

# Correlation of HLA-A02\* genotype and HLA class I antigen down-regulation with the prognosis of epithelial ovarian cancer

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Received: 28 October 2011 / Accepted: 7 January 2012 / Published online: 19 January 2012  
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## Abstract

**Background** In recent years, evidence is accumulating that cancer cells develop strategies to escape immune recognition. HLA class I HC down-regulation is one of the most investigated. In addition, different HLA haplotypes are known to correlate to both risk of acquiring diseases and also prognosis in survival of disease or cancer. We have previously shown that patients with serous adenocarcinoma of the ovary in advanced surgical stage disease have a particularly poor prognosis if they carry the HLA-A02\* genotype. We aimed to study the relationship between HLA-A02\* genotype in these patients and the subsequent HLA class I HC protein product defects in the tumour tissue.

**Materials and methods** One hundred and sixty-two paraffin-embedded tumour lesions obtained from Swedish women with epithelial ovarian cancer were stained with HLA class I heavy chain (HC) and  $\beta_2$ -microglobulin ( $\beta_2$ -m)-specific monoclonal antibodies (mAb). Healthy ovary and tonsil tissue served as a control. The HLA genotype

of these patients was determined by PCR/sequence-specific primer method. The probability of survival was calculated using the Kaplan–Meier method, and the hazard ratio (HR) was estimated using proportional hazard regression.

**Results** Immunohistochemical staining of ovarian cancer lesions with mAb showed a significantly higher frequency of HLA class I HC and  $\beta_2$ -m down-regulation in patients with worse prognosis (WP) than in those with better prognosis. In univariate analysis, both HLA class I HC down-regulation in ovarian cancer lesions and WP were associated with poor survival. In multivariate Cox-analysis, the WP group (all with an HLA-A02\* genotype) had a significant higher HR to HLA class I HC down-regulation.

**Conclusions** HLA-A02\* is a valuable prognostic biomarker in epithelial ovarian cancer. HLA class I HC loss and/or down-regulation was significantly more frequent in tumour tissues from HLA-A02\* positive patients with serous adenocarcinoma surgical stage III–IV. In multivariate analysis, we show that the prognostic impact is reasonably correlated to the HLA genetic rather than to the expression of its protein products.

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**Keywords** HLA-A02\* · Ovarian cancer · Serous adenocarcinoma · Immunohistochemistry · MHC · Prognosis

## Abbreviations

APM Antigen processing machinery  
BP Better prognosis  
 $\beta_2$ -m  $\beta_2$ -microglobulin  
BSA Bovine serum albumin  
CTL Cytotoxic T lymphocyte  
EOC Epithelial ovarian cancer  
FFPE Formalin fixed paraffin-embedded  
HF Haplotype frequency

HFS	High frequency stained The constitutional allele characteristicsHLA genotype
HR	Hazard ratio
KIR	Killer cell immunoglobulin-like receptors
HLA	Human leucocyte antigen
IHC	Immunohistochemistry
FIGO	International federation of gynaecology and obstetrics
LD	Linkage disequilibrium
LFS	Low frequency stained
MHC	Major histocompatibility complex
HC	Heavy chain
Mab	Monoclonal antibody
NK	Natural killer HLA-otherwiseNon-HLA-A02*
PLC	Peptide loading complex
PCR	Polymerase chain reaction
tpn	Tapasin
TAP	Transporter associated with antigen processing
TBS	Tris-buffered saline
TA	Tumour antigen
WP	Worst prognosis

## Introduction

EOC consists of different histological tumour entities where the most important are serous, endometrioid, mucinous and clear cell carcinoma [1]. Serous histology accounts for approximately 45–55% of EOC cases and is the most frequent type in Sweden. More than 75% of the cases are detected at late disease stage (stages III–IV) when they are inoperable. Despite excellent initial responses, most patients [2] relapse early after treatment [3, 4]. After the development of chemotherapy-resistant disease, the majority of patients with serous EOC will succumb to their disease [5, 6]. The development of new therapies for serous EOC is central to improving the prognosis of this cancer. Immune-based therapies have shown promise in the treatment of EOC, but more work is needed to understand the role of immune surveillance in ovarian cancer progression [7].

Recently, HLA-A02\* genotype has been described as an independent poor prognostic factor in Swedish women with advanced EOC of serous histology [8–11]. Different HLA haplotypes have been linked to patient prognosis in several types of cancers such as lung carcinoma [12, 13], malignant melanoma [14] and head and neck squamous carcinoma [15]. In addition, the HLA phenotype has been correlated with different outcomes in immune-based therapies in melanoma [16–20], renal cell carcinoma (RCC) [21–23], cervical carcinoma [24, 25] and chronic myeloid leukaemia (CML) [26].

The prognostic relationship between high malignancy and HLA-A02\* challenges us to analyse underlying mechanisms. In this respect, we focused on that loss or down-regulation of HLA class I HC has been reported in tumour lesions of distinct histology [27, 28], including EOC [29]. Defects in HLA class I expression may play a role of tumour progression through avoidance of detection and elimination by cytotoxic T-lymphocytes (CTL) [30] resulting in a worse clinical outcome.

The purpose of this study is to determine whether the negative prognostic relationship of HLA-A02\* genotype of patients with serous adenocarcinoma of the ovary in stage III–IV is associated with a higher frequency of HLA class I and  $\beta_2$ -m defects in the tumour.

## Materials and methods

### Patients

Patient information and histopathology samples were accessed with approval of the local ethics committee. One hundred and eighty-two patients admitted to the gynaecological Oncology unit at the Karolinska University Hospital Department of Oncology between 1995 and 2004 were initially considered for inclusion in this study. Twenty of these patients have been previously described [8]. The diagnosis of EOC as well as tumour stage and grade were confirmed by histopathology of formalin-fixed paraffin-embedded (FFPE) tumour specimens. The HLA genotype of the patients was the primary inclusion criteria. After inclusion, pertinent clinical information was entered into a database. For 162 patients, FFPE tumour tissues were available for immunohistochemical analysis. The date of diagnosis, FIGO stage (I–IV), histological type of the patient's epithelial ovarian carcinoma (serous, mucinous, endometrioid, clear cell, undifferentiated, mixed epithelial, unclassified) and grade of differentiation (well, G1; moderately, G2; poorly, G3 differentiated) were recorded. Non-epithelial ovarian cancers, borderline ovarian tumours, cancers of genital tract (except ovaries) and of extra genital localization were excluded from the analysis [31, 32]. FFPE healthy tissue from ovary (two cases) and tonsil (two cases) were used as controls. One block of FFPE tissue from each case and control was serially cut into 4- $\mu$ m-thick slices and mounted onto super frost slides.

