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Association of *HLA-DQB1* alleles with risk of follicular lymphoma

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Abstract

In a recent genome-wide association study of follicular lymphoma (FL), we identified novel risk alleles on chromosome 6p21.33 that appeared to be part of an extended haplotype including *HLA-DRB1**0101, *DQA1**0101, and *DQB1**0501. To follow up on these findings, we obtained 2–4 digit *HLA-DQB1* allelotypes on a subset of 265 FL cases and 757 controls using a novel assay that applies multiplexed ligation-dependent probe amplification (MLPA). We confirmed a positive association between FL and the *HLA-DQB1**05 allele group (OR=1.70, 95% CI 1.28–2.27; adjusted *p*-value=0.013) and also identified an allele group inversely associated with FL risk, *HLA-DQB1*06* (OR=0.51, 95% CI 0.38–0.69; adjusted *p*-value=4.46×10^{-5}). Although these findings require verification, the role of *HLA* class II proteins in B-cell survival and proliferation make this a biologically plausible association.

Keywords

follicular lymphoma; MHC; HLA; DQ; NHL

INTRODUCTION

Follicular lymphoma (FL) is the second most common subtype of non-Hodgkin lymphoma (NHL), comprising up to 30% of all NHL cases in populations worldwide[1]. Thus the more than 300,000 incident NHL cases diagnosed each year[2], are comprised of approximately 24,000–90,000 incident cases of FL. Although FL has a relatively long median survival of 8–10 years, more than 20% of FL patients transform to an aggressive lymphoma with a poor clinical outcome[3].

The “defining event” for FL is the t(14;18) chromosomal translocation. However this signature translocation also is present in greater than 50% of healthy individuals, and is not sufficient to cause the full transformation of a B-cell to a FL tumor[4]. Common gene variants appear to play a significant role in lymphomagenesis[5–7]. Candidate gene studies have indentified single nucleotide polymorphisms (SNPs) associated with FL susceptibility in pathways related to oxidative stress, energy regulation, detoxification, and

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DECLARATION OF INTERESTS

The authors have no conflicts of interest to declare.

immunoregulation[7,8]. In a recent genome-wide association study (GWAS) of 1,465 cases and 6,958 controls, we identified and validated highly significant associations between FL and non-coding SNPs in strong linkage disequilibrium (LD) (rs10484561 odds ratio (OR)=1.95, 95% confidence interval (CI)=1.72–2.22, trend p-value=1.12×10^{-29}; rs7755224 OR=2.07 95%CI=1.76–2.42, trend p-value=2.00×10^{-19}; D′=1.0, r^2=0.99) in the human leukocyte antigen (*HLA*) region on chromosome 6p21.33[4]. Using tag SNPs for *HLA* alleles[9], we identified an extended haplotype containing the alleles *HLA-DRB1**0101—*HLA-DQA1**0101—*HLA*DQB1**0501 that was in strong LD with rs108484561 and rs7755224 and associated with increased FL risk.

To confirm these findings, our group developed a novel assay to type the *HLA-DQB1* locus using the multiplexed ligation-dependent probe amplification (MLPA) technique[10]. Allelotyping of 265 FL cases and 757 controls from a larger NHL case-control study based in the San Francisco Bay Area (2,050 cases and 2,081 controls)[11] confirmed the high LD between rs10484561/rs7755224 and *HLA-DQB1**05 alleles, and revealed a strong inverse association between FL and *HLA-DQB1**06 alleles.

