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Research Paper

Targeted next generation sequencing of mucosal melanomas identifies frequent *NF1* and *RAS* mutations

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ABSTRACT

Purpose: Mucosal melanoma represents ~1% of all melanomas, frequently having a poor prognosis due to diagnosis at a late stage of disease. Mucosal melanoma differs from cutaneous melanoma not only in terms of poorer clinical outcome but also on the molecular level having e.g. less *BRAF* and more frequent *KIT* mutations than cutaneous melanomas. For the majority of mucosal melanomas oncogenic driver mutations remain unknown.

Experimental Design and Results: In our study, 75 tumor tissues from patients diagnosed with mucosal melanoma were analyzed, applying a targeted next generation sequencing panel covering 29 known recurrently mutated genes in melanoma. *NF1* and *RAS* mutations were identified as the most frequently mutated genes occurring in 18.3% and 16.9% of samples, respectively. Mutations in *BRAF* were identified in 8.4% and *KIT* in 7.0% of tumor samples.

Conclusions: Our study identifies *NF1* as the most frequently occurring driver mutation in mucosal melanoma. *RAS* alterations, consisting of *NRAS* and *KRAS* mutations, were the second most frequent mutation type. *BRAF* and *KIT* mutations were rare with frequencies below 10% each. Our data indicate that in mucosal melanomas *RAS*/*NF1* alterations are frequent, implying a significant pathogenetic role for MAPK and potentially PI3K pathway activation in these tumors.

INTRODUCTION

Mucosal melanomas arise from melanocytes of the mucosal membrane and represent a rare subgroup of melanoma, accounting for around 1% of all melanomas [1]. Frequently diagnosed at an advanced tumor stage, they generally have a poor prognosis [2]. In contrast to cutaneous melanomas, where exogenous or endogenous risk factors such as UV-exposure or genetic predisposition are well known and studied in a high number of patients [3], no comparable factors have been identified for mucosal melanomas. Additionally, little information is

available with regard to the molecular pathogenesis of mucosal melanoma. In general, mucosal melanomas seem to have a lower overall mutational burden as compared to cutaneous melanomas (8193 vs. 86495 somatic single nucleotide variants per tumor) [4, 5] but a higher number of chromosomal aberrations [6]. However, *BRAF* V600 mutations, the most frequent and therapeutically best-targetable gene alteration in cutaneous melanoma (present in ~50% of cases) is only rarely found in mucosal melanomas (≤ 10% of cases), which limits clinical treatment options [6-8]. Other activating oncogenic events, e.g. gene amplifications or gain-of-function mutations of *KIT* are more frequently detected in mucosal melanomas. Existing literature reports *KIT* activation in 15-39% of mucosal melanomas [9-13]. However, *KIT*-targeting therapies, e.g. with imatinib, have failed to show convincing therapeutic efficiency in mucosal melanoma in larger studies [14, 15]. *NRAS* mutations are somewhat less frequent in mucosal (10-20%) [6, 8] than cutaneous melanomas (20-30%) [6, 16-18]. Activating mutations in *GNAQ* and *GNA11*, which are commonly detected in uveal melanoma [19], have recently been reported to occur in 9.5% of mucosal melanomas [20], a finding not reported in previous studies [21]. Many existing studies have been performed on cohorts with limited sample numbers.

In summary, mucosal melanoma represents a clinically aggressive cancer entity rarely harboring known therapeutically targetable driver mutations. Our study aimed to identify additional oncogenic driver mutations in mucosal melanoma in a larger cohort of patients to recognize additional molecular pathways with the potential to be exploited for establishing future therapeutic strategies.

RESULTS

Patient characteristics

Samples were obtained from 75 patients, 46 females and 29 males. Clinic-pathological characteristics are summarized in Table 1. In 4 cases, sequencing analysis was not possible due to poor sequencing quality; clinico-pathological characteristics of these patients are not included in Table 1.

