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Short report

DNA methylation changes in CD4$^+$ T cells isolated from multiple sclerosis patients on dimethyl fumarate

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Vicki E Maltby, Rodney A Lea, Karen A Ribbons, Katherine A Sanders, Daniel Kennedy, Myintzu Min, Rodney J Scott and Jeannette Lechner-Scott

Abstract

Background: Dimethyl fumarate is an oral treatment for multiple sclerosis, whose mechanism of action is not fully understood.

Objective: To investigate the effects of dimethyl fumarate on DNA methylation in the CD4$^+$ T cells of multiple sclerosis patients.

Methods: We performed Illumina EPIC arrays to investigate the DNA methylation profiles of CD4$^+$ T cells derived from multiple sclerosis patients before and after dimethyl fumarate treatment.

Results: Treatment with dimethyl fumarate resulted in 97% of differentially methylated positions showing hypermethylation. Four genes, *SNORD1A*, *SHTN1*, *MZB1* and *TNF* had a differentially methylated region located within the transcriptional start site.

Conclusion: This study investigates the effect of dimethyl fumarate on DNA methylation in multiple sclerosis patients.

Keywords: Multiple sclerosis, dimethyl fumarate, immunology, DNA methylation, relapsing–remitting, CD4$^+$ T cells, tumour necrosis factor

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Introduction

Although increasing numbers of treatments are available for multiple sclerosis (MS), the exact mechanism of action of these therapies is often unclear. Patients are frequently required to trial several treatments to identify which is most suitable for their disease activity. Dimethyl fumarate (DMF; Tecfidera, Biogen Idec, Cambridge MA, USA) is approved in Europe and Australia as a first-line oral drug for the treatment of relapsing–remitting multiple sclerosis, and its use is associated with a reduction in disease activity and a variable effect on progression.[1,2]

Although the exact mode of action is not fully elucidated, DMF has been shown to have both anti-inflammatory and anti-oxidative properties. Decreased absolute lymphocyte counts and a shift in T lymphocyte polarisation from T helper (Th)1 and Th17 (pro-inflammatory) to Th2 phenotype (anti-inflammatory) has been reported after DMF treatment in MS patients.[3] DMF also promotes translocation of nuclear factor erythroid 2-related factor 2 into the nucleus, which upregulates the transcription of anti-oxidative enzymes.[3]

DNA methylation refers to the epigenetic modification whereby the addition/removal of methyl groups to CpG dinucleotides regulates gene transcription. We, and others, have assessed global methylation profiles in CD4$^+$ and CD8$^+$ T cells from MS patients compared to healthy controls.[4–6] Our studies have demonstrated altered methylation profiles in the CD4$^+$ T cells of treatment-naive patients or in the absence of treatment. However, the effect of disease-modifying therapies (DMTs) on methylation

Correspondence to:
Jeannette Lechner-Scott, Department of Neurology, John Hunter Hospital, Australia.
jeannette.lechner-scott@hnehealth.sw.gov.au

Vicki E Maltby, School of Medicine and Public Health, University of Newcastle, Australia
Brain and Mental Health, Hunter Medical Research Institute, Australia

Rodney A Lea, Brain and Mental Health, Hunter Medical Research Institute, Australia
Institute of Health and Biomedical Innovation, Queensland University of Technology, Australia

Karen A Ribbons, Brain and Mental Health, Hunter Medical Research

Institute, Australia
Department of Neurology, John Hunter Hospital, Australia

Katherine A Sanders, Faculty of Health Sciences and Medicine, Bond University, Australia

Daniel Kennedy, ARC Centre of Excellence for the Mathematical and Statistical Frontiers, Queensland University of Technology, Brisbane, Australia

Myintzu Min, Brain and Mental Health, Hunter Medical Research Institute, Australia
Department of Neurology, John Hunter Hospital, Australia

Rodney J Scott, Brain and Mental Health, Hunter Medical Research Institute, Australia
School of Biomedical Sciences and Pharmacy, University of Newcastle, Australia
Department of Medical Genetics, John Hunter Hospital, Australia

Jeannette Lechner-Scott, School of Medicine and Public Health, University of Newcastle, Australia
Brain and Mental Health, Hunter Medical Research Institute, Australia
Department of Neurology, John Hunter Hospital, Australia

profiles remains unclear. Neither group found significant changes in $CD8^+$ T cells.[5,6]

Here we performed a longitudinal study of the genome-wide methylation profiles of $CD4^+$ T cells in MS patients before and after DMF treatment.

