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Multiple sclerosis as a generalized CNS disease—comparative microarray analysis of normal appearing white matter and lesions in secondary progressive MS

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Abstract

We used microarrays to compare the gene expression profile in active lesions and donor-matched normal appearing white matter (NAWM) from brain autopsy samples of patients with secondary progressive multiple sclerosis (MS) with that from controls who died from non-neurological diseases. The 123 genes in lesions, and 47 genes in $NAWM_{MS}$ were differentially expressed. Lesions distinguished from $NAWM_{MS}$ by a higher expression of genes related to immunoglobulin synthesis and neuroglial differentiation, while cellular immune response elements were equally dysregulated in both tissue compartments. Current results provide molecular evidence of a continuum of dysfunctional homeostasis and inflammatory changes between lesions and $NAWM_{MS}$, and support the concept of MS pathogenesis being a generalised process that involves the entire CNS.

Keywords: Multiple sclerosis; Lesion; Normal appearing white matter; Differential gene expression; Microarray

1. Introduction

Multiple sclerosis (MS) is believed to result from an autoimmune reaction against myelin components of the CNS. The disease is characterized by the formation of disseminated areas of demyelination and neuronal damage, called lesions or plaques (Lassmann, 1998). Relapses and residual disability after relapses are clinical correlates of plaque formation (Confavreux et al., 2000). However, the lesion load as depicted by standard MRI sequences and the number of relapses are only weak determinants for the long-term clinical outcome, as the disease tends to evolve into a progressive course in 90% of patients (Weinshenker et al., 1989), independent of relapses (Confavreux et al., 2000). Modern MRI techniques reveal that the so-called normal appearing white matter (NAWM), the part of the brain that do not show abnormalities in standard MRI scans, indeed harbour abnormalities, indicative of a process of *diffuse* myelin damage and neuronal loss that may result in progressive generalized brain atrophy (Filippi et al., 1998; van Waesberghe et al., 1999; Werring et al., 2000; Lucchinetti et al., 2000; Bjartmar et al., 2001; Griffin et al., 2002; Fulton et al., 1999; Filippi et al., 2003). These NAWM abnormalities commence early in disease, but are much more pronounced in the phase of secondary progression. The underlying molecular mechanisms that lead to these extralesional changes remain largely unknown. Although current MS therapies, i.e. IFN-β and glatiramer acetate, have a partially beneficial effect on the disease based on clinical and MRI measures, they may have little or no impact on the more diffuse changes, and hence long-term disability (European Study Group on Interferon beta-1b in Secondary Progressive MS, 1998; Secondary Progressive Efficacy Clinical

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Trial of Recombinant Interferon-beta-1a in MS (SPECTRIMS) Study Group, 2001; Molyneux et al., 2000).

The advent of DNA microarray technology and other high-throughput methods made it possible to measure transcriptional regulation of thousands of known and expressed-sequence-tags (ESTs) simultaneously (Steinman, 2001). This technique has allowed to define altered gene expression in PBMC of MS patients (Bomprezzi et al., 2003), and to delineate genomic effects of MS therapy with IFN-β (Weinstock-Guttman et al., 2003). To date, seven studies using large-scale gene expression profiling in MS lesions (Becker et al., 1997; Whitney et al., 1999, 2001; Chabas et al., 2001; Lock et al., 2002; Mycko et al., 2003; Tajouri et al., 2003) have been performed. Quantitative real-time PCR technique was used in another study to compare the transcriptional expression of 56 cytokines and related targets in MS lesions of different evolution states (Baranzini et al., 2000). Both approaches revealed a complex pattern of mostly known genes related to inflammatory processes that were differentially expressed in plaques. In contrast, there is only one study in which NAWM of MS ($NAWM_{MS}$) has specifically been studied (Graumann et al., 2003), and no comparative analysis with lesional tissue has been performed with a comprehensive gene expression analysis method so far. Based on earlier findings showing an overexpression of certain matrix metalloproteinases both in $NAWM_{MS}$ and lesions (Lindberg et al., 2001), we hypothesized that other pathogenetically relevant changes may be shared in between these two tissue compartments.

In this study, we compared the gene expression pattern between active-type lesions and donor-matched NAWM from brain autopsy samples of patients with secondary progressive MS using Affymetrix® oligonucleotide microarrays and quantitative real-time PCR (RT-PCR). We categorized differentially expressed genes according to possible disease-relevant functions, and defined their specificity for MS tissue by comparison with control brain white matter from patients, who were not affected by primary neurological diseases.

2. Materials and methods

2.1. Brain tissue samples

Autopsy material was obtained via the rapid autopsy system of the Netherlands Brain Bank, which supplies postmortem specimens from clinically well documented and neuropathologically confirmed cases. Once the brain was removed it was macroscopically examined and immediately dissected following a standard protocol (Lindberg et al., 2001). Specimens were rapidly frozen in liquid nitrogen and stored at −80 °C. From four MS patients, matched lesion and NAWM ($NAWM_{MS}$) tissue were studied, from one patient (96-040) only active lesion and from one patient (95-057) only $NAWM_{MS}$ tissue was available. All patients and their next-of-kin had given written consent for autopsy, and for use of their brain for scientific research.

2.2. Neuropathological evaluation

Staging of MS lesions was performed according to the classification described (Van der Valk and De Groot, 2000). Five-micrometer-thick brain tissue sections were stained with anti-myelin basic protein antibodies (MBP; IgG, Boehringer; Mannheim, Germany) and with the neutral lipid marker Oil Red O (ORO) to delineate areas of myelin breakdown and demyelination. Mouse-anti-human KP1 (CD68; IgG1, Dako; Copenhagen, Denmark) and mouse anti-human leukocyte common antigen (LCA) (CD45; IgG1, Dako) antibodies were used to detect leukocyte infiltration and microglial activation. Markers to detect activated lymphocytes and astrogliosis were MHC class II antigen (HLA-DR, Biotest; Dreieich, Germany) anti-cow glial fibrillary acidic protein (anti-GFAP, Dako), respectively. Sections were stained by the avidin–biotin horseradish peroxidase complex method (ABC, Vector Laboratories; Burlingame, CA) using 3,3′ -diaminobenzidine tetrachloride (DAB) as a chromogen.

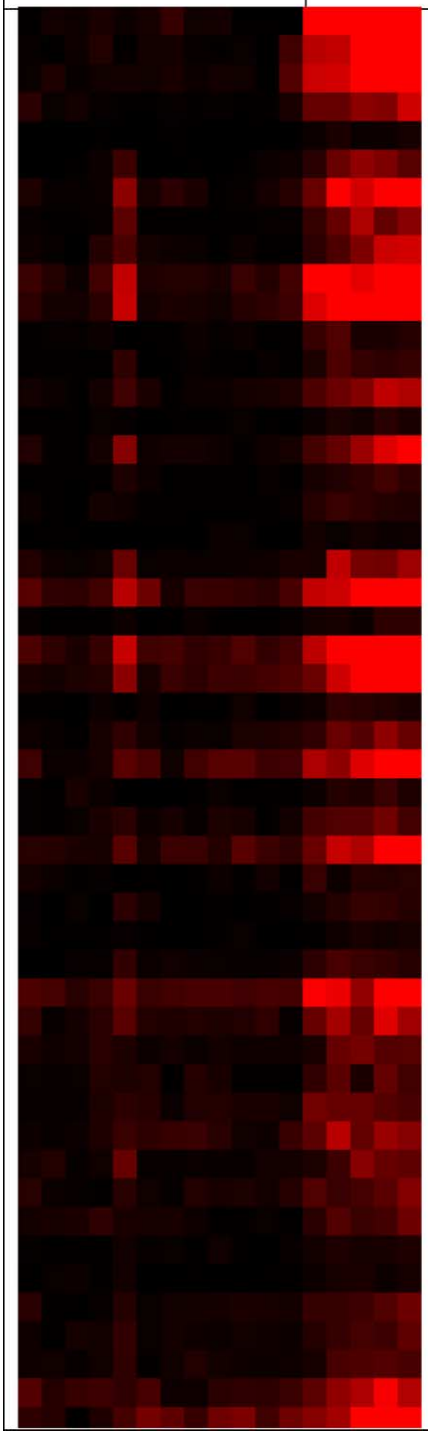
2.3. Isolation of RNA from brain tissue

Approximately 100 mg of brain tissue was suspended in 1 ml RNAzol (AMS Biotechnology; Bioggio, Switzerland) and disrupted for 20 s using FastPrep tubes and a Savant homogenizer (Bio101; Buena Vista, CA). Total RNA was isolated according to the manufacturer's instruction (AMS). DNAse I treatment (Promega; Wallisellen, Switzerland) was applied to eliminate genomic DNA interference. Gel electrophoresis and spectrophotometry ($\lambda_{260}/\lambda_{280}$) were used to assess RNA quality.