Targeted next generation sequencing

Mutations were identified in 50 samples (Figure 1, Table 2, Supplementary Table 1). *NF1* and *RAS* were the most frequently mutated genes (Figure 2, Supplementary Figure 1 and 2, Table 2, Supplementary Table 1), with 15 *NF1* mutations identified in 13 samples (18.3.%) and 12 *RAS* mutations identified in 12 samples (16.9%). In 9 out of 13 samples (69.2%) a clearly inactivating *NF1* mutation was present resulting in non-sense (synthesis stop) or frameshift mutations. Examples of some inactivating *NF1* mutations detected in our cohort are shown in Supplementary Figure 1. Two samples harbored multiple *NF1* mutations (Table 2, Supplementary Table 1); one of them having two inactivating mutations, the other one having an inactivating and a D896N missense mutation. *RAS* gene alterations were found in 12 out of 71 samples (16.9%), including 8 *NRAS* and 4 *KRAS* mutations. Sanger sequencing was performed to validate the identified *KRAS* mutations (Supplementary Figure 3). Six samples (8.4%) harbored *BRAF* mutations, 5 of which were activating V600 mutations (4 V600E and 1 V600K) and 1 N188S mutation (Supplementary Table 2). The mutation pattern on protein level for the identified *NRAS, KRAS* and *NF1* mutations are shown in Figure 2. *KIT* mutations were detected in 5 samples (7%), but only 1 was a known activating mutation. Another 4 samples carried known activating *TERT*-promoter mutations. One *GNA11* S267F mutation and 1 *GNAQ* R183Q mutation were identified. Examples of the most frequent activating mutations identified are illustrated in Supplementary Figure 2. Three samples harboring *KRAS* mutations also had concurrent *NF1* mutations, 2 of which were clearly functionally inactivating. Additionally, 5 *TP53*, 7 *SF3B1,* 4 *MITF* and 3 *PTEN* mutations were identified. Other less frequent mutations were identified in various genes including *SMARC, BAP1, TERT, WT1, PIK3CA, MAP2K2, CDK4, CTNNB1, RAC1, ARID2 and ARID1A*. In 21 samples no non-synonymous protein coding mutation were identified in the 29 genes analyzed.

Statistical analysis

We performed a statistical analysis to assess possible associations of clinical parameters such as gender, location of the primary tumor and sample type (primary, metastasis or recurrence) with the *NF1*, *RAS* and *RAF* mutational status. A statistically significant association was determined between *RAS* mutational status and male sex (p=0.024, Table 1).

DISCUSSION

In this study, 71 mucosal melanoma samples were screened for mutations in known recurrently mutated genes in cutaneous and uveal melanoma. The most frequent mutations were identified in the *NF1* gene and *RAS* gene family members, indicating that mucosal melanomas have a genetic mutation profile which is different from that of cutaneous or uveal melanomas. Our study identified an unexpectedly high number of *NF1* mutations. In 13 (18.3,%) out of 71 samples, *NF1* mutations were identified. Nine of those (69.2%) harbored clearly inactivating mutations, i.e. nonsense or frameshift mutations.

To our knowledge, there is no previous data demonstrating that mucosal melanomas express such a high frequency of *NF1* mutations. Yang *et al.* recently

Table 1: Clinicopathological characteristics of the patients

Variable	Total (n=71)	*NF1*	WT	p-value	*RAS*	WT	p-value	*BRAF*	WT	p-value
Median age (range)	64 (33-84)									
Gender										
Male	26	3	23	0.348	8	18	0.024	2	24	1.0
Female	45	10	35		4	41		4	41	
Anatomical site										
Head and neck	28	3	25	0.386	9	19	0.224	2	26	0.672
Genital area	25	5	20		3	22		2	23	
Anorectum	9	3	6		0	9		1	8	
Digestive tract	3	1	2		0	3		1	2	
Urinary tract	3	0	3		0	3		0	3	
Data missing	3	1	2		0	3		0	3	
Sample type										
Primary tumor	41	8	33	0.798	8	33	0.936	5	36	0.837
Metastasis	22	5	17		3	19		1	21	
Recurrence	3	0	3		0	3		0	3	
Data missing	5	0	5		1	4		0	5	

