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Two Single-Nucleotide Polymorphisms in the 5′ and 3′ Ends of the Osteopontin Gene Contribute to Susceptibility to Systemic Lupus Erythematosus

S. D'Alfonso,[1] N. Barizzone,[1] M. Giordano,[1] A. Chiocchetti,[1] C. Magnani,[1] L. Castelli,[1] M. Indelicato,[2] F. Giacopelli,[3] M. Marchini,[4] R. Scorza,[4] M. G. Danieli,[5] M. Cappelli,[5] S. Migliaresi,[6] B. Bigliardo,[6] M. G. Sabbadini,[7] E. Baldissera,[7] M. Galeazzi,[8] G. D. Sebastiani,[9] G. Minisola,[9] R. Ravazzolo,[10] U. Dianzani,[1] and P. Momigliano-Richiardi[1]

Objective. **To test the association of osteopontin (OPN) polymorphisms with systemic lupus erythematosus (SLE).**

Methods. **The coding 5′ and 3′ flanking regions of the OPN gene were scanned for polymorphisms by denaturing high-performance liquid chromatography. A case–control association study was performed in 394 Italian SLE patients and 479 matched controls. OPN serum levels were determined by enzyme-linked immunosorbent assay in 40 patients and 124 controls, and the mean levels were compared between the different OPN genotypes.**

Results. **Among the 13 detected single-nucleotide polymorphisms (SNPs), alleles −156G (frequency 0.714 versus 0.651; $P = 0.006$, corrected P [P_{corr}] = 0.036) and +1239C (0.377 versus 0.297; $P = 0.00094$, $P_{corr} = 0.0056$) were significantly increased in the SLE patients compared with the controls. The presence of the associated allele in single or double dose conferred an odds ratio (OR) of 2.35 (95% confidence interval [95% CI] 1.38–4.02) for SNP −156 and an OR of 1.57 (95% CI 1.16–2.13) for SNP +1239. These effects were independent of each other, i.e., not a consequence of linkage disequilibrium between the 2 alleles. The risk associated with a double dose of susceptibility alleles at both SNPs was 3.8-fold higher (95% CI 2.0–7.4) relative to the complete absence of susceptibility alleles. With regard to individual clinical and immunologic features, a significant association was seen between lymphadenopathy and −156 genotypes (overall $P = 0.0011$, $P_{corr} = 0.046$). A significantly increased OPN serum level was detected in healthy individuals carrying +1239C ($P = 0.002$), which is indicative of an association between the SLE susceptibility allele and OPN levels.**

Conclusion. **These data suggest the independent effect of a promoter (−156) and a 3′-untranslated region (+1239) SNP in SLE susceptibility. We can speculate that these sequence variants (or others in perfect linkage disequilibrium) create a predisposition to high production of OPN, and that this in turn may confer susceptibility to SLE.**

Systemic lupus erythematosus (SLE) is an autoimmune disease with a multifactorial etiology that is characterized by impaired T cell responses and dysregulation of B cell activation, leading to B cell hyperactivity and production of autoantibodies. Several lines of evi-

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[1]S. D'Alfonso, PhD, N. Barizzone, BD, M. Giordano, PhD, A. Chiocchetti, PhD, C. Magnani, MD, L. Castelli, BD, U. Dianzani, MD, P. Momigliano-Richiardi, PhD: Department of Medical Sciences and IRCAD, University of Eastern Piedmont, Novara, Italy; [2]M. Indelicato, BD: University of Eastern Piedmont, Novara, and University of Catania, Catania, Italy; [3]F. Giacopelli, PhD: G. Gaslini Institute, Genoa, Italy; [4]M. Marchini, PhD, R. Scorza, MD: IRCCS Ospedale Maggiore, and Milano University, Milan, Italy; [5]M. G. Danieli, MD, M. Cappelli, MD: Università Politecnica delle Marche, Ancona, Italy; [6]S. Migliaresi, MD, B. Bigliardo, MD: Napoli Second University, Naples, Italy; [7]M. G. Sabbadini, MD, E. Baldissera, MD: "Vita e Salute" University, and IRCCS San Raffaele Hospital, Milan, Italy; [8]M. Galeazzi, MD: Siena University, Siena, Italy; [9]G. D. Sebastiani, MD, G. Minisola, MD: San Camillo-Forlanini Hospital, Rome, Italy; [10]R. Ravazzolo, MD: G. Gaslini Institute, and Genova University, Genoa, Italy.

Address correspondence and reprint requests to S. D'Alfonso, PhD, Department of Medical Sciences, Eastern Piedmont University, Via Solaroli 17, 28100 Novara, Italy. E-mail: dalfonso@med.unipmn.it.

