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ORIGINAL RESEARCH

TERT-CLPTM1 locus polymorphism (rs401681) is associated with the prognosis of hepatocellular carcinoma

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Hye Won Lee[1,*]
Won-Jin Park[2,*]
Yu-Ran Heo[2]
Tae In Park[3]
Soo Young Park[4]
Jae-Ho Lee[2,*]

[1]Department of Pathology, Keimyung University School of Medicine, Daegu, Republic of Korea; [2]Department of Anatomy, Keimyung University School of Medicine, Daegu, Republic of Korea; [3]Department of Pathology, Kyungpook National University School of Medicine, Daegu, Republic of Korea; [4]Department of Internal Medicine, Kyungpook National University School of Medicine, Daegu, Republic of Korea

*These authors contributed equally to this work

Abstract: Telomere length is associated with the development of hepatocellular carcinoma (HCC), and recent studies have focused on the genetic alteration or polymorphism in telomere-maintaining genes. We examined the clinicopathologic and prognostic value of rs401681 polymorphism, located in the *TERT-CLPTM1L* locus, in HCC. The relationship between rs401681 variants and telomere length was also analyzed in 156 HCC patients. The rs401681 polymorphism had the following genotype frequencies: C/C in 51.3% of the samples, C/T in 39.7%, and T/T in 9.0%. Telomeres in the tumor samples were 4.04-fold longer, on average, than the telomeres in matched normal samples (SD =1.32), and there were no differences in telomere length according to rs401681 polymorphism (p=0.802). Our results indicate that the rs401681 C allele was significantly associated with increased T and International Union for Cancer Control stages (p<0.01). Univariate and multivariate survival analyses showed that HCC with C allele had poorer prognosis (p<0.01). In conclusion, our findings suggest that rs401681 is a possible prognostic biomarker for HCC patients.

Keywords: CLPTM1L polymorphism, hepatocellular carcinoma, *TERT-CLPTM1L* locus, telomere length

Introduction

Telomeres are repetitive (TTAGGG)n sequences into arrays of up to 25 kb and cap the end of linear chromosomes in human cells. They play a key role in counteracting the end-replication losses that occur as a consequence of semiconservative replication of linear DNA molecules. Telomere length (TL) is maintained mainly by telomerase, a ribonucleoprotein that forms part of a protein complex that allows the addition of repetitive sequences to the 30 chain during DNA replication as a protective action against chromosome erosion. Particularly, TL showed a significant association with the prognosis of patients in various malignancies, although the prognosis according to length varies according to the type of tumor.[1,2]

A large number of genes and gene products are involved in this process. Specifically, the telomerase contains two key genes: telomerase reverse transcriptase (*TERT*) gene and cleft lip and palate transmembrane 1 like gene (CLPTM1L; alias CRR9; MIM612585). The *TERT* gene encodes for the telomerase enzyme, and the CLPTM1L gene product seems to participate in *TERT* regulation through some regions in the 30 end, called the TERT-CLPTM1L locus.[3–5] According to the large single-nucleotide polymorphism (SNP) database of TERT-CLPTM1L locus, rs401681 (C.T, located in the intron 13 of CLPTM1L and 27 kb from the *TERT* gene) is one of

Correspondence: Jae-Ho Lee
Department of Anatomy, Keimyung University School of Medicine, 2800, Dalgubeoldaero, Dalseo-Gu, Daegu, 42601, Republic of Korea
Tel +82 53 580 3833
Fax +82 53 580 3835
Email anato82@dsmc.or.kr

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the most extensively studied SNPs. However, effects of this SNP varied extremely across cancer types. In lung cancer and melanomas, T allele (minor allele) was associated with cancer risk, whereas C allele (major allele) increased the risk of lung, bladder, and prostate cancers.[6]

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer in adults and is a leading cause of death from cancer worldwide. Moreover, the incidence and mortality rates of HCC still continue to increase.[7] Up to now, there have been many studies on diagnostic genes, prognostic factors, and genes for target therapy. There was a previous SNP study of TERT-CLPTM1 locus in HCC demonstrating that the T allele was associated with a significantly increased risk of HCC.[8]

