Comparison of MART-1 Frozen Sections to Permanent Sections Using a Rapid 19-Minute Protocol

Basil S. Cherpelis, MD, Richard Moore, MD, Sharron Ladd, BS, Ren Chen, MD, MPH, and L. Frank Glass, MD*

BACKGROUND The use of melanoma-associated antigen recognized by T cells (MART-1) immunostain has been proposed as a useful adjunct to overcome the inherent difficulties in the use of frozen sections during Mohs surgery for the treatment of melanoma, but no studies have compared MART-1 frozen sections with MART-1 permanent sections. Current MART-1 1-hour protocols add significant time to the procedure.

OBJECTIVE To determine whether there is a significant difference between frozen and permanent MART-1 immunostained sections using a rapid 19-minute protocol.

METHODS Frozen and permanent sections stained with MART-1 were made from dog-ears excised during 25 reconstructions. A rapid 19-minute protocol was used to stain the frozen tissue. The sections were examined blinded, and statistical analysis was performed to analyze the data.

RESULTS No significant difference was found in number of keratinocytes, nuclear diameter of keratinocytes, number of melanocytes, melanocytic nuclear diameter, confluence, pagetoid spread, melanocytic nesting, or atypical melanocytes.

CONCLUSIONS The 19-minute protocol is a rapid and effective MART-1 immunostain. Frozen sections stained with MART-1 provide information equivalent to that obtained from MART-1 stained permanent sections. Mohs surgeons using MART-1 can feel confident that they have the same information as they would have obtained using permanent sections using the slow Mohs method.

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Lentigo maligna (LM) is melanoma in situ that presents as a slowly growing pigmented macule on chronically sun-damaged skin of the head and neck, especially on the face of elderly patients. LM can often extend far beyond the clinical extent of the lesion.[1,2] For this reason, intra-operative margin control would be the ideal method of treating these cancers. Although delayed permanent sections can be used, the delay is inconvenient for patient and physician. Mohs surgery has been shown to be effective in treating these cancers,[3–5] but it can be difficult to identify melanocytes and to distinguish chronic sun-damaged skin from melanoma using hematoxylin and eosin (H&E)-stained frozen sections. This difficulty led to the use of melanoma-associated antigen recognized by T cells (MART-1) immunostaining on frozen sections. The addition of MART-1 immunostaining has improved the ability to identify melanocytes on frozen sections,[6,7] but some still question the validity of frozen-section immunostains.[8] Although a rapid 11.5-minute protocol has been published, neither statistical analysis nor comparison of this protocol with permanent sections was reported.[9] The most common protocol reported in the literature is 1 hour long.[10] We report the use of a novel rapid 19-minute MART-1 immunostain protocol that can be used to stain frozen sections quickly. This frozen-section immunostain was compared with the permanent-section immunostain to see whether there was a significant difference.

**All authors are affiliated with the University of South Florida, Department of Dermatology and Cutaneous Surgery, Tampa, Florida*

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Methods

Our institutional review board approved this study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Surplus tissue from patients undergoing surgery for basal and squamous cell carcinomas of the head and neck over a 3-month period was used for additional processing and immunohistochemical staining. For each patient, age, sex, location, type of tumor excised, personal history of dysplastic nevi or melanoma, and history of recent intense sun exposure were recorded. Recent intense sun exposure was defined as outdoor exposure for longer than 15 minutes within 2 weeks before surgery. Recent intense sun exposure can increase the number and activity of melanocytes.

Dog-ears excised during the reconstruction of the defects left after the extirpation of the tumors were collected and submitted for study. Dog-ears were excluded if a skin lesion was present or if the patient had a history of radiation to the face or neck. Frozen and permanent sections (formalin fixed, paraffin embedded) were made by dividing the dog-ear tissue in half and using half for each protocol. A thickness of 4 μm was used for all sections. Sections were stained for H&E and MART-1 immunostain (Figures 1–4). The standard dermatopathology laboratory protocol for MART-1 immunostaining on formalin-fixed, paraffin-embedded sections was used. This protocol uses citrate buffer for heat-induced epitope retrieval and uses the Biogenex (San Ramon, CA) automated stainer to apply the same reagents used in the rapid protocol, only with extended times. Signet Pathology Systems (Orlando, FL) makes the humid slide-staining chamber in our laboratory. A rapid 19-minute protocol was used to stain the frozen sections (Table 1).