2.4. Synthesis and hybridization of cRNA

Double strand cDNA was synthesized from 20 μg of total RNA using a cDNA Synthesis System (Roche Diagnostics; Rotkreuz, Switzerland) with the T7-$(T)_{24}$ primer. The MEGAScript T7 kit (Ambion; Austin, TX) was used to transcribe the cDNA into cRNA in the presence of Biotin-11-CTP and Biotin-16-UTP (Enzo; Farmingdale, NY) according to the instructions supplied with the kit. After purification with the RNeasy kit (Qiagen; Hilden, Germany), integrity of the cRNA was checked using gel electrophoresis. 10–20 μg fragmented cRNA were used for hybridization to the Human U95A array (Affymetrix GeneChip® array; Santa Clara, CA). The array contained 12633 probe sets, consisting of 12566 sequences for known or EST genes, and 67 sequences for control oligonucleotides.

Hybridization and staining were performed as described previously (Lockhart et al., 1996; De Saizieu et al., 1998). Arrays were scanned with a confocal laser scanner (Hewlett-Packard).



Affymetrix Probe set	Mean intensity in control WM	Mean intensity in lesions	Change factor	p-value	Mean intensity in $NAWM_{MS}$
38194_s_at	228	11885	51.23	0.015	8468
33274_f_at	222	3942	16.76	0.007	6687
33273_f_at	294	4228	13.39	0.005	7126
41827_f_at	156	1848	10.81	0.003	2484
37006_at	22	232	9.68	0.015	312
33764_at	134	1366	9.17	0.006	199
32138_at	411	3753	8.13	0.009	465
32093_at	175	1543	7.8	0.006	204
36563_at	227	1796	6.91	0.025	529
38800_at	740	5824	6.88	0.020	1780
38484_at	636	4982	6.83	0.001	985
38870_at	89	621	6	0.018	168
32090_at	104	699	5.75	0.002	117
41675_at	284	1850	5.52	0.005	419
35081_at	57	358	5.33	0.009	115
40314_at	351	2183	5.23	0.017	558
33630_s_at	95	570	4.98	0.005	114
34208_at	96	559	4.85	0.001	149
39974_at	41	219	4.34	0.002	68
40075_at	313	1640	4.25	0.022	335
36591_at	945	4889	4.17	0.024	1455
35711_at	79	392	3.94	0.025	246
36049_at	948	4664	3.92	0.017	1265
31901_at	721	3530	3.9	0.010	1443
39930_at	91	446	3.89	0.010	112
41107_at	269	1313	3.89	0.006	318
39619_at	737	3582	3.86	0.016	1356
36556_at	93	450	3.84	0.020	126
41559_at	232	1117	3.81	0.001	463
33712_at	665	3092	3.65	0.018	846
936_s_at	106	486	3.57	0.015	210
33404_at	119	534	3.49	0.003	104
35949_at	52	233	3.46	0.018	135
40378_at	160	713	3.45	0.001	231
38719_at	919	4054	3.41	0.014	1543
39395_at	485	2052	3.23	0.006	644
33728_at	272	1127	3.14	0.015	348
37214_g_at	226	906	3.01	0.019	597
39698_at	325	1290	2.97	0.0001	650
39026_r_at	458	1794	2.92	0.003	828
34412_s_at	296	1140	2.86	0.022	305
330_s_at	306	1157	2.79	0.006	392
32652_g_at	256	962	2.76	0.009	487
39646_at	92	344	2.75	0.0001	98
693_g_at	72	268	2.72	0.006	134
31887_at	267	975	2.65	0.005	329
173_at	240	856	2.56	0.006	320
32606_at	250	884	2.53	0.009	282
38516_at	648	2281	2.52	0.008	850
32105_f_at	977	3424	2.51	0.015	1519

Fig. 1. Differentially expressed genes in five lesions as compared to white matter from 12 non-neurologic control patients. Expression levels are reported as the mean intensity measured using GeneChip software (Affymetrix). The scale extends from the highest mean intensity value (indicated with the strongest red color) to the lowest value (indicated with black color). Change factor and *P* values refer to the comparison of mean intensity in lesion vs. mean intensity in control WM. Sequences are indicated with Affymetrix probe sets. The gene list is truncated for the last 79 genes, change factors for up- and downregulated genes are marked with blue and yellow, respectively. Genes with significant differential expression both in lesions and $NAWM_{MS}$ are highlighted with light green.

2.5. Expression data analysis and filtering criteria

Fluorescence intensities were analyzed with the Microarray Suite software, version 5.0 (Affymetrix) and expression data were analyzed with the RACE-A program (Hoffmann-La Roche; Basel, Switzerland). The expression level, "mean intensity", for each gene was determined by calculating the mean of average differences of all replicates (perfect match–mismatch oligonucleotides) for each probe set. Absolute call (score values for present: 1–>0.5, marginal: 0.5, or absent: <0.5–0) was defined by Microarray Suite software. The data filtering criteria applied for the analysis were as follows: for lesions: (a) change factor >1.5 or <−1.5, (b) statistical significance (unpaired *t*-test) $P<0.025$, (c) mean intensity >200 fluorescence units, indicating that the gene is expressed, (d) call >0.2 (=more than 20% present calls). For $NAWM_{MS}$, we used identical filtering criteria as for lesions, except for the change factor (>1.0 or <−1.0), due to smaller quantitative differences in gene expression levels as compared to controls. Data analysis software, RACE-A (Hoffmann-La Roche) calculates change factors based on the differences of mean intensities between two conditions (comparative study), therefore for example change factor 1 corresponds to a twofold change.

We used cluster and TreeView software (Eisen et al., 1998) (http://rana.lbl.gov) to create graphical presentation of microarray data shown as normalized mean intensities (Fig. 1). Affymetrix gene descriptions are used in Fig. 1, whereas in Tables 2–5 the sequences are presented both with respective gene accession numbers and gene symbols. Probe sets for differentially expressed sequences are categorized and numbered in the order of magnitude of change factor for lesions (#1–129) and for $NAWM_{MS}$ (#130–177) (Tables 2 and 3). For easier identification of genes, this numbering is used throughout the entire manuscript.

2.6. Validation of microarray data with real-time PCR

Total RNA (100 ng/μl final concentration) was incubated with 0.5 μg random hexamer at 70 °C (2 min) and then reverse-transcribed at 37 °C (1 h) in reaction mix containing of final concentration of 1× first-strand buffer, 500 μM of each deoxynucleotide triphosphates, 1 U/μl of Moloney murine leukemia virus reverse transcriptase and 1 U/μl of ribonuclease inhibitor (RNAsin) (all reagents from Promega; Madison, WI). cDNA was used as a template for the realtime RT-PCR analysis based on the 5′ -nuclease assay (Gibson et al., 1996) with ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, PE Europe; Rotkreuz, Switzerland) using Assay-on-Demand primer/probe sets for all targets (Applied Biosystems). Ribosomal 18S RNA was amplified from all samples on each plate as a housekeeping gene to normalize expression levels of targets between different samples, and to monitor assay reproducibility. Relative quantitation of all targets was calculated by the comparative cycle threshold method outlined in user bulletin No. 2 provided by Applied Biosystems. A nontemplate control (NTC) was included for each target analyzed.

2.7. Statistical analysis of patient data, and of correlation of microarray and RT-PCR data

Age and gender of patients, pH of CSF and the net number of 'present' oligonucleotide sequences in-between patient groups were compared using the Kruskal–Wallis and the Mann–Whitney test. *P* values <0.05 were considered significant. Linear regression analysis was used to calculate the correlation coefficiency for comparison of expression levels measured by microarray and RTPCR. Quantitative significance of r was calculated by ANOVA. *F* values <0.01 were considered significant.

3. Results

3.1. Characterization of patient material

The clinical characteristics of MS patients and control subjects are described in Table 1. MS patients (5 women, 1 man; mean age (range) 52 (40–81) years) were all in the secondary progressive phase of clinically definitive MS (Lublin and Reingold, 1996), the mean disease duration (range) was 20 (10–49) years. None of these patients has been treated with specific immunomodulatory therapies, such as IFN-β or glatiramer acetate. The pH of CSF (average [range]) were similar in the MS group and control group (6.58 [6.30–6.95] and 6.55 [6.22–7.04], respectively; $P=0.96$) ensuring equal conditions for RNA stability and quality (Preece and Cairns, 2003).

The control group consisted of patients (8 women, 4 men) who died from non-neurological diseases and whose mean age (72 (range 51–91) years) was not different from MS patients ($P>0.05$).

All MS lesions were of active demyelinating type as identified by immunohistochemical staging criteria previously described (Van der Valk and De Groot, 2000) (not shown). In $NAWM_{MS}$ and white matter tissue from controls, no signs of inflammation or myelin loss were detectable using the markers described in Methods (not shown).

