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Article

Predicting Toxicity and Response to Pembrolizumab Through Germline Genomic HLA Class 1 Analysis

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Abstract

Background: Human leukocyte antigen class 1 (HLA-1)–dependent immune activity is linked to autoimmune diseases. HLA-1–dependent $CD8^+$ T cells are required for immune checkpoint blockade antitumor activity. It is unknown if HLA-1 genotype is predictive of toxicity to immune checkpoint blockade. **Methods:** Patients with advanced solid tumors stratified into 5 cohorts received single agent pembrolizumab (anti-programmed cell death-1) 200 mg intravenously every 3 weeks in an investigator-initiated phase II trial (Investigator-Initiated Phase II Study of Pembrolizumab Immunological Response Evaluation study, NCT02644369). Germline whole-exome sequencing of peripheral blood mononuclear cells was performed using the Illumina HiSeq2500 platform. HLA-1 haplotypes were predicted from whole-exome sequencing using HLAminer and HLAVBSeq. Heterozygosity of HLA-A, -B, and -C, individual HLA-1 alleles, and HLA haplotype dimorphism at positions −21 M and −21 T of the HLA-A and -B leader sequence were analyzed as predictors of toxicity defined as grade 2 or greater immune-related adverse events and clinical benefit defined as complete or partial response, or stable disease for 6 or more cycles of pembrolizumab. Statistical significance tests were 2-sided. **Results:** In the overall cohort of 101 patients, the frequency of toxicity and clinical benefit from pembrolizumab was 22.8% and 25.7%, respectively. There was no association between any of the HLA-1 loci or alleles with toxicity. HLA-C heterozygosity had an association with decreased clinical benefit relative to HLA-C homozygosity when controlling for cohort (odds ratio = 0.28, 95% confidence interval = 0.09 to 0.91, $P = .04$). HLA-A and -B haplotype −21 M/T dimorphism and heterozygosity of HLA-A, -B, and -C were not predictive of outcomes. **Conclusions:** HLA-C heterozygosity may predict decreased response to pembrolizumab. Prospective validation is required.

Inhibitors of programmed cell death-1 or its ligand (anti-PD-1/L1) have demonstrated robust clinical outcomes in several poor-prognostic malignancies (1,2). However, they are limited by modest response rates of approximately 10%-30% (with lower rates in other tumor types) (3,4) and risk of all-grade and high-grade immune-related adverse events (irAEs) approaching 40% and 14%, respectively (5-8). Many putative predictive markers of response to immune checkpoint blockade (ICB) require molecular and immune profiling from either archival tumor specimen or invasive biopsy (9). With the exception of prior history of autoimmune disease (10), there are no validated biomarkers to identify which patients are likely to develop irAEs.

Human leukocyte antigen class 1 (HLA-1) molecules display intracellular nonself-peptide fragments to $CD8^+$ T cells (11) and natural killer (NK) cells (12) and thus play a critical role in the interface between malignant cells and the host immune system (13). ICB antitumor activity is largely dependent on HLA-1 interaction with $CD8^+$ T cells (14-16) and possibly to some degree

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with NK cells (17,18). Because each HLA-1 locus binds a finite quantity of peptide fragments, HLA-1 locus homozygosity limits the number of putative neoantigens presented to $CD8^+$ T cells and NK cells and potentially diminishes tumor immunogenicity (11). Similarly, loss of heterozygosity in HLA-1 loci on tumor cells may be a mechanism of immune evasion because of its association with increased PD-L1 staining on tumor-infiltrating leukocytes (19). The potential immune evasion from increased PD-L1 expression may explain the poor clinical outcomes in cancer patients with HLA expression downregulation (20-22). Further, HLA-1–dependent immune activity is also linked to autoimmune diseases and inflammatory conditions (23), implying a possible explanation behind the development of irAEs from ICB (24,25). This may partially be a result from educating NK cells in the concept of "self" by the HLA haplotype dimorphism at positions −21 M and −21 T of the HLA-A and -B leader sequence (26).

We hypothesize that toxicity and/or response to PD-1 inhibition is related to germline HLA-1 genotype. This primary objective was explored using 2-digit resolution HLA-1 haplotypes predicted from germline whole-exome sequence (WES) data within a prospective investigator-initiated phase II trial examining single-agent pembrolizumab (anti-PD-1) in advanced solid tumors.

