ORIGINAL ARTICLE

Impact of germline HLA genotypes on clinical outcomes in patients with urothelial cancer treated with pembrolizumab

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Abstract
Human leukocyte antigen class I (HLA-I) genotypes are suggested to influence the cancer response to checkpoint blockade immunotherapy. This study assessed the impact of germline HLA genotypes on clinical outcomes in patients with chemoresistant advanced urothelial cancer (UC) treated with pembrolizumab. Zygosity, supertypes, evolutionary divergence, and specific alleles of germline HLA-I and -II were evaluated using the Luminex technique in 108 patients with chemoresistant metastatic or locally advanced UC treated with pembrolizumab. Among the 108 patients, 69 died and 83 showed radiographic progression during follow-up. Homozygous for at least one HLA-I locus, absence of the HLA-A03 supertype, and high HLA-I evolutionary divergence were associated with a radiographic response, but were not associated with survival outcomes. Patients with the HLA-DQB1*03:01 allele had significantly lower disease control rates than patients without the allele (17.4% vs. 53.8%, \( p = 0.002 \)); its presence was also an independent risk factor for progressive disease (hazard ratio 4.35, 95% confidence interval 1.03–18.46). Furthermore, patients with the HLA-DQB1*03:01 allele had significantly worse progression-free survival than patients without the allele (median progression-free survival 3.1 vs. 4.8 months, \( p = 0.035 \)). There was no significant relationship between any HLA status and the incidence of severe adverse events. Several germline HLA genotypes, especially HLA-DQB1*03:01, may be associated with radiographic progression. However, their impact on treatment response is limited, and germline HLA genotypes was not independently associated with survival outcomes. Further prospective studies are needed to confirm the

Abbreviations: AE, adverse event; BOR, best overall response; CI, confidence interval; CR, complete response; DCR, disease control rate; ECOG, Eastern Cooperative Oncology Group; GC, gemcitabine plus cisplatin; HED, HLA evolutionary divergence; HLA-I, human leukocyte antigen class I; HR, hazard ratio; ICI, immune checkpoint inhibitor; LDH, lactate dehydrogenase; MVAC, methotrexate, vinblastine, doxorubicin, and cisplatin; NLR, neutrophil-to-lymphocyte ratio; ORR, objective response rate; OS, overall survival; PD, progressive disease; PD-1, programmed death 1; PFS, progression-free survival; PR, partial response; PS, performance status; RECIST, Response Evaluation Criteria in Solid Tumors; SD, stable disease; UC, urothelial cancer.

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relationship between germline HLA genotypes and clinical outcomes in patients with chemoresistant advanced UC treated with pembrolizumab.

KEYWORDS
checkpoint blockade immunotherapy, HLA evolutionary divergence, human leukocyte antigen, pembrolizumab, urothelial cancer

1 | INTRODUCTION

Urothelial cancers (UCs) include a variety of tumors from the urinary tract, bladder, renal pelvis, ureter, and urethra. Combination chemotherapy such as gemcitabine plus cisplatin (GC) or methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) is the standard option for first-line systematic therapy in patients with locally advanced and metastatic UC. Several new immunotherapeutic agents that act by blocking immune checkpoints have dramatically changed the treatment strategy of advanced UC in recent years. Pembrolizumab, a humanized monoclonal antibody targeting antiprogrammed death 1 (PD-1), can be used to treat chemoresistant, surgically unresectable UC and improve overall survival (OS) in these patients. Therefore, pembrolizumab is used worldwide to treat a large number of patients with advanced UC. However, a recently published study with >2 years of follow-up found that pembrolizumab had 12- and 24-month OS rates of 44.2% and 26.9%, respectively, and the objective response rate (ORR) was only 21.2%. In addition, it was reported that 62% of patients experienced treatment-related adverse events (AEs), whereas 12% experienced serious AEs.

Considering the high heterogeneity in the treatment responses mentioned above, there is an urgent need for biomarkers to predict better oncological outcomes and AEs of pembrolizumab treatment in advanced UC. Several factors, including tumor mutation burden, PD-L1 expression, copy number variation, and gut microbial diversity, were known to be potential biomarkers for the response to immune checkpoint inhibitors (ICIs) in patients with cancer. However, there are no promising biomarkers to predict treatment response, prognosis, and AEs in patients with advanced UC receiving pembrolizumab.

Germline genetic factors can potentially be good biomarkers since they influence immune traits in many diseases. Human leukocyte antigens (HLAs) are expressed in a variety of cells, including cancer cells and immune cells, whereas HLA molecules play critical roles in triggering cytotoxic T lymphocytes (CTL)-mediated tumor cell killing, T cell priming, and clinal expansion. HLA-I molecules are also highly polymorphic, with variations located in the peptide-binding region, and this polymorphism plays a critical role in the interface between malignant cells and the host immune system. Moreover, HLA class-II proteins, which were found in the extracellular matrix of cluster of differentiation 4+ (CD4+) T-helper cells, are known to be associated with response to monotherapy and combination immune checkpoint therapies. Recent studies have shown that germline HLA gene zygosity, supertype, evolutional divergence, and individual HLA genotypes are associated with the prognosis of checkpoint blockade immunotherapy. However, the aforementioned studies included patients who received a variety of immune checkpoint drugs and did not focus on those with UC.

