Inflammation and immunomodulation in uveal melanoma

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Chapter 3

Monosomy of chromosome 3 and an inflammatory phenotype occur together in uveal melanoma

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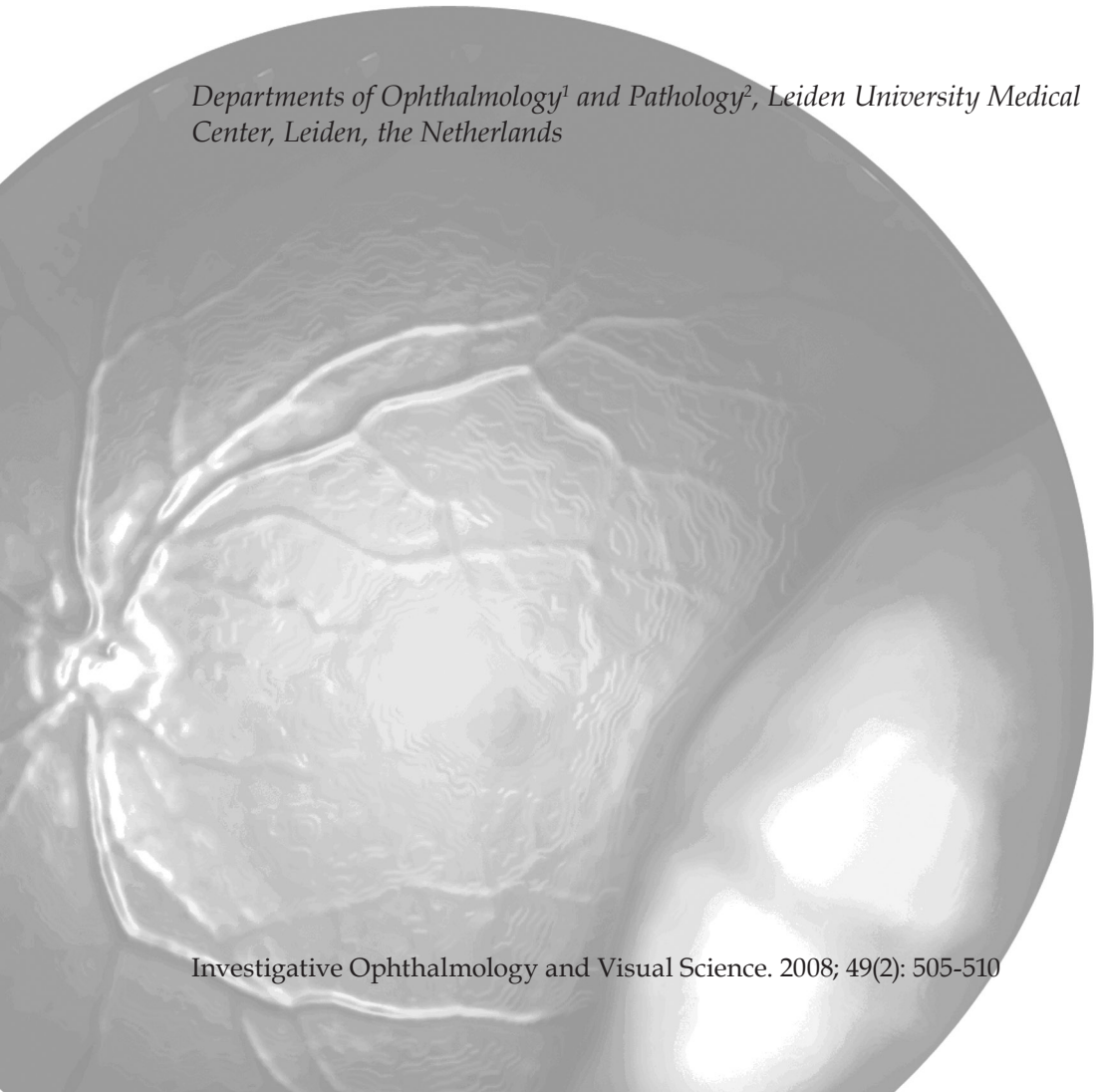

