Journal of Pain Research

Dovepress
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ORIGINAL RESEARCH

DNA Methylation Changes in Blood Cells of Fibromyalgia and Chronic Fatigue Syndrome Patients

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Purpose: Fibromyalgia (FM) and Chronic Fatigue Syndrome (CFS) affect 0.4% and 1% of society, respectively, and the prevalence of these pain syndromes is increasing. To date, no strong association between these syndromes and the genetic background of affected individuals has been shown. Therefore, it is plausible that epigenetic changes might play a role in the development of these syndromes.

Patients and Methods: Three previous studies have attempted to elaborate the involvement of genome-wide methylation changes in blood cells in the development of fibromyalgia and chronic fatigue syndrome. These studies included 22 patients with fibromyalgia and 127 patients with CFS, and the results of the studies were largely discrepant. Contradicting results of those studies may be attributed to differences in the omics data analysis approaches used in each study. We reanalyzed the data collected in these studies using an updated and coherent data-analysis framework.

Results: Overall, the methylation changes that we observed overlapped with previous results only to some extent. However, the gene set enrichment analyses based on genes annotated to methylation changes identified in each of the analyzed datasets were surprisingly coherent and uniformly associated with the physiological processes that, when affected, may result in symptoms characteristic of fibromyalgia and chronic fatigue syndrome.

Conclusion: Methylomes of the blood cells of patients with FM and CFS in three independent studies have shown methylation changes that appear to be implicated in the pathogenesis of these syndromes.

Keywords: epigenetics, chronic pain, microarray studies, cytosine methylation

Introduction

Fibromyalgia (FM) and Chronic Fatigue Syndrome (CFS) are characterized by chronic pain, fatigue, and weakness. Patients with these symptoms also suffer from sleep abnormalities and report affected cognitive processes such as memory. The diagnosis of these two syndromes is challenging and is based on questionnaires that make the diagnosis rather difficult and prone to be subjective. Currently, the American College of Rheumatology (ACR) criteria are the most widely used in the diagnosis of FM[1–4] and for CFS diagnosis the National Academy of Medicine (NAM)[5] criteria, which recently replaced 1999 Fukuda and 2003 Canadian criteria,[5,6] are the most widely adopted. Morbidity statistics show that FM affects 0.4–9.3% of people in different geographical regions[7,8] and even 1% of the worldwide population may suffer from CFS.[9] Women are three times more affected by each disease than men.[4,9] The most frequent age of onset for FM is between 50 and 60 years,[4] while CFS is most often diagnosed in two age groups: 10–19 and 30–39 years old.[5] The treatment of both syndromes is challenging and depends on patient-specific symptoms such as post-exertional malaise, orthostatic intolerance, sleep issues, cognitive dysfunction, fatigue, immune dysfunction, pain, and gastrointestinal issues.[5] As both diseases affect young and middle-aged people, and a large proportion of FM and CFS patients require continuous medical attention and are frequently unable to work, the management of FM and CFS presents a significant challenge for both healthcare systems and the labor market. To date, only weak evidence of the genetic background of FM and CFS has been reported in the literature. In the case of FM, the results of an observational study indicate a familiar aggregation of FM and odds ratio of FM in relatives of FM probands was found to

Received: 8 September 2023
Accepted: 13 November 2023
Published: 30 November 2023

be 8.5 (95% CI: 2.8–26).[10] A more recent GWAS-based study estimated the heritability of FM in different age groups; however, the estimates differed significantly between the groups.[11] Other studies on the genetic background of FM or chronic widespread musculoskeletal pain (CWP), which is a symptom of fibromyalgia, also suggested a genetic predisposition to this condition, but the results of these studies are largely discrepant.[12,13]

Similar to FM, a genetic contribution to CFS has been suggested by familial aggregation.[14–16] Here, disease-associated genetic variants *GRIK2* and *NPAS2*[17] have been reported, but these findings were not corroborated by large-scale GWASes.[18,19]

Considering rather weak evidence for a genetic predisposition to FM and CFS, it is plausible that aberrations in epigenetic mechanisms of gene expression regulation are involved in the development of these syndromes. Here, we reanalyzed available methylomics data for patients with FM and CFS from three independent studies using an updated and uniform bioinformatics data analysis framework. We found remarkable coherence of the physiological processes potentially affected by the identified methylation changes between those studies. Our results add to the body of evidence that epigenetic changes play a key role in the development of fibromyalgia and chronic fatigue syndromes.

