Association of *TERT* promoter mutations with telomerase expression in melanoma

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Keywords

TO THE EDITOR

Recurrent mutations in the promoter of the telomerase reverse transcriptase (*TERT*) gene were first discovered in melanoma (Horn et al., 2013; Huang et al., 2013) and subsequently in several other cancer types (Killela et al., 2013). These mutations occur mainly at positions −124 bp (chr5, 1,295,228 C>T hg19 coordinate) and −146 bp (chr5, 1,295,250 C>T hg19 coordinate) from the ATG translation start site (Heidenreich et al., 2014; Horn et al., 2013; Huang et al., 2013), and are hereafter termed as −124C>T and −146C>T, respectively (Figure 1a). Mutually exclusive −124C>T and −146C>T mutations have been detected in more than 65% of melanomas (Network, 2015). These mutations contribute to *TERT* transcriptional upregulation by recruiting the GABPA/B1 transcription factor (Bell et al., 2015). The association of *TERT* promoter mutations with increased *TERT* expression has been demonstrated in various tumor types such as bladder cancer and glioblastoma (Borah et al., 2015; Heidenreich et al., 2015), but the functional consequences of these mutations have not yet been clarified in melanoma.

In a study of 39 primary melanoma samples, Heidenreich et al. (2014) reported higher *TERT* expression levels in aggregate in 10 melanomas with various types of mutations compared to 29 samples with the wild-type *TERT* promoter (Heidenreich et al., 2014). Their study

provided circumstantial evidence that all cancer-associated *TERT* promoter mutations upregulate *TERT* expression in melanoma. However, an mRNA expression analysis by The Cancer Genome Atlas (TCGA) network, using RNA sequencing data from a large set of melanoma samples, found that only the −124C>T mutation was associated with increased *TERT* expression (Network, 2015). Therefore, the effect of the −146C>T mutation or the −138/−139CC>TT (chr5, 1,295,242–243 CC>TT) tandem mutation on telomerase expression in melanoma is still unclear.

To assess *TERT* mRNA expression levels in melanoma samples harboring −146C>T or −138/−139CC>TT mutations, in this study we used real-time quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) in a sample set of metastatic pediatric melanocytic tumors for which the methylation status and the mutational profile of the *TERT* promoter were known. We previously showed that in a subset of melanomas, *TERT* expression is mediated epigenetically by promoter hypermethylation rather than by promoter point mutations (Fan et al., 2016). For RT-qPCR, we used formalin-fixed paraffin-embedded (FFPE) tissues from 10 melanomas (7 conventional; 1 fatal spitzoid; 2 arising in giant congenital nevi) and included 9 atypical Spitz tumors with a wild-type unmethylated *TERT* promoter for comparison. The *TERT* promoter mutation and methylation data on these samples have been previously published (Fan et al., 2016; Lu et al., 2015). The conventional and spitzoid melanomas each harbored a hotspot *TERT* promoter mutation (−146C>T in 4; −124C>T in 3; −138/−139CC>TT in 1), and the two melanomas arising in giant congenital nevi (samples MM9, MM10) carried a wild-type hypermethylated *TERT* promoter.

For RT-qPCR, total RNA was isolated from FFPE tumor tissues by using the Maxwell® 16 LEV RNA FFPE Purification Kit (Promega, AS1260) according to the manufacturer's protocol. To quantify full-length *TERT* mRNA expression levels, 2 μg of total RNA from each sample was converted to cDNA by using the SuperScript® VILO cDNA Synthesis Kit (Invitrogen, 11754-010). RT-qPCR was performed in triplicate by using the TaqMan® Gene Expression Assays with gene-specific primers (Life Technologies) for *TERT* (Hs00972656_m1), and normalized with *GAPDH* (Hs02758991_g1) as the endogenous control. Relative expression levels were determined by using transcript levels of an atypical Spitz tumor as the reference.

TERT mRNA was undetectable or expressed at low levels by RT-qPCR in the atypical Spitz tumor samples (Figure 1c). Thus, we selected an atypical Spitz tumor with the highest *TERT* mRNA expression (sample # AST9) as the reference sample (Figure 1c). *TERT* mRNA in all melanomas, including the four samples with the −146C>T *TERT* promoter mutation (Figure 1b), was expressed at higher levels than those in atypical Spitz tumors with a 3- to 71-fold (average, 26-fold) increase relative to the control value (Figure 1c). RT-qPCR showed a wide range of expression across samples with similar *TERT* promoter mutations, whereas the range of expression overlapped between −124C>T (3- to 68-fold increase, average 33-fold) and −146C>T (5- to 71-fold, average 27-fold) mutant melanomas (Figure 1c). The two melanomas arising in congenital nevi showed a 20- and 27-fold increase relative to the control value.

