Table 1
Primers utilized for PCR-based cloning and site-directed mutagenesis

Genes	Proteins (amino acid residues)	GenBank accession no.	Sense primers	Antisense primers	Cloning vector
YWHAE	14-3-3ε Isoform (2-255)	NM_006761	5'gatgatgagaggatctggtgtac3'	5'ctgatttcgtcttccacgtcctg3'	pSecTag/FRT/V5-His TOPO
EAP30	EAP30 subunit of ELL complex (2-258)	NM_007241	5'caccgccgcgggggtgggagctggc3'	5'tcagggggaggggcttcctgccctc3'	pcDNA4/HisMax-TOPO
DDX54	Dead box polypeptide 54 (2-881)	NM_024072	5'gcggcgacaagggccgggcggct3'	5'tccatcctcttccgcatcttgcc3'	pcDNA4/HisMax-TOPO
STAC	src homology three and cysteine rich domain (2-402, full length)	NM_003149	5'atccctccgagcagccccgcgag3'	5'tcagatgtttctagttacatcaag3'	pcDNA4/HisMax-TOPO
STAC	src homology three and cysteine rich domain (2-402 with S172A: SMT)	NM_003149	5'gtttcggcggttactacgcctcccccttgctcattc3'	5'gaatgagcaaggggagggagcgtagtaacgccgaaac3'	pcDNA4/HisMax-TOPO modified by site-directed mutagenesis
STAC	src homology three and cysteine rich domain (2-402 with S172A and S173A: DMT)	NM_003149	5'cggcgttactacgcgccccctgctcattcat3'	5'atgaatgagcaagggggggcgcgtagtaacgccg3'	pcDNA4/HisMax-TOPO modified by site-directed mutagenesis
STAC	src homology three and cysteine rich domain (2-233, N-terminal half: NTF)	NM_003149	5'atccctccgagcagccccgcgag3'	5'tcaaagatctgaagtagaggttct3'	pcDNA4/HisMax-TOPO
STAC	src homology three and cysteine rich domain (234-402, C-terminal half: CTF)	NM_003149	5'gtggaggttcctgaggaagccaat3'	5'tcagccacctgatcgagaccagc3'	pcDNA4/HisMax-TOPO
STAC	src homology three and cysteine rich domain (2-164, truncated form A: TR-A)	NM_003149	5'atccctccgagcagccccgcgag3'	5'tcatggcagcttgccatgccaccg3'	pcDNA4/HisMax-TOPO
STAC	src homology three and cysteine rich domain (2-105, truncated form B: TR-B)	NM_003149	5'atccctccgagcagccccgcgag3'	5'tcagccacctggatcgagaccagc3'	pcDNA4/HisMax-TOPO

The PCR product was cloned into a vector pSecTag/FRT/V5-His TOPO to express a fusion protein with a V5 tag or into a vector pcDNA4/HisMax-TOPO to express a fusion protein with a Xpress tag in HEK293 cells.

tion gradient of bovine serum albumin (BSA) (row 1; columns 3–8), four spots of a concentration gradient of a rabbit anti-GST antibody (row 1; columns 9–12), four spots of a concentration gradient of calmodulin (row 1; columns 13–16), 16 spots of a concentration gradient of GST (row 2; columns 1–16), two spots of buffer only (row 8; columns 9,10), and two spots of an anti-biotin antibody (row 8; columns 11,12). The complete list of 1752 target proteins immobilized on the microarary is shown in Supplementary Table 1 online.

Non-specific binding was blocked by incubating the microarray for 90 min in the PBST blocking buffer composed of 1% BSA and 0.1% Tween 20 in phosphate-buffered saline (PBS). Then, it was incubated for 30 min at 4 °C with the probe described above at a concentration of 50 μg/ml in the probing buffer composed of 1% BSA, 5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.05% Triton X-100, and 5% glycerol in PBS. The array was washed three times with the probing buffer, followed by incubation for 30 min at 4 °C with mouse monoclonal anti-V5 antibody conjugated with Alexa Fluor 647 (Invitrogen) at a concentration of 260 ng/ml in the probing buffer. The array was washed three times with the probing buffer, and then scanned by the GenePix 4200A scanner (Axon Instruments, Union City, CA) at a wavelength of 635 nm. The data were analyzed by using the ProtoArray Prospector software v2.0 (Invitrogen) following acquisition of the microarray lot-specific information online, including inter-lot variations in protein concentrations (http://www.invitrogen.com/protoarray). According to the default setting of the software, the spots showing the background-subtracted signal intensity value greater than the median plus three standard deviations of all the fluorescence intensities were considered as having a significant binding. The Z-score, an indicator for statistical evaluation of binding specificity, was calculated as the background-subtracted signal intensity value of the target protein minus the average of the background-subtracted signal intensity value from the negative control distribution, divided by the standard deviation of the negative control distribution. All the procedure described above could be accomplished within 5 h. The 14-3-3-binding consensus motif mode I (RSXpSXP) sequence located in target proteins was surveyed by the Scansite Motif Scanner, which assesses the probability of a site matching the candidate motif under high, medium, or low stringent conditions (Obenauer et al., 2003). The information on known 14-3-3 interactors was obtained from Biomolecular Interaction Network Database (BIND; http://www.bind.ca) and PubMed database search.

2.3. Transient expression of 14-3-3-binding proteins in HEK293 cells

To verify the results of microarray analysis, the ORF of the genes encoding EAP30 subunit of ELL complex (EAP30), dead box polypeptide 54 (DDX54), and src homology three (SH3) and cysteine rich domain (STAC) were amplified by PCR using Pfu-Turbo DNA polymerase and the primer sets listed in Table 1. They were then cloned into a mammalian expression vector pcDNA4/HisMax-TOPO (Invitrogen) to produce a fusion protein with an N-terminal Xpress tag. To express the STAC mutant

(a) Human protein microarray

(b) 14-3-3ε-V5

(c) subarray **(d) EAP30** **(e) DDX54** **(f) STAC**

subarray 1. row 7
columns 11, 12

subarray 27. row 3
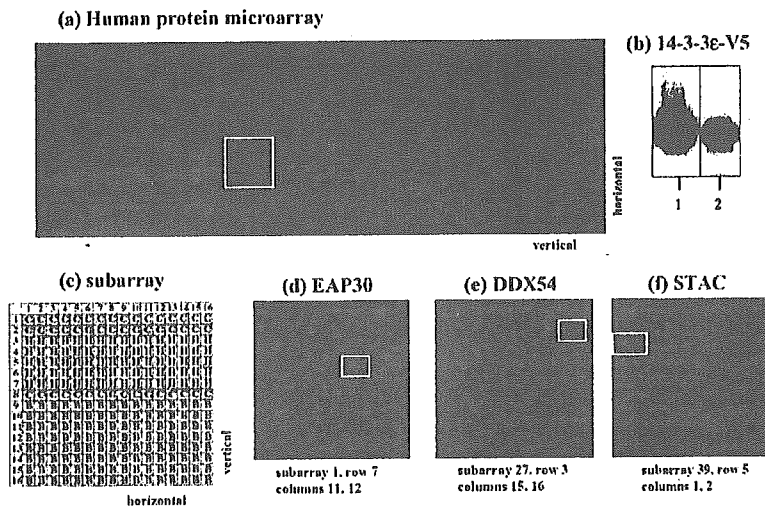columns 15, 16

subarray 39. row 5
columns 1, 2

Fig. 1. Protein microarray analysis. (a) Human protein microarray. The microarray contains 1752 distinct human proteins of various functional classes spotted in duplicate on a nitrocellulose-coated glass slide. They are printed in an arrangement of 4 × 12 subarrays equally spaced in vertical and horizontal directions. A representative subarray is indicated by an enclosed yellow line. (b) Recombinant human 14-3-3ε protein tagged with V5. One microgram of the protein was processed for Western blot analysis using anti-V5 antibody (lane 1) or anti-14-3-3ε antibody (lane 2). (c) Layout of the subarray. Each subarray includes 16 × 16 spots composed of 48 control spots (C), 80 human proteins (H), and 128 blanks (B). The positive control spots include an Alexa Fluor 647-labeled antibody (rows 1, 8; columns 1, 2; strong signals), a concentration gradient of a biotinlyated anti-mouse antibody with a capacity to bind to mouse monoclonal anti-V5 antibody conjugated with Alexa Fluor 647 (row 8; columns 3–8; signals visible on the higher concentration), and a concentration gradient of V5 protein (row 8; columns 13–16; signals visible on the higher concentration). (d) EAP30. (e) DDX54. (f) STAC. The three proteins indicated by an enclosed yellow line located on different subarrays (d, f) represent an example identified as showing significant binding to the probe.

