Visualization of Circulating Melanoma Cells in Peripheral Blood of Patients with Primary Uveal Melanoma

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Abstract

Purpose: In patients with uveal melanoma, tumor cell dissemination and subsequent formation of metastases are confined mainly to the hematogenous route. Here, we sought to isolate circulating melanoma cells in peripheral blood of patients with primary uveal melanoma and clinically localized disease.

Experimental Design: Blood samples from 52 patients with clinically localized uveal melanoma and from 20 control individuals were prospectively collected before therapy of the primary tumor. Tumor cells expressing the melanoma-associated chondroitin sulfate proteoglycan were enriched by immunomagnetic cell sorting and visualized by immunocytologic staining. Results were compared with clinical data at presentation.

Results: In 10 of 52 patients [19%; 95% confidence interval (95% CI), 10-33%], between 1 and 5 circulating melanoma cells were detected in 50 mL peripheral blood. No melanoma-associated chondroitin sulfate proteoglycan-positive cells were detected in any of the 20 controls examined. The presence of tumor cells in peripheral blood was associated with ciliary body invasion [odds ratio (OR), 20.0; 95% CI, 3.0-131.7], advanced local tumor stage (OR, 6.7; 95% CI, 1.8-25.4), and anterior tumor localization (OR, 4.0; 95% CI, 1.2-12.7), all established factors for uveal melanoma progression.

Conclusions: Immunomagnetic enrichment enables detection of intact melanoma cells in peripheral blood of patients with clinically localized ocular disease. Visualization and capturing of these cells provide a unique tool for characterizing potentially metastasizing tumor cells from a primary melanoma at an early stage of the disease.

Uveal melanoma is the most common primary intraocular malignant tumor in adults and 98% of these patients present with clinically localized disease (1, 2). Despite successful local tumor control, one half of the patients with primary uveal melanoma will die from metastatic disease (3). The 10-year survival rates in patients with nonmetastatic tumors range from 81% (T1N0M0) to 15% (T4N0M0; ref. 4). Metastasis-related deaths occur as long as 35 years after diagnosis, indicating that disseminated tumor cells may stay quiescent for decades and change their biological behavior even after this time (5). As therapeutic options in metastatic disease are poor, with a mean survival of 12 to 14 months (6, 7), there is an urgent need for early identification of patients at increased risk for metastases. This will allow evaluation of adjuvant therapeutic strategies in high-risk patients.

Established prognostic factors for clinically localized ocular disease include largest basal tumor diameter (LBD), ciliary body involvement, and extracocular growth (8–17). A number of staging classifications use combinations of the above-mentioned criteria (for review, see ref. 4). However, neither of these factors alone or in combination is adequate for predicting the occurrence of metastatic disease in an individual patient. Other prognostic factors such as histologic subtype (11–14, 16, 18) as well as genomic changes (19–21) of the primary tumor also correlate with clinical outcome. Yet, these factors are increasingly difficult to determine as patients are currently often treated with radiotherapy. Although a diagnostic biopsy may be done before treatment, in most cases tumor tissue is not available for analysis.

Metastatic spread in uveal melanoma is confined to the hematogenous route as long as the conjunctiva is not infiltrated trans-sclerally (22). The presence of tumor cells in peripheral
blood is an obligate (although not sufficient) event in the metastatic cascade. Detection of circulating tumor cells might therefore represent a unique tool to identify patients at increased risk for metastatic disease. A recent systematic meta-analysis (23) of the prognostic value of tumor cell detection in peripheral blood in melanoma patients identified and evaluated two major approaches, PCR-based detection of melanoma-associated mRNA \( (n = 52 \) studies) and our recently published cytometric assay that allows visualization of intact tumor cells in peripheral blood \( (24) \).

The analysis revealed that circulating tumor cells might have a clinically valuable prognostic power in patients with melanoma and should be further evaluated on more homogeneous series of patients. A broader implementation of cytometric methods was suggested as mRNA analysis is extremely susceptible to false positives resulting from contamination, amplification of pseudogenes, and illegitimate transcription \( (23) \).

