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Lymphatic Invasion Revealed by Multispectral Imaging is Common in Primary Melanomas and Associates with Prognosis

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Abstract

Lymphatic invasion by tumor cells has been noted infrequently in primary melanomas. Our primary hypotheses were that using immunohistochemical markers of lymphatic vessels and of tumor cells would improve detection of lymphatic invasion and that lymphatic invasion would correlate with regional nodal metastatic disease. This study included 106 patients who were diagnosed between 1972 and 1991 and who had ≥ 10 years of follow up. We performed dual immunohistochemical stains for podoplanin (for lymphatic vessels) and S-100 (for melanoma cells). Lymphatic invasion was identified by light microscopy and confirmed by multispectral imaging analysis. Lymphatic invasion was detected by morphology alone in 5 cases (4.7%) in contrast to immunohistochemical staining augmented by multispectral imaging analysis where 35 cases (33%) were identified ($p < 0.0001$). Lymphatic invasion was significantly associated with time to regional nodal metastatic disease, as well as first metastasis and melanoma-specific death. "Local metastasis", defined by immunohistochemistry-detected lymphatic invasion, satellites or neural invasion, identified 64% of those who had regional nodal metastatic disease within 5 years of diagnosis. Lymphatic invasion is an under-observed phenomenon in primary melanomas that can be better detected by immunohistochemical staining. The presence of lymphatic invasion may be a clinically useful predictor of regionally metastatic disease.

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Introduction

Primary cutaneous melanomas are well known for their propensity for early metastatic spread via lymphatic vessels to regional lymph nodes [1]. Lymphangiogenesis appears to occur early in melanoma progression [2] and regional nodal metastasis is a prognostic factor strongly associated with disseminated disease and death [3]. However, lymphatic invasion (LI) within primary melanomas has not been rigorously studied because of the difficulties in recognizing actual tumor cells in unequivocal lymphatic vessels [4]. This has been due to the lack of specific lymphatic endothelial markers for paraffin embedded tissues as well as a methodology to confirm the presence of tumor cells within lymphatic vessels.

Using antibodies specific to lymphatic endothelium, such as podoplanin and the lymphatic endothelial hyaluronan receptor-1 (LYVE-1), lymphatic vessels have been identified in paraffin-embedded samples of cervical, ovarian, breast cancers and melanoma [1,5,6]. In patients with melanoma, Valencak, et al recently showed that those whose primary lesions had high lymphatic density had shorter overall and disease-free survival [7]. Detmar, et al then noted that intratumoral lymphatics detected by immunohistochemical (IHC) staining of LYVE-1 were found more frequently in primary melanomas excised from patients whose sentinel lymph nodes (SLN) had metastases than in those taken from SLN-negative patients [2]. These studies provide evidence that lymphangiogenesis is a potential prognostic marker for regional metastasis and melanoma-related death.

Using a monoclonal antibody (D2-40), which specifically detects a fixation resistant epitope on podoplanin, and a sensitive melanocytic lineage marker (S-100), we frequently found lymphatic vessels within and at the periphery of primary melanomas and noted LI by melanoma cells. In contrast, LI had been detected in only a few melanomas on a formal review of hematoxylin and eosin (H&E) stained lesions that included a protocol-specified search for lymphatic and blood vessel invasion [8]. We also found that apparent LI detected by podoplanin immunostaining alone often appeared as single or only a few cells in lymphatic channels and that these cells were often difficult to distinguish from such non-neoplastic cells as histiocytes/monocytes, lymphocytes or even plump lymphatic endothelial cells within the lymphatic channels. LI could, however, be confirmed by visualizing simultaneously specifically marked tumor cells and lymphatic vessels using multispectral imaging (MSI), a technique that can detect spectral differences of the chromogens used to visualize antibodies directed at different markers, as well as the other colorimetric labels present on the slides, such as hematoxylin.

We had two primary hypotheses. First, LI would be more frequently detected with double IHC staining than with routine H&E histology. Second, LI would be associated with regional nodal metastatic disease, a step in tumor progression of particular interest to the clinician considering whether or not to stage patients with sentinel node biopsies. In a secondary analysis we evaluated another potential prognostic factor, a composite variable designated “local metastasis”. LI, lymphatic density, and “local metastasis”, were evaluated in tumors from a well-characterized cohort of patients with stage I and II melanomas with more than ten years of follow up and tested as prognostic biomarkers. We also addressed the clinical application of MSI to detect these potential prognostic factors. Finally, in an exploratory analysis we examined these factors and lymphatic density for their association with first metastases and melanoma-specific death.

