Chromosome 3 Status in Uveal Melanoma: A Comparison of Fluorescence In Situ Hybridization and Single-Nucleotide Polymorphism Array

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Purpose. To compare fluorescence in situ hybridization (FISH) using a centromeric probe for chromosome 3 (CEP3) and 3p26 locus-specific probe with single-nucleotide polymorphism array (SNP-A) analysis in the detection of high-risk uveal melanoma.

Methods. Fifty cases of uveal melanoma (28 males, 22 females) treated by enucleation between 2004 and 2010 were analyzed. Fresh tissue was used for FISH and SNP-A analysis. FISH was performed using a CEP3 and a 3p26 locus-specific probe. Tumor size, location, and clinical outcome were recorded during the 7-year study period (median follow-up: 35.5 months; mean: 38.5 months). The sensitivity, specificity, positive predictive value, and negative predictive value were calculated.

Results. Monosomy 3 was detected by FISH-CEP3 in 27 tumors (54%), FISH-3p26 deletion was found in 30 (60%), and SNP-A analysis identified 31 (62%) of the tumors with monosomy 3. Due to technical failures, FISH and SNP-A were noninterpretable in one case (2%) and two cases (4%), respectively. In both cases of SNP-A failure, tumors were positive for FISH 3p26 deletion and in a single case of FISH failure, monosomy 3 was found using SNP-A. No statistically significant differences were observed in any of the sensitivity or specificity measures.

Conclusions. For prediction of survival at 36 months, FISH CEP3, FISH 3p26, and SNP-A were comparable. A combination of prognostication techniques should be used in an unlikely event of technical failure (2%–4%). (*Invest Ophthalmol Vis Sci.* 2012;53:3331–3339) DOI:10.1167/iovs.11-9027

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Supported in part by National Cancer Institute/National Institutes of Health Grant R01CA136776, a Falk Medical Research Trust grant, an unrestricted grant from Research to Prevent Blindness, Department of Ophthalmology, Cleveland Clinic Learner College of Medicine, the Sondra J. and R. Hardis Endowed Chair in Cancer Genomic Medicine (Cleveland Clinic), and an F. M. Kirby Foundation grant (CE).

Submitted for publication November 5, 2011; revised April 1 and April 9, 2012; accepted April 9, 2012.

Disclosure: **A.D. Singh**, None; **M.E. Aronow**, None; **Y. Sun**, None; **G. Bebek**, None; **Y. Saunthararajah**, None; **L.R. Schoenfield**, None; **C.V. Biscotti**, None; **R.R. Tubbs**, None; **P.L. Triozzi**, None; **C. Eng**, None

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In the early 1990s, nonrandom genetic abnormalities involving chromosomes 1, 3, 6, and 8 were identified in uveal melanoma tumor samples.[1,2] These aberrations were later shown to correlate with poor prognosis.[3–5] Of various cytogenetic abnormalities observed, monosomy 3 is the strongest predictor of metastatic risk.[5–14] Several techniques are currently being used to detect monosomy 3 and other chromosomal changes associated with the development of metastatic disease. Gene-expression profiling is also being used in prognostication.[15,16] Fluorescence in situ hybridization (FISH) is a rapid and economical assay commonly used in the molecular prognostication of cancer that uses fluorochromes linked to DNA probes, enabling determination of chromosome copy number and location of specific DNA sequences. It is primarily a visual technique that requires the use of a fluorescence microscope and readily allows for coincident cytologic confirmation of malignancy.

In general, three basic types of DNA probes are used: centromeric (chromosome enumeration probes [CEPs]), whole chromosome probes (whole chromosome paints), and locus-specific probes.[17] Single-nucleotide polymorphism array (SNP-A) analysis is an automated DNA microarray. Whereas SNP-A analysis requires specialized instrumentation, it does offer several advantages to FISH. SNP-A analysis detects loss of heterozygosity of large numbers of moderately polymorphic DNA segments, providing more comprehensive characterization of genomic data. SNP-A analysis is also useful in identifying uniparental disomy and deletions that may be functionally equivalent to monosomy 3.[18]

Rapid development and adoption of prognostication assays has led to wide variation in practice patterns. To date, there have been few direct comparisons between available techniques. In the majority of uveal melanoma cytogenetic studies using FISH, CEP3 probes have been used.[1,7–11,18–41] The prognostic accuracy of SNP-A has been reported to be superior to that of FISH with a CEP3 probe.[18,38] Deletion-mapping studies have identified 3p24–26 as a commonly affected region in patients with metastatic uveal melanoma. These loci can be detected by FISH using 3p24 and 3p26 probes.[41,42] The manner in which locus-specific FISH analysis of chromosome 3

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in patients with uveal melanoma compares with CEP3 and with SNP-A status has not been reported.

The overall purpose of prognostication is to enter high-risk patients into an adjuvant treatment trial aimed at reducing tumor-specific mortality.[43] The purpose of this study was to compare techniques of FISH using CEP3, FISH using 3p26 locus-specific probe, and SNP-A in assessing chromosome 3 status within the tumor. Additionally, we wanted to compare predictive values for survival using these techniques.