In this study, we investigated whether rs401681 of TERT-CLPTM1 locus is closely associated with survival outcome in HCC, together with the clinicopathologic characteristics. The associations between this polymorphism and *TERT* promoter mutation and TL were also investigated based on our previous report.[9]

Patients and methods

Patients and tissue samples

We evaluated 156 HCC patients who underwent surgical resection as first-line treatment at the Kyungpook National University Hospital from January 2006 to December 2010. HCC was diagnosed and treated according to the American Association for the Study of Liver Diseases guidelines. The clinicopathologic parameters of the patients were re-evaluated by a review of the patients' medical records, including age, sex, tumor size, laboratory results, and etiology of underlying liver disease. The age of the patients was based on the time of curative surgical resection. We have included this in the text. The TNM stage was evaluated according to the seventh edition of the American Joint Committee on Cancer staging system. Patients dying of causes other than HCC, patients with cancer at other sites, and patients lost to follow-up were excluded from this study. We also excluded patients who had received prior treatments such as local ablation therapy and transarterial chemoembolization. The study was approved by the institutional review board (KNUH-2014-04-056-001). All patients provided written informed consent at the time of their treatment for the use of material and medical records in future research.

SNP genotyping

Peripheral venous blood samples were collected, and DNA was extracted by using a QIAamp DNA Mini Kit (Qiagen NV, Venlo, the Netherlands). The polymerase chain reaction (PCR) amplification of the TERT-CLPTM1 locus for SNP study was performed as described previously, with minor modification.[4] PCR was performed using AmpliTaq Gold (Thermo Fisher Scientific, Waltham, MA, USA). The PCR products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide to confirm the size of the bands. Then, direct DNA sequencing was performed using the ABI 3730 DNA sequencer (Bionics Inc., Korea).

TERT promoter mutation and TL analysis

Surgically removed HCC specimens and corresponding nonmalignant liver tissues were formalin fixed and paraffin embedded. Paraffin blocks containing representative tumor lesions were selected after review of the corresponding hematoxylin and eosin slides. Representative lesions from each case were marked on the paraffin blocks and cored with a 3.0 mm diameter cylindrical device manually. Genomic DNA was extracted by using a QIAamp DNA Mini Kit (Qiagen). PCR amplification of the *TERT* promoter region was performed as described previously. Then, the PCR products were directly sequenced in both forward and reverse directions. All mutations were verified by analysis of an independent PCR isolate.

TL was examined using a real-time PCR assay. For the quantitative determination of content relative to DNA, primers for specific amplification of telomere and β-globin gene were selected according to a previous study.[9] Real-time PCR was then carried out on the LightCycler 480 II system (Hoffman-La Roche Ltd., Basel, Switzerland) with a total volume of 20 μL of the reaction mixture. Each measurement was repeated in triplicate, and five serially diluted control samples were included in each experiment. The relative TL was calculated with the $2^{-\Delta\Delta CT}$ method. First, TL was normalized with β-globin gene; the TL of the tumor sample was compared with the mean level of TL in normal liver tissues and expressed as an n-fold ratio.

Statistical analysis

Chi-square, Fisher's exact test, Mann–Whitney *U* test, Kruskal–Wallis, and simple correlation analysis were used to analyze the relationship between variables. Survival curves, estimated with the Kaplan–Meier method (univariate analysis), were compared by log-rank test. Overall survival (OS) was defined as the time between diagnosis and death from either disease or from other causes. Disease-free survival (DFS) was defined as the time between diagnosis and disease recurrence or development of distant metastasis.

We used Cox proportional hazard modeling to calculate the hazard ratios (HRs) and 95% CIs, using time since diagnosis as the underlying time metric. For the multivariate models, we adjusted for age at diagnosis, sex, tumor size, aspartate transaminase, and alanine transaminase (ALT). All p-values <0.05 were considered statistically significant.

