F221Y mutation in hepatitis B virus reverse transcriptase is associated with hepatocellular carcinoma prognosis following liver resection

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Abstract. Hepatitis B virus (HBV) reverse transcriptase (RT) is encoded by the polymerase gene in the reverse transcriptase region, which overlaps with the S gene. The association between mutations of HBV RT and the pathobiological features of hepatocellular carcinoma (HCC) remain to be elucidated. The present study aimed to examine mutations in this region of the HBV genome and its clinical significance. Briefly, HBV total DNA was extracted from 84 pairs of HCC tumor tissue and corresponding adjacent non-tumor tissue samples. The RT/S regions (nt130-1161) were amplified and sequenced using the Sanger method, and associations between RT mutations and the clinical characteristics of patients with HCC were analyzed. Finally, 27 and 29 mutations with frequencies >5% were identified in the RT and S regions, respectively. The rtF221Y variation and a tumor size >8 cm were found to be independent risk factors for the postoperative recurrence of HCC, with hazard ratios of 2.345 (95% CI, 1.391-3.953; P=0.001) and 1.838 (95% CI, 1.069-3.161; P=0.028), respectively. rtF221Y was also an independent risk factor for poor overall survival rates (HR=2.557; 95% CI, 1.344-4.866; P=0.004). The mutation of R122 K in the HBV S protein was closely associated with tumor recurrence (P<0.001). As a result, rtF221Y was identified as a risk factor for poor prognosis and may be a potential viral marker for predicting prognosis in HCC.

Introduction

Hepatitis B virus (HBV) infection is one of the major etiological factors of HCC in China (1). Almost 360,000,000 individuals are chronic HBV carriers worldwide (2). HBV infection can lead to liver diseases, including hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC) (3). The HBV genome has a partially double stranded circular structure, consisting of four overlapping open reading frames (ORFs) (4) encoding the virus polymerase, S protein, X protein and core protein, respectively. The ORF of polymerase contains four regions: A carboxy terminal region (nt2307-2840), spacer region (nt2841-129), reverse transcriptase (RT) region (nt130-1161) and an RNase H region (nt1129-1621) (5), of which the RT region is crucial for HBV replication. Several mutations occur in the HBV genome due to deficiency in the proofreading function in RT and HBV replication through RNA-intermediated reverse transcription. Therefore, in the course of HBV infection, mutations continuously accumulate, and a number of these mutations may be used as viral markers for evaluating the development and prognosis of HBV-associated HCC.

Numerous studies in previous decades have focused on the association between HBV mutations, including point mutations, deletions and structure variation, and the risk of HCC. It has been shown that nucleotide mutations in the S gene and pre C/C gene are closely associated with increasing risks of HCC (6-9). In the basal core promoter/enhancer II region, A1762T/G1764A has been found to be significantly associated with HCC (10-12). Increasing studies are focusing on the association between HBV gene variation and the prognosis with HCC. Yeh *et al* (13) reported that the presence of the A1762T/G1764A mutation in liver tissue within the BCP was an independent predictor for disease-free survival (DFS) and overall survival (OS) rates in HCC. A pre-S deletion located between codons 107 and 141 was

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found to be associated with poorer postoperative prognosis, and Su *et al* (14) showed that the pre-S deletion was crucial for post-operative tumor recurrence. However, studies investigating mutations in RT associated with the HCC prognosis are limited.

In the present study, HBV DNA was extracted from liver tissues of patients with HCC, and viral quasispecies within the RT/S region (RT overlapped with the S gene) were analyzed using Sanger sequencing. Cox proportional hazard model analysis was used to investigate the association between variations in the HBV RT/S region and the prognosis of HCC.

Materials and methods

Patients and samples. A total of 84 patients with HCC were recruited between March 2007 and May 2009, who received complete surgical resection at the Eastern Hepatobiliary Surgery Hospital (Shanghai, China). Serum samples, tumor tissue (TT) and paired adjacent non-tumor tissue (ANTT) samples were collected. Written informed consent was obtained from all patients. The present study was approved by the Ethics Committee of Human Resources at the Second Military Medical University (Shanghai, China).

