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Mutations of the *TERT* promoter are common in basal cell carcinoma and squamous cell carcinoma

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Telomerase is frequently expressed in cancer and contributes to carcinogenesis. Two recent publications report the identification of a set of recurrent mutations in melanoma in the promoter of the telomerase reverse transcriptase gene (*TERT*) that appears to be the result of mutagenesis from ultraviolet (UV) radiation. Both groups reported that the mutations increase the transcription of *TERT*. This prompted our search for similar mutations in two other UV-related skin cancers, basal cell carcinoma, and squamous cell carcinoma. We found that the activating *TERT* promoter mutations reported in melanoma are also frequent in squamous cell carcinoma (50%) and basal cell carcinoma, the latter including both sporadic tumors (78%) and tumors from patients with nevoid basal cell carcinoma syndrome (68%). These mutations were found in only 1 of 11 Bowen's disease (squamous cell carcinoma *in situ*) specimens, and in none of 15 non-malignant skin specimens and 57 blood specimens. The mutations were frequently homozygous or hemizygous, with little or no normal signal at the mutated positions. These data suggest that *TERT* promoter mutations are the most frequent putative oncogenic mutations in cutaneous cancer.
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Basal cell carcinoma and squamous cell carcinoma are the most common cancers in light-skinned individuals, and account for the majority of all human cancers.[1–3] Both tumors are strongly associated with chronic ultraviolet (UV) radiation exposure and occur primarily, but not exclusively, on sun-exposed areas of the body.[4–8] The most commonly mutated gene in basal cell carcinoma and squamous cell carcinoma is *TP53*, with the majority of mutations consistent with UV as the mutagen.[9,10] Inactivating mutations in the *PTCH* gene, the gene responsible for nevoid basal cell carcinoma syndrome, have also been identified in sporadic basal cell carcinoma, also with evidence for UV causation.[11,12]

Telomerase is a ribonucleoprotein that adds telomeric sequences (TTAGGG hexamers) to the ends of chromosomes. Increased telomerase activity is thought to promote carcinogenesis and immortalization of cells by preventing senescence induced by telomere shortening.[13] Telomerase activity is not detectable in the majority of normal skin samples tested by the telomerase rapid amplification protocol, whereas basal cell carcinoma, squamous cell carcinoma, and some premalignant lesions, including Bowen's disease and actinic keratosis, have detectable telomerase activity.[14–16]

Horn *et al*[17] and Huang *et al*[18] recently reported the frequent occurrence of a set of C>T mutations in the promoter of the telomerase reverse transcriptase gene (*TERT*) in melanoma. These mutations were consistent with UV-induced mutagenesis, and created binding sites for the ETS/TCF transcription factors. Both groups also reported that the mutations caused a 2- to 4-fold increase in transcriptional activity of the promoter, using reporter gene assays. These findings prompted us to investigate the possibility that similar mutations would be found in other cutaneous cancers in which UV has a causal role, basal cell carcinoma and squamous cell carcinoma. We analyzed sporadic basal cell carcinoma and basal cell carcinoma from patients with nevoid basal cell carcinoma syndrome,

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squamous cell carcinoma, as well as its precursor lesion Bowen's disease, for mutations in the *TERT* promoter. We show that a high percentage of basal cell and squamous cell carcinomas carry mutations in the *TERT* promoter, that the mutations are consistent with UV exposure as the mutagen, and that many of the mutations are homozygous. Because of the high prevalence of basal cell and squamous cell carcinomas in the population, *TERT* promoter mutations may represent one of the most common mutations in human cancer.