Method

Study Patients and Study Design

Patients eligible for this retrospective nested cohort study had vertical growth phase (VGP) primary melanomas and no apparent metastases at the time of definitive treatment and were seen between 1972 and 1991 at the Pigmented Lesion Clinic of the University of Pennsylvania. The protocol was approved by Institutional Review Board at University of Pennsylvania. One hundred and forty patients were selected from a total of 489 patients who had at least 10 years of follow-up and had paraffin blocks available for IHC staining [9]. Subjects were selected using stratified random sampling where strata were defined by the occurrence of a ten-year metastasis and tumor thickness (three groups were defined using AJCC T1–T3 criteria as:

< 1mm, 1–2 mm and > 2 mm). The statistical analysis was based on 106 patients after 34 patients were excluded because their tissue sections lacked VGP on the slides examined. The patients analyzed included 36 patients (34%) who had a metastasis within ten years of diagnosis (cases) and 70 patients (66%) who were disease-free for at least ten years (controls).

IHC staining

Lymphatic endothelium was visualized with the chromogen DAB (DakoCytomation, Carpinteria, CA) and melanoma cells with Nova Red (Vector Laboratories, Burlingame, CA). The D2-40 antibody (mouse monoclonal, 1:25 dilution, Signet Laboratories, Dedham, MA) that specifically detects a fixation resistant epitope on podoplanin was used to decorate lymphatic epithelium. Melanoma cells were identified using S-100 antibody (rabbit polyclonal, 1:50, DakoCytomation). IHC assays were done on 5 µm-thick formalin-fixed paraffin-embedded sections. Heat induced epitope retrieval was performed by boiling the slides in 1X EDTA buffer (LabVision, Fremont, CA) for 20 minutes. Slides were incubated with the D2-40 and S-100 antibodies for 1 hour at room temperature. IHC staining was done on a DakoCytomation Autostainer using the EnVision+ HRP DAB system (DakoCytomation) according to manufacturer's recommendations. Normal mouse serum (1:1000 dilution) was substituted for the primary antibody in each case as a negative control.

Pathology Definitions

All slides initially were reviewed for routine histological attributes by two pathologists (D.E.E. and W.H. Clark Jr, MD) without knowledge of patients' outcomes [8]. Attributes included Breslow thickness; Clark level, dermal mitotic rate (MR), expressed in terms of mitoses per square millimeter; VGP tumor infiltrating lymphocytes (TIL), classified as either brisk, nonbrisk, or absent; regression, if present in the invasive or in situ radial growth phase adjacent to the VGP; microscopic satellites; ulceration; and vascular (blood or lymphatic) invasion. We defined the composite variable "local metastasis" as the presence of any of the following: vascular invasion (blood or lymphatic), microscopic satellites, or neural invasion. IHC stained slides were reviewed independently by two pathologists (X. X, H. L, or K. M) blinded to clinical outcome. LI was defined by S-100 positive cell(s) present in lumens highlighted by podoplanin staining as determined by MSI analysis. Intratumoral lymphatic density was taken as a measure of "lymphangiogenesis" and the density was determined by counting the number of vessels in 1 square millimeter in "hot spots" within the tumor mass; peritumoral lymphatic density was determined by counting the number of vessels in a hot spot within a 0.5 mm radius around the tumor mass. "Hot spots" were discrete areas with easily visible lymphatic vessels. Disagreements were resolved by consensus. The averages of the intratumoral and peritumoral lymphatic density readings were used in the analysis.

Multispectral imaging analysis

Slides were examined using a Leica DMRA2 microscope (Leica Microsystems Inc., Bannockburn, IL) equipped with planapochromatic lenses. Pictures of each potential focus of lymphatic invasion were imaged at 200x through a liquid crystal filter using the Nuance Multispectral Imaging System (Cambridge Research and Instrumentation Inc., Woburn, MA). This imaging system is based on a tunable liquid crystal technology that is linked to a charge-coupled device (CCD) camera and a personal computer (PC). The MSI system was used at full chip resolution without data binning. Spectral data were acquired from 420–720 nm in 10 nm increments. Spectral unmixing was accomplished by Nuance software v1.42 and pure spectral libraries of individual chromogens (slides stained with only DAB, Nova red or hematoxylin). Nonspecific background staining was subtracted from each image individually. To visualize several spectral markers simultaneously, images were then evaluated for the presence of LI using unmixed images generated by the Nuance system.

