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Relative telomere length: a novel non-invasive biomarker for the risk of non-cirrhotic hepatocellular carcinoma in patients with chronic hepatitis B infection

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Abstract ***Background and aims:*** Telomere length has emerged as a promising risk predictor of various cancers including hepatocellular carcinoma (HCC). However, the majority of studies in this area measured telomere length in hepatocytes and one in lymphocytes with conflicting results. Moreover, no studies have been reported on using circulating DNA telomere length as a non-invasive HCC biomarker.

Methods: We conducted a nested case-control study to determine the relative telomere length (RTL) in serum DNA from 140 hepatitis B virus (HBV)-related HCC cases and 280 frequency-matched cancer-free HBV controls.

Results: Cases had a significantly longer RTL (median, 0.31; range, 0.02–2.31) than controls (median, 0.20; range, 0.01–1.60) ($P = 0.003$). Consistently, longer RTLs conferred a significantly increased HCC risk compared to short RTLs in a univariate logistic regression analysis (odds ratio [OR] = 1.55, 95% confidence interval [CI] = 1.02–2.33, $P = 0.038$). This association attenuated after multivariate adjustment (OR = 1.40, 95% CI = 0.90–2.19, $P = 0.132$). In a quartile analysis, a significant dose-response relationship was noted in univariate analysis ($P_{trend} = 0.017$) which was again attenuated in multivariate analysis ($P_{trend} = 0.079$). Further analyses revealed that the significant association between serum RTL and HCC risk was evident in non-cirrhotic (OR = 3.54, 95% CI 1.58–7.93 $P = 0.002$), but not cirrhotic

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(OR = 0.95, 95% CI 0.55–1.64, $P = 0.860$) HBV patients. Moreover, the significantly increased HCC risk conferred by cirrhosis was modulated by RTL with a significant interaction effect ($P_{interaction} = 0.013$).

Conclusions: RTL in circulating cell-free serum DNA could potentially be used as a novel non-invasive biomarker for non-cirrhotic HCC. Prospective cohort studies are warranted to validate this finding and assess its clinical significance in HCC prevention.

1. Introduction

In the United States, the incidence of new acute hepatitis B virus (HBV) infection decreased over the past two decades, however, the number of patients living with chronic HBV infection has grown significantly. This development is partly due to the trend of increasing immigration from areas with high HBV endemicity such as Asian and Sub-Arab countries.[1] Among the more than 40 million Americans born outside of the United States, about 1.5 million have chronic HBV infection. Moreover, over 60% of current HBV patients in the United States are relatively young and as these patients age, significant increases in HBV-induced hepatocellular carcinoma (HCC) and corresponding medical costs are expected.[1] Thus, continuous monitoring of these patients and identification of high-risk individuals for more targeted and intensive intervention are important to the reduction of HBV-induced burden.

Telomeres consist of small tandem nucleotide repeats (TTAGGG in humans) that form the physical ends of eukaryotic chromosomes.[2] The main function of telomeres is to stabilise the linear chromosome ends and protect them from degradation and other insults resulting from DNA damage response as well as end-to-end fusion.[3,4] Due to the end replication inefficiency of DNA polymerase, telomeres shorten by 50–200 base pairs within each cell division cycle in normal somatic cells.[5] When the shortening of telomeres reaches a critical point, loss of telomere protection will lead to replicative senescence and/or apoptosis in normal cells, which in turn prevents tumour initiation caused by genomic instability.[6,7] In addition to the end replication issue, other factors such as oxidative stress, chronic inflammation, and loss of telomere binding proteins may also contribute to telomere shortening and lead to the tumourigenesis of many solid cancers including HCC.[8–10] Furthermore, several studies have demonstrated a significant correlation between decreased telomere length of tissue DNAs and promotion of hepatocarcinogenesis.[11–14]

Recently, Liu et al. conducted a case-control study in a Chinese population and reported that longer telomeres in peripheral blood leukocytes (PBLs) conferred an increased risk of HCC.[15] This finding was consistent with several epidemiologic reports indicating a positive correlation between longer telomere length in blood cells and a greater susceptibility to breast cancer,[16] melanoma[17] and non-Hodgkin lymphoma,[18] whereas contradictory findings of other studies demonstrated an association between shorter telomeres and an increased risk of various cancers.[19] In HCC, mounting evidence has substantiated the correlation between short telomere length in liver tissues and increased cancer risk.[20] These paradoxical findings regarding telomere length in cancer predisposition suggest that the previously observed associations might be tissue and/or tumour specific and further evaluation is warranted.

