

The Phenomenon of Gene Rearrangement is Frequently Associated with TP53 Mutations and Poor Disease-Free Survival in Hepatocellular Carcinoma

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Purpose: Gene rearrangements (GRs) have been reported to be related to adverse prognosis in some tumours, but the relationship in hepatocellular carcinoma (HCC) remains less studied. The objective of our study was to explore the clinicopathological characteristics and prognosis of HCC patients (HCCs) with GRs (GR-HCCs).

Patients and Methods: This retrospective study included 297 HCCs who underwent hepatectomy and had their tumours sequenced by next-generation sequencing. Categorical variables between groups were compared by the chi-square test. The impact of variables on disease-free survival (DFS) and survival after relapse (SAR) was analysed by the Kaplan–Meier method and Cox regression.

Results: We observed four repetitive GR events in 297 HCCs: BRD9/TERT, ARID2/intergenic, CDKN2A/intergenic and OBSCN truncation. GR-HCCs frequently presented with low tumour differentiation, tumour necrosis, microvascular invasion, elevated AFP and gene mutations (TP53, NTRK3 and BRD9). The 1-, 2-, and 3-year cumulative DFS rates in GR-HCCs were 45.1%, 31.9%, 31.9%, respectively, which were significantly lower than those of GR-negative HCCs (NGR-HCCs) (72.5%, 57.9%, and 49.0%, respectively; $P = 0.001$). GR was identified as an independent risk factor for inferior DFS in HCCs (HR = 1.980, 95% CI = 1.246–3.147; $P = 0.004$). However, there was no significant difference in SAR between GR-HCCs and NGR-HCCs receiving targeted therapy or immunotherapy.

Conclusion: GR is frequently associated with TP53 mutations and significantly affects DFS following radical resection for HCC. We recommend that GR-HCCs should be closely followed up as a high-risk group for postoperative recurrence.

Keywords: hepatocellular carcinoma, gene rearrangement, next-generation sequencing, prognosis, risk factors

Introduction

According to the latest global cancer statistics in 2020, primary liver cancer accounted for 4.7% of all new malignancies worldwide, and its mortality rate (8.3%) ranked third in tumour-specific mortality.¹ Hepatocellular carcinoma (HCC) accounts for the majority of primary liver cancer cases, and liver transplantation (LT), liver resection (LR) and local ablation are currently effective treatments for early HCC.² However, due to the complexity of the genetic background of HCC, the high recurrence rate after surgery is still a challenge for the prognosis of

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HCC patients (HCCs). Fortunately, with the large-scale adoption of next-generation sequencing (NGS), an increasing number of molecular alterations (TP53, TERT and CTNNB1 mutations, etc) that promote the development of HCC have been revealed.³ In addition, several clinical trials have shown improved overall survival in unresectable patients treated with molecular targeting drugs (sorafenib, lenvatinib, regorafenib, etc) and immune checkpoint inhibitors (nivolumab, pembrolizumab, etc).⁴ Regrettably, at present, there is still a lack of actionable and characteristic biomarkers for HCC to evaluate therapeutic efficacy and prognosis in clinical practice.

In recent years, however, gene rearrangements (GRs) or fusions have been confirmed as one of the driving factors of malignant tumours.^{5,6} GR occurs when some gene fragments change their original order of articulation through insertion, deletion, translocation, inversion, etc, and eventually rearrange themselves into a new transcription unit by a DNA repair mechanism.⁷ GRs can lead to a variety of consequences, including gene activation, silencing, or fusion. Some fusion proteins expressed by rearranged genes have become distinctive targets of treatment, which has led to a remarkable improvement in patient prognosis.^{8,9} Imatinib, dasatinib and nilotinib have been successively approved by the US Food and Drug Administration (FDA) for the treatment of leukaemia with BCR/ABL rearrangement.¹⁰ Crizotinib, ceritinib and alectinib have been approved one after another by the FDA for the treatment of non-small cell lung cancer (NSCLC) with ALK rearrangement.¹¹ The value of GR is exemplified by the clinical benefits obtained by patients, and the approval of the aforementioned drugs has also greatly encouraged researchers to investigate the phenomenon of GR in other malignancies. Some GR events were described by The Cancer Genome Atlas in HCC;^{12,13} however, there are few studies on the clinical effects of GR on HCCs. Thus, we conducted a retrospective analysis based on data from 297 HCCs who underwent surgery in our hospital to explore the clinicopathological characteristics and prognosis of HCCs with GR (GR-HCCs) and then provide new ideas for the clinical management of HCC with different gene statuses.

Patients and Methods

Enrolled Cases

We collected the specimens and corresponding clinical materials of patients who underwent surgical resection for HCC in the Affiliated Hospital of Qingdao University

from April 2016 to October 2020. A total of 297 patients were ultimately enrolled in our research study after excluding patients who had received preoperative treatment and patients with a history of primary malignancy in other tissues or organs. Inflammation grading and fibrosis staging of each specimen were determined by Scheuer's method, and grades 3–4 or stages 3–4 were considered as advanced stages. Radical (R0) resection refers to the complete removal of the tumour and postoperative microscopic confirmation that the margins are free of tumour cells, no tumour lesions should be found by postoperative imaging examination, and the dynamic detection of tumour markers should indicate that the quantitative level gradually decreased to the normal range within two months after surgery.

NGS

Specimens: All formalin-fixed paraffin-embedded (FFPE) specimens were confirmed by pathological examination to be larger than 1 cm² in area and more than 20% in tumour cell density.

Sequencing: DNA was extracted from FFPE tumour tissues and paired plasma according to the instructions of the QIAamp[®] DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) and sequenced by the Illumina NextSeq500 (Illumina Incorporated, San Diego, CA) platform with an average coverage of 1000×. Single-nucleotide variants (SNVs), copy number variations (CNVs), short insertions/deletions (InDels), GRs and tumour mutation burden (TMB) were the main events of our concern. We performed all sequencing work with Yuansu 450[™] in OrigiMed[®] (a laboratory certified by the CAP and CLIA in Shanghai, China).

Immunohistochemistry (IHC)

Programmed cell death 1 ligand 1 (PD-L1) IHC staining of all FFPE (4 µm thick) sections was completed using an automatic sample pretreatment instrument (PT link, Dako) and the EnVision[™] FLEX Kit (Cat# K800221-2, Dako, DK) on the automated immunostainer Dako Autostainer Link 48 (Agilent Technologies Inc., CA, USA) according to the manufacturer's instructions. In addition to the primary antibody (FLEX RTU, Dako), other important detection reagents were provided with the kits, including Peroxidase-Blocking Reagent (DM841), Monoclonal Mouse Anti-Human PD-L1 (clone 22C3, Cat# M3653), EnVision FLEX DAB + Chromogen (DM847), and EnVision FLEX Substrate Buffer (DM843). Placental

tissue and phosphate buffered saline were used as positive and negative controls, respectively, in each staining run. All sections were interpreted by 2 trained diagnostic pathologists, and the expression of HCC PD-L1 protein was interpreted by the combined positive score (CPS), which is the number of PD-L1 stained cells (tumour cells, lymphocytes, and macrophages) divided by the total number of viable tumour cells and multiplied by 100.¹⁴ According to previous studies, the positive expression of PD-L1 was defined as $CPS \geq 1$, while $CPS \geq 20$ was defined as high expression.^{14,15}

Systemic Treatment After Recurrence

Sorafenib (400 mg twice daily, Bayer HealthCare Pharmaceuticals Inc, Germany) was applied to patients with well-preserved liver function (Child-Pugh A) and with tumour staging (BCLC B-C), as well as to those who were unsuitable for local therapies. Patients who were intolerant to sorafenib and had positive expression of PD-L1 protein or TMB >6 Muts/Mb (the median of our data) were treated with camrelizumab (an anti-PD-1 agent, Hengrui Pharmaceuticals Inc, China) at a dose of 200 mg every 3 weeks.

