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ORIGINAL ARTICLE

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Genetic polymorphism of NK receptors and their ligands in melanoma patients: prevalence of inhibitory over activating signals

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Abstract Antitumor cytotoxicity of NK cells and T cells expressing NK-associated receptors is regulated by interaction between their cell surface killer immunoglobulin-like receptors (KIRs) and CD94/NKG2 heterodimers with MHC class I ligands on target cells. To test the hypothesis that KIR and/or HLA polymorphisms, and KIR/HLA combinations could contribute to the tumorigenesis, association studies were performed in 50 patients with malignant melanoma (MM) in different stages of disease and 54 controls. Our data showed that the frequency of inhibitory and activating KIR genes and KIR genotypes did not differ significantly between healthy individuals and melanoma patients. HLA haplotype distribution showed statistically significant increased frequencies of A*01-B*35-Cw*04 (0.069 vs 0.000; $pc < 0.05$; OR = 19.9), A*01-B*08-DRB1*03 (0.079 vs 0.019; $pc < 0.05$; OR = 4.5), and A*24-B*40-DRB1*11 (0.026 vs 0.000; $pc < 0.05$; OR = 7.1) in melanoma patients compared with healthy controls. Individuals homozygous for group 2 HLA-C ligands were less frequent in the patient group compared with the control cohort (12% vs 31.5%; $p < 0.017$). In addition, we observed an increased frequency (88.0% vs 68.5%; $p = 0.017$; OR = 2.80) of KIR2DL2/2DL3 in combination with their group 1 HLA-C ligands, while the presence of these KIRs in the absence of the putative ligands was decreased (12.0% vs 31.5%; $p = 0.017$) in the patient group. Furthermore, an increased frequency of activating KIR2DS1 in the absence of the putative HLA-C^{Lys80} ligands was found in melanoma patients (16.0% vs 9.2%). In contrast, KIR2DS2 was absent in patients more often (38.0% vs 25.9%) when the presumptive HLA-C^{Asn80} ligands were present. A slightly higher incidence of KIR3DL1 in combination with the less effective $Bw4^{Thr80}$ ligands was seen in patients with primary (20.8%) compared with metastatic (4.2%) disease. The data obtained in this study imply that there may not be a direct association between KIR gene content in the genome and the presence of malignant melanoma, or melanoma progression. However, some HLA haplotypes could be predisposing to MM in the Bulgarian population. Furthermore, distinct KIR/HLA ligand combinations may be relevant to the development of malignancy whereby inhibition overrides activation of NK cells and T cells expressing NK-associated receptors, which in turn might facilitate tumor escape and progression.

Keywords HLA · KIR · Malignant melanoma · Tumor escape

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Introduction

The immune surveillance hypothesis proposed that the immune system surveys the body for nascent malignancies, eliminating many or most tumors, and possibly slowing the growth of others. However, tumor cells succeed in eluding immune surveillance using several different routes. In this tumor-host scenario, MHC class I antigens are emerging as leading players, because of the crucial interaction of HLA molecules with T- and NK-specific receptors present in both types of immune effector cells [33]. Tumors can escape MHC class I–restricted cytotoxic T-lymphocyte responses by down-regulating the expression of class I molecules on the tumor cell surface [13–15, 40]. However, loss of HLA class I expression renders tumor cells susceptible to lysis

by natural killer (NK) cells when inhibitory signals provided by HLA molecules disappear [16, 29]. Thus, regulation of HLA class I expression appears to determine whether self-tolerance or antitumor immunity occurs. NK cells and T cells expressing NK cell receptors (NKRs) can kill tumor cells in vitro and release cytokines that can activate and regulate other antitumor effector cells [19, 30, 34]. They could either be directly antimetastatic [11, 35] or suppress antitumor immunity [26, 36]. Antitumor cytotoxicity by NK cells and NKR^+ T cells is regulated by interaction between their cell surface killer immunoglobulin-like receptors (KIRs) and CD94/NKG2 heterodimers with MHC class I ligands on target cells.

