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Quantification of hTERT Splice Variants in Melanoma by SYBR Green Real-time Polymerase Chain Reaction Indicates a Negative Regulatory Role for the β Deletion Variant[1]

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Abstract

Telomerase activity is primarily determined by transcriptional regulation of the catalytic subunit, human telomerase reverse transcriptase (hTERT). Several mRNA splice variants for hTERT have been identified, but it is not clear if telomerase activity is determined by the absolute or relative levels of full-length (functional) and variant hTERT transcripts. We have developed an SYBR green–based reverse transcription–quantitative polymerase chain reaction assay for the enumeration of the four common hTERT mRNA variants and correlated these with telomerase activity and telomere length in 24 human melanoma cell lines. All except five of the lines expressed four hTERT transcripts, with an overall significant level of co-occurrence between absolute mRNA levels of full-length α+/β+ hTERT and the three splice variants α−/β+, α+/β−, and α−/β−. On average, α+/β+ made up the majority (48.1%) of transcripts, followed by α+/β− (44.6%), α−/β− (4.4%), and α−/β+ (2.9%). Telomerase activity ranged from 1 to 247 relative telomerase activity and correlated most strongly with the absolute amount of α+/β+ ($R = 0.791$, $P = .000004$) and the relative amount of α+/β− ($R = -0.465$, $P = .022$). This study shows that telomerase activity in melanoma cells is best determined by the absolute expression of full-length hTERT mRNA and indicates a role for the hTERT β deletion variant in the negative regulation of enzyme activity.

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Introduction

Telomerase is a ribonucleoprotein complex that maintains chromosome length by synthesizing and adding repetitive $(TTAGGG)_n$ DNA sequences to the ends of telomeres [1]. Its absence from most normal somatic cells is believed to contribute to eventual senescence and limited cellular life span [2], whereas its reactivation in immortalized cells has been associated with the unlimited growth potential required for malignancy [3–5]. In particular, progression of melanoma is known to be accompanied by a steady increase in telomerase activity during the transformation of isolated naevi to metastatic disease [6,7], with virtually all melanoma cell lines exhibiting some degree of telomerase activity [6]. These discriminating properties have made telomerase an attractive target for cancer therapy [8], and there has been much emphasis on methods for detecting and determining the regulatory mechanisms of this important enzyme.

Telomerase is made up of two essential components: a constitutively expressed human telomerase RNA (hTR), which acts as a transcription template [9,10], and a catalytic human telomerase reverse transcriptase (hTERT), whose expression controls enzymatic activity

Abbreviations: hTERT, human telomerase reverse transcriptase; RTA, relative telomerase activity; hTR, human telomerase RNA

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[11,12]. The transcriptional and posttranscriptional regulation of hTERT is complex and remains to be fully elucidated. Studies in human development have revealed the presence of multiple hTERT RNA transcripts occurring in patterns that are both tissue-specific and gestational stage–dependent [13]. Such nonrandom alternative splicing is a common method of genetic regulation in eukaryotes [14], and to date, 10 different splice variants of hTERT have been identified [13,15–17]. The most widely studied variants involve splicing at two main sites: the α splice site, which produces a 36-bp in-frame deletion within the conserved reverse transcript motif A; and the β site, which results in a 183-bp deletion and non–sense mutation that truncates the protein, effectively deleting the remaining three reverse transcriptase motifs [13,18]. Splicing at either site can occur independently or in combination to produce three variants from the full-length α+/β+: α−/β+, α+/β−, and α−/β−, which have been shown to occur at approximate proportions of 5%, 1%, 80% to 90%, and 5% to 15%, respectively, within various cancer cell lines [19]. To date, only the α−/β+ variant has been shown to exhibit any regulatory function, acting as a dominant-negative inhibitor of telomerase activity when overexpressed in either normal or tumor cells [20,21]. It is unclear whether the ratio of full length to spliced hTERT is important in determining telomerase activity [22], because some studies have shown that the absolute expression of hTERT is well correlated with telomerase activity [23–27], and still others have found no correlation with either relative or absolute amounts of variant transcripts [28]. The regulatory functions of various hTERT transcripts may well be cell type–specific; however, the many different methods used to quantify hTERT mRNA have made it difficult to interpret these findings.

