



# Bioinformatic-based mechanism identification of *E2F1*-related ceRNA and *E2F1* immunoassays in hepatocellular carcinoma

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**Background:** *E2F1* is an important transcription factor. Previous studies have shown that the overexpression of *E2F1* is closely related to the occurrence and development of hepatocellular carcinoma (HCC). However, the current research on the regulatory mechanism of *E2F1* is still insufficient. This study sought to identify valuable therapeutic *E2F1*-related targets for HCC.

**Methods:** HCC-related transcriptome data and patient clinical information downloaded from The Cancer Genome Atlas (TCGA) database. The expression of the *E2F1* gene in pan-cancer was analyzed using the Tumor Immune Estimation Resource (TIMER) 2.0 database, and the expression level of *E2F1* in HCC was verified using the Gene Expression Profiling Interactive Analysis database. The overall survival (OS) and progression-free survival (PFS) in HCC patients were also analyzed. Subsequently, based on the Encyclopedia of RNA Interactomes (ENCORI) database, we adopted *E2F1* as the research objective and identified the target long non-coding RNAs (lncRNAs) and microRNAs that suggested the competing endogenous RNA (ceRNA) mechanisms related to *E2F1*. We also performed a correlation analysis of *E2F1* using the R language package that contained immune cell and immune checkpoint information. Finally, the drug sensitivity of *E2F1* was detected using the R language package, “pRRophetic.”

**Results:** Ultimately, the following 6 potential ceRNA-based pathways targeting *E2F1* were identified—lncRNA: *LINC01224*, *PCBP1-AS1*, and *ITGA9-AS1-miR-29b-3p-E2F1*; lncRNA: *SNHG7* and *THUMPD3-AS1*, and *LINC02323-miR-29c-3p-E2F1*. Cluster of differentiation (CD)4 memory activated T cells, memory B cells, eosinophils, and T follicular helper cells were positively correlated with *E2F1* ( $P < 0.05$ ), and monocytes, naïve B cells, and CD4 memory resting T cells were negatively correlated with *E2F1* ( $P < 0.05$ ). The immune checkpoint analysis showed that *E2F1* was positively correlated with *PDCD1*, *CTLA4*, and *LAG3* ( $P > 0.2$ ). According to the drug sensitivity analysis, *E2F1* may be sensitive to 39 drugs ( $P < 0.05$ ).

**Conclusions:** This study provides a valuable direction for researching transcription factor *E2F1*, which may be conducive in identifying research targets for HCC-related molecular biological therapy and immunotherapy in future.

**Keywords:** Competing endogenous RNA (ceRNA); *E2F1*; bioinformatics; hepatocellular carcinoma (HCC); immunoassay

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## Introduction

Hepatocellular carcinoma (HCC) accounts for about 80% of primary liver cancer cases (1). The major pathogenic causes of HCC include hepatitis [e.g., hepatitis B virus (HBV) and hepatitis C virus (HCV)] alcoholism, smoking, obesity, and congenital inheritance. In the United States, the latest cancer statistics showed that the number of deaths from liver cancer reached 30,230 in 2021 (2). HCV infection is the leading cause of liver cancer in Western countries and causes approximately 1/4 of all the HCC cases. In developing countries (e.g., China), HBV infection is the predominant cause of liver cancer (3). Compared to other cancers, the prognosis of liver cancer is still relatively poor, and it has a 5-year survival rate of only 20% (4). Thus, research urgently needs to be conducted to identify effective biological targets related to liver cancer, especially HCC.

Most long non-coding RNAs (lncRNAs) do not encode proteins (5), and have even been considered junk DNA; however, in-depth research on non-coding RNAs have revealed that many lncRNAs regulate gene expression during or after transcriptional processes. LncRNAs affect a series of pathological and physiological processes by participating in the biological regulation, such as chromosome imprinting, epigenetic regulation, cell proliferation and cell cycle (6,7). Under the recently proposed potential competing endogenous RNA (ceRNA) theory, lncRNA competes to occupy a large number of micro RNAs (miRNAs) in the cell and acts like a sponge to buffer and interfere with the protein encoded by the target gene messenger RNA (mRNA) (8). This kind of mechanism also provides a good entry point for researchers to explore the mechanism of tumorigenesis and development and find effective tumor therapy targets.

*E2F1* is an important transcription factor involved in multiple steps, including DNA damage response and cell-cycle regulation (9). Previous studies have shown that the overexpression of *E2F1* is closely related to the occurrence and development of various malignant tumors, including HCC (10-12). The abnormal activation of *E2F1* affects its downstream transcriptional targets, resulting in DNA replication stress (13). The above mechanisms play an important role in the occurrence and development of liver cancer. At present, there is still a lot of room for exploration on the regulatory mechanism upstream of *E2F1*. By finding out the effective regulatory mechanism related to *E2F1*, and then inhibiting the expression of *E2F1*, it is helpful to finally achieve the purpose of inhibiting the development of

HCC. As our current understanding of *E2F1* is insufficient, we sought to study the mechanism and related regulation of *E2F1* in HCC. Thus, based on bioinformatics, we adopted the ceRNA mechanism as an entry point to analyze the lncRNAs and miRNAs related to *E2F1* and explore the correlation between *E2F1* and immune infiltration levels of various types of immune cells in HCC to identify potential biological targets and to prepare for subsequent basic research. We present the following article in accordance with the TRIPOD reporting checklist (available at <https://jgo.amegroups.com/article/view/10.21037/jgo-22-674/rc>).

