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

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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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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.

Table 1
Major demographic characteristics and SF-36, MFI and SI scales of subjects in analysis.

Factors	Classification[a]	
	NF (N = 121)	CFS (N = 50)
Age (years)		
Mean ± SD (range)	48.50 ± 9.46 (24–62)	48.02 ± 10.11 (22–63)
Female (%)	64.50	92.00***
BMI ± SD		
Both men and female	26.79 ± 5.13	28.84 ± 5.27*
(Men only)	(29.06 ± 4.47)	(27.75 ± 3.95)
(Female only)	(25.45 ± 5.04)	(28.93 ± 5.39)
SF-36 scales (mean ± SD)		
Physical function	96.07 ± 5.67	61.60 ± 23.80***
Role physical	99.59 ± 3.20	29.50 ± 36.66***
Body pain	84.11 ± 14.55	38.62 ± 14.54***
General health	85.50 ± 12.10	44.12 ± 18.65***
Vitality	80.04 ± 12.54	22.90 ± 16.07***
Social function	98.76 ± 3.75	49.75 ± 22.66***
Role emotional	100 ± 0.00	55.33 ± 42.92***
Mental health	89.59 ± 7.87	58.64 ± 22.17***
MFI scales (mean ± SD)		
General fatigue	6.41 ± 2.17	16.78 ± 2.49***
Physical fatigue	5.67 ± 1.58	13.92 ± 3.36***
Mental fatigue	6.07 ± 2.58	13.14 ± 4.02***
Reduced activity	5.30 ± 1.45	12.62 ± 4.41***
Reduced motivation	5.53 ± 1.81	12.40 ± 3.63***
SI scales (mean ± SD)		
Number of symptoms	1.87 ± 1.91	10.26 ± 2.96***
Number of CFS symptoms	0.91 ± 1.05	5.32 ± 1.15***
CFS defining symptom score	3.12 ± 4.45	44.21 ± 14.17***
Non-CFS symptom score	3.10 ± 4.33	31.51 ± 14.89***
SI-summary score	6.21 ± 7.69	77.05 ± 26.72***

[a] Subject classification: NF, non-fatigued with no medical/psychiatric exclusion; CFS, chronic fatigue syndrome subjects with no medical/psychiatric exclusions.
* Significantly different between NF and CFS at $p < 0.05$.
*** Significantly different between NF and CFS at $p < 1.0 \times 10^{-4}$. Higher MFI and lower SF-36 scores indicate more severe conditions respectively.

3.2. Potentially functional SNPs associated with CFS

Affymetrix results identified 427 SNPs associated with CFS at allele and 405 at genotype levels. The 38 SNPs with potential functional significance that were identified at both allele and genotype levels, along with 14 of their proxy SNPs that were not on the Affymetrix chip, were genotyped by pyrosequencing. After adjusting pyrosequencing results for covariates, 32 of 38 SNPs and 10 of 14 proxy SNPs remained significantly associated with CFS (Supplementary Table 2). The 32 functional SNPs were grouped, either directly or through their proxy SNPs, as non-synonymous variants (11 SNPs), synonymous variants (4 SNPs), located in the untranslated regulatory regions (UTR) of genes (11 SNPs), or intronic (6 SNPs). The genetic location, allele frequency, pathways, and function/mechanism for SNPs are summarized by category in Tables 2–5 and will be discussed in turn.

The minor alleles of 6 of 11 non-synonymous SNPs were risk alleles for CFS (Table 2). While most changes were predicted to be benign, probable (rs6115 in *serpin peptidase inhibitor* clade *A member 5*, *SERPINA5*) or possible (rs4151667 in *complement factor B*, *CFB*; rs3803568 in *lectin mannose binding 1 like*, *LMAN1L*) damaging changes were noted in three genes, two of which are in the complement pathway. For both of these complement genes the minor alleles were more common in CFS; G of rs6115 in *SERPINA5* (50% CFS vs 31% NF; $p = 1.58 \times 10^{-3}$) and A of rs4151667 in *CFB* (10.0% CFS vs 2.9% NF; $p = 5.94 \times 10^{-3}$). In 5 genes splicing regulation was a predicted effect of SNPs in the non-synonymous group. The minor alleles of 3 of the 4 synonymous SNPs were risk alleles for CFS. All four synonymous SNPs had predicted effect in splicing regulation of 4 genes (Table 3), two of which function in the complement cascade (rs1061147 in *complement factor H*, *CFH* and rs3774268 in *mannan-binding lectin serine peptidase 1*, *MASP1*). For one of the complement pathway genes the minor allele was more common in CFS; A of rs3774268 in *MASP1* (22.9% CFS vs 10.7% NF; $p = 3.88 \times 10^{-3}$). For the other complement pathway gene, the minor allele was less common in CFS; A of rs1061147 in *CFH* (28.0% CFS vs 43.8% NF, $p = 6.47 \times 10^{-3}$).

