

# Mutational dichotomy in desmoplastic malignant melanoma corroborated by multigene panel analysis

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**Desmoplastic malignant melanoma is a distinct melanoma entity histologically subtyped into mixed and pure forms due to significantly reduced lymph node metastases in the pure form. Recent reports investigating common actionable driver mutations have demonstrated a lack of *BRAF*, *NRAS*, and *KIT* mutation in pure desmoplastic melanoma. In search for alternative driver mutations next generation amplicon sequencing for hotspot mutations in 50 genes cardinal to tumorigenesis was performed and in addition the *RET* G691S polymorphism was investigated. Data from 21 desmoplastic melanomas (12 pure and 9 mixed) were retrieved. Pure desmoplastic melanomas were either devoid of mutations (50%) or displayed mutations in tumor suppressor genes (*TP53*, *CDKN2A*, and *SMAD4*) singularly or in combination with the exception of a *PIK3CA* double-mutation lacking established biological relevance. Mixed desmoplastic melanomas on the contrary were frequently mutated (89%), and 67% exhibited activating mutations similar to common-type cutaneous malignant melanomas (*BRAF*, *NRAS*, *FGFR2*, and *ERBB2*). Separate analysis of morphologically heterogeneous tumor areas in four mixed desmoplastic malignant melanomas displayed no difference in mutation status and *RET* G691S status. *GNAQ* and *GNA11*, two oncogenes in *BRAF* and *NRAS* wild-type uveal melanomas, were not mutated in our cohort. The *RET* G691S polymorphism was found in 25% of pure and 38% of mixed desmoplastic melanomas. Apart from *RET* G691S our findings demonstrate absence of activating driver mutations in pure desmoplastic melanoma beyond previously investigated oncogenes (*BRAF*, *NRAS*, and *KIT*). The findings underline the therapeutic dichotomy of mixed versus pure desmoplastic melanoma with regard to activating mutations primarily of the mitogen-activated protein kinase pathway.**

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Desmoplastic malignant melanoma is a rare subtype of spindle cell malignant melanoma composed of spindled tumor cells interspersed in a characteristic, dense, paucicellular fibrous stroma, or fibromyxoid stroma.<sup>1</sup> A variable admixture of an epithelioid or nondesmoplastic-spindled tumor cell component resembling conventional malignant melanoma is frequently seen. Presence of a conventional (nondesmoplastic) malignant melanoma component of >10% has been used as a cut-off to define mixed desmoplastic malignant melanoma as opposed to

pure desmoplastic malignant melanoma. Desmoplastic malignant melanomas account for <4% of cutaneous melanoma cases and patients tend to present with advanced stage of disease due to lack of lesional pigmentation and occasional initial misdiagnosis.<sup>2</sup> Clinically, desmoplastic malignant melanoma is frequently associated with lentigo maligna. When compared to conventional malignant melanoma of identical thickness, desmoplastic malignant melanoma has been associated with a reduced rate of lymph node metastases but higher local recurrence rates, most likely reflecting incomplete excisions of these tumors.<sup>3,4</sup> In addition, desmoplastic malignant melanoma exhibits a propensity for hematogenous spread primarily to the lung.<sup>5</sup> Histological subclassification into pure and mixed desmoplastic malignant melanoma has been advocated on grounds of a purportedly negligible risk for lymph node metastases in pure desmoplastic

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malignant melanoma and restriction to sentinel node biopsy to patients with clinically conspicuous lymph nodes.<sup>6,7</sup> Targeted therapies (vemurafenib and imatinib) available for patients harbouring *BRAF*,<sup>8</sup> *NRAS*,<sup>9</sup> and *KIT*<sup>10</sup> mutations have added therapeutic relevance to mutational subtyping of malignant melanoma. Increasing evidence demonstrated that mutational characteristics segregate with currently accepted subtyping based on histology and tumor location:<sup>11–16</sup> uveal melanomas present the prototypical case of a melanoma entity with a distinct mutation profile as they typically lack *BRAF* mutations,<sup>17</sup> but instead frequently display *GNAQ* or *GNA11* mutations.<sup>18,19</sup> Spitzoid melanomas demonstrate a reduced *BRAF* mutation frequency compared to general melanoma,<sup>11</sup> and mucosal melanomas are frequently *KIT* mutated but *BRAF* wild type.<sup>20</sup> Regarding desmoplastic malignant melanoma a complete lack of *BRAF* mutations was reported in one publication<sup>21</sup> and a markedly decreased frequency of *BRAF* mutations in another.<sup>22</sup> Subclassification of desmoplastic malignant melanoma into pure and mixed type is not only clinically relevant, but also apparently reflects different mutation patterns as well. Miller *et al*<sup>23</sup> have recently shown that pure desmoplastic malignant melanoma is devoid of *BRAF* mutations as opposed to mixed desmoplastic malignant melanoma, which did exhibit *BRAF* mutations albeit at a low level (6% of cases). In addition, absence of *BRAF* mutations in an investigation including 10 desmoplastic melanomas (not further subclassified) has been reported by another group.<sup>24</sup> To identify other, yet unrecognized oncogenic mutations in desmoplastic malignant melanoma we investigated 23 desmoplastic malignant melanomas by next generation amplicon sequencing using a mutation panel (IonAmpliSeq Cancer Hotspot Panel v2, Life Technologies), which covers 2855 COSMIC-annotated hotspot mutations of 50 cardinal tumor-related genes (for genes and respective amplicons included, as well as their genomic coordinates see Supplementary Table 1). This panel includes among others *BRAF*, *CDKN2A*, *BRAF*, *TP53*, *PTEN*, *KIT* as well as *GNAQ*, and *GNA11*, the latter reported in *BRAF* wild type uveal melanomas, but to our knowledge not yet investigated in desmoplastic malignant melanoma.

