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Prognostic Impact of Telomere Maintenance Gene Polymorphisms on Hepatocellular Carcinoma Patients With Chronic Hepatitis B

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Our goal was to determine whether single-nucleotide polymorphisms (SNPs) of telomere maintenance genes influence the development and clinical outcomes of patients with hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC). We evaluated 20 SNPs of five telomere maintenance genes in 702 patients with HCC and 351 hepatitis B virus surface antigen-positive controls without HCC. Significant SNPs were then validated in an independent cohort of 857 HCC patients and 429 controls. We assessed the association of each SNP with the development of HCC and overall survival through a multivariate Cox proportional analysis. A significantly increased risk of HCC development was identified for the telomerase-associated protein 1 (*TEP1*) rs1713449 SNP in both the discovery and replication phases (combined odds ratio = 1.42, $P = 9.378 \times 10^{-5}$). In addition, the *TEP1* rs1713449, *TEP1* rs872072, protection of telomeres 1 homolog rs7784168, telomerase reverse transcriptase rs13167280, and telomeric repeat binding factor 1 rs2306494 SNPs had a significant effect on the overall survival, and a similar survival effect was validated in the replication cohort. Moreover, there was a significant dose-dependent association between the number of putatively high-risk genotypes of the five aforementioned SNPs and overall survival. The median survival time was significantly prolonged for patients with HCC with two or fewer putatively high-risk genotypes versus those with three or more high-risk genotypes (85 versus 44 months, log-rank $P = 4.483 \times 10^{-5}$), and this was demonstrated in the replication cohort (52 versus 37 months, log-rank $P = 0.026$). *Conclusion*: These observations suggest that the SNPs of telomere maintenance genes play a potential role in the development of HCC and the survival of HCC patients with chronic HBV infections. (HEPATOLOGY 2014;59:1912-1920)

Chronic hepatitis B virus (HBV) infection is one of the established etiological agents of hepatocellular carcinoma (HCC) worldwide, and it is also an important contributing factor to the majority of HCC cases in Korea.[1,2] However, only a small proportion of chronic HBV carriers develop HCC in their lifetime, and this suggests that other risk factors may contribute to interindividual variations in the susceptibility to hepatocarcinogenesis.[3] In addition, various genetic factors appear to influence the outcome of HBV infections.[4,5] On the basis of our current knowledge of the molecular pathogenesis of cancer, polymorphic variations such as single-nucleotide polymorphisms (SNPs) within several gene classes are considered to be important for tumor development.[6-8]

By regulating the length of telomeres and the higher order structure of the protein complex at the ends of linear chromosomes, telomere maintenance genes are

Abbreviations: CH, chronic hepatitis; CI, confidence interval; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HR, hazard ratio; HWE, Hardy-Weinberg equilibrium; MAF, minor allele frequency; MST, median survival time; OR, odds ratio; POT1, protection of telomeres 1 homolog; RTL, relative telomere length; SNP, single-nucleotide polymorphism; TEP1, telomerase-associated protein 1; TERF, telomeric repeat binding factor; TERT, telomerase reverse transcriptase

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essential for maintaining genomic integrity and stability.[9-11] A dysfunctional telomere pathway may lead to less efficient DNA damage repair and genomic instability, which may ultimately initiate carcinogenesis. Previous studies have suggested that telomere dysfunction, leading to telomere-based chromosomal instability, may be associated with the early stage of hepatocarcinogenesis when telomerase activation takes place.[12,13] Theoretically, functional genetic variants in telomere maintenance genes, which potentially influence the length of telomeres, the activity of telomerase, and the protein complex, might play a key role in the process of HCC development. Although a few studies have been reported on the associations between SNPs of telomere maintenance genes and the risks for various cancers,[14-18] a study has not yet been reported on the associations between SNPs of telomere maintenance genes and HCC. Thus, we investigated the association between selective SNPs of telomere maintenance genes and the risk of the development of HCC and its clinical outcomes.

Patients and Methods

Study Population. This study included 1559 patients with HBV-associated HCC and 780 HBV-positive controls from two sources in Korea from January 1999 to December 2010, and the clinical outcomes of HCC were recorded until October 2012. The subjects were categorized into discovery and replication cohorts. The discovery cohort consisted of 702 HCC patients and 351 controls from Ulsan University Hospital. The impact of telomere maintenance genes was further tested in a replication cohort of 857 patients with HCC and 429 hepatitis B virus surface antigen (HBsAg)-positive controls from Asan Medical Center. The diagnosis of HCC was made by liver biopsy or by the combination of an increased alpha-fetoprotein level ($\geq$200 ng/mL) and the typical vascular pattern on angiography or dynamic imaging.[19] The control group consisted of HBsAg carriers without HCC. Clinical information was collected from medical records with patients' consent. Tumor progression was defined as major vessel invasion, distant metastasis, and postoperative recurrence. Major vessel invasion was defined as the presence of tumor growth or thrombi in major vessels (e.g., the portal vein, hepatic artery/vein, or inferior vena cava) on the imaging study. The tumor stage was based on the Barcelona Clinic Liver Cancer staging system.[20] The overall survival time was calculated from the date of tumor resection or the first local treatment to the date of death. The ethics committee of Ulsan University Hospital and Asan Medical Center approved the study, and written informed consent was obtained from all of the participants.

