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HLA superfamily assignment is a predictor of immune response to cancer testis antigens and survival in ovarian cancer

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Abstract

Objectives—To characterize the association between major histocompatibility complex (MHC) types and spontaneous antibody development to the cancer testis (CT) antigen NY-ESO-1.

Methods—Tumor expression of NY-ESO-1 and serum antibodies to NY-ESO-1 were characterized in addition to human leukocyte antigen (HLA) type for patients with epithelial ovarian cancer. HLA types were assigned to structure-based superfamilies and statistical associations were examined. HLA types were compared to existing reference libraries of HLA frequencies in a European-Caucasian American population.

Results—Out of 126 patients identified, 81% were expression positive and 48% had spontaneous antibody responses to NY-ESO-1. There was an association between HLA-B superfamily and seropositivity among patients with tumors expressing NY-ESO-1 ($p < 0.001$). The differences in HLA-B superfamily assignment were driven by HLA-B44. Among all patients, the B27 superfamily was over-represented compared with the general population ($p < 0.001$).

Conclusions—HLA type appears to be associated with spontaneous anti-CT antigen antibodies, as well as with the overall risk of ovarian cancer.

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Conflict of Interest Statement

The other authors declare that there are no conflicts of interest.

Introduction

Epithelial ovarian cancer is the most lethal gynecologic malignancy, with more than 75% of patients dying of their disease within five years of diagnosis. Despite numerous clinical trials investigating chemotherapeutic agents, dosing schedules, and routes of administration, minimal improvements have been made in the outcome of patients with this disease. In search for suitable alternatives, considerable interest has recently focused on immunotherapy as a potential strategy for ovarian cancer [1–2]. The potential for immunotherapy in ovarian cancer is based on evidence that tumor infiltration by CD8⁺ T cells leads to improved survival [3–5].

The anti-tumor effects of effector cells of the immune system are mediated by specific recognition of tumor-associated antigens (TAAs). The cancer testis antigens (CTA) are a subclass of TAA encoded by approximately 140 genes [6]. Expression of these antigens are known to be restricted in immune privileged sites such as the testes, placenta and fetal ovary, but not in other normal tissues. Abnormal expression of these germ-line genes in malignant tumors may reflect the activation of a silenced “gametogenic program”, which ultimately leads to tumor progression [7]. The immunogenicity of CTA has led to the widespread development of cancer vaccines targeting these antigens in many solid tumors. Among CT antigens, NY-ESO-1 is the most immunogenic and is expressed by approximately 40% of epithelial ovarian cancers [2–8]; and has emerged as a promising candidate for ovarian cancer immunotherapy [9–11]. Similar to other antigens, CT antigens are processed by antigen presenting cells (APCs) and presented in the context of HLA molecules to CT antigen-specific T cells that mediate tumor destruction [12].

Interestingly, a subset of ovarian cancer patients develop spontaneous immune responses to NY-ESO-1 [2–8]. This response is frequently integrated, consisting of antibody as well as CD4 and CD8 T-cell responses. The mechanism by which only a subset of patients with NY-ESO-1-expressing tumors develop this spontaneous immune response is currently unknown. Among factors thought to determine induction of immune response, individually different combinations of major histocompatibility complex (MHC) types are likely to play a crucial role [13–14]. This is because after antigen-processing, only selective peptide fragments bind on specific MHC molecules for presentation to T cells [14]. Therefore, it is possible that the immunogenicity of NY-ESO-1 is largely affected by patients' HLA types. HLA type has previously been implicated in the risk for and outcomes of patients with HIV, viral hepatitis, tuberculosis, and other cancers [15–18]. It has also been associated with certain autoimmune diseases, such as ankylosing spondylitis, autoimmune hepatitis, type I diabetes, and autoimmune polyendocrine syndrome [19–21]. Moreover, MHC has been associated with certain solid tumors, such as cervical and colorectal cancers [22–23]. The goals of this study were to determine if HLA haplotypes differ between (i) ovarian cancer patients with or without spontaneous immune response to NY-ESO-1; (ii) patients with and without tumor expression of NY-ESO-1; and (iii) patients with different outcomes.