Follow-Up

All HCCs were followed up once a month in the first 3 months of the first year after surgery and then every 3 months until 2 years after surgery and every 6 months thereafter. All enrolled populations were followed up until 30 November 2020 or death. The follow-up items included laboratory tests (alpha-fetoprotein (AFP) and liver function tests) and imaging evaluations (upper abdominal ultrasonography or computed tomography (CT)). If HCC recurrence or metastasis was suspected, one or two of the following examination methods were selected for confirmation: enhanced CT, magnetic resonance imaging (MRI), Gd-EOB-DTPA-enhanced MRI, bone scan or positron emission tomography/CT (PET/CT). In addition, if the imaging manifestation of the lesion was atypical, ultrasound-guided biopsy might be required to further clarify the diagnosis.

The date of HCC recurrence or metastasis was the date of diagnosis by imaging. Disease-free survival (DFS) and survival after relapse (SAR) were calculated from the date of specimen acquisition and the date of recurrence or metastasis, respectively. The follow-up data of all patients were obtained through the HIS system of our hospital and telephone calls.

Statistical Analysis

TMB and mutation number were nonnormally distributed and are expressed as the median (interquartile range (IQR)); their differences between groups were compared by the Mann-Whitney *U*-test. Categorical variables between groups were compared by the Pearson's chi-square test. DFS and SAR were analysed by the Kaplan–Meier method and compared by the Log rank test. Based on factors with a *P* value < 0.1 in univariate analysis, the independent risk factors for postoperative DFS in HCC were determined by a Cox proportional hazards regression model. Receiver operating characteristic (ROC) curves and the areas under the curve (AUCs) were calculated to evaluate the prognostic abilities of the risk factors. *P* values < 0.05 (two-tailed) were considered to be statistically significant. The GR events observed in this study were demonstrated by a cluster heatmap (produced by R software 3.6.1). All data were statistically analysed by SPSS software (version 24.0, IBM Corp, NY, USA), Kaplan–Meier curves and ROC curves were drawn with GraphPad Prism software (version 8.3, San Diego, CA, USA).

Results

Baseline Characteristics

A total of 297 postoperative patients were included in this study, including 60 (20.2%) GR-HCCs and 237 (79.8%) GR-negative HCCs (NGR-HCCs). In this study, there were 253 males (85.2%) and 44 females (14.8%), with an average age of 56.3 ± 9.8 years. Compared with NGR-HCCs, GR-HCCs had higher levels of AFP (*P* = 0.041). Tumours in the GR-HCCs were less differentiated (*P* = 0.026) and more frequently accompanied by necrosis (*P* = 0.005) and microvascular invasion (MVI) (*P* = 0.023).

Overall, the PD-L1 positivity rate in this study was 47.5% (141/297). There were 156 patients (52.5%) with $CPS < 1$, 117 patients (39.4%) with $CPS 1-19$, and 24 patients (8.1%) with $CPS \geq 20$. Nevertheless, the expression of PD-L1 protein showed no significant difference between GR-HCCs and NGR-HCCs. The detailed clinico-pathological features of GR-HCCs and NGR-HCCs are shown in Table 1.

Profile of Gene Variations

A total of 81 GR events involving 115 genes were observed in 297 HCC samples. Repetitive GR events were defined as GR events detected in at least 2 samples where the breakpoints of genes from different samples were in the same region, such as

Table 1 Demographics and Clinical Characteristics of GR-HCCs and NGR-HCCs [n (%)]

Variables	GR-HCC (n=60)	NGR-HCC (n=237)	P
Sex (male)	51(85.0)	202(85.2)	0.964
Age (≤ 60 years)	38(63.3)	148(62.4)	0.899
Smoking (Yes)	25(41.7)	123(51.9)	0.157
Diabetes (Yes)	10(16.7)	30(12.7)	0.417
Hepatitis virus			0.126
HBV	49(81.7)	214(90.3)	
HCV	2(3.3)	4(1.7)	
None	9(15.0)	19(8.0)	
HBsAg (+)	48(80.0)	204(86.1)	0.241
HBeAg (+)	12(20.0)	42(17.7)	0.683
Antivirals (Yes)	24(51.1)	108(52.9)	0.816
Alcoholism (Yes)	22(36.7)	81(34.2)	0.717
FH of cancer (Yes)	13(21.7)	67(28.3)	0.303
Hypertension (Yes)	16(26.7)	52(21.9)	0.436
PHT (Yes)	13(21.7)	49(20.8)	0.878
ALB (<35 g/L)	4(6.7)	24(10.1)	0.413
ALT (>50 U/L)	9(15.0)	46(19.4)	0.432
AST (>40 U/L)	13(21.7)	39(16.5)	0.343
TB (>22 μ mol/L)	22(36.7)	72(30.4)	0.350
HBV-DNA ($\geq 10^3$ copies/ml)	17(28.3)	60(25.3)	0.634
Child-Pugh score			0.702
A	54(90.0)	217(91.6)	
B	6(10.0)	20(8.4)	
Non-R0 resection (+)	4(7.7)	30(13.3)	0.268
Steatosis (Yes)	9(15.0)	24(10.1)	0.283
PVT (Yes)	3(5.0)	7(3.0)	0.701
HCI (Yes)	28(46.7)	95(40.1)	0.355
LNI (Yes)	2(3.3)	4(1.7)	0.350
MVI (+)	39(65.0)	115(48.5)	0.023
PD-L1 protein			0.757
CPS <1	29(48.3)	127(53.6)	
CPS 1–19	26(43.3)	91(38.4)	
CPS ≥ 20	5(8.3)	19(8.0)	
MTD (>5.0 cm)	18(30.0)	82(34.6)	0.501

(Continued)

Table 1 (Continued).

Variables	GR-HCC (n=60)	NGR-HCC (n=237)	P
AFP (≥ 400 ng/ml)	24(40.0)	63(26.6)	0.041
TNM stage (III–IV)	18(30.0)	69(29.1)	0.893
BCLC stage (B or C)	23(38.3)	99(41.8)	0.629
No. of tumours (≥ 2)	23(38.3)	99(41.8)	0.629
Encapsulation (No)	13(21.7)	46(19.4)	0.695
Fibrosis score (S3–4)	41(68.3)	172(72.6)	0.515
Satellite lesion (Yes)	14(23.3)	38(16.0)	0.184
Tumour necrosis (Yes)	24(40.0)	53(22.4)	0.005
Vascular invasion (Yes)	7(11.7)	21(8.9)	0.506
Distant metastasis (Yes)	4(6.7)	15(6.3)	1.000
Inflammation score (G3–4)	3(5.0)	22(9.3)	0.286
Tumour differentiation ($>II$)	37(61.7)	108(45.6)	0.026

Abbreviations: AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer stage; FH, family history; HCI, Hepatic capsule involvement; LNI, lymph node involvement; MTD, maximal tumour diameter; MVI, microvascular invasion; No., number; PD-L1, programmed cell death 1 ligand 1; PHT, portal hypertension; R0, radical; PVT, portal vein thrombosis; TNM, Tumour-Node-Metastasis.

promoter, exon, intron or intergenic regions, and we found 4 repetitive GR events in this study: BRD9/TERT (NC_000005.9:g.892748_1295893dup), CDKN2A/intergenic (NC_000001.10:g.90508893_90508894ins[NC_000009.11:g.21967750_21974544inv]), OBSCN truncation (NC_000001.10:g.228538022_232211839del), and ARID2/intergenic (NC_000012.11:g.46123275_46124923del). The differences in the above four repetitive events between GR-HCCs and NGR-HCCs were statistically significant ($P < 0.05$). In addition, genes that were present in at least 3 GR events were annotated as the most frequent genes involved in rearrangement events, including NTRK3 ($n = 6$), TERT ($n = 4$), CDKN2A ($n = 4$), CCND1 ($n = 3$), and BRD9 ($n = 3$). The detailed GR events observed in this study are presented in Figure 1.

