Vascular Endothelial Growth Factor C mRNA Expression Correlates with Stage of Progression in Patients with Melanoma

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ABSTRACT

Purpose: **Vascular endothelial growth factor (VEGF)-C promotes the ingrowth and invasion of lymphatics in many different tumor types, including melanoma. To determine whether expression of VEGF-C correlates with stage of progression, we measured VEGF-C mRNA levels in melanomas representing different stages of progression and from the vertical and horizontal growth-phase of individual primary melanomas.**

Experimental Design: **Total RNA was extracted from human melanoma specimens taken from operative specimens and subjected to quantitative real-time PCR. VEGF-C levels were determined for 54 melanoma samples, including primary melanomas ($n = 15$), local recurrences ($n = 6$), regional dermal metastases ($n = 11$), nodal metastases ($n = 12$), and distant metastases ($n = 10$). As a surrogate for lymphatic density, we also measured the expression of the lymphatic endothelial marker LYVE-1 and correlated its expression with previously measured VEGF-C levels.**

Results: **Vertical growth phase melanomas expressed significantly higher levels of VEGF-C than horizontal growth phase melanomas. Nodal metastases expressed the highest level of VEGF-C, followed by regional dermal metastases. Primary and local recurrences expressed a relatively low level of VEGF-C, as did negative lymph nodes and distant metastases. In addition, VEGF-C expression correlated well with LYVE-1 expression ($r = 0.611$; $P < 0.0001$).**

Conclusions: **These data suggest that high levels of VEGF-C may be important in regional lymphatic disease in melanoma and that VEGF-C and LYVE-1 levels may identify tumors with a high risk for nodal metastases, for which antilymphangiogenic therapy may be more effective.**

INTRODUCTION

Melanoma tends to metastasize early and almost always spreads first through the local lymphatics to regional lymph nodes. Initially, the primary tumor grows horizontally through the epidermis, but later, for reasons poorly understood, it will begin to invade vertically, with a direct correlation between the thickness of this vertical growth phase component of the tumor and the likelihood of metastasis (1). Hematogenous spread usually occurs later, either when lymphatic metastatic disease is already present or, much less commonly, when a primary tumor reaches a relatively large size, although on rare occasions thin melanomas have been observed to result in hematogenous spread, even with negative sentinel lymph nodes (2–4). One hypothesis to explain this pattern of metastatic spread is that vertical growth phase melanoma cells develop the ability to produce prolymphangiogenic substances at an early stage of development, leading to the ingrowth of local lymphatic vessels. VEGF-C[1] is a potent prolymphangiogenic peptide that has been implicated in lymphatic metastases in several different tumor types, including melanoma (5–7). However, clinically, it has yet to be determined whether VEGF-C plays a significant role in the proliferation and invasion of lymphatic vessels in primary melanomas or correlates with regional metastatic spread.

Because of the orderly pattern of metastasis to the regional lymph nodes typically seen in patients with melanoma, we hypothesized that VEGF-C expression is involved in melanoma progression. Therefore, to determine whether VEGF-C expression correlates with melanoma progression, we used QRT-PCR to measure VEGF-C mRNA levels in melanoma specimens representing different stages of progression. Because vertical growth phase spread appears to be necessary for lymphatic metastases in most melanomas (4), we also measured VEGF-C levels in tumor specimens taken from the horizontal and vertical growth phases of the same primary melanomas to determine whether the vertical growth phase of a tumor produces more VEGF-C than its horizontal growth phase. Finally, because levels of the mRNA for LYVE-1, receptor for hyaluronan whose expression is restricted primarily to lymphatic tissue and the sinusoids of the liver (8, 9), measured by by QRT-PCR have been reported to correlate with lymphatic density (10), we studied whether VEGF-C expression might correlate with lym-

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[1] The abbreviations used are: VEGF, vascular endothelial growth factor; QRT-PCR, quantitative real-time PCR; BHQ-1, Black Hole Quencher-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 6-FAM, 6-carboxyfluorescein.

phatic density in clinical melanoma samples by using QRT-PCR to measure LYVE-1 levels in the same melanoma samples. We report here that VEGF-C expression increases as melanoma progresses regionally to lymphatic metastases and correlates with LYVE-1 expression in melanoma, suggesting that the measurement of VEGF-C and LYVE-1 may be helpful in determining the likelihood of a melanoma to metastasize to the regional lymphatics and identifying patients for whom antilymphangiogenic therapies may be effective.

