Stereologic Estimation of Volume-Weighted Mean Nuclear Volume as a Predictor of Prognosis in “Thin” Malignant Melanoma

Michael Binder, Irene Dolezal, Klaus Wolff, and Hubert Pehamberger

Department of Dermatology I, University of Vienna, Vienna, Austria

At present, tumor invasion represents the most reliable prognostic factor for primary malignant melanoma. The 10-year survival rate of “thin” melanomas (Breslow < 0.76 mm) is more than 95%, but approximately 5% of these low-risk tumors do metastasize.

In an attempt to determine prognostic markers for “thin” melanomas we investigated the volume-weighted mean nuclear volume ($V_v$) of primary melanomas with tumor invasions below 0.76 mm in 32 patients. Ten of these patients had developed melanoma metastases within a mean follow-up period of 49 months; 22 patients who had not developed metastases and who were comparable with regard to clinical and histologic criteria as well as to follow-up period were selected as a comparison group. $V_v$ was determined by computer-assisted image analysis (IBAS 20, Kontron, Germany) on hematoxylin-eosin–stained sections employing stereologic estimation of the volume-weighted mean nuclear volume. In addition, two-dimensional morphometric parameters (nuclear area and shape factors) as well as clinical (sex, age, location) and histologic characteristics (Breslow’s thickness, Clark’s level, and growth patterns) were recorded.

The mean $V_v$ (±SD) of primary melanomas with subsequent metastatic course was 273 $\mu$m$^3$ (± 81.3), whereas primary melanoma lesions without subsequent metastases exhibited a significantly lower $V_v$ of 154 $\mu$m$^3$ (± 25.3) ($p = 0.0008$). On the other hand, two-dimensional morphometric and clinical and histologic parameters did not correlate with prognosis.

$V_v$ thus seems to represent a powerful and independent prognostic marker for “thin” primary melanomas. Assessment of $V_v$ may provide a valuable tool in selecting patients with high-risk stage I, Breslow < 0.76 mm, melanoma for adjuvant therapy. J Invest Dermatol 99:180–183, 1992

The increasing incidence and mortality rate of malignant melanoma is well documented [1–4] and so is the seemingly paradoxical improvement of prognosis, which, however, is due to earlier recognition of this tumor [5,6]. In general, the prognosis of “thin” malignant melanoma, i.e., tumors with a Breslow thickness of less than 0.76 mm, is excellent [7,8] with a 5-year survival rate of approximately 95% [7–12].

The actual rate of metastases occurring in these patients is still a matter of debate. The incidence of metastases in patients with “thin” malignant melanoma has been reported in different studies to range from 0 to 22% with an estimated mean of 5% [7,8,11–14]. Thus, there must exist a subset of “thin” malignant melanoma that is lethal and the recognition of patients with such high-risk “thin” malignant melanomas appears to be of paramount importance.

In the present study, 32 patients with “thin” malignant melanomas, ten of whom had developed subsequent metastases, were investigated for morphometric and stereologic criteria that might predict the outcome of metastatic disease. We found the stereologic estimation of volume-weighted mean nuclear volume ($V_v$) a reliable predictive criterion. Here we report that the $V_v$ of patients with “thin” melanomas developing metastatic disease is significantly higher than in comparable, non-metastatic tumors and thus permits discrimination of high-risk from low-risk “thin” melanomas.