### Patients groups

Patients have been grouped by surgical stage, histopathology and HLA-A genotype according to our previous findings [8, 10]. Worst prognosis (WP) is defined by stage III–IV, serous adenocarcinoma HLA-A02\*. Better prognosis (BP)

**Table 1** IHC staining of healthy ovarian tissue by antibodies against receptors for the MHC

Antibody/target antigen	Epithelium	Tubar epithelium	Follicle	Endothelium	Mononuclear cells	Fibroblasts
HC10/MHC class I heavy chain	Strongly positive	Negative	Positive	Strongly positive	Strongly positive	Negative
HCA/MHC class I heavy chain	Strongly positive	Positive	Strongly positive	Strongly positive	Strongly positive	Negative
L368/ $\beta$ 2 microglobulin	Strongly positive	Weakly positive	Positive	Positive	Strongly positive	Negative

includes stage I–II, all the histological type and all HLA-A genotypes including HLA-A02\*.

#### Sample preparation for DNA extraction

Peripheral blood mononuclear cells were obtained by separating blood samples on lymphoprep according to the manufacturer's instructions (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, and Mannheim, Germany) according to the manufacturer's protocol. Two hundred microlitres/sample was 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  $\mu$ l binding buffer and 80  $\mu$ l proteinase K. This mixture was further incubated for 10 min at 72°C adding 200  $\mu$ l 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  $\mu$ l wash buffer at 5,000×g centrifugation for 2 min. After the final wash step, samples were dried by a 10-min centrifugation at max speed. DNA in the filter was then eluted with 50  $\mu$ l elution buffer into a new reaction tube by a 1-min centrifugation at 5,000×g. Finally, the DNA amount and purity were determined by NanoDrop technology (GE lifesciences, Uppsala, Sweden).

#### HLA genotyping

DNA analysis was used for HLA genotyping of both FFPE material from deceased patients and of peripheral blood samples from consented patients, and HLA genotyping in FFPE extracted DNA was performed using primers only for HLA-A02\* (provided by Olle Olerup, SSP AB, Saltsjöbaden, Sweden) as previously described [8]. For the DNA extracted from peripheral blood, complete allele HLA genotype was performed using the Olerup SSP HLA Typing Kit (Olerup SSP AB, Stockholm, Sweden) [10]. 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. An additional PCR was performed with primers for the control housekeeping gene S14.

#### Monoclonal antibodies for immunohistochemistry

The mAb HCA-2 (HCA), which recognizes  $b_2m$ -free HLA-A (excluding -A24), -B7301 and -G heavy chains (1, 2), the mAb HC-10, which recognizes  $b_2m$ -free HLA-A3, -A10, -A28, -A29, -A30, -A31, -A32, -A33 and -B (excluding -B5702, -B5804 and -B73) heavy chains (1–3) and the  $b_2m$ -specific mAb L368 (4) were developed and characterized as described [33–36].

#### Immunohistochemical staining and analysis

Tissue sections were de-paraffined, re-hydrated and rinsed with water. The samples were heated in a microwave for 20 min for antigen retrieval. The slides were left for 30 min in 0.5%  $H_2O_2$  in water, finally rinsed in water and twice for 5 min in tris-buffered saline (TBS). Blocking was performed with 1% bovine serum albumin (BSA) in TBS in a moist chamber for 30 min before the sections were stained with the primary antibody at +8°C over night. Avidin–biotin–peroxidase complex (ABC) kit (Vectastain, Vector Laboratories) was used for antigen detection. The sections were rinsed three times for 10 min each in TBS followed by incubation with the secondary antibody for 40 min. After three washing steps for three min in TBS, an ABC reagent was added for 40 min before the slides were again rinsed three times for 10 min in TBS. The immunolabelling was developed with the chromogen 3'-diaminobenzidine (15 mg/50 ml TBS for 6 min), and haematoxylin was applied as a counter stain. Staining of tissue sections with isotypes IgG1, IgG2a mouse mAb or secondary antibody, respectively, served as negative controls. FFPE healthy ovarian tissues, obtained from two oophorhysterectomies due to uterine myomas as well as tonsillar tissues, were used as positive controls (Table 1). When present, healthy epithelium adjacent to tumour tissue and/or infiltrating lymphocytes in the stroma were used as internal control.

The percentage of stained malignant cells in each lesion was evaluated twice by two investigators (EA and GM). In case of discordant results (more than two score levels), a third evaluator (AH) was consulted and consensus was made. Scoring to ensure detection of any significant differences was performed as follows: 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%) or 4 (76–100%) [37, 38]. This extended evaluation system gave the possibility to merge the results

**Table 2** Characteristics of the cohort and groups

	Cohort <sup>a</sup>		WP <sup>a</sup>		BP <sup>a,c</sup>	
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
Patients eligible for analysis	162	100	47	29	115	71
Age median (range)	60	30–86	61	36–83	58	30–86
Age <60	88	50.8	21	46 <sup>b</sup>	67	58 <sup>b</sup>
HLA-A2 positive	92	57	47	100	45	40
Surgical staging						
I a, b, c	38	23	0	0	38 (22)	33
II a, b, c	17	10	0	0	15 (9)	13
III a, b, c	82	51	32	68	52 (9)	45
IV	25	15	15	32	10 (5)	9
Stage merged						
A	53	33	0	0	53 (31)	46
B	109	67	47	100	62 (14)	54
Histopathology						
Serous	87	52	47	100	40 (6 <sup>d</sup> )	35
Endometrioid	45	28	0	0	45 (24)	39
Mucinous	14	9	0	0	14 (7)	12
Clear cells	10	6	0	0	10 (7)	9
Others (mixed epithelial, undifferentiated)	6	5	0	0	6 (1)	5
Grade						
1	21	13	1	2	20 (11)	17
2	34	21	10	21	24 (12)	21
3	103	63	35	75	68 (21)	59
Unknown	4	3	1	2	3 (1)	3
Treatments						
Primary surgery	138	85	35	76	103	89
Radical surgery	55	34	4	9	51	44
Secondary surgery	22	14	8	17	14	12
Platinum-based chemotherapy	152	94	45	97	107	92
Number of chemotherapy cycles						
6 and less	97	60	15	33	82	71
More than 6	65	40	31	67	34	29
Radiotherapy	7	4	3	7	4	3

<sup>a</sup> Cohort: all patients included in the study. *WP* worst prognosis group, *BP* better prognosis group