Methods

Patients and Clinical Outcomes

The single-center, investigator-initiated biomarker phase II clinical trial called Investigator-Initiated Phase II Study of Pembrolizumab Immunological Response Evaluation (INSPIRE; NCT02644369) prospectively enrolled patients from March 21, 2016, to May 9, 2018, into 5 cohorts: squamous cell cancer of the head and neck, triple-negative breast cancer, epithelial ovarian cancer, malignant melanoma (cutaneous and noncutaneous), or mixed solid tumors. See Supplementary Methods (available online) for key inclusion and exclusion criteria.

All patients received single-agent pembrolizumab (anti-PD-1 antibody) 200 mg intravenously every 3 weeks. Clinical assessments included laboratory tests of organ function every 3 weeks and restaging computed tomography scans every 9 weeks. Clinical benefit was defined a priori as achieving Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 complete response or partial response, or stable disease after 6 or more cycles of pembrolizumab. Toxicity was defined a priori as developing grade 2 or greater Common Terminology Criteria for Adverse Events (CTCAE) 4.03 (27) irAE with at minimum possible attribution to pembrolizumab that the investigator ascribed to be of autoimmune etiology secondary to pembrolizumab treatment. Grade 2 or higher irAEs were chosen as a threshold because of their clinical significance (28). Only the highest grade of a specific irAE was recorded, but the same event could be recorded more than once in the event the irAE occurred on a separate occasion and was not a flare during immunosuppression taper. INSPIRE data coordinators annotated both response and toxicity data, and 1 author (M.I.) subsequently verified the accuracy. Two authors (M.I. and L.L.S.) verified that irAEs were autoimmune in causality from pembrolizumab exposure.

This study was reviewed and approved by the Research Ethics Board of the Princess Margaret Cancer Centre, University Health Network (Toronto, Canada), and patient-informed consent was obtained. The study was carried out in accordance with the Declaration of Helsinki.

Germline Genomic HLA-1 Analysis

Patients received baseline blood work within 28 days before first pembrolizumab exposure. A total of 30-40 mL of blood was collected into ethylenediaminetetraacetic acid (EDTA)tubes. Whole blood was processed within 18 hours, and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Paque Premium (GE Healthcare, Chicago, IL). PBMCs designated for DNA or RNA extraction were snap frozen on dry ice and stored at −80°C (median frozen time = 48 days, range = 0-529 days, thawed once).

PBMC DNA exomes were sequenced at the Princess Margaret Genomic Centre (www.pmgenomics.ca) and the Princess Margaret - Ontario Institute of Cancer Research Translational Genomics Laboratory (https://labs.oicr.on.ca/translational-genomics-laboratory) in Toronto, Canada. Please see Supplementary Methods (available online) for details on WES library construction, HLA-1 consensus, and genomic-inferred ethnicity.

PD-L1 Status

PD-L1 status was determined from formalin-fixed paraffin-embedded blocks of INSPIRE screening biopsies using the PD-L1 immunohistochemistry clone 22C3 applied to 4- to 5-μm sections mounted on positively charged ProbeOn slides (QualTek, Goleta, CA). QualTek generated a modified proportion score (MPS) indicating the proportion of PD-L1–expressing tumor cells and mononuclear inflammatory cells within tumor nests. Further details are found in Supplementary Methods (available online) and in the INSPIRE interim report (29).

Statistical Analysis

Clinical benefit and toxicity, as defined above, were the coprimary endpoints. Heterozygosity of HLA-A, -B, and -C, individual HLA alleles, and HLA haplotype dimorphism at positions −21 M and −21 T were tested to investigate if they were predictors of clinical benefit and toxicity. The testing was performed in 3 phases. The Fisher exact test was applied to obtain unadjusted P values. Conditional logistic regression was used to test the effects of HLA features on clinical benefit and toxicity while adjusting for cohort considered as stratum. The following covariates were chosen for inclusion into the multivariable model because of their possible association with ICB outcomes: age, sex, genomic-inferred ethnicity, and PD-L1 status. Propensity scores based on these covariates were used in the conditional logistic models to adjust the effect of HLA status on the coprimary endpoints. Odds ratios and 95% confidence intervals (CIs) were obtained from these models. HLA-1 alleles that were heterozygous in more than 10 patients were also explored as predictors of clinical benefit and toxicity. The multiple correlative analyses performed on the INSPIRE dataset precluded the use of correction for multiple testing.

