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ORIGINAL ARTICLE

The common Scandinavian human leucocyte antigen ancestral haplotype 62.1 as prognostic factor in patients with advanced malignant melanoma

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Abstract
Purpose We have previously demonstrated an association of the human leukocyte antigen (HLA), HLA-A2 allele with ovarian and prostate cancer mortality as well as a segregation of the ancestral HLA haplotype (AHH) 62.1 [(A2) B15 Cw3 DRB1*04] in patients with stage III–IV serous ovarian cancer. The objective of the present study was to determine the role of the HLA phenotype on the prognosis in stage III–IV malignant melanoma patients.
Patients and methods A cohort of metastatic malignant melanoma patients ($n = 91$), in stage III ($n = 26$) or IV ($n = 65$) were analysed for HLA-A, -B, -Cw and -DRB1 types by PCR/sequence-specific primer method. The frequencies of HLA alleles in the patients were compared to that of healthy Swedish bone marrow donors. The effect of HLA types on prognosis was defined by Kaplan–Meier and Cox analysis.
Results The presence of the AHH 62.1 in clinical stage IV patients was significantly and independently associated with the worst survival rate recorded from the appearance of metastasis (HR = 2.14; CI = 1.02–4.4; $P = 0.04$). In contrast, the period from the primary diagnosis to metastasis was the longest in patients with this haplotype (HR = 0.40; CI = 0.17–0.90; $P = 0.02$).
Conclusions Melanoma patients in our cohort with 62.1 AHH which is associated with autoimmune diseases have an initial strong anti-tumour control with longer metastasis-free period. These patients have rapid progression after the appearance of metastasis, responding poorly to chemo- or/and immunotherapy. This apparently paradoxical clinical process could be due to the interplay between tumour clones escape and immune surveillance ending up with a rapid disease progression.

Keywords HLA · Ancestral haplotype 62.1 · Malignant melanoma · Survival · Cox analysis

Abbreviations

HLA	Human leucocyte antigen
MHC	Major histocompatibility complex
AHH	Ancestral HLA haplotype
PCR	Polymerase chain reaction
HSD	Healthy Swedish donors
MM	Malignant melanoma
OS	Overall survival
TTM1	Time from primary diagnosis to first metastasis
SFM1	Survival time from metastasis

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Introduction

Metastatic malignant melanoma (MM) remains a highly lethal malignancy with 5-year survival rate of 45% in stage III and 10% in stage IV patients. In spite of several predictors are available, there is still a need to extend the investigation about more specific prognostic factors.

The inconsistent clinical outcome of malignant melanoma, may be correlated to the complex interactions between the different human leukocyte antigens (HLAs) and antigen-specific cytolytic T cells (CTLs) or natural killer (NK) cells, which are determinant in eliciting an effective immune response against melanoma [6, 22, 36]. The strong evidence, suggesting the existence of a spontaneous antitumor T cell response in melanoma patients preceded the identification of human tumour antigens [37, 9].

Human major histocompatibility complex (MHC) locus is characterized by an extreme degree of polymorphism of the genes encoding peptide-presenting molecules (HLADPB1, -DQB1, -DQA1, -DRB1, -Cw, -B and -A) and a high degree of linkage disequilibrium (LD). Haplotype block is an algorithmic definition of a region in the human genome characterized by strong LD. Most of the common HLA haplotypes are either ancestral HLA haplotypes (AHH) (about 25–30% in Caucasians) or recombinants (estimated around 25–50%) of ancestral haplotypes [12]. Due to linkage disequilibrium, ancestral HLA haplotypes are conserved en bloc within related populations and encompass MHC class I, II and III genes. The MHC locus is arranged into conserved, extended haplotypes of variable length but a minimum size of 3.2 Mb [39, 40, 47].

Several investigators have highlighted the various functions potentially mediated by HLA class I and class II in immune surveillance against tumours [3]. As a prognostic factor, HLA has been studied in association with head and neck tumours as well as lung cancer [29, 42]. In addition HLA has also been studied as a factor that could predict the response to immunological treatments, such as vaccine or cytokine therapy, particularly in melanoma, renal cell carcinoma and in chronic myelogenous leukaemia [2, 8, 43]. In patients with advanced ovarian cancer we have previously reported a poor survival of HLA-A2 positive patients as well as an overrepresentation of the common Scandinavian ancestral haplotypes (AHH) 62.1 ([A2] B15 Cw3 DRB1*04) and 8.1 ([A1]) B8 Cw7 DRB1*03) [11, 17, 25]. The goal of this study was to assess the relevance of HLA phenotype on the survival of a cohort of stages III and IV melanoma patients.

