The Use of Immunocytochemical Study in the Cytologic Diagnosis of Melanoma:
Evaluation of Three Antibodies

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There are limited studies on the utility of immunostaining in cytologic specimens suspected of melanoma. In this study, we examined the performance of the most commonly used antibodies including monoclonal antibodies against Melan-A (A103), S-100, and HMB-45 antigens. Immunostains were performed on formalin-fixed, paraffin-embedded cell blocks prepared from 100 cytologic specimens. The specimens consisted of 57 melanomas and 43 nonmelanocytic neoplasms. Of 57 melanomas, 53 showed positive reaction to Melan-A antibody while 51 and 41 revealed positive immunostaining for S-100 and HMB-45, respectively. Of 43 nonmelanocytic neoplasms, 10, 4, and 8 specimens stained positive with an antibody against S-100, HMB-45, and Melan-A, respectively. However, the false-positive immunostaining for Melan-A was eliminated in seven of the eight specimens after applying the pretreatment with avidin/biotin blocking reagents. Overall, the highest sensitivity and negative predictive value (NPV) were achieved in Melan-A antibody (93 and 90%) compared with antibodies to S-100 (89 and 85%), and HMB-45 (72 and 71%). Initially, an intermediate specificity and positive predictive value (PPV) were obtained for Melan-A antibody (81 and 87%) that were greater than S-100 (77 and 84%), and lower than HMB-45 (91 and 91%). However, the aforementioned treatment with avidin/biotin blocking reagents improved both specificity (98%) and PPV (98%) for Melan-A antibody. In conclusion, by blocking endogenous biotin, Melan-A antibody offers the greatest performance. In terms of cost-effectiveness, we suggested that Melan-A antibody should be used as the first-line antibody for detecting melanoma in the cytologic specimens. Diagn. Cytopathol. 2013;41:126–130.

Key Words: melanoma; cytology; melan-A; S-100; HMB-45

Malignant melanoma is notorious for its great variety of cytohistomorphological features which may be seen in almost any nonmelanocytic malignant neoplasm and rarely non-neoplastic/benign conditions. Cytologically, melanoma cells may present as dispersed, single cells pattern or in discohesive clusters. Occasionally, it may present as large cohesive or papillary-like groups. The majority of melanoma cells are polygonal or plasmacytoid shaped with eccentric, hyperchromatic nuclei, as well as occasional reddish prominent nucleoli and intranuclear inclusions. However, it is not uncommon to see other cell types including spindle cells, lymphoma-like small round cells, and undifferentiated/anaplastic cells. The latter are often large, pleomorphic cells with bi- or multinucleation.[1,2] Thus, immunostaining has been extensively used under the following circumstances: (1) confirmation of recurrent melanoma which shows unusual morphological features; (2) work-up of differential diagnoses in a patient who has known history of more than one malignant neoplasm including melanoma; and (3) establishment of diagnosis for a neoplasm of unknown origin.

Melan-A/MART-1 gene was identified as a gene encoding a melanocyte lineage-specific antigen that is recognized by autologous cytotoxic lymphocytes.[3,4] Ohsie et al. undertook a literature review on immunohistochemical studies with Melan-A/MART-1 antibody that were performed on paraffin-embedded histologic specimens. Accordingly, Melan-A/MART-1 antibody has demonstrated a sensitivity ranging from 75 to 92% and a specificity ranging from 95 to 100% in detecting melanoma.[5] There are limited studies regarding the utilization of Melan-A/MART-1 antibody in cytologic materials.[6–8] In this study, we assessed our experience with sensitivity, specificity, positive (PPV), and negative (NPV) predictive value of Melan-A monoclonal

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Table I. Nonmelanocytic Neoplasms