(a) **(c)** **(d)**

14-3-3 WT WT

STAC NTF

    1 2 3   CTF

(b) SMT SMT

EAP30 DMT

EAP30
(+PP1) TR-A

DDX54 TR-B DMT
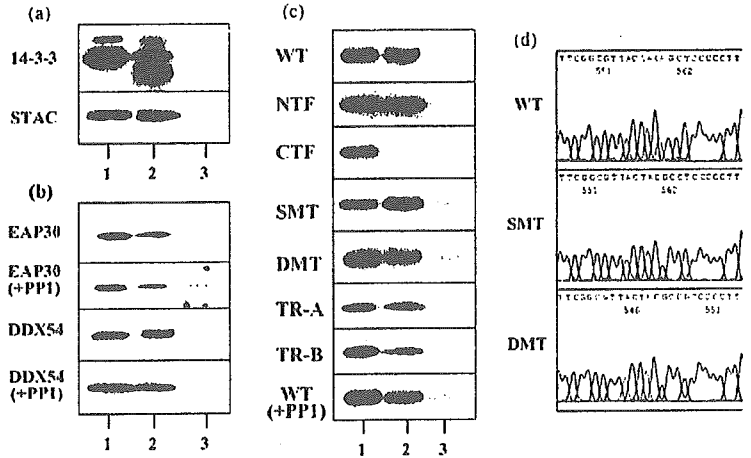
DDX54
(+PP1) WT
(+PP1)

    1 2 3   1 2 3

Fig. 2. Immunoprecipitation analysis of 14-3-3-binding proteins. (a) Binding of STAC to 14-3-3. Total protein extract of HEK293 cells expressing Xpress-tagged recombinant STAC was processed for immunoprecipitation (IP) with rabbit polyclonal antibody reacting with all 14-3-3 isoforms (K-19) or with normal rabbit IgG. The immunoprecipitates were then processed for Western blot analysis using mouse monoclonal antibody reacting with all 14-3-3 isoforms (H-8) (upper panel) or mouse monoclonal anti-Xpress antibody (lower panel). Lanes (1–3) represent (1) the input control, and IP with (2) K-19 and (3) normal rabbit IgG. (b) Binding of EAP30 and DDX54 to 14-3-3. Total protein of HEK293 cells expressing Xpress-tagged recombinant EAP30 or DDX54 extracted by using the lysis buffer with inclusion of phosphatase inhibitors or with inclusion of protein phosphatase-1 (PP1) instead of phosphatase inhibitors (+PP1) was processed for IP with K-19 or with normal rabbit IgG. The immunoprecipitates were then processed for Western blot analysis using anti-Xpress antibody. Lanes (1–3) represent (1) the input control, and IP with (2) K-19 and (3) normal rabbit IgG. (c) Binding of mutant and truncated STAC to 14-3-3. Total protein was extracted from HEK293 cells expressing a panel of Xpress-tagged recombinant STAC proteins. They include the full-length wild-type (WT) STAC, the N-terminal half (NTF), the C-terminal half (CTF), the S172A mutant (SMT), the S172A and S173A double mutant (DMT), the truncated form lacking the 14-3-3-binding consensus motif RYYSSP (TR-A), the truncated form lacking the cysteine-rich domain (CRD) (TR-B), and WT STAC isolated by using the lysis buffer with inclusion of PP1 instead of phosphatase inhibitors (WT + PP1). Primers utilized for PCR-based cloning and site-directed mutagenesis are listed in Table 1. The lysate was processed for IP with K-19 or with normal rabbit IgG. The immunoprecipitates were then processed for Western blot analysis using anti-Xpress antibody. Lanes (1–3) represent (1) the input control, and IP with (2) K-19 and (3) normal rabbit IgG. (d) The sequence of the 14-3-3-binding consensus motif located in amino acid residues 169-174 in expression vectors of STAC. The panels indicate WT (nucleotide sequence CGT-TAC-TAC-AGC-TCC-CCC: the corresponding amino acid sequence RYYSSP), SMT (CGT-TAC-TAC-GCC-TCC-CCC: RYYASP), and DMT (CGT-TAC-TAC-GCC-GCC-CCC: RYYAAP).

with a single amino acid substitution S172A (the single mutant; SMT) or with double amino acid substitutions S172A and S173A (the double mutant; DMT), the pcDNA4/HisMax-TOPO vector containing the wild-type (WT) STAC gene was modified by consecutive site-directed mutagenesis using QuikChange II site-directed mutagenesis kit (Stratagene) and the primer sets listed in Table 1. The mutations introduced in the vector were verified by sequencing analysis (Fig. 2d). All these vectors were transfected in HEK293 cells by Lipofectamine 2000 reagent.

2.4. Immunoprecipitation analysis

To prepare total protein extract, the cells were homogenized and incubated at room temperature for 30 min in M-PER lysis buffer (Pierce, Rockford, IL) supplemented with a cocktail of protease inhibitors (Sigma), with inclusion of phosphatase inhibitors (Sigma) to maintain the protein phosphorylation status or with inclusion of recombinant protein phosphatase-1 (PP1) catalytic subunit α-isoform (5 U/ml; Sigma) instead of phosphatase inhibitors to induce the protein dephosphorylation reaction (Ichimura et al., 2005), followed by centrifugation at 12,000 rpm at 4 °C for 20 min. After preclearance, the supernatant was incubated at 4 °C for 3 h with 30 μg/ml rabbit polyclonal anti-14-3-3 protein antibody (K19)-conjugated agarose (Santa Cruz Biotechnology, Santa Cruz, CA) or the same amount of normal rabbit IgG-conjugated agarose (Santa Cruz Biotechnology). After several washes, the immunoprecipitates were processed for Western blot analysis using mouse monoclonal anti-14-3-3 protein antibody (H-8, Santa Cruz Biotechnology) and mouse monoclonal anti-Xpress antibody (Invitrogen). K-19 and H-8 antibodies recognize all 14-3-3 isoforms. The specific reaction was visualized using a chemiluminescent substrate (Pierce).

3. Results

3.1. Protein microarray analysis identified 20 distinct 14-3-3-binding partners

To analyze a high-density human protein microarray, the recombinant 14-3-3ε protein tagged with V5 was purified form the supernatant of 293 eV5 cells secreting the recombinant protein in the culture medium. Western blot analysis verified the purity and specificity of the probe (Fig. 1b). Among 1752 proteins on the microarray, 20 were identified as the proteins showing significant binding to the probe (Table 2). All of these previously unreported 14-3-3-binding partners by the BIND search. Seven were hypothetical clones of uncharacterized function, derived from the mammalian genome collection (MGC) or the full-length long Japan (FLJ). Thirteen annotated proteins included EAP30 subunit of ELL complex (EAP30) (Fig. 1d), lymphocyte cytosolic protein 2 (LCP2), methionine aminopeptidase 2 (METAP2), melanoma antigen family B, 4 (MAGEB4), chondroitin 4 sulfotransferase 11 (CHST11), nuclear interacting partner of anaplastic lymphoma kinase (ZC3HC1), minichromosome maintenance deficient 10 (MCM10), DEAD box polypeptide 54 (DDX54) (Fig. 1e), heterogeneous nuclear ribonucleo-

protein C (HNPRC), fibroblast growth factor 12 (FGF12), glutathione S-transferase M3 (GSTM3), src homology three (SH3) and cysteine rich domain (STAC) (Fig. 1f), and ATPase, H+ transporting, lysosomal, 21 kDa, V0 subunit C″ (ATP6V0B). The 14-3-3-binding consensus motif mode I (RSXpSXP) was found only in STAC (pS172) and HNRPC (pS125) by the Scansite Motif Scanner search under the high stringent condition, while 15 of 20 proteins have one or several motifs when a query with the medium or low stringency was performed (Table 2).

3.2. Immunoprecipitation analysis validated the specific binding to 14-3-3

EAP30, DDX54, and STAC were selected to verify the results of microarray analysis, in view of their higher Z-scores. The recombinant proteins were expressed in HEK293 cells, which constitutively express a substantial amount of endogenous 14-3-3 protein. Total protein was extracted by using the lysis buffer with inclusion of phosphatase inhibitors to maintain the protein phosphorylation status or with inclusion of recombinant protein phosphatase-1 (PP1) instead of phosphatase inhibitors to induce the protein dephosphorylation reaction, followed by processing for immunoprecipitation (IP) with rabbit polyclonal antibody reacting with all 14-3-3 isoforms (K-19) or with normal rabbit IgG. K19 coimmunoprecipitated 14-3-3 and STAC from the lysate of HEK293 cells expressing the recombinant STAC protein, whereas normal rabbit IgG did not pull down these proteins (Fig. 2a). K-19 immunoprecipitated EAP30 and DDX54 from the lysate of HEK293 cells expressing the recombinant EAP30 or DDX54 protein, respectively, under both phosphorylated and dephosphorylated conditions (Fig. 2b). These results indicate that EAP30, DDX54, and STAC could interact with the endogenous 14-3-3 protein in HEK293 cells where the corresponding recombinant proteins were expressed.

