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Research Article

Genetic Variants in Apoptosis and Immunoregulation-Related Genes Are Associated with Risk of Chronic Lymphocytic Leukemia

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Abstract

To identify low-penetrance susceptibility alleles for chronic lymphocytic leukemia (CLL), we performed a case-control study genotyping 768 single-nucleotide polymorphisms (SNP) in 692 cases of CLL and 738 controls. We investigated nonsynonymous SNPs, SNPs with potential functional effect, and tag SNPs in regulatory gene regions in a total of 172 genes involved in cancer biology. After adjustment for multiple testing, we found a strong association between CLL risk and six genetic variants: *CCNH* (rs2266690, V270A), *APAF1* (rs17028658, 3′region), *IL16* (rs4505265, first intron), *CASP8* (rs1045485, D302H), *NOS2A* (rs2779251, promoter), and *CCR7* (rs3136687, intron 1). We found association with CLL susceptibility and 22 haplotypes in *APAF1, IL6, TNFRSF13B, IL16, CASP3, CCR7, LTA/TNF, BAX, BCL2, CXCL12, CASP10/CASP8, CASP1, CCL2, BAK1*, and *IL1A* candidate genes. Finally, we evaluated using public data sets the potential functional effect on gene expression levels of the CLL associated genetic variants detected in regulatory regions. Minor alleles for *APAF1* and *IL16* were associated with lower mRNA levels; no expression differences were observed for *CCR7*, whereas *NOS2A* could not be assessed. This study suggests that common genetic variation in apoptosis- and immunoregulation-related genes is associated with the CLL risk. [Cancer Res 2008;68(24):10178–86]

Introduction

Chronic lymphocytic leukemia (CLL) is the most prevalent type of human leukemia in Western countries and accounts for about 40% of all leukemias in adults over the age of 65 years. This neoplasm is characterized by the progressive accumulation of B lymphocytes that express CD5 (1). The genetic and molecular mechanisms involved in the development of the disease are not well known, but recent observations suggest that the modulation of survival signals interfering with programmed cell death pathways and stimulating proliferation may be a pivotal mechanism in the pathogenesis of CLL (2). Microenvironment signals mediated by stromal cells and extracellular matrix, chemokines, cytokines, other ligands, and their receptors create a network of interactions that also modulate the biology of the tumor cells (2).

Family and epidemiologic studies have shown the existence of an inherited susceptibility to CLL and other B-cell lymphoid neoplasms (3–7). However, genome-wide screening of families with CLL has not identified clear candidate genes (8–11). Although part of the familial incidence of CLL could be due to high-penetrance mutations in unidentified genes, it seems plausible that common genetic variants with modest effects on disease susceptibility may be related to this risk.

Multiple association studies are been carried out to identify common causal variants to CLL, comparing the frequency of some single-nucleotide polymorphisms (SNP) in candidate genes between patients and control subjects (12). Recent developments in high-throughput genotyping technology allow the analysis of thousands of SNPs (13). Usually, these studies include nonsynonymous SNPs, but recently, other approaches have been done investigating functional SNPs, SNPs in the coding and noncoding regions, or/and tag SNPs that may maximize the recognition of the common variation across a given gene (14, 15).

To evaluate the genetic susceptibility for CLL, we performed a case-control study genotyping 768 SNPs in 692 CLL patients and 738 healthy controls from the Spanish population. The study includes nonsynonymous SNPs selected from genes involved in cancer biology, SNPs in potentially functional regions (coding and regulatory gene regions), and tag SNPs from 48 genes involved in apoptosis, immune response, DNA repair, and cell adhesion pathways. Our results suggest that common genetic variants in apoptosis and immunoregulation related genes are associated with risk of CLL.

Materials and Methods

Study population. This study includes 692 incident CLL and 738 controls. Subjects were recruited in three medical centers from Spain: two centers from Barcelona (Hospital Clínic and Institut Català d'Oncologia) and one from Alava (Txagorritxu Hospital). Cases were defined as consecutive patients with the diagnosis of chronic lymphocytic leukemia and with an available blood sample for DNA extraction before any treatment. The diagnosis of CLL was established using standard clinicopathologic and immunophenotypic criteria in accordance with the 2001 WHO classification guidelines (16). For the Hospital Clinic and Txagorritxu Hospital, controls were selected from a population-based sample repository of healthy subjects residing in the same area as the cases, whereas for the Institut Català d'Oncologia patients, the controls were clinic-based selected among patients with no hematologic cancer that were

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Table 1. Age and gender distribution of the CLL patients and control population included in the genotyping analysis

	Overall	Control	Cases
Participants, *n* (%)	1412	723 (51.2)	689 (48.8)
Age, mean (SD)	65.2 (12.6)	65.3 (12.2)	65.2 (13.0)
Age, *n* (%)*			
≤56	354 (25.2)	176 (24.3)	178 (26.0)
57–66	350 (24.9)	184 (25.5)	166 (24.3)
67–74	372 (26.4)	197 (27.3)	175 (25.6)
≥75	331 (23.5)	166 (23.0)	165 (24.1)
Sex, *n* (%)*			
Female	598 (42.4)	310 (42.9)	288 (41.9)
Male	813 (57.6)	413 (57.1)	400 (58.1)

*Patients and controls had a similar gender and age distribution ($P = 0.95$ and $P = 0.70$, respectively). Age and gender were not available in five cases and one case, respectively.

also residents in the same area as the cases. All controls were matched by age, sex, and center and were unrelated to other participants. All cases and control subjects were Spanish Caucasians. All cases and controls signed an informed consent and the study was approved by the Institutional Board.

