DNA replication stress stratifies prognosis and enables exploitable therapeutic vulnerabilities of HBV-associated hepatocellular carcinoma: An in-silico precision oncology strategy

Xiaofan Lu,1,2,11 Jialin Meng,3,11 Haitao Wang,3,11,# Yujie Zhou,3 Jian-Guo Zhou,3,6 Xinjia Ruan,1,8 Yi Chen,1 Yuqing Ye,1 Liwen Su,1 Xiaole Fan,9 Hangyu Yan,1 Liyun Jiang,1,10,* and Fangrong Yan1,*

*Correspondence: lijang.cpu@foxmail.com (L.J.); f.r.yan@outlook.com (F.Y.)
Received: February 20, 2023; Accepted: May 12, 2023; Published Online: June 6, 2023; https://doi.org/10.59717/j.xinn-med.2023.100014
© 2023 The Author(s). This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

GRAPHICAL ABSTRACT

PUBLIC SUMMARY
- Host genome-integrated hepatitis B virus (HBV) causes chronic DNA replication stress.
- Prognostic DNA replication stress contributes heterogeneity of HBV+ hepatocellular carcinoma (HCC).
- A tailored prognostic index ($P_{RS}$) improves population-based prognostication.
- $P_{RS}$ enables exploitable therapeutic vulnerabilities.
- Four therapeutic targets and five agents were identified for HBV+ HCC.
Novel therapies target DDR and often replication stress. Replication stress exacerbates genome instability response (DDR) which prompts DNA repair, cell cycle checkpoints, and enrichment in DNA replication stress, cell cycle pathways, and increased responsiveness to immunotherapy. Conversely, higher $P_{\text{RS}}$ scores correlated with elevated Ki-67 marker, cancer stemness, and enrichment in DNA replication stress, cell cycle pathways, and chromatin remodelers, resulting in an ‘immune-cold’ phenotype and unfavorable clinical outcomes. Through large-scale in-silico drug screening, potential therapeutic targets (TOP2A, PRMT1, CSNK1D, and PP2H) and five agents, including topoisomerase and CDK inhibitors, were identified for patients with high $P_{\text{RS}}$ scores. These findings hold promise for optimizing therapeutic strategies in HCC and providing insights into the management of HBV carriers. In summary, our machine-learning approach yielded $P_{\text{RS}}$ as a powerful predictor for assessing prognosis in HBV-associated HCC. This analytic framework improves population-based therapeutic strategies, facilitates personalized treatment, and ushers in a new era of precision medicine in HCC.

RESULTS

Study design

A total of 606 cases of HBV-associated HCC were included from five clinical cohorts, with complete clinical follow-up available for 536 patients. Detailed information is summarized in Table S1, and the entire study design is delineated in Figure 1.

Development of a nine replication stress-related gene-based $P_{\text{RS}}$ in HBV-associated HCCs

Based on genes within 21 replication stress signatures, we preliminarily identified 302 replication stress-related genes that were tightly associated with overall survival (OS) ($P<0.01$). To enhance the prognostic robustness, a bootstrap approach was conducted, resulting in 69 genes ($P<0.01$ in more than 80% resampling processes). Subsequently, random survival forest further narrowed down the list to a final panel of nine genes with the largest C-index (Figure S2, Table S2). A score of $P_{\text{RS}}$ was then individually calculated, ranging from 0 to 10. We developed the R package “hccPIRS” to calculate $P_{\text{RS}}$ from a single-sample perspective, which is documented and freely available at https://github.com/xlucpu/hccPIRS.

