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ARTICLE

TERT Promoter Mutation Status as an Independent Prognostic Factor in Cutaneous Melanoma

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Background Recently, *TERT* promoter mutations were identified at high frequencies in cutaneous melanoma tumor samples and cell lines. The mutations were found to have a UV-signature and to lead to increased *TERT* gene expression. We analyzed a large cohort of melanoma patients for the presence and distribution of *TERT* promoter mutations and their association with clinico-pathological characteristics.

Methods 410 melanoma tumor samples were analyzed by Sanger sequencing for the presence of *TERT* promoter mutations. An analysis of associations between mutation status and various clinical and pathologic variables was performed.

Results *TERT* promoter mutations were identified in 154 (43%) of 362 successfully sequenced melanomas. Mutation frequencies varied between melanoma subtype, being most frequent in melanomas arising in nonacral skin (48%) and melanomas with occult primary (50%), and less frequent in mucosal (23%), and acral (19%) melanomas. Mutations carried a UV signature (C>T or CC>TT). The presence of *TERT* promoter mutations was associated with factors such as *BRAF* or *NRAS* mutation ($P < .001$), histologic type ($P = .002$), and Breslow thickness ($P < .001$). *TERT* promoter mutation was independently associated with poorer overall survival in patients with nonacral cutaneous melanomas (median survival 80 months vs 291 months for wild-type; hazard ratio corrected for other covariates 2.47; 95% confidence interval [CI] = 1.29 to 4.74; $P = .006$).

Conclusions UV-induced *TERT* promoter mutations are one of the most frequent genetic alterations in melanoma, with frequencies varying depending on melanoma subtype. In nonacral cutaneous melanomas, presence of *TERT* promoter mutations is independently associated with poor prognosis.

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Melanomas are characterized by recurrent mutations of oncogenes such as *BRAF* (v-Raf murine sarcoma viral oncogene homolog) (1), *NRAS* (neuroblastoma RAS viral oncogene homolog) (2), and *KIT* (v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog) (3), and tumor suppressor genes including *CDKN2A* and *PTEN* (4,5). Recent whole-exome sequencing studies have identified a host of additional genetic events (6,7), many of which occur only in a small proportion of tumors, or in combination with other genetic events. The clinical relevance of these recently identified genetic events, such as putative activating mutations in *PPP6C*, *STK19*, *RAC1* (6,7), and *TRRAP* (8), remains to be seen. Whole-exome sequencing approaches focus on enriching and sequencing protein-coding regions of DNA, neglecting most noncoding DNA sequences. This could be the reason why only a few mutations in regulatory DNA domains have been described to date.

Recently, two independent studies identified frequent mutations in the promoter region of the *TERT* (telomerase reverse transcriptase) gene, encoding the catalytic subunit of the telomerase holoenzyme (9,10). Horn et al. identified *TERT* promoter mutations in a melanoma-prone family, in which affected members developed melanomas at a very young age with near 100% penetrance (10). Subsequently, recurrent mutations at other locations in the *TERT* promoter were identified in 33% of sporadic primary melanomas and 74% of melanoma cell lines. Huang et al. screened whole-genome sequencing data of melanomas and found that, apart from mutations in *BRAF* and *NRAS*, recurrent *TERT* promoter mutations were the most frequent genomic alterations (9). They validated their findings in a cohort of 70 melanoma samples and short-term cultures, of which 50 (71%) harbored recurrent *TERT* promoter mutations (9). Functional studies by both groups

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showed that the promoter mutations led to a 2–4–fold increase in gene expression, most likely a result of the mutations creating ETS transcription factor binding sites (9,10).

Subsequent studies have identified *TERT* promoter mutations in a wide array of human cancers, including bladder cancer, hepatocellular carcinoma, thyroid cancer, and different types of gliomas (11–14). Killela et al. suggested that high frequencies of *TERT* promoter mutations occurred in tumors arising in tissues with low rates of self-renewal (11).

The goals of our study were to analyze the frequency of *TERT* promoter mutations in a large cohort of melanomas: a) to determine if the types and frequency of mutations varied between melanoma subtypes, b) to establish whether *TERT* promoter mutations were associated with prognosis, and c) to ascertain their prevalence in the germ line of patients with sporadic cutaneous melanoma.

Methods

Sample Selection and Histopathology

Melanoma tumor samples were obtained from the tissue archives of the Departments of Dermatology of the University Hospital Essen, Germany, and the Hospital Clinic Barcelona, Spain. Additionally, peripheral blood mononuclear cells were obtained from patients with cutaneous melanoma (Hospital Clinic Barcelona, Spain). Only one sample per individual was included. Sample characteristics, obtained from medical records, are listed in Table 1. The study was performed with written informed consent from participating patients and in accordance with the guidelines of the Ethics Committees of both institutions.

DNA Isolation

10 µm-thick sections were cut from formalin-fixed, paraffin-embedded tumor tissues. The sections were deparaffinized and manually microdissected according to standard procedures. Genomic DNA was isolated using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In rare cases in which frozen tissue was available, the tissue was directly applied to the Qiagen Kit for DNA purification. Constitutional DNA was isolated from peripheral blood mononuclear cells (PBMC), applying the salting-out method as previously described (15).

Direct (Sanger) Sequencing

Sequencing for *BRAF*, *NRAS*, and *KIT* was frequently performed sequentially, as these mutations are almost always found to be mutually exclusive in melanoma (4). If no mutation in *BRAF* was found, *NRAS* was sequenced. *KIT* was sequenced primarily in mucosal and acral melanomas lacking *BRAF* and *NRAS* mutations.

