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Clinical Relevance of Detection of Lymphovascular Invasion in Primary Melanoma Using Endothelial Markers D2-40 and CD34

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Abstract

Immunohistochemistry (IHC) using endothelial markers may facilitate the detection of lymphovascular invasion (LVI) in primary melanoma; however, the clinical implications of enhanced detection are unknown. We evaluated whether the use of lymphatic endothelial marker D2-40 and panvascular marker CD34 increases LVI positivity relative to routine histology alone and then evaluated the prognostic relevance of LVI detected using these markers in terms of disease-free (DFS) and overall survival (OS). A total of 246 primary melanomas were assessed for LVI using D2-40, CD34, and routine histology. Associations between LVI positivity and clinicopathologic variables, DFS, and OS were compared using univariate and multivariate analyses. The use of endothelial markers increased the rate of LVI positivity (18% using D2-40 and/or CD34 vs. 3% by routine histology, $P < 0.0001$). On univariate analysis, IHC-detected LVI was significantly associated with more adverse clinicopathologic variables (thickness, ulceration, mitoses, and nodular subtype) compared with LVI detected by routine histology (thickness and ulceration only). In a multivariate model controlling for stage, LVI detected using IHC markers remained a significant marker of both reduced DFS [hazard ratio (HR), 2.01; 95% confidence interval (CI): 1.27–3.18; $P = 0.003$] and OS (HR, 2.08; 95% CI: 1.25–3.46; $P = 0.005$). Results show that D2-40 and CD34 increase the detection of LVI in primary melanoma and that cases missed by routine histology have prognostic relevance.

Keywords

melanoma; lymphovascular; invasion; D2-40; CD34

The lymphatic vasculature is the primary route of dissemination of melanoma cells from the skin to the regional lymph nodes and distant organs.¹² The presence of lymphovascular invasion (LVI) in a primary tumor is generally regarded as an unfavorable prognostic

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indicator and has been shown to be an independent predictor of worse survival in several cancers.^{2,13,14,17} However, in the current American Joint Committee on Cancer-staging system for melanoma, only thickness, ulceration, and the presence of mitotic figures are included as validated tumor (T) parameters.¹ Intralymphatic metastases (satellites and intransit metastases), together with nodal status, comprise the N category of the staging system. Thus, LVI represents a potentially informative clinicopathologic variable for prognostication because it serves as a link between the T and N categories of the staging system.

The incidence of LVI in primary melanoma, however, is disproportionately low, relative to the incidence of sentinel lymph node (SLN) positivity. It has been shown in several studies that the rate of LVI positivity ranges from 0% to 6% compared with 19% to 47% for SLN positivity, leading to speculation that these reports may be underestimating the true incidence of LVI in primary melanoma.^{5,16,19,20} The relatively low incidence of LVI may be due to difficulties in detecting it by routine histology. Factors such as the misinterpretation of stromal retraction artifact as LVI and difficulties in the identification of intratumoral LVI when tumor cells completely fill the lumen may contribute to the low rate of definitive LVI reported in primary melanoma. It is also possible that the discrepancy between the incidence of LVI and SLN positivity is due to the use of immunohistochemistry (IHC) in the evaluation of SLNs (but not all primary melanomas), resulting in increased sensitivity.

Thus, it would be expected that IHC, particularly the use of endothelial markers, would increase the sensitivity of LVI detection in primary melanoma. D2-40 is a monoclonal antibody against a 40-kD sialoglyco-protein that has been shown to be a specific marker for lymphatic endothelial cells.^{8,9,15} CD34, a panvascular marker, detects the endothelial cells of both lymphatic channels and blood vessels. Previous investigations have shown that the use of IHC endothelial markers can increase the rate of LVI detection in primary melanoma; however, assessments of the clinical relevance of this increased detection with respect to associations with adverse clinicopathologic variables, SLN status, disease-free survival (DFS), and overall survival (OS) have yielded conflicting results.^{5,15,16,19,20} The objectives of this study were to: (1) compare the incidence of LVI detected using D2-40 and/or CD34 with that detected by routine histology in a large cohort of melanoma patients with prospective clinical follow-up; (2) investigate the association between IHC-detected LVI and clinicopathologic variables compared with routine histology-detected LVI; and (3) evaluate the prognostic relevance of LVI positivity in terms of DFS and OS in a multivariate model.