The association between covariates and HLA-1 was investigated using the Fisher exact test for categorical variables or Mann-Whitney test for continuous variables. Median survival times and survival probabilities were calculated from Kaplan-Meier curves. Overall survival (OS) and progression-free survival (PFS) were measured as the duration between the day of first

Table 1. Patient demographics (n = 101) stratified by those who did or did not develop clinical benefit (CB) or toxicity to pembrolizumab

Covariates	Patients, No. (%)			Patients, No. (%)		
	Without CB (n = 75)	With CB (n = 26)	*P*	Without toxicity (n = 78)	With toxicity (n = 23)	*P*
Sex, female	50 (79.4)	13 (20.6)	.16[a]	53 (84.1)	10 (15.9)	.05[a]
Ethnicity, Caucasian	63 (75.0)	21 (25.0)	.76[a]	63 (75.0)	21 (25.0)	.35[a]
Age at first pembrolizumab infusion, median (range), y	57 (21-78)	62 (34-82)	.27[b]	58 (21-81)	61 (27-73)	.30[b]
Cohorts						
A: HNSCC	13 (72.2)	5 (27.8)	<.001[a]	12 (66.7)	6 (33.3)	.006[a]
B: TNBC	21 (95.5)	1 (4.5)		21 (95.5)	1 (4.5)	
C: HGSOC	17 (89.5)	2 (10.5)		15 (78.9)	4 (21.1)	
D: Melanoma	4 (33.3)	8 (66.7)		5 (41.7)	7 (58.3)	
E: Mixed solid tumor	20 (66.7)	10 (33.3)		25 (83.3)	5 (16.7)	
PD-L1 MPS-positive cells ≥1%[c]	31 (63.3)	18 (36.7)	.02[a]	35 (71.4)	14 (28.6)	.24[a]
PD-L1 MPS-positive cells, median (range)[c], %	0.0 (0.0-95.0)	6.0 (0.0-100.0)	.003[b]	0.0 (0.0-95.0)	12.0 (0.0-100.0)	.01[b]

[a]*P* value calculated by Fisher exact test, unadjusted. HGSOC = high-grade serous ovarian carcinoma; HNSCC = head and neck squamous cell carcinoma; MPS = modified percent score; PD-L1 = programmed cell death-1 or its ligand; TNBC = triple-negative breast cancer.
[b]*P* value calculated by Mann-Whitney test, unadjusted.
[c]Two patients had unknown PD-L1 MPS status and were removed from the PD-L1 MPS analysis.

pembrolizumab infusion and death owing to any cause, and death from any cause or progression of disease, respectively. The hazard ratios (HRs) and 95% confidence intervals were calculated within the Cox proportional hazards model using the cohort as a stratum. The association between the development of toxicity with OS was analyzed using toxicity and progressive disease (PD) as time-dependent covariates.

All *P* values were 2-sided, and *P* values less than .05 were considered statistically significant. The analysis was performed using R 3.4 (The R Foundation for Statistical Computing).

Results

Patient Characteristics and Outcomes

WES sequencing and sequence prediction algorithms determined the HLA-1 haplotypes for 102 of 106 participants in the INSPIRE clinical trial. One patient withdrew consent after 1 cycle of pembrolizumab before any clinical outcomes were recorded and was not included in the analysis. In total, 101 patients were analyzed in this study.

The frequency of toxicity and clinical benefit from pembrolizumab were 22.8% and 25.7%, respectively. Clinical-pathologic data and their association with clinical benefit and toxicity are summarized in Table 1. The majority of patients were female (n = 63, 62.4%), the median age was 59 years (range = 21-81 years), and most patients were Caucasian (n = 84, 83.2%). Triple-negative breast cancer (n = 22, 21.8%) was the most common tumor type. PD-L1 data were missing for 2 patients; median PD-L1 positive cells were 0% (range = 0%-100%; 50 patients had 0%). Unadjusted univariate analysis suggested both clinical benefit and toxicity were different between the cohorts ($P < .001$ and $P = .006$, respectively). Patients who attained clinical benefit tended to have a larger percentage of PD-L1 MPS-positive cells in comparison with those without clinical benefit (medians = 69.1% vs 42.4%, $P = .02$).

