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Enhanced Immunohistochemical Detection of Neural Infiltration in Primary Melanoma: is there clinical value?

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Abstract

Background—Neural infiltration in primary melanoma is a histopathologic feature that has been reported to be associated with desmoplastic histopathologic subtype and local recurrence. We tested the hypothesis that improved detection and characterization of NI into peritumoral or intratumoral location and perineural or intraneural involvement could have prognostic relevance.

Methods—We studied 128 primary melanoma cases prospectively accrued and followed at NYU using immunohistochemical detection with anti-human neurofilament protein (NF) and compared it to routine histology. Neural infiltration, defined as the presence of tumor cells involving or immediately surrounding nerve foci, was identified and characterized.

Results—Neural infiltration rate of detection was enhanced by IHC in matched pair design (47% by IHC vs. 25% by routine histology). Immunohistochemical detection of neural infiltration was significantly associated with ulceration ($p=0.021$), Desmoplastic and Acral Lentiginous histologic subtype ($p=0.008$), as well as the head/neck/hands/feet location ($p=0.037$). Histologically detected NI was significantly associated with local recurrence ($p=0.010$). IHC detected more intratumoral NI cases compared to routine histology (30% vs. 3%, respectively). Using a multivariate model controlling for stage, routinely detected NI and enhanced IHC characterization of NI was not significantly associated with disease free or overall survival.

Conclusions—Our data demonstrate that routinely detected NI is associated with local recurrence in all histologic subtypes, but the improved detection and characterization of NI with IHC in primary melanoma does not add to prognostic relevance.

Introduction

Neural infiltration (NI) is a pathologic feature, detected by routine histology, which has an impact on local recurrence.[1-3] NI can be characterized by tumor cells directly invading

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neural structures (intraneural), surrounding the nerve (perineural).[2, 4-6] The association of NI with the outcome is contradictory. While the majority of the studies do not show any association between NI and sentinel lymph node metastasis, [7, 8] distant recurrence [5, 9, 10] and overall survival[3, 11] some studies have shown NI to be associated with distant metastasis[3] and decreased survival.[11] Neurotropic melanoma (NM), is most commonly considered under the umbrella of spindle cell melanoma[1] or desmoplastic melanoma (DM) as desmoplastic neurotropic melanoma.[5, 12] DM, a variant of spindle cell melanoma, [5, 13] is an uncommon [14, 15] and often locally aggressive tumor [3, 8, 12, 13, 15-17] largely attributed to its propensity for NI.[9, 10, 14, 18-20] Nevertheless, NI is not limited to these tumor histologies and appears in other melanoma subtypes with comparative infrequency. [11, 14, 19]

The implications of NI in primary melanoma have been confounded with the grouping of NM and DM in most studies [1, 3, 5, 8, 12, 15-17, 21, 22] which may account for the conflicting findings of NI with prognosis. In literature, NI has been reported in 30%-40% of DM.[3, 23] However, this phenomenon is scarcely noted in other more common epithelioid histologic subtypes such as superficial spreading and nodular melanomas.[18] Our own database identified NI in only 64 of 1948 (3%) primary melanomas on routine histology. The identification of NI in other histologies could serve as a predictor of local recurrence and can help identify the patients most likely to benefit from wider excision margins [3, 10, 14, 19, 20] or adjuvant radiation therapy.[6, 9, 10, 24-26]

The modest prevalence of NI across melanoma subtypes could be partially attributed to difficulties in the detection of NI during routine histology, particularly with regards to intratumoral location. The malignant cells within the tumor can either mechanically or chemically distort the entangled nerve branches making the diagnosis of intratumoral NI on routine histology potentially challenging. In addition, intratumoral hypercellularity is another confounding factor in detecting NI compared to the less encumbered peritumoral region. Immunohistochemistry (IHC) has been used successfully to enhance the intratumoral and overall detection of lymphatic vessel invasion in malignant melanomas.[27] Immunolabeling using P75 nerve growth factor receptor (P75 NGFR) has been used to identify NI in melanoma, particularly in DM and spindle cell melanoma.[18] P75 NGFR has also been identified as a potential stain to facilitate the diagnosis and differentiation of DM and NM from other neoplasms and melanoma histologic subtypes.[19, 28, 29] Concerns regarding diffuse staining of P75 NGFR in all neural-crest derived lesions, [28] non-neurotropic melanomas, benign melanocytic tumors after mild fixation, [4] and regular staining of spindle cell melanoma [29, 30] directed our use of neurofilament antibody (NF) to unambiguously and specifically stain neurons of the central and peripheral nervous system[31, 32]. To our knowledge, NF has not been utilized in cutaneous melanoma to detect NI but has demonstrated a compelling capacity to locate neural filaments, including intratumoral axons, in schwannomas.[33, 34] Our aim was to exploit NF to not only detect the presence of neurofilament but also to distinguish peritumoral/intratumoral as well as intraneural/perineural involvement.

