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Polymorphisms in the Interferon- γ /Interleukin-26 Gene Region Contribute to Sex Bias in Susceptibility to Rheumatoid Arthritis

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Objective. To determine whether polymorphisms in the interferon- γ (IFN γ)/interleukin-26 (IL-26; formerly, AK155) gene cluster contribute to sex-based differential susceptibility to rheumatoid arthritis (RA).

Methods. Four microsatellite markers, located in a 118-kb interval that contains both the IFN γ and IL-26 genes on chromosome 12q15, were typed in 251 patients with RA and 198 unrelated healthy controls (all of whom lived in Northern Ireland) by means of polymerase chain reaction–based fragment analysis.

Results. Marker *D12S2510*, which is located 3 kb 3' from the IL-26 gene, was significantly associated with RA in women (corrected P [P_{corr}] = 0.008, 2 degrees of freedom [2 df]) but not in men (P = 0.99, 2 df). A 3-marker haplotype, *IFNGCA*13;D12S2510*8;D12S2511*9*, was inferred that showed significant underrepresentation in women with RA (odds ratio 0.50, 95% confidence interval 0.32–0.78; P = 0.002, P_{corr} = 0.03) but not in men with RA.

Conclusion. Our results demonstrate that common polymorphisms in the IFN γ /IL-26 gene region may contribute to sex bias in susceptibility to RA, by distorting the propensity of female carriers versus male carriers to contract this disease. These results conform to

our recent observations of a role for this gene cluster in sex-based differential susceptibility to another Th1-type inflammatory disease, multiple sclerosis.

Rheumatoid arthritis (RA) is a multifactorial disease in which genetic factors are thought to account for 60% of disease susceptibility (1). Current thinking suggests a model in which the accumulated or epistatic effects of multiple genes may predispose to RA. Genes located in the HLA cluster have been consistently confirmed as predisposing factors in RA, although this region likely accounts for no more than one-third of the total genetic component (1). One widely used approach to the identification of additional susceptibility genes in RA consists of association studies performed with polymorphic markers located within or near candidate genes that are inferred from the etiopathogenesis of the disorder.

The interferon- γ (IFN γ) gene has recently attracted attention for its role as a plausible candidate in susceptibility to RA. This interest is generally based on the observation that T cell clones established from RA synovium typically produce Th1-related proinflammatory cytokines, including IFN γ , but are deficient in the production of Th2 cytokines (2). Different groups of investigators have used a microsatellite polymorphism, located in the first intron of this gene, as a marker to assess the association of the IFN γ gene with susceptibility to, and the clinical course of, RA. An initial report by Khani-Hanjani et al (3) claimed a strong association of this marker with disease severity; however, this association may have occurred erroneously because of the misassignment of alleles (4,5). Indeed, further confirmatory studies failed to confirm the association between the IFN γ gene polymorphism and RA susceptibility or severity (6,7).

Notwithstanding these negative findings, indications that the IFN γ gene region has a role in determin-

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ing the differential susceptibility patterns of men and women to RA justify further scrutiny. In a linkage study on cytokine gene polymorphisms, linkage of borderline significance with the $IFN\gamma$ microsatellite was found in male/male and female/male affected sibling pairs (logarithm of odds score = 0.96; $P = 0.03$), but not in female/female affected sibling pairs (8). Ollier reported an odds ratio (OR) of 1.8 (95% confidence interval [95% CI] 1.0–3.1) for single-nucleotide polymorphisms in the $IFN\gamma$ gene in men with RA (4).

An indication for a more general role of the $IFN\gamma$ gene region in sex-restricted differential susceptibility patterns to inflammatory diseases comes from our research on multiple sclerosis (MS). Like rheumatoid arthritis, MS is a chronic inflammatory disease characterized by endogenous production of Th1-type cytokines, and epidemiologic surveys reveal a preponderance of women affected by MS. We recently demonstrated that the intronic microsatellite in the $IFN\gamma$ gene is differentially associated with MS in men compared with women (9,10). We performed a linkage disequilibrium screen and haplotype analysis of the $IFN\gamma$ region to narrow the interval for location of this sex-associated factor (11). This allowed us to identify a 118-kb stretch that contains both the $IFN\gamma$ and $IL-26$ genes (separated by 40 kb) as the most probable interval for location of this factor (11). Although these initial studies were performed in the Sardinian population, we recently confirmed the occurrence of similar sex-associated differences in this gene region in case-control studies in the US and Northern Ireland (12).

