data indicated that *E2F1* mRNA and protein were overexpressed in most solid tumors including LIHC.

The relationship between *E2F1* expression and survival in LIHC

Kaplan-Meier plotter was utilized to investigate survival data in accordance with *E2F1* expression level. Results

revealed that OS, DSS, PFS, and RFS were significantly in favor of *E2F1* low expression cohort, and the hazard ratios (HR) were 1.75 (1.23–2.49, $P=0.0016$), 1.91 (1.21–3.01, $P=0.0046$), 1.60 (1.19–2.15, $P=0.0018$), and 1.68 (1.21–2.35, $P=0.002$) respectively (see Figure 5A–5D).

Moreover, subgroup analysis according to different clinicopathological characteristics indicated that the OS was significantly negatively correlated with *E2F1*



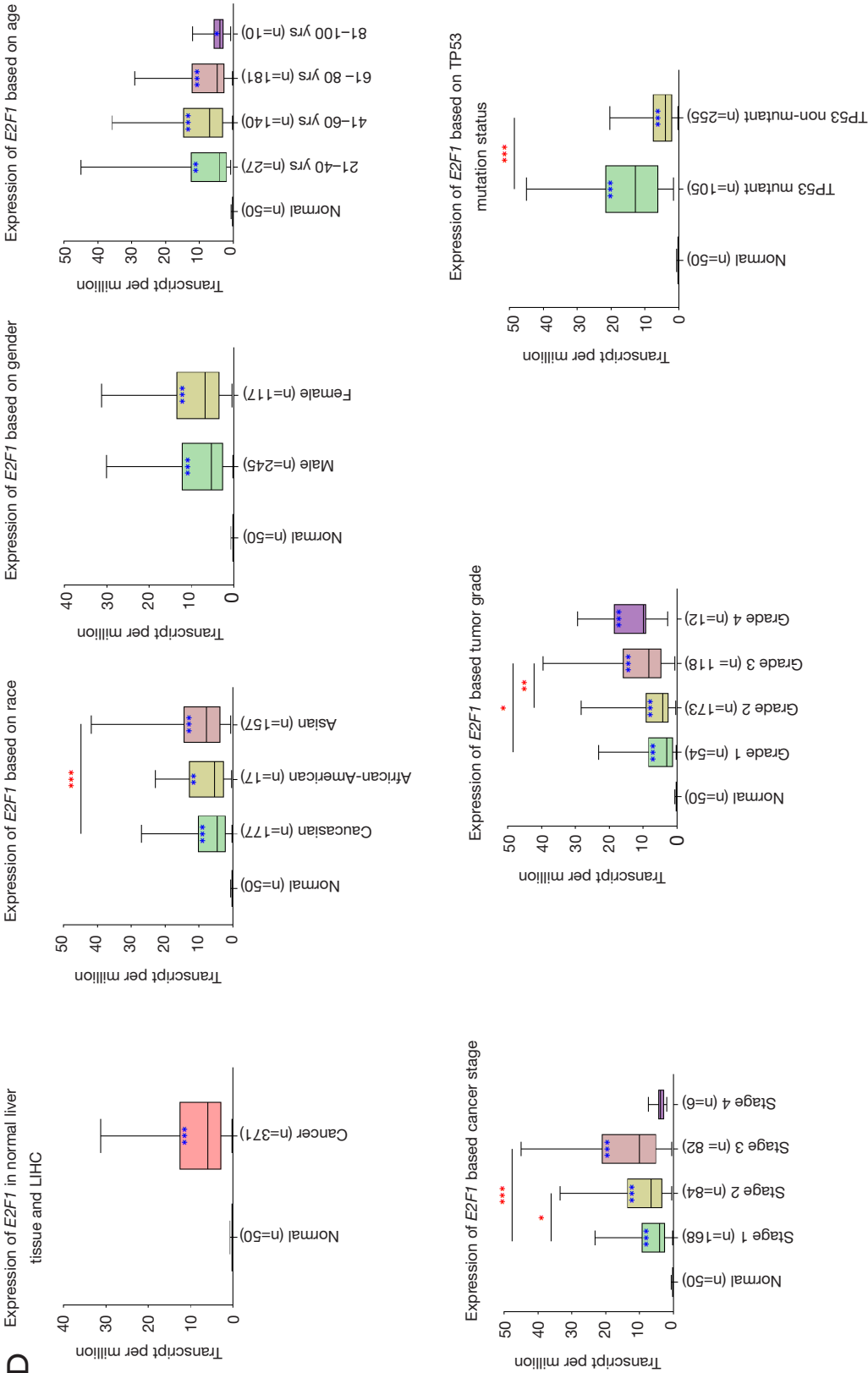


Figure 4 *E2F1* mRNA and protein expression levels in various cancers including LIHC. (A-C) *E2F1* mRNA expression levels in various cancers via Oncomine, GEPIA2, and TIMER2.0; (D) UALCAN showed *E2F1* mRNA expression levels in LIHC and corresponding normal liver tissue, associated with clinicopathological characteristics of race, gender, age, cancer stage, tumor grade, as well as *TP53* mutation status; (E) HPA database showed *E2F1* protein expression level in various cancers examined by different antibodies including HPA008003, CAB000329, and CAB019308. Blue * in box represents comparison to normal liver tissue group; red * above whisker represents comparison between indicated groups. *P<0.05, **P<0.01, ***P<0.001. LIHC, liver hepatocellular carcinoma; HPA, Human Protein Atlas.

