Original Article

E2F-mediated POLA2 upregulation is correlated with macrophage infiltration and poor prognosis in hepatocellular carcinoma

Yao Teng[1]^, Ran Liu[2]^

[1]Department of Rheumatology and Immunology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China; [2]Department of Hepatobiliary Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

Contributions: (I) Conception and design: Both authors; (II) Administrative support: R Liu; (III) Provision of study materials or patients: Y Teng; (IV) Collection and assembly of data: Y Teng; (V) Data analysis and interpretation: Y Teng; (VI) Manuscript writing: Both authors; (VII) Final approval of manuscript: Both authors.

Correspondence to: Ran Liu, MD. Department of Hepatobiliary Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1277 Jiefang Avenue, Wuhan 430022, China. Email: 1210745343@qq.com.

Background: Hepatocellular carcinoma (HCC) is the major histological type of primary liver cancer with a relatively poor prognosis, because most HCC cases are usually diagnosed at an advanced stage. Thus, it is essential to explore novel candidate biomarkers for early diagnosis and prognosis predication of HCC.

Methods: HCC transcription sequencing data were downloaded and analyzed. POLA2 expression pattern was characterized in tumor development process. POLA2 expression was evaluated by western blotting. Kaplan-Meier plot was utilized to evaluate POLA2's ability for prognosis predication. Correlation analysis and enrichment analysis were performed to explore the functional role of POLA2 in HCC development. Knockdown of E2F1 or E2F4 was used to evaluate their ability in regulating POLA2 expression. CYBERSORT (https://cibersortx.stanford.edu/) was used to estimate the infiltration levels of immune cells.

Results: POLA2 was overexpressed in HCC. Moreover, western blotting results showed that POLA2 was upregulated by transcription factors E2F1 rather than E2F4 in HCC. POLA2 facilitated cell cycle progression, DNA replication and DNA repair. High POLA2 expression was correlated with poor overall survival in HCC patients. POLA2 induced macrophage infiltration in the tumor microenvironment by upregulating the expression CSF1 and VEGFA expression.

Conclusions: POLA2 is a novel diagnostic and prognostic biomarker of HCC with potential clinical value.

Keywords: POLA2; hepatocellular carcinoma (HCC); macrophage; cell cycle; biomarker

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Introduction

Liver cancer, predominantly hepatocellular carcinoma (HCC), is the third leading cause of cancer-related mortality globally (1). Due to the lack of specific symptoms and markers for early detection, the great majority of HCC patients are often diagnosed in an advanced stage, which limits treatment options and lead to poor prognosis (2). Therefore, exploring new reliable biomarkers for early diagnosis and prognosis monitoring of HCC is urgently needed.

Sustained proliferation and replicative immortality are hallmarks of cancer (3), both of which rely heavily

^ ORCID: Ran Liu, 0009-0002-8851-3670; Yao Teng, 0000-0001-6877-5417.

on DNA replication. Human DNA replication is a highly complex and coordinated process, which needs cooperation of numerous proteins. A replication initiating machine composed of origin recognition complex (ORC), minichromosome maintenance (MCM), cell division cycle 45 (CDC45) and the go-ichi-ni-san (GINS) heterotetramer unwinds DNA parent chains, followed by DNA polymerases-mediated DNA replication (4). Human encodes 15 DNA polymerases (Pols) belonging to the A, B, X, and Y families (5). B family members including Polα-primate, Polδ and Polε finish the majority of DNA synthesis tasks, in which Polα-primate synthesizes primers on both leading and lagging strands followed by DNA extension mediated by Polδ on lagging strand and Polε (6) and Polδ (7) on leading strand. Polα-primate is composed of four members including DNA polymerase POLA1, RNA primase catalytic enzyme PRIM1 and regulatory subunits POLA2 and PRIM2 (5). Therefore, it is not surprising that DNA polymerases participate in tumor progression. High POLA2 expression was reported to correlate with poor prognosis of bladder cancer (8) and glioblastoma multiforme (9), while the opposite was observed in ovary cancer (10). Recently, POLA2 was confirmed to be involved in HCC progression (11,12). Nevertheless, the mechanism of POLA2 involved in HCC has not been explored.

In the present study, we found POLA2 is upregulated by E2F1 and E2F4 in HCC. POLA2 facilitates cell cycle progression, DNA replication and DNA repair and leads to poor overall survival in HCC patients. POLA2 is also positively correlated with macrophage infiltration in the tumor microenvironment, probably due to elevating CSF1 and VEGFA expression. Thus, POLA2 is a novel candidate of diagnostic and prognostic biomarker of HCC. We present this article in accordance with the TRIPOD reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2145/rc).

Highlight box

Key findings

- POLA2 is correlated to cell cycle progression, DNA replication and DNA repair, indicating its critical role in carcinoma progression. We demonstrated POLA2 is upregulated by E2F1 in hepatocellular carcinoma (HCC) patients and can predict prognosis of HCC.

What is known and what is new?

- POLA2 expression is correlated with poor prognosis of bladder cancer, glioblastoma multiforme and ovary cancer.
- High POLA2 expression is correlated with poor overall survival in HCC patients.

What is the implication, and what should change now?

- This study established specific diagnostic and prognostic predictions for HCC patients. Moreover, the potential mechanism of POLA2 involved in HCC should be further clarified in the future study.

Methods

Data download and processing

The Cancer Genome Atlas-Liver Hepatocellular Carcinoma (TCGA-LIHC) data were downloaded from TCGA website (https://portal.gdc.cancer.gov/). Chinese HCC patients with hepatitis B virus (HBV) infection (CHCC-HBV) data were downloaded from https://www.biosino.org/node/project/detail/OEP000321 (13). Liver Cancer-RIKEN, Japan (LIRI-JP) data were downloaded from International Cancer Genome Consortium (ICGC) website (https://dcc.icgc.org/). GSE105130, GSE77509, GSE94660 were downloaded from Gene Expression Omnibus (GEO) website (https://www.ncbi.nlm.nih.gov/geo/). All sequencing data were normalized to transcripts per million (TPM) for analysis. Immunohistochemistry (IHC) images of POLA2 were from The Human Protein Atlas (HPA) website (https://www.proteinatlas.org/). Gene sets for enrichment analysis were from MsigDB (http://www.gsea-msigdb.org/gsea/msigdb/index.jsp). E2F1 or E2F4 ChIP-seq data were from ENCODE (https://www.encodeproject.org/). The data were processed and analyzed in R (4.1.2) software.

Clinical sample collection

Human HCC tumor and adjacent non-tumor tissue (n=12) were collected from HCC patients who underwent hepatectomy at the Department of Hepatobiliary Surgery, Union Hospital affiliated to Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China) from October 2020 to April 2022. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). All procedures involving patient specimen were approved by the Ethics Committee of Union Hospital affiliated to Tongji Medical College, Huazhong University of Science and Technology (No. 0322-01). Written informed consent was obtained from all patients prior to surgery. Samples were kept in liquid

nitrogen for protein extraction.

Cell culture and transfection

Human HCC cell lines HepG2, Huh7, SNU182 and MIHA were cultured in high glucose DMEM (Procell, Wuhan, China) medium supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS). SiRNAs targeting E2F1 (5'-TAACTGCACTTTCGGCCCTTT-3') and E2F4 (5'-CCCTCTCTTCATTTCGGCTTT-3') and negative control (5'-UUCUCCGAACGUGUCACGUTT-3') were obtained from Gene Pharma company (Suzhou, China), and transfected cells using lipofectamine 3000 (#3000015, Invitrogen, USA).

Protein extraction and western blot

Proteins of HCC tissue and adjacent normal tissue were extracted with western RIPA lysis buffer (Beyotime, Shanghai, China) followed by. The proteins were then separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and transferred from gel onto membranes. The membranes were blocked by skim milk and were incubated with primary antibodies against POLA2 (Proteintech, 21778-1-AP, 1:1,000 dilution), anti-E2F1 (Proteintech, 66515-1-Ig, 1:1,000 dilution) , anti-E2F4 (Proteintech, 10923-1-AP, 1:1,000 dilution) , β-actin (Proteintech, 66009-1-Ig, 1:5,000 dilution) or anti-GAPDH (#8884, Cell Signaling Technology, USA, dilution 1:10,000). overnight. Then the membranes were incubated with corresponding HRP-conjugated AffiniPure Goat Anti-rabbit (mouse) IgG (Boster Biological Technology, BA1055, 1:5,000 dilution) and visualized by the ChemiDoc imaging system (Bio-Rad, California, USA). ImageJ was used to quantify the western blot results, β-actin or glyceraldehyde-3-phosphate dehydrogenas (GAPDH) was used to be the control.

Gene set enrichment analysis (GSEA)

Genes were preranked by descending their log2 fold change (FC) values. GSEA was performed using 'GSEA' function in 'clusterProfiler' package with gene sets from MsigDB and all parameters were set to default. The GSEA plot were plotted with 'gseaplot' function in 'clusterProfiler' package.

Enrichment of co-expressed genes

All genes were tested for the expression correlation with POLA2 in TCGA-LIHC, CHCC-HBV and LIRI-JP data sets. The genes that had Pearson correlation coefficients greater than 0.4 were intersected and defined as genes co-expressed with POLA2. These genes were used in enrichment analysis with 'enricher' function and visualized with 'dotplot' function in 'clusterProfiler' package.

