function, molecular weight, and subcellular localization was obtained from Biomolecular Interaction Network Database (BIND; www.bind.ca), Human Protein Reference Database (HPRD; www.hprd.org), Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequence Database (PSORT II; psort.ims.u-tokyo.ac.jp), and PubMed Database (www.pubmed.gov). The 14-3-3-binding consensus motif mode I (RSXpSXP) located in target proteins was surveyed by the Scansite 2.0 Motif Scanner (scansite.mi.edu),[58] which assesses the probability of a site matching the candidate motif under high, medium or low stringent conditions (Figure 13.3).

RESULTS

Protein Microarray Analysis Identified 20 Distinct 14-3-3-Interactors

Western blot analysis verified the purity and specificity of the recombinant 14-3-3ε protein tagged with V5 (Figure 13.1). Among 1752 proteins on the microarray, 20 were identified as the proteins showing significant binding to the probe, all of which were previously unreported 14-3-3-binding partners by the BIND search.[56] Seven were categorized into hypothetical clones of uncharacterized function, derived from either the Mammalian Genome Collection (MGC) or the Full-Length Long Japan (FLJ). They include FLJ10415 (GenBank accession number NM_018089), LOC57228 (NM_020467), MGC17403 (NM_152634), LOC137781 (BC032347), LOC92345 (NM_138386), FLJ10156 (NM_019013), and FLJ25758 (NM_001011541). Thirteen proteins with annotation are as follows:

1. *EAP30 subunit of ELL complex (EAP30; NM_007241)* (Figure 13.2b). This is a 30-kDa component of the ELL complex (estimated MW is 28,866 suggested by HPRD; putative subcellular location is cytoplasmic suggested by PSORT II), which confers derepression of transcription by RNA polymerase II.[59] EAP30 is also named VPS22, a component of the ESCRT-II endosomal sorting complex that plays a key role in the multivesicular body (MVB) pathway.[60] The 14-3-3-binding consensus motif mode I is not identified by the Scansite Motif Scanner, although the Z-Score of two corresponding spots on the array shows the highest values, 22.9 and 24.6 respectively. The similarity in the scores between distinct spots supports the reproducibility of the results of protein microarray analysis.
2. *Lymphocyte cytosolic protein 2 (LCP2; NM_005565).* This is a 72-kDa protein (MW 60,191; nuclear), alternatively named SH2 domain-containing leukocyte protein of 76kD (SLP76), which associates with the Grb2 adaptor protein and provides a substrate of the ZAP-70 protein tyrosine kinase.[61] LCP2 plays a key role in promoting T cell development and activation. It contains three mode I motifs with low stringency; pS297 (TTERHERSSPLPGKK), pS376 (SSFPQSASLPPYFSQ), and pT456 (DSSKKTTTNPYVLMV).

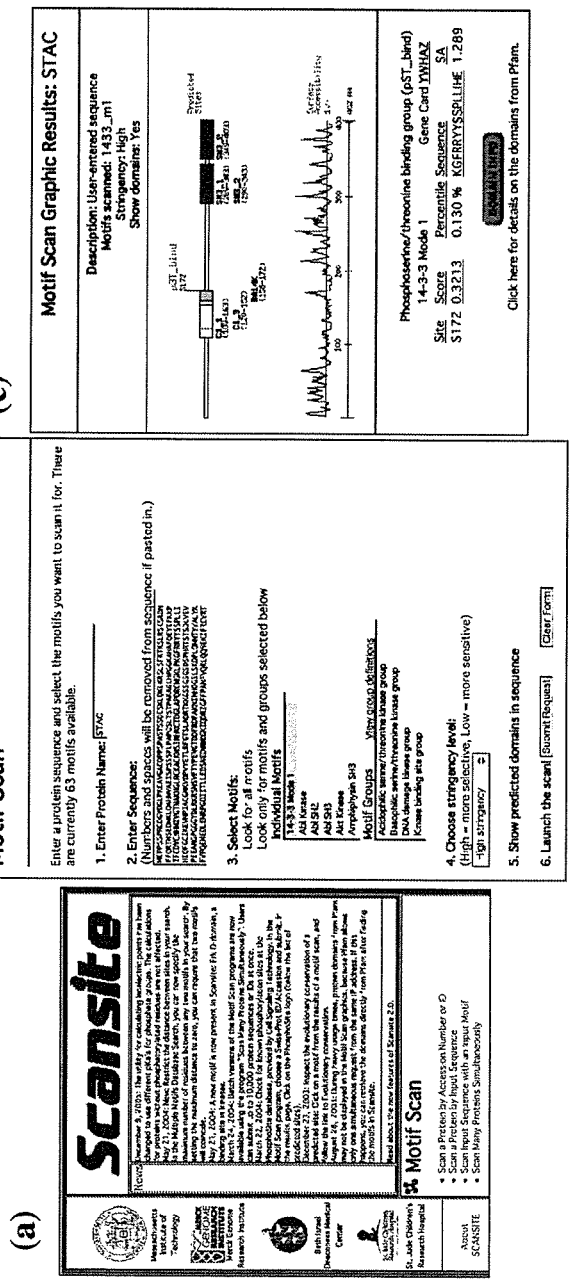

FIGURE 13.3 The Scansite Motif Scanner. The 14-3-3-binding consensus motif mode I (RSXpSXP) located in target proteins was surveyed by the Scansite Motif Scanner which assesses the probability of a site matching the candidate motif under high, medium or low stringent conditions. (a) The opening menu (cited from the website of http://scansite.mi.edu). (b) The motif scan menu. After entering the protein sequence of STAC, 63 different motifs including 14-3-3 mode I could be processed for searching. (c) The graphic view of the results. The candidate for the 14-3-3-binding motif of STAC under the high stringent condition (pST_bind S172) is indicated on the predicted domain structure of the protein (upper panel), accompanied by the relative scores for the interaction of pS172 domain: KGFRRYYSSPLLIHE with 14-3-3 protein (lower panel). A plot of the surface accessibility suggests the residues located near the protein surface with a capacity to interact with target proteins (upper panel).

