SHORT COMMUNICATION

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TERT promoter mutations in ocular melanoma distinguish between conjunctival and uveal tumours

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Background: Recently, activating mutations in the *TERT* promoter were identified in cutaneous melanoma. We tested a cohort of ocular melanoma samples for similar mutations.

Methods: The *TERT* promoter region was analysed by Sanger sequencing in 47 uveal (ciliary body or choroidal) melanomas and 38 conjunctival melanomas.

Results: Mutations of the *TERT* promoter were not identified in uveal melanomas, but were detected in 12 (32%) conjunctival melanomas. Mutations had a UV signature and were identical to those found in cutaneous melanoma.

Conclusion: Mutations of *TERT* promoter with UV signatures are frequent in conjunctival melanomas and favour a pathogenetic kinship with cutaneous melanomas. Absence of these mutations in uveal melanomas emphasises their genetic distinction from cutaneous and conjunctival melanomas.

Cutaneous and ocular melanomas affect people worldwide and are associated with significant mortality (Singh *et al*, 2005; Siegel *et al*, 2012; Flaherty *et al*, 2012a). Currently, cure is achievable only in patients with localised disease. Once metastatic spread occurs, the prognosis is poor.

Cutaneous melanoma is characterised by activating driver mutations in genes such as *BRAF* (Davies *et al*, 2002), *NRAS* (Ball *et al*, 1994), and *KIT* (Curtin *et al*, 2006), and recurrent losses of specific tumour suppressors such as *CDKN2A* and *PTEN* (Curtin *et al*, 2005). In patients with certain genetic subsets of melanoma, recently introduced therapies such as BRAF inhibitors improve survival, even in advanced disease (Chapman *et al*, 2011; Hauschild *et al*, 2012; Flaherty *et al*, 2012b).

Uveal melanomas arise from the iris, ciliary body, or choroid of the eye. They comprise ~90% of ocular melanomas and are genetically distinct from cutaneous melanoma. They are characterised by activating somatic mutations in either *GNAQ* or *GNA11* (Van Raamsdonk *et al*, 2009, 2010). Based on gene expression profiles, uveal melanomas may be subdivided into two prognostic groups, termed class 1 (good prognosis) or class 2 (poor prognosis) (Tschentscher *et al*, 2003; Onken *et al*, 2004). Class 1 tumours frequently harbour *SF3B1* mutations (Harbour

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et al, 2013), whereas class 2 tumours typically show chromosomal monosomy 3 (Prescher *et al*, 1996), as well as inactivating *BAP1* mutations (Harbour *et al*, 2010).

Conjunctival melanomas, unlike uveal melanomas, frequently harbour *BRAF* and *NRAS* mutations and copy number alterations reminiscent of cutaneous and mucosal melanoma (Gear *et al*, 2004; Spendlove *et al*, 2004; Goldenberg-Cohen *et al*, 2005; Lake *et al*, 2011; Griewank *et al*, 2013). Their clinical behaviour also differs from uveal melanoma and is similar to that of cutaneous melanomas (Zembowicz *et al*, 2010; Harooni *et al*, 2011; Shields *et al*, 2011).

Recently two studies showed that up to 71% of cutaneous melanomas harboured novel mutations in the promoter region of *TERT*, coding for the catalytic subunit of the telomerase holoenzyme (Horn *et al*, 2013; Huang *et al*, 2013). These mutations were shown to lead to increased *TERT* expression, most likely by creating ETS transcription-factor-binding sites (Horn *et al*, 2013; Huang *et al*, 2013). An additional study found *TERT* promoter mutations in a wide array of different human cancers, including bladder cancer, hepatocellular carcinoma, and different types of gliomas (Killela *et al*, 2013). They associated high frequencies of *TERT* promoter mutations, with tumours arising in tissues having low rates of self-renewal.

The frequency of *TERT* promoter mutations in ocular melanomas has not been analysed. Here, we investigated the presence of *TERT* promoter mutations in ocular melanomas including conjunctival and uveal melanomas.

MATERIALS AND METHODS

Sample selection. Ocular melanoma samples were obtained from patients treated in the Department of Ophthalmology for conjunctival or uveal (choroidal or ciliary body) melanoma, as well as from the tissue archives of the Departments of Ophthalmology, Pathology, and Dermatology, University Hospital Essen, Germany, and the Department of Ophthalmology, University Hospital Tübingen, Germany. The study was carried out in accordance with the guidelines set forth by the ethics committee of the University of Duisburg-Essen.

