Genomic Profile of 320 Uveal Melanoma Cases: Chromosome 8p-Loss and Metastatic Outcome

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Purpose. Uveal melanoma (UM) was a fatal malignancy in 40% to 50% of cases. The aim of this study is to evaluate the independent contributions of chromosome 1, 3, 6, and 8 abnormalities for prognostication of metastasis, and to define multichromosome copy number aberration (CNA) signatures that can be used to evaluate risk.

Methods. A series of 320 UM were analyzed for chromosome 1, 3, 6, and 8 abnormalities using whole genome single-nucleotide polymorphism arrays. Results for changes in six chromosomal regions were analyzed using univariate and multivariate Cox proportional hazard modeling to identify significant predictors of metastasis and CNA signatures.

Results. Univariate Cox analysis indicated that losses of chromosome 3, 1p, 6q, and 8p and gain of 8q, as well as sex, source of tumor tissue (fine-needle aspiration biopsy [FNAB] compared with tumor from an enucleated eye), tumor basal diameter and height, and ciliary body involvement were all significant predictors of poor metastatic outcome. In the multivariate analysis, loss of chromosome 3 and 8p remained significant after adjusting for the effects of all other variables, as did sex, tissue source, and basal diameter. Multivariate analysis of the joint effects of changes in the six chromosomal regions showed that six signatures, including chromosome 3-loss, 1p-loss, 8p-loss, and/or 8q-gain had hazard ratios (HR) ranging from 7.90 to 37.25.

Conclusions. In UM, tumor size and location, tissue source, and sex were all significantly associated with increased metastasis. In addition, chromosome 3-loss and 8p-loss were found to be independent predictors of poor metastatic outcome and CNA signatures were identified that can add a specific HR value for classification of risk categories.

Keywords: uveal melanoma, chromosome changes, prognosis, SNP arrays, marker

Uveal melanoma (UM) is a deadly tumor associated with loss of vision occasionally accompanied by loss of the eye, and a high rate of mortality of 40% to 50%.[1,2] Most mortality occurs within 2 to 15 years following diagnosis, and results from metastatic disease predominantly in the liver, as well as in the lung, spleen, and bone.[3,4] The incidence of UM increases with age, and most cases are diagnosed in patients during the fifth decade of life. UM is primarily considered to be a sporadic cancer with familial predisposition being reported only rarely.[5,6]

UM prognosis has been shown to be correlated with clinical and histologic features of the tumor, including large tumor size, ciliary body involvement, extraocular extension, epithelioid cell type, and increased mitotic activity, as well as nonrandom chromosome aberrations.[7–10] The chromosomal losses and gains have been assessed by a variety of methods including conventional cytogenetics,[11] fluorescence in situ hybridization,[12,13] comparative genomic hybridization,[14–16] microsatellite analysis,[17,18] multiplex ligation-dependent probe amplification assays,[19,20] and whole genome single-nucleotide polymorphism arrays.[21,22] Over the past two decades, the association of chromosome 3-loss with metastatic death in UM patients has dominated research[11,23,24] and remains the most commonly used cytogenetic predictor of disease-specific mortality. More recently, loss or gain of chromosomes 1p, 6q, 8p, and 8q have also been found to be associated with poor prognosis for UM.[13,15,19,25–30] It has further been shown that chromosome 3-loss together with 8q-gain is more highly correlated with metastatic death than either chromosome abnormality alone.[19,30] During the last few years, an alternative approach to cytogenetic classification of UM by Harbour and colleagues[31–34] and van Gils et al.[35] has used gene expression profiling to classify UM into tumors at high risk or low risk for metastasis.

In this manuscript, genome-wide chromosomal copy numbers of a large series of 320 UM samples were analyzed to test the validity of chromosome 3-loss and 8q-gain as the most significant prognostic genetic markers in the context of other patient demographic and tumor characteristics.

Methods

Patients

The UM samples described in this study were primary tumors obtained from 320 individuals managed by the Ocular Oncology Service at Wills Eye Hospital, Philadelphia, Pennsyl-

vania, between October 1990 and October 2011. Among these patients, 254 samples were obtained by fine-needle aspiration biopsy (FNAB) prior to ^{125}Iodine plaque radiotherapy[17,36–38] beginning in 2005. The remaining 66 samples were obtained by solid open biopsy from enucleated globes. The samples were submitted to the Genetic Diagnostic Laboratory at the University of Pennsylvania for chromosomal copy number analysis.

Information on age at time of UM diagnosis, sex (male/female), tissue source (FNAB or biopsy of enucleated tumor), tumor location (choroid [CH], ciliary body [CB], ciliochoroid [CB-CH], iris, iris-CB, or iris-CB-CH), and largest basal diameter and height were obtained by a retrospective review of medical charts for all patients. Information on metastasis (yes/no) was available for all patients and was ascertained either by chart review or by phone interview if the patient had not had a clinic visit within 6 to 12 months of when the chart review was carried out.

