ORIGINAL RESEARCH

The mutation of hepatitis B virus and the prognosis of hepatocellular carcinoma after surgery: a pilot study

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Background: Although hepatitis B virus (HBV) is still one of the most common etiological factors for hepatocellular carcinoma (HCC), the association between the HBV mutations and the clinical characteristics and prognosis of HBV-related HCC patients (HBV-HCC) after surgical resection remains largely unknown.

Materials and methods: A cohort of 131 consecutive patients who received hepatectomy for HBV-HCC were retrospectively enrolled. The HBV genotype and 14 genomic mutations, which have been reported to relate to HCC in liver samples, were sequenced. The associations between the genomic mutations and clinical characteristics and outcomes were analyzed.

Results: Both A1762T/G1764A mutation and Pre S deletion related to worse overall survival (OS, $p=0.040$ and $p<0.001$, respectively) and disease-free survival (DFS, $p=0.040$ and $p<0.001$, respectively), G1899A mutation related to worse OS ($p=0.030$), A1762T/G1764A mutation correlated with tumor size ($r=0.204$, $p=0.019$), G1899A mutation correlated with vascular invasion ($r=0.332$, $p<0.001$), and Pre S deletion correlated with alpha-fetoprotein (AFP; $r=0.254$, $p=0.003$) positively. Multivariate analysis with Cox proportional hazards model revealed that both A1762T/G1764A mutation and Pre S deletion were independent prognostic factors for OS (hazard ratio [HR]=3.701, 95% CI=1.390–9.855, $p=0.009$, and HR=4.816, 95% CI=2.311–10.032, $p<0.001$, respectively) and DFS (HR=3.245, 95% CI=1.400–7.521, $p=0.006$, and HR=2.437, 95% CI=1.311–4.530, $p<0.001$, respectively), and patients with dual mutations were found to have the worst OS and DFS ($p<0.001$ and $p<0.001$, respectively). Patients with A1762T/G1764A mutation or Pre S deletion were more likely to have early recurrence ($p=0.042$ and $p=0.019$, respectively).

Conclusion: HBV DNA genomic mutations in A1762T/G1764A and Pre S deletion were associated with worse prognoses and early recurrence for HBV-HCC patients after surgery.

Keywords: hepatitis B virus, mutation, hepatocellular carcinoma, prognosis

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer mortality worldwide. Approximately 700,000 people die of HCC each year.[1,2] The prognosis of HCC is poor because the disease is often at a fairly advanced stage at the time of diagnosis.[3,4] Liver resection remains the main curative option for HCC with a 5-year overall survival (OS) rate of 50–70% after curative hepatectomy. However, the long-term prognosis is still unsatisfactory due to the high recurrence rate.[3,5] To improve prognosis, it is crucial to identify the risk factors affecting hepato-carcinogenesis and post-surgical recurrence of HCC.

Despite successful vaccination programs, hepatitis B virus (HBV) is still the most common etiological factor of HCC.[6,7] HBV is a hepatotropic virus that chronically

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infects approximately 400 million people worldwide and is thought to be responsible for a million deaths annually.[8,9] Especially in the People's Republic of China, HBV infection is a challenging health issue with approximately 93 million people being HBV carriers and 30 million people being chronic hepatitis B patients.[10,11] HBV infection is associated with a large spectrum of liver diseases including chronic hepatitis, cirrhosis, and HCC. The pathogenesis of HCC in chronic HBV infections has been investigated comprehensively in recent decades.[12–15] Studies[12–15] have demonstrated a significant relationship between serum HBV DNA levels and the risk of developing HCC in chronic HBV carriers. In addition to viral load, HBV genotype and mutations are also correlated with the development of HCC.[14–17] However, only a few studies have focused on the prognostic value of these mutations in HBV-related HCC (HBV-HCC) patients.[18,19]

The HBV genome contains four overlapping open reading frames that encode the surface protein (S), the core protein, a polymerase, and a multifunctional nonstructural protein called X.[13,20] HBV has a mutation rate that is more than 10-fold higher than that of other DNA viruses due to the deficiency of the proofreading function for HBV polymerase. The HBV mutations are clustered in a particular region of the HBV genome such as the basal core promoter (BCP)/preCore region and the Pre S/S region. The most frequent BCP mutation is a double mutation involving an A to T substitution at nucleotide 1762 and a G to A substitution at nucleotide 1764. The A1762T/G1764A BCP double mutation was associated with a hazard ratio (HR) of 1.73 for developing HCC.[12–15] Other BCP mutations, notably T1753V and T1768A, have also been reported to be associated with an increased risk of HCC. The X gene mutations (two of the most common being C1653T and T1753V) and Pre S2 gene deletions have been associated with increased incidence of HCC.[13–15] Accumulation of mutations reflects both the activity of virus infection and the strength of the immune response. However, relatively little is known about the association between the genomic changes in HBV DNA and the clinical outcomes of patients with HBV-HCC after surgical resection.

In present study, we sought to determine whether the genomic mutations in HBV DNA are associated with the clinical characteristics and outcomes of HBV-HCC patients after surgical resection.