<sup>b</sup> Difference in % between *WP* and *BP* is NS

<sup>c</sup> HLA-A02\* genotype in paraneoplasms

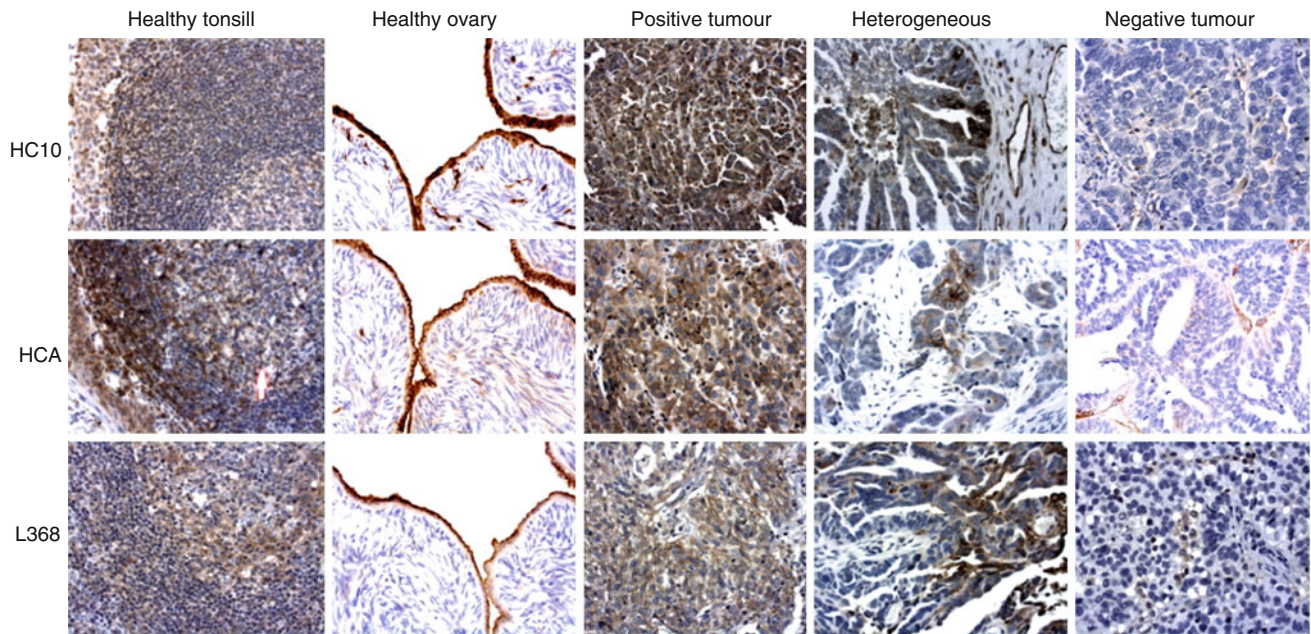
<sup>d</sup> HLA-A02\* genotype patients: 4 in stage I and 2 in stage II

in fewer, but relevant, groups for statistical analysis. In parallel, the staining intensity was also evaluated and separately scored as weak, moderate or intense, but statistical analysis revealed no patterns or significant differences (data not shown).

### Statistics

The  $\chi^2$  test was used to examine patient characteristics for discrete categorical variables or factors. Time to death from any cause was defined as the primary end point in the study. Survival time for deceased patients was calculated using the date of first diagnosis to the date of death. For surviving patients, the survival time was calculated from the date of

first diagnosis to the date of the last clinical follow-up. HLA-A02\* genotype was scored as 1 and HLA-otherwise as 0. Cumulative survival plots and time-to-event Kaplan–Meier curves were constructed with the log-rank test applied to detect univariate differences between groups. Diagnostic data were collected during the year 1995–2004 and were censored at 1 June 2010. Univariate Cox regression analyses were performed for each prognostic factor we studied. These factors were clinical stage, histological type and HLA-A02\* genotype grouped in *WP* and *BP*. Results from the Cox regression models are presented as Hazard Ratios (HR) together with 95% confidence intervals (CI). *P* values from the regression model refer to the Wald test. Multivariate Cox regression analyses were performed



**Fig. 1** Representative examples of immunohistochemical staining patterns of different MHC components in healthy tissues using the mAbs HC-10 and HCA detecting the HLA class I HC and mAb L368 detecting  $\beta_2$ -m (reading the figure horizontally). Vertically and from

left to right; healthy tonsill and ovary, tumour tissue with the majority of stained cells (*positive*), heterogeneous and *negative* (clearly *positive* infiltrating cells in the stroma) staining. Original magnification  $\times 400$

including binary coding of all factors (WP vs. BP, HC-10, HCA, L368, low vs. high staining percentages).  $P$  values  $<0.05$  were considered statistically significant. Interactions were evaluated by including product terms in the model. All analyses were performed with program StatView for Windows, SAS Institute Inc. Version 5.0.1.

## Results

### Patient population

The patients' characteristics and the HLA genotype are summarized in Table 2. Age, stage, grade of differentiation and histology distribution of the population did not differ from the figures released by the FIGO annual report for European countries [39]. There is no significant difference in the distribution of patients younger than 60 in the two prognostic groups ( $P$  value 0.12). Forty-seven serous adenocarcinoma patients surgical stages (III–IV) HLA-A02\* genotype were grouped for statistical analysis as worse prognosis (WP). The remaining 115 patients were categorized as BP group. Only four (9%) of the WP patients received radical surgery compared to 51 (44%) of the BP. The majority of the patients received platinum-based chemotherapy (97% vs. 92%) but 67% of the patients with WP required more than six therapy cycles compared to only 29% of the BP group.

### HLA class I HC and $\beta_2$ -m expression in healthy ovaries

The staining of the tissues from two healthy ovaries and two healthy tonsils with HLA class I HC and  $\beta_2$ -m mAb served as positive controls. Immunoreactive expression was detected in all cells of the parenchyma (follicle and tuba epithelium), endothelium and in the inflammatory cells of the stroma. In contrast, the staining of stromal fibroblasts was negative for the mAb HC10 and was only weakly positive for HCA mAb (Fig. 1).

### HLA class I HC and $\beta_2$ m expression in tumour tissue

Forty-seven lesions from the WP group and 115 lesions from the BP group were stained with the HC-10, HCA and L368 mAbs (Table 3) and evaluated by five grade scoring system. Most lesions in the BP group were score 3 or 4. On the contrary, most lesions in the WP group were grade 0, 1 or 2.

Scores were summarized in  $\geq 50\%$  (high) and  $<50\%$  (low) (Table 4). In the WP group, the numbers of lesions exhibiting a low staining by mAb HC10, HCA and L368 were significantly higher than in the BP group (HC10: 48% vs. 19%; HCA: 53% vs. 36%; L368: 81% vs. 57.5%). Interestingly, 40 tumours did not stain for mAb L368, but scored high for HC10 and HCA. They were equally distributed between the BP and WP groups. In the BP group, 45 patients were HLA-A02\*. Forty-three of them were distinguished by

a greater percentage of high staining compared to BP with other HLA genotypes.