Furthermore, the top ten genes with the highest mutation incidences in this study were TP53 (163/297, 54.9%),

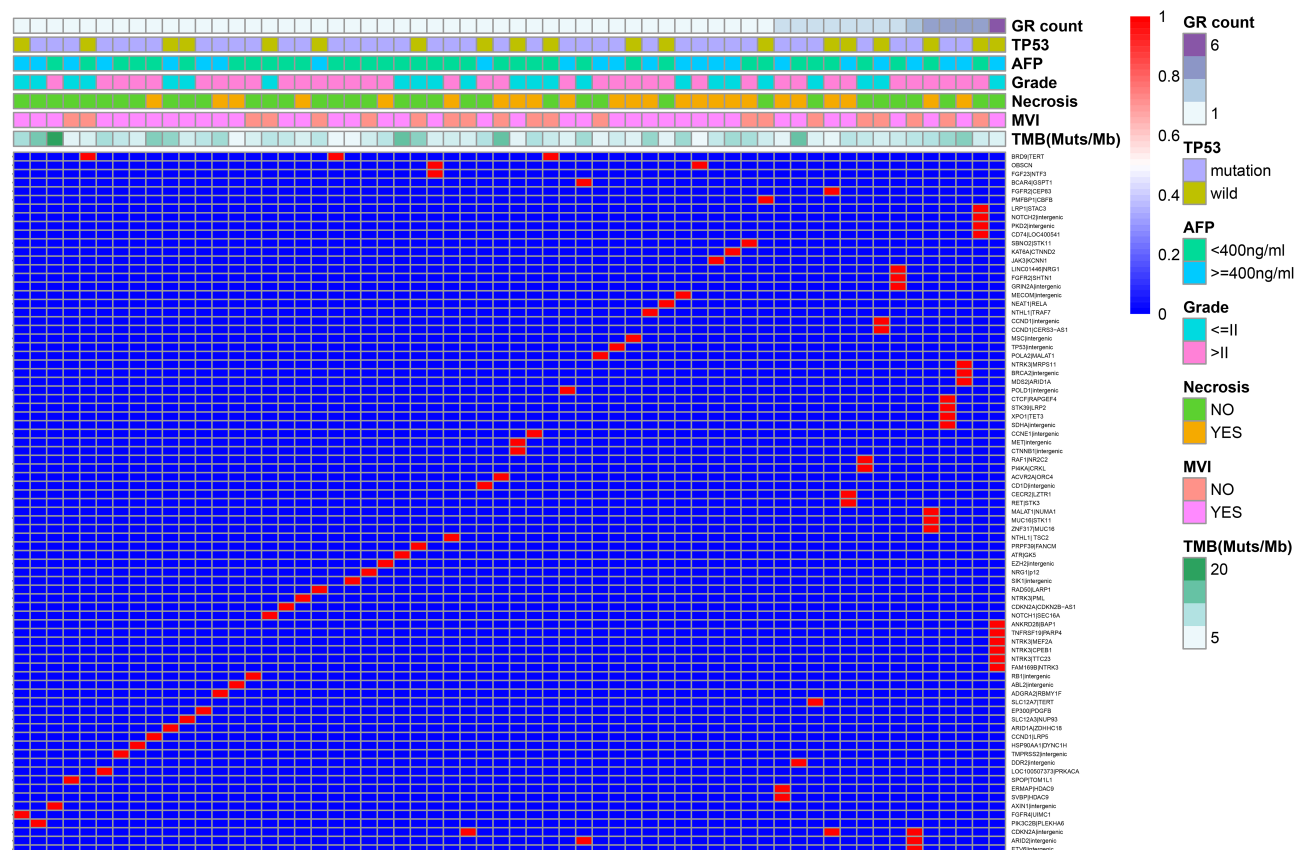


Figure 1 Detailed GR events observed in this research. The rows and columns represent gene rearrangement events and samples, respectively. The red mark in each column of the figure represents the rearrangement event of the sample. Clinicopathological information corresponding to each sample is displayed at the top of the figure, including the number of rearrangement events (GR count), TP53 mutation, AFP value, tumour differentiation (grade), tumour necrosis, microvascular invasion (MVI) and tumour mutation burden (TMB). The meaning of the various colours is shown on the far right side of the diagram.

TERT (127/297, 42.8%), CTNNB1 (65/297, 21.9%), AXIN1 (46/297, 15.5%), TSC2 (43/297, 14.5%), LRP1B (41/297, 13.8%), RB1 (38/297, 12.8%), SPTA1 (35/297, 11.8%), MUC16 (32/297, 10.8%), CCND1 (28/297, 9.4%), CDKN2A (28/297, 9.4%), and FAT3 (28/297, 9.4%). The mutation frequencies of TP53, NTRK3 and BRD9 in GR-HCCs were 66.7% (40/60), 8.3% (5/60), and 5.0% (3/60), respectively, which were significantly higher than those in NGR-HCCs (51.9%, 2.1%, and 0%, respectively) ($P < 0.05$). Details of TP53 mutations are summarized in [Supplementary Table 1](#). In addition, we found that CCND1 and FGF19 often coamplified on chromosome 11q13.3 ($\chi^2=228.6$, $P = 0.000$). The relationships between GR events and the above high-frequency variant genes are shown in [Table 2](#).

Prognosis of GR-HCCs and NGR-HCCs

The median follow-up time for this group of patients was 25.5 months (range, 6.1–55.8 months). Excluding patients with nonradical resection (34 cases) and patients with less

than 6 months of follow-up (19 cases), a total of 244 patients (48 GR-HCCs and 196 NGR-HCCs) were finally included in the survival analysis.

The cumulative DFS rates of GR-HCCs at 1, 2, and 3 years were 45.1%, 31.9%, and 31.9%, respectively, which were significantly lower than those of NGR-HCCs (72.5%, 57.9%, and 49.0%, respectively) ($P = 0.001$). The probability of DFS in the NGR-HCC group dropped abruptly to zero at the end because one patient with the longest DFS (43.6 months) in the NGR-HCC group had pulmonary metastasis. Overall, the median DFS (mDFS) of GR-HCCs was significantly lower than that of NGR-HCCs (10.5 months vs 31.9 months, $P = 0.001$) ([Figure 2](#)).

Predictive Factors for DFS

Univariate analysis revealed that the following factors were associated with short DFS after R0 resection in HCCs: (a) clinical features, including Child-Pugh score and hypertension; (b) GR; and (c) tumour features, including number of lesions, maximum tumour diameter (MTD),

Table 2 The Distribution of Genetic Variants in GR-HCCs and NGR-HCCs [n (%)]

Variation	GR-HCC (n=60)	NGR-HCC (n=237)	P
TP53 mutation	40(66.7)	123(51.9)	0.040
TERT mutation	27(45.0)	100(42.2)	0.686
TSC2 mutation	13(21.7)	30(12.7)	0.076
RBI mutation	10(16.7)	28(11.8)	0.315
CTNBN1 mutation	9(15.5)	56(23.2)	0.201
LRPIB mutation	8(13.3)	33(13.9)	0.906
SPTA1 mutation	8(13.3)	27(11.4)	0.677
MUC16 mutation	7(11.7)	25(10.5)	0.803
OBSCN mutation	6(10.0)	12(5.1)	0.259
AXIN1 mutation	5(8.6)	41(17.0)	0.112
FAT3 mutation	5(8.6)	23(9.7)	0.745
CDKN2A mutation	5(8.3)	23(9.7)	0.745
NTRK3 mutation	5(8.3)	5(2.1)	0.047
BRD9 mutation	3(5.0)	0(0.0)	0.008
FGF19 amplification	3(5.0)	22(9.3)	0.286
CCND1 amplification	3(5.0)	25(10.5)	0.189
BRD9/TERT GR	3(5.0)	0(0.0)	0.008
CDKN2A/Intergenic GR	3(5.0)	0(0.0)	0.008
ARID2/intergenic GR	2(3.3)	0(0.0)	0.040
OBSCN truncation GR	2(3.3)	0(0.0)	0.040
Number of mutant gene ^a	11(8, 16)	11(7, 15)	0.092
TMB ^a	5.2(3, 8.4)	6.1(3.7, 9.2)	0.075

Note: ^aMann-Whitney U-test.