MATERIALS AND METHODS

Tissue Harvesting and Total RNA Isolation. Tumor samples were obtained through The Tissue Retrieval Service, a shared resource of The Cancer Institute of New Jersey, following UMDNJ-Robert Wood Johnson Medical School Institutional Review Board guidelines. Tissue samples were collected such that no patient-identifiable information was attached to the specimens, but tumor location and stage were recorded. The negative lymph nodes used as a control in this project were from completion lymphadenectomy specimens from patients with melanoma. Normal-appearing lymph nodes, distant from the site of the involved lymph nodes, were dissected out from the lymphadenectomy specimen in the operating room. One-fourth of each normal node was preserved in liquid nitrogen and used in these experiments. The remainder of each node was examined using routine histopathological techniques to confirm the absence of metastatic deposits of melanoma. The remainder of each node that was used in the experiments was tested by reverse transcription-PCR to detect tyrosinase and MART-1 (melanoma antigen recognized by T cells-1), and all samples were found to be negative for these two markers, confirming that no melanoma was present in the samples. In five patients, it was possible to obtain separate specimens from the same tumor representing its horizontal and vertical growth phases. To identify specimens from the vertical and horizontal growth phases of each tumor, a 4-mm punch was taken of what clinically appeared to be either a horizontally or vertically growing portion of the tumor. The punch biopsy holes were inked to mark their location in the primary tumor. Histological confirmation of the presence of a vertical or horizontal growth phase at the inked punch biopsy site was then made by a dermatopathologist specializing in melanoma. A local recurrence was defined as occurring in the original wide excision scar or in the epidermis immediately adjacent to the scar. Regional dermal metastatic disease was defined as nodules arising in the regional lymphatic bed outside of the original scar and regional lymph nodes (2, 3). Tumor specimens were obtained in the operating room, flash-frozen in liquid nitrogen within 5 min of resection, and then stored in liquid nitrogen until RNA extraction. Total RNA was isolated from samples using a standard, spin column-based kit (RNAeasy; Qiagen). First-strand synthesis was performed on total RNA using another standard kit (Superscript kit; Invitrogen) and oligo(dT) primers to reverse transcribe mRNA.

QRT-PCR Measurement of VEGF-C and LYVE-1 mRNA Levels. Before QRT-PCR, mRNA samples were subjected to standard reverse transcription-PCR with previously described primers to amplify β-actin, MART-1, and tyrosinase (11). All tumor samples were positive for these three genes. All normal lymph nodes were positive for β-actin but negative for MART-1 and tyrosinase. VEGF-C and LYVE-1 message levels were then determined by QRT-PCR using TaqMan probes, with the carboxyfluorescein fluorescent dye 6-FAM as the 5′-fluorophore and BHQ-1 (Biosearch Technologies, Inc.) as the 3′-quencher (12). QRT-PCR was carried out using a Cepheid SmartCycler thermocycler, and the associated SmartCycler version 1.2 software was used to analyze the data and determine the threshold count (C_t).

Primers and probes were designed using the MacVector 7.1 software package (Accelrys, San Diego, CA). Sequences of the VEGF-C primers and probe were as follows: 5′-TGC-CGA-TGC-ATG-TCT-AAA-CT-3′ (forward); 5′-TGA-ACA-GGT-CTC-TTC-ATC-CAG-C-3′ (reverse); product size = 252 bp; and 5′-(6-FAM)-CAG-CAA-CAC-TAC-CAC-AGT-GTC-AGG-CA-3′-(BHQ-1) [TaqMan probe]. Sequences of primers and probe for LYVE-1 were as follows: 5′-CCA-GTG-AGC-CGA-CAG-TTT-GGA-G-3′ (forward); 5′-CAG-GTA-TTG-TAG-AGT-AAG-GGG-ATG-CC-3′ (reverse); product size = 184 bp; and 5′-(6-FAM)-AAC-TCA-TCT-GAT-ACT-TGG-ACT-AAC-TCG-C-3′-(BHQ-1) [TaqMan probe]. To correct for differences in RNA quality and quantity between samples, the target gene was normalized to β-actin message levels. Sequences of the β-actin primer and probe set were as follows: 5′-TCA-GCA-AGC-AGG-AGT-ATG-ACG-AG-3′ (forward); 5′-ACA-TTG-TGA-ACT-TTG-GGG-GAT-G-3′ (reverse), product size = 265 bp; 5′-(6-FAM)-ACG-GTG-AAG-GTG-ACA-GCA-GTC-G-3′-(BHQ-1) [TaqMan probe].

