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ORIGINAL RESEARCH

RAD51AP1 as an Immune-Related Prognostic Biomarker and Therapeutic Response Predictor in Hepatocellular Carcinoma

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Background: *RAD51* associated protein 1 (*RAD51AP1*) is shown to regulate cell proliferation and cancer progression. However, the immune-infiltrating correlation and the therapeutics guidance of *RAD51AP1* in hepatocellular carcinoma (HCC) still need further investigation.

Methods: In this study, comprehensive bioinformatic analysis of *RAD51AP1* on differential expression, clinicopathologic correlation, prognostic value, and function enrichment were performed in The Cancer Genome Atlas (TCGA), Gene Expression Omnibus (GEO; GSE14520 and GSE76427), and International Cancer Genome Consortium (ICGC) datasets. Besides, the Guangxi cohort containing 50 pairs HCC and adjacent non-cancerous samples from First Affiliated Hospital of Guangxi Medical University was served as validation cohort. Moreover, we explored the predictive value of *RAD51AP1* to therapeutics response and its underlying correlation with HCC immunoinfiltration.

Results: *RAD51AP1* was significantly overexpressed in HCC tissues and had a high diagnostic value of HCC. The shorter survival time and poorer clinical features were showed when *RAD51AP1* upregulated, and then a nomogram featuring *RAD51AP1* expression and other clinicopathologic factors was established to predict prognosis. In CIBERSORT analysis, higher T cells follicular helper but lower T cells CD4+ memory resting infiltration levels were exhibited when *RAD51AP1* upregulated. The ssGSEA analysis demonstrated that high-*RAD51AP1* expression subgroup had higher macrophages, Th2 and Treg cells infiltration levels, but lower type II IFN response function. Furthermore, high-*RAD51AP1* expression subgroup exhibited the upregulated expression levels of immune-related checkpoint genes, but lower IPS and TIDE scores which suggested a possibly better immunotherapy response. The drug sensitivity analysis showed the high-expression subgroup may be more susceptible to Bexarotene, Doxorubicin, Gemcitabine and Tipifarnib.

Conclusion: Taken together, *RAD51AP1* is a potential diagnostic and prognostic biomarker. It may be related to the immunosuppressive microenvironment and could be an underlying HCC treatment strategy. However, the conclusions still require further validation studies.

Keywords: hepatocellular carcinoma, *RAD51AP1*, prognostic signature, bioinformatics, immune filtration, drug sensitivity

Introduction

Liver cancer is the sixth most widespread cancer and the fourth primary cause of cancer-related death.[1] Its incidence and mortality may keep rising by 2030.[2] Hepatocellular carcinoma (HCC) with a dismal prognosis is the most prominent type of primary liver cancer.[3] The important risk factors of HCC include chronic B or C viral hepatitis, heavy alcohol consumption, metabolic associated fatty liver disease, and aflatoxins.[4] Serum alpha fetoprotein (AFP) is a traditional

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index for the diagnosis and postoperative follow-up of HCC patients. But the diagnosis sensitivity of AFP is only 40–60%.[5] For HCC treatment, the development of systemic therapies of HCC has quickly accelerated.[6] Nevertheless, the treatment response for advanced HCC is still not optimistic due to tumor heterogeneity and drug resistance.

The DNA damage response plays an anti-cancer role in early human tumorigenesis, and the mutations in DNA repair pathways may increase genomic instability and tumor progression.[7] Deregulation of nucleotide pathway and alteration of cGAS/STING gene, which induced DNA damage, could be the crucial mechanisms in the development of non-alcoholic steatotic liver disease, an underlying risk factor of HCC.[8] It is demonstrated that full-length hepatitis B virus X protein could decrease the expression of *RAD51* expression and impaired apoptosis.[9] Exploring and identifying new molecular biomarkers with a satisfactory diagnostic performance is critical to improving the clinical outcome of HCC patients. The human *RAD51AP1* gene, located on chromosomes 12p13.1 to 13.2,[10] was identified in 1997[11] and mainly functions in DNA homologous recombination repair by stimulating *RAD51* activity.[12] It may play a role in promoting cancer by meditating TGF-β/Smad signaling pathway.[13] In the treatment of ionizing radiation for glioblastoma, the elevated phosphorylation of *PTEN* on tyrosine 240 contributed to DNA repair by the recruitment of *RAD51*. Moreover, *RAD51AP1* may regulate breast cancer stem cell self-renewal, which contributes to the progression of disease. Furthermore, high *RAD51AP1* expression was also related to poor cancer prognosis of ovarian cancer[13] and lung cancer.[14] By contrast, down-regulation of *RAD51AP1* was shown to suppress the metastasis and proliferation of lung carcinoma cells[15] and retard the growth of intrahepatic cholangiocarcinoma cells.[16] Besides, *RAD51AP1* gene has been also reported to be overexpressed in HCC[17] and Zhuang et al demonstrated that HCC patients with overexpressed *RAD51AP1* had the poor clinical features and dismal prognosis.[18]

However, the molecular mechanism and detailed clinical significance of *RAD51AP1* in HCC have not been completely investigated, especially its immunoinfiltration correlation and therapeutics response guidance, including possible effect of different *RAD51AP1* expression levels on immune cell infiltration levels in tumor microenvironment and predicted drug sensitivities of HCC treatment-related chemotherapeutic and immunotherapeutic agents. In this project, we aimed to authenticate the diagnostic, prognostic and therapeutic value of *RAD51AP1*.

Materials and Methods

Data Source and HCC Samples Collection

The mRNA expression arrays and corresponding clinicopathologic info of HCC patients were retrieved from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/repository). The GSE14520, GSE76427 and ICGC cohorts, including gene expression profiling and survival info of HCC patients, were retrieved from Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) and International Cancer Genome Consortium (ICGC; http://dcc.icgc.org) databases. Besides, a total of 50 pairs HCC and corresponding adjacent non-cancerous tissue samples from the First Affiliated Hospital of Guangxi Medical University were served as the Guangxi cohort. Every pair (cancer and non-cancer) sample came from the same patient. Then, the corresponding clinical info, including age, gender, serum AFP, tumor size, histologic grade, portal vein tumor thrombus, China Liver Cancer Staging (CNLC), and Barcelona Clinic Liver Cancer (BCLC) staging were also collected (Supplementary Table 1).

Differential Expression and Prognostics Value Analysis

The differential *RAD51AP1* expression analyses between HCC and non-cancerous liver tissues were carried out in the TCGA, GSE14520, GSE76427, ICGC and Guangxi cohorts, respectively. The diagnostic ability of *RAD51AP1* for HCC was evaluated through plotting diagnostic receiver operating characteristic (ROC) curves. The median expression of *RAD51AP1* was used to categorize the subgroups as high- and low-expression. The prognostic value of *RAD51AP1* was explored preliminarily by Kaplan–Meier survival analysis in TCGA-HCC cohort and was validated in the GSE14520 and ICGC cohorts. Using the "ggpubr" R package,[19] the difference of *RAD51AP1* expression level between different clinicopathological characteristics was investigated in the TCGA and Guangxi cohorts.

Nomogram Construction

Univariate and multivariate Cox regression analyses were performed in TCGA cohort to analyze the prognostic factors of HCC. In the Cox regression analysis, overall survival (OS), including survival time and survival state, was the dependent variable. OS is the period from the date of diagnosis until the date of death from any cause. Furthermore, nomogram is an effective approach to measure the specific risk by integrating multiple variables and establishing a multivariate Cox proportional risk regression model, in which the regression coefficient represents the contribution degree of each influencing factor to the outcome variable. Subsequently, the predicted probability of individual outcome event is determined by the accumulated score of multiple influencing variables. Utilizing the "survival" and "rms" R package,[20,21] a nomogram combining *RAD51AP1* expression and easily accessible and widely accepted clinicopathological parameters (gender, age, BMI, AFP, histologic grade, Tumor Node Metastasis (TNM) stage, and vascular invasion) was established based upon the TCGA-HCC data to determine the likelihood of 1-, 3-, and 5-year OS for HCC patients. The analysis removed the patients with incomplete clinical data and follow-up periods of fewer than 30 days. The bootstrap method was applied to calculate concordance index (C-index) with 1000 resamples and the discrimination performance of nomogram was examined based on the consistency degree of calibration curves.

