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Comparative Genomic Hybridization in the Detection of DNA Copy Number Abnormalities in Uveal Melanoma[1]

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ABSTRACT

Genomic instability appears to play an important role in the development, growth, invasiveness, and eventual metastasis of the neoplastic cell. We have used a powerful new technique, comparative genomic hybridization, to evaluate genetic alterations in 10 fresh frozen uveal melanomas. Comparative genomic hybridization utilizes dual fluorescence *in situ* hybridization to characterize chromosome deletions and duplications, allowing for simultaneous evaluation of the entire human genome. Several consistent chromosomal abnormalities were detected. This study confirmed previous findings obtained using standard cytogenetic techniques but demonstrated an increased incidence in abnormalities of chromosomes 3 and 8; there was loss of chromosome 3 and duplication of 8q. In addition, we identified, although less frequently, other recurrent abnormal regions including alterations on chromosomes 6p, 7q, 9p, and 13q.

INTRODUCTION

Specific genetic alterations can transform normal cells and induce progression toward a malignant phenotype (1, 2). In some cases, such genetic changes can be characterized as abnormalities in gene copy number, due to either amplifications and over-expressions of protooncogenes (3) or loss of specific loci, such as those encoding tumor suppressor genes (3, 4).

Uveal melanoma is the most common primary intraocular tumor of adults and can produce both blindness and death (5). Several prognostically relevant characteristics have been identified, including patient age, tumor size, location, cell type, and variability of nucleolar size (5–7). Little is known about the genetic events that result in transformation of uveal melanocytes. Cytogenetic abnormalities observed in the few uveal melanomas reported are trisomy of chromosome arm 8q and monosomy of chromosome 3; each has been reported in about one-half of cases studied. Other genomic alterations include deletions of 6q, extra copies of 6p, and abnormalities of chromosomes 1 and 7 (8–14). Limitations of traditional karyotyping have compromised characterization of uveal melanoma chromosomal abnormalities (15).

CGH[3] (16) is based on dual FISH. Tumor DNA and DNA from normal peripheral blood lymphocytes are labeled with different reporter molecules and then simultaneously hybridized to normal metaphase spread chromosomes. An image acquisition system is used to quantitate signal intensities contributed by tumor and reference DNA along the entire length of each chromosome. Regions of duplication and deletion within tumor DNA are demonstrated as quantifiable alterations.

CGH adapts molecular biological techniques for simultaneous analysis of the entire genome. Novel loci important in neoplasia can be identified, and the possibility that more than one locus is involved in tumor initiation and progression can be assessed. Genomic DNA from tumor specimens is used so that genetic alterations identified with CGH are not artifactually altered by propagation in cell culture. In the present study, we used CGH to detect alterations in gene copy number in ten fresh frozen uveal melanomas.

MATERIALS AND METHODS

Clinical Data. Ten uveal melanomas were evaluated after primary enucleation. The tumors were classified histologically according to the modified Callender classification (5). Three tumors were spindle cell type, five were mixed cell type, and two were epithelioid melanomas. All tumors were large; the basal diameters ranged from 13 to 21 mm, and the tumor heights from 9.2 to 15.3 mm. All tumors involved some of the posterior fundus; the anterior margin of three cases was between the equator and the pars plana. There was extension into the ciliary body in five cases and into the iris in two cases. Six patients developed metastatic disease within 5 to 29 months (mean, 19.6 months) from the time of treatment. The other patients remained free of metastases with a follow-up ranging from 33 to 69 months (mean, 49 months).

Tissue Samples. All fresh tumor samples were immediately split, an aliquot of tumor samples was snap frozen and stored at −80°C, and the remaining samples were submitted for histological analysis. The tissue was thawed, minced using sterile scissors, and incubated for 12–16 h at 50°C in 10 ml of a digestion buffer, 0.05% proteinase K (0.25-ml proteinase K stock, 8.75-ml 1× sodium tris EDTA buffer-1.00-ml 10% sodium dodecyl sulfate). High molecular weight DNA was obtained and labeled as described previously (16). Tumor DNA samples were labeled using biotin-14-dATP, and normal DNA samples were labeled using digoxigenin-11-dUTP (Boehringer Mannheim Corp., Indianapolis, IN) using the Bionick Labeling System and supplemental DNA polymerase I (BRL). The amount of DNase and DNA polymerase I was modified for each DNA sample to produce labeled probe fragments of 600–2000 base pairs, as observed on a nondenaturing 1% agarose gel.