Clinical Definitions

Regional nodal disease (RND) was identified by either pathologically confirmed or clinically evident malignant melanoma in the regional nodal basin associated with the primary lesion, as previously described and validated [10]. Patients with RND included those who 1) developed evident lymphadenopathy that was confirmed pathologically subsequent to wide excision of their primary lesion, 2) underwent prophylactic (“elective”) lymphadenectomy at or around the time of their definitive excision and who had melanoma found in the pathology specimen, and/or 3) developed either pathological or clinical evidence of regional lymphadenopathy within six months of the diagnosis of systemic (super-regional) metastatic disease. For some patients, elective lymph node dissection was part of definitive treatment for the primary melanoma in this cohort that pre-dates the era of routinely performing SLN biopsies. Time to RND was the time between definitive treatment and the diagnosis of RND. Patients with a positive elective lymph node dissection at or around the time of surgery of the primary were assumed to have had RND in the month of the procedure. Censored patients for the analysis of RND were those who had a distant metastasis (without RND or with RND noted ≥ 6 months after the diagnosis of super-regional metastasis), who died without evidence of RND, or who were lost to follow-up. Patients without RND were followed per protocol for at least 10 years; their median follow-up was 16.1 years ($n=76$, 0.4 to 29.1 years). The median time to RND was 3.6 years ($n=30$ patients, 0.1 to 17.4 years). The median follow-up for those without metastasis and for those who were alive at the time of last follow up was 18.7 years ($n=63$, 0.3 to 29.1 years) and 18.9 years ($n=70$, 6.8 to 29.1 years), respectively. The median time to first metastasis (regional nodal metastasis with or without super-regional metastases or melanoma-related death) was 3.6 years ($n=43$) and the median time to melanoma-related death was 4.9 years ($n=36$).

Statistical Methods

Pearson’s chi-square statistic or Fisher’s exact test were used to identify differences between study patients included in the analysis ($n=106$) and those excluded ($n=34$) from study, as well as to evaluate differences in rates of LI and local metastasis by patient and tumor characteristics available for this analysis. The proportions of patients with LI identified using H&E at the time of initial diagnosis and LI identified using double IHC staining were compared using McNemar’s test. Kaplan-Meier curves were estimated for the time to RND and the log-rank test was used to identify significant differences. Univariate Cox models were used to obtain unadjusted hazard ratios and their 95% confidence intervals. Analyses were performed using SAS Version 9.0. Except for chi-square tests, p-values are based on two-sided tests.

With a sample size of 106 the two-group continuity-corrected chi-square test with a significance level of 0.05 has 83% power to detect an odds ratio of 4.0. The study was powered

to screen for biomarkers that were strongly associated with RND and that might therefore be more informative than the best of our current morphological biomarkers.

Results

Patient and Lesion Characteristics

The patients and their lesions included in this analysis (see Table 1) were similar to those excluded, although patients excluded were somewhat more likely to be female, were less likely to have had an elective lymph node dissection, and had thinner primary lesions, with lower mitotic rates and more frequent ulceration (data not shown).

MSI distinguished luminal melanoma cells from non-tumor cells

Since other cell types can mimic melanoma cells within lymphatic vessels, we used double stains to distinguish melanoma cells from such simulants as lymphocytes, macrophages, and endothelial cells (Figure 1A–1D). It was often difficult by routine light microscopy to distinguish DAB stained lymphatic endothelial cells from Nova Red stained melanoma cells (Figure 1E and 1G). Double stained slides were analyzed using MSI for the presence of tumor cells (S100+, DAB chromogen) in lymphatic vessels (podoplanin+, Nova Red chromogen). Using the distinctive spectral signatures of DAB and Nova Red, we readily distinguished luminal non-tumor cells from melanoma cells (Figure 1F and 1H).