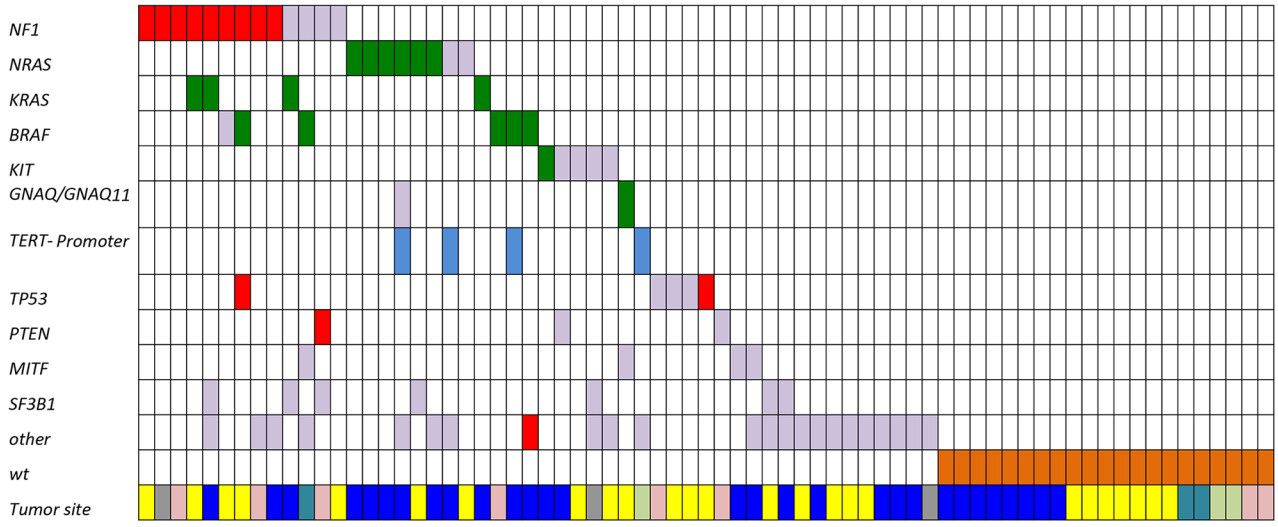

Figure 1: Mutation distribution in mucosal melanomas. Green: mutations known or assumed to be activating; red: loss of function mutations; blue: mutations in the *TERT* promoter region; grey: missense mutation (frequently with unknown functional consequences); brown: wild-type samples (showing no mutation in the analyzed gene panel). Tumor location: Yellow, genital area; light pink, anorectum; dark blue, head and neck; light green, urinary tract; petrol, digestive tract; grey, data missing.

Table 2: List of identified mutations

Nr.	Type	Location primary	NF1	RAS	BRAF	Other Mutations
1	M	G	E2174fs; L151fs			
2	M	DM	R106*			
3	M	G	R2258*	KRAS G12D		
4	P	A	V1308fs			TERT S663N
5	P	G	G1425fs		V600E	TP53 Q165*
6	P	HN	H553fs			PIK3CA E109del
7	P	G	T889fs		N188S	
8	M	A	R1306*			
9	P	HN	T1184fs; D896N	KRAS G12A		ARID1A V700A; SF3B1 D894N
10	M	G	H55R			
11	P	A	I183N			PTEN K163fs; SF3B1 R625H
12	P	D	M1376V		V600E	ARID1A R1202Q ; ARID2 T1208A; MITF A401S
13	P	HN	V1308L	KRAS E63K		SF3B1 R625H
14	P	HN		KRAS G12F		
15	P	HN		NRAS Q61R		
16	P	HN		NRAS Q61R		
17	P	HN		NRAS Q61K		
18	P	HN		NRAS G13R		TERT Ser1104Thr
19	P	HN		NRAS Q61K		TERT P C228T; RAC1 N92K; GNA11 S267F;
20	U	G		NRAS Q61L		SF3B1 V634A
21	M	HN		NRAS A59D		TERT P C243T; TERT P C252T; SMARCA4 A152T
22	M	V		NRAS I46M		
23	M	HN			V600E	TERT P C250T
24	P	HN			V600E	PIK3CA L896fs
25	P	A			V600K	
26	M	HN				KIT L576P
27	M	Ur				TERT P C228T; WT1 D497N
28	M	G				GNAQ R183Q; MITF V487I
29	P	G				TP53 P58fs
30	P	G				ARID2 Y612C
31	M	HN				TERT L1002V
32	M	A				PTEN L108R
33	M	G				PIK3R1 T239M

