Prediction of Metastasis of Uveal Melanoma: Comparison of Morphometric Determination of Nucleolar Size and Spectrophotometric Determination of DNA

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We studied 95 cases of uveal melanoma using cytomorphometry to measure the standard deviation of nucleolar area (SD-NA) based on 200 cells per tumor and microspectrophotometry to determine the quantity of DNA in the nucleus of 100 cells per tumor. The 95 cases of uveal melanoma, in which the eye was enucleated between 1970 and 1973, were selected from the files of the Registry of Ophthalmic Pathology. Forty-nine patients survived with a median follow-up of 15 years without evidence of metastasis at the time of the last follow-up, and 46 patients died of metastatic melanoma. Statistical analysis indicated that SD-NA was a better predictor of metastasis than DNA determination in this data set. Invest Ophthalmol Vis Sci 29:507-511, 1988

In the evaluation of a patient with cancer, the role of the pathologist is twofold. First, he must correctly diagnose the patient’s tumor and then, based on that diagnosis, he must estimate the degree of malignancy. For uveal melanoma, the risk of metastasis has traditionally been estimated subjectively using modifications of the classification scheme proposed by Callender in 1931.1,2 We have shown that the Callender classification is effective in predicting outcome in groups of patients with uveal melanoma, but because of great variability among skilled ophthalmic pathologists in the classification of a given tumor, this schema suffers from limited reproducibility.3 We believe that the lack of reproducibility among pathologists results from the subjective nature of Callender’s classification. To reduce subjectivity in classifying uveal melanomas, we have proposed the measurement of cytologic features. In previous reports we have documented that measurement of the standard deviation of the nucleolar area (SD-NA) in uveal melanomas is both more effective and more reproducible in predicting patient outcome than the Callender classification.4-6

Investigators working with a variety of cancers have shown that measurement of the amount of deoxyribonucleic acid (DNA) in the cells of the tumor can be used to estimate the tumor’s malignancy. Alternative methods have been developed for the determination of the amount of DNA in the nuclei of tumor cells. The first of these techniques is flow cytometry.7 Flow cytometric determination of DNA is performed by suspending the nuclei of the tumor cells and reacting them with a fluorescent dye that binds stoichiometrically with the nuclear DNA. The suspended nuclei are passed through the flow cytometer and the fluorescent dye is activated using a laser. The amount of fluorescence is then proportional to the quantity of DNA in the nucleus. The second of these techniques is microspectrophotometry of Feulgen-stained histologic sections.8-9 With this method the
optical density measured over the area of the nucleus has been shown to correlate with the quantity of DNA in the nucleus.

Both flow cytometry and microspectrophotometry have advantages and disadvantages. The main advantage of flow cytometry is the large number of cells that can be quickly analyzed, which allows the presence of aneuploid peaks to be precisely determined. On the other hand, in flow cytometry the cells are not examined histologically and thus nontumor cells and clumped cells cannot be excluded. In microspectrophotometry the technician views each cell to be measured and can eliminate nontumor cells and overlapping nuclei; however, the procedure is slow and only a few hundred cells can be analyzed, compared to the tens of thousands of cells that can be analyzed by flow cytometry.

In this paper we will compare four methods of predicting the outcome of patients following enucleation for uveal melanoma: (1) morphometric determination of the SD-NA; (2) microspectrophotometric determination of the quantity of DNA in the tumor cells; (3) subjective evaluation of Callender’s cell type; and (4) measurement of the largest dimension of the tumor (LTD) from the histological microslide.

**Materials and Methods**

The cases of posterior uveal melanoma used in this study were selected from the Registry of Ophthalmic Pathology. In order to optimize our statistical analysis, selected cases were equally divided between patients who died of metastatic melanoma and patients who experienced long-term survival. Beginning with the first case in 1970, the following criteria were used: The “fatal” group consisted of the first 55 cases in which the patient’s cause of death was believed to be metastatic melanoma and the paraffin block containing the tumor was available for the preparation of histologic sections. The “nonfatal” group consisted of the first 55 cases in which the patient had not died of metastatic melanoma and a paraffin block containing the tumor was available. Patients that died of other causes were coded for survival analysis as withdrawn at the time of death. To increase the percentage of cases with long-term follow-up, patients lost to follow-up within the first 5 years were excluded; however, two patients who died of other causes within the first 5 years were included in the nonfatal group.

Serial sections from the paraffin blocks were prepared at a thickness of 6 μm. One section was stained with hematoxylin and eosin and the next section was stained with Feulgen.