Most reverse transcription–polymerase chain reaction (RT-PCR)–based assays for hTERT variants use primers that flank the α and β subunits and thus amplify all transcripts in one reaction (Figure 1). The products are then scanned by densitometry to give the relative distribution of hTERT variants in each sample [19,29,30], the results of which are largely biased by the competitive nature of PCR. A few techniques have been developed using dual-labeled probe technology on a real-time PCR platform [31–35]; however, most of these assays use primers designed to the region downstream from the α and β subunits and, therefore, do not discriminate between the deletion variants [31–33]. A commercially available Light Cycler kit (TeloTAGGGG hTERT Quantification Kit; Roche Diagnostics, Basel, Switzerland) has proven popular, but the exact primer sequences are not revealed, thus it is not clear which transcripts are measured. Therefore, results from these types of studies need to be interpreted with caution.

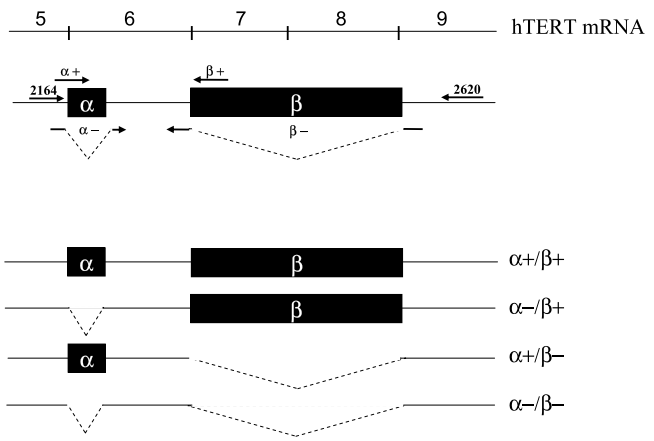

Figure 1. hTERT mRNA α and β deletion variants. Relevant hTERT exons are numbered and corresponding location of α and β sequences are illustrated by shaded boxes. Deleted sequences are designated by dashed lines. Locations of PCR primers are indicated by arrows and labeled according to the notation used in Tables 1 and 2.

The aim of the present study was to establish whether absolute or relative levels of hTERT variant transcripts determine telomerase activity. We developed an SYBR green–based real-time PCR assay as a more affordable option to labeled probes, with the further advantage of enabling melt curves to confirm the presence of specific transcripts. The assay was then used to determine the absolute and relative hTERT variant expression in a series of melanoma cell lines. In doing so, we have revealed the importance of overall levels of full-length hTERT mRNA in determining telomerase expression, and a possible role for the relative amount of β deletion variant in the regulation of telomerase activity.

Materials and Methods

Cell Lines

A total of 24 human melanoma cell lines were used for this study. ME4405 was a kind gift from Dr. Parmiani (Milan, Italy). MM200 was supplied by Dr. Parsons (Queensland Institute of Medical Research, Brisbane, Australia), and IGR3 was provided by Dr. Hope (Institute of Medical and Veterinary Science, Adelaide, Australia). Clones of the latter two lines along with the remaining lines, which were developed from primary melanoma tissue biopsies after receiving informed patient consent, were kindly provided by the Newcastle Melanoma Unit, Calvary Mater Newcastle. HL60 was obtained from the American Type Culture Collection (Manassas, VA) for use as a control. All melanoma cells were cultured in DMEM (JRH Biosciences, Australia), and HL60 was maintained in RPMI 1640 (JRH Biosciences), supplemented with 10% fetal bovine serum at 37°C and 5% CO_2.

Telomerase Activity

Telomerase activity was determined using the Telo TAGGG Telomerase PCR ELISA^{Plus} kit (Roche, Mannheim, Germany), which is based on the Telomeric Repeat Amplification Protocol assay with a nonradioactive ELISA detection. The procedure was performed on 3 ng of total cell protein in accordance with the manufacturer's instructions and results normalized to the cell line MM200 C3E8. All assays were performed singly and repeated on three separate occasions.