3.2. Microarray analysis of MS tissue

Of the 12,566 probe set sequences surveyed, 35.8%, 36.5% and 35.0% were present (abs call ≥0.5) in lesions, $NAWM_{MS}$ and controls, respectively ($P>0.05$, Kruskal–Wallis and Mann–Whitney). In MS lesions, we identified 129 sequences that were differentially regulated (94 up- and 35 downregulated) (Table 2) as compared to controls (Fig. 1, only the 50 genes with the highest up-regulation are depicted). Accordingly, in $NAWM_{MS}$, 48 sequences were

Table 1
Details of multiple sclerosis and control autopsy tissues

Patient case no.	Diagnosis	Duration of disease (years)	Age (years)	Gender	Postmortem delay (hh:min)	pH of CSF	Cause of death	Tissue type used for the analysis	
97-160[a]	spMS	11	40	F	7:00	6.33	aspiration pneumonia, cardiac failure	lesion	$NAWM_{MS}$
96-121[a]	spMS	19	53	F	7:15	6.54	pneumonia	lesion	$NAWM_{MS}$
96-040	spMS	11	35	F	5:45	6.41	cachexia	lesion	–
96-076	spMS	49	81	F	4:15	6.93	cachexia	lesion	$NAWM_{MS}$
99-054	spMS	20	58	F	8:10	6.30	suicide	lesion	$NAWM_{MS}$
95-057	spMS	10	46	M	8:10	6.95	cachexia	–	$NAWM_{MS}$
98-125[a]	Control	–	58	F	6:15	6.31	septicaemia, pneumonia	WM	
94-125[a]	Control	–	51	M	6:00	6.50	liposarcoma, ileus	WM	
98-127[a]	Control	–	56	M	5:25	6.55	cardiac infarction	WM	
98-143	Control	–	91	F	4:50	6.49	cachexia	WM	
98-148	Control	–	54	F	5:35	6.40	liver cirrhosis, vascular	WM	
98-157	Control	–	85	M	5:13	6.23	cardiac	WM	
95-092	Control	–	63	F	6:25	6.67	suicide	WM	
94-063	Control	–	74	F	5:35	7.04	mesenterial thrombosis	WM	
95-062	Control	–	80	M	4:30	6.22	renal failure	WM	
94-074	Control	–	85	F	5:11	6.95	pneumonia	WM	
94-123	Control	–	76	F	9:20	6.50	respiratory failure	WM	
97-008	Control	–	88	F	3:45	6.74	cachexia	WM	

[a] Same samples used previously (Lindberg et al., 2001).

differentially expressed (31 up- and 17 downregulated) (Table 3). Thirty-seven of the 125 sequences (30%) upregulated in lesions or $NAWM_{MS}$, were not detectable in control brain tissue.

As a number of genes are represented with two or more probe sets on the microarray, current results demonstrate differential regulation of five genes (α-tubulin (#55,62), β-tubulin (#69,73,78), CAP2 (#59,64,72), CAMK2G (#7,12) (Table 2) and MAF (#130,133) (Table 3)) with independent sequences; change factors and *P* values of redundant sequences (indicated by brackets on Table 2 and 3) were highly congruent which supports that measurements of gene expression levels with microarrays are technically consistent and of quantitative character.

Seventy percent (123/177) of sequences that were significantly regulated in either lesions or $NAWM_{MS}$ showed transcriptional changes into the same direction in the respective other tissue compartment. However, only eight genes reached the set significance levels for differential expression in both lesions and $NAWM_{MS}$ (marked with green in Fig. 1 and Tables 2–4). In contrast, up-regulation was specific for 51 genes in lesions and for 3 genes in $NAWM_{MS}$, as expression was not different from levels in control in the respective other MS tissue compartment (gene numbers marked with brown background in Tables 2–4). We could not identify any gene that showed significant inverse regulation in lesions as compared to $NAWM_{MS}$.

3.3. Validation of microarray data with real-time PCR

In order to validate the results of microarray analysis, we measured the transcriptional regulation of 22 randomly chosen genes regulated in lesions or $NAWM_{MS}$ using quantitative real-time PCR (Table 4). Among those, four out of eight targets were included that showed significant regulation in both lesions and $NAWM_{MS}$. Table 4 summarizes the results of statistical analysis of all genes comparatively analysed: RT-PCR-based measurements confirmed those of microarray analysis for more than 92% (20/22) of genes, including those four, which were significantly downregulated in both lesions and $NAWM_{MS}$. The two genes whose differential expression could not be confirmed by RT-PCR were FANCC (#139) and IRF5 (#145); in both, the sequences of primer/probe sets used for RT-PCR did not overlap with those on the gene chip.

3.4. Gene categorization

We categorized differentially expressed genes into four functional groups, i.e. (a) transcriptional control, (b) immune response, (c) neural homeostasis and (d) unknown, to delineate specific patterns of gene regulation linked to inflammation and degeneration in the two tissue compartments of MS (Tables 2 and 3).

3.4.1. Lesions

Inflammatory changes are believed to be the pathoanatomical hallmark of active lesions as analyzed here. Hence, genes involved in immune response were expected to be the prevailing category in this tissue compartment. However, only 21% (26/123) of differentially regulated genes fell in this category which we subcategorized into genes involved in humoral and cellular immune response (Table 2). Although 77% (20/26) belonged to the latter group, the four genes with the highest change factor (#21,22,23,24) as compared to controls encode all for various immunoglobulin sequences, emphasizing the preeminent role of a specific humoral immune response in lesions. Quantitatively, their