STAC has the highly stringent 14-3-3-binding consensus motif RYYSSP in amino acid residues 169–174 (pS172), as suggested by the Scansite Motif Scanner (Table 2). Therefore, a possible involvement of this motif in binding to 14-3-3 was investigated by IP analysis of a series of mutant and truncated STAC proteins (Table 1). K-19 immunoprecipitated the full-length wild-type (WT) STAC comprised of amino acid residues 2–402 (Fig. 2a and c). K-19 also pulled down the S172A mutant (SMT), and the S172A and S173A double mutant (DMT), and the N-terminal half (NTF; amino acid residues 2–233) from the lysate of HEK293 cells expressing the corresponding recombinant proteins (Fig. 2c). In contrast, K-19 did not pull down the C-terminal half (CTF; amino acid residues 234–402) (Fig. 2c). These observations indicate that the RYYSSP motif is not essential for binding of STAC to 14-3-3. This was confirmed by the observations that K-19 immunoprecipitated the truncated form lacking the RYYSSP sequence (TR-A; amino acid residues 2–164) and the shortest form lacking both the RYYSSP sequence and the cysteine-rich domain (CRD) (TR-B; amino acid residues 2–105) from the lysate of HEK293 cells expressing the corresponding recombinant proteins (Fig. 2c). Finally, the full-length WT STAC interacted with 14-3-3 under the dephosphorylated condition (Fig. 2c). These observations indicate that the 14-

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Table 2
Twenty 14-3-3-binding proteins identified by protein microarray analysis

No.	Symbol	Database ID	Protein name	Putative biological function	14-3-3-binding consensus motif mode I	Stringency level of the binding motif	Subarray	Row	Column	Z-score
1	EAP30	NM_007241	EAP30 subunit of ELL complex	a 30-kDa component of the ELL complex that confers derepression of transcription by RNA polymerase II	No sites	NA	1	7	11	22.8593
									12	24.60829
2	FLJ10415	NM_018089	Hypothetical protein, cDNA clone MGC:969	Unknown	S258: ARGGPSHSAGANLRR	Low	5	4	11	4.16265
					S405: SPKQGSGSEGEDGFQ S525: PADPRVLSLLSAPLG S690: VNTRRCWSCGASLQG	Low Low Low			12	4.35203
3	LOC57228	NM_020467	Hypothetical protein	Unknown	S28: AARKRNISSNDSQAP	Low	5	6	9	16.84741
									10	17.1519
4	MGC17403	NM_152634	Hypothetical protein	Unknown	T274: KQLRASYTESCIQEH	Low	11	3	1	4.04754
									2	4.08838
5	LCP2	NM_005565	Lymphocyte cytosolic protein 2	A 72-kDa protein (SLP76) that associates with the Grb2 adaptor protein, provides a substrate of the ZAP-70 protein tyrosine kinase, and plays a role in promoting T cell development and activation	S297: TTERHERSSPLPGKK	Low	13	5	11	4.47457
					S376: SSFPQSASLPPYFSQ T456: DSSKKTITINPYVLMV	Low Low			12	4.46343
6	METAP2	NM_006838	Methionine aminopeptidase 2	A 67-kDa protein that interacts with eukaryotic initiation factor-2 (eIF-2) and regulates protein synthesis	T113: KRGPKVQTDPPSVPI	Low	15	6	11	3.54252
					S152: TAAWRTTSEEKKALD	Low			12	3.31601
7	MAGEB4	NM_002367	Melanoma antigen family B, 4	A member of the MAGEB family expressed in testis whose function remains unknown	T18: AREKRQRTRGQTQDL	Medium	18	3	7	9.50614
					T194: GNQSSAWTLPRNGLL S339: SAYSRATSSSSQPM	Low Low			8	9.30562
8	CHST11	NM_018413	Chondroitin 4 sulfotransferase 11	A member of HNK-1ST family GalNAc 4-O-sulfotransferase that plays a role in chondroitin sulfate and dermatan sulfate biosynthesis	S93: TDTCRANSATSRKRR	Medium	20	5	3	3.97327
					S56: DICRKGSRSPLQEL S194: EPFERLVSAYRNKFT	Low Low			4	3.92871
9	ZC3HC1	NM_016478	Nuclear interacting partner of anaplastic lymphoma kinase (ALK)	A 60-kDa protein that interacts with ALK and plays an antiapoptotic role in nucleophosmin-ALK signaling events	No sites	NA	23	3	3	3.33458
									4	3.55366

No.	Symbol	Accession	Gene name	Function	Phosphorylation site	Level				Score
10	MCM10	NM_018518	Minichromosome maintenance deficient 10	A key component of the pre-replication complex (pre-RC) that is essential for the initiation of DNA replication	S90: AQPPRTGSEPPRLEG	Medium	25	3	13	4.26291
					S35: KPAIKSISASALLKQ	Low			14	4.12552
					S55: LEMRRKSEEIQKRF	Low				
					S302: PCGNRSISLDRLPNK	Low				
					T329: DGMLKEKTGPKIGGE	Low				
11	DDX54	NM_024072	DEAD (Asp-Glu-Ala-Asp) box polypeptide 54	A 97-kDa RNA helicase (DP97) that interacts with estrogen receptor (ER) and represses the transcription of ER-regulated genes	T95: EDKKKIKTESGRYIS	Low	27	3	15	9.2425
12	HNPRC	NM_004500	Heterogeneous nuclear ribonucleoprotein C	A member of heterogeneous nuclear ribonucleoproteins (hnRNPs) involved in pre-mRNA processing, mRNA metabolism and transport	S102: TESGRYISSSYKRDL	Low			16	9.10882
					S125: DYYDRMYSYPARVPP	High	28	6	9	4.81248
					S158: NTSRRGKSGFNSKSG	Low			10	5.18382
					S170: KSGQRGSSKSGKLKG	Low				
					S240: ETNVKMESEGGADDS	Low				
13	LOC137781	BC032247	Hypothetical gene, cDNA clone MGC:40429	Unknown	No sites	NA	30	5	11	3.56109
									12	3.47568
14	LOC92345	NM_138386	Hypothetical protein	Unknown	S339: QGRKKLKSEFNEPGE	Low	32	5	13	3.55366
					T374: GYRNREFTRGFSRAR	Low			14	3.73933
					S467: PLLNLPYSLPPPPP	Low				
15	FGF12	NM_004113	Fibroblast growth factor 12, transcript variant 2	A member of the FGF family that plays a role in nervous system development and function	S150: VCMYREQSLHEIGEK	Low	34	6	5	5.73339
16	GSTM3	NM_000849	Glutathione S-transferase M3 (brain)	A cytoplasmic glutathione S-transferase of the mu class that plays a role in detoxification of carcinogens, therapeutic drugs, environmental toxins, and products of oxidative stress	S165: QGRSRKSSGTPTMNG	Low			6	5.75567
					S64: GIKLRSFSV	Low	38	5	15	7.82029
17	STAC	NM_003149	src homology three (SH3) and cysteine rich domain	A 47-kDa protein with a SH3 and a cysteine-rich domain that plays a role in the neuron-specific signal transduction pathway	S172: KGFRRYYSSPLLIHE	High	39	5	16	7.70889
									1	16.63575
					S56: TKSLRSKSADNFFQR	Medium				
					S255: DLRKRSNSVFTYPEN	Medium				
					S46: QKLKRSLSFKTKSLR	Low			2	16.64318
					S51: SLSFKTKSLRSKSAD	Low				
					S66: NFFQRTNSEDMKLQA	Low				
					S253: GYDLRKRSNSVFTYP	Low				

Table 2 (*Continued*)

No.	Symbol	Database ID	Protein name	Putative biological function	14-3-3-binding consensus motif mode I	Stringency level of the binding motif	Subarray	Row	Column	Z-score
18	FLJ10156	NM_019013	Hypothetical protein, cDNA clone MGC:961	Unknown	S16: GTSVRRSLQHQEQL	Low	41	3	7	7.31156
					T190: FRSPYSSTEPLCSPS	Low			8	6.94023
19	ATP6V0B	NM_004047	ATPase, H+ transporting, lysosomal, 21 kD, V0 subunit C"	A 23-kDa component of vacuolar ATPase that mediates acidification of intracellular organelles	No sites	NA	43	7	3	3.3123
									4	3.60565
20	FLJ25758	NM_001011541	Hypothetical protein, clone MGC:33355	Unknown	No sites	NA	48	7	7	4.18864
									8	4.18122

Among 1752 proteins on the microarray, 20 were identified as showing a significant interaction, based on the signal intensity value exceeding the median plus three standard deviations of all the fluorescence intensities by analyzing with ProtoArray Prospector software. They are listed with the 14-3-3-binding consensus motif (putative phosphoserine and phosphothreonine indicated by underline) and its stringency level by the Scansite Motif Scanner, the position on the array, and the Z-score calculated as described in Section 2. Abbreviations: FLJ, the full-length long Japan; MCG, mammalian gene collection; NA, not available.

3-3-interacting domain is located in the N-terminal segment spanning amino acid residues 2-105 of STAC, and the interaction is independent of serine/threonine-phosphorylation of the binding domain of STAC.

4. Discussion

The present study was designed to rapidly and systematically identify 14-3-3-binding proteins by analyzing a high-density protein microarray. The array included 1752 proteins derived from multiple gene families of biological importance, including cell-signaling proteins, kinases, membrane-associated proteins, and metabolic proteins. In general, protein microarray has its own limitations associated with the expression and purification of a wide variety of target proteins. In the microarray we utilized, the target proteins were expressed in a baculovirus expression system, purified under native conditions, and spotted on the slides to ensure the preservation of native structure, posttranslational modifications, including glycosylation and serine phophorylation (Culleré et al., 1998; Tennagels et al., 1999), and proper functionality. Immunolabeling of the array with anti-phosphotyrosine (pTyr) antibody indicated that approximately 10-20% of the proteins on the array are phosphorylated (the unpublished data of Invitrogen Technical Service). When this microarray was utilized for kinase substrate identification, most of known kinases immobilized on the array are enzymatically active with the capacity of some degree of autophosphorylation, suggesting that they are certainly phosphorylated on tyrosine, serine, and threonine residues (see the Protoarray application note on http://www.invitrogen.com/protoarray). However, we could not currently validate the precise levels of phosphorylation of individual proteins, because of a lack of anti-phosphoserine (pSer) and anti-phosphothreonine (pThr) antibodies suitable for detection of pSer and pThr residues of the proteins on glass slide.