Abbreviations: GR, gene rearrangement; TMB, tumour mutation burden.

necrosis, AFP, MVI, vascular invasion, hepatic capsule involvement (HCI), satellite lesions, Edmondson grade, Barcelona Clinic Liver Cancer (BCLC) stage, Tumour-Node-Metastasis (TNM) stage, and PD-L1 expression. Multivariate analysis indicated that AFP ≥ 400 ng/ml ($P = 0.049$) and GR ($P = 0.004$) were independent risk factors for adverse DFS after R0 resection for HCC (Table 3).

For GR-HCCs, albumin (ALB) < 35 g/L, AFP ≥ 400 ng/ml, MTD > 5.0 cm, multiple tumours, tumour differentiation (III–IV), vascular invasion, MVI, portal vein thrombosis (PVT), satellite lesions, distant metastasis, BCLC stage (B/C), TNM stage ($>II$), PD-L1 expression, and CCND1/FGF19 coamplification were important factors leading to inferior DFS after R0 resection ($P < 0.05$); HCI ($P=0.058$) and MUC16 mutations ($P=0.054$) were also of marginal

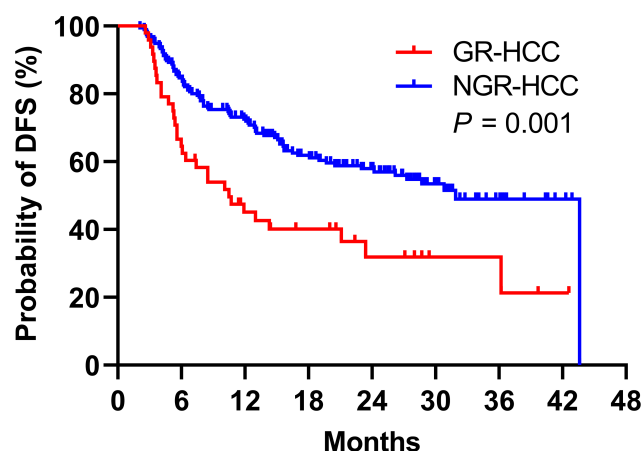


Figure 2 Kaplan-Meier plot for postoperative DFS of HCC. GR-HCCs showed significantly lower mDFS than NGR-HCCs (10.5 months vs 31.9 months, $P = 0.001$). Abbreviations: GR-HCCs, HCC patients with gene rearrangement; NGR-HCCs, HCC patients with gene rearrangement-negative; DFS, disease-free survival.

significance (as shown in Table 4). The survival curves for the abovementioned factors are displayed in Figure 3.

The Prognostic Performance of the Predictive Factors for DFS

The predictive power of the above factors for predicting recurrence was compared by ROC curve analysis. The AUCs of GR and AFP for predicting postoperative recurrence of HCC were 0.572 (95% CI: 0.500–0.645) and 0.598 (95% CI: 0.526–0.670), respectively (Figure 4A). Nevertheless, the AUCs of MTD, multiple tumours, BCLC stage (B/C), and TNM stage ($>II$) for predicting postoperative recurrence of GR-HCC were 0.680 (95% CI: 0.530–0.831), 0.715 (95% CI: 0.568–0.863), 0.729 (95% CI: 0.586–0.871), and 0.710 (95% CI: 0.567–0.852), respectively ($P < 0.05$) (Figure 4B). The AUCs of all predictors are listed in Table 5.

Therapies and Survival After Recurrence

A total of 113 patients (46.3%, 113/244) presented with recurrence during our follow-up period, including 31 GR-HCCs and 82 NGR-HCCs. The median SAR (mSAR) of 113 patients with recurrent HCC (r-HCCs) was 32.4 months (95% CI: 18.42–46.4 months). There was no statistically significant difference in the mSAR between GR-HCCs (not reached) and NGR-HCCs (32.4 months, 95% CI: 21.48–43.32 months) ($\chi^2 = 0.58$, $P = 0.4$).

Among the 113 r-HCCs, 38 patients (7 GR-HCCs and 31 NGR-HCCs) received targeted therapy, and the cumulative SAR rates appeared to be higher in r-GR-HCCs at 1 and 2 years (75% and 75%, respectively) than in r-NGR-HCCs

Table 3 Univariate and Multivariate Analysis of DFS Predictors in HCCs (n=244)

Variables	n/n	Univariate		Multivariate	
		mDFS (Months)	P	HR(95% CI)	P
Age, y ($\leq 60/>60$)	150/94	23.0/31.9	0.124		
Gender (male/female)	205/39	28.7/30.8	0.859		
Somking (no/yes)	121/123	26.2/31.9	0.325		
Alcoholism (no/yes)	159/85	26.2/31.9	0.789		
HBsAg (-/+)	34/210	31.9/26.2	0.365		
HBeAg (-/+)	201/43	30.8/28.7	0.933		
Antivirals (no/yes)	95/114	19.7/25.7	0.599		
Diabetes (no/yes)	211/33	28.7/26.9	0.751		
PHT (no/yes)	190/54	28.7/27.3	0.515		
Hypertension (no/yes)	184/60	23.0/40.0	0.024	0.599(0.346–1.036)	0.067
FH of cancer (no/yes)	183/61	27.3/31.9	0.702		
ALB, g/L ($<35/\geq 35$)	18/226	23.0/30.8	0.290		
ALT, U/L ($\leq 50/>50$)	201/43	31.9/17.0	0.301		
AST, U/L ($\leq 40/>40$)	207/37	31.9/17.0	0.092	0.896(0.546–1.472)	0.665
TB, $\mu\text{mol/L}$ ($\leq 22/>22$)	175/69	30.8/24.2	0.622		
HBV-DNA, copies/ml ($<10^3/\geq 10^3$)	185/59	30.8/23.0	0.291		
Child-Pugh score (A/B)	226/18	31.9/10.1	0.005	1.699(0.862–3.347)	0.126
Steatosis (no/yes)	214/30	28.7/36.2	0.776		
Fibrosis ($\leq 2/>2$)	63/181	39.0/21.8	0.130		
Inflammation ($G\leq 2/>2$)	226/18	30.8/19.0	0.419		
No. of tumours ($< 2/\geq 2$)	148/96	43.6/10.7	0.000	1.641(0.817–3.296)	0.164
Encapsulation(no/yes)	51/193	20.5/30.8	0.414		
MTD, cm ($\leq 5/>5$)	175/69	43.6/10.4	0.000	1.569(0.838–2.937)	0.160
PVT (no/yes)	236/8	30.8/7.7	0.069	2.168(0.751–6.260)	0.153
MVI (no/yes)	122/122	43.6/15.7	0.000	1.025(0.641–1.640)	0.917
HCI (no/yes)	146/98	42.0/15.0	0.000	1.357(0.860–2.139)	0.189
LNI (no/yes)	242/2	28.9/23.0	0.146		
TNM stage ($\leq \text{II}/>\text{II}$)	188/56	43.6/6.8	0.000	0.820(0.355–1.893)	0.642
BCLC stage (0/A:B/C)	154/90	43.6/8.5	0.000	1.472(0.660–3.281)	0.345
Differentiation ($\leq \text{II}/>\text{II}$)	126/118	36.2/15.7	0.005	1.115(0.735–1.691)	0.608
Satellite lesion (no/yes)	204/40	36.2/7.8	0.000	1.382(0.799–2.390)	0.248
Tumour necrosis (no/yes)	189/55	36.2/12.4	0.002	1.223(0.756–1.980)	0.412
AFP, ng/ml ($< 400/\geq 400$)	179/65	43.6/10.7	0.000	1.586(1.001–2.512)	0.049
PD-L1 ($< 1/1-19/\geq 20$)	132/92/20	36.2/19.0/10.5	0.032	1.217(0.543–2.723)	0.634
Distant metastasis(no/yes)	236/8	28.7/4.3	0.080	1.285(0.393–4.196)	0.678
Vascular invasion (no/yes)	226/18	30.8/5.3	0.000	1.248(0.555–2.805)	0.592
GR (no/yes)	196/48	31.9/10.5	0.001	1.980(1.246–3.147)	0.004
TMB, Muts/Mb ($\leq 6.0/>6.0$)	121/123	36.2/24.2	0.395		
TP53 mutation (no/yes)	113/131	30.8/28.7	0.596		
TERT mutation (no/yes)	140/104	30.8/23.0	0.176		