MATERIALS AND METHODS

Study Population

A population-based case-control study of NHL (2055 cases, 2081 controls) was conducted in the San Francisco Bay Area that included incident cases diagnosed from 2001 through 2006. Details of the process and criteria for subject selection have been described elsewhere[11]. Briefly, eligible patients were aged 20–85 years and residents of one of the six Bay Area counties at the time of diagnosis. Controls were frequency matched to patients by age in five-year groups, sex and county of residence. Blood and/or buccal specimens were collected from eligible cases and controls that participated in the laboratory portion of the study (participation rates, 87% and 89%, respectively). Patient diagnostic pathology materials were re-reviewed by the study's expert pathologist to confirm NHL diagnosis and histology. For this study, an HIV-negative subset of 265 FL cases and 757 controls restricted to white, non-Hispanics was selected for allelotyping. Of these, 203 FL cases (77%) and 160 controls (21%) were genotyped in our recent GWAS.

HLA-DQB1 Typing

Subjects were typed for *HLA-DQB1* using a novel in-house designed assay based on the multiplexed ligation-dependent probe amplification (MLPA) methodology developed by MRC-Holland[10] (www.mlpa.com). To verify the assay, 36 control DNAs were allelotyped using a DQB1 exon 2 sequencing-based typing technique adapted from van Dijk et al[12] and sequences were compared to MLPA results. Agreement was 100% between both techniques.

Briefly, 50ng of genomic DNA from blood or buccal cells with a mix of probes to discern *HLA-DQB1* alleles (see below) was incubated overnight at 60°C to allow the probes to anneal. The probes were designed such that two would meet directly at a key SNP site. A ligation step then used *Taq* DNA ligase (NEB, Ipswich, MA) to connect any probe pair that matched at the SNP site. Because each probe was designed at a known length and each probe had identical PCR-primer ends, ligated probes were amplified using PCR, and different sized PCR products were identified using capillary fragment analysis on an ABI 3730xl (UC Berkeley DNA Sequencing Facility).

The novel *HLA-DQB1* typing method developed for this study (manuscript submitted) using our probes (for sequences see http://gel.berkeley.edu/pdf/238.pdf) was able to distinguish the following alleles: DQB1*02, DQB1*03, DQB1*04, DQB1*05, DQB1*0601, DQB1*0602, DQB1*0603, DQB1*0604, and DQB1*0609. The following allele

combinations were indistinguishable by our MLPA method: (*0601/*0601) vs. (*0601/*0602); (*0603/*0603) vs. (*0602/*0603); (*0604/*0604) vs. (*0604/*0609); and (*0602/*0604) vs. (*0603/*0604) vs. (*0603/*0609). Sequencing-based typing[12] was used to distinguish these cases.

Statistical Analysis of Allele Associations

For each *HLA-DQB1* allele, a 3×2 contingency table was constructed that contained the number of subjects carrying zero, one, or two copies of the tested allele among the cases and controls. The number of subjects carrying one or two copies of an allele was compared to the number carrying zero copies of that allele (Presence/Absence—Table I). Then the number of subjects carrying two copies of an allele was compared to the number of subjects carrying zero copies of that allele (Homozygous—Table I). The fisher.test function from the stat package in R (http://stat.ethz.ch/R-manual/R-devel/library/stats/html/fisher.test.html) was used to estimate ORs and 95% CIs using conditional maximum likelihood estimation, and to determine the significance of the distribution of the alleles between cases and controls. The *p*-values for the allelotype tests were adjusted using the Bonferroni correction with the p.adjust function from the same package in R.

Haploview (http://www.broadinstitute.org/haploview/haploview) was used to determine LD between previously discovered SNPs and *HLA-DQB1* alleles. Using sequence information from the IMGT/HLA database (http://www.ebi.ac.uk/imgt/hla/), genotype information was inferred for 32 SNPs in exons 2 and 3 of *HLA-DQB1* based on our determined allelotypes. These SNPS can act as unique tags for specific alleles, or groups of alleles. The allele-based SNP genotypes were combined with the corresponding GWAS genotype data (203 cases, 160 controls),[5] including rs10484561 and rs7755224. Haploview was used to analyze this combined data file and determine LD between rs10484561, rs7755224, and allele-specific SNPs of *HLA-DQB1.* We then reported those allele groups in *HLA-DQB1* whose SNPs had the strongest LD with rs10484561/rs7755224.