Nested polymerase chain reaction (PCR) was performed to amplify *BRAF* exon 15 and *NRAS* exon 1 and 2 and sequenced as previously described (16). Sequencing of *KIT* exons 9, 11, 13, 17 and 18 was performed in a similar fashion for a number of samples. Primers and conditions used for *KIT* sequencing have been previously published (17). *GNAQ* and *GNA11* were only sequenced in select uveal melanoma samples, as previously described (18). PCR amplification of the *TERT* promoter region was performed using primers: hTERT_F ACGAACGTGGCCAGCGGCAG and hTERT_R CTGGCGTCCCTGCACCCTGG (474 bp product),

Table 1. Summary of clinical, pathologic and genetic characteristics of 362 melanoma patients*

Characteristics	N (%)
Sex	
Female	170 (47)
Male	192 (53)
Age, y	
Median	58
Range	16–94
≤60	179 (49)
>60	173 (48)
Missing data	10 (3)
Mutant oncogene	
BRAF	128 (36)
NRAS	68 (19)
KIT	2 (1)
TERT promoter mutations†	
All mutations	154/362 (43%)
250C>T	77 (50)
228C>T	47 (31)
242CC>TT	20 (13)
228CC>TT	10 (6)
228C>T 230C>T	2 (1)
228C>T 242C>T	1 (1)
250C>T 253C>T	1 (1)
228C>T 242CC>TT	1 (1)
242CC>TT 250C>T	1 (1)
228CCC>TTT	1 (1)
228CC>TT 242CC>TT 250C>T	1 (1)
Stage at diagnosis‡	
I	59 (16)
II	109 (30)
III	109 (30)
IV	24 (7)
Missing data	62 (17)
Anatomic distribution of primary	
Nonacral skin	248 (69)
Acral	42 (12)
Mucosal	26 (7)
Occult	34 (9)
Conjunctival	7 (2)
Uveal	3 (1)
Missing data	2 (0)
Anatomic site of skin and acral tumors	
Head & neck	43 (12)
Upper limbs	24 (7)
Trunk	108 (30)
Lower limbs	83 (23)
Missing data	104 (29)
Histologic type (skin and acral tumors)	
ALM	37 (10)
LMM	6 (2)
NM	79 (22)
SSM	66 (18)
Not classified	85 (24)
Missing data	89 (25)
Breslow thickness (skin and acral tumors), mm	
Median	3.7
Range	0.1–55.0
0.01–1.00	58 (16)
1.01–2.00	66 (18)
2.01–4.00	83 (23)
>4.00	76 (21)
Missing data	79 (22)

(Table continues)

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Table 1. (Continued).

Characteristics	N (%)
Clark level (skin and acral tumors)	
I	3 (2)
II	9 (3)
III	66 (19)
IV	88 (24)
V	23 (6)
Missing data	173 (48)
Sample type sequenced	
Primary	145 (40)
Metastasis	145 (40)
Recurrence	9 (3)
Occult	34 (9)
Missing data	29 (8)
Ulceration present	71/252 (28)
SLN positive	75/161 (47)

* ALM = acral lentiginous melanoma; BRAF = v-Raf murine sarcoma viral oncogene homolog; KIT = v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog; LMM = lentigo maligna melanoma; NM = nodular melanoma; NRAS = neuroblastoma RAS viral oncogene homolog; SLN = sentinel lymph node; SSM = superficial spreading melanoma; TERT = telomerase reverse transcriptase.

† Mutations are annotated applying the last three digits of the first nucleotide mutated in the chromosome location according to hg19: Chr.5: 1295xxx (where xxx is a place holder for the mutation number).

‡ Staging according to the American Joint Committee on Cancer (AJCC) Melanoma Staging System 2009 (32).

or primers hTERT_short_F CAGCGCTGCCTGAAACTC and hTERT_short_R GTCCTGCCCCTTCACCTT (163bp product) as previously described (10). PCR reaction products were purified with the QIAquick PCR Purification Kit (Qiagen) and then used as templates for sequencing. The sequencing chromatogram files were examined, and mutations were identified using Chromas (version 2.01, University of Sussex, Brighton, UK) or Sequencher (Gene Codes Corporation, Ann Arbor, MI) software.

Statistical Analyses

Associations of TERT Promoter Mutation Status With Clinical and Pathologic Variables. We used univariate logistic regression analyses to explore associations of *TERT* promoter mutation carrier status with available clinical and pathologic variables, including age, sex, *BRAF* and *NRAS* mutation status, anatomical distribution of primary tumor, histologic subtype, Breslow thickness, Clark level, ulceration and sentinel lymph node status. Further details are listed in Table 2 and in Supplementary Tables 1 and 2 (available online).

Associations of TERT Promoter Mutation Status and Clinico-Pathologic Variables With Survival. We investigated associations between clinico-pathologic factors, *TERT* promoter status, and oncogene mutation status with overall survival, defined as the interval from time of diagnosis of primary melanoma to death. Cases in which the endpoint was not reached at the time of the last follow-up were censored. Univariate results were displayed by the Kaplan-Meier method and hazard ratio estimates and *P* values were derived from Cox proportional hazards models. Model diagnostics included both graphical and formal checks of the proportional hazards assumption. Subgroup analyses were performed selecting non-acral cutaneous melanoma patients only (ie, we excluded patients with acral, ocular, and mucosal melanomas). Multivariable analyses included two steps with a focus on *TERT* promoter status. In the extended model, all main effects with univariate *P* values less than or equal to .1 were investigated simultaneously (Tables 3 and 4, Model 1). To avoid overfitting, a restricted model (Tables 3 and 4, Model 2) with only those variables showing evidence of independent effects in Model 1 ($P \leq .2$) in addition to *TERT* promoter status was jointly assessed afterwards. In addition, we also performed automatic forward and backward selection strategies, which had no impact on our conclusions. Model diagnostics included graphical and formal checks.

Confidence intervals (CI) were calculated with coverage of 95%. All reported *P* values are nominal and two-sided. We applied a significance level of 5%. All statistical analyses were performed using SPSS Statistics software (version 20.0; SPSS Chicago, IL) or R 3.0.2.

RESULTS

Tumors and Patients

In total, 410 melanoma samples were obtained, 369 from the University Hospital Essen, Germany, and 41 from the Hospital Clinic Barcelona, Spain. The cohort further analyzed consisted of samples from 362 patients in whose tumors *TERT* promoter sequencing was successful. There were 170 women and 192 men with a median age of 58 years (range 16–94 years). Follow-up data was available in 353 of 362 cases, with a median follow-up duration of 34.6 months (interquartile range = 13.3–75.9 months). The patient demographics and clinico-pathologic features of the cohort are summarized in Table 1.

