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Clinical, environmental and histological distribution of *BRAF*, *NRAS* and *TERT* promoter mutations among patients with cutaneous melanoma: a retrospective study of 563 patients*

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Summary

Background The distinct somatic mutations that define clinical and histopathological heterogeneity in cutaneous melanoma could be dependent on host susceptibility to exogenous factors like ultraviolet radiation.
Objectives Firstly, to characterize patients with cutaneous melanoma clinically and pathologically based on the mutational status of *BRAF*, *NRAS* and *TERT* promoter. Secondly, to elucidate the modified features due to the presence of *TERT* promoter mutations over the background of either *BRAF* or *NRAS* mutations.
Methods We performed a retrospective study on 563 patients with melanoma by investigating somatic mutations in *BRAF*, *NRAS* and *TERT* promoter.
Results We observed co-occurrence of *TERT* promoter mutations with *BRAF* and *NRAS* mutations in 26.3% and 6.9% of melanomas, respectively. Multivariate analysis showed an independent association between *BRAF* mutations and a decreased presence of cutaneous lentigines at the melanoma site, and an increased association with the presence of any MC1R polymorphism. We also observed an independent association between *TERT* promoter mutations and increased tumour mitotic rate. Co-occurrence of *BRAF* and *TERT* promoter mutations was independently associated with occurrence of primary tumours at usually sun-exposed sites, lack of histological chronic sun damage in surrounding unaffected skin at the melanoma site, and increased tumour mitotic rate. Co-occurrence of *NRAS* and *TERT* promoter mutations was independently associated with increased tumour mitotic rate. The presence of *TERT* promoter together with *BRAF* or *NRAS* mutations was associated with statistically significantly worse survival.
Conclusions The presence of *TERT* promoter mutations discriminates *BRAF*- and *NRAS*-mutated tumours and indicates a higher involvement of ultraviolet-induced damage and tumours with worse melanoma-specific survival than those without any mutation. These observations refine classification of patients with melanoma based on mutational status.

What is already known about this topic?

- Mutations in *TERT* promoter, *BRAF* and *NRAS* constitute the most frequent driver alterations in melanoma.

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Conflicts of interest
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- Noncoding *TERT* promoter mutations increase *TERT* expression and synergistically influence the effect of *BRAF* and *NRAS* on patient survival and modify associations with different clinical and tumour characteristics.

What does this study add?

- We have shown differences in *BRAF*- and *NRAS*-mutated tumours associated with concomitant presence of *TERT* promoter mutations.
- The presence of *TERT* promoter mutations together with either *BRAF* or *NRAS* mutation resulted in an increased association with ultraviolet-induced damage and poor prognosis.
- The poor prognosis was more pronounced in tumours with *TERT* promoter mutations over the background of *NRAS* than *BRAF* mutations.

What is the translational message?

- Activating *BRAF* and *NRAS* mutations are frequent in melanomas but are not sufficient to drive malignant transformation, and require additional genetic events.
- Co-occurrence of *TERT* promoter mutation with *BRAF* and *NRAS* alterations correlates with poor prognosis, with a demonstrated functional link between mitogen-activated protein kinase signalling and telomerase reactivation, which could have potential clinical implications.
- Pertinently, the largest subgroup of patients had no mutations at any of the three investigated loci and had demonstrably better disease outcome.

Some studies have shown that young individuals tend to present melanomas mainly on body parts that are intermittently exposed to the sun, like the trunk, arms and legs, and have adjacent melanocytic naevi.[1] Most of such tumours and precursor lesions harbour *BRAF* mutations.[2] In contrast, melanomas in older patients occur frequently at chronically sun-exposed sites like the neck and head. These older patients with melanoma carry decreased numbers of melanocytic naevi and frequently have nonmelanoma skin cancers.[3] Different mutational profiles characterize the two clinically distinct postulated melanoma types.[2,4,5] Upregulation of the mitogen-activated protein kinase (MAPK) pathway, mainly due to *BRAF* and *NRAS* mutations, represents a common feature in melanoma.[6]

Frequent oncogenic *BRAF* mutations, which are initiating events in melanoma, result in increased basal levels of the protein and abrogate the requirement of exogenous mitogenic signalling.[7] Current therapies are based on targeting of constitutive kinase activity due to mutant *BRAF* using targeted inhibitors for treatment of patients with metastasized disease.[8] The proportion of *NRAS* mutations ranges between 0% and 50% depending on the tumour subtype, and these tumours are less amenable to treatment.[9,10] Frequent mutations within the promoter of the telomerase reverse transcriptase gene (*TERT*) are documented to associate with aggressive tumours and poorer prognosis.[11–14]

We hypothesize that the observed clinical and histopathological heterogeneity of melanoma can be partly explained by frequently mutated loci, which include *BRAF*, *NRAS* and *TERT* promoter. Identification of the mutation-associated characteristics that confer increased aggressiveness would be useful in the development of specific strategies for prevention of melanoma progression.[15] To date there are a few studies that have evaluated the effect on survival in combination.[11–14]

Our first objective was to characterize, clinically and pathologically, patients with cutaneous melanoma based on the mutational status of *BRAF*, *NRAS* and *TERT* promoter. The next objective was to elucidate the modified features due to the presence of *TERT* promoter mutations over the background of either *BRAF* or *NRAS* mutations.

