Multiple HLA class I and II associations in classical Hodgkin lymphoma and EBV status defined subgroups

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The pathogenesis of classical Hodgkin lymphoma (cHL) involves environmental and genetic factors. To explore the role of the human leukocyte antigen (HLA) genes, we performed a case-control genotyping study in 338 Dutch cHL patients and more than 5000 controls using a PCR-based sequence-specific oligonucleotide probe hybridization approach. HLA-A68 and HLA-DR11 (5) were significantly increased in the cHL patient population compared with the controls. Three class II associations were observed in the EBV$^-$ cHL population with an increase of HLA-DR15 (2) and a decrease of HLA-DR4 and HLA-DR7. Allele frequencies of HLA-A1, HLA-B37, and HLA-DR10 were significantly increased in the EBV$^+$ cHL population; these alleles are in strong linkage disequilibrium and form a common haplotype in whites. The allele frequency of HLA-A2 was significantly decreased in the EBV$^+$ cHL population. Sequence-specific oligonucleotide probe analysis revealed significant differences between EBV$^+$ and EBV$^-$ cHL patients for 19 probes that discriminate between HLA-A*01 and HLA-A*02. In conclusion, the HLA-A1 and HLA-A2 antigens and not specific single nucleotide variants shared by multiple alleles are responsible for the association with EBV$^+$ cHL. Furthermore, several new protective and predisposing HLA class I and II associations for the EBV$^+$, the EBV$^-$, and the entire cHL population were identified. (*Blood*. 2011;118(19):5211-5217)

Introduction

Classical Hodgkin lymphoma (cHL) is a typical multifactorial disease with both environmental and genetic factors acting together to cause disease.[1,2] Epidemiologic studies reporting familial clustering of cHL[3] and racial variation in the incidence of cHL[4] give substantial support for an inherited risk to cHL. Genetic associations with specific HLA alleles have been reported in both sporadic and familial cHL.[5-7]

EBV is a well-established causal factor in a subset of cHL patients.[8] The expression pattern of EBV genes in Hodgkin Reed-Sternberg cells is restricted to the 2 latent membrane proteins (LMP1 and LMP2) and the EBV nuclear antigen 1 (EBNA1).[8] Despite the lack of immunodominant EBV proteins, LMP and EBNA1-specific T-cell responses can be efficiently induced in the context of specific HLA class I or class II molecules.[9-11] The extreme diversity of HLA genes influences both the affinity and specificity of antigenic peptide binding and is responsible for variations in host antiviral immune defenses. Genetic variation in host antiviral immune responses related to HLA polymorphisms might be an important contributor to the development of virally induced malignancies.

Initial HLA association studies in cHL were performed without taking EBV status into account, and associations of HLA-A1, HLA-B5, HLA-B8, and HLA-B18 with cHL have been described, although the degree of reproducibility has been low.[6,12-14] More recently, we focused on the EBV$^+$ cHL subgroup in a genetic screening study of the entire HLA region.[5] In a subsequent fine-screening analysis, we found a strong association of specific HLA-A alleles with susceptibility to EBV$^+$ cHL in Dutch and English patients. HLA-A1 was associated with an increased risk for EBV$^+$ cHL, whereas the HLA-A2 was associated with a decreased risk for EBV$^+$ cHL.[15,16] This association was confirmed in 934 Scandinavian and English cHL patients in a study by Hjalgrim et al.[17] In a recently performed genome-wide association study (GWAS) in cHL patients, the most significantly associated SNP (rs6903608) was located within the HLA class II region.[18]

In the present study, we performed an extensive screening of the HLA class I and II genes to investigate possible associations of HLA alleles in the total, the EBV$^+$, and the EBV$^-$ cHL (sub)populations. Furthermore, we intended to establish whether specific single nucleotide polymorphism (SNP) positions in the HLA genes that might be shared by multiple HLA alleles are responsible for the observed genetic associations rather than specific HLA alleles.

Methods

Patients and controls

A total of 183 cHL patients from the northern region of The Netherlands who participated in the previous population-based genotyping study[5] were included in the present study. A total of 155 additional patients diagnosed and/or treated between 2000 and 2010 at the University Medical Center

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Table 1. Distribution of sex, histologic subtype, and age in cHL population stratified by EBV status

	No. of patients	EBV$^+$, %
Sex		
Male	177	36
Female	161	13
Histologic subtype		
NS	256	19
MC	29	64
LR	10	30
NOS	43	31
Median age, y (range)	35 (13-81)	

NS indicates nodular sclerosis; MC, mixed cellularity; LR, lymphocyte rich; and NOS, not otherwise specified.

