Immunostaining in Mohs Micrographic Surgery: A Review

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BACKGROUND With the advent of incorporating the immunoperoxidase staining technique into the processing of frozen tissue, the use of Mohs micrographic surgery (MMS) has been expanded to include several high-risk tumors such as lentigo maligna, malignant melanoma, and dermatofibrosarcoma protuberans.

OBJECTIVES To thoroughly review the English medical literature pertaining to the use of immunohistochemical staining techniques on frozen sections during MMS and to summarize the basic relevant outcomes from the different relevant studies.

MATERIALS AND METHODS Medline search was conducted, with the following words used in the search criteria: ''Mohs surgery,'' ''staining,'' ''immunostaining,'' and ''immunoperoxidase.''

RESULTS Generally, all immunostains showed advantage over the traditional hematoxylin and eosin approach. Studies of MART-1 in melanoma chemosurgery indicated that it is typically crisp and has less background staining than MEL-5 and better staining consistency than HMB-45. In cases of desmoplastic melanomas, S100 is the stain of choice.

CONCLUSION Immunostaining offers an advantage in MMS. Large, randomized, prospective studies comparing the different immunostains are still lacking in the literature.

The authors have indicated no significant interest with commercial supporters.

Mohs micrographic surgery (MMS) is historically used for the treatment of basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). It has been occasionally used for the treatment of other types of tumors such as malignant melanoma[1] and microcystic adnexal carcinoma,[2] as well as other malignant eccrine neoplasms,[3] malignant follicular tumors,[4] Merkel cell carcinoma,[5] sebaceous carcinoma,[6] atypical fibroxanthoma,[7] malignant fibrous histiocytoma,[8] dermatofibrosarcoma protuberans,[9] leiomyosarcoma,[10] and extramammary Paget's disease.[11]

Immunoperoxidase technique, using formalin-fixed and paraffin-embedded tissue, normally takes several hours to process. The technique works well for regular excisions when the specimen can be processed in 24 hours but is not suitable for Mohs surgery, when tissue is regularly processed using the frozen section technique. Initially, the Mohs technique was modified to include "rush" permanent sections, particularly in instances in which frozen section reading was difficult to interpret, as in cases of lentigo maligna[12] or lentigo maligna melanoma.[13] Subsequently, the immunohistochemical staining technique was modified to allow for rapid staining and hence was performed on frozen tissue in Mohs surgery.[14–16] One advantage of immunostaining frozen sections rather than formalin-embedded sections is the prevention of loss of antigens. In formalin-fixed tissue, the cell membrane is generally disrupted as a result of

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 • Published by Wiley Periodicals, Inc. • ISSN: 1076-0512 • Dermatol Surg 2010;36:275–290 • DOI: 10.1111/j.1524-4725.2009.01432.x

autolysis, and cell-surface antigen staining is not reliable In contrast, with frozen section staining, cytoplasmic and cell membrane antigen-staining can be displayed.[17]

Although previous techniques of immunostaining required immunofluorescence, necessitating the use of a specialized microscope, the current immunohistochemical staining techniques allow for examination of the slide under standard light microscopy.

The Immunohistochemical Staining Technique

The original, or direct, method of immunostaining allowed a single antibody, which is conjugated to an enzyme, to interact with an antigen present on the cell of interest. A substrate is then added, and after a reaction mediated by the conjugated enzyme, the substrate will fluoresce or form an insoluble color product deposited near the antibody (Figure 1). The pitfall of this technique is that it requires high concentrations of the antibody to obtain the staining.[18] In other words, the technique has low sensitivity.

The indirect, or amplifying, technique has proven to be more efficient than the direct technique, particularly in the setting of frozen section tissue examination. In this method, the tissue is initially incubated with a primary antibody and then washed and incubated with a secondary antibody. The secondary antibody is commonly peroxidase labeled. A chromogen is added afterwards and, in the presence of the peroxidase-labeled antibody, will form an insoluble colored product[18] (Figure 2). Commonly used chromogens include 3′-diaminobenzidine tetrachloride, forming a brown product, or 3-amino-9-ethylcarbazole, forming a red product. The advantage of this technique is the requirement for a lower antibody concentration to detect the antigen on formalin sections, but to shorten the time required for processing of frozen sections to 30 to 90 minutes, it is necessary to increase the concentration of the antibody. Although this enhances the sensitivity of the technique, it compromises its specificity by increasing background nonspecific staining. Hence, the use of negative controls in these instances is typically advised.

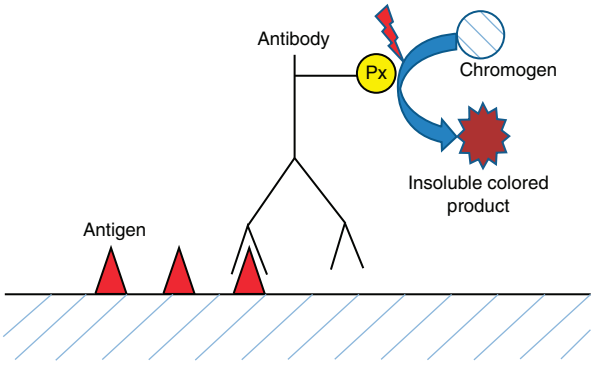

Figure 1. Direct conjugate method. The enzyme, peroxidase (Px) in this example, is attached directly to the antibody that is specific to the antigen under study.