MATERIALS AND METHODS

Patient Characteristics

The study cohort consisted of 246 primary melanoma tissues from 246 patients prospectively enrolled in the Interdisciplinary Melanoma Cooperative Group of the New York University with a date of initial melanoma diagnosis between January 20, 1989 and April 1, 2008.²⁵ The median age at initial melanoma diagnosis was 59 years (range, 20 to 88 y), and 61% (N = 150) were men. The majority of patients (N = 204, 83%) were of the American Joint Committee on Cancer clinical stage I or II at the time of initial diagnosis, and the median thickness of all tumors assessed was 1.2 mm (range, 0.2 to 30.0 mm). Additional patient characteristics are summarized in Table 1. Survival data were recorded prospectively for all patients. Median follow-up time calculated on the basis of survivors (N = 164 of 246) was 6.0 years, with all cases having at least 2 years of follow-up (range, 2.3 to 17.3 y). DFS was defined as survival of the patient from the date of primary melanoma diagnosis to the date of first recurrence, and OS was defined as survival from the date of primary melanoma diagnosis to the date of last follow-up or death. Informed consent for the

use of clinical data and tissue was obtained from each patient under a protocol approved by the New York University Institutional Review Board.

IHC

IHC was performed on formalin-fixed, paraffin-embedded sections using commercially available endothelial markers D2-40 (monoclonal, SIG-730; Signet, MA) and CD34 (monoclonal, clone QBEnd/10; Ventana, AZ). In brief, after the sections were deparaffinized and rehydrated, they were pretreated with 0.01M citrate buffer (pH 6.0) in a microwave oven at 1200W at 90% power for 10 minutes, followed by cooling for 30 minutes, and then rinsed in distilled water. Antibody incubations and detection were carried out at 37°C on a NEXes instrument (Ventana Medical Systems, AZ) using Ventana reagent buffer and detection kits. D2-40 (diluted 1:50) was applied for 30 minutes at room temperature, followed by incubation with Ventana biotinylated goat anti-mouse secondary antibody and a streptavidin alkaline phosphatase detection. The complex was visualized with naphthol-AS-MX phosphatase and Fast Red complex, and nuclei were counterstained with hematoxylin. Appropriate positive and negative controls were included with each run. For CD34, heat was applied without an antigen retrieval step for 30 minutes, followed by incubation with Ventana biotinylated goat anti-mouse secondary antibody and a streptavidin alkaline phosphatase detection. Three representative cases were selected for dual staining with anti-S100 (Dako, Carpinteria, CA; dilution 1:1000) and D2-40 antibodies with 60 minutes of heat pretreatment, followed by antibody incubation for 44 minutes and 24 minutes for D2-40 and S-100, respectively. The Ultraview Universal Alkaline Phosphatase Red Detection kit was used for S-100 (red reaction), and the Ultraview Universal DAB Detection kit was used for D2-40 (brown reaction).

Assessment of LVI

Separate slides from the same block stained with D2-40, CD34, and hematoxylin and eosin (H&E) were evaluated by the attending pathologist (F.D.) for LVI, which was defined as the presence of tumor cells within the endothelium-lined spaces without distinguishing between lymphatic channels and blood vessels. The term “routine histology” is used to refer to a single representative H&E-stained section. The H&E-stained sections were initially read over the course of several weeks with the investigator blinded to the clinical information. The IHC-stained slides were subsequently reviewed, again with the investigator blinded to the clinical data and to the previous H&E interpretation. Cases were designated as either LVI positive or negative, with the number of channels per case recorded. In addition, the localization of each positive channel was recorded and defined as follows: intrautemoral (confined to the tumor border), tumor edge (1 high-power field from the tumor border), and peritumoral (> 1 high-power field from the tumor border). Only unequivocal cases of LVI were recorded.