Results

Demographics of patients according to rs401681 genotype

The study population was composed of 117 males and 39 females with a median age of 57 years (range, 37–78 years). The genotype frequencies of rs401681 polymorphisms were as follows: C/C, 51.3%; C/T, 39.7%; T/T, 9.0%. The frequency of the rs401681 C allele was 0.71 (T allele 0.29), consistent with previously described values for Korean controls.[10] The clinicopathologic characteristics of rs401681 polymorphism in HCC are presented in Table 1. The rs401681 C allele was statistically associated with T stage (p=0.003) and International Union for Cancer Control stage (p=0.008). *TERT* promoter mutation was found in 26.9% of the patients (42/156). TL was analyzed in 156 patients with HCC. Average of TL in HCC was 4.04-fold longer than that in matched normal tissues (SD =1.32). The mean TLs of HCC patients with C/C, T/T, and C/T alleles were 3.45-fold (SD =1.01), 2.60-fold (SD =0.83), and 5.11-fold (SD =1.56), respectively, which showed no statistical difference (p=0.802; Figure 1). The rs401681 polymorphisms did not have any relationship with other clinical parameters, TL, and *TERT* promoter mutation.

Prognostic value of rs401681 polymorphisms in HCC

We then conducted survival analysis to clarify the prognostic significance of rs401681 polymorphisms in HCC. The median follow-up of patients for survival analysis was 70.6 months (range, 3–101). Univariate survival analysis performed by Kaplan–Meier curve showed a shorter DFS in HCC patients with C/C allele (40.05 versus 65.7 [T/T] and 60.0 [C/T] months, χ^2=8.33, p=0.016), as shown in Figure 2. However, OS was not different according to this polymorphism in HCC (67.8 [C/C] versus 50.6 [T/T] and 74.8 [C/T] months, χ^2=0.68, p=0.710), as shown in Figure 2. Then, we compared the C/C genotype with T allele carriers (C/T + T/T). C allele (40.05 months) was significantly associated with poor DFS (versus 58.0 [T/T and C/T] months, χ^2=7.47, p=0.006), though OS result was

Table 1 Clinicopathologic characteristics of rs401681 polymorphism in hepatocellular carcinoma

	rs401681 (TERT-CLPTM1L locus)			*p*-value
	CC	CT	TT	
Total	**80 (51.3)**	**62 (39.7)**	**14 (9.0)**	
Age	57.6±11.9	56.8±10.0	56.0±10.5	0.626
Sex				0.564
Male	58 (49.6)	47 (40.2)	12 (10.3)	
Female	22 (56.4)	15 (38.5)	2 (5.1)	
Infection				0.165
Alcohol	8 (38.1)	12 (57.1)	1 (4.8)	
HBV	31 (56.4)	16 (29.1)	8 (14.5)	
HCV	1 (25.0)	3 (75.0)	0 (0)	
Others	40 (52.6)	31 (40.8)	5 (6.6)	
AST				0.655
<40 U/L	51 (52.6)	36 (37.1)	10 (10.3)	
≥40 U/L	29 (50.0)	25 (43.1)	4 (6.9)	
ALT				0.637
<40 U/L	58 (52.7)	41 (37.3)	11 (10.0)	
≥40 U/L	22 (48.9)	20 (44.4)	3 (6.7)	
Tumor size				0.927
<5 cm	47 (50.5)	37 (39.8)	9 (9.7)	
≥5 cm	33 (52.4)	25 (39.7)	5 (7.9)	
AJCC T stage				0.003
T1	4 (30.8)	4 (30.8)	5 (38.5)	
T2	55 (49.5)	50 (45.0)	6 (5.4)	
T3	20 (66.7)	7 (23.3)	3 (10.0)	
T4	1 (50.0)	1 (50.0)	0 (0)	
UICC				0.008
I	5 (35.7)	4 (28.6)	5 (35.7)	
II	54 (49.5)	49 (45.0)	6 (5.5)	
III	20 (62.5)	9 (28.1)	3 (9.4)	
IV	1 (100)	0 (0)	0 (0)	
TERT mutation				0.486
(+)	23 (54.8)	14 (40.5)	5 (11.9)	
(–)	54 (48.6)	48 (43.2)	9 (8.1)	

Notes: Values are presented as number (%) unless otherwise indicated. *p*-values of χ^2 tests are indicated.

