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Mixed *versus* pure variants of desmoplastic melanoma: a genetic and immunohistochemical appraisal

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Desmoplastic melanoma is subclassified into pure and mixed variants with a higher rate of lymph node metastasis in the latter. Given that reasons for these biological differences are not currently known, we investigated these subtypes with techniques that included genetic and immunohistochemical analyses of 43 cases of desmoplastic melanoma (24 pure, 19 mixed). Direct DNA sequencing was performed on *BRAFV600E*, *RET* gene (coding region on exon 11) and *KIT* (exons 11, 13 and 17). Immunohistochemical stains were performed with antibodies to markers of significance with respect to biological potential of nevomelanocytic proliferations and/or desmoplastic melanoma (Ki-67, CD117, nestin, clusterin, SOX10 and CD271/p75NTR). Polymorphism at the *RET* coding region (*RETp*) was noted in 33% of pure (8/24 cases) *versus* 24% of mixed (4/17 cases); *BRAFV600E* was absent in all cases of pure (0/24 cases) *versus* 6% of mixed (1/17 cases); no mutations were found in any of the cases on analyses of exons 11, 13 and 17 of the *c-KIT* gene (P = NS for all). For immunohistochemical analyses of pure *versus* mixed: mean percentage of Ki-67 nuclear positivity was 5% (s.d. = 5.6) *versus* 28% (s.d. = 12.6, $P < 0.001$); CD117 stained 26% (6/23 cases) *versus* 78% (14/18 cases, $P < 0.01$); nestin stained 83% ($n = 19/23$ cases) *versus* 89% (16/18 cases, P = NS); clusterin stained 4% (1/23 cases) *versus* 6% (1/18 cases, P = NS); SOX10 87% (20/23 cases) *versus* 94% (17/18 cases, P = NS) and CD271 stained 61% (14/23 cases) *versus* 67% (12/18 cases, P = NS). Increased CD117 staining in the mixed variant suggests that alterations in the *KIT* protein may be involved in tumor progression. In addition, the proliferative index of the mixed variant was higher than that of the pure variant.
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Desmoplastic melanoma is a rare variant of invasive cutaneous melanoma, with an annual incidence rate of approximately 2 per 1 000 000.[1] Features unique to this melanoma type include delayed diagnosis, deep invasion, increased perineural invasion and local recurrence.[2,3] George *et al*[4] recently reviewed 87 cases of desmoplastic melanoma and identified a significant difference in propensity for regional lymph node metastasis in the variant composed of a mixed population of cells, that is, cells resembling conventional melanoma in addition to spindled cells, compared with the variant composed predominantly of spindled cells, lending credence to the concept of subclassifying desmoplastic melanoma into mixed and pure subtypes.

Although several molecular pathways are important in the tumorigenic progression of melanoma, the *RAS/MAP* kinase pathway appears to be the most important in conventional types. Approximately 60% of conventional melanomas have *BRAF* mutations, but genetic profiling studies indicate that desmoplastic melanoma does not exhibit mutations

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in *BRAF* to this extent.[5–9] Recently, the frequency of a polymorphism of RET, a receptor tyrosine kinase whose ligand is glial cell line-derived neurotrophic factor (GDNF), at codon G691S has been shown to be significantly increased in desmoplastic *versus* conventional melanoma, a finding which in part explains the more prominent neurotropism seen in the desmoplastic variant.[10] Gene expression profiling has also demonstrated multiple genetic differences between desmoplastic and conventional melanoma; many of these differences cluster to specific phenotypic families, including melanin pigment synthesis and neurotrophic factors.[11]

Although several studies have demonstrated the genetic and immunohistochemical profile of desmoplastic melanoma, the histopathological subtypes (mixed and pure) have not been characterized in this manner. In the current study, we sought to characterize these two subtypes genetically and immunohistochemically. Gene sequencing studies included analyses of *RET*, *BRAF* and *KIT* genes. Rationale for the investigation of *RET* and *BRAF* was based on previous results described above, in which these two genes have different expression in conventional and desmoplastic melanoma; the initial hypothesis was that they may also be differentially expressed in the pure and mixed desmoplastic subtypes. *KIT* was investigated on the basis of the rationale that melanomas lacking *BRAF* mutations may have mutations or copy number alterations in *c-KIT*.[12] Immunohistochemical markers included the following: nestin, an intermediate filament protein, a melanocyte stem cell marker and a marker for melanoma-initiating cells, associated with poor prognosis in conventional melanoma;[13] clusterin, an 80-kDa glycoprotein of undefined biological significance shown to be significantly increased in desmoplastic melanoma;[11] SOX10, a neural crest stem cell marker, expressed in melanocytes, specifically, strongly expressed by desmoplastic melanomas;[14] CD117, a stem cell growth factor receptor encoded by the *KIT* gene, a receptor tyrosine kinase involved in cell survival and proliferation of melanocytes; CD271 (p75NTR), a neural crest stem cell factor, which has been shown to correlate with higher metastatic potential and worse clinical outcomes in melanoma;[15] and Ki-67 protein, a marker of cellular proliferation, which is expressed during of all phases of cell cycling (G1, S, G2 and mitosis), but absent during the resting phase.[16]

Materials and methods

This study was approved by the Institutional Review Board of Boston Medical Center (H-30085). Reports of cases from the Skin Pathology Laboratory, Boston University School of Medicine, Boston, MA, between 2000 and 2010, with a diagnosis of desmoplastic melanoma were perused and 43 cases (24 pure and 19 mixed desmoplastic melanomas) identified as meeting criteria for inclusion in this study with sufficient material for genetic and immunohistochemical analyses. Archival material from all 43 cases was retrieved. Histological sections of all cases were re-reviewed and the diagnoses confirmed. All patient data were de-identified. Tissue samples used were not selected for outcome measurements; hence, annotations regarding patient clinical data including follow-up/outcome are not included.