Abbreviations: AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer stage; GR, gene rearrangement; HCI, Hepatic capsule involvement; LNI, lymph node involvement; MTD, maximal tumour diameter; MVI, microvascular invasion; No., number; PHT, portal hypertension; PVT, portal vein thrombosis; TNM, Tumour-Node-Metastasis; TMB, tumour mutation burden.

(69% and 56%, respectively), but the difference was not statistically significant ($P = 0.284$) (Figure 5A). In addition, 17 r-HCC patients (4 GR-HCCs and 13 NGR-HCCs) in this study underwent immunotherapy, and r-GR-HCCs showed slightly higher cumulative SAR rates at 1 and 2 years (100% and 100%, respectively) than r-NGR-HCCs (92% and 78%,

respectively), but the difference was not statistically significant ($P = 0.464$) (Figure 5B).

Discussion

Initially, the phenomenon of GR was mainly observed in haematologic disorders, such as leukaemia¹⁶ and

Table 4 Factors Affecting DFS After Radical Treatment in GR-HCCs

Variables	n/n	DFS (Months)	P
Age, y (≤ 60 / >60)	31/17	8.5/10.7	0.246
Gender (male/female)	39/9	10.7/5.6	0.474
Smoking (no/yes)	27/21	6.4/21.1	0.294
Alcoholism (no/yes)	31/17	10.5/10.7	0.613
HBsAg (-/+)	9/39	24.9/10.1	0.178
HBeAg (-/+)	39/9	10.5/10.7	0.319
Antivirals (no/yes)	18/20	10.7/6.1	0.816
Diabetes (no/yes)	41/7	11.9/8.5	0.748
Hypertension (no/yes)	34/14	10.1/23.4	0.209
ALB, g/L (≤ 35 / ≥ 35)	4/44	5.3/11.9	0.019
ALT, U/L (≤ 50 / >50)	39/9	10.7/10.1	0.889
AST, U/L (≤ 40 / >40)	34/14	8.5/10.7	0.727
TB, $\mu\text{mol/L}$ (≤ 22 / >22)	32/16	8.5/10.5	0.830
HBV-DNA, copies/ml ($<10^3$ / $\geq 10^3$)	34/14	8.5/10.7	0.939
Child-Pugh score (A/B)	42/6	11.9/5.3	0.079
PHT (no/yes)	36/12	10.7/5.6	0.189
Steatosis (no/yes)	41/7	8.5/36.2	0.143
Fibrosis score (≤ 2 / >2)	15/33	8.5/10.7	0.621
Inflammation score (≤ 2 / >2)	46/2	10.1/10.7	0.611
PD-L1 (<1 / $1-19$ / ≥ 20)	25/20/3	21.1/6.0/3.4	0.002
MVI (0/1/2)	16/21/11	36.2/10.7/5.3	0.001
HCI (no/yes)	24/24	36.2/6.4	0.058
PVT (no/yes)	46/2	10.7/2.9	0.005
MTD, cm (≤ 5 / >5)	34/14	23.4/6.1	0.022
TNM stage ($\leq \text{II}$ / $> \text{II}$)	35/13	23.4/5.3	0.000
BCLC stage (0/A/B/C)	31/17	36.2/6.0	0.000
Encapsulation (no/yes)	11/37	10.5/21.1	0.247
No. of tumours (<2 / ≥ 2)	29/19	21.1/6.1	0.004
Tumour necrosis (no/yes)	31/17	11.9/8.5	0.377
AFP, ng/ml (<400 / ≥ 400)	29/19	13.0/5.6	0.049
Satellite lesion (no/yes)	37/11	21.1/6.1	0.011
Distant metastasis (no/yes)	46/2	10.7/2.6	0.000
Vascular invasion (no/yes)	41/7	13.0/5.3	0.000
Tumour differentiation ($\leq \text{II}$ / $> \text{II}$)	16/32	36.2/7.4	0.015
TP53 mutation (no/yes)	17/31	10.7/10.1	0.842
ARID2 mutation (no/yes)	46/2	10.5/2.9	0.262
AXIN1 mutation (no/yes)	43/5	10.7/10.1	0.711
BRD9 mutation (no/yes)	45/3	10.5/29.3	0.255
TERT mutation (no/yes)	27/21	11.9/10.1	0.883
LRP1B mutation (no/yes)	43/5	10.1/13.0	0.588
TSC2 mutation (no/yes)	37/11	10.7/7.4	0.764
FAT3 mutation (no/yes)	43/5	10.5/14.3	0.921
RBI mutation (no/yes)	39/9	10.7/10.1	0.388
SPTA1 mutation (no/yes)	40/8	10.1/14.3	0.803
MUC16 mutation (no/yes)	43/5	13.0/8.5	0.054
OBSCN mutation (no/yes)	44/4	10.5/8.5	0.648
NTRK3 mutation (no/yes)	43/5	10.7/10.1	0.425

(Continued)

Table 4 (Continued).

Variables	n/n	DFS (Months)	P
CTNNB1 mutation (no/yes)	40/8	8.5/19.7	0.358
CDKN2A mutation (no/yes)	43/5	10.1/23.4	0.553
TMB, Muts/Mb (≤ 6.0 / >6.0)	27/21	23.4/8.5	0.105
CCND1/FGF19 coamplification (no/yes)	45/3	11.9/3.6	0.007

Abbreviations: AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer stage; HCI, hepatic capsule involvement; LNI, lymph node involvement; MTD, maximal tumour diameter; MVI, microvascular invasion; No., number; PHT, portal hypertension; PVT, portal vein thrombosis; TNM, Tumour-Node-Metastasis.

lymphoma.¹⁷ With the continuous maturity of sequencing technology, many studies found that GRs were also present in a variety of solid tumours, such as the RET/CCDC6 rearrangement in thyroid cancer,⁸ the TMPRSS2/ERG rearrangement in prostate cancer,⁹ the BCOR/CCNB3 rearrangement in osteosarcoma,¹⁸ ALK rearrangements in renal cell carcinoma,¹⁹ and the EML4/ALK rearrangement in NSCLC.²⁰ Most of these rearranged genes played a carcinogenic role. GRs also exist in malignant liver tumours. FGFR2 fusion or rearrangement was found in approximately 10% to 16% of intrahepatic cholangiocarcinoma patients, and the FGFR kinase inhibitor pemigatinib was approved by the FDA for the clinical treatment of the aforementioned patients in April 2020.²¹ The DNAJB1-PRKCA rearrangement has also been frequently reported in fibroblastic HCC;²² however, the specificity of GR in ordinary HCC remains unclear.^{12,23}