Abbreviations: AJCC, American Joint Committee on Cancer; ALT, alanine transaminase; AST, aspartate transaminase; HBV, hepatitis B virus; HCV, hepatitis C virus; TERT, telomerase reverse transcriptase; UICC, Union for International Cancer Control.

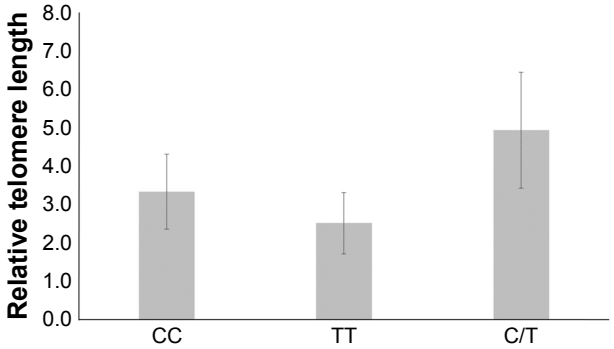

Figure 1 Telomere length difference according to rs401681 polymorphisms. Telomere length was not different according to rs401681 polymorphisms (p=0.802).

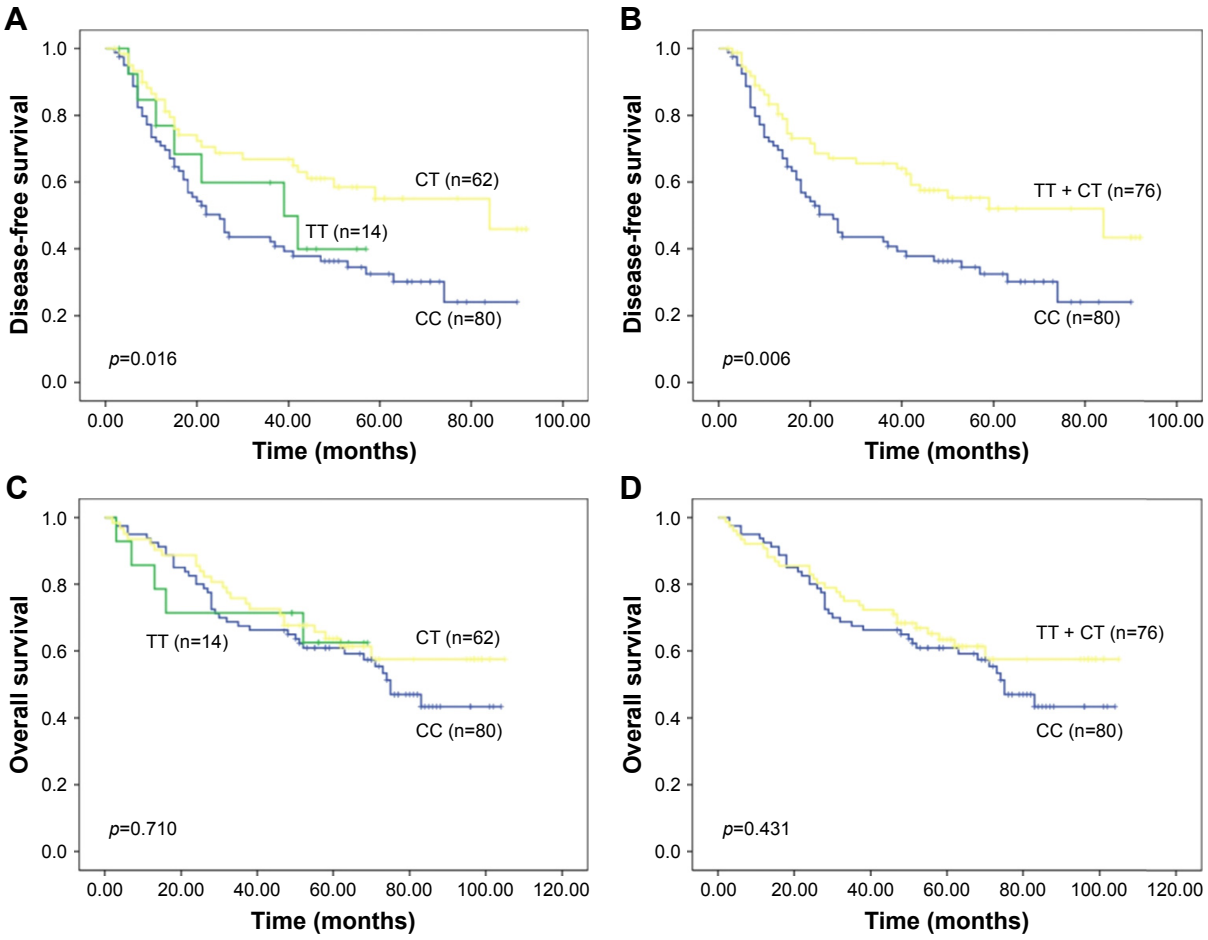

Figure 2 Kaplan–Meier cures for disease-free survival (**A**, **B**) and overall survival (**C**, **D**) of HCC patients according to rs401681 polymorphisms.
Abbreviation: HCC, hepatocellular carcinoma.

not different (67.8 [C/C] versus 74.1 [T/T and C/T] months, χ^2=0.62, p=0.431), as shown in Figure 2.

To evaluate whether rs401681 polymorphism is an independent prognostic predictor in HCC, we further analyzed the data by using the Cox proportional hazards regression model after adjusting for possible confounders of survival (Table 2). Multivariate analysis showed that rs401681 polymorphism is a potential marker for independent prognostic factors (HR for DFS =1.88, 95% CI: 1.03–3.44, p=0.040; HR for OS =1.07, 95% CI: 0.63–1.83, p=0.800).

Table 2 Multivariate analysis of the prognostic values of various factors in HCC

Variable	DFS			OS		
	p-value	Hazard ratio	95% CI	*p*-value	Hazard ratio	95% CI
rs401681	0.040	1.88	1.03–3.44	0.80	1.07	0.63–1.83
Age	0.824	1.00	0.98–1.03	0.570	1.01	0.98–1.04
Sex	0.032	1.93	1.06–3.53	0.007	3.02	1.36–6.68
Tumor size	0.036	1.09	1.00–1.19	0.001	1.18	1.08–1.29