Table 2
Categorization of differentially expressed genes in lesions

No	Probe set Affymetrix	Gene symbol	Accession no.	Description	Function [related disease]		Change factor
				1. Transcriptional control			
1	33630_s_at	SPTBN2	AF026488	beta iii spectrin (sptbn2) mrna	vesicle protein, cytoskeletal structural protein, actin binding		4.98
2	39974_at	ENTPD3	AF039917	cd39l3 mrna, ectonucleoside triphosphate diphosphorylase 3	catabolism of extracellular nucleotides		4.34
3	36049_at	CPLX2	W27899	39c4 cdna, homolog to rat complexin 2	regulates SNAP receptor function		3.92
4	33712_at	SULT4A1	N63574	yy63f05.s1 cdna	sulfotransferase 4A family		3.65
5	38719_at	NSF	U03985	n-ethylmaleimide-sensitive factor	ATPase activity of NSF is for membrane fusion, complexed with SNAPs and SNAREs		3.41
6	37214_g_at	DNASE1L1	X90392	dnase x gene	transcriptional regulation		3.01
7	32105_f_at	CAMK2G	U66063	calcium/calmodulin-dependent protein kinase ii gamma	phosphorylation of neurotransmitters, activation-induced T-cell differentiation		2.51
8	37749_at	MEST	D78611	mest mrna	expressed ubiquitously during development		2.21
9	39829_at	ARL7	AB016811	adp ribosylation factor-like protein	GTP-binding protein, GTPase, hydrolase, endocytosis, vesicle traffic		2.2
10	40808_at	CHGA	U03749	chromogranin a (chga) gene	regulation of hormone secretion		2.19
11	34847_s_at	CAMK2B	AF112471	calcium/calmodulin-dependent protein kinase ii beta	phosphorylation of neurotransmitters, activation-induced T-cell differentiation		2.13
12	650_s_at	CAMK2G	I07044	calcium/calmodulin-dependent protein kinase (camk) gamma	phosphorylation of neurotransmitters, activation-induced T-cell differentiation		2.05
13	407_at	PTPRB	X54131	hptp beta mrna for protein tyrosine phosphatase beta	dephosphprylation		1.72
14	40182_s_at	CARM1	AF055027	clone 24658 mrna sequence, coactivator of transcription	regulation of hormone-responsive genes		1.7
15	426_at	PGR	X51730	mrna and promoter dna for progesterone receptor	transcriptional activation		1.51
16	37752_at	EIF4E	M15353	cap-binding protein mrna, translation initiation factor	protein synthesis		–1.51
17	34721_at	FKBP5	U42031	54 kda progesterone receptor-associated immunophilin fkbp54	transcriptional regulation, inhibition of calcineurin		–1.67
18	35811_at	RNF13	AF037204	ring zinc finger protein (rzf) mrna	regulation of transcription		–1.7
19	40202_at	BTEB1	D31716	GC box bindig protein	transcriptional regulation		–1.78
20	39059_at	DHCR7	AF034544	delta7-sterol reductase	cholesterol metabolism		–2.14
				2. Immune response			
21	38194_s_at	IGKC	M63438	ig rearranged gamma chain mrna, v-j-c region	Ig synthesis	H	51.23
22	33274_f_at	IGL	M18645	ig rearranged lambda-chain mrna vjc-region subgroup lambda-iv	Ig synthesis	H	16.76
23	33273_f_at	IGGL1	X57809	rearranged immunoglobulin lambda light chain	Ig synthesis	H	13.39
24	37006_at	IGJ	AI660656	wf23c07.x1 cdna, immunoglobulin J	Ig synthesis	H	9.68
25	35081_at	FGF9	D14838	fgf-9, fibroblast growth factor 9, glia activating factor	signal tranduction for cell proliferation	C	5.33
26	39395_at	THY1	AA704137	ag47g01.s1 cdna, Thy-1 (CD90) T-cell antigen	cell-cell interaction in T-cells and neuroglial cells	C	3.23
27	34412_s_at	PNUTL1	U59632	h5 mrna, partial cds; and platelet glycoprotein ib beta chain	GTPase activity, cell division	C	2.86
28	173_at	CDH18	U59325	cadherin-14, Ca2+ dependent cell adhesion molecule	cell adhesion, neural cell-cell interaction	C	2.56
29	985_s_at	IL6R	X12830	interleukin-6 (il-6) receptor	signaling	C	2.37
30	31586_f_at	-	X72475	rearranged ig kappa light chain variable region	Ig synthesis	H	2.36
31	33800_at	ADCY9	AF036927	adenylyl cyclase type ix	intracellular signaling	C	2.29
32	41544_at	SNK	AF059617	serum-inducible kinase	regulates M phase in cell cycle, functions in wound healing [neoplasia]	C	1.85
33	39288_at	NECL1	AI951798	nectin like protein1, brain immunoglobulin receptor precursor	adhesion molecule?, Ig superfamily	H	1.76
34	34922_at	CDH19	AF047826	cadherin-7 (cdh7)	cell adhesion, neural cell-cell interaction	C	–1.55
35	1585_at	ERBB3	M34309	epidermal growth factor receptor (her3), receptor tyrosine kinase	growth regulation, signalling	C	–1.61
36	32526_at	JAM3	AA149644	zl39d08.s1 cdna, 3 end, junctional adhesion molecule 3	leukocyte emigration	C	–1.62
37	41124_r_at	ENPP2	L35594	autotaxin	cell motility and migration	C	–1.65
38	670_s_at	H_GS165L15.1	I05515	camp response element-binding protein (cre-bp1)	intracellular TGF-beta signaling	C	–1.69
39	33596_at	HSAJ1454	AJ001454	testican-3, serine protease inhibitor	inhibition of proteases including matrix metalloproteinases	C	–1.77
40	38402_at	LAMP2	U36336	lysosome-associated membrane protein-2b	protection of lysosomal membrane from proteolysis	C	–1.79
41	39100_at	SPOCK	X73608	testican	cell adhesion, migration, proliferation	C	–1.89
42	659_g_at	THBS2	I12350	thrombospondin 2 (thbs2)	functions in cell adhesion and migration	C	–1.94
43	32001_s_at	PACE4	M80482	subtilisin-like protein (pace4)	proteolysis	C	–1.97
44	40425_at	EFNA1	M57730	b61 mrna, induced by cytokines	immediate-early response gene of endothelium	C	–1.97
45	1767_s_at	TGFB3	X14885	transforming growth factor-beta 3 (tgf-beta 3)	negative MMP-regulation, ECM deposition, axonal guidance?	C	–3.05
46	32521_at	SFRP1	AF056087	secreted frizzled related protein	up-regulated in apoptosis	C	–3.23
				3. Neural homeostasis			
47	33764_at	GPR51	AF056085	gaba-b receptor	neurotransmission	F	9.17
48	32138_at	DNM1	I07807	dynamin, GTP-binding protein	receptor mediated endocytosis, interacts e.g. with endophilin, cell trafficking	F	8.13
49	38800_at	STMN2	D45352	humhg17416 cdna, stathmin-like 2	neuronal growth-associated protein, regulation of microtubule dynamics	S	6.88
50	38484_at	SNAP25	D21267	synaptosome-associated protein	synaptic vesicle membrane docking and fusion	F	6.83
51	38870_at	GFRA2	U97145	ret ligand 2 (retl2)	activates GDNF/RET-complex, neuroprotection [familial Hirschsprung disease]	F	6
52	40314_at	SYNGR3	AJ002309	synaptogyrin 3, associated with presynaptic vesicles	regulator of Ca2+ dependent exocytosis in vesicles	F	5.23
53	34208_at	SLC12A5	U79245	clone 23586 mrna, potassium-chloride transporter	synaptic inhibition	F	4.85
54	40075_at	SYT1	M55047	synaptotagmin	Ca2+ sensor in vesicular trafficking and exocytosis	F	4.25
55	36591_at	TUBA	X06956	halpha44 gene for alpha-tubulin	cytoskeletal element of microtubules	S	4.17
56	35711_at	GA	AF038170	clone 23817 mrna sequence	glutamate metabolism	F	3.94
57	31901_at	KCNAB2	AF044253	potassium channel beta 2 subunit (hkvbeta2.2), alternatively spliced	regulatory voltage-gated channel in neurons	F	3.9
58	39930_at	EPHB6	D83492	eph-family protein, kinase defective Eph-like receptor	neural development	S	3.89
59	33404_at	CAP2	U02390	adenylyl cyclase-associated protein homolog cap2	interacts with actin, regulatory protein	S	3.49
60	40378_at	SH3GLB1	AF036268	een-b1 mrna	synaptic vesicle recycling, interacts with dynamin, synaptojanin and huntingtin	F	3.45
61	33728_at	BSN	AF052224	neuronal double zinc finger protein (znf231), bassoon	presynaptic cytometrix protein	S	3.14
62	330_s_at	-	HG2259-HT2348	TIGR, tubulin alpha 1, isoform 44	cytoskeletal element of microtubules	S	2.79
63	39646_at	CACNB2	S60415	voltage-dependent Ca2+ channel (Lambert-Eaton syndrome)	cell surface molecule, receptor, signalling	F	2.75
64	693_g_at	-	HG2530-HT2626	TIGR, Adenylate cyclase-associated protein2	interacts with actin, regulatory protein	S	2.72
65	31887_at	CKMT1	J04469	mitochondrial creatine kinase (ckmt)	energy metabolism	F	2.65
66	32606_at	BASP1	AA135683	brain abundant, membrane attached signal protein 1	intracellular Ca2+ signalling	F	2.53
67	38516_at	SCN1B	I10338	sodium channel beta-1 subunit (scn1b), voltage-gated	synaptic transmission, Na+ transport	F	2.52
68	39780_at	PPP3CB	M29551	calcineurin a2	activates dendritic cells, Ca2+ signalling, endocytosis in nerve terminals	F	2.43
69	296_at	-	HG4322-HT4592	TIGR, tubulin	cytoskeletal element of microtubules	S	2.31
70	33942_s_at	STXBP1	AF004563	hunc18b alternatively-spliced mrna, syntaxin binding protein	vesicle trafficking and neurotransmitter release	F	2.22
71	39665_at	GLRB	U33267	glycine receptor beta subunit (glrb)	neurotransmission, hyperpolarisation, inhibition of neuronal firing	F	2.19
72	33405_at	CAP2	N90755	adenylyl cyclase-associated protein 2	interacts with actin, regulatory protein	S	2.11
73	39331_at	TUBB	X79535	beta tubulin, clone nuk_278	cytoskeletal element of microtubules	S	1.94
74	31814_i_at	LRP3	AB009462	hlrp105 mrna for ldl receptor related protein 105	receptor mediated endocytosis (?)	F	1.88
75	34382_at	DCX	AJ003112	doublecortin	cortical neuronal migration and differentiation, microtubule stabilization, [DC syndrome]	S	1.87
76	34408_at	RTN2	AF004222	rtn2-a, reticulon	inhibition of neuronal regeneration	F	1.86
77	41435_at	PPFIA3	AB014554	protein tyrosine phosphatase, receptor type	axon guidance, cell-matrix interaction	F	1.79
78	297_g_at	-	HG4322-HT4592	TIGR, tubulin beta	cytoskeletal element of microtubules	S	1.79
79	37780_at	PCLO	AB011131	piccolo, presynaptic cytomatrix protein	involved in the cycling of synaptic vesicles	F	1.76
80	34520_at	HCN2	AJ012582	hyperpolarization-activated cation channel hcn2	cation transport	F	1.54
81	33345_at	KIF3C	AF035621	kinesin-related protein (kif3c)	transport of vesicles in axons	F	1.53
82	35978_at	PRRG1	AF009242	proline-rich gla protein 1 (prgp1), vitamin K-dependent membrane protein	modification of glutamate	F	–1.51
83	36532_at	SYNJ2	AF039945	synaptojanin 2b	synaptic vesicle recycling and actin function	F	–1.52
84	41688_at	TM4SF11	AI688299	wc87h10.x1 cdna, 3 end, plasmolipin	myelin protein, induces formation of ion channels [Bardet-Biedl syndrome]	S	–1.56
85	32094_at	CHST3	AB017915	chondroitin 6-sulfotransferase, ECM-protein	maintenance of naive T lymphocyte	S	–1.57
86	34972_s_at	DNEL2	AJ000522	dynein heavy chain	cell trafficking, transport of vesicles in axons	F	–1.58
87	33809_at	GNAI1	AL049933	guanine nucleotide binding protein	G-protein signaling, inhibitory	F	–1.6
88	38403_at	LAMP-2	X77196	lysosome-associated membrane protein-2	protects the lysosomal membrane from proteolysis in neurons	F	–1.69
89	37580_at	SH3GL3	AF036271	een-b2-l3, SHS-domain, GRB2-like 3	signal transduction for cell polarisation and motility, interacts with huntingtin	F	–1.79
90	38243_at	NCAM1	X16841	nontransmembrane isoform of n-cam from skeletal muscle	neural cell adhesion among neurons and between neurons and muscle	F	–1.81

