Ocular responses to foreign corneal and tumor issue

Essen, T.H. van

CHAPTER 5

A Comparison of HLA Genotype with Inflammation in Uveal Melanoma

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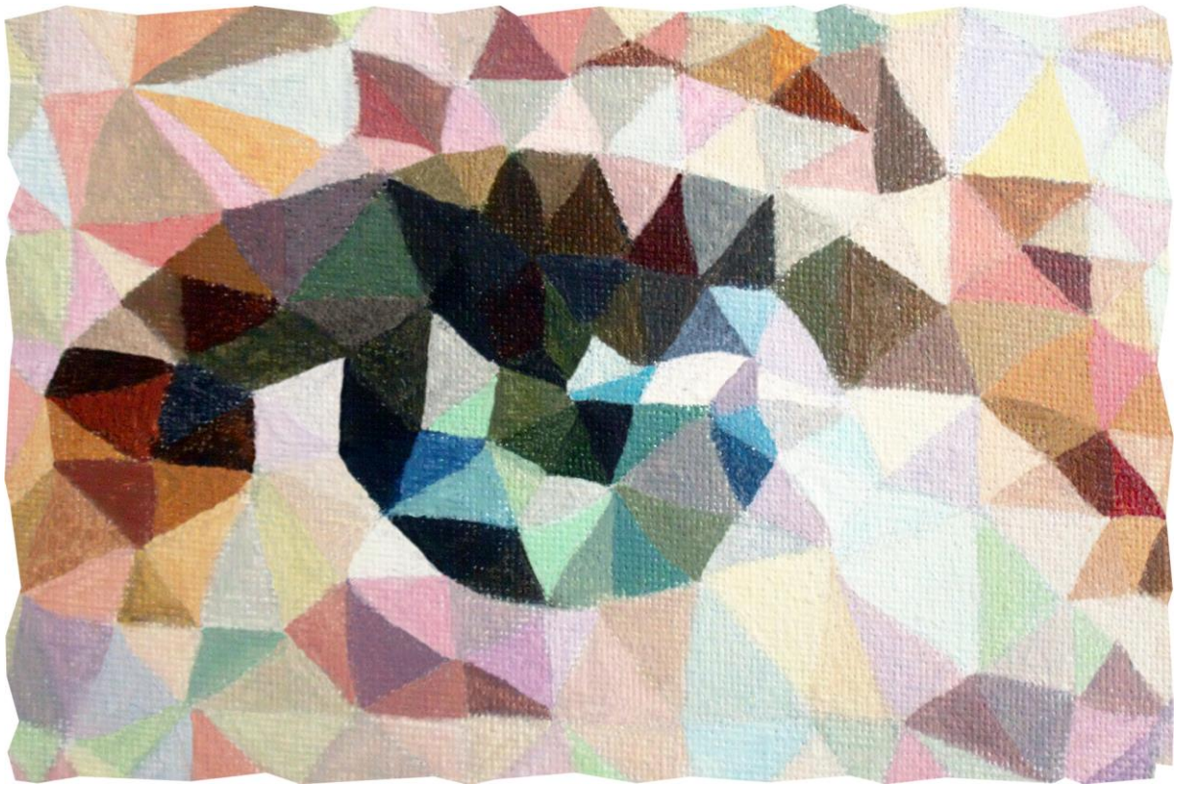

Abstract

Purpose: Human leukocyte antigen (HLA) polymorphisms have been associated with the development of autoimmune diseases. In uveal melanoma, a high expression of HLA classes I and II, and infiltration with lymphocytes and macrophages are associated with a bad prognosis. Inflammation has an important role in this malignancy. The goal of our study was to determine whether specific HLA alleles are associated with increased inflammation.

Methods: Records were analyzed of 45 patients who underwent enucleation for uveal melanoma. HLA typing, tumor HLA expression and tumor macrophage infiltration were determined in each case.

Results: Before correction for multiple testing, macrophage infiltration was less in HLA-A2 positive patients. Patients with HLA-DR6 had a higher tumor cell expression of HLA-DR. After correction for the number of analyses, no associations remained statistically significant.

