The rs5743836 polymorphism in TLR9 confers a population-based increased risk of non-Hodgkin lymphoma

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Abstract

Non-Hodgkin lymphoma (NHL) has been associated with immunological defects, chronic inflammatory and autoimmune conditions. Given the link between immune dysfunction and NHL, genetic variants in toll-like receptors (TLRs) have been regarded as potential predictive factors of susceptibility to NHL. Adequate antitumoral responses are known to depend on TLR9 function, such that the use of its synthetic ligand is being targeted as a therapeutic strategy. We investigated the association between the functional rs5743836 polymorphism in the TLR9 promoter and risk for NHL in three independent case-control association studies from Portugal (1160 controls, 797 patients), Italy (468 controls, 467 patients) and the U.S. (972 controls, 867 patients). We found that the rs5743836 polymorphism was significantly over-transmitted in both Portuguese [odds ratio (OR), 1.85; P=7.3e-9] and Italian [OR, 1.71; *P*=5.2e-4] and not in an U.S. cohort of NHL patients. Our findings suggest the relevance of this functional *TLR9* polymorphism on NHL susceptibility in a population dependent manner.

Introduction

Non-Hodgkin's lymphoma (NHL) includes a heterogeneous group of malignant lymphoproliferative diseases whose incidence has substantially increased over the past decades in Western countries [1]. Individuals with specific immune deficiencies associated with immune suppressive therapy after transplantation, human immunodeficiency virus (HIV) infection and congenital conditions are among those with higher incidence rates of lymphoid malignancies. Additionally, clinical and experimental data consistently associated several autoimmune and chronic inflammatory disorders with increased risk of NHL [2-3].

Toll-like receptors (TLRs) are widely studied members of the pattern recognition receptor (PRR) family with a major role in activation and homeostasis of the immune system upon pathogen recognition [4]. Among the TLRs that bind nucleic acids, TLR9 recognizes unmethylated CpG DNA motifs (present at a much higher frequency in the genomes of prokaryotes than of eukaryotes) as a "danger signal" that activates the innate immune system. In humans, this receptor is expressed in plasmacytoid dendritic cells (pDCs) and B lymphocytes, known to have a diverse TLR repertoire, where TLR9 predominates [5]. A role for TLR9 on viral, fungal, mycobacterial and *Helicobacter pylori* infections has been described [6-10]. TLR9 (and TLR7) has been also involved in the recognition of self-nucleic acids, which is believed to play an important role in the pathogenesis of autoimmune diseases, particularly in systemic lupus erythematosus [11] and psoriasis [12].

In addition to self-recognition, inappropriate TLR9 activation leading to disease may also involve mechanisms of transcriptional deregulation of the *TLR9* gene. Consistently, cells from different lymphoma types (mantle cell, B-cell small lymphocytic, follicular and diffuse large B-cell) have been reported to overexpress *TLR9* and to show an increased proliferation upon CpG stimulation, although with a heterogeneous pattern when comparing either different lymphomas or different patients [5, 13]. More specifically, the highly prevalent rs5743836 polymorphism in the *TLR9* gene (1237T>C), has been demonstrated to predispose to Hodgkin

lymphoma as well as to several autoimmune and chronic inflammatory diseases, including asthma and Crohn's disease [14-18].

Given the substantial weight of evidence for immune dysfunction as the underlying basis of lymphomagenesis, a genetic variant unbalancing the regulation and expression of TLR9-associated mechanisms may have a major impact upon host antitumoral responses and therefore, an important role in NHL pathogenesis. In the present study, we evaluated the association of the rs5743836 polymorphism in the *TLR9* promoter with the risk of NHL.

Results and Discussion

Epidemiological and genetic studies have shown that inflammatory and infectious conditions, particularly bacterial and viral infections, are associated with increased NHL risk [3, 19-22], suggesting that chronic immune stimulation plays a role in NHL development [3, 21, 23-26].

Given the role of TLR9 in bacterial and viral recognition, and the link of the *TLR9* rs5743836 polymorphism with Hodgkin lymphoma, we hypothesized that rs5743836 could also influence NHL risk. We therefore analyzed whether this polymorphism was associated with NHL susceptibility in a large-scale, case-control study. Genotype frequencies of Portuguese control individuals (n=1160) for rs5743836 were in Hardy-Weinberg equilibrium (P>0.05). A comparison of genotype distribution between the NHL patients (n=797) and controls revealed a positive association between the presence of rs5743836 and risk of NHL (Table 1), using the dominant (P=7e-9, odds ratio (OR)=1.85, 95% confidence interval (CI), [1.50-2.27]) or the additive (P=1e-7, OR=1.66) genetic models. To validate our findings, two independent Caucasian populations were used: an Italian cohort (467 NHL cases and 468 controls) and a U.S. population of European ancestry (972 NHL cases and 867 controls) [27]. In both populations, rs5743836 frequencies for controls were in Hardy-Weinberg equilibrium (P>0.05). Whereas the association was confirmed in the Italian cohort, with either the dominant (P=5.2e-4, OR=1.71, CI = [1.26-2.33]) or the additive (P=1.1e-3, OR=1.49) genetic models, no association between NHL susceptibility and the presence of rs5743836 was found in the U.S. patients (Table 1).

