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Expression of Interferon-gamma Receptor Genes in PBMCs is Associated with Rheumatoid Arthritis and Its Radiographic Severity in African Americans

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Abstract

BACKGROUND—Factors responsible for radiographic severity of rheumatoid arthritis (RA) in African-Americans are poorly understood. We sought to identify genes whose expression in peripheral blood mononuclear cells (PBMCs) is associated with radiographic severity of RA.

METHODS—We initially performed quantitative real-time (qRT)-PCR for 182 genes based on plausible immune pathways in 40 African-American RA patients with extremes of radiographic damage (low versus high radiographic scores) and disease duration (early versus late) and 20 healthy African-American controls. In the second phase, we analyzed the expression of significantly associated candidate genes (*IFNGR2* and its biological partner *IFNGR1*) with radiographic scores in 576 African-American RA patients and 51 controls not previously analyzed, accounting for autoantibody status and disease duration.

RESULTS—We found significant differences in *IFNGR1* expression between RA and controls ($P=6 \times 10^{-14}$) and in *IFNGR2* expression between those with erosions vs no erosions (P=0.01) (Wilcoxon sum test). We also found A significant correlations between *IFNGR2* expression and radiographic scores (P=0.03 for erosions, P=0.04 for joint space narrowing, and P=0.03 for total radiographic score, zero-inflated negative binomial model) and annualized progression rate (P=0.0024, Spearman correlation).

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CONCLUSIONS—These findings have important implications with respect to IFNγ for the pathogenesis of RA and may lead to identification of a biomarker for radiographic damage. Additional studies are needed to define cell subsets responsible for the association of IFNγ receptor gene expression with radiographic finding, which downstream mechanisms are involved, and generalizability to other RA populations.

Rheumatoid arthritis (RA) often leads to joint damage [1], but there are no robust biomarkers to identify patients who will develop radiographic damage. Furthermore, molecular mechanisms influencing the radiographic damage have not been examined in detail in African-Americans. To investigate the hypothesis that gene expression in PBMCs of African-American patients with RA is associated with radiographic severity, we performed a two phase, candidate gene study. A total of 616 African-Americans with RA and 71 healthy African-American controls were studied. We found that *IFNGR2* expression was independently associated with RA and its radiographic severity, and that *IFNGR1* expression was associated with the presence of RA. These findings provide novel insights into the pathways involved in radiographic damage in RA and provide a possible biomarker of future damage. Additional studies will be required to define the cell types responsible for this finding and to determine if these findings are consistent across different populations.

MATERIALS AND METHODS

Study Design

We initially analyzed 40 RA patients with extremes of phenotypes based on disease duration (≤ 2 years versus > 2 years) and radiographic severity (severe versus none) and 20 controls (Supplemental Table 1). The 10 early disease/high damage participants were those with < 2 years disease duration and the highest scores; the 10 early disease/low damage were chosen from among those with normal radiographs with positive anti-CCP antibody; the 10 late disease/high damage were those with the highest (most severe) scores and disease duration > 2 years; the 10 late disease/low damage were 10 randomly chosen participants from among those with normal radiographs who were anti-CCP positive. We chose 182 genes for analysis (Supplemental Table 2) based on our previous findings [2], literature review, and available panels of low density arrays focused on pathways relevant to RA. The second (replication) phase sought to validate associations of these findings in a previously unstudied sample of 576 African-Americans with RA and 51 African-American controls.

Study Subjects and Controls

All RA subjects and controls from the cross-sectional arm of the Consortium for the Longitudinal Evaluation of African-Americans with Rheumatoid Arthritis (CLEAR) Registry [3] (see http://medicine.uab.edu/rheum/70918/). The method of Sharp, as modified by van der Heijde, was used to score the radiographs, as previously described [3]. This study included African-American RA patients for whom total radiographic scores were available, a total of 616 patients.