3. *Methionine aminopeptidase 2 (METAP2; NM_006838).* This is a 67-kDa protein (MW 52,894; cytoplasmic) that interacts with eukaryotic initiation factor-2 (eIF-2) and regulates protein synthesis [62]. It contains two mode I motifs with low stringency; pT113 (KRGPKVQTDPPSVPI) and pS152 (TAAWRTTSEEKKALD).
4. *Melanoma antigen family B, 4 (MAGEB4; NM_002367).* This is a member of the MAGEB family (MW 38,925; nuclear) expressed abundantly in testis whose function remains unknown.[63] It contains three mode I motifs; T18 (AREKRQRTRGQTQDL) with medium stringency, and pT194 (GNQSSAWTLPRNGLL) and pS339 (SAYSRATSSSSSQPM) with low stringency.
5. *Chondroitin 4 sulfotransferase 11 (CHST11; NM_018413).* This is a member of the HNK1 sulfotransferase family GalNAc 4-*O*-sulfotransferase (MW 41,557; endoplasmic reticulum and mitochondria) that plays a role in chondroitin sulfate and dermatan sulfate biosynthesis.[64] It contains three mode I motifs; pS93 (TDTCRANSATSRKRR) with medium stringency, and pS56 (DICCRKGSRSPLQEL) and S194 (EPFERLVSAYRNKFT) with low stringency.
6. *Zinc finger, C3HC-type containing 1 (ZC3HC1; NM_016478).* This is a 60-kDa protein (MW 55,258; nuclear) that interacts with anaplastic lymphoma kinase (ALK) and plays an antiapoptotic role in nucleophosmin-ALK signaling event.[65] The 14-3-3-binding consensus motif mode I is not found.
7. *Minichromosome maintenance deficient 10 (MCM10; NM_018518).* This is a key component of the pre-replication complex (pre-RC) (MW 98,188; nuclear) essential for the initiation of DNA replication.[66] It contains five mode I motifs; pS90 (AQPPRTGSEFPRLEG) with medium stringency, and pS35 (KPAIKSISASALLKQ) S55 (LEMRRRKSEEIQKRF), pS302 (PCGNRSISLDRLPNK), and T329 (DGMLKEKTGPKIGGE) with low stringency.
8. *DEAD box polypeptide 54 (DDX54; NM_024072)* (Figure 13.2c). This is a 97-kDa RNA helicase (DP97) (MW 98,601; nuclear) that interacts with estrogen receptor (ER) and represses the transcription of ER-regulated genes.[67] It contains two mode I motifs with low stringency; pT95 (EDKKKIKTESGRYIS) and pS102 (TESGRYISSSYKRDL).
9. *Heterogeneous nuclear ribonucleoprotein C (HNPRC; NM_004500).* This is a member of heterogeneous nuclear ribonucleoproteins (hnRNPs) (MW 33,291; nuclear) involved in pre-mRNA processing, mRNA metabolism and transport.[68] It contains four mode I motifs; pS125 (DYYDRMYSYPARVPP) with high stringency, and pS158 (NTSRRGKSGFNSKSG), pS170 (KSGQRGSSKSGKLKG), and pS240 (ETNVKMESEGGADDS) with low stringency.
10. *Fibroblast growth factor 12 (FGF12; NM_004113).* This is a member of the FGF family (MW 27,401; nuclear) that plays a role in nervous system development and function.[69] It contains two mode I motifs with low

stringency; pS150 (VCMYREQSLHEIGEK) and pS165 (QGRSRKSS-GTPTMNG).

11. *Glutathione S-transferase M3 (GSTM3; BC030253).* This is a cytoplasmic glutathione *S*-transferase of the mu class (MW 26,561; cytoplasmic) that plays a role in detoxification of carcinogens, therapeutic drugs, environmental toxins, and products of oxidative stress.[70] It contains one mode I motif with low stringency; pS64 (GIKLRSFSV).
12. *Src homology three (SH3) and cysteine rich domain (STAC; NM_003149)* (Figure 13.2d). This is a 47-kDa protein containing a SH3 domain and a cysteine-rich domain (MW 44,556; nuclear) that plays a role in the neuron-specific signal transduction pathway.[71] It contains seven mode I motifs; pS172 (KGFRRYYSSPLLIHE) with high stringency (Figure 13.3c), pS56 (TKSLRSKSADNFFQR) and pS255 (DLRKRSNSVFTYPEN) with medium stringency, and pS46 (QKLKRSLSFKTKSLR), pS51 (SLSFK-TKSLRSKSAD), pS66 (NFFQRTNSEDMKLQA), and pS253 (GYDL-RKRSNSVFTYP) with low stringency.
13. *ATPase, H^+ transporting, lysosomal, 21 kD, V0 subunit C" (ATP6V0B; NM_004047).* This is a 23-kDa component of vacuolar ATPase (MW 21,408; endoplasmic reticulum) that mediates acidification of intracellular organelles.[72] The 14-3-3-binding consensus motif mode I is not found.

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 higher Z-Score values.[56] The recombinant proteins were expressed in HEK293 cells that constitutively express a substantial amount of endogenous 14-3-3 protein. The cells were homogenized in the lysis buffer either with inclusion of phosphatase inhibitors or with inclusion of recombinant protein phosphatase-1 (PP1) instead of phosphatase inhibitors. Total cell lysate was processed for immunoprecipitation (IP) with rabbit anti-14-3-3 protein antibody (K-19) or with normal rabbit IgG. K19 coimmunoprecipitated 14-3-3 and STAC from the lysate of HEK293 cells that express the recombinant STAC protein, whereas normal rabbit IgG did not pull down these proteins (Figure 13.1). K-19 immunoprecipitated EAP30 and DDX54 from the lysate of HEK293 cells that express the recombinant EAP30 or DDX54 protein, respectively (Figure 13.1). These results indicate that EAP30, DDX54 and STAC 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 to 174 (pS172) by the Scansite Motif Scanner search (Figure 13.3). Therefore, a possible involvement of this motif in binding to 14-3-3 was further investigated by IP analysis of a panel of mutant and truncated STAC proteins. K-19 immunoprecipitated the full-length wild-type (WT) STAC consisting of amino acid residues 2 to 402 (Figure 13.1). K-19 also pulled down the S172A mutant (SMT), and the S172A and S173A double mutant (DMT) from the lysate of HEK293 cells that express the corresponding recombinant proteins.[56] K-19 immunoprecipitated

the N-terminal half (NTF; amino acid residues 2 to 233) but not the C-terminal half (CTF; amino acid residues 234 to 402) of STAC (Figure 13.1). These observations indicate that the RYYSSP motif is not involved in binding of STAC to 14-3-3. This was confirmed by the observations that K-19 immunoprecipitated the truncated STAC protein lacking the RYYSSP motif (TRA; amino acid residues 2 to 164)[56] and the shortest form lacking both the RYYSSP sequence and the cysteine-rich domain (CRD) (TRB; amino acid residues 2 to 105) (Figure 13.1). Finally, K-19 pulled down the full-length WT STAC, EAP30, and DDX54 under the dephosphorylated condition (PP1) (Figure 13.1). These observations indicate that the 14-3-3-interacting domain is located in the N-terminal segment spanning amino acid residues 2 to 105 of STAC. The interaction of 14-3-3 with STAC, EAP30, and DDX54 is independent of serine/threonine-phosphorylation of the binding domains.

DISCUSSION

Protein Microarray Analysis Effectively Identifies 14-3-3-Binding Proteins

Protein microarrays provide a valuable tool for global proteome analysis with a wide range of applications, particularly to identification and characterization of protein function and molecular pathways closely associated with disease markers and therapeutic targets.[39–43] The great advantage of this technology exists in low reagent and sample consumption, rapid interpretation of the results, and the ability to easily manipulate experimental conditions.

