

# The Mechanism and Prognostic Value of DNA Polymerase $\delta$ Subunits in Hepatocellular Carcinoma: Implications for Precision Therapy

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**Purpose:** Polymerase  $\delta$  (POLD) proteins is a pivotal B-family DNA polymerase in the process of genome replication and repair and are comprised of POLD1-4. The predictive value of POLDs in hepatocellular carcinoma (HCC) has not been evaluated until now.

**Patients and Methods:** A total of 369 hepatocellular carcinoma samples and 50 adjacent normal samples were enrolled from the TCGA-LIHC database, and the GSE10186 database was also used. Transcription, methylation and genetic alteration status of HCC patients were evaluated by GEPIA, Kaplan–Meier plotter, cBioPortal, MethHC, MethSurv. SurvExpress was employed to generate the overall prognosis prediction signature of POLDs. POLDs coexpressed genes were explored and enriched in potential pathways. K-M curves were generated to compare the different survival results in different groups, while ROC curves were used to validate the efficiency of the POLD signature.

**Results:** All four POLD subunits were highly expressed in HCC tumor tissues. POLD1-3 and increased mRNA levels were also positively associated with advanced tumor stage and OS prognosis. Methylation in the promoter of POLDs affects mRNA expression and OS, especially for some specific CpG sites. Meanwhile, POLDs could preferably predict the prognosis for patients who suffered from a high gene mutation burden. We evaluated the combined prognostic predictive value of four POLD subunits in both the TCGA-LIHC and GSE10186 databases and recognized the statistically significant HR of the high-risk group, along with the reliable predictive value. The coexpressed gene sets and annotation results showed that the POLD coexpressed genes were mostly associated with DNA repair and cell cycle regulation pathways.

**Conclusion:** POLD is an essential predictive factor for the prognosis of HCC. The united signature could precisely identify unfavorable clinical outcome of HCC.

**Keywords:** DNA polymerase  $\delta$  subunits, hepatocellular carcinoma, DNA repair, prognosis

## Introduction

Hepatocellular carcinoma (HCC) is the 5th and 9th most frequent malignant carcinoma in males and females worldwide and is the 3rd leading cause of cancer-specific death.<sup>1</sup> There were approximately 42,030 new cases and 31,780 estimated deaths of HCC patients in 2019 around America.<sup>2</sup> Due to the increased infection risk of hepatitis B or C virus (HBV or HCV), regions with traditional low HCC incidence have experienced more cases in recent years, such as the United States and Canada.<sup>3</sup> China might face the largest number of HCC patients based on the large size of the population, as well as the high frequency of hepatitis B virus (HBV); almost 10% of the population is infected with HBV.<sup>4–6</sup> A favorable survival prognosis of HCC patients after surgical or medicinal treatment has been observed; however, effective prognostic predictive factors are still scarce.<sup>7</sup> Although serum alpha-fetoprotein (AFP) is regarded as the

diagnostic and prognostic factor for HCC patients, it is always affected by side diseases, such as chronic hepatitis, cirrhosis and stetosis.<sup>8–10</sup> On the other hand, up to 40% of HCC patients are AFP negative.<sup>11,12</sup> Therefore, it is necessary to study new biomarkers to illustrate the prognosis of HCC.

Polymerase  $\alpha$ ,  $\epsilon$  and  $\delta$  are the three pivotal B-family DNA polymerases in the process of genome duplication.<sup>13</sup> Polymerase  $\delta$  (POLD) is reported to synthesize DNA in both the leading strand and lagging strand.<sup>14,15</sup> Polymerase  $\delta$  could also control the process of break-induced replication and homologous recombination to safeguard the stability of the genome.<sup>16,17</sup> Polymerase  $\delta$  is comprised of four subunits: POLD1 is the catalytic subunit, and the large p125 subunit provides the essential catalytic subunit and performs its function in protein assembly by combining with the accessory subunits POLD2, POLD3 and POLD4.<sup>18</sup> The mechanism of POLDs in tumorigenesis has been reported in several tumors. Wang et al<sup>19</sup> illustrated that POLE/POLD1 mutations are promising potential predictive biomarkers for positive immune checkpoint inhibitor therapy outcomes. Elgaaen et al<sup>20</sup> reported that POLD2 increased approximately 2–20-fold compared with moderately to poorly differentiated serous carcinomas and was linked with poor prognosis. Qin et al<sup>21</sup> found that increased expression of POLD1 was associated with shorter recurrence-free survival and acted as a potential prognostic marker for invasive breast carcinoma.

However, the predictive value of POLDs in HCC has not been evaluated until now. In the current study, we assessed the transcription, methylation and genetic alteration of POLDs and printed the overall prognostic results by POLDs based on TCGA and GEO databases.

## Materials and Methods

### Genetic and Clinical Data of HCC Patients

A total of 369 hepatocellular carcinoma samples and 50 adjacent normal samples were enrolled in the current study. Data of transcription profiling (mRNA SeqV2), genetic alterations (gene level) and methylation (Methylation450k) for POLDs were all from The Cancer Genome Atlas (TCGA) project (<https://www.cancer.gov>). Overall survival (OS) time and status were also considered to represent the prognosis of each patient, as well as the tumor stage.

### Gene Expression, Genetic Alteration and DNA Methylation Effect on Prognosis

The pancancer expression of POLDs was assessed by ULCAN (<http://ualcan.path.uab.edu/analysis.html>).<sup>22</sup> We further evaluated the prognostic value of POLDs in pancancer by GEPIA (<http://gepia.cancer-pku.cn>) and compared POLDs mRNA expression between normal and tumor groups and between different tumor stages.<sup>23</sup> The prognostic evaluation of POLDs was assessed via the Kaplan–Meier (K-M) plotter Pancancer RNA-seq platform ([https://kmplot.com/analysis/index.php?p=service&cancer=pancancer\\_mnaseq](https://kmplot.com/analysis/index.php?p=service&cancer=pancancer_mnaseq)), while the best separation determined by algorithms was chosen as the method to separate patients into two groups.<sup>24</sup> The genetic alterations of POLDs were illustrated by the cBioPortal platform (<http://www.cbioportal.org/>), and OncoPrinter was employed to determine the frequency of genetic alterations among the recorded databases in cBioPortal.<sup>25,26</sup> The MethHC database (<http://methhc.mbc.nctu.edu.tw/php/index.php>) was used to evaluate the association between DNA promoter region methylation and gene expression.<sup>27</sup> We also characterized the methylation effect on OS. The prognostic predictive value of DNA methylation CpG sites on POLDs was analyzed by the TCGA database-based methylation-prognosis visualization web tool MethSurv (<https://biit.cs.ut.ee/methsurv/>).<sup>28</sup>

### Prognostic Signature Based on Four POLDs

To comprehensively appraise the prognostic predictive value of the POLDs, we employed the public resource-based survival assessment platform SurvExpress (<http://bioinformatica.mty.itesm.mx:8080/Biomatec/SurvivaX.jsp>) to determine the characteristics of the signature.<sup>29</sup> We chose the LIHC-TCGA-Liver hepatocellular carcinoma database (n=361) and Hoshida Golub Liver GSE10186 database (n=80) to evaluate the POLD signature. Based on the multivariate Cox analysis, the coefficient (coef) value of each POLD was determined, and then each patient obtained a risk score generated by the mRNA expression of POLDs and the coef value. Subsequently, patients were divided into high- and low-risk groups, K-M curves were conducted to illustrate the different OS between the two groups, and receiver operating characteristic (ROC) curves were used to evaluate the survival curves.

