Pre-S deletions of hepatitis B virus predict recurrence of hepatocellular carcinoma after curative resection

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Abstract

The relationship between hepatitis B virus (HBV) and the prognosis of hepatocellular carcinoma (HCC) after surgery remains uncertain. A retrospective cohort study was performed to evaluate the impact of pre-S deletions, T1762/A1764, and A1896 mutations on prognosis of HCC after curative resection. A total of 113 patients with positive serum HBV DNA (>200 IU/mL) who had underwent curative resection of pathologically proven HCC were recruited to determine the risk factors affecting the prognosis.

The median follow-up time was 36.5 months and recurrence was detected in 67 patients (59.3%). The cumulative recurrence rates and overall survival rates at 1-, 3-, and 5-year after curative resection were 18.0%, 49.7%, 70.3%, and 93.7%, 61.0%, 42.5%, respectively. Patients with pre-S deletions showed significantly higher recurrence rates compared with those with wild type infection (HR: 1.822, $P = .018$), but not related with a significantly poor survival (HR: 1.388, $P = .235$). Subgroup analysis indicated that the patients with type III deletion had significant higher tumor recurrence rates than other deletion types (HR: 2.211, 95% confidence intervals [CI]: 1.008–4.846, $P = .048$). Multivariate analysis revealed that pre-S deletion, tumor size >3 cm in diameter, and the presence of microvascular invasion were independent risk factors for tumor recurrence. HBV pre-S deletions were found to be clustered primarily in the 5′ end of pre-S2 region and were more often found between amino acids 120 and 142 of the pre-S2 domain. The domains most frequently potentially involved were the transactivator domain in pre-S2 and polymerized human serum albumin binding site.

Our cohort showed that pre-S deletions at the time of resection could predict tumor recurrence in HCC patients after curative resection.

Abbreviations: CI = confidence intervals, HBV = hepatitis B virus, HCC = hepatocellular carcinoma, HR = hazard ratio.

Keywords: hepatitis B virus, hepatocellular carcinoma, pre-S deletion, recurrence

1. Introduction

Hepatocellular carcinoma (HCC) is a worldwide health threat, as it is the fifth most common malignant tumor and the third-place cancer associated death.[1] Etiologically, over half HCC occurs in chronic hepatitis B virus (HBV) infected subjects, especially in East Asia and sub-Saharan Africa, where HBV infection is highly popular.[2,3] For these HCC cases, curative surgery was commonly considered the most effective treatment and the prognosis of HCC had been greatly improved during past decades. Nevertheless, high incidence of postoperative recurrence remains a major hindrance for making further improvement of the postoperative prognosis in HCC subjects. A previous study has documented that the postoperative tumor recurrence incidence at 3 years was calculated to be 39% in men and 37% in women who had underwent curative HCC tumor surgery.[4] It has been speculated that 2 different mechanisms were related with tumor intrahepatic recurrence, including intrahepatic metastasis and de novo multicentric carcinogenesis.[5,6] The latter is distinct from the primary tumor clones.[7] However, majority of previous studies mainly aimed on the traditional potentially risk factors associated with intrahepatic metastasis, such as tumor size, nodule number, a positive surgical margin, TNM stage, macroscopic vascular invasion, and Edmondson grade.[8–10] In theory, it is probable that viral replication and integration of sub-genomic HBV DNA fragments into the host liver cells might play a key role in contributing to the process of carcinogenesis.[11,12] Recently, the pathogenesis of HCC in HBV infection has been extensively investigated, and several viral risk factors have been ascertained, including seropositivity of hepatitis B e antigen (HBeAg), high serum levels of hepatitis B surface antigen (HBsAg), and HBV DNA, certain viral genotype, C1653T mutation in the enhancer II, T1753V mutation, and A1762T/G1764A double mutations in basal core promoter (BCP), and pre-S deletions.[13–17] However, the relationship between such viral factors with tumor recurrence after surgery have not been discussed comprehensively and so far little was known of the significance of the virologic status of

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patients with prognosis after resection of HCC, perhaps in part owing to the lack of interest in hepatitis virology among liver surgeons who handled these patients. The role of viral status in prognosis of HCC after curative resection remains uncertain and controversial.

As we known, the lower reach of Yangtze River is one of the highest endemic regions for chronic HBV infection and HCC in China. In a previous study, we have reported that serum HBV DNA level $\geq 10^4$ copies/mL at tumor resection was independently associated with HCC recurrence in a retrospective cohort with a median follow-up period of 33.7 months after surgery.[18] Based on this result, we further planed to conduct this cohort study to assess the effect of viral status, especially certain viral mutations in pre-S and BCP regions, on the prognosis of HCC among patients who receiving curative surgery.