Selection of Candidate Genes. Twenty SNPs in five candidate telomere maintenance genes were selected for testing in this study (Table 1). These SNPs were chosen on the basis of previous reports of their association with the risk for cancer and a comprehensive tag SNP approach.[21-23] Tag SNPs were selected from the set of common SNPs genotyped in the Asian (CHB [Han Chinese in Beijing] and JPT [Japanese in Tokyo]) populations of the international HapMap project (phase III) with the Tagger algorithm in Haploview with a minor allele frequency (MAF) of at least 5%.[24,25] Three SNPs (rs10250202, rs10263573, and rs7784168) were selected from protection of telomeres 1 homolog (*POT1*), eight SNPs (rs2075786, rs13167280, rs2853677, rs2736099, rs2736098, rs2853669, rs2735940, and rs2736109) were selected from telomerase reverse transcriptase (*TERT*), five SNPs (rs1713449, rs1760904, rs872072, rs1760898, and rs1760897) were selected from telomerase-associated protein 1 (*TEP1*), three SNPs (rs34742076, rs2306494, and rs3863242) were selected from telomeric repeat binding factor 1 (*TERF1*; also known as *TRF1*), and one SNP (rs251796) was selected from *TERF2* (also known as *TRF2*).

Laboratory Methods. DNA was extracted from frozen white blood cells with standard methods. Genotype identification was performed with the GenomeLab SNPstream ultrahigh-throughput system, which uses multiplexed polymerase chain reaction in conjunction with tag array single-base extension genotyping (Beckman Coulter, Fullerton, CA).[26] This system and its accompanying SNPstream software have been described previously by Denomme and Van Oene.[27] The 20 individual SNPs were identified by their position and

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Additional Supporting Information may be found in the online version of this article.

Table 1. SNPs of the Telomere Maintenance Genes Evaluated in This Study

Gene	SNP	Polymorphism	Chromosome	MAF	HWE	Call Rate (%)
POT1	rs10250202	IVS13-98 G > T	7q31.33	0.502	0.854	99.05
	rs10263573	IVS12+41 T > A	7q31.33	0.496	0.951	96.30
	rs7784168	IVS6-33 A > G	7q31.33	0.216	0.179	98.90
TERT	rs2075786	IVS10+269 T > C	5p15.33	0.148	0.717	98.69
	rs13167280	IVS3-24 C > T	5p15.33	0.162	0.814	99.05
	rs2853677	IVS2-4455 T > C	5p15.33	0.309	1	98.74
	rs2736099	IVS2-4601 C > T	5p15.33	0.365	0.601	99.34
	rs2736098	Ex2-659 G > A	5p15.33	0.301	0.666	98.96
	rs2853669	−244 T > C	5p15.33	0.312	0.368	99.43
	rs2735940	−1381 C > T	5p15.33	0.404	0.137	99.43
	rs2736109	−1654 G > A	5p15.33	0.245	0.801	99.53
TEP1	rs1713449	Ex45+36 G > A	14q11.2	0.266	0.385	98.80
	rs1760904	Ex24+49 C > T	14q11.2	0.420	0.395	97.44
	rs872072	IVS13+84 C > T	14q11.2	0.238	0.505	98.12
	rs1760898	Ex4+51 C > A	14q11.2	0.381	0.477	98.27
	rs1760897	Ex1-222 T > C	14q11.2	0.169	0.521	99.05
TERF1	rs34742076	IVS7+82 C > T	8q13	0.138	0.897	97.06
	rs2306494	IVS8-124 G > A	8q13	0.289	1	99.37
	rs3863242	IVS9-163 C > T	8q13	0.149	0.901	97.44
TERF2	rs251796	IVS7-41 T > C	16q22.1	0.374	0.148	97.91

fluorescent color in each well according to the position of the tagged oligonucleotides. Genotype data were generated on the basis of the relative fluorescent intensities for each SNP. Graphical reviews and operator adjustments of the genotype clusters were performed to refine fluorescent cutoff values. Genotyping data were reviewed and manually confirmed by experienced researchers. In addition, the relative telomere length (RTL) was measured on extracted genomic DNA in duplicates with a previously described quantitative real-time polymerase chain reaction-based method to evaluate the association of the RTL with SNPs of telomere maintenance genes and clinical outcomes of patients who underwent surgical resection.[28]