(*Continued*)

Nr.	Type	Location primary	NF1	RAS	BRAF	Other Mutations
34	P	G				KIT Y553del; MAP2K2 G286R
35	P	G				TERT R819H
36	P	HN				MITF N267K
37	P	A				TP53 C135R
38	R	HN				KIT V50L; PTEN C136R
39	P	G				TP53 P151A
40	U	G				ARID2 M545I; SF3B1 R625H
41	P	G				TERT R819H
42	U	A				MITF V487I; SMARCA4 R1260S
43	R	HN				SF3B1 T916S; CK4 R209C
44	P	HN				TP53 R175G
45	P	G				KIT, L783I
46	M	DM				KIT, I748T; BAP1, G579R: SF3B1, R625L
47	P	HN				BAP1 Y646C
48	P	HN				CTNNB1 Y331C
49	P	HN				TERT S953F
50	M	DM				CK4 V174M

Green, mutations known to be activating; red, loss of function mutations; black, missense mutation with unknown functional consequences. Abbreviations: Nr. sample number; M metastasis; P primary tumor; R recurrence; U unknown; fs frame shift; * = stop codon (nonsense mutation); HN Head and Neck, G Genital area, A Anorectum, D Digestive tract, Ur Urinary tract, DM data missing.
For more details including allele frequencies and cDNA annotations, see Supplementary Table 1.

conducted a targeted sequencing analysis on 15 anorectal melanomas and identified that 3 of these tumors harbored an *NF1* mutation [22]. In recent years, *NF1* has been recognized as the third most commonly mutated gene (after *BRAF* and *RAS*) resulting in activation of the MAPK pathway with a reported mutation frequency of 14% [18]. In cutaneous melanomas, more than half of the mutations reported are loss-of-function (LoF) events. In the mucosal melanomas studied here, 9 out of 13 cases carried *NF1* LoF mutations (69.2%). It is known that NF1 is a GTPase-activating protein which downregulates the activity of the RAS protein [18]. As such, LoF mutations in *NF1* are an important genetic mechanism for constitutive MAPK pathway activation.

A relevant role for *NF1* mutations in cutaneous melanomas lacking conventional (i.e. *BRAF* or *NRAS*) activating mutations has been already highlighted by other studies. Wiesner *et al.* demonstrated that desmoplastic melanomas frequently harbor *NF1* mutations [23]. Desmoplastic melanomas are typically associated with high UV-exposure and high mutational loads. Congruently, Krauthammer *et al.* described *NF1* mutations in association with mutations in RASopathy genes (e.g. *RASA2*, *PTPN11,* etc.) in cutaneous melanomas with evidence of high sun-exposure [24]. The association of UV-exposure with *NF1* mutations observed in cutaneous melanoma is not to be expected in mucosal melanoma [4, 5]. Although mutational mechanisms may differ, these studies and our data support *NF1* mutations being highly relevant in melanoma subgroups that rarely harbor *BRAF* or *NRAS* mutations.

