Significantly high lymphatic vessel density in cutaneous metastasizing melanoma

Špirić Z¹, Erić M², Eri Ž³, Skrobić M⁴

¹Department of Nuclear Medicine and Thyroid Diseases, Clinical Centre Banja Luka, Republic of Srpska, Bosnia and Herzegovina
²Department of Anatomy, Faculty of Medicine, University of Novi Sad, Novi Sad, Serbia
³Department of Pathology, Institute for Pulmonary Diseases of Vojvodina, Sremska Kamenica, Faculty of Medicine, University of Novi Sad, Novi Sad, Serbia
⁴Department of Nuclear Medicine, Faculty of Medicine, University of Banja Luka, Banja Luka, Republic of Srpska, Bosnia and Herzegovina

Abstract

Background: Cutaneous melanoma has the propensity to early metastatic spread via the lymphatic vessels. Recent studies have found a positive correlation between an increased number of tumor-associated lymphatics and lymph node metastasis. The aim of this study was to determine whether there was a difference in the lymphatic vessel density (LVD) when cutaneous metastasizing melanomas were compared with nonmetastasizing melanomas and nevi.

Methods: Ninety-five melanoma specimens (45 with lymph node metastasis, 50 nonmetastasizing) and 22 nevi specimens (7 compound, 5 intradermal, 4 blue, and 6 dysplastic) were investigated by immunostaining for the lymphatic endothelial marker D2-40. The quantification of lymphatics was conducted by computer-assisted morphometric analysis. Metastasizing and nonmetastasizing melanoma specimens were matched according to their thickness into three classes ≤2.0 mm, 2.01 – 4.0 mm, >4.0 mm.

Results: Metastasizing melanomas thick 2.01–4.0 mm and thicker than 4.0 mm, showed a significantly higher intratumoral and peritumoral LVD compared with nonmetastasizing melanomas (2.01–4.0 mm, p =0.006 and p =0.032, respectively; >4.0 mm, p =0.045 and p =0.026, respectively). No significant difference in intratumoral and peritumoral LVD was found between metastasizing and nonmetastasizing melanomas of thickness ≤2.0 mm. Metastasizing melanomas showed a significantly higher intratumoral LVD compared with compound, intradermal, blue and dysplastic nevi (p <0.001, p =0.002, p =0.002 and p <0.001, respectively), and significantly higher peritumoral LVD compared with compound nevi (p =0.039). Total average LVD was significantly higher in metastasizing melanomas than in nonmetastasizing melanomas (p <0.001), compound, intradermal, blue and dysplastic nevi (p <0.001, p <0.001, p =0.001 and p <0.001, respectively).

Conclusions: This study shows higher LVD in metastasizing melanomas compared with nonmetastasizing melanomas and nevi. In melanomas with intermediate thickness and in thick melanomas, higher intratumoral and peritumoral LVD are significantly associated with lymph node metastasis. This finding suggests that LVD can be a useful marker for identifying melanomas which are at a higher risk for the metastasis development. Hippokratia 2015; 19 (3): 210-215.

Key words: Melanoma, nevi, lymphatic vessel density, D2-40, metastasis

Introduction

Cutaneous melanoma is characterized by a high malignant potential and the ability to metastasize via the lymphatic vessels at an early stage of the disease.

Many issues related to metastatic spreading via lymphatic vessels, as well as the interaction between the tumor and surrounding lymphatic vessels, are not yet fully explained¹³.

The research of the lymphatic system has been enabled only recently owing to the discovery of specific immunohistochemical markers that distinguish blood and lymphatic vessels in conventionally processed formalin-fixed, paraffin-embedded tissue specimens. Most widely used markers for specific stain and quantification of lymphatic vessels have been the LYVE-1 (lymphatic vessel endothelial hyaluronan receptor-1) and D2-40 (podo-planin, mucin-type transmembrane glycoprotein)²⁴⁵.

Until recently, the lymphatic system of the melanoma surrounding skin has been believed to have a passive role in the progression of the disease and that lymphatic invasion occurs via tumor cells infiltrating pre-existing lymphatic vessels³⁶. However, since it was found that
melanoma secrets vascular endothelial growth factor (VEGF)-C and VEGF-D, and thus leads to lymphangiogenesis - formation of new lymphatic vessels - intratumoral, and, in particular, peritumoral, the clinical knowledge that progression of the disease is associated with lymphangiogenesis has become more refined3-9.

