

Immunodetection of phosphohistone H3 as a surrogate of mitotic figure count and clinical outcome in cutaneous melanoma

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In the American Joint Committee on Cancer (AJCC)-TNM (2009) staging system, the key prognostic factor in cutaneous melanoma is the depth of dermal invasion (Breslow thickness) with further refinement according to the presence of epidermal ulceration or dermal mitoses. Immunodetection of phosphohistone H3 has been shown to facilitate the identification of mitotic figures in various neoplasms. We selected 120 cases of primary cutaneous melanoma with completely annotated histopathologic parameters and clinical outcomes and performed double immunohistochemical staining for MLANA (Mart-1/Melan-A) and phosphohistone H3. One hundred and thirteen cases were amenable to antiphosphohistone H3 staining from 66 men and 47 women, with mean age of 64 years (9–93), including 61 superficial spreading type, 24 nodular, 6 lentigo maligna, 8 acral lentiginous, and 14 unclassified. The mean Breslow thickness was 2.53 mm (0.20–25), ulceration was present in 25/113 (22%) and the mean mitotic count was 3.2/mm² (<1–29/mm²). In 27/113 (24%) of the cases, antiphosphohistone H3 failed to highlight mitotic figures anywhere in the tissue (normal or tumor cell), whereas in 86/113 (76%) antiphosphohistone H3 detected at least one mitotic figure. Among the latter, antiphosphohistone H3 did not detect mitotic figures in dermal tumor cells in 37/86 cases (43%), whereas anti-PHH3 identified at least one melanocytic mitotic figure in the other 49/86 cases (57%; range: 1–66/mm²). The relationship between phosphohistone H3 and manual mitotic count was statistically significant (Pearson correlation = 0.59, $P < 0.0001$). Logistic regression analyses demonstrated an association between the development of subsequent metastatic disease and the following variables: mitotic figures (odds ratio (OR) = 5.7; $P = 0.0001$); phosphohistone H3-positive mitotic figures (OR = 3.0; $P = 0.008$); Breslow thickness (OR = 4.0 per mm; $P = 0.0002$); ulceration (OR = 3.94; $P = 0.008$). The application of phosphohistone H3 immunohistochemistry to the description of primary cutaneous melanoma is useful in identifying mitotic figures, improves upon the specificity of this designation when used together with MLANA, and correlates with an increased risk for metastasis in univariate analyses.

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Melanoma is among the most deadly forms of skin cancer with a steadily rising incidence and poor survival in advanced disease: 10-year survival ranges

from ~6–15% among patients with distant metastases (stage IV).¹ Therefore, there is a critical need to identify clinically significant biomarkers to delineate those patients at highest risk for an aggressive disease course. The 2009 AJCC staging system for melanoma determined that the primary tumor (pT) should be classified according to tumor thickness (mm) with further refinement according to the presence of ulceration (for all tumors) or dermal mitotic figures (for tumors ≤ 1.0 mm).² In addition, patients with cutaneous melanoma typically undergo sentinel

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lymph node biopsy for tumors greater than 1 mm in thickness or for tumors ≤ 1.0 mm when other adverse features are present: ulceration, dermal mitotic figures or a positive deep margin in the original biopsy specimen.^{3–5} Thus, the presence of dermal mitotic figures in primary melanoma has emerged as a critical variable in subsequent management decisions and prognostic algorithms.

Phosphorylation of histone H3 at serine10 (H3S10P) is an important event in cell cycle progression, first emerging in pericentromeric chromatin in late G2 phase. Subsequently, it spreads in a systematic, non-random fashion throughout the condensing chromatin during prophase and persists through anaphase of the cell cycle. It is typically no longer detectable when mitosis is completed.^{6–8} Furthermore, phosphohistone H3 is a specific marker of mitotic figures, as it is not expressed in apoptotic bodies—common morphologic mimics of mitotic figures in routine light microscopy.