Functional Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) software program (v4.1.0) was employed to seek the putative regulatory mechanisms of high- and low-*RAD51AP1* expression subgroups in virtue of the gene set data "c2.all.v7.0.symbols.gmt" and "c5.all.v7.0.symbols.gmt".[22] The number of permutations was set at 1000. Functional terms that met the criteria of a nominal $P < 0.05$ and a false discovery rate (FDR) <0.05 were regarded as significant enrichment pathways.

Additionally, based on the entire mRNA expression profile of the TCGA-HCC dataset, the genome-wide correlation analysis of *RAD51AP1* was performed. Genes with correlation coefficient >0.7 and P <0.05 were incorporated to implement the gene ontology terms (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses utilizing the "clusterProfiler" R package.[23]

Immune Cell Infiltration Analysis

Based on a novel analytical methodology, namely cell-type identification by estimating relative subsets of RNA transcripts (CIBERSORT)[24] and TCGA-HCC expression profiling, the proportions of various immune cell in each sample were assessed. Then, the immune infiltration levels in different *RAD51AP1* expression subgroups were uncovered. Additionally, the correlation analyses of *RAD51AP1* expression and multiple immune cells were also conducted. Tumor Immune Estimation Resource 2 (TIMER2) (http://timer.cistrome.org/), a data repository for measuring immune cell infiltrations of distinct cancers. Spearman correlation analyses in TIMER2 were performed between *RAD51AP1* and immune cell subsets to validate the results of CIBERSORT analysis.[25] To further illustrate the relationship between *RAD51AP1* expression and cancer immunity of HCC, the immune-associated cell infiltration levels and the relative expression activities of various function pathways of the different *RAD51AP1* expression subgroups were analyzed and compared in the TCGA and ICGC datasets using the "gsva" R package which was based on an algorithm called single-sample Gene Set Enrichment Analysis (ssGSEA).[26] Additionally, the stromal score, immune score and ESTIMATE (Estimation of Stromal and Immune cells in Malignant Tumor tissues using Expression data) score of each TCGA-HCC samples were calculated using the 'estimate' R package,[27] and the relationships between *RAD51AP1* expression and these scores were also excavated.

Drug Sensitivity Prediction Analysis

In the TCGA-HCC cohort, the association between *RAD51AP1* expression and tumor mutation burden (TMB), established immune checkpoint genes were explored using the Spearman correlation analysis. The R package "pRRophetic" was applied to determine the half-maximal inhibitory concentration (IC50) of HCC-treatment related chemotherapeutic and immunotherapeutic drugs by ridge regression method.[28] Additionally, a computation-based online program called Tumor Immune Dysfunction and Exclusion (TIDE, http://tide.dfci.harvard.edu/)

stimulates the immune system of human body to further forecast the response to immune checkpoint inhibitor. The calculated score of TIDE, immune dysfunction, immune exclusion, and microsatellite instability (MSI) data of each TCGA-HCC sample were acquired from TIDE website. In addition, the immunophenotypic score (IPS) of tumor sample can be obtained from the Cancer Immunome Atlas (TCIA, https://tcia.at/), which contains the info of solid cancers from TCGA dataset. IPS contributes to estimate the therapeutic effect of immune-checkpoint inhibitors (ICIs), including anti-programmed cell death protein 1 (*PD-1*) and anti- cytotoxic T lymphocyte antigen-4 (*CTLA-4*) antibodies,[29] and higher IPS indicates potentially fine response. Subsequently, these immunotherapy-related indicators, including drug IC50, TIDE score and IPS, were compared between the different *RAD51AP1* expression subgroups in the TCGA-HCC cohort. Besides that, IMvigor210 dataset, a sizable immunotherapy dataset containing the information of 298 urothelial carcinoma patients at advanced stage who were administered with anti-*PD1* inhibitor, was used to grope the immunotherapy response predictability of *RAD51AP1* in other cancer.

Reverse Transcription Quantitative PCR

The method of specimen preservation, RNA extraction, and reverse transcription quantitative PCR was conducted as previously described.[30] Clinical sample collecting and handling procedures strictly adhered to the standard protocol. The sequence of primers was as follows: GAPDH, forward: GTCAGCCGCATCTTCTTT, reverse: CGCCCAATACGACCAAAT. *RAD51AP1*, forward: AGTGAAGGTAAAATCCCCAGTAGA, reverse: TGGCAAGGACTGAGATTCTGAT. Using the 2 ΔΔCT approach, the relative mRNA expressions of *RAD51AP1* were measured.[31]

Statistical Analysis

R (v4.1.1) was applied to complete data analysis and result visualization. The Wilcoxon rank-sum test was conducted between the different subgroups with continuous data, while the chi-square test was used for categorical data. Paired *t*-test was utilized to explore the discrepancy of *RAD51AP1* expression levels between HCC samples and adjacent normal tissues of the Guangxi cohort. The association of two variables was probed using the Spearman correlation analysis. Unless otherwise indicated, $P < 0.05$ was provided as the threshold cutoff value.

Results

The Upregulated Expression Level of *RAD51AP1* in HCC

Compared with normal liver tissues, *RAD51AP1* gene expression level was markedly over-expressed in HCC in various cohorts (Figure 1A–E). The ROC curve indicated that *RAD51AP1* had a high predictive accuracy of HCC diagnosis with the area under curve (AUC) >0.90 in the TCGA, GSE14520 and ICGC cohorts (Figure 1F) and the AUC values of GSE76427 and Guangxi cohorts were 0.82 and 0.72, respectively. These results showed that *RAD51AP1* carried a decent diagnostic significance for HCC.

RAD51AP1 Correlated with Dismal Survival and Poor Clinical Features

The survival analysis suggested that the high *RAD51AP1*-expression group showed a poorer OS and recurrence free survival (RFS) than that of the low expression group in the TCGA cohorts (Figure 2A and B). Furthermore, the dismal prognosis of high *RAD51AP1*-expression subgroup was confirmed in the GSE14520 and ICGC cohorts (Figure 2C and D). Moreover, a significantly higher *RAD51AP1* gene expression was observed in HCC with serum AFP > 400 ng/mL in the TCGA cohorts. Along with the advance of clinicopathologic features (histologic grade, TNM stage, T staging and vascular invasion degree) which were believed to get poorer prognosis, the expression level of *RAD51AP1* presented an upward trend (Figure 3). In Guangxi cohort, we further confirmed that *RAD51AP1* was significantly upregulated in HCC patients with AFP > 400 ng/mL, poor histologic grade, large tumor size and more advanced clinical stage (CNLC Ia vs Ib) (Figure 4).