In the present study, we assessed the impact of multiple germline HLA-I and -II genotypes on the clinical outcomes of patients with chemoresistant advanced UC treated with pembrolizumab in a Japanese multicenter cohort.

2 | MATERIALS AND METHODS

2.1 | Patients and treatment

This multicenter retrospective study included Japanese patients whose blood samples were available with an opt-out approach to secondary use of residual blood specimens in each institution. This study was approved by each Institutional Review Board. The clinicopathological data of study participants, defined as patients with advanced UC who received pembrolizumab from 2015 to 2020 after being resistant to treatment with chemotherapy, were collected from the medical records of each institution. No patients received prior immunotherapy.

2.2 | Data collection

The following data were collected using a standardized case report form: age, gender, body mass index, serum hemoglobin/lactate dehydrogenase (LDH)/C-reactive protein levels, neutrophil-to-lymphocyte ratio (NLR), Eastern Cooperative Oncology Group (ECOG) performance status (PS), primary tumor site, tumor pathology, treatment indication, history of surgery and/or radiotherapy of primary tumors, number of prior treatment regimens, sites of metastasis, and number of target sites. The NLR was calculated by dividing the number of neutrophils by the number of lymphocytes in the patient’s complete blood count. The best overall response (BOR) was defined as the best response noted in radiographic findings from the start of pembrolizumab until disease progression or until discontinuation of treatment for any reason. The BORs are divided into complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD), as defined by the Response Evaluation Criteria in Solid Tumors (RECIST) criteria. There was no central review to define the RECIST responses, and imaging responses were
determined according to the RECIST criteria. The ORR was defined as the percentage of patients with CR or PR, whereas the disease control rate (DCR) was defined as the percentage of patients with CR, PR, or SD among all patients.

2.3 | Serum sample collection and HLA typing

Genomic DNA was extracted from whole-blood samples and stored at −80°C prior to analysis. Genotyping of 108 samples in HLA-I (A, B, C) and HLA-II (DRB1, DQB1, DPB1) alleles was conducted via polymerase chain reaction with a sequence-specific oligonucleotide probe using the WAKFlow HLA typing kit (Wakunaga Pharmaceutical Co.) and the LABType SSO (One Lambda, Inc.) as described previously.16 HLA homozygosity was defined as a patient with two alleles with the same serological classification. Supertypes in the HLA-A and HLA-B loci were classified according to the method previously described.20 HLA evolutionary divergence (HED) was calculated as previously described.18,19 Briefly, the divergence between allele sequences was calculated using the Grantham distance, which is a quantitative one-to-one distance that takes into account the physicochemical properties of amino acids, hence the functional similarity between sequences.20 For a particular HLA-I, HLA-DRB1 or HLA-DQB1 with two-allele loci, the peptide-binding domain sequences of each allele are aligned,21 and the Grantham distance is calculated as the sum of the amino acid differences (i.e., biochemical composition, polarity, and volume of each amino acid) along the peptide-binding domain sequence.20 The final Grantham distance is calculated by normalizing the value in this equation by the length of the alignment between the peptide-binding domains of the two alleles of a particular HLA-I or HLA-DRB1 genotype. All HED analyses were performed using R-4.1.3 for Windows. All germline HLA data in this study are available in Table S1.

2.4 | Statistical analyses

To assess the impact of each allele of HLA-I and -II on clinical outcomes, alleles observed in more than 10% of the patients were used for further analyses. We statistically compared patient characteristics and BORs among the groups using Student’s t-test, chi-squared test, or Fisher’s exact test as appropriate. Regarding the association between each HLA allele and its clinical outcomes, we also analyzed the data using the Benjamini–Hochberg method, a conventional method of correcting for multiple comparison. Multivariate logistic regression analyses were performed to yield adjusted odds ratios. Survival analysis was performed using the Kaplan–Meier method and the log-rank test. Univariate and multivariate analyses were performed using the Cox hazard proportional model. Hazard ratios (HRs) were estimated with 95% confidence intervals (CIs). p values were all two-sided and p < 0.05 was considered significant. Statistical analyses were performed using EZR version 1.53 and SPSS version 19.0.

3 | RESULTS

3.1 | Patient characteristics, germline HLA genotypes, and clinical outcomes

A total of 108 patients were included in the analysis. Patient characteristics are shown in Table 1. The median age was 71 (63.8–78.3) years and 75.9% were male. The primary sites of cancer were the upper urinary tract in 41 (38.0%) patients, the lower urinary tract (i.e., bladder and urethra) in 66 (61.1%) patients, and both in one (0.9%) patient. As for metastatic sites, 63 (58.3%) had lymph node metastasis, 33 (30.6%) had lung metastasis, 13 (12.0%) had liver metastasis, 14 (13.0%) had bone metastasis, and 24 (22.2%) had metastasis in other organs.