Materials and methods

Specimen Selection

Cutaneous tumors, normal skin, benign skin lesions, and anonymized DNA from blood specimens were obtained according to a protocol approved by the University of Rochester Research Subject Review Board. Formalin-fixed paraffin-embedded samples were retrieved from the Surgical Pathology Division of the Department of Pathology, University of Rochester. Samples in which tumor cells occupied >50% of the paraffin block were used for *TERT* promoter mutation analysis, without microdissection. All samples were biopsied from 2011 to 2013, with the exception of two tumor samples from a patient with nevoid basal cell carcinoma syndrome which were obtained between 2007 and 2010. Tables 1–4 summarize the demographic information of the patients whose tumors were analyzed. A total of 18 patients with sporadic basal cell carcinoma ($n=23$ tumors, Table 1), 4 patients with nevoid basal cell carcinoma syndrome ($n=19$ tumors, Table 2), 19 subjects with squamous cell carcinoma ($n=26$ tumors, Table 3), and 11 subjects with Bowen's disease tumors ($n=11$, Table 4) were tested. Controls included 57 anonymized DNAs from blood specimens that had been collected for unrelated genetic studies, and 15 benign skin specimens (Table 5) with a variety of diagnoses including 7 seborrheic keratosis, 2 hemangiomas, 2 histologically normal skin, 2 scar tissue (cicatrix), and 1 each of lymphoid hyperplasia and soft fibroma.

DNA Preparation, PCR, and Sequencing

Material was cut from the paraffin block of each sample (30 μM) and genomic DNA was purified using the QIAamp system (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. PCR was performed in a total volume of 12 μl with primers at a final concentration of 1 μM each, 50 μM of each dNTP, 5% dimethylsulfoxide, 0.75 units of HotStar *Taq* DNA polymerase, 1.2 μl of the 10X buffer provided by the enzyme manufacturer (Qiagen) and 50–100 ng of template DNA. The gene-specific parts of the upstream and downstream primers were 5′-GCCGGGCTCCCAGTGGATTCG-3′ and 5′-GCTTCCCACGTGCGCAGCAGGA-3′, respectively. The PCR primers were synthesized with M13 tail sequences appended to the 5′-end to facilitate sequencing, and were targeted to amplify sequence

Table 1 Sporadic basal cell carcinomas: mutations and demographic information

Specimen no. BCC-	*Classic mutation*[a]	*Other mutations*[a]	*Age/gender*	*Site*
1	−146C>T	−101C>T, heterozygous	51/M	Nose
2A[b]	−138/−139CC>TT	N	81/M	Cheek
2B[b]	N	N	81/M	Back
3	−138/−139CC>TT	N	81/M	Neck
4	−146C>T	N	48/F	Neck
5	−146C>T	N	82/M	Forehead
6A[b]	−124/−125CC>TT	−101C>T	71/M	Shoulder
6B[b]	N	−159C>T + −101C>T both heterozygous	71/M	Nose
6C[b]	−138/−139CC>TT + −124/−125CC>TT	−100/−101CC>TT	71/M	Nose
6D[b]	N	−111C>T	71/M	Temple
7	−124C>T, heterozygous	N	63/M	Cheek
8	−124/−125CC>TT	−145C>T	92/M	Neck
9	−146C>T, heterozygous	N	71/F	Temple
10	−146C>T	N	91/M	Ear
11	−146C>T	N	63/M	Shoulder
12	−138/−139CC>TT	−101C>T	70/M	Lip
13A[b]	−124C>T	N	76/M	Ear
13B[b]	−124C>T	N	76/M	Back
14	N	N	47/F	Nose
15	−138/−139CC>TT	N	66/M	Nose
16	−146C>T	−101C>T, heterozygous	66/M	Temple
17	N	N	82/M	Shoulder
18	−138/−139CC>TT, heterozygous	N	82/M	Ear

Abbreviation: N, no mutation identified in this category.
[a]All mutations were homozygous unless indicated otherwise. Classic mutations are the recurrent mutations in the TERT promoter discovered in melanoma (−124C>T, −146C>T, and the double mutations −124/−125CC>TT and −138/−139CC>TT).[17,18]
[b]Letter subcodes in the first column indicate tumors taken from the same patient, on the same day.