Methods

Patients

Fifty consecutive patients with uveal melanoma treated by primary enucleation at the Cleveland Clinic Cole Eye Institute were enrolled between 2004 and 2010. The study was approved by the Institutional Review Board, and this research adhered to the tenets of the Declaration of Helsinki. Patients were followed over the study period ending in October 2011 (median follow-up: 35.5 months; mean: 38.5 months). At the time of diagnosis, each patient underwent comprehensive ophthalmic examination with supporting diagnostic studies including fundus photography, ultrasonography, and in some cases optical coherence tomography or indocyanine green angiography. Computed tomography (CT) scans of the chest, abdomen, and pelvis were initially performed to rule out metastatic disease. Following enucleation, all patients underwent scheduled surveillance for the development of metastases every 6 months, with clinical evaluation, hepatic ultrasound, and liver function testing. The cause of death was established (metastatic or nonmetastatic) by evaluation of medical records, imaging studies, and biopsy results. Where necessary, the patient's family or primary care practitioners were contacted as part of ongoing data collection efforts.

Tumor Sampling

Immediately following enucleation, transillumination was used to mark the tumor margins. Dissection was carried out through a scleral flap overlying the tumor base. In all cases, impression smears were made from fresh (or previously frozen) tumor tissue for FISH analysis. Fresh tumor tissue was also further processed for SNP-A analysis.

FISH

Chromosome 3 status was assessed by FISH using both directly labeled enumeration probes (X SpectrumGreen/Y SpectrumOrange Direct Labeled Fluorescent DNA Probe Kit; Abbott Molecular Diagnostics, Des Plaines, IL) for the alphacentromeric locus of chromosome 3 (CEP3) and a locus-specific probe. The locus-specific probe, 3p26 (TelVysion 3p; Abbott Molecular Diagnostics) used for this study is commercially available (Vysis FISH Chromosome Search Tool, Abbott Molecular Diagnostics), having specificity for locus D3S4559 spanning a large number of genes.[44]

We excluded triploidy/tetraploidy/polyploidy through the use of CEP8 performed using a previously described interphase FISH method for touch preparations.[45] Probes were hybridized to fixed fresh cells (Carnoy's solution for cytogenetic investigation) affixed to touch preparations from frozen tissue. A total of 200 interphase cells were scored using a FISH workstation (Carl Zeiss Workstation) to determine the percentage of signals for each locus. Based on frequency distribution of the percentage of cells demonstrating monosomy in each tumor by FISH-CEP3 (Fig. 1) and the fact that many prior studies in the literature have used such a cutoff value,[18,30,34,35,37,40] we chose to use a cutoff value of 20% to define monosomy for chromosome 3. Nevertheless, we explored alternative cutoff points using the sum of sensitivity and specificity values. Bootstrap methods were used to evaluate the robustness of the best cutoff point. Statistical analyses were performed using R software (version 2.8; R Foundation for Statistical Computing, Vienna, Austria; available at http://www.r-project.org/). A 0.05 significance level was assumed for all tests.

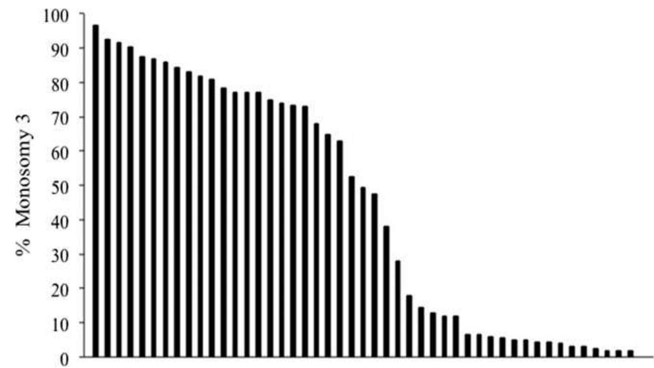

Figure 1. Frequency distribution of the percentage of cells demonstrating monosomy 3 in each tumor by FISH-CEP3. Each vertical bar represents a single case. Data are shown from 49 cases because FISH was not informative in a single case.

SNP-A

SNP-A analysis was performed on fresh-frozen tumor tissue that was permeabilized in RNA protective reagent (RNAlater-ICE; Ambion, Austin, TX) in accordance with the manufacturer's protocol. Permeabilized tissue was minced, equilibrated in cold phosphate-buffered saline (PBS), then digested with proteinase-K prior to DNA extraction (DNeasy Kit; Qiagen, Germantown, MD). Using approximately 200 ng of DNA per sample, a labeling and detecting whole-genome genotyping sample kit (Illumina Human 660W-Quad v1; Illumina, San Diego, CA) was used to analyze >660,000 SNP and copy number variation loci to provide high-resolution analysis of chromosome structure. Probe preparation and hybridization were performed by the Cleveland Clinic Genomics Core. Chromosomal aberrations were identified using commercial data analysis software (GenomeStudio with KaryoStudio module; Illumina). Contiguous regions (gain or loss of heterozygosity) of a minimum group size (default 20 adjacent SNPs of the same polarity) and a minimum sequence length of 200 kb were designated as abnormality and quantified by 20-SNP blocks (this is a size of abnormality that can be validated using FISH analysis). A tumor was considered to manifest monosomy 3 if there was contiguous deletion of an entire copy of chromosome 3.

