Chromosome 3 Intratumor Heterogeneity in Uveal Melanoma

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PURPOSE. To investigate the presence of focal or diffuse heterogeneity of monosomy 3 in uveal melanoma, by using fluorescence in situ hybridization (FISH).

METHODS. Direct interphase FISH in a series of 151 uveal melanomas revealed 82 tumors with loss of chromosome 3. Tumors with monosomy 3 were suspected to be heterogeneous if there were low percentages of monosomy 3, triploid clones, inconsistencies between FISH on centromere 3 and the long arm of chromosome 3, or discrepancies between fine-needle-aspiration biopsies (FNABs) and the main tumor. These tumors (n = 16), all choroidal melanomas, were selected and analyzed for intratumor heterogeneity by using FISH on paraffin-embedded tissue sections.

RESULTS. Different sections of each tumor were evaluated with FISH: 6 tumors showed monosomy 3 in the same percentage throughout the tumor, and 10 showed multiple clones with different percentages of monosomy 3. However, these tumors did not show focal heterogeneity with respect to chromosome 3 status, and differences in monosomy 3 distribution between the base and apex of the tumor could not be identified.

CONCLUSIONS. Although a small number of uveal melanomas show heterogeneity for chromosome 3, it does not affect survival. In the presence of triploid clones, the loss of chromosome 3 is more difficult to interpret. In general, tumor biopsies in uveal melanoma provide an accurate prediction of the patient’s prognosis. In this study we investigated intratumor heterogeneity by using FISH on paraffin-embedded tissue sections.

Uveal melanoma (UM) is the most common primary intraocular malignant tumor in adults and has a predilection for hematogenous dissemination to the liver. There is no effective treatment for liver metastasis, which result in tumor-related death in approximately 45% of patients with UM.1 Prognostic factors to identify patients at risk for metastasizing disease include clinical (tumor location, largest tumor diameter), histologic (cell type, vascular mimicry), and genetic (chromosomal aberrations, gene expression profiling) parameters.2–4

Gene expression profiling identifies two distinct classes of UM: with a class II expression profile that predicts metastatic disease.5 Loss of chromosome 3 (monosomy 3) and a class II expression profile are considered to be the most accurate in selecting high-risk patients.6 A correlation between gene expression profile and monosomy of chromosome 3 has also been established.7

Identification of high-risk patients has implications for follow-up and would allow the use of adjuvant therapy in the future. Most small and medium-sized UMs are currently managed by eye-saving treatments, and consequently, the available tissue for assessment of prognosis is limited. Depending on tumor location, biopsies can be obtained via a transscleral or transvitreous route. Recent studies reveal that fine-needle-aspiration biopsy (FNAB) provides sufficient material for FISH analysis.8–10

In our laboratory, all UMs treated with enucleation are screened for the classic genetic parameters loss of the short arm of chromosome 1, monosomy 3, and chromosome 6 and 8 abnormalities by fluorescence in situ hybridization (FISH) and karyotyping.

However, we could not demonstrate loss of chromosome 3 in all cases of UM with metastatic disease (12/51 metastatic tumors had disomy 3). It is possible that some tumors evolved in a different manner, but it could also be an inability to detect, for example, partial loss of chromosome 3 (often screening is done by FISH-analysis of the centromere), isodisomy 3 (duplication of one copy of a chromosome), or intratumor heterogeneity. Intratumor heterogeneity is considered a consequence of cancer pathogenesis. Cancer development is often associated with genomic instability and acquisition of genomic heterogeneity,11 generating both clonal and nonclonal tumor cell populations.12 Morphologic heterogeneity is well recognized in UM showing variable proportions of epithelioid and spindle cells. Epithelioid cells lose chromosome 3 more frequently than do spindle cells.13,14 White et al.13 and Sandinha et al.15 reported cytogenetic heterogeneity in UM. However both these studies describe morphologic heterogeneity corresponding with cytogenetic heterogeneity. In the case of FNAB, intratumor heterogeneity would interfere with a correct prediction of the patient’s prognosis. In this study we investigated intratumor heterogeneity of chromosome 3 in different areas of a tumor (posterior, anterior, base, and apex) in UM. Furthermore, we wanted to know whether a transscleral or a transvitreous approach for FNAB is preferable.