All studies adhered to the tenants of the Declaration of Helsinki and informed consent for the use of tissues and data for research was obtained from all individuals who participated in genetic testing. This research was approved by the institutional review boards of the University of Pennsylvania and the Wills Eye Hospital.

DNA Extraction and Determination of Chromosomal Alterations

FNAB samples were stored at 4°C in Hanks Balanced Salt Solution (Gibco, Life Technologies, Grand Island, NY) solution immediately following the procedure.[37] Biopsy samples were obtained from fresh-frozen tumor tissue from enucleated eyes or from archived, formalin-fixed, paraffin-embedded blocks. Genomic DNA was isolated from either fresh-frozen or archived tumor samples using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) and from FNAB samples using the DNA Microkit (Qiagen) following manufacturers' protocols.

Whole genome copy number variation was analyzed in the 320 tumors using Affymetrix Human 100K, SNP-5.0, or SNP-6.0 genotyping arrays (Affymetrix, Santa Clara, CA). Cel files generated with GCOS software (Affymetrix) were imported into Partek Genomic Suite v6.5 (Partek Inc., St. Louis, MO) and analyzed using the Copy Number Analysis workflow option of Partek Genomics Suite, version 6.5, which creates copy number from allele intensities using HapMap controls as the baseline. The segmentation analysis option was used to define regions of amplifications and deletions as described in the software manual. To determine the reliability of chromosome copy number results in FNAB samples compared with biopsies of the solid tumor following enucleation, we assayed nine matched FNAB and biopsy samples from the tumor of an enucleated eye by microsatellite analysis and four by SNP arrays; all but one of the tumors assayed by microsatellite analysis were concordant. These data are in accord with results reported by Sisley et al.[39] and Naus et al.[40]

Data Analyses

Individual differences in clinical features and prevalence of chromosomal alterations between patients with and without metastasis were analyzed using univariate Cox proportional hazard modeling. This analytical method takes into account the observed differences in the time that individuals who did, or did not, develop metastases contribute to the study. Individuals without metastases were censored at the time of last known follow-up (months), and those with metastases were censored at the time of diagnosis of metastasis. Univariate analysis was used to assess the individual contributions chromosomes 3, 1, 6, and 8 aberrations, as well as sex and age and tumor tissue source, TNM stages (T, N and M describe tumor size, lymph node involvement, and distant metastasis), diameter, height, and location make to metastasis-free survival. TNM tumor staging was done according to the American Joint Committee on Cancer TNM system (http://www.cancer.org/cancer/eyecancer/detailedguide/eye-cancer-staging). Results are reported as hazard ratios (HR) and corresponding 95% confidence intervals (CI). When the variable such as tumor location and TNM staging or chromosome 3 had more than two categories, they were included in the regression models as indicator variables. In the TNM analysis, because there were no metastatic events among individuals with Stage 1 disease, we used Stage IIA as the reference group. For the chromosomal gains or losses, univariate metastasis-free survival was visualized with Kaplan-Meier (K-M) survival curves.

Multivariable Cox regression analysis was used to assess the independent contributions of chromosome 3, 1, 6, and 8 aberrations to metastasis-free survival after adjusting simultaneously for the effects of all other variables indicated above. The multivariate analysis included 293 UM samples since data for one or more chromosomal regions was missing for 27 of the 320 tumor samples.

We further explored the joint effect of all chromosomal alterations by creating a multichromosome copy number aberration (CNA) signature indicating normal disomy status, loss, or gain at each of six genomic regions: chromosome 3 (disomy/partial loss/loss), 1p (disomy/loss), 6p (disomy/gain), 6q (disomy/loss/gain), 8p (disomy/loss/gain), and 8q (disomy/gain). Normal disomy was coded as 0, loss as 1, and gain as 2. We defined the reference multichromosome CNA signature as normal disomy at all six chromosome regions (coded 000000). Multivariate Cox proportional hazard modeling was used to identify those signatures that were statistically significant predictors of metastatic outcome. Cox regression and K-M analyses were carried out using SPSS 19 (IBM, New York, NY) or SAS v9.3 (SAS, Cary, NC). P values less than 0.05 were considered statistically significant.

Results

A description of the 320 individuals with UM and characteristics of the tumor samples is presented in Table 1. Among these 320 individuals, 60 (18.8%) developed metastases. The median time to metastasis was 17.5 months (mean 21.6, range 0–66 months). In our patient cohort, 93% (56 of 60) of the metastases that occurred did so within 48 months from treatment of the primary tumor. The proportion of female and male individuals in the cohort are similar (48.8%, $N = 156$ and 51.3%, $N = 164$, respectively) and the median age of diagnosis was 58 years (mean, 56.3; range, 12.3–91.0 years).