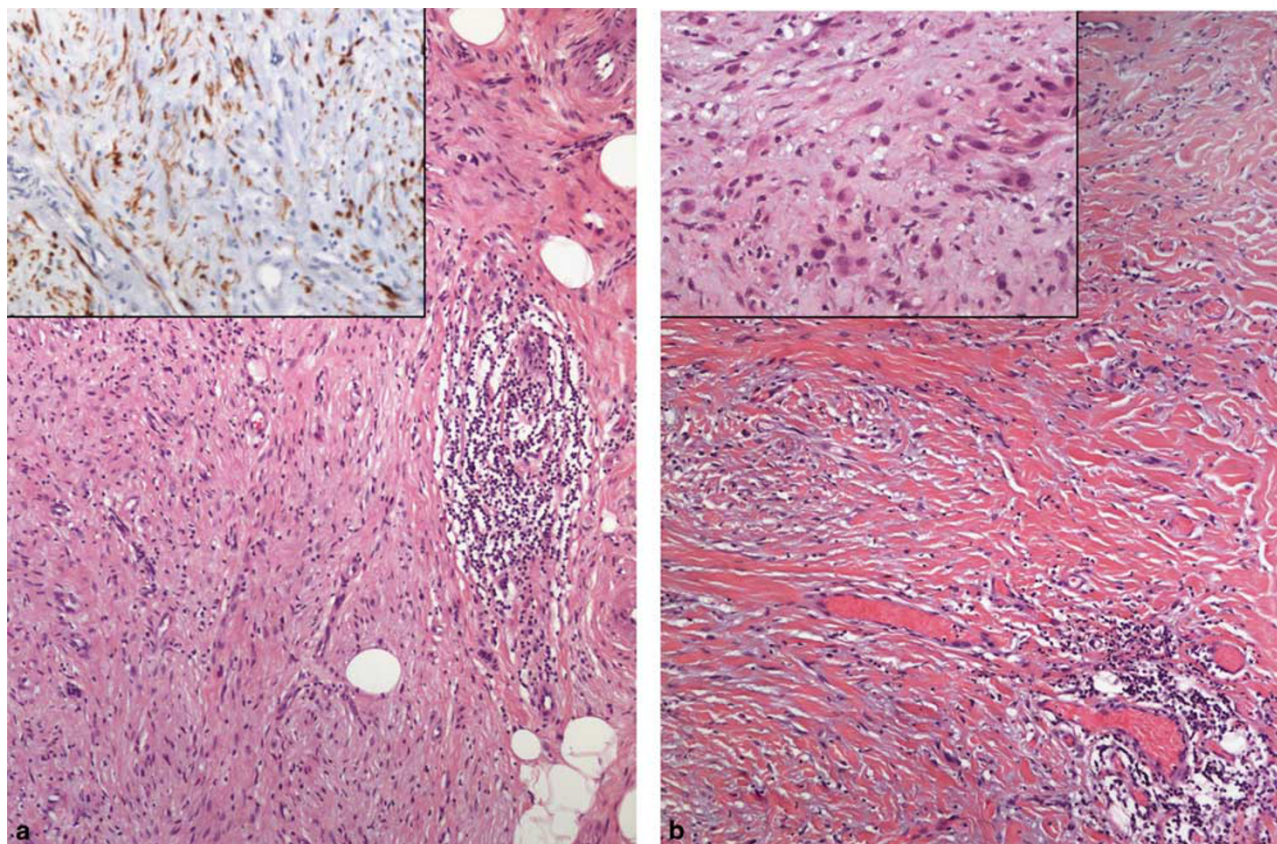
The mutation panel also includes several mutation hotspots of the receptor tyrosine kinase *RET*, a protooncogene with an amino-acid changing single-nucleotide polymorphism in the juxtamembrane region of *RET*, namely G691S (*RET*<sub>p</sub>). This polymorphism enhances the response of *RET* to glial cell line-derived neurotrophic factor, which activates both the *RET*-*RAF*-*RAS*-*MEK*-*ERK* pathway and the *RET*-*PIK3*-*AKT* pathway.<sup>25,26</sup> This activation has been linked to the observed neurotropism in desmoplastic melanoma and pancreatic ductal adenocarcinoma among others. Narita *et al*<sup>25</sup> have described a significantly higher frequency of *RET*<sub>p</sub> in desmoplastic melanomas (61%) compared to nondesmo-

