

Glucocorticoid receptor polymorphisms and haplotypes associated with chronic fatigue syndrome

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Chronic fatigue syndrome (CFS) is a significant public health problem of unknown etiology, the pathophysiology has not been elucidated, and there are no characteristic physical signs or laboratory abnormalities. Some studies have indicated an association of CFS with deregulation of immune functions and hypothalamic–pituitary–adrenal (HPA) axis activity. In this study, we examined the association of sequence variations in the glucocorticoid receptor gene (*NR3C1*) with CFS because *NR3C1* is a major effector of the HPA axis. There were 137 study participants (40 with CFS, 55 with insufficient symptoms or fatigue, termed as ISF, and 42 non-fatigued controls) who were clinically evaluated and identified from the general population of Wichita, KS. Nine single nucleotide polymorphisms (SNPs) in *NR3C1* were tested for association of polymorphisms and haplotypes with CFS. We observed an association of multiple SNPs with chronic fatigue compared to non-fatigued (NF) subjects ($P < 0.05$) and found similar associations with quantitative assessments of functional impairment (by the SF-36), with fatigue (by the Multidimensional Fatigue Inventory) and with symptoms (assessed by the Centers for Disease Control Symptom Inventory). Subjects homozygous for the major allele of all associated SNPs were at increased risk for CFS with odds ratios ranging from 2.61 (CI 1.05–6.45) to 3.00 (CI 1.12–8.05). Five SNPs, covering a region of approximately 80 kb, demonstrated high linkage disequilibrium (LD) in CFS, but LD gradually declined in ISF to NF subjects. Furthermore, haplotype analysis of the region in LD identified two associated haplotypes with opposite

alleles: one protective and the other conferring risk of CFS. These results demonstrate *NR3C1* as a potential mediator of chronic fatigue, and implicate variations in the 5' region of *NR3C1* as a possible mechanism through which the alterations in HPA axis regulation and behavioural characteristics of CFS may manifest.

Keywords: Chronic fatigue syndrome, glucocorticoid receptor gene (*NR3C1*), haplotypes, hypothalamic–pituitary–adrenal axis, polymorphisms

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Introduction

Chronic fatigue syndrome (CFS) is a complex and debilitating disorder that affects between 400 000 and 900 000 people in the USA, resulting in national productivity losses comparable to digestive, immune, nervous system and skin diseases (Reynolds *et al.* 2004). CFS has no diagnostic physical signs or laboratory abnormalities so the syndrome is a diagnosis of exclusion based on medically unexplained self-reported fatigue lasting for 6 months or longer accompanied by specific accompanying symptoms (Fukuda *et al.* 1994). While the etiology of CFS remains unknown, several clinical studies suggest dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis and perturbations of the immune system and both autonomic and central nervous systems (Cleare 2004; Freeman 2002; Haddad *et al.* 2002; Unger *et al.* 2004). More recently, investigators have described altered expression of genes associated with immune function in persons with CFS (Kaushik *et al.* 2005; Powell *et al.* 2003; Steinau *et al.* 2004; Vernon *et al.* 2002). In addition, others have found an association of polymorphisms in the genes of people with CFS; specifically polymorphisms in the genes for the corticosteroid binding protein (*SERPINA6*), serotonin transporter (*SLC6A4*), angiotensin converting enzyme (*ACE*), and human leucocyte antigen (HLA) class II antigens (Narita *et al.* 2003; Smith *et al.* 2005; Torpy *et al.* 2001, 2004; Vladutiu & Natelson 2004).

The HPA axis is a promising candidate for further molecular studies. Glucocorticoids (cortisol in humans), the end product of the HPA axis, exert marked effects on metabolism, immune function and the brain, adjusting physiological functions and behaviour in response to the stressor (Heim

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the funding agency.

et al. 2004; Swaab et al. 2005). Cortisol is synthesized *de novo* and secreted from the adrenal cortex in response to adrenocorticotrophin (ACTH) secreted from the anterior pituitary gland. The secretion of ACTH is synergistically stimulated by corticotrophin-releasing hormone and arginine vasopressin released from the hypothalamic paraventricular nucleus. Glucocorticoids exert negative feedback control on the HPA axis by regulating hippocampal and paraventricular nucleus neurons. The effects of glucocorticoids are mediated via two types of adrenal steroid receptors, the mineralocorticoid receptor (*NR3C2*) and the glucocorticoid receptor (*NR3C1*). While the *NR3C2* is implicated in HPA axis regulation under basal conditions, *NR3C1* is involved in mediating glucocorticoid feedback under challenge conditions and also mediates cortisol effects on immune function.