Patients were included in the cohort for examination if they fulfilled following inclusion criteria: i) serum hepatitis B surface antigen (HBsAg)-positive for at least 6 months; ii) HBV DNA levels >1,000 IU/ml; iii) nucleos(t)ide analogue (NA) naïve prior to surgical resection. The exclusion criteria included hepatitis C virus or human immunodeficiency virus co-infection, or a history of liver transplantation, autoimmune liver diseases, metastatic liver cancer, other malignancies, drug-associated liver diseases, alcoholic hepatitis or other causes of chronic liver disease diagnosed prior to enrollment.

HBV nucleic acid extraction and polymerase chain reaction (PCR) amplification. HBV genomes were extracted from frozen TTs and ANTTs with a QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. Due to the limitation of sequencing length, the RT region was amplified as two overlapping segments, respectively. The primers for the first segment were as follows: Forward 5'-CTG CTGGTGGCTCCAGTTC-3' (nucleotides 57-75) and reverse 5'-TGGCTCAGTTTACTAGTGCCA-3' (nucleotides 668-688). The primers for the second segment were as follows: Forward 5'-TCAGTCCGTTTCTCCTGGCTCAG-3' (nucleotides 653-675) and reverse 5'-GAGTTCCGCAGTATGGATCG-3' (nucleotides 1,281-1,262). The RT segments were amplified with Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The final composition of 20 μl PCR mixtures contained 5 μl DNA template, 0.5 μl Phusion High-Fidelity DNA polymerase, 4 μl 5X Phusion HF buffer, 0.5 μl 10 mM dNTPs, 1 μl forward primer (1 μM), 1 μl reverse primer (1 μM) and 8 μl DNase-Free water. The RT segments were amplified with the following thermocycling conditions: 95°C for 5 min followed by 35 cycles of 98°C for 30 sec, 57°C for 30 sec and extension at 72°C for 1 min. The mixtures were subjected to further extension at 72°C for 10 min. The TaqMan probes for covalently closed circular (ccc) DNA and intrahepatic HBV total DNA (tDNA) quantification were FAM-ATCTGC CGGACCGTGTGC-TAMARA and FAM-CTCACCAACCTC CTGTCCTCCA-TAMARA, respectively.

Table I. Clinical characteristics of patients with HBV-associated hepatocellular carcinoma.

Characteristic	Cohort (n=84)	P-value
Gender		
Male	72 (86%)	
Female	12 (14%)	
Age (years)	50 (28-70)	
AFP (ng/ml)	1,067 (1.5->1,210)	
TBIL (μmol/l)	14.7 (7.1-50.5)	
DBIL (μmol/l)	5.4 (1.7-18.4)	
ALT (U/l)	49 (21-1,067)	
AST (U/l)	51 (22-1041)	
Serum HBV DNA($\log_{10}$IU/ml)	5.2 (3.0-7.6)	
HBV tDNA ($\log_{10}$copies/10^6cells)		0.029
TT	6.6±1.2	
ANTT	7.0±0.9	
cccDNA ($\log_{10}$copies/10^6cells)		0.544
TT	4.9±1.4	
ANTT	5.0±1.0	
HBsAg ($\log_{10}$IU/ml)	3.1 (0.32-4.11)	
Tumor size (cm)	8.0 (1.1-25.0)	
HBeAg		
Positive	40 (48%)	
Negative	44 (52%)	
HBV genotype		
B	22 (26%)	
C	62 (74%)	
Ascites		
Yes	13 (15%)	
No	67 (80%)	
Tumor number		
Single	59 (70%)	
Multiple	22 (26%)	
Cirrhosis		
Present	72 (86%)	
Absent	12 (14%)	
Capsule		
Complete	23 (27%)	
Incomplete	47 (56%)	
TNM stage		
I-II	49 (58%)	
III-IV	23 (27%)	

HBV, hepatitis B virus; HbsAg, hepatitis B surface antigen; AFP, α-fetoprotein; TBIL, total bilirubin; DBIL, direct bilirubin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; tDNA, total DNA; cccDNA, covalently closed circular DNA; TT, tumor tissue; ANTT, adjacent non-tumor tissue; HBeAg, hepatitis B e antigen; TNM, tumor-node-metastasis.

Sanger sequencing and sequence alignment. The products of the RT region segmented PCR amplification were gel purified

Table II. Univariate and multivariate analyses of clinicopathologial and virological characteristic for DFS and OS in patients with HBV-associated hepatocellular carcinoma.