Materials and Methods

Patients' and Samples Characteristics

The results presented in this manuscript are based on data from published studies and were thus performed under appropriate ethical approval. The original datasets were obtained from the Gene Expression Omnibus database (Table 1). Specifically, the microarray-based genome-wide methylation profiling data included methylation profiles of blood cells from 22 women diagnosed with FM,[20] profiles of T CD3+ cells from 15 male and 46 female CFS patients[21] and profiles of peripheral blood mononuclear cells (PBMC) from 66 females with CFS.[22,23] FM was diagnosed using the 2010 ACR criteria.[2,3,20] Additionally, pain intensity and the effect of pain on daily activity patients were assessed using three

Table 1 Detailed Description of the Patient Cohorts Used in the Study

	FM	CFS	CFS
Tissue type	Peripheral blood	T CD3+	PBMC
Case samples (M = male/F = female)	22 (all F)	61 (F/M: 46/15)	66 (all F)
Healthy control samples	23 (all F)	48 (F/M: 36/12)	24 (all F)
Age	>18 years old	32.2	49.4 ± 1.9
BMI	–	27.2	23.3 ± 0.5
Diagnostic criteria	ACR criteria for FM	1994 Fukuda and 2003 Canadian criteria for CFS	
Evaluation of controls with FM/CFS diagnostic criteria	Information not available	Information not available	Information not available
Pain dimensions measurement	McGill Pain Questionnaire	–	
Pain intensity measurement	11-point numerical rating scale	Rand-36 (patients and controls)	Rand-36 (patients and controls)
Pain interference with daily activity measurement	The Brief Pain Inventory		
Effects of pain on the health domains, eg, shopping, driving, walking	FM impact questionnaire		
Any pain treatment	Information not available	Information not available	Information not available

(*Continued*)

Table 1 (Continued).

	FM	CFS	CFS
Pharmacological pain treatment	Information not available	Information not available	Information not available
Medication use quantification score	Information not available	Information not available	Information not available
Sleep quality in patients	Information not available	Information not available	Information not available
Fatigue in patients	Information not available	Information not available	Information not available
Mood disturbances in patients	Information not available	Information not available	Information not available
Exclusion criteria	1. Primary psychiatric or neurological disorders 2. Contraindications to TMS	1. Tested positive for HIV or Hepatitis C 2. History of immunomodulatory or immunosuppressive medications intake	1. Tested positive for HIV, AIDS, and/or Hepatitis C 2. Were not white non-obese females (BMI < 30) with history of immunomodulatory and/or epigenetic active medication consumption
Technology	Infinium Human Methylation 450k BeadChip		
Geo accession	GSE85506	GSE156792	GSE93266 and GSE102504
Reference - PMID	28621701	30516085	28231836

questionnaires, as described in a previous study.[24] CFS was diagnosed using the 1994 Fukuda and 2003 Canadian criteria, and the RAND-36 questionnaire was used to measure pain intensity and its impact on daily activity.[21,22] The controls in the experiment were healthy counterparts of the cells used in specific methylation screening experiments and included methylation profiling data from 23 peripheral blood, 48 T CD3+, and 24 PBMC cells.

Unified Genome-Wide Methylation Analysis

The main goal of our study was to analyze the Infinium HumanMethylation 450 K BeadChip (450 K, Illumina Inc.) methylation profiling data from three independent studies with one coherent and up-to-date bioinformatics data analysis framework that would allow us to compare the results across those studies. Briefly, raw data were processed using the ChAMP package[25,26] and normalized using the BMIQ method. Subsequently, we used ComBat to correct for the batch effect in all datasets; this procedure was not used previously in the data analysis of data from reference.[20] Correction of the cell composition of individual samples has become a standard procedure for studies based on blood samples.[27,28] In two of the analyzed in our study datasets,[21,22] cell-type proportion correction was not used or was used as a covariate,[22] which has been shown to be less precise than cell fraction correction according to a previous study.[29] We adjusted all our analyses for cell fraction differences with the EpiDISH R package modified as described by Bińkowski et al[30] with reference restricted only to cell types present in individual samples. We then used linear regression (function in ChAMP) to identify differentially methylated probes (DMPs) between cases and controls. In the gene GSEA and enrichment analyses, we considered only DMPs displaying more than 0.05 absolute mean β-value difference between cases and controls, with adjusted p-value (Benjamini-Hochberg) of less than 5%. All analyses were performed using R 4.1.2.

