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Normal HLA Class I, II, and MICA gene distribution in uveal melanoma

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Purpose: The molecules of the HLA class I and II molecules as well as the MHC class I chain-related gene A (MICA), a polymorphic and stress-induced cell surface molecule, are involved in T-cell and natural killer-cell (NK-cell) mediated immune responses. In this study we looked for any genetic susceptibility contributed by HLA class I, class II, or MICA genes with regard to the development of uveal melanoma.
Methods: Between 1998 and 2001, 159 uveal melanoma patients were typed for HLA class I and II, and 168 uveal melanoma patients were evaluated for MICA by microsatellite typing. The HLA antigen and MICA allele frequencies were compared with control groups of, respectively, 2,440 and 247 healthy Dutch individuals.
Results: HLA class I, HLA class II, and MICA gene frequencies in uveal melanoma patients and healthy Dutch controls showed no significant deviations after correction for the number of comparisons.
Conclusions: We conclude that there is no genetic susceptibility or increased risk attributed to any HLA class I, class II, and MICA polymorphism with regard to the development of uveal melanoma.

The human leukocyte antigen (HLA) complex, located on chromosome 6, plays an important role in the cellular immune response to tumor antigens. The function of cytotoxic T lymphocytes (CTLs) and of natural killer (NK) cells is directly influenced by expression of HLA antigens on tumor cells [1-3]. Many types of tumors use downregulation of HLA expression as an effective mechanism to escape immune surveillance [1,4]. NK cells on the other hand are able to kill tumor cells that have lost their specific HLA class I cell-surface molecules. The most successful tumor cells are those cells that downregulate HLA in such a way that they are not recognized by specific CTLs but still have sufficient HLA expression to avoid NK cells. Uveal melanoma cells, however, are in this matter completely different from most other tumors. In uveal melanoma, downregulation of HLA class I is not associated with tumor cell escape and disease progression, but with a favorable patient outcome [5,6]. This suggests that NK cells play a protective role in the development of metastatic disease by killing tumor cells with a low HLA class I expression. In addition, not only expression of HLA class I, but also expression of HLA class II was found to be related to a worse survival in uveal melanoma [6].

The outstanding feature of the HLA genes is their extensive polymorphism. This polymorphism defines the repertoire of (tumor-derived) peptides that bind to HLA allotypes and subsequently influences the activation of specific CTLs. In the literature, a relatively small, but growing number of studies indicates that, apart from the quantitative level of HLA expression, also HLA polymorphisms mediate susceptibility to several neoplastic diseases [7,8]. In nonmelanoma skin cancer, HLA-A3, HLA-B27, and HLA-DR7 have been associated with an increased risk of developing disease, whereas HLA-A11 and HLA-DR4 were associated with a decreased risk [9,10]. The presence of HLA-B40 was observed to be related to both the development and the clinical progression of cutaneous melanoma [11]. In uveal melanoma, associations with certain HLA class I and II antigens have been described, too. However, the findings are contradictory, with the exception for a significantly increased incidence of HLA-A32 in two studies [12-15]. Similar to the situation in cutaneous melanoma, the presence of HLA-B40 was correlated with poor survival in uveal melanoma patients [16].

Recently, a new family of HLA class I-like molecules has been identified, MHC class I chain-related gene A (MICA) [17,18], located approximately 50 kb centromeric to the HLA-B gene. Similarly to the HLA class I genes, the MICA gene encodes for an α chain with three extracellular domains (a1, a2, and a3), a transmembrane segment, and a cytoplasmic tail. However, MICA is highly divergent from the other HLA class I molecules: MICA proteins lack a CD8 binding site; they do not associate with β2 microglobulin and do not need to be stabilized by peptide binding. MICA molecules are frequently expressed on monocytes, endothelial cells, epithelial cells, fibroblasts, and keratinocytes [19]. Expression of MICA has been described for several epithelial tumors [20] and for cutaneous melanoma [21]. As MICA expression is upregulated after heat shock treatment, MICA molecules are considered to play a role in the immunological elimination of stress-induced damaged cells [18,22,23]. Recently, MIC-A/B expression was

