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Pathway-focused genetic evaluation of immune and inflammation related genes with chronic fatigue syndrome ☆

Mangalathu S. Rajeevan *, Irina Dimulescu, Janna Murray [1], Virginia R. Falkenberg, Elizabeth R. Unger

Division of High-Consequence Pathogens & Pathology, Centers for Disease Control and Prevention, Atlanta, GA, USA

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ABSTRACT

Recent evidence suggests immune and inflammatory alterations are important in chronic fatigue syndrome (CFS). This study was done to explore the association of functionally important genetic variants in inflammation and immune pathways with CFS. Peripheral blood DNA was isolated from 50 CFS and 121 non-fatigued (NF) control participants in a population-based study. Genotyping was performed with the Affymetrix Immune and Inflammation Chip that covers 11 K single nucleotide polymorphisms (SNPs) following the manufacturer's protocol. Genotyping accuracy for specific genes was validated by pyrosequencing. Golden Helix SVS software was used for genetic analysis. SNP functional annotation was done using SPOT and GenomePipe programs. CFS was associated with 32 functionally important SNPs: 11 missense variants, 4 synonymous variants, 11 untranslated regulatory region (UTR) variants and 6 intronic variants. Some of these SNPs were in genes within pathways related to complement cascade (*SERPINA5*, *CFB*, *CFH*, *MASP1* and *C6*), chemokines (*CXCL16*, *CCR4*, *CCL27*), cytokine signaling (*IL18*, *IL17B*, *IL2RB*), and toll-like receptor signaling (*TIRAP*, *IRAK4*). Of particular interest is association of CFS with two missense variants in genes of complement activation, rs4151667 (L9H) in *CFB* and rs1061170 (Y402H) in *CFH*. A 5′ UTR polymorphism (rs11214105) in *IL18* also associated with physical fatigue, body pain and score for CFS case defining symptoms. This study identified new associations of CFS with genetic variants in pathways including complement activation providing additional support for altered innate immune response in CFS. Additional studies are needed to validate the findings of this exploratory study.

1. Introduction

Chronic fatigue syndrome (CFS) is a severely debilitating condition characterized by multi-system symptoms including chronic fatigue, pain, post-exertional malaise, muscle weakness, un-refreshing sleep, and cognitive impairment. While the cause of CFS is not known, many proposed risk factors including infection, environmental exposures, allergies, physiological and psychosocial stress, act through the immune system and inflammatory response [1–8]. Inflammatory markers have been associated with specific symptoms common in CFS; chronic fatigue, heart rate variability, sleep quality, cognitive problems and post-exertional malaise [9–17]. Changes in cytokine profiles have been suggested as biomarkers of CFS [18–22].

Polymorphisms that impact gene function, either directly or interaction through other risk factors, may contribute to genetic susceptibility for CFS. Only a small number of polymorphisms in a few genes involved in immune and inflammatory response have been studied [23–25]. The Affymetrix Human Immune and Inflammation Chip was developed to facilitate a systematic genetic evaluation of immune and inflammation pathways [26]. We used this platform to explore the genetics of the immune and inflammation response in CFS.

2. Materials and methods

2.1. Subjects

This study was approved by the Centers for Disease Control & Prevention (CDC) Human Subjects Committee and adhered to the

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* Corresponding author at: Division of High-Consequence Pathogens & Pathology, Centers for Disease Control & Prevention, 1600 Clifton Road, Atlanta, GA 30333, USA.

E-mail address: mor4@cdc.gov (M.S. Rajeevan).

[1] Current address: Influenza Division, Centers for Disease Control and Prevention, USA.

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human experimental guidelines of the US Department of Health and Human Services. All participants gave written informed consent for the study.

Participants in the follow-up study of a population based surveillance of CFS in Georgia, USA (Georgia CFS surveillance study) were clinically evaluated as described in the baseline surveillance [27], and classified as CFS if they met the 1994 international research definition as previously described. The clinical evaluation included physical examination, laboratory screening and Structured Clinical Interview for DSM-IV (SCID) to identify exclusionary conditions and completion of the Multidimensional Fatigue Inventory (MFI), the SF-36® Health Survey (SF-36), and the CDC Symptom Inventory (SI). Subjects meeting none of the criteria for CFS were classified as non-fatigued (NF) controls. The current analysis included all 171 Non-Hispanic White participants with no medical/psychiatric exclusions: 121 NF controls and 50 CFS. The decision to restrict non-Hispanic Whites was based on the limited power to detect association in other racial/ethnic groups (only 14 CFS and 41 NF participants were non-White or Hispanics).

2.2. Highly multiplex Affymetrix targeted genotyping

Peripheral blood was collected via venipuncture in PAXgene blood DNA tubes (Qiagen, Valencia, CA). Specimen handling, storage, transportation, and extraction of genomic DNA using PAXgene blood DNA Kit (Qiagen) were done following the manufacturer's instructions. DNA quality and quantity were determined using agarose gel (1%) electrophoresis and Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) respectively.