Statistical Analysis

Associations between LVI positivity and clinicopathologic variables were assessed separately for each endothelial marker (D2-40 and CD34) and for the H&E-stained slides. Thus, each statistical test was performed 3 times for each cohort of 246 tumors (LVI positive or negative by D2-40, LVI positive or negative by CD34, and LVI positive or negative by routine histology). A combined marker variable, defined as LVI positive by D2-40 and/or CD34 vs. negative for both markers, was also evaluated in the univariate analyses. As 11 demographic and clinicopathologic variables were analyzed in the univariate analyses (ie, multiplicity of outcomes), the assessment of statistical significance for these analyses was adjusted using a modified α level of $0.05/11 = 0.005$. For categorical variables, the frequency distributions between LVI-positive and LVI-negative cases were evaluated using the χ^2 test or Fisher exact test, as appropriate. The 2-sample *t* test was used

to compare mean age at diagnosis between patients with LVI-positive and LVI-negative tumors. For analyses pertaining to primary tumor characteristics, patients without complete information for a given variable were excluded. The Wilcoxon rank sum test was used to compare median primary tumor thickness between LVI-positive and LVI-negative tumors. DFS and OS were estimated by the Kaplan-Meier survival analysis, and the log-rank test was used to compare OS and DFS between patients with LVI-positive and LVI-negative tumors. The independent effect of LVI and other significant clinicopathologic variables identified by univariate analysis was assessed by multivariate Cox proportional hazards regression analysis. Adjusted hazard ratios were computed, and 95% confidence intervals (95% CI) for the hazard ratio are presented to assess the precision of the obtained estimates. Median follow-up time was computed based on survivors. All *P* values are 2-sided, with statistical significance evaluated at the 0.05 α level (except for the univariate analyses as noted above). All analyses were performed in SAS Version 9.2 and Stata Version 10.0 (Stata Corporation, College Station, TX).

RESULTS

D2-40 and/or CD34 Detect More Cases of LVI in Primary Melanoma Compared With Routine Histology

Upon review of H&E-stained sections, LVI was identified in 7 of 246 (3%) cases, representing a total of 8 positive vascular channels (7 intratumoral and 1 tumor edge) (Fig. 1A and Fig. 2). In cases stained with endothelial markers, the rate of LVI positivity was 7% (*N* = 16 of 246) using CD34 (Figs. 1B, C) and 15% (*N* = 38 of 246) using D2-40 (Fig. 1D). Forty-four cases (18%) were positive for LVI by D2-40 and/or CD34; thus, the use of the 2 markers resulted in a significantly higher rate of LVI detection in primary melanoma compared with routine histology (18% vs. 3%, respectively; *P* < 0.0001) (Fig. 2). In 6 of 246 (2%) cases, LVI was detectable only using CD34 (and not D2-40), thus possibly representative of tumors with pure blood vascular invasion. Selective positive cases (*N* = 3) were double stained for D2-40 and S-100, which confirmed the presence of LVI in all cases examined (Fig. 3).

Overall, the rate of LVI positivity in the cohort as detected using either of the endothelial markers or by routine histology was 45 of 246 (18%). Of these 45 LVI-positive cases, 38 (84%) were undetectable or “missed” by routine histology. Only 1 case (0.4%) of LVI identified by routine histology was not detected by D2-40 or CD34.

LVI Detected Using D2-40 and/or CD34 Is Associated With More Adverse Clinicopathologic Variables Compared With LVI Detected by Routine Histology

The presence of LVI as detected using D2-40 and/or CD34 was significantly associated with 4 clinicopathologic variables: thickness (*P* < 0.0001), ulceration (*P* < 0.0001), the presence of mitotic figures (*P* = 0.003), and histologic subtype (*P* = 0.0006) (Table 1). Tumors with LVI detected using endothelial markers were thicker than those without LVI (2.3 vs. 1.0 mm, respectively), more likely to be ulcerated (*N* = 24, 55% vs. *N* = 33, 16%, respectively), had at least 1 mitotic figure (*N* = 41, 93% vs. *N* = 140, 69%, respectively), and were more likely to be of nodular histologic subtype (*N* = 24, 55% vs. *N* = 56, 28%, respectively). Patients who were LVI positive by D2-40 and/or CD34 were older than patients with negative markers (63 vs. 58 y); however, this did not reach statistical significance (*P* = 0.06). No association was noted between the presence of LVI detected by D2-40 and/or CD34 and sex, site of primary, or SLN status, nor with the development of distant metastases (Table 1).