The distributions of germline HLA homozygosity, supertype, evolutionary divergence, and each allele of HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 are described in Tables S2 and S3. Figures S1 and S2. The HED values of one patient with HLA-A, -B, -C, and -DRB1 and four patients with HLA-DQB1 were not evaluated due to a calculation error. The HED of HLA-DRB1 was not assessed due to the lack of program availability. There were 43 (39.8%) patients homozygous for at least one HLA-I locus, whereas 42 (38.9%) were homozygous for at least one HLA-II locus. The mean HED scores of HLA-A, -B, -C, -DRB1, and -DQB1 were 8.1 (1.0–10.7), 7.4 (6.1–9.4), 5.2 (3.5–6.0), 10.2 (7.5–14.5), and 10.3 (5.2–13.9), respectively (Figure S2). The mean HED scores of HLA-A+B+C and HLA-A+B+C+DRB1 were 6.4 (4.6–7.7) and 7.3 (6.0–8.5), respectively. The five most frequent alleles (in order) among HLA-A, -B, -C -DRB1, -DQB1, and -DPB1 were DPB1*05:01 (66.7%), A*24:02 (57.4%), DPB1*02:01 (37.0%), C*07:02 (31.5%), and C*03:03 (27.8%) (Table S3 and Figure S1).

One patient who died of rapid cancer progression after administration of pembrolizumab was excluded from the analyses for radiographic response. The profile of AEs in all patients is described in Table S4. Excluding the patient mentioned above, the BORs were CR in 6 (5.6%) patients, PR in 23 (21.5%), SD in 21 (19.6%), and PD in 57 (53.2%). Among 108 patients, 69 died and 83 showed radiographic progression during the follow-up period. The median PFS and OS were 4.5 and 11.2 months, respectively (Figure S3).

3.2 | Germline HLA genotypes, treatment response, and prognosis

Since a previous study reported that the germline homozygosity of HLA-I was associated with survival in patients with cancer,10 we first examined the impact of germline HLA-I and -II homozygosity on clinical outcomes (Table 2). The DCR was significantly lower in patients with at least one homozygosity of HLA-A, -B, or -C than the others (34.9% vs. 54.7%, p = 0.044; Table 2 and Figure S4A), but there was no significant difference in the rate of ORR (18.6% vs. 32.8%, p = 0.105; Table 2 and Figure S4A). Furthermore, there were no significant differences in PFS and OS either with or without homozygosity of HLA class I loci (Figures 1A and 2A).
In the melanoma cohort, several HLA supertypes, such as B44 and B62, have been significantly associated with patient survival. Therefore, we assessed the impact of eight supertypes of HLA-A or -B on BOR, PFS, and OS (Table 2). The presence of HLA-A3 supertype was significantly associated with higher DCR than the absence of HLA-A3 (62.8% vs. 35.9%, \( p = 0.006 \); Table 2 and Figure S4B). However, there were no significant differences in PFS and OS between the presence and absence of HLA-A3 (Table 2, Figures 1B and 2B). Moreover, B44 and B62 supertypes were not statistically associated with ORR and DCR (Table 2).

Sequence divergence between the peptide-binding domains of patient HLA alleles is potentially associated with response to ICIs. A recent study showed that HLA-I evolutional divergence has a strong impact on the response to ICIs and high mean HLA-I evolutional divergence was significantly associated with improved OS in patients. Therefore, we also investigated the impact of the evolutionary divergence of HLA-A, -B, -C, -DRB1, and -DQB1 on BOR, PFS, and OS. After subcategorizing the patients into two groups based on the median of Grantham scores of HLA-I and -II, we found that patients with high mean HLA A+B+C evolutionary divergence had

| Number of patients | % |
|--------------------|---|
| N                  | 108 100.0|
| Age (median, IQR)  | 71.0 (63.8–78.3)|
| Gender (male:female) | Male 82 75.9 Female 26 24.1|
| BMI (median, IQR)  | 21.7 (19.5–23.6)|
| Hemoglobin         | 10.6 (8.9–12.0)|
| PLT                | 27.2 (19.0–57.0)|
| NLR                | 3.14 (217.02–5.)|
| LDH                | 186.5 (162.0–222.5)|
| CRP                | 0.84 (0.20–3.53)|
| ECOG-PS            | 0 59 54.6 1 34 31.5 2 10 9.3 3 5 4.6|
| Primary tumor site | Upper tract 41 38.0 Lower tract 66 61.1 Both 1 0.9|
| Pathology          | UC 98 90.7 UC with variant 9 8.3 Other 1 0.9|
| Indication         | Metastatic 96 88.9 Locally advanced 12 11.1|
| Surgical treatment | 63 58.3|
| Radiation therapy  | 51 28.7|
| Number of prior regimens | 0 34 31.5 1 57 52.8 \( \geq 2 \) 17 15.7|
| Metastatic site    | Liver 13 12.0 Lung 33 30.6 Bone 14 13.0 Lymph node 63 58.3 Other 24 22.2|
| Number of target sites | 1 16 14.8 2–5 37 34.3 Multiple 54 50.0|

Table 1 Baseline characteristics in patients with advanced urothelial carcinoma who received pembrolizumab.