Oncogene Mutations

Sequencing for *BRAF* and *NRAS* was successful in 324 (90%) of cases. *KIT* was successfully sequenced in 88 (24%) cases. One hundred and twenty-eight (36%) tumors harbored *BRAF* mutations, including 118 (92%) V600E, 9 (7%) V600K, and 1 (0.7%) D594N mutations. *NRAS* mutations were found in 68 (19%) cases, including 30 (44%) Q61R, 22 (32%) Q61K, 10 (15%) Q61L, 3 (4%) Q61H exon 2, and rare exon 1 mutations, including 1 (1.5%) G13R, 1 (1.5%) G13D, and 1 (1.5%) G12S mutation. Two samples had *KIT* mutations, both in exon 11, consisting of 1 (50%) W557G and 1 (50%) L576P mutation.

TERT Promoter Mutations

The *TERT* promoter was successfully PCR-amplified and sequenced in 362 cases. The tumors were: primary (145), metastases (145), recurrences (9), and occult (34). In 29 cases, definitive classification into one of these categories was not possible. Recurrent mutations identified were located at the previously described hotspots: Chr.5:1295228C>T, Chr.5:1295228_1295229CC>TT, Chr.5:1295242_1295243CC>TT, or Chr.5:1295250C>T (annotated according to human genome assembly hg19). Mutations can alternatively be denoted with respect to their upstream location of the *TERT* gene ATG initiation codon, as c.-124C>T, c.-124_125CC>TT, c.-138_139CC>TT, and c.-146C>T, respectively. For simplicity, the mutations will further be referred to using solely the last three digits of the chromosome location nomenclature, ie, as 228C>T, 228CC>TT, 242CC>TT, and 250C>T.

In total, the *TERT* promoter region showed wild-type reads in 208 tumors (57%) and harbored at least one mutation in 154 cases (43%). There were 77 (50%) 250C>T mutations, followed by 47

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Table 2. Associations of *TERT* mutation status with clinical and pathological variables in 362 melanoma patients*

Variables	*TERT* WT N (%)	*TERT* mut N (%)	Total	OR† (95% CI)	*P* ‡
Sex					
Female	103 (61)	67 (39)	170	1.0 (referent)	.26
Male	105 (55)	87 (45)	192	1.27 (0.89 to 1.94)	
Age					
per 5 y			352	1.01 (0.95 to 1.08)	.77
≤47 y	53 (60)	36 (40)	89	1.00 (referent)	.18
47–60 y	50 (56)	40 (44)	90	1.18 (0.65 to 2.13)	
60–70 y	37 (47)	42 (53)	79	1.67 (0.91 to 3.08)	
>70 y	59 (63)	35 (37)	94	0.87 (0.48 to 1.58)	
Missing data			10		
Mutant oncogene					
WT	85 (68)	41 (33)	126	1.00 (referent)	.001
BRAF	58 (45)	70 (55)	128	2.50 (1.50 to 4.17)	
NRAS	31 (46)	37 (54)	68	2.47 (1.35 to 4.53)	
KIT	2 (100)	0	2	NA	
Missing data			38		
BRAF and *NRAS*					
Both WT	86 (68)	41 (32)	127	1.00 (referent)	<.001
Either mutant	89 (45)	107 (55)	196	2.52 (1.58 to 4.02)	
Missing data			39		
Stage at diagnosis §					
I	38 (66)	20 (35)	58	1.00 (referent)	.06
II	49 (45)	60 (55)	109	2.33 (1.20 to 4.50)	
III	60 (55)	49 (45)	109	1.55 (0.80 to 3.00)	
IV	15 (63)	9 (38)	24	1.14 (0.42 to 3.06)	
Missing data			62		
Anatomic distribution of primary					
Nonacral skin	129 (52)	119 (48)	248	1.00 (referent)	.001
Acral	34 (81)	8 (19)	42	0.25 (0.11 to 0.57)	
Mucosal	20 (77)	6 (23)	26	0.33 (0.13 to 0.84)	
Occult	17 (50)	17 (50)	34	1.08 (0.53 to 2.22)	
Conjunctival	3 (43)	4 (57)	7	NA	
Uveal	3 (100)	0	3	NA	
Missing data			2		
Anatomic site of skin and acral tumors					
Head & neck	26 (61)	17 (39)	43	1.00 (referent)	.41
Upper limbs	14 (58)	10 (42)	24	1.09 (0.40 to 3.02)	
Trunk	51 (47)	57 (53)	108	1.71 (0.83 to 3.51)	
Lower limbs	46 (55)	37 (45)	83	1.23 (0.58 to 2.60)	
Missing data			104		
Histologic type					
ALM	27 (73)	10 (27)	37	1.00 (referent)	.002
LMM	6 (100)	0	6	NA	
NM	32 (41)	47 (60)	79	3.97 (1.69 to 9.31)	
SSM	43 (65)	23 (35)	66	1.44 (0.60 to 3.50)	
Unclassified	48 (57)	37 (43)	85	2.08 (0.90 to 4.83)	
Missing data			89		
Breslow thickness					
0.01–1.00 mm	47 (81)	11 (19)	58	1.00 (referent)	<.001
1.01–2.00 mm	32 (49)	34 (52)	66	4.54 (2.01 to 10.26)	
2.01–4.00 mm	44 (53)	39 (47)	83	3.79 (1.73 to 8.31)	
>4.00 mm	36 (47)	40 (53)	76	4.75 (2.14 to 10.52)	
Missing data			79		
Clark level (skin tumors only)					
I	3 (100)	0	3	NA	.12
II	9 (100)	0	9	NA	
III	43 (65)	23 (35)	66	1.00 (referent)	
IV	43 (49)	45 (51)	88	1.96 (1.02 to 3.77)	
V	12 (52)	11 (48)	23	1.71 (0.66 to 4.49)	
Missing data			173		
Ulceration					
Absent	102 (56)	79 (44)	181	1.00 (referent)	.11
Present	32 (45)	39 (55)	71	1.57 (0.91 to 2.73)	
Missing data			110		

(Table continues)

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Table 2. (Continued).

