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Common single nucleotide polymorphisms in immunoregulatory genes and multiple myeloma risk among women in Connecticut

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Abstract

In light of the relationship between immune system dysregulation and multiple myeloma (MM) risk, we investigated whether genetic variation in 92 immune function genes among 77 gene regions are associated with MM susceptibility in a population-based case-control study (108 cases and 482 controls) conducted among Caucasian women in Connecticut. Tagging single-nucleotide polymorphisms (SNPs; N=870) were selected using a pairwise linkage-disequilibrium based algorithm. Odds ratios (ORs) and 95% confidence intervals (CIs) for SNP genotypes were estimated using unconditional logistic regression. Tests of association for gene regions were conducted using the minP test. We applied the false discovery rate (FDR) method to the minP test results as a means of controlling for multiple comparisons. The *CD4* gene region located on 12p13-q13 (minP=0.0009), had an FDR value < 0.1. In this region, a total of six tag SNPs in two genes (*CD4* and *LAG3*) were significantly associated with MM risk (P_{trend}<0.05), with the strongest association observed for the *CD4* variant rs11064392 ($OR_{AG/GG}$=2.53, 95% CI=1.59–4.02). Our findings suggest that genetic variation in *CD4* may influence susceptibility to MM. Additional studies are needed to replicate these findings and, more generally, to explore the manner in which genes receptors may influence the pathogenesis of this poorly understood malignancy.

Keywords

immunoregulatory genes; multiple myeloma; single nucleotide polymorphism; *CD4*; *LAG3*

1. Introduction

Multiple myeloma is a B-cell malignancy characterized by an accumulation of predominantly immunoglobulin G (IgG)-producing monoclonal plasma cells in the bone marrow, and accounts for 10–15% of all hematologic malignancies and 1% of all cancers in Western countries. The etiology of multiple myeloma is poorly understood; the established risk factors are African-American ethnicity, obesity, and family history of multiple myeloma.

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Increased risk of multiple myeloma has been observed among those who have a family history of multiple myeloma, suggesting a genetic component in multiple myeloma development [1–2]. Also, a number of studies have shown that changes in the immuoregulatory role of T cells may contribute to the development of multiple myeloma [3–5]. Several case-control studies, including our study of multiple myeloma in Connecticut women [6], have investigated genetic variants in immune-related genes as possible susceptibility loci [6–10]. Associations have been reported with *IL1A −889C>T* [9], *IL4R −28120T>C* [6], *IL6 −572G>C* and *−373* A_nT_n [8], *FCGR2A −120A>G* [6], *TNFA −308G>A* [7,10], and *LTA 252A>G* [10]; however, findings across studies are inconsistent. These studies typically genotyped only a limited number of candidate SNPs, and did not include comprehensive investigations of genetic variation across the gene of interest through the use of tag SNPs.

To comprehensively investigate whether genetic variations in immune pathway genes play a role in multiple myeloma development, we genotyped 870 tag SNPs representing genetic variation across 92 genes.

2. Materials and Methods

2.1. Subjects

This population-based case-control study has been previously described [11]. Briefly, cases were women residing in Connecticut newly diagnosed between 1996 and 2000 with histologically-confirmed multiple myeloma, aged 21 to 84 years, with no previous diagnosis of cancer except nonmelanoma skin cancer and alive at time of interview. In-person interviews were obtained from 183 of the 323 eligible cases. Population-based female controls (n=691) aged 21 to 84 years, were recruited for a parallel study of non-Hodgkin lymphoma, by random digit dialing methods for those below age 65 and from Health Care Financing Administration (Center for Medicare and Medicaid Services) files for those 65 years and older. The participation rates for cases, controls below age 65, and controls aged 65 years and older were 57%, 69% and 47%, respectively. The study was approved by the Yale University School of Medicine's Human Investigations Committee, the Connecticut Department of Public Health, and the National Cancer Institute's Special Studies Institutional Review Board.