MATERIALS AND METHODS

Patients, Tumor Selection, and Follow-up

Between March 1992 and May 2006, tumor tissue was collected from 151 patients with UM who underwent enucleation. Informed consent was given before enucleation, and the study was performed according...
to the tenets of the Declaration of Helsinki. A conventional histopathologic examination was performed on all tumors and confirmed the origin of the tumor, as well as the tumor size, cell-type, and vascular pattern. Follow-up data regarding metastases and tumor-related death were obtained by reviewing each patient’s charts and contacting the general physician. Three patients were lost to follow-up. The primary clinical endpoint was metastatic disease measured from the time of enucleation.

**Fluorescence In Situ Hybridization (FISH)**

Fresh tumor tissue from enucleated eyes containing UM were routinely cultured for karyotyping or directly used for FISH (chromosomes 1, 3, 6, and 8) as previously described. Cutoff limits for deletion was >15% of the nuclei with one signal and for amplification was >10% of the nuclei with three or more signals. Chromosome abnormalities are described according to the recommendations for human cytogenetic nomenclature.

Tumors were considered of possible intratumor heterogeneity if FISH showed a low percentage of monosomy 3 (n = 8) or inconsistencies between centromere 3 and the long arm of chromosome 3 (n = 2). Furthermore, when triploid clones were present (n = 4) or if FISH results differed between ex vivo fine-needle-aspiration biopsies (FNABs) and the main tumor (n = 3). The latter were selected from a group of 40 UM, in which FISH analysis on chromosomes 1, 3, 6, and 8 was compared between ex vivo FNAB material and single cell suspension from fresh tumor material. In three tumors (6/249 hybridizations) the results were incongruent.

From 16 tumors, with possible heterogeneity, 5-μm paraffin-embedded tissue sections were pretreated by dewaxing with xylene, permeabilizing with sodium thiocyanate, proteolysis, and de-naturation. Dual-color FISH was performed with the following probes: Pa3.5 (centromere 3) and RP11-1059N10 (5q12.1). Chromosome 5 is rarely involved in genetic changes in UM and was used as a control for aneuploidy and truncation or cutting artifacts. In some tumors, additional FISH was performed for 1p36 (probe RP11-48E9), 6p22 (probe RP11-356B3), 6q21 (probe RP11-78722), and 8q21 (probe RP11-88822). The concentration of the centromeric probe was 5 ng per slide, whereas for the BAC-clones, 15 to 25 ng per slide was used. After hybridization and washing, the slides were counterstained with 4',6-diamidino-2-phenylindole and mounted in antifade solution (1:1; Dabco-Vectashield; Vector Laboratories, Burlingame, CA). Tumor sections were screened for the difference in signals between the tumor base and the apex, and the anterior and posterior parts of the tumor. Two hundred nuclei per sample were counted.

**Statistical Analysis**

The influence of single prognostic factors on metastasis-free survival was assessed by using log-rank analysis (for categorical variables) or Cox proportional hazards analysis (for continuous variables). The statistical analyses were performed with commercial software (SPSS 14.0 software; SPSS, Chicago, IL).

**RESULTS**

Interphase FISH on direct fresh tumor material was performed routinely in 151 UM, to screen for chromosomal aberrations. The variation in the status of 1p, 3, 6p, 6q, and 8q, assessed using FISH analysis, was subdivided into three categories: loss of one chromosome, two copies of a chromosome (disomy), and gain of one or more chromosomes. Univariate analysis of the single prognostic risk factors showed a significant decreased survival for patients with UM consisting of epithelioid cells, closed vascular loops, monosomy 3, and gain of 8q (Table 1). Tumor diameter and patient age were significant prognostic risk factors as well. The other chromosomal changes loss of 1p, gain of 6p, and loss of 6q did not reach significance. In addition, tumor thickness, tumor location and patient gender did not influence metastasis-free survival significantly.

