Journal of
Clinical & Cellular Immunology

Brenu et al., J Clin Cell Immunol 2014, 5:3
DOI: 10.4172/2155-9899.1000228

Research Article Open Access

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

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Received date: March 28, 2014, **Accepted date:** June 13, 2014, **Published date:** June 20, 2014

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 differential 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 concrete diagnostic biomarkers have not been identified.

DNA methylation is an 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 assessed using the 1994 Centre for Disease Control and Prevention case definition (1994 CDC) Criteria for CFS/ME [3]. Majority of the CFS/ME cases were women, which is reflective of the high female to male ratio observed in CFS/ME cases. All patients reported active CFS/ME with low to moderate levels of physical activity and full time employment. A non-fatigued control group comprising 18 healthy participants with no incidence of CFS/ME or other medical conditions was also included in the study (10 Females and 8 males; age= 47.44 ± 2.16). Participants with autoimmune diseases, psychosis or smoking were excluded from the study.

$CD4^+$ 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 manufacture'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.

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, Irvine, CA) was used in the Bisulfite conversion of DNA. This was then hybridized onto a beadchip. The Illumina HumanMethylation450K BeadChip assay (Illumina Inc., San Diego, CA) was the method of choice for performing the whole genome methylation. This assay contains 485,577 CpG targets. Enzymatic end-point fragmentation, precipitation and re-suspension were used to amplify all the DNA samples. These samples were then hybridized for 16 hours at 48°C on to BeadChips. Dioxy nucleotide extension was used to achieve single nucleotide extensions. 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.

HRM analysis

HRM analysis was performed for genes including *NINJ2*, *HSPD1*, *TEX14*, *HLA-C*, *RAD51C*, *FMN2*, *DGKQ*, *LIPT1*, *GJA9* and *MYCBP*. HRM analysis was performed on the LightCycler 480 II system (Roche Diagnostics, Mannheim, Germany). Each reaction mixture contained 5 µl of precision melt supermix (Bio-rad), 0.5 µl of each primer (200 nM) and 1-50 ng of DNA. The total reaction volume was 10 µl. The analyses were performed on a 96 well plate in triplicates. An initial pre-incubation step was set at 95°C for 10 minutes, followed by 45 cycles of 95°C for 10 seconds, with annealing at 58°C for 15s and extension at 72°C for 25s. Melting analysis was designed to cover temperatures from 65 to 95°C where temperature was increases at 0.1°C increments. Melt curve data was performed on the LightCycler software.

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 Ontolology 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 either the age or full blood count parameters examined in the CFS/ME and control groups.

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

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 dmCpG sites and these can be classified in to 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-DQB1*). Genetic pathways specific to these sites were determined using the Kegg pathway analysis. The Kegg pathway analysis identified *HLA-C* and *HLA-DQB1* as genes in the pathway with the highest enrichment score (Table 1).

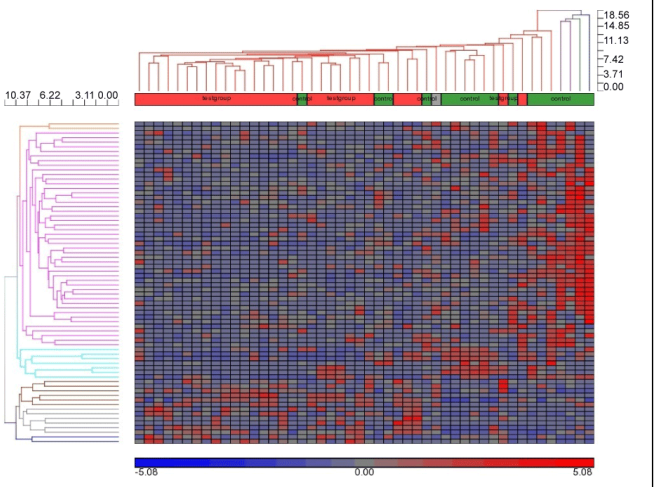

Figure 1: DNA methylation profiles of CD4$^+$T cells in CFS/ME patients and controls. The heat map and hierarchical clustering results of methylated regions in CD4$^+$T cells from CFS/ME patients and control. The blue regions represent hypomethylation while the red regions represent hypermethylation.