Evaluation and validation of the prognostic potential of $P_{\text{RS}}$

We reasoned that the area under the receiver operating characteristic (ROC) curves was eligible (Figure 2A-D) given the time-dependent ROC curve at 1-, 3- and 5-year survival. Univariate and multivariate Cox regression were considered to construct a multivariate model (Figure 2E). In this manner, we...
Discovery cohort from TCGA-LIHC
Primary tumors: n=359
Adjacent normal tissues: n=50

Virus detection by VirusSeq algorithm

103 HBV-associated HCCs with expression of 4 HBV oncoproteins

Screening of prognostic genes
i. Extract DNA replication stress-related genes
ii. Univariate Cox proportional hazards regression
iii. Bootstrap resampling to enhance prognostic robustness
iv. Random survival forest with variable hunting procedure
v. Independent searching for model with largest C-index

Development of a replication stress-related prognostic index ($P_{\text{Rs}}$)

Large-scale in silico screening for therapeutic targets and agents

Figure 1. Experimental design This study enrolled a total of 606 HBV-associated HCC cases and developed and validated a nine replication stress-related gene-based prognostic index ($P_{\text{Rs}}$), which was further leveraged to predict potential therapeutic targets and agents.

found that $P_{\text{Rs}}$ remained an independent prognostic factor after adjusting for other variables in the TCGA-LIHC (HR: 1.25, 95% confidence interval [CI]: 1.01−1.48, $P=0.01$), CN-LIHC (HR: 1.23, 95% CI: 1.07−1.43, $P=0.005$), and GSE14520 cohorts (HR: 1.14, 95% CI: 1.02−1.27, $P=0.024$) but did not reach significance in the LIRI-JP cohort, which was likely due to a relatively small sample size (HR: 1.16, $P=0.05$). Interestingly, alpha-fetoprotein (AFP) only served as an independent prognostic factor in CN-LIHC, which might question its robustness in predicting clinical outcomes in clinical settings. The simultaneous statistical significance and independent prognostic value of both the $P_{\text{Rs}}$ and pathological stage in most of the cohorts suggested that $P_{\text{Rs}}$ contributed additional predictive power beyond what was already accounted for by the pathological stage. Additionally, $P_{\text{Rs}}$ significantly stratified patients into low- and high-risk groups ($P_{\text{Rs}}$ and $P_{\text{Rs5}}$ according to the top tertile cutoff in TCGA-LIHC (HR: 5.07, 95% CI: 2.43−10.6, $P<0.001$; Figure 2F, Figure S3A), CN-LIHC (HR: 3.18, 95% CI: 1.87−5.38, $P<0.001$; Figure 2G, Figure S3B), LIRI-JP (HR: 3.96, 95% CI: 1.15−13.6, $P=0.018$; Figure 2H, Figure S3C) and GSE14520 (HR: 2.13, 95% CI: 1.38−3.3, $P<0.001$; Figure 2I, Figure S3D) cohorts. The expression landscape of the nine genes was also validated in the GSE121248 cohort (Figure S3E). Restrained mean survival (RMS) ratios ranging from 0.54 to 0.78 were observed in the different cohorts (Table S3).

To test the universal prognostic value of $P_{\text{Rs}}$, a general cutoff of 5.6 was determined using the top tertile $P_{\text{Rs}}$ among 606 HBV-associated HCCs. Using this cutoff, four of the cohorts were reseparated into $P_{\text{Rs}}^*$ and $P_{\text{Rs*}}^*$ groups, and a strong association existed between the general cutoff-based new groups and cohort-specific cutoff-based groups ($P<0.001$; Figure S4A), implying the universal applicability of $P_{\text{Rs}}$ in different cohorts/sequencing platforms (Figure S4B). Consistently, the $P_{\text{Rs*}}^*$ groups presented with significantly poorer OS than the matched $P_{\text{Rs*}}$ groups (all, $P<0.05$; Figure S4C-F).

We then compared $P_{\text{Rs}}$ to other prognostic signatures in HCC. After 10,000 resamplings, $P_{\text{Rs}}$ exhibited comparable predictive performance compared to other signatures in the TCGA-LIHC cohort but demonstrated comparable or superior performance in other HCC cohorts. Additionally, $P_{\text{Rs}}$ maintained stable predictive performance across different cohorts (all, C-index>0.6), while other signatures lost power in at least one cohort (Figure S5, Table S4).

