Classifying Melanoma by TERT Promoter Mutational Status

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Although TERT promoter mutations have been associated with a worsened prognosis in melanoma, the relationship between mutation status and downstream telomerase activity and telomere length remains convoluted. Using Sanger sequencing and techniques based on quantitative reverse transcriptase in real time, we evaluated 60 melanoma cell lines for TERT promoter mutational status, copy number, gene expression, and telomere length to provide a comprehensive analysis of the TERT/telomere pathway and establish a classification system whereby the associations between TERT mutations and their downstream molecular manifestations can more easily be ascertained. Mutations at positions -124/125 and -146 were associated with the highest levels of TERT gene expression but had no appreciable impact on absolute telomere length. In contrast, the common variant rs2853669 (at position -245) was significantly associated with longer telomere length via a recessive model in our cohort (P = 0.003). Our results, which are from assays performed on purified melanoma cell lines, suggest that the TERT promoter harbors a more complex mutational landscape than previously thought. Furthermore, the failure of TERT promoter mutations to consistently correlate with TERT expression and telomere length suggests an alternative method whereby tumor cells escape the critical shortening of telomeres.


INTRODUCTION

Mutations in the promoter region of TERT are associated with a decreased disease-free survival, increased tumor recurrence, and an increased rate of metastasis in melanoma (Hugdahl et al., 2018; Nagore et al., 2016a; Nagore et al., 2016b). Long known to be crucial for the maintenance of telomere length and the immortalization of cancer cells of all types, TERT promoter (TERTp) mutations are found in over 90% of aggressive malignancies and over 60% of melanomas (Cancer Genome Atlas Network, 2015; Bell et al., 2015). For these reasons, the TERT/telomerase/telomere pathway has been identified as a promising avenue for the targeted therapy, prognosis, and management of tumors of all types, melanoma included.

Several studies have confirmed a relationship between the most commonly observed mutations in TERTp (C>T transitions at positions -124 and -146 from the ATG start site) and TERT mRNA expression, telomerase activity, and telomere length (Cancer Genome Atlas Network, 2015; Chiba et al., 2017; Heidenreich et al., 2014; Huang et al., 2015; Lee et al., 2016; Vinagre et al., 2013). Despite these findings, no clinically actionable knowledge has been ascertained, and there exists only one drug (imetelstat, a telomerase inhibitor) that has demonstrated moderate success in phase 1 and 2 clinical trials in various cancers (clinicaltrials.gov identifiers NCT00510445 and NCT02011126). Furthermore, the functional relevance of several additional TERTp variants (particularly at positions -138/139 and -245) and the correlation between any of these mutations and their expected phenotypic manifestations has not been firmly established.

To address this inconsistency, we comprehensively characterized the TERT/telomere pathway in 60 cutaneous melanoma cell lines. What we observed was a clustering pattern based on a mutual exclusivity among the three most commonly mutated TERTp loci (-124 or -125, -146, and -138/139), which established a framework for characterizing the downstream effects of these mutations. Our results confirm a relationship between the 124/125 and 146 variants and elevated TERT gene expression, while presenting data that suggest an inverse relationship for the 138/139 variant. We also present evidence that expands upon the suggestion that the rs2853669 common variant (at position 245) contributes to the role of TERT in the survival and recurrence of melanomas by being significantly associated with longer telomere length (P = 0.003) (Nagore et al., 2016b).

**RESULTS**

We identified seven unique mutations in TERTp in 58 of 60 (96.7%) cell lines: -124C>T (43.3%), -146C>T (33.3%), -124/125C>TT (10.0%), -138/139C>TT (10.0%), -136C>T (1.7%), -100C>T (1.7%), and the familial variant -57A>C (1.7%) (Supplementary Table S1 and Supplementary Figure S1). We additionally identified the previously described single-nucleotide polymorphism rs2853669 (at position -245) in 51.7% of our cohort. The observed mutual
exclusivity at the most frequently occurring loci in our cohort (-124 or -125, -146, and -138/139) revealed a clustering pattern whereby four unique subgroups were established (Figure 1).