RESULTS

The population two-digit *HLA-DQB1* allele frequencies are presented in Table I. These two-digit alleles represent major groupings of *DQB1* alleles, which were used to screen for associations. We observed a statistically significant increased risk of FL associated with the *DQB1*05 allele group (OR=1.70, 95% CI 1.28–2.27; adjusted *p*-value=0.013). A strong association with reduced risk for FL also was found with the *DQB1*06 allele group (OR=0.51, 95% CI 0.38–0.69; adjusted *p*-value=4.46×10^{-5}).

Our assay allowed the separation of the *06 group into its component alleles, thus we were able to determine the alleles within the *06 group that contributed to the observed inverse association (Table II). Of the 5 major alleles that comprise the *06 group in Caucasians, *0602, *0603, *0604, and *0609, all were associated with reduced FL risk, whereas the *0601 allele was associated with increased FL risk (OR=2.04, 95%CI=0.72–5.44). However, for *0601, statistical power was limited due to the low frequency of this allele. There was a decreasing trend in ORs associated with each *06 allele carried (1 allele: OR=0.51, 95% CI 0.38–0.69, 2 alleles: OR=0.16, 95% CI=0.04–0.45, Table I). For the *05 risk allele, one allele was associated with a 1.7-fold increased risk of FL (OR=1.70, 95% CI=1.28–2.27) and two alleles with a 2-fold increased risk (OR=2.04, 95%CI=1.00–4.00; Table I).

Measures of LD (Haploview v4.1) were used to verify that our disease-associated alleles were the same genetic signal that was reported previously. In Table III, our findings showed that the risk alleles, rs10484561 and rs7755224, identified in our GWAS[13] were in strongest

LD with SNPs found only in the *HLA-DQB1**05 allele (r^2=0.71). LD also was examined between allele-specific SNPs and SNPs near *HLA-DQB1* including rs9274614, that was inversely related to FL susceptibility (OR=0.57 95%CI=0.40–0.80, trend *p*-value=7.04×10^{-4}) in our earlier study[5]. This SNP showed strong LD with the *0602, *0603, *0604, and *0609 groups (r^2=0.94), suggesting that the observed signal for rs9274614 could be driven by *HLA-DQB1**06 alleles.

DISCUSSION

Here, we present evidence of positive and negative associations between *HLA-DQB1**05 and *06 alleles and risk of FL, respectively. These results confirm our previous findings of an association between FL risk and SNPs in the *HLA-DRB1**0101—*HLA-DQA1**0101—*HLA-DQB1**0501 extended haplotype. A recent study reported that *HLA-DRB1**0101 was associated with increased risk for FL, whereas *HLA-DRB1**13 was inversely associated with FL risk[14]. These alleles are in strong LD with *HLA-DQB1**0501 and *HLA-DQB1**06, respectively. Thus, our results highlight the need for further studies to determine whether *HLA-DQB1* alleles, *HLA-DRB1* alleles, or some other gene variant(s), are causal in the pathogenesis of FL. Due to extended LD in the *HLA* region, a typing study of non-Europeans with different LD patterns will aid in differentiating disease association signals in *HLA-DRB1* from those in *HLA-DQB1*.

HLA alleles associated with disease risk may alter the presentation of specific antigens of autoimmune or infectious origin, and thereby influence the immune response. For example, with type-1 diabetes the significant association between *HLA* class II alleles and disease risk[15] is attributed to differential presentation of insulin by specific *HLA* class II protein isoforms[16,17]. This, in turn, effects T-cell mediated destruction of insulin-secreting pancreatic β-cells. Similar models have been proposed to explain associations of *HLA* alleles with narcolepsy, celiac disease, and rheumatoid arthritis[17]. Although it is possible that FL pathogenesis is initiated by a similar mechanism dependent on a single antigen, a more generalized mechanism may be at play. Comparisons of the strength of associations between risk alleles and disease implicate a unique causal pathway for FL. In the cases of narcolepsy, celiac disease, and rheumatoid arthritis, >90% of patients are carriers of a risk allele[17]. In contrast, 43% of FL cases in this study carried an *HLA-DQB1**05 allele. This implies a more subtle increase in risk per allele, or perhaps heterogeneity in the causal pathways in FL patients.

