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LETTER TO THE EDITOR

Dual Immunohistochemical Detection of Mitoses in Melanoma

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Background

Mitotic rate in melanoma is the second most powerful independent predictor of survival outcome after tumor thickness [1]. The mitotic rate plays a critical role in the 2008 American Joint Committee on Cancer (AJC) TMN staging on melanoma [2]. Mitosis can be obscured by mitotic mimickers such as apoptosis, karyorrhectic debris, and mitosis in non-melanocytic cells make the definitive determination of mitotic rate difficult. The immunohistochemical stain phosphohistone H3 is expressed nearly exclusively by cells during the metaphase component of the cell cycle [3, 4]. The metaphase component of the cell cycle is characterized chromosomal condensation and coiling. The metaphase component of the cell cycle is recognizable in H&E stained tissue through identification of mitotic figures. The antigen expression during this exclusive period of cell division creates an ideal nuclear target for immunohistochemical detection of mitotic figures. PHH3 is expressed in nearly all cells during metaphase so an additional marker is necessary to properly identify the mitotically active cells of interest. Melanoma-associated antigen recognized by T cells (MART-1) provides another useful target as this antigen's target is expressed in the cytoplasm of melanocytes [5]. Combining the sensitivity and specificity of PHH3 for mitotic figures and the sensitivity of MART-1 for melanosomes, cells that are dual stained with both antigens can reliably be quantified as mitotic figures in melanocytes. This dual stain approach allows the user to ignore the cells only staining with PHH3 during the mitotic count as they represent mitotically active cells that are not melanocytes.

Methods

Employing PHH3 and MART-1 we analyzed 20 melanomas from our institution. The melanomas were subdivided into two groups. Thin melanomas comprising tumors less than 1 mm thick (pTMN stage pT1a or pT1b) and thick melanomas with tumors greater than 1 mm thick (pTMN stage pT2 or greater). These tumors were stained utilizing dual immunohistochemical stain and compared to the original H&E stained slides for statistical significance. The original diagnosis of melanoma was determined by a board certified dermatopathologist and confirmed by a second board certified dermatopathologist. The mean patient age was 61.4 years (57 and 69 for the thin and thick melanomas, respectively. The mean tumor thickness was 1.73 mm, (0.51 and 3.47 mm thin and thick melanomas, respectively)

The immunohistochemical slides were prepared with the following method. Paraffin embedded tissue were cut at 4 μm thickness and mounted on Path Supply charged glass slides (Path Supply, Brentwood TN). The Ventana Benchmark XT automated staining system (Ventana Medical Systems, Tucson AZ) provided automating staining utilizing the Mart-1 pre-diluted antibody (Ventana product 790–2990, A103 clone) with an incubation time of 60 min and a retrieval time of 90 min. The phosphohistone H3 rabbit polyclonal stain (Ventana product number 760–4591) was used. The enhanced alkaline phosphatase red detection kit (Ventana 760–031) was used to

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highlight the MART-1 Antibodies and the ultraview universal dab detection kit (Ventana 760–500) incubation time 17 min highlighted the nuclear staining phosphohistone H3.

The dual immunohistochemical stained slides and hematoxylin and eosin stained slides were randomized and assessed by five different individuals blinded to the original diagnosis and mitotic count. The participants included a pathology resident, two dermatopathology fellows, a junior dermatopathology faculty member and a senior dermatopathology faculty member. Participants were initially trained to identify mitoses using a matched pair of H&E slide and a dual immunohistochemical stained slide from a dermal deposit of metastatic melanoma and instructed to familiarize identifying mitotic activity with the dual MART-1 and PHH3 stained sections. Figure 1.

The participants determined the mitotic rates for the thin and think melanomas in the 20 randomized cases analyzing both the H&E slides and the dual stained slides. The mitotic counts for the thin and thick melanomas are displayed seen in Tables 1 and 2, respectively.

