TERT promoter mutations in B viral human hepatocarcinogenesis : Suppression of PROX1-induced TERT transcription by stable HBx expression

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TERT promoter mutations in B viral human hepatocarcinogenesis : Suppression of PROX1-induced TERT transcription by stable HBx expression

Directed by Professor Young Nyun Park

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This certifies that the Doctoral Dissertation of Young-Joo Kim is approved.

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마지막으로, 박사학위 공개발표 전 할아버지 만나러 하늘나라로 가신 할머니께 보고 싶다는 말씀과 함께 감사 드립니다. 이제는 할머니, 할아버지가 된 부모님, 장모님께도 머리 숙여 감사 드립니다. 나의 분신들, 은희, 민준이, 그리고 뱃속의 아이야. 고맙고 사랑한다.

어떤 말로 많은 분들께 감사의 마음을 전해야 하는지는 논문을 쓰는 것보다 어렵고 조심스럽게 느껴집니다. 제 표현의 열 배, 스무 배 이상의 마음을 모든 분들께 전해 드리며 앞으로 더욱 정진하겠습니다. 감사합니다.

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ABSTRACT

TERT promoter mutations in B viral human hepatocarcinogenesis : Suppression of PROX1-induced TERT transcription by stable HBx expression

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(Directed by Professor Young Nyun Park)

Telomerase reverse transcriptase (*TERT*) promoter somatic mutations, related to telomerase activation, have been known to frequently occur at two hot spots located at -124 and -146 bp upstream of ATG in various cancers. In the present study, we investigated the occurrence and implications of the genetic alterations of the *TERT* promoter in B viral hepatocarcinogenesis. *TERT* promoter mutations, especially -124C>T mutation, obviously enhanced *TERT* promoter activity in hepatocellular

carcinoma (HCC) cell lines. We identified prospero homeobox protein 1 (PROX1) as a novel transcriptional activator for *TERT* gene through binding to the promoter regions containing two hot spots. PROX1 binding affinity was strong to the mutant *TERT* promoter harboring a consensus E-twenty six/ ternary complex factor (ETS/TCF) binding sequence (CCGGAA). The incidence of *TERT* promoter mutations gradually increased according to the progression of human B viral multistep hepaticarcinogenesis, which was found in 9.0% of low grade dysplastic nodules (LGDNs), 13.5% of high grade dysplastic nodules (HGDNs), 27.3% of early HCCs (eHCCs) and 28.4% of progressed HCCs (pHCCs). The occurrence of *TERT* promoter mutations correlated with lower levels of alpha-fetoprotein (AFP) (p=0.046) and a poor overall survival (p=0.012) in B viral HCC patients. On the contrary to *in vitro* data, *TERT* mRNA expression was lower in B viral HCCs with the mutant *TERT* promoter compared to those without. In addition, mRNA level of *PROX1* was not correlated with that of *TERT* in B viral HCCs, in contrast that such correlation was evident in non-B viral HCCs. Interestingly, induction of stable *HBx* expression inhibited PROX1-mediated *TERT* expression *in vitro* study. In conclusion, our findings suggest that *TERT* promoter somatic mutations are early events in B viral human multistep hepatocarcinogeneis and HBx can induce a loss of PROX1 function as transcriptional activator for *TERT* expression.

Key words: *TERT* promoter mutation, B viral hepatocarcinogenesis, *PROX1*, transcription factor, *HBx*

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I. INTRODUCTION

Telomerase activation is an important event implicated in cellular immortality and carcinogenesis, by which cancer cells are able to maintain short and stable telomere through addition of telomeric repeats (TTAGGG) to the telomeres.[1-3] Telomerase reverse transcriptase (*TERT*), a catalytic subunit of telomerase, is closely related to telomerase activation.[4-6] *TERT* expression is regulated by several transcription factors such as c-Myc, Sp1, AP-1 and MAZ as well as epigenetic

changes such as DNA methylation of the *TERT* promoter.[7-11] Recently, it has been reported that somatic mutations (cytosine-to-thymine transition) in the *TERT* core promoter occurred frequently at two hot spots located at -124 and -146 bp upstream of ATG start site and triggered an increase of *TERT* transcription in various cancers including hepatocellular carcinoma (HCC).[12-15] Interestingly, both of the mutations create a de novo binding motif (CCGGAA) for E-twenty six /ternary complex factor (ETS/TCF) transcription factors, which upregulate *TERT* mRNA expression in tissues expressing ETS/TCF.[16,17] A common single nucleotide polymorphism (SNP) rs2853669 located at -245 bp upstream of ATG start site, is also reported to contribute to dysregulation of *TERT* promoter activity in bladder cancer and glioblastoma where variant C allele of the rs2853669 (TC heterozygotes and CC homozygotes) interfered with ETS2 binding.[14,18] Thus, the genetic alterations in the *TERT* promoter are considered to be tightly associated with *TERT* transcription, however, their precise regulatory mechanism remains obscure.

HCC is the seventh most common malignancy worldwide and the third greatest cause of cancer related mortality. Hepatitis B virus (HBV) is a main etiology in Asia and sub-Saharan Africa, whereas HCV and alcohol intake are major etiological factors in Japan and Western countries, respectively. Chronic HBV infection is one of the high risk factors for HCC development. HBV can promote hepatocarcinogenesis through HBV X protein (HBx) expression and HBV integration into the host genome which interfere cell proliferation, function of endogenous genes and chromosomal integrity.[19] Recently, *TERT* promoter mutations were reported to be found in 54% of HCCs by whole-genome sequencing

survey,[20] and those were early genetic events in C viral and alcoholic hepatocarcinogenesis.[21] *TERT* is the most frequent gene integrated by HBV in HCC.[22] HBV genome integration is well described to cause high expression of *TERT*,[22] however, that is mutually exclusive with *TERT* promoter mutation.[20] Several previous studies showed HBx also modulated telomerase activity although effects of its expression on telomerase have been controversial.[12,23,24] We previously reported that telomerase activation occurred in dysplastic nodules (DNs) of precancerous lesions, and *TERT* mRNA levels increased with the progression of B viral hepatocarcinogenesis.[25] Therefore, telomerase activation via *TERT* upregulation is crucial for B viral hepatocarcinogenesis, however, the detailed mechanism of *TERT* expression by promoter somatic mutations and/or *HBx* expression remains to be determined.

In the present study, we aimed to study regulatory mechanism of *TERT* expression by its promoter somatic mutations in B viral hepatocarcinogenesis. We found that *TERT* promoter somatic mutations were early events in B viral human multistep hepatocarcinogeneis. Prospero homeobox protein 1 (PROX1) was found to be a novel transcriptional activator for *TERT* gene through dominantly binding to the mutant *TERT* promoter, whose function was suppressed by stable *HBx* expression.

II. MATERIALS AND METHODS

1. Cell culture

The human hepatocellular carcinoma cell lines, HepG2, Hep3B and PLC/PRF/5 purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and SNU423 purchased from the Korean Cell Line Bank (Seoul, Korea), were routinely grown in DMEM (Gibco, Carlsbad, MD, USA) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

2. Cloning

For reporter constructs, the *TERT* promoter region (from the position -424 to +65 bp from ATG start site) was amplified using genomic DNA extracted from normal liver tissue with rs2853669 TT and CC genotype, respectively. Each PCR was performed using the specific primers containing 5'-extension and SacI and XhoI restriction sites (Table 1) and Q5® High-Fidelity DNA Polymerase (NEB, Ipswich, MA, USA). Subsequently, the amplicons digested with SacI (NEB) and XhoI (NEB) were cloned into the pGL3-enhancer vector (Promega, Madison, WI, USA), respectively.

Table 1. Primer sequences and conditions used for cloning, mutagenesis, ChIP, RT-PCR, Sanger sequencing

Primer	Sequence (5'→3')	Annealing
TERT -424 *Sac*I F.	GATC GAGCTC GGCCGATTCGACCTCTCT	66°C
TERT +65 *Xho*I R.	GATC CTCGAG AGCACCTCGCGGTAGTGG	
TERT -124C<T F.	CGGCCCAGCCCC T TCCGGGCCCTCC	55°C
TERT -124C<T R.	GGAGGGCCCGGA A GGGGCTGGGCCG	
TERT -146C<T F.	GTCCCGACCCCT T CCGGGTCCCCGG	55°C
TERT -146C<T R.	CCGGGGACCCGG A AGGGGTCGGGAC	
TERT amplicon 1 F.	CTGCCCCTTCACCTTCCAG	58°C
TERT amplicon 1 R.	AGCGCTGCCTGAAACTCG	
TERT amplicon 2 F.	CTCCCAGTGGATTCGCGG	60°C
TERT amplicon 2 R.	CTGCCTGAAACTCGCGCC	
TERT amplicon 3 F.	ATTCGCCATTGTTCACCCCT	60°C
TERT amplicon 3 R.	CTGTGTACAGGGCACACCTT	
HBx F.	ATGGCTGCTAGGGTGTGCTG	62°C
HBx R.	TTAGGCAGAGGTGAAAAAGTTGCAT	
GAPDH F.	CGGAGTCAACGGATTTGGTCGTAT	62°C
GAPDH R.	AGCCTTCTCCATGGTGGTGAAGAC	
TERT -424 F.	GGCCGATTCGACCTCTCT	64°C
TERT +65 R.	AGCACCTCGCGGTAGTGG	

Underlined bases correspond to the restriction sites.

3. Site-directed mutagenesis

Single-nucleotide substitutions from C to T at the positions -124 and -146 in the cloned *TERT* promoter region were generated using complementary primers converting the sites (Table 1) and the QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's recommendations. A total of six *TERT* reporter constructs, wild-type (WT), WT with CC genotype of rs2853669, -124C>T mutant, -124C>T mutant with CC genotype of rs2853669, -146C>T mutant and -146C>T mutant with CC genotype of rs2853669, were generated. All of the reporter constructs were analyzed by Sanger sequencing (Macrogen, Seoul, Korea) to confirm the respective sequences of the mutations and a SNP.

4. Dual-luciferase assay

One hundred nanograms of each reporter construct and 0.25 ng of pNL1.1.TK vector (Promega) per well were cotransfected in 96-well white plates (SPL Life Science, Pocheon, Korea) using 0.5 μl of FuGENE® HD Transfection Reagent (Promega). To determine the effect of *PROX1* knockdown on *TERT* promoter activity, 100 ng of each reporter construct, 0.25 ng pNL1.1.TK vector and the indicated concentration of Silencer® Select Pre-designed siRNAs (n331082 and n331083, Thermo Fisher Scientific, San Jose, CA, USA) per well were cotransfected in 96-well white plates using 0.4 μl of DharmaFECT™ Duo Transfection Reagent (GE Healthcare, Little Chalfont, UK). To determine the effect of *PROX1* overexpression on *TERT* promoter activity, 50 ng of each reporter

construct, 0.125 ng pNL1.1.TK vector and 50 ng pCMV6-PROX1 vector (RC201140, OriGene, Beijing, China) per well were cotransfected in 96-well white plates using 0.5 μl of FuGENE® HD Transfection Reagent. Forty-eight hours post-transfection, luciferase activity was measured by Nano-Glo® Dual-Luciferase® Reporter Assay System (Promega) according to manufacturer's recommendations. Relative firefly luciferase activity was normalized to NanoLuc™ luciferase expression to adjust for variation in the transfection efficiency.