While several studies suggest that CFS is related to low circulating cortisol (hypocortisolism), others found elevated circulating cortisol levels (hypercortisolism) in CFS (Cleare 2003, 2004; Demitrack 1998). Hypo- and hyper- levels of cortisol are also observed with fibromyalgia, sleep disorders, post-traumatic disorder, anxiety and major depression (Buckley & Schatzberg 2005; Calis et al. 2004; Cleare 2004; Heim et al. 2000; Kunugi et al. 2006) all of which are frequent co-morbid conditions in CFS and share risk factors similar to those for CFS. These disorders may be related to inter-individual variations in HPA axis activity and altered glucocorticoid effects.

A major determinant of glucocorticoid signalling is the sensitivity of the glucocorticoid receptor (*NR3C1/hGR*) to its ligand. The *NR3C1* gene maps to chromosome 5q31 and spans approximately 128 kb in length with 12 exons, multiple promoters and differentially spliced isoforms. The GR α and GR β isoforms are generated by alternative splicing between exons 9 α and 9 β . Imbalances in the number, processing and function of *NR3C1* gene products can alter the level of negative feedback influence on HPA axis activity and the immune system associated with various medical conditions (Jurueña et al. 2004; Pariante & Miller 2001; Raison & Miller 2003). Since *NR3C1* is a major effector of the HPA axis through which cortisol signals to other physiological systems and the central nervous system, we examined the association of sequence variation in *NR3C1* with CFS.

Materials and methods

Subjects

Enrollment

This study adhered to the human experimentation guidelines of the Helsinki Declaration and was approved by the Centers for Disease Control (CDC) Institutional Review Board. All subjects were volunteers who gave informed consent.

We enrolled subjects who were identified from 1997 through 2000 in the Wichita CFS Surveillance Study (Reyes et al. 2003). In brief, the surveillance study used a random-

digit-dialling telephone survey to screen 56 146 adult residents, aged 18–69 years, of Wichita, Sedgwick County, KS and followed a cohort of 3528 fatigued and 3634 non-fatigued adults at 12-, 24- and 36-month intervals with telephone interviews and clinical evaluations.

We enrolled 227 people from the surveillance cohort for this 2-day in-hospital study (Reeves et al. 2005). In brief, we enrolled 58 people with CFS diagnosed at least once during surveillance by 1994 criteria, 55 non-fatigued control subjects who did not have medical or psychiatric exclusions and who had not reported fatigue of at least 1-month duration, and 114 subjects from the surveillance cohort with other unexplained fatiguing illness, not CFS (we term this insufficient symptoms or fatigue; i.e. ISF). Non-fatigued (NF) controls were matched to CFS cases based on sex, race/ethnicity, age and body mass index (BMI).

Clinical evaluation

During the clinic stay, we re-evaluated all 227 subjects in terms of CFS symptoms and exclusionary medical and psychiatric conditions. Members of the hospital staff were unaware of the subjects' enrollment status; as were the subjects themselves. To identify exclusionary medical conditions of CFS (Fukuda et al. 1994; Reeves et al. 2003), subjects completed a standardized past medical history and review of systems, brought all current prescribed and over-the-counter medications to the hospital, underwent a standardized physical examination and provided blood and urine for routine clinical laboratory analyses. To identify exclusionary psychiatric conditions, licensed and specifically trained psychiatric interviewers conducted the Diagnostic Interview Schedule to diagnose current and lifetime Axis I psychiatric disorders (Robbins et al. 1995). Exclusionary psychiatric illnesses were current or lifetime bipolar disorder, psychosis, substance abuse within 2 years and eating disorders within 5 years.

During the hospital stay, participants also completed three instruments to evaluate the major domains of CFS (impairment, fatigue and accompanying symptoms). We used the Medical Outcomes Study 36-Item Short-Form Health Survey (SF-36) to measure impairment (Ware & Sherbourne 1992). The SF-36 assesses functional impairment in eight areas: (1) limitations in physical activities because of health problems (physical function), (2) limitations in social activities because of physical or emotional problems (social function), (3) limitations in usual role activities because of physical health problems (role physical), (4) bodily pain, (5) general mental health, (6) limitations in usual role activities because of emotional problems (role emotional), (7) vitality (energy and fatigue), and (8) general health perceptions (general health). Scores in each area reflect function and well-being and lower values indicate more impairment. We used the 20-item self-report Multidimensional Fatigue Inventory (MFI) to measure fatigue (Smets et al. 1995). The MFI measures five dimensions of fatigue: (1) general fatigue, (2) physical

fatigue, (3) mental fatigue, (4) reduced motivation and (5) activity. The score in each dimension reflects fatigue severity and higher values indicate more severe fatigue. We used the CDC Symptom Inventory (SI) case definition score to measure occurrence, frequency, and severity of the eight CFS case-defining symptoms (Wagner *et al.* 2005).