Materials and methods

Study description

This paper is a historical cohort study of 91 unrelated Swedish patients with metastatic MM analysed for HLA-A, HLA-B, HLA-Cw and HLA-DRB1. Confirmed histopathology or cytology for metastatic MM and tested for HLA phenotype were the main recruitment criteria. At the oncology clinic at Karolinska University Hospital, HLA genotyping is routinely performed on all patients recruited to treatments that include immunotherapy and they are informed about the HLA typing. Patients were analysed in order to be included into a vaccination trial (Karolinska Institute Ethical committee KI 01:376), or an immunochemotherapy trial (Karolinska Institute Ethical committee KI 99:139) or simply to receive cytokine based immunotherapy as part of regular clinical proposed treatment. If recruited to one of the trials, the patients signed a written informed consent including performance of HLA analysis. Date of recruitment was the date when HLA typing was preformed. Patients were recruited between September 2001 and February 2007 and censored in September 2007. The control group comprised 40,189 healthy Swedish bone marrow donors (HSD), 22,916 females (57%) and 17,276 males (43%) with age between 18 and 55, median 43, typed for the HLA loci A, B, and DRB1 and 350 for the HLA locus Cw, respectively. Most of HSD in the registry are typed by genomic analysis as the patients in the study. The early entries in the registry between 1990 and 1998 were serologically defined. This control cohort reflects by all means the HLA phenotype distribution in the Swedish population [7]. These data were provided by the coordinating centre of the Bone Marrow Donors Worldwide Registries (Leiden, The Netherlands) [39].

Blood sample preparation and HLA genotyping

Blood samples were drawn from the patients in 10 ml citrate treated tubes for HLA-A, -B, -C and HLA-DRB1 testing [17]. Blood samples were drawn from the patients in 10 ml citrate treated tubes for HLA-A, -B, -C and HLA-DRB1 testing. Peripheral lymphocytes were isolated from collected blood samples by addition of Lymphoprep according to manufacturer's protocol (Axis-Shield PoC AS, Oslo, Norway). Genomic DNA was extracted from isolated lymphocytes using "Roche High-Pure DNA extraction kit procedures 030625" (Roche, Molecular Biochemicals, Manheim, Germany) according to the manufacturer's protocol. Samples of 200 µl were treated with 1 ml extraction buffer, mixed, incubated for 30 min at 80°C and then centrifuged for 10 min at 12,000*g*. Supernatant was collected into a new

reaction tube with 400 µl binding buffer and 80 µl proteinase K. This mixture was further incubated for 10 min in 72°C adding 200 µl Isopropanol and then loaded onto a filter tube placed on a collection tube and centrifuged for 1 min at 5,000 g. These samples were washed twice with 450 wash buffer at 5,000*g* centrifugation for 2 min. After the final wash step, samples were dried by a 10 min centrifugation at maximum speed. DNA in the filter was then eluted with 50 µl elution buffer into a new reaction tube by a 1 min centrifugation at 5,000*g*. Finally the DNA amount and purity were measured by NanoDrop technology.

Human leucocyte antigen genotyping was performed using the Olerup SSP HLA Typing Kit (Olerup SSP AB, Stockholm, Sweden). PCR products were separated by electrophoresis at 150 V for 30 min on a 3% agarose gel, stained with ethidium bromide, and visualized under UV light.

Statistics

HLA gene and haplotype frequency calculation

Human leucocyte antigen class I and II phenotypes frequency were calculated by dividing the number of alleles present for each HLA type by the total number of patients. Homozygotes were tallied once. Haplotype frequency was determined using the maximum likelihood method [39, 40].

The statistical significance of the frequencies of individual HLA alleles or haplotypes with the cohort of patients compared to HSD was calculated using Fisher's exact test. Two-tailed *P* values were utilized to detect positive and negative associations. Results with a *P* value of <0.05, adjusted for Bonferroni correction [44], were regarded to be significant.