## POLDs Related Genes and Pathways

POLD-related genes were generated with UALCAN (<http://ualcan.path.uab.edu/index.html>).<sup>22</sup> We downloaded the coexpressed genes to each POLD, and the cut-off value was  $|R| > 0.3$ . Gene enrichment annotation was also performed on GO molecular functions, GO biological processes, GO cellular components and KEGG pathways by Metascape (<http://metascape.org>).<sup>30</sup>

## POLDs Related Immunocytes

Immunocytes and immune microenvironment have important impacts on tumorigenesis and patient prognosis.<sup>31</sup> We evaluated the potential association between POLDs and immunocytes by TIMER (<https://cistrome.shinyapps.io/timer/>).<sup>32</sup> In addition, we evaluated the correlation of POLD expression and immune checkpoint levels by ENCORI (<https://starbase.sysu.edu.cn/index.php>).<sup>33</sup>

## Immunohistochemical Staining

Tissue microarrays containing 10 HCC samples and 10 adjacent normal liver samples with associated clinicopathological information were purchased from Shanghai Outdo Biotech Co. Ltd. (Cat# HLivH020PG02-M-255, China). Immunohistochemical staining (IHC) assays were performed on a tissue microarray using anti-POLD1 antibody (Cat#383045, dilution 1:200, ZEN-BIO, Chengdu, China), anti-POLD3 antibody (Cat#bs-2755R, dilution 1:200, Bioss, Boston, USA) and anti-PDL1 antibody (Cat#GB14132, dilution 1:200, Servicebio, Wuhan, China). The IHC staining score (H-score) can be divided into two parts: staining intensity and percentage of positive tumor cells. Staining intensity was classified as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). The percentage of positive tumor cells was classified as 1+ ( $\leq 25\%$ ), 2+ (26–50%), 3+ (51–75%), and 4+ ( $> 75\%$ ). The final expression scores were calculated by multiplying the two variables together.

## Prediction of Potential Target Drugs

The potential treatment drugs prediction for hepatocellular carcinoma was also evaluated in the current study. We compared the clinical response of common chemotherapy drugs used in hepatocellular carcinoma via the “MOVICS” packages.<sup>34</sup> Moreover, we also searched for new drugs through the GSEA online website.<sup>35</sup>

## Statistical Analysis

Univariate Cox regression analysis was used to assess the CpG site effect on OS. Multivariate Cox regression analysis of POLDs was performed in OS to generate the coef and risk score formula. K-M curves were generated to compare the different survival results in different groups, while ROC curves were used to validate the efficiency of the POLD signature. A P value less than 0.05 was considered a significant result in all cases.

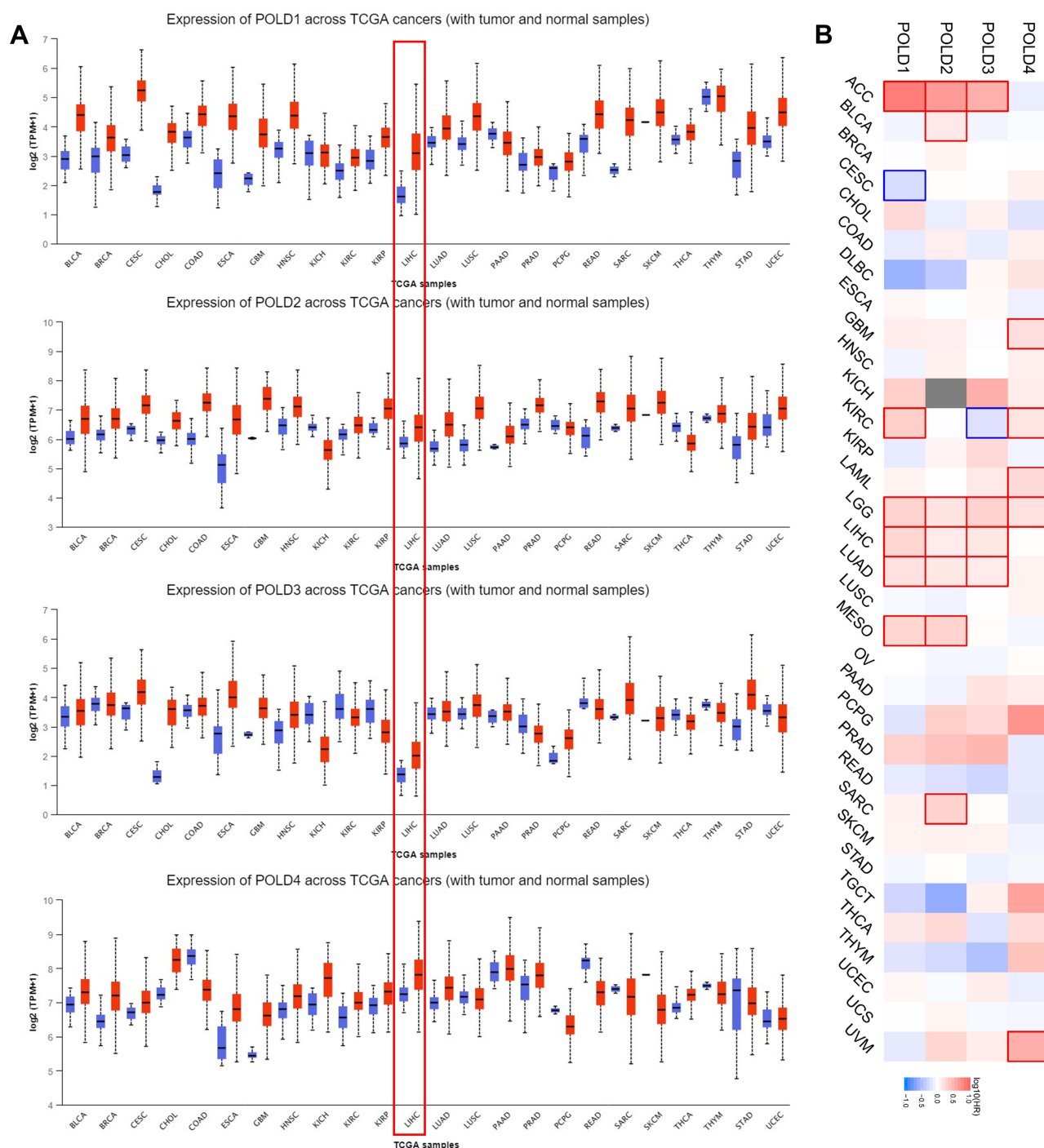
## Results

### POLDs Increased in Tumor Tissues and Indicated Poor Prognosis in Pan Cancer

We evaluated the expression of POLDs in tumor tissues compared with normal tissues across cancers and revealed that POLDs were elevated in most cancer types, POLD1 (20/24, 83.33%), POLD2 (18/24, 75.00%), POLD3 (10/24, 41.67%), and POLD4 (14/24, 58.33%), especially in HCC patients (Figure 1A). Furthermore, we evaluated the prognostic value of overall survival of POLDs and observed that the increased expression of POLDs was associated with poor clinical outcome in several cancer types, especially ACC, LGG, LIHC, LUAD, and MESO (Figure 1B).