Comparative Genomic Hybridization. This technique was performed in a manner described previously (16). Briefly, lymphocyte metaphase slides were prepared from phytohemagglutinin-stimulated peripheral blood lymphocytes from normal males. Then 120–240 ng each of the biotinylated tumor DNA, the digoxigenin-labeled normal DNA and 10–20 μg of human Cot-1 DNA (BRL) were precipitated with ethanol, resuspended in a hybridization buffer, heated at 73–75°C for 5 min, and then applied immediately to the denatured lymphocyte metaphase slides. The slides were incubated for 3 days at 37°C in a humid environment. The biotinylated DNA was detected using 5-μg/ml fluorescein isothiocyanate-avidin and the digoxigenin-labeled DNA with 2 μg/ml anti-digoxigenin rhodamine in 1% bovine serum albumin 4× standard saline-citrate.

After hybridization, the slides were washed, and the biotinylated DNA was detected using 5 μg/ml fluorescein isothiocyanate-avidin and the digoxigenin-labeled DNA with 2-μg/ml anti-digoxigenin rhodamine in 1% bovine serum albumin 4× standard saline-citrate. The hybridized slides were analyzed using a digital image analysis system that was based on a Nikon SA microscope equipped with a cooled-CCD camera (Photometrics, Inc., Tucson, AZ). Sequential images of a single metaphase were acquired using three excitation wavelengths with registration shifts. The three color images were collected and processed with a SUN IPX workstation using Scil-Image software (TNO, Delft, the Netherlands) for pseudo-color display of the images. The contrast-stretched three-color images were used to visually inspect the color changes along the metaphase chromosomes. A 4′,6-diamidino-2-phenylindole counterstained image was contrast stretched and used to identify the chromosomes from the banding patterns. A quantitative analysis of the green and red fluorescence intensities was performed for each chromosome using the

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[3] The abbreviations used are: CGH, comparative genomic hybridization; FISH, fluorescence *in situ* hybridization.

XWoolz software (MRC, Edinburg, United Kingdom). The contour and medial axis of the chromosomes were defined based on collective three-color staining. Local background fluorescence was determined for each chromosome and subtracted from the green and red images. Green- and red-fluorescence intensities were determined along an axis from p telomere to the q telomere by integrating fluorescence across the medial axis at 1-pixel intervals. Green:red-fluorescence intensity ratio profiles were then calculated for each chromosome. All profiles were normalized so that the overall green:red ratio for each metaphase was set at 1.0.

Five to 8 metaphases for each DNA sample were analyzed to detect deletions or amplifications on each chromosome. At least two metaphases for each sample were analyzed using the XWoolz program, and the profiles were averaged to decrease artifactual fluctuations. Deletions and amplifications detected by the green:red ratio measurement were noted for all chromosomes and verified on at least three other metaphase spreads. Definition of deletions as green:red ratios less than 0.8 and definition of amplifications was green:red ratios that exceeded 1.2.

Two tumors that showed the combination of no gain or loss of chromosome 17, a gain of chromosome 8, and loss of chromosome 3 had verification of these abnormalities with FISH using chromosome-specific probes which target the α-satellite repetitive sequence regions of the chromosomes (Oncor, Gaithersburg, MD). Tumor-touch preparations were made onto glass slides and stored (−20°C). The slides were fixed 3 times for 5 min each in 8 ml Carnoy's fixative (6 ml methanol, 2 ml glacial acetic acid) and dried on a 60–70°C plate. FISH was performed as described previously (17, 18). The number of chromosome-specific probes as counted in 200 nuclei for each tumor sample.