Gene	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 combs like 2 (Drosophila)	-3.11677	0.028253	hypomethylation	2	
HSPE1	Heatshock 10kDa protein 1	-3.07642	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

NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	-2.66399	0.008423	hypomethylation	5	Intracellular signalling cascade,
ADAMTSL1	ADAMTS (a disintegrin and metalloproteinase with thrombospondin motif)	-2.65750	0.005077	hypomethylation	9	
SELT	Selenoprotein T	-2.57892	0.002665	hypomethylation	3	
MX1	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	-2.53843	0.045739	hypomethylation	21	Purine ribonucleotide binding,
MACF1	Microtubule-actin crosslinking factor 1	-2.53607	0.001098	hypomethylation	1	Protein localization in organelle, cellular macromolecule localization, cellular protein localization, microtubule cytoskeleton,
FRMD4A	FERM domain containing 4Ap	-2.47480	0.003371	hypomethylation	10	
DMXL1	Dmx-like 1	-2.43990	0.029074	hypomethylation	5	
PGD	Phosphogluconate dehydrogenase	-2.43270	0.003595	hypomethylation	1	Ribonucleotide binding
MARK1	MAP/microtubule affinity-regulating kinase 1	-2.42785	0.016744	hypomethylation	1	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	5	GTPase activator activity
ATM	Ataxia telangiectasia mutated	-2.41147	0.020951	hypomethylation	11	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	11	
SDCCAG10	CWC27 spliceosome-associated protein homolog (*S. cerevisiae*)	-2.39996	0.004538	hypomethylation	5	
RSBN1	Round spermatid basic protein 1	-2.38842	0.016944	hypomethylation	1	
MED13	Mediator complex subunit 13	-2.32252	0.009664	hypomethylation	17	VitaminD receptor binding, intracellular signalling cascade,
MATN2	Matriline 2	-2.31173	0.021316	hypomethylation	8	
RAD51	RAD51 recombinase	-2.28733	0.025275	hypomethylation	15	Purine ribonucleotide binding, ribonucleotide binding, nucleotide binding,
SLC4A5	Solute carrier family 4 (sodium bicarbonate cotransporter), member 5	-2.27009	0.015108	hypomethylation	2	Anion transmembrane transporter activity,
WBSCR17	Williams-Beuren syndrome chromosome region 17	-2.26845	0.015743	hypomethylation	7	
RBM25	RNA binding motif protein 25	-2.24768	0.046929	hypomethylation	14	Ribonucleotide binding, nucleotide binding,
DOCK4	Dedicator of cytokinesis 4	-2.22303	0.023445	hypomethylation	7	Rho GTPase binding, RacGTPase activator activity, RacGTPase binding,
CCDC148	Coiled-coil domain containing 148	-2.19913	0.014485	hypomethylation	2	