Variables	*TERT* WT	*TERT* mut			
	N (%)	N (%)	Total	OR† (95% CI)	*P*‡
SLN					
Negative	44 (51)	42 (49)	86	1.00 (referent)	.78
Positive	40 (53)	35 (47)	75	0.92 (0.49 to 1.70)	
Missing data			201		

* ALM = acral lentiginous melanoma; BRAF = v-Raf murine sarcoma viral oncogene homolog; KIT = v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog; LMM = lentigo maligna melanoma; NM = nodular melanoma; NRAS = neuroblastoma RAS viral oncogene homolog; SLN = sentinel lymph node; SSM = superficial spreading melanoma; TERT = telomerase reverse transcriptase. All statistical tests were two-sided.

† The odds ratio (OR) displays the odds for being a *TERT* promoter mutation carrier as compared with being a *TERT* promoter wild-type carrier.

‡ *P* value for the omnibus model test of the univariate predictors (cases with sparse observations for which no estimator is provided are also not included in the test).

§ Staging according to the American Joint Committee on Cancer (AJCC) Melanoma Staging System 2009 (32).

(31%) 228C>T mutations. Di-pyrimidine mutations were also frequent with 20 (13%) 242CC>TT and 10 (6%) 228CC>TT mutations. Rare tumors harbored more than one mutation in the *TERT* promoter (Table 1). Details of cases in which *TERT* promoter sequencing failed (either because of failure of PCR amplification or mixed or ambiguous reads) are listed in Supplementary Table 2 (available online). Clinical and pathologic characteristics of primary and metastatic tumors with regard to *TERT* promoter mutation status are detailed in Supplementary Table 3 (available online).

Germ-Line *TERT* Promoter Analysis

Constitutional DNA was isolated from peripheral blood mononuclear cells obtained from 196 patients with cutaneous melanoma. Only in two (1%) samples were non-SNP germ-line variants noted. One was a Chr.5:1295229C>A (c.-125C>A), the other a Chr.5:1295319G>A (c.-215G>A) nucleotide change.

Associations of *TERT* Promoter Status With Clinical and Pathologic Variables

TERT promoter mutations were statistically significantly more common in tumors harboring either a *BRAF* or *NRAS* mutation ($P < .001$), in tumors of nonacral skin than in those of acral or mucosal locations ($P = .001$), in nodular and superficial spreading histologic types ($P = .002$), and thicker tumors ($P < .001$) (Table 2). The *TERT* mutation frequency differed between primary (36.6%; 53 of 145) and metastatic tumor samples (50.3%; 73 of 145; $P = .02$ for the frequency difference). For occult and recurrent or local metastatic tumor samples, the frequencies were 50% (17 of 34) and 56% (5 of 9), respectively.

Associations of *TERT* Promoter Mutation Status and Clinico-Pathologic Variables With Overall Survival

Survival analyses were performed for all patients with tumors (Table 3) as well as for the largest subgroup, namely patients with cutaneous melanomas arising in nonacral skin (excluding occult melanomas, melanomas in acral locations, and ocular and mucosal melanomas) (Figure 1; Table 4).

In all tumors (Table 3), univariate predictors of survival were Breslow thickness ($P < .001$), Clark level (skin tumors only) ($P = .001$), presence of ulceration ($P = .03$), increasing stage at diagnosis ($P < .001$), and anatomic location of primary (poorest in occult and intermediate in acral, compared with nonacral skin; $P = .01$). Patients with *TERT* promoter-mutant tumors showed a trend toward worse prognosis (median survival 106 months, compared with 291 months for wild-type tumors; $P = .06$). The multivariable analyses indicated that only *BRAF* or *NRAS* mutations and to some extent tumor stage at diagnosis (in particular stage III or IV compared with lower stages or alternatively increased Breslow thickness or Clark levels) were independent predictors of patient survival. This finding was robust across various model choices (Table 3, Model 1, including footnotes) and similarly found using automatic variable selection strategies (data not reported). In a restricted model (Table 3, Model 2) including *TERT* mutation status in addition to *BRAF* or *NRAS* mutation status and tumor stage, only tumor stage was an independent factor.

In nonacral cutaneous melanomas (Table 4), factors statistically significantly associated with poorer patient survival in the univariate models were: *TERT* promoter mutation ($P = .002$), increasing stage at diagnosis ($P = .002$), anatomic location of primary (poorer for tumors on head/neck and trunk than for those on lower limbs; $P = .02$), increasing Breslow thickness ($P < .001$), increasing Clark level ($P = .009$), presence of *BRAF* or *NRAS* mutation ($P = .04$), and presence of ulceration ($P = .02$). Multivariate analyses (Table 4, Model 2) robustly showed that *TERT* promoter mutation status ($P = .006$), increasing stage at diagnosis ($P = .001$) (or Breslow thickness or Clark level), and anatomic location of primary ($P = .009$) were independently associated with poorer survival. *TERT* promoter mutation carriers had a median survival of 80 months compared with 291 months for noncarriers. The estimated adjusted hazard ratio was 2.47 (95% CI = 1.29 to 4.74, $P = .006$) for *TERT* promoter mutation carriers compared with noncarriers.

Finally, we performed a sensitivity analysis of patient survival stratified by primary and metastatic nonacral cutaneous melanoma samples with *TERT* promoter mutation carrier status (Supplementary Figure 1, available online). The hazard ratio estimates for *TERT* promoter mutation carriers compared to noncarriers were relatively similar, 2.18 (95% CI = 0.82 to 5.76, $P = .11$) for patients with primary samples and 2.01 (95% CI = 1.04 to 3.86, $P = .04$) for patients with metastatic samples.