Clinical and pathological parameters. Clinical and pathological details were obtained from patient records. A review of pathological slides was also performed to confirm the conjunctival origin of tumours and to assess the following: site(s) of tumour involvement (primarily or secondarily); pathological stage; the presence of associated lesions such as conjunctival naevus and primary acquired melanosis; and pigmentation (presence of melanin pigment in the cytoplasm of tumour cells). Conjunctival naevus was defined as an acquired junctional or compound proliferation of benign melanocytes, usually in a nested pattern, in the conjunctiva. Primary acquired melanosis was defined clinically as flat, speckled brown lesions of the conjunctiva, and histologically as hyperpigmentation of conjunctival epithelium without melanocytic hyperplasia, or melanocytic hyperplasia in conjunctival epithelium with or without cytologic atypia.

DNA isolation. Ten-micrometre thick sections were cut from formalin-fixed, paraffin-embedded tumour tissues. The sections were deparaffinised and manually macrodissected according to the standard procedures. Genomic DNA was isolated using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Samples of fresh frozen tissue were directly applied to the QIAamp DNA Mini Kit. Uveal melanoma sample DNA isolation and determination of chromosome 3 status by microsatellite analysis were performed as previously described (Thomas *et al*, 2012).

Table 1. Associations of *TERT* mutation status with clinical and pathological parameters in conjunctival melanoma

Parameter	Level	*TERT* wild-type (*n* = 26)	*TERT* mutant (*n* = 12)	*P*-value
Median age (years)		67.2	73.3	0.72[a]
Sex	Female	15	6	1.00
	Male	10	5	
Sites of involvement[b]				
Bulbar	No	11	8	0.13
	Yes	12	2	
Caruncle	No	20	5	0.043
	Yes	2	4	
Fornix	No	16	6	1.00
	Yes	6	2	
Sclera	No	23	9	0.51
	Yes	1	1	
Palpebra	No	19	6	0.32
	Yes	3	3	
Multifocal	No	16	4	0.14
	Yes	7	6	
Clinical stage	Ia	5	1	0.20
	Ib	7	1	
	IIa	0	1	
	IIb	6	2	
	IIc	0	2	
	IId	1	0	
	IIIa	1	1	
	IIIb	3	2	
Pathological stage	I	1	0	0.19
	Ia	5	2	
	Ib	3	0	
	Ic	3	0	
	IIa	4	1	
	IIb	1	0	
	IIc	1	4	
	III	4	3	
Associated lesion	PAM	15	6	0.47
	Naevus	3	1	
	De novo	5	1	
Cell type	Mixed	13	3	0.22
	Spindle	3	3	
	Epithelioid	3	3	
Pigmentation	Yes	20	8	0.52
	No	1	1	
NRAS mutation	No	24	9	0.30
	Yes	2	3	
BRAF mutation	No	20	8	0.69
	Yes	6	4	
Disease-free survival	1.25 (0.47–3.35)[c]	24[d]	18[d]	0.65[e]
Overall survival	1.92 (0.60–6.08)[c]	NR[d]	89[d]	0.27[e]

Abbreviations: NR = not reached; PAM = primary acquired melanosis. *P*-values are derived from χ^2 or Fisher's exact tests, as appropriate, unless otherwise specified. Clinical and pathological stage is according to the American Joint Committee on Cancer staging system for conjunctival melanoma, 7th edition. Note: The sum of the numbers of cases for individual parameters may not equal the total number of cases because data for some parameters was not available in all cases.

[a]Kruskal–Wallis test.

[b]Sites of involvement refer to structures affected by the tumour, either primarily or secondarily. Only tumours of conjunctival origin were included in the study.

[c]Hazard ratio (95% confidence interval for hazard ratio).

[d]Estimates of median survival (in months), derived from Kaplan–Meier method.

[e]*P*-values, derived from univariate Cox regression analysis.

Direct (Sanger) sequencing. Nested PCR was performed to amplify *BRAF* exons 11 and 15 and *NRAS* exons 1 and 2, and sequenced as previously described (Houben *et al*, 2004). A 474-base pair region of the *TERT* promoter region was amplified using the following primers: hTERT_F 5′-ACGAACGTGGCCAGCGGCAG-3′ and hTERT_R 5′-CTGGCGTCCCTGCACCCTGG-3′. For amplification of DNA from formalin-fixed material, primers hTERT_short_F 5′-CAGCGCTGCCTGAAACTC-3′ and hTERT_short_R 5′-GTCCTGCCCCTTCACCTT-3′, which amplify a 163-bp fragment, were applied as previously described (Horn *et al*, 2013). After purification with the QIAquick PCR Purification Kit (Qiagen), PCR products were used as templates for sequencing. The sequencing chromatogram files were examined, and mutations were identified using Chromas software (version 2.01, University of Sussex, Brighton, UK).