The presence of LVI as detected using routine histology was significantly associated only with thickness (*P* = 0.003) and ulceration (*P* < 0.0001) (Table 1). Lesions with LVI on

routine histology were almost 3-times thicker than those without LVI (3.2 vs. 1.1 mm, respectively), and almost all cases were ulcerated ($N = 6$, 86% vs. $N = 51$, 21%, respectively). There was no association noted between routine histology detected LVI cases and SLN status, nor with the development of distant metastases. Among tumors that were positive for LVI, those with >1 positive channel identified ($N = 14$) had a higher rate of SLN positivity ($N = 4$, 28.6%) relative to tumors with only 1 positive channel ($N = 1$ of 17; 5.9%); however, the association did not reach statistical significance ($P = 0.15$; Fisher Exact test).

LVI Detected Using D2-40 and/or CD34 Is Associated With Both DFS and OS on Univariate Analysis

The overall median survival of the cohort ($N = 246$) was 9.4 years (95% CI = 8.3 y, 11.0 y). At the end of the study period, 164 (67%) patients were alive (139 without melanoma, 9 with melanoma, and 16 with melanoma status unknown), and there were 82 (33%) deaths (70 with melanoma, 8 without melanoma, and 4 with melanoma status unknown). The univariate Kaplan-Meier analysis revealed a significant association between the presence of LVI detected using D2-40 and/or CD34 and DFS ($P = 0.0002$) (Fig. 4A). Patients with LVI-positive tumors had a median DFS of 4.8 years (95% CI = 1.5 years, upper limit not estimated) compared with 7.6 years (95% CI = 7.2 y, 9.4 y) for patients with LVI-negative tumors. The difference in median DFS was also significant ($P = 0.003$) when stratified by LVI positivity based on routine histology [LVI negative, 7.6 y (95% CI = 6.9 y, 9.1 y) vs. LVI positive, 3.9 y (95% CI = 0.4 y, 5.3 y)] (Fig. 4B). The association between LVI and OS was statistically significant only for cases in which LVI was detected using D2-40 and/or CD34 ($P = 0.001$, Fig. 4C), not for cases in which LVI was detected by routine histology ($P = 0.30$). The median OS was 8.9 years (95% CI = 3.0 y, upper limit not estimated) for patients with LVI-positive tumors detected by D2-40 and/or CD34 and 10.3 years (95% CI = 8.3 y, 13.9 y) for patients with LVI-negative tumors.

Among LVI-positive cases (as detected using IHC markers or routine histology), tumors with more positive channels (> 1) had significantly worse DFS relative to tumors with only 1 positive channel [1+ channel: $N = 24$, median DFS=NR vs. >1 + channel: $N = 20$, median DFS = 1.5 y (95% CI = 0.5 y, 5.0 y); $P = 0.02$]. There was no difference in OS between tumors with only 1 LVI-positive channel and those with >1 LVI-positive channel [1 + channel: $N = 24$, median OS NR vs. >1 + channel: $N = 20$, median OS, 3.8 y (95% CI = 1.6 y, 8.9 y); $P = 0.17$].

LVI detected using D2-40 and/or CD34 remains a significant predictor of reduced DFS and OS in a multivariate model controlling for clinical stage. In a multivariate model controlling for clinical stage at diagnosis (I/II vs. III/IV), LVI detected using IHC markers remained a significant predictor of reduced DFS [hazard ratio (HR) 2.01; 95%CI: 1.27–3.18; $P = 0.003$] and OS (HR 2.08; 95% CI: 1.25–3.46; $P = 0.005$) (Table 2).