Statistical Methods. The genotype frequencies of each of the SNPs were tested for Hardy-Weinberg equilibrium (HWE) in both HCC cases and HBsAg-positive controls without HCC with the permutation test. A comparison of the ages of the cases and the controls was performed with the Student *t* test. Categorical variables were compared with the χ^2 test. An unconditional logistic regression, which was used to evaluate significant associations between the distribution of SNPs and clinical variables as well as associations with disease (HCC patients versus controls), was undertaken to estimate the odds ratios (ORs) and their 95% confidence intervals (95% CIs) after adjustments for age, sex, Child-Pugh class, alpha-fetoprotein level, and HBV DNA level. The relationship of SNPs with overall survival was identified via the Kaplan-Meier method with the log-rank test and a Cox proportional hazards model. The data analysis was performed with SAS 8.0 computer software (SAS, Inc., Cary, NC). All tests were two-tailed, and $P < 0.05$ was considered statistically significant.

Results

Characteristics of the Study Population. The baseline characteristics of the studied population were similar for the HCC cases and the controls (Table 2). The mean age at enrollment was 53.3 years for the HCC cases and 53.4 years for the control group ($P = 0.775$). The male/female ratios were also similar for the HCC patients (80%/20%) and the controls (79%/21%, $P = 0.785$). Patient characteristics and clinical tumor features are summarized in Table 3. There were 702 patients in the discovery cohort: 334 had their primary tumor surgically resected, and the remaining 368 patients underwent local therapy (e.g., transarterial embolization or radiofrequency ablation). All 334 patients who underwent hepatic resection had a grossly complete resection; however, postoperative recurrence was observed in 150 cases (45%). The median follow-up period was 23 months (range = 1-152 months). In the replication cohort, postoperative recurrence was detected in 56 cases (47%), and the median follow-up period was 24 months (range = 1-156 months).

Genotype Frequency and Effects on the Development of HCC. The observed genotype frequencies of all 20 SNPs in the case and control subjects were in agreement with HWE ($P > 0.05$). Among the telomere maintenance gene SNPs of the discovery cohort, the polymorphism of *TEP1* rs1713449 was significantly associated with the development of HCC in the multivariate logistic regression analysis. The allele frequency of the GA/AA genotypes was significantly increased in patients with HCC versus controls (47.9% versus 38.6%), and this was also demonstrated in the replication cohort (46.4% versus 38.5%). The

Table 2. Baseline Characteristics of the Study Patients

Characteristic	HCC Patients (n = 1559)	Controls (n = 780)	*P* Value
Age (years)*	53.3 ± 8.4	53.4 ± 7.9	0.775
Sex: male/female [n/n (%/%)]	1247/312 (80/20)	620/160 (79/21)	0.785
Child-Pugh class: CH/A/B/C (n)	230/894/342/93	367/226/133/54	0.042
Median alpha-fetoprotein level (ng/mL)	98.7	3.3	<0.001
Mean HBV DNA level (copies/mL)	1.51×10^7	5.67×10^7	0.129

*The data are presented as means and standard deviations.
Abbreviation: CH, chronic hepatitis.

Table 3. Clinical Characteristics of the Enrolled Patients With HCC

Classification	Discovery Cohort (n = 702)	Replication Cohort (n = 857)
Age (years)*	53.1 ± 8.3	53.4 ± 8.6
Sex: male/female (n/n)	579/123	668/189
Child-Pugh class: CH/A/B/C (n)	109/422/126/45	121/472/216/48
Model for End-Stage Liver Disease score [n (%)]		
<10	448 (64)	476 (56)
≥10	254 (36)	381 (44)
Tumor size (cm)*	5.1 ± 3.3	5.8 ± 4.2
Tumor type [n (%)]		
Single nodular	412 (59)	476 (55)
Multinodular	156 (22)	204 (24)
Diffuse	134 (19)	177 (21)
Tumor staging [n (%)]		
Very early (0)	105 (15)	125 (14)
Early (A)	362 (52)	434 (51)
Intermediate (B)	115 (16)	142 (17)
Advanced (C)	99 (14)	121 (14)
Terminal (D)	21 (3)	35 (4)
Major vessel invasion [n (%)]	200 (29)	325 (38)
Distant metastasis [n (%)]	191 (27)	314 (37)
Surgical resection [n (%)]	334 (48)	119 (14)
Postoperative recurrence [n (%)]	150 (45)	56 (47)
Follow-up period (months)†	23 (1-152)	24 (1-156)

*The data are presented as means and standard deviations.
†The data are presented as medians and ranges.
Abbreviation: CH, chronic hepatitis.

adjusted OR between the GG and GA/AA genotypes of *TEP1* rs1713449 was 1.438 (95% CI = 1.072-1.929, $P = 0.015$) for the discovery stage, 1.369 (95% CI = 1.071-1.751, $P = 0.012$) for the replication stage, and 1.395 (95% CI = 1.163-1.672, $P = 3.248 \times 10^{-4}$) for all samples combined (Table 4).