Source of specimen	*Number of specimen*
Poorly differentiated squamous cell carcinoma	3
Small cell carcinoma of lung	2
Breast carcinoma	6
Renal cell carcinoma	5
Hepatocellular carcinoma	3
Carcinoma of unknown origin	3
Metastatic adenocarcinoma	2
Lymphoma	8
Sarcoma	9
Spindle cell neoplasm	1
Germ cell neoplasm	1

Table II. Description of the Antibodies

Antibody	*Dilution*	*Pretreatment*
Melan-A	1:12.5	Tris-EDTA at PH 9.0, microwaved for 15 min
S-100	1:500	None
HMB-45	1:25	Protease

antibody (A103) for detecting melanoma in cytologic specimens, in comparison with antibodies to S-100 and HMB-45.

Materials and Methods

A SNOMED search of the electronic pathology database in our institution for the last 10-year period retrieved a total of 150 cases with an established diagnosis of melanoma or its mimics. Upon assessing the original hematoxylin and eosin-stained cell block slides prepared from the individual cases, 50 cases were excluded from the study due to the absence or the presence of scant cells of interest. The study employed remaining 100 cases consisting of 57 melanomas and 43 nonmelanocytic neoplasms that need to be considered in the differential diagnosis (Table I). The specimens of melanoma were prepared from 54 fine-needle aspirates (FNAs) and three effusions. The sources of nonmelanocytic neoplasms were 35 FNAs and 8 effusions.

Immunostaining was performed on paraffin-embedded cell blocks. Briefly, 4-μm sections were cut, mounted on positively charged PLUS™ slides (Baxter cat#M6146-PLUS) and heated at 60°C for 30–60 min. Using the standard avidin-biotin-peroxidase complex technique, immunostaining was performed on the Ventana ES automated immunohistochemistry system and the Ventana DAB detection kit (Ventana Medical Systems, Tucson, AZ). Antibodies used for immunostaining (Dako Corporation, Carpinteria, CA) were Melan-A (A103), S-100, and HMB-45. Dilutions and methods of pretreatment for the individual antibodies are shown in Table II. Appropriate positive and negative controls were obtained.

All cases were reviewed blindly and the immunostaining was assessed based on the approximate percentage of neoplastic cells showing positive cytoplasmic staining (Melan A and HMB-45) or positive nuclear/cytoplasmic staining (S-100) and scored as follows: 0 (complete lack of or blush staining), 1 (5–25%), 2 (25–50%), 3 (50–75%), and 4 (75–100%). The immunostaining was considered to be positive when a score of 1 or greater was obtained. Using the established cytologic and/or histologic diagnosis as the gold standard, sensitivity, and specificity, as well as PPV and NPV were calculated.

Table III. Immunostaining Reactions to Antibodies Against Melan-A, S-100, and HMB-45

Antibody	*Staining Intensity*	*Melanoma (n = 57)*	*Nonmelanoma (n = 43)*
Melan-A	3	27	2
	2	13	4
	1	13	2
	Blush	4	5
	0	0	30
S-100	3	15	0
	2	17	0
	1	19	10
	Blush	0	0
	0	6	33
HMB-45	3	20	0
	2	15	0
	1	6	4
	Blush	0	0
	0	16	39

Results

Among the 57 melanoma specimens, six specimens stained positive for only one of the three markers, including three for Melan-A and three for S-100 while none of the specimens show positive staining for HMB-45 alone; 11 specimens stained positive for two markers, including nine for Melan-A and S-100 and two for Melan-A and HMB-45; and the remaining 39 specimens showed positive staining for all three markers. Table III summarizes immunostaining reactions to antibodies against Melan-A, S-100, and HMB-45. Of the 57 specimens of melanoma, 53 revealed positive reactions (a score of 1 or greater) with immunostaining for Melan-A while 51 and 41 specimens showed positive immunostaining for S-100 and HMB-45, respectively (Fig. 1). None of the antibodies produced a positive reaction in more than 75% (an immunostaining score of 4) of melanoma cells. However, positive immunostaing with a score of 3 (50–75% cells stained) was observed in a greater number of melanoma specimens for Melan-A ($n = 27$) compared with S-100 ($n = 15$) and HMB-45 ($n = 20$). Of the 43 nonmelanocytic specimens, positive immunostaining for Melan-A was observed in eight specimens (seven FNAs and one effusion), including rhabdomyosarcoma 1, adenocarcinoma of the breast origin 2 (including one pleural effusion), leiomyosarcoma 1, lymphoma 1, hepatocellular carcinoma 2, and renal cell carcinoma 1. The majority