Data Analysis

The data was analyzed using Graph Pad Prism 6.0 statistical software. A Wilcoxon matched paired test was used to compare the mitotic counts using the H&E and Dual IHC stained slides using a p value of <0.05 as significant.

The mitotic rates for the thin and thick melanoma groups between all participants utilizing both methods were averaged and results are displayed Figs. 2 and 3, respectively.

The overall mitotic rate average from each reviewer for the thin and thick melanoma groups is displayed in Tables 3 and 4, respectively.

Results

The average mitotic rate detected by all individuals for the group of thin melanomas was 0.5 mitosis per mm^2 utilizing hematoxylin and eosin and 0.74 mitosis per mm^2 utilizing the dual immunohistochemical stain. This is a statistically non-significant ($p = 0.176$) 48% increase in mitotic rate detection. There is one notable case in which the mitotic rate changed utilizing the dual immunohistochemical stain method. The original H&E stained slides failed to reveal any mitoses. The dual immunohistochemical stain highlighted a mitotic figure that was appreciated by three of the five reviewers. This case initially was diagnosed as pT1a lesion with a mitotic rate of 0 mitoses/mm^2.

The mitotic rates determined by the reviewers from the thick melanomas yielded an average mitotic rate of 7.12 mitosis / mm^2 for the H&E stained sections and 11.84 mitosis per mm^2 for the dual immunohistochemical stained sections. This correlates to a statistically significant ($p = 0.008$) 60.1% increase in mitotic rate.

Discussion

Mitotic rate in melanoma is the second most powerful independent predictor of survival outcome after tumor thickness [3, 6]. Mitotic rate is a critical component of the 2008

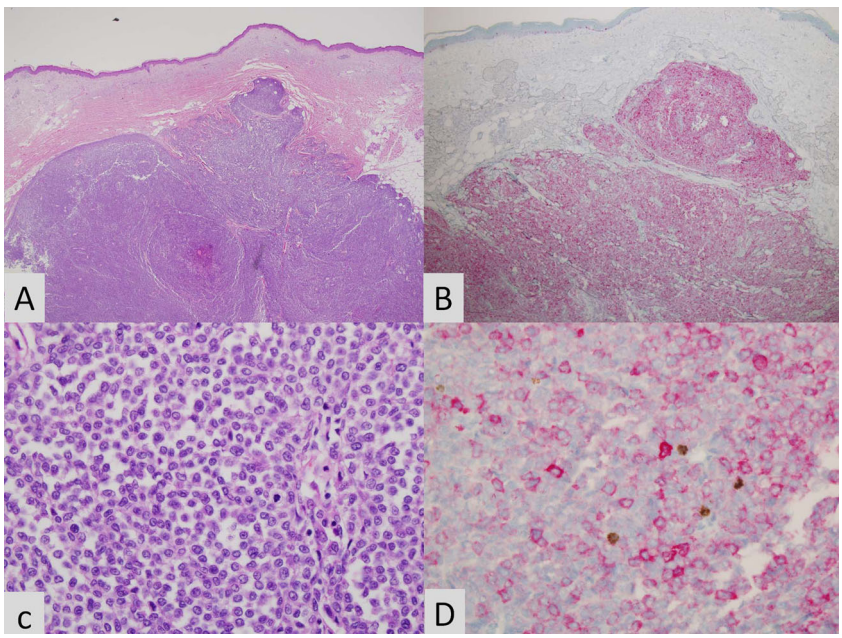

Fig. 1 Dermal deposit of melanoma stained with H&E (A 40×, C 200×) and a dual immunohistochemical stain with PHH3 (brown chromogen) and Mart-1 (Red chromogen) (B 40×, D 200×)

Table 1 Mitotic rate per square millimeter per reviewer utilizing H&E and dual IHC stains on melanomas less than 1 mm thick