2. Methods

2.1. Study patients

Between January 2010 and December 2012, 113 patients with HBV-related HCC who underwent curative resection as primary therapy in the Affiliated Hospital of Nantong University were recruited in this retrospective cohort based on the following inclusion criteria: positive serum HBsAg for at least 12 months with positive serum HBV DNA (>200 IU/mL); all tumor nodules were completely removed with a surgical free margin of at least 5 mm by pathological inspection; absence of portal vein tumor thrombus (in main trunk or major branches), no cancerous thrombus found in hepatic veins or bile duct; ≤3 tumor nodules; absence of extrahepatic metastasis; no adjuvant antiviral therapy was given before or after the operation; death in the hospital not due to postoperative hepatic failure; and presence of early recurrence at least 3 months postoperatively (in order to exclude preoperative metastases). Maximal tumor size, nodule number, liver cirrhosis, capsular formation around the tumor, microvascular invasion, histological grade proposed by Edmondson and Steiner, and extent of resection were also determined. Serum samples collected before surgery were stored at −70°C before analysis. The protocol was approved by the Ethics Committee of the Affiliated Hospital of Nantong University in accordance with the tenets of the Declaration of Helsinki (date of approval, May 18, 2009; permission code, 2,009,025).

2.2. Follow-up and treatment for tumor recurrences

After surgical resection of HCC, all 113 patients were assessed every 3 to 6 months with dynamic contrast-enhanced computer tomography (CT) and/or magnetic resonance imaging (MRI), as well as with measurement of a serum tumor marker such as AFP. Hepatic angiography was performed when recurrence was suspected in only 1 patient according to strong personal requirement. In this case, the tumor recurrence was finally confirmed by elevated serum AFP level and typical image in CT examination 3 months after hepatic angiography. The identification of intrahepatic recurrence was based upon histopathologic examination of tumor tissue in 17 patients receiving second time tumor resection and on the typical imaging features on CT and MRI in 50 patients. No included patients received liver transplantation. Once recurrence was detected, patients obtained most suitable therapy on the basis of the location and number of recurrent nodules and hepatic function. Treatment of HCC recurrence contained a second time tumor resection, transcatheter arterial chemoembolization (TACE), and hepatic arterial infusion chemotherapy, percutaneous ethanol injection (PEI), radiofrequency ablation (RFA), and chemotherapy or radiotherapy.

2.3. Evaluation of prognostic clinicopathological factors

All clinicopathological factors of present study were enrolled for their potential relevance to the prognosis based on previous studies, including sex (man vs woman), age, alanine aminotransferase (ALT), albumin, total bilirubin, prothrombin time, HBeAg status (positive vs negative), serum HBV DNA (≤2000 vs >2000 IU/mL), serum AFP level (≤400 vs >400 ng/mL), coexisting hepatitis C virus (HCV) infection (yes vs no), liver cirrhosis (yes vs no), BCLC stage (B or C vs A), Child-Pugh grade (A vs B), tumor size (≤3 vs >3 cm in diameter), number of tumor nodules (single vs multiple), microvascular invasion (yes vs no), tumor capsular formation (yes vs no), differentiation of tumor cells (Edmondson classification I/II vs III/IV), and extent of hepatic resection (major, ≥3 segments vs minor, <3 segments) based on Couinaud nomenclature.

2.4. Nested polymerase chain reaction and direct sequencing of the BCP/precore (PC) and pre-S regions

HBV DNA was extracted from 200 μL serum samples using the commercial Kit (Shanghai Shenyou Biotech Company, China). HBV genes of the BCP/PC regions were amplified by nested PCR. First-round PCR primers were 5′-CAGCTTG TTTTGCTCG CAGC-3′ (nt 1286–1305) and 5′-GAGTAACTCCACAGAAGC TCC-3′ (nt 2083–2063). PCR reaction was carried out in 50 μL containing 5 μL 10× buffer, 4 μL 2.5 mmol/L deoxynucleoside triphosphates (dNTP), 2 μL 10 μmol/L sense and antisense primers, 1.5U PlatinumTaq DNA polymerase (Invitrogen, Shanghai, China). First-round PCR was performed as follows: 95°C for 2 minutes; 95°C for 30 seconds, 56°C for 30 seconds, and 68°C for 3 minutes for 35 cycles; and finally, 68°C for 10 minutes. Two microliter of the first-round PCR product was reamplified by the same PCR condition as the first-round reaction. Second-round PCR primers were 5′-GTGCACTTCGC TTCACCTCT-3′ (nt 1579–1598) and 5′-TCCACAGAAGCTCC GAATTC-3′ (nt 1941–1922). For pre-S region sequence analysis, pre-S genes were amplified under the same PCR condition described above, except the primers were used. First-round PCR primers were 5′-AAAATTAATTATCCTGCTAGG-3′ (nt 2627–2647) and 5′-GAGAAGTCCACCACGAGTC-3′ (nt 269–251). Second-round PCR primers were 5′-TTTACAACTCTGTG-GAAGGC-3′ (nt 2747–2766) and 5′-GAGTCTAGACTCTGTG GTATTGTG-3′ (nt 255–232). All required prophylactic measures to prevent cross-contamination were taken, and negative controls were comprised in each run. Both strands of amplified products were directly sequenced by using an ABI 3700 sequencer and commercial kit (Applied Biosystems, Foster City, CA). Sequences analysis of the genome and deduced amino acid sequences were aligned and compared with the software MEGA version 4.1.

