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Increased promoter methylation of the immune regulatory gene SHP-1 in leukocytes of multiple sclerosis subjects

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Abstract

The protein tyrosine phosphatase, SHP-1, is a negative regulator of proinflammatory signaling and autoimmune disease. We have previously reported reduced SHP-1 expression in peripheral blood leukocytes of subjects with multiple sclerosis (MS). Recent evidence indicates that virus-induced DNA methylation of the SHP-1 promoter is responsible for aberrant silencing of SHP-1 expression and function in hematopoietic cells that might relate to inflammatory diseases. In the present study, bisulfite sequencing of the SHP-1 promoter demonstrated that over a third of MS subjects had abnormally high promoter methylation. As SHP-1 is deficient in MS leukocytes and SHP-1-regulated proinflammatory genes are correspondingly upregulated, we propose that increased SHP-1 promoter methylation may relate in part to decreased SHP-1 expression and increased leukocyte-mediated inflammation in MS.

Keywords

Bisulfite sequencing; epigenetics; immune regulation; multiple sclerosis; PBMC; protein tyrosine phosphatase

1. Introduction

Multiple sclerosis is a chronic inflammatory demyelinating disease of the central nervous system (Kornek and Lassmann, 2003). A recent analysis of multiple sclerosis (MS) patient tissue discovered that activated transcription factors, such as NF-κB and STATs, are present

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in MS lesions and peripheral blood mononuclear cells (PBMC) of MS patients (Gobin et al., 2001, Frisullo et al., 2006, Christophi et al., 2008b, Christophi et al., 2011), suggesting that altered regulation of cytokine signaling that occurs in MS patients may be responsible for promoting expression of proinflammatory genes and inflammatory demyelination in MS lesions.

SHP-1, a protein tyrosine phosphatase, contains two SH2 domains and functions as a negative regulator of cytokine signaling through both NF-κB and STATs (David et al., 1995, Jiao et al., 1996). When motheaten mice (me/me), which genetically lack SHP-1 gene expression, are subjected to either virus-induced or autoantigen-induced demyelinating diseases, an increased level of inflammatory demyelination relative to that seen in wild type mice is observed (Deng et al., 2002, Massa et al., 2002). Abnormally low levels of SHP-1 in mice (hypomorphic mutations) also cause increased susceptibility to autoimmune and innate inflammatory disease (Croker et al., 2008, Croker et al., 2011). Moreover, reduced SHP-1 in lymphocytes has been related to human lymphoproliferative diseases (Oka et al., 2002, Oka et al., 2001, Zhang et al., 2000, Koyama et al., 2003). These results indicate that SHP-1 plays an important role in lymphocyte activation and proliferation, which may be important to inflammatory demyelinating immune reactions in white matter observed in MS.

We and others previously described that the anti-inflammatory gene SHP-1 is reduced in PBMC of MS compared to normal subjects both at the mRNA and protein levels which may relate to increased inflammatory activity of these cells in the CNS. Further analysis indicated that both lymphocytes and myeloid cells of MS patients have lower amounts of SHP-1 protein (Christophi et al., 2008c, Feng et al., 2002). Moreover, decreased SHP-1 correlated with specific repression of promoter 2 (hematopoietic specific) relative to promoter 1 (epithelial specific) (Tsui et al., 2002) transcripts (Christophi et al., 2008a, Christophi et al., 2009a). Therefore, reduced promoter 2 transcriptional activity may be responsible for reduced expression of SHP-1 in PBMC in MS.

Several studies have described the reduction of SHP-1 expression in various inflammatory and lymphoproliferative disorders. In the latter, DNA methylation within promoter 2 of the SHP-1 gene was responsible for a strong reduction of SHP-1 mRNA and protein (Howell et al., 2000, Koyama et al., 2003, Oka et al., 2002, Nakase et al., 2009, Ruchusatsawat et al., 2006, Oka et al., 2001). In the present study, we addressed the question of whether similar methylation patterns may relate to our previous observations of decreased expression of promoter 2 transcripts in MS. To do this, we used bisulfite genomic sequencing to analyze DNA methylation of specific and functionally relevant CpG sites within the human SHP-1 promoter 2 in MS subject peripheral blood leukocytes. We discovered that SHP-1 promoter 2 in more than a third of MS subjects is modified by extensive CpG methylation, and that this DNA methylation occurs in a region responsible for reduction of SHP-1 promoter 2 activity.