Statistical Analysis

Kaplan–Meier curves were used to assess the degree of correlation between assay results (monosomy 3 determined separately by FISH-CEP3 and SNP-A, as well as 3p26 deletion detected by FISH) and patient survival. Within each method, the statistical significance of differences in disease-free survival was determined using the log-rank test. In these analyses, patients who died without metastases were censored at the time of death, whereas those alive and dead with metastases were considered to have an event at the time of the diagnosis of metastases. Patients who remained alive without metastases were censored at the date of last follow-up.

For both FISH-CEP3 and FISH-3p26, the sensitivity, specificity, positive predictive value, and negative predictive value were calculated using SNP as the gold standard. Then FISH CEP3, FISH 3p26, and SNP were evaluated as predictors of survival at 36 months. McNemar's test was performed to compare sensitivities and specificities.

Results

Patient Characteristics

Tumors from the primary enucleation specimens of 50 white patients, of northern and western European origin, with uveal

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Table 1. Patient and Tumor Characteristics

Patient ID	Sex	Age (y)	Tumor		COMS	AJCC	Location
			LBD (mm)	HGT (mm)			
1	F	86	18.0	11.0	Large	3b	Ciliochoroidal
2	M	44	20.0	10.1	Large	4a	Choroidal
3	F	74	10.0	2.7	Medium	1a	Choroidal
4	F	75	18.0	9.8	Large	3b	Ciliochoroidal
5	F	83	16.5	12.5	Large	3b	Iridociliochoroidal
6	M	49	22.0	11.0	Large	4b	Ciliochoroidal
7	M	79	15.0	7.2	Medium	2b	Ciliochoroidal
8	F	79	17.7	8.1	Large	2a	Choroidal
9	F	76	18.0	5.6	Large	2b	Iridociliochoroidal
10	F	69	15.6	12.2	Large	3b	Ciliochoroidal
11	M	58	14.0	12.0	Large	3a	Choroidal
12	F	70	17.7	6.7	Large	3a	Choroidal
13	F	79	18.5	14.6	Large	4b	Iridociliochoroidal
14	M	90	21.5	7.6	Large	4b	Ciliochoroidal
15	M	90	12.3	11.8	Large	3b	Ciliochoroidal
16	M	59	20.0	10.0	Large	4b	Ciliochoroidal
17	M	75	17.1	8.4	Large	2b	Iridociliochoroidal
18	M	51	18.0	7.3	Large	2a	Choroidal
19	M	60	19.0	11.2	Large	4b	Ciliochoroidal
20	M	62	19.0	8.4	Large	4b	Ciliochoroidal
21	M	53	18.5	10.1	Large	4a	Choroidal
22	F	80	15.0	9.3	Medium	3a	Choroidal
23	F	43	18.5	10.5	Large	4b	Iridociliochoroidal
24	M	66	15.0	13.1	Large	3b	Iridociliochoroidal
25	F	24	11.7	11.1	Large	3a	Choroidal
26	F	78	19.4	3.9	Large	4b	Ciliochoroidal
27	F	91	16.3	11.0	Large	3a	Choroidal
28	F	64	16.6	11.1	Large	3a	Choroidal
29	M	51	11.0	4.0	Medium	1a	Choroidal
30	F	67	15.5	12.2	Large	3b	Ciliochoroidal
31	F	50	19.6	7.0	Large	4a	Choroidal
32	M	26	17.7	13.0	Large	3b	Iridociliochoroidal
33	M	75	18.0	14.8	Large	3b	Ciliochoroidal
34	M	52	19.0	6.5	Large	4b	Ciliochoroidal
35	F	68	15.0	12.2	Large	3a	Choroidal
36	F	76	17.6	10.4	Large	3a	Choroidal
37	F	67	15.0	7.6	Medium	2a	Choroidal
38	M	57	15.0	12.3	Large	3a	Choroidal
39	M	54	14.3	12.0	Large	3a	Choroidal
40	M	56	18.0	7.8	Large	2a	Choroidal
41	F	50	16.0	8.0	Medium	2a	Choroidal
42	M	58	20.0	11.9	Large	4b	Ciliochoroidal
43	F	82	17.5	12.2	Large	3a	Choroidal
44	M	73	17.3	9.0	Large	2a	Choroidal
45	M	61	12.7	11.6	Large	3b	Ciliochoroidal
46	M	50	15.4	10.6	Large	3b	Ciliochoroidal
47	M	57	24.0	4.2	Large	4b	Iridociliochoroidal
48	M	53	19.0	8.0	Large	4a	Choroidal
49	M	43	20.0	8.2	Large	4a	Choroidal
50	M	77	22.5	9.7	Large	4b	Ciliochoroidal