Materials and methods
Patients and clinical treatment
A cohort of 131 consecutive patients who received hepatectomy for HCC in our department from January 2010 to December 2010 were retrospectively enrolled. The inclusion criteria for the present study were: 1) no previous treatment for HCC before surgery; 2) histologic confirmation of HCC; 3) HBV positive; 4) R0 resection; 5) no lymph node or extrahepatic metastasis; and 6) follow-up period ≥6.0 months. Patients co-infected with immunodeficiency virus (HIV), hepatitis C virus, or hepatitis D virus were excluded. All the preoperative parameters were recorded and evaluated as possible predictors of survival including sex, age, HBV DNA level, platelet count, AFP, total bilirubin level (TBil), albumin, liver function status, tumor size and number, and tumor thrombus. Liver functional reserve was also assessed using Child-Pugh classification.

Hepatectomy was carried out under general anesthesia using a right subcostal incision with a midline extension. Intraoperative ultrasonography was routinely performed to confirm resectability and major vascular structures. Pringle's maneuver was routinely used with a clamp/unclamp time of 10 minutes/5 minutes. The liver parenchyma was divided with clamp-crushing technique or ultrasonic dissector according to the surgeon's preference. Anatomic resection was our preferred surgical method in hepatic resection for multiple nodules in one segment or in neighboring segments. For anatomic resection, the hepatic parenchyma was transected at the intersegmental plane as described by Couinaud et al.[30] For multiple bilobar nodules, anatomic resection was preferred for the main tumor, while satellite nodules were resected nonanatomically with intent for a negative resection margin. When an inadequate liver remnant was suspected, nonanatomic resection was performed to try to achieve a negative resection margin. Hemostasis on the raw liver surface was done with suturing and fibrin glue.

After surgery, patients were followed up every 3 months for the first 2 years and every 6 months thereafter with physical examination, blood tests for AFP and liver function, and contrast abdominal computed tomography. Anti-virus treatment with lamivudine, entecavir or adefovir was given according to the APASL guideline.[6] Causes of death and sites of recurrence were determined from death certificates, medical interviews, and radiological findings. OS was defined as the interval between surgery and time of either death or last follow-up. Disease-free survival (DFS) was defined as the length of time after liver resection for HCC to detectable intrahepatic and/or extrahepatic recurrence. The treatment for recurrent tumors was determined by our multidisciplinary team including surgeons, oncologists, radiologists, hepatologists, and pathologists. The last follow-up date for patients still alive was in December 2016.

Ethics statement

The research was approved by the institutional review board of Sun Yat-sen University Cancer Center, and written informed consent was obtained from each patient involved in the study.

All the procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments, or comparable ethical standards.

Virological assay

The methods have been described previously by others.[18,19,21–23] All the samples were collected within 30 minutes after resection; the matched paired tumor and corresponding surrounding non-tumorous liver tissue samples were immediately frozen at −80°C. The Wizard Genomic DNA extraction kit (Promega, Madison, WI, USA) was used to extract genomic DNA in surrounding non-tumorous liver tissue. The ABI 7300 TaqMan platform (Life Technologies, Carlsbad, CA, USA) was used to determine the HBV DNA concentration, and the concentration was calculated as copies per microgram of genomic DNA. The DNA sequences were identified by using fluorescence-labeled primers with 3700 Automatic Sequencer (ABI, Foster City, CA, USA). According to the sequencing outcome, HBV genotype and mutations (including A1752T/G, T1753C, G1757A, A1762T/G1764A, C1766T, T1768A, A1775G, C1799G, A1846T, T1858C, G1896A, G1898A, G1899A, and Pre S deletion) were confirmed by the BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical analyses

The statistical analyses were performed using the SPSS 13.0 statistical software (SPSS Company, Chicago, IL, USA). We compared the presence of genomic changes in HBV DNA as well as the clinical characteristics with the clinical outcomes of these patients after surgical resection. Comparisons between the two groups were done using the Student's t-test for continuous data and the chi square test for categorical data. The OS and DFS were calculated by Kaplan-Meier method and compared by log-rank test. The prognostic variables in predicting OS and DFS were assessed by multivariate Cox proportional hazards regression analysis. Results were given as mean ± SD. All statistical tests were two sided, and a significant difference was considered when $p < 0.05$.

Results

Baseline characteristics

The baseline characteristics of all patients are described in detail in Table 1. In brief, there were 118 male and 13 female patients, with a median age of 50 years (mean ± SD: 49.6±10.5, range: 16–73 years). Tumor size ranged from 1.0 cm to 18.0 cm (mean ± SD: 5.6±3.8); 78 patients (59.5%) had tumor ≤5.0 cm and 53 (40.5%) had tumor >5.0 cm. A total of 99 patients (75.6%) had a single tumor and 32 (24.2%) had multiple tumors. All patients were HBV positive and HIV/HCV/HDV negative. The median HBV DNA was 1.3×10^5 IU/L, ranging from 0 to 2.57×10^8 IU/L. According to the 7th edition of the tumor-node-metastasis (TNM) classification of the American Joint Committee on Cancer, 94 patients (71.8%) had stage I–II disease, and 37 (28.2%) had stage III disease (Table 1).

Anatomic resections based on the segments defined by Couinaud et al[30] were performed in 72.5% of patients (95/131). Major hepatectomy, defined as resection of more than two anatomic segments, was required in 68.7% of patients (80/131). The mean operative time was 126.7±37.5 minutes (interquartile range [IQR] 90–240); the mean blood loss was 147.5±35.5 mL (IQR 50–1500). The median length of hospital stay after surgery was 7 days (IQR 6–15). In all, 36 of 131 (27.5%) patients developed postoperative complications. The 30-day mortality rate was 0%.