Here, we present the first study using a cytometric analysis to detect circulating melanoma cells in patients with primary nonmetastatic uveal melanoma. We show that intact tumor cells may be found in a subset of patients and show that their presence is closely associated with established clinical risk factors for tumor progression.

**Materials and Methods**

**Patient selection.** Between July 2003 and May 2006, 52 consecutive patients with clinically localized uveal melanoma presenting at the University Eye Hospital, Centre for Ophthalmology, Eberhard-Karls University of Tuebingen, Germany, were enrolled in the study. The diagnosis of uveal melanoma was based on clinical and ultrasound examination done by a specialized ophthalmologist. Patients with clinically suspicious lesions were not included in the study. The study conforms to the guidelines of the Declaration of Helsinki as revised in Tokyo and Venice and was authorized by the local ethic committee \( (411/2003; 412/2003) \). All patients gave their informed consent before their inclusion in the study.

To exclude metastatic disease, patients had completed a clinical examination; liver function tests (alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, bilirubin); ultrasound of the abdomen; and computer tomography of the head, chest, and abdomen. Standardized A and B scans \( (13 \) System-ABD, Innovative Imaging Inc.) and ultrasound biomicroscopy \( (Dicon, Ultrasound Biomicroscope Plus, Model P45 UBM Plus, Paradigm Medical Industries, Inc.) \) were done to obtain size measurements of the tumor and the exact intraocular localization, and to document ciliary body involvement. Posterior tumors were defined as lesions localized posterior to the equator. Peripheral tumors were located between the equator and the ora serrata. Anterior lesions were localized anterior to the ora serrata.

The data were coded and analyzed according to the modified classification of Augsburger no. 1 and the 6th edition of tumor-node-metastasis \( (TNM) \) classification \( (4) \). Due to a technical problem, the LBD was not recorded in one patient without circulating tumor cells. TNM and Augsburger staging in this single patient was based on tumor height alone, whereas in all other patients LBD and tumor height were used for classification.

**Controls.** Twenty control samples included 18 nonmelanoma patients treated at the Department of Dermatology, Eberhard-Karls University of Tuebingen, and two healthy volunteers.

**Immunomagnetic melanoma cell enrichment.** Fifty milliliters of heparinized peripheral blood were collected from the patients before treatment and from the control group. The blood samples were processed immediately. The technique of enrichment was done as described previously \( (25, 26) \) with the following modifications: 100 \( \mu l \) per \( 5 \times 10^7 \) total cells of the melanoma-associated chondroitin sulfate proteoglycan \( (MCSP) \) antibody conjugated to microbeads \( (clone\ 9.2.27 \) directly conjugated to microbeads; Miltenyi Biotec) were used for enrichment of melanoma cells. Positive cells were selected on a MS column and stained on the column with a MCSP antibody conjugated to alkaline phosphatase \( (clone\ EP1 \) conjugated to alkaline phosphatase; Miltenyi Biotec). For this purpose, 1.5 \( \mu l \) of the MCSP antibody were added to 100 \( \mu l \) buffer and applied to the column. After elution from the column, MCSP-positive cells were visualized by using 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium as substrate for the alkaline phosphatase. Isotype controls were performed for each patient \( ( \)isotype control antibody directly conjugated to alkaline phosphatase kindly provided by Miltenyi Biotec\) \). The sensitivity of the modified assay was in the same range as published before \( (25) \). Thus, using spiking experiments, one melanoma cells per milliliter of whole blood could be detected reliably with the modified assay.

**Evaluation criteria.** In all cases, the entire positive fraction was analyzed under a Zeiss light microscope. Evaluation was done without knowledge of the clinical data. Cells had to show blue immunostaining to be judged as positive. If one or more positive cells were found, the sample was considered positive. The absolute number of cells detected per sample was recorded.