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observed on 50% of primary uveal melanomas [24]. It was not expressed on most metastatic lesions. Yet there was one intriguing case: A subcutaneous metastasis taken from a patient prior to chemotherapy showed no MIC-A/B expression; however, following treatment with systemic fotemustine, a second metastasis was removed. This metastasis showed a positive MIC-A/B expression and an abundant infiltrate with NKG2D positive lymphocytes. Since MICA activates immune cells via the NKG2D receptor, expressed on γδ T cells, CD8 αβ T cells, and NK cells, this suggests that chemotherapy may lead to phenotypical changes which stimulate immunological surveillance, helping the removal of transformed, infected, or damaged cells, and that MIC-A/B may be involved in this process [22,25].

Associations between MIC genes and several types of cancer have been described: In cervical intraepithelial neoplasia and nonmelanoma skin cancer, this polymorphism was not associated with an increased cancer risk, while in breast cancer a certain extended haplotype that included MICA was associated with an increased risk of developing this malignancy [26-28]. In oral squamous cell carcinoma, an increase of the MICA A6 allele frequency was observed, while in gastric cancer an association with the genetically determined presence of MICA type A9 was observed [29,30]. In uveal melanoma, to our best knowledge, MICA polymorphism has not yet been investigated.

The aim of the present study was to examine the role of HLA class I, class II, and MICA polymorphism in uveal melanoma. Early studies on this topic, presenting contradictory results [12-15], may be outdated due to low patient numbers, limited number of HLA antigens tested, and a less accurate determination of HLA type. In addition, during the last decade, the introduction of molecular HLA typing methods resulted in a better definition and characterization of specific HLA antigens and alleles. We set out to determine if a susceptibility gene or a protective gene for uveal melanoma is located among the HLA class I or II region or within the MICA genes. Therefore, we determined the HLA-A, HLA-B, HLA-C, HLA-DR, HLA-DQ, and MICA allele distribution in, respectively, 159 and 168 Dutch uveal melanoma patients in comparison to a large panel of healthy Dutch individuals.

METHODS

HLA-typed patients and controls: Between 1999, and 2001, 159 consecutive Dutch patients diagnosed with primary choroidal and/or ciliary body melanoma at the Department of Ophthalmology of the Leiden University Medical Center (LUMC) were typed for HLA class I and II, either by a complement-dependent cytotoxicity test (60 patients) or by DNA-based techniques (99 patients). Of the 159 patients, 92 were women and 67 were men. Age range was 26-84 years, with a mean age at diagnosis of 60.85 years. Sixty-six of these patients were treated by enucleation. The other patients received ruthenium brachytherapy in combination with transpupillary thermotherapy. The control group consisted of 2,440 healthy Dutch blood donors. For this group, the HLA gene frequencies are extensively analyzed and well controlled. This group is considered to be an excellent representation of the HLA gene distribution in the Dutch population [31].

MICA-typed patients and controls: The 168 uveal melanoma patients analyzed for MICA gene variants visited the Department of Ophthalmology of the LUMC between April 1998 and March 1999. All patients, 73 male and 95 female, were diagnosed as having primary choroidal and/or ciliary body melanoma at the age of 7-82 years, with a mean age of 58.96 years. Twenty-one of these patients were treated by enucleation. The other patients received ruthenium brachytherapy in combination with transpupillary thermotherapy. The MICA-typed population shared 114 patients with the HLA-typed group (see Figure 1). The control group of 247 healthy persons was recruited from the Ophthalmology Outpatient Clinic of the LUMC. Exclusion criteria were the xeroderma pigmentosum (XP) syndrome, presence of cutaneous melanoma, and being non-Caucasian. This control group has been used in several LUMC cancer studies [27,32]. In both studies the research protocol followed the tenets of the Declaration of Helsinki.

HLA-typing: Sixty patients were typed for HLA class I (A, B, C) and II (DR, DQ) by complement-dependent cytotoxicity test. In the remaining 99 patients, HLA-genotyping was performed on genomic DNA, isolated from peripheral blood leukocytes by ARMS PCR.