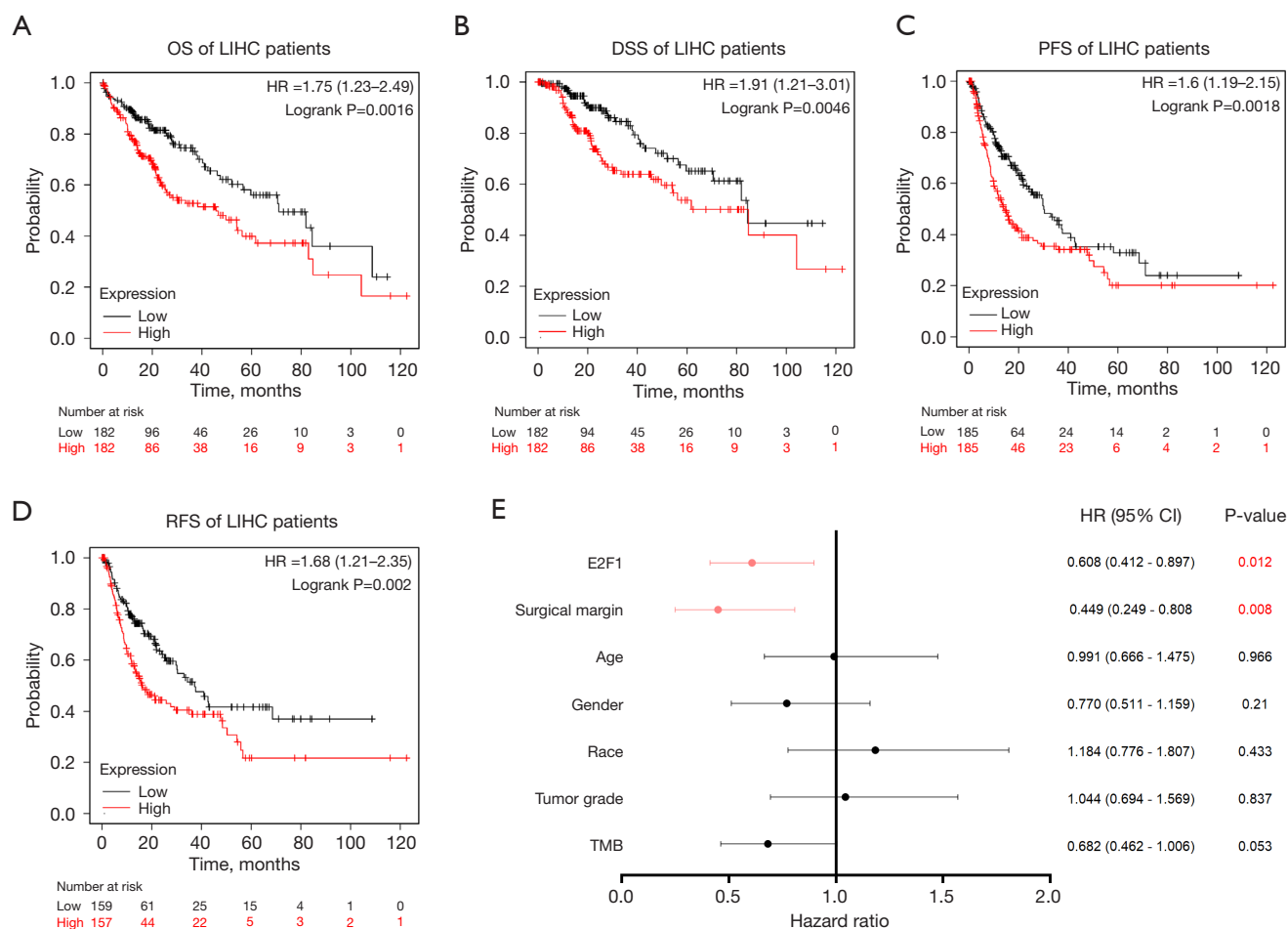


Figure 5 The relationship between *E2F1* expression level and survival. (A–D) The Kaplan-Meier survival curves for overall survival (OS), disease-specific survival (DSS), progression-free survival (PFS), relapse-free survival (RFS) respectively; (E) multivariate analyses of overall survival by Cox regression analysis. LIHC, liver hepatocellular carcinoma.

expression level in the following subgroups: male, Asian, Caucasian, stage 1, stages 1 and 2, stages 2 and 3, grade 2, no hepatitis virus infection, no alcohol consumption (see Figure S1A–S1I). In general, the results indicated that high levels of *E2F1* were associated with poor prognosis in LIHC.

In addition, 366 LIHC patients were retrieved from TCGA database. Nineteen cases were excluded due to lack of complete data. Therefore, 347 cases were subject to analysis. Multivariate Cox regression analysis indicated that *E2F1* expression level and surgical margin are independent factors influencing OS for LIHC. Specifically, low *E2F1* expression level (HR = 0.608, 95% CI: 0.412–0.897, P = 0.012) and negative surgical margin (HR = 0.449, 95% CI: 0.249–0.808, P = 0.008) indicated better overall survival.

Other factors such as age, gender, race, tumor grade, TMB (tumor mutation burden) were not related to prognosis (see Figure 5E).