The studies are based on the quantification of lymphangiogenesis and the determination of intratumoral and peritumoral lymphatic vessel density (LVD) give a new insight of the melanoma metastasis and suggest an active role of the lymphatic system of the skin in the progression of the disease11. Recent clinicopathological studies have found a positive correlation between an increased number of tumor-associated lymphatics and lymph node metastasis, and also suggested that LVD might be used as a novel prognostic indicator for the risk of lymph node metastasis in cutaneous melanoma3-2,6-7,11.

The aim of this study was to determine whether there was a difference in intratumoral and peritumoral LVD between melanomas with lymph node metastasis and melanomas without developed metastasis. In addition, the aim was to determine whether there was any difference in intratumoral and peritumoral LVD between melanomas and benign cutaneous melanocytic tumors (nevi), as it was assumed that the LVD in and around the nevi was expected to be low.

For this purpose, we have used monoclonal antibody D2-40, the most reliable marker for lymphatic endothelial cells27.

Material and Methods

Conventionally processed formalin-fixed, paraffin-embedded tissue specimens of 117 cutaneous melanocytic tumors were taken from the Department of Pathology archives, Clinical Centre of Banja Luka. There were melanocytic nevi (n =22) and malignant melanomas (n =95). The group of melanocytic nevi included the compound (n =7), dysplastic (n =6), blue (n =4), and intradermal nevi (n =5). After reviewing the complete medical records of patients in the Oncology Clinic, Clinical Centre of Banja Luka, melanoma specimens were divided into metastasizing melanomas (n =45) and nonmetastasizing melanomas (n =50). Melanoma specimens taken from patients with lymph node metastasis were labeled as metastasizing, while specimens of patients who have not developed metastatic disease for over five years after the primary biopsy were labeled as nonmetastasizing. Melanoma thickness was categorized as in AJCC (American Joint Committee on Cancer) staging using the T (tumor) classes (≤1.0 mm, 1.01-2.0 mm, 2.01-4.0 mm, >4.0 mm). Metastasizing and nonmetastasizing melanoma specimens were matched according to the tumor thickness. Considering that only two metastasizing melanomas were thick ≤1.0 mm, for the analysis we chose the division into three classes ≤2.0 mm, 2.01-4.0 mm, >4.0 mm.

Immunohistochemistry

Immunohistochemical staining was performed using the streptavidin-biotin-peroxidase conjugate method. Three-µm paraffin sections were deparaffinized in xylol, rehydrated through a graded ethanol series and water, and were submitted to microwave antigen retrieval in 0.01M citrate buffer, pH 6.0, for 20 minutes at 800 W.

After inactivation of endogenous peroxidase activity with 3% hydrogen peroxide in water for five minutes, sections were washed in phosphate buffered saline (PBS 0.1 M). Sections were then incubated with primary D2-40 monoclonal mouse anti-human antibody (clone D2-40, Code M3619, Dako, Denmark) at a 1/100 of dilution for 45 minutes at room temperature, and then were washed in 0.1 M PBS pH 7.2.

Subsequently, the sections were incubated with biotinylated anti-mouse IgG secondary antibody (LSAB+/HRP, Biotinylated Link, Code K0690, Dako, Denmark) for 20 minutes at room temperature in a humid chamber, and were washed in 0.1 M PBS. Then, sections were incubated with streptavidin-peroxidase for 20 minutes at room temperature in a humid chamber (LSAB+/HRP, Streptavidin HRP, Code K0690, Dako, Denmark) and then were washed in 0.1 M PBS.

Sections were visualized with diaminobenzidine (DAB+) and washed in water, counterstained Mayer’s hematoxylin, dehydrated and mounted.

Double staining of melanomas was performed for detection two antigens (D2-40 and second antigen S-100). Melanoma cells were identified using S-100 antibody (rabbit polyclonal, 1/400 dilution; Code Z0311 Dako, Denmark), with AP (alkaline phosphatase) labeled polymer and permanent red chromogen (DakoCytomation EnVision G2 Doublestain system, HRP-AP, Code K5361, Dako, Denmark).