Statistical analyses

For expression level comparison, Wilcoxon test was used as POLA2 expressions were generally not normally distributed and variances were not equal. For survival analysis, log-rank test was used. P values of Cox proportional hazards model was provided by the model fit. P values, adjusted P values and false discovery rate (FDR) of enrichment analysis were provided by corresponding analysis function. Pearson correlation coefficients and corresponding P values were provided by Pearson correlation test. All statistical analyses were performed in R (4.1.2) software and $P<0.05$ were considered statistically significant.

Results

POLA2 is highly upregulated in HCC

To explore whether POLA2 is upregulated in HCC, we downloaded six HCC transcriptome sequencing data sets (TCGA-LIHC, CHCC-HBV, LIRI-JP, GSE105130, GSE77509, GSE94660) and compared POLA2 expression between tumors and normal liver tissues. We found that POLA2 was significantly increased in HCC (*Figure 1*, Figure S1). Specifically, we found that in the six independent data sets, POLA2 expression was significantly higher in HCC tumor than in normal liver tissue (*Figure 1A,1D,1G,1J*, Figure S1A,S1D). Since sequencing data of some patients' normal liver tissues were not provided by TCGA-LIHC and LIRI-JP, we compared POLA2 expression level between HCC tumor and adjacent normal liver tissue to further rule out individual difference. We found that in most patients, POLA2 expression increased during the transition from normal tissue to tumor tissue (*Figure 1B,1E,1H,1K*, Figure S1B,S1E). For the patients that had both tumor and normal tissue sequencing data, POLA2 expression showed good efficacy to distinguish tumor tissue from normal tissue in all the six data sets, with area under curve (AUC) ranging from 0.891 to 0.993 in receiver operating characteristic (ROC) curve analysis (*Figure 1C,1F,1I,1L*, Figure S1C,S1F). Consistently, IHC

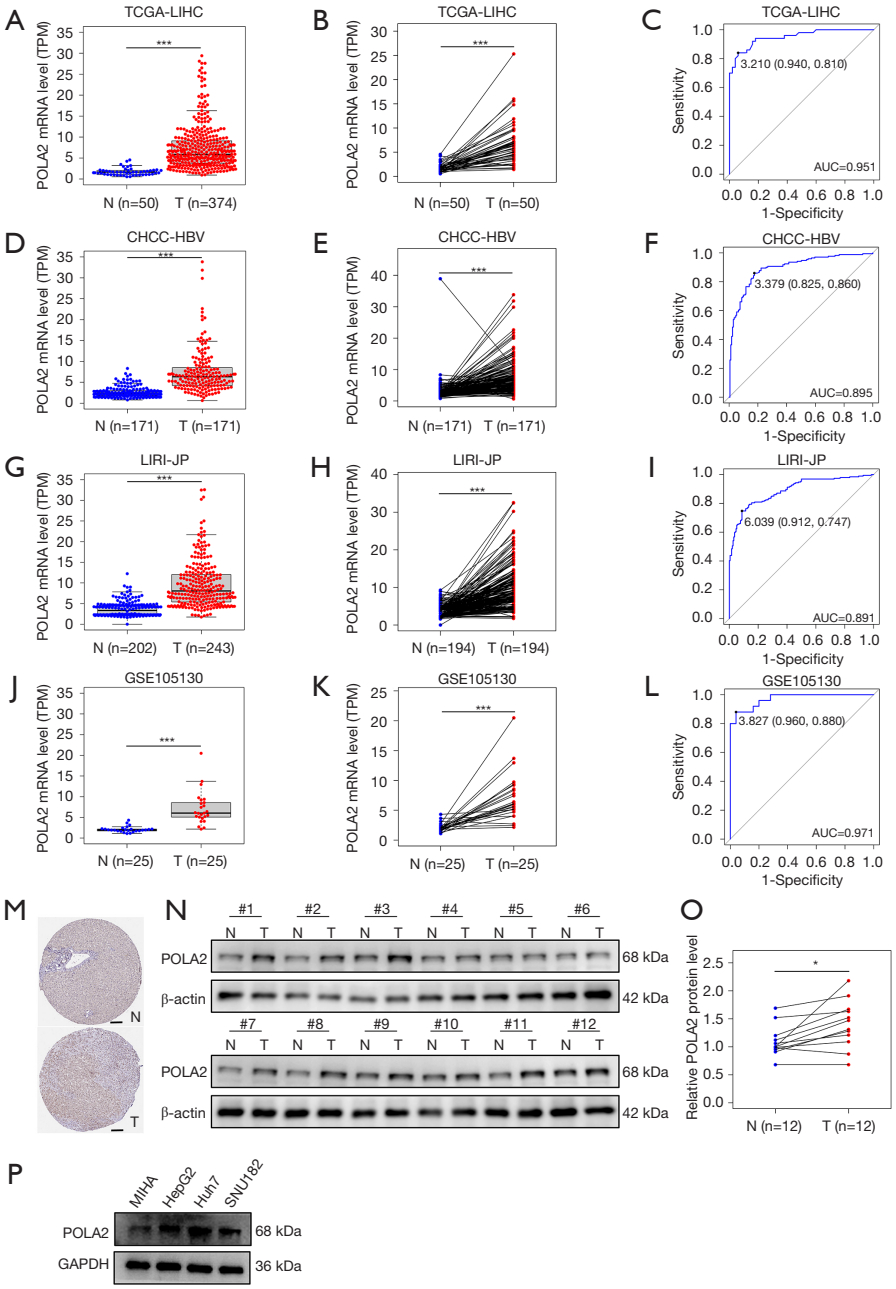

Figure 1 POLA2 is highly upregulated in HCC. (A-L) Scatter plots, pair plots and diagnosis ROC curves to demonstrate POLA2 expression difference between tumor tissue and normal liver tissue in TCGA-LIHC (A-C), CHCC-HBV (D-F), LIRI-JP (G-I), GSE105130 (J-L) data sets. (M) POLA2 IHC images from HPA website (https://www.proteinatlas.org/). The link to the normal liver and tumor tissues of POLA2 was provided (https://www.proteinatlas.org/ENSG00000014138-POLA2/tissue/liver#img). (N) POLA2 protein expression level was determined by western blot. (O) The quantitative result of relative POLA2 protein levels. (P) POLA2 protein expression level in HCC cell line and normal liver cell line. Scale bar, 100 μm. *, P<0.05; ***, P<0.001. TCGA-LIHC, The Cancer Genome Atlas-Liver Hepatocellular Carcinoma; TPM, transcripts per million; N, normal; T, tumor; AUC, area under curve; CHCC-HBV, Chinese HCC patients with HBV infection; HBV, hepatitis B virus; LIRI-JP, Liver Cancer-RIKEN, Japan; GAPDH, glyceraldehyde-3-phosphate dehydrogenas; HCC, hepatocellular carcinoma; ROC, receiver operating characteristic; IHC, immunohistochemistry; HPA, Human Protein Atlas.

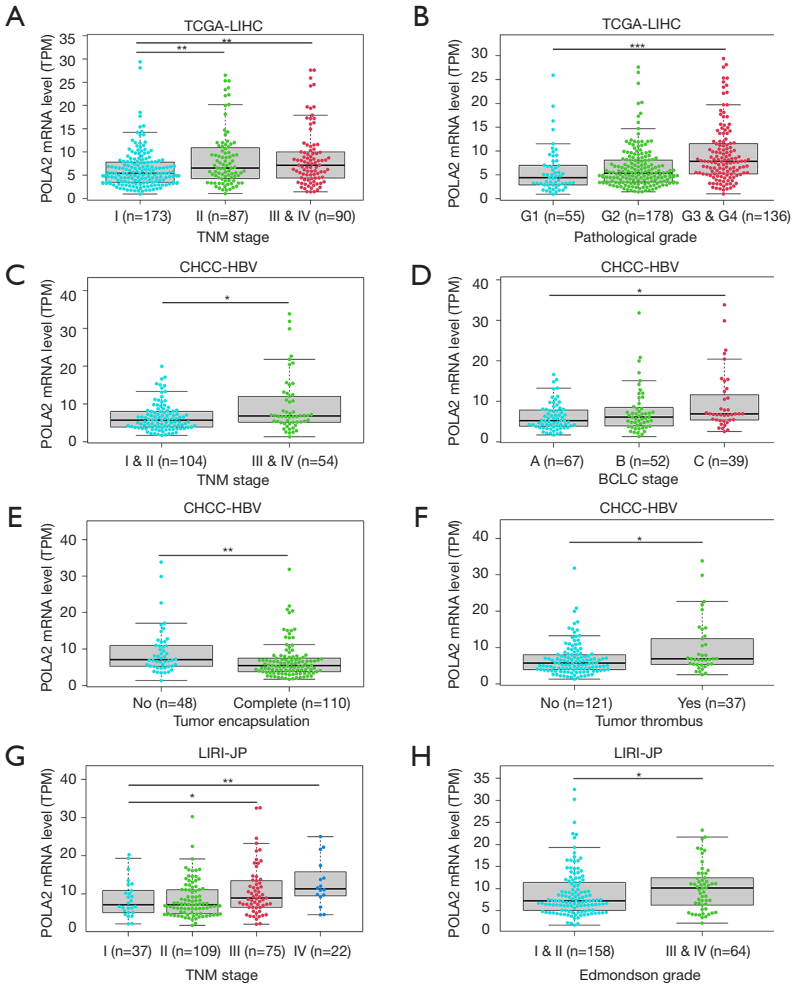

Figure 2 POLA2 expression is correlated with HCC malignant degree. (A,B) Tumor POLA2 expression in different TNM stage (A) or pathological grade (B) in TCGA-LIHC data set. (C,D) Tumor POLA2 expression in different TNM stage (C) or BCLC stage (D) in CHCC-HBV data set. (E,F) Tumor POLA2 expression in tumors of different encapsulation (E) or thrombus (F) conditions in CHCC-HBV data set. (G,H) Tumor POLA2 expression in different TNM stage (G) or Edmondson grade (H) in LIRI-JP data set. *, P<0.05; **, P<0.01; ***, P<0.001. TCGA-LIHC, The Cancer Genome Atlas-Liver Hepatocellular Carcinoma; TPM, transcripts per million; TNM, tumor node metastasis; CHCC-HBV, Chinese HCC patients with HBV infection; HBV, hepatitis B virus; BCLC, Barcelona Clinic Liver Cancer; LIRI-JP, Liver Cancer-RIKEN, Japan; HCC, hepatocellular carcinoma.