SNP selection. Candidate SNPs were identified following three strategies, mainly focused to include SNPs with putative functional effect in protein structure and gene expression. The initial step was to search for nonsynonymous SNPs with possible functional effect in genes that could be relevant in CLL pathogenesis. These nonsynonymous SNPs were searched in an initial list of 618 genes involved in hematologic system development and function, immune response, cell death, DNA replication, DNA recombination, DNA repair, cell cycle, cell signaling and interaction, cell motility, and cell growth. These genes were obtained from a systematic Medline search to identify the reports published on lymphoid neoplasm and CLL using these categories as key words in the queries.

In a second step, we selected SNPs located within putative functional regions [5′ upstream, 5′-untranslated region (UTR), coding region, splicing sites, first intron, 3′-UTR, and downstream]. These SNPs were searched in 48 genes that we prioritized from the genes obtained in step 1. These genes were included based on their involvement in immune response, cell death, and DNA repair pathways that were considered of potential relevance in CLL pathogenesis according to the literature review (Supplementary Table S1). The SNPs in these 48 candidate genes were searched in the 10 kb 5′upstream from the start codon, first intron, and 1 kb downstream from the stop codon of each candidate gene. The genetic polymorphisms included in this group were SNPs disrupting potential transcription factor binding sites predicted by the web software Pupas View[7] (17) and SNPs located in conserved sequences in several species determined using the UCSC Genome Browser Database.[8] In addition, several tag SNPs were selected to cover those SNPs located in these putative regulatory gene regions that did not show evidence of functionality. In this study, tag SNPs are defined as variants with $r^2 \geq 0.98$ with other SNPs in HapMap individuals with European ancestry (CEU; Tagger, Haploview 3.2; ref. 18). Finally, we selected all genes that contained SNPs previously reported in the literature to be associated with CLL and other lymphoid neoplasms.

We selected only SNPs with a minor allele frequency >5% (≥0.05) in Caucasic population of the HapMap-CEU Europe (19) and PERLEGEN-EUR-Panel North America reported in public available ENSEMBL genome browser[9] (v33-36) working with dbSNP build 124 and National Center for Biotechnology Information (NCBI) genome build 35.1. All the SNPs selected were then filtered by Illumina technology criteria (score ≥0.6 or GoldenGate validated status, Illumina, Inc.).

A total of 768 SNPs from 172 genes were genotyped (Supplementary Table S2), 42.5% were located in 5′ upstream and first intron regions, 40.5% in coding regions (all nonsynonymous SNP), 4.8% in intronic regions (except first intron), and 12.2% in 3′ downstream gene regions.

Genotyping. Genomic DNA was isolated from peripheral blood lymphocytes using conventional DNA extraction procedures. Blood samples and written informed consent were obtained in accordance with the Institutional Ethical Committees and the Hospital Clinic Human Investigation Review Committee for Genetics Procedures. Genomic DNA was quantified using PicoGreen and diluted to a final concentration of 50 ng/μL. Genotyping was carried out at the Spanish National Genotyping Centre (CeGen) using a high-throughput platform of Illumina Bead Array System (Illumina). The results obtained for blinded duplicate blood DNA samples were concordant for all SNPs.

Statistical analysis. Genotypic frequencies in control subjects for each SNP were tested for departure from Hardy-Weinberg equilibrium using a Fisher's exact test (20).

Gene-disease associations. To test the hypothesis of association between genetic polymorphisms and CLL, multivariate methods based on logistic regression analyses were used. To quantify the degree of the association, odds ratios (OR) and 95% confidence intervals (95% CI) were calculated for each group under the codominant, dominant, recessive, and log-additive inheritance models. All analyses were adjusted for age, sex, and center of recruitment.

Because many SNPs have been explored and several inheritance models have been used, some may show significant results by chance. We assessed the robustness of the findings by calculating an estimate of the false discovery rate for significant results. False discovery rate is the expected proportion of false positives (erroneous rejections) among the significant tests (rejections). For this purpose, Q values were computed for each SNPs. Furthermore, to select a set of significant SNPs with an estimated false discovery rate <5%, SNPs with $Q < 0.05$ were selected (21).

Population stratification. We applied the method of Genomic Control to estimate quantitatively the amount of population stratification (22). The Genomic Control model allows for extra variance by assuming that the statistic test is inflated by a factor λ that can be estimated for each of the inheritance model. The Genomic Control model approach is based on the assumption that the variance inflation factor λ is approximately constant across the genome for all loci that are not associated with the disease.

Haplotype analysis. For 82 genes in which more than one SNPs was genotyped, haplotype block structure was determined with Haploview 3.32 program using the confidence intervals algorithm, which defines pairs to be

[7] http://www.pupasnp.org
[8] http://genome.cse.ucsc.edu
[9] http://www.ensembl.org

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in "strong linkage disequilibrium" if the one-sided upper 95% confidence bound on D' is >0.98 (i.e., consistent with no historical recombination) and the lower bound is >0.7 (18, 23). For each block, haplotypes were analyzed using the haplo.stats package for R statistical software (24), which implements the expectation maximization algorithm to estimate the haplotype frequencies. For each individual, the compatible haplotypes and their posterior probabilities were computed and coded with dummy indicator variables. The posterior probabilities were used as weights in the logistic regression models to account for uncertainty in the identification of phase-unknown haplotypes.

Genotype-gene expression association analysis. Association between genetic variants and germ-line mRNA expression differences was assessed using experimental data of 60 HapMap CEU unrelated individuals, U.S. residents with northern and western European ancestry (25). HapMap genotypes were downloaded from the release 22 (NCBI build 36) and transcript measurements from the Gene Expression Omnibus record GSE6536, which used the Illumina Sentrix Human-6 Expression BeadChip. Thus, measurements belong to immortalized lymphocytes of healthy CEU individuals. Data were compiled and processed using the R programming language, and associations were assessed with the SNPStats web software (26) by fitting linear equations and P values obtained based on the F test. Results were corroborated using two independently generated data sets in immortalized lymphocytes (27).