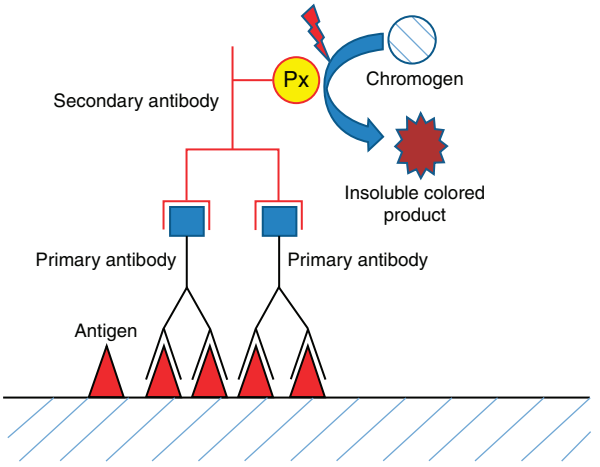

Figure 2. Indirect conjugate or sandwich method. The peroxidase (Px) is attached to the secondary antibody that is specific to the primary antibody.

Other methods of enhancing the sensitivity of the direct and indirect techniques are antigen retrieval and microwave heating in combination with heavy metal–containing solutions or citrate buffers.[18] Fixation of the tissue affects many antigens, and fixed tissue may not stain with the most sensitive techniques. These modifications allow for better staining, or greater sensitivity, but again at the expense of specificity and an increase in background staining. Again, the use of negative controls is advised.

The steps for the indirect technique are as follows:

(1) Cut frozen section 4 to 6 μm thick

(2) Fix specimen in acetone

(3) Air dry or heat

(4) Rehydrate with Tris buffered saline (TBS)

(5) Apply antibody

(6) Rinse with TBS

(7) Apply linking agent

(8) Rinse with TBS

(9) Apply labeling agent

(10) Rinse with TBS

(11) Apply chromogen

(12) Rinse, dehydrate, clear, and mount

Some authors have demonstrated an enhancement in the technique by combining some steps or shortening the length of each step.

Methods of Review

A review of the English literature pertaining to immunohistochemical staining technique use in Mohs surgery was done using a Medline search. The following words were used in the search criteria: "Mohs surgery," "staining," "immunostaining," and "immunoperoxidase." Articles relevant to the use of immunohistochemical staining for frozen sections in Mohs surgery of malignant skin cancers were retrieved (Tables 1–3).

Lentigo Maligna and Malignant Melanoma

The use of MMS for treatment of lentigo maligna and malignant melanoma has historically been somewhat controversial. These disorders can be difficult to diagnose under light microscopy, particularly when it comes to differentiating atypical melanocytic proliferation from melanoma.[19,20] Upon comparing hematoxylin and eosin (H&E)-stained frozen sections to permanent sections in melanoma, Cohen and colleagues found a sensitivity of 73% and a specificity of 68%.[21] In contrast, other authors have reported that the sensitivity and specificity of H&E-stained frozen sections for the evaluation of surgical margins of lentigo maligna are 100% and 90%, respectively.[22] These results can be achieved only using excellent-quality sections and experienced reviewers, yielding more than a 90% survival rate at 5 years of follow-up.[23]

The introduction of immunostaining has significantly decreased the controversy surrounding the use of Mohs surgery for lentigo maligna and malignant melanoma. It has greatly facilitated the detection of melanocytes and the diagnosis of melanoma on permanent sections and can be used on frozen sections as well (Table 1).[24]

According to Gross and colleagues,[25] to make MMS successful for melanoma, the following four criteria must be met: the tumor cells must be visually identifiable in the sections, the tumor must be contiguous to avoid false-negative margins, the mapping and staining component must be technically feasible, and the total tissue processing time should be short enough to allow for a staged excision and repair on the same day.

Several types of immunostains have been used to identify abnormal melanocytes.

Human melanoma, black-45 (HMB-45) is a mouse monoclonal antibody that recognizes a 30- to 35-kDa melanosome-associated sialated glycoprotein.[26] It is present in stage I and II melanosomes of neoplastic melanocytes and stage II and III melanosomes of fetuses and infants.[26,27] Staining is cytoplasmic and granular and is independent of tyrosinase activity.[27] The sensitivity of HMB-45 for melanocytes in melanoma on paraffin-embedded sections has been reported to be 86% to 97%.[28] HMB-45 staining is often negative in spindle cell melanomas, including desmoplastic and neurotropic melanomas, although newer antigen retrieval techniques have been reported to increase sensitivity to 75% in spindle cell melanomas.[29]

TABLE 1. Summary of Immunostains Used on Frozen Sections During Mohs Micrographic Surgery for Melanoma In Situ (MIS) and Malignant Melanoma (MM)