HLA class I ligands have been identified for some KIRs. HLA-Bw4 are ligands for KIR3DL1, and thereby inhibit the lysis of target cells expressing Bw4 [17, 32]. The Bw4 molecules can be divided into two groups on the basis of whether isoleucine or threonine is present at position 80 ($Bw4^{Ile80}$and $Bw4^{Thr80}$). The ligand for 3DS1 has not been identified, but 3DS1 and 3DL1 share about 97% amino acid sequence similarity in their extracellular domains and they might share a similar set of ligands, as do other inhibitory and activating KIRs that have high sequence similarity [3]. In addition, residue 80 of the HLA-C molecule is crucial in determining binding specificity for receptors containing two immunoglobulin domains (KIR2D) [5, 9]. The genomic plasticity of the KIR region generates a remarkable source of individuality for the number, combination, and quality of KIR genes in different individuals. Since NK cells can express multiple KIR and/or CD94/NKG2 complexes, selection of NK cells may be based on the total set of receptors expressed, requiring the expression of at least one receptor recognizing a self-HLA class I allotype. Consequently, the balance between inhibitory and activating receptors may influence the ability of NK cells to kill tumor cells. In addition, recent studies have demonstrated that KIR receptor-ligand pairs are involved in the pathogenesis and progression of autoimmune [24, 39, 42] and infectious [23] diseases. To test the hypothesis that KIR and/or HLA polymorphisms, and KIR/HLA combinations could contribute to tumorigenesis, association studies were performed in patients with malignant melanoma (MM) in different stages of disease.

Material and methods

Patient and control characteristics

Fifty patients (26 men and 24 women) with malignant melanoma and 54 ethnically matched healthy controls (23 men and 31 women) from the Bulgarian population were studied for NK receptor polymorphism. HLA typing was performed on the melanoma patients and on 128 healthy individuals (68 men, 60 women), including the above-mentioned 54 control participants. The age was 41.1 ± 10.4 (mean ± SD) years for the patients and 43.9 ± 12.9 years for the controls. All patients sampled had a histological diagnosis of malignant melanoma and were treated and followed up at the Department of Dermatology at the National Center of Oncology, Sofia, Bulgaria. At the time of inclusion, according to the staging classification of the AJCC [1], patients were classified as follows: stage I ($n=9$), stage II ($n=20$), stage III ($n=15$), and stage IV ($n=6$). Exclusion criteria were history of other malignancy, or of autoimmune, inflammatory, or infectious chronic disease. All individuals included in the present study were unrelated and randomly selected. Samples were collected after obtaining prior informed consent.

DNA isolation

Genomic DNA was extracted from whole venous blood using the standard proteinase K digestion method followed by salting-out extraction and ethanol precipitation [25].

KIR genotyping

Generic KIR typing of genomic DNA was performed by the PCR-SSP method as described by Uhrberg et al. [38], with modifications [37]. Ten genes encoding inhibitory KIR (2DL1–2DL3 and 3DL1–3DL2) and activating KIR (2DS1–2DS4 and 3DS1) were detected.

HLA genotyping

HLA-A, HLA-B, HLA-C, and HLA-DRB genotyping was performed by PCR-SSP method using Olerup SSP typing kits.

Statistical analysis

HLA-A, HLA-B, HLA-C, and HLA-DRB1/3/4/5 allele frequencies were estimated by maximum-likelihood analysis using the Arlequin program version 1.1. The software provides a large set of methods and statistical tests, in order to extract the main genetic features of population samples. Standard deviations were calculated from 100 bootstrap iterations. Hardy–Weinberg equilibrium was tested by two methods: a hidden Markov chain with 100,000 steps, implemented in the Arlequin program and a conventional χ^2 heterozygosity test. Arlequin software was also used to estimate maximum-likelihood three-locus haplotype frequencies (HF) from genotypic data through an expectation-maximization algorithm. This procedure is an iterative process aiming at obtaining maximum-likelihood estimates of haplotype frequencies from multilocus genotype data. The principle of the EM algorithm is the following: (1) arbitrary (random) estimates of haplotype frequencies; (2) these

estimates are used to compute expected genotype frequencies for each phenotype, assuming Hardy–Weinberg equilibrium (the E-step); (3) the relative genotype frequencies are used as weights for their two constituting haplotypes in a gene-counting procedure leading to new estimates of haplotype frequencies (the M-step); (4) steps 2–3 are repeated until the haplotype frequencies reach equilibrium (do not change more than a predefined ε value). Comparison of the HLA allele and haplotype frequencies and KIR between the patients and the control group were made by χ^2-test and with the Fisher exact test when appropriate. The correction of Bonferroni for multiple tests was applied by multiplying p by the number of comparisons. Probability values were considered significant if $pc < 0.05$. Odds ratio (OR) estimate was calculated using the methods of Woolf and Haldane. Ninety-five percent confidence intervals were given.