2. Materials and Methods

Human subjects

Genomic DNA was isolated from buffy coats of sixty-nine MS subjects from the Multiple Sclerosis Research Center of New York (MSRCNY), New York, NY (archival DNA samples provided by Dr. Bernadette Kalman) and nineteen normal subjects (Tables 1 and 2, respectively). All MS subjects had definite MS (McDonald et al., 2001). Six out of the 42 relapsing-remitting MS (RR) subjects (specimen# 2, 7, 13, 21, 23, and 41) (Table 1) were classified as active at the time of drawing blood (defined as having a moderate to severe relapse within the last 6 months). The remaining 36 RR MS subjects were classified as stable.

Cell lines and PCR primers

Genomic DNA from 293t cells was used as a positive control for SHP-1 promoter 2 methylation (Nakase et al., 2009). The cells were cultured in DMEM (HyClone, Cat. No. SH30243.01) supplemented with 10% FBS and 2mM glutamine. Genomic DNA was isolated using QIAamp DNA mini kit (Qiagen, Cat. No.51304). A 1,738 bp synthetic PCR product that covers the relevant portion of SHP-1 promoter 2 under investigation was used as a negative control of SHP-1 promoter 2 methylation. The latter was amplified using primer pairs SHP1-F (5'-CAC AGT GGC ATG GAC AAA CTG CAT-3') and SHP1-R (5'-AGC AGG GAG GAG GGA CAT TGA GA-3'). Platinum Taq DNA polymerase, High Fidelity (Invitrogen, Cat. No.11304-011), was used for the PCR. The cycle conditions were 5' at 94C° for the initial denaturation, 35 cycles of 30 seconds at 94 C°; 30seconds at 65 C°; 30 seconds at 68 C°, followed by 5' at 68 C° of the final extension.

Cloning and bisulfite sequencing of genomic promoter 2 DNA

One microgram of genomic DNA from MS subjects, normal control subjects, 293t cells, or twenty nanograms of the synthetic 1,738 bp PCR product that covers the SHP-1 promoter 2 was treated with bisulfite using EpiTect Bisulfite Kit (Invitrogen, Cat. No.59104). To analyze the DNA methylation pattern at the SHP-1 promoter 2, three pairs of PCR primers were used as indicated in the text to amplify promoter 2 sequences: Bis-F (CG2)/Bis-R (CG23) pair covers CpG 2 to 23 of SHP-1 promoter 2, Bis-F (CG13)/Bis-R (CG25) pair (Nakase et al., 2009) covers CpG 13 to 25, and Bis-F (CG2)/Bis-R (CG25) pair covers CpG 2 to 25. The sequence and the position of each primer pair are listed in Table 3 and Figure 1, respectively. The PCR mixture consisted of 3 microliters of bisulfite-modified DNA, 1x PCR buffer, 200nM of dNTP mix, 2mM $MgSO_4$, 500nM of each forward and reverse primer, and 1 unit of Platinum Taq DNA polymerase, High Fidelity (Invitrogen, Cat. No. 11304-011) in a final volume of 75μl. To avoid possible PCR bias, the final volume of 75μl was divided into 4 tubes. The PCR conditions used were 3 minutes at 94°C for the initial denaturation, 45 cycles of 30 seconds at 94°C; 30seconds at 55°C for Bis-F (CG2)/Bis-R (CG23) pair, 57°C for Bis-F (CG13)/Bis-R (CG25) pair, and 60°C for Bis-F (CG2)/Bis-R (CG25) pair; 1' at 68°C, was followed by 5' at 68°C for the final extension. PCR products were gel purified using QIAEX II Gel Extraction kit (Qiagen, Cat. No.20021), cloned into pGEM-T vector (Promega, Cat. No.A3600), transformed into NEB 5-alpha competent E. coli (High Efficiency) (NEB, Cat. No.C2987). Approximately twenty bacteria colonies from multiple sclerosis and normal control subjects, or ten colonies from 293t cells (positive control) and the synthetic 1,738 bp PCR product that covers SHP-1 promoter 2 (negative control), were picked for DNA bisulfite sequencing. Plasmid DNA was isolated with Fast Plasmid mini Kit (5 prime, Cat. No.2300010) and was commercially sequenced (Genewiz, South Plainfield, NJ). BiQ analyzer software (http://biq-analyzer.bioinf.mpiinf.mpg.de/index.php) was used for methylation analysis of each clone. Up to 20 clones for each MS and control subject were produced for analysis in multiple rounds of bisulfite sequencing of sixty-nine MS and nineteen normal control subjects. To assess reliability of each bisulfite sequencing, we included 293t cell genomic DNA (positive methylation control) and a 1,738 bp synthetic PCR-generated sequence (negative methylation control) for each round.