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Abstract

Purpose: In uveal melanoma, different predictors of poor prognosis have been identified, including monosomy of chromosome 3, HLA expression, and the presence of infiltrating leukocytes and macrophages. As each of these parameters can be used to differentiate prognostically favorable tumors from unfavorable ones, we hypothesized that they should be related, and that monosomy of chromosome 3 should occur in the same tumors as the unfavorable inflammatory phenotype.
Methods: Material was obtained from 50 cases of uveal melanoma treated between 1999 and 2004. After enucleation, nuclei were isolated from paraffin-embedded tissue for fluorescence in-situ hybridization in order to determine chromosome 3 copy number. Each tumor-containing globe was further processed for conventional histopathological examination and for immunohistochemical analysis with HLA Class I and II-specific antibodies and with macrophage marker CD68.
Results: Out of 50 uveal melanomas, 62% (31/50) could be categorized as having monosomy of chromosome 3. Monosomy 3 was associated with the presence of epithelioid cells, an increased density of tumor-infiltrating macrophages, and a higher HLA Class I and II expression. Survival analysis showed that monosomy 3 was correlated with decreased survival and identified monosomy 3, ciliary body involvement, and largest basal tumor diameter as the best prognostic markers.
Conclusions: Monosomy 3 in uveal melanoma is associated with the presence of an inflammatory phenotype, consisting of a high HLA Class I and II expression as well as increased numbers of tumor-infiltrating macrophages. In a multivariate Cox regression analysis, the presence of monosomy 3 was one of the best prognostic markers for metastatic disease and survival, although we have a relatively short follow-up time.

Introduction

Uveal melanoma is the most frequent primary intraocular tumor in the adult Caucasian population, having an incidence of 0.7 per 100,000 per year [1,2]. Although a lot of progress has been made in the local treatment of the intraocular tumor, survival rates have not improved. Tumor dissemination occurs in 30-50% of cases [3,4] and shows a predilection for the liver. Once metastases are clinically discernable, survival is poor [5].
Several prognostic factors, based on clinical and histological features, show a correlation with survival. Important clinical prognostic factors are tumor diameter and tumor location in the eye, while others are related to tissue characteristics (cell type, antigen expression, karyotype). One of the most important factors known to be correlated with metastatic disease in uveal melanoma is loss of one copy of chromosome 3, i.e. monosomy 3 [6,7]. This chromosomal aberration occurs in over 50% of all uveal melanomas and several studies have shown that its presence is highly correlated with survival and the development of metastatic disease [8-10]. Usually, the presence of monosomy 3 is determined by karyotyping or by the application of fluorescence in situ hybridization (FISH) on cultured cells, or by FISH analysis on tissue sections or cells obtained by fine needle aspiration biopsy (FNAB). We recently reported that studying isolated nuclei instead of cultured cells or sections increases the number of tumors positive for monosomy 3 from 38 to 62%, as many tumors show tissue heterogeneity for this chromosome loss [11].
Other important parameters related to prognosis include immunological determinants such as human leukocyte antigen (HLA) expression [12-14], leukocyte [15] and macrophage infiltration [16,17] Although in general oncology, downregulation of HLA-antigen expression is considered an important tumor escape mechanism, several studies reported that in uveal melanoma a high HLA expression is an unfavorable prognostic sign [12-14,16]. Increased expression of HLA Class I as well as of HLA Class II expression carries an unfavorable prognosis, occurrs more frequently in epithelioid tumors, and is associated with increased numbers of CD3+ and CD4+ T lymphocytes, as well as with an increased density of CD11b macrophages [17]. Ericcson [14] observed that HLA Class II too was expressed at a higher level in uveal melanomas containing epithelioid cells. In an independent study, Mäkitie et al. showed that high numbers of tumor-infiltrating CD68+ macrophages were related to an unfavorable prognosis and were associated with the presence of epithelioid cells and an increased microvascular density [18,19]. Other markers of inflammation also show an association with leukocyte infiltration, such as COX-2 [20]. Overall, one can identify a series of markers that identify an inflammatory phenotype.
In this study, we hypothesized that tumors with an unfavorable prognosis would not only have monosomy of chromosome 3, but would also demonstrate the inflammatory phenotype, consisting of a high HLA Class I and Class II expression, and relatively high numbers of macrophages. Therefore, we determined the presence of monosomy for chromosome 3 in 50 uveal melanomas by applying FISH on nuclei isolated from paraffin-embedded tissue and compared the results with HLA Class I and II expression and macrophage