Genotype Frequency and Effects on the Tumor Progression of HCC. The polymorphisms of *POT1* rs7784168, *TERT* rs13167280, and *TEP1* rs1760898 were significantly associated with a lower likelihood of major vessel invasion by HCC, and this was validated in the replication cohort except for the *TEP1* rs1760898 SNP (Supporting Table 1). Although the *TERT* rs13167280, rs2075786, and rs2853677 SNPs were significantly associated with distant HCC metastasis in the discovery cohort, only *TERT* rs13167280 was validated in the replication cohort (Supporting Table 2). Patients with one or two copies of the *TERT* rs13167280 T allele were at a lower risk for distant HCC metastasis than those who were homozygous for the C allele. The adjusted OR was 0.577 (95% CI = 0.389-0.852, $P = 0.006$) for the discovery cohort, 0.683 (95% CI = 0.49-0.953, $P = 0.025$) for the replication cohort, and 0.623 (95% CI = 0.484-0.801, $P = 2.239 \times 10^{-4}$) for all samples combined. As for the association between the SNPs of telomere maintenance genes and the postoperative recurrence of HCC, only *POT1* rs7784168 was significantly associated with the postoperative recurrence of HCC (Supporting Table 3). The adjusted OR between the AA and AG/GG genotypes of *POT1* rs7784168 was 0.518 (95% CI = 0.321-0.836, $P = 0.007$) for the discovery cohort, 0.359 (95% CI = 0.159-0.811, $P = 0.014$) for the replication cohort, and 0.461 (95% CI = 0.306-0.694, $P = 2.057 \times 10^{-4}$) for both cohorts combined.

Genotype Effects on the Overall Survival of Patients With HCC. There were 269 deaths among the 702 HCC cases (38.3%) in the discovery cohort and 500 deaths (58.3%) in the replication cohort. The median survival time (MST) for the enrolled cases was 55 months in the discovery cohort and 41 months in the replication cohort. According to a Kaplan-Meier analysis of the overall pooled population, a younger age, a tumor size larger than 5 cm, diffuse HCC, advanced tumor staging, the presence of major vessel invasion and distant metastasis, an unresected tumor, and postoperative recurrence were factors that were significantly associated with reduced overall survival (Supporting Table 4). The genotype frequencies and the MST by each genotype are shown in Table 5. Five of

Table 4. Multivariate Logistic Regression Analysis of the *TEP1* rs1713449 Polymorphism in Patients With HCC and Controls

Cohort	Genotype	HCC Patients [n (%)]	Controls [n (%)]	OR	95% CI	*P* Value
Discovery	GG	363 (52.1)	215 (61.4)	Referent		
	GA	276 (39.6)	115 (32.9)	1.450	1.066-1.973	0.018
	AA	58 (8.3)	20 (5.7)	1.394	0.760-2.559	0.284
	GA/AA	334 (47.9)	135 (38.6)	1.438	1.072-1.929	0.015
Validation	GG	449 (53.6)	262 (61.5)	Referent		
	GA	323 (38.5)	138 (32.4)	1.380	1.065-1.787	0.015
	AA	66 (7.9)	26 (6.1)	1.325	0.804-2.182	0.270
	GA/AA	389 (46.4)	164 (38.5)	1.369	1.071-1.751	0.012
Combined	GG	812 (52.9)	477 (61.5)	Referent		
	GA	599 (39.0)	253 (32.6)	1.396	1.153-1.689	0.001
	AA	124 (8.1)	46 (5.9)	1.424	0.984-2.061	0.061
	GA/AA	723 (47.1)	299 (38.5)	1.395	1.163-1.672	3.248×10^{-4}

Table 5. Overall Survival by the Genotype of Telomere Maintenance Genes

	Discovery Cohort				Replication Cohort				Overall Pooled Population	
Variable	Cases (n)	Deaths (n)	MST (Months)*	*P* Value	Cases (n)	Deaths (n)	MST (Months)*	*P* Value	MST (Months)*	*P* Value
TEP1 rs1713449				0.007				0.016		3.500×10^{-4}
GG	363	122	85 (56-114)		449	237	52 (41-63)		57 (48-66)	
GA/AA†	334	145	48 (36-60)		389	249	36 (28-44)		41 (35-47)	
TEP1 rs872072				0.022				0.005		3.223×10^{-4}
CC	391	137	70 (48-92)		491	271	53 (44-62)		57 (50-64)	
CT/TT†	289	123	48 (33-63)		358	224	32 (25-39)		37 (31-43)	
POT1 rs7784168				0.024				0.069		4.661×10^{-3}
AA†	428	178	48 (28-68)		449	237	39 (32-46)		43 (38-48)	
AG/GG	264	88	67 (45-89)		389	249	47 (36-58)		58 (49-67)	
TERT rs13167280				0.029				0.053		2.267×10^{-3}
CC†	477	194	52 (38-66)		610	371	39 (33-45)		44 (39-49)	
CT/TT	217	72	72 (44-100)		225	117	47 (36-58)		56 (44-68)	
TERF1 rs2306494				0.044				0.042		3.083×10^{-3}
GG	350	121	85 (58-112)		411	219	48 (39-57)		54 (48-60)	
GA/AA†	348	145	48 (34-61)		441	277	37 (30-44)		40 (35-45)	