Survival analysis

The χ^2 trend test was used to examine patients' characteristics for discrete categorical variables or factors. Three time-related statistical events have been considered in this study. (i) Overall survival (OS) with a statistical event defined as death from any cause; survival time was calculated using the date of first diagnosis and the date of the last follow-up or date of death. The overall survival was split into two time variables. (ii) Time from primary diagnosis to first metastasis (TTM1) with a statistical event defined as metastatic relapse of MM and the time variable calculated using the date of first diagnosis to the date of the verified first metastasis. (iii) Survival time from metastasis (SFM1) with a statistical event defined as death from any cause and survival time calculated using the date of metastasis and the date of the last follow-up (censored) or the date of death (uncensored). Haplotype positive was regarded as 1 and negative as 0. Cumulative survival plots and time-to-event curves were constructed using the Kaplan–Meier product-limit method, with the log-rank test applied to detect differences between groups. Univariate Cox regression analyses were performed for prognostic factors such as age, gender, tumour thickness according to Breslow, Clark level of invasion, clinical stage as well as the expression of HLA haplotypes. Hazard ratios (HR) and 95% confidence intervals (CI) were estimated. To test the assumption of proportional hazards, an interaction term of a prognostic variable and a time-dependent covariate were added. A significant effect of that interaction term denotes the presence of a time dependent effect and thus a violation of the proportional hazards assumption. Multivariate Cox regression analyses were performed including binary coding of all factors with a stepwise procedure. *P* values <0.05 were considered statistically significant. All analyses were performed using the statistical software StatView™ for Windows, SAS Institute Inc. Version 5.0.1 and STATISTICA™ StatSoft Inc. Version7.

Results

Patient population

A total of 91 patients were included in the analysis (Table 1). The gender distribution was almost equal (48% females and 52% males). The median age of the patients at the time of recruitment was 59 years (range 27–88 years). The tumour thickness according to Breslow ranged between 0.4 and 21.1 mm (median 3 mm). The level of invasion of the primary tumours were Clark level II 3%, level III 23%, level IV 41% and level V 13%. The level of invasion was not defined in 9% of tumours, whereas 11% of patients presented with metastatic disease with an unknown primary tumour. The majority of the tumours were defined as SSM (33%), followed by NM (22%). Very few patients (less than 2%) at the diagnosis went through sentinel node examination since this procedure was not yet performed routinely. At recruitment, 30% of patients had a melanoma of tumour stage III and 70% of stage IV. Thirteen patients with an initial stage III disease developed a stage IV disease during the study. Five of these were AHH 62.1 block positive (see below). The most frequent sites of metastasis were subcutaneous tissue and lymph nodes (49%), lung (38%), liver (22%), brain (20%) and bone (16%).

A total of 40% of the analysed patients received chemotherapy and 35% chemoimmunotherapy. A total of 15% of stage III patients and 7% of stage IV patients did not receive any chemo- or immunotherapy, but were treated with surgery and radiotherapy.

Table 1 Patient and tumour characteristics

	Patients ($n = 91$)	Percentage
Sex: F/M	44/47	48/52
Age median (minimum–maximum)	59 (27–88)	–
Classification of the primary tumour		
Tumour thickness		
Median (minimum–maximum) (mm)	3.0 (0.4–21.1)	
Level of invasion		
Clark II	3	3
Clark III	21	23
Clark IV	37	41
Clark V	12	13
Primary tumour unknown	10	11
Not available	8	9
Histological type		
SSM	30	33
NM	20	22
Unclassifiable	19	21
Mucosal melanoma	4	4
Tumour stage at recruitment		
Stage III	27	30
Stage IV	64	70
Tumour stage at censor date		
Stage III	14	15
Stage IV	77	85
Distant metastasis sites at censor date		
Subcutaneous tissue and lymph nodes	45	49
Lung	35	38
Liver	20	22
Brain	18	20
Bone	15	16
Systemic treatment received		
Chemotherapy only	36	40
Chemo and Immunotherapy	35	35
No chemo or immunotherapy—stage III patients	14	15
No chemo or immunotherapy—stage IV patients[a]	6	7

F Female, *M* male, *SSM* superficial spreading melanoma, *NM* nodular melanoma

[a] Five patients received successful surgery/radiotherapy for distant metastasis. One patient died before he could start planned chemotherapy

Analysis of HLA distribution in the cohort and clinical stage subgroups

The distribution of HLA-A, -B, -Cw loci as well as the HLA class II locus DRB1 was determined for all patients (see supplementary table). No significant differences were found between patients and HSD comparing frequencies for individual HLA alleles. The frequencies of the most common Scandinavian haplotype blocks are presented in Table 2. The distribution of the patients in clinical stage III and IV groups demonstrated an increased frequency ($P = 0.0412$, corrected $P = 0.44$) of the B15 DRB1*04 haplotype block in the complete cohort compared to HSD. Noteworthy, this 62.1 block was only present in the stage IV patients at censor date ($P = 0.009$, corrected $P = 0.118$). The same trend was observed for the AHH blocks 8.1 and 60.1, but the differences are not statistically significant before or after Bonferroni correction. In contrast, A2 B27 haplotype was significantly enriched in the stage III group ($P = 0.001$, corrected $P = 0.011$).