Thus, immunodetection of phosphohistone H3 has become a sensitive and specific marker of mitotic figures and correlates with outcome in a variety of different human tumor types,⁹ including breast carcinoma,^{10,11} colorectal adenocarcinoma,¹² ovarian serous adenocarcinoma,¹³ pulmonary neuroendocrine carcinoma,¹⁴ uterine smooth muscle tumors,¹⁵ astrocytomas,¹⁶ and meningiomas.¹⁷ In melanoma, immunodetection of phosphohistone H3 has been shown to increase the sensitivity and accuracy for mitotic figure detection; it improves inter-observer variability; and it reduces the time required to identify mitoses in primary cutaneous and uveal melanomas.^{18–23} In relatively thick nodular melanomas, the presence of phosphohistone H3-positive mitotic figures has been shown to correlate with known prognostic indicators (like depth of invasion and ulceration), but it also serves as an independent prognostic indicator in multivariate analyses.²²

In the current study, we sought to expand on these latter observations using a broad spectrum of histopathologic subtypes of primary cutaneous melanoma. We showed that immunodetection of mitotic figures with a cocktail of antibodies for MLANA (Mart-1/Melan-A) and phosphohistone H3 provides a sensitive and specific approach to identifying mitotic figures in melanocytes and strongly correlates with manual detection of mitotic figures on routine H&E-stained sections. Furthermore, in univariate analyses, the identification of phosphohistone H3-positive mitotic figures in MLANA-positive melanocytes correlates with the development of subsequent metastases.

Materials and methods

Case Selection

We selected 120 cases of primary cutaneous melanoma from our archives (2002–2005) with

completely annotated histopathologic parameters and clinical outcomes for immunohistochemical studies using an antibody cocktail against MLANA and phosphohistone H3 (see below). The study was approved by the Institutional Review Board at The University of Texas MD Anderson. Routine H&E-stained sections were previously reviewed by at least one of the authors and pertinent histopathologic parameters (including tumor thickness, ulceration, and mitotic figure counts) were collated for each case. Mitotic figures were calculated by at least two dermatopathologists in the study using the ‘hot spot’ method. We first identified a single high-power field with the greatest number of mitotic figures and began a formal count from this field. Mitotic figures in 4.5 sequential high-power fields (each = $\times 400$), equivalent to 1 mm² in our microscope, was counted and reported as mitotic figures/mm². Phosphohistone H3-positive mitotic figures in tumor cells were counted in a similar fashion and reported as phosphohistone H3-positive mitotic figures/mm².

Immunohistochemical Studies

Immunohistochemical studies were performed using an antibody cocktail for MLANA (Thermo-Scientific; 1:400; 3-Amino-9-EthylCarbazole chromogen) and phosphohistone H3 (Ser10; Millipore; 1:400; 3,3'-diaminobenzidine chromogen) using a Bond stainer, according to the manufacturer's instructions.¹⁵ In brief, following deparaffinization with heat and alcohol wash, antigen retrieval was performed with citrate buffer (5 min, 100 °C). Antibodies were added and developed sequentially as follows: phosphohistone H3 with diaminobenzidine chromogen (including Diaminobenzidine chromogen enhancer)—> followed by MLANA antibodies with 3-amino-9-ethylcarbazole chromogen. For both antibodies, a post-primary antibody polymer enhancer was used. In order to be considered for further analysis, we required the presence of at least one phosphohistone H3-positive mitotic figure in either a tumor cell or in a non-tumor cell.

Statistical Analyses

Pearson correlation was used to assess the association between mitotic counts on histology and by phosphohistone H3. A phosphohistone H3 test was performed, which only worked for a subset of patients. Analyses of phosphohistone H3 are restricted to the subset of patients for whom this test worked (ie, where at least one phosphohistone H3-positive mitosis was identified). For all analyses except mitotic counts by phosphohistone H3, all 87 patients with information on metastases were included. Logistic regression was used to assess the association between the probability of metastasis and the following variables: manual mitotic counts

on routine H&E-stained sections, phosphohistone H3-positive mitoses, Breslow thickness and ulceration (yes/no). A multivariate logistic regression model was fit to the probability of metastasis to assess the joint effect of mitotic counts from phosphohistone H3, Breslow thickness, and ulceration (yes/no). For the analysis of mitotic counts by phosphohistone H3, analysis only included patients with data on both metastasis and phosphohistone H3 (69 patients). No adjustment was made for multiple testing. All statistical tests were performed two-sided.