Prevalence of HBV genotype and genomic mutations

Among the 131 cases, 51 cases (38.9%) were HBV genotype type B and 80 (61.1%) were type C. Most mutation regions that have been reported (including Pre S1, Pre S2, part of S region, part of X region, BCP region, part of C region) were covered in the sequencing. According to different mutation regions, A1752T/G, T1753C, G1757A, A1762T/G1764A, C1766T, T1768A, A1775G, C1799G, A1846T, T1858C, G1896A, G1898A, G1899A, and Pre S deletion mutations were recognized in 9.8% (39/131), 31.3% (41/131), 42.7% (56/131), 74.0% (97/131), 7.6% (10/131), 5.3% (7/131), 43.5% (57/131), 38.9% (51/131), 35.1% (46/131), 47.3% (62/131), 33.6% (44/131), 17.6% (23/131), 12.2% (16/131), and 16.8% (22/131) cases, respectively (Table 2).

HBV genomic mutations related to survival and clinical characteristics

As shown in Table 2, most of the HBV genomic mutations (including A1752T/G, T1753C, G1757A, C1766T, T1768A,

Table 1 Patients characteristics and univariate analysis for OS and DFS

Variables	Cases	Mean OS (month)	p-value	Mean DFS (month)	p-value
Sex					
Male	118	55.2	0.898	46.1	0.729
Female	13	52.2		41.6	
Age (years)					
≤50	62	57.3	0.278	48.9	0.338
>50	69	51		41.8	
Child-Pugh					
A	129	56.1	<0.001	47	<0.001
B	2	5.7		2.7	
Tumor size (cm)					
≤5	78	60.2	0.037	50.9	0.071
>5	53	47.6		36.4	
Tumor number					
Single	99	59.8	0.001	51.2	<0.001
Multiple	32	38.7		27.4	
Macrovascular invasion					
Yes	10	19	<0.001	16.6	0.001
No	121	58		48.5	
PLT (×10⁹/L)					
<100	22	54.9	0.619	45.6	0.73
≥100	109	54.8		46	
ALT (U/L)					
≤40	68	53	0.731	46.6	0.366
>40	63	54.3		43.2	
AST (U/L)					
≤40	65	57.9	0.015	49.7	0.032
>40	66	49		40.1	
ALB (g/L)					
≥35	124	56.7	0.009	47.4	0.101
<35	7	32.2		28.8	
TBil (umol/L)					
≤40	128	56.5	<0.001	47.4	<0.001
>40	3	4.5		2.5	
AFP (ng/mL)					
<400	78	55.9	0.093	47.4	0.111
≥400	53	49.8		41.5	
Prothrombin time (s)					
≤12	82	59.6	0.019	51.9	0.006
>12	49	46.2		35.1	
HBV DNA (IU/L)					
≤10⁵	63	57.4	0.575	50.1	0.225
>10⁵	68	49.5		40.2	
TNM stage					
I-II	94	59.7	0.002	47.7	0.020
III	37	41.2		30.6	

Abbreviations: AFP, alpha-fetoprotein; ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DFS, disease-free survival; HBV, hepatitis B virus; PLT, platelet count; TBil, total bilirubin level; TNM, tumor-node-metastasis; OS, overall survival.

A1775G, C1799G, A1846T, T1858C, G1896A, and G1898A) did not make a significant statistical difference in OS and DFS. However, A1762T/G1764A mutation related to worse OS and DFS (p=0.040 and p=0.006, respectively), G1899A mutation related to worse OS (p=0.030), and Pre S deletion mutation related to worse OS and DFS (p<0.001 and p<0.001, respectively, Table 2, Figure 1).

The relationships between A1762T/G1764A, G1899A, and Pre S deletion mutations and clinical parameters were analyzed respectively. As shown in Tables S1, S2, and S3, the A1762T/G1764A mutation is correlated with tumor size (r=0.204, p=0.019), G1899A mutation with vascular invasion (r=0.332, p<0.001), and Pre S deletion with AFP (r=0.254, p=0.003), positively.

Table 2 HBV genomic mutations and the relation to patient survival

| Mutations	Cases	Mean OS (month)	*p*-value	Mean DFS (month)	*p*-value
A1752T/G					
Wild type	92	57.1	0.186	47.6	0.453
Mutant type	39	48.2		41.0	
T1753C					
Wild type	90	53.9	0.365	46.1	0.813
Mutant type	41	55.6		42.6	
G1757A					
Wild type	75	51.7	0.097	45.6	0.785
Mutant type	56	56.7		42.4	
A1762T/G1764A					
Wild type	34	64.1	0.040	60.9	0.006
Mutant type	97	49.9		38.1	
C1766T					
Wild type	121	56.2	0.234	46.7	0.736
Mutant type	10	40		35.7	
T1768A					
Wild type	124	56.1	0.138	46.8	0.558
Mutant type	7	33.6		31.6	
A1775G					
Wild type	74	51.6	0.085	44.7	0.472
Mutant type	57	57.2		44.2	
C1799G					
Wild type	80	52.7	0.183	42.8	0.111
Mutant type	51	56		49	
A1846T					
Wild type	85	53.1	0.186	44.6	0.38
Mutant type	46	56.7		43.2	
T1858C					
Wild type	69	54.9	0.935	46.7	0.995
Mutant type	62	53.2		41.6	
G1896A					
Wild type	87	55.3	0.950	46.1	0.948
Mutant type	44	53.1		39.8	
G1898A					
Wild type	108	56.3	0.386	46.8	0.851
Mutant type	23	43.4		38.6	
G1899A					
Wild type	115	56.9	0.030	47.8	0.095
Mutant type	16	39.4		32.3	
Pre S deletion					
Wild type	109	59.3	<0.001	50.3	<0.001
Mutant type	22	33.3		23.2	
HBV gene type					
B	51	57.1	0.556	36.9	0.604
C	80	51.7		44.3	