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dence suggest that early T cell activation gene 1, or osteopontin (OPN), may have a role in the pathogenesis of SLE as well as other autoimmune disorders.

OPN is a 60-kd secreted phospho-protein functioning as a free cytokine in body fluids or as an immobilized extracellular matrix molecule in mineralized tissues (1,2). OPN may influence autoimmune diseases through its immunoregulatory effects, enhancing the proinflammatory Th1 cell response and inhibiting the Th2 responses (3,4). Moreover, OPN stimulates T cell proliferation, interferon-γ production, and CD40 ligand expression, which in turn sustains the proliferation of B cells and production of antibodies (5). In mice, transgenic overexpression of OPN on a nonautoimmune background produces an autoimmune pattern with accumulation of B1 lymphocytes, hypergammaglobulinemia, and production of autoantibodies, including anti–double-stranded DNA antibodies (6). In humans, the serum level of OPN is increased in patients with SLE (7), and lupus nephritis is associated with increased OPN expression in renal tissue (8,9). A synonymous sequence variation in exon 7 ($Ala^{236}Ala$) of the OPN gene was significantly associated with SLE in a sample of 81 American patients with SLE (10).

A pathogenetic role of OPN has been strongly suggested in MRL-*lpr*/*lpr* mice (11–14) and in patients affected by the autoimmune lymphoproliferative syndrome (ALPS) (15); in both mice and patients with ALPS, an autoimmune pattern partially similar to SLE develops, associated with hypergammaglobulinemia, lymphadenopathy and/or splenomegaly, and peripheral expansion of CD4/CD8 double-negative T cells. In both mice and humans, the disease has been attributed to inherited mutations targeting the function of the Fas death receptor involved in switching of the immune response (16,17). Several studies have suggested that high OPN levels contribute to the disease. Observations in mice revealed that 1) CD4/CD8 double-negative T cells constitutively expressed high levels of OPN, and this elevation of OPN coincided with the appearance of immunologic abnormalities (11–13), and 2) onset of polyclonal B cell activation was delayed by crossing MRL-*lpr*/*lpr* with $opn^{-/-}$ mice (13,14), while observations in humans demonstrated that 3) high OPN levels directly correlated with hypergammaglobulinemia (15), and 4) 2 SNPs in the OPN 3′-untranslated region (3′-UTR) (+1083A/G and +1239A/C) were associated with high production of OPN and increased the risk of ALPS by 8-fold (15). Intriguingly, families of ALPS patients display an increased frequency of several common autoimmune diseases, including SLE, which suggests that they carry an autoimmune-prone genetic background.

The present study sought to test the involvement of OPN in SLE. To this purpose, the coding 5′ and 3′ flanking regions of the OPN gene were screened for sequence variations in SLE patients. The identified SNPs were tested for an association with SLE in a large panel of Italian patients and controls. The frequencies of the associated SNPs were then compared in patient subsets subdivided according to their clinical and immunologic features. Finally, the association of SLE-associated OPN genotypes with OPN serum production was investigated.

PATIENTS AND METHODS

Subjects. A total of 394 Italian patients with SLE (7.5:1 female:male ratio) were included in this study. All patients fulfilled ≥4 of the American College of Rheumatology 1997 revised criteria for the classification of SLE (18). Enrollment was completed when the patients provided their informed consent. The clinical and immunologic features of the SLE patients are shown in Table 1. A random panel of 479

Table 1. Clinical and immunologic features of the patients with SLE*

Feature	% of patients
Clinical manifestation	
Malar rash	40
Photosensitivity	35
Discoid lesion	12
Serositis†	30
Oral ulcers	27
Arthritis	65
Glomerulonephritis	31
Autoimmune hemolytic anemia	11
Raynaud's phenomenon	32
Lymphadenopathy	17
Leukopenia and/or lymphopenia‡	53
Thrombocytopenia§	23
Neurologic involvement¶	16
Antiphospholipid syndrome	9
Autoantibodies	
ANA	89
Anti-dsDNA	76
Anti-La(SSB)	6
Anti-Ro(SSA)	23
Anti-RNP and/or Sm	23
Antiphospholipids#	31
Rheumatoid factor	19

* SLE = systemic lupus erythematosus; ANA = antinuclear antibodies; anti-dsDNA = anti–double-stranded DNA.
† Includes pericarditis and/or pleuritis.
‡ Leukocyte count $<4{,}000/mm^3$; lymphocyte count $<1{,}500/mm^3$.
§ Platelet count $<100{,}000/mm^3$.
¶ Includes seizures and/or psychosis and/or transient ischemic attack.
# Presence of anticardiolipin antibodies and/or lupus anticoagulant activity.