Genetic Analyses

DNA was extracted by proteinase K digestion of formalin-fixed, paraffin-embedded archival tissue. Direct DNA sequencing was performed on the *BRAF* gene (forward gene coding strand only) spanning codon 600. *RET G691S* functional polymorphism was detected by direct DNA sequencing on the RET coding region on exon 11 with the forwarding primer. Sequencing of the *KIT* gene was performed using forward and reverse primers for coding regions of exons 11, 13 and 17 as described previously.[17,18] Codon regions studied included the following: for exon 11, regions 550–591 (forward: 5′-TCCAGAGTGCTCTAATGAC-3′, reverse: 5′-AGGTGGAACAAAACAAAGG-3′), for exon 13, regions 627–664 (forward: 5′-TACTGCATGCGCTTG ACATC-3′, reverse: 5′-CCAAGCAGTTTATAATCT AGC-3′) and for exon 17, regions 788–828 (forward: 5′-GTGAACATCATTCAAGGCGT-3′, reverse: 5′-CCT TTGCAGGACTGTCAAGCA-3′). Sequencing data were collected with ABI genetic analyzer 3130 and analyzed with ABI sequencing analysis software v5.3.1. For both *BRAF* and *RET* sequencing, the reagents were ABI BigDye TerV3.1 cycle sequencing terminator ready reaction kit. Sequencing reactions were performed on an ABI 9700 thermocycler utilizing the ABI recommended protocol (Applied Biosystems, Foster City, CA, USA). A positive and/or negative control was included in each batch of sequencing analysis.

Immunohistochemical Analyses

Sections of 5μm thick were obtained for immunohistochemical studies, which were performed on formalin-fixed, paraffin-embedded tissue, using standard peroxidase immunohistochemical techniques, heat-induced epitope-retrieval buffer and primary antibodies against Ki-67 (clone MIB-1; 1:150; Dako, Carpinteria, CA, USA), CD117 (NB120-956; 1:50; Novus Biologicals, Littleton, CO, USA), clusterin (anti-clusterin alpha chain, clone 41D; 1:150; Millipore, Billerica, MA, USA), nestin (MAB5326, 1:200, Millipore, Temecula, AZ, USA), SOX10 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and CD271 (p75NTR; clone EP1039Y, 1:100, Biocare Medical, LLC, Concord,

CA, USA). Appropriate positive and negative controls were included. All stained slides were reviewed and scored by a dermatopathologist (MM) in a blinded fashion, to ensure consistency of interpretation.

Ki-67 nuclear reactivity was scored as a percentage on the basis of a manually performed 500-cell count in the most densely staining area in the dermis per previously established criteria.[19] For CD117, nestin, clusterin, SOX10 and CD271 (p75NTR), cut-off values for scoring were the following: negative or $0 = <10\%$, $1 = 11–25\%$, $2 = 26–50\%$ and $3 = >50\%$. For purposes of statistical analyses, cases with 11% or more were considered positive.

Statistical Analysis

For analysis of direct DNA sequencing results, Fischer's exact test was used to compare categorical variables. For the results of immunostaining with Ki-67, CD117, Sox10 and p75NTR, Wilcoxon rank sums were used to compare means and s.d. of Ki-67 proliferation rates. Fisher exact test was used to compare the proportions of positivity to CD117, Sox 10 and p75NTR. All statistical analyses were performed with Statistical Analysis Software (SAS) version 9.2 (SAS Institute, Cary, NC, USA), and a *P*-value of less than 0.05 was considered statistically significant.

Results

Clinical Data

The demographics of the patients with pure and mixed desmoplastic melanoma are listed in Table 1 and compared in Table 2. Briefly, 22 patients were male and 19 were female, with no significant difference in gender distribution between the two

Table 1 Demographics of patients with pure (Cases 1–23) and mixed (Cases 24–41) desmoplastic melanoma

Case number	*Subtype*	*Age*	*Sex*	*Site*	*Clinically pigmented*
1	Pure	68	F	R lower leg	Yes
2	Pure	47	M	R cheek	Yes
3	Pure	81	M	Sternal notch	Yes
4	Pure	62	F	R eyebrow	No
5	Pure	65	F	L orbital rim	No
6	Pure	67	M	L great toe	Unknown
7	Pure	71	F	R post arm	Yes
8	Pure	55	F	L upper back	Yes
9	Pure	64	M	Back	Yes
10	Pure	45	M	R chest	No
11	Pure	74	M	R lat arm	No
12	Pure	75	F	L nasal sidewall	No
13	Pure	68	M	L ant neck	Yes
14	Pure	82	M	Scalp	No
15	Pure	85	M	L cheek	Yes
16	Pure	80	M	L temple	No
17	Pure	81	M	L hip	Yes
18	Pure	70	F	L dorsal hand	No
19	Pure	64	F	R arm	Yes
20	Pure	91	M	Vertex scalp	Yes
21	Pure	77	F	L cheek	Yes
22	Pure	67	M	Back	No
23	Pure	82	M	R cheek	No
24	Mixed	91	M	Vertex scalp	No
25	Mixed	67	M	L cheek	Yes
26	Mixed	79	F	R forearm	No
27	Mixed	61	F	L upper back	No
28	Mixed	63	M	R preauric	Yes
29	Mixed	84	M	Mid-forehead	No
30	Mixed	38	M	L lat cheek	No
31	Mixed	79	F	L leg	Yes
32	Mixed	71	F	L arm	Yes
33	Mixed	16	F	R ear	No
34	Mixed	62	M	L abdomen	Yes
35	Mixed	83	F	L shin	No
36	Mixed	92	F	Post scalp	No
37	Mixed	92	F	Post scalp	No
38	Mixed	92	F	R distal arm	Yes
39	Mixed	73	M	R forearm	No
40	Mixed	77	M	R ant scalp	Unknown
41	Mixed	61	F	L shoulder	No

study groups ($P=0.35$). Patients with both subtypes each had an average age of 71 years at the time of diagnosis, and in both tumors presented in predominantly sun-exposed sites. A total of 20 of 41 were located on the head and neck, 13 of 41 on extremities, and 8 of 41 on the trunk; no significant difference in distribution was observed between the two groups. When available, data regarding clinically apparent pigmentation was recorded; 12 of 22 pure-type tumors were clinically suspected to be pigmented lesions, *versus* 6 of 17 mixed desmoplastic melanomas ($P=0.33$).