E2F1 promotor methylation level in LIHC

To explore factors contributing to *E2F1* overexpression in LIHC, promotor methylation was firstly investigated from TCGA database via UALCAN web. Results showed that there was no significant difference of *E2F1* promotor methylation level between LIHC and normal liver tissue, and were generally very low (see Figure 6A). More specifically, *E2F1* promotor methylation level exhibited no statistical difference regardless of race, gender, age, stage, grade, nodal metastasis status, and TP53 mutation status.

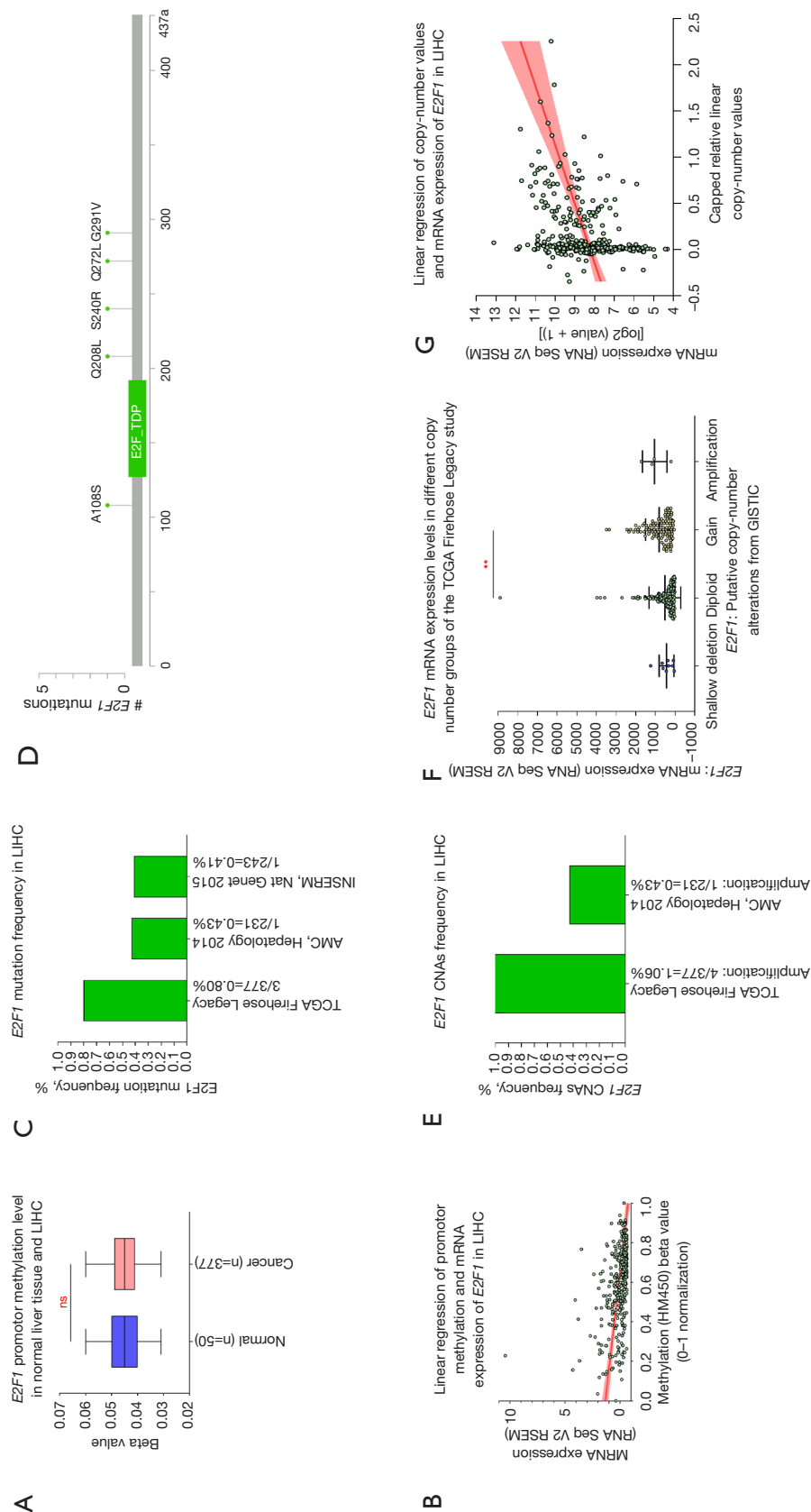


Figure 6 *E2F1* promoter methylation level, mutations and CNAs in LIHC. (A) *E2F1* promoter methylation level in LIHC and normal liver tissue. (B) The correlation between promoter methylation level and mRNA expression level of *E2F1*. (C) *E2F1* mutation frequency of 3 LIHC cohort. (D) *E2F1* mutation types. (E) *E2F1* CNAs frequency of 2 LIHC cohort. (F) *E2F1* mRNA expression level in different CNAs status groups in TCGA Firehose Legacy study. (G) The correlation between CNAs and mRNA expression level of *E2F1* in TCGA Firehose Legacy Study. ns, P>0.05; **P<0.01. CNAs, copy number alterations; LIHC, liver hepatocellular carcinoma; HPA, Human Protein Atlas.

Nevertheless, the simple linear regression showed that *E2F1* promotor methylation level could negatively affect *E2F1* mRNA expression level in LIHC with the intercept of 1.362 and the slope of -2.069 (ANOVA P value <0.001, Coefficients P value <0.001) (see Figure 6B).

