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Molecular network analysis of T-cell transcriptome suggests aberrant regulation of gene expression by NF-κB as a biomarker for relapse of multiple sclerosis

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Abstract. Molecular mechanisms responsible for acute relapse of multiple sclerosis (MS) remain currently unknown. The aim of this study is to identify the relapse-specific biomarker genes in T lymphocytes of relapsing-remitting MS (RRMS). Total RNA of $CD3^+$ T cells isolated from six RRMS patients taken at the peak of acute relapse and at the point of complete remission was processed for DNA microarray analysis. We identified a set of 43 differentially expressed genes (DEG) between acute relapse and complete remission. By using 43 DEG as a discriminator, hierarchical clustering separated the cluster of relapse from that of remission. The molecular network of 43 DEG investigated by KeyMolnet, a bioinformatics tool for analyzing molecular interaction on the curated knowledge database, showed the most significant relationship with aberrant regulation of gene expression by the nuclear factor-kappa B (NF-κB) in T cells during MS relapse. These results support the logical hypothesis that NF-κB plays a central role in triggering molecular events in T cells responsible for induction of acute relapse of MS, and suggest that aberrant gene regulation by NF-κB on T-cell transcriptome might serve as a molecular biomarker for monitoring the clinical disease activity of MS.

Keywords: KeyMolnet, multiple sclerosis, nuclear factor-kappa B, relapse, T cells

1. Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) white matter mediated by an autoimmune process triggered by a complex interplay of both genetic and environmental factors [1]. The great majority of MS patients show a relapsing-remitting (RR) clinical course. Intravenous administration of interferon-gamma (IFNγ) to MS patients provoked acute relapses accompanied by activation of the systemic immune response, indicating a pivotal role of proinflammatory T helper type 1 (Th1) lymphocytes in the immunopathogenesis of RRMS [2]. More recent studies proposed the pathogenic role of Th17 lymphocytes in sustained tissue damage in MS [3]. Several studies showed an etiological implication of viral infections for induction of acute relapse of MS [4]. However, the involvement of any viruses in MS relapse is not fully validated. A recent study showed that a methylprednisolone pulse therapy immediately reduces the levels of activated p65 subunit of the nuclear factor-kappa B (NF-κB) in lymphocytes of MS patients, suggesting a key role of NF-κB in induction of acute relapse of MS [5]. Furthermore, IFNγ is identified as one of NF-κB target genes, while IFNβ treatment attenuates proinflammatory responses in T cells by inhibiting the NF-κB activity [6,

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7]. At present, the precise molecular mechanism underlying MS relapse remains almost unknown. If the molecular biomarkers for MS relapse are identified, we could predict the timing of relapses, being highly valuable to start the earliest preventive intervention.

DNA microarray technology is a novel approach that allows us to systematically monitor the expression of a large number of genes in disease-affected tissues and cells. It has given new insights into molecular mechanisms promoting the autoimmune process in MS, and has made it possible to identify biomarkers for monitoring the clinical outcome [8]. The comprehensive gene expression profiling of MS brain tissues and peripheral blood lymphocytes identified a battery of genes deregulated in MS, whose role has not been previously predicted in its pathogenesis [9–12]. By microarray analysis, we recently identified a set of interferon-responsive genes expressed in highly purified peripheral blood $CD3^+$ T cells of RRMS patients receiving treatment with interferon-beta (IFNβ) [13]. IFNβ immediately induces a burst of expression of chemokine genes with potential relevance to IFNβ-related early adverse effects in MS [14]. The majority of differentially expressed genes in $CD3^+$ T cells between untreated MS patients and healthy subjects were categorized into apoptosis signaling regulators [15]. Furthermore, we found that T-cell gene expression profiling classifies a heterogeneous population of Japanese MS patients into four distinct subgroups that differ in the disease activity and therapeutic response to IFNβ [16].