Groningen in the northern region of The Netherlands were also included. Data on gender, age, and histopathologic diagnosis were available for all these patients. No data were available on family history. Classification according to the WHO was performed consistently with the previous study, and cHL cases that could not be unequivocally subtyped (usually because of little tissue) were classified as cHL not otherwise specified (Table 1). The presence of EBV in tumor cells was detected in formalin-fixed paraffin-embedded tissue sections by in situ hybridization with a fluorescein-conjugated PNA probe specific for the EBV-encoded EBER RNAs (Dako Denmark). The control group consisted of blood bank donors from the same geographic region typed for HLA-A (n = 7099), HLA-B (n = 7283), and HLA-DR (n = 5922) by serologic methods and, in case of unclear results or apparent class II homozygosity, additionally by DNA-based methods. All patients gave written informed consent in accordance with the Declaration of Helsinki, and the protocols were approved by the medical ethics board of the University Medical Center, Groningen.

HLA genotyping

Blood samples of cHL patients were collected, and genomic DNA was extracted from the peripheral blood mononuclear cell pellets using standard laboratory protocols. The HLA genotype was analyzed at medium resolution by a PCR-based sequence-specific oligonucleotide probe hybridization (SSOP) approach using commercial kits (Gen-Probe) and Luminex xMAP technology (Luminex Corp). The assays were performed according to the manufacturer's instructions in a European Federation for Immunogenetics accredited laboratory. Briefly, biotin-labeled amplification products were generated for exon 2 (for HLA-DRB and HLA-DPB1) and exon 3 (for HLA-A, HLA-B, HLA-Cw, and HLA-DQB1) of the HLA genes, followed by a hybridization reaction with a series of SSOPs. Of the 401 PCR-SSOP probes, 43 were either positive or negative for all samples and were therefore excluded from the analyses. Of the 358 included probes, 63 were for the HLA-A locus, 83 for the HLA-B locus, 53 for the HLA-C locus, 72 for the HLA-DRB locus, 41 for the HLA-DQB1 locus, and 46 for the HLA-DPB1 locus. Each probe covered 1 to 3 SNP positions. The HLA alleles were defined by specific hybridization patterns of multiple probes. HLA genotype was ascertained according to the manufacturer's instructions using the manufacturer's software and additionally by the SCORE 4.0204F software,[19] enabling the exclusion of probes with borderline hybridization signals. The presumed antigen or T-cell receptor binding function of the SNPs was identified according to Bjorkman and Parham.[20]

HLA allele frequencies of the cHL patients were deducted from the PCR-SSOP genotyping data based on the nomenclature for factors of the HLA system (http://hla.alleles.org/nomenclature/naming.html). Split antigens were used as indicated in the tables. In case of ambiguous results, the allele combination only consisting of common and well-documented alleles[21] were used. For HLA-DPB1, 1 ambiguity remained (DPB1*04:02/105:01), which was analyzed as 1 group of alleles.

Haplotype analyses of rs6903608 and HLA typings

Our patient cohort was used in the replication series of the recently published GWAS, which identified the HLA class II SNP rs6903608 as a highly significant susceptibility marker for cHL.[18] We now compared the SNP alleles (C and T) with the HLA typing data to investigate their linkage. Combined HLA typing and SNP data were available for 278 cHL patients. Two-marker haplotypes of the SNP rs6903608 and the HLA typing were constructed per gene using PHASE Version 2.1.[22,23]

Statistical analysis

For each person, PCR-SSOP association with EBV status was tested by logistic regression in PLINK Version 1.07[24] (http://pngu.mgh.harvard.edu/purcell/plink/) with EBV status as a dependent variable, SSOP as an independent variable, and age and histopathologic diagnosis of cHL as confounding covariates. Linkage disequilibrium (LD) between the SSOPs was determined using the measures D' and r^2. The first measure reflects the evolutionary history of the SSOP pairs in which a D' of 1 implies no recombination between the 2 SSOPs. The latter measure is the correlation between SSOPs and reflects whether SSOP associations are independent of each other (higher r^2 means less independence).

Allele frequencies of HLA-A, HLA-B, and HLA-DR of the total cHL group, the EBV$^+$ subgroup, and the EBV$^-$ subgroup were compared with allele frequencies of the controls, and significant differences were assessed by χ^2 tests. Allele frequencies for HLA-C, HLA-DP, and HLA-DQ of the controls were not available. Significant differences in allele frequencies between EBV$^+$ and EBV$^-$ cHL patients were also assessed by χ^2 tests. Alleles with a frequency $< 1\%$ in our population were excluded for all these analyses.