Author	*Immunostain Used*	*Patients Tested*	*Positive Results*	*False-Positive Results*	*False-Negative Results*	*Comments and Results*	*Duration of Follow-Up*	*Location*	*Primary/Recurrent*	*Clinical Outcome*
Griego and Zitelli[44]	HMB-45	1	1	0	0	Stained margins that were negative according to H&E	22 months	Acral	1/0	No recurrence
Menaker et al.[45]	HMB-45	20 (18 MIS, 2 MM)	11	1	0	Compared with paraffin sections: Sensitivity = 100%, Specificity = 95%	NA	NA	18 MIS and 2 MM/0 MIS and 0 MM	NA
Zalla et al.[46]	HMB-45	59 (distribution nondefined)	50	0	2	Missed 2 desmoplastic neurotropic melanoma	1–32 months (average 16 months)	Head	46 MIS and 22 MM/0 MIS and 0 MM	No recurrences
	MEL-5	13 (distribution nondefined)	12	0	1	Missed 1 desmoplastic neurotropic melanoma				
	Melan-A	27 (distribution nondefined)	26	0	1	Missed 1 desmoplastic neurotropic melanoma				
	S100	5 (distribution nondefined)	5	0	0	More background staining				
Gross et al.[25]	MEL-5	2 lentigo maligna	2	0	0	Positive controls from tumors and negative controls from contralateral site used	15 and 16 months	Left earlobe and right preauricular cheek	2 lentigo maligna/0 lentigo maligna	No recurrence
Bhardwaj et al.[47]	Mel-5	200 (158 MIS, 42 MM)	200	0	0	Detected all cases	Average 38.4 months (6–58 months)	25 periorbital, 49 on nose, 21 on ear, 5 on lip, 17 on scalp, 35 on cheeks, 17 on forehead and temple, 19 on arms, 4 on hands, 5 on legs, 1 on feet, 2 on back	97 MIS and 26 MM/39 MIS and 10 MM	One recurrence; 3 patients had postoperative radiation therapy

Kelley and Starkus[48]	MART-1	7 (all lentigo maligna)	7	0	0	Compared with paraffin sections with 100% accordance	NA	Face	7 MIS/0 MIS	NA
Albertini et al.[49]	MART-1	10 cases (7 MIS, 3 MM)	NA	NA	NA	MART-1 was more sensitive than HMB-45	NA	Head and neck	NA	NA
	HMB-45	10 cases (7 MIS, 3 MM)	NA	NA	NA	HMB-45 failed to demonstrate many foci of melanocytic proliferation	NA	Head and neck	NA	NA
	S-100	10 cases (7 MIS, 3 MM)	NA	NA	NA	Poor controls were achieved with S-100	NA	Head and neck	NA	NA
Bricca et al.[50]	MART-1	40 (24 MIS, 16 MM)	40	0	0	Detected 3 cases equivocal on H&E and 1 case negative by H&E	NA	23 on face, 6 on scalp, 6 on trunk, 2 on extremities, 3 on hands or feet	31 (distribution unknown)/9 (distribution unknown)	NA
Kimyai-Asadi et al.[51]	MART-1	30 MM	30	0	0	Detected all cases	NA	NA	NA	NA

NA, not available.

MEL-5 is a mouse monoclonal antibody that recognizes gp75, the most abundant glycoprotein in melanocytes. It is a member of the tyrosinase-related family of proteins (TRP-1) and is an integral membrane protein of melanosomes, present primarily in stage III and IV melanosomes.[30–32] Also designated TA99, MEL-5 stains melanosome-containing cells, including normal epidermal melanocytes, epidermal components of benign nevi, and 95% of melanomas. However, it also stains epithelial cells in the basal layer of the epidermis through transfer of melanosomes.[30–33] MEL-5 may be negative in amelanotic or desmoplastic melanoma and in the dermal component of melanoma.[31,32]

The Melan-A antigen, or melanoma antigen recognized by T cells (MART-1), is a melanocyte differentiation antigen like gp100 and gp75 (recognized by HMB-45 and MEL-5, respectively) and tyrosinase.[34,35] It is a 22-kDa cytoplasmic melanosome-associated glycoprotein recognized by murine monoclonal antibodies A-103 and M2-7C10. It is present in 80% to 100% of melanomas, resting adult melanocytes, and nevus cells in epidermal and dermal compartments.[34–39] Although one study found a slightly higher sensitivity of HMB-45 for melanoma than Melan-A,[40] most have noted the reverse. Blessing and colleagues[41] found a 97% positivity for Melan-A and 90% positivity for HMB-45 in primary melanomas. In metastatic melanomas, Melan-A stains 81% to 89% of tumors, and HMB-45 stains 75% to 76%.[35,42]

S100 is a 21-Kd protein and was originally given its name because of its solubility in 100% saturated ammonium sulfate solution. It was first found to stain human melanoma cells in 1981. Its function is nor completely understood, but it is thought to function with intracellular calcium trafficking, microtubular assay, or both. Its specificity is low because antibody to S100 stains Schwannomas, ependymomas, astrogliomas, and almost all benign and malignant melanocytic lesions and their metastases. S100 protein is also expressed in the antigen-presenting cells such as the Langerhans cells