density.

Material and methods

Patients and specimens

Formalin-fixed, paraffin-embedded tissue was obtained from 50 cases of uveal melanoma, treated at the Leiden University Medical Center between 1999 and 2004. In this study, a substantial proportion of tumors were medium and large, according to the COMS criteria (48% and 50% of the cases, respectively). The average age of the 23 female and 27 male patients was 60 years (range 25 – 87 years). The research protocol followed the current revision of the tenets of the Declaration of Helsinki (world medical association declaration of Helsinki 1964; ethical principles for medical research involving human subjects).

Histopathological examination

Histological sections were prepared from tissues fixed in 10% buffered neutralized formalin for 48 hours and embedded in paraffin. Hematoxylin- and eosin-stained 4μm sections were reviewed by one ocular pathologist [DdWR] for confirmation of diagnosis, intraocular localization, cell type, largest basal diameter, prominence and scleral invasion (none, superficial (< ½ of the sclera), deep (½ to ¾ of the sclera), extrascleral and total scleral invasion) (see Table 1). PAS staining was used to assess loops and networks.

Interphase FISH on nuclei isolated from paraffin-embedded tissue

Interphase FISH was performed on nuclei isolated from 50 μm paraffin-embedded tissue sections as described earlier [11]. In brief, after enzymatic digestion with pepsin, and two additional washing steps with phosphate-buffered saline (PBS), cells were filtered through a 70 micrometer pore size nylon filter (Verseidag-Industrietextilen GmbH, Kempen, Germany). Nuclei were fixed with methanol: acetic acid (3:1). Cell density was adjusted with fixative to ensure that 400-500 nuclei were present on each slide. The slides were air-dried and used for hybridization. DNA probe CEP3 SpectrumOrange: CEN 3, specific for the centromere region of chromosome 3 (band 3p11.1-q11.1) was used for hybridization according to the manufacturer's protocol (Vysis Inc., Des Plaines, IL, USA). Three healthy tonsils from different individuals were used as controls. The tonsil sections were treated in exactly the same manner as the tumor samples. The cut-off level was set at the mean of these controls plus three times the SD, i.e. at 5%, for detecting monosomy 3.
Slides were analyzed using a Leica DMRXA fluorescence microscope. Image capture was performed by a monochrome CCD camera (COHU, San Diego, CA) attached to the fluorescence microscope and Leica Q-FISH software (Leica Imaging Systems, Cambridge, UK).

