

Identification of novel expressed sequences, up-regulated in the leucocytes of chronic fatigue syndrome patients

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Summary

Background Chronic fatigue syndrome (CFS) is an increasing medical phenomenon of unknown aetiology leading to high levels of chronic morbidity. Of the many hypotheses that purport to explain this disease, immune system activation, as a central feature, has remained prominent but unsubstantiated. Supporting this, a number of important cytokines have previously been shown to be over-expressed in disease subjects. The diagnosis of CFS is highly problematic since no biological markers specific to this disease have been identified. The discovery of genes relating to this condition is an important goal in seeking to correctly categorize and understand this complex syndrome.

Objective The aim of this study was to screen for changes in gene expression in the lymphocytes of CFS patients.

Methods 'Differential Display' is a method for comparing mRNA populations for the induction or suppression of genes. In this technique, mRNA populations from control and test subjects can be 'displayed' by gel electrophoresis and screened for differing banding patterns. These differences are indicative of altered gene expression between samples, and the genes that correspond to these bands can be cloned and identified. Differential display has been used to compare expression levels between four control subjects and seven CFS patients.

Results Twelve short expressed sequence tags have been identified that were over-expressed in lymphocytes from CFS patients. Two of these correspond to cathepsin C and MAIL1 – genes known to be upregulated in activated lymphocytes. The expression level of seven of the differentially displayed sequences have been verified by quantifying relative level of these transcripts using TAQman quantitative PCR.

Conclusion Taken as a whole, the identification of novel gene tags up-regulated in CFS patients adds weight to the idea that CFS is a disease characterized by subtle changes in the immune system.

Keywords chronic fatigue syndrome, differential display, expressed sequence tags, leucocytes, lymphocyte activation, RNA, TAQman™

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Introduction

Chronic fatigue syndrome (CFS) is a multi-factorial disease of complex pathology and unknown aetiology [1–3]. This is reflected in the broad range of disciplines that have studied this disease and the numerous hypotheses that exist to explain the primary feature of this illness i.e. persistent and relapsing fatigue [2, 4]. Opinion has been divided as to the fundamental nature of this syndrome for many years, and much needed validation for this disease has only recently been forthcoming [5]. CFS has been shown to follow a large range of acute infections, an observation supported by the occasional occurrence of localized epidemics [6]. The diversity of infectious agents that may pre-dispose to CFS suggests that an aberrant host response to infection may be the central

feature of this disease [7–9]. This remains one of a number of models for CFS and is supported by empirical observations of CFS patients and other disorders of the immune system. The over-expression of pro-inflammatory cytokines such as INF- γ and IL-1 has been reported in CFS [10, 11], and when these mediators are administered clinically, they can reproduce some of the clinical features of CFS [12, 13]. Analysis for CFS subsets, with better defined aetiologies, such as B19 associated fatigue or Post Q-fever syndrome have also shown that the pro-inflammatory cytokines TNF- α and INF- γ are the likely mediators of these syndromes [14–16]. TGF β , an immunosuppressive cytokine, has been found to be up-regulated in a number of studies [14]. This may represent a host attempt to reign in an over active or inappropriate immune response, or may be induced by pathogens trying to overcome the host response.

An alternative model for CFS places the neuroendocrine system in the driving seat of chronic disease [17–21]. Other cytokines up-regulated in CFS, such as IL-6, have targets

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within the hypothalamic–pituitary–adrenal axis (HPA axis) and numerous studies have documented subtle perturbations in this system that may be highly relevant to CFS pathology [17–21]. As well as providing an explanation for the neuro-endocrine aspects of CFS such as depression, insomnia and photo-phobia, the increased negative regulation of glucocorticoids may also favour a Th2 response tending towards allergic reactions which are elevated in CFS [17, 18].

Although both the cause and relevance of all these observations are still indeterminate, collectively these papers support a model for CFS that involves an immune system pathology induced by infection and/or disruption of the balance of the HPA axis. We hypothesized that if these findings are of any consequence in CFS, there would likely be other host genes that may be differentially regulated in CFS patients compared to controls. The aim of this study was to identify new target genes that are up or down regulated in chronically ill CFS patients.