5. Protein extraction

For preparation of total protein, cells were harvested and resuspended in 1× RIPA buffer (Cell Signaling Technology, CST, Danvers, MA, USA) supplemented with 1× protease inhibitor cocktail and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 30 min on ice. The supernatant, which contained the total protein, was collected by centrifugation at 13,200 rpm for 30 min at 4°C. For isolation of cytoplasmic and nuclear extracts, cells were resuspended in a hypotonic buffer (20 mM Tris-HCl [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$) supplemented with 1× protease inhibitor cocktail, 1 mM PMSF and 1 mM Na_3VO_4 for 15 min on ice, after which appropriate volumes of 10% Nonidet P-40 (USB, Cleveland, OH, USA) were added. The supernatant, which contained the cytosolic fraction, was collected by centrifugation at 13,200 rpm for 1 min at 4°C. The remaining pellet was rinsed three times with cold PBS and resuspended in 1× RIPA buffer supplemented with 1× protease inhibitor cocktail and 1 mM PMSF by rocking at 4°C for 15 min. The supernatant, which contained the nuclear fraction, was collected by centrifugation at 13,200 rpm for 5

min at 4°C.

6. Oligo pull-down assay

Biotinylated oligonucleotides (Fig. 1) corresponding to the region spanning the two hot spots on the *TERT* promoter were synthesized from Integrated DNA Technologies Pte. Ltd. (IDT, Singapore, Singapore). Equal volumes of both complementary oligos were resuspended in annealing buffer (10 mM Tris-HCl [pH 7.4], 50 mM NaCl, 1 mM EDTA), heated at 95°C for 5 min and annealed by slow cooling to room temperature. Streptavidin-coated magnetic beads (Dynabeads® M-280 Streptavidin, Invitrogen, Carlsbad, CA, USA) were incubated with the annealed oligos (for LC-MS/MS analysis: 100 pmol, for immunoblot analysis: 50 pmol) for 30 min at room temperature by rotation. Subsequently, precleared nuclear extracts (for LC-MS/MS analysis: 1 mg, for immunoblot analysis: 500 μg) were added to the beads, followed by incubation for 2 h at 4°C by rotation. Eluted proteins from the beads were then analyzed by SDS-PAGE and LC-MS/MS.

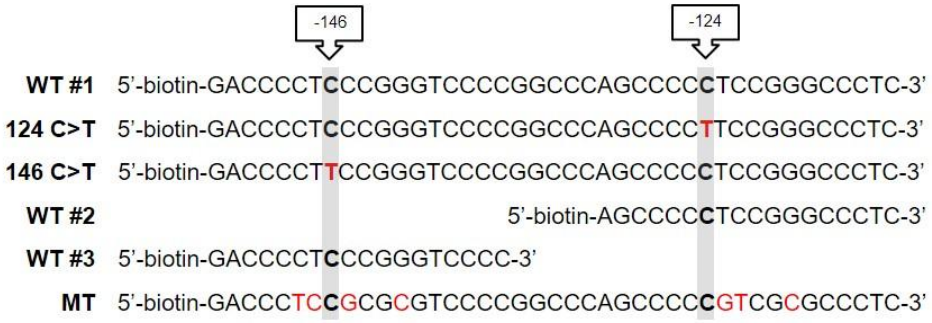


Fig. 1. Biotinylated oligonucleotide sequences used for oligo pull-down assay corresponding to the *TERT* promoter, which differ in length or sequence harboring ETS/TCF motif. WT, wild-type; MT, mutant. Only the top strands are shown.

7. LC-MS/MS analysis and protein identification

Each oligo pull-down sample was separated on a NuPAGE 12% Bis-Tris gel (invtrogen) and subjected to a conventional in-gel digestion procedure with minor modifications.[26] The prepared peptide samples were analyzed using a LTQ XL linear trap mass spectrometer (Thermo Fisher Scientific) equipped with a nano-HPLC system (Eksigent, Dublin, CA, USA). Tandem mass spectra were analyzed using SEQUEST module of Proteome Discoverer (Thermo Fisher Scientific, Version 1.4.1.14) and X! Tandem (The GPM, thegpm.org; Version CYCLONE (2010.12.01.1)). Scaffold (Proteome Software Inc., Portland, OR, Version Scaffold_4.4.1.1) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability. Peptide Probabilities from X! Tandem were assigned by the Scaffold Local FDR algorithm. Peptide Probabilities from SEQUEST were assigned by the Peptide Prophet algorithm with Scaffold delta-mass correction.[27] Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm.[28] Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Common contaminants such as keratins were excluded manually.

For semi-quantification of identified proteins, total spectrum count (TSC) value was used. First, the TSC data for each samples were normalized using the most

abundant protein, PARP1. Using those normalized TSC data, the fold-change ratio (R_{sc}) was calculated to compare the relative abundance of each protein. R_{sc} was calculated using the following formula (eq. 1).

$$R_{sc} = \log_2(n_s/n_c) \qquad (1)$$

where, for each protein, R_{sc} is the $\log_2$ ratio of the protein abundance between the mutant immunoprecipitated group and the WT group, and n_c and n_s are the normalized TSC data of the WT control group and the mutant group, respectively. Proteins with an Rsc above 1 or below -1 were considered up- or down-regulated, respectively.

8. Immunoblot analysis

Samples were separated by electrophoresis on a NuPAGE 4-12% Bis-Tris gel (Invitrogen), blotted onto a polyvinylidene difluoride (PVDF) transfer membrane and analyzed with epitope-specific primary and secondary antibodies. The bound antibodies were visualized using SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and ImageQuant™ LAS 4000 mini (GE Healthcare). Primary antibodies used were rabbit anti-PROX1 (11067-2-AP, Proteintech, Rosemont, IL, USA), rabbit anti-ETS1 (6258, CST), mouse anti-GABPA (ab55052, Abcam, Cambridge, UK), mouse anti-Flag (F3165, Sigma-Aldrich, St. Louis, MO, USA), rabbit anti-GAPDH (2118, CST), rabbit anti-COX IV (4844, CST) and rabbit anti-Lamin B1(ab16048, Abcam).

9. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was carried out using the SimpleChIP® Plus Enzymatic Chromatin IP Kit (CST) according to manufacturer's recommendations. For immunoprecipitation, 5-10 μg of cross-linked and digested chromatin was used with 1 μg of PROX1 antibody (11067-2-AP, Proteintech). Primers used for amplicon 1, 2 and 3 on the *TERT* promoter by semi-quantitative RT-PCR are listed in Table 1.

10. RNA interference

PROX1 siRNAs (Silencer® Select Pre-designed siRNAs; n331082 and n331083, Thermo Fisher Scientific) and Silencer® Select Negative Control No. 1 siRNA (Thermo Fisher Scientific) were transfected into Hep3B and HepG2 cells using Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's recommendations. To confirm the specificity and efficiency of *PROX1* knockdown, we checked mRNA and protein levels by quantitative real-time PCR and immunoblot analysis, respectively (Fig. 2).

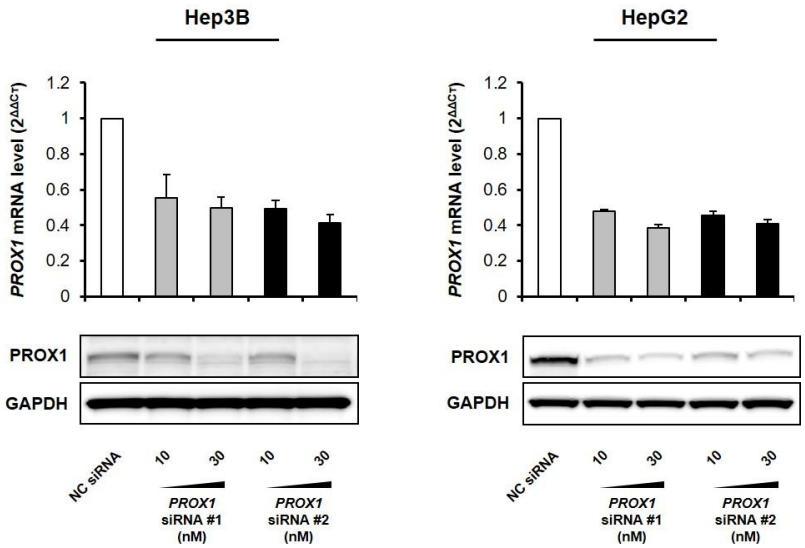


Fig. 2. Validation of *PROX1* knockdown efficiency. Hep3B and HepG2 cells were transfected with *PROX1* siRNAs at the indicated concentrations. The knockdown efficiency was validated by *PROX1* mRNA expression using quantitative real-time PCR and PROX1 protein level using immunoblotting. Thirty nanomolar of *PROX1* siRNA #2 showed higher efficacy of knockdown in the both cells. *18S* rRNA was used as an internal control for mRNA expression analysis. The data for quantitative real-time PCR represent mean ± SD. GAPDH was used as an internal control for protein expression analysis.

11. Tissue samples and pathological examination

A total of 242 liver specimens from 132 HBV-related patients who were all serum HBsAg-positive and anti-HCV-negative were investigated, including 33 cases of liver cirrhosis (LC), 6 cases of large regenerative nodules (LRNs), 31 cases of low-grade dysplastic nodules (LGDNs), 37 cases of high-grade dysplastic nodules (HGDNs), 33 cases of early HCCs (eHCCs) and 102 cases of progressed HCCs (pHCCs). The patient population consisted of 105 males and 27 females, and their ages were 53 ± 9.6 years. Non-B viral HCCs were collected from 14 cases of C viral and 11 cases of alcoholic HCC patients. The patient population consisted of 19 males and 6 females, and their ages were 67 ± 8.4 years. Non-neoplastic liver tissues (normal livers) were obtained from 5 patients with metastatic carcinoma. Fresh frozen liver specimens were provided by the Liver Cancer Specimen Bank, National Research Resource Bank program by the Korea Science and Engineering Foundation under the Ministry of Science and Technology. This study was approved by the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine.

12. Total RNA isolation and RT-PCR analysis

Total RNA was isolated from cells and tissues using the RNeasy Mini Kit (QIAGEN, Germantown, MD, USA) and reversetranscribed into cDNA using random hexamer primers and SuperScript® III Reverse Transcriptase (Invitrogen) according to the manufacturer's recommendations. For amplification of *TERT* and *PROX1*, quantitative real-time PCR was performed using TaqMan® Gene

Expression Assay (*TERT*: Hs00972656_m1, *PROX1*: Hs00896294_m1, *18S*: Hs99999901_s1, Applied Biosystems, Foster City, CA, USA) and TaqMan® Fast Advanced Master Mix (Applied Biosystems). For amplification of *HBx*, semi-quantitative RT-PCR was performed using Solg™ 2× Taq PCR Pre-Mix (Solgent, Daejeon, Korea). Primers used to amplify full-length *HBx* and *GAPDH* by semi-quantitative RT-PCR are listed in Table 1.