**PATIENTS AND METHODS**

**Patients** Since 1970 more than 1800 melanoma patients have been registered in our computerized pigmented-lesion database. In the last 10 years, 255 patients with primary melanoma lesions less than 0.76 mm according to Breslow [13] were identified [4]. Ten of these patients had been selected because they had developed metastases within an observation period of 6 years. Twenty-two patients who had not developed metastatic disease and who were comparable with regard to sex, age, site, type of melanoma, invasion level, and duration of follow-up served as a comparison group. All study patients had received surgical treatment only, consisting of wide local excision without elective regional lymph-node dissection. Adjuvant immunotherapy and/or chemotherapy had not been given prior to the development of metastases.
Table I. Patient Characteristics*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients with Metastases</th>
<th>Patients without Metastases</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>10</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Sex Male (percent)</td>
<td>5 (50)</td>
<td>7 (32)</td>
<td></td>
</tr>
<tr>
<td>Female (percent)</td>
<td>5 (50)</td>
<td>15 (68)</td>
<td>NS</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>55</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>Minimum/maximum</td>
<td>33/68</td>
<td>26/74</td>
<td>NS</td>
</tr>
<tr>
<td>Site</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trunk</td>
<td>7 (70%)</td>
<td>10 (45%)</td>
<td></td>
</tr>
<tr>
<td>Limbs</td>
<td>3 (30%)</td>
<td>12 (55%)</td>
<td>NS</td>
</tr>
<tr>
<td>Growth pattern</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSM</td>
<td>9 (90%)</td>
<td>21 (95%)</td>
<td></td>
</tr>
<tr>
<td>LMM</td>
<td>1 (10%)</td>
<td>1 (5%)</td>
<td>NS</td>
</tr>
<tr>
<td>Clark's level</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>5 (50%)</td>
<td>10 (45%)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>4 (40%)</td>
<td>11 (50%)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>1 (10%)</td>
<td>1 (5%)</td>
<td>NS</td>
</tr>
<tr>
<td>Mean Thickness (mm)</td>
<td>0.57</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>0.16</td>
<td>0.09</td>
<td>NS</td>
</tr>
<tr>
<td>Minimum/maximum</td>
<td>0.30/0.75</td>
<td>0.50/0.75</td>
<td></td>
</tr>
<tr>
<td>Mean duration of follow-up (month)</td>
<td>49.1</td>
<td>65.5</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Percent values are rounded to the nearest integer.

In all patients the diagnosis of primary malignant melanoma had been confirmed by histopathology. Date recorded and available for evaluation included age, sex, localization of primary tumor, histopathologic type of melanoma, level of invasion according to Clark et al [15], and tumor thickness according to Breslow [13].

Metastases diagnosed at follow-up were recorded as regional lymph-node metastases, cutaneous/subcutaneous metastases, and/or visceral disease. Patients had been examined for evidence of metastatic disease every 3 months for 5 years and subsequently every 6 months. In addition, chest X-ray, abdominal ultrasound, and bone scan were done every 6 or 12 months, respectively. Clinical and pathologic parameters evaluated are listed in Table I.

Histopathologic Specimens Histologic sections (4–5 μm) were cut from routinely processed, paraffin-embedded tissue blocks, and were stained with hematoxylin and cosin and processed for stereologic and morphometric evaluation.

Stereologic Methods An IBAS 20 automatic image analyzer (Zeiss, Kontron, Germany) was equipped with a Zeiss Axiosmat microscope. Using this system with a 40× lens the fields of vision were grabbed into the computer by a Sony DXC 325-P three CCD chip color camera at a final resolution of 768 × 512 pixels. The absolute magnification on the high-resolution screen was about 1300 X. The system was calibrated to measure microns of lengths and was corrected for distortion between the x- and y-axes.

After appropriate noise reduction and contrast enhancement, ten microscopic fields in each tumor were studied in two to four sections, excluding tumor areas with necrosis, heavy pigmentation, or massive inflammation. Selection of measured fields was done by random assignment over the whole tumor area. Both superficial and deeper portions of the melanomas were included in the sampling procedure by the computer. Measurements were performed without knowledge of data and the outcome of patients.

The methodologic and mathematical basis of estimation of the three-dimensional volume-weighted mean Vv has been described previously by Gundersen and Jensen [16,17].

An orientation frame was superimposed on the TV screen with its left-hand edge perpendicular to the cutaneous surface of the tumor, ensuring that the measurements were carried out on so-called vertical sections [18].

A random number between 0 and 97 was generated by the computer, and this number determined the starting orientation number of the linear elements on a superimposed test probe. For each field of vision the number 10 was added to the initial number so that the direction of the test system was weighted by the sine of its angle from the vertical axis. If a point of the test probe hits a nuclear profile the orientated test line was measured using the digitizer tablet interactively. By multiplying the mean of individually cubed intercept lengths by π/3 an unbiased estimate of the volume-weighted mean Vv was obtained. The mean number of point sampled nuclear intercepts was 132 per melanoma (range, 68–153) from ten fields of vision.

Almost every step of the procedure, including data sampling and computation of data described above, was automatically executed by the computer, but the measurement of point-sampled intercept lengths was done in an interactive way using the digitizer tablet (see Fig 1).