Computer-assisted morphometric analysis

LVD was defined as the number of vessels per square millimeter. To determine LVD, sections were examined using an Olympus CX21 microscope (Olympus Corp., Tokyo, Japan) at x100 magnification seeking the fields with the highest LVD (hot spots). Digital images were taken at x400 magnification for each section - for six hot spots, three intratumoral and three peritumoral within an area of 200 µm from the tumor border.

The digital images were analyzed with Image Tool software (UTHSCSA, San Antonio, Texas, USA), while the number of lymphatics in 1 mm² was determined with the program addition “count and tag”. The mean values of the measurements in three fields were used for statistical analysis.

Statistical analysis

All statistical tests were performed using SPSS software version 21.0 (IBM, Armonk, NY, USA). The results were presented as a mean and standard deviation. Comparison of the LVD between the two groups was performed by Student’s t-test. For comparison of LVD among more groups one-way analysis of variance (ANOVA) was applied, and subsequent Tukey’s post hoc test for multiple comparisons, and a nonparametric Kruskal-
Wallis test. For the determination of the difference between the peritumoral and intratumoral LVD a two-way ANOVA was employed. Correlations between LVD and melanoma thickness were analyzed by the Spearman’s rho test. $p < 0.05$ was considered significant.

**Results**

Immunohistochemistry of D2-40 showed stained endothelial cells of lymphatic vessels. Lymphatic vessels were visible in tumor mass and peritumoral area, in all cases. They had a thin wall consisting of a single layer of endothelial cells. Intratumoral lymphatics were smaller, often compressed, with a narrow, partially collapsed or collapsed lumen. Peritumoral lymphatics were larger, and with a more dilated open lumen (Figure 1). LVD in peritumoral mass was higher in comparison with intratumoral LVD in all observed groups, but this difference was not statistically significant (two-way ANOVA, $p = 0.892$).

There was no significant correlation between LVD and melanoma thickness (Spearman’s rho test, $r = 0.143$, $p = 0.170$ intratumoral; $r = 0.080$, $p = 0.446$ peritumoral LVD) (Table 1). Metastasizing melanomas between 2.01 mm and 4.0 mm thick, as well as melanomas thicker than 4.0 mm, showed a significantly higher intratumoral and peritumoral LVD compared with nonmetastasizing melanomas (2.01-4.0 mm, $p = 0.006$ and $p = 0.032$, respectively; >4.0 mm, $p = 0.045$ and $p = 0.026$, respectively) (Table 1). In the group of melanoma thickness $\leq 2.0$ mm, intratumoral and peritumoral LVD in metastasizing melanomas was higher than in nonmetastasizing melanomas, but the difference was not statistically significant (Student’s t-test, $p = 0.139$ and $p = 0.825$, respectively) (Table 1).

For all the specimens, regardless of their division according to thickness, metastasizing melanomas showed a significantly higher intratumoral and peritumoral LVD compared with nonmetastasizing melanomas (Student’s t-test, $p < 0.001$ and $p = 0.004$, respectively) (Table 2).

Among the observed metastasizing melanomas, nonmetastasizing melanomas, compound nevi, blue, dysplastic and intradermal nevi, one-way ANOVA showed a statistically significant difference in intratumoral LVD ($p < 0.001$), as well as a statistically significant difference in peritumoral LVD ($p < 0.001$). Tukey’s *post hoc* test showed a significantly higher intratumoral LVD in metastasizing melanomas than in nonmetastasizing melanomas ($p < 0.001$), compound, intradermal, blue and dysplastic nevi ($p < 0.001$, $p = 0.002$, $p = 0.002$ and $p < 0.001$, respectively). Also, in case of peritumoral LVD, there was a significantly higher peritumoral LVD in metastasizing melanomas than in nonmetastasizing melanomas ($p = 0.022$) and compound nevi ($p = 0.039$) (Figure 2, Table 2).

Additional comparisons using Tukey’s HSD test showed a significantly higher mean (total average) LVD in metastasizing melanomas compared to nonmetastasizing melanomas ($p < 0.001$), compound, intradermal, blue and dysplastic nevi ($p < 0.001$, $p < 0.001$, $p = 0.001$, and
There was no significant difference in intratumoral and peritumoral LVD among compound, intradermal, blue and dysplastic nevi (p=0.513 and p=0.754, respectively; Kruskall-Wallis test) (Figure 2, Table 2).