Table 2 (*continued*)

No	Probe set Affymetrix	Gene symbol	Accession no.	Description	Function [related disease]		Change factor
91	39654_at	ASPA	S67156	asp=aspartoacylase	NAA metabolism in oligodendrocytes, myelin synthesis	F	–1.97
92	40387_at	EDG2	U80811	lysophosphatidic acid receptor homolog	G-protein-coupled receptor, LPA signalling during cortical neurogenesis	F	–2.03
93	642_s_at	PS1	I76528	presenilin (ps1)	neuronal degeneration	S	–2.07
94	31525_s_at	-	J00153	alpha globin gene cluster on chromosome 16- zeta gene	O2-homeostasis in neurons, marker for neuronal damage	F	–2.65
				4. Unknown			
95	41827_f_at	-	AI932613	wo05c02.x1 cdna, 3 end			10.81
96	32093_at	KIAA0444	AB0079139	kiaa0444 protein			7.8
97	36563_at	DKFZP761N09	AF038190	clone 23582 mrna sequence			6.91
98	32090_at	KIAA0479	AB007948	kiaa0479 protein			5.75
99	41675_at	KIAA0656	AB014556	kiaa0656 protein			5.52
100	41107_at	KIAA0374	AB002372	kiaa0374 gene			3.89
101	39619_at	LOC51308	AF070551	clone 24515 mrna sequence			3.86
102	36556_at	KIAA0672	AB014572	kiaa0672 protein			3.84
103	41559_at	KIAA1201	AA434319	zw24f03.r1 cdna, 5 end			3.81
104	936_s_at	-	HG3570-HT3773	TIGR, protein phosphatase inhibitor homolog			3.57
105	35949_at	KIAA0774	AB018317	kiaa0774 protein, partial cds			3.46
106	39698_at	SMAP31	U51712	hsu51712 cdna, lung cancer-associated Y protein			2.97
107	39026_r_at	MEG3	AF052114	clone 23887 mrna sequence			2.92
108	32652_g_at	NP25	W28770	51d8 cdna, neuronal protein			2.76
109	32345_at	-	AL09696	cdna clone euroimage 21920			2.35
110	35780_at	KIAA0657	AF035292	clone 23584 mrna sequence, immunoglobulin like			2.15
111	37595_at	-	AF038201	clone 23555 mrna sequence			2.14
112	36750_at	KIAA0318	AB002316	kiaa0318 gene, partial cds			2.06
113	40500_at	NDGR4	AF007138	clone 23631 mrna sequence			2.05
114	32712_at	KIAA1106	AB029029	kiaa1106 protein, partial cds			1.93
115	41704_at	-	I35592	germline mrna sequence			1.88
116	37067_at		L10374	clone ctg-a4, neuronal			1.88
117	37566_at	KIAA1045	AB028968	kiaa1045 protein, partial cds			1.87
118	37839_at	-	AL109700	cdna clone euroimage 51358			1.85
119	36025_at	KIAA0337	AB002335	kiaa0337 gene, complete cds			1.85
120	36155_at	KIAA0275	D87465	kiaa0275 gene, complete cds			1.8
121	40900_at	-	AI382123	te30a09.x1 cdna, non-muscle myosin heavy chain B, immunogenic			1.8
122	38008_at	D4S234E	M98528	neuron-specific protein gene, last exon, clone d4s234			1.68
123	39650_s_at	KIAA0435	AB007895	kiaa0435 mrna, complete cds			1.65
124	33353_at	-	W26466	32f11 cdna			1.56
125	39572_at	-	AI401567	tg28f02.x1 cdna, 3 end, EST			1.54
126	34809_at	KIAA0999	H53921	yq87g03.r1 cdna, 5 end			–1.54
127	35310_at	-	D45288	humhg2121 cdna			–1.89
128	37169_at	KIAA0390	AB002388	kiaa0390 gene, complete cds			–2.41
129	39526_at	KIAA1155	AA648931	ns41c12.s1 cdna, 3 end			–2.7

change factors ranged highest among all upregulated genes in MS tissue. Half (13/26) of immune response genes were downregulated. Here, the most striking feature is that many of them, like cre-bp1 (#38), testican-3 (#39), LAMP 2 (#40), SPOCK (#41), and TGF-β3 (#45), exert anti-inflammatory properties, suggesting a functional deficit of genes counter-regulating excessive inflammation.

Differentially regulated genes operative in neural homeostasis formed the largest category (45/123, 37%) and outnumbered immune-related genes by almost a factor of 2. Of those, two thirds regulated specific functional systems, such as vesicle recycling, cell trafficking and synaptic neurotransmission as opposed to genes coding for cytoskeleton proteins (tubulins #55,62,69,73,78), and genes functionally related to microtubules (STMN2 #49, doublecortin #75) and actin filaments (CAP2 #59,64,72). Interestingly, reticulon (#76), which is known to inhibit neuronal regeneration, was upregulated, whereas presenilin (#93), which is involved in neuronal degeneration, was downregulated. Genes for neurofilament light (NF-L) and medium (NF-M) subunits were highly upregulated (change factors 30.99 and 9.24, respectively), but did not match the set significance levels ($P = 0.048$ and 0.037, respectively) on microarrays. With RT-PCR analysis instead, differential regulation in lesions could be confirmed for both genes ($P < 0.006$).

3.4.2. $NAWM_{MS}$

Immune-related genes represented the predominant category regulated in $NAWM_{MS}$; with 40% (19/47), the score was almost double as high as in lesions (Table 3). Most of these genes are involved in signaling and effector functions of the cellular immune response, i.e. blood–brain barrier (BBB) disruption and leukocyte extravasation (e.g. MMP-2 #146) (Rosenberg, 2002) and lymphocyte activation (e.g. IL-1β-convertase #141), whereas only few upregulated genes are involved in specific humoral immune response processes. As in lesions, the anti-inflammatory genes TGF-β3 (#156) and cre-bp-1 (#154) that downregulate MMP activities (Blavier et al., 2001) showed decreased expression levels. Of note, of the eight genes significantly regulated both in $NAWM_{MS}$ and lesions, all but one (87%) were downregulated.

3.4.3. Genes involved in transcriptional control or with unknown functions

Many differentially regulated genes could not be assigned to a specific function in MS pathogenesis. This applies for the category of pleiotropic genes involved in transcriptional control and a large series of genes whose protein product (28% (35/123) in lesions, and 17% (8/47) in NAWM) remains currently unknown.