Based on whole-genome sequencing, Fernandez-Banet et al observed the first recurrent rearrangement event (ABCB11/LRP2) in 88 HCC cases and found that similar events frequently induced the aberrant overexpression of genes on the 3' side of the 3' breakpoint in DNA.¹² However, Zhu et al identified 43 recurrent rearrangement events in HCC from their own database and four public RNA sequencing (RNA-seq) databases via STAR software, three (HLA-DPB2-HLA-DRB1, CDH23-HLA-DPB1, and C15orf57-CBX3) of which were interpreted as disease-related.¹³ Due to the limited sample size and the use of different analysis strategies, the above events were not identified in our study. However, some of the rearrangement events identified in our study may have an important role in HCC. We identified 4 repetitive GR events (BRD9/TERT, ARID2/intergenic, CDKN2A/

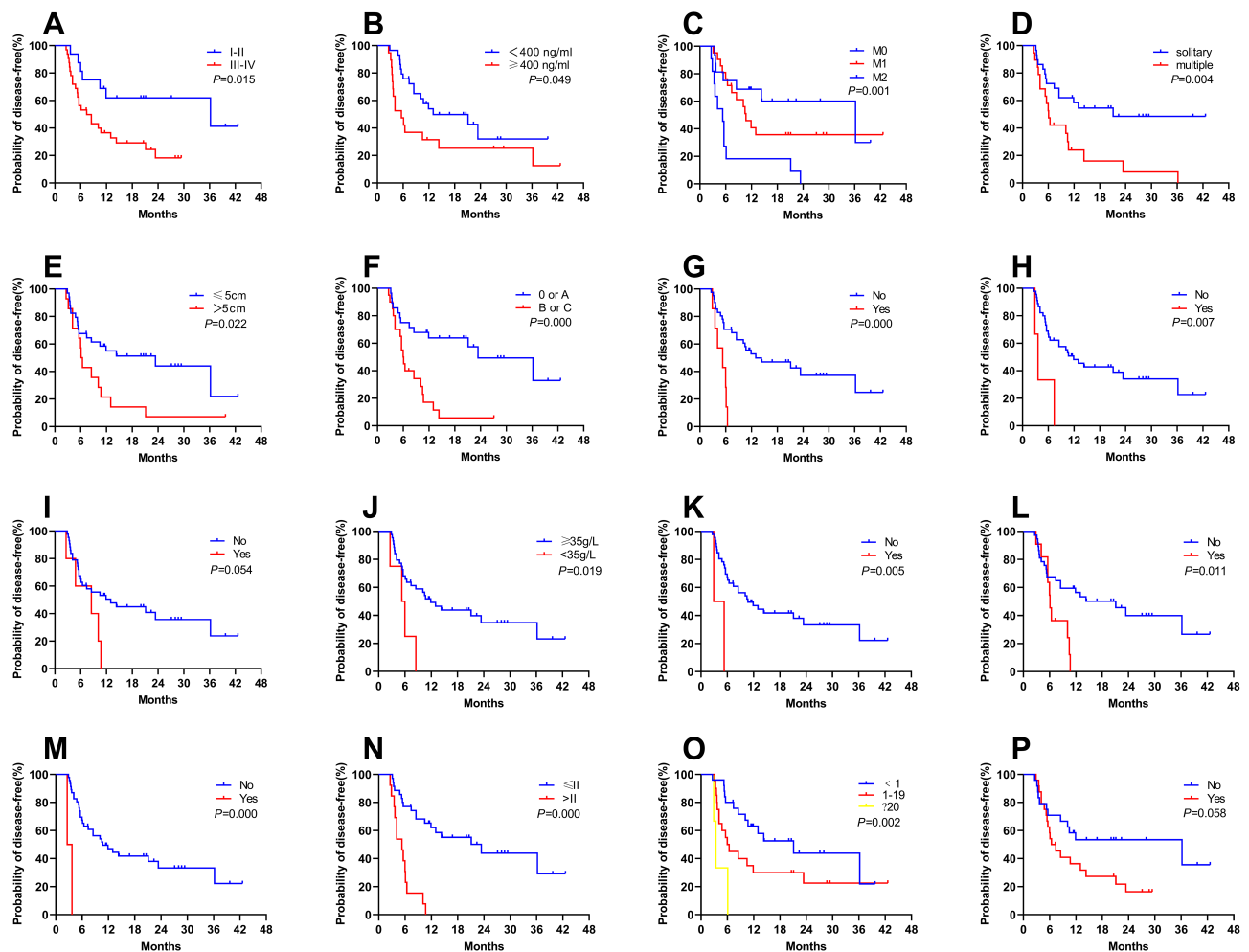


Figure 3 Survival curves of factors influencing DFS in GR-HCCs. (A) Tumour differentiation (grade III–IV), (B) serum AFP values $\geq 400\text{ng/ml}$, (C) microvascular invasion, (D) multiple tumours, (E) maximal tumour diameter $> 5.0\text{cm}$, (F) BCLC stage (B or C), (G) Vascular invasion, (H) CCND1/FGF19 coamplification, (I) MUC16 mutation, (J) ALB $< 35\text{g/L}$, (K) portal vein thrombosis, (L) satellite lesion, (M) distant metastasis, (N) TNM stage ($> \text{II}$), (O) PD-L1 expression, and (P) hepatic capsule involvement were important factors leading to inferior DFS in GR-HCCs.

intergenic and OBSCN truncation) in 60 GR-HCCs. TERT activation is necessary to maintain the unlimited proliferative capacity of tumour cells. Bromodomain-containing protein 9 (BRD9) has been shown to promote the growth and metastasis of HCC cells by activating the TUFT1/AKT pathway.²⁴ We found that the breakpoint of BRD 9 was located in the 3' UTR, the breakpoint of TERT was located in its upstream promoter sequence, and this variant retained the complete coding region of TERT. It has been reported in the literature that the expression level of TERT mRNA in liposarcoma samples with other TERT rearrangements (such as TRIO-TERT) is more than 100 times higher than that in samples without TERT rearrangements,²⁵ and the above study indicated that the BRD9/TERT rearrangement may lead to sustained high expression of TERT mediated by the BRD9 promoter.

Cyclin-dependent kinase inhibitor 2A (CDKN2A) encodes two cell cycle inhibitory proteins, p16INK4a and p14ARF. CDKN2A deficiency leads to the inactivation of Rb and p53 function, which in turn leads to uncontrolled cell proliferation.²⁶ We found that the breakpoint in the first intron of CDKN2A resulted in abnormal mRNA splicing, abnormalities or functional deletion of the p16INK4a protein. AT-rich interaction domain 2 (ARID2) encodes a protein that is a member of the DNA-binding protein family, a subunit of the PBAF (SWI/SNF-B) chromosome remodelling complex, which maintains the stability of PBAF and is involved in transcriptional regulation and chromatin structural modifications.²⁷ We observed that the ARID2/intergenic rearrangement resulted in the deletion of the promoter region, transcription start site and exons 1–2 of ARID2, which might lead to abnormal