Results

KIR frequencies

Analysis of KIR gene distribution in melanoma patients and healthy controls in the Bulgarian population revealed that gene frequencies ranged from 32% to 100%. The most frequent KIR genes in Bulgarians (Table 1) are KIR2DL1, 2DL3, 3DL1, and 2DS4, followed by 2DS2 and 2DL2. The remainder (KIR2DS1, 2DS3, 3DS1) were found in less than half of the samples. Our data showed that the frequency of inhibitory and activating KIR genes did not differ significantly between healthy individuals and melanoma patients (Table 1). To define further KIR patterns we determined all putative genotypes according to the A and B haplotype groups of Uhrberg et al. [38] and the model of Witt et al. [41]. The AA genotypes include 2DL1 and 2DL3, but not 2DL2, the BB genotypes include 2DL2, but not 2DL1 and 2DL3, and in the AB genotypes, all of these genes are present [41]. The results obtained showed that the genotype distribution was similar in both melanoma patients and control individuals. Half of the individuals had AA genotypes, followed by AB genotypes (Table 1). The BB genotypes were less frequent. No significant differences were found for KIR gene and genotype distribution between patients with primary (stages I and II) and metastatic (stages III and IV) melanoma (data not shown).

Table 1 Frequencies (%) of KIR genes in the Bulgarian population

	Melanoma patients ($n = 50$)	Control group ($n = 54$)
Inhibitory KIR		
2DL1	90.0	90.7
2DL2	54.0	50.0
2DL3	90.0	90.7
3DL1	90.0	90.7
3DL2	100	100
Activating KIR		
2DS1	38.0	37.0
2DS2	56.0	53.7
2DS3	32.0	27.8
2DS4	94.0	96.3
3DS1	34.0	38.9
KIR genotype		
AA	50.0	50.0
AB	42.0	40.7
BB	8.0	9.3

HLA associations

Since HLA molecules are implicated in many diseases, we compared the HLA-A, HLA-B, HLA-C, and HLA-DRB1 alleles and haplotypes between melanoma patients and healthy individuals (Table 2). No deviations from Hardy–Weinberg equilibrium were observed for HLA-A, HLA-B, HLA-C, and HLA-DRB1 loci in both patients and controls. The most frequent HLA alleles found in patients with MM were HLA-A*02, *01, *24; B*35, *51, *08; Cw*07, *04, *01; and DRB1*11, *16, *03, while in the healthy individuals the alleles A*02, *24, *01; B*35, *51, *18; Cw*07, *04, *12; and DRB1*11, *16, *03 were the most frequent. Increased frequencies of HLA-A*01, B*08, Cw*01, DRB1*11 alleles were observed in melanoma patients compared with controls. However, the differences were not statistically significant after Bonferroni p-value correction.

Study of HLA class I haplotypes showed 74 possible haplotypes in melanoma patients and 156 in the control group. The most frequent HLA-A-B-C haplotypes among patients were A*01-B*08-Cw*07 (HF, 0.079), A*01-B*35-Cw*04 (HF, 0.069), and A*02-B*51-Cw*01 (HF, 0.035), while the most common haplotypes among the controls were A*01-B*18-Cw*07 (HF, 0.043), A*03-B*35-Cw*04 (HF, 0.045), and A*01-B*08-Cw*07 (HF, 0.027). Seventy-seven possible HLA-A-B-DRB1 haplotypes were observed in melanoma patients and 172 in controls, respectively. The most frequent haplotypes found among the patient group were A*01-B*08-DRB1*03 (HF, 0.079), A*01-B*35-DRB1*11 (HF, 0.035), and A*02-B*51-DRB1*16 (HF, 0.035). Haplotypes A*02-B*18-DRB1*11 (HF, 0.027), A*02-B*51-DRB1*13 (HF, 0.023), and A*02-B*44-DRB1*01 (HF, 0.023) were observed with higher frequency in the controls. Comparisons of HLA haplotype distribution showed statistically significant increased frequencies of A*01-B*35-Cw*04 (HF, 0.069 vs 0.000; $pc < 0.05$; OR = 19.9), A*01-B*08-DRB1*03 (HF, 0.079 vs 0.019; $pc < 0.05$; OR = 4.5), and A*24-B*40-DRB1*11 (HF, 0.026 vs 0.000; $pc < 0.05$; OR = 7.1) in melanoma patients compared with healthy controls. These haplotypes might be considered to be predisposing to MM for our population.