Reaction mixtures for QRT-PCR were 25 μl, containing 0.75 unit of Taq polymerase (Invitrogen), reaction buffer, and 0.2 mM deoxynucleotide triphosphates, plus optimized concentrations of $MgCl_2$, probe, and primers. For all reactions, negative controls were run with no template present. In addition, total RNA from patient samples was used for sham reverse transcription reactions with no reverse transcriptase present and then subjected to standard PCR using β-actin primers to verify that no bands were produced. The PCR cycle started with an initial 1.5-min denaturation step at 95°C, followed by 30–40 cycles of denaturation at 95°C for 10 s, annealing at the appropriate annealing temperature for each primer set (56°C for VEGF-C, 62°C for LYVE-1, and 54°C for β-actin) for 20 s, and extension at 72°C for 30 s.

Each sample was run in triplicate, and C_t was determined for VEGF-C and β-actin. We chose β-actin as the housekeeping gene to which we normalized our specimens because it has long been used as an internal standard for the measurement of mRNA levels in Northern blots and then later used in QRT-PCR reactions. It is expressed in a relatively stable fashion in nearly every cell type and is only weakly regulated (13, 14). We chose it over other commonly used housekeeping genes, particularly GAPDH, because of recent evidence that GAPDH levels can vary significantly between individuals when compared with β-actin, making GAPDH inappropriate for experiments comparing gene mRNA levels between individuals (15, 16). The

VEGF-C:β-actin ratio was estimated using the ΔC_t method (16). For each sample, $C_t^{VEGF\text{-}C}$ and $C_t^{\beta\text{-actin}}$ were determined, and $\Delta C_t = C_t^{\beta\text{-actin}} - C_t^{VEGF\text{-}C}$. Differences in the relative level of VEGF-C normalized to β-actin can be estimated by differences in the ratio:

$$\frac{\text{VEGF-C}}{\beta\text{-Actin}} \propto 2^{\Delta Ct},$$

This ratio was determined for each sample and used to compare relative levels of VEGF-C normalized to β-actin between experimental groups. LYVE-1 levels were determined using the same method. After being subjected to the Kolmogorov-Smirnov test to verify Gaussian distributions of the data sets, differences between the means of multiple experimental groups were calculated by one-way ANOVA using Prism 3.0 (Graph Pad, San Diego, CA), followed by the Bonferroni multiple comparison test to perform pairwise comparisons when $P < 0.05$ for the initial ANOVA. Differences between pairs were considered significant when P was <0.05. Because each pair of samples came from the same patient, differences between horizontal and vertical growth phase tumors were determined using Student's paired t test, with differences being considered significant if P was <0.05. Correlation between VEGF-C and LYVE-1 mRNA expression was determined by Pearson correlation.

RESULTS

VEGF-C levels were determined for 54 melanoma samples, including primary melanomas ($n = 15$), local recurrences ($n = 6$), regional dermal metastases ($n = 11$), nodal metastases ($n = 12$), and distant metastases ($n = 10$). We also examined normal lymph lymph nodes ($n = 12$). There was a significant difference between the mean VEGF-C mRNA levels for all groups (Fig. 1; $P = 0.0215$). Mean VEGF-C levels increased systematically with level of progression from primary melanomas to regional dermal metastases to nodal metastases, but the difference was statistically significant only between primary melanomas and nodal metastases (Fig. 1). Similarly, there was a significant difference in VEGF-C expression between normal lymph nodes and nodal metastases. Surprisingly, VEGF-C levels in distant metastases were not statistically different from those measured in primary melanomas, regional dermal metastases, or negative lymph nodes (Fig. 1). The level of scatter in the data was high, particularly for regional dermal metastases and nodal metastases (Fig. 1; data not shown). For five patients, during the harvesting of tissue from the primary melanoma, it was possible to separately sample the vertical and horizontal