Caucasian individuals from Italy (comprising medical students, university and hospital staff, and blood donors) were used as control subjects (2.6:1 female:male ratio). The regional origin of the patients and controls was similar and included ~65% of individuals residing in northern Italy and the remaining in central-southern Italy. Individuals with Sardinian ancestors were excluded.

Search for sequence variations in the OPN gene. The OPN gene was amplified from genomic DNA as 12 polymerase chain reaction (PCR) fragments covering a total of 4,401 bp, including the 7 exons, the intron-exons boundaries, 2,083 bp upstream of the transcription start site, and 383 bp in the 3′-UTR region. PCR primers were designed on the genomic DNA GenBank sequence D14813 (version GI: 506341). The search for sequence variations was performed by denaturing high-performance liquid chromatography (DHPLC) on an automated HPLC instrument (WAVE; Transgenomic, Santa Clara, CA) as previously reported (19).

The temperature required for successful resolution of heteroduplex molecules was determined using a specific program (available at the Web site http://insertion.stanford.edu/melt.html). Samples were analyzed at the predicted temperatures (RTm) and at RTm +2°C, as recommended by the software authors (20), and were eluted from the column using a linear acetonitrile gradient at a constant flow rate of 0.9 ml/minute. The gradient was created by mixing elution buffers A (0.1*M* triethylamine acetate buffer [TEAA], pH 7) and B (25% acetonitrile in 0.1*M* TEAA, pH 7). The start and end points of the gradient depended on the size of the PCR fragments. Primers used for amplification and DHPLC conditions are available from the authors upon request. The PCR products displaying a heteroduplex peak were sequenced and compared with the sequence of a homozygous sample.

Sequencing. Prior to sequencing, unincorporated dNTPs and primers were removed by 0.5 units of shrimp alkaline phosphatase and 5 units of exonuclease I (both from Amersham, Arlington Heights, IL) at 37°C for 30 minutes, after which the enzymes were inactivated by incubation at 80°C for 15 minutes. Samples were sequenced in both directions on an Applied Biosystems 3100 Genetic Analyzer using the BigDye terminator cycle-sequencing reaction kit (Applied Biosystems, Foster City, CA).

Genotyping of OPN SNPs. In a preliminary screening on a small panel of patients and controls, all of the detected SNPs were genotyped by primer extension followed by HPLC analysis on the WAVE (Transgenomic) instrument (21). The 6 SNPs selected after this analysis were tested on a larger panel by the SNaPshot method on an ABI 3100 Genetic Analyzer (Applied Biosystems). Because of random technical reasons, different numbers of individuals, ranging from 366 to 394 patients and from 420 to 479 controls, were genotyped for each SNP. Two SNaPshot reactions were designed, one for the 4 SNPs in the 5′ region (−616G/T, −443T/C, −156G/GG, −66T/G) amplified in the same amplicon, and one for the 2 SNPs in the 3′ region (+1083A/G and +1239A/C), also amplified in one fragment. Samples were electrophoresed on the ABI 3100 Genetic Analyzer. Resulting electropherograms were analyzed using ABI GeneScan, version 3.7 software. SNaPshot conditions are available from the authors upon request.

Enzyme-linked immunosorbent assay (ELISA). Serum OPN concentrations were evaluated by a capture ELISA in accordance with the protocol provided by the manufacturer (Calbiochem, La Jolla, CA). The optical density was measured at 450 nm with a microplate reader (Bio-Rad, Hercules, CA). The I-smart program was used to create a regression curve.

Statistical analysis. The statistical significance of the difference in gene and genotype frequencies between patients and controls was evaluated for each polymorphism using the chi-square test with Yates' correction. When required by the small number of expected cases, Fisher's 2-tailed exact test was used. When specified, P values were corrected (P_{corr}) for the number of comparisons, according to the Bonferroni method.

Maximum-likelihood estimation of haplotype frequencies and corresponding standard deviations were calculated from data with unknown gametic phase, using an expectation-maximization (EM) algorithm (Arlequin, version 2.0 [22]). The significance of the difference in single haplotype frequencies between patients and controls was evaluated by analyzing the standard deviation, in which a P value of less than 0.05 corresponds to patient/control haplotype frequencies that do not overlap when considering frequencies of ±2 SD. The significance of the overall difference in haplotype frequencies between patients and controls was evaluated by estimating the value as follows: L= (L1 + L2) − L3, where L1, L2, and L3 are the −2 log likelihood estimated in the patients (L1), the controls (L2), and patients + controls (L3). The L value follows a chi-square distribution with degrees of freedom (df) corresponding to the total number of estimated haplotypes −1. Pairwise linkage disequilibrium was evaluated by calculating the D′ (23) and r^2 (24) values.

