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High-density genome array is superior to fluorescence in-situ hybridization analysis of monosomy 3 in choroidal melanoma fine needle aspiration biopsy

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Purpose: Using fluorescence in situ hybridization (FISH) and high-density single nucleotide polymorphism (SNP) mapping genome array, we comparatively evaluated chromosome 3 status and other chromosomal aberrations within a series of choroidal melanomas biopsied by fine needle aspiration (FNAB).

Methods: Transscleral FNAB was performed in 59 patients (59 eyes) who had a clinical diagnosis of choroidal melanoma. Biopsies were processed for chromosome 3 status by centromeric interphase FISH, cytopathology, cell culture, and simultaneous genomic DNA and RNA mapping array analysis.

Results: FISH yielded chromosome 3 status in 38 of 59 (64%) eyes, while high-density SNP mapping array yielded chromosome 3 status in 43 of 59 (73%) eyes. Monosomy 3 was detected by FISH in 15 of 38 (39%) cases, and high-density SNP mapping array data confirmed the finding in 13 of the 15 cases. Furthermore, high-density SNP mapping array revealed five additional cases of significant chromosome 3 aberration not detected by FISH. High-density genomic mapping also provided detailed patterns of chromosomal gain and loss on chromosomes 1, 6, 8, and 9 which segregated into two groups characterized by either monosomy 3 or chromosome 6p gain.

Conclusions: High-density SNP mapping array was better than FISH in detecting chromosome 3 aberrations and monosomy in our melanoma samples. More importantly, the mapping arrays detected additional patterns of chromosomal aberration, which suggest specific pathways for cytogenetic rearrangements in choroidal melanoma and may improve prognostic testing.

Choroidal melanoma is the most common primary intraocular cancer in adults. Despite successful local ocular therapies at least 50% of all patients with choroidal melanoma will die from metastasis within 15-25 years of disease onset [1-4]. Recently, cytogenetic and molecular testing for metastatic risk has become an increasing part of the management of patients with choroidal melanoma. Loss of one copy of chromosome 3 has been reported in approximately 50% of choroidal melanomas. Monosomy 3 of the tumor tissue is the strongest known predictor of metastatic death. Longitudinal reports have indicated that of the patients with monosomy 3, 50-70% identified at the time of ocular treatment will develop metastasis within five years [3,5-8]. Accurate identification of patients with a poor prognosis may allow for the detection of metastasis at an earlier stage and will help select those for whom clinical trials for metastatic therapies may be best suited.

Chromosome 3 status of choroidal melanoma may be determined through a combination of surgical and analytic techniques. Tumor material may be obtained from an enucleated globe, as well as in vivo with fine needle aspiration biopsy (FNAB) at the time of plaque brachytherapy. This latter technique has been shown to be a feasible method to obtain material for prognostic testing [9-13]. Intraoperative FNAB enables patients with smaller tumors who undergo globe-conserving surgery to benefit from prognostic testing. Centromeric probing of chromosome 3 by fluorescent in-situ hybridization (FISH) is the most common method for detecting monosomy 3 and is feasible in material obtained from FNAB. Chromosome 3 status may also be determined by genome-wide studies including conventional karyotype analysis [8,14-17], comparative genomic hybridization (CGH) [4,18-22], microsatellite analysis [4,23-26], and more recently, single nucleotide polyphormism (SNP) high-density genome-wide array mapping techniques [27]. These latter techniques, which provide more comprehensive genome-wide data, have been described in enucleated specimens but little has been reported in specimens obtained in vivo from FNAB of smaller choroidal melanomas.

Using a combination of FISH analysis and genome-wide high-density chromosomal mapping by SNP array, we compared the abilities of these methods to detect monosomy 3 and other chromosomal aberrations in choroidal melanoma specimens obtained via intraoperative transscleral FNAB.