Association among $P_{\text{Rs}}$, replication stress signatures and chromatin remodeling regulators

We showed that $P_{\text{Rs}}$ in the TCGA-LIHC cohort displayed significantly activated replication stress signatures at the transcriptome level (Figure S6). To further investigate transcriptomic differences, potential cancerous chromatin remodeling regulators were analyzed, reinforcing the biological pertinency of the top tertile cutoff due to the remarkably shifted regulon activity pattern (Figure S6). Chromatin remodeling-associated regulon activity highlighted other possible differential regulatory schemes, suggesting that transcriptional networks driven by the epigenome might be differentiators of great importance. Differential methylation analysis might further sustain the potential epigenetic differences between $P_{\text{Rs5}}$ and $P_{\text{Rs}}$ because $P_{\text{Rs5}}$ harbored increased hypermethylated promoters compared to $P_{\text{Rs}}$ (2.867 vs. 1.36, FDR<0.05; Table S5).

Investigation of $P_{\text{Rs}}$ associated clinical characteristics and biological processes

Considering that both TCGA-LIHC and CN-LIHC cohorts provide detailed...
clinicopathological information, we next investigated the association between \( P_{\text{RS}} \) and clinical variables. We found that a higher \( P_{\text{RS}} \) was tightly associated with higher AFP levels and more aggressive clinical stages, including pathological stage and Barcelona Clinic Liver Cancer (BCLC) staging systems (Figure 3, Table S5–7). Interestingly, we observed a mild and negative correlation between expression of the oncoproteins HBVgp2-5 (\( R=0.24, P=0.015 \)) and HBVgp3X (\( R=-0.28, P=0.005 \)) with \( P_{\text{RS}} \), as well as the HBV activity in the TCGA-LIHC cohort (\( R=-0.2, P=0.036 \); Figure S7).

To investigate the biological relevance of \( P_{\text{RS}} \), we first quantified the deregulation of cancer hallmarks using transcriptome-based Pathifier analysis based on TCGA-LIHC and CN-LIHC cohorts considering their available normal samples. We found that almost all hallmarks presented a significantly elevated degree of deregulation as \( P_{\text{RS}} \) increased (Figure 3). Given that Pathifier did not specify the deregulation direction, we next performed gene-level protein abundance-based gene set enrichment analysis (GSEA) in the CN-LIHC cohort; the results indicated that DDR-relevant and proliferation-level protein abundance-based gene set enrichment analysis (GSEA) in the CN-LIHC cohort (\( R=-0.2, P=0.036 \); Figure S7).

We observed a mild and negative correlation between expression of the oncoproteins HBVgp2-5 (\( R=0.24, P=0.015 \)) and HBVgp3X (\( R=-0.28, P=0.005 \)) with \( P_{\text{RS}} \), as well as the HBV activity in the TCGA-LIHC cohort (\( R=-0.2, P=0.036 \); Figure S7).

To investigate the biological relevance of \( P_{\text{RS}} \), we first quantified the deregulation of cancer hallmarks using transcriptome-based Pathifier analysis based on TCGA-LIHC and CN-LIHC cohorts considering their available normal samples. We found that almost all hallmarks presented a significantly elevated degree of deregulation as \( P_{\text{RS}} \) increased (Figure 3). Given that Pathifier did not specify the deregulation direction, we next performed gene-level protein abundance-based gene set enrichment analysis (GSEA) in the CN-LIHC cohort; the results indicated that DDR-relevant and proliferation-related hallmarks were significantly upregulated as \( P_{\text{RS}} \) increased, including G2-M checkpoint, E2F and MYC targets (Figure 3). Moreover, several metabolism-related processes were significantly activated as \( P_{\text{RS}} \) decreased, including glycolysis and oxidative phosphorylation (Figure 3). To validate the \( P_{\text{RS}} \)-relevant biological process across different cohorts, we conducted a random effects model (REM) meta-analysis based on differential expression between \( P_{\text{RS}} \) and \( P_{\text{RS}} \) tumors in five cohorts (Figure S8A). We then performed transcriptome-based GSEA using a pre-ranked gene list according to the summary fold change, the results of which verified the robustness of the underlying biology of \( P_{\text{RS}} \) (Figure S8B).