2.5. Identification of HBV genotype and mutations

HBV genotypes were determined by comparing the sequence of BCP/PC and pre-S regions with a set of standard sequences obtained from GenBank. HBV nucleotide sequences were aligned with the Clustal X software program and the phylogenetic trees were constructed by software MEGA version 4.1 software, as described previously.[16,17]

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2.6. Statistical analysis

Data values were expressed as proportions, means $\pm$ SD, and median (range). Clinical and pathological data at the time of resection were analyzed to identify factors that influenced the prognosis by the Cox proportional hazards model. The overall survival was analyzed from the date of surgery to the date of last follow-up or death. The cumulative recurrence was analyzed from the date of surgery to the date of recurrent tumor detection, if HCC recurrence was not detected within the time of follow-up, the patients were censored on the last date of follow-up or the date of death. Survival curves were calculated by the Kaplan–Meier method and differences were statistically compared using the Log-Rank test. Multivariate analysis was performed by the Cox proportional hazards regression model to identify the independent factors on HCC prognosis. Two-tailed *P* values of <.05 were considered as statistically significant. All statistical analyses were performed using the Statistical Program for Social Sciences (SPSS v17.0 for Windows; SPSS, Inc., Chicago, IL).

3. Results

Sixty seven patients (59.3%) had intrahepatic tumor recurrence during the median observation period of 36.5 months (range, 3–72 months). Among these recurrent cases, 34 received TACE, 17 underwent a repeat tumor resection, 6 received RFA, 5 received postoperative radiotherapy or chemotherapy or PEI, and the reminder accepted a supportive therapy, respectively. Fifty eight patients died during the follow-up duration. Among them, 19 patients without HCC recurrence died from other reasons, mainly containing esophageal-gastric varices bleeding and chronic hepatic failure. In these unrecurrence related death, 10 died of gastrointestinal bleeding (2 with pre-S deletions, 8 with wild type), 7 hepatic failure (1 with pre-S deletions, 6 with wild type), and 2 brain hemorrhage (1 with pre-S deletions, 1 with wild type). The 1-, 3-, and 5-year overall survival rates after curative surgery in this study were 93.7%, 61.0%, and 42.5%, respectively. Meanwhile, the 1-, 3-, and 5-year cumulative recurrence rates after curative surgery were 18.0%, 49.7%, and 70.3%, respectively. The baseline demographic, clinicopathological, and virologic features of this retrospective cohort study were listed in Table 1. Moreover, there was no significant difference between patients with pre-S deletions (n=33) and patients with wild type infection (n=80) in terms of these features (data not shown). Only the incidence of liver cirrhosis (occurring in 63.6% [21/33] of pre-S deletions and 77.5% [62/80] of wild type) exhibited a borderline difference by univariate analysis (P=.129).

3.1. Factors associated with tumor recurrence after curative resection

Serum HBV DNA >2000 IU/mL (HR: 1.625, 95% CI: 1.004–2.630, P=.048), AFP >400 ng/mL (HR: 2.038, 95% CI: 1.137–3.655, P=.017), tumor size >3 cm in diameter (HR: 1.912, 95% CI: 1.111–3.292, P=.019), microvascular invasion (HR: 1.939, 95% CI: 1.133–3.316, P=.016), and the presence of pre-S deletion (HR: 1.822, 95% CI: 1.109–2.994, P=.018) at the time of resection were the significant risk factors for recurrence in univariate analyses. Other factors including serum albumin, total bilirubin, ALT, HBeAg statue, coexisting hepatitis C virus (HCV) infection, presence of cirrhosis, tumor number, Child-Turcotte-Pugh grade, BCLC stage, and the presence of A1896 mutation

Table 1

Patient characteristics.

Characteristics	No. (%)	Values
No. of patients	113 (100)	
Median age, years (range)		53 (29–71)
Male: female ratio	97:16 (85.8:14.2)	
Coexisting HCV infection	4 (3.5)	
Presence of cirrhosis	83 (73.5)	
HBeAg seropositivity	40 (35.4)	
HBV DNA >2000 IU/mL	46 (40.7)	
AFP >400 ng/mL	19 (16.8)	
Median baseline biochemistry and hematology (range)		
Total bilirubin, μmol/L		16.0 (5.3–37.8)
Albumin, g/L		42 (30–51)
Alanine aminotransferase, IU/L		43.0 (12–224)
Prothrombin time, s		11.4 (9.8–15.1)
Tumor size (≤3 cm: >3 cm)	41:72 (36.3:63.7)	
Tumor number (single: multiple)	91:22 (80.5:19.5)	
Microvascular invasion	25 (22.1)	
Capsular formation	63 (55.8)	
Differentiation of tumor (I/II:III/IV)	79:34 (69.9:30.1)	
Child-Turcotte-Pugh grade		
A	108 (95.6)	
B	5 (4.4)	
BCLC stage		
A	77 (68.1)	
B or C	36 (31.9)	
Type of surgical procedure (major: minor)	46:67 (40.7:59.3)	
Genotype (C: B)	105:8 (92.9:7.1)	
Pre-S deletion	33 (29.2)	
T1762/A1764 mutations	79 (69.9)	
A1896 mutation	50 (44.2)	
Median follow-up time, mo		36.5 (3–72)
Median time of recurrence, mo		24.0 (3–66)
Median time of survival, mo		42.6 (4–68.5)

