Methylation Profile of CD4+ T Cells in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis

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Abstract

Objective: Methylation is known to regulate biological processes and alterations in methylation patterns have been associated with a variety of diseases. Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) is an unexplained disorder associated with immunological and molecular changes. CD4+ T cells specifically, regulatory T cells (Tregs) have been implicated in CFS/ME patients where significant increases in Tregs have been observed in these patients. Therefore the objective of this study was to examine methylation in CD4+ T cells from CFS/ME patients.

Methods: The study comprised twenty-five CFS/ME participants and eighteen controls aged between 25-60 years. A volume of 20 ml whole blood was collected from each participant and peripheral blood mononuclear cells were isolated via density gradient centrifugation. A negative isolation kit was used to isolate the CD4+ T cells from the peripheral blood samples. Genome wide methylation studies were performed on isolated CD4+ T cells using the Illumina Infinium 450 K Human methylation array system. Data analysis was performed using Genome studio and Partek Enrichment software.

Results: 120 CpGs were observed to be differentially methylated in the CFS/ME patients in comparison to the controls. Of these 70 were associated with known genes. The majority of the differentially methylated regions in the CFS/ME patients were hypomethylated. Additionally, most of the genes with differentially methylated regions in the CFS/ME patients were responsible for apoptosis, cell development, cell function and metabolic activity.

Conclusion: The present study demonstrates that epigenetic changes in CD4+ T cells may have a potential role in the immunological changes observed in CFS/ME patients.

Keywords: Chronic Fatigue Syndrome; CD4+ T cells; Methylation; miRNA

Introduction

Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) is an inexplicable disorder that affects 1–4% of individuals worldwide [1,2]. CFS/ME patients are subject to severe incapacitating fatigue, post-exertional sickness, discrepancies in cognition, painful lymph nodes, muscle aches and unbalanced sleep patterns [3]. CFS/ME involves disruption to immunological processes including reduced cytotoxic activity and elevated levels of regulatory T cells [4-6]. Furthermore, CFS/ME patients may exhibit differential expression in genes that regulate various physiological processes known to be abnormal in CFS/ME [4,7-12]. To date a succinct pathomechanism for CFS/ME and non-fatigued controls.

DNA methylation is a epigenetic modification process where DNA methyltransferases adds a methyl group to the 5’ position of cytosines of CpG dinucleotides [13]. The process of methylation has many consequences on gene expression as the extent and pattern of 5-methylcytosines dictates the rate of gene expression in a particular region [14]. In particular, DNA methylation recruits transcriptional co-repressors of the methyl-DNA binding domain family which repress the transcription of certain genes [13]. Regulation of DNA methylation is important, especially, during embryonic development, chromatin condensation and genomic imprinting [15]. Epigenetic modifications may have great value in various diseases including inflammatory and autoimmune diseases. Importantly, epigenetic modifications have been suggested to account for certain immune related diseases [16]. Increases and decreases in DNA methylation has been shown to regulate the expression of T cell related cytokines genes such as IL-2 and IL-6 [17].

In CFS/ME changes in gene expression has been reported for a number of mRNA and microRNA genes involved in various immune processes. However, to date, the role of cell specific methylation in CFS/ME has not been explored. This study performed a differential genome wide methylation analysis on CD4+ T cells in CFS/ME patients and non-fatigued controls.
Materials and Methods

Ethical approval

Approval for the study was granted by the Institutional Ethics Review Board at Griffith University. Written informed consent was obtained from all participants prior to the study.

CFS/ME patients and controls

Twenty-five patients with CFS/ME (21 Females and 4 males; age=50.31 ± 2.27) were enrolled into the study. These patients were excluded from the study.

DNA extraction and methylation

Genomic DNA was extracted using the QIAamp DNA extraction kit according to the manufacturer's instructions. Genome wide methylation was performed at the Australian Genome Research Facility (AGRF; The Walter and Eliza Hall Institute of Medical Research, Sydney). Assessment of integrity, quality and quantity was determined by the Nanodrop Spectrophotometer and electrophoresis on a 0.8% agarose gel. The EZ DNA Methylaiton kit (Zymo Research, Sydney) was used in the Bisulfite conversion of DNA. This was followed by a series of staining steps to differentiate biotin and DNP. The Illumina Human Methylation 450K Bead Chip assay (Illumina Inc., San Diego, CA) also covers miRNA promoter regions therefore we also examined methylation of the miRNA promoter regions with 200bp proximity.

CD4+ T cell isolation

A total volume of 20 ml of venous peripheral blood was collected from each participant in to EDTA containing tubes. Ficoll-hypaque density gradient centrifugation was used to isolate the peripheral blood mononuclear cells (PBMCs) from whole blood samples. Isolation of the CD4+T cells was performed according to the manufacturer’s directive, using a negative selection protocol which required the use of magnetic beads labelled with markers that exclude the CD4+ T cell population (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated PBMCs were resuspended in a buffer solution of PBS, stained and incubated with a biotin solution at 4°C for 10 minutes. Following incubation samples were resuspended in PBS and stained with microbeads at 4°C for 15 minutes. The samples were washed by adding PBS solution and centrifuging at 300 x g for 5 minutes. The supernatant generated was discarded and samples were resuspended in a buffer. The cells in suspension were applied to columns attached to a magnetic stand, the flow through fluid containing the cells of interest were collected and snap frozen in liquid nitrogen and stored at -80°C until required for further processing.

Overall methylation pattern in CD4+T cells

The present study compared and examined DNA methylation subtleties in CD4+ T cells from CFS/ME patients and non-fatigued controls. A total of 485 577 methylation sites in the genome were examined. Principle component analysis and hierarchical clustering of differentially methylated genes were used to determine the transcriptome profile and sources of variance in the groups (Figure 1). A predominant trend of genome hypomethylation was observed in the CD4+ T cells from the CFS/ME patients compared with the controls. We detected 120 CpGs that were differentially methylated between the

HRM analysis

HRM analysis was performed for genes including NINJ2, HSPD1, TES14, HLA-C, RAD51C, PHLN2, DQKQ, LIPT1, GJA9 and MYCBP.

Statistical analysis

Quality control checks were performed for target removal, staining for non-polymorphic probes, staining for negative controls, target removal, bisulfide conversion efficiency, hybridization efficiency and specificity. Analysis of the methylation data was performed with the GenomeStudio Illumina methylation module (version 1.8). Methylation levels of the different CpG loci were determined by the value of β (which is the ratio of the intensities between methylated and unmethylated alleles). The detection p value was set to <0.001, to eliminate poorly detected CpGs. Differentially methylated genes were characterized as genes with a fold difference ≥ 2.0. Gene Ontology and Pathways enrichment analysis was performed using Partek Genomic Suite™ software, version 6.6 (Partek Inc., St. Louis, MO) where significant differences in expression were determined at enrichment scores ≥ 3 [18]. ANOVA was used to calculate significance of variation in normalized expression values between sample groups, fold change of gene expressions was calculated as mean ratio.