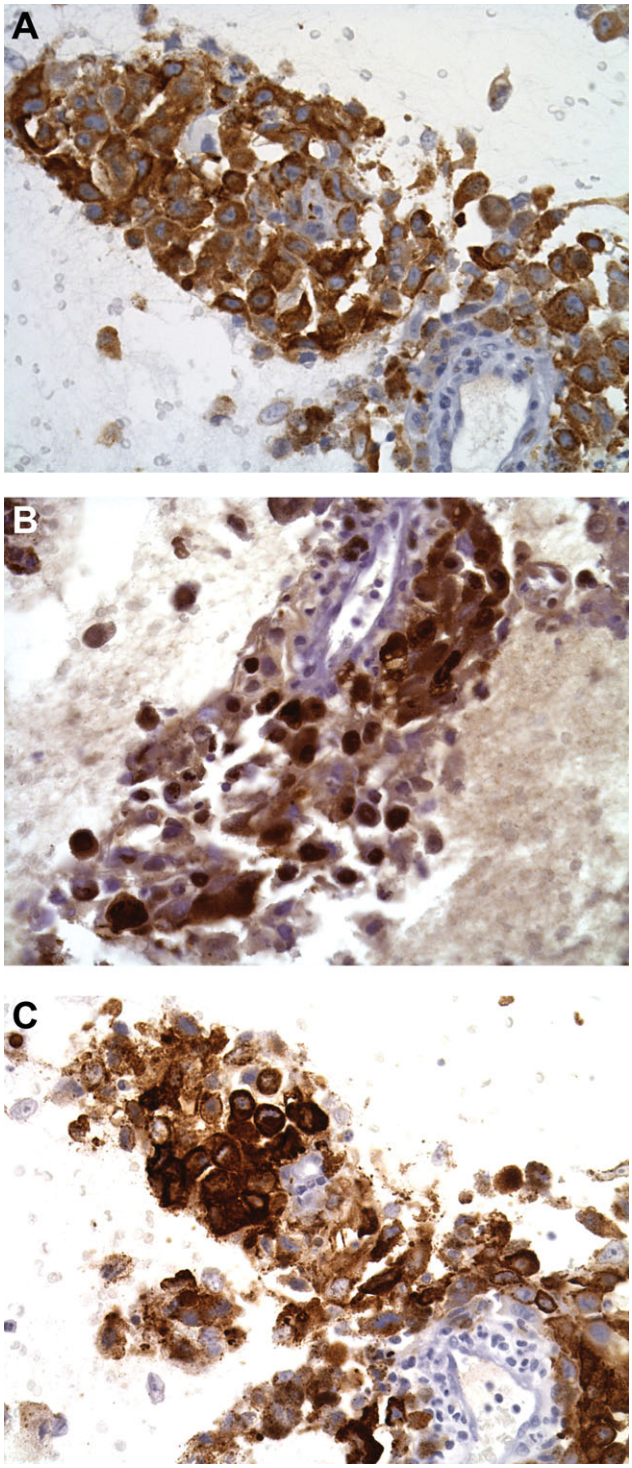

Fig. 1. Positive immunostaining for (**A**) Melan A, (**B**) S-100, (**C**) HMB-45 in the melanoma specimen (immunostain, 400×). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