A total of 41 of 60 cell lines (68.3%) additionally harbored BRAF and 15 of 60 (25.0%) NRAS alterations, with no significant variability in the mutational frequency between classes (BRAF: $X^2 = 0.97; \ P = 0.81$; NRAS: $X^2 = 2.26; \ P = 0.52$). The loss of heterozygosity in TERT was detected at a rate of 33.9% in those cell lines with a mutated TERT promoter, both suggesting a mechanism of selection and reinforcing a possible functional advantage for TERTp mutations.

Consistent with the results of previous studies, TERTp mutations at -124/-125 and -146 were associated with the highest levels of TERT gene expression with median-fold changes of 442.2 and 423.5, respectively, relative to a fibroblast control value. In contrast, mutations at position -138/139 were associated with a fold change of 242.5, which was considerably lower than the TERT Wild Type gene expression of 390.9-fold change.

There was no measurable relationship between telomere length and TERT gene expression (Pearson coefficient $R^2 = 0.005; \ P = 0.59$) or TERT copy number amplification ($R^2 = 0.016; \ P = 0.33$, linear regression analysis), despite 50.0% of the cell lines displaying copy gain. However, the TERT Wild Type subgroup was associated with the longest telomere length with a median value of 64.3 kb compared with values of 20.58 kb, 28.93 kb and 22.67 kb in -124/125, -146, and -138/139, respectively. This paradoxical result may be explained by one proposed strategy whereby TERTp mutations allow for the maintenance of critically short telomeres that promote genomic instability (Chiba et al., 2017). Despite this finding in the Wild Type subgroup, the lack of relationship between telomere length and TERT gene expression remained consistent even when analyzing only those cell lines with mutated TERT promoters (Pearson coefficient $R^2 = 0.006; \ P = 0.58$).

The rs2853669 variant, which was recently found to modify the effects of TERTp mutations on both survival and recurrence in melanoma (Nagore et al., 2016b), was significantly associated with a longer absolute telomere length via a recessive model in our cohort ($P = 0.003$, linear regression analysis), suggesting a possible molecular explanation for these clinical consequences. None of the remaining mutations revealed any statistically significant relationship with TERT gene expression or telomere length (Figure 2).

**DISCUSSION**

Mutations in the promoter region of TERT are nearly ubiquitous in cancer. The role of TERT in tumorigenesis and as a harbinger of poorer outcomes in melanoma is relatively unquestioned, but a precise mechanism behind this association...
remains elusive. In their retrospective study of 116 melanoma tumor samples, Pópolo et al. (2014) reported that TERTp mutations are significantly associated with both disease-free and overall survival, despite there being no measurable relationship between actual TERT gene expression and either promoter mutational status or survival (Pópolo et al., 2014). In this study, we sought to develop a better understanding of this pathway by studying cell lines that lack the contamination from fibroblasts, lymphocytes, and endothelial cells that likely alters the results of similar studies on human- or animal-derived tissues. What we found was a lack of consistent association between the most well-studied mutations and their molecular phenotype, which is partially in contrast to the published literature on this topic. Furthermore, our data, which are based on in vitro studies of purified melanoma cell lines, reveal a more complex mutational landscape of the TERTp compared with that of similar studies on primary tumor samples. Although we cannot rule out the
possibility of similar complexity within The Cancer Genome Atlas Program’s analysis without access to their primary data, their mention of just two TERTp variants (-124 and -146) can only be seen as a portion of the picture. The loss of heterozygosity present in our cell lines further suggests a growth advantage to the TERTp mutations, which appear to have accumulated in several generations of in vitro selection.

The lack of a clear link between TERTp mutations and telomere length makes it more plausible that there are auxiliary genetic phenomena playing a role in the way tumor cells escape the critical shortening of telomeres. Alternative lengthening of telomeres (ALT), TERT gene rearrangement, and DNA hypermethylation have all been proposed as possible additional mechanisms, the last of which having been identified as a possible driver, and thus therapeutic target, of pediatric and adolescent melanomas by Fan et al. (Fan et al., 2016). Any one of these alternative mechanisms of preventing telomere attrition might explain the disappointing and inconclusive attempts at targeting the TERT/telomerase/telomere pathway to date (Ivancich et al., 2017; Reyes-Uribe et al., 2018).