Selection of Candidate Genes

Criteria for candidate genes were the strength of association with RA, the biological relevance or known role of the gene in RA and its severity, and availability of assays. Genes in the SABiosciences Innate and Adaptive Immune Signaling Array (SAB) and Applied Biosystems Human Immune Array (ABI) were analyzed, as were 17 genes from our preliminary analysis of early erosive disease using a globe gene expression approach [2] (Supplemental Table 2). The SAB array has 84 genes involved in the host response to bacterial infection and sepsis, and 5 housekeeping genes (http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-052A.html). The ABI array contains 90 genes involved in stress response, signal transduction, cytokines/receptors, cell surface receptors, oxidoreductase, chemokines, protease, and cell cycle, and six housekeeping genes (http://tools.lifetechnologies.com/content/sfs/brochures/cms_042394.pdf). In the first phase, the strongest association with radiographic severity expression levels of *IFNGR2* (data not shown). Thus, our next analyses focused on validation of association of expression of this gene and its biological partner, *IFNGR1* with radiographic severity, in an independent set of subjects and controls (Table 1).

Quantitative Real-time PCR analysis

Total RNA from whole blood was obtained from PAXgene Blood RNA Tubes (Qiagen) using PAXgene Blood RNA Kit (Qiagen), including treatment with RNase-free DNase Set (Qiagen) to eliminate genomic DNA. Total RNA was reverse-transcribed into cDNA, and then quantitative real-time PCR (qPCR) was performed on an Applied Biosystems QuantStudio™ 6 Flex Real-Time PCR System. The 40S ribosomal protein S9 (RPS9) was used as endogenous control in relative gene quantification using the comparative cycle threshold method ($2^{-\Delta\Delta Ct}$).

Statistical analysis

The median of three technical replicates was used for analyses. For the low density RT-PCR array data analysis, two samples were removed due to low overall signal. Genes that were undetectable in > 60% of samples were also removed. Standard comparisons based on housekeeping controls were used for Ct value normalization. For genes found on both low density arrays, ABI data were used. Normalized radiographic data were used to compare groups using Wilcoxon Rank-Sum analysis. False discovery rate (FDR) of 0.15 was used to select candidate genes for further validation [4]. For the validation (second phase), a Wilcoxon Rank-Sum test was used to compare gene expression between patients and controls. Spearman correlation was used to examine the relationship between gene expression and radiographic severity. A zero-inflated negative binomial model was fit to severity scores using gene expression, age, body mass index, smoking status, disease duration, and anti-CCP antibody status, rheumatoid factor status, current or ever use of methotrexate, current or ever use of biologic agent as covariates. Adjustment for multiple testing was performed using family-wise error rate by the method of Holm [5]. The statistical software R (version 3.0.0) was used for all analyses.

RESULTS

We found significantly higher *IFNGR2* expression in RA compared to controls (Table 2), and significantly higher *IFNGR2* expression in patients with more severe radiographic damage. Figure 1 shows a direct correlation between level of *IFNGR2* expression and degree of damage. *IFNGR2* expression levels were associated with multiple radiographic outcomes, including erosion score (P=0.01), joint space narrowing score (P=0.04), total radiographic score (sum of erosion and JSN scores) (P=0.01), and radiographic annualized progression rate (APR), defined as the radiographic score divided by the disease duration (P=0.0006) (Table 2). Results of the zero-inflated negative binomial model demonstrated a significant association of *IFNGR2* gene expression (adjusting for covariates) with radiographic erosion score (P=0.01), JSN score (P=0.04), and overall score (P=0.02) (Table 2). We also found a significant difference in *IFNGR1* expression between RA and controls (P=0.01). However, *IFNGR1* expression levels were not statistically significantly associated with erosion status (presence versus absence) or other radiographic outcomes (Table 2).

Compared with the healthy control group, there was a slightly higher proportion of current smoking (a known risk factor for RA) in the 574 RA patients for whom smoking status was available (25.2% vs 17.6%), but this difference failed to reach statistical significance. There was a statistically significant difference between the mean age at blood sample collection, gender and CCP and RF status between RA cases and healthy controls. As expected, there were statistically significant differences between the groups with radiographic damage and those without radiographic damage, including differences in age, body mass index, disease duration, RF and anti-CCP status and methotrexate use (Table 1). In the zero-inflated binomial model, none of these covariates affected the significance of the relationship between *IFNGR2* expression and radiographic severity, although as expected, some were significantly associated with radiographic severity (data not shown).

