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ORIGINAL ARTICLE

Polymorphisms and haplotypes in *TLR9* and *MYD88* are associated with the development of Hodgkin's lymphoma: a candidate–gene association study

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Toll-like receptors (TLRs) and myeloid differentiation primary response protein 88 (MYD88) gene polymorphisms may be involved in the pathogenesis of Hodgkin's lymphoma (HL) through altered immunoregulatory and inflammatory responses. A candidate–gene association study was conducted to investigate the association between *TLR9* −1237T>C, *TLR9* 2848A>G, *MYD88* −938C>A and *MYD88* 1944C>G gene polymorphisms and the risk for HL. The impact of haplotypes was also examined. The study showed that carriership for −1237C and 2848A was associated with an increased risk for HL (odds ratio (OR)=2.53 (1.36–4.71) and OR=6.20 (1.3–28.8)). The MYD88 polymorphisms produced nonsignificant results. The estimated frequencies of the TLR9/1237C-2848A and MYD88/938C-1944G haplotypes were also significantly different between HL and controls ($P<0.01$). In addition, a significant difference between HL and controls was observed for the TLR9/1237C-TLR9/2848A-MYD88/938C-MYD88/1944C haplotypes ($P<0.01$). In conclusion, our study showed that TLR polymorphisms, and TLR9 and MYD88 haplotypes are related to the development of HL.
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Keywords: gene; Hodgkin's lymphoma; HL; MYD88; polymorphism; TLR9

INTRODUCTION

Hodgkin's lymphoma (HL) is an uncommon neoplasm with an age-adjusted incidence of 2.5–3.1 per 10E5 in the Western world.[1,2] Involved lymph nodes show an inflammatory cellular background and rare Reed Sternberg (RS) cells, which are the malignant cells of HL. The pathogenesis of HL remains unclear. A transcription factor that has been implicated in the pathogenesis of HL is nuclear factor-κB (NF-κB), a pleiotropic mediator of inflammation. Strong constitutive NF-κB activity is a common feature of HL cell lines[3] and is required for the proliferation and survival of RS cells.[3] However, the cause of NF-kB triggering is still elusive, but there is evidence that it may be linked to the Epstein Bar Virus (EBV), a herpes virus that has been associated with HL.[4–9] In addition, one of the EBV proteins, the Latent Membrane Protein 1 (LMP1), activates NF-κB, stimulates cell proliferation and prevents apoptosis.[10]

Family studies have shown that inherited factors may also have a role in the development of HL; individuals related to HL patients have more than a three-fold increased risk of developing HL, as shown by population[11–15] and twin studies.[16] Factors that are involved in EBV recognition and NF-kB activation are the Toll-like receptors (TLRs). Specifically, TLR9 recognizes viral CpG islands and, once activated, it signals through myeloid differentiation primary response protein 88 (MYD88) and leads to NF-κB activation.[17–19] Considering the importance of TLRs in NF-κB activation, one could hypothesize that these receptors may have a role in the abundant inflammatory background present in HL. To test this hypothesis, we chose to examine whether genetic variations in *TLR9* and *MYD88* are associated with the development of HL. Previous studies in HL[20] showed an odds ratio (OR) of 0.82 for *TLR9*-1237C that was of borderline significance, whereas weak associations were also detected for follicular lymphoma and chronic lymphocytic leukemia.

TLR9 is located on chromosome 3p21.3;[21] it consists of two exons and two single-nucleotide polymorphisms (SNPs) define the four most common haplotypes in Caucasians.[22] These include the −1237T>C (dbSNP rs5743836) and the synonymous 2848A>G (P545P) (dbSNP rs352140) SNP located in exon 2. *TLR9* is in close proximity to the *MYD88* gene, which is located on chromosome 3p22 and consists of five exons.[23] The −938C>A SNP (dbSNP rs4988453)

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and the 1944C>G (dbSNP rs4988457) SNP define the two most common haplotypes in Caucasians.[24] In this analysis, we undertook a gene–candidate association study to investigate whether the above-mentioned polymorphisms contribute to the risk of HL.

MATERIALS AND METHODS

Subjects

A total of 90 formalin-fixed paraffin-embedded tissue sections collected from HL patients were analyzed from the Pathology Laboratories of Hygeia Hospital (Athens, Greece), the Evaggelismos Hospital (Athens, Greece) and from the University Hospital of Larissa (Larissa, Greece). All patients were of Caucasian origin and met the World Health Organization criteria for classic HL. Peripheral blood samples were obtained from 92 healthy subjects from the University Hospital of Larissa (Larissa, Greece), after receiving informed consent. The control subjects were healthy individuals of Caucasian origin, with no known history of HL or any other disorder.