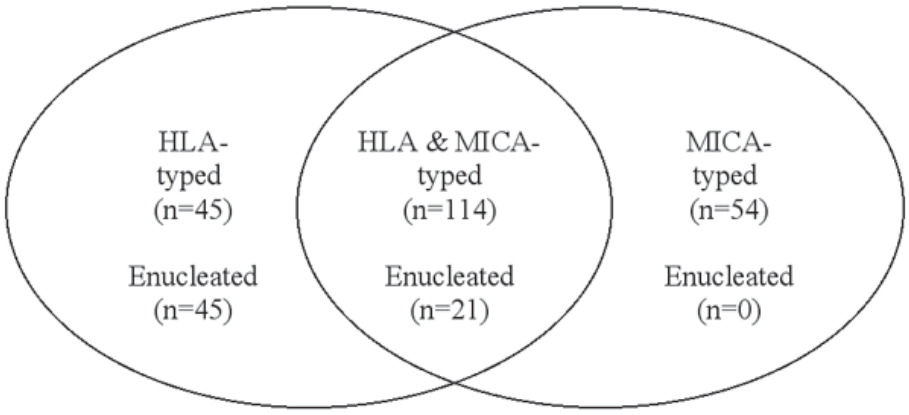

Figure 1. Composition of study populations. The HLA-typed study group consisted of 159 uveal melanoma patients, of which 66 were enucleated. The MHC class I chain-related gene A (MICA)-typed study group consisted of 168 uveal melanoma patients, of which 21 were enucleated.

MICA-microsatellite typing: Genomic DNA from 168 uveal melanoma patients was extracted from peripheral blood leukocytes and adjusted to 100 ng/µl. The following primers were used: flanking primer, MICA forward 5F 5'-CCT TTT TTT CAG GGA AAG TGC-3'; reverse MICA 5R 5'-CCT TAC CAT CTT CCA GAA ACT GC-3'. The 5F primer was labeled with fluorescent amadite (Isogen Bioscience, Maarssen, The Netherlands). The forward primer corresponds to the boundary region with 12 nucleotides in intron 4, whereas the reverse primer is located in intron 5 of the transmembrane region.

The amplification was performed in 50 µl reaction volume containing 1 µl genomic DNA, 25 pmol of each primer, 2.5 units Taq Polymerase (Perkin Elmer Nieuwerkerk a/d IJssel, the Netherlands). The amplification buffer contained 50 mM KCl, 10 mmol Tris-HCl (pH 8.4, at room tempera-

Table 1. Distribution of HLA-A, HLA-B, and HLA-C antigens in uveal melanoma patients and healthy Dutch blood donors