Mutations and CNAs of *E2F1* in LIHC

Data indicated that *E2F1* mutation frequency is low in LIHC. Only 0.41% to 0.80% of the LIHC samples contained mutations in the *E2F1* gene (see Figure 6C). Five missense mutations were identified, including A108S, Q208L, S240R, Q272L, and G291V. All these 5 mutations were not located in the E2F/DP family winged-helix DNA-binding domain and the significance was unknown. No other mutations such as truncating mutations, in-frame mutations, splice mutations, or fusion mutations were found (see Figure 6D).

The frequency of CNAs ranged from 0.43% to 1.06% (see Figure 6E). Through analyzing 360 samples from the TCGA Firehose Legacy Study, it is indicated that *E2F1* mRNA expression level was significantly higher in gain group than that in diploid group (P value =0.005) (see Figure 6F). In spite of no statistical significance due to small sample size, amplification group also showed higher *E2F1* mRNA expression level compared to other groups (see Figure 6F). Moreover, the simple linear regression showed that relative linear copy-number values could positively affect *E2F1* mRNA expression level in LIHC with the intercept of 8.239 and the slope of 1.567 (ANOVA P value <0.001, Coefficients P value <0.001) (see Figure 6G).

Possible ceRNAs of *E2F1*

Since lncRNAs could negatively affect miRNAs which then negatively affect *E2F1* levels, those lncRNAs and miRNAs positively and negatively correlated with *E2F1* expression level respectively could be considered as ceRNAs. ENCORI showed that 8 lncRNAs are considered as possible ce-lncRNAs. Three out of 8 lncRNAs showed significantly positive correlation with *E2F1* expression, named *AC025048.4*, *AC090114.2*, and *AC092171.5* (see Figure 7A-7C). Thus, these 3 lncRNAs are supposed to be ce-lncRNAs. Twenty-six miRNAs that could be regulated by the above 3 ce-lncRNAs were found. Thirteen out of 26 miRNAs exhibited lower expression in LIHC than in normal liver tissue. Moreover, 4 out of 13 miRNAs displayed favorable OS in LIHC patients and were included into ceRNAs network, namely miR-150-5p, miR-302c-3p,

miR-520d-3p, and miR-330-5p (see Figure 7D-7G). Thus, the ceRNAs network was established (see Figure 7H).

The association between *E2F1* and tumor immune microenvironment

Through exploring single cell sequencing data via TISCH, 1 dataset (LIHC_GSE125449_aPDL1aCTLA4) including malignant cell cluster showed that *E2F1* was expressed in malignant cell cluster (see Figure 8A,8B). The other 3 datasets including various immune cell clusters showed that *E2F1* was mainly expressed in proliferating T cells (see Figure 8A,8C-8E). Gene set enrichment analysis also indicated that the hallmark of upregulated genes of proliferating T cells mainly focused on E2F targets, G2M checkpoints, mitotic spindle, and MYC targets (see Figure 8C-8E). Notably, E2F targets were almost exclusively enriched in proliferating T cells (see Figure 8C-8E). Furthermore, *E2F1* was positively correlated with specific types of immune cells, including CD8(+) T cells, CD4(+) T cells, B cells, macrophages, dendritic cells (see Figure 8F). Therefore, these findings suggest that *E2F1* could affect immune functions and thereby might influence the tumorigenesis and the development of LIHC.

Discussion

LIHC is one of the most lethal cancers. Most of LIHC patients are diagnosed at advanced stage and have poor prognosis with roughly equivalent incidence rate and mortality rate (2,45). It is indicated that some inner molecular mechanisms may play important roles leading to relatively high malignancy of LIHC. Thus, exploring molecular mechanisms is of potential significance for a deeper understanding into LIHC. Nevertheless, although studies have unveiled substantial amounts of knowledge regarding mutations, epigenetic drivers, aberrant gene-expression profiles, disrupted signaling pathways and chromosomal aberrations (46), the efficacy of treatments regarding those mechanisms remains limited, and it desperately awaits a better understanding of the cancer's molecular biology (47). Hereby, this study adopted bioinformatics to merge a great amount of data to explore the possible roles of *E2F1* in LIHC.

The reasons for choosing *E2F1* as the research objective are as follows: *E2F1* as one of hub genes in LIHC was demonstrated by this study and was verified by several other studies (48,49). Moreover, the roles of *E2F1* in