The protein microarray utilized in the present study includes 11 known 14-3-3-binding proteins, such as PCTAIRE protein kinase 1 (PCTK1) (Graeser et al., 2002), protein kinase C zeta (PRKCZ) (van der Hoeven et al., 2000), keratin 18 (KRT18) (Ku et al., 1998), myosin light polypeptide kinase (MYLK) (Haydon et al., 2002), v-abl Abelson murine leukemia viral oncogene homolog 1 (ABL1) (Yoshida et al., 2005), v-akt murine thymoma viral oncogene homolog 1 (AKT1) (Powell et al., 2002), epidermal growth factor receptor (EGFR) (Oksvold et al., 2004), cell division cycle 2 (CDC2) (Chan et al., 1999), mitogen-activated protein kinase kinase kinase 1 (MAP3K1) (Fanger et al., 1998), mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2) (Powell et al., 2003), and stratifin (SFN) (Benzinger et al., 2005) (Table 3). All of these were not identified as a 14-3-3-binding protein in the present study. Therefore, the possibility could not be excluded that some 14-3-3 binding partners were not detected due to imperfect phosphorylation of the proteins on the array or due to 14-3-3 isoform-specific binding. Calmodulin, another known 14-3-3 interactor (Luk et al., 1999), was included as a negative control on the array and identified as negative in the present study, because the calcium-dependent interaction between 14-3-3 and calmodulin could not be detected under the calcium-free conditions we employed.

Table 3
Eleven known 14-3-3-binding proteins immobilized on the protein microarray utilized in the present study

Gene name	Database ID	Reference
PCTAIRE protein kinase 1 (PCTK1), transcript variant 3	NM_033019	Graeser et al. (2002)
Protein kinase C, zeta (PRKCZ)	NM_002744	van der Hoeven et al. (2000)
Keratin 18 (KRT18), transcript variant 1	NM_000224	Ku et al. (1998)
Myosin, light polypeptide kinase (MYLK), transcript variant 6	NM_005965	Haydon et al. (2002)
V-abl Abelson murine leukemia viral oncogene homolog 1 (ABL1), transcript variant a	NM_005157	Yoshida et al. (2005)
V-akt murine thymoma viral oncogene homolog 1 (AKT1), transcript variant 1	NM_005163	Powell et al. (2002)
Epidermal growth factor receptor (EGFR), transcript variant 1	NM_005228	Oksvold et al. (2004)
Cell division cycle 2 (CDC2), transcript variant 1	NM_001786	Chan et al. (1999)
Mitogen-activated protein kinase kinase kinase 1 (MAP3K1)	XM_042066	Fanger et al. (1998)
Mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2), transcript variant 1	NM_004759	Powell et al. (2003)
14-3-3 Sigma, stratifin (SFN)	NM_006142	Benzinger et al. (2005)

The known 14-3-3-binding proteins, which were spotted on the protein microarray but were not detected in the present study are listed. The 14-3-3-binding proteins validated by definitive evidence are selected and shown with references.

Increasing studies indicate that 14-3-3-binding phosphorylation sites do not exactly fit the consensus motif (Aitken et al., 2002; Ku et al., 1998) and a second site is required to enhance a stable 14-3-3-target interaction (MacKintosh, 2004), and show that the 14-3-3 protein interacts with a set of target proteins in a phosphorylation-independent manner (Dai and Murakami, 2003; Henriksson et al., 2002; Zhai et al., 2001). Supporting the latter possibility, the present observations showed that the interaction of 14-3-3 with target proteins is independent of serine/threonine-phosphorylation of the binding sites of EAP30, DDX54, and STAC. This suggests that substantial numbers of 14-3-3 binding partners identified by protein microarray analysis, if not all, employ phosphorylation-independent binding domains.

All the procedure required for microarray analysis takes approximately 5 h. This analysis identified a set of 20 human proteins as 14-3-3 interactors, most of which were previously unreported except for glutathione S-transferase M3 (GSTM3) that was found as one of binding partners by 14-3-3 affinity purification of HeLa cell protein extracts (Pozuelo Rubio et al., 2004). Unexpectedly, the highly stringent 14-3-3-binding consensus motif was identified only in two, such as STAC and HNPRC, by the Scansite Motif Scanner search, while 15 of 20 proteins have one or several motifs when a query with the medium or low stringency was performed (Table 2). The specific binding to 14-3-3 of EAP30, DDX54, and STAC was verified by immunoprecipitation analysis of the recombinant proteins expressed in HEK293 cells. These results indicate that protein microarray is a powerful tool for rapid identification of protein–protein interactions, including those unpredicted by the Database search.

Among the 14-3-3-binding partners we identified, several proteins could be categorized as a component of multimolecular complexes involved in transcriptional regulation. ELL is a human oncogene encoding a RNA polymerase II (Pol II) transcription factor that promotes transcription elongation (Schmidt et al., 1999). EAP30 is a 30-kDa component of the ELL complex where EAP30 confers derepression of transcription by Pol II (Schmidt et al., 1999). A recent study showed that EAP30 could interact with the tumor susceptibility gene TSG101 product, a cellular factor that plays a key role in packaging of HIV

virions (von Schwedler et al., 2003). DDX54 is a 97-kDa RNA helicase (DP97) that interacts with estrogen receptor (ER) and represses the transcription of ER-regulated genes (Rajendran et al., 2003). A recent study by using chromatin immunoprecipitation (ChIP) assay combined with promoter microarray analysis showed that hepatocyte nuclear factor 4-alpha (HNF4α), a master regulator of hepatocyte gene expression, interacts with the DDX54 gene promoter, together with Pol II (Odom et al., 2004). HNPRC is a member of heterogeneous nuclear ribonucleoproteins (hnRNPs) involved in pre-mRNA processing, mRNA metabolism and transport (Nakagawa et al., 1986). Increasing evidence indicates that the 14-3-3 protein and its targets are widely distributed in various subcellular compartments, including the nucleus (Dougherty and Morrison, 2004; Meek et al., 2004).

STAC is a 47-kDa cytosolic protein that has a cysteine-rich domain (CRD) of the protein kinase C family in the N-terminal half (NTF), and a src homology three (SH3) domain in the C-terminal half (CTF), suggesting its function as an adapter on which divergent signaling pathways converge (Hardy et al., 2005; Suzuki et al., 1996). STAC is expressed predominantly in the brain with the distribution in a defined population of neurons (Suzuki et al., 1996). IP analysis of mutant and truncated forms argued against an active involvement of the most stringent motif RYYSSP (pS172) of STAC in its binding to 14-3-3. The present observations indicated that the 14-3-3-interacting domain is located in the N-terminal segment spanning amino acid residues 2-105 of STAC and the interaction is serine/threonine phosphorylation-independent.

In conclusion, protein microarray is a useful tool for rapid and comprehensive profiling of 14-3-3-binding proteins, although the validation of the results by different methods is highly important.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.jneumeth.2005.09.015.

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DNA マイクロアレイによる多発性硬化症の免疫病態の解析

佐藤　準一

＜特集Ⅰ　サイトカイン・ケモカインからみた多発性硬化症の病型と病態＞

DNA マイクロアレイによる多発性硬化症の免疫病態の解析

佐藤　準一[1,2]

DNA Microarray Analysis Clarifies Immunopathogenesis of Multiple Sclerosis

Jun-ichi Satoh[1,2]

Abstract

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) white matter mediated by an autoimmune process whose development is triggered by a complex interplay of both genetic and environmental factors. MS shows a great range of phenotypic variability in terms of the disease course, lesion distribution, therapeutic response to IFNβ, and pathological aspects, suggesting that MS is a kind of neurological syndrome caused by different immunopathological mechanisms leading to the final common pathway that provokes inflammatory demyelination. DNA microarray technology is a novel approach to systematically monitoring the expression of a large number of genes. It gives us new insights into the complexity of molecular interactions promoting the autoimmune process in MS. By microarray analysis followed by hierarchical clustering analysis, we found that T cell gene expression profiling is valuable to identify distinct subgroups of MS associated with differential disease activity and therapeutic response to IFNβ. These observations suggest that microarray analysis is highly valuable for designing personalized treatment for heterogeneous populations of MS.

Key words : DNA microarray, gene expression profile, multiple sclerosis, personalized medicine

はじめに

多発性硬化症（multiple sclerosis; MS）は中枢神経系白質に炎症性脱髄巣が多発し，様々な神経症状が再発を繰り返して進行する難病である。MS 発症機序は十分解明されていないが，多数の遺伝的要因と環境因子の存在下で，脳炎惹起性髄鞘抗原に分子相同性を示すウイルスなどの外来抗原を認識し活性化した自己反応性 CD4+ Th1 T 細胞が，血液脳関門を通過して中枢神経系組織内に浸潤し，マクロファージ・ミクログリアを活性化して TNFα などの炎症増強因子の産生を誘導し，脱髄が惹起されると考えられている（自己免疫機序）[1]。回復期には髄鞘再生を認めるが，炎症が高度で遷延化すると髄鞘再生不全・軸索傷害・神経変性を来して不可逆的な機能障害を残す。

近年欧米・本邦で実施された大規模臨床試験により，インターフェロンベータ（interferon-beta; IFNβ）の MS 再発抑制効果が立証され，現在では急性増悪期に副腎皮質ステロイド短期間大量静脈内投与を行い，回復期に IFNβ の継続的皮内・筋肉内投与を行う方法が，最も一般的な治療法として選択されている。しかし IFNβ が全く効果を示さない症例も多い[2,3]。すなわち MS は均一な疾患ではなく多様な病態（phenotypic heterogeneity）を呈する疾患群である可能性が高い。実際 MS は臨床経過から再発寛解型（relapsing-remitting MS; RRMS），2 次進行型（secondary-progressive MS; SPMS），1 次進行型（primary-progressive MS; PPMS），病巣分布から脳型（conventional MS; CMS）と視神経脊髄型（opticospinal MS; OSMS），