Twelve out of 71 (16.9%) mucosal melanomas analyzed harbored *RAS* mutations, 8 in the *NRAS* and 4 in the *KRAS* gene. No mutations were identified in *HRAS*. Previous studies have focused primarily on *NRAS*, where the mutation frequency ranges from 5% to 15% [6, 8, 25-27]. The frequency of *NRAS* mutations detected in our study with 11.2% is comparable. *KRAS* mutations have not been assessed in most previous studies of mucosal melanoma, however, accounted for one third of the mutations in *RAS* genes observed in our cohort. A slightly

significant association of *RAS* mutation status with male gender was noted (p=0.024). This potential association will need be to assessed in future studies with considerably larger sample numbers.

BRAF was the third most frequently mutated gene identified in our study. Of the 6 mutations (8.4%) identified, *5* were well known V600 activating mutations, consisting of 4 V600E and 1 V600K mutation. The other identified mutation, resulting in an N188S exchange, is of unclear significance and could be a non-relevant passenger mutation. These findings are in accordance with reports stating that activating *BRAF* mutations in mucosal melanomas are rare with a frequency between 5 and 17% [8, 9, 26, 28, 29]. Only one patient in our cohort received BRAF inhibitor therapy showing a partial response (Supplementary Table 2). The efficacy of BRAF inhibitor therapies in mucosal melanomas will need to be further assessed in larger studies.

Genetic alterations of *KIT*, including mutations and copy number increases, have been reported to occur in up to 39% of mucosal melanomas [9-13]. In our study, 5 out of 71 (7.0%) samples had a *KIT* mutation; 2 mutations in tumors of the head and neck region and 2 in vulvar melanomas. Omholt et al. [27] reported *KIT* mutations in 17% (n=12) of primary mucosal melanomas. They found that 35% (8 out of 23) of vulvar melanomas harbored *KIT* mutations. In our study, 2 out of 9 (22.2%) vulvar melanomas had a *KIT* mutation. Generally, our findings support existing literature stating that *KIT* mutations are more frequent in melanomas of the genital area followed by melanomas of the head and neck and the anorectal area [8, 26, 30, 31].

TERT promoter mutations resulting in increased transcription of the *TERT* gene have been identified as the most common mutation in cutaneous melanoma [32-34]. In congruence with previous studies reporting low mutation rates in mucosal melanomas [35, 36], the mutation frequency in our cohort was 5.6%. All *TERT* mutations were C>T mutations. As C>T alterations are classically associated with UV-exposure [32, 37, 38], the lower *TERT* mutation frequency may be due to the very limited UV-exposure of tumors arising in mucosal locations.