Patients and methods

Patient selection

We carried out an observational study on the data collected prior to design and development from the institutional melanoma database of the dermatology department of the Instituto Valenciano de Oncología (IVO), Valencia, Spain. The database contains comprehensive information on clinical, epidemiological and histological parameters collected prospectively from 2000.[16] Patients diagnosed with a histologically confirmed primary cutaneous melanoma and registered between January 2000 and December 2019 were deemed eligible for the study. Only patients with documented mutational status of *BRAF*, *NRAS* and *TERT* promoter were included in the study. We included patients who were aged 18 years or older at diagnosis. We excluded patients with mucosal melanoma or melanoma with unknown primaries, patients with multiple primary melanomas, and those with both *BRAF* and *NRAS* mutations together. The study was approved by the IVO's research ethics board. Informed patient consent had been obtained previously.

Clinical and pathological data collection

To correlate the mutational status with clinical, phenotypic and histopathological features, the patients were classified into six different subgroups based on mutational status: (i) triple wildtype (TWT) and mutations in (ii) *BRAF*, (iii) *NRAS*, (iv) *TERT* promoter, (v) *BRAF* + *TERT* promoter and (vi) *NRAS* + *TERT* promoter.

The following variables were correlated with mutational status. Demographic, clinical and phenotypic variables included age (< 50, 50–65 or > 65 years), sex, melanoma location (head/neck, upper extremities, trunk, lower extremities, and acral locations), past personal lifetime history of severe sunburns (no or yes), past personal history of sunburns at the melanoma site (no or yes), pattern of photoexposure at the melanoma site (rare, occasional or usual), past personal history of nonmelanoma skin cancer (basal cell carcinoma and/or squamous cell carcinoma), presence of actinic keratosis (no or yes), solar lentigines at the melanoma site (no or yes), number of melanocytic naevi (< 20 or ≥ 20), predominant type of common melanocytic naevi (junctional/flat, yes or no; or compound/intradermal, yes or no), cherry angiomas (no, ≤ 50 or > 50) and seborrhoeic keratoses (no, ≤ 50 or > 50). Melanocortin 1 receptor (MC1R) variants, described elsewhere,[17] were classified based on presence or absence of any nonsynonymous variant.

Histopathological variables included the histological subtype (lentigo maligna melanoma, superficial spreading melanoma, nodular melanoma, acral lentiginous melanoma, not specified or other), presence of chronic sun damage (CSD) (no or yes),[18] Breslow tumour thickness (≤ 2 mm or > 2 mm), presence of ulceration (no or yes), remnants of pre-existent naevus (no or yes), tumour mitotic rate (TMR) (mitoses per mm^2 categorized into TMR1, 0; TMR2, 1–5; TMR3, > 5) and melanoma stage (*in situ*, localized, locoregional or metastatic disease).

Mutational screening

Mutations within the *TERT* promoter, *BRAF* and *NRAS* genes were screened by polymerase chain reaction and Sanger sequencing as described previously.[14,19]

Statistical analysis

The initial analysis was carried out by comparing the presence of mutations in *BRAF*, *NRAS* and *TERT* promoter separately and in combinations. TWT melanomas were used as the reference. The associations between different mutations or combinations and clinical and pathological variables were determined by χ^2-test. The effect size was estimated using odds ratio (ORs) and 95% confidence interval (CIs). A multinomial multivariate logistic regression analysis was performed to determine the independent association between the mutational status and clinical, phenotypic or histopathological features; variables with a P-value ≤ 0.1 in univariate analysis were included in the multivariate analysis.

A second analysis was performed where patients with *BRAF* + *TERT* mutations or *NRAS* + *TERT* mutations were compared with patients with only *BRAF* or *NRAS* mutations in tumours, respectively.

The effects of mutational status on disease-free and melanoma-specific survival (MSS) for stage I and II melanomas were determined by the Kaplan–Meier method. The events were the appearance of the first relapse or death due to melanoma for disease-free survival (DFS) or MSS, respectively. The size of the effect was determined by univariate Cox regression analysis. Multivariate stepwise forward Cox proportional hazard models included the patient age at diagnosis, sex, Breslow thickness, ulceration, TMR, site of primary and tumour stage as covariates.

All statistical analyses were performed using SPSS software for Windows version 21.0 (IBM, Armonk, NY, USA).

Results

Clinical features

Following all inclusion and exclusion criteria, 563 cases were included in the present study. The clinical, phenotypic and environmental, and histopathological characteristics of the patients are described in Table S1 (see Supporting Information).

Mutation frequencies

BRAF mutations were present in 241 (42.8%) tumours, *NRAS* mutations in 65 (11.5%) and *TERT* promoter mutations in 272 (48.3%). *BRAF* mutations alone were present in 93 tumours (16.5%), only *NRAS* mutations in 26 (4.6%) and only *TERT* promoter mutations in 85 (15.1%) (Table S1; see Supporting Information). *BRAF* and *TERT* promoter mutations were present together in 148 tumours (26.3%), *NRAS* and *TERT* promoter mutations were detected together in 39 (6.9%), and 172 (30.6%) were TWT.

The most prevalent *BRAF* mutations were V600E (36.8%), V600K (4.5%) and V600R (0.5%); the most common *NRAS* mutations were Q61R (4.6%), Q61K (1.8%) and Q61L (1.8%); and the most common *TERT* promoter mutations were −146C>T (24.8%), −124C>T (17.9%) and −138_139CC>TT (3.4%) (Table S2; see Supporting Information).