Abbreviations: CRP, C-reactive protein; ECOG-PS, Eastern Cooperative Oncology Group performance status; IQR, interquartile range; LDH, lactate dehydrogenase; NLR, neutrophil-to-lymphocyte ratio; PLT, platelet count; UC, urothelial cancer.
### Table 2: Association of HLA homozygosity and supertype with response of IO drugs in all patients

|                     | CR   | PR  | SD  | PD  | p value | CR + PR | SD + PD | p value | CR + PR + SD | PD   | p value |
|---------------------|------|-----|-----|-----|---------|---------|---------|---------|-------------|------|---------|
| All (n, %)          | 6 (5.6) | 23 (21.5) | 21 (19.6) | 57 (53.3) |         | 29 (27.1) | 77 (72.9) |         | 50 (46.7) | 57 (53.3) |         |
| Homozygosity HLA A  | 1 (3.1) | 7 (21.9) | 5 (15.6) | 19 (59.4) | 0.754 | 8 (25.0) | 24 (75.0) | 0.749 | 13 (40.6) | 19 (59.4) | 0.408 |
| Heterozygosity      | 5 (6.7) | 16 (21.3) | 16 (21.3) | 38 (50.7) |         | 21 (28.0) | 54 (72.0) |         | 37 (49.3) | 38 (50.7) |         |
| HLA B               | 0 (0.0) | 1 (20.0) | 2 (40.0) | 2 (40.0) | 0.663 | 1 (20.0) | 4 (80.0) | 1.000* | 3 (60.0) | 2 (40.0) | 0.663* |
| Homozygosity        | 6 (58.8) | 22 (21.6) | 19 (18.6) | 55 (53.9) |         | 28 (27.5) | 74 (72.5) |         | 47 (46.1) | 55 (53.9) |         |
| Heterozygosity      | 5 (6.7) | 16 (21.3) | 16 (21.3) | 38 (50.7) |         | 21 (28.0) | 54 (72.0) |         | 37 (49.3) | 38 (50.7) |         |
| HLA C               | 0 (0.0) | 2 (16.7) | 2 (16.7) | 8 (66.7) | 0.701 | 2 (16.7) | 10 (83.3) | 0.507* | 4 (33.3) | 8 (66.7) | 0.373* |
| Homozygosity        | 6 (6.3) | 21 (22.1) | 19 (20.0) | 49 (51.6) |         | 27 (28.4) | 68 (71.6) |         | 46 (48.4) | 49 (51.6) |         |
| Heterozygosity      | 5 (6.7) | 16 (21.3) | 16 (21.3) | 38 (50.7) |         | 21 (28.0) | 54 (72.0) |         | 37 (49.3) | 38 (50.7) |         |
| HLA A or B          | 1 (2.9) | 7 (20.0) | 6 (17.1) | 21 (60.0) | 0.715 | 8 (22.9) | 27 (77.1) | 0.491 | 14 (40.0) | 21 (60.0) | 0.331 |
| Homozygosity        | 6 (6.5) | 20 (21.5) | 17 (18.3) | 50 (53.8) |         | 26 (28.0) | 67 (72.0) |         | 43 (46.2) | 50 (53.8) |         |
| Heterozygosity      | 5 (7.8) | 16 (25.0) | 14 (21.9) | 29 (45.3) |         | 21 (32.8) | 43 (67.2) |         | 35 (54.7) | 29 (45.3) |         |
| HLA DRB1            | 0 (0.0) | 3 (21.4) | 4 (28.6) | 7 (50.0) | 0.661 | 3 (21.4) | 11 (78.6) | 0.754 | 7 (50.0) | 7 (50.0) | 1.000 |
| Homozygosity        | 6 (6.5) | 20 (21.5) | 17 (18.3) | 50 (53.8) |         | 26 (28.0) | 67 (72.0) |         | 43 (46.2) | 50 (53.8) |         |
| Heterozygosity      | 5 (6.3) | 17 (21.3) | 12 (15.0) | 46 (57.5) |         | 22 (27.5) | 58 (72.5) |         | 34 (42.5) | 46 (57.5) |         |
| HLA DQB1            | 0 (0.0) | 4 (22.2) | 3 (16.7) | 11 (61.1) | 0.705 | 4 (22.2) | 14 (77.8) | 0.639 | 7 (38.9) | 11 (61.1) | 0.467 |
| Homozygosity        | 5 (6.3) | 17 (21.3) | 12 (15.0) | 46 (57.5) |         | 22 (27.5) | 58 (72.5) |         | 34 (42.5) | 46 (57.5) |         |
| Heterozygosity      | 5 (5.7) | 19 (21.8) | 18 (20.7) | 45 (51.7) |         | 24 (27.6) | 63 (72.4) |         | 42 (48.3) | 45 (51.7) |         |
| HLA DRB1 or DQB1 or DPB1 | 0 (0.0) | 10 (23.8) | 10 (23.8) | 22 (52.4) | 0.198 | 10 (23.8) | 32 (76.2) | 0.523 | 20 (47.6) | 22 (52.4) | 0.737 |
| Homozygosity        | 5 (8.2) | 13 (21.3) | 9 (14.8) | 34 (55.7) |         | 18 (29.5) | 43 (70.5) |         | 27 (44.3) | 34 (55.7) |         |