**Lower \( P_{\text{RS}} \) is tightly associated with activated immune/metabolism pathways and an increased likelihood of responding to immunotherapy**

Among 606 cases, Désert’s ECM/STEM and Boyault’s S3 subtypes exhibited significantly higher \( P_{\text{RS}} \) than other subtypes (both, \( P<0.001 \)), which consistently mirrored frequent TP53 mutations as \( P_{\text{RS}} \) increased (\( P<0.001 \); Figure 4A-B). Additionally, Désert’s peritumoral and Hoshida’s S3 subtype presented significantly lower \( P_{\text{RS}} \) compared to other categories within the corresponding classification system (both, \( P<0.001 \); Figure 4B).

Our previous study demonstrated that highly expressed human papillomavirus in cervical squamous cell carcinoma may stimulate the inflammatory/immune response of the host, leading to favorable prognosis.\(^{16} \) We therefore examined whether this situation could be mirrored in HBV-associated HCC cases. We quantified the infiltration level of 24 tumor microenvironment immune cells among 606 HCCs, and strikingly, we observed globally activated immunocyte infiltration in the \( P_{\text{RS}} \) **group (Figure 4A, Figure S9)**, which motivated us to investigate whether a lower \( P_{\text{RS}} \) was associated with a higher likelihood of responding to immunotherapy. Considering that immune checkpoint inhibitors are not yet approved for HCC management by regulatory agencies, we estimated the Tumor Immune Dysfunction and Exclusion (TIDE) prediction score, which represents the potential of tumor immune invasion (a higher value indicates reduced likelihood of benefiting from anti-PD1/CTLA4).\(^{17} \) We revealed that the \( P_{\text{RS}} \) group contained a significantly higher proportion of TIDE-predicted responders than the %D
group ($P<0.001$; Figure 4A). Additionally, $P_{\text{IRES}}$ displayed a significant and positive correlation with the TIDE prediction score in all five HCC cohorts (Figure 4D), suggesting that HBV-associated HCC patients with lower $P_{\text{IRES}}$ may respond to immune checkpoint inhibitors.

Additionally, the cancerous genomic landscape has been shown to be related to antitumor immunity; for instance, the presence of neoantigens triggers T-cell responses.\(^{15}\) whereas aneuploidy may cause immune evasion and attenuation of the immunotherapy response.\(^{15}\) In this context, we investigated the TCGA-LIHC cohort and found that $P_{\text{IRES}}$ exhibited a strong and negative correlation with the number of predicted neoantigens ($R=-0.38$, $P=0.027$; Figure S9B) but was positively correlated with the number of broad-level deletions ($R=0.44$, $P<0.001$; Figure S9C) and the fraction of lost genome ($R=0.28$, $P=0.005$; Figure S9D); no statistical significance was observed regarding the fraction of gained genome or broad-level amplification (both, $P>0.25$, not shown) using MOVICS pipeline.\(^{20}\) Integrative analysis between $P_{\text{IRES}}$ and gene-level copy number variation revealed that increased $P_{\text{IRES}}$ was globally correlated with copy number deletion (Figure 4D); specifically, copy number deletion of genes that showed strong correlation with $P_{\text{IRES}}$ ($R>0.5$, $P<0.05$) were frequently located in chromosome 1q and 8q (Table S8). Gene Ontology analysis on genes within these two regions demonstrated enrichment of immune response-related, and catabolic/metabolic processes in chromosome 1q and 8q, respectively (Figure 4E), suggesting impairment of these functional pathways in patients with high $P_{\text{IRES}}$.