Conclusion: The results before correction suggest that the HLA genotype may influence inflammation as indicated by HLA expression and macrophage infiltration in uveal melanoma. However, after correction this association did not prove significant.

Introduction

Mechanisms by which tumor cells evade the immune system have an important role in tumor growth, as emphasized by the cancer immunosurveillance theory.[1] Among these escape mechanisms, alterations in the expression of classical and non-classical human leukocyte antigen (HLA) classes I and II antigens on tumor cells are of a particular interest. These antigens have a crucial role in the induction of specific immune responses, and can modulate the interactions of natural killer (NK) cells and T cell subpopulations with their target cells.[2–4] The clinical relevance of HLA class I expression in oncology is evident from the finding that a reduced expression often correlates with a worse prognosis,[5–10] while the relevance of HLA class II expression in cancer remains ambivalent.[1,11,12]

Unlike most tumors, in uveal melanoma a high HLA class I expression is considered to be a bad prognostic sign.[13–16] This observation may be explained by the role of NK cells. Tumors with high HLA class I expression are more resistant to destruction by NK cells, as these cells specifically kill cells that lack certain HLA class I antigens.[16–18] Metastases of primary uveal melanoma show a high HLA-A and B expression,[19] thus suggesting evasion of NK cells by the tumor is necessary in the development of metastases. In uveal melanoma, a high expression of HLA class II also is related to a bad prognosis.[15]

A small, but growing number of studies have indicated that, apart from HLA expression, HLA polymorphisms mediate susceptibility to certain neoplastic diseases.[20,21] In several uveal melanoma studies an increased incidence of HLA-A32 has been found,[22–25] and another study revealed that patients with HLA-B40 died more often from metastases.[26] Nevertheless, a study on 235 cases could not confirm this association between HLA- B40 and metastasis; however, the study did find an association between HLA-B44 and metastasis before correction. Therefore, a role for HLA genotype in prognosis is not excluded.[27]

Other important immunological parameters associated with prognosis in uveal melanoma are lymphocyte[28] and macrophage[29] infiltration. High numbers of tumor-infiltrating $CD68^+$ and $CD163^+$ macrophages are associated with an unfavorable prognosis,[29–31] and $CD68^+$ macrophages have been associated with increased HLA classes I and II expression as well.[32] Several specific associations are known between HLA antigens and ocular diseases. It may well be that certain local immune responses may protect against the development of uveal melanoma by providing a local immunosurveillance system, for example against aberrant melanocytes. Choroidal auto-immune responses are noticed in birdshot chorioretinopathy (BCR), which is characterized by multiple hypopigmented chorioretinal lesions, and is associated with HLA-A29.[33] In Vogt-Koyanagi-Harada syndrome (VKH), a bilateral, chronic, diffuse panuveitis with late stage depigmentation of the fundus occurs, with a genetic association with primarily HLA-DR4.[34] Finally, uveitis in relation to autoimmune diseases has been related to HLA-B27.[35,36] Therefore, we investigated whether the most common as well as these specific HLA genotypes are related to the level of HLA expression on tumor cells and intra-tumoral macrophage infiltration, which can be regarded as parameters of inflammation in uveal melanoma.

Material and Methods

Study Population and Data Set

Records were analyzed of 50 patients with uveal melanoma who underwent enucleation between 1999 and 2004 at the Leiden University Medical Center in the Netherlands.

Table 1. Distribution of Sex, Age, and Pathological TNM Classification for Each Group

	Original Group* (*n* = 50)	**HLA Expression (*n* = 45)**	**Macrophage Infiltration (*n* = 38)**
Males (mean age)	23 (60 y)	22 (59 y)	19 (60 y)
Females (mean age)	27 (61 y)	23 (62 y)	19 (62 y)
Stage I	10%	9%	5%
Stage IIA	22%	22%	21%
Stage IIB	28%	24%	26%
Stage IIIA	34%	38%	40%
Stage IIIB	6%	7%	8%
Stage IV	0%	0%	0%

*Maat et al.[32]

For 45 patients, the HLA genotype was available. Information on the presence or absence of one chromosome 3 in the tumor was available for all 45 cases.[37] The research protocol followed the tenets of the Declaration of Helsinki (World Medical Association Declaration of Helsinki 1964, ethical principles for medical research involving human subjects). All patients signed an informed consent form.

