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Telomerase Expression by Aberrant Methylation of the *TERT* Promoter in Melanoma Arising in Giant Congenital Nevi

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TO THE EDITOR

Telomeres are tandem repeats of the non-coding DNA structures at the end of human chromosomes that protect the coding DNA and the integrity of the genome (Blackburn, 1991). The ability to sustain telomere length confers unlimited proliferative capacity to cancer cells. In a majority of cancers, telomere length is maintained by the activity of the enzyme telomerase (Kim *et al.*, 1994), whose catalytic subunit is encoded by the *telomerase reverse transcriptase* (*TERT*) gene. However, until recently, the underlying mechanisms for telomerase activation in cancer cells were largely unknown.

Recurrent transcription activating mutations of the *TERT* promoter were first described in melanoma and subsequently in other tumor types (Horn *et al.*, 2013; Vinagre *et al.*, 2013). These mutations upregulate *TERT* expression by recruiting the multimeric GA-binding protein transcription factor that specifically binds to the mutant promoter (Bell *et al.*, 2015). In addition to the mutations, DNA methylation of the *TERT* promoter is likely to play a role in *TERT* expression (Guilleret and Benhattar, 2004). Recently, it was shown that a region of the *TERT* promoter upstream of the transcription start site is methylated in malignant

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telomerase-expressing pediatric brain tumors but not in telomerase-negative normal brain tissues or low-grade tumors (Castelo-Branco *et al.*, 2013).

We previously showed that an aggressive form of pediatric melanoma developing within giant congenital nevi (GCN) retains the wild-type *TERT* promoter (Lu *et al.*, 2015). To determine whether epigenetic modifications may play a role in telomerase expression in this melanoma subtype, we analyzed the DNA methylation profile of a CpG-rich region of the *TERT* promoter, shown previously to be differentially methylated between normal and malignant tissues (Castelo-Branco *et al.*, 2013), in 13 melanomas (3 arising in GCN; 7 conventional; and 3 spitzoid) and 10 benign or borderline melanocytic tumors (1 GCN; 3 GCN with nodular proliferation; and 6 borderline spitzoid melanocytic neoplasms) from 23 pediatric and adult patients. The human investigations were performed after approval by the local institutional review boards. Written, informed patient consent was waived because the research involved no more than minimal risk to the subjects. The status of the *TERT* promoter, *BRAF* and *NRAS* mutations, and kinase fusions was available for the spitzoid tumors and a subset of melanoma samples from our prior studies (Lee *et al.*, 2015; Lu *et al.*, 2015).

Supplementary Table S1 and Figure 1 summarize the demographic and outcome data and the primary driver oncogene for the 23 study subjects. PCR–Sanger sequencing identified a hotspot *TERT* promoter mutation [4 C228T (chr5:1,295,228); 3 C250T (chr5: 1,295,250), and 2 CC242/243TT (chr5: 1,295,242-3)] in 9 of 13 melanoma samples (6/7 conventional; 3/3 spitzoid) but not in the 10 benign or borderline melanocytic neoplasms (Figure 1). The DNA methylation status of a region of the *TERT* promoter, from 482 bp to 667 bp upstream of the ATG start site [chr5:1295586–1295771 (GRCh37/hg19)] (Supplementary Figure S1 online), encompassing 26 CpG sites, was assayed by next-generation bisulfite sequencing (Methods, Supplementary Material online). For each CpG site, the methylation ratio (Beta-value) was measured in the range of 0 to 1 (Supplementary Table S2 online). The methylation status was defined as follows: >0.7, methylated (Figure 1, red); 0.5–0.7, partially methylated (orange); 0.3 to <0.5, partially unmethylated (cyan); and <0.3, unmethylated (blue). Supplementary Table S3 shows the total number of methylated Cs and unmethylated Cs in the sequenced region for each sample. Remarkably, almost all 26 CpG sites in the sequenced region were highly methylated in the 3 melanomas arising in GCN (S1, S2, S21), and the one conventional melanoma bearing wild-type *TERT* promoter (S22), whereas the CpG sites remained predominantly unmethylated in the 9 mutant *TERT* promoter melanomas and the 10 benign or borderline melanocytic neoplasms (Figure 1).