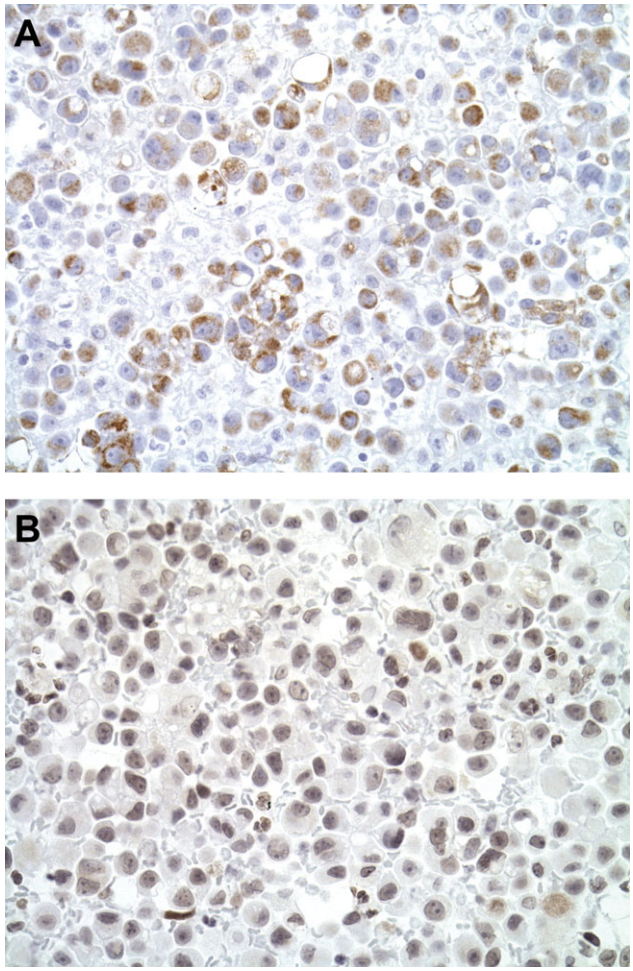

Fig. 2. A: The cell block material prepared from effusion of metastatic breast carcinoma showed false-positive immunostaining for Melan A. **B**: The false-positive staining was eliminated upon applying pretreatment with avidin/biotin blocking reagents (immunostain, 400×). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

of the nonmelanocytic specimens with a false-positive reaction for Melan-A antibody exhibited an immunostaining scores 1 or 2 while a score of 3 was seen in one hepatocellular carcinoma and 1 breast adenocarcinoma. In contrast to the crisp, well-defined staining pattern in melanoma, nonmelanocytic specimens showed low intensity, faint staining. While repeating immunostaining for Melan-A in these eight specimens with false-positive reaction after the specimens were pretreated using avidin/biotin blocking reagents prior to the immunostaining procedure, the false-positive reaction with Melan-A antibody was eliminated in seven specimens (an example shown in Fig. 2). Positive immunostaining for S-100 and HMB-45 was also observed with a score of 1 in 10 and 4 nonmelanocytic neoplasms, respectively.

The highest sensitivity and negative predictive value (NPV) were achieved in Melan-A antibody (93 and 90%) compared with antibodies to S-100 (89 and 85%), and HMB-45 (72 and 71%). Initially, an intermediate specificity and positive predictive value (PPV) were obtained for Melan-A antibody (81 and 87%) that were greater

Table IV. Comparison of Immunotaining Performance of the Antibodies

Antibody	*Melanoma (n = 57)*	*Nonmelanoma (n = 43)*	*Sensitivity (%)*	*Specificity (%)*	*PPV (%)*	*NPV (%)*
Melan-A	53	8	93	81	87	90
Melan-A (Biotin blocked)	–	1	–	98	98	91
S-100	51	10	89	77	84	85
HMB	41	4	72	91	91	71

than S-100 (77 and 84%), and lower than HMB-45 (91 and 91%). However, the aforementioned treatment with avidin/biotin blocking reagents improved both specificity (98%) and PPV (98%) for Melan-A antibody.

