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Association Between the Telomerase rs2736098_TT Genotype and a Lower Risk of Chronic Hepatitis B and Cirrhosis in Chinese Males

Guanghui Cheng, MSc[1,5], Xiaotian Yuan, BSc[1,2,5], Fang Wang, MD[3], Qing Sun, BSc[1], Qian Xin, PhD[1], Kailin Li, MSc[1], Chao Sun, MSc[1], Zhaomin Lin, PhD[1], Yun Luan, PhD[1], Yiteng Xu, MD[1], Ping Li, PhD[4], Feng Kong, MD[1] and Dawei Xu, MD, PhD[1,2]

OBJECTIVES: Chronic hepatitis B (CHB) is caused by infection of hepatitis B virus (HBV) and liver cirrhosis (LC) is its most common complication. The accumulated evidence indicates a genetic context of HBV infection phenotypes. Here we determine a potential association of CHB/LC with the genetic variant of telomerase reverse transcriptase (TERT), a key player in aging including immune-senescence.

METHODS: The study included 227 Chinese CHB patients and 315 sex/age-matched healthy controls. TERT rs2736098 and rs2736100 genotyping was performed using pre-designed TaqMan SNP genotyping assay kits. Leukocyte telomere length (LTL) was determined using quantitative PCR.

RESULTS: The rs2736098_CT/CC genotypes were significantly associated with risk of CHB compared to the TT one (OR 2.265, 95% CI 1.202–4.269, $P = 0.015$). A similar association was also found in CHB patients with cirrhosis (CT/CC vs TT: OR 2.398, 95% CI 1.168–4.922, $P = 0.02$). Further analyses showed that the rs2736098_TT genotype difference occurred between male controls and patients ($P = 0.008$) and male CT/CC-carriers exhibited highly increased risk of CHB compared to male controls (CT+CC vs TT, OR 3.182, 95% CI 1.350–7.500, $P = 0.01$). There was no difference in the rs2736100 variants between controls and CHB patients. LTL was not different between cases and controls.

CONCLUSIONS: The *TERT* rs2736098_TT genotype is associated with a lower CHB and LC risk in Chinese males, which may have implications in CHB pathogenesis and prevention.

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INTRODUCTION

Close to 400 million individuals are chronically infected with hepatitis B virus (HBV) worldwide and approximately one million of them die from HBV-related liver diseases including chronic hepatitis B (CHB), liver cirrhosis (LC) and hepatocellular carcinoma (HCC) annually.[1] The accumulated evidence has indicated the host-genetic background of susceptibility to HBV infection and CHB. Indeed, early epidemiological studies strongly suggested a genetic context of infection phenotypes, while recent GWAS and genetic analyses further identified a panel of genetic variants associated with HBV infection and CHB susceptibility. Because the host immune system responsive for both innate immune and adaptive immunity plays a key role in controlling HBV infection and outcomes,[2,3] its association with genetic variants of immuno-modulatory factors has been extensively explored. So far, variations in genes encoding IL-6, IL-1, IL-10, PPARγ, HLAs, TNFα, IFNγ, TIM3, CTLA4 and others have been found to significantly influence HBV infection and CHB predisposition.[4–19]

Telomerase is a RNA-dependent DNA polymerase that elongates telomeric DNA.[20] Most normal human somatic cells lack telomerase activity due to the tight transcriptional repression of its rate-limiting, catalytic component *telomerase reverse transcriptase (TERT)* gene.[20–22] This, together with the end-replication problem, leads to progressive telomere shortening with each round of cell division or with increased age, and when they become too short (dysfunctional) to protect chromosomes, the DNA damage response is activated, thereby triggering the permanent growth arrest of cells (replicative senescence).[20,22] By regulating telomere length and many other biological activities, telomerase or TERT plays critical parts in human health.[20] Importantly, it has been shown that abnormal telomere length is associated with chronic liver diseases, and telomerase provides a protection against liver fibrosis.[23–26] In addition, shorter telomere in immune cells is associated with a lower activity against viral infection.[27] These findings suggest that TERT or telomerase may be involved in HBV infection-related CHB and LC.