RESULTS

Fig. 1, a and b shows one of the metaphase and corresponding green:red-fluorescence intensity ratio profiles after hybridization with DNA from a uveal melanoma. A decrease in tumor DNA copy number is seen on both copies of chromosome 3 and is reflected on the histogram as a decreased green:red ratio. A highly increased tumor DNA copy number is seen on chromosome arm 8q. Fig. 2 shows a metaphase after hybridization with a different uveal melanoma. There are large DNA copy number increases in chromosomal regions 6p and 8q observed on both the metaphase image and the ratio profile, as well as a focal increase on chromosome 9p. The metaphase in Fig. 3 demonstrates several deletions involving both copies of chromosome 1p, 3, and 15, all of which were corroborated by the ratio profile. These profiles also demonstrate two focal increases on 7q and an increase in partial 13q.

Consistent chromosome copy number abnormalities were found in the uveal melanomas studied; an increase in chromosome arm 8q was observed in all 10 cases. A loss of chromosome 3 was demonstrated in 7 of 10 cases. Other frequent copy number abnormalities included an increase in both chromosomes 2 and 13q in 70% of cases (Fig. 4).

The fluorescence ratio profiles identified some nonrandom focal abnormalities. Two focal regions of increased copy number were seen on chromosome arm 7q in seven tumor cases. Chromosomal copy number abnormalities are summarized in Fig. 4. Many alterations were consistent across several or all uveal melanomas studied.

Fewer copy number abnormalities were demonstrated in the more benign spindle cell tumors compared to mixed and epithelioid tumors. These findings are summarized by cell type in Table 1.

FISH with chromosome-specific probes was performed for 2 cases to verify the confirmed decreased copy number of chromosome 3 and the increased copy number in chromosome 8 (data not shown).

DISCUSSION

We used CGH to detect alterations in gene copy number in ten fresh frozen uveal melanomas. Several consistent chromosomal abnormalities were found. A decrease in copy number of chromosome 3 was seen in seven cases, and an increase in 8q was analyzed in all ten