Results

Data and genotyping success. A successful genotyping was obtained in 1,412 of the 1,430 submitted DNA samples (98.7%), 689 of 692 cases (99.5%), and 723 of 738 controls (98.0%). SNP call rates per sample for each of the 1,412 DNA samples were 88.0% (95% CI, 87.7–88.2) in cases and 87.4% (95% CI, 87.2–87.6) in controls. No differences were observed in the age and gender distribution of the CLL patients and controls with a successful genotyping (Table 1).

Of the 768 SNPs submitted for analysis, 85 SNPs failed in the genotyping process (no PCR amplification, insufficient intensity for cluster separation, or poor or no cluster definition) and 683 (89%) SNPs were genotyped satisfactorily. Of these, 34 SNPs turned out to be monomorphic, leaving 649 SNPs for which genotype data were informative. For each SNP, the average percentage of samples for which a genotype could be obtained was 98.9% (95% CI, 98.8–99.1) and 98.3% (95% CI, 98.0–98.6) among cases and controls, respectively. Thirty-six SNPs that violated the Hardy-Weinberg equilibrium in controls and 35 SNPs that had a minor allele frequency <1% in the controls were removed from the study, leaving 578 SNPs for further study. In control samples, 43 SNPs had a

Table 2. SNPs showing a significant association with risk of CLL with a $P < 0.010$ for the best inheritance model

SNPs	Gene	Alleles	Region	MAF	HWE	Codominant model				Best model		
						Heterozygote	Rare homozygote					
						OR 95%CI	OR 95%CI	*P*	Q	P	Q	IM
rs2266690	*CCNH*	*T>C*	V270A	0.26	0.14	0.59 (0.47–0.74)	0.42 (0.29–0.61)	0.0000	0.0000	0.0000	0.0000	A
rs17028658	*APAF1*	*T>C*	3′	0.05	0.60	0.42 (0.29–0.61)	NA	0.0000	0.0008	0.0000	0.0002	A
rs4505265	*IL16*	*A>C*	intron 1	0,00	NA	NA	NA	0.0000	0.0005	0.0000	0.0005	cD
rs1045485	*CASP8*	*G>C*	D302H	0.11	0.09	0.57 (0.44–0.73)	0.53 (0.22–1.28)	0.0000	0.0028	0.0000	0.0006	D
rs2779251	*NOSA2*	*G>A*	5′	0.16	0.06	0.68 (0.54–0.87)	0.51 (0.30–0.87)	0.0009	0.0730	0.0002	0.0184	A
rs3136687	*CCR7*	*A>G*	intron 1	0.02	1.00	0.38 (0.22–0.64)	NA	0.0005	0.0526	0.0003	0.0311	D
rs8079130	*TNFRSF13B*	*C>T*	5′	0.17	0.07	0.99 (0.77–1.26)	3.43 (1.59–7.40)	0.0029	0.1766	0.0006	0.1764	R
rs3181092	*VCAM1*	*G>A*	3′	0.29	0.37	0.71 (0.56–0.89)	0.66 (0.45–0.95)	0.0040	0.2120	0.0010	0.0705	D
rs7805828	*IL6*	*G>A*	5′	0.44	0.65	0.78 (0.61–1.01)	0.61 (0.45–0.83)	0.0063	0.2536	0.0015	0.0930	A
rs2228014	*CXCR4*	*C>T*	I138I	0.14	0.11	1.76 (1.24–2.49)	1.47 (0.53–4.13)	0.0061	0.2536	0.0015	0.0941	D
rs2227306	*IL8*	*C>T*	intron 1	0.41	0.36	1.32 (1.04–1.67)	0.79 (0.57–1.10)	0.0024	0.1641	0.0024	0.1642	cD
rs4987853	*BCL2*	*A>G*	3′-UTR	0.23	0.25	0.71 (0.57–0.89)	0.81 (0.51–1.29)	0.0109	0.3629	0.0031	0.1705	D
rs2857653	*CCL2*	*C>T*	5′	0.24	0.07	1.04 (0.83–1.31)	2.15 (1.27–3.63)	0.0147	0.3951	0.0040	0.5620	R
rs11574665	*CCR7*	*C>A*	intron 1	0.01	1.00	0.36 (0.18–0.72)	NA	0.0046	0.2210	0.0041	0.1994	D
rs11674246	*CASP10*	*C>T*	intron 1	0.45	0.05	1.12 (0.87–1.44)	0.74 (0.54–1.00)	0.0108	0.3629	0.0041	0.5620	R
rs2009658	*LTATNF*	*C>G*	5′	0.14	0.90	0.75 (0.59–0.95)	0.55 (0.28–1.08)	0.0180	0.4074	0.0046	0.1881	A
rs1546762	*IL6*	*T>C*	5′	0.43	1.00	1.15 (0.91–1.46)	1.6 (1.17–2.19)	0.0132	0.3951	0.0048	0.1881	A
rs1009316	*BAX*	*C>T*	intron 1	0.13	0.89	0.71 (0.55–0.91)	0.73 (0.36–1.50)	0.0210	0.4336	0.0055	0.2409	D
rs1880242	*IL6*	*T>G*	5′	0.43	0.87	1.11 (0.88–1.41)	1.57 (1.14–2.15)	0.0186	0.4074	0.0072	0.5756	R
rs1800067	*ERCC4*	*G>A*	R415Q	0.12	0.11	1.1 (0.84–1.43)	0.26 (0.08–0.79)	0.0217	0.4336	0.0074	0.5756	R
rs3917366	*IL1B*	*G>T*	3′	0.25	1.00	1.24 (0.99–1.55)	1.73 (1.07–2.80)	0.0268	0.4506	0.0078	0.1979	A
rs4645878	*BAX*	*G>A*	5′	0.12	0.88	0.72 (0.55–0.94)	0.58 (0.24–1.39)	0.0313	0.4506	0.0087	0.1979	A
rs4802527	*BAX*	*C>G*	5′	0.15	1.00	0.74 (0.58–0.94)	0.7 (0.37–1.31)	0.0316	0.45056	0.0087	0.2685	D
rs2307389	*ORC3L*	*G>A*	V217L	0.09	0.51	1.38 (1.01–1.88)	6.71 (0.79–56.87)	0.0153	0.3951	0.0091	0.1979	A
rs2844482	*LTATNF*	*G>A*	5′	0.14	0.70	0.74 (0.58–0.94)	0.67 (0.33–1.36)	0.0334	0.4506	0.0095	0.2685	D
rs2302427	*Ezh2*	*C>G*	D185H	0.06	0.25	0.72 (0.53–0.99)	0.26 (0.05–1.26)	0.0244	0.4506	0.0095	0.1979	A
rs4647698	*CASP3*	*G>A*	3′-UTR	0.01	1.00	0.42 (0.22–0.80)	NA	0.0113	0.3629	0.0100	0.2686	D