results from HPA website indicated that POLA2 intensity was higher in tumor tissue than normal tissue, locating mainly in nucleus (*Figure 1M*). Western blot analysis also showed POLA2 protein level was higher in tumor tissue than adjacent normal tissue in our clinical samples (*Figure 1N,1O*). POLA2 protein expression was increased in HCC lines (HepG2, Huh7) compared to the immortal normal liver cell line (MIHA) (*Figure 1P*). Taken together, these data suggest that POLA2 expression is highly upregulated in HCC on both mRNA and protein levels.

POLA2 expression is correlated with HCC malignant degree

Now that POLA2 is upregulated in HCC tumor compared with normal tissue, we continued to investigate whether tumor POLA2 expression is correlated with malignant clinicopathological parameters. In TCGA-LIHC cohort, we found POLA2 expression increased along with elevated tumor node metastasis (TNM) stage (*Figure 2A*) or pathological grade (*Figure 2B*). Likewise, in CHCC-HBV

cohort, POLA2 was upregulated as TNM stage (*Figure 2C*) or Barcelona Clinic Liver Cancer (BCLC) stage (*Figure 2D*) increased and POLA2 expression was positively correlated with tumor size (Figure S1G). Moreover, in CHCC-HBV cohort, POLA2 expression was higher in the tumors that had no complete encapsulation (*Figure 2E*) or the tumors that developed tumor thrombus (*Figure 2F*), both of which are associated with tumor progression and poor prognosis. These results were further validated in LIRI-JP cohort, in which POLA2 expression were elevated in tumors of higher TNM stage (*Figure 2G*) or Edmondson grade (*Figure 2H*). Taken together, these results show that POLA2 expression continue to elevate when HCC tumors progress to more malignant phenotypes.

High POLA2 expression is correlated with poor prognosis

Since POLA2 is upregulated as tumors developed more malignant potential, we wondered whether POLA2 expression is associated with patients' prognosis. In *Figure 3*, we found that the high level of POLA2 was correlated with poor prognosis of HCC. As expected, we found that in TCGA-LIHC cohort, patients that had high tumor POLA2 expression had bad prognosis (*Figure 3A*). The time dependent ROC curve showed that POLA2 expression had the best performance to predict survival at one year (*Figure 3B*). We then analyzed survival difference using Cox proportional hazards model. In univariate Cox model, we investigated the effects of gender, age, TNM stage, pathological grade and POLA2 expression on survival. We found that TNM stage [stage I&II as reference; stage III&IV hazard ratio (HR) =2.9; 95% confidence interval (CI): 1.98–4.25; P value <0.001] and POLA2 expression (low POLA2 as reference; high POLA2 HR =1.85; 95% CI: 1.25–2.74; P value =0.002), as well as female gender (male as reference; female HR =1.36; 95% CI: 0.92–2, P value =0.13) were risk factors for HCC patients, with P values less than 0.2 (Table S1). These three factors were then analyzed with multivariate Cox model and the result showed only TNM stage (stage I&II as reference; stage III&IV HR =2.61; 95% CI: 1.76–3.86; P value <0.001) and POLA2 expression (low POLA2 as reference; high POLA2 HR =1.6; 95% CI: 1.03–2.3, P value =0.03) showed P value less than 0.05 (*Figure 3C*, Table S1).

We also constructed a prognostic model consisting of TNM stage and POLA2 expression, which showed that high POLA2 was correlated with unfavorable prognosis (*Figure 3D*). Consistently, in CHCC-HBV and LIRI-JP cohorts, high POLA2 was associated with poor prognosis (*Figure 3E,3G*), although P value was greater than 0.05 in LIRI-JP cohort (probably because the patients had relatively good overall survival and the follow-up time was not long enough). Different from TCGA-LIHC cohorts, POLA2 expression showed the best predictive efficacy at 4 years in CHCC-HBV and LIRI-JP cohorts (*Figure 3F,3H*). Overall, these data suggest that high POLA2 expression is correlated with poor prognosis for HCC patients.

POLA2 participates in cell cycle progression and DNA replication and repair

After confirming POLA2 as a diagnostic and prognostic biomarker for HCC, we next sought to detect the possible mechanism of POLA2 in the regulation of HCC progression. Tumor samples were divided into two groups according to POLA2 expression level with POLA2 median expression level as cutoff value, and GSEA was performed. We performed the same analysis process in TCGA-LIHC, CHCC-HBV and LIRI-JP cohorts independently and intersect the results that had FDR less than 0.25 in each cohort. In GSEA-Gene Ontology (GO)-biological progress (BP) analysis, there were 131 gene sets in the intersection (*Figure 4A*, Table S2). Gene sets including 'positive regulation of cell cycle', 'DNA replication', 'chromatin remodeling', 'recombinational repair' were significantly enriched in high POLA2 group (*Figure 4B-4E*), indicating POLA2 participated in these processes. Consistently, in GSEA-Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, there were 29 gene sets in the intersection (*Figure 4F*, Table S3), including 'cell cycle', 'DNA replication', 'mismatch repair', 'homologous recombination' (*Figure 4G-4J*), which demonstrated that POLA2 regulated cell cycle, DNA replication and DNA repair. These results were further validated by enrichment of the genes co-expressed with POLA2. The genes whose expression had Pearson correlation coefficients greater than 0.4 with POLA2 expression in TCGA-LIHC, CHCC-HBV, LIRI-JP were intersected (Figure S2A, table available at https://cdn.amegroups.cn/static/public/tcr-23-2145-1.pdf) and GO-BP, KEGG enrichment were conducted for them. In GO-BP and KEGG analysis, gene sets related to cell cycle, DNA replication, DNA repair were significantly enriched (*Figure 4K,4L*, tables available at https://cdn.amegroups.cn/static/public/tcr-23-2145-2.xlsx and https://cdn.amegroups.cn/static/public/tcr-23-2145-3.xlsx). Taken together, these results suggest that POLA2 participates in