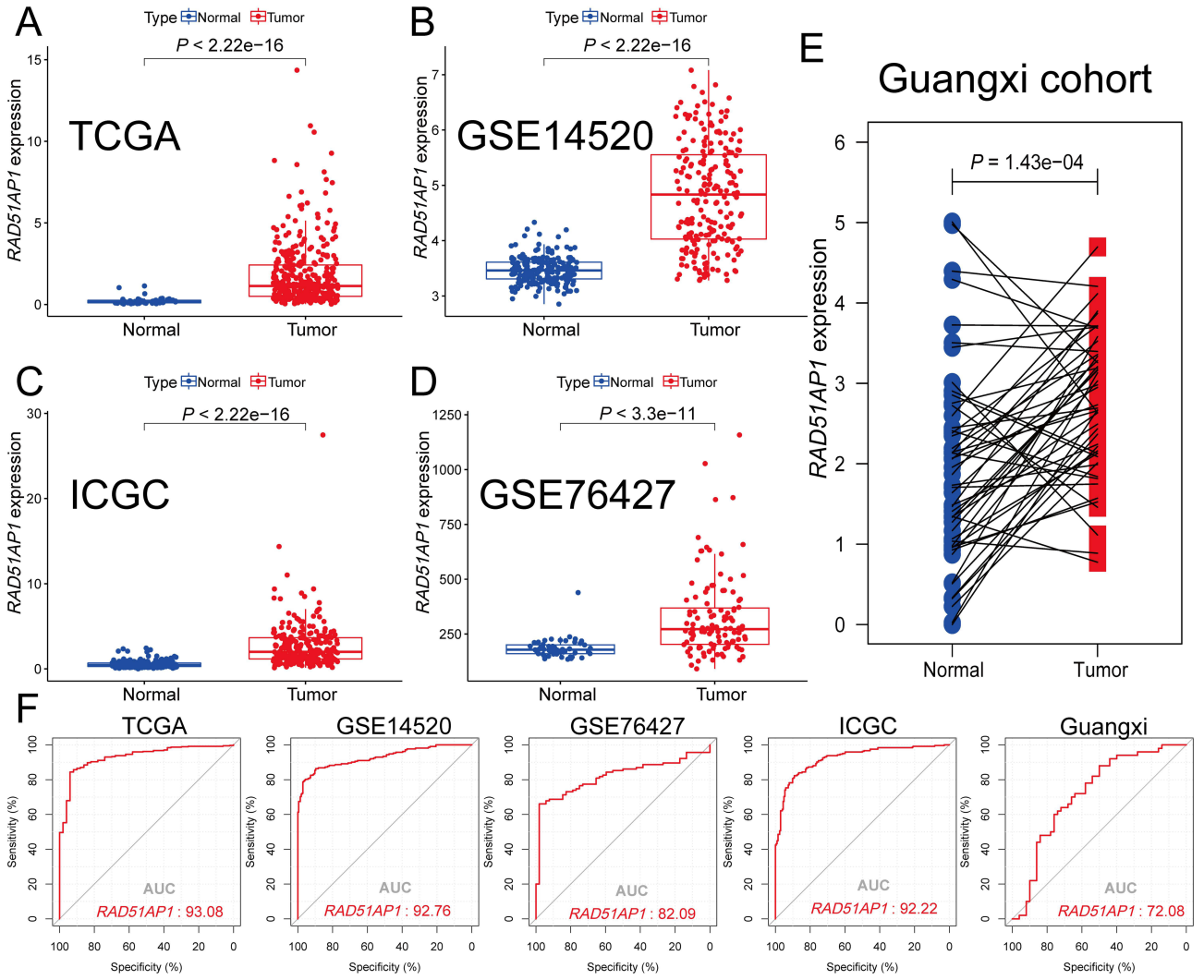

Figure 1 (**A**–**E**) Scatter plots illustrated the higher *RAD51AP1* expression in HCC compared with normal liver tissues in the TCGA, GEO (GSE14520 and GSE76427), ICGC and Guangxi cohorts. (**F**) Diagnostic receiver operator curves with decent AUC values of *RAD51AP1* for HCC diagnosis in various cohorts.
Abbreviations: *RAD51AP1, RAD51* associated protein 1; HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; ICGC, International Cancer Genome Consortium; AUC, area under curve.

Construction of a Nomogram

Univariate Cox analysis discovered that *RAD51AP1* expression (HR = 1.218; 95% CI = 1.055–1.407), TNM stage, T staging, and vascular invasion were the high-risk factors for poor OS in TCGA-HCC patients (Figure 5A). Furthermore, *RAD51AP1* expression (HR = 1.159; 95% CI = 1.002–1.341) remained statistically significant in multivariate Cox analysis. Therefore, we deduced that *RAD51AP1* could be identified as an independent prognostic factor (Figure 5B). The established nomogram, featuring *RAD51AP1* expression and easily accessible and widely accepted clinicopathological parameters (gender, age, BMI, AFP, histologic grade, Tumor Node Metastasis (TNM) stage, and vascular invasion), contributed to predicting the 1-, 3-, and 5-year OS of HCC patients. The C-index of nomogram is 0.725 (Figure 5C). The calibration curves of nomogram exhibited a satisfactory consistency between the estimated and the actual OS rates at 1, 2, and 3 years (Figure 5D).

RAD51AP1 Was Involved in Cancer-Promoting and Cell Cycle Related Pathways

GO terms enrichment analysis discovered that *RAD51AP1* up-regulation was significantly related to "ATPASE_ACTIVITY, CELL_CYCLE_G1_S_PHASE_TRANSITION, CHROMOSOME_SEGREGATION,

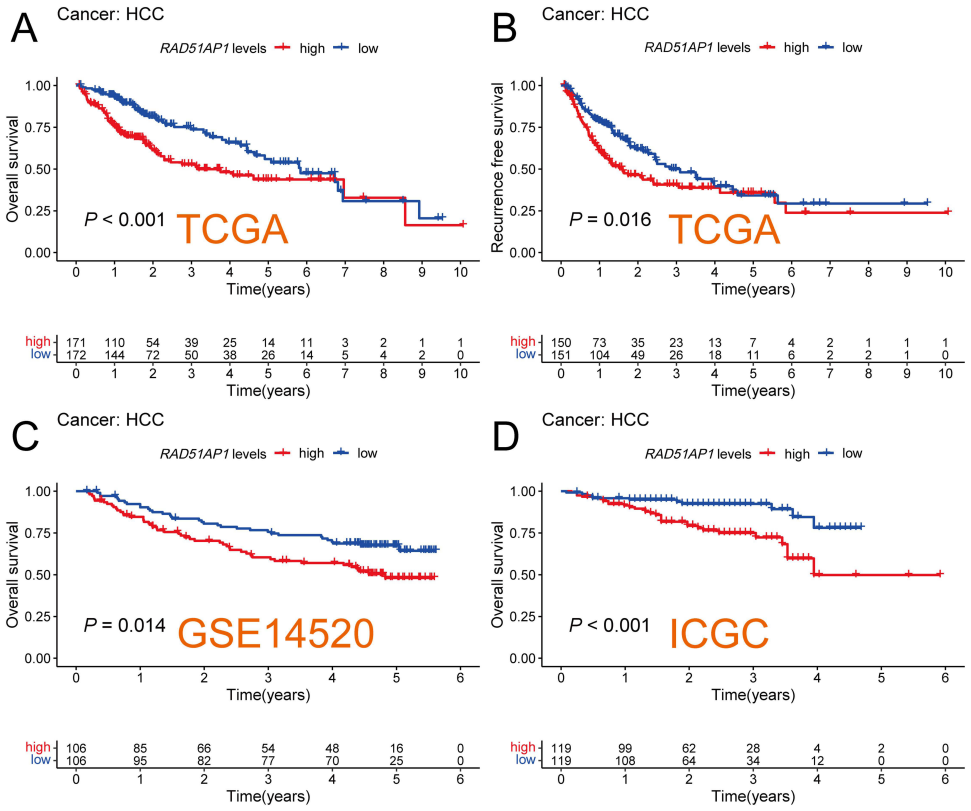

Figure 2 Kaplan–Meier curves for OS and RFS with Log rank test between high- and low-*RAD51AP1* expression subgroups in the TCGA (**A** and **B**). Kaplan–Meier curves for OS with Log rank test between high- and low-*RAD51AP1* expression subgroups in the GSE14520 (**C**) and ICGC (**D**) cohorts.

Abbreviations: OS, overall survival; RFS, recurrence free survival; *RAD51AP1*, *RAD51* associated protein 1; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; ICGC, International Cancer Genome Consortium.

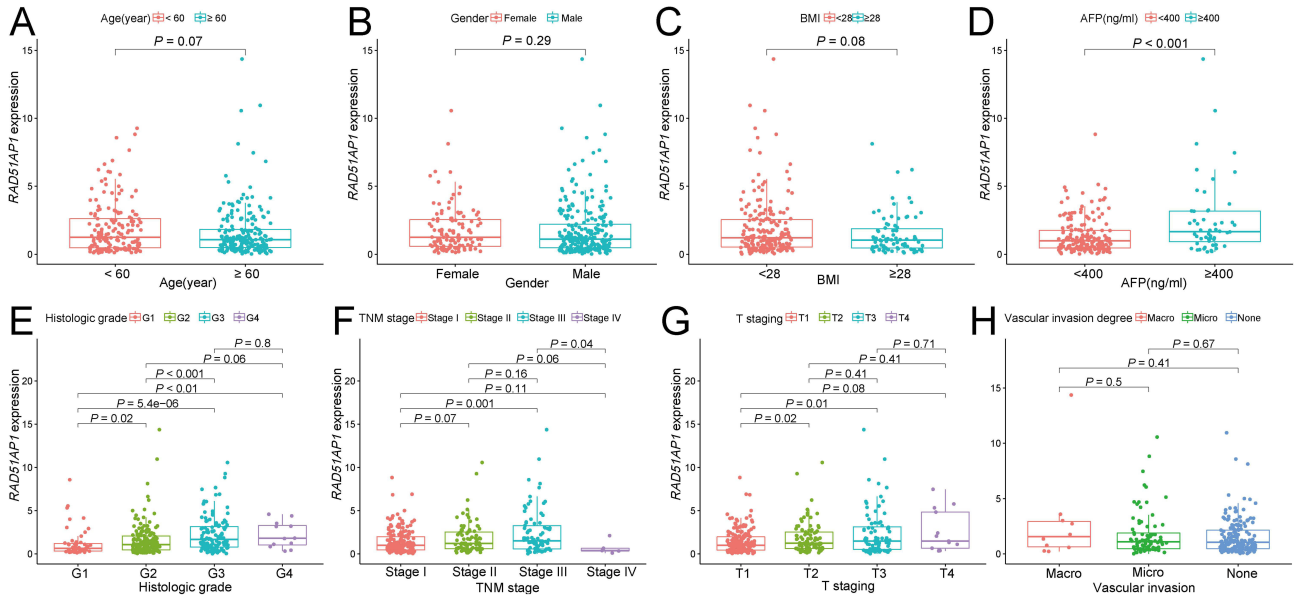

Figure 3 (**A**–**H**) The relationship between *RAD51AP1* expression and the different clinicopathological characteristics in TCGA cohort, including age, gender, BMI, serum AFP level, histologic grade, TNM stage, T staging and vascular invasion degree.