### POLDs Increased Significantly in Hepatocellular Carcinoma and Correlated with Poor Prognosis

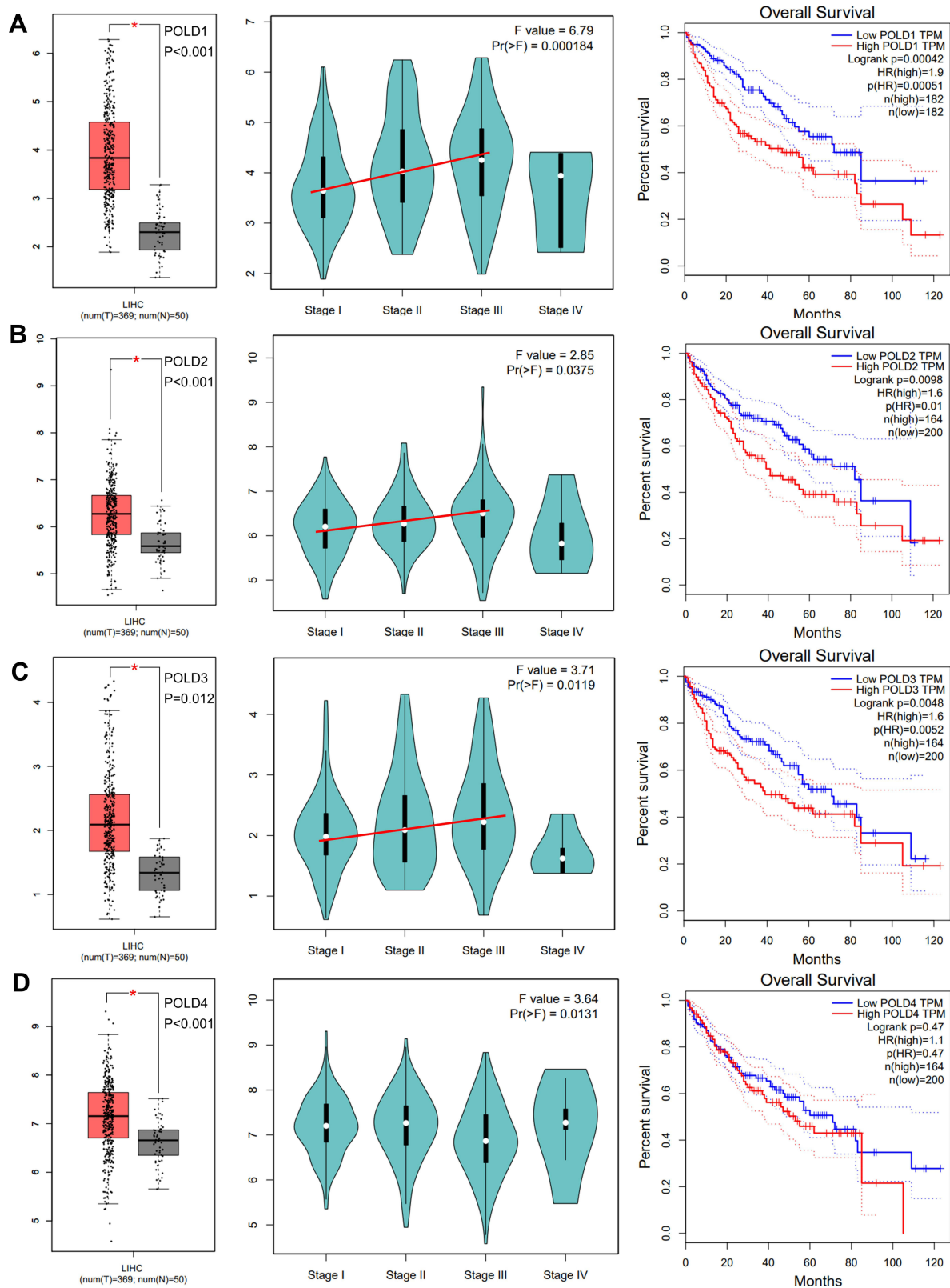
Based on the mRNA expression data of HCC tissue and adjacent normal tissue, we evaluated the difference in POLDs and overall survival. POLD1 increased dramatically in tumor tissues compared to normal tissues and increased with advanced tumor stage ( $P < 0.001$ ), while high POLD1 expression also indicated poor OS ( $P < 0.001$ ) (Figure 2A).



**Figure 1** Expression and prognostic value of POLDs in pancreatic cancer: **(A)** Expression of POLDs among tumor and normal tissues in pancreatic cancer; **(B)** Prognostic value of POLDs in pancreatic cancer.

Regarding POLD2, increased expression was also observed in tumor tissues, as well as advanced tumor stage ( $P=0.0375$ ). High POLD2 expression was associated with poor OS ( $P<0.001$ ) (Figure 2B). POLD3 expression in tumor tissues was higher than normal, and the evaluated level was associated with tumor stage ( $P=0.0119$ ) and linked to unfavorable OS ( $P<0.001$ ) (Figure 2C). However, we only observed the increased mRNA level of POLD4 in tumors, not the story with advanced tumor stage and OS (Figure 2D).

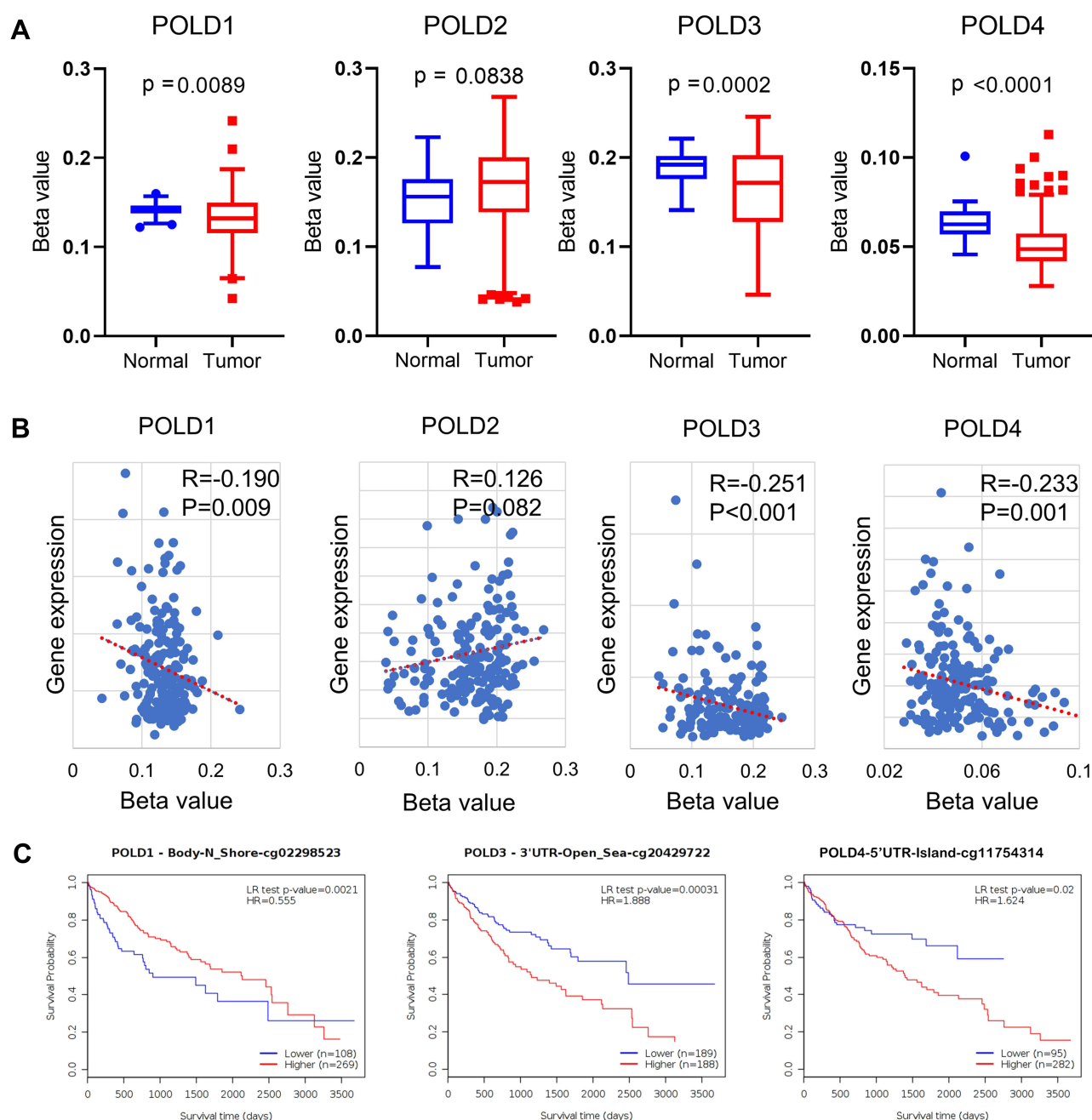




**Figure 2** mRNA expression and prognostic value of POLDs. The increased mRNA expression of POLD1 (A), POLD2 (B), and POLD2 (C) in tumor tissues and advanced tumor stage indicated poor prognosis but not POLD4 (D). \*P < 0.05.