Considering induction of the non-infiltrated phenotype in the $P_{\text{IRES}}^{\text{Cancer}}$ group, a recent study reported that the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) contributes to an “immune-cold” phenotype by inducing COX2/PGE2 and inhibiting the DNA-sensing innate immune response.\(^{21}\) NRF2 also accumulates in the nucleus and forms foci at DNA damage sites, leading to increased COX2/PGE2 production and impaired DNA-sensing immune response.
facilitating DDR and DNA repair. Therefore, we investigated the expression of NRF2 and COX2 in three of the cohorts (i.e., TCGA-LIHC, CN-LIHC, and GSE121248) in which both genes were matchable. We found that $P_{\text{RBS}}$ groups demonstrated significantly higher expression of NRF2 and its downstream marker COX2 in TCGA-LIHC ($P=0.009$ for NRF2; $P=0.001$ for COX2; Figure S9E) and CN-LIHC cohorts ($P<0.001$ for NRF2; $P<0.001$ for COX2; Figure S9F). However, we did not observe statistical significance of NRF2 in the GSE121248 cohort ($P=0.15$, not shown), while COX2 was dramatically upregulated in the $P_{\text{RBS}}$ group ($P=0.042$; Figure S9G).

Because we already demonstrated that metabolic pathways could be suppressed with increasing $P_{\text{RBS}}$, we next investigated the metabolic landscape among 606 HCCs. As expected, patients belonging to the $P_{\text{RBS}}$ group presented with significant activation of metabolism-relevant signatures, including amino acid metabolism-relevant signatures, lipid metabolism-relevant signatures, and drug metabolism-relevant signatures, while only a few metabolic pathways were enriched in the $P_{\text{RBS}}$ group (Figure 4A, Figure S9H). The activation of metabolic pathways and enrichment of the periporal HCC subtype indicated that HCC cases in the $P_{\text{RBS}}$ group preserved the default metabolic program (e.g., gluconeogenesis, amino acid catabolism, and urea cycle) in the normal liver and were well differentiated and non-proliferative, which may synergistically contribute to a favorable prognosis.

We verified the non-proliferative nature of tumors with lower $P_{\text{RBS}}$ as evidenced by their significantly reduced expression of the proliferation marker Ki-67 (all, $P<0.001$; Figure S10A). Moreover, we observed a strong positive correlation between $P_{\text{RBS}}$ and mRNA expression-based stemness index (mRNAIs) scores ($R=0.45$, $P<0.001$, Figure S10B-C), suggesting that tumors with high $P_{\text{RBS}}$ values were more likely to lead to cancer cell dissemination to distant organs, resulting in disease progression and poor prognosis. This is especially concerning, as metastatic cancer cells are often resistant to currently available therapies. These findings highlighted the essentiality of tailoring therapeutic strategies for patients at high risk.

![Figure 4. Association between immune/metabolism pathways, molecular features and $P_{\text{RBS}}$](image)

(A) Heatmap showing the landscape of the tumor immune microenvironment and metabolic pathways in 606 HBV-associated HCC cases from four independent cohorts. Samples are arranged in ascending order according to the $P_{\text{RBS}}$ and the corresponding TP53 mutation status was annotated. TIDE prediction and other previous molecular classifications of HCC are annotated at the top of the heatmap. (B) Boxplot showing the distribution of $P_{\text{RBS}}$ in four molecular classifications of HCC. (C) Scatter plot showing the correlation between $P_{\text{RBS}}$ and TIDE prediction score in four HBV-associated HCC cohorts. A higher TIDE prediction score indicates a lower likelihood of benefiting from anti-PD1/CTLA4 therapy. (D) Pearson’s correlation between $P_{\text{RBS}}$ and gene-level copy number deletion; the coefficient was arranged according to the sorted gene loci in the entire chromosome. The deletion level was presented as absolute value (positive measurement), and a positive correlation coefficient indicates that when $P_{\text{RBS}}$ increases, the level of copy number deletion increases. (E) Gene Ontology analysis on genes within chromosome 1q and 19q that presented strong and positive correlation ($R>0.5$, $P<0.05$) with $P_{\text{RBS}}$.
Correlation analysis between PRMT1, CSNK1D, PPIH, SPTLC2, UBE2N, and PRMT3.