Distribution of HLA-C KIR ligands in MM patients

Receptors of the KIR2DL and KIR2DS subfamilies recognize polymorphisms on HLA-C molecules.

Table 2 HLA class I and class II allele frequencies in melanoma patients and healthy controls. *AF* Allele frequency, *SD* standard deviation

Allele	Patients		Controls	
	AF	SD	AF	SD
HLA-A				
*01	0.1929	0.0169	0.0978	0.0162
*02	0.2456	0.0339	0.3086	0.0284
*03	0.1053	0.0339	0.0859	0.0191
*11	0.0439	0.0254	0.0547	0.0144
*23	0.0439	0.0010	0.0469	0.0133
*24	0.1754	0.0254	0.1367	0.0207
*25	0.0175	0.0042	0.0117	0.0064
*26	0.0351	0.0127	0.0625	0.0395
*29	0.0088	0.0000	0.0234	0.0077
*30	0.0175	0.0042	0.0195	0.0091
*31	0.0088	0.0042	0.0391	0.0140
*32	0.0263	0.0042	0.0429	0.0121
*33	0.0263	0.0169	0.0234	0.0094
*36	0.0000	0.0000	0.0078	0.0059
*68	0.0526	0.0042	0.0273	0.0111
*69	0.0000	0.0000	0.0039	0.0035
*80	0.0000	0.0000	0.0039	0.0038
HLA-Cw				
*01	0.1404	0.0123	0.0703	0.0170
*02	0.0965	0.0301	0.1016	0.0174
*03	0.0439	0.0012	0.0586	0.0124
*04	0.2016	0.0651	0.1679	0.0245
*05	0.0088	0.0011	0.0316	0.0117
*06	0.0789	0.0098	0.0898	0.0178
*07	0.2807	0.0256	0.2188	0.0227
*08	0.0351	0.0091	0.0273	0.0096
*12	0.0526	0.0074	0.1211	0.0211
*14	0.0351	0.0111	0.0313	0.0125
*15	0. 0175	0.0055	0.0391	0.0117
*16	0.0088	0.0011	0.0234	0.0104
*17	0.0000	0.0000	0.0156	0.0072
HLA-B				
*07	0.0702	0.0000	0.0469	0.0129
*08	0.0965	0.0037	0.0469	0.0134
*13	0.0175	0.0185	0.0156	0.0085
*14	0.0175	0.0037	0.0156	0.0069
*15	0.0351	0.0032	0.0235	0.0061
*18	0.0614	0.0185	0.0821	0.0201
*27	0.0351	0.0037	0.0468	0.0133
*35	0.2018	0.0000	0.1642	0.0226
*37	0.0088	0.0037	0.0000	0.0000
*38	0.0000	0.0000	0.0469	0.0144
*39	0.0088	0.0037	0.0234	0.0096
*40	0.0614	0.0185	0.0703	0.0094
*41	0.0000	0.0000	0.0117	0.0063
*44	0.0439	0.0037	0.0781	0.0171
*45	0.0088	0.0000	0.0000	0.0000
*47	0.0000	0.0000	0.0117	0.0061
*48	0.0088	0.0000	0.0000	0.0000
*49	0.0439	0.0074	0.0313	0.0111
*50	0.0263	0.0037	0.0195	0.0085
*51	0.1842	0.0259	0.1564	0.0232
*52	0.0088	0.0011	0.0273	0.0091
*53	0.0000	0.0000	0.0156	0.0082
*55	0.0088	0.0000	0.0156	0.0084
*56	0.0351	0.0037	0.0078	0.0062
*57	0.0175	0.0155	0.0273	0.0098
*58	0.0000	0.0000	0.0156	0.0071
DRB1				
*01	0.0965	0.0012	0.1211	0.0139
*03	0.1140	0.0211	0.0938	0.0111
*04	0.0614	0.0043	0.0820	0.0098
*07	0.0702	0.0051	0.0625	0.0031
*08	0.0175	0.0011	0.0273	0.0009
*09	0.0000	0.0000	0.0039	0.0037
*10	0.0088	0.0000	0.0117	0.0012
*11	0.3158	0.0211	0.2109	0.0309
*12	0.0175	0.0009	0.0078	0.0011
*13	0.0614	0.0051	0.0898	0.0131
*14	0.0351	0.0021	0.0703	0.0023
*15	0.0702	0.0032	0.0781	0.0013
*16	0.1316	0.0031	0.1406	0.0351

