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CpG Island Methylation Patterns in Relapsing-Remitting Multiple Sclerosis

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Abstract

DNA methylation may predispose to multiple sclerosis (MS), as aberrant methylation in the promoter regions across the genome seems to underlie several processes of MS. We have currently determined the methylation status of eight genes in relapsing-remitting MS patients. Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) was used to determine the status of 31 CpG islands, located across eight genes, in 33 healthy individuals and 66 MS patients (33 in relapse and 33 in remission). The methylation levels in the examined sites ranged from 0 to 31%. Methylation positivity for RUNX3 and CDKN2A differed significantly between MS patients and healthy controls. Maximum methylation in RUNX3, CDKN2A, SOCS1, and NEUROG1 genes was significantly different between patients and controls. Roc curves demonstrated that the appropriate cut-offs to distinguish patients from healthy controls were 2% for RUNX3 (OR 3.316, CI 1.207–9.107, $p = 0.024$) and 3% for CDKN2A (OR 3.077, CI 1.281–7.39, $p = 0.018$). No difference in methylation was observed between patients in relapse and patients in remission, in any of the genes examined. Methylation patterns of RUNX3 and CDKN2A may be able to distinguish between MS patients and healthy controls, but not between MS patients in relapse and in remission.

Keywords Epigenetics · DNA methylation · Multiple sclerosis · RUNX3 · CDKN2A · CpG islands

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Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), characterized by axonal demyelination and degeneration. The etiology of the disease remains obscure, and several genetic (Dardiotis et al. 2017b) and environmental factors (Dardiotis et al. 2017a; Mentis et al. 2017b) seem to be implicated. Epigenetics may represent the bridge between genotypes, environmental exposures, and phenotypes (Ramagopalan et al. 2010; Sokratous et al. 2016).

Epigenetic modifications are reversible alterations in gene expression that are dependent on biological and environmental factors (Renaudineau and Youinou 2011). The best described epigenetic mechanism is DNA methylation, which has been extensively studied in cancer (Gonzalo 2010). It is essential for genome integrity, proliferation, and normal cell development (Weber and Schubeler 2007). The methylation process usually takes place at CpG islands in gene promoters and is mediated by enzymes called DNA methyltransferases. Hypermethylation of the promoter CpG islands leads to

transcriptional silencing of a gene, while hypomethylation results in increased expression of the gene (Klose and Bird 2006).

A great effort has been made over the last decade to define the genetic background, which predisposes to MS. Until now, collaborative studies have reported several HLA and 110 non-HLA genetic risk factors of MS (Hilven and Goris 2015). However, these variants can account for only 27% of MS heritability (Lill 2014) and have failed to explain the low MS concordance rate between monozygotic twins (Hansen et al. 2005). This could only be explained by a prevalent role of epigenetics in MS (Sawcer et al. 2011).

Different methylation patterns, in different loci, have been observed in MS. The genetic loci studied until today are the ones regulating the function of oligodendrocytes, MHC class II alleles, and CD4+/CD8+ T cells (Bos et al. 2015; Huynh et al. 2014; Sawcer et al. 2011; Shen and Casaccia-Bonnefil 2008), suggesting that methylation could be used as a potential biomarker for the disease (Sawcer et al. 2014). However, in MS, each gene follows its own methylation pattern; thus, it is difficult to conclude that a particular gene methylation pattern results in disease alone, rather than in combination with other factors (Mastronardi et al. 2007).

In the present study, we investigated the methylation pattern of eight genes: RUNX3, MLH1, NEUROG1, IGF2, SOCS1, CDKN2A, CACNA1G, and CRABP1 in MS patients in relapse or remission and in healthy individuals. These genes were selected because there is evidence that they participate in oligodendrocyte differentiation, CD4/CD8 T cell differentiation, and neuroinflammation (de Pagter-Holthuizen et al. 1988; Flagiello et al. 1997). Furthermore, most of them have been found to be overexpressed in active and chronic MS lesions (de Pagter-Holthuizen et al. 1988; Krishnamurthy et al. 2004; Lu et al. 2000).

