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Association between gene expression classification, proliferation and metastasis in uveal melanoma

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Abstract

Purpose—Uveal melanomas cluster into two molecular groups based on their gene expression profile. Tumors with the class 1 signature rarely metastasize, whereas those with the class 2 signature have a very high rate of metastasis. However, the biological basis for this metastatic propensity of class 2 tumors remains unclear. Towards such an explanation, this study was conducted to determine whether class 2 tumors proliferate more rapidly than class 1 tumors.

Design—Experimental study.

Participants—The study included 28 primary uveal melanomas with extensive clinical, pathologic and genetic annotation.

Methods—Clinical and pathologic features were recorded, including age, gender, ciliary body involvement, tumor basal diameter, thickness, cell type and final patient status. Gene expression profile was performed using a previously described multi-gene assay. Status of chromosome 3 was determined using a single nucleotide polymorphism (SNP) assay. Aneuploidy was calculated using global array-based comparative genomic hybridization.

Main Outcome Measures—Immunopositivity for Ki-67 was determined by counting positive nuclei per unit area (12.5 unit areas per 40X high power field).

Results—Class 2 gene expression profile signature, loss of chromosome 3 and increased aneuploidy were significantly associated with Ki-67 positivity ($P=0.04$, $P=0.004$ and $P=0.03$, respectively). Tumor thickness and epithelioid cell type showed a borderline significant association with Ki-67 positivity ($P=0.06$ and $P=0.07$, respectively). Using Receiver Operating Characteristic (ROC) analysis of Ki-67 positivity, and using the class 2 gene expression profile signature as a surrogate endpoint for metastasis, a Ki-67 score of 1.5 cells per unit area (approximately 20 cells per high power field) was identified as the optimal cutoff to distinguish between patients at low versus high risk for metastasis. Using this dichotomous classification, Ki-67 positivity exhibited a significant association with metastasis (log rank test, $P=0.01$).

Conclusions—On average, class 2 uveal melanomas have a higher proliferation rate than class 1 tumors. This finding may explain, at least in part, the strong tendency for class 2 tumors to metastasize. Further work is needed to determine whether loss of chromosome 3, increased aneuploidy, or other factors may be responsible for the increased proliferation rate.

Keywords

gene expression profile; uveal melanoma; eye; choroid; ciliary body; proliferation; metastasis; survival

Uveal melanoma is the most common primary malignancy of the eye and spreads hematogenously to the liver and other sites in up to half of patients. Once overt metastasis develops, the disease is uniformly fatal with no effective treatments. Improvements in the survival of patients with uveal melanoma will likely require a better understanding of the biology and genetics of this cancer in order for more targeted and effective treatments to be developed. In recent years, there have been important breakthroughs in unraveling the molecular underpinnings of uveal melanoma and its propensity for metastasis. Recently, primary uveal melanomas were found to cluster into two groups of roughly equal proportions based on their gene expression profile. Tumors with the class 1 gene expression profile rarely metastasize, whereas those with the class 2 gene expression profile have a very high rate of metastasis.^{1,2} The genes that discriminate between class 1 and class 2 tumors provide insights into the underlying biology of metastasis. The transcriptome of class 1 tumors is only slightly different than normal uveal melanocytes, suggesting relatively few genetic changes have occurred. In contrast, the class 2 transcriptome is vastly different from melanocytes and resembles primitive stem-like ectodermal cells.³ Among class 2 tumors, loss of chromosome 3 is a frequent finding. Even though chromosome 3 loss is not as accurate as the gene expression profile for predicting metastasis,² the strong relationship between chromosome 3 loss and the class 2 gene expression profile suggests that alterations on chromosome 3 may be responsible for the global change in gene expression in class 2 tumors. Class 2 tumors also have significantly greater chromosomal aneuploidy, an indicator of generalized genomic instability, compared to class 1 tumors.⁴ Despite these insights, it remains unclear why class 2 tumors metastasize more readily than class 1 tumors. One possibility is that class 2 tumors have greater capacity than class 1 tumors for migration and invasion. In an earlier study, we showed that deletion of a metastasis modifier locus on chromosome 8p was associated with increased migration and invasion of uveal melanoma cells, but this deletion occurred in only a quarter of class 2 tumors, which would leave unexplained the high metastatic rate of the other three quarters of class 2 tumors.⁵ Further, class 2 tumor cells have not shown increased migratory behavior compared to class 1 cells in cultures established from over 200 enucleated eyes (O. Agapova, personal communication). Another possibility is that class 2 tumors proliferate more rapidly, which can promote metastasis by accelerating the accumulation of new mutations that convey a selective advantage to the evolving tumor. The Ki-67 antibody recognizes a nuclear antigen present in proliferating cells, but absent in resting cells.⁶ Ki-67 labeling has been studied in uveal melanoma by several groups.⁷⁻¹¹ Factors that have been associated Ki-67 positivity include p53 expression, larger tumor size, epithelioid cells, microcirculation patterns and shorter survival.⁹⁻¹² To our knowledge, however, the association between Ki-67 and gene expression profile and 6 other genetic prognostic factors has not been investigated. Thus, we evaluated Ki-67 labeling in a cohort of uveal melanomas with detailed clinical, pathologic, transcriptomic and chromosomal annotation. We found a significant association between increased Ki-67 positivity and class 2 signature, loss of chromosomes 3, increased aneuploidy and metastasis. These findings may lead to a better understanding of class 2 tumors and why they have such a strong tendency for metastasis.