Abbreviations: *RAD51AP1*, *RAD51* associated protein 1; TCGA, The Cancer Genome Atlas; BMI, Body Mass Index; AFP, alpha-fetoprotein. TNM, Tumor Node Metastasis; Macro represents macrovascular invasion; Micro represents microvascular invasion.

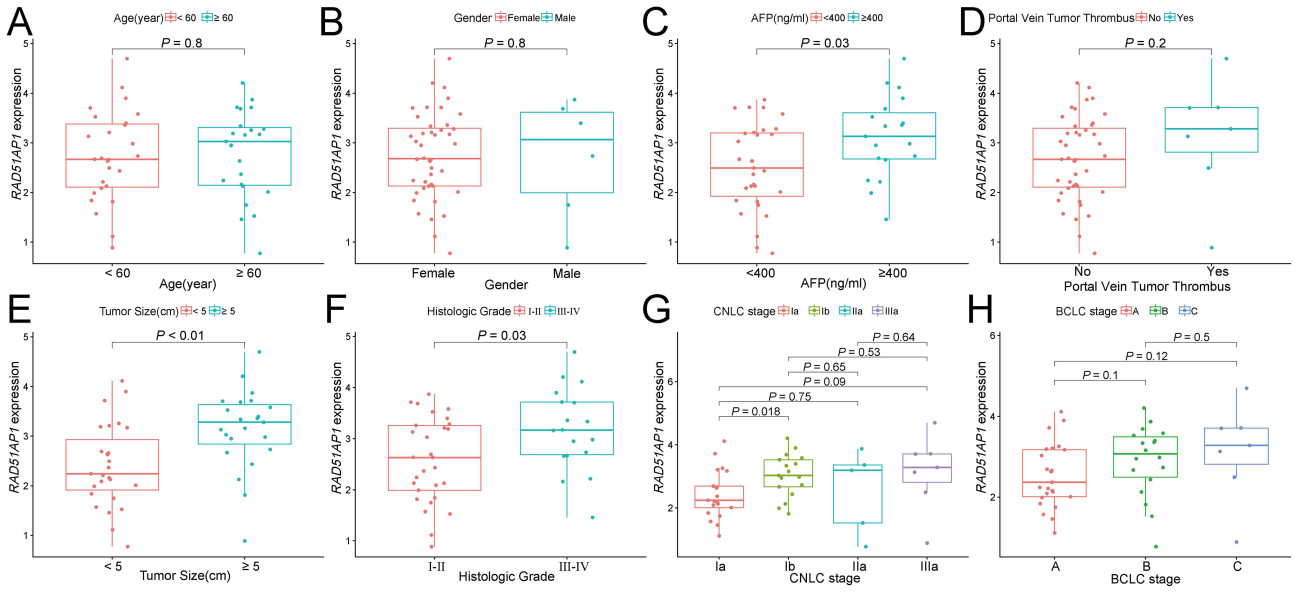

Figure 4 (**A**–**H**) The relationship between *RAD51AP1* expression and the different clinicopathological characteristics in Guangxi cohort, including age, gender, serum AFP level, portal vein tumor thrombus, tumor size, histologic grade, CNLC and BCLC stage.
Abbreviations: *RAD51AP1*, *RAD51* associated protein 1; AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; CNLC, China Liver Cancer Staging.

DNA_REPLICATION, NUCLEAR_INNER_MEMBRANE, REGULATION_OF_GENE_EXPRESSION_EPIGENETIC, SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR" pathways (Figure 6A). Furthermore, KEGG pathway analysis highlighted that "REGULATION_OF_AUTOPHAGY, NOTCH_SIGNALING_PATHWAY, P53_SIGNALING_PATHWAY, PATHWAYS_IN_CANCER, ERBB_SIGNALING_PATHWAY" pathways were enriched in the up-regulated *RAD51AP1* subgroup, while "COMPLEMENT_AND_COAGULATION_CASCADES, DRUG_METABOLISM_CYTOCHROME_P450, FATTY_ACID_METABOLISM, METABOLISM_OF_XENOBIOTICS_BY_CYTOCHROME_P450" pathways were enriched in the down-regulated *RAD51AP1* subgroup (Figure 6B).

The results of genome-wide correlation analysis showed that 92 genes were significantly relevant to *RAD51AP1* in the TCGA-HCC cohort. All these genes, the absolute correlation coefficient values of which were all greater than 0.7, were positively correlated with *RAD51AP1* expression (Figure 7A). By the GSEA analysis on these correlated genes, the *RAD51AP1*-associated HCC hallmarks, including regulation of cell cycle, cell cycle-phase transition, nuclear chromosome, tubulin binding, cellular senescence, FoxO signaling pathways, were identified (Figure 7B and C).

Correlation Between *RAD51AP1* and the Immune Infiltration of Tumor Microenvironment in HCC

CIBERSORT analysis revealed higher T cells follicular helper but lower T cells $CD4^+$ memory resting significantly infiltrated in the high-*RAD51AP1* expression subgroup ($P < 0.05$; Figure 8A). In line with the above results, a correlation heatmap displayed the positive and negative connections of *RAD51AP1* expression and different immunizing cells (Figure 8B and Supplementary Table 2). In TIMER2, *RAD51AP1* expression negatively correlated to the infiltration levels of T cells $CD4^+$ memory resting, but positively correlated to B cell memory, cell $CD4^+$ T memory activated and Myeloid dendritic cell activated (Supplementary Figure 1). The ssGSEA analysis showed that the up-regulated *RAD51AP1* expression exhibited the higher macrophages, Th2 and Treg