(Continues)
| Supertype | Presence | CR (0.0) | PR (20.0) | SD (16.0) | PD (64.0) | p value | CR + PR | SD + PD | p value |
|-----------|----------|----------|-----------|-----------|-----------|---------|---------|---------|---------|
| A01 | 0 (0.0) | 5 (20.0) | 4 (16.0) | 16 (64.0) | 0.426 | 5 (20.0) | 20 (80.0) | 0.361 | 9 (36.0) | 16 (64.0) |
| Absence | 6 (7.3) | 18 (30.0) | 17 (20.7) | 41 (50.0) | 24 (29.3) | 58 (70.7) | 41 (50.0) | 41 (50.0) |
| A02 | 2 (4.2) | 11 (22.9) | 7 (14.6) | 28 (58.3) | 0.583 | 13 (27.1) | 35 (72.9) | 0.997 | 20 (41.7) | 28 (58.3) |
| Absence | 4 (6.8) | 12 (20.3) | 14 (23.7) | 29 (49.2) | 16 (27.1) | 43 (72.9) | 30 (50.8) | 29 (49.2) |
| A03 | 5 (11.6) | 9 (20.9) | 13 (30.2) | 16 (37.2) | 0.007 | 14 (32.6) | 29 (67.4) | 0.298 | 27 (62.8) | 16 (37.2) |
| Absence | 1 (1.6) | 14 (21.9) | 8 (12.5) | 41 (64.1) | 15 (23.4) | 49 (76.6) | 23 (35.9) | 41 (64.1) |
| A24 | 2 (3.3) | 14 (23.0) | 11 (18.0) | 34 (55.7) | 0.637 | 16 (26.2) | 45 (73.8) | 0.761 | 27 (44.3) | 34 (55.7) |
| Absence | 4 (8.9) | 9 (20.0) | 9 (20.0) | 23 (51.1) | 13 (28.9) | 32 (71.1) | 22 (48.9) | 23 (51.1) |
| B07 | 3 (5.1) | 16 (27.1) | 13 (22.0) | 27 (45.8) | 0.286 | 19 (32.2) | 40 (67.8) | 0.188 | 32 (54.2) | 27 (45.8) |
| Absence | 3 (6.3) | 7 (14.6) | 8 (16.7) | 30 (62.5) | 10 (20.8) | 38 (79.2) | 18 (37.5) | 30 (62.5) |
| B27 | 1 (4.2) | 6 (25.0) | 2 (8.3) | 16 (62.5) | 0.424 | 7 (29.2) | 17 (70.8) | 0.796 | 9 (37.5) | 15 (62.5) |
| Absence | 5 (6.0) | 17 (20.5) | 19 (22.9) | 42 (50.6) | 22 (26.5) | 61 (73.5) | 41 (49.4) | 42 (50.6) |
| B44 | 3 (6.5) | 7 (15.2) | 10 (21.7) | 26 (56.5) | 0.584 | 10 (21.7) | 36 (78.3) | 0.278 | 20 (43.5) | 26 (56.5) |
| Absence | 3 (4.9) | 16 (26.2) | 11 (18.0) | 31 (50.8) | 19 (31.1) | 42 (68.9) | 30 (49.2) | 31 (50.8) |
| B62 | 4 (8.2) | 9 (18.4) | 8 (16.3) | 28 (57.1) | 0.528 | 13 (26.5) | 36 (73.5) | 0.903 | 21 (42.9) | 28 (57.1) |
| Absence | 2 (3.4) | 14 (24.1) | 13 (22.4) | 29 (50.0) | 16 (27.6) | 42 (72.4) | 29 (50.0) | 29 (50.0) |

**HED**

| Mean HED A + B + C | Low | 2 (3.8) | 8 (15.1) | 11 (20.8) | 32 (60.4) | 0.277 | 10 (18.9) | 43 (81.1) | 0.080 | 21 (39.6) | 32 (60.4) |
| High | 4 (7.5) | 15 (28.3) | 9 (17.0) | 25 (47.2) | 19 (35.8) | 34 (64.2) | 28 (52.8) | 25 (47.2) |

| Mean HED A + B + C + DRB1 | Low | 3 (5.6) | 9 (16.7) | 11 (20.4) | 31 (57.4) | 0.640 | 12 (22.2) | 42 (77.8) | 0.278 | 23 (42.6) | 31 (57.4) |
| High | 3 (5.8) | 14 (26.9) | 9 (17.3) | 26 (50.0) | 17 (32.7) | 35 (67.3) | 26 (50.0) | 26 (50.0) |

| Mean HED A + B + C + DQB1 | Low | 0 (0.0) | 8 (17.4) | 10 (21.7) | 28 (60.9) | 0.108 | 8 (17.4) | 38 (82.6) | 0.045 | 18 (39.1) | 28 (60.9) |
| High | 5 (8.8) | 15 (26.3) | 9 (15.8) | 28 (49.1) | 20 (35.1) | 37 (64.9) | 29 (50.9) | 28 (49.1) |

| Mean HED A + B + C + DRB1 + DQB1 | Low | 1 (2.3) | 10 (23.3) | 9 (20.9) | 23 (53.5) | 0.739 | 11 (25.6) | 32 (74.4) | 0.757 | 20 (46.5) | 23 (53.5) |
| High | 4 (6.7) | 13 (21.7) | 10 (16.7) | 33 (55.0) | 17 (28.3) | 43 (71.7) | 27 (45.0) | 33 (55.0) |