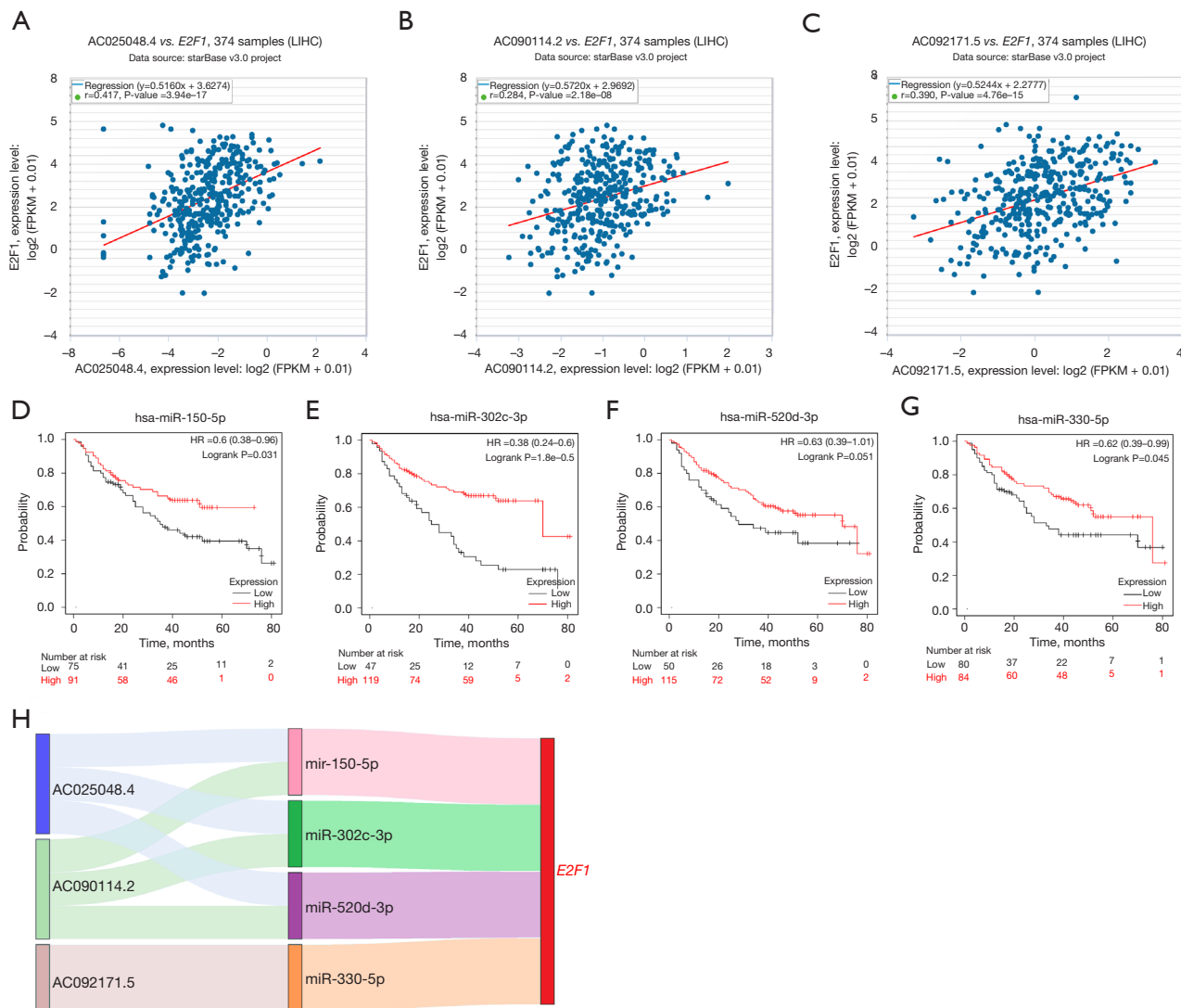
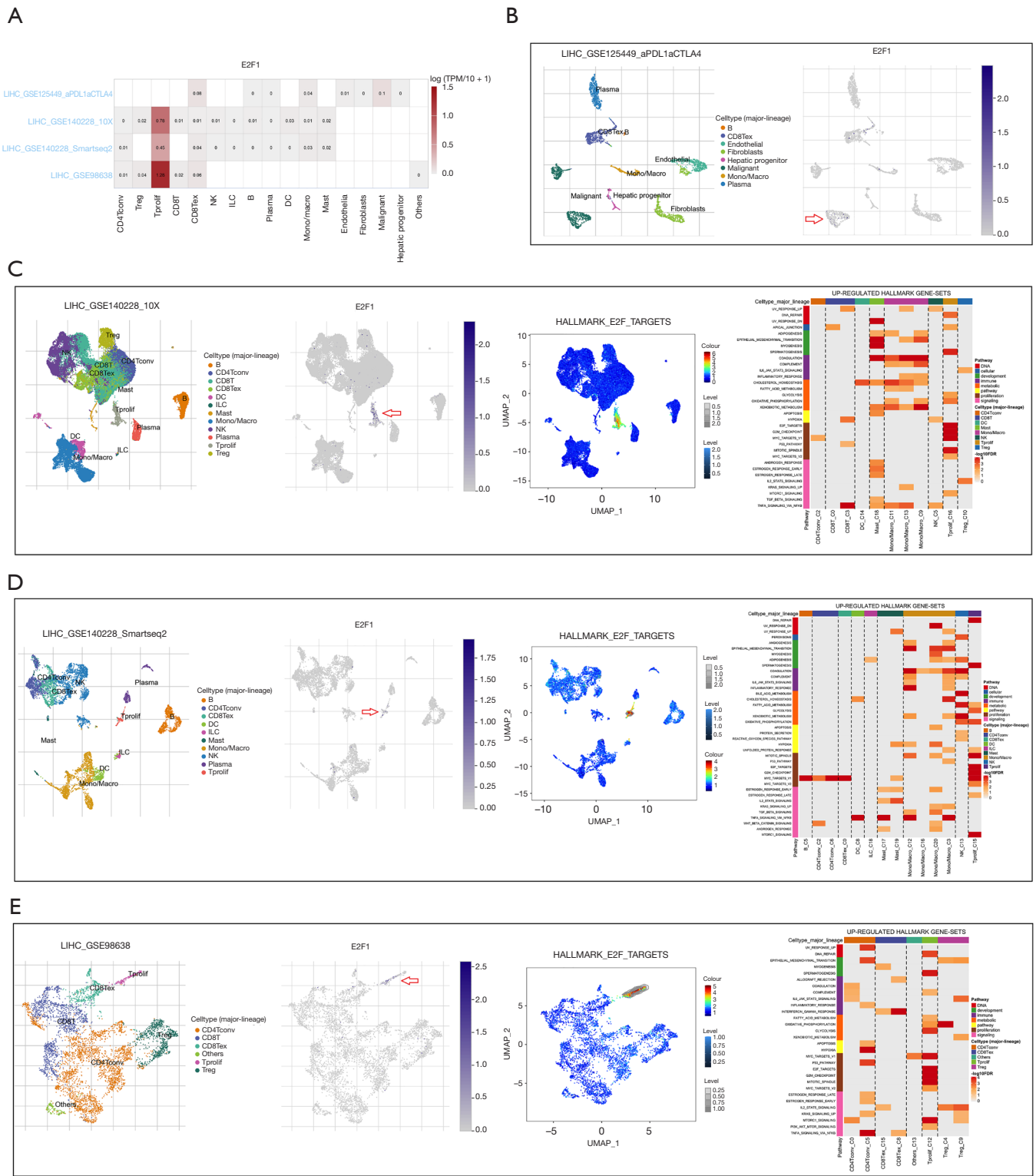