Results

Participant characteristics

The age of the participants was (CFS/ME: 50.31 ± 2.27 years; non-fatigued controls: 47.44 ± 2.16 years) and full blood counts were performed on whole blood samples from all participants prior to CD4+T cell isolation. There were no significant differences observed in the CFS/ME patients compared with the controls.

Overall methylation pattern in CD4+T cells

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two groups and of these 85% were hypomethylated while 14.17% were hypermethylated. 75 of these methylated regions were linked to known genes while the remainder were unknown. These differentially methylated (dmCpG) sites were detected on chromosomes 1-22 with no particular concentration on one chromosome. Structurally these dmCpGs were located on the 3’UTR, 5’UTR and transcription start sites with a large proportion located within the gene. 30% of the CpG islands were associated with gene promoters.

Differential methylated genes

The dmCpGs were found in 75 genes in the CD4+ T cells. The gene with the most dmCpG site was NINJ2 where three CpG sites in this region were hypomethylated. Incidentally this gene contained CpG sites that were highly hypomethylated in the CFS/ME patients compared with the controls. This gene has not previously been associated with CFS/ME. Additionally, none of the genes identified in the present study has been previously associated with CFS/ME.

miRNA methylation analysis generated 176 miRNAs with significant dmCpGs, of these miRNAs, 82 miRNA genes were hypermethylated while 94 were hypomethylated.

Gene ontology and pathway enrichment profile

To determine whether the dmCpGs were biologically significant, the Partek ontology enrichment tool was applied to all genes with dmCpGs that were significantly altered (p<0.05). Gene enrichment analysis detected 59 different functional terms (p<0.05) that were associated with dmCpGs and these can be classified into three groups, biological, cellular and molecular processes (Table 1). The most defining factor to suggest that these genes were detected in CD4+ T cells was the observation that some of these genes were related to MHC Class II receptor activity (HLA-DQBI). Genetic pathways specific to these sites were determined using the Kegg pathway analysis. The Kegg pathway analysis identified HLA-C and HLA-DQBI as genes in the pathway with the highest enrichment score (Table 1).

| Gene      | Full name                                      | Fold change | P-value    | Methylation          | Chromosome | Function                                                                 |
|-----------|------------------------------------------------|-------------|------------|----------------------|------------|--------------------------------------------------------------------------|
| NINJ2     | Ninjurin (for nerve injury induced)            | -6.01644    | 0.025353   | hypomethylation      | 12         | Neuron adhesion                                                          |
| TXNRD1    | Thioredoxinreductase 1                         | -3.55680    | 0.000165   | hypomethylation      | 12         | Ribonucleotide binding, nucleotide binding, oxidation reduction          |
| BRWD1     | Bromodomain and WD repeat domain containing 1  | -3.31289    | 0.000813   | hypomethylation      | 21         |                                                                             |
| ATP9B     | ATPase, class II, type 9B                      | -3.26934    | 0.014517   | hypomethylation      | 18         | Nucleotide biosynthetic process, purine biosynthetic process             |
| ASXL2     | Additionalsex comb b 2 (Drosophila)            | -3.11677    | 0.028253   | hypomethylation      | 2          |                                                                             |
| HSP70     | Heatshock 10kDa protein 1                      | -3.07842    | 0.002192   | hypomethylation      | 2          | Purine nucleotide binding, Ribonucleotide binding, nucleotide binding     |
| HSPD1     | Heatshock 60kDa protein 1 (chaperonin)         | -3.03374    | 0.002294   | hypomethylation      | 2          | Purine nucleotide binding, Ribonucleotide binding, nucleotide binding     |
| KDM2B     | Lysine(K)-specific demethylase 2B              | -3.01638    | 0.002514   | hypomethylation      | 12         | Oxidation reduction                                                      |
| COG3      | Component of oligomeric golgi complex 3        | -2.96826    | 0.025353   | hypomethylation      | 13         | Protein localization in organelle, intra-Golgi vesicle-mediated transport, cellular macromolecule localization, cellular protein localization, retrograde vesicle-mediated transport, Golgi to ER, Golgi transport complex, protein transporter activity |

