

Two HLA Class II Gene Variants Are Independently Associated with Pediatric Osteosarcoma Risk

Chenan Zhang^{1,2}, Joseph L. Wiemels^{1,2}, Helen M. Hansen², Julio Gonzalez-Maya², Alyson A. Endicott², Adam J. de Smith¹, Ivan V. Smirnov², John S. Witte¹, Libby M. Morimoto³, Catherine Metayer³, and Kyle M. Walsh^{1,4,5}



Abstract

Background: The genetic etiology of osteosarcoma remains poorly understood despite the publication of a genome-wide association study. Association between HLA genetic variants and risk of several cancers has been observed, but HLA variation is not well captured by standard SNP arrays.

Methods: We genotyped 207 Californian pediatric osteosarcoma cases and 696 controls of European ancestry using a custom genome-wide array supplemented with approximately 6,000 additional probes across the MHC region. We subsequently imputed 4-digit classical HLA alleles using a reference panel of 5,225 individuals who underwent high-resolution HLA typing via next-generation sequencing. Case-control comparisons were adjusted for ancestry-informative principal components, and top associations from the discovery analysis underwent replication in an independent dataset of 657 cases and 1,183 controls.

Results: Three highly correlated HLA class II variants ($r^2 = 0.33$ – 0.98) were associated with osteosarcoma risk in discovery analyses, including *HLA-DRB1*0301* (OR = 0.52; $P =$

3.2×10^{-3}), *HLA-DQA1*0501* (OR = 0.74; $P = 0.031$), and *HLA-DQB1*0201* (OR = 0.51; $P = 2.7 \times 10^{-3}$). Similar associations were observed in the replication data ($P_{\text{range}} = 0.011$ – 0.037). Meta-analysis of the two datasets identified *HLA-DRB1*0301* as the most significantly associated variant (OR_{meta} = 0.62; $P_{\text{meta}} = 1.5 \times 10^{-4}$), reaching Bonferroni-corrected statistical significance. The meta-analysis also revealed a second significant independent signal at *HLA-DQA1*01:01* (OR_{meta} = 1.33, $P_{\text{meta}} = 1.2 \times 10^{-3}$), and a third suggestive association at *HLA-DQB1*0302* (OR_{meta} = 0.73, $P_{\text{meta}} = 6.4 \times 10^{-3}$).

Conclusions: Multiple independent HLA class II alleles may influence osteosarcoma risk.

Impact: Additional work is needed to extend our observations to other patient populations and to clarify the potential causal mechanisms underlying these associations. Understanding immunologic contributions to the etiology of osteosarcoma may inform rational therapeutic targets. *Cancer Epidemiol Biomarkers Prev*; 27(10); 1151–8. ©2018 AACR.

Introduction

Osteosarcoma is the most commonly diagnosed primary malignant bone tumor, with peak incidence occurring during adolescence. In the United States, approximately 400 new cases are diagnosed annually in individuals younger than 20 years of age (1), with similar rates of childhood and adolescent-onset osteosarcoma observed worldwide (2, 3). Risk factors for osteosarcoma include taller stature and male sex (4–6), but the genetic etiology of the disease remains poorly understood.

Although increased osteosarcoma risk is associated with Paget disease of the bone and inherited cancer predisposition syndromes (e.g., Li-Fraumeni syndrome), the majority of cases are sporadic (7, 8). Candidate gene studies have suggested a role for common genetic variants in the DNA repair pathway (9), growth hormone pathway (9, 10), and telomere maintenance pathway (11) in contributing to risk of osteosarcoma, but only a single genome-wide association study (GWAS) has been published to date (12). This GWAS reported two risk loci associated with osteosarcoma, one of which was located in the *GRM4* gene at 6p21.3, approximately 1 Mb from the MHC class II region.

The MHC region, encompassing approximately 7.6 Mb on chromosome 6p21, is one of the most polymorphic and gene-dense regions of the genome, including the highly variable *HLA* genes. HLA variants have been associated with cancer risk in previous studies of chronic lymphocytic leukemia, lung squamous cell carcinoma, ovarian, breast, cervical, and nasopharyngeal cancers (13–18). Various mechanisms linking HLA genetic variation to cancer development have been hypothesized, including differential tumor tolerance by the immune system, efficiency of immunosurveillance, and cancer immunoeediting resulting in certain HLA variants predisposing to particular malignancies (18–21). Investigating the role of heritable HLA variation in osteosarcoma risk may

¹Department of Epidemiology and Biostatistics, University of California, San Francisco, San Francisco, California. ²Division of Neuroepidemiology, Department of Neurological Surgery, University of California, San Francisco, San Francisco, California. ³School of Public Health, University of California, Berkeley, Berkeley, California. ⁴Division of Neuro-epidemiology, Department of Neurosurgery, Duke University, Durham, North Carolina. ⁵Children's Health and Discovery Institute, Duke University, Durham, North Carolina.

Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (<http://cebp.aacrjournals.org/>).

Corresponding Author: Kyle M. Walsh, Duke University, Durham, NC 27510. Phone: 919-684-8732; Fax: 919-681-9775; E-mail: Kyle.Walsh@Duke.edu

doi: 10.1158/1055-9965.EPI-18-0306

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inform disease etiology, and inform potential strategies for immunotherapy.

Although the previous osteosarcoma GWAS did not report any signals in the MHC region, genetic variation and haplotype structure in *HLA* genes are not well captured by standard genome-wide SNP arrays due to the unique characteristics of sequence and copy-number variation across the MHC region. Using a custom Affymetrix Axiom Array supplemented with approximately 6,000 additional probes spanning the MHC region, we typed classical HLA alleles at 4-digit resolution (e.g., *HLA-DRB1*1501*) and tested for association with pediatric osteosarcoma risk. We subsequently attempted replication in an independent set of osteosarcoma cases and controls and performed meta-analysis of these two datasets, totaling 864 cases and 1,879 controls. We further assessed nonadditive genetic associations in the MHC region as a complementary approach given the observation of widespread nonadditive and epistatic effects among HLA variants for a number of diseases (22–24), as well as any potential modification by subject sex or clinical presentation (e.g., tumor location, presence of metastasis, and age at diagnosis) to seek further insight into the etiology, progression, and prognosis of this aggressive cancer.

Materials and Methods

Discovery sample

The study was approved by the institutional review boards at the Universities of California at Berkeley and San Francisco, and the California Department of Public Health (CDPH, Sacramento, CA). The biospecimens and/or data used in this study were obtained from the California Biobank Program (SIS request number 550). The CDPH, Genetic Diseases Screening Branch obtains newborn blood samples from all neonates born within the state for the purpose of disease screening. The bloodspots that remain after screening have been archived at -20°C since 1982 and made available for approved research. We linked statewide birth records maintained by the CDPH (for years 1982–2009) to cancer diagnosis data from the California Cancer Registry (CCR, for years 1988–2011). Included in this analysis were non-Hispanic white children born in California during 1982 to 2009 and diagnosed with osteosarcoma (ICD-O-3 codes 9180–9183, 9185–9187, and 9192–9195) by age 19, per CCR record. Children born in California during the same period and not reported to CCR as having any childhood cancer were selected as controls. Characteristics of osteosarcoma cases appear in Supplementary Table S1.

DNA extraction

A one-third portion of a 12-mm dried bloodspot was partitioned into three uniform segments and placed in a 2-mL microcentrifuge tube prior to extraction, which was carried out using the QIAamp DNA Investigator Kit (Qiagen). In brief, 280 μL of Buffer ATL and 20 μL of Proteinase K were added to each sample. Samples were vortexed and then incubated in a dry-bath shaker at 900 rpm and 56°C for 1 hour. After incubation, samples were briefly centrifuged and the lysate solution was transferred to a new 2-mL microcentrifuge tube, while the solid remnants were discarded. One microliter of 1 ng/ μL carrier RNA was added to the lysate and then briefly vortexed. After adding carrier RNA, samples were placed in the Qiagen

Qiacube automated work station for DNA isolation. The result of the Qiacube extraction protocol was a purified DNA sample in ATE buffer.

Discovery sample genotyping and quality control

DNA specimens were assigned to genotyping plates using blocked randomization according to case–control status, reported ethnicity, and sex. DNA was genotyped on the Affymetrix Axiom Latino Array (25), with high coverage of European ancestries, supplemented with approximately 6,000 additional SNP probes in the MHC region. DNA samples were genotyped on an Affymetrix TITAN system, and raw image files were processed with Affymetrix Genetools to call genotypes.

Duplicate samples included on the array ($n = 34$) had average genotype concordance $>99\%$. Call-rate filtering for SNPs and samples was performed iteratively as described previously (26), excluding SNPs with call rates $<97\%$ and samples with call rates $<97\%$. SNPs displaying significant departure from Hardy–Weinberg equilibrium ($P < 1.0 \times 10^{-5}$) among European-ancestry controls and samples with mismatched reported versus genotyped sex were excluded. One individual from each subject pair with identity-by-descent (IBD) proportion >0.18 was excluded in the discovery data (27, 28). Using genome-wide SNP array data from 1184 HapMap phase III samples, we performed principal component analysis using unlinked autosomal biallelic SNPs with allele frequency >0.05 and removed from analysis any sample showing evidence of non-European ancestry (>3 SDs from mean CEPH values on PCs 1–3).