Collection of Specific Data

The specific HLA genotype of each patient had been determined previously[27] and was retrieved from the patients' chart. All results of DNA-based techniques were transformed to the serological classification to obtain unified data, usable for statistical analysis.

Immunostaining of tumor cells of these patients for expression of HLA-A, HLA-B and HLA-DR had been performed previously and described by Maat et al.,[32] using the monoclonal antibodies (mAbs) HCA2, which stains exclusively HLA-A, and HC10, which binds to HLA-B and HLA-C.[38,39] Both antibodies were obtained from the Dutch Cancer Institute (Amsterdam, The Netherlands). For HLA-DR staining Tal.1B5 was used, obtained from DakoCytomation (Glostrup, Denmark).

Phenotypic characterization of macrophages and digital counting were performed previously by Bronkhorst et al.[31] using immunofluorescence double-staining with mAbs against CD68 and CD163. The amount of positive staining was calculated as pixels per mm^2.

Comparison of Data

To minimize loss of significance after correction for the number of tests performed, only common and well-defined HLA alleles with the highest frequency in 2440 healthy Dutch blood donors were selected for statistical analysis.[40] In detail, we selected three alleles for HLA-A (A1, A2, A3) and the two major grouping alleles for HLA-B (Bw4, Bw6), while for HLA-DR two alleles (DR4 associated with VKH, DR6) were used. Additionally, we selected alleles associated previously with uveal melanoma and bad prognosis (HLA-B40 and -B44), and two alleles associated with ocular autoimmune diseases (HLA-A29 and HLA-B27).

All statistical analyses were performed with a statistical software program (SPSS for Microsoft Windows, version 17.0.2; SPSS Inc., Chicago, IL). The hypothesis was tested with the Mann-Whitney U test (Wilcoxon rank test) for non-normal distribution for each specific HLA allele. For the comparison of each HLA allele with chromosome 3 status, the v^2 test was used. Statistical significance was assumed for a P value <0.05. Correction for multiple testing was performed according to the Bonferroni correction for the number of alleles (n = 8) tested.

Results

General Results

The HLA genotype of 45 patients was collected. Tissue specimens for macrophage staining were available for 38 patients. Tumor staging according to the pathologic TNM staging of the 7th edition of the American Joint Committee on Cancer International Union on Cancer (AJCC-UICC),[41] and sex and age distribution of these patients is shown in Table 1. The frequencies of the studied HLA alleles in patients with uveal melanoma were compared to those of healthy Dutch blood donors and showed a comparable distribution (Table 2).

The VKH-associated HLA-A29 was present only in four cases, while the autoimmune uveitis-related HLA allele B27 was not present in the population being studied. HLA-B40, associated previously with bad prognosis in uveal melanoma, is now split into B60 and B61. Only five patients carried B60, while no patients were positive for HLA-B61. Therefore, the relationship between these alleles and the degree of HLA expression or macrophage infiltration was not determined.

Expression of HLA I and II Molecules

Sections of uveal melanoma stained with HCA2 (HLA-A), HC10 (HLA-B and HLA-C) and Tal.1B5 (HLA-DR) had been analyzed previously. Figure 1 shows an example of a section stained with HCA2. The percentage of cells that reacted positively with the anti-HLA class I antibodies HC10 and HCA2 varied widely, with a mean of 43% for HCA2 (SD 29%) and 37% for HC10 (SD 31%). The mean percentage of HLA-DR-positive cells was 20% (SD 23%).

Staining for HCA2 correlated positively with HC10 staining ($P < 0.001$). Staining for Tal.1B5 was correlated with HCA2 and HC10 staining ($P = 0.01$ and $P = 0.005$, respectively, Spearman test).