The association of each polymorphism with the disease was measured by the odds ratio (OR) and its 95% confidence interval (95% CI), while the joint effect of different polymorphisms was tested by multivariate logistic regression (25) using SAS Proc Logistic (SAS Institute, Cary, NC). The departure of the observed ORs from those predicted under additivity of effects (additive-effects model) was measured by computing the RERI index and its 95% CI (26). The departure from a multiplicative model was tested by inclusion of an interaction term in the logistic regression model (27). In addition, data were made to fit a parametric model (reparametrization) by creating a set of dummy variables for the different possible combinations of genotypes (26); this procedure was used for descriptive purposes (as in Table 6), for computing the RERI as suggested by Hosmer and Lemeshow (26), and also for estimating the departure from a multiplicative model when the product term could not fit because of lack of data.

The distribution of OPN serum levels in the study population was right-skewed. A logarithmic transformation of the OPN serum levels was introduced to approximate a normal distribution. Relevant comparisons were performed using Student's t-test.

RESULTS

Analysis of the OPN gene-sequence variations. The 7 exons, the exon-intron boundaries, 2,083 bp of the 5′ flanking region, and 383 bp of the 3′-UTR of the OPN

Table 2. Osteopontin single-nucleotide polymorphisms (SNPs) identified in patients with systemic lupus erythematosus*

Variation	Location	Sequence	No. of heterozygotes	Accession no. (ref.)†
−1748A/G‡	5′flanking	aattttcA/Ggacttcc	7	rs2728127 (28)
−1282A/G‡	5′flanking	tagtccaA/Gtctgcat	1	This study
−616G/T‡	5′flanking	tcatcctG/Tctctcag	4	rs2853744 (28)
−443T/C‡	5′flanking	gcaagttT/Ctctgaac	10	(28)
−156G/GG‡	5′flanking	cgtttttG/GGtttttttt	7	rs7687316 (28)
−66T/G‡	5′flanking	caatctcT/Gccgcctc	5	This study
IV3−42A/C§	Intron 3	tccctacA/Ctttcttt	1	This study
+282T/C (Asp^{80}Asp)¶	Exon 6	atgatgaT/Cgaccatg	14	rs4754 (29)
+351T/C (Thr^{103}Thr)¶	Exon 6	ttttcacT/Cccagttg	2	This study
+750 C/T (Ala^{236}Ala)¶	Exon 7	ggaaagcC/Taatgatg	14	rs11226616 (29)
+1083A/G¶	3′-UTR	aataactA/Gatgtgtt	11	rs1126772 (29)
+1158A/G¶	3′-UTR	tatgtctA/Gtgttcat	1	This study
+1239A/C¶	3′-UTR	atgtagaA/Cgcaaaca	14	rs9138 (29)

* Sequence variations are shown in upper case. Heterozygotes were detected by denaturing high-performance liquid chromatography in 23 patients.
† Accession number in the National Center for Biotechnology Information SNP data bank (http://www.ncbi.nlm.nih.gov/SNP). Thirteen additional SNPs are reported in the database in the sequences screened in this report (namely, rs3891314, rs2853745, rs2853747, rs2859696, rs2728128, rs2853748, rs2859697, rs4660, rs1126859, rs112688, rs1126893, rs6812524, and rs7435825), but these were not detected in the tested individuals.
‡ Numbering is relative to the transcription start site (+1) corresponding to nucleotide 2268 in the GenBank sequence number D14813 (GI: 506341).
§ Numbering is relative to the last (−1) nucletotide of intron 3.
¶ Position in the cDNA considering the A nucleotide of the first ATG as +1.

gene were screened for sequence variations by DHPLC in 23 SLE patients. A total of 13 SNPs were identified (6 in the 5′ flanking region, 1 in intron 3, 3 synonymous variations in exons 6 and 7, and 3 in the 3′-UTR) (Table 2). Five of these SNPs were newly identified, whereas the remaining were previously reported in the literature (28,29) or in SNP databases.

About 70 patients and 100 controls were then genotyped for all of the SNPs reported in Table 2 and, in addition, for a recently published TG/TGTG biallelic polymorphism in the first intron (30). Four SNPs (namely, −1282A/G, IV3−42A/C, +351T/C, and +1158A/G) with a minor allele frequency of <3% in both patients and in controls were not tested further. SNPs at positions +282, +750, and +1239 showed a perfect linkage disequilibrium between each other, and therefore given the redundant genetic information, only 1 of them, namely +1239A/C, was further considered. The same was true for −1748A/G, −156G/GG, and the intron 1 polymorphism, and consequently, only −156G/GG was further considered.