*95% confidences intervals are shown in parentheses.
†High-risk genotype.

the 20 SNPs evaluated in the discovery cohort showed a significant effect on overall survival. The strongest genetic effect on survival was observed for *TEP1* rs1713449 with MSTs of 85 and 48 months for the GG and GA/AA genotypes, respectively (log-rank $P = 0.007$). The *TEP1* rs872072, *POT1* rs7784168, *TERT* rs13167280, and *TERF1* rs2306494 SNPs also showed significant effects on the overall survival of patients with HCC. Even if two SNPs (*POT1* rs7784168 and *TERT* rs13167280) showed only a marginal effect on survival, the other three SNPs remained significant predictors of survival for patients with HCC in the replication cohort. In an analysis of the overall pooled population, all five SNPs showed a strong association with the overall survival of patients with HCC.

Association of the RTL With SNPs of Telomere Maintenance Genes and Clinical Outcomes. RTL measurements were available for 274 patients who underwent surgical resection for HCC. The median RTL for the resected HCC patients was 0.689 (range = 0.052-2.089). To investigate whether the RTL had prognostic significance, we divided the HCC patients who underwent surgical resection into two groups according to the median value (0.689) of their RTLs. The RTL for resected HCC was significantly associated with a genetic variant of *TEP1* rs1713449 (Supporting Table 5). A shorter RTL showed a significantly higher distribution of the GA/AA genotype of *TEP1* rs1713449 (OR = 1.897, 95% CI = 1.171-3.073, $P = 0.009$). However, the RTL for resected HCC was not significantly associated with postoperative recurrence (Supporting Table 6) or the overall survival of patients with HCC (Supporting Table 4).

Combined Genotype Effects on the Overall Survival of Patients With HCC. A strong gene dosage effect was observed when the *TEP1* (rs1713449) GA/AA, *TEP1* (rs872072) CT/TT, *POT1* (rs7784168) AA, *TERT* (rs13167280) CC, and *TERF1* (rs2306494) GA/AA genotypes, which were suggested as high-risk genotypes by the Kaplan-Meier analysis, were analyzed in combination (Table 6). In the discovery cohort, patients with no high-risk genotypes, one high-risk genotype, or two high-risk genotypes had an MST of 85 months, whereas those with three to five high-risk genotypes had an MST of 44 months (log-rank $P = 4.483 \times 10^{-5}$; Fig. 1A). In an analysis of the replication cohort, HCC patients with no high-risk genotypes, one high-risk genotype, or two high-risk genotypes also showed significantly prolonged survival (MST = 52 months) in comparison with patients with three or more high-risk genotypes (MST = 37 months, log-rank $P = 0.026$; Fig. 1B). Furthermore, the combined genotypic effect of five SNPs on the overall survival of HCC was strongest in the pooled analysis of the two cohorts with MSTs of 59 and 39 months (log-rank $P = 1.663 \times 10^{-5}$).

Cox Regression Models. Finally, we conducted a multivariate analysis of the effects of genotype on survival with Cox proportional hazards models adjusted for other clinical factors. As shown in Table 7, the *TEP1* rs1713449, *TEP1* rs872072, *POT1* rs7784168, *TERT* rs13167280, and *TERF1* rs2306494 SNPs remained significant independent predictors of survival among HCC patients. The hazard ratios (HRs) for the *TEP1* rs1713449, *TEP1* rs872072, *POT1* rs7784168, *TERT* rs13167280, and *TERF1* rs2306494 SNPs were

Table 6. Combined Effects of High-Risk Genotypes on the Overall Survival of Patients With HCC

	Discovery Cohort				Replication Cohort				Overall Pooled Population	
Variable	Cases (n)	Deaths (n)	MST (Months)*	*P* Value	Cases (n)	Deaths (n)	MST (Months)*	*P* Value	MST (Months)*	*P* Value
Number of high-risk genotypes†				4.277×10^{-4}				1.968×10^{-6}		2.129×10^{-8}
0	23	5	92 (74-110)‡		20	8	66 (18-114)		68 (57-79)	
1	106	29	94 (75-114)‡		120	56	52 (35-69)		80 (54-106)	
2	184	63	59 (35-83)		246	140	50 (37-63)		55 (44-66)	
3	213	85	48 (30-66)		236	129	50 (38-62)		49 (40-58)	
4	117	60	33 (20-46)		164	111	33 (22-44)		33 (25-41)	
5	59	27	37 (9-65)		71	56	18 (10-26)		27 (20-34)	
Number of high-risk genotypes divided into two groups				4.483×10^{-5}				0.026		1.663×10^{-5}
0-2	313	97	85 (60-110)		386	204	52 (41-63)		59 (50-68)	
3-5	389	172	44 (33-55)		471	296	37 (30-44)		39 (34-44)	

*95% confidences intervals are shown in parentheses.