The present study was designed to identify 14-3-3-binding proteins by using a high-density human protein microarray. The array contains 1752 proteins derived from multiple gene families of biological importance, including cell-signaling proteins, kinases, membrane-associated proteins, and metabolic proteins. The entire procedure could be accomplished within five hours of obtaining a specific probe. By probing with V5-tagged 14-3-3ε, we identified twenty 14-3-3 interactors, most of which were previously unreported except for glutathione *S*-transferase M3 (GSTM3) that was reported previously.[36] Unexpectedly, the highly stringent 14-3-3-binding consensus motifs (STAC and HNPRC) were identified only in two by the Scansite Motif Scanner search. The specific binding to 14-3-3 of EAP30, DDX54 and STAC was validated by coimmunoprecipitation analysis of the recombinant proteins expressed in HEK293 cells. These results indicate that protein microarray is an effective tool for the rapid and systematic identification of protein–protein interactions, including those not predicted by the Database searching.

Potential Problems Remain to Be Solved in the Present Study

In general, protein microarray has its own limitations associated with the efficient expression and purification of native target proteins.[40,41] The target proteins spotted on the microarray we utilized were expressed by a baculovirus expression system and purified under non-denaturating conditions to maximize the preservation of native folding, posttranslational modifications, and proper functionality. In contrast,

bacterially expressed proteins lack glycosylation and phosphorylation moieties, and are often misfolded during purification. Post-translational modifications play a pivotal role in a range of protein–protein interactions. Immuno-labeling with anti-phosphotyrosine (pTyr) antibody showed that approximately 10 to 20% of the proteins on the array are phosphorylated (Invitrogen, unpublished data). When it was utilized for kinase substrate identification, most of known kinases immobilized on the array are enzymatically active with the capacity of autophosphorylation, suggesting that they are certainly phosphorylated on tyrosine residues, probably on serine and threonine residues (Invitrogen application note). However, we could not currently validate the precise level of serine and threonine phosphorylation of individual target proteins due to a lack of anti-phosphoserine (pSer) and anti-phosphothreonine (pThr) antibodies suitable for detection on glass slides.

The protein microarray we utilized includes 11 known 14-3-3-binding proteins, such as PCTAIRE protein kinase 1 (PCTK1),[73] protein kinase C zeta (PRKCZ),[74] keratin 18 (KRT18),[75] myosin light polypeptide kinase (MYLK),[76] v-abl Abelson murine leukemia viral oncogene homolog 1 (ABL1),[77] v-akt murine thymoma viral oncogene homolog 1 (AKT1),[78] epidermal growth factor receptor (EGFR),[79] cell division cycle 2 (CDC2),[80] mitogen-activated protein kinase kinase kinase 1 (MAP3K1),[81] mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2),[82] and stratifin (SFN).[37] However, none of these were identified as positive. Therefore, there exists the possibility that some 14-3-3 binding partners were not detected due to imperfect phosphorylation of target proteins, inaccessibility by a sterical hindrance of epitope tags,[83] or a 14-3-3 isoform-specific binding ability. Calmodulin, another known 14-3-3 interactor,[84] is included as a negative control on the array. It was found 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. Recently, by using two dimensional (2-D)-gel electrophoresis and mass spectrometry, we showed that vimentin, an intermediate filament protein, interacts with 14-3-3ε in cultured human astrocytes.[20] More recently, we found that heat shock protein Hsp60 and the cellular prion protein PrPC interact with 14-3-3ζ in human neurons in culture and brain tissues.[85] Unfortunately, the protein microarray we examined here includes neither vimentin, Hsp60 nor prion protein.

Repetition of "kinase" OK here?

Recent evidence indicates that 14-3-3-binding phosphorylation sites do not exactly fit the consensus motif,[1,25,75] and an accessory site is required to enhance a stable 14-3-3-target interaction.[4,86] Furthermore, 14-3-3 interacts with a set of target proteins in a phosphorylation-independent manner.[26–29] We found that the interaction is independent of serine/threonine-phosphorylation of the binding sites of EAP30, DDX54 and STAC, supporting this possibility.

Biological Roles of 14-3-3-Interacting Proteins

Among the 14-3-3 interactors we identified, several proteins are 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. EAP30 is a component of the ELL complex where EAP30 mediates derepression of transcription by Pol II,[59] although the PSORT

II search suggests that its putative location is cytoplasmic. A recent study showed that EAP30 interacts with the tumor susceptibility gene TSG101 product, a cellular factor that mediates packaging of HIV virions.[87] DDX54 is a RNA helicase that interacts with estrogen receptor (ER) and represses the transcription of ER-regulated genes.[67] A chromatin immunoprecipitation (ChIP) assay 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.[88] HNPRC belongs to a member of heterogeneous nuclear ribonucleoproteins (hnRNPs) involved in pre-mRNA processing, mRNA metabolism and transport.[68] Increasing evidence indicates that the 14-3-3 protein and its targets are widely distributed in nearly all subcellular compartments, including the nucleus.[3,35]

STAC 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 role as an adapter on which divergent signaling pathways converge.[71,89] STAC is expressed predominantly in the brain with the distribution in a defined population of neurons.[71] IP analysis of mutant and truncated forms of STAC argued against an active involvement of the most stringent motif RYYSSP (pS172) in its binding to 14-3-3, and indicated that the interacting motif is located in the N-terminal amino acid residues 2 to 105 without requirement of serine/threonine phosphorylation.

FUTURE DIRECTIONS

Protein microarrays are a powerful tool for the rapid and systematic identification of protein–protein and other biomolecule interactions. However, they are still under development in methodological aspects. The strict quality controls of analytical procedures,[90] validation of the results by different methods, and evaluation of enormous data by bioinformatic approaches are highly important. The applications of protein microarrays include characterization of antibody specificity and autoantibody repertoire, and identification of novel biomarkers and molecular targets associated with disease type, stage and progression, leading to establishment of personalized medicine.[44–51] Theoretically, this technology could determine all of the binding partners at once, consisting of "the whole interactome" in a subset of cells responding to specific treatment. It would open up a new avenue of drug discovery research. Development of an ultrahigh-density protein microarray containing all spliced variants of target proteins could facilitate achievement of this purpose. A cell-free transcription and translation-coupled system might provide an effective tool for producing ideal proteins.[83] At present, the most advanced version of human protein microarray contains approximately 5000 GST-tagged proteins, commercially available from Invitrogen (ProtoArray v3.0), accompanied by an upgraded version of the analytical software (ProtoArray Prospector). It seems highly efficient to screen a large number of protein–protein interactions in human cells, including those unrecognized by the conventional methods such as Y2H.[91,92] However, when faced with a huge amount of data, bioinformatic and statistical analyses become crucial (visit the useful website of Pathguide for a comprehensive pathway resource list; cbio.mskcc.org/prl). Recently, an ultrahigh sensitive detection method armed with

silicon-nanowire field-effect sensors has come into use with its application to protein microarray analysis.[93] This promising technology could detect the low-femtomolar range of interacting proteins, and greatly increase the detection sensitivity and specificity.