ID, study identification number; F, female; M, male; LBD, largest basal diameter; HGT, height; COMS, Collaborative Ocular Melanoma Study; AJCC, American Joint Commission Classification, 7th edition.

melanoma were analyzed. This series included 28 males (56%) and 22 females (44%). The median age at diagnosis was 65 years (range: 24–91 years). Using the Collaborative Ocular Melanoma Study (COMS) size criteria, tumors were classified as large in 44 cases (88%), medium in 6 cases (12%), and small in 0 cases.[46] The median largest basal diameter was 17.7 mm (range: 11.0–24.0 mm). The median tumor height was 10.1 mm (range: 2.7–14.8 mm). Tumor location was choroidal in 24 (48%), ciliochoroidal in 18 (36%), and iridociliochoroidal in 8 (16%) patients. Patient and tumor characteristics are summarized in Table 1.

Cutoff Point

The highest combined sensitivity and specificity value was observed with cutoff point at 8%, although several other cutoff

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TABLE 2. The Sensitivity, Specificity, and Sum of the Two Measures for Various Levels of FISH-CEP3–Positive Cells

Positive Cells	Sensitivity	Specificity	Sum
2	94.6%	0.0%	0.946
2.5	89.2%	8.3%	0.975
3	89.2%	16.7%	1.059
4	89.2%	33.3%	1.225
4.5	86.5%	33.3%	1.198
5	81.1%	41.7%	1.227
5.5	75.7%	50.0%	1.257
6	75.7%	58.3%	1.340
6.5	75.7%	66.7%	1.423
8	**75.7%**	**83.3%**	**1.590**
9	73.0%	83.3%	1.563
12	70.3%	83.3%	1.536
13	67.6%	83.3%	1.509
14.5	64.9%	83.3%	1.482
43.5	64.9%	91.7%	1.565
47.5	62.2%	91.7%	1.538
49.5	59.5%	91.7%	1.511
52.5	56.8%	91.7%	1.484
63	54.1%	91.7%	1.457
68	51.4%	91.7%	1.430
73	48.6%	91.7%	1.403
73.5	45.9%	91.7%	1.376
74	43.2%	91.7%	1.349
75	40.5%	91.7%	1.322
77	37.8%	91.7%	1.295
78.5	29.7%	91.7%	1.214
81	27.0%	91.7%	1.187
82	24.3%	91.7%	1.160
83	21.6%	91.7%	1.133
84.5	18.9%	91.7%	1.106
86	16.2%	91.7%	1.079
87	16.2%	100.0%	1.162
87.5	13.5%	100.0%	1.135
90.5	10.8%	100.0%	1.108
91.5	8.1%	100.0%	1.081
92.5	5.4%	100.0%	1.054
96.5	2.7%	100.0%	1.027

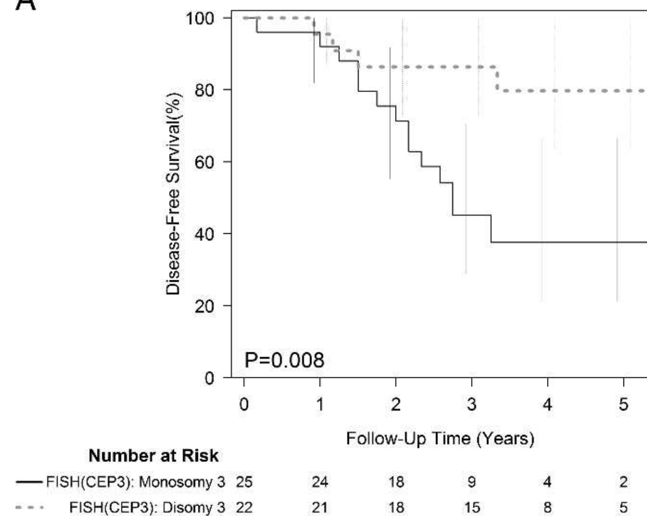
Data of 37 cases that were positive by SNP-A are included in the analysis.

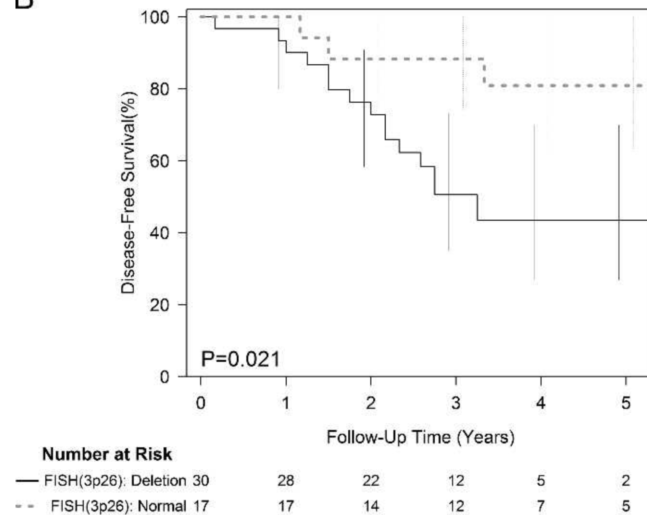
points (as high as 49%) provided combined values that were within 0.1 of those observed with a cutoff point of 8% (Table 2). When compared against the traditionally used cutoff point (20%), neither the sensitivity ($P = 0.13$) nor specificity ($P = 0.99$) significantly differed between the two cutoff points.