#### Association of HLA class I heavy chain expression with patients' survival

The overall survival was estimated by Kaplan–Meir analysis of the cohort studied (Fig. 2a). Patients with tumours with high staining scores for HLA class I HC had a significantly longer survival than patients with low or absent HLA class I HC staining (HC10,  $P = 0.007$ ; HCA,  $P = 0.02$ ; Fig. 2b, c). On the other hand, no difference in survival between cases with  $\beta_2$ -m high- or low-stained tumours could be detected (L368  $P = 0.21$ ; Fig. 2d). When the 40 patients with low L368 but high HC10/HCA staining were excluded from the analysis, the difference in survival almost reached the significance threshold ( $P = 0.07$ ) (data not shown).

**Table 3** Percentage of tumours stained with antibodies detecting MHC molecules

Antibodies detecting MHC molecules on tumour tissue	Scoring <i>N</i>	Cohort (%)	WP (%)	BP (%)
HC-10	0	12.3	21	8.6
	1	6.1	10	4.3
	2	8.6	17	5.2
	3	36.4	26	40.5
	4	36.4	26	40.5
HCA	0	17.9	27.6	14
	1	9.8	12.7	8.6
	2	12.9	12.7	13
	3	43.2	34	47
	4	16.2	13	17.4
L368	0	24	34	20.2
	1	18.5	28	14.7
	2	21.6	19	22.6
	3	19.1	13	21.7
	4	16.6	6	20.8

**Table 4** Distribution of mAbs detecting <50% or equal/more than 50% MHC molecules on the tumour tissue

<sup>a</sup> <50% staining scores (low) and >50% staining scores (high)

<sup>b</sup> In parenthesis are presented the fraction of HLA-A02\* patients

<sup>c</sup> Comparison between % of WP and BP for low and high respectively.  $P$  values and  $\chi^2$

Antibodies detecting MHC molecules on tumour tissue	Cumulative % of positive tumour tissue <sup>a</sup>	Cohort	WP	BP (HLA-A02*) <sup>b</sup>	$P$ ( $\chi^2$ ) <sup>c</sup>
	<i>N</i>	162%	47%	115 (45)%	
HC-10	<50% (low)	27	48	19 (6.5)	<0.001 (14.6)
	≥50% (high)	73	52	81 (93.5)	<0.001 (18.8)
HCA	<50% (low)	41	53	35.5 (11)	0.04 (4.5)
	≥50% (high)	59	47	64 (8.9)	0.02 (5.1)
L368	<50% (low)	64	81	57.5 (30.4)	0.02 (5.7)
	≥50% (high)	36	19	42.5 (69.6)	<0.001 (11.3)

The probability of survival was higher in the BP than in the WP group ( $P > 0.001$ , Fig. 2e). This confirms previous results obtained with a different patients cohort [8]. BP HLA-A02\* patients are characterized by significant longer survival compared with the other groups ( $P = 0.02$ , Fig. 2e) and also a tendency of higher staining scores for HLA class I HC.

The introduction of low and high HLA class I HC or  $\beta_2$ -m staining variables could not further improve the probability of survival of WP and BP groups (Fig. 2f–h).

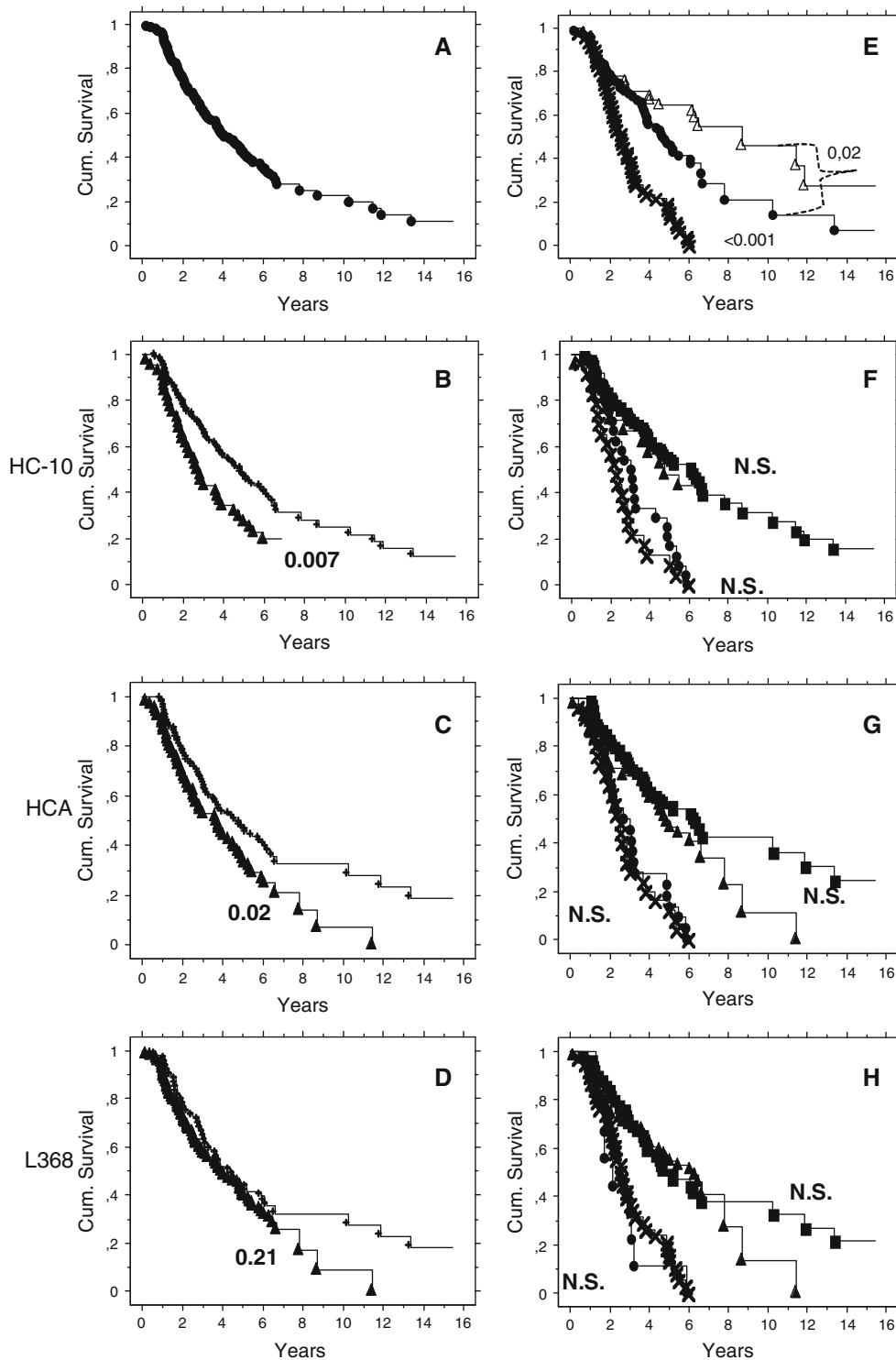
These observations were completed by time to death validation using the proportional hazard regression method (Table 5). Prognostic groups (WP and BP) with high and low HLA class I HC and  $\beta_2$ -m expression were tested in univariate and multivariate analyses. The HR in univariate analysis for WP versus BP was 3.3 (CI 2.2–4.9  $P = <0.001$ ) and for low versus high staining for HC10 was 1.7 (CI 1.1–2.5,  $P = 0.01$ ) and for HCA 1.5 (CI 1.5–2.2,  $P = 0.02$ ). As expected from the results presented in the Kaplan–Meir analysis, the HR for mAb L368 low versus high staining was 1.27 (CI 0.86–1.9,  $P = 0.22$ ).