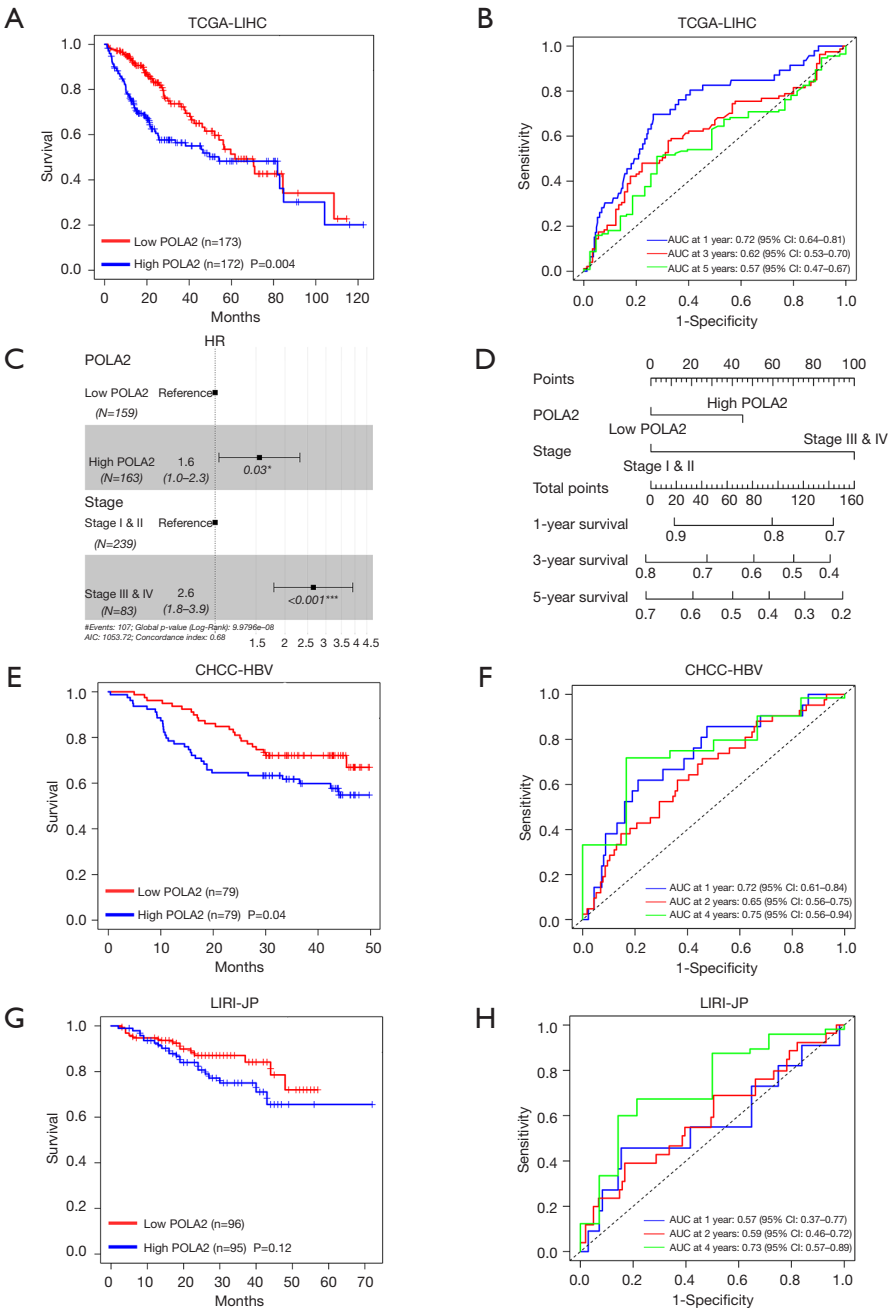

Figure 3 High POLA2 expression is correlated with poor prognosis. (A,B) Kaplan-Meier plot (A) and time dependent ROC curve (B) to show the prognosis prediction efficacy of POLA2 on HCC patients in TCGA-LIHC data set. (C) Forest plot of multivariate Cox proportional hazards model involving POLA2 expression and TNM stage. (D) Nomogram to predict overall survival based on POLA2 expression and TNM stage. (E,F) Kaplan-Meier plot (E) and time dependent ROC curve (F) to show the prognosis prediction efficacy of POLA2 on HCC patients in CHCC-HBV data set. (G,H) Kaplan-Meier plot (G) and time dependent ROC curve (H) to show the prognosis prediction efficacy of POLA2 on HCC patients in TCGA-LIHC data set. *, P<0.05; ***, P<0.001. TCGA-LIHC, The Cancer Genome Atlas-Liver Hepatocellular Carcinoma; AUC, area under curve; HR, hazard ratio; AIC, Akaike information criterion; CHCC-HBV, Chinese HCC patients with HBV infection; HBV, hepatitis B virus; LIRI-JP, Liver Cancer-RIKEN, Japan; ROC, receiver operating characteristic; HCC, hepatocellular carcinoma; TNM, tumor node metastasis.

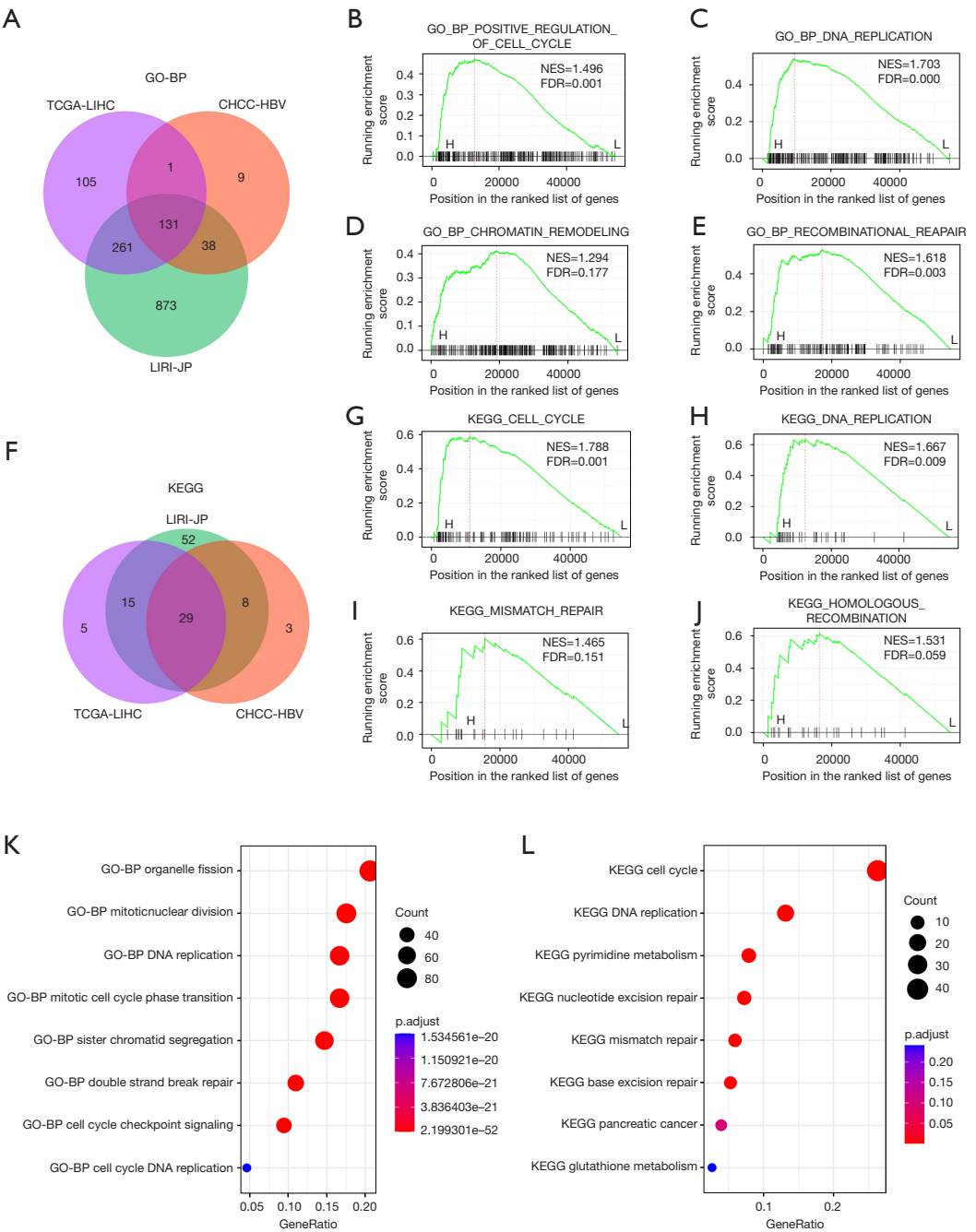

Figure 4 POLA2 participates in cell cycle progression and DNA replication and repair. (A) Venn diagram to show the overlap of GO-BP gene sets whose FDR <0.25 in GSEA analysis in TCGA-LIHC, CHCC-HBV, LIRI-JP data sets. (B-E) The representative results in the intersection of GSEA results in TCGA-LIHC data set. (F) Venn diagram to show the overlap of KEGG gene sets whose FDR <0.25 in GSEA analysis in TCGA-LIHC, CHCC-HBV, LIRI-JP data sets. (G-J) The representative results in the intersection of GSEA results in TCGA-LIHC data set. (K,L) Dot plot to show GO-BP (K) and KEGG (L) enrichment analysis of genes co-expressed with POLA2 in TCGA-LIHC, CHCC-HBV, LIRI-JP data sets. TCGA-LIHC, The Cancer Genome Atlas-Liver Hepatocellular Carcinoma; GO, Gene Ontology; BP, biological progress; CHCC-HBV, Chinese HCC patients with HBV infection; HBV, hepatitis B virus; LIRI-JP, Liver Cancer-RIKEN, Japan; NES, normalized enrichment score; FDR, false discovery rate; H, high POLA2 expression group; L, low POLA2 expression group; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, gene set enrichment analysis.

modulating cell cycle progression, DNA replication and repair in HCC.

POLA2 is regulated by E2F1 transcription factor

Since tumor progression was regulated by various transcription factors (14), we wondered which transcription factors might interact with POLA2 in HCC. We found that POLA2 is regulated by E2F1 transcription factor rather than E2F4 (*Figure 5*). To this end, we conducted GSEA-transcription factor target (TFT) analysis in TCGA-LIHC, CHCC-HBV, LIRI-JP and intersected the gene sets with FDR less than 0.25. The results showed 12 gene sets were in the intersection (*Figure 5A*, table available at https://cdn.amegroups.cn/static/public/tcr-23-2145-4.xlsx) and all of them were related to E2F transcription factor family, especially E2F1 and E2F4 (*Figure 5B-5E*). TFT analysis also showed gene sets related to E2F family were significantly enriched in the genes co-expressed with POLA2 (*Figure 5F*, table available at https://cdn.amegroups.cn/static/public/tcr-23-2145-5.xlsx). Therefore, we focused on the regulation relationship between E2F family and POLA2. We found there was a sharp peak at the transcription start site in E2F1 ChIP-seq data conducted with HepG2, a human HCC cell line (*Figure 5G*). POLA2 expression was highly positively correlated with E2F1 expression in the three cohorts (*Figure 5H-5J*). Moreover, knockdown of E2F1 inhibited POLA2 expression in HCC cell (*Figure 5K*,*5M*). E2F4 transcription factor showed similar results (Figure S2B-S2E), while western blot results showed that knockdown of E2F4 had no effects on POLA2 expression, indicting E2F4 was not involved in the transcription of POLA2 (*Figure 5L-5M*). Altogether, these results suggest POLA2 is regulated by E2F1 in HCC.