For each phenotype and PCR-SSOP, a test was performed; hence, a correction for multiple testing was required. Because LD exists between the PCR-SSOPs and between the HLA phenotypes, and because the HLA phenotypes are derived from the PCR-SSOPs, a Bonferroni test for 401 PCR-SSOPs and 74 phenotypes would be too conservative. Therefore, we considered P values $< .001$ to be significant at a level of 5% and P values $< .003$ to be suggestive for association.

Frequencies of HLA typing among haplotypes with a C allele and a T allele at rs6903608 were compared per HLA typing using a Student t test.

Results

Clinicopathologic characteristics

Characteristics of the patient population in terms of age, sex, and histopathologic subtype in relation to EBV status are summarized in Table 1. EBV was present in the tumor cells in 78 (25%) of the 311 cHL patients (for 27 patients, EBV status was unknown). In the total group, median age at diagnosis was 35 years (range, 13-81 years) and the percentage of males was 52%. Age at time of diagnosis was similar in the EBV$^+$ and EBV$^-$ groups: 37 years (range, 17-70 years) in EBV$^+$ and 32 years (range, 13-81 years) in EBV$^-$. Forty-one of 338 cHL patients could not be subtyped and were designated cHL not otherwise specified. In the remaining patients, nodular sclerosis (NS) was the most common subtype accounting for 87%. Mixed cellularity and lymphocyte-rich subtypes were less common with frequencies of 10% and 3%, respectively. The lymphocyte depletion subtype was absent in this patient group. These characteristics are largely consistent with those published for white cHL populations.

Allele frequency differences compared with controls

An overview of all allele frequency data, odds ratios, and P values is given in supplemental Table 4 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). HLA phenotype frequencies (frequencies of allele carriers), including odds ratios and P values, are given in supplemental Table 4. This paper focuses on the HLA allele frequency analysis. Overall,

Table 2. HLA allele frequencies of HLA-A, -B, and -DR alleles with a (nearly) significant difference between controls and cHL patients

	Controls		cHL patients		Controls vs cHL (*P*)
	n	%	n	%	
HLA-A68(28)	124	0.9	34	5.0	< .000001*
HLA-B51(5)	745	5.1	52	8.0	.0012†
HLA-B60(40)	1037	7.1	26	4.0	.0023†
HLA-DR4	2146	18.1	85	12.6	.00031*
HLA-DR7	1320	11.1	35	5.2	< .00001*
HLA-DR11(5)	87	0.7	77	11.5	< .000001
HLA-DR15(2)	1690	14.3	142	21.1	< .000001

*Significant difference ($P < .001$).
†Suggestive difference ($P < .003$).

the results were similar to the HLA phenotype frequency analysis (supplemental Tables 1-3; supplemental Figure 1).

In the cHL patient group, the allele frequency of HLA-A68(28), HLA-DR11(5), and HLA-DR15(2) was significantly increased compared with the controls (5.0% vs 0.9%, $P < .000001$; 12% vs 0.7%, $P < .000001$; and 21% vs 14%, $P = .000001$; respectively). In contrast, HLA-DR4 and HLA-DR7 allele frequencies were significantly decreased (13% vs 18%, $P = .00031$; and 5.2% vs 11%, $P < .00001$; respectively; Table 2; Figure 1).

In EBV$^-$ cHL patients, a significantly decreased allele frequency was found for HLA-DR4 and HLA-DR7 (12% vs 18%, $P = .00063$; 5.6% vs 11%, $P = .00019$) compared with the controls and a significantly increased frequency for HLA-A68(28) HLA-DR11(5) and HLA-DR15(2) compared with controls (5.6% vs 0.9%, $P < .000001$; 13% vs 0.7%, $P < .000001$; 23% vs 14%, $P < .000001$; Table 3; Figure 1).

For the EBV$^+$ cHL subgroup, strong associations were observed for HLA-A1, HLA-A2, HLA-A68(28), HLA-B37, HLA-DR10, and HLA-DR11(5) (Table 3; Figure 1). The HLA-A1 frequency was increased compared with the controls (33% vs 19%, $P < .00001$), whereas the HLA-A2 frequency was decreased (16% vs 34%, $P < .00001$) in EBV$^+$ cHL. For HLA-A68(28) (3.2 vs 0.9, $P = .0021$), HLA-B37 (8.4% vs 1.9%, $P < .000001$), and HLA-DR10 (3.8% vs 1.0%, $P = .00047$), an increased frequency was observed in the EBV$^+$ cHL patients compared with the controls. For HLA-DR11(5), the allele frequency was increased in the EBV$^+$ cHL population compared with the controls (4.5% vs 0.7%, $P < .000001$).