SNP genotyping

Case and control subjects were genotyped for the *TLR9* (GenBank Accession No. NM_017442) and *MYD88* (GenBank Accession No. NM_002468) SNPs of interest. After consecutive attempts, genotyping was successful for 86 HL cases and for 84 control subjects for the *TLR9* −1237T>C SNP, for 90 cases and 90 controls for the *TLR9* 2848A>G SNP, for 90 cases and 87 controls for the *MYD88* −938C>A SNP and for 86 cases and 89 controls for the *MYD88* 1944C>G SNP. PCR was used to amplify DNA fragments encompassing SNPs of interest in a typical reaction containing the 1× PCR buffer, ~30 ng genomic DNA, 1–1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM of each primer and 1.25 Units Go Taq Flexi Polymerase (Promega, Southampton, UK). Samples were denatured for 5 min at 96 °C and subjected to 35 cycles of denaturation at 96 °C for 1 min, annealing at 56–64 °C (depending on the primers used) for 1 min and extension at 72 °C for 1 min, followed by an extension step at 72 °C for 5 min.

The *TLR9* −1237T>C genotypes were determined by amplification of a 456 bp fragment of the 5′-flanking region of *TLR9* using primers 5′-AAG AAG CTG ACA TTC CAG CAG GG-3′ (forward) and 5′-CTA GGT CCC TCC TCT GCT CAG AC-3′ (reverse) at 64 °C annealing, followed by *Bst*NI digestion of the PCR product. Digestion resulted in fragments of 31, 38, 48, 89, 112 and 186 bp in the presence of the T allele, and in fragments of 31, 38, 48, 89, 112 and 138 bp in the presence of the C allele. The *TLR9* 2848A>G genotypes were identified by amplification of a 371 bp fragment of exon 2 using primers 5′-TGG ATC TGC CAC GGA ACA ACC-3′ (forward) and 5′-CAG CGA CGT ACT GCA GAG CTG-3′ (reverse) at 62 °C annealing, followed by *Bst*UI digestion. Digestion of the PCR product resulted in fragments of 226 and 145 bp in the presence of the G allele, and 371 bp in the presence of the A allele. The *MYD88* −938C>A genotypes were determined by PCR amplification of a 503 bp fragment of the 5′-flanking region of *MYD88* using primers 5′-GCA GCC AGG ACC GCT TACT GC T-3′ (forward) and 5′-GCA CGT GGC CTT GCC CTT GCC CTT TAG G-3′ (reverse) at 62 °C annealing, followed by *Bsr*I digestion of the PCR product. The expected fragment sizes after digestion were 23, 97, 165 and 218 bp in the presence of the C allele, and 97, 165 and 241 bp in the presence of the A allele. The *MYD88* 1944C>G genotypes were identified by amplification of a 448 bp fragment of intron 3 using primers 5′-GCC CCA GCG ACA TCC AGT TTG TG-3′ (forward) and 5′-TCT TGC CAG AGC AGG GTT GAG CTT-3′ (reverse) at 56 °C annealing, followed by *Cac8*I digestion. Digestion of the PCR products resulted in fragments of 92, 135 and 221 bp in the presence of the G allele, and in fragments of 135 and 313 bp in the presence of the C allele. At least 10% of the samples were randomly selected as an internal control for repeated genotyping.

Data analysis

The genotypes were tested for Hardy–Weinberg (HW) equilibrium and the loci were tested for linkage disequilibrium using exact tests according to Weir.[25,26] The haplotype frequencies were estimated and compared using PHASE v2.1 (Seattle, WA, USA) (http://www.stat.washington.edu/stephens/). The disequilibrium and correlation coefficients were estimated according to Weir.[26] The genotype distribution, allele contrast and the dominant model (risk allele carriers) of the HL patients was compared with the control group using a log-linear model.[27] The comparisons were expressed in terms of OR unadjusted and adjusted for age and sex. A *P*-value of <0.05 was considered statistically significant.