Abbreviations: DFS, disease-free survival; HBV, hepatitis B virus; OS, overall survival.

A1762T/G1764A mutation and Pre S deletion predict survival

The median follow-up period was 55.0 months. The 1-, 3-, and 5-year OS was 84%, 73.3%, and 64.9%, respectively. The 1-, 3-, and 5-year DFS was 71.8%, 57.3%, and 48.3% respectively.

The univariate and multivariate analyses of prognostic factors for OS and DFS were analyzed (Tables 1–3). All clinical parameters were included in univariate analysis for OS and DFS (Table 1). All variables with *p*<0.10 in univariate analysis, as well as A1762T/G1764A, G1899A and Pre S deletion mutations, were further included in multivariate analysis with Cox proportional hazards model. As shown in Table 3, macrovascular invasion (HR=4.994, 95% CI 2.060–12.107, *p*<0.001), TBil level (HR=18.188, 95% CI 4.153–79.644,
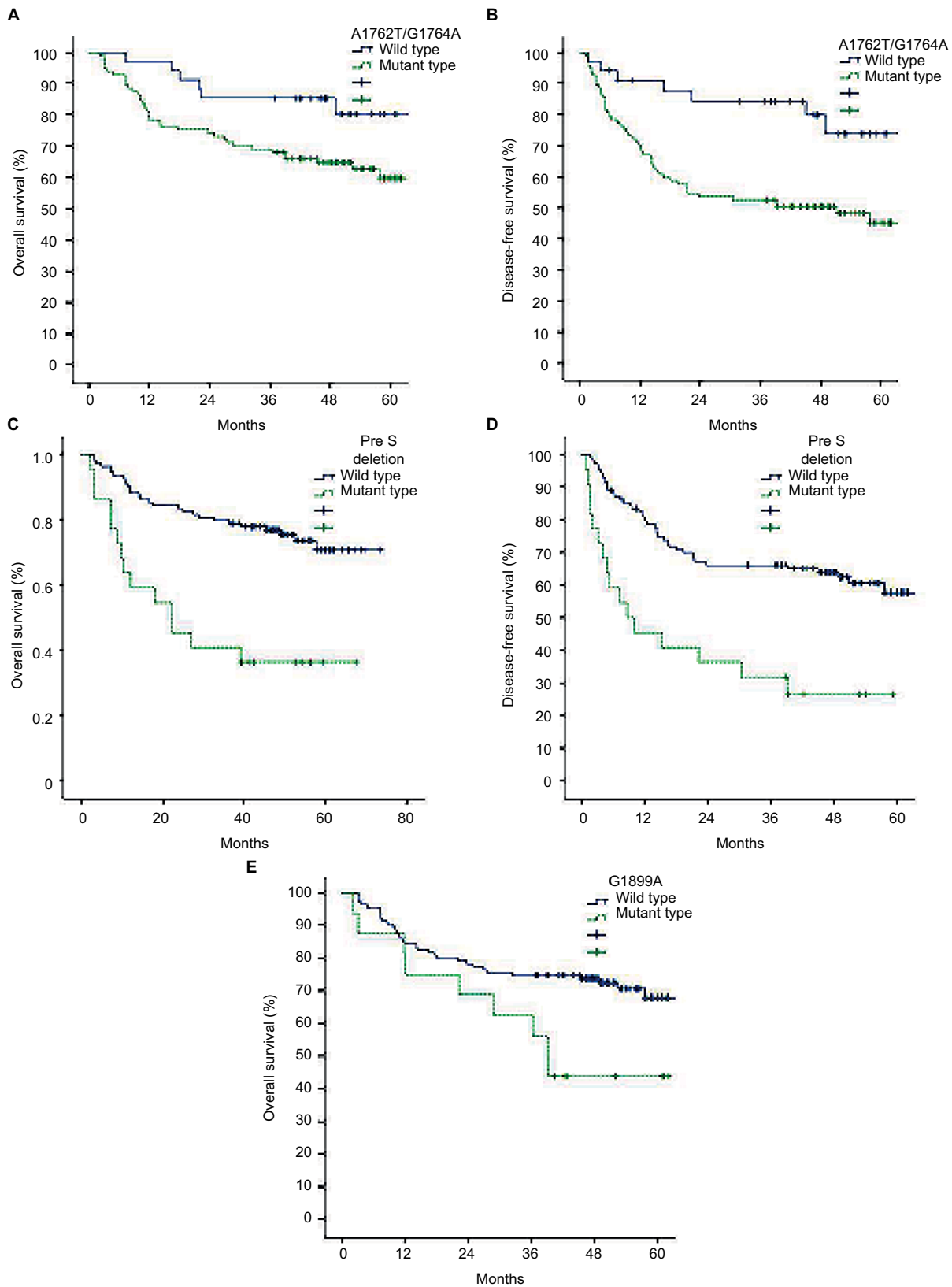
Figure 1 OS and DFS curves of 131 HCC patients after hepatectomy assessed by Kaplan-Meier analysis according to HBV genomic mutations.
Notes: A1762T/G1764A mutation related to worse OS (**A**, p=0.040) and DFS (**B**, p=0.006), Pre S deletion mutation related to worse OS (**C**, p<0.001) and DFS (**D**, p<0.001), and G1899A mutation related to worse OS (**E**, p=0.030).
Abbreviations: OS, overall survival; DFS, disease-free survivial; HCC, hepatocellular carcinoma; HBV, hepatitis B virus.