To enhance the credibility of these drug inferences, we searched these candidate agents in DepMap, leading to 20 matched compounds. Next, we calculated Spearman’s correlation coefficient between primary drug response (measured as log_{10}(FoldChange)) and $P_{\text{res}}$ in 19 HCCs, which yielded six drugs whose sensitivity was significantly elevated with increasing $P_{\text{res}}$ (all, $R<0.4$, $P<0.05$; Figure 6B), including JNJ-7706621, teniposide, PHA-793887, doxorubicin, epirubicin, and givinostat. Of note, teniposide, a topoisomerase inhibitor, targets TOP2A, which we have already considered potentially druggable, suggesting that our in-silico strategy for drug screening could be reliable. Additionally, we investigated the association between the $P_{\text{res}}$s of HCCs and the drug sensitivity of the most common chemotherapies for treating HCC, including sorafenib, gemcitabine, oxaliplatin, cisplatin, 5-fluorouracil, capecitabine, leucovorin, and cyclophosphamide. None of them showed a significant correlation with $P_{\text{res}}$ (all, $P>0.05$; Figure 6B), implying that these routine interventions might fail to pose a prognosis-dependent effect in HBV-associated HCC patients.

**Therapeutic response prediction of targeted chemotherapy**

To screen for potentially effective chemical compounds for HBV-associated HCCs with high $P_{\text{res}}$, we performed The Connectivity Map (CMap) analysis as a preliminary method to investigate the therapeutic potential of candidate agents. To this end, 150 upregulated and 150 downregulated genes with the most significant fold changes in REM meta-analysis were submitted to CMap, which identified 85 chemical compounds under pharmacologic CMap classes (Table S12). A total of 30 agents exhibited perturbagens with enrichment scores below -95, including topoisomerase inhibitors, CDK inhibitors, HDAC inhibitors, PI3K inhibitors, etc. (Figure 5A).

To enhance the credibility of these drug inferences, we searched these candidate agents in DepMap, leading to 20 matched compounds. Next, we calculated Spearman’s correlation coefficient between primary drug response (measured as log_{10}(FoldChange)) and $P_{\text{res}}$ in 19 HCCs, which yielded six drugs whose sensitivity was significantly elevated with increasing $P_{\text{res}}$ (all, $R<0.4$, $P<0.05$; Figure 6B), including JNJ-7706621, teniposide, PHA-793887, doxorubicin, epirubicin, and givinostat. Of note, teniposide, a topoisomerase inhibitor, targets TOP2A, which we have already considered potentially druggable, suggesting that our in-silico strategy for drug screening could be reliable. Additionally, we investigated the association between the $P_{\text{res}}$s of HCCs and the drug sensitivity of the most common chemotherapies for treating HCC, including sorafenib, gemcitabine, oxaliplatin, cisplatin, 5-fluorouracil, capecitabine, leucovorin, and cyclophosphamide. None of them showed a significant correlation with $P_{\text{res}}$ (all, $P>0.05$; Figure 6B), implying that these routine interventions might fail to pose a prognosis-dependent effect in HBV-associated HCC patients.

Additionally, we also matched 3 out of 6 drugs (i.e., teniposide, JNJ-7706621, and givinostat) with a second measurement for dose finding in 14 HCCs; as expected, as the dose increased, the responsiveness of HCCLs associated with $P_{\text{res}}$ was also validated (Figure 5D-H).

**Figure 5. Identification of $P_{\text{res}}$-related therapeutic targets** (A) Volcano plot showing the correlation coefficient against statistical significance derived from Pearson’s correlation analysis between $P_{\text{res}}$ and therapeutic target expression at the transcriptome level in clinical tumors. Light-colored points represent potential targets that pass the threshold ($R>0.3$ and $P<0.05$), and dark-colored points represent targets that were also identified from CERES analysis. Target name in gray color indicates potential disease progression markers associated with $P_{\text{res}}$, suggesting that high $P_{\text{res}}$ might synergistically promote the antitumor activity of these compounds (Figure 6C).
givinostat) using a model-based strategy. Generally, in addition to the observation where correlation analysis between PIRES and predicted area under the curve (AUC) demonstrated widely negative associations in HBV-associated HCCs, patients belonging to PIRES groups exhibited remarkably lower estimated AUCs than those in PIURS groups (Figure 6D).