13. Genomic DNA isolation and sequencing

Genomic DNA was isolated from tissues and cells using the QIAamp® DNA Mini Kit (QIAGEN) according to the manufacturer's recommendations. The *TERT* promoter region 489 bp (from the position -424 to +65 from ATG start site) was screened for somatic mutations and a SNP using PCR and Sanger sequencing. PCR was carried out using the specific primers (Table 1) and Dr.MAX DNA Polymerase (Doctor Protein, Seoul, Korea). PCR products purified with Millipore plate MSNU030 (Millipore SAS, Molsheim, France) were then analyzed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and a 3730xl DNA Analyzer (Applied Biosystems) at Macrogen.

14. Establishment of stable HBx-expressing cells

The pcDNA3.1-Flag-HBx plasmid obtained from Addgene (Plasmid 42596, Cambridge, MA, USA) were transfected into Hep3B and HepG2 cells using the Lipofectamine® 3000 Transfection Reagent (Invitrogen) according to the manufacturer's recommendations. Stable *HBx*-expressing cells were selected with

Geneticin® Selective Antibiotic (G418 Sulfate, Gibco) at 0.6 mg/ml (for Hep3B cells) or 1 mg/mL (for HepG2 cells) for 14 days. To confirm stable *HBx* expression, we checked mRNA and protein levels by semi-quantitative RT-PCR and immunoblot analysis, respectively (Fig. 3).

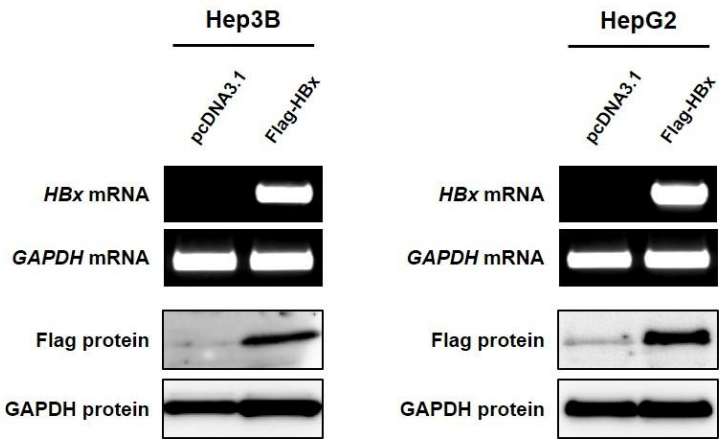


Fig. 3. Establishment of stable *HBx*-expressing cells. Stable *HBx* expression in Hep3B and HepG2 cells was validated by *HBx* mRNA expression using semi-quantitative RT-PCR and Flag protein expression using immunoblotting. *GAPDH* was used as an internal control for mRNA and protein expression analysis.

15. Statistical analysis

Student's t test was used to identify significant differences in comparison of *TERT* and *PROX1* mRNA expression in HBV-related HCC patients and a correlation between *TERT* and *PROX1* mRNA expression in HCC patients. The survival data were calculated using the Kaplan–Meier method. All p values were two-tailed and a p value of less than 0.05 was considered significant.

III. RESULTS

1. Elevation of *TERT* transcriptional activity in HCC cell lines by sequence changes at -124 and -146 bp from ATG in the *TERT* promoter

To verify whether the sequence changes in the *TERT* promoter modulate *TERT* transcription in HCC, various *TERT* reporter constructs containing *TERT* promoter mutations and the allelic variants of the rs2853669 were transfected into 4 HCC cell lines and luciferase reporter assay was subsequently carried out. The constructs with mutations, especially -124C>T mutation, increased the promoter activity compared to those with WT in the HCC cell lines except SNU423 (Fig. 4). The increase in the promoter activity, however, slightly diminished in the presence of variant C allele of the rs2853669. The allelic variants of rs2853669, on the other hand, were not significant for the promoter activity in the absence of mutations. Therefore, genetic events in the *TERT* promoter are considered to directly contribute to telomerase activation through modulation of *TERT* transcription in HCC.

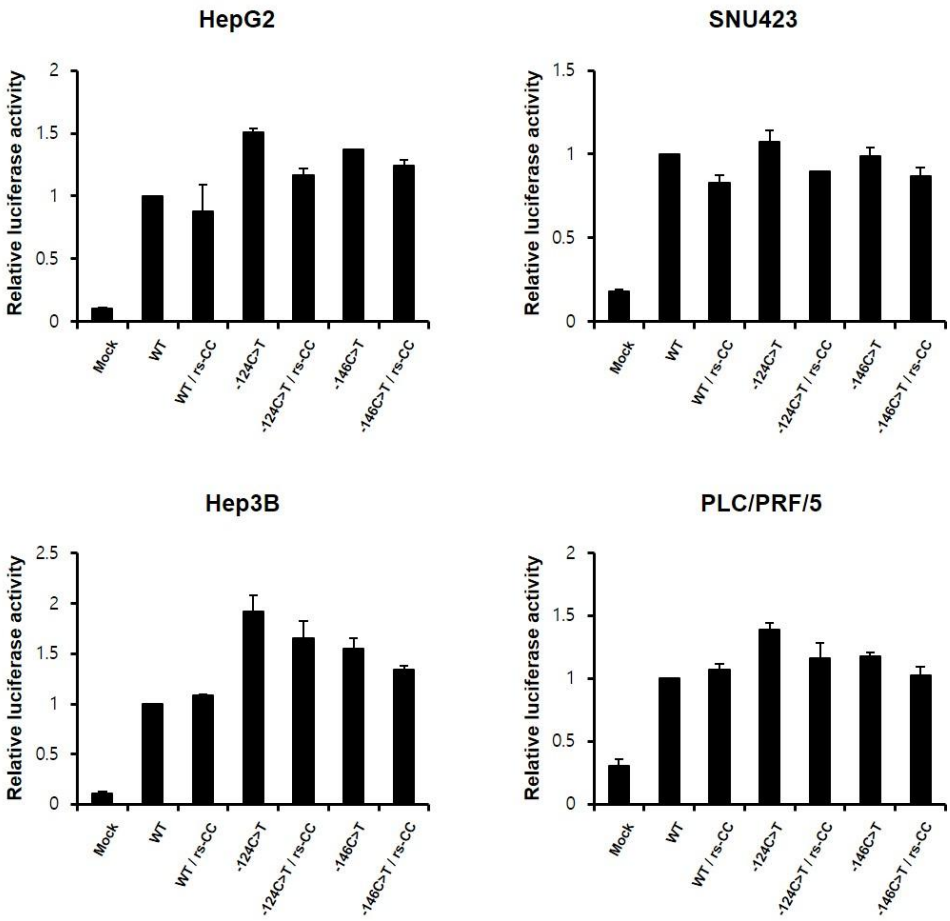


Fig. 4. Genetic alterations in the *TERT* promoter are associated with *TERT* promoter activity in HCC cell lines. Relative luciferase activity of various *TERT* promoter constructs, WT, WT with CC genotype of rs2853669 (WT/rs-CC), -124C>T mutant (-124C>T), -124C>T mutant with CC genotype of rs2853669 (-124C>T/rs-CC), -146C>T mutant (-146C>T) and -146C>T mutant with CC genotype of rs2853669 (-146C>T/rs-CC). The data represent mean ± SD.

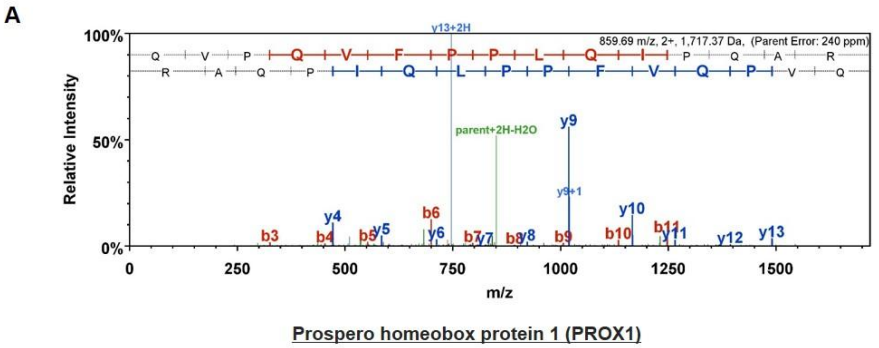
2. Identification of PROX1 as *TERT* promoter-binding protein and increase of its binding affinity to the site created by mutations

A consensus ETS/TCF binding site (CCGGAA) has been reported to be generated by *TERT* promoter somatic mutations, where ETS/TCF transcription factors bound resulting in elevation of *TERT* transcription.[12-15] Nevertheless, it has not been yet demonstrated experimentally what kind of proteins, ETS/TCF family members or others, bind to the *de novo* sites, and to find them, oligo pull-down experiment combined with LC-MS/MS analysis was performed. We prepared nuclear extracts from Hep3B cells because the promoter activities of -124C>T and -146C>T mutant constructs were higher in Hep3B cells compared to the other HCC cell lines. The biotinylated double-stranded oligos shown in Fig. 1 were incubated nuclear extracts and protein-DNA affinity was then analyzed by comparing the spectrum count values of each sample using mass spectrometry. Based on detection of multiple peptides, several proteins were identified having significantly higher interaction toward 124C>T and 146C>T substitution oligos compared to WT #1 oligos (Table 2). Among these proteins, PROX1 (molecular weight of 83 kDa, Fig. 5A) was selected owing to the fact that it has been known as an essential transcription factor in the development of multiple organs and tissues.[33-37] The binding affinity of PROX1 to the *TERT* promoter with somatic mutations was further confirmed by immunoblot assay using the samples from the oligo pull-down assay. As shown in Fig. 5B, enhanced PROX1 bindings to C to T substitution oligos, especially -124C>T oligos, were observed in common with the results of mass spectrometry. Interestingly, PROX1 recruitment was also detected in WT #1 oligos

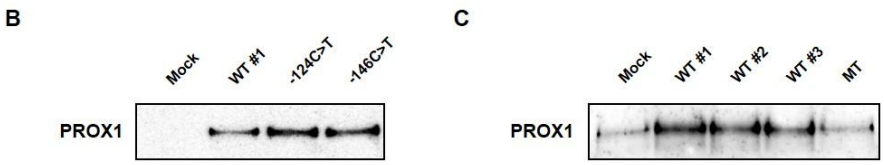
although its level was lower than one in the C to T substitution oligos. To further validate the PROX1 binding to the WT *TERT* promoter sequences harboring hot spots, we examined the oligo pull-down assay using various WT oligos (WT #1, WT #2 and WT #3) and artificial mutant oligos (MT) which caused loss of ETS/TCF binding motif. Fig. 5C showed that PROX1 specifically bound the WT *TERT* promoter sequences (CCGGAG and CCGGGA). According to public repositories of cDNA microarray data (BioGPS, http://biogps.org), *PROX1* mRNA expression is higher in liver tissue (GeneAtlas U133A, gcrma; probe 207401_at). Endogenous PROX1 was also detected abundantly in the nucleus of HCC cell lines Hep3B and HepG2 except SNU423 by immunoblot analysis (Fig. 5D). Of ETS/TCF family members, GA binding protein transcription factor alpha subunit (GABPα) and ETS1 were also checked for their endogenous levels in the HCC cell lines, however, the expression levels of them were not valid for the results of mutation-induced promoter activation in the respective cells shown in Fig. 4. To confirm whether endogenous PROX1 binds the *TERT* promoter with or without mutation, we carried out ChIP assay using Hep3B (with the WT *TERT* promoter) and HepG2 (with the -124C<T mutant *TERT* promoter) cells. As shown in Fig. 5E, PROX1 was recruited to the regions of the *TERT* promoter spanning the hot spots only (amplicon 1) and the rs2853669 besides those (amplicon 2) in the both cells. In the 3' UTR (amplicon 3), however, PROX1 was not. By these approaches, it is demonstrated that PROX1 binds the *TERT* promoter, and the recruitment is dramatically increased by the occurrence of somatic mutations at the two hot spots in HCC.