Characteristics by each mutational profile compared with triple-wildtype melanomas

BRAF mutation

Patients with *BRAF* mutations were younger at diagnosis than those with TWT tumours (> 65 vs. < 50 years: OR 0.48, 95% CI 0.26–0.90, P = 0.02). *BRAF* mutations were less frequent in tumours at acral sites than on the trunk in comparison with TWT (OR 0.41, 95% CI 0.29–0.86, P = 0.02). Patients with *BRAF* mutations in tumours had reduced presence of solar lentigines at the melanoma site (OR 0.41, 95% CI 0.02–0.73, P = 0.002) and increased TMR (TMR2, OR 2.70, 95% CI

1.40–5.20, P = 0.003; TMR3, OR 3.20, 95% CI 1.24–8.25, P = 0.016) (Table S3; see Supporting Information).

Multinomial multivariate logistic regression analysis showed that tumours with *BRAF* mutations were less frequent on the lower extremities (OR 0.23, CI 0.08–0.63, P = 0.004) and at acral sites (OR 0.13, CI 0.04–0.42, P < 0.001) than on the trunk. Similarly, tumours with *BRAF* mutations were less frequent in tumours with solar lentigines at the melanoma site (OR 0.17, CI 0.07–0.41, P < 0.001) and were positively associated with nonsynonymous MC1R variants (OR 2.46 CI 1.23–4.94, P = 0.01) (Table 1).

NRAS mutation

In univariate analysis, tumours with *NRAS* mutations had statistically significantly increased TMR (TMR3, OR 4.80, 95% CI 1.29–17.9, P = 0.01) compared with TWT tumours (Table S3; see Supporting Information). In a multinomial multivariate logistic regression analysis no statistically significant association was observed with *NRAS* mutations (Table 1). The number of patients with *NRAS* mutations in tumours was only 26, which limited the statistical power.

TERT promoter mutation

TERT promoter mutations were more frequent in tumours at head and neck sites (OR 3.07, 95% CI 1.46–6.48, P = 0.003) and less frequent at acral locations (OR 0.05, 95% CI 0.00–0.35, P = 0.003) than on the trunk. *TERT* promoter mutations were statistically significantly associated with the presence of CSD (OR 3.70, 95% CI 1.77–7.76, P < 0.001), actinic keratosis (OR 2.38, 95% CI 1.18–4.82, P = 0.01), usual photoexposure at the site of melanoma (OR 14.1, 95% CI 4.36–45.8, P < 0.001), increased solar lentigines at the melanoma site (OR 2.33, 95% CI 1.45–4.52, P < 0.001) and an increased number of seborrhoeic keratoses (OR 3.73, 95% CI 1.18–11.8, P = 0.03). *TERT* promoter mutations were also associated with increased tumour thickness (OR 1.78, 95% CI 1.04–3.05, P = 0.04) (Table S3; see Supporting Information).

In multivariate analysis, *TERT* promoter mutations were independently associated with increased TMR (TMR3, OR 3.62, 95% CI 1.20–11.0, P = 0.02), and occurred at a lower frequency in tumours at acral sites than in those on the trunk (OR 0.09, 95% CI 0.01–0.92, P = 0.04) (Table 1).

Combined *BRAF* and *TERT* promoter mutation

BRAF and *TERT* promoter mutations together were less frequent in tumours at acral sites (OR 0.11, 95% CI 0.04–0.29, P < 0.001). The simultaneous presence of those mutations was also associated with occasional and usual photoexposure at melanoma areas (OR 2.33, 95% CI 1.23–4.41, P = 0.009 and OR 2.93, 95% CI 1.27–6.72, P = 0.011, respectively), the presence of any nonsynonymous MC1R variant (OR 2.76, 95% CI 1.64–4.65, P < 0.001), increased tumour thickness (OR 1.71, 95% CI 1.08–2.71, P = 0.02), nodular histological

Table 1 Multivariate multinomial logistic regression analysis for characteristics associated with *BRAF*-, *NRAS*- and *TERT*-mutated melanomas vs. triple-wildtype (TWT) melanomas