Abbreviations: CR, complete response; HED, HLA evolutionary divergence; PD, progressive disease; PR, partial response; SD, stable disease.

*Fisher’s direct probability test.*
FIGURE 1 Kaplan–Meier curves of progression-free survival according to HLA status. (A) HLA-I homozygosity, (B) HLA-A3 supertype, (C) HLA-I evolutionary divergence, (D) HLA-DQB1*03:01, (E) HLA-DRB1*13:02

FIGURE 2 Kaplan–Meier curves of overall survival according to HLA status. (A) HLA-I homozygosity, (B) HLA-A3 supertype, (C) HLA-I evolutionary divergence, (D) HLA-DQB1*03:01, (E) HLA-DRB1*13:02
a higher rate of ORR than those with low mean HLA A+B+C evolutionary divergence (35.8% vs. 18.9%, p = 0.080; Table 2). However, there was no significant relationship between any HED status and survivals (Figures 1C and 2C). Other HLA evolutionary divergence parameters including mean HLA-A, -B, -C, -A+B+C+DRB1, and -A+B+C+DQB1 evolutionary divergence were not statistically associated with DCR, PFS, and OS.

We then examined whether 43 specific alleles of HLA-I and -II were associated with BOR, PFS, and OS (Table S5). Patients with HLA-DQB1*03:01 had significantly lower DCRs than patients without the allele (91.7% vs. 41.1%, p = 0.002; Table S5 and Figure S4D), and the difference remained significant after multiple testing corrections (p = 0.029). Patients with HLA-DRB1*13:02 or HLA-DPB*04:01 had significantly higher DCRs than patients without these alleles, but this significance diminished after multiple testing corrections (p = 0.043 and p = 0.043, respectively). In the multivariate analysis, the presence of HLA-DQB1*03:01 was an independent risk factor of progressive disease (HR 4.35, 95% CI 1.03–18.46; Figure 1D), although no significant relationship was observed between the presence of HLA-DQB1*03:01 and OS (Figure 2D). No statistical relationship was observed between HLA-DRB1*13:02/HLA-DPB*04:01 and survival outcomes; however, patients with the HLA-DRB1*13:02 allele tended to have better PFS than patients without the allele (median PFS 7.9 vs. 3.7 months, p = 0.083; Figure 1E). Patients with A*33:03, B44:03:01, or C*14:03 had significantly better DCRs than those without these alleles, but this significance diminished after multiple testing corrections (Table S5). The other alleles were not statistically associated with BOR.

With respect to risk factors for survival outcomes, multivariable analysis of risk factors for PFS showed that the presence of bone metastasis and high serum LDH of >186.5 U/L were independent prognostic factors for PFS (HR 2.51, 95% CI 1.21–5.19; HR 1.94, 95% CI 1.17–3.22, respectively), whereas the presence of HLA-DQB1*03:01 was not significant (HR 1.36, 95% CI 0.79–2.35) (Table S6). Furthermore, an ECOG-PS of ≥2, the presence of bone metastasis, and high serum LDH of >186.5 U/L were independent prognostic factors for OS (HR 2.10, 95% CI 1.03–4.27; HR 2.77, 95% CI 1.31–5.86; HR 2.36, 95% CI 1.38–4.02, respectively) (Table S7).

TABLE 3  Univariate and multivariable analysis of risk factors for progressive disease