Replication dataset

The osteosarcoma replication dataset was obtained from dbGaP study accession phs000734.v1.p1 (GWAS of risk for osteosarcoma) and phs000381.v1.p1 (eMERGE Geisinger eGenomic Medicine MyCode Project Controls). Cases and controls were genotyped on the Illumina OmniExpress array and underwent quality-control filtering as described previously (11). Briefly, SNPs with call rates <0.98 and subjects with genotyping call rates <0.97 were removed. Ancestry-informative principal components were calculated using Eigenstrat (29) and HapMap reference samples and mean values of the first five principal components were calculated among HapMap CEPH samples. Subjects with evidence of non-European ancestry (>3 SDs from the mean CEPH values) were excluded. SNPs with Hardy–Weinberg equilibrium $P < 1.0 \times 10^{-5}$ among controls were removed. Osteosarcoma cases from the discovery and replication datasets were compared and both duplicated and cryptically related (IBD > 0.18) samples were excluded from the replication dataset ($n = 22$ duplicated patients, 0 cryptically related patients). A total of 657 nonoverlapping European-ancestry cases and 1,183 controls were included in the final replication dataset. Patients with osteosarcoma from dbGaP were predominantly children and adolescents (age < 21), although individual-level age data were unavailable, and the original publication did not restrict to a specific age range. These patients with osteosarcoma are a subset of those included in a previous GWAS publication (12).

Imputation of HLA genotypes

We imputed 4-digit classical HLA alleles of the three main class I genes (*HLA-A*, *HLA-B*, and *HLA-C*) and the five class II genes (*HLA-DRB1*, *-DPA1*, *-DPB1*, *-DQA1*, and *-DQB1*), as well as 5,695

HLA intragenic SNPs using SNP2HLA (30), which uses a reference panel of 5,225 individuals of European ancestry from the Type 1 Diabetes Genetics Consortium who underwent high-resolution HLA typing via next-generation sequencing. We used the SNP2HLA imputed allele dosage data for the primary MHC-wide association analyses, and phased best guess genotypes for the nonadditive effect analyses, as described previously (22). Association analyses were restricted to classic 4-digit HLA class I and class II alleles with imputation quality score INFO > 0.80, allele frequency > 0.05 (19 class I alleles and 27 class II alleles). For nonadditive effect analyses, we restricted to individuals who had two best-guess haplotypes at each HLA gene analyzed.

Statistical analyses

We performed logistic regression analyses for each of the HLA class I and class II alleles, using the imputed allele dosages output from SNP2HLA and the "logistic" command in PLINK 1.9, adjusting for the first 10 ancestry-informative principal components generated by Eigenstrat. As a secondary exploratory investigation, we also performed association analyses for 5,695 imputed HLA intragenic SNPs in the MHC region. The analyses were performed separately in the discovery and replication datasets and meta-analyzed using the "meta-analysis" command in PLINK. We conducted conditional analyses by repeating the logistic regressions adjusting for the top signals. We also adjusted for SNPs 1906953, a previously identified osteosarcoma GWAS hit at 6p21.3 that is located approximately 1 Mb from the MHC region to assess any attenuation of the top signals present in our analyses. For sex-stratified analyses, we examined association signals separately in males and females and tested for heterogeneity of effect. We also conducted case-only analyses, assessing whether top HLA association signals identified in case-control analyses were associated with differences in clinical presentation, including age at diagnosis, tumor location, tumor size, extension, differentiation, and presence of metastasis. Information on clinical variables was available for only the discovery dataset, coded as described previously (6).

We investigated nonadditive effects of the HLA alleles by assessing dominance effect models as described in prior studies (22, 24). In brief, we tested the improvement in model fit comparing the additive effect model with a model that additionally included a dominance term representing heterozygous status for each HLA allele investigated. A *P* value for model improvement from the χ^2 test represents the significance of the deviation from an additive model. We additionally tested for interaction between top variants in each independent signal detected and each of the remaining class II variants.

HLA class I and II alleles that achieved nominal statistical significance in the discovery analysis (*P* < 0.05) were carried

forward for replication. Variants that achieved nominal significance in both datasets and a *P* value surpassing Bonferroni correction in the meta-analysis were considered to be statistically significant. Because of linkage disequilibrium (LD) between HLA variants, a traditional Bonferroni correction threshold would be overly conservative as the tests are not independent. We estimated independent tests performed after adjusting for LD to correct for multiple comparisons (31), reducing the number of "effective" tests from 46 to 39 primary HLA class I and II allele analyses and from 5,695 to 1,713 secondary HLA intragenic SNP analyses. Corresponding Bonferroni corrections of significance thresholds were then performed using the "effective" number of tests for HLA class I and II alleles ($0.05/39 = 1.3 \times 10^{-3}$) and HLA intragenic SNPs ($0.05/1713 = 2.9 \times 10^{-5}$).