Next, we evaluated the association of *TERT* promoter CpG methylation with telomerase expression by *TERT* mRNA in situ hybridization (ISH) and by gene expression analysis (Methods, Supplementary Material online). *TERT* mRNA ISH revealed distinct, intracellular punctate signals in melanomas arising in GCN (Figure 2c and 2f) but not in the proliferative nodules in GCN (Figures 2i and 2l). The *TERT* promoter methylation level was calculated as the log2 ratio of the total number of methylated Cs versus the total number of unmethylated Cs in the sequenced region [logit (B-value)]. The *TERT* expression level was measured by using RNA sequencing data available for a subset of samples. An association analysis

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revealed a strong correlation between *TERT* promoter methylation and *TERT* expression level (P = 0.0422, adjusted r^2 = 0.5145; Supplementary Figure S2 online).

Our data demonstrate that epigenetic modification through *TERT* promoter CpG methylation is an alternative pathway for *TERT* reactivation in melanoma. Although epigenetic remodeling by promoter methylation is generally considered a signature of gene silencing, *TERT* expression is paradoxically increased by promoter methylation (Guilleret and Benhattar, 2004). Although the exact mechanism underlying CpG DNA methylation in *TERT* upregulation is not known, one possible mechanism is by inhibiting transcriptional repressors such as CTCF (Renaud *et al.*, 2007), SIN3A, or MAZ (Xu *et al.*, 2013) from binding to the target DNA-binding sites in the region (Figure S1). Also, even when the promoter is largely methylated, a small region of the core promoter upstream of the transcription start site remains unmethylated to allow for the continued transcriptional activity of *TERT* (Renaud *et al.*, 2007; Zinn *et al.*, 2007).

Individuals with GCN are at increased risk for developing melanoma (Figures 2a-b and 2d-e) that occurs most frequently in the first decade of life (Bittencourt *et al.*, 2000). A much more frequent change in these nevi than melanoma is the development of clonal proliferations often in the form of nodules (Figures 2g-h and 2j-k), which may suggest or mimic melanoma on clinical or histological grounds (Yelamos *et al.*, 2014). The differential pattern for *TERT* promoter methylation and telomerase expression between melanomas in GCN and proliferative nodules demonstrated in our study is consistent with the benign clinical course of proliferative nodules compared with the invariably aggressive behavior of melanoma arising in GCN. Further studies in a larger number of patients are needed to determine the potential diagnostic value of *TERT* promoter methylation assays for ambiguous proliferative lesions within GCN.

In our cohort, *TERT* promoter hypermethylation or promoter mutations occurred in all melanoma samples but in none of the the benign or low-grade melanocytic lesions, suggesting that a panel incorporating *TERT* promoter methylation and mutation assays may help discriminate between benign/borderline and overtly malignant melanocytic neoplasms. Future studies need to assess the potential use of these assays for diagnostic or prognostic purposes in the clinic.

In summary, we demonstrate that in subsets of malignant melanoma, *TERT* is upregulated epigenetically by a methylation-dependent mechanism. These findings have potential therapeutic implications. *TERT* promoter CpG hypermethylation is a reversible phenomenon. Treatment with DNA demethylating agents reduced *TERT* expression and telomerase activity in telomerase-positive cell lines (Guilleret and Benhattar, 2003; Renaud *et al.*, 2007). Together, these findings provide a rationale for developing a therapeutic strategy in preclinical studies through epigenetic modifications at *TERT* promoter regulatory sites in melanomas with the CpG island methylator phenotype.