Results from Cox univariate analysis shown in Table 1 revealed a significant association between sex and incidence of metastases with males having a significantly poorer prognosis (HR = 1.90, 95% CI = 1.12-3.22, $P = 0.018$), but no significant association between patient age and incidence of metastases was found (HR = 1.0, 95% CI = 0.98–1.02). The average basal diameter and thickness of all tumors was 12.3 and 6.0 mm, respectively, and both size measures were highly predictive of metastatic outcome (HR = 1.23, 95% CI = 1.14–1.33, $P < 0.001$; and HR = 1.24, 95% CI = 1.15–1.34, $P < 0.001$, respectively), as was tumor location (CH: HR = 1.00; CB: HR = 3.58, 95% CI = 1.25–10.22, $P = 0.017$; and CB-CH: HR = 3.50, 95% CI = 2.02–6.07, $P < 0.001$). In most cases, the tumor size and location contributed substantially to the decision of whether the tumor was treated by globe-sparing procedures such as plaque radiotherapy or enucleation.[41,42] Thus, it was

Table 1. Patient Demographics and Uveal Melanoma Characteristics and Genomic Profile

Variable	Total (Frequency) $N = 320$	Patients With No Metastases During Follow-up Interval* (Frequency) $N = 260$	Patients With Metastases† (Frequency) $N = 60$	HR‡ (P Value)§
Patient sex				
Female	156 (0.488)	135 (0.519)	21 (0.350)	1.0
Male	164 (0.513)	125 (0.481)	39 (0.650)	**1.90 (0.018)**
Patient age median (mean ± SD, range)	320	57 (56.1 ± 14.7, 12.3-91.0)	60.9 (57.3 ± 13.5, 22.1-79)	1.00 (0.804)
Tissue source				
Fine-needle aspiration biopsy	254 (0.794)	228 (0.877)	26 (0.433)	1.0
Tumor biopsy-enucleated eye	66 (0.206)	32 (0.123)	34 (0.567)	**5.03 (<0.001)**
Tumor size median (mean ± SD, range)				
Basal diameter, mm	308	12 (11.7 ± 3.9, 4-24)	14 (14.8 ± 3.6, 6-22)	**1.23 (<0.001)**
Height, mm	313	4.5 (5.5 ± 3.0,1-14)	8.7 (8.4 ± 3.3, 0.7-15)	**1.24 (<0.001)**
Location of tumor				
Choroid (CH)	237 (0.748)	208 (0.803)	29 (0.500)	1.0
Ciliary body (CB)	7 (0.022)	3 (0.012)	4 (0.069)	**3.58 (0.017)**
Ciliochoroid (CB-CH)	58 (0.183)	35 (0.135)	23 (0.397)	**3.50 (<0.001)**
Iris, Iris-CB or Iris-CB-CH	15 (0.047)	13 (0.050)	2 (0.034)	1.05 (0.949)
Tumor stage‖	293			
Stage I	92 (0.315)	92 (0.383)	0	0
Stage IIA	66 (0.225)	60 (0.250)	6 (0.113)	1.0
Stage IIB	74 (0.253)	55 (0.229)	19 (0.358)	**3.16 (0.014)**
Stage IIIA	43 (0147)	28 (0.117)	15 (0.283)	**3.90 (0.005)**
Stage IIIB	11 (0.038)	5 (0.021)	6 (0.113)	**10.26 (<0.001)**
Stage IIIC	0	0	0	-
Stage IV	7 (0.024)	0	7 (0.132)	n.e.
Chromosome 3, 1, 6, 8 abnormalities				
Chromosome 3				
Disomy	157 (0.491)	147 (0.565)	10 (0.167)	**1.0**
Loss	144 (0.450)	101 (0.388)	43 (0.717)	**5.54 (<0.001)**
Partial loss	19 (0.059)	12 (0.046)	7 (0.117)	**5.48 (0.001)**
Chromosome 1p				
Normal	250 (0.812)	213 (0.842)	37 (0673)	1.0
Loss	58 (0.188)	40 (0.158)	18 (0.327)	**2.32 (0.004)**
Chromosome 6p				
Normal	216 (0.679)	176 (0.680)	40 (0.678)	1.0
Gain	102 (0.321)	83 (0.320)	19 (0.322)	0.93 (0.804)
Chromosome 6q				
Normal	247 (0.772)	207(0.796)	40(0.667)	1.0
Loss	55 (0.172)	37 (0.142)	18 (0.300)	**1.94 (0.020)**
Gain	18 (0.056)	16 (0.062)	2 (0.033)	0.56 (0.420)
Chromosome 8p				
Normal	212 (0.665)	186 (0.718)	26 (0.433)	1.0
Loss	57 (0.179)	33 (0.127)	24 (0.400	**4.72 (<0.001)**
Gain	50 (0.157)	40 (0.154)	10 (0.167)	1.52 (0.264)
Chromosome 8q				
Normal	156 (0.488)	145 (0.558)	11 (0.183)	1.0
Gain	164 (0.513)	115 (0.442)	49 (0.817)	**4.80 (<0.001)**

* Median interval follow-up (months) (mean ± SD, range) = 21 (25.4 ± 18.1, 1–86).