Histopathological Evaluation

Tumors were classified as mixed desmoplastic melanoma according to the criteria established in multiple previous studies,[4,20,21] in which the tumor contained two morphologically distinct populations of melanocytes with at least 10% of cells resembling conventional melanoma. Pure desmoplastic melanoma was defined as a predominantly spindled-cell tumor, with >90% of tumor cells consistent with the typical desmoplastic melanoma morphology. Forty-three cases of desmoplastic melanoma were found in our records; of these, two cases represented re-excisions after initial biopsies, which were also identified on initial blinded search. Thus, a total 41 cases were included in the histological comparisons, of which 18 were classified as mixed (44%) and 23 as pure desmoplastic melanoma (56%). Histopathological features of the two groups are compared in Table 2. Average Breslow depth (for tumors not transected at their deep margin) was 4.2 mm; mixed-type tumors had an average depth of 5.5 mm ($n=11$ cases), whereas pure-type tumors averaged 3.74 mm ($n=19$ cases; $P=0.76$). Of 41 tumors, 28 contained a junctional component and 21 were pigmented microscopically, with no significant difference noted for the two groups for either of these features. A statistically significant difference was observed for mitotic rate in the two groups; pure-subtype cases had an average of 1.6 mitoses per mm^2, *versus* 3.73 per mm^2 for mixed desmoplastic melanomas ($P=0.045$).

Table 2 Comparison of clinical and selected microscopic features of the two study groups. Only mitotic rate was significantly different between the two groups

	All cases (n = *41*)	*Pure* (n = *23*)	*Mixed* (n = *18*)	P-*value*
Average age	71	71	71	0.59
Male gender	22	14	8	0.36
Clinically pigmented[a]	18	12	6	0.33
Breslow depth (mm)	4.2	3.74[b]	5.5[c]	0.76
Junctional component	28	14	14	0.32
Pigmentation	21	12	9	1
Neurotropism	26	12	14	0.11
Mitotic rate (per mm^2)	2.54	1.6	3.73	0.045

[a]For one case from each subgroup, presence of pigmentation clinically was unknown.
[b]A total of 4 of 23 specimens were transected at their deep margin and were excluded.
[c]A total of 7 of 18 specimens were transected at their deep margin and were excluded.

Genetic Analyses

Polymorphism at the RET Coding Region (RETp)
The results of genetic analysis and immunohistochemical staining are summarized in Table 3 and examples are shown in Figures 1 and 2. For genetic analysis, a total of 41 of the 43 initially identified cases were analyzed; for two of the initial group (both of the mixed subtype), the DNA extracted was suboptimal, precluding genetic analysis. In all, 12 of 41 of these cases (29%) exhibited *RETp* mutations. Of 24 cases of pure desmoplastic melanoma, 8 (33%) exhibited the G691S *RETp*, whereas in 16 (67%), the polymorphism was absent. In 17 cases of mixed desmoplastic melanoma, *RETp* was present in 4 (24%) and absent in 13 (76%) cases; there was no statistically significant difference between the two groups ($P=$NS).

BRAF
Of 41 cases tested, only 1 (2%) exhibited BRAFV600E mutation; this was 1 of the 17 mixed-subtype cases (6%). Of note, this case was negative for *RETp*. No pure-subtype cases exhibited *BRAF* mutation. There was no statistically significant difference between the two groups in this respect ($P=$NS).

KIT
No mutations were identified in any of the 41 cases on analysis of exons 11, 13 and 17 of the *c-KIT* gene. Testing revealed wild-type genotype in all instances for all cases.

Table 3 Comparison of genetic and immunohistochemical profiles of the two groups

	All cases (n = *41*)	*Pure* (n = *24*)	*Mixed* (n = *17*)	P-*value*
Genetic analysis				
RET polymorphism	12	8	4	NS
BRAFV600E	1	0	1	NS
c-KIT mutation	0	0	0	NS
	All cases (n = *41*)	*Pure* (n = *23*)	*Mixed* (n = *18*)	P-*value*
Immunohistochemistry				
Ki-67%	15	5	28	<0.0001
Positive CD117	20	6	14	0.002
Positive nestin	35	19	16	NS
Positive Sox-10	37	20	17	NS
Positive clusterin	2	1	1	NS
Positive CD271	26	14	12	NS