plastic melanomas (31%) analyzing *RET*<sub>p</sub> from tumor DNA, but Miller *et al*<sup>23</sup> have only detected *RET*<sub>p</sub> in 33% of pure and in 24% of mixed desmoplastic malignant melanoma. In the light of occasional, somatic, *de novo* *RET*<sub>p</sub> occurrences reported in pancreatic adenocarcinoma,<sup>27</sup> Bar *et al*<sup>28</sup> have investigated germline DNA for *RET*<sub>p</sub> in melanoma patients and found germline *RET*<sub>p</sub> in 30% of patients with desmoplastic melanomas, compared to 21% in nondesmoplastic malignant melanoma patients, but this difference was not significant at the investigated sample size. As *RET* codon 691 is not covered by the abovementioned hotspot panel, tumor DNA was subjected to Sanger sequencing for additional evaluation of this *RET* codon in order to encompass germline and possible somatic *RET* G691S occurrences.

## Materials and Methods

Twenty-three patients with histologically confirmed desmoplastic malignant melanomas were included in the study. Formalin-fixed paraffin-embedded tumor tissue of the primary tumor was retrieved from the archives of the Biobank of the Medical University of Graz, the Dermatopathologie Bodensee, Friedrichshafen, Germany, the Department of Dermatology and Venereology of the Medical University of Graz and from Austrian district hospital pathologies in three consultation cases. H&E and immunohistochemical slides were reevaluated to confirm the diagnoses by two independent pathologists (BL-A and SWJ) prior to inclusion in the study. Immunohistochemically, all tumors were positive for S100. When stratified for the presence of a desmoplastic tumor component of at least 90%, 13 desmoplastic malignant melanoma were classified as pure and 10 tumors were classified as mixed desmoplastic malignant melanomas. See Figure 1 for exemplary tumor morphology of cases analyzed.

Serial sections with first and last levels H&E stained for topographic reference were obtained. Unstained, intermediate sections were mounted on glass slides for selective manual microdissection with a scalpel. DNA was extracted on a Maxwell, MDx Research System (Promega, Fitchburg, Wisconsin, USA). In cases with limited tumor tissue content laser microdissection on a Veritas microdissection instrument (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to standard protocols was performed and DNA was purified using the QIAmp DNA mini kit (Qiagen, Hilden, Germany). Primers for selected target regions of 50 tumor-associated genes (Table 1), corresponding to 2855 COSMIC-annotated hotspot mutations are included in the Ion AmpliSeq Cancer Hotspot Panel v2 (Thermo Fisher Scientific). Library preparations were performed using the Ion AmpliSeq library kit 2.0 (Thermo Fisher Scientific). Sequencing was performed on an Ion PGM Sequencer (Thermo Fisher



**Figure 1** (a) Pure desmoplastic malignant melanoma with spindled tumor cells embedded in fibrotic stroma; inset (a) tumor cells with S100 reactivity (high power). (b) Mixed desmoplastic malignant melanoma with areas of spindle cells and desmoplastic stroma as well as nondesmoplastic areas with epithelioid tumor cells (inset (b), high power).

Scientific). Emulsion PCR and sequencing were performed with the appropriate kits (Ion One Touch Template Kit v2 and Ion PGM 200 sequencing kit, Thermo Fisher Scientific) multiplexing four or eight samples on 316 or 318 chips, respectively. Sequencing length was set to 500 flows and yielded reads ranging from 111 to 187 base pairs consistent with the expected size range; 350,000 to 500,000 reads were obtained for each sample with >80% of bases at AQ20.