Results

A total of 13,103 SNPs in the MHC region were successfully genotyped on-array in the discovery dataset, which included 207 pediatric osteosarcoma cases and 696 controls of European ancestry. Using these data to impute HLA class I and class II alleles, we observed 19 class I alleles and 27 class II alleles with frequency > 0.05 (Supplementary Table S2). In logistic regression analyses, three class I alleles were nominally associated with osteosarcoma risk, including: *HLA-A*0201* [OR = 1.35; 95% confidence interval (CI), 1.05–1.74; *P* = 0.020], *HLA-A*0301* (OR = 0.66; 95% CI, 0.46–0.94; *P* = 0.023), and *HLA-B*0801* (OR = 0.60; 95% CI, 0.38–0.92; *P* = 0.021; Table 1). In addition, three class II alleles were nominally associated with osteosarcoma risk, including *HLA-DRB1*0301* (OR = 0.52; 95% CI, 0.34–0.81; *P* = 3.2×10^{-3}), *HLA-DQA1*0501* (OR = 0.74; 95% CI, 0.56–0.97; *P* = 0.031), and *HLA-DQB1*0201* (OR = 0.51; 95% CI, 0.33–0.79; *P* = 2.7×10^{-3} ; Table 1).

In the replication dataset of 657 nonoverlapping osteosarcoma cases and 1,183 controls of European ancestry, a total of 5,401 SNPs in the MHC region were successfully genotyped on-array and used to impute 4-digit HLA variants. None of the HLA class I variants identified in the discovery set were associated with osteosarcoma risk in the replication data (*P*_{range} = 0.12–1.0). However, all three HLA class II variants identified in the discovery dataset showed evidence of association in the replication data (*P*_{range} = 0.011–0.037; Table 1).

Meta-analysis of the two studies identified *HLA-DRB1*0301* as top signal (OR_{meta} = 0.62; 95% CI, 0.48–0.79; *P*_{meta} = 1.5×10^{-4}), reaching Bonferroni-corrected significance (Fig. 1). Other class II variants identified in the discovery analysis (i.e., *HLA-DQA1*0501* and *HLA-DQB1*0201*) had similar *P* values and effect sizes (Table 2). We observed that subject genotype at

Table 1. Association results (*P* < 0.05) for discovery stage of 207 osteosarcoma cases and 696 controls, and replication stage of 657 cases and 1,183 controls

Class	HLA allele	Discovery			Replication		
		Freq ^a	OR (95% CI)	<i>P</i>	Freq ^b	OR(95% CI)	<i>P</i>
I	<i>HLA-A*0201</i>	0.26	1.35 (1.05–1.74)	0.020	0.28	1.07 (0.89–1.28)	0.48
	<i>HLA-A*0301</i>	0.15	0.66 (0.46–0.94)	0.023	0.14	1.00 (0.79–1.27)	1.0
	<i>HLA-B*0801</i>	0.11	0.60 (0.38–0.92)	0.021	0.10	0.75 (0.52–1.08)	0.12
II	<i>HLA-DRB1*0301</i>	0.12	0.52 (0.34–0.81)	3.2×10^{-3}	0.12	0.67 (0.50–0.91)	0.011
	<i>HLA-DQA1*0501</i>	0.25	0.74 (0.56–0.97)	0.031	0.28	0.81 (0.67–0.99)	0.037
	<i>HLA-DQB1*0201</i>	0.12	0.51 (0.33–0.79)	2.7×10^{-3}	0.12	0.70 (0.52–0.94)	0.018

^aAllele frequency in discovery dataset controls.

^bAllele frequency in replication dataset controls.

Table 2. Meta-analysis results for HLA class II variants nominally associated with osteosarcoma ($P < 0.05$) in 3 statistically independent loci

HLA allele	Freq _{discovery} ^a	OR _{meta} (95% CI)	P_{meta} ^b	I^2 ^c (%)	Signal
<i>HLA-DRB1*0301</i>	0.12	0.62 (0.48–0.79)	1.5×10^{-4}	0	1
<i>HLA-DQA1*0501</i>	0.25	0.79 (0.67–0.92)	3.2×10^{-3}	0	1
<i>HLA-DQB1*0201</i>	0.12	0.63 (0.49–0.81)	2.6×10^{-4}	23.8	1
<i>HLA-DRB1*0101</i>	0.08	1.37 (1.10–1.71)	4.9×10^{-3}	0	2
<i>HLA-DQA1*0101</i>	0.15	1.33 (1.12–1.58)	1.2×10^{-3}	0	2
<i>HLA-DQB1*0501</i>	0.13	1.34 (1.11–1.61)	2.5×10^{-3}	0	2
<i>HLA-DQB1*0302</i>	0.11	0.73 (0.58–0.91)	6.4×10^{-3}	0	3

^aAllele frequency in discovery dataset controls, with similar frequencies in replication set.