In view of these findings, we were anxious to verify whether the $IFN\gamma/IL-26$ gene region may contribute to sex bias in RA as well. In addition to the microsatellite polymorphism in the $IFN\gamma$ gene ($IFNGCA$), we typed 3 other markers spanning the 118-kb sex-associated interval that emerged from our MS study (11), i.e., $D12S313$, $D12S2510$, and $D12S2511$. Typing and analysis were performed in a case-control study of >250 Northern Irish patients with RA and 198 unrelated control subjects living in Northern Ireland. The markers $D12S2510$ and $D12S2511$ were originally identified and characterized in our laboratory and are located at the $IL-26$ locus (13). We specifically investigated the association of individual markers and imputed haplotypes with RA, in patients stratified according to sex.

PATIENTS AND METHODS

After informed consent was received, blood samples were obtained from patients with RA who were seen by a

rheumatologist (MR) at Musgrave Park Hospital and Belfast City Hospital. This study was approved by the ethics committee of Queen's University. In order to recruit similar numbers of men and women (in consideration of the increased prevalence of the disease in females), approximately every second female patient and every male patient were prospectively recruited. All patients were assessed by a rheumatologist, and all fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for RA (14). A total of 251 patients (143 women and 108 men) were recruited. The mean age of the patients was 57 years (range 23–82 years); the mean age of the female patients was 56 years (range 25–81 years) and that of the male patients was 58 years (range 23–82 years). The mean disease duration was 15 years (range 1–56 years); the mean duration for female patients was 17 years (1–56 years) and that for male patients was 12 years (range 1–37 years). All samples were coded at the source, in a blinded manner. DNA samples were obtained from 198 healthy subjects (97 men and 101 women) at the Belfast City Hospital and were used as a control. Both control subjects and patients were of Northern Irish descent.

Polymorphic markers in the $IFNG/IL26$ gene region.

All DNA samples were typed for 4 distinct microsatellite markers that span a 118-kb interval encompassing both the $IFNG$ and $IL26$ (previously called AK155) genes, i.e., from centromere to telomere $D12S313$, $IFNGCA$, $D12S2510$, and $D12S2511$. $D12S313$ is a multiallelic dinucleotide polymorphism located at a distance of 51 kb downstream from $IFNG$. $IFNGCA$ is a CA repeat polymorphism located in the first intron of the $IFN\gamma$ gene. $D12S2510$ and $D12S2511$ are novel microsatellite polymorphisms in the $IL-26$ gene, discovered in our laboratory (13). $D12S2510$ is a trinucleotide repeat polymorphism located 3 kb 3' to $IL26$, and $D12S2511$ is a pentanucleotide repeat polymorphism located in the third intron of the $IL-26$ gene (13). $D12S2510$ consists of 2 frequent alleles (137 bp and 143 bp, respectively) and a very rare 146-bp allele, corresponding to 6, 8, and 9 repeat units, respectively. For $D12S2511$, 3 frequent alleles of 175 bp, 180 bp, and 186 bp corresponding to 8, 9, and 10 repeat units were found, together accounting for ~95% of the allele pool. Extreme care was taken to ensure the correct assignment of alleles. Amplification products were sequenced to determine the exact number of repeats and were compared with reference samples. Alleles of all markers are named according to the number of repeat units, except $D12S313$, for which alleles are named according to the length in basepairs.

Genotyping procedures. Details on primers and amplification procedures can be found online at The Genome Database (www.gdb.org). Fragment analysis was performed by electrophoresis through POP-6 polymer-filled capillaries on an ABI Prism 3100 Sequencer with GeneScan software (Applied Biosystems, Foster City, CA). Forward primers were labeled with fluorophores, and GeneScan 400 HD (Applied Biosystems) was used as an internal size standard. In addition, we used marker amplicons from an identical DNA pool comprising 20 individual DNA samples as an internal control on each run. Such a pool reflects both the frequencies and sizes of all alleles present for each marker. Comparison of individual samples with this control ensured consistent and correct allele assignment.

Table 1. Allele frequencies of markers in the *IFNG* and *IL26* gene region in patients and controls*