Characteristic	DFS				OS			
	Univariate	Multivariate			Univariate	Multivariate		
	P-value	HR	(95% CI)	P-value	P-value	HR	(95% CI)	P-value
Age >50 years	0.717				0.985			
Gender (male)	0.680				0.952			
AFP >400 ng/ml	0.736				0.735			
ALT >40 U/l	0.274				0.475			
Tumor size >8 cm	<0.001	2.345	1.391-3.953	0.001	0.688			
HBV DNA ($\log_{10}$ IU/ml)	0.497				0.375			
ANTT HBV tDNA ($\log_{10}$copies/10^6cells)	0.673				0.452			
ANTT HBV cccDNA ($\log_{10}$copies/10^6cells)	0.892				0.695			
N13R/S	0.024				0.130			
I16T	0.002				0.015			
S53N	0.002				0.020			
H55R/Q/K	0.509				0.056			
I91L	<0.001				0.017			
P109S	0.419				0.612			
T118N	0.118				0.321			
N121I	0.023				0.084			
Y124N/H	0.004				0.160			
G127R	0.005				0.084			
N131D	0.001				0.058			
D134N/E	0.994				0.405			
N139K	0.211				0.197			
L145M	0.190				0.119			
F151Y	0.003				0.033			
F221Y	<0.001	1.838	1.069-3.161	0.028	0.003	2.557	1.344-4.866	0.004
T222A	0.003				0.033			
S223A	0.210				0.311			
I224V	0.784				0.638			
N238H	0.272				0.543			
S256C	0.959				0.092			
Q267L	0.146				0.679			
L269I	0.012				0.272			
R280P	0.077				0.097			
S317A	0.027				0.056			
C332S/R	0.007				0.085			
K333Q	0.002				0.154			

HBV, hepatitis B virus; DFS, disease-free survival; OS, overall survival; CI, confidence interval; ANTT, adjacent non-tumor tissue; tDNA, total DNA; cccDNA, covalently closed circular DNA; AFP, α-fetoprotein; ALT, alanine aminotransferase.

and sequenced using an ABI PRISM BigDye sequencing kit on an ABI 3500 genetic analyzer (Applied Biosystens; Thermo Fisher Scientific, Inc.). HBV genotypes were identified using an online genotyping tool (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi). The sequences from each of the samples were compared with the RT sequence in the NCBI database (HBVgp1; NC_003977.2), which was performed using DNAMAN software (version 4.0; Lynnon Corporation, Pointe-Claire, QC, Canada).