DISCUSSION

Our study of LVI in a large prospectively accrued cohort of melanoma patients demonstrated that the use of endothelial markers D2-40 and CD34 significantly increased the rate of LVI detection over routine histology alone. We also showed that the additional LVI-positive cases identified using IHC markers were significantly associated with adverse clinical variables, DFS, and OS. These results suggest that routine histology fails to detect a substantial number of LVI-positive cases in primary melanoma, which may have clinically relevant prognostic implications for the patient.

Although previous studies have reported increased rates of LVI positivity with IHC markers, it was unclear whether these additional cases had additional prognostic relevance. In many studies evaluating IHC-detected LVI, there were no cases of LVI detectable by routine histology, which thus precluded a comparison between the 2 methodologies.^{15,19,21} Most IHC-detected cases of LVI remained undetectable even upon review of the H&E-stained sections; thus, it is not known whether these additional cases are true or false positives. Some studies have used SLN positivity as a proxy for the gold standard in evaluating the sensitivity and specificity of IHC-detected LVI.⁵ However, there are many molecular events that occur between vascular invasion and the ultimate seeding of the SLN, and many patients with LVI-positive tumors do not undergo SLN biopsy, which limits the applicability of SLN status as an appropriate benchmark. Our data demonstrate that the use IHC markers enhances the detection rate of LVI in primary melanoma. Furthermore, LVI-positive cases that would have been “missed” by routine histology are associated with poor DFS and OS on univariate analysis (Fig. 4). These “missed” LVI-positive cases also have unfavorable clinicopathologic features such as increased thickness and ulceration; thus, the association loses significance in the multivariate analysis that includes all of these variables. However, the enhanced rate of LVI detection using markers may help to identify patients at higher risk, tailor treatment accordingly, and possibly improve outcomes.

All LVI-positive channels in our melanoma cohort were either intratumoral or localized to the tumor edge. The clinical and biological relevance of intratumoral LVI has been contested. Previous studies have shown a relatively high incidence of intratumoral LVI that correlates with adverse clinical outcome¹⁹ and with SLN positivity,¹⁶ whereas others have reported that LVI is more common¹¹ or only detected²¹ in the peritumoral area. The predominance of intratumoral LVI noted in our cohort and in others may explain, at least in part, the difficulty in detecting LVI by routine histology and the subsequent increase in detection noted with the use of endothelial markers. This observation is similar to previously reported data in breast carcinoma in which LVI is more reliably detected in peritumoral tissue by routine histology but more frequently detected in intratumoral tissue by IHC methods.²⁴ However, only peritumoral LVI is considered in the St Gallen criteria for adjuvant treatment of breast cancer.⁶ Similarly, functional studies of xenograft mice have suggested that intratumoral lymphatics are not functional and that lymphatic vessels at the tumor margin are solely responsible for metastatic dissemination.¹⁸ As the rate of peritumoral LVI noted in our cohort was so low, we were not able to compare outcomes between patients with peritumoral versus intratumoral LVI. However, our results showing that intratumoral LVI is associated with worse DFS and OS independent of stage suggest that intratumoral lymphatics in primary melanoma are functional and that the detection of LVI in an intratumoral location has important prognostic implications.

Our results demonstrated a strong association between LVI and ulceration, which was significant for both the IHC-detected cases and the cases detected by routine histology. A previous study of primary melanomas evaluated for LVI by routine histology alone found that ulceration and LVI had virtually the same effect on risk of relapse and death.¹⁰ Another study of IHC-detected LVI in melanoma reported the same association between ulceration and LVI as noted in our study,²¹ and previous investigations have also noted an association between ulceration and the lymphatic vessel area.¹¹ Although current evidence strongly suggests a link between the 2 variables, it is currently unknown whether one is the cause and the other the effect or whether there is an underlying molecular mechanism simultaneously driving both processes. It has been demonstrated in vitro that melanoma cells overexpress proangiogenic factors such as vascular endothelial growth factor in response to hypoxia but not to other external stimuli such as ultraviolet radiation.²² Thus, it is possible that ulceration is indicative of a hypoxic state in primary melanoma that promotes lymphangiogenesis, which in turn results in a larger vessel area that provides more conduits

by which melanoma cells can spread to regional and distant sites. Also unknown is the relative contribution of the melanoma cell versus the lymphatic endothelial cell in promoting invasion. Previous studies have concluded that intratumoral vessels play a passive role in the process.¹⁸ Other investigations that emphasize the role of the tumor microenvironment, however, suggest that the endothelium may also secrete cytokines that attract tumor cells and facilitate vascular invasion.^{7,26} An important finding from our study and from others⁵ is that the lymphatic endothelial cells in LVI-positive cases are usually intact, suggesting that the process of invasion is more intricate and not merely an effect of mass or volume.