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METHODS

Fine needle aspiration biopsy specimens: Fifty-nine patients (59 eyes) who had a clinical diagnosis of choroidal melanoma were treated at the Jules Stein Eye Institute between April 2006 and May 2007. Two of the 59 patients required enucleation

and 57 patients were treated with iodine-125 plaque brachytherapy. Tumors ranged in height from 2.0-12.7 mm with a mean tumor height of 4.6 mm. Each eye was biopsied by fine needle aspiration either immediately prior to iodine-125 plaque placement or immediately after enucleation.

All studies were approved by the Institutional Review Board of the University of California, Los Angeles (UCLA) and work was in compliance with the Health Insurance Portability and Accountability Act of 1996 (HIPAA). Prior to treatment, evaluation of each patient included comprehensive ophthalmic examination, ultrasonography, photography, optical coherence tomography, and fluorescein angiography. All patients had systemic evaluation, usually by an oncologist at the Jonsson Comprehensive Cancer Center at UCLA, and were offered psychologic support by a clinical psychologist or social worker with particular expertise in choroidal melanoma.

Intraoperative transscleral FNAB and iodine-125 plaque brachytherapy were performed by a single surgeon (TAY). The extent of the choroidal melanoma was confirmed with indirect ophthalmoscopy. Following localization of the melanoma with transillumination, FNAB was performed with a 30-gauge needle via a tangential transscleral approach [13]. The first aspirate of the biopsy was immediately passed off to the ocular pathologist (BJG), who was present in the operating room. This was immediately smeared on glass slides in the operating room, fixed in ethanol, stained with hematoxylin and eosin, then evaluated for cytologic evidence of melanoma [28]. Two pooled aspirates were obtained for FISH processing. Additional aspirates were obtained and pooled for nucleic acid stabilization in RNAprotect cell reagent (Qiagen, Valencia, CA). Residual material in the needles was immediately rinsed into RNAprotect cell reagent. A range of five to nine aspirates was obtained from each patient with a mean of seven aspirates. Indirect binocular ophthalmoscopy was performed following the biopsy. Samples insufficient for an immediate cytologic diagnosis were later tested by immunohistochemistry for HMB-45 [29]. The plaque was sutured in place upon receipt of a preliminary cytologic diagnosis, and optimal plaque position was confirmed with intraoperative ultrasonography.

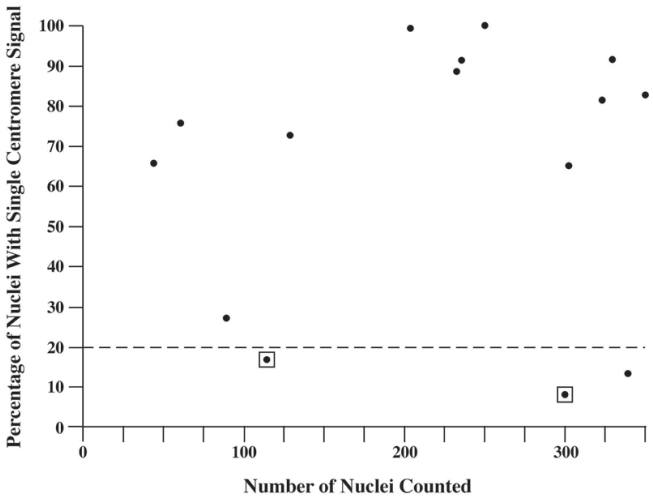

Figure 1. Monosomy 3 ratios by fluorescence in-situ hybridization in fine needle aspiration specimens. The scatter plot shows the distribution of scoring for the 15 samples determined to be monosomy 3 by fluorescence in-situ hybridization (FISH). The vertical axis represents sample heterogeneity with respect to monosomy 3. The 20% level is the threshold below which FISH and single nucleotide polymorphism (SNP) mapping array results diverge. Boxed data points indicate monosomy 3 by FISH which is inconsistent with mapping array.