Table 2 Nevoid basal cell carcinoma syndrome: mutations and demographic information

Specimen no. NBCCS-	*Classic mutation*[a]	*Other mutations*[a]	*Age/gender*	*Site*
Subject 1				
1A[b]	N	−125/−126, heterozygous	52/F	Eyelid
1B[b]	−124/−125CC>TT	−101C>T	52/F	Ear
2A[b]	−146C>T, heterozygous	−101C>T, heterozygous	52/F	Scalp
2B[b]	N	N	52/F	Scalp
3	N	N	52/F	Scalp
Subject 2				
1A[b]	−146C>T	N	81/F	Neck
1B[b]	−146C>T	N	81/F	Scalp
2A[b]	N	N	81/F	Scalp
2B[b]	N	N	81/F	Scalp
2C[b]	−138/−139CC>TT	−101C>T	81/F	Chin
Subject 3				
1A[b]	−124C>T	−100C>T	48/F	Neck
1B[b]	−124C>T	N	48/F	Shoulder
1C[b]	−124C>T	N	48/F	Arm
1D[b]	−146C>T	N	48/F	Back
2	N	N	48/F	Nose
3A[b]	−124C>T	N	48/F	Back
3B[b]	−124C>T	N	48/F	Back
Subject 4				
1	−124C>T	N	49/M	Leg
2	−124/−125CC>TT	N	49/M	Ear

Abbreviation: N, no mutation identified in this category.
[a]All mutations were homozygous unless indicated otherwise. Classic mutations are the recurrent mutations in the TERT promoter discovered in melanoma (−124C>T, −146C>T, and the double mutations −124/−125CC>TT and −138/−139CC>TT).[17,18]
[b]Letter subcodes in the first column indicate tumors taken from the same patient, on the same day.

from −270 to −50 bps upstream from the initiator ATG within the promoter region of the *TERT* gene. The reactions were cycled 42 times between 95 °C for 15 s, 63 °C for 15 s, and 72 °C for 45 s, preceded by 15 min at 95 °C, and followed by 5 min at 72 °C. The primers were purchased from Integrated DNA Technologies (Coralville, IA). The 306-bp amplicon (including tails) was treated with ExoSap (Amersham Biosciences, Piscataway, NJ, USA) to remove the primers and dNTPs; then sequenced using the M13 tails as sequencing primers and Applied Biosystems (ABI, Foster City, CA, USA) BigDye Terminator v.3.1 chemistry. The sequencing reactions were purified using the CleanSeq system (Agencourt Bioscience, Beverly, MA, USA) and then resolved by capillary electrophoresis on the ABI 3500XL Genetic Analyzer. All of the mutations were confirmed by repeat analysis starting with the PCR step.

Statistical Analysis

Groups were compared for mutation frequencies using the χ^2 statistics. $P<0.05$ was considered as significant.

Results

TERT Promoter Mutation Analysis

We analyzed squamous cell carcinoma ($n=26$), Bowen's disease ($n=11$), sporadic basal cell carcinoma ($n=23$), and 19 basal cell carcinomas from 4 patients with nevoid basal cell carcinoma syndrome. Patients with nevoid basal cell carcinoma syndrome were diagnosed based on the characteristic clinical features, including the presence of multiple basal cell carcinomas starting at a young age. Figure 1 shows the representative photomicrographs of hematoxylin and eosin-stained sections of the tumors studied. We obtained sequence from −270 to −50 bps upstream from the initiator ATG, which includes all of the recurrent *TERT* promoter mutations described in melanoma.[17,18] These mutations include: −124C>T (chr 5: 1 295 228), −146C>T (1 295 250), and the double mutations −124/−125CC>TT (1 295 228_1 295 229) and −138/−139CC>TT (1 295 242_1 295 243). We will refer to this set of recurrent mutations as the classic mutations. A full description of the mutations found in each specimen, and demographic information for each patient, is presented in Tables 1–5. The results are summarized in Table 6.