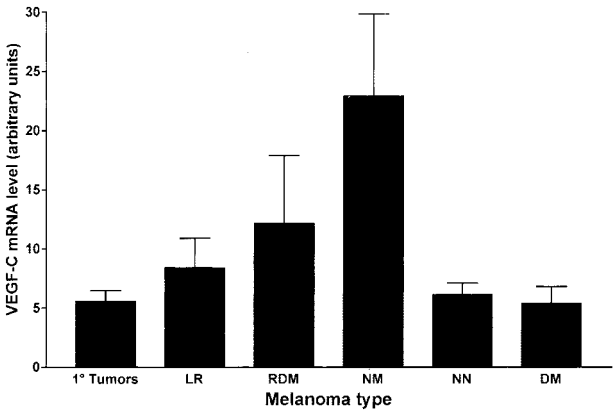

Fig. 1 VEGF-C mRNA expression in melanoma specimens representing different stages of progression. VEGF-C levels were measured using QRT-PCR, and each sample was run in triplicate. VEGF-C mRNA expression was normalized to β-actin expression and expressed in arbitrary units as described in "Materials and Methods." Each *bar* represents the mean ± SE for the measured VEGF-C levels in each group. There was a significant difference between the means of all of the groups ($P = 0.0215$, ANOVA). A significant difference in VEGF-C levels was observed between nodal metastases and primary tumors ($P < 0.05$) and between nodal metastases and negative lymph nodes ($P < 0.05$). There was not a statistically significant difference between any of the other data pairs. *1° Tumors*, primary tumors; *LR*, local recurrences; *RDM*, regional dermal metastases; *NM*, nodal metastases; *NN*, negative lymph nodes; *DM*, distant metastases.

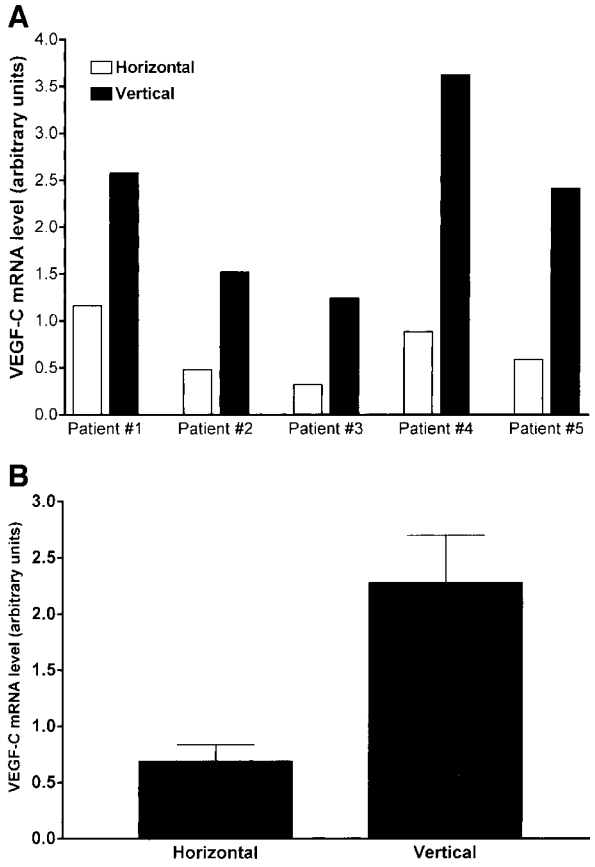

Fig. 2 VEGF-C expression in horizontal and vertical growth phase melanomas. VEGF-C levels were measured using QRT-PCR, and each sample was run in triplicate. VEGF-C mRNA expression was normalized to β-actin expression and expressed in arbitrary units as described in "Materials and Methods." *A*, VEGF-C expression in the horizontal and vertical growth phases from patients 1–5. The difference between VEGF-C levels in horizontal and vertical growth phase melanomas was statistically significant ($P = 0.0003$, paired Student's t test). *B*, mean VEGF-C expression in the horizontal and vertical growth phases of melanoma. Each *bar* represents the mean ± SE for the measured VEGF-C levels in each group.

growth phase. We therefore measured VEGF-C mRNA levels in the vertical and horizontal growth phases of these primary melanomas (Fig. 2, *A* and *B*). VEGF-C message levels were significantly higher in vertical growth phase melanomas than in horizontal growth phase melanomas, both in each patient (Fig. 2*A*; $P = 0.0003$) and in overall group means (Fig. 2*B*). The difference is striking, even though the number of tumors examined was small.