Thus, starting from 14 validated SNPs, a set of 6 SNPs was further tested with a larger panel. A significant pairwise linkage disequilibrium was observed among these 6 SNPs, as shown in the healthy controls (Table 3); similar values were also observed in the patients (data

Table 3. Pairwise linkage disequilibrium (LD) between osteopontin single-nucleotide polymorphisms in controls*

Allele	−616 (G)	−443 (C)	−156 (G)	−66 (T)	+1083 (G)	+1239 (C)
−616 (G)		0.125	0.259	NS†	0.053	0.072
−443 (C)	**1.00**‡		**0.519**	0.317	0.229	0.134
−156 (G)	**1.00**‡	**1.00**‡		**0.660**	0.185	0.232
−66 (T)	NS†	**1.00**‡	**1.00**‡		0.104	0.146
+1083 (G)	**1.00**§	0.861‡	**0.966**‡	0.929‡		**0.687**
+1239 (C)	**1.00**§	0.524‡	0.942‡	0.942‡	**1.00**‡	

* Values for r^2 and D′ (Lewontin D values) are reported in the upper-right triangle and lower-left triangle of the table, respectively. All values refer to the variant allele indicated in parentheses. A minimum of 345 individuals were analyzed for each comparison. All r^2 values ≥0.5 and D′ values ≥0.95 are shown in boldface.
† Linkage disequilibrium between −66T and −616G was not significant (NS). A significant linkage disequilibrium was detected between −66T and the −616T allele ($P = 0.006$, $D' = 1$, $r^2 = 0.045$) (not shown).
‡ $P < 0.05$.
§ $P < 0.001$.

Table 4. Estimated SNP haplotype combinations in patients with SLE and controls*

Haplotype	−616	−443	−156	−66	+1083	+1239	SLE (n = 330)† Frequency	SLE (n = 330)† Standard deviation	Controls (n = 345)† Frequency	Controls (n = 345)† Standard deviation
1	G	T	GG	G	A	A	0.183	0.0134	0.224	0.0169
2	T	T	GG	T	A	A	0.078	0.0089	0.102	0.0104
3	G	T	G	T	A	A	0.115	0.0138	0.118	0.0116
4	G	C	G	T	A	A	0.241	0.0176	0.246	0.0146
5	G	T	G	T	A	C	0.062	0.0095	0.043	0.0065
6	G	T	G	T	G	C	0.020	0.0073	0.020	0.0053
7	G	C	G	T	G	C	0.280	0.0193	0.227	0.0183

* The displayed 7 haplotypic combinations account for 98% of the total haplotype frequencies. The remaining 2% were distributed in 5 rare haplotypes in both patients and controls. SLE = systemic lupus erythematosus.
† Number of individuals tested with the complete set of the 6 selected single-nucleotide polymorphisms (SNPs).

not shown). The frequency of the 7 haplotype combinations, accounting for 98% of the total (Table 4), were estimated in both patients and controls by the maximum-likelihood method based on the EM algorithm (22).

Association with SLE susceptibility. The overall haplotype distribution was significantly different between patients and controls ($P = 0.024$, 11 df). However, none of the specific haplotype frequencies was significantly distorted. The haplotype showing the highest frequency in the patients (haplotype 7 in Table 4) carried alleles that were all individually increased in the patients. The association with SLE was significant for 3 of the alleles, namely, −156G (frequency in SLE patients versus controls 0.714 versus 0.651; $P = 0.0060$, $P_{corr} = 0.036$), +1083G (0.303 versus 0.244; $P = 0.0077$, $P_{corr} = 0.046$), and +1239C (0.377 versus 0.297; $P = 0.00094$, $P_{corr} = 0.0056$), withstanding Bonferroni correction for the number of tested SNPs (n = 6). For simplicity, alleles significantly increased in the patients (−156G, +1083G, and +1239C) were named 1 (i.e., susceptibility alleles), whereas alleles significantly decreased in the patients (−156GG, +1083A, and +1239A) were named 0 (i.e., protective alleles).