† Median time to metastasis (months) (mean ± SD, range) = 17.5 (21.6 ± 16.0, 0–66).

‡ HR.

§ P value derived from Cox univariate proportional hazards model.

‖ Tumor staging according to American Joint Committee on Cancer (AJCC) TNM system (http://www.cancer.org/cancer/eyecancer/detailedguide/eye-cancer-staging). Our staging does not take into account information of lymph node involvement since this information was not available. n.e. = not estimable.

not surprising that tissue sourced from either FNAB or biopsy of the tumor from an enucleated eye was also a highly significant predictor of metastatic outcome (HR = 5.03, 95% CI = 3.01–8.40, $P < 0.001$) with the enucleated tumor having a significantly poorer outcome. Similarly, since TNM staging is based primarily on tumor size and location, stages IIB, (HR = 3.16, 95% CI = 1.26–7.91, $P = 0.014$), IIIA (HR = 3.90, 95% CI = 1.51–10.09, $P = 0.005$), and IIIB (HR = 10.26, 95% CI = 3.29–31.98, $P < 0.001$) were all found to be associated with poor metastatic outcome. (The HR for Stage IV tumors is inestimable since all individuals had metastatic events in the category).

Table 1 shows that complete or partial loss of chromosome 3 (HR = 5.54, 95% CI = 2.78–11.04, $P < 0.001$; and HR = 5.48, 95% CI = 2.08–14.43, $P = 0.001$, respectively), 1p-loss (HR = 2.32, 95% CI = 1.31–4.05, $P = 0.004$), 6q-loss (HR = 1.94, 95% CI = 1.11–3.39, $P = 0.020$), 8p-loss (HR = 4.72, 95% CI = 2.70–8.24, $P < 0.001$), and 8q-gain (HR = 4.80, 95% CI = 2.49–9.22, $P < 0.001$) were all significantly associated with poor metastatic outcome in univariate analysis, while 6p-gain, 6q-gain, and 8p-gain were not. The K-M survival plots provided in the Figures A through F present a graphical view of these results, again showing that in addition to conventional chromosomal changes including 3-loss, 8q-gain, and 1p-loss, chromosome 8p-loss was also a significant prognostic indicator of metastatic outcome.

Table 2A shows the results of multivariable modeling predicting metastasis-free survival for each chromosome aberration, including complete or partial loss of chromosome 3, 1p, 6q, and 8p, and gain of 6p, 6q, 8p, and 8q after adjusting for the effects of the other chromosomal changes. Loss of chromosome 3 (HR = 3.19, 95% CI = 1.27–8.02, $P = 0.014$), 1p (HR = 2.16, 95% CI = 1.14–4.06, $P = 0.018$), and 8p (HR = 1.97, 95% CI = 1.03–3.77, $P = 0.04$) were all significant prognostic factors for poor metastatic outcome. Chromosome 8q-gain was marginally significant in this model (HR = 2.30, 95% CI = 0.99–5.30, $P = 0.052$), but had the second highest HR after chromosome 3-loss. Table 2B shows the results of the multivariate modeling of chromosomal abnormalities after further adjustment for demographic and tumor characteristics. In these models, sex (HR = 2.68, 95% CI = 1.40–5.09, $P = 0.003$), tissue source (HR = 2.70, 95% CI = 1.32–5.52, $P = 0.006$), and basal diameter (HR = 1.13, 95% CI = 1.04–1.23, $P = 0.006$) remained significant after adjusting for the effects of other tumor characteristics and chromosomal losses or gains. Comparison of the results of the effect of chromosome gains or losses without (Table 2A) and with (Table 2B) adjustment for demographic and tumor characteristics showed that chromosome 3-loss (HR = 3.19, $P = 0.014$ in Table 2A; and HR = 4.16, $P = 0.007$ in 2B) and 8p-loss (HR = 1.97, $P = 0.040$ in 2A; and HR = 2.66, $P = 0.010$ in 2B) had increased HR values, becoming more significant after adjusting for the effects of the other covariates. In contrast, both chromosome 1p-loss and 8q-gain had lower HR values and were no longer significant after adjusting for the other variables.