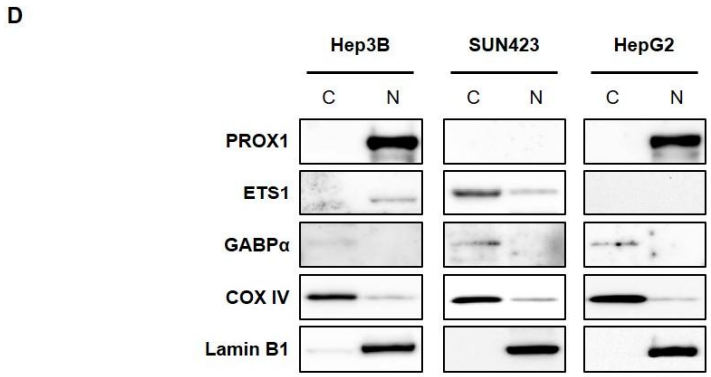
Table 2. R_{sc} value of proteins that are enhanced in mutant groups compared to WT group

Identified Protein Name	R_{sc}	
	-124C<T	-146C<T
Prospero homeobox protein 1	3.9	4.3
cDNA FLJ76127, highly similar to Homo sapiens replication factor C (activator 1) 5	3.5	4.3
Nuclease-sensitive element-binding protein 1	2.3	3.5
Double-strand-break repair protein rad21 homolog	2.2	3.4
Nucleoporin NUP188 homolog	2.2	2.9
Titin, isoform CRA_a	2.2	2.1
SMARCA1 protein	1.8	3.0



Prospero homeobox protein 1 (PROX1)





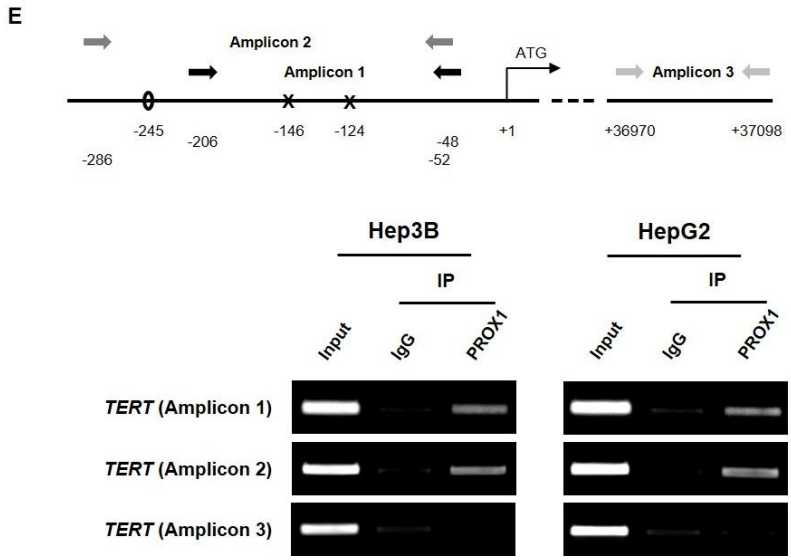


Fig. 5. Identification of PROX1 as *TERT* promoter-binding protein and increase of its binding to the site created by mutations. (A) Representative LC-MS/MS spectrum of identified proteins in nuclear extracts from Hep3B cells which were pulled down by -124C>T mutant oligonucleotide. The parent ion has an m/z of 859.69 and the spectrum was identified as the tryptic peptide QVPQVFPPLQIPQAR from PROX1. (B) The oligonucleotide-protein affinity was confirmed by immunoblotting. PROX1 could bind to not only mutant oligonucleotides but WT oligonucleotide although more PROX1 bound to mutant sequences, expecially -124C>T. (C) Oligo pull-down assay elucidated that PROX1 specifically bound the WT *TERT* promoter sequences (CCGGAG and CCGGGA) harboring hot spots. (D) Immunoblot analysis elucidated that endogenous PROX1 was detected in the nucleus of Hep3B and HepG2 cells except SNU423 cells. GABPα was not detected in the nucleus of Hep3B, SNU423 and HepG2 cells, and low level of ETS1 detected in the nucleus of Hep3B and SNU423 cells. Lamin B1 and COX IV were used as a nuclear and cytosolic control, respectively. (E) ChIP assay elucidated that PROX1 directly bound the *TERT* promoter in Hep3B and HepG2 cells. Immunoprecipitated chromatin was subjected to PCR analysis using the primer sets indicated on the schematic diagram of the *TERT* promoter.

3. Function of PROX1 as a novel transcriptional activator for *TERT* gene

To evaluate whether PROX1 regulates *TERT* transcription by recruitment to the region containing the hot spots on the *TERT* promoter in HCC, endogenous *PROX1* expression was depleted by siRNA in Hep3B and HepG2 cells. Silencing *PROX1* obviously induced suppression of *TERT* mRNA expression in the both cells (Fig. 6A). To elucidate the mechanism how PROX1 regulates *TERT* transcription, various *TERT* reporter constructs along with *PROX1* siRNA were transfected in Hep3B and HepG2 cells. *PROX1* knockdown dramatically inhibited the enhanced promoter activities of -124C>T and -146C>T mutant constructs as well as basal activity of WT construct (Fig. 6B). On the other hand, in SNU423 cells, the promoter activities of the respective constructs with or without mutations increased when *PROX1* was overexpressed (Fig. 6C). Especially, the PROX1-induced promoter activation of -124C>T mutant construct was markedly higher than that of WT construct. Therefore, PROX1 is one of the transcription machineries for *TERT* gene and plays important role in a transcriptional activator through dominantly binding to the mutant *TERT* promoter in HCC.

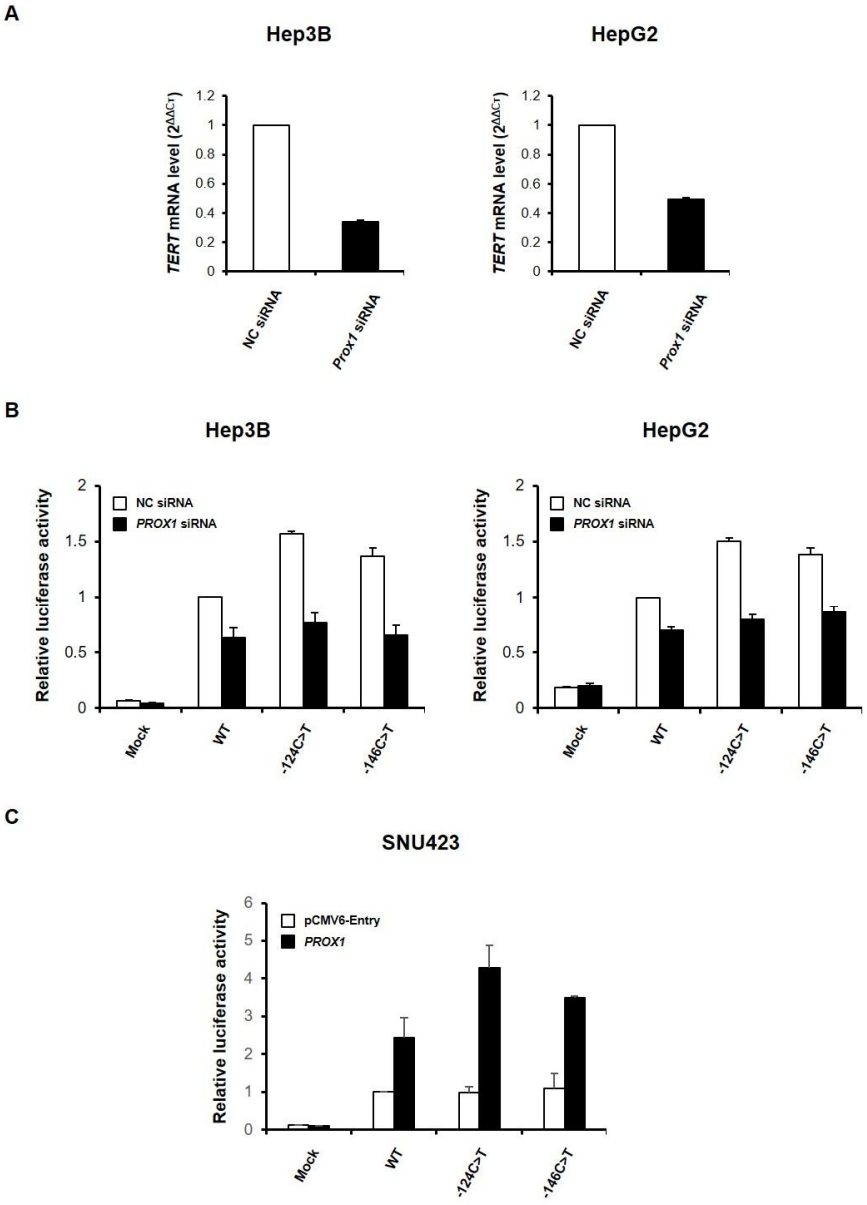


Fig. 6. Function of PROX1 as a transcriptional activator for *TERT* expression. (A) Quantitative real-time PCR elucidated that *PROX1* knockdown led to significant reduction of *TERT* mRNA expression in Hep3B and HepG2 cells. *18S rRNA* was used as an internal control. The data represent mean ± SD. (B) Dual-luciferase assay elucidated that *PROX1* knockdown in Hep3B and HepG2 cells

inhibited the enhanced *TERT* promoter activities of -124C>T and -146C>T mutant constructs as well as basal activity of WT construct. The data represent mean ± SD. (C) Dual-luciferase assay elucidated that *PROX1* overexpression in SNU423 cells resulted in 2.4, 4.3 and 3.5- fold enhanced *TERT* promoter activities of WT, -124C>T and -146C>T mutant constructs compared with the corresponding controls, respectively. The data represent mean ± SD.

4. *TERT* promoter mutations and the rs2853669 in B viral human multistep hepatocarcinogenesis

Based on our *in vitro* findings, we verified the relationship between the genetic alterations in the *TERT* promoter and *TERT* expression in B viral human multistep hepatocarcinogenesis. Genomic DNA was isolated from human liver tissue samples including 33 LC, 6 LRNs, 31 LGDNs, 37 HGDNs, 33 eHCCs and 102 pHCCs collected from the 132 patients, and the *TERT* promoter region from -424 to +65 bp was sequenced. The incidence of *TERT* promoter mutations gradually increased with the progression of hepatocarcinogenesis, which detected in 3/31 LGDNs (9%), 5/37 HGDNs (13.5%), 9/33 eHCCs (27.3%) and 29/102 pHCCs (28.4%) (Fig. 7A). Most mutations are -124C>T, except 1/5 mutations in HGDNs (-124C>A) and 3/9 mutations in eHCCs (-146C>T), and these mutations occurred mutually exclusive either at -124 or -146 bp upstream ATG. On the contrary, *TERT* promoter mutations were not found in 5 normal livers, 33 LC and 6 LRNs.