Figure 7 ceRNAs network of *E2F1*. (A–C) The correlation between expression level of *E2F1* mRNA and expression level of lncRNAs including AC025048.4, AC090114.2, and AC092171.5 respectively; (D–G) overall survival in different expression level groups of miRNAs including miR-150-5p, miR-302c-3p, miR-520d-3p, and miR-330-5p; (H) ceRNAs network of *E2F1* in LIHC. ceRNAs, competing endogenous RNAs; lncRNAs, long non-coding RNAs; miRNAs, microRNAs; LIHC, liver hepatocellular carcinoma.

tumorigenesis were investigated in other cancers. For instance, *E2F1* was overexpressed in breast cancer and facilitated stemness and tumorigenesis through binding with the promoter region of Nanog gene and promoting its transcription (50). *E2F1* mRNA expression level is related to pathological parameters and is an independently unfavorable factor for OS in clear cell renal cell carcinoma (51). Although *E2F1* is one of the most researched *E2Fs* family members (52), the exact role of *E2F1* in LIHC remains controversial and unsettled. It is

reported that *E2F1* is upregulated in LIHC and promotes the development of LIHC via targeting *MYBL2* or through being regulated by *SIRT5* (15,53). *E2F1* might also function as a critical anti-apoptotic factor by counteracting c-myc-initiated apoptosis via concomitant induction of PIK3CA/Akt/mTOR and c-Myb/COX-2 pathways (18). On the other hand, *E2F1* could inhibit hepatitis B virus (HBV) associated LIHC by suppressing HBV life cycle through interfering with the control of HBx on *p53* promoter as well as directly binding and activating *p53* promoter (54). Also, *E2F1*



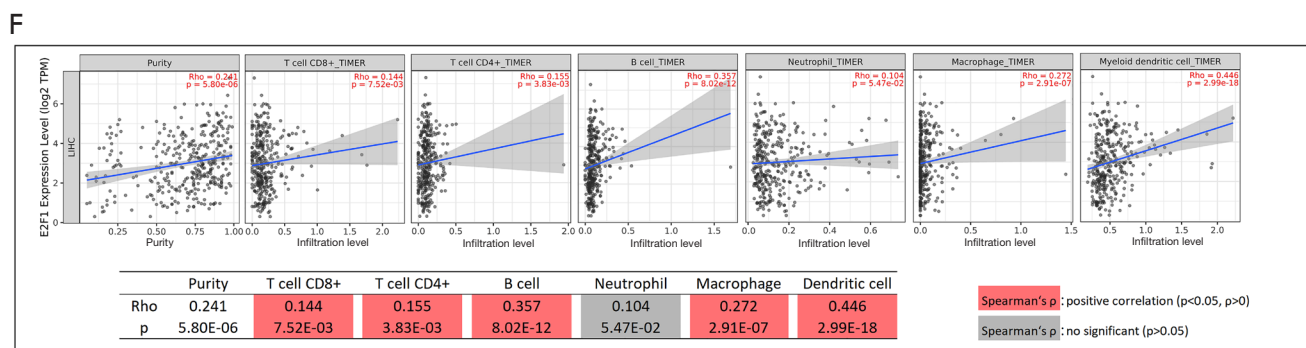


Figure 8 The association between *E2F1* and tumor immune microenvironment explored via single cell sequencing data from TISCH database. (A) The heatmap of *E2F1* expression levels in different types of cell clusters of 4 LIHC datasets; (B) *E2F1* expression pattern in the LIHC_GSE125449_aPDL1aCTLA4s dataset showed that *E2F1* expressed in malignant cell cluster; (C-E) the *E2F1* expression pattern, the enrichment of *E2F* targets, and the hallmark of upregulated genes in different cell clusters investigated from 3 datasets, namely LIHC_GSE140228_10X, LIHC_GSE140228_Smartseq2, and LIHC_GSE98638; (F) the correlations between *E2F1* expression and infiltrations of different immune cells in LIHC analyzed via TIMER2.0 database. Red arrows indicate *E2F1* expression in malignant cell cluster or proliferating T cell cluster. LIHC, liver hepatocellular carcinoma.

expression level proved to be positively related to tumor apoptotic index in LIHC (55). Since the proliferative and suppressive functions of *E2F1* in LIHC may coexist, it is worth exploring the relationship between *E2F1* and LIHC from translationally and clinically relevant viewpoints.