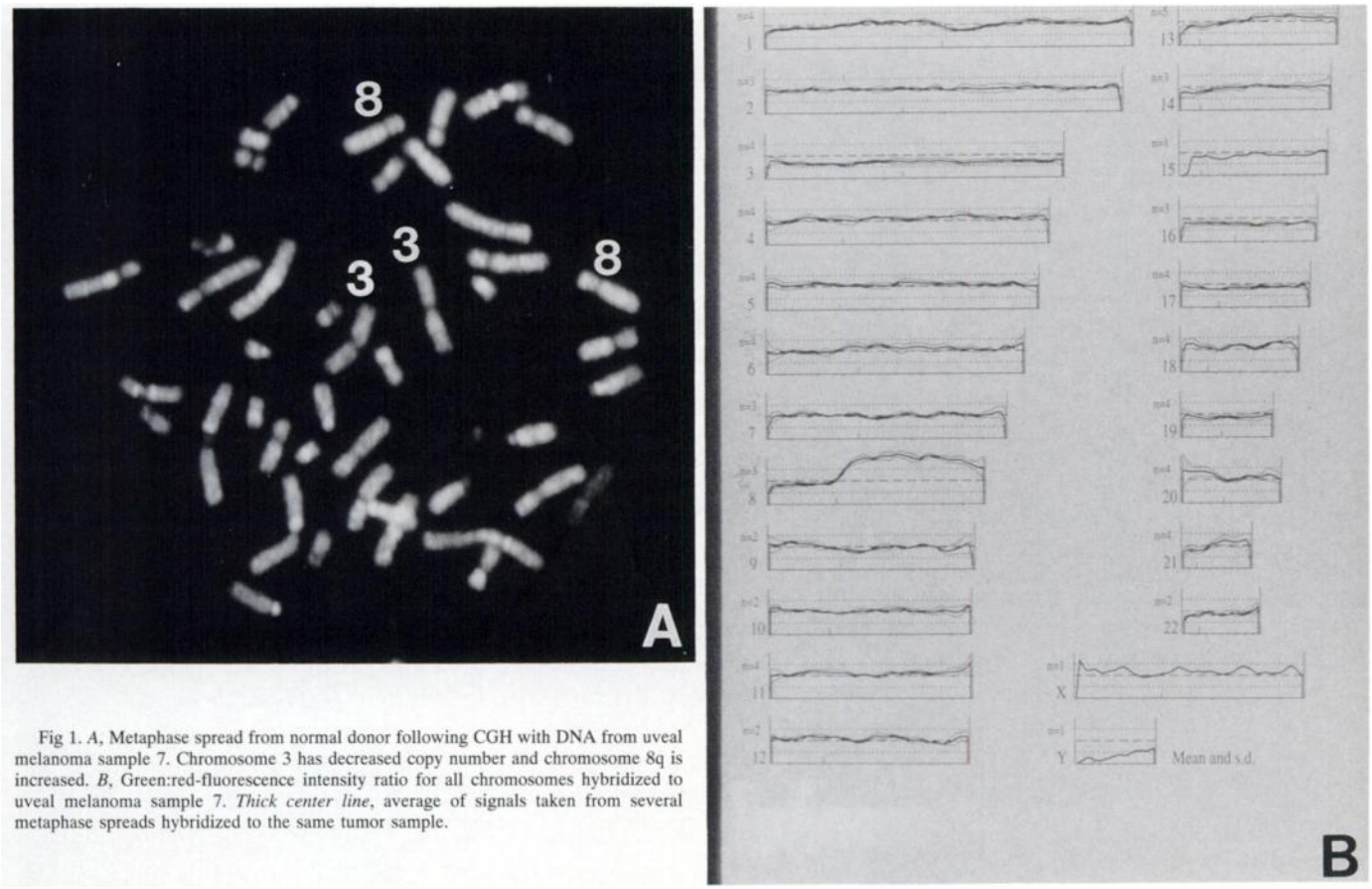

Fig 1. *A*, Metaphase spread from normal donor following CGH with DNA from uveal melanoma sample 7. Chromosome 3 has decreased copy number and chromosome 8q is increased. *B*, Green:red-fluorescence intensity ratio for all chromosomes hybridized to uveal melanoma sample 7. *Thick center line*, average of signals taken from several metaphase spreads hybridized to the same tumor sample.

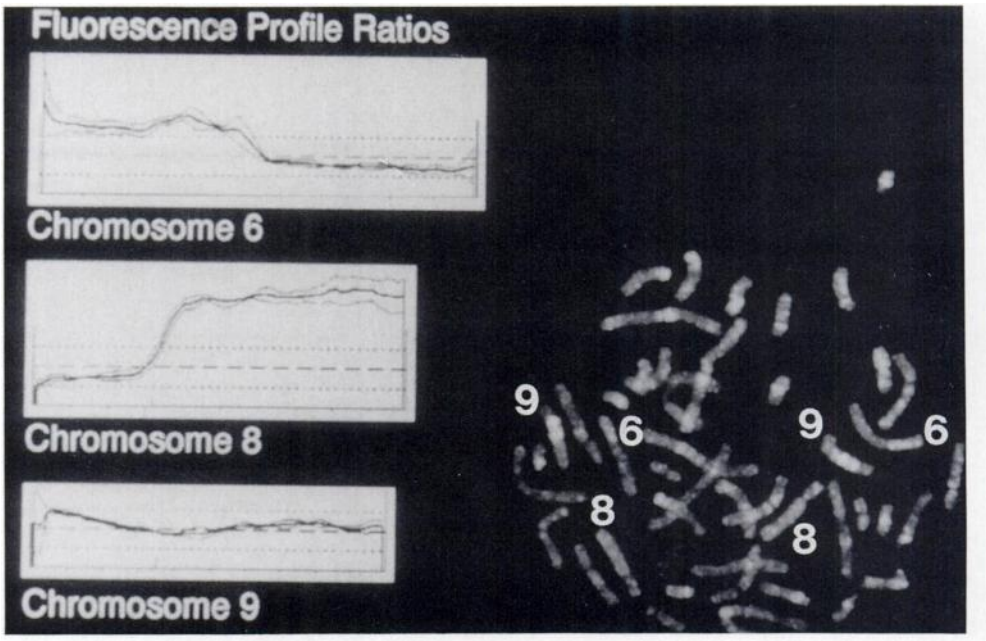

Fig 2. Metaphase spread from normal donor following CGH with DNA from sample 9. Note on both metaphase and *graph* a large increase in hybridization by tumor DNA on both chromosome arms 6p and 8q. The *graph* also demonstrates a focal amplification on 9p.

cases. Several other nonrandom alterations were noted less frequently, including an increased copy number of regions on chromosomes 6p, 7q, 9p, and 13q.