LIMITATIONS

We acknowledge several limitations in this study. Our results are based on TERT gene expression rather than the enzymatic activity of telomerase, which prevents us from detecting any variability in translational or posttranslational processing, in addition to any unforeseen discrepancies in protein function. However, the two measures should otherwise be tightly correlated, and none of the variants in the TERTp are known to alter the active site of telomerase. Our cell lines are likely heterogeneous populations with variable mutational complications. It is safe to assume that this genetic variability has hindered our ability to appreciate a direct relationship between TERTp mutations and TERT expression or telomere length, and this may also explain the observed higher mRNA expression, more variable telomere length, and higher copy number than might be expected. In addition, we were unable to determine whether the amplified copies of TERT were associated with a Wild Type or a mutant promoter in those cell lines with TERT gene amplification, which may explain the failure of gene amplification to modify gene expression in our cohort.

CONCLUSIONS

We believe the results presented here, although in contrast to those of other studies, are a significant contribution to the overall depiction of a TERT/telomerase/telomere pathway that continues to trouble researchers with its convoluted, yet undeniable, connection with worsening outcomes in melanoma. Although the data herein are not independently relevant in a clinical setting, we hope that this newfound information will help to guide further research into the alternative pathways underlying this crucial tumorigenic process. By respecting the complexity of these pathways, we might be better able to understand where our efforts are best put forth to improve clinical outcomes. Given that all attempted strategies of targeting TERT have proven unfruitful, more research is needed to uncover the true relevance of alternate mechanisms to the TERT/telomerase/telomere pathway.

MATERIALS AND METHODS

Melanoma cell lines

We adopt stringent criteria for establishing the veracity and identity of our cell lines. All new lines are purchased directly from trusted repositories (e.g., ATCC). Legacy cell lines in our laboratory (e.g., those gifted from collaborators) are stratified into three levels of confidence:

1. All our lines have been short tandem repeat genotyped. The lines that demonstrated an unequivocal match between our designation and the short tandem repeat database were considered “CONFIRMED,” whereas those that showed an unequivocal mismatch to another cell line were discarded. All new ATCC cell lines were considered “CONFIRMED.”

2. For those with “no hits” in the short tandem repeat database, we performed a manual search of key melanoma mutations (e.g., BRAF/V600E, NRAS/Q61, CDKN2A, and TP53) reported for each line in either Catalogue of Somatic Mutations in Cancer, Cancer Cell Line Encyclopedia, or in individual publications in the literature. We subsequently compared Sanger or whole-exome sequence information generated in our laboratory for these lines. If there is a direct match between our sequence information and the public domain data, we designated these lines as “CONSISTENT.”

3. For cell lines without public domain information because they had been recently derived from melanoma patients and had not been published, we analyzed levels of MITF (M-isoform) to determine if the cells were compatible with melanocytic cells. Those that expressed significant M-isoform MITF were designated as “COMPATIBLE.”

When choosing cell lines for experimentation, the order of preferential selection was “CONFIRMED” > “CONSISTENT” > “COMPATIBLE.”

Furthermore, all cell lines were tested for common pathogens, such as mycoplasma, and were pathogen-free. All lines were cultured in DMEM (Corning Cellgro, Corning, NY) with 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) and 1% penicillin/streptomycin (Gibco).

Sanger sequencing

DNA of sufficient quality (confirmed using spectrophotometry and the ratio of 260:280 nm) was extracted from cultures of 60 human cutaneous melanoma cell lines using the QIAspin Miniprep Kit (QIAGEN, Valencia, CA). The TERTp region was first amplified by PCR using primer sequences targeting positions 27 to 286 from the ATG start site (See Supplementary Tables S2–S4 for primer sequences, PCR settings, and reagents). The same was done using primers targeting exons 11 and 15 of BRAF and exons 2 and 3 of NRAS. The amplicons were size confirmed by gel electrophoresis and then treated with ExoSAP-IT (Affymetrix/USB Corporation, Cleveland, OH) before being submitted to the institutional sequencing core. Sequencing data were analyzed using Chromas software (Version 2.6, Technelysium Pty Ltd, South Brisbane, Australia).