Discussion
Cutaneous melanoma has the propensity to early metastatic spread to regional lymph nodes. Prognosis is currently based predominantly on tumor thickness13,14.

An essential step in the metastatic process is tumor secretion of VEGF-C and VEGF-D, which induces the formation of new lymphatic vessels and their enlargement1-3,9,10.

New lymphatic vessels are formed intratumorally and peritumorally, close to the edge of the melanoma. According to current knowledge, peritumoral lymphatic vessels have a critical role in the metastatic process. They are more densely positioned than intratumoral lymphatic vessels; they are large, dilated vessels, with an open, tortuous lumen and have a greater capacity for fluid and cell transport. The importance of intratumoral lymphatics for cancer metastasis has remained unclear. Intratumoral vessels may collapse due to the increased pressure within the tumor, and thus may be non-functional1,8,15,16. Padera et al found lymphatic metastasis in the absence of func-

Table 1: Lymphatic vessel density in relation to tumor thickness and lymphatic metastasis in cutaneous melanoma.

<table>
<thead>
<tr>
<th>Thickness (mm)</th>
<th>n</th>
<th>Mean peritumoral LVD ± SD</th>
<th>p value</th>
<th>Mean intratumoral LVD ± SD</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 1.0</td>
<td>14</td>
<td>48.18 ± 14.81</td>
<td>0.446</td>
<td>46.50 ± 19.20</td>
<td>0.170</td>
</tr>
<tr>
<td>1.01 - 2.0</td>
<td>20</td>
<td>47.78 ± 19.05</td>
<td></td>
<td>45.77 ± 19.86</td>
<td></td>
</tr>
<tr>
<td>2.01 – 4.0</td>
<td>30</td>
<td>49.52 ± 20.56</td>
<td></td>
<td>47.63 ± 15.76</td>
<td></td>
</tr>
<tr>
<td>&gt; 4.0</td>
<td>31</td>
<td>53.10 ± 22.59</td>
<td></td>
<td>50.12 ± 15.13</td>
<td></td>
</tr>
</tbody>
</table>

| ≤ 2.0         | 825 | 51.39 ± 15.65             | 0.825   | 54.20 ± 17.81             | 0.139   |
| Metastasizing melanoma | 5  | 49.30 ± 11.24             |         | 43.16 ± 12.71             |         |
| Nonmetastasizing melanoma | 29 |                  |         |                           |         |

| 2.01 – 4.0    | 0.032 | 56.40 ± 23.91             | 0.032   | 55.14 ± 16.31             | 0.006   |
| Metastasizing melanoma | 16 | 38.86 ± 13.52             |         | 39.57 ± 10.66             |         |
| Nonmetastasizing melanoma | 14 |                  |         |                           |         |

| > 4.0         | 0.026 | 57.88 ± 22.83             | 0.026   | 53.03 ± 14.26             | 0.045   |
| Metastasizing melanoma | 24 | 39.97 ± 12.14             |         | 40.15 ± 14.66             |         |
| Nonmetastasizing melanoma | 7  |                  |         |                           |         |

| LVD: lymphatic vessel density, SD: standard deviation, n: number of specimens.

Figure 2: Distribution of mean peritumoral (A) and mean intratumoral (B) lymphatic vessel density (LVD) in a series of metastasizing melanomas (MM), nonmetastasizing melanomas (NM), compound nevi (CN), blue nevi (BN), dysplastic nevi (DN) and intradermal nevi (IN) - Tukey’s post hoc test showed a significantly higher peritumoral LVD in metastasizing melanomas compared to nonmetastasizing melanomas (p =0.022) and compound nevi (p =0.039); and significantly higher intratumoral LVD in metastasizing melanomas compared to nonmetastasizing melanomas (p <0.001), compound nevi (p <0.001), blue (p =0.002), dysplastic (p <0.001) and intradermal nevi (p =0.002).
tional intratumor lymphatics.