HBeAg = hepatitis B e antigen, HBV = hepatitis B virus, HCV = hepatitis C virus.

and T1762/A1764 mutations were not significantly associated with HCC recurrence (Table 2). Among 67 recurrence patients, 26 (38.8%) had pre-S deletion at the time of tumor resection. While, 7 (15.2%) of 46 non-recurrence patients had pre-S deletion (P=.007). Patients with pre-S deletion had significantly higher cumulative recurrence rates compared with those with wild type infection (P=.015; Fig. 1). In multivariate analysis, tumor size >3 cm in diameter (HR: 1.827, 95% CI: 1.052–3.174, P=.032), the presence of microvascular invasion (HR: 1.929, 95% CI: 1.114–3.340, P=.019) and pre-S deletion (HR: 1.781, 95% CI: 1.065–2.976, P=.028) were independent risk factors for tumor recurrence (Table 2).

3.2. Factors associated with overall survival after curative resection

In univariate analysis, Child-Pugh grade B (HR: 3.358, 95% CI: 1.194–9.439, P=.022), BCLC stage B or C (HR: 1.837, 95% CI: 1.085–3.111, P=.024), and the presence of microvascular invasion (HR: 2.072, 95% CI: 1.182–3.634, P=.011) were significantly associated with poor overall survival after surgery. Other clinical factors including sex, age, coexisting HCV infection, serum albumin, total bilirubin, ALT, prothrombin time, HBeAg statue, HBV-DNA levels, presence of cirrhosis, tumor number, extent of resection, and status of viral mutations

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Table 2

Factors identified on univariate and multivariate Cox regression analysis that influenced recurrence in HCC patients undergoing curative resection.

Factors	P value	Hazard ratio	95% CI
Univariate analysis			
Sex (male vs female)	.614	0.841	0.428–1.651
Age, y	.594	0.993	0.968–1.019
Coexisting HCV infection	.365	1.596	0.580–4.395
Presence of cirrhosis	.063	1.784	0.968–3.286
HBeAg seropositivity	.265	1.325	0.808–2.173
HBV DNA >2000 IU/mL	.048	1.625	1.004–2.630
AFP >400 ng/mL	.017	2.038	1.137–3.655
Total bilirubin	.769	1.005	0.973–1.038
Albumin	.651	1.013	0.957–1.072
Alanine aminotransferase	.968	1.000	0.994–1.006
Prothrombin time, s	.512	0.934	0.762–1.145
Tumor size (>3 cm vs ≤3 cm)	.019	1.912	1.111–3.292
Tumor number (multiple vs single)	.391	1.288	0.723–2.295
Microvascular invasion	.016	1.939	1.133–3.316
Capsular formation	.955	0.986	0.609–1.597
Differentiation (III/IV vs I/II)	.720	1.104	0.643–1.896
Child-Pugh grade (B vs A)	.346	1.757	0.544–5.675
BCLC stage (B or C vs A)	.491	1.509	0.467–4.870
Extent of resection (major vs minor)	.657	1.118	0.683–1.830
Genotype (C vs B)	.727	0.849	0.338–2.132
Pre-S deletion	.018	1.822	1.109–2.994
T1762/A1764 mutations	.912	0.971	0.579–1.628
A1896 mutation	.393	0.809	0.498–1.315
Multivariate analysis			
Pre-S deletion	.028	1.781	1.065–2.976
Tumor size >3 cm	.032	1.827	1.052–3.174
Microvascular invasion	.019	1.929	1.114–3.340

CI = confidence interval, HBeAg = hepatitis B e antigen, HBV = hepatitis B virus, HCC = hepatocellular carcinoma, HCV = hepatitis C virus.

were not statistically associated with the survival (Table 3). The overall survival rates in group without pre-S deletion were higher than that in the patients with pre-S deletion mutation, but the difference was not significant (P = .230; Fig. 2). In multivariate analysis, the presence of microvascular invasion (HR: 2.035, 95% CI: 1.160–3.572, P = .013) and BCLC stage B or C (HR: 1.085, 95% CI: 1.066–3.059, P = .010) were independent risk factors for overall survival (Table 3).

Figure 1. Comparison of cumulative recurrence rates between patients with and those without pre-S deletions (log rank test, P = .015).