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IFNβ 治療反応性から responder (RMS), nonresponder (NRMS) に分類される。病理学的にも T 細胞浸潤, 抗体沈着, oligodendrocyte apoptosis の観点から 4 型に分類される[4]。近年 MS の免疫病態の多様性を解析する手法として遺伝子アレイが用いられている。ヒトゲノムプロジェクトの完結によりヒト全遺伝子の塩基配列が解明された結果, 遺伝子アレイを用いて個々の細胞における数万遺伝子（ヒト全遺伝子約 30,000）の発現情報を包括的・網羅的・系統的に解析可能になった。RNA 発現解析を transcriptome 解析, タンパク質発現解析を proteome 解析と呼ぶ。網羅的発現解析 (global expression analysis) により, 従来の少数分子に焦点を向けた研究 (pinpoint study) では予期しなかった遺伝子群の MS 発症機序における役割が次々明らかになった[5]。また治療による遺伝子発現変化を経時的に解析することにより薬物反応性遺伝子を同定し（薬理ゲノミクス pharmacogenomics）, 有効性や副作用を治療開始前に予知することにより, テーラーメイド医療 (personalized medicine) に道が開かれた。本稿では DNA マイクロアレイ解析の基本原理と MS の免疫病態解析における応用に関して最近の知見を概説する。

1. DNA マイクロアレイ解析の基本原理

遺伝子アレイはスライドグラスやナイロン膜などの基盤上に, 機能既知または未知の数千・万の cDNA または oligonucleotide が貼付けてあるチップである。主として cDNA をスポッターで基盤上にスポットしてある DNA マイクロアレイ (DNA microarray) と光オリゴヌクレオチド合成により基盤上で直接高密度の oligonucleotide を伸長合成している GeneChip (Affymetrix) に分類される（表1)[6]。スライドグラスを DNA microarray, ナイロン膜を DNA macroarray と総称することもある。最近では約 3,000 種類のタンパク質をスライドグラスに固定してあるプロテインチップ (protein microarray) も普及しており, タンパク質間相互作用 (interactome) やシグナル伝達系の網羅的解析に用いられている[7]。遺伝子アレイは遺伝子多型・変異解析にも応用可能であるが本稿では割愛する。

遺伝子アレイ解析ではまず遺伝子発現レベルの異なる 2 種類以上の細胞・組織, 例えば IFNβ 投与前後の細胞などから mRNA を抽出し増幅する（図 1）。DNA マイクロアレイでは一般的に別々の螢光色素 (Cy3, Cy5) でラベルした cDNA または cRNA を作成して同一チップ上で競合的ハイブリダイゼーションを行い, 2 色法と呼ばれる。GeneChip では in vitro transcription (IVT) により cDNA から biotin 標識 cRNA を作成, fragment に切断してハイブリダイゼーションを行い, streptoavidin-phycoerythrin (SAPE) を添加して螢光標識する。GeneChip では 1 サンプルに 1 枚のアレイが必要で, アレイ間の比較になる。どちらの場合もスキャナーで螢光シグナルを検出し, 得られたデータ (dataset) を正規化 (normalization) して統計学的検定を行い, サンプル間の遺伝子発現プロフィール (gene expression profile) を比較解析する。同

表 1 cDNA microarray と GeneChip の比較

	cDNA/Oligonucleotide Microarray	GeneChip
基盤	スライドグラス (microarray) またはナイロン膜 (macroarray)	半導体チップ
固定化法	スポッティングまたは化学合成	オンチップフォトリソグラフ合成
遺伝子	300–1000 bp cDNA or 30–80 mer oligonucleotide	25 mer oligonucleotides of perfect match (PM) and mismatch (MM)
集積度	40,000/slide 程度	>500,000/chip
Tm	不均一	一定
螢光標識	2 色法 (Cy3, Cy5)	単色法
定量原理	競合的ハイブリダイゼーションによる比較	個々のチップのデーターを正規化して比較
代表的なヒト遺伝子発現解析用アレイ（遺伝子数；Commercial Supplier）	Whole Human Genome G4112A Array (41,000; Agilent), Human Whole Genome Bioarray (55,000; Amersham)	Human Genome U133 Plus 2.0 Array (47,000; Affymetrix)
カスタム性	高い 汎用マイクロアレイスキャナーが使用可能	低い 専用のハイブリダイゼーションオーブンや洗浄装置とスキャナーが必要

文献 6 より引用改変.
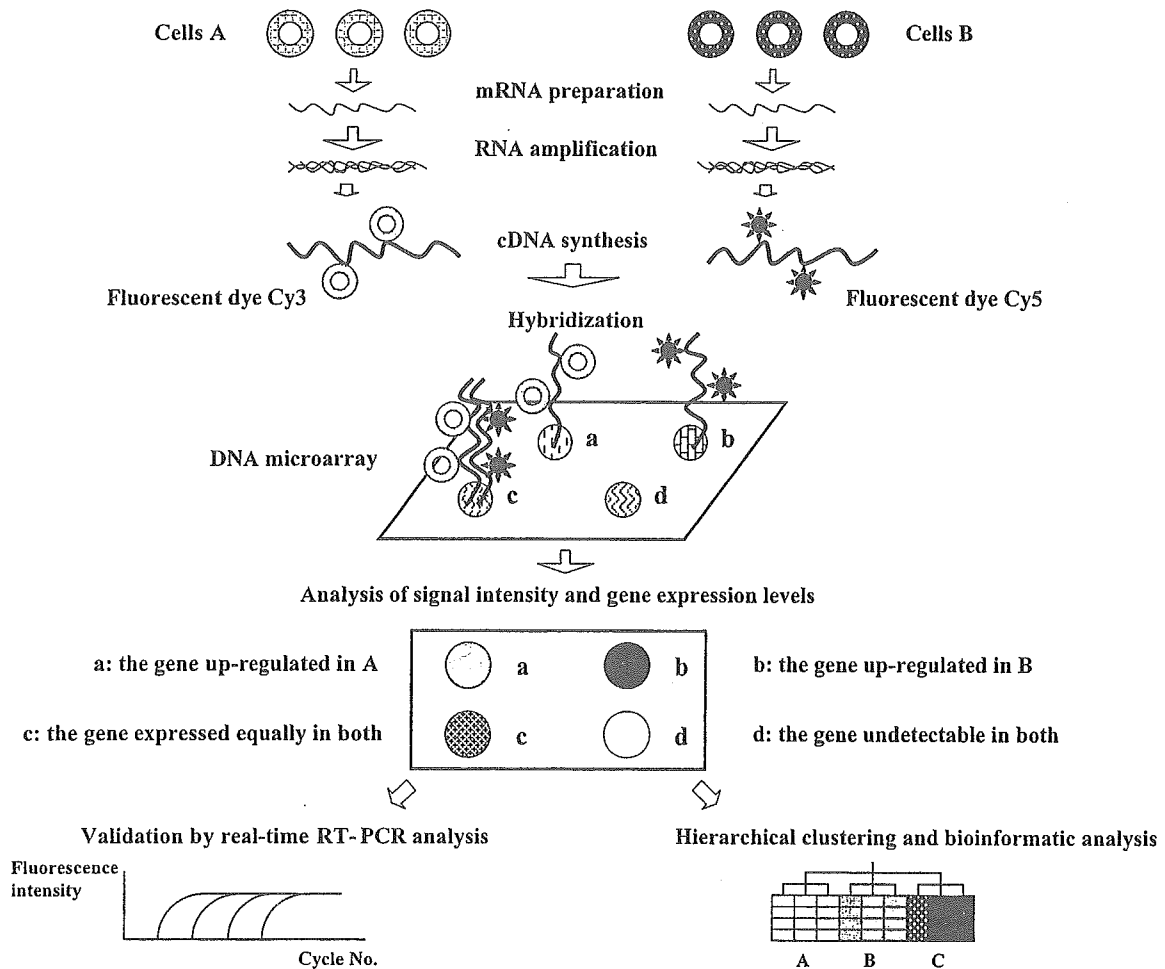
図1 DNA マイクロアレイ解析の概要。
2 種類の細胞から mRNA を抽出増幅し，別々の螢光色素 (Cy3, Cy5) でラベルした cDNA probe を作成して，cDNA がスポットさ れたチップ上で競合的ハイブリダイゼーションを行う。スキャナーで螢光シグナルを検出し，データを正規化して統計学的検定 を行い，サンプル間の遺伝子発現プロフィール (gene expression profile) を比較解析する。さらに階層的クラスター解析 (hierarchical clustering analysis) を行って，類似した発現パターンを呈する遺伝子やサンプルをグループに分類する。有意な発 現差異を呈する遺伝子は real-time RT-PCR で mRNA を定量して検証する。

定した遺伝子の機能・構造の注釈情報 (annotation) は遺 伝子リストの ID から Web 上で database を検索可能であ る（表2）。既に様々な細胞・組織の遺伝子発現データが Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/ geo) に登録されているが，実験に用いたチップの format が異なるとデータ間の互換性がなくなると考えられ，大 規模な meta-analysis を実施する場合に支障となる[8]。サ ンプル数が多い場合はデータセットの要素の特性を抽出 するため，階層的クラスター解析 (hierarchical clustering analysis) を行う[9]。すなわちサンプルに関する事前情報 なしに（教師なし法 unsupervised method），類似した発

現パターンを呈する遺伝子やサンプルをグループに分類 して，樹状図 (dendrogram) と発現レベルの2次元マト リックスで表示する。またサンプルをいくつかのグルー プに分類する代表な遺伝子 (discriminator genes) を抽出 し，これらを3次元に圧縮投射する主成分解析 (principal component analysis) を行う[9]。
　我々は薬物応答遺伝子アレイ 1,258 cDNA microarray (Hitachi Life Science) を用いて，MS 患者末梢血 T 細胞 の遺伝子発現プロフィールを解析している。健常者3名 の RNA mixture を universal reference として Cy3 で標識 し，患者や健常者のサンプルを全て Cy5 で標識し，各サ