	Uveal melanoma patients (n=159)		Dutch blood donors (n=2440)				
HLA allele	Number positive	Percent positive	Number positive	Percent positive	Odds ratio	p uncorrected	p corrected
A1	50	31	747	31	1.044	0.8592	1.0000
A2	88	55	1284	53	1.114	0.5132	1.0000
A3	37	23	700	29	0.760	0.1473	1.0000
A11	16	10	281	12	0.880	0.6994	1.0000
A23 (A9)	4	3	60	2	1.132	1.0000	1.0000
A24 (A9)	36	23	403	17	1.481	0.0630	0.9970
A25 (A10)	5	3	46	2	1.832	0.2375	1.0000
A26 (A10)	8	5	107	4	1.218	0.6889	1.0000
A28	9	6	244	10	0.566	0.0735	0.9989
A29 (A19)	7	4	119	5	0.955	1.0000	1.0000
A30 (A19)	5	3	85	3	0.979	1.0000	1.0000
A31 (A19)	11	7	146	6	1.211	0.6060	1.0000
A32 (A19)	7	4	149	6	0.753	0.4903	1.0000
A33 (A19)	2	1	32	1	1.174	1.0000	1.0000
B5	21	13	289	12	1.154	0.6131	1.0000
B7	37	23	668	27	0.812	0.2710	1.0000
B8	43	27	554	23	1.270	0.2069	1.0000
B12	36	23	617	25	0.872	0.5092	1.0000
B13	10	6	109	4	1.495	0.3230	1.0000
B14	4	3	70	3	0.972	1.0000	1.0000
B17	14	9	162	7	1.397	0.3257	1.0000
B18	15	9	158	6	1.545	0.1412	1.0000
B21	2	1	54	2	0.694	0.5791	1.0000
B27	12	8	157	6	1.229	0.6168	1.0000
B35	25	16	429	18	0.887	0.5916	1.0000
B37	2	1	99	4	0.373	0.0880	0.9997
B38 (B16)	7	4	98	4	1.169	0.8340	1.0000
B39 (B16)	9	6	79	3	1.873	0.1104	1.0000
B41	3	2	28	1	1.892	0.4338	1.0000
B55 (B22)	7	4	105	4	1.085	0.8422	1.0000
B56 (B22)	3	2	33	1	1.601	0.4826	1.0000
B60 (B40)	16	10	361	15	0.659	0.1045	0.9999
B61 (B40)	5	3	73	3	1.132	0.8130	1.0000
B62 (B15)	29	18	370	15	1.263	0.3065	1.0000
B63 (B15)	2	1	12	0	3.079	0.2101	1.0000
CW1	12	8	119	5	1.550	1.1990	1.0000
CW2	15	9	240	10	0.919	0.7884	1.0000
CW3	44	28	795	35	0.728	0.0836	0.9996
CW4	31	19	514	22	0.855	0.4889	1.0000
CW5	22	14	312	14	1.034	0.9053	1.0000
CW6	28	18	373	17	1.026	0.9141	1.0000
CW7	93	58	1102	51	1.344	0.0840	0.9996

The “p corrected” column represents the correction of p values for the number of comparisons (93) conducted according to the method of Edwards [35].

ture), 2 mM $MgCl_2$, 1.25% glycerol, and 200 μM of each dNTPs. The amplification was performed in two steps to increase the specificity of primer annealing during the first 8 cycles on a Peltier Thermal Cycler (PTC-200 MJ research). The PCR was carried out as a touch down amplification. The reaction mixture was subjected to one cycle of denaturation at 95 °C for 5 min followed by 8 cycles of denaturation at 94 °C for 30 s, and the annealing temperature of 68 °C was decreased by 1 °C per cycle in the first 8 cycles. Extension was performed at 72 °C for 30 s. After the 8 cycles the amplification was done for 30 cycles at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. Finally, the PCR was finished by an extension at 72 °C for 5 min. The PCR products were visualized on 2% agarose gels.

For accurate allele sizing, four internal allelic ladders and two external size markers covering the range of the MICA microsatellites were used. The reaction product was loaded into the wells of a standard denaturing sequencing gel (6.0% polyacrylamide, 0.6X TBE, 280x30x0.5 mm) in an Automated Laser Fluorescence-DNA Sequencer (ALF™, Amersham Pharmacia Biotech, Roosendaal, the Netherlands). Electrophoresis (2000 V, 50 mA, 50 W, and 3 mW laser power at 50 °C) and data collection was carried out using the ALF Manager software (Amersham Pharmacia Biotech). The data were analyzed by Fragment Manager software (Amersham Pharmacia Biotech).

Statistical analysis: The significance of differences in the HLA antigen frequencies in uveal melanoma patients compared to the control population was assessed using the Woolf-Haldane analysis [33,34]. Correction of p values for the number of comparisons (93) was done using the method of Edwards [35]. Odds ratios were calculated as an estimation of the relative risks. Data were further analyzed using an exact (stratified) 2x2 analysis and by stepwise logistic regression (multivariate analysis).

Fisher's exact test was used to compare MICA allele frequencies in uveal melanoma patients and healthy controls. To correct for the number of comparisons, the p values were multiplied by five (number of MICA alleles).