## POLDs Methylation Affects Gene Expression and Prognosis

To study the potential reason for the increased mRNA expression of POLDs in tumor tissue, we evaluated the methylation status of the promoter regions of POLDs. As shown in Figure 2A, we found that promoter methylation decreased in tumor tissues compared to normal tissues in POLD1 ( $P=0.0089$ ), POLD2 ( $P=0.0002$ ), and POLD4 ( $P<0.0001$ ) but not POLD2 ( $P=0.0838$ ) (Figure 3A). The methylation  $\beta$  score in the promoter region was negatively associated with the gene expression of POLD1 ( $R=-0.190$ ,  $P=0.009$ ), POLD3 ( $R=-0.251$ ,  $P<0.001$ ), and POLD4 ( $R=-0.233$ ,  $P=0.001$ ) (Figure 3B). Furthermore, we assessed how CpG site methylation affects the OS of hepatocellular carcinoma patients. We found that methylation of cg02298523 and cg1906387 in POLD1 was linked to favorable OS, as well as the methylation of cg00453717 and cg01173485 sites in POLD3. Meanwhile, methylation of the cg20428722,



**Figure 3** POLDs methylation affects gene expression and prognosis. **(A)** The methylation  $\beta$  value of the POLDs promoter region compared in normal and tumor tissues; **(B)** The correlation between the methylation  $\beta$  value of the POLDs promoter and gene expression; **(C)** The representative K-M plotter for the OS of HCC patients with different methylation status of CpG sites.

**Table 1** The Significant Prognostic Value of CpG Sites in POLDs

CpG Site	HR	95% CI	P value
POLD1-N_Shore-Body-cg02298523	0.592	(0.405–0.865)	0.006768
POLD1-N_Shore-TSS1500-cg19063397	0.645	(0.444–0.936)	0.020856
POLD3-Open_Sea-Body-cg00453717	0.603	(0.418–0.871)	0.007026
POLD3-N_Shore-TSS1500-cg01173485	0.601	(0.412–0.876)	0.008095
POLD3-Open_Sea-3'UTR-cg20429722	1.888	(1.329–2.682)	0.00039
POLD3-Open_Sea-Body-cg20469507	1.831	(1.272–2.634)	0.001124
POLD3-Open_Sea-Body-cg26564815	1.833	(1.256–2.675)	0.001673
POLD3-S_Shore-Body-cg26717758	1.725	(1.080–2.753)	0.02245
POLD4-Island-1stExon-Body-5'UTR-cg11754314	1.624	(1.060–2.487)	0.025802
POLD4-S_Shore-TSS200-Body-cg18235480	1.415	(1.004–1.994)	0.047599

cg20469507, cg26564815, and cg26717758 sites in POLD3 was associated with poor OS, and methylation of the cg11754314 and cg18235480 sites indicated poor OS (Table 1, Figure 3C).

## Genetic Alterations of POLDs and the Effect of POLDs in Patients with Different Tumor Mutant Burdens

With the help of OncoPrinter, we analysed the frequency of genetic alterations among the POLDs. The overall genetic alteration rate was very low in POLDs; for example, only approximately 1.2% of patients had genetic alterations in POLD4 (Figure 4A). Due to the pivotal role of POLDs in DNA replication and repair, we appraised their effect on OS in HCC patients with high and low tumor mutational burdens and found that higher expression of POLDs was significantly linked to poor prognosis in patients with a high mutational burden, especially POLD1 ( $P=0.0022$ ) and POLD3 ( $P=0.04$ ) (Figure 4B), but not in patients with a low mutational burden (Figure 4C).

## United Prognosis Predicting Value of Four POLDs

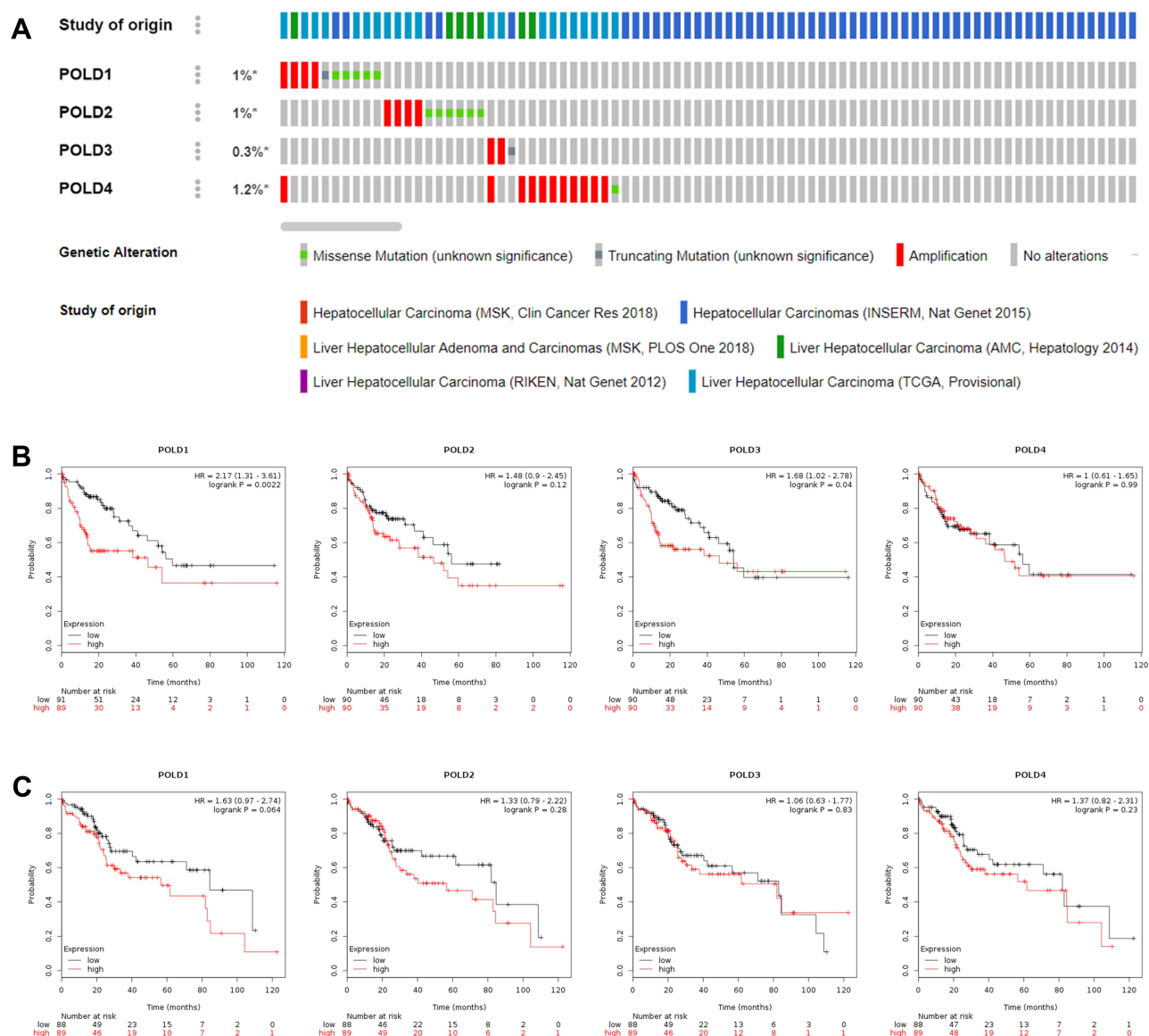
To estimate the overall predictive value of POLD for OS, we employed SurvExpress to generate the results. For patients in the TCGA-LIHC database, the risk score was generated by the coef of each POLD with the different expression levels for each patient. Patients in the high-risk group illustrated higher mRNA levels of POLD1 ( $P<0.001$ ), POLD2 ( $P<0.001$ ) and POLD3 ( $P<0.001$ ) than those in the low-risk group (Figure 5A and B), which is consistent with the expression pattern in tumor and normal tissues. We also observed that a high risk indicated an unfavorable OS ( $HR=2.56$ , 95% CI: 1.71–3.84,  $P<0.001$ ) (Figure 5C), and the AUC value was 0.655 (Figure 5D). Moreover, we also detected the predictive value of POLDs in the GSE10186 database, and similar results were obtained. Patients with high-risk scores also had high levels of POLD1 ( $P=0.0269$ ), POLD2 ( $P<0.001$ ), and POLD3 ( $P<0.001$ ) (Figure 5E and F). The high risk was also associated with poor prognosis in GSE10186 patients ( $HR=2.17$ , 95% CI: 1.05–4.47,  $P=0.03576$ ), while the AUC value was 0.709 (Figure 5G and H).