In the present study, to identify MS relapse-specific biomarker genes, we conducted DNA microarray analysis of peripheral blood $CD3^+$ T cells isolated from RRMS patients taken at the peak of acute relapse and at the point of complete remission of the identical patients. We focused highly purified $CD3^+$ T cells because autoreactive pathogenic and regulatory cells, which potentially play a major role in MS relapse and remission, might be enriched in this fraction. Since microarray analysis usually produces a large amount of gene expression data at one time, it is often difficult to find out the meaningful relationship between gene expression profile and biological implications from such a large quantity of available data. To overcome this difficulty, we have made a breakthrough to identify the molecular network most closely associated with DNA microarray data by a novel data-mining tool of bioinformatics named KeyMolnet.

2. Subjects and methods

2.1. Blood samples

The present study included 6 Japanese women presenting with clinically active RRMS, diagnosed by certified neurologists of Department of Neurology, Musashi Hospital, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), according to the established criteria [17]. Written informed consent was obtained from all the patients. The Ethics Committee of NCNP approved the present study. The patients showed the mean age of 41 ± 12 years and the mean Expanded Disability Status Scale (EDSS) score of 1.8 ± 0.5 (Fig. 1). None of the patients received treatment with glatiramer acetate, mitoxantrone or other immunosuppressants at any time in the entire clinical course. None of them were given methylprednisolone or interferons at least for three weeks before the time point of blood sampling. Blood samples of individual patients taken at two time points were compared, one at the point of complete remission and the other at the peak of acute relapse, usually on the day of onset or one day after acute relapse, just before starting treatment with intravenous methylprednisolone pulse (IVMP) or oral administration of high dose prednisolone (Fig. 1). The comprehensive clinical and neuroradiological evaluation on each case satisfied characteristics of either relapse or remission of MS.

2.2. Microarray analysis

The present study utilized a custom microarray containing duplicate spots of 1,258 cDNA, which were carefully designed by excluding any cross-hybridization, generated by PCR, and immobilized on a poly-L-lysine-coated slide glass (Hitachi Life Science, Kawagoe, Saitama, Japan) [13–16]. The array contains well-annotated biologically important human genes of various functional classes, which cover a wide range of cytokines/growth factors and their receptors, apoptosis regulators, oncogenes, transcription factors, signal transducers, cell cycle regulators and housekeeping genes (see the reference [14] for the complete gene list).

Peripheral blood mononuclear cells (PBMC) were isolated from 30 ml of heparinized blood by centrifugation on a Ficoll density gradient. They were labeled with anti-CD3 antibody-coated magnetic microbeads (Miltenyi Biotec, Auburn, CA), and $CD3^+$ T cells were separated by AutoMACS (Miltenyi Biotec). The puri-