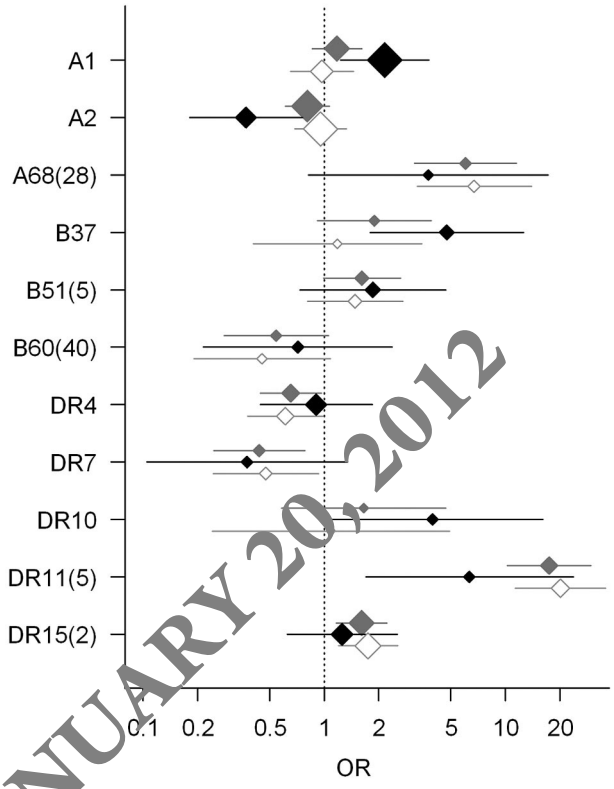

Figure 1. Odds ratios and 99.9% confidence intervals of the genotype allele frequencies. The graph shows the (nearly) significant differences between the controls and the total cHL patient group (gray), the EBV$^-$ (white), or the EBV$^+$ (black) subgroup of patients. The size of the diamonds reflects the allele frequency.

Differences in HLA allele frequencies between EBV$^+$ and EBV$^-$ cHL patients

Comparison of the HLA allele frequencies revealed 4 significant differences between the EBV$^+$ and EBV$^-$ cHL subgroups (see supplemental Table 4 for the phenotype frequencies). The HLA-A1 frequency was significantly increased (33% vs 13%, $P < .0001$), and the HLA-A2 frequency was significantly decreased (16% vs 33%, $P < .0001$). In addition, we observed a significantly increased frequency for HLA-B37 (8.4% vs 2.2%, $P = .00055$) and HLA-Cw6 (14% vs 5.9%, $P = .0022$) in EBV$^+$ cHL patients versus EBV$^-$ cHL patients (Table 4).

Table 3. Allele frequencies of HLA-A, HLA-B, and HLA-DR alleles with (nearly) significant difference between controls and EBV$^+$ or EBV$^-$ cHL subgroups

	Controls		EBV$^+$ cHL		EBV$^-$ cHL		Controls vs EBV$^+$ (*P*)	Controls vs EBV$^-$ (*P*)
	n	%	n	%	n	%		
HLA-A1	2667	18.8	52	33.3	85	13.0	< .00001†	NS
HLA-A2	4828	34.0	25	16.0	153	33.0	< .00001†	NS
HLA-A68(28)	124	0.9	5	3.2	26	5.6	.0021†	< .000001†
HLA-B37	277	1.9	13	8.4	10	2.2	< .000001†	NS
HLA-B51(5)*	745	5.1	14	7.4	33	9.1	NS	NS
HLA-B60(40)	1037	7.1	8	5.2	15	3.4	NS	.0022‡
HLA-DR4	2146	18.1	26	16.7	55	11.9	NS	.00063†
HLA-DR7	1320	11.1	7	4.5	26	5.6	NS	.00019†
HLA-DR10	118	1.0	6	3.8	5	1.1	.00047†	NS
HLA-DR11(5)	87	0.7	7	4.5	60	13.0	< .000001†	< .000001†
HLA-DR15(2)	1690	14.3	27	17.3	104	22.5	NS	.000001†

NS indicates not significant.
*HLA-B51 is included because it had significantly different frequencies in the total group of cases compared with controls (Table 2).
†Significant difference ($P < .001$).
‡Suggestive difference ($P < .003$).

Table 4. HLA allele frequencies with a (nearly) significant difference between EBV+ and EBV− cHL patients

	EBV+ cHL		EBV− cHL		
	n	%	n	%	*P*
HLA-A1	52	33.3	85	13.0	< .0001*
HLA-A2	25	16.0	153	33.0	< .0001*
HLA-B37	13	8.4	10	2.2	.00055*
HLA-Cw6	21	13.6	27	5.9	.0022†

*Significant difference ($P < .001$).
†Suggestive difference ($P < .003$).