Patients and Methods

Patients

A total of 66 MS patients (66.6% female), admitted to the University Hospital of Larissa, and 33 healthy individuals were recruited for the study. MS patients were either on relapse ($n = 33$) or during remission ($n = 33$). MS patients were either receiving immunomodulatory treatment ($n = 43$) or were drug-naïve patients ($n = 23$). Blood collection in the subgroup of MS patients on relapse was performed before corticosteroid treatment. Demographic and clinical characteristics, such as gender, current age, age of disease onset, disease duration and disability measures, using Expanded Disability Status Scale (EDSS) score were collected. Unrelated healthy controls, matched for age and sex, were recruited mainly from patients' spouses, from adult visitors to the hospital and hospital employees. Eligibility criterion for the control subjects was the negative medical history for any previous neurological and autoimmune disease. The study was approved by the University of Thessaly Ethics Committee and informed consent was provided by all participants.

DNA Isolation, Methylation-Specific Multiple Ligation Probe Amplification PCR, and MLPA Data Analysis

Genomic DNA was extracted from participants' peripheral whole blood, using a "salting out" method, as previously described (Dardiotis et al. 2015; Dardiotis et al. 2016).

CpG island methylation in the selected genes was studied using the methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) method, which allows the simultaneous detection both of the copy number and methylation status of up to 50 DNA sequences in a single reaction. Specific MS-MLPA probe-mixes (SALSA MLPA Probemix ME042-C1 CIMP and SALSA Binnig DNA SD029-SO2) were used according to the manufacturer instructions (MRC-Holland, Amsterdam, Netherlands). Copy number and methylation PCR fragments were separated by capillary electrophoresis using an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA) and were visualized by the GeneMapper software v5.0.

The Coffalyser.NET™Software, beta version, (MRC-Holland, Amsterdam, the Netherlands) with default settings was used to evaluate raw data from fragment and comparative analysis and to estimate the methylation of each probe. All quality control parameters were within satisfactory range and intra-sample normalization was accomplished. During intra-sample normalization, each probe signal was divided by the signal of every reference probe provided and the median of the ratios was the normalization constant of each probe. The DNA methylation for each probe (ratio of peak height of digested vs undigested) was calculated by diving the normalization constant of the digested sample by the respective normalization constant of its undigested counterpart. The outcome was expressed as a percentage.

Statistical Analysis

We evaluated differences in methylation positivity and in maximum methylation levels for each gene between MS patients and healthy individuals and between patients in relapse and in remission. Methylation positivity was considered when methylation levels were $\geq 1\%$ in at least one of the tested probes-CpG islands for each gene. Maximum methylation levels were defined as the highest methylation percentage detected in any of the tested probes-CpG islands for each gene.

Differences in methylation positivity were evaluated using the chi-square test. Maximum methylation levels were assessed for normality using graphs with Kolmogorov-Smirnov. This analysis showed that maximum methylation levels were not normally distributed. Therefore, differences in the maximum

methylation levels between MS patients and healthy individuals or between MS patients in relapse and remission (two groups) were evaluated with the Mann-Whitney *U* test. In addition, differences in the maximum methylation levels between MS patients in remission, in relapse, and healthy controls (three groups) were evaluated with the Kruskal-Wallis test. Positive results from this analysis were subjected to post hoc analysis using Mann-Whitney for every group combination (control vs relapse, control vs remission, relapse vs remission), in order to specify which groups differed statistically. ROC analysis was performed to identify the methylation level cut-offs, where the higher sensitivity and specificity for MS risk were observed. The significance of the identified cut-offs was evaluated with cross tabulation analysis. For statistical analysis, the IBM SPSS v21 statistical software was used.

Results

Demographic and clinical characteristics of the participating individuals are shown in Table 1.

Mean and maximum methylation levels of each group, per probe, are shown in Table 2.

The methylation levels varied from 0 to 31% and in particular, from 0 to 12% in healthy individuals and from 0 to 31% in patients. The maximum value for healthy controls was observed in probe 3 of the CRABP1 gene and for patients in probe 2 of the SOCS1 gene. The number of positive, for methylation, probes (methylation > 1%) varied from 10 (out of 31) in a healthy individual to 27 in a patient in relapse. Two sites in the SOCS1 gene (probes 1 and 4) and one site in the MLH1 gene (probe 2) were not at all methylated, neither in healthy individuals nor in patients.