Discussion

Overall we identified recurrent *TERT* promoter mutations in 43% (154/362) of all melanomas analyzed and in 48% (119/248) of

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Table 3. Associations of *TERT* mutation status and clinico-pathologic variables with overall survival, which was available for 353 of the 362 melanoma patients*

	N†	Median survival	Univariate Cox models		Multivariable Cox Model 1‡ (extended model)		Multivariable Cox Model 2§ (restricted model+*TERT*)	
Variables			**HR (95% CI)**	***P*‖**	**HR (95% CI)**	***P*‖**	**HR (95% CI)**	***P*‖**
Sex								
Female	166	210	1.00 (referent)	.08	1.00 (referent)	1.00		
Male	187	107	1.44 (0.96 to 2.15)		1.00 (0.51 to 1.96)			
Age¶								
per 5 y	348		1.07 (1.00 to 1.14)	.07	1.05 (0.95 to 1.17)	.34		
≤47 y	86	205	1.00 (referent)	.18				
47–60 y	90	141	1.15 (0.66 to 1.98)					
60–70 y	79	NA	1.33 (0.74 to 2.39)					
>70 y	93	NA	1.91 (1.05 to 3.48)					
Mutant oncogene								
WT	126	108	1.00 (referent)	.26				
BRAF	125	28	1.43 (0.90 to 2.28)					
NRAS	68	14	1.46 (0.82 to 2.60)					
KIT	2	NA	NA					
BRAF and *NRAS*								
Both WT	127	290	1.00 (referent)	.10	1.00 (referent)	.01	1.00 (referent)	.13
Either mutant	193	111	1.45 (0.94 to 2.24)		3.41 (1.28 to 8.53)		1.45 (0.90 to 2.33)	
TERT								
WT	200	291	1.00 (referent)	.06	1.00 (referent)	.37	1.00 (referent)	.13
Mutant	153	106	1.47 (0.99 to 2.19)		1.38 (0.69 to 2.76)		1.39 (0.90 to 2.16)	
Stage at diagnosis#								
I	58	NA	1.00 (referent)	<.001	1.00 (referent)	.15	1.00 (referent)	<.001
II	109	111	1.80 (0.95 to 3.40)		1.24 (0.40 to 3,88)		1.80 (0.93 to 3.47)	
III	109	76	2.97 (1.54 to 5.70)		3.75 (0.83 to 16.90)		3.05 (1.56 to 5.97)	
IV	24	16	12.58 (5.70;27.76)		NA		13.11 (5.85 to 29.38)	
Anatomic location of primary								
Nonacral skin	239	206	1.00 (referent)	.01	1.00 (referent)	.44		
Acral	42	93	1.84 (0.99;3.44)		1.40 (0.60 to 3.25)			
Mucosal	26	NA	1.01 (0.41;2.52)		NA			
Occult	34	34	2.58 (1.38 to 4.82)		NA			
Conjunctival	7	130	NA		NA			
Histologic type								
ALM	37	NA	1.00 (referent)	.16				
LMM	6	NA	NA					
NM	79	81	1.09 (0.50 to 2.36)					
SSM	60	210	0.56 (0.25 to 1.30)					
Unclassified	85	428	0.65 (0.29 to 1.45)					

(Table continues)

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Table 3. (Continued).

Variables	N†	Median survival	Univariate Cox models		Multivariable Cox Model 1‡ (extended model)		Multivariable Cox Model 2§ (restricted model+*TERT*)	
			HR (95% CI)	*P* ǁ	HR (95% CI)	*P* ǁ	HR (95% CI)	*P* ǁ
Breslow thickness, mm								
0.01–1.00	51	NA	1.00 (referent)	<.001	**		**	
1.01–2.00	66	107	2.05 (0.93 to 4.52)					
2.01–4.00	83	206	1.55 (0.71 to 3.36)					
>4.00	76	61	4.44 (2.13 to 9.27)					
Clark level (skin tumors only)								
I	3	NA	NA	.001	**		**	
II	8	NA	NA					
III	60	NA	1.00 (referent)					
IV	88	NA	1.93 (0.92 to 4.04)					
V	23	71	4.64 (1.96 to 11.00)					
Ulceration								
Absent	181	142	1.00 (referent)	.03	1.00 (referent)	.80		
Present	71	77	1.82 (1.08 to 3.06)		1.10 (0.24 to 2.03)			
SLN								
Negative	86	110	1.00 (referent)	.09	1.00 (referent)	.47		
Positive	75	68	1.72 (0.92 to 3.20)		0.66 (0.21 to 2.03)			

* ALM = acral lentiginous melanoma; BRAF = v-Raf murine sarcoma viral oncogene homolog; KIT = v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog; LMM = lentigo maligna melanoma; NM = nodular melanoma; NRAS = neuroblastoma RAS viral oncogene homolog; SLN = sentinel lymph node; SSM = superficial spreading melanoma; TERT = telomerase reverse transcriptase. All statistical tests were two-sided.

† The number of available data for a particular variable in the univariate analysis.

‡ Model in which all predictors with univariate *P* values ≤.10 are included—for “Age” see †; see also **; no interactions were considered.

§ Restricted model which includes “*BRAF* and *NRAS*,” “Stage at diagnosis,” and “TERT;” no interactions were considered.

ǁ *P* value for the omnibus model test of the univariate predictors (cases with sparse observations for which no estimator is provided are also not included in the omnibus test).

¶ Multivariable Cox regression analysis results for “Age” are displayed for the continuous linear predictor, as other transformations including those displayed or “Age” as continuous quadratic predictor or “Age” as both linear and quadratic continuous predictor, had no impact on the conclusions.

Staging according to the American Joint Committee on Cancer (AJCC) Melanoma Staging System 2009 (32).

** There was strong multicollinearity between “Stage at diagnosis,” “Breslow thickness,” and “Clark level,” so we decided to only include “Stage at diagnosis,” given that this resulted in the largest number of complete data sets (n = 121 for model 1 and n = 282 for model 2) with no impact on the conclusions.