Methods

We recruited seven MS patients (three men and four women) who were either treatment naive or had been off DMT for at least 3 months and were planning to start DMF therapy (Table 1). The majority of patients had not had steroid use for at least 2 months prior to entry into this study (Table 1). Samples were collected and processed as previously described.[7] Blood was collected prior to the first dose of DMF and 6 months following treatment initation. At 6 months, all patients remained on therapy and had no change in Expanded Disability Status Score (EDSS). Two patients had evidence of disease activity as assessed by the appearance of new lesions on magnetic resonance imaging (MRI). However, both of these patients showed no new disease activity at their next MRI and remain on therapy.

$CD4^+$ T cells were extracted using magnetic isolation kits (Stem Cell Technologies, Canada) and purity (mimimum threshold $\geq$90%) was assessed using the FACS CantoII (BD Biosciences) system. Purified DNA was bisulphite converted and hybridised to Illumina EPIC arrays. Raw fluorescence data were processed using a combination of R/Bioconductor and custom scripts. Differences in mean methylation before and after the 6-month treatment period were tested using a paired samples *t*-test for each CpG. A CpG was considered a differentially methylated position (DMP) if the *P* value was less than 0.0005 and the absolute difference in mean methylation between groups was greater than 5%. A differentially methylated region (DMR) was defined as two or more contiguous DMPs located within 500 bp of each other, whose methylation changes were in the same direction. If a DMP was located outside of the 500 bp region but was less than 500 bp from the last DMP it was also included in the DMR.

Results and discussion

In total, 945 DMPs were identified when comparing the 6-month time point to baseline, the majority of which were hypermethylated after treatment (912; 97%) (see Supplementary Table 1). The most altered DMP between baseline and treatment was 17.5% hypermethylated (cg14048158); however, this site maps to an area with no known gene association. To identify sites of potential functional consequence, we filtered DMPs to include only those with a DMR, gene name and position annotation. Table 2 shows the DMPs with the largest percentage change for each of the resulting 64 genes.

Four genes had at least two adjacent DMPs located in the transcriptional start site (TSS) (Table 3). *SNORD1A* (small nucleolar RNA, C/D box 1A) encodes for an uncharacterised small nucleolar RNA. *SHTN1* encodes shootin1, a protein involved in neuronal polarisation of axons.[8] *MZB1* (marginal zone B and B1 cell-specific protein) codes for an endoplasmic reticulum calcium regulator. While it has not previously been linked to MS, a study by Belkaya et al. (2013) found that overexpression of miR-185 resulted in a nearly five-fold decrease of *MZB1* in mice.[9] This decrease corresponded with lymphopenia and a reduced proliferative response in $CD4^+$ T

Table 1. MS cohort demographic/clinical features at baseline (prior to DMF treatment).

Sex	Age (years)	Prior DMT	Prior steroids (days prior to collection)	EDSS at baseline	EDSS at 6 months
M	23	Naive	312	1	1
M	40	Naive	7	3	3
M	35	Naive	54	1.5	1.5
F	32	Naive	3400	2.5	2.5
F	42	(Fingolimod)	1224	3	3
F	31	(Glatiramer acetate)	72	1.5	1.5
F	43	(Peginterferon beta-1a, interferon beta-1a)	95	2	2

MS: multiple sclerosis; DMF: dimethyl fumarate; DMT: disease-modifying therapy; EDSS: Expanded Disability Scale Status.

Table 2. DMRs with gene name and annotation.