Enrichment of DMPs in Genomic Regulatory Regions

Statistically significant enrichment or depletion of methylation changes in specific functional genomic regions is likely to suggest the function of these changes. Thus, we analyzed the distribution of DMPs identified with our data analysis frame work in regions related to genes as defined in the Infinium Human Methylation 450 Bead Chip manifest v. 1.2, including TSS1500, TSS200, 5'UTR, 1stExon, Gene body, 3'UTR, and Unknown regions as well as genomic regions related to CpG islands (CGI), including N-shelf, N-shore, Island, S-shore, S-shelf, and Opensea (both according to UCSC

coordinates). We also assessed the distribution of identified methylation changes within regions defined in the Ensembl database,[31] including Promoters, Promoter Flanking Region, TF binding site, open chromatin, CTCF binding site, Enhancer and Unknown regions. The background was set as the region in which all the processed CpGs were annotated.

Gene Set Enrichment Analysis

To approximate physiological processes potentially affected by identified methylation changes, we performed the Gene Set Enrichment Analysis (GSEA) using the "GENE2FUNC" function of Functional Mapping and Annotation of Genome-Wide Association Studies platform (FUMA GWAS).[32]

UCSC Genome Browser Analysis

The functional context of the loci that displayed identical methylation changes in all analyzed datasets was assessed using UCSC Genes,[33] Open Regulatory Annotation (ORegAnno),[34] GeneHancer,[35] and UCSC CpG Islands track[36] from the UCSC Genome Browser on Human (GRCh37/hg19) (https://genome.ucsc.edu/).

Results

FM and CFS Specific Methylation Changes

Overall, our analysis identified 1256 DMPs (hypomethylated: 940,74.84%; hypermethylated: 316, 25.16%) in peripheral blood from FM patients, 510 DMPs (108, 21.18% hypomethylated and 402, 78.82% hypermethylated) in T CD3+ cells from CFS patients, and 1751 DMPs (947 CpGs, 54.08% hypomethylated and 804, 45.92% hypermethylated) in PBMCs of CFS patients (the list of the identified DMPs with annotations are shown in Supplementary Tables 1–3). The unsupervised hierarchical clustering based on DMPs that we identified for each dataset showed a clear separation between patients and controls (Figure 1a–c), indicating that, although methylation changes observed in both syndromes were small (between 5% and 10%) (Supplementary Figure 1), all patients in each of the studies displayed these changes. The identified methylation changes overlapped only to a certain extent with previously identified methylation changes in each of the datasets we used. In patients with FM, 16.55% of the methylation changes that we identified with the new analysis framework were those previously identified and for the CFS study based on T CD3+ and CFS based on PBMC