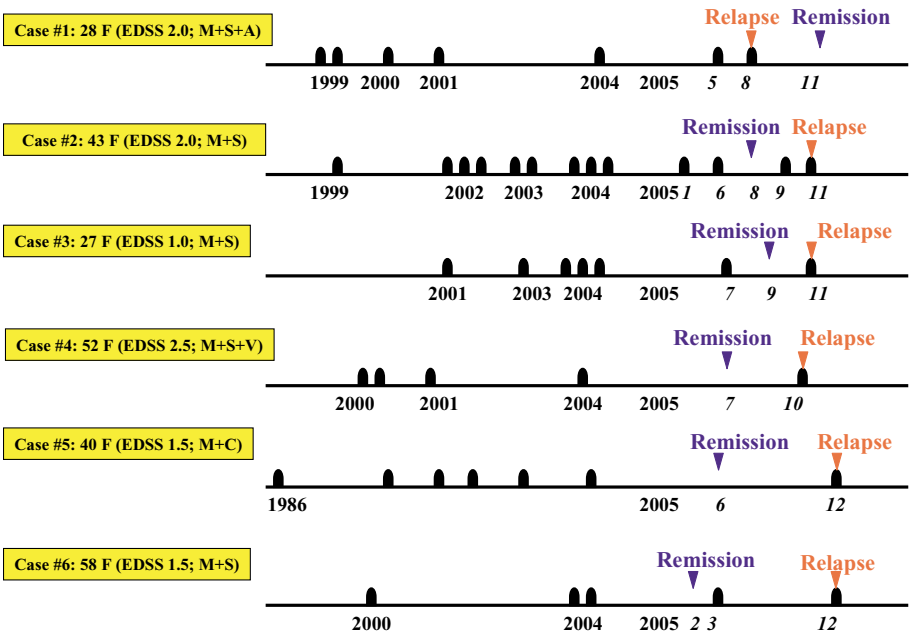

Fig. 1. Blood sampling from six RRMS patients in relapse and remission. Blood samples were taken from six patients with RRMS at the peak of acute relapse (red arrowhead) and at the time of complete remission (blue arrowhead). $CD3^+$ T cells were purified and processed for DNA microarray analysis. The relapses of MS (bell shape) specified by year and month (italic), age, sex, Expanded Disability Status Scale (EDSS) score, and cardinal clinical symptoms (M, motor impairment; S, sensory impairment; A, autonomic impairment; C, cognitive impairment; and V, visual impairment) are shown. The cases #3, 4, and 6 have a past history of short-term IFNβ treatment that was discontinued at the time point long enough to wash out the immunomodulatory effects of IFNβ on T-cell transcriptome.

ty of $CD3^+$ cells generally exceeded 90–95% by flow cytometric analysis. Total RNA was isolated from the cells by using RNeasy Mini Kit (Qiagen, Valencia, CA). Five μg of purified RNA was *in vitro* amplified, and the antisense RNA (aRNA) was labeled with a fluorescent dye Cy5, while universal reference aRNA was labeled with Cy3 to standardize the gene expression levels. The arrays were hybridized at 62°C for 10 hours 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. The gene expression level (GEL) was calculated according to the formula: GEL = FI (Cy5) of the sample/FI (Cy3) of the universal reference.

2.3. *Statistical analysis, hierarchical clustering, and molecular network analysis*

The genes differentially expressed between the samples of acute relapse and those of remission were identified by statistical evaluation with Student t-test via TTEST function of Excel, by comparing the log ratio of GEL of each gene at the two time points. The genes with a p value of < 0.05 were considered significant. Hierarchical clustering was performed on all the samples. The set of differentially expressed genes (DEG) was utilized as a discriminator to separate clusters following the "Gene Tree" algorithm on GeneSpring 7.2 (Agilent Technologies, Palo Alto, CA). This unsupervised approach arranged the genes and samples with a similar expression pattern to separate distinct clusters on the dendrogram.

The molecular network of DEG was analyzed by using a data-mining tool named KeyMolnet originally developed by the Institute of Medicinal Molecular Design, Inc. (IMMD), Tokyo, Japan [18], and the English version is currently utilized worldwide, including European Molecular Biology Laboratory (EMBL), Heidelberg, Germany (see the website of www.immd.co.jp/en/news/news20051222.html). KeyMolnet constitutes a knowledge-based content database of numerous interactions among human genes, molecules, diseases, pathways and drugs. They have been manually collected and carefully curated from selected review articles, literature, and public databases

by expert biologists of IMMD. The KeyMolnet contents, composed of approximately 12,000 molecules, are focused on human species, and categorized into either the core contents collected from selected review articles or the secondary contents extracted from abstracts of the PubMed database.