Survival and prognostic factors

Overall survival (OS), time to first metastasis (TTM1) and survival from appearance of metastasis (SFM1) represented time variables in the study (Table 3). Age was included in the univariate analysis as a continuous time factor and correlated at a statistically significant level to OS. Both OS and TTM1 were favourable for patients aged 50 or younger compared to older patients. A slight advantage for women over men was also noted but the difference did not reach statistical significance. Breslow's and Clark's staging correlated significantly with OS and TTM1, but not with the SFM1. As expected, clinical stage correlated significantly with SFM1. The statistic for sentinel node is not shown due to the low number of patients that underwent this analysis.

The haplotypes identified by the frequency analysis (Table 3) were tested for hazard ratio. Univariate analysis for the haplotypes is presented by two strata, all the patients in the cohort and all clinical stage IV patients. The stage III stratum was composed of a small number of patients resulting in a lower power for computing HR. With regard to the OS, none of the AHHs showed a significant HR, however, dividing the OS into TTM1 and SFM1 revealed that patients with ancestral haplotype 62.1 block B15 Cw3 DRB1*04 and A2 B15 Cw3 DRB1*04, had longer time interval from diagnosis to the first metastasis (HR 0.60; CI 0.30–1.06; $P = 0.07$ and HR 0.32; CI 0.12–0.84; $P = 0.02$, respectively). On the contrary these patients had shorter SFM1 (HR 1.9; CI 1.04–3.59; $P = 0.03$ and HR 1.94; CI 0.88–4.3; $P = 0.09$, respectively). SFM1 for the AHH 60.1 block A2 B40 Cw3 DRB1*04 also showed an adverse prognosis (HR 2.58; CI 1.01–6.57; $P = 0.04$). The 8.1 AHH had no significant impact on survival. Contrary to AHH 62.1 and 60.1, the SFM1 for patients with the haplotype A2 B27 was longer (HR 0.4; CI 0.14–1.13; $P = 0.08$). This finding however did not reach statistical significance, possibly due to the limited number of patients and thus needs to be confirmed in a larger cohort.

The stratification underscored that within the stage IV patient group, the AHHs 62.1 and 60.1 had significantly

Table 2 Frequencies of most common haplotype blocks in malignant melanoma patients and healthy Swedish donors