Morphometric Methods The two-dimensional parameters were determined in a combined approach (automatically and interactively). Briefly, image processing was performed as follows. For acquisition of images the red channel of the color camera was used; the signal of this channel obtains the highest contrast objects of interest (blue stained) and background. Image enhancement was performed by normalization of the image and application of a contour-enhancement algorithm. Image segmentation itself was done using threshold segmentation. Contours extracted from the original image were superimposed over the original image. In most cases more than 60% of nuclei were correctly identified. Using the original image the final segmentation was done interactively (i.e., completion of incorrectly segmented nuclei). At this step rejection of artifacts was possible using the digitizer board mouse. Derived from previous experiments we found that the combined approach of segmentation on routine slides is less time consuming and shows a very good correlation with strictly manually segmented nuclei.

The same orientation frame as applied for stereologic measurements was used for measuring the following two-dimensional morphometric parameters: nuclear area, circularity shape factor (Fshape defined as (4π area)/perimeter^2) and aspect ratio of the nucleus (FSHAPE defined as D_MIN/D_MAX) [19–21].

Statistics Comparisons of grouped data in Table I were analyzed by chi-square test and U test. Statistical significance of differences of data in Table II was tested with the pooled data of the two groups by the chi-square test and U test. Statistically significant differences of data in Table I were analyzed using the nonparametric Wilcoxon rank-sum test [22–24]. p values of less than 0.05 were considered to indicate statistical significance.

![Figure 1](image-url) Screen copy of a vertical section of a “thin” malignant melanoma at high-power field (magnification ×1113). The vertical axis has been aligned with the left-hand edge perpendicular to the cutaneous surface at low-power magnification. The starting angle of the test probe is generated randomly by the computer. If a test point hits a nuclear profile the nuclear intercept is measured using a digitizer board.
RESULTS

Clinical Parameters  The clinical parameters of the two groups of patients studied are listed in Table I. As is shown, no significant difference was present between both groups with regard to clinical and pathologic features. Thus from a statistical point of view the groups were comparable.

Two-Dimensional Morphometric Estimations  The mean nuclear area ranged from 18 to 36 μm² with a mean of 25.5 μm² (SD ± 6.08) in the group of patients with metastatic disease and was 14 to 32 μm², with a mean of 22.5 μm² (SD ± 4.18), in the group without metastases. The mean nuclear area thus was larger in patients with subsequent metastatic disease than in patients without metastases, but there was no statistically significant difference (see Table II). In addition, aspect ratio of the nucleus (Fshape) as well as stereologic estimates of mean nuclear area, Fv, and Vv did not prove to differ in both groups of patients investigated (see Table II).

Table II. Morphometric (Two-Dimensional) and Stereologic (Three-Dimensional) Parameters

<table>
<thead>
<tr>
<th>Parameter (±SD)</th>
<th>Patients with Metastases</th>
<th>Patients without Metastases</th>
<th>Statistics (p values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>10</td>
<td>22</td>
<td>NS</td>
</tr>
<tr>
<td>Nuclear area (μm²)</td>
<td>25.48 (±6.08)</td>
<td>22.45 (±4.18)</td>
<td>NS</td>
</tr>
<tr>
<td>SD nuclear area</td>
<td>10.46 (±2.60)</td>
<td>9.64 (±2.05)</td>
<td>NS</td>
</tr>
<tr>
<td>Fshape</td>
<td>0.62 (±0.04)</td>
<td>0.62 (±0.06)</td>
<td>NS</td>
</tr>
<tr>
<td>SD Fshape</td>
<td>0.14 (±0.02)</td>
<td>0.15 (±0.03)</td>
<td>NS</td>
</tr>
<tr>
<td>Fcine</td>
<td>0.81 (±0.03)</td>
<td>0.82 (±0.05)</td>
<td>NS</td>
</tr>
<tr>
<td>SD Fcine</td>
<td>0.12 (±0.02)</td>
<td>0.12 (±0.02)</td>
<td>NS</td>
</tr>
<tr>
<td>Vv (μm³)</td>
<td>272.63 (±81.36)</td>
<td>153.73 (±25.30)</td>
<td>0.0008</td>
</tr>
</tbody>
</table>

Stereologic Estimation of Volume-Weighted Mean Vv  The Vv of “thin” primary melanomas with subsequent metastatic course varied from 155 to 457 μm³ with a mean of 272.63 μm³ (SD ± 81.3). In the group of patients without metastases, Vv varied from 102 to 199 μm³ with a mean of 153.73 μm³ (SD ± 25.3). This difference was found to be highly significant (p = 0.0008). “Thin” melanomas with subsequent metastases exhibit a significantly higher Vv than melanomas that do not metastasize (see Table II, Fig 2).