## Methods

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This research is divided into the following parts. First, we preliminarily verified that *E2F1* is significantly overexpressed in malignant tumors including HCC through the TCGA database. Kaplan-Meier analysis was used to discuss the relationship between *E2F1* expression and survival. Second, a retrospective analysis of 364 patients was performed to explore the relationship between *E2F1* and clinicopathological parameters. The specificity and sensitivity of *E2F1* as a prognostic indicator were also evaluated. Second, a retrospective analysis of 364 patients was performed to explore the relationship between *E2F1* and clinicopathological parameters. The specificity and sensitivity of *E2F1* as a prognostic indicator were also evaluated. Third, bioinformatics analysis was used to explore potential ceRNA mechanisms, and a total of 6 potential *E2F1*-related signaling pathways were screened. Then, the relationship between *E2F1* and tumor-related immunity was explored using the Tumor IMmune Estimation Resource (TIMER) database. Finally, the drug sensitivity of *E2F1* as a therapeutic target in HCC was explored.

## Differential expression and survival analyses of *E2F1*

HCC-related transcriptome data and patient clinical information downloaded from The Cancer Genome Atlas (TCGA) database (<https://portal.gdc.cancer.gov/>). A differential expression map of *E2F1* in pan-cancer was obtained from the TIMER2.0 database (<http://timer.cistrome.org/>). Based on the magnitude of the P value of *E2F1* for different cancer types, the P value was divided into  $P < 0.001$ ,  $P < 0.01$ , and  $P < 0.05$ . The differential expression analysis of *E2F1* in HCC was analyzed and plotted using the “limma”, “ggplot2”, and “ggpubr” packages. Prognosis-

related survival curves were downloaded from the Gene Expression Profiling Interactive Analysis (GEPIA) database (<http://gepia.cancer-pku.cn>).

### *Analysis of clinical prognostic factors*

The correlation between *E2F1* expression and each prognostic factor was analyzed and plotted using the “limma” and “ggpubr” packages. Heatmaps for each clinical prognostic factor were drawn using the “limma” and “ComplexHeatmap” packages. The receiver operator characteristic (ROC) curves, calibration curves, and nomograms were made using the “survival,” “survminer,” “timeROC,” “regplot,” and “rms” analysis packages. Univariate and multivariate Cox analyses were conducted, and forest plots were generated using the “survival” package.

### *Establishment of mRNA-miRNA-lncRNA co-expression network and survival analysis of miRNA and lncRNA*

The mRNA-miRNA and miRNA-LncRNA interaction data were downloaded from the starBase database (<http://starbase.sysu.edu.cn/>), with a programNum  $\geq 2$  as one of the mRNA-miRNA screening criteria. The correlation coefficient values (an R value  $>0.2$  was defined as a positive correlation, and an R value  $<-0.2$  was defined as negative correlation), differential expression values (a P value  $<0.01$  was considered statistically significant), and survival curve values (a P value  $<0.05$  was considered statistically significant) were screened out and plotted using the R language package. A conceptual diagram of the potential ceRNA mechanisms associated with *E2F1* was drawn with BioRender.

### *E2F1 immune correlation analysis*

The correlation analyses between *E2F1* and various immune cells and immune checkpoints were visualized using various R language packages, including “limma,” “reshape2,” “ggplot2,” “ggpubr,” “vioplot,” “ggExtra,” and “corrplot.” The p values were calculated using the Spearman statistical method. A positive correlation was defined as a P value  $<0.05$ , an R value  $>0.2$ , a negative correlation was defined as a P value  $<0.05$ , an R value  $<-0.2$ , and a P value  $>0.05$  was defined as not significant. *E2F1*, PDCD1, CD274, CTLA4, and LAG3 were analyzed using the TIMER 2.0 database (<http://timer.cistrome.org/>).

### *E2F1-related drug sensitivity evaluation*

half maximal inhibitory concentration (IC<sub>50</sub>) represents the concentration required for the 50% inhibition of drug concentration. We calculated the IC<sub>50</sub> of drugs using the “pRRophetic” R package with its dependencies “car, ridge preprocessCore, genefilter, and sva,” which contained information on the effects of 138 drugs. Boxplots were drawn using the “ggplot2” R package. A P value  $<0.05$  indicated a statistically significant difference.

### *Statistical analysis*

Wilcoxon rank-sum test was used to compare the difference between the two groups. Differential expression data were analyzed by “DESeq2” and “survival” R software. KM survival analysis was used for ROC curve analysis, univariate and multivariate Cox regression analysis. Spearman’s test was used to measure correlations between *E2F1* and immune functions. And P value  $<0.05$  was regarded as the significant threshold.