AST	0.699	1.00	0.99–1.02	0.602	1.00	0.99–1.02
ALT	0.812	1.00	0.68–1.08	0.563	1.01	0.99–1.02

Notes: Hazard ratio (95% CI) of multivariate analysis. p-values of χ^2 tests are indicated; $p<0.05$, statistically significant.
Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; DFS, disease-free survival; HCC, hepatocellular carcinoma; OS, overall survival.

Discussion

Telomeres are involved in maintaining genomic stability. Telomere biology plays a critical role in the initiation and progression of cancer in various aspects.[3] There are various genes and proteins associated with the synthesis of telomerase (*TERT*, *TERC*, *CLPTM1L*, and so on) and telomere binding protein (such as TRF1, TRF2, POT1, TIN2, RAP1, and so on).[3] The TL is controlled by the interaction of these various telomere-related genes.[11] Among these, two novel telomere-associated genes, *TERT* and CLPTM1L, have been widely studied in cancer research.[6,8] *TERT* gene encodes the catalytic subunit of telomerase reverse transcriptase, and this promoter mutation and dysregulated expression were proved in diverse human malignancies.[3] Our previous study has demonstrated that *TERT* promoter mutation is frequent in HCC, and TL has some association with age, American Joint Committee on Cancer T stage, OS, and infection status. Also, TL might have a potential value as a

prognostic factor in HCC. According to many reports, while shortened tumor TL shows poorer prognosis in most solid tumors, HCC has been observed with favorable prognosis as in colorectal cancer.[1,9]