DISCUSSION

HCC, especially HBV-associated HCC, remains the leading cause of cancer-related mortality worldwide. Chronic replication stress induced by HBV integration can provide a therapeutic vulnerability in HCC. Herein, we aimed to develop a prognostic predictor, PIRES, for HBV-associated HCC based on DNA replication stress signatures. We also explored tailored therapeutic strategies for patients with high mortality risk. PIRES is a machine-learning trained predictor that not only informs about prognosis but can also guide targeted treatment. Specifically, four potential therapeutic targets (i.e., TOP2A, PRMT1, CSNK1D, and PP1H) and five agents, including three topoisomerase inhibitors (i.e., teniposide, doxorubicin, and epirubicin) and two CDK inhibitors (i.e., JNJ−7706621 and PHA−793887), were identified for patients with high PIRES.

Type II topoisomerases (TOP2) are pervasive enzymes that can alter DNA superhelicity and unlink replicating DNA. In HCC, Panvichian et al. revealed that TOP2A is significantly overexpressed in tumor tissues compared to adjacent normal tissue, and another meaningful result included high TOP2A expression being more frequently observed in patients with a positive serum HBsAg test. Protein arginine methyltransferase 1 (PRMT1) may play a pivotal role in multiple cellular processes, including proliferation, transformation, invasiveness, and survival of malignancies, through methylation of arginine residues that underlie these processes. PRMT1 promotes the tumorigenesis and progression of HCC by activating the STAT3, TGF-β1/Smad and HNF4α pathways. Moreover, it has recently been reported that genetic knockdown and pharmacological inhibition of PRMT1 by DCPT1061, a novel potent inhibitor, drastically induced G1-phase cell cycle arrest and suppressed cell growth of clear cell renal cell carcinoma. Another PRMT1 inhibitor (GSK3368715) impaired replication restart of pancreatic ductal adenocarcinoma, thus inhibiting tumor growth. Casein kinase 1 delta (CK1δ, CSNK1D) is a member of the serine/threonine protein kinase family that comprises six isoforms (i.e., α, δ, ε, γ1, γ2 and γ3) that are involved in several signaling pathways (e.g., Hedgehog, Wnt, and Hippo) and mediate numerous cellular processes (e.g., DNA replication, DDR, RNA metabolism, membrane trafficking, cytoskeleton maintenance, and circadian rhythm). Rosenberg et al. revealed that silencing or inhibition of CSNK1D using SR-3029 provokes potent antitumor effects in breast cancer cells in vivo. PP1H is a member of the peptidyl-prolyl cis-trans isomerase (PPIase) family. Although limited
evidence directly demonstrated the antitumor effect of inhibiting PP1H, Uchida et al. revealed that inhibition of PIN1, a popular member of the PPlase family, impaired the growth of several cancer cell lines.11 Taken together, emerging evidence indicates that the four targets we identified all play special roles in malignant development, and several inhibitors have demonstrated potent antitumor effects in specific cancer types, suggesting the feasibility of developing corresponding targeted therapies for high-risk HBV-associated HCC.

Inhibition of TOP2 (topoisomerase inhibitor) is a therapeutic strategy for cancer treatment and has been applied to treat cancers for many years through agents such as etoposide and teniposide, which target TOP2A. Several clinical trials are ongoing to evaluate the efficacy of topoisomerase inhibitors in HCC (e.g., NCT0351195, NCT03533582, NCT03017326). Doxorubicin and epirubicin are traditional topoisomerase inhibitors that have been widely used for the treatment of HCC. Even though they have never been recommended for systemic treatment of HCC, these two primary drugs used for transarterial chemoembolization (TACE) in HCC.14 Unfortunately, a recent result from the phase III Alliance/CALGB 80802 trial failed to observe a benefit from the addition of doxorubicin treatment to sorafenib, and the safety concerns of doxorubicin raised a cautious attitude for its limited application due to the presence of underlying cirrhosis, hematologic toxicity, and cardiac toxic events.15 such disappointing reports inversely place more emphasis on the development of robust biomarkers for precision medicine, enhancing efficacy and reducing adverse reactions. Based on our findings, HBV-associated HCC patients with high PRS are more susceptible to these inhibitors, which might guide future clinical trial designs.