Table 2. Distribution of the Selected HLA Alleles in the Study Group and a Dutch Blood Donor Database

	Study Group (*n* = 45)		Dutch Blood Donors (*n* = 2440)*		
HLA Allele	**Positive (*n*)**	**Positive (%)**	**Positive (n)**	**Positive (%)**	***P* Value**
A1	14	31	747	31	0.94
A2	24	53	1284	53	0.93
A3	16	36	700	29	0.31
A29	4	9	119	5	0.22
B27	0	0	157	6	0.11†
B44	10	22	586	24	0.78
B60 (B40)	5	11	361	15	0.49
B61 (B40)	0	0	73	3	0.64
Bw4	23	51	1346	55	0.59
Bw6	38	84	2143	88	0.49
DR4	13	29	679	28	0.88
DR6	14	31	796	34	0.83

P values for each allele are given (χ^2 test).

*Schipper et al.[40]

†Fisher's exact test was used due to zero positive cases in the study group.

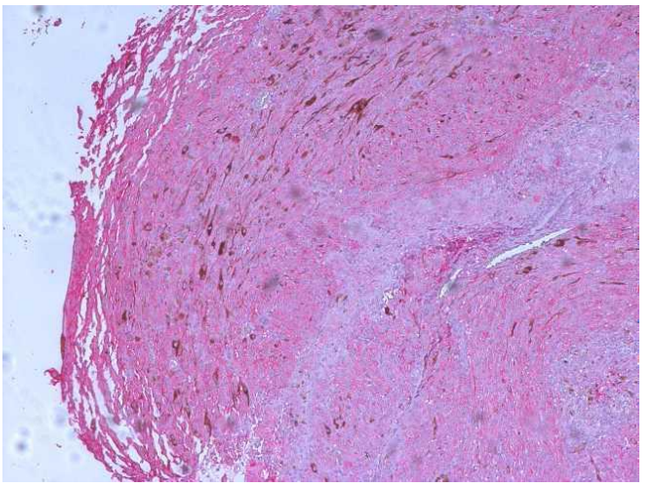

Figure 1. Immunohistochemical staining with the antibody HCA2 for HLA class I expression in uveal melanoma. 100x magnification.

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HLA Allele versus HLA Expression

As in autoimmune diseases some HLA alleles are associated with increased inflammation, we wondered if this were the case in uveal melanoma. Therefore, we compared staining of uveal melanoma cells for HCA2, HC10 and Tal.1B5 with the presence of frequently-occurring HLA alleles in 45 patients, as well as with alleles associated previously with prognosis in uveal melanoma, and alleles well-known for their association with ocular auto-immune diseases. HLA-A29 was present in only four cases, HLA-B27 in none, and HLA-B60 in five, and comparisons, therefore, were not feasible. HLA-B44 was present in 10 cases, but showed no correlation with HLA expression.

HLA-DR4 was associated almost significantly with a decreased expression of HLA-DR ($P = 0.06$), while HLA-DR6 was associated with a higher HLA-DR expression ($P = 0.04$). Significance was lost after correction for the total number of tests. An overview of HLA alleles versus HLA expression is shown in Table 3.

Macrophage Infiltration

Infiltration of macrophages had been assessed previously using immunofluorescence staining on paraffin-embedded sections of uveal melanomas from 38 patients. An example of this staining is shown in Figure 2. The presence of positive cells was measured as total amount of staining per section. The area of $CD163^{+}CD68^{+}$ double-staining associated with the area of $CD68^{+}$ (Spearman test, $P < 0.001$).

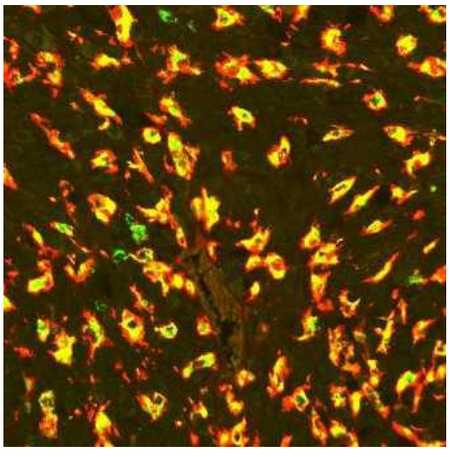

Figure 2. Immunofluorescence staining of macrophages in uveal melanoma using two antibodies directed against CD68 or CD163. Fluorescent staining of CD68 is shown as green and of CD163 as red. Overlay of both stainings showing double-positive cells are visualized in yellow. 250x magnification.