2.2. Genotyping

Genomic DNA for genotyping was extracted from peripheral blood or buccal cells using a phenol-chloroform-extraction method. Based on previous experimental as well as epidemiological studies, immune related genes and gene-regions were composed of three main categories, i.e., Th1–Th2, TNF-NFKB, other immune related including innate immunity genes. Tag SNPs were chosen from the designable set of common SNPs (minor allele frequency >5%) that were genotyped in the Caucasian population sample of the HapMap Project (Data Release 20/Phase II, NCBI Build 35 assembly, dpSNPb125) using Tagzilla (http://tagzilla.nci.nih.gov), which implements a tagging algorithm based on the pairwise binning method. For each gene, SNPs between 20 kb 5' of the start of transcription (exon 1) and 10 kb 3' of the end of the last exon were grouped using a binning threshold of r^2>0.8. When multiple transcripts were available for loci, the primary transcript was assessed. Tag SNPs were genotyped using a GoldenGate assay (www.illumina.com), at the National Cancer Institute's Core Genotyping Facility (Gaithersburg, MD). The GoldenGate assay included 1536 tag SNPs from candidate genes in multiple pathways. Only the tag SNPs (n=926) in immune related genes were selected for this analysis. Blinded duplicate and replicate samples were interspersed throughout the genotyping plates to assess quality control. After 56 tag SNPs with a discordant rate >5% for QC samples or a completion rate

<90% were excluded, the remaining 870 tag SNPs (Supplementary Table 1) in 92 targeted genes (77 gene regions, Supplementary Table 2) were included in this study.

Genotyping for immune related genes was successful for 125 cases and 515 controls (2 controls were excluded by the criteria of <90% sample completion rate). After restricting the study to Caucasian subjects only, 108 non-Hispanic female cases and 482 non-Hispanic female controls remained for analysis. Hardy-Weinberg equilibrium (HWE) for each tag SNP was tested among the controls with a Pearson χ^2-test or a Fischer's exact test if any of the controls' cell counts were less than five. A total of 23 out of 870 tag SNPs (2.6 %) deviated from HWE ($p<0.05$). Quality control data were rechecked for all of the assays not in HWE, of which all were found to be accurate and consequently included in the final analysis.

2.3. Statistical analysis

All statistical procedures were conducted using SAS (Cary, NC) unless otherwise indicated.

To assess the significance of the association between each gene region and multiple myeloma, we used a minP test to assess the minimal p-value in each gene region using a permutation procedure that adjusts for correlations between SNPs as well as the number of SNPs genotyped for each gene region [12]. Under the complete null hypothesis, case-control status and age were permuted 10,000 times to generate a set of permutation datasets which then could be used to obtain a reasonable and accurate estimate of the minP, which is the proportion of generated p-values that are equal to or smaller than the observed p-value. Additionally, the false discovery rate (FDR) method of Benjamini and Hochberg [13] was applied to the minP test results to account for multiple comparisons. We considered FDR <0.1 as being noteworthy.

Unconditional logistic regression was used to estimate the age-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for the association between selected SNPs and multiple myeloma risk. Independently for each tag SNP, the homozygote of the common allele was used as the reference group while adjusting for age (<50, 50–59, 60–69, and ≥70). Gene-dose effects were estimated by a linear trend test based on the number of variant alleles present (0, 1, and 2).

To consider linkage disequilibrium between SNPs, we reviewed the haplotype structure for each significant gene region among controls using Haploview version 4.0 (www.broad.mit.edu/mpg/haploview). Haplotype analyses among subjects were performed using the haplo.stats statistical package (http://mayoresearch.mayo.edu/mayo/research/schaid_lab/software.cfm) in the R program (v. 2.2.1: http://www.r-project.org). Haplotype frequencies were estimated from genotype data using the expectation-maximization algorithm, excluding those with frequencies less than 1%, and were evaluated by the global score test. Age-adjusted (<50, 50–59, 60–69, and ≥70 years) haplotype ORs and 95% CIs were also calculated in a co-dominant effect model. We also performed analyses of consecutive 2-, 3- and 4-SNP haplotype windows for the gene regions with minP values < 0.05.