There are multiple single-nucleotide polymorphisms in the *TERT* gene, among which rs2736100 and rs2736098 are most

[1]Central Research Laboratory, The Second Hospital of Shandong University, Jinan, PR China; [2]Department of Medicine, Division of Hematology and Center for Molecular Medicine, Karolinska University Institutet and Karolinska University Hospital, Stockholm, Sweden; [3]Clinical Laboratory, The Second Hospital of Shandong University, Jinan, PR China and [4]School of Nursing, Shandong University, Jinan, PR China

Correspondence: Feng Kong, MD, The Second Hospital of Shandong University, Jinan 250033, PR China or D Xu, MD, PhD, Center for Molecular Medicine, Karolinska University Institutet, Stockholm 17176, Sweden.

E-mail: kongfeng@sdu.edu.cn or Dawei.Xu@ki.se

[5]These authors contributed equally to this work.

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studied. These variants have been shown to be associated with cancer, atherosclerosis, depression and other health problems.[28–40] Given the findings documented above, we sought to ask whether the rs2736100 and rs2736098 genotypes modify susceptibility to CHB and its LC complication. Our results reveal a significant association between the *TERT* rs2736098_TT genotype and reduced risk of CHB and LC in Chinese males.

METHODS

Study populations. The case-control individuals include 542 unrelated Chinese adults consisting of 227 CHB patients and 315 healthy controls. All 227 patients were active CHB and recruited from Infectious Diseases Inpatient, the Shandong University Second Hospital, between January 2014 and October 2015. Three hundred and fifteen healthy adults who were age- and sex-matched to cases were recruited as controls, from Physical Examination Center of the Shandong University Second Hospital. The study was approved by the Ethics Review Committee of Shandong University Second Hospital and written informed consent was obtained from the participants. All experiments were performed in accordance with relevant guidelines and regulations.

DNA extraction and genotyping of the TERT rs2736100 and rs2736098 variants. Genomic DNA was extracted from peripheral blood cells using QIAGEN DNA extraction kits. The *TERT* rs2736100 (AC) and rs2736098 (T/C) genotyping was carried out using pre-designed TaqMan SNP genotyping assay kits on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA), as described.[30,39] Both positive and negative controls were included in all assays and the running condition was as followed: 95 °C for 10 min, followed by 40 cycles of 92 °C for 15 s and 60 °C for 1 min.

Leukocyte telomere length assay. Genomic DNA was isolated from peripheral blood cells as above and leukocyte telomere length (LTL) was assessed using real-time PCR as previously described.[41,42] Briefly, 2 ng of DNA were used for each PCR reaction. The primer sequences for human telomere (Tel 1b and Tel 2b) and β-globin (HBG3 and HBG4) were: Tel1b: 5′-CGGTTTGTTTGGGTTTGGGT-TTGGGTTTGGGTTTGGGTT-3′; Tel2b: 5′-GGCTTGCCTTACCCTTACCCTTACCC-TTACCCTTACCCT-3′; HBG3: 5′-TGTGCTGGCCCATCACTTTG-3′, and HBG4: 5′-ACCAGCCA-CCACTTTCTGATAGG-3′. T/HBG values were determined using the formula $T/S = 2^{-\Delta Ct}$, where ΔCt = average $Ct_{telomere}$ − average $Ct_{\beta\text{-globin}}$. The *T*/*S* ratio was arbitrarily expressed as LTL.