'Differential Display' (DD) is a method for amplifying and comparing mRNA populations between control and test samples by means of the polymerase chain reaction [22]. Contrasting banding patterns produced on a polyacrylamide gel are indicative of differential gene expression and these bands can be cloned and sequenced to give 'expressed sequence tags' (ESTs) corresponding to the gene of interest. To test our hypothesis, we harvested mononuclear cells (principally monocytes and lymphocytes) from CFS patients and controls and compared them for differential gene expression. We report the sequencing of a number of ESTs that have been cloned using DD. Five of these mapped to previously identified genes with some known function [23–25], three further ESTs mapped to predicted genes, and the remaining 8 to uncharacterized, but transcriptionally active regions of the genome. Semi-quantitative TAQman™ (ABI, Foster City, CA, USA) RT-PCR has confirmed over-expression of five of these sequences in the CFS samples from which they have been identified. These preliminary findings could lead to the cloning of important genes associated with CFS, and the cloning of MAIL in this study suggests an underlying mechanism for IL-6 upregulation in CFS.

Methods

Patient selection

CFS patients were selected using the criteria set out by US Centres for Disease Control and Prevention [2]. In addition to these, patients were required to have been diagnosed with CFS for over 2 years and therefore to be established in the chronic stage of this disease. They were also obliged to report an acute onset to their disease suggestive of an infection as the initiating event to CFS [26]. These extra inclusion criteria were added to exclude patients in the acute phase of illness, who may not be typical of the CFS population as whole, or who may be misdiagnosed. Furthermore, since DD has the potential to identify genes from pathogens, this was also an attempt to select patients that may have CFS as a post-viral syndrome and who may therefore be chronically infected with a virus. The age range of patients and controls was limited to between 18 and 50; pregnant women as well as those on steroid treatments were excluded from participating. All

patients were of Caucasian ethnicity. In total, seven CFS patients were recruited of which two were female and samples obtained from four healthy controls, of which 2 were female. Blood samples were taken in the morning at the patient's home, or within a clinical setting for the control subjects. Endotoxin-free tubes were used for the collection and the blood was stored on ice for transportation to the laboratory. Ethical approval for these studies was obtained the Joint Ethics Committee of Southampton University and General Hospital.

Preparation of total RNA from mononuclear cells

Mononuclear cells were isolated from red blood cells using separation on Ficoll gradients. Peripheral blood was collected in heparinized tubes, and stored immediately on ice, before dilution 1:2 in serum-free RPMI and overlaying onto 15 mL of Ficoll in a 50 mL falcon tube. Samples were centrifuged for 25 min at 400 *g*. Mononuclear cells were harvested from the top of the Ficoll, washed in PBS and total RNA immediately extracted using Tri reagent (Sigma, St Louis, MO, USA) and a modified method of the guanidine isothiocyanate extraction protocol [27]. Since DD is very sensitive to genomic DNA contamination, samples were DNase treated by incubation with 5 U of DNase A (Promega, Madison, WI, USA) in RQ1 buffer for 30 min at 37 °C. RNA was extracted and precipitated using standard protocols and quantified by spectrophotometry.

Differential display

Differential display was performed using the delta differential display kit (Clontech, Palo Alto, CA, USA). Detailed protocols can be obtained from <http://www.clontech.com/techinfo/manuals/PDF/PT1173-1.pdf> [22]. In brief, 2 µg of each total RNA sample was reverse transcribed using a poly-T primer and MMLV reverse transcriptase. Radioactively labelled PCR fragments were then generated using [³⁵S]ATP and combinations of the 10 'P' and nine 'T'. Negative control RNA samples, which lacked the RT step, were run in parallel to prove that DD band profiles were derived from nascent cDNA and not due to DNA carried over during the RNA extraction. Radioactively labelled bands were resolved on a standard 12.5% polyacrylamide 'sequencing gel' and differentially displayed bands identified by eye using two independent observers. Selected bands were first excised from the gel by overlaying the autoradiogram and then re-amplified using Taq polymerase in conjunction with the original 'P' and 'T' primers. Re-amplified bands were T/A cloned using the Topo II cloning kit (Invitrogen, Paisley, UK), sequenced in both directions and aligned with publically available DNA databases (Table 1).

TAQman™ semi-quantitative RT-PCR

Taqman™ RT-PCR is a quantitative system for accurately measuring the relative number of nucleic acid templates in biological solutions. The PCR forward and reverse primers are designed either side of a fluorochrome labelled probe that also has a 3' quencher. During the extension phase, the fluorochrome is cleaved away from the quencher and the rate of liberation is dependent on the number of starting templates.

