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Focused Microarray Analysis of Peripheral Mononuclear Blood Cells from Churg–Strauss Syndrome Patients

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Abstract

DNA diagnostics are useful but are hampered by difficult ethical issues. Moreover, it cannot provide enough information on the environmental factors that are important for pathogenesis of certain diseases. However, this is not a problem for RNA diagnostics, which evaluate the expression of the gene in question. We here report a novel RNA diagnostics tool that can be employed with peripheral blood mononuclear cells (PBMCs). To establish this tool, we identified 290 genes that are highly expressed in normal PBMCs but not in TIG-1, a normal human fibroblast cell. These genes were entitled *PREP* after predominantly expressed in PBMC and included 50 uncharacterized genes. We then conducted *PREP* gene-focused microarray analysis on PBMCs from seven cases of Churg–Strauss syndrome (CSS), which is a small-vessel necrotizing vasculitis. We found that *PREP135* (coactosin-like protein), *PREP77* (prosaposin), *PREP191* (cathepsin D), *PREP234* (*c*-fgr), and *PREP136* (lysozyme) were very highly up-regulated in all seven CSS patients. Another 28 genes were also up-regulated, albeit more moderately, and three were down-regulated in all CSS patients. The nature of these up- and down-regulated genes suggest that the immune systems of the patients are activated in response to invading microorganisms. These observations indicate that focused microarray analysis of PBMCs may be a practical, useful, and low-cost bedside diagnostics tool.

Key words: focused microarray; RNA diagnosis; PMBC; allergic granulomatosis angiitis; Churg–Strauss syndrome

1. Introduction

The advent of array technology and the subsequent development of high-density oligonucleotide arrays[1] have been enormously helpful in improving our understanding of the genome-wide transcriptional profiles of many biological systems in both basic and applied research.[2] Array technology has also been extremely useful for discovering and developing diagnostic gene markers for disease subcategories, disease prognosis, and treatment outcome; this has paved the way for effective pharmaceutical drug discovery, the development of novel strategies for molecular (DNA, RNA) diagnostics, and the design of personalized drug regimens.[2]

Oligonucleotide microarrays, which were initially designed to analyze genome-wide gene expression levels, have turned out to be particularly useful in DNA diagnostics as they can be used for many different

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applications, including discovering polymorphisms and genotyping patients by identifying inheritable genetic markers.[3] With regard to the latter applications, such DNA diagnostics have greatly improved our understanding of and ability to detect the causative mutations of various diseases. However, this technology is hampered by major ethical and privacy issues since the genome sequence of a person carries unchangeable private information whose discovery can affect the life of not only that person, but also to some extent their family members. The need to protect patient privacy upon DNA diagnostics testing and the practical problems this constitutes also imposes extra costs on the testing procedure. Moreover, many kinds of diseases are caused not only by the DNA polymorphism, but also by environmental status. These environmental factors can be reflected in the RNA status, but DNA diagnostics usually cannot provide enough information on the environments. Thus, the activity of the disease can be detected by RNA or protein level but not by DNA level.

These problems are not faced by RNA diagnostics, in which the genome-wide mRNA levels are monitored by oligonucleotide (or cDNA) microarrays. Ethical issues of the nature described above do not apply here because the information obtained from RNA diagnostics does not necessarily relate to the DNA sequence. Instead, this technology monitors the dynamic changes in gene activities, namely, the increased or decreased transcriptional (mRNA) levels in the samples provided by the individual. The transcriptional levels would vary in the same individual depending on the health of the individual. In this sense, DNA diagnostics is a static test of the genome, whereas RNA diagnostics is a dynamic test of the genome.