A pathway analysis has been conducted using GSEA-SNP program to investigate which gene sets and pathways are significantly associated with multiple myeloma. The GSEA-SNP method has been implemented in R (www.r-project.org) by extending the original GSEA code [14]. After excluding SNPs with unknown gene annotation, 827 SNPs remained for the GSEA-SNP analysis. The SNPs-in pathways analysis was conducted using the C2 curated gene sets for pathway annotation (http://www.broad.mit.edu/gsea/msigdb/collections.jsp#C2). This catalogue contained 1892

gene sets, which was converted into 1982 SNP sets. SNP sets with less than 15 SNPs or more than 500 SNPs were excluded, leaving 407 sets for the analysis.

3. Results

Selected characteristics of our subjects are shown in Table 1. Cases were slightly older than the controls in our study population of white females (P=0.04). Cases and controls had similar body mass index, education, marital status and smoking status (P>0.05).

Six out of 77 gene regions were significantly (P<0.05) associated with multiple myeloma using the minP test (Table 2). However, only the *CD4* gene region remained noteworthy after accounting for multiple tests using the FDR method (min$P$=0.0009; noteworthy at a level of FDR control of 0.07).

In this region, a total of six SNPs in two genes (*CD4* and *LAG3*) out of 20 SNPs were significantly associated with risk (P_{trend}<0.05) (Table 3), with the strongest association observed for rs11064392 (−10912A>G; $OR_{AG/GG}$=2.53, 95% CI=1.59–4.02) located at −259 base pairs upstream from the starting point of transcription. The three SNPs in the *CD4* (rs7296859, rs2707212, and rs107538) were all in linkage disequilibrium with closely located rs11064392 (D'=1.0, 1.0, and 0.95, respectively; r^2=0.06, 0.05, and 0.18, respectively). Supplementary Table 3 provides the allele distribution among cases and controls, and multiple myeloma risk associated with all analyzed tag SNPs.

Haplotype analyses supported the single SNP analysis and did not yield new results (Supplementary Tables 4 and 5).

Additional GSEA-SNP analysis to evaluate which gene sets and pathways are significantly associated with multiple myeloma showed that total 5 gene sets or pathways were significant at the nominal significance level of 0.01; MOREAUX_TACI_HI_VS_LOW_UP (*TNFRSF13B*, *REL*, *SCNN1A*, *TNF*, and *TNFRSF8*), TALL1PATHWAY (*TRAF2*, *RELA*, *TNFRSF13B*, *TNFRSF17*, *NFKB1*, *TNFSF13B*, *TRAF6*, and *CHUK*), IL2PATHWAY (*JAK3* and *IL2*), CHESLER_BRAIN_NEURAL_HIGH_GENES (*GPR162*, *CD4*, *LAG3*, and *LEPREL2*), IL22BPPATHWAY (*IL10RA* and *JAK3*). We note that the results of GSEA-SNP analysis are basically consistent with those from gene- or gene region-level analysis; the significant gene sets and pathways tended to include significant genes or gene-regions shown in Table II, III, and Supplementary Table 5. Detailed results are shown in Supplementary Table 6.

4. Discussion

From our investigation of variation in immune function genes in a case-control study of multiple myeloma, we observed strong evidence that genetic variants in the *CD4* region are associated with disease risk.

The CD4 receptor is crucial for appropriate antigen responses of CD4+ T cells including regulatory T cells [15] and anti-tumor immune response [16]. Multiple myeloma has been reported to accompany various T cell abnormalities including quantitative and functional defects of CD4+ T cells [3–5,17,18]. Therefore, it is biologically plausible that genetic variations in the *CD4* gene may be related to dysfunctional T-cell responses resulting in a loss of control over B-cell proliferation.