SUMMARY AND CONCLUSIONS

The 14-3-3 protein family consists of acidic 30-kDa proteins composed of seven isoforms in mammalian cells, expressed abundantly in neurons and glial cells of the CNS. The 14-3-3 isoforms form a dimer that acts as a molecular adaptor interacting with key signaling components involved in cell proliferation, transformation, and apoptosis. Until present, more than 300 proteins have been identified as 14-3-3-binding partners, although most of previous studies focused on a limited range of 14-3-3-interacting proteins. In this chapter we describe a comprehensive profile of 14-3-3-binding proteins by analyzing a high-density protein microarray (1752 proteins; ProtoArray v1.0) using recombinant human 14-3-3ε protein as a probe. We identified twenty 14-3-3 interactors, most of which were previously unreported 14-3-3-binding partners, although eleven known 14-3-3-binding proteins on the array, including KRT18 and MAPKAPK2, were undetected. The assay required less than five hours. Unexpectedly, highly stringent 14-3-3-binding consensus motifs, such as STAC and HNPRC, were identified only in two proteins by the Scansite Motif Scanner search. The specific binding to 14-3-3 of EAP30, DDX54 and STAC was verified by coimmunoprecipitation analysis of the recombinant proteins expressed in HEK293 cells. These results suggest that protein microarray is a valuable tool for rapid and comprehensive profiling of 14-3-3-binding proteins.

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Microarray analysis identifies an aberrant expression of apoptosis and DNA damage-regulatory genes in multiple sclerosis

Jun-ichi Satoh,[a] Megumi Nakanishi,[a] Fumiko Koike,[a] Sachiko Miyake,[a] Toshiyuki Yamamoto,[b] Mitsuru Kawai,[b] Seiji Kikuchi,[c] Kyouichi Nomura,[d] Kazumasa Yokoyama,[e] Kohei Ota,[f] Takashi Kanda,[g] Toshiyuki Fukazawa,[h] and Takashi Yamamura[a,*]

[a]*Department of Immunology, National Institute of Neuroscience, NCNP, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan*
[b]*National Center Hospital for Mental, Nervous and Muscular Disorders, NCNP, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan*
[c]*Department of Neurology, Hokkaido University Graduate School of Medicine, Kita-15 Nishi-7, Kita-ku, Sapporo 060-8638, Japan*
[d]*Department of Neurology, Saitama Medical School, 38 Morohongo, Moroyamachou, Saitama 350-0495, Japan*
[e]*Department of Neurology, Juntendo University School of Medicine, Hongo 2-1-1, Bunkyo-ku, Tokyo 113-841, Japan*
[f]*Department of Neurology, Tokyo Women's Medical University, 8-1 Kawadachou, Shinjuku-ku, Tokyo 162-8666, Japan*
[g]*Department of Neurology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan*
[h]*Hokuyukai Neurology Hospital, Niju-Yon-Ken 2-2-4-30, Nishi-ku, Sapporo 063-0802, Japan, Sapporo, Japan*

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To clarify the molecular mechanisms underlying multiple sclerosis (MS)-promoting autoimmune process, we have investigated a comprehensive gene expression profile of T cell and non-T cell fractions of peripheral blood mononuclear cells (PBMC) isolated from 72 MS patients and 22 age- and sex-matched healthy control (CN) subjects by using a cDNA microarray. Among 1258 genes examined, 173 genes in T cells and 50 genes in non-T cells were expressed differentially between MS and CN groups. Downregulated genes greatly outnumbered upregulated genes in MS. More than 80% of the top 30 most significant genes were categorized into apoptosis signaling-related genes of both proapoptotic and antiapoptotic classes. They included upregulation in MS of orphan nuclear receptor Nurr1 (NR4A2), receptor-interacting serine/threonine kinase 2 (RIPK2), and silencer of death domains (SODD), and downregulation in MS of TNF-related apoptosis-inducing ligand (TRAIL), B-cell CLL/lymphoma 2 (BCL2), and death-associated protein 6 (DAXX). Furthermore, a set of the genes involved in DNA repair, replication, and chromatin remodeling was downregulated in MS. These results suggest that MS lymphocytes show a complex pattern of gene regulation that represents a counterbalance between promoting and preventing apoptosis and DNA damage of lymphocytes.

Keywords: Apoptosis; Gene expression profile; Microarray; Multiple sclerosis

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) white matter. Although the etiology of MS remains unknown, immunological studies have suggested that MS is an autoimmune disease mediated by T-lymphocytes secreting proinflammatory T helper type 1 (Th1) cytokines, whose development is triggered by a complex interplay of both genetic and environmental factors (Compston and Coles, 2002). Increasing evidence indicates that the elimination of autoreactive T cells via apoptosis, a common regulatory mechanism for normal development and homeostasis of the immune system, is impaired in MS (Zipp et al., 1999). The mRNA levels of Fas, Fas ligand, and TNF-related apoptosis-inducing ligand (TRAIL) are elevated in peripheral blood mononuclear cells (PBMC) of relapsing–remitting MS (RRMS) patients, while T cell lines established from these patients show a functional defect in the Fas signaling pathway (Comi et al., 2000; Gomes et al., 2003; Huang et al., 2000). The expression of B-cell CLL/lymphoma 2 (BCL2) family proteins is dysregulated in lymphocytes of clinically active MS patients in a manner that promotes resistance to apoptosis (Sharief et al., 2003). Furthermore, apoptosis-regulatory proteins are aberrantly expressed in active MS brain lesions (Bonetti et al., 1999; D'Souza et al., 1996). However, the precise implication of these observations in immunopathogenesis of MS is fairly limited, because most of these studies have focused on a limited range of apoptosis-signaling regulators.

The DNA microarray technology is a novel approach that allows us to systematically and simultaneously monitor the expression of a great number of genes. Application of this

* Corresponding author. Fax: +81 42 346 1753.
E-mail address: yamamura@ncnp.go.jp (T. Yamamura).
Available online on ScienceDirect (www.sciencedirect.com).

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technique has begun to give us new insights into the complexity of molecular interactions involved in the MS-promoting autoimmune process (Steinman and Zamvil, 2003). Actually, microarray analysis identified upregulation of a set of genes in active MS brain lesions, whose pathological role has not been previously predicted in MS (Lock et al., 2002). Recently, we have studied the gene expression profile of T cells and non-T cells derived from RRMS before and after treatment with interferon-beta (IFNβ) (Koike et al., 2003). IFNβ altered the expression of 21 genes, including nine with IFN-responsive promoter elements, thereby contributing to the therapeutic effects of IFNβ in MS. Supporting our observations, different studies using distinct cDNA microarrays identified IFNβ-responsive genes expressed in PBMC of RRMS patients receiving IFNβ (Stürzebecher et al., 2003; Weinstock-Guttman et al., 2003). Importantly, a recent study showed that a battery of the genes relevant to development of MS include those encoding apoptosis regulators, although this study enrolled only four MS patients (Maas et al., 2002).