Methylation positivity differed significantly between MS patients and healthy individuals for the RUNX3 and CDKN2A genes (Pearson chi-square 5.73, $p = 0.017$ and 6.55, $p = 0.032$, respectively). No significant difference in the methylation positivity and levels was observed between MS patients in relapse and in remission.

Regarding maximum methylation levels, statistical analysis of the means revealed that the RUNX3 was significantly more methylated in MS patients compared to controls (2.8 vs 0.8%, $p = 0.013$). Similarly, the CDKN2A and SOCS1 maximum methylation was significantly different between patients and controls (4.1 vs 1.4%, $p = 0.003$ and 6.2 vs 4.4%, $p = 0.023$, respectively). The RUNX3 and CDKN2A maximum methylation levels were also different between healthy controls, MS patients in remission, and MS patients in relapse, when the Kruskal-Wallis test was performed ($p = 0.033$ and $p = 0.011$, respectively). However, according to post hoc analysis, this difference arose from the difference between controls and MS patients in relapse ($p = 0.011$ and $p = 0.03$). We also detect a marginally significant difference in maximum methylation levels of NEUROG1 (Kruskal-Wallis test, $p = 0.044$). This difference arose from the difference between controls and MS patients in remission (Kruskal-Wallis test, $p = 0.017$). ROC curve analysis revealed that the maximum methylation level of RUNX3 could distinguish between healthy individuals and MS groups ($p = 0.036$, area under the curve = 63%), with appropriate positivity cut-off point at 2%. Using this cut-off point, a statistically significant difference between healthy individuals and MS patients was observed (OR 3.316, CI 1.207–9.107, $p = 0.024$). Moreover, the maximum methylation level of CDKN2A could also distinguish between healthy individuals and MS groups ($p = 0.005$, area under the curve = 67.5%) with appropriate cut-off point at 3%. With this cut-off point, there was a statistically significant difference between healthy individuals and MS patients (OR 3.077, CI 1.281–7.39, $p = 0.018$).

For the rest of the genes studied (MLH1, NEUROG1, IGF2, GRABP1, SOCS1, CACNA1G), no significant differences in maximum methylation levels were observed between MS patients in relapse and in remission. Results from the Mann-Whitney *U* test, Kruskal-Wallis test, and post hoc, ROC, and crosstabs analyses are presented in Table 3.

Discussion

Epigenetics has been suggested to be the link between the contribution of genetics (Aggelakis et al. 2010) and the contribution of environmental factors (Efthymiou et al. 2017;

Table 1 Summary of the demographic and clinical characteristics of the participants at the time of sample collection

Variable	Healthy individuals	Patients (in total)	Patients in relapse	Patients in remission
n (%)	33 (33.3%)	66 (66.6%)	33 (33.3%)	33 (33.3%)
Female, *n* (%)	22 (66.6%)	44 (66.6%)	22 (66.6%)	22 (66.6%)
Age of onset, mean (range)	NA	33.18 (17–56)	33.33 (17–49)	33.03 (17–56)
Current age, mean (range)	33.06 (20–67)	39.08 (21–65)	37.91 (21–58)	40.24 (23–65)
Disease duration, mean (range)	NA	5.89 (1–15)	4.58 (1–14)	7.21 (1–15)
EDSS, mean (range)	NA	2.39 (0–5)	2.61 (0–5)	2.00 (1–4)

MS multiple sclerosis, *EDSS* Expanded Disability Status Scale, *NA* non-applicable