The multivariate analysis ranked WP versus BP highly, superior to the low versus high HLA class I HC and  $\beta_2$ -m staining. Thus, the combination of variables that characterizes WP patients (HR 3.2, CI 2.1–4.9,  $P = <0.001$ ) is an independent predictor of risk of death regardless of HLA class I HC or  $\beta_2$ -m staining (HC10 1.14,  $P = 0.64$ ; HCA 1.37,  $P = 0.64$ ; L368 0.79,  $P = 0.34$ ).

Stratification for HLA-A02\* in univariate and multivariate strongly corroborates the data presented for the all cohort.

## Discussion

Cancer patients with serous adenocarcinoma of the ovary in advanced stage have a significant worse prognosis if they carry HLA-A02\* genotype; however, this is not a risk factor [8, 10]. The distribution of HLA haplotypes is different over the world and mirrors populations migration as well as



**Fig. 2** Kaplan–Meier and log-rank *P* values. **a** All the cohort. **b** Immunohistochemistry staining with HC10 mAb. High (*plus symbol*) and low (*filled triangle*). **c** Stained HCA mAb. **d** Stained with L368 mAb. **e** Kaplan–Meier and log-rank *P* values of patients stratified for stage III–IV and serous histology and HLA-A02\*<sup>+</sup> WP (*times*

*symbol*) versus BP (*filled circle*) and BP HLA-A02\*<sup>+</sup> (*open triangle*). **f** As in E but in the log-rank calculation are considered also the subsets defined by HC10, HCA (**g**) and L368 (**h**). BP: high (*filled square*); low (*filled triangle*). WP: high (*filled circle*); low (*times symbol*)

selection due to environmental pressure; HLA-A02\* genotype is most common in Scandinavia, Asia and among American Indians [40–42]. We investigated the correlation

of HLA-A02\* genotype with HLA class I HC and  $\beta_2$ -m expression in EOC patients. Since many other studies demonstrated HLA class I HC down-regulation as an

**Table 5** Proportional hazard regression and time to death. Comparison between MHC class I products and HLA genotype

Strata	Factors	Univariate analysis			Multivariate analysis		
		HR	95% CI	P	HR	95% CI	P
All patients not stratified	WP versus BP	3.3	2.2–4.9	<0.001	3.2	2.1–4.9	<0.001
	HC10 low versus high	1.7	1.1–2.5	0.01	1.14	0.65–1.99	0.64
	HCA low versus high	1.50	1.01–2.2	0.02	1.37	0.8–2.3	0.24
	L368 low versus high	1.27	0.86–1.9	0.22	0.79	0.49–1.3	0.34
HLA-A02*	WP versus BP	5.4	3–10	<0.001	5.8	3.1–10.9	<0.001
	HC10 low versus high	1.8	1–3.2	0.05	1.3	0.5–3.2	0.54
	HCA low versus high	1.5	0.9–2.6	0.1	0.9	0.4–2.3	0.99
	L368 low versus high	1.34	0.83–2.1	0.23	0.7	0.4–1.3	0.31

independent prognostic marker [7, 43, 44], we investigated whether this could be a possible explanation for the poor prognostic impact of HLA-A02\* as previously described by us [8, 10].

Firstly, we could confirm our previous observation that the WP group identified by serous histology, advanced surgical stage and HLA-A02\* genotype had an impaired prognosis [8].

Our results show that HLA-A02\* genotype plays the leading role in the natural course of EOC of serous histology. HLA-A02\* links a prognostic factor to the aggressiveness of the disease. We have no evidence of HLA-A02\* as a predictive risk factor before the tumour arises. One might speculate that HLA-A02\* or genes closely connected to the HLA-A02\* locus on chromosome 6 are related to other known or unknown molecules involved in proliferation, metastases and migration or NK cell resistance of tumour cells. It has recently been shown that clonal chromosomal imbalances detected by FISH with probes targeting 6p25, centromer 6 and 6q23 identify melanomas with metastatic potential [45].

HLA-G and HLA-E are also relevant, they are HLA class I paralogues and are both involved in suppression of NK cell-mediated lysis [46]. In particular, HLA-G has been described as a diagnostic and poor prognostic factor in various cancers including advanced stages of ovarian cancer [47]. Both HLA-G and HLA-E genes are in close proximity of the HLA-A gene. We are therefore currently investigating the relationship between HLA-A02\* genotype and the expression of HLA-E and HLA-G antigens. This could add crucial information to explain the relationship between poor survival and HLA-A02\* genotype.

Our second conclusion is that altered expression of HLA class I HC in tumour tissues predicts a lower overall survival in univariate analysis, which is in agreement with a previous study describing MHC I APM component expression and intratumoral infiltrating T-cells as independent prognostic markers in ovarian carcinoma [29]. However,  $\beta_2$ -m down-regulation showed no impact on survival in our study.  $\beta_2$ -m is typically co-expressed with HLA class I HC and both are needed for the trimeric HLA/ $\beta_2$ -m/peptide complex that is presented on the cell surface. The trimeric

complex is only recognized by the mAb W6/32, which does not work in formalin fixed, paraffin-embedded tissues. With that, in our study, there only exists indirect evidence of whole HLA molecule expression. On the other hand, investigation of the individual products offers very detailed information. We found that mAb L368 directed against  $\beta_2$ -m showed more “low stained” cases compared to both mAbs HC10 and HCA. However, a significant correlation to survival in univariate or multivariate analysis was not detected. Mutations or deletions in the  $\beta_2$ -m gene have been described in colon carcinoma, melanoma and other tumours [48, 49], and our interpretation of the 40 cases with low  $\beta_2$ -m expression, but high expression of HLA class I HC, is that it most possibly reflect  $\beta_2$ -m regulation at the gene level independently from HLA class I HC expression. Cases, with low staining for all three mAbs, had HR comparable to the HR observed for HLA class I HC loss alone and were more likely to represent co-down-regulation.