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Table 4. Associations of *TERT* mutation status and clinico-pathologic variables with overall survival, which was available for 239 of 248 with melanomas of nonacral skin*

			Univariate Cox models		Multivariable Cox Model 1‡ (extended model)		Multivariable Cox Model 2§ (restricted model+*TERT*)	
Variables	**N†**	**Median survival**	**HR (95% CI)**	***P*‖**	**HR (95% CI)**	***P*‖**	**HR (95% CI)**	***P*‖**
Sex								
Female	103	210	1.00 (referent)	.09	1.00 (referent)	.75		
Male	136	138	1.53 (0.93 to 2.54)		0.91 (0.51 to 1.63)			
Age¶								
per 5 y	236		1.02 (0.94 to 1.11)	.57				
≤47 y	67	210		.70				
47–60 y	58	177	0.96 (0.50 to 1.85)					
60–70 y	55	NA	1.29 (0.65 to 2.55)					
>70 y	56	NA	1.42 (0.67 to 3.02)					
Mutant oncogene								
WT	73	291	1.00 (referent)	.13				
BRAF	96	114	1.84 (1.01 to 3.33)					
NRAS	49	138	1.60 (0.75 to 3.38)					
BRAF and *NRAS*								
Both WT	73	291	1.00 (referent)	.04	1.00 (referent)	.04	1.00 (referent)	.04
Either mutant	145	114	1.77 (1.00 to 3.13)		2.26 (1.03 to 4.97)		2.11 (1.03 to 4.33)	
TERT								
WT	121	291	1.00 (referent)	.002	1.00 (referent)	.01	1.00 (referent)	.006
Mutant	118	80	2.15 (1.29 to 3.58)		2.31 (1.18 to 4.50)		2.47 (1.29 to 4.74)	
Stage at diagnosis#								
I	51	NA	1.00 (referent)	.002	1.00 (referent)	.002	1.00 (referent)	.001
II	83	138	1.84 (0.92 to 3.70)		1.45 (0.7 to 2.93)		1.63 (0.79 to 3.35)	
III	66	59	3.77 (1.79 to 7.94)		3.67 (1.63 to 8.27)		4.07 (1.86 to 8.91)	
IV	5	16	NA		NA		NA	
Anatomic location of primary								
Head/neck	43	111	1.00 (referent)	.02	1.00 (referent)	.009	1.00 (referent)	.009
Upper limbs	22	114	0.09 (0.01 to 0.71)		0.09 (0.01 to 0.74)		0.02 (0.01 to 0.72)	
Trunk	103	291	0.77 (0.41 to 1.43)		0.58 (0.25 to 1.34)		0.63 (0.29 to 1.38)	
Lower limbs	68	106	0.40 (0.19 to 0.84)		0.26 (0.10 to 0.68)		0.29 (0.12 to 0.71)	
Histologic type								
ALM	12	NA	1.00 (referent)	.19				
LMM	5	NA	NA					
NM	73	138	1.51 (0.45 to 5.07)					
SSM	55	210	0.74 (0.21 to 2.61)					
Unclassified	78	428	0.89 (0.26 to 3.05)					
Breslow thickness, mm								
0.01–1.00	41	NA	1.00 (referent)	<.001	**		**	
1.01–2.00	58	107	2.25 (0.93 to 5.43)					
2.01–4.00	61	210	1.58 (0.66 to 3.83)					
>4.00	61	46	5.77 (2.55 to 13.05)					

(Table continues)

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Table 4. (Continued).

Variables	N†	Median survival	Univariate Cox models HR (95% CI)	Univariate Cox models P‖	Multivariable Cox Model 1‡ (extended model) HR (95% CI)	Multivariable Cox Model 1‡ P‖	Multivariable Cox Model 2§ (restricted model+*TERT*) HR (95% CI)	Multivariable Cox Model 2§ P‖
Clark level								
I	NA	NA	NA	.009	**		**	
II	NA	NA	NA					
III	52	NA	1.00 (referent)					
IV	72	NA	2.08 (0.91 to 4.77)					
V	19	71	4.67 (1.74 to 12.52)					
Ulceration								
Absent	147	177	1.00 (referent)	.02	1.00 (referent)	.29		
Present	57	68	2.02 (1.13 to 3.59)		1.41 (0.75 to 2.66)			
SLN								
Negative	67	138	1.00 (referent)	.21				
Positive	59	68	1.56 (0.78 to 3.28)					

* ALM = acral lentiginous melanoma; BRAF = v-Raf murine sarcoma viral oncogene homolog; KIT = v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog; LMM = lentigo maligna melanoma; NM = nodular melanoma; NRAS = neuroblastoma RAS viral oncogene homolog; SLN = sentinel lymph node; SSM = superficial spreading melanoma; TERT = telomerase reverse transcriptase. All statistical tests were two-sided.

† The number of available data for a particular variable in the univariate analysis.

‡ Model in which all predictors with univariate *P* values ≤.10 are included—for "Age" see †; see also **; no interactions were considered.

§ Restricted model, which includes "*BRAF* and *NRAS*," "Stage at diagnosis," and "*TERT*," no interactions were considered.

‖ *P* value for the omnibus model test of the univariate predictors (cases with sparse observations for which no estimator is provided are also not included in the omnibus test).

¶ Multivariable Cox regression analysis results for "Age" are displayed for the continuous linear predictor, as other transformations including those displayed or "Age" as continuous quadratic predictor or "Age" as both linear and quadratic continuous predictor, had no impact on the conclusions.

Staging according to the American Joint Committee on Cancer (AJCC) Melanoma Staging System 2009 (32).

** There was strong multicollinearity between "Stage at diagnosis," "Breslow thickness," and "Clark level," so we decided to only include "Stage at diagnosis," given that this resulted in the largest number of complete data sets (n = 158 for model 1 and n = 161 for model 2) with no impact on the conclusions.