3.3. Deletion patterns in the pre-S region

Similar to our previous study,[16] the position and size of pre-S deletions were depicted in Table 4. Of these 33 patients with pre-S deletions, the deletion pattern could be divided into 5 major types on the basis of the deletion site: type I (4 patients; pre-S1 deletions [5′ terminus predominant; range from aa 1 to 57]); type II (5 patients; pre-S1 deletions [3′ terminus predominant; range from aa 58 to 119]); type III (16 patients; pre-S2 deletions only); type IV (2 patients; deletions spanning both pre-S1 and pre-S2 regions; type V [2 patients; deletion at 2 diverse locations, one in the pre-S1 and the other in the pre-S2 separately]); and combined deletion type (4 patients). Overall, the number of pre-S nucleotide deletions altered from 15 to 86 bps. HBV pre-S deletions were found to be primarily clustered in the N-half of pre-S2 region (16 [48.5%] of 33). Pre-S deletions were more frequently detected in the pre-S2 domain coding amino acids 120 to 142. Loss of pre-S2 start codon was detected in 4 subjects (12.1%). Genotype C (30/33, 90.9%) dominated the HBV isolates with pre-S deletions in this cohort (Table 4). We also analyzed the correlation of pre-S deletion types and tumor prognosis in the subgroup with pre-S deletions. For the limited patients with pre-S deletions (33 patients) in this cohort, we divided this subgroup into type III (16 patients) and other types (17 patients) deletions. The data showed that the patients with type III deletion had significant higher tumor recurrence rates than other deletion types (HR: 2.211, 95% CI: 1.008–4.846, P = .048). However, we did not observer the significant difference in overall survival rates between patients with type III and other types deletions (HR: 1.170, 95% CI: 0.494–2.769, P = .721).
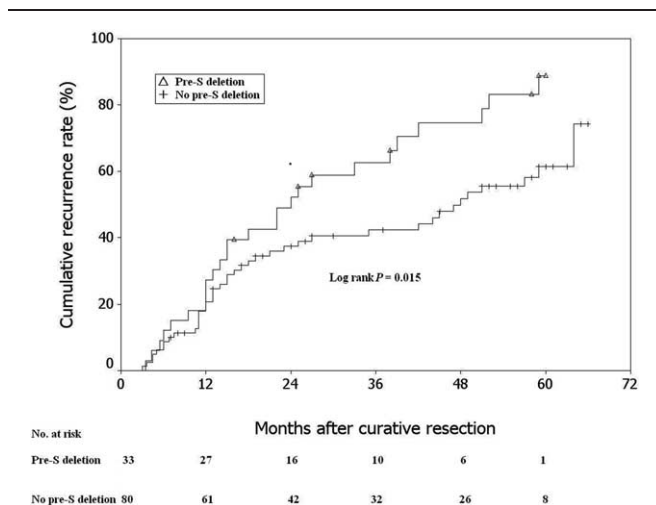
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Factors identified on univariate and multivariate Cox regression analysis that influenced overall survival in HCC patients undergoing curative resection.

Factors	*P* value	Hazard ratio	95% CI
Univariate analysis			
Sex (male vs female)	.259	0.684	0.353–1.323
Age, y	.526	0.991	0.964–1.019
Coexisting HCV infection	.278	0.334	0.046–2.417
Presence of cirrhosis	.290	1.410	0.746–2.665
HBeAg seropositivity	.410	1.251	0.734–2.134
HBV DNA >2000 IU/mL	.166	1.442	0.859–2.420
AFP >400 ng/mL	.474	1.273	0.658–2.465
Total bilirubin	.066	1.032	0.998–1.066
Albumin	.709	0.988	0.930–1.051
Alanine aminotransferase	.234	1.003	0.998–1.009
Prothrombin time, s	.293	1.117	0.909–1.371
Tumor size (>3 cm vs ≤3 cm)	.251	1.386	0.794–2.422
Tumor number (multiple vs single)	.929	0.970	0.489–1.922
Microvascular invasion	.011	2.072	1.182–3.634
Capsular formation	.843	0.949	0.566–1.591
Differentiation (III/IV vs I/II)	.264	0.731	0.422–1.267
Child-Pugh grade (B vs A)	.022	3.358	1.194–9.439
BCLC stage (B or C vs A)	.024	1.837	1.085–3.111
Extent of resection (major vs minor)	.359	1.279	0.756–2.164
Genotype (C vs B)	.568	0.765	0.305–1.920
Pre-S deletion	.235	1.388	0.808–2.385
T1762/A1764 mutations	.648	1.144	0.643–2.035
A1896 mutation	.159	0.685	0.404–1.160
Multivariate analysis			
Microvascular invasion	.013	2.035	1.160–3.572
BCLC stage B or C	.028	1.085	1.066–3.059

CI = confidence interval, HCC = hepatocellular carcinoma, HBeAg = hepatitis B e antigen, HBV = hepatitis B virus, HCV = hepatitis C virus.