Interphase fluorescence in-situ hybridization: For FISH analysis, we used a directly labeled centromeric probe to assess the status of chromosome 3. This probe was hybridized to fixed cultured cells following the manufacturer's protocol (Abbott-Vysis, Downers Grove, Illinois). Briefly, cells were fixed in a 3:1 solution of methanol:glacial acetic acid, placed on slides and stored at -20 °C until hybridization. Slides were denatured in 70% formamide at 70 °C for 2 to 4 min, dehydrated in a 70%, 85%, 100% ethanol series, and air-dried. A Spectrum Orange conjugated probe (Abbott-Vysis, Des Plaines, IL) specific for chromosome 3 was used for interphase FISH. Hybridization was performed overnight at 37 °C in a humidified chamber and the nuclei counterstained with 0.2 mM diamino-2-phenylindole dihydrochloride (DAPI) in 90% glycerol/10% PBS, pH 8.0. Hybridization signals were manually counted in non-overlapping nuclei of cells under a fluorescence microscope (Zeiss Axiophot, Zeiss, Jena, Germany) equipped with a triple filter (DAPI/FITC/Texas-Red) [13].

Isolation of DNA for microarray analysis: Pooled aspirates stabilized in RNA protect cell reagent were pelleted and DNA and RNA were simultaneously isolated from the same sample using an AllPrep DNA/RNA Mini Kit (Qiagen) as per manufacturer's instructions. Isolated DNA was quantitated using a NanoDrop ND-1000 (NanoDrop, Wilmington, DE). No DNA samples were subjected to whole genome amplification techniques. RNA was stored for future analyses, and the data were not generated for inclusion in this report.

Single nucleotide polymorphism analysis: DNA copy number was assessed using 500k NSPI Mapping Arrays (Affymetrix, Santa Clara, CA). Probe preparation, hybridization, and reading were performed by the UCLA DNA Microarray Core (Los Angeles, CA), according to the standard 96-well protocol published by Affymetrix. Copy number variation was computed using CNAT v4.0.1 software from Affymetrix.

Statistical analysis: Chromosome aberration frequency analysis was performed using Fisher's exact test. Chromosomal aberration clustering for each biopsy was performed using (1-Pearson correlation matrix) as input of average linkage hierarchical clustering to arrive at a dendrogram (clustering tree).

RESULTS

Comparison of sample recoveries for chromosome 3 fluorescence in-situ hybridization assay and mapping array analysis: Of the 59 patients who underwent FNAB, FISH results were obtained in 38 (64%) of the cases. Parallel, pooled aspirates (range; 2-4) from each patient were processed for simultaneous isolation of DNA and RNA, and the nucleic acid re-

coveries were determined. Where DNA recoveries exceeded 350 ng, samples were determined to be adequate for mapping array analysis. Of the 59 patients who underwent FNAB, 49 (83%) of the cases yielded adequate DNA, ranging 380-3040 ng. Six of these 49 failed to generate adequate probe for microarray due to melanin coprecipitation. Mapping array data were successfully obtained in the remaining 43 cases (73%) of the total cases. Mapping arrays not only provided data in all 38 cases where FISH data were obtained, but also provided data in five patients in whom FISH data were not obtained.

Comparison of findings of chromosome 3 fluorescence in-situ hybridization assay and mapping array analysis: Of the 38 cases where FISH results were obtained, monosomy 3 was reported in 15 (39%) of these cases, as shown in Figure 1. Normal signal pattern (disomy 3) was reported in the remainder. Of the 43 cases where SNP mapping array results were obtained, 18 (42%) of the cases demonstrated either monosomy 3 or significant aberrations in chromosome 3. In 13 cases, FISH and high-density mapping results were in agreement.

In one case where FISH yielded insufficient material (MEL20-06-022), SNP mapping array resulted in a finding of monosomy 3. There were six discrepancies between data reported by FISH and by SNP mapping array: Two were cases of monosomy 3 by FISH, which had disomy 3 by SNP, and four were cases of disomy 3 by FISH, which had chromosome aberration or monosomy 3 by SNP.