Methods

Study population

128 Primary melanoma tissues were retrieved from patients enrolled in the Interdisciplinary Melanoma Cooperative Group (IRB#10362), a prospectively collected clinicopathological-biospecimen database at New York University Langone Medical Center (August 2002 to December 2011).[35] Informed consent for the use of clinical data and tissue was obtained from each patient at the time of enrollment, and all demographic, clinicopathological, and follow-up data were recorded prospectively. Survival data were recorded prospectively for all patients. Median follow-up time calculated on the basis of survivors (N=76) was 3.6 years, with all cases having at least 1.4 years of follow up (range, 1.4 to 13.7 y).

Patients with available primary melanoma tissues were identified, and NI positive patients in routine histology were matched to NI negative patients for stage at initial diagnosis and histologic subtype. Clinicopathological features collected from these cases included primary tumor thickness, ulceration, mitotic rate, sentinel lymph node status, the American Joint Committee on Cancer stage at pathological diagnosis, histological subtype, primary tumor location, and follow up data.

Immunohistochemistry

IHC was performed using anti-human neurofilament protein clone 2F11 (Dako, Denmark) on formalin fixed, paraffin embedded primary melanoma tissues to detect NI. In brief, after deparaffinization and rehydration, heat induced epitope retrieval was performed in 0.1M Tris-EDTA buffer (pH 9.0) in a microwave oven at 1200W at 90% power for 10 minutes. Sections were cooled in tap water for 5 minutes, washed with PBS, and incubated with blocking serum (VECTASTAIN ABC Kit-Mouse IgG, Vector Laboratories, Burlingame, CA, USA) for 30 minutes. The primary antibody was diluted in buffer (1:200) and the sections were incubated at room temperature for 1 hr and at 4 °C overnight. Slides were washed in buffer and incubated with diluted biotinylated secondary antibody solution (1:200) for one hour at room temperature followed by VECTASTAIN ABC-AP Reagent. The complex was visualized with ultraView Universal Alkaline Phosphatase Red Detection kit. Slides were washed in distilled water and counterstained with hematoxylin. Appropriate positive and negative controls were included with the study sections.

Assessment of Tissues

Separate slides from the same block stained with NF and hematoxylin and eosin (H&E) were evaluated for NI by the attending pathologist (FD) who was blinded to patient's clinical data. NI was defined as the presence of tumor cells involving or immediately surrounding nerve foci. Routine histology is defined as a single representative H&E stained section which was initially reviewed for NI and other pathologic characteristics at the time of patient enrollment. The IHC-stained slides were subsequently reviewed for NI blinded to the original H&E interpretation. The H&E was used to clarify the presence of tumor cells. Cases were read as NI positive or negative, with the positive cases also read quantitatively for the number of foci located intratumorally (within the tumor perimeter) or peritumorally (outside the tumor perimeter). Each involved nerve focus was evaluated for perineural

invasion (tumor surrounding the perineurium of the nerve) or intraneural invasion (tumor directly penetrating the endoneurium of the nerve). Only unambiguous NI positive cases were recorded. Furthermore, only pure desmoplastic melanoma cases were studied.

Statistical analysis

Associations between NI positivity and clinicopathologic variables were assessed separately for NF and for the H&E-stained slides. For categorical variables, the frequency distributions between NI-positive and NI-negative cases were evaluated using the Chi-square test or Fisher exact test, as appropriate. Statistical significance of tests was indicated when P values were <0.05. The 2-sample t test was used to compare mean age at diagnosis between patients with NI-positive and NI-negative tumors. The Wilcoxon rank sum test was used to compare median primary tumor thickness between NI-positive and NI-negative tumors. Then the associations between NI status (peritumoral or intratumoral) and clinicopathologic variables were assessed by NF antibody. Assessment between NF detected NI status with clinical outcomes including SLN status, local recurrence, organ metastasis, recurrence free survival, or overall survival were tested by univariate association tests at statistical significance level 0.05. All analyses were performed in SPSS Version 18.