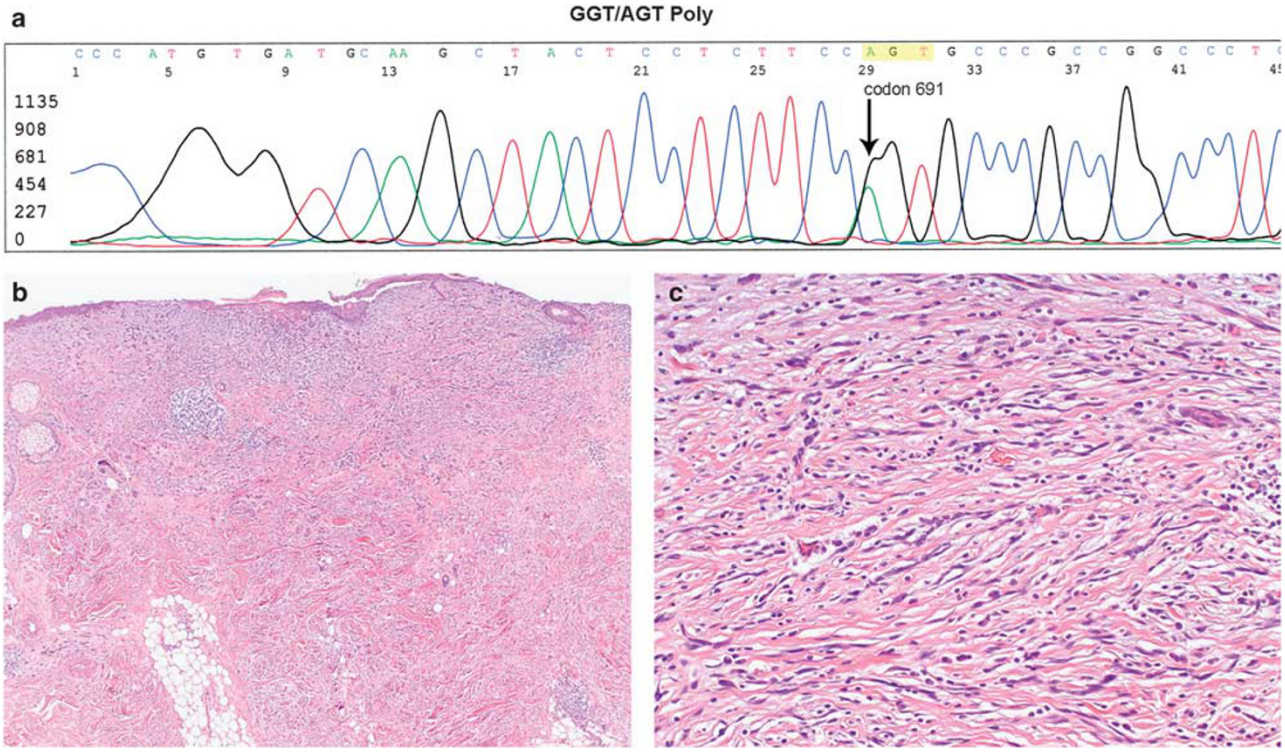

Figure 1 Pure desmoplastic melanoma (Case 16). (**a**) Results of direct DNA sequencing of exon 11 of the *RET* coding region, demonstrating *RETp* at codon 691 (GGT/AGT). (**b**) Hematoxylin–eosin stained section showing a diffuse infiltrate of spindled cells filling and expanding the dermis (low power). (**c**) High-power view, demonstrating a monomorphous spindle cell population (high power).

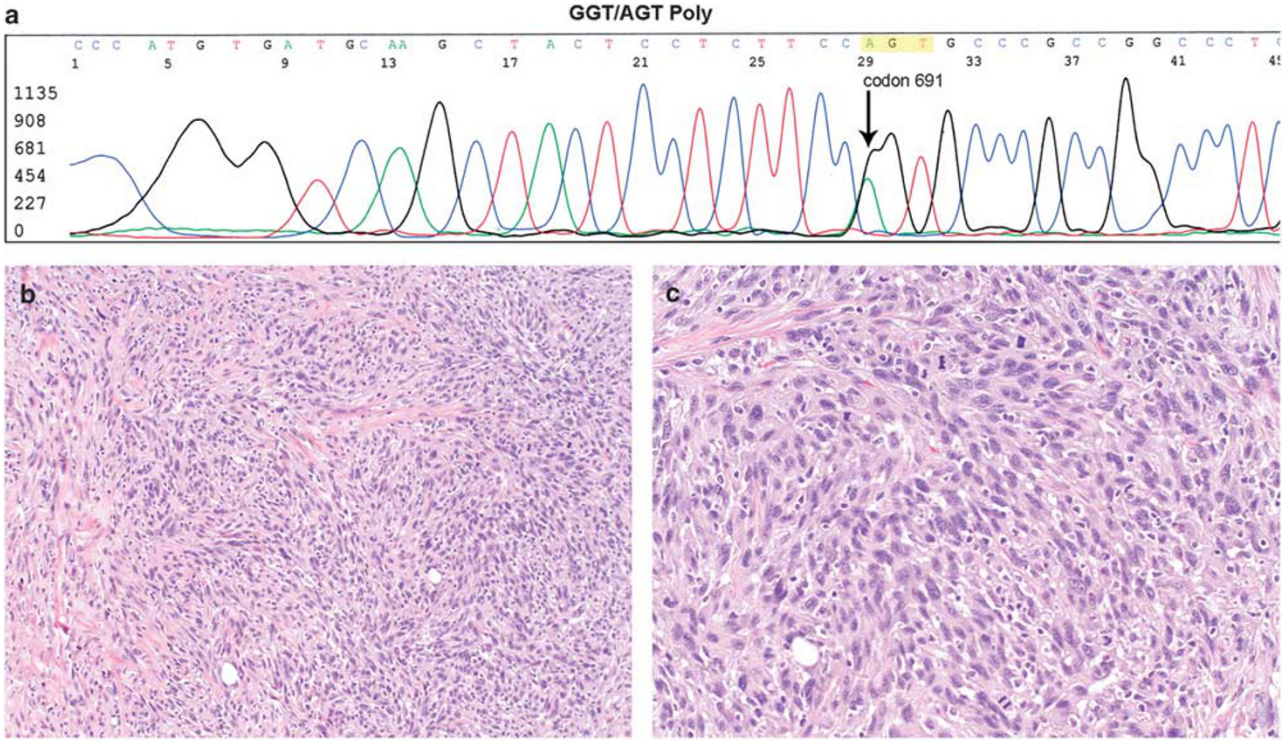

Figure 2 Mixed desmoplastic melanoma (Case 27). (**a**) Results of direct DNA sequencing, also with *RETp* at codon 691 (GGT/AGT). (**b**) Hematoxylin–eosin stained section demonstrating a mixed population of spindled and epithelioid melanocytes (low power). (**c**) High-power view, with a dense epithelioid cell population and multiple mitotic figures (high power).