	TWT	*BRAF*			*NRAS*			*TERT*		
	n	n	OR (95% CI)	P-value	n	OR (95% CI)	P-value	n	OR (95% CI)	P-value
Melanoma location										
Head/neck	10	10	0.23 (0.05–1.13)	0.07	6	0.30 (0.02–4.95)	0.40	27	1.21 (0.21–7.06)	0.83
Upper extremities	22	9	0.51 (0.15–1.73)	0.28	1	0.30 (0.03–3.06)	0.31	14	0.76 (0.27–2.12)	0.60
Trunk	60	45	Reference		9	Reference		31	Reference	
Lower extremities	31	16	0.23 (0.08–0.63)	0.004	5	0.57 (0.12–2.61)	0.47	12	0.74 (0.27–2.07)	0.57
Acral	42	13	0.13 (0.04–0.42)	< 0.001	5	0.24 (0.03–1.86)	0.17	1	0.09 (0.01–0.92)	0.04
Photoexposure of melanoma site										
Rare	39	23	Reference		4	Reference		4	Reference	
Occasional	113	58	0.38 (0.13–1.15)	0.88	16	1.74 (0.21–14.3)	0.60	50	2.62 (0.45–15.2)	0.28
Usual	20	12	3.93 (0.78–11.8)	0.11	6	6.17 (0.63–60.7)	0.05	29	5.03 (0.91–27.7)	0.06
Solar lentigines at melanoma site										
No	97	69	Reference		15	Reference		27	Reference	
Yes	72	21	0.17 (0.07–0.41)	< 0.001	9	0.45 (0.12–1.71)	0.24	52	1.28 (0.56–2.93)	0.55
Melanocortin 1 receptor										
Wildtype	70	23	Reference		11	Reference		29	Reference	
Any variant	84	58	2.46 (1.23–4.94)	0.01	9	0.53 (0.17–1.63)	0.27	45	1.19 (0.60–2.37)	0.61
Tumour mitotic rate										
0 mitosis per mm^2	60	15	Reference		5	Reference		25	Reference	
1–5 mitosis per mm^2	86	58	2.13 (0.99–4.59)	0.05	12	1.42 (0.39–5.23)	0.61	37	1.11 (0.53–2.30)	0.79
> 5 mitosis per mm^2	15	12	2.34 (0.76–7.43)	0.15	6	5.15 (0.99–26.8)	0.12	20	3.62 (1.20–11.0)	0.02
Chronic sun damage										
No	132	73	Reference		18	Reference		56	Reference	
Yes	14	4	0.55 (0.09–3.06)	0.49	4	2.31 (0.20–26.3)	0.50	22	1.68 (0.48–5.90)	0.42

CI, confidence interval; OR, odds ratio.

subtype (OR 4.88, 95% CI 1.52–15.7, P = 0.008), increased TMR (TMR2, OR 2.24, 95% CI 1.27–3.93, P = 0.005; TMR3, OR 7.00, 95% CI 3.29–14.9, P < 0.001) and the presence of locoregional disease (stage III vs. I–II) (OR 2.41, 95% CI 1.45–4.01, P < 0.001) (Table S3; see Supporting Information).

In multivariate analysis, the simultaneous presence of BRAF and TERT promoter mutations was associated with usual photoexposure at the melanoma site (OR 5.62, CI 95% 1.52–20.8, P = 0.01) and the absence of CSD (OR 0.22, 95% CI 0.06–0.89, P = 0.03). The presence of mutations at both loci was also associated inversely with tumours at acral locations (OR 0.09, 95% CI 0.02–0.35, P < 0.001), increased presence of nonsynonymous MC1R variants (OR 2.70, 95% CI 1.47–4.97, P = 0.002) and increased TMR (TMR2, OR 2.77, 95% CI 1.42–5.40, P = 0.003; TMR3, OR 10.9, 95% CI 4.07–28.9, P < 0.001) (Table 2).

Combined *NRAS* and *TERT* promoter mutation

The presence of both NRAS and TERT promoter mutations, in univariate analysis, was statistically significantly associated with melanoma at usually sun-exposed sites (OR 3.51, 95% CI 1.04–11.9, P = 0.04), increased tumour thickness (OR 4.44, 95% CI 2.11–9.35, P < 0.001) and increased TMR (TMR2, OR 5.35, 95% CI 1.54–18.6, P = 0.008; TMR3, OR 17.3, 95% CI 4.37–68.7, P < 0.001) (Table S3; see Supporting Information). In multinomial multivariate logistic regression analysis, NRAS and TERT promoter mutations together remained independently associated with increased TMR (TMR3, OR 21.1, 95% CI 4.53–98.3, P < 0.001) (Table 2).

The effect of *TERT* promoter mutations over background *BRAF* or *NRAS* mutations

The presence of BRAF + TERT promoter mutations was inversely associated with tumours at an acral location (OR 0.17, 95% CI 0.47–0.60, P = 0.006) compared with tumours with only BRAF mutations. A similar comparison also showed the presence of the two mutations to be associated with occasional and usual photoexposure at the tumour site (OR 2.68, 95% CI 1.31–5.46, P = 0.007 and OR 2.87, 95% CI 1.12–7.37, P = 0.028, respectively), with the presence of solar lentigines at the melanoma site (OR 2.26, 95% CI 1.25–4.09, P = 0.007) and with the presence of locoregional disease (stage III) (OR 1.86, 95% CI 1.04–3.34, P = 0.038) (Tables S4 and S5; see Supporting Information).

In a multinomial multivariate logistic regression analysis, the presence of both NRAS and TERT promoter mutations was inversely associated with acral locations vs. head/neck (OR 0.14, 95% CI 0.03–0.63, P = 0.01), with occasional photoexposure at the melanoma site (OR 3.69, CI 95% 1.29–10.6, P

Table 2 Multivariate multinomial logistic regression analysis for characteristics associated with BRAF + TERT promoter- and NRAS + TERT promoter-mutated melanomas vs. triple-wildtype (TWT) melanomas