表2 Transcriptome・proteome 解析に有用な database

Name	Website	Contents
★統合 Database 検索システム		
Entrez	www.ncbi.nlm.nih.gov/Entrez/index.html	The Life Sciences Search Engine
Gene	www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=gene	A Searchable Database of Genes
OMIM	www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=omim	Online Mendelian Inheritance in Man
DBGET	www.genome.ad.jp/dbget/dbget.links.html	Web of Molecular Biology Databases
KEGG	www.genome.ad.jp/kegg	Kyoto Encyclopedia of Genes and Genomes
SRS	srs6.ebi.ac.uk	European Bioinformatics Institute Database
HGMD	www.hgmd.cf.ac.uk/hgmd0.html	Human Gene Mutation Database
★配列解析		
UniProt	www.genome.jp/dbget-bin/www_bfind?uniprot	SWISS-PROT Protein Sequence Database
PIR	www.genome.jp/dbget-bin/www_bfind?pir	PIR Protein Sequence Database
BLAST	blast.genome.jp	Sequence Similarity Search
dbSNP	www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=snp	Single Nucleotide Polymporphism Database
CLUSTALW	align.genome.jp	Multiple Sequence Alignment
TraceSuite II	www-cryst.bioc.cam.ac.uk/~jiye/evoltrace/evoltrace.html	Evolutionary Trace Server
ORF Finder	www.ncbi.nlm.nih.gov/gorf/gorf.html	Open Reading Frame Finder
PROSCAN	thr.cit.nih.gov/molbio/proscan	Web Promoter Scan
★タンパク質立体構造解析		
PDB	www.rcsb.org/pdb	The RCSB Protein Data Bank
RasMol	www.rcsb.org/pdb/help-graphics.html#rasmol_download	Molecular Graphics
GRASS	honiglab.cpmc.columbia.edu/cgi-bin/GRASS/surfserv_enter.cgi	Graphical Representation and Analysis of Structure Server
SWISS-MODEL	swissmodel.expasy.org//SWISS-MODEL.html	An Automated Comparative Protein Modelling Server
ERRAT	nihserver.mbi.ucla.edu/ERRAT	A Protein Structure Verification Algorithm
Verify3D	nihserver.mbi.ucla.edu/Verify_3D	A Crystal Structure Evaluation Server
SCOP	scop.mrc-lmb.cam.ac.uk/scop/index.html	Structural Classification of Proteins
DBAli	salilab.org/DBAli	A Database of Structure Alignments
★タンパク質機能予測		
SOSUI	sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html	Classification and Secondary Structure Prediction of Membrane Proteins
PSORT II	psort.ims.u-tokyo.ac.jp	Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences
SignalP 3.0	www.cbs.dtu.dk/services/SignalP	Prediction of Signal Peptide Cleavage Sites in Amino Acid Sequences
InterPro	www.ebi.ac.uk/interpro	A Database of Protein Families, Domains and Functional Sites
PredictProtein	www.embl-heidelberg.de/predictprotein/predictprotein.html	Structure Prediction and Sequence Analysis
BIND	www.bind.ca	The Biomolecular Interaction Network
DIP	dip.doe-mbi.ucla.edu	Databases of Interacting Proteins
MINT	160.80.34.4/mint/index.php	A Molecular Interaction Database
PPID	www.anc.ed.ac.uk/mscs/PPID/cgi-bin/ppid_search.pl	Protein-Protein Interaction Database
PROCAT	www.biochem.ucl.ac.uk/bsm/PROCAT/PROCAT.html	A Database of 3D Enzyme Active Site Templates
Scansite	scansite.mit.edu	A Motif Scan
PhosphoSite	www.phosphosite.org/Login.jsp	An In Vivo Phosphorylation Site Database
ExPASy	au.expasy.org	Expert Protein Analysis System Proteomics Server

2005 年 8 月の時点でのサイト.

ンプルで遺伝子ごとに Cy5/Cy3 signal intensity ratio を測定している. 比較する 2 群間 (MS vs 健常者など) で有意な発現差異を呈する遺伝子は, Bayesian t test または R 解析 (www.cran.r-project.org) と Bonferroni 補正で統計学的有意性を検定することにより同定している. さらに有意な遺伝子に関しては, LightCycler (Roche) による

real-time RT-PCRで定量してアレイ解析の結果を検証 (validation) している。階層クラスター解析と主成分解析は GeneSpring (Silicon Genetics-Agilent) で行っている。末梢血リンパ球の遺伝子アレイ解析の問題点は，特定の遺伝子の発現レベルが年齢・性・喫煙・飲酒・常用薬・嗜好品・精神的ストレスなどの個人差や採血時刻（日内変動）の影響を受けることである（interindividual and intraindividual variation)[10]。また脳組織の遺伝子アレイ解析の問題点は死後脳凍結までに要する時間（RNA degradation time) で，組織の pH がある程度参考になる。

2. DNA マイクロアレイによる多発性硬化症の免疫病態の解析

2.1. MS 脳組織の網羅的遺伝子発現解析

　DNA マイクロアレイによる MS の病態解析の最初の報告はWhitney らによる研究である[11]。彼らは独自の cDNA microarray を用いて MS 急性期炎症性病巣と正常白質 (normal-appearing white matter; NAWM) を比較し，MS 病巣における interferon-regulatory factor IRF-2, 5-lipooxygenase 発現上昇を報告した（表 3)[11,12]。Chabas らは MS brain cDNA ライブラリーの網羅的シークエンス解析で osteopontin (OPN) 発現レベルの上昇を認めた[13]。さらにラット EAE 脊髄のカスタム oligonucleotide microarray 解析で OPN の発現上昇を確認した。OPN は主として T 細胞が産生し macrophages による IL-12 産生を促進して IL-10 産生を抑制する Th1 cytokine で，活動性 RRMS 患者血清で上昇している[14]。OPN 遺伝子欠損マウスは EAE 惹起に対して抵抗性を示す[13]。Lock らは GeneChip を用いて MS 急性炎症性病巣と慢性非活動性病巣を比較し，活動性病巣での granulocyte colony stimulating factor (G-CSF) 発現上昇と非活動性病巣での IgG Fc receptor, IgE receptor, histamine receptor type 1 の発現上昇を認めた[15]。さらに彼らは G-CSF 投与で EAE を軽症化出来ること，immunoglobulin FcR γ-chain 遺伝子欠損マウスでは EAE 慢性化が抑制されることを証明し，アレイ解析の結果を裏付けた。Chabas らや Lock らの報告により，MS, EAE の病巣形成における allergic response mediators の役割が認識されるようになった[16]。

　Mycko らは cDNA microarray (Clontech) を用いて SPMS の慢性活動性病巣と非活動性病巣，脱髄巣辺縁部と中心部を比較し，活動性病巣辺縁部における炎症・免疫応答遺伝子群（TNFα など）の発現上昇を認めた[17]。Graumann

らは cDNA macroarray (Clontech) を用いて MS の NAWM と非神経疾患のコントロール白質を比較し，NAWM における脳虚血関連遺伝子（hypoxia-inducible factor 1 alpha; HIF-1α など）の発現上昇を認めた[18]。Lindberg らは GeneChip でSPMSの活動性病巣とNAWMを比較し，活動性病巣での immunoglobulin 産生亢進の所見を見出した[19]。Tajouri らは独自の cDNA microarray を用いて SPMS の急性・慢性活動性病巣を non-MS コントロール白質と比較し，活動性病巣における αB-crystallin, superoxide dismutase SOD1の発現上昇を報告した[20]。上述のMS脳組織のマイクロアレイ解析は各々症例数が少なく，RNA 抽出部位が必ずしも全体像を反映していない可能性は否定出来ない。EAE 脳・脊髄の網羅的遺伝子発現解析に関しては割愛する[21-23]。

2.2. 末梢血リンパ球を用いたMSと健常コントロールの比較解析

　Ramanathan らはResearch Genetics (Invitrogen) のGene-Filter membrane array を用いて，MSと健常者のmonocyte-depleted peripheral blood lymphocytes (PBL)を比較し，MS における lymphocyte-specific protein tyrosine kinase (LCK), IL-7R の発現上昇を報告した[24]。LCK は Airla らによる RRMS の PBMC の cDNA macroarray (Clontech) 解析で，intravenous methylprednisolone pulse (IVMP) で発現低下する遺伝子として報告されている[25]。Bomprezzi らは独自の cDNA microarray を用いて，24 例の RRMS と 21 名の健常者の peripheral blood mononuclear cells (PBMC) を比較し，発現差異を示した 53 遺伝子を同定した[26]。MS では自己反応性 T 細胞活性化に関連する IL-7R, ZAP70, TNFRSF7 (CD27) の発現上昇およびサイトカイン mRNA の ubiquitin-proteasome 系による分解を制御する HSPA1A (HSP70) の発現低下を認めた。Mayne らはRRMSと健常者のCD4陽性T細胞をnegative selection で分離し，cDNA membrane array (NIA) を用いて解析し，MS における cytoplasmic FMR1 interacting protein 2 (CYFIP2) の発現上昇を認めた[27]。