To identify those patient or tumor characteristics that most affected the association between individual chromosomal changes and metastatic outcome, we completed multivariate modeling separately for each chromosomal change in which we adjusted for demographic and tumor covariates one at a time (Table 3). Chromosome 1p-loss was no longer significant after adjusting for either tissue source or diameter, while 8q-gain lost statistical significance after adjusting for tissue source, diameter, height, or location. In contrast, the significance and HRs remained constant for chromosome 3-loss and 8p-loss after adjusting for each of the other covariates individually.

To investigate the joint effects of changes in six chromosomal regions in predicting metastatic outcome, we created multichromosome CNA signatures indicating the status of chromosomes 3, 8q, 1p, 8p, 6p, and 6q (normal, loss, and/or gain) in the 293 UM samples with complete genotyping data available. A total of 59 multichromosome CNA signatures were observed at least once and there were 21 that were observed at least 3 times. Six of these 21 signatures were significantly associated with metastasis when compared with the presence of the reference signature (Table 4). All the significant signatures contained chromosome-3 loss; in addition, five included 8q-gain, four included 8p-loss, and three showed 1p-loss. Each significant signature contained at least two chromosome changes (e.g., loss of chromosomes 3 and 1p seen in signature 101000). The HR for these multichromosome CNA signatures ranged from 7.90 to 37.25 (95% CI range, 1.88–160.65).

Discussion

The primary tumor cure rate for UM is typically greater than 90%,[43] however, the risk of metastasis within a short time of the primary diagnosis makes UM a deadly disease. The goals of this manuscript were to determine the independent and joint contributions of individual chromosome abnormalities to UM metastasis and to develop a multichromosome CNA signature that can be used as a reliable prognostic genetic marker. Numerous studies on UM have indicated that tumor size and location, as well as aberrations on chromosomes 1p, 6, and 8 have prognostic relevance in addition to that of chromosome 3.[13,15,19,25–30,35] Among these studies, those by Onken et al.,[28] van Gils et al.,[35] and Damato et al.[25] have all presented results that show, using different methods, that loss of 8p is associated with poor metastatic outcome. In the largest series of UM analyzed to data, Damato and colleagues[19] showed that loss of chromosome 3 and 1p and 8q-gain were all correlated with increased mortality in 452 individuals with UM, with the combined presence of 3-loss and 8q-gain having the poorest prognosis.

In order to re-evaluate the significance of this set of chromosomal aberrations as prognostic markers, we performed statistical comparisons of the chromosome losses and gains in a series of 320 primary tumors. Among the six chromosomal regions that were analyzed and showed loss or gain in our study sample, chromosome 3-loss was the most strongly associated with poor metastatic outcome (HR = 5.54, Table 1) in the univariate Cox analysis. However, in the multivariate analysis (Table 2A), the HR value for chromosome 3-loss decreased from 5.54 to 3.19 indicating the confounding effects of one or more of the other chromosomal changes present in the tumor. A similar decrease in HR was seen comparing the effect measures from the univariate model of 8p-loss and 8q-gain to those determined in the multivariate analysis (HR = 4.72 compared with 1.97, and 4.80 compared with 2.30, respectively), suggesting that individual chromosomal changes need to be analyzed in the context of other chromosomal aberrations. In contrast, the HR for 1p-loss was more stable (HR = 2.32 compared with 2.16) indicating little impact on the risk of metastasis after adjustment for the other genetic factors. It is of note that in addition to the conventional expectation that chromosome 3-loss, 1p-loss, and 8q-gain are independent risk factors for metastasis, this analysis shows that 8p-loss is also an independent risk factor. This is evident in the multivariate analysis where 8p-loss retains significance after accounting for the effects of the other chromosomal changes (Table 2A). Furthermore, chromosome 8p-loss is the only chromosomal change, along with 3-loss, that remains significant after adjusting for six different demographic and tumor variables (Tables 2B and 3). Our data does not provide any