Two cases of monosomy 3 by fluorescent in situ hybridization with disomy 3 by single nucleotide polymorphism: MEL20-06-004 was found to have monosomy 3 by FISH with a count of 39 nuclei out of 300 nuclei reporting a single centromeric signal. MEL20-07-068 was reported as monosomy 3 with 18 of 113 nuclei reporting a single centromeric signal. Mapping arrays reported disomy 3 in both cases (Figure 1).

Four cases of disomy 3 by fluorescent in situ hybridization which had chromosome 3 aberration or monosomy 3 by single nucleotide polymorphism: Two samples, MEL20-06-013 and MEL20-06-038, were reported by FISH to be normal

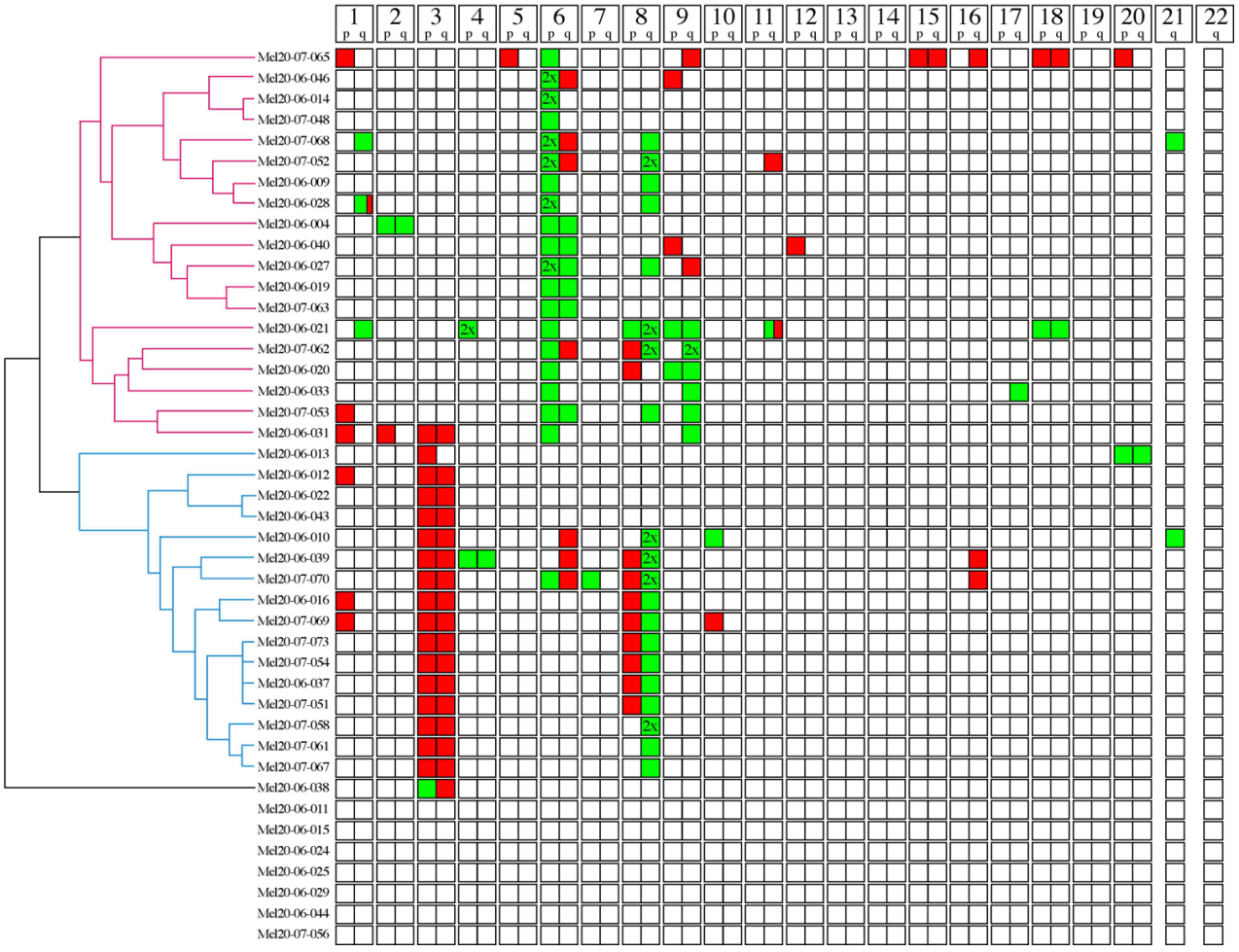

Figure 2. Chromosomal aberration determined by high-density genome single nucleotide polymorphism mapping array in fine needle aspiration specimens. Summary of accumulated chromosomal gain and loss data from GeneChip 500k *Nsp*I mapping arrays for 43 biopsies is demonstrated. Array data were processed using CNAT v4.0.1. Sample organization into a dendrogram used (1-Pearson correlation matrix) as input of average linkage hierarchical clustering. Red boxes denote whole or partial loss; green boxes denote a whole or partial gain; green boxes labeled 2X denote a two-copy gain.

signal pattern yet were found by SNP mapping array to have significant aberrations in chromosome 3. MEL20-06-013 was shown by SNP mapping array to have lost the majority of the p-arm of one copy of chromosome 3. This result was confirmed by reprobing the FISH slide with a cocktail of CEP-3 Spectrum Orange and a BAC probe (RP11584A6) spanning the microphthalmia transcription factor (MITF) locus at 3p12. The majority of cell nuclei showed two