METHODS

Patients and Tumor Samples

The study was approved by the Institutional Review Board of Washington University and included a cohort of 28 primary uveal melanomas that have been extensively evaluated.^{1,2,13,14} All primary tumors were treated by enucleation without prior radiation therapy or other local treatment. Clinical features were recorded for each patient, including age, gender, ciliary body involvement, tumor basal diameter and thickness, and final status (alive and disease-free, alive with metastasis, dead of metastasis, or dead of other causes).

Gene expression profile analysis and assignment of a class 1 or class 2 signature was performed as previously reported.²

Immunohistochemistry

Immunohistochemistry was performed using the streptavidin-biotin method with the Vector ABC Elite kit (Vector Laboratories, Inc., Burlingame, CA) and blue stain to avoid confusion with brown melanin pigment. Nuclear fast red was used for counterstain. Four-micron sections were obtained, deparaffinized, rehydrated with ethanol, and treated with 0.3% hydrogen peroxide and methanol to inhibit endogenous peroxidase activity. Heat induced epitope retrieval was performed using microwave treatment in citrate buffer. Primary antibody against Ki-67 (Catalog#556003; BD Pharmingen; San Jose, CA) was applied at 4°C overnight. The secondary antibody without primary antibody was used as a negative control. All stained tumor sections were reviewed by at least two independent observers masked to patient outcome and variables. Images from immunostained tumor sections were obtained on a microscope-mounted digital imager at 40X magnification. Positive nuclei were marked manually with black dots of uniform size using Adobe Photoshop software (Adobe, San Jose, CA). Resulting images were analyzed in a masked fashion with ImageJ software (available at <http://rsb.info.nih.gov/ij>) using the line tool to define the outline of the tumor and the plot profile function to calculate the total number of marked nuclei within the outlined region and the total area of the outlined region. The ratio of these values was calculated as nuclei per unit area of 100×100 pixels (approximately $104 \times 104 \mu\text{m}$ at scale).

Statistical Analysis

Statistical associations between variables were evaluated using non-parametric methods, as indicated. Metastasis free survival was analyzed using Kaplan-Meier analysis. All calculations were performed using MedCalc® version 9.3.6.0 (<http://www.medcalc.be>).

RESULTS

Clinical, histopathologic, immunohistochemical and molecular data are summarized in Table 1. The study included 28 patients who underwent enucleation for previously untreated posterior uveal melanoma, including 15 males and 13 females. Mean patient age was 61.9 years (median 63.0 years, range 25 to 82 years). Mean tumor thickness was 10.4 mm (median 10.4 mm, range 2.4 to 22.0 mm). Mean tumor largest basal dimension was 17.5 mm (median 18.5 mm, range 8.0 to 24.3 mm). The tumor involved the ciliary body in 17 cases (61%). Predominant histopathologic cell type was spindle in 8 cases (29%), mixed in 7 cases (25%), and epithelioid in 13 cases (46%). Scleral invasion was scored histopathologically as absent in 8 cases (29%), intrascleral in 13 cases (46%), and extrascleral in 7 cases (25%). Loss of heterozygosity for chromosome 3 was detected in 17 cases (61%). The gene expression profile was class 1 in 13 cases (46%) and class 2 in 15 cases (54%). Mean follow-up was 33.2 months (median 24.1 months, range 1.0 to 82.3 months). The mean number of cells per unit area with positive nuclear immunostaining for Ki-67 was 2.2 (median 1.3 cells, range 0.17 to 9.2 cells) (Figure 1). At last follow-up, nine (32%) patients had developed metastatic disease, all of whom had a class 2 tumor with loss of heterozygosity for chromosome 3. Statistical analysis was performed to identify variables associated with Ki-67 positivity (Table 2). Class 2 gene expression profile and loss of heterozygosity of chromosome 3 (Mann-Whitney test, $P=0.04$ and $P=0.004$, respectively), and increased aneuploidy (Spearman correlation, $P=0.03$) were significantly associated with increased Ki-67 positivity (Figure 2). Decreased tumor thickness (Spearman correlation, $P=0.06$) and spindle cell type (Mann-Whitney test, $P=0.07$) showed a trend toward statistical significance. Receiver Operating Characteristic (ROC) analysis was performed using Ki-67 positivity as the variable and gene expression class 2 as a surrogate endpoint for metastasis.