Results

Query of the IMCG database identified 64 of 1948 (3%) primary melanoma tumors to be positive for NI based on routine histology. Tissue was available for 32 of these melanoma patients with NI. These 32 cases were matched by stage at primary diagnosis and histological subtype to 96 NI negative patients in routine histology for a total of 128 patients in the study cohort. Primary tumor stage was similar between the two groups ($p=0.523$; table 1). An effort was made to include appropriately matched DM and acral lentiginous melanoma (ALM), but because of the observed propensity for NI in DM and the scarcity of ALM after accounting for stage, the matched cases were overrepresented with superficial spreading and nodular melanoma. There were no significant differences in the matched group for age at melanoma diagnosis, sex, thickness, ulceration, and mitotic figures ($p=0.05$; Table 1). There were no significant differences between the site of primary (extremity vs axial $p=0.475$; Table 1). Subanalysis revealed a trend that head/neck and hands/feet were associated with NI ($p=0.102$ table 1). ALM also tended to be associated with local recurrence when compared to other histologies (44% vs. 25%, respectively; $p=0.107$)

Neurofilament antibody detects more cases of neural infiltration in primary melanoma compared with routine histology

NI was reviewed and confirmed on H&E stained sections for the 32 cases with tissue. The use of IHC significantly increased the rate of NI detection in primary melanoma compared with routine histology (60 out of 128 (47%) vs. 32 out of 128 (25%) respectively; $p=0.0004$).

In the cases with both intratumoral and peritumoral NI, the location with the greater number of nerve foci and stronger signal was used for statistical analysis. All NI cases identified in routine histology were also detected using NF. The 32 cases with NI identified in routine

histology represented 4 intratumoral and 28 peritumoral nerves. The 60 cases with IHC-detected nerves represented 39 intratumoral and 21 peritumoral nerves (Figures 1, 2, and 3).

The majority of IHC-detected NI cases identified more perineural (N=46) tumor cells than intraneural (N=18). The classification of perineural and intraneural tumor cells was not performed using routine histology because this feature could not be read unequivocally without the use of IHC.

Neural infiltration detected by neurofilament antibody is associated with distinct clinicopathologic variables compared with neural infiltration detected by routine histology

IHC-detected NI was significantly associated with 3 clinicopathologic variables: ulceration (P=0.021), histologic subtype (P=0.008), and the site of primary (P=0.037) (Table 1).

Tumors with NI detected with IHC were less likely to be ulcerated than those without NI (N = 18, 30% vs. N = 34, 50%, respectively), less likely to be of common histologic subtypes nodular and superficial spreading (N = 23, 38% vs. N = 42, 61%, respectively), and were less likely to be located on the torso, arms, or legs (N= 26, 43% vs. N=42, 61%, respectively). Patients with IHC-detected NI were younger than those without NI (61 vs 68 y); however, this was not statistically significant. Tumors with IHC-detected NI had no difference in tumor thickness than those without NI (3.53 mm vs. 3.50 mm). There was no association of IHC-detected NI with sex, stage at diagnosis, or mitotic figures.

Neural infiltration detected by neurofilament antibody is not associated with adverse clinical outcomes

The presence of routinely detected NI was significantly associated with local recurrence (P=0.010; Table 1). Local recurrence was defined as a tumor regrowth within 2 cm of the excised site of primary melanoma or an in-transit metastasis en route to the regional lymph node basin. This association was not observed in IHC-detected NI. IHC-detected NI was not associated with SLN status, local recurrence, visceral metastasis, recurrence free survival, or overall survival. The location of the nerve (peritumoral/intratumoral) and the tumor cell infiltration (perineural/intraneural) also had no association with any clinical outcomes.

Discussion

This study of NI in a heterogeneous cohort of melanoma patients indicates that while NF enhances the detection of NI in primary melanoma in comparison to routine histology, the improved neural discernment does not have prognostic relevance. We also demonstrate that NI observed in routine histology is significantly associated with local recurrence. Our results convey that while IHC facilitates the detection of NI, it is the nerves that are not obscured by mechanical or chemical deterioration from tumor cells that are readily apparent in routine histology. This may contribute to the local recurrence that is associated with DM and NM in the literature. While the IHC-detected NI not observed in routine histology could potentially be false positives, the stain specifically identified the neurofilaments of the NI positive cases read in routine histology and, in general, had very little nonspecific staining.