Immunohistochemical Analyses

Ki-67

Positive staining was noted by ascertaining positive nuclear staining, and any cytoplasmic staining was considered background artifact. Positive staining of cells in the basal layer of the epidermis and the follicular epithelium in cases where the same was visualized served as positive internal control. Mean percentage of Ki-67 nuclear positivity was 5%

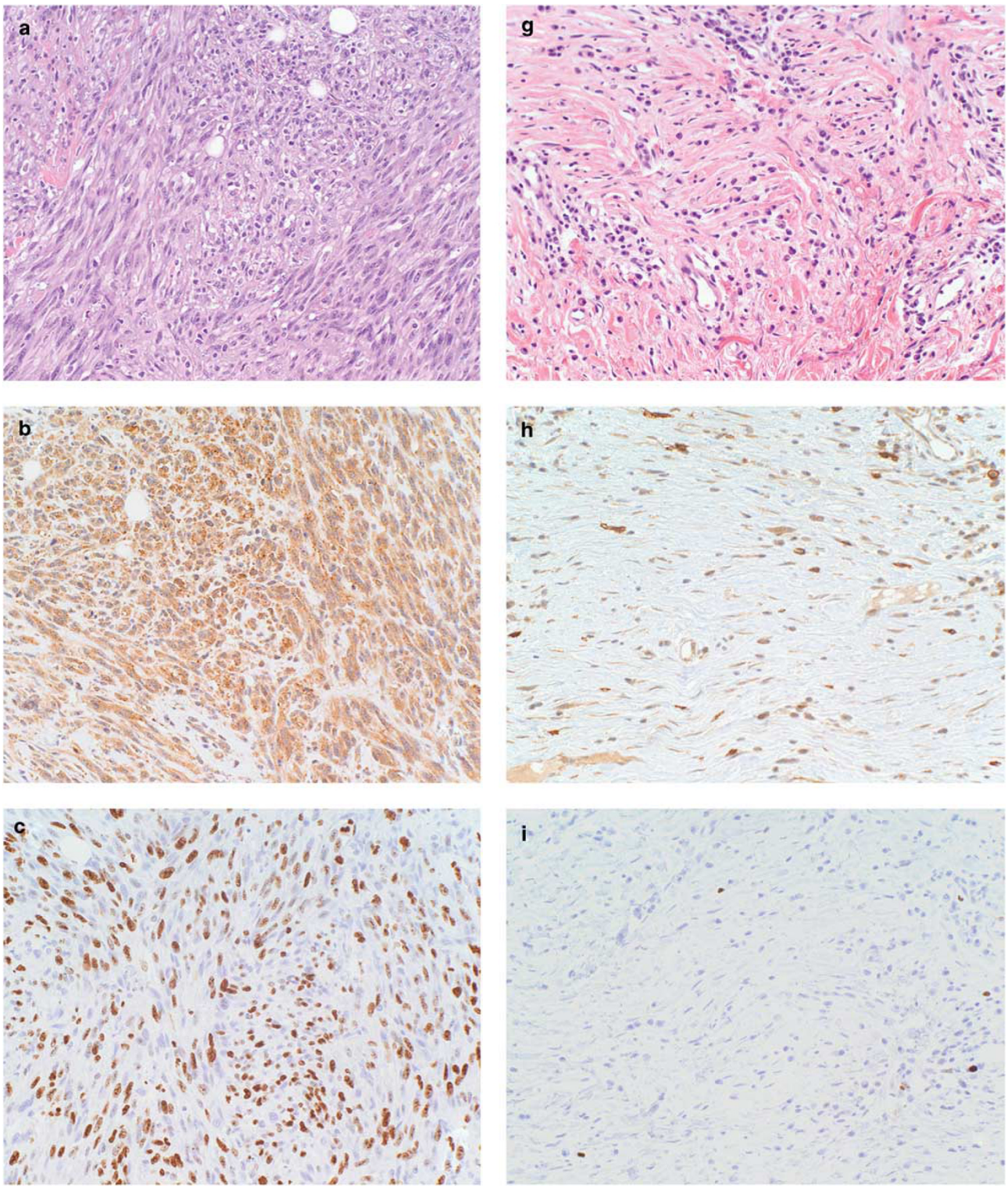

Figure 3 For Figure caption see next page.