Specimen	Reviewer 1 H&E	Reviewer 2 H&E	Reviewer 3 H&E	Reviewer 4 H&E	Reviewer 5 H&E	Reviewer 1 Dual	Reviewer 2 Dual	Reviewer 3 Dual	Reviewer 4 Dual	Reviewer 5 Dual	H&E Average	Dual Average	Original Mitotic Rate
1	1	0	0	0	0	0	0	0	2	0	0.2	0.4	1
2	0	0	0	0	0	0	1	1	2	0	0	0.8	0
3	3	0	1	1	0	0	0	0	0	0	1	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	1
5	0	1	0	0	1	1	1	1	3	3	0.4	1.8	2
6	1	1	1	2	1	2	0	0	0	0	1.2	0.4	1
7	1	0	1	1	1	1	1	1	1	2	0.8	1.2	1
8	2	0	0	0	0	0	0	0	0	0	0.4	0	0
9	0	1	0	0	1	0	1	2	0	0	0.4	0.6	1
10	0	1	1	0	1	3	0	2	4	2	0.6	2.2	1

Table 2 Mitotic rate per square millimeter per reviewer utilizing H&E and dual IHC stains on melanomas greater than 1 mm thick

Specimen	Reviewer 1 H&E	Reviewer 2 H&E	Reviewer 3 H&E	Reviewer 4 H&E	Reviewer 5 H&E	Reviewer 1 Dual	Reviewer 2 Dual	Reviewer 3 Dual	Reviewer 4 Dual	Reviewer 5 Dual	H&E Average	Dual Average	Original Mitotic Rate
1	13	21	15	4	13	15	29	19	15	26	13.2	20.8	16
2	3	4	0	4	10	5	11	9	19	9	4.2	10.6	9
3	0	0	0	0	0	1	0	0	0	0	0	0.2	2
4	2	3	0	1	6	3	2	7	5	8	2.4	5	2
5	12	14	9	6	20	15	32	13	17	20	12.2	19.4	10
6	8	21	13	14	12	14	35	15	9	51	13.6	24.8	20
7	12	1	6	0	4	10	4	7	4	7	4.6	6.4	2
8	2	0	1	1	1	2	1	1	4	4	1	2.4	1
9	11	8	10	7	13	20	11	7	10	22	9.8	14	12
10	18	6	11	6	10	35	7	12	8	12	10.2	14.8	5

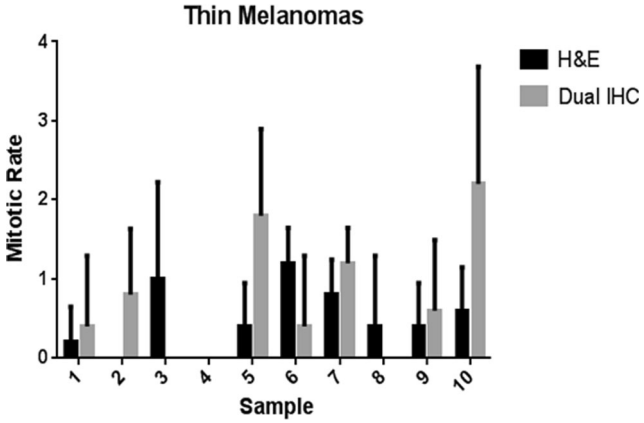

Fig. 2 Average mitotic rate per square millimeter of melanomas less than 1 mm thick utilizing H&E and dual IHC stains

American Joint Committee on Cancer (AJCC) of the TMN staging of melanoma [2]. A mitotic rate of one mitosis per square millimeter is significant in thin melanomas as defined by tumor thickness less than 1 mm. In thin melanomas, the presence of a single mitosis alters the stage from a pT1a lesion to a pT1b thus meriting more aggressive treatment and surveillance [6, 7]. Accurate mitotic rates in melanoma are challenging due to various mitotic mimickers such as karyorrhectic debris, apoptosis, and mitosis in non-melanocytic cells. Inter observer reproducibility is variable in counting mitosis and an additional tool to assist in identifying mitosis in melanoma may be helpful.