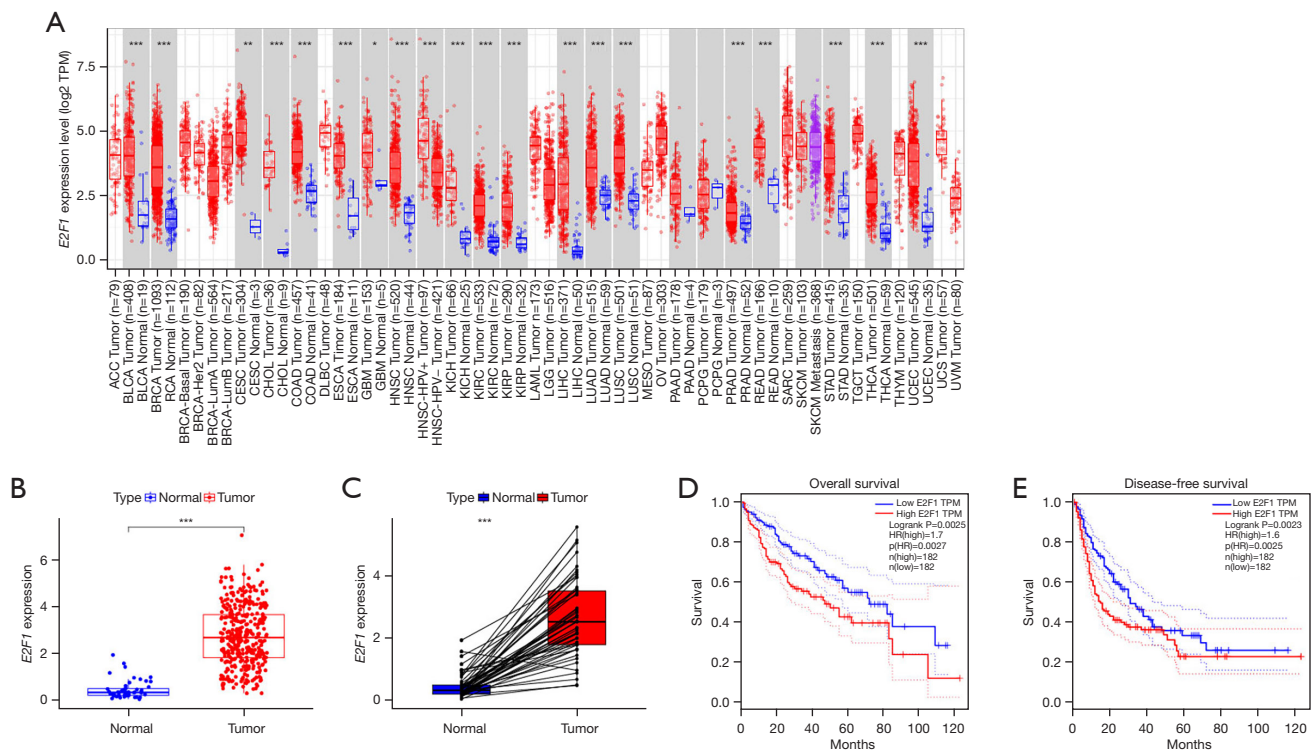
## **Results**

### *Differential expression and survival analyses of E2F1 in HCC*

We analyzed the expression of *E2F1* in 38 cancer types in the TIMER2.0 database and found that *E2F1* was significantly differentially expressed between the tumor group and the normal group in terms of 20 malignant tumors, including HCC (P $<0.001$ ; see *Figure 1A*). We downloaded the HCC-related transcriptome data and patient clinical information downloaded from TCGA database, and found that *E2F1* was significantly differentially expressed in malignant tumors (P $<0.01$ ; see *Figures 1B,1C*). We also searched *E2F1*-related disease-free survival (DFS) and overall survival (OS) of HCC patients in the GEPIA database and found that there were significant differences in the prognosis of the high-risk group (182 cases) and the low-risk group (182 cases) (DFS: P=0.0027, OS: P=0.0025), and the prognosis of the low-risk group was significantly better than that of the high-risk group in terms of both OS (see *Figure 1D*) and DFS (see *Figure 1E*).

### *Analysis of prognostic factors of E2F1 in HCC*

Subsequently, we analyzed the key prognostic factors related to *E2F1* and found that there was a significant difference