^bFixed effects meta-analysis P value adjusted for ancestral components. Bold highlighting indicates significance after Bonferroni correction with adjustment for linkage disequilibrium between variants ($P < 1.3 \times 10^{-3}$).

^c I^2 heterogeneity index.

these three class II genes (*HLA-DQB1*0201*, *HLA-DRB1*0301*, and *HLA-DQA1*0501*) were highly correlated ($r^2 = 0.33$ – 0.98 ; Supplementary Table S3). In logistic regression analyses conditioned on genotype at *HLA-DRB1*0301*, neither *HLA-DQA1*0501* nor *HLA-DQB1*0201* remained significantly associated with osteosarcoma risk in the meta-analysis ($P_{range} = 0.35$ – 0.97), indicating that these three alleles represent a single protective haplotype.

The meta-analysis also revealed another association signal at three additional class II alleles: *HLA-DRB1*0101* (OR_{meta} = 1.37; 95% CI, 1.10–1.71; $P_{meta} = 4.9 \times 10^{-3}$), *HLA-DQA1*0101* (OR_{meta} = 1.33; 95% CI, 1.12–1.58; $P_{meta} = 1.2 \times 10^{-3}$), and *HLA-DQB1*0501* (OR_{meta} = 1.34; 95% CI, 1.11–1.61; $P_{meta} = 2.5 \times 10^{-3}$). High LD between these three alleles ($r^2 = 0.57$ – 0.99), together with conditional analyses showing attenuation of signals, suggests that they represent a single risk haplotype. Conditional analyses adjusting for *HLA-DRB1*0301* located on the protective haplotype described above did not substantially attenuate the associations with these three alleles ($P_{range} = 6.3 \times 10^{-3}$ – 0.014), suggesting that they represent a second independent signal (Supplementary Fig. S1).

We observed a third independent risk locus at *HLA-DQB1*0302* that was suggestively associated with osteosarcoma (OR_{meta} = 0.73; 95% CI, 0.58–0.91; $P_{meta} = 6.4 \times 10^{-3}$). Conditional analyses adjusting for *HLA-DRB1*0301* (the top signal in the protective haplotype) and *HLA-DQA1*0101* (the top signal in the risk haplotype) did not substantially attenuate the association at *HLA-DQB1*0302* ($P_{conditional} = 0.01$; Supplementary Fig. S1). In addition, all three HLA class II association signals remained when adjusting for rs1906953, the *GRM4* variant on 6p21.3 previously identified as an osteosarcoma risk locus (Supplementary Fig. S2; ref. 12), which showed no LD with any of our HLA class II risk variants ($r^2 < 0.01$).

Although the replication dataset genotyping array did not contain the additional approximately 6,000 probes for the MHC region included on the discovery dataset custom array, imputation accuracy was similarly high in both discovery and replication datasets for the nominally significant osteosarcoma risk loci reported above (Supplementary Table S4).

Sex-specific analyses of the combined discovery and replication data suggested that the effect of the third HLA class II signal, *HLA-DQB1*0302*, may be modified by subject sex ($P_{interaction} = 0.02$). Specifically, we observed a greater magnitude of effect in females ($n = 397$; OR_{meta} = 0.54; $P_{meta} = 2.4 \times 10^{-3}$) than in males ($n = 506$; OR_{meta} = 0.90; $P_{meta} = 0.49$). No differences across strata of subject sex were observed for the other association signals. In case-only analyses of the discovery sample where clinical data were available, the lead HLA variant

of the top signal (*HLA-DQB1*0201*) was not significantly associated with clinical features, although it was suggestively associated with higher tumor grade ($P_{trend} = 0.09$).