A comparison of $CD68^+$ cells with HLA expression showed that an increased density of macrophages was associated with an increased number of cells positive for staining with mAbs HCA2, HC10, and Tal1.B5 (respectively, $P = 0.02$, $P < 0.001$, and $P < 0.001$, Spearman test).

HLA Allele versus Macrophage Infiltration

The most common HLA alleles together with HLA B44 were compared with macrophage infiltration and phenotype. Of the class I HLA alleles, only where the HLA-A2 allele was present, less $CD68^+$staining ($P = 0.03$) as well as less $CD68^+CD163^+$ staining ($P = 0.02$) was observed. With regard to HLA-DR4 and -DR6, there was no increased or decreased macrophage infiltration compared to other alleles. When corrected for the total number of tests, significance was lost for all associations between HLA genotype and macrophage infiltration. An overview of the distribution of macrophage infiltration per HLA allele is shown in Figures 3 and 4, and the P values are displayed in Table 3.

HLA Allele versus Monosomy 3

Since we had shown previously that an inflammatory phenotype is associated with loss of one chromosome 3 in uveal melanoma tissue, we performed a comparison between the presence of HLA alleles and monosomy 3 in these 45 cases. No significant associations were found between any of the HLA alleles and monosomy 3 (supplementary data, Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11–8901/-/ DCSupplemental).

Discussion

Certain HLA polymorphisms predispose to immunological and neoplastic diseases. The frequencies of HLA alleles found in our study group correlated well with known data of HLA frequencies in the Caucasian population.[40,42–44] We analyzed the most common HLA alleles, as well as HLA alleles that were associated previously with bad prognosis in uveal melanoma, and some alleles that have a known association with ocular inflammation. Individuals with local autoimmune disease might be protected against the development of melanoma due to local immunosurveillance. However, the alleles HLA-A29 and HLA-B40 were very infrequent, and HLA-B27 was absent in the 45 patients studied. Patients positive for HLA-DR4 (a gene with an association with VKH) had a trend toward a lower HLA-DR expression on their tumor cells, while the presence of the HLA-DR6 allele was associated with a higher HLA-DR expression on uveal melanoma tumor cells. With regard to macrophage infiltration, only the presence of the HLA-A2 allele correlated with less macrophage infiltration. After correction for the number of analyses, statistical significance was no longer present. Our study was performed to determine a possible relationship between HLA genotype and the level of HLA expression, and the amount of macrophage infiltration or loss of chromosome 3 in uveal melanoma. To study these relationships, we analyzed common alleles, as the results otherwise most likely would be insignificant due to necessary corrections for the number of analyses. Focusing on a