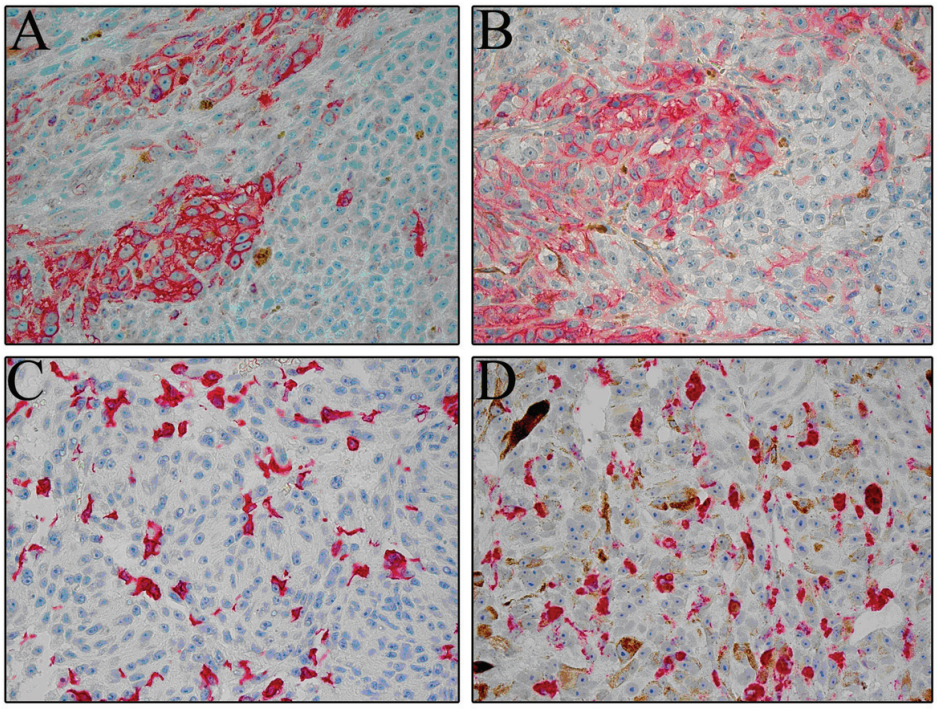

Figure 1. HLA Class I and II expression and the presence of macrophages in uveal melanoma. **(A)** Uveal melanoma stained with mAb HC10. **(B)** mAb HCA2 staining. **(C)** mAb PG-M1 against macrophages epitope CD68 was used to label macrophages. **(D)** Uveal melanoma stained against HLA-DR with mAb clone Ta1.1B5. (Magnification, x400).

Immunostaining protocol

Immunohistochemistry was performed using the alkaline phosphatase-monoclonal anti-alkaline phosphatase (APAAP) method [21]. In brief, 4µm paraffin-embedded sections, mounted on coated slides (Knittel Gläser, Braunschweig, Germany), were deparaffinized in xylene (four times, 5 minutes each) and with ethanol (three times, 5 minutes each), followed by one rinse with distilled water at room temperature. Incubation of the slides with methanol/ H_2O_2 0.3 % for 20 minutes blocked the endogenous peroxidase activity. After the slides were washed, antigen retrieval was performed by boiling in citrate buffer (DakoCytomation, Glostrup, Denmark) for 10 minutes. After three additional washing steps with PBS, the primary monoclonal antibodies were added to the sections, which were then incubated at room temperature for 60 minutes. Monoclonal antibodies were diluted in PBS/1% bovine serum albumin and optimal antibody concentrations were determined by titration studies. Optimal dilutions ranged from 1:100 – 1:150. After three 5-minutes washes with PBS, sections were incubated with Poly-AP anti-Mouse IgG (Powervision, ImmunoVision Technologies Co., Dale City, CA, USA) for 60 minutes at room temperature. Slides were washed three times with PBS for 5 minutes each and staining was visualized using Fast Red (Scytec, Logan, UT, USA) in naphthol-

phosphate buffer (Scytec, Logan, UT, USA) and levamisole 50 mM, according to the manufacturer's guidelines. Sections were counterstained with Mayer's hematoxylin (Klinipath, Duiven, The Netherlands) and finally embedded in Kaiser's glycerin.
Antibodies used were HC10 and HCA2 from the Dutch Cancer Institute [22,23], Tal.1B5, recognizing HLA-DR, from DakoCytomation, Glostrup, Denmark [24], and PG-M1, recognizing PG-M1, also from DakoCytomation [25] (Figure 1).