AHH	Haplotypes	Patients cohort				HSD			Woolf Haldane 95% CI			Fisher Bonferroni		
		Groups	Positive	Negative	hf (%)	Positive	Negative	hf (%)	OR	Lower	Upper	*P* value	*P* corrected	
62.1	B15 DRB1*04	Complete cohort	16	166	9	2,242	41,203	5	1.82	1.10	3.03	**0.041**	0.445	1
		Stage III	0	28	0	2,242	41,203	5	0.32	0.02	5.28	0.400	0.999	0
		Stage IV	16	138	10	2,242	41,203	5	2.19	1.31	3.65	**0.009**	0.118	1
62.1	B15 Cw3 DRB1*04	Complete cohort	14	168	8	42	658	6	1.33	0.72	2.48	0.396	0.999	0
		Stage III	0	28	0	42	658	6	0.27	0.02	4.53	0.398	0.999	0
		Stage IV	14	140	9	42	658	6	1.60	0.86	2.98	0.206	0.960	0
62.1	A2 B15 Cw3 DRB1*04	Complete cohort	8	174	4	26	674	4	1.09	0.48	2.49	1.000	1.000	0
		Stage III	0	28	0	26	674	4	0.45	0.03	7.51	0.618	1.000	0
		Stage IV	8	146	5	26	674	4	1.29	0.56	2.97	0.644	1.000	0
60.1	B40 Cw3	Complete cohort	16	166	9	59	641	8	1.05	0.90	1.22	0.800	1.000	0
		Stage III	1	27	3	59	641	8	0.40	0.06	2.73	0.720	1.000	0
		Stage IV	15	139	10	59	641	8	1.17	0.78	1.75	0.630	1.000	0
60.1	(A2)B40 (Cw3)	Complete cohort	5	177	3	19	671	3	1.00	0.97	1.03	0.990	1.000	0
		Stage III	0	28	0	19	671	3	0.25	0.01	5.26	0.990	1.000	0
		Stage IV	5	149	3	19	671	3	1.19	0.43	3.23	0.780	1.000	0
8.1	B8 DRB1*03	Complete cohort	16	166	9	3,508	39,937	8	1.13	0.68	1.87	0.683	1.000	0
		Stage III	2	26	7	3,508	39,937	8	1.07	0.29	3.93	1.000	1.000	0
		Stage IV	14	140	9	3,508	39,937	8	1.17	0.68	2.02	0.655	1.000	0
8.1	B8 Cw7 DRB1*03	Complete cohort	16	166	9	51	649	7	1.25	0.70	2.23	0.530	1.000	0
		Stage III	2	26	7	51	649	7	1.19	0.32	4.49	1.000	1.000	0
		Stage IV	14	140	9	51	649	7	1.30	0.71	2.40	0.501	1.000	0
8.1	A1 B8 Cw7 DRB1*03	Complete cohort	12	170	7	41	659	6	1.17	0.61	2.24	0.727	1.000	0
		Stage III	0	28	0	41	659	6	0.28	0.02	4.65	0.396	0.999	0
		Stage IV	12	142	8	41	659	6	1.39	0.72	2.69	0.359	0.998	0
	A2 B27	Complete cohort	10	170	4	2,128	77,632	3	1.78	0.89	3.54	0.160	0.912	0
		Stage III	5	23	18	2,128	77,632	3	8.54	3.37	21.62	**0.001**	**0.011**	2
		Stage IV	5	147	2	2,128	77,632	3	0.84	0.29	2.43	0.802	1.000	0

The values in bold are significant values

P value is corrected for 14 haplotypes

0 = not significant, 1 = significant before, but not after Bonferroni correction, 2 = significant after correction

longer time to metastasis formation and significantly shorter survival following onset of metastatic disease.

AHH 62.1 and 60.1 represent independent prognostic variables with regard to the TTM1 and SFM1 when computed for the proportional hazard ratio by multivariate analysis together with age, Breslow and Clark. On the other hand, these AHHs were not independent of the clinical stage since almost all of these patients had stage IV disease (Table 3). The curves of probability for TTM1 and SFM1 in stage IV patients with or without AHH 62.1 (B15 Cw3 DRB1*04) are presented in Fig. 1.

Effect of treatments in the survival of patients with AHH 62.1 block

In order to avoid possible bias due to the treatments, a log rank Mantel–Cox analysis of the survival in the AHH 62.1 block versus otherwise from the TTM1 to censor date was performed. The number of patients with or without 62.1 phenotype was equally represented in the chemotherapy and immunochemotherapy group. This therapeutic stratification did not affect specifically the survival ($P = 0.20$ and $P = 0.93$) (Table 4).

Discussion

This study emphasizes the role of the MHC in the progress of malignant melanoma and correlates certain common Scandinavian haplotypes with disease prognosis. These findings are in accordance with previous analyses demonstrating the relevance of HLA alleles or haplotypes to the clinical outcome of various malignancies including MM [6, 16, 17, 21, 26, 28–30, 32, 35, 42]. Our results in this paper provide evidence that the presence of the ancestral haplotype 62.1 in clinical stage IV melanoma patients is related