Other studies have explored the potential of antibodies to facilitate detection of neurotropism in melanoma[18]. However, we are not aware of any that has attempted to use

an antibody to highlight specific neurofilaments for the purpose of enhancing both the detection and characterization of NI, with respect to location and type in a large enough cohort of histologically diverse melanoma nor to associate these findings with prognosis. To date, there has been discordance on the characterization of NM and DM.[5, 14] Most studies either combine the phenotypes of desmoplasia and neurotropism as a singular histology [1, 3, 5, 8, 12, 15-17, 21, 22] under a more comprehensive spindle cell melanoma [12] or identify NM as a unique melanoma subtype. This practice fails to acknowledge the potential of NI as a pathologic feature with possible prognostic relevance found in diverse underlying histologic subtypes.[14] Other studies have focused on risk factors for developing local recurrence, [36] but have not included the influence of NI beyond what has been readily observed in DM or NM. This study attempted to elucidate the influence of NI across all melanoma subtypes, beyond what can be routinely observed in pathologic diagnosis, on clinical outcome.

The biological relevance of intratumoral versus peritumoral LVI has been explored in the literature with contradictory findings.[27] The St Gallen Expert Consensus meeting on the treatment of early breast cancer only considers peritumoral LVI as a significant pathologic consideration for clinical treatment decisions.[37] Furthermore, the assessment of functional intratumoral lymphatics in mice revealed that lymphatics in the tumor margin alone drive metastasis.[38] However, conflicting evidence of intratumoral LVI correlating with adverse clinical outcome [27, 39] and the unique neural biology with similar potential for tumor extension abilities [11] directed us to explore the prognostic significance of intra/peritumoral nerve location in NI. Consistent with IHC examinations of LVI in breast carcinoma and melanoma, our study indicates that peritumoral NI is much more commonly detected in routine histology with intratumoral NI only reliably observed with the use of IHC.[27, 40] Most of the neural involvement detected by IHC was located intratumorally (N=39) whereas only a few intratumoral NI were identified on routine histology (N=4). The correlation of NI-detected by routine histology with local recurrence and the loss of this significance in IHC-detected NI would indicate that intratumoral NI, similar to intratumoral LVI, is biologically irrelevant. Intratumoral nerves are likely to have been misshapen and obscured by the tumor, which may render them structurally unfit to harbor tumor growth. Therefore, tumors with foci of structurally stable peritumoral NI are plausibly more likely to function as conduits for tumor spread and harbor remote malignant deposits left behind in surgical excision leading to local recurrence. Our data suggest that peritumoral NI can be reliably detected by routine histology.

Tumor cells with neural involvement can either surround the perineurium of the nerve (perineural invasion) or directly penetrate the endoneurium of the nerve (intraneural/endoneurial invasion).[6, 10] The use of immunohistochemistry in this study facilitated the classification of neural involvement as peri/intraneural by clearly identifying the neural boundaries. Our analysis revealed that peri/intraneural involvement by the tumor cells does not have any prognostic significance for local recurrence, sentinel lymph node status at diagnosis, or visceral recurrence. This reinforces our finding that the use of immunohistochemistry to better identify intratumoral, perineural, and intraneural features is not justified in the pathologic diagnosis of primary melanoma.

Our results indicate that there was a strong association between IHC-detected NI with ulceration. IHC-detected NI was more likely to be observed in primary tumors without ulceration. The hypoxia undermining the epithelium in ulcerated melanomas [41] can lead to less viable intratumoral nerve branches, or it could be postulated that the hypoxic environment may reduce the antigenicity of the nerves.