Classification of CFS

A medical or psychiatric condition that excluded CFS was identified at the time of the hospital study in 63 study participants. As recommended by the International CFS Study Group, we classified the remaining 164 eligible subjects as CFS, ISF, or NF at the time of the hospital study by clinically empirical criteria (Reeves *et al.* 2003, 2005). In brief, we considered substantial reduction in recreational, occupational, educational or social activities as a score ≤ 70 on the physical function, **or** ≤ 50 on the role physical, **or** ≤ 75 on the social function, **or** ≤ 66.7 on the role emotional subscales of the SF-36. We defined severe fatigue as a score ≥ 13 on the general fatigue **or** ≥ 10 on the reduced activity scales of the MFI. Finally, patients reporting four or more symptoms **and** scoring ≥ 25 on the CDC SI Case Definition Subscale were considered to have substantial accompanying symptoms. Forty-three study participants who met all three criteria (SF-36, MFI and SI) at the time of the in-hospital study were classified as CFS; 61 who met some but not all of the three criteria were considered as ISF and 60 who met none of the criteria were classified as NF. Seventeen of these 60 NF participants had been identified with CFS or ISF during surveillance and could therefore be considered in remission so the present analysis was limited to the NF controls who had been consistently non-fatigued during surveillance. We also restricted the present analysis to Caucasians since most (93%) of the CFS cases were Caucasians and allele frequencies of *NR3C1* polymorphisms vary with ethnicity (Hawkins *et al.* 2004). After all exclusions, the genetic study contained 137 participants (40 CFS, 55 ISF and 42 NF).

Genotyping

DNA was extracted from peripheral blood mononuclear cells using the Trizol RNA/DNA extraction method (Invitrogen, Carlsbad, CA, USA) and was subjected to genome-wide amplification using the Genomiphi DNA amplification kit (Amersham Biosciences, Piscataway, NJ, USA). The yield of amplified DNA was determined by quantitative TaqMan polymerase chain reaction for β -globin, assuming two copies of β -globin per cell and 5 pg DNA per cell (Rajeevan *et al.* 2005).

NR3C1 is approximately 128 kb in length and we genotyped nine single nucleotide polymorphism (SNP) markers as shown schematically in Fig. 1. Seven markers (A:rs1866388, B:rs2918419, C:rs860458, D:rs852977, E:rs6188, F:rs258750 and G:rs6196) were selected using an Applied Biosystems (ABI) SNP Browser™ with the criteria of linkage disequilibrium unit (LDU) ≤ 0.5 and minor allele frequency (MAF) $\geq 10\%$. The other two markers (H:rs6191 and I:rs6198) with MAF $> 10\%$ were from the dbSNP of National Center for Biotechnology Information (NCBI). Of the nine markers tested, six (A to F) are intronic with three of these (markers A to C) located in the intron between exons 2 and 3 (Fig. 1). Marker G resides in exon 9 α resulting in synonymous substitution (Asn766Asn) in the ligand-binding domain and binding site for heat-shock proteins in *NR3C1*. Markers H and I are located in the 9 β 3' untranslated region and marker I has been shown to affect mRNA stability (Schaaf & Cidlowski 2002).

Markers A to G were typed using a validated TaqMan genotyping assay kit from ABI (Ranade *et al.* 2001) and markers H and I were typed by pyrosequencing (Ronaghi 2003). Primers, probes and other reagents for TaqMan genotyping were obtained from ABI, and typing followed the manufacturer's protocol using the ABI 7900 Sequence Detection System (Foster City, CA, USA). For pyrosequencing, PCR and sequencing primers were designed using the Assay Design Software (Biotage, Inc. Foxboro, MA) and assays were conducted using PyroGold chemistry, PSQ 96MA instrument system and protocols from Biotage, Inc.

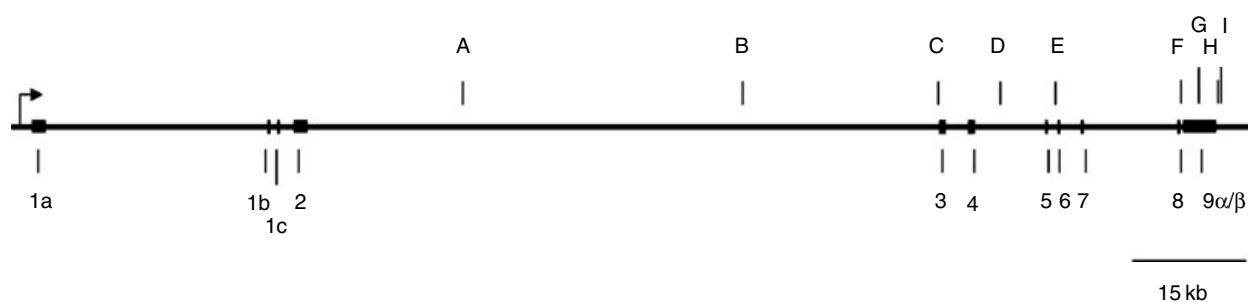


Figure 1: Location of selected SNPs in *NR3C1*. *NR3C1* is approximately 128 kb in length. Exons are numbered and SNP markers are designated with letters. Marker G results in synonymous change (Asn766Asn); markers A to F are intronic, and markers H and I are in the 3' untranslated region.