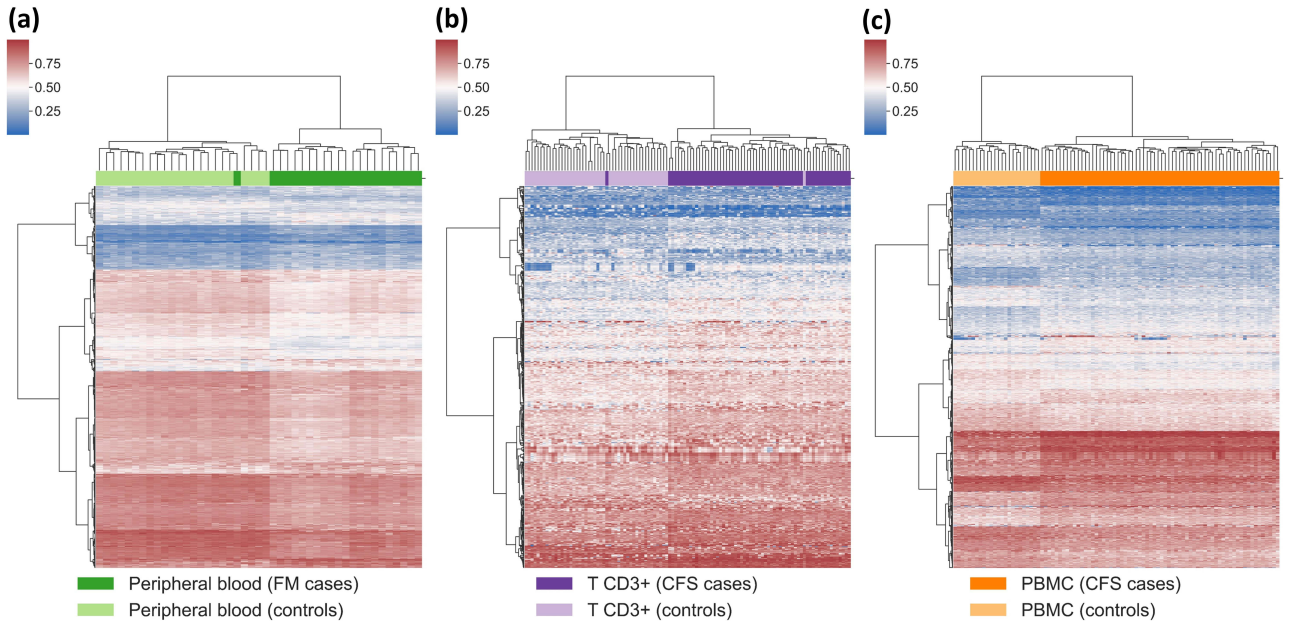

Figure 1 Heatmaps based on of beta values at identified DMPs, illustrating unsupervised hierarchical clustering of samples from each of the analyzed cohorts. Rows and columns of each heatmap indicate specific DMPs and samples, respectively. The color on heatmap indicates the level of methylation at specific CpG sites and samples (from blue: lower to red: higher). This analysis demonstrates clear separation of case (darker color) and control groups (lighter color) in all cohorts, including: (**a**) FM peripheral blood (green), (**b**) CFS T CD3+ (violet), and (**c**) CFS PBMC cohort (Orange).

cells, common changes accounted to 10.1% and only 1.01%, respectively. The magnitude of the changes was also different between each of these analyses, with the largest absolute methylation changes observed in the analysis of PBMCs from CFS patients (12.16% of identified methylation changes were higher than 10%), significantly smaller in the case of peripheral blood from FM patients (1.75% of methylation changes higher than 10%) and T CD3+ from CFS cases (7.25% of the identified changes over 10%). This may indicate that different cell types harbor distinct methylation changes. Nevertheless, regardless of the analyzed cell type, the genes annotated to the top hits in each of the analyses were those with described functions in neurological processes (Supplementary Table 4).

FM and CFS Specific Methylation Changes Affect Regions Indirectly Involved in Transcription Regulation

Next, to approximate physiological function of the identified methylation changes, we analyzed the enrichment of these changes in specific genomic regulatory regions (Figure 2).

According to the Ensembl regions, DMPs identified in each of the analyses were enriched in TF binding sites, open chromatin, and enhancers (Figure 2a, red boxes) and were depleted in promoters (Figure 2a, blue box). The direction of enrichment was not consistent between datasets for the Promoter Flanking Region, and CTCF Binding Site (Figure 2a). Similarly, analysis of UCSC gene regions showed that identified DMPs were consistently enriched in unknown regions (Figure 2b, red box) and depleted in TSS1500, TSS200, 5'UTR, and 1st Exon (Figure 2b, blue box). We also analyzed the enrichment of the identified DMPs within regions in relation to CpG Islands, and as expected, CpG islands (Figure 2c, blue box) were depleted of these changes. The N_Shelf, N_Shore, S_Shore, and S_Shelf regions have

