Desmoplastic melanoma (DM) is an uncommon variant of malignant melanoma characterized by spindle cells and a prominent stromal fibrous response [1] (Fig. 1). The long spindle cells are present in varying density, often diffusely present and may be arranged in a parallel or scattered fashion. The surrounding fibrosis is characteristically diffuse and pericellular rather than forming septa. Lesions are currently classified as desmoplastic if the lesion is wholly desmoplastic or contains a recognizable desmoplastic component [2]. A more recent definition defines DM as predominantly (> 90%) showing spindle cell morphology with prominent fibrosis throughout the tumour [3]. Other components commonly identified are described as junctional and epithelioid. The junctional component, if present, is commonly in the overlying epidermis whereas the epithelioid component consists of oval cells often in the same layer as the desmoplastic cells (Fig. 1). Occasionally, an overlying epidermal component with a superficial spreading histology is present (Fig. 2). Neurotropism is a common associated feature characterized by perineural extension, specifically within the neural sheath endoneurium or perineurium [4]. This subset is also termed desmoplastic neurotropic melanoma (Fig. 2b). This has led to the identification of p75 neurotrophin receptor as an additional marker that may be used to distinguish this subset [5]. This variant is more aggressive with a higher local recurrence rate [6].

The density and distribution of lymphocytes infiltrating the vertical growth phase of primary cutaneous melanomas have been suggested by several studies to be of prognostic significance, with a 'brisk' infiltrate of tumour infiltrating lymphocytes (TILs) reported to be associated with better clinical outcomes [7,8]. This possibly reflects immune control by lymphocytes, which have the capacity to recognize tumour [9]. In DM, lymphoid aggregates are often seen; however, the significance of these is unclear [10–12].

Clinically, DM may occur at any site but has a predilection for the head and neck [1,13,14]. It is characterized by a higher local recurrence rate and a lower incidence of regional lymph node metastasis than other forms of malignant melanoma [6,13–15]. DM tends to occur in an older group of patients and more commonly in males [11,13,14]. As a result of its atypical features, DM may be misdiagnosed on histological grounds and, if
missed, the diagnosis is often made only after recurrence following incomplete excision. Many (44–77%) are amelanocytic [14,15]. Because of the extensive fibrous response, DM may also be confused with scar tissue, and a variety of benign and malignant mesenchymal proliferations [15,16]. Differential diagnoses include atypical fibrohistiocytoma, spindle cell variant of squamous cell carcinoma, angiosarcoma and leiomyosarcoma.

Immunohistochemical analysis is an important adjunct in the diagnosis of DM; however, there is currently no set of markers that are both sensitive and specific for DM. It has been demonstrated that the immunohistochemical features of DM differ from those of other types of melanoma; while the vast majority (95–100%) stain positive for S100 antigens (Ags), DM stains less frequently for differentiation Ags [15,17–19]. These include glycoprotein 100 (gp100), Melan-A/MART 1 and tyrosinase in 0–30% [3,11,15,18–21], 0–29% [16,19] and 0–55% [3,19] of cases, respectively. In one report, five patients with DM had 100% staining with tyrosinase, gp100 and S100, which is an atypical result [22]. The majority of these studies involve small numbers and there is often variability in the staining pattern and intensity noted. These Ags are relatively specific for the melanocyte lineage but because they are often not present in “DM”, they lack sensitivity. S100 Ag is the most consistently positive antigen expressed [3,14,15,18,19,22]. It is not specific however, as it stains neural tissue. Dendritic cells, Langerhans cells and Schwann cells which are also present in the skin [16,23]. Other recently discovered and less routinely used differentiation Ags include microphthalmia transcription factor and multiple myeloma 1, with variable reported staining frequencies of 7–55% [3,19,21,24,25] and 13% [26], respectively. This distinction between DM and non-DM has also recently been demonstrated by gene expression profiling. Hierarchical cluster analysis showed a decrease in the expression of the number of melanocyte differentiation genes in DM compared with non-DM [27].

Cancer testis (CT) Ags are a relatively new group of Ags that are expressed in germ cells, trophoblast and malignant tumours of different origins [28]. They include Mage-A1, Mage-A3, Mage-A4 and NY-ESO-1. They are of particular interest as they also serve as targets for immune recognition and therefore immunotherapy [29–32].

The difficulty in diagnosing DM indicates that there is a need both for a high clinical index of suspicion and to improved diagnostic techniques. Currently, little data exist on the expression patterns of CT Ag, NY-ESO-1 and Mage-A4 in DM. In order to assess the expression of
these for diagnostic purposes, which also have implications on their suitability for enrolment into melanoma immunotherapy trials, we have analysed 32 DMs for expression of NY-ESO-1, MAGE-A1 and MAGE-A4.