A systematic search for nonadditive effects in the region identified a departure from additivity at *HLA-DQB1*0302*. Inclusion of a dominance term improved the model fit in both the discovery and the replication datasets ($P_{improve} = 0.04$ and $P_{improve} = 0.03$, respectively). The dominance term (OR = 0.48; 95% CI, 0.25–0.92) suggests that for *HLA-DQB1*0302*, heterozygosity confers 2-fold greater protection than expected with the effect observed in individuals homozygous for this allele. Given that *HLA-DQB1*0302* had a female-specific association, we carried out sex-stratified nonadditive analysis, observing the nonadditive protective effect in male subjects ($P_{improve} = 0.09$ and $P_{improve} = 6.4 \times 10^{-3}$ for the discovery and replication datasets), but not female subjects ($P_{improve} = 0.23$ and $P_{improve} = 0.51$ for the discovery and replication datasets). Nonadditive effects were not observed for any other DQB1 alleles (Supplementary Fig. S3) or any other HLA class II alleles. Epistasis analyses of the top signal, represented by *HLA-DRB1*0301*, identified suggestive interactions with linked alleles *HLA-DRB1*1301* ($P_{interaction} = 3.0 \times 10^{-3}$), *HLA-DQA1*0103* ($P_{interaction} = 2.5 \times 10^{-3}$), and *HLA-DQB1*0603* ($P_{interaction} = 4.8 \times 10^{-3}$), although these interactions were not significant after adjustment for all interactions tested (i.e., $0.05/26 = 1.9 \times 10^{-3}$; Supplementary Table S5). No evidence of interaction at the other top loci appearing in Table 2 was observed.

A previous study on the association between HLA variants and irritable bowel disease demonstrated that electrostatic potential differences at the peptide-binding groove correlate with the directionality of effect of DRB1 alleles on disease risk (23). We queried their published electrostatic potential data for *HLA-DRB1*0301* and *HLA-DRB1*0101*, our two top signals showing opposing effects. We observed an electrostatic potential difference between these two alleles of approximately 0.8 on a scale ranging from 0 (identical) to 1 (maximum difference), indicating that the osteosarcoma risk allele (*HLA-DRB1*0101*) and protective allele (*HLA-DRB1*0301*) have substantially different electrostatic potentials, supporting a potential functional difference between these alleles.

In secondary analyses of imputed HLA intragenic SNPs, we observed several SNPs downstream of *HLA-A* showing evidence of association with osteosarcoma risk, including lead SNP rs2517612 (OR_{meta} = 0.60, $P_{meta} = 5.5 \times 10^{-5}$). An additional independent signal was observed upstream of *HLA-DRB1* at rs3129890 (OR_{meta} = 0.71, $P_{meta} = 5.7 \times 10^{-5}$). The intragenic SNPs were in moderate LD with the top HLA class II signal *DRB1*301* ($r^2 = 0.41$ – 0.42). However, neither SNP survived

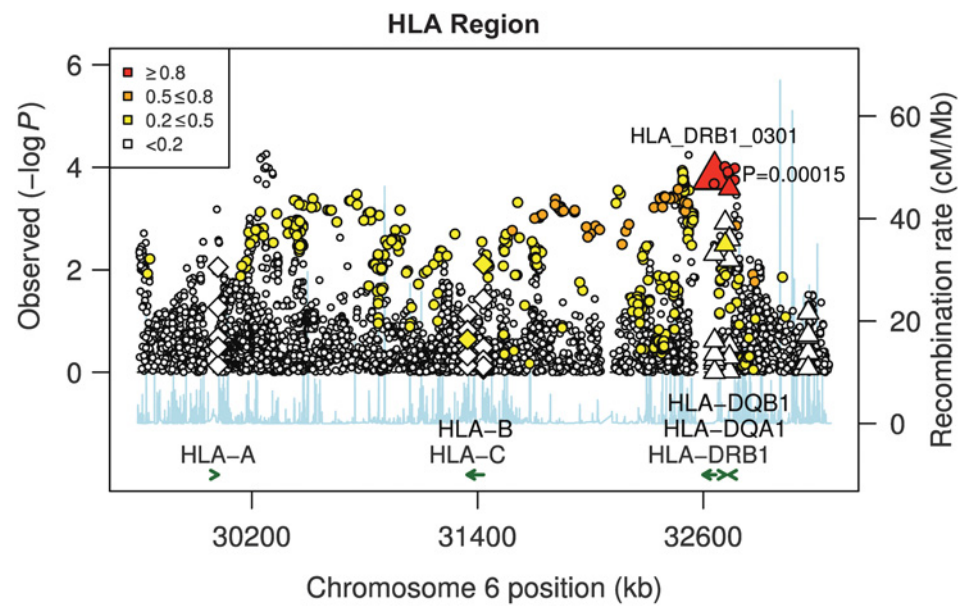


Figure 1. Regional association plot of the meta-analysis for HLA variants. Diamonds represent HLA class I variants, triangles represent HLA class II variants, and circles represent HLA intragenic SNPs. The large triangle represents the top signal in the region.

corrections for multiple testing (i.e., $<2.9 \times 10^{-5}$). Association statistics, stratified by dataset, for SNPs with meta-analysis P values $<1.0 \times 10^{-4}$ appear in Supplementary Table S6.