Association analysis of individual PCR-SSOPs in EBV+ and EBV− cHL patients

Analysis of each of the 358 PCR-SSOPs revealed a significant difference between EBV− and EBV+ cHL patients for 19 HLA-A PCR-SSOPs (Figure 2; supplemental Table 1). Ten of the PCR-SSOPs were specific for the *HLA-A*01* allele and were more common in EBV+ cHL patients, whereas the other 9 PCR-SSOPs specific for the *HLA-A*02* allele were less common in this patient group (Table 2). Interestingly, the most significant SSOP (C295) is not strongly correlated with any of the other SSOPs ($r^2 < 0.4$ except for pair C295-C331 [$r^2 = 0.70$]; supplemental Figure 2). This implies that the association of C331 is probably caused by LD with C295. These probes were the only ones that were specific for *A*23* and *A*24* next to *HLA-A*01*. In addition, we observed a second independent association for the SSOPs C395 and C211, which were specific for *HLA-A*03* and *HLA-A*11*. A third independent association was shown for the SSOPs C378, C325, C375, C281, and C215. Specificity of these SSOPs is restricted to *HLA-A*01*. The C332 SSOP showed an intermediate association. The *HLA-A*02* specific SSOPs are strongly correlated with each other (most pairs have $r^2 > 0.8$; for all pairs, $r^2 > 0.3$); hence, there is probably only one signal that is causing these associations. No LD is observed between the *HLA-A*01* and the *HLA-A*02* specific SNPs.

The 19 SSOPs with significant differences contained polymorphic residues within 8 of the *HLA-A*02*-specific and 10 of the *HLA-A*01*-specific SSOPs that are located at key positions in the peptide binding pockets of the HLA molecules[20] (Table 5). PCR-SSOPs that were less specific for the *HLA-A*01* or *HLA-A*02* alleles showed a similar trend in the odds ratio as the significant PCR-SSOPs but with lower *P* values (supplemental Table 4).

Analysis of the NS and the non-NS subgroups separately for the most significant PCR-SSOPs revealed trends similar to the total group, albeit with lower *P* values. This is caused by the smaller group sizes. Remarkably, the odds ratios in the non-NS group were more pronounced (0.21-0.23 for the *HLA-A*02* SSOPs and 4.74-8.06 for *HLA-A*01* SSOPs) than the odds ratios in the NS subgroup (0.36-0.44 for *HLA-A*02* SSOPs and 2.31-3.49 for the *HLA-A*01* SSOPs). Nevertheless, there was no significant difference between the NS and the non-NS subgroups for these SSOPs (*P* values for interaction effects of SSOP by subtype on EBV status were all > .05).

Association of rs6903608 alleles with HLA alleles

Association of the rs6903608 alleles with the HLA typing revealed 16 significant associations (Table 6; supplemental Figure 2). Five of these associations were in agreement with the HLA allele frequency results: HLA-A1, HLA-DR4, HLA-DR7, HLA-DR11(5), and HLA-DR15(2). The T allele was significantly associated with HLA-A1, HLA-DR4, and HLA-DR7 alleles (28% vs 13%, $P < .00001$; 28% vs 0%, $P < .000001$; and 9% vs 0%, $P < .000001$). The C allele was significantly associated with HLA-DR11 and HLA-DR15 (24% vs 1%, $P < .000001$; and 47% vs 1%, $P < .000001$). Six SNP/HLA allele associations (ie, HLA-B7, HLA-B8, HLA-Cw15, HLA-DR1, HLA-DR3, and HLA-DR8) were not significantly associated with cHL in the HLA allele frequency analysis. The 5 SNP/HLA-DQB associations have not been analyzed in the HLA allele frequency association analysis because of lack of data from the control population.

Discussion

Inherited variations within the HLA genes as well as variations in host immune responses have long been recognized to be associated with susceptibility to disease, including cHL.[25] A number of HLA genes, alleles, and serotypes have previously been reported to be involved in the pathogenesis of cHL,[5-7] and a recent GWAS indicated the most significant association for cHL to be within the HLA locus.[18] In this study, we observed multiple associations that are specific for the EBV+, the EBV−, or the total cHL (sub)populations. HLA-A68(28) and HLA-DR11(5) are risk alleles for cHL irrespective of the EBV status. HLA-DR4 and HLA-DR7 are associated with decreased susceptibility and HLA-DR15(2) with an increased susceptibility in the EBV− cHL groups. In the EBV+ cHL population, an increased susceptibility was observed for HLA-A1, HLA-B37, and HLA-DR10, whereas resistance to disease development was observed for HLA-A2.