Figure 1: DNA methylation profiles of CD4+ T cells in CFS/ME patients and controls. The heat map and hierarchical clustering results of methylated regions in CD4+ T cells from CFS/ME patients and control. The blue regions represent hypomethylation while the red regions represent hypermethylation.
| Gene   | Description                                                                 | Log2 Fold Change | P-value   | Methylation Type | Enriched Pathways                                                                                           |
|--------|------------------------------------------------------------------------------|------------------|-----------|------------------|------------------------------------------------------------------------------------------------------------|
| NR3C1  | Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)     | -2.66399         | 0.008423  | hypomethylation  | Intracellular signalling cascade,                                                                          |
| ADAMTS1| ADAMTS (a disintegrin and metalloproteinase with thrombospondin motif)       | -2.65750         | 0.005077  | hypomethylation  |                                                                                                            |
| SELT   | Selenoprotein T                                                               | -2.57892         | 0.002665  | hypomethylation  |                                                                                                            |
| MX1    | Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse) | -2.53843         | 0.045739  | hypomethylation  | Purine ribonucleotide binding,                                                                             |
| MACF1  | Microtubule-actin crosslinking factor 1                                      | -2.53607         | 0.001098  | hypomethylation  |                                                                                                            |
| FRMD4A | FERM domain containing 4Ap                                                    | -2.47480         | 0.003371  | hypomethylation  | Purine ribonucleotide binding,                                                                             |
| DMXL1  | Dmx-like 1                                                                   | -2.43990         | 0.029074  | hypomethylation  |                                                                                                            |
| PGD    | Phosphogluconate dehydrogenase                                                | -2.43270         | 0.003595  | hypomethylation  |                                                                                                            |
| MARK1  | MAP/microtubule affinity-regulating kinase 1                                 | -2.42785         | 0.016744  | hypomethylation  | Nucleotide binding, protein kinase cascade, protein amino acid phosphorylation, intracellular signalling cascade, microtubule cytoskeleton, protein kinase activity, phosphotransferase activity, alcohol group as acceptor |
| FAM13B | Family with sequence similarity 13, member B                                  | -2.41743         | 0.006546  | hypomethylation  | GTPase activator activity                                                                                 |
| ATM    | Ataxia telangiectasia mutated                                                 | -2.41147         | 0.020951  | hypomethylation  | Purine ribonucleotide binding, ribonucleotide binding, nucleotide binding, protein amino acid phosphorylation, intracellular signalling cascade, microtubule cytoskeleton, |
| NPAT   | Nuclear protein, ataxia-telangiectasia locus                                  | -2.40925         | 0.020950  | hypomethylation  |                                                                                                            |
| SDCCAG10| CWC27 spliceosome-associated protein homolog (S. cerevisiae)                  | -2.39996         | 0.004538  | hypomethylation  |                                                                                                            |
| RSBN1  | Round spermatid basic protein 1                                               | -2.38842         | 0.016944  | hypomethylation  |                                                                                                            |
| MED13  | Mediator complex subunit 13                                                  | -2.32252         | 0.009664  | hypomethylation  | VitaminD receptor binding, intracellular signalling cascade,                                             |
| MATN2  | Matrilin 2                                                                   | -2.31173         | 0.021316  | hypomethylation  |                                                                                                            |
| RAD51  | RAD51 recombinase                                                             | -2.28733         | 0.025275  | hypomethylation  | Purine ribonucleotide binding, ribonucleotide binding, nucleotide binding,                               |
| SLC4A5 | Solute carrier family 4 (sodium bicarbonate cotransporter), member 5         | -2.27009         | 0.015108  | hypomethylation  | Anion transmembrane transporter activity,                                                                 |
| WBCS17 | Williams-Beuren syndrome chromosome region 17                                 | -2.26845         | 0.015743  | hypomethylation  |                                                                                                            |
| RBM25  | RNA binding motif protein 25                                                  | -2.24768         | 0.046929  | hypomethylation  | Ribonucleotide binding, nucleotide binding,                                                                |
| DOK4   | Dedicator of cytokinesis 4                                                    | -2.22303         | 0.023445  | hypomethylation  | Rho GTPase binding, RacGTPase activator activity, RacGTPase binding,                                       |
| CCDC148| Coiled-coil domain containing 148                                             | -2.19913         | 0.014485  | hypomethylation  |                                                                                                            |
| Gene Symbol | Description | Fold Change | p-value | DNA Methylation Status | Pathway Comments |
|-------------|-------------|-------------|---------|------------------------|------------------|
| PHF19 | PHD finger protein 19 | -2.19496 | 0.010693 | hypomethylation | 9 |
| RPS6KA2 | Ribosomal protein S6 kinase, 90kDa, polypeptide 2 | -2.19420 | 0.004795 | hypomethylation | 6 |
| CTSO | Cathepsin O | -2.18726 | 0.035781 | hypomethylation | 4 |
| STK17B | Serine/threonine kinase 17b (apoptosis-inducing) | -2.18165 | 0.029008 | hypomethylation | 2 |
| SGK1 | Serum glucocorticoid regulated kinase 1 | -2.14645 | 0.013318 | hypomethylation | 6 |
| STK17C | Major histocompatibility complex, class I, C | -2.1388 | 0.006649 | hypomethylation | 6 |
| ATP13A3 | ATPase type 13A3 | -2.12355 | 0.024860 | hypomethylation | 17 |
| PHF12 | PHD finger protein 12 | -2.10335 | 0.024860 | hypomethylation | 17 |
| A2BP1 | RNA binding protein, fox-1 homolog (C. elegans) 1 | -2.10170 | 0.006649 | hypomethylation | 16 |
| ANKH | ANKH inorganic pyrophosphate transport regulator | -2.07819 | 0.041987 | hypomethylation | 5 |
| ERICH1 | Glutamate-rich 1 | -2.07436 | 0.013699 | hypomethylation | 8 |
| PHC3 | Polyhomeotic homolog 3 (Drosophila) | -2.05013 | 0.016319 | hypomethylation | 3 |
| LOC404266 | HomeoboxB5 | -2.04707 | 0.018899 | hypomethylation | 17 |
| KHDRBS2 | KH domain containing, RNA binding, signal transduction associated 2 | -2.04276 | 0.030130 | hypomethylation | 2 |
| CRIM1 | Cysteine rich transmembrane BMP regulator 1 (chordin-like) | -2.04223 | 0.015920 | hypomethylation | 5 |
| AMACR | Alpha-methylacyl-CoA racemase | -2.03930 | 0.010834 | hypomethylation | 19 |
| ASNA1 | Arsarsenite transporter, ATP-binding, homolog 1 (bacterial) | -2.03726 | 0.004322 | hypomethylation | 15 |
| NDUFA5 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5 | -2.0395 | 0.020507 | hypomethylation | 7 |
| UNC84A | SAD1 and UNC84 domain containing 1 | -2.03190 | 0.000411 | hypomethylation | 7 |
**Table 1:** A list of genes with differential methylated regions in the patient group.