When the list of either GenBank accession number or probe ID of the genes extracted from microarray data was imported into KeyMolnet, it automatically provided corresponding molecules as a node on networks [18]. Among four different modes of the molecular network search, the "common upstream" search enables us to extract the most relevant molecular network composed of the genes coordinately regulated by putative "common upstream" transcription factors. The extracted molecular network was compared side by side with 346 distinct canonical pathways of human cells of the KeyMolnet library. They include a broad range of signal transduction pathways, metabolic pathways, and transcriptional regulations. The statistical significance in concordance between the extracted network and the canonical pathway was evaluated by the algorithm that counts the number of overlapping molecular relations between both. This makes it possible to identify the canonical pathway showing the most significant contribution to the extracted network. The calculation of significance score is based on the following formula, where O = the number of overlapping molecular relations between the extracted network and the canonical pathway, V = the number of molecular relations located in the extracted network, C = the number of molecular relations located in the canonical pathway, T = the number of total molecular relations installed in KeyMolnet, and X = the sigma variable that defines coincidence.

$$\text{Score} = -\log_2 (\text{Score (p)})$$

$$\text{Score (p)} = \sum_{x=O}^{Min(C,V)} f(x)$$

$$\text{f(x)} = {}_{\text{C}}\text{C}_{\text{x}} \cdot {}_{\text{T}-\text{C}}\text{C}_{\text{V}-\text{x}} / {}_{\text{T}}\text{C}_{\text{V}}$$

3. Results

3.1. Microarray analysis identified 43 genes differentially expressed in peripheral blood T cells between relapse and remission of MS

Among 1,258 genes on the microarray, 43 genes were expressed differentially in peripheral blood $CD3^+$ T cells of 6 RRMS patients at the peak of acute relapse and at the point of complete remission (Table 1). Among 43 differentially expressed genes (DEG), 18 genes were upregulated, whereas 25 genes were downregulated at the time of relapse. Next, by using the set of 43 DEG as a discriminator, hierarchical clustering analysis was performed on total 12 samples, comprised of 6 relapse and 6 remission samples. Although the difference between the two groups was not so apparently huge, the clustering method effectively separated the cluster of relapse samples from the cluster of remission samples based on gene expression profile of 43 DEG, except for one remission sample included in the cluster of relapse samples (Fig. 2). These observations suggest that the gene network of signaling molecules located upstream of 43 DEG in T cells might be substantially different between two distinct clinical phases of MS.

3.2. Molecular network analysis suggests a key role of NF-κB in relapse of MS

To clarify the molecular network of 43 DEG regulated coordinately in T cells during acute relapse, their GenBank accession numbers and expression levels were imported into KeyMolnet. In the first step, GenBank accession numbers were converted into Keymolnet ID numbers. Then, the common upstream search of these generated a complex network composed of 128 fundamental nodes with 315 molecular relations. Among them, 25 nodes were included in the list of 43 DEG (Table 1), and 103 additional nodes outside the list were automatically incorporated from both core and secondary contents of KeyMolnet following the network-searching algorithm. The extracted molecular network was arranged with respect to subcellular location of the molecules by the editing function of KeyMolnet (Fig. 3). Finally, the statistical evaluation of the extracted network showed the most relevant relationship with transcriptional regulation by the nuclear factor NF-κB with the score of 11.036 and score (p) = 4.764E-004. No other canonical pathways (n = 345) than NF-κB-regulated gene transcription were identified as statistically significant in the extracted molecular network, judged by the scoring system involving the pathway based on molecular relations.

4. Discussion

To clarify molecular mechanisms underlying acute relapse of MS, we conducted DNA microarray analysis

Table 1
Fourty-three differentially expressed genes in T-cells between relapse and remission of MS