RESULTS

Sample cohort. The study cohort consisted of 50 uveal melanoma samples (which included 22 tumours with chromosome 3 disomy, and 28 tumours with chromosome 3 monosomy) randomly selected from a cohort of 374 tumours described previously (Thomas *et al*, 2012), and 43 conjunctival melanoma samples. All the samples were primary or recurrent tumours. Available clinical data of conjunctival melanoma are listed in Table 1 and Supplementary Table 1.

***TERT* promoter mutation analysis.** Sequence analysis failed in one and was ambiguous in two of the 50 uveal melanoma samples. In the remaining 47 samples (21 disomy 3, 26 monosomy 3), no *TERT* promoter mutations were identified. Sequence analysis was successful in 38 of the 43 conjunctival melanomas, and *TERT* promoter mutations were identified in 12 (32%) tumours. The mutations were located at positions Chr.5:1295228C>T ($n=2$, 5%), Chr.5:1295242_43delinsTT ($n=2$, 5%), and Chr.5:1295250C>T ($n=8$, 21%), as shown in Figure 1. Mutations will be further annotated using the last three digits of their chromosome location as 228C>T, 242CC>TT, and 250C>T respectively. All identified *TERT* promoter mutations had a UV signature (C>T and CC>TT) (Pleasance *et al*, 2010). In eight cases, matched constitutional DNA isolated from peripheral blood was sequenced. None of these samples harboured the *TERT* promoter mutations detected in the corresponding tumour DNA, verifying that the detected mutations were somatically acquired (Supplementary Figure 1).

***BRAF* and *NRAS* mutations.** Successful sequencing of *BRAF* and *NRAS* was performed in the conjunctival melanoma samples. Ten of the thirty-eight tumours (26%) harboured *BRAF* mutations: nine p.V600E (c.1799T>A) and one p.G469A (c.1406G>C). Four (40%) *BRAF*-mutant samples had a concomitant *TERT* promoter mutation (250C>T, $n=2$ and 228C>T, $n=2$). Mutations of *NRAS* were present in five (13%) conjunctival melanomas, including three p.Q61R (c.182A>G) and two p.Q61K (c.181C>A) mutations. Three (60%) *NRAS*-mutant samples had concomitant *TERT* promoter mutations (250C>T, $n=3$).

Associations of clinical and pathological parameters with *TERT* mutation status in conjunctival melanoma. An analysis with the available clinicopathological data was performed. Median follow-up duration was 39.2 months (3.2–171 months). Five (13%) patients were lost to follow-up. No statistically significant associations of *TERT* promoter mutation status with clinical and pathological parameters (age, sex, site of tumour involvement, clinical stage, pathological stage, associated lesion, and pigmentation) or with disease-free survival or overall survival were found (Table 1).

DISCUSSION

Mutations of the *TERT* promoter were quite frequent (32%) in conjunctival melanoma, but were not seen in uveal melanomas. The mutations identified in conjunctival melanoma are identical to those described in cutaneous melanoma (Horn *et al*, 2013; Huang *et al*, 2013). They were found at the same hotspots and were mutually exclusive of each other. Additionally, all mutations showed a UV signature, consisting of C>T or CC>TT nucleotide changes (Pleasance *et al*, 2010). The frequencies of *TERT* promoter mutations reported in cutaneous melanoma vary significantly, probably in part due to varying sample selection (type of melanomas, primary or metastatic samples, and so on). The mutation frequency of 33% reported by Horn *et al* (2013) in primary cutaneous melanomas is very similar to the mutation rate of 32% detected in our cohort of

