Expression Profile of a γ-Deletion Variant of the Human Telomerase Reverse Transcriptase Gene[1]

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Abstract

The human telomerase reverse transcriptase (hTERT) is an essential component of the holoenzyme complex that adds telomeric repeats to the ends of chromosomes. The hTERT transcript has been shown to have two deletion type alternative splicing sites. One deletion site induces the α-deletion variant, lacking 36 bp from exon 6, and the other induces the β-deletion variant, lacking 182 bp from exons 7 and 8. Here, we identified a novel deletion variant of the hTERT transcript in hepatocellular carcinoma cell lines. The deleted transcript was characterized by an in-frame deletion of 189 bp, spanning nucleotides 2710 to 2898, corresponding to the complete loss of exon 11 (γ-deletion). The region lacking in the γ-deletion lies within RT motifs D and E, suggesting that it is missing conserved residues from the catalytic core of the protein. Both γ- and α-deletion variants were occasionally detected, but the β-deletion variant was frequently observed. Our results may provide important information for more detailed studies on the regulation of telomerase activity.

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Keywords: telomerase; hTERT; alternative splicing; γ-deleton ASV; telomere.

Introduction

Pre-mRNA splicing is a fundamental biological process involved in the expression of genes, and up to a third of human genes are thought to be alternatively spliced [1,2]. General alternative splicing takes place in variant proteins and expression patterns as products of different genes. Recently, Serine–Arginine (SR) proteins have been shown to play a role in constitutive and alternative splicing, and the characters and functions of SR proteins have now been extensively investigated [3,4]. Some alternatively spliced variants (ASVs) have been associated with a subset of human diseases [5–8]; however, the pathological importance of ASVs in most human diseases is still uncertain.

Human telomerase reverse transcriptase (hTERT) [9–11], which has two deletion-type ASVs [12,13], is the protein component for telomerase. Telomerase adds DNA repeats to the ends of chromosomes [14], and the enzyme telomerase plays a crucial role in cellular proliferation, development, and tumorigenesis [15,16]. The *hTERT* gene consists of 16 exons and the transcript is about 4.0 kb long [17]. The protein has a molecular mass of 127 kDa and contains a telomerase-specific motif and seven reverse transcriptase (RT) motifs [17,18]. The deletion-type ASVs are α- and β-deletion types [19–21]. The α-deletion ASV lacks 36 nucleotides from exon 6 including motif A, and the β-deletion ASV lacks 182 nucleotides from exons 7 and 8, including motif B′. The α-deletion ASV mRNA has occasionally been detected, whereas the β-deletion mRNA is frequently observed irrespective of the telomerase activity [19–21].

We have now identified a novel ASV of hTERT mRNA. The ASV identified lacks the entire exon 11, and the protein encoded by this ASV was conserved with no frameshift mutation. Although ASVs are transcripts from a single gene, it is important to discriminate the full-length isoform from ASVs. The aim of the present investigation was to demonstrate evidence of a novel ASV of *hTERT* gene expression in hepatocellular carcinoma (HCC) cell lines. Here, we developed a polymerase chain reaction (PCR)–based specific assay for the quantification of alternatively spliced hTERT expression.

Materials and Methods

Total RNA was extracted from three cell lines [Japan Health Science Foundation (HSRRB), Osaka, Japan] derived from HCC, Huh 7, HLE, and Huh 6–clone 5, and from two normal cell lines (HSRRB) LI 90 (derived from normal Ito cells) and HUV-EC-C (derived from normal endothelial cells) (Table 1) using the RNAzolB reagent (Sawady, Shinjuku, Japan) following the

Abbreviations: hTERT, human telomerase reverse transcriptase; ASV, alternatively spliced variant; HCC, hepatocellular carcinoma; RT real-time PCR, reverse transcriptase real-time polymerase chain reaction; TRAP, telomeric repeat amplification protocol

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[1]The sequences reported in this paper have been deposited in the GenBank database (accession nos. AB085628).

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Table 1. Description of Cell Lines in this Study.