## Enrichment Annotation Among POLDs Coexpressed Genes

We downloaded the POLDs coexpressed genes from the UCLAN online database with the cut-off value of  $|R|>0.3$ . Finally, 6468 genes were reported to be associated with POLD1, 1247 genes with POLD2, 7094 genes with POLD3 and 47 genes with POLD4. We merged the four groups and chose the genes correlated with at least three POLDs to the gene pool, and 1009 POLD coexpressed genes were chosen for the next step of enrichment annotation (Figure 6A). The enrichment annotation results showed that POLDs mostly affected the cell cycle, DNA repair, DNA replication, chromosome segregation and cell cycle regulation pathways (Figure 6B and C).

## POLDs Link with Immunocytes and Immunotherapy

We evaluated the association between POLDs and immunocytes and observed that POLD1 and POLD3 were significantly associated with the infiltration of immunocytes, especially B cells (POLD1,  $R = 0.468$ ,  $P < 0.001$ ; POLD3,

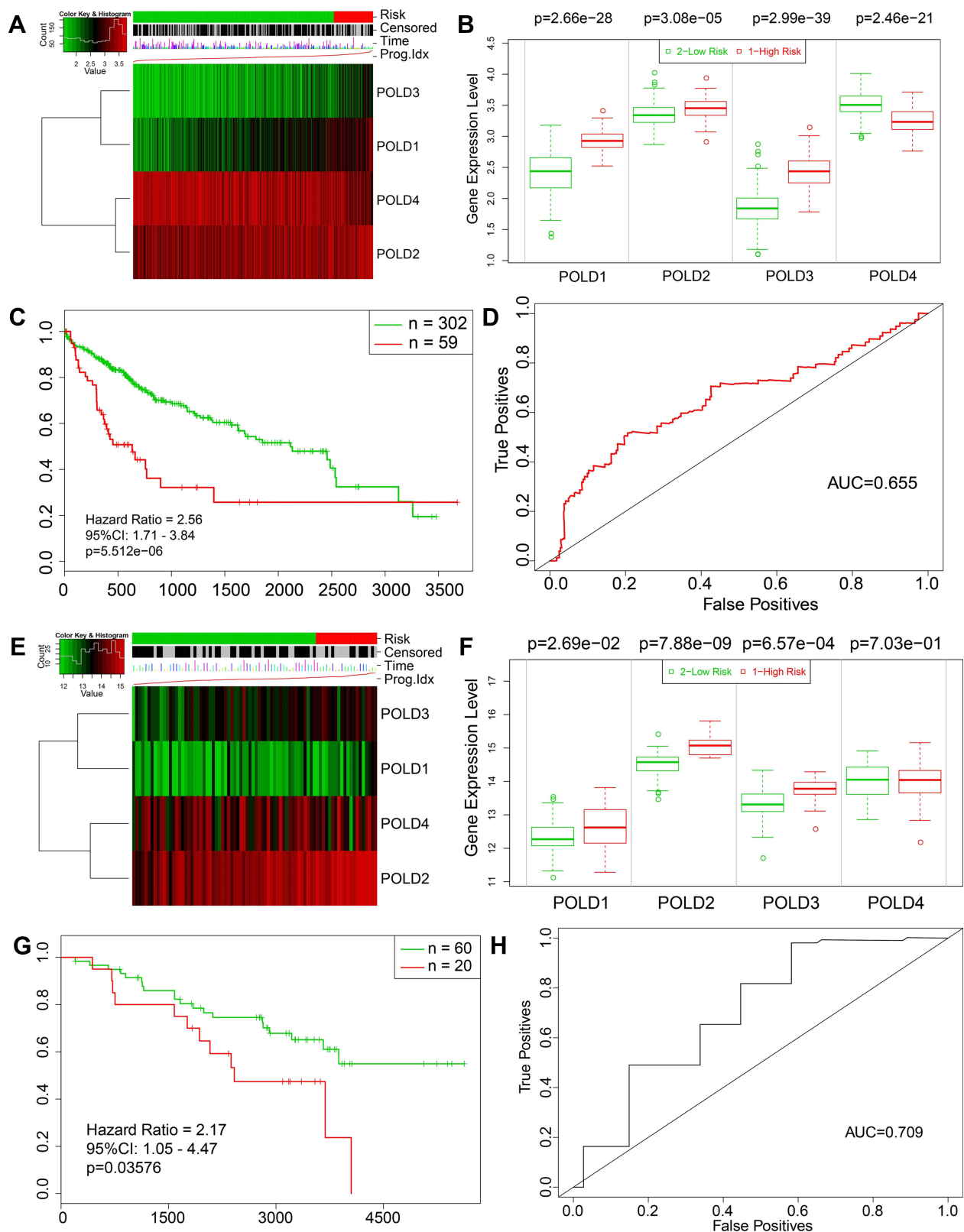


**Figure 4** Genetic alterations of POLDs and the effect of POLDs in patients with different tumor mutational burdens. **(A)** The frequency of genetic alterations among the POLDs; **(B)** The OS prognosis of POLDs with a high tumor mutation burden; **(C)** The OS prognosis of POLDs with a low tumor mutation burden. \* $P < 0.05$ .

$R = 0.442$ ,  $P < 0.001$ ) and macrophages (POLD1,  $R = 0.397$ ,  $P < 0.001$ ; POLD3,  $R = 0.504$ ,  $P < 0.001$ ) (Figure 7A). Meanwhile, POLD2 and POLD3 did not show a significant association with the infiltration of immunocytes (Figure S1). In addition, we also assessed and observed that POLD1 (PDCD1,  $R = 0.318$ ,  $P < 0.001$ ; CD274,  $R = 0.172$ ,  $P < 0.001$ ; PDCD1,  $R = 0.277$ ,  $P < 0.001$ ) and POLD3 (PDCD1,  $R = 0.277$ ,  $P < 0.001$ ; CD274,  $R = 0.278$ ,  $P < 0.001$ ; PDCD1,  $R = 0.232$ ,  $P < 0.001$ ) were significantly positively associated with the expression of immune checkpoints (Figure 7B).