Methods

Patients & Specimens

Patients who underwent HLA testing as part of a test for inclusion in one of several clinical trials between January 1, 2002 and December 31, 2012 were included in this study. All tissue specimens and health record information were accessed under an institutional review board approved protocol at the Roswell Park Cancer Institute (Buffalo, NY). All pathology specimens were reviewed by experienced gynecologic pathologists and classified according to WHO guidelines. The detailed handling protocol has been described elsewhere [24]. Briefly, formalin fixed paraffin embedded and flash frozen tumor specimens were obtained prospectively from patients diagnosed with ovarian, Fallopian tube, and primary peritoneal carcinoma (here referred to as EOC owing to their common origin of Müllerian tissues). Peripheral blood was additionally collected after surgery and serum was obtained by centrifugation. Medical records were reviewed from a prospectively maintained database to determine stage (assigned to FIGO 2014), three-tiered grade, debulking status, platinum status, and progression-free and overall survival. For patients with no disease-free interval, the date of progression was the earliest date of (a) twice the upper normal CA 125 (twice the nadir if the CA 125 never normalized), (b) biopsy proven disease at a second-look surgery, or (c) radiographic evidence of disease progression.

Tissue total RNA isolation

The methods of tissue RNA isolation have previously been described [24]. Briefly, frozen tissues were treated with the TRI Reagent (Molecular Research Center Inc, Cincinnati, OH) according to the manufacturer's protocol. DNase I (Baehringer-Mannheim, Mannheim, Germany) was used to remove any DNA contamination and RNA was suspended and quantified; quality was checked by electrophoresis on a 1.5% agarose gel.

Determination of NY-ESO-1 status

Tumor positivity for NY-ESO-1 expression status was determined by both RT-PCR and immunohistochemistry (IHC) using methods previously described [2-4]. Briefly, deparaffinized, formalin-fixed sections were treated with an antigen-retrieval system (DAKO high pH solution), incubated at 95 °C for 20 minutes, and cooled. Exogenous peroxidase activity was blocked using 0.3% hydrogen peroxide and 0.1% sodium azide containing PBS [4]. Mouse monoclonal antibodies for anti-human NY-ESO-1 (clone ES121) were used as the primary antibody and the EnVision Plus system (DAKO) was used for detection. Negative control slides omitting the primary antibody were included in all assays [4]. For RT-PCR, 2 µg of each RNA sample was reverse transcribed using the Ready-to-Go first strand synthesis kit (Pharmacia, Uppsala, Sweden). Primers for NY-ESO-1 were ESO1A (5'-CACACAGGATCCATGGATGCTGCAGATGCGG-3') and ESO1B (5'-CACACAAAGCTTGGCTTAGCGCCTCTGCCCTG-3'). Amplification was performed for 1 min at 94 °C, 1 min at 60 °C, and 1.5 min at 72 °C for 35 cycles. There was a 10-min elongation step at 72 °C following the cycles. The PCR products were 341 bp in length and visualized using ethidium bromide staining after separation over a 1.5% agarose gel [2]. Seropositivity for NY-ESO-1 was determined using methods previously described [2]. Briefly, Recombinant NY-ESO-1 protein at a concentration of 1 µg/mL in PBS was adsorbed

to 96-well half area plates (Corning) at 30 μ L/well overnight at 4°C. Plates were washed with PBS and blocked with 5% NF milk for 2 hours at room temperature. After washing, 30 μ L/well of serum dilutions in 5% NF milk were added and incubated overnight at 4°C. Plates were washed, and 30 μ L/well diluted secondary antibody-5% NF milk were added (goat anti-human IgG-AP; Southern Biotechnology, Birmingham, AL) and incubated for 1 h at room temperature. Plates were washed, incubated with 30 μ L/well of substrate solution (Attophose substrate; JBL Scientific, San Louis Obispo, CA) for 30 min at room temperature, add 15 μ L of 3N NaOH, and immediately read by microplate reader (Perseptive Biosystems, Cytofluor Series 4000, Forster City, CA) set for Excitation 450/50, Emission 580/50 and gain 25. Sera were tested over a range of 4-fold dilutions from 1:100 to 1:100,000. NY-ESO-1 seropositivity was defined as a reciprocal titer > 100.

HLA Typing

HLA typing was performed at the HLA typing laboratory of the Roswell Park Cancer Institute using sequence-specific primer pairs obtained from Genovision [25]. Of the 126 patients included in the present study, HLA-A was tested in 121 (96.0%) and HLA-B in 90 (71.4%) patients and all alleles were assigned to HLA superfamilies as defined by Sidney et al [26]. MHC class II was additionally tested and assigned to superfamilies in 120 (95.2%) patients.