Statistical analysis of SHP-1 promoter 2 methylation

After bisulfite sequencing of DNA clones, the percent methylation (# methylated sites per total number of CpG sites) of each clone was scored. For each subject, the frequency of clones that were methylated at 30%, 40%, 50%, and 60% of the total CpG residues was determined, in the region spanning CpG 2–23. We did not determine methylation status beyond the 60% level because previous reports have reported that this is a stringent functional criterion (Nakase et al., 2009). Statistical comparisons of the relative frequencies

of subjects that met these criteria in the MS and control groups were performed using a Fisher's Exact Test. We also calculated an odds ratio to determine if there was an increased probability of MS subjects achieving different levels of methylation compared to controls using the Haldane modification when necessary to account for zero values in the ratios. These odds ratios were determined to be significant at the $p < 0.05$ level when the lower limit of the 95% confidence interval exceeded 1.0 (Table 4A). For comparison, we also determined the relative frequencies of positive and negative control DNA clones that met the same criteria (Table 4B). In addition to the subject-level analyses, we also compared the MS and control groups using the Fisher's Exact Test and Haldane odds ratios based on the results of individual clones, in the region spanning CpG 13–25 (Table 5A), and listed the results from the positive and negative control DNA clones separately (Table 5B).

3. Results

Subjects

The total of sixty-nine MS subjects (twenty males and forty-nine females) and nineteen normal subjects (ten males and nine females) were included in the study (Tables 1 and 2). The average age of normal subjects was 33.4 and the average age of MS subjects studied was 42.2. The MS subjects included seven primary progressive type (PP), fifty relapsing/remitting type (RR) and twelve secondary progressive type (SP) MS.

Significant Difference in SHP-1 Promoter Methylation Between MS and Normal Subjects

Previous studies have indicated that abnormally high CpG DNA methylation of the SHP-1 gene promoter 2 (Figure 1) was associated with a reduction of SHP-1 expression in B and T cell lymphoma (Zhang et al., 2005, Koyama et al., 2003). Therefore, CpG methylation in this region in fifty-eight MS subjects (1,428 total clones) and nineteen normal subjects (568 total clones) was determined and a frequency distribution analysis at the subject level was performed. The analysis revealed that 39.7% of MS subjects possessed at least one clone with >30% methylation of the promoter compared with 10.5% of control subjects (Table 4A). An odds ratio (OR) and Fisher's exact test was used to compare the relative numbers of subjects within the >30% methylation category. Overall, MS subjects were estimated to be nearly 5 times more likely to meet this criterion compared with control subjects (OR = 4.6, 95th confidence interval (CI), 1.1 – 19.2, $p < 0.05$). The results of the Fisher's exact test confirmed a highly significant increase in the relative frequency of >30% promoter 2 methylation in the MS compared to normal control subjects ($p = 0.015$) (Table 4A). The differences between MS and control subjects were even more significant at the 40%, 50% and 60% methylation level, where the odds of an MS subject meeting these criteria were more than 20 times that of control subjects (Table 4A). Specifically, 38% of MS subjects (and 0% of normal subjects) had clones with 40% methylation of promoter 2, and 36% of MS patients contained clones with either 50% or 60% methylation of promoter 2 compared to 0% of normal subjects. In agreement with the odds ratio analysis, these differences were highly significant by Fisher's exact test (40% cutoff $p = 0.00055$, 50% or 60% cutoff $p = 0.00084$) (Table 4A). Notably, 77% of the clones from the positive 293t cell control DNA sample and 0% of the negative control DNA sample had clones meeting the 30% methylation criteria. Thus, MS subjects possessed slightly more than one half the frequency of promoter 2 methylated clones seen in the hypermethylated 293t cells (Table 4B).

Significant methylation within the functional core of SHP-1 promoter 2

A previous analysis that related the extent of DNA methylation of individual promoter 2 clones from transformed lymphoblastoid cells to silencing of SHP-1 expression demonstrated that, though 30% methylation of CpG13–25 of SHP-1 promoter 2 was sufficient to suppress SHP-1 expression by 30% of normal, 60% methylation of this region

was sufficient to substantially repress transcription of the SHP-1 gene to a third of normal levels (Nakase et al., 2009). To determine whether DNA methylation observed in clones of the MS subjects reached this functional level, percent DNA methylation within the CpG13–25 region of SHP-1 promoter 2 within the different groups was analyzed. This group analysis consisted of 870 clones from twenty-nine MS subjects, 518 clones from fourteen normal subjects, 204 clones of methylation positive control (293t cell DNA) and 147 clones of the synthetic DNA negative control. We observed rare clones (<1%) from the normal subject group having greater than 50% methylation (5 clones out of 518 clones examined) (Table 5A). In contrast, approximately 5% of clones from the MS subject groups had greater than 50%, or even 60% methylation, in contrast to the <1% from the normal subject group. The odds ratio analysis produced an OR for 50% cutoff of 5.3 (with 95th CI, 2.2–12.8, $p < 0.05$), and an OR for 60% methylation cutoff level of 5.0 (with 95th CI, 2.1 – 12.3, $p < 0.05$). Thus, MS subjects are 5 times more likely to have clones with at least 60% methylation at CpG13–25 compared with normal control subjects. The result of the odds ratio analysis was confirmed by Fisher's exact test ($p<0.00001$ for 50% cutoff and $p < 0.00002$ for 60% cutoff) (Table 5).