Table 3 Multivariate analyses of prognostic factors for OS and DFS

Variables	OS		
	HR	95.0% CI	*p*-value
Macrovascular invasion	4.994	2.060–12.107	<0.001
TBil	18.188	4.153–79.644	<0.001
A1762T/G1764A mutation	3.701	1.390–9.855	0.009
Pre S deletion	4.816	2.311–10.032	<0.001
Variables	**DFS**		
	HR	**95.0% CI**	***p*-value**
Macrovascular invasion	2.565	1.052–6.253	0.038
Tumor number	2.033	1.147–3.606	0.015
TBil	4.902	1.084–22.162	0.039
A1762T/G1764A mutation	3.245	1.400–7.521	0.006
Pre S deletion	2.437	1.311–4.530	0.005

Abbreviations: DFS, disease-free survival; HR, hazard ratio; OS, overall survival; TBil, total bilirubin level.

p<0.001), A1762T/G1764A mutation (HR=3.701, 95% CI 1.390–9.855, *p*=0.009), and Pre S deletion (HR=4.816, 95% CI 2.311–10.032, *p*<0.001) were independent prognostic factors for OS. Macrovascular invasion (HR=2.656, 95% CI 1.052–6.253, *p*=0.038), tumor number (HR=2.033, 95% CI 1.147–3.606, *p*=0.015), TBil level (HR=4.902, 95% CI 1.084–22.162, *p*=0.039), A1762T/G1764A mutation (HR=3.245, 95% CI 1.400–7.521, *p*=0.006), and Pre S deletion (HR=2.437 95% CI 1.311–4.530, *p*<0.001) were independent prognostic factors for DFS.

Since both A1762T/G1764A mutation and Pre S deletion were independent prognostic factors for OS and DFS, the 131 patients were divided into three groups according to A1762T/G1764A mutation and Pre S deletion status: no mutation (26 cases), single mutation (77 cases), and dual mutations (16 cases). The baseline clinical characteristics are listed in Table S4, and there were no significant differences between groups. As it was shown in Figure 2, the dual mutation group related to the worst OS and DFS, then the single mutation, and lastly the no mutation group (*p*<0.001 and *p*<0.001, respectively).

## A1762T/G1764A mutation and Pre S deletion related to early recurrence

During the follow-up, a total of 74 patients experienced tumor recurrence, 54 cases within 2 years (early recurrence) and 20 cases after 2 years (late recurrence). As shown in Table 4, patients with A1762T/G1764A mutation or Pre S deletion were more likely to have early recurrence (*p*=0.042 and *p*=0.019, respectively). Especially for patients with A1762T/G1764A and Pre S deletion dual mutation, the early recur-

rence rate (62.5%, 10/16) was much higher than for those with single mutation (44.8%, 39/87) or no mutation (17.9%, 5/28, *p*=0.008). No relationships can be identified between A1762T/G1764A mutation and/or Pre S deletion with late recurrence (data not shown).

Discussion

Although HBV is still one of the most common etiological factors of HCC, relatively little is known about the association between the HBV genomic mutations and the clinical characteristics and outcomes of HBV-HCC patients after surgical resection.[22,24] In the present study, most mutation regions that have been reported in the literature previously, were sequenced,[20,23] and the results demonstrated that HBV DNA genomic mutations in A1762T/G1764A and Pre S deletion were associated with worse prognosis and early recurrence for HBV-HCC patients after surgery. Our results highlight the importance of anti-virus treatment for HBV-HCC after surgery.