Abbreviations: MAF, minor allele frequency in CLL group; HWE, Hardy-Weinberg P value in control group; IM, inheritance model; A, additive; NA, not available; cD, codominant; D, dominant.

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Table 3. Genotype frequencies of SNPs with strong association with CLL risk ($Q < 0.05$)

SNP (gene)	Model	Genotype	Controls n (%)	Cases *n* (%)	OR (95%CI)	*P*
rs2266690 (*CCNH*)	Codominant	*TT*	311 (43)	382 (57)	1.00	5.70e−08
		TC	312 (43)	238 (35)	0.59 (0.47–0.74)	
		CC	100 (14)	56 (8)	0.42 (0.29–0.61)	
	Dominant	*TT*	311 (43)	382 (57)	1.00	3.74e−08
		TC-CC	412 (57)	294 (43)	0.55 (0.44–0.68)	
	Recessive	*TT-TC*	623 (86)	620 (92)	1.00	0.00040
		CC	100 (14)	56 (8)	0.54 (0.38–0.76)	
	Log-additive	0, 1, 2	723 (100)	676 (100)	0.63 (0.53–0.74)	1.62e−08
rs17028658 (*APAF1*)	Codominant	*TT*	372 (80)	611 (90)	1.00	5.27e−06
		TC	87 (19)	65 (10)	0.42 (0.29–0.61)	
		CC	3 (1)	0 (0)	NA	
	Dominant	*TT*	372 (80)	611 (90)	1.00	1.79e−06
		TC-CC	90 (20)	65 (10)	0.41 (0.28–0.59)	
	Recessive	*TT-TC*	459 (99)	676 (100)	1.00	0.09069
		CC	3 (1)	0 (0)	NA	
	Log-additive	0, 1, 2	462 (100)	676 (100)	0.41 (0.28–0.59)	1.2e−06
rs4505265 (*IL16*)	Codominant	*AA*	695 (98)	684 (100)	1.00	2.2e−06
		AC	15 (2)	0 (0)	NA	
		CC	0 (0)	0 (0)	NA	
	Log-additive	0, 1, 2	710 (100)	684 (100)	NA	
rs1045485 (*CASP8*)	Codominant	*GG*	485 (68)	533 (79)	1.00	0.00002
		GC	217 (30)	138 (20)	0.57 (0.44–0.73)	
		CC	14 (2)	8 (1)	0.53 (0.22–1.28)	
	Dominant	*GG*	485 (68)	533 (79)	1.00	3.95e−06
		GC-CC	231 (32)	146 (21)	0.56 (0.44–0.72)	
	Recessive	*GG-GC*	702 (98)	671 (99)	1.00	0.02675
		CC	14 (2)	8 (1)	0.61 (0.25–1.48)	
	Log-additive	0, 1, 2	716 (100)	679 (100)	0.60 (0.48–0.75)	6.32e−06
rs2779251 (*NOS2A*)	Codominant	*GG*	460 (64)	491 (72)	1.00	0.00091
		GA	221 (31)	165 (25)	0.68 (0.54–0.87)	
		AA	40 (5)	23 (3)	0.51 (0.30–0.87)	
	Dominant	*GG*	460 (64)	491 (72)	1.00	0.00032
		GA-AA	261 (36)	188 (28)	0.66 (0.52–0.83)	
	Recessive	*GG-GA*	681 (95)	656 (97)	1.00	0.03548
		AA	40 (5)	23 (3)	0.57 (0.34–0.97)	
	Log-additive	0, 1, 2	721 (100)	679 (100)	0.70 (0.57–0.84)	0.00019
rs3136687 (*CCR7*)	Codominant	*AA*	584 (93)	649 (97)	1.00	0.00054
		AG	45 (7)	22 (3)	0.38 (0.22–0.64)	
		GG	0 (0)	1 (0)	NA	
	Dominant	*AA*	584 (93)	649 (97)	1.00	0.00035
		AG-GG	45 (7)	23 (3)	0.40 (0.24–0.67)	
	Recessive	*AA-AG*	629 (100)	671 (100)	1.00	0.26561
		GG	0 (0)	1 (0)	NA	
	Log-additive	0, 1, 2	629 (100)	672 (100)	0.43 (0.26–0.71)	0.00072

NOTE: Abbreviations are explained in Table 2.

minor allele frequency between 1% and 5%, and 535 higher than 5% (Supplementary Table S2).