However, it is not possible to monitor all 44 000 kinds of mRNA species that are transcribed from the human genome for many samples at the same time. A novel way to circumvent this problem is to develop a 'focused array' in which a limited number of mRNAs are tested in a low-density array. In the present study, we developed a focused oligonucleotide (or cDNA) array for use with patient peripheral blood mononuclear cells (PBMCs). For this, we selected the genes that are predominantly expressed in normal PBMCs, as determined by stepwise subtractive hybridization[4] and by genome-wide cDNA microarray analysis. From the 290 'PBMC-focused' genes we identified, we can prepare the PBMC-focused cDNA array. We examined the expression levels of these *PREP* genes to analyze the PBMC RNAs obtained from patients suffering the autoimmune disease Churg–Strauss syndrome (CSS), which is an alternative name of allergic granulomatosis angiitis, because the autoimmune response of CSS patients is expected to disturb the expression levels of immune-related genes in PBMC. Indeed, we identified several genes whose expressions are markedly up- or down-regulated in all CSS patients tested. These observations suggest that this low-cost RNA diagnostics test is useful, practical, and can be used at the bedside.

2. Patients, Materials and methods

2.1. Human subjects: patients and healthy controls

Blood was obtained from eight healthy volunteers (four males and four females; aged 25–49 years) for the cDNA library preparation. Blood was also obtained from seven cases of CSS patients whose profiles are shown in Supplementary Table S1 and 18 healthy controls (six males and 11 females; aged 25–86 years) for focused microarray analysis. CSS patients were diagnosed according to the diagnostic criteria of the American College of Rheumatology.[5] This study was reviewed and approved by the Internal Review Board of the Research Institute for Microbial Diseases, Osaka University. In accordance with the requirements of the Board, a written informed consent was obtained from each participant before venous blood samples were obtained. Serum samples were consecutively obtained regardless of the patient's symptom, active, or inactive phase.

2.2. Preparation of RNA

The RNA of the PBMCs obtained from healthy volunteers was prepared as described previously.[6] Briefly, heparinized venous blood (10 mL) was mixed with an equal volume of 2% dextran/saline solution and incubated at room temperature for 30 min to precipitate the red blood cells. Total RNA was extracted from the PBMC pellet by adding guanidine–thiocyanate solution and the samples were used for cDNA library preparation and subtractive hybridization.[7] Total RNA was also prepared by acid guanidinium–phenol–chloroform extraction for the DNA microarray, northern blot, and RT–PCR analyses. In some experiments, total RNA was synthesized using Ribo Max kit (Promega, Madison, WI) from the PBMC cDNA library. Total RNA or mRNA from human fibroblast TIG-1 cells was prepared as described previously.[7] Ex*Taq* DNA polymerase for RT–PCR was purchased from TaKaRa Co. Ltd. (Otsu, Japan). Probe labeling and detection for northern blots were performed by using the Gene Images Random-Prime Labelling and Detection System (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

2.3. Preparation of the subtracted cDNA library and stepwise subtraction

Poly(A)$^+$ RNA was purified from total RNA by oligo(dT) cellulose chromatography. A cDNA library

with eight million independent clones was constructed from the PBMC mRNAs by using the linker-primer method and the pAP3neo vector as described previously.[7] The poly(A)$^+$ RNAs from exponentially growing TIG-1 cells that had been incubated with 10% serum in tissue culture plates were also purified and biotinylated by using photobiotin. After converting the cDNA library to a single-stranded form by transfection with an f1 helper phage, we hybridized it with the biotinylated mRNAs and subtracted it by biotin–avidin interactions.[7] The unhybridized clones were converted to the double-stranded form and used to transform competent *Escherichia coli* cells. This generated a first-stage subtracted cDNA library of 11 million independent clones.