TABLE 2. Summary of Immunostains Used on Frozen Sections During Mohs Micrographic Surgery for Basal Cell Carcinoma (BCC) and Squamous Cell Carcinoma (SCC)

Author	*Immunostain Used*	*Patients Tested*	*Positive Results*	*False-Positive Results*	*False-Negative Results*	*Comments and Results*	*Duration of Follow-Up*	*Location*	*Primary/Recurrent*	*Clinical Outcome*
Robinson and Gottschalk[57]	Polyclonal antibodies to fibrous keratin	10 nodular BCC/10 MBCC	10 nodular BCC/10 MBCC	0	0	Good staining of tumors with polyclonal antibodies; poor staining when switched to monoclona	NA	NA	NA	NA
		5 WDSCC/5 PDSCC	5 WDSCC/5 PDSCC	0	0		NA	NA	NA	NA
		3 KA	3 KA	0	0		NA	NA	NA	NA
		2 DT	2 DT	0	0		NA	NA	NA	NA
Robinson[58]	AE1, AE3, AE1-AE3, EKH4, Miles	36 NA BCC	36 NA BCC only to AE1, AE1-AE3, and EKH4	0	0	Recommended AE1-AE3 for aggressive tumors	NA	NA	NA	NA
		36 ABCC	36 AE1-AE3	0	0		NA	NA	NA	NA
		10 WDSCC	Miles, AE1-AE3	0	0		NA	NA	NA	NA
		10 PDSCC	AE1-AE3 (superficial edge of tumor)	0	0		NA	NA	NA	NA
Jimenez et al.[16]	AE1/AE3 and CK14	25 BCC/11 SCC	14 BCC/8 SCC	0 BCC/0 SCC	0 BCC/0 SCC	Detected 1 of 12 (only inflammation seen), 8 morpheaform, 5 BCC with perineural involvement/3 of 6 SCC (only inflammation seen), 5 SCC with perineural involvement	NA	NA	NA	NA
Zachary et al.[14]	AE1	20 SCC	8 SCC	0 SCC	0 SCC	Detected 8 SCC where only inflammation was seen	NA	NA	NA	NA
Setoyama et al.[59]	TNKH1	32 BCC/2 SCC	32 BCC/1 SCC	0 BCC/0 SCC	0 BCC/1 SCC	BCC stained in entirety; SCC stained in periphery; 1 SCC missed	NA	NA	NA	NA

Kist et al.[61]	Ber-EP4	15 NBCC, 11 MBCC, 1 AdBCC	15 NBCC, 11 MBCC, 1 AdBCC	0 BCC	0 BCC	Ber-EP4 stained 2 cases that were negative according to H&E and enhanced the visualization of 13 cases, particularly MBCC type	NA	NA	NA	NA
Jimenez et al.[62]	Ber-EP4	NA	NA	NA	NA	BCC stained on frozen section	NA	NA	NA	NA
Krunic et al.[63]	Anti-Dsg	18 BCC	18 BCC	0 BCC	0 BCC	Capable of staining 4 BCC cases where differentiation of BCC from follicular basaloid proliferation was difficult	Average: 14 months (6-24 months)	18 on-o-se	NA	No recurrences

ABCC, aggressive BCC; AdBCC, adenoid BCC; CK, cytokeratin; Dsg, desomglein; DT, desmoplastic trichoepitheliomas; KA, keratoacanthoma; MBCC, morpheaform BCC; NBCC: nodular BCC; NABCC, non-aggressive BCC; PDSCC, poorly differentiated SCC; WDSCC, well-differentiated SCC.

in skin and interdigitating reticulum cells in the paracortex of lymph nodes.[43]

Griego and Zitelli reported a case of MMS using HMB-45 for a recurrent acral melanoma.[44] The authors noted that HMB-45 stained portions of the margins that were clear in frozen and permanent sections. The patient was followed up for 22 months with no recurrence seen.

Menaker and colleagues described a 90-minute protocol for HMB-45 staining on frozen section, comparing it to similar staining on permanent sections.[45] Twenty patients underwent MMS with HMB-45 staining. Eleven patients were positive. One patient had a false positive result on the HMB-45. Accordingly, the HMB-45 performance on frozen sections, in comparison with permanent sections, had a sensitivity of 100% and a specificity of 95%. The authors acknowledge that the specificity was decreased because of staining of non-malignant melanocytic neoplasms.