We did not observe a significant correlation between LVI and SLN status. Previous studies using endothelial markers have shown that the extent of lymphangiogenesis in the primary melanoma, irrespective of the identification of tumor cells inside the vasculature, is predictive of SLN positivity and worse OS.^{3,4,23} However, previous studies investigating the association between vascular invasion and SLN status have yielded conflicting results, with some studies showing a correlation between the 2 variables^{5,10,16,19} and others showing no correlation^{20,21} (Table 3). Our data show that neither IHC-detected LVI nor LVI detected by routine histology was correlated with SLN status, suggesting that it should not figure prominently in the decision to perform SLN biopsy in a patient who would otherwise not be a candidate based on currently accepted criteria. Nonetheless, we did note that LVI was a negative prognostic variable in terms of its association with worse DFS and OS. Thus, it is possible that, although LVI is an early event in the metastatic cascade, it is a better predictor of later events and the ultimate outcome rather than of more immediate events such as SLN positivity. Further study is warranted to evaluate why unequivocal evidence of LVI in the primary tumor does not necessarily translate into SLN positivity.

Another consideration to be taken into account is that previous investigations demonstrating an association between LVI and SLN positivity were retrospective and only included cases in which an SLN was performed. Thus, the melanoma cases included in these studies are often thicker (>1.00 mm) and the rate of SLN positivity higher than what would be expected in a consecutively accrued patient cohort from an academic medical center. The cases in our study were prospectively followed up and selected for inclusion without regard to SLN status; thus, many of the cases were thin, and only 11% of patients had a positive SLN biopsy. The selection criteria reflect the aims of the study, which were not to assess the ability of IHC-detected LVI to predict SLN status but to evaluate IHC-detected LVI in terms of the association with adverse clinicopathologic variables and survival.

In a multivariate model inclusive of stage, IHC-detected LVI was significantly associated with worse DFS and OS. One previous survival analysis reported a significant association between IHC-detected LVI and OS in a multivariate model in which ulceration also remained significant.¹⁹ Another study, however, consisting of a larger cohort of cases noted no association between IHC-detected LVI and DFS or OS on univariate analysis.⁵ It is possible that case selection and the length of follow-up contribute to the disparate results noted among these 3 studies. Our results suggest that LVI detected using IHC adds prognostic information beyond that obtainable by conventional staging alone.

In conclusion, the use of endothelial markers significantly increased the rate of detection of LVI in primary melanoma. Thus, the use of endothelial markers should be considered in primary melanoma cases.

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FIGURE 1.

Examples of LVI in primary melanoma detected using routine histology, panvascular marker CD34, and lymphatic endothelial marker D2-40. A, Evidence of LVI by routine histology in primary melanoma. B, Stain for CD34 highlights the presence of tumor embolus in the same case. C, CD34 stains both lymphatic channels (arrow) and blood vascular endothelium-lined spaces (arrowhead), whereas D2-40 (D) stains the lymphatic channels that contain melanoma cells (arrow) but not the blood vascular channels that contain only red blood cells (arrowhead). Magnification: $\times 200$.

FIGURE 2.

Rates of LVI in primary melanoma stratified by method of detection used: D2-40, CD34, or routine histology. * $P < 0.0001$; CD34 and/or D2-40 vs. routine histology; t test.

FIGURE 3.