Statistical analysis. Mutations between TT and ANTT were analyzed using the χ^2 test. Forward stepwise multivariate regression analysis was performed to obtain the hazard ratios of potential risk factors for HCC prognosis. Matched
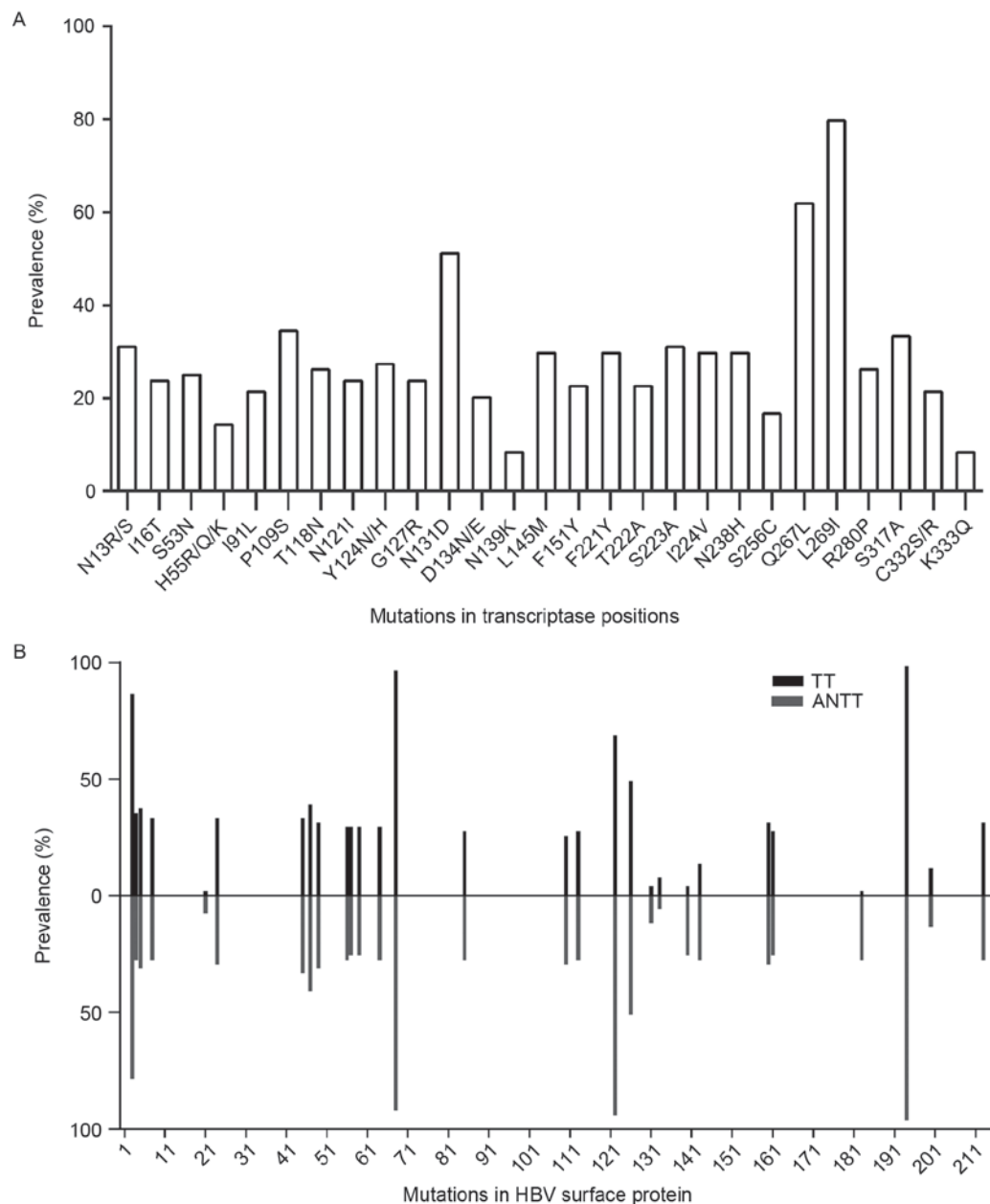
Figure 1. Amino acid mutations in reverse transcriptase and HBsAg. (A) 27 amino acid substitutions within reverse transcriptase. (B) Overall distributions of amino acid mutations and their relative frequencies within the HBV HBsAg. HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; TT, tumor tissue; ANTT, adjacent non-tumor tissue.

clinicopathological characteristics were subjected to stratified analysis. P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed on SPSS software (version 18.0; SPSS, Inc., Chicago, IL, USA).

Results

Patient characteristics. The baseline characteristics of the 84 patients included in the present study are presented in Table I. The cohort, comprised of 72 (86%) male subjects, had a median age of 50 years, and 74% were infected with HBV genotype C. Over half of the patients (58%) were histologically diagnosed with early stage tumors (tumor-node-metastasis stages I-II). In addition, the median duration of HCC recurrence was 10.0 months (range 0.43-50.9 months).

Association between intrahepatic HBV DNA and serum HBV DNA. The levels of HBV tDNA in the ANTTs were higher, compared with those in the TTs (7.0±0.9, vs. 6.6±1.2 $\log_{10}$copies/10^6cells; P=0.029; Table I). Serum HBV DNA was moderately correlated with ANTT tDNA (r=0.419; P<0.001) and cccDNA (r=0.370; P<0.001), but not with TT tDNA (r=0.154; P=0.166) or cccDNA (r=0.123; P=0.281). These results suggested that serum HBV DNA may derive from ANTTs, rather than TTs, therefore, serum HBV DNA levels may not reflect the real level of HBV DNA in TTs.

Characteristics of amino acid mutations in RT and HBsAg. According to the criteria of 5% frequency, a total of 27 amino acid mutations in the RT domain were included (Fig. 1A). The majority of these mutations occurred with a frequency of

Table III. Comparison of clinical characteristics of patients with and without the rtF221Y mutation.