The minor alleles of 6 of the 11 UTR SNPs were risk alleles for CFS (Table 4). Nearly all (10 of 11) were located in the 3′ region and predicted to function in microRNA binding to 10 genes, two of which function in the complement cascade (rs9200 in *C6* and rs6108 (proxy rs9113) in *SERPINA5*. The minor allele T of rs9113 in *SERPINA5*, proxy SNP for rs6108, was more common in CFS (49% vs 33.5% NF; $p = 7.12 \times 10^{-3}$) with predicted disruption of a binding site for hsa-miR1258. The minor allele A of rs353254, proxy SNP for CFS-associated rs372402, was predicted to create a binding site for hsa-miR-607 in *prenylcysteine oxidase 1 like* (*PCYOX1L*). This minor allele was more frequent in CFS (53%) than NF (43.27%), with statistical significance only in a dominant model adjusted for sex and BMI ($p = 1.63 \times 10^{-3}$). The minor allele C of rs1051007 in the overlapping 3′ UTR of *mediator complex subunit 11* (*MED11*) and *chemokine (C-X-C motif) ligand 16* (*CXCL16*) was more common in CFS (20% CFS vs 6.6% NF; $p = 2.43 \times 10^{-4}$). One of the 11 UTR SNPs was near the 5′ end of one gene in PI3K/AKT signaling pathway and predicted to affect transcription factor binding.

The minor alleles of 3 of the 6 intronic SNPs were risk alleles for CFS (Table 5), creating transcription factor binding sites (TFBS) (matrix similarity scores 0.912–0.957). None of the intronic SNPs were located in genes in the complement pathway. The minor allele A of rs11257804 in intron 1-alternative promoter of *calcium/calmodulin-dependent protein kinase ID* (*CAMK1D*) was more common in CFS and created a binding site for the initiator response element (INRE) (44% CFS vs 24.4% NF; $p = 3.21 \times 10^{-4}$). The minor allele G of rs7616342 in intron 6 of *potassium voltage gated channel subfamily H member 8* (*KCNH8*) was more common in CFS and created a binding site for *STAT6* (*signal transducer and activator of transcription 6 interleukin 4 induced*) (53.0% CFS vs 35.1% NF; $p = 2.18 \times 10^{-3}$).

3.3. C2/CFB and CFH alleles and haplotypes associated with CFS

We used pyrosequencing to genotype an additional 6 SNPs in complement genes (rs12614 and rs641153 in *CFB*; rs9332739 in *C2*; rs800292, rs1061170 and rs10801555 in *CFH*). Fig. 1 shows the LD analysis of these SNPs with CFS and the haplotype analysis for markers in LD. For *C2*/*CFB*, two SNPs, rs9332739 (G/C polymorphism) and rs4151667 (T/A polymorphism) were in high LD (Fig. 1A). Their respective minor alleles C and A were associated with CFS in both allele and haplotype analyses. Haplotype CA was nearly 4 times more likely to be associated with CFS than NF (OR = 3.73; CI = 1.38–10.10; $p = 6.0 \times 10^{-3}$). For *CFH*, four SNPs, rs1061147 (G/T polymorphism), rs7529589 (C/T polymorphism), rs1061170 (T/C polymorphism) and rs10801555 (G/A polymorphism), were in high LD (Fig. 1B). Their respective major alleles G, C, T and G were associated with CFS in both allele (72–73% CFS vs 56% NF; $p = 4.81–6.5 \times 10^{-3}$) and haplotype analyses. Haplotype GCTG was 2 times more likely to be associated with CFS than NF (OR = 2.07; CI = 1.24–3.45; $p = 4.8 \times 10^{-3}$).

Three of these CFS-associated SNPs in the complement pathway were non-synonymous [rs1061170 (Fig. 1B); rs9332739 and rs4151667 (Fig. 1A)]. The T/C polymorphism in rs1061170 changes tyrosine with histidine at position 402 (Y402H) in exon 9 of *CFH*. The G/C polymorphism of rs9332739 replaces glutamate with aspartate at position 318 (E318D) in exon 7 of *C2*. The T/A polymorphism of rs4151667 replaces leucine to histidine at position

Table 2
Summary of SNPs associated with CFS: non-synonymous group.