Changes in specific amino acids in HLA proteins are one reason that different immune systems may respond differently to the same antigen. Polymorphisms in the polypeptide binding groove can affect preference of the polypeptides to be presented to T-cells[18] and thereby influence the immune response. For example, if we compare *HLA-DQB1**0501 and *HLA-DQB1**0602 (http://www.ebi.ac.uk/imgt/hla/), seven amino acid changes are in pockets known to influence antigen binding or interaction with the T-cell receptor[17–19]. If these amino acid changes affect the immune response to an antigen, it could conceivably influence susceptibility to FL via several mechanisms such as chronic immune activation, T-cell receptor stimulation or effects on HLA gene expression on antigen presenting cells.

Chronic immune activation is one proposed mechanism of lymphomagenesis that may explain the association of *HLA* class II alleles with FL risk. B-cell proliferation is dependent on two signals, the first resulting from B-cell receptor interactions with antigen, and the second resulting from HLA class II-bound antigen-peptide interactions with T-cells[20]. This two-part signal leads to clonal proliferation, class switch recombination and somatic hypermutation of B-cells. Chronic immune activation ensues when an autoantigen or a chronic infectious agent repeatedly provides growth signals to B-cells. Because class switch

recombination and somatic hypermutation have been associated with DNA strand breaks, this process is potentially oncogenic and has been proposed as a mechanism of B-cell lymphomagenesis[21]. The extent that a given antigen will cause chronic B-cell stimulation may be modified by the polymorphisms which define HLA class II alleles.

Interactions with regulatory T-cells (T_{regs}) represent a second mechanism in which a differentially presented antigen could affect FL risk. B-cell NHL tumors contain high levels of T_{regs} compared to control tissues, and these T_{regs} suppress proliferation of tumor fighting CD4+ and CD8+ T-cells[22,23]. Recent evidence indicates that T-helper cells can be converted to T_{regs} by malignant FL B-cells in a process involving T-cell receptor stimulation[24]. Because HLA class II molecules interact with the T-cell receptor, this suggests a role for HLA alleles in the generation of T_{regs}. If an antigen, unique to FL, were differentially presented by the *HLA* class II alleles, it could possibly affect T_{regs} levels, thus modifying FL risk. The anti-apoptotic protein, *BCL-2*, which is up-regulated in FL due to the t(14:18) translocation, may be a plausible candidate antigen that is unique for FL. Further studies will be needed to determine the potential role of T_{regs} as a possible causal intermediate between *HLA* class II alleles and FL risk.

FL immune evasion may also be caused by decreased *HLA* class II protein expression. Decreased HLA class II protein expression has been linked to poor survival in DLBCL patients, likely the result of decreased immune surveillance[25]. Additional research will be needed to assess the role of survival and *HLA* class II expression in FL, and the role that HLA allelotypes play on expression of *HLA* class II proteins.

In summary, this paper provides further evidence that *HLA-DQB1**05 is associated with FL risk, and demonstrates a novel inverse association between *HLA-DQB1**06 alleles and FL risk. Currently, it is unclear whether these *HLA-DQB1* alleles are causal or merely markers of association. Here, we propose several mechanisms to support the biological plausibility of an association between *HLA* class II alleles with FL risk. Further genetic studies will be needed that include a large number of participants to provide in-depth coverage of the entire *HLA* class II region to elucidate the role of HLA alleles in the pathogenesis of FL.