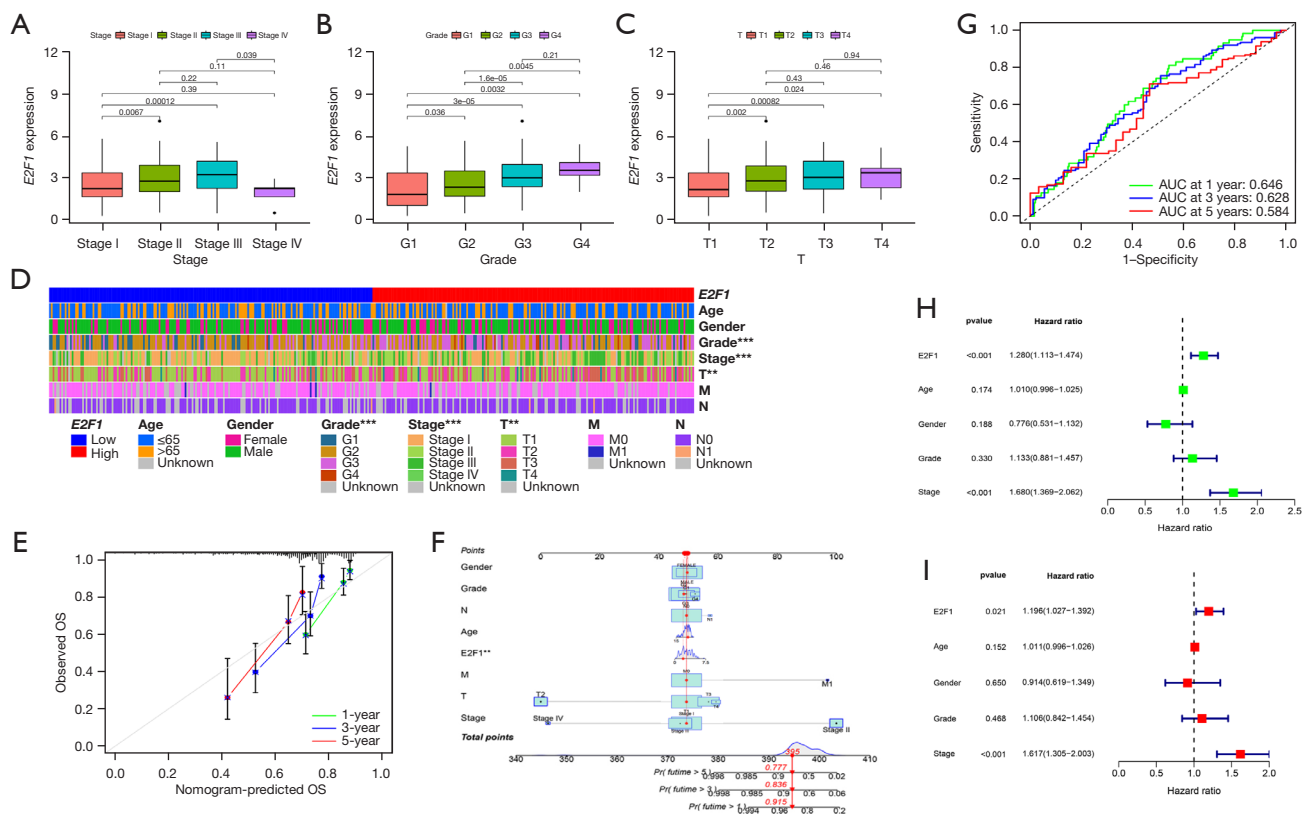
**Figure 1** Differential expression and prognostic survival curve of *E2F1* (A) Differential expression of *E2F1* in HCC from the TIMER2.0 database; (B) differential expression of *E2F1* between the tumor group and the normal group in HCC; (C) pairwise differential analysis of *E2F1* in HCC (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ); (D) overall survival curve from the GEPIA database; (E) disease-free survival curve from the GEPIA database. HCC, hepatocellular carcinoma; TIMER, Tumor Immune Estimation Resource; GEPIA, Gene Expression Profiling Interactive Analysis.

between stage I, stage II, and stage III, and between stage III and IV ( $P < 0.01$ ; see *Figure 2A*). Significant differences were found among all the grades, except grade 3 and grade 4 (see *Figure 2B*). The expression of *E2F1* differed significantly between T1 and T2, T3, and T4 ( $P < 0.01$ ; see *Figure 2C*), and there were significant differences in tumor (T) stage, stage, and grade in the high- and low-expression groups ( $P < 0.001$ ; see *Figure 2D*). In the sensitivity and specificity analyses, the ROC curve of the target gene *E2F1* showed that the areas under the curve (AUCs) at 1, 3, and 5 years were 0.646, 0.628, and 0.584, respectively (see *Figure 2E*). A nomogram was drawn to assess whether *E2F1* could predict survival time in HCC (see *Figure 2F*), and the feasibility of this prediction method was validated with a calibration curve (see *Figure 2G*). Finally, we concluded that *E2F1* expression and HCC stage were independent risk factors for prognosis through univariate and multivariate Cox regression analyses (see *Figure 2H, I*).

### Establishment of mRNA-miRNA co-expression network and related miRNA survival analysis

After downloading the *E2F1*-miRNA interaction data from the starBase database and using the R language package for the analysis, we screened 2 groups of miRNAs that were co-expressed and negatively correlated with *E2F1* (i.e., had a correlation coefficient <value  $-0.2$ , and a P value  $< 0.001$ ) in preparation for the subsequent screening of potential targets and pathways. The subsequent analysis showed that the 2 groups of miRNAs were differentially expressed in the normal group and the tumor group ( $P < 0.001$ ), and the above-mentioned miRNA-related prognosis survival curve analysis showed that the high-expression group had a better result than the low-expression group ( $P < 0.05$ ; see *Figure 3A, 3B*).

Next, we downloaded the lncRNA data that interacted with *miR-29b-3p* and *miR-29c-3p* from the starBase database, and screened and analyzed the correlation coefficients (those



**Figure 2** *E2F1* prognostic factors (A) stage; (B) grade; (C) T stage; (D) heat map; *E2F1* was found to be correlated with diagnosis and prognosis; (E) calibration curve (OS); (F) nomogram; (G) the predictive effect of *E2F1* (AUC); (H) univariate Cox regression analysis of prognosis-related risk factors; and (I) multivariate Cox regression analysis of prognosis-related risk factors. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . OS, overall survival; AUC, area under curve.

with a correlation coefficient value  $>0.2$ ), log fold change (FC) values (those with a log FC value  $>0$ ), survival curves, and the differential expression between the tumor group and the normal group ( $P < 0.01$ ) by R language. We also selected lncRNAs whose expression levels were positively correlated with *E2F1* according to the above screening results (those with a correlation coefficient value  $>0.2$ , and a  $P$  value  $< 0.01$ ). Finally, the *miR-29b-3p*-related lncRNAs (i.e., *LINC01224*, *PCBP1-AS1*, and *ITGA9-AS1*), and the *miR-29c-3p*-related lncRNAs (i.e., *SNHG7*, *THUMP3-AS1*, and *LINC02323*) were screened (see Figure 4A,4B). Based on the above results, the possible potential ceRNA mechanism diagram for *E2F1* was constructed (see Figure 5).