SNP ID[a]	Chr#	Allele	MAF[b] (NF)	MAF (CFS)	*p*-Value (allele)	*p*-Value (adjusted)[c]	Gene (pathway)	SNP function/mechanisms[d]
rs6112	14	C/T	0.289	0.460	2.42E-03	8.98E-03 (A)	*SERPINA5* (complement cascade)	Missense (S64N); probable damaging effect by codon change
rs6115		A/G	0.318	0.500	1.58E-03	7.98E-03 (A)		
rs9550987	13	T/A	0.178	0.330	2.06E-03	1.61E-03 (A)	*TNFRSF19* (TNF super family signaling)	Missense (S31T); splicing regulation; benign effect by codon change
rs3802814	11	G/A	0.103	0.190	2.94E-02	3.42E-02 (A)	*TIRAP* (toll-like receptor signaling)	Missense (S180L); benign effect by codon change. May have regulatory potential
rs8177374		C/T	0.128	0.190	NS	NS		
rs4151667	6	T/A	0.029	0.100	5.94E-03	3.88E-02 (A)	*CFB* (complement cascade)	Missense (L9H); possible damaging effect by codon change
rs2278831	19	A/G	0.037	0.110	9.05E-03	7.73E-03 (D)	*SIGLEC5* (T-cell activation)	Missense (F322S); benign effect on protein structure; splicing regulation
rs12439525	15	C/T	0.074	0.020	NS	2.78E-02 (A)	*LMANIL* (protein processing in endoplasmic reticulum)	Missense (R105Q); possible damaging by codon change; splicing regulation
rs3803568		C/T	0.062	0.010	3.84E-02	4.10E-02 (A)		
rs2277680	17	A/G	0.504	0.390	NS	3.20E-02 (A)	*CXCL16* (chemokines)	Missense (I142T; A200V); benign effect by both codon changes
rs1050998		T/C	0.500	0.390	NS	4.71E-02 (A)		
rs4251545	12	G/A	0.112	0.040	3.60E-02	1.28E-02 (A)	*IRAK4* (toll-like receptor signaling)	Missense (A428T); benign effect by codon change; splicing regulation
rs1801058	4	C/T	0.424	0.276	1.07E-02	2.60E-03 (A)	*GRK4* (G-protein coupled receptor signaling)	Missense (V486A); benign effect on protein structure; splicing regulation
rs17500510	6	G/A	0.074	0.150	3.12E-02	2.47E-02 (A)	*HLA-DQA2* (phagocytosis-Ag presentation)	Missense (G247D); benign effect on protein structure
rs2071800		C/T	0.054	0.092	NS	NS		
rs11575584	9	G/A	0.058	0.100	NS	2.73E-02 (D)	*CCL27* (chemokines)	Missense (L96F); benign effect on protein structure

[a] Bold font indicates non-synonymous variant; bold-italics font indicates non-synonymous proxy SNPs; other SNPs in non-synonymous group because of proxy SNP.
[b] MAF = minor allele frequency.
[c] *p*-Values after adjusting for sex and BMI. NS = non-significant. (A) = additive and (D) = dominant genetic models.
[d] Based on SPOT/GenomePipe annotations.

Table 3
Summary of SNPs associated with CFS: synonymous group.

SNP ID[a]	Chr#	Allele	MAF[b] (NF)	MAF (CFS)	*p*-Value (allele)	*p*-Value (adjusted)[c]	Gene (pathway)	SNP function/mechanisms[d]
rs2228428	3	C/T	0.273	0.430	4.52E-03	4.21E-04 (R)	*CCR4* (chemokines)	Codon-synonymous (Y338Y); splicing regulation
rs11214105	11	G/A	0.198	0.320	1.56E-02	3.31E-02 (D)	*IL18/TEX12* (cytokines)	rs11214105 near gene-5' with potential transcription factor binding.
rs549908		T/G	0.231	0.320	NS	NS		rs549908 results in codon synonymous change (S35S); splicing regulation
rs7529589	1	C/T	0.446	0.280	4.26E-03	7.79E-03 (A)	*CFH* (complement cascade)	Codon-synonymous (A307A); splicing regulation
rs1061147		C/A	0.438	0.280	6.47E-03	1.49E-02 (A)		
rs3774268	3	G/A	0.107	0.229	3.88E-03	1.26E-03 (A)	*MASP1* (complement cascade)	Codon-synonymous (S445S); splicing regulation

[a] Bold font indicates synonymous variant; bold-italics font indicates synonymous proxy SNPs; other SNPs in synonymous group because of proxy SNP.
[b] MAF = minor allele frequency.
[c] *p*-Values after adjusting for sex and BMI. NS = non-significant. (A) = additive; (D) = dominant; (R) = recessive genetic models.
[d] Based on GenomePipe and FastSNP annotations.