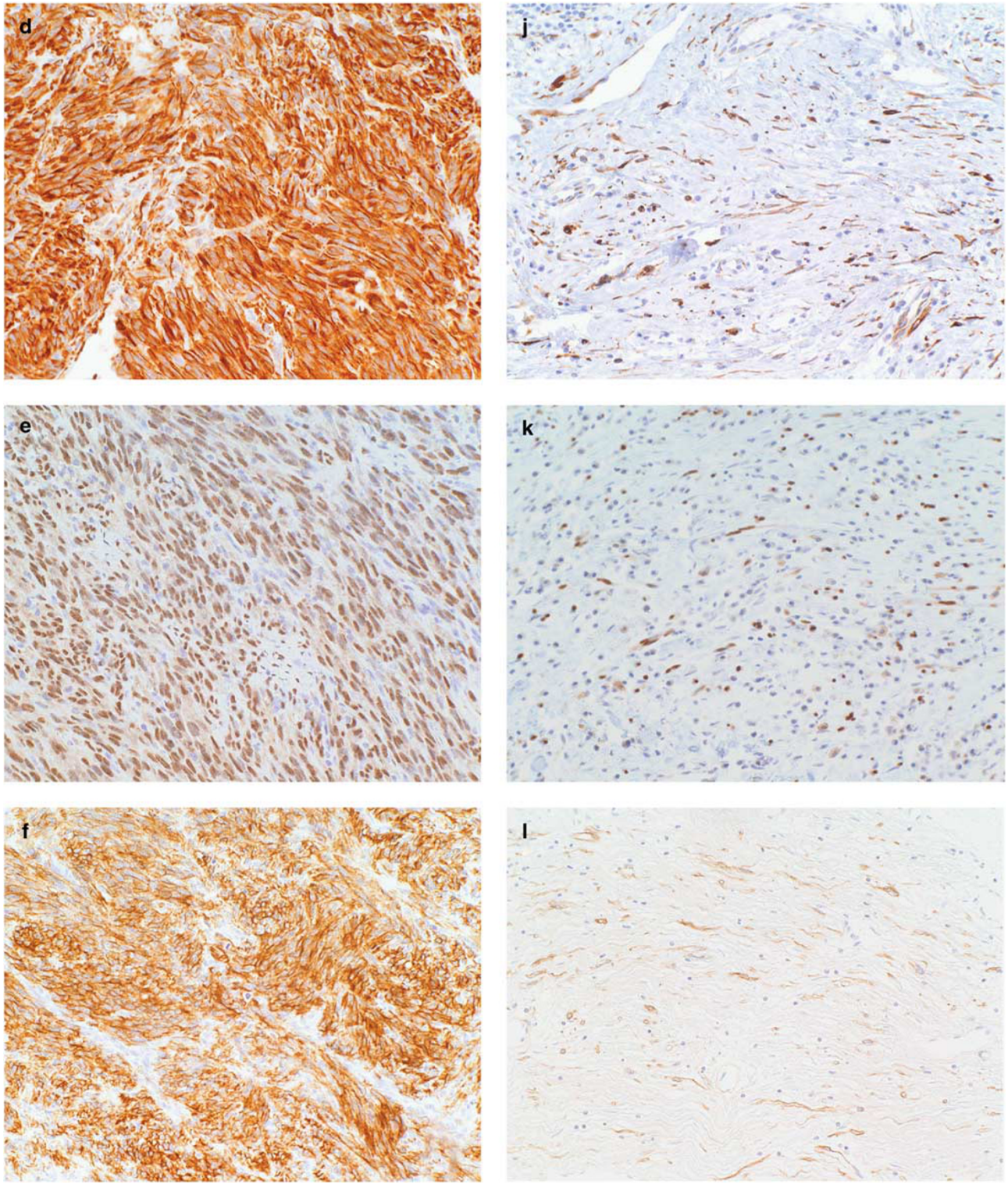

Figure 3 Immunohistochemical results of representative samples of mixed (**a–f**, Case 41) and pure (**g–l**, Case 22) desmoplastic melanoma. (**a**, **g**) Hematoxylin–eosin stained sections of mixed and pure tumors, respectively. (**b**, **h**) CD117 staining, with positive staining of both epithelioid and spindled cells in mixed desmoplastic melanoma. (**c**, **i**) Ki-67 staining. Proliferative index is markedly increased in the mixed-type case. (**d**, **j**) nestin, (**e**, **k**) SOX10 and (**f**, **l**) CD271 immunostaining, respectively.

(s.d. = 6) in the pure subtype, *versus* 28% (s.d. = 12) in mixed-subtype tumors ($P<0.001$). Figure 3 illustrates examples of immunohistochemical profiles of each tumor subtype.

CD117

Positive staining was noted by ascertaining cytoplasmic expression and any nuclear staining was considered the background artifact. Positive staining

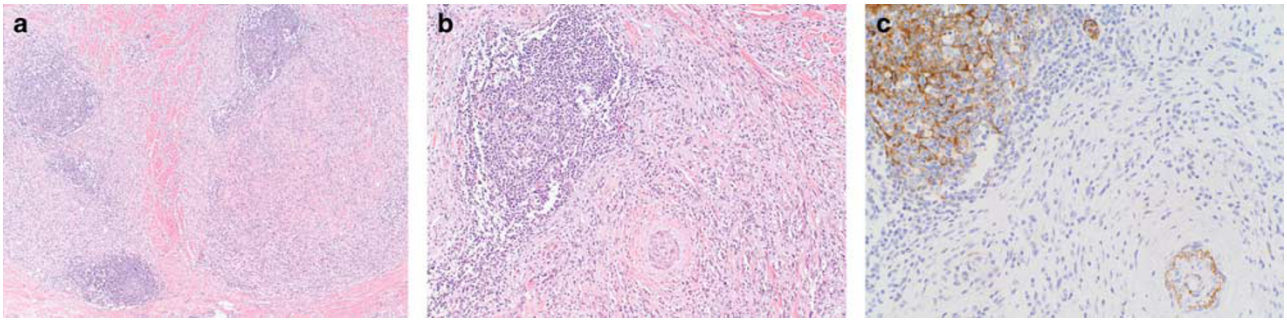

Figure 4 Mixed desmoplastic melanoma (Case 41), hematoxylin–eosin stained section (**a**; low power). (**b**) Focus of lymphocytic inflammation with germinal center formation (medium power). (**c**) Clusterin immunohistochemical stain, highlighting follicular dendritic cells, but without staining of lesional melanocytes (high power).

of melanocytes in the basal layer of the epidermis, basal layer of sebaceous glands, eccrine glands and mast cells (when present) served as the positive internal control. CD117 stained 26% (6 of 23 cases) of pure- *versus* 78% (14 of 18 cases) of mixed-subtype cases ($P<0.01$), with equal intensity staining of both cell populations in the positive mixed-subtype cases.

Nestin
Positive staining was noted by ascertaining cytoplasmic expression and any nuclear staining was considered the background artifact. Positive staining of endothelial cells and/or in the inner aspect of the outer root sheath (below attachment of the pilar muscle) in cases where the same was visualized served as the positive internal control. Nestin stained 83% (19/23) of pure- *versus* 89% (16/18) of mixed-subtype cases ($P=$NS).