Rank	Gene symbol	KeyMolnet node	Full name	GenBank accession number	P value	Regulation in relapse versus remission
1	*PPARG*	PPARg	peroxisome proliferative activated receptor gamma	NM_005037	9.78E-04	up
2	*RND3*	Rnd3	Rho family GTPase 3	NM_005168	1.26E-03	down
3	*IL6*	IL-6	interleukin 6	NM_000600	1.97E-03	down
4	*AKT2*	AKT2	v-akt murine thymoma viral oncogene homolog 2	NM_001626	2.73E-03	up
5	*DCC*	DCC	deleted in colorectal carcinoma	NM_005215	3.80E-03	up
6	*CREBBP*	CBP	CREB binding protein	NM_004380	6.05E-03	down
7	*ATF5*	ATFx	activating transcription factor 5	NM_012068	6.99E-03	down
8	*PLCG1*	PLCg1	phospholipase C gamma 1	NM_002660	9.36E-03	up
9	*CDK3*	CDK3	cyclin-dependent kinase 3	NM_001258	1.01E-02	up
10	*RIPK1*	RIP1	receptor-interacting serine-threonine kinase 1	NM_003804	1.15E-02	up
11	*TNFRSF4*	OX40	TNF receptor superfamily, member 4	NM_003327	1.21E-02	down
12	*ABCC9*	SUR2	ATP-binding cassette, sub-family C, member 9	NM_005691	1.40E-02	down
13	*STAT2*	STAT2	signal transducer and activator of transcription 2	NM_005419	1.49E-02	up
14	*PTEN*	PTEN	phosphatase and tensin homolog	NM_000314	1.80E-02	down
15	*AVP*	AVP, VP, NPII, copeptin	arginine vasopressin	NM_000490	1.82E-02	up
16	*FADD*	FADD	Fas-associated via death domain	NM_003824	1.93E-02	up
17	*ELF2*	NERF2	E74-like factor 2 (ets domain transcription factor)	NM_006874	2.10E-02	down
18	*NFKB2*	p100NFkB, p52NFkB	NF-kappa B subunit 2 (p52/p100)	NM_002502	2.11E-02	up
19	*ERBB4*	Erb4, ERBB4	v-erb-a erythroblastic leukemia viral oncogene homolog 4	NM_005235	2.18E-02	down
20	*BCL2L1*	Bcl-XL	BCL2-like 1	NM_001191	2.53E-02	up
21	*BTRC*	b-TRCP, Fbw1	beta-transducin repeat containing protein	NM_003939	2.65E-02	up
22	*SULT1B1*	SULT1B1	sulfotransferase family, cytosolic, 1B, member 1	NM_014465	2.79E-02	down
23	*EP300*	p300	E1A binding protein p300	NM_001429	2.86E-02	down
24	*GJA4*	Cx37	gap junction protein alpha 4 (connexin 37)	NM_002060	2.87E-02	down
25	*PDGFB*	PDGF-B, PDGF-BB	platelet-derived growth factor beta polypeptie	NM_002608	2.92E-02	up
26	*ARID4A*	ARID4A	AT rich interactive domain 4A (RBP1-like)	NM_002892	3.05E-02	down
27	*CYP2C19*	CYP2C19	cytochrome P450, family 2, subfamily C, polypeptide 19	NM_000769	3.07E-02	down
28	*FGF1*	FGF-1	fibroblast growth factor 1	NM_000800	3.17E-02	down
29	*MMP2*	MMP-2	matrix metallopeptidase 2	NM_004530	3.27E-02	up
30	*ARHGAP1*	Cdc42GAP	Rho GTPase activating protein 1	NM_004308	3.35E-02	down
31	*TOP3B*	TOP3B	DNA topoisomerase III beta	NM_003935	3.97E-02	up
32	*SUB1*	PC4	SUB1 homolog	NM_006713	4.33E-02	down
33	*ZMYND8*	PRKCBP1	zinc finger, MYND-type containing 8	NM_183047	4.34E-02	down
34	*TGFB2*	TGFb2	transforming growth factor beta 2	NM_003238	4.36E-02	up
35	*SMAD7*	SMAD7	SMAD, mothers against DPP homolog 7	NM_005904	4.37E-02	down
36	*TCF4*	E2-2	transcription factor 4	NM_003199	4.40E-02	down
37	*NOS1*	nNOS	nitric oxide synthase 1 (neuronal)	NM_000620	4.42E-02	down
38	*TSC22D1*	TSC22	TSC22 domain family, member 1	NM_183422	4.54E-02	down
39	*GNB1L*	(none)	G protein beta subunit-like protein	NM_053004	4.57E-02	down
40	*IFNA8*	IFNA8	interferon alpha 8	NM_002170	4.60E-02	down
41	*IL1A*	IL-1a	interleukin 1 alpha	NM_000575	4.77E-02	up
42	*CD3D*	CD3d	CD3 delta	NM_000732	4.92E-02	up
43	*IL1R1*	IL-1R1	interleukin 1 receptor type I	NM_000877	4.95E-02	down