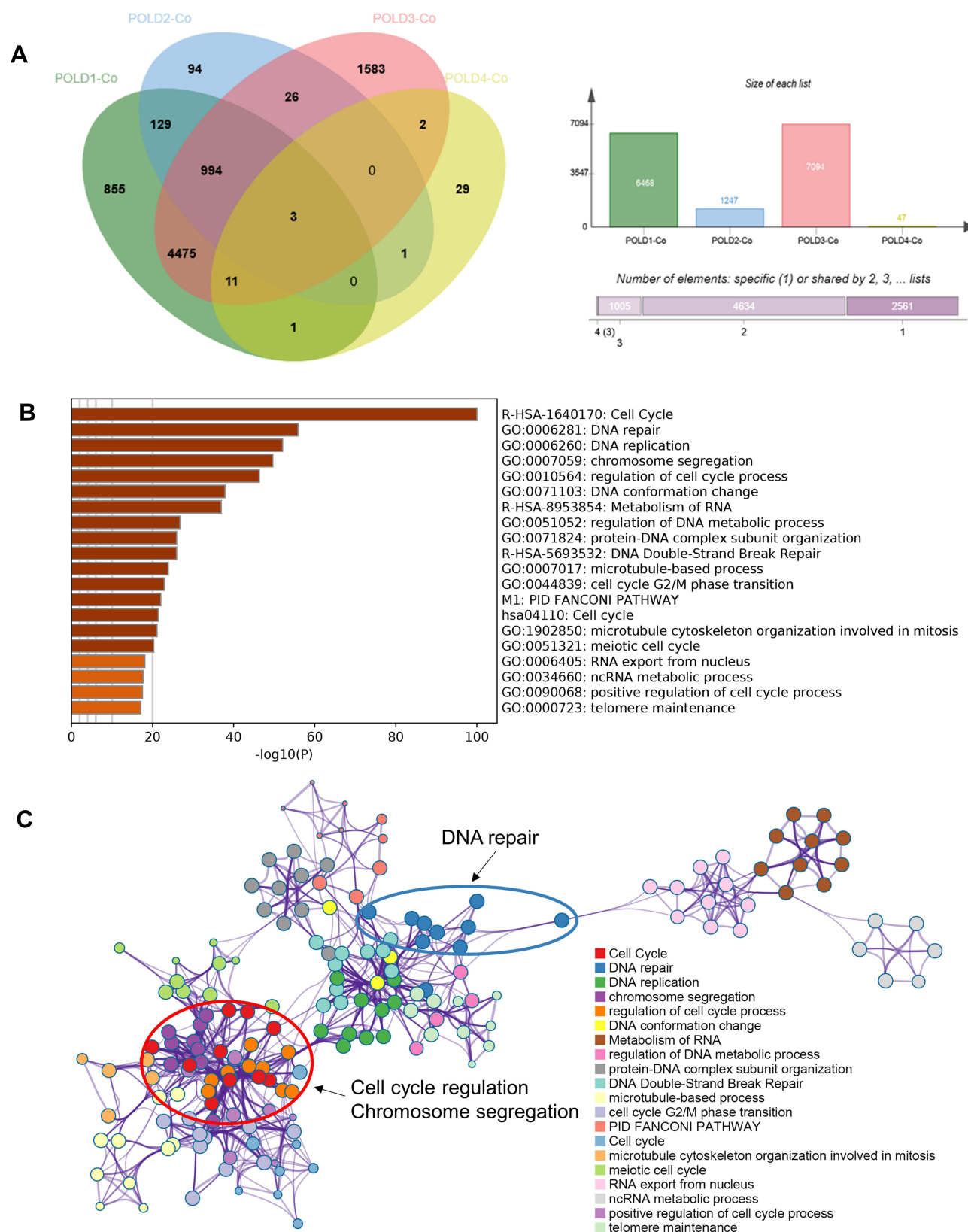
## Potential Drugs Targeting the POLDs for Treatment

We revealed that patients in the POLDs-determined high-risk group were more suitable for chemotherapy, which is commonly applied in clinical treatment, including 5-fluorouracil, cisplatin, doxorubicin, gemcitabine, and sorafenib (all  $P < 0.001$ , Figure 8). Moreover, we also searched for potential new drugs via the GSEA online website, and the top effective drugs for POLDs are listed in Table 2. The negative correlation indicated that the higher the level of POLDs was, the more sensitive the drug was to the target drug.

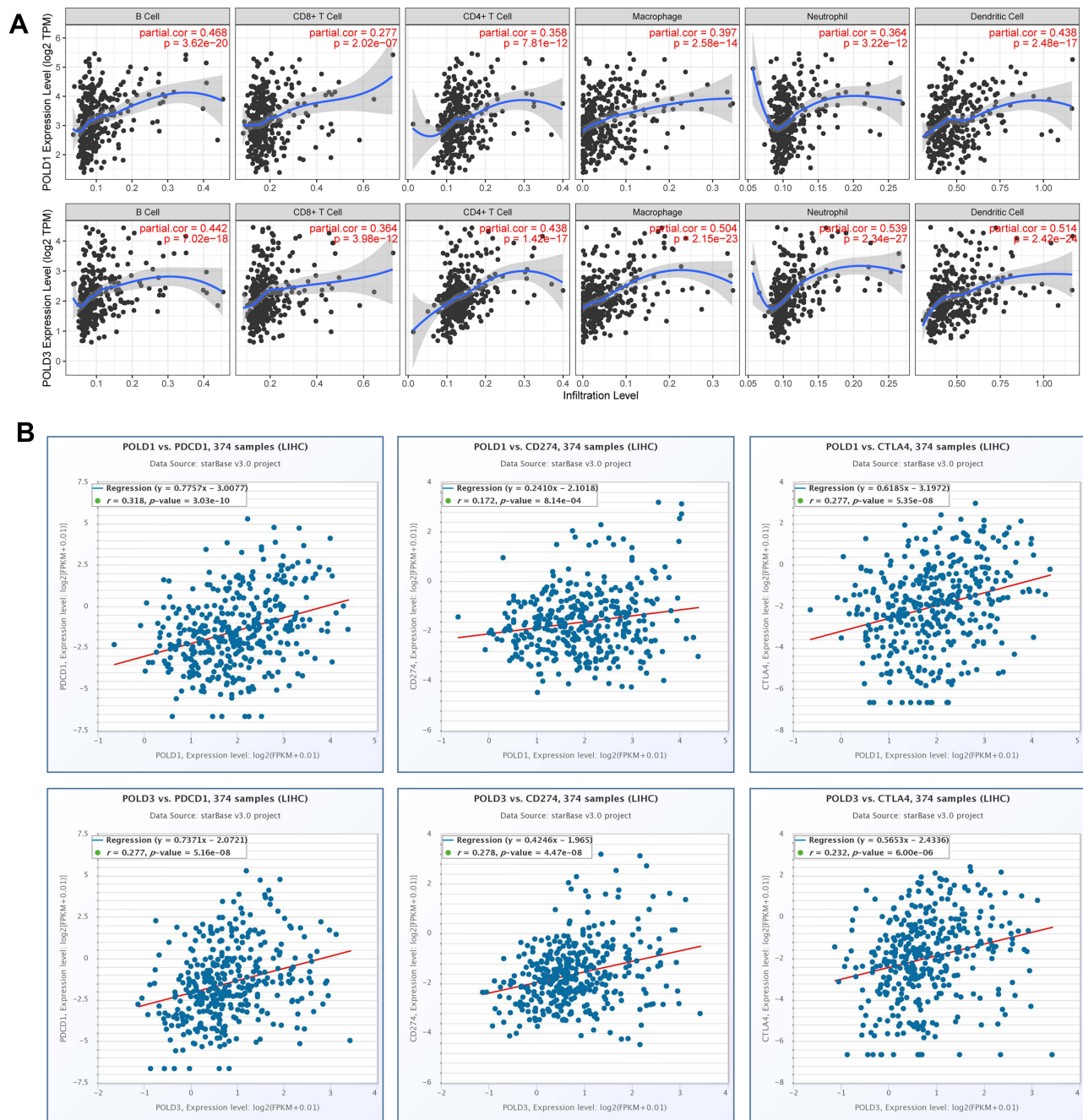


**Figure 5** The comprehensive prognostic value of four: (A) Heatmap of POLDs expression in low- and high-risk patients in TCGA-LIHC; (B) The differential expression of POLDs in low- and high-risk patients in TCGA-LIHC; (C) K-M plotter showing the comprehensive prognostic value of POLDs in TCGA-LIHC; (D) ROC curve showing the prognostic accuracy of POLDs in TCGA-LIHC. (E) Heatmap of POLDs expression in low- and high-risk patients in GSE10186. (F) Differential expression of POLDs in low- and high-risk patients in GSE10186. (G) K-M plotter showing the comprehensive prognostic value of POLDs in GSE10186. (H) ROC curve showing the prognostic accuracy of POLDs in GSE10186.