The events important in the malignant transformation of uveal melanocytes and melanoma progression are unclear. Several studies delineate common chromosomal abnormalities associated with cutaneous melanoma. Familial-associated melanoma has been linked to loci on chromosomes 9 and 1 (19, 20). Abnormalities of chromosome 6 have become implicated in tumor progression and metastatic potential (21–23), and a worsened prognosis has been associated with aberrations of chromosomes 7 and 11 (24).

In uveal melanoma, molecular events important in malignant transformation and progression are less clear. Mukai and Dryja reported loss of heterozygosity of chromosome 2 in 2 of 15 patients (25). They postulated the existence of a putative tumor suppressor gene important in uveal melanoma progression on this chromosome. Other investigators were unable to confirm involvement of chromosome 2 with standard cytogenetics (14, 26). Our findings demonstrated abnormalities on this chromosome in 70% of cases. The most common cytogenetic abnormalities reported from previous karyotyping studies of uveal melanoma are trisomy of 8q and monosomy of chromosome 3 observed in approximately 55% of cases. Using CGH we detected these aberrations in a much higher frequency. CGH may have increased sensitivity over standard cytogenetics allowing for identification of aberrations not detectable with conventional karyotyping.

Several other nonrandom copy number abnormalities were detected with CGH. These involved chromosome arms 13q, 9p, and two focal regions on 7q (Fig. 4). Inherent tumor genome instability may lend to the accumulation of multiple genetic aberrations. The occurrence of each of these alterations in over 50% of cases suggests that multiple genes may contribute to either neoplastic transformation or tumor progression or both.

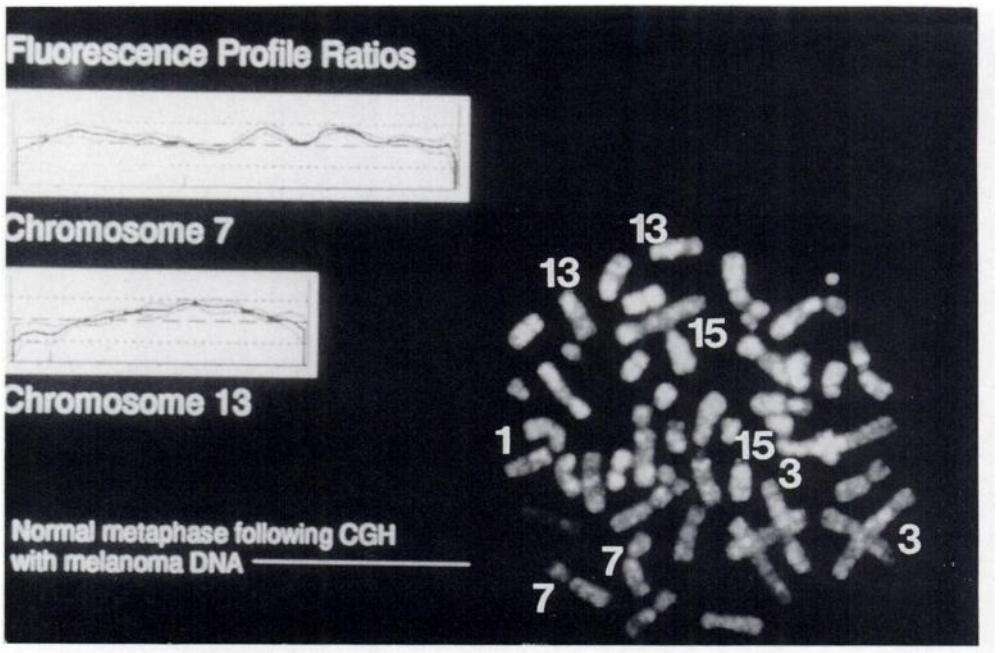

Fig 3. Metaphase spreads from normal donor following CGH with DNA from melanoma sample 2. Note decreased copy number by tumor to both copies of chromosomes 1p, 3, and 15. The fluorescence intensity profiles also demonstrate two focal increases on chromosome arm 7q and an increase in hybridization by tumor DNA to chromosome arm 13q.