**Statistical analysis.** The data set was analyzed using SAS statistical software \( (SAS Institute, Inc.) \) under Windows XP. Statistical analysis to show the association between exploratory variables and the presence of circulating tumor cells was done using univariate logistic regression models, providing odds ratios with the corresponding Wald confidence intervals and Wilcoxon rank sum test. Further Clopper Pearson confidence intervals and confidence intervals for zero events were given to the proportions. To perform a comparative evaluation of the univariate distinctive effects \( (selecte\ on the basis of P values <0.25)\), a \( \)multivariate\) logistic regression model was adapted to the data. All numbers are rounded by the first decimal digit. All \( P \) values are explorative.

**Results**

**Patient cohort characteristics.** The study included 52 patients with primary uveal melanoma without clinically detectable metastatic disease. Clinical data of the patient cohort are summarized in Table 1.

**Detection of circulating melanoma cells.** Circulating melanoma cells were detected in 10 of the 52 melanoma patients \( [19\%; 95\% \) confidence interval \( (95\% CI)\), 10-33\%] \) examined. The absolute cell number varied between 1 and 5 cells \( (median 2.5) \) per 50 \( \mu l \) whole blood. All positive cells showed bright blue staining and were larger in size than the surrounding unstained lymphocytes \( (Fig. \ 1A-C) \). Isotype controls were negative in all patients. No MCSP-positive cells were found in any of the 20 control samples \( (0\%; 95\% CI, 0-14\%) \). Faint positivity of granulocytes as noted with our previously assay was no longer detectable with the modified assay used for this study \( (Fig. \ 1D) \).

**Association of circulating tumor cells with established markers for tumor progression.** Next, we analyzed the association between the presence of circulating tumor cells and clinical risk factors for tumor progression in patients with primary uveal melanoma \( (Tables \ 1 \) and \ 2) \). We found that ciliary body involvement was closely associated with the presence of circulating tumor cells \( [P = 0.0018, \ Wald^2 \) test; odds ratio \( (OR), 20.0; 95\% CI, 3.0-131.7] \). Advanced primary tumor stage was significantly associated with the presence of detectable
circulating melanoma cells, whether we used the TNM classification ($P = 0.0005$, Wald $\chi^2$ test; OR, 6.7; 95% CI, 1.8-25.4) or the Augsburger classification ($P = 0.0004$, Wald $\chi^2$ test; OR, 8.4; 95% CI, 1.9-36.4). Median LBD ($P = 0.0030$, Wilcoxon test) and, to a lesser extent, tumor height ($P = 0.0249$, Wilcoxon test) showed statistically significant higher values in patients with circulating melanoma cells compared with patients without tumor cells (Table 1). Four of 35 patients (11%; 95% CI, 3-27%) with basal diameter $\leq 14$ mm were tested positive compared with 6 of 16 patients (38%; 95% CI, 15-65%) with a LBD $>14$ mm ($P = 0.0065$, Wilcoxon test).

We also found a marked association between anterior tumor localization and the presence of circulating tumor cells ($P = 0.0018$, Wald $\chi^2$ test; OR, 20.0; 95% CI, 3.0-131.7) given that in 4 of 5 patients (80%; 95% CI, 28%-99%) with anterior localized tumors, circulating melanoma cells were detectable. We found no association between circulating tumor cells and age ($P = 0.8368$, Wald $\chi^2$ test; OR, 1.0; 95% CI, 1.0-1.1) or sex ($P = 0.5710$, Wald $\chi^2$ test; OR, 1.5; 95% CI, 0.4-6.1; Table 1).

In a multivariate sensitivity analysis, we studied the possible simultaneous influence on the occurrence of circulating tumor cells. We included the factors tumor height, TNM stage, ciliary body involvement, and classified LBD in the model and observed that ciliary body involvement was predominantly associated with circulating tumor cells ($P = 0.0335$).