Here we investigated a comprehensive gene expression profile of $CD3^+$ T cells and $CD3^-$ non-T cells isolated from 72 MS patients and 22 healthy subjects by using a cDNA microarray containing 1258 genes of various functional classes. We found that 173 genes in T cells and 50 genes in non-T cells were differentially expressed between MS and control (CN) groups. Unexpectedly, more than 80% of the top 30 most significant genes were categorized into apoptosis signaling-related genes of both proapoptotic and antiapoptotic classes, reflecting a counterbalance between resistance and susceptibility of lymphocytes toward apoptosis in MS.

Materials and methods

The study populations

The present study enrolled 72 Japanese, clinically active MS patients and age- and sex-matched 22 Japanese healthy control (CN) subjects. Their demographic characteristics are listed in Table 1. The MS patients were diagnosed according to the established criteria (McDonald et al., 2001). No patients had a past history of treatment with interferons, glatiramer acetate, or mitoxantrone. No patients had received corticosteroids or other immunosuppressants at least 1 month before blood sampling. Written informed consent was obtained from all subjects.

RNA isolation from T cell and non-T cell fractions

Thirty milliliters of heparinized blood was taken in the morning. Within 6 h, PBMCs were isolated by centrifugation on a Ficoll density gradient. Immediately, they were labeled with anti-CD3 antibody-coated magnetic microbeads and separated by AutoMACS (Miltenyi Biotec, Auburn, CA) into a $CD3^+$ T cell fraction and a $CD3^-$ non-T cell fraction, the latter composed of monocytes, B cells, and NK cells. The viability of the cells and the purity of each fraction were verified by trypan blue dye exclusion test and flow cytometric analysis. Total RNA was isolated from each fraction by using RNeasy Mini Kit (Qiagen, Valencia, CA). Five micrograms of purified RNA was in vitro amplified within a linear range of the amplification, and the antisense RNA (aRNA) was processed for cDNA microarray analysis as described previously (Koike et al., 2003).

cDNA microarray analysis

The present study utilized a custom microarray containing duplicate spots of 1258 cDNA immobilized on a poly-L-lysine-coated slide glass (Hitachi Life Science, Kawagoe, Saitama, Japan). They were prepared by PCR of sequence-known genes of various functional classes, including cytokines/growth factors and their receptors, apoptosis regulators, oncogenes, transcription factors, cell cycle regulators, and housekeeping genes. The complete gene list of the microarray is available upon request (express@ls.hitachi.co.jp). Individual aRNA of MS patients and CN subjects was labeled with a fluorescent dye Cy5 by reverse transcriptase reaction. Pooled aRNA of three independent healthy volunteers who were not included in the study was labeled with Cy3 and used as a universal reference to standardize the gene expression levels throughout the experiments as described previously (Koike et al., 2003). The arrays were hybridized at 62°C for 10 h in the hybridization buffer containing equal amounts of Cy3- or Cy5-labeled cDNA, and they were then scanned by the ScanArray 5000 scanner (GSI Lumonics, Boston, MA). The data were analyzed by using the QuantArray software (GSI Lumonics). The average of fluorescence intensities (FI) of duplicate spots was obtained after global normalization between Cy3 and Cy5 signals.

Table 1
Demographic characteristics of the study populations

Characteristics	Multiple sclerosis (MS) patients	Healthy control (CN) subjects
The number of the study population (*n*)	72	22
Age (average ± SD, year)	36.1 ± 10.3	38.6 ± 12.3
Sex (male vs. female)	17 vs. 55	6 vs. 16
Disease course (RRMS vs. SPMS)	65 vs. 7	(–)
Disease subtype (conventional MS vs. non-conventional MS)	57 vs. 15	(–)
Disease duration (average ± SD, year)	7.7 ± 5.4	(–)
EDSS score (average ± SD, score)	2.8 ± 2.0	(–)
Number of lesions on T2-weighted MRI (average ± SD, number)	24.7 ± 31.9	(–)
Number of relapses during 2 years before blood sampling (average ± SD, number)	1.9 ± 1.5	(–)
Day of IVMP treatment during 2 years before blood sampling (average ± SD, day)	5.9 ± 5.8	(–)
Day of hospitalization during 2 years before blood sampling (average ± SD, day)	49.7 ± 70.0	(–)

Based on the lesion distribution pattern, MS was separated into two subtypes, that is, the conventional MS that affects various regions of the CNS white matter and non-conventional MS that affects chiefly the optic nerve and the spinal cord. Abbreviations: RRMS, relapsing–remitting MS; SPMS, secondary progressive MS; EDSS, expanded disability status scale; and IVMP, intravenous methylprednisolone pulse.

The impact of inter-experiment variability was verified by analyzing a scatter plot. The genes exhibiting the average FI smaller than the level of 1000 were omitted to be processed for further analysis. The gene expression level (GEL) was calculated according to the formula: GEL = FI (Cy5) of the sample/FI (Cy3) of the universal reference. Some results were expressed as box and whisker plots.

The genes were categorized into the group of apoptosis signaling-related genes, when their involvement in regulation of apoptosis was identified through computerized searches in PubMed.

Statistical analysis

The statistical significance of differences in GEL between MS and CN samples was evaluated by a regularized *t* test (Cyber-T) using the Bayesian inference of variance, where they were considered as significant when the error rate of this test was smaller than 0.05 (Baldi and Long, 2001).

Northern blot analysis

Unfractionated PBMCs of a healthy subject were suspended at 5×10^6 cells/ml in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 55 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were then incubated in a 5%CO_2/95% air incubator at 37°C for 6 h in medium with inclusion of both 25 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO) and 1 μg/ml ionomycin (IOM; Sigma), or incubated for 24 h in the plate coated with 1 μg/ml mouse monoclonal antibody (mAb) against human CD3 (OKT3) or in the medium containing 100 ng/ml recombinant human IFN-gamma (IFNγ) (a specific activity of $\geq 2 \times 10^7$ units/mg, PeproTech, London, UK). They were processed for RNA preparation as described previously (Satoh and Kuroda, 2001). Three micrograms of total RNA was separated on a 1.5% agarose–6% formaldehyde gel and transferred onto a nylon membrane. After prehybridization, the membranes were hybridized at 54°C overnight with the DIG-labeled DNA probe synthesized by the PCR DIG probe synthesis kit (Roche Diagnostics, Mannheim, Germany) using the sense and antisense primer sets listed in Supplementary Table 1 online. The specific reaction was visualized on Kodak X-OMAT AR X-ray films by the DIG chemiluminescence detection kit (Roche Diagnostics).

Results

Microarray analysis identified differentially expressed genes in peripheral blood lymphocytes between MS and controls

Among 1258 genes examined, 173 genes in T cell fraction and 50 genes in non-T cell fraction were expressed differentially between 72 MS patients and 22 CN subjects (see Supplementary Table 2 online for all data set). In T cell fraction, 25 genes were upregulated, while 148 genes were downregulated in MS. In non-T cell fraction, 11 genes were upregulated, while 39 genes were downregulated in MS. Thus, downregulated genes greatly outnumbered upregulated genes in MS. No genes showed an opposed pattern of regulation between T cell and non-T cell fractions. The top 30 most significant genes are listed in Tables 2 and 3, and among them, top 10 are expressed as box and whisker plots (Figs. 1 and 2). Among top 30 genes, six genes, such as regulator of G protein signaling 14 (RGS14), SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 3 (SMARCA3), transcription factor 17 (TCF17), carbohydrate sulfotransferase 4 (CHST4), cytochrome *c* oxidase assembly protein (COX15), and death-associated protein 6 (DAXX), were downregulated coordinately in both cell fractions.