	TWT	TERT + BRAF			TERT + NRAS		
	n	n	OR (95% CI)	P-value	n	OR (95% CI)	P-value
Melanoma location							
Head/neck	10	23	1.14 (0.27–4.86)	0.86	9	0.65 (0.82–5.23)	0.69
Upper extremities	22	19	0.93 (0.38–2.26)	0.87	11	1.76 (0.53–5.79)	0.35
Trunk	60	67	Reference		13	Reference	
Lower extremities	31	34	0.85 (0.39–1.87)	0.69	3	3.12 (0.06–1.67)	0.17
Acral	42	5	0.09 (0.02–0.35)	< 0.001	3	0.27 (0.05–1.51)	0.14
Photoexposure of melanoma site							
Rare	39	16	Reference		5	Reference	
Occasional	113	108	1.77 (0.56–5.57)	0.33	25	1.30 (0.24–14.6)	0.76
Usual	20	24	5.62 (1.52–20.8)	0.01	9	21.1 (4.53–98.3)	0.16
Solar lentigines at melanoma site							
No	97	83	Reference		17	Reference	
Yes	72	57	0.54 (0.27–1.07)	0.076	20	0.81 (0.27–2.37)	0.69
Melanocortin 1 receptor							
Wildtype	70	29	Reference		10	Reference	
Any variant	84	96	2.70 (1.47–4.97)	0.002	25	2.18 (0.83–5.70)	0.11
Tumour mitotic rate							
0 mitoses mm^2	60	24	Reference		3	Reference	
1–5 mitoses mm^2	86	77	2.77 (1.42–5.40)	0.003	23	3.88 (1.03–14.6)	0.05
> 5 mitoses mm^2	15	42	10.9 (4.07–28.9)	< 0.001	13	21.1 (4.53–98.3)	< 0.001
Chronic sun damage							
No	132	133	Reference		27	Reference	
Yes	14	6	0.22 (0.06–0.89)	0.03	5	0.58 (0.09–3.62)	0.56

CI, confidence interval; OR, odds ratio.

= 0.015) and with the presence of solar lentigines at the melanoma site (OR 2.18, 95% CI 1.07–4.44, P = 0.03) (Table 3).

The simultaneous presence of *NRAS* and *TERT* promoter mutations in multivariate analysis showed association with MC1R variants (OR 3.40, 95% CI 1.06–10.9, P = 0.04) (Table 3).

Survival analysis

After a median follow-up time of 85 months, 102 patients (24.6%) relapsed and 54 (12.9%) died because of their melanoma. Survival analysis showed that the *NRAS* + *TERT*-mutated melanomas were associated with the worst DFS and MSS, followed by *NRAS* mutated, *BRAF* + *TERT* mutated, *TERT* mutated and *BRAF* mutated (log-rank test, P = 0.020 for DFS and P = 0.001 for MSS) (Figure 1 and Table 4). The data for MSS remained statistically significant after multivariate analysis but not for DFS.

Discussion

In this study based on a large series of patients with melanoma, we observed differences in clinical and pathological characteristics based on *BRAF*, *NRAS* and *TERT* promoter mutational status. The presence of a mutation at any of those three loci imparted characteristics of tumour aggressiveness that were enhanced in tumours that had either simultaneous *BRAF* and *TERT* promoter or *NRAS* and *TERT* promoter mutations (Figure 2). Simultaneous *BRAF* + *TERT* promoter or *NRAS* + *TERT* promoter mutations were independently associated with poor MSS. The effect of melanomas with *NRAS* + *TERT* promoter mutation was the worst, driven by their aggressive pathological profile and as indicated by the worst 5-year and 10-year MSS.

Cutaneous melanoma carries one of the highest mutation burdens and the mutation signatures reflect the dominant effect of radiation (UVR). Epidemiological data have shown involvement of at least two distinct pathways involved in the disease genesis.[2,20] The first pathway is related to cumulative sun damage due to chronic sun exposure; the second pathway is based on intrinsic susceptibility to melanocyte proliferation, with limited involvement of UVR.[21] The latest World Health Organization classification differentiates cutaneous melanomas based on the degree of solar elastosis (CSD vs. non-CSD), independently of the clinical phenotype vis-à-vis proneness to naevi, based on the distinct mutational profiles.[22] In the new classification CSD melanomas are described as harbouring neurofibromin 1, *NRAS*, non-p.V600E *BRAF* and, to a lesser extent, *KIT* mutations, while non-CSD melanomas harbour quintessential BRAF mutations, mainly p.V600E.

In this study, the inclusion of *TERT* promoter mutations has further allowed us to refine differences between tumours with *BRAF* and *NRAS* mutations. *TERT* promoter mutations in melanoma and keratinocyte cancers indicate a UVR aetiology, due to their over-representation in tumours from sun-exposed sites coupled with a substantial proportion of CC>TT tandem mutations, and differences in profiles from other cancers.[23] We observed that *BRAF*-mutated melanomas were associated with the absence of solar lentigines at the melanoma site and showed decreased presentation in the lower extremities and acral locations, as well as having an increased presence of nonsynonymous MC1R variants. In contrast, *BRAF*-mutated tumours that also harboured *TERT* promoter mutations occurred mainly at usually sun-exposed areas and were associated more with nonsynonymous MC1R variants compared with TWT tumours.