A single observer, a board-certified dermatopathologist, examined the slides in a blinded fashion. The sections were examined with × 20 and × 40 objectives for number, density, contiguity, pagetoid spread of melanocytes, and atypical melanocytes.

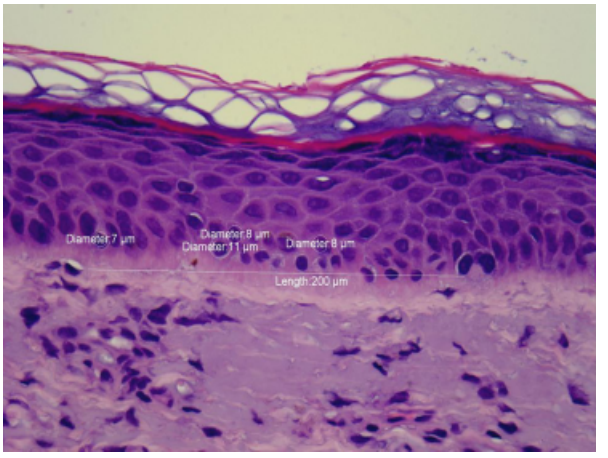

Figure 1. Permanent section stained hematoxylin and eosin (× 20 magnification).

A melanocytic nest was defined as three or more melanocytes clustered together in direct contact with each other but not necessarily the basement membrane. Contiguous melanocytes were defined as three or more adjacent melanocytes in direct contact with the basement membrane.[11] Atypical melanocytes were defined as the presence of atypical nuclei. Atypical melanocytic nuclei were larger than keratinocytic nuclei or exhibited significant pleomorphism. Pagetoid melanocytes were defined as melanocytes above the dermal epidermal junction.

Five representative areas of each section measuring 200 μm were examined and photographed for

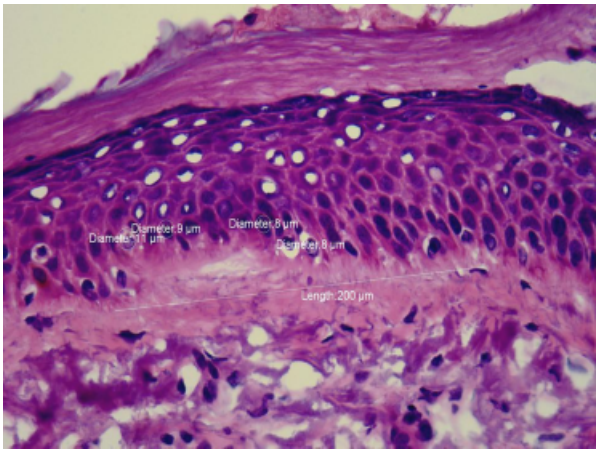

Figure 2. Frozen section stained with hematoxylin and eosin (× 20 magnification).

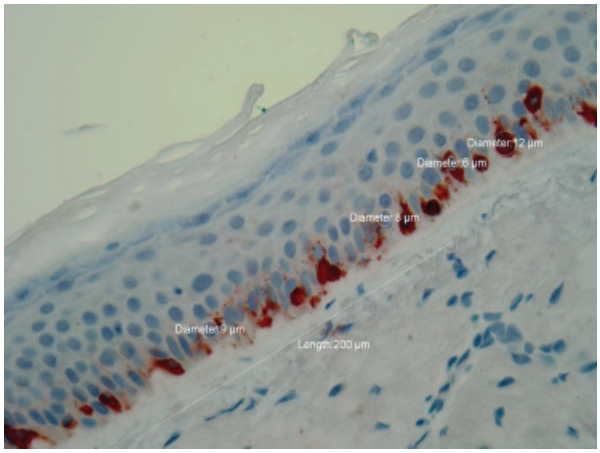

Figure 3. Permanent section stained with melanoma-associated antigen recognized by T cells (× 20 magnification).

recording of data. Photomicrographs were obtained using a high-resolution digital camera, and histopathologic parameters were recorded using SPOT imaging software (Diagnostic Instruments, Inc., Sterling Heights, MI). Melanocyte and keratinocyte densities were recorded on 200-µm segments and extrapolated to the customary 500 µm, or 1 high power field (hpf) designation. These parameters were recorded as the median of five representative 200-µm segments. Unusually thick sections were not used because of inconsistencies in immunostaining and because overlapping melanocytes may give the appearance of greater melanocyte density. Only melanocytes and keratinocytes that were clearly in focus and easily identifiable were counted, to avoid inflating the figures.