Chr.	CpG ID	Position	Gene name	Element	Mean methylation		% Change	T stat.	*P* value
					Baseline	6 Months			
1	cg16144718	23115066	*EPHB2*	Body	0.50	0.62	11.47	7.06	4.05×10^{-4}
1	cg06808725	32264502	*SPOCD1*	Body	0.44	0.56	11.74	6.64	5.64×10^{-4}
1	cg24533227	42145514	*HIVEP3*	5′UTR	0.65	0.76	10.63	6.71	5.32×10^{-4}
1	cg02410801	55046065	*ACOT11*	Body	0.56	0.66	9.02	8.87	1.15×10^{-4}
1	cg25130912	201982886	*ELF3*	Body	0.66	0.76	9.67	7.11	3.89×10^{-4}
2	cg05333614	1168186	*SNTG2*	Body	0.72	0.77	5.36	7.77	2.40×10^{-4}
2	cg03771015	15831147	*LOC101926966*	Body	0.62	0.70	7.78	6.35	7.16×10^{-4}
2	cg14501323	31279457	*GALNT14*	Body	0.81	0.87	6.37	6.96	4.38×10^{-4}
2	cg10796691	65135899	*LOC400958*	Body	0.60	0.69	8.36	9.16	9.53×10^{-5}
2	cg16603943	121614683	*GLI2*	Body	0.51	0.63	11.66	6.35	7.13×10^{-4}
2	cg20772458	158983130	*UPP2*	Body	0.74	0.80	5.89	7.26	3.46×10^{-4}
2	cg18707238	218688237	*TNS1*	Body	0.73	0.78	5.52	6.47	6.49×10^{-4}
3	cg15756415	14932169	*FGD5*	Body	0.42	0.54	12.05	6.48	6.40×10^{-4}
3	cg02790932	23373256	*UBE2E2*	Body	0.65	0.72	7.55	8.65	1.31×10^{-4}
3	cg00049674	123058535	*ADCY5*	Body	0.61	0.68	7.51	7.01	4.20×10^{-4}
5	cg27073488	14262157	*TRIO*	Body	0.70	0.75	5.39	8.81	1.19×10^{-4}
5	cg16375820	55289001	*IL6ST*	5′UTR	0.31	0.23	–7.30	–9.25	9.03×10^{-5}
5	cg27346756	90431802	*ADGRV1*	Body	0.58	0.64	6.52	7.38	3.18×10^{-4}
5	cg16558774	132579360	*FSTL4*	Body	0.68	0.75	6.66	9.33	8.58×10^{-5}
5	cg11988321	138725622	*MZB1*	TSS200	0.42	0.54	12.21	16.30	3.39×10^{-6}
6	cg04095776	31106941	*PSORS1C1*	Body	0.66	0.72	6.25	7.34	3.28×10^{-4}
6	cg19978379	31542671	*TNF*	TSS1500	0.54	0.67	13.00	7.09	3.95×10^{-4}
6	cg15496866	40491590	*LRFN2*	5′UTR	0.61	0.72	11.04	7.47	2.97×10^{-4}
6	cg01473948	148823785	*SASH1*	Body	0.59	0.66	7.47	6.91	4.54×10^{-4}
7	cg13800949	47343103	*TNS3*	Body	0.79	0.85	5.90	8.14	1.85×10^{-4}
7	cg14797899	69882555	*AUTS2*	Body	0.68	0.78	9.85	7.70	2.51×10^{-4}
7	cg02170577	104939331	*SRPK2*	Body	0.72	0.77	5.03	6.94	4.44×10^{-4}
7	cg05476934	133859100	*LRGUK*	Body	0.52	0.60	8.64	6.74	5.18×10^{-4}
7	cg09891341	138619424	*KIAA1549*	Body	0.78	0.84	6.24	6.81	4.90×10^{-4}
7	cg06679384	158049077	*PTPRN2*	Body	0.60	0.66	6.23	7.57	2.76×10^{-4}
9	cg08290373	8633541	*PTPRD*	Body	0.68	0.78	10.02	6.66	5.52×10^{-4}
9	cg17557530	90193634	*DAPK1*	Body	0.61	0.73	12.22	6.62	5.74×10^{-4}
9	cg06749278	97662692	*C9orf3*	Body	0.75	0.83	7.50	6.91	4.55×10^{-4}
10	cg16203213	45398814	*TMEM72-AS1*	Body	0.66	0.74	7.24	9.01	1.05×10^{-4}
10	cg26754789	49857879	*ARHGAP22*	Body	0.74	0.79	5.36	9.19	9.34×10^{-5}
10	cg13312268	50019744	*WDFY4*	ExonBnd	0.72	0.78	6.84	6.68	5.44×10^{-4}
10	cg12552633	71573337	*COL13A1*	Body	0.45	0.55	10.40	6.72	5.29×10^{-4}
10	cg24587741	79313774	*KCNMA1*	Body	0.68	0.75	7.07	6.89	4.62×10^{-4}
10	cg17753789	81026766	*ZMIZ1*	Body	0.69	0.76	7.74	6.52	6.21×10^{-4}
10	cg16035098	118886914	*SHTN1*	TSS1500	0.46	0.55	9.16	9.66	7.04×10^{-5}
10	cg01613414	126693304	*CTBP2*	Body	0.52	0.62	10.31	7.40	3.13×10^{-4}
11	cg09731767	503628	*RNH1*	5′UTR	0.53	0.61	7.79	9.85	6.32×10^{-5}
11	cg11922498	4936427	*OR51G2*	1stExon	0.65	0.71	6.09	9.45	7.99×10^{-5}
11	cg00842359	10686144	*MRVI1*	5′UTR	0.67	0.77	9.37	9.28	8.85×10^{-5}
11	cg14595291	35993855	*LDLRAD3*	5′UTR	0.50	0.65	14.89	6.42	6.76×10^{-4}
11	cg00964019	117593395	*DSCAML1*	Body	0.76	0.82	5.81	7.08	3.97×10^{-4}
12	cg11439695	2561024	*CACNA1C*	Body	0.46	0.56	9.93	8.25	1.71×10^{-4}
12	cg17451712	122293122	*HPD*	Body	0.68	0.75	7.13	6.57	5.95×10^{-4}

