Correlation of Heterogeneity for Chromosome 3 Copy Number with Cell Type in Choroidal Melanoma of Mixed-Cell Type

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PURPOSE. To study heterogeneity for chromosome 3 copy number in mixed choroidal melanoma with discrete populations of spindle and epithelioid cells using chromosome in situ hybridization (CISH) and to correlate chromosomal loss with cell type.

METHODS. Twenty-two archival cases of choroidal melanoma with discrete populations of spindle and epithelioid cells were identified. CISH was used to identify chromosome 3 copy number in spindle and epithelioid areas.

RESULTS. Monosomy 3 was detected in 12 (55%) of 22 choroidal melanomas. Of these, 10 (45%) had two copies of chromosome 3 in both epithelioid and spindle cells, 7 (32%) showed monosomy 3 in the epithelioid areas only, and 5 (23%) showed monosomy 3 in both epithelioid and spindle areas.

CONCLUSIONS. CISH is a useful technique for analyzing chromosome copy number in different cell populations within a tumor. In mixed choroidal melanomas with discrete spindle and epithelioid cell populations, there may be heterogeneity for chromosome 3 copy number that correlates with areas of different cell type. (Invest Ophthalmol Vis Sci. 2006;47: 5177–5180) DOI:10.1167/iovs.06-0332

Uveal melanoma has an overall mortality of approximately 50% due to liver metastasis with the peak mortality from metastatic disease occurring within 3 years of diagnosis.1–2 The prognosis is difficult to predict from clinical and pathologic features, but large tumors composed predominantly of epithelioid cells appear to carry a poorer prognosis.3

Cytogenetic studies have shown that loss of an entire chromosome 3 homologue (monosomy 3) often associated with an increased copy number of the long arm of chromosome 8, has been linked to metastatic death.4–6 Earlier studies have attempted to predict the presence of monosomy 3 from histology and have shown that the presence of epithelioid cells can predict monosomy 3 but only in association with large tumor size3 or the presence of closed vascular loops.7 More recently, gene expression profiling studies have shown that uveal melanoma clusters into two distinct molecular classes. In this study, high-risk tumors were distinguishable from low-risk tumors by the presence of downregulated genes on chromosome 3 and upregulated genes on the long arm of chromosome 8.8

High-risk melanomas also contained more epithelioid cells than did low-risk melanomas, when melanomas were ranked according to the proportion of epithelioid cells.

In most cytogenetic studies, uveal melanoma has been considered to be a homogeneous entity, detected by the use of DNA extracted from whole tumors or by performing classic cytogenetics on selected samples of tumor cells. However, morphologic heterogeneity is well recognized in uveal melanoma. Most tumors are composed of variable proportions of epithelioid and spindle-shaped cells.9 There have been few studies of cytogenetic heterogeneity in uveal melanoma. Monosomy 3 has been associated with a reduction in the 5-year survival from 100% to 30%, because of metastasis-related death.3 Therefore, cytogenetic heterogeneity carries significant clinical implications for the sampling of tumors for cytogenetic studies.

The purpose of this study was to evaluate the presence of cytogenetic heterogeneity of chromosome 3 in choroidal melanomas with morphologically distinct spindle and epithelioid cell types, by using the technique of chromosome in situ hybridization (CISH) on archival paraffin-embedded tissue.

MATERIAL AND METHODS

Case Selection

Sixty-four cases of choroidal malignant melanoma of mixed cell type (modified Callender system10) were identified from the eye pathology files (Western Infirmary, Glasgow) between the years 1975 and 2002. From these 64 tumors, the cases in which the populations of spindle and epithelioid cells were relatively discrete were identified. All tissues had been fixed in glutaraldehyde or formalin and embedded in paraffin wax. This project received full approval from the West Ethics Committee, North Glasgow Trust, and adhered to the tenets of the Declaration of Helsinki.

Survival Status

The survival status of the patients and cause of death were obtained from cases notes and the cancer registry. The time to death was calculated from the date of treatment, and survival status was determined up to August 2003.

Chromosome In Situ Hybridization

The copy number of chromosome 3 was assessed by CISH, with chromosome-specific centromeric probes, as previously described.11 Briefly, 4-μm sections were pretreated by microwaving followed by digestion in pepsin (0.4% pepsin in 0.2 M hydrochloric acid) for 30 minutes at 37°C. The tissue sections and probe were then simultaneously denatured for 5 minutes at 80°C to obtain single-stranded DNA and hybridized overnight at 37°C. Sites of hybridization were detected using anti-digoxigenin alkaline phosphatase (AP) Fab fragments (Roche Molecular Biochemicals, Indianapolis, IN). Chromosome 18 was used as a control chromosome, as it rarely shows abnormalities in uveal...
melanoma. The chromosome 3 copy number was assessed in both spindle and epithelioid areas. A minimum of 200 nuclei were counted in both spindle and epithelioid areas. The number with chromosomess was assessed using both the chromosome index (CI) and signal distribution (SD), as previously described. The chromosome index gives an average chromosome copy number and is calculated by dividing the number of hybridization sites by the number of nuclei counted. Chromosome loss is defined as a CI < 3 standard deviations from the mean for retina (normal tissue). The SD is defined as the percentage of nuclei with only one hybridization site. An SD of > 60% was the cutoff point used to define chromosome loss. The region tumors had to show chromosome loss by both CI and SD to be regarded as monosomic. The cutoff points were selected in accordance with our laboratory's previous experience of the technique.