DISCUSSION

We present strong evidence that in African-Americans, expression levels of genes encoding IFNγ receptors (*IFNGR1* and *IFNGR2*) are highly associated with RA, and that *IFNGR2* expression is associated with radiographic damage. Interferon gamma (IFNγ), a type II interferon, is secreted predominantly by activated T cells and natural killer cells. The interferon gamma receptor complex consists of a heterodimer of two chains, *IFNGR1* and *IFNGR2* [6]. Upon binding of IFNγ, the receptor subunits dimerize, and signal transduction occurs through the *JAK1/JAK2-STAT1* pathway [7]. *JAK* mediated phosphorylation of *STAT1* induces a change in conformation of *STAT1* to its activated form, and the activated *STAT1* homodimer translocates to the nucleus to promote transcription of IFNγ-induced genes. The *IFNγ-STAT1* pathway is known to play a role in RA via macrophage activation, enhanced MHC expression on neighboring cells, balancing Th1/Th2 differentiation, and priming secretion of pro-inflammatory cytokines [6] [8, 9].

The association of expression of IFNγ receptor genes with the presence of RA and the joint damage phenotype is biologically plausible, based on other findings in RA and other autoimmune diseases such as systemic lupus erythematosus (SLE) and multiple sclerosis

(MS). A recent multi-ethnic meta-analysis showed that a genetic variant in the *IFNGR2* region, is a risk locus for RA [10]. Other studies have implicated IFNγ receptor genetic variants or gene expression with SLE [11] or MS [12].

IFNGR1 gene expression is constitutive and the protein is found at moderate levels on the surface of nearly all cells. It is unclear whether the higher expression level of *IFNGR2* is due to enrichment of a subset of cells in which *IFNGR2* is constitutively expressed, or whether its expression is upregulated in one or more subsets of cells. Cell subsets potentially important in the pathogenesis of RA and that may help to explain mechanistically the association of *IFNGR2* expression with RA and radiographic damage include T lymphocytes, B lymphocytes, and monocytes. *IFNGR2* is expressed on Th2 cells, but not on Th1 cells, and is constitutively expressed at extremely low levels; expression in certain cell types (e.g. Th17 cells) can be affected by external stimuli [13]. The ability of certain T cells to respond to IFNγ is highly regulated through the modulated expression of the *IFNGR2* gene. Regulation of the *IFNGR2* gene thus becomes a critical factor in determining IFN responsiveness in T cells, B cells, and macrophages [6] [13]. Future studies will be focused on identifying the circulating cell types in which *IFNGR* gene expression is up-regulated in RA.

The mechanisms whereby up-regulated expression of *IFNGR2* may increase radiographic severity in RA need further exploration. Interestingly, we did not find an association between elevated expression of IFN-gamma and radiographic severity. Expression of *IFNG* is not dependent on expression of *IFNGR*, so our finding suggests that the rate limiting factor in IFNγ signaling is at the level of IFNγ receptors. Alternatively, this might be an indication that the association between *IFNGR* expression and radiographic damage may be mediated by a pathway other than IFNγ binding to IFNγ receptors, but there are no data to suggest that other cytokines bind to IFNg receptors. In RA, increased expression of *IFNGR2* gene results in a denser distribution of *IFNGR* complex on cell surfaces and more IFNγ binding. *IFNGR2* also makes association between IFNγ and *IFNGR1* more stable, a necessary step for IFNγ-dependent activation of the *JAK-STAT1* signaling pathway and for initiation of the signaling cascade leading to the expression of IFNγ-inducible genes such as *CXCL9*, *CXCL10*, and *CCL5*. Qiao et al. recently reported synergistic activation of inflammatory cytokine genes (TNF, IL-6) by IFNγ-induced chromatin remodeling and toll-like receptor signaling [8], which may be highly relevant to RA. Other possible mechanisms by which elevated expression of *IFNGR2* may promote bone erosion include enhancing MHC expression on cells of the innate immune system, alterations in Th1/Th2 differentiation, activation of macrophages, and/or increased differentiation of osteoclasts.