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	Proteinkinase cascade, protein amino acid phosphorylation, intracellular signalling cascade, proteinserine/threonine kinase activity
CTSO	Cathepsin O	-2.18726	0.035781	hypomethylation	4	
STK17B	Serine/threonine kinase 17b (apoptosis-inducing)	-2.18165	0.029008	hypomethylation	2	Proteinserine/threonine kinase activity, protein kinase activity, phosphotransferase activity, alcohol group as acceptor
SGK1	Serum glucocorticoid regulated kinase 1	-2.14645	0.013318	hypomethylation	6	Phosphotransferase activity, alcohol group as acceptor, protein amino acid phosphorylation
HLA-C	Major histocompatibility complex, class I, C	-2.13388	0.006649	hypomethylation	6	MHC class II receptor activity
C20orf3	Chromosome 3 open reading frame, human	-2.13244	0.025289	hypomethylation	20	
HLA-C		-2.12459	0.027134	hypomethylation	6	MHC class II receptor activity
ATP13A3	ATPase type 13A3	-2.12378	0.047204	hypomethylation	3	Nucleotide biosynthetic process, purine nucleotide biosynthetic process,
PHF12	PHD finger protein 12	-2.10335	0.024860	hypomethylation	17	
A2BP1	RNA binding protein, fox-1 homolog (*C. elegans*) 1	-2.10170	0.000697	hypomethylation	16	
MCC	Mutatedin colorectal cancers	-2.09595	0.028307	hypomethylation	5	
ANKH	ANKH inorganic pyrophosphate transport regulator	-2.07819	0.041987	hypomethylation	5	Locomotory behaviour, regulation of bone mineralization, inorganic anion transmembrane transporter activity
ERICH1	Glutamate-rich 1	-2.07436	0.013699	hypomethylation	8	
PHC3	Polyhomeotichomolog 3 (Drosophila)	-2.05013	0.016319	hypomethylation	3	
LOC404266		-2.04836	0.018899	hypomethylation	17	
H**OXB5**	HomeoboxB5	-2.04707	0.018899	hypomethylation	17	
KHDRBS2	KH domain containing, RNA binding, signal transduction associated 2	-2.04293	0.022498	hypomethylation	6	
CRIM1	Cysteine rich transmembrane BMP regulator 1 (chordin-like)	-2.04276	0.030130	hypomethylation	2	Proteinkinase activity,
AMACR	Alpha-methylacyl-CoA racemase	-2.04223	0.015920	hypomethylation	5	
ASNA1	arsAarsenite transporter, ATP-binding, homolog 1 (bacterial)	-2.03930	0.010834	hypomethylation	19	Inorganic anion transmembrane transporter activity
BNIP2	BCL2/adenovirus E1B 19kDa interacting protein 2	-2.03728	0.040322	hypomethylation	15	GTPase activator activity,
NDUFA5	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5	-2.03695	0.020507	hypomethylation	7	Oxidation reduction, mitochondrial respiratory chain, respiratory chain,
UNC84A	Sad1 and UNC84 domain containing 1	-2.03190	0.000411	hypomethylation	7	

BTAF1	BTAF1 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	Geminin, 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	
NUDT1	Nudix (nucleoside diphosphate linked moiety X)-type motif 1	2.11897	0.002342	hypermethylation	7	
FTSJ2	FtsJ RNA methyltransferase homolog 2 (*E. coli*)	2.12453	0.002342	hypermethylation	7	
HLA-DQB1	Majorhistocompatibility complex, class 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	
MED12L	Mediator complex subunit 12-like:	2.40530	0.033766	hypermethylation	3	
GPR171	G protein-coupled receptor 171	2.42853	0.033766	hypermethylation	3	
TEX14	Testis expressed 14	3.09392	0.035419	hypermethylation	17	Protein amino acid phosphorylation, protein kinase activity, transferase activity, transferring phosphorus-containing groups
RAD51C	RAD51 paralog C	3.47220	0.035419	hypermethylation	17	Purine ribonucleotide binding
MYCBP	MYC binding protein;	3.52884	0.000838	hypermethylation	1	
GJA9	Gap junction protein, alpha 9, 59kDa	3.95041	0.000838	hypermethylation	1	
LIPT1	Lipoyl transferase1	5.93865	0.029695	hypermethylation	2	
TSGA10	Testis specific, 10	-6.01644	0.029695	Hypermethylation	2	
DGKQ	Diacylglycerol kinase, theta 110kDa	-3.66709	0.013574	Hypermethylation	4	Proteinkinase cascade, intracellular signalling cascade
FMN2	formin 2	-3.55680	0.019258	Hypermethylation	1	Establishment of spindle localization, metaphase, cytokinesis during cell cycle, establishment of chromosome localization, intracellular signalling cascade
C1orf52	Chromosome1 open reading frame 52	-3.34680	0.015531	Hypermethylation	1	
PSMD2	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 2	-3.31289	0.023962	Hypermethylation	3	Proteasome regulatory particle
DGKQ	Diacylglycerol kinase, theta 110kDa	-3.26934	0.016689	hypermethylation	4	Protein kinase cascade, intracellular signalling cascade

Table 1: A list of genes with differential methylated regions in the patient group.