Population stratification. To determine whether the observed association results could be due to a stratification of the population, we applied the genomic control method. Estimation of population stratification inflation factor (λ) under codominant model was 1.11 (95% CI, 0.81–1.40). Supplementary Fig. S1 shows the *Q-Q* plot based for the adjusted (considering the λ inflation parameter) and unadjusted χ^2 test for all inheritance models. Unadjusted and adjusted χ^2 plots show very similar distribution, with inflation parameter λ being greater in the additive model. Despite this, after adjustment for population stratification, the 27 SNPs with significant association with CLL risk continue ($P \leq 0.01$) to be significant (data not shown).

SNPs and risk of CLL. The comparison between CLL patients and controls revealed that 95 SNPs had a significant statistical association with CLL risk ($P < 0.05$ for any of the inheritance models; Supplementary Table S2). The 27 SNPs with a $P \leq 0.01$ are shown in Table 2. After the stringent false discovery rate adjustment to take into account the multiple comparisons, six SNPs remain significantly associated with CLL risk ($Q < 0.05$). These SNPs were *CCNH* (rs2266690, V270A), *APAF1* (rs17028658,

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3′region), *IL16* (rs4505265, first intron), *CASP8* (rs1045485, D302H), *NOS2A* (rs2779251, promoter), and *CCR7* (rs3136687, intron 1; Table 3). The presence of the minor allele of the *CCNH, APAF1, CASP8, NOS2A*, and *CCR7* variants resulted in a protective effect for the risk of CLL (ORs <1; Table 3). Although the *IL16* variant (rs4505265) was strongly associated with CLL risk (P = 2.2 10^{-6}, Q = 0.0005), the OR could not be estimated because the minor allele was not observed in cases and was only present in the heterozygous genotype in controls. The homozygote genotypes for the risk allele of the *APAF1* and *CCR7* variants were not detected in the CLL group.

Haplotypes and risk of CLL. To test the association between haplotypes and risk of CLL, we determined the linkage disequilibrium block structure for each candidate gene. Variants in *CASP10/CASP8* and *LTA/TNF* genes were analyzed together because these genes are consecutive in the genome. Twenty-two haplotypes were

Table 4. Haplotypes showing a statistical significant association with risk of CLL (P < 0.05)

Gene	SNPs	Haplotype	Controls (%)	Cases (%)	OR (95%CI)*	P
APAF1	rs10860361; rs17028658	*GC*	10.32	4.79	0.43 (0.31–0.61)	0.0000
CASP10/CASP8	rs1035140; rs17649845	*TG*	31.40	38.72	1.32 (1.12–1.56)	0.0008
IL6	rs1546762; rs7801617; rs7805828	*CGG*	37.81	43.07	1.29 (1.10–1.51)	0.0019
IL6	rs1880242; rs4719714	*TT*	25.50	21.59	0.76 (0.62–0.92)	0.0043
TNFRSF13B	rs4985726; rs12051889; rs4985694; rs3818716	*CCGA*	24.24	29.37	1.29 (1.07–1.55)	0.0062
IL16	rs7182786; rs931963; s1509557; rs4505265; rs17337098; rs8029937;	*AGGAAA*	8.72	11.99	1.40 (1.09–1.80)	0.0096
CASP3	rs1049216; rs4647698; rs1049253	*CAT*	2.35	1.17	0.46 (0.25–0.84)	0.0120
CCR7	rs3136687; rs11574665	*GA*	2.14	1.07	0.45 (0.24–0.84)	0.0124
LTA/TNF	rs928815; rs3131637; rs2844484; rs2009658; rs915654; rs2844482	*CACCAG*	20.37	24.77	1.28 (1.50–1.56)	0.0150
CCR7	rs3136687; rs11574665	*GC*	1.59	0.69	0.38 (0.17–0.84)	0.0175
BAX	rs11667200; rs11667229; rs11667351; rs4645878; rs1009316	*ACGAT*	14.62	11.61	0.76 (0.60–0.95)	0.0188
BCL2	rs1807999; rs12961976	*CC*	8.30	6.14	0.71 (0.53–0.95)	0.0227
CXCL12	rs2297630; rs2236534; rs2839693; rs4948878; rs2839690; rs3780891; rs2839685; rs6593412	*GGGATGCG*	2.91	4.77	1.62 (1.05–2.50)	0.0275
CASP10/CASP8	rs11674246; rs17468215; rs13010627; rs13425163; rs12693932; rs6747918	*TTGGCA*	42.81	38.18	0.83 (0.71–0.98)	0.0276
LTA/TNF	rs909253; rs2857713; rs1041981; rs1799964; rs1800610	*CAATC*	22.85	25.53	1.25 (1.02–1.52)	0.0287
LIG4	rs3093772; rs10131	*GG*	10.82	13.25	1.30 (1.03–1.64)	0.0302
CASP1	rs1792774; rs2282659	*CG*	32.19	28.88	0.83 (0.70–0.98)	0.0316
CCL2	rs7223422; rs8068314; rs4795893; rs2857653; rs11867200; rs1860190; rs1860189; rs3917878; rs1024611; rs3760399; rs3760396; rs991804	*CAGTCATCTTCG*	20.62	23.99	1.27 (1.02–1.59)	0.0354
POLL	rs3730463; rs3730477	*AT*	28.19	25.49	0.83 (0.70–0.99)	0.0364
SELL	rs3177980; rs4987310	*TT*	17.39	14.08	0.80 (0.64–0.99)	0.0386
BAK1	rs5745568; rs17627049	*AC*	20.11	23.35	1.23 (1.01–1.49)	0.0391
IL1A	rs1878318; rs1878320; rs3783521; rs1800587; rs2856837; rs1609682; rs17561; rs3783546; rs2856836	*CGCTTATGC*	24.25	28.95	1.20 (1.01–1.44)	0.0431

*Using as reference 1.00 the major haplotype.