Classic mutations were found in 18/23 sporadic basal cell carcinomas from a total of 18 patients (Table 1), 13/19 basal cell carcinomas from 4 patients with nevoid basal cell carcinoma syndrome (Table 2), 13/26 squamous cell carcinomas from a total of 19 patients (Table 3), and 1/11 Bowen's disease specimens from a total of 11 patients (Table 4). Figure 2 shows Sanger sequencing data for representative tumors. As shown in Figure 2, detailed in Tables 1–4, and summarized in Table 6, many of the mutations were homozygous, with little

Table 3 Squamous cell carcinomas: mutations and demographic information

Specimen no. SCC-	*Classic mutation*[a]	*Other mutations*[a]	*Age/Gender*	*Site*
1A[b]	N	N	86/F	Shin
1B[b]	N	N	86/F	Shin
2	N	N	74/M	Arm
3A[b]	−146C>T	N	90/F	Scalp
3B[b]	−146C>T	−126C>T	90/F	Scalp
3C[b]	−146C>T	−126C>T	90/F	Scalp
4	−146C>T	N	55/M	Ear
5	N	−150C>T	71/M	Temple
6	−138/−139CC>TT, heterozygous	N	79/F	Arm
7	N	N	71/M	Scalp
8	N	−126/−127CC>TT, heterozygous	61/M	Back
9	−146C>T, heterozygous	N	79/M	Ear
10	−124C>T	N	62/M	Cheek
11A[b]	−138/−139CC>TT	N	52/M	Scalp
11B[b]	N	N	52/M	Shin
11C[b]	N	N	52/M	Thigh
11D[b]	N	N	52/M	Shin
12A[b]	−124C>T, heterozygous	N	76/M	Jaw
12B[b]	−146C>T, heterozygous	N	76/M	Arm
13	N	N	63/F	Arm
14	N	N	76/M	Cheek
15	N	N	63/M	Ear
16[c]	−146C>T	N	70/M	Cheek
17[c]	N	−101C>T, heterozygous	65/M	Shoulder
18[c]	−124C>T, heterozygous	N	81/M	Forearm
19[c]	−124C>T	N	67/M	Lip

Abbreviation: N, no mutation identified in this category.
[a]All mutations were homozygous unless indicated otherwise. Classic mutations are the recurrent mutations in the TERT promoter discovered in melanoma (−124C>T, −146C>T, and the double mutations −124/−125CC>TT and −138/−139CC>TT).[17,18]
[b]Letter subcodes in the first column indicate tumors taken from the same patient, on the same day.
[c]Poorly differentiated SCC.

Table 4 Bowen's disease (squamous cell carcinoma *in situ*): mutations and demographic information

Specimen no. BD-	*Classic mutation*[a]	*Other mutations*[a]	*Age/ Gender*	*Site*
1	N	N	79/F	Leg
2	N	N	77/F	Arm
3	N	N	88/F	Shin
4	N	N	82/M	Shoulder
5	N	N	63/F	Thigh
6	N	N	55/M	Thigh
7	N	N	87/F	Thigh
8	−146C>T	N	62/M	Cheek
9	N	N	58/F	Shoulder
10	N	N	71/M	Temple
11	N	−101C>T, heterozygous	61/M	Neck

Abbreviation: N, no mutation identified in this category.
[a]All mutations were homozygous unless indicated otherwise. Classic mutations are the recurrent mutations in the TERT promoter discovered in melanoma (−124C>T, −146C>T, and the double mutations −124/−125CC>TT and −138/−139CC>TT).[17,18]

or no signal from the normal nucleotide at the position of the mutation. None of the classic mutations was found in a survey of 57 anonymized blood specimens that had been collected for unrelated genetic studies (data not shown) or in 15 benign skin specimens (Table 5). Previous work on *TERT* promoter mutations in melanoma noted that the classic mutations were mutually exclusive, with no tumor having more than one of the recurrent C>T or CC>TT mutations.[17,18] Our results followed this pattern of mutual exclusivity with one exception, a sporadic basal cell carcinoma (BCC 6C) that had both the −138/−139CC>TT and −124/−125CC>TT mutations, as well as a −100/−101CC>TT mutation, all homozygous (Figure 2c). The −57A>C *TERT* promoter mutation that co-segregated with disease in a pedigree with familial melanoma[17] was not found in any of the specimens.