(continued)

Table 2. Continued

Chr.	CpG ID	Position	Gene name	Element	Mean methylation		% Change	T stat.	*P* value
					Baseline	6 Months			
14	cg03725784	61992305	*PRKCH*	Body	0.41	0.54	12.42	7.14	3.81×10^{-4}
14	cg11198334	75040680	*LTBP2*	Body	0.63	0.74	10.81	6.90	4.56×10^{-4}
14	cg07399096	91050031	*TTC7B*	Body	0.69	0.75	6.38	6.58	5.91×10^{-4}
14	cg15325186	102562217	*HSP90AA1*	Body	0.51	0.60	8.52	7.79	2.36×10^{-4}
15	cg25814224	51572976	*CYP19A1*	5′UTR	0.65	0.71	6.39	10.93	3.49×10^{-5}
16	cg02260059	78262124	*WWOX*	Body	0.72	0.78	5.78	6.62	5.74×10^{-4}
17	cg04456720	54250143	*ANKFN1*	Body	0.67	0.77	10.60	8.93	1.10×10^{-4}
17	cg19439071	74557625	*SNORD1A*	TSS200	0.56	0.67	10.87	8.68	1.29×10^{-4}
17	cg11476241	78866235	*RPTOR*	Body	0.56	0.66	10.10	6.45	6.58×10^{-4}
18	cg13297582	13288627	*LDLRAD4*	5′UTR	0.51	0.62	10.73	7.79	2.36×10^{-4}
18	cg03385871	46311648	*CTIF*	Body	0.45	0.56	11.75	6.56	6.00×10^{-4}
19	cg07345937	1175444	*SBNO2*	TSS1500	0.53	0.63	9.74	10.58	4.19×10^{-5}
20	cg10453816	37499530	*PPP1R16B*	Body	0.51	0.64	13.41	6.85	4.74×10^{-4}
20	cg04991444	50057438	*NFATC2*	Body	0.41	0.53	12.25	9.96	5.94×10^{-5}
21	cg10919441	44143035	*PDE9A*	5′UTR	0.67	0.73	6.59	7.18	3.68×10^{-4}

DMT: disease-modifying therapy; Chr.: chromosome.

Table 3. Genes with DMRs in the transcriptional start site.

Chr.	CpG ID	Position	Gene name	Element	Mean methylation		% Change	T stat.	*P* value
					Baseline	6 months			
5	cg11988321	138725622	*MZB1*	TSS200	0.421426	0.543513	12.20872	16.29927	3.39×10^{-6}
5	cg04359635	138725975	*MZB1*	TSS1500	0.513568	0.633291	11.97221	6.619687	5.72×10^{-4}
6	cg19978379	31542671	*TNF*	TSS1500	0.537903	0.667883	12.99794	7.088953	3.95×10^{-4}
6	cg24452282	31542740	*TNF*	TSS1500	0.470376	0.584539	11.41626	6.396426	6.88×10^{-4}
10	cg16035098	118886914	*SHTN1*	TSS1500	0.458233	0.549798	9.156483	9.664102	7.04×10^{-5}
10	cg23251794	118886883	*SHTN1*	TSS1500	0.641729	0.707866	6.613676	9.553839	7.51×10^{-5}
17	cg19439071	74557625	*SNORD1A*	TSS200	0.562719	0.671405	10.86864	8.67833	1.29×10^{-4}
17	cg07180212	74557703	*SNORD1A*	TSS200	0.62678	0.726408	9.962796	6.422776	6.73×10^{-4}
17	cg13664588	74557494	*SNORD1A*	TSS1500	0.527718	0.624507	9.678917	6.578915	5.92×10^{-4}