Biomarkers based on circulating cell-free serum or plasma samples have unique advantages over other specimens due to their non-invasive nature, especially in prospective studies with extended followed-up periods with only serum or plasma samples collected at study initiation. Circulating serum DNA as a non-invasive predictive, diagnostic and prognostic biomarker has been extensively investigated in many cancers.[21–23] In the current study, we sought to evaluate the association between serum DNA telomere length and the risk of HBV-related HCC (HBV-HCC) using a nested case-control approach in a clinic-based cohort of Korean HBV patient population.

2. Materials and methods

2.1. Study population

The subjects in this study were selected from an existing and ongoing clinic-based cohort. The patients were consecutively enrolled from those who visited the Liver Disease Prevention Center at Thomas Jefferson University Hospital for treatment of liver diseases, such as chronic HBV or HCV infection, fibrosis, cirrhosis or HCC. There were no restrictions on age, sex, ethnicity, and disease aetiology in patient enrolment. Enrolment started in 1988 and is ongoing. As of October 2010, the cohort included more than 2600 patients, of which 90% were of Korean ancestry. More than 90% of the patients in this cohort had HBV infection. To minimise confounding effects from disease aetiology and ethnicity, we restricted the current study to Korean HBV patients. This study has been approved by the Institutional Review Board of Thomas Jefferson University.

2.2. Epidemiologic and clinical data collection

Demographic and clinical data were obtained for each patient through medical chart review and consulting with

the treating physicians. Demographic variables collected in this research included age, sex, ethnicity, smoking status, drinking status, cirrhosis and family history of cancer. An individual who smoked more than 100 cigarettes in lifetime was defined as an ever smoker, otherwise as a never smoker. Never drinkers were defined as those who never consumed alcohol or consumed less than or equal to one drink per month. Ever drinkers were those who consumed more than one drink per month. Liver cirrhosis and HCC were determined by the combined use of clinical diagnosis and imaging studies (ultrasound, computed tomography or magnetic resonance imaging). A blood sample was drawn for each participant for clinical laboratory testing, and the remaining serum sample was stored at –80 °C for research purposes.

2.3. Measurement of relative telomere length

Circulating cell-free serum DNA was extracted from a 200 μL serum sample using QIAamp DNA Blood Mini kit (Qiagen, CA) according to the manufacturer's protocol. The relative telomere length (RTL) of each DNA sample was determined by quantitative real-time polymerase chain reaction (qRT-PCR) using a protocol described by Cawthon,[24] which measured the ratio of the copy number of telomere repeats to the copy number of a human single copy gene, *36B4*, with minor modifications. Briefly, the PCR reaction (10 μL) for the telomere or *36B4* amplification consisted of 1× SYBR Green Master Mix (Applied Biosystems), 200 nmol/L each telomere or *36B4* specific primers, and 2 μL purified serum DNA sample. The thermal cycling conditions for both telomere and *36B4* were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 58 °C for 1 min with signal collection. The primer sequences were as follows: forward telomere primer (Tel-1), 5′-CGGTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′; reverse telomere primer (Tel-2), 5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3′; forward human *36B4* primer, 5′-CAGCAAGTGGGAAGGTGTAATCC-3′; reverse human *36B4* primer, 5′-CCCATTCTATCATCAACGGGTACAA-3′. All samples were assayed in duplicate on a 0.1 mL fast plate using a StepOnePlus real-time PCR system (Applied Biosystems, CA). The same negative controls and calibrator DNAs were included on each plate for quality control and calibration of PCR efficiency. A reference DNA sample was used to construct a standard curve for RTL measurement on each plate. The reference DNA was extracted from 5 ml pooled serum from 25 randomly selected patients. For each standard curve, the reference DNA sample was diluted using a 3-fold increment per dilution to produce a seven-point standard curve, between 40 and 0.05 ng DNA in each reaction. The R^2 for each standard curve was $\geqslant 0.98$, with acceptable standard deviation set at 0.25 (for the C_t values). If the RTL data for a tested sample were found to be out of the acceptable range of the standard curve, the sample was repeated.