Figure 1 is a Consolidated Standards of Reporting Trials (CONSORT) flow diagram of patient assignment, HLA processing, and clinical outcomes of each cohort. The last clinical outcome update was May 3, 2019. At that time, patients were followed up for a median of 27 months from date of first pembrolizumab infusion (range = 9-35 months), and 68 (67.3%) patients died. Two patients lost to follow-up had clinical benefit and toxicity outcomes obtained and were included in this analysis. The median number of pembrolizumab infusions was 3 (range = 1-35 infusions). Three patients (3.0%) were RECIST non-evaluable because of early clinical progression and were tallied as a PD event. Clinical benefit was achieved in 26 patients (25.7%). RECIST progression was the major reason for stopping treatment (n = 57, 56.4%). Toxicity occurred in 23 patients (22.8%), and 11 patients (10.9%) developed more than 1 toxicity. Median time from first dose of pembrolizumab to toxicity onset was 85 days (range = 3-482 days). Grade 3 colitis (n = 2, 2.0%) and grade 3 pneumonitis (n = 2, 2.0%) were the most common toxicity events requiring treatment discontinuation. In total, 1 patient (1.0%) developed grade 4 lipase elevation, 9 patients (8.9%) developed grade 3 toxicity, and 3 patients (2.9%) developed more than 1 grade 3 toxicity. There were no grade 5 events. Progression occurred in 71 of 78 (91.0%) patients without toxicity and in 15 of 23 (65.2%) patients with toxicity.

HLA-1 Results

Heterozygosity of HLA-A, -B, or -C was present in 89 (88.1%), 92 (91.1%), and 80 (79.2%) patients, respectively. Heterozygosity of all 3 HLA loci (HLA-A, -B, and -C) was present in 66 patients (65.3%). In total, 5 patients (5.0%) had homozygosity in 2 loci, and 1 patient (1.0%) had homozygosity in all 3 loci. The following alleles were heterozygous and present in more than 10 patients: A*01, A*02, A*03, A*11, A*24, B*07, B*08, B*35, B*40, B*44, B*51, C*03, C*04, C*05, C*06, C*07, and C*12. The most frequent alleles were A*02 (n = 49, 48.5%), C*07 (n = 36, 35.6%), B*44 (n = 29, 28.7%), A*04 (n = 25, 24.8%), C*04 (n = 23, 22.8%), and B*35 (n = 21, 20.8%).

Our HLA prediction method was compared with HLA typing by Sanger Sequencing of 15 patients as an independent validation: the WES prediction and Sanger Sequencing results showed high concordance (13 of 15, 86.7%); the Sanger Sequencing results were used in this study when available.