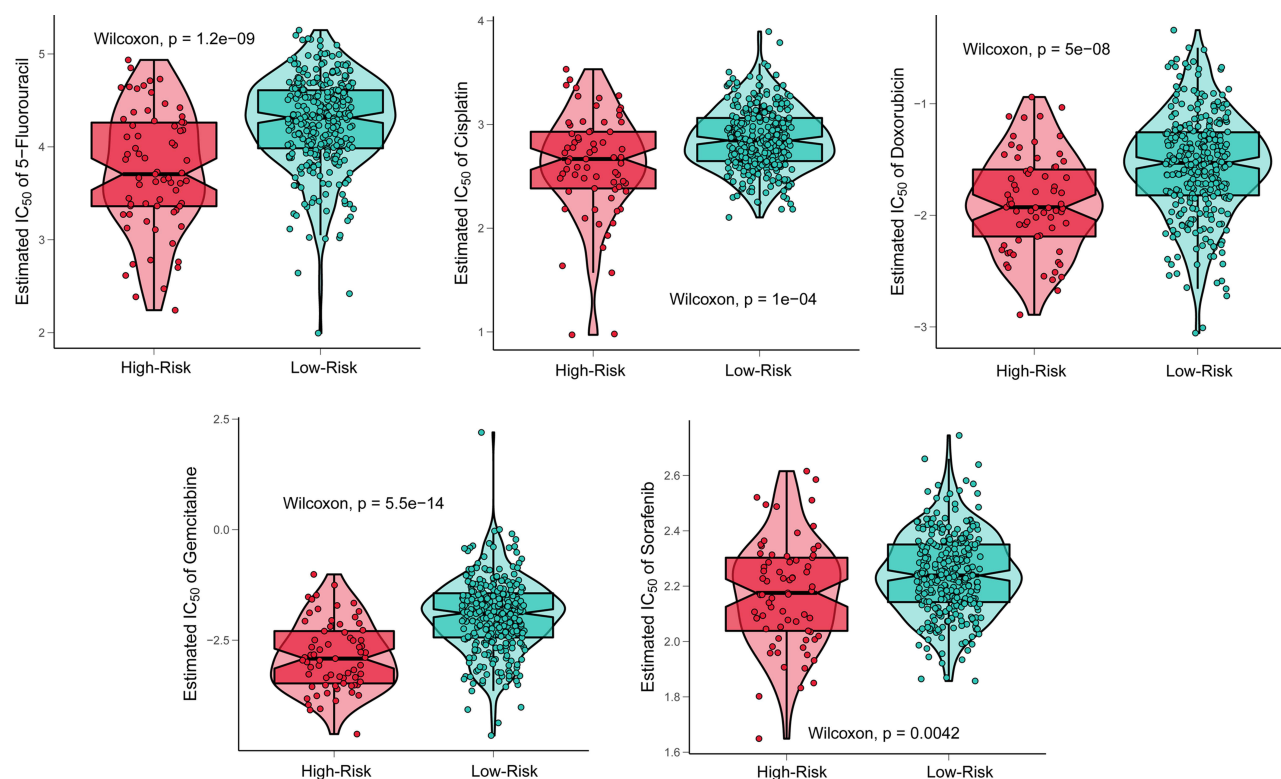
**Figure 6** Enrichment annotation among POLDs coexpressed genes. **(A)** The numbers of each POLD coexpressed gene obtained from the UCLAN online database. **(B and C)** Pathway enrichment of POLD coexpressed genes.



**Figure 7** POLDs expression with immune microenvironment. **(A)** Correlation between POLD1, POLD3 and six immunocytes; **(B)** Correlation between POLD1, POLD3 and the immune checkpoints PDCD1, CD274 and CTLA4.

## Validation of the Expression of POLD1 and POLD3 in a Tissue Microarray

To further confirm the expression of POLDs in HCC, we employed a tissue microarray, and the proteins POLD1 and POLD3 were detected due to their higher index in the prognostic formula. Notably, we observed a higher level of POLD1 protein in HCC tissues than in adjacent normal liver tissues ( $P = 0.0109$ , Figure 9A), as well as a higher protein level of POLD3 ( $P = 0.0009$ , Figure 9B). Moreover, we also observed an increased level of POLD1 in stage III HCC tissues compared with stage I–II HCC tissues ( $P = 0.0027$ , Figure 9D). In the above mentioned results, we revealed the positive correlation of POLD1/3 and PD-L1, implicating potential links with immunotherapy. We added IHC staining of PD-L1



**Figure 8** Response to common chemotherapy drugs in POLDs risk groups.

and further confirmed the positive correlation between POLD1 and PD-L1 ( $R = 0.314$ , Figure 9E) and between POLD3 and PD-L1 ( $R = 0.788$ , Figure 9F).

## Discussion

DNA duplication is a pivotal process in cell proliferation that can transmit and pass genetically hereditary information. DNA damage response pathways are the safeguard of genetic replication in cells, while DNA polymerase  $\delta$  works in both DNA replication and repair processes.<sup>36</sup> Alterations in POLDs in humans are associated with several disorders. Ferrer-Avargues et al<sup>37</sup> reported a novel Leu474Pro mutation in POLD1 associated with the increased occurrence of colorectal cancer among Spain families. Elouej et al<sup>38</sup> reported mandibular hypoplasia, deafness, progeroid features, and lipodystrophy syndrome caused by Ile1070Asn in the ZnF2 (Zinc Finger 2) domain of the POLD1 protein. Zhou et al<sup>39</sup> found that Pold3 plays important roles in DNA double strand break repair, telomere maintenance and genomic stability of both embryonic stem cells and spermatocytes in mice. Hung et al<sup>40</sup> revealed that the decrease in POLD4 leads to blockade of the cell cycle at G1-S by suppression of the AKt-Skp2-p27 pathway. Therefore, POLDs play an important role in cell progression and tumorigenesis.