TERT gene expression and amplification

Total RNA was isolated using the QIAGEN RNeasy kit. It was converted to cDNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions (Supplementary Table S2–S4). Using the TaqMan Gene
Expression Assay (Applied Biosystems). TERT mRNA expression levels were quantified in triplicate relative to the housekeeping gene, GUSB, on the LightCycler 480 (Roche, Indianapolis, IN) (Supplementary Tables S2–S4).

Assessment of copy number amplification affecting the TERT gene was performed in triplicate using qPCR with the TaqMan Copy Number Assay (Applied Biosystems). RPPH1 (RNase P) was employed as a single copy gene for the purposes of normalization and copy number calculation. The TERT copy number in fibroblasts was used as a normal control with an expected copy number of 2 (Supplementary Tables S2–S4). Calculation of the copy number status was performed using CopyCaller software (Version 2.1, Applied Biosystems).

**Absolute telomere length**

After DNA extraction as described above, the absolute telomere length was quantified in triplicate using the SYBR green–based qPCR method defined by O’Callaghan and Fenech (O’Callaghan and Fenech, 2011). Two separate qPCR reactions were run for each sample, one to quantify the total amount of telomere length and one to quantify the number of genomic copies in the DNA extracted from each cell line (Supplementary Tables S2–S4). Two sets of primers, one set that is designed to target the TTAGGG telomeric repeat segments and the other targeting the hemoglobin subunit beta (HBB) gene, a single copy gene, were used for the telomere length and genomic copy reactions, respectively. Two standard curves were developed using known concentrations of telomeric DNA and a known number of genomic copies that were serially diluted. Experimental values were extrapolated from these curves using Roche LightCycler 480 software (see Supplementary Table S5 for the calculation of standard values, standard curves, and sample PCR-plate setup). Absolute telomere length was calculated as the ratio of telomeric DNA (kilobases [kb]) to genomic copies, which represents the total amount of telomeric sequence in one genome.

**Statistical analysis**

Initially, the dataset was subjected to several quality-check steps. First, we analyzed observed distributions of copy number, telomere length, and normalized expression. Several significantly outlying data points were removed, as they would strongly bias downstream analysis. Specifically, a data point was removed if its value on one or more metrics exceeded the value of the next largest data point by more than 20%. We constructed a model for linear regression: \[ y = \beta_1 x + \beta_0 \], where \( y \) is a measured “phenotype” (i.e., expression, telomere length, and copy number variation), and \( x \) is an observed genotype (taking values of 0, 1, 2 with respect to count of alternative alleles). The significance threshold of phenotypic associations was estimated using Bonferroni correction: 0.05/8 DNA variants tested = 0.00625.

**Data availability statement**

The authors confirm that the data supporting the findings of this study are available within the article and its Supplementary Materials.

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**CONFLICT OF INTEREST**

The authors state no conflict of interest.

**ACKNOWLEDGMENTS**

This study was supported in part by NIH grant K24CA149202 to HT.

**AUTHOR CONTRIBUTIONS**

Conceptualization: MS, CN, MA, HT; Data Curation: MS, CN, MA, HT; Formal Analysis: MS, CN, MA, HT; Funding Acquisition: HT; Investigation: MS, CN, MA, HT; Writing - Original Draft Preparation: MS, CN, MA, HT.

**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2019.06.149.

**REFERENCES**


Supplementary Figure S1. Sanger sequencing chromatograms. TERT Sanger sequencing chromatograms from selected cell lines with heterozygous mutations (left) and homozygous mutations (right). Variant alleles are identified with an orange arrow. TERT, telomerase reverse transcriptase.