A few studies have focused on the prognostic value of the HBV genomic mutations in HBV-HCC patients, but the results remain controversial. By assessing HBV DNA in liver tissue specimens, in 2010, Yeh et al[25] from Taiwan showed for the first time that the BCP A1762T/G1764A mutation is independently predictive of postoperative survival in HCC patients. In 2011, Ryu et al[26] from Korea reported that Pre S deletion and BCP mutations in HBV of genotype C2 did not affect the initial tumor features, but Pre S deletion was an independent risk factor affecting HCC survival. However, in 2013 Mathews et al[23] from Korea revealed that the postoperative recurrence or survival was not affected by the genomic changes at the preCore, BCP, X, and Pre S2 regions in HBV of genotype C2 in patients with HBV-HCC treated with curative surgical resection, by assessing serum HBV DNA in a total of 247 patients. Su et al[19] from Taiwan demonstrated that among patients with higher serum HBV DNA levels, those with Pre S deletion had significantly higher rates of recurrence after surgery. More recently, Guo et al[18,21] from People's Republic of China identified that HBV-HCC survival after surgery is associated with HBV mutations in the X protein region, preCore regions, and small S genes. In the present study, we sequenced most mutation regions that have been reported in the literature previously, and found that both A1762T/G1764A and Pre S deletion mutations related to worse OS and DFS. Compared to previous studies, our study has the following advantages: 1) our study covered the 14 mutation regions, more regions than previous studies; 2) both genotype B and C HBV-HCC, which are common in
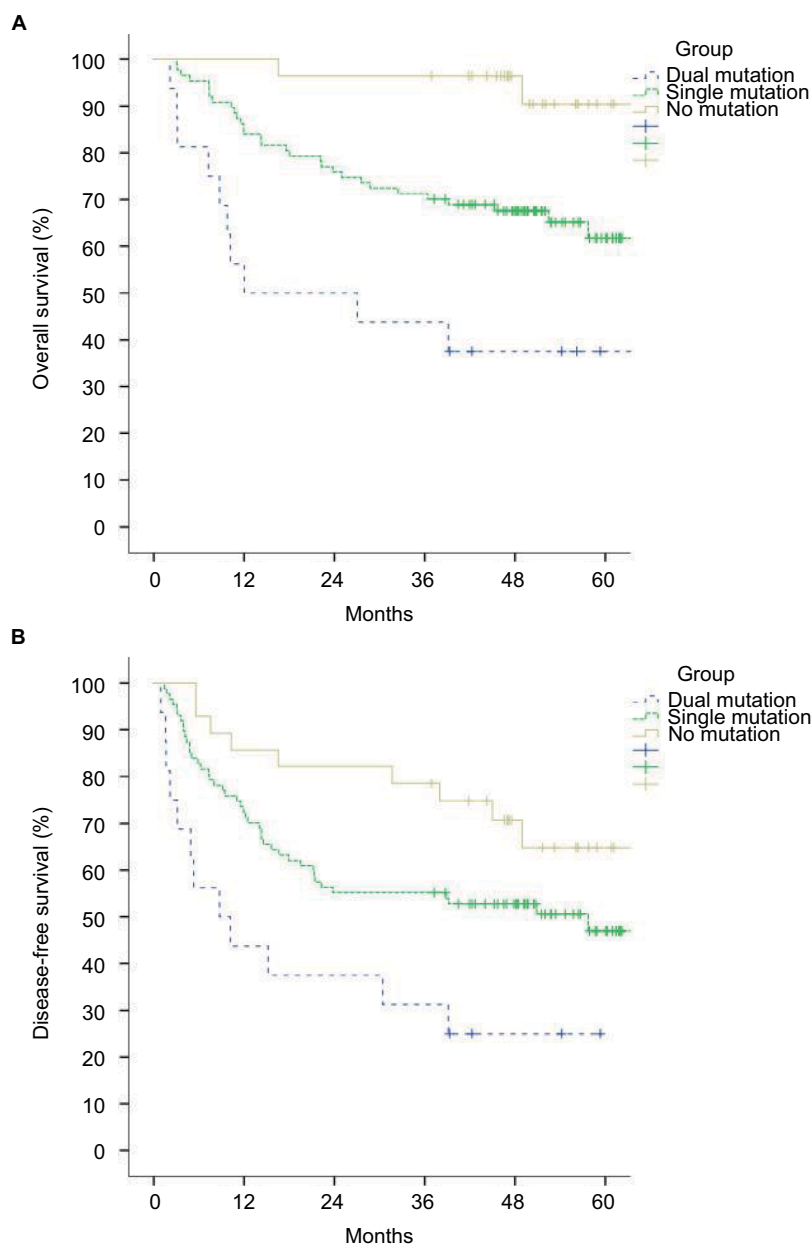
Figure 2 OS and DFS curves of 131 HCC patients after hepatectomy assessed by Kaplan–Meier analysis according to A1762T/G1764A and Pre S mutation status.
Notes: A1762T/G1764A and Pre S dual mutations related to the worst OS (**A**, $p<0.001$) and DFS (**B**, $p<0.001$), compared to the single mutation and no mutation group.
Abbreviations: OS, overall survival; DFS, disease-free survivial; HCC, hepatocellular carcinoma.

Table 4 A1762T/G1764A mutation and Pre S deletion relate to early recurrence

Mutations	Cases	Early recurrence	p-value
A1762T/G1764A			
Wild type	34	9	0.042
Mutant type	97	45	
Pre S deletion			
Wild type	109	40	0.019
Mutant type	22	14	
A1762T/G1764A mutation and Pre S deletion			
Dual mutation	16	10	0.008
Single mutation	87	39	
No mutation	28	5	

East Asia, were included in our study; 3) the median follow-up period was 55.0 months, which is sufficient; and 4) we detected the HBV DNA directly from liver tissue, which is believed to be more reliable.