†The high-risk genotypes were GA/AA for *TEP1* rs1713449, CT/TT for *TEP1* rs872072, AA for *POT1* rs7784168, CC for *TERT* rs13167280, and GA/AA for *TERF1* rs2306494.

‡The mean survival time was provided when the MST could not be calculated.

1.294 (95% CI = 1.121-1.495), 1.279 (95% CI = 1.108-1.477), 0.759 (95% CI = 0.653-0.883), 0.707 (95% CI = 0.599-0.835), and 1.240 (95% CI = 1.075-1.431), respectively, after adjustments for clinical factors. Furthermore, having three or more putatively high-risk genotypes also remained a significant independent predictor for survival (HR = 1.372, 95% CI = 1.186-1.587, $P < 0.001$).

Discussion

In this study, we evaluated the effect of 20 SNPs of five telomere maintenance genes on the development, tumor progression, and overall survival of patients with HBV-associated HCC. To the best of our knowledge, this is the first study to describe the association between SNPs of telomere maintenance genes and the occurrence and clinical outcomes of HCC. We demonstrated that the *TEP1* rs1713449 variant genotype was significantly associated with the development of HCC. Moreover, the *TEP1* rs1713449 SNP, both alone and in combination with other genes, was associated with significantly decreased overall survival in two independent cohorts. In addition, four SNPs of the *TEP1*, *POT1*, *TERT*, and *TERF1* genes were also found to significantly affect the overall survival of HCC patients.

The *TEP1* gene, a telomerase that adds new telomeres to the ends of chromosomes, is known for its function in the protection of telomeres from degradation. In our study, the polymorphism of *TEP1* rs1713449 was found to be associated with the development of HCC and the overall survival of patients with HCC. This SNP is a nonsynonymous SNP (Val2214Ile). Changing the aliphatic amino acid (valine) to isoleucine is likely to affect TEP1 synthesis and influence telomerase activity. Participants with one or two copies of the A allele of *TEP1* rs1713449 had a 1.42-fold higher risk for HCC than participants homozygous for the G allele. Moreover, a significant genetic effect on the overall survival was observed with both the Kaplan-Meier and multivariate Cox regression methods. These data suggest that the genetic variant of *TEP1* rs1713449 can be a potential genetic marker indicative of an increased risk for the development of HCC and reduced survival. In addition, rs872072 of *TEP1* also showed a significant association with overall survival in patients with HCC. Because the *TEP1* rs872072 SNP is located within the intron, it is unclear what effect rs872072 may have on the *TEP1* transcript. However, the rs872072 SNP could potentially affect splicing of the *TEP1* transcript or change the expression of *TEP1* by altering transcription factor binding sites.

Previous studies have reported on the role of telomere length in the prediction of HCC risk, and they have demonstrated that a short telomere length is associated with a higher risk of HCC development and postoperative recurrence.[29,30] In our study, we demonstrated that a shorter RTL was significantly associated with the *TEP1* rs1713449 SNP, which showed a higher risk of HCC development and reduced survival for patients with HCC. These results suggest that the *TEP1* rs1713449 SNP may contribute to the genetic control of telomere length and influence the risk of HCC development; this is consistent with the hypothesis that