RESULTS

Demographic characteristics of the study population

A total of 91 cases and 92 controls were analyzed in this study. The mean age (± s.d.) was 42.9 ± 18.5 and 35.5 ± 10.1 years for cases and controls, respectively; there were 44 (50.6%) males and 43 (49.4%) females in the case group, whereas the control group comprised 27 (29.3%) males and 65 (70.7) females. HL samples were of the following histological subtypes: nodular sclerosis (73 cases), mixed cellulary (15 cases) and lymphocyte rich (3 cases). The control subjects were younger and comprised a higher percentage of females than did the cases.

Genotype distributions and study quality

The distributions of the *TLR9* −1237T>C, *TLR9* 2848A>G, MYD88 −938C>A and MYD88 1944C>G genotypes in both cases and controls are shown in Table 1. Significant differences were detected in the distribution of genotypes of the two TLR polymorphisms between cases and controls (P=0.01 for *TLR9* −1237T>C and P=0.03 for *TLR9* 2848A>G). In contrast, there were no differences for the MYD88 polymorphisms (P=0.16 for MYD88 −938C>A and P=0.25 for MYD88 1944C>G). No individuals homozygous for the mutant allele in either cases or controls were present for the MYD88 polymorphisms.

The controls were in HW equilibrium for all investigated polymorphisms, indicating the lack of stratification and/or genotyping error[28] (P=0.11 for *TLR9* −1237T>C, P=0.37 for *TLR9* 2848A>G, P=0.99 for MYD88 −938C>A and P=0.60 for MYD88 1944C>G).

Polymorphisms in *TLR9* and association with HL

Table 2 shows the association results for the *TLR9* polymorphisms. The allele contrast showed that for the −1237C allele there was a two-fold risk for HL relative to the −1237T allele (OR=1.99 (1.18–3.36)). Furthermore, −1237C-carrier individuals were associated with a higher risk of HL unadjusted and adjusted for age and sex (OR=2.53 (1.36–4.71) and OR=2.02 (1.04–3.89), respectively).

With regard to the *TLR9* 2848A>G polymorphism, the allele contrast produced a marginal association (OR=1.54 (1.00–2.39)).

Table 1 Distribution (in %) of the *TLR9* −1237T>C, *TLR9* 2848A>G, *MYD88* −938C>A and *MYD88* 1944C>G genotypes among cases and control subjects

SNPs	*Genotypes*	*Cases (n=90)*	*Controls (n=92)*	P-*value*
TLR9 −1237T>C	TT	43.5	66.7	**0.01**
	TC	55.3	33.3	
	CC	1.2	0	
TLR9 2848 A>G	AA	43.3	33.3	**0.03**
	AG	54.5	54.5	
	GG	2.2	12.2	
MYD88 −938C>A	CC	90	82.8	0.16
	CA	10	17.2	
	AA	0	0	
MYD88 1944C>G	CC	67.1	75.3	0.25
	CG	32.9	24.7	
	GG	0	0	

Abbreviations: HL, Hodgkin's lymphoma; SNP, single-nucleotide polymorphism.
The *P*-values for testing the association between genotype distribution of each SNP and susceptibility to HL are shown.
Bold values emphasize statistical significance.

Table 2 Odds ratio (OR) and the corresponding 95% confidence intervals (CIs) for testing the association between HL and SNPs of the *TLR9* and *MYD88* genes for the allele contrast and the dominant model

SNP	*Genetic contrast*	*OR (95% CIs)*	P-*value*	*OR adjusted (95% CIs)*
TLR9 −1237T>C	C vs T	1.99 (1.18–3.36)	**0.01**	
	Dominant for C	2.53 (1.36–4.71)	<**0.01**	2.02 (1.04–3.89)
TLR9 2848 A>G	A vs G	1.54 (1.00–2.39)	0.06	
	Dominant for A	6.20 (1.33–28.8)	**0.01**	4.66 (1.00–22.9)
MYD88 −938C>A	C vs A	1.77 (0.75–4.16)	0.28	
MYD88 1944C>G	G vs C	1.40 (0.77–2.56)	0.36	
	Dominant for G	1.47 (0.76–2.84)	0.25	1.38 (0.68–2.78)

Abbreviations: HL, Hodgkin's lymphoma; SNP, single-nucleotide polymorphism.
The ORs adjusted for age and sex are also shown.
Bold values emphasize statistical significance.