In single SNP analysis, the strongest association we observed was CD4 rs11064392, which is located in the enhancer region. The *CD4* enhancer and promoter are known as the major regions for regulation of CD4 transcription in the mature Th cell [19,20]; thus CD4

rs11064392 may affect the promoter activity and level of expression of CD4. Using HapMap data (http://www.hapmap.org/), we verified that SNPs ranging from enhancer/promoter to intron 1 in CD4 were in linkage disequilibrium. Although CD4 rs11064392 has not been investigated for its functional significance, another linked variant in promoter (*CD4 −181C>G* (rs2855534); D'=1.0 and r^2=0.38 with rs11064392: calculated using HapMap data) was reported to affect stimulated promoter activity *in vitro* [21]. In addition, SNPs other than rs11064392 or a pyrimidine-rich pentanucleotide repeat polymorphism (CD4-1188 (TTTTC)) in enhancer/promoter region have been reported to be associated with autoimmune diseases (i.e., rheumatoid arthritis, systemic lupus erythematosus, and vitiligo) [22,23], multiple sclerosis that is a T-cell mediated disease of the central nervous system [24], and insulin-dependent diabetes mellitus (IDDM) [25]. It is also noteworthy that recent reports have shown that a non-synonymous SNP (*CD4* Trp240Arg), common among individuals of African descent but non-polymorphic among Caucasian, influence human immunodeficiency virus (HIV-1) infection risk [26,27]. Further study is warranted to verify the exact locus with functional significance in this region, and to elucidate the molecular mechanisms with regard to multiple myeloma development.

We also observed associations with two SNPs in intron regions of *LAG3* (lymphocyte activation gene-3), which are located within ~20k base pairs 5' upstream of the candidate *CD4* gene. Given that the intron regions do not belong to enhancer regions for *CD4* transcription [21], and the two SNPs are not in linkage disequilibrium with the SNPs in the enhancer/promoter of CD4, it is plausible that variant in *LAG3* gene itself may play a role in susceptibility of multiple myeloma. The *LAG3* gene encodes a suspected inhibitor of CD4+ and CD8+ T cell activation, which has recently been suggested as a new marker of T cell induced B cell activation [28]. However, no available data on functional significance of any *LAG3* SNPs prevents us from further inference.

To our knowledge, this study represents the most comprehensive investigation of immune pathway genes and multiple myeloma risk conducted to date, and is the first to report an association with *CD4* and *LAG3*. Given the small sample size of our study and the large number of genes investigated, we must consider the possibility that these findings are due to chance. The fact that our observed association with the *CD4* region remained noteworthy upon FDR adjustment argues against a chance finding. The results from GSEA-SNP analysis might also support the role of *CD4* region in multiple myeloma development. Nonetheless, these results require confirmation in other study populations before meaningful inferences regarding causation can be drawn.

On the other hand, important associations may have been missed due to low statistical power caused by small sample size. We found no association between the SNPs genotyped in our study (i.e., *IL1A* rs2856836, *IL4R* rs2239347 and rs3024578, *IL6* rs6949149 and *FCGR2A* rs12139150) that are in high LD (r^2>0.8; Caucasian data of the SNP500 Cancer database (http://snp500cancer.nci.nih.gov/)) with the SNPs suggested to play a role in multiple myeloma development in previous studies (i.e., *IL1A −889C>T*, *IL4R −28120T>C*, *IL6 −572G>C*, and *FCGR2A −120A>G*, respectively) and multiple myeloma risk (p>0.1). Although the associations suggested previously were not consistent across the studies, low statistical power may at least partly explain no significant association between the *IL1A* rs2856836, *IL4R* rs2239347 and rs3024578, *IL6* rs6949149 and *FCGR2A* rs12139150, and multiple myeloma risk. Additionally, only women subjects may be another limitation.

In conclusion, our results suggest that genetic polymorphisms in *CD4* and *LAG3* are associated with multiple myeloma risk. Additional studies are needed to replicate these findings and, more generally, to explore the manner in which these genes may influence the pathogenesis of this poorly understood malignancy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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TABLE I

Selected characteristics of multiple myeloma cases (n=108) and population controls (n=482)