Supplementary Material

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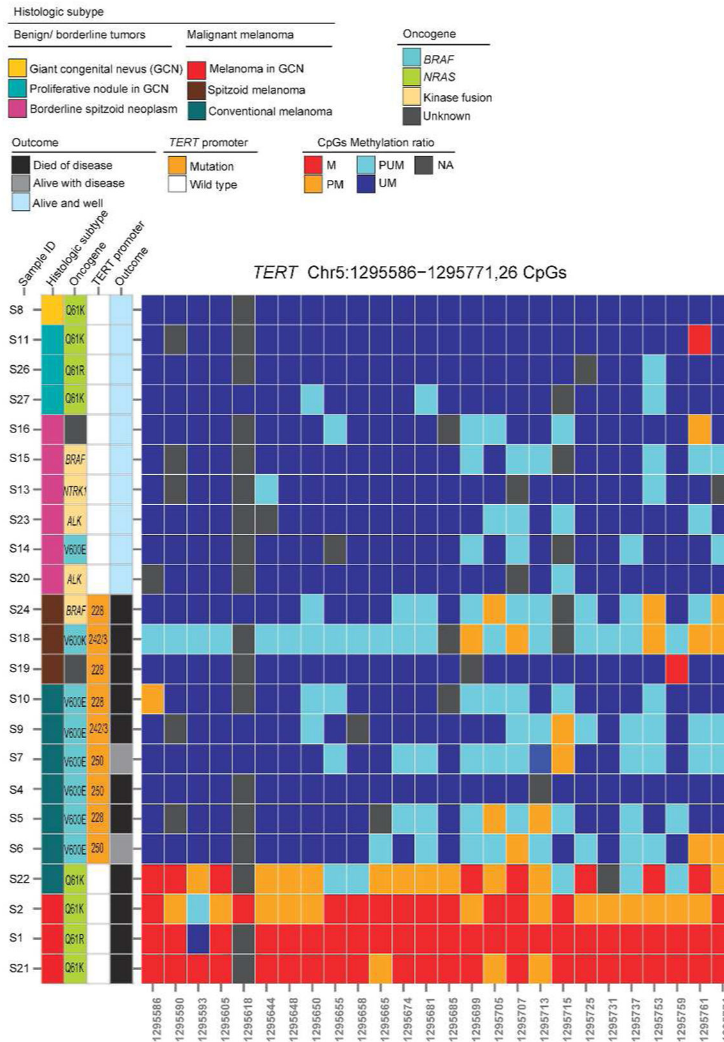

Figure 1.

Association of the mutational status and the methylation profile of the *TERT* promoter with disease characteristics and outcome data for 23 patients with melanocytic tumors. The 26 CpG sites were aberrantly methylated in wild-type *TERT* promoter melanomas (the last 4 rows) but were predominantly unmethylated in low-grade or benign melanocytic tumors (atypical spitzoid neoplasms and GCN with proliferative nodules) and in mutant *TERT* promoter melanomas. Methylation panel color code: M, methylated; PM, partially methylated; PUM, partially unmethylated; UM, unmethylated; NA, not available.

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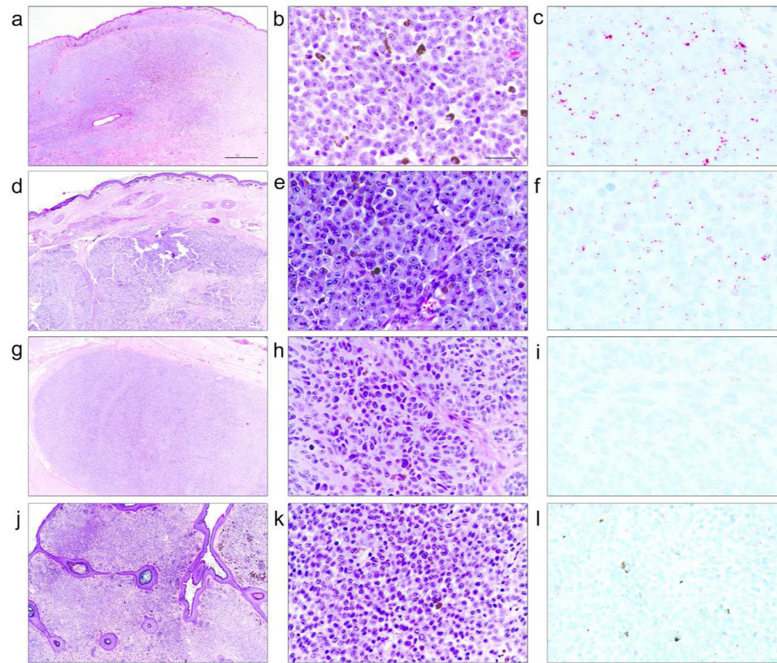

Figure 2.

Photomicrographs (H&E–stained) and *TERT* mRNA in situ hybridization (ISH) for 2 melanomas in GCN (2 top panels) and 2 proliferative nodules in GCN (2 bottom panels). mRNA ISH shows numerous high-resolution red intracellular punctate signals in malignant melanocytes (c and f) and no signals above the background level in melanocytes of proliferative nodules (i and l). Scale bars= 1000 μm (a) and 50 μm (b).