Cell lines	Source	JCRB number	Establisher
Huh 6–clone 5	Well-differentiated hepatoma	JCRB0401	I. Doi, J. Sato
Huh 7	Differentiated hepatoma	JCRB0403	H. Nakabayashi, J. Sato
HLE	Nondifferentiated hepatoma	JCBR0404	I. Doi, J. Sato
LI 90	Normal Ito cell	JCBR0160	K. Murakami, et al.
HUV-EC-C	Normal endothelial cell	IFO50271	H. Hoshi

manufacturer's instructions. The final RNA preparations were resuspended in diethylpyrocarbonate-treated water and quantified by absorbance analysis at 260 nm. Complementary DNA (cDNA) was prepared by incubating DNase-treated total RNA (1.0 μg) with M-MLV RT (Invitrogen, Carlsbad, CA, USA) in the presence of random primers.

ASV Identification

The primer set for the amplification of an hTERT mRNA was designed according to GenBank AF128894, using forward primers at the exon 10 region (5′-CTG CAG GAG ACC AGC CCG C-3′) and reverse primers at the exon 13 region (5′-ACA CCG TCT GGA GGC TGT TCA-3′). The reaction parameters were 95°C for 30 seconds, 60°C for 40 seconds, and 72°C for 30 seconds for 45 cycles, followed by a 10-minute extension at 72°C using AmpliTaq Gold DNA polymerase (PE Applied Biosystems, Foster City, CA). Half of the PCR product was separated by electrophoresis in TBE buffer in a 3.0% agarose gel, stained with ethidium bromide, and then detected with ultraviolet light (Figure 1*a*).

The PCR products of hTERT were purified using a High Pure PCR Product Purification Kit (Roche Molecular Biochemicals Diagnostics, Indianapolis, IN), cloned into a pCR2.1 vector (Invitrogen) and then sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) with an ABI PRISM 3100 Genetic Analyzer (PE Applied Biosystems). Finally, the sequence was compared with the full-length hTERT mRNA sequence (Figure 1*b*).

Specific Quantitative System (RT Real-Time PCR)

The real-time PCR reaction mixture was prepared using a TaqMan PCR Core Reagents Kit (PE Applied Biosystems). The primers and probes to amplify the mRNA of the full-length isoform and ASVs mRNA are shown in Table 2. The real-time PCR reaction was performed for 55 cycles (95°C for 30 seconds, 60°C for 45 seconds, 72°C for 30 seconds)

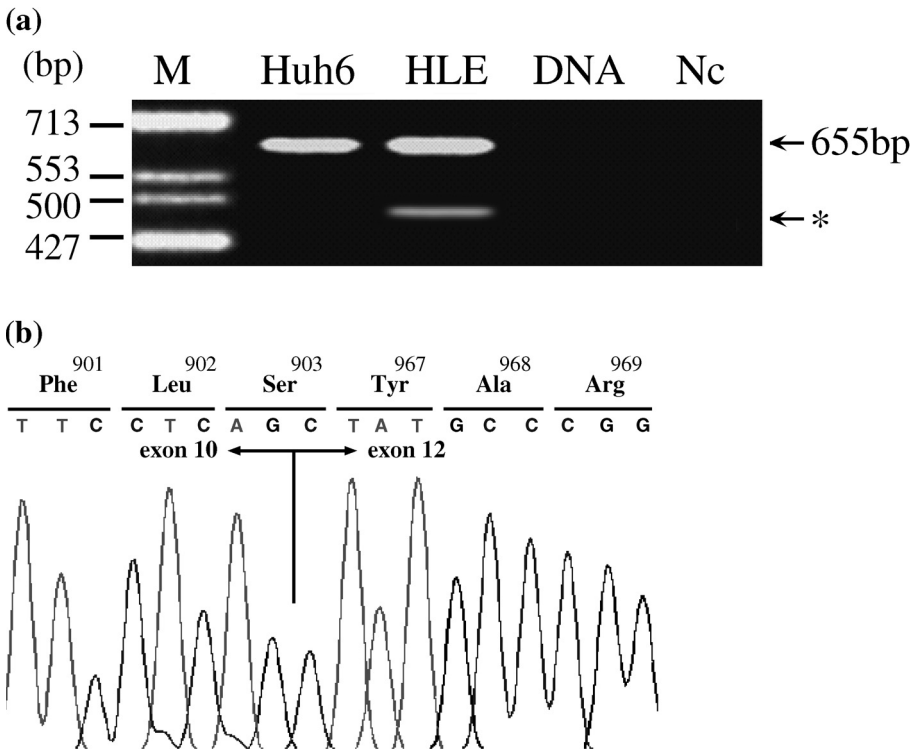

Figure 1. *Alternative splicing of hTERT mRNA. (a) RT-PCR products of the hTERT gene spanning exons 10 to 13. An obvious shorter signal (*arrow) was observed in the HLE sample. Huh 6, Huh 6–clone 5 cell line; HLE, HLE cell line; DNA, genomic DNA; Nc, negative control (RNase-free water); M, molecular weight markers φX174 DNA/HinfI digest. (b) Sequence analysis of the transcript bearing the deletion of exon 11 of the hTERT gene. The sequence in (a) shows the in-frame deletion spanning codons 904 to 966, corresponding to the complete loss of exon 11. An arginine-to-serine (Arg → Ser) transition is revealed at codon 903.*

Table 2. Primers and Probes for Quantification of hTERT mRNA.