To investigate whether gene copy number gains or amplification contributed to the observed variations in mRNA expression levels, we evaluated the 10 melanoma samples for *TERT* copy number alterations by interphase fluorescence in situ hybridization (FISH), using a probe specific to the *TERT* gene together with a control probe containing a specific sequence at 5q11.2. The probe sets were developed using bacterial artificial chromosome clones (BACPAC Resources, Oakland, CA) CTD-3080P12 covering the *TERT* sequence and RP11-350C7 covering the sequence of the *PLK2* control gene at 5q11.2. FISH analysis revealed that the *TERT* to *PLK2* ratio was >1 (1.6 to 2) in at least 40% of cells in four melanomas and there was a normal ratio of 1 in the remaining six melanomas. Interestingly, FISH showed a *TERT*/*PLK2* ratio of 1.9 in each of the two samples that had the highest *TERT* expression levels (MM4 and MM8) (Figure 1c and 1d). Although the findings suggested that gene copy gains might have contributed to the observed variations in telomerase expression, these assays could not determine whether the extra copies of *TERT* had a normal or a mutant promoter. It is conceivable that additional unaccounted biological mechanisms other than promoter mutations, copy number variations, or promoter hypermethylation contributed to *TERT* transcriptional upregulation. It is also possible that confounding factors secondary to tissue processing and formalin fixation affected the *TERT* transcript levels. One major shortcoming of our study is that our comparison is based on telomerase expression rather than the enzymatic activity of *TERT* mRNA. The direct enzymatic activity of *TERT* could not be evaluated in these tumors for which the only available tissue was FFPE material. Also, the small sample size (n=10) precluded statistical analysis and therefore the results need to interpreted with caution. Further, because all the melanoma samples were from pediatric patients only, it is necessary to reproduce these findings in a larger number of melanoma samples from adults.

The lack of an association between −146C>T mutations and elevated *TERT* mRNA expression in the melanoma TCGA data might be because the expression levels were compared relative to melanomas with wild-type promoters, of which some could be telomerase positive due to a different mechanism, such as promoter hypermethylation, rather than point mutations. There are several possible reasons for higher *TERT* expression in the −124C>T mutant melanomas than in other melanomas in the TCGA study. It has been shown that the −124C>T and −146C>T mutations activate *TERT* by distinct mechanisms (Li et al., 2015). The −146C>T promoter, unlike the −124C>T promoter, requires non-canonical NF-κB signaling in cooperation with ETS factors to induce *TERT* reactivation (Li et al., 2015). Although once differentiated into somatic cells both −146C>T and −124C>T mutations increased telomerase activity, a study in which embryonic stem cells were engineered to harbor a *TERT* promoter mutation reported that only the −124C>T mutation increased *TERT* transcription in pluripotent stem cells (Chiba et al., 2015). Also, an mRNA expression analysis in glioma samples showed that tumors with the −124C>T mutation had higher *TERT* expression levels than those with the −146C>T mutations (Heidenreich et al., 2015). Altogether, these findings suggest that the −124C>T genotype is more potent than the −146C>T genotype in inducing *TERT* expression, which could explain the observed difference seen in the TCGA data for *TERT* expression levels.

In conclusion, our RT-qPCR findings demonstrate that in melanoma, *TERT* promoter hotspot −146C>T and −138/−139CC>TT mutations, similar to the −124C>T mutation,

correlate with *TERT* overexpression. Our findings also support that the −146C>T and −138/−139CC>TT mutations contribute biologically to tumorigenesis in melanoma.