We used the Affymetrix Human Immune and Inflammation Chip that interrogates 11 K SNPs in 1000 genes representing 38 sub-pathways. Selection of pathways, genes and SNPs for the inflammation chip has already been reported [26]. The assay used the Affymetrix Targeted Genotyping system with Molecular Inversion Probe assay (MIP) read with four color GC3000 TG scanner 7G 4C, and GeneChip Targeted Genotyping Analysis Software (GTGS) following the manufacture's protocol [26,28,29]. Each array was scanned four times to measure the signal from label on each of the 4 nucleotides, and the data were stored as .cell files after gridding. Genotype calls were made automatically by GTGS following the standard criteria: SNP call rate $\geqslant$ 80% (estimate of assays clearly genotyped); half rate $\leqslant$ 10% (estimate of assays marginally genotyped); signal noise ratio (S/N) $\geqslant$ 20 (median ratio of assay allele/non-allele channel signals); coefficient of variation (CV) of control feature signals for hybridization $\leqslant$ 30%.

2.3. Genotyping by pyrosequencing

Pyrosequencing was used to validate and supplement Affymetrix data for 57 SNPs of interest. Pyrosequencing assays were designed using the Assay Design Software (Qiagen), and SNPs were detected using the PyroMark PCR kit, PyroMark Q96MD instrument system and other assay instructions from Qiagen. Supplementary Table 1 provides the primer sequences, primer concentrations, annealing temperature and "sequence to analyze" for each of the SNPs. In brief, each 12.5 μl PCR contained 1× PyroMark PCR Mastermix, 1× CoralLoad Concentrate, either 0.2 μM or 0.4 μM each of forward and reverse primers and 25 ng of DNA. PCR consisted of an initial denaturation step of one cycle for 95 °C for 15 min followed by 45 cycles of 95 °C for 30 s, the respective annealing temperature for 30 s, and 72 °C for 30 s. Each sequencing reaction used 5 μl of biotinylated PCR product, 0.3 μM of sequencing primer in a total annealing buffer volume of 12 μl and sequenced using the "sequence to analyze" generated for each SNP by the Assay Design Software. We used Coriell DNA (Coriell Institute for Medical Research, Camden, NJ) with known genotype to validate the pyrosequencing assays optimized in this study.

2.4. Data analysis and bioinformatics

Differences between CFS and NF with respect to demographic characteristics and MFI, SF-36 and SI scales were assessed using chi-square test to compare proportions or independent sample *t*-test to compare means using SPSS version 19. Golden Helix SVS software was used for comprehensive genetic analysis with categorical (subject classification) and quantitative (SF-36, MFI and SI scores) variables, including quality control of SNP data in terms of Hardy–Weinberg Equilibrium (HWE), population stratification and adjustments for covariates. Based on SNP quality control criteria, 2353 SNPs (representing markers with call rate < 80%, MAF < 5%, HWE < 0.01 and 84 SNPs on X chromosome) were removed, leaving 9146 (79.5%) autosomal SNPs for genetic association analyses with CFS. Population stratification was not detected by the genomic control method as applied in the SVS software. Chi-square test was used to assess basic allele and genotypic associations between SNP and CFS (compared with NF). SNPs associated with CFS with a *p*-value of $\leqslant$0.05 were selected for functional annotation. We used bioinformatics tools SPOT (https://spot.cgsmd.isi.edu/submit.php) [30], GenomePipe (http://snpinfo.niehs.nih.gov/snpfunc.htm) [31] FastSNP (http://fastsnp.ibms.sinica.edu.tw/), PolymiRTS (http://compbio.uthsc.edu/miRSNP/) and Genomatix to identify SNPs with potential functional roles in affecting protein structure and functions (synonymous or non-synonymous SNPs), splicing regulation (enhancers or silencers), gene expression regulation (create or abolish transcription factor binding site [TFBS]), or affecting microRNA binding sites (create/delete). Genotypic calls of SNPs with predicted functional significance and proxy SNPs in high linkage disequilibrium (LD $\geqslant$ 0.8) were validated by pyrosequencing. These refined genotyping results were re-evaluated for association with CFS using allele test (chi-square test) and specific genetic models (by logistic regression). For analysis of genetic models, the genotypes were coded into numeric values (additive: dd = 0, Dd = 1, DD = 2; dominant: dd = 0, Dd = 1, DD = 1; recessive: dd = 0; Dd = 0, DD = 1 where d is the major allele and D is the minor allele). Linear regression was used to test the association of SNPs with quantitative measures of function (SF-36), fatigue (MFI) and symptoms (number of CFS case defining symptoms and SI score for CFS case defining symptoms [32]) in CFS subjects only using the numerically recoded genotypes in additive model. *p*-values were adjusted for covariates using SVS software. SVS software was also used for LD and haplotype analyses of markers in selected genes. Confidence interval (CI) values were estimated at 95% confidence level.

3. Results

3.1. Demographics of participants included in this analysis

The major demographic characteristics and MFI, SF-36 and SI scales of participants included in this analysis are shown in Table 1. The median time since onset of fatigue for CFS participants was 8.97 years (range 0.39–40.2 years) and 82.2% had gradual onset of illness. Body mass index (BMI) and sex were both associated with CFS compared to NF. Because of their association with CFS, BMI and sex were included as covariates in genetic models. As expected, all MFI, SF-36 and SI scales were significantly different between NF and CFS subjects, with higher MFI and lower SF-36 scores indicating more severe conditions respectively.

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