	Primers and probes	Positions
ex6-F primer	5′-ATG TGA CGG GCG CGT ACG A-3′	Exon 6, nt 2135–2154
Adel-F primer	5′-CTG AGC TGT ACT TTG TCA AGG ACA GG-3′	Exon 5, nt 2111–2130; exon 6, nt 2167–2172
ex11-R primer	5′-GGA AGT TCA CCA CTG TCT TCC GC-3′	Exon 11, nt 2699–2721
Gdel-R primer	5′-CTT CCT CAG CTA TGC CCGGAC CT-3′	Exon 10, nt 2646–2654; exon 12, nt 2843–2856
ex7 probe	5′-CCT GCA GGA GAC CAG CCC GCT GAG G-3′	Exon 7, nt 2337–2361
Bdel probe	5′-CTT CAA GAG CCA CGT CCT ACG TCC AGT GCC-3′	Exon 6, nt 2274–2286; exon 9, nt 2369–2385

using a real-time PCR system (ABI PRISM 7700 Sequence Detection System; PE Applied Biosystems).

To prepare standard RNA for the quantification, PCR products were cloned into a pBluescript vector (Stratagene, La Jolla, CA) and standard RNA was synthesized using T7 RNA polymerase and purified by RNAzolB and DNase I (TaKaRa, Shiga, Japan) treatment.

Quantification of the mRNA samples was carried out by relating the PCR threshold cycle obtained from the cell line samples to the amplicon-specific standard curves. Serial dilution of standard RNA was carried out in duplicate from 10^7 copies to 10^2 copies and used in triplicate RT real-time PCR reactions. The mRNA expression levels are presented as the mRNA copy number per microgram of total RNA [22].

Telomerase Activity

The telomerase activities of all cells were assessed using a TRAP-eze Kit (Intergen, New York, NY) with a Cy-5–labeled TS primer as described elsewhere [23,24].

Results

Identification of a Novel ASV of hTERT

The PCR mixture presented two bands on electrophoresis (Figure 1*a*). The sizes of these PCR products were 655 bp (no-deletion isoform) and 466 bp. The sequence of the 466-bp product lacked 189 bp from the entire exon 11 (γ-deletion; Figure 1*b*). For the γ-deletion ASV, longer cDNA were sequenced based on 5′ and 3′ rapid amplification of cDNA ends (RACE) with long-distance PCR. The protein encoded by the novel γ-deletion ASV mRNA was 63aa shorter than the full-length isoform. Amino acid sequences without the missing region were conserved because no frameshift mutation was observed.

To verify whether the exon deletion observed in our samples was caused by genomic hTERT alteration, we amplified the region of the hTERT genomic DNA that included the splicing site of exon 11. Single-strand conformation polymorphism (SSPC) analysis of exon 11 did not demonstrate germline mutations responsible for the deletion