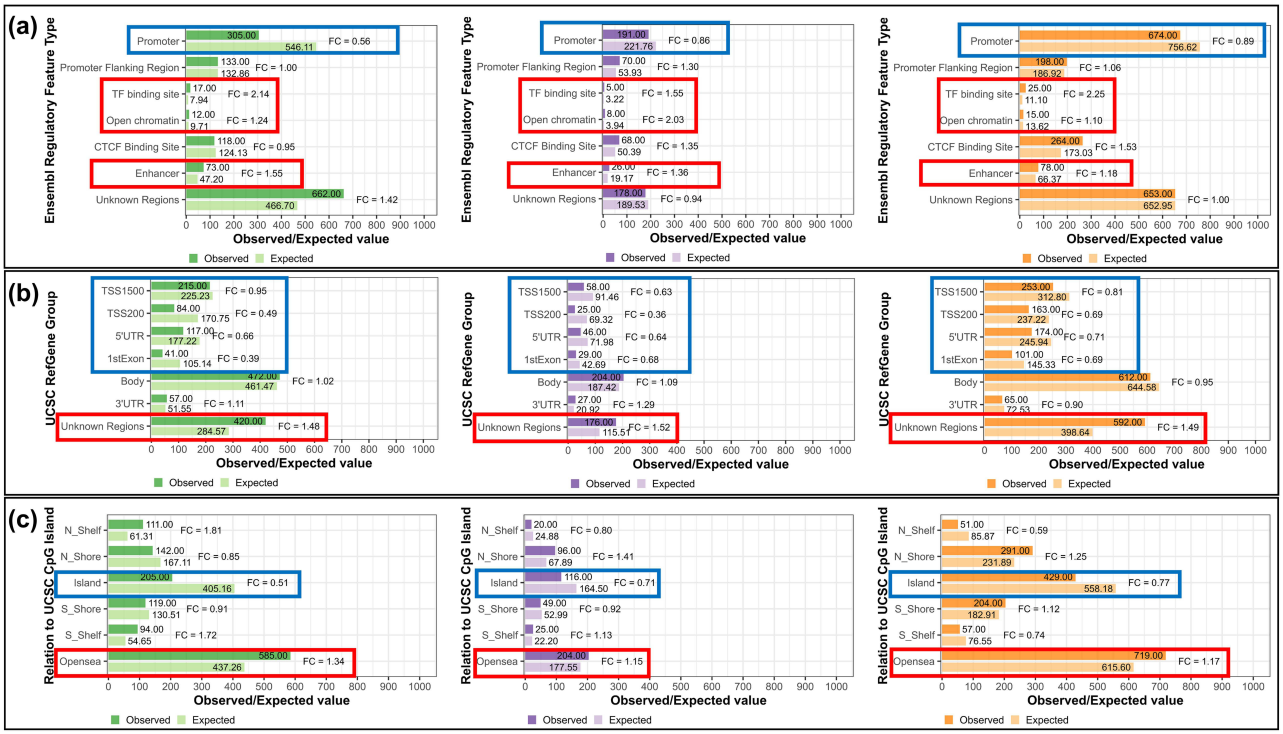

Figure 2 The results of the enrichment analysis of DMPs identified in each of the analyzed cohorts within genomic regions, including (**a**) Ensembl regulatory regions, (**b**) Gene elements (UCSC), and (**c**) UCSC CpG Island related elements. Green, violet, and Orange color indicate results for FM peripheral blood, CFS T CD3+ and CFS PBMC cohort, respectively. In each of the figures, the lighter bar illustrates expected number of CpG sites within assessed region (with informative probes obtained after data processing selected as a background in analyzed cohorts: FM peripheral blood: 411,150, CFS T CD3+: 411,537, CFS PBMC: 404,545) and the darker bar shows the observed number of DMPs that were annotated to specific regions. The red box indicates enrichment, whereas the blue box indicates depletion of DMPs within a specific region. This analysis revealed that in each of the analyzed cohorts methylation changes occur predominantly within regions not directly involved in gene expression regulation ((**a**) – TF binding site, Open chromatin and Enhancer, (**b**) – Unknown regions, (**c**) – Opensea) and that they are depleted within promoter-associated regions ((**a**) – Promoter, (**b**) – TSS1500, TSS200, 5'UTR and 1stExon, (**c**) – Island).

different directions of the enrichment throughout three datasets (Figure 2c) and we also observed enrichment of identified DMPs in OpenSea (Figure 2c, red box).

Overall, the analysis of genomic annotation of the identified methylation changes suggests uniformly across all analyzed patient groups that those changes do not affect the promoters of genes, but rather affect regions that do not directly regulate the transcription of genes.

Methylation Changes in FM and CFS Appear to Affect Similar Biological Processes

As already mentioned, the analysis of each dataset included in our study resulted in different numbers of DMPs, and only four of the identified methylation changes were common across all analyses. Interestingly, however, when we performed GSEA based on the genes to which the identified methylation changes were annotated, the physiological processes associated with these methylation changes were remarkably coherent between all analyzed datasets (Supplementary Table 5).

Analysis of the association of the identified gene set signatures with tissue-specific gene expression profiles showed that genes annotated to methylation changes identified in both CFS patient groups were expressed in several pain perception-related brain structures, including the amygdala, anterior cingulate cortex, cerebellum, and nucleus accumbens basal ganglia. Studies in which these neurological structures were previously associated with pain have involved both rodents[37] and humans.[38] The same analysis for FM did not show significant results.