POLA2 induces macrophage infiltration by upregulating CSF1 and VEGFA in HCC

It is well-established that tumor cells interact with immune cells to form a microenvironment that facilitates tumor progression (15). Therefore, we explored whether POLA2 expression might regulate immune infiltration in HCC tumors. Immune cell abundance was calculated with CIBERSORT algorithm (16) and 22 cell types were analyzed (*Figure 6A-6C*). We then intersected the cell types whose relative abundance significantly correlated with POLA2 expression with P value less than 0.05 in the three cohorts and macrophage M0 was the only cell type in the intersection (*Figure 6D*). In all the three cohorts, POLA2 positively correlated with macrophage M0 infiltration (*Figure 6E-6G*). It was reported that several cytokines play important roles in recruiting macrophage, such as CCL2, CSF1, VEGF, ANGPT2 and CXCL12. Among them, CSF1 and VEGFA showed significant positive correlation with POLA2 expression in all the three cohorts (*Figure 6H-6J*, Figure S2F-S2H). Altogether, these results suggest that POLA2 might regulate macrophage infiltration through upregulating CSF1 and VEGFA in HCC.

Discussion

In the present study, we found POLA2 was upregulated in HCC tumors compared with adjacent normal tissue and its expression was higher in the tumors that had more malignant characteristics. Furthermore, high POLA2 expression was closely correlated with poor prognosis of HCC patients and unregulated by E2F1. Since these results were validated in multiple independent data sets, we believe POLA2 acts as an oncogene that could be utilized for early diagnosis and prognosis prediction.

In enrichment analysis, we found POLA2 participated in DNA replication and cell cycle progression. As DNA replication is necessary for cell cycle progression, and POLα-primate complex played a crucial role in initiating DNA replication by synthesizing primers, this result is not surprising. We also found POLA2 play a role in DNA repair such as mismatch repair and homologous recombination repair, which is in accordance with recent study. POLA2 was reported to contribute to double strand breaks repair after etoposide treatment (9) and facilitate telomere maintenance (17). These results highlight POLA2's function in DNA repair besides relatively well-known DNA replication function, and the exact underlying mechanism demands further research.

Tumor microenvironment is shaped by tumor cells, generally through altering secretion of cytokines or extracellular vesicles or changing the expression pattern of membrane molecules (18). Macrophages in the tumor microenvironment are predominantly M2 polarized, which is due to tumor cell 'education' (19). M2 polarized macrophages facilitate tumor progression through various mechanisms and typically represent a pro-tumor cell type (20). In our study, we found POLA2 expression was positively correlated with macrophage infiltration as well as expression of CSF1 and VEGFA, two cytokines that play chemoattractant role for macrophage (21). These results

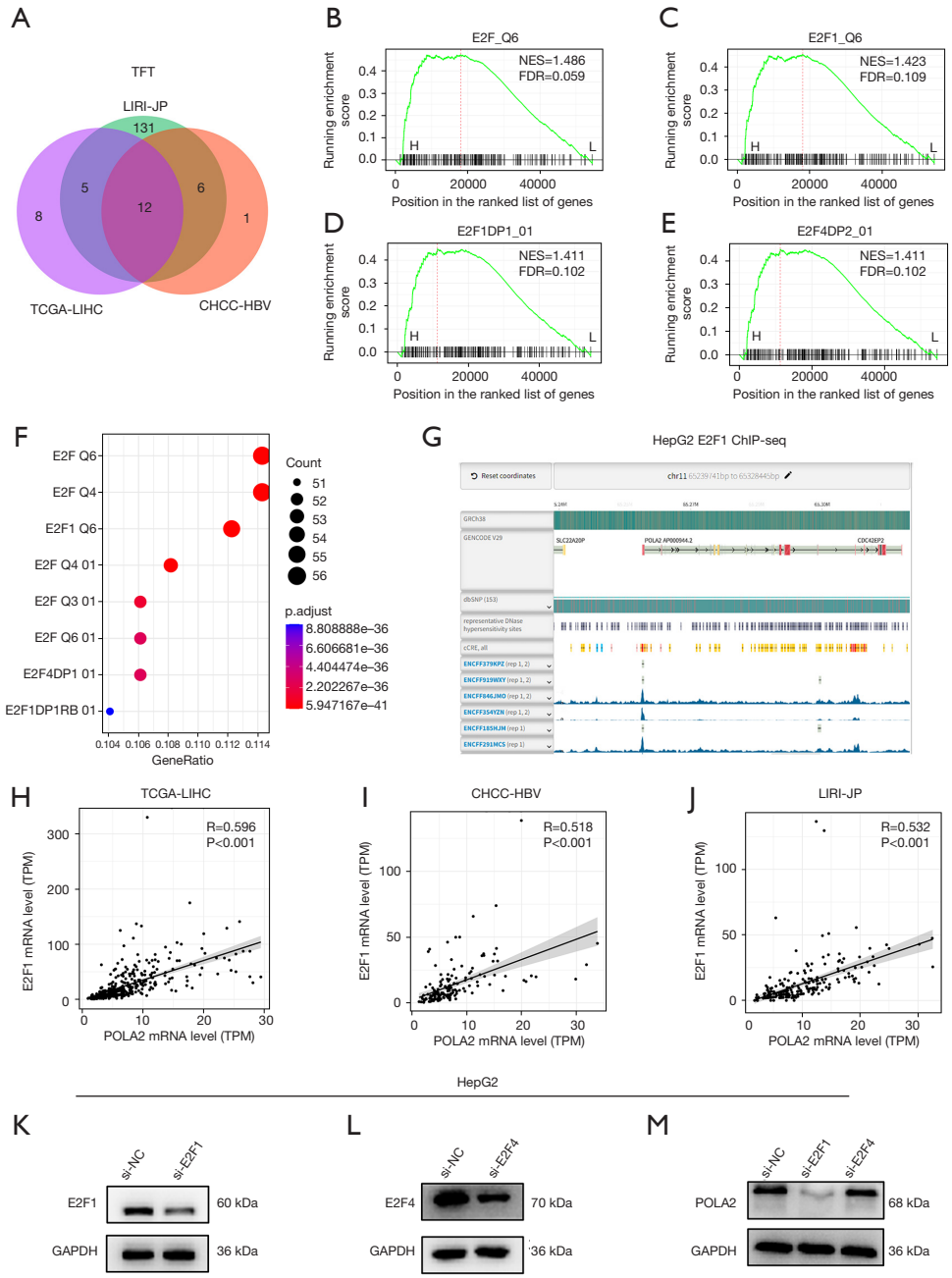

Figure 5 POLA2 is regulated by E2F1 transcription factor. (A) Venn diagram to show the overlap of TFT gene sets whose FDR <0.25 in GSEA analysis in TCGA-LIHC, CHCC-HBV, LIRI-JP data sets. (B-E) The representative results in the intersection of GSEA results in TCGA-LIHC data set. (F) Dot plot to show TFT enrichment analysis of genes co-expressed with POLA2 in TCGA-LIHC, CHCC-HBV, LIRI-JP data sets. (G) Screen shot of E2F1 ChIP-seq peaks near POLA2 transcription start site conducted in HepG2 cell line on ENCODE website. (H-J) Expression correlation of E2F1 and POLA2 in TCGA-LIHC (H), CHCC-HBV (I), LIRI-JP (J) data sets. (K-L) Knockdown E2F1 or E2F4 expression by transfecting siRNAs targeting E2F1 or E2F4 in HepG2 cell. (M) POLA2 protein level in E2F1 or E2F4 knockdown HepG2 cell. TFT, transcription factor target; TCGA-LIHC, The Cancer Genome Atlas-Liver Hepatocellular Carcinoma; LIRI-JP, Liver Cancer-RIKEN, Japan; CHCC-HBV, Chinese HCC patients with HBV infection; HBV, hepatitis B virus; NES, normalized enrichment score; FDR, false discovery rate; H, high POLA2 expression group; L, low POLA2 expression group; TPM, transcripts per million; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSEA, gene set enrichment analysis.