We then prepared plasmid DNA from ~800 randomly selected cDNA clones and numbered and digested an aliquot of each plasmid DNA with *Sma*I and *Not*I restriction enzymes to prepare 10 sheets of Southern blots, each of which included 80 clones arranged in order. We then purified the cDNA inserts of clones 1–20 on 1% agarose gels by digesting them with *Eco*RI and *Not*I; these inserts were labeled with fluorescent dye and then used as probes for northern analysis with PBMC and TIG-1 RNAs to determine which clones contained *PREP* genes (data not shown). The DNA sequences of the *PREP* clones from the 5′ end of the cDNA inserts were determined by the dideoxy-chain termination reaction using an automatic DNA sequencer (Licor 4000L; Lincoln, NE). After these analyses, we selected the next 20 unhybridized clones on the Southern blot (from 21 onwards) for the next round of cDNA insert preparation, fluorescent labeling, and northern analysis. This procedure was repeated until we finished testing all 800 unhybridized clones. The *PREP* genes identified in the preceding step were then converted to RNA, biotinylated with photobiotin, and used to subtract the first-stage subtracted cDNA library. The second-stage subtracted cDNA library was then analyzed as described above and subtracted again. This process was repeated three times as described previously.[4]

After the clones whose transcription was conspicuously up-regulated in PBMCs as compared with TIG-1 cells were sequenced, the DNA sequences were used to search the EST database by using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/).

2.4. Human cDNA microarray analysis for identification of PREP genes

The total RNAs (500 ng) from the normal human PBMCs were pooled, as were the TIG-1 cell RNAs, after which both pools were reverse-transcribed by using oligo-dT primers containing the T7 RNA polymerase promoter sequence. The cDNAs were then subjected to *in vitro* transcription using T7 RNA polymerase to label the complementary RNAs (cRNAs) with cyanine 3 (Cy3)-CTP or cyanine 5 (Cy5)-CTP (Amersham Pharmacia Biotech, Piscataway, NJ). The Cy-labeled cRNAs from the normal human PBMCs (1 μg) were then mixed with the same amount of reverse color Cy-labeled TIG-1 cell-derived cRNAs. Hybridizations, rinsing, scanning, and gene analysis on the Agilent's all human cDNA microarray (Hu44K) were conducted according to the manufacturer's protocol (G2940BA; Agilent Technologies, Inc., Palo Alto, CA). Fluorophore reversal (dye swap) duplicates were used in two-color DNA microarray experiments. The 399 genes that showed the highest level of up- or down-regulation were selected and subjected to RT–PCR analysis using the normal human PBMC and TIG-1 RNAs (Fig. 1) with relevant oligonucleotide primers (Supplementary Table S2). Of these, 122 genes were identified as *PREP* genes, 33 of which were already identified as *PREP* genes by stepwise subtraction.[4]

2.5. Individual microarray analysis on CSS patients

The quality of the RNA samples obtained from PBMCs of each CSS patient was examined by using the RNA 6000 Nano LabChip Kit (p/n 5065-4476) on the Agilent 2100 Bioanalyzer. To conduct the individual cDNA microarray (Hu44K) analysis on CSS patients and normal volunteers, we generated fluorescently labeled cRNA by *in vitro* transcription with T7 RNA polymerase in the presence of Cy5-CTP or Cy3-CTP using a Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies, Inc.) as described above. The Cy5-labled cRNA of each patient was then mixed with the Cy3-labled cRNA of normal volunteers to probe the cDNA microarray. *PREP* gene expression analysis was then conducted by using the Gene Spring software 7.3.1 (Agilent Technologies, Inc.) by setting appropriate parameters to select 33 or 3 *PREP* genes whose expressions are up-regulated (>1.2-fold change) or down-regulated (<1.0-fold change) in all seven CSS patients as compared with healthy volunteers. We also conducted similar analysis to select nine conspicuously up-regulated *PREP* genes (>2.0-fold change).