Assessment of staining

Scores were determined by two independent observers without knowledge of the results obtained by the other investigator to ensure accuracy of quantification of immunohistochemical slides. For assessment of HLA Class I and II staining, the number of HLA-positive cells was estimated at 100x magnification and expressed as percentage of the total number of tumor cells. In case of a difference of 10% or more between the two investigators, consensus could be reached during a simultaneous session. For grading the density of infiltrating macrophages, we used a semi-quantitative scoring system [17]. The number of infiltrating macrophages was assessed by comparing 10 high-power fields at 250x magnification with three standard photographs showing a low, moderate and high macrophage density.

Statistical methods

All statistical analyses were performed by computer using the statistical software program SPSS for Windows, release 12.0.1 (SPSS Inc., US). Statistical significance was assumed for P-values of 0.05 or less. Bivariate correlations were analyzed with Pearson's coefficient. ANOVA test was used for analyzing the distribution among various numerical groups. Overall survival distributions were estimated using Kaplan-Meier methodology and comparisons between categorical variables in survival were assessed using the log-rank test. A Cox proportional hazard model was used to determine the best prognostic factor and to perform multivariate regression analyses.

Results

Determination of monosomy for chromosome 3 by interphase FISH

Interphase FISH was performed on nuclei isolated from paraffin-embedded tissue from uveal melanomas and was successful in all cases. Signals were bright and intense and easily recognizable. Percentages of nuclei carrying only one chromosome 3 varied between 0 and 94% [11]. With a threshold value of 5% (based on normal controls), 19 uveal melanomas (38%) were categorized as being disomic for chromosome 3, and 31 (62%) as having monosomy for chromosome 3. Monosomy of chromosome 3 was associated with the presence of epithelioid cells (P=0.006, Chi-Square Test) (Table 2).

Table 1. Distribution of HLA class I and II expression in relation to clinical and histological parameters.
The medians for tumor prominence and LBD were 8.0 and 13.0 mm, respectively. The data are the mean (SD) percentage of positively-staining cells. CB, ciliary body. *Significant at P ≤ 0.05.

			Antibody		
		N	**HC10**	**HCA2**	**Ta1.1B5**
Categorical variables					
Gender	Male	27	36.5 (30.7)	43.2 (31.0)	18.3 (20.3)
	Female	23	38.0 (34.2)	34.5 (30.1)	22.0 (24.5)
Cell type	Spindle	15	19.3 (25.1)*	25.7 (24.9)*	13.0 (14.4)
	Mixed + epithelioid	35	44.9 (32.3)	50.9 (29.5)	23.0 (24.4)
CB involvement	Not present	29	38.5 (34.3)	46.2 (33.5)	17.2 (19.2)
	Present	21	35.5 (30.1)	39.3 (25.5)	23.8 (25.7)
Macrophage density	Low	16	24.7 (28.2)*	36.3 (33.3)	8.4 (5.4)*
	Medium	20	33.5 (27.9)	41.0 (29.6)	18.0 (18.6)
	High	14	56.8 (35.3)	54.6 (26.2)	36.1 (29.4)
Chromosome 3	Disomy	19	21.6 (27.4)*	28.7 (32.1)*	9.7 (7.0)*
	Monosomy	31	46.8 (31.6)	52.2 (25.8)	26.3 (25.8)
Scleral ingrowth	None	3	8.3 (10.4)	5.0 (5.0)	6.67 (2.89
	Superficial	25	38.8 (34.4)	42.2 (30.2)	16.6 (14.7)
	Deep	12	47.5 (29.3)	53.3 (27.7)	32.1 (32.4)
	Extrascleral	7	23.6 (27.3)	42.1 (29.1)	22.9 (26.3)
	Total	3	43.3 (40.4)	53.3 (41.6)	6.7 (2.9)
Loops	Not present	12	22.1 (26.2)	20.8 (17.2)*	16.3 (18.8)
	Present	38	42.0 (32.9)	50.4 (30.2)	21.2 (23.2)
Networks	Not present	19	30.3 (32.6)	30.3 (28.4)*	19.7 (28.0)
	Present	31	41.5 (31.9)	51.3 (29.0)	20.2 (18.3)
Numerical variables					
Tumor prominence	Prominence ≤ 8.0 mm	31	36.0 (32.4)	44.0 (32.3)	20.5 (23.8)
	Prominence > 8.0 mm	19	38.1 (33.3)	42.8 (28.2)	17.0 (17.7)
Largest basal diameter	LBD ≤ 13.0 mm	29	34.3 (33.6)	43.6 (30.7)	16.0 (19.7)
	LBD >13.0 mm	21	41.2 (30.7	42.9 (30.5)	25.5 (24.6)