DISCUSSION

At present the prognosis of malignant melanoma is evaluated on the basis of clinical and histopathologic criteria. Sex [25,26], age [27], anatomic site [28] of the tumor, and clinical evidence of ulceration [29] have been shown to influence prognosis. Histopathologically, the growth pattern [30], cell type of melanoma [31], evidence of regression or ulceration [27,31,32], mitotic rate [33], and lymphocytic infiltration [34] are considered to be of prognostic importance.

However, so far the most important predictive value is assigned to the evaluation of the micro-invasion level of the tumor as assessed by the invasion levels described by Clark et al [15] and measurement of tumor thickness according to Breslow [13]. Multivariable analyses of single prognostic values enable the identification of independent predictors of survival [27,35]. Based on a model of tumor progression in stage I melanoma, Clark et al [36] have shown that patients with radial growth-phase tumors have survived for a minimum of 8 years and the identification of independent prognostic predictors such as mitotic rate, tumor-infiltrating lymphocytes, tumor thickness, anatomic site, gender, and regression allows an 84.1% accuracy in the prediction of survival in vertical growth-phase tumors. However, there is consensus that tumor thickness according to Breslow is the single most powerful prognostic variable [37].

On the other hand, there is sufficient evidence that in stage I melanoma with tumor invasion of less than 0.76 mm, the tumor thickness of Breslow does not seem to provide infallible prognostic information [7,36]. There is no question that metastasis and death from “thin” melanomas is rare, but several reports exist documenting that an estimated mean of 5% [7,8,11–14] of patients with “thin” melanoma develop metastases. So far, studies have failed to define significant clinical, histologic, and/or immunologic markers for this subgroup.

Sorensen [38] has already shown the predictable value of Vv determination in the assessment of prognosis in clinical stage I and II melanomas. In the present study we now show that the stereologic estimates of mean Vv of patients with “thin” melanomas (< 0.76 mm) and subsequent metastases is significantly larger (Vv = 273 μm³) than the Vv of melanoma patients (Vv = 154 μm³) who do not develop metastases. In contrast, two-dimensional morphometric parameters such as nuclear area, aspect ratio, or circularity shape factor did not prove to be statistically different between the two groups of patients. The two-dimensional morphometric parameters have already been shown to be of greater value in classification than in prognostication [39–41].

In malignant melanoma vertical and horizontal growth patterns cause directional orientation of tumor cells. In our study, anisotropy was eliminated by individually weighting the lines of the test probe as a function of their angle from the vertical axis. Shape and orientation-independent estimates of Vv are therefore more accurate than two-dimensional parameters. Estimates of Vv are shape and orientation-independent three-dimensional estimates and thus combine information about both volume and variation of volume. Thus, Vv increases not only when the ordinary number-weighted mean Vv increases; a rise in Vv is also observed with an increasing variability or heterogeneity of nuclear volume itself.

We do not know the mechanisms that lead to a large nuclear Vv in certain melanomas with a subsequent detrimental course and not in others that biologically appear more “benign” and do not metastasize so rapidly. A recent study shows that there is a correlation of karyometric parameters and proliferative activity in cutaneous melanocytic tumors, as defined by the immunohistochemical activity of Ki-67 antibody [42].

In conclusion, our data show that 1) patients with “thin” malignant melanomas do carry a risk of developing metastatic disease, and that 2) estimation of volume-weighted mean Vv is a sensitive parameter for identification of high-risk “thin” melanomas. Thus, vol-
ume-weighted mean $V_r$ seems to represent a powerful and independent prognostic marker for “thin” primary melanomas. Assessment of $V_r$ may therefore prove a valuable tool in selecting patients with high-risk melanoma for adjuvant therapy.

REFERENCES


26. Kuehnl-Petzoldt C, Keil H, Schoepf E: Prognostic significance of the patient's sex, tumor site, and mitotic rate in thin (less than or equal to 1.5 mm) melanoma. Arch Derm 276:151–155, 1984