### Data Analysis

Initial data analysis was performed using the Ion Torrent Suite Software Plug-ins (Thermo Fisher Scientific, open source, GPL, <https://github.com/iontorrent/>). Briefly, this included base calling, alignment to the reference genome (HG19) using the TMAP mapper and variant calling by a modified diBayes approach taking into account the flow space information. All called variants were annotated using open source software (ANNOVAR, <http://www.openbioinformatics.org/annovar/>;<sup>29</sup> SnpEff, <http://snpeff.sourceforge.net/><sup>30</sup>) and custom Perl scripts. All coding, nonsynonymous mutations were further evaluated and visually inspected in IGV

(<http://www.broadinstitute.org/igv/>) and variant calls resulting from technical read errors or sequence effects were excluded from the analysis. Mutations were subsequently assessed for biological relevance by computerized prediction inferred by a particular amino acid change. ‘Sorting intolerant from tolerant’ algorithm (SIFT) was used to generate SIFT-scores.<sup>31</sup> Amino acid substitutions with scores below 0.05 were interpreted as functionally relevant (prediction ‘damaging’), scores between 0.05 and 0.1 were regarded as possibly relevant (‘possibly damaging’) and scores >0.1 were regarded as irrelevant (‘functionally neutral’) by computerized prediction. Final assessment of biological relevance was interpreted in conjunction with data from the COSMIC database<sup>32</sup> (accessed November 2014) and literature search as explained in the text.

Sequence analysis for evaluation of codon 691 of *RET* exon 11 by Sanger sequencing was performed according to standard procedures from forward and reverse primer. PCR reactions were carried out in a final volume of 20  $\mu$ l with the HotStarTaq Plus Master Mix kit (Qiagen, Hilden, Germany), 0.4  $\mu$ mol/l primers (F-5′-GTCTGCCTTCTGCATCCA CT-3′, R-5′-CCTCACCAGGATCTTGAAGG-3′; VBC-Biotech Service GmbH, Vienna, Austria), PCR-grade water (Roche Diagnostics, Mannheim, Germany) and

25 ng tumor DNA. For purification and sequencing PCR products were sent to the VBC-Biotech Service GmbH. Sequence analysis was performed with the CodonCode Aligner sequence analysis software (CodonCode Corporation, Centerville, MA, USA).

SPSS Statistics 22, IBM, was used to calculate Fisher's exact tests for statistical analyses of *RET*<sub>p</sub> and mutation frequencies between pure and mixed desmoplastic malignant melanomas.

## Results

Twenty-three patients were included in the study. After generation of amplicon libraries, 21 of the 23 samples yielded sequencing raw data suitable for further analysis (12 pure and 9 mixed desmoplastic malignant melanoma from 14 male and 7 female patients). Two of the three laser microdissected samples yielded sequencing data unsuitable for further evaluation due to incomplete amplicon coverage and were excluded from further analysis. Tumors were located at the upper torso/arm in nine cases, in the face/at the head in eight cases and at the neck/shoulder in four cases. Median age at tumor presentation was 72 years (mean 70.9 years). Table 2 details histological type of desmoplastic malignant melanoma (pure/mixed), sex, patient age, tumor location, mutations detected, *RET*<sub>p</sub> status, presence of perineural invasion, histological evidence of UV damage and concomitant *in situ* lesions. All tumors were at least TNM stage pT4 with the exception of one case (case 12 in Table 2) that was pT3a. Nonsynonymous (amino acid changing) mutations were found in 14 out of 21 desmoplastic malignant melanomas (67%) with a total of 25 mutations detected (see Table 3 for details). All mutations were point mutations, comprising three stopgain mutations in *CDKN2A* and *TP53*. When subclassified into pure and mixed desmoplastic malignant melanoma 6 out of 12 (50%) pure desmoplastic malignant melanomas were devoid of mutations in our analysis. Pure desmoplastic malignant melanomas with mutations exhibited mutated tumor suppressor genes in five out of six cases mainly affecting *TP53* and *CDKN2A* as well as *SMAD4* in one case. Only one pure desmoplastic malignant melanoma displayed a mutated protooncogene (*PIK3CA*). In contrast, all but one mixed desmoplastic malignant melanoma were mutated with oncogenic mutations in two-thirds of cases. The observed difference of oncogenic mutations between pure and mixed desmoplastic malignant melanomas is statistically significant at  $P=0.016$  by Fisher's exact test and this difference is even more pronounced ( $P=0.0015$ ) under the assumption of the one *PIK3CA* double-mutation detected in pure desmoplastic melanoma being in fact a functionally irrelevant passenger mutation as explicated in the discussion. Activating mutations were found in *BRAF* (V600E and V600R), in *NRAS*, *FGFR2* (twice), and in *ERBB2*. Tumor