Eighty two choroidal melanomas showed monosomy 3. As commonly observed in UM patient populations, monosomy 3 was identified as a significant prognostic factor for metastatic disease by log-rank analysis of Kaplan-Meier curves (P < 0.001). Whereas 15% is the detection border for chromosome loss, in the majority of UM monosomy 3 was detected in ~90% of the counted nuclei. Sixteen tumors were selected for further analysis of intratumor heterogeneity. Six UM showed monosomy 3 in the same percentage throughout the tumor (EOM 193, 206, 207, 208, 237, and 305). The other melanomas showed clones with various percentages of monosomy 3 (Table 2). No structural difference in monosomy 3 distribution between the base and
apex of the tumor was identified. Figure 1 shows a paraffin-embedded tissue section of a choroidal melanoma with low mitotic activity and a more pigmented area of the tumor composed of spindle and epithelioid cells, whereas the other part of the tumor was composed of epithelioid cells only. FISH showed a higher percentage of monosomy 3 in the pigmented area, but also the other part had loss of chromosome 3 (Fig. 2). When comparing the UMs with a low percentage of monosomy 3 with UMs with monosomy 3 in most nuclei, no significant difference in metastasis-free survival was observed ($P = 0.573$).

In all 16 tumors, monosomy 3 was found in every part of the tumor. In four UMs, we observed a difference in chromosome 3 status between interphase FISH on single-cell suspension from fresh tumor tissue and FISH on paraffin-embedded tissue. In the tissue sections, we observed a 14% to 96% loss of

![Figure 1](image1.png)

**FIGURE 1.** Heterogeneity of UM EOM 303: section stained with hematoxylin and eosin staining. (A) Choroidal melanoma located at the equator extending to the pars plana. (B, C) Epithelioid and spindle cells with melanin-containing macrophages in between, (D, E) less pigmented part of the tumor with predominantly epithelioid cells. Magnification: (A) $\times 10$; (B, D) $\times 25$; (C, E) $\times 200$.

![Figure 2](image2.png)

**FIGURE 2.** FISH of EOM 303 paraffin-embedded tissue section. The posterior part (A, B) shows 60% monosomy 3 and the anterior part (C, D) shows 19% monosomy 3 (green signals, centromere 3; red signals, 5q).
chromosome 3, whereas interphase FISH revealed only a relative loss of chromosome 3. These four UMs contained triploid subpopulations of tumor cells with only two copies (instead of three) of chromosome 3.

**DISCUSSION**

In the study presented in this article, we attempted to gain insight into intratumor heterogeneity. Subclones with different percentages of monosomy 3 could be identified within the tumor in 10 cases. None of the analyzed choroidal melanomas required adjustment of risk calculation for metastasizing disease based on chromosome 3 status throughout the tumor. Intratumor heterogeneity should be considered in tumor biopsies, although it does not seem to influence survival. UMs with a low percentage of chromosome 3 loss also result in reduced survival; therefore, it would be interesting to examine the liver metastases to investigate which clone or clones have led to tumor progression. Identifying high-risk patients based on examining tumor biopsies appears reliable, because UM do not demonstrate abundant chromosomal focal heterogeneity.

Another group of interesting UMs are the ones with disomy 3 that do metastasize. Of 51 metastasized primary tumors, 12 showed disomy 3. Additional analysis such as karyotyping and comparative genomic hybridization (CGH) did not suggest loss of chromosome 3 or heterogeneous loss of chromosome 3. Currently, we are investigating these tumors by high-density array CGH.

Sandinha et al. have examined morphologic and cytogenetic heterogeneity in UM. They found monosomy 3 in 12 of 22 mixed cell-type UMs. In seven of these tumors, monosomy 3 was found only in the epithelioid cells and not in the spindle cells. In the present study, we were more interested in cytogenetic differences between tumor areas, because that might influence the route (transscleral or transvitreal) to choose for FNAB. We found monosomy 3 in nine spindle cell-type UMs, four epithelioid cell-type UMs, and three mixed cell-type UMs.