Therefore, their functional relevance depends on the HLA-C genotype of the individual. Specificity of inhibitory KIRs for HLA-C allotypes is dictated to a large extent by the presence of asparagine (group 1 HLA-C) or lysine (group 2 HLA-C) at position 80 of the HLA-C molecules. To examine whether patients with MM differ from the healthy subjects in the genomic representation of the presumptive KIR ligands, we performed comparative analysis according to the presence of group 1 or group 2 HLA-C alleles. Individuals homozygous for group 2 HLA-C ligands were less frequent in the patient group (12% vs 31.48%; $p < 0.017$) compared with the control cohort (Table 3). The patients bearing only group 1 HLA-C alleles (36.0% vs 29.63%) and those having both groups HLA-C ligands (52.0% vs 38.9%) were slightly increased in comparison with healthy individuals. Further, we analyzed the distribution of these alleles between the patients with primary and with regional lymph node and distant metastasis (data not shown). However, no significant differences were observed.

KIR/HLA combinations in MM patients

Since the NK receptor/HLA ligand pairs control NK-cell function, we evaluated their status in melanoma patients on the basis of presumptive KIR ligands. Thus, we analyzed the receptors 2DL1 and 2DS1 having as their ligands HLA alleles with the C2 epitope; the receptors 2DL2, 2DL3, and 2DS2 and the respective C1 epitope ligands; KIR3DL1 and its ligands—HLA-B alleles with HLA-Bw4 motif. Our data (Table 4) showed an increased frequency of activating KIR2DS1 in the absence of the putative HLA-C^{Lys80} ligands in melanoma patients (16.0%) compared with the healthy individuals (9.2%). In addition, KIR2DS2 was absent in patients more often (38.0%) than in controls (25.9%), when the presumptive HLA-C^{Asn80} ligands were present. However, these differences did not achieve statistical significance. With respect to the inhibitory KIRs we observed an increased frequency (88.0% vs 68.5%; $p = 0.017$; OR = 2.80) of KIR2DL2/2DL3 in combination with their group 1 HLA-C ligands, while the presence of these KIRs in the absence of the putative ligands

Table 3 HLA-C^{Lys80} and HLA-C^{Asn80} group KIR ligands in melanoma patients and healthy controls

Ligands	Frequency	
	Patients ($n=50$)	Controls ($n=54$)
HLA-C^{Asn80}	18 (36.0%)	16 (29.63%)
HLA-C^{Lys80}	6 (12.0%)*	17 (31.48%)
HLA-$C^{Lys80;Asn80}$	26 (52.0%)	21 (38.89%)

*$p=0.017$; OR = 0.270; 95% CI, 0.09–0.91

Table 4 KIR/ligand combinations in melanoma patients and healthy controls

KIR/ligand	Frequency	
	Patients ($n=50$)	Controls ($n=54$)
Inhibitory KIR		
2DL1 + /L +	30 (60.0%)	35 (64.8%)
2DL1 + /L-	15 (30.0%)	14 (25.9%)
2DL1-/L +	3 (6.0%)	3 (5.6%)
2DL2 and/or 2DL3 + /L +	44 (88.0%)*	37 (68.5%)
2DL2 and/or 2DL3 + /L-	6 (12.0%)**	17 (31.5%)
2DL2 and/or 2DL3-/L +	0	0
3DL1 + /L +	24 (48.0%)	33 (61.1%)
3DL1 + /L-	21 (42.0%)	16 (29.6%)
3DL1-/L +	5 (10.0%)	5 (9.3%)
Activating KIR		
2DS1 + /L +	11 (22.0%)	13 (24.1%)
2DS1 + /L-	8 (16.0%)	5 (9.2%)
2DS1-/L +	21 (42.0%)	24 (44.4%)
2DS2 + /L +	22 (44.0%)	23 (42.6%)
2DS2 + /L-	6 (12.0%)	11 (20.0%)
2DS2-/L +	19 (38.0%)	14 (25.9%)