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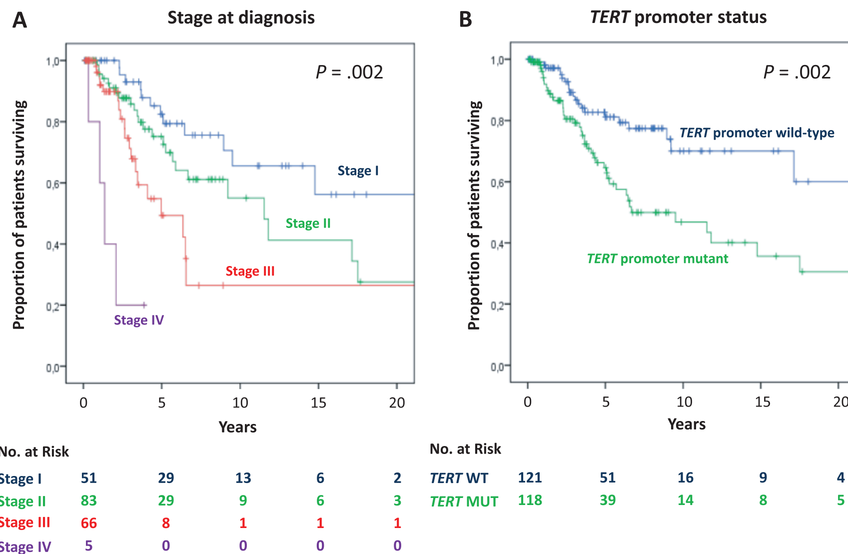

Figure 1. Associations of tumor stage at diagnosis and *TERT* promoter status with overall survival in 239 patients with melanomas of nonacral skin. Kaplan-Meier survival curves of overall survival according to **A)** tumor stage at diagnosis and **B)** *TERT* promoter status (mutant vs wild type). All statistical tests were two-sided. *TERT* = telomerase reverse transcriptase.

melanomas in nonacral skin. This verifies the original reports of *TERT* promoter mutations in melanoma and highlights these as common genetic alterations in this tumor.

The recurrent mutations we found in the *TERT* promoter were at previously reported hotspots (9,10) and had a UV-signature with C>T or CC>TT changes (19,20), supporting an etiologic role of UV exposure. Both 228C>T and 250C>T mutations have been detected in various cutaneous tumors (9,10,21–23), but have also been identified in cancers of internal organs, such as hepatocellular cancer, bladder cancer, thyroid cancer, and gliomas, in which UV-exposure is not a factor (11,13,14). This implies that while 228C>T and 250C>T mutations can be UV-induced, they may also occur by other means. In contrast to C>T mutations, di-pyrimidine CC>TT mutations are considered virtually pathognomonic of UV induction (19,20). Although identified in a range of UV-induced cutaneous tumors (10,12,21), di-pyrimidine CC>TT alterations have only very rarely been described in tumors arising in internal organs. The presence of CC>TT substitutions in 30 of 154 (19%) identified mutations in the *TERT* promoter underscores the role of UV-exposure in inducing these mutations in melanoma. This is further supported by the distribution of *TERT* promoter mutations among melanoma subtypes. The frequency (48%) of *TERT* promoter mutations found in UV-exposure-prone nonacral cutaneous melanomas was considerably higher than that seen in acral (19%) or mucosal (23%) melanomas, tumors arising in areas with minimal or absent sun exposure. Additionally, the majority of UV-pathognomonic CC>TT mutations identified (26 of 30, 87%) were found in nonacral cutaneous melanoma (Supplementary Table 1, available online). Overall, the type and distribution of *TERT* promoter mutations identified supports a major role for UV induction. The high *TERT* promoter mutation frequency (50%) found in occult melanoma suggests a cutaneous origin for many of these tumors.

Frequent genomic amplifications of the *TERT* gene locus in acral melanomas have been described (24–26). These findings are intriguing, further supporting an important role for *TERT* alterations in melanoma and could mean that other genetic mechanisms beside promoter mutations are responsible for increased *TERT* expression in tumors arising from locations with little or no UV exposure.

One of the major questions we aimed to address was whether *TERT* promoter mutation status could be a prognostic marker, as suggested by preexisting data. Horn et al. detected *TERT* promoter mutations in 33% of primary melanomas and at considerably higher frequencies in melanoma cell lines (74%) and corresponding tissue from metastases (85%) (10). Similar frequencies were reported by Huang et al. (9). The increased mutation frequencies in metastases or cell lines (which are frequently derived from metastatic tumors) could indicate an association of mutation status with more aggressive disease.

The samples analyzed in our patient cohort were a combination of primary and metastatic tumor samples. The mutation frequency detected in primary samples was 36.6% (53 of 145), which fits well with the 33% mutation rate previously reported by Horn et al. (10). We also detected a statistically significantly higher mutation rate of 50.3% (73 of 145, P = .02) in the metastatic samples we analyzed. Occult and recurrent or local metastatic samples had comparably high mutation frequencies of 50% (17 of 54) and 55.6% (5 of 9), respectively. In concordance with the previous Horn et al. study, this distribution points toward a prognostic implication for *TERT* promoter mutations.

When analyzing all patient samples jointly to increase statistical power, a trend for patients with *TERT* promoter-mutant tumors having a poorer survival was observed in univariate analysis. Further subset analyses showed that in nonacral cutaneous melanomas, in addition to a statistically significant association between the presence of *TERT* promoter mutations with increasing Breslow thickness, the presence of *TERT* promoter mutations was also found to be independently associated with poorer patient survival. Stratifying samples from patients with nonacral

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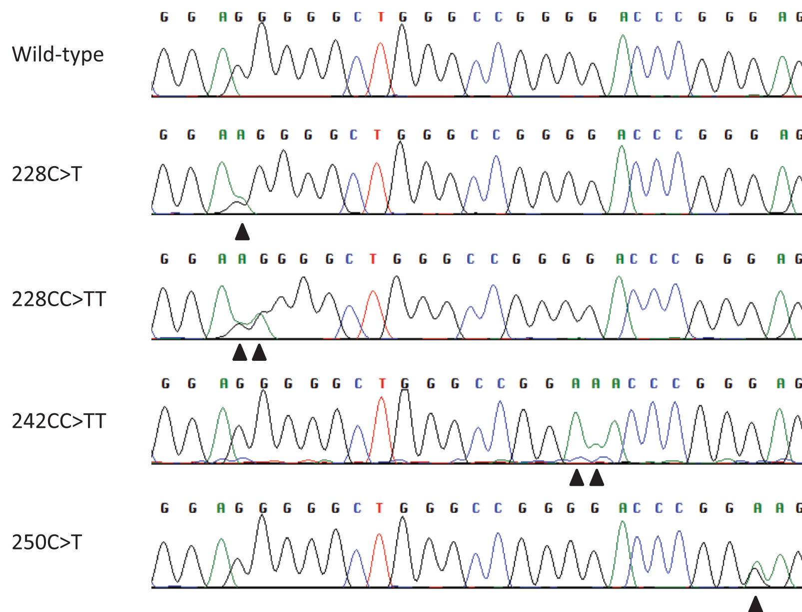