**Materials and methods**

**Patients**

Thirty-two samples were evaluated from patients attending the Ludwig Institute Melanoma Clinic at Austin Health, Melbourne, Australia. All were analysed using standardized methods in the Department of Anatomical Pathology at Austin Health by the authors. All slides were scored by E.L. and J.B. under the supervision of an experienced histopathologist (D.M.). They were also reviewed by a case conference attended by all authors.

**Histology**

The diagnoses were based on accepted histological criteria, including spindle cell proliferation separated by abundant collagen, cytologic atypia of the spindle cells, atypical melanocytic proliferation at the dermo-epidermal junction and neurotropism [1]. Histological information on the tumour site, thickness, Clark level, and presence or absence of ulceration was recorded when information was available (Table 1).

TILs were scored as absent, non-brisk or brisk in accordance with the criteria of Clark et al. [33]. ‘Brisk’ was defined as lymphocytes present throughout the substance of the vertical growth phase or invasive component, or present and infiltrating across the entire base of the vertical growth phase. ‘Non-brisk’ was defined as lymphocytes present in one or more foci of the vertical growth phase, either dispersed throughout or situated focally in the periphery. ‘Absent’ was defined as no lymphocyte present or if the lymphocytes present were only in stroma and did not infiltrate the melanoma.

**Antibodies**

Antibodies to NY-ESO-1, Melan-A, tyrosinase and MAGE-A1 were produced by the Biological Production facility at the Ludwig Institute of Cancer Research and used at concentrations of 3 μg/ml for E978 (NY-ESO-1) [34], 9.4 μg/ml for ES121 (NY-ESO-1) [35], 2.67 μg/ml for A103 (Melan-A) [36], 5.59 μg/ml for T311 (tyrosinase) [37], and 1:50 dilution for MAGE-A1. The monoclonal antibody supernatant 57B, which recognizes MAGE-A4 [38], was kindly supplied by Dr G. Spagnoli (Surgical Research Centre, Basel, Switzerland) and used at a 1:100 dilution. Polyclonal anti-S100 and anti-gp100 (HMB-45) was purchased from DakoCytomation (Carpinteria, California, USA) and used at a 1:20 dilution and 1:400 dilution, respectively [39].

Specimens of known positive tumours were used as a positive control, and negative substitution controls, in...
which the antibody diluent solution (10% fetal calf serum in phosphate-buffered saline, pH 7.6) replaced the primary antibody, were included with every immunohistochemical test.

**Immunohistochemistry**

Immunohistochemical staining was performed for the differentiation Ag (Melan-A/MART-1, gp100, tyrosinase) and CT Ag (NY-ESO-1, MAGE-A1, MAGE-A4). Formalin-fixed paraffin sections were prepared and dried overnight at 37°C. After dewaxing in xylene and rehydration with alcohol, microwave Ag retrieval was carried out for 10 min using ethylenediaminetetraacetic acid buffer pH 8.0 (NeoMarkers, Fremont, California, USA) for E978 (NY-ESO-1) and MAGE-A1, and citrate buffer pH 6.0 (NeoMarkers) for S100, gp100, Melan-A, tyrosinase and 57B. Immunohistochemistry was performed using the Dako Envision +kit (DakoCytomation) [40] or Vectastain Elite Universal ABC kit (Vector Laboratories, Burlingame, California, USA). All sections were submitted to 3% H2O2/phosphate-buffered saline for 10 min to block endogenous peroxidase. Endogenous biotin activity was quenched by sequential application of egg white and skim milk according to published methods for the Vectastain Elite Universal ABC kit [41,42]. All incubations were carried out at room temperature using the Shandon Sequenza immunostainer. 3-Amino-9-ethylcarbazole (Sigma A-5754) was used as the chromogen and slides were counterstained with Mayer's haematoxylin (Amber Scientific MH-5L). Application of CrystalMount (Biomedica M03) preceded dehydration and mounting in DePeX (BDH 36125).

The immunohistochemical staining of the junctional, epithelioid and spindle cell components was recorded separately. The relative percentages of cells stained were scored in the range brackets of none (−), < 5% (○), < 25% (●), 25−50% (●●), 50−75% (●●●) and > 75% (●●●●), consistent with other series in the literature [3]. Controls using S100 Ag staining were used (Table 2 and Fig. 3).