The genotype distribution at the 3 positions (Table 5) was significantly different between patients and controls. The presence of the disease-associated allele, in single or in double dose, conferred an OR of 2.35 (95% CI 1.38–4.02) for SNP −156, an OR of 1.56

Table 5. Genotype distribution in patients with SLE and controls for osteopontin SNPs −156, +1083, and +1239*

SNP site, genotype†	SLE, % (n = 330)‡	Controls, % (n = 401)‡	Crude OR (95% CI)	Adjusted OR (95% CI)§
−156				
00	0.07	0.15	1.00 (referent)	1.00 (referent)
01	0.40	0.40	2.19 (1.29–3.73)	1.95 (1.12–3.38)
11	0.53	0.45	2.58 (1.53–4.35)	2.04 (1.16–3.59)
+1083				
00	0.49	0.60	1.00 (referent)	1.00 (referent)
01	0.40	0.33	1.46 (1.07–2.00)	1.08 (0.63–1.85)
11	0.11	0.07	1.82 (1.08–3.06)	0.80 (0.27–2.34)
+1239				
00	0.40	0.51	1.00 (referent)	1.00 (referent)
01	0.45	0.39	1.49 (1.09–2.04)	1.21 (0.70–2.10)
11	0.15	0.10	2.07 (1.29–3.33)	2.03 (0.74–5.56)

* Odds ratios (ORs) and 95% confidence intervals (95% CIs) are relative to the referent genotype. See Table 4 for other definitions.
† Alleles significantly increased in the patients (−156G, +1083G, and +1239C) are named 1, while alleles significantly decreased in the patients (−156GG, +1083A, and +1239A) are named 0.
‡ The total comprises the number of tested individuals. Only individuals tested for all 3 SNPs are included. Genotypes were distributed according to Hardy-Weinberg equilibrium.
§ Calculated with the use of a multivariate logistic regression analysis adjusted for −156, +1083, and +1239 SNPs. Results adjusted for the remaining 2 SNPs are reported for each SNP.

Table 6. Association of SLE susceptibility with different genotype combinations for the 3′ (+1239) and 5′ (−156) SNPs*

Genotype combination†		No. of subjects with combination			
+1239	−156	SLE	Controls	OR (95% CI)‡	*P*†
00	00	21	59	1.00 (referent)	–
00	01	62	86	2.03 (1.2–3.7)	0.02
00	11	48	61	2.21 (1.2–4.1)	0.01
01	01	70	74	2.66 (1.5–4.8)	0.001
01	11	77	82	2.64 (1.5–4.7)	0.001
11	11	49	36	3.82 (2.0–7.4)	<0.0001

* See Tables 4 and 5 for definitions.
† Alleles significantly increased in the patients (−156G and +1239C) are named 1, while alleles significantly decreased in the patients (−156GG and +1239A) are named 0. Two further rare combinations were detected: genotypes +1239-11, −156-01 (1 patient and 2 controls), and +1239-01, −156-00 (2 patients and 2 controls):
‡ Relative to the referent genotype.

(95% CI 1.15–2.12) for SNP +1083, and an OR of 1.57 (95% CI 1.16–2.13) for SNP +1239. For each SNP, a double dose conferred a higher OR than a single dose, but the difference was not statistically significant. A multivariate analysis (Table 5) adjusted for genotypes at positions +1239 and −156 indicated that +1083 was not an independent susceptibility factor (adjusted OR ~1.00). Conversely, the OR conferred by −156 and +1239 after adjustment for the other 2 polymorphisms remained similar to the crude OR, although the OR for +1239 was no longer statistically significant. This analysis suggests that each of these 2 SNPs, one in the 5′ flanking region and the other in the 3′-UTR, contributes to susceptibility independent of their linkage disequilibrium relationship.

In order to fully describe the effect of the different combinations of the 5′ and the 3′ sequence variations, we analyzed the ORs for all of their possible genotype combinations (Table 6). Due to linkage disequilibrium, 6 genotype combinations accounted for 99% of the samples. Two rare combinations accounted for the remaining 1%. The overall distribution was significantly different between patients and controls ($P = 0.0069$). Relative to the 00 genotype (absence of susceptibility allele) at both SNPs, all of the other genotype combinations conferred a significantly increased OR. The highest OR was observed in individuals homozygous for allele 1 at both SNPs (OR 3.82, 95% CI 2.0–7.4). More precisely, the risk conferred by each genotype carrying 1 or 2 susceptibility alleles at position −156 increased with an increasing number of the +1239 susceptibility allele. The complementary analysis was not possible, since +1239-1 was present only in haplotypes also carrying −156-1 (Table 4).