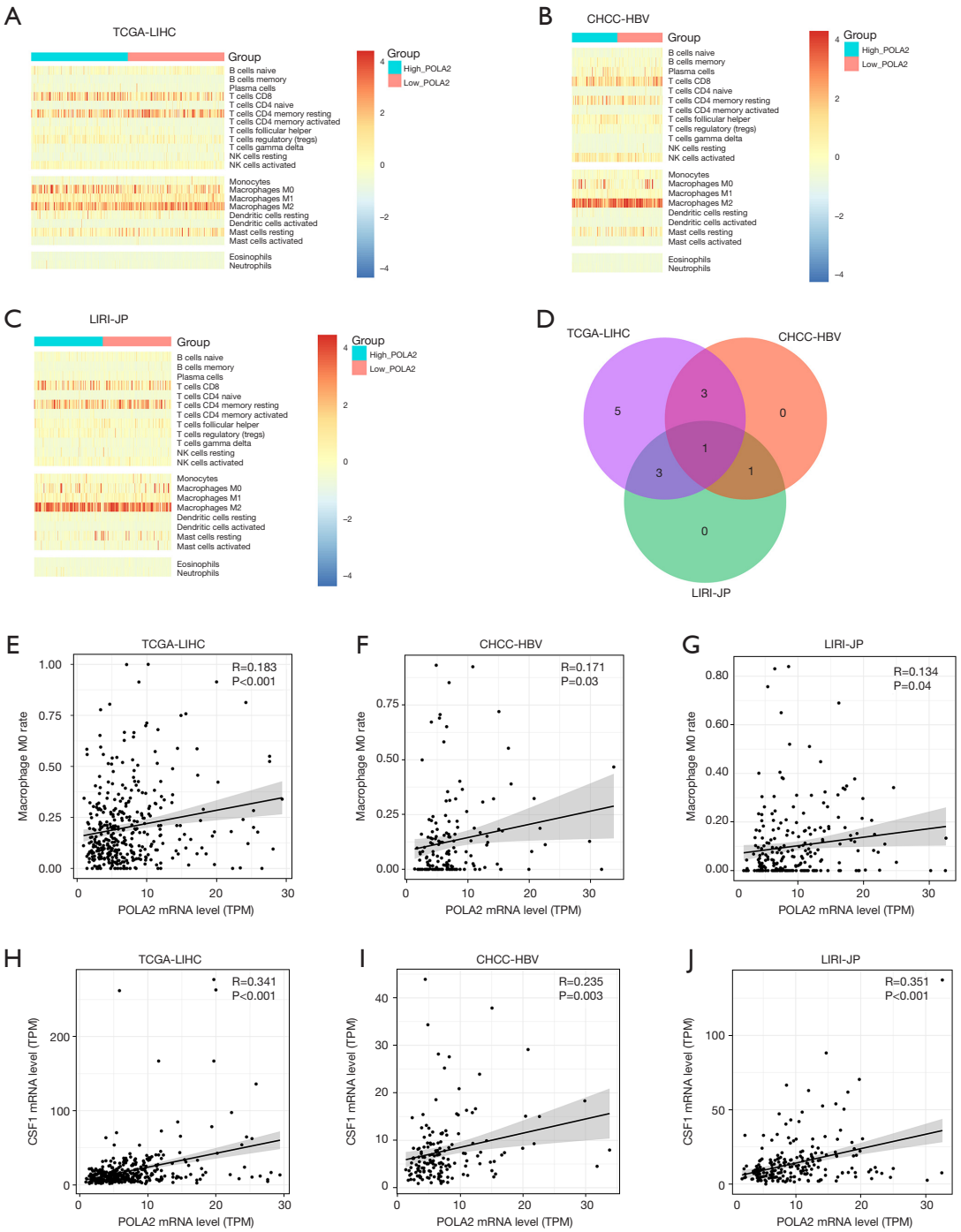

Figure 6 POLA2 induces macrophage infiltration by upregulating CSF1 and VEGFA in HCC. (A-C) Heat map to show immune cell infiltration level in TCGA-LIHC (A), CHCC-HBV (B), LIRI-JP (C) data sets estimated by CYBERSORT algorithm. (D) Venn diagram to show the overlap of cell types whose infiltration level had significant correlation with POLA2 expression in the three data sets. (E-G) The correlation between macrophage M0 and POLA2 expression level in TCGA-LIHC (E), CHCC-HBV (F), LIRI-JP (G) data sets. (H-J) Expression correlation between CSF1 and POLA2 in TCGA-LIHC (H), CHCC-HBV (I), LIRI-JP (J) data sets. TCGA-LIHC, The Cancer Genome Atlas-Liver Hepatocellular Carcinoma; CHCC-HBV, Chinese HCC patients with HBV infection; HBV, hepatitis B virus; LIRI-JP, Liver Cancer-RIKEN, Japan; TPM, transcripts per million; HCC, hepatocellular carcinoma.

may reflect the fact that rapidly proliferating tumor cells may attract and educate macrophages to further promote tumor cell proliferation and POLA2 plays a role in this process by upregulating expression of CSF1 and VEGFA.

E2F family of transcription factors play a critical role in HCC (22). E2F transcription factor 3 (E2F3) was reported to be overexpressed and indicated poor prognosis in HCC (23). In this study, we found E2F1 increased POLA2 expression in HCC.

Our study has obvious limitations. DNA polymerase family contains 15 subunit members and only POLA2 was discussed here. In our preliminary study, we observed that among these polymerase subunits, POLA1, PRIM1, PRIM2, POLD1, POLE2 showed similar results as POLA2 in diagnosis and prognosis prediction (data not shown). In these genes, POLA2 had the best efficacy across data sets, thus we focused on POLA2. This suggests that results of POLA2 in this study can be extended to these polymerase genes to some extent. It should be further investigated whether combination of these DNA polymerase subunit genes would perform better in early diagnosis and prognosis prediction. Another limitation of this study is the lack of experimental validation. Our conclusions about POLA2's biological functions and its potential as a therapeutic target are based on bioinformatics analysis, which needs to be consolidated by further experimental investigation.

Conclusions

In summary, we found POLA2 expression is upregulated by E2F1 in HCC and is further elevated in the tumors of more malignant phenotypes. High POLA2 expression is closely correlated with poor overall survival. POLA2 facilitates cell cycle progression, DNA replication and DNA repair. Its expression is positively correlated with CSF1 and VEGFA expression and macrophage infiltration. POLA2 is a novel diagnostic and prognostic biomarker of HCC with potential clinical value.

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Footnote

Reporting Checklist: The authors have completed the TRIPOD reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2145/rc

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Conflicts of Interest: Both authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2145/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). All procedures involving patient specimen were approved by the Ethics Committee of Union Hospital affiliated to Tongji Medical College, Huazhong University of Science and Technology (No. 0322-01). Written informed consent was obtained from all patients prior to surgery.

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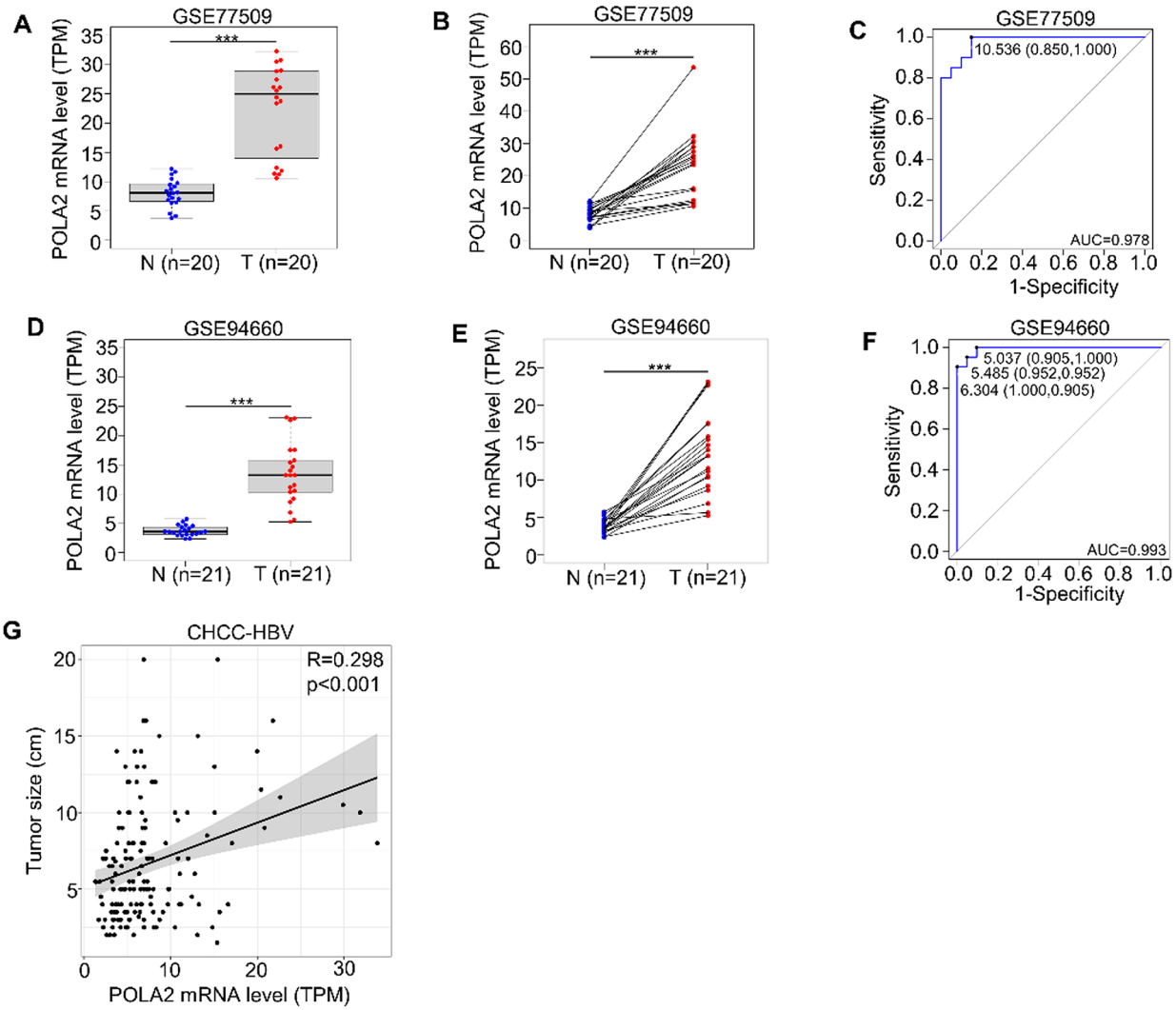

Figure S1 The mRNA level of POLA2 in HCC. (A-F) Scatter plots, pair plots and diagnosis ROC curves to demonstrate POLA2 expression difference between tumor tissue and normal liver tissue in GSE77509 (A-C), GSE94660 (D-F) data sets. (G) Correlation between tumor size and POLA2 expression in CHCC-HBV data set. ***, P<0.001. TPM, transcripts per million; N, normal; T, tumor; AUC, area under curve; CHCC-HBV, Chinese HCC patients with HBV infection; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; ROC, receiver operating characteristic.