Results

Clinical-Pathologic Parameters

Of the 120 cases initially selected, 113 cases were amenable to antiphosphohistone H3 staining (Table 1). These included primary melanomas from 66 men and 47 women with mean age of 64 years (range: 9–93 years) with the following histopathologic subtypes: 61 superficial spreading, 24 nodular, 6 lentigo maligna, 8 acral lentiginous, and 14 unclassified. The mean Breslow thickness was 2.53 mm (0.20–25 mm), ulceration was present in 25/113 lesions (22%) and the mean mitotic count was 3.2/mm² (range: <1–29 mitoses/mm²).

Immunodetection of Phosphohistone H3

To be included in the study, we required immunohistochemical detection of a phosphohistone H3-positive mitotic figure in either a non-tumor cell or a tumor cell (Figure 1). In 27/113 (24%) of the cases, antiphosphohistone H3 failed to highlight

mitotic figures anywhere in the tissue (including in non-tumor cells); these cases were excluded from further analyses. In contrast, antiphosphohistone H3 detected mitotic figures somewhere in the tissue (in at least one normal and/or tumor cell) in 86/113 (76%) cases (Figure 1). Among the latter, antiphosphohistone H3 did not detect mitotic figures in tumor cells in 37/86 cases (43%; Figure 1a), whereas antiphosphohistone H3 identified at least one dermal melanocytic mitotic figure in 49/86 cases (57%; range: 1–66/mm²; Figures 1b–d).

Discrepancies between manual mitotic count and antiphosphohistone H3 mitotic count were identified in nine cases: antiphosphohistone H3 failed to identify mitotic figures in five cases where they were previously identified in routine sections, whereas antiphosphohistone H3 detected mitoses in four cases previously described as <1 mitosis/mm². Among the former, additional H&E-stained slides were reviewed together with the antiphosphohistone H3 study. In 3/5 cases, there were no mitotic figures identified on either the deeper H&E-stained sections or on the antiphosphohistone H3 stained sections. In one case, no mitotic figures (antiphosphohistone H3-positive or otherwise) were identified in melanocytes of the antiphosphohistone H3 slide, although they were present on deeper H&E-stained tissue levels. In the final case, there were phosphohistone H3-positive mitotic figures highlighted, but these did not appear to be of melanocytic origin as the cell containing the phosphohistone H3-positive mitotic figure did not react with anti-MLANA (Figure 2). Review of the latter four cases failed to reveal mitotic figures in dermal melanocytes from the original tissue levels. Taken together, these results underscore the utility of MLANA/phosphohistone H3 immunohistochemistry to improve the accuracy of mitotic figure identification in melanoma: phosphohistone H3 highlighted mitotic figures in dermal melanocytes in four cases where these were previously not identified, and use of the cocktail improved the specificity of this designation, demonstrating that a previously reported dermal mitotic figure was not melanocytic in origin.

Table 1 Clinical and histopathologic parameters of cases used for Mart-1/PHH3 immunohistochemistry

<i>Gender</i>	
Male	66
Female	47
<i>Age (years)</i>	
Mean	64
Range	9–93
<i>Subtype</i>	
SST	61
NOD	24
LM	6
ALM	8
UNCL	14
<i>Histopathologic parameters</i>	
Mean breslow thickness (mm)	2.53
Range	0.20–25
Ulceration	25/113 (22%)
Mean mitotic count (per mm ²)	3.2
Range (per mm ²)	<1–29

Abbreviations: ALM, acral-lentiginous/mucosal; LM, lentigo Maligna; NOD, nodular; SST, superficial spreading type; UNCL, unclassified.

Association between Mitotic Figures by Phosphohistone H3 and by Histopathology

This analysis was restricted to the subset of 86 patients for whom the antiphosphohistone H3 test worked. To determine whether the relationship between phosphohistone H3 and manual mitotic count was statistically significant, a plot of the mitotic count as assessed by pathology and the mitotic count as assessed by antiphosphohistone H3 was constructed (Figure 3). There was a highly statistically significant relationship between these two variables (Pearson correlation = 0.59, $P < 0.0001$).