Relationships between DNA methylation and subject parameters

Finally, we analyzed a potential correlation between frequency of highly methylated SHP-1 promoter clones in MS subjects with different MS disease type, years with MS, and expanded disability status scale (EDSS) score (Table 6). For each subject, we calculated % clone frequency (the number of clones that had >30% methylation / the total number of clones studied in a particular subject). From regression analysis of scatter plots (not shown), no association between the frequency of highly methylated clones in particular MS subjects with any subject parameter including EDSS (R^2= 0.039), subject age (R^2= 0.01), or years with MS (R^2= 0.029) could be detected. Finally, there was no significant difference in the average frequency of clones with high promoter methylation (>30% methylation) between MS subjects with either active (n=6) or stable (n=36) relapsing-remitting MS analyzed by Student's t-test (3.8% vs. 4.9%; p=0.79). Further, there was no significant difference in the proportion of subjects with either active or stable relapsing-remitting MS that displayed clones >30% versus <30% methylation (Active 1/5 vs. Stable 16/20; Fisher's exact test p=0.37).

4. Discussion

DNA methylation is an epigenetic alteration that results in both acquired and heritable gene silencing and is critical for developmental progression, cell fate determination, and various diseases including hematopoietic cancers (Rush and Plass, 2002, Kanai, 2010, Issa, 2004, Egger et al., 2004, Tost, 2010, Vaissiere et al., 2008) and autoimmunity including MS (Burrell et al., 2011, Liggett et al., 2010, Meda et al., 2011, Brooks et al., 2010, Pedre et al., 2011, Casaccia-Bonnefil et al., 2008, Baranzini et al., 2010). Studies indicate that the extent of promoter methylation that inhibits gene expression differs depending on type of gene, ranging from 9% for RB1 gene (Ohtani-Fujita et al., 1997) to 84% for MLH1 gene in microsatellite-unstable colorectal tumor (Herman et al., 1998). In hematopoietic cancers, CpG methylation at the SHP-1 core promoter of approximately 60% substantially reduced SHP-1 gene expression to a third of normal levels (Oka et al., 2002, Koyama et al., 2003, Nakase et al., 2009, Zhang et al., 2005). The present studies have revealed a similar level of methylation of the SHP-1 promoter in a fraction of hematopoietic cells from MS subjects which may have functional relevance to disease.

Although SHP-1 is believed to be an important tumor suppressor in hematopoietic cells and transcriptional silencing promotes lymphoblastoid cell proliferation, the main function of SHP-1 determined from animal studies with null or hypomorphic mutations in the SHP-1

gene is to attenuate leukocyte activation and immune-mediated inflammation (Deng et al., 2002, Massa et al., 2002, Pao et al., 2007, Croker et al., 2008). As such, it was of particular interest that we previously described that PBMC of MS subjects had reduced levels of SHP-1 mRNA (specifically of promoter 2 transcripts) and protein relative to those of normal subjects (Christophi et al., 2009b, Christophi et al., 2008c). Consequently, it was of particular interest to determine whether DNA methylation of transcriptionally relevant CpG sites within the SHP-1 promoter 2 (Nakase et al., 2009) may be more common in MS compared to normal subjects. Statistical analysis of methylation with the CpG2–23 region performed at the subject level indicated that MS patients are nearly 5 times more likely to possess 30% or greater methylation within the SHP-1 promoter 2 compared to normal subjects. At more stringent levels, the group differences were even more robust with a highly-significant 20-fold elevation in the likelihood of having 40, 50, or 60% methylation in the SHP-1 promoter in MS subjects versus controls. Importantly, we also observed a highly-significant increase in percent DNA methylation in MS patients compared to normal subjects within the critical CpG13–25 region of the SHP-1 promoter 2. Of particular note, 60% methylation in this restricted region of the proximal promoter is sufficient to repress transcription from promoter 2 to a third of normal levels (Nakase et al., 2009). Assuming that individual clones of methylated genomic DNA are derived from a fraction of individual leukocytes in buffy coat samples from MS subjects, we propose that MS subjects are more likely to contain leukocytes in which SHP-1 is functionally repressed. Further examination of this question will require comparative analysis of percent methylation of promoter 2 in both silenced leukocytes and leukocytes with normal levels of SHP-1 expression within individual subjects.