DMR: differentially methylated region; Chr.: chromosome.

cells.[9] The observed increase in DNA methylation identified in the *MZB1* TSS in our dataset may result in a similar decrease in *MZB1* transcription. A resulting decrease in $CD4^+$ T cells would be consistent with the known anti-inflammatory action of DMF.

The fourth DMR identified is located at the TSS of tumour necrosis factor (*TNF*). *TNF* is a pro-inflammatory cytokine that is produced by many cell types, including lymphocytes (reviewed in Wajant *et al*).[10] TNF binding to its receptor activates the nuclear factor kappa B (NF-κB) pathway, which activates the transcription of genes involved in cell survival and proliferation, inflammatory response and anti-apoptotic factors. Hypermethylation at the *TNF* TSS may result in decreased TNF production, and a decrease in activation of the NF-κB pathway. One known mechanism of action for DMF is preventing translocation of NF-κB to the nucleus, resulting in a decrease of pro-inflammatory cytokines and an increase of anti-inflammatory cytokines (reviewed in Pistono et al.).[3] It is possible that altered DNA methylation profiles at the *TNF* TSS may contribute to this mechanism.

DMF has previously been linked to other epigenetic mechanisms in a study by Kalinin et al. (2013), in which they reported that DMF increased expression of histone deacetylases in cultured rat astrocytes.[11] Both DNA methylation and histone deacetylation are associated with gene repression.[12] Taken together there is now evidence that DMF may act as an epigenetic modifier with the function of shutting down transcription associated with pro-inflammatory activity.

One limitation of this study is that we only assessed patients who started DMF treatment. Also, athough the majority of patients were stable at the time of baseline collection, two patients had recently had a relapse, only one of whom was treated with steroids. We are therefore unable to determine for certain if the changes in methylation profiles are due to treatment effects or stabilisation of disease. Future studies comparing changes following different therapies and different disease severities are required. A further limitation is the small sample size and lack of transcriptional data. Future studies characterising treatment responses in larger populations that also investigate the functional changes at the transcriptional level are warranted.

This is the first longitudinal study to investigate the effect of DMF on the DNA methylation of $CD4^+$ T cells of MS patients. Of the most interest, the DMRs identified at *TNF* and *MZB1* provide a potential novel mechanism of action for DMF. Treatment with DMF resulted in overall hypermethylation suggesting that DMF may act to promote DNA methylation. Larger studies are warranted to elucidate further the functional link between DMF and epigenetic mechanisms.

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Author contribution

VEM, KAS, RAL, JLS, RJS and KAR initiated and designed the original study. VEM and KAS performed all laboratory experiments. VEM wrote the final manuscript and revised all versions of the manuscript. RAL and DK performed the statistical analysis. RAL, KAS, JLS, KAR, MM and RJS helped interpret the data and critically reviewed the manuscript.

Availability of data and material

The datasets generated or analysed during the current study are included in this published article (Supplementary Table 1). Raw data files are available from Rodney A Lea.

Conflict on Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: JLS's institution receives non-directed funding as well as honoraria for presentations and membership on advisory boards from Sanofi Aventis, Biogen Idec, Bayer Health Care, Merck Serono, Teva and Norvatis Australia.

Ethics approval and consent to participate

The Hunter New England health research ethics committee and University of Newcastle ethics committee approved this study (05/04/13.09 and H-505-0607, respectively), and methods were carried out in accordance with institutional guidelines on human subject experiments. Written and informed consent was obtained from all patient and control subjects.

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ORCID iD

Vicki E Maltby http://orcid.org/0000-0002-3785-4742

Supplemental Material

Supplementary material is available for this article online.

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