Chromosome	miRNA	Start	End	Methylation	Fold Change	P-value	Target Gene
chr2	hsa-mir-7845	208031124	208031222	Hypomethlation	-1.17746	0.000205	KLF7
chr2	hsa-mir-5001	233415184	233415283	Hypomethlation	-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-3928	31556048	31556105	hypermethylation	1.14670	0.001707	
chr19	hsa-mir-6795	15290094	15290161	Hypomethlation	-1.00656	0.002333	
chr22	hsa-mir-3653	29729147	29729256	hypermethylation	1.01139	0.002345	
chr19	hsa-mir-520b	54204481	54204541	Hypomethlation	-1.04360	0.002389	
chr17	hsa-mir-33b	17717150	17717245	hypermethylation	1.01560	0.002862	
chr19	hsa-mir-639	14640355	14640452	Hypomethlation	-1.11001	0.003032	TECR;MIR639
chr9	hsa-mir-4674	139440625	139440711	Hypomethlation	-1.14622	0.003294	NOTCH1
chr15	hsa-mir-3175	93447629	93447705	Hypomethlation	-1.11911	0.003765	CHD2
chr14	hsa-mir-4710	105144031	105144086	Hypomethlation	-1.12202	0.003959	
chr8	hsa-mir-124-2	65291706	65291814	Hypomethlation	-1.13882	0.004364	
chr6	hsa-mir-6891	31323001	31323093	Hypomethlation	-1.10710	0.004376	
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	Hypomethlation	-1.14561	0.006109	
chr11	hsa-mir-139	72326107	72326174	Hypomethlation	-1.01062	0.008066	
chr16	hsa-mir-1225	2140196	2140285	Hypomethlation	-1.00302	0.008162	
chr1	hsa-mir-760	94312388	94312467	hypermethylation	1.52577	0.008974	
chr17	hsa-mir-142	56408593	56408679	hypomethylation	-1.1211	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;DALRD 3; MIR425
chr13	hsa-mir-320d-1	41301964	41302011	hypermethylation	1.05393	0.011027	
chr19	hsa-mir-523	54201639	54201725	hypomethylation	-1.01830	0.011236	
chr6	hsa-mir-6891	31323001	31323093	hypomethylation	-1.34121	0.011631	
chr19	hsa-mir-498	54177451	54177574	hypomethylation	-1.01044	0.011972	
chr20	hsa-mir-124-3	61809852	61809938	hypomethylation	-1.12224	0.012418	
chr10	hsa-mir-2110	115933864	115933938	hypomethylation	-1.09673	0.012533	
chr9	hsa-mir-6853	35732919	35732992	hypomethylation	-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	

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	179442303	179442397	hypermethylation	1.01906	0.014508	
chr8	hsa-mir-596	1765397	1765473	hypomethylation	-1.19681	0.014675	MIR596
chr6	hsa-mir-6834	33258022	33258102	hypermethylation	1.07284	0.015375	
chr2	hsa-mir-375	219866367	219866430	hypomethylation	-1.56597	0.015549	
chr19	hsa-mir-181c	13985513	13985622	hypermethylation	1.00944	0.015609	
chr19	hsa-mir-181d	13985689	13985825	hypermethylation	1.00944	0.015609	
chr4	hsa-mir-5091	13629489	13629581	hypermethylation	1.35117	0.016711	BOD1L
chr20	hsa-mir-647	62573984	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	hypomethylation	-1.00382	0.018569	
chr2	hsa-mir-375	219866367	219866430	hypomethylation	-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-let-7g	52302294	52302377	hypomethylation	-1.15178	0.021046	
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	61809852	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	