**Table 1** Genes included for hotspot mutation analysis in the Ion AmpliSeq Cancer Hotspot Panel v2, Thermo Fisher Scientific

ABL1	EZH2	JAK3	PTEN
AKT1	FBXW7	IDH2	PTPN11
ALK	FGFR1	KDR	RB1
APC	FGFR2	KIT	RET
ATM	FGFR3	KRAS	SMAD4
BRAF	FLT3	MET	SMARCB1
CDH1	GNA11	MLH1	SMO
CDKN2A	GNAS	MPL	SRC
CSF1R	GNAQ	NOTCH1	STK11
CTNNB1	HNF1A	NPM1	TP53
EGFR	HRAS	NRAS	VHL
ERBB2	IDH1	PDGFRA	
ERBB4	JAK2	PIK3CA	

suppressor gene mutations pertained to *TP53* in 4, *CDKN2A* in 1, *SMARCB1* in 1, and to *PTPN11* in 1 out of 9 mixed desmoplastic malignant melanomas. None of the tumors analyzed exhibited mutations in *KIT*, *GNAQ*, or *GNA11*. *RET* codon 691 status was retrieved in all but one case, which even on increased DNA input failed to amplify. Overall, *RET*<sub>p</sub> was seen in 30% of melanomas. *RET*<sub>p</sub> frequency was 25% for pure desmoplastic malignant melanomas and 38% for mixed desmoplastic malignant melanomas. Perineural invasion could be assessed in 19 out of 21 cases, while in two cases not enough peritumoral tissue was resected for evaluation. Seven tumors demonstrated perineural invasion of which three (43%) were *RET*<sub>p</sub>. Twelve cases lacked perineural invasion of which again three (25%) were *RET*<sub>p</sub>. No statistically significant difference was found between *RET*<sub>p</sub> frequencies of pure versus mixed desmoplastic malignant melanomas ( $P=NS$ ) at the given sample size.

## Discussion

Malignant melanoma is currently subtyped based on location and histomorphology. Analysis for mutations currently amenable to therapy (ie, *BRAF*, *NRAS*, and *KIT*) have revealed mutational patterns segregating with the traditional subgroups. Regarding desmoplastic malignant melanoma a complete absence of *BRAF* mutations<sup>21</sup> as well as a reduced mutation rate<sup>22</sup> has been reported. Recently, Miller et al<sup>23</sup> have observed a lack of *BRAF* mutations in pure desmoplastic malignant melanoma compared to a low frequency in the mixed subtype and a trend toward an increased *RET*<sub>p</sub> frequency in pure desmoplastic malignant melanoma. Kim et al<sup>24</sup> extended mutation analysis in desmoplastic malignant melanoma to *BRAF* and *KIT* reporting wild-type *BRAF* and *KIT* in all tumors investigated.

Evidence that activating *BRAF* and *NRAS* mutations do not represent the main driver mutations in desmoplastic malignant melanoma prompted us to search for other, yet unaccounted mutations that