Table 2 Mean and maximum methylation level per probe, at each sample group

Probes per gene	Healthy individuals mean, max (%)	Patients (in total)	Patients in relapse	Patients in remission
RUNX3				
Probe 1 (01-025.128720)	0, 0	0.32, 6	0.36, 6	0.27, 5
Probe 2 (01-025.128920)	0.88, 9	2.71, 18	2.85, 18	2.58, 17
Probe 3 (01-025.129596)	0.52, 8	1.20, 9	1.52, 8	0.88, 9
MLH1				
Probe 1 (03-037.009361)	3.21, 7	4.67, 28	5.03, 28	4.30, 27
Probe 2 (03-037.009621)	0, 0	0, 0	0, 0	0, 0
Probe 3 (03-037.009769)	0.12, 4	0.27, 6	0.33, 6	0.21, 4
Probe 4 (03-037.010228)	0.09, 3	0.36, 6	0.55, 6	0.18, 3
NEUROG1				
Probe 1 (05-134.898938)	10.36, 12	11.89, 26	11.21, 24	12.58, 26
Probe 2 (05-134.899244)	8.73, 12	9.32, 17	8.82, 17	9.82, 16
Probe 3 (05-134.899351)	7.85, 25	7.56, 11	7.27, 11	7.85, 11
Probe 4 (05-134.899479)	8.48, 12	9.27, 19	9.09, 19	9.45, 18
Probe 5 (05-134.899537)	10.03, 15	9.79, 14	9.61, 12	9.97, 14
Probe 6 (05-134.899663)	1.55, 7	3.09, 18	3.48, 18	2.70, 14
CDNK2A				
Probe 1 (09-021.964676)	1.42, 8	4.11, 25	4.42, 25	3.79, 22
Probe 2 (09-021.965200)	0.12, 4	0.92, 13	1.09, 13	0.76, 9
Probe 3 (09-021.984269)	0, 0	0.08, 5	0.15, 5	0, 0
Probe 4 (09-021.985277)	0, 0	0.23, 5	0.33, 5	0.12, 2
IGF2				
Probe 1 (11-002.117590)	5.70, 11	5.36, 8	5.39, 8	5.33, 8
Probe 2 (11-002.118681)	7.30, 9	8.58, 30	8.45, 29	8.70, 30
Probe 3 (11-002.118895)	16.97, 30	16.38, 26	16.18, 26	16.58, 23
SOCS1				
Probe 1 (16-011.256544)	0, 0	0, 0	0, 0	0, 0
Probe 2 (16-011.256960)	4.39, 8	6.23, 31	6.55, 31	5.90, 27
Probe 3 (16-011.257200)	0, 0	0.17, 5	0.33, 5	0, 0
Probe 4 (16-011.257552)	0, 0	0, 0	0, 0	0, 0
CRABP1				
Probe 1 (15-076.419820)	1.48, 5	1.80, 7	1.61, 7	2, 6
Probe 2 (15-076.420033)	0.76, 3	2.21, 24	2.21, 24	2.21, 24
Probe 3 (15-076.420493)	8.67, 15	9.72, 30	9.61, 30	9.85, 27
Probe 4 (15-076.420701)	4.64, 8	4.58, 10	4.70, 10	4.45, 8
CACNA1G				
Probe 1 (17-045.993509)	0.12, 4	0.18, 5	0.36, 5	0, 0
Probe 2 (17-045.993745)	2.70, 11	3.30, 12	3.91, 12	2.70, 12
Probe 3 (17-045.993972)	1.06, 7	3.35, 27	3.70, 27	3, 23

Mentis et al. 2017a) in the risk for MS (Ramagopalan et al. 2010). Studies on DNA methylation in MS are relatively few and sometimes with discordant results (Baranzini et al. 2010). Nevertheless, they have revealed that genetic loci, previously un-linked to MS, may play a crucial role (Maltby et al. 2015). In addition, gene methylation patterns may be different among MS phenotypic subtypes (RRMS, PPMS, and SPMS) (Fagone et al. 2016; Kulakova et al. 2016).

We have currently showed that methylation patterns of RUNX3, CDKN2A, SOCS1, and NEUROG1 differ significantly between healthy individuals and MS patients, but not between patients in relapse and in remission. Moreover, methylation levels of RUNX3 and CDKN2A can discriminate between healthy controls and MS patients. With a cut-off at 2% for RUNX3, the relative risk for MS is 3.316, while with cut-off at 3% for CDKN2A, the respective relative risk is 3.077.