GWAS indicated that SNP rs6903608 was strongly associated with cHL.[18] To explore associations of the T and C allele with certain HLA alleles, we compared the SNP data of our cohort to the HLA typing data and observed a significant LD for 10 HLA alleles (Table 6). Five of the 16 HLA alleles (HLA-A1, HLA-DR4, HLA-DR7, HLA-DR11[5], and HLA-DR15[2]) were also significantly associated with cHL in both the HLA allele and phenotype frequency analysis. HLA-A1 and HLA-DR15 were specifically

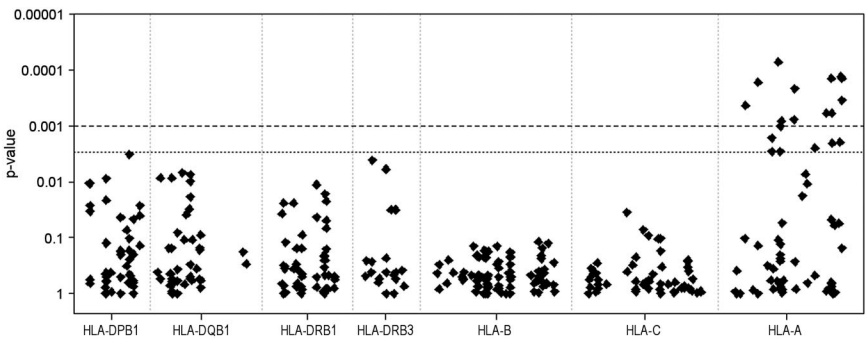

Figure 2. Genetic association of individual PCR-SSOP in EBV+ cHL. The *P* value of each SSOP for differences in frequencies between EBV+ and EBV− cHL cases is plotted on the y-axis. Genes are ordered according to their relative positions on the short arm of chromosome 6 (6p-telomere to 6p-centromere). Strong associations with EBV status were present only for part of the HLA-A PCR-SSOPs.

Table 5. Overview of PCR-SSOPs with significant differences between EBV+ and EBV− cHL population

SSOP	Specificity of probe*	AA positions (IMGT)	Potential contact position†	EBV+, %	EBV−, %	*P*	OR
c279	*A*02*	9-12,23-25	Peptide (9,24)	23.0	50.0	.00043‡	0.32
c348	*A*02,A68*	141-142, 144-146	Peptide (146), TCR (145,146)	34.6	60.3	.0006‡	0.37
c373	*A*02,A*68*	143-146, 150-153	Peptide (143,152),TCR (145,146,150,151)	34.6	60.3	.0006‡	0.37
c349	*A*02*	94-98	Peptide (95,97)	26.9	53.0	.00076‡	0.36
c273	*A*02,A*31*	69-72, 76-79	Peptide (70,77),TCR (69,72,76,79)	31.6	58.2	.00081‡	0.37
c339	*A*02,A*24,A*68*	150-154	Peptide (152),TCR (150,151,154)	48.7	71.6	.002§	0.41
c397	*A02,A*23,A*24,A*68*	124-128		51.3	72.8	.0025§	0.41
c208	*A*02*	60-65	Peptide (62,63), TCR (61,62,65)	29.5	53.0	.0029§	0.41
c241	*A*02*	70-71, 73-74, 76	Peptide (70,73 74), TCR (76)	29.5	53.0	.0029§	0.41
c295	*A*01,A*23,A*24,A*26, A*30, A*32*	70-74	Peptide (70,73,74), TCR (72)	80.8	53.9	.00007‡	3.79
c378	*A*01*	156, 158, 166-168	Peptide (156,167),TCR (158,166,167)	56.4	29.9	.00013‡	3.05
c325	*A*01*	150-152	Peptide (152)TCR (150,151)	56.4	30.2	.00014‡	3.03
c375	*A*01*	161-163, 166-168	Peptide (163,167),TCR (162,163,166,167)	56.4	30.2	.00014‡	3.03
c281	*A*01*	41-44		55.8	29.7	.00017‡	3.02
c395	*A*01,A*03,A*11,A*30*	93-98	Peptide (95,97)	84.6	61.2	.00021‡	3.79
c331	*A*01,A*23,A*24*	164, 166-168	Peptide (167), TCR (166,167)	71.8	47.9	.00034‡	2.96
c215	*A*01,A*26,A*29*	74-77	Peptide (74,77), TCR (76)	59.0	35.5	.001§	2.58
c211	*A*01,A*03,A*11,A*30,A*31,A*32*	59-60, 62-64	Peptide (59,62,63), TCR (62)	87.2	68.0	.0016§	3.36
c332	*A*01,A*11,A*26*	160-163, 165	Peptide (163), TCR (162,163)	66.1	45.9	.002§	2.49