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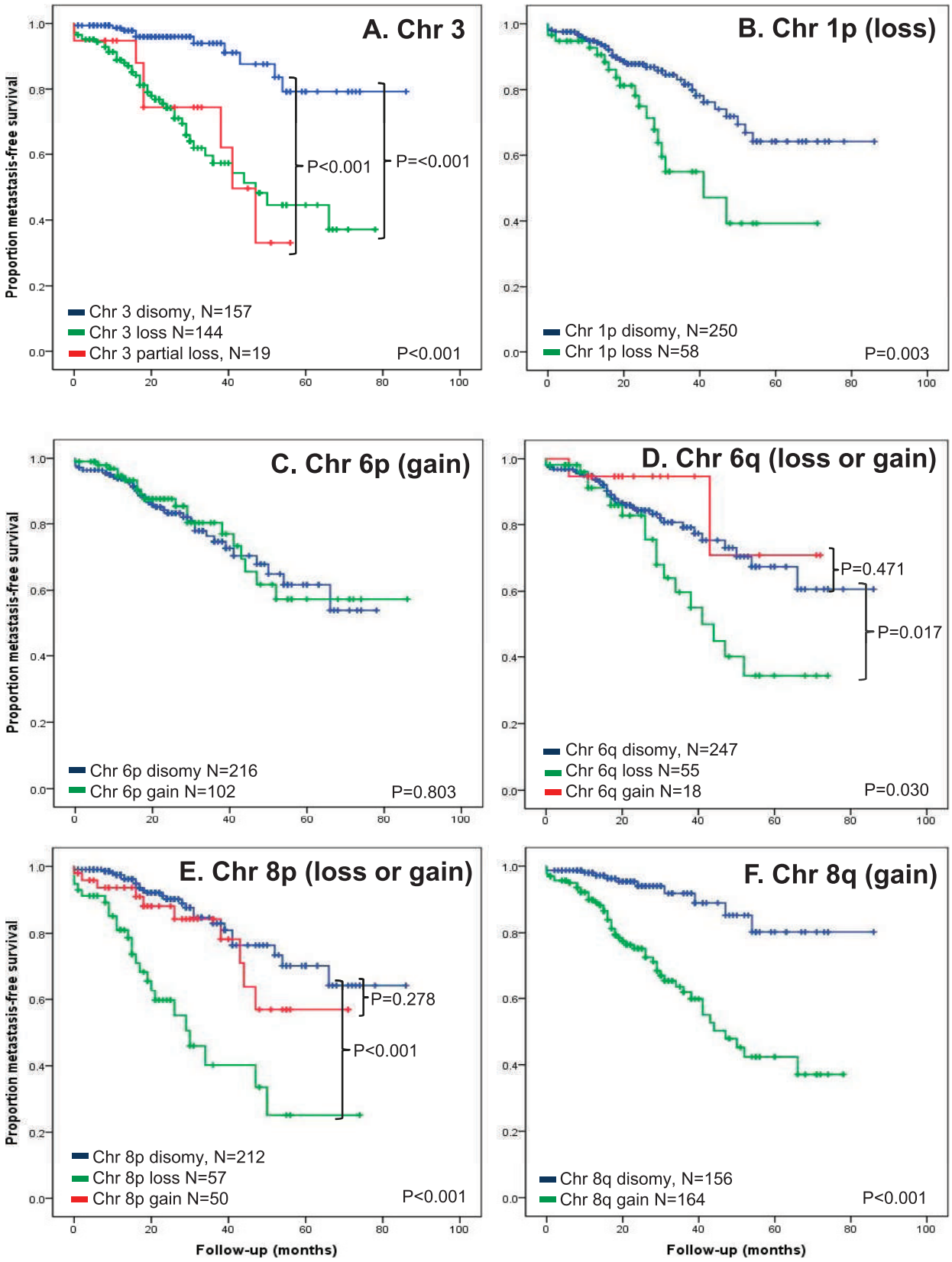

Figure. (**A**–**F**) K-M analysis of UM metastasis-free survival and presence of chromosome (chr) 3-disomy, loss or partial loss (**A**), chr 1p-disomy or loss (**B**) chr 6p-disomy or gain (**C**), 6q-disomy, loss or gain (**D**), 8p-disomy, loss or gain (**E**), and 8q-disomy or gain (**F**). Significance values are expressed as log-rank *P* values. Vertical tick marks indicate individuals whose data were censored by the period of last follow-up; each step down in the curve represents an incidence of one or more metastatic events.

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Table 2. Cox Multivariate Regression Analysis Correlating the Incidence of Metastasis in UM with Chromosome (Chr) Copy Number Changes and Patient and Tumor Characteristics ($N = 293$)