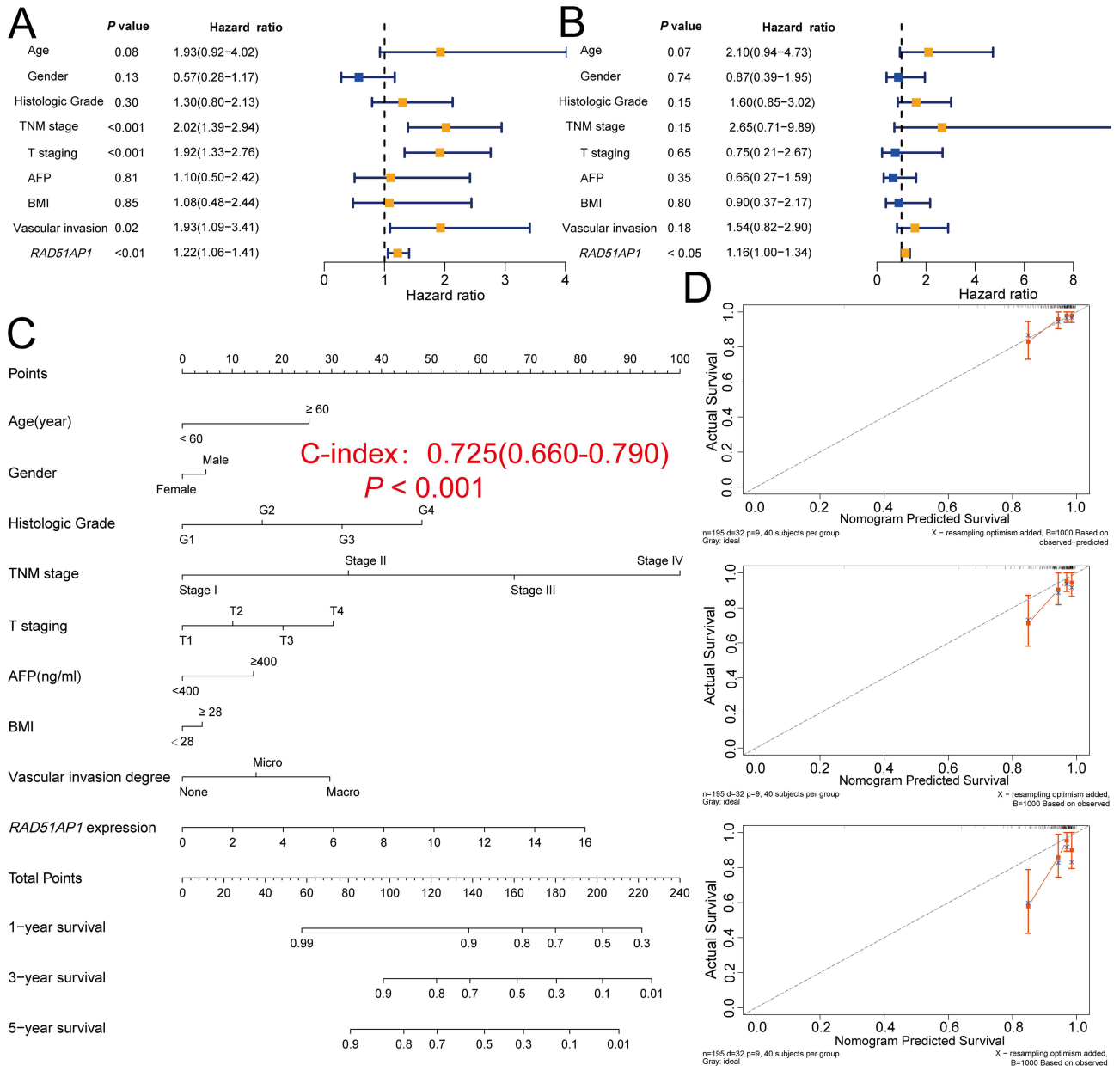

Figure 5 (**A** and **B**) Univariate and multivariate Cox regression analyses of *RAD51AP1* and clinicopathologic factors were used to analyze the prognostic factors of HCC in the TCGA datasets. (**C** and **D**) A nomogram featuring *RAD51AP1* expression and easily accessible and widely accepted clinicopathological parameters (gender, age, BMI, AFP, histologic grade, TNM stage, and vascular invasion) and its corresponding calibration curves for the OS rates at 1, 2, and 3 years.
Abbreviations: *RAD51AP1, RAD51* associated protein 1; HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; BMI, Body Mass Index; AFP, alpha-fetoprotein; TNM, Tumor Node Metastasis; OS, overall survival.

cells infiltration levels but lower immune function of type II IFN response in both TCGA and ICGC cohort ($P < 0.05$; Figure 9A–D).

Predictive Value of Therapeutic Efficacy in HCC

Based on gene expression profiling in TCGA-HCC dataset, the up-regulated expression levels of immune-related checkpoint genes, including *CD44, HAVCR2, LGALS9, PDCD1, TNFRSF4, TNFRSF14, TNFRSF18, CD27, CD48, CD40, IDO1* and *LAG3*, were uncovered in the high-*RAD51AP1* expression subgroup (Figure 10A). Moreover,

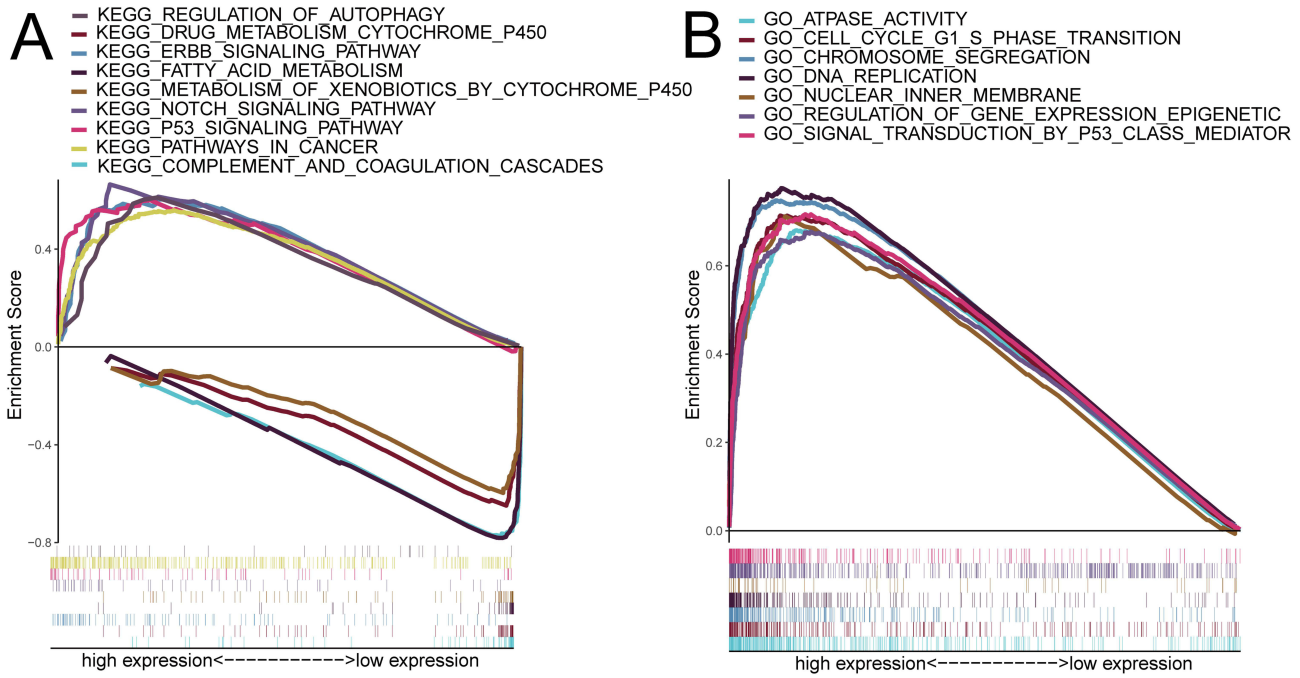

Figure 6 (**A** and **B**) The KEGG and GO function enrichment signaling pathways of the high- and low-*RAD51AP1* expression subgroups in GSEA analysis based on the TCGA-HCC cohort.

Abbreviations: KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; GSEA, Gene Set Enrichment Analysis; *RAD51AP1*, *RAD51* associated protein 1; HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas.