**Table 2** Overview of clinical data and mutations detected

Case	Type of DMM	Sex	Age of patient (years)	Perineural infiltration	Solar elastosis	Associated in situ lesion	Site	Mutations detected (percentage of mutated reads)	RET G691 status in tumor
1	Pure	W	90	Yes	Yes	No	Cheek	<i>CDKN2A</i> (28%)	RETwt
2	Pure	M	76	Yes	No	Yes	Sternum (local recurrence)	<i>TP53</i> (25%)	RETwt
3	Pure	M	81	Yes	Yes	Yes	Neck	<i>TP53</i> (62%)	RETwt
4	Pure	M	85	No	Yes	No	Clavícula	<i>SMAD4</i> (13%), <i>CDKN2A</i> (19%), <i>TP53</i> (17%)	RETwt
5	Pure	M	72	No	Yes	Yes	Head (capillitium)	<i>CDKN2A</i> (12%), <i>TP53</i> (6%)	RETwt
6	Pure	W	73	Yes	NA	NA	Upper arm	<i>PIK3CA</i> (9%), <i>PIK3CA</i> (7%)	RETp
7	Pure	M	43	No	No	NA (no epidermis)	Neck	None	RETwt
8	Pure	M	82	Yes	Yes	No	Temple	None	RETwt
9	Pure	M	67	No	Yes	No	Shoulder	None	RETp
10	Pure	W	71	No	No	No	Supraorbital	None	RETwt
11	Pure	M	63	Yes	No	No	Cubita	None	RETp
12	Pure	M	85	No	Yes	Yes	Back	None	RETwt
13	Mixed	W	54	No	Yes	Yes	Upper arm	<i>BRAF</i> V600R (21%)	RETp
14	Mixed	M	86	No	No	NA (ulcerated)	Lower lip	<i>ERBB2</i> (20%), <i>CDKN2A</i> (32%), <i>TP53</i> (35%)	RETp
15	Mixed	M	47	No	Yes	NA	Shoulder	<i>BRAF</i> V600E (8%)	RETwt
16	Mixed	W	71	NA	NA	NA	Cheek	<i>TP53</i> (21%)	RETwt
17	Mixed	W	59	NA	NA	NA	Upper thorax	<i>FGFR2</i> (18%), <i>NRAS</i> (29%), <i>TP53</i> (33%)	F
18	Mixed	W	81	No	Yes	Yes	Head (capillitium)	<i>FGFR2</i> (40%), <i>PTPN11</i> (28%)	RETwt
18a	Desmoplastic component							<i>FGFR2</i> (26%), <i>PTPN11</i> (15%)	RETwt
18b	Nondesmoplastic component								RETwt
19	Mixed	M	66	No	Yes	Yes	Neck	<i>SMARCB1</i> (27%), <i>SMO</i> (18%), <i>TP53</i> (42%)	RETwt
19a	Desmoplastic component							<i>SMARCB1</i> (36%), <i>SMO</i> (12%), <i>TP53</i> (34%)	RETwt
19b	Nondesmoplastic component								RETwt
20	Mixed	M	58	Yes	Yes	No	Cheek	<i>BRAF</i> V600E (42%)	RETp
20a	Desmoplastic component							<i>BRAF</i> V600E (17%)	RETp
20b	Nondesmoplastic component								RETp
21	Mixed	M	78	No	No	Yes	Back	None	RETwt
21a	Desmoplastic component							None	RETwt
21b	Nondemoplastic component							None	RETwt

Abbreviations: F: failed DNA amplification; NA: not available for histological evaluation.

**Table 3** Details of mutations detected

Case	Type of DMM	Gene	Mutation: Amino acid change	Chromosome	Type of alteration	Percentage of mutated reads <sup>a</sup>
1	Pure	<i>CDKN2A</i>	NM_000077:c.T161A:p.M54K	9	Missense mutation	28
2	Pure	<i>TP53</i>	NM_001126115:c.A319G:p.N107D	17	Missense mutation	25
3	Pure	<i>TP53</i>	NM_001126115:c.G460A:p.E154K	17	Missense mutation	62
4	Pure	<i>SMAD4</i>	NM_005359:c.C572T:p.S191L	18	Missense mutation	13
4	Pure	<i>CDKN2A</i>	NM_000077:c.C238T:p.R80X	9	Stoppain mutation	19
4	Pure	<i>TP53</i>	NM_001126115:c.G404A:p.R135Q	17	Missense mutation	17
5	Pure	<i>CDKN2A</i>	NM_000077:c.C341T:p.P114L	9	Missense mutation	12
5	Pure	<i>TP53</i>	NM_001126115:c.C55T:p.P19S	17	Missense mutation	6
6	Pure	<i>PIK3CA</i>	NM_006218:c.A1034G:p.N345S	3	Missense mutation	9
6	Pure	<i>PIK3CA</i>	NM_006218:c.T249G:p.F83L	3	Missense mutation	7
13	Mixed	<i>BRAF</i>	NM_004333:c.GT1798-99AG:p.V600R	7	Missense mutation	21
14	Mixed	<i>ERBB2</i>	NM_004448:c.C2301G:p.I767M	17	Missense mutation	20
14	Mixed	<i>CDKN2A</i>	NM_000077:c.C172T:p.R58X	9	Stoppain mutation	32
14	Mixed	<i>TP53</i>	NM_001126115:c.C241T:p.R81X	17	Stoppain mutation	35
15	Mixed	<i>BRAF</i>	NM_004333:c.T1799A:p.V600E	7	Missense mutation	8
16	Mixed	<i>TP53</i>	NM_001126115:c.G443C:p.R148T	17	Missense mutation	21
17	Mixed	<i>FGFR2</i>	NM_001144914:c.C776T:p.A259V	10	Missense mutation	18
17	Mixed	<i>NRAS</i>	NM_002524:c.A182G:p.Q61R	1	Missense mutation	29
17	Mixed	<i>TP53</i>	NM_001126115:c.C76G:p.R26G	17	Missense mutation	33
18	Mixed	<i>FGFR2</i>	NM_001144914:c.C776T:p.A259V	10	Missense mutation	40 and 26
18	Mixed	<i>PTPN11</i>	NM_002834:c.C1472T:p.P491L	12	Missense mutation	28 and 15
19	Mixed	<i>SMARCB1</i>	NM_001007468:c.C536T:p.P179L	22	Missense mutation	27 and 36
19	Mixed	<i>SMO</i>	NM_005631:c.G625A:p.D209N	7	Missense mutation	18 and 12
19	Mixed	<i>TP53</i>	NM_001126115:c.T7C:p.C3R	17	Missense mutation	42 and 34
20	Mixed	<i>BRAF</i>	NM_004333:c.T1799A:p.V600E	7	Missense mutation	42 and 17