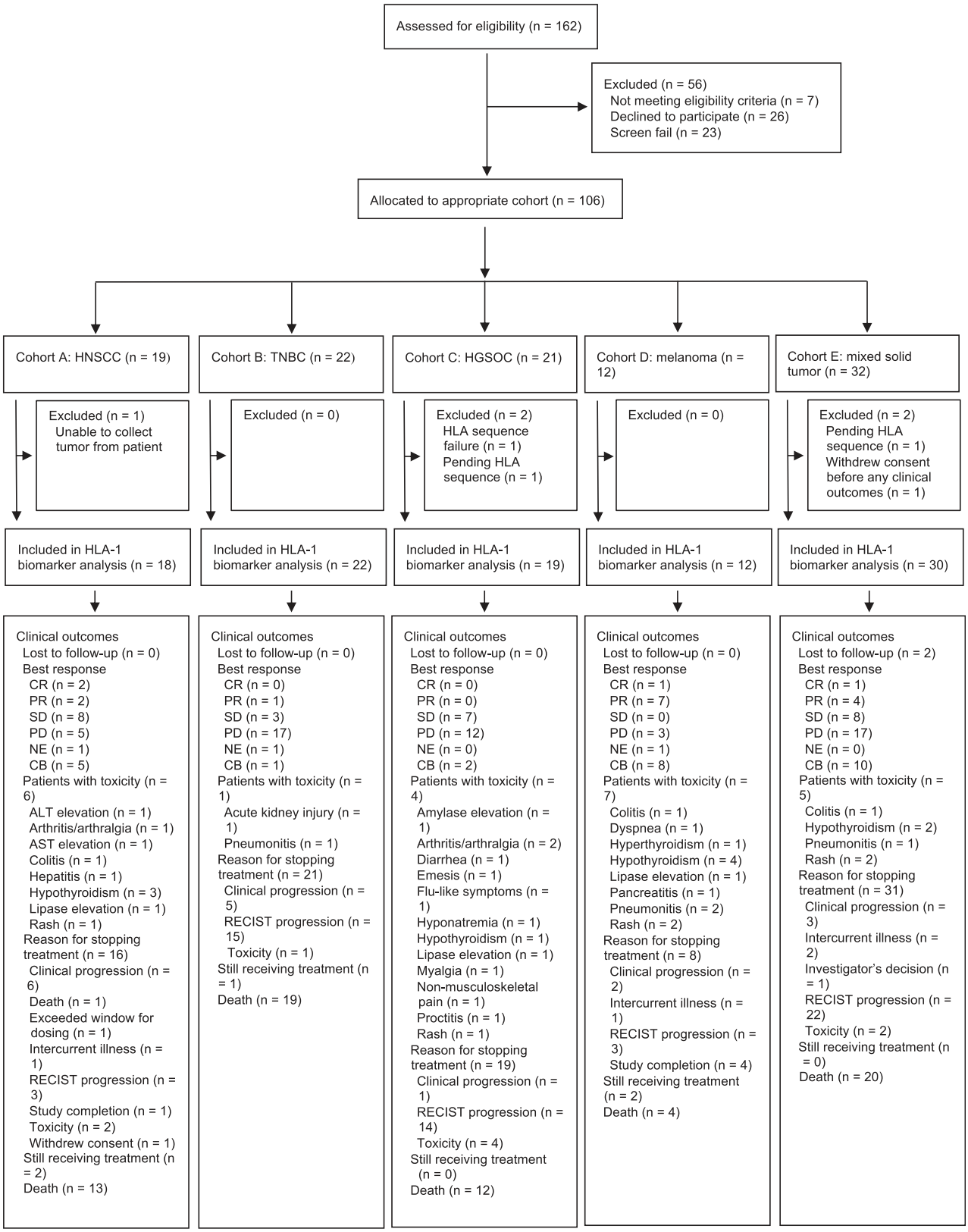

Figure 1. Consolidated Standards of Reporting Trials (CONSORT) diagram of patient allocation and outcomes. CONSORT diagram displaying all relevant details regarding the 101 patients used in this human leukocyte antigen class 1 (HLA-1) analysis from the Investigator-Initiated Phase II Study of Pembrolizumab Immunological Response Evaluation (INSPIRE) study. Note that patients can develop more than 1 type of toxicity event. CB = clinical benefit; CR = complete response; HGSOC = high-grade serous ovarian carcinoma; HNSCC = head and neck squamous cell carcinoma; NE = nonevaluable; PD = progressive disease; PR = partial response; SD = stable disease; TNBC = triple-negative breast cancer.

Table 2. Association of germline HLA-1 with clinical benefit and toxicity

Event	HLA class I loci	Zygosity	Unadjusted (n = 101) Without event, No. (%)	With event, No. (%)	P[a]	Adjusted for cohort (n = 101) OR (95% CI)	P[b]	Multivariable (n = 99) OR (95% CI)	P[c]
Clinical benefit	HLA-A	Heterozygous	66 (74.2)	23 (25.8)	>.99	1.12 (0.24 to 5.22)	.89	0.67 (0.12 to 3.62)	.64
		Homozygous	9 (75.0)	3 (25.0)					
	HLA-B	Heterozygous	70 (76.1)	22 (23.9)	.23	0.36 (0.08 to 1.67)	.19	0.21 (0.03 to 1.45)	.11
		Homozygous	5 (55.6)	4 (44.4)					
	HLA-C	Heterozygous	63 (78.8)	17 (21.2)	.05	0.28 (0.09 to 0.91)	.04	0.28 (0.07 to 1.17)	.08
		Homozygous	12 (57.1)	9 (42.9)					
	HLA-A, -B, and -C	All loci heterozygous	53 (80.3)	13 (19.7)	.09	0.38 (0.14 to 1.05)	.06	0.42 (0.13 to 1.33)	.14
		At least 1 loci homozygous	22 (62.9)	13 (37.1)					
Toxicity	HLA-A	Heterozygous	69 (77.5)	20 (22.5)	>.99	1.03 (0.23 to 4.62)	.97	0.38 (0.07 to 2.04)	.26
		Homozygous	9 (75.0)	3 (25.0)					
	HLA-B	Heterozygous	71 (77.2)	21 (22.8)	>.99	1.32 (0.24 to 7.26)	.75	1.37 (0.15 to 12.70)	.78
		Homozygous	7 (77.8)	2 (22.2)					
	HLA-C	Heterozygous	62 (77.5)	18 (22.5)	>.99	0.81 (0.24 to 2.78)	.74	0.93 (0.20 to 4.28)	.93
		Homozygous	16 (76.2)	5 (23.8)					
	HLA-A, -B, and -C	All loci heterozygous	53 (80.3)	13 (19.7)	.33	0.58 (0.21 to 1.62)	.30	0.53 (0.16 to 1.77)	.30
		At least 1 loci homozygous	25 (71.4)	10 (28.6)					

[a]Calculated using Fisher exact test. CI = confidence interval; OR = odds ratio; PD-L1 = programmed cell death-1 or its ligand.
[b]Based on conditional logistic regression with the cohort as strata.
[c]Determined using propensity scores (age, sex, ethnicity, and PD-L1 status) with cohort as strata. Two patients were removed because of unknown PD-L1 status.

