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Macrophage Migration Inhibitory Factor and Stereotypical Behavior in Autism Spectrum Disorders

Elena L. Grigorenko^{1,2,3,4,*}, Summer S. Han^{4,#}, Carolyn M. Yrigollen^{1,#}, Lin Leng^{3,6}, Courtney McDonald^{3,6}, Yuka Mizue⁷, George M. Anderson¹, Erik J. Mulder⁸, Annelies de Bildt⁸, Ruud B. Minderaa⁸, Fred R. Volkmar^{1,2}, Joseph T. Chang⁴, and Richard Bucala^{3,6,*}

¹Child Study Center, Yale University, 230 South Frontage Rd, New Haven, CT 06519, USA

²Department of Psychology, Yale University, 2 Hillhouse Ave, New Haven, CT 06520, USA

³Department of Epidemiology and Public Health, Yale University, 300 Cedar St, New Haven, CT 06520

⁴Department of Psychology, Moscow State University, 11/5 Mokhovaia St, Moscow 125009, Russia

⁵Department of Statistics, Yale University, 24 Hillhouse Avenue, New Haven, CT 06520, USA

⁶Departments of Internal Medicine and Pathology, Yale University, 300 Cedar St, New Haven, CT 06520

⁷Sapporo Immuno Diagnostic Laboratory, 12-20, Shinkawa 2-2, Kita-ku, Sapporo 001-0922, Japan

⁸Accare/University Medical Center Groningen, University Center for Child and Adolescent Psychiatry, P.O. Box 660, 9700 AR Groningen, the Netherlands

Abstract

Background—Autism-spectrum disorders (ASD) are childhood neurodevelopmental disorders characterized by social and communicative impairment and repetitive and stereotypical behavior. Macrophage migration inhibitory factor (MIF) is an upstream regulator of innate immunity that promotes monocyte/macrophage activation responses by increasing the expression of Toll-like receptors and inhibiting activation-induced apoptosis. Based on results of prior genetic linkage studies and reported altered innate immune response in ASD, we hypothesized that *MIF* could represent a candidate gene for ASD or its diagnostic components.

Methods—Genetic association between ASD and *MIF* was investigated in two independent sets of families of probands with ASD, from USA (527 participants from 152 families) and Holland (532 participants from 183 families). Probands and their siblings, when available, were evaluated with clinical instruments used for ASD diagnoses. Genotyping was performed for two polymorphisms in the promoter region of the *MIF* gene in both samples sequentially. In addition, MIF plasma analyses were carried out in a subset of Dutch patients from whom plasma was available.

Results—There were genetic associations between known functional polymorphisms in the promoter for *MIF* and ASD-related behaviors. Also, probands with ASD exhibited higher circulating MIF levels than did their unaffected siblings; the amount of MIF in the plasma correlated with the severity of multiple ASD symptoms.

Conclusions—These results identify *MIF* as a susceptibility gene for ASD. Further research is warranted on the precise relationship between *MIF* and the behavioral components of ASD, the mechanism by which MIF contributes to ASD pathogenesis, and the clinical utility of *MIF* genotyping.

*To whom correspondence should be addressed: Telephone: 203-737-1453, 203-737-2316, Fax: 203-785-7053, 203-785-3002, richard.bucala@yale.edu, elena.grigorenko@yale.edu.

#These authors contributed to the work equally.

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The etiopathogenesis of autism-spectrum disorders (ASD) is unknown. ASD are considered among the most heritable neurodevelopmental disorders¹ and are observed in 34 to 62.6 per 10,000 children². There is also evidence for genetic transmission of milder autism-associated phenotypes and autism components³. Although multiple genome-wide scans have been conducted, results generally have been inconsistent. Chromosomal regions 7q and 10p are supported by meta-analytic⁴ and high-resolution scanning studies, respectively⁵, but other regions, including 22q^{6, 7}, may confer susceptibility to ASD. Multiple candidate genes, including those controlling brain growth⁸, glutamatergic and GABAergic synaptogenesis⁵, and immune function⁹, also have been proposed to play a role. In appreciation of this mosaic of findings, the consensus hypothesis is that the etiology of ASD is predominantly oligogenic and likely includes gene–gene and gene–environment interactions.