Discussion

As mentioned previously, immunostaining plays a very vital role in order to confirm recurrent melanoma, work up of differential diagnoses and establish diagnosis for a neoplasm of unknown origin. The most commonly used melanocytic markers include S-100 and HMB 45 proteins. S-100 belongs to a calcium binding group of proteins. S-100 protein is considered a sensitive but not a specific melanocytic marker due to the fact that it presents in not only high proportion of melanomas, but also nonmelanocytic neoplasms such as gliomas, Schwannomas, and neurofibromas.[9] On the other hand, HMB-45, a cytoplasmic premelanosomal glycoprotein is considered a more specific but less sensitive melanocytic marker compared with S-100.

MART-1/Melan-A antigen is a marker of melanosomal differentiation that is recognized by autologous cytotoxic lymphocytes.[3,4] MART-1/Melan-A is a relatively newer melanocytic marker and two clones of antibody are available: MART-1 (M2-7C10) and Melan-A (A103).[10] According to the literature review on immunohistochemical studies by Ohsie et al., Melan-A/MART-1 antibody demonstrated a sensitivity ranging from 75 to 92% and a specificity ranging from 95 to 100% in detecting melanoma while sensitivity and specificity of S-100 ranged from 97 to 100% and 75–87%, respectively; HMB-45 showed a sensitivity of 69–93% and a specificity of 56–100%.[5]

There are a few studies comparing the performance of MART-1/Melan-A, S-100m and HMB-45 in cytology specimens. In this regard, MART-1 was reported as a potentially preferable antibody over HMB-45 for the diagnosis of metastatic melanoma using cytospin preparation of FNAs.[8] While testing immunostainig on melanoma cells of effusions (mainly cell block and a few cytospin preparations), MART-1 antibody showed the rate of positive staining (78%) which was compatible with that of S-100 or HMB-45 (81%).[6] A study consisting of cell blocks prepared from 40 melanoma (mainly FNAs) and 32 nonmelanocytic neoplasms demonstrated that in comparison with S-100 and HMB-45, Melan-A has the greatest sensitivity (95%) and NPV (94%). Both specificity (97%) and PPV (97%) of Melan-A are compatible with that of HMB-45, which were greater than that of S-100.[7]

In this study, immunostaining was performed on the cell blocks which were prepared predominantly from FNAs. Similar to the previously reported data, Melan-A also demonstrated the greatest sensitivity (93%) and NPV (90%). Initially, an intermediate specificity and positive predictive value (PPV) were obtained for Melan-A antibody (81 and 87%) that were relatively lower than the aforementioned data.[7] The relatively lower specificity and PPV for Melan-A is thought to be associated with the false-positive immunostaining for Melan-A seen in 8 nonmelanocytic neoplasms. The false-positive staining was caused by retrieved endogenous biotin, a product of the heating procedure, which is a known factor causing pitfalls in immunostaining.[11] However, applying the pretreatment with avidin/biotin blocking reagents eliminated the false-positive reaction with Melan-A antibody in seven cases and therefore improved both specificity (98%) and PPV (98%) for Melan-A antibody. It has been reported that false-positive reaction resulting from endogenous biotin can be effectively avoided by utilization of newer, nonavidin-biotin polymer-based methods.[12,13] Currently, immunostaining for Melan-A is performed in our laboratory using the biotin-free method.

It is noteworthy to mention that a few specimens of melanoma group in the current study showed positivity staining for Melan-A or S-100 only ($n = 3$ each). On the other hand, none of the melanoma specimens reacted with the antibody against HMB-45 alone.

In an effort to continue to explore the most cost-effective approach of utilization of ancillary tests as a complement in the cytologic diagnosis of melanoma, identification of the optimal panel of stains is desired. On the basis of our results and the results of other studies, Melan-A can be used by itself and S-100 can be used subsequently when Melan-A is not informative. HMB-45 seldom adds additional information.

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