HLA-1 Genotype as a Predictive Biomarker

Table 2 summarizes HLA-A, -B, and -C zygosity and their association with clinical benefit and toxicity. Patients with HLA-C heterozygosity had an association with decreased clinical benefit relative to HLA-C homozygosity when controlling for cohort (odds ratio = 0.28, 95% CI = 0.09 to 0.91, $P = .04$), but this association was not statistically significant in multivariable analysis ($P = .08$). HLA-C zygosity was not associated with the development of toxicity, nor was HLA-A or -B zygosity associated with clinical benefit or toxicity in any of the statistical models. Similarly, patients with heterozygosity of all 3 loci (HLA-A, -B, and -C) did not have any association with clinical benefit or toxicity compared with homozygosity of at least 1 locus.

Individual HLA alleles that were heterozygous and present in more than 10 patients were also explored. There was no association of any HLA allele with either clinical benefit or toxicity in any of the statistical models (Supplementary Tables 1 and 2, available online). HLA haplotype dimorphism at positions −21 M and −21 T of the HLA-A and -B leader sequence was also explored as a predictor of clinical benefit and toxicity; as shown in Supplementary Table 5 (available online), these haplotypes were not associated with either endpoint.

HLA-1 Genotype as a Prognostic Biomarker

When adjusting for cohort, there was no difference in OS or PFS for heterozygosity of HLA-A, -B, and -C compared with patients with homozygosity of at least 1 locus (Figure 2). Similarly, there was no association in OS and PFS when comparing patients with heterozygosity of HLA-A (Supplementary Figure 1, available online), HLA-B (Supplementary Figure 2, available online), or HLA-C (Supplementary Figure 3, available online) relative to homozygosity of the corresponding loci.

Association Between Toxicity and Survival

After adjusting for cohort and analyzing toxicity as a time-dependent covariate, patients who developed toxicity from pembrolizumab had longer OS (HR = 0.41, 95% CI = 0.19 to 0.99, $P = .02$) compared with patients who did not develop toxicity. However, we subsequently analyzed toxicity and PD as time-dependent covariates due to the increased likelihood of developing toxicity with longer pembrolizumab exposure and the fact that early progressors are less likely to have experienced toxicity and more likely to have died. When adjusting for cohort and analyzing toxicity and PD as time-dependent covariates, there was no difference in OS with the development of toxicity compared with patients who did not develop toxicity (HR = 0.58, 95% CI = 0.27 to 1.23, $P = .15$).

Association of HLA-1 Genotype With Standard Prognostic Variables

HLA-1 zygosity was explored for possible associations with confounding covariates. HLA-A, -B, and -C zygosity was not associated with age (Supplementary Table 6, available online), sex (Supplementary Table 7, available online), ethnicity (Supplementary Table 8, available online), or PD-L1 MPS when analyzed as a categorical (Supplementary Table 9, available online) or continuous (Supplementary Table 10, available online) variable. Although HLA-B haplotype dimorphism at positions −21 M and −21 T of the HLA-B leader sequence was associated with ethnicity ($P = .03$; Supplementary Table 11, available online), there was no association of HLA-A and -B haplotype dimorphism with any of the remaining covariates (Supplementary Tables 12-15, available online).

Discussion

This is the first study, to our knowledge, to analyze germline HLA-1 status as a predictor of clinical benefit and toxicity in a