Individuals with ASD can exhibit additional disorders, including seizures, gastrointestinal and neurological symptoms, and immunological deficiencies. The presence of immune abnormalities in patients with ASD has long been noted¹⁰. The relevant literature can be traced back some three decades to a report of a high frequency of autoimmune diseases in a family with a proband with ASD¹¹. This report triggered family studies of the co-occurrence of ASD and autoimmune diseases and confirmation of the observation of elevated counts of autoimmune diseases when compared with control families^{12, 13}. Additionally, indicators of chronic neuroinflammation have been reported in the brain^{14–17} and blood and urine^{18–21} of probands with ASD. The literature further describes abnormal cellular immune responses^{22–26} and other autoimmune abnormalities in probands with ASD^{27, 28}. Also of interest are a variety of hypotheses linking the well-established hyperserotoninemia and immune abnormalities in ASD²⁹. Finally, it is important to note that there have been a number of genetic studies investigating allelic association and linkage among genes or in the region of the major histocompatibility complex (MHC) and ASD; however, results of these studies are mixed, with some pointing to the presence of the association^{30, 31} and others excluding linkage³². Some studies suggest that innate rather than adaptive neuroimmune responses are associated with ASD. Thus, the literature on the presence of immune problems in probands with ASD and their families contains a critical mass of information for generating a hypothesis regarding the involvement of autoimmune genes in the etiopathogenesis of ASD. At present, views of possible immune dysfunction in ASD range from conclusions that it may contribute to manifestations of the disorder in some patients¹⁸ to hypotheses that neuroimmunopathogenic responses play a fundamental role in ASD³³. Studies suggest that innate rather than adaptive neuroimmune responses are associated with ASD³⁴.

Given a complex model of the inheritance of ASD, one possible etiopathic mechanism might involve an immunologic insult to the central nervous system (CNS) in individuals with a susceptible genetic background. We evaluated the genetic association between the innate mediator, macrophage migration inhibitory factor³⁵ (MIF), and different behavioral components of ASD. MIF is encoded in a functionally polymorphic locus on chromosome 22q11.2 (OMIM 153620, GenBank accession no. NM_002415) that has been associated with the incidence or severity of different autoimmune inflammatory conditions³⁶. Given its location in a previously identified locus of interest and its upstream action in immunity, *MIF* may represent a candidate gene for ASD or its components. Thus, the central hypothesis underlying this research was that a genetic predisposition to a particular level of MIF production, may lead to a pro-inflammatory profile of cell activation that, if present during a neurodevelopmentally sensitive period, might contribute to the etiopathogenesis of autism.

The *MIF* gene (see Figure 1) spans less than 1 Kb and is highly conserved. Several functional *MIF* alleles exist in the general population and differ in the structure of their promoter region. A CATT repeat (–794 CATT_{5–8}) influences basal and stimulus-induced

transcriptional activity such that transcription increases in an almost proportional fashion with repeat number³⁷. The CATT₅*MIF* allele is typically referred to as a “low expression” allele, and the CATT₆, CATT₇, and (rare) CATT₈ *MIF* alleles are considered “higher expression” alleles. A single nucleotide polymorphism (SNP, –173 G/C) is located within the same haplotype block as the CATT site and may exert a regulatory function by means of linkage disequilibrium or functional interaction with the repeat³⁸.

METHODS

Patients

Two sets of families, one recruited through the Yale University Child Study Center (YCSC) in the United States and the other through the University Center for Child and Adolescent Psychiatry, Accare/University Medical Center Groningen in the Netherlands, contributed to this work. Clinical characterization of these families has previously been described³⁹. All research was approved by corresponding Institutional Review Boards and appropriate consent forms were obtained.

The US samples included 527 individuals from 152 families ascertained through a proband with ASD assessed at the clinics of the YCSC³⁹. The Dutch sample included 532 participants from 183 families who approached the Accare Center for evaluation purposes⁴⁰. For more details on the samples, see Table 1. In the analyses presented here, only families of probands with autism and Asperger’s disorder were included.