Multiple cellular proliferation markers are commercially available. The Ki-67 antigen is commonly used in surgical pathology as a marker for cellular proliferation but due to the low specificity for mitosis, Ki-67 is a poor marker for mitotic rate [3]. A reliable correlation between mitotic rate and Ki-67 staining does not exist. Phosphohistone H3 is a more specific marker for mitosis as the histone H3 is phosphorylated during the last portion of telophase and entirely through metaphase correlating well with the visualization of mitotic spindles [8].

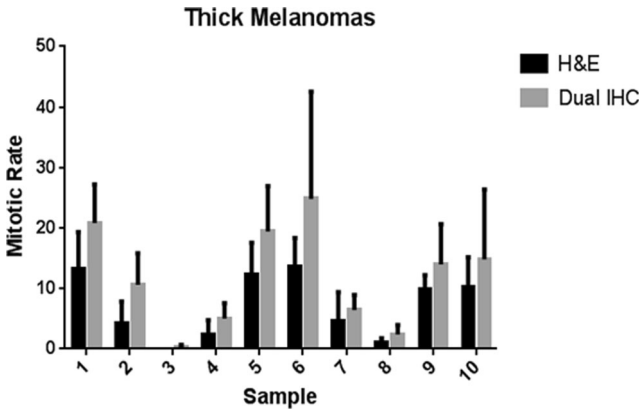

Fig. 3 Average mitotic rate per square millimeter of melanomas greater than 1 mm thick utilizing H&E and dual IHC stains

Table 3 Average mitotic rate per reviewer utilizing H&E and dual IHC stains on melanomas less than 1 mm thick

	H&E Mitotic Rate	Dual IHC Mitotic Rate
Reviewer 1	0.8	0.7
Reviewer2	0.4	0.4
Reviewer 3	0.4	0.7
Reviewer 4	0.4	1.2
Reviewer 5	1.5	1.7
Average	0.5	0.74

Other studies analyzed the utility of immunohistochemical aids in determining mitotic rate [9, 10]. Ikenberg et al. assessed the speed mitotic figures were identified using a dual immunohistochemical stain approach in 15 thin melanoma cases. Shimming et al. found that mitotic rates were more consistent amongst reviews in thin melanomas when utilizing PHH3 immunohistochemical stains. Our study is unique in that we analyzed thin melanomas (tumor thickness < 1 mm as well as thick melanomas tumor thickness ≥ 1 mm). The significance of detecting mitotic rate in thick melanomas is not as distinct as in thin melanomas but these tumors were included to test the consistency and reliability of a dual immunohistochemical stain in determining mitotic rate in larger more mitotic active tumors. The more mitosis detected on H&E the larger the mitotic rate determined by dual IHC detection. This increase in mitotic rate in the thick melanomas detected by IHC is statistically significant. This finding should be considered if assessing mitotic rate utilizing IHC in highly mitotically active tumors.

The clinical significance of utilizing immunohistochemical stains to aid in the detection of mitosis is not fully proven. The AJCC staging manual makes no mention of using immunohistochemistry when determining mitotic rate. Increasingly, recent studies are showing the utility of immunohistochemical detection of mitosis and outcomes [11–13]. Although the data from this study shows no statistically significant increase in mitotic rate utilizing the dual IHC approach there was one case that three of the five reviewers would have increased the mitotic rate from 0 to 1 thus increasing the stage from a pT1a

Table 4 Average mitotic rate per reviewer utilizing H&E and dual IHC stains on melanomas greater than 1 mm thick

	H&E Mitotic Rate	Dual IHC Mitotic Rate
Reviewer 1	8.1	12
Reviewer2	7.8	13.2
Reviewer 3	6.5	9
Reviewer 4	4.3	9.1
Reviewer 5	8.9	15.9
Average	7.1	11.8

lesion to a pT1b. The exact reasoning for this increase is likely multifactorial. A more mitotically active area of the tumor may have been sampled when making the slides for the dual IHC staining. Sampling likely played a role in a separate thin melanoma case where the average mitotic rate was 1 mitotic figure per square millimeter utilizing the H&E but dropped to 0 mitotic figures per square millimeter when analyzed by all reviewers utilizing the dual IHC.