NJN-7706621, a potent CDK inhibitor targeting CDK1/2, blocks tumor progression through the cell cycle, causing cells to accumulate in G2/M phase, preventing cells from entering mitosis and activating apoptosis, which could be useful for treating various cancers.16-18 PHA-793887 is another type of multiple CDK inhibitor with activity against CDK1/2/4/6/7/9, which was also revealed in our study as a potential drug for high-risk HBV-associated HCC. Brasca et al. demonstrated that PHA-793887 exhibited good efficacy in human ovarian A2780, colon HCT-116 and pancreatic Bx-PC3 xenograft models and was well tolerated via daily intravenous administration, suggesting that PHA-793887 is promising as a drug candidate for clinical evaluation.19 In fact, inhibitors of the CDK pathway have been widely reported to induce apoptosis, and there are ongoing clinical trials for HCC targeting this pathway include palbociclib (NCT01356628, CDK4/CDK6 inhibitor), flavopiridol (NCT00087282, CDK1/2/4/6/7 inhibitor) and mlicillic (NCT03109886, CDK2/4/6/7 inhibitor). Additionally, Ehrlich et al. revealed that the combinational value of the CDK5 inhibitor roscovitine with DNA damage-inducing chemotherapeutics synergistically inhibited HCC tumor progression.20 Tourneau et al. also observed a partial response to selicicib, a CDK1/2/4/7 inhibitor, in one HCC patient from a phase I evaluation. Therefore, pan-CDK inhibitor PHA-793887 might achieve a better response for high-risk patients.

Despite high HBV viral load being associated with worse survival,46-48 PRS negatively correlated with HBV oncoprotein expression. We suggest this may indicate the presence of a compensatory mechanism countering the effects of HBV oncoproteins. Further investigations are necessary to clarify this relationship.

We acknowledge several limitations, including variations in cohort size, composition, and sequencing technology, and incomplete treatment records. Additionally, bulk sequencing and microarray profiles can be confounded by signals from mixed cell populations. Future studies should consider combining our findings with multiplex immunohistochemistry and possibly adapting PRS for use with a qPCR assay.

In summary, we developed PRS, a DNA replication stress signature for HBV-associated HCC. This single-sample survival predictor may guide prognosis stratification and personalized therapeutic strategies. Physicians could use PRS to avoid overtreatment in low-risk patients and identify potential therapeutic targets and agents in high-risk patients. Overall, this work provides a roadmap for the clinical development of personalized treatment.

**AVAILABILITY OF DATA AND MATERIAL**

The raw data for this study were generated at the corresponding archives and were detailed for source in the section of **Materials and Methods.** We developed the R package, “heccPIRS,” which is documented and freely available at https://github.com/xlucpu/heccPIRS. This package calculates replication stress-related prognostic index (PRS) for HBV-associated HCC patients, and estimates the enrichment of 21 replication stress signatures. If specified, a heatmap will be generated to show the landscape of the replication stress signatures in an ascending order of PRS score. Core R codes of prognostic model development, functional enrichment, REM-meta analysis, and drug target identification have been uploaded to GitHub (https://github.com/xlucpu/heccPIRS/tree/main/code). Derived data and other code supporting the findings are available from the corresponding author [F. Y] on reasonable request.

**MATERIALS AND METHODS**

Methodology details were included in the **SUPPLEMENTAL INFORMATION.**

**REFERENCES**


ACKNOWLEDGMENTS

We greatly appreciate the patients and investigators who participated in the corresponding medical project for providing data. This work was supported by the National Natural Science Foundation of China (No. 81973145, No. 82273735) (F.Y.), and Key R&D Program of Jiangsu Province (Social Development) (BE2020694) (F.Y.).

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

It can be found online at https://doi.org/10.5971/j.xinn-med.2023.100014