Table 3 Mann-Whitney *U* test, Kruskal-Wallis test, ROC analysis, and crosstabs comparing the maximum gene methylation levels between MS patients and healthy individuals

	Mann-Whitney *U* test	Kruskal-Wallis test	Post hoc	ROC			Cross tabulation		
	p value	*p* value	*p* value	Area under the curve	*p* value	Cut-off	*p* value	Risk	95% CI
RUNX3	*0.013*	*0.033*	*0.011**	*0.63*	*0.036*	*1.5*	*0.024*	*3.316*	*1.207–9.107*
MLH1	0.335	0.345		0.557	0.355				
NEUROG1	0.168	*0.044*	*0.017*[#]	0.584	0.177				
CDKN2A	*0.003*	*0.011*	*0.03**	*0.675*	*0.005*	*2.5*	*0.018*	*0.007*	*1.281–7.39*
IGF2	0.328	0.21		0.44	0.335				
GRABP1	0.801	0.936		0.485	0.806				
SOCS1	*0.023*	0.071		0.632	*0.033*	*4.5*	0.088		
CACNA1G	0.232	0.235		0.572	0.245				

Statistically significant values are given in italic

*The statistically significant results arose from the difference between controls and MS patients in relapse

[#] The statistically significant results arose from the difference between controls and MS patients in remission

RUNX3 is a gene located on chromosome 1. The gene encodes the Runt-related transcription factor 3 protein; it acts as a tumor suppressor gene and is frequently silenced or deleted in cancer. It regulates many molecular pathways, including the Wnt/Hedgehog/Notch and the Dendritic Cells Developmental Lineage Pathway (Levanon et al. 1994). RUNX3 is mainly expressed in T and NK cells (Hirahara et al. 2013). In MS, the RUNX3 transcription factor has been shown to regulate CD4/CD8 differentiation. Its dysregulated expression in leukocytes is supposed to be a potential risk factor for the disease (Wang et al. 2017). Rs11129295 of the EOMES gene, which is a member of the recently published list of the 110 non-MHC MS susceptibility loci, is very close to RUNX3 and affects the transcription of this gene (Parnell et al. 2014). Parnell et al. have shown that this gene is under-expressed in MS patients (Parnell et al. 2014). However, in our study, MS patients were found to have greater methylation positivity and higher methylation levels, compared to healthy individuals.

CDKN2A (Cyclin-dependent Kinase Inhibitor 2A) is located on chromosome 9. It encodes two proteins, p16 and p14arf, which regulate the cell cycle and act as tumor suppressors. The first protein inhibits CDK4 and CDK6, while the other one activates the p53 tumor suppressor gene (Nobori et al. 1994). Overexpression of p53 has been shown to induce oligodendrocyte apoptosis in active MS lesions (Wosik et al. 2003). Upregulation of this gene is also found to promote growth arrest, through the cellular senescence tumor suppressor mechanism. Studies in all mammalian species have shown increased expression of CDKN2A with aging (Krishnamurthy et al. 2004). Furthermore, a cell-free plasma DNA study showed that the CDKN2B gene, which is adjacent to CDKN2A, was the most differentially methylated between patients in remission and healthy individuals (Liggett et al. 2010).

SOCS1 (suppressor of cytokine signaling-1) gene, on chromosome 16, appears to have a significant role in demyelinating conditions via a number of signaling pathways (Baker et al. 2009). In addition, SOCS1 polymorphisms were found to confer susceptibility to MS development (Vandenbroeck et al. 2012). NEUROG1 is on chromosome 5. It is a transcriptional regulator and is mainly involved in procedures of neuronal differentiation (Dixit et al. 2014).

DNA methylation has not yet been established as a biomarker for MS, since candidate-gene and genome wide methylation analyses have come upon discordant results (Levenson and Melnikov 2012). It has been clarified that DNA methylation affects gene expression (Mangano et al. 2014), but it remains unclear at which level or how many sites need to be methylated to suppress gene expression. Furthermore, it is not yet clear which technique is more appropriate to detect aberrant methylation (Berg et al. 2014). In addition, cut-offs should be carefully set and evaluated, in order to be indicative of aberrant methylation and simultaneously distinguish between MS patients and healthy individuals. Finally, methylation patterns of certain genes may be population-specific contributing to the natural human variation (Heyn et al. 2013).

In conclusion, our study shows that methylation patterns of RUNX3 and CDKN2A may be able to discriminate healthy individuals from MS patients. However, further investigation in larger groups is warranted to elucidate if these methylation patterns can be used as important site-specific biomarkers for MS.

Compliance with Ethical Standards

The study was approved by the University of Thessaly Ethics Committee and informed consent was provided by all participants.

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