The A1762T/G1764A double mutation is the most frequent BCP mutation. It downregulates HBeAg expression, promotes immunological tolerance, accelerates HBV copying and HBsAg expression, and finally leads to serious liver damage.[20,27] Previous studies[23,25] had confirmed that A1762T/G1764A double mutation is not only an independent risk factor for HCC but also a predictor for poor survival in

HBV-HCC patients. Patients with A1762T/G1764A double mutation tend to have poor liver function and serious cirrhosis, which will reduce time of survival after surgery. In the present study, we also revealed that the A1762T/G1764A mutation is correlated with tumor size, which indicates that A1762T/G1764A mutation might accelerate tumor proliferation through some unknown mechanism. More recently, Chen et al[22] reported that A1762T/G1764A double mutation and phosphorylated AKT (pAKT) correlated with proliferation and microvascularization but inversely correlated with apoptosis in HCC samples; levels of p21 and p27 were decreased in A1762T/G1764A double mutation or pAKT overexpressing HCC due to SKP2 upregulation; levels of E2F1, and both mRNA and protein of SKP2, were increased in A1762T/G1764A double mutation HCC. Further studies are needed to uncover the underlying mechanism.

The Pre S region of HBV is crucial in mediating the attachment of the virus to host hepatocytes.[20] Moreover, it is important for interacting with the host immune responses. It has been reported that the proportions of Pre S deletion mutants are significantly higher in patients with HBV-related HCC than in those with inactive HBV carrier status, implying that these mutants may be associated with HCC carcinogenesis.[15] Su et al[19] reported that patients with Pre S deletion mutants have a higher proportion of positive HBeAg in sera and a trend of higher HBV DNA levels than their counterparts; the presence of Pre S deletion mutants correlates to a higher rate of tumor recurrence in patients with serum HBV DNA levels >10^5 copies/mL or HBsAg >500 IU/mL. However, the correlations cannot be observed in patients with low viral loads or in those receiving antiviral therapy after resection. It implies that the hepatocarcinogenesis and tumor recurrence induced by Pre S deletion mutants may be based on active HBV viral replication, the accumulation of oxidative stress, and subsequent chromosomal instability.[19]

More importantly, for the first time, we demonstrated that HBV-HCC with dual mutations in A1762T/G1764A and Pre S deletion related to the worst OS and DFS, and patients tended to have early recurrence. Accumulation of mutations reflects both the activity of virus infection and the strength of the immune response.[22] Previous studies[15] have demonstrated that the accumulated mutations in HBV DNA increase the risk to develop HCC in chronic HBV carriers. Huang et al[28] reported that the combo mutant accelerated p21 (WAF1/CIP1) degradation by the upregulated expression of SKP2, and increased cyclin E expression in primary hepatocytes and HepG2 cells, resulting in the combo mutant accelerating cell cycle progression. Chen et al[22] also reported that the accumulation of A1762T/G1764A combined with other mutation(s) was more common in patients with poor prognosis than in patients with better prognosis. But the underlying mechanism remains unclear. However, these findings highlight the importance of anti-HBV treatment for the HBV-HCC patients. HBV infection contributes to not only hepatocarcinogenesis and tumor progression but also tumor recurrence. Clinical trials[29] have shown that patients who receive anti-virus treatment have significantly decreased early recurrence and improved liver function 6 months after surgery, and anti-virus treatment significantly improved both OS and DFS. Our findings also indicated that more aggressive anti-HBV therapy and more frequent follow-up should be suggested for those HBV-HCC patients with dual mutations in A1762T/G1764A and Pre S deletion. However, our findings should be verified by prospective studies.

Other HBV mutations, which have been reported to relate to liver cirrhosis and HCC, including A1752T/G, T1753C, G1757A, C1766T, T1768A, A1775G, C1799G, A1846T, T1858C, and G1898A, failed to show the association with patients' prognosis in the present study. Therefore, no further analysis was performed.

There are several limitations in the present study that must be taken into account. Firstly, sample size was relatively small with only 131 patients included, although the follow-up period was relatively long. Secondly, HBV DNA in serum was unavailable in the present study and the relationship between liver tissue HBV DNA and serum HBV DNA cannot be analyzed. Thirdly, the HBV DNA statuses in recurrent tumors are not available.

Conclusion

The present study demonstrated that HBV DNA genomic mutations in A1762T/G1764A and Pre S deletion were associated with worse prognosis and early recurrence for HBV-HCC patients after surgery. Anti-virus treatment might benefit HBV-HCC patients.

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

Table S1 Patient characteristics according to A1762T/G1764A mutation

Variables	HCC without A1762T/G1764A mutations (n=34)	HCC with A1762T/G1764A mutations (n=97)	*p*-value
Sex (male/female)	32/2	86/11	0.364
Age (years)[a]	50 (16–68)	50 (21–73)	0.878
Child-Pugh (A/B)	34/0	95/2	0.399
Tumor size (cm)	3.6±3.6	6.2±3.6	0.019
Tumor number (single/multiple)	27/7	72/25	0.548
Macrovascular invasion (yes/no)	2/32	8/89	0.658
PLT ($\times10^9$/L)	198.5±95.9	169.0±76.8	0.366
ALT (U/L)	48.2±37.3	45.8±21.0	0.593
AST (U/L)	49.9±33.7	47.6±25.1	0.959
ALB (g/L)	42.5±3.6	41.2±3.8	0.109
TBil (umol/L)	13.3±4.6	17.0±12.6	0.303
AFP (ng/mL)[a]	230.2 (2.9–>121,000)	115.0 (1.4–>121,000)	0.617
Prothrombin time (s)	11.9±1.1	12.0±1.4	0.128
HBeAg (±)	18/16	40/57	0.112
HBV DNA level (<10^5/≥10^5)	20/14	43/54	0.148
TNM stage (I–II/III)	24/10	70/27	0.505
HBV gene type (B/C)	13/21	38/59	0.838

Note: [a]This value is a median (range).
Abbreviations: AFP, alpha-fetoprotein; ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; PLT, platelet count; TBil, total bilirubin level; TNM, tumor-node-metastasis.