Genetic analysis

Polymorphisms were assessed to determine if the observed genotype frequencies were consistent with Hardy–Weinberg proportions using χ^2 -tests, as determined by SAS GENETICS (SAS Institute, Cary, NC, USA). Any marker out of Hardy–Weinberg equilibrium in the control subjects was excluded from further analysis. Linkage disequilibrium (LD) was assessed using both r^2 and Lewontin's D' statistics (Devlin & Risch 1995; Lewontin 1995) and plotted using GOLD (Abecasis & Cookson 2000).

To test for association between a marker and fatigue status (CFS, ISF or both CFS and ISF combined) allele frequencies of cases and controls were compared by χ^2 -test. Association between a marker and measures of the SF-36, MFI and SI scores was examined in all participants ($n = 137$) using the Wilcoxon rank sum test. Genotypes were first tested for general association by using a two-degree-of-freedom test for each outcome. If the P -value was found to be < 0.10 , or if there was an *a priori* hypothesis for a certain inheritance mode, further testing of the SNP effects were performed assuming specific modes of inheritance. For example, in the event that subjects homozygous for the minor allele had a mean score similar to heterozygous subjects, a recessive model was applied which combined the two groups. Results are reported for best mode of inheritance only. A recessive model was also applied for the estimation of genotype-specific risk measured as odds ratios (OR) with 95% confidence interval (CI) using SAS version 8 (SAS Institute). All nominal P -values represent point-wise significance, and were adjusted using the Westfall and Young permutation test (Westfall & Young 1993).

Haplotype analysis was performed using a score test developed by Schaid (Schaid *et al.* 2002), as implemented by HAPLO.STAT (<http://mayoresearch.mayo.edu/mayo/research/biostat/schaid.cfm>). Statistical differences in overall haplotype frequencies were tested for association with CFS, ISF, or CFS and ISF subjects combined. Specific haplotype effects were examined if the test for the overall frequency distribution was significant. All nominal P -values with haplotype analysis were also adjusted by permutation test.

Results

Demographics of study subjects

Because matching was based on diagnosis during a surveillance study and some subjects were reclassified into another diagnostic category or excluded at the time of the in-hospital study, matching was not maintained. Nevertheless, the characteristics of CFS, ISF and NF subjects were similar (Table 1). Of the 137 subjects in this study, over 75% of them were female in each fatigue status. The mean age and BMI of all subjects were 50.5 years and 28.8 (overweight), respectively. Potentially confounding factors such as age, sex and BMI were examined for interaction

Table 1: Demographic characteristics of non-fatigued controls (NF), and subjects with insufficient fatigue (ISF) and chronic fatigue syndrome (CFS)

Characteristics	NF ($n = 42$)	ISF ($n = 55$)	CFS ($n = 40$)	All ($n = 137$)*
Age (mean \pm SD)	51.0 \pm 7.5	49.9 \pm 8.4	50.7 \pm 8.7	50.5 \pm 8.2
Female (%)	86.0	76.6	82.5	81.7
BMI (mean \pm SD)	29.2 \pm 5.3	28.0 \pm 5.4	29.4 \pm 4.5	28.8 \pm 5.1

*All Caucasian subjects

with each marker and outcome, but no confounders were identified for adjustment.

Linkage disequilibrium

The extent of LD, as measured both by D' and r^2 , varied with respect to illness classification (Fig. 2). Patients with CFS had a region of higher LD between markers A through E (D' 0.916–1.000; r^2 0.410–0.872) with lower LD between markers F through I (D' 0.245–0.495; r^2 0.030–0.072). The extent of LD between the majority of the markers was less in ISF than in CFS and least in NF subjects.