9 (L9H) in the signal peptide of *CFB*. *C2* and *CFB* are paralogous genes separated only by 500 bp. Therefore for a two-locus combined genotype analysis of *C2*/*CFB* and *CFH* only rs9332739 in *C2* (genotypes, GG/GC/CC) was used with rs1061170 (genotypes, TT/TC/CC) in *CFH*. Fig. 2 shows the distribution of CFS and NF subjects in 5 genotype combinations (GG-CC, GG-CT, GG-TT, GC-CT and GC-TT) of these SNPs. No instances of *C2* genotype CC were identified. The distribution of CFS and NF subjects was significantly different for all genotype combinations except GC-CT. Since the CFS risk alleles were the minor allele C of rs9332739 in *C2* and the major allele T of rs1061170 in *CFH*, the least likely genotype combinations in CFS were GG-CC (CFS, 13.7% [4/29]; NF, 86.2% [25/29]; $p = <1.0 \times 10^{-4}$) and GG-CT (CFS, 22.7% [15/66]; NF, 77.2% [51/66]; $p = <1.0 \times 10^{-4}$). CFS patients were significantly less likely to have these two genotype combinations compared to the rest of the genotype combinations (adjusted odds ratio 0.410 (CI = 0.198–0.849; $p = 1.5 \times 10^{-2}$).

3.4. SNPs associated with quantitative measures of CFS

The 32 SNPs associated with CFS (Tables 2–5) were tested for association in additive models with measures of function (SF-36), fatigue (MFI) and symptoms (SI). Age, sex and BMI were examined as co-variates with each measure, and those with significant association were used to adjust the association. Four SNPs each associated with a different SF-36 subscale (rs11214105 in *interleukin 18/testis expressed 12* (*IL18/TEX12*) with body pain, $p = 8.0 \times 10^{-3}$ adjusted for age; rs6112 in *SERPINA5* with physical function, $p = 1.8 \times 10^{-2}$ adjusted for age; rs227680 in *CXCL16* with general health, $p = 2.0 \times 10^{-3}$ adjusted for age; and rs1801058 in *G protein-coupled receptor kinase 4* (*GRK4*) with social function, $p = 1.2 \times 10^{-2}$). Two of these same SNPs were associated with MFI physical fatigue score (rs11214105 in *IL18/TEX12* and rs6112 in *SERPINA5*; $p = 3.0–6.0 \times 10^{-3}$). The *IL18/TEX12* SNP (rs11214105) was also associated with CDC SI score for CFS case

Table 4
Summary of SNPs associated with CFS: UTR group.

SNP ID[a]	Chr#	Allele	MAF[b] (NF)	MAF (CFS)	*p*-Value (allele)	*p*-Value (adjusted)[c]	Gene (pathway)	SNP function/mechanisms[d]
rs1051007	17	T/C	0.066	0.200	2.43E-04	2.64E-05 (A)	*MED11*/*CXCL16* (chemokines)	UTR-3; miRNA binding
rs11658971		G/A	0.075	0.190	1.92E-03	2.02E-04 (A)		
rs13010656	2	G/T	0.397	0.560	5.70E-03	5.92E-03 (A)	*BMPR2* (NF-& kappa; B signaling)	UTR-3; miRNA binding[f]
rs1048829		G/T	0.397	0.560	5.70E-03	4.35E-03 (A)		
rs3020729	2	T/C	0.157	0.040	2.71E-03	1.88E-03 (A)	*CD8A* (lymphocyte signaling)	UTR-3; miRNA binding
rs9200	5	G/A	0.517	0.340	2.92E-03	6.41E-03 (A)	*C6* (complement cascade)	UTR-3; miRNA binding[e]
rs228945	22	A/G	0.310	0.170	7.95E-03	5.88E-03 (A)	*IL2RB* (cytokines)	UTR-3; miRNA binding
rs228941		C/G	0.298	0.190	4.07E-02	3.65E-02 (A)		
rs6108	14	T/A	0.331	0.490	5.66E-03	1.27E-02 (A)	*SERPINA5* (complement cascade)	UTR-3; miRNA binding[e,f]
rs9113		C/T	0.335	0.490	7.12E-03	1.41E-02 (A)		
rs3751488	14	G/A	0.298	0.146	4.16E-03	3.47E-03 (D)	*MRPL52* (migration)	UTR-3; miRNA binding[f]
rs733590	6	T/C	0.364	0.500	1.94E-02	2.36E-02 (A)	*CDKN1A* (P13K/AKT signaling)	Near gene 5'; transcription factor binding site
rs2395655		A/G	0.397	0.550	9.40E-03	3.61E-02 (A)		
rs2016483	4	T/A	0.376	0.510	2.22E-02	3.75E-02 (A)	*SMARCAD1* (helicase protein)	UTR-3; miRNA binding[e]
rs8336		G/A	0.360	0.440	NS	NS		
rs4819388	21	C/T	0.314	0.200	3.28E-02	3.44E-03 (D)	*ICOSLG* (B7 family)	UTR-3; miRNA binding[f]
rs372402	5	C/T	0.413	0.530	4.82E-02	4.45E-04 (D)	*IL17B*/*PCYOX1L* (cytokines)	UTR-3; miRNA binding[e,f]
rs353254		G/A	0.433	0.530	NS	1.63E-03 (D)		