Table 1. Characterization of DD bands up-regulated in CFS sufferers

Name*	Size	Accession number	Identification
F1(P4)	816	BQ5803379	Uncharacterized Unigene clusters
M3(P4)1	440	BQ5803384	Uncharacterized Unigene cluster
M3(P4)3	434	BQ5803385	Hypothetical protein KIAA1025
M7(P1T1)	500	BQ5803387	Uncharacterized Unigene cluster
F5(P5T5)	454	BQ5803380	MAD1L1
F5(P6T6)	650	BQ5803381	Uncharacterized Unigene cluster
M7(P5T5)	186	BQ5803388	Hypothetical protein FLJ10876
M2(P2T7)	367	BQ5803383	Phosphatidylinositol glycan, class K (PIGK)
M2(P2T1)	293	BQ5803382	Molecule possessing ankyrin repeats induced by lipopolysaccharide (MAIL)
M3(P6)	843	BQ5803389	Cathepsin C (dipeptidyl-peptidase I)
M6(P1T1)	358	BQ5803386	Hypothetical protein MGC3329
F1(P1)	287	BQ5803378	Moesin (cyto-skeletal protein)

*Bands have been named according to the individual they were cloned from e.g. F1/M7, and the 'P' and 'T' primers used for the amplification in the DD protocol.

Primer Design The TAQmanTM primers and probes were designed using the 'primer express' software (ABI) and the additional criteria set out in the standard protocols. Database sequences that matched the differentially displayed ESTs, were used to construct consensus sequences for each EST and the TAQmanTM primers and probes designed to target this consensus sequence. This was to eliminate sequence errors that may have accumulated in the ESTs through the two long cycles of PCR that were required to 'display' and then clone the bands. In addition to quantifying the CFS associated ESTs identified by DD, the positive control genes TNF- α and protein kinase R (PKR), were also included for quantification. These genes have previously been reported to be over-expressed in the lymphocytes of CFS patients [28, 29]. The primers used for TAQmanTM RT-PCR for each EST and the control genes are shown in Table 2.

TAQmanTM RT-PCR

Standard protocols, from Applied Bio-systems were used throughout in a multiplex assay system that simultaneously measured target gene expression and 18S rRNA levels (<http://www.appliedbiosystems.com/support/>). A standard curve was also produced for 18S rRNA and target gene by measuring 2-fold serial dilutions of the pooled samples. The amounts of these two species were then calculated from their respective standard curves and the level of the target gene normalized to the level of the 18S rRNA (target value/18S rRNA value). The data shown are therefore, relative values plotted in arbitrary units (Fig. 1). In brief, 1 μ g of RNA was reverse transcribed using a random hexamer primer and avian leukaemia virus reverse transcriptase (Promega). Each TAQmanTM amplification contained approximately 25 ng of template, core reagents (Oswell, Romsey, UK), 900 nM forward and reverse primers to the target gene and 18S rRNA, 250 nM 5' FAM (6-carboxy-fluorescein) and 3' TAMRA (6-carboxy-N,N,N',N'-tetramethyl-rhodamine) labelled probe complementary to the target gene and 250 nM 5'

VICTM and 3' TAMRA labelled probe to the 18S rRNA (Applied Biosystems). The average fold increase between patient and controls samples is plotted, and the error bars represent one standard deviation with *P*-values calculated using a Student's *t*-test.

Results

Differential display

Each DD gel contained the healthy and subject RT reaction and one combination of 'P' and 'T' primers. In total, over 60 of these gels were run and from these, a total of 12 different DD bands were identified and cut from the most intensely displayed band in each case. All of these bands were induced or up-regulated in CFS patient and no bands could be identified that were over-expressed in control samples.

Sequence alignments

Of the 12 clones identified, four aligned to genes previously identified, three to predicted/hypothetical genes and four to poorly characterized regions of the genome within Unigene clusters (Table 1). For the poorly characterized clones, overlapping, or adjacent ESTs (within 2 kb) could be located using BLAST searches and although it could not always be determined exactly to which Unigene cluster a particular EST belonged, it was clear that all sequences mapped to transcriptionally active regions of the genome. This suggested that seven of the ESTs corresponded to unknown genes, of which one (M7(P5T5)) was also found to have a defined intron/exon structure that matched a computer predicted gene. The current search results are shown in Table 1.