Zalla and colleagues described a 90-minute protocol for HMB-45, MEL-5, Melan-A, and S100 stains.[46] They performed immunostaining on 68 tumors (46 melanomas in situ and 22 invasive melanomas). In their study, sections were stained with H&E together with one or more immunostains to compare stain quality and results. HMB-45, MEL-5, and Melan-A all exhibited areas of crisp positive staining in areas involved by tumor. When equivocal areas were noted with one immunostain, another immunostain was performed, or the area was considered positive, and further layers were taken. In this study, HMB-45 was positive in 50 of 59 tumors (85%). It was negative in two desmoplastic neurotropic melanomas. The two tumors were detected using immunostaining and not on H&E-stained frozen sections. MEL-5 was performed on 13 cases and stained positive 12 of the 13 tumors (92% of cases), including six of seven HMB-45-negative tumors. One desmoplastic neurotropic melanoma stained negative with MEL-5. MEL-5 was found to be better than S100 in intensity and specificity, with less background

TABLE 3. Summary of Immunostains Used on Frozen Sections During Mohs Micrographic Surgery for Other Kinds of Tumors

Author	*Immunostain Used*	*Patients Tested*	*Positive Results*	*False-Positive Results*	*False-Negative Results*	*Comments and Results*	*Duration of Follow-Up*	*Location*	*Primary/ Recurrent*	*Clinical Outcome*
Robinson [67]	Keratin, GFAP, Desmin	4	0	0	0	DFSP does not stain with keratin, GFAP or desmin	60 months	Left anterior shoulder, right buttock, left scapular area, left anterior chest	4 primary/0 recurrent	No recurrence in 5 years
Jimenez et al.[68]	CD 34	1	1	0	0	Tumor cells stained strongly for CD-34	NA	Medial left breast	1 primary/0 recurrent	NA
Garcia et al.[69]	CD34	1	1	0	0	Tumor cells stained strongly for CD-34	NA	abdomen	1 primary/0 recurrent	NA
Harris et al.[15]	CEA	1	1	0	0	Staining enabled the affected cells to be clearly seen in bright red	8 weeks	Scrotum and left thigh	1 primary/0 recurrent	Complete healing, no recurrence
Smith et al.[70]	S-100	1	1	0	0	Granular cell tumor with extension along nerves/S-100 detected tumor when H&E did not	NA	Right plantar foot	1 primary/0 recurrent	NA
Albertini et al.[71]	Actin	1	1	0	0	Infantile digital fibromatosis	24 months	Right second toe	1 primary/0 recurrent	No recurrence
Marra et al.[72]	Low molecular weight cytokeratin/pan-cytokeratin (AE1/AE3)/CEA/EMA/vimentin	1 low molecular weight cytokeratin/1 pan-cytokeratin/0 CEA/1 EMA/0 vimentin	1	0	0	Primary cutaneous mucinous carcinoma	36 months	Left inferior lateral canthus	0 primary/1 recurrent	No recurrence
Jimenez et al.[73]	AE1/AE3	1	1	0	0	Lymphoepithelioma-like carcinoma of the skin that is difficult to delineate with the reactive infiltrate	12 months	Left nasal ala	1 primary/0 recurrent	No recurrence

Allee et al.[74]	Cytokeratin 17	1	1	0	0	Trichilemmal carcinoma	24 months	Left cheek	0 primary/1 recurrent	No recurrence
Hardaway et al.[75]	HMB-45/S-100/vimentin/desmin/pankeratin/34 BE-12/Cam-5.2/CD-68/CD34/Factor VIIIa/actin/MyoD1	0 HMB-45/1 S-100/1 vimentin/1 desmin/0 pankeratin/0 34 BE-12/0 Cam 5.2/0 CD-68/0 CD34/0 Factor VIIIa/1 Actin/1 MyoD1	1	0	0	Embryonal rhabdomyosarcoma	22 months	cheek	1 primary/0 recurrent	Underwent radiation and 2 cycles of chemotherapy after surgery

H&E, hematoxylin and eosin; DFSP, dermatofibrosarcoma protuberans; CEA, carcinoembryonic antigen; EMA, epithelial membrane antigen.

staining, although the authors concur with another study, in which it was found to be less specific than HMB-45, staining non-melanocytic lesions such as pigmented actinic keratoses, pigmented Bowen's disease, and lichen planus-like keratoses.[32] Melan-A was performed on 27 cases, 26 of which were positive, including one case that was negative according to HMB-45. One desmoplastic neurotropic melanoma was weakly positive, and another was negative with this stain.

Gross and colleagues performed MMS on two cases of lentigo maligna using MEL-5.[25] A 75-minute protocol was followed. Positive controls were taken from the center of the tumors, and negative controls were taken from the contralateral sites. In the authors' opinion, MEL-5 was superior to the other immunohistochemical stains because it reliably stained epidermal melanocytes and melanoma cells. Follow-ups of 15 and 16 months revealed no recurrence of tumor.

Bhardwaj and colleagues[47] studied the use of MEL-5 on 200 cases of primary or recurrent lentigo maligna and malignant melanoma. MEL-5 was done in parallel with H&E staining. The MEL-5 staining technique required approximately 40 minutes, but with the use of an autostainer, the time needed was shortened to 20 minutes. There was no mention of MEL-5 highlighting areas not previously highlighted with H&E staining. Of the 200 patients, three needed postoperative radiation therapy. Only one recurrence has been observed. The mean duration of follow-up in this study was 38.4 months. there was no mention of the sensitivity or the specificity of the 200 tumors studied although the authors mention a previous retrospective study that used MMS for the treatment of melanoma in situ. In that study, the sensitivity and specificity were 59% and 81%, respectively, when comparing Mel-5-stained frozen sections with Mel-5-stained paraffin sections.