Discussion

In this first study to date of the association between HLA variation and osteosarcoma risk, we observed a signal associated with reduced risk consisting of the three linked alleles *HLA-DQB1*0201*, *HLA-DRB1*0301*, and *HLA-DQA1*0501*, which was replicated in an independent dataset and remained significant after Bonferroni correction. Two additional independent signals, one of reduced risk at *HLA-DQB1*0302* and one of increased risk at three linked alleles *HLA-DRB1*0101*, *HLA-DQA1*0101*, and *HLA-DQB1*0501*, were revealed in meta-analysis of the discovery and replication datasets. None of the three signals were attenuated in conditional analyses adjusting for rs1906953, indicating that the effects are independent of a previously identified GWAS signal in *GRM4* at 6p21.3.

A growing body of literature has emerged on the complex interactions between bone and immune cells, with shared signal transduction pathways, common hematopoietic precursors, and cross-talk between the bone and the immune systems (32). The expression of HLA class II molecules on human osteoblasts has been observed in multiple studies (33, 34), as has upregulation of the HLA class II receptor activity pathway in osteosarcoma (35), supporting a biological role for HLA class II genes in osteosarcoma development and progression. Indeed, in addition to immunotherapy targets currently in clinical development for patients with osteosarcoma (e.g., PD1 and PDL1), HLA class II molecules, IL10, and TGF β are currently considered potential targets for breaking immune tolerance to the tumor (36).

A recent study found an association between variation in immune cell transcripts, specifically in monocytes and T cells, and osteosarcoma metastasis and survival (37). A role for immune cell infiltration and immune monitoring in bone is consistent with our results, suggesting potential immunologic contributions to osteosarcoma etiology, potentially operating

through differences in antigen presentation. Although we were unable to assess patient survival, a suggestive association between *HLA-DQB1*0201* and tumor grade suggests that germline variation in immune-related genes may influence patient outcomes. This complements a recent GWAS, which identified an allele associated with reduced IL33 expression and poorer osteosarcoma patient survival (38). IL33 induces production of Th2-associated cytokines and is associated with atopic conditions (39, 40). Because it is expressed by both osteoblasts and lymphocytes, the interaction of local and systemic immunologic changes in impacting osteosarcoma risk and outcome and interplay between IL33 and HLA class II variation merit further attention.

Studies of other tumor sites have also revealed the involvement of the HLA alleles highlighted in our results, raising an interesting possibility of this variant as a source of genetic pleiotropy. Notably, the extended haplotype *HLA-DRB1*0301:DQA1*0501:DQB1*0201* corresponding to our top association signal was previously associated with increased risk of ovarian cancer (19). Increased risk of nasopharyngeal carcinoma, associated with the Epstein–Barr virus, was found with the extended haplotype *HLA-A*3303-B*5801/2-DRB1*0301-DQB1*0201/2-DPB1*0401*, specific to the Chinese ethnic group of the Taiwanese study (17). Previous examples of pleiotropic associations within the MHC region have been described among B-cell malignancies, suggesting the possibility of shared biological pathways influencing the development of different cancer types (41). Furthermore, opposing risk associations in different B-cell malignancies have previously been noted for pleiotropic risk loci in the MHC region (41), similar to our observation of protection against osteosarcoma compared with increased risk for other cancer types for the same HLA variant. Individual alleles, *HLA-DRB1*0101*, *HLA-DQA1*0101*, and *HLA-DQB1*0501* of our second signal have also been linked to a variety of cancers including renal cell carcinoma, breast cancer, cervical cancer, and childhood acute lymphoblastic leukemia (42–46). Our third signal, *HLA-DQB1*0302*, has been linked to HPV-related cervical cancer, hypothesized to be due to

differential affinity to HPV antigen binding associated with protection against infection (47).

Exploratory analyses of HLA intragenic SNPs identified lead SNP rs2517612, an expression quantitative trait locus for a large number of genes, including *BTN3A2*, *SLC15A3*, *FAM20B*, *HLA-B*, *HLA-C*, *HLA-DQA1*, *HLA-DQA2*, among others. Because of the large number of HLA intragenic SNPs analyzed, no single-SNP association survived Bonferroni correction.

The potential interaction between DQB1*0302 and subject sex is intriguing given the well-documented increased incidence of osteosarcoma in males versus females (5). The larger and more significant decrease in risk for osteosarcoma seen in females with the *HLA-DQB1*0302* allele than males, despite a smaller sample size, is consistent with this variant potentially contributing to lower incidence of osteosarcoma in girls. It is unclear why the association would be stronger in females, although sex-specific HLA associations were previously observed for gastric cancer and acute lymphoblastic leukemia (48, 49). One possibility may relate to sex differences in rates of bone growth during adolescence, previously suggested to impact osteosarcoma risk due to the susceptibility of rapidly proliferating cells to oncogenic agents and mitotic errors (50). However, the observed male-specific nonadditive protective effect complicates interpretations and suggests a role for *HLA-DRB1*0302* in osteosarcoma susceptibility across sexes. Because this HLA variant did not remain significant after Bonferroni correction, additional follow-up studies are necessary to confirm the association and explore any effect modification by sex.