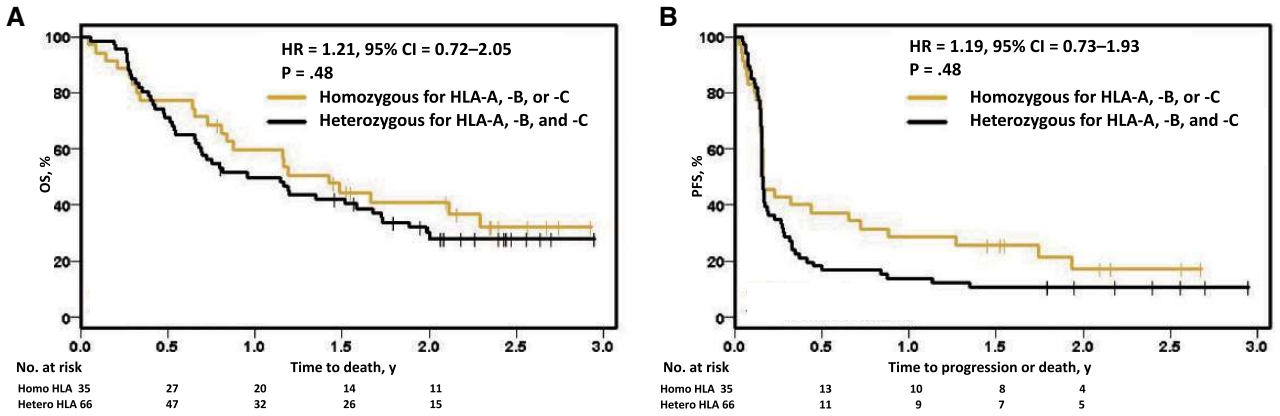

Figure 2. Survival and progression outcomes. Kaplan-Meier curves of (**A**) overall survival (OS) and (**B**) progression-free survival (PFS) after dichotomizing patients with germline heterozygosity of human leukocyte antigen (HLA)-A, -B, and -C compared with homozygosity of at least 1 HLA loci. *P* values are adjusted for cohort and calculated using the Wald test. CI = confidence interval; HR = hazard ratio.

prospective clinical trial of patients with mixed solid tumors treated with anti-PD-1 monotherapy. We found that HLA-C heterozygosity was associated with decreased clinical benefit relative to homozygosity of this locus after adjustment for cohort. Each HLA-1 molecule can bind a specific repertoire of intracellular antigens for cell surface presentation to cytotoxic $CD8^+$ T cells (11). In theory, HLA-1 heterozygosity should increase neoantigen presentation to cytolytic lymphocytes and hence increase tumor immunogenicity and confer better antineoplastic response. Our results contradict recently validated data showing germline heterozygosity of all 3 loci (HLA-A, -B, and -C) was associated with improved OS in patients treated with anti-CTLA4, anti-PD-1/L1, or a combination of both when compared with homozygosity of at least 1 HLA-1 locus (30). We suspect the mixed histologies in our trial and the small number of responses in each cohort may obscure our ability to detect any statistically significant association between clinical benefit and germline HLA-1 status. Further, our inability to correct for multiple testing raises the possibility that the HLA-C heterozygosity association with decreased clinical benefit could be a false positive.

Somatic changes within the tumor HLA-1 machinery can act as a mechanism of immune evasion. Loss of HLA-1 heterozygosity in non-small cell lung cancer (NSCLC) tumor samples showed nearly 92% of predicted neoantigens would bind to the lost haplotype, leading to reduced tumor neoantigen presentation to cytotoxic lymphocytes and decreased response to ICB (19). Although somatic HLA-1 mutations were associated with larger tumor $CD8^+$ cytotoxic T-cell infiltration (31), loss of HLA-1 heterozygosity was accompanied by a statistically significant increase in PD-L1 staining on tumor infiltrative immune cells (19). Future investigations studying alterations in other HLA-1 mechanisms, such as beta-2-microglobulin mutation (32,33) and expression of the HLA-1 transcriptional coactivator NLRC5 (34), will likely identify other pathways of ICB response augmentation.

Somatic tumor HLA-1 mutations are also associated with increased tumor mutation burden. Analysis from The Cancer Genome Atlas data demonstrated higher overall mutation burden in microsatellite-stable tumors that harbored somatic tumor HLA-1 mutations when compared with non-HLA-1 mutated tumors (19,32). However, it remains unclear if somatic HLA-1 alterations create different magnitudes of tumor mutation burden in different tumor types (31,33). Given the association between tumor mutational burden and response to PD-1 inhibition (35-38), studies controlling for tumor mutational burden enriched in a specific tumor type will allow better understanding of how either HLA-1 germline zygosity and/or somatic mutation status can alter response to ICB.

HLA class-II (HLA-2) proteins are typically found on professional antigen-presenting cells and allow presentation of antigens found in the extracellular matrix to $CD4^+$ T-helper cells. Several cancers also express HLA-2 antigens (39), and its expression was associated with increased response to both monotherapy (40,41) and combination ICB (42) secondary to increased expression of CXCR3-binding $CD4^+$ T-helper cell-recruiting cytokines (43). Tumors antagonize this HLA-2 antineoplastic effect through acquisition of Lymphocyte-Activation Gene 3 (LAG-3) and/or Fc Receptor Like 6 upregulation (43). As a result, several early-phase clinical trials are now investigating agents antagonizing LAG-3 (NCT03489369, NCT02061761, NCT01968109), although anti-Fc Receptor Like 6 agents are not yet undergoing human testing.