The genes differentially expressed between relapse and remission were identified by comparing the log ratio of gene expression level of each gene at two time points, evaluated by Student t-test. The genes with a p value of < 0.05 was selected. The gene symbol, KeyMolnet node name, full name, GenBank accession number, p value, and regulation in relapse versus remission are shown.

of peripheral blood $CD3^+$ T cells of 6 RRMS patients taken at the peak of acute relapse and at the point of complete remission of the identical patients. The battery of 43 genes was expressed differentially between relapse and remission. By using 43 DEG as a discriminator, hierarchical clustering analysis separated the cluster of relapse samples and the cluster consisting mainly of remission samples. Then, we for the first time intensively studied the molecular network of DEG between MS relapse and remission by using a bioinformatics tool for analyzing molecular interaction on the curated knowledge database named KeyMolnet. The common upstream search of 43 DEG on KeyMolnet indicated the central role of transcriptional regulation

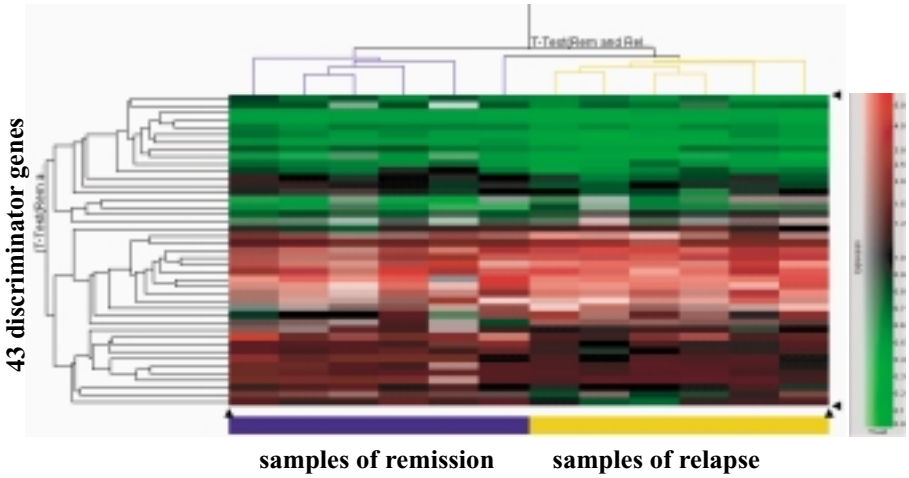

Fig. 2. Hierarchical clustering of 43 genes differentially expressed in T cells between relapse and remission of MS. Hierarchical clustering was performed on total 12 samples, consisting of 6 relapse (orange) and 6 remission (blue) samples, by using the set of 43 differentially expressed genes in T cells between relapse and remission (Table 1) as a discriminator. This separated two clusters, one composed of 5 remission samples and the other composed of 6 relapse samples and one remission sample. The matrix is labeled by a pseudo-color, with red expressing upregulation, green expressing downregulation, and the color intensity representing the magnitude of the deviation from GEL 1.0 as shown on the right.

by NF-κB in aberrant gene expression in T cells during MS relapse. We have recently characterized 286 genes differentially expressed in purified CD3$^+$ T cells between 72 untreated clinically-active MS patients and 22 age- and sex-matched healthy subjects [16]. When the set of 286 DEG was imported into KeyMolnet, the common upstream search illustrated the complex molecular network composed of 335 nodes. We found that the generated network showed again the most significant relationship with transcriptional regulation by NF-κB [19]. These observations, taken together, suggested that aberrant gene regulation by NF-κB on T-cell transcriptome might serve as a surrogate biomarker not only for discriminating MS from healthy subjects but also for monitoring the clinical disease activity of individual MS patients. This hypothesis warrants further evaluation by including a large cohort of MS patients whose blood samples are taken at acute relapse and during remission of the identical patients.