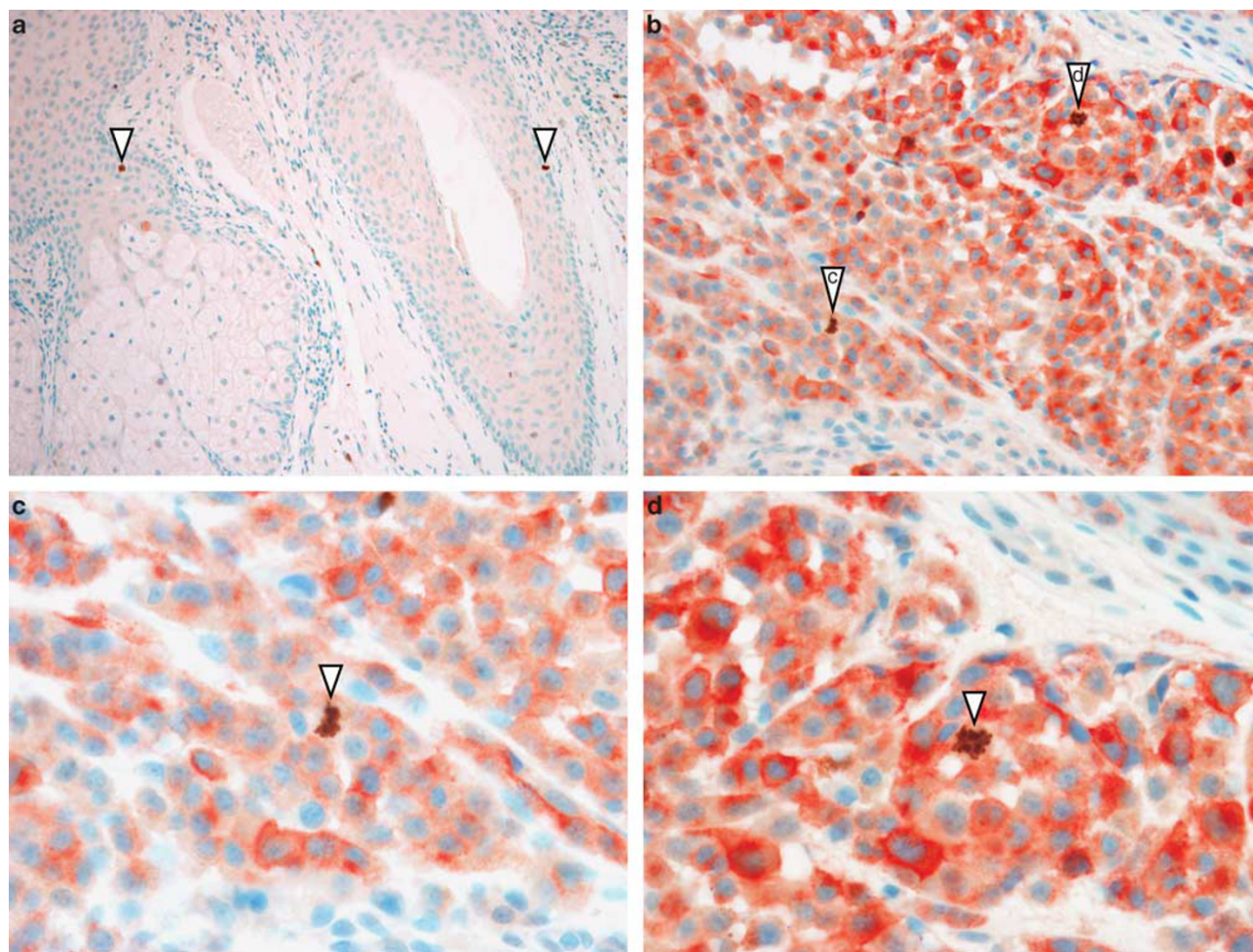


Figure 1 Immunohistochemistry for MLANA and phosphohistone H3. (a, b) Open arrowheads highlight two phosphohistone H3-positive mitoses (brown stain) occurring in follicular epithelium ($\times 10$). (b) MLANA/phosphohistone H3 immunohistochemistry highlights two mitoses (phosphohistone H3; brown) in tumor cells (MLANA; red) ($\times 20$). Arrowheads indicate the mitoses highlighted in (c, $\times 40$) and (d, $\times 40$), respectively.