IHC-detected NI was associated with both histologic subtype and tumor location. NI was more commonly observed in DM and ALM as well as the head/neck and hands/feet regions, the typical respective anatomic locations for these particular subtypes.[3, 15, 23] DM has been well established as having neurotropic tendencies, however few studies have implicated ALM with this feature. Nagore *et al* observed that NI was more frequently found in ALM than other common variants.[42] Local recurrence was also more commonly observed in ALM than the other histologic subtypes in our cohort. Egger *et al* reported that of their 21 ALM patients who had a melanoma recurrence, 17 had a local recurrence.[43] While ALM is a distinct biological entity with distinct genomic profiles and presentations, [42] the common propensity of ALM and DM for NI could elucidate their shared tendency for local recurrence observed in this cohort and previous studies.[36, 42, 43] Moreover, Messegue F, *et al* identified primary tumors in the head/neck and the lower limbs (particularly acral sites) as a predictor of locoregional cutaneous metastasis.[36] Melanomas of head/neck region has been well identified to have a predilection for neurotropism [2, 3, 5, 15, 21] and we found that the tumors in acral regions as having an analogous proclivity for NI. This could be attributed to a denser network of superficial nerves in the head/neck and hand/feet regions than in the trunk and extremities. Thus, nerves could be incidentally involved by the tumor cells as the latter proliferate. Alternatively, it can be posited that DM and ALM have an inherent biologic proclivity for neurotropism. Regardless, the prevalence of NI in tumors of these regions and their strong association for local recurrence indicate that tumor involvement of neural tissue has biological significance when implicated.

Our analysis did not reveal any significant associations in IHC-detected and routinely detected NI with disease free or overall survival. However, the association of routinely detected NI with local recurrence has important clinical implications. The literature recognizes the potential of skip areas separating neurotropic tumor cells for the main tumor in a 2-dimensional view making the assessment of appropriate margins difficult in these tumors. [9] Tumors can recur from persistent foci left behind on neural structures undetected in surgical margins posing a clinical challenge to prevent local recurrence. While the literature generally associates NM with better recurrence free survival and overall survival rates, a propensity for local recurrence confers increased risk of further recurrence.[20] Locoregional cutaneous metastasis rarely occurs without other forms of progression and potentially systemic disease.[36]

The literature calls for wider margins on DM and its commonly implicated neurotropic melanoma subtype.[3, 10, 14, 19, 20] Radiotherapy has been shown to provide local control of disease, but no prospective randomized trials have been completed to date. A randomized study is currently underway comparing surgery alone with surgery and adjuvant radiation therapy for patients with completely resected NM of the head and neck with an estimated study completion date in 2018 (NCT00975520). While this is an important trial to

potentially standardize and improve the treatment of NM, our study has identified the importance NI foci in other histologic subtypes and anatomic locations, especially in ALM on the hands/feet, to better predict local recurrence. Furthermore, our study proposes the important consideration and clarification that a singular focus of NI is enough to portend increased risk of local recurrence and, as such, should be considered in a radiation study (NCT00975520). Radiation therapy is a reasonable option for melanoma in the cosmetically sensitive head/neck region where the wider excisions recommended in desmoplastic and neurotrophic histologies can be challenging.[3, 5, 9, 14] Conservation of tissue is also an important consideration for ALM[44] where wide excisions can sometimes only be achieved through amputation and significant loss in hand/foot function. Furthermore, the increased morbidity, resection costs, and impact on quality of life in locally recurrent melanoma are important considerations for treatment regimens.[5] A radiation therapy trial would also be valuable for patients with ALM presenting with routinely detected NI to potentially control local recurrence with a proactive approach and consideration for the prevention of disfigurement. NI, regardless of histologic subtype and anatomic location, confers increased risk for local recurrence. Therefore, while radiation therapy may be appropriately used in delicate locations, wider excisions are appropriate for melanoma tumors with NI identified in the torso or extremities, where tissue removal is less morbid.

In conclusion, this study has shown that the relevant features of NI in primary melanoma can be readily identified through routine histology, making the routine use of IHC to enhance detection unnecessary. Our data indicate that routinely observed NI is associated with local recurrence across all histologic subtypes and is observed commonly in melanomas of head/neck and hands/feet regions and in desmoplastic and acral subtypes. We support a routine assessment of NI in all primary melanoma to guide therapeutic decisions regarding margins and the use of radiation therapy depending on anatomic location.

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Figure 1. Nerve location scoring of NI

IHC was proficient in detecting intratumoral NI. Routine histology primarily identified NI in the peritumoral location. IHC: Immunohistochemistry; NI: Neural infiltration.