Characteristic	MT (n=25)	WT (n=59)	P-value
Age (years)	49.2±8.5	49.2±9.8	0.748
ALT (U/l)	50 (21.2-360.5)	49 (21.4-1,067.5)	0.773
DBIL (μmol/l)	4.9±1.8	6.4±3.0	0.021
AFP (ng/ml)	1,210 (39.7-1,210)	496 (10-1,210)	0.048
CREA (μmol/l)	71.5±11.1	65.6±9.5	0.018
Serum HBV DNA ($\log_{10}$IU/ml)	5.1±1.3	5.3±1.0	0.525
ANTT tDNA ($\log_{10}$copies/10^6cells)	6.8±1.0	7.0±0.9	0.461
ANTT cccDNA($\log_{10}$copies/10^6cells)	5.3±0.9	4.8±1.1	0.090
Tumor diameter (cm)			
≤8	4	24	0.001
>8	18	35	
Ascites			
Yes	3	10	0.487
No	22	45	
Capsule			
Complete	15	32	0.994
Incomplete	8	17	
Tumor number			
Single	14	45	0.255
Multiple	8	24	

Data are presented as the mean ± standard deviation or median (range). MT, rtY221 mutant type; WT, rtF221, wild-type; ALT, alanine amino-transferase; DBIL, direct bilirubin; AFP, α-fetoprotein; CREA, creatinine; HBV, hepatitis B virus; ANTT, adjacent non-tumor tissue; tDNA, total DNA; cccDNA, covalently closed circular DNA.

>20%, particularly rtQ267 L and rtL269I, which had frequencies >50%. Common NA resistance mutations, including rtI169T, rtA181T/V, rtT184A/C/F/G/I/L/M/S, rtA194T, rtS202C/G/I, rtM204I/V/S, rtN236T, rtM250I/L/V, rtV173 L and rtL80I/V were not detected during sequence alignment. The substitutions of rtS53N, rtI91L, rtF221Y and rtN238H detected were putative NA-resistance mutations. The pretreatment mutations, rtY124 N/H and rtN139K, were also found in the cohort. As the HBV S gene overlaps with the RT gene, the S gene originating from 51 pairs of TTs and ANTTs were analyzed, and 29 amino acid mutations within HBsAg met the 5% frequency inclusion criteria (Fig. 1B). The frequencies of sA194V, sT68I, sR122K, sS3N and sI126T were >50%, and 10 amino acid mutations were located in the major hydrophilic region (MHR; aa99-169) of HBsAg. No significant differences were found in HBsAg mutations between TTs and ANTTs, with the exception of three mutations, sR122 K (P=0.004), sT140S (P=0.001) and sF183V (P=0.001), which occurred more frequently in ANTTs.

Mutations associated with the prognosis of HCC. The Cox proportional hazard model was used to analyze the association between clinicopathological and virological factors associated with DFS and OS following surgical resection of HBV-associated HCC (Table II). Tumor sizes >8 cm (HR=2.345; P=0.001) and rtF221Y (HR=1.838; P=0.028) were associated with shorter DFS. rtF221Y (HR=2.557; P=0.004)

was also found to be an independent risk factor for OS. Kaplan-Meier survival analysis indicated that rtF221Y was significantly associated with poorer DFS (P=0.0027) and OS (P<0.001; Fig. 2A and B). The DFS and OS rates were significantly shorter in those with the rtF221Y mutation, compared with those with the wild-type amino acid. In addition, when Kaplan-Meier survival analysis and log-rank tests were used to compare survival probability, it was found that the HBsAg R122 K mutation in the TT was closely associated with tumor recurrence (P<0.001; Fig. 2C) and patients with the sR122 K mutation had a higher rate of recurrence (P=0.002), which was determined using the χ^2 test.

Association between HBV mutations and the clinical characteristics of patients with HCC. The levels of α-fetoprotein (AFP) in patients with the F221Y mutation were higher, compared with those without the mutation (P=0.048). In addition, patients harboring the F221Y mutation had larger tumor sizes, compared with patients without the F221Y mutation (P=0.001; Table III). No significant differences in age, alanine aminotransferase (ALT), aspartate aminotransferase, γ-glutamyltransferase, serum HBV DNA levels or tumor numbers were found between the patients with or without theF221Y mutation.