Association between Development of Metastasis and Mitotic Figures, Tumor Thickness, Ulceration and Phosphohistone H3-positive Mitotic Figures

Univariate logistic regression analyses demonstrated an association between the subsequent development of metastatic disease and the following variables: mitotic figures (OR = 5.7; $P = 0.0001$); antiphosphohistone H3-positive mitotic figures (OR) = 3.0; $P = 0.008$); Breslow thickness (OR = 4.0/mm; $P = 0.0002$); ulceration (OR) = 3.94; $P = 0.008$). Thus, higher mitotic counts (manual and antiphosphohistone H3-positive), an increased Breslow thickness, and the presence of ulceration each associated with a significantly higher likelihood of metastasis (Table 2).

A multivariate logistic regression model was fit to the probability of metastasis to assess the joint effect of mitotic counts from antiphosphohistone H3 (per mitotic figure/mm²), Breslow thickness (per mm), and ulceration (present or absent). When all three variables were included in a multivariate model, only Breslow thickness was significantly associated with the probability of metastasis (Table 3).

Discussion

In the current study, we demonstrate that the application of antiphosphohistone H3 immunohistochemistry to a broad spectrum of primary cutaneous melanomas facilitates the identification of mitotic figures and correlates with both mitotic figures identified under routine light microscopy and an increased risk for metastasis.

Our findings are consistent with previous studies demonstrating a strong correlation between mitotic figures identified by phosphohistone H3 immunohistochemistry and mitotic figures identified by light microscopy (Pearson correlation = 0.59, $P < 0.0001$). In a study of 92 thin melanomas (Breslow thickness ≤ 1.0 mm), immunodetection of phosphohistone H3 resulted in increased sensitivity for the detection of mitotic figures and reduced inter-observer variability in comparison to conventional determination of mitoses by examination of H&E-stained sections.²³ In a study of 15 primary cutaneous melanomas (including various histopathologic subtypes), dual staining for Melan-A