Bootstrap methods were also used to evaluate the robustness of the best cutoff point. Two sensitivity analyses were performed. First, the choice of best cutoff point was evaluated. Across 2000 samples, 8% was the best cutoff point in 45% of the bootstrap samples, whereas 9%, 43.5%, and 47% were the best in at least 10% of the bootstrap samples. Again, although 8% provides the best combination of sensitivity and specificity in a given sample, it did not perform significantly better than the currently used cutoff point (20%).

Chromosome 3 Status

FISH-CEP3 identified 27 tumors (54%) with monosomy 3. FISH-3p26 revealed a deletion in 30 cases (60%), including every case detected by FISH-CEP3. SNP-A analysis detected 31 cases (62%) with monosomy 3. Triploidy/tetraploidy/polyploidy was not observed. In two cases (tumors 24 and 25), SNP-A





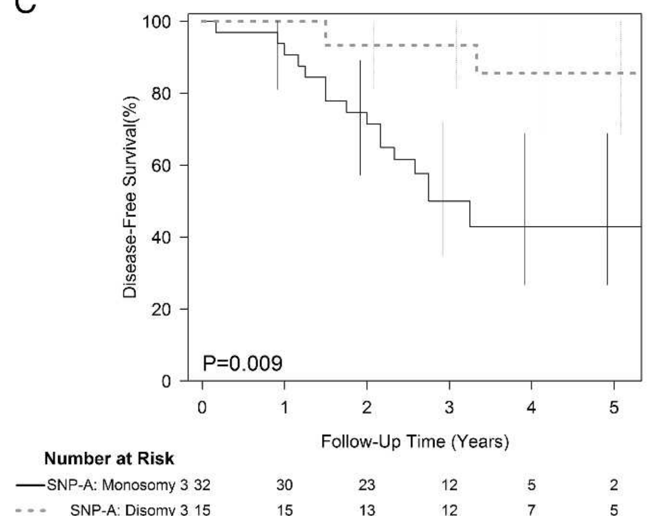


FIGURE 2. Disease-free survival and chromosome 3 status defined by the FISH-CEP3 (**A**), FISH-3p26 (**B**), and by SNP-A (**C**).

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Table 3. Chromosome 3 Copy Number by FISH (using CEP3 and 3p26 probes) and by SNP-A (overall chromosome 3 status, centromeric, and 3p26 regions)