Measurement of Mean Telomere Length

The mean telomere length of each cell line was determined using Southern blot techniques as previously reported [36,37]. Briefly, genomic DNA was isolated and digested with restriction enzymes, *Rsa*I and *Msp*I (Amersham Pharmacia Biotech, Uppsala, Sweden), electrophoresed through a 0.6% agarose gel and transferred to Hybond N^+ membrane (Amersham Pharmacia Biotech). A fluorescein-labeled oligonucleotide probe $(CCCTAA)_3$ was hybridized to the membrane and detected using enhanced chemiluminescent detection (Amersham Pharmacia Biotech). Kodak Digital Science documentation software was used to determine the net intensity at 1-kbp intervals along the length of the telomere smear. The mean telomere

length was calculated as: $\Sigma(MW_i \times NI_i)/\Sigma(NI_i)$, where MW_i = molecular weight (kbp) at interval *i* and NI_i = net intensity (pixels) at interval *i* [38]. Assessment of the accuracy of the methodology showed a mean ± SE within-day variation of telomere length of 6 ± 1% and between-day variation of 9 ± 2% [36]. For each sample, the average of three mean telomere lengths calculated from three separate blots was used for statistical analysis.

Polymerase Chain Reaction

The full-length hTERT and the three splice variants were amplified from the HL60 cell line by PCR using previously reported primers and methods [16,39]. HL60 was used for optimization because it has been shown to express all four hTERT variants [29]. Products were resolved by agarose gel electrophoresis and identified based on size (Table 1). Bands were extracted from the gel and were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI). These fragments were used to establish specificity and sensitivity of the qPCR method.

Establishment of the qPCR Assay

Purified PCR products of each variant were used to optimize the qPCR cycling conditions. Specific primers were designed to the boundary spanning regions of the α and β subunits of hTERT, because this has been shown to be an effective way to detect splice variants without the use of probes [40]. These primers were combined with previously reported primers designed to hybridize to the α (hTERT 2172 [39]) and β subunits (variation of hTERT 2350 [39]) and thus only amplify variants containing these sequences (Table 2). Standard curves were generated, and the minimal detection limit was determined to be one copy per reaction. The specificity of the assay was confirmed by testing each primer pair against the purified PCR products for all variants, and in each case, the primers amplified only the splice variant to which they had been designed, with minimal cross-reactivity.

Reverse Transcription–Quantitative Polymerase Chain Reaction

Lysates prepared for telomerase activity analysis (previously mentioned) were pooled for each cell line and RNA extracted using the Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA) and quantified using the RiboGreen RNA Quantification Kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Reverse transcription reactions were standardized to the lowest RNA concentration, so that equivalent amounts of RNA (112 ng) from each cell line were synthesized into cDNA using Superscript III first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA). The cDNA equivalent of 5.6 ng of RNA was then amplified by qPCR in 20-μl reactions using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen) with 10 μmol of each appropriate primer. Reactions were run on a Rotor-Gene 3000 (Corbett Life Science, Sydney, Australia) using a four-step cycling program that consisted of the following: 50°C for 2 minutes, 95°C for 2 minutes followed by 60 cycles of 95°C for 10 seconds, 53°C or 58°C for 15 seconds, 72°C for 20 seconds, and data acquisition on the FAM/SYBR channel at 80°C for 10 seconds. This final acquisition was set to eliminate background fluorescence generated below this temperature. For GAPDH, a two-step cycling program was used: 50°C for 2 minutes, 95°C for 2 minutes followed by 60 cycles of 95°C for 10 seconds, and 60°C for 1 minute with data acquisition on the FAM/SYBR channel. A melt curve (57-95°C) was generated at the end of each run to verify specificity.

Table 1. Primers and Expected PCR Products for Amplification of hTERT Splice Variants.

Forward Primer	Reverse Primer	Annealing Temperature (°C)	Possible Amplicons (bp)
2164	2620	50–65 (gradient)	α+/β+ = 457
			α−/β+ = 421
			α+/β− = 275
			α−/β− = 239
α+	β+	53	α+/β+ = 202
α−	β+	53	α−/β+ = 172
α+	β−	53	α+/β− = 189
α−	β−	58	α−/β− = 159

Table 2. Primers Used for hTERT and GAPDH Amplification.