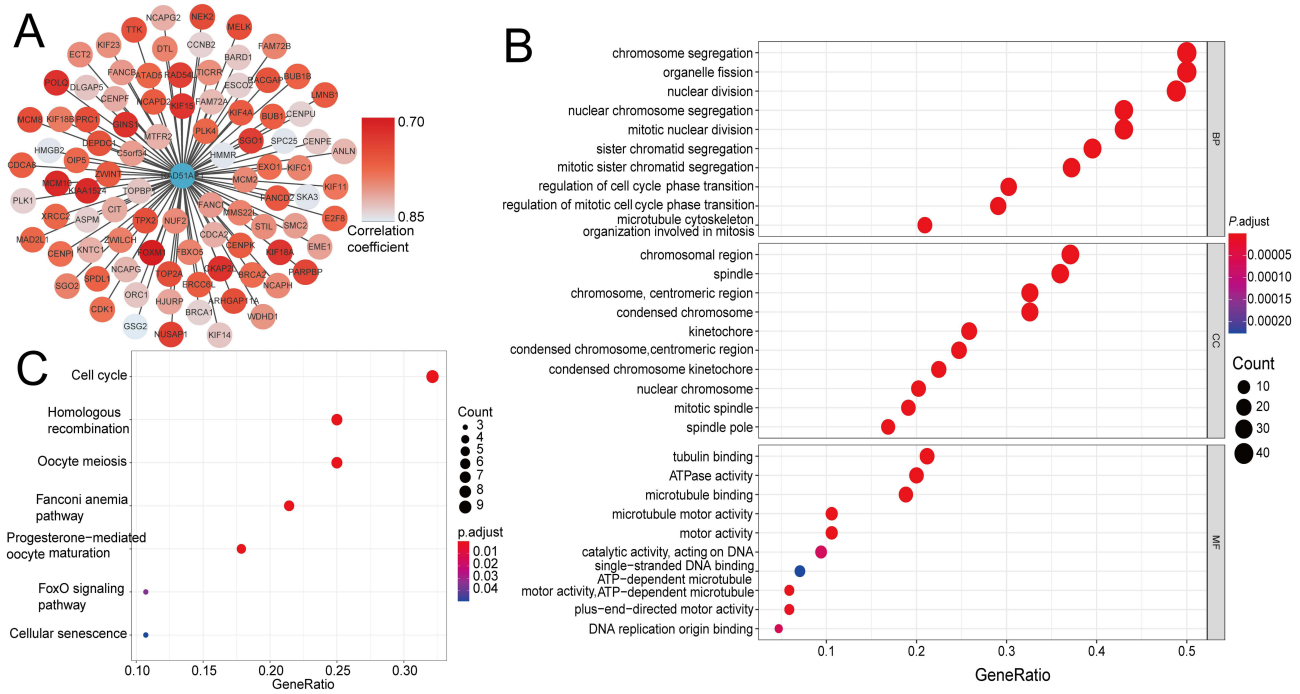

Figure 7 (**A**) Regulatory network of *RAD51AP1* gene containing its co-expressed genes in the TCGA-HCC cohort. (**B** and **C**) The bubble diagrams displaying the significant GO and KEGG function enrichment signaling pathways of *RAD51AP1* and its co-expressed genes.

Abbreviations: *RAD51AP1*, *RAD51* associated protein 1; HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

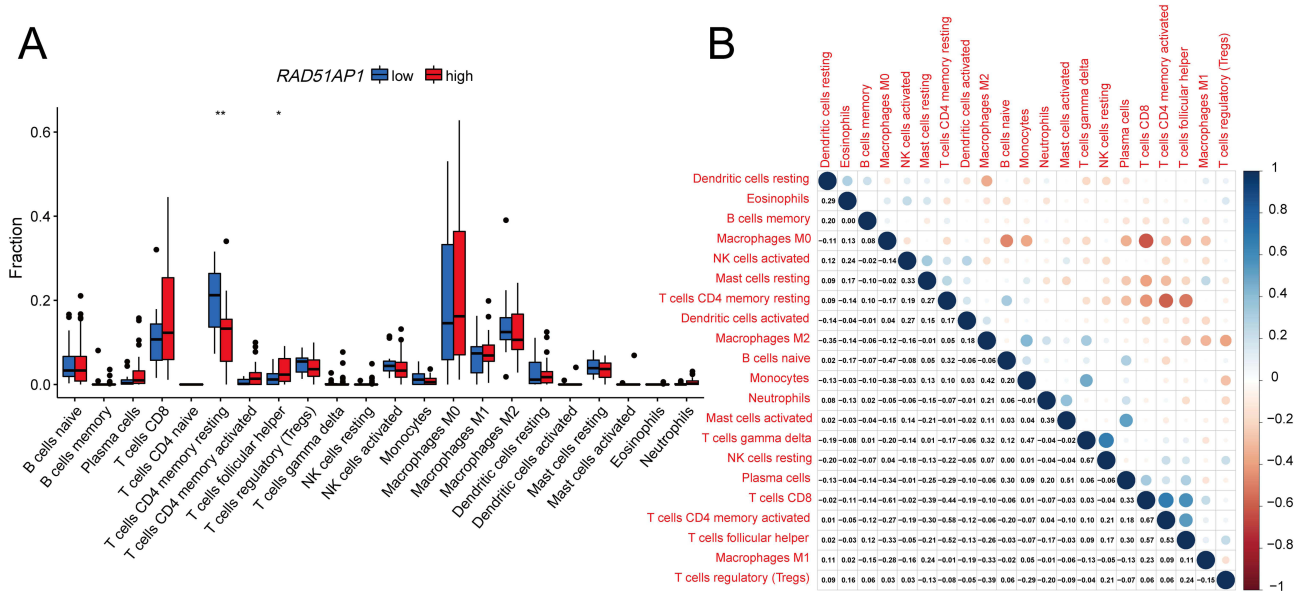

Figure 8 (**A**) The percentages of 22 immune cell infiltration profiles based on CIBERSORT analysis were compared between the high- and low-*RAD51AP1* expression subgroups. (**B**) The correlation heatmap displaying the correlation between *RAD51AP1* expression and various immunocyte levels. *$P < 0.05$; **$P < 0.01$.
Abbreviations: CIBERSORT, cell-type identification by estimating relative subsets of RNA transcripts; *RAD51AP1*, *RAD51* associated protein 1.

RAD51AP1 expression was negatively related to stromal score ($R = -0.20$, $P < 0.001$, Figure 10C), ESTIMATE score ($R = -0.13$, $P < 0.05$, Figure 10E). However, the correlations of *RAD51AP1* and immune score or TMB were statistically insignificant (Figure 10B and D). The predictive findings based on operational simulation in drug sensitivity analysis showed that eight medications (Sorafenib, Nilotinib, Axitinib, Erlotinib, Dasatinib, Docetaxel, Pazopanib, Pyrimethamine, Rapamycin, Sunitinib and Temsirolimus) displayed higher IC50s in the high-*RAD51AP1* expression subgroup, whereas Bexarotene, Doxorubicin, Gemcitabine and Tipifarnib had lower IC50s, which suggested that the HCC patients with high-*RAD51AP1* expression may be more susceptible to the treatment Bexarotene, Doxorubicin, Gemcitabine and Tipifarnib (all $P < 0.05$, Figure 11).

Violin plots showed the high-*RAD51AP1* expression subgroup exhibited relatively lower IPSs, representing a possibly dismal reaction to immunotherapy of *PD-1* and *CTLA-4* inhibitors ($P < 0.05$; Figure 12A–D). Moreover, the subgroup with higher TIDE score represents immunotherapy non-responders whose suppressive cells in the immune microenvironment inhibit the infiltration of T cells. We observed that the high-*RAD51AP1* expression subgroup had markedly lower TIDE and immune dysfunction scores but higher immune exclusion scores than those of low expression subgroup ($P < 0.001$, 12F-I). Furthermore, the responders in the IMvigor210 dataset had significantly the higher *RAD51AP1* expression levels, which demonstrated that *RAD51AP1* could reliably predict the therapeutic response of immunotherapy not just for HCC but for other cancer types as well.

Discussion

Genetic alteration is a hallmark characteristic of hominine solid tumors[32] and can greatly affect the formation and progression of HCC.[33] DNA damage can activate DNA damage repair system, which increases the risk of DNA modifications and genome defection.[34] Homologous recombination DNA repair (HR), especially operating in double-strand DNA breaks,[35] is identified as an efficient repair mechanism.[36] *RAD51* recombinase can catalyze HR[37] and form a nucleoprotein filament to promote HR DNA repair.[38] *RAD51AP1* acts as a necessary protein to activate *RAD51* recombinase.[39] Thus, *RAD51AP1* functions to keep genomic integrity via *RAD51* recombinase enhancement.[40] Furthermore, Gonzalez et al showed that *RAD51AP1* functions to maintain telomere length and