Probands and their siblings were evaluated with several clinical instruments used for ASD diagnosis [*Autism Diagnostic Interview*, ADI, multiple versions⁴¹; and *Autism Diagnostic Observational Schedule*, ADOS, multiple modules⁴²], from which six phenotypes that capture different facets of ASD were generated as described below. In addition, all patients received clinical diagnoses (see Table 1).

Genotyping

Standard methods were used to extract DNA from blood collected in EDTA or from buccal mucosa cells. Analysis of the collected samples for the CATT *MIF* polymorphism was performed as previously described³⁷, and the fluor-labeled amplicons were resolved using an ABI 3100 Genetic Analyzer. DNA from previously genotyped homozygous individuals was used to generate control amplicons for size calibration for capillary electrophoresis. The –173 G/C alleles were determined by TaqMan SNP Genotyping Assays on the ABI Prism 7900HT and analyzed with SDS software. The probe was obtained from Applied Biosystems (Foster City, CA). The two samples were genotyped sequentially, with the US sample considered the initial discovery sample and the Dutch sample the replication sample⁴³. The genotyping was separated by about eight months and was carried out on the same machinery with the same control DNAs.

Variable Description

As prescribed by corresponding manuals, using all designated items, the administration of the ADI resulted in three subscores (ADI social, ADI communication, and ADI stereotypical behaviors). The administration of the ADOS resulted in four subscores (ADOS social, ADOS communication, ADOS stereotypical behaviors, and ADOS imaginative skills). For each of the three presumed latent traits (social, communication, and stereotypical facets of ASD), we formed combinations of measured scores capturing that latent trait and analyzed the resulting vectors of measured scores with multivariate association models (phenotypes 1–3, see Table 3). Thus, the social phenotype was a vector of ADI social and ADOS social; the communication phenotype consisted of the two communication scores from the ADI and

ADOS, and finally, the stereotypical behaviors phenotype consisted of the two summative scores from ADI (restricted/repetitive behaviors) and ADOS (stereotyped behaviors). This construction of phenotypes was done for two reasons: (1) to maximize on the convergence between different instruments to decrease the amount of error in assessing different facets of ASD; and (2) to minimize the number of phenotypes investigated and, correspondingly, to decrease the probability of Type I error increasing as the number of multiple comparisons increased. Before conducting genetic association tests, we regressed each trait on age and gender and transformed the phenotypes using rank transformation. For phenotypes 1–3 (Table 3), we used these residuals as phenotypes in input data files for association analyses. In addition, we regressed each of these three phenotypes on two other domain phenotypes and used the residuals as the phenotype in input data (e.g., regressing ADOS stereotypical behaviors on ADOS communication and ADOS social; all three were already regressed on age and gender). Specifically, phenotype (1) was considered when the variance attributable to indicators of language/communication impairments and stereotypical behaviors (as captured by the corresponding variables described earlier: 2 from the ADOS and 2 from the ADI) were removed (the resulting residualized phenotype is designated as phenotype 4 in Table 3). Similarly, phenotype (2), impairment in language/communication, was considered when we residualized measures of social interaction and stereotypical (or restricted interests/repetitive) behaviors from the ADOS and ADI (see phenotype 5 in Table 3). Finally, phenotype (3) was analyzed when the ADOS and ADI indicators of social interaction and communication/language functioning were removed (see phenotype 6 in Table 3). Such residualization permitted us to (1) focus on specific variance associated with a particular facet of ASD, when variance attributable to other facets was regressed out; and (2) once again, minimize the number of multiple comparisons.

With these phenotypes, we carried out multivariate linkage and association analyses for the two polymorphisms in the *MIF* gene promoter separately for the two samples.

Association Analyses

We used the Family Based Association Tests (FBAT)⁴⁴ software for univariate and haplotype tests. We also used the Tools for Family-Based Association Studies (PBAT)⁴⁵ software, specifically the R library pbatR, for multivariate tests of the null hypothesis of “no association in the presence of linkage.”

Three rounds of analyses were performed. First, we executed an exploratory set of analyses on the US dataset with the six phenotypes described earlier. Because these analyses involved multiple comparisons, they were followed by a simulation study that generated new threshold empirical P-values for interpretation of the nominal P-values (see Table 2). We then analyzed the Dutch data as a confirmatory sample; correspondingly, we did not use adjustments for multiple comparisons. Finally, we completed a summative analysis of both samples, using a method initially proposed by Fischer⁴⁶ and currently being advocated⁴⁷ for combining results from multiple samples to establish gene-based association.