chr17	hsa-mir-1288	16185328	16185402	hypermethylation	1.03806	0.023703	
chr2	hsa-mir-4444-1	178077454	178077527	hypermethylation	1.10018	0.023789	HNRNPA3
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	hypomethylation	-1.17464	0.025691	ZNF207;MIR632
chr11	hsa-mir-1304	93466840	93466930	hypermethylation	1.11806	0.026275	
chr15	hsa-mir-3175	93447629	93447705	hypomethylation	-1.14724	0.026841	CHD2
chr20	hsa-mir-663a	26188822	26188914	hypomethylation	-1.16412	0.026892	MIR663
chr9	hsa-mir-4672	130631694	130631774	hypomethylation	-1.00712	0.027149	
chr3	hsa-mir-885	10436173	10436246	hypomethylation	-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	hypomethylation	-1.01026	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	hypomethylation	-1.29192	0.029281	
chr10	hsa-mir-2110	115933864	115933938	hypomethylation	-1.09997	0.029535	MIR2110;C10orf118
chr10	hsa-mir-1296	65132717	65132808	hypermethylation	1.01367	0.029866	
chr19	hsa-mir-23a	13947401	13947473	hypermethylation	1.06274	0.029990	MIR27A;MIR24-2
chr19	hsa-mir-24-2	13947101	13947173	hypermethylation	1.06274	0.029990	
chr19	hsa-mir-27a	13947254	13947331	hypermethylation	1.06274	0.029990	MIR27A;MIR24-2
chr17	hsa-mir-152	46114527	46114613	hypomethylation	-1.30883	0.030070	MIR152;COPZ2
chr17	hsa-mir-6516	75085499	75085579	hypermethylation	1.14486	0.030396	SCARNA16; C17orf86
chr8	hsa-mir-320a	22102475	22102556	hypermethylation	1.08542	0.030987	POLR3D;MIR320A
chr1	hsa-mir-320b-2	224444706	224444843	hypomethylation	-1.00847	0.031140	
chr1	hsa-mir-5087	147806603	147806678	hypomethylation	-1.47707	0.031743	
chr17	hsa-mir-4521	8090263	8090322	hypomethylation	-1.10388	0.032254	
chr14	hsa-mir-496	101526910	101527011	hypomethylation	-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	ATF2;MIR933
chr12	hsa-mir-7107	121882076	121882155	hypermethylation	1.01076	0.033806	KDM2B
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	hypomethylation	-1.00845	0.034456	

chr6	hsa-mir-6832	31601564	31601635	hypermethylation	1.00795	0.034535	
chr15	hsa-mir-627	42491768	42491864	hypomethylation	-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	hypomethylation	-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	hypomethylation	-1.10764	0.040469	
chr17	hsa-mir-365b	29902430	29902540	hypomethylation	-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	RPS5
chr5	hsa-mir-1229	179225278	179225346	hypomethylation	-1.00370	0.041022	
chr15	hsa-mir-7706	85923827	85923893	hypomethylation	-1.10880	0.041546	AKAP13
chr11	hsa-mir-4492	118781417	118781496	hypomethylation	-1.13724	0.042569	BCL9L
chr18	hsa-mir-4741	20513312	20513401	hypomethylation	-1.13235	0.042592	RBBP8
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	hypomethylation	-1.00568	0.044221	KDM2B
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	TRIM41
chr11	hsa-mir-4687	3877292	3877371	hypomethylation	-1.06416	0.046474	STIM1
chr19	hsa-mir-1909	1816158	1816237	hypermethylation	1.01084	0.046474	
chr10	hsa-mir-1307	105154010	105154158	hypermethylation	1.01492	0.046703	
chr19	hsa-mir-769	46522190	46522307	hypermethylation	1.02118	0.046976	
chr3	hsa-mir-6828	170140891	170140950	hypomethylation	-1.00978	0.047242	
chr7	hsa-mir-339	1062569	1062662	hypermethylation	1.02186	0.047576	