*$p=0.017$; OR = 2.80; 95% CI, 0.09–0.91; **$p=0.017$; OR = 0.27; 95% CI, 0.09–0.91

decreased (12.0% vs 31.5%; $p=0.017$) in the patient group. Moreover, given the ligand-receptor relationship between HLA class I and KIR molecules, we also tested whether the presence of inhibitory KIR/HLA combinations in the absence of their closely related activating KIRs could be associated with MM (Table 5). However, no statistically significant difference was found between melanoma patients and healthy controls, or between the different stages of malignant progression (data not shown). In addition, we analyzed the HLA-Bw4 ligands for inhibitory KIR3DL1 in the context of the presence of isoleucine ($Bw4^{Ile}$) or threonine ($Bw4^{Thr}$) at position 80 (Table 6), but again no difference between patients and controls was observed. A slightly higher incidence of the KIR3DL1/$Bw4^{Thr}$ combination was seen in patients with primary (20.8%) compared with those with metastatic (4.2%) malignant disease.

Discussion

With the discovery of NK-cell receptors, present on both T and NK immune effector cells and capable of recognizing MHC molecules, studies have been devoted to highlighting their relevance in tumor immunity. It is conceivable that KIR-gene content in the genome could contribute to the function of NK cells and T lymphocytes by modulating immune responses to malignant cells. Consequently, a genetic imbalance between activating and inhibitory KIR genes may facilitate the development of malignancies. On the other hand, the extent to which KIRs can be used as inhibitory self-receptors depends upon a person's HLA type [28]. Thus, if the polymorphisms of both HLA and the KIR-gene complex are considered together, dissimilar numbers and qualities of KIR/HLA pairs appear to function in different individuals. In addition, the independent segregation of HLA and KIR genes raises the possibility that any given individual can express the receptor or the ligand only, or both receptor and ligand. Since the NK receptor/HLA ligand pairs control the NK-cell function, their status from "none to too many" may have a role in the immune response to different challenges [31]. Thus, KIR and HLA polymorphisms could behave as factors of susceptibility to, progression of, or protection from malignancies. With these concepts in mind, we carried out HLA class I and KIR genotyping in melanoma patients in order to evaluate the associations of ligands only, receptors only, and/or receptor/ligand combinations with the presence of malignancy.

The KIR gene frequencies observed in our control group closely resembled those found in previous studies in Caucasian populations [10, 27, 41]. Similar results for the KIR gene repertoire were obtained in our patients with MM. The profile distribution of KIR genes also did not demonstrate a prevalence of a given haplotype (A or B) in the patients compared with the controls. Our data indicate that the genetic polymorphism within the family of MHC class I–recognizing receptors is associated

Table 5 Distribution of inhibitory KIRs/HLA-C ligands in the absence of the corresponding activating KIRs in melanoma patients and healthy controls

KIR	Ligands	Frequency	
		Patients	Controls
KIR2DL2/2DL3	HLA-C^{Asn80}	7 (38.9%)	3 (18.8%)
KIR2DL1	HLA-C^{Lys80}	3 (60.0%)	11 (68.8%)
KIR2DL1; KIR2DL2/2DL3	HLA-$C^{Lys80;Asn80}$	12 (46.2%)	7 (33.3%)

Table 6 Distribution of HLA-$Bw4^{Ile80}$and HLA-$Bw4^{Thr80}$ ligands in KIR3DL1/HLA-Bw4 positive patients ($n=24$) and controls ($n=33$)

	$Bw4^{Ile80}$	$Bw4^{Ile80;Thr80}$	$Bw4^{Thr80}$
Patients	17 (70.8%)	1 (4.2%)	6 (25.0%)
Primary MM	7 (29.2%)	1 (4.2%)	5 (20.8%)
Metastatic MM	10 (41.6%)	0	1 (4.2%)
Controls	26 (78.8%)	2 (6.0%)	5 (15.2%)

neither with the presence of MM nor with the progression of malignant disease.