Table 3 Univariate and multivariate Cox–Mantel analysis

Univariate analysis		Overall survival			TTM1			SFM1		
Strata	Factors	Hazard ratio	95% CI	P	Hazard ratio	95% CI	*P*	Hazard ratio	95% CI	*P*
Complete cohort	Age (continuous)	1.37	1.14–1.67	0.001	1.1	0.99–1.28	0.16	1.1	0.92–1.31	0.26
	Age <50 versus >50	0.43	0.24–0.77	0.004	0.4	0.28–0.7	0.0006	0.92	0.55–1.55	0.78
	Gender F versus M	0.7	0.43–1.2	0.21	0.97	0.64–1.47	0.89	1.2	0.7–1.98	0.51
	Breslow >1 mm versus <1 mm	2.2	0.96–4.9	0.06	3.6	1.7–7.7	0.0007	0.8	0.39–1.8	0.67
	Clark 4–5 versus 2–3	2.4	1.23–4.48	0.009	3.6	2.04–6.5	0.0001	0.8	0.49–1.69	0.76
	Clinical stage at recruitment IV versus III	1.6	0.81–3.2	0.16	0.6	0.40–1.02	0.06	2.3	1.17–4.5	0.01
	B15 Cw3 DRB1*04 versus other	1.0	0.52–1.87	0.97	0.6	0.30–1.06	0.07	1.9	1.04–3.59	0.03
	A2 B15 Cw3 DRB1*04 versus other	0.68	0.29–1.63	0.39	0.32	0.12–0.84	0.02	1.94	0.88–4.3	0.09
	B8 Cw7 DRB1*03 versus other	1.2	0.45–3.45	0.67	0.9	0.36–2.22	0.81	1.2	0.51–3.2	0.58
	A1 B8 Cw7 DRB1*04 versus other	0.7	0.29–1.63	0.39	1.1	0.58–2.06	0.78	0.96	0.44–2.17	0.97
	(A2) B40 Cw3 DRB1*04 versus other[a]	0.94	0.33–2.6	0.9	0.56	0.20–1.56	0.26	2.58	1.01–6.57	0.04
	A2 B15 Cw3 DRB1*04/A2 B40 Cw3 DRB1*04 versus other	1.0	0.44–2.1	0.96	0.5	0.21–0.98	0.04	2.1	1.01–4.07	0.04
	A2 B27 versus other	0.5	0.17–1.35	0.16	1.0	0.52–1.99	0.94	0.4	0.14–1.13	0.08
Stage IV at recruitment	B15 Cw3 DRB1*04 versus other	0.68	0.31–1.4	0.68	0.4	0.17–0.90	0.02	2.14	1.02–4.4	0.04
	A2 B15 Cw3 DRB1*04/A2 B40 Cw3 DRB1*04 versus other	0.65	0.29–1.4	0.3	0.38	0.16–0.91	0.03	2.003	0.93–4.3	0.07
Multivariate analysis										
Complete cohort	Age <50 versus >50	0.43	0.24–0.77	0.004	0.47	0.29–0.75	0.001	1.01	0.59–1.7	0.95
	A2 B15 Cw3 DRB1*04 -A2 B40 Cw3 DRB1*04 versus other	1.03	0.49–2.14	0.93	0.54	0.25–1.16	0.11	2.05	1.02–4.15	0.04
	Breslow >1 mm versus <1 mm	3.2	1.23–8.55	0.1	4.1	1.75–9.9	0.001	1	0.44–2.2	0.99
	A2 B15 Cw3 DRB1*04-A2 B40 Cw3 DRB1*04 versus other	2.3	0.92–5.8	0.07	1.3	0.52–3.3	0.55	1.98	0.88–4.4	0.09
	Clark 4–5 versus 2–3	2.5	1.28–4.8	0.006	3.5	1.9–6.2	0.0001	0.87	0.47–1.6	0.17
	A2 B15 Cw3 DRB1*04-A2 B40 Cw3 DRB1*04 versus other	1.4	0.65–3.2	0.35	0.7	0.31–1.59	0.4	1.98	0.91–4.3	0.08
	Clinical stage at recruitment IV versus III	1.6	0.83–3.3	0.15	0.69	0.43–1.1	0.12	2.3	1.17–4.6	0.01
	A2 B15 Cw3 DRB1*04-A2 B40 Cw3 DRB1*04 versus other	0.84	0.40–1.7	0.64	0.49	0.23–1.01	0.07	2.01	1.03–4.08	0.03
	Age <50 versus >50	0.57	0.26–1.2	0.16	0.99	0.62–1.8	0.97	0.85	0.4–1.8	0.67
	Breslow >1 mm versus <1 mm	1.7	0.52–5.6	0.37	3.2	1.1–8.9	0.02	1.1	0.37–2.7	0.98
	Clark 4–5 versus 2–3	1.4	0.66–3.1	0.34	2.9	1.4–5.7	0.001	0.75	0.36–1.5	0.43
	Clinical stage at recruitment IV versus III	1.2	0.54–2.6	0.64	0.4	0.21–0.7	0.001	2.1	1.01–4.5	0.048
	A2 B15 Cw3 DRB1*04-A2 B40 Cw3 DRB1*04 versus other	1.8	0.71–4.8	0.2	1.6	0.62–4.2	0.31	2.01	0.84–4.7	0.11

TTM1 Time from primary diagnosis to first metastasis, *SFM1* survival from first metastasis to censor date