3.5. Additional genes found to be differentially regulated in MS in other largescale expression analysis studies

Microarray analysis could confirm upregulated expression in lesions and/or $NAWM_{MS}$ for only five genes (Igλ chain (#23), CACNB2 (#63), SPOCK (#41), TGF-β3

Table 3
Categorization of differentially expressed genes in $NAWM_{MS}$

No	Affymetrix Probe set	Gene symbol	Accession no.	Description	Function [related disease]		Change factor
				1. Transcriptional control			
130	41505_r_at	MAF	AF055376	short form transcription factor c-maf (c-maf) mrna	transcriptional regulation		1.81
131	37214_g_at	DNASE1L1	X90392	dnase x gene	transcriptional regulation		1.64
132	40604_at	DYRK2	Y13493	protein kinase dyrk2	phosphorylates protein-synthesis initiation factor, eIF2B epsilon		1.53
133	41504_s_at	MAF	AF055376	short form transcription factor c-maf (c-maf)	transcriptional regulation		1.43
134	40742_at	HCK	M16591	hemopoietic cell protein-tyrosine kinase (hck) gene, clone lambda-a2/1a	signaling		1.38
135	34254_at	RALGDS	U14417	ral guanine nucleotide dissociation stimulator	transcriptional regulation		1
136	125_r_at	CDK10	X78342	pisslre, cyclin-dependent kinase 10	negative control of cell proliferation		–1.28
137	39059_at	DHCR7	AF034544	delta7-sterol reductase	cholesterol metabolism		–1.4
138	40202_at	BTEB1	D31716	GC box binding protein, Zinc finger protein	transcriptional regulation		–1.49
				2. Immune response			
139	1982_s_at	FANCC	X66893	facc mrna from complementation group c (fa(c))	suppresses IFN-g-caspase-mediated apoptosis, DNA repair [Fanconi anemia]	C	2.4
140	1319_at	DDR2	X74764	receptor protein tyrosine kinase	collagen receptor, MMP up-regulation, [dwarfism in knockout mice]	C	1.7
141	574_s_at	CASP1	M87507	interleukin-1 beta convertase (il1bce), caspase 1, apoptosis-related cysteine protease	activation of interleukin-1 beta, cytokine signaling	C	1.62
142	36889_at	FCER1G	M33195	fc-epsilon-receptor gamma-chain	immunoreceptor tyrosine-based activation	H	1.43
143	37918_at	ITGB2	M15395	integrin beta2 subunit (CD18)	ligand for ICAM-1 (CD54), -2 (CD102), and -3 (CD50), fibrinogen, iC3b, [leukocyte adhesion deficiency]	C	1.42
144	36131_at	CLIC1	AJ012008	chloride intracellular channel 1	nuclear membrane associated, transport of small molecules, chloride intracellular channel protein 1	H	1.39
145	478_g_at	IRF5	U51127	interferon regulatory factor 5 (humirf5)	induction of Type I IFN genes, mediates T cell recruitment via stimulation of chemokine transcription	C	1.33
146	39007_at	MMP-2	M55593	collagenase type iv (clg4) gene, matrix metalloproteinase 2	proteolysis, ECM-modelling	C	1.2
147	38894_g_at	NCF4	AL008637	ncf4 (p40phox) protein,cytokine receptor common beta chain precursor csf2rb	defense response	C	1.19
148	34433_at	DOK1	AF035299	clone 23863 mrna, docking protein 1, 62kDa (downstream of tyrosine kinase 1)	cell surface receptor linked signal transduction	C	1.16
149	1278_at	-	HG162-HT3165	TIGR-sequence, tyrosine kinase, receptor Axl	signaling	C	1.1
150	35869_at	MD-1	AB020499	bcg-regulated mrna for md-1 homologue	activates B cell proliferation, associated with B-cell surface molecule RP-105	H	1.06
151	39038_at	FBLN5	AF093118	up50 mrna, fibulin 5, ECM-protein	cell-cell matrix adhesion, integrin ligand	C	1.05
152	31875_at	ASB1	AF055024	clone 24763, ankyrin repeat, SOCS box-containing 1	suppression of cytokine signaling (socs)	C	–1.15
153	1731_at	PDGFRA	M21574	platelet-derived growth factor receptor alpha (pdgfra)	signal transduction, cell proliferation	C	–1.18
154	670_s_at	H_GS165LI5.1	I05515	camp response element-binding protein (cre-bp1)	intracellular TGF-beta signaling	C	–1.29
155	37676_at	PDE8A	AF056490	camp-specific phosphodiesterase 8a (pde8a)	regulation of intracellular concentration of cyclic nucleotides	C	–1.47
156	1767_s_at	TGFb3	X14885	transforming growth factor-beta 3 (tgf-beta 3)	negative MMP-regulation, ECM deposition, axonal guidance?	C	–1.69
157	35680_r_at	DPP6	M96860	dipeptidyl aminopeptidase like protein	Similarity with T-cell activation antigen CD26	C	–2.04
				3. Neural homeostasis			
158	33871_s_at	FOLR2	J02876	placental folate binding protein	folate transport	S	3.76
159	39043_at	ARPC1B	AF006084	arp2/3 protein complex subunit p41-arc (arc41), actin related protein 2/3 complex	promotes actin assembly, cell motility	S	1.65
160	37845_at	HEM1	M58285	membrane-associated protein (hem-1)	hematopoiesis	F	1.12
161	33412_at	LGALS1	AI535946	vicpro2.d07.r cdna, lectin, galactin1	regulation of cell proliferation and cell adhesion	F	1.11
162	34091_s_at	VIM	Z19554	vimentin, actin interactin protein	maintainance of intermediate filaments	S	1.1
163	33626_at	CACNA1E	I27745	voltage-operated calcium channel, alpha-1E subunit	transport of small molecules, synaptic transmission	F	1.01
164	33535_at	P2RX1	U45448	P2x1 purinergic receptor, ligand-gated ion channel 1	transport of small molecules, ATP-mediated Ca2+ influx, signal transduction	F	1.01
165	39654_at	ASPA	S67156	asp=aspartoacylase	NAA metabolism in oligodendrocytes, myelin synthesis	F	–0.99
166	36121_at	EPN2	AB028988	kiaa1065 protein, epsin 2	clathrin mediated endocytosis in vesicles in nerve terminals	F	–1.12
167	37580_at	SH3GL3	AF036271	een-b2-l3, SHS-domain, GRB2-like 3	signal transduction for cell polarisation and motility, interacts with dynamin, synaptojanin and huntingtin	F	–1.19
168	31687_f_at	HBB	M25079	beta-globin	neuronal oxygen-homeostasis	F	–1.73
169	31525_s_at	-	J00153	alpha globin gene cluster on chromosome 16- zeta gene	O2-homeostasis in neurons, marker for neuronal damage	F	–2.23
				4. Unknown			
170	32502_at	PP1665	AL041124	dkfzp434d0316_s1 cdna, hypothetical protein PP1665			1.28
171	38754_at	P8	AI557295	pt2.1_16_d02.r cdna			1.25
172	40330_at	-	AL050031	cdna dkfzp566e0224 (from clone dkfzp566e0224)			1.06
173	422_s_at	MAX	X66867	max gene			1.04
174	40478_at	DJ971N18.2	AL021396	clone 971n18 on chromosome 20p12 contains processed pseudogene phkbp1			1
175	34921_at	KIAA0420	AB007880	kiaa0420 mrna			–1.19
176	39120_at	MT1L	AA224832	nc33b06.s1 cdna, metallothionein 1L			–1.46
177	35959_at	KIAA0844	AB020651	kiaa0844 protein			–1.81

Table 4
Validation of microarray data with real-time PCR

No #	Target tissue	Gene	Gene symbol	Change factor in microarray	p-value in microarray	Change factor in RT-PCR	p-value in RT-PCR	Correlation coefficient
50	Lesion	SNAP25	SNAP25	6.83	0.001	100.50	0.006	0.931
51	″	Receptor tyrosine kinase RET ligand 2	GFRA2	6.00	0.018	12.34	0.003	0.891
52	″	Synaptogyrin 3	SYNGR3	5.23	0.017	46.43	0.005	0.820
54	″	Synaptotagmin	SYT1	4.25	0.022	90.14	0.006	0.975
59	″	Adenylyl cyclase-associated protein	CAP2	3.49	0.003	6.54	0.003	0.653
68	″	Calcineurin A2	PPP3CB	2.43	0.007	7.48	0.003	0.874
31	″	Adenylyl Cyclase type IX	ADCY9	2.29	0.024	2.05	0.003	0.671
71	″	Glycine receptor beta subunit	GLRB	2.19	0.005	1.72	0.003	0.877
32	″	Serum-inducible kinase	SNK	1.85	0.023	1.80	0.028	0.628
36	″	cAMP response element binding protein	H_GS165LI5.1	–1.69	0.0002	–2.41	0.020	0.771
89	″	Endophilin	SH3GL3	–1.79	0.00001	–20.94	0.003	0.823
41	″	Testican	SPOCK	–1.89	0.003	–1.23	0.057	0.682
91	″	Aspartoacylase	ASPA	–1.97	0.002	–5.40	0.007	0.507
45	″	TGF-beta 3	TGFb3	–3.05	0.0001	–4.71	0.010	0.939
139	$NAWM_{MS}$	Complementation group C	FANCC	2.40	0.001	0.08	NS	0.176
140	″	Discoidin domain receptor 2	DDR2	1.7	0.013	2.16	0.039	0.600
143	″	Leukocyte adhesion protein	ITGB2	1.42	0.006	0.91	0.064	0.606
145	″	Interferon regulatory factor 5	IRF5	1.33	0.024	0.60	NS	0.201
146	″	Matrix metalloproteinase-2	MMP-2	1.20	0.016	0.37	0.041	0.412
161	″	Galactin 1	LGALS1	1.11	0.007	0.78	0.055	0.434
162	″	Vimentin	VIM	1.1	0.008	0.87	0.014	0.663
163	″	Voltage-operated calcium channel	CACNA1E	1.01	0.023	3.37	0.042	0.557
165	″	Aspartoacylase	ASPA	–1.00	0.015	–1.29	0.020	0.507
167	″	Endophilin	SH3GL3	–1.19	0.00004	–0.54	0.025	0.700
154	″	cAMP response element binding protein	H_GS165LI5.1	–1.29	0.0002	–1.22	0.020	0.771
156	″	TGF-beta 3	TGFb3	–1.69	0.001	–1.74	0.010	0.939

(#45,156), vimentin (#162)) identified by others, but failed to do so for 15 other genes operative for immune functions and neural homeostasis (Table 5) (Becker et al., 1997; Whitney et al., 1999, 2001; Chabas et al., 2001; Lock et al., 2002; Baranzini et al., 1999). We therefore evaluated our microarray data more closely for the latter group and measured, where possible, expression levels by RT-PCR. Table 5 shows that in 93% (14/15) of these genes transcriptional changes in MS tissue, as measured by RT-PCR, pointed into the same direction as described in earlier publications, although they were not identified by microarray due to their failure to match set levels for statistical significance ($P < 0.025$) or change factor ($-1.0 > x > 1.0$).