Our final and most important conclusion in our work highlights the association between loss of HLA class I HC expression and HLA-A02\* genotype in the WP group. In multivariate analysis, the combination of variables that characterizes WP patients is an independent predictor of risk of death regardless of HLA class I HC or  $\beta_2$ -m expression.

This is the first study, to our knowledge, examining the prognostic influence of both HLA genotype and HLA class I HC protein expression in EOC patients.

Limitations in this study include both the nature of paraffin-embedded tissues and the mAbs.

Immunohistochemical analyses of paraffin-embedded tissues are limited by the availability of locus-specific mAbs and interpretation must be made with these restrictions in mind. The two mAbs used recognize HLA class I HC of different loci: the HC10 mAb detects mainly HLA-B and HLA-C, the HCA mAb mainly HLA-A and HLA-G antigens [36, 50].

In our study, mAb HC10 stained the tumour tissues with higher frequency than mAb HCA. This could be either due to a higher sensitivity of mAb HC10 or to a higher



frequency of HLA-B and HLA-C antigen expression [51, 52]. If the latter would be the case, further studies on the underlying molecular mechanisms of HLA class I antigen alterations are motivated with focus on the HLA-A locus, in particular HLA-A02\*.

Immunohistochemical results give a good indication whether any alterations exists at all. They represent the basis for studies on larger patient populations and for further investigations regarding the underlying molecular mechanisms, such as loss of heterozygosity, deletions or mutations at different HLA loci. It is noteworthy that all the specimens analysed in this study were obtained from primary and not metastatic tumour lesions. Hence, this material represents different degrees of aggressiveness at the beginning of the clinical care, enabling comparative analysis of clinical outcome.

In conclusion, EOC is a deadly disease with poor outcome in general. Very few prognostic tools are available. Consequently, most patients receive indiscriminate treatment. WP patients progress early and rapidly during treatment and are likely to suffer more than they benefit from therapies today. Thus, according to our findings, it is essential to define the HLA-A genotype as soon as the patient is classified as serous adenocarcinoma of the ovary in surgical stage III–IV since additional treatment strategies need to be applied.

Investigations of loss or down-regulation of HLA class I HC have only a relative consequence in patient care or prognostic assessment. We conclude that analysis of HLA I HC expression should be pertinent when immune-based treatments are considered, and our observations point out the need for development of immunotherapies not solely relying on HLA class I restricted CD8 T-cells.

Further studies with focus on the genetics are necessary to understand the underlying mechanisms and hopefully find leads towards new therapeutic strategies.

**Acknowledgments** The statistics was performed under the supervision of Dr. Henning Johansson, Department of Oncology, Radiumhemmet, Karolinska Hospital, Stockholm, Sweden: hemming.johansson@karolinska.se. We thank Dr. Anders Höög, Department of Oncology-Pathology, Karolinska Institutet, Karolinska University Hospital, SE-171 76 Stockholm, Sweden, anders.hoog@karolinska.se as third pathologist consultant in the diagnosis and read out of immunohistochemistry. This study was supported by grants from the Cancer Society in Stockholm and the King Gustaf V Jubilee Fund and the Swedish Cancer Society, the Karolinska Institute/Stockholm County ALF grant and the Deutsche Forschungsgemeinschaft (DFG) Se-585-10-1 (Barbara Seliger). We gratefully acknowledge the motility grant #040226# from “NordForsk, Holbergs gate 1, NO-0166 Oslo, Norway” to the NCV (Emilia Andersson, HH, Lisa Villabona, Kjell Bergfeldt, Barbara Seliger, Rolf Kiessling) network project. We thank: Dr. Elisabeth Åvall-Lundqvist for her suggestions; Mrs. Liss Garber, Mrs. Gunilla Hagberg and the personnel at the Pathology Unit for assistance with the histological samples.

**Conflict of interest** The authors have no conflicts of interest.

## References

- Auersperg N, Wong AS, Choi KC, Kang SK, Leung PC (2001) Ovarian surface epithelium: biology, endocrinology, and pathology. *Endocr Rev* 22(2):255–288. doi:10.1210/er.22.2.255
- Seidman JD, Horkayne-Szakaly I, Haiba M, Boice CR, Kurman RJ, Ronnett BM (2004) The histologic type and stage distribution of ovarian carcinomas of surface epithelial origin. *Int J Gynecol Pathol* 23(1):41–44. doi:10.1097/01.pgp.0000101080.35393.16
- Agarwal R, Linch M, Kaye SB (2006) Novel therapeutic agents in ovarian cancer. *Eur J Surg Oncol* 32(8):875–886. doi:10.1016/j.ejso.2006.03.041
- Parkin DM, Bray F, Ferlay J, Pisani P (2002) Global cancer statistics. *CA Cancer J Clin* 55(2):74–108. doi:55/2/74
- Herbst AL (1994) The epidemiology of ovarian carcinoma and the current status of tumor markers to detect disease. *Am J Obstet Gynecol* 170(4):1099–1105. doi:S0002937894000955; Discussion 1105–1097
- Coukos G, Rubin SC (1998) Chemotherapy resistance in ovarian cancer: new molecular perspectives. *Obstet Gynecol* 91(5 Pt 1):783–792
- Callahan MJ, Nagymanyoki Z, Bonome T, Johnson ME, Litkouhi B, Sullivan EH, Hirsch MS, Matulonis UA, Liu J, Birrer MJ, Berkowitz RS, Mok SC (2008) Increased HLA-DMB expression in the tumor epithelium is associated with increased CTL infiltration and improved prognosis in advanced-stage serous ovarian cancer. *Clin Cancer Res* 14(23):7667–7673. doi:10.1158/1078-0432.CCR-08-0479
- Gamzatova Z, Villabona L, Dahlgren L, Dalianis T, Nillson B, Bergfeldt K, Masucci GV (2006) Human leucocyte antigen (HLA) A2 as a negative clinical prognostic factor in patients with advanced ovarian cancer. *Gynecol Oncol* 103(1):145–150. doi:10.1016/j.ygyno.2006.02.004
- De Petris L, Bergfeldt K, Hising C, Lundqvist A, Tholander B, Pisa P, Van Der Zanden HG, Masucci G (2004) Correlation between HLA-A2 gene frequency, latitude, ovarian and prostate cancer mortality rates. *Med Oncol* 21(1):49–52. doi:10.1385/MO:21:1:49
- Gamzatova Z, Villabona L, van der Zanden H, Haasnoot GW, Andersson E, Kiessling R, Seliger B, Kanter L, Dalianis T, Bergfeldt K, Masucci GV (2007) Analysis of HLA class I–II haplotype frequency and segregation in a cohort of patients with advanced stage ovarian cancer. *Tiss Antigens* 70(3):205–213. doi:10.1111/j.1399-0039.2007.00875.x
- Masucci GV, Andersson E, Villabona L, Helgadottir H, Bergfeldt K, Cavallo F, Forni G, Ferrone S, Aniruddha C, Seliger B, Kiessling R (2010) Survival of the fittest or best adapted-HLA-dependent tumour development. *J Nucl Acids Investig*. doi:10.4081/jnai.2010.e9
- So T, Takenoyama M, Sugaya M, Yasuda M, Eifuku R, Yoshimatsu T, Osaki T, Yasumoto K (2001) Unfavorable prognosis of patients with non-small cell lung carcinoma associated with HLA-A2. *Lung Cancer* 32(1):39–46
- Rogentine CN Jr, Dellon AL, Chretien PB (1977) Prolonged disease-free survival in bronchogenic carcinoma associated with HLA-Aw19 and HLA-B5. A 2-year prospective study. *Cancer* 39(6):2345–2347
- Helgadottir H, Andersson E, Villabona L, Kanter L, van der Zanden H, Haasnoot GW, Seliger B, Bergfeldt K, Hansson J, Ragnarsson-Olding B, Kiessling R, Masucci GV (2009) The common Scandinavian human leucocyte antigen ancestral haplotype 62.1 as prognostic factor in patients with advanced malignant melanoma. *Cancer Immunol Immunother* 58(10):1599–1608. doi:10.1007/s00262-009-0669-8