Due to the negative relevance between HBsAg mutations in ANTTs with clinicopathological characteristics, amino acid substitutions within HBsAg in TTs were subjected to stratified analysis (Table IV). The results revealed that the

Table IV. Association between TT HBsAg mutations and patient characteristics.

Characteristic	sS3N			sR122K			sI126T		
	WT n=7	MT n=44	P-value	WT n=16	MT n=35	P-value	WT n=26	MT n=25	P-value
Age (years)	50.4	49	0.710	51.1	48.3	0.296	50.3	48.1	0.392
Gender									
Male	1	2	0.364	15	30	0.999	25	23	0.610
Female	6	42		1	2		1	2	
ALT (U/l)	148	65	0.925	97.1	70.2	0.948	95	58	0.105
AFP (ng/ml)	866	730	0.620	767	772	0.736	570	935	0.007
HBsAg (S/CO)	2,696	1,434	0.378	1,809	1,455	0.543	2,006	1,134	0.162
Serum DNA (log$_{10}$IU/ml)	4.86	5.46	0.201	5.07	5.51	0.166	5.53	5.24	0.354
HBV tDNA (log$_{10}$copies/10^{6}cells)	6.56	7.02	0.215	7.1	6.9	0.428	7.12	6.79	0.204
cccDNA (log$_{10}$copies/10^{6}cells)	3.96	5.09	0.004	4.8	4.9	0.687	4.88	5	0.675
Tumor size (cm)	9.7	8.4	0.565	5.9	9.7	0.017	7.12	10.04	0.049
Recurrence									
Yes	7	33	0.323	8	32	0.002	21	19	0.679
No	0	11		8	3		5	6	
Cirrhosis									
Present	6	39	1.000	13	31	0.999	23	22	1.000
Absent	0	5		1	4		2	3	
Tumor number									
Single	4	33	0.643	10	27	0.493	18	19	0.747
Multiple	2	11		5	8		7	6	
Capsule									
Complete	3	26	1.000	4	25	0.019	14	15	0.936
Incomplete	2	15		8	9		8	9	
TNM stage									
I-II	5	29	0.573	9	25	0.411	16	18	0.734
III-IV	0	10		1	9		4	6	

Continuous variables are presented as the mean ± standard deviation or median (range). MT, mutant type; WT, wild-type; ALT, alanine aminotransferase; DBIL, direct bilirubin; AFP, α-fetoprotein; CREA, creatinine; HBV, hepatitis B virus; TT, tumor tissue; tDNA, total DNA; cccDNA, covalently closed circular DNA; TNM, tumor-node-metastasis.

occurrence of the sS3 N mutation was associated with higher TT cccDNA levels (3.96±0.7, vs. 5.09±1.0 log$_{10}$copies/10^{6}cells; P=0.004). In addition, patients with sI126T had higher AFP levels, compared with those with wild-type HBsAg (P=0.007). Patients with sR122 K or sI126T had larger tumor sizes (P=0.017 and P=0.049, respectively). However, no associations were found between HBsAg mutations in the ANTTs and the clinicopathological features.