Primer	Orientation	Sequence	Reference
2164	Sense	gcctgagctgtactttgtcaa	Krams et al. [39]
2620	Antisense	cgcaaacagcttgttctccatgtc	Krams et al. [39]
α+	Sense	tgtactttgtcaaggtggatgtg	Krams et al. [39]
α−	Sense	ctgagctgtactttgtcaaggac	Lincz et al. (this study)
β+	Antisense	gtacggctggaggtctgtcaa	Variation of [39]
β−	Antisense	ggcactggacgtaggacgtgg	Lincz et al. (this study)
GAPDHA.69 f	Sense	ctctctgctcctcctgttcgac	Carraro et al. [54]
GAPDHA.69 r	Antisense	tgagcgatgtggctcggct	Carraro et al. [54]

Data Analysis

Each reaction was performed in duplicate on three separate occasions. Raw fluorescence values were exported and analyzed by Data Analysis for Real-Time PCR, a freely available excel file containing an algorithm that calculates the amplification efficiency of each sample from its amplification profile [41]. This has been validated and found to give comparable results to other methods of quantitation without the need for standard curves [41]. We used the average amplification efficiency for our calculations, because this has been shown to give the most accurate results [42]. Outliers were excluded and the threshold was set based on the mean midpoint (*M*) of the transformed signal range. Expression values (in arbitrary fluorescence units) were obtained for each hTERT transcript and normalized by dividing them by individual expression values for GAPDH to produce R_0. The mean of three experiments was determined for each transcript and expressed as a percentage of the reference cell line, MM200 C3E8 (absolute expression) or as a percentage of the total number of hTERT transcripts for each cell line (relative expression). Spearman's rank and Pearson's correlation coefficients were calculated to assess linear relationships among telomere length, telomerase activity, and hTERT mRNA expression. P values < .05 were considered statistically significant.

Results

The results are summarized in Table 3. Mean telomere length of the cell lines ranged from 2.19 to 7.37 kbp, with an overall average of 3.97 kbp. Telomerase activity was detected in all cells and expressed as a percentage of the MM200 C3E8 reference cell line that was arbitrarily set as 100. Relative telomerase activity (RTA) varied widely (1-247 RTA), with a mean of 112. Similarly, the absolute expression of hTERT mRNA transcripts was highly variable. When the

Table 3. Summary of Results for 24 Melanoma Cell Lines.

Analysis		Mean	Range
Telomere length (kbp)*		3.97	2.19–7.37
RTA (% of reference cell line)		112	1–247
Absolute hTERT transcript mRNA (% of reference cell line)	α+/β+	116	3–430
	R_0	6.1×10^{-4}	1.6×10^{-5} to 2.2×10^{-3}
	α−/β+	115	0–520
	R_0	4.0×10^{-5}	0.0 to 1.9×10^{-4}
	α+/β−	96	15–247
	R_0	4.3×10^{-4}	7.3×10^{-5} to 1.6×10^{-3}
	α−/β−	60	0–271
	R_0	7.7×10^{-5}	0.0 to 7.9×10^{-4}
Relative hTERT transcript mRNA (% of all transcripts)	α+/β+	48.1	3.94–69.3
	α−/β+	2.9	0.0–8.2
	α+/β−	44.6	21.9–96.1
	α−/β−	4.4	0.0–21.8
Ratio α+/β+:α−/β+, α+/β−, α−/β−		1.11	0.04–2.26

*n = 22.

transcripts were expressed as a percentage of the total amount of hTERT mRNA in each cell line, on average, the full-length α+/β+ made up the majority (48.1%) of transcripts, followed closely by the α+/β− variant (44.6%). The α−/β− and dominant-negative α−/β+ variants made up the smallest proportion of transcripts (4.4% and 2.9%, respectively). The average ratio of full-length transcript to spliced transcripts was 1.11 (0.04-2.26).

Individually, the full-length hTERT α+/β+ transcript and its α+/β− variant were detected in all cell lines (Figure 2). Only six cell lines did not express all of the variants; of these, three did not express either of the α deletion variants α−/β+ and α−/β−, two lacked only the α−/β+ transcript, whereas only one failed to express the double-deletion variant α−/β−.