We tested whether this pattern was better explained by an additive model as compared with a multiplicative model, by comparing the observed ORs with those predicted under additivity of effects (additive-effects model) and with those predicted under a multiplicative model. Departure from additivity was measured by the RERI index and its 95% CI (26). Departure from a multiplicative scale was tested by comparing a model including −156 and +1239 susceptibility alleles without interaction and a reparametrized model as in Table 6 (see ref. 26 for details on parametrization). This latter procedure was used since a product term could not fit because of the absence of some relevant combinations due to linkage disequilibrium. However, these analyses did not detect a departure of the results from those predicted by either an additive or a multiplicative model, so that the best model to describe the combined effect of the 2 genotypes remains uncertain.

Association with disease features. Genotype frequencies of the −156 and +1239 SNPs were compared between patients who were positive or negative for each of the clinical and immunologic features shown in Table 1. A statistically significant association, withstanding correction for the number of comparisons (n = 21 clinical and immunologic features × 2 SNPs), was detected only for the −156 polymorphism with the presence of lymphadenopathy (overall $P = 0.0011$; $P_{corr} = 0.046$). The frequencies of −156-11, -01, and -00 genotypes were 0.714, 0.238, and 0.048, respectively, in the 63 patients with lymphadenopathy and 0.458, 0.472, and 0.070, respectively, in the 286 patients without lymphadenopathy.

Correlation with OPN serum levels. In order to investigate a possible functional basis for the observed genetic association, we analyzed OPN protein production. OPN serum levels measured by ELISA were significantly higher in 40 SLE patients than in 124 controls (mean ± SD of logarithm-transformed values 5.32 ± 0.55 ng/ml versus 4.94 ± 0.53 ng/ml, corresponding to a geometric mean of 204.8 ng/ml versus 140.1 ng/ml; $P < 0.001$). We then analyzed the association of OPN genotypes with OPN protein levels in the serum. In a previous study, we reported that among 50 healthy controls, individuals carrying the +1239C allele (i.e., the SLE susceptibility allele) produced significantly more OPN serum protein than did individuals negative for this allele (15). We now extended the analysis to a total of 124 healthy individuals (including the previously reported 50) and to the −156 polymorphism.

The association of OPN serum levels with +1239 was confirmed in the larger panel of healthy subjects. In particular, individuals carrying allele 1 for +1239 (in single or in double dose) displayed higher OPN serum levels than did individuals negative for this allele (mean ± SD of logarithm-transformed values 5.09 ± 0.527 ng/ml versus 4.79 ± 0.495 ng/ml, corresponding to a geometric mean of 162.4 ng/ml versus 120.3 ng/ml; $P = 0.002$). A similar trend was observed in individuals positive for −156-1 (mean ± SD of logarithm-transformed values 4.96 ± 0.536 ng/ml versus 4.86 ± 0.512 ng/ml, corresponding to a geometric mean of 141.9 ng/ml versus 129.4 ng/ml). However, this difference was not statistically significant and completely disappeared when considering only the +1239-1–negative samples (n = 62). Thus, it appears that only the 3′ variation has an influence on the OPN serum level in healthy individuals. In contrast with the findings in controls, no significant association of OPN serum levels with +1239 or −156 genotypes was detected in the SLE patients (mean ± SD of logarithm-transformed values 5.27 ±0.57 ng/ml for the 20 +1239-1–positive patients versus 5.37 ± 0.55 ng/ml for the 20 +1239-1–negative patients, and 5.29 ± 0.53 ng/ml for the 36 −156-1–positive patients versus 5.56 ± 0.69 for the 4 −156-1–negative patients).

DISCUSSION

The association of OPN gene polymorphisms with SLE susceptibility was tested in a large group of Italian patients and controls. Sequence variations were searched by a systematic screen of the coding regions as well as the 3′-UTR and 2,083 bp of the 5′ flanking OPN regions in 23 patients. This search was designed to ensure that potentially causative variants were considered among those tested, given that the SNPs available in the public database and in the literature may not include those specifically relevant to SLE. A total of 13 SNPs were detected, of which 5 are described for the first time herein. Six SNPs (none of which have been previously tested for association with SLE) were selected for further testing in a large panel of Italian patients.

Two sequence variations located in the 5′ flanking region (−156G/GG) and in the 3′-UTR (+1239A/C) were significantly associated with the disease. SNP at position +1239 was in perfect linkage disequilibrium with the synonymous variation in exon 7 ($Ala^{236}Ala$), which was associated with SLE in a group of 81 American individuals (10). Thus, the previously reported result has been confirmed in a larger and different population.