15. Tisch M, Kyrberg H, Weidauer H, Mytilineos J, Conradt C, Opelz G, Maier H (2002) Human leukocyte antigens and prognosis in patients with head and neck cancer: results of a prospective follow-up study. *Laryngoscope* 112(4):651–657. doi:[10.1097/00005537-200204000-00011](https://doi.org/10.1097/00005537-200204000-00011)
16. Hoon DS, Okamoto T, Wang HJ, Elashoff R, Nizze AJ, Foshag LJ, Gammon G, Morton DL (1998) Is the survival of melanoma patients receiving polyvalent melanoma cell vaccine linked to the human leukocyte antigen phenotype of patients? *J Clin Oncol* 16(4):1430–1437
17. Lee JE, Abdalla J, Porter GA, Bradford L, Grimm EA, Reveille JD, Mansfield PF, Gershenwald JE, Ross MI (2002) Presence of the human leukocyte antigen class II gene DRB1\* 1101 predicts interferon gamma levels and disease recurrence in melanoma patients. *Ann Surg Oncol* 9(6):587–593
18. Marincola FM, Venzon D, White D, Rubin JT, Lotze MT, Simonis TB, Balkissoon J, Rosenberg SA, Parkinson DR (1992) HLA association with response and toxicity in melanoma patients treated with interleukin 2-based immunotherapy. *Cancer Res* 52(23):6561–6566
19. Mitchell MS, Harel W, Groshen S (1992) Association of HLA phenotype with response to active specific immunotherapy of melanoma. *J Clin Oncol* 10(7):1158–1164
20. Sosman JA, Unger JM, Liu PY, Flaherty LE, Park MS, Kempf RA, Thompson JA, Terasaki PI, Sondak VK (2002) Adjuvant immunotherapy of resected, intermediate-thickness, node-negative melanoma with an allogeneic tumor vaccine: impact of HLA class I antigen expression on outcome. *J Clin Oncol* 20(8):2067–2075. doi:[10.1200/jco.2002.08.072](https://doi.org/10.1200/jco.2002.08.072)
21. Bain C, Merrouche Y, Puisieux I, Blay JY, Negrier S, Bonadona V, Lasset C, Lanier F, Duc A, Gebuhrer L, Philip T, Favrot MC (1997) Correlation between clinical response to interleukin 2 and HLA phenotypes in patients with metastatic renal cell carcinoma. *Br J Cancer* 75(2):283–286
22. Franzke A, Buer J, Probst-Kepper M, Lindig C, Framzle M, Schrader AJ, Ganser A, Atzpodien J (2001) HLA phenotype and cytokine-induced tumor control in advanced renal cell cancer. *Cancer Biother Radiopharm* 16(5):401–409
23. Onishi T, Ohishi Y, Iizuka N, Imagawa K (1996) Phenotype frequency of human leukocyte antigens in Japanese patients with renal cell carcinoma who responded to interferon-alpha treatment. *Int J Urol* 3(6):435–440
24. Mehta AM, Jordanova ES, Kenter GG, Ferrone S, Fleuren G (2008) Association of antigen processing machinery and HLA class I defects with clinicopathological outcome in cervical carcinoma. *Cancer Immunol Immunother* 57(2):197–206
25. Montoya L, Saiz I, Rey G, Vela F, Clerici-Larradet N (1998) Cervical carcinoma: human papillomavirus infection and HLA-associated risk factors in the Spanish population. *Eur J Immunogenet* 25(5):329–337
26. Cortes J, Fayad L, Kantarjian H, O'Brien S, Lee MS, Talpaz M (1998) Association of HLA phenotype and response to interferon-alpha in patients with chronic myelogenous leukemia. *Leukemia* 12(4):455–462
27. Del Campo A, Mendez R, Aptsiauri N, Rodriguez T, Zinchenko S, Garcia A, Gallego A, Gaudernack G, Ward S, Ruiz-Cabello F, Garrido F (2007) HLA total loss: different molecular mechanisms found in different tumor cell lines. *Tiss Antigens* 69(5):412
28. Cabrera T, Lopez-Nevot MA, Gaforio JJ, Ruiz-Cabello F, Garrido F (2003) Analysis of HLA expression in human tumor tissues. *Cancer Immunol Immunother* 52(1):1–9. doi:[10.1007/s00262-002-0332-0](https://doi.org/10.1007/s00262-002-0332-0)
29. Han LY, Fletcher MS, Urbauer DL, Mueller P, Landen CN, Kamat AA, Lin YG, Merritt WM, Spannuth WA, Deavers MT, De Geest K, Gershenson DM, Lutgendorf SK, Ferrone S, Sood AK (2008) HLA class I antigen processing machinery component expression and intratumoral T-cell infiltrate as independent prognostic markers in ovarian carcinoma. *Clin Cancer Res* 14(11):3372–3379. doi:[10.1158/1078-0432.CCR-07-4433](https://doi.org/10.1158/1078-0432.CCR-07-4433)
30. Leffers N, Lambeck AJ, de Graeff P, Bijlsma AY, Daemen T, van der Zee AG, Nijman HW (2008) Survival of ovarian cancer patients overexpressing the tumour antigen p53 is diminished in case of MHC class I down-regulation. *Gynecol Oncol* 110(3):365–373. doi:[10.1016/j.ygyno.2008.04.043](https://doi.org/10.1016/j.ygyno.2008.04.043)
31. Benedet JL, Bender H, Jones H III, Ngan HY, Pecorelli S (2000) FIGO staging classifications and clinical practice guidelines in the management of gynecologic cancers. FIGO committee on gynecologic oncology. *Int J Gynaecol Obstet* 70(2):209–262
32. Tavassoli F, Devilee P (2003) Tumours of the breast and female genital organs. IARC Press, Lyon. ISBN 9283224124
33. Lampson LA, Fisher CA, Whelan JP (1983) Striking paucity of HLA-A, B, C and beta 2-microglobulin on human neuroblastoma cell lines. *J Immunol* 130(5):2471–2478
34. Perosa F, Luccarelli G, Prete M, Favoino E, Ferrone S, Dammacco F (2003) Beta 2-microglobulin-free HLA class I heavy chain epitope mimicry by monoclonal antibody HC-10-specific peptide. *J Immunol* 171(4):1918–1926
35. Sernee MF, Ploegh HL, Schust DJ (1998) Why certain antibodies cross-react with HLA-A and HLA-G: epitope mapping of two common MHC class I reagents. *Mol Immunol* 35(3):177–188. doi:[S0161589098000261](https://doi.org/S0161589098000261)
36. Stam NJ, Spits H, Ploegh HL (1986) Monoclonal antibodies raised against denatured HLA-B locus heavy chains permit biochemical characterization of certain HLA-C locus products. *J Immunol* 137(7):2299–2306
37. Lee YS, Kim TE, Kim BK, Park TG, Kim GM, Jee SB, Ryu KS, Kim IK, Kim JW (2002) Alterations of HLA class I and class II antigen expressions in borderline, invasive and metastatic ovarian cancers. *Exp Mol Med* 34(1):18–26
38. Garrido F, Cabrera T, Accolla RS et al. (1997) HLA and cancer. In: 12th International histocompatibility workshop study—HLA, Jun, St. Malo, France, vol I, 1996 edn, pp 445–452
39. Pecorelli S, Odicino F, Maisonneuve P, Creasman W, Shepherd J, Sideri M, Benedet J (1998) Carcinoma of the ovary. *J Epidemiol Biostat* 3(1):75–102
40. Cavalli-Sforza LL, Menozzi P, Piazza A (eds) (1994) The history and geography of human genes. Princetown University Press, Princetown
41. Jones PN (ed) (2004) American Indian MtDNA, Y chromosome genetic data, and the peopling of North America. Bäu Institute, Boulder
42. Parham P, Adams EJ, Arnett KL (1995) The origins of HLA-A, B, C polymorphism. *Immunol Rev* 143(1):141–180. doi:[10.1111/j.1600-065X.1995.tb00674.x](https://doi.org/10.1111/j.1600-065X.1995.tb00674.x)
43. Leffers N, Gooden MJ, de Jong RA, Hoogeboom BN, ten Hoor KA, Hollema H, Boezen HM, van der Zee AG, Daemen T, Nijman HW (2009) Prognostic significance of tumor-infiltrating T-lymphocytes in primary and metastatic lesions of advanced stage ovarian cancer. *Cancer Immunol Immunother* 58(3):449–459. doi:[10.1007/s00262-008-0583-5](https://doi.org/10.1007/s00262-008-0583-5)
44. Rolland P, Deen S, Scott I, Durrant L, Spendlove I (2007) Human leukocyte antigen class I antigen expression is an independent prognostic factor in ovarian cancer. *Clin Cancer Res* 13(12):3591–3596. doi:[10.1158/1078-0432.CCR-06-2087](https://doi.org/10.1158/1078-0432.CCR-06-2087)
45. North JP, Vetto JT, Murali R, White KP, White CR Jr, Bastian BC (2011) Assessment of copy number status of chromosomes 6 and 11 by FISH provides independent prognostic information in primary melanoma. *Am J Surg Pathol* 35(8):1146–1150. doi:[10.1097/PAS.0b013e318222a634](https://doi.org/10.1097/PAS.0b013e318222a634)
46. Menier C, Rouas-Freiss N, Favier B, LeMaoult J, Moreau P, Carosella ED (2010) Recent advances on the non-classical major histocompatibility complex class I HLA-G molecule. *Tiss Antigens* 75(3):201–206. doi:[10.1111/j.1399-0039.2009.01438.x](https://doi.org/10.1111/j.1399-0039.2009.01438.x)