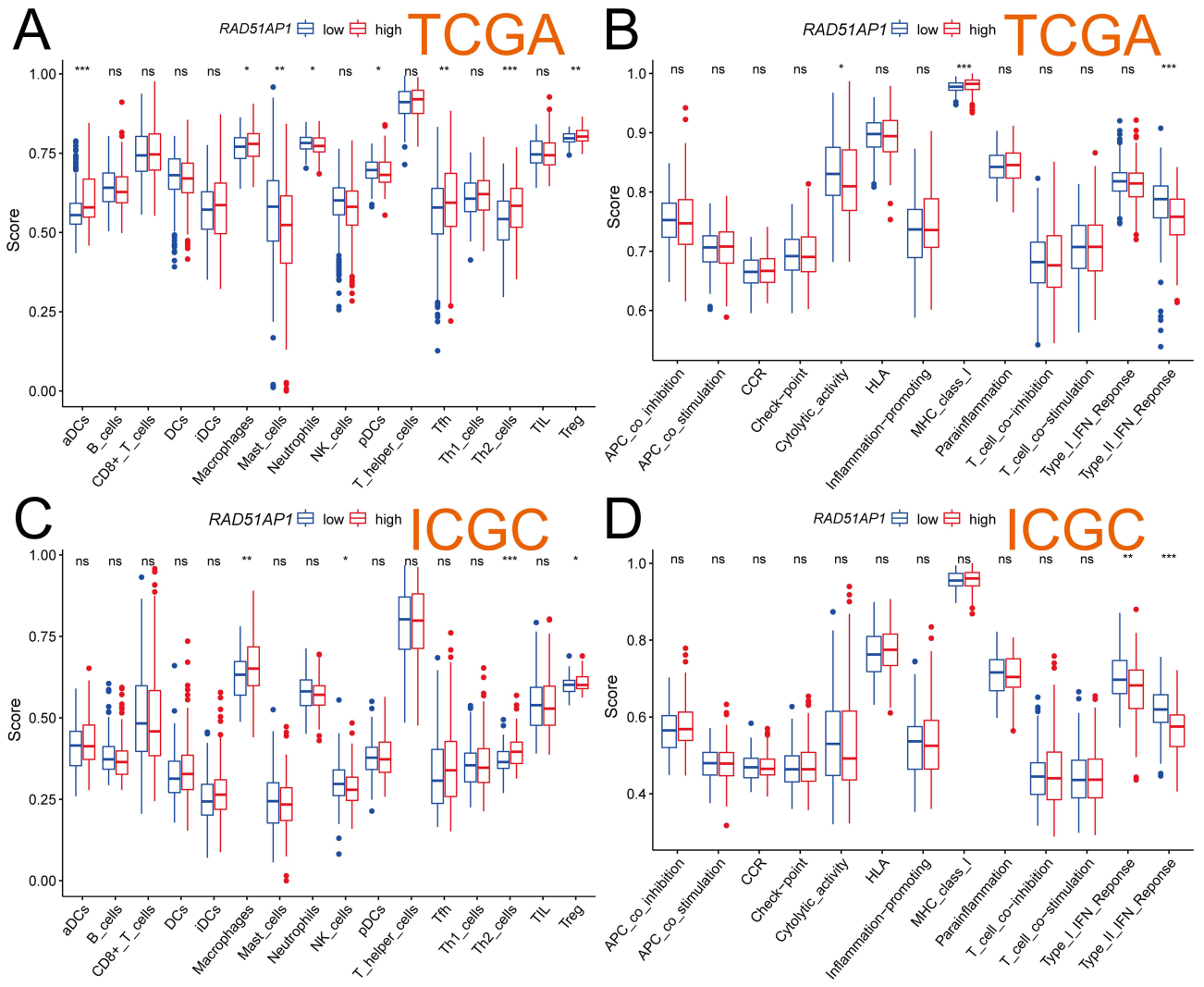

Figure 9 The results of ssGSEA analysis demonstrated the differences of the infiltration levels of immune cells and the relative expression activities of various function pathways between the high- and low-*RAD51AP1* expression groups in the TCGA (**A** and **B**) and ICGC (**C** and **D**) cohorts. ns, No significance, **P* < 0.05; ***P* < 0.01; and ****P* < 0.001.

Abbreviations: ssGSEA, single-sample Gene Set Enrichment Analysis; *RAD51AP1, RAD51* associated protein 1; TCGA, The Cancer Genome Atlas; ICGC, International Cancer Genome Consortium.

protect against the proliferation of alternative lengthening of telomeres in cancer cells.[41] It is worth mentioning that Wu et al demonstrated an up-regulated *RAD51AP1* in non-small cell lung cancer (NSCLC). In addition, the metastasis, proliferation, invasion, and migration of NSCLC cell line were inhibited under circumstance of *RAD51AP1* silencing.[15] Obama et al showed that downregulation of *RAD51AP1* retarded growth of intrahepatic cholangiocarcinoma cells.[16] Likewise, *RAD51AP1* knockout experiments showed that *RAD51AP1* knockout in human breast cancer cells and syngeneic mouse models reduced tumor growth and metastasis by increasing breast cancer stem cell self-renewal.[42] A mouse model experiment using U87MG cells revealed that knocking-down *RAD51AP1* inhibited glioma.[43]

In our study, the upregulated *RAD51AP1* expression was related to shorter OS and RFS, histologic grade, TNM stage, T staging and vascular invasion degree and higher serum AFP level and was involved in cancer-promoting pathways in HCC, indicating that *RAD51AP1* represents increased malignancy and poor prognosis. Immune

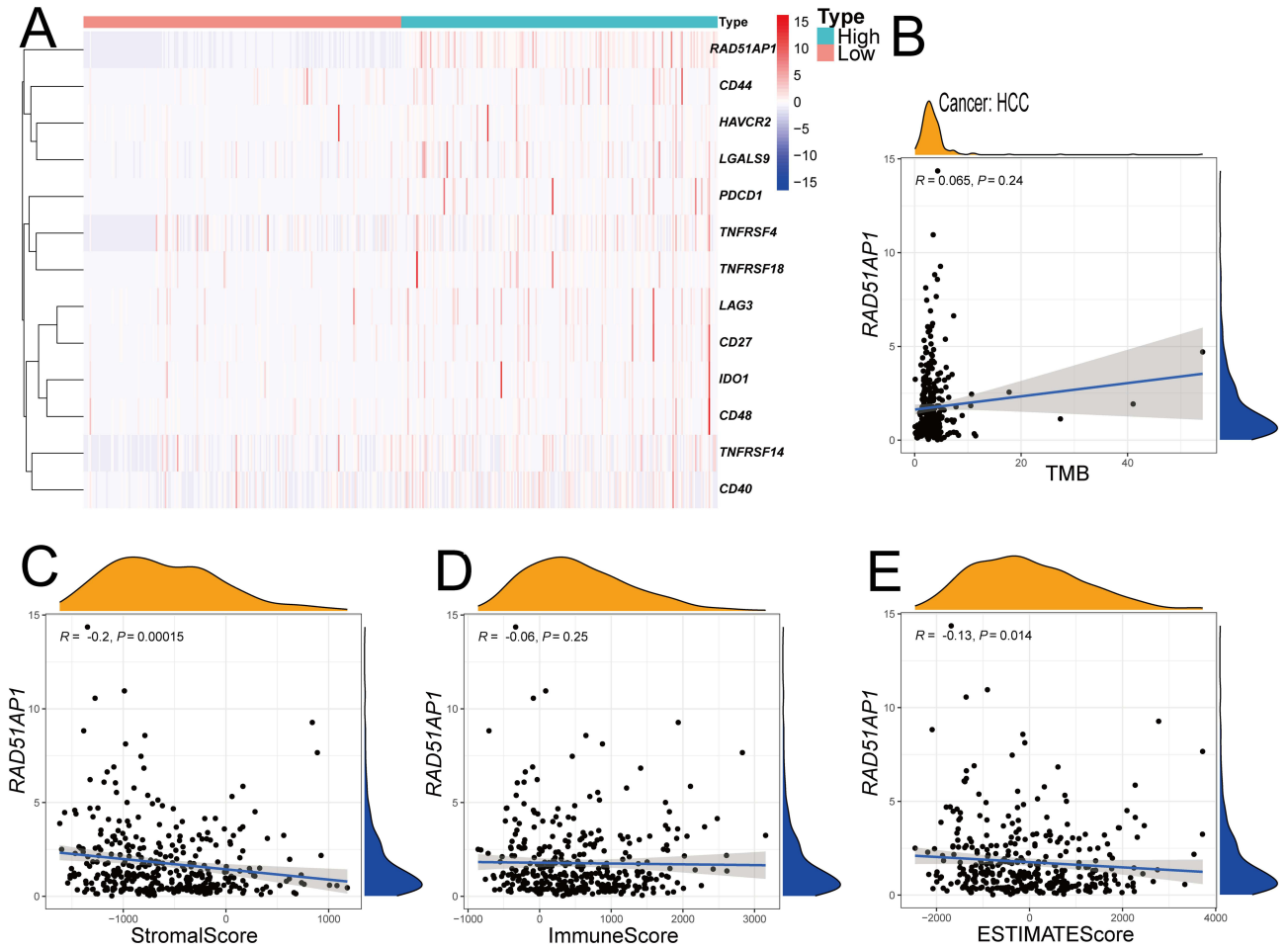

Figure 10 (**A**) The heatmap illustrating the significantly differential expression immune-related checkpoint genes between the high- and low-*RAD51AP1* expression groups based on the TCGA-HCC database. (**B**–**E**) Spearman analysis of *RAD51AP1* expression level and TMB (**B**), stromal score (**C**), immune score (**D**) and ESTIMATE score (**E**). **Abbreviations**: *RAD51AP1*, *RAD51* associated protein 1; TCGA, The Cancer Genome Atlas; HCC, hepatocellular carcinoma; TMB, tumor mutation burden; ESTIMATE, Estimation of Stromal and Immune cells in Malignant Tumor tissues using Expression data.