centromeric signals and a single MITF signal (data not shown). MEL20-06-038 was shown by mapping array to have lost a 3q and gained a 3p, which may be explained by isochromosome formation of 3p. The configuration of the aberrations in chromosome 3 detected in each of these two samples is consistent with the centromere data provided by FISH. Therefore, FISH was technically not in conflict, but it was inadequate to detect significant chromosome 3 aberrations in these cases. Finally, two cases reported as disomy 3 by FISH, (MEL20-07-058 and MEL20-07-61), were found to have monosomy 3 by mapping array.

Other chromosomal aberrations detected by single nucleotide polymorphism mapping array analysis: Beyond chromosome 3 analysis, we found mapping arrays were able to detect additional genomic aberrations in choroidal melanoma that occurred with a high frequency and were consistent with aberrations reported by other cytogenetic means. Figure 2 summarizes the chromosomal aberration found in each biopsy for which SNP mapping array data were reported. The biopsies were sorted using (1-Pearson correlation) clustering. Seven of the 43 biopsies had no detectable chromosomal aberrations and were excluded from the cluster analysis. The cluster analysis revealed two distinct sets of chromosomal aberrations based on monosomy 3 or chromosome 6p gain. MEL20-06-038 was found to be the sole outlier, having what appeared to be isochromosome 3p formation.

The frequency and extent of chromosomal gain and loss are shown in Figure 3 and reveal instabilities in chromosomes 1, 3, 6, 8, and 9. Of particular note were recurring double gains in both the telomeric region of 6p and the entire arm of 8q.