　我々は cDNA microarray (Hitachi Life Science) を用いて，72 例の MS (65 RRMS, 7 SPMS) と 22 名の健常者の末梢血 CD3 陽性 T 細胞，CD3 陰性 non-T 細胞の遺伝子発現プロフィールを解析した（表 3)[28]。その結果 T 細胞で 173 遺伝子，non-T 細胞で 50 遺伝子の発現差異を認め，上位 30 遺伝子（the most significant genes）を抽出すると，T 細胞で 25 遺伝子（NR4A2, TCF8 の上昇と

表 3　Microarray による MS の免疫病態の解析

Authors (Reference No.)	Year	No of MS Patients and Controls	RNA Samples
Whitney et al. (11)	1999	PPMS (n=1)	acute lesion vs NAWM
Ramanathan et al. (24)	2001	RRMS (n=15) vs HC (n=15)	monocyte-depleted PBL
Wandinger et al. (35)	2001	RRMS (n=1) plus HC (n=2)	PBMC incubated with IFNβ in vitro
Whitney et al. (12)	2001	PPMS (n=1), RRMS (n=1), EAE vs HC (n=3)	acute or chronic lesions of MS and EAE vs white matter of non-MS controls
Lock et al. (15)	2002	CPMS and SPMS (n=4)	acute or chronic active lesions vs chronic silent lesions
Mass et al. (32)	2002	RA (n=20), SLE (n=24), IDDM (n=5), and MS (n=5) vs HC before and after influenza vaccination (n=9)	PBMC
Bomprezzi et al. (26)	2003	RRMS (n=18), SPMS (n=6) vs HC (n=21)	PBMC (fresh or frozen)
Graumann et al. (18)	2003	SP/PP/RRMS (n=10) vs non-neurological controls (n=7)	NAWM vs control white matter
Koike et al. (36)	2003	RRMS (n=13) before and at 3 and 6 months after IFNβ treatment	T and non-T cells separated from PBMC
Mycko et al. (17)	2003	SPMS (n=4)	chronic active vs silent lesions and the lesion margin vs center
Stürzebecher et al. (46)	2003	RRMS before and after IFNβ treatment for 6 months (n=10; 6 responders vs 4 non-responders)	frozen PBMC ex vivo or incubated with IFNβ in vitro
Tajouri et al. (20)	2003	SPMS (n=5) vs non-MS	acute and chronic active lesions
Weinstock-Guttman et al. (44)	2003	RRMS before and at 1, 2, 4, 8, 24, 120, and 160 h after IFNβ treatment (n=8)	monocyte-depleted PBL
Achiron et al. (29)	2004	RRMS (n=26; 14 with treatment) vs HC (n=18)	PBMC
Achiron et al. (30)	2004	RRMS treated (n=13) vs untreated (n=13)	PBMC
Airla et al. (25)	2004	RRMS (n=6) before and after IVMP	PBMC
Hong et al. (47)	2004	RRMS/SPMS treated with IFNβ (n=18), GA (n=12) or untreated (n=15)	PBMC
Iglesias et al. (33)	2004	RRMS (n=17) vs HC (n=7)	PBMC
Lindberg et al. (19)	2004	SPMS (n=6) vs non-neurological controls (n=12)	active lesions vs NAWM
Mandel et al. (31)	2004	RRMS (n=13) vs SLE (n=5) vs HC (n=18)	PBMC
Mayne et al. (27)	2004	RRMS (n=21) vs HC (n=19)	CD4+ T cells
Satoh et al. (28)	2005	RRMS (n=65) plus SPMS (n=7) vs HC (n=22)	T and non-T cells separated from PBMC

Abbreviations: RRMS, relapsing-remitting MS; SPMS, secondary progressive MS; PPMS, primary progressive MS; CPMS, chronic progressive MS; HC, healthy controls; IDDM, insulin-dependent diabetes mellitus; NAWM, normal appearing white matter; PBMC, peripheral blood mononuclear cells; PBL, peripheral blood lymphocytes; IFN, interferon; GA, glatiramer acetate; IVMP, intravenous methylprednisolone pulse.

MAPK1, SMARCA3, HSPA1A, TRAIL, TOP1, CCR5, BAG1, DAXX, TSC22, PARP の低下など), non-T 細胞で 27 遺伝子 (ICAM1, CDC42, RIPK2, SODD, TOP2A の上昇と BCL2, RPA1, NFATC3, HSPA1L, RBBP4, PRKDC の低下など) が apoptosis 制御遺伝子に属していた。すなわち apoptosis 促進遺伝子 (proapoptotic genes)

と抑制遺伝子 (antiapoptotic genes) の発現上昇・低下の拮抗的バランス (counterbalance) を認め, MS 免疫病態における apoptosis 制御機構の異常が示唆された。Achiron らは GeneChip を用いて, 26 例の RRMS と 18 名の健常者の PBMC を比較解析した[29]。両群間で 1,109 遺伝子の発現差異を認め, MS における T 細胞活性化関連遺伝子

Type of Microarray	No of Genes on Microarray	Key Findings
Original cDNA Glass Array	1,344 or 5,000	Upregulation of IRF-2 and TNFRp75 in acute lesions
GeneFilters GF211 Membrane Array (Research Genetics)	5,184	Upregultion of LCK, IL-7R and MMP-19 and downregulation of CCR6 and DFFA in MS
Mini-Lymphochip cDNA Array	6,432	Upregulation of proinflammatory genes such as CCR5, IP-10, and IL-15RA by IFNβ treatment
Original cDNA Glass Array	2,798	Upregulation of 5-lipoxygenase in MS and EAE lesions
HuGene FL Oligonucleotide Array (Affymetrix)	7,026	Upregulation of G-CSF in active lesions and upregulation of IgG FcR in silent lesions, and amelioration of EAE in FcRγ-chain-KO mice and by treatment with G-CSF
GeneFilters GF211 Membrane Array (Research Genetics)	4,329	Indistinguishable profiles between MS and IDDM and downregulation of apoptosis-regulatory genes in autoimmune diseases
Original cDNA Array (Modified Lymphochip)	6,500 or 7,500	Upregulation of PAFAH1B1, IL-7R, ZAP70, and TNFRSF7(CD27) and downregulation of HSPA1A (HSP70) and CKS2 in MS
Atlas Human cDNA Memebrane Array 1.2 (Clontech)	3,528	Upregulation of ischemic preconditioning genes such as HIF-1α in NAWM of MS
Human cDNA Array (Hitachi Life Science)	1,258	Upregulation of 15 IFN-responsive genes in MS after IFNβ treatment
Atlas Human 1.0 Glass Microarray (Clontech)	588	Upregulation of inflammation/immune-related genes in the margin of active lesions
Mini-Lymphochip cDNA Array	6,432 or 12,672	Downregulation of IL-8 in responders after IFNβ treatment
Custom-made cDNA Glass Array	5,000	Upregulation of αB-crystallin and SOD in acute lesions
GeneFilters GF211 Membrane Array (Research Genetics)	5,184	Time-dependent upregulation of IFN-responsive genes
Human U95Av2 Oligonucleotide Array (Affymetrix)	12,000	Upregulation of T cell activation genes and downregulation of IL-1 and TNF signaling genes in MS
Human U95Av2 Oligonucleotide Array (Affymetrix)	12,000	Identification of SCYA4, IL2RG, and TNFRSF6(Fas) as immunomodulatory treatment-associated genes
Atlas Human Hematology/Immunology Membrane Array (Clontech)	448	Downregulation of LCK, TCF7, CD5, and ISGF3 by IVMP
Original Membrane Array	36	Distinct gene expression profile between MS patients treated with IFNβ and GA
HuGene FL Oligonucleotide Array (Affymetrix)	6,800	Upregulation of E2F transcription factor pathway genes in MS
Human U95A Oligonucleotide Array (Affymetrix)	12,633	Upregulation of genes related to Ig synthesis in active lesions of MS
Human U95Av2 Oligonucleotide Array (Affymetrix)	12,000	Downregulation of NR4A1 and NR4A3 as the autoimmunity-specific signature
Immune Membrane Array (National Institute on Aging)	1,152	Upregulation of CYFIP2 in MS
Human cDNA Array (Hitachi Life Science)	1,258	Aberrant expression of apoptosis and DNA damage-regulatory genes in MS

(LEF1, TCF3, SLAM, ITGB2, CTSB) の発現上昇および IL-1β, TNFα シグナル伝達系遺伝子の発現低下を認めた。我々の結果[28]に反し, MS における orphan nuclear receptor NR4A2 の発現低下を報告した。彼らの研究では MS 14 例は採血時に IFNβ, glatiramer acetate (GA), IVIg 治療中である点が問題である。彼らは同じ症例の治療中 13 例と未治療 13 例の PBMC の比較解析を行い, 治療関連 7 遺伝子 (TNFRSF6; Fas など) を同定した[30]。さらに 13 例の RRMS と 5 例の SLE を 18 名の健常者と比較して, 自己免疫疾患共通遺伝子 (autoimmunity-specific signature) を探索し, 自己免疫疾患における apoptosis, matrix metalloproteinase (MMP) 制御系遺伝子の発現異

常を発見した[31]。Maas らも 20 例の RA, 24 例の SLE, 5 例の IDDM, 5 例の MS と 9 名の健常者 (influenza ワクチン接種前後) の PBMC を比較解析した[32]。ワクチンに対する免疫応答と自己免疫疾患の遺伝子発現プロフィールは全く異なるが, RA と SLE, MS と IDDM は極めて類似し, 自己免疫疾患では共通して apoptosis 制御遺伝子群の発現低下を認めることを報告した。Iglesias らは GeneChip を用いて 17 例の RRMS と 10 名の健常者の PBMC を比較し, MS における E2F transcription factor pathway 遺伝子群の発現上昇を見出し, E2F1 遺伝子欠損マウスでは EAE が軽症化することを報告した[33]。