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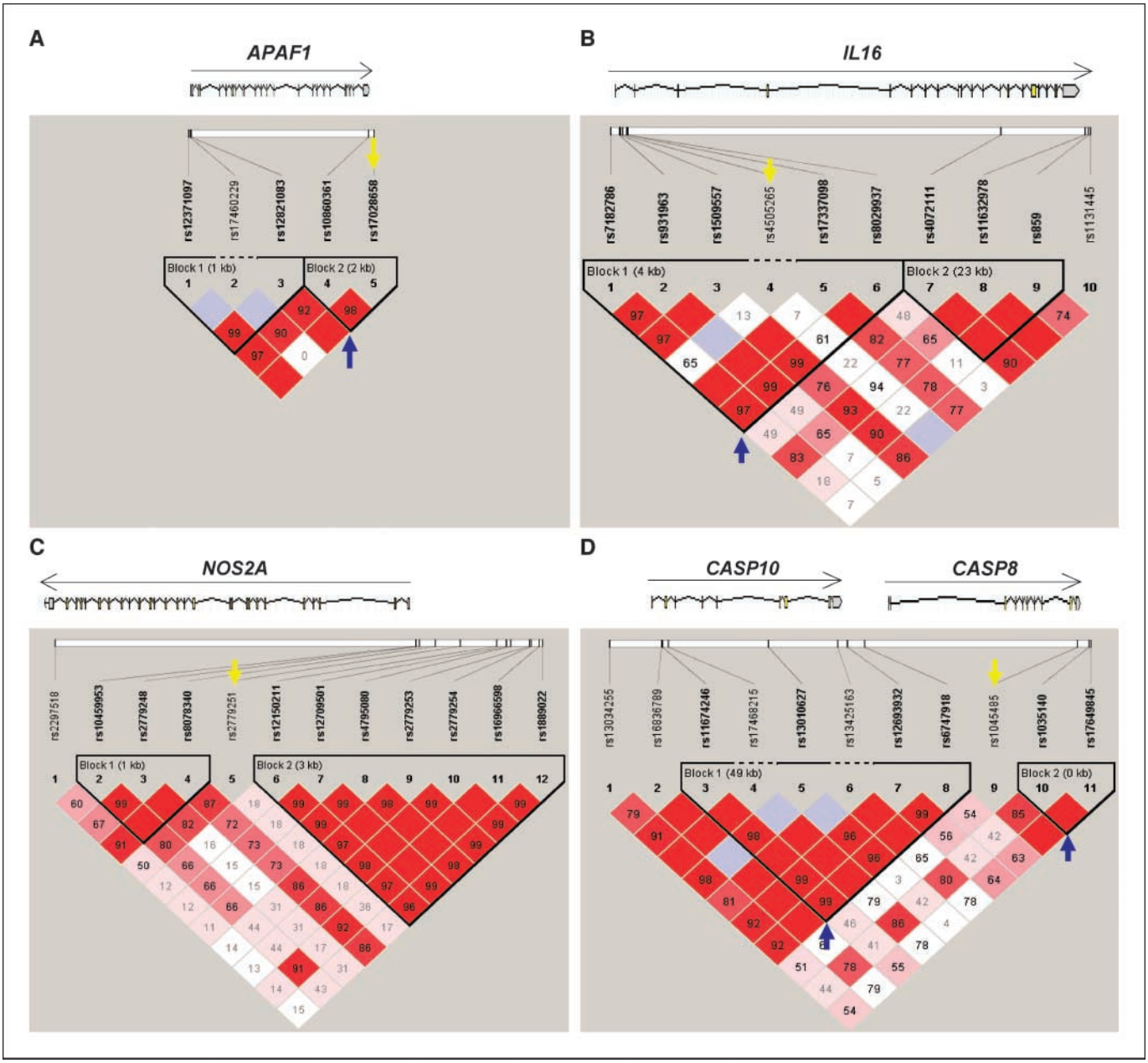

Figure 1. Gene map and linkage disequilibrium plot of *APAF1* (*A*), IL1*6* (*B*), *NOS2A* (*C*), and *CASP10/CASP8* (*D*) genes and flanking regions. Boxes above represent gene structure. Color scheme is based on *D′* and LOD score values: *white, D′* < 1 and LOD < 2; *blue, D′* = 1 and LOD < 2; *shades of pink/red, D′* < 1 and LOD ≥ 2; *bright red, D′* = 1 and LOD ≥ 2. Numbers in squares are *D′* values (values of 1.0 are not shown). Block definition is based on the Gabriel et al. method (23). Arrows show haplotypes (*blue*; $P \leq 0.01$) or SNPs (*yellow*; $P \leq 0.001$ and $Q \leq 0.05$) in association with CLL risk.

significantly associated with CLL risk ($P < 0.05$). Frequencies, ORs, 95% CIs, and *P* values for each haplotype are shown in Table 4. Six of these haplotypes show $P < 0.01$.

The rs17028658 (*APAF1*), rs4505265 (*IL16*), and rs3136687 (*CCR7*) variants significantly associated with increased CLL risk were also included in haplotypes significantly related to CLL susceptibility (Fig. 1). However, the rs1045485 (*CASP8*) and rs2779251 (*NOS2A*) variants were not included in any linkage disequilibrium block. *CCNH* rs22666690 was the only SNP genotyped in this gene.