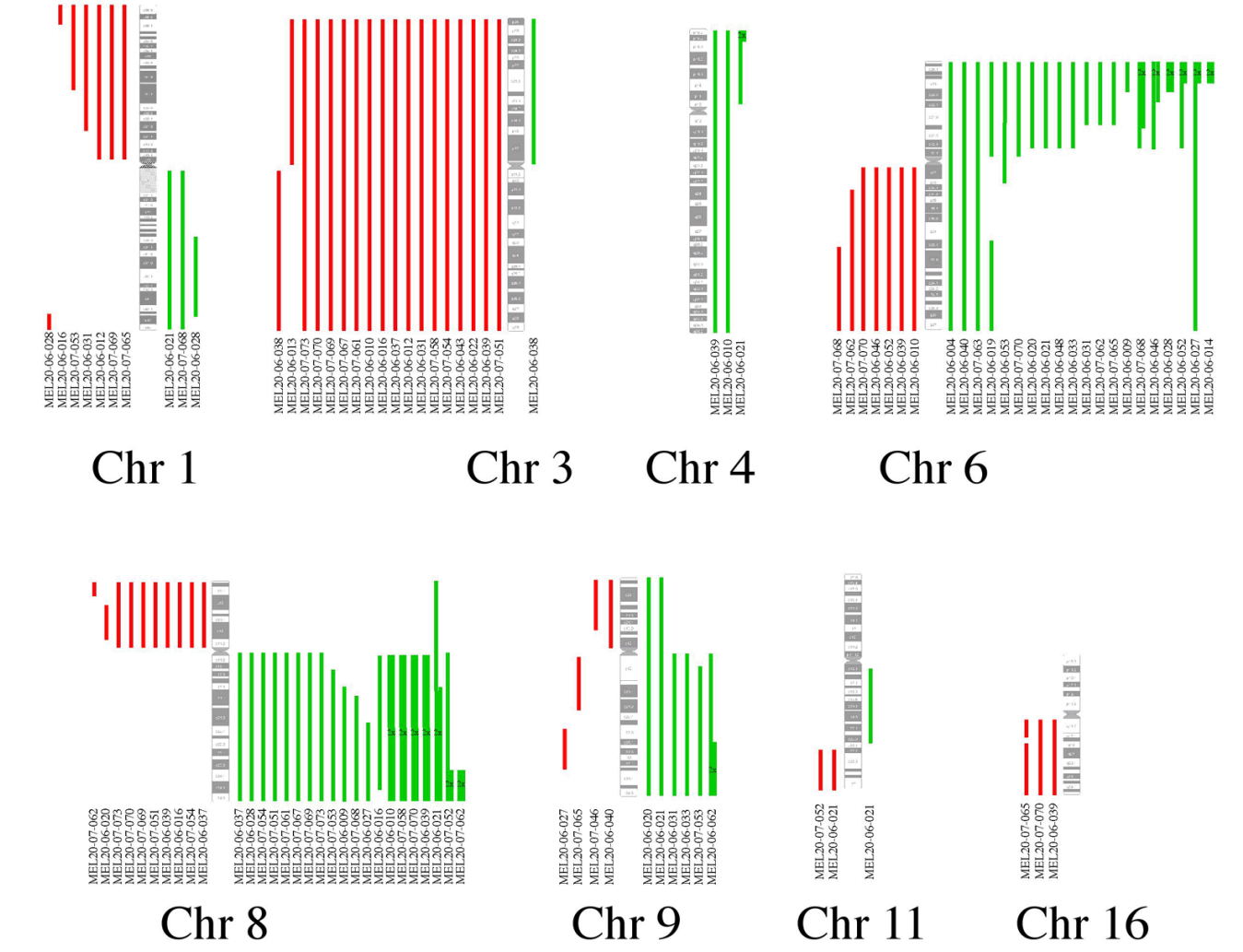

Figure 3. Frequency and extent of chromosomal gains and losses by high-density genome single nucleotide polymorphism mapping array in fine needle aspiration specimens. Graphical alignment of chromosomal gain and loss data from GeneChip 500k *Nsp*I mapping arrays is shown. Losses are to the left of each ideogram and are denoted in red. Gains are to the right and are denoted in green. Wide green bars labeled 2X denote regions of two-copy gain. Only those chromosomes that demonstrated instability in three or more tumors are depicted.

Chromosome 1p loss occurred in six cases, but always in association with either monosomy 3 or 6p gain. Chromosome 8q gain or double-gain occurred in 20 (47%) of the cases, but always in association with either monosomy 3 or 6p single- or double-gain. The combination of 8p loss and concomitant 8q gain or double gain occurred in nine (21%) of the cases and was strongly associated with monosomy 3 (Fisher's exact test, p=0.008).