The influence of UVR exposure was further evident when tumours with *BRAF* and *TERT* promoter mutations were compared with *BRAF*-mutated tumours. Those tumours had

Table 3 Multivariate regression analyses for characteristics associated with melanomas mutated for *BRAF* + *TERT* promoter vs. *BRAF* and *NRAS* + *TERT* promoter vs. *NRAS*

BRAF + *TERT* promoter vs. *BRAF*[a]				
	BRAF n	*BRAF* + *TERT* n	OR (95% CI)	P-value[c]
Melanoma location				(0.055)
Head/neck	10	23	Reference	
Upper extremities	9	19	0.40 (0.09–1.62)	0.20
Trunk	45	67	0.33 (0.09–1.09)	0.07
Lower extremities	16	34	0.63 (0.19–2.25)	0.48
Acral	13	5	0.14 (0.03–0.63)	0.01
Photoexposure of melanoma site				(0.052)
Rare	23	16	Reference	
Occasional	58	108	3.69 (1.29–10.6)	0.015
Usual	12	24	2.16 (0.68–6.90)	0.19
Solar lentigines at melanoma site				
No	69	83	Reference	
Yes	21	57	2.18 (1.07–4.44)	0.03
Tumour mitotic rate				(0.007)
0 mitoses per mm^2	15	24	Reference	
1–5 mitoses per mm^2	58	77	0.65 (0.29–1.42)	0.28
> 5 mitoses per mm^2	12	42	2.40 (0.88–6.54)	0.08

NRAS + *TERT* promoter vs. *NRAS*[b]				
	NRAS n	*NRAS* + *TERT* n	OR (95% CI)	P-value
Melanocortin 1 receptor				
Wildtype	11	10	Reference	
Any variant	9	25	3.40 (1.06–10.9)	0.04

CI, confidence interval; OR, odds ratio. [a]For *BRAF* vs. *BRAF* + *TERT* promoter the melanoma stage was excluded from the final model. [b]For *NRAS* vs. *NRAS* + *TERT* promoter the histological subtype and Breslow thickness were excluded from the final model. [c]The P-value for the variable is given in brackets where there are more than two categories.

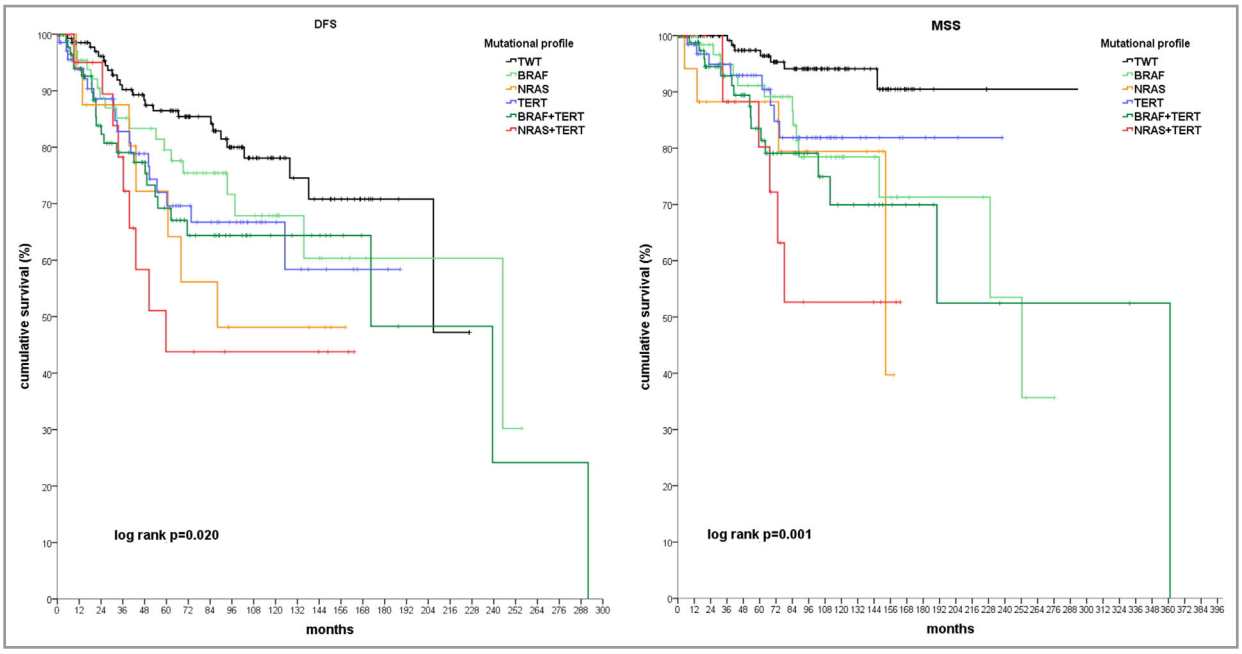

Figure 1 Kaplan–Meier curves by mutational status for disease-free survival (DFS) and melanoma-specific survival (MSS). TWT, triple wildtype.

Table 4 Survival analyses. Estimated 5-year and 10-year survival by mutational status, and univariate Cox regression analysis for disease-free survival (DFS) and melanoma-specific survival (MSS) for mutated melanomas vs. triple-wildtype (TWT) melanomas