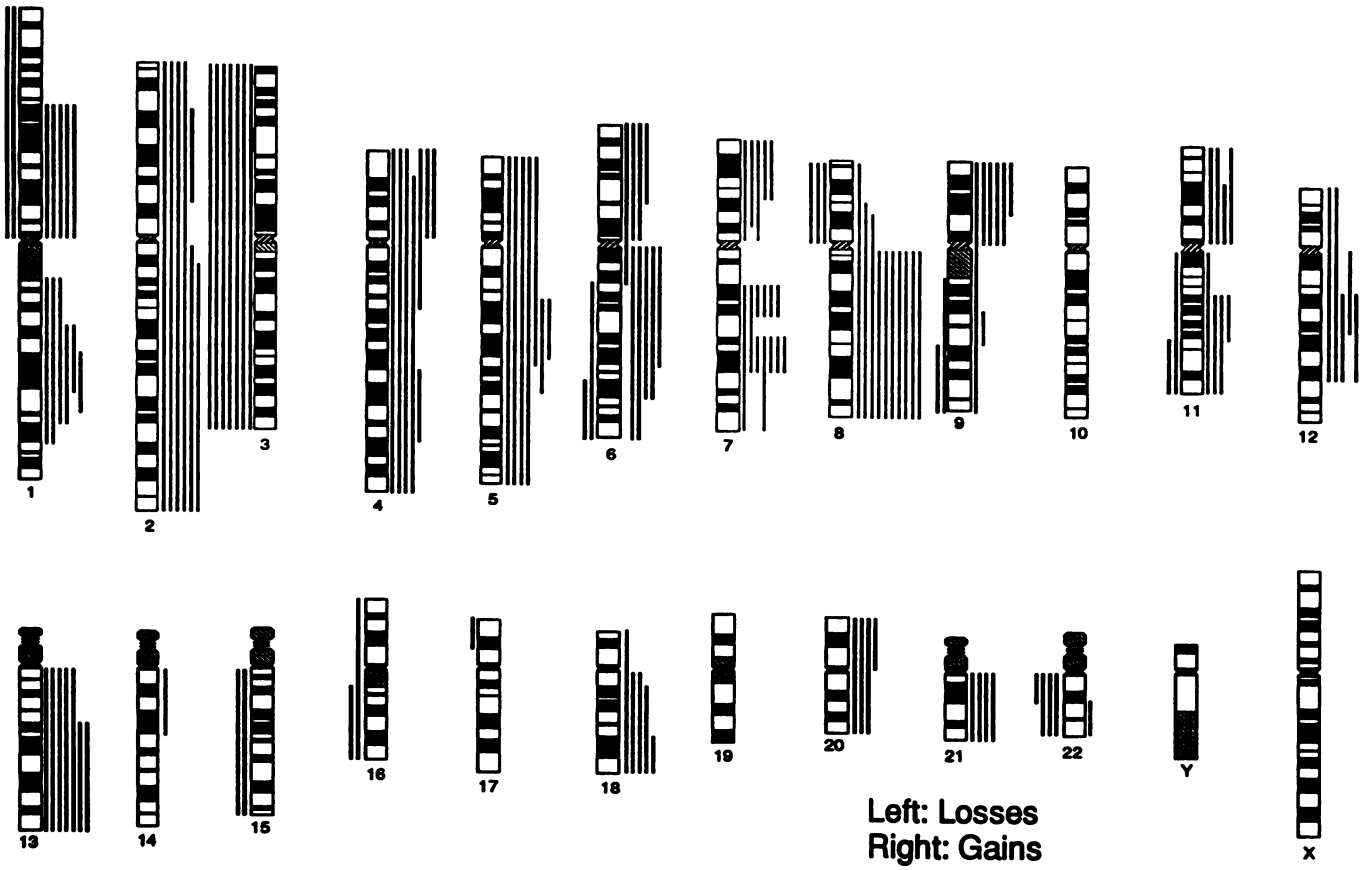

Fig 4. Summary of copy number abnormalities demonstrated by CGH in 10 cases of uveal melanoma. Banded karyotype, chromosomes 1-22, X, and Y. *Lines* to the *left* of the chromosome, copy number decreases; *lines* to the *right* of the chromosome, increases. Their location reflects those regions in which abnormalities were demonstrated.