In the GSEA based on the "GO molecular functions" category, the term common for gene sets from all the analyses that we performed was "GO_CALCIUM_ION_BINDING", which was the only term identified in the analysis of T CD3 + and PBMC cells from CFS patients and peripheral blood cells from FM patients. This process is essential for voltage-gated calcium channels (VGCCs), and their malfunction is widely related to pain physiology. A study of mice with neuronal overexpression of *Cacna2d1* (encoding a subunit of VGCCs) revealed that upregulation of this gene correlates with enhanced activity of VGCCs in sensory neurons and with hyperexcitability of dorsal horn neurons, both of which are crucial for pain modulation.[39] Moreover, a study of chronic constriction injury to the infraorbital nerve (CCI-ION) rat model showed that CCI-ION correlated with elevated levels of Cavα2δ1 protein in trigeminal ganglion neurons and increased excitatory synaptogenesis compared to non-injured rats.[40] VGCCs have also been implicated in pain hypersensitivity, as an injection of T-type VGCC antagonists has been shown to reverse CFA-induced allodynia in rats.[41] VGCCs have also been used as therapeutic targets for the treatment of neuropathic pain. For example, gabapentin, a drug that binds to the α2δ-1 VGCC subunit and indirectly blocks injury-evoked synaptogenesis, was shown to decrease α2δ-1 protein levels in spinal cord tissue collected from neuropathic pain model rats after spinal nerve ligation (SNL), but not in spinal cord tissue from sham rats.[42]

In the GSEA based on the "GO cellular components" category, "GO_INTRINSIC_COMPONENT_OF_PLASMA_MEMBRANE" was the ontology term common to all the datasets. This term was also the only term identified in the analysis of T CD3+ cells from patients with CFS and one of the two terms identified in the analysis of peripheral blood cells from patients with FM. Aberrations in the excitability of the intrinsic membrane in neurons play an important role in chronic pain development; for example, a recent study showed that SNL-induced neuropathic pain model mice exhibited increased intrinsic excitability of thalamic neurons in vitro, when compared to sham mice.[43]

Furthermore, the top statistically significant ontology terms in the "GO biological processes" category that were common to all three analyses of genes associated with methylation changes in both FM and CFS included terms related to cell adhesion and homophilic cell adhesion via "plasma membrane adhesion molecules", "cell adhesion via plasma membrane adhesion molecules", "cell adhesion", and "biological adhesion". Altered serum concentrations of 11 angiogenic factors, including soluble platelet endothelial cell adhesion molecule-1 (sPECAM-1), have been observed in patients with acute and subacute pain syndromes, such as postherpetic neuralgia, low back pain, and trigeminal neuralgia, compared with healthy controls.[44] sPECAM-1 is a protein closely related to neuroinflammation, as *PECAM-1* transcript was recently found to be overexpressed in the brain tissue of patients with multiple sclerosis compared to controls without neuropathological conditions.[45]

Also interestingly, we found that three of the motifs targeted by human mature RNA, including "ATATGCA_MIR448", "CGGGACCA_MIR133A_MIR133B", and "TCCAGAG_MIR518C" were associated with the methylation signatures identified in each of the analyses and were the only terms identified in analysis of T CD3+ cells. Higher expression of

microRNA448 in the spinal cord tissue of rats after CCI of the sciatic nerve has been associated with neuropathic pain development by decreasing the microglial expression of *SIRT1*, a neuroprotective target of microRNA448, and with higher spinal cord levels of inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, when compared to sham rat tissue counterparts.[46] Also, lower expression of this microRNA in PC-12 cells induced the expression of Janus kinase 1 (JAK1), which is involved in the JAK/STAT signaling pathway, regulating diverse processes, such as cell migration, cellular responses, inflammation, and angiogenesis.[47]

The plasma expression levels of microRNA133A in patients with acute chest pain were higher in patients diagnosed with acute myocardial infarction than in those diagnosed with other diagnoses of chest pain and in the plasma of healthy controls.[48] This mRNA was also overexpressed in the serum of patients undergoing coronary artery bypass grafting due to unstable angina pectoris, compared to patients undergoing aortic valve replacement due to aortic stenosis.[49]

MicroRNA133B was shown to be overexpressed in the dorsal root ganglion of rats that did not develop chronic pain after peripheral nerve injury in comparison to its tissue counterpart in sham rats.[50] A negative correlation between microRNA-133B thalamic, plasma, and cerebrospinal fluid expression levels and the severity of allodynia has also been observed in rats with central post-stroke pain (CPSP) and selective overexpression of miR-133b-3p. These findings were confirmed in a study that included patients with CPSP who exhibited decreased plasma levels of circulating miR-133b-3p when compared with control participants without pain.[51] Moreover, a study investigating the mechanisms underlying the regulation of neuronal differentiation by morphine reported a morphine-induced decrease in miR-133b expression in zebrafish model embryos and immature mammalian neurons, suggesting that morphine acts as an analgesic agent by inducing dopaminergic neuron differentiation in mammals.[52]