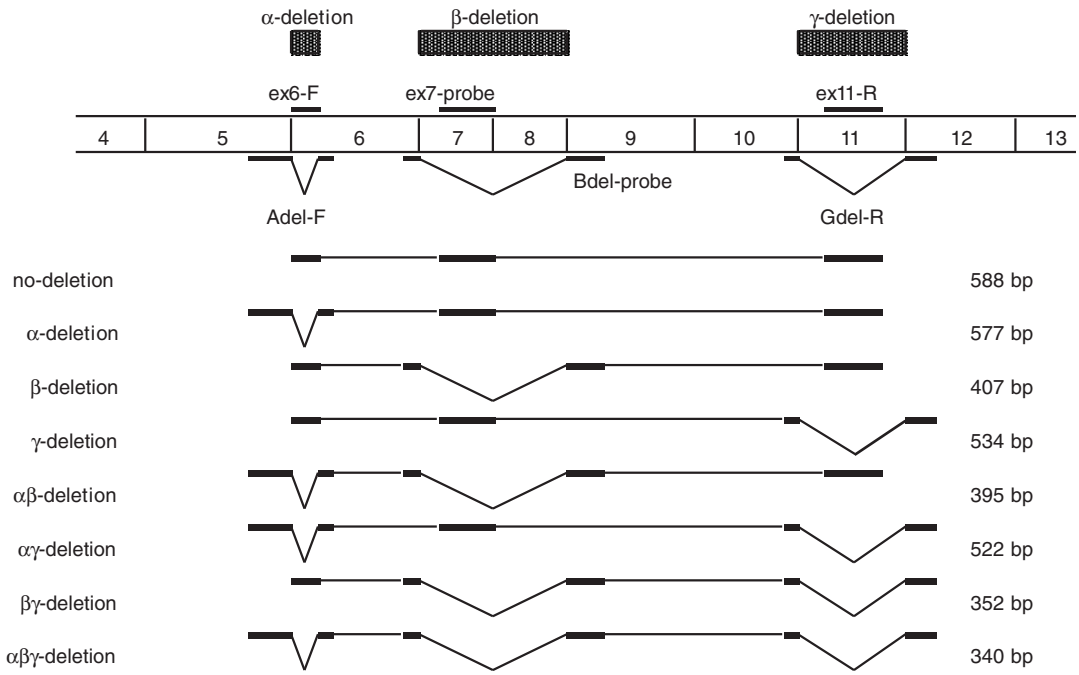

Figure 2. *Theoretically, alternative splicing of the hTERT transcript may lead to many isoforms. The α site causes a 36-base deletion resulting in a no-frameshift mutation, and the β splice site results in a 182-base deletion resulting in a nonsense mutation. The γ splice site induces a 189-base deletion resulting in a no-frameshift mutation. The size of the PCR products produced by each primer set depends upon the alternative splicing of the hTERT transcript in the samples. The Adel-F and ex6-F primers were forward primers, and ex11-R and Gdel-R were reverse primers.*

Table 3. Expression and Relative Levels of the hTERT and Alternatively Spliced Isoforms.

Cell lines	hTERT mRNA*										Telomerase† activity
	No deletion	α	β	γ	αβ	αγ	βγ	αβγ	No deletion + β	Total hTERT	
Huh 6	2.77	2.23	2.95	0.00	1.98	0.00	1.77	0.00	3.17	3.26	44
	(32.5%)	(9.4%)	(49.5%)	(0.0%)	(5.3%)	(0.0%)	(3.3%)	(0.0%)	(82.0%)	(100%)	
Huh 7	3.28	1.93	4.03	1.79	3.08	1.57	2.06	0.00	4.10	4.15	76
	(13.4%)	(0.6%)	(75.9%)	(0.4%)	(8.6%)	(0.3%)	(0.8%)	(0.0%)	(89.3%)	(100%)	
HLE	3.29	2.30	3.84	2.32	2.89	0.00	2.04	1.93	3.95	4.01	69
	(18.9%)	(1.9%)	(67.7%)	(2.0%)	(7.5%)	(0.0%)	(1.1%)	(0.8%)	(86.6%)	(100%)	
LI 90	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0
	(0.0%)	(0.0%)	(0.0%)	(0.0%)	(0.0%)	(0.0%)	(0.0%)	(0.0%)	(0.0%)	(0.0%)	
HUV-EC-C	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0
	(0.0%)	(0.0%)	(0.0%)	(0.0%)	(0.0%)	(0.0%)	(0.0%)	(0.0%)	(0.0%)	(0.0%)	

Upper row: Each mRNA was calculate as log copies per microgram of total RNA; lower row: relative level of each mRNA was calculated.
*The means of triplicate determinations are shown.
† The means of duplicate determinations are shown (TPG units).

(data not shown). The possibility that the deleted transcript could have resulted from PCR false priming or other artifacts was excluded because triplicate experiments based on independent RNA extractions and cDNA preparations yielded consistent results.

Here, we have identified a novel ASV, γ-deletion ASV. The sequence was compared with nonredundant sequences in GenBank, and no identical sequences were found. The sequence of the γ-deletion ASV was submitted to GenBank (access no. AB085628).