There was high disparity in the relative proportions of hTERT mRNA variants among different cell lines (Figure 3). The full-length transcript was predominantly expressed in most cell lines, whereas the α+/β− was the main transcript in six lines.

Absolute Expression of the Full-length hTERT Transcript Is the Best Predictor of Telomerase Activity

The absolute level of each individual hTERT variant was positively correlated with telomerase activity, with the full-length message showing the strongest association (R = 0.791, P = .000004; Table 4). The relative amount of full-length hTERT was also positively correlated with telomerase activity, although this was not statistically significant (R = 0.403, P = .051), whereas the ratio of full-length hTERT to variant hTERT was significantly correlated with telomerase activity (R = 0.414, P = .044). The relative expression of α+/β− was negatively correlated with telomerase activity (R = −0.465, P = .022), whereas there was no significant correlation between telomerase activity and relative expression of either of the α deletion variants (α−/β+, α−/β−).

A High Level of Co-occurrence Exists between hTERT Variants

Despite some cell lines completely lacking expression of particular variants, there was an overall significant level of co-occurrence between absolute mRNA levels of the full length hTERT α+/β+ transcript and the three splice variants α−/β+ (R = 0.731), α+/β− (R = 0.559), and α−/β− (R = 0.825). Between variants, there was correlation between absolute expression of all hTERT transcripts except for α−/β+ and α+/β−. When expressed as relative proportions of hTERT, the amount of α+/β− variant was significantly inversely related to that of all other transcripts.

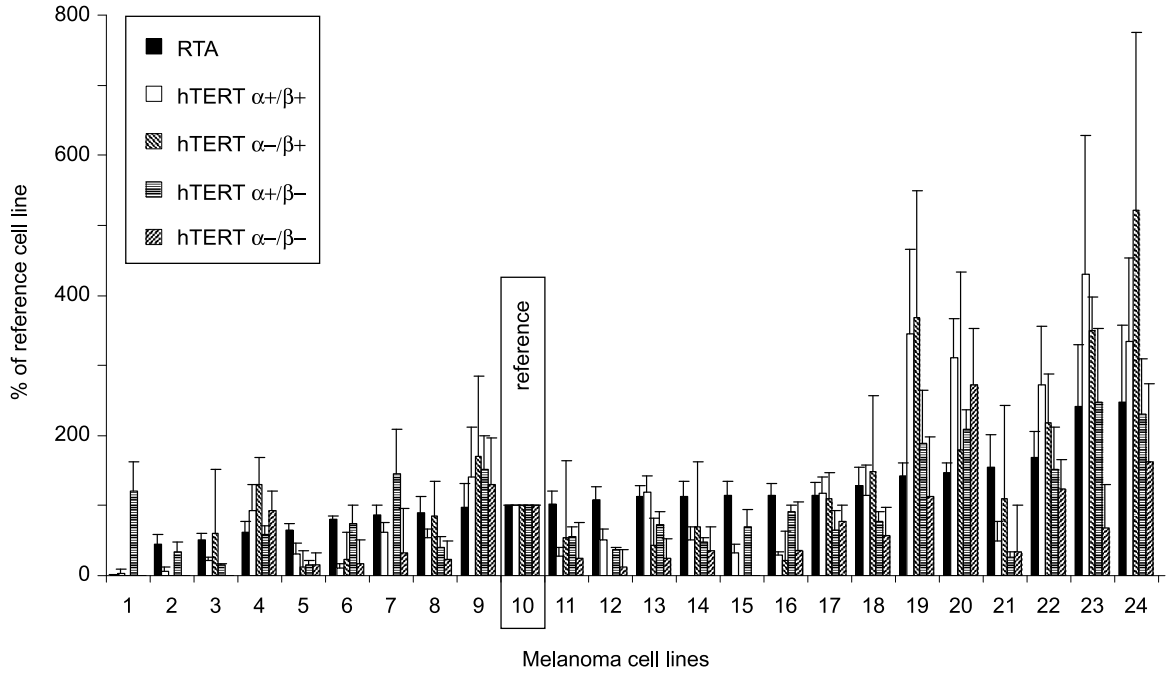

Figure 2. RTA and absolute expression of hTERT mRNA transcripts in melanoma cell lines. Levels of RTA and each variant mRNA are expressed as a percentage of the reference cell line, MM200 C3E8, arbitrarily designated as 100%, and error bars indicate SDs between three separate experiments.