4. Discussion

Since the first descriptions of MS by Cruveilhier and Carswell in the early 19th century, the disseminated formation of focal lesions, was believed to be the pathoanatomical hallmark of MS (Compston, 1998). In contrast, the surrounding white matter seemed to be unaffected on macroscopic inspection. However, subtle histopathological alterations, such as edema (Dousset et al., 1992), demyelination (Griffin et al., 2002), gliosis and axonal damage (Bjartmar et al., 2003), can be detected there and imply an active disease process outside of lesions which led to the introduction of the term ‘normal appearing white matter’ (NAWM) in the early 1970s (Suzuki et al., 1973). These NAWM changes develop independently of BBB damage and contribute eventually to progressive brain atrophy which is the preeminent determinant for long-term disability (for review, see Fisher and Rudick, 2003). Microarray technology provides an ideal tool to study the network of transcriptional gene regulation in MS (Dyment and Ebers, 2002). Earlier studies using microarrays or other high-throughput techniques have established pronounced differences in transcriptional gene expression in MS lesions (Becker et al., 1997; Whitney et al., 1999, 2001; Chabas et al., 2001; Lock et al., 2002; Tajouri et al., 2003). These reports did not reveal a consistent pattern of gene regulation as they based on the analysis from only one (Becker et al., 1997; Whitney et al., 1999) or few patients (Whitney et al., 2001; Chabas et al., 2001; Lock et al., 2002) of mixed (relapsing–remitting/secondary progressive and primary progressive) types of MS, and of different stages of plaque evolution (Whitney et al., 1999, 2001; Chabas et al., 2001; Lock et al., 2002; Tajouri et al., 2003). Lock et al. (2002) were the first to define specific genes upregulated during certain phases of plaque formation: the immunoglobulin Fc receptor was found to be upregulated in chronic inactive lesions, while G-CSF was upregulated in acute lesions. However, all these studies focused on lesional changes, as compared to normal control brain tissue (Becker et al., 1997; Whitney et al., 2001; Chabas et al., 2001, 2002), or donor-matched NAWM (Whitney et al., 1999; Tajouri et al., 2003), which precluded the definition of a specific gene expression pattern in NAWM in MS.

Table 5
Comparison of the genes, which were reported in the literature to be differentially expressed in MS tissue compared to control WM

Affymetrix Probe set	Gene symbol	Accession no.	Description	Mean intensity in control WM in microarray	Mean intensity in lesions in microarray	Change factor in lesion in microarray	p-value in lesion in microarray	Change factor in lesion in RT-PCR	p-value in lesion in RT-PCR	Mean intensity in $NAWM_{MS}$ in microarray	Change factor in $NAWM_{MS}$ in microarray	p-value in $NAWM_{MS}$ in microarray	Change factor in $NAWM_{MS}$ in RT-PCR	p-value in $NAWM_{MS}$ in RT-PCR	Ref	Expression change in lesion found by the others
			Immune response													
37864_s_at	IGG1L	Y14737	immunoglobulin lambda heavy chain, constant	329	13351	39.58	0.047	NA	NA	10171	29.92	0.103	NA	NA	Becker et al., 1997; Lock et al., 2002	↑
34095_f_at	V4-31	U80114	immunoglobulin heavy chain variable region, v4-31	154	715	3.64	0.188	NA	NA	422	1.73	0.132	NA	NA	Baranzini et al., 1999	↑
31472_s_at	CD44	AF098641	cd44 isoform rc (cd44)	373	580	0.55	0.325	NA	NA	547	0.46	0.158	NA	NA	Becker et al., 1997;	↑
39728_at	IP-30	J03909	gamma-interferon-inducible protein (ip-30)	2390	3288	0.38	0.364	−1.91	0.464	3256	0.36	0.464	0.11	0.143	Becker et al., 1997;	↑
1563_s_at	TNFR1	M58286	tumor necrosis factor receptor 1	1708	2260	0.32	0.129	−0.4	0.770	2617	0.53	0.033	−0.37	0.942	Becker et al., 1997; Whitney et al., 1999 Baranzini et al., 2000	↑
1583_at	TNFR2	M32315	tumor necrosis factor receptor 2	1128	1427	0.26	0.157	−1.32	0.770	1852	0.64	0.069	−0.34	0.462	Becker et al., 1997; Whitney et al., 1999 Lock et al., 2002 Baranzini et al., 2000	↑
1830_s_at	TGFB1	M38449	transforming growth factor-beta1	794	936	0.18	0.179	−0.46	0.558	1084	0.37	0.079	0.77	0.107	Becker et al., 1997; Baranzini et al., 2000	↑
2092_s_at	SPP1	J04765	osteopontin	17458	12493	−0.4	0.175	−1.1	0.188	18703	0.07	0.757	−0.79	0.394	Lock et al., 2002	↑
307_at	ALOX5	J03600	lipoxygenase	336	447	0.33	0.071	NA	NA	630	0.87	0.012	NA	NA	Whitney et al., 2001	↑
38081_at	LTA4H	J03459	leukotriene a-4 hydrolase	1506	1893	0.26	0.049	NA	NA	2186	0.45	0.048	NA	NA	Whitney et al., 1999	↑
			Neural homeostasis													
41158_at	PHRET1	M54927	myelin proteolipid protein (PLP)	36575	36205	−0.01	0.960	−2.35	0.124	33952	−0.08	0.647	−0.88	0.769	Becker et al., 1997; Chabas et al., 2001 Lock et al., 2002 Baranzini et al., 2000	↓
38558_at	MAG	M29273	myelin-associated glycoprotein (mag)	16517	12957	−0.27	0.215	−10.26	0.003	17303	0.05	0.713	−1.13	0.143	Lock et al., 2002 Baranzini et al., 2000	↓
35817_at	MBP	M13577	myelin basic protein (mbp)	29278	22015	−0.33	0.047	−3.9	0.003	25440	−0.15	0.238	−0.06	0.770	Becker et al., 1997; Whitney et al., 2001 Chabas et al., 2001 Baranzini et al., 2000	↓
38653_at	PMP-22	D11428	peripheral myelin protein 22	16999	9861	−0.72	0.00001	−4.34	0.005	13631	−0.25	0.021	−0.67	0.242	-	–
35784_at	VAMP3	U64520	synaptobrevin-3	1013	488	−1.08	0.005	−4.57	0.003	752	−0.35	0.061	−0.48	0.380	Chabas et al., 2001	↓

Here we present a comparative study of gene expression analysis in active lesions and NAWM from patients with spMS, vis-à-vis brain tissue from age-matched control subjects. Special emphasis was put on stratified sample cohorts to exclude possible confounding factors such as a tissue pH, gender and postmortem delay that could interfere with gene expression measurements. The goal was to delineate clusters of functionally interconnected genes that are differentially regulated in lesions and NAWM of MS, and to compare their relative expression between these two compartments. Real-time PCR, the current gold standard method for quantitation of transcriptional gene regulation, was used to validate results of the semiquantitative microarray. Statistically congruent results were obtained in over 92% of comparative analysis which confirms the technical reliability of gene chip analysis. Our main finding is that in MS, NAWM and lesions do not form discrete compartments with respect to transcriptional expression patterns, but show a continuum of differential regulation. Over 70% of genes significantly regulated in one location showed similar changes, although quantitatively less pronounced, in the other, whereas only a minority demonstrated transcriptional changes restricted to either tissue compartment.