Estimation of hTERT Expression

Theoretically, alternative splicing of the hTERT transcript may lead to eight isoforms, as illustrated in Figure 2. The α-deletion ASV, γ-deletion ASV, and (an ASV combination of these) the αγ deletion ASV were characterized by in-frame deletion, whereas the β-deletion ASV, αβ deletion ASV, βγ deletion ASV, and αβγ deletion ASV were characterized by out-of-frame deletion, followed by premature termination. All the ASVs were observed in this study.

The results of the relative levels are shown in Table 3. No hTERT mRNA were detected in LI 90 and HUV-EC-C cells. In the three cell lines with hTERT signals, the average intensities of the no-deletion isoform, α-deletion, β-deletion, γ-deletion, αβ deletion, αγ deletion, βγ deletion, and αβγ deletion ASVs were 22%, 4%, 64%, 1%, 7%, < 1%, 2%, and < 1%, respectively. The total hTERT level was more than 10^4 copies in the Huh 7 and HLE cell lines, and about 10^3 copies in the Huh 6−clone 5 cell line. The β-deletion ASV showed the highest expression of all the hTERT isoforms. The γ-deletion ASV and the combination ASVs of the αγ, βγ, and αβγ deletion were of very low intensities.

The average telomerase activities in the Huh 6−clone 5, Huh 7, and HLE cells were 44, 76, and 69 units, respectively.

Discussion

Telomerase is activated in a variety of malignant tumors [25]. Thus, regulation of telomerase activity could be an important mechanism to limit the growth of cancer cells. Telomerase activity has been shown to correlate well with the expression level of hTERT [26−28]. Furthermore, it has been reported that alternative splicing plays an important role in hTERT regulation [29,30]. The β-deletion ASV causes premature translation termination, whereas the α-deletion ASV is 36 bp and lies within RT motif A, suggesting that it may be a candidate as a dominant-negative inhibitor of telomerase [20,21]. The ASVs of the *hTERT* gene have been reported not only as deletion types, but also as insertion types. The insertion types of ASVs are the 38-nucleotide insertion of intron 4, the partial insertion of intron 11, the 159-nucleotide insertion of intron 14, and the replacement of the complete exon 15 and the 5′ part of exon 16 with the first 600 nucleotides of intron 14 [12]. Both the α- and γ-deletion ASVs were in-frame deletions, whereas the others were out-of-frame mutations. The novel γ-deletion ASV will cause serious defects because the γ-deletion causes the loss of RT motifs D and E. Although the authors investigated only a limited number of hTERT-expressing cell lines, it is possible that the γ-deletion ASV may be a candidate as a dominant-negative inhibitor of telomerase, along with the α-deletion ASV.

It was clear that the intensity of telomerase activity in HCC nodules was closely associated with tumor differentiation [31]. The telomerase activity in well-differentiated HCC tissues was lower than that in poorly differentiated HCC tissues [32]. The total hTERT mRNA level in Huh 6−clone 5, a well-differentiated cell line, was one sixth of that in Huh 7, a differentiated cell line, and one eighth of that in HLE, a nondifferentiated cell line. In this study, the hTERT expression in the well-differentiated HCC cell line was also lower than that in the not well-differentiated HCC cell lines.

In the HCC cell lines, our results complement those of Yi et al. [33] who described that the β-deletion ASV showed the highest expression of all the hTERT isoforms. In this study, the amount of both the β-deletion ASV and the no-deletion isoform also accounted for more than 80% of the total hTERT levels. If the γ-deletion ASV was a dominant-negative inhibitor, the hTERT mRNA that introduced telomerase activity were both the β-deletion ASV and the no-deletion isoforms. In this study, there was a statistically significant difference between the amount of the no-deletion isoform as well as the β-deletion ASV expression and the telomerase activity

($r^2 = 0.983$). Thus, researchers who utilize a genetic marker instead of the telomerase activity [34,35] should measure both hTERT levels.

Although the γ-deletion ASV showed a low expression in the cell lines with high telomerase activity, this ASV may occasionally be detected in samples with low telomerase activity, such as HCC nodules. This ASV was not necessarily specific for tumor cell lines; however, it may have some roles as a dominant-negative inhibitor, along with the α-deletion ASV.

While there are still concerns, our results might provide the basis for more detailed studies on the regulation of telomerase activity, and may lead to the development of new cancer therapies. Because this study on the associations between these ASVs and the clinicopathological features have only been developing recently, it will provide useful information not only for developing prevention strategies for HCC but also for clarifying the biological mechanism of ASVs in human diseases.

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