checkpoint inhibitors (ICIs) and molecularly targeted therapy have achieved a remarkable survival benefit to the HCC managements.[6,44,45] HCC has a high tumor heterogeneity and different molecule characteristics.[46,47] Tumor associated macrophages (TAMs), contributing to tumor growth and progression, are the essential elements of tumor microenvironment.[48] *RAD51AP1* was demonstrated to regulate colorectal cancer stem cell self-renewal and promote colorectal cancer growth and drug resistance.[49] We demonstrated that *RAD51AP1* may be associated with an immunosuppressive microenvironment with higher TAMs infiltration in HCC. Interestingly, the high *RAD51AP1* expression may suggest a better immunotherapy response and could be more susceptible to Bexarotene, Doxorubicin, Gemcitabine and Tipifarnib. Collectively, *RAD51AP1* may be a potential molecular therapeutic target. The dominating analyses were bioinformatics research based on the public datasets. Therefore, immunohistochemistry is urgently needed to confirm the *RAD51AP1* protein expression level in HCC tissue. To decipher the molecular mechanism, the impact on HCC immune infiltration, and therapeutic sensitivity of *RAD51AP1* still requires further validated researches in vitro and in vivo.

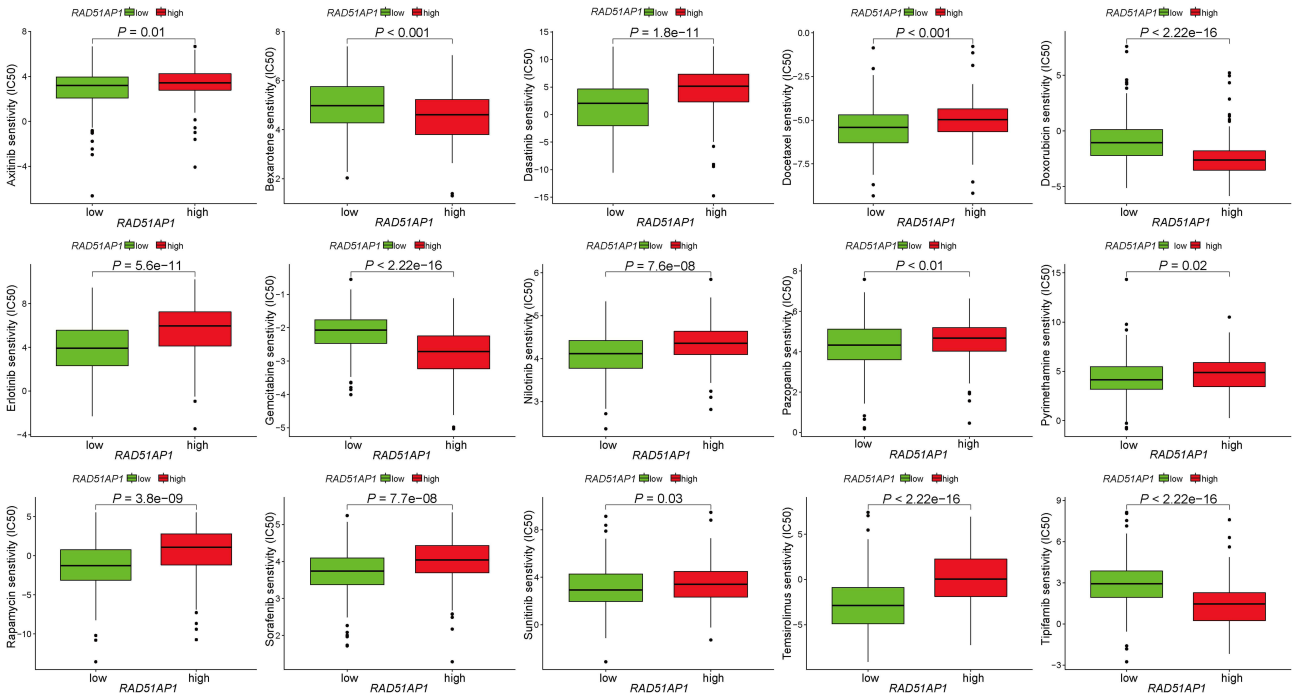

Figure 11 Based on R package "pRRophetic" and the gene expression profile of the TCGA-HCC dataset, the predicted IC50 values of anti-HCC therapies, including chemotherapeutic and molecular targeted drugs, in the high- and low-*RAD51AP1* expression groups were determined and then compared.
Abbreviations: TCGA, The Cancer Genome Atlas; HCC, hepatocellular carcinoma; IC50, half-maximal inhibitory concentration; *RAD51AP1, RAD51* associated protein 1.

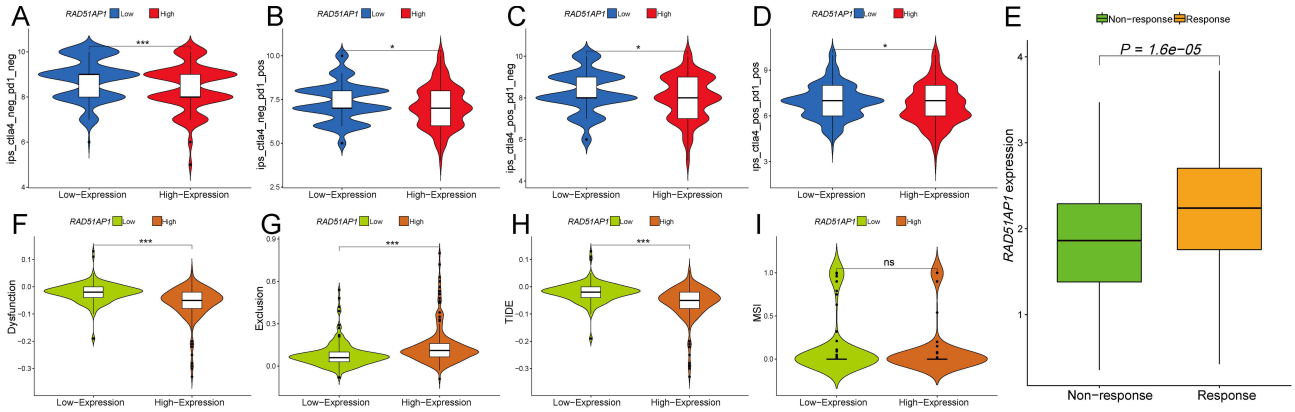

Figure 12 (**A**–**D**) Violin plots showed the different levels of IPSs between the different *RAD51AP1* expression groups. (**E**) Differential *RAD51AP1* expression level between non-response and response groups was demonstrated in IMvigor210 dataset. (**F**–**I**) Immune dysfunction, immune exclusion, TIDE, and MSI scores were compared between the high- and low-*RAD51AP1* expression groups. *$P < 0.05$; ***$P < 0.001$.
Abbreviations: IPS, immunophenotypic score; *RAD51AP1, RAD51* associated protein 1; TIDE, Tumor Immune Dysfunction and Exclusion; MSI, microsatellite instability.

Conclusions

Taken together, *RAD51AP1* mediating the immunosuppressive microenvironment is a potential diagnostic and prognostic biomarker and could be an underlying therapeutic target for HCC. However, this conclusion still requires further validations form additional cohorts.

Data Sharing Statement

All raw online RNA sequencing dataset and clinical information of HCC patients, which were included in the current study, can be downloaded from the TCGA (https://portal.gdc.cancer.gov/), ICGC (http://dcc.icgc.org), and GEO database (https://www.ncbi.nlm.nih.gov/geo/). Further inquiries can be directed to the corresponding author.

Ethics Statement

This experiment was authorized by the Ethical Review Committee of the First Affiliated Hospital of Guangxi Medical University [Approval Number: 2021 (KY-E-032)]. Written informed consent was agreed and signed by all HCC patients. This trial was conducted in accordance with the ethical principles in the Declaration of Helsinki.

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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. Yongguang Wei and Chenlu Lan should be regarded as co-first authors.

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Disclosure

The authors declare that they have no conflict of interest.

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