A Ki-67 score of 1.5 positive cells per unit area (approximately 20 positive cells per high power field at 40X magnification) was identified as the optimal cutoff to distinguish between patients at low versus high risk for metastasis. Using this dichotomous classification, Ki-67 positivity exhibited a significant association with metastasis (log rank test, $P=0.002$) (Figure 3).

DISCUSSION

In this study, class 2 uveal melanomas exhibited more Ki-67 positivity and hence, greater proliferative activity (on average) than class 1 tumors. Chromosome 3 loss and increasing aneuploidy, which are closely associated with the class 2 signature, also showed significant correlation with increased Ki-67 positivity. What does this relationship between proliferation and the class 2 tumor type mean from a cancer biology standpoint? One possibility is that more rapidly proliferating tumors are more likely to acquire the mutation(s) that are responsible for the class 2 tumor type. In support of this possibility was the observed association between Ki-67 positivity and increased aneuploidy. Further, most chromosomal abnormalities occur during cell division,¹⁵ so rapidly dividing cells would be more vulnerable to chromosomal instability and aneuploidy. Conversely, it may be that the mutation(s) that bring about the class 2 tumor type concomitantly (but independently) increase proliferation. Further studies will be necessary to clarify this issue. Counterintuitively, there was not a significant association between Ki-67 and tumor size (basal diameter or thickness). One potential explanation is that the tumors included in this study were obtained by enucleation, which is a treatment generally reserved for larger tumors. As such, these tumors represent an endpoint of tumor growth and do not reflect the rate of tumor growth leading up to detection and treatment. Another important consideration is that tumor size is a reflection of the sum of many factors besides proliferation, including apoptosis, senescence, necrosis, autophagy, and immunologic response. In two earlier studies investigating Ki-67 in uveal melanoma, no association was found between Ki-67 positivity and clinicopathologic prognostic factors.^{7, 8} In more recent studies, Ki-67 positivity was associated with p53 expression, larger tumor size, epithelioid cells, microcirculation patterns and shorter survival.^{9–12} In this study, we took advantage of the class 2 gene expression profile signature, a highly accurate predictor of metastasis,² as a surrogate endpoint for metastasis. This allowed us to identify an optimal value of Ki-67 positivity for dichotomous classification into low and high groups. This technique allowed us to confirm that Ki-67 positivity is strongly associated with metastatic risk. Nevertheless, Ki-67 did not perform as well as the gene expression profile and, hence, is not recommended as a substitute prognostic biomarker.

Strengths of this study include a cohort of uveal melanoma samples with detailed clinical, pathologic, chromosomal and gene expression profile data and long clinical follow-up. Weaknesses include a relatively small sample set and lack of experimental studies, which will be challenging to perform given the small number of appropriate cell lines available. Nevertheless, these results provide important new information to aid in laying a groundwork for hypothesis driven investigations into the relationship between gene expression class, proliferation and metastasis.

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Figure 1. Representative photomicrographs of tumors with low (**A**), medium (**B**) and high(**C**) Ki-67 positivity. Positive cells are indicated by dark blue nuclei. Sections are counterstained with nuclear fast red. Brown pigment is melanin.

Figure 2. Kaplan-Meier survival plot showing relationship between Ki-67 positivity and uveal melanoma metastasis. High Ki-67 was defined as ≥ 1.5 positive cells per unit area (approximately 20 positive cells per high power field).

Figure 3.

Graphs showing relationship between Ki-67 positivity and gene expression class (**A**), loss of chromosome 3 (**B**) and aneuploidy (**C**). Significance levels calculated by Mann-Whitney test are indicated for **A** and **B**. Linear regression of data in **C** (line) indicates a positive relationship between tumor mitotic activity and aneuploidy.