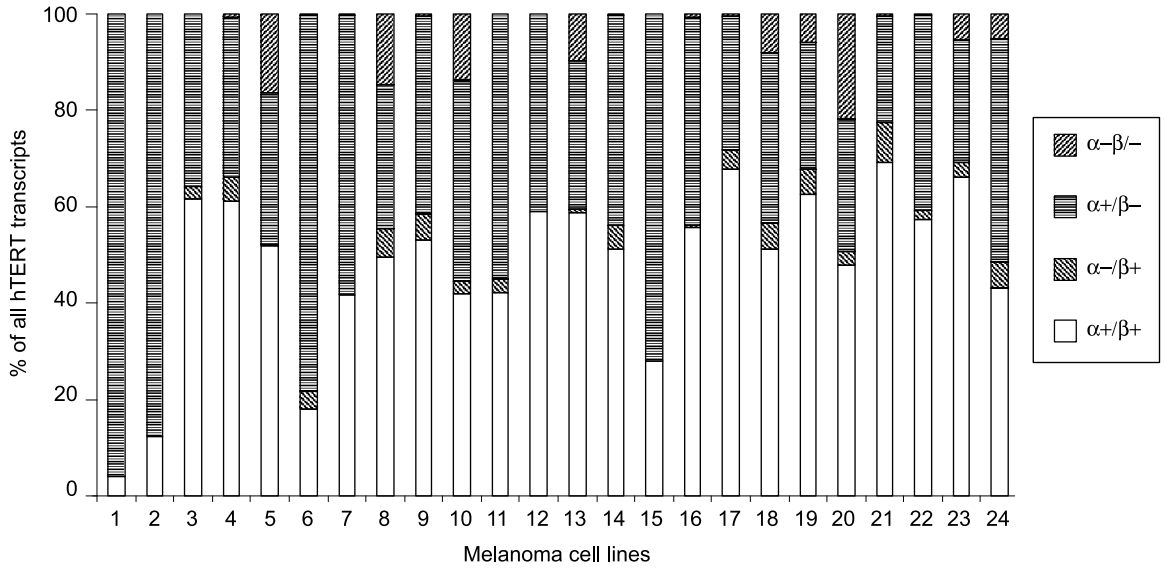

Figure 3. Relative expression of hTERT mRNA transcripts in melanoma cell lines. Levels of each variant mRNA are expressed as a percentage of the total four hTERT transcripts per cell line.

Telomere Length Was Not Correlated to Telomerase Activity

Telomere length was not correlated to telomerase activity (R = −0.233, P = .297) or to absolute or relative expression of any of the individual hTERT transcripts.

Discussion

The only comprehensive method of hTERT splice variant quantification to emerge so far has been a technique developed by Ohyashiki et al. [35], which uses TaqMan primer/probe sets to determine both absolute and relative transcript numbers for each of the four main hTERT variants. A similar approach by Mavrogiannou et al. [34] also seems to be highly specific for each variant but perhaps not as quantitatively reliable because it does not use a reference gene. Ours is the first report of an SYBR green–based assay that can distinguish between the four hTERT variant transcripts, providing a more economical option for such studies.

Unlike results reported for other cell types, the hTERT α+/β+ variant accounted for the largest fraction of hTERT message in this series of melanoma cells [19,34]. Our data confirms similar results from the only other investigation of these variants in a series of 52 melanoma lesions, which also found that expression of the full-length transcript was generally equal or slightly higher than the spliced variants, with a prevalence toward expression of the β deletion variant [22]. Taken together, these results suggest that the relative proportions of variants in melanoma cells do not follow the pattern described previously for other immortalized human cells, including mammary, prostate, renal, and non–small lung carcinoma cell lines [19]. In particular, we found higher-than-expected proportions of the full-length α+/β+, similar to a recent report on 6 cell lines and 28 non–small cell lung cancer tissue

Table 4. Correlations with Absolute and Relative Expression of hTERT Variants.