The method for determining SD-NA has been well described in previous publications. Briefly, the computer selected on the basis of random numbers 200 fields scattered throughout the tumor. Any field that fell outside the tumor section or was uninterpretable because of hemorrhage, artifact or necrosis was rejected by the technician and an additional field was selected by the computer. When each acceptable field was presented by the computer-driven scanning stage, the technician selected the nucleus nearest the center that contained a nucleolus. A concentric transparent reticle placed in one eyepiece of the microscope assisted in this selection. The computer calculated the area of the largest nucleolus within the selected nucleus as the technician outlined it on the digitizer. From these 200 measurements, the standard deviation of nucleolar area (SD-NA) for each tumor was computed.

The amount of DNA in the nuclei of the tumor cells was measured using the Leitz (Rockleigh, NJ) MPV-DADS-560 Data Acquisition and Display System (DADS). The use of a microspectrophotometer to determine nuclear DNA content has been described previously. To analyze uveal melanomas the technician first measured the amount of DNA in 20 cells from the inner nuclear layer of the retina. The mode of this measurement provided an estimate of the diploid complement of DNA (2c value) and was used to calibrate the system for each histologic section. This corrected for variation in section thickness or variation in the density of the Feulgen stain. The technician then moved the microscopic field to one edge of the tumor. In this field and adjacent fields, stepping across the tumor, the technician selected the nucleus located nearest the center of the microscopic field that did not overlap adjacent nuclei. Fields that were extensively necrotic or heavily pigmented were rejected by the technician. Using this selection process, the central nucleus in 100 fields was measured for each tumor.

To investigate the correlation between the quantity of DNA in the nuclei of a uveal melanoma and patient survival, the distribution of DNA in the 100 cells measured with the DADS had to be converted to a parameter. Since studies have not been done to determine which parameter should be used, we investigated a number of parameters to determine their prognostic value. These included simple parameters such as the mode, mean, median and standard deviation of the DNA distribution. More complex parameters were also investigated since the possibility existed that tumors with a significant proportion of cells falling within a given range of the DNA distribution may be the most malignant. We divided the DNA histogram into four regions. The first we defined as the
diploid range which consisted of nuclei with a DNA content of 2c ± 2 standard deviations of the control cell population. The third we defined as the tetraploid range by making the assumption that the relative variance is constant. This region consisted of nuclei with a DNA content of 4c ± 2.8 standard deviations of the control cell population. The second region, which was located between the diploid and tetraploid regions, we termed the near diploid aneuploid range. The fourth region was the hypertetraploid range. The frequency of cells with DNA contents within each of these four ranges was calculated for each tumor.

The measurement of the largest dimension of the tumor (LTD) was done on the microslide using a caliper. The determination of cell type was done by one of the authors (IWM) using the current AFIP modification of Calender's classification.2 Measurement of all of the variables was done without knowledge of the patient's outcome.

The Cox Proportional Hazard Model was used to analyze the correlation between the prognostic factors and survival of the patients. This model allowed for incomplete follow-up and quantified with a chi-square test the relationship between the prognostic factors and force of mortality (hazard rate). This analysis was performed using the P2L program in the package of BMDP Statistical Software (University of California, Berkeley, CA).10

Results

Only 95 of the original 110 cases could be measured by both morphometry and microspectrophotometry. Heavily pigmented or extensively necrotic tumors could not be measured by either technique. An additional problem with microspectrophotometry was the need to measure retinal bipolar cells for calibration. Some large tumors were eliminated because they had destroyed the overlying retina and there was no retinal tissue present in the remainder of the histologic sections. Because large or necrotic tumors are more likely to metastasize, some fatal (nine) than nonfatal (six) tumors were unmeasurable.

The presence of metastatic melanoma was documented by biopsy or autopsy in 19 of the 46 fatal tumors. Correct prediction of the patient's outcome in 72.3% of the cases and SD-DNA correctly predicted the patient's outcome in 64.1% of the cases.

Table 1. Association between DNA measurements and force of mortality in patients with uveal melanoma

<table>
<thead>
<tr>
<th>Measurement from DNA distribution</th>
<th>Chi-square value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>15.1</td>
<td>0.0001</td>
</tr>
<tr>
<td>Median</td>
<td>13.5</td>
<td>0.0002</td>
</tr>
<tr>
<td>Mode</td>
<td>4.5</td>
<td>0.03</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>19.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Near diploid</td>
<td>4.6</td>
<td>0.03</td>
</tr>
<tr>
<td>Tetraploid</td>
<td>9.4</td>
<td>0.002</td>
</tr>
<tr>
<td>Hypertetraploid</td>
<td>11.2</td>
<td>0.0008</td>
</tr>
</tbody>
</table>

The Cox Model, the chi-square value for SD-DNA dropped to a nonsignificant level (2.06, P = 0.15) indicating that SD-DNA did not have additional prognostic value.