Lesional lymphatic vessels were prevalent and lymphatic invasion was common

Supporting our first primary hypothesis, LI made evident by using double IHC staining was identified in 33% (35/106) of cases, significantly higher than the 4.7% (5/106) of cases observed at the time of initial prospective review of H&E stained slides (McNemar's Test, $p < 0.0001$). By routine histology 16% (17/106) of cases had "local metastasis". By double IHC staining, there were 44 (41.5%) with local metastasis. Of these patients the majority ($n=27$, 61%) had only LI, (i.e. no evidence of blood vessel invasion, satellites and/or neurotropism).

We found that intratumoral and peritumoral lymphatic vessels were detectable in 93.3% (99 out of 106) and 98.9% (96 out of 97) of patients, respectively. The average densities of the intratumoral and peritumoral lymphatic vessels were 8.1 and 13.1, respectively. Most lymphatic vessels could not be appreciated by routine histological examination.

LI was significantly associated with increased peritumoral lymphatic density but was not associated with intratumoral lymphatic density (Wilcoxon test, $p=0.036$ and 0.140 , respectively; data not shown). With the exception of thickness, LI was not associated with the commonly reported prognostic factors in melanoma (see Table 1); similar results were obtained for local metastasis as defined with double IHC staining.

Lymphatic density, LI, local metastasis and prognosis

Higher intratumoral lymphatic density was significantly associated with metastasis ($HR=1.05$, $p\text{-value}=0.019$) and melanoma-related death ($HR=1.05$, $p=0.016$) but was only marginally associated with RND ($HR=1.04$, $p=0.083$). In contrast, peritumoral lymphatic density was associated only with melanoma-specific death ($HR=1.06$, $p=0.009$).

Supporting our second primary hypothesis, there was a significant difference in the time to RND for those with and without LI (Log-rank test, $p=0.026$). This was also true for patients with and without local metastasis (Log-rank test, $p=0.005$). The five-year RND rates were 33% (95% CI=17%–50%) and 17% (95% CI=8%–26%) for those with and without LI, respectively (Figure 2A). With better separation between risk groups, the respective five-year RND rates were 36% (95% CI=22%–51%) and 13% (95% CI=5%–21%) for those with and without local

metastasis (Figure 2B). The Cox regression analyses of RND identified statistically significant unadjusted hazard ratios for LI, local metastasis and seven other prognostic factors, thickness $\geq 2.00\text{mm}$, VGP mitotic rate ≥ 6.0 per mm^2 , age 60 years, presence of brisk or non-brisk VGP tumor infiltrating lymphocytes, ulceration, satellites, and VGP vascular invasion (Table 2). Similar patterns were seen in the unadjusted hazard ratios from the Cox regression models for metastasis and disease-specific survival (Table 2).

LI and local metastasis had better sensitivity for RND than routine histology

Both LI using double IHC and MSI and local metastasis were better able to identify those with and without regional nodal failure than LI using routine histology (Table 3). The composite prognostic factor local metastasis identified 64% of the patients who had RND within 5 years of their definitive treatment (sensitivity=64%, 95% CI = 47%–80%) and it identified 69% of the patients who free of RND at 5 years (specificity=69%; 95% CI = 58%–79%). The sensitivity of both prognostic factors was significantly higher than LI from routine histology that identified only 9% of those who had RND within 5 years of definitive treatment. Local metastasis improved sensitivity with a slight decrease in specificity compared to LI alone. Positive and negative predictive values were slightly higher for local metastasis compare to LI (Table 4).

Discussion

Intratumoral lymphatic vessels have been noted in certain human cancers, including some characterized by frequent metastases to regional nodes such as melanomas, head and neck carcinomas, papillary thyroid and breast cancers [5,11,12]. It has been established that dissemination of metastases may occur via the lymph nodes [13]. In melanoma and head and neck cancer, it has been postulated that cure might follow from the removal of regional metastases in a minority of those without evidence of disseminated disease [14]. Our use of specific IHC staining for lymphatic vessels highlighted the near ubiquity of lymph vessels within VGP melanomas.