Because LYVE-1 expression levels have previously been correlated with lymphatic density (10), we next used QRT-PCR to measure LYVE-1 message levels in the same tumor samples (Fig. 3). Again, there was a statistically significant difference between the mean LYVE-1 levels of the groups (Fig. 3*A*; $P = 0.015$). However, for LYVE-1, the pattern was somewhat different than that for VEGF-C. Distant metastases expressed the highest level of LYVE-1 message ($P < 0.01$), and no statistically significant difference in LYVE-1 expression between primary melanomas, regional dermal metastases, local recurrences, or nodal metastases was observed (Fig. 3*A*). We therefore examined LYVE-1 expression in the same vertical and horizontal growth phase specimens (Fig. 3*B*). For two of the specimens, VEGF-C levels were severalfold greater in the vertical phase than in the horizontal phase, and for one it was approximately 2-fold greater. However, for two of them, there was no difference. Overall, unlike the case for the expression of VEGF-C, the difference in LYVE-1 expression between the two groups approached statistical significance but did not achieve it ($P = 0.0937$). This could be due to the small number of specimens, inherent biological variability, or a combination of the two. Additional patients for whom both vertical and horizontal phase melanoma can be harvested would clarify which is the more likely case.

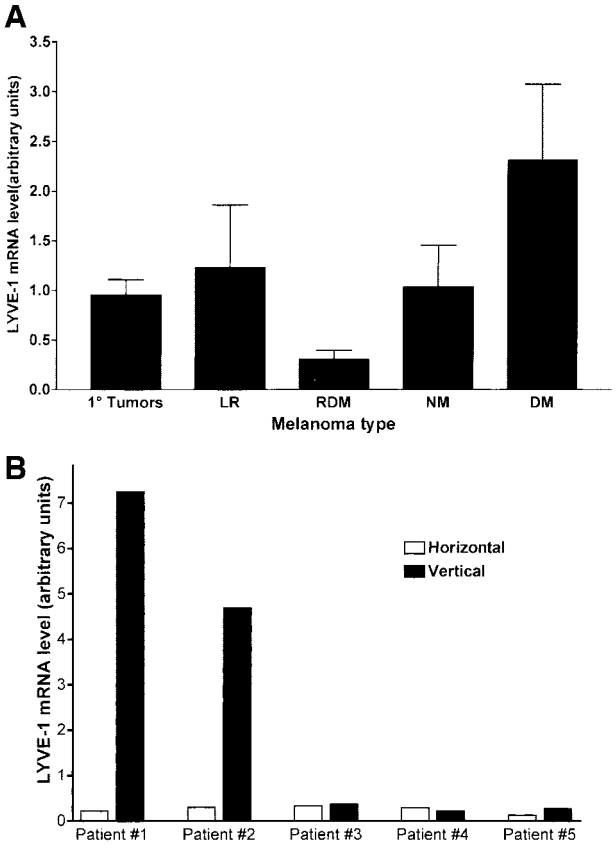

Fig. 3 LYVE-1 mRNA expression in melanoma specimens representing different stages of progression. LYVE-1 levels were measured using QRT-PCR, and each sample was run in triplicate. LYVE-1 mRNA expression was normalized to β-actin expression and expressed in arbitrary units as described in "Materials and Methods." *A*, LYVE-1 expression by melanoma stage of progression. Each *bar* represents the mean ± SE for the measured VEGF-C levels in each group. There was a significant difference between the means of all of the groups ($P = 0.015$, ANOVA). There was a statistically significant difference between LYVE-1 mRNA levels in primary tumors and distant metastases ($P < 0.01$). There was not a statistically significant difference between any of the other data pairs. *1° Tumors*, primary tumors; *LR*, local recurrences; *RDM*, regional dermal metastases; *NM*, nodal metastases; *DM*, distant metastases. *B*, LYVE-1 expression in the horizontal and vertical growth phases from patients 1–5. The difference between LYVE-1 levels in horizontal and vertical growth phase melanomas approached but did not reach statistical significance ($P = 0.0937$, paired Student's *t* test).