Superfamily assignment

HLA superfamily classification can be performed using multiple methods including binding pocket peptide sequence homology [27, 28], global sequence homology [29], structural interaction patterns [30], and pocket chemical specificity [26]. After an initial exploration of the frequency distribution of MHC class I alleles, we adopted superfamily classification as defined by Sidney et al [26]. Briefly, HLA alleles were compared for peptide sequence homology and similarity of peptide binding pockets specificities before assignment to various superfamilies. The other classification schemes were also explored and found to be inadequate due to significant numbers of unclassified alleles [27–30]. We further evaluated associations between zygosity of allele and allelic families to determine if the heterozygosity of MHC class I is associated with ovarian cancer incidence or survival. For comparison between ovarian cancer patients and the general population, a standard reference library of allelic frequencies was downloaded from HLA frequencies.net, using the USA NMDP European Caucasian (n = 1,242,890) accessed 3/31/15) [31].

Statistical Analysis

All statistical analyses were performed using SAS Software (Cary, NC) version 9.4 and the R 3.1.2 statistical computing language. A nominal significance threshold of 0.05 was used unless otherwise specified. A 2×2 contingency table was used to evaluate the concordance of RT-PCR and ELISA in detection of the NY-ESO-1 status.

Statistical testing included Student's t-test, χ^2 and Fisher's exact tests, and Kaplan-Meier survival analysis with log-rank testing. The multivariate analysis included stage, categorized as early (I or II) or late (III or IV), grade (1 vs. 2/3), debulking status (optimal vs. suboptimal), and platinum status. Progression free survival (PFS) and overall survival (OS)

were computed from the date of diagnosis to the date of initial recurrence for PFS and date of death for OS. Patients who did not experience a recurrence or death were censored at the date of last visit for PFS and OS, respectively.

Results

Patient Population and expression of immune responses to NY-ESO-1

We identified 139 patients with matched tissue and serum specimens of sufficient quantity for analysis. Thirteen patients were excluded for lack of definitive NY-ESO-1 expression-related information (1 missing IHC, 4 missing ELISA, and 8 missing PCR); in total 126 patients were included in the analysis, 125 of whom were identified as non-Hispanic Caucasians (99.2%). The NY-ESO-1 mRNA transcript was expressed in 61 of 99 patients tested (61.6%); IHC was performed on 116 patients, including the remaining 27 who did not undergo RT-PCR testing, and 76 of 116 (65.5%) stained positive for protein NY-ESO-1 expression. As production of antibody requires NY-ESO-1 protein expression in tumor, 6 seropositive patients with negative IHC and RT-PCR testing were considered expression positive. The absence of antigen expression in seropositive patients may reflect immune selection pressure. In total, NY-ESO-1 expression was thus detected in 102 of 126 (81%) patients and 19% (24/126) of patients were NY-ESO-1 expression negative. Because of the selection criteria for this study the difference in prevalence of NY-ESO-1 protein expression from prior reported prevalence in ovarian cancer patients may be explained by these selection forces [2, 8]. The expression positive group (102/126) was further divided into seronegative (41/102, 40.2%) and seropositive (61/102, 59.8%) for a total of three analysis groups.

Patient characteristics associated with NY-ESO-1 expression and seroreactivity groups are summarized in Table 1. Seropositive patients were an average of 10 years older (58 vs. 68 years, $p < 0.001$) with higher fraction of grade 3 disease (80% vs. 93%, $p = 0.025$) and two-fold higher platinum resistant/refractory disease than seronegative patients (23% vs. 51%, $p = 0.002$). Difference in FIGO stage, histotype (serous vs. non-serous), and debulking status were not different among the groups.

NY-ESO-1 specific results

The HLA superfamilies stratified by NY-ESO-1 analysis classes (Table 2) show no significant difference in the distribution of superfamily assignment between patients with spontaneous seropositivity to NY-ESO-1 and those without, for either HLA-A (chi-square $p=0.52$) or HLA-B superfamilies ($p=0.85$). Among seronegative patients, 23 had tumors that were expression negative for NY-ESO-1 by both IHC and RT-PCR and 38 were expression positive. When only patients with NY-ESO-1 expression positive tumors were considered, there were significant differences in HLA-B superfamily assignments between patients who were seropositive and seronegative ($p < 0.001$). This difference in superfamily assignment was driven by HLA-B44, which appeared to be associated with more frequent spontaneous antibody responses.