2.4. Statistical analysis

All statistical analyses were performed using the SAS software version 9.2 (SAS Institute, Cary, NC). The differences in the distribution of host characteristics between cases and controls were compared using the chi-square test for categorical variables, and the Student's t test for continuous variables. Wilcoxon rank-sum test was used to evaluate the differences of RTL between cases and controls. RTL was analysed as categorical variable based on a cut-off point at the median, tertile or quartile value of the control group. The association between RTL and HCC risk was estimated using unconditional univariate and multivariate logistic regressions to determine the unadjusted and adjusted odds ratios (ORs), respectively, and 95% confidence intervals (95% CIs). The multivariate analysis controlled for age, sex, smoking status, drinking status, cirrhosis and family history of cancer, where appropriate. The test for interaction between demographic factors and RTL on HCC risk was conducted by including a cross-product term into the logistic regression model. All statistical tests were two sided, and $P < 0.05$ was considered as the threshold of statistical significance.

3. Results

3.1. Demographic characteristics of the study subjects

The distributions of patient characteristics are summarised in Table 1. This study included 140 HBV-HCC cases and 280 cancer-free HBV controls that were 1:2 frequency-matched to cases based on age and gender. All participants were restricted to Korean HBV patients to control the confounding effect of ethnicity and HCC aetiology. As shown in Table 1, there were no significant differences between cases and controls on age (mean ± standard deviation [SD], 55.3 ± 8.8 years versus 53.7 ± 9.6 years, respectively, $P = 0.099$) and gender ($P = 0.327$). No significant differences were identified between cases and controls with regard to smoking status ($P = 0.096$) and drinking status ($P = 0.334$). As expectedly, a significantly higher percentage of cirrhotic patients were identified in HCC cases (75.0%) versus controls (41.4%) ($P < 0.001$). Additionally, there was a significantly higher percentage of patients with a family history of cancer among cases (47.9%) than controls (32.9%) ($P = 0.003$).

3.2. The distribution of RTL in cases and controls by host characteristics

As shown in Table 2, the overall serum DNA RTL was significantly longer in HCC cases than in cancer-free HBV controls [median, 0.31 (range, 0.02–2.31) versus median, 0.20 (range, 0.01–1.60), P=0.003]. We

Table 1
Distribution of host characteristics in all cases and controls.

Variable	Hepatocellular carcinoma (HCC) cases ($n = 140$)	Hepatitis B virus (HBV) controls ($n = 280$)	P value
Age: mean ± SD	55.3 ± 8.8	53.7 ± 9.6	0.099
Gender			
Female	22(15.7)	55(19.6)	
Male	118(84.3)	225(80.4)	0.327
Smoking status			
Never	69(49.3)	162(57.9)	
Ever	71(50.7)	118(42.1)	0.096
Drinking status			
Never	67(47.9)	148(52.9)	
Ever	73(52.1)	132(47.1)	0.334
Cirrhosis			
No	35(25.0)	164(58.6)	
Yes	105(75.0)	116(41.4)	**< 0.001**
Family cancer			
No	73(52.1)	188(67.1)	
Yes	67(47.9)	92(32.9)	**0.003**

Significant P values (< 0.05) were in bold fonts.

further conducted a stratified analysis to evaluate the RTL distribution differences between cases and controls by host characteristics. The differences of RTL between cases and controls remained at least borderline significant in patients regardless of age, smoking status and drinking status. In comparison, the difference was only evident in males ($P = 0.001$) but not females ($P = 0.757$), in non-cirrhotic patients ($P = 0.007$) but not cirrhotic patients ($P = 0.423$), and in patients without a family history of cancer ($P = 0.002$) but not in those with a family history of cancer ($P = 0.264$). Moreover, we also compared the RTL differences between the strata of each variable in cases and controls separately, and identified a significant difference between cirrhotic and non-cirrhotic HBV controls. The median (range) of RTL was 0.28 (0.01–1.60) and 0.18 (0.01–1.11) in cirrhotic and non-cirrhotic HBV controls, respectively ($P = 0.004$) (Table 2).