Figure 2. Intratumoral neural infiltration (NI)

(A) Hematoxylin and eosin (H&E) preparation shows uninvolved nerve structures in the peritumoral region (asterisk) while NI is not readily identifiable in the intratumoral region; (B) Neurofilament antibody preparation facilitates intratumoral NI detection (arrow). Peritumoral uninvolved nerve structures, observed in the corresponding H&E, serve as benchmark. (10×)

Figure 3. Peritumoral neural infiltration (NI)

Hematoxylin and eosin preparation shows NI in the peritumoral region (arrow) (A); Neurofilament antibody preparation detects the same focus of NI in the peritumoral region (arrow) (B). ($\times 25$)

TABLE 1
Comparison of the Correlation Between Clinicopathologic Variables and NI in Primary Melanoma Detected Using Neurofilament Antibody vs. Routine Histology in a Cohort of Melanoma Patients With Prospective Clinical Follow-Up (N = 128)

	NI-Negative NF Ab (N=68)	NI-Positive NF Ab (N=60)	<i>P</i> ^a	NI-Negative Histology (N=96)	NI-Positive Histology (N=32)	<i>P</i> ^a	NFAB Positive NI Cases Missed by Routine Histology (N=28)
<i>Age(y)</i>			0.075			0.917	
Mean(SD)	66 (SD 16)	61 (SD 18)		64 (SD 17)	64 (SD 19)		58 (SD 17)
<i>Sex</i>			0.678			0.604	
Female	27 (40)	26 (43)		41 (43)	12 (38)		14 (50)
Male	41 (60)	34 (57)		55 (57)	20 (42)		14 (50)
<i>Stage^b</i>			0.564			0.523	
I	11 (16)	7 (12)		14 (15)	4 (12)		3 (11)
II	31 (46)	33 (55)		49 (51)	15 (47)		18 (64)
III	25 (37)	20 (33)		32 (33)	13 (41)		7 (25)
IV	1 (1)	0 (0)		1 (1)	0 (0)		0 (0)
<i>Thickness(mm)^c</i>			0.675			0.356	
Median(range)	3.50(1-31)	3.53(1-30)		3.30(1-31)	4.12(1-30)		2.90(1-24)
<i>Ulceration</i>			0.021			0.212	
Absent	34 (50)	42 (70)		54 (56)	22 (69)		20 (71)
Present	34 (50)	18 (30)		42 (44)	10 (31)		8 (29)
<i>Mitotic Figures</i>			0.375			0.744	
Absent	9 (13)	5 (8)		11 (11)	3 (9)		2 (7)
Present	59 (87)	55 (92)		85 (89)	29 (91)		26 (93)
<i>Histologic subtype^d</i>			0.008			0.185	
Common(SSM or NodM)	42(61)	23(38)		52(54)	13(41)		10 (36)
ALM	10 (15)	15 (25)		18 (19)	7 (22)		8 (29)
Desmoplastic	14 (21)	19 (32)		22 (23)	11 (34)		8 (29)
Other	2 (3)	3 (5)		4 (4)	1 (3)		2 (7)
<i>Site of primary</i>			0.037			0.102	
Extremity and Axial	42(62)	26(43)		55(57)	13(41)		13 (46)
Head/Neck/Hand/Feet	26(38)	34(57)		41(43)	19(59)		15 (53)
<i>SLN status</i>			0.400			0.743	
Negative	44 (65)	43 (72)		66 (69)	21 (66)		22 (79)
Positive	24 (35)	17 (28)		30 (31)	11 (34)		6 (21)
<i>Local recurrence</i>			0.299			0.010	
Yes	17 (25)	20 (33)		22 (23)	15 (47)		5 (18)
No	51 (75)	40 (67)		74 (77)	17 (53)		23 (82)
<i>Organ Recurrence</i>			0.991			0.462	
Yes	26 (38)	23 (38)		35 (36)	14 (44)		9 (32)
No	42 (62)	37 (62)		61 (64)	18 (56)		19 (68)

Baseline variables for cases of NI that were only detectable using the neurofilament antibody and not routine histology are also presented (N = 28).

ALM indicates acral lentiginous melanoma; LMM, lentigo maligna melanoma; NodM, nodular melanoma; SD, standard deviation; SLN, sentinel lymph node; SSM, superficial spreading melanoma.

^a By t test, Wilcoxon rank sum test, χ^2 test, or Fisher exact test, as appropriate

^b Chi-square or Fisher exact P value calculated based on comparison between clinical categories stage I/II vs III/IV

^c Median thickness (mm) of all melanomas (n = 128) was 3.50 mm (range, 1 to 31 mm)

^d Chi-square or Fisher exact P value calculated based on comparison between SSM /NM versus all other types

^e Chi-square or Fisher exact P value calculated based on comparison between Extremity/Axial vs. All other types