Hence, this study assessed the clinical significance of *E2F1* in LIHC using a variety of databases to consolidate the results. It is confirmed that *E2F1* mRNA and protein were overexpressed in LIHC regardless of clinicopathological characteristics. Moreover, those harboring more malignant features, such as advanced stage and high grade, exhibited higher *E2F1* expression level, potentially indicate that *E2F1* may serve as a vital molecule in LIHC development. Furthermore, this study revealed that *E2F1* overexpression is associated with poor OS and PFS, denoting that *E2F1* may be employed as an unfavorable prognostic factor for LIHC. These findings are, to some extent, in accordance with the standpoint of Farra *et al.* (56), proposing that the proliferative and apoptotic functions of *E2F1* in LIHC may coexist but the proliferative effect seems to be more pronounced than the apoptotic one.

Possible mechanisms contributing to the upregulation of *E2F1* were probed. Previous studies reported that *E2F1* showed methylation differences between LIHC and healthy liver tissue, as well as between LIHC and cirrhosis tissue, suggesting *E2F1* promotor methylation plays an important role in hepatocarcinogenesis (57,58). Gao *et al.* (59,60) suggested that *E2F1* could be epigenetically regulated by

PcG via EZH2-mediated H3K27me3 in LIHC. However, this study indicated promotor methylation may not be considered as a vital factor that could influence *E2F1* expression.

Gene amplification is also closely linked to the pathogenesis and anti-tumor drug sensitivity in various cancers (61). Chromosome 1q21-23 amplification has been identified as the most frequent chromosomal alteration associated with LIHC (62). Besides, CCN2/MAPK/Id-1 loop feedback amplification is involved in oxaliplatin resistance, and CCN2 or MAPK signaling inhibitor could ameliorate oxaliplatin resistance in LIHC (63). This study revealed amplification is one of the contributing factors to the overexpression of *E2F1*. Kent *et al.* have shown that copy number gains in *E2F1* or *E2F3b* resulted in dosage-dependent spontaneous LIHC in mice without the involvement of additional organs (64), suggesting that *E2F1* is a vital molecule for LIHC.

Dysregulation of ceRNAs network has been implicated in the pathogenesis of cancers like LIHC (65). An LIHC-specific lncRNA-miRNA-mRNA ceRNAs network is being expanded in recent years and is deemed as a potential prognostic marker (66). It is also found that ceRNAs could be harnessed to support LIHC treatment and improve drug sensitivity (65,67). Nonetheless, the ceRNAs network of *E2F1* in LIHC has not been well established. Here, a ceRNAs network of *E2F1* including 3 lncRNAs and 4 miRNAs was mapped out. The 3 lncRNAs

named *AC025048.4*, *AC092171.5*, and *AC090114.2* were reported to be associated with poor prognosis in other cancers such as lung adenocarcinoma, pancreatic cancer, and cholangiocarcinoma, and the first two are related to immunoregulatory pathways (68-71). The carcinostatic role of the 4 miRNAs including miR-150-5p, miR-302c-3p, miR-520d-3p, and miR-330-5p in LIHC were verified by wet-lab experiment (72-75). Besides, miR-150-5p is supposed to be one of the segments regarding sorafenib resistance in LIHC (76).

Liver is a special immune-tolerant organ that can effectively escape the immune response (77), while the LIHC neoplasm contains a large number of immune cells, forming a complex immune microenvironment (78,79). The recent years have witnessed the emerging and unprecedented progression of immunotherapy, however, low response rate and consequent drug-resistance have largely limited the efficacy of immunotherapy (80). Exploring immune microenvironment to improve the efficacy of immunotherapy is needed. By analyzing single cell sequencing data, this study revealed that *E2F1* was not only expressed in malignant cell cluster but also expressed in specific immune cell clusters. Yim *et al.* revealed that LIHC from transgenic mice expressing Myc/*E2f1* or *E2f1* exhibited poor prognosis. Moreover, 88% of LIHC from *E2f1* model were classified into high immune activity subgroup with high Pd-1 expression, which can predict the response to Pembrolizumab (81). Mechanically, one study indicated that CASC11 and *E2F1* could impact the activation of the NF- κ B signaling and PI3K/Akt/mTOR pathway and further regulate the expression PD-L1 (82). Together with our findings, those evidence suggests that *E2F1* could affect immune microenvironment and thereby might be employed as a potential therapeutic target for immunotherapy.

E2F1/E2F4 inhibitor HLM006474 has been preclinically applied to treat several kinds of cancers. Rouaud *et al.* (83) demonstrated that HLM006474 had an anti-proliferative effect on melanoma cells. Some relevant *E2F1* target genes, such as *BIRC5*, *CDC6*, or *BRCA1* were inhibited by HLM006474. Moreover, HLM006474 could induce cell cycle arrest in the G2/M phase, apoptosis via caspase-3 and p53, and senescence. Meanwhile, it proved that HLM006474 could reduce the viability of lung cancer cell lines (84) and potentially alter the progression of myelodysplastic syndrome into acute myeloid leukemia (85). Meanwhile, Sheldon (86) indicated that in breast cancer, arsenic trioxid could inhibit the dissociation of *E2F1* from

the tumor suppressor, retinoblastoma protein (pRB) due to changes in pRB phosphorylation which leads to decreased *E2F1* transcriptional activity. There are also a few studies about *E2F1* inhibitors for hepatocellular carcinoma. The small molecule BTYNB could interfere with *E2F*-driven gene expression and tumor growth in experimental mouse tumor models (87). Liu *et al.* (88) reported that hepatocellular carcinoma tissues with embryonic stem cell-like signature were sensitive to HLM006474. Although preliminary data strengthen the hypothesis that *E2F* inhibitors could be useful as a new therapeutic approach for hepatocellular carcinoma, there is still a long way to go prior to clinical application.