3.3. RTL and HCC risk

We estimated the association between serum DNA RTL and the risk of HCC by univariate and multivariate logistic regression analyses, through treating RTL as a categorical variable based on a cut off value of median, tertile or quartile distribution in cancer-free HBV controls. As shown in Table 3, individuals with longer RTL by median cut-off had a significantly increased risk of HCC in univariate analysis (unadjusted OR = 1.55, 95% CI, 1.02–2.33, $P = 0.038$), but not in multivariate analysis after adjusting all host variables including age, gender, smoking status, drinking status, cirrhosis and family history of cancer (adjusted OR = 1.40, 95% CI 0.90–2.19, $P = 0.132$). When patients were further categorised according to the tertile or quartile distribution of RTL values in controls, we found that, using the first (shortest RTL) group as reference, there was a significant dose-response relationship between longer RTL and increased HCC risk in the univariate analysis ($P_{trend} = 0.013$ by tertile; $P_{trend} = 0.017$ by quartile), which was attenuated in the multivariate analysis ($P_{trend} = 0.067$ by tertile; $P_{trend} = 0.079$ by quartile). The OR for the second or third group in the tertile analysis was 1.15 (95% CI 0.67–1.96, $P = 0.615$) and 1.85 (95% CI 1.12–3.06, $P = 0.017$), respectively in the univariate analysis, and 1.18 (95% CI 0.67–2.08, $P = 0.577$) and 1.64 (95% CI 0.96–2.82, $P = 0.072$), respectively in the multivariate analysis. Similar results were obtained in the quartile analysis (Table 3). Because we suspected that the attenuated association between RTL and HCC risk was due to a strong confounding from cirrhosis, we conducted another multivariate analysis adjusting for all other host variables but cirrhosis. The result of this analysis was similar to that of the univariate analysis: individuals with longer RTL by median cut-off had a significantly increased HCC risk (OR = 1.59; 95% CI 1.04–2.42, $P = 0.031$) and a significant dose-response effect was observed between longer RTL and increased HCC risk in the tertile and quartile analysis ($P_{trend} = 0.007$ and 0.012, respectively). The results of similar multivariate analysis without adjusting for other host characteristics including age, gender, smoking status, drinking status and family history of cancer yielded similar results to those of the multivariate analysis adjusting for all the variables (data not shown).

3.4. The association of RTL with HCC risk stratified by host characteristics

To further address the apparent attenuation of the association between RTL and HCC risk in the multivariate analysis, we conducted a stratified analysis to evaluate the potential modulating effect of each host variables. Consistent with the results of Tables 2 and 3, we found a significant association between long RTL and increased HCC risk in non-cirrhotic HBV patients (adjusted OR = 3.54, 95% CI 1.58–7.93, $P = 0.002$), but not in cirrhotic HBV patients (adjusted OR = 0.95, 95% CI 0.55–1.64, $P = 0.860$) (Table 4). A significant association was also observed in never drinkers (adjusted OR = 1.93, 95% CI 1.04–3.60, $P = 0.038$). In addition, borderline significant associations were observed in males ($P = 0.076$), and patients without a family history of cancer ($P = 0.078$) (Table 4).

3.5. Association between cirrhosis and HCC risk modulated by serum DNA RTL

Because longer RTL conferred a significantly increased risk of HCC in non-cirrhotic but not in cir-

rhotic patients, we sought to evaluate whether the effect of cirrhosis on HCC risk was also modulated by RTL. As shown in Table 5, as expected, cirrhosis was significantly associated with an increased HCC risk (adjusted OR = 4.11, 95% CI 2.60–6.51, $P = 1.48 \times 10^{-9}$). This effect was much more prominent in patients with short RTL (adjusted OR = 8.04, 95% CI 3.82–16.90, $P = 3.89 \times 10^{-8}$), compared to those with long RTL (adjusted OR = 2.35, 95% CI 1.27–4.33, $P = 0.006$). Moreover, we observed a statistically significant interaction effect between cirrhosis and RTL on the prediction of the risk of HBV-HCC (P for interaction = 0.013).