[a] Bold font indicates UTR variant; bold-italics font indicates UTR proxy SNPs; other SNPs in UTR group because of proxy SNP.
[b] MAF = minor allele frequency.
[c] *p*-Values after adjusting for sex and BMI. NS = non-significant. (A) = additive and (D) = dominant genetic models.
[d] Based on GenomePipe and PolyMir annotations.
[e] Score difference due to allele change is $\geqslant 16$ (by GenomePipe), suggesting an SNP effect on miRNA binding.
[f] Supported also by the PolyMir database.

Table 5
Summary of SNPs associated with CFS: intronic group.

SNP ID	Chr#	Allele	MAF[a] (NF)	MAF (CFS)	*p*-Value (allele)	*p*-Value (adjusted)[b]	Pathway	Gene/SNP location	Associated allele	Created TFBS[c] (score[d])
rs2014012	5	A/T	0.355	0.160	2.22E-02	2.27E-03 (A)	G-protein coupled receptor signaling	*PDE4D*/intron 7	A (major)	*FOXQ1* (0.877)
rs11257804	10	G/A	0.244	0.440	3.21E-04	1.62E-03 (A)	Calcium signaling	*CAMK1D*/intron 1-alternative promoter	A (minor)	INRE (0.957)
rs829370	1	T/C	0.033	0.120	1.83E-03	3.07E-04 (A)	ERK/MAPK signaling	*RAP1GAP*/intron 8	C (minor)	*FOXH1* (0.912)
rs7616342	3	A/G	0.351	0.530	2.18E-03	1.98E-05 (D)	P38 MAPK signaling	*KCNH8*/intron 6	G (minor)	*STAT6* (0.921)
rs17591814	1	C/T	0.450	0.300	9.99E-03	1.87E-03 (D)	ERK/MAPK signaling	*PLA2G4A*/intron 3	C (major)	*ZEB1* (0.994)
rs10498445	14	C/G	0.306	0.160	5.35E-03	5.59E-04 (D)	Eicosanoid signaling	*PTGDR*/intron 1	C (major)	*PAX* (0.854)