In 2002, Kelley and Starkus reported a series of seven patients with lentigo maligna on whom MMS was performed, and frozen sections stained with

H&E and MART-1 were compared with permanent sections.[48] MART-1 staining on frozen sections correlated 100% with MART-1 on permanent sections and detected the atypical melanocytes in all seven cases. In this study, the authors emphasized the difficulty in discerning atypical melanocytes of lentigo maligna from the surrounding atypical melanocytic hyperplasia of sun exposure. For that purpose, sections from nine cases of normal photodamaged skin and five photoprotected cases were evaluated and used as a baseline for comparison. The authors concluded that, when the margin of lentigo maligna is evaluated, confluence of atypical melanocytes alone is not enough to label margins as positive. At minimum, there must be crowding of the atypical melanocytes.

In the same year, Albertini and colleagues reported their experience comparing MART-1, HMB-45, and S100.[49] Their protocol required approximately 2 hours for the immunostaining to be completed. Some patients needed more than 1 day to achieve negative margins. Ten cases were stained with H&E and the three mentioned stains. Positive and negative control stains were processed for each immunostain for each layer. MART-1 had better sensitivity than HMB-45 and S100 and was the preferred stain in the final pathologic determination. HMB-45 failed to demonstrate many foci of melanocytic proliferation visible with MART-1. S100 had poor controls on frozen sections, although good controls were achieved when permanent fixation was used.

In 2004, Bricca and colleagues suggested a shorter protocol for Melan-A staining.[50] In brief, the protocol decreased the duration from a 90-minute to a 60-minute protocol. In comparison with other protocols, the 1-hour protocol eliminated the linking step and shortened the protein blocking step. The protein blocking step was reduced by using a blocking agent that has high quantities of nonspecific immunoglobulin (Ig)G. The linking step was omitted by using a special secondary antibody that is bound to a spherical polymer that is directly attached to horseradish peroxidase (HRP). In this study, 40 patients with lentigo maligna and malignant melanoma were evaluated. The MART-1 stain delineated the malignant cells in three equivocal cases and one negative case according to H&E staining.

In 2008, Kimyai-Asadi and colleagues[51] reported a protocol for rapid MART-1 staining that needed only 20 minutes to perform. The heating period, fixation phase, drying phase, rehydration phase, blocking phase, antibody application phase, buffering phases, HRP application phase, and mounting phase were shorter in the 20-minute protocol than in the 1-hour protocol by Bricca and colleagues. MART-1 was able to delineate all 30 malignant melanomas studied, although there was no follow-up on the patients. The authors mention that MART-1 enhances the sensitivity and specificity of melanoma detection on frozen section, although it is not a reliable stain for spindle cell melanoma.

Melan-A epidermal staining on frozen section is intense and crisp, with regular staining of normal basilar cells and less background staining than MEL-5, thus giving less equivocal results than MEL-5. The consistency of Melan-A stain is better than that of HMB-45, and more cases are detected than with HMB-45.[46,49] However, in the case of desmoplastic melanomas, S100 offers advantages over the other stains.

BCC and SCC

Although the histologic recognition of BCC and SCC in frozen sections is straightforward in most cases, situations exist in which clear delineation and mapping of the tumor infiltration can be difficult. For example, the presence of a dense inflammatory lymphocytic infiltrate in tissue sections may make it difficult, if not impossible, to detect the malignant cells.

Cytokeratins are one of the five types of intermediate filaments that constitute the cytoskeleton of the cell. At least 20 types of cytokeratins have been characterized. Glandular epithelium is composed mainly of

low- (LMW) to intermediate-molecular-weight keratins, and squamous epithelium consists primarily of the more complex high-molecular-weight (HMW) keratins. BCCs express cytokeratins 5, 14, 15, and 17,[52–55] and cutaneous SCCs express cytokeratins 5, 6, 8, 14, 17, and 18.[53,56] In routine practice, a cocktail that recognizes a wide spectrum of keratins is used. AE1 detects the HMW cytokeratins 10, 14, 15, and 16, and also the LMW cytokeratin 19 (acidic keratin). AE3 detects the HMW cytokeratins 1, 2, 3, 4, 5, and 6 and the LMW cytokeratins 7 and 8 (basic keratin). By combining these two reagents, a single reagent with a broad spectrum of reactivity against HMW and LMW cytokeratins is obtained.

In 1984, Robinson and Gottschalk[57] evaluated several tumors using cytokeratin antibodies using two kinds of techniques: immunofluorescence and immunoperoxidase (Table 2). Their study indicated that immunoperoxidase is sensitive in recognizing BCCs, keratoacanthomas, and desmoplastic trichoepitheliomas. Monoclonal and polyclonal antibodies were used. Although polyclonal antikeratin antibodies stained all tumors mentioned previously, the intensity of the staining correlated with the degree of differentiation of the tumor. Monoclonal antibodies, on the other hand, stained BCC cells with varying intensity and did not stain poorly differentiated SCC.