Confirming data from Lock et al. (2002), many of the 'usual suspects' considered important in MS, e.g. TNF-α/β, IFN-γ, IL-12, were not upregulated in the present MS tissue specimen. The importance of comparing lesions and NAWM in MS side-by-side is exemplified by caspase-1 (IL-1β-convertase). Others have found upregulation of this gene on the transcriptional and protein level in different stages of plaques as compared to normal control tissue (Ming et al., 2002). Present results demonstrate transcriptional upregulation in $NAWM_{MS}$ and we conclude that this gene may not be specific for lesion formation. The principal difference that distinguishes lesions from $NAWM_{MS}$ is a far higher upregulation of genes that are directly (Ig synthesis) or indirectly (IL-6R) related to a specific humoral immune response. Cellular immune response elements instead are similarly upregulated in both lesions and $NAWM_{MS}$ and represent the prevailing category in the latter. This supports the concept that the cellular immune response is not sufficient for lesion formation and that full-scale demyelination may be precipitated where focal antibody synthesis reaches a certain threshold (Linington and Lassmann, 1987). Accordingly, others have postulated that demyelination in MS results from autoantibodies against MOG on the basis of their detection in acute lesions of MS and EAE in marmosets, and the induction of the latter by immunization with MOG (Genain et al., 1999). The microarray used here is not able to detect idiotypic rearrangement diversity and deviation from the normal Ig repertoire. However, the pattern of heavy and light chain gene expression in present results is congruent with the detection of an increase of VHIg transcripts in lesions, but not in $NAWM_{MS}$ (Baranzini et al., 1999).

In lesions and in $NAWM_{MS}$, most of the upregulated cellular immune response genes alert pro-inflammatory, while downregulated genes exert mainly anti-inflammatory properties (testican #41), or trophic functions (EGF receptor #35). Of special interest, two functionally linked immune response genes, TGF-β3 and cre-bp1, were downregulated in lesions and NAWM. Cre-bp1 serves as signal transduction element for TGF-β (Zhang et al., 2000). In return, the transcription of TGF-β3 is dependent on the activity of cre-bp1 (Zhang et al., 2000). Astrocyte-derived TGF-β3 is an important regulator of neuronal survival (Krieglstein et al., 2002), as it enhances the effect of neurotrophic factors. TGF-β is protective against the development of EAE (Crisi et al., 1995), while anti-TGF-β or genetic deficiency for TGF-β leads to exacerbation of disease (Kjellen et al., 1998). Mice lacking cre-bp1 and the related CREM undergo progressive neurodegeneration, as neurotrophin-dependent survival and axon extension in cultured neurons from cre-bp1 null mice is compromised (Lonze et al., 2002). This downregulation of anti-inflammatory immune response genes in lesions and NAWM of MS reveal a generalized pattern of dysinhibition of inflammation.

In active lesions, the largest number of regulated genes is involved in neural homeostasis. Here, functional genes (#48, dynamin; #50, synaptosome-associated protein; #60, eenb1) which are essential for cell trafficking and exocytosis in nerve terminals, as well as genes involved in cytoskeleton homeostasis (α/β-tubulin, NF-L/M, STMN2, CAP2, doublecortin) were upregulated. Increased intrathecal release of tubulin and neurofilament *proteins* correlates with clinical disability and is considered a marker of axonal damage (Semra et al., 2002; Eikelenboom et al., 2003). On the other hand, *transcriptional* overexpression of these genes may indicate early neuroglial development (Poddar et al., 1996; Gloster et al., 1999) and regeneration (Sotelo-Silveira et al., 2000), and we speculate that recruitment and differentiation of neuronal (Evans et al., 2002) and glial (Simpson and Armstrong, 1999) progenitor cells may exert reparative functions in order to compensate for evolving cellular damage. An additional feature is that ten upregulated genes were coding for molecules involved in the formation or function of Na/Ca/K/Cl channels (#52,54,57,63,66,67,68,80,163,164). For instance, the pore-forming alpha1B subunit of the N-type Ca channel is transcriptionally upregulated in $NAWM_{MS}$. Others have demonstrated its ectopic overexpression in axons of actively demyelinating lesions and in EAE which may lead to excessive Ca influx, and eventually axonal degeneration (Kornek et al., 2001). Moreover, axons exposed to nitric oxide, one of the major endogenous toxins produced in the course of a neuroinflammatory response as encountered in MS (Smith et al., 1999), undergo conduction block and degeneration, especially when electrically active, but may be protected by ion channel blockers (Kapoor et al., 2003; Smith et al., 2001). Thus, current results corroborate the concept of pathways of axonal damage in MS that are

independent of those classical inflammatory processes that involve BBB damage and immune cell extravasation.

α- and β-globins were downregulated in MS tissue; for the former, this was significant in lesions and NAWM. Hemoglobin is a physiologic product of neurons (Ohyagi et al., 1994), but its specific function in this cell type is poorly understood. α-Globin expression is suppressed in the peripheral nerve after injury (De Leon et al., 1991) and we speculate its downregulation in MS tissue may similarly be a marker of neuronal damage.

Our study has some obvious limitations. Technically, the dynamic measuring range of microarrays is smaller than for RT-PCR and may fail to detect subtle expressional changes for genes with very high or very low numbers of transcripts. This may be the reason, why we could not confirm previous findings on expression of MMP-7 and -9 by microarray. However, upregulation of these genes was found in the fraction of present samples analysed with RT-PCR, confirming our earlier results (Lindberg et al., 2001) (not shown). Similarly, the reported downregulation of highly expressed myelin components such as PLP, MAG and MBP could be confirmed only by RT-PCR, due to oversaturation of fluorescence signals in microarrays and hence measurements in a non-dynamic range. Of interest in this context, aspartoacylase, which is almost exclusively expressed in oligodendrocytes, is downregulated in lesions and NAWM. This enzyme uses neuron-derived NAA as a source for the production of free acetate for myelin lipid synthesis (Chakraborty and Ledeen, 2003) and its downregulation may reflect impaired myelin synthesis in lesions and $NAWM_{MS}$. Conceptionally, our study is restricted to active types of plaques from patients with long-lasting spMS. The formation of plaques is heterogeneous and has been categorized into four different patterns according to distinct mechanisms of demyelination (macrophage-mediated, Ab-mediated, distal oligodendrogliopathy, primary oligodendroglia degeneration) (Lassmann et al., 2001). Additional studies to investigate gene expression in different types and stages of plaque formation are therefore needed. Recently, Graumann et al. reported upregulation of genes in $NAWM_{MS}$ induced by ischemic preconditioning, such as HIF-1α, which could not be confirmed in the current study. A possible explanation for these discrepancies may be the selection of types of MS: present data bases on tissue specimen that derive exclusively from spMS, whereas Graumann et al. analyse a mixture of primary progressive, relapsing–remitting, and secondary-progressive types of MS in one group. Another source for conflicting results may be the differences in postmortem delay of tissue sampling, and possibly tissue pH, known determinants of mRNA degradation (Bauer et al., 2003; Preece and Cairns, 2003). Although we cannot rule out that some targets may show altered expression due to postmortem changes, similar pH and equally short postmortem delay in control and MS tissues in our study speak against artefactual relative changes between these groups in current results. In contrast, the average postmortem delay of the tissue samples used by Graumann et al. is more than two times longer and significantly different between MS (11.4 h) and control (18.3 h) group ($P=0.035$), the latter ranging up to 26 h. Interestingly, others have found that the protein expression of HIF-1a is upregulated specifically in pattern III lesions, but not in other types of active lesion and not at all in $NAWM_{MS}$ (Aboul-Enein et al., 2003; W. Bruck, personal communication).

Upregulation of osteopontin (OPN) in brain tissue of MS and EAE has been reported (Chabas et al., 2001), but could not be confirmed by us and others (Lock et al., 2002) in MS using different analytical platforms. The role of OPN is further in doubt after a report that OPN knock-out mice had a similar course of EAE as wild-type animals, as well as in two other related model diseases (Blom et al., 2003). Others described upregulation of OPN in relapsing–remitting MS, specifically during relapses, but not in primary and secondary progressive MS (Vogt et al., 2003). Present results go along with the latter report, showing no differential regulation of this target in brain tissue of secondary progressive MS. The current study revealed data of 43 differentially regulated genes of unknown function. These genes could be highly relevant for pathogenesis of MS and the development of novel diagnostic and therapeutic approaches, but further studies are needed to delineate their function and cellular expression.

In summary, present results reveal a complex pattern of gene regulation in lesions and NAWM in brain tissue from spMS. Besides a dysbalanced regulation of immune genes leading to excessive activity of pro-inflammatory principles, there is evidence that tissue damage may be mediated via dysregulation of ion channel functions, and hence independent of classical pathways of inflammation. On the other hand, there is strong evidence for the recruitment of reparative functions that may represent future targets for novel therapeutic approaches in MS.

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