[a] B40 Cw3 DRB1*04 are all A2

to a poor survival following onset of mestastatic disease. Surprisingly the period from initial diagnosis to metastasis was the longest in patients with this haplotype. Our results also demonstrate that the ancestral block B40 Cw3 (AHH 60.1) might be associated with the outcome of melanoma patients. The 60.1 haplotype was mainly present in patients with stage IV disease, hence there is a similarity in the distribution of the 60.1 haplotype block with the 62.1 block. Ancestral haplotypes are of interest due to their association with autoimmune diseases, immune responses to HIV and malignancies [10, 14, 15, 38]. We have previously reported a possible clinical relevance of ancestral haplotypes 62.1 and 8.1 in patients with advanced ovarian cancer [17]. It is evident that in the subgroup of melanoma patients

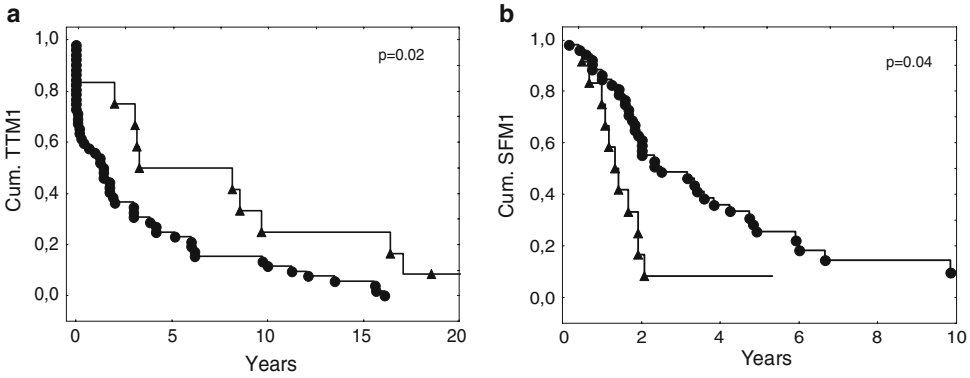

Fig. 1 Kaplan–Meier analysis of time from primary diagnosis to first metastasis (TTM1) and survival from first metastasis (SFM1), with log rank Mantel–Cox test. **a** TTM1, stage IV patients, AHH 62.1 (B15 Cw3 DRB1*04) positive ($n = 14$, *filled triangles*) and negative ($n = 62$, *filled circles*); **b** SFM1, stage IV patients, AHH 62.1 positive ($n = 14$, *filled triangle*) and negative ($n = 62$, *filled circle*)

Table 4 Type of treatments and survival in AHH 62.1 block versus otherwise

Therapy[a]	AHH62.1 block		Otherwise		Kaplan–Meier survival by log rank Mantel–Cox analysis	
	n	%[b]	*n*	%[b]	χ^2	*P*
Chemotherapy only	7	8	29	32	1.50	0.2[c]
Chemoimmunotherapy	6	7	29	32	0.01	0.93[c]

[a] Twenty patients did not receive chemo or chemoimmunotherapy

[b] Percentage/100 referred to the all population

[c] Not significant

with AHH 62.1, the period from the first diagnosis of melanoma to the appearance of the first metastasis takes several years longer compared to other haplotypes. On the other hand the survival time following relapse is curtailed since 80% of melanoma patients with this AHH die on an average of 2 years earlier and 91% relaps within 6 months on first line systemic therapy compared to 54% of the patients with other HLA types. This raises the question whether the long time to relapse is due to the patients immune defence and if so, why is this efficient control lost in the metastasis.

The patients with the A2 B27 haplotype exhibit a pattern opposite to that described for the AHHs, i.e. segregation within stage III and favourable prognosis after first metastasis. HLA-B27 is a well-known predisposing factor for autoimmune disease such as ankylosing spondylitis, Reiter's syndrome and Crohn's disease [4, 34]. In our cohort B27 was almost always present with A2. The association of A2 B27 in cancer has not been described previously and merits further investigation.

The evidence that subgroups of patients with certain HLA haplotypes have the ability to delay tumor relaps might be explained by an immunological control. HLA restriction has a strong impact on immune recognition of tumour cells and anti-tumour effector function [3]. The information acquired in this cohort of patients might be due to the interacting ability of T lymphocytes to recognize tumour antigens in the context of MHC class I molecules which are essential components of the adaptive immune system [45].