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	MIR196B
chr11	hsa-mir-675	2017989	2018061	hypomethylation	-1.01792	0.048989	
chr19	hsa-mir-638	10829080	10829179	hypomethylation	-1.15170	0.049180	DNM2;MIR638
chr11	hsa-mir-34c	111384164	111384240	hypomethylation	-1.04702	0.049253	
chr2	hsa-mir-5703	228336848	228336903	hypomethylation	-1.11947	0.049776	AGFG1
chr8	hsa-mir-6850	146017316	146017376	hypomethylation	-1.25334	0.050049	RPL8
chr6	hsa-mir-6891	31323001	31323093	hypomethylation	-1.16699	0.050185	
chr2	hsa-mir-5001	233415184	233415283	hypomethylation	-1.08397	0.050224	EIF4E2;TIGD1
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	-1.01120	0.051369	
chr19	hsa-mir-519a-2	54265598	54265684	hypermethylation	1.02831	0.051921	
chr14	hsa-mir-369	101531935	101532004	hypomethylation	-1.00538	0.052194	
chr14	hsa-mir-409	101531637	101531715	hypomethylation	-1.00538	0.052194	
chr14	hsa-mir-412	101531784	101531874	hypomethylation	-1.00538	0.052194	
chr19	hsa-mir-638	10829080	10829179	hypomethylation	-1.07948	0.052329	DNM2;MIR638
chr7	hsa-mir-550a-2	32772593	32772689	hypomethylation	-1.03930	0.052335	
chr7	hsa-mir-550b-2	32772593	32772689	hypomethylation	-1.03930	0.052335	
chr14	hsa-mir-1247	102026624	102026759	hypomethylation	-1.09325	0.052954	DIO3
chr8	hsa-mir-1208	129162362	129162434	hypermethylation	1.02168	0.053031	
chr22	hsa-mir-1286	20236657	20236734	hypermethylation	1.01313	0.053109	
chr1	hsa-mir-4259	159869769	159869869	hypomethylation	-1.06762	0.053249	
chr1	hsa-mir-200b	1102484	1102578	hypermethylation	1.01675	0.053309	MIR200B;MIR200A
chr9	hsa-mir-204	73424891	73425000	hypermethylation	1.00784	0.053460	
chr16	hsa-mir-662	820183	820277	hypomethylation	-1.01052	0.053469	
chr4	hsa-mir-572	11370451	11370545	hypermethylation	1.08956	0.054065	
chr2	hsa-mir-933	176032361	176032437	hypermethylation	1.28796	0.054611	ATF2;MIR933
chr16	hsa-mir-762	30905224	30905306	hypomethylation	-1.08783	0.054681	BCL7C;MIR762

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 *DGKQ* (Figure 2).

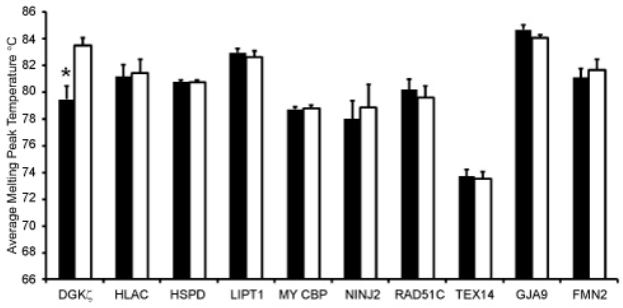

Figure 2: HRM Validation of some methylated genes in the CD4+T cells from the CFS/ME patients and controls. The bar graphs represent the average melting temperatures of the genes examined, where the black bars are results from the CFS/ME patients and the white bars are results from the controls. * denotes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

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 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 (MHCI) 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 MHCII 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 *OXA1L*. 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*, *BRWD1*, *ASXL2*, *MED13*, *NPAT*, *C20ORF3* and *PHF19*) 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.

DGKζ (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 *DGKζ* 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 DGKζ 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.

Acknowledgement

This project was funded by grant contributions from the Alison Hunter Memorial Foundation, Mason Foundation and Queensland Government Co-Investment Funding Grant Program.

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