4. Discussion

In the current study, we evaluated the association between RTL in circulating cell-free serum DNA and the risk of HBV-related HCC in a clinic-based patient population. We found that longer RTL was associated with an increased risk of HBV-HCC, an effect that was much more evident in patients without cirrhosis than those with cirrhosis. Further analysis revealed a significant interaction effect between RTL and cirrhosis in the modulation of HCC risk in HBV patients.

The use of telomere length as a potential predictor for cancer risk and prognosis has been extensively investigated during the past decade. The vast majority of these studies have been focused on evaluating telomere length in tumour tissues, whereas an increasing number of studies were conducted recently assessing this relationship through measuring telomere length in PBLs using qRT-PCR.[24,25] In HCC, many studies that measured telomere length in tumour and surrounding normal tissue yielded mixed results. The majority of these studies reported that telomere shortening was involved in hepatocarcinogenesis, while others reported that longer telomeres contribute to increased HCC risk.[11–13,20,26–28] In addition to these discrepant findings, these studies focused mainly on hepatic tissues, which is more reflective of the primary tumour characteristics than the genetic background of the patients. Recently, Liu et al. measured RTL in PBLs in a Chinese population, and found that longer RTL was significantly associated with an increased risk of HBV-HCC, comparing to both healthy controls and cancer-free controls with chronic liver diseases such as cirrhosis or fibrosis.[15] As a surrogate specimen for individual's genetic background, PBL has been widely used in epidemiological studies. However, the use of PBL as a non-invasive biomarker has been limited, although they carry considerable clinical value, especially in those prospective cohort studies with several decades of clinical follow-up that collected serum or plasma samples at the time of study initiation. To the best of our knowledge, there has not been a population-based study determining the association of serum DNA telomere length with cancer risk or clinical outcome. In the current study, we measured the RTL in circulating cell-free serum DNA in a Korean American HBV-infected population, and observed a significant correlation between long RTL and increased risk of HBV-HCC. This finding is consistent with the results of the study of Liu et al.[15] The overall median value of RTL in our study was lower than that of *Liu* et al., which is possibly accounted for by the difference between circulating cell-free DNA and PBL DNA. Circulating cell-free DNA is a heterogeneous mixture including DNA molecules released from various sources with broad variations in content and concentrations. It consists of DNAs from a wide spectrum of cells such as necrotic and apoptotic cells, active blood cells, and circulating tumour cells in cancer patients,[22] In comparison, the DNA from PBLs is highly homogeneous and to a larger extent reflects the constitutional genetic background of the subjects.

Cirrhosis is a major risk factor for HCC and between 60% and 90% of patients with primary HCC have underlying cirrhosis.[29–31] In our study, the increased HCC risk conferred by long serum DNA RTL was only evident in non-cirrhotic patients. This could be due to the possibility that the strong effect of cirrhosis on HCC might have overshadowed the association conferred by long telomere length in our study population. Alternatively, there may be different molecular mechanisms underlying the development of HCC with and without cirrhosis. For instance, HBV viral genome can directly integrate into the host human genome and act as an oncogenic factor, a process that is independent of the chronic inflammation that commonly characterises cirrhosis.[32] In line with this notion, recent *in vitro* and *in vivo* studies suggested that the HBx protein, encoded by the HBV viral genome, increases both the expression of telomere reverse transcriptase and telomerase activity, the enzyme responsible for the maintenance of telomere length, thus prolonging the lifespan of hepatocytes and contributing to malignant transformation.[33,34] Partly due to these properties of HBV genome, liver cirrhosis in patients with chronic HBV infection is only present in about 60–70% of HBV-HCC cases and not a prerequisite step for tumourigenesis.[35] These lines of evidence are consistent with our data linking telomere length and the risk of non-cirrhotic HBV-HCC. However, the molecular mechanism underlying these observations still needs further investigation.