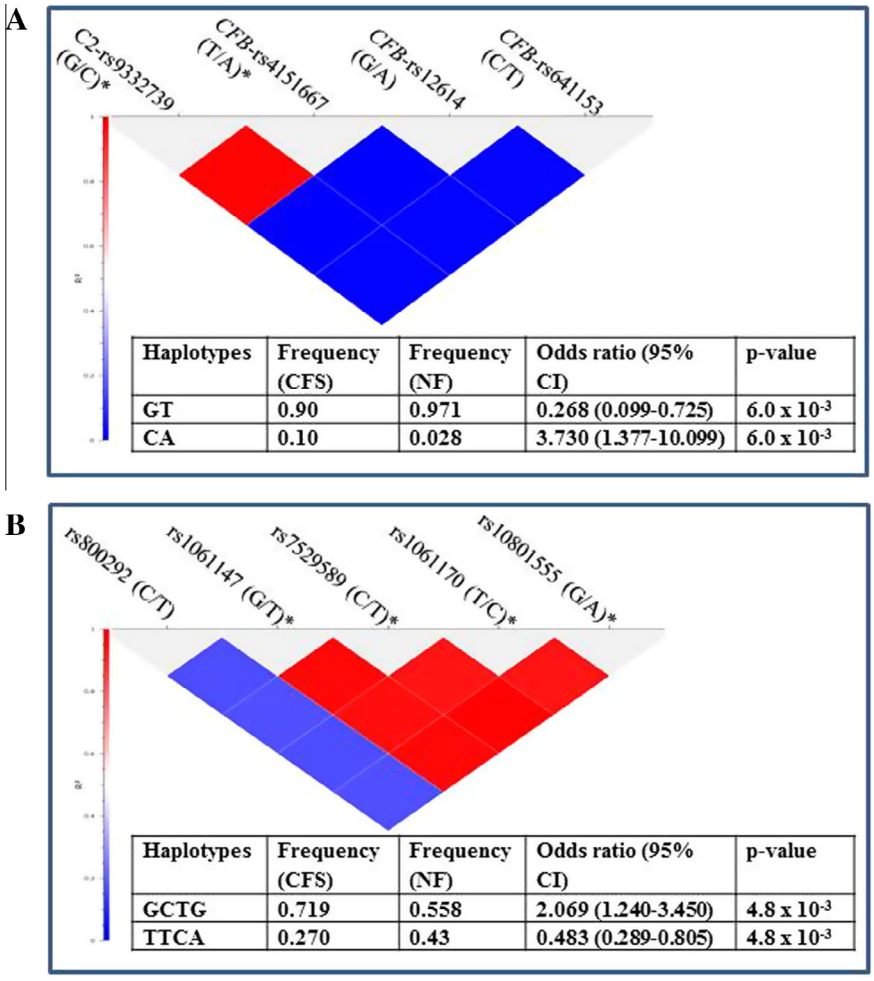
[a] MAF = minor allele frequency.
[b] *p*-Values after adjusting for sex and BMI. (A) = additive, and (D) = dominant genetic models.
[c] FOXQ1, forkhead box Q1; INRE, initiation response element in the promoter; FOXH1, forkhead box H1; STAT6, signal transducer and activator of transcription 6; ZEB1, zinc finger E-box binding homeobox 1; PAX6, paired box 6.
[d] Matrix similarity score by Genomatix.

defining symptoms ($p = 6.06 \times 10^{-5}$) and the number of CFS symptoms ($p = 4.1 \times 10^{-4}$). In each instance, homozygosity for the minor allele A of rs11214105 in *IL18/TEX12* was associated with more severity for each of the measures. rs6112 in *SERPINA5* was also associated with the number of CFS symptoms ($p = 8.1 \times 10^{-4}$). The non-synonymous variant, rs2278831 in *sialic acid binding Ig-like lectin 5* (*SIGLEC5*), was also associated with CDC SI score for CFS case defining symptoms ($p = 4.7 \times 10^{-3}$).

4. Discussion

This study identified several previously unrecognized genetic associations with CFS that are worthy of further study for validation. If validated, these associations support the hypothesis that immune and inflammatory mechanism may be involved in CFS. Many of the SNPs associated with CFS were located in genes involving complement activation, chemokines and cytokines and toll-like receptor (TLR) signaling. The findings in the complement system are of a particular interest, as a role for complement activation in CFS has been suggested by a recent case report and prior gene expression and proteomic studies. In the case report, the patient remained chronically fatigued while the levels of complement split products were elevated but CFS symptoms resolved within two months of normalization of split products [33]. Gene expression studies indicated differential expression of complement protein *MASP2* contributing to the C4a split product in CFS following exercise [14,15]. Complement proteins in the cerebrospinal fluid of subjects with CFS were elevated compared with healthy controls [34].

The complement system plays major roles in defending host against infection, coordinating events during inflammation and bridging innate and adaptive immune responses [35]. Complement activation is initiated by three sub-pathways (classical, alternate and lectin pathways), all of which lead to the formation of C3 convertase required for the cleavage of C3 [36]. Both *CFH* and *CFB* play pivotal but opposing roles in the alternate pathway. *CFB* activates the complement cascade by contributing to the formation C3 convertase and *CFH* inhibits the same pathway by accelerating the decay of C3 convertase [37]. *C2* is similar to *CFB*, but contributes to the formation of C3 convertase through the classical pathway. Interestingly two CFS-associated missense variants in genes of complement activation, [rs4151667 (L9H) in *CFB* and rs1061170 (Y402H) in *CFH*] have previously been associated with