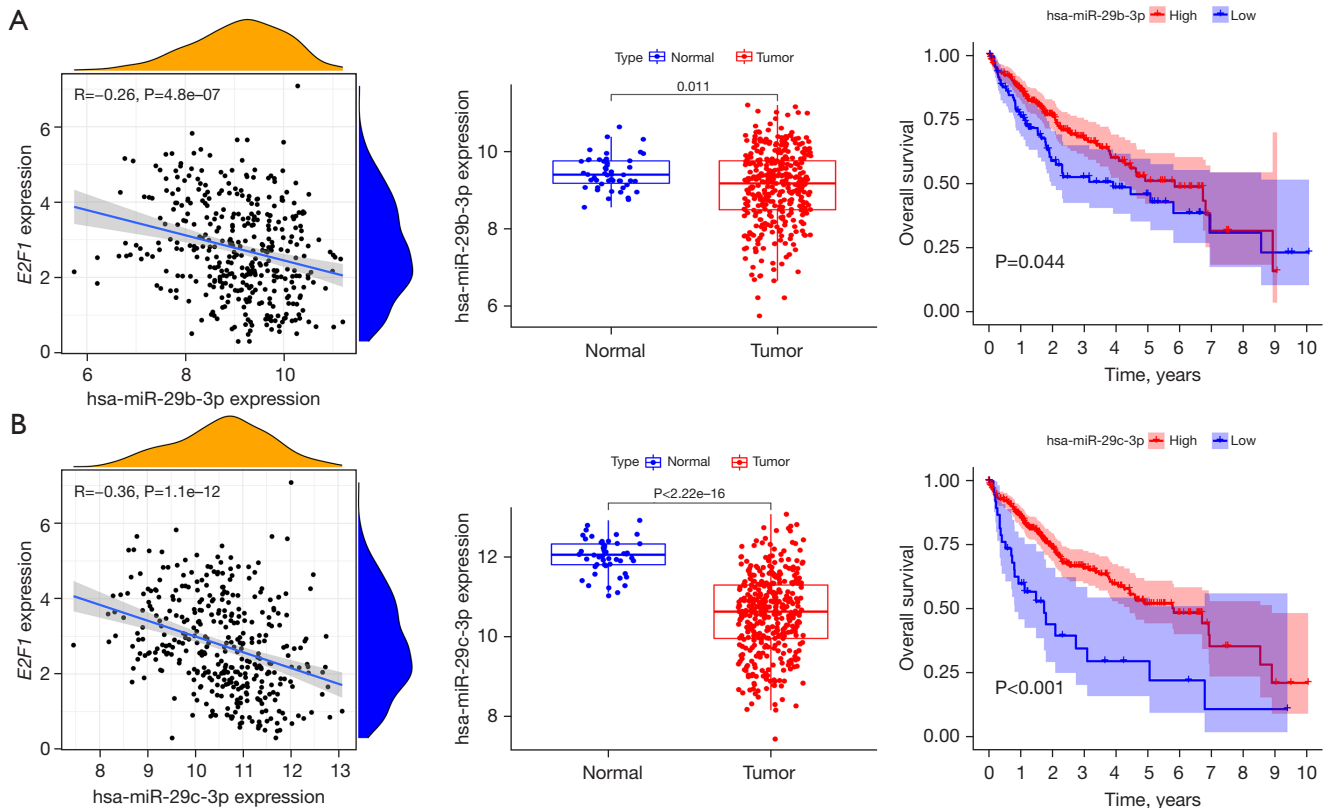
### Correlation analyses of *E2F1* with various immune cells and immune checkpoints

Additionally, we analyzed the correlations between immune

cells and the levels of immune infiltration for *E2F1*, and found that cluster of differentiation (CD)4 memory activated T cells, memory B cells, eosinophils, and follicular helper T cells were positively correlated with *E2F1* ( $R > 0.2$ ,  $P < 0.01$ ), and monocytes, naïve B cells, and CD4 memory resting T cells were negatively correlated with *E2F1* ( $R < -0.2$ ,  $P < 0.01$ ; see Figure 6A,6B). The TIMER database-related immune checkpoint analysis showed that *E2F1* was positively correlated with *PDCD1*, *CTLA4*, and *LAG3* ( $R > 0.2$ ,  $P < 0.01$ ; see Figure 6C,6D).

### Drug sensitivity evaluation

We examined the relationship between the risk score and the IC50 of various drugs used in the clinical treatment of HCC, including imatinib, etoposide, and paclitaxel. Patients in the high-expression group appeared to be more susceptible to most drugs than those in the low-expression



**Figure 3** Correlation, difference, and survival curve analyses between *E2F1* and 2 miRNAs. (A) miR-29b-3p; (B) miR-29c-3p.