In 1987, Robinson used several monoclonal antikeratin antibody immunostains to compare invasive BCC and SCC with their less aggressive counterparts.[58] AE1, AE3, EKH4, Miles, and AE1-AE3 combination antibodies were used. EKH4 is a monoclonal mouse antibody derived from human trichilemmoma cells. Miles antibodies are derived from bovine hoof prekeratin antibodies and will stain the granular and corneal epidermal layers. Nodular BCC exhibited staining with AE1, EKH4, and an AE1-AE3 combination but not Miles antibodies. As the tumor becomes more aggressive, AE1-AE3 still stain the tumor, but EKH4 and AE1 will stain only peripheral cells and ultimately becomes occasional and nonreliable. Well-differentiated SCC displayed strong staining for the Miles antibody in keratin pearls and AE1-AE3 staining throughout the tumor. In contrast to nodular BCC, AE1 and EKH4 only weakly and occasionally stained the malignant cells. As the SCC became more invasive, there was loss of the AE1-AE3 stain at the deeper margins.

In 1994, Zachary and colleagues used cytokeratin AE1 in 20 cases of SCC.[14] The immunostain detected all 20 SCCs. In eight of 20 cases, small clumps or single cells of residual tumor were identified using cytokeratin-positive staining. The residual tumor often resided in areas of inflammation. Positive controls were taken from the vertical sections of the primary tumor. In the papillary dermis, the occasional presence of globular material that stained positive with the procedure was noted and might have beeen mistaken for a false-positive result if no positive controls had been used.

Jimenez and colleagues[16] used a broad-spectrum anticytokeratin (AE1/AE3) together with anticytokeratin 14 in the detection of BCC and SCC during MMS. They described a 1-hour protocol for the anticytokeratin stain. In this study, the immunostaining technique was able to pick up one of 12 BCCs, and three of six SCCs where only dense inflammatory infiltrate was seen on H&E. The stain was also able to delineate and map out more accurately subtle tumor islands in eight cases of morpheaform BCCs and highlighted perineural involvement in five cases of BCC and five cases of SCC with perineural involvement.

Immunostains other than cytokeratins have also been used. TNKH1, an antiglycoprotein antibody, is a monoclonal antibody that was primarily developed in mice to recognize differentiation antigen of a human melanoma cell line (A375). It was also found to stain epithelial tumors considered to be derived from or differentiating toward hair follicle. In their study, Setoyama and colleagues[59] showed that TNKH1 stained 32 of 32 BCCs, labeling the tumors in their entirety. The antibody was useful in delineating the

upper hair follicle and eccrine duct from BCC tumors. The antibody also irregularly stained one of two SCCs over the periphery of the tumor.

Ber-EP4 is an antibody raised against an epitope of the protein region of two human epithelial membrane glycoproteins.[60] Its ability to differentiate BCC from SCC has been demonstrated previously. Kist and colleagues[61] and Jimenez and colleagues[62] found Ber-EP4 to be useful during MMS for BCC tumors.

Krunic and colleagues used the monoclonal antibody 33-3D, a mouse IgM monoclonal antibody that recognizes the cytoplasmic domains of human desmoglein, during MMS for 18 cases of BCC.[63] The protocol described for staining required approximately 60 minutes to perform. The antibody shows intense pericellular staining around normal cells and will show general or diffuse cytoplasmic staining or a reduction in staining in areas of tumors. The antibody helps to differentiate BCC from hair follicles or from folliculocentric basaloid proliferation (FBP) in horizontal sections.[63] FBP is vertically oriented basaloid proliferations that can radiate outward and downward from the hair follicle and up toward the skin edges. It surrounds BCC and may extend inward to the proliferating border of the neoplasm.[64] In another study, Krunic and colleagues stained 24 SCCs and 12 keratoacanthomas (KAs) using the antidesmoglein antibodies on frozen sections from excised lesions.[65] All 12 KAs showed extensive, uniform pericellular staining for desmoglein throughout the nonkeratinized layers of the tumor, whereas all five SCCs showed only focal pericellular staining, and seven SCCs showed absence of staining.

Other Kinds of Tumors

Dermatofibrosarcoma Protuberans

Dermatofibrosarcoma protuberans (DFSP) is a tumor that is commonly removed using MMS. The problem is that its histologic differentiation on H&E-stained frozen sections can be difficult, particularly at the margin of the tumor and at areas of scarring from previous surgical procedures. Traditional surgical excision usually fails to treat the microscopic extensions, and in most series, recurrence rates of 49% to 53% have been reported, with the majority developing 1 to 2 years after therapy.[66] When surgical margins of 3 cm have been used, the recurrence rate drops to 10% to 20%.[8]

In 1985, Robinson reported a 5-year prospective study of four cases of DFSP treated using MMS.[67] Staining with keratin, glial fibrillary acid protein, and desmin were attempted but were negative in all four tumors (Table 3).