Statistical analyses. Required sample size for association studies was calculated using QUANTO (Version 1.2, University of Southern California) and a power >0.80 is acceptable. The evaluation of distribution differences of selected variables and alleles of the *TERT* rs2736100 and rs2736098 between CHB patients and healthy controls were done using χ^2 test. Hardy–Weinberg equilibrium of the genotype distribution among the controls was tested by a goodness-of-fit χ^2 test. Unconditional univariate and multivariate logistic regression analyses were used to estimate ORs for risk of CHB or LC and their 95% CIs. The LTL difference between patients and healthy controls was assessed using the Mann–Whitney *U*-tests. All the tests were computed using SigmaStat3.1 software (Systat Software, Inc., Richmond, CA, USA). For comparison of rs2736098 and rs2736100 genotypes between healthy controls and CHB patients, *P* values were adjusted by Bonferroni correction and a statistical significance should be <0.025. *P* values of <0.05 were considered as statistically significant for all other comparisons.

Table 1 TERT rs2736098 and 2736100 genotyping in healthy adults and CHB patients

	HA	CHB	Odds ratio (95% CI)	*P* value[a]
N	315	227		
Sex				
M	210	153		
F	105	74		
M/F Ratio	2.0	2.0		
Age (years)	44 ± 15	43 ± 13		
Genotypes				
rs2736098 (*N*)	304 (100%)	219 (100%)		
TT	41 (13.4)	14 (6.4)	1.0 (ref.)	
CT	146 (47.7)	111 (50.7)	2.227 (1.157–4.286)	0.022
CC	119 (38.9)	94 (42.9)	2.313 (1.191–4.495)	0.018
CT+CC	265	205	2.265 (1.202–4.269)	0.015
rs2736100 (*N*)	306 (100%)	227 (100%)		
AA	115 (37.3)	94 (41.4)	1.0 (ref.)	
CA	139 (45.5)	101 (44.5)	0.889 (0.612 to 1.292)	0.602
CC	52 (17.2)	32 (14.1)	0.753 (0.449–1.264)	0.344
CA+CC	191	133	0.852 (0.600–1.210)	0.421

CHB, chronic hepatitis B; CI, confidence interval; HA, Healthy adults; ref., reference.
[a]$P<0.025$ as statistically significant according to Bonferroni correction.

Table 2 Sex difference in the association of rs2736098 with CHB

	HA	CHB	Odds ratio (95% CI)	*P* value
	Male			
Genotypes (N)	204 (100%)	147 (100%)		
TT	28 (13.7)	7 (4.8)	1.0 (ref.)	
CT	75 (36.8)	75 (51.0)	4.000 (1.646–9.720)	0.002
CC	101 (49.5)	65 (44.2)	2.574 (1.063–6.237)	0.051
CT+CC	176	140	3.182 (1.350–7.500)	0.010
	Female			
Genotypes (N)	102 (100%)	72 (100%)		
TT	13 (12.7)	7 (9.7)	1.0 (ref.)	
CT	48 (47.1)	36 (50.0)	1.122 (0.401–3.137)	0.968
CC	41 (40.2)	29 (40.3)	1.631 (0.587–4.532)	0.490
CT+CC	89	65	1.356 (0.513–3.588)	0.708

CHB, chronic hepatitis B; CI, confidence interval; HA, healthy adults; ref., reference.

Table 3 The association of rs2736098 with cirrhosis in CHB

	HA	CHB with cirrhosis	Odds ratio (95% CI)	*P* value
Genotypes (N)	304 (100%)	165 (100%)		
TT	41 (13.4)	10 (6.1)	1.0 (ref.)	
CT	146 (47.7)	88 (53.3)	2.471 (1.179–5.180)	0.020
CC	119 (38.9)	67 (40.6)	2.308 (1.087–4.908)	0.041
CT+CC	265	155	2.398 (1.168–4.922)	0.020

CHB, chronic hepatitis B; CI, confidence interval; HA, healthy adults; ref., reference.