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Table I

Odds ratios (OR) and 95% confidence intervals (CI) for two-digit *HLA-DQB1* alleles associated with risk of follicular lymphoma (FL) from a population-based case-control study of non-Hodgkin lymphoma in the San Francisco Bay Area.

	Cases (N= 265) Number of Copies			Controls (N=757) Number of Copies						
Allele	**2**	**1**	**0**	**2**	**1**	**0**	[a] **Presence/Absence** [b] **OR (95% CI)**	[a] **Homozygous OR (95% CI)**	***p*-value**	**Bonf. *p***
*02	18	86	161	40	245	472	1.07 (0.80–1.43)	1.33 (0.72–2.35)	0.621	1.000
*03	34	120	111	87	340	330	1.07 (0.80–1.43)	1.16 (0.73–1.82)	0.787	1.000
*04	1	11	253	0	42	715	0.82 (0.40–1.52)	NA	0.189	1.000
*05	14	101	150	24	211	522	1.70 (1.28–2.27)	2.04 (1.00–4.00)	1.08×10^{-3}	0.013
*06	3	71	191	44	283	430	0.51 (0.38–0.69)	0.16 (0.04–0.45)	3.71×10^{-6}	4.46×10^{-5}

[a] Presence/Absence groups homozygotes and heterozygotes for the allele of interest as one group and compares against carriers of zero copies. Homozygous compares homozygotes for the allele of interest against those carrying zero copies of the allele.

[b] ORs and 95% CIs were estimated by conditional maximum likelihood using the fisher.test function in the stats package of R.

Table II

ORs and 95% CIs for four-digit alleleotypes within the *HLA-DQB1*06* group associated with risk of follicular lymphoma (FL) from a population-based case-control study of non-Hodgkin lymphoma in the San Francisco Bay Area.

	Cases (N=265)		Controls (N=757)				
Allele	**Present**	**Absent**	**Present**	**Absent**	**[a] OR (95% CI)**	***p*-value**	**Bonf. *p***
*0601	7	258	10	747	2.04 (0.72–5.44)	0.208	1.000
*0602	37	228	166	591	0.58 (0.39–0.85)	0.016	0.192
*0603	17	248	91	666	0.51 (0.29–0.85)	0.010	0.125
*0604	10	255	55	702	0.51 (0.24–0.97)	0.136	1.000
*0609	3	262	23	734	0.38 (0.09–1.12)	0.112	1.000
*0602/0603[b]	1	264	11	746	0.29 (0.01–1.52)	0.316	1.000
*0604/0609[b]	0	265	2	755	NA	1.000	1.000

[a] ORs and 95% CIs were estimated by conditional maximum likelihood using the fisher.test function in the stats package of R. For these alleles only presence (1 or 2 copies) vs. absence (0 copies) was compared.

[b] These alleles were left ambiguous by our MLPA based assay (see methods) and failed to provide a clear sequence for unambiguous sequence-based typing.

Table III

Linkage disequilibrium (LD) values[a] between previously published data[4] and alleles of *HLA-DQB1* using overlapping data from 203 FL cases and 160 controls.

GWAS risk SNPs (rs10484561/rs7755224[b])		
Alleles	**D′**	**r^2**
*05	0.96	0.71
*05, *04[c]	0.96	0.60
*05, *0604, *0609	0.96	0.59
GWAS protective SNPs (rs9274614)		
Alleles	**D′**	**r^2**
*0602, *0603, *0604, *0609	0.99	0.94
*0602, *0603	0.99	0.74

[a] Haploview v.4.1 was used to assess D′ and r^2 using the "Linkage Format" function.

[b] rs10484561 and rs7755224 are in high LD in our dataset (D′=1.0, r^2=0.991). Thus the LD values given are similar for either SNP.

[c] As an example, *05, *04 indicates that these LD values were calculated between rs104845651/rs7755224 and a SNP present only in *05 and *04 alleles.