CD34 antigen is typically found in hematopoietic stem cells, endothelium, dermal and periadnexal dendritic cells, and endoneuronal dendritic cells. Its detection helps in differentiating DFSP from keloids, dermatofibroma, atypical fibroxanthoma, and malignant fibrous histiocytoma. There is greater variability of CD34 staining in nodular areas than in plaque areas of DFSP.[67]

In 1994, Jimenez and colleagues used CD-34 immunostaining on MMS for a DFSP over the left medial breast of a 13-year-old girl.[68] The protocol described took approximately 1 hour. The tumor immunostaining was strongly positive. Positive control was used from the first stage, where tumor was present.

In 1996, Garcia and colleagues reported the use of CD-34 immunostaining during MMS on another patient with a DFSP over the abdomen.[69] The tumor again strongly stained with CD-34, although it was negative on the initial biopsy. The authors recommend biopsies from the plaque areas of the tumor with the inclusion of fatty tissue to improve the diagnostic yield of the CD-34 immunostain.

Miscellaneous Tumors

Harris and colleagues reported the treatment of extramammary Paget's disease on one patient using

carcinoembryonic antigen (CEA) during MMS.[15] The use of a quick-staining immunoalkaline phosphatase kit that, when combined with high-affinity primary antibodies, reduced the total staining time to 30 minutes, producing moderate-intensity cytoplasmic staining. As an internal control, the CEA stains eccrine and apocrine cells uniformly throughout the cytoplasm. Although Periodic Acid-Schiff stain can be used during MMS for extramammary Paget's disease, the authors find CEA to be a superior diagnostic method.

Smith and colleagues reported the treatment of one case of granular cell tumor on the right plantar foot.[70] In their case report, the tumor was tracking along the nerves, and the last three layers during the surgery showed positivity only with S100 but not with the H&E staining.

Albertini and colleagues have used actin stain in addition to H&E and Masson trichrome in a case of a tender infantile digital fibromatosis.[71] Although the tumor involved the joint capsule and was left positive at that margin, 2-year follow-up showed no recurrence of disease.

Marra and colleagues described a 45- to 60-minute protocol using automated staining for LMW cytokeratin, pancytokeratin (AE1/AE3), CEA, epithelial membrane antigen (EMA), and vimentin during MMS for one case of primary cutaneous mucinous carcinoma.[72] In this report, the tumor was positive for LMW cytokeratin, AE1/AE3, and less clearly EMA but was negative for CEA and vimentin. The authors commented that the adnexal structures normally stained positive with the LMW cytokeratin and could thereby confound the interpretation of immunostained slides, whereas the tumor cells lacked histopathologic characteristics of acinar or ductal elements.

Jimenez and colleagues used pan-cytokeratin staining (AE1/AE3) during MMS for lymphoepithelioma-like carcinoma of the skin.[73] The staining was particularly helpful in some areas where the tumor cells were difficult to delineate within the reactive infiltrate. Normal epidermis was used as positive internal control, and negative control runs were performed in parallel. The patient was free of recurrence after a follow-up of 12 months.

Allee and colleagues have used cytokeratin-17 during MMS of a recurrent trichilemmal carcinoma over the left cheek.[74] Positive and negative margins were taken. The tumor showed strong cytoplasmic staining of the tumor cells for cytokeratin-17 and failed to stain for cytokeratin-15. Cytokeratin-17 is an intermediate filament expressed constitutively in the outer root sheath of the hair follicles but not in the interfollicular epidermis, whereas cytokeratin-15 is expressed in a subpopulation of keratinocytes in the bulge area of the outer root sheath.

Hardaway and colleagues[75] have used HMB-45, S100, vimentin, desmin, pankeratin, 34 BE-12, Cam-5.2, CD-68, CD34, Factor VIIIa, actin, and MyoD1 during MMS for an embryonal rhabdomyosarcoma of the cheek. S100, vimentin, desmin, actin and MyoD1 were the only positive stains. The authors emphasize that MyoD1 and myogenin are DNA-binding proteins that are involved in the differentiation of mesenchymal progenitor cells and have been used as markers in characterizing rhabdomyosarcoma.

Prevalence and Costs

In 2001, Robinson conducted a survey of 108 Mohs surgery laboratories.[76] Thirteen laboratories were doing immunostaining at the time (13%). Performance of immunostaining was associated with resections of melanoma or DFSP, with only one laboratroy using an automated immunostainer. HMB-45 was used by 50% of the labs, S100 by 42%, Mart-1 and MEL-5 by 42%, antikeratin by 42%, and anti-CD34 by 33%. The author emphasized that, by using polyclonal antibodies instead of monoclonal antibodies and by using higher antibody titers, the processing time would be further shortened and would fall within the expectations of Mohs

surgeons. The *International Classification of Diseases, Ninth Revision,* code for immunostaining sections in Mohs surgery is 88,342, with a current Medicare reimbursement of approximately $100. The current estimate of cost per slide is between $20 and $25.

Conclusion

The use of immunohistochemical staining during MMS is definitely on the rise, particularly in cases of lentigo maligna and malignant melanoma, but large randomized prospective studies comparing the different immunostains are lacking in the literature. As a result of increasing familiarity with the stains, faster processing, and lowering costs of the antibodies, Mohs surgeons should be encouraged to integrate immunostaining into their laboratory routine.

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