Mait et al. have pointed out that heterogeneity of monosomy 3 is a frequent event in UM. In their study, 7 of 50 UMs were found to be heterogeneous, but FISH was unsuccessful in 17 UMs and difficult to interpret in 8. Furthermore, FISH was only performed for chromosome 3, without a control probe for aneuploidy and/or truncation artifacts, and no cutoff values for loss or gain of a chromosome were reported. Possible caveats include truncation artifacts in paraffin-embedded sections leading to cutting of nuclei and as a result to the deletion of chromatin material from a section, sampling errors in FISH (e.g., looking at macrophages instead of tumor cells), and signal reduction due to degradation. However, truncation artifacts can be controlled by assessing a chromosome not involved in UM for aneuploidy. Few stroma are present in UMs and melanoma nuclei are larger than nuclei of macrophages and fibroblasts, making identification of tumor cell nuclei easy and probe signals distinguishable.

A more important drawback in interphase FISH of paraffin-embedded tissue sections and other techniques, as well, is that the tumor is observed in one dimension, which limits insight into the entire lesion, possibly missing intratumor heterogeneity. Previous studies by our group demonstrate that FISH analysis of FNAB biopsy specimens gives reliable results of cytogenetic changes within the tumor. Chromosome status in the biopsy specimens was consistent with the results in the main tumor tissue. In 6 of 249 hybridizations FNAB showed normal copy numbers while a direct single-cell suspension of the main tumor showed gain or loss of chromosomes in small subclones. In our present study, the incongruent chromosome 3 status of four tumors in different experiments might be the result of relative loss in triploid clones or genetic imbalance. Two of these tumors metastasized within 19 months of diagnosis. All four tumors have triploid clones, and tissue was taken from different parts of the tumors. Yet, the results were obtained from different FISH techniques, and consequently, conclusions about focal heterogeneity may be indefinite. Different available FISH techniques need further attention and standardization in ocular oncology research.

Monosomy of chromosome 3 is the most frequently found nonrandom chromosomal aberration in UM and is predominantly found in metastasizing tumors. In the majority of tumors with chromosome 3 loss, there is complete monosomy, although occasionally, isodisomy of this chromosome is acquired. Rarely, melanomas with partial aberrations on chromosome 3 or translocations have been described, making it difficult to map putative tumor suppressor genes. Loss-of-heterozygosity studies demonstrate common regions of allelic loss located at 3p25 and on the long arm spanning from 3q24 to 3q26.

Monosomy 3 is considered to be a primary event, because it is seen in combination with all other chromosomal aberrations in UMs such as loss of 1p, gain of 6p, and gain of 8q. Tumors with gain of 6p have been proposed to represent a separate group of UMs with an alternative genetic pathway in carcinogenesis.

Reviewing our Erasmus ocular melanoma (EOM) database of 151 UMs, we found that 61 tumors with loss of chromosome 3 gained 8q concurrently, whereas no tumors with disomy 3 gained 8q. Tumors with monosomy 3 had gained 6p concurrently in 17 (21.5%) cases and had lost 6p in one case. Gain of 6p was also identified in 32 tumors with disomy 3. In a recent study by Ehlers et al., integrative genomic analysis of 49 UMs also revealed a concurrent gain of 6p and loss of chromosome 3 in 4% of the tumors. Gain of 6p did not reach significance as a single prognostic factor in our study population. Furthermore, gain of 6p occurred more often in tumors with monosomy 3, suggesting that it is unlikely to represent an alternative pathway of UM pathogenesis. We do agree that gaining 6p is probably an early event in carcinogenesis.

This study is the first to systematically evaluate genetic heterogeneity, and it confirmed that monosomy 3 remains an important prognostic factor in UM that can be determined with relative easy cytogenetic techniques. Overall, our findings support the use of tumor biopsies for diagnosis, prognosis, and therapeutic decision-making. A transscleral or transvitreal approach can be chosen, depending on tumor location. To reduce the possible impact of heterogeneity on identification of high-risk patients, FNABs should be obtained from several areas of the tumor.

**References**