			Estimated survival (% ± SE)	
DFS[a]	HR (95% CI)	P-value	5-year	10-year
TWT	Reference	Reference	86.5 ± 3.2	78.1 ± 4.4
BRAF	1.48 (0.80–2.72)	0.21	79.5 ± 5.3	67.9 ± 7.3
NRAS	2.61 (1.13–6.04)	0.03	72.2 ± 12.0	48.1 ± 13.9
TERT	1.87 (1.02–3.43)	0.04	72.0 ± 6.3	66.7 ± 6.9
BRAF + *TERT*	2.02 (1.15–3.54)	0.02	69.2 ± 6.1	64.4 ± 6.4
NRAS + *TERT*	3.10 (1.44–6.66)	0.004	43.8 ± 12.9	43.8 ± 12.9
MSS[b]	HR (95% CI)	P-value		
TWT	Reference	Reference	97.4 ± 1.8	94.1 ± 2.4
BRAF	3.69 (1.47–9.28)	0.006	91.1 ± 3.8	78.4 ± 6.3
NRAS	5.33 (1.56–18.2)	0.008	88.2 ± 7.8	79.4 ± 10.9
TERT	2.96 (1.07–8.18)	0.036	92.9 ± 3.4	81.9 ± 6.0
BRAF + *TERT*	4.69 (1.91–11.5)	0.001	83.5 ± 4.9	69.9 ± 7.8
NRAS + *TERT*	8.04 (2.69–24.0)	< 0.001	80.2 ± 10.4	52.6 ± 14.8

CI, confidence interval; HR, hazard ratio. [a]For DFS, only Breslow thickness was retained as significant after multivariate analysis (> 2 mm vs. ≤ 2 mm: HR 3.71, 95% CI 2.42–5.69, P < 0.001). Mutational profile, tumour mitotic rate (TMR), sex, age, ulceration and location were excluded from the final model. [b]For MSS, Breslow thickness (> 2 mm vs. ≤ 2 mm: HR 5.99, 95% CI 3.17–11.3, P < 0.001) and mutational status (*BRAF* vs. TWT: HR 2.71, 95% CI 0.94–7.83, P = 0.066; *NRAS* vs. TWT: HR 5.96, 95% CI 1.68–21.2, P = 0.006; *TERT* vs. TWT: HR 2.04, 95% CI 0.65–6.69, P = 0.22; *BRAF* + *TERT* vs. TWT: HR 4.43, 95% CI 1.69–11.6, P = 0.003; and *NRAS* + *TERT* vs. TWT: HR 4.64, 95% CI 1.46–14.7, P = 0.009) were retained as significant in the final model. TMR, sex, age, ulceration and location were excluded from the final model.

increased presentation at sun-exposed areas and were rarely present at acral sites compared with *BRAF*-mutated tumours. The limited number of cases masked such differences in *NRAS*-mutated tumours. However, a statistically significant association with nonsynonymous MC1R variants probably indicates genetic susceptibility to UVR damage.

The genetic profile was also related to the tumour aggressiveness. *TERT* promoter mutations, as also reported earlier, associate with different markers of poor prognosis and poor patient survival. Noncoding *TERT* promoter mutations result in increased *TERT* expression through binding of ETS transcription factors at *de novo* sites.[19,24] In melanoma and keratinocyte cancers, unlike