		Telomere Length	RTA	Absolute		
				α+/β+	α−/β+	α+/β−
Absolute	α+/β+	−0.266 (*0.231*)	**0.791** (***0.000***)			
	α−/β+	−0.231 (*0.299*)	**0.603** (***0.002***)	**0.731** (***0.000***)		
	α+/β−	−0.276 (*0.214*)	**0.535** (***0.007***)	**0.559** (***0.004***)	0.309 (*0.141*)	
	α−/β−	−0.257 (*0.249*)	**0.614** (***0.001***)	**0.825** (***0.000***)	**0.749** (***0.000***)	**0.547** (***0.006***)
				Relative		
				α+/β+	α−/β+	α+/β−
Relative	α+/β+	−0.059 (*0.793*)	0.403 (*0.051*)			
	α−/β+	−0.247 (*0.268*)	0.371 (*0.074*)	0.389 (*0.061*)		
	α+/β−	0.143 (*0.526*)	**−0.465** (***0.022***)	**−0.810** (***0.000***)	**−0.479** (***0.018***)	
	α−/β−	−0.232 (*0.298*)	0.320 (*0.127*)	0.218 (*0.305*)	**0.411** (***0.046***)	**−0.632** (***0.001***)
Ratio	α+/β+: α−/β+, α+/β−, α−/β−	0.047 (*0.837*)	**0.414** (***0.044***)			
RTA		−0.233 (*0.297*)				

Numbers given are correlation coefficients with associated P values italicized in parenthesis. Statistically significant values are highlighted in bold.

samples, which found that the α+/β+ made up approximately 50% of the total transcripts in these cells [34]. These authors also used a quantitative real-time PCR analysis that was specific for each variant and attributed their findings to this more sensitive approach. Thus, it is not clear if our findings are unique to melanoma cells or simply due to the different methods used in individual studies.

We found a strong level of co-expression of all three deletion variants with the full-length transcript, suggesting that up-regulation of hTERT transcription affects all variants to a similar extent. This phenomenon has also been reported for other cell types, where copy numbers of full-length hTERT mRNA correlated with all but the α deletion variant in acute leukemia cells [35] and specifically with the β deletion variant in cirrhotic liver [25] and melanoma lesions [22]. Chromosomal imbalances are common in melanoma, and in particular, the sequences on 5p15.33, which harbor the gene for hTERT, have been found to be overrepresented in 33% of cases [43]. This could result in dysregulation of the gene and may account for the widespread overexpression of all variants. The present study shows that it is this absolute increase in transcription of hTERT mRNA that is most important for determining telomerase activity.

Since the discovery that the α deletion variant has dominant-negative regulatory influences on telomerase [20,21], some studies have focused on this variant [44]. Only one has specifically addressed the β deletion variant and found that overexpression does not cause down-regulation of telomerase activity in immortalized lung fibroblasts, prostate carcinoma, or non–small cell lung cancer cell lines [20]. It is plausible that telomerase regulation by its splice variants is cell type–specific, as suggested by the precise patterns of splicing that occur in particular cell types during development [13]. It is conceivable that these same patterns become reactivated during tumorigenesis and, if so, could have profound implications for the development of therapeutic telomerase inhibitors.

In the present study, telomere length was not related to telomerase activity or hTERT gene expression, suggesting that the maintenance of chromosome length may not be the primary function of telomerase in these neoplastic cells. This theory is supported by studies showing that inhibition of telomerase can induce apoptosis of cancer cells in a manner that is independent of both p53 and telomere shortening [45–47]. Furthermore, therapeutic induction of differentiation in some cancer cells is accompanied by a reduction in telomerase activity [48–51], and recent gene expression profiling has revealed that this is more likely to be a causative rather than consequential factor of cell maturation [52]. These alternative roles for telomerase may be regulated through the splicing of hTERT, and further investigation into these mechanisms will require the use of sensitive quantitation methods.

Finally, it is becoming clear that alternative splicing of pre-mRNA is a common process, affecting at least 74% of human genes and effectively increasing the coding potential of the genome [53]. Missplicing of cellular genes often occurs in cancer, and deciphering the regulatory mechanisms governing splice site selection is a rapidly emerging area of research [53]. Cells such as the melanoma lines described herein should provide valuable tools to further this field of knowledge.

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