Adjustments for Multiple Comparisons

The tests we performed for the several traits for each of several alleles in our discovery (US) sample led to multiple P values. To adjust for multiple comparisons and account for dependence among traits, we conducted a Monte Carlo simulation study designed to assess our results relative to the smallest P values that would arise by chance, assuming the truth of the null hypothesis, with our particular suite of statistical tests and with our particular trait and parental genotype data. We simulated 1,000 synthetic datasets according to the null hypothesis of no association, conditional on the minimal sufficient statistics identified by Rabinowitz and Laird⁴⁸. To create each of the datasets, we simulated new random genotypes

for the participants from the appropriate conditional distributions⁴⁸ while fixing the trait measurements and parental genotypes at the values observed in our data. For each synthetic dataset, we then carried out the same statistical tests applied to our original dataset, creating a new table of P values. For each of the resulting 1,000 tables, we recorded the minimum P value, giving an empirical distribution of the minimum P value. The 5% quantile of that minimum P value distribution was 0.0071. Thus, the procedure of rejecting each of the null hypotheses whose P value in Table 3 (US portion) was less than 0.0071 has an overall significance level of 0.05, taking into account multiple testing.

Plasma MIF Analysis

MIF was measured by sandwich ELISA using specific antibodies and native sequence human MIF that was prepared in the Bucala laboratory as a standard⁴⁹.

RESULTS

The allele frequencies in each sample and for both samples combined are shown in Table 2. The results of the association analyses are shown in Table 3. Because the CATT₈ allele was not observed in the US data and had low frequency in the Dutch sample, it was not tested for association. We tested alleles CATT₅, CATT₆, and CATT₇. Also of note is the degree of linkage disequilibrium between the -794 CATT and the -173 SNP, which was repeatedly high in both samples ($D = .57$ and $.72$ in the US and Dutch samples, respectively).

US Sample

Our findings indicated the presence of a genetic association between the -794 CATT site and ASD. The strongest associations were between the -794 CATT₆ *MIF* alleles and the stereotypical components of ASD, residualized (phenotype 6, P -value=0.00009), and non-residualized (phenotype 3, P -value=0.00259). We also performed univariate PBAT tests for each trait, which provided confirmatory information for the multivariate findings. Specifically, the CATT₆ allele showed P -values lower than 0.05 for both ADOS stereotypical behaviors scores ($P=0.00059$ and 0.00022 , before and after residualization, respectively).

Dutch Sample

The Dutch sample was treated as a confirmatory sample analysis; a number of multivariate phenotypes provided support to the genetic association with *MIF*. Specifically, consistently with the results of the US sample, the stereotypical components of ASD, residualized (phenotype 6), gave a P -value of 0.02973 with CATT₆. In this sample, the -173 SNP also generated statistically significant or borderline P -values for nonresidualized multivariate phenotypes of social impairment (phenotype 1, $P=0.05278$), communication impairment (phenotype 2, $P=.03700$), and stereotypical behaviors (phenotype 3, $P=0.05638$). The genetic associations with *MIF* were further supported in the Dutch sample by univariate analyses. Specifically, the -173 SNP polymorphism appeared to be associated with all ADI and ADOS unresidualized phenotypes (ADI: P -values of 0.01698, 0.02208, and 0.017103 and ADOS: 0.025062, 0.011041, and 0.024524, for social impairment, communication impairment, and stereotypical behaviors, respectively).

To summarize the patterns of results across the two samples, we applied Fisher's product criterion^{46, 50}, which allows unifying different P -values obtained for different alleles across the two samples in a meta-analytic fashion. Specifically, we combined P -values for the two residualized multivariate phenotypes (stereotypical behaviors and stereotypical behaviors-residualized) that survived the correction for multiple comparisons in the US sample and for the two univariate phenotypes (ADI and ADOS stereotyped behaviors) that contributed to

these two multivariate phenotypes. The corresponding US–Dutch combined Fisher P-values were $P=0.01649$ and $P=0.00047$ for multivariate phenotypes. For univariate phenotypes, the ADOS-based indicators gave statistically significant P-values ($P=0.00217$ and $P=0.01749$, for stereotypical behaviors and stereotypical behaviors–residualized).