3.4. Functional domains potentially related to HCC-related pre-S deletions

To explore the possible effect of pre-S deletions on the viral function, we further analyzed the specific modes of all HCC postoperative recurrence involved pre-S deletions. Evidently, we found that the genome location spanning from the 3′-terminal half of pre-S1 to the 5′-terminal half of pre-S2 looked like to be the favorite target position of these deletions. Some well-informed T- and B-cell epitopes overlap with this area. Epitope mapping of our 33 pre-S deletion mutations revealed that most of the deletion regions encompassed T and B-cell epitopes. Of them, the deletions in B-cell epitope coding amino acids from 120 to 145 were most frequently detected (66.7%, 22 of 33), followed by the T-cell epitope coding amino acids from 140 to 149 (57.6%, 19 of 33). Additionally, we further observed which of the well-informed functional sites of HBV genome were possibly involved by these deletions. Functional mapping of these pre-S deletion sequences showed that most of such deletion mutation types lost ≥1 important functional sites. The domains most usually involved were the transactivator domain in pre-S2 (TD-2, aa 120–172, 23 of 33, 69.7%), and the polymerized human serum albumin binding site (PHSA, aa 122–135, 21 of 33, 63.6%), followed by the nucleocapsid binding site (NBS, aa 107–127, 13 of 33, 39.4%), and heat shock protein 70-binding site (HSC70, aa 74–118, 11 of 33, 33.3%) (Table 5).

4. Discussion

The prognosis of HCC is still not satisfactory, although it has been greatly enhanced in the past decades. Even after curative HCC resection, the incidence of postoperative tumor recurrence remains extremely high.[19] It has been reported that tumor size, vascular invasion, and intrahepatic metastasis were related significantly to predict HCC recurrence.[8,10,20–22] Meanwhile, the linkage between serum HBV DNA levels, status of HBsAg and HBeAg, certain viral genotypes, and specific viral sequence mutations and the risk of HCC development has been extensively confirmed. Despite convincing results supplied by previous
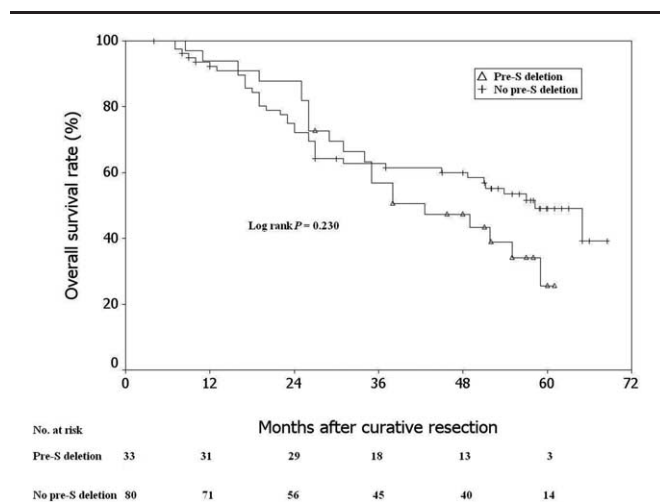
Figure 2. Comparison of overall survival rates between patients with and those without pre-S deletions (log rank test, *P* = .230).

Table 4

Characteristics of pre-S deletions in 33 HCC patients.

Case	Age (y)	Genotype	Region (aa)	Region (nts)	Size of deletion (bp)	Deletion type*
No. 5	46	C	130–141	27–55	29	III
No. 9	55	C	124–131	2–23	22	III
No. 11	60	B	23–30	2916–2935, 3028–3065	20	
			61–73		38	I+II
No. 13	56	C	128–142	14–57	44	III
No. 14	46	C	76–83	3075–3096	22	II
No. 19	50	C	22–30	2911–2937	27	
			109–123	3172–3215	44	I+IV
No. 22	62	C	23–28	2916–2930	15	I
No. 27	39	C	127–141	11–55	45	III
No. 31	45	C	131–140	23–52	30	III
No. 33	41	C	112–130	3182–21	54	IV
No. 39	47	C	132–142	26–57	32	III
No. 42	53	C	59–80	3023–3086	64	
			131–140	23–52	30	V
No. 46	48	C	1–10	2849–2875	27	I
No. 48	45	C	64–74	3039–3068	30	II
No. 52	56	C	126–143	10–59	50	III
No. 53	69	C	130–145	22–67	46	III
No. 59	57	C	22–30	2912–2936	25	
			132–141	26–55	30	I+III
No. 62	49	C	100–117	3147–3196	50	II
No. 67	65	C	130–141	22–54	33	III
No. 69	52	C	19–28	2902–2931	30	
			41–49	2968–2992	25	I
No. 71	64	C	125–142	5–58	54	III
No. 74	59	C	113–141	3185–55	86	IV
No. 75	43	C	123–134	3214–33	35	III
No. 79	31	C	138–151	45–83	39	III
No. 81	62	C	131–140	24–50	27	III
No. 86	58	C	109–122	3172–3212	41	
			132–141	27–55	29	III+IV
No. 87	63	B	69–76	3052–3074	19	
			132–141	25–55	31	V
No. 93	48	C	45–60	2981–3025	45	I
No. 94	57	B	94–116	3128–3195	68	II
No. 98	52	C	132–141	28–55	27	III
No. 102	61	C	124–141	4–54	51	III
No. 106	52	C	101–116	3148–3195	48	II
No. 111	71	C	145–159	65–107	43	III

aa = amino acid, bp = base pair, nt = nucleotide, HCC = hepatocellular carcinoma.