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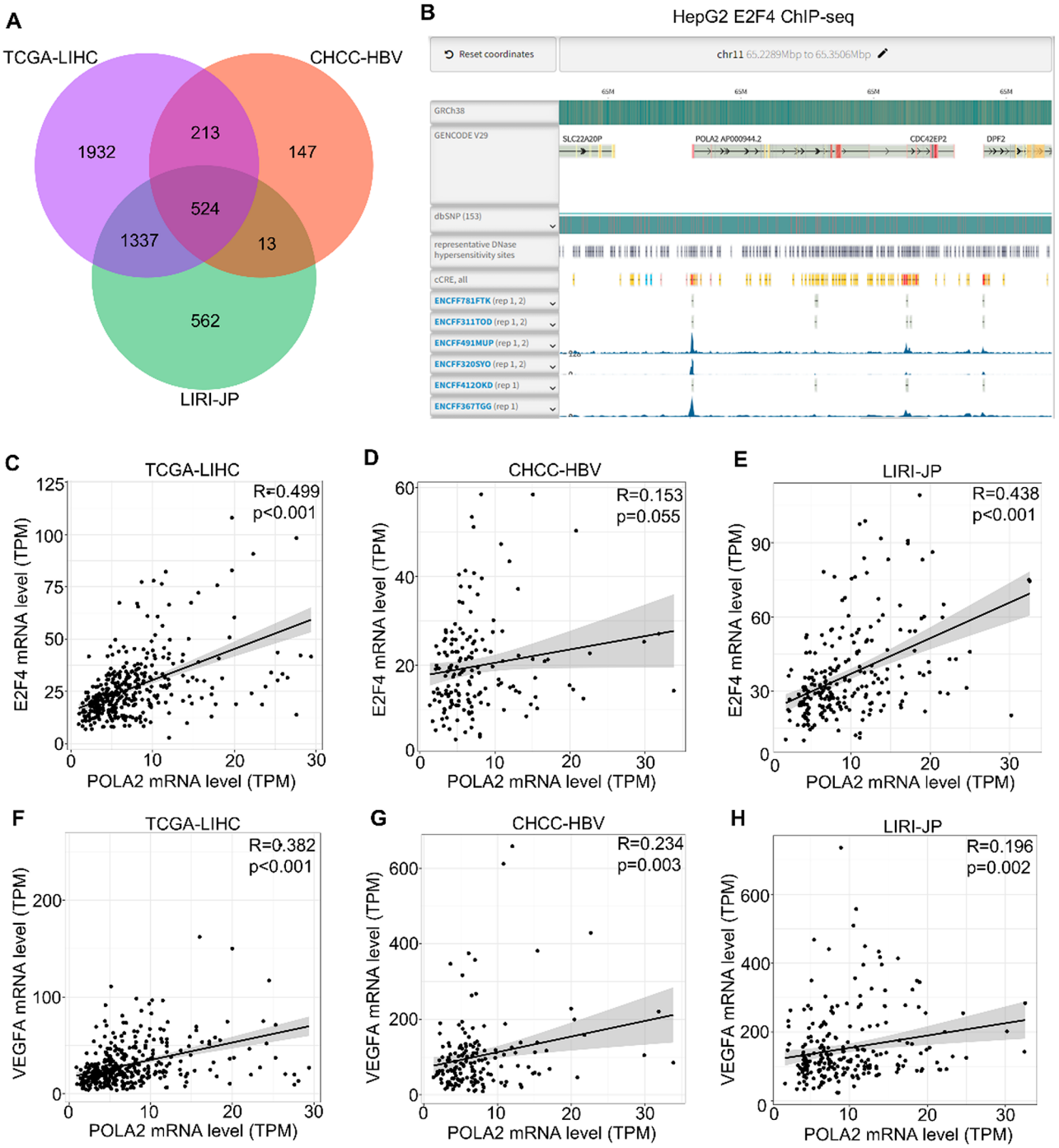

Figure S2 POLA2 is regulated by E2F transcription factor. (A) Venn diagram to show the overlap of genes co-expressed with POLA2 with Pearson correlation coefficients >0.4 in TCGA-LIHC, CHCC-HBV, LIRI-JP data sets. (B) Screen shot of E2F4 ChIP-seq peaks near POLA2 transcription start site conducted in HepG2 cell line on ENCODE website. (C-E) Expression correlation between E2F4 and POLA2 in TCGA-LIHC (C), CHCC-HBV (D), LIRI-JP (E) data sets. (F-H) Expression correlation between VEGFA and POLA2 in TCGA-LIHC (F), CHCC-HBV (G), LIRI-JP (H) data sets. TCGA-LIHC, The Cancer Genome Atlas-Liver Hepatocellular Carcinoma; CHCC-HBV, Chinese HCC patients with HBV infection; HBV, hepatitis B virus; LIRI-JP, Liver Cancer-RIKEN, Japan; TPM, transcripts per million.

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Table S1 TCGA Cox regression results

Variables	Univariate			Multivariate		
	HR	95% CI	P	HR	95% CI	P
Age	1.15	0.78–1.7	0.481	NA	NA	NA
Gender	1.36	0.92–2	0.125	1.25	0.85–1.84	0.264
Grade	1.06	0.72–1.57	0.756	NA	NA	NA
POLA2	1.85	1.25–2.74	0.002	1.54	1.03–2.3	0.035
Stage	2.9	1.98–4.25	0	2.61	1.76–3.86	0

TCGA, The Cancer Genome Atlas; HR, hazard ratio; CI, confidence interval; NA, not applicable.