Metastatic spread of tumor cells is responsible for the majority of cancer deaths. In this study, we tested the hypothesis that LI and other evidence of intraprimary metastasis (“local metastasis”) are associated with regional nodal metastatic disease, as well as disease disseminated beyond the region and melanoma death. Several previous studies using antibodies recognizing LYVE-1 or podoplanin to highlight the lymphatic vessels in melanomas have showed that the extent of tumor lymphatic density was significantly associated with regional lymph node and disseminated metastasis [2,15–18]. It has been proposed that the increase in lymphatic surface area increases the chances of intravasation and subsequent dissemination of neoplastic cells [19]. A recent study by Shields et al described both lymphatic endothelial cell (LEC) chemotaxis and proliferation in response to metastatic melanoma cells and secretion by endothelial cells of chemotactic agents that attract melanoma cells, suggesting that bidirectional interactions between these partners may promote lymphatic invasion [20]. Consistent with this hypothesis, the present study unequivocally showed that lymphatic invasion is common in primary VGP melanomas. Interestingly, Shields reported that peritumoral lymphatic density for melanomas is no greater than that found in normal skin. Although we did not address whether intra- and peritumoral lymphatic invasion are associated with lymph node metastasis, we showed that intra-tumoral and peri-tumoral lymphatic vessels are prevalent and that they and tumoral lymphatic invasion are associated with both metastasis and melanoma-related death.

These considerations raise the important biological and clinical questions of whether some primary melanomas initially metastasize solely via a regional lymphatic route and whether this is translatable into a therapeutic advantage, that is, whether removal of “early” metastatic disease by sentinel lymphadenectomy is associated with improved overall survival (as suggested

by Morton et al's secondary analysis of their randomized trial of the impact the procedure on survival [14] or only with informative prognostic information as suggested by Gervasoni et al [21]. We preliminarily addressed this issue in an analysis that examined whether patients with regional metastatic failure would more frequently have lymphatic invasion than those who failed with disseminated disease. In the present study, patients with evidence of regional skin/nodal metastatic disease (n=30) were more likely to have lymphatic invasion compared to those with disseminated disease and no evidence of skin/nodal disease (n=10). The lymphatic invasion rates for these two groups were 50% and 30%, respectively. As expected in this small dataset, these percentages were not significantly different (Fisher's exact test, two-sided, $p=0.4645$). Patients with evidence of regional skin/nodal disease also more frequently had "local metastasis" compared to those with disseminated disease and no evidence of skin/nodal disease. The local metastasis rates for these two groups were 63% and 40%, respectively. These percentages were not significantly different (Fisher's exact test, two-sided, $p=0.2743$). LI in the form of tumor emboli has been reported in up to 5–8% of primary lesions, similar to our study's rate of 5% using H&E stained slides. Most studies only stain lymphatic vessels without simultaneously staining for tumor cells. This would be expected both to miss the single or non-aggregated and infrequent intralymphatic tumor cells [1,2,19,22] and to misinterpret other intralymphatic cells as tumor cells. With double IHC staining and MSI, we found a surprising 35% of the cases had LI. We believe it is potentially of clinical importance to identify LI. Its presence likely reflects a step in tumor progression beyond lymphangiogenesis explaining the association of lymphangiogenesis with worse prognosis. It, perhaps as part of a composite biomarker "local metastasis", may also be an independent predictive factor for regional metastasis. The test of the latter hypothesis and of the hypothesis of a therapeutically exploitable lymphatic pathway awaits larger studies that also include makers of blood vascular invasion, that specifically address the status of SLN biopsies, and that have long term follow-up for outcomes that include patterns of metastatic failure and disease-specific survival.

This study demonstrates the potential utility of MSI in quantifying biomarkers (e.g., with image analysis) and interpreting the spatial and anatomical distribution of biomarkers of high order processes (e.g., lymphatic vascular invasion). We have demonstrated that MSI can be used effectively to separate multiple dyes on archival formalin-fixed paraffin-embedded histological sections of melanomas. With the development of new visualization methods, such as quantum dot labeled antibodies [23], it will be possible to extensively phenotype tumor cells and their relationship with their microenvironment on a single histological section.

We conclude that LI is an under-observed phenomenon in primary melanomas. Detection of LI in primary melanomas can be significantly improved by IHC and MSI. LI is a strong candidate as a clinically useful predictor of the state of regional nodes that may influence clinical decision-making (e.g., with respect to whether or not to offer patients with primary melanomas SLN biopsies). The use of MSI to detect biomarkers and to reveal them in an anatomical context can be widely generalized to address other issues in tumor biology and prognostication.