### Discussion

The data provided here show for the first time that intact circulating melanoma cells are detectable in patients with $T_{2,4}N_{3}M_{0}$ uveal melanoma before therapy. Using the immunomagnetic melanoma cell enrichment assay, we have previously shown that the presence of intact circulating melanoma has an effect on survival in patients with metastatic melanoma (24). In the present study, we examined patients with nonmetastatic uveal melanoma before treatment for the presence of intact melanoma cells in peripheral blood. The rationale for this study was 2-fold.

First, there is an urgent need to define risk factors for tumor progression in patients with nonmetastatic uveal melanoma. Half of the patients with clinically localized tumors will die from metastatic disease. Although some authors suggest to perform biopsies (27), there is no reliable early predictor for the occurrence of metastasis in individual patients. Second, the circulation of tumor cells may be considered an obligate event preceding the development of metastatic disease in uveal melanoma. In contrast to patients with cutaneous melanoma where early dissemination of tumor cells most frequently occurs via the lymphatic system (26), metastatic spread in uveal melanoma is confined to the hematogenous route as long as the conjunctiva is not infiltrated trans-sclerally (22). The detection of tumor cells in peripheral blood may therefore represent an ideal tool for early detection of systemic spread in patients with clinically localized tumors.

Here, we show that intact tumor cells can be detected in patients with uveal melanoma and clinically localized disease at the time of initial diagnosis. In this prospective study, we were thus able to isolate tumor cells from peripheral blood in 10 of 52 patients (19%; 95% CI, 10-33%), whereas 20 controls were negative. Moreover, we found a statistically significant positive association between the presence of circulating tumor cells and established prognostic factors associated with increased risk of metastasis.

### Table 1. Association of established clinical prognostic markers with the presence of circulating tumor cells

<table>
<thead>
<tr>
<th>Variable</th>
<th>Positive for CTC ($n = 10$)</th>
<th>Negative for CTC ($n = 42$)</th>
<th>All patients ($N = 52$)</th>
<th>$P$</th>
<th>OR (95% Wald confidence limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>72</td>
<td>64</td>
<td>65</td>
<td>0.8368</td>
<td>1.0 (1.0, 1.1)</td>
</tr>
<tr>
<td>Min, max</td>
<td>18, 81</td>
<td>26, 82</td>
<td>18, 82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>6</td>
<td>21</td>
<td>27</td>
<td>0.5710</td>
<td>1.5 (0.4, 6.1)</td>
</tr>
<tr>
<td>Male</td>
<td>4</td>
<td>21</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LBD (mm)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>16.15</td>
<td>11.51</td>
<td>12.2</td>
<td>0.0030</td>
<td>NA</td>
</tr>
<tr>
<td>Min, max</td>
<td>10.12, 19.27</td>
<td>4.22, 23.0</td>
<td>4.22, 23.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$&gt;14$</td>
<td>6</td>
<td>10</td>
<td>16</td>
<td>0.0065</td>
<td>1.4 (1.1, 1.8)</td>
</tr>
<tr>
<td>$&lt;14$</td>
<td>4</td>
<td>31</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor height (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>8.84</td>
<td>6.87</td>
<td>7.76</td>
<td>0.0249</td>
<td>NA</td>
</tr>
<tr>
<td>Min, max</td>
<td>4.65, 13.0</td>
<td>1.3, 18.0</td>
<td>1.3, 18.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ciliary body infiltration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>5</td>
<td>2</td>
<td>7</td>
<td>0.0018</td>
<td>20.0 (3.0, 131.7)</td>
</tr>
<tr>
<td>No</td>
<td>5</td>
<td>40</td>
<td>45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Localization</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior</td>
<td>4</td>
<td>1</td>
<td>5</td>
<td>0.0206</td>
<td>4.0 (1.2, 12.7)</td>
</tr>
<tr>
<td>Peripheral</td>
<td>3</td>
<td>20</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Posterior</td>
<td>3</td>
<td>21</td>
<td>24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: All tests are based on fitting a univariate logistic regression model (Wald $\chi^2$ test) except for median tumor height and LBD, which were analyzed using the Wilcoxon rank sum test.