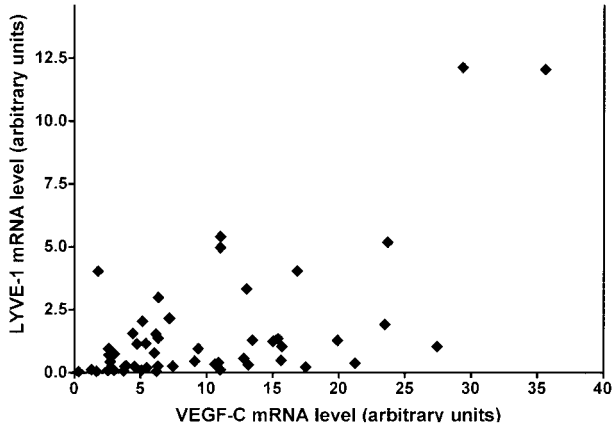

Fig. 4 Correlation between VEGF-C and LYVE-1 mRNA expression in melanomas. VEGF-C and LYVE-1 levels were measured by QRT-PCR and correlated with each other. There was a strong correlation between VEGF-C and LYVE-1 expression in all melanomas ($P < 0.0001$ and $P = 0.6108$, Pearson's correlation). See "Results" for details.

Finally, given the biological variability of our specimens, we decided to determine how closely the expression of VEGF-C correlates with the expression of LYVE-1 in melanoma. We therefore directly compared VEGF-C and LYVE-1 mRNA levels in the melanoma specimens studied (Fig. 4). There was a strong correlation between VEGF-C and LYVE-1 mRNA levels ($P < 0.0001$; $r = 0.6108$; Pearson correlation) for all stages of melanoma. Moreover, when we divided our samples into groups based on the level of progression, this correlation was strongest for regional dermal metastases ($P = 0.0006$; $r = 0.754$; Pearson correlation; data not shown) and nodal metastases ($P = 0.0010$; $r = 0.7702$; Pearson correlation; data not shown). The correlation was not as strong for primary melanomas ($P = 0.042$; $r = 0.498$; Pearson correlation; data not shown), and for distant metastases, it approached but did not achieve statistical significance ($P = 0.083$; $r = 0.601$; Pearson correlation).

DISCUSSION

Melanoma is usually produced by the transformation of an epidermal melanocyte into a malignant cell. The primary tumor grows horizontally through the epidermis. However, over time, a vertical growth phase component develops in the primary tumor, and the melanoma begins to thicken and invade the lower levels of the dermis. Once a vertical grow phase has developed, metastasis becomes more likely, and there is a direct correlation between the thickness of the vertical growth phase component of a primary melanoma and the likelihood of metastasis (1). The initial metastatic pathway is almost always through regional lymphatics to the regional lymph nodes, although metastatic melanoma cells can halt along the lymph vessels and grow as regional dermal metastases (1, 4, 17, 18).

Why melanoma can spread so easily through lymphatic vessels but is less able to spread hematogenously is poorly understood, as is why it acquires the ability to metastasize hematogenously after forming nodal metastases. Recently, the production of proangiogenic and lymphangiogenic factors by cancer cells has been described, and many of these factors have been implicated in the production of metastatic disease by a variety of different tumor types (19, 20). One of these factors, VEGF-C, is produced by melanoma cells and has been implicated in the production of lymph node metastases in other tumor types (6, 7, 21). VEGF-C is a ligand for Flt-4 (VEGF receptor-3), a tyrosine kinase receptor mainly expressed by the endothelial cells of lymphatic vessels (5). VEGF-C and VEGF-D (22) are the only growth factors thus far identified whose primary function appears to be to simulate the growth and development of lymphatic vessels (7, 20). Consistent with this function, VEGF-C expression correlates with lymph node metastases in many different tumor types (6, 7, 21, 23). In melanoma, overexpression of VEGF-C in human xenografts in nude mice results in enhanced lymphangiogenesis, angiogenesis, and macrophage chemotaxis (24). These observations all suggest a role for VEGF-C in melanoma progression, given how dependent melanoma is on the lymphatic system for its metastases.

Consistent with an important role for VEGF-C in melanoma progression, our data suggest that as a melanoma progresses from the horizontal to the vertical growth phase, the expression of VEGF-C increases significantly (Fig. 2, *A* and *B*), producing a concurrent but much more variable increase in LYVE-1 expression (Fig. 3*B*), although this variability could be due mainly to sampling. Also, as a melanoma progresses from the primary tumor through the regional lymphatics to the regional lymph nodes, VEGF-C expression tends to increase until the regional nodal bed is reached (Fig. 1). However, distant metastases produce little VEGF-C (Fig. 1), although VEGF-C levels correlate well with LYVE-1 levels as measured by QRT-PCR (Fig. 4). We noted a wide scatter of levels of VEGF-C expression at all stages of progression (Fig. 1) and even more variability in LYVE-1 expression (Fig. 3*A*). This variability in VEGF-C expression was most marked for regional dermal metastases and nodal metastases (Fig. 1; data not shown), and the variability in LYVE-1 expression was most marked for nodal and distant metastases (Fig. 3*A*). The biological variability we observed in the expression of VEGF-C may reveal how it could regulate melanoma lymphangiogenesis and metastasis and thus suggest how measuring VEGF-C levels might be clinically useful.