For those patients with multiple tumors ($n=11$), three were concordant for the classic mutations (same mutation in all tumors), and eight were non-concordant, with at least some diversity for the mutations among the tumors (see Tables 1–3). Of the three concordant sets, two were sets of only two tumors, limiting the opportunity to find different mutations. Thus, the mutations appeared to be independent events as expected for independent primary tumors. The frequency of mutations in sporadic basal cell carcinoma and nevoid basal cell carcinoma syndrome was similar, with all four patients with the inherited form exhibiting *TERT* promoter mutations in two or more of the tumors.

Table 5 Non-malignant skin controls: mutations and demographic information

Specimen no. Cntrl-	*Diagnosis*	*Classic mutation*[a]	*Other mutations*[a]	*Age/gender*	*Site*
1	Lymphoid hyperplasia	N	N	35/M	Neck
2	Normal	N	N	46/M	Shoulder
3	Normal	N	N	27/F	Back
4	SK	N	N	55/M	Shoulder
5	SK	N	N	71/M	Temple
6A[b]	SK	N	N	50/M	Penis
6B[b]	SK	N	N	50/M	Shoulder
7	Cicatrix	N	N	70/F	Abdomen
8	Hemangioma	N	−126C>T, heterozygous	51/F	Chest
9	Cicatrix	N	N	31/F	Shoulder
10	Soft fibroma	N	N	69/F	Arm
11	SK	N	N	82/F	Arm
12	Hemangioma	N	N	53/F	Leg
13A[b]	SK	N	N	55/M	Chest
13B[b]	SK	N	N	55/M	Neck

Abbreviations: SK, seborrheic keratosis; N, no mutation identified in this category.
[a]All mutations were homozygous unless indicated otherwise. Classic mutations are the recurrent mutations in the TERT promoter discovered in melanoma (−124C>T, −146C>T, and the double mutations −124/−125CC>TT and −138/−139CC>TT).[17,18]
[b]Letter subcodes in the first column indicate tumors taken from the same patient, on the same day.

Other Mutations

In addition to the classic mutations identified in melanoma, C>T mutations were identified at positions −100, −101, −111, −126, −145, −150, and −159 (see Tables 1–5). Dual CC>TT mutations were also found at −100/−101, −125/−126, and −126/−127. As for the classic mutations, many of these non-classic mutations were also homozygous (Table 6). These mutations differ from the classic mutations in that they do not create a putative TCF binding motif. These non-classic mutations were found in some tumors concurrently with a classic mutation (12 cases) and in some independently (7 cases) (Table 6). Only one non-classic mutation was found in more than two cases, C>T at −101 (Figure 2b), which was found in eleven cases, but was accompanied by a classic mutation in all but three cases. Mutations at positions −101 and −126, as well as several other positions in the *TERT* promoter, have been described in melanoma.[17] One of the benign skin lesions, a hemangioma, had a heterozygous −126C>T mutation (Table 5).