The last mRNA identified across all analyzed datasets, microRNA518C, was not associated with pain. However, another member of the microRNA518 family, MIR518b, has been shown to be abnormally expressed in patients with complex regional pain syndrome in terms of their response to plasma exchange treatment.[53]

Interestingly also, enrichment analysis of genes annotated to DMPs that we identified in PBMCs of CFS patients returned only processes, aberrations of which may result in the development of chronic pain syndromes (Supplementary Table 5).

Methylation Changes Common for FM and CFS Annotate to Loci Occupied by Genes Potentially Involved in Pain Regulation

Our analysis also identified four CpG sites, cg08155325 (chr5:140857813), cg22838050 (chr19:872690), cg02454025 (chr1:11042201), and cg19643109 (chr6:160697626), displaying uniform methylation level changes in all studded data sets.

cg08155325 is located in the 1st intron of various transcripts of protocadherin gamma (*PCDHG*) family genes. Protocadherins are neural adhesion proteins that play a crucial role in the establishment and function of specific cell–cell trans-interactions between neurons and between neurons and astrocytes. In addition, these proteins promote dendrite growth and arbor complexity.[54,55] For example, gamma protocadherins are involved in the patterning of axon terminals.[56] Moreover, *PCDHG* genes regulate the generation of functional neural circuits in the brain,[57] including the modulation of dendrite arborization, neuronal survival, and synaptogenesis.[58] Moreover, the expression of the *PCDHGA2* gene has been shown to be essential for the establishment of neuronal connections and signal transduction at the synaptic membrane in the neocortex, hippocampus, cerebellum, and olfactory bulb via cadherin-related neuronal receptors and binding to tyrosine kinase Fyn.[59] The expression of *PCDHGA11* in hippocampal neurons of congenitally helpless animals was significantly higher (with a 2.6-fold change) than that in non-learned helpless rats, and its overexpression could affect transduction signaling and lead to depression.[60] Whole-exome sequencing of blood DNA revealed deletion of the *PCDHGB1* gene in two patients with dystonia-plus phenotype[61] characterized by fatigue and headache symptoms also occurring in CFS.[62] Moreover, mice deficient in *PCDHG* gene cluster lacks inhibitory interneurons in the cortex and cerebellum and displays motor deficits and seizure.[63]

Interestingly, the U343 cell-line knockout for *PCDHGC3* gene displayed downregulation of the several genes from WNT signaling pathway. This is a major pathway involved in embryogenesis, and its disruption is common in cancer.[64] In addition, the cg08155325 locus is a binding site for several transcription factors (TF) described in the JASPAR Transcription Factors Database, including RXRA:VDR, SOX12, SOX14, and PHOX2B, and according to the ORegAnno database CTCF binds to

two bases close to this locus. Most of these TF do not appear to be involved in the physiology of pain, but *PHOX2B* is expressed in cranial neurons. Moreover, research on recombinant mouse embryos shows that *PHOX2B* is essential for neuronal development of the trigeminal, superior, and jugular ganglia and the trigeminal sensory nuclei and damage to these brain parts leads to pain sensation in the oro-facial region,[65] a symptom observed in FM and CFS.[66]

cg22838050 is located within the binding site of *EGR1* transcription factor and enhancers within *MED16* and *CFD* genes. CFD is a crucial regulator of inflammation, as it is involved in the activation of the innate immune response[67] and has been shown to be upregulated in dorsal root ganglia (DRG) neurons of rats with paclitaxel-induced peripheral neuropathy.[68] Increased *EGR1* expression in anterior cingulate cortex neurons was observed in response to chronic inflammatory pain, similar to that in the spinal dorsal horn, and was significantly ($p < 0.001$) reduced in Egr1-knockout mice.[69] Also, increased levels of EGR1 protein have been reported in a study of rat neurons of the insular cortex after nerve injury.[70] *EGR1* expression is significantly reduced in the blood of patients with Irritable Bowel Syndrome (IBS) after placebo treatment, and reduced levels of EGR1 protein could be a biomarker for IBS, a syndrome with gastro-intestinal symptoms similar to those observed in FM and CFS.[71]

cg02454025 is localized in the TSS200 of *C1orf127* and RARA::RXRG TF binding sites. Interestingly, missense mutations in *C1orf127* have been detected in patients with primary ciliary dyskinesia (PCD), but the precise function of this gene remains unknown.[72] *RARA* and *RXRG* were overexpressed in DRG (*RARA*) and trigeminal ganglion (*RXRG*) neurons of recombinant Nav1.8^{Cre}/Rosa26^{fsTRAP} mice in a study on ribosome-bound, sensory neuron-specific, and nociceptor-enriched RNAs, suggesting the involvement of these genes in pain development.[73]