HLA Class I and II expression

For each antibody, the number of positively-staining tumor cells was estimated and expressed as the percentage of the total number of tumor cells in the sections. The percentage of cells that reacted positively with the anti-HLA Class I antibodies HC10 and HCA2 varied widely, with a mean of 37% for HC10 (range 0 to 100%) and 43% for HCA2 (range 0 to 100%). The mean percentage of HLA-DR positive cells was 20%, with a range of 5 to 100%. When compared to the chromosome analysis, the group with monosomy of chromosome 3 had a significantly higher expression of HLA Class I (mAbs HC10 and HCA2) and II (mAb Tal.1B5) than the group with disomy for chromosome 3 (P= 0.006, P= 0.006, and P=0.002, respectively, Table 2). Tumors with a mixed or epithelioid cell type contained more cells that stained positively with the anti-HLA Class I antibodies HC10 (mean difference 26%) and HCA2 (mean difference 25 %) compared to tumors with spindle cells only (P = 0.009; P = 0.006, respectively). For HLA-DR, there was a mean difference of 12% positively-staining cells between mixed and/or epithelioid cell type compared to spindle, which difference was not significant (P = 0.146) (Tables 1 and 3).

Table 2. Distribution of monosomy 3 status
For antibody staining, data indicate mean ± standard deviation for the parameter concerned. For other variables, data are the number of cases with monosomy or disomy 3. The medians for tumor prominence and LBD were 8.0 and 13.0 mm, respectively.

		Chromosome 3 status			
		N	**Monosomy**	**Disomy**	**p-value**
Gender	Male	27	16	11	0.665
	Female	23	15	8	
Cell type	Spindle	15	5	10	*0.006*
	Mixed + epithelioid	35	26	9	
CB involvement	Not present	29	14	15	*0.019*
	Present	21	17	4	
Macrophage density	Low	16	4	12	*0.001*
	Medium	20	15	5	
	High	14	12	2	
Tumor prominence	Prominence ≤ 8.0 mm	31	20	10	0.535
	Prominence >8.0 mm	19	11	8	
Largest basal diameter	LBD ≤ 13.0 mm	29	16	13	0.242
	LBD >13.0 mm	21	15	6	
Scleral ingrowth	None	3	0	3	0.137
	Superficial	25	18	7	
	Deep	12	6	6	
	Extrascleral	7	5	2	
	Total	3	2	1	
Loops	Not present	12	5	7	0.096
	Present	38	26	12	
Networks	Not present	19	9	10	0.095
	Present	31	22	9	
HC10		50	46.8 (31.6)	21.6 (27.4)	*0.006*
HCA2		50	52.2 (25.8)	28.7 (32.1)	*0.006*
Ta1.1B5		50	26.3 (25.8)	9.7 (7.0)	*0.002*

Table 3. Probabilities for correlations and associations between clinical and histological parameters, HLA expression and Chromosome 3 status.

	HC10	**HCA2**	**Ta1.1B5**	**Chromosome 3**
Gender	0.867	0.970	0.570	0.655
Tumor prominence	0.504	0.292	0.491	0.788
Largest basal diameter	0.881	0.339	0.212	0.119
Cell Type	*0.009**	*0.006**	0.146	*0.006**
CB involvement	0.752	0.411	0.306	*0.019**
Macrophage density	*0.017**	0.233	*0.001**	*0.001**
Chromosome 3	*0.006**	*0.006**	*0.002**	X
Scleral invasion	0.288	0.163	0.167	0.137
Extravascular Matrix Patterns (Loops)	0.062	*0.002**	0.507	0.096
Extravascular Matrix Patterns (Networks)	0.238	*0.016**	0.948	0.095

CB, ciliary body. *Significant at P ≤ 0.05.