Table S2 Patient characteristics according to G1899A mutation

Variables	Patients without G1899A mutation (n=115)	Patients with G1899A mutation (n=16)	*p*-value
Sex (male/female)	103/12	15/1	0.603
Age (years)[a]	49 (16–71)	55 (22–73)	0.247
Child-Pugh (A/B)	113/2	16/0	0.595
Tumor size (cm)	5.4±3.7	6.9±4.4	0.777
Tumor number (single/multiple)	90/25	9/7	0.055
Macrovascular invasion (yes/no)	110/5	11/5	<0.001
PLT ($\times10^9$/L)	181.7±84.2	140.6±63.7	0.100
ALT (U/L)	46.8±27.2	43.6±16.3	0.872
AST (U/L)	48.2±28.1	48.1±23.4	0.974
ALB (g/L)	41.5±3.8	42.1±3.4	0.314
TBil (umol/L)	15.4±9.9	20.5±17.8	0.262
AFP (ng/mL)[a]	152.1 (1.37–>121,000)	118.3 (1.8–>121,000)	0.181
Prothrombin time (s)	12.0±1.4	12.3±1.2	0.270
HBeAg (±)	49/66	9/7	0.205
HBV DNA level (<10^5/≥10^5)	52/63	11/5	0.079
TNM stage (I–II/III)	84/31	10/6	<0.001
HBV gene type (B/C)	43/72	8/8	0.332

Note: [a]This value is a median or mean (range).
Abbreviations: AFP, alpha-fetoprotein; ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBV, hepatitis B virus; PLT, platelet count; TBil, total bilirubin level; TNM, tumor-node-metastasis.

Table S3 Patient characteristics according to Pre S deletion mutation

Variables	HCC without Pre S deletion (n=109)	HCC with Pre S deletion (n=22)	p-value
Sex (male/female)	97/12	21/1	0.359
Age (years)[a]	51 (16–73)	46 (21–64)	0.418
Child-Pugh (A/B)	107/2	22/0	0.522
Tumor size (cm)	5.4±3.7	6.2±3.8	0.963
Tumor number (single/multiple)	85/24	14/8	0.156
Macrovascular invasion (yes/no)	5/104	5/17	0.003
PLT (×10⁹/L)	178.4±86.9	168.0±59.8	0.293
ALT (U/L)	47.4±27.6	41.9±16.2	0.464
AST (U/L)	49.5±29.0	41.8±17.2	0.334
ALB (g/L)	41.7±4.0	40.9±2.4	0.225
TBil (umol/L)	15.6±10.2	18.1±15.0	0.442
AFP (ng/mL)[a]	110.9 (1.37–>121,000)	3709 (3.09–>121,000)	0.003
Prothrombin time (s)	12.0±1.4	12.1±1.0	0.396
HBeAg (±)	50/59	8/14	0.608
HBV DNA level (<10⁵/≥10⁵)	52/57	11/11	0.846
TNM stage (I–II/III)	81/28	13/9	0.024
HBV gene type (B/C)	42/67	9/13	0.873

Note: [a]This value is a median or mean (range).
Abbreviations: AFP, alpha-fetoprotein; ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; PLT, platelet count; TBil, total bilirubin level; TNM, tumor-node-metastasis.

Table S4 Patient characteristics according to A1762T/G1764A mutation and Pre S deletion status

Variables		Cases	Dual mutation group	Single mutation group	No mutation group	p-value
Sex	Male	118	15	77	26	0.696
	Female	13	1	10	2	
Age (years)	<50	62	10	38	14	0.624
	≥50	69	6	49	14	
Child-Pugh	A	129	16	85	28	0.598
	B	2	0	2	0	
Tumor size (cm)	≤5	78	9	47	22	0.068
	>5	53	7	40	6	
Tumor number	Single	99	10	66	23	0.343
	Multiple	32	6	21	5	
Macrovascular invasion	Yes	10	3	7	0	0.077
	No	121	13	80	28	
PLT (×10⁹/L)	<100	22	2	16	4	0.780
	≥100	109	14	71	24	
ALT (U/L)	≤40	68	9	44	15	0.899
	>40	63	7	43	13	
AST (U/L)	≤40	65	10	41	14	0.527
	>40	66	6	46	14	
ALB (g/L)	≥35	124	16	80	28	0.154
	<35	7	0	7	0	
TBil (umol/L)	≤40	128	15	85	28	0.411
	>40	3	1	2	0	
AFP (ng/mL)	<400	78	5	56	17	0.046
	≥400	53	11	31	11	
Prothrombin time (s)	≤12	82	8	53	21	0.220
	>12	49	8	34	7	
HBV DNA (IU/L)	<10³	19	4	9	6	0.156
	10³–10⁵	44	4	29	11	
	>10⁵	68	8	49	11	
TNM stage	I–II	94	10	63	21	0.601
	III	37	6	24	7	

Abbreviations: AFP, alpha-fetoprotein; ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBV, hepatitis B virus; PLT, platelet count; TBil, total bilirubin level; TNM, tumor-node-metastasis.