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Figure 1. Multispectral imaging (MSI) analysis enhances detection of tumor cells in lymphatic lumens

All the sections were reacted with D2-40 that recognizes podoplanin (a marker for lymphatic vessels), S-100 (a marker for melanoma cells) and hematoxylin. Podoplanin was labeled with DAB and was pseudo-colored as yellow; S-100 was labeled with Nova Red and was pseudo-colored as red; and hematoxylin is blue and was pseudo-colored as blue. A) There are many hematoxylin stained cells within a podoplanin stained lymphatic vessel (light microscopy, 400X). Bar represents 50 μ M. B) Unmixed MSI image of the same field shows that the cells in the lymphatic lumen depicted in A are blue and are therefore not tumor cells. The arrowhead points to lymphatic epithelium; arrows point to cells in the lymphatic lumen. C) There are cells

in a lymphatic lumen and it is difficult to tell whether these cells are DAB or Nova Red stained (light microscopy, 400X). Bar represents 50 μ M. D) Unmixed MSI image of the same field as shown in C demonstrates that the cells are podoplanin positive and are therefore lymphatic epithelial cells. An arrowhead points to lymphatic epithelium and an arrow points to the lymphatic epithelial cell projecting into the lymphatic lumen. E) There is one cell in the lymphatic lumen and it is difficult to tell whether this cell is DAB or Nova Red stained (light microscopy, 400X). Bar represents 50 μ M. F) Unmixed MSI image of the field shown in E shows the cell in the lymphatic vessels is red, S-100 positive, and therefore a melanoma cell. An arrowhead points to lymphatic epithelium and an arrow points to the tumor cell in the lymphatic lumen. G) There are many cells in a lymphatic lumen and it is difficult to tell which cells are DAB or Nova Red stained (light microscopy, 600X). Bar represents 75 μ M. H) Unmixed MSI image of the field shown in G demonstrates that the one red colored cell in the lymphatic vessel is a melanoma cell. The arrowhead points to lymphatic epithelium and an arrow points to the tumor cell in the lymphatic lumen.

Figure 2. Kaplan-Meier Survival Curves for Time to Regional Nodal Disease

Panel A shows survival curves for patients where lymphatic invasion was present or absent in their melanomas. Panel B shows survival curves for patients where local metastasis was present or absent within their melanomas.

Table 1

Lymphatic Invasion and Local Metastasis Rates, by Factor

Prognostic Factor	Frequency n=106	Percent	Lymphatic Invasion		Local Metastasis	
			Rate (%)	p-value	Rate (%)	p-value
Thickness						
≤1.00 mm	35	33.0	17.1	0.006	20.0	<0.001
1.01–2.00 mm	32	30.2	28.1		34.4	
> 2.00 mm	39	36.8	51.3		66.7	
Mitotic Rate						
0	12	11.3	25.0	0.184	25.0	0.061
0.1–5.9	60	56.6	27.9		36.1	
≥ 6.0	34	32.1	45.5		57.6	
Gender						
Male	49	46.2	26.5	0.188	49.1	0.086
Female	57	53.8	38.6		32.7	
Age						
< 60 Years	78	73.6	28.2	0.079	38.5	0.288
≥ 60 Years	28	26.4	46.4		50.0	
Anatomic Site						
Axial	66	62.3	39.4	0.073	48.5	0.061
Extremity	40	37.7	22.5		30.0	
VGP TIL						
Absent	34	32.1	35.3	0.732	38.9	0.426
Present	72	67.9	31.9		47.1	
RGP Regression						
Present	32	30.2	34.4	0.854	34.4	0.327
Not Present	74	69.8	32.4		44.6	
Ulceration						
Present	17	16.0	41.2	0.435	58.8	0.114
Absent	89	84.0	31.5		38.2	
Satellites						
Present	15	14.2	46.7	0.225	100.0	<0.001
Absent	91	85.8	30.8		31.9	
Vascular-Lymphatic Invasion **						
Present	5	4.7	60.0	0.189	100.0	0.011
Not Present	101	95.3	31.7		38.6	
Neurotropism						
Present	3	2.8	33.3	1.000	100.0	0.069
Not Present	103	97.2	33.0		39.8	
Lymphatic invasion						
Present	37	34.9	---	---	100.0	<0.001
Absent	69	65.1	---	---	12.7	
Local Metastasis						
Present	44	41.5	0.0	<0.001	---	---
Absent	62	58.5	79.6		---	