The genotype distribution of the rs2853669 was evaluated in B viral HCC patients (Fig. 7B). The rs2853669 with carrier variants was detected in 55/97 patients (56.7%; TC heterozygotes: 46.4%, CC homozygotes: 10.3%), similar to those in the 1000 Genome database of Asian population (52.4%, data available for 286 individuals).[29] There was no significant effect of rs2853669 status on frequency of *TERT* promoter mutations in B viral HCC patients.

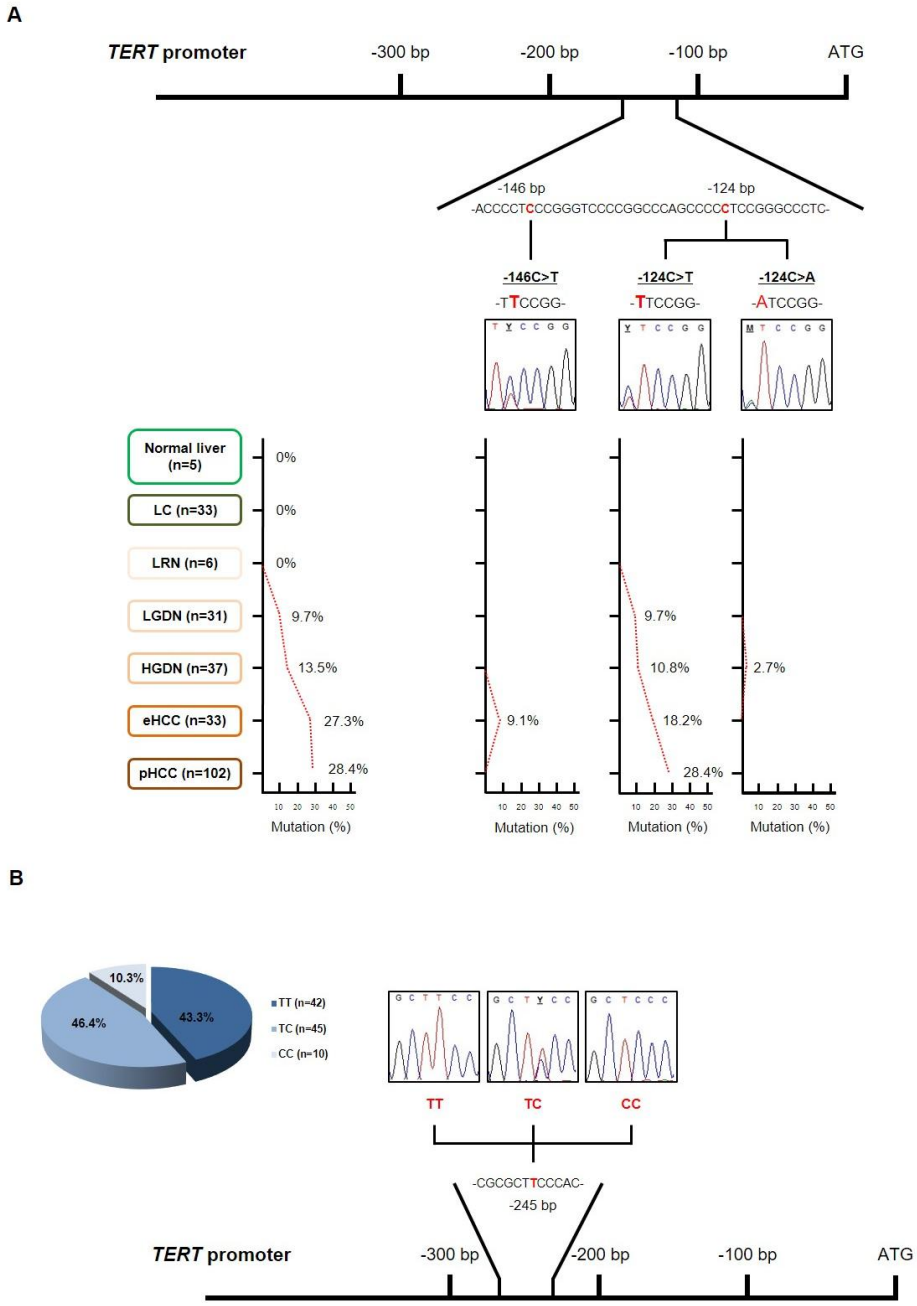


Fig. 7. Genetic events of the *TERT* promoter in the progression of B viral hepatocarcinogenesis. (A) Somatic mutation spectrum of the *TERT* promoter in B viral multistep hepatocarcinogenesis. *TERT* promoter mutations located at -124 and

-146 bp from ATG start site were early genetic events and gradually increased with the progression of B viral hepatocarcinogenesis. (B) The genotype distribution of a SNP rs2853669 in patients with B viral HCC.

5. *TERT* and *PROX1* expression in B viral human multistep hepatocarcinogenesis

TERT mRNA expression was evaluated in B viral human multistep hepatocarcinogenesis (Fig. 8A). *TERT* transcript levels gradually increased according to the progression of multistep hepatocarcinognesis, and a significant increase of *TERT* mRNA expression occurred in HGDNs (p=0.0626) and eHCCs (p=0.0060) with the highest levels in pHCCs (p=0.0016) compared to LC. The induction of *TERT* mRNA was not found in normal livers, LC and LRNs, and *TERT* mRNA level was very low in LGDNs.

PROX1 mRNA expression was also evaluated in the same liver tissue samples (Fig. 9A). A significant increase of *PROX1* mRNA expression occurred in pHCCs (p<0.0001) compared to LC, and most pHCCs (14/18) showed higher levels of *PROX1* mRNA expression compared to their adjacent non-neoplastic liver (non-HCC) (Fig. 9B). Whereas, such difference was not found among LRNs, DNs and eHCCs.

As a next step, a correlation between the genetic alterations in the *TERT* promoter and *TERT* mRNA expression was assessed. On the contrary to the *in vitro* data, *TERT* mRNA expression was significantly lower in HGDNs (p=0.0686), eHCCs (p=0.0058) and pHCCs (p=0.0024) with the mutant *TERT* promoter compared to those with the WT *TERT* promoter (Fig. 8B and Fig. 10A). Furthermore, *TERT* mRNA levels in DNs and eHCCs were lower in carriers (TC or CC genotype of the rs2853669) compared to non-carriers (TT genotype of the rs2853669), whereas those in pHCCs were higher in carriers compared to non-

carriers (Fig. 10B). However, there are not statistically significant (p>0.05 for all).

The correlation between *TERT* and *PROX1* mRNA expression was further assessed in B viral pHCCs, and there was no significant correlation (Fig. 11A). This finding was not consistent with *in vitro* experiment, which showed PROX1-mediated *TERT* expression. To query whether this phenomenon is limited to B viral HCC, same experiments was performed in non-B viral HCCs (14 C viral HCCs and 11 alcoholic HCCs). Interestingly, there was a positive correlation between *TERT* and *PROX1* mRNA expression unlike B viral HCCs (Fig. 11B).

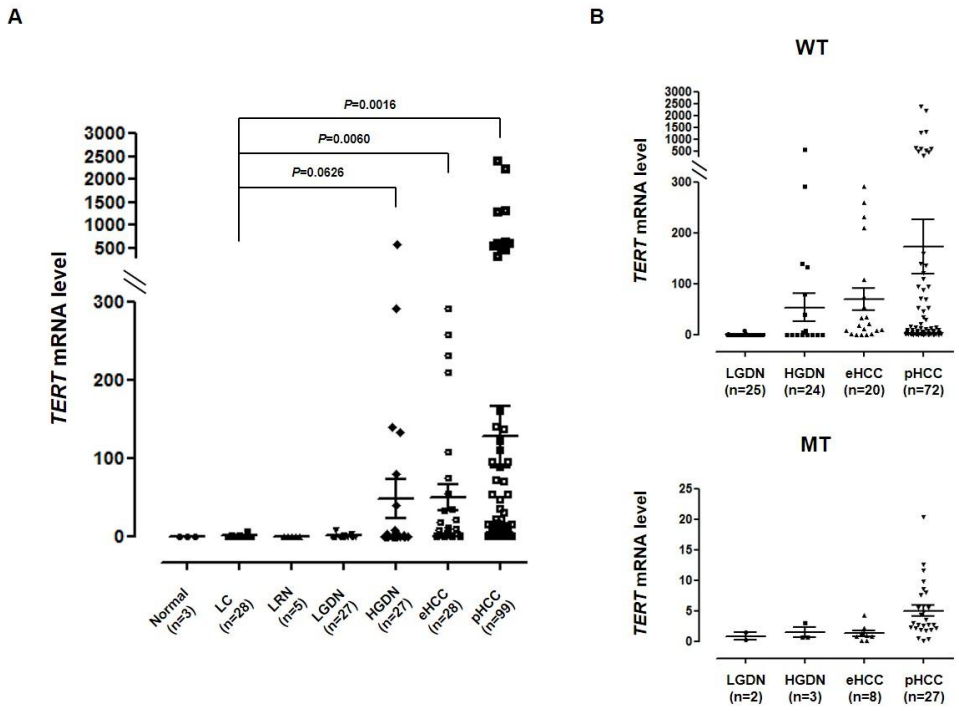


Fig. 8. *TERT* mRNA levels in B viral multistep hepatocarcinogenesis. (A) Quantitative real-time PCR elucidated that *TERT* mRNA expression was gradually enhanced with the progression of hepatocarcinogenesis, especially HGDNs (p=0.0626), eHCCs (p=0.0060) and pHCCs (p=0.0016) compared to LC. *18S rRNA* was used as an internal control. Each bar represents mean ± SEM. (B) Quantitative real-time PCR elucidated that the remarkably enhanced *TERT* mRNA expression was observed in HGDNs, eHCCs and pHCCs only with the WT *TERT* promoter. *18S rRNA* was used as an internal control. Each bar represents mean ± SEM.