Mixed findings have been identified from the numerous studies that have extensively investigated the relationship between altered telomere length and cancer risk. Wentzensen et al. conducted a meta-analysis and found that long telomere length was associated with the increased risk of breast cancer, lung cancer, colorectal cancer and non-Hodgkin's lymphoma, but reduced risk of bladder cancer, oesophageal cancer, gastric cancer, head and neck cancer, ovarian cancer and renal cell car-

Table 2
RTL distribution by host characteristics in cases and controls.

Variable	Relative telomere length (RTL): median (range)		*P* value
	Hepatocellular carcinoma (HCC) cases ($n = 140$)	Hepatitis B virus (HBV) controls ($n = 280$)	
Overall	0.31 (0.02–2.31)	0.20 (0.01–1.60)	**0.003**
Age, years			
⩽54.1	0.32 (0.02–2.22)	0.22 (0.02–1.15)	0.071
>54.1	0.30 (0.02–2.31)	0.18 (0.01–1.60)	**0.016**
P value	0.998	0.414	
Gender			
Female	0.28 (0.02–2.22)	0.26 (0.01–1.60)	0.757
Male	0.32 (0.02–2.31)	0.19 (0.01–1.15)	**0.001**
P value	0.866	0.102	
Smoking status			
Never	0.29 (0.02–2.30)	0.20 (0.01–1.60)	**0.045**
Ever	0.32 (0.02–2.31)	0.21 (0.02–1.15)	**0.021**
P value	0.997	0.674	
Drinking status			
Never	0.28 (0.02–2.21)	0.20 (0.01–1.60)	0.077
Ever	0.34 (0.02–2.31)	0.22 (0.02–1.15)	**0.011**
P value	0.611	0.912	
Cirrhosis			
No	0.29 (0.02–2.21)	0.18 (0.01–1.11)	**0.007**
Yes	0.33 (0.02–2.31)	0.28 (0.01–1.60)	0.423
P value	0.904	**0.004**	
Family cancer			
No	0.34 (0.02–2.30)	0.20 (0.01–1.60)	**0.002**
Yes	0.27 (0.02–2.31)	0.20 (0.01–1.35)	0.264
P value	0.120	0.947	

Significant *P* values (< 0.05) were in bold fonts.

cinoma.[19] In HCC, our finding was consistent with the study of Liu et al., who conducted a case-control analysis in a Chinese population and reported that HCC patients had significantly longer RTL measured using DNA samples of peripheral blood leukocytes, compared to both liver disease-free normal controls and controls with chronic liver diseases such as chronic HBV infection and liver cirrhosis.[15] However, the findings are contradictory to several previous studies reporting that cancerous liver tissues had a shorter telomere length compared to paired normal tissues or non-cancerous liver tissues.[14,27,36,37] Several potential reasons may account for these discrepancies. Ours and Liu's studies were conducted in Asians whereas almost all other studies used Caucasian subjects. It has been demonstrated there were significant racial differences in the distribution of telomere length.[38–40] Whether these differences have an impact on the role of telomere length in the progression of HBV-HCC remains a task for further evaluation. Moreover, our study used circulating cell-free DNA from serum samples and Liu's study used PBLs, whereas the majority of other studies used hepatocytes.[14,27,36,37] The RTL measurement in hepatocytes from liver tissue was more likely a reflection of the characteristics of telomere homoeostasis, rather than a simple constitutive non-invasive biomarker of risk prediction measured from leukocytes or serum.[15] Consistent with this notion and the results of our study, Wiemann et al. reported that in cirrhotic patients, telomere shortening was mainly restricted to hepatocytes of cirrhotic liver tissues whereas lymphocytes and stellate cells in areas of cirrhosis had significantly longer telomere length.[14] Furthermore, telomere dysfunction has been reported to have dual roles in liver carcinogenesis. Short telomere length appears to have a tumour-initiating effect through inducing chromosomal instability.[27,41] In comparison, the elongation of telomeres by the activation of telomerase has also been reported to contribute to tumour growth and progression.[20] Various independent studies have demonstrated a significantly elevated activity of telomerase at the stage of severe liver diseases such as cirrhosis and HCC,[42–44] which is consistent with the findings of our and Liu's studies that telomere length increases along with liver disease progression. Collectively, these lines of evidence indicate the requirement of a balanced state of telomere length in the normal physiological liver functions, since either extremely short or long telomeres lead to liver pathogenesis. Additional biomarker and basic studies are needed to further explain these paradoxical findings and reveal the underlying mechanisms.