* RGP is the radial growth phase adjacent to the VGP

** Determined by H&E at the time of diagnosis

Table 2
Unadjusted Hazard Ratios for Regional Nodal Disease, Metastasis and Melanoma-related Death

Prognostic Factor	Regional Nodal Disease Unadjusted Hazard Ratio	p-value	First Metastasis Unadjusted Hazard Ratio	p-value	Melanoma-specific Death Unadjusted Hazard Ratio	p-value
Thickness						
≤1.00 mm	1.0	---	1.0	---	1.0	---
1.01–2.00 mm	1.7	0.359	2.1	0.113	2.1	0.156
> 2.00 mm	5.2	0.001	5.2	<0.001	4.1	0.002
Mitotic Rate						
<6	1.0	---	1.0	---	1.0	---
≥ 6.0	5.3	<0.001	3.7	<0.001	3.3	0.001
Gender						
Male	1.6	0.218	2.5	0.006	2.7	0.007
Female	1.0	---	1.0	---	1.0	---
Age						
<60 Years	2.1	0.049	1.8	0.698	1.7	0.156
≥ 60 Years	1.0	---	1.0	---	1.0	---
Anatomic Site						
Axial	2.0	0.086	2.5	0.011	2.7	0.014
Extremity	1.0	---	1.0	---	1.0	---
Dermal TIL						
Absent	3.4	0.001	2.1	0.015	1.8	0.094
Present	1.0	---	1.0	---	1.0	---
RGP Regression						
Present	0.9	0.750	1.2	0.634	1.3	0.499
Not Present	1.0	---	1.0	---	1.0	---
Ulceration						
Present	2.5	0.027	2.5	0.009	3.0	0.003
Absent	1.0	---	1.0	---	1.0	---
Satellites						
Present	4.0	0.001	3.4	0.001	1.8	0.179
Absent	1.0	---	1.0	---	1.0	---
Vascular-Lymphatic Invasion*						
Present	7.1	<0.001	4.0	0.009	2.1	0.216
Not Present	1.0	---	1.0	---	1.0	---
Neurotropism						
Present	2.6	0.184	1.8	0.409	1.2	0.876
Not Present	1.0	---	1.0	---	1.0	---
Lymphatic invasion						
Present	2.2	0.031	2.1	0.018	2.0	0.037
Absent	1.00	---	1.00	---	1.00	---
Local Metastasis						
Present	2.7	0.007	2.4	0.004	1.9	0.051
Absent	1.0	---	1.0	---	1.0	---
Intratumoral Lymphatic Density	1.04	0.083	1.05	0.019	1.05	0.016
Pertumoral Lymphatic Density	1.04	0.184	1.05	0.072	1.06	0.009

* Determined by H&E at the time of diagnosis

Table 3
Sensitivity and Specificity for 5-Year Regional Nodal Failure

Biomarker	Five-Year RND	
	Yes n=33 Sensitivity (95% CI [*])	No n=73 Specificity (95% CI [*])
Vascular-Lymphatic Invasion (Routine histology)	9.1 (0.0 – 18.3)	97.3 (93.6 – 1.00)
Lymphatic Invasion (Double IHC and MSI)	48.5 (31.4 – 65.6)	74.0 (63.9 – 84.1)
Local Metastasis	63.6 (47.2 – 80.0)	68.6 (58.0 – 79.2)

* Confidence interval

Table 4
Predictive Values for Five-Year Regional Nodal Failure

	Present Frequency	Positive Predictive Value (95% CI [*])	Absent Frequency	Negative Predictive Value (95% CI [*])
Vascular-Invasion	5	60.0 (17.1 – 100)	101	70.3 (61.4 – 79.2)
Lymphatic (routine histology)				
Lymphatic Invasion	35	45.7 (29.2 – 62.2)	71	76.1 (66.1 – 86.0)
Local Metastasis	44	47.7 (33.0 – 62.5)	62	80.7 (70.8 – 90.5)

* Confidence Interval