Macrophages

Immunostaining with mAb PG-M1 specific for the CD68 epitope which identifies macrophages was satisfactory in all specimens of uveal melanoma. Immunopositive cells were easily recognized. The number of CD68-positive cells was low in 16 tumors (32%), moderate in 20 (40%) and high in 14 tumors (28%). Tumors with monosomy 3 contained significantly more macrophages than the group without monosomy 3 (P=0.001, Table 2).
The number of infiltrating macrophages was positively correlated with HC10 expression (P=0.017) as well as with Tal.1B5 expression (P=0.001) (Table 1 and 3).

Scleral invasion and extravascular matrix patterns

Monosomy 3 was not associated with scleral invasion, when tested with the Chi-square test (P = 0.137) (Table 2 and 3). There was also no significant difference among the different classification groups of scleral invasion (none, superficial, deep, extrascleral and total scleral invasion) concerning the expression of HC10 (P = 0.288), HCA2 (P = 0.163), and HLA-DR (P = 0.167) (ANOVA-tests).
Histopathological occurrence of loops and networks patterns was not associated with the presence of monosomy 3 (P = 0.096 and P = 0.095, respectively, Chi-square test). Tumors with loops and network patterns showed a relatively higher expression of HCA2 expression than tumors without such patterns (P = 0.002 for loops and P = 0.016 for networks patterns, ANOVA test), but not with HC10 and Ta1.1B5 expression (Table 3, Figure 1).

Survival analysis

The mean follow-up at the time of analysis was 36 months (range 12 - 73 months), which is quite short. During this time period, 16 patients had died, 14 due to metastatic disease (28%). There was one patient with metastasis (2%), who was still alive at the end of follow-up. Even after this relatively short follow-up time, Kaplan-Meier analysis and Log Rank Test showed that the presence of monosomy 3 was correlated with a decreased survival (P= 0.003). Other significant associations with death due to metastases were seen with regard to involvement of the ciliary body (P = 0.002) and the presence of epithelioid cells (P = 0.002). Scleral invasion status was not significantly associated with a higher percentage of death due to metastases (Kaplan-Meier survival analysis, P = 0.719). Gender was not significantly different with respect to survival as analysed by log rank analysis (P = 0.153). Expression of HLA-DR (P = 0.798, ratio, 1.00), HC10 (P = 0.751, ratio = 1.00) or HCA2 (P = 0.808, ratio 1.00) were not significantly correlated with death due to metastases (Cox univariate analysis). Multivariate analysis with Cox regression showed three parameters, which were significant predictors for death due to metastatic disease, namely largest basal diameter (P = 0.017, ratio 5.70), monosomy 3 (P = 0.017, ratio 5.70), and ciliary body involvement (P = 0.008, ratio 7.04) (Table 4).

Table 4. Probabilities for Kaplan Meier and Cox proportional hazard survival. Analysis of different parameters with death due to metastasis as the end point.

	Cox univariate	LR	Cox multivariate	LR	Kaplan-Meier
Gender	0.175	2.17	-		0.153
Age	0.237	1.01	-		-
Largest tumor diameter	*0.003*	1.55	*0.017*	5.70	-
Tumor prominence	0.499	1.08	-		-
Ciliary body involvement	*0.007*	6.00	*0.008*	7.04	*0.002*
Histopathologic cell type	0.085	6.06	-		*0.002*
Macrophage density	0.663	1.17	-		0.151
Scleral invasion	0.452	1.20	-		0.719
HCA2	0.808	1.00	-		-
HC10	0.751	1.00	-		-
HLA-DR	0.798	1.00	-		-
Monosomy 3 (FISH on nuclei)	0.098	48.20	*0.017*	5.70	*0.003*

LR = likelihood ratio.