47. Menier C, Prevot S, Carosella ED, Rouas-Freiss N (2009) Human leukocyte antigen-G is expressed in advanced-stage ovarian carcinoma of high-grade histology. *Hum Immunol* 70(12):1006–1009. doi:[10.1016/j.humimm.2009.07.021](https://doi.org/10.1016/j.humimm.2009.07.021)
48. Atkins D, Ferrone S, Schmahl GE, Storkel S, Seliger B (2004) Down-regulation of HLA class I antigen processing molecules: an immune escape mechanism of renal cell carcinoma? *J Urol* 171(2 Pt 1):885–889. doi:[10.1097/01.ju.0000094807.95420.fe](https://doi.org/10.1097/01.ju.0000094807.95420.fe)
49. Hirata T, Yamamoto H, Taniguchi H, Horiuchi S, Oki M, Adachi Y, Imai K, Shinomura Y (2007) Characterization of the immune escape phenotype of human gastric cancers with and without high-frequency microsatellite instability. *J Pathol* 211(5):516–523. doi:[10.1002/path.2142](https://doi.org/10.1002/path.2142)
50. Stam NJ, Vroom TM, Peters PJ, Pastoors EB, Ploegh HL (1990) HLA-A and -B-specific monoclonal antibodies reactive with free heavy chains in western blots, in formalin-fixed, paraffin-embedded tissue sections and in cryo-immuno-electron microscopy. *Int Immunol* 2(2):113–125
51. Menon AG, Morreau H, Tollenaar RA, Alphenaar E, Van Puijnenroek M, Putter H, Janssen-Van Rhijn CM, Van De Velde CJ, Fleuren GJ, Kuppen PJ (2002) Down-regulation of HLA-A expression correlates with a better prognosis in colorectal cancer patients. *Lab Invest* 82(12):1725–1733
52. Demanet C, Mulder A, Deneys V, Worsham MJ, Maes P, Claas FH, Ferrone S (2004) Down-regulation of HLA-A and HLA-Bw6, but not HLA-Bw4, allospecificities in leukemic cells: an escape mechanism from CTL and NK attack? *Blood* 103(8):3122–3130. doi:[10.1182/blood-2003-07-25002003-07-2500](https://doi.org/10.1182/blood-2003-07-25002003-07-2500)