Statistical Analysis
The relationship of the pattern of monosomy 3 distribution with tumor diameter, age, and survival was tested by pair-wise t-test and pair-wise rank sum test.

RESULTS
Case Selection
Twenty-two suitable cases were identified from the original group of 64 mixed choroidal melanomas. Nineteen of the cases were from patients who had died of metastatic melanoma and three were from living patients or those who had died of other causes.

Chromosome 3 Copy Number in Spindle and Epithelioid Areas
The tumors separated into three groups based on the copy number of chromosome 3 in spindle and epithelioid areas. Ten (45%) melanomas displayed two copies of chromosome 3 in both spindle and epithelioid cells and were defined as balanced for chromosome 3 (BB), including the three cases in which the patients were alive or had died of causes other than metastases. Five (23%) displayed monosomy 3 in both spindle and epithelioid cell areas (M3M3). In the remaining seven cases (32%) the epithelioid areas displayed monosomy 3, but the spindle cell areas contained two copies of chromosome 3 (M3B; Fig. 1).

Relationship of the Pattern of Monosomy 3 Distribution with Clinical Features and Survival
The pattern of monosomy 3 (BB, M3M3, M3B) was not significantly related to clinical features of the patients such as tumor size, age at surgery, or time to death.

DISCUSSION
Monosomy 3 in choroidal melanoma is a significant predictor of metastases related death and has been associated with a reduction in the 5-year survival from 100% to 30%. Although monosomy 3 is an important predictor of metastatic death we have shown that there is also a small but significant number of people with metastasizing melanoma whose tumors are balanced for chromosome 3. One possible explanation for our cases of metastasizing melanoma without monosomy 3 is the presence of genetic heterogeneity. Morphologic heterogeneity of choroidal melanoma is well recognized, with most cases being of mixed-cell type. The objective of this study was to evaluate corresponding loss of heterozygosity of chromosome 3 in areas of spindle and epithelioid cell type using CISH. In 23% of all melanomas, monosomy 3 was identified in both spindle and epithelioid cell areas, whereas in 32% it was present only in the epithelioid cell areas. Forty-five percent of melanomas were balanced in both spindle and epithelioid areas. This latter group included seven cases of metastasizing melanoma. Therefore, cytogenetic heterogeneity for chromosome 3 does not explain metastases in cases balanced for chromosome 3 in both spindle and epithelioid areas. White et al. have previously reported a case of clonal heterogeneity in a uveal melanoma. In this case the tumor was found to have distinct pigmented and nonpigmented areas on gross examination. Tissue samples were collected from both of these areas for standard cytogenetics. Histologic examination revealed small regular epithelioid cells in the pigmented area and large pleomorphic epithelioid cells in the nonpigmented area. Standard cytogenetic analysis showed two copies of chromosome 3 in the pigmented area compared with monosomy 3 in the nonpigmented area. It is notable that gross differences in pigmentation are not frequently present to alert the sampler to morphologic and cytogenetic heterogeneity when cells are to be cultured for standard cytogenetics. Techniques that involve extraction of DNA from tissue sections should theoretically represent the tumor cell population more accurately. However, some subclones or even contaminating normal DNA may be preferentially amplified during polymerase chain reaction. The CISH technique differs from other technique used to assess monosomy 3 because chromosomal losses are assessed in both interphase and metaphase nuclei within a population of cells in a tissue section. This allows the direct correlation of genotype with phenotype.

In our study we selected tumors with discrete populations of spindle and epithelioid cells to aid in counting the different areas. The CISH technique can be applied only to a defined population of tumor cells, and the assessment of chromosomal loss cannot be made on individual tumor cells. These 22 cases were selected from 64 choroidal melanomas of mixed-cell type. The size of this sample supports the authors’ experience that such morphologic subclones are not uncommon. However, because these cases do not represent our entire archive, there may be an element of selection bias. In the other 42 tumors there were insufficiently large areas of each cell type to allow accurate counting. Although the number of cases studied was small, we have demonstrated heterogeneity for chromosome 3 copy number in 7 (32%) of 22 cases. This finding has important implications for other methods of cytogenetic analysis, such as short-term culture for metaphase spreads, since often only a sample of tumor is submitted for analysis. For example, a small biopsy of the tumor may be taken before submitting the remaining tumor for histopathologic examination. Furthermore, since the importance of cytogenetic assessment of uveal melanoma will increase as new therapies become available, tumors may be biopsied by fine needle aspiration (FNA) to obtain cytogenetic information before treatment with modalities other than surgery. The possibility of morphologically nonrepresentative material in FNA has already been reported by Folberg et al. in a study comparing average nucleolar area in FNA with enucleation specimens of uveal melanoma. Similarly, Augustburger et al. demonstrated a needle track that just missed an epithelioid region in a tumor removed after FNA. Based on the results of our study, the confidence attributed to any prognostic assessment undertaken on a small sample of tumor would be greater if a morphologic assessment showed the tissue sampled to contain epithelioid cells.

In conclusion, mixed choroidal melanomas with discrete spindle and epithelioid cell populations may display heterogeneity for chromosome 3 copy number that correlates with populations of different cell type. CISH is a useful technique to identify this clonal heterogeneity in excision specimens. However, the genetic information obtained from small samples using other techniques such as classic cytogenetics may not be...
representative. This drawback in turn will affect the degree of certainty in patient counseling and potentially in patient selection for the use of novel treatments.

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**References**


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