Abbreviations: CTC, circulating tumor cells; $n$, number; min, minimum; max, maximum; mm, millimeter; LBD, largest basal diameter; NA, not applicable.

*Missing data: $n = 1$.

† Analyzed using the Wilcoxon rank sum test.
metastatic disease (8–17). Here, we could show an effect of the primary tumor size (reflected by advanced local tumor stage and LBD >14 mm) on the presence of circulating tumor cells for univariate analysis. Certainly, larger studies are required to finally establish the effect of tumor size on the presence of circulating tumor cells.

In particular, we disclosed a positive association between ciliary body involvement and the presence of circulating tumor cells both in the univariate and multivariate analysis. One might speculate that the high vascularization of the ciliary body might account for this effect.

The follow-up time is too short to evaluate the effect of circulating tumor cells on survival; however, the association of established prognostic factors with the presence of circulating tumor cells is strongly supportive for their clinical relevance. Previous studies focusing on PCR-based techniques for isolation of circulating melanoma cells in primary uveal melanoma basically detected melanoma-associated transcripts (28–32). Most assays used multiple markers such as tyrosinase mRNA and Melan A/Mart 1 mRNA to increase sensitivity. The reported detection rates range from 0% to 39% (28, 29) in patients with nonmetastatic uveal melanoma. In a prospective study recently published in the journal, it could even be shown that the presence of circulating melanoma cells, defined as Melan A or tyrosinase mRNA positivity, was an independent predictor for survival (33). The study included 110 patients; five patients tested positive for each marker alone, whereas one patient expressed both markers (total positivity rate 10%).

Our immunomagnetic assay is based on the detection of intact cells expressing the melanoma-associated chondroitin sulfate proteoglycan on their cell surface. We used monoclonal antibody 9.2.27 directly conjugated to microbeads for positive enrichment of tumor cells. Alkaline phosphatase–conjugated monoclonal antibody EP1 that recognizes a different epitope of the MCSP complex was used for immunocytologic staining of the enriched cell fraction.

There are several advantages of using immunomagnetic melanoma cell enrichment for melanoma cell detection. First, immunomagnetic melanoma cell enrichment allows visualization of whole tumor cells and not only melanoma-associated transcripts at a sensitivity of one melanoma cell per milliliter of blood (25). Second, the MCSP antigen is expressed on 95% of uveal melanomas (34). Loss of cells due to lack of antigen expression may therefore be minimized. Normal blood cells, on the other hand, are negative. Thus, no MCSP-positive cells were detected in 20 controls examined. Faint positive staining of granulocytes as noted with our previous assay (24) was no longer present using monoclonal antibody Ep1 conjugated to alkaline phosphatase for immunostaining. Sensitivity, on the other hand, was not affected by the modified assay.

Third, the method allows further characterization of the isolated cells with respect to their phenotypic and genotypic properties. Such analysis will be especially interesting for patients treated with primary radiotherapy, where no tumor tissue is available for analysis.

In our previous study, we were able to show by single-cell comparative genomic hybridization that MCSP-positive cells isolated from peripheral blood display multiple genomic aberrations (24, 35). Cells isolated from patients with metastatic cutaneous melanoma had a different genomic profile compared with cells isolated from metastatic uveal melanoma.

As future melanoma therapies may become more individualized, the assay might also add to identify patients who could benefit from targeted delivery of therapy. Most interestingly, one potential therapy target is the MSCP complex itself. Recent preclinical studies using the MSCP antigen for targeted delivery of $^{132}$Bi-labeled 9.2.27 monoclonal antibody were able to induce tumor regression in preclinical studies in uveal melanoma (34).