Table 3. HLA allele versus HLA expression and macrophage infiltration

			HLA-A expression (% HCA2+)			HLA-B and C expression (% HC10+)			HLA-DR expression (% Tal.1B5+)				CD68+ cells (mm^2x10^4)			CD68+CD163+ (mm^2x10^4)		
		n=45										*n*=38						
HLA allele		*n*	**Mean**	***P***	***P****	**Mean**	***P***	***P****	**Mean**	***P***	***P****	*n*	**Mean**	***P***	***P****	**Mean**	***P***	***P****
A1	**neg**	31	40			35			22			27	12.9			10.3		
	pos	14	51 ↑	.23	1.00	44 ↑	.57	1.00	18 ↓	.82	1.00	11	12.7↓	.83	1.00	9.1 ↓	.55	1.00
A2	**neg**	21	43			45			23			19	15.4			12.3		
	pos	24	44 ↑	.96	1.00	31 ↓	.12	.95	19 ↓	.55	1.00	19	10.3↓	**.03**	.26	7.6 ↓	**.02**	.14
A3	**neg**	29	41			34			19			23	11.9			8.8		
	pos	16	49 ↑	.30	1.00	43 ↑	.24	1.00	24 ↑	.36	1.00	15	14.4↑	.24	1.00	11.7 ↑	.14	1.00
B44	**neg**	35	43			38			19			31	13.0			9.9		
	pos	10	45 ↑	.82	1.00	34 ↓	.73	1.00	26 ↑	.33	1.00	7	12.0↓	.99	1.00	10.2 ↑	.87	1.00
Bw4	**neg**	22	44			38			18			20	12.7			9.7		
	pos	23	43 ↓	1.0	1.00	37 ↓	.67	1.00	23 ↑	.68	1.00	18	13.1	.75	1.00	10.1 ↑	.93	1.00
Bw6	**neg**	7	42			36			31			5	10.1			8.6		
	pos	38	44 ↑	.99	1.00	38 ↑	.90	1.00	19 ↓	.55	1.00	33	13.3↑	.59	1.00	10.1 ↑	.62	1.00
DR4	**neg**	32	44			40			22			29	13.8			10.5		
	pos	13	43 ↓	.88	1.00	32 ↓	.46	1.00	17 ↓	.06	.46	9	9.9↓	.19	1.00	8.0 ↓	.20	1.00
DR6	**neg**	31	47			37			16			26	13.2			9.8		
	pos	14	36 ↓	.32	1.00	39 ↑	.70	1.00	31 ↑	**.04**	.33	12	12.2↓	.85	1.00	10.3 ↑	.68	1.00

HLA expression and amount of macrophage infiltration per HLA allele. All tests are performed with the Mann-Withney-U (Wilcoxon rank) test.

Arrows represent graphically whether there was a higher or lower expression or a higher or lower amount of cells, per positive HLA allele.

* P-value corrected for number of tests (*n*=8).

limited number of frequently-occurring alleles would reveal population-relevant correlations.

The relationship that we observed between the HLA-DR4 and the HLA-DR6 allele, and level of HLA-DR expression could suggest that certain HLA genotypes influence the expression of HLA on tumor cells. Since we know that a high expression of HLA classes I and II are prognostic factors for metastasis in uveal melanoma,[13,15] this would be expected. However, the tendency to a lower expression associated with HLA-DR4 is surprising, as this gene is associated with inflammation through its link with VKH.

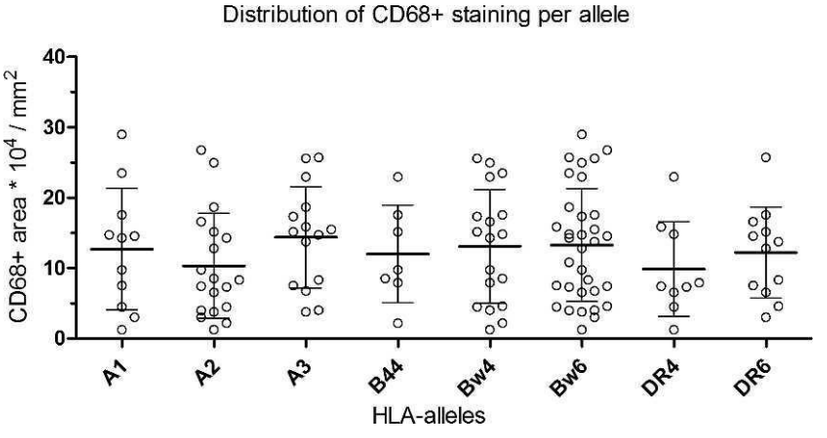

Figure 3. Distribution of CD68-positive staining for the most common HLA alleles. Error bars represent the SD of the mean.