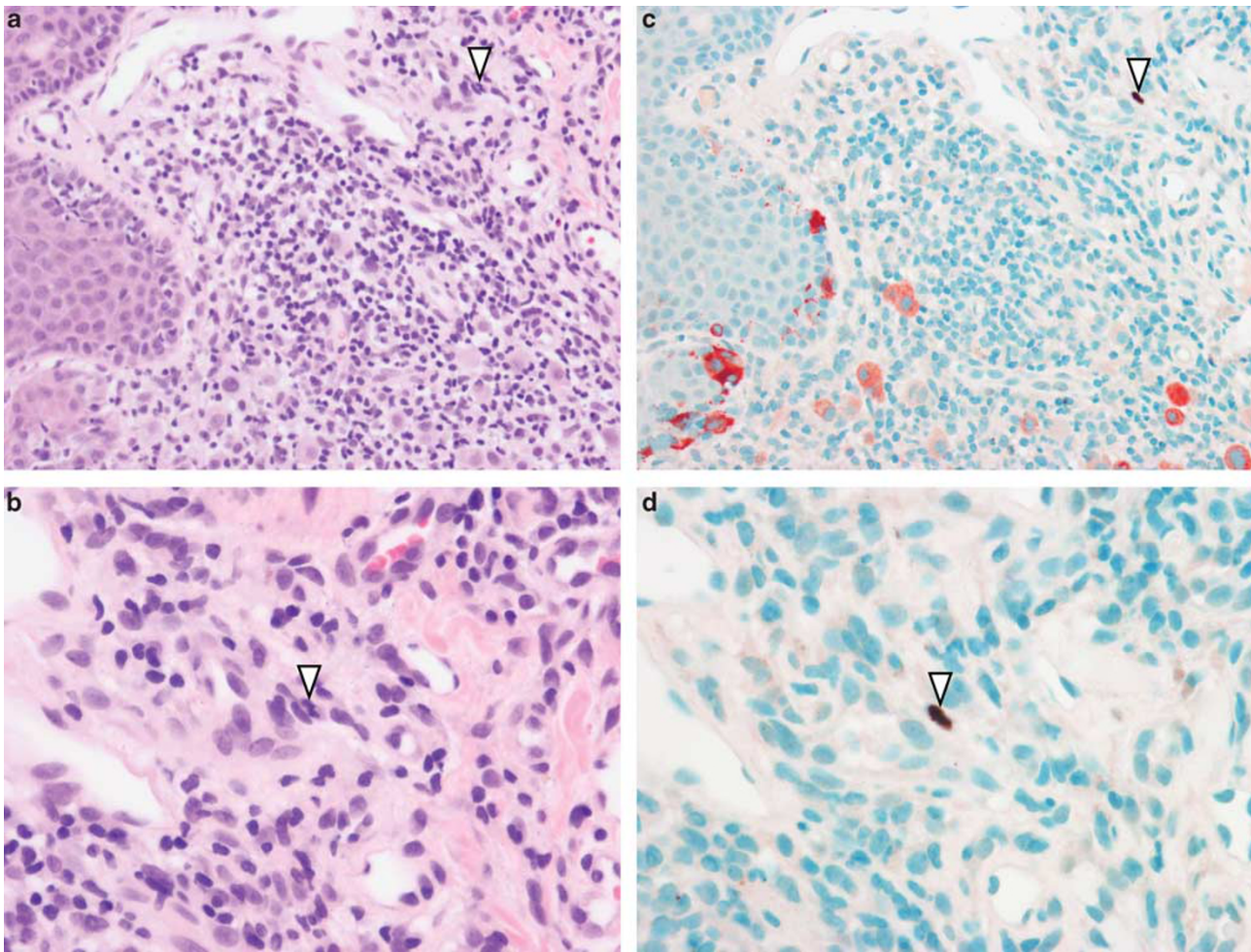


Figure 2 Immunohistochemistry for MLANA and phosphohistone H3 highlight a mitosis in a non-tumor cell. (a, b) Examination of routine H&E-stained sections of a primary invasive melanoma reveals a mitotic figure (arrowhead) in an area where tumor cells are admixed with inflammatory cells (a, $\times 20$; b, $\times 40$). (c, d) MLANA/phosphohistone H3 immunohistochemistry highlights the mitotic figure (phosphohistone H3; brown), which occurs in a non-tumor cell (MLANA; red). Arrowheads indicate the mitotic figure in question. (c, $\times 20$) and (d, $\times 40$).

and phosphohistone H3 together reduced the time required to detect mitotic figures by 63% and improved accuracy of mitotic figure detection by precisely delineating the dermal mitotic figures that occurred in melanocytes.²⁰ In a study of 30 primary cutaneous melanomas immunodetection of phosphohistone H3 strongly correlated with mitotic figure count (Spearman coefficient = 0.88; $P < 0.0001$).¹⁹ In a study of 202 nodular melanomas using tissue microarrays, immunodetection of phosphohistone H3 correlated with mitotic count and percentage Ki-67 positivity and significantly correlated with tumor thickness, ulceration and necrosis.²¹ In this study, phosphohistone H3 did not independently correlate with outcome—a result likely attributable to the use of tissue microarrays rather than whole slides where there is a greater likelihood of identifying a mitosis. Finally, in a subsequent landmark study, the same authors used whole slides and demonstrated in 345 cases of nodular melanoma that immunodetection of phos-

phohistone H3-positive mitotic figures strongly correlated with mitotic figures identified on routine light microscopy (Spearman coefficient = 0.62; $P < 0.001$), and antiphosphohistone H3-positive mitotic figures correlated with adverse histopathologic features, like increased Breslow thickness and ulceration. In both univariate and multivariate analyses, phosphohistone H3 emerged as a factor which significantly correlated with adverse clinical outcome.²² The primary limitation of the latter study was its focus on only cutaneous melanomas of the nodular subtype, whereas superficial spreading type is the most commonly encountered subtype in our practice (Table 1). Finally, identification of both mitotically active cells and cells in G2 phase of the cell cycle as highlighted with another histone marker (H3K79me3T80P) may be important in identification of a subset of melanomas with more aggressive clinical course.²⁴