Functional consequences of risk alleles. Our results suggest that variants affecting the germ-line transcription regulation of *APAF1, CCR7, IL16*, or *NOSA2* may contribute to the risk of CLL. To determine the potential functional effect of these changes, we examined the possible consequences of the association between risk or protective alleles and mRNA expression differences in lymphocytes.

The rs4505265 and rs17337098 variants are located within the first intron of *IL16* and included in the same linkage disequilibrium block. The heterozygosis of these SNP are too low to be examined in the HapMap data set (25), but the analysis of larger sample series of gene expression in lymphocytes and paired genetic data shows that variation in these *cis* elements correlates with germ-line mRNA expression differences of *IL16* [*cis* logarithm of the odds of linkage (LOD) scores of 20 and 2.5 and heritability estimates for

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gene expression $P < 10^{-10}$]. Based on these analyses, the risk allele may be associated with lower expression levels of *IL16* (27, 28).

The rs17028658 variant is located a few kilobases distal from the 3′-UTR region of *APAF1*. Examination of HapMap data set identified significant expression differences between genotypes. The minor allele C seems to be associated with relative lower levels of *APAF1*; each copy of this allele decreases *APAF1* expression in lymphocytes with 0.35 log 2 ratio units (95% CI, −0.65 to 0.05; log-additive model $P = 0.023$). The association with this allele is protective for the risk of CLL (OR, 0.42; 95% CI, 0.29–0.61). No differences in the expression levels were observed for the *CCR7* variant, and the *NOS2A* variant could not be evaluated because it is not expressed in normal lymphocytes.

In addition, we performed a similar analysis for the 93 SNPs with positive association with CLL risk ($P < 0.05$) that did not pass multiple testing corrections. SNP variants in *BAX, BCL2, ERCC2, GSTM3, IL1A, IL6*, and *LTA* correlated significantly with mRNA expression differences (data not shown). Some of these variants were included in haplotypes that also showed an increased risk for CLL. These data suggest that these additional candidates should be prioritized for replication.

Discussion

In this study, we have successfully genotyped 613 SNPs in 172 genes of potential relevance in the pathogenesis of lymphoid neoplasms in 689 patients with CLL and 723 healthy controls. We have identified 27 SNPs in association with CLL risk ($P \leq 0.01$), 6 of them with a strong statistical significant relationship after correction for multiple testing ($P \leq 0.0004$ and $Q \leq 0.05$). In addition, 22 haplotypes in 17 of these genes were also significantly associated with an increased risk for CLL. These genes were mainly related to apoptosis and immunoregulatory pathways.

Several studies have evaluated the risk of CLL using a restricted number of genetic variants in a limited number of candidate genes, placed in the coding or in the putative promoter sequences (29–39). To date, only one study has published results of high-throughput genotyping to the study of CLL risk, analyzing a large number of nonsynonymous SNPs in 992 CLL patients and 2,707 controls (13). In our study, we not only investigated a series of nonsynonymous SNPs that change the coded amino acid but also expanded the selection strategy to tag SNPs to optimize the genotyping analysis and to SNPs located in regulatory regions, including variants in the promoter, first intron, and 3′-UTR gene regions, which may modify gene expression. This approach enriches the study with putative functional SNPs located in regulatory regions. However, it has also the limitation of including a high number of candidate genes with a restricted SNP coverage. Finally, the selection of several SNPs across the same gene allowed us to explore the effect of haplotypes in CLL risk.

Our study showed a strong association with CLL risk after adjustment for multiple testing in six gene variants: *CCNH* (rs2266690, V270A), *APAF1* (rs17028658, 3′region), *IL16* (rs4505265, first intron), *CASP8* (rs1045485, D302H), *NOS2A* (rs2779251, promoter), and *CCR7* (rs3136687, intron 1).

The *CCNH* rs22666690 variant (V270A) was associated with a reduced risk for CLL (OR, 0.63; 95% CI, 0.53–0.74). Cyclin H forms a cyclin-dependent kinase (CDK)–activating kinase complex with CDK7 kinase and ring finger protein MAT1 (40).This complex participates in the regulation of the cell cycle by activating CDK/cyclin complexes and also facilitates the transcriptional activity of RNA polymerase II (40). The functional role of the alanine-to-valine substitution in cyclin H protein is not known, and further studies are required to establish whether this variant itself or another variant in strong linkage disequilibrium may play a role in CLL pathogenesis.

Regulation of programmed cell death (apoptosis) has a relevant role in CLL pathogenesis and response to therapy (2). Caspase-8 and apoptotic protease activating factor 1 (Apaf1) are important initiators of the apoptosis in lymphoid cells. Ours findings suggest that the C variant of rs1045485 in *CASP8*, which results in an aspartic acid-to-histidine substitution, is associated with a reduced CLL risk (OR, 0.56; 95% CI, 0.44–0.72). The functional consequences of this change are not known, but it is interesting that this polymorphism has been recently found to be strongly associated with reduced breast cancer risk (41), suggesting a potential common effect of this variant in human cancer susceptibility. Although we have investigated other SNPs in *CASP8* gene, rs1045485 was not in linkage disequilibrium with other SNPs, indicating that this variant itself could have a pathogenetic role in CLL. The minor allele *C* of the *APAF1* rs17028658, located a few kilobases distal from the 3′-UTR region, was associated with a protective effect of CLL (OR, 0.42; 95% CI, 0.29–0.61). The functional expression analysis suggests that this allele is associated with significantly lower levels of *APAF1* expression. However, this SNP was in linkage disequilibrium with rs10860361, and therefore it is not clear whether the risk effect may be influenced by other SNPs in the same block of linkage disequilibrium.