Discussion

Although several studies have focused on the association between HBV variation and HCC, the majority of these have been performed using serum samples (11). Previous data have reported that serum HBV DNA shows moderate correlation with ANTT viral DNA, but no correlation with that of TT, which suggests that the presence of variants in serum HBV DNA may not reveal the actual status in the liver. Therefore, resected tissue specimens may have preponderance when designing investigations. Furthermore, the quantitative results of HBV tDNA and cccDNA from the liver tissues in the present study suggested that the levels of tDNAs in ANTT were significantly higher, compared with those in paired TT samples (P=0.029). The microenvironment in ANTT may be more suitable for HBV replication, and viruses in TT may have deficient replication activity due to an unfavorable microenvironment. Therefore, numerous missense mutations are likely to accumulate during the replication of HBV in the ANTT. HBV DNA in TT is more likely to integrate with the hepatocyte genome, which may also contribute to the lower level of replication of viral DNA (15). HBV integration can be detected at any stage of HBV infection and it is reasonable to suggest that fewer mutations in the
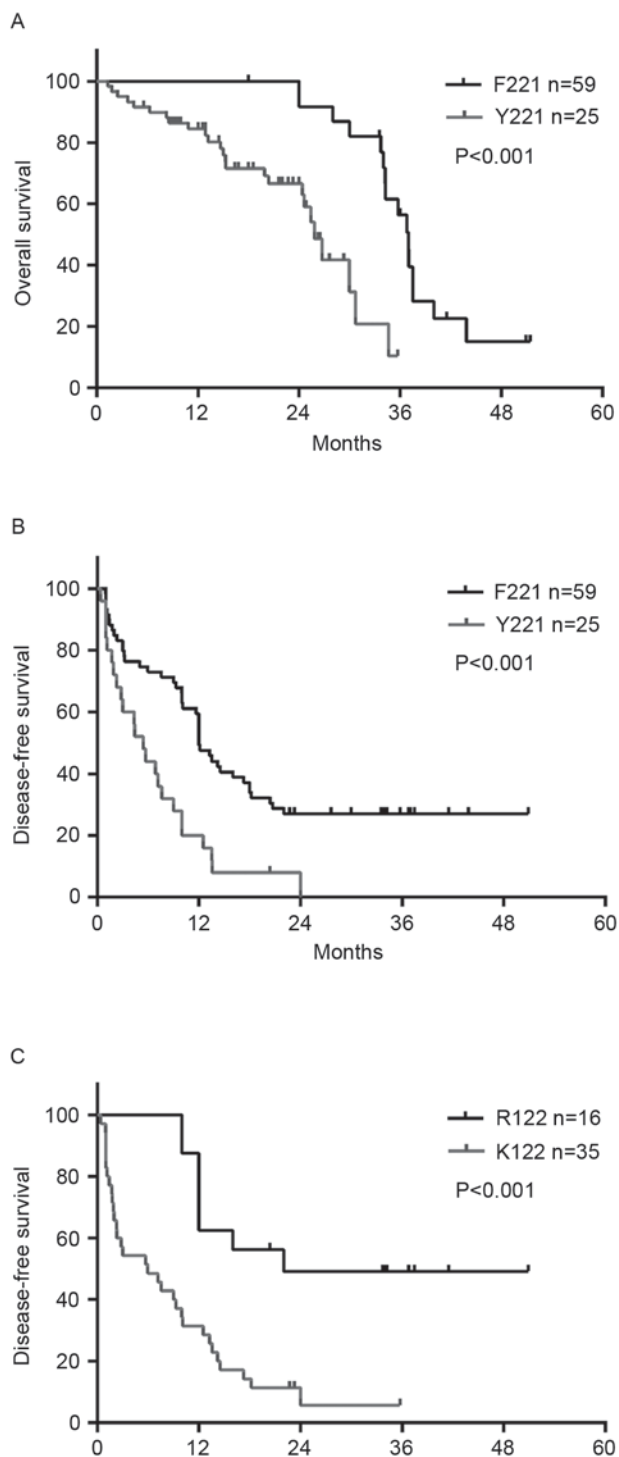
Figure 2. Association between survival and mutations. Comparison of postoperative (A) overall survival and (B) disease-free survival between patients with or without rtF221Y. (C) Comparison of disease-free survival between two group characterized by sR122 and sK122. The log-rank test was used to compare the survival curves between groups.

integrated form of the HBV genome occur in TT, compared with the replicative form of the HBV genome in ANTT (16,17). When the distribution of HBV mutations between the TT and ANTT samples were compared in the present study, there were only nuance were detected. As a result, gene variations in the HBV genome derived from ANTT may more suitable for investigations on the effect of HBV mutations on HCC.

Several studies have indicated that mutations in the S, C and X genes of the HBV genome are associated with a high risk of HCC (18,19), however, there are few reports on mutations in the RT region of the HBV genome, particularly in TTs and ANTTs, and its effect on development and prognosis of HCC. The present study focused on the genetic diversity in RT sequences isolated from liver tissues. When the alignment was completed, 27 mutations were obtained when the occurrence rate threshold was set as 5%. In addition, there was minimal observation of common NAs resistance mutations in sequence alignment, including L80I/V, I169T, V173L, A181T/V, T184A/C/F/G/I/L/M/S, A194T, S202C/G/I, M204I/V/S, N236T and M250I/L/V (20,21), which indicated that these variants were absent or were present at a low frequency due to the limitations of Sanger sequencing. This can also be attributed to the lack of regular NAs therapy. In the present study, S53N, I91L, N139K, F221Y and N238H were identified as putative drug resistance mutations; however, their functions remain to be fully elucidated.