Because we can detect on average a 5% frequency of highly methylated clones (>60% methylation) in the MS subject group (Table 5), we may hypothesize that SHP-1 is functionally reduced in approximately 5% of the leukocytes in the blood of those with MS. In assessing the functional significance of this methylation, whether these leukocytes constitute autoreactive cells with increased inflammatory potential would be particularly important (Deng et al., 2003, Stefanova et al., 2003). For instance, the frequency of myelin reactive T cells in MS to any particular antigenic epitope is generally less than 1 in 10,000 of total T cells in the blood (0. 01%) (Hellings et al., 2001, de Rosbo and Ben-Nun, 1998). As a consequence, functional repression of SHP-1 in 5% of the peripheral leukocytes may potentially accommodate increased inflammatory activity in multiple lineages of pathogenic T cells directed towards hundreds of different myelin antigenic epitopes that could potentially be sufficient to mediate inflammatory demyelinating disease in MS. In future studies, it will be important to determine whether SHP-1-deficient leukocytes significantly overlap with these pathogenic populations of lymphocytes in the MS population.

We observed that DNA methylation seen in MS subjects includes substantial methylation within the critical CpG13–25 region containing the binding sites for various transcription factors, such as Sp1, Oct-1, NF-κB, PU.1 and CREB-1 important for the expression of SHP-1 (Cheng et al., 2007, Nakase et al., 2009, Wlodarski et al., 2007). Because many of these CpG sites are either adjacent or overlapping these transcriptional elements, increased methylation blocks factor binding and interferes with transcriptional activation of the SHP-1 gene (Nakase et al., 2009). Consistent with this hypothesis, the CpG13–25 region is critical for HTLV-1 Tax-mediated suppression of SHP-1 expression which acted at a NF-κB site to recruit NF-κB, Tax, histone deacetylase (HDAC1), and DNA methyltransferase (DNMT1) to increase methylation of promoter 2 (Nakase et al., 2009, Cheng et al., 2007). These studies suggest similar pathways for acquired DNA methylation at the SHP-1 promoter 2 that may lead to gene repression during development of inflammatory diseases. We propose that such acquired SHP-1 deficiency in leukocytes may impart susceptibility to autoimmunity as recently described in the mouse (Croker et al., 2008) rather than being

directly related to changes in disease activity as suggested in Table 6. The latter possibility is supported by a lack of significant difference in high promoter methylation between active and stable relapsing-remitting MS subjects suggesting that SHP-1 promoter methylation may be independent of active disease in this group. However, we hesitate to draw any firm conclusions from this exploratory analysis due to the small number of active relapsing-remitting MS subjects included in our study.

Even if SHP-1 is not a primary susceptibility gene for MS, SHP-1 could play a subsequent role in some aspects of MS disease because SHP-1 controls the expression of multiple proinflammatory pathways in both lymphocytes and myeloid cells (Kruger et al., 2000, Zhou et al., 2010, Pao et al., 2007, Christophi et al., 2009b, Sathish et al., 2007, Johnson et al., 1999). Epigenetic alteration is potentially a reversible process, and some epigenetic drugs are being tested in clinical trials for the treatment of hematopoietic malignancies (Egger et al., 2004). Studies demonstated that the activity of proinflammatory signaling molecules, such as NF-κB and STATs, which are negatively regulated by SHP-1, are increased in MS leukocytes and CNS lesions (Christophi et al., 2009b, Frisullo et al., 2006, Gobin et al., 2001). Even though such repression may be limited to a subset of MS patients, if MS patients were screened for epigenetic modification and specific ways to reverse epigenetic changes were discovered, it may be possible to alter the inflammatory disease process in some MS subjects.