Our findings confirm but also expand upon these initial observations. Using melanomas of varying

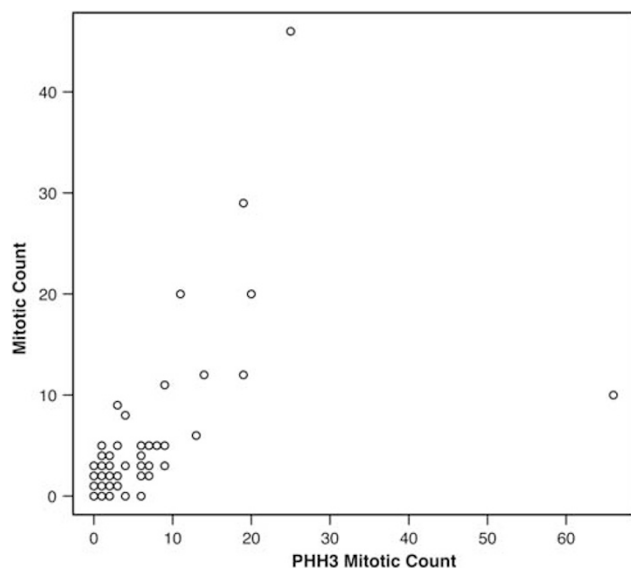


Figure 3 Scatter plot indicating the relationship between manual mitotic count and phosphohistone H3-positive mitotic count. A plot of the mitotic count as assessed by pathology and the mitotic count as assessed by phosphohistone H3. There is a highly statistically significant relationship between these two variables (Pearson correlation = 0.59, $P < 0.0001$). This analysis was restricted to the subset of 86 patients for whom the phosphohistone H3 test worked.

histopathologic subtypes, we demonstrate in univariate analyses that the presence of phosphohistone H3-positive mitotic figures—like increased Breslow thickness, ulceration and mitotic figures—correlates with an increased risk of metastasis. Only Breslow thickness proved to be independently predictive of subsequent metastases in multivariate analyses, suggestive that larger sample sets are necessary to demonstrate the independent utility of parameters like ulceration and mitoses.

Our discrepant cases also underscored the utility of dual MLANA/phosphohistone H3 immunohistochemistry to improve the accuracy and specificity of mitotic figure identification in melanoma: anti-phosphohistone H3 highlighted mitotic figures in dermal melanocytes in four cases where these were previously not identified, and use of the dual MLANA/phosphohistone H3 cocktail demonstrated that a previously reported dermal mitotic figure was not melanocytic in origin. Although it is formally possible that the latter discrepancy is due to the presence of a non-melanocytic (endothelial or inflammatory cells) mitotic figure on deeper sectioning, we believe this to be less likely as the section examined by dual MLANA/phosphohistone H3 immunohistochemistry was a sequential tissue section, the mitotic figure in question was in the same location in the immunohistochemical study as the original H&E-stained slide, and there were apparently no MLANA-positive melanocytes in this area of the tissue (Figure 2).

Despite this, however, a significant observation in our study (and a potentially important limitation to the application of phosphohistone H3 immunohis-

Table 2 Association between the presence of metastasis and mitotic counts, PHH3-positive mitoses, Breslow thickness, and ulceration

Variable	Group	N	No. of metastases	OR	95% CI (OR)	P-value
Mitotic counts (Pathology)	Per count	87	34	5.72	2.33, 14.0	0.0001
Mitotic counts (PHH3)	Per count	69	27	3.03	1.33, 6.90	0.008
Breslow thickness	Per mm	87	34	4.01	1.92, 8.39	0.0002
Ulceration	No	65	20	—	—	0.008
	Yes	22	14	3.94	1.43, 10.9	

Abbreviations: 95% CI, 95% confidence interval; N, number of cases; OR, odds ratio.