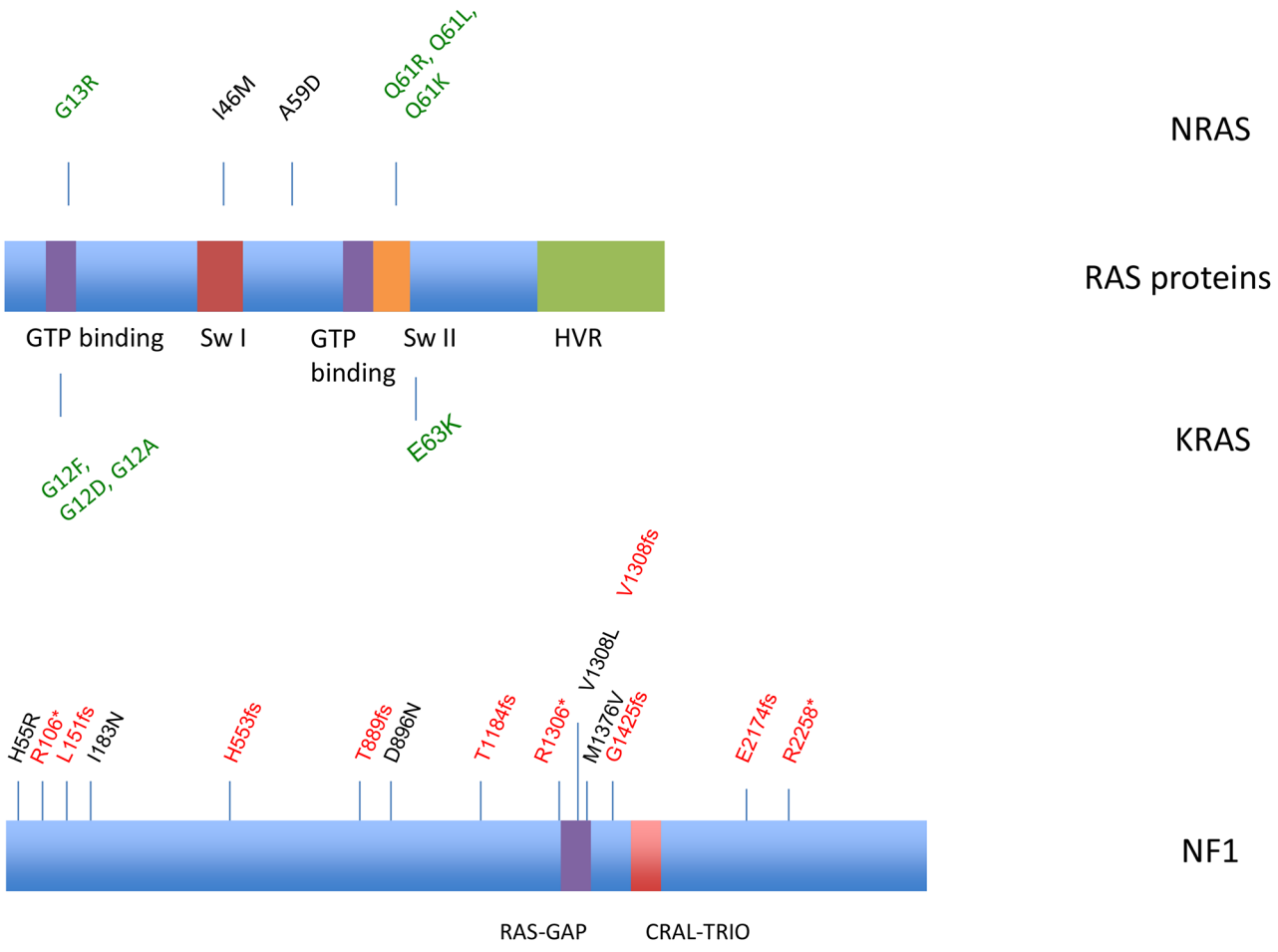

Figure 2: Distribution of identified *NRAS, KRAS* and *NF1* mutations. Frameshift and nonsense mutations are annotated in red, activating mutations are demonstrated in green. Missense mutations are black. Switch I, effector/GAP interaction; Switch II, EF interaction; HVR, hypervariable region. NF1 contains a Ras-GAP domain (GTPase-activator protein for Ras-like GTPase) and a CRAL-TRIO domain.

Two alterations were identified in *GNAQ* and *GNA11*, mutations that are usually associated with uveal melanomas and blue nevi [19]. Of those, only the *GNAQ* R183Q mutation is known to be functionally activating, resulting in increased mitogenic signaling in melanocytic tumors and rare vascular diseases such as Sturge-Weber syndrome and phakomatosis pigmentovascularis [39, 40]. The other mutation identified (*GNA11* S267F) is not known to be functionally relevant and probably represents a bystander mutation considering the sample also harbored a *NRAS* Q61K hot-spot mutation. While *GNAQ* and *GNA11* mutations were recently reported to occur in 9.5% of mucosal melanomas [20], our study suggests these mutations are less frequent in this tumor entity.