In our study, the group of individuals with radiographic damage were diagnosed at a younger age, were more likely to be RF and anti-CCP antibody positive, had more years of disease and a lower BMI and more likely to have used methotrexate, but not biologic agents. As can be seen in Table 1, these differences were either relatively small (age 48 years vs 44 yrs; BMI 34 vs 31) or expected, due to known differences in variables associated with more severe radiographic findings such as disease duration (6 vs 13 years); RF positivity (73% vs 84%); anti-CCP positivity (59% vs 80%) and methotrexate use (74% vs 85%). There are no data to suggest that MTX or biologic agents influence IFNGR expression. None of the other

factors is known or suspected to be associated with *IFNGR* expression and none appeared to explain the association of *IFNGR2* expression with radiographic severity (data not shown).

It is unclear whether our findings are generalizable to other populations. There are no known differences with regard to IFNGR gene expression between healthy individuals of difference ethnicities (e.g. European vs African ancestry). Examples of population differences in autoimmunity include the dependence of IFN-induced gene expression on the presence of autoantibodies in African-American, but not European, patients with SLE [14], and multiple ethnicity-associated variations in signaling responses in discrete cell subsets [15].

In conclusion, we found that among African-Americans with RA, there is increased expression of *IFNGR1* and *IFNGR2* and an association of *IFNGR2* with increased radiographic severity. *IFNGR2* expression in peripheral blood is a potential biomarker for RA radiographic severity and disease prognosis. These findings have important implications for a pathogenic role of IFNγ in susceptibility, and severity of RA and other autoimmune diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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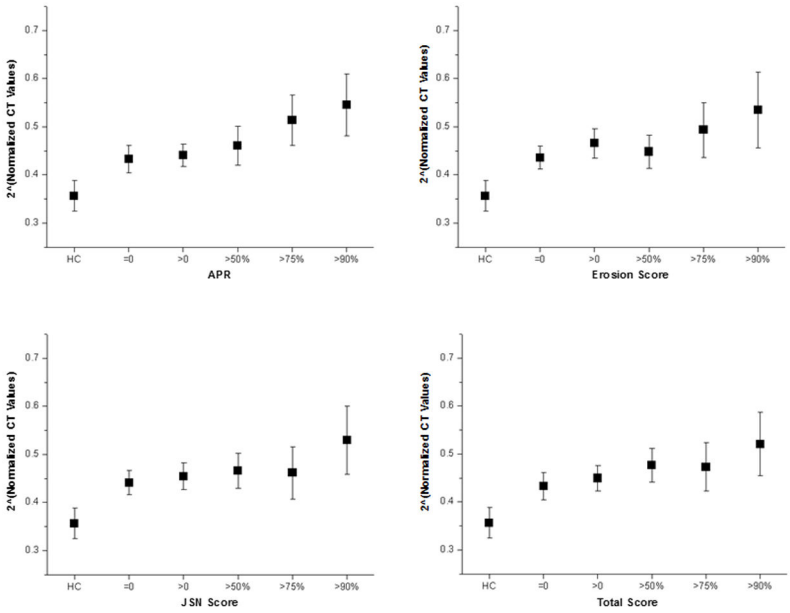

Figure 1.

IFNGR2 gene expression is associated with radiographic severity of RA: Mean normalized gene expression (2Exp-) for IFNGR2 measured from PBMCs of RA patients and controls. RA patients categorized by radiographic severity as measured in four ways: erosion score; joint space narrowing (JSN) score; total score; and annual progression rate (APR, defined as total score/disease duration in years). “0” = no damage, “>0” = score at least 1 but less than the median score, “>50%” = greater than the median score but less than 75 percentile of scores, “>75%” = greater than 75 percentile of scores but less than 90 percentile of scores, “>90%” =greater than 90 percentile of scores. Healthy controls (N = 51). Sample size for joint Erosion score: 0 (273), >0 (150), >50 (78), >75 (48), >90 (31). Sample size for joint space narrowing: 0 (246), >0 (165), >50 (86), >75 (51), >90 (32). Sample size for total score: 0 (198), >0 (192), >50 (96), >75 (56), >90 (38). Sample size for annual progression rate: 0 (198), >0 (191), >50 (96), >75 (57), >90 (38).