Table 1 *Copy number abnormalities with relation to tumor cell type. +, increase in copy number of a chromosome or chromosome arm: −, decrease*

	Sample Chromosomal abnormality									
Spindle cell tumor										
#7	3	8q	13q	16	18q	20p				
	−	+	+	−	+	+				
8	2p	6p	7p	8	9	11q	12q	18q		
	+	+	+	+	+	−	+	+		
10	1	2q	3	4	5	6	7q	8	9p	11p
	+	+	−	+	+	+	+	+	+	+
	13q	17p	21q	22q						
	+	−	+	−						
Mixed cell tumor										
2	1p	1q	2	3	4p	4q	5	6q	7	8
	−	+	+	−	+	−	+	+	+	+
	9p	11	12	13q	14q	15	18q	21q	22q	
	+	+	+	+	+	−	+	+	−	
3	1p	4	5	6p	6q	7q	8q	9q	11q	20
	+	+	+	+	−	+	+	−	−	+
4	1	2	4	5q	6q	7	8p	8q	9p	11
	+	+	+	+	+	+	−	+	+	+
	12q	13q								
	+	+								
6	1	2	3	4	5	6q	7	8q	9p	11
	+	+	−	+	+	+	+	+	+	+
	12	13q	16q	18	20	21q				
	+	−	+	+	+					
9	1	2	3	4q	5	6p	6q	7	8p	8q
	+	+	−	+	+	+	−	+	−	+
	9	11q	13q	15	20	21q	22q			
	+	+	+	−	+	+	+			
Epithelioid cell tumor										
1	1p	1q	2q	3	4p	5q	6q	7q	8q	9q
	−	+	+	−	+	+	+	+	+	−
	12q	13q	18q	22						
	+	+	+	−						
5	3	4p	8p	8q	22q					
	−	+	−	+	−					

Approximately 50% of uveal melanoma patients develop metastatic disease. Epithelioid cell tumors and tumors that straddle or are located entirely anterior to the equator of the eye, such as those in the anterior choroid or ciliary body, are associated with a worse prognosis. Prescher *et al.* (11) reviewed the cytogenetics of 13 uveal melanomas and found that significantly more ciliary body tumors were associated with either an increase in copy number of 8q or monosomy of chromosome 3. Horsman and White (14) evaluated 20 uveal melanomas using standard cytogenetics. They observed that the combination of the copy number abnormalities involving chromosome 3 and 8q occurred more frequently in ciliary body tumors and epithelioid tumors, and they postulated that the combination of these chromosomal aberrations defined a subset of tumors with a higher metastatic potential.

In the ten cases analyzed with CGH, the abnormalities of chromosomes 3 and 8 were not associated with tumor location or cell type. An increase in the long arm of chromosome 8 was found in all cases and a decrease in copy number of chromosome 3 in 7 of 10 cases was found. Our findings may differ from previous studies because all 10 tumors studied were relatively large and at least partially located anterior to the equator. The nonrandom abnormalities of chromosomes 3 and 8 may be associated with tumor progression. Alternatively, CGH may afford greater sensitivity in the detection of copy number abnormalities. The use of fresh frozen tissue may eliminate selection artifact contributed by propagation of cells in culture and may circumvent the limitations of karyotypic-banding analysis. Any of these explanations could similarly account for differences in copy number observed in less frequently involved chromosomes in this study compared with other uveal melanoma studies.

Our sample size was too small to correlate prognostic data and histological parameters with specific copy number aberrations. However, there were fewer abnormalities associated with the spindle cell tumors compared to the mixed and epithelioid tumors suggesting, perhaps, a role for genetic instability in the progression of a more malignant phenotype (Table 1).

The molecular abnormalities associated with the chromosome copy number alterations we have observed are uncertain. The protooncogene c-*myc* located on 8q24 has been shown to promote abnormal proliferation in some neoplasms (3). When present in increased copy number it can result in both uncontrolled growth and genetic instability. The partial increase of 13q in two of our specimens needs further explanation since the tumor suppressor gene associated with the formation of retinoblastoma, another intraocular neoplasm, is located near the involved region. CGH cannot specifically delineate either chromosomal rearrangements or translocations, although breakpoints may be identified if there are differences in DNA copy number across the regions. Currently, we are using chromosome-specific probes with FISH to characterize such alterations.

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