Haplotypes	Frequency (CFS)	Frequency (NF)	Odds ratio (95% CI)	p-value
GT	0.90	0.971	0.268 (0.099-0.725)	6.0×10^{-3}
CA	0.10	0.028	3.730 (1.377-10.099)	6.0×10^{-3}

Haplotypes	Frequency (CFS)	Frequency (NF)	Odds ratio (95% CI)	p-value
GCTG	0.719	0.558	2.069 (1.240-3.450)	4.8×10^{-3}
TTCA	0.270	0.43	0.483 (0.289-0.805)	4.8×10^{-3}

Fig. 1. LD plot and haplotype association results with selected markers in *C2*/*CFB* (A) and *CFH* (B). Selected markers are shown at the top of each LD plot. LD was measured by pair-wise R^2 values indicated by colors ranging from blue (no LD) to red (high LD). Haplotype association was restricted to markers (denoted with asterisk) in LD. Haplotypes significantly different between CFS and NF are given under the LD plots. Haplotype GT comprises major alleles G and T and haplotype CA comprises minor alleles C and A of rs9332739 in *C2* and rs415667 in *CFB* respectively. Haplotype GCTG consists of the major alleles G, C, T and G and haplotype TTCA consists of the minor alleles T, T, C and A of rs1061147, rs7529589, rs1061170 and rs10801555 respectively in *CFH*.

age-related macular degeneration (AMD) [38,39]. The haplotype analysis that included the rs9332739 (E318D) in *C2*, a SNP that is in high LD with rs4151667 in *CFB*, reported to be associated with AMD, was also associated with CFS in our study; however, the risk and protective variants were switched (i.e. alleles protective for AMD were associated with risk for CFS). A recent report indicates that protective alleles of *CFH* and *CFB* for AMD associate with lower levels of complement activation [40]. Since the same alleles are associated with risk for CFS in this study, we hypothesize lower levels of complement activation may be associated with at least a subset of CFS subjects.

To our knowledge, this is the first study that associates CFS with polymorphisms in chemokine ligands/receptors, molecules that regulate leukocyte trafficking. One of the strongest associations was with a synonymous risk variant rs2228428 (Y338Y) potentially regulating splicing in *CCR4*, a CC chemokine receptor reported to be expressed in Th2 and Treg cells [41]. Associations with variants in *CXCL16*, a member of the CXC family of chemokines were also found. rs1051007 in the 3′ UTR of *CXCL16*, reported to impact its gene expression, was associated with risk for CFS [42] whereas a missense polymorphism (rs2277680) in *CXCL16* was associated with protection. These results suggest that further studies of *CCR4* and *CXCL16* in CFS are warranted.

Prior studies have reported a few polymorphisms in cytokines to be associated with CFS [23,25]. These previously reported SNPs were not included in the Affymetrix Human Immune and Inflammation Chip we used, however we identified additional cytokine polymorphisms, particularly rs11214105 in *IL18*, a pleotropic cytokine that enhances perforin mediated T-cell and NK-cell cytotoxicity. *IL18* has been reported to contribute to the development and pathogenesis of infectious and neuro-inflammatory diseases with immune and cognitive dysfunctions [43,44]. Animal studies linked *IL18* to diseases with sex-specific prevalence and complex, regional and sex-specific parental effects in the brain [45]. We found this *IL18* polymorphism to be associated with CFS and quantitative measures of CFS symptoms: body pain, physical fatigue, symptom summary scores and number of CFS symptoms. Our results suggest additional study of *IL18* as a candidate gene in CFS pathophysiology.

Several CFS associated 3′ UTR polymorphisms merit further analyses since they may impact binding sites for microRNAs that have not yet been examined in CFS. While the impact of these SNPs on microRNA binding remains to be verified experimentally, our results suggest a mechanism for microRNA regulation of genes associated with CFS. Some of the microRNAs impacted by CFS associated SNPs include hsa-miR-580 by rs9200 in *C6*, hsa-miR-607 by rs353254 in *IL17B*, and hsa-miR-1258 by rs9113 in *SERPINA5*. A missense variant in *SERPINA5* (rs6115) was also associated with CFS. Since these *SERPINA5* variants are not in LD, they may confer independent risk for CFS. *SERPINA5*, an inhibitor of activated