* Deletion mutations are divided into the following 5 types: type I, pre-S1 deletion (N-half predominant; range, aa 1–57); type II, pre-S1 deletion (C-half predominant; range, aa 58–119); type III, pre-S2 deletion only; type IV, border between pre-S1 and pre-S2 region; type V, deleted at 2 separated sites, one in the pre-S1 region, the other in the pre-S2 region.

studies, the relationship between certain HBV mutations and carcinogenesis has not been identified by evaluating the effect of such virologic factors on prognostic value in clinical postoperative models.

According to prior published documents, there are strong and adequate proofs that high serum HBV DNA levels was associated with an increased risk of tumor recurrence in the remaining liver in HCC patients who underwent curative resection.[23–26] However, there has been only a few of studies aiming at the viral mutations in patients receiving HCC resection. Based on our previous findings of HBV risk in the HCC development, the present cohort focused primarily on the linkage between classical HBV sequence mutations and the prognosis of HCC after curative resection. Our data distinctly revealed that tumor size >3 cm in diameter, the presence of microvascular invasion, and pre-S deletions were independent risk factors for postoperative recurrence. Additionally, other typical hot spot mutations comprising of the presence of A1896 mutation and T1762/

A1764 double mutations were not significantly associated with HCC prognosis. However, the current study did not show a significant effect of pre-S deletion on the overall survival rates for this retrospective recruited cohort. In the subgroup analysis, we further indicated that the patients with pre-S deletions had significant lower rate of dying from other reasons but not tumor recurrence than those with wild type infection, mainly comprising chronic deterioration of hepatic function and severe complications of advanced liver disease (4/33 vs 15/80, $P < .05$). We speculated that the distribution of un-recurrence related deaths in different groups might explain the discrepant effect of pre-S deletions on overall survival and tumor recurrence. Till date, present knowledge with regard to the role of certain HBV sequence mutations in HCC prognosis after surgery remains limited and controversial. At the beginning of the 20th century, Kubo et al[27] first described that the possibility of HCC recurrence after hepatic resection in cases with G1896A was statistically lower than those with wild-type infection. But this

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Table 5

The distribution of HCC-related pre-S deletions in HBV functional domains.

Case	HSC70	CAD	NBS	TD-1	TD-2	S-P	CBF	VS	PHSA
No. 5	−	−	−	−	+	−	−	−	+
No. 9	−	−	+	−	+	−	−	+	+
No.11	−	−	−	+	−	+	−	−	−
No. 13	−	−	−	−	+	−	−	−	+
No. 14	+	+	−	+	−	+	−	−	−
No. 19	+	−	+	+	+	+	+	+	+
No. 22	−	−	−	+	−	−	−	−	−
No. 27	−	−	+	−	+	−	−	−	+
No. 31	−	−	−	−	+	−	−	−	+
No. 33	+	−	+	−	+	−	−	+	+
No. 39	−	−	−	−	+	−	−	−	+
No. 42	+	−	−	+	+	+	−	−	+
No. 46	−	−	−	−	−	−	−	−	−
No. 48	+	−	−	+	−	+	−	−	−
No. 52	−	−	+	−	+	−	−	−	+
No. 53	−	−	−	−	+	−	−	−	+
No. 59	−	−	−	−	+	−	−	−	−
No. 62	+	+	+	−	−	+	+	−	−
No. 67	−	−	−	−	+	−	−	−	+
No. 69	−	−	−	+	−	−	−	−	−
No. 71	−	−	+	−	+	−	−	−	+
No. 74	+	−	+	−	+	−	−	+	+
No. 75	−	−	+	−	+	−	−	+	+
No. 79	−	−	−	−	+	−	−	−	−
No. 81	−	−	−	−	+	−	−	−	+
No. 86	+	−	+	−	+	+	−	+	+
No. 87	+	−	−	+	+	+	−	−	+
No. 93	−	−	−	+	−	−	−	−	−
No. 94	+	+	+	−	−	+	+	−	−
No. 98	−	−	−	−	+	−	−	−	+
No. 102	−	−	+	−	+	−	−	+	+
No. 106	+	+	+	−	−	+	+	−	−
No. 111	−	−	−	−	+	−	−	−	−
Involved (%)*	33.3	12.1	39.4	30.3	69.7	30.3	12.1	21.2	63.6

CAD = cytosolic anchorage determinant (aa 81−105), CBF = CCAAT binding factor-binding site (aa 97−100), HBV = hepatitis B virus, HCC = hepatocellular carcinoma, HSC70 = heat shock protein 70-binding site (aa 74−118), NBS = nucleocapsid binding site (aa 107−127), PHSA = polymerized human serum albumin-binding site (aa 122−135), S-P = S-promoter (aa 66−111), TD-1 = transactivator domain in the pre-S1 region (aa 21−90), TD-2 = transactivator domain in the pre-S2 region (aa 120−172), VS = viral secretion (aa 120−124); +, − = indicate presence of deletion and absence of deletion.
* The percentage of deletion clones with the indicated functional domain involved.