Discussion

We hypothesized that tumors with a poor prognosis, would not only have monosomy of chromosome 3, but would also have a high HLA Class I and II expression and many macrophages. Our data show that, indeed, in general the same tumors that carry only one chromosome 3 also show the inflammatory phenotype. Data obtained by several centers on RNA micro arrays have suggested the presence of two tumor types, one of which is associated with the lack of one chromosome 3 [26,27]. Singh. et al (Cleveland Clinic, Ohio) stated that HLA Class II expression should be added to the markers that can be derived from array studies and which identify prognostically bad tumors [28]. Our data show that monosomy of chromosome 3 and the inflammatory phenotype are indeed part of the same infaust tumor phenotype. To our surprise, in our study, the presence of vascular loops and networks was not significantly associated with monosomy of chromosome 3, although most previous studies did find such a correlation [10,29], and it would therefore be logical if loops and networks would be part of the same phenotype.

It is well known that uveal melanomas are often heterogeneous, both in cell type as well as in the expression of many antigens. In addition, different areas of the tumor may be heterogeneous with regard to loss of chromosome 3, but it may be that the same cells in a tumor carry both characteristics (monosomy 3 as well as a high HLA expression). Recently, Sandinha et al. [30] reported that one tumor can have one area with an epithelioid cell type with monosomy of chromosome 3, and another area with spindle cells, which carry two chromosomes 3. Interestingly, Meir et al. [31] performed a similar study, now using laser capture dissection, and microsatellite analysis. Meir isolated areas with and without specific vasculogenic mimicry patterns, but did not find any difference with regard to the number of chromosome 3 signals in areas with and without networks. It is clear that the presence of a network vasculogenic mimicry pattern carries a bad prognosis, but the specific location of the network was not correlated with loss of one chromosome 3.

The reason that loss of one copy of monosomy 3 is related to death due to metastases is subject to speculation. One of the possibilities is the presence of tumor suppressor genes, which have been suggested to occur on chromosome 3 [27]. Another option may be that an expression regulator, that antagonizes inflammatory responses, is located in this area. In macrophages, a regulator of activation that is located on chromosome 3 is the peroxisome proliferators-activated receptor γ (PPARγ). PPARγ plays a role in regulating a number of inflammatory response genes [32], and loss of the activity of such a regulator might result in general upregulation of factors such as NF-κB, and thus in an inflammatory phenotype. However, this is pure speculation, but might explain the association between monosomy 3 and the inflammatory phenotype.
Monosomy of chromosome 3 was correlated with decreased survival (P = 0.003) and metastatic disease (P = 0.001). In addition, ciliary body involvement and the presence of epithelioid cells were also correlated with the development of metastases (P= 0.002 and P = 0.002, respectively). These findings correspond to most studies in the literature. However, Cox regression analysis showed that monosomy 3 was one of prognostic factors predicting death due to metastatic disease, but it did not come to a conclusion, which of the three parameters (largest basal diameter, monosomy 3, ciliary body involvement) is the best predictor. Damato et al.[33] recently published that one can obtain the best predictive index by not using one parameter only, but by using monosomy of chromosome 3, basal tumor diameter, as well as epithelioid cellularity and creating a combined prognostic index.
In our study we showed that tumors showing monosomy 3 have a higher HLA Class I and II expression than tumors without this aberration, but that HLA expression is not an independent prognostic factor predictive for metastatic disease. Tumors with monosomy 3 in general have an increased HLA expression. As NK cells are unable to lyse tumor cells with a high HLA Class I expression that migrate through the blood stream, it may well be that if cells from highly malignant tumors manage to break away from the eye, they cannot be lysed before reaching the liver, thereby circumventing one of the immunological defense systems of the body [16]. Thus, the association between chromosome 3 monosomy and HLA expression may have biological relevance.

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