CLPTM1L gene may be involved in apoptotic response and its expression level affects the sensitivity of cancer cell lines to anticancer drugs.[4] Various genome-wide association studies have identified SNPs in the CLPTM1L-*TERT* gene whose locus is at chromosome 5p15.33, and rs401681 is one of the significant cancer-associated SNPs.[6,8,10] It has been reported to increase the risk of various malignancies, including lung cancer, which is the most widely studied tumor type, basal cell carcinoma of skin, prostate cancer, and urinary bladder cancer.[6,12] But other studies showed various genetic effects according to cancer type. The individuals with rs401681 polymorphism are rather at lower risk of melanoma and colorectal cancer.[6] Also, there was no association between rs401681 and breast and endometrial cancer risk.[5]

However, to date, very little is known about the genetic polymorphisms of TERT-CLPTM1L on the HCC. Only one study has been conducted on the TERT-CLPTM1L HCC susceptibility variant in Chinese patients. Su et al found that rs401681 T allele of CLPTM1L was associated with a significantly increased risk of HCC (odds ratio, 1.399; 95% CI: 1.002–1.955). Also, this study showed the interaction of rs401681 polymorphism and other polymorphisms in HCC.[4] This study focused on the association between rs401681 genotype and clinicopathologic parameters including survival analysis in HCC patients. To our knowledge, this is the first study describing evidence on the association between SNP rs401681 and clinical behavior of HCC.

The distribution of allele frequency in HCC was similar to that reported in a previous study conducted in Korean population.[10] Another study has shown that there is a link between rs401681 C allele and telomere shortening,[6] but several reports have not demonstrated an association between the risk allele and mean TL in a variety of malignancies.[13,14] A recent study also suggested that the combination of the rs2853669 and *TERT* mutation indicates poor prognosis in HCC.[15] However, we found no evidence that SNP rs401681 is associated with mean TL or *TERT* mutation. In our previous study, we found that ALT level and TL were significantly related.[9] But in this study, aspartate transaminase and ALT were not associated with *TERT*-CLPTM1 locus polymorphism.

Interestingly, we did find significant associations between SNP rs401681 and TNM stage and survival, that is, worse prognosis in patients with the C allele. Our findings revealed that rs401681 C/C or C/T genotype was significantly related to an increased risk of disease relapse in HCC patients, compared with those carrying the T/T genotype. Furthermore, Cox multivariate analysis demonstrated that rs401681 polymorphism was an independent prognostic factor in HCC. In other cancers, the possibility of using it as a genetic marker has been suggested,[9,16,17] and this is the first study demonstrating that in HCC. Further larger scale studies are needed to confirm the role of this possible genetic marker, and then, its molecular mechanism should be clarified.

To summarize, HCC patients with SNP rs401681 allele C had a tendency to develop advanced disease and poor DFS. Our data suggest that the genetic variant in *TERT*-CLPTM1L is implicated in HCC aggressiveness. This study had some limitations. TL may also be affected by lifestyle and environmental factors, such as alcohol and obesity, as well as socioeconomic factors and psychologic stress.[18,19] Therefore, a carefully designed study with subdivided patients is needed. The biologic functions of rs401681 polymorphism are of great interest for HCC research.

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Author contributions

Conceptualization: Lee HW, Lee JH; data curation: Park WJ, Heo YR, Lee HW; formal analysis: Lee HW, Lee JH; funding acquisition: Lee JH; investigation: Park WJ, Park TI, Park SY, Heo YR; methodology: Park WJ, Park TI, Park SY, Heo YR; project administration: Lee HW, Lee JH; resources: Lee HW, Park WJ, Park TI, Park SY, Heo YR; supervision: Lee JH; validation: Lee HW, Lee JH; visualization: Park WJ, Heo YR, Lee JH; writing original draft: Lee HW, Lee JH; writing review and editing: Lee HW, Park WJ, Park TI, Park SY, Heo YR, Lee JH. All authors contributed toward data analysis, drafting and critically revising the paper, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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