KeyMolnet stores the highly reliable content database of human proteins, small molecules, molecular relations, diseases, and drugs, carefully curated by experts from the literature and public databases [18]. This software makes it possible to effectively extract the most relevant molecular interaction from large quantities of gene expression data [19,20]. Our results indicate that the combination of DNA microarray and molecular network analysis is more effective to establish a biologically-relevant logical working model than the conventional microarray data analysis [21].

NF-κB is a central regulator of innate and adaptive immune responses, cell proliferation, and apoptosis [22,23]. The NF-κB family consists of five members, such as NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB, and c-Rel. NF-κB exists in an inactive state in unstimulated cells, being sequestrated in the cytoplasm via non-covalent interaction with the inhibitor of NF-κB (IκB) proteins. Viral and bacterial products, cytokines, and stress-inducing agents activate specific IκB kinases that phosphorylate IκB proteins. Phosphorylated IκBs are ubiquitinated and processed for proteasome-mediated degradation, resulting in nuclear translocation of NF-κB that regulates the expression of target genes by binding to the consensus sequence located in the promoter.

Previous studies identified more than 150 NF-κB target genes, including those involved in not only immune, inflammatory and antiapoptotic responses, but also antiinflammatory and proapoptotic responses [24]. It is worthy to note that *BTRC*, β-transducin repeat containing protein, listed as one of upregulated genes in T cells of MS relapse (Table 1), acts as a RING E3 protein that mediates ubiquitination of IκBα [25]. Importantly, a number of NF-κB target genes activate NF-κB itself, providing a positive regulatory loop that amplifies and perpetuates inflammatory responses [22]. These observations raise the scenario that even subclinical levels of infections and stresses affecting the immune and neuroendocrine systems [4,26] could induce the persistent oscillation between activation and inactivation of