	FISH				SNP-A			Clinical Data		
ID	Copy #	CEP3	%M	3p26	Copy #	Centromere	3p26	F/U	Status	Mets
1	Monosomy 3	Del	73.0	Del	Monosomy 3	Del	Del	43	Dead	No
2	Monosomy 3	Del	75.0	Del	Monosomy 3	Del	Del	59	Alive	No
3	Monosomy 3	Del	49.5	Del	Monosomy 3	Del	Del	49	Alive	No
4	Monosomy 3	Del	73.5	Del	Monosomy 3	Del	Del	67	Alive	No
5	Monosomy 3	Del	84.5	Del	Monosomy 3	Del	Del	83	Dead	No
6	Monosomy 3	Del	47.5	Del	Monosomy 3	Del	Del	37	Dead	No
7	Monosomy 3	Del	92.5	Del	Monosomy 3	Del	Del	36	Alive	No
8	Monosomy 3	Del	87.0	Del	Monosomy 3	Del	Del	28	Dead	Yes
9	Monosomy 3	Del	77.0	Del	Monosomy 3	Del	Del	35	Alive	No
10	Monosomy 3	Del	63.0	Del	Monosomy 3	Del	Del	21	Dead	Yes
11	Monosomy 3	Del	65.0	Del	Monosomy 3	Del	Del	28	Alive	No
12	Monosomy 3	Del	81.0	Del	Monosomy 3	Del	Del	26	Dead	Yes
13	Monosomy 3	Del	91.5	Del	Monosomy 3	Del	Del	16	Dead	No
14	Monosomy 3	Del	82.0	Del	Monosomy 3	Del	Del	37	Dead	No
15	Monosomy 3	Del	74.0	Del	Monosomy 3	Del	Del	18	Dead	Yes
16	Monosomy 3	Del	68.0	Del	Monosomy 3	Del	Del	24	Dead	Yes
17	Monosomy 3	Del	83.0	Del	Monosomy 3	Del	Del	2	Dead	Yes
18	Monosomy 3	Del	52.5	Del	Monosomy 3	Del	Del	33	Dead	Yes
19	Monosomy 3	Del	96.5	Del	Monosomy 3	Del	Del	12	Dead	Yes
20	Monosomy 3	Del	78.5	Del	Monosomy 3	Del	Del	31	Dead	Yes
21	Monosomy 3	Del	90.5	Del	Monosomy 3	Del	Del	33	Dead	Yes
22	Monosomy 3	Del	77.0	Del	Monosomy 3	Del	Del	39	Dead	Yes
23	Monosomy 3	Del	87.5	Del	Monosomy 3	Del	Del	26	Dead	Yes
24	Monosomy 3	Del	77.0	Del	Chip Failure	NA	NA	27	Dead	Yes
25	Monosomy 3	Del	86.0	Del	No Tumor DNA	NA	NA	17	Dead	No
26	Monosomy 3	Del	28.0	Del	Monosomy 3	Del	Del	15	Dead	Yes
27	Disomy 3	Normal	0.0	Normal	Monosomy 3	Del	Del	28	Dead	No
28	Disomy 3	Normal	13.0	Del	Monosomy 3	Del	Del	38	Alive	No
29	Sample Debris	NA	NA	NA	Monosomy 3	Del	Del	82	Alive	No
30	Disomy 3	Normal	2.0	Del	Monosomy 3	Del	Del	26	Alive	No
31	Disomy 3	Normal	18.0	Del	Monosomy 3	Del	Del	11	Dead	Yes
32	Disomy 3	Normal	12.0	Normal	Monosomy 3	Del	Del	14	Dead	Yes
33	Monosomy 3	Del	38.0	Del	Monosomy 3	Del	Del	18	Dead	Yes
34	Disomy 3	Normal	2.5	Normal	Disomy 3	Normal	Normal	69	Alive	No
35	Disomy 3	Normal	4.5	Normal	Disomy 3	Normal	Normal	50	Alive	No
36	Disomy 3	Normal	3.0	Normal	Disomy 3	Normal	Normal	46	Alive	No
37	Disomy 3	Normal	14.5	Normal	Disomy 3	Normal	Normal	44	Alive	No
38	Disomy 3	Normal	6.5	Normal	Disomy 3	Normal	Normal	90	Alive	No
39	Disomy 3	Normal	5.0	Normal	Disomy 3	Normal	Normal	95	Alive	No
40	Disomy 3	Normal	6.0	Normal	Disomy 3	Normal	Normal	41	Alive	No
41	Disomy 3	Normal	6.5	Normal	Disomy 3	Normal	Normal	33	Alive	No
42	Disomy 3	Normal	3.0	Normal	Disomy 3	Normal	Normal	67	Alive	No
43	Disomy 3	Normal	4.0	Normal	Disomy 3	Normal	Normal	70	Dead	No
44	Disomy 3	Normal	5.5	Normal	Disomy 3	Normal	Normal	48	Dead	No
45	Disomy 3	Normal	2.0	Normal	Disomy 3	Normal	Normal	40	Dead	Yes
46	Disomy 3	Normal	4.5	Normal	100% Del 3q	Normal	Normal	43	Alive	No
47	Disomy 3	Normal	5.0	Del	100% Del 3q 66.5% Gain 3p 33.5% Del 3p	Del	Del	51	Alive	No
48	Disomy 3	Normal	2.0	Del	10% Del 3p	Normal	Del	36	Alive	No
49	Disomy 3	Normal	0.0	Normal	50% Gain 3q	Normal	Normal	18	Dead	Yes
50	Disomy 3	Normal	12.0	Del	75% Gain 3p	Normal	Gain	23	Dead	No

ID, identification; %M, percentage of monosomy 3 cells by FISH-CEP3; F/U, follow-up (months); Mets, metastases; Del, deletion; NA, not applicable.

analysis was not interpretable due to a chip failure in one case and sampling of nontumor DNA in the second. In a single case (tumor 29), FISH was not interpretable secondary to sample debris. In both cases of SNP-A failure, tumors were positive for FISH-3p26 deletion and in a single case FISH failure, monosomy 3 was detected using SNP-A. Chromosome 3 status determined by each technique (FISH-CEP3, FISH-3p26, and SNP-A) is listed in Table 3.

The sensitivity, specificity, positive predictive value, and negative predictive value for FISH-CEP3 and FISH-3p26, calculated using SNP-A as the gold standard (Table 4). For prediction of survival at 36 months, the sensitivity and

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Table 4. Predictors of Chromosome 3 Status

Variable	Levels	SNP Disomy 3	SNP Monosomy 3	Sensitivity	Specificity	PPV	NPV
FISH- CEP3	Disomy 3	15	7	0.78 (0.61, 0.89)	1.00 (0.80, 1.00)	1.00 (0.87, 1.00)	0.68 (0.47, 0.84)
	Monosomy 3	0	25				
FISH- 3p26	Normal	15	2	0.94 (0.80, 0.98)	1.00 (0.80, 1.00)	1.00 (0.89, 1.00)	0.88 (0.66, 0.97)
	Deleted	0	30				

For both FISH CEP3 and FISH 3p26, the sensitivity, specificity, positive predictive value, and negative predictive value were calculated using SNP-A as the gold standard. PPV, positive predictive value; NNP, negative predictive value. Values within parentheses indicate 95% confidence limits.

specificity for FISH-CEP3, FISH-3p26, and SNP-A were also calculated (Table 5).