Several possible explanations exist to explain the wide variation in VEGF-C expression in tumor samples at the same level of progression. For example, only a small fraction of the cells in a primary melanoma may need to express VEGF-C for lymphatic growth and invasion to take place. Sampling errors in the selection of the region of the tumor from which RNA was extracted could explain the variations in VEGF-C expression levels observed in our study. A random piece of the tumor is selected in the operating room during tissue banking, and a random area of the preserved specimen is used in standard RNA extractions. More specific sampling of the perilymphatic cells within a tumor, using techniques such as laser capture dissection, may lead to a better correlation between VEGF-C expression and stage of progression. We also point out that our data do not rule out other sources of melanoma-associated VEGF-C. However, for purposes of tumor-induced lymphangiogenesis, it may be a combination of tumor cell secretion of VEGF-C and/or stromal or inflammatory cell secretion that accounts for the differences. A definitive determination of whether the increased VEGF-C production noted in lymph node metastases comes from melanoma cells themselves or from inflammatory or stromal cells in the tumor will be important.

An alternate explanation might be that the tumors expressing quantities of VEGF-C far above mean levels are the ones more likely to produce lymph node metastases. This hypothesis might explain why VEGF-C levels tended to be higher in lymph node metastases than in recurrent primary tumors or regional dermal metastases, with cells expressing high levels of VEGF-C having been selected by the time they reach regional lymph nodes. On the other hand, tumors that metastasize to the regional dermal lymphatics but do not reach the regional lymph node basins demonstrate an intermediate level of VEGF-C expression (Fig. 1) but a comparatively low level of LYVE-1 expression (Fig. 3*A*). It may be that intermediate levels of VEGF-C expression are insufficient to produce significant ingrowth of lymphatic tissue in tumor nodules that have lodged in regional dermal tissues instead of the regional lymphatic bed. Furthermore, cells in the vertical growth phase of a primary melanoma that do not produce large quantities of VEGF-C but are in close proximity to cells that do could potentially invade the lymphatics along with the VEGF-C-producing cells and form regional dermal metastases that do not produce high levels of this factor, whereas the cells that produce the highest levels of VEGF-C are the ones that ultimately continue migrating along the dermal lymphatic channels to form lymph node metastases.

Finally, we observed that distant metastases, while expressing high levels of LYVE-1 (Fig. 3*A*), expressed VEGF-C at levels not detectably different than levels observed in primary melanomas (Fig. 1). This observation suggests that melanoma cells that metastasize distantly may change their phenotype to express either different prolymphangiogenic factors such as VEGF-D (22) or more proangiogenic factors. Given that there is overlap between the binding affinities of the VEGF isoforms for different VEGF receptors, leading to the activation of both prolymphangiogenic and proangiogenic signaling by different VEGF isoforms to varying degrees (7, 20), it may be that melanoma cells that acquire the capability of metastasizing

distantly have acquired the ability to express more proangiogenic isoforms of VEGF to promote hematogenous metastases. Alternatively, they may express different proangiogenic and prolymphangiogenic factors than melanoma cells forming lymph node metastases. A final possibility is that melanoma metastasis results in the increased expression of VEGF-C by stromal and immune cells in the lymph node; however, because the nodes we chose were all chosen because they were grossly positive for melanoma, this possibility seems less likely. Further clarifying which proangiogenic and prolymphangiogenic factors are important in promoting both early lymph node metastases and late hematogenous metastases will be important as the basis for the development of targeted therapies of melanoma directed at these two processes. Furthermore, the biological variability of melanoma that results in such scatter in the levels of VEGF-C expression implies that determining the relationship between VEGF-C expression by an individual melanoma and that melanoma's ability to induce lymphangiogenesis and, more importantly, metastasis could lead to targeted antilymphangiogenic therapies that could be offered to patients with the highest levels of VEGF-C expression and, presumably, the highest risk of regional nodal metastases.

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