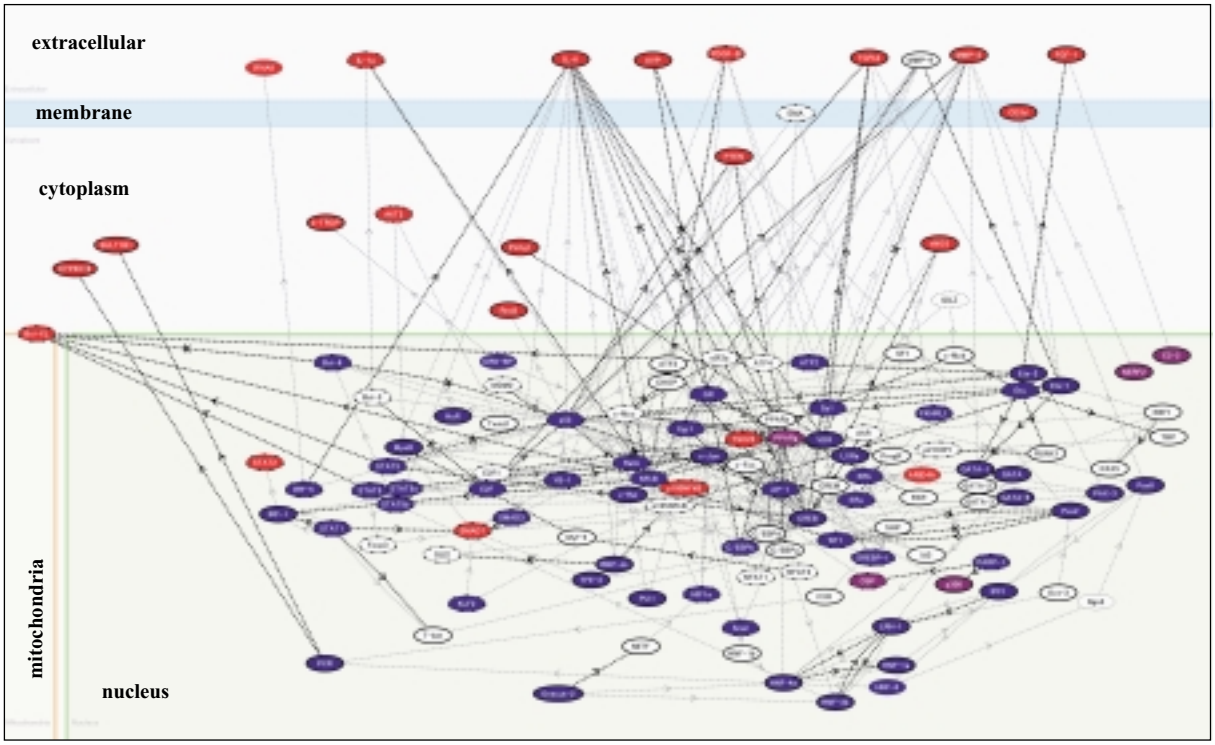

Fig. 3. The common upstream search of differentially expressed genes in T cells between relapse and remission of MS. The GenBank accession numbers and expression levels of 43 genes were imported into KeyMolnet. The "common upstream" search of these generated a network composed of 128 fundamental nodes with 315 molecular relations. It is shown with respect to subcellular location of molecules. Red nodes represent starting point molecules, whereas blue nodes represent common upstream molecules. Purple nodes express characteristics of both starting point and common upstream molecules. White nodes exhibit additional molecules extracted automatically from KeyMolnet core and secondary contents incorporated in the network to establish molecular connections. The direction of molecular relation is indicated by dash line with arrow (transcriptional activation) or dash line with arrow and stop (transcriptional repression). Thick lines indicate the core contents, while thin lines indicate the secondary contents of KeyMolnet. The statistical evaluation of the extracted molecular network indicated the principal relationship with transcriptional regulation by the nuclear factor NF-κB.

NF-κB in autoreactive T cells, thereby cause the fluctuation of disease activity from relapse to remission in RRMS patients. Unusual posttranslational modification of IκB and of NF-κB proteins occasionally causes aberrant NF-κB activation [27].

Increasing evidence supports a central role of aberrant NF-κB activation in development of MS. Pathologically, RelA, c-Rel, and p50 subunits of NF-κB are overexpressed in macrophages in active demyelinating lesions of MS [28], while RelA is activated in oligodendrocytes that survive in the lesion edge [29]. Genetically, a predisposing allele in the NFKBIL gene is closely associated with development of RRMS [30]. We previously showed that the orphan nuclear receptor NR4A2, a direct target gene of NF-κB, is upregulated at the highest level in $CD3^+$ T cells of untreated MS patients [15, 16]. Targeted disruption of the NFKB1 gene confers resistance to development of experimental autoimmune encephalomyelitis (EAE), an animal model of MS [31]. *In vivo* administration of selective inhibitors of NF-κB protects mice from EAE [32]. Furthermore, the CNS-restricted inactivation of NF-κB ameliorates EAE, accompanied by a defect in induction of proinflammatory genes in astrocytes [33]. These results suggest that development of drugs aimed at fine-tuning of NF-κB function in T lymphocytes could provide a promising approach to suppress the clinical activity of MS.

In conclusion, the molecular network analysis of T-cell transcriptome suggests the logical hypothesis that abnormal transcriptional regulation by NF-κB plays a central role in aberrant gene expression in T cells during MS relapse, and that aberrant gene regulation by NF-κB on T-cell transcriptome might serve as a molecular biomarker for monitoring the clinical disease activity of individual MS patients. Although the study population ($n = 6$) is relatively small, our observations warrant further evaluation by using a large cohort of RRMS patients.

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