To illustrate and compare the prognostic value of SD-DNA, SD-NA and LTD the cases of uveal melanoma were divided into two groups using each of the three variables. Cases with values less than or equal to the median were assigned to the good prognosis group and cases with values greater than the median were assigned to the poor prognosis group. The life-table survival calculated using only melanoma-related deaths for each of the groups is shown in Figure 1. SD-NA provided the best discrimination and SD-DNA provided the worst discrimination. At 15 years, SD-NA correctly predicted the patient's outcome in 79.5% of the cases, LTD correctly predicted the patient's outcome in 72.3% of the cases and SD-DNA correctly predicted the outcome in 64.1% of the cases.

Table 2. Association between four risk factors and force of mortality in patients with uveal melanoma

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Chi-square value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard deviation—DNA</td>
<td>19.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Standard deviation—nucleolar area</td>
<td>34.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Largest tumor dimension</td>
<td>24.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Calender's cell type</td>
<td>23.1</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

The ability to predict patient outcome was compared for SD-DNA content, SD-NA, cell type, and LTD in a second Cox model (Table 2). In this analysis SD-NA was the best predictor, while LTD and cell type were of intermediate prognostic value and SD-DNA was the worst predictor. In a stepwise analysis, when both SD-NA and LTD were entered into the Cox Model, the chi-square value for SD-DNA dropped to a nonsignificant level (2.06, P = 0.15) indicating that SD-DNA did not have additional prognostic value.
YEARS AFTER ENUCLEATION

Fig. 1. Comparisons of life-table survival, with deaths from cases other than melanoma treated as withdrawn alive at the time of death. Patients divided into good and poor prognosis groups using SD-NA (solid curves), LTD (dashed curves) and SD-DNA (dotted curves). SD-NA provides better discrimination than LTD or SD-DNA.

Discussion

In this study we have shown that for assessing the malignancy of uveal melanomas, SD-NA measured by a technician using computer-assisted morphometry is superior to SD-DNA measured by a technician using the DADS microspectrophotometer. It is important to realize that this is a comparison of two techniques. The fact that SD-NA correlated better with outcome than did SD-DNA does not prove that variability of nucleolar size in a uveal melanoma is more related to the ability of the cells to metastasize than variability in ploidy. It is quite possible that the differences observed may be due to the methods employed. Measurements of SD-NA and SD-DNA differed in at least two important aspects: first, 200 cells per tumor were used to calculate SD-NA versus 100 cells per tumor used to calculate SD-DNA. Second, cells were more randomly selected using morphometry than microspectrophotometry. Measuring a greater number of cells per tumor and selecting them more randomly would favor the morphometric approach over the microspectrophotometric method. A third aspect relating to the methodology is measurement error. Since both of the techniques that we employed provided imperfect measurements of biological variables, it is possible that the size of the nucleolus was determined more accurately using morphometry than was the quantity of DNA in the nucleus using microspectrophotometry.

We believe the methods we have used to measure SD-NA and SD-DNA are still in the experimental stage. Measurement of SD-NA requires approximately one-half hour for the technician to laboriously trace 200 nucleoli. The measurement of SD-DNA is less labor intensive, but requires approximately 1.5 hr for the technician to measure the DNA content of 100 tumor cells and 20 control cells. Both techniques require moderately expensive equipment. The technologies of computer-assisted morphometry and microspectrophotometry are both undergoing rapid evolution. Also, flow cytometry provides an alternative to microspectrophotometry for the determination of DNA content. Since flow cytometry can also measure the quantity of ribonucleic acid in cells it may provide an alternative to measuring the size of the nucleolus. For these reasons, neither computer-assisted morphometry nor microspectrophotometry have evolved to the stage where they can be employed routinely by practicing ophthalmic pathologists.

Our observations that variability of the size of the nucleolus and variability in the quantity of DNA in the cells of the uveal melanoma are strongly associated with the ability of the tumor to metastasize suggests that biologic heterogeneity within a uveal melanoma is important in the process of metastasis. A current working hypothesis concerning metastasis is that malignant tumors disseminate large numbers of cells but only a few of these cells have the appropriate genotype to survive and form a new tumor. While one might expect that tumors most likely to metastasize would be composed of a clone or clones with uniformly high metastatic potential, Poste et al have shown the opposite to be true using the B16 melanoma in the mouse. They found that tumors that produced a large number of metastatic foci tended to be genetically more heterogeneous than tumors with a low metastatic potential. The heterogeneity resulted because the metastasizing clones tended to be more genetically unstable than the nonmetastasizing clones. Cell sublines from metastasizing tumors, even when clonally derived, quickly developed polyclonality for metastatic capability.

Despite the limitations of current technology, parameters can be measured that are sufficiently related to malignant potential that they can predict death from uveal melanoma with a high degree of accuracy. SD-NA, for example, was able to correctly predict patient outcome in 79.5% of the cases. Given that there is variability among patients in immunity against tumors, 80% accuracy may represent the best prediction possible unless host response can be measured and factored into the prognostic equation.

Key words: uveal melanoma, prognosis, aneuploidy, morphometry, microspectrophotometry

References