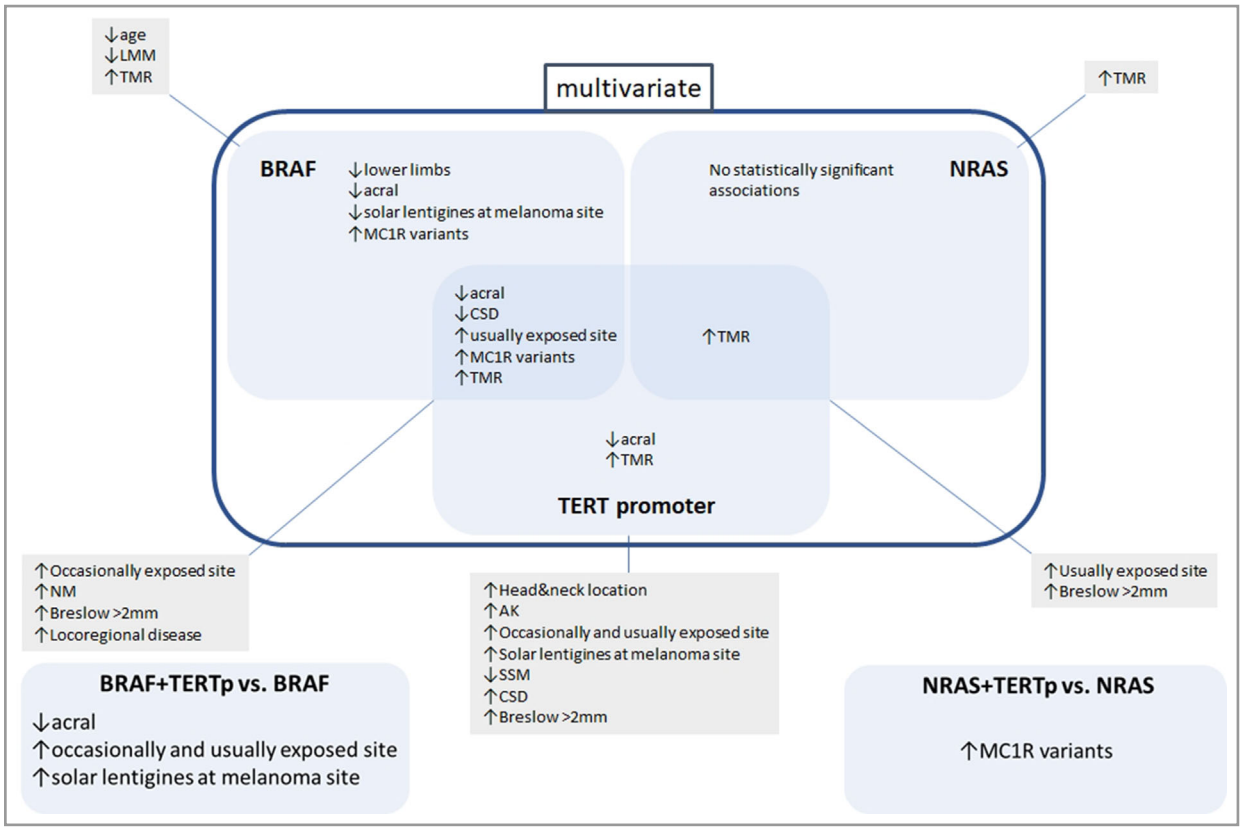

Figure 2 Summary of associations with each mutational profile obtained by univariate and multivariate regression analyses. The characteristics significantly associated after multivariate analysis are included in the charts inside the dark blue line. The characteristics that were statistically significant in univariate but not in multivariate analysis are outside the dark blue line in light-grey-coloured boxes. The characteristics that were statistically significantly associated with the addition of *TERT* promoter (TERTp) mutations in each oncogene (*BRAF* or *NRAS*) are included in light-blue-coloured boxes below both corners. AK, actinic keratosis; CSD, chronic sun damage; LMM, lentigo maligna melanoma; MC1R, melanocortin 1 receptor; NM, nodular melanoma; SSM, superficial spreading melanoma; TMR, tumour mitotic rate.

nonskin cancers, the −146C>T *TERT* promoter mutation is more frequent than the −124C>T alteration.[23] The two main *TERT* promoter mutations upregulate *TERT* through distinct mechanisms and affect melanoma survival differently.[11,25,26]

The effect of *TERT* promoter mutations remained markedly conspicuous in tumours that also simultaneously carried *BRAF* mutations, as indicated clinically by the sun-exposure profile of the affected patients. Although this subgroup of patients displayed some of the significant specific clinical features associated with *BRAF* mutations, the predominantly observed clinical and environmental features were related to the *TERT* promoter-mutated melanomas. *TERT* promoter mutations have been shown to impart a distinctive tumour environment characterized by an epithelial-to-mesenchymal transition gene expression signature and MAPK signalling.[27]

TERT promoter and *BRAF* mutations together occur in about 20–25% of melanomas, which represent the cases with aggressive disease and poor outcome.[12,14,19] The co-occurrence of *BRAF* and *TERT* promoter mutations renders *TERT* expression dependent on MAPK activation.[28] RAS–extracellular signal-regulated kinase (ERK) signalling regulates the active chromatin state through physical binding of ERK2 to the *TERT* promoter through displacement of histone deacetylase 1, leading to the recruitment of RNA polymerase II.[29] The interaction between MAPK and *TERT* activation with a mutant promoter has also been shown to involve activation of *GABPA* promoter through phosphorylated FOS.[30]

The pronounced effect of *NRAS* mutations on survival, particularly in combination with *TERT* promoter mutations, can probably be attributed to its involvement in multiple pathways. Besides being upstream of the RAF–MAPK pathway, RAS also affects metabolic pathways and promotes aerobic glycolysis and glutamine metabolism to provide energy and facilitate autophagy and macropinocytosis, which generate building blocks for tumour growth and strengthen the antioxidant defence in tumour cells.[17,31] However, due to the dependence of *TERT* expression on RAS signalling in mutant tumours, targeting with the telomere-uncapping agent 6-thio-2′-deoxyguanosine in combination with the mitochondrial inhibitor gamitrinib has been shown to suppress *NRAS*-mutant melanoma effectively in cells and animal models.[32]

In conclusion, our data show the relationship between different mutated melanoma subtypes and relevant clinical and pathological parameters. Activating *BRAF* and *NRAS* mutations are frequent in melanomas but are not sufficient to drive malignant transformation and require additional genetic events. Frequent co-occurrence of the *TERT* promoter with *BRAF* and, particularly, with *NRAS* alterations correlates with poor prognosis and MSS, implying a functional link between *BRAF* signalling and telomerase reactivation in melanomas. The synergistic effect due to combined *TERT* promoter and *BRAF* and *NRAS* mutations could have an implication for patients treated with MAPK inhibitors. However, the largest subgroup of melanomas had no mutations at any of the three investigated loci, which was associated with comparatively improved patient survival.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1 Distribution of characteristics according to the presence of *BRAF*, *NRAS* and *TERT* promoter mutations.

Table S2 Summary of mutations in *BRAF*, *NRAS* and *TERT* promoter in the melanoma study population.

Table S3 Univariate multinomial regression analyses for characteristics associated with *BRAF*, *NRAS*, *TERT* promoter, *BRAF* + *TERT* promoter and *NRAS* + *TERT* promoter mutated melanomas vs. triple-wildtype melanomas.

Table S4 Distribution analyses by contingency tables and c^2-test (or Fisher test where appropriate) of characteristics according to the presence of *BRAF* vs. *BRAF* + *TERT* and *NRAS* vs. *NRAS* + *TERT* mutations.

Table S5 Univariate multinomial regression analyses for characteristics associated with *BRAF* vs. *BRAF* + *TERT* promoter and *NRAS* vs. *NRAS* + *TERT* promoter mutated melanomas.