In out cohort, 31 samples presented mutations in the MAPK pathway (some of them harboring more than one mutation): 13 *NF1*, 12 *RAS,* 6 *RAF,* 5 *KIT,* 1 *GNAQ* and 1 *GNA11* mutation. In 23 of those samples mutations resulting in MAPK activation were found suggesting that this is a critical event in the pathogenesis of mucosal melanoma. This is similar to cutaneous melanoma, where constitutive activation of the MAPK pathway is a known critical event. The genetic alterations leading to this activation however vary between these melanoma subtypes [18].

Although our study represents the most comprehensive genetic analysis of 75 mucosal melanomas presented to date, it does have some limitations. Mutations occurring in genes not covered by our panel could not be identified. Additionally, our approach did not allow us to reliably detect copy number variations.

Our results underline that mucosal melanomas are genetically distinct from cutaneous and uveal melanomas with frequent inactivating mutations in *NF1* and activating mutations in *RAS* genes. Our findings suggest that similar to cutaneous melanoma, activation of the MAPK pathway is a pivotal event in mucosal melanoma. Taken into consideration that both *NF1* and *RAS* alterations can additionally activate PI3K signaling, this pathway could be of particular significance in mucosal melanomas. It stands to reason that other, so far unidentified, mutations are present in mucosal melanomas and future whole-exome or whole-genome studies of larger tumor cohorts will be required to fully elucidate the landscape of genetic alterations involved.

MATERIALS AND METHODS

Sample selection

Samples of mucosal melanomas were retrieved from the biobank of the Department of Dermatology, Essen, Germany, from the TRIM (Tissue Registry in Melanoma) project of the German DeCOG (Dermatological Cooperative Oncology Group) as well as from patients enrolled into the DeCOG trial ChemoSensMM [ClinicalTrials.gov: NCT00779714 [41]. Samples were considered mucosal melanoma if the tumor originated in a mucosal site, was diagnosed as melanoma histopathologically (confirmed by at least one immunohistochemical marker [Melan-A, HMB-45 or S100]) and no other preexisting, e.g. cutaneous, melanoma was reported (to exclude potential mucosal metastasis). The study was performed with informed patient consent in accordance with the guidelines of the Ethics Committee of the Medical Faculty of the University Duisburg-Essen (ethical approval no. 15-6473-BO, no. 15-6566-BO).

DNA isolation

FFPE tissue was prepared in 10 µm sections and deparaffinized according to standard procedures. In brief, 2 steps of 5 min xylene, 5 min 100% ethanol, 5 min 95% ethanol, 5 in 70% ethanol, 5 min 50% ethanol, rinsing in water. After air drying, the tumor tissue was manually macrodissected from the sections. The tumor content in the area of macrodissection was required to be at least 25%, however, was generally considerably higher (documented values are listed in Supplementary Table 1). Genomic DNA was isolated applying the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Targeted sequencing

A custom designed amplicon-based sequencing panel covering the *TERT* promoter and the complete coding regions of 29 known recurrently mutated genes in cutaneous and uveal melanoma (Supplementary Table 3) was applied. This panel was initially clinically validated over a period of 3 months, where Sanger and NGS panel sequencing were performed in parallel for all samples analyzed. All known recurrent mutations in melanoma (incl. *BRAF* V600, *NRAS* G12, G13 and Q61 and *KIT* L576, K642 and N822) were repeatedly picked up by our NGS panel which showed a 100% concordance with mutations identified by Sanger sequencing, however demonstrated a higher level of sensitivity. After successful validation, our routine clinical sequencing effort has solely relied on the NGS panel, which in over 2 years has been applied to more than 1300 melanocytic tumors. Our panel sequencing approach is not able to reliably detect copy number variations.