TAQmanTM RT-PCR

Six ESTs were arbitrarily selected for further study using TAQmanTM quantitative RT-PCR using primers designed to

Table 2. Primers used for TAQman™ PCR of differentially expressed ESTs and positive control genes

Target	Forward	Reverse	Probe (5' FAM and 3' TAMRA)
TNFA*	Pre-developed (ABI)	Pre-developed (ABI)	Pre-developed (ABI)
PKR	CCAACCAGGATACGGGAAGA	CCTGCTTCTGACGGTATGTATTAAGT	AAGAAACCTGCTGAAAGATCACCAGCCAT
M7(P1T1)	GCCTCTGCTCTGGACCATCTCT	GTAATCCTGTAGGGATTTAAGCCAGTT	TCCACTCTCCATCTTTGCCTGATTCCAT
M3(4)3	GTCAGCTTCTGTGCCACAGTTT	CCCATCTGCAAAGTGCTACGT	TCCCACGCGCATGATCCCG
F1(P4)	TGGGATAAGGAATATCTGTAAAGCAGT	GAAGCTGACACATTGTTGTTTTCTATTA	AACATGAAACAGGCCCTCAATAAATGAAACT
F5(P6T6)	ATGCCAGCTTCCCTCATTCC	AAGCAAGGTCATTTTGCTGAGAGT	AGGATGTTTGCTCCTGGCCACC
M3(P4)1	CTGGTAGCGGGAAATTCTG	GCCATTAACACAATCAGTGCAAA	TGGCAAAGTCAAGTTGCCAGAAACAG
M7(P5T5)	TTTCTAGATACTGAGGCTGCGAACT	GCTAACTGGAGGCTTTTTGTTGTAA	AATTTCTTAGACATCCACTTATGCTAGTCCACTGCCA
Moesin	TTTCTCCACCTGGCTGAAACTC	TCGGACACATCCTCAGGGTAG	CCCCTGCTCTTTAAGTTCCGTGCCA

*The TNFA primers were purchased as a 'pre-developed' assay and the sequences are not made available by ABI.