Clusterin
Positive staining of elastic tissue fibers and follicular dendritic cells in cases where the same was visualized served as the positive internal control (Figure 4). Clusterin stained 4% (1/23) of pure- *versus* 6% (1/18) of mixed-subtype tumors ($P=$NS).

SOX10
Positive staining was noted by ascertaining nuclear expression and any cytoplasmic staining considered the background artifact. Positive staining of melanocytes in the basal layer of the epidermis and follicular epithelium in cases where the same was visualized served as the positive internal control. SOX10 stained 87% (20/23) of pure- *versus* 94% (17/18) of mixed-subtype tumors ($P=$NS).

CD271 (p75NTR)
Positive staining was noted by ascertaining cytoplasmic expression and any nuclear staining considered the background artifact. Positive staining of nerve fascicles, eccrine glands and the follicular epithelium (outer root sheath) in cases where the same was visualized served as the positive internal control. CD271 stained 61% (14/23) of pure- *versus* 67% (12/18) of mixed-subtype tumors ($P=$NS).

Discussion

Desmoplastic melanoma is an uncommon variant of cutaneous malignant melanoma; various estimates place its frequency between 1 and 4% of all diagnosed melanomas.[21–23] Although desmoplastic melanoma was first characterized in the medical literature in 1971,[2] only recently (2004) has the concept of two morphological subtypes been put forth.[20] Owing to the rarity of desmoplastic melanomas in general and this nascent classification system, the relative incidence of the two subtypes is not well established. Mixed desmoplastic melanoma appears to constitute between 40 and 60% of all cases obtained from histopathological archives, an estimate that appears to concur with findings from the current study in which 44% of cases (18/41 cases) were of the mixed subtype.[4,22,24]

The increased propensity for regional nodal metastasis of the mixed subtype has been previously established in several studies.[4,25,26] In one study of approximately equal numbers of cases of pure and mixed desmoplastic melanomas, regional lymph node involvement was notably higher in the mixed subgroup (14 *vs* 3%; $P=0.02$), and overall 5-year survival was significantly worse in this variant (61 *vs* 80%; $P=0.001$).[27] Furthermore, in the pure subtype cases, local recurrence was usually the first event, but in mixed desmoplastic melanoma, distant metastasis was usually the first event, without a preceding history of local recurrence.[27] In another case series, systemic metastases appeared in 4 of 29 mixed desmoplastic melanoma patients (14%) *versus* 1 of 26 with a pure-type tumor (4%), and 5 patients with mixed-type tumors had disease-related death *versus* 2 with the pure type (although the authors did make note of the fact that the small sample size precluded any conclusions being drawn about the significance).[22] In yet another series of patients with metastatic desmoplastic melanoma, the mixed subtype (hazard ratio 6.17; CI 1.61–23.81;

$P = 0.019$) was an independent predictor of poorer overall survival in a multivariate analysis.[28] Thus, the higher mortality of the mixed subtype appears to be well established. However, immunohistochemical and genetic differences between these groups have not been previously reported.

Early genetic studies revealed that desmoplastic melanoma does not typically demonstrate mutations in *BRAFV600E*, which is found in up to 60% of conventional melanomas.[7] Our study further supports this observation, as only 1 of 41 cases exhibited *BRAF* mutation. A more recent finding explains, in part, phenotypic properties of desmoplastic melanoma.[10] Given that this variant is commonly neurotrophic, Narita *et al*[10], investigated the rates of polymorphism in *RET,* which encodes a receptor tyrosine kinase for which the ligand is GDNF, and identified a statistically significant increased frequency of *RETp* mutation in desmoplastic *versus* conventional melanoma (61 *vs* 31%). Functionally, mutations in *RETp* in cutaneous and non-cutaneous malignant tumors (including pancreatic cancer and melanoma) have been thought to alter responsiveness to GDNF and promote cellular proliferation via signaling through the *RAS–BRAF–ERK*, *PI3K* and *MAPK* pathways.[10,29,30] Of the 41 cases in the current study, 12 demonstrated mutations in *RETp*, though statistically significant differences in the frequency of *RETp* between pure and mixed desmoplastic melanoma (8/24 *vs* 4/17; $P = $ NS) were not noted. Of the 12 cases, 10 (83%) with *RETp* exhibited neurotropism microscopically, *versus* 17 of 29 cases (59%) without *RET* polymorphism; although this difference did not reach statistical significance, there was a trend toward *RETp* being predictive of neurotropism ($P = 0.165$). The only case that exhibited the *BRAF V599E* mutation, a mixed-subtype tumor (Case 36), did not have a coexistent *RETp* mutation.

Immunohistochemistry results for CD117 were the most significant finding in the current study. In the only two previous studies documenting expression of CD117 in desmoplastic melanoma, the frequency of expression ranged from 21 to 29%.[31,32] In the more recent of the two, CD117 stained the epidermal component in 8 of the 10 cases, in which 1 was present, and the dermal component in 3 of 14 cases (21%).[31] Although the subtypes were not specified, it seems reasonable to extrapolate that these cases were all of the pure variant, as all were described as 'clear cut' cases of desmoplastic melanoma. Frequency of CD117 expression in the pure subtype in our series (26%) was similar to this finding.

Curtin *et al*[12] found that melanomas with *c-KIT* mutation or copy number increase were significantly more likely to have a vertical growth phase component, and concluded that *KIT* expression is enhanced in tumors of more advanced stage (though none of the melanomas included their study were of desmoplastic type). We noted significantly enhanced expression of CD117 in 79% of cases of the mixed subtype, with uniform staining of lesional cells in positive cases, with staining in both the spindled and 'epithelioid' components of the tumors. We observed no significant difference in either depth or mitotic rate for the positive and negative groups, though we suspect this may relate to the relatively small sample size. These findings should be validated by further studies on larger sample sizes of mixed desmoplastic melanoma cases.