RESULTS

Distribution of HLA class I and II alleles: The frequencies of HLA class I (A, B, C) and class II (DR, DQ) alleles were analyzed in 159 Dutch uveal melanoma patients and in 2440 healthy Dutch individuals. In total, 93 different alleles (including the split antigens) were determined. A tendency ($p<0.1$) for an increased allele frequency in uveal melanoma patients was observed for HLA-A24 and HLA-Cw7, while the frequencies of HLA-A28, HLA-B37, HLA-Cw3, and HLA-DQ5 tended to be decreased (Table 1, Table 2). The HLA-DQ4 allele was significantly decreased ($p=0.0129$) in the patient group, but the number of healthy individuals tested for this antigen was only 915. However, after correction for the number of comparisons, the p value was close to 1.00 for all HLA alleles. Also in the multivariate analysis, no significances were found. In Table 1 and Table 2, the distribution of the HLA antigens is summarized.

We also analyzed the presence of certain HLA class I or II alleles in relation to several clinicopathological parameters (e.g., cell type, tumor diameter, prominence, mitotic rate, pres-

Table 2. Distribution of HLA-DR and HLA-DQ antigens in uveal melanoma patients and healthy Dutch blood donors

	Uveal melanoma patients (n=159)		Dutch blood donors (n=2440)				
HLA allele	Number positive	Percent positive	Number positive	Percent positive	Odds Ratio	p uncorrected	p corrected
DR1	24	15	473	20	0.734	0.1782	1.0000
DR3	46	29	599	25	1.228	0.2995	1.0000
DR4	44	28	679	28	0.974	0.9276	1.0000
DR7	33	21	459	19	1.117	0.6044	1.0000
DR8	13	8	128	5	1.624	0.1483	1.0000
DR10	4	3	100	4	0.661	0.4074	1.0000
DR11 (DR5)	21	13	340	14	0.937	0.8145	1.0000
DR12 (DR5)	5	3	108	5	0.743	0.5508	1.0000
DR13 (DR6)	48	30	669	28	1.096	0.6502	1.0000
DR14 (DR6)	9	6	127	5	1.094	0.8566	1.0000
DR15 (DR2)	47	30	414*	26	1.231	0.2964	1.0000
DR16 (DR2)	3	2	43	2	1.177	1.0000	1.0000
DQ2	64	40	881	1479	1.134	0.4985	1.0000
DQ4	12	8	29*	3	2.547	0.0129	0.6856
DQ5 (DQ1)	39	25	300*	35	0.619	0.0132	0.6945
DQ6 (DQ1)	83	52	453*	50	1.087	0.6674	1.0000
DQ7 (DQ3)	40	25	652	28	0.877	0.5216	1.0000
DQ8 (DQ3)	27	17	184*	20	0.817	0.3882	1.0000
DQ9 (DQ3)	14	9	71*	8	1.177	0.6340	1.0000

The "p corrected" column represents the correction of p values for the number of comparisons (93) conducted according to the method of Edwards [35]. The asterisk (*) indicates that the number of healthy individuals typed for this specific allele is fewer than 2,440.

ence of vascular loops) of 64 uveal melanomas obtained by enucleation. After correction for the number of comparisons, no significant associations were present (data not shown).

Distribution of MICA gene variants: As MICA associations have been found with regard to other malignancies, we wondered whether any MICA antigen occurred more frequently in uveal melanoma patients.

The MICA allele frequencies were determined in 168 uveal melanoma patients and 247 healthy controls (Table 3). Triplet repeat polymorphisms in the transmembrane region identified as A4, A5, A5.1, A6, and A9 were evaluated in both groups. Compared to the other alleles, the A5.1 allele was the most common allele present. The MICA A5 allele frequency was significantly decreased in the patient group (p=0.030). However, after correction of the p value for the number of comparisons, this difference was no longer significant (Table 3). Between the two groups, no other significant differences were observed, suggesting that MICA gene polymorphism is not associated with an increased risk for the occurrence of uveal melanoma.