Table 1

Clinical and demographic characteristics of the 40 African-American RA patients and 20 African-American controls used in the first phase, and the 576 RA patients and 51 healthy controls used in the second phase. The p-values presented in superscript reflect statistical significant differences between RA patients with radiographic damage vs those without radiographic damage (columns 1 and 2) and RA vs controls (columns 3 and 4).

Variable	*RA without Radiographic Damage (n=196)*	*RA with Radiographic Damage (n=380)*	RA combined (n=576)	Controls (n=51)
Gender (F, %)	89.8%	85.8%	87.1% **	64.7%
BMI, mean (SD)	33.7 (7.8)**	31.0 (7.2)	31.9 (7.5)	NA
Age at diagnosis, years, mean (SD)	48.0 (9.7)**	43.5 (13.4)	45.0 (12.4)	NA
Disease duration years, mean (SD)	6.3 (5.3)**	13.1 (9.7)	10.8 (9.1)	NA
Age at time of PBMC collection, years, mean (SD)	54.1 (9.3)*$*	56.7 (12.0)	55.8 (11.8)*	58.7 (7.3)
RF IgM positive, %	73%*$*	84%	80%**	22%
Anti-CCP antibody positive, %	59%**	80%	73%**	6%
Current Smoking status, %	24%	26%	25%	18%
Methotrexate (ever used), %	74%*$$*	85%	81%	NA
RA Biologic agent (ever used), %	28%	36%	33%	NA
Corticosteroids (ever used), %	91%	95%	94%	NA
Total score, median (IQR)	0	21.5 (47)	6 (31.0)	NA
JSN score, median (IQR)	0	13 (29)	4 (21.0)	NA
Erosion score, median (IQR)	0	6 (17.5)	1 (11.0)	NA

* p <0.05,

** p< 0.0001,

$ p<0.005,

$$ p<0.001.

Table 2

Results of statistical analyses for *IFNGR1* and *IFNGR2* expression and RA and its radiographic severity. The Ct values from qRT-PCR experiments were normalized against *RPS9* expression to obtain dCt values. The RA group (N=576) was compared with the control group (n=51), and RA patients with no erosion (defined as total score = 0) was compared with those with erosions (defined as total score > 0). For the zero inflated negative binomial model for *IFNGR2* expression, the Ct values were normalized against *RPS9* expression to obtain dCt values. The model was tested for association of *IFNGR2* expression with erosion score, JSN score, and total score, adjusting for covariates as described in the text. APR – annualized progression rate (total radiographic score divided by number of years' disease duration). P values were adjusted for family-wise error rate using Holm's method and those less than 0.05 are shown in italics.

Statistical Tests	**Variables**	**Estimates value**		**P value (adjusted)**	
		IFNGR1	***IFNGR2***	***IFNGR1***	***IFNGR2***
Wilcoxon test	Controls vs. Patients	24149.5	18204.5	*3 x 10^{-14} ($6x10^{-14}$)*	*0.003 (0.06)*
	No erosion vs Erosion	44835	46418.5	0.07 (0.07)	*0.01 (0.01)*
Spearman correlation test	Erosion score	−0.04492	v0.10146	0.28 (0.54)	*0.01 (0.03)*
	JSN score	−0.05633	−0.0838	0.18 (0.54)	*0.04(0.04)*
	Total score	−0.05476	−0.10432	0.19 (0.54)	*0.01 (0.03)*
	APR	−0.0631	−0.14132	0.13 (0.52)	*0.0006 (0.0024)*

Zero Inflated Negative Binomial model

Radiographic Outcome	**Estimate Value**	**Standard Error**	**z value**	**P value (adjusted)**
Erosion Score	0.51993	0.20401	2.549	*0.01 (0.03)*
JSN Score	0.263780	0.128363	2.055	*0.04 (0.04)*
Total Score	0.336566	0.148415	2.268	*0.02 (0.04)*