| Gene Symbol | Description                                           | Methylated Region | p Value | Methylation Type | Function                                                                 |
|-------------|--------------------------------------------------------|-------------------|---------|-----------------|--------------------------------------------------------------------------|
| BTAF1       | BTA F1 RNA polymerase II, B-TFIID transcription factor-associated, 170kDa | -2.02822          | 0.042294| hypomethylation | 10 Ribonucleotide binding,                                               |
| OXA1L       | Oxidase (cytochrome c) assembly 1-like                 | 2.00760           | 0.034505| hypomethylation | 14 Oxidation reduction, negative regulation of ATPase activity, proton-transporting two-sector ATPase complex assembly, mitochondrial respiratory chain complex assembly, aerobic respiration, negative regulation of hydrolase activity, cellular macromolecule localization, cellular protein localization |
| GMNN        | Geminiin, DNA replication inhibitor                   | 2.02374           | 0.012055| hypomethylation | 6 Negative regulation of DNA replication, nuclear export, Cajal body    |
| LSG1        | Large60S subunit nuclear export GTPase 1              | 2.07421           | 0.019916| hypermethylation| 3 Oxidation reduction, negative regulation of ATPase activity, proton-transporting two-sector ATPase complex assembly, mitochondrial respiratory chain complex assembly, aerobic respiration, negative regulation of hydrolase activity, cellular macromolecule localization, cellular protein localization |
| NUDT1       | Nudix (nucleoside diphosphate linked moiety X)-type motif 1 | 2.11897           | 0.002342| hypermethylation| 7 Negative regulation of DNA replication, nuclear export, Cajal body    |
| FTSJ2       | FtsJ RNA methyltransferase homolog 2 (E. coli)        | 2.12453           | 0.002342| hypermethylation| 7 Oxidation reduction, negative regulation of ATPase activity, proton-transporting two-sector ATPase complex assembly, mitochondrial respiratory chain complex assembly, aerobic respiration, negative regulation of hydrolase activity, cellular macromolecule localization, cellular protein localization |
| HLA-DQB1    | Major histocompatibility complex II, DQ beta 1        | 2.29574           | 0.033972| hypermethylation| 6 MHC class II receptor activity                                         |
| ADAMTS12    | ADAM metallopeptidase with thrombospondin type 1 motif, 12 | 2.32516           | 0.033508| hypermethylation| 5 Oxidation reduction, negative regulation of ATPase activity, proton-transporting two-sector ATPase complex assembly, mitochondrial respiratory chain complex assembly, aerobic respiration, negative regulation of hydrolase activity, cellular macromolecule localization, cellular protein localization |
| MED12L      | Mediator complex subunit 12-like                      | 2.40530           | 0.033766| hypermethylation| 3 Oxidation reduction, negative regulation of ATPase activity, proton-transporting two-sector ATPase complex assembly, mitochondrial respiratory chain complex assembly, aerobic respiration, negative regulation of hydrolase activity, cellular macromolecule localization, cellular protein localization |
| GPR171      | G protein-coupled receptor 171                        | 2.42853           | 0.033766| hypermethylation| 3 Oxidation reduction, negative regulation of ATPase activity, proton-transporting two-sector ATPase complex assembly, mitochondrial respiratory chain complex assembly, aerobic respiration, negative regulation of hydrolase activity, cellular macromolecule localization, cellular protein localization |
| TEX14       | Testis expressed 14                                   | 3.09392           | 0.035419| hypermethylation| 17 Purine ribonucleotide binding                                        |
| RAD51C      | RAD51 paralog C                                       | 3.47220           | 0.035419| hypermethylation| 17 Oxidation reduction, negative regulation of ATPase activity, proton-transporting two-sector ATPase complex assembly, mitochondrial respiratory chain complex assembly, aerobic respiration, negative regulation of hydrolase activity, cellular macromolecule localization, cellular protein localization |
| MYCBP       | MYC binding protein                                   | 3.52884           | 0.000838| hypermethylation| 1 Oxidation reduction, negative regulation of ATPase activity, proton-transporting two-sector ATPase complex assembly, mitochondrial respiratory chain complex assembly, aerobic respiration, negative regulation of hydrolase activity, cellular macromolecule localization, cellular protein localization |
| GJA9        | Gap junction protein, alpha 9, 59kDa                 | 3.95041           | 0.000838| hypermethylation| 1 Oxidation reduction, negative regulation of ATPase activity, proton-transporting two-sector ATPase complex assembly, mitochondrial respiratory chain complex assembly, aerobic respiration, negative regulation of hydrolase activity, cellular macromolecule localization, cellular protein localization |
| LIPT1       | Lipooyl transferase1                                  | 5.93865           | 0.020965| hypermethylation| 2 Oxidation reduction, negative regulation of ATPase activity, proton-transporting two-sector ATPase complex assembly, mitochondrial respiratory chain complex assembly, aerobic respiration, negative regulation of hydrolase activity, cellular macromolecule localization, cellular protein localization |
| TSGA10      | Testis specific, 10                                  | -6.01644          | 0.029695| hypermethylation| 2 Oxidation reduction, negative regulation of ATPase activity, proton-transporting two-sector ATPase complex assembly, mitochondrial respiratory chain complex assembly, aerobic respiration, negative regulation of hydrolase activity, cellular macromolecule localization, cellular protein localization |
| DGKQ        | Diacylglycerol kinase, theta 110kDa                  | -3.66709          | 0.013574| Hypermethylation| 4 Oxidation reduction, negative regulation of ATPase activity, proton-transporting two-sector ATPase complex assembly, mitochondrial respiratory chain complex assembly, aerobic respiration, negative regulation of hydrolase activity, cellular macromolecule localization, cellular protein localization |
| FMN2        | Formin2                                              | -3.55880          | 0.019258| Hypermethylation| 1 Oxidation reduction, negative regulation of ATPase activity, proton-transporting two-sector ATPase complex assembly, mitochondrial respiratory chain complex assembly, aerobic respiration, negative regulation of hydrolase activity, cellular macromolecule localization, cellular protein localization |
| C1orf52     | Chromosome1 open reading frame 52                    | -3.34680          | 0.015531| Hypermethylation| 1 Oxidation reduction, negative regulation of ATPase activity, proton-transporting two-sector ATPase complex assembly, mitochondrial respiratory chain complex assembly, aerobic respiration, negative regulation of hydrolase activity, cellular macromolecule localization, cellular protein localization |
| PSMD2       | Proteasome (prosome, macropian) 26S subunit, non-ATPase, 2 | -3.31289          | 0.023962| Hypermethylation| 3 Oxidation reduction, negative regulation of ATPase activity, proton-transporting two-sector ATPase complex assembly, mitochondrial respiratory chain complex assembly, aerobic respiration, negative regulation of hydrolase activity, cellular macromolecule localization, cellular protein localization |
| DGKQ        | Diacylglycerol kinase, theta 110kDa                  | -3.26934          | 0.016689| Hypermethylation| 4 Oxidation reduction, negative regulation of ATPase activity, proton-transporting two-sector ATPase complex assembly, mitochondrial respiratory chain complex assembly, aerobic respiration, negative regulation of hydrolase activity, cellular macromolecule localization, cellular protein localization |