The majority of top 30 differentially expressed genes between MS and controls were categorized into apoptosis signaling-related genes

In T cell fraction, the top 30 contained 25 genes closely related to apoptosis signaling (Table 2). They included upregulation in MS of nuclear receptor subfamily 4, group A, member 2 (NR4A2; No. 1), transcription factor 8 (TCF8; No. 2), and cytochrome *P*450 family 1, subfamily A, polypeptide 2 (CYP1A2; No. 3). They also included downregulation in MS of RGS14 (No. 4), mitogen-activated protein kinase 1 (MAPK1; No. 6), SMARCA3 (No. 7), TCF17 (No. 9), heat shock 70-kD protein 1A (HSPA1A; No. 10), TRAIL (No. 12), topoisomerase 1 (TOP1; No. 13), protein tyrosine phosphatase, non-receptor type 6 (PTPN6; No. 14), chemokine, CC motif, receptor 5 (CCR5; No. 15), v-erb-a erythroblastic leukemia viral oncogene homolog 4 (ERBB4; No. 17), transcription factor 21 (TCF21; No. 18), ATPase, hydrogen-transporting, lysosomal, 56/58 kDa, V1 subunit B, isoform 2 (ATP6V1B2; No. 19), cAMP responsive element-binding protein 1 (CREB1; No. 20), integrin, beta 1 (ITGB1; No. 21), COX15 (No. 22), Myc protooncogene (MYC; No. 23), BCL2-associated athanogene 1 (BAG1; No. 24), cell division cycle 16 (CDC16, No. 25), DAXX (No. 27), TGFβ-stimulated gene 22 (TSC22; No. 28), GA-binding protein transcription factor, beta subunit 1 (GABPB1; No. 29), and poly(ADP-ribose) polymerase (PARP; No. 30). Surprisingly, the top 30 included none of Th1-specific marker genes except for CCR5. The concurrent downregulation of proapoptotic and antiapoptotic genes such as TRAIL, DAXX, and BAG1 suggests that the gene expression pattern in T cells of MS represents a counterbalance between promoting and preventing apoptosis.

In non-T cell fraction, the top 30 contained 27 apoptosis signaling-related genes (Table 3). They included upregulation in MS of cell division cycle 42 (CDC42; No. 2), receptor-interacting serine/threonine kinase 2 (RIPK2; No. 3), Max dimerization protein (MAD; No. 5), chemokine, CXC motif, ligand 2 (CXCL2; No. 6), silencer of death domains (SODD; No. 7), topoisomerase 2 alpha (TOP2A; No. 8), and intercellular adhesion molecule-1 (ICAM1; No. 1). ICAM1 was listed as an apoptosis signaling-related gene because it provides a costimulatory signal to protect T cells from apoptosis by upregulation of BCL2 (Kohlmeier et al., 2003). They also included downregulation in MS of SMARCA3 (No. 9), RGS14 (No. 10), COX15 (No. 11), A-kinase anchor protein 11 (AKAP11; No. 12), TCF17 (No. 13), cell division cycle 25B (CDC25B; No. 14), granzyme A (GZMA; No. 15), BCL2 (No. 17), complement component receptor 2 (CR2; No. 18), replication protein A1 (RPA1; No. 19), RNA polymerase II, subunit H (POLR2H; No. 20), E2F transcription factor 5 (E2F5; No. 21), Ras associated protein RAB7-like 1 (RAB7L1; No. 22), nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 3 (NFATC3; No. 23), heat shock 70-kD protein-like 1 (HSPA1L; No. 24), retinoblastoma-binding protein 4 (RBBP4; No.

Table 2
Top 30 genes expressed differentially in T cells between MS and controls

No.	Symbol	GenBank accession number	Description	Presumed function	Possible involvement in apoptosis regulation	Significance (p-log)
The upregulated genes						
1	NR4A2	NM_006186	Nuclear receptor subfamily 4, group A, member 2	an orphan nuclear receptor of the steroid–thyroid hormone receptor superfamily designated Nurr1	(+)	2.55E−12
2	TCF8	NM_030751	Transcription factor 8	a transcription repressor for IL-2 expression in T cells designated ZEB	(+)	1.17E−09
3	CYP1A2	NM_000761	Cytochrome P450, family 1, subfamily A, polypeptide 2	a monooxygenase involved in the metabolism of drugs, toxic chemicals, and carcinogens	(+)	1.64E−08
The downregulated genes						
4	RGS14	NM_006480	Regulator of G protein signaling 14	a downregulator of signaling through G protein-coupled receptors	(+)	1.51E−13
5	CHST2	NM_004267	Carbohydrate sulfotransferase 2	an *N*-acetylglucosamine-6-*O*-sulfotransferase	unknown	6.43E−13
6	MAPK1	NM_002745	Mitogen-activated protein kinase 1	a protein kinase designated ERK2 (p42) that regulates diverse cellular functions	(+)	6.02E−12
7	SMARCA3	NM_003071	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 3	a DNA helicase-like chromatin remodeling enzyme	(+)	1.70E−11
8	TPST2	NM_003595	Tyrosylprotein sulfotransferase 2	a tyrosylprotein sulfotransferase	unknown	2.31E−11
9	TCF17	NM_005649	Transcription factor 17 designated Kid-1	a transcriptional repressor of renal genes	(+)	3.14E−11
10	HSPA1A	NM_005345	Heat shock 70kD protein 1A	an inducible member of the HSP70 family	(+)	4.67E−11
11	AGTRL2	NM_005162	Angiotensin receptor-like 2	a protein homologous to the angiotensin II receptor type 1	unknown	3.51E−10
12	TRAIL	NM_003810	TNF-related apoptosis-inducing ligand	an apoptosis-inducing ligand of the TNF family for DR4 and DR5	(+)	5.19E−10
13	TOP1	NM_003286	Topoisomerase 1	a DNA topoisomerase	(+)	7.03E−10
14	PTPN6	NM_080549	Protein tyrosine phosphatase, non-receptor type 6	a protein tyrosine phosphatase with SH2 domains designated SHP-1	(+)	7.77E−10
15	CCR5	NM_000579	Chemokine, CC motif, receptor 5	a chemokine receptor for RANTES, MIP1α, MIP1β and MCP2	(+)	1.10E−09
16	CHST4	NM_005769	Carbohydrate sulfotransferase 4	an *N*-acetylglucosamine 6-O sulfotransferase	unknown	1.84E−09
17	ERBB4	NM_005235	V-erb-a erythroblastic leukemia viral oncogene homolog 4	an EGF receptor-related receptor tyrosine kinase interacting with neuregulins	(+)	2.22E−09
18	TCF21	NM_003206	Transcription factor 21	a transcription factor designated Capsulin	(+)	4.99E−09
19	ATP6V1B2	NM_001693	ATPase, hydrogen-transporting, lysosomal, 56/58kD, V1 subunit B, isoform 2	a vacuolar ATPase that mediates acidification of intracellular organelles	(+)	5.10E−09
20	CREB1	NM_134442	Cyclic AMP responsive element-binding protein 1	a CRE-binding transcription factor for cAMP-responsive genes	(+)	6.58E−09
21	ITGB1	NM_002211	Integrin, beta-1	a common beta chain of the VLA protein family	(+)	7.16E−09
22	COX15	NM_078470	Cytochrome c oxidase assembly protein COX15	a protein essential for assembly of COX	(+)	1.13E−08
23	MYC	NM_002467	Myc protooncogene	a transcription factor that regulates diverse cellular functions	(+)	1.18E−08
24	BAG1	NM_004323	BCL2-associated athanogene 1	an enhancer of the antiapoptotic effects of BCL2	(+)	1.51E−08
25	CDC16	NM_003903	Cell division cycle 16	a component of the anaphase-promoting complex essential for mitosis	(+)	1.99E−08
26	SLC35A1	NM_006416	Solute carrier family 35, member A1	a CMP-sialic acid transporter	unknown	2.06E−08
27	DAXX	NM_001350	Death-associated protein 6	a protein that interacts with the death domain of Fas	(+)	2.23E−08
28	TSC22	NM_006022	TGFβ-stimulated gene 22	a transcription factor induced by TGFβ	(+)	2.34E−08
29	GABPB1	NM_005254	GA-binding protein transcription factor, beta subunit 1	a nuclear transcription factor for cytochrome *c* oxidase	(+)	6.16E−08
30	PARP	NM_001618	Poly(ADP-ribose) polymerase	a chromatin-associated enzyme that catalyzes polyADP-ribosylation of nuclear proteins	(+)	6.72E−08