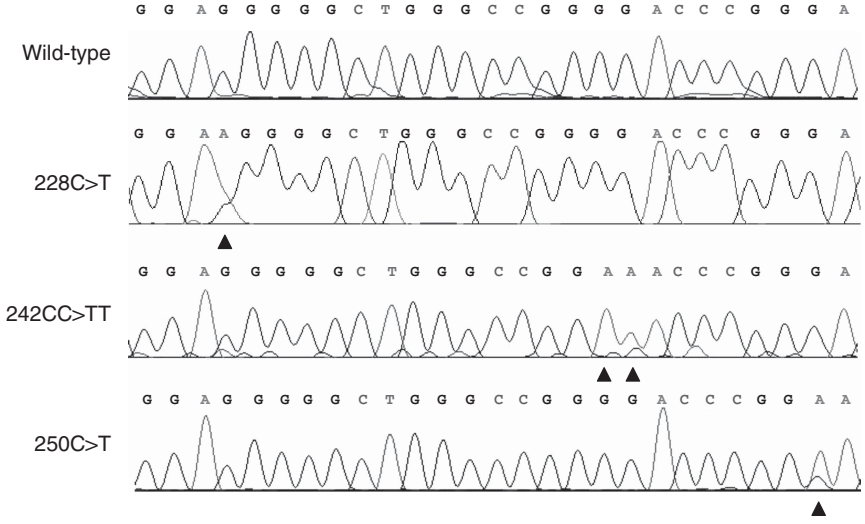

Figure 1. Mutations identified in the *TERT* promoter. Representative electropherograms showing the wild-type sequence from a uveal melanoma sample (on top) and the mutations identified in three conjunctival melanoma samples –Chr.5:1295228C>T, Chr.5:1295242_43delinsTT, and Chr.5:1295250C>T (hg19 assembly). Mutations in the figure are labeled using the last three digits of the first nucleotide mutated (underlined). The black arrowheads indicate the respective nucleotide changes.

conjunctival melanoma samples. This finding provides further evidence for a pathogenetic link between cutaneous and conjunctival melanomas. Further support for such a link derives from the frequency of *BRAF* and *NRAS* mutations, and similarity of clinical behaviour between these melanoma types.

In our cohort of 38 conjunctival melanomas, there were no significant associations between *TERT* promoter mutation status and clinicopathological parameters. Future studies with larger tumour cohorts will be required to validate these findings.

Our results underline the distinct pathogenesis of uveal melanoma compared with other forms of melanoma. Mutations of the *TERT* promoter join the list of genetic events such as mutations in *BRAF* or *NRAS* and losses of *PTEN* or *CDKN2A,* which are very common in cutaneous melanoma but are virtually never seen in uveal melanoma. Considering that correspondingly, almost all genetic events identified in uveal melanoma, such as mutations in *GNAQ* (Van Raamsdonk *et al*, 2009), *GNA11* (Van Raamsdonk *et al*, 2010), *BAP1* (Harbour *et al*, 2010), and *SF3B1* (Harbour *et al*, 2013), are rarely seen in cutaneous or mucosal melanoma; uveal melanomas potentially will be found to harbour their own unique set of recurrent mutations in regulatory DNA regions (that is, promoters, enhancers, and so on).

Killela *et al* (2013) found high frequencies ($>15\%$) of *TERT* promoter mutations in tumours arising from tissues with low self-renewal capability. Our results suggest that this phenomenon may apply to conjunctival melanoma, as well as cutaneous melanoma. In certain cancers, Killela *et al* (2013) reported alternative lengthening of telomeres (ALT) with inactivating mutations in *ATRX* and *DAXX* as a mechanism for telomere maintenance in *TERT* promoter mutation-negative samples. Alternative lengthening of telomeres has generally not been reported to be relevant in melanoma. Additionally, larger whole-exome sequencing studies both for cutaneous melanoma ((Hodis *et al*, 2012; Krauthammer *et al*, 2012), $n>250$) and uveal melanoma ((Harbour *et al*, 2013) and our own unpublished data, $n=40$) did not find recurrent mutations in *ATRX* or *DAXX*. This argues against a relevant role for ALT in ocular melanoma.

The role of UV-mediated tumourigenesis in ocular melanomas is yet to be resolved (Pane and Hirst, 2000; Guenel *et al*, 2001; Singh *et al*, 2004). The UV signature in the mutations identified in the *TERT* promoter of conjunctival melanomas suggests a potential role for UV-induced genetic alterations in the pathogenesis of these tumours. Whether UV radiation contributes to the development of uveal melanomas is yet to be determined.

In summary, the distribution of *TERT* promoter mutations in ocular melanoma provides further evidence that ocular melanomas comprise genetically distinct tumour groups. The presence of *TERT* promoter mutations with UV signatures in conjunctival melanomas supports an UV-induced pathogenesis and a pathogenetic kinship with cutaneous melanomas. Absence of these mutations in uveal melanomas emphasises their genetic distinction from cutaneous and conjunctival melanomas.

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CONFLICT OF INTEREST

Dirk Schadendorf is on the advisory board and has received honararia from Roche, Genetech, Novartis, Amgen, GSK, BMS, Boehringer Ingelheim, and Merck. Lisa Zimmer has received honoraria from Roche, Bristol-Meyers Squibb, and Amgen, and travel support from Merck Sharp & Dohme and Bristol-Meyers Squibb. Bastian Schilling has received travel support from BMS. The remaining authors declare no conflict of interest.

DISCLAIMER

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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