Plasma Analyses

We also measured levels of plasma MIF protein⁴⁹ in 10 probands⁵¹ and their unaffected siblings from the Dutch sample. A significantly higher level of circulating MIF in the ASD-affected group (ASD: 13.12 ± 9.18 ng/ml; unaffected siblings: 6.87 ± 2.75 ng/ml; Mean $\pm$ SD, $P=0.0323$, Figure 2) provided independent corroboration of the genetic association findings.

Moreover, when the plasma MIF protein in 29 probands (10 from the previous sib comparison and 19 assayed additionally) was correlated with behavioral indicators, statistically significant positive correlations were obtained between plasma MIF levels and ADOS scores on social impairment, imaginative skills, and total score ($r=0.41$, 0.41 , and 0.39 , respectively, $P<0.05$ for all). The correlation with the ADOS stereotypical behavior score did not reach statistical significance ($r=0.15$, $P>0.10$), but trended in the same direction.

DISCUSSION

Collectively, these data identify *MIF* as a potential ASD susceptibility gene and support earlier suggestions of a role for innate immunity in the etiopathogenesis of this disease. These data include evidence for neuroglial and innate immune activation in brain tissue and cerebrospinal fluid¹⁷ and increased expression of pro-inflammatory cytokines in the CNS or blood of patients with ASD⁵². Persistent elevation of cytokines in the CNS may reflect an ongoing inflammatory process, microglial activation, or developmental arrest, as some cytokine levels increase during phases of neurodevelopment³⁴. Because MIF regulates the expression of innate cytokines³⁵, we hypothesize that a genetic predisposition at the *MIF* locus may lead to an inappropriate level of MIF production during a neurodevelopmentally sensitive period, contributes to the pathogenesis of ASD. Our data add to the evidence that some innate immunity genes may play an important role in the development of ASD⁹.

Although numerous studies have linked high-expression *MIF* alleles with autoimmune diseases^{35, 37, 49, 53}, a specific role for MIF in CNS disorders has not been described previously and it may be relevant that MIF is also expressed by neuronal cells. In vitro studies have shown that the intracellular content of MIF increases during neuronal firing and then acts to reduce the chronotropic effects of further stimulation⁵⁴. Thus, the role of the known variant *MIF* alleles in ASD may involve neuronal functions that extend beyond the protein's well-described immunologic actions³⁵. Pharmacologic inhibitors of MIF are presently in pre-clinical development^{55, 56} and therapies aimed specifically at MIF pathways in patients with ASD might be feasible.

In summary, we present evidence for an association between the *MIF* locus and ASD, specifically with the stereotypical components of ASD. Because ASD are heterogeneous in presentation and etiology, it is possible that different genes underlie the manifestation of different facets of ASD. Given that distinctive diagnostic components of ASD show differential heritability estimates and patterns of familial transmission and, thus, may be fractionable^{3, 57}, these components may be associated with different etiopathogeneses. In the overwhelming majority of (if not all) studies of immunological abnormalities in ASD, phenotyping was restricted to clinical diagnoses (affected vs. unaffected) based on DSM criteria. Some inconsistencies in the immunological research results on ASD may be due to cross-study differences in phenotyping and failure to study specific phenotypes of ASD. If

our findings, in which immunological abnormalities are mostly associated with particular facets of ASD, are replicated, this inconsistency may be explained. More generally, evidence of higher rates of autoimmunity (rheumatoid arthritis, lupus, asthma, allergies, thyroid disorders)³⁴ in relatives of individuals with ASD suggests that additional immunologic response genes also should be tested for involvement in ASD.

These initial findings will require further study in other samples of probands with ASD in order to determine their replicability. In addition, an examination of samples of probands with other developmental disorders can provide an assessment of the specificity of the association between *MIF* genotype and ASD. These results also prompt a reconsideration of prior observations and stimulate the investigation of new hypotheses regarding relationships between early immune function and ASD, and possibly other developmental neuropsychiatric disorders. Determination of an *MIF* genotype or protein levels may assist in better defining ASD phenotypes, thereby improving the prognosis of behavioral abnormalities, and potentially enabling new pharmacologic interventions.