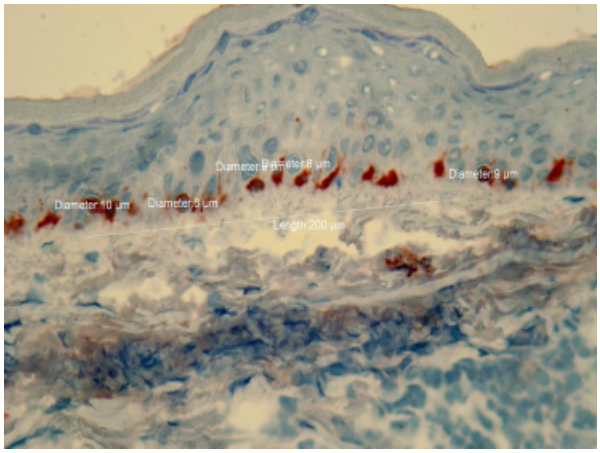

Figure 4. Frozen section stained with melanoma-associated antigen recognized by T cells (× 20 magnification).

TABLE 1. Rapid 19-Minute Immunostain Protocol for Frozen Sections

1	Cut 4-µm sections and mount on positively charged barrier slides.
2	Fix in acetone for 30 seconds. A small coplin staining jar can be used for fixing and rinsing steps.
3	Rehydrate in 1 × citrate buffer (Biogenex HK080-9K) for 30 seconds.
4	Remove excess buffer by shaking slides or placing the slide edge on a paper towel, then place slides in a humid slide-staining chamber at 37°C.
5	Apply primary antibody (melanoma-associated antigen recognized by T cells)* for 5 minutes
6	Quick rinse in citrate buffer for 10 seconds
7	Apply link (Super-Sensitive MultiLink Biogenex HK340-9K)* for 5 minutes
8	Quick rinse in citrate buffer for 10 seconds
9	Apply label (Super-Sensitive Label-Streptavidin peroxidase Biogenex HK330-9K)* for 5 minutes
10	Quick rinse in citrate buffer for 10 seconds
11	Apply AEC chromogen (3-amino-9-ethyl-carbazole in N,N-dimethyl formamide) for ~1 minute (watch color develop)
12	Rinse in water for 10 seconds
13	Counterstain with Mayer's hematoxylin for 30 seconds
14	Rinse and blue in warm tap water for 10 seconds
15	Mount with aqueous mounting medium

*With heat (37°C) and humidity. AEC, amino ethyl carbazol.

Three forms of statistical analyses were performed on the results to compare the frozen and permanent-section groups: *t*-test, Fisher exact test, and multivariate analysis of variance (MANOVA) procedures. For measurements that follow an approximately normal distribution and are independent events, a simple *t*-test was used to test whether MART-1-staining paraffin and frozen sections would give significantly different measurements. The Fisher exact test was used for dichotomous variables. MANOVA is a multivariate significance test that controls for inflated type I errors (false-positive results) and analysis of all of the measurements simultaneously.

Results

All 25 patients were Caucasian. Of these 25 patients, 17 (68%) had basal cell carcinoma and eight (32%) had squamous cell carcinoma. The mean age of the patients was 65 (range 41–84); 50% were men, and 50% were women. Eight (32%) patients had Fitzpatrick skin Type I, 13 (52%) Type II, and five (16%) Type III. Three (12%) patients had a prior history of melanoma, and 12 (48%) had a prior history of nonmelanoma skin cancer. A family history of melanoma was reported in two (8%) patients and a family history of nonmelanoma skin cancer in six (24%). A prior history of atypical (dysplastic) nevi were reported in three (12%) patients. No patients had a history of recent intense sun exposure. All skin samples were from sun-exposed areas of the head and neck.