2.3. MS におけるインターフェロンベータ治療反応性の解析

我々は cDNA macroarray (Invitrogen) を用いて, ヒト胎児脳より樹立したアストロサイト (astrocytes) 純培養で IFNβ, IFNγ により発現変動する遺伝子群を解析した[34]。IFNβ による interferon-regulatory factor IRF-7 と pleiotrophin の発現上昇, IFNγ による IRF-1 と ICAM-1 の発現上昇を発見した。Wandinger らは RRMS と健常者の PBMC を IFNβ で刺激して cDNA microarray (Mini-Lymphochip) を用いて解析した[35]。彼らは proinflammatory molecules である CC chemokine receptor 5 (CCR5), interferon-inducible cytokine IP-10 (CXCL10), IL15 receptor alpha (IL-15RA) の発現上昇を認めた。我々は cDNA microarray (Hitachi Life Science) を用いて, 13 例の RRMS の末梢血 CD3 陽性 T 細胞と CD3 陰性 non-T 細胞で, IFNβ1b 治療開始後に発現変動したインターフェロン応答遺伝子群 (IFN-responsive genes; IRG) を同定した[36]。21 遺伝子が有意な変動を呈し, T 細胞で 8 IRG (IRF-7, ISG15, IFI56, IFI6-16, IFI60, IFI30, ATF3, TLR5) の発現上昇, IL-3, monokine induced by IFNγ (MIG) などの発現低下を認め, non-T 細胞では 12 IRG (IRF-7, ISG15, IFI56, IFI6-16, IFI27, IFI17, TAP1, TNFAIP6, TSC22, SULT1C1, RPC39, RAB11A) の発現上昇, IL-3 の発現低下を認めた。ISG15, IFI56, IFI6-16, IFI27, TSC22, SULT1C1 に関しては, 治療開始後 3-6 ヶ月の持続的な上昇を認めた。一方統計学的有意差は見られなかったが, 治療後に Th1 関連遺伝子 CCR5 (T), IFNγ (T), TNFα (non-T) の発現上昇傾向を認めた。このことは MS において IFNβ 治療は必ずしも明確な Th2 shift を誘導しないという見解[35]に一致する。上記のうち 9 遺伝子 (IRF7, ISG15, IFI56, IFI6-16, IFI60, IFI17, TAP1, TNFAIP6,

MIG) はプロモーター領域に IFN-stimulated response element (ISRE) や IRF element (IRF-E) が同定されている既知の IRG であり, IFNβ 治療に直接反応して上昇し治療効果発現に深く関与していると考えられる。興味深いことに培養系では多くの IRG は IFNγ によっても発現が誘導される[36,37]。IRF-7 はウイルス感染時に IFNα/β 産生を増幅する正の制御因子である[38]。IFI30 は class II MHC 拘束性抗原提示に働くチオール還元酵素であり, IFI30 遺伝子欠損マウスでは抗原呈示能低下を来す[39]。TAP1 は class I MHC 拘束性抗原提示を司るペプチド輸送因子で, TAP1 遺伝子欠損マウスでは CD8+ T 細胞を介する結核菌への抵抗力が減弱する[40]。TNFAIP6 は TNFα, IL-1β により発現誘導される分泌蛋白質で, マウス関節炎に投与すると抗炎症作用を呈する[41]。以上より MS において IFNβ は antiviral and antiinflammatory mediator 遺伝子群の発現上昇を誘導することが明らかになった。非常に興味深いことに SLE では治療に関わらず PBMC における IRG の発現レベルが高い[42,43]。

Weinstock-Guttman らは GeneFilter membrane array を用いて, 8 例の RRMS で IFNβ1a 投与後経時的に monocyte-depleted PBL を解析して IRG を同定したが, その多くは我々の結果とオーバーラップしている[44]。また Liang らは Weinstock-Guttman らのデータを再解析し, IFNβ により発現誘導される IRG は early-onset (within 8 hours), intermediate-onset (24 hours), late-onset (48 hours) の 3 群に分類されることを報告した[45]。Stürzebecher らは cDNA microarray (Mini-Lymphochip) を用いて, 10 例の RRMS で IFNβ1b 治療前後の PBMC を解析した[46]。治療開始前 6 ヶ月から開始 12 ヶ月後まで毎月 Gd 造影 MRI で活動性病巣数を算出し, 治療後に病巣数が 60% 以上減少した症例を responder と定義した。また nonresponder を治療開始後から効果のない nonresponder from initiation of therapy (INR) と, 治療開始後一定期間は効果があり neutralizing antibody (NAb) 出現とともに効果が減弱した nonresponder with development of NAb (NAbNR) の 2 群に分類した。さらに ex vivo 解析と同時に IFNβ で刺激した in vitro 解析も行った。Responder で治療後 2 倍以上変動した遺伝子は ex vivo では 25 遺伝子 (IFI17, OAS, Stat1 の上昇と IL-8, CD69, c-fos, TSC22 の低下など) で, そのうち IL-8 発現低下は responder の指標となる可能性が示唆された。一方 in vitro IRG は 87 遺伝子で, responder と nonresponder の間で発現レベルに差異を認めなかっ

た。彼らの結果に反して，我々は IFNβ 治療後に non-T 細胞で TGFβ-stimulated protein TSC22 の発現上昇を認めている[36]。彼らの研究の問題点は responder 6 例・INR 2 例・NAbNR 2 例と症例数が少なく，PBMC を凍結保存後に解凍して刺激しており，実験操作で遺伝子発現が変化し得ることである[46]。さらに 1 例の responder では治療前に約 90 個の Gd 造影病巣を呈しているが，これほど多数の造影病巣を示す症例は日本人 MS では極めて異例である。Hong らは免疫応答に極めて重要な 36 遺伝子に絞った cDNA macroarray を作成し，未治療 MS と IFNβ1a または GA 治療症例の PBMC を解析し，治療反応性遺伝子群の相違を明らかにした[47]。興味深いことに活性化 T 細胞の血液脳関門通過に重要な MMP-9 の発現は IFNβ により低下したが GA では上昇した。

　van Boxel-Dezire らは26例の IFNβ1b 治療中の RRMS で PBMC のサイトカイン遺伝子発現レベルを半定量的 RT-PCR で経時的に解析した[48]。治療前後 2 年間の再発回数・IVMP 回数・Extended Disability Status Scale (EDSS) スコアから 16 例の responder と 10 例の nonresponder に分けて比較すると，responder は治療前に IL-12p35 発現レベルが低い傾向を呈した。Wandinger らは RRMS で IFNβ1a 治療後 1 年間一度も再発がなく，EDSS スコア悪化の見られない症例を responder，一度以上再発が見られた症例を nonresponder と定義し，20 例の responder と 19 例の nonresponder の PBMC を比較し，responder では TNF-related apoptosis-inducing ligand (TRAIL; TNFSF10) が持続的高値を呈することを報告した[49]。TRAIL は IRG の 1 つで我々は MS の T 細胞における発現低下を認めている[28]。TRAIL 遺伝子欠損マウスは胸腺細胞の apoptosis に異常を来たし，コラーゲン関節炎などに高感受性になることが報告されている[50]。Baranzini らは 52 例の IFNβ 治療中の RRMS で経時的に PBMC の 70 遺伝子の発現レベルを RT-PCR で定量的に解析した。彼らは治療後 2 年間一度も再発がなく，EDSS スコア悪化の見られない症例を responder，2 回以上再発が見られた症例を nonresponder と定義し，両者は 3 遺伝子 (caspase2, caspase10, FLICE inhibitory protein; FLIP) の発現レベルの 3 次元解析で 86％区別可能と報告している[51]。

　最近我々は前述[28]の 72 例の IFNβ 未治療 MS (46 例は初回採血後 2 年間 IFNβ 治療開始) と 22 名の健常者の末梢血 CD3 陽性 T 細胞を cDNA microarray (Hitachi Life Science) を用いて解析したデータを，両群間で発現差異

を示す 286 遺伝子を指標にして階層的クラスター解析で再解析した (Satoh et al. Manuscript in preparation)。この解析により 286 遺伝子は class #1–#5 に分類され，MS は健常者から分離されてさらに 4 つのサブグループに分類された。すなわち遺伝子発現プロフィールが健常者に近似した A 群，治療導入前後 2 年間の再発回数・IVMP 日数・入院日数の点で最も活動性が高い B 群，大脳限局病変が多い C 群，最も EDSS スコアが高値の D 群に分類された。B 群は chemokine 遺伝子を多く含む class #5 の発現レベルが高かった。また IFNβ 治療前後 2 年間の再発回数・IVMP 日数・入院日数・EDSS スコア・MRI T2 強調画像病巣数の比較と患者満足度から算出した IFNβ 治療反応スコアで評価すると，responder は A 群と B 群に集積していた。また responder では nonresponder に比較して治療開始後 6ヶ月の時点でも IRG (ISG15, IFI27, MCP-1, TNFRp75) の発現レベルが高く保持される傾向を示した。

3. 結　　語
　我々は DNA マイクロアレイ解析を用いて MS が T 細胞の遺伝子発現プロフィールに基づき 4 群に分類され，各群は疾患活動性・病変分布・IFNβ 治療反応性との対応を認めることを報告した。現在，欧米人 MS にも同様の結果が当てはまるかどうか症例数を増加して解析中である。このような研究成果を積み重ねることにより MS のテーラメイド医療樹立に貢献出来ると思われる。

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