The genes were categorized into the group of apoptosis signaling-related genes, when their involvement in regulation of apoptosis was identified through computerized searches in PubMed. The average signal intensity and average increase (fold change) of the genes listed are shown in Supplementary Table 2 online.

Table 3
Top 30 genes expressed differentially in non-T cells between MS and controls

No.	Symbol	GenBank accession number	Description	Presumed function	Possible involvement in apoptosis regulation	Significance (p-log)
The upregulated genes						
1	ICAM1	NM_000201	Intercellular adhesion molecule-1	a cell surface glycoprotein ligand (CD54) for LFA-1 and Mac-1	(+)	1.11E−09
2	CDC42	NM_001791	Cell division cycle 42	a small GTPase that regulates diverse cellular functions	(+)	1.49E−08
3	RIPK2	NM_003821	Receptor-interacting serine/threonine kinase 2	a protein kinase interacting with CLARP in the Fas-signaling pathway	(+)	1.88E−07
4	IL1R2	NM_004633	IL-1 receptor, type II	a decoy receptor for IL-1 that inhibits IL-1 activity	unknown	4.56E−07
5	MAD	NM_002357	Max dimerization protein	a transcriptional repressor that competes with MYC for binding to MAX	(+)	1.00E−06
6	CXCL2	NM_002089	Chemokine, CXC motif, ligand 2	a chemokine designated MIP2 binding to CXCR2	(+)	1.91E−06
7	SODD	NM_004874	Silencer of death domains	an adaptor protein designated BAG4 associated with HSP70 and the death domain of TNFR1 and DR3	(+)	3.13E−06
8	TOP2A	NM_001067	Topoisomerase 2 alpha	a DNA topoisomerase	(+)	4.82E−06
The downregulated genes						
9	SMARCA3	NM_003071	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 3	a DNA helicase-like chromatin remodeling enzyme	(+)	3.95E−08
10	RGS14	NM_006480	Regulator of G protein signaling 14	a downregulator of signaling through G protein-coupled receptors	(+)	5.44E−08
11	COX15	NM_078470	Cytochrome c oxidase assembly protein COX15	a protein essential for assembly of COX	(+)	6.43E−08
12	AKAP11	NM_016248	A-kinase anchor protein 11	a protein anchoring the regulatory subunit of protein kinase A	(+)	1.68E−07
13	TCF17	NM_005649	Transcription factor 17	a transcriptional repressor of renal genes designated Kid-1	(+)	1.92E−07
14	CDC25B	NM_021874	Cell division cycle 25B	a tyrosine phosphatase that activates the cyclin dependent kinase CDC2	(+)	2.40E−07
15	GZMA	NM_006144	Granzyme A	a cytotoxic T cell- and NK cell-specific serine protease	(+)	2.49E−07
16	CHST4	NM_005769	Carbohydrate sulfotransferase 4	an *N*-acetylglucosamine 6-O sulfotransferase	unknown	3.46E−06
17	BCL2	NM_000633	B-cell CLL/lymphoma 2	a mitochondrial membrane protein that blocks the apoptotic death	(+)	4.81E−07
18	CR2	NM_001877	Complement component receptor 2	a membrane receptor (CD21) for C3d	(+)	5.88E−07
19	RPA1	NM_002945	Replication protein A1	a single-stranded DNA-binding protein that regulates DNA replication	(+)	6.72E−07
20	POLR2H	NM_006232	RNA polymerase II, subunit H	a subunit of RNA polymerase II	(+)	7.28E−07
21	E2F5	NM_001951	E2F transcription factor 5	a transcription factor of the E2F family	(+)	1.00E−06
22	RAB7L1	NM_003929	Ras associated protein RAB7-like 1	a RAS-related small GTP-binding protein	(+)	1.49E−06
23	NFATC3	NM_173165	Nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 3	a component of DNA-binding transcription complex that regulates the gene expression in T cells	(+)	1.66E−06
24	HSPA1L	NM_005527	Heat shock 70-kD protein-like 1	a constitutive member of the HSP70 family	(+)	1.87E−06
25	RBBP4	NM_005610	Retinoblastoma-binding protein 4	a nuclear protein binding to RB1	(+)	3.13E−06
26	PRKDC	NM_006904	Protein kinase, DNA-activated, catalytic subunit	a nuclear serine/threonine protein kinase	(+)	3.36E−06
27	RASSF1	NM_170714	Ras association domain family 1	a lung tumor suppressor gene having a Ras-association domain	(+)	3.49E−06
28	DAXX	NM_001350	Death-associated protein 6	a protein that interacts with the death domain of Fas	(+)	5.16E−06
29	EGF	NM_001963	Epidermal growth factor	a potent mitogenic factor for the cells of both ectodermal and mesodermal origin	(+)	5.74E−06
30	NPR2L	NM_006545	Nitrogen permease regulator 2-like	a possible tumor suppressor gene	unknown	1.13E−05

The genes were categorized into the group of apoptosis signaling-related genes, when their involvement in regulation of apoptosis was identified through computerized searches in PubMed. The average signal intensity and average increase (fold change) of the genes listed are shown in Supplementary Table 2 online.