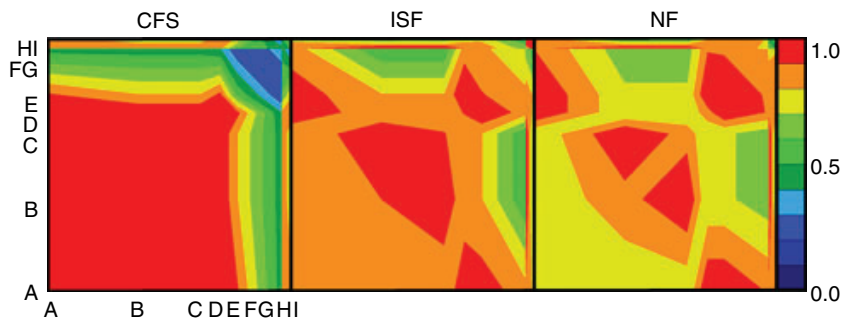
Single marker association with fatigue

The proportions of alleles for markers A to E (Table 2) differed significantly between fatigued (CFS and ISF combined) and NF subjects ($P = 0.0164$ to $P = 0.0383$). This significant difference in allele frequency also occurred with markers A, B, C and E when analysis compared CFS to NF subjects ($P = 0.0233$ to $P = 0.0466$). With all associated markers, the proportions of the major alleles were significantly higher in fatigued than non-fatigued subjects. Genotypic frequencies for markers A, B, C, D, E and G were also significantly different between all fatigued (CFS and ISF) and NF subjects ($P = 0.0218$ to $P = 0.0434$) with OR ranging from 1.93 (CI 0.90–4.13) to 2.64 (CI 1.20–5.82). Genotypic associations were also maintained for markers A, B, C and E when analysis was restricted to CFS subjects only ($P = 0.034$ to $P = 0.042$) with OR ranging from 2.61 (CI 1.05–6.45) to 3.00 (CI 1.12–8.05). None of the differences between NF and ISF subjects in either allele or genotype tests for any of the markers reached significance levels.

Association of polymorphisms in NR3C1 with SF-36, MFI and SI scores

SF-36 scores in four of the eight measured domains (bodily pain, general health, vitality and social function) demonstrated significant associations with one or more of the SNP markers (Table 3) whereas no association was found with SF-36 measures for physical function, role physical, role emotional and mental health. Marker A was associated with scores in all four of these SF-36 domains (P -values 0.007–0.026). Social

Figure 2: Linkage disequilibrium (LD) among SNP in *NR3C1* with respect to diagnostic status. LD measured by D' is shown in a GOLD plot. Pairwise D' between markers A–I is indicated by colours from no LD (blue) to high LD (red). GOLD plot by r^2 showed similar trend with respect to diagnosis.



function was associated with six markers, followed by vitality (four markers), body pain and general health (three markers). In each instance of association between SF-36 and genotype, subjects homozygous for the major allele had lower scores indicative of higher disability.

Scores in four of the five fatigue domains measured in MFI showed a significant association with one or more markers (Table 4). Marker A was associated with scores in all four of these MFI domains. Reduced activity and mental fatigue were both associated with seven or eight markers, followed by general fatigue (four markers) and physical fatigue (one marker). In each instance of association between MFI and genotype, subjects homozygous for the major allele showed higher scores indicative of higher disability. Symptom inventory scores resulted in association with six markers (A through E and G; Table 4) and in each case subjects homozygous for the major allele were associated with a higher score indicative of greater disability.

Haplotype association

Haplotype analysis was performed with all nine markers or with the five markers (A to E) in high LD in CFS subjects (Table 5). Global P -values indicated significant overall frequency distribution when analysis was conducted with CFS subjects either alone or with ISF subjects but not when

analysis involved ISF subjects alone. Four haplotypes accounted for nearly 80% of all nine marker combinations, and of these haplotypes 9–3 and 9–4 accounted for nearly 50% of the subjects. Haplotype 9–1 (GCAGAGGTA) was more frequent in NF when analysed with CFS subjects alone (score -2.91 , $P = 0.004$) or with ISF subjects (score -3.008 , $P = 0.003$). No nine-marker haplotype was associated with increased risk of CFS.

When analysis was restricted to five markers in high LD, three haplotypes accounted for 95% of the subjects with haplotype 5–3 being the most common (60–63%). Haplotype 5–3 (ATGAC), comprised of all major alleles for the five markers and was more frequent in fatigued subjects when analysed with both CFS subjects alone (score 2.62, $P = 0.005$) or with ISF subjects (score 2.529, $P = 0.019$). On the other hand, haplotype 5–1 with all minor alleles (GCAGA) appeared to be protective when analysed with CFS subjects alone (score -2.31 , $P = 0.026$) or combined with ISF subjects (score -2.420 , $P = 0.020$).