Correlation with Clinical Outcome

Of 33 patients with monosomy 3 tumors, there were 17 (52%) who developed clinically detectable metastases. All of these patients were deceased (median time from initial diagnosis to death of 21 months). Of the 17 patients with disomy 3 tumors, 12 were alive without metastatic disease at a median follow-up of 47 months. Three patients with disomy-3 tumors died from causes unrelated to uveal melanoma. In two patients (ID 27 and ID 32), FISH-CEP3 and FISH-3p26 indicated normal chromosomal status, whereas SNP-A revealed monosomy 3. Both patients died, one with confirmed metastases (ID 32). Two additional patients (ID 45 and ID 49) with tumor characterized as disomy 3 by FISH-CEP3, FISH-3p26, and SNP-A developed metastases. These patients died from metastases 18 and 40 months following initial diagnosis. Kaplan–Meier survival curves for patients with and without chromosome 3 abnormalities detected by each technique (FISH-CEP3, FISH-3p26, and SNP-A) are shown (Fig. 2).

For prediction of survival at 36 months, the sensitivity and specificity for FISH-CEP3, FISH-3p26, and SNP-A were 0.78 (0.55, 0.91), 0.64 (0.45, 0.80); 0.83 (0.61, 0.94), 0.52 (0.33, 0.70); 0.89 (0.67, 0.97), 0.48 (0.30, 0.67), respectively (Table 5). No significant differences between measures were observed in any of the sensitivity or specificity measures.

Discussion

For the past decade, prognostication techniques have been a major focus of ophthalmic oncology research. As a result, technologies for identifying high-risk tumors have evolved rapidly. Each treatment center has adopted preferred methodologies and, at times, there has been a lack of consensus regarding standards for performing prognostication studies. FISH in particular is a flexible technology with many adjustable parameters including: type of tissue analyzed (fresh or frozen versus paraffin-embedded), number and type of probes used, cutoff point value used to determine the presence of monosomy 3, and the number of cells scored. We chose a cutoff value of 20% to define monosomy for chromosome 3 based on frequency distribution of the percentage of cells demonstrating monosomy in each tumor (Fig. 1) and because of the fact that many prior studies in the literature have used such a cutoff value.[18,30,34,35,37,40] Additionally, although 8% cutoff provided the best combination of sensitivity and specificity, it did not perform significantly better than the currently used cutoff point (20%). Of note, the cutoff point of 8% to define monosomy 3 observed in our study is similar to the cutoff value of 5% (using a nuclear enrichment technique) that has been reported to correlate significantly with the risk of metastases at 5 years.[47]

There is a paucity of studies that directly compare prognostic techniques in uveal melanoma. A study based on microsatellite analysis (MSA) done on a large number of cases reported a 3-year metastasis rate of 24% in the complete monosomy group and 3% in the disomy group.[48] A more recent MSA-based study reported disease-specific mortality rates for tumors with disomy 3 of 13.2% compared with 75.1% in the monosomy 3 group.[49] These studies did not include other techniques for comparison.[48] In a study using multiplex ligation-dependent probe amplification (MLPA) to detect chromosome 3 loss, 10-year disease-specific mortality was 0% in 133 tumors without chromosome 3 loss.[50] Although these authors did not directly compare MLPA with other techniques, Vaarater et al.[51] demonstrated that MLPA was equivalent to FISH for the identification of patients at risk for metastatic disease in uveal melanoma. The sensitivity of MLPA to detect patients at risk for metastatic disease was higher than that of FISH (0.795 vs. 0.692) but the specificity was equal for both techniques (0.840).[51]

Onken et al.[18] reported that loss of heterozygosity of chromosome 3 detected by SNP-A was superior to that of FISH in predicting metastatic outcome. However, FISH analysis was suboptimal in that study because investigators used paraffin-embedded tissue (replete with truncation artifacts) and counted only 100 cells. A threshold of 30% (higher than the conventional cutoff of 20%) was used to define monosomy 3 status.[18] Young and colleagues reported that monosomy 3 status could be successfully determined in choroidal melanoma in only 64% of cases analyzed by FISH, compared with 73% of cases evaluated by SNP-A. In their series, fresh tissue was

Table 5. Predictors of Survival at 36 Months

Variable	Levels	Alive	Dead	Sensitivity	Specificity	PPV	NPV
FISH- CEP3	Disomy 3	16	4	0.78 (0.55, 0.91)	0.64 (0.45, 0.80)	0.61 (0.41, 0.78)	0.80 (0.58, 0.92)
	Monosomy 3	9	14				
FISH- 3p26	Normal	13	3	0.83 (0.61, 0.94)	0.52 (0.33, 0.70)	0.56 (0.37, 0.72)	0.81 (0.57, 0.93)
	Deleted	12	15				
SNP-A	Disomy 3	12	2	0.89 (0.67, 0.97)	0.48 (0.30, 0.67)	0.55 (0.38, 0.72)	0.86 (0.60, 0.96)
	Monosomy 3	13	16				

FISH-CEP3, FISH-3p26, and SNP-A were evaluated as predictors of survival. This analysis was performed on 43 patients, since 4 patients were alive but had not yet reached 36 months of follow-up. Values within parentheses indicate 95% confidence limits.