RESULTS

Demographic and/or clinical characteristics of study subjects. A total of 227 patients with CHB were genotyped for TERT rs2736100 and rs2736098 variants. A total of 165 CHB patients had LC complication. Active CHB diagnosis was made according to abnormal levels of alanine aminotransferase and/or aspartate transaminase, higher amounts of HBV DNA quantification and positive HBSeAg. The presence of LC complication was diagnosed based on liver Ultrasound B and Computer Tomography examination. None of the patients developed HCC. Patient age and sex are summarized in Table 1. In total, 315 healthy adults used as controls were age- and sex-matched (Table 1), and all of them were negative for HBV surface antigen and had normal levels of alanine aminotransferase and aspartate transaminase. The power for the number of cases and controls in this study, as made using QUANTO, was 0.968 (>0.80 acceptable).

The TERT rs2736100 variant and CHB risk. The TERT rs2736100 genotyping was successfully performed on DNA from 306 of 315 healthy adults and all 227 CHB patients, and the frequency was summarized in Table 1. The genotype distributions were 37.3, 45.5, and 17.2% for AA, AC, and CC, respectively, in healthy adults, while 41.4, 44.5, and 14.1% for AA, AC, and CC, respectively, in CHB patients (Table 1). There was no significant difference in the rs2736100 genotype frequency between healthy controls and CHB patients, which indicates the lack of rs2736100 association with CHB susceptibility (Table 1).

The TERT rs2736098 variant and CHB risk. The TERT rs2736098 genotyping was performed on DNA from those same individuals, and the analysis was successfully in 304 of 315 healthy adults and 219 of 227 CHB patients. As shown in Table 1, 41 of 304 controls had TT (13.4%), 146 CT (47.7%), and 119 CC genotype (38.9%), while there were 14 TT (6.4%), 111 CT (50.7%), and 94 CC genotype (42.9%) among 219 cases. The TT genotype was presented at a significantly higher frequency in the healthy controls compared to that in the CHB patients ($P=0.015$). The CT and CC genotypes exhibited significantly increased risk of CHB (TT vs CT: OR, 2.227, 95% CI, 1.157–4.286, $P=0.022$; TT vs CC: OR, 2.313, 95% CI, 1.195–4.495, $P=0.018$). These results remained same even when P value <0.025 was considered as statistically significant after Bonferroni correction.

We further compared the rs2736098 allele distribution in male and female individuals separately. The highly significant lower TT genotype was observed in male cases (4.8%) compared to that in male controls (13.7%; Table 2, TT vs CT: OR, 4.0, 95% CI, 1.646–9.720, $P=0.002$; TT vs CC: OR, 2.574, 95% CI, 1.063–6.237, $P=0.018$). In contrast, a slight difference in the TT genotype was seen between female controls (12.7%) and patients (9.7%), which was not significant (controls vs cases in female, $P>0.05$ in all the comparisons; Table 2).

The TERT rs2736098 variant and LC risk in CHB patients. Telomerase is known to protect liver from non-HBV-related cirrhosis,[25] and we are thus interested in the relationship of rs2736098 variants with CHB-mediated LC. Among 165 CHB patients with the LC complication, 155 of them were available for the rs2736098 genotyping. Compared with healthy controls, patients with LC exhibited a significantly lower

frequency of the TT type (Tables 3, 13.4 vs 6.1%). Both CT and CC genotypes were significantly associated with a higher risk of LC development in CHB patients (Table 3, TT vs CT: OR, 2.471, 95% CI, 1.179–5.180, $P=0.02$; TT vs CC: OR, 2.309, 95% CI, 1.087–4.908, $P=0.041$).

Leukocyte telomere length in CHB patients. Finally, we determined telomere length in leukocytes from healthy adults and CHB patients, and then compared its difference between two groups. LTL was 1.01 ± 0.47 and 0.94 ± 0.60 for HA and CHB, respectively (Figure 1a), and there was no significant difference ($P=0.182$). Because the rs2376098 variants were previously shown to be correlated with LTL,[32,43] we further made a comparison among individuals with different rs2736098 alleles. In healthy controls, TT-, CT-, and CC-carriers had LTL 0.85 ± 0.36, 0.89 ± 0.36, and 1.00 ± 0.44, respectively (age-corrected values; Figure 1b). LTL in CHB patients was: TT: 0.83 ± 0.40, CT: 0.89 ± 0.49, and CC: 1.03 ± 0.689 (Figure 1). There was a trend of LTL increase from TT-, CT- to CC-carriers in both healthy controls and CHB patients, however, none of these differences were significant (Figure 1b). LTL between controls and patients bearing the same genotype did not differ significantly either (Figure 1b).