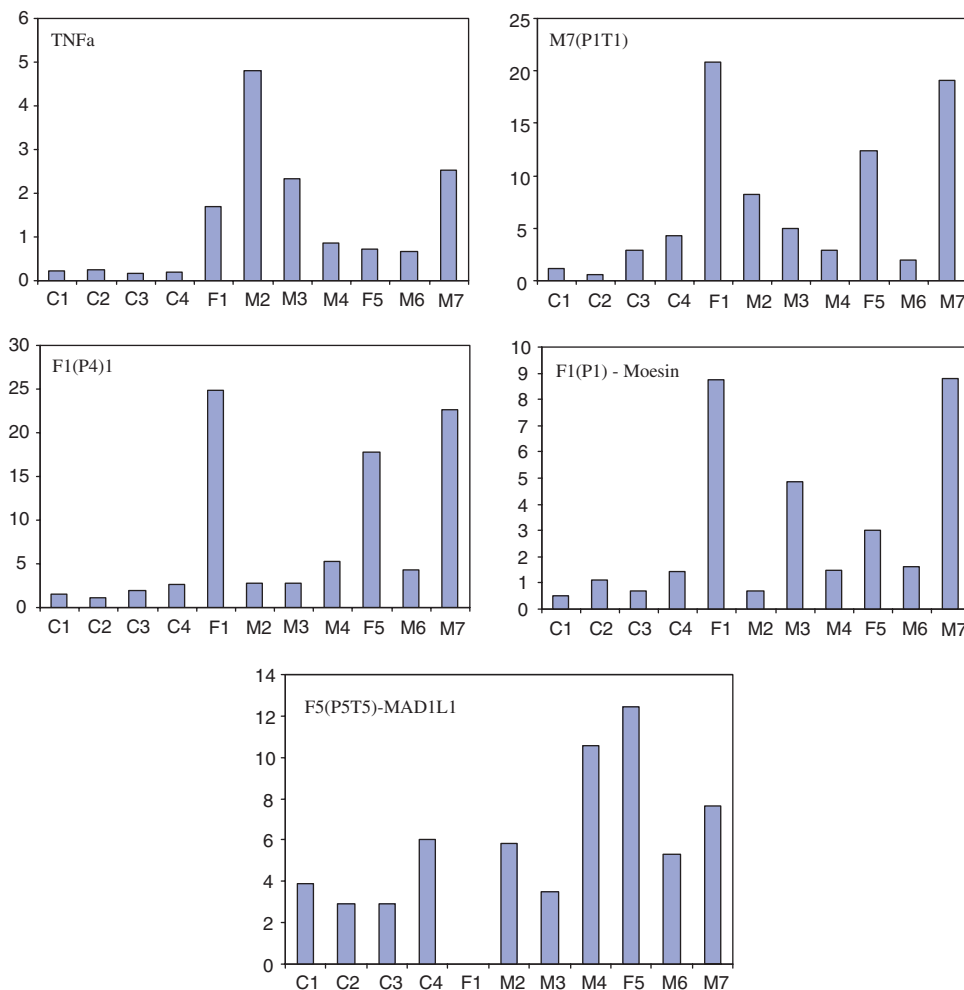


Fig. 1. Individual data for patients and controls showing expression of TNF- α and 4 of the six expressed sequence tags that were identified by differential display. Control samples (C1–C4) and CFS patients (F1–M7) were quantified by TAQman™ and normalized to the levels of 18S rRNA in each sample. The values are plotted in arbitrary units.

measure the relative expression of these ESTs between patients and controls. Two positive control genes, previously reported to be over-expressed in CFS (TNF- α and PKR) were also included for study [28, 29]. All sets of primers demonstrated an RT dependent signal in trial experiments indicating that all of the putative ESTs tested by TAQman™ RT-PCR are indeed derived from mRNA molecules

expressed in mononuclear cells (data not shown). In CFS patients relative to controls, TNF- α and PKR and five out of the six ESTs tested, revealed an overall higher level of expression (Fig. 1). Patients F1, F5 and M7 showed the most dramatic changes in expression profile and were the most consistent over-expressers of the genes in this study. The pattern of increased expression seen between CFS patients is

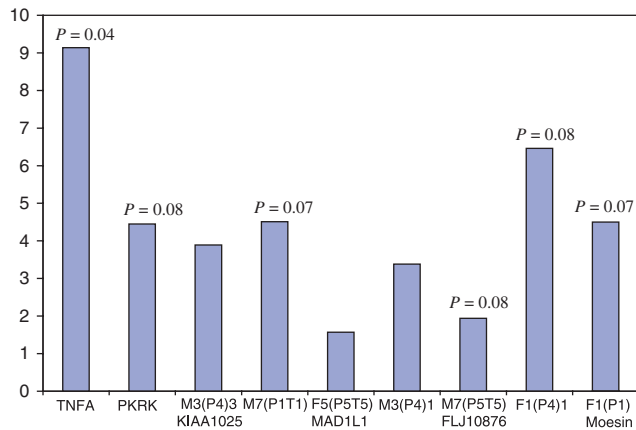


Fig. 2. Average fold increases for all genes quantified by TAQman™ including TNF- α and PKR included as positive controls. The data obtained by relative quantification for patients was divided by the average value obtained in control samples. The average fold increase between patient and controls samples is plotted and the error bars represent one standard deviation with *P*-values calculated using a Student's *t*-test.

similar for the ESTs M7(P1T1), F1(P4)1, M3(P4)3 and moesin. By contrast to this, M7(P5T5) and TNF- α gave unique patterns of expression and F5(P5T5) showed virtually no increase in CFS patients relative to controls. These variations serve as useful internal controls for all the ESTs measured from the same RT reaction on the same day. When the standard error of the mean fold increases were calculated from this data, TNF- α , PKR, moesin and three ESTs showed a significant increase in patients relative to controls at the 10% level (Fig. 2).

Discussion

In a small but well-characterized population of CFS patients, differential display has been used to clone and sequence genetic markers that are over-expressed in the mononuclear cells of CFS patients [30]. Five of these sequence tags map to genes with at least one known function and the rest to predicted genes or uncharacterized regions of the genome. The results of differential display analysis have been substantiated by performing quantitative RT-PCR using a subset of these EST sequences as design templates for TAQman™ RT-PCR assays. Six of the seven sequences analysed in this way were expressed more abundantly by CFS patients than controls, of which 4 sequences were expressed marginally significantly higher. The control genes TNF- α and PKR were also over-expressed in CSF subjects consistent with previous studies [28, 29, 31].