<sup>a</sup>Double values correspond to results of separate analyses of heterogeneous tumor areas in mixed desmoplastic malignant melanomas.

might be responsible for tumor development and potentially be amenable to therapeutic intervention. Data from 21 desmoplastic malignant melanomas (12 pure and 9 mixed) were retrieved by next generation amplicon sequencing for hotspot mutations in 50 cancer-related genes employing the IonAmpliSeq Cancer Hotspot Panel v2 (Thermo Fisher Scientific).

Two-thirds (67%) of pure desmoplastic malignant melanomas were devoid of mutations. Within the group of pure desmoplastic malignant melanomas only a single case demonstrated a mutated proto-oncogene (*PIK3CA*). The remaining pure DMMs displayed mutations in tumor suppressor genes only. The activating mutation present in that case was a *PIK3CA* double mutation, of which we regard one as an unequivocal passenger mutation (p.F83L, no COSMIC entries, SIFT score 0.6), while the other one (p.N345S, SIFT score 0.09—‘possibly damaging’) we view to be likely nonpathogenic as well, with only two COSMIC entries unconfirmed for somatic occurrence together with the fact that *PIK3CA* is a widely investigated gene. The remaining five pure desmoplastic malignant melanomas mutated displayed mutations in two recurrent tumor suppressor genes namely *TP53* and *CDKN2A*. The *SMAD4* mutation in case 4 appears to be a passenger mutation without biological relevance (no COSMIC entries, SIFT score 0.16). As *RET*<sub>p</sub> could only account for proliferative activation in 38% of pure desmoplastic malignant melanomas in our series, the absence of canonical activating mutations, even after analysis with an extended multigene tumor panel,

points to a still elusive proliferative stimulus in pure desmoplastic malignant melanoma. Irrespective of the activating pathogenic alterations driving these tumors, mutations in well-known tumor suppressor genes could be identified as relevant to neoplastic transformation in ~40% of pure desmoplastic malignant melanomas.

Results in mixed desmoplastic malignant melanoma were markedly different. All but one mixed desmoplastic malignant melanoma (89%) were mutated, frequently displaying oncogenic mutations of a spectrum similar to that seen in melanoma in general (Tables 2 and 3). The activating mutation spectrum encompassed *BRAF* V600 E and R, *NRAS*, the receptor tyrosine kinases *FGFR2*, *ERBB2* as well as a passenger mutation in the protooncogene *SMO* (no COSMIC database entry, SIFT score 0.69). *ERBB2* and *FGFR2* mutations are seen in malignant melanoma, but have not yet been reported in desmoplastic malignant melanoma with the exception of a single case of an *FGFR2* mutation in a desmoplastic malignant melanoma with additional sarcomatoid dedifferentiation.<sup>33</sup> The two *FGFR2* mutations seen in cases 17 and 18 are identical. This mutation (p.A259V) is a confirmed somatic mutation in metastatic renal cell carcinoma<sup>34</sup> with a SIFT score of 0.07 and we regard it as a plausible, activating driver mutation. Finally, the *ERBB2* mutation in case 14 is a previously confirmed somatic oncogenic mutation (SIFT score of 0) in breast, ovarian, and bladder carcinoma.<sup>35–37</sup> The observed *BRAF* mutation frequency in mixed desmoplastic malignant

melanomas at 30% is above what we expected from the literature<sup>38</sup> with Miller *et al*<sup>23</sup> reporting a 6% BRAF mutation rate in mixed desmoplastic malignant melanoma (but an absence of BRAF mutations in pure desmoplastic malignant melanoma in accordance with our data). The higher percentage of BRAF mutations in the present study in mixed desmoplastic malignant melanoma might be explained by an increased sensitivity due to manual microdissection of tumors. Desmoplastic tumor areas usually present with decreased cellularity owing to intermixed fibrosis as illustrated by one BRAF V600E mutations at a frequency at 8% of alleles (Table 2), which most likely would have been missed without microdissection. Regarding RET codon 691 status, 38% of mixed desmoplastic malignant melanomas were RET<sup>p</sup> and interestingly all three also displayed concomitant canonical activating mutations in BRAF and ERBB2.