Logistic regression was used to assess the association between the probability of metastasis and mitotic counts on routine H&E-stained sections, mitotic counts from PHH3 immunohistochemistry, Breslow thickness, and the presence of ulceration. For all analyses except mitotic counts by PHH3, all 87 patients with information on metastases were included. For the analysis of mitotic counts by PHH3, only patients with data on both metastasis and for whom PHH3 immunohistochemistry were included. Higher mitotic counts (both those detected on H&E (per mitotic figure/mm²) and those with PHH3 immunohistochemistry (per PHH3-mitotic figure/mm²), a larger Breslow thickness (per mm), and the presence (vs absence) of ulceration each associated with a significantly higher likelihood (OR) of metastasis.

Table 3 Multivariate analysis of the association between the presence of metastasis and mitotic counts, PHH3-positive mitoses, Breslow thickness, and ulceration

Variable	Group	N	No. of metastases	OR	95% CI (OR)	P-value
Mitotic counts (PHH3)	Per count	69	27	1.70	0.79, 3.66	0.17
Breslow thickness	Per mm	69	27	2.73	1.33, 5.61	0.006
Ulceration	No	52	18	—	—	0.57
	Yes	17	9	0.64	0.14, 2.93	

Abbreviations: 95% CI, 95% confidence interval; N, number of cases; OR, odds ratio.

A multivariate logistic regression model was fit to the probability of metastasis to assess the joint effect of mitotic counts from PHH3 (per PHH3-mitotic figure/mm²), Breslow thickness (per mm), and ulceration (yes/no). When all three variables are included in a multivariate model, only Breslow thickness is significantly associated with the probability of metastasis (OR = 2.73, $P = 0.006$). Only the 69 patients for whom mitotic counts by PHH3 were available were included in this analysis.

tochemistry) was the relatively high frequency of cases where we failed to identify phosphohistone H3-positive mitotic figures anywhere in the tissue. In total, ~24% of the cases in our study failed to react with antiphosphohistone H3 immunohistochemistry altogether (and in approximately half of these there were *bona fide* phosphohistone H3-negative mitotic

figures present). Each negative case was subjected to a repeat antiphosphohistone H3 immunohistochemical study without a change in the original results. Ladstein *et al*²² also reported phosphohistone H3-negative mitoses in their series, albeit at a lower rate (~7.5% cases). Of note, all of the cases in the current study were cases sent to our institution in consultation from referring institutions, spanning our archives from 2002–2005. There was no apparent enrichment of phosphohistone H3-negativity observed as a function of specimen age, but we have not observed a similarly high failure rate of phosphohistone H3 immunohistochemistry on specimens processed in our own laboratory (data not shown). Thus, one possible explanation for the apparent high failure rate may be explained by variations in tissue processing in different laboratories. The protocol for this antibody was standardized using tissues processed in our own laboratory and immunodetection of PHH3 may be susceptible to differences in tissue handling and processing. Taken together, there is a potentially important limitation to the utility of antiphosphohistone H3: namely, the absence of tumor cell mitoses by phosphohistone H3 immunohistochemistry should be interpreted with careful scrutiny to ensure that antiphosphohistone H3 staining detects mitoses elsewhere in the tissue—particularly in cases where tissue has been processed at a different institution.

Together with the previous studies in melanoma,^{19,20,22,23} the findings presented in the current work, provide definite support for the application of MLANA/phosphohistone H3 immunohistochemistry as an adjunctive test in the evaluation of primary melanomas. In particular, if performed successfully, this immunohistochemical study could potentially add important value to the analysis and characterization of primary cutaneous melanoma. This would be particularly true for the description of thin melanomas (≤ 1.0 mm)—where mitotic figures can be difficult to identify and for which the identification of a mitotic figure would have the most profound implications for staging and patient management. In our practice, we routinely apply dual immunohistochemical staining for MLANA and phosphohistone H3 in all cases where mitotic figures are not readily identified on the initial tissue sections as well as in cases reviewed from referring institutions in which there are discrepancies of mitotic figure count apparent.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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