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| Chromosome | miRNA     | Start      | End        | Methylation      | Fold Change | P-value   | Target Gene |
|------------|-----------|------------|------------|------------------|-------------|-----------|-------------|
| chr2       | hsa-mir-7845 | 208031124  | 208031222  | Hypomethylation | -1.17746    | 0.000205  | KLF7        |
| chr2       | hsa-mir-5001 | 233415184  | 233415283  | Hypomethylation | -1.10555    | 0.000806  | EIF4E2;TIGD1 |
| chr14      | hsa-mir-376c | 101506027  | 101506092  | Hypermethylation | 1.42619     | 0.001340  |
| chr3       | hsa-mir-4444-2 | 75263627   | 75263700   | Hypermethylation | 1.07197     | 0.001379  |
| chr22      | hsa-mir-3828 | 31566048   | 31566105   | Hypermethylation | 1.14670     | 0.001707  |
| chr19      | hsa-mir-6795 | 15290094   | 15290161   | Hypomethylation | -1.00656    | 0.002333  |
| chr22      | hsa-mir-3653 | 29729147   | 29729256   | Hypermethylation | 1.01139     | 0.002345  |
| chr19      | hsa-mir-520b | 54204481   | 54204541   | Hypomethylation | -1.04360    | 0.002389  |
| chr17      | hsa-mir-33b  | 17717150   | 17717245   | Hypermethylation | 1.01560     | 0.002862  |
| chr19      | hsa-mir-639  | 14640355   | 14640452   | Hypomethylation | -1.11001    | 0.003032  | TECR;MIR639 |
| chr9       | hsa-mir-4674 | 139440625  | 139440711  | Hypomethylation | -1.14622    | 0.003294  | NOTCH1      |
| chr15      | hsa-mir-3175 | 93474829   | 9347705    | Hypomethylation | -1.11911    | 0.003765  | CHD2        |
| chr14      | hsa-mir-4710 | 105144031  | 105144086  | Hypomethylation | -1.12202    | 0.003959  |
| chr8       | hsa-mir-124-2 | 65291706   | 65291814   | Hypomethylation | -1.13882    | 0.004364  |
| chr6       | hsa-mir-6891 | 31323001   | 31323093   | Hypomethylation | -1.10710    | 0.004378  |
| chr5       | hsa-mir-143  | 148808481  | 148808586  | Hypermethylation | 1.05776     | 0.005115  |
| chr11      | hsa-mir-130a | 57408671   | 57408759   | Hypermethylation | 1.02682     | 0.005571  |
| chr11      | hsa-mir-34c  | 111384164  | 111384240  | Hypomethylation | -1.14561    | 0.006109  |
| chr11      | hsa-mir-139  | 72326107   | 72326174   | Hypomethylation | -1.01062    | 0.008066  |
| chr16      | hsa-mir-1225 | 2140196    | 2140285    | Hypomethylation | -1.00302    | 0.008162  |
| chr1       | hsa-mir-760  | 94312388   | 94312467   | Hypermethylation | 1.52577     | 0.008974  |
| chr17      | hsa-mir-142  | 56048593   | 56408679   | Hypomethylation | -1.12111    | 0.009029  |
| chr19      | hsa-mir-638  | 10829080   | 10829179   | Hypermethylation | 1.23848     | 0.009762  | MIR638;DNM2 |
| chr14      | hsa-mir-496  | 101526910  | 101527011  | Hypomethylation | -1.01214    | 0.009849  |
| chr11      | hsa-mir-1908 | 61582633   | 61582712   | Hypomethylation | -1.08932    | 0.010624  | MIR1908;FADS1 |
| chr3       | hsa-mir-425  | 49057581   | 49057667   | Hypermethylation | 1.12229     | 0.010864  | NDUFAF3;DALRD3;MIR425 |
| chr13      | hsa-mir-320d-1 | 41301964  | 41302011   | Hypermethylation | 1.05393     | 0.011027  |
| chr19      | hsa-mir-523  | 54201639   | 54201725   | Hypermethylation | -1.01830    | 0.011236  |
| chr6       | hsa-mir-6891 | 31323001   | 31323093   | Hypomethylation | -1.34121    | 0.011631  |
| chr19      | hsa-mir-498  | 54177451   | 54177574   | Hypermethylation | -1.01044    | 0.011972  |
| chr20      | hsa-mir-124-3 | 61809852  | 61809938   | Hypermethylation | -1.12224    | 0.012418  |
| chr10      | hsa-mir-2110 | 115933864  | 115933938  | Hypermethylation | -1.09673    | 0.012533  |
| chr9       | hsa-mir-6853 | 35732919   | 35732992   | Hypermethylation | -1.05786    | 0.012640  | CREB3;TLN1 |
| chr3       | hsa-mir-944  | 189547711  | 189547798  | Hypermethylation | 1.02836     | 0.012847  |
| chr11      | hsa-mir-192  | 64658609   | 64658718   | Hypermethylation | 1.00819     | 0.013255  |
| Chr | Hsa-mir | Start | End | Methylation | Log2 Ratio | p-value |
|-----|---------|-------|-----|-------------|------------|---------|
| chr1 | hsa-mir-6742 | 228584749 | 228584810 | hypermethylation | 1.01184 | 0.013631 |
| chr19 | hsa-mir-4321 | 2250638 | 2250717 | hypermethylation | 1.06948 | 0.013643 |
| chr8 | hsa-mir-4664 | 144815253 | 144815323 | hypermethylation | 1.04538 | 0.014353 |
| chr17 | hsa-mir-3184 | 28444104 | 28444178 | hypermethylation | 1.44860 | 0.014434 | MIR423;CCDC55 |
| chr17 | hsa-mir-423 | 28444097 | 28444190 | hypermethylation | 1.44860 | 0.014434 | MIR423;CCDC55 |
| chr5 | hsa-mir-340 | 17942303 | 17942397 | hypermethylation | 1.01906 | 0.014508 |
| chr8 | hsa-mir-596 | 1765397 | 1765473 | hypomethylation | -1.19681 | 0.014675 | MIR596 |
| chr6 | hsa-mir-6834 | 33250222 | 33258102 | hypermethylation | 1.07284 | 0.015375 |
| chr2 | hsa-mir-375 | 219866347 | 219866430 | hypermethylation | -1.56597 | 0.015549 |
| chr19 | hsa-mir-181c | 13896513 | 13896522 | hypermethylation | 1.00944 | 0.015609 |
| chr19 | hsa-mir-181d | 13896589 | 138965825 | hypermethylation | 1.00944 | 0.015609 |
| chr4 | hsa-mir-5091 | 13629489 | 13629581 | hypermethylation | 1.35117 | 0.016711 | BOD1L |
| chr20 | hsa-mir-647 | 62579384 | 62574079 | hypermethylation | 1.01182 | 0.017086 |
| chr1 | hsa-mir-6733 | 43637323 | 43637383 | hypomethylation | -1.04073 | 0.017516 | WDR65;EBNA1BP2 |
| chr19 | hsa-mir-330 | 46142252 | 46142345 | hypomethylation | -1.11000 | 0.018314 | MIR330;EML2 |
| chr8 | hsa-mir-6876 | 25202918 | 25202990 | hypermethylation | 1.26651 | 0.018319 |