Tumor suppressor gene mutations were found in five mixed desmoplastic malignant melanomas, namely in TP53, CDKN2A, and two tumor suppressor genes not yet reported in desmoplastic malignant melanoma: SMARCB1 and PTPN11. The mutation in SMARCB1 (case 19) is a confirmed somatic mutation in malignant melanoma<sup>39</sup> with a SIFT score of 0, clearly arguing for biological relevance. SMARCB1 is a highly conserved subunit of the SWI/SNF ATP-dependent chromatin-remodeling complex. Its functions include facilitation of UV-induced DNA damage repair and regulatory activity of transcription and cell cycle progression.<sup>40</sup> In accordance with its tumor suppressor function immunohistochemical loss of SMARCB1 expression has been reported as an independent prognostic factor of reduced survival in malignant melanoma.<sup>41</sup> Lastly, the PTPN11 mutation in case 18 is an unequivocal, confirmed somatic mutation (SIFT score 0.04) previously observed in a melanoma<sup>42</sup> and in acute lymphoblastic leukemia.<sup>43</sup>

Of note the mutation panel employed in our study also included hotspot mutation analysis for GNAQ and GNA11 two G-protein subunits leading to constitutive activation of the ERK signalling cascade. Either of these two G-protein subunits was found to be mutated in over 80% of uveal melanomas, which are almost exclusively BRAF and NRAS wild type.<sup>18,44,45</sup> To the best of our knowledge this is the first study to extend GNAQ and GNA11 mutation analysis to desmoplastic malignant melanoma and our data provide evidence that tumorigenesis in desmoplastic malignant melanoma (whether of pure or mixed form) is not driven by GNAQ and GNA11 mutations.

In addition, we explored mutational heterogeneity in mixed desmoplastic malignant melanomas by separate analysis of desmoplastic and nondesmoplastic tumor areas. In four mixed desmoplastic malignant melanomas these areas were sufficiently separated to allow for selective manual microdissection. In all four cases identical mutation patterns

were seen in both components, and RET polymorphism at codon 691 was identical as well.

Clinically, desmoplastic malignant melanoma as a whole is associated with a favourable prognosis, and a significantly reduced rate of lymph node metastases and this prognostic advantage has specifically been linked to the pure form. Safae Ardekani *et al*<sup>46</sup> have demonstrated a 1.7-fold reduction of overall survival in patients suffering from BRAF-mutated melanomas in their comprehensive meta-analysis. This clinical observation correlates well with the molecular findings of our study demonstrating activating mutations in the vast majority of mixed desmoplastic malignant melanomas but none in pure desmoplastic malignant melanoma. The activating mutations detected essentially all converge on the same pathways, namely activation of the MAP-kinase pathway (BRAF and RET), and additional induction of the PIK3/Akt pathway in the case of FGFR2, ERBB2, and RET. Furthermore, our study provides evidence that mixed desmoplastic malignant melanomas are homogeneous regarding their mutation profile and RET<sup>p</sup> status. RET<sup>p</sup> was detected at about the same frequency as in the general healthy population but may explain the proliferative stimulus in a subset (25% in our study) of pure desmoplastic malignant melanomas devoid of canonical oncogenic mutations and may add to or modify tumor proliferation and neurotropism in tumors with already a baseline oncogenic drive, as seen in a subset of our mixed desmoplastic malignant melanomas.

Our study thus underscores the validity of subtyping desmoplastic malignant melanoma into pure and mixed forms from a molecular perspective. Lack of canonical oncogenes in pure desmoplastic malignant melanoma might in principle be attributable to inherent limitations of a restricted hotspot mutation analysis. Specifically, complex mutations of potential relevance such as activating translocations of the anaplastic lymphoma kinase gene (ALK) have recently been described in KIT- and BRAF-negative mucosal melanomas.<sup>47</sup> Alternatively, oncogenes amplified by copy number changes (eg, CCND1 and MYC1) were shown to exhibit site-specific frequencies in malignant melanoma.<sup>48</sup> Regarding clinical management the dichotomy between pure and mixed desmoplastic malignant melanoma further extends to prognosis as previously reported and limits therapy options in pure desmoplastic malignant melanoma. In conclusion, especially pure desmoplastic malignant melanoma appears to be the desmoplastic malignant melanoma subentity in need for further investigation regarding additional molecular driver mechanism and structural as well as numeric genome analysis to elucidate the oncogenic changes propagating tumor growth in this special melanoma subtype.

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## Disclosure/conflict of interest

The authors declare no conflict of interest.

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