Discussion

Maintenance of telomere length, most frequently by activation of telomerase, is a hallmark of cancer.[19] The recent discovery of activating mutations in the promoter of the *TERT* gene, at first in melanoma[17,18] and then in several other malignancies,[18,20] suggests that these mutations may be one of the most common mechanisms contributing to maintenance of telomeres in cancer. We examined mutations in the *TERT* promoter in two UV-related skin cancers, basal cell carcinoma, and squamous cell carcinoma, and in a cohort of patients with basal cell carcinomas arising in association with nevoid basal cell carcinoma syndrome. We detected promoter mutations associated with *TERT* gene activation to be very frequent in these cancers, particularly in basal cell carcinomas (74%). While squamous cell carcinomas also displayed a high frequency of mutations (50%), mutations were significantly more frequent in the basal cell tumors ($P=0.046$). Mutations in sporadic basal cell carcinomas (78%) and tumors from individuals with nevoid basal cell carcinoma syndrome (68%) were similar. The classic *TERT* promoter mutations were more frequent in squamous cell carcinomas compared with the precursor lesion Bowen's disease ($P=0.019$). While the poorly differentiated squamous cell carcinomas were more likely to have the classic mutations than the well-differentiated tumors, this difference did not reach statistical significance, possibly due to the small sample size. A recent publication found a *TERT* promoter mutation in one of the five skin squamous cell carcinomas studied.[20]

All mutations identified were C>T substitutions or CC>TT double substitutions at dipyrimidine sites, characteristic of mutations associated with exposure to UV.[21] This includes the classic mutations[17,18] as well as the non-classic mutations. We speculate that most of the non-classic mutations were UV-induced passenger mutations that were present in the skin cells before tumorigenesis, with the possible exception of the −101C>T mutation that was found more frequently than the other non-classic mutations. The non-classic mutations are likely not pathogenic because they do not create the TCF binding motif (TTCCGG) that is created by the classic mutations, they co-occur frequently with the classic mutations, and with the exception of -101C>T, are not recurrent. Considering the