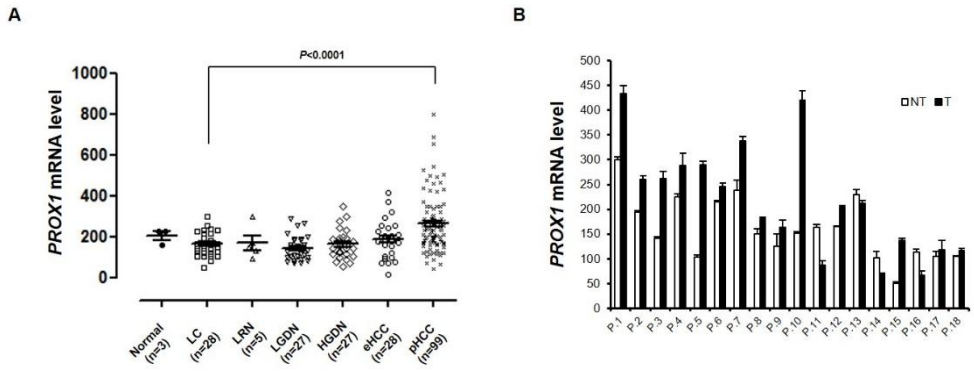


Fig. 9. *PROX1* mRNA levels in B viral multistep hepatocarcinogenesis. (A) Quantitative real-time PCR elucidated that a significant increase of *PROX1* mRNA expression occurred in pHCCs (p<0.0001) compared to LC. *18S rRNA* was used as an internal control. Each bar represents mean ± SEM. (B) Quantitative real-time PCR elucidated that most of HBV-related HCC patients showed increase of *PROX1* mRNA expression in pHCCs compared to their adjacent non-HCC. *18S rRNA* was used as an internal control. The data represent mean ± SD.

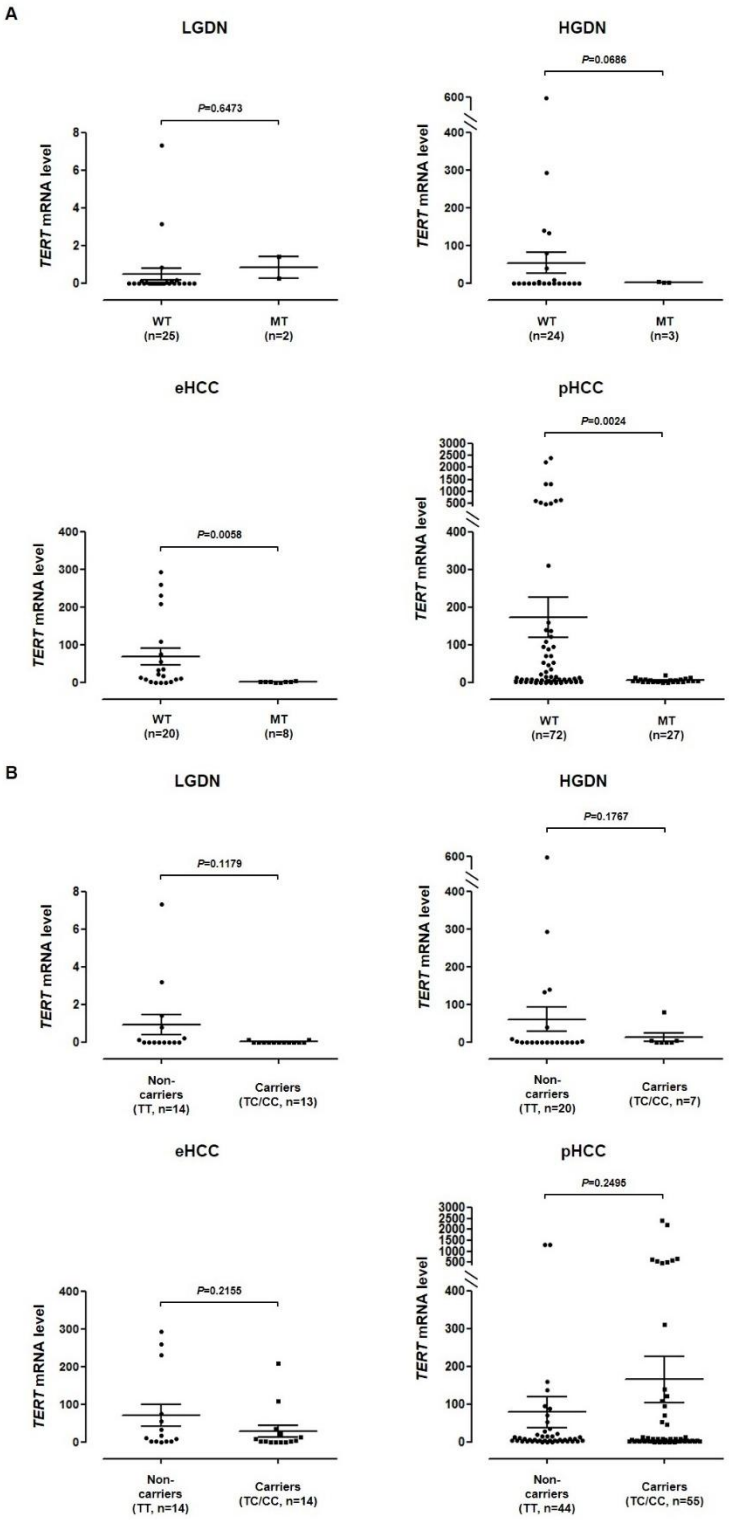


Fig. 10. *TERT* mRNA expression according to genetic events of the *TERT* promoter in the progression of B viral hepatocarcinogenesis. (A) Quantitative real-time PCR elucidated that *TERT* mRNA level was lower in HGDNs (p=0.0686), eHCCs (p=0.0058) and pHCCs (p=0.0024) with the mutant *TERT* promoter compared to the WT *TERT* promoter. *18S rRNA* was used as an internal control. Each bar represents mean ± SEM. (B) Quantitative real-time PCR elucidated that there is no statistically significant difference between *TERT* mRNA level and allelic variants of rs2853669. *18S rRNA* was used as an internal control. Each bar represents mean ± SEM.

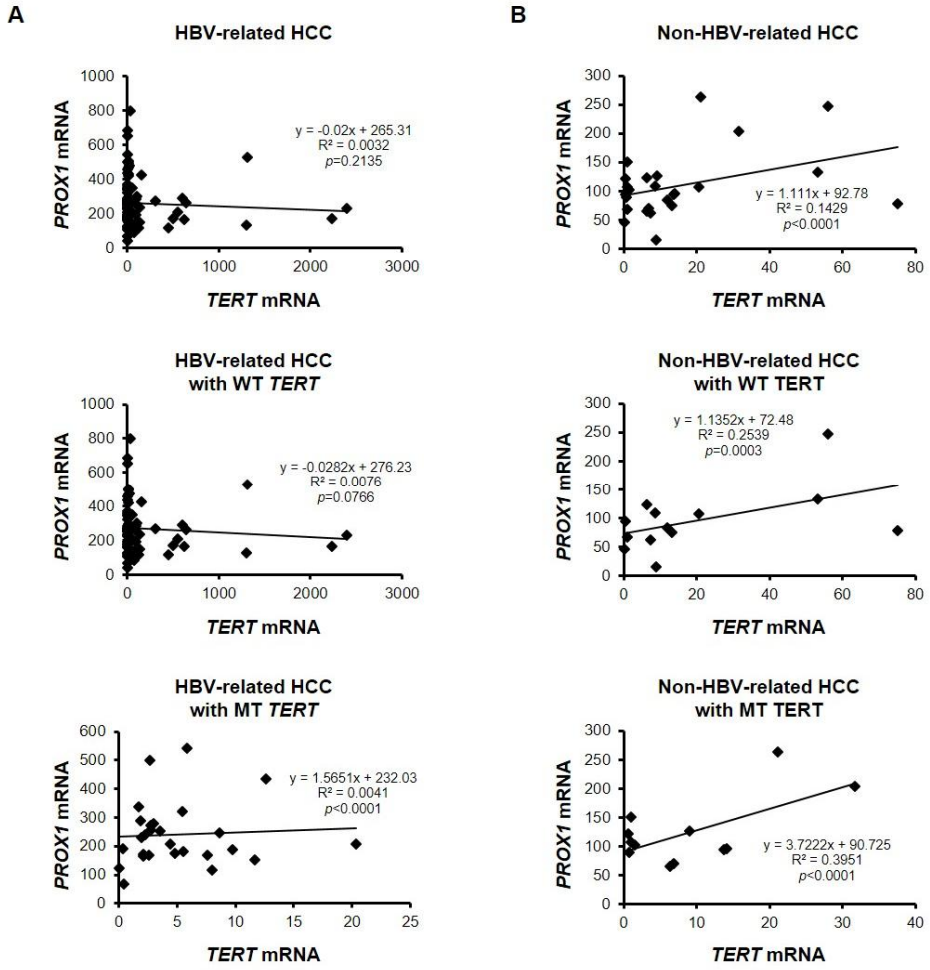


Fig. 11. Comparison between *TERT* and *PROX1* mRNA expression. (A) There is little correlation between *TERT* and *PROX1* mRNA expression in B viral HCCs (n=99). (B) However, positive correlation between the two was observed in non-B viral HCCs (n=25).

6. Suppression of PROX1-mediated *TERT* expression by *HBx*

In our human data of B viral hepatocarcinogenesis, there was little elevation of *TERT* transcription by somatic mutations activating the *TERT* promoter unlike our *in vitro* data and no correlation between *TERT* and *PROX1* mRNA expression unlike non-B viral HCCs. Based on that *HBx* was not expressed in the HCC cell lines used as well as non-B viral HCC, *w*e hypothesized that HBx might be involved in the regulation of PROX1-mediated *TERT* expression. To study influence of HBx on PROX1-mediated *TERT* expression, stable *HBx*-expressing cell lines using Hep3B and HepG2 were established. Interestingly, stable *HBx* expression caused significant suppression of *TERT* mRNA expression in the both cells compared with the corresponding control (Fig. 12A). Considering that PROX1 binding was more sensitive to the mutant *TERT* promoter resulting in increase of promoter activity when *HBx* expression was absent, we queried whether HBx hindered PROX1 to stimulate *TERT* promoter activity. First, dual-luciferase assays were performed by transfection of *TERT* reporter constructs with WT and -124C>T mutation (the majority of *TERT* promoter somatic mutations in B viral HCCs) into the *HBx*-expressing cells. We found that stable *HBx* expression significantly inhibited the enhanced *TERT* promoter activity of mutant construct as well as basal promoter activity of WT construct (Fig. 12B). This finding is consistent with the result of dual-luciferase assay revealing disruption of activating the *TERT* promoter by *PROX1* knockdown shown in Fig. 6B. We next checked whether HBx was involved in the loss of PROX1-mediated *TERT* activation. Oligo pull-down assay was carried out using nuclear extracts from *HBx*-expressing cells. Enhanced PROX1 binding

affinity to -124C>T substitution oligos and basal PROX1 binding affinity to WT #1 oligos were markedly inhibited by stable *HBx* expression (Fig. 12C). To confirm the malfunction of PROX1 by HBx, we carried out ChIP assay using the *HBx*-expressing cell lines. Stable *HBx* expression inhibited the direct physical association of PROX1 to the *TERT* promoter with or without mutation (Fig. 12D). These findings suggest that HBx contributes to defunctionalization of PROX1 on *TERT* transcription by hindering the potential of PROX1 from binding and regulating the *TERT* promoter.