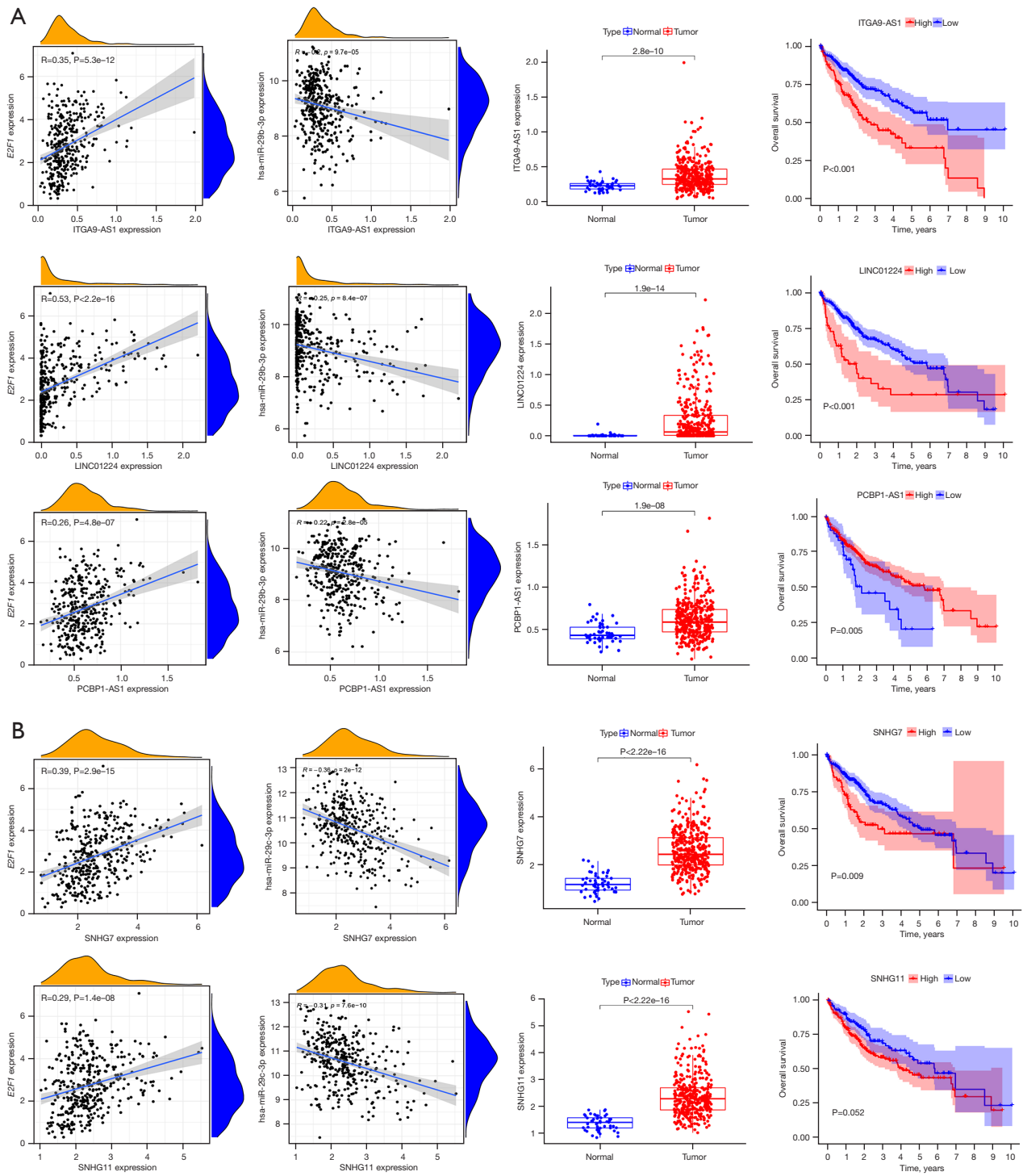
group ( $P < 0.001$ ; see *Figure 7*).