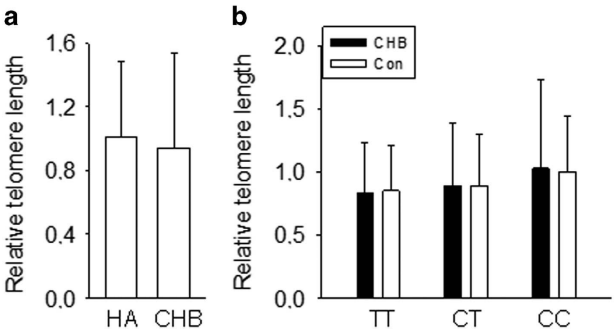

Figure 1 Leukocyte telomere length (LTL) in healthy adults (HA) and patients with chronic hepatitis B (CHB) and relation with the rs2736098 genotyping. Genomic DNA from HA and CHB peripheral leukocytes analyzed for telomere length using qPCR, and relative LTL was calculated as described in Methods. (**a**) Comparison of LTL between HA and CHB patients. (**b**) LTL in HA and CHB patients bearing different rs2736098 genotypes. The LTL values shown were age-corrected.

DISCUSSION

The variants of the *TERT* gene have been observed to be associated with a significantly higher susceptibility to cancer and aging-related disorders,[29,33] however, their relationship with chronic virus infection is unclear. In the present study, we investigated the influence of the TERT genetic variants on risk of CHB and LC. Our results reveal significantly higher frequencies of the rs2736098_TT in healthy controls than in CHB patients, which suggest that the *TERT* rs2736098_TT genotype exerts a protective effect on susceptibility to CHB and LC. To our knowledge, this is the first report demonstrating an association between the rs2736098 variant and CHB or its complications.

During HBV infection, complete HBV clearance occurs in more than 90% of infected adults, while 5–10% of them has viral persistence, thereby leading to CHB, and then further development of cirrhosis or even HCC.[1] Cell-mediated immune response is believed to contribute to variable clinical outcomes of HBV infection. Telomerase or TERT and telomeres have long been recognized to play pivotal parts in regulating immunological activity.[44,45] Activation of telomerase via induction of TERT expression occurs in activated lymphocytes for their expansion in response to infectious challenge.[44,45] Shorter LTL was associated with increased susceptibility to experimentally induced acute upper respiratory infection and clinical illness in adults.[27] Mechanistically, shorter telomeres limit proliferation potentials of immune cells and compromise immune response to pathogens, thereby lowering host resistance to infection.[44,45] It is thus likely that TERT variants modify risk of CHB by influencing host immune function.

However, the LTL comparison between healthy controls and CHB patients did not reveal a significant difference, which indicates that telomere homeostasis is not significantly impaired in CHB patients. In addition, the rs2736098 TT and CC genotype-carriers were previously shown to harbor shortest and longest LTL, respectively, while the CT variant was correlated with medium levels of LTL.[32] Consistently, Atzmon *et al.* found a significant association between rs2736098 and LTL.[43] However, we did not find this scenario in both healthy controls and CHB patients, indicating that the association between rs2736098_TT allele and a lower CHB risk is unlikely attributable to telomere length regulation. However, the sample size in the present study is relatively small, which might be a potential reason behind the dissociation between the rs2736098 genotype and phenotype (LTL). On the other hand, telomere lengthening is the canonical function of telomerase or TERT, but recent studies have demonstrated its multiple properties independently of telomere homeostasis.[46–51] Ectopic expression of TERT was shown to promote mobilization of stem cells and proliferation of many types of cells via upregulation of stem cell factors, growth factors or their receptor expression;[52–54] telomerase and TERT is capable of protecting cells from apoptosis mediated by various insults.[49,50,55,56] Mechanistically, TERT can act as co-factors to stimulate gene transcription.[48] It is thus likely that the rs2736098_TT allele boosts host immune response for HBV clearance via a telomere lengthening-independent manner. However, the exact underlying mechanism calls for further investigations.