Notably, one limitation of this study is that only bioinformatic methods were adopted to integrate and analyze numerous data. Further wet-lab verification is needed to make the findings more solid.

Conclusions

Bioinformatic analysis indicated that, as a hub gene, *E2F1* was overexpressed in LIHC and related to poor prognosis. Amplification and ceRNAs regulation may contribute to the overexpression of *E2F1*. *E2F1* could affect immune microenvironment. Together, this study indicated that *E2F1* is one of the key genes and could be used as a potential prognostic biomarker and therapeutic target for LIHC.

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Footnote

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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).

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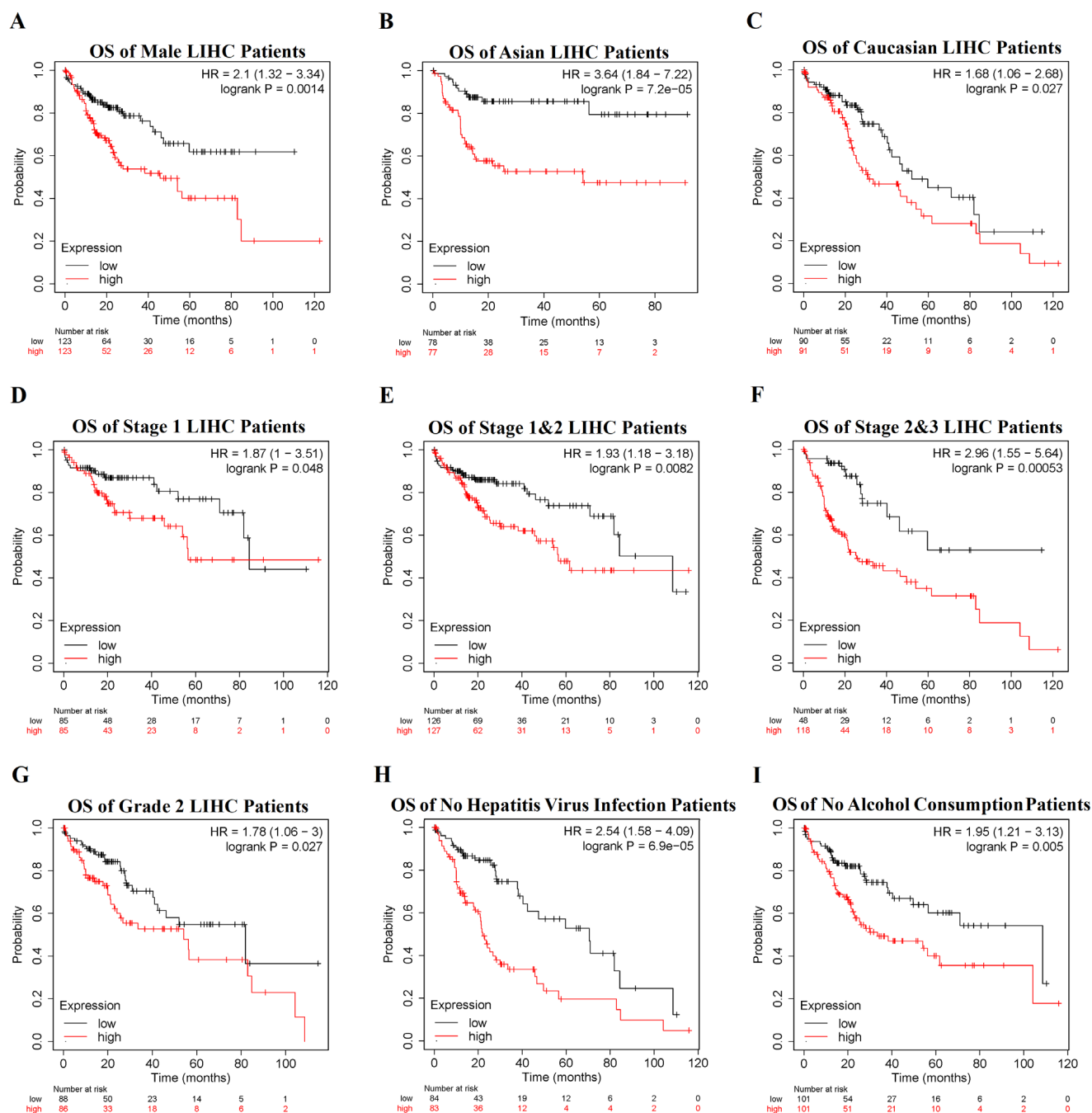


Figure S1 The relationship between E2F1 expression and survival in subgroups of LIHC. (A-I) The Kaplan-Meier overall survival curves in subgroups of male, Asian, Caucasian, cancer stage 1, cancer stage 1 & 2, cancer stage 2 & 3, tumor grade 2, No hepatitis virus infection, No alcohol consumption.