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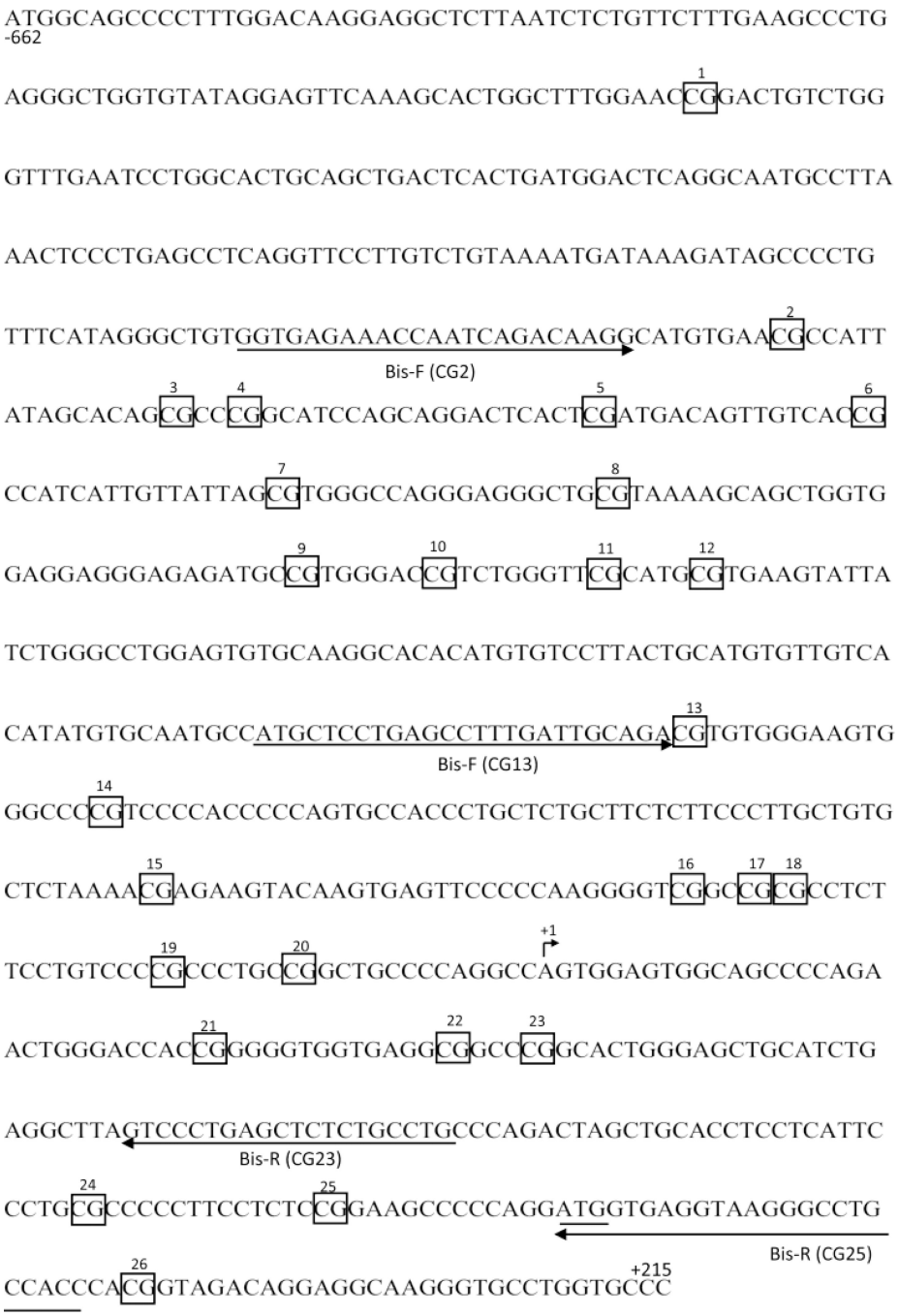

Figure 1.

Assay designs for analysis of SHP-1 gene promoter 2 methylation by Bisulfite genomic sequencing. Arrows indicate placement and direction of PCR primers. Three primer pairs were used to analyze 26 potential methylation sites in the human SHP-1 gene promoter 2 (boxed CpGs are numbered 1–26). Primers, Bis-F (CG13) and Bis-R (CG25) are from Nakase, K et al. 2009. Bis-F (CG2) and Bis-R (CG23) are designed using BiSearch online software. Both the transcriptional and translational start sites are labeled +1 and by underlining, respectively. The nucleotide numbering at the beginning and end of the promoter region displayed is relative to the transcriptional start site (+1).

Table 1

MS subjects.

Specimen No.	Gender	Patient's age	Disease course	Current EDSS	No. of years with MS
1	F	57	SP	7.5	20
2	F	47	RR	1.5	12
3	M	58	RR	4	6
4	F	30	RR	3	5
5	F	35	RR	0.5	3
6	M	33	RR	1	4
7	M	27	RR	2	7
8	F	52	RR	0	2
9	F	57	SP	1	5
10	F	59	SP	4	26
11	F	54	PP	7	8
12	M	42	PP	6.5	10
13	F	27	RR	0.5	0
14	M	48	PP	6	8
15	F	47	RR	2	3
16	M	69	SP	4.5	17
17	F	49	SP	5	14
18	F	39	RR	1	5
19	F	30	RR	1	11
20	M	60	PP	6.5	27
21	F	50	RR	3.5	4
22	M	35	RR	0.5	3
23	M	37	RR	2.5	3
24	M	37	RR	1	6
25	F	33	RR	1.5	6
26	F	57	RR	3.5	8