There are several possible reasons for the discrepant results obtained for the association of rs5743836 with NHL. It is noteworthy that several other studies have found minor allele control frequencies of rs5743836 similar to those assessed in our European populations [8, 28-29]. This suggests that, despite the fact that the populations enrolled in our study shared the same population bottleneck expanding out of Europe, the decreased minor allele frequency detected in the Portuguese and Italian cohorts likely reflects a greater genetic drift in Europe [30]. On the other hand, familial aggregation and twin studies for lymphoproliferative disorders have ruled out the role of highly penetrant genes in affecting risk of the majority of NHL cases [31]. It is therefore likely that the additive effects of genetic variants and interactions with environmental and infectious agents may play a significant role in the differential relevance of the rs5743836 polymorphism in the risk for NHL. This hypothesis is in agreement with epidemiological and genetic studies showing that inflammatory and infectious conditions are associated with increased NHL risk, suggesting that chronic immune stimulation plays a role in NHL development [3, 20-22].

The rs5743836 polymorphism in TL*R9* has been consistently associated with increased transcriptional activity [9, 32-33]. Therefore, it is possible to predict that the complex immunological response of various cell types to CpG, involving both direct and indirect effects of TLR9 is likely deregulated in individuals carrying the rs5743836 polymorphism. In fact, it has been shown that increased B-cell proliferation upon CpG stimulation is a common feature of different NHL subtypes, even though the proliferation pattern varies among NHL subtypes and individual patients [13]. Signal transduction initiated by TLR9 activation results in nuclear translocation of NF-κB [34] that may enhance lymphocyte neoplastic transformation by promoting proliferation and survival of mutated cells [35]. The neoplastic process leading to the development of NHL may be usurping impairments in TLR signaling pathways to advance cancer progression, which suggests that targeting these pathways may open novel therapeutic avenues. CpG molecules are now regarded as highly promising for cancer therapy, mostly due to their direct effect on TLR9 activation on immune cell subpopulations that play an important role in anti-tumor immunity,

including B cells [36]. However, therapeutic applications of these CpG should be individually tailored since individual genetic variations may affect the outcome of the TLR9 signaling pathway.

In summary, our results suggest a potential role for the host genetic background in NHL susceptibility. However, deeper insights into the pathogenetic mechanisms underlying TLR9-mediated mechanisms in NHL are needed. Ultimately, functional studies dissecting the role of the rs5743836 may allow the identification of potential therapeutic targets.

Conflict of interest

The authors declare no conflict of interest.

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Table 1. Analysis of rs5743836 in non-Hodgkin lymphoma (NHL) patients and controls.

Population [a]	Study Groups	rs5743836 genotype [b] N (% frequency)				Statistical analysis [c]			
		TT	TC	CC	TC + CC	*P* value*	OR [95% CI]*	*P* value**	OR**
Portuguese	Controls (1160)	934 (80.5)	217 (18.7)	9 (0.8)	226 (19.5)				
	NHL (797)	551 (69.1)	244 (30.6)	2 (0.3)	246 (30.9)	7.3e-9*	1.85 [1.50-2.27]	1.1e-6**	1.66
Italian	Controls (468)	379 (81.0)	81 (17.3)	8 (1.7)	89 (19)				
	NHL (467)	333 (71.3)	123 (26.3)	11 (2.4)	134 (28.7)	5.2e-4*	1.71 [1.26-2.33]	1.0e-3**	1.49
U.S.	Controls (972)	674 (69.3)	275 (28.3)	23 (2.4)	298 (30.7)				
	NHL (867)	629 (72.5)	224 (25.8)	14 (1.6)	238 (27.5)	1.3e-1*	0.86 [0.70-1.0]	9.3e-2**	

[a] Portuguese and Italian patients had a mean age of 60.4±15.8 (51.7% males, 48.3% females) and 62.8±12.4 (58.9% males, 41.1% females), respectively. Controls were unrelated healthy blood donors frequency-matched to cases by gender and age. Patients and controls with history of transplantation, hematological malignancy or HIV infection were excluded from the study. U.S. patients derived from a study conducted in the San Francisco Bay Area (California, U.S.) that included incident cases diagnosed from 2001 through 2006. Details of the process and criteria for subject selection have been described elsewhere [27]. All study participants provided written informed consent.

[b] For the Portuguese and Italian studies, genotyping was performed using bi-directional PCR amplification of specific alleles (Bi-PASA) as described [37]. For the U.S. study, genotyping was performed using a TaqMan SNP Genotyping Assay (Applied Biosystems, Foster City, CA) on a GeneAmp PCR System 9700 and a post-PCR plate read on the 7700 Sequence Detection System was used to determine genotype. Reactions were done with the following protocol: 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Probes and primer sets used were as follows: forward primer, GCCTTGGGATGTGCTGTTC; reverse primer, CAGAGACATAATGGAGGCAAAGGA; T probe, CCTGAAAACTCCC; and C probe, CTGGAAACTCCC.

[c] Association tests were conducted using a dominant, recessive and an additive model (Cochran-Armitage trend test) tests. For the Portuguese and Italian studies, Fisher's exact test and Pearson's $\chi 2$ test were used to compare genotype frequencies between patients and controls. Unconditional logistic regression was used to compute odds ratios (ORs) and corresponding 95% confidence intervals (CI) adjusted for age and sex. For the U.S. study, association tests were conducted using the PLINK 1.04 software. ORs and 95% CI were calculated by median-unbiased estimation using the mid-p method from the epitools R package (http://sites.google.com/site/medepi/epitools). *P* values were calculated regarding dominant* or the additive** genetic model.