**Fig. 1.** Blue immunostaining of MCSP-positive tumor cells in peripheral blood of patients with primary uveal melanoma (A–C). Surrounding lymphocytes (B) and granulocytes enriched from peripheral blood of a healthy volunteer (D) do not express the antigen (5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium, alkaline phosphatase–anti-alkaline phosphatase; original magnification, ×400).
Double staining of MCSP-positive cells could possibly help to identify further antigens suitable for targeted therapy. Thus, immunomagnetic melanoma cell enrichment allows visualization of disseminated melanoma cells in peripheral blood of patients with $T_2N_0M_0$ uveal melanoma. Although the repeatability of the test was not examined due to the large amount of blood required, the clinical data established thus far suggest that the assay has the potential to define patients at increased risk for metastatic spread.

An additional compelling aspect of the assay comes from the possibility to further characterize the isolated cells especially in patients where no tissue from the primary melanoma is available for analysis. Examination of the biological characteristics of the isolated cells might add valuable prognostic information and help to identify patients who could benefit from targeted therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Ursula Schiebel and Oliver Hallmaier for excellent technical support.

References

18. Al Jamal RT, Makite T, Kivela T. Nucleolar diameter

Table 2. Association between tumor stage according to TNM 6th edition (A) and modified Augsburger classification no. 1 (B) with the presence of circulating tumor cells

<table>
<thead>
<tr>
<th>Tumor stage according to TNM 6th edition*</th>
<th>No. pts examined</th>
<th>No. pts positive</th>
<th>Positivity rate, % (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_1N_0M_0$ LBD ≤ 10 mm and height ≤ 2.5 mm regardless of extraocular extension</td>
<td>15</td>
<td>0</td>
<td>0.0 (0.0-18.0)</td>
</tr>
<tr>
<td>$T_2N_0M_0$ $10 &lt; LBD ≤ 16$ mm and/or $2.5 &lt; height ≤ 10$ mm regardless of extraocular extension</td>
<td>30</td>
<td>5</td>
<td>17.0 (6.0-35.0)</td>
</tr>
<tr>
<td>$T_3N_0M_0$ LBD &gt; 16 mm and/or height &gt; 10 mm without extraocular extension</td>
<td>5</td>
<td>4</td>
<td>80.0 (28.0-99.0)</td>
</tr>
<tr>
<td>$T_4N_0M_0$ LBD &gt; 16 mm and/or height &gt; 10 mm with extraocular extension</td>
<td>2</td>
<td>1</td>
<td>50.0 (1.0-99.0)</td>
</tr>
</tbody>
</table>

Table 2. Association between tumor stage according to TNM 6th edition (A) and modified Augsburger alternative classification no. 1 (B) with the presence of circulating tumor cells

<table>
<thead>
<tr>
<th>Modified Augsburger alternative classification no. 1, ciliary body and choroid*</th>
<th>No. pts examined</th>
<th>No. pts positive</th>
<th>Positivity rate, % (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_1N_0M_0$ LBD ≤ 10 mm and height ≤ 5 mm regardless of extraocular extension</td>
<td>13</td>
<td>0</td>
<td>0.0 (0.0-21.0)</td>
</tr>
<tr>
<td>$T_2N_0M_0$ $10 &lt; LBD ≤ 15$ mm and/or $5 &lt; height ≤ 8$ mm regardless of extraocular extension</td>
<td>20</td>
<td>1</td>
<td>5.0 (0.0-25.0)</td>
</tr>
<tr>
<td>$T_3N_0M_0$ LBD &gt; 15 mm and/or height &gt; 8 mm without extraocular extension</td>
<td>17</td>
<td>8</td>
<td>47.0 (23.0-72.0)</td>
</tr>
<tr>
<td>$T_4N_0M_0$ LBD &gt; 15 mm and/or height &gt; 8 mm with extraocular extension</td>
<td>2</td>
<td>1</td>
<td>50.0 (1.0-99.0)</td>
</tr>
</tbody>
</table>

NOTE: All 95% CI values are Clopper Pearson confidence intervals except for the data in bold, which are confidence intervals for zero events.

Abbreviations: T, tumor stage; no., number; pts, patients; height, tumor height, LBD, largest basal diameter.

*See ref. 4.


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