The *HLA-DQB1*0302* association displayed a nonadditive dominance effect in both the discovery and replication datasets, implying a stronger protective effect in heterozygotes than homozygotes. Nonadditive effects in the MHC region are hypothesized to play an important role in the necessary functional redundancy of the immune system (51), and given the overlapping signal transduction pathways and cross-talk between the bone and immune system, the detection of such effects in relation to osteosarcoma is intriguing.

Because our genotyping data were generated with custom SNP arrays rather than direct typing of HLA alleles via targeted sequencing, our genotyping is limited by imputation accuracy. However, imputation error would only lead to reduced power to detect signals and should not lead to any increase in type I error rate. In addition to rare variants, HLA imputation may also fail to detect variants in paralog genes and copy-number variable regions (52), as such variants could be excluded from analyses due to deviation from Hardy-Weinberg equilibrium. Higher resolution HLA sequencing will be necessary to more conclusively identify the causative variants underlying our observed associations, potentially enabling specific protective HLA variants to guide approaches for breaking immune tolerance to the tumor.

The array-based platform utilized also limits our ability to detect rare or private germline mutations in genes associated with hereditary cancer predisposition syndromes, including *TP53*. Although previous studies have not observed strong associations between common *TP53* variants and osteosarcoma risk (53), a recent study found that 9.5% of patients with osteosarcoma diagnosed before age 30 harbored at least one rare exonic *TP53* variant (7). Given recent reports of a higher-than-expected population prevalence of *TP53* mutations in controls unselected for cancer history (54), and potential

polygenic contributions from multiple rare variants to sarcoma risk (55), the role of HLA class II variants and other immune factors in modifying cancer penetrance among *TP53* mutation carriers should be explored.

Our study has several additional limitations. Our analyses were performed in subjects of European ancestry and findings may not be generalizable to other ethnicities. The SNP2HLA method requires an ethnic-specific reference panel, and non-European reference datasets of adequate sample size are currently lacking (30). We also cannot confidently ascertain the likeliest causative allele among multiple linked HLA variants in our top two signals, although our query of the electrostatic properties of *HLA-DRB1* variants suggests that the *HLA-DRB1* alleles of the two signals are strong candidates for functional relevance. However, additional functional studies are necessary to infer causality. Finally, although our two top signals remained significant after LD-adjusted Bonferroni correction for multiple testing, additional datasets will be necessary to confirm our findings.

In summary, we genotyped HLA class I and class II alleles at 4-digit resolution and observed multiple independent class II alleles associated with risk of pediatric osteosarcoma, including two different alleles of DRB1. These findings improve our understanding of potential immunologic contributions to osteosarcoma etiology and may also provide insights for the development of future immunotherapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

The California Department of Public Health is not responsible for the results or conclusions drawn by the authors of this publication.

Authors' Contributions

Conception and design: C. Zhang, J.L. Wiemels, K.M. Walsh
Development of methodology: C. Zhang, J. Gonzalez-Maya, K.M. Walsh
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H.M. Hansen, J. Gonzalez-Maya, A.A. Endicott, L.M. Morimoto, C. Metayer, K.M. Walsh
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Zhang, I.V. Smirnov, J.S. Witte, C. Metayer, K.M. Walsh
Writing, review, and/or revision of the manuscript: C. Zhang, J.L. Wiemels, A.J. de Smith, L.M. Morimoto, C. Metayer, K.M. Walsh
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.L. Wiemels, H.M. Hansen, A.A. Endicott, I.V. Smirnov, L.M. Morimoto
Study supervision: J.L. Wiemels, K.M. Walsh

Acknowledgments

Funding for the MyCode Project was provided by a grant from the Commonwealth of Pennsylvania and the Clinic Research Fund of Geisinger Clinic. Funding support for the genotyping of the MyCode cohort was provided by a Geisinger Clinic operating funds and an award from the Clinic Research Fund. This work was supported by the NIH T32CA151022-06 (to C. Zhang), R25T CA112355 (to J.S. Witte), R01 CA155461 (to J.L. Wiemels), and by "A" Awards from The Alex's Lemonade Stand Foundation (to A.J. deSmith, and K.M. Walsh).

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Received March 23, 2018; revised May 29, 2018; accepted July 9, 2018; published first July 23, 2018.

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