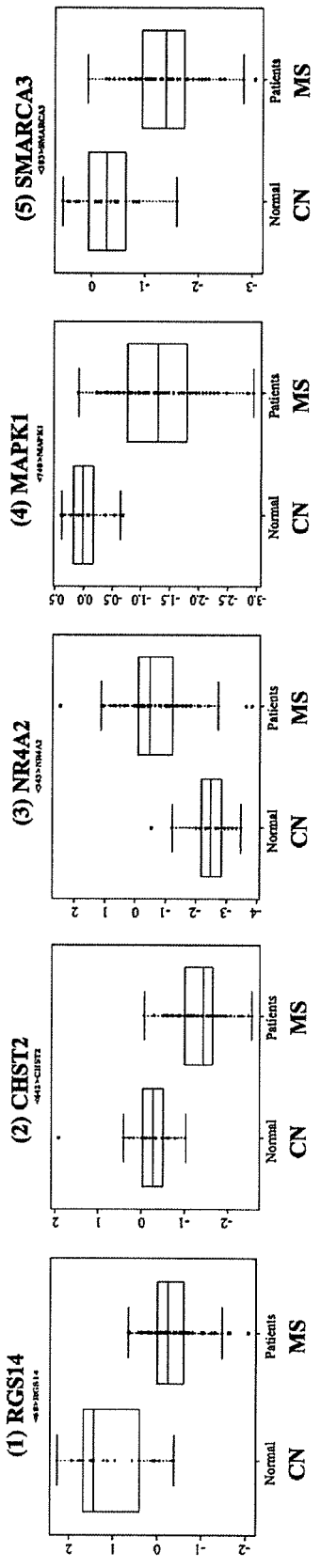

Fig. 1. Top 10 differentially expressed genes in T cell fraction between MS and CN groups. The gene expression profile was studied in $CD3^+$ T cell fraction isolated from 72 MS patients and 22 healthy control (CN) subjects by analyzing a cDNA microarray containing 1258 genes. RNA of MS and CN samples was labeled with Cy5, while RNA of a universal reference was labeled with Cy3. The genes differentially expressed between both groups were identified by Cyber-T test. Top 10 significant genes are shown as box and whisker plots where the longitudinal axis indicates log gene expression level (GEL). They are arranged in order of the significance listed in Table 2. The plots represent the following genes: (1) regulator of G protein signaling 14 (RGS14), (2) carbohydrate sulfotransferase 2 (CHST2), (3) nuclear receptor subfamily 4, group A, member 2 (NR4A2), (4) mitogen-activated protein kinase 1 (MAPK1), (5) SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 3 (SMARCA3), (6) tyrosylprotein sulfotransferase 2 (TPST2), (7) transcription factor 17 (TCF17), (8) heat shock 70-kDa protein 1A (HSPA1A), (9) angiotensin receptor-like 2 (AGTRL2), and (10) TNF-related apoptosis-inducing ligand (TRAIL).

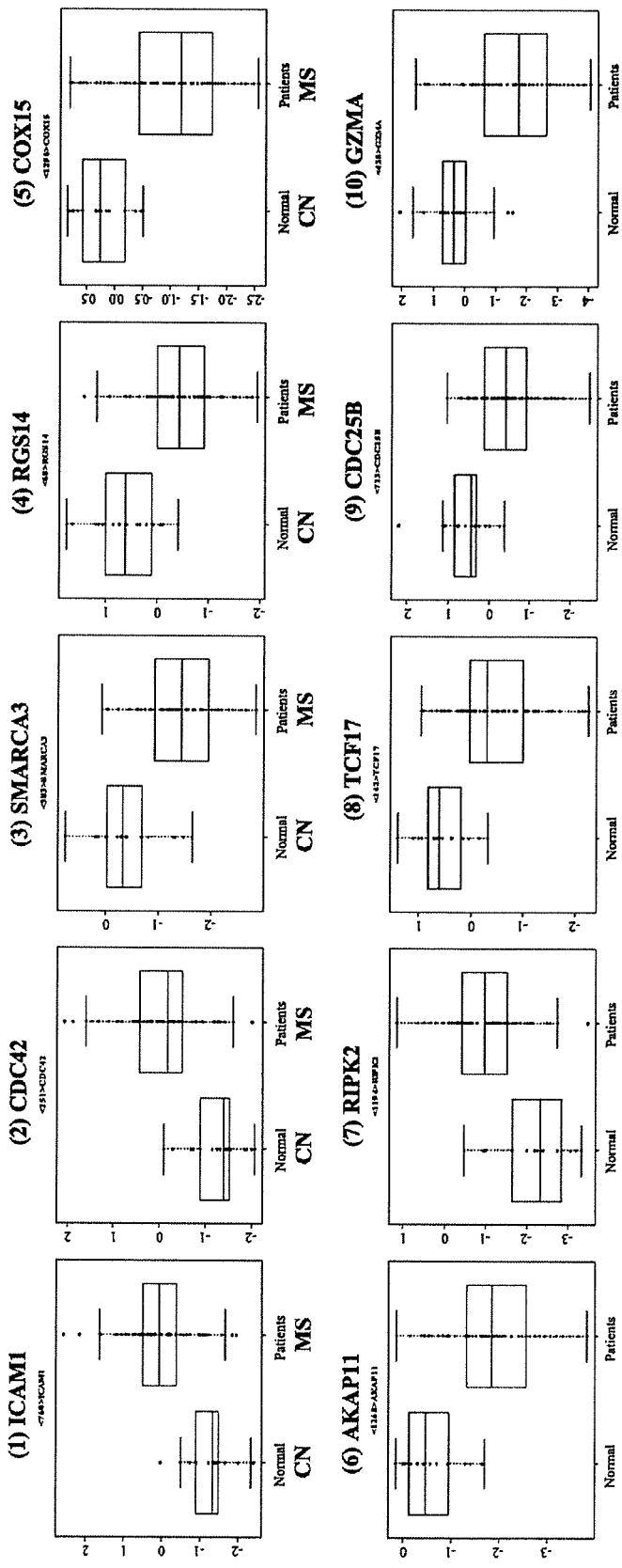

Fig. 2. Top 10 differentially expressed genes in non-T cell fraction between MS and CN groups. The gene expression profile was studied in $CD3^-$ non-T cell fraction isolated from 72 MS patients and 22 CN subjects by analyzing 1258 cDNA microarrays. See Fig. 1. Top 10 genes are shown as box and whisker plots where the longitudinal axis indicates log GE. They are arranged in order of the significance listed in Table 3. The plots represent the following genes: (1) intercellular adhesion molecule-1 (ICAM1), (2) cell division cycle 42 (CDC42), (3) SMARCA3, (4) RGS14, (5) cytochrome *c* oxidase assembly protein (COX15), (6) A-kinase anchor protein 11 (AKAP11), (7) receptor-interacting serine/threonine kinase 2 (RIPK2), (8) TCF17, (9) cell division cycle 25B (CDC25B), and (10) granzyme A (GZMA).