When *c-KIT*, a transmembrane receptor tyrosine kinase, is activated via binding by its ligand (stem cell/steel factor), downstream events including activation of *RAS/BRAF* in the MAP kinase pathway are initiated, leading to cell proliferation.[12,31] Given this, it makes sense that melanomas with *c-KIT*-activating mutations would not necessarily require *BRAF* or *RAS* mutations for oncogenesis. Desmoplastic melanoma would seem to share features in common with several melanoma types in this *c-KIT* group, including a predilection for sun-exposed sites and generally a lack of *BRAF* mutations. Head and neck locations are the most common sites for development of primary desmoplastic melanoma, ranging from 52 to 72% of all locations.[22,33] Also of interest is the observation that in desmoplastic melanomas that have an associated intra-epidermal component, the majority exhibit a lentigo maligna-like phenotype (56 of 91 cases, 61% in one series;[22]), with generally small, monomorphous melanocytes in a lentiginous growth pattern and minimal pagetoid scatter.[22,34,35] This also suggests homology with other melanomas on chronically sun-damaged skin, which commonly exhibit *c-KIT* mutations. According to Curtin *et al*,[12] 'melanoma types that have frequent genetic alterations of *KIT* typically have a lentiginous growth pattern, characterized by melanocytes lined up as single cells along the epidermis in the progression stage preceding invasive growth. By contrast, non-CSD [chronically sun exposed] melanomas, which have no *KIT* mutations or copy number increases, typically show a pagetoid growth pattern with melanocytes scattered throughout the epidermis.' Of the 14 cases, 11 (79%) of CD117-positive mixed subtype had an overlying lentiginous junctional component, though this percentage was not significantly different than the CD117-negative mixed cases, nor the entire sample set as a whole (see Table 2).

We identified no mutations in *c-KIT* exons 11, 13 and 17 on genetic analysis of all tumors. Although the precise reasons for lack of correlation between genetic sequencing and c-*KIT* immunohistochemical expression are not clear, ours is not the first study to note lack of correlation.[36,37] We recently observed a poor correlation between CD117 staining and *c-KIT* sequencing in atypical acral nevi.[38] Briefly, we noted that CD117 stained 80% of acral nevi (with and without atypia), but genomic analyses of *KIT* exon regions 11, 13 and 17 revealed no abnormalities in 'hotspots' frequently associated with point

mutations in acral melanomas.[38] One proposed theory is that the *c-KIT* product identified on positive immunostaining may be a non-mature protein rather than the active membranous receptor form.[18] Another explanation may lie in the fact that copy number increases or gene amplifications may account for increased immunohistochemical expression without genetic mutations;[39] copy number amplification of KIT can be involved in the upregulation of the MAP kinase pathway. Additionally, given that the coding region of the *c-KIT* gene is large, from a scientific perspective, it seems reasonable to speculate that a novel mutation that does not involve the examined exons, that is, 11, 13 and 17 may be responsible.

Busam *et al*[11] have demonstrated that, compared with conventional melanoma, desmoplastic tumors show a significantly decreased expression of select genes related to melanin synthesis, and enhanced expression of multiple neurotrophic factors, observations consistent with the decreased pigmentation of desmoplastic melanoma, as well as its propensity for perineural invasion.[21] In their initial screen, this group found that the gene coding for clusterin, believed to be involved in apoptotic pathways, was significantly increased in desmoplastic melanoma, with a 31-fold higher expression *versus* conventional melanoma on gene expression profiling.[11] However, in a subsequent study, the same group noted that clusterin expression could only be identified in a minority of desmoplastic melanomas (7 of 21; 33%) on immunohistochemical staining.[40] We observed staining with clusterin in only two cases (one pure and one mixed variant). Although a possible explanation is that the results are reflective of differences relating to staining technique, both studies utilized the same clone of antibody, although dilutions were different. In addition, the scoring system incorporated here appears to be different from the one utilized by Busam *et al*,[40] with different thresholds for positivity.

Additionally, we found a significantly increased Ki-67 proliferative rate in the mixed desmoplastic melanoma variant. Higher Ki-67 index has been associated with poorer overall and disease-free survival in cutaneous melanoma.[41] Our finding here is similar to previous reports, in which higher mitotic rate was observed in mixed *versus* pure desmoplastic tumors, and correlated with more aggressive biological behavior.[4,24]

Nestin is an intermediate filament protein and marker for melanoma-initiating cells; immunohistochemical expression has been identified in desmoplastic melanoma[42] and is associated with poor prognosis in conventional melanomas.[13] CD271 (p75NTR) is a neural crest stem cell marker expressed in melanoma cells, which is associated with higher metastatic potential and worse prognosis.[15] In the current study, nestin and CD271 stained 86 and 65%, respectively, of all cases of desmoplastic melanoma with no statistically significant differences noted in the two variants, indicating that the populations of stem cells harbored appear to be similar in the two subtypes. Similar findings were obtained with respect to expression of SOX10, which stained 91% of cases in the current series with no significant difference between the two subtypes. SOX10 is a transcription factor, which is important in melanocyte development. It is downregulated upon terminal differentation of melanocytes, and appears to be a necessary cofactor for the expression of nestin, which has been correlated with tumor progression.[43–45] Given this, we hypothesized that SOX10 and/or nestin may have been differentially expressed in the two subtypes; the data did not support this theory.

A subclassification system of desmoplastic melanoma into pure and mixed subtypes has recently evolved on the basis of the clinical observation that these tumors behave in a heterogeneous fashion, some with limited lymph node metastasis and propensity for local recurrence, and others with higher nodal and distant metastasis and a poorer prognosis. In this study, although we noted many similarities between these two subtypes on genetic analysis and immunohistochemistry, we also noted a distinct difference in CD117 expression, which has not been previously recognized. This observation requires further study and validation, but may, in part, begin to explain the significant phenotypic differences between these two groups.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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