Table 3 *P*-values/D'/r^2 for testing linkage disequilibrium between pairs of SNPs for HL patients and controls (in brackets)

	TLR9 −1237T>C	*TLR9 2848A>G*	*MYD88 −938C>A*
TLR9 2848A>G	<0.01/0.11/0.01 (0.35/0/0)		
MYD88 −938C>A	0.04/0.02/0 (0.32/0.07/0)	<0.01/0.02/0 (0.63/0.07/0)	
MYD88 1944C>G	0.05/0/0 (0.62/0.02/0)	0.06/0.08/0 (0.78/0.01/0)	0.47/0.78/0.39 (<0.01/0.87/0.63)

Abbreviations: HL, Hodgkin's lymphoma; SNP, single-nucleotide polymorphism.

2848A carriers had an increased risk for HL, as the unadjusted OR was significant (OR=6.20 (1.3–28.8)); however, the adjusted OR was marginally significant (OR=4.66 (1.00–22.9)).

Polymorphisms in the *MYD88* gene and association with HL

Table 2 shows that the MYD88 polymorphisms produced nonsignificant results. The allele contrasts −938C vs A and 1944G vs C were not significant (OR=1.77 (0.75–4.16) and OR=1.40 (0.77–2.56)). In addition, 1944G carriers were not associated with increased risk of HL (unadjusted OR=1.47 (0.76–2.84) and adjusted OR=1.38 (0.68–2.78)).

TLR9 and *MYD88* haplotype frequency in HL patients and control subjects

Table 3 shows the *P*-values, and the respective D' and r^2, for testing linkage disequilibrium between pairs of SNPs for HL patients and controls. In the patient population, *TLR9* −1237T>C was in linkage disequilibrium with *TLR9* 2848A>G ($P<0.01$), *MYD88* −938C>A ($P=0.04$) and *MYD88* 1944C>G ($P=0.05$). *TLR9* 2848A>G was also found in linkage disequilibrium with *MYD88* −938C>A ($P<0.01$) in HL patients. In controls, the two *MYD88* polymorphisms were in linkage disequilibrium ($P<0.01$) (Table 3).

The distribution of the estimated haplotype frequency of the two *TLR9* polymorphisms and the two *MYD88* polymorphisms for the HL and controls is presented in Table 4. Regarding the *TLR9* haplotypes, there was an overall significant difference between the HL and controls ($P=0.03$). This difference was due to the 1237C-2848A haplotype (or the 1237T-2848G haplotype) ($P<0.01$). For the MYD88 haplotypes, the overall difference was not significant ($P=0.20$). However, the estimated frequency of the haplotype 938C-1944G was different in HL and controls; this difference might be due to the linkage disequilibrium with the *TLR9* polymorphisms or because of the interaction between the two polymorphisms, given that the individual *MYD88* polymorphisms were not significant.

When the estimated haplotypes of the four investigated polymorphisms were considered (Table 5), a significant difference between HL and controls was observed for the 1237T-2848G-938C-1944C and the 1237C-2848A-938C-1944C haplotypes ($P<0.01$), further indicating the association of 1237T and 2848G alleles (or allele carriers) with HL susceptibility.

Table 4 Estimated haplotype frequencies for the two *TLR9* SNPs (*TLR9* −1237T>C and *TLR9* 2848A>G) and the two *MYD88* SNPs (*MYD88* −938C>A and *MYD88* 1944C>G)

	Haplotype frequency			
	Cases	*Controls*	P-*value*	P-*value overall*
TLR9 haplotype (5′–3′)				
TG	0.272	0.379	<0.01	0.03
TA	0.443	0.454	0.63	
CG	0.025	0.020	0.83	
CA	0.260	0.147	<0.01	
MYD88 haplotype (5′–3′)				
CC	0.802	0.824	0.09	0.20
CG	0.144	0.088	<0.01	
AC	0.039	0.0529	0.28	
AG	0.0139	0.0353	0.10	

Abbreviation: SNP, single-nucleotide polymorphism.
The *P*-values for comparing each haplotype between cases and controls, and the *P*-values for comparing the difference overall the haplotypes are shown.