	(A) Multivariate Analysis Considering Chromosomal Abnormalities as Variables				(B) Multivariate Analysis Considering Chromosomal Abnormalities and Demographic and Tumor Variables			
		95% CI for HR*				95% CI for HR*		
Variables	**HR***	**Lower**	**Upper**	**P Value**	**HR***	**Lower**	**Upper**	***P* Value**
Sex								
Female					1.00			
Male					**2.68**	1.40	5.09	**0.003**
Age					0.98	0.96	1.01	0.261
Tissue source								
Fine-needle aspiration biopsy					1.00			
Tumor biopsy-enucleated eye					**2.70**	1.32	5.52	**0.006**
Basal diameter					**1.13**	1.04	1.23	**0.006**
Height					1.05	0.93	1.18	0.432
Location								
CH					1.00			
CB					3.08	0.62	15.43	0.171
CB-CH					1.85	0.88	3.88	0.105
Iris, Iris-CB, Iris-CB-CH					2.86	0.56	14.67	0.207
Chr 3								
Chr3-disomy	1.00				1.00			
Chr3-loss	**3.19**	1.27	8.02	**0.014**	**4.16**	1.47	11.78	**0.007**
Chr3-partial loss	1.67	0.49	5.66	0.411	2.31	0.67	7.95	0.184
Chr1p								
Chr1p-disomy	1.00				1.00			
Chr1p-loss	**2.16**	1.14	4.06	**0.018**	*1.41*	0.67	2.95	*0.361*
Chr6p								
Chr6p-disomy	1.00				1.00			
Chr6p-gain	0.98	0.41	2.35	0.961	1.08	0.39	3.00	0.884
Chr6q								
Chr6q-disomy	1.00				1.00			
Chr6q-loss	1.61	0.71	3.68	0.256	0.81	0.31	2.16	0.678
Chr6q-gain	0.00	0.00	n.e.†	0.977	0.00	0.00	n.e.†	0.972
Chr8p								
Chr8p-disomy	1.00				1.00			
Chr8p-loss	**1.97**	1.03	3.77	**0.040**	**2.66**	1.26	5.62	**0.010**
Chr8p-gain	0.42	0.15	1.17	0.097	0.66	0.22	1.95	0.449
Chr8q								
Chr8q-disomy	1.00				1.00			
Chr8q-gain	**2.30**	0.99	5.30	**0.052**	*0.99*	0.36	2.68	*0.982*

(**A**) Multivariate analysis of chromosome 3, 1p, 6p, 6q, 8p, and 8q loss or gain in predicting metastatic outcome after adjusting for the other chromosomal changes. (**B**) Multivariate analysis including patient and tumor variables in addition to chromosomal abnormalities. (Values in bold are significant; those in *italics* indicate *P* values which are no longer significant when demographic and tumor covariates are included in the regression model.)

* HR

† n.e., not estimable.

evidence for a protective role of chromosome 6p-gain as has been shown by others.[19,30,35]

Tumor heterogeneity has been predicted to be present in UM based on analysis of multiple biopsy samples from the same tumor from an enucleated eye.[44,45] This finding indicates the potential for skewing of the results of the analysis of chromosome copy number changes as revealed by analysis of single FNAB samples.[39,40] In our limited data set, which includes 13 matched FNAB and biopsy samples of tumors from enucleated eyes, only one discordant sample was observed. We concluded from this small sample that less than 10% of the UM samples are potentially heterogeneous, and therefore likely to give erroneous results based on a single FNAB sample. When tumors from enucleated eyes are tested, multiple samples can be examined to gage tumor heterogeneity; however, our result indicates FNAB sampling is accurate in the majority of UM samples.

The multichromosome CNA signature analysis demonstrates the significant role of 8p-loss beyond that of 3-loss and 8q-gain. For example, the signature showing only 3 loss and 8q-gain

Table 3. Significance of Chromosomal Copy Number Changes After Separately Adjusting for Demographic and Tumor Variables ($N = 293$)

Additional Covariate	None*		Sex		Age		Tissue Source		Basal Diameter		Height		Tumor Location	
	HR†	*P* Value	HR*	*P* Value	HR*	*P* Value	HR*	*P* Value	HR*	*P* Value	HR*	*P* Value	HR*	*P* Value
Chr3-disomy	1		1		1		1		1		1		1	
Chr3-loss	**3.19**	**0.014**	**3.26**	**0.015**	**3.41**	**0.009**	**3.80**	**0.006**	**3.02**	**0.016**	**2.40**	**0.074**	**2.94**	**0.022**
Chr3-partial loss	1.67	0.411	1.74	0.378	1.46	0.547	2.02	0.261	1.45	0.547	1.64	0.424	1.86	0.323
Chr1-disomy	1		1		1		1		1		1		1	
Chr1p-loss	2.16	0.018	2.16	0.015	2.02	0.033	*1.72*	*0.113*	*1.88*	*0.071*	2.06	0.029	2.13	0.021
Chr6p-disomy	1		1		1		1		1		1		1	
Chr6p-gain	0.98	0.961	1.00	0.999	0.90	0.812	1.23	0.658	0.97	0.937	0.94	0.892	1.10	0.843
Chr6q-disomy	1		1		1		1		1		1		1	
Chr6q-loss	1.61	0.256	1.29	0.545	1.66	0.240	1.44	0.410	1.19	0.693	1.26	0.607	1.38	0.455
Chr6q-gain	0.00	0.977	0.00	0.966	0.00	0.977	0.00	0.971	0.00	0.977	0.00	0.968	0.00	0.968
Chr8p-disomy	1		1		1		1		1		1		1	
Chr8p-loss	**1.97**	**0.040**	**2.02**	**0.033**	**2.09**	**0.029**	**2.02**	**0.037**	**2.01**	**0.048**	**2.40**	**0.011**	**1.99**	**0.039**
Chr8p-gain	0.42	0.097	0.43	0.105	0.44	0.121	0.52	0.210	0.49	0.179	0.54	0.241	0.38	0.106
Chr8q-disomy	1		1		1		1		1		1		1	
Chr8q-gain	2.30	0.052	2.36	0.054	2.36	0.044	*1.47*	*0.374*	*1.61*	*0.283*	*1.54*	*0.328*	*1.94*	*0.156*