Clinicopathological studies based on the quantification of lymphangiogenesis have found a correlation between an increased number of tumor-associated lymphatic vessels and lymph node metastasis. The study by Shields et al found statistically significant differences in peritumoral and intratumoral LVD in metastasizing and nonmetastasizing melanomas. No statistically significant differences in intratumoral LVD was found. In that study, peritumoral LVD was significantly higher in metastasizing melanomas. In the case-control study done by Massi et al both peritumoral and intratumoral LVD were significantly higher in melanomas with sentinel lymph node (SLN) metastasis. Results from several other studies (Dadras et al, Liu et al, Emmet et al) showed a significantly higher peritumoral LVD in melanomas with lymph node metastasis compared with nonmetastasizing melanomas. The above studies strongly suggest that the extent of lymphangiogenesis in the primary cutaneous melanoma can serve as a prognostic indicator to predict the presence of lymph node metastasis.

However, studies by Sahni et al and Gallega et al found no significant difference in intratumoral and peritumoral LVD in SLN-negative and SLN-positive cases. The results of our study showed a significant difference in LVD between metastasizing and nonmetastasizing melanomas thicker than 2 mm. Metastasizing melanomas showed significantly higher intratumoral and peritumoral LVD. These findings clearly show that with the increase in the density of lymphatic vessels, the risk for metastasis increases as well and that the results of the quantitative analysis of tumor lymphangiogenesis can be used as a prognostic factor. Thus, our study supports the studies that found a correlation between an increased number of tumor-associated lymphatic vessels and lymph node metastasis. However, we have not found statistically significant correlation between LVD and lymph node metastasis in thinner melanomas. Perhaps, a small number of metastasizing melanomas thinner than 2.01 mm could have affected statistical significance in our study.

In contrast to the results shown here, there has been one report that LVD may be decreased in patients with metastasis. The authors suggested that large and aggressive melanomas might compress and destroy the lymphatics and possibly make them less detectable.

This discrepancy between the results of different studies is likely due to methodological variability, such as the use of different markers for lymphatics, LVD determination by the hot spot selection versus the median absolute and computer-assisted morphometric analysis and non-computerized counting.

In addition, we have investigated and compared the LVD of benign melanocytic tumors (nevi) and melanomas. We found two studies dealing with this issue with different results. In the study by Massi et al, peritumoral LVD was significantly higher in melanomas. On the other hand, in the study by Giorgdaze et al, as well as in our study, intratumoral LVD in metastasizing melanomas was significantly higher compared with all observed types of nevi (compound, blue, dysplastic, intradermal). Also, in our study, peritumoral LVD in metastasizing melanomas was significantly higher than in compound nevi.

Significantly higher total average LVD in metastasizing melanomas than in nonmetastasizing melanomas and all observed types of nevi reveals that metastasizing melanomas strongly stimulate the formation of new lymphatic vessels.

In conclusion, this study, based on the quantitative analysis of tumor-associated lymphatic vessels network, shows significantly high LVD in cutaneous metastasizing melanomas. In melanomas with intermediate thickness and in thick melanomas, higher intratumoral and peritumoral LVD are significantly associated with lymph node metastasis. This findings suggest that LVD can be a useful marker for identifying melanomas which are at a higher risk for the metastasis development.

Conflicts of interest
All authors have no conflicts of interest.

References

Table 2: The quantitative results of peritumoral, intratumoral and mean lymphatic vessel density in various melanocytic tumors.

<table>
<thead>
<tr>
<th></th>
<th>Mean peritumoral LVD ± SD</th>
<th>Mean intratumoral LVD ± SD</th>
<th>Mean LVD ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>55.85 ± 22.05</td>
<td>53.88 ± 14.95</td>
</tr>
<tr>
<td>Metastasizing melanomas</td>
<td>45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonmetastasizing melanomas</td>
<td>50</td>
<td>43.68 ± 17.93</td>
<td>41.58 ± 12.26</td>
</tr>
<tr>
<td>Compound nevi</td>
<td>7</td>
<td>33.27 ± 7.22</td>
<td>26.77 ± 4.15</td>
</tr>
<tr>
<td>Blue nevi</td>
<td>4</td>
<td>29.44 ± 4.64</td>
<td>28.11 ± 4.64</td>
</tr>
<tr>
<td>Dysplastic nevi</td>
<td>6</td>
<td>34.80 ± 8.29</td>
<td>25.70 ± 3.59</td>
</tr>
<tr>
<td>Intradermal nevi</td>
<td>5</td>
<td>32.12 ± 5.68</td>
<td>30.51 ± 6.72</td>
</tr>
</tbody>
</table>

LVD: lymphatic vessel density, SD: standard deviation, n: number of specimens.