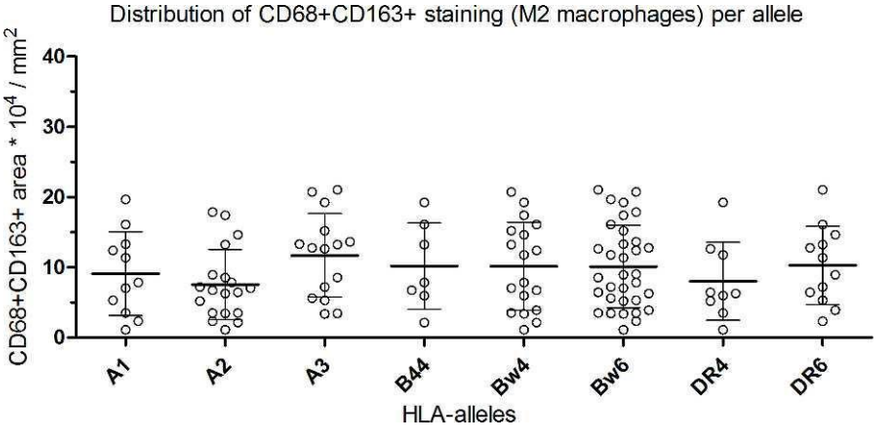

Figure 4. Distribution of CD68 and CD163 positive staining, representing the M2 macrophages, for the most common HLA alleles. Error bars represent the SD of the mean.

It is known that different mechanisms lead to HLA down regulation, which may be allele specific or haplotype specific.[45] While mutations in the β2-microglobulin (β2M) gene can be responsible for lack of HLA class I expression in colon carcinoma and cutaneous malignant melanoma,[46–48] in uveal melanoma no complete loss of β2M has been found, although a significantly decreased expression of

β2M has been described[15] and is associated with a favorable outcome.[49] Loss of chromosome material due to defective chromosome segregation or mitotic recombination may be another mechanism for loss of HLA expression. However, a study on the presence of loss of heterozygosity (LOH) in the area on chromosome 6 that codes for the HLA class I genes did not find a correlation of LOH with HLA-A or -B expression.[50] Up regulation of HLA classes I and II expression also is mediated by cytokines, such as interferon-alpha and –gamma,[45,51] which are released in the presence of an inflammatory infiltrate. Inflammation often is found in uveal melanoma and is associated with a worse prognosis.[18,52]

The observed correlation between HLA-A2 and fewer infiltrating macrophages in uveal melanoma is difficult to interpret. Less macrophage infiltration is associated with a better prognosis.[29–31] Thus, the association between being positive for the HLA-A2 allele and fewer infiltrating macrophages pleads for a protective function of the HLA-A2 allele in uveal melanoma. However, studies have shown that a direct association for HLA-A2 with survival in uveal melanoma does not exist.[26,27] This does not exclude any influence of HLA-A2 on macrophage infiltration and, to prove this, studies with a larger patient population should be undertaken.

No cases were positive for HLA-B27, which is known for its association with several autoimmune diseases, that is ankylosing spondylitis and anterior uveitis. This most likely is due to the relatively small study group, as a previous association study showed that 7% of 235 uveal melanoma patients were positive for HLA-B27.[27] We also looked at HLA-A29 because of its association with VKH, and at the HLA-B alleles B44 and B40 (now B60 and B61), as earlier studies showed an increased risk for metastases with these alelles,[26,27] although no association with an increased susceptibility to uveal melanoma was seen.[53] However, most of these alleles occurred with a frequency that was too low for a useful comparison. Where possible, we did analyze relations with these alleles, and did not find a correlation with HLA expression and macrophage infiltration or chromosome 3 status.

Understanding the pathological mechanism of HLA expression and macrophage infiltration in uveal melanoma is important, since both are related to prognosis. Our study shows that with the possible exception of an association between HLA-A2 and fewer infiltrating macrophages, and between HLA-DR6 and increased HLA-DR expression, HLA genotype does not determine the amount of HLA expression and macrophage infiltration or chromosome 3 status in uveal melanoma. It is important to look at these findings in the light of multiple testing, as these associations were observed before correction of the P value because of the number of tests performed. A second study with larger patient population, analyzing HLA genotypes with HLA expression and macrophage infiltration, will be essential to determine whether the associations we observed were valid.

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