Specimen No.	Gender	Patient's age	Disease course	Current EDSS	No. of years with MS
27	F	33	RR	0	2
28	F	35	RR	0	2
29	M	39	RR	1.5	8
30	F	36	RR	0	8
31	F	41	RR	0	2
32	F	37	RR	5.5	9
33	F	53	RR	3	8
34	F	54	SP	6.5	19
35	F	34	SP	4.5	8
36	F	48	RR	2	7
37	F	57	SP	4	12
38	M	45	SP	7	13
39	F	48	RR	1.5	9
40	M	38	RR	0.5	4
41	F	36	RR	1	10
42	F	51	RR	0.5	8
43	M	32	RR	0	1
44	F	34	RR	0	7
45	M	48	PP	7	15
46	F	31	RR	0.5	3
47	F	45	RR	3	7
48	F	38	RR	0	3
49	M	23	RR	1	4
50	F	31	RR	4.5	6
51	F	41	RR	3	7
52	M	44	PP	4	7
53	F	37	RR	0	2
54	F	37	RR	2	6

Specimen No.	Gender	Patient's age	Disease course	Current EDSS	No. of years with MS
55	F	49	SP	2.5	5
56	F	39	RR	1.5	7
57	F	57	SP	6.5	8
58	M	43	RR	1	8
59	F	27	RR	0	1
60	M	31	RR	1	4
61	F	40	RR	0	6
62	F	52	RR	0	1
63	F	30	RR	0	1
64	F	57	PP	6	4
65	F	27	RR	0	0
66	F	35	RR	1	6
67	F	31	RR	0	3
68	M	28	RR	4.5	6
69	F	71	SP	5	8
Summary of MS patients information					
Number of Males					20
Number of Females					49
Average age (total) (years)					42.2
Average EDSS (total)					2.44
Average years with MS					7.07
Number of Relapsing/Remitting (RR) MS					50
Number of Primary Progressive (PP) MS					7
Number of Secondary progressive (SP) MS					12

DNA was isolated from buffy coat leukocytes. F = female. M = male. SP = Secondary Progressive type. RR = Relapsing/Remitting. PP = Primary Progressive. No. of years of MS was calculated by subtracting year of diagnosis of MS from the year of DNA collection.

Table 2

Normal Subjects

Specimen Number	Normal subject code	Gender	Age
1	C1	M	26
2	C2	F	34
3	C3	F	42
4	C4	M	23
5	C5	M	25
6	C6	F	25
7	C7	M	29
8	C8	F	26
9	C11	M	56
10	C12	F	25
11	C13	M	56
12	C14	M	71
13	C15	M	25
14	C16	F	25
15	C17	M	31
16	C18	M	25
17	C19	F	27
18	C23	F	25
19	C24	F	39
Summary of normal subject			
Number of Male Number of Female Average Age (total)		10 9 33.4	

DNA from all of the samples was isolated from buffy coat. M = male. F = female

Table 3

Primers used for Bisulfite sequencing.

Primers	Primer sequence
Bis-F (CG2)	5’-GGT GAG AAA TTA ATT AGA TAA GG-3’
Bis-F (CG13)	5’-ATG TTT TTG AGT TTT TGA TTG TAG A-3’
Bis-R (CG23)	5’-CAA ACA AAA AAC TCA AAA AC-3’
Bis-R (CG25)	5’-AAT AAC AAA CCC TTA CCT CAC CAT-3’

Three combinations of primer pairs used for Bisulfite genomic sequencing experiments are 1) Bis-F (CG13)/Bis-R (CG25), 2) Bis-F (CG2)/Bis-R (CG23) and 3) Bis-F (CG2)/Bis-R (CG25). CG of CG2, CG13, CG23 and CG25 indicates the position of CpG sites on promoter 2 studied for the assay (see Figure 1 for primer orientation and targets).

Table 4

A Percentage of subjects with various levels of promoter 2 methylation				
	Subjects (Ss)			
	Normal (N=19)	MS (N=58)	Fisher's Exact Test P value	Haldane Odds Ratio (95th CI)
Total # clones sequenced	568	1428		
Ss w/clones > 30% meth	10.5% (2/19)	39.7% (23/58)	0.01522	4.6 (1.1–19.2)
Ss w/clones > 40% meth	0.0% (0/19)	37.9% (22/58)	0.00055	24.0 (1.4–418.1)
Ss w/clones > 50% meth	0.0% (0/19)	36.2% (21/58)	0.00084	22.4 (1.3–389.2)
Ss w/clones > 60% meth	0.0% (0/19)	36.2% (21/58)	0.00084	22.4 (1.3–389.2)

B Percentage of clones with various levels of promoter 2 methylation in NegCtrl (1,738) and PosCtrl (293t) DNA		
	% NegCtrl (1738) clones	% PosCtrl (293) clones
Total # clones sequenced	178	216
clones > 30% meth	0.0% (0/178)	77.3% (167/216)
clones > 40% meth	0.0% (0/178)	76.9% (166/216)
clones > 50% meth	0.0% (0/178)	75.9% (164/216)
clones > 60% meth	0.0% (0/178)	72.2% (156/216)

Bisulfite sequencing results from primer pairs that span CpG 2–23 and 2–25 were combined for this analysis. Note the increased percentage of MS subjects with clones displaying elevated promoter 2 methylation compared to normal subjects. PosCtrl=Positive Control. NegCtrl=1738bp synthetic DNA negative control. Meth=methylation. 95th CI=95th percentile confidence interval. Ss=subjects.