|                      | Univariate |                   | Multivariable |                   |
|----------------------|------------|-------------------|---------------|-------------------|
|                      | OR         | 95% CI        | p value       | OR               | 95% CI        | p value       |
| Age                  | 1.01       | 0.98–1.05      | 0.520         |                   |               |               |
| Sex                  | 1.03       | 0.43–2.50      | 0.946         |                   |               |               |
| ECOG-PS ≥2 vs. 0 or 1 | 0.24       | 0.06–0.91      | 0.035         | 0.29             | 0.60–19.57    | 0.168         |
| Primary site UUT or not | 1.34       | 0.61–2.93      | 0.464         |                   |               |               |
| Number of lines First or not | 0.90      | 0.40–2.05      | 0.808         |                   |               |               |
| Oligometastases Yes vs. no | 0.29      | 0.13–0.64      | 0.002         | 0.51             | 0.17–1.57     | 0.242         |
| Lung metastasis Yes vs. no | 0.37      | 0.16–0.88      | 0.025         | 0.51             | 0.15–1.71     | 0.274         |
| Liver metastasis Yes vs. no | 0.68 | 0.21–2.23      | 0.526         |                   |               |               |
| LN metastasis Yes vs. no | 3.39      | 1.50–7.70      | 0.003         | 2.31             | 0.78–6.79     | 0.129         |
| Bone metastasis Yes vs. no | 0.08     | 0.01–0.61      | 0.015         | 0.11             | 0.81–105.82   | 0.074         |
| CRP >Equal median vs. <median | 0.72 | 0.34–1.55      | 0.401         |                   |               |               |
| Hemoglobin >Equal median vs. <median | 1.07 | 0.50–2.30      | 0.854         |                   |               |               |
| NLR >Equal median vs. <median | 0.53 | 0.24–1.14      | 0.102         |                   |               |               |
| LDH >Equal median vs. <median | 0.37 | 0.17–0.80      | 0.012         | 0.44             | 0.81–6.46     | 0.116         |
| HLA-DRB1*13:02 Absence vs. presence | 15.80 | 1.96–127.39   | 0.010         | 3.82             | 0.14–107.17   | 0.431         |
| HLA-DQB1*03:01 Presence or absence | 5.52 | 1.72–17.69    | 0.004         | 4.35             | 1.03–18.46    | 0.046         |
| HLA-DPB1*04:01 Absence vs. presence | 17.84 | 2.22–143.09   | 0.007         | 8.41             | 0.66–107.07   | 0.101         |

Abbreviations: CI, confidence interval; CRP, C-reactive protein; ECOG-PS, Eastern Cooperative Oncology Group performance status; LN, lymph node; LDH, lactate dehydrogenase; NLR, neutrophil-to-lymphocyte ratio; OR, odds ratio; PLT, platelet count; UUT, upper urinary tract.
3.3 | Germline HLA genotypes and adverse events

Finally, we investigated the relationship between germline HLA genotypes and the presence of AEs (Tables S8 and S9). The heterozygosities of HLA-A and HLA-A or B were significantly associated with the occurrence of any AE ($p = 0.047$ and $p = 0.011$, respectively), whereas HLA homozygosity, evolutionary divergence, and supertypes were not statistically associated with ≥3 AEs. Although HLA-B*35:01, HLA-DRB1*01:03, HLA-DRB1*08:03, and HLA-DPB1*04:02 were significantly associated with the occurrence of any ≥3 AEs ($p = 0.039, 0.041, 0.021, 0.004$, respectively), the differences were not significant after multiple testing corrections.

4 | DISCUSSION

Chowell et al. previously investigated the impact of germline HLA-I status in two cohorts of patients with cancer (cohort 1 with melanoma and non-small-cell lung cancer and cohort 2 with multiple cancers, including 87 with bladder cancer) on their survival outcomes after immune checkpoint therapies. They found that HLA-I homozygosity in at least one locus was significantly associated with reduced survival in the two cohorts and this remained significant in the multivariate analysis. Moreover, HLA-I homozygosity and low mutation burden in tumors were strongly associated with decreased survival compared to patients who were heterogenous at each class I locus. In the present study, we showed that the homozygosity of at least one locus of HLA-I was associated with a lower DCR than the heterozygosity of the HLA-I locus on crude analysis. However, the homozygosity of at least one locus of HLA-I was not an independent factor for DCR on multivariable analysis; it was not significantly associated with PFS and OS. In line with this, a previous study including 101 patients with advanced solid tumors who received single-agent pembrolizumab revealed no association between the heterozygosity of HLA-A, -B, and -C and outcomes. Currently, the impact of germline HLA genotypes including HLA homozygosity on clinical outcomes appears to be limited in patients with UC treated with pembrolizumab. Thus, it is reasonable to combine germline HLA genotypes with tumor genetic factors such as tumor mutation burden and/or loss of heterozygosity of the HLA locus in tumors as a biomarker for predicting outcomes.

On the basis of similar peptide-anchor-binding specificities, individual HLA alleles were divided into eight supertypes in the present study based on the previous literature. In the present Japanese cohort, 42.6% and 46.3% of the patients in the present Japanese cohort had B44 and B62 supertypes, respectively. These are known to be associated with survival outcomes in patients with cancer treated with ICIs; these germline genetic supertypes were not associated with clinical outcomes. By contrast, our study found that patients with the A3 supertype had significantly better DCR than those without A3 (62.8% vs. 35.9%, $p = 0.006$; Table 2), but this supertype had no influence on survival outcomes. Functional differences among supertypes in patients with advanced UC based on their overlapping peptide-binding repertoires should be elucidated in future studies, since the functional roles of each supertype, including HLA-A3 in the immune milieu of UC progression, are still unclear.