Data on HLA associations with solid tumors are limited and have shown predisposition conferred by different HLA alleles. HLA-B*08 was found to be protective for breast cancer, while A2, B14, and Cw6 were found to be predisposing [3]. Previous studies on HLA and MM were focused mainly on the DQB1 locus [12, 22]. DQB1*0301 was found to be a risk factor for development of metastatic disease in Caucasian MM patients [20, 21]. In the Japanese population, such a risk factor is DQB1*0302 [18]. Currently, few data on HLA class I associations with malignant melanoma are available [2]. Our study in the Bulgarian population showed slightly increased frequencies of HLA-A*01, B*08, and Cw*01 alleles in melanoma patients. Similar results were observed for DRB1*11, which is in strong linkage disequilibrium with DQB1*0301. Interestingly, the DRB1*11 allele was found to be protective for different autoimmune diseases in our population (unpublished data). Three haplotypes—A*01-B*35-C*04, A*01-B*08-DRB1*03, and A*24-B*40-DRB1*11—conferred significant predisposition to MM in the Bulgarian population. Therefore, we could speculate that both HLA class I and class II molecules, encoded by certain alleles could be involved in the immune escape mechanisms of this solid tumor.

KIR2D receptors predominantly recognize HLA-C molecules and are able to distinguish between subtle allelic polymorphisms [7, 8]. The inhibitory receptors, KIR2DL2 and KIR2DL3, and the stimulatory receptor, KIR2DS2, recognize HLA-C alleles which share asparagine at position 80. In contrast, KIR2DL1 and KIR2DS1 are specific for HLA-C alleles having lysine at position 80. Thus, the individual's HLA-C type is closely related to the functioning of KIR2D receptors. Although no differences were found for the KIR gene frequencies, the HLA genotyping demonstrates differences in the group 1 and group 2 HLA-C ligand distributions between melanoma patients and healthy controls. HLA-C types including only HLA-C^{Lys80} were significantly less frequent in patients than in controls. Since those individuals can use KIR2DL1 as an inhibitory receptor, a minority of melanoma patients in our study group (homozygous for HLA$-C^{Lys80}$) could have a limited number of functional inhibitory KIR/HLA pairs. In contrast, the majority of the patients (homozygous for HLA-C^{Asn80} or heterozygous for HLA-C at position 80) could use more inhibitory receptor/ligand combinations.

The biological significance of KIRs in vivo depends on whether these receptors are present in individuals simultaneously with their ligands. Thus, any effect of KIR on disease susceptibility or progression might depend on the presence of putative HLA ligands within an individual. We established frequencies for both activating and inhibitory KIR genes in the presence of an individual's predicted HLA ligands. The activating KIR/ligand associations were not significantly different between patients and controls. In contrast, the observation of inhibitory KIR2DL2/2DL3 in association with their putative ligands was more frequent in melanoma patients. Therefore, it could be speculated that the tumor dissemination and escape from immunosurveillance might be due to a prevalence of inhibitory over activating signals in melanoma patients. In addition, HLA-B molecules containing $Bw4^{Thr80}$ may be less effective ligands for KIR3DL1 than those containing $Bw4^{Ile80}$ [6, 19]. Although not statistically significant, our data showed higher incidence of KIR3DL1/$Bw4^{Thr80}$ combinations in primary compared with metastatic MM. This finding seems to support the suggestion that more effective inhibition of killer cell function via KIR3DL1/$Bw4^{Ile80}$ pairs could interfere with occurrence of metastasis.

The data obtained in this study imply that there may not be a direct association between KIR gene content in the genome and the presence of malignant melanoma, or melanoma progression. However, some HLA haplotypes could be predisposing to MM in the Bulgarian population. Furthermore, distinct KIR/HLA-ligand combinations may be relevant to the development of malignancy in a manner whereby inhibition overrides activation of NK and T cells expressing NK-associated receptors, which in turn might facilitate the tumor escape and progression.

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