DISCUSSION

This study investigated whether certain *TLR9* and *MYD88* gene polymorphisms were associated with the development of HL. Our results showed that the *TLR9* −1237C allele confers a two-fold increased risk of HL and that the *TLR9* 2848A allele increased HL risk by ~4.5-fold in the Caucasian population examined. None of the *MYD88* SNPs analyzed (*MYD88* −938C>A and *MYD88* 1944C>G) were found to alter the susceptibility to HL. The genotype distributions of the SNPs examined were all in HW in the control group, indicating no population stratification. Haplotype analysis also

Table 5 Estimated haplotype frequencies for the four SNPs (*TLR9* −1237T>C, *TLR9* 2848A>G, *MYD88* −938C>A and *MYD88* 1944C>G)

	Haplotype frequency		
TLR9–MYD88 haplotype (5′–3′)[a]	*Cases*	*Controls*	P-*value*
TGCC	0.176	0.290	**<0.01**
TGCG	0.039	0.032	0.63
TGAC	0.015	0.022	0.52
TGAG	0.010	0.024	0.25
TACC	0.397	0.402	0.86
TACG	0.063	0.035	0.11
TAAC	0.009	0.015	0.54
TAAG	0.002	0.013	0.26
CGCC	0.041	0.037	0.85
CGCG	0.007	0.002	0.46
CGAC	0.002	0.0003	0.66
CGAG	0.004	0.0008	0.57
CACC	0.192	0.109	**<0.01**
CACG	0.031	0.014	0.21
CAAC	0.009	0.001	0.24
CAAG	0.002	0.003	0.83

Abbreviation: SNP, single-nucleotide polymorphism.
The *P*-values for comparing each haplotype between cases and controls are shown.
Bold values emphasize statistical significance.
[a]*TLR9* T-1237C/*TLR9* A2848G/*MYD88* C-938A/*MYD88* C1944G.

showed that the distribution of the *TLR9* 1237C-2848A haplotypes were different between cases and controls.

Others have also examined the role of TLRs and particularly that of *TLR9* gene polymorphisms in susceptibility to human diseases associated with altered immune responses and inflammation. Among the other TLR polymorphisms, Nieters *et al.*[20] investigated the contribution of the *TLR9* −1486T>C and −1237T>C SNPs to the development of lymphomas, including 115 cases of HL. A 20% decreased risk of all lymphomas was associated with the *TLR9* −1237C allele, but this was of borderline significance. In contrast to our study, no contribution of the −1237T>C SNP to HL was found.

Although significant associations were detected in our study, the size was relatively small. However, candidate–gene association studies have the tendency to lack the power to detect a statistically significant association. For example, to achieve a power of >80% to identify a modest genetic effect (OR=1.2) of a polymorphism present in 10% of individuals, a sample size of ⩾10 000 subjects would be required.[28] Therefore, the sample sizes required to predict association have to be far beyond what is currently available and no single institution or entity alone will be able to provide a reasonable number of patients. However, a future meta-analysis of multiple studies clearly has a role in offering an analysis with the potential for higher power.[28,29] Future collaborative studies may allow the pooling of data, providing more power to detect significant associations. Furthermore, consortia performing gene–candidate or genome-wide association studies will be able to replicate the validity of the present findings.

This study was based on the hypothesis that genetic variations in *TLR9* and *MYD88*, both of which have crucial roles in immunity and inflammation, could alter the downstream immunological responses after NF-κB activation. Given that increased NF-κB activation is a characteristic of HL cell lines and is required for the proliferation and survival of RS cells,[3] TLR expression variability could participate in the pathogenesis of HL. This hypothesis was further supported by the fact that EBV has been repeatedly associated with the development of HL and that, on EBV infection, LMP1 constitutively transmits signals that activate NF-κB.[10]

So far, the functional role of the *TLR9* −1237T>C polymorphism seems controversial. Deletions spanning the −3.2 to −0.7 kb promoter region did not alter the *TLR9* promoter activity,[30] although the region was shown to be important for the Human Papillomavirus 16-mediated inhibition of *TLR9* expression.[31] *In silico* analysis of the *TLR9* promoter showed that the −1237T>C change introduces a putative c-Rel/NF-κB transcription factor binding site;[32] *in vitro* gene reporter assays showed that the *TLR9* −1237T>C SNP alters the *TLR9* promoter activity, with the −1237T allele having a higher promoter activity (P=0.018), suggesting the existence of regulatory elements across the polymorphic site[33] and implying that the −1237C allele is associated with lower *TLR9* expression levels.

In conclusion, this study showed that a genetic variation in *TLR9* may alter the susceptibility to HL. Although the functional significance, if any, of the *TLR9* −1237T>C and the *TLR9* 2848A>G SNPs remains to be elucidated, our results suggest that TLR9 polymorphisms and TLR9 and MYD88 haplotypes may be involved in the pathogenesis of HL.

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