Table 5

A Incidence of highly-methylated promoter 2 clones in MS and Normal Ss				
	Subjects (Ss)			
	Normal (N=14)	MS (N=29)	Fisher's Exact Test P value	Haldane Odds Ratio (95th CI)
Total # clones sequenced	568	870		
clones > 50% meth	0.97% (5/518)	5.29% (46/870)	0.00001	5.3 (2.2–12.8)
clones > 60% meth	0.97% (5/518)	5.06% (44/870)	0.00002	5.0 (2.1–12.3)

B Percentage of clones with various levels of promoter 2 methylation in NegCtrl (1,738) and PosCtrl (293t) DNA		
	% NegCtrl (1738) clones	% PosCtrl (293) clones
Total # clones sequenced	147	204
clones > 50% meth	0.0% (0/147)	83.3% (170/204)
clones > 60% meth	0.0% (0/147)	81.9% (167/204)

The results from bisulfite sequencing using the two primer sets (Figure 1) that span CpG 13–25 were used for the analysis. PosCtrl: Positive Control. NegCtrl=1738bp synthetic DNA negative control. Ss=subjects.

Table 6

% DNA methylation from bisulfite sequencing and MS subject parameters.

Specimen No.	% freq of clones with >30% met	Gender	Patient's age	Yrs with MS	Disease course	Current EDSS
65	33%	F	27	0	RR	0
42	5%	F	51	8	RR	0.5
47	0%	F	45	7	RR	3
36	0%	F	48	7	RR	2
29	0%	M	39	8	RR	1.5
26	19%	F	57	8	RR	3.5
46	0%	F	31	3	RR	0.5
48	7%	F	38	3	RR	0
21	0%	F	50	4	RR	3.5
41	23%	F	36	10	RR	1
63	0%	F	30	1	RR	0
27	0%	F	33	2	RR	0
59	5%	F	27	1	RR	0
44	7%	F	34	7	RR	0
23	0%	M	37	3	RR	2.5
35	0%	F	34	8	SP	4.5
30	10%	F	36	8	RR	0
61	0%	F	40	6	RR	0
43	0%	M	32	1	RR	0
60	8%	M	31	4	RR	1
69	0%	F	71	8	SP	5
33	0%	F	53	8	RR	3
28	8%	F	35	2	RR	0
39	0%	F	48	9	RR	1.5
31	0%	F	41	2	RR	0

Specimen No.	% freq of clones with >30% met	Gender	Patient's age	Yrs with MS	Disease course	Current EDSS
22	5%	M	35	3	RR	0.5
62	19%	F	52	1	RR	0
38	4%	M	45	13	SP	7
45	0%	M	48	15	PP	7
64	5%	F	57	4	PP	6
7	0%	M	27	7	RR	2
1	0%	F	57	20	SP	7.5
6	0%	M	33	4	RR	1
17	0%	F	49	14	SP	5
25	0%	F	33	6	RR	1.5
15	0%	F	47	3	RR	2
34	0%	F	54	19	SP	6.5
20	5%	M	60	27	PP	6.5
5	0%	F	35	3	RR	0.5
32	5%	F	37	9	RR	5.5
14	0%	M	48	8	PP	6
2	0%	F	47	12	RR	1.5
13	0%	F	27	0	RR	0.5
3	15%	M	58	6	RR	4
12	2%	M	42	10	PP	6.5
8	0%	F	52	2	RR	0
11	2%	F	54	8	PP	7
37	9%	F	57	12	SP	4
19	0%	F	30	11	RR	1
10	0%	F	59	26	SP	4
9	0%	F	57	5	SP	1
16	0%	M	69	17	SP	4.5
18	2%	F	39	5	RR	1

Specimen No.	% freq of clones with >30% met	Gender	Patient's age	Yrs with MS	Disease course	Current EDSS
67	26%	F	31	3	RR	0
4	0%	F	30	5	RR	3
40	0%	M	38	4	RR	0.5
66	4%	F	35	6	RR	1
24	0%	M	37	6	RR	1

% of colonies that had greater than 30% methylation from bisulfite sequencing analysis of CpG2–23 for each MS patient was tested for correlation with various MS subject parameters.