The thick melanomas showed a statistically significant increase in mitotic rate when the dual IHC approach was used to determine mitotic rate compared to the original H&E. This result may have limited clinical application but it provides useful information that the mitotic rates tend to increase when the dual stain is used. The thick melanomas are more mitotically active compared to the thin melanomas. The larger the mitotic rate detected utilizing H&E stained sections the greater the increase in mitotic rate detected utilizing the dual IHC sections.

Our institution routinely reviews new diagnosis of melanoma among three board certified dermatopathologists. The time spent quantifying mitotic figures in tumors can be significant. All reviews agreed the dual IHC stained sections provided a more rapid assessment of mitotic figures. We hope that our experience with dual IHC stained slides can be implemented in other dermatopathology settings to provide a helpful tool for the solo practitioner or large academic practice.

This dual immunohistochemical method provides another tool for determining mitotic rate in melanoma. The current ambiguity surrounding mitotic figures only detected after immunohistochemical staining is problematic. Future staging manuals will hopefully provide guidance regarding the utilization of immunohistochemical stains for determining mitotic rate.

Compliance with Ethical Standards

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References

1. Blach C, Soong SJ, Gershenwal JE (2001) Prognostic factors analsyis of 17,600 melanoma patients: validation of the AMerican joint Commision on cancer melanoma staging system. Am J Clin Oncol 19:3622–3634
2. Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A, editors. AJCC cancer staging manual (7th ed). New York, NY: Springer; 2010
3. Gerdes J, Schwab U, Lemke H, Stein H (1983) Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 31(1):13–20
4. Hendzel M (1997) Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. Chromosoma 6:348–360
5. Kawakami Y, Eliyahu S, Delgado C, Robbins P (1994) Cloning of the Gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. Proc Natl Acad Sci U S A 91(9):3515–3519
6. Ponti G, Pollio A, Cesinaro AM, Pellecani G (2012) Value and prognostic significance of mitotic rate in a retrospective series of pT1 cutaneous malignant melanoma patients. Cancer Epidemiol 36:303–305
7. Kesmodel S, Karakouisis C, Botbyl J, Canter R (2005) Mitotic rate as a predictor of sentinel lymph node positivity in patients with thin melanomas. Ann Surg Oncol 12:449–458
8. Shibata K, Ajiro K (1993) Cell cycle-dependent suppressive effect of histone H1 on mitosis-specific H3 phosphorylation. J Biol Chem 25:18431–18434
9. Ikenberg K, Pfaltz M, Rakozy C, Kempf W (2012) Immunohistochemical dual staining as an adjunct in assessment of mitotic activity in melanoma. J Cutan Pathol 324-330
10. Schimming TT, Grabellus F, Roner M, Pechlivanis S (2012) pHH3 immunostaining improves Interobserver agreement of mitotic index in thin melanomas. Am J Dermatopathol 34:266–269
11. Thareja S, Zager J, Sadhwani D (2014) Analysis of tumor mitotic rate in thin metastatic melanomas compared with thin melanomas without metastasis using both the hematoxylin and eosin and anti-phosphohistone 3 IHC stain. Am J Dermpathol 64-67
12. Hale CS (2013) Mitotic rate in melanoma prognostic value of immunostaining and computer-assisted image analysis. Am J Surg Pathol 37(6):882–889
13. Tatzlaff M, Curry J, Ivan D (2013) Immunodetection of pshophoistone H3 as a surrogate of mitotic figure count and clincial outcome in cutaneous melanoma. Mod Pathol 26:1153–1160