Comparison with General Population

The distribution of alleles into the HLA-A and HLA-B superfamilies was significantly different from that expected based on the distribution obtained at HLAfrequencies.net [31]. Upon subgroup analysis, patients seronegative and expression negative or seropositive and expression positive were not significantly different from the expected HLA-A distribution ($p = 0.36$ and $p = 0.07$, respectively), but their HLA-B superfamily distribution did still differ from expected with more ovarian cancer patients being in the HLA-B27 superfamily than expected ($p = 0.019$). In contrast, HLA-B7 and -B44 occurred at lower frequency than the general population ($p < 0.001$). In another subgroup comparison, the seronegative/expression positive group was significantly different from expected in both HLA-A ($p = 0.002$) and HLA-B ($p < 0.001$) superfamily distributions. The HLA differences between these cancer patients and the general population were driven by the A2, B27, and B44 superfamilies. In the present study A2 and B44 superfamilies were less common and the B27 was more common in cancer patients than in the general population. We also evaluated the effects of superfamily homozygosity/heterozygosity on tissue and serum NY-ESO-1, but did not find any association (data not shown).

MHC Class II

There was no significant difference in the distribution of MHC Class II alleles when we compared the three subgroups with each other or with the general population [31] (data not shown).

Survival Analysis

Expression of HLA-B44 superfamily was associated with a worse PFS (median 18.8 vs. 13.9 months, $p = 0.030$), as well as a non-significant drop in OS (median 61.8 vs. 38.0 months, $p = 0.22$). However, it should be noted that there was significant heterogeneity in therapy and the observed associations with PFS and OS may be explained by differences in treatment received.

Discussion

HLA-superfamily is associated with NY-ESO-1 seropositivity in patients with epithelial ovarian cancer expressing NY-ESO-1. Specifically, we found that HLA B44 patients with tumor NY-ESO-1 expression were more likely to have spontaneous antibody responses than those with other HLA-B superfamily types. Furthermore, although it is possible that the association of HLA with survival is confounded by vaccine therapy in a subset of our study population [9], seropositivity was independently associated with platinum status, suggesting potential for a true association to be identified in a larger study. Prior studies among Japanese and Dutch patients have identified associations between HLA and ovarian cancer prognosis [32, 33]. Mariya et al identified an association between epithelial ovarian cancer cell expression of HLA-class I molecules and prognosis in Japanese patients [33]. They also identified a weak positive association between HLA class I expression and tumor infiltration with T-cells, a marker of host anti-tumor immune response [4].

A small study of HLA-class II antigens and cervical cancer in the United States implicated HLA-DQw3 in cancer susceptibility, given that it was found more frequently than expected in cancer cases vs. non-cases [22]. However, similar associations were not identified within the current dataset with respect to ovarian cancer. Existing data are conflicting with respect to HLA-DPB1*0401 and NY-ESO-1 positivity [34–35]. Zeng and colleagues found spontaneous seropositivity for NY-ESO-1 in 16 of 17 HLA-DP4 patients [34]; however in a larger study by Huarte et al the association was not identified [35]. The present study confirms the findings of Huarte et al of no association between DP4 positivity and spontaneous NY-ESO-1 seropositivity ($p = 0.53$; data not shown). In addition, we did not observe any association between HLA class II subtype and humoral anti-NY-ESO-1 response (data not shown). This is surprising because MHC class II-restricted CD4⁺ helper T cells are in general known to help induce antibody production. It is possible that the sample size in this study is too small to achieve statistical significance for each HLA class II subtype.

When exploring this patient population in general, HLA superfamily is associated with a diagnosis of ovarian cancer. Specifically, ovarian cancer patients were more than twice as likely to have alleles from the B27 superfamily compared with what was expected in the general US population of European Caucasians [31], and more than five-fold greater than the population prevalence reported in the US National Health and Nutrition Examination Survey (NHANES) 2009 report [36]. This is particularly interesting because of the association with HLA-B27 with multiple inflammatory conditions, including ankylosing spondylitis. Chronic inflammation has been proposed as an etiologic factor for ovarian cancer. HLA-B27 has been shown to control viral infections such as HCV, EBV, HIV, and influenza [19–37]. However, in certain circumstances the host immune system becomes sensitized and auto-immune activation ensues. It is plausible that a similar inflammatory environment may pre-dispose women with HLA-B27 alleles to ovarian cancer given the recent attention to PID and endometriosis [38–39] as possible precursors or predisposing factors to some types of ovarian cancer.