The modulating effects of telomere length by environmental factors such as cigarette smoking and alcohol use have been reported in studies of HBV-HCC,[15] and some other solid tumours.[45,46] In our study, we only observed a borderline significant association between an increased HCC risk with long serum RTL among never drinkers but not among ever drinkers (Table 4). In addition, no significant interaction effect was identified between RTL and smoking or drinking status (data not shown). These results may likely be explained by the adequate matching between cases and controls on smoking and drinking status. Moreover, due to the limitation of a retrospective clinic-based study, we did not have detailed data of the intensities of smoking and drinking which could also contribute to the paradoxical findings. Further larger prospectively designed cohort studies are warranted to test the cumulative and interaction effects between RTL and these environmental exposures on the risk of HCC.

A major strength of this study is the unique and highly homogenous HBV patient population. Our study is restricted to Korean American HBV patients only, eliminating the confounding effects of ethnicity and disease aetiology. The vast majority of patients were infected with HBV at birth or childhood, making this population an ideal resource to study the long-term outcome of HBV infection at the population level. Strict matching criteria were implemented between cases and controls to minimise the potential confounding effects from other major risk factors. To the best of our knowledge, this is the first study to demonstrate that RTL in circulating cell-free serum DNA could potentially be

Table 3
Hepatocellular carcinoma (HCC) risk as estimated by telomere length on hepatitis B virus (HBV) controls.

Relative telomere length (RTL)	HCC cases	HBV controls	Unadjusted		Multivariate-adjusted[a]		Multivariate-adjusted[b]	
			OR (95% confidence interval)	*P* Value	OR (95%CI)	*P* Value	OR (95%CI)	*P* Value
By median								
Short	55	140	1.00		1.00		1.00	
Long	85	140	**1.55 (1.02–2.33)**	**0.038**	1.40 (0.90–2.19)	0.132	**1.59 (1.04–2.42)**	**0.031**
By tertile								
First tertile	35	93	1.00		1.00		1.00	
Second tertile	41	95	1.15 (0.67–1.96)	0.615	1.18 (0.67–2.08)	0.577	1.16 (0.68–2.01)	0.583
Third tertile	64	92	**1.85 (1.12–3.06)**	**0.017**	1.64 (0.96–2.82)	0.072	**1.99 (1.18–3.32)**	**0.009**
P trend				**0.013**		0.067		**0.007**
By quartile								
First quartile	26	70	1.00		1.00		1.00	
Second quartile	29	70	1.12 (0.60–2.08)	0.732	1.26 (0.65–2.46)	0.492	1.15 (0.61–2.17)	0.665
Third quartile	35	70	1.35 (0.73–2.47)	0.336	1.43 (0.75–2.72)	0.283	1.37 (0.74–2.54)	0.316
Fourth quartile	50	70	**1.92 (1.08–3.43)**	**0.027**	1.72 (0.93–3.19)	0.085	**2.06 (1.14–3.72)**	**0.017**
P trend				**0.017**		0.079		**0.012**

Significant *P* values (< 0.05) were in bold fonts.
[a] Adjusted for age, gender, smoking status, drinking status, cirrhosis and family history of cancer.
[b] Adjusted for age, gender, smoking status, drinking status, and family history of cancer.

Table 4
Hepatocellular carcinoma (HCC) risk estimated by relative telomere length (RTL) on hepatitis B virus (HBV) controls stratified by host characteristics.