Figure 2. Recurrent mutations identified in the *TERT* promoter. Sanger sequencing chromatograms of representative mutations are shown. Recurrent mutations identified were found at location Chr.5:1295228C>T, Chr.5:1295228_1295229CC>TT, Chr.5:1295242_1295243CC>TT, and Chr.5:1295250C>T (according to human genome assembly hg19). A wild-type promoter sequence is shown at the top for comparison. Mutations presented in the figure are highlighted by **black arrowheads** and labeled applying the last three nucleotides of the first base mutated (**underlined** above). *TERT* = telomerase reverse transcriptase.

cutaneous melanomas by primary or metastatic origin revealed comparable effect size estimates.

Tumors with *BRAF* or *NRAS* mutations were found to harbor *TERT* promoter mutations statistically significantly more often than tumors lacking *BRAF* or *NRAS* mutations. Previous reports have shown *BRAF* or *NRAS* mutations to be associated with a poorer prognosis in patients with stage III or IV melanoma (27–29). In agreement with these studies, patients in our cohort with nonacral cutaneous melanomas harboring either a *BRAF* or *NRAS* mutation (compared with those with tumors that were wild type for both genes) showed statistically significant poorer survival (P = .04).

Interestingly, even looking at subgroups, we failed to find the comparably high percentages (>70%) of *TERT* promoter mutations that have previously been reported in cell lines, short-term cultures, or matched metastasis (9,10). One explanation could be cell-line specificity. Successful establishment of cell lines is only achieved with certain melanoma samples. The high percentage of *TERT* promoter mutations identified in cell lines could be because of a survival advantage conferred by these mutations in cell culture. This hypothesis could potentially be addressed experimentally in future studies.

In germ-line analysis, we only identified two samples with single nucleotide alterations in a cohort of 196 samples taken from cutaneous melanoma patients. The clinical significance of these single nucleotide exchanges is unclear. The Chr.5:1295229C>A (c.-125C>A) alteration is a nucleotide also frequently altered in somatic mutations, however, only in conjunction with an alteration of the adjacent 228 residue. Considering that the 228C>T mutation is frequently found alone and in a range of different cancers, it is likely that 228 is the critically mutated residue, with the concurrent 229 mutations representing bystanders resulting from UV-induced CC>TT dipyrimidine mutations. To our knowledge, the other nucleotide change we identified, Chr.5:1295319G>A (c.-215G>A), has not been previously reported. In summary, germ-line variations of the *TERT* promoter were rare (~1%) and of unclear functional significance in patients with sporadic melanoma.

Functional studies showed that the identified hotspot *TERT* promoter mutations induce a 2–4 fold increase in gene expression (9,10), most likely by introducing additional ETS transcription binding sites (9–11). Although many adjacent nucleotides could acquire C>T or CC>TT mutations (Figure 2), the *TERT* promoter mutations identified in our cohort almost exclusively affected the previously described, functionally relevant hotspots. This clearly implies a selection pressure for these mutations, resulting in overexpression of the enzymatic subunit of the telomerase holoenzyme. Increased telomerase expression is thought to enable tumors to maintain telomere length and chromosomal stability, allowing cells to continuously proliferate without becoming genetically unstable, and thereby to avoid apoptosis or senescence (30,31).

In certain cancers, Killela et al. described *DAXX* and *ATRX* mutations associated with alternative lengthening of telomeres (ALT) as a mechanism for telomere maintenance in tumors lacking *TERT* promoter mutations (11). To our knowledge, ALT is not recognized as a relevant mechanism in melanoma. Additionally, larger whole-exome sequencing studies have not reported recurrent mutations in *DAXX* and *ATRX* (6,7), also arguing against a substantial role for ALT in cutaneous melanoma. However, we cannot exclude the possibility that ALT plays a role in a subset of melanoma cases lacking *TERT* promoter mutations.

Limitations of the study include potential sample selection bias; our cohort contained a large number of thick melanomas, many of which metastasized and had a poor prognosis. Additionally, the melanomas analyzed in our study were a combination of primary and metastatic tumors; nevertheless, independent survival analysis

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of each sample set (primaries and metastases) showed similar results to the overall cohort, supporting our findings. It will be interesting to explore, in future studies, whether *TERT* promoter mutation status changes during the course of tumor progression and whether this affects patient survival. An additional possible technical caveat is the use of Sanger sequencing, which is robust and specific, but can miss mutations present at low frequencies in tumor samples (<20% of allelic DNA). Overall, future studies of other sample cohorts, potentially applying more sensitive next-generation sequencing-based assays, could be valuable to confirm our findings and further delineate the role *TERT* promoter mutations play in melanoma.

In summary, our findings indicate that *TERT* promoter mutations are common genetic events in cutaneous melanoma. Mutations were considerably more frequent in nonacral cutaneous melanomas than in mucosal or acral melanomas. This finding is consistent with a role for UV-induction, further supported by the UV signature of mutations (C>T and in particular CC>TT) identified. The presence of *TERT* promoter mutations was found to be an independent marker of poor prognosis in nonacral cutaneous melanomas in our patient cohort. Analysis of independent, prospectively collected data sets will be needed to validate our findings. Additional studies could further investigate whether *TERT* promoter mutations are of therapeutic relevance, either in terms of influencing the efficacy of established therapies (ie, BRAF inhibitors or immunotherapies) or whether they might even prove to be valuable direct therapeutic targets.

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Notes

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