On the other hand, HLA-B44 has been associated with worse prognosis, both PFS and OS, in patients with diffuse large B-cell lymphoma [18]. In the present study HLA-B44 was also associated with worse progression-free and overall survival. Associations between HLA type and the presence of tumor infiltrating lymphocytes (TILs) have previously been reported [32–33]; however, because of heterogeneity in treatments received among patients included in the present study, these differences in outcome should be interpreted with caution.

Limitations of this study include inferred classification of superfamily assignment according to HLA alleles and small sample size. The assignment was made using the Sidney et al [26] classification due to complete coverage of all HLA allele results with one superfamily or another and the judgment that their methods were the most comprehensive with respect to true *in vivo* behavior of MHC. Despite small sample size, which is typically a threat when non-significant results are found, several associations could be identified including that with the HLA-B27 superfamily. This family is especially striking because the 31% prevalence in this population is five times the reported population prevalence from NHANES 2009 [36], suggesting further investigation into this population of patients may be warranted.

In conclusion, HLA superfamily is associated with ovarian cancer risk and generation of spontaneous immunity for tumor-expressed CT antigens. The clinical significance of an association between the B27 superfamily and risk of developing ovarian cancer cannot be over-stated, as this may represent a new population of patients at increased risk of developing ovarian cancer. Similarly, ovarian cancer patients harboring alleles in the B44 superfamily, with their propensity to have baseline seropositivity to NY-ESO-1, may be particularly sensitive to immune modulators [40]. Larger studies are needed to confirm these findings and should be undertaken given the potential impact of the results.

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Highlights

- HLA-B27 superfamily assignment is associated with an increased risk of ovarian cancer compared with the general population
- Patients with specific HLA superfamily assignments may be more sensitive to immune modulating therapy

Table 1

Summary Statistics

Seroreactivity Expression		Negative		Positive		All groups (p)	Expression positive-only (p)
		Negative	Positive	Negative	Positive		
n		24	41	61			
Age	Years (at diagnosis)	60.5	57	68.4	<0.001	<0.001	
Stage	% IIIC or IV	96%	90%	89%	0.562	1.00	
Grade	% Grade 3	88%	75%	93%	0.033	0.022	
Histology	% Serous, Pap Serous	83%	83%	82%	0.386	0.857	
Debulking	% Optimal	83%	83%	77%	0.721	0.380	
Platinum Status	% Resistant/Refractory	17%	27%	51%	0.005	0.048	
Alleles	HLA-A Alleles	46	76	120			
	HLA-B Alleles	40	48	92			

Comparison of basic demographic/clinicopathologic information. All groups (seronegative/tumor expression negative, seronegative/tumor expression positive, and seropositive/tumor expression positive) were compared with a chi-square test. Tumor expression positive patients were compared on the basis of seroreactivity using Student's t test.

Table 2**Superfamily Results**

Distribution		Expression Frequency							
	REF	All Patients	SERO NEG	SERO POS	SERO NEG, EXPR NEG	SERO NEG, EXPR POS	SERO POS, EXPR POS		
	N1	244*	122	122	46	76	122		
A1	29%	32%	32%	33%	28%	37%	33%		
A2	30%	23%	18%	27%	22%	18%	27%		
A3	28%	27%	28%	27%	33%	28%	27%		
A24	11%	15%	16%	13%	17%	17%	13%		
AOTHER	2%	0%	0%	0%	0%	0%	0%		
Within Patient Comparison			0.52		N/A		0.53		
Comparison with Population		< 0.001	0.001	0.007	0.36	0.002	0.07		
	N2	180	88	92	40	48	92		
B7	30%	25%	25%	26%	25%	25%	26%		
B8	11%	11%	10%	12%	10%	10%	12%		
B27	12%	31%	32%	30%	28%	35%	30%		
B44	30%	18%	16%	21%	20%	12%	21%		
B58	5%	4%	5%	4%	0%	8%	4%		
B62	7%	6%	8%	3%	10%	6%	3%		
BOTHER	5%	4%	5%	3%	8%	2%	3%		
Within Patient Comparison			0.85		N/A		< 0.001		
Comparison with Population		< 0.001	< 0.001	< 0.001	0.019	< 0.001	< 0.001		

Comparison of HLA superfamily assignment to the frequency expected in the general population. AOTHER and BOTHER are alleles not assigned to any other superfamilies.

* - Numbers reported are the frequency of alleles for the 126 patients in the study. Numbers do not sum to 252 (126 patients $\times$ 2 alleles) because not all patients were tested for both HLA-A and HLA-B.