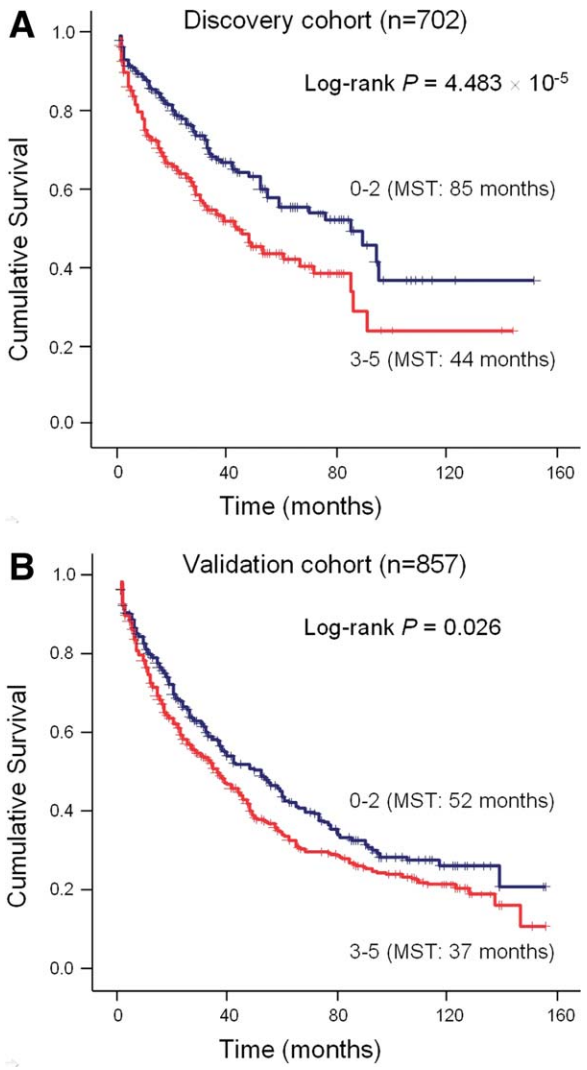

Fig. 1. Kaplan-Meier curves of the overall survival of patients with HBV-associated HCC with the combined effect of *TEP1* rs1713449, *TEP1* rs872072, *POT1* rs7784168, *TERT* rs13167280, and *TERF1* rs2306494: (A) the discovery cohort and (B) the replication cohort. The numbers 0 to 5 indicate the number of high-risk genotypes.

shorter telomeres predispose people to an increased cancer risk.[31]

A telomeric DNA binding protein (POT1) is essential to telomere integrity because it is responsible for recruiting telomerase to the single-stranded 3′-telomere overhang and consecutively limiting telomere elongation by telomerase.[32] Little is known about the impact of *POT1* genetic variations on telomere function and cancer risk. Although POT1 might function as a negative regulator of telomere length by directly inhibiting telomerase activity or controlling telomeric DNA substrate access to telomerase in human cells,[33,34] two previous population studies did not observe any association between *POT1* polymorphisms and breast or lung cancer risk.[35,36] Although our study failed to demonstrate an association between *POT1* polymorphisms and the risk of HCC development, the *POT1* rs7784168 SNP was significantly associated with a decreased risk of major vessel invasion and postoperative recurrence. Moreover, the *POT1* rs7784168 AG/GG genotype showed a significant effect on improved overall survival, and this suggests that the G allele of *POT1* rs7784168 may be associated with favorable prognoses for patients with HCC.

TERT is an important catalytic subunit of telomerase and determines telomerase activity.[37,38] In a report of 66 non-small cell lung cancer tissues, telomere activity was significantly decreased in carriers of the *TERT* rs2853669 CC genotype versus those with the TT genotype.[39] Another study of breast cancer risk and *TERT* polymorphisms indicated that the CC genotype was correlated with a significantly decreased breast cancer risk.[14] Our data, however, did not show any significant association between *TERT* rs2853669 and HCC risk. Until now, no experimental data have been reported in an attempt to show an association between *TERT* polymorphisms and HCC in the English literature. In our study, we found that the *TERT* rs13167280 SNP (TC/TT genotype) was strongly associated with a lower likelihood of major vessel invasion and distant metastasis. Accordingly, the *TERT* rs13167280 SNP was associated with significantly improved survival, and this suggests that the T allele of *TERT* 13167280 acts as a preventive factor for the progression of HCC.

TERF1 encodes the TERF1 protein, which is a member of the shelterin telomere protection complex that protects telomeres from degradation and inappropriate DNA repair.[40] A recent study has demonstrated that an SNP of *TERF1* is significantly associated with the risk of melanoma,[16] whereas other studies have failed to demonstrate an association between *TERF1* polymorphisms and cancer risk.[18,35,41] In our study, the *TERF1* rs2306494 SNP was found to be associated with the overall survival of patients with HCC in both the discovery and replication cohorts with an MST of 54 months for the GG genotype and an MST of 40 months for the GA/AA genotypes in the overall pooled population.

Because genetic interactions are complex, it is unlikely that a single SNP is responsible for susceptibility to HCC. However, the combined influence of several SNPs may exert natural combined or synergistic protection against the development or progression of HCC. In this study, SNPs of *TEP1*, *POT1*, *TERT*, and *TERF1* were found to be significantly associated with overall survival across the two study populations. In addition, the results of this study show the combined effect of individual SNPs of telomere maintenance