Variable	RTL (by median in controls)	HCC cases	HBV controls	Odd ratios (OR) (95%CI)[a]	*P* value
Age, years					
⩽53.1	Short	27	77	1.00	
	Long	35	72	1.21 (0.63–2.32)	0.574
>53.1	Short	31	75	1.00	
	Long	47	56	1.64 (0.88–3.04)	0.120
Gender					
Female	Short	10	27	1.00	
	Long	12	28	0.96 (0.30–3.07)	0.939
Male	Short	48	125	1.00	
	Long	70	100	1.55 (0.96–2.52)	0.076
Smoking status					
Never	Short	29	88	1.00	
	Long	40	74	1.49 (0.81–2.76)	0.200
Ever	Short	29	64	1.00	
	Long	42	54	1.40 (0.73–2.70)	0.312
Drinking status					
Never	Short	28	84	1.00	
	Long	39	64	**1.93 (1.04–3.60)**	**0.038**
Ever	Short	30	68	1.00	
	Long	43	64	1.06 (0.55–2.06)	0.857
Cirrhosis					
No	Short	12	101	1.00	
	Long	23	63	**3.54 (1.58–7.93)**	**0.002**
Yes	Short	46	51	1.00	
	Long	59	65	0.95 (0.55–1.64)	0.860
Family cancer					
No	Short	28	101	1.00	
	Long	45	87	1.71 (0.94–3.09)	0.078
Yes	Short	30	51	1.00	
	Long	37	41	1.21 (0.61–2.38)	0.587

Significant *P* values (< 0.05) were in bold fonts.
[a] Adjusted for age, gender, smoking status, drinking status, cirrhosis and family history of cancer, where appropriate.

used as a simple, inexpensive, and non-invasive biomarker of HCC risk and the findings are highly consistent with that obtained using PBLs.[15] This proof-of-concept finding makes serum a valuable resource for RTL-based biomarker research, especially in prospective longitudinal studies that have been followed-up for extended time periods but only collected serum samples at the time of study initiation.

Table 5
Association of cirrhosis with the risk of hepatitis B virus-related hepatocellular carcinoma (HBV-HCC) modulated by relative telomere length (RTL).

Cirrhosis and RTL	HCC cases	HBV controls	Odd ratio (OR) (95% confidence interval) [a]	*P* value
In all patients				
Non-cirrhosis	35	164	1.00	
Cirrhosis	105	116	**4.11 (2.60–6.51)**	**1.48×10^{-9}**
In patients with short RTL				
Non-cirrhosis	12	101	1.00	
Cirrhosis	46	51	**8.04 (3.82–16.90)**	**3.89×10^{-8}**
In patients with long RTL				
Non-cirrhosis	23	63	1.00	
Cirrhosis	59	65	**2.35 (1.27–4.33)**	**0.006**
P for interaction				**0.013**

Significant *P* values (< 0.05) were in bold fonts.
[a] Adjusted for age, gender, smoking status, drinking status, and family history of cancer.

There are also limitations in this study. First, because of the heterogeneous nature of circulating serum DNA, the mechanism underlying the associations observed in this study remains a task for further evaluation. The finding of our study is in line with that of Liu et al. whereas contradictory to several other studies showing a positive correlation between short RTL measured in hepatocytes and an increased HCC risk. Although there have been studies indirectly showing that this discrepancy might be due to the differences between blood cells and hepatocytes, further in-depth characterisations at the molecular level will provide more definitive evidence on this issue. In addition, it is worth noting that although the average RTL was measured in this study, the determination of the longer and shorter telomere lengths would be more clinically relevant and meaningful in the identification of individuals with an increased risk of HCC. Future independent studies with more homogeneous DNA sources are needed to conduct these evaluations. Second, this study was restricted to a highly homogeneous group of Korean Americans. Although this eliminated the confounding effect of patient ethnicity, the generalisability of our findings in other ethnic groups remains to be assessed. Moreover, variations of common serum biomarkers such as alpha-fetoprotein in different ethnic groups have been indicated in previous studies.[47–49] Although such ethnic difference in serum RTL has not been reported, the possibility cannot be ruled out. Therefore, validations of our findings in HBV patient populations of different ethnicities are warranted. Third, due to the nature of retrospective case-control design, our study also has the reverse-causation limitation that is inherent in most case-control studies, and may not differentiate the causal relationship between RTL alteration and HCC development. Prospective and longitudinal studies are needed to address this issue.

In conclusion, our study reported for the first time that longer RTL in circulating cell-free serum DNA was significantly associated with an increased risk of non-cirrhotic HBV-HCC, a finding that warrants further retrospective and prospective validations, in-depth molecular characterisations and assessments to determine the clinical value in the risk prediction and early detection of HCC.

Conflict of interest statement

None declared.

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