Marker, allele	Total		Male		Female	
	RA (n = 502)	Controls (n = 396)	RA (n = 216)	Controls (n = 194)	RA (n = 286)	Controls (n = 202)
<i>D12S313</i>						
188	8.0	9.8	8.3	9.3	7.7	10.4
190	5.2	6.6	4.2	5.2	5.9	7.9
192	19.1	15.7	15.3	14.4	22.0	0.8
194	24.9	27.0	25.9	29.9	24.1	24.3
200	4.6	7.1	5.6	5.2	3.8	8.9
202	24.7	18.7†	26.4	19.1	23.4	18.3
204	5.2	5.6	5.1	5.7	5.2	5.4
X	8.4	9.6	9.3	11.2	7.4	8.0
<i>IFNGCA</i>						
12	46.0	44.9	42.1	42.8	49.0	47.0
13	43.6	47.0	49.1	47.4	39.5	46.5
X	10.4	8.0	8.8	9.8	11.4	6.5
<i>D12S2510</i>						
6	70.1	62.6†	65.3	65.5	73.8	59.9‡
8	29.1	35.9†	33.8	33.5	25.5	38.1§
X	0.8	1.5	0.9	1.0	0.7	2.0
<i>D12S2511</i>						
8	20.9	21.0	20.4	23.2	21.3	18.8
9	57.8	60.4	58.8	58.2	57.0	62.4
10	16.5	14.9	16.2	13.4	16.8	16.3
X	4.8	3.9	4.7	5.1	4.8	2.5

* Values are the percentage of allele counts. X represents low-frequency alleles (occurring in <5% of total controls) grouped together. RA = rheumatoid arthritis.

† $P \leq 0.03$, corrected $P (P_{\text{corr}}) > 0.05$.

‡ $P = 0.001$, $P_{\text{corr}} = 0.006$.

§ $P = 0.002$, $P_{\text{corr}} = 0.01$.

Statistical analysis. Rare alleles (frequency <5%) were grouped together. The global and individual allele distributions of each marker were compared between unstratified and sex-stratified patients and controls by means of chi-square analysis. Patients and controls were also compared for carrier, homozygote, and heterozygote rates. Associations are also expressed as ORs with logit 95% CIs. Maximum-likelihood estimations of the haplotype frequencies were calculated with an expectation-maximization algorithm implemented using Arlequin software (available online at <http://anthro.unige.ch/arlequin>). Frequent haplotypes (>5%) were compared between patients and controls. P values are uncorrected. Corrected $P (P_{\text{corr}})$ values are Bonferroni-corrected for multiple comparisons of genotypes, alleles, or haplotypes, and sex strata per locus. Using the Arlequin software likelihood ratio, both tests for pairs of markers in linkage disequilibrium and tests for the population in Hardy-Weinberg equilibrium at individual markers were applied.

RESULTS

The succession from centromere to telomere of the markers typed in the present study is *D12S313*, *IFNGCA*, *D12S2510*, and *D12S2511* (with the latter 2 markers spanning the IL-26 locus), with spacing of 51 kb between *D12S313* and *IFNGCA*, 40 kb between *IFNGCA* and *D12S2510*, and 27 kb between *D12S2510*

and *D12S2511* (11,13). Both the IFN γ and IL-26 genes are transcriptionally oriented from telomere to centromere (13). Table 1 summarizes the allele frequencies of all 4 markers in RA patients and controls before and after stratification according to sex.

In the unstratified group, only the marker *D12S2510* showed a weak global association with RA ($P = 0.048$, P_{corr} not significant, 2 df). The global allele distribution of marker *D12S2510* was significantly different in female RA patients compared with female controls ($P = 0.004$, $P_{\text{corr}} = 0.008$, 2 df) but not in male patients versus male controls ($P = 0.99$, 2 df). More specifically, the frequency of the 6-repeat allele was higher and that of the 8-repeat allele was lower in female patients compared with female controls ($P_{\text{corr}} = 0.006$ and $P_{\text{corr}} = 0.01$, respectively). This is reflected in significantly different rates of *D12S2510**6/*6 homozygotes (55.2% versus 32.7%; OR 2.54, 95% CI 1.50–4.32, $P = 0.0004$, $P_{\text{corr}} = 0.002$) and *D12S2510**8 carriers (44.1% versus 64.4%; OR 0.44, 95% CI 0.26–0.74, $P = 0.002$, $P_{\text{corr}} = 0.01$) in the female, but not male, patient-control strata (Table 2).

With *D12S2510* being de facto a biallelic marker,

Table 2. Genotype distribution of *IFNGCA* and *D12S2510* in patients with RA and controls, stratified by sex*

Locus, genotype	Male		<i>P</i> (<i>P</i> _{corr})	Female		<i>P</i> (<i>P</i> _{corr})
	RA (n = 108)	Controls (n = 97)		RA (n = 143)	Controls (n = 101)	
<i>IFNGCA</i>						
Carrier 12	73	65	NS	101	73	NS
12/12	18	18	NS	39	22	NS
12/X	55	47	NS	62	51	NS
X/X	35	32	NS	42	28	NS
Carrier 13	81	69	NS	87	71	NS
13/13	25	23	NS	26	23	NS
13/X	56	46	NS	61	48	NS
X/X	27	28	NS	56	30	NS
<i>D12S2510</i>						
Carrier 6	97	89	NS	132	88	NS
6/6	44	38	NS	79	33	0.0004 (0.002)†
6/X	53	51	NS	53	55	NS
X/X	11	8	NS	11	13	NS
Carrier 8	63	58	NS	63	65	0.002 (0.01)‡
8/8	10	7	NS	10	12	0.03 (NS)
8/X	53	51	NS	53	53	0.004 (0.02)
X/X	45	39	NS	80	36	NS