Table 7. HRs for Overall Survival in Multivariate Cox Regression Models

	Discovery Cohort			Replication Cohort			Overall Pooled Population		
Variable	**HR**	**95% CI**	***P* Value**	**HR**	**95% CI**	***P* Value**	**HR**	**95% CI**	***P* Value**
Clinical variables									
Age: ≤50 versus >50 years	0.785	0.608-1.014	0.063	0.909	0.752-1.099	0.325	0.863	0.741-1.004	0.057
Sex: male versus female	0.868	0.610-1.235	0.432	0.995	0.779-1.271	0.967	0.946	0.775-1.154	0.583
Child-Pugh class: CH/A versus B/C	1.760	1.269-2.442	0.001	1.605	1.319-1.954	<0.001	1.663	1.386-1.994	<0.001
Model for End-Stage Liver Disease score: <10 versus ≥10	1.605	1.169-2.204	0.003	1.599	1.291-1.982	<0.001	1.602	1.345-1.907	<0.001
Tumor size: ≤5 versus >5 cm	1.096	0.713-1.685	0.677	1.029	0.745-1.420	0.864	1.056	0.818-1.365	0.675
Tumor type: nodular versus diffuse	1.829	1.312-2.549	<0.001	1.421	1.087-1.857	0.009	1.592	1.296-1.956	<0.001
Tumor staging: 0/A versus B/C/D	2.534	1.608-3.993	<0.001	3.326	2.351-4.707	<0.001	2.877	2.189-3.781	<0.001
Major vessel invasion: no versus yes	2.279	1.703-3.050	<0.001	2.653	2.160-3.260	<0.001	2.503	2.117-2.959	<0.001
Distant metastasis: no versus yes	1.903	1.451-2.496	<0.001	2.077	1.717-2.513	<0.001	2.031	1.742-2.369	<0.001
Surgical resection: no versus yes	0.593	0.437-0.805	0.001	0.564	0.395-0.803	0.002	0.581	0.467-0.723	<0.001
Postoperative recurrence: no versus yes	5.629	2.114-14.990	0.001	56.82	7.44-434.12	<0.001	11.21	4.767-26.35	<0.001
Telomere maintenance gene SNP									
TEP1 rs1713449: GG versus GA/AA	1.483	1.162-1.893	0.002	1.227	1.026-1.466	0.025	1.294	1.121-1.495	<0.001
TEP1 rs872072: CC versus CT/TT	1.314	1.028-1.680	0.029	1.266	1.060-1.512	0.009	1.279	1.108-1.477	0.001
POT1 rs7784168: AA versus AG/GG	0.697	0.538-0.901	0.006	0.794	0.658-0.959	0.016	0.759	0.653-0.883	<0.001
TERT rs13167280: CC versus CT/TT	0.712	0.542-0.935	0.015	0.721	0.584-0.890	0.002	0.707	0.599-0.835	<0.001
TERF1 rs2306494: GG versus GA/AA	1.285	1.009-1.636	0.042	1.196	1.001-1.428	0.048	1.240	1.075-1.431	0.003
High-risk genotype: 0-2 versus 3-5*	1.542	1.197-1.986	0.001	1.281	1.070-1.534	0.007	1.372	1.186-1.587	<0.001

*The high-risk genotypes were GA/AA for *TEP1* rs1713449, CT/TT for *TEP1* rs872072, AA for *POT1* rs7784168, CC for *TERT* rs13167280, and GA/AA for *TERF1* rs2306494.

Abbreviation: CH, chronic hepatitis.

genes. This combined effect, which was measured by the number of putatively high-risk genotypes, was significantly associated with the overall survival of patients with HCC. Overall survival was reduced in the step-down phase with the increasing frequency of the high-risk genotypes. This result suggests that telomere maintenance genes may act together and influence the survival of patients with HCC. Therefore, screening for these SNPs may provide useful information in clinical practice for stratifying patients with HBV-associated HCC into lower and higher risk groups.

The multivariate survival analysis showed that advanced cirrhosis, a diffuse tumor, advanced tumor staging, the presence of major vessel invasion and distant metastasis, an unresected tumor, and postoperative recurrence remained significant predictors of the overall survival of HCC patients. Thus, the results of this study are strengthened by these well-known prognostic factors of HCC, which were also significant. This study, however, was limited by the retrospective nature of the analysis and the potential selection bias of the control subjects. The controls were selected from hospital-based, HBsAg-positive, inactive carriers. In addition, our study had the limitations of multiple comparisons and the possibility of false-positive associations (limitations common to genetic variant studies). To overcome these issues, we used a two-stage study design in the independent patient population, which provided a high likelihood of true-positive findings. Because our study did not include all genes involved in the telomere pathway, possibly significant SNPs of other telomere maintenance genes might have been missed. Another limitation was the variety of tumor characteristics and treatment modalities, which may have influenced the survival of patients with HCC, although we adjusted for these factors in the multivariate analysis.

In conclusion, a polymorphism of *TEP1* rs1713449 was associated with an increased risk of HCC development. In addition, SNPs of *TEP1*, *POT1*, *TERT*, and *TERF1*, both alone and in combination with other SNPs of telomere maintenance genes, were significantly associated with the overall survival of patients with HBV-associated HCC. These results suggest that the polymorphisms of telomere maintenance genes may act as prognostic factors for predicting clinical outcomes of Korean HCC patients with chronic HBV infections.

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