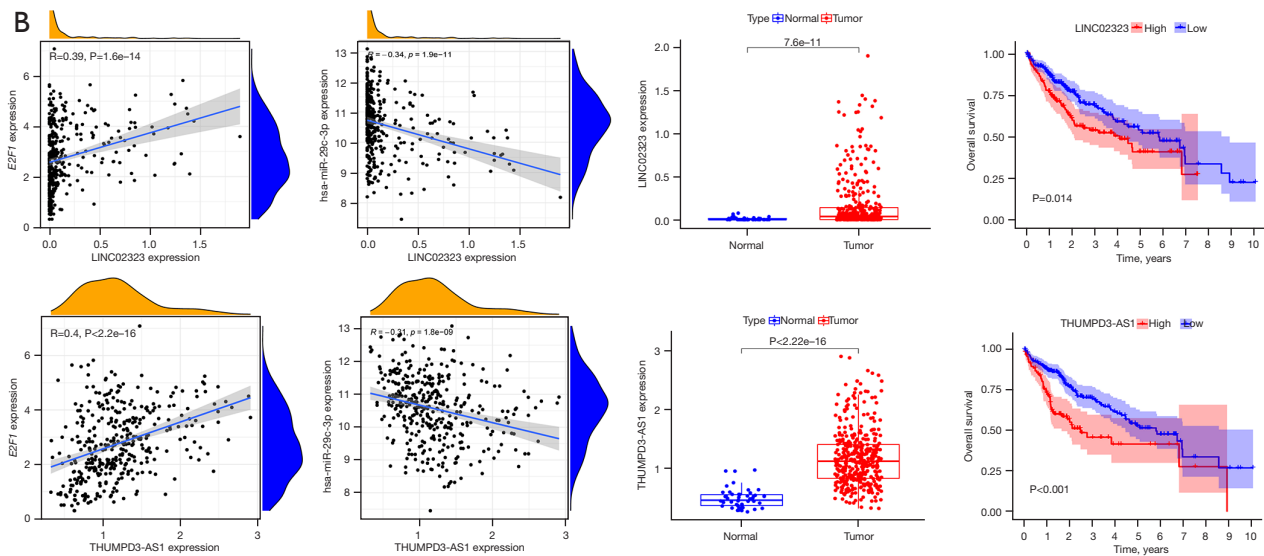
## Discussion

*E2F1* was the first member of the *E2F* transcription factor family, which comprises 8 proteins, to be discovered (14). Based on their different functions, they are usually classified as activators (*E2F1-e2f3a*) or inhibitors (*E2F3b-E2F8*) (15). Studies have shown that *E2F1* mainly regulates the transcription of S-phase cyclins and related genes required for DNA replication, DNA repair, and apoptosis (16). At present, the common genes that cause the abnormal activation of *E2F1* mainly include retinoblastoma (*Rb*), *Ras*, and *PI3K*. The abnormal activation of *E2F1* affects its downstream transcriptional targets, resulting in DNA replication stress (16). Its transcriptional targets include cyclin E and *RRM2*. Cyclin E promotes the phosphorylation of essential DNA replication factors to initiate and allow the progression of bidirectional DNA synthesis. Cyclin E overexpression results in enhanced *CDK2* activity and cell cycle progression, thereby reducing the ability of cells

to regulate the G1 (DNA prophase)-S (DNA replication period) transition (17). This regulatory mechanism has been widely observed in a number of malignancies (17-19). In addition, another important transcriptional target of *E2F1* that could contribute to DNA replication stress is *RRM2* (20), and the above signaling pathway of *E2F1* has been reported in adrenocortical carcinoma (21), colorectal cancer (22), pancreas cancer (23), and other malignant tumors.

Using the TIMER database, we sought to identify the immune cells correlated with *E2F1* in terms of the level of immune infiltration in HCC. We found that the expression of *E2F1* was positively correlated with CD4 memory activated T cells, memory B cells, eosinophils, and follicular helper T cells, and negatively correlated with monocytes, naïve B cells, and CD4 memory resting T cells. Studies have shown that HTLV-1 basic leucine zipper factor (HBZ) is a related viral factor required for the viral replication and transformation of infected cells. HBZ protein interacts with the *Rb/E2F-1* complex and induces the transcription of *E2F* target genes. The activation of the *Rb/E2F* pathway by the HBZ protein accelerates G1/S transition and apoptosis in

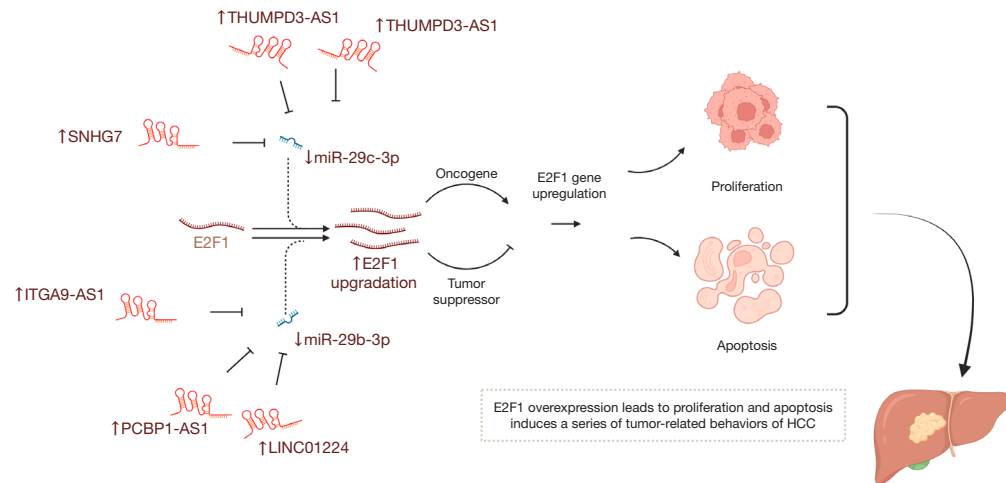




**Figure 4** Correlation, difference, and survival curve analyses between miR-29b-3p-*E2F1*-related lncRNA and miRNA and *E2F1*, respectively (A). (B) Correlation, difference, and survival curve analyses between miR-29c-3p-*E2F1*-related lncRNA and miRNA and *E2F1*, respectively.

***E2F1* related lncRNA-miRNA Gene Expression Regulation in HCC**

Diagram of *E2F1*-related ceRNA mechanism (hypothesis)



**Figure 5** Conceptual diagram of the *E2F1*-related ceRNA mechanism in HCC. HCC, hepatocellular carcinoma.

primary CD4<sup>+</sup> T cells (24). The downregulation of *E2F1* decreases the susceptibility of CD8<sup>+</sup> T cells. *E2F1* has been shown to be a transcription factor for *TBX21*, a Th1 cell-specific transcription factor that controls the expression of the hallmark Th1 cytokine and interferon gamma (IFN- $\gamma$ ) (25). Thus, *E2F1* plays an important role in tumor immunity by affecting the activation of effector CD8<sup>+</sup> T cells (26).