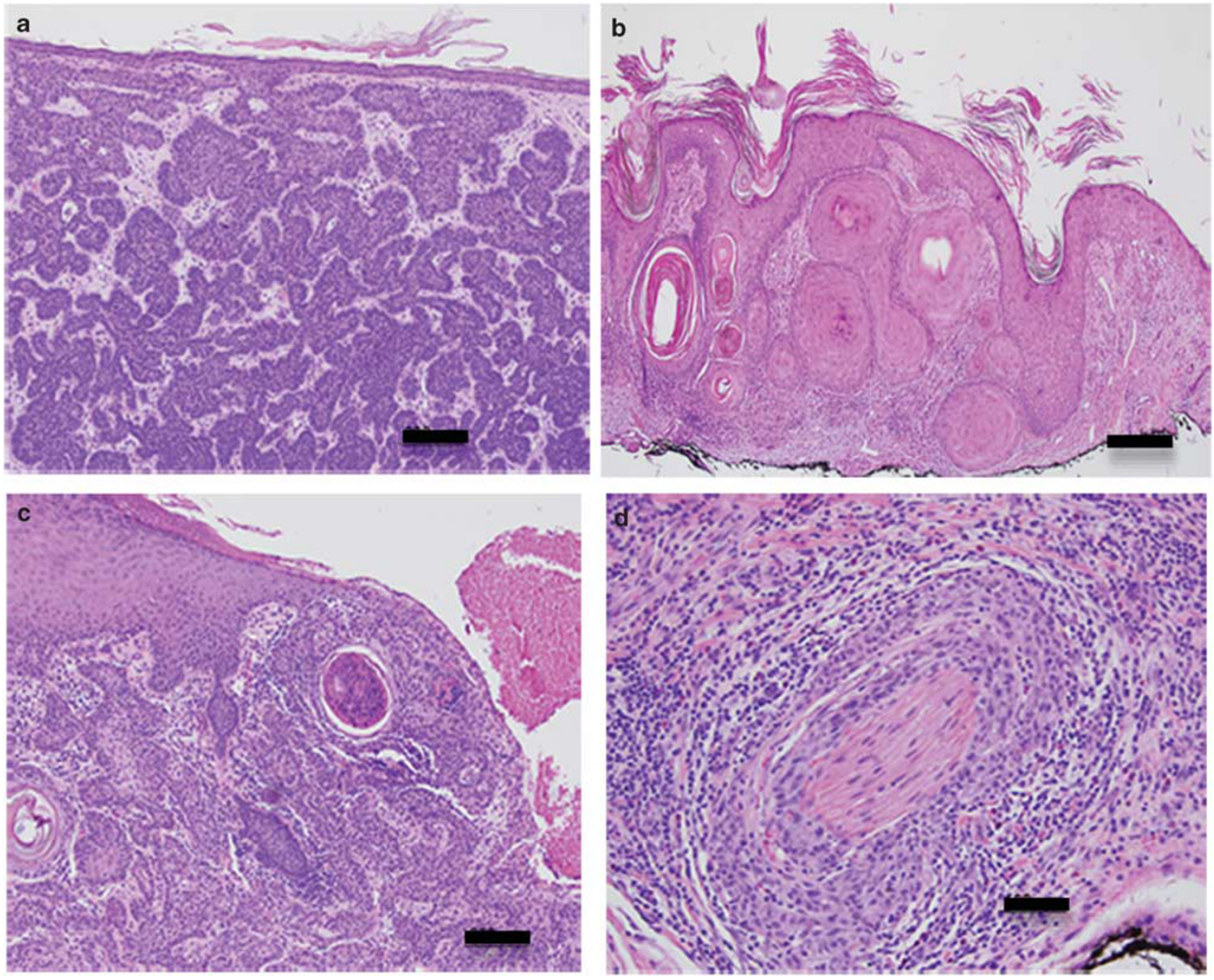

Figure 1 Representative tumors used in the study. Hematoxylin and eosin-stained sections of representative tumors used in these studies. (**a**) Basal cell carcinoma (bar = 200 μm). (**b**) Well-differentiated squamous cell carcinoma (bar = 500 μm). (**c**) Poorly differentiated squamous cell carcinoma (bar = 200 μm). (**d**) Perineural involvement by tumor in a poorly differentiated squamous cell carcinoma (bar = 100 μm).

Table 6 Distribution of *TERT* gene promoter mutations in the study specimens

		Classic mutations[a]		*Non-classic mutations*		
Diagnosis	*N*	*Number (%)*	*Homozygous (%)*[b]	*Number (%)*	*Homozygous (%)*[b]	*Co-occurrence*[c]
Sporadic basal cell carcinoma	23	18 (78%)	15 (83%)	8 (35%)	5 (62%)	6 (75%)
Nevoid basal cell carcinoma syndrome	19	13 (68%)	12 (92%)	5 (26%)	3 (60%)	4 (80%)
Squamous cell carcinoma	26	13 (50%)	8 (62%)	5 (19%)	3 (60%)	2 (40%)
Bowen's disease	11	1 (9%)	1 (100%)	1 (9%)	0	0
Benign	15	0	NA	1	0	NA

Abbreviations: N, total number of specimens; NA, not applicable.
[a]The classic mutations are −124C>T, −146C>T, and the double mutations −124/−125CC>TT and −138/−139CC>TT.
[b]Number and percent of mutations that were homozygous.
[c]Number and percent of specimens with a non-classic mutation that also had a classic mutation.

high frequency of the −101C>T mutation in BCC, it may be worthy of further investigation with functional studies. Interestingly, we also found a heterozygous −126C>T mutation in one of the two hemangiomas studied. This is a preliminary finding that also may be worthy of further investigation.

Although we did not have germline DNA from the patients to directly determine whether the