Discussion

To our knowledge, this is the first report of both single marker and haplotype-based analysis of *NR3C1* with CFS. We chose *NR3C1* as a candidate gene because of its key role in cortisol-exerted negative feedback on the HPA

Table 2: Association of *NR3C1* polymorphisms with CFS

Marker*	SNP ID	Alleles	NF	CFS	P -value**	ISF	P -value	CFS + ISF	P -value**
			Allele frequency	Allele frequency		Allele frequency		Allele frequency	
A	rs1866388	A/G	0.57/0.43	0.74/0.26	0.0233	0.67/0.33	0.1300	0.71/0.29	0.0335
B	rs2918419	T/C	0.73/0.27	0.88/0.12	0.0410	0.84/0.16	0.0744	0.85/0.15	0.0164
C	rs860458	G/A	0.73/0.27	0.88/0.12	0.0375	0.84/0.16	0.0766	0.85/0.15	0.0180
D	rs852977	A/G	0.57/0.43	0.71/0.29	0.0809	0.70/0.30	0.0931	0.70/0.30	0.0365
E	rs6188	C/A	0.57/0.43	0.73/0.27	0.0466	0.69/0.31	0.1319	0.70/0.30	0.0383
F	rs258750	A/G	0.59/0.41	0.71/0.29	0.1485	0.67/0.33	0.2270	0.69/0.31	0.1312
G	rs6196	A/G	0.76/0.24	0.88/0.12	0.1231	0.85/0.15	0.1918	0.86/0.14	0.0719
H	rs6191	G/T	0.41/0.59	0.53/0.47	0.2025	0.53/0.47	0.1398	0.53/0.47	0.1105
I	rs6198	A/G	0.79/0.21	0.83/0.17	0.5512	0.87/0.13	0.2587	0.84/0.16	0.2887

*Markers listed in order of 5' to 3' direction.

** P -values in bold indicate significant differences in allele frequencies between NF and fatigued (CFS or CFS plus ISF) subjects.

Table 3: Association of *NR3C1* polymorphisms with SF-36 scores*

Marker	Genotypes (n)	Physical function	Role physical	Bodily pain	General health	General mental health	Role emotional	Vitality	Social function
A	AA (59)	59.66	61.82	58.60	57.10	60.03	62.95	58.80	56.80
	AG (57)	71.83	67.94	72.80	73.40	68.80	69.62	72.30	71.60
	GG (15)	68.73	75.07	69.10	72.70	78.87	64.23	70.50	80.80
	<i>P</i> -value [†]	NS	NS	0.021	0.008	NS	NS	0.025	0.005
B	TT (96)	65.90	66.47	65.56	65.58	66.88	69.34	64.20	64.50
	TC (31)	72.98	71.23	72.25	70.94	72.23	71.90	80.00	79.40
	CC (10)	86.40	86.35	91.85	86.20	79.35	56.65	80.70	79.70
	<i>P</i> -value [†]	NS	NS	NS	NS	NS	NS	0.016	0.017
C	GG (96)	65.90	66.47	65.56	66.58	66.86	69.34	64.20	64.50
	AG (31)	72.98	71.23	72.25	70.94	72.22	71.94	80.00	79.40
	AA (10)	86.40	86.35	91.85	86.20	79.35	56.65	80.70	79.70
	<i>P</i> -value [†]	NS	NS	NS	NS	NS	NS	0.016	0.017
D	AA (62)	62.07	64.51	59.90	60.80	64.31	66.39	62.08	60.10
	AG (55)	74.83	70.07	76.10	74.80	68.85	70.15	74.85	73.70
	GG (18)	67.56	73.69	71.10	71.90	78.11	66.97	67.50	77.80
	<i>P</i> -value [†]	NS	NS	0.013	0.026	NS	NS	NS	0.013
E	CC (61)	61.29	64.30	59.90	61.34	62.16	64.56	62.75	59.10
	AC (57)	74.66	69.76	75.40	73.77	70.59	71.64	72.68	73.80
	AA (17)	69.76	75.35	72.40	72.62	80.29	68.14	71.15	80.50
	<i>P</i> -value [†]	NS	NS	0.014	NS	NS	NS	NS	0.008
F	AA (61)	62.78	64.95	62.87	60.90	64.13	65.96	61.45	62.91
	AG (55)	72.84	68.10	71.84	73.00	70.24	70.18	74.39	70.51
	GG (19)	70.76	77.50	73.37	76.10	73.94	68.24	70.53	77.08
	<i>P</i> -value [†]	NS	NS	NS	0.030	NS	NS	NS	NS
G	AA (94)	63.47	64.84	64.34	63.86	65.53	68.42	62.30	62.80
	AG (33)	76.39	71.58	72.56	74.09	70.91	67.21	80.90	78.30
	GG (6)	70.58	75.75	78.17	77.25	68.42	43.58	64.40	70.30
	<i>P</i> -value [†]	NS	NS	NS	NS	NS	NS	0.014	0.025
H	GG (34)	56.81	58.96	60.44	58.22	56.94	61.80	59.36	60.28
	GT (62)	70.66	69.91	67.07	70.05	67.63	70.37	69.62	67.39
	TT (36)	68.47	67.75	71.23	58.22	73.58	64.25	67.84	70.85
	<i>P</i> -value [†]	NS	NS	NS	NS	NS	NS	NS	NS
I	AA (94)	66.27	66.71	64.41	65.52	65.47	65.77	67.48	65.78
	AG (35)	73.19	70.50	77.11	74.50	75.13	73.39	69.90	71.70
	GG (6)	64.92	73.66	71.08	69.00	66.08	71.58	65.00	81.25
	<i>P</i> -value [†]	NS	NS	NS	NS	NS	NS	NS	NS

*All scores are expressed as least squared means. Low SF-36 scores correlate with higher disability.

