Telomerase expression by aberrant methylation of the TERT promoter in melanoma arising in giant congenital nevi

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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 noncoding 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 most 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 to the TERT promoter (Bell et al., 2015). In addition to the mutations, DNA methylation 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 (online) 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; 0/3 melanomas in GCN) but not in the 10 benign or borderline 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 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 sequenced region for each sample. Remarkably, almost all 26 CpG sites in the sequenced region were highly methylated in the three melanomas arising in GCN (S1, S2, S21) and in 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 and by gene expression analysis (Supplementary Methods). TERT mRNA in situ hybridization revealed distinct, intracellular punctate signals in melanomas arising in GCN (Figure 2c and f) but not in the proliferative nodules in GCN (Figure 2i and l). The TERT promoter methylation level was calculated as the log 2 ratio of the total number of methylated Cs versus the total number of unmethylated Cs in the sequenced region (logit [beta value]). The TERT expression level was measured by using RNA sequencing data available for a subset of samples. An association analysis revealed a strong correlation between TERT promoter methylation and TERT expression level ($P = 0.0422$, adjusted $R^2 = 0.5145$; see 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

Abbreviations: GCN, giant congenital nevi; TERT, telomerase reverse transcriptase (gene)

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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 (Supplementary 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 (Figure 2a, b, d, and 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 (Figure 2g, h, j, and k), which may suggest or mimic melanoma on clinical or histologic grounds (Yelamos et al., 2015). 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 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 Benhatar, 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.

**CONFLICT OF INTEREST**
The authors state no conflict of interest.

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**Figure 2.** Representative photomicrographs of H&E-stained histological sections and TERT mRNA in situ hybridization. The two top panels represent two melanomas in GCN and the two bottom panels represent two proliferative nodules in GCN. mRNA in situ hybridization 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).
Diabetic Wounds Exhibit Distinct Microstructural and Metabolic Heterogeneity through Label-Free Multiphoton Microscopy

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TO THE EDITOR
Cutaneous wound healing is a complex process requiring the coordination of inflammatory cells, endothelial cells, fibroblasts, and keratinocytes (Gurtner et al., 2008). Chronic wounds fail to progress through this coordinated process, and are often characterized by prolonged inflammation, poor vascularization, callus formation, and infection (Brem et al., 2007; Martin et al., 2010). A wide variety of advanced wound care products have been developed to target specific characteristics of chronic wounds (Apelqvist, 2012). However, no single therapy has yielded widespread clinical success, and there is a critical need to develop new biomarkers and diagnostic technologies to evaluate wound status and guide care.

Currently, wound closure is the only accepted objective endpoint to evaluate wound treatments, and there is a lack of quantitative surrogate biomarkers to predict healing at earlier stages. Multiphoton microscopy techniques, such as two-photon excited fluorescence (TPEF), have emerged as useful approaches to evaluate epithelial tissues and offer advantages in imaging depth and signal collection over confocal microscopy (Balu et al., 2013; Varone et al., 2014). Through TPEF imaging, the intrinsic fluorescence of nicotinamide and flavin adenine dinucleotides (NADH and FAD) can be measured without the application of exogenous stains (Georgakoudi and Quinn, 2012; Zipfel et al., 2003). An optical redox ratio of FAD/NADH autofluorescence has been computed from a variety of cells and tissues and is correlated with the intracellular concentration ratio of NADH/NAD+ (Quinn et al., 2013; Varone et al., 2014). This noninvasive measure of the relative rates of glucose catabolism to oxidative phosphorylation has been used to help diagnose disease and evaluate tissue development (Georgakoudi and Quinn, 2012).

Although metabolic stress at the systemic and tissue level often coincides with chronicity, the dynamics of metabolism in the wound at the cellular level are not well understood. Using label-free multiphoton microscopy, the objective of this study was to provide an initial quantitative assessment of metabolic biomarkers with sensitivity to differences between nondiabetic and diabetic wounds. To this end, multiphoton imaging was performed on unstained sections of full-thickness excisional wounds from the dorsum of a streptozotocin-induced diabetic mouse model. Animal studies were conducted in accordance with Beth Israel Deaconess Medical Center IACUC protocol #072-2012 and Tufts IACUC protocol #M2014-58. Additional methods’

Abbreviations: FAD, flavin adenine dinucleotide; NADH, nicotinamide adenine dinucleotide; TPEF, two-photon excited fluorescence

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