The present study demonstrated that rtF221Y was an independent risk factor for DFS and OS in HCC. rtF221Y was also linked to a higher level of AFP and larger tumor size. To the best of our knowledge, the present study is the first to show that rtF221Y was associated with HCC in a relatively large cohort of patients. Therefore, rtF221Y may be used as a potential viral marker for predicting HCC prognosis following surgery. However, previous studies have suggested that rtF221Y may belong to putative antiviral drug resistance mutations. Mirandola et al (22) and Lee et al (23) reported that rtF221Y was associated with adefovir dipivoxil (ADV) or lamivudine+ADV experience. Of note, data have shown that one point mutation, L213I, observed in the surface protein, which leads to F221Y and A222T dual mutations in the RT domain of polymerase, in combination with the classical BCP mutations, A1762T/G1764A, are associated with the development of HCC in HBeAg-positive patients (24). In the present study, stratified analysis revealed that patients with the rtF221Y mutation had lower intrahepatic cccDNA levels (P=0.090), suggesting viral replication was less active. rtF221 is located at the nucleic acid binding domain, based on three-dimensional modeling analysis, therefore the rtF221Y mutation is likely to affect the enzyme structure and impair its polymerase activity. Patients with rtF221Y may require regular monitoring and caution when using ADV.

For HBV-associated HCC, a high copy number of HBV DNA is one of the important factors promoting the development of HCC (25,26). RT is important in the process of HBV replication and key mutations may occur during the immune response, affecting the activity of RT. When a guanine was substituted by cytosine at nucleotide 162 in the RT region, which induced the S3 N mutation in HBsAg synchronously, the levels of cccDNA were found to be significantly elevated, compared with those in the wild-type group (P=0.004). NAs treatment is considered to be an effective method to suppress HBV replication, normalize liver function, reduce HBV-associated HCC recurrence and improve postoperative survival rates. The occurrence of mutations association with the prognosis of HCC, including rtF221Y, may also decrease when receiving NAs therapy. Several meta-analyses have also revealed that regular antiviral treatment prior to or following

surgery can significantly prolong OS and decrease recurrence rates (27-29). It is necessary for patients with HBV-associated HCC to adhere to standard antiviral therapy.

The frequency of sR122K, sT140S and sF183 V within the HBsAg between ANTTs and TTs were significantly different, and these mutation sites were all located in the MHR, the primary B-cell epitope of HBV. Of note, the amino acid at position 122 within an antigenic loop determinates the serological HBs subtype. Subtype determinant d have a lysine (K) at this positions, whereas an arginine (R) indicates subtype determinant y. The positive charged amino acid R at position 122 primarily contributes to electrostatic interaction with negatively charged heparan sulfate proteoglycans at the plasma membrane of hepatocytes during infection (30). However, sR122K is closely associated with immune evasion (31). Patients who harbored R122 K in TTs suffered from larger tumor size and had higher recurrence rates in the present study. Further investigations are required to clarify the association between R122K and HCC. There were certain limitations in the present study; a lack of liver biopsies from patients with chronic hepatitis prevented deeper probing into sequence discrepancies in the HBV genome between patients suffering from HCC and chronic hepatitis. Therefore, the exact role of mutations, particularly those located in RT/S, during the development of HCC requires further investigation. In addition, due to the methodological limitations of Sanger sequencing, limited sensitivity hinders the detection of low abundance mutations (32). Therefore, next-generation sequencing technology is required to dissect viral quasispecies at the RT/S region, to understand the variation of the whole virus population in patients, to analyze minor antiviral resistance mutations and to provide a guide for HBV treatment.

In conclusion, the use of Sanger sequencing of virus derived from TT and ANTT samples revealed two novel substitutions, rtF221Y and sR122K, which were found to be associated with HBV-associated HCC recurrence. In particular, rtF221Y may serve as a viral marker for predicting HCC prognosis. In view of HBV RT mutations being one of the important factors linked to recurrence, it is necessary for NAs to be timely and selected deliberately for limiting the copy numbers of HBV DNA and preventing drug resistance. This can assist in decreasing recurrence rate and improving postoperative survival rates of patients with HBV-associated HCC.

Acknowledgements

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