**Results**

A total of 32 tissue samples were studied (Table 1). All had desmoplastic features. Spindle cell melanomas without desmoplasia were excluded. Fourteen patients had perineural extension, two with neural-like differentiation and six patients with a superficial spreading component identified (Fig. 2a). TILs were reported in 27 patients: TILs were absent in eight patients, brisk in six patients and non-brisk in the remainder (Fig. 4). The patients with a brisk and non-brisk response stained positive more frequently for differentiation Ag than the patients with absent TILs; however, the staining of CT Ag was poor in all three categories (Table 3). No association was found between the presence of a brisk TIL response and the type or pattern of Ag staining.

One patient did not have an epithelioid component and 12 patients did not have a junctional component identified. The Ag staining for the whole tumour was assessed according to the three morphological components: junctional, epithelioid and spindle cell. Overall, Ag expression was not always congruent across all three cellular subtypes, as one subtype often stained positive whereas the other two cell subtypes stained negatively for the same Ag. When the immunohistochemical staining for all three subtypes was considered collectively, 100% of the 32 samples stained for S100, 56% stained for gp100 and tyrosinase, and 66% stained for Melan-A/MART-1. For the CT Ag, 19% stained for MAGE-A1 and MAGE-A4 while only 9% stained for NY-ESO-1.

The Ag staining pattern for the three cellular subtypes revealed consistent positive staining with S100, particularly in the spindle cell type where all 32 cases stained positive in > 75% of cells. The percentage of the spindle cell subtype stained with gp100 was 59%, Melan-A/MART-1 66%, tyrosinase 59%, MAGE-A1 19%, MAGE-A4 19% and NY-ESO-1 9% (Fig. 5).

Spindle cells were consistently less likely to stain than epithelioid or junctional cells for antigens other than S100. Immunohistochemical staining was positive in only 34.5% for gp100, 46.9% for Melan-A/MART-1, 40.6% for tyrosinase, 12.5% for MAGE-A1 and MAGE-A4, and 6.2% for NY-ESO-1 (Table 2). The junctional component, when present, was more likely to stain for the differentiation Ag than the other two cell types, ranging between 80% for tyrosinase and 95% for Melan-A. Staining of the epithelioid component for differentiation Ag ranged between 39 and 50%, depending on the Ag.

As for the CT Ag, the overall percentage of samples stained was significantly lower than the differentiation Ag, with less than 20% staining positive. The percentage of patients staining positive in the spindle cell component for CT Ag was in general lower than for the differentiation Ag, ranging from 6% for NY-ESO-1 antigen to 13% for MAGE-A1 and MAGE-A4; in the junctional component, 5% for MAGE-A4 and 21% for the MAGE-A1 antigen; and in the epithelioid component, 7% for NY-ESO-1 and 20% for MAGE-A4. CT Ag rarely stained across all three cell subtypes and, when present, stained in tumours that were also positive for the differentiation Ag. The addition of CT Ag to the Ag ‘panel’ of differentiation Ag did not increase the diagnostic yield for DM when compared with those identified using differentiation Ag alone.

**Discussion**

We studied a series of DMs to evaluate expression of six Ags, four of which have previously been reported [3,11,15,17–20,22]. The study was undertaken to assess
<table>
<thead>
<tr>
<th>Ag100</th>
<th>MAGE A1</th>
<th>MAGE A4</th>
<th>NY-ESO-1</th>
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</thead>
<tbody>
<tr>
<td><strong>Differentiation Ag</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S100</td>
<td></td>
<td></td>
<td></td>
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<td>Ep</td>
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<td></td>
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<td>SC</td>
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<td></td>
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</tr>
<tr>
<td>Juno</td>
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<tr>
<td>Tyrosinase</td>
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<tr>
<td>MAGE A1</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>MAGE A4</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>NY-ESO-1</td>
<td></td>
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</tbody>
</table>

|                | NP | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | **Total** |
|----------------|----|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| **Desmoplastic melanoma** |    |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 32/32 |

Ag, antigen; SC, spindle cell component; Epi, epithelioid component; Juno, junctional component; NP, not present (grey boxes); number of cells stained: –, none; ●, < 5% cells stained; ●●, 5–25% cells stained; ●●●, 25–50% cells stained; ●●●●, 50–75% cells stained; ●●●●●, > 75% cells stained.
whether the additional Ag improved the therapeutic yield of immunohistochemistry for the diagnosis of DM and to evaluate the potential of this subtype to be targeted by immunotherapy. As the cellular composition of DM comprises junctional, spindle and epithelioid cells, the distribution of Ag expression within each of these components was studied. Previous publications do not make such a distinction [14–16]. It is unclear whether the behaviour of the different cell components differs; the study of metastases from DM may provide valuable insight.