Acknowledgments

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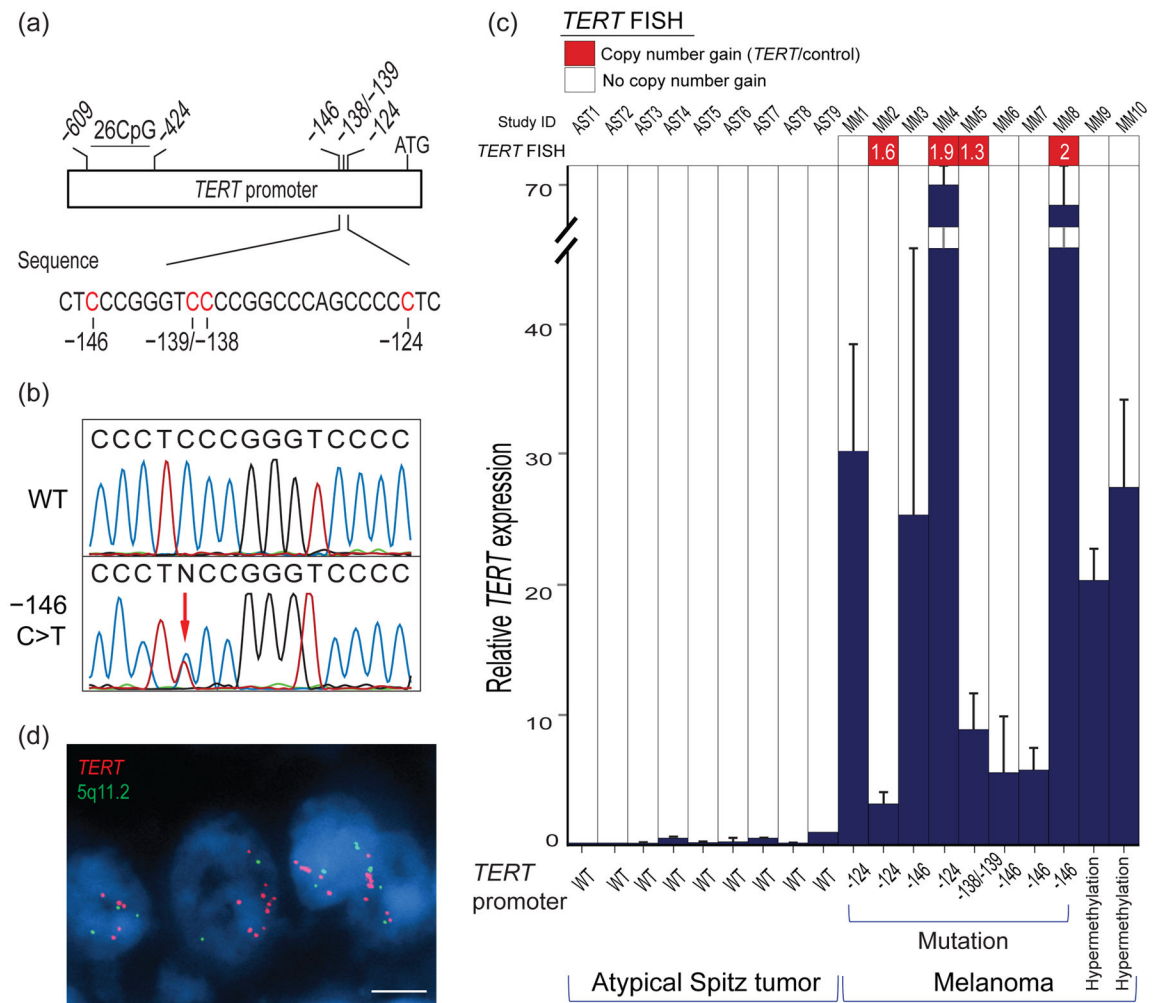
Figure 1.
TERT mRNA expression by RT-qPCR in 10 melanomas with *TERT* promoter alterations and 9 atypical Spitz tumors with the wild-type (WT) *TERT* promoter. a) Schematic of the *TERT* promoter showing the position of hotspot mutations. b) Sequence chromatogram of a wild-type (top) and a mutated (bottom) *TERT* promoter sequence. The bottom sequence is heterozygous at the chr5, 1,295,250 residue, indicated as an N in the printed sequence (red arrow), representing 2 overlapping peaks, a C (WT allele) and a T (mutant allele). c) *TERT* copy number and relative *TERT* mRNA expression data in melanomas with *TERT* promoter −146C>T, −124C>T, or −138/−139CC>TT mutations or *TERT* promoter hypermethylation versus atypical Spitz tumors with the WT *TERT* promoter. The *TERT* expression value in sample AST9 was used as the reference. The numbers in red boxes represent the *TERT* to *PLK2* ratio. d) Representative image of interphase fluorescence in situ hybridization (DAPI counterstain) in a melanoma sample (MM4) showing *TERT* copy gains in tumor nuclei (scale bar = 10 μm).