The other aspect of our findings is the very short time registered from the detection of the first metastasis to the final outcome in these frequent haplotypes. One of the possible mechanisms underlying this aggressive tumour progression is escape from immunesurveillance [13, 23, 24]. We might consider the possibility that a haplotype, such as 62.1, which is associated with Diabetes type I and Rheumatoid Arthritis, Felty's syndrome, may efficiently control the presence of possible residual tumour cells after surgery as long as they are discernible to the immune system. Paradoxically this high efficacy might induce a selection and overgrowth of tumour cells that become "invisible" due to the loss of HLA expression which is often associated with deficient expression of components of antigen processing and presentation machinery [5]. In particular TAP and tapasin are often down regulated in tumours of distinct histology, which has also been shown to correlate with a poor prognosis and patients' survival. To this escape scenario it should be considered that patients 62.1 AHH may also select tumour cells by loss of heterozygosis (LOH) [1, 31]. The influence of NK cell mediated killer immunoglobulin-like

receptor (KIR)/HLA ligand interactions on disease susceptibility and/or progression in malignant melanoma should also be considered; although it has to kept in mind that there is still a paucity of information on how this may favour certain HLA haplotypes [33].

These immune escape variants may also bear mutated oncogenes, which contribute to the more fulminant nature of the tumour manifested by rapidly disseminating disease [1]. Haplotypes other than the ones described above may stimulate a lower level of antitumour effector activity ensuing in a shorter time to relapse. However, the lower selection pressure exerted on the tumour cells by this immune haplotype sustains the immune recognition of the tumours cells. Consequently, these cells may be more susceptible to therapy which translates to longer survival.

The genetic background of an individual has been postulated as crucial for the dissemination, progression and survival of malignant diseases, but this has been mainly attributed to alterations in genes not directly associated with the immune system [20, 27]. Our findings underscore the importance of the immunological background (HLA alleles frequency). Moreover, an allele-specific down regulation of HLA and aberrant antigen processing might play a role in the malignant processes [41]. Disease-associated imbalances in HLA allele frequencies between patients and healthy populations presumably arising from genetic selection favouring effective immune responses have been reported for infectious diseases like malaria [18, 19] but has not been established for malignancies till now. In theory, any HLA-restricted epitope derived from a reasonably common tumour associated antigen (TAA), that induces an effective antitumour response should influence the frequency of the HLA allele in the patient population. However, it is difficult to validate this hypothesis without information pertaining to (i) the nature and (ii) the level of expression of the corresponding TAA, and (iii) the percentage of patients mounting a protective immune response against the TAA. In a immunotherapeutic context, the negative role of 62.1 AHH in controlling the tumour and inducing escape could possibly be circumvented by stimulation with TAA peptides presented by the other haplotype.

Human leucocyte antigen imbalances could possibly also be explained by linkage to genetic factors influencing disease progression. In this respect, it should be noted that many non-MHC genes encoding immunological relevant molecules such as TNF-α or components of the complement system are located within the HLA locus. Furthermore, some alleles of these non-MHC genes are genetically linked to certain HLA alleles [46].

The relevance of HLA haplotypes in malignancies has not been widely examined. This may be due to heterogeneity within populations of patients and healthy individuals as well as sometimes limited access to relevant clinical data. Our results reveal that in the complete cohort we observed no differences in haplotype frequency compared to HSD. Only upon comparison of stage III to stage IV patients the difference in haplotype expression was resolved. Additionally no significant differences were apparent in overall survival, whereas significant differences were evident on stratifying the total time interval (TTM1 and SFM1). We hypothesize that by defining these time frames the natural history of the disease progression and its association to HLA type as well as possible role of immune surveillance will become apparent. In this context it is noteworthy that one of the limiting factors in this analysis is the small subgroups, which occasionally does not provide adequate statistical power.

In conclusion melanoma patients with the 62.1 ancestral HLA haplotype associated with autoimmune diseases, initially present a significantly longer metastasis-free period suggesting an efficient anti-tumour control. On the other hand these patients exhibit high incidence of relapses with a rapid progression, poor response to chemo- and/or immunotherapy and require intensive clinical management. This could be explained by a selection of highly malignant clones that escape immunosurveillance or due to a potential interaction of HLA with other genes. Further molecular and immunohistochemical analysis are needed to understand the aetiology behind these findings. Finally, different approaches should be considered in the clinical management of these patients as well as in the design of chemo- and/or immunotherapeutic trials.

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