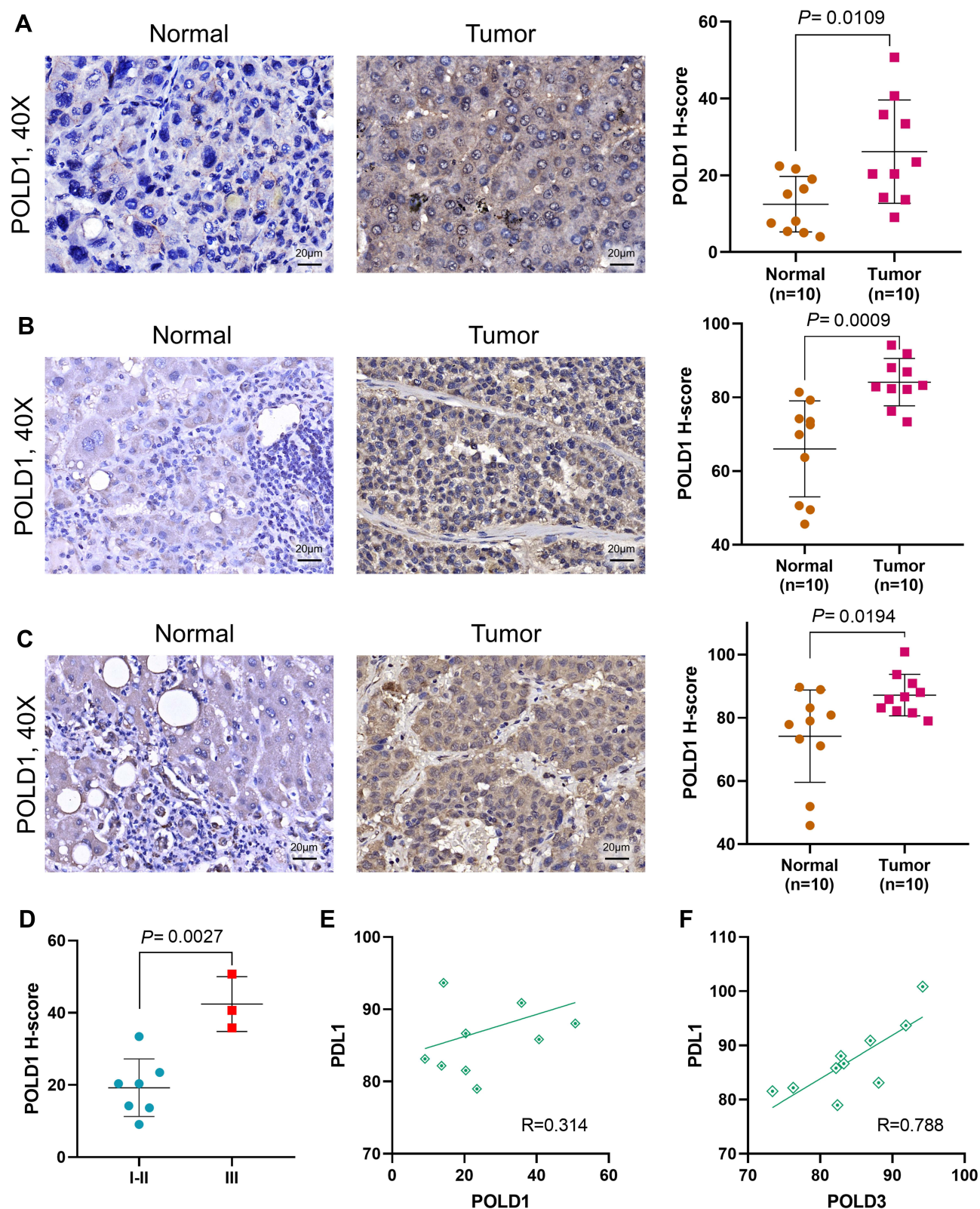
The association between HCC risk and DNA replication and repair has been widely reported. Shen et al<sup>41</sup> reported that facilitating chromatin transcription complex (FACT), a DNA repair-related and transcription-related histone chaperone, could promote the progression of HCC through the NRF2-driven oxidative stress response. Ji et al<sup>42</sup> exposed the deficiency of histone deacetylase 3 (HDAC3) promote HCC via the defect of H3K9ac/H3K9me3 transition, HDAC3 is the essential components for DNA damage control. To maintain the stability of genetic information, the stability of the replication fork is very important in the process of DNA replication, which can strongly inhibit the development of cancer. Ge et al<sup>43</sup> found that ubiquitin conjugate BIR repeat containing ubiquitin-conjugating enzyme (BRUCE) down-regulation in HCC promotes the activation of ATR and the phosphorylation of its downstream targets CHK1 and RPA, subsequently leading to the inhibition of tumorigenesis in HCC.

**Table 2** Potential Target Drugs for POLDs Treatment

Gene Symbol	Drug Name	Correlation	FDR
POLD1	BRD-K30748066	-0.45	<0.01
POLD1	Teniposide	-0.44	<0.01
POLD1	Cytarabine hydrochloride	-0.42	<0.01
POLD1	GSK461364	-0.41	<0.01
POLD1	Clofarabine	-0.41	<0.01
POLD1	Docetaxel	-0.41	<0.01
POLD1	Triazolothiadiazine	-0.41	<0.01
POLD1	CD-437	-0.4	<0.01
POLD1	GSK-J4	-0.39	0.01
POLD1	BI-2536	-0.39	<0.01
POLD2	Dinaciclib	-0.16	<0.01
POLD2	SB-743921	-0.15	<0.01
POLD2	GW-843682X	-0.15	<0.01
POLD2	Triazolothiadiazine	-0.15	<0.01
POLD2	Methotrexate	-0.15	<0.01
POLD2	Valdecixib	-0.15	<0.01
POLD2	Docetaxel	-0.15	0.01
POLD2	BI-2536	-0.14	<0.01
POLD2	Vincristine	-0.14	<0.01
POLD2	Tivantinib	-0.13	0.02
POLD3	GSK-J4	-0.42	<0.01
POLD3	BRD-K01737880	-0.35	0.04
POLD3	Teniposide	-0.34	<0.01
POLD3	Clofarabine	-0.29	<0.01
POLD3	Cytarabine hydrochloride	-0.28	<0.01
POLD3	Topotecan	-0.27	<0.01
POLD3	Decitabine	-0.27	<0.01
POLD3	COL-3	-0.27	<0.01
POLD3	Necrosulfonamide	-0.27	<0.01
POLD3	Gemcitabine	-0.26	<0.01
POLD4	Dasatinib	-0.22	<0.01
POLD4	Saracatinib	-0.19	<0.01
POLD4	Trametinib	-0.15	0.02
POLD4	IC-87114	-0.14	0.01
POLD4	Vandetanib	-0.11	0.01
POLD4	Pluripotin	-0.1	0.04
POLD4	Selumetinib	-0.09	0.05
POLD4	PD318088	-0.09	0.05

In the current study, we examined the predictive value and potential mechanism of POLDs in HCC. POLD1-4, one catalytic subunit and accessory subunit, are all highly expressed in HCC tumor tissues compared to adjunct normal tissues. For POLD1-3, the increased mRNA level was also positively associated with the advancement of tumors, as well as OS prognosis. Methylation in the promoter of POLDs is also different in tumors and normal tissues, which affects mRNA expression and OS, especially for some specific CpG sites. Because POLDs play a pivotal role in DNA replication and repair, we generated results that could preferably predict the prognosis for patients who suffer from a high gene mutation burden. Then, we evaluated the combined prognostic predictive value of four POLD subunits in both the TCGA-LIHC and GSE10186 databases and recognized the statistically significant HR of the high-risk group, along with the reliable predictive value. We also confirmed the increased protein levels of POLD1 and POLD3 in HCC tissues compared with adjacent normal tissues with a tissue microarray, validated the positive correlations among PD-L1 and POLDs, and provided insight into POLDs and immunotherapy. By exploring the potential mechanism impacted by





**Figure 9** Validation of POLD1, POLD3 and PD-L1 levels in tissue microarray. (A) Protein level of POLD1 in HCC and normal liver tissues; (B) Protein level of POLD3 in HCC and normal liver tissues; (C) Protein level of PD-L1 in HCC and normal liver tissues; (D) Protein level of POLD1 in Stage I-II and Stage III HCC tissues; (E) Correlation between POLD1 and PD-L1 expression; (F) Correlation between POLD3 and PD-L1 expression.



POLDs in HCC patients, we obtained coexpressed gene sets and annotated the related pathways. As expected, POLDs coexpressed was mostly enriched in DNA repair and cell cycle regulation pathways.

In summary, POLDs are essential predictive factors for the prognosis of HCC. The united signature could precisely identify unfavorable prognoses.

## Data Sharing Statement

The data used to support the findings of this study are included within the article. The gene expression data can be accessed on The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO).

## Ethics Approval and Consent to Participate

The need for further ethics approval was waived by The First Affiliated Hospital of Shantou University Medical College Ethics Committee.

## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

## Disclosure

The authors report no conflicts of interest in this work.

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