[†]Indicates recessive model. *P*-values in bold indicate significance; NS, no significance.

axis that appears to be deregulated in CFS (Cleare 2003, 2004; Demitrack 1998). Allele frequencies of multiple SNPs particularly located more to the 5' region of *NR3C1* showed significant differences between fatigued subjects (either CFS alone or with ISF) and non-fatigued controls. These data were supported when quantitative measures of wellness, fatigue and symptoms were examined. With all associated SNPs, the major allele was consistently more frequent in fatigued subjects, and subjects homozygous for the major allele of markers A, B, C and E were at two- to three-fold increased risk for ISF or CFS.

The extent of LD differed for subjects grouped by fatigue classification, with extent of LD decreasing with decreasing fatigue (CSF > ISF > NF) providing a genetic basis for the rigorous classification of subjects into CFS, ISF and NF in this study. The region of high LD in CFS subjects spans approximately 80 kb. While haplotype analysis with nine markers identified only a protective haplotype (GCAGAGGTA), analysis restricted to the five markers in high LD in CFS identified haplotypes that were both protective (GCAGA) and risk-conferring (ATGAC) for CFS either alone or with ISF.

Several markers in the region of high LD, particularly marker A (rs1866388), were also associated with scores

Table 4: Association of *NR3C1* polymorphisms with MFI and SI scores*

Marker	Genotypes (n)	General fatigue	Physical Fatigue	Mental fatigue	Reduced activity	Reduced motivation	Symptom inventory
A	AA (59)	74.50	73.30	73.20	75.20	72.28	76.70
	AG (57)	58.40	60.40	63.80	60.70	61.29	59.40
	GG (15)	60.60	58.70	46.00	49.70	59.20	48.80
	<i>P</i> -value [†]	0.010	0.024	0.026	0.007	NS	0.002
B	TT (96)	72.90	71.47	73.40	73.10	70.93	74.00
	TC (31)	63.20	67.54	61.40	59.40	64.21	60.50
	CC (10)	49.90	49.80	50.50	59.90	65.25	47.60
	<i>P</i> -value [†]	0.041	NS	0.023	0.033	NS	0.012
C	GG (96)	72.90	71.47	73.40	73.10	70.94	74.00
	AG (31)	63.20	67.55	61.40	59.40	64.21	60.50
	AA (10)	49.50	49.80	50.50	59.90	65.25	47.60
	<i>P</i> -value [†]	0.041	NS	0.023	0.033	NS	0.012
D	AA (62)	75.30	73.73	74.30	77.20	73.52	76.90
	AG (55)	60.10	62.51	66.60	62.70	63.05	62.20
	GG (18)	67.00	65.06	50.80	52.40	64.11	55.00
	<i>P</i> -value [†]	0.023	NS	0.044	0.007	NS	0.009
E	CC (61)	74.77	73.54	74.50	76.90	73.74	77.30
	AC (57)	62.14	64.04	66.70	64.40	64.02	62.90
	AA (17)	63.35	61.44	49.30	48.10	60.76	51.40
	<i>P</i> -value [†]	NS	NS	0.041	0.009	NS	0.007
F	AA (61)	74.51	74.80	74.90	75.30	73.40	73.30
	AG (55)	60.92	62.53	64.90	64.00	64.24	65.05
	GG (19)	67.57	62.03	54.90	56.00	61.55	59.52
	<i>P</i> -value [†]	NS	NS	0.032	0.025	NS	NS
G	AA (94)	71.16	70.65	71.70	71.10	68.79	71.20
	AG (33)	55.39	57.37	54.30	55.40	60.29	56.40
	GG (6)	65.66	62.66	64.00	66.20	75.83	59.00
	<i>P</i> -value [†]	NS	NS	0.0165	0.029	NS	0.027
H	GG (34)	78.88	74.94	75.90	77.47	77.82	78.44
	GT(62)	62.89	62.52	66.31	65.40	60.04	63.25
	TT (36)	65.75	65.39	57.94	58.03	66.92	56.94
	<i>P</i> -value [†]	NS	NS	NS	NS	NS	NS
I	AA (94)	70.14	69.90	70.09	71.90	70.71	70.30
	GA (35)	62.66	63.71	66.33	61.90	63.79	60.64
	GG (6)	65.58	63.17	45.08	41.90	50.08	51.83
	<i>P</i> -value [†]	NS	NS	NS	0.0394	NS	NS

*All scores are expressed as least squared means. High MFI and SI scores correlate with higher disability.