DISCUSSION

In the present study, we examined the role of HLA class I polymorphism in uveal melanoma. A direct disease association with a specific HLA allele suggests that the function of the HLA molecule encoded by this allele may differ significantly from the molecules encoded by alternative alleles in its ability to present tumor antigens to T lymphocytes [8]. Therefore, it is not surprising that numerous associations have been established between HLA type and immune-mediated diseases. Also in cancer, a relatively small number of studies have established HLA-related susceptibility [7,8]. However, in these publications relative risks are low, even for virally associated cancers, such as cervical neoplasia (human papillomavirus) and Hodgkin's lymphoma (Epstein-Barr virus) [7,8,36,37]. In a number of studies, associations found between HLA type and cancer lack significance after correction of the p value for the number of antigens studied [11,13,14,38]. Our study on HLA polymorphisms and uveal melanoma contained the largest number of patients tested up to now. Studying 159 uveal melanoma patients and 2,440 healthy Dutch blood donors, we did not find any significant HLA associations. This is in agreement with a previous study by Völker-Dieben et al. [14], but in contrast with two other studies that described an increased frequency of HLA-A32 [13,15]. In our study, the frequency of HLA-A32 was low (4% in the patient group and 6% in the control group) with no significant difference (uncorrected p=0.4903, Table 1). It should be noted again that direct comparison of these studies is complicated because of differences in sample sizes, experimental designs, statistical analyses, composition of control groups and improved laboratory methods for HLA typing.

However, these findings do not rule out an important role for HLA class I or II in the development of metastases. An increased expression of HLA class I is associated with a worse prognosis, and a high HLA class I expression has been found to inhibit NK-cell mediated lysis [5,6,39]. As NK cells express a wide range of killing receptors that recognize specific HLA alleles only, it is most likely that when one looks specifically at such HLA alleles and survival, associations will appear.

The present study also analyzed the role of MICA polymorphism in uveal melanoma. A comparison of the MICA allele distribution of 168 uveal melanoma patients with 247 healthy controls revealed no significant deviations, suggesting that MICA gene polymorphisms do not contribute to the susceptibility to develop uveal melanoma. However, this does not exclude a possible role of MICA molecules in the progression or metastatic development of uveal melanoma. MICA has been shown to activate NK cells via engagement of the NKG2D receptor [22,40]. Moreover, in a recent study, resistance of melanoma cell lines to NK cell-mediated lysis was associated with a lack of MICA expression [41]. However, MICA-triggered NK cell-mediated lysis of tumor cells can be inhibited by co-expression of the nonclassical HLA-G molecule [40]. As already mentioned in relation to HLA class I expression, NK cells are believed to play a protective role in the development of metastatic disease in uveal melanoma [5,6,42,43]. Fortunately, uveal melanoma cells do not express HLA-G [44,45], but according to Vetter et al., primary tumors do often express MIC-A/B [24]. MICA expression by uveal melanoma cells may be a positive tumor characteristic because it can provide additional stimulation of NK cells [46], and while we did not see an association between the development of uveal melanoma and the presence of any HLA or MICA polymorphism, the important role of NK cells in removing uveal melanoma metastases prior to spreading may potentially indicate a role for polymorphisms in the formation of metastases. This can only be analyzed in future studies, as death from uveal melanoma metastases occurs quite late.

In conclusion, although HLA antigens, and possibly also MICA antigens, are important players in the immune surveillance against uveal melanoma, our data show that HLA and MICA polymorphisms do not contribute to an increased ge-

Table 3. Allele frequency of the MICA gene in uveal melanoma patients and in healthy controls

MICA allele	Uveal melanoma patients (n=168)	Healthy controls (n=247)
A4	40 (11.9%)	44 (8.9%)
A5	23 (6.8%)	56 (11.3%)
A5.1	172 (51.2%)	256 (51.8%)
A6	52 (15.5%)	69 (14.0%)
A9	49 (14.6%)	69 (14.0%)
	336 (100%)	494 (100%)

The data in this table show that no significant differences were found in the distribution of MICA gene variants between uveal melanoma patients and healthy controls.

netic susceptibility to this tumor. This does not exclude a role for HLA antigens in the development of metastases.

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