There was no statistical correlation between melanocyte density and anatomic location or patient age. In addition, no association was found between melanocyte density and Fitzpatrick skin type, personal history of dysplastic nevi, or family history of melanoma or non-melanoma skin cancer.

The mean number of melanocytes per high-powered field ($\times 40$) was 16.3 ± 6.9 (median 15, range 2.5–35.0).

Based on $\alpha = 0.05$, no significant difference was found in any of the measurements: numbers of keratinocytes at the dermal-epidermal junction per 200 μm ($t = 1.05$, $p = .30$), nuclear diameter of keratinocytes ($t = -1.19$, $p = .24$), numbers of melanocytes per 200 μ ($t = -0.05$, $p = .96$), melanocyte nucleus diameter ($t = 0$, $p = 1.00$), and melanocyte cytoplasm diameter ($t = -0.26$, $p = .79$) (Table 2).

No significant difference was found in contiguity (chi-square (χ^2) = .015, $p = .50$), pagetoid spread ($\chi^2 = 0.15$, $p = .19$), melanocytic nesting ($\chi^2 = 0.03$, $p = .97$), or atypical melanocytes ($\chi^2 = 0.02$, $p = .3$). Linear regression analysis was used to compare these coincident parameters obtained using the two staining methods, and no significant difference was found ($f = 0.06$, $p = .81$). Nesting was noted in two cases, and atypical melanocytes were noted in two cases. Pagetoid spread of only a single isolated melanocyte was found in three cases, and contiguous melanocytes consisting of three to four melanocytes were noted in two cases.

To control inflation of type I error resulting from the multiple comparisons, MANOVA was also used to test these measurements simultaneously. All of the

TABLE 2. Comparison of Frozen and Permanent Melanoma-Associated Antigen Recognized by T Cells (MART-1) Immunostains

Method	*Variable*	*Mean*	*Standard Deviation*	*Median*	*Minimum*	*Maximum*
MART-1 paraffin	Number of keratinocytes at the DEJ (per 200 μm)	29.84	6.24	30	16	43
	Nuclear diameter of keratinocytes (μm)	7.98	0.92	8	6	10.5
	Number of melanocytes (per 200 μm)	6.68	3.42	7	1	14
	Melanocyte nucleus diameter (μm)	6.90	0.84	7	5	8
	Melanocyte cytoplasm diameter (μm)	10.72	1.39	11	8	13
MART-1 frozen	Number of keratinocytes at the DEJ (per 200 μm)	27.76	7.64	27	16	48
	Nuclear diameter of keratinocytes (μm)	8.26	0.74	8	7	10
	Number of melanocytes (per 200 μm)	6.72	2.62	7	2	12
	Melanocyte nucleus diameter (μm)	6.90	1.00	7	5	9
	Melanocyte cytoplasm diameter (μm)	10.82	1.31	11	8.5	14

DEJ = dermal-epidermal junction.

four statistics from MANOVA gave the same nonsignificant result ($p = .77$).

There was no statistically significant difference in the measured variables between frozen sections and permanent sections stained with MART-1.

Discussion

The primary goal of this study was to compare MART-1 frozen sections with permanent sections. This is important because controversy exists regarding the use of frozen sections in the treatment of melanoma. It can be difficult to identify melanocytes and to distinguish chronic sun-damaged skin from melanoma on frozen sections stained with routine H&E.[7,8] This difficulty led to the use of MART-1 immunostain on frozen sections.

Some of the conventional diagnostic criteria used for melanoma in situ include widespread contiguous melanocytes, widespread melanocytic atypia, multiple nested melanocytes, and numerous pagetoid melanocytes, yet a comparable increase in the number of melanocytes, contiguous melanocytes, and even atypical melanocytes can be found in chronic sun-damaged skin.[11] The spectrum of histologic changes seen at the periphery of melanoma in situ and the baseline features of sun damaged skin often blend, making distinguishing one from the other difficult.[11] We looked for the presence of these individual characteristics in frozen and permanent sections in chronically photodamaged skin. In addition, we recorded certain cytologic parameters such as melanocyte and keratinocyte nuclear and cytoplasmic diameter. We sought to determine whether there was a difference in the ability of one method and the other to identify these characteristics.