Table S2 Intersection of GSEA_GO_BP results

GOBP_CELLULAR_AMINO_ACID_CATABOLIC_PROCESS
GOBP_ALPHA_AMINO_ACID_CATABOLIC_PROCESS
GOBP_MONOCARBOXYLIC_ACID_CATABOLIC_PROCESS
GOBP_FATTY_ACID_CATABOLIC_PROCESS
GOBP_ALPHA_AMINO_ACID_METABOLIC_PROCESS
GOBP_SISTER_CHROMATID_SEGREGATION
GOBP_NUCLEAR_CHROMOSOME_SEGREGATION
GOBP_CHROMOSOME_SEGREGATION
GOBP_ORGANELLE_FISSION
GOBP_SECONDARY_ALCOHOL_METABOLIC_PROCESS
GOBP_MEIOTIC_CELL_CYCLE
GOBP_XENOBIOTIC_CATABOLIC_PROCESS
GOBP_CELLULAR_RESPONSE_TO_XENOBIOTIC_STIMULUS
GOBP_XENOBIOTIC_METABOLIC_PROCESS
GOBP_DNA_TEMPLATED_DNA_REPLICATION
GOBP_MEIOTIC_CELL_CYCLE_PROCESS
GOBP_MITOTIC_NUCLEAR_DIVISION
GOBP_CELLULAR_RESPONSE_TO_ZINC_ION
GOBP_DETOXIFICATION_OF_COPPER_ION
GOBP_CELLULAR_RESPONSE_TO_COPPER_ION
GOBP_REGULATION_OF_MITOTIC_SISTER_CHROMATID_SEGREGATION
GOBP_REGULATION_OF_MITOTIC_NUCLEAR_DIVISION
GOBP_STRESS_RESPONSE_TO_METAL_ION
GOBP_PRIMARY_ALCOHOL_METABOLIC_PROCESS
GOBP_DNA_CONFORMATION_CHANGE
GOBP_DNA_REPLICATION
GOBP_RESPONSE_TO_ZINC_ION
GOBP_NEGATIVE_REGULATION_OF_NUCLEAR_DIVISION
GOBP_RESPONSE_TO_COPPER_ION
GOBP_REGULATION_OF_CHROMOSOME_SEGREGATION
GOBP_REGULATION_OF_CHROMOSOME_SEPARATION
GOBP_CHROMOSOME_SEPARATION
GOBP_TRIGLYCERIDE_METABOLIC_PROCESS
GOBP_MITOTIC_CELL_CYCLE_CHECKPOINT_SIGNALING
GOBP_CELL_CYCLE_CHECKPOINT_SIGNALING
GOBP_REGULATION_OF_MITOTIC_CELL_CYCLE_PHASE_TRANSITION
GOBP_MITOTIC_METAPHASE_PLATE_CONGRESSION
GOBP_MEIOSIS_I_CELL_CYCLE_PROCESS
GOBP_CELL_CYCLE_DNA_REPLICATION
GOBP_REGULATION_OF_NUCLEAR_DIVISION
GOBP_MICROTUBULE_CYTOSKELETON_ORGANIZATION_INVOLVED_IN_MITOSIS
GOBP_DNA_REPLICATION_INITIATION
GOBP_NEGATIVE_REGULATION_OF_CHROMOSOME_ORGANIZATION
GOBP_REGULATION_OF_MITOTIC_CELL_CYCLE
GOBP_OPSONIZATION
GOBP_BENZENE_CONTAINING_COMPOUND_METABOLIC_PROCESS
GOBP_POSITIVE_REGULATION_OF_CELL_CYCLE_PROCESS
GOBP_METAPHASE_ANAPHASE_TRANSITION_OF_CELL_CYCLE
GOBP_REGULATION_OF_CELL_CYCLE_PHASE_TRANSITION
GOBP_MITOTIC_CELL_CYCLE_PHASE_TRANSITION
GOBP_COMPLEMENT_ACTIVATION_LECTIN_PATHWAY
GOBP_CHROMOSOME_LOCALIZATION
GOBP_CELLULAR_RESPONSE_TO_CADMIUM_ION
GOBP_SPINDLE_ELONGATION
GOBP_REGULATION_OF_DNA_TEMPLATED_DNA_REPLICATION
GOBP_METAPHASE_PLATE_CONGRESSION
GOBP_MITOTIC_SPINDLE_ASSEMBLY
GOBP_POSITIVE_REGULATION_OF_CELL_CYCLE
GOBP_REGULATION_OF_CHROMOSOME_ORGANIZATION
GOBP_DNA_RECOMBINATION
GOBP_HOMOLOGOUS_CHROMOSOME_SEGREGATION
GOBP_NEGATIVE_REGULATION_OF_CELL_CYCLE_PROCESS
GOBP_TETRAPYRROLE_METABOLIC_PROCESS
GOBP_MEIOTIC_CHROMOSOME_SEGREGATION
GOBP_MITOTIC_SPINDLE_ORGANIZATION
GOBP_MALE_MEIOTIC_NUCLEAR_DIVISION
GOBP_CELLULAR_PROCESS_INVOLVED_IN_REPRODUCTION_IN_MULTICELLULAR_ORGANISM
GOBP_SPINDLE_MIDZONE_ASSEMBLY
GOBP_BRANCHED_CHAIN_AMINO_ACID_CATABOLIC_PROCESS
GOBP_REGULATION_OF_OPSONIZATION
GOBP_NEGATIVE_REGULATION_OF_MITOTIC_CELL_CYCLE_PHASE_TRANSITION
GOBP_ATTACHMENT_OF_SPINDLE_MICROTUBULES_TO_KINETOCHORE
GOBP_DOUBLE_STRAND_BREAK_REPAIR
GOBP_CELL_CYCLE_G2_M_PHASE_TRANSITION
GOBP_REGULATION_OF_DNA_REPLICATION
GOBP_KINETOCHORE_ASSEMBLY
GOBP_OMEGA_HYDROXYLASE_P450_PATHWAY
GOBP_NEGATIVE_REGULATION_OF_METAPHASE_ANAPHASE_TRANSITION_OF_CELL_CYCLE
GOBP_RECOMBINATIONAL_REPAIR
GOBP_MONOCARBOXYLIC_ACID_TRANSPORT
GOBP_FEMALE_MEIOTIC_NUCLEAR_DIVISION
GOBP_HOMOLOGOUS_RECOMBINATION
GOBP_SERINE_FAMILY_AMINO_ACID_METABOLIC_PROCESS
GOBP_NEGATIVE_REGULATION_OF_MITOTIC_CELL_CYCLE
GOBP_AMINO_ACID_BETAINE_METABOLIC_PROCESS
GOBP_CENTROMERE_COMPLEX_ASSEMBLY
GOBP_PROTEIN_LOCALIZATION_TO_CHROMOSOME_CENTROMERIC_REGION
GOBP_BILE_ACID_AND_BILE_SALT_TRANSPORT
GOBP_CELLULAR_AMINO_ACID_BIOSYNTHETIC_PROCESS
GOBP_EPOXYGENASE_P450_PATHWAY
GOBP_REGULATION_OF_CELL_CYCLE_G2_M_PHASE_TRANSITION
GOBP_UREA_METABOLIC_PROCESS
GOBP_NEGATIVE_REGULATION_OF_CELL_CYCLE
GOBP_REGULATION_OF_CELL_CYCLE_CHECKPOINT
GOBP_BILE_ACID_METABOLIC_PROCESS
GOBP_CHROMOSOME_CONDENSATION
GOBP_UREA_CYCLE
GOBP_CHROMOSOME_ORGANIZATION_INVOLVED_IN_MEIOTIC_CELL_CYCLE
GOBP_2_OXOGLUTARATE_METABOLIC_PROCESS
GOBP_KINETOCHORE_ORGANIZATION
GOBP_OXIDATIVE_DEMETHYLATION
GOBP_DNA_UNWINDING_INVOLVED_IN_DNA_REPLICATION
GOBP_REGULATION_OF_SYSTEMIC_ARTERIAL_BLOOD_PRESSURE_MEDIATED_BY_A_CHEMICAL_SIGNAL
GOBP_DNA_INTEGRITY_CHECKPOINT_SIGNALING
GOBP_REGULATION_OF_CYCLIN_DEPENDENT_PROTEIN_KINASE_ACTIVITY
GOBP_MITOTIC_DNA_INTEGRITY_CHECKPOINT_SIGNALING
GOBP_PROTEIN_LOCALIZATION_TO_CONDENSED_CHROMOSOME
GOBP_DNA_TEMPLATED_DNA_REPLICATION_MAINTENANCE_OF_FIDELITY
GOBP_ARGININE_METABOLIC_PROCESS
GOBP_POSITIVE_REGULATION_OF_CELL_CYCLE_G2_M_PHASE_TRANSITION
GOBP_POSITIVE_REGULATION_OF_CHROMOSOME_SEGREGATION
GOBP_POSITIVE_REGULATION_OF_CHROMOSOME_SEPARATION
GOBP_AROMATIC_AMINO_ACID_FAMILY_METABOLIC_PROCESS
GOBP_GLUTAMATE_METABOLIC_PROCESS
GOBP_DNA_REPLICATION_CHECKPOINT_SIGNALING
GOBP_FEMALE_GAMETE_GENERATION
GOBP_ATTACHMENT_OF_MITOTIC_SPINDLE_MICROTUBULES_TO_KINETOCHORE
GOBP_PIRNA_METABOLIC_PROCESS
GOBP_HOMOLOGOUS_CHROMOSOME_PAIRING_AT_MEIOSIS
GOBP_REPLICATION_FORK_PROCESSING
GOBP_REGULATION_OF_DNA_DIRECTED_DNA_POLYMERASE_ACTIVITY
GOBP_DNA_STRAND_ELONGATION_INVOLVED_IN_DNA_REPLICATION
GOBP_MALE_MEIOSIS_I
GOBP_CELLULAR_RESPONSE_TO_GLUCAGON_STIMULUS
GOBP_REGULATION_OF_CELL_DIVISION
GOBP_MESODERM_DEVELOPMENT
GOBP_RESPONSE_TO_GLUCAGON
GOBP_CHROMATIN_REMODELING
GOBP_INTERSTRAND_CROSS_LINK_REPAIR
GOBP_POSITIVE_REGULATION_OF_DNA_METABOLIC_PROCESS
GOBP_PROTEIN_DNA_COMPLEX_SUBUNIT_ORGANIZATION

GSEA, gene set enrichment analysis; GO, Gene Ontology; BP, biological progress.

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Table S3 Intersection of GSEA_KEGG results

KEGG_FATTY_ACID_METABOLISM
KEGG_COMPLEMENT_AND_COAGULATION_CASCADES
KEGG_PEROXISOME
KEGG_DRUG_METABOLISM_CYTOCHROME_P450
KEGG_GLYCINE_SERINE_AND_THREONINE_METABOLISM
KEGG_RETINOL_METABOLISM
KEGG_VALINE_LEUCINE_AND_ISOLEUCINE_DEGRADATION
KEGG_METABOLISM_OF_XENOBIOTICS_BY_CYTOCHROME_P450
KEGG_STEROID_HORMONE_BIOSYNTHESIS
KEGG_TRYPTOPHAN_METABOLISM
KEGG_ARGININE_AND_PROLINE_METABOLISM
KEGG_PRIMARY_BILE_ACID_BIOSYNTHESIS
KEGG_PROPANOATE_METABOLISM
KEGG_CELL_CYCLE
KEGG_LINOLEIC_ACID_METABOLISM
KEGG_CITRATE_CYCLE_TCA_CYCLE
KEGG_DRUG_METABOLISM_OTHER_ENZYMES
KEGG_TYROSINE_METABOLISM
KEGG_LYSINE_DEGRADATION
KEGG_DNA_REPLICATION
KEGG_HISTIDINE_METABOLISM
KEGG_BUTANOATE_METABOLISM
KEGG_PRION_DISEASES
KEGG_BETA_ALANINE_METABOLISM
KEGG_HOMOLOGOUS_RECOMBINATION
KEGG_RENIN_ANGIOTENSIN_SYSTEM
KEGG_ALANINE_ASPARTATE_AND_GLUTAMATE_METABOLISM
KEGG_MISMATCH_REPAIR
KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION

GSEA, gene set enrichment analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes.

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