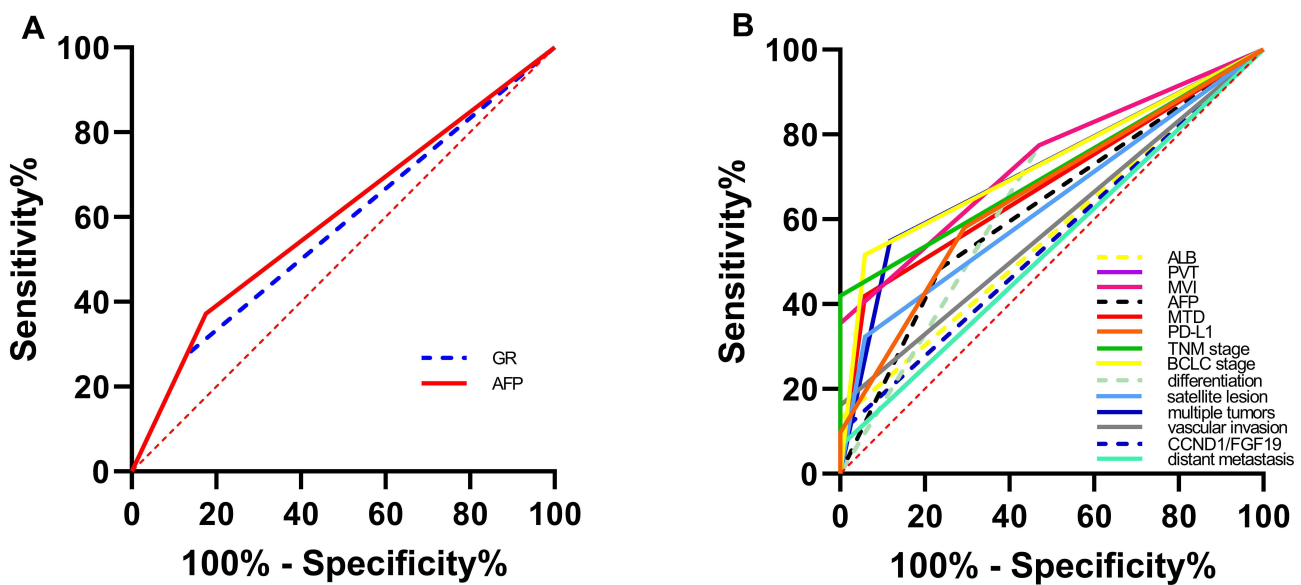


Figure 4 Receiver operating characteristic curves of factors predicting DFS for HCC (A) and GR-HCC (B).

ARID2 protein function. Moreno et al indicated through functional experiments that ARID2 deficiency affected DNA repair and enhanced the sensitivity of cells to DNA-damaging agents.²⁸ The truncation of OBSCN resulted in the deletion of amino acid residues (6106–7968) from obscurin, and the deletion region contained part of the Ig-

like structural domain, protein kinase structural domain and fibronectin type III structural domain. Obscurin is a protein (720 kDa) encoded by OBSN, which belongs to the family of giant sarcomeric signalling proteins. Knockdown of OBSCN significantly affects the growth and biological properties of epithelial cells, such as cell adhesion and intercellular communication, leading to increased cellular carcinogenesis.²⁹ Thus, BRD9/TERT, ARID2/intergenic, CDKN2A/intergenic and OBSCN truncation may be involved in tumourigenesis, and further validation is needed in HCC.

Although the genetic characteristics of GRs in HCC have been described in previous studies, reports on the clinical impact of GRs on HCC are still scarce. Therefore, we performed this research and found that GR-HCCs had lower tumour differentiation ($P = 0.026$), higher serum AFP levels ($P = 0.041$), and higher incidences of tumour necrosis ($P = 0.005$) and MVI ($P = 0.023$) than NGR-HCCs. Previous studies have found that GRs can lead to gene fusions and the aberrant expression of some oncogenes or tumour suppressor genes^{7,13} and then induce the oncogenic activation of the WNT,²³ RAS/MAPK and PI3K/AKT signalling pathways,³⁰ resulting in the low differentiation and rapid proliferation of tumours. The rapid proliferation of tumours often causes relative hypoxia, microangiogenesis and vascular invasion around the tumour. Once the tumour proliferates faster than microangiogenesis, tumour necrosis will occur due to relative ischaemia.

Table 5 Prognostic Performance of Predictors of DFS

Predictive Factors	AUC	95% CI	P-value
In HCC cohort			
GR	0.572	0.500–0.645	0.05
AFP ≥ 400 ng/ml	0.598	0.526–0.670	< 0.01
In GR-HCC cohort			
MVI	0.652	0.484–0.820	0.085
PVT	0.532	0.363–0.701	0.714
TNM stage	0.710	0.567–0.852	0.017
BCLC stage	0.729	0.586–0.871	0.009
Satellite lesion	0.632	0.474–0.790	0.134
MTD > 5.0 cm	0.680	0.530–0.831	0.041
ALB < 35 U/L	0.565	0.399–0.730	0.464
AFP ≥ 400 ng/ml	0.624	0.461–0.788	0.158
Multiple tumours	0.715	0.568–0.863	0.014
Vascular invasion	0.581	0.417–0.744	0.360
Distant metastasis	0.532	0.363–0.701	0.714
Tumour differentiation	0.652	0.484–0.820	0.085
PD-L1 expression	0.657	0.500–0.815	0.074
CCND1/FGF19 coamplification	0.548	0.381–0.716	0.583

Abbreviations: ALB, albumin; GR, gene rearrangement; MTD, maximal tumour diameter; MVI, microvascular invasion; PVT, portal vein thrombosis.

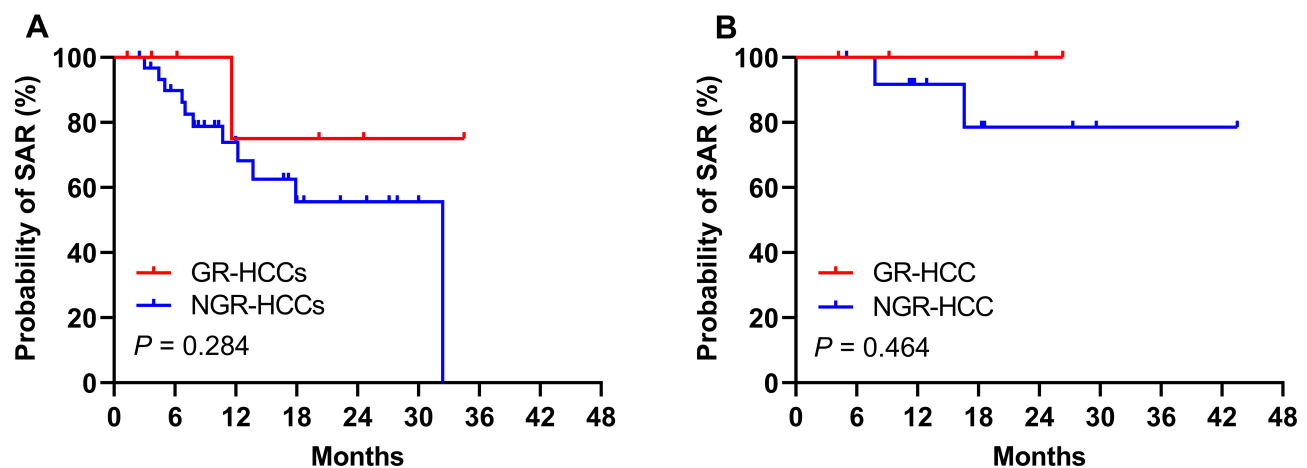


Figure 5 Kaplan-Meier plot for SAR of recurrent HCC patients according to different treatments. (A) Targeted therapy, (B) immunotherapy.

PD-L1, a transmembrane protein expressed on the membranes of immune cells and tumour cells, can inhibit immune cells and promote the development of tumours by binding to the PD-1 receptor on the surface of immune cells. The combination of atezolizumab (an immunosuppressive agent targeting PD-L1) and bevacizumab was approved by the FDA for first-line treatment in patients with unresectable HCC in May 2020. Therefore, confirmation of PD-L1 expression is potentially valuable for HCC. To date, the FDA has approved four PD-L1 assays (22C3, 28-8, SP142 and SP263) as companion or complementary diagnostics of cancer immunotherapy. Shi et al found that three assays (22C3, 28-8 and SP263) were highly consistent in terms of PD-L1 scoring in HCC.¹⁵ This study used the 22C3 assay to assess PD-L1 expression, and the positive rate of PD-L1 expression was 47.5%, which was higher than the results of Huang et al (19%, 78/411).³¹ A meta-analysis revealed that the expression of PD-L1 in HCC was significantly correlated with low histological grade and poor survival.³² Although this study found that GR-HCCs often presented with poorly differentiated tumours, there was no significant difference in the expression of PD-L1 between GR-HCCs and NGR-HCCs. These differences may be mainly attributed to different threshold settings and whether the positive immune cells expressing PD-L1 in tumour areas are calculated. Notably, it has been reported that pathologists are highly inconsistent when evaluating the expression of PD-L1 at a lower cut-off.¹⁵ Therefore, it is suggested that trained pathologists are used and that the samples are deglycosylated before staining to estimate the expression of PD-L1 protein more accurately.