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acquired by fine-needle aspiration biopsy (FNAB) using a 30-gauge needle via a transscleral approach. For FISH analysis, a CEP3 probe was used, although the threshold for determining monosomy 3 status and the exact number of cells counted were not specified.[38]

The manner in which FISH using a locus-specific probe compares with SNP-A analysis has not been previously reported. Monosomy 3 was detected in 33 cases (66%) by either FISH or SNP-A status, which is commensurate with the previous reports involving large tumors or those treated by enucleation.[11,19,21,22,26,32] SNP-A analysis was the most sensitive technique in identifying 31 of these cases, yielding a detection rate of 62%. When the locus-specific FISH probe was used, 3p26 deletion was found in 60% of monosomy 3 tumors. Of particular importance, FISH-3p26 deletion was present in every case of monosomy 3 detected by FISH-CEP3 (54%). There was a very high correlation between cases of monosomy 3 identified using SNP-A and those with FISH-3p26 deletion. The sensitivity of FISH-3p26 (0.78 [0.61, 0.89]) was higher than that of FISH-CEP3 (0.94 [0.80, 0.98]) using SNP-A as the gold standard.

The detection of 3p26 deletion signifies only that a particular locus is absent, and therefore does not definitively indicate the absence of whole chromosome 3 (monosomy). FISH-3p26 is also limited by its ability to differentiate disomic tumors from tumors harboring iso disomy 3 (duplication of one remaining chromosome is equivalent to functional monosomy). Isodisomy 3 can be inferred in two patients (ID 27 and ID 32), wherein FISH-CEP3 and FISH-3p26 indicated normal chromosomal status, whereas SNP-A revealed monosomy 3. Both patients died, one with confirmed metastases (ID 32). Some of the variability in the results is influenced by differences in methodology for detection and interpretation of changes in parts of chromosome 3 (gains and losses).[49,52] Data from several studies have indicated that partial loss of chromosome may not be as deleterious as total loss of chromosome 3 in influencing the risk of metastasis because tumors with partial chromosome 3 loss behave similar to tumors classified as disomic.[48,49,52] Our study further underscores the need for standardization of techniques, if results from various centers are to be directly compared.

The promise of molecular prognostication lies in the possibility that it may identify the subpopulation of patients with uveal melanoma in whom the risk of metastatic disease is sufficiently high and the disease-free interval is sufficiently short to be considered for adjuvant therapy. Although the sensitivity for prediction of survival at 36 months did not reach a statistically significant difference, the sensitivity was highest with SNP-A (0.89 [0.67, 0.97]) and least with FISH-CEP3 (0.78 [0.55, 0.91]). FISH-3p26 gave intermediate sensitivity (0.83 [0.61, 0.94]). It is also important to assess technical failures (i.e., an inability to determine risk status despite a patient's desire and false-negative rates of these techniques) because a patient with such a result would be denied potentially life-saving adjuvant therapy.[16] In our series, a technical failure rate of 2–4% (SNP-A analysis [2 cases]) and FISH [1 case]) was comparable to that of a commercially available gene-expression profiling technique.[15] However, in both cases of SNP-A failure, tumors were positive for FISH-3p26 deletion and in a single case FISH failure, monosomy 3 was found using SNP-A. Our observations support using a combination of techniques rather than relying on a single method in an unlikely event of a technical failure.

Negative predictive values for survival at 36 months were comparable for FISH-CEP3 (0.80 ([0.58, 0.92]), FISH-3p26 (0.81 [0.57, 0.93]), and SNP-A (0.86 [0.60, 0.96]), suggesting any of these tests can be used to identify patients for adjuvant treatment trial. Our data are based on a small number of patients that had large tumors and so these observations may not be directly applicable to smaller tumors that undergo eye conservative treatments in conjunction with prognostic FNAB.[48] Metastatic events may occur late[53] and therefore with longer follow-up, additional metastatic events may occur in the disomy group increasing the false-negative rates. Moreover, it is always challenging to determine the exact cause of death despite all efforts.[54]

The shortfall in detecting tumors with poor prognosis provides an opportunity for improvements in the power of the techniques used. As we move forward in our endeavor to improve prognostication in uveal melanoma, it will be of paramount importance to use a standard methodology so that results from various study centers can be meaningfully compared.

Acknowledgments

The authors thank James Bena, MS, for assistance with statistical analysis.

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