NK cells assist in removal of neoplastic cells (44,45) and may succumb to immune exhaustion through expression of PD-1 (46,47). Although their efficacy may be influenced by exposure to anti-PD-1/L1 agents, the exact therapeutic effect of PD-1 inhibition on NK cells remains unclear (17). However, we were unable to demonstrate any association of germline HLA haplotype dimorphism at positions −21 M and −21 T of the HLA-A and -B leader sequence with clinical outcomes after pembrolizumab treatment. This may be secondary to alternative mechanisms of HLA immune augmentation on NK cells; although somatic loss of HLA-1 heterozygosity in NSCLC tumors was associated with a statistically significant increase in tumor-infiltrative NK cells (19), the HLA-2–antagonizing signals LAG-3 and FCLR6 decreased NK cytolytic activity (43). Because loss of HLA-1 heterozygosity in NSCLC tumors was associated with a statistically significant increase in PD-L1 staining on tumor infiltrative immune cells (19), any therapeutic benefit gained by increased tumor-infiltrative NK cells from HLA-1 loss of heterozygosity may be overshadowed by the potential loss of T-cell cytolytic activity. The inhibitory NKG2A antibody monalizumab has recently been combined with anti-PD-L1 therapy in a phase I trial (48), and preliminary data have indicated promising activity against microsatellite stable colorectal cancer. Future studies

examining the degree of intra- and peri-tumoral PD-1 positive NK cells and controlling for HLA-1 and -2 status will allow appraisal of both the quantity of NK cells and their state of immune exhaustion in an effort to determine the role haplotype leader sequences may play in response and/or toxicity to anti-PD-1.

We were unable to show any association between HLA-1 loci or alleles with toxicity. A prospective analysis of NSCLC and melanoma patients treated with ICB demonstrated a link between HLA alleles associated with autoimmune diseases and the risk of developing organ-specific irAEs; after controlling for sex and age, there was a positive association of HLA-DQB1*03:01 with colitis [an HLA class-II protein with a strong relationship to inflammatory bowel disease (49)] and HLA-DRB1*11:01 with pruritus (50). Due to the organ-specific nature of both irAEs and autoimmune diseases, future investigations that enrich for a specific irAE and/or analyze HLA class-II status may better elucidate the role of HLA as a biomarker for irAEs.

Several studies reported increased survival and/or response to ICB for patients who develop irAEs; however, few studies (51,52) have actually proven this association with appropriate statistical methods to prevent bias from the time dependence of both predictor and outcome variables (53,54). Although we demonstrated an association between toxicity and OS when adjusting for cohort and analyzing toxicity as a time-dependent covariate, this association was lost when also including PD as a time-dependent covariate in the model. To better ascertain the association between toxicity and OS, future meta-analyses using time dependency should be considered.

Our study has several limitations. HLA analysis was limited to 2-digit typing, and thus higher resolution typing may identify a larger proportion of heterozygous patients. Our small sample size precluded the ability to assess for various allelic combinations as a possible predictive biomarker. HLA was only 1 of several potential biomarkers tested from the INSPIRE data. Hence, we were unable to effectively correct for multiple testing of the various HLA loci and alleles, producing an increased risk of false-positive results. The mixed tumor histologies in small numbers, in addition to not controlling for somatic HLA-1 mutations, HLA-2 expression, tumor-infiltrating lymphocytes, and tumor mutational burden, created a challenge in interpreting the clinical benefit results. Our irAEs are heterogeneous, and the small sample size precluded determination of whether HLA genotypes are associated with organ-specific irAEs. Despite our long follow-up, the toxicity data may be incomplete due to the possibility of developing new irAEs from PD-1 inhibition many months after treatment initiation (51).

In conclusion, we identified an association between germline HLA-C heterozygosity and decreased response to pembrolizumab when adjusting for cohort. Clinical trials performing serial tumor biopsies may allow better understanding of the role HLA-1 zygosity plays in the mechanism of tumor-immune evasion. Although further prospective validation in a larger and more homogenous cohort is required, our findings highlight the role of host genetic variability as a predictive biomarker for clinical outcome during ICB therapy.

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Data Availability

Anonymized germline whole exome sequence data can be found at European Genome-Phenome Archive https://ega-archive.org/datasets/EGAD00001006562.

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