TCR indicates T-cell receptor.
*Only CWD alleles were included.
†Contact position according to Bjorkman and Parham (1990).[20]
‡Significant difference ($P < .001$)
§Suggestive difference ($P < .003$)

associated with EBV+ cHL, whereas HLA-DR4 and HLA-DR7 were associated with EBV− cHL and HLA-DR11 with both EBV− and EBV+ cHL. The strong association of the SNP with cHL is probably caused by its strong linkage with these 5 HLA alleles, whereas the observed linkage with HLA-B7, HLA-B8, HLA-Cw15, HLA-DR1, HLA-DR3, and HLA-DR8 does not seem to have an impact on cHL susceptibility. For the HLA-DQB alleles, we cannot draw any conclusions because we do not have HLA typing data of the control population for this gene. Thus, by applying HLA typing, we were able to identify the HLA genes and alleles that underlie the strong genetic association identified in a recent GWAS.[18]

The previously reported association of the HLA-A gene with EBV+ cHL[5,15,16] was confirmed compared with both EBV− cHL and healthy controls with an increased risk for *HLA-A*01* and a reduced risk for *HLA-A*02*. A tentative explanation for this association is the known presence of multiple cytotoxic T-cell epitopes for LMP2-derived antigenic peptides restricted through *HLA-A*02*,[9] whereas *HLA-A*01*-restricted epitopes to the latent EBV peptides have not been found.[26] Analysis of the individual PCR-SSOPs revealed a significant difference for 19 of the 358 probes in EBV+ compared with EBV− cHL. These probes discriminated between the *HLA-A*01* and *HLA-A*02* alleles and showed only a limited number of cross-reacting other alleles. The other, less significant, *HLA-A*01* and *HLA-A*02* identifying probes showed a similar risk pattern. The less significant level of these probes can be explained by their broader specificities, including some common HLA-A alleles. For example, several probes share specificity for both *HLA-A*1* and *HLA-A*11*. *HLA-A*11* was previously reported to be a protective allele in EBV-associated undifferentiated nasopharyngeal carcinoma in both endemic and nonendemic regions.[27,28] Undifferentiated nasopharyngeal carcinoma has the same EBV latency pattern as cHL with expression of only LMPs and EBNA1, and it was suggested that *HLA-A*11* can efficiently present antigenic peptides from these proteins.[28,29] Therefore, specificity of a probe for both *HLA-A*11* and *HLA-A*01* might diminish the strength of its association with EBV+ cHL.

Overall, our genotype analysis showed no evidence that individual SNPs detected by the PCR-SSOPs are important for the *HLA-A*02* association, indicating that the complete *HLA-A*02* allele is important for this association. For HLA-A*01, 3 clear independent signals were observed, representing different allele specificities in addition to the *HLA-A*01* allele: *A*23/A*24* for C295 and C331, *A*03/A*11* for C395 and C211, and no additional specificities for 4 of the 5 SSOPs of the third signal. However, the frequencies of these 5 HLA-A alleles were similar between EBV− and EBV+ cases. Nevertheless, it might indicate that SNPs at the positions that overlap between these 5 HLA-A alleles are important with binding

Table 6. Association of the rs6903608 SNP alleles with HLA alleles in 278 cHL patients

HLA allele	C-allele*	T-allele*	*P*
HLA-A	n = 244	n = 310	
1	13%	28%	< .00001
HLA-B	n = 244	n = 307	
7	34%	7%	.000001
8	8%	21%	.000015
HLA-Cw	n = 238	n = 302	
15	52%	31%	.00001
HLA-DR	n = 244	n = 310	
1	0%	18%	.000001
3	5%	21%	.000001
4	0%	22%	.000001
7	0%	9%	.000001
8	0%	6%	.00001
11	24%	1%	.000001
15	47%	1%	.000001
HLA-DQB	n = 243	n = 307	
2	4%	26%	.000001
4	0%	5%	.00001
6	62%	15%	.000001
8(3)	0%	14%	.000001
9(3)	0%	5%	.00001

*The total number of alleles is 556.

EBV-derived peptides and for defining the immune responses against EBV.