*E2F1* also significantly represses the transcriptional activity of the interleukin (IL)-6 promoter, while the overexpression of *E2F1* promotes this activity. *E2F1* regulates macrophage cytokine expression via IL-6 in nasopharyngeal carcinoma (NPC) cell supernatants, which supports its utility in the tumor microenvironment (TME). In a xenograft tumorigenesis model, small interfering-





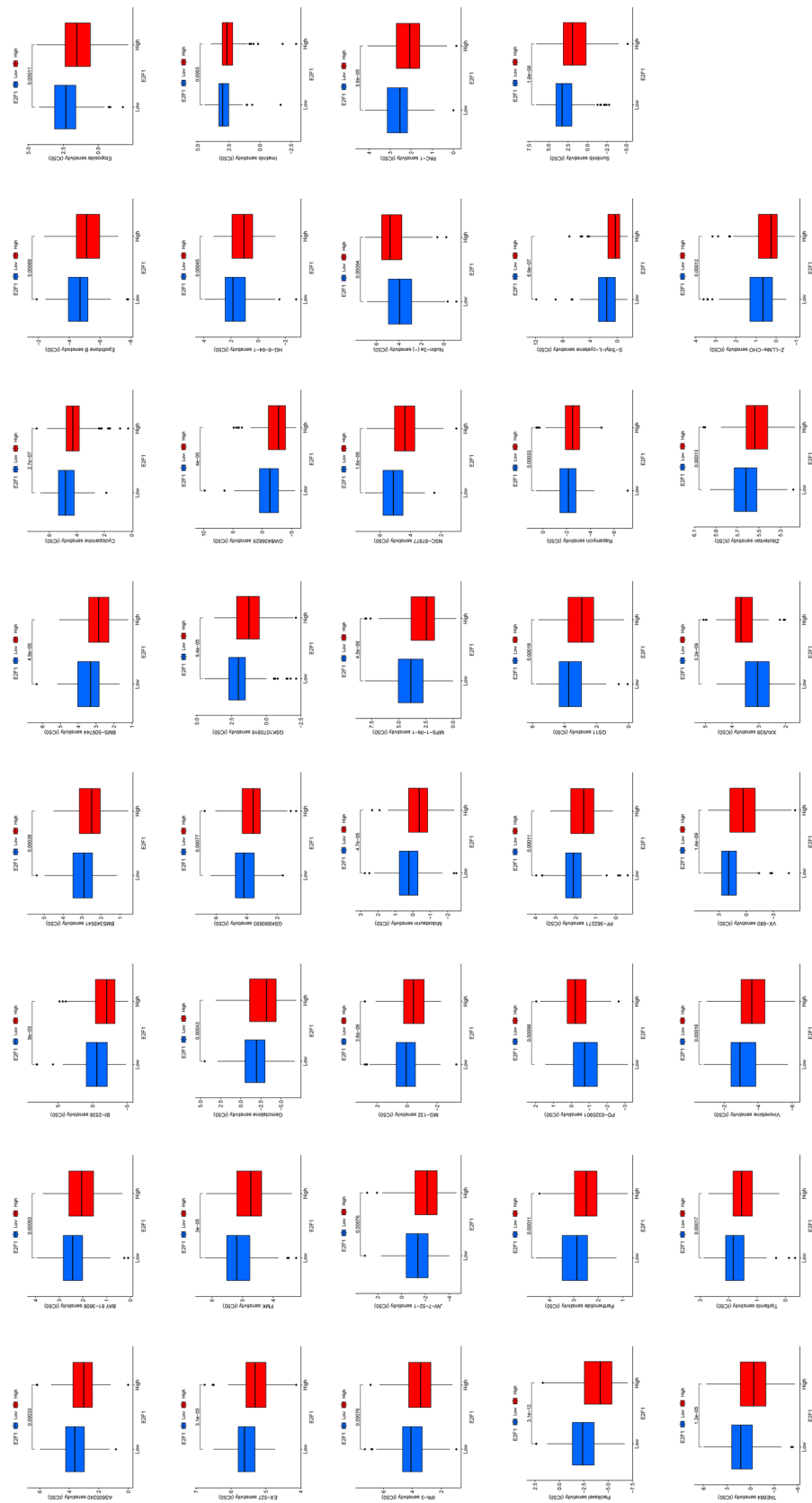


Figure 7 The IC50 of 39 drugs in *E2F1* high- and low-expression groups (P<0.001).

based on a bioinformatics analysis; thus, the validity of the findings needs to be further verified by basic experimental research. However, our results still provide a very valuable direction and reference for research on transcription factor *E2F1*, which may be helpful in identifying research targets for future HCC-related molecular biological therapy and immunotherapy.

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### Footnote

**Reporting Checklist:** Both authors have completed the TRIPOD reporting checklist. Available at <https://jgo.amegroups.com/article/view/10.21037/jgo-22-674/rc>

**Conflicts of Interest:** Both authors have completed the ICMJE uniform disclosure form (available at <https://jgo.amegroups.com/article/view/10.21037/jgo-22-674/coif>). Both authors report this study was supported by the Fundamental Research Funds for the Provincial Universities (No. 2018-KYYWF-0534). Both authors have no other conflicts of interest to declare.

**Ethical Statement:** The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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