* Values are the number of subjects with each genotype. RA = rheumatoid arthritis; *P*_{corr} = corrected *P* value; NS = not significant at the 5% level.

† Compared with subjects not homozygous for 6/6 (i.e., 6/X and X/X).

‡ Compared with noncarriers; this is a weighted average of the risk of homozygotes versus noncarriers and the risk of heterozygotes versus noncarriers.

it is impossible to distinguish between either a primary susceptibility effect of the 6/6 genotype (etiologic fraction [EF] or attributable risk = 0.34) or a primary protective effect conferred by carriage of at least one 8-repeat allele (prevented fraction [PF] = 0.36). Similarly, the frequency of 6/6 homozygotes was significantly higher ($P = 0.023$, EF = 0.24) and that of *D12S2510**8 carriers lower ($P = 0.025$, PF = 0.26) in female patients compared with male patients. It is worth noting that a tendency for a difference in global allele distribution ($P = 0.09$) for underrepresentation of the 13 CA-repeat allele of the IFN γ polymorphism (39.5% versus 46.5%; $P = 0.12$) and for a lower carriage rate for this allele (60.8% versus 70.3%; $P = 0.13$) was also seen in women with RA compared with female controls, although these associations were not significant at the 5% level (Tables 1 and 2). Neither the *D12S313* nor the *D12S2511* markers showed a sex-specific association with RA. Except for a limited deviation caused by a smaller-than-expected number of men heterozygous for one of the rare alleles of *IFNGCA*, the control population was in Hardy-Weinberg equilibrium for all markers.

In the total study population, linkage disequilibrium between pairs of markers was found over the whole 118-kb interval studied. Because linkage disequilibrium with a disease/protective locus may be stronger for haplotypes than for single alleles, 2-marker and

multiple-marker haplotype frequencies were estimated by means of an expectation-maximization algorithm. Several haplotypes were identified that were specifically associated with RA in either male or female strata, although none of these was associated with RA in the unstratified group (Table 3, and data not shown). Of these, the 3-marker haplotype *IFNGCA**13; *D12S2510**8; *D12S2511**9 was highly significantly underrepresented in women with RA (15.4% versus 26.6%; OR 0.50, 95% CI 0.32–0.78, $P = 0.002$). Even after correction for 7 compared trimarker haplotypes in 2 sex strata, this association remained significant ($P_{\text{corr}} = 0.03$). This association was absent in the male patient-control stratum.

DISCUSSION

We recently identified a 118-kb interval spanning the IFN γ /IL-26 loci that is involved in male versus female differential susceptibility to the inflammatory disease MS (11). The present study was undertaken to assess whether this interval is similarly associated with another inflammatory disease, namely RA, in which sex-specific susceptibility is also observed. Previous studies have indicated that polymorphisms at the IFN γ locus show suggestive evidence for linkage and association with RA in a sex-specific manner (4,8).

Table 3. Estimated haplotype frequencies for the most frequent haplotypes (>5%) in sex-stratified RA patients and controls*