Table 1

Summary of clinical, histopathologic and molecular features

Patient Number	Age	Gender	Ciliary body involvement	Largest tumor basal dimension	Tumor thickness	Pathologic cell type	Scleral Invasion	Gene expression class	Loss of chr 3	Loss of chr 8p	Aneuploidy score	Ki67 score	Months to last follow-up	Metastasis
MM23	74	Male	Yes	20	11.0	Spindle	None	Class 2	Yes	No	N/A	1.64	22.1	Yes
MM35	74	Male	No	14	11.9	Spindle	Intrasceral	Class 1	No	No	0.15	0.17	76.7	No
MM37	25	Female	Yes	20	15.0	Spindle	Intrasceral	Class 1	No	No	0.00	0.31	82.3	No
MM48	50	Male	Yes	24	9.0	Mixed	None	Class 1	No	No	0.13	0.45	66.8	No
MM50	50	Female	Yes	19	8.9	Epithelioid	None	Class 1	No	No	N/A	1.49	71.4	No
MM51	62	Female	No	16	6.6	Mixed	Intrasceral	Class 2	Yes	No	0.10	0.84	51.5	No
MM54	80	Female	No	15	6.7	Mixed	Extrascleral	Class 2	Yes	No	0.08	1.73	34.6	Yes
MM55	82	Female	Yes	19	8.6	Epithelioid	Extrascleral	Class 2	Yes	No	0.23	2.25	81.3	No
MM57	53	Male	Yes	22	11.6	Epithelioid	Intrasceral	Class 1	No	No	0.03	0.78	39.6	No
MM60	67	Male	Yes	14	9.5	Epithelioid	Intrasceral	Class 2	Yes	No	0.13	1.27	37.4	Yes
MM63	42	Male	Yes	16	15.9	Spindle	None	Class 1	No	No	0.13	0.75	56.7	No
MM70	62	Male	Yes	24	15.6	Epithelioid	Extrascleral	Class 2	Yes	Yes	0.13	2.42	31.5	Yes
MM71	63	Female	No	N/A	12.5	Spindle	Intrasceral	Class 2	Yes	No	0.10	0.80	46.3	No
MM74	77	Male	No	N/A	22.0	Mixed	Extrascleral	Class 1	No	No	0.05	0.27	24.4	No
MM82	63	Female	No	10	5.2	Epithelioid	Extrascleral	Class 1	No	No	0.00	1.28	50.8	No
MM85	67	Male	Yes	21	13.4	Epithelioid	Extrascleral	Class 1	No	Yes	0.28	4.26	22.9	No
MM86	47	Male	No	14	14.0	Spindle	None	Class 1	No	No	0.08	1.18	17.7	No
MM88	60	Male	Yes	16	10.6	Epithelioid	None	Class 2	Yes	Yes	0.13	4.67	22.5	No
MM105	71	Male	Yes	14	10.7	Epithelioid	Intrasceral	Class 1	Yes	No	N/A	1.12	9.3	No
MM107	79	Male	Yes	20	10.3	Epithelioid	None	Class 2	Yes	No	N/A	2.38	23.8	Yes
MM110	48	Female	No	14	8.0	Epithelioid	Extrascleral	Class 2	Yes	Yes	N/A	3.66	0.0	No
MM113	54	Male	No	14	11.0	Mixed	Intrasceral	Class 1	No	No	N/A	1.21	8.0	No
MM116	64	Female	Yes	21	9.9	Epithelioid	Intrasceral	Class 2	Yes	Yes	N/A	1.40	16.7	Yes
MM120	68	Female	Yes	20	10.4	Spindle	Intrasceral	Class 2	Yes	No	N/A	2.35	1.4	Yes
MM121	52	Female	Yes	19	5.8	Spindle	Intrasceral	Class 2	Yes	No	N/A	0.92	6.0	No
MM122	52	Male	Yes	24	10.0	Epithelioid	Intrasceral	Class 1	Yes	No	N/A	9.16	1.0	No

Patient Number	Age	Gender	Ciliary body involvement	Largest tumor basal dimension	Tumor thickness	Pathologic cell type	Scleral Invasion	Gene expression class	Loss of chr 3	Loss of chr 8p	Aneuploidy score	Ki67 score	Months to last follow-up	Metastasis
MM125	79	Female	No	18	3.4	Mixed	Intrasceral	Class 2	Yes	No	N/A	3.14	7.4	Yes
MM128	69	Female	No	8	2.4	Mixed	None	Class 2	Yes	No	N/A	8.77	18.5	Yes

Table 2

Summary of statistical analysis

Variable	Number of cases	P value
Age	28	0.320
Gender	28	0.407
Ciliary body involvement	28	0.757
Largest tumor basal dimension	26	0.848
Tumor thickness	28	0.055
Histopathologic cell type	28	0.073
Extraocular extension	28	0.143
Gene expression class	28	0.040
Loss of chromosome 3	28	0.004
Aneuploidy	16	0.025