	Cases (%)	Controls (%)	*p*[a]
Age (years)			
<50	8 (7.4)	91 (18.9)	0.04
50–59	22 (20.4)	89 (18.5)	
60–69	30 (27.8)	109 (22.6)	
≥70	48 (44.4)	193 (40.0)	
Body mass index (kg/m^2)			
<25	55 (50.9)	277 (57.5)	0.46
25–30	33 (30.6)	128 (26.6)	
≥30	20 (18.5)	77 (16.0)	
Education			
<12 yrs	15 (13.9)	52 (10.8)	0.09
high school graduate	37 (34.3)	119 (24.7)	
vocational/ some college	32 (29.6)	144 (29.9)	
college degree	15 (13.9)	97 (20.1)	
graduate/ professional degree	9 (8.3)	70 (14.5)	
Marital status			
married / living as married	58 (53.7)	285 (59.1)	0.20
widowed	28 (25.9)	119 (24.7)	
separated/ divorced	13 (12.0)	60 (12.4)	
never married	9 (8.3)	18 (3.7)	
Smoking (pack years)			
never smoker	57 (52.8)	221 (45.9)	0.59
<6.3	11 (10.2)	65 (13.5)	
6.3–16.5	10 (9.3)	61 (12.7)	
16.5–33.0	13 (12.0)	60 (12.4)	
>33.0	14 (13.0)	74 (15.4)	

[a] chi-square test

TABLE II

Immunoregulatory gene regions that are significantly associated with multiple myeloma

Target Gene Region	Chromosome	# SNPs	Lowest P_{trend}[a]	min$P$[b]	FDR[c]
CD4	12 p13-q13	20	0.0001	**0.0009**	**0.07**
JAK3	19 p13.1-p12	14	0.0021	**0.022**	0.61
TNFRSF13B	17 p11.2	26	0.0022	**0.031**	0.61
CHUK	10 q24-q25	4	0.013	**0.040**	0.61
RELA	11 q13	4	0.014	**0.043**	0.61
IL12RB2	1p31.3-p31.2	13	0.0056	**0.047**	0.61

[a] adjusted for age;

[b] case-control status and age were permutated 10,000 times;

[c] The false discovery rate (FDR) method was applied to the minP test results to account for multiple comparisons.

Note. Those gene regions with minP less than 0.05 are listed, but only one gene region was noteworthy (FDR<0.1)

Table III

Tag SNPs that are associated with multiple myeloma in the *CD4* gene region

Gene	SNP (rs#)	Allele (reference genotype)	Heterozygotes			Homozygotes			Dominant Model			Trend		
			OR[a]	95% CI	*P*	OR[a]	95% CI	*P*	OR[a]	95% CI	*P*	OR[a]	95% CI	*P*
LAG3	rs2365094	*IVS3−91G>C (GG)*	1.44	0.92–2.25	0.11	2.29	1.12–4.72	**0.02**	1.57	1.03–2.39	**0.04**	1.49	1.08–2.04	**0.01**
	rs3782735	*IVS5+362G>A (GG)*	0.80	0.51–1.24	0.32	0.36	0.16–0.81	**0.01**	0.69	0.45–1.07	0.09	0.67	0.48–0.93	**0.02**
CD4	rs7296859[b]	*−15140C>G (CC)*	0.64	0.41–1.00	**0.05**	0.48	0.22–1.06	**0.07**	0.61	0.40–0.93	**0.02**	0.67	0.48–0.94	**0.02**
	rs11064392	*−10912A>G (AA)*	2.48	1.55–3.98	**0.0002**	3.68	0.60–22.6	0.16	2.53	1.59–4.02	**0.0001**	2.36	1.53–3.63	**0.0001**
	rs2707212[b]	*IVS1+2306C>T (CC)*	0.49	0.31–0.77	**0.002**	0.74	0.36–1.52	0.41	0.53	0.35–0.81	**0.004**	0.68	0.49–0.96	**0.03**
	rs1075838[b]	*IVS1− 3523T>C (TT)*	1.04	0.66–1.66	0.86	2.46	1.31–4.60	**0.005**	1.27	0.82–1.96	0.29	1.44	1.05–1.97	**0.02**

[a] adjusted for age;

[b] These three SNPs (rs7296859, rs2707212, and rs107538) were in linkage disequilibrium with rs11064392 (D'=1.0, 1.0, and 0.95, respectively; r^2=0.06, 0.05, and 0.18, respectively).