| chr19 | hsa-mir-4746 | 4445975 | 4446045 | hypermethylation | -1.00382 | 0.018569 |
| chr2 | hsa-mir-375 | 219866367 | 219868430 | hypermethylation | -1.38810 | 0.019266 |
| chr19 | hsa-mir-638 | 10829080 | 10829179 | hypomethylation | -1.09026 | 0.020249 | DNM2 |
| chr21 | hsa-mir-155 | 26946292 | 26946356 | hypomethylation | -1.67121 | 0.020383 |
| chr3 | hsa-mir-15b | 160122376 | 160122473 | hypermethylation | 1.07586 | 0.020474 |
| chr3 | hsa-mir-16-2 | 160122533 | 160122613 | hypermethylation | 1.07586 | 0.020474 |
| chr19 | hsa-mir-642a | 46178186 | 46178282 | hypermethylation | 1.00701 | 0.020574 |
| chr19 | hsa-mir-642b | 46178190 | 46178266 | hypermethylation | 1.00701 | 0.020574 |
| chr22 | hsa-mir-658 | 38240279 | 38240378 | hypomethylation | -1.16552 | 0.020627 | ANKRD54;MIR658 |
| chr3 | hsa-lets-7g | 52302294 | 52302377 | hypomethylation | -1.15178 | 0.021048 |
| chr15 | hsa-mir-4515 | 83736087 | 83736167 | hypomethylation | -1.05581 | 0.021243 | BTBD1 |
| chr17 | hsa-mir-4523 | 27717680 | 27717748 | hypomethylation | -1.14820 | 0.021784 |
| chr7 | hsa-mir-6840 | 99954274 | 99954344 | hypermethylation | 1.01168 | 0.022112 |
| chr17 | hsa-mir-10a | 46657200 | 46657309 | hypermethylation | 1.14698 | 0.022855 |
| chr20 | hsa-mir-124-3 | 61809652 | 61809938 | hypomethylation | -1.10597 | 0.023198 |
| chr17 | hsa-mir-4523 | 27717680 | 27717748 | hypermethylation | 1.32887 | 0.023291 |
| chr2 | hsa-mir-3679 | 134884696 | 134884763 | hypomethylation | -1.02140 | 0.023375 |
| chr1 | hsa-mir-6733 | 43637323 | 43637383 | hypomethylation | -1.47410 | 0.023379 | WDR65;EBNA1BP2 |
| chr3 | hsa-mir-4792 | 24562853 | 24562926 | hypomethylation | -1.11756 | 0.023586 |
| Chromosome | Gene Symbol | Start | End | Methylation Type | Methylation Value | P Value |
|------------|-------------|-------|-----|-----------------|------------------|---------|
| chr17      | hsa-mir-1288| 16185328 | 16185402 | hypermethylation | 1.03806 | 0.023703 |
| chr2       | hsa-mir-4444-1 | 178077454 | 178077527 | hypermethylation | 1.10018 | 0.023789 |
| chr17      | hsa-mir-378j | 35974976 | 35975084 | hypomethylation | -1.00674 | 0.024333 |
| chr2       | hsa-mir-1471 | 232756952 | 232757008 | hypomethylation | -1.00916 | 0.024522 |
| chr17      | hsa-mir-632 | 30677128 | 30677221 | hypermethylation | -1.17464 | 0.025691 |
| chr11      | hsa-mir-1304 | 93466640 | 93466930 | hypermethylation | 1.11806 | 0.026275 |
| chr15      | hsa-mir-3175 | 93447629 | 93447705 | hypermethylation | -1.14724 | 0.026841 |
| chr20      | hsa-mir-663a | 26188822 | 26188914 | hypermethylation | -1.16412 | 0.026892 |
| chr11      | hsa-mir-1304 | 93466640 | 93466930 | hypermethylation | 1.11806 | 0.026275 |
| chr9       | hsa-mir-4672 | 130631694 | 130631774 | hypermethylation | -1.00712 | 0.027149 |
| chr3       | hsa-mir-885  | 10436173 | 10436246 | hypermethylation | -1.00941 | 0.027299 |
| chr7       | hsa-mir-183  | 129414745 | 129414854 | hypermethylation | 1.01084 | 0.027378 |
| chr7       | hsa-mir-96   | 129414532 | 129414609 | hypermethylation | 1.01084 | 0.027380 |
| chr12      | hsa-mir-141  | 7073260  | 7073354  | hypermethylation | 1.01399 | 0.027805 |
| chr7       | hsa-mir-590  | 73605528 | 73605624 | hypermethylation | -1.10206 | 0.028022 |
| chr12      | hsa-mir-1178 | 120151439 | 120151529 | hypermethylation | 1.01277 | 0.028608 |
| chr4       | hsa-mir-1973 | 117220881 | 117220924 | hypermethylation | 1.08332 | 0.028757 |
| chr2       | hsa-mir-375  | 219866367 | 219866430 | hypermethylation | -1.29192 | 0.029281 |
| chr10      | hsa-mir-2110 | 115933884 | 115933938 | hypermethylation | -1.09997 | 0.029535 |
| chr10      | hsa-mir-1296 | 65132717 | 65132808 | hypermethylation | 1.01367 | 0.029866 |
| chr19      | hsa-mir-23a  | 13947401 | 13947473 | hypermethylation | 1.06274 | 0.029990 |
| chr19      | hsa-mir-24-2 | 13947101 | 13947173 | hypermethylation | 1.06274 | 0.029990 |
| chr19      | hsa-mir-27a  | 13947254 | 13947331 | hypermethylation | 1.06274 | 0.029990 |
| chr17      | hsa-mir-152  | 46114527 | 46114613 | hypermethylation | -1.30883 | 0.030070 |
| chr17      | hsa-mir-6516 | 75085499 | 75085579 | hypermethylation | 1.14486 | 0.030396 |
| chr8       | hsa-mir-320a | 22102475 | 22102556 | hypermethylation | 1.08542 | 0.030987 |
| chr1       | hsa-mir-320b-2 | 224444706 | 224444843 | hypermethylation | -1.00847 | 0.031140 |
| chr1       | hsa-mir-5087 | 147806063 | 147806678 | hypermethylation | -1.47707 | 0.031743 |
| chr17      | hsa-mir-4521 | 8090263  | 8090322  | hypermethylation | -1.10388 | 0.032254 |
| chr14      | hsa-mir-496  | 101526910 | 101527011 | hypermethylation | -1.01232 | 0.033237 |
| chr1       | hsa-mir-320b-1 | 117214371 | 117214449 | hypermethylation | 1.01642 | 0.033722 |
| chr2       | hsa-mir-933  | 176032361 | 176032437 | hypermethylation | 1.15198 | 0.033760 |
| chr12      | hsa-mir-7107 | 121882076 | 121882155 | hypermethylation | 1.01076 | 0.033806 |
| chr14      | hsa-mir-6717 | 21491473 | 21491545 | hypermethylation | 1.03920 | 0.033889 |
| chr7       | hsa-mir-4648 | 2566708  | 2566779  | hypermethylation | 1.00823 | 0.034164 |
| chr10      | hsa-mir-938  | 29891193 | 29891275 | hypermethylation | -1.00845 | 0.034456 |
| Chr | Gene | Start | End | Methylation | p-value |
|-----|------|-------|-----|-------------|---------|
| chr6 | hsa-mir-6832 | 31601564 | 31601635 | hypermethylation | 1.00795, 0.034535 |
| chr15 | hsa-mir-627 | 42491768 | 42491864 | hypermethylation | -1.01772, 0.035449 |