Representative case of LVI in primary melanoma detected using D2-40/S-100 dual IHC. Lymphatic endothelium (brown, arrow) surrounds an intratumoral melanoma cell that stains positive for S-100 (red, arrowheads), thus clearly differentiating it from surrounding lymphocytes (asterisk) that are negative for S-100. Magnification: $\times 400$.

FIGURE 4.

Kaplan-Meier survival curves showing DFS stratified by the presence of LVI in the primary melanoma as detected using endothelial markers D2-40 and/or CD34 (A) compared with routine histology (B). OS stratified by LVI positivity as detected using D2-40/and or CD34 (C).

Comparison of the Correlation Between Clinicopathologic Variables and LVI in Primary Melanoma Detected Using Endothelial Markers CD34 and/or D2-40 vs. Routine Histology in a Cohort of Melanoma Patients With Prospective Clinical Follow-Up (N = 246)

TABLE 1

	LVI-Negative D2-40/CD34 (N = 202)*	LVI-Positive D2-40/CD34 (N = 44)	LVI-Negative Histology (N = 239)	LVI-Positive Histology (N = 7)	P [†]	P [‡]	D2-40/CD34- Positive LVI Cases Missed by Routine Histology (N = 38)
Age (y)					0.06	0.63	
Mean (SD)	58 (SD 16)	63 (SD 15)	59 (SD 16)	56 (SD 11)			64 (SD 15)
Sex					0.69	1.00	
Female (n = 96)	80 (40)	16 (36)	93 (39)	3 (43)			14 (37)
Male (n = 150)	122 (60)	28 (64)	146 (61)	4 (57)			24 (63)
Stage[‡]					0.047	0.10	
I (n = 146)	133 (66)	13 (30)	146 (61)	0 (0)			13 (34)
II (n = 58)	39 (19)	19 (43)	54 (23)	4 (57)			16 (42)
III (n = 40)	29 (14)	11 (25)	37 (15)	3 (43)			8 (21)
IV (n = 2)	1 (0.5)	1 (2)	2 (0.8)	0 (0)			1 (3)
Thickness (mm)[§]					<0.0001	0.003	
Median (range)	1.0 (0.16–13.0)	2.3 (0.40–30)	1.1 (0.16–30)	3.2 (2.20–8.0)			2.1 (0.40–30)
Ulceration					<0.0001	<0.0001	
Absent (n = 188)	169 (84)	19 (43)	187 (78)	1 (14)			18 (47)
Present (n = 57)	33 (16)	24 (55)	51 (21)	6 (86)			19 (50)
Mitotic figures					0.003	0.13	
Absent (n = 57)	54 (27)	3 (7)	57 (24)	0 (0)			3 (8)
Present (n = 181)	140 (69)	41 (93)	174 (73)	7 (100)			35 (92)
Histologic subtype					0.0006	0.36	
SSM (n = 140)	124 (61)	16 (36)	138 (58)	2 (29)			14 (37)
NM (n = 80)	56 (28)	24 (55)	77 (32)	3 (43)			22 (58)
ALM (n = 9)	6 (3)	3 (7)	7 (3)	2 (29)			1 (3)
LMM (n = 6)	5 (2)	1 (2)	6 (3)	0 (0)			1 (3)
Desmoplastic (n = 6)	6 (3)	0 (0)	6 (3)	0 (0)			0 (0)

	LVI-Negative D2-40/CD34 (N = 202)*	LVI-Positive D2-40/CD34 (N=44)	LVI-Negative Histology (N = 239)	LVI-Positive Histology (N = 7)	P [†]	P [‡]	D2-40/CD34- Positive LVI Cases Missed by Routine Histology (N = 38)
Other (n = 5)	5 (2)	0 (0)	5 (2)	0 (0)			0 (0)
Site of primary							
Extremity (n = 102)	85 (42)	17 (39)	99 (41)	3 (43)	0.75	0.99	14 (37)
Axial (n = 105)	84 (42)	21 (48)	3 (43)				19 (50)
Head/neck (n = 39)	33 (16)	6 (14)	1 (14)				5 (13)
SLN status[§]							
Negative (n = 102)	76 (38)	26 (59)	98 (41)	4 (57)	0.51	0.61	23 (61)
Positive (n = 26)	21 (10)	5 (11)	24 (10)	2 (29)			3 (8)
Not performed (n = 118)	105 (52)	13 (30)	117 (49)	1 (14)			12 (32)
Distant metastases							
Yes (n = 83)	64 (32)	19 (43)	79 (33)	4 (57)	0.14	0.23	16 (42)
No (n = 163)	138 (68)	25 (57)	160 (67)	3 (43)			22 (58)
Brain metastases							
Yes (n = 65)	48 (24)	17 (39)	62 (26)	3(43)	0.04	0.38	14 (37)
No (n= 181)	154 (76)	27 (61)	177 (74)	4 (57)			24 (63)