DISCUSSION

High-density SNP mapping array provided a more robust and sensitive determination of monosomy 3 than FISH in prognostic testing of choroidal melanoma tissue obtained by FNAB. High-density mapping revealed five additional patients whose tumors contained a high degree of chromosome 3 aberration or monosomy 3 not detected by FISH. In addition, high-density SNP mapping array provided a wealth of detailed genome-wide information with respect to chromosomal gains and losses. These findings revealed a dichotomy of patients who had monosomy 3 and those with gains in 6p; these two groups were mutually exclusive. Finally, each of the eight cases that showed a loss of the entire 8p arm also had a gain of the entire 8q arm, suggesting that 8q isochromosome formation may be an important and common instability in choroidal melanoma.

We have demonstrated that the use of intraoperative transscleral FNAB to establish a molecular karyotype by means of high-density chromosome mapping with SNP is feasible in patients with choroidal melanoma. With this method, our yield for information on chromosome 3 status was 73%. This is comparable to Shields et al., who reported a yield of 75% using microsatellite analysis to determine chromosome 3 status in transscleral FNABs [11]. Furthermore, our data are consistent with the findings reported by Parrella et al in which microsatellite analysis of material obtained from enucleated choroidal melanoma resulted in similar results with patients segregating into two mutually exclusive groups: those with monsomy 3 and those with gains in chromosome 6p; both groups demonstrated aberrations in chromosome 8 [26]. Additionally, gains in the long arm of chromosome 9 have also been reported by Magauran et al [30]. We have shown that our high-resolution data indicate consistent large-scale gains and losses often encompassing entire chromosome arms.

Heterogeneity is a known characteristic of all solid tumors including choroidal melanoma. Sampling a tumor with any biopsy technique may reveal information that does not reflect the entire tumor composition or may miss a small clone of highly malignant cells. Although Sandinha et al [31] and Maat et al [32], using different specimen preparations and techniques, reported intra-tumor differences with respect to monosomy 3, Meir et al [33] did not report any heterogeneity in the two areas per tumor sampled in a study evaluating correlative histologic factors with monosomy 3. Our method of FNAB involved multiple aspirates from variable sites within the tumor: pooled aspirates for FISH, as well as two or more pooled aspirates for DNA and RNA analyses. Perhaps this may have reduced inconsistent data, which could arise from heterogeneity. Yet, we did have two samples for which the FISH aspirates may have contained either a different clonal region of the tumor or a non-tumor tissue. We observed a wide range of cell number and sample heterogeneity by FISH with respect to monosomy 3. Relatively homogenous samples were found to have good agreement with SNP mapping array data. However, below the 20% threshold the results became divergent. We believe that this is a limitation of FISH, rather than high-density SNP mapping array. In contrast to previous reports of tumor heterogeneity which refer to FISH or microsatellite analyses, we found that the incorporation of high-resolution genome-wide SNP array resulted in tumors of two distinct lineages-the monosomy 3 genotypic pattern or the chromosome 6p gain pattern.

As we continue to follow the metastatic outcome of our patients, the mortality associated with specific chromosomal aberrations will be elucidated. At a genome-wide level, our data suggest a finite level of genetic complexity within choroidal melanomas. The ability to narrowly define specific chromosomal aberration sets between tumor groups with SNP mapping may allow us to more accurately prognosticate on the morbidity and mortality of our patients than with conventional FISH testing for monosomy 3 alone.

In summary, we report that high-density SNP mapping arrays of choroidal melanoma FNAB material are feasible and provide more complete information regarding genome-wide variations than FISH testing for monosomy 3 alone. In addition, patient chromosomal aberrations in this cohort were aligned into two discrete groups-monosomy 3 and chromosome 6p gains-which were mutually exclusive. Continued investigation of the impact of these findings on genomic expression and ultimately choroidal melanoma phenotype is needed to better understand the molecular biology of this form of cancer.

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