Lastly, the cg19643109 is localized in IRF3 TF binding site and inhibition of IRF3 with dexmedetomidine in rats was associated with reduction of visceral pain,[74] and this type of pain is frequently reported by both FM cases[4] and CFS patients.[75]

Discussion

Genetic studies have shown only a weak association of the genetic background with fibromyalgia, and chronic fatigue syndrome. This suggests that epigenetic changes may play key roles in the development of these diseases. To date, only three studies have reported genome-wide methylation changes in fibromyalgia and chronic fatigue syndrome, but the results of these studies are coherent to a very limited extent. We re-analyzed datasets from those studies with a uniform data analysis framework and additional data analysis modules, such as cell fraction and batch effect corrections, which were not always used in previous analyses and are currently considered state-of-the-art in blood-based EWAS studies.

Our analysis identified different numbers of methylation changes in each dataset, the majority of which were not found in previous studies. However, GSEA based on genes annotated to those changes consistently in all datasets associated those changes with physiological processes, disturbances of which may lead to the symptoms reported by patients with fibromyalgia and chronic fatigue syndrome. The discrepancies between methylation signatures that we identified can at least partly be attributed to the type of cells used, study design, and data quality. Despite these limitations, the surprising coherence of physiological processes associated with the signatures identified in each of the analyses clearly suggests that methylation changes may play a key role in the development of syndromes characterized by chronic pain.

We were unable to access data from five other studies on genome-wide methylation changes in FM,[76] CFS,[77–79] and chronic widespread pain.[80] However, we were able to access the list of genes annotated to methylation changes from these studies. Although none of these studies were based on a unified data analysis pipeline that we implemented, there were common genes between these analyses and our study, despite the fact that each of those analyses found different numbers of genes (75 genes in T CD4+ cells in study,[77] 6835 genes in PBMCs in study,[78] and 829 genes in PBMCs in study[79]). In addition, closer analysis of the data from these studies showed that the poor overlap of our results with data from two of those studies was likely attributed to the low magnitude of the methylation changes identified in those studies, which was below 5%[80] and in our analyses, we only considered methylation changes higher than 5% as meaningful in the context of the limitations of bead array technology. Also in one of the previous studies, DMPs were identified using M-values (a derivative of the beta values used in our data analysis), and we were not able to directly compare our results with the results reported in that study.[76]

Additionally, we identified four studies that analyzed methylation changes in FM and CFS using the NGS platform, of which two used the RRBS protocol[81,82] and two others used targeted bisulfite sequencing.[83,84] Despite the different

technologies used and different hypotheses tested, there were common genes between our study and RRBS-based studies. Our results did not include any genes identified by targeted bisulfite NGS sequencing. However, these studies reported smaller methylation differences than those we aimed to identify in our data analysis.

There are also a number of studies that analyzed methylation changes at single genes in FM and CFS.[85–92] However, in most of these studies, pyrosequencing was used for methylation screening and identified gene-specific methylation changes were smaller than 5%; thus, they could not have been identified in our analysis. Nevertheless, we identified differentially methylated probes within the *BDNF* gene that were reported to undergo hypermethylation in patients with CFS and comorbid FM in one of the gene-centric methylation studies.[92]

Conclusion

Overall, our results, especially when analyzed in the context of results reported in similar studies that utilized different data analysis and experimental approaches, clearly indicate that symptoms of chronic pain may be caused by the methylation changes, but further and better controlled studies are required to elaborate the role of DNA methylation in the development of chronic pain syndromes.

Data Sharing Statement

All analyzed datasets are publicly available in the Gene Expression Omnibus database under accession numbers GSE85506, GSE156792, GSE93266, and GSE102504.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This study was funded by the Polish Return Grant Program of the Polish National Agency for Academic Exchange (grant ID: PPN/PPO/2018/1/00088).

Disclosure

The authors report no conflicts of interest in this work.

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