In addition, we found that GR-HCCs were frequently accompanied by TP53 mutations. The predominant type of TP53 mutation in this study was SNV, with C:G>T:A and G:C>T:A transitions being the most common forms. 52.8% of TP53 mutations showed loss of function (LOF), 22.6% of TP53 mutations showed gain of function (GOF), 22.1% of TP53 mutations were of unknown significance, and 2.5% of TP53 mutations were nonsense (see [Supplementary Table 1](#)). The specific mechanism of GRs combined with TP53 mutation in HCC is still unclear, but this phenomenon also exists in other tumours, such as leukaemia,³³ NSCLC,³⁴ and paediatric medulloblastoma.³⁵ We assume that this may be due to GRs causing mutations in the tumour suppressor gene TP53 or TP53 mutations leading to increased chromosomal instability, which in turn creates GRs.³⁵ Although TP53 mutations have been reported to be associated with poor prognosis in HCC in previous studies, our study failed to confirm this finding. This may be related to the fact that we identified TP53 mutations only at the DNA level rather than based on the expression or function of the p53 protein. Because some genetic mutations (eg, MDM2, MDM4, PPM1D, or CDKN2A) other than TP53 mutations can also inactivate the p53 protein in tumours, resulting in wild-type TP53 tumours exhibiting the same malignancy as TP53-mutated tumours.³⁶ This is one of the important reasons for the inconsistency between TP53 mutation status and clinical outcome in many studies. Moreover, several studies have reported that concurrent TP53 mutations in ALK-rearranged NSCLC predict little benefit from systemic therapy.³⁷ Unfortunately, we were unable to validate this phenomenon in GR-HCC due to the small sample size.

We also found that GR-HCCs were frequently accompanied by NTRK3 and BRD9 mutations, however, only a small number of cases with NTRK3 ($n=7$) and BRD9 ($n=3$) mutations were observed, and this result remains to be further validated.

As previously mentioned, HCC patients have high recurrence rates after R0 treatment. Although a number of previous studies revealed some risk factors associated with postoperative recurrence,^{38–40} we found that GR-HCCs showed significantly lower DFS than NGR-HCCs (10.5 months vs 31.9 months, $P = 0.001$) and identified that GR ($P = 0.004$) and AFP ($P = 0.049$) were independent risk factors for poor DFS after R0 resection for HCC. We further revealed that MTD, multiple tumours, vascular invasion, satellite lesions, distant metastasis, PVT, ALB, AFP, BCLC stage (B/C), TNM stage ($>II$), tumour differentiation (grade III/IV), MVI, PD-L1 protein expression, and FGF19/CCND1 coamplification were important risk factors for adverse DFS after R0 resection in GR-HCCs ($P < 0.05$). GRs reflect the high instability of tumour genomes,^{34,41} which could give rise to tumourigenesis by generating gene fusions and causing abnormal gene expression. CCND1 amplification is a positive regulatory element in the G1 to S phase of the cell cycle and usually acts synergistically with other oncogenes or suppressor genes to induce tumour development.⁴² Our data revealed a significant correlation between CCND1 amplification and FGF19 amplification ($P=0.000$). Fibroblast growth factor 19 (FGF19) is an oncogene that can play a driving role in carcinogenesis through its receptor (FGFR4), and its amplification has been reported to be independently associated with poor survival and tumour recurrence in HCCs.⁴³ Sawey et al confirmed that co-overexpression of CCND1 and FGF19 was more tumourigenic than single gene overexpression.⁴⁴ Therefore, multiple mutations with synergistic effects rather than any single dominant mutation are more likely to be a potential risk for postoperative recurrence in GR-HCCs. Meanwhile, we recommend that patients with gene variations, especially those with GRs, should be closely followed up as a high-risk group for postoperative recurrence.

Although GR-HCCs exhibited poorer DFS than NGR-HCCs, no significant difference in SAR was observed between GR-HCCs and NGR-HCCs. We considered that this might be related to the regular postoperative follow-up of patients, early detection of recurrence, and timely access to individualized treatment. Similar to the findings of Tan et al,⁴⁵ we did not observe a significant improvement in SAR of GR-HCCs in r-HCCs treated with immunotherapy.

This may be related to the LOF in TP53, which promotes the recruitment of suppressor regulatory T (Treg) cells in tumours and attenuates CD4⁺ T helper 1 (Th1) and CD8⁺ T cell responses in vivo.⁴⁶ In addition, it has been reported that some epithelial malignancies with abnormal activation of tyrosine kinase caused by GR respond well to targeted drugs, such as neurotrophic receptor tyrosine kinase (NTRK) rearrangement.^{20,30,47} However, our study found that there was no significant difference between GR-HCCs and NGR-HCCs in response to targeted therapy. We speculated that this might be related to the type of tumour, specific mutation targets, the small size of our sample and the duration of follow-up.

The limitations of this study lie in its single-centre design and retrospective nature. Our study only included patients from Asian, and detailed genetic comparisons were not performed between populations with different aetiologies and ethnicities, so there might be potential bias in our study with respect to patient populations. Additional experiments are needed to reveal the functional significance of the GR events identified from our research. Large-scale clinical trials are needed in GR-HCCs to screen populations that would benefit from targeted therapies.

Conclusions

Our work identified four repetitive GR events (BRD9/TERT, ARID2/intergenic, CDKN2A/intergenic and OBSCN truncation) in HCC. We found that GR-HCCs were frequently accompanied by TP53 mutations, NTRK3 mutations, and BRD9 mutations and had lower tumour differentiation, higher serum AFP levels and higher incidences of tumour necrosis and MVI than NGR-HCCs. In addition, GR was identified as an independent risk factor for DFS after R0 resection for HCC. Therefore, we recommend that GR-HCCs should be closely followed up as a high-risk group for postoperative recurrence. In addition, our study revealed that there was no significant difference between GR-HCCs and NGR-HCCs in the response to targeted therapy and immunotherapy. Our research provides new information on the phenomenon of GR in HCC. These findings may expand our understanding of rearrangement events in HCC and contribute to the clinical management of HCC.

Abbreviations

AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer stage; CAP, the College of American Pathologists; CLIA, the Clinical Laboratory Improvement Amendments;

DFS, disease-free survival; FDA, the US Food and Drug Administration; FH, family history; GRs, gene rearrangements; GR-HCCs, HCC patients with gene rearrangement; HBsAg, hepatitis B virus surface antigen; HCC, hepatocellular carcinoma; HCCs, hepatocellular carcinoma patients; r-HCCs, recurrent hepatocellular carcinoma patients; HCl, Hepatic capsule involvement; IQR, inter-quartile range; LNI, lymph node involvement; MDT, multidisciplinary team; MTD, maximal tumour diameter; MVI, microvascular invasion; NGR-HCCs, HCC patients with gene rearrangement-negative; NGS, next-generation sequencing; No., number; NSCLC, non-small cell lung cancer; PD-L1, programmed cell death 1 ligand 1; PHT, portal hypertension; PVT, portal vein thrombosis; SAR, survival after relapse; SLT, salvage liver transplantation; TMB, tumour mutation burden; TNM, Tumour-Node-Metastasis.

Ethics Approval and Informed Consent

This study was conducted in accordance with the criteria of the Declaration of Helsinki, and was sanctioned by the Research Ethics Committee of the Affiliated Hospital of Qingdao University (approval number: QDFYKYLLL-20,161,212). The informed consent was obtained from all patients.

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Disclosure

The authors have no conflict of interests related to this publication.

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