Baseline variables for cases of LVI that were only detectable using the endothelial markers and not routine histology are also presented (N = 38).

* Sum and percentages may be less than total due to lack of data for a given variable.

[†] By *t* test, Wilcoxon rank sum test, χ^2 test, or Fisher exact test, as appropriate.

[‡] Chi-square or Fisher exact *P* value calculated based on comparison between clinical categories stage I/II vs. III/IV.

[§] Median thickness (mm) of all melanomas (n = 246) was 1.2 mm (range, 0.16 to 30 mm).

^{||} Chi-square or Fisher exact *P* value calculated based on comparison between SSM vs. NM only. Chi-square *P* value for SSM vs. NM/other: *P* = 0.002 (D2-40 and/or CD34), Fisher exact *P* value for SSM vs. NM/other: *P* = 0.14 (histology).

[¶] Chi-square or Fisher exact *P* value calculated only for patients who had the procedure performed (n = 128 of 246).

Bold underlined figures indicate statistically significant associations (*P* < 0.005) based on a correction for multiple tests.

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

Univariate and Multivariate Models Demonstrating a Significant Association Between LVI Positivity Using D2-40 and/or CD34 and DFS and OS

TABLE 2

	Univariate			Multivariate		
	HR	95% CI	P	HR	95% CI	P
DFS:						
LVI-Positive, D2-40 and/or CD34	2.31	1.47–3.65	<0.0001	2.01	1.27–3.18	0.003
Clinical Stage (III/IV vs. I/II)	3.29	2.14–5.05	<0.0001	3.02	1.96–4.65	<0.0001
OS:						
LVI Positive, D2-40 and/or CD34	2.29	1.38–3.81	0.001	2.08	1.25–3.46	0.005
Clinical Stage (III/IV vs. I/II)	3.51	2.21–5.61	<0.0001	3.33	2.08–5.32	<0.0001

The associations remain statistically significant when controlling for clinical stage (III/IV vs. I/II).

TABLE 3
Summary of Published Reports Using Lymphatic Endothelial Markers to Detect LVI in Primary Melanoma

Series (Reference)	Markers	No. Cases	LVI+Histology and LVI+IHC (%)	LVI and Clinicopathologic Variables	LVI and SLN Status	LVI and DFS/OS
Sahni et al ²¹	LYVE-1	36 tumors	0	NA	Not significant	NA
			17			
Niakosari et al ¹⁵	D2-40	44 tumors	0	No associations noted	NA	NA
			16			
Niakosari et al ¹⁶	D2-40	96 tumors	NA	Clark Level, Thickness	Significant, multivariate	NA
			33			
Xu et al ²⁷	D2-40	106	5	Thickness	NA	DFS, univariate
	S-100	tumors	33			
Doeden et al ⁵	D2-40	94 tumors	6	Stage, Histologic subtype	Significant, univariate	No association noted
	LYVE-1		16			
	CD31					
Petersson et al ¹⁹	D2-40	74 tumors	0	NA	Significant, univariate	DFS and OS, multivariate
			23			
Pettitt et al ²⁰	D2-40	27 tumors	4	No associations noted	Not significant	NA
	S-100		37			
Present series	D2-40	246	3	Thickness	Not significant	DFS and OS, multivariate *
	CD34	tumors	18	Ulceration		
				Mitotic figures		
				Histologic subtype		

* Multivariate model inclusive of clinical stage.

NA indicates not assessed.