We examined frozen and permanent sections of dog-ear skin specimens obtained from patients undergoing excisional surgery for basal and squamous cell carcinomas of the head and neck. Four-μm sections were stained for H&E and rapid 19-minute MART-1 immunostains, and a board-certified dermatopathologist examined them in a blinded fashion. Statistical analysis was performed on recorded histologic parameters to compare the frozen and permanent methods of slide preparation.

We found that normal chronically sun-damaged skin has an average of 16.33 ± 6.9 (median 15, range 2.5–35) melanocytes per high-power field (in 0.5 mm of skin). Our data are comparable with those of Hendi and colleagues, who found a mean of 15.60 ± 4.38 (median 15.0, range, 6–29) melanocytes per high-power field.[12]

Our study showed no statistical correlation between melanocyte density, anatomic location, and patient age. We also found no association between Fitzpatrick skin type, personal history of dysplastic nevi, and family history of melanoma or non-melanoma skin cancer. Our data are similar to those reported by Barlow and colleagues, who also found no association with melanocyte density or the above parameters.[11]

There was no statistically significant difference between frozen and permanent sections in terms of melanocyte density, number of keratinocytes, and cytologic parameters such as melanocytic nuclear diameter and keratinocyte nuclear diameter.

Rare focal areas of contiguous melanocytes, atypical melanocytes, nests of melanocytes, and pagetoid spread were noted in some sections. These features have been reported to occur commonly in areas of melanocytic hyperplasia associated with chronically sun-damaged skin.[11,13] These findings, sometimes referred to as "field effect," may result in confusion when evaluating margins of melanoma in situ involving photodamaged skin. There was no difference between frozen and permanent MART-1 sections in identifying these histopathologic findings.

Our data indicate that frozen sections of sun-damaged skin stained with MART-1 provide the

same information as MART-1-stained permanent sections, although it cannot be inferred from the results that Mohs surgeons using MART-1 on frozen tissue will have no difficulty in determining where chronic sun damage ends and malignant melanoma in situ begins during excisional surgery. Rather, it is likely that this problem is common to all methods of immunohistochemistry regarding melanoma in situ on photodamaged skin. Yet one would infer that the information obtained from rapid frozen sections would be similar to the permanent-section methods of melanoma removal, or "slow Mohs."

A secondary goal of this study was to evaluate the effectiveness of a rapid 19-minute protocol to perform MART-1 immunostaining on frozen tissue. This method has proven to be significantly shorter than the previously reported 1-hour protocol.[10] We found no statistically significant differences between frozen- and permanent-section slides using MART-1. We surmise that the rapid turnaround time of the 19-minute protocol would allow melanoma in situ to be treated using Mohs surgery within a matter of hours as opposed to days or weeks. This protocol has the practical advantage of increasing the number of layers that can be taken in a single day and processed using immunohistochemistry. This increase in efficiency would likely affect the cost-effectiveness of the Mohs surgery unit by increasing the volume of cases, decreasing histotechnician workload, and ultimately improving the experience for the patient.

Immunohistochemistry staining is often perceived to be time consuming and cost ineffective. The 2008 Medicare code 88342 reimburses $92.78 per specimen for immunohistochemistry. This reimbursement includes labor, supply costs, and physician fees. The cost per slide in this study was $30.00. This cost can be less if one is part of an institution or organization with collective buying power. Therefore, immunostaining with MART-1 is cost effective for Mohs surgery frozen-section laboratories.

In summary, we describe a rapid and effective MART-1 immunostain protocol for frozen tissue that could ultimately eliminate the need for time-consuming surgical procedures using permanent sections for margin assessment during melanoma excision. Our study shows that frozen sections stained using the rapid MART-1 protocol provide equivalent information to MART-1-stained permanent sections on chronically photodamaged skin. More study is required, but Mohs surgeons are likely to be using a protocol similar to ours to obtain information in minutes, rather than days or weeks, on which to base their decisions about melanoma margins. Further study is also required to solve problems that remain in distinguishing between melanoma in situ and melanocytic hyperplasia associated with sun-damaged skin that occur regardless of how the tissue is processed and stained.

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Address correspondence and reprint requests to: Basil S. Cherpelis, MD, 12901 Bruce B. Downs Blvd. MDC 79, Tampa, FL 33647, or e-mail: bcherpel@health.usf.edu