The strong LD observed in the HLA region might affect the results of our HLA association studies. The HLA-A1 antigen is known to be in strong LD with HLA-B8; and in contrast to HLA-A1, HLA-B8 is capable of presenting EBV-derived peptides.[30] We observed that, in the EBV$^+$ cHL population, 53% (23 of 43) of the HLA-A1$^+$ patients also possessed the HLA-B8 allele, whereas in the EBV$^-$ cHL population, this percentage was 66% (46 of 70). In theory, the presence of HLA-B8 in HLA-A1 carrying EBV$^+$ cHL might overcome the less effective presentation of EBV-derived peptides by HLA-A1. Nevertheless, we found a very significant effect for HLA-A1 in this study consistent with previous studies[5,15,16] and no significant differences for HLA-B8.

The predisposing effects of HLA-B37 and HLA-DR10 for EBV$^+$ cHL might at least be partly attributed to their strong LD with HLA-A1 because HLA-A1-B37-Cw6-DR10-DQ5 is a rather common haplotype in the white population. In our study, 13 of 14 HLA-B37$^+$ and 5 of 6 HLA-DR10$^+$ EBV$^+$ cHL patients also carried HLA-A1. Although we do not have haplotype data available, 7 patients were found to potentially possess the HLA-A1-B37-DR10 haplotype, in which 5 were EBV$^+$.

Two associations (HLA-A68[28] and HLA-DR11[5]) were present in both the EBV$^-$ and EBV$^+$ cHL subgroups and are thus specific for the total cHL group irrespective of the EBV status. These associations have not been reported previously. An association with HLA-DR5 has been reported previously by Robertson et al in familial cHL.[31] Significant effects of HLA-A1, HLA-B8, and HLA-B18 for cHL that have been reported in previous publications[12-14] could not be confirmed in the total patient group in our study, whereas the previously reported association with HLA-B*29 was borderline significant in the overall cHL group in our study. These differences might be explained by differences in sample size, patient selection, and proportion of EBV$^+$ cases. The previous studies were carried out in relatively small numbers of patients ranging from 11 to 137 within different countries, such as Egypt, Czech Republic, Sweden, Denmark, and the United States. Distribution of HLA alleles varies widely by ethnicity and geography, which might lead to differences in disease-associated HLA alleles among different ethnic groups and geographic locations. In addition, most case-control studies used low-resolution serologic HLA typing. A limitation of this approach is the inability to discriminate between specific allele variants, which might be more or less common in different populations.

The HLA-DR5, HLA-DR7, and HLA-DR15(2) class II associations were not observed in the EBV$^+$ subgroup and represent associations specific for the EBV$^-$ cHL subgroup. An association with *DRB1*15:01* has been reported previously to be associated with familial NS subtype cHL.[7] HLA-DR15(2) is the serologic name for the *HLA-DRB1–*15:01* typing, and this association is thus similar to the association that we found to be specific for EBV$^-$ cHL.

The HLA-A1 and HLA-A2 class I associations were specific for the EBV$^+$ cHL subgroup, whereas the HLA class II associations were most pronounced in the EBV$^-$ cHL subgroup. HLA class II molecules present exogenous antigenic peptides to CD4$^+$ helper T cells, whereas HLA class I molecules bind and present peptides derived from endogenous proteins to CD8$^+$ cytotoxic T lymphocytes.[32] Our findings thus support a differential immunogenic basis with a more prominent role in the effector phase of the immune response for HLA class I in EBV$^+$ cHL and an immunoregulatory role for HLA class II in the pathogenesis of EBV$^-$ cHL. This suggests the presence of a specific HLA class II-restricted antigen involved in the pathogenesis of EBV$^-$ cHL. Whether this is a pathogen-derived antigen or an antigen derived from a mutated protein remains unknown.

In conclusion, the current study confirms the previously reported genetic association of the HLA-A1 and HLA-A2 in EBV$^+$ cHL. The genetic influence of these 2 HLA antigens is more important than individual SNPs that might be shared by multiple HLA-A alleles. In addition, we demonstrated 2 significant associations (HLA-A68 and HLA-DR11[5]) for the cHL population, 3 significant HLA class II associations (HLA-DR5, HLA-DR7, and HLA-DR15[2]) specific for the EBV$^-$ cHL population, and one additional association (HLA-DR10) for the EBV$^+$ cHL subgroup. This implies an influence of the interaction between environmental and genetic risk factors in the development of cHL and supports heterogeneity in the genetic predisposition to EBV$^+$ and EBV$^-$ cHL.

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Authorship

Contribution: H.X., W.K., I.B., N.K., R.V., and L.V. performed experiments; L.V., G.v.I., A.D., and A.v.d.B. performed patient collection; K.K. and I.N. analyzed data; I.N., B.O., and R.S.H. conducted SNP analysis and linkage to HLA typing; A.D. and S.P. reclassified HL cases; B.H. conducted HLA typing analysis; A.v.d.B., B.H., and A.D. designed the study; and all authors supervised writing of the manuscript.

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