| chr3 | hsa-mir-128-2 | 35785968 | 35786051 | hypermethylation | 1.01399, 0.035564 |
| chr3 | hsa-mir-4444-2 | 75263627 | 75263700 | hypermethylation | 1.06916, 0.035786 |
| chr14 | hsa-mir-300 | 101507700 | 101507782 | hypermethylation | 1.40643, 0.036924 |
| chr6 | hsa-mir-6891 | 31323001 | 31323093 | hypomethylation | 1.13122, 0.037499 |
| chr17 | hsa-mir-142 | 56408593 | 56408679 | hypermethylation | 1.10981, 0.038468 |
| chr14 | hsa-mir-409 | 101531637 | 101531715 | hypomethylation | 1.01538, 0.038623 |
| chr14 | hsa-mir-412 | 101531784 | 101531874 | hypomethylation | 1.01538, 0.038623 |
| chr15 | hsa-mir-628 | 55665138 | 55665232 | hypermethylation | 1.06044, 0.038658 |
| chr13 | hsa-mir-8073 | 110993305 | 110993376 | hypomethylation | 1.12176, 0.039730 |
| chr4 | hsa-mir-572 | 11370451 | 11370545 | hypermethylation | 1.13340, 0.040330 |
| chr22 | hsa-mir-3928 | 31556048 | 31556105 | hypermethylation | 1.20598, 0.040468 |
| chr14 | hsa-mir-4505 | 74225450 | 74225522 | hypermethylation | 1.10764, 0.040469 |
| chr17 | hsa-mir-365b | 29902430 | 29902540 | hypermethylation | 1.00811, 0.040829 |
| chr17 | hsa-mir-4725 | 29902288 | 29902377 | hypomethylation | 1.00811, 0.040829 |
| chr19 | hsa-mir-4754 | 58898137 | 58898225 | hypomethylation | 1.12062, 0.040839 |
| chr5 | hsa-mir-1229 | 179225278 | 179225346 | hypermethylation | 1.00370, 0.041022 |
| chr15 | hsa-mir-7706 | 85923893 | 85923893 | hypermethylation | 1.10880, 0.041546 |
| chr11 | hsa-mir-4492 | 118781417 | 118781496 | hypermethylation | 1.13724, 0.042569 |
| chr18 | hsa-mir-4741 | 20513312 | 20513401 | hypermethylation | 1.13235, 0.042592 |
| chr13 | hsa-mir-19a | 92003145 | 92003226 | hypermethylation | 1.03689, 0.043500 |
| chr13 | hsa-mir-19b-1 | 92003446 | 92003532 | hypermethylation | 1.03689, 0.043500 |
| chr13 | hsa-mir-20a | 92003319 | 92003389 | hypermethylation | 1.03689, 0.043500 |
| chr13 | hsa-mir-92a-1 | 92003568 | 92003645 | hypermethylation | 1.03689, 0.043500 |
| chr12 | hsa-mir-7107 | 121882076 | 121882155 | hypermethylation | 1.00568, 0.044221 |
| chr8 | hsa-mir-661 | 145019359 | 145019447 | hypermethylation | 1.01970, 0.044401 |
| chr14 | hsa-mir-4308 | 55344831 | 55344911 | hypermethylation | 1.00911, 0.044475 |
| chr12 | hsa-mir-1251 | 97885687 | 97885756 | hypermethylation | 1.02643, 0.045113 |
| chr5 | hsa-mir-4638 | 180649566 | 180649633 | hypermethylation | 1.03734, 0.045950 |
| chr11 | hsa-mir-4687 | 3877292 | 3877371 | hypermethylation | 1.06416, 0.048474 |
| chr19 | hsa-mir-1909 | 1816158 | 1816237 | hypermethylation | 1.01084, 0.048474 |
| chr10 | hsa-mir-1307 | 105154010 | 105154158 | hypermethylation | 1.01492, 0.048703 |
| chr9 | hsa-mir-769 | 46522190 | 46522307 | hypermethylation | 1.02118, 0.048976 |
| chr3 | hsa-mir-6828 | 170140891 | 170140950 | hypermethylation | 1.00978, 0.047242 |
| chr7 | hsa-mir-339 | 1062569 | 1062662 | hypermethylation | 1.02186, 0.047576 |
| Chromosome | miRNA Gene | Start Position | End Position | Methylation Type | Log2 Fold Change | p-Value |
|------------|------------|----------------|--------------|-----------------|-----------------|---------|
| chr1       | hsa-mir-181a-1 | 198828173 | 198828282 | hypermethylation | 1.02246 | 0.048357 |
| chr1       | hsa-mir-181b-1 | 198828002 | 198828111 | hypermethylation | 1.02246 | 0.048356 |
| chr14      | hsa-mir-369 | 101531935 | 101532004 | hypomethylation | -1.00698 | 0.048589 |
| chr14      | hsa-mir-409 | 101531637 | 101531715 | hypomethylation | -1.00698 | 0.048589 |
| chr14      | hsa-mir-412 | 101531784 | 101531874 | hypomethylation | -1.00698 | 0.048589 |
| chr7       | hsa-mir-196b | 27209099 | 27209182 | hypomethylation | -1.09179 | 0.048613 |
| chr11      | hsa-mir-675 | 2017989 | 2018061 | hypomethylation | -1.01792 | 0.049180 |
| chr19      | hsa-mir-638 | 10829080 | 10829179 | hypomethylation | -1.15170 | 0.049180 |
| chr11      | hsa-mir-34c | 111384164 | 111384240 | hypomethylation | -1.04702 | 0.049253 |
| chr8       | hsa-mir-6850 | 146017316 | 146017376 | hypomethylation | -1.11947 | 0.050049 |
| chr11      | hsa-mir-194-2 | 64658827 | 64658911 | hypomethylation | -1.00694 | 0.051008 |
| chr17      | hsa-mir-33b | 17717150 | 17717245 | hypermethylation | 1.01486 | 0.051177 |
| chr17      | hsa-mir-6777 | 17716794 | 17716859 | hypermethylation | 1.01486 | 0.051177 |
| chr22      | hsa-mir-3653 | 29729147 | 29729256 | hypermethylation | 1.01870 | 0.051352 |
| chr14      | hsa-mir-127 | 101349316 | 101349412 | hypomethylation | -1.16899 | 0.050185 |
| chr19      | hsa-mir-5001 | 233415184 | 233415283 | hypomethylation | -1.08397 | 0.050224 |
| chr11      | hsa-mir-194-2 | 64658827 | 64658911 | hypomethylation | -1.00694 | 0.051008 |
| chr17      | hsa-mir-33b | 17717150 | 17717245 | hypermethylation | 1.01486 | 0.051177 |
| chr17      | hsa-mir-6777 | 17716794 | 17716859 | hypermethylation | 1.01486 | 0.051177 |
| chr22      | hsa-mir-3653 | 29729147 | 29729256 | hypermethylation | 1.01870 | 0.051352 |
| chr14      | hsa-mir-127 | 101349316 | 101349412 | hypomethylation | -1.16899 | 0.050185 |
| chr19      | hsa-mir-5001 | 233415184 | 233415283 | hypomethylation | -1.08397 | 0.050224 |
| chr11      | hsa-mir-194-2 | 64658827 | 64658911 | hypomethylation | -1.00694 | 0.051008 |
| chr17      | hsa-mir-33b | 17717150 | 17717245 | hypermethylation | 1.01486 | 0.051177 |
| chr17      | hsa-mir-6777 | 17716794 | 17716859 | hypermethylation | 1.01486 | 0.051177 |
| chr22      | hsa-mir-3653 | 29729147 | 29729256 | hypermethylation | 1.01870 | 0.051352 |