A multivariate analysis showed that the effect on SLE susceptibility of the 2 SNPs in the 5′ and 3′ regions was independent of each other, i.e., not a consequence of linkage disequilibrium between them. Moreover, individuals carrying susceptibility alleles for both SNPs had a higher risk of developing SLE than did individuals carrying susceptibility alleles only at position −156 (Table 6): the risk associated with homozygosity for susceptibility alleles at both SNPs was 1.7-fold higher relative to homozygosity only at −156, and almost 4-fold higher relative to the complete absence of susceptibility alleles. These data suggest a synergism of the 2 sequence variations for SLE susceptibility, which is consistent with a growing number of other examples reported in the literature (31,32), and indicates that both variations should be tested in any study investigating the role of OPN in disease etiology. The remaining haplotype background does not seem to influence the effect of these 2 SNPs.

The perfect linkage disequilibrium between alleles at positions −156, −1748, and intron 1 as well as between +1239, +282, and +750 does not allow us, in the absence of functional data, to determine which, if any, of these sequence variations is primarily involved in SLE susceptibility. The polymorphism in intron 1 does not seem to have a functional relevance, according to recently published observations (30). The SNPs in the 5′ flanking region may affect the transcription capability of the OPN promoter by a differential binding of transcription factors. Recent evidence suggests that position −156 falls in a putative binding site for a component of the RUNX family of transcription factors (33). Interestingly, SNPs in RUNX binding sites in 3 different genes

were recently associated with 3 autoimmune diseases, namely SLE, psoriasis, and rheumatoid arthritis, suggesting an important role for this family of DNA binding proteins in autoimmunity (34).

As for the 3 associated SNPs in the 3′ part of the OPN gene, 2 of them, namely, +282T/C and +750C/T, are synonymous variations located in exons 6 and 7, respectively. Synonymous variations that modify exonic splicing enhancer or exonic splicing silencer sites have been reported to be causative mutations of genetic diseases (35). However, the involvement of OPN exon 6 and exon 7 synonymous SNPs in this mechanism seems unlikely, since the 2 known alternatively spliced isoforms of human OPN involve exons 4 and 5 (36).

The third SNP (+1239A/C) is located in the 3′-UTR region. This region is strongly implicated in the regulation of gene expression because it specifically controls stability, translational activity, and nuclear export of messenger RNA (mRNA) (37–39). Sequence variations in the 3′-UTR may affect some of these functions and can be causative mutations of genetic diseases (38,39). Interestingly, even if this SNP is not part of sequences known to bind regulative factors, it falls in an 18-bp sequence conserved in the human, bovine, ovine, and porcine OPN 3′-UTR, suggesting a possible functional role of this region. Preliminary mRNA quantitative analysis performed in 3 individuals heterozygous for +1239A/C and homozygous for all of the promoter SNPs showed that mRNA carrying the SLE-associated allele (+1239C) was 4.4-fold more expressed than mRNA carrying +1239A. These data suggest a possible influence of this SNP in the control of mRNA stability. Accordingly, the +1239C allele was significantly associated with a higher serum protein level in healthy controls, directly relating OPN levels to this OPN gene variation and to SLE susceptibility. Conversely, no association with the baseline protein serum level was detected for position −156.

Thus, the mechanism by which this polymorphism contributes to SLE susceptibility is less clear. We can speculate that it might be relevant in the regulation of OPN production in response to the initial immunostimulating trigger. The fact that neither SNP was correlated with the protein level in SLE patients could be explained by an overwhelming effect of the immune activation in the patients following the initial trigger.

Intriguingly, the −156 susceptibility allele (−156G) was also associated, albeit with borderline significance ($P_{corr} = 0.046$), with development of lymphadenopathy in SLE patients. This pattern recalls the characterization of ALPS as involving autoimmunities partly similar to SLE and lymphadenopathy, and as displaying high OPN levels and association with the +1239C allele (the −156 SNP was not evaluated in ALPS patients [15]). Since OPN favors proliferation and inhibits death of lymphocytes in vitro (15), it was suggested that OPN acts in synergy with the inherited Fas defect, which is considered the main cause of ALPS. This synergy may favor lymphocyte accumulation in the secondary lymphoid tissues and development of autoimmunity. A similar synergy might also work in SLE patients, who do not carry inherited defects of Fas function, but rather, overproduce a soluble form of Fas that may inhibit Fas function (40). Alternatively, OPN might act in synergy with other apoptosis defects not identified to date in SLE.

In summary, these data strongly suggest that OPN genetic variations have a key role in building up an autoimmune-prone background favoring lymphocyte accumulation in peripheral lymphoid tissues and leading to the development of autoimmunity. For at least one of these genetic variations, an association with increased OPN levels was demonstrated. OPN may exert its preferential effect through its capacity to stimulate proliferation and inhibit death of lymphocytes (15,41) or through its capacity to modulate the immune response by inducing Th1 responses and potentiating polyclonal activation of B cells (3–5).

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