Haplotype	Male		<i>P</i> (<i>P</i> _{corr})	Female		<i>P</i> (<i>P</i> _{corr})
	RA (n = 216)	Controls (n = 194)		RA (n = 286)	Controls (n = 202)	
<i>D12S313*192;IFNGCA*12</i>	9.3	8.6	NS	17.5	10.1	0.02 (NS)
<i>D12S313*194;IFNGCA*12</i>	17.4	16.8	NS	12.2	10.7	NS
<i>D12S313*202;IFNGCA*12</i>	9.6	8.8	NS	7.8	7.8	NS
<i>D12S313*188;IFNGCA*13</i>	7.4	8.8	NS	6.1	6.8	NS
<i>D12S313*194;IFNGCA*13</i>	4.3	9.0	NS	9.5	12.2	NS
<i>D12S313*202;IFNGCA*13</i>	16.7	8.7	0.02 (NS)	13.7	9.5	NS
<i>IFNGCA*12;D12S2510*6</i>	39.8	39.4	NS	47.1	42.7	NS
<i>IFNGCA*13;D12S2510*6</i>	16.6	16.8	NS	15.7	11.6	NS
<i>IFNGCA*13;D12S2510*8</i>	31.5	29.6	NS	23.1	33.7	0.01 (NS)
<i>D12S2510*6;D12S2511*8</i>	15.7	17.4	NS	14.7	14.6	NS
<i>D12S2510*6;D12S2511*9</i>	35.5	34.6	NS	39.9	31.6	NS
<i>D12S2510*6;D12S2511*10</i>	11.0	10.3	NS	16.2	11.7	NS
<i>D12S2510*8;D12S2511*9</i>	23.2	23.0	NS	17.0	30.8	0.0004 (0.003)
<i>IFNGCA*12;D12S2510*6;D12S2511*8</i>	8.4	7.3	NS	6.9	8.3	NS
<i>IFNGCA*12;D12S2510*6;D12S2511*9</i>	20.6	23.5	NS	27.7	24.4	NS
<i>IFNGCA*12;D12S2510*6;D12S2511*10</i>	8.0	7.6	NS	11.1	8.0	NS
<i>IFNGCA*13;D12S2510*6;D12S2511*8</i>	2.7	6.0	NS	5.4	6.2	NS
<i>IFNGCA*13;D12S2510*6;D12S2511*9</i>	14.0	7.9	0.05 (NS)	6.3	4.6	NS
<i>IFNGCA*13;D12S2510*8;D12S2511*8</i>	6.9	6.3	NS	5.3	2.0	NS
<i>IFNGCA*13;D12S2510*8;D12S2511*9</i>	18.1	18.3	NS	15.4	26.6	0.002 (0.03)

* Values are the percentage. RA = rheumatoid arthritis; *P*_{corr} = corrected *P* value; NS = not significant at the 5% level.

Of the 4 markers tested in this interval, *D12S2510* was individually associated with RA in women, but not with RA in men. Importantly, the construction and evaluation of 2- and 3-marker haplotypes reinforced linkage disequilibrium with this putative sex-associated locus. More specifically, 2- and 3-marker haplotypes composed of allele combinations of *IFNGCA*13*, *D12S2510*8*, and *D12S2511*9* were significantly under-represented in female patients with RA. It is worth noting that the same haplotypes showed, identically, underrepresentation in Northern Irish female patients with MS versus female controls (Vandenbroeck K, et al: unpublished observations). This finding is somehow in contrast with previous studies on the IFN γ locus in RA, in which the effects observed were stronger in male patients (4,8). Remarkably, in our family-based study on MS in Sardinian patients, we found a strong reduced transmission of the haplotype *IFNGCA*12;D12S2510*6* from heterozygous parents to male patients with MS, with little evidence for association in female patients (11). It is, however, likely that such discrepancies may be at least partially explained by genetic differences between these study populations.

In the present study on RA, as well as in 2 MS case-control studies (12), the *IFNGCA*12* allele, the *D12S2510*6* allele, and the combination of the 2 alleles in the haplotype *IFNGCA*12;D12S2510*6*, were less frequent in male patients than in female patients,

whereas the inverse was true for alleles *IFNGCA*13* and *D12S2510*8* and their haplotype *IFNGCA*13;D12S2510*8*. These ratios were different from what was seen in male versus female controls and were of similar magnitudes in the 3 studies.

Pending further analysis, the present study indicates that polymorphisms in the IFN γ /IL-26 gene region are likely to be associated with susceptibility to autoimmune diseases such as RA and MS in a sex-specific manner. The single- and multiple-locus sex-specific associations with RA observed in the present study are strongest for markers *D12S2510* and *D12S2511*, both of which are located at the IL-26 locus. Thus, the effects seen previously (4,8) could possibly be attributable to linkage disequilibrium with a sex-associated susceptibility locus that occurs closer to the IL-26 gene than to the IFN γ gene. Typing of an extended number of RA patients and controls for all markers analyzed in the present study is warranted to confirm and refine the present observations.

Ours is the first study to investigate the role of the whole IFN γ /IL-26 gene region in susceptibility to RA. A growing body of evidence suggests the presence of a locus on chromosome 12q15 in proximity to the IFN γ and IL-26 genes that is specifically involved in sex-restricted patterns of susceptibility to inflammatory diseases such as RA and MS (4,8,10–12). As a first step toward the characterization of this locus, we have iden-

tified a multiple-marker haplotype specifically associated with RA in women. Determining the genetics and biology of this locus may profoundly increase our knowledge of the mechanisms underlying sex bias in autoimmune diseases and may have important implications in terms of future strategies for therapeutic interventions in RA.

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