Each variable was considered separately with the individual chromosomal changes in the Cox multivariate model to determine which chromosomal changes remained significant predictors of poor metastatic outcome after adjusting for the six demographic and tumor variables. (Values in bold remain significant after adjusting for individual tumor variables, those in italics indicate P values which are no longer significant after adjusting for individual variables.)

* HR and significance after adjusting for other chromosomal changes (Table 2A).

† HR.

(110000) imparts a nonsignificant 2-fold increased risk ($P = 0.435$ seen in Supplementary Table S1); in comparison, signatures that include a 1p- and/or 8p-loss impart a significant increase in risk (14- to 37-fold, Table 4). Thus, it appears that even in tumors acquiring chromosome 3-loss and 8q-gain, additional acquisition of chromosome 1p-loss and 8p-loss seems to significantly increase the risk of metastasis and needs to be considered in determining individual patient risk. The approach to defining multichromosome CNA signatures presented in Table 4 reinforces this finding by showing that loss of chromosome 3 is necessary, but not sufficient for UM metastasis and other independent changes in 1p and 8p contribute significantly to determining the individual patient's risk of developing future metastases. The results of Onken et al.[28] showed that chromosome 8p-loss, in the presence of chromosome 3-loss, is a more important prognostic indicator of metastatic outcome than 8q-gain. This was integral to the model presented by Harbour[46] that indicates that the class 2 tumors with the highest risk of metastasis have chromosome 3-loss, 1p-loss, and 8p-loss (8q-gain), which mirrors the results of our analysis. We have been able to attribute specific HR values associated with different CNA signatures where we capture the combined contributions of chromosome 1p, 8p, and 8q changes on the background of chromosome 3 status.

In conclusion, we have developed a multichromosome CNA signature that can be used to better characterize the UM tumors with respect to future risk of metastasis. While this includes the chromosome 3-loss and 8q-gain, the finding of the independent contributions of 1p-loss and 8p-loss to the risk of metastasis are novel and suggests the presence of important

Table 4. Cox Multivariate Analysis of the Association of Multichromosome CNA Signatures and Metastatic Outcome on UM

Chr 3	Chr 8q Gain	Chr 1p Loss	Chr 8p Loss/Gain	Chr 6p Gain	Chr 6q Loss/Gain	Multichromosome CNA Signature*	Number (Frequency)	Number Metastases (Frequency)	*P* Value	HR†	95% CI for HR† Lower	95% CI for HR† Upper
0	0	0	0	0	0	000000‡	85 (0.29)	5 (0.059)		1.00		
Loss	Gain	0	Loss	0	0	120100	28 (0.096)	13 (0.464)	<0.001	14.74	5.18	42.01
Loss	Gain	Loss	Loss	0	0	121100	3 (0.010	3 (1)	<0.001	37.25	8.64	160.65
Loss	Gain	Loss	0	0	0	121000	5 (0.017)	3 (0.6)	<0.001	13.91	3.25	59.44
Loss	Gain	0	loss	0	Loss	120101	3 (0.010)	2 (0.667)	<0.001	18.66	3.54	98.19
Loss	Gain	0	Loss	Gain	Loss	120121	4 (0.014)	2 (0.5)	<0.001	18.01	3.40	95.37
Loss	0	Loss	0	0	0	101000	8 (0.027)	3 (0.375)	0.005	7.90	1.88	33.18

The multilocus signature is based on copy number gain or loss for chromosome 3, 8q, 1p, 8p, 6p, and 6q ($N = 293$ tumors). Only signature classes with ≥3 samples and a significant association with metastatic outcome are shown. (For a complete list of the results for all 59 CNA signatures, see Supplementary Table S1.)

* Multichromosome CNA signature designations in the order chromosome 3, 8q, 1p, 8p, 6p, 6q: 0 = normal; 1 = chromosome 3-loss/partial loss, 1p-loss, 8p-loss or 6q-loss; 2 = 8q-gain, 8p-gain, 6p-gain, 6q-gain.

† HR.

‡ Reference class with baseline risk of metastasis of approximately 6%.

functional changes in these chromosomal regions. This combined CNA signature encompasses the changes previously known, but adds a specific HR value for classification of risk categories.

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