Adapter ligation and barcoding of individual samples occurred applying the NEBNext Ultra DNA Library Prep Mastermix Set and NEBNext Multiplex Oligos for Illumina from New England Biolabs. Sequencing analysis was performed using CLC Cancer Research Workbench from QIAGEN as previously described [42]. In brief, analysis included the following steps: The CLC workflow included adapter trimming and read pair merging before mapping to the human

reference genome (hg19). Insertions and deletions as well as single nucleotide variant detection, local realignment and primer trimming followed. Additional information was then obtained regarding potential mutation type, known single nucleotide polymorphisms and conservation scores by cross-referencing various databases (COSMIC, ClinVar, dbSNP, 1000 Genomes Project, HAPMAP and PhastCons_Conservation_scores_hg19). The CLC generated csv files were further analyzed manually with mutations affecting the protein-coding portion of the gene considered if predicted to result in non-synonymous amino acid changes. The average fold coverage was 2585x. To eliminate questionable low frequency background mutation calls, not uncommon in our experience with FFPE amplicon sequencing approaches [43], mutations were reported if overall coverage of the mutation site was ≥30 reads, ≥10 reads reported the mutated variant and the frequency of mutated reads was ≥10 %.

Statistical analysis

The associations of mutation status with clinical parameters such as gender, location of the primary tumor and sample origin (primary, metastasis or recurrence), was investigated using chi-squared tests and Fisher exact tests as appropriate. Statistical analyses were performed using SPSS 23.0 (IBM Corp., Armonk NY, USA), considering a p-value of $p \leq 0.05$ as statistically significant.

Author contributions

Study concept and design was performed by K. Griewank and A. Roesch. Involved in data acquisition were S. Ugurel, L. Zimmer, M. Ziemer, C. Pföhler, J. Utikal, P. Mohr and C. Pfeiffer. Quality control of data was performed by A. Sucker, I. Cosgarea, S. Horn, U. Hillen. Data analysis was covered by I. Cosgarea and K. Griewank. Involved in the statistical analysis was L. Livingstone and I. Cosgarea. Manuscript preparation was covered by I. Cosgarea, K. Griewank, A. Roesch, D. Schadendorf. In charge with manuscript editing were I. Cosgarea, K. Griewank and A. Roesch. Manuscript review was performed by all the authors.

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CONFLICTS OF INTEREST

Ioana Cosgarea has received travel support from Novartis, Bristol-Meyers Squibb, TEVA. Selma Ugurel is on the advisory board or has received honoraria from Bristol-Myers Squibb, Merck Sharp & Dohme and Roche, and received grant support or travel support from Bristol-Myers Squibb, medac and Roche. Lisa Zimmer has received honoraria from Roche, Bristol-Myers Squibb, MSD Sharp & Dohme, Merck, GlaxoSmithKline, and Novartis, and travel support from MSD Sharp & Dohme, Novartis and Bristol-Meyers Squibb. Elisabeth Livingstone has received honoraria from Roche, Bristol-Myers Squibb, Amgen, Novartis, Boehringer-Ingelheim, Merck Sharp & Dohme and Merck, and travel support from Amgen, Merck Sharp & Dohme, Bristol-Myers Squibb, Novartis and Boehringer-Ingelheim. Mirjana Ziemer is on the advisory board or has received honoraria from Bristol-Myers Squibb, Merck Sharp & Dohme and Roche, as well as received travel support from Bristol-Myers Squibb. Jochen Utikal is on the advisory board or has received honoraria and travel support from Amgen, BMS, GSK, LeoPharma, MSD, Novartis, Roche. Claudia Pföhler has received honoraria and travel support from Novartis, BMS, Roche, Merck Serono, MSD Merck Sharp & Dohme, Bencard and Celgene and is on the advisory board from Novartis and Merck Serono. Dirk Schadendorf is on the advisory board or has received honararia from Roche, Genentech, Novartis, Amgen, GSK, BMS, Boehringer Ingelheim, and Merck. Klaus Griewank has received travel support from Roche. Alexander Roesch received travel grants and honoraria from Roche, TEVA, Bristol-Myers Squibb, MSD Sharp & Dohme, Amgen, and Novartis and a research grant from Novartis.

All other authors have nothing to declare.

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