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What's Known on this Subject

ASD are clinically complex and characterized by many etiological pathways involving multiple genetic and environmental risk factors. Although a number of such risk factors have been proposed, no single factor has sustained the scrutiny of replications in multiple diverse samples.

What This Study Adds

The study contributes data, generated in two independent samples of ASD families, that suggests for the first time a role for the innate cytokine, macrophage migration inhibitory factor (*MIF*), in the etiology of ASD.

Figure 1.

Diagram of the human MIF gene showing its three exons, predicted transcription factor binding sites, and the -173 SNP and -794 CATT polymorphisms.

Figure 2.

Boxplots of the plasma concentrations of MIF in probands and their unaffected siblings (n=10 per group). The bottom, median, and top lines of the box demarcate the 25th, 50th, and 75th percentiles, respectively. The vertical lines show the minimum and maximum values. *P=0.0323 for autistic group vs. unaffected siblings (T-test, unpaired).

Table 1

Descriptive Statistics on the Samples of US and Dutch Families of the ASD Probands

	US	Dutch
Total number of people genotyped	472	528
Male (%)	62.3	61.7
Female (%)	37.7	38.3
Ethnic background (%) ¹		
Caucasian	95%	100%
Ethnic minority	5%	0%
Number of probands per family		
1	129	183
2	21	0
3	2	0
Average age of the probands	10.97	9.85
Probands' Clinical Diagnoses ²		
Autism	84	88
Asperger's Disorder	46	12
CDD	7	0
PDD-NOS	34	57

Notes:

¹The analyses for the US sample were carried out with and without families of minority ethnic background. Because the results were virtually identical, we here present the results for the ethnically admixed sample

²Clinical diagnoses were obtained by consensus among practicing clinicians at both sites, respectively, based on opinions of at least two independent evaluators.

Table 2Allele frequencies at two *MIF* polymorphisms in the US and Dutch samples

Allele frequencies	US	Dutch
<i>CATT</i> ₅	0.267	0.258
<i>CATT</i> ₆	0.627	0.571
<i>CATT</i> ₇	0.104	0.171
<i>CATT</i> ₈		0.002
<i>SNP</i> _C	0.155	0.190
<i>SNP</i> _G	0.845	0.810

Table 3

Results (P values) of multivariate association analyses for the six autism-related phenotypes and two *MIF* markers in the US sample under the null hypothesis of no association by PBAT.

Phenotypes	Additive Model			
	<i>CATT₅</i>	<i>CATT₆</i>	<i>CATT₇</i>	<i>SNP (C vs. G)</i>
<i>Discovery (US) Sample</i>				
Social Impairment (1)	.21977	.07191	.41436	.21733
Communication Impairment (2)	.25319	.13250	.40131	.23507
Stereotypical Behaviors (3)	.00584	.00259	.56716	.81854
Social Impairment–R (4)	.07207	.05609	.51668	.82883
Communication Impairment–R (5)	.23247	.37857	.17543	.27731
Stereotypical Behaviors–R (6)	.00373	.00009	.28669	.44593
<i>Replication (Dutch) Sample</i>				
Social Impairment (1)	0.61215	0.77958	0.21100	<i>0.05278</i>
Communication Impairment (2)	0.72255	0.48094	0.15405	<i>0.03700</i>
Stereotypical Behaviors (3)	0.45537	0.84091	0.30765	<i>0.05638</i>
Social Impairment–R (4)	0.91088	0.99357	0.95776	0.89561
Communication Impairment–R (5)	0.61608	0.18231	0.36073	0.82589
Stereotypical Behaviors–R (6)	0.11373	<i>0.02973</i>	0.34349	0.87544

Note:

We performed the analyses under the null hypotheses “no linkage and no association” under additive genetic models. The P-values shown in bold (US Sample) are less than 0.0071; the null hypothesis can be rejected at a simultaneous significance level of 0.05, corrected for multiple testing. The P-values shown in italics (Dutch Sample) are less than 0.05.