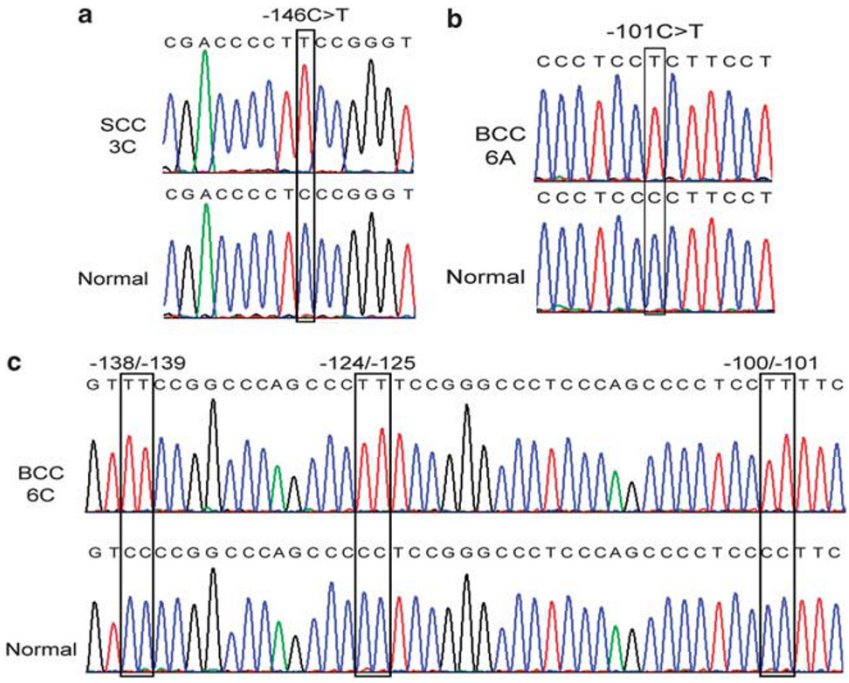

Figure 2 Sanger sequencing results showing several of the mutations found in the *TERT* promoter. (**a**) A homozygous −146C>T mutation in squamous cell carcinoma SCC 3C (Table 3). (**b**) A homozygous −101C>T mutation in sporadic basal cell carcinoma BCC 6A (Table 1). (**c**) A homozygous −124/−125CC>TT, −138/−139CC>TT, and −100/−101CC>TT from sporadic basal cell carcinoma BCC 6C (Table 1).

mutations were somatic, the detection of discordant mutations in multiple tumors from the same patient is consistent with a somatic origin. This conclusion is also consistent with results on TERT promoter mutations in melanoma and glioma, which have also been shown to be somatic.[17,18,20]

The majority of mutations were homozygous or hemizygous with all, or almost all, of the signal representing the mutant nucleotide (Table 6). This novel finding may have been overlooked previously due to differences in tumor purity between studies (contaminating normal cells in tumor samples would make detection of a homozygous mutation difficult) or may be a unique feature of these tumors. We speculate that the apparent homozygosity is due to mitotic recombination, a common event resulting in loss of the normal copy of tumor-suppressor genes and oncogenes,[22–24] which would likely cause even greater *TERT* activation than having only one copy with an activating mutation. For the *TERT* promoter, there may be selection for even greater expression than can be supported by an activating mutation in one copy alone. Alternative explanations include deletion of a nearby tumor suppressor, or a gene conversion event. Further studies will be needed to determine the cause of the homozygosity observed in these tumors.

To summarize, we show that the somatic activating mutations in the *TERT* promoter, initially identified in melanoma, are also frequently present in the two most common types of skin cancer, basal cell and squamous cell carcinoma. The mutations are consistent with UV radiation mutagenesis, and are very frequently homozygous. The high recurrence of these mutations in skin cancer, as well as several other types of cancer,[17,18,20] suggests that they are significant, and may have a role in initiation or progression of both melanoma and non-melanoma skin cancer. Considering the importance of maintaining telomere length in the pathogenesis of cancer,[19] and the role of telomerase in carrying out this function in normal cells,[13] we suspect that the *TERT* promoter mutations contribute to carcinogenesis by increasing expression of telomerase.[17,18] However, functional studies, beyond the scope of this report, including reporter and transformation assays, are necessary to test this. Because of the high frequency of basal cell carcinoma in the human population, and the identification of *TERT* promoter mutations in the majority of these tumors, these mutations may be the most common cancer-associated mutations yet discovered.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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