Telomere and telomerase or TERT have been shown to be involved in the pathogenesis of chronic liver diseases including LC.[23–25] Telomerase-deficient mice at late generations exhibit shortened telomeres and diminished capacity for liver regeneration, and undergo accelerated development of cirrhosis after liver injury, whereas re-expression of telomerase activity is shown to improve liver function and protect mice from development of hepatic steatosis and fibrosis.[25] Similar pathological alterations including accelerated telomere attrition and regenerative exhaustion of liver cells were observed in patients with loss-of-function of telomerase genes.[23,24] On the other hand, telomere over-erosion also occurs in CHB-related cirrhosis due to a higher turn-over of hepatocytes to compensate for liver damage. Conceivably, HBV infection and shorter telomeres cooperate to accelerate the development of LC. However, it is currently unclear whether this mechanism is involved in the rs2736098_CT/CC-related risk of LC.

Intriguingly, we only observed the effect of rs2736098 variants on risk of male CHB patients. It is well-known that estrogen protects females from HBV infection and CHB by

directly inhibiting HBV replication.[57] On the other hand, however, estrogen is also a strong activator of TERT transcription and telomerase.[58,59] There may thus be a possibility that a potent controlling of TERT expression by estrogen masks the effect of the rs2736098 genotypes in female CHB patients. Further studies are required to elucidate this issue.

In summary, the finding presented here demonstrates that the TERT rs2736098 variants significantly contribute to the susceptibility of CHB and its cirrhosis complication in Chinese males, providing new insights into telomerase biology and etiology of CHB and LC. These results also raise a number of intriguing questions: How those different TERT genotypes modify the risk of CHB and LC, and why their influence only occurs in males. In addition, because CHB is a key factor that drives development of HCC, while telomerase and TERT are essential in oncogenesis, it is important to further dissect the relationship between rs22736098 variants and CHB or HBV-related HCC. Elucidation of all these issues will certainly contribute to the management and prevention of CHB and its complications.

CONFLICT OF INTEREST

Guarantor of the article: Feng Kong, MD.
Specific author contributions: Planning the study: Guanghui Cheng, Xiaotian Yuan, Feng Kong, Dawei Xu. Conducting the study: Guanghui Cheng, Xiaotian Yuan, Fang Wang, Qing Sun, Qian Xin, Kailin Li, Chao Sun, Zhaomin Lin, Yun Luan, Yiteng Xu, Ping Li. Collecting and/or interpreting data: Guanghui Cheng, Xiaotian Yuan, Fang Wang. Drafting the manuscript: Guanghui Cheng, Xiaotian Yuan, Yiteng Xu, Feng Kong, Dawei Xu. All the authors have approved the final draft submitted.
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Potential competing interests: None.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ The accumulated evidence has indicated the host-genetic background of susceptibility to chronic hepatitis B (CHB).
- ✓ Telomerase is involved in liver inflammation and cirrhosis.

WHAT IS NEW HERE

- ✓ The telomerase (TERT) rs2736098_TT genotype is associated with a lower risk of CHB in Chinese males.
- ✓ The TERT rs2736098_TT genotype may reduce the onset of cirrhosis resulting from CHB.

TRANSLATIONAL IMPACT

- ✓ The TERT rs2736098 genotyping may identify individuals with increased susceptibility to CHB, thereby allowing precision prevention of CHB.

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