beneficial impact of G1896A mutation was not validated by the following researches carried out in Hong Kong and mainland of China.[24,28] Meanwhile, prior studies had reported that BCP double mutations was related with significantly increased risk of HCC recurrence after curative hepatic resection.[24,29] However, this high tumor recurrence risk caused by A1762T/G1764A double mutations was not certified by the study conducted by Wu et al.[25] In present retrospective cohort, A1896 mutation and T1762/A1764 double mutations were not correlated with either tumor recurrence or overall survival. Furthermore, as another common mutation in pre-S sequence, the impact of pre-S deletions has been widely discussed. Su et al[30] had indicated that HCC subjects harboring pre-S deletions suffered obviously higher risk of tumor recurrence and multivariate analysis showed pre-S deletion was one of the independent risk factors associated with postoperative recurrence. Nevertheless, the data from other studies performed in Taiwan and Korea did not verify the point that the presence of pre-S deletions was related with HCC postoperative intrahepatic recurrence.[31,32] A potential reason for these discrepant findings may attribute to the distinct viral and clinical feature of the chronic HBV infection had been introduced in different geographical regions of the world and is increasingly

related with genetic diversity of the viral infection.[33] Moreover, Yeh et al[29] had displayed that only HCC subjects with short in-frame or rearrangement pre-S deletion patterns (<100 bp) demonstrated a lower recurrence-free survival after tumor resection and all short pre-S deletions situated in a particular region ranging from codon 107 to 141. In current study, we also attempted to analyze the location and sizes of pre-S deletions. The pre-S2 deletions displayed a higher frequency than pre-S1 deletions in HBV isolates among HCC recurrent patients. The region spanning from the 3′-terminal half of pre-S1 to the 5′-terminal half of pre-S2 appeared to be the favored location for these deletions. Some familiar T- and B-cell epitopes overlap within this position. Therein, the B-cell epitope encoding amino acids between 120 and 145 was most usually deleted, subsequently followed by amino acids from 140 to 149 at the T-cell epitope. Additionally, we have identified which of the important functional regions of viral sequence were possibly involved by these deletion mutations. The pre-S region is essential for mediating the attaching HBV virus to host hepatocytes and is crucial in interaction with the host immune responses. Such deletions may provide a convenience in allowing HBV mutations to escape from immune attack. Meanwhile, this region has

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overlapped 9 important functional sites in HBV genome.[34,35] These regions were most frequently related with the trans-activator domain in pre-S2 (TD-2, aa 120–172), and followed by the polymerized human serum albumin binding site (PHSA, aa 122–135) in our study. It has been documented that functional damage of PHSA binding induced by pre-S deletions might lead to viral immaturities and create mutation viruses harboring changed envelope proteins.[36] Such mutant or immature viral particles may tend to be retained intracellularly, formation of ground glass hepatocytes, inducing endoplasmic reticulum stress, oxidative DNA impairment and overall genomic instability, which may cause eventually hepatocarcinogenesis.[37–39] Our opinion of this connection between pre-S deletions and HCC progression had also been verified by animal experiments, in which transgenic mice embodying pre-S2 mutation plasmids have been prone to induce dysplastic variants in hepatocytes and subsequent liver cancer.[40]

In addition, tumor size and microvascular invasion were confirmed as independent risk factors affecting postoperative recurrence. Meanwhile, the presence of microvascular invasion and BCLC stage B or C were independently associated with poor overall survival after surgery. These findings were similar to that described previously.[41,42]

The strengths of present study included that we only enrolled HBV related-HCC patients after curative resection not experiencing antiviral therapy in this retrospective cohort, and thus these data are important in clarifying the impact of viral sequence mutation on tumor recurrence in the natural course of HBV. There are also several limitations of the current study that should be noticed. First of all, for the limited number of subjects with genotype B infection in population of this area, it is difficult to evaluate the risk of different viral genotype in HCC prognosis. Second, the method of direct sequencing just displays the predominant viral strains in the host and it tends to underestimate the real mutation level in cases, for in most chronic HBV carriers, mixed infection of multiple viral strains is common. Third, because of the detection limit of our nested PCR assay, we just recruited patients with positive serum HBV DNA into our study, it tends to underestimate rather than overestimate the risk of viral load. Fourth, we found that many included patients received conservative drug therapy several months before tumor resection, such as diuretic, albumin, plasma, and cryoprecipitate and prothrombin complex. After above treatment, some Child-Pugh grade B patients could achieve preoperative grade A. Unfortunately, all demographic data of subjects in this study were examined within 1 week before the operation. It tends to underestimate the real degree of liver function damage by Child-Pugh grade preoperation. Finally, as a retrospective cohort study, it is difficult to draw a firm conclusion and a large-scale, well-designed prospective study with long-term follow-up should be performed in future.

5. Conclusion

In summary, by conducting this postoperative prognostic model, our study supported the opinion that pre-S deletions were related with recurrence after curative tumor resection. If the role of pre-S deletion in predicting HBV-related HCC prognosis after curative resection is further confirmed, this new potential biomarker could be incorporated into the HCC recurrence risk surveillance strategy. More sophisticated, larger population-based cohort studies may be required to elucidate this issue in future.

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