Ours results suggest that the allele *A* of the rs2779251 polymorphism in the promoter region of *NOS2A* is protective for CLL risk (OR, 0.70; 95% CI, 0.57–0.84). *NOS2A* encodes for the inducible nitric oxide synthase, which produces nitric oxide. Nitric oxide is a reactive free radical that acts as a biological mediator in many physiologic processes, including immune response and antitumoral activity (42). Although inducible nitric oxide synthase is not expressed in normal lymphocytes, it is up-regulated in CLL cells and the nitric oxide produced by the activity of the enzyme confers resistance to apoptosis in these cells (43). This SNP is not in linkage disequilibrium with other polymorphisms, suggesting that this variant itself could have a causal effect.

The *IL16* rs4505265 variant was strongly related to CLL risk. This SNP is in linkage disequilibrium with the rs17337098 variant also located in the first intron, and a haplotype including both SNPs was also associated with CLL risk. The *in silico* analysis suggests that these genetic variants and others in the same linkage disequilibrium block influence the germ-line *IL16* expression differences in lymphocytes. Although the frequency of this variant in the population examined is relatively low, it is interesting that a nonsynonymous SNP (rs4072111; P434S) of this gene that is not in linkage disequilibrium with the rs4505265 variant has been recently associated with a favorable prognosis in CLL patients, suggesting that variants of this gene may play a role in the pathogenesis of the disease (44).

The last variant in association with CLL risk detected after multiple testing adjustment was the *G* allele of rs3136687 polymorphism in the *CCR7* gene (OR, 0.40; 95% CI, 0.24–0.67). This variant is located in the first intron and it is in linkage disequilibrium with rs11574665, and the *GA* haplotype was also associated with CLL risk. CCR7 is a chemokine receptor and the interaction between chemokines and chemokine receptors plays a critical role in B lymphocyte migration and survival (45).

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In addition to the previous six variants, we identified 27 SNPs that showed a significant statistical association with CLL risk (P < 0.01 for someone of the inheritance models; Table 2). Among them, the rs1800067 variant (R415Q) in the *ERCC4* gene predicts for a possibly damaging effect on the protein structure (46). *ERCC4*, also known as *XPF*, is involved in the nucleotide excision repair pathway and plays an important role in recombination repair, mismatch repair, and possibly immunoglobulin class switching. We also found that the rs4645878 variant (G125A) of the *BAX* promoter showed association with decreased CLL risk (OR, 0.73; 95% CI, 0.58–0.92; P = 0.0087), and it is included in a haplotype with significant association with low CLL risk (OR, 0.76; 95% CI, 0.60–0.95; P = 0.0188). The presence of this gene variant has been correlated with lower *BAX* transcript and protein levels in CLL cells (33, 36), and in our study the rs11667351 variant, which is in linkage disequilibrium with rs4645878, was also associated with lower *BAX* mRNA levels in lymphocytes. The role of BAX in the CLL pathogenesis is not clear because CLL cells seem to have higher BAX levels than normal B lymphocytes (35). On the other hand, our observation of a protective effect for the G125A change on CLL risk is paradoxical with its apparent relationship with more advanced stage, treatment resistance, and short overall survival of CLL patients observed in some studies (36–38), suggesting a potential different role for this variant in the development and progression of the disease. Among these 27 SNPs (Table 2), it is interesting that the rs2228014 variant in the *CXCR4* gene, rs4647698 in *CASP3*, and rs2009658 in *LTATNF* are located at 2q21, 4q35.1, and 6p21.3, respectively, in genomic positions close to the chromosome regions that have been recently identified as susceptibility loci for CLL (8, 11).

Finally, 22 haplotypes in *APAF1, BAK1, BAX, BCL2, CASP1, CASP3, CASP10/CASP8, CCL2, CCR7, CXCL12, IL16, IL6, IL1A, LTA/TNF*, and *TNFRSF13B* genes were significantly associated with CLL risk. Allelic variants in some of these genes may play a role in CLL pathogenesis because they were associated with different transcript levels in our analysis and previous studies have also observed the association of individual SNPs in these genes with CLL risk (29–32, 34).

Rudd and colleagues (13), in a large study of nonsynonymous SNP in CLL patients, detected association of three gene variants implicated in the DNA damage response axis and CLL risk. The design of our study and that of Rudd and colleagues have some differences in the gene and SNP selection. Thus, in addition to a number of nonsynonymous SNP, we included also polymorphisms in regulatory gene regions, tag SNPs, and SNPs with a minor allele frequency >5%. Therefore, the two studies are complementary in the pathways and strategies investigated. The findings in our study emphasizing the association between allelic variants in apoptotic and immunomodulatory genes may reflect the complexity of the genetic variants involved in the susceptibility for CLL. However, the *CASP8* and *CCNH* nonsynonymous SNPs included in both studies were associated with CLL risk only in our study. This association was very strong ($P < 4.10^{-6}$) even after correction for multiple testing. These differences between the two studies done suggest that CLL genetic susceptibility may be related to population differences. In fact, the minor allele frequencies of the *CASP8* and *CCNH* variants in our control group (17% and 35%, respectively) were significantly higher than in the control group of Rudd's study based on a British population (13% and 21%, respectively; $P < 10^{-5}$). This finding may be similar to the geographic variation observed in the immunoglobulin gene usages in CLL patients of Mediterranean and Northern European areas, highlighting the potential population-based differences in the molecular pathogenesis of the disease (47–49).

In summary, the strong association between the allelic variants of genes related to apoptosis and immunoregulation and CLL risk observed in our study suggests that they may influence the development of the disease. The confirmation of these results requires its replication in larger studies including pooled analyses of larger data sets.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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