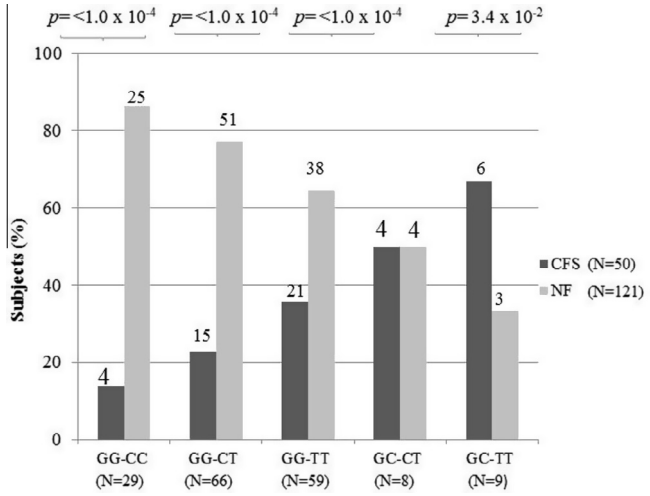


Fig. 2. Two-locus combined genotype analysis of *C2*/*CFB* and *CFH*. CFS and NF subjects were stratified by genotype combinations of rs9332739 (G/C) in *C2* and rs1061170 (T/C) in *CFH*. CFS risk alleles of rs9332739 and rs1061170 are C and T respectively. *X*-axis shows the five genotype combinations between these two markers in the CFS/NF subjects and *Y*-axis represent the proportion of CFS/NF subjects within each genotype combinations. Numbers on the top of each bar are observed numbers of CFS/NF in each genotype combinations. The distributions of CFS/NF were significantly different in all genotype combinations except for GC-CT.

protein C, is located in chromosome 4 with other members of the serpent protease family including *corticosteroid binding globulin* (*SERPINA6*) which has been reported to be associated with CFS [46].

The CFS associated intronic variant rs11257804 in *CAMK1D* is of interest. *CAMK1D* is a protein kinase in the calcium signaling pathway. It plays a major role in granulocyte function and has little or no expression in monocytes and lymphocytes [47]. This result suggests further exploration of granulocyte function and host defense in CFS is warranted.

The small sample size along with the lack of correction for multiple tests is the major limitation of this exploratory study. To mitigate these limitations we used a focused selection of genes and gene-centric SNPs in immune and inflammation pathways [26], supplemented with functional annotation using bioinformatics tools like SPOT and Genome Pipe [30,31], and validation of genotyping accuracy of SNP results by pyrosequencing. Because we did not correct for multiple testing, we used SNP prioritization tools to identify functionally significant associations. This approach has been successful in identifying replicated associations that would have been considered false positive after correction for multiple testing [30,31]. The power of study to detect associations is dependent on sample size as well as the genetic model, minor allele frequencies and effect size of the SNP [48]. With the sample size used in this study, some of the functionally annotated SNPs with high statistical significance in dominant model (rs372402 in Table 4 and rs7616342 in Table 5) provide 76–99% power to detect association with CFS at a pooled disease prevalence of 0.31% and controls screened for not having the disease (based on the Genetic Power Calculator [48]). In addition, based on genotyping of a selected set of 52 SNPs by pyrosequencing, the association of two SNPs with CFS (rs1051007 in Table 4 and rs11257804 in Table 5) met the threshold ($p \leqslant 9.62 \times 10^{-4}$) for multiple testing by Bonferroni correction. Our study identified 11 missense variants, 4 synonymous variants, 11 3′ UTR and 6 intronic variants with potential regulatory functions by incorporating the predictive power of biological information in the SNP prioritization process. In addition, the specificity of the associations for CFS cannot be determined in this study since the comparison was only made to non-fatigued controls. Identifying CFS-associated abnormalities in a case control study design is an important first step in identifying candidate genes and pathways that may be implicated in the pathogenesis of CFS.

In conclusion, this study identified several new genetic associations of CFS with variants in the complement activation, chemokine's, cytokines and toll-like receptor signaling. Associations in these pathways provide additional support for a role of altered innate immune response in CFS. Additional studies in larger patient cohorts are needed to validate the findings of this exploratory study.

Author contribution

M.S.R. conceived, designed and participated in all phases of the study including analysis and the first draft of the manuscript. M.S.R. and E.R.U. participated in manuscript writing, interpretation and discussion of the results. I.D. and J.M. conducted the genotyping experiments using the Affymetrix platform and pyrosequencing. V.R.F. provided bioinformatics support on the identification of SNP dependent transcription factor binding sites. All authors read and approved the manuscript for publication.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.humimm.2015.06.014.

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