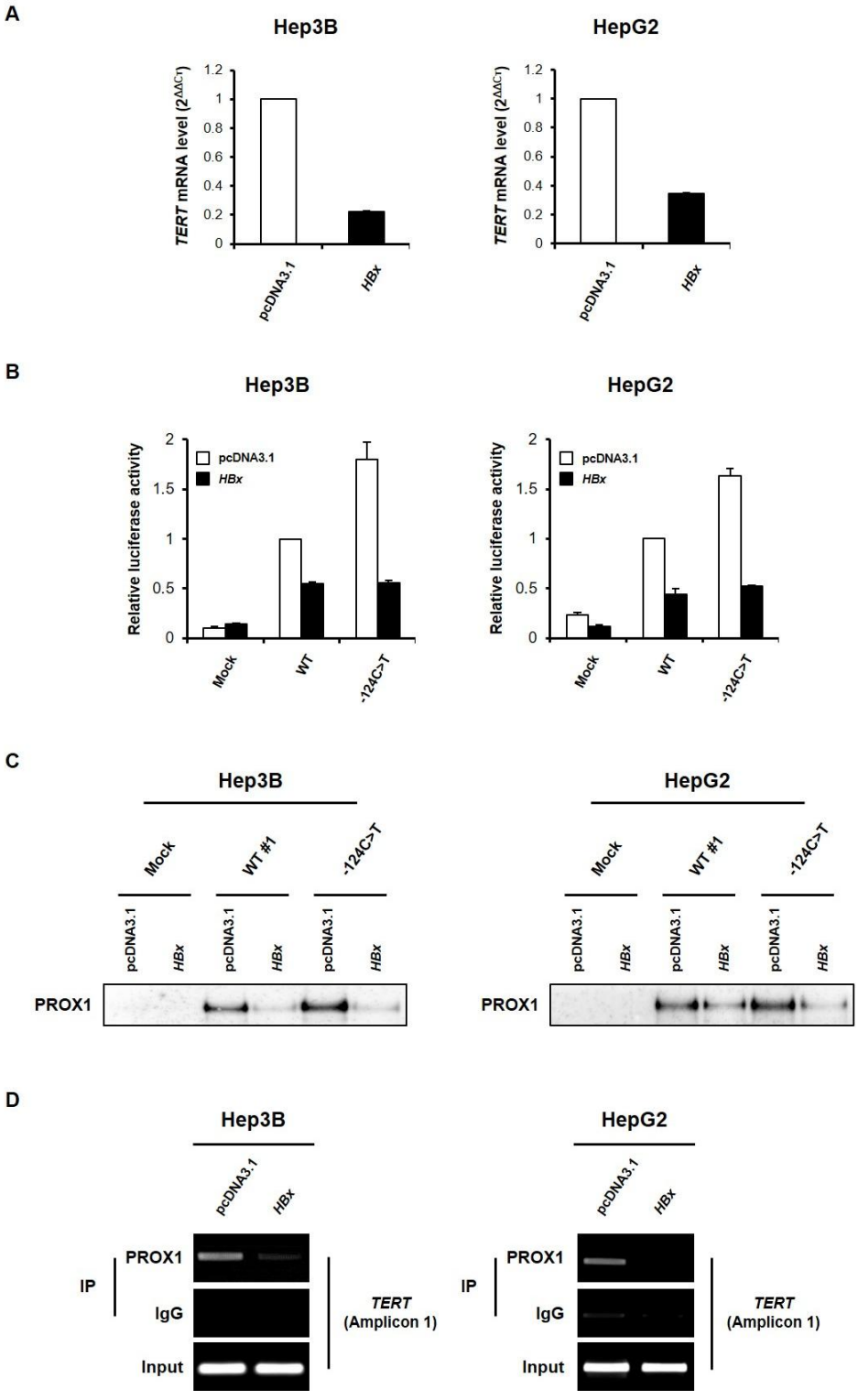


Fig. 12. Stable *HBx* expression inhibits PROX1-mediated *TERT* Expression. (A) Quantitative real-time PCR elucidated that *TERT* mRNA expression significantly was reduced in stable *HBx*-expressing cell lines. *18S rRNA* was used as an internal control. The data represent mean ± SD. (B) Dual-luciferase assay elucidated that the enhanced promoter activity of -124C>T mutant construct and basal promoter activity of WT construct were suppressed in stable *HBx*-expressing cell lines. The data represent mean ± SD. (C) Oligo pull-down assay elucidated that stable *HBx* expression triggered inhibition of PROX1 binding affinity to both -124C>T substitution and WT #1 oligonucleotide. (D) Disruption of the direct association between PROX1 and the *TERT* promoter by stable *HBx* expression was confirmed by ChIP assay. Immunoprecipitated chromatin was subjected to PCR analysis using the primer sets against amplicon 1.

7. Prognostic significance of *TERT* promoter mutations, the rs2853669 and *TERT* mRNA level in B viral HCC patients

The relationship between *TERT* promoter mutations/ a SNP rs2853669 and clinicopathological features in B viral HCC patients is summarized in Table 3. Interestingly, the patients with the mutant *TERT* promoter significantly had lower alpha-fetoprotein (AFP) levels (p=0.046) than those with WT. To determine whether the genetic alterations in the *TERT* promoter and *TERT* expression influence on the prognosis of B viral HCCs, overall survival and disease-free survival rates were evaluated in B viral HCC patients (n=78) excluding liver transplantation patients. The occurrence of *TERT* promoter mutations was correlated with a poor overall survival (p=0.012) in B viral HCC patients (Fig. 13A), and the high *TERT* mRNA level (the upper 20%) was correlated with a poor overall survival (p=0.042) and a disease-free survival (p=0.004) in B viral HCC patients with non-carrier rs2853669 (Fig. 13B).

Table 3. Clinicopathological characters of B viral HCC patients according to *TERT* promoter mutations and rs2853669 variant

Variable	All (n=78)	*TERT* promoter mutations		*p*-value	SNP rs2853669		*p*-value
		Present (n=21)	Absent (n=57)		Carrier (TC+CC) (n=47)	non-Carrier (TT) (n=31)	
Age (year, mean ± SD)	51.79 ± 10.33	52.14 ± 8.78	51.67 ± 10.91	0.858	51.23 ± 8.54	52.65 ± 12.68	0.558
Gender (male:female)	61:17	17:4	44:13	0.721	35:12	26:5	0.325
Tumor size (cm, mean ± SD)	5.52 ± 3.59	5.61 ± 3.87	5.49 ± 3.52	0.904	5.72 ± 3.73	5.23± 3.41	0.561
Tumor number (mean ± SD)	1.08 ± 0.35	1.19 ± 0.51	1.04 ± 0.27	0.084	1.09 ± 0.35	1.06 ± 0.36	0.802
ICG R15 (%, mean ± SD)	9.54 ± 6.11	9.91 ± 5.31	9.49 ± 6.43	0.751	9.79 ± 6.91	9.18 ± 4.86	0.678
AFP (IU/mL, mean ± SD)	1909 ± 5792	500 ± 1612	2427 ± 6647	0.046	1362 ± 5047	2737 ± 6773	0.308
PIVKA-II (mAU/mL, mean ± SD)	645 ± 812	639 ± 844	648 ± 807	0.969	608 ± 779	697 ± 865	0.651
Portal vein invasion (n, %)	23 (29.5)	4 (19.0)	19 (33.3)	0.220	10 (21.3)	13 (41.9)	0.050
Vascular invasion							
Macrovascular (n, %)	10 (12.8)	3 (14.3)	7 (12.3)	>0.999	5 (10.6)	5 (16.1)	0.507
Microvascular (n, %)	55 (70.5)	13 (61.9)	42 (73.7)	0.312	32 (68.1)	26 (74.2)	0.563
Bile duct invasion (n, %)	4 (5.1)	1 (4.8)	3 (5.3)	>0.999	0	4 (12.9)	0.022
Differentiation (major)				0.030			0.744
Edmonson grade I-II (n, %)	52 (66.7)	18 (85.7)	34 (59.6)		32 (68.1)	20 (64.5)	
Edmonson garde III-IV (n, %)	26 (33.3)	3 (14.3)	23 (40.4)		15 (31.9)	11 (35.5)	

ICG, indocyanine green; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonist-II.

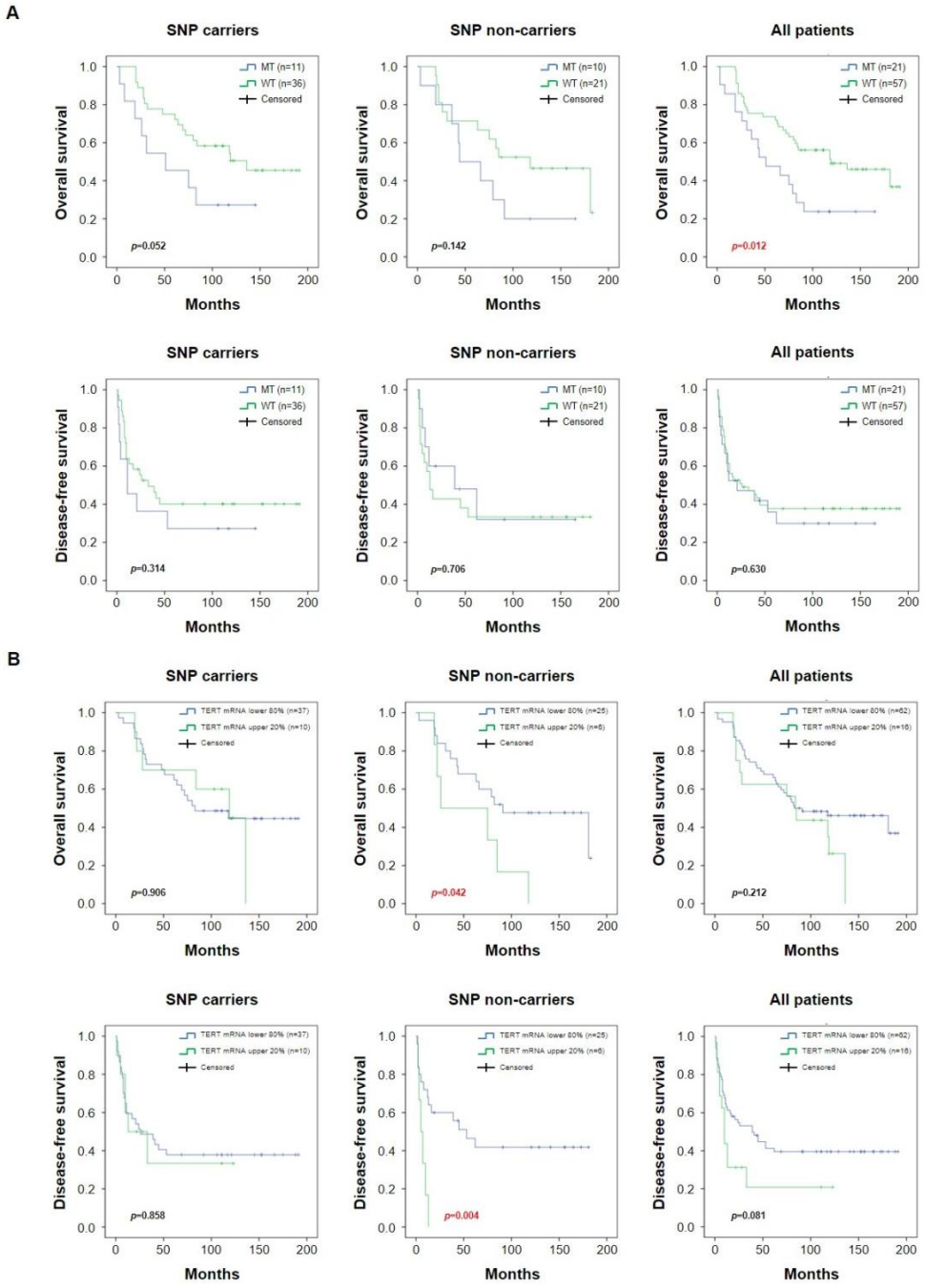


Fig. 13. Kaplan–Meier analysis of differences in overall survival and disease-free survival based on (A) a combination between the rs2853669 status and

***TERT* promoter mutations and (B) a combination between the rs2853669 status and *TERT* mRNA level.**

IV. DISCUSSION

The *TERT* core promoter consists of regulatory sequences with the E-boxes, GC-boxes and various motifs for transcription machinery.[30,31] Recently, genetic alterations in the *TERT* core promoter have been reported to be essential for modulation of *TERT* expression in various cancers.[12-15] In the present study, sequence changes (-124C>T and -146C>T) induced increase of *TERT* promoter activity in HCC cell lines Hep3B, HepG2 and PLC/PRF/5. Meanwhile, variant C allele of the rs2853669 diminished the mutation-induced *TERT* promoter activation *in vitro*. Therefore, specific sequences created in *TERT* core promoter by genetic alterations are considered one of mechanisms for telomerase activation in HCC.