[†]Indicates recessive model. *P*-values in bold indicate significance; NS, no significance.

assessing functional impairment (SF-36 scores), general dimensions of fatigue (MFI) and with SI scores with CFS. It is interesting to note that these markers in high LD that were associated with these various quantitative measurements of the illness domains of CFS were also significant at the qualitative syndrome level of analysis. The association of *NR3C1* polymorphisms with only certain illness domains as measured by SF-36 and MFI may be related to the differing dependences of these traits on HPA axis function. There are no prior reports of *NR3C1* polymorphisms associated with fatigue except for the case report of primary cortisol resistance presenting with fatigue as the only symptom that was associated with a thermolabile glucocorticoid receptor

(variant status unknown) in a patient and her son (Bronnegard *et al.* 1986).

Since deregulation in cortisol signalling is implicated in a number of pathophysiological conditions, the association of sequence variations in the *NR3C1* gene was examined with a variety of other complex phenotypes that include glucocorticoid resistant syndrome, HPA axis regulation, metabolism, cardiovascular control, immune function and behaviour (Bray & Cotton 2003; DeRijk *et al.* 2002). *NR3C1* polymorphisms were examined in association with the HPA axis traits of circadian rhythm, negative feedback and activation, and specific associations were noted with the *Tth111L* variant located in the 5' flanking region, N363S, and the *Bcl I* random fragment

Table 5: Association of NR3C1 haplotypes with CFS

Test*	Haplotype	Haplotype number	CFS			CFS + ISF		
			Frequency	Score	P-value†	Frequency	Score	P-value†
Nine-marker	GCAGAGGTA	9-1	12.6	-2.910	0.004	10.8	-3.008	0.003
	GTGGAGATG	9-2	12.6	-1.390	0.174	13.9	-0.645	0.525
	ATGACAATA	9-3	16.3	0.599	0.599	17.5	0.441	0.666
	ATGACAAGA	9-4	36.7	0.810	0.428	38.6	1.296	0.204
	ATGACGAGA	9-5				5.0	1.367	0.190
Five-marker	GCAGA	5-1	17.4	-2.310	0.026	16.1	-2.420	0.020
	GTGGA	5-2	16.6	-0.930	0.361	16.3	-0.780	0.435
	ATGAC	5-3	60.7	2.620	0.005	62.8	2.529	0.019

*Indicates haplotypes generated with all nine markers or five markers (A to E) in high LD

†Indicates significance of haplotype-specific score.

length (intronic) polymorphisms respectively (DeRijk *et al.* 2002). Carriers of 363S showed significantly increased salivary cortisol whereas the *Bcl* homozygotes exhibited diminished cortisol in response to psychosocial stress (Wust *et al.* 2004a,b). Recently, two studies of *NR3C1* found association of a three-marker haplotype across intron B (between exons 2 and 3) with low post-dexamethasone cortisol (Stevens *et al.* 2004), and also association of haplotypes with polymorphisms in the 5' region with genetic vulnerability for recurrent major depression (van West *et al.* 2005). The results from *NR3C1* haplotype analyses from three independent studies, including this study, (Stevens *et al.* 2004; van West *et al.* 2005) indicate that the 5' region of *NR3C1* may harbour a functional sequence variation. This is supported by the observation of a key role for the 5' untranslated region in determining tissue-specific expression of *NR3C1* isoforms through splice variants (Turner & Muller 2005).

The observed *NR3C1* associations do not specifically indicate an underlying biological mechanism. Genetic variants in *NR3C1* could contribute to the genetic programming of the individual's set point of HPA axis activity or to the perpetuation of deregulation of HPA axis activity by biological, psychosocial stress, trauma, or early life experiences. This study is limited by the small sample size and by the use of mostly intronic markers that have unknown functional impact on the HPA axis. It is interesting to note that SNPBrowser software, which was used for SNP selection, showed > 90% power to detect an association with *NR3C1* polymorphisms in Caucasian subjects with 250 cases and 250 controls, assuming a disease allele frequency of 0.20 (De La Vega *et al.* 2005a,b). This study employed rigorous hospital-based clinical evaluation of both case and control subjects who were identified from the general population, and were prospectively followed over 7 years for fatigue status. Thus, the clinically and epidemiologically well-matched cases and controls add considerable strength to our study.

In conclusion, our results demonstrate that *NR3C1* is a potential mediator of chronic fatigue. Further studies of

sequence variations associated with CFS should focus on fine mapping efforts in the 5' region, which includes the promoter and intron/exon boundaries.

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