Table 2: A list of miRNA genes with differential methylated regions in the CFS/ME patients in comparison to controls.
MicroRNA methylation pattern in CD4\(^+\) T cells

MicroRNA methylation patterns were examined in the CD4\(^+\) T cells from CFS/ME patients and non-fatigued controls. A total of 2291 methylation sites observed. Of these, 133 were differentially methylated between the two groups where 51.9% were hypomethylated while 48.1% were hypermethylated (Table 2).

Validation of methylated genes via HRM

Of the genes that were selected for HRM analysis we observed significant changes in the melting peak temperatures of DKGK (Figure 2).

![Figure 2: HRM Validation of some methylated genes in the CD4\(^+\) T cells from the CFS/ME patients and controls. The bar graphs represent the average melting temperatures of the genes examined, where the black bars are results from the CFS/ME patients and the white bars are results from the controls. * denotes significance at p-value <0.05.](image)

Discussion

This is the first study to report on a genome-wide DNA methylation analysis in CD4\(^+\) T cells from CFS/ME patients. This is also the first study to demonstrate significant hypo-and hyper- methylation sites in CD4\(^+\) T cells in CFS/ME patients. A predominant hypomethylation was observed in the CFS/ME patients and these were mostly located in the promoter regions of the genes. The genes with dmCpG sites were associated with a number of gene ontology terms and pathways which were significantly enriched in the CFS/ME group.

Pathway enrichment analysis showed that most of the genes with significant dmCpG sites were involved in forty seven different pathways. Among these pathways the most significantly enriched were involved in type I diabetes mellitus, autoimmune thyroid disease, viral myocarditis, antigen processing and presentation and cell adhesion molecules. The genes related to these pathways were HLA-C and HLA-DQB1. HLA-C is a major histocompatibility complex I (MHC) gene which has been associated with the progression of Human Immunodeficiency Virus (HIV) [19,20]. The HLA-C molecule is recognized by Killer Immunoglobulin-like Receptors (KIR)s, KIR2DL1 and KIR2DL2,3. Amongst the CD4\(^+\) T cells there is a subgroup of cells characterized by non-MHC restricted cytotoxicity, with Natural Killer (NK)-like activity and HLA-Cw7 dependent inhibition of cytotoxic activity [21-24]. Methylation in HLA-C may therefore affect cytotoxic activity mediated by CD4\(^+\) T cells and may suggest a role of HLA-C restricted T cell response in CFS/ME. In CFS/ME patients, cytotoxic activity is known to be reduced in both NK and CD8\(^+\) T cells, a global reduction in NK activity may persist in CFS/ME patients and this may be related to changes in the epigenetic patterns that regulate cytotoxic activity. Additionally, methylation in HLA-C may represent diversity in HLA-C restricted T cells as a consequence of reduced HLA-C expression on dendritic cells in the thymus during T cell thymic development [25]. HLA-DQB1 is a MHCI gene. Polymorphisms in a number of HLA-DQB1 haplotypes have been associated with susceptibility to certain types of cancers [26]. In particular, HLA-DQB1 has been identified as a risk factor for oesophageal cancer [27,28]. HLA-DQ alleles, in particular HLA-DQA1*01 and HLA-DQB1*06 have been observed to be increased in CFS/ME patients although the increase in HLA-DQB1*06 was only minimal [29].

Methylation in genes related to molecular processes such as membrane transport, kinase activity, nucleotide and ribonucleotide binding, GTPase activity and transferase activity, may suggest breakdown in molecular processes specific to CD4\(^+\) T cells in these patients. For example STK17B is involved in calcium and ROS signalling in CD4+ T cells [30] and hypomethylation in this gene may alter this process. Additionally, methylation in TXNRD1 may affect the antioxidant capacity of T cells [31]. RPS6KA2 and SGK1 are associated with the regulation of the mechanistic target of rapamycin (mTOR) signalling which is important in lymphocyte survival, growth, differentiation and proliferation of T cells ensuring efficient metabolism, cytoskeletal organization and apoptosis [32-34]. Importantly, RPS6KA2 is also observed to be associated with the MAPK signalling pathway and these are important in T cell mediated responses. During T cell activation HSPE1 and HSPD1 form a complex which regulates the activity of pro-caspase 3 [35]. Differential methylation in these genes may therefore be detrimental to caspase activity. Importantly, mitochondrial dysfunction has been proposed to be involved in the CFS/ME disease presentation [36-38], this may be associated with changes in NDUFA and OXACT. Similarly, genes responsible for DNA repair, including ATM, RAD51 and RAD51C, were also differentially methylated in the CFS/ME patients.

Although, the precise function of a number of these genes in T cells is unknown (DOCK4, BWD1, ASXL2, MED13, NPA3, C20ORF3 and PPH19) the observation that most of these genes are related to intracellular processes suggest potential abnormalities within the cell that may present in the form of increased cell numbers and changes in cytokine levels.

DGK\(\alpha\) (Diacylglycerol kinase, theta) is expressed in T cells and is known to regulate the magnitude of the TCR response by inhibiting MAPK activation and the expression of co-stimulatory molecules such as CD69 and CD25 [39]. In CFS/ME little is known about the status of the TCR. However, modulations in the expression of DGK\(\alpha\) may be important in the mechanism of the disorder.

Of the 176 miRNAs with significant dmCpGs, eight were CD4\(^+\) T cell specific miRNA genes including miR-124, miR-155, miR-181a, miR-142, miR-27a, miR-339, miR-340 and miR-425. Mir-155 is necessary for the differentiation and proliferation of CD4\(^+\) T cells in to the four main subsets (Th1, Th2, Th17 and Tregs) [40-42]. In FOXP3 specific Tregs, miR-155 is upregulated and a decrease in miR-155 decreases the number of these cells [40]. Changes in miR-155 regulation may account for the increases in Tregs observed in CFS/ME patients and the shifts in Th1/Th2 immune related responses [43]. Activation of the T cell receptor (TCR) involves a number of signalling pathways whose genes were methylated in the present study. Importantly, TCR signalling is modulated by miR-181a, as it inhibits
autoreactivity by promoting central tolerance and enhancing TCR responsiveness [44,45]. In the absence of miR-181a, autoreactive immune responses occur resulting in autoimmunity. miR-124 and miR-27a are over expressed in central memory and effector memory CD4+ T cells respectively during differentiation [46]. Among the CD4+ helper T cells, miR-425 is reduced in Th1 cells while miR-142 is increased in expression in Tregs [46].

In conclusion, the present study has identified for the first time potential disruption in epigenetic pathways in CD4+ T cells and this may contribute to the pathogenesis of CFS/ME. The most important finding in the present study is the dmCpG in the DGKI gene and CD4+ T cell specific miRNAs. As previous studies have observed changes in these molecules in CFS/ME patients it is possible to posit that these molecules may be important in deciphering a mechanism for CFS/ME. Epigenetic changes in immune cells may be an important component of CFS/ME.

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