So far, *de novo* sequence (CCGGAA) generated by mutations was reported to be a consensus binding motif for ETS/TCF transcription factors. However, their association has not been experimentally demonstrated yet. Recently, Bell *et al*. revealed that GABPα, one of the ETS transcription factors, directly bound and modulated the mutant *TERT* promoter across various cancer types.[32] Based on this report, we screened endogenous levels of GABPα and ETS1 in Hep3B, SNU423 and HepG2 cells. GABPα was not detected in the nucleus of all three HCC cells, and low level of ETS1 was detected in the nucleus of Hep3B and SNU423, which was not valid for mutation-induced promoter activation in the respective cells. We, meanwhile, found that the protein having higher interaction toward the ETS/TCF-consensus sequence was PROX1, not ETS/TCF family proteins, by oligo pull-down

experiment combined with LC-MS/MS analysis from nuclear extract of Hep3B cells. Interestingly, PROX1 also bound the WT *TERT* promoter regions harboring CCGGAG and CCGGGA sequences although the binding affinity was lower than that to the mutant sequence (CCGGAA). *PROX1* has been known as a transcription factor controlling development of multiple organs including lymphatic vessel, eye and liver and cancer progression.[33-37] Additionally, PROX1 was reported to be involved in HCC proliferation and metastasis by activating the Wnt/β-catenin pathway and HIF-1α signaling, respectively.[38,39] However, the molecular mechanisms how PROX1 regulates the transcription of *TERT* genes have not been reported yet. In the present study, we observed that endogenous PROX1 was detected in the nucleus of Hep3B and HepG2 cells but SNU423 cells, which was consistent with the pattern of modulating the mutant *TERT* promoter activation in these cells. With *PROX1* knockdown experiments, we detected a significant decrease in *TERT* mRNA expression through inhibition of promoter activity in HepG2 and Hep3B cells with or without the mutant *TERT* promoter, respectively. On the other hand, ectopic expression of *PROX1* in SNU423 cells induced significant elevation of *TERT* promoter activity, especially -124 C>T mutant *TERT* promoter activity. Taken together, we first found the potential of PROX1 as one of the transcription machineries for *TERT* gene in HCC which served as a transcriptional activator through dominantly binding to the mutant *TERT* promoter.

Recently, Nault *et al.* demonstrated that somatic mutation in the *TERT* promoter was an early genetic event in hepatocarcinogenesis and occurred in 2/32 LGDNs (6%), 3/16 HGDNs (19%), 14/23 eHCCs (61%) and 7/17 small and progressed

HCCs (42%), in which main etiological factors of the patients were HCV (41%) and alcohol intake (44%).[21] Whereas, *TERT* promoter mutation in B viral hepatocarcinogenesis has been poorly understood yet. In the present study, we sequenced the promoter region from the tissue samples, 33 LC, 6 LRNs, 31 LGDNs, 37 HGDNs, 33 eHCCs and 102 pHCCs from B viral cirrhotic patients. We revealed that frequency of the somatic mutations gradually increased with the progression from LGDN (9%) to pHCC (28.4%) although it was lower in B viral hepatocarcinogenesis, especially in pHCCs, compared to non-B viral hepatocarcinogenesis. In addition, we discovered that *TERT* mRNA expression increased with the progression of hepatocarcinogenesis, and this pattern in multistep was similar to the occurrence of somatic mutations. Therefore, we speculated that *TERT* transcription could be related to its promoter mutations in HBV-related hepatocarcinogenesis as well. However, we found that *TERT* mRNA expression was significantly lower in patients with the mutant *TERT* promoter compared to those with the WT *TERT* promoter. Theoretically and actually, somatic mutations in the *TERT* promoter upregulate *TERT* expression by PROX1 recruitment, as seen in our *in vitro* data. However, those phenomena were not observed in our human data. Moreover, a positive correlation between *TERT* and *PROX1* mRNA expression was observed in only non-B viral HCCs. Thus, we hypothesized that HBV infection, especially *HBx* expression, hindered the PROX1-mediated *TERT* transcription. The reason for focusing *HBx* expression is that HBV genome integration, another factor induced by HBV infection, is known to be mutually exclusive with *TERT* promoter mutations. Moreover, HBV integration into the *TERT* promoter has a close relation

to high level of *TERT* mRNA expression.[40] To date, the effects of HBx on telomerase activity have been debated. Several studies suggested that HBx upregulated *TERT* mRNA expression,[23,24] however, Su *et al.* showed that HBx induced telomere shortening by acting as a transcriptional corepressor of MAZ on the *TERT* promoter.[11] In the present study, stable *HBx* expression also inhibited the physical binding of PROX1 to the *TERT* promoter resulting in hindrance of *TERT* promoter activation in HCC cell lines. This regulatory mechanism eventually led to downregulation of *TERT* mRNA expression. Further study is necessary to elucidate how to modulate the PROX1 binding affinity to ETS/TCF binding motifs by stable *HBx* expression.

A previous study on bladder cancer has reported the significance of a SNP rs2853669 for interfering *TERT* mRNA expression, which affected patient survival and disease recurrence when coupled with existing *TERT* promoter mutations.[14] As observed in our *in vitro* study, variant C allele of the rs2853669 slightly disrupted *TERT* promoter activation induced by mutations in HCC cell lines. However, there are no statistically significant difference between *TERT* mRNA expression and rs2853669 status in B viral hepatocarcinogenesis. Meanwhile, in those with non-carrier rs2853669, the high *TERT* mRNA level has a close relation to a poor overall survival and a disease-free survival. These data indicate that a SNP rs2853669 alone is of little importance for *TERT* mRNA expression and clinical outcomes in B viral hepatocarcinogenesis.

AFP is a biomarker used for the diagnosis and observation of HCC. Serum AFP levels are abnormally elevated in HCC, but this is not always the case. In the

present study, *TERT* promoter mutations were significantly frequent in B viral HCC patients with lower levels of AFP. In addition, there is impact on a poor overall survival by *TERT* promoter mutations regardless of rs2853669 status. These findings imply that occurrence of *TERT* promoter mutations can be a potential marker in the clinic through early detection in high-risk patients and monitoring of HCC patients who have low AFP levels.

V. CONCLUSION

TERT promoter somatic mutations, especially -124C>T mutation, are early events in B viral human multistep hepatocarcinogeneis and can be clinical biomarkers to make a prognosis of the patient survival. PROX1 is identified as a novel transcriptional activator for *TERT* gene through dominantly binding to the mutant *TERT* promoter sequence harboring hot spots in HCC. HBx contributes to downregulation of *TERT* mRNA expression through defunctionalization of PROX1.

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ABSTRACT (in Korean)

HBV 유래 간암발생과정에서의 TERT promoter 돌연변이
: 안정적인 HBx 발현에 따른 PROX1 유도 TERT 전사의 억제

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Telomerase 활성화에 관련된 Telomerase reverse transcriptase (*TERT*) 유전자의 promoter 체세포 돌연변이는 다양한 암에서 발견되며, 돌연변이 다발점으로 알려진 ATG 개시 codon의 상류 (-124 bp와 -146 bp) 염기 서열에서 빈번히 발생한다고 알려져 있다. 본 연구에서는 이러한 *TERT* promoter의 유전적 변이가 HBV 유래 간암 발생에서 어떠한 빈도로 나타나며 어떠한 작용 기전을 보이는지에 대하여 밝히고자 하였다. 간암 세포주를 이용한 실험을 통하여, *TERT* promoter의 돌연변이, 특히 염기 서열 -124 bp 지역의 변이는 *TERT* promoter 활성을 명백히 증가시켰다. 이러한 결과는 prospero homeobox protein 1 (PROX1) 단백질이 *TERT* promoter의 돌연변이

다발점에 결합하여 promoter 활성을 증가시키는 전사 활성인자로서의 기능에 관련된 것임을 새로이 확인하였으며, 특히 PROX1의 결합력은 *TERT* promoter의 돌연변이 결과로 생성된 E-twenty six/ ternary complex factor (ETS/TCF) 결합 서열 (CCGGAA)에서 증가함을 확인하였다. 또한 HBV 유래 간암 환자의 간 조직을 이용한 실험을 통하여, *TERT* promoter의 체세포 돌연변이 발생은 저등도 형성이상결절에서 9.0%, 고등도 형성이상결절에서 13.5%, 조기 간세포암종에서 27.3% 그리고 진행성 간세포암종에서 28.4%의 발생 빈도를 보여 간암으로 진행 시 *TERT* promoter의 체세포 돌연변이 발생이 점진적으로 증가함을 확인하였다. 그리고 이러한 *TERT* promoter의 돌연변이 발생은 HBV 유래 간암 환자에서 낮은 alpha-fetoprotein (AFP) 수치와 불량한 전체 생존율을 보였다. 하지만 생체외 실험 결과와는 달리, *TERT* promoter의 돌연변이를 지닌 HBV 유래 간암 환자에서 *TERT* mRNA 발현은 돌연변이를 지니지 않는 환자보다 낮았다. 또한 *TERT* mRNA 발현 정도는 *PROX1* mRNA 발현 정도와 상관 관계를 보이지 않았다. 그러나 비 HBV 유래 간암 환자에서는 그 둘 간에 양의 상관 관계를 보였다. 이러한 현상은 흥미롭게도 HBx와 관련된 것으로, *HBx*가 안정 발현되는 간암 세포주에서는 PROX1 유도 *TERT* mRNA 발현이 저해됨을 확인하였다. 요약하면, *TERT* promoter의 체세포 돌연변이는 HBV 유래 간암 발생

과정 중 초기 단계에 나타나는 현상이며, HBx는 전사 활성인자 PROX1의 기능 저하를 야기하여 *TERT* mRNA 발현을 조절할 수 있음을 본 실험을 통하여 밝혔다.

핵심되는 말: *TERT* promoter mutation, B viral hepatocarcinogenesis, *PROX1*, transcription factor, *HBx*