Our results show a higher staining frequency for differentiation Ag than for the majority of reported series [3,15,16,18,19], with the frequency generally above 50%. A number of factors can influence immunohistochemical results and therefore affect comparative studies. Firstly, antibody source, concentrations and Ag retrieval methods are not standardized across studies and are not always described in published accounts. Secondly, during different stages of tumour development, different isoforms of the same Ag may be expressed that may vary in its detection with the antibody [43]. These technical factors need to be taken into account when assessing reports of Ag expression that differ between reported series.

Our results show that the staining pattern with CT Ag was generally low in DM, with less than 20% staining positive. This is particularly so for NY-ESO-1, which only had 15% of the samples staining positive. This contrasts with the percentages found in other melanoma types, ranging from 9% for MAGE-A4, 20% for the MAGE-A1 and 45% for NY-ESO-1, a reversal in the relative prevalence of NY-ESO-1 staining compared with DM [34].

By analysing the three cell types separately, we found that the staining pattern was often not congruent. Nonetheless, the overall diagnostic yield for DM was only slightly increased when all three components are considered together as opposed to an analysis based on the more common spindle cell subtype alone. This highlights the importance of not relying only on the spindle cell type for the diagnosis of DM. Interestingly, the junctional subtype, although not always present, had a higher percentage of cells stained than the spindle and epithelioid cellular subtypes, a pattern perhaps more in keeping with patterns seen with non-DM. This gives rise to the possibility that the different cell subtypes may be biologically different.

We have studied the presence of TILs in an attempt to identify any relationship with Ag expression. We note that when TILs are present, there is a higher frequency of staining with differentiation Ag than when TILs are absent, although there is little difference in immunohistochemical staining with CT Ag. It is, however, not possible to discern such a meaningful relationship because of the small numbers. The potential role of Ag-specific TILs to modify the natural history of this subtype of melanoma therefore requires further elucidation.

The immunohistochemical identification of Ag is important in melanoma for two main reasons. Firstly, it serves as a diagnostic tool, which can assist in the diagnosis of lesions that have atypical morphology. Secondly, it establishes the pattern of Ag distribution, which may have an important bearing on the selection of patients and Ag for immunotherapy trials. Currently, there is major interest in developing therapies that target melanoma Ag, and the success of clinical trials may depend on the suitability of patient selection. Clearly, tumour variants with poor expression of key Ags are likely to be unsuitable for such studies [29–32]. The determination of Ag expression by immunohistochemistry is limited by its sensitivity and perhaps other techniques such as polymerase chain reaction should be considered in assessing the eligibility for such studies.
Although the Ag staining in DM is generally expressed at a lower frequency than in typical melanoma, it is a vital adjunct in the diagnosis of DM. Our series demonstrates that other than S100, the staining frequencies for all the other identified Ags were generally low. It was also found that DM expresses differentiation Ag more commonly than CT Ag. The addition of CT Ag staining did not provide additional diagnostic value as these antigens were generally absent and, when present (9–19%), were found only in those tumours that also expressed differentiation Ag. As a result of the low frequency of CT Ag expression, patients with DM will often be unsuitable for immunotherapies that target these Ag. Nonetheless, a minority of patients will be suitable, and so DM need not be an exclusion criterion.

Table 3  Relationship of tumour infiltrating lymphocytes to antigen staining in desmoplastic melanoma

<table>
<thead>
<tr>
<th>TILs</th>
<th>brisk</th>
<th>Non-brisk</th>
<th>Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>6</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td>S100</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>gp100</td>
<td>83%</td>
<td>85%</td>
<td>38%</td>
</tr>
<tr>
<td>Melan-A/MART-1</td>
<td>83%</td>
<td>92%</td>
<td>62%</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>67%</td>
<td>85%</td>
<td>50%</td>
</tr>
<tr>
<td>MAGE-A1</td>
<td>33%</td>
<td>15%</td>
<td>25%</td>
</tr>
<tr>
<td>MAGE-A4</td>
<td>33%</td>
<td>8%</td>
<td>38%</td>
</tr>
<tr>
<td>NY-ESO-1</td>
<td>0%</td>
<td>23%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Various stains demonstrating percentages of positive staining (S100 controls on the right panel), (a) Fewer than 5% of spindle cells stained for gp100; (b) 5–25% spindle cells stained for NY-ESO-1; (c) 25–50% epithelioid cells stained for Mage-4; (d) 50–75% cells stained for Melan-A with brisk TILs and (e) more than 75% of spindle and epithelioid cells stained for S100.
Immunohistochemistry staining patterns in 32 Ludwig Institute of Cancer Research–Austin Health desmoplastic melanoma samples.