A high HED allows for the presentation of a more diverse immunopeptidome, resulting in the ability to present tumor-associated antigens. One study among patients with late-stage melanoma and non-small-cell lung cancer who received anti-CTLA-4 or PD-1/PD-L1 blockade demonstrated that patients with HED in the upper quartile were more responsive to ICIs than those with low HED. Subsequently, the same group also showed that the high mean HED was significantly associated with longer PFS and greater durability of clinical response in patients with renal cell carcinoma who received a combination therapy with ICI and TKI. In the present study, although patients with high HED tended to have a higher radiographic response than those with low HED (Table 2), HED was not associated with clinical outcomes (Figures 1C and 2C). Although this discrepancy could be attributed to racial differences, the distribution of HLA-I evolutionary divergence in our Japanese cohort (mean 6.4, range 0–10.1) was in accordance with that of a previous study encompassing distinct racial cohorts (mean 6.9, range 0.69–9.0). Therefore, HED, homozygosity, and specific supertype only had a limited impact on clinical outcomes in patients with UC who received pembrolizumab.

Among the specific alleles of HLA-I and -II, the presence of HLA-DQB1*03:01 was associated with a lower DCR and PFS (Table 2 and Figure 1D). Previous studies have suggested that the DQB1*03:01 allele is associated with increased risk of gastric adenocarcinoma and melanoma. Moreover, patients with melanoma and the DQB1*03:01 allele had more stage (III/IV) tumors than those without the allele (44% vs. 5%). Lee et al. claimed two mechanisms underlie the aggressive phenotype in patients with melanoma and the DQB1*03:01 allele. One is that the allele lacks presentation of class II-associated tumor-specific peptides to T cells. The second is that the expression of the allele stimulates CD4-inducer Suppressor or CD4-effector Suppressor T cells that can suppress the development of an effective immune response against melanoma. Although the mechanism behind the impact of the DQB1*03:01 allele on clinical outcomes is largely unknown, it is possible that these immune environment features in patients with the DQB1*03:01 allele may influence treatment effects and survival in patients with UC treated with pembrolizumab. Notably, candidate HLA alleles that are associated with radiographic progression in the present study, including A*33:03, B*44:03:01, DRB1*13:02, and DPB1*04:01, belong to the most common six loci haplotype (4.4%) in the Japanese population. Although the combination of alleles in the haplotype was not associated with PFS (Table S10), patients with all four alleles had a 100% DCR and a significantly better DCR than those without the alleles (100% vs. 41.8%, $p < 0.001$). Since a recent meta-analysis claimed that Asian patients with cancer have a significantly improved survival rate compared to non-Asian patients receiving PD-1/PD-L1 inhibitor-based therapy, it is imperative to assess the impact of specific haplotype blocks on clinical outcomes in patients treated with immune checkpoint inhibitors in future studies.
There are several limitations in the current study. First, the treatment setting varied according to the retrospective study, making it difficult to conclude the survival impact of each HLA genotype on survival outcomes. Second, although it may be advantageous to assess HLA genotypes in a Japanese population (a rather homogeneous race), it is still unknown whether our findings can be applied to other populations. Third, our study did not exclude the impact of pseudoprogression on radiographic responses after pembrolizumab administration. Specific evaluation of radiographic findings with consideration of pseudoprogression may be important to evaluate the actual impact of immunotherapy drugs on clinical outcomes in future studies. Finally, this study had a small sample size and a short follow-up duration; these factors potentially limited the evaluation of survival.

In conclusion, we identified whether several germline HLA genotypes, especially HLA-DQB1*03:01, were associated with the treatment response in patients with UC after pembrolizumab treatment. However, none of these germline HLA genotypes, including HLA-DQB1*03:01, statistically influenced survival outcomes after adjusting the covariates. Although further prospective studies with a larger number of patients and a longer follow-up are required, our finding provides a basis for the impact of germline HLA genotypes as a host-related biomarker on clinical outcomes in patients with advanced UC.

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CONFLICT OF INTEREST
The authors declare no conflicts of interest associated with this manuscript.

DISCLOSURES
Shintaro Narita received honoraria from Janssen Pharmaceutical KK. Shingo Hatakeyama received honoraria from Janssen Pharmaceutical KK and Nipro Corporation. Chikara Ohyama received honoraria from Astellas Pharma Inc., Nippon Shinyaku Company Ltd, AstraZeneca KK, Janssen Pharmaceutical KK, Takeda Pharmaceutical Company Ltd, Novartis Pharma KK, ONO Pharmaceutical Company Ltd, Chugai Pharmaceutical Company Ltd, Sanofi SA, Bayer AG, Pfizer Inc., Bristol Myers Squibb, Otsuka Pharmaceutical Company Ltd, Kissei Pharmaceutical Company Ltd, Kyowa Kirin Company Ltd, Daiichi Sankyo Company Ltd, Kaneka Corporation, and Nipro Corporation. Tomonori Habuchi also received honoraria from Janssen Pharmaceutical KK, Takeda Pharmaceutical Company Ltd, Astellas Pharma Inc., Daichi Sankyo Company, Ltd, AstraZeneca KK, Sanofi SA, and Bayer AG. The other authors have no disclosures.

ETHICS STATEMENT
This study was approved by the ethics committee of Akita University School of Medicine (No. 2251) and each institutional review board. All patients gave opt-out consent for inclusion after being informed of the study and provided information on the institution’s website.

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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