Two of the known genes, cathepsin C and MAIL, identified in this study, are directly supportive of the notion that CSF is a disease characterized by immune system activation. Cathepsin C has been shown to be up-regulated in activated T cells and is also involved in the development and differentiation of CD8⁺ cells [24]. Likewise the transcription factor MAIL is associated with activation of lymphocytes. This novel member of the I kappa B family of proteins, has demonstrated nuclear localization and enhances the transcription of IL-6, especially in response to stimulation with

LPS [25, 32]. IL-6 messenger RNA levels have been shown to be up-regulated in CFS and this study suggests that induction via MAIL maybe the mechanism by which this is achieved. This would be a novel target for CFS intervention since hyper-secretion of IL-6 has been proposed as a cause of daytime sleepiness [33], and may underlie other mechanisms that are fundamental to CFS symptoms via interaction with the HPA axis [18, 21, 33]. Since IL-6 is stimulatory of the HPA axis and this favours a Th2 response, this observation may also underlie the increase in atopy seen in CFS patients. MAIL is highly likely to have other promoter targets and these genes may also be up-regulated, and contributory to CFS pathology.

The identification of moesin as a candidate gene, is surprising since it is often used as a normalizing control in measurements of gene expression. However, it has also been identified by differential display in HIV infected cells cultures [34]. This is especially interesting since there is some evidence for retroviral infection in the aetiology of CFS [35]. However, since PCR screens for known retroviruses have produced negative results, it would have to be a hitherto unknown virus [36, 37]. For the remaining known genes, over expression of MAD1L1 could not be confirmed by TAQman and has most likely been cloned as a false positive (Fig. 2). A link between PIGK to current thinking on CFS is not obvious.

The average increases in gene expression, which vary between two and ninefold are not dramatic (Fig. 2), however, the individual data for each patient are more informative since there is a wide range of values within the CFS population (Fig. 1). Samples from F1, M5 and M7 demonstrated consistently higher expression levels than the other patients, which showed only modest increases in expression. Such a finding is typical of gene expression measurements performed on CFS patients, and is most likely explained by the subjective methods used to select patients. Many researchers have recognized that current methods of diagnosis lead to the selection a heterogeneous sample and these data support that view. An alternative explanation may be derived from the fluctuating severity of CFS seen over time. For many, CFS is episodic, featuring cycles of relapse and remission and it may be found that modulation of these genes mirrors these exacerbations. It is encouraging that the wide 'spread' of data seen in CFS patients is not seen in the control samples which gave much more uniform results throughout.

Interestingly, in no CFS subjects were down regulated differentially displayed bands identified. This is consistent with other publications on CFS, where many more genes have been reported to be up, than down-regulated in mononuclear cells. The data presented here adds weight to the idea that CFS is a disease characterized by over-expression of genes some of which are known to be associated with immune system activation. Identifying the triggering events for the induction of these genes will increase our understanding of this disease and their precise role in it. Some interesting possibilities include viral infection, neuroendocrine disturbances, and allergen exposure. [38]. A link with allergy may be especially pertinent since approximately 80% of CFS patients are atopic compared to 20–55% of the general population [39, 40]. Some of the genes identified in this study, and in particular MAIL, may therefore be linked with the

increase in allergic effects seen in CFS. If this is confirmed, then further characterization of these genes may be relevant to a range of chronic diseases that are associated with allergy [39, 40].

Unfortunately, the number of patients in this study was limited by the methodology of differential display. Indeed before a role for any of these candidate genes can be firmly established, quantification must be performed in a larger CFS population, and also in other related disease conditions such as fibromyalgia. A study of expression levels over a suitable time course will show whether these genes are stably over-expressed in the CFS population and therefore useful as diagnostic markers. It will also be important to relate these findings to the clinical features of CFS and establish which cell type(s) within the mononuclear cell population are responsible for expression of these genes, and how these genes are regulated.

Although differential display was the leading technology at the start of this project, this is no longer the case. Gene chip technologies offer a more thorough way of analysing changes in gene expression and the most natural progression of this study would be the application of these techniques to CFS. This preliminary study shows that such an approach is well justified and predicts that a more detailed characterization would identify more candidate genes for this disease.

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