3. Results

3.1. Isolation of putative PBMC-specific genes

To isolate putative human PBMC-specific genes, we first prepared mRNAs from the PBMCs of healthy volunteers and generated a human PBMC cDNA library with 80 million independent clones by using the linker-primer method described previously.[7] We also prepared mRNAs from exponentially growing normal

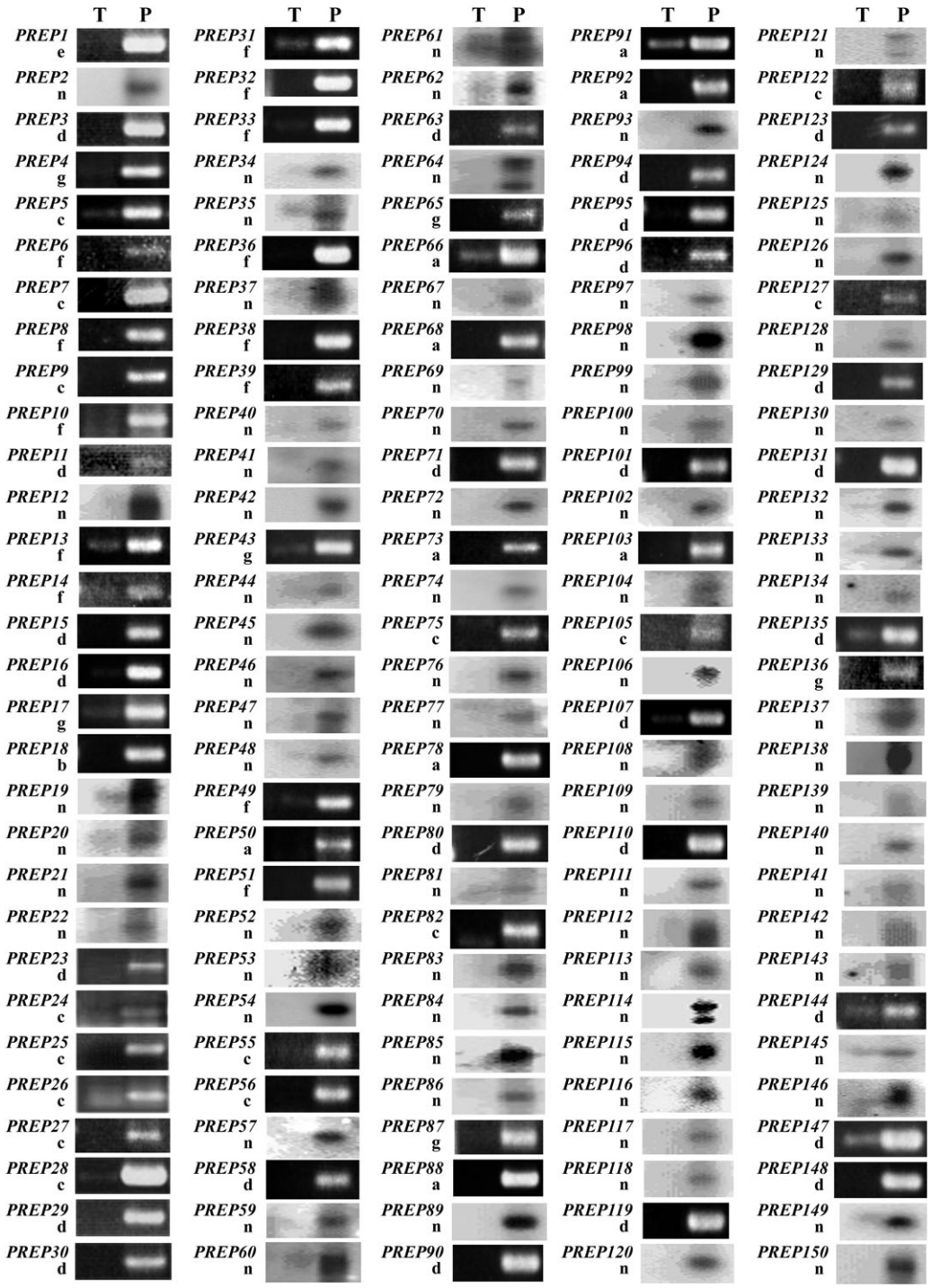

Figure 1. Identification of *PREP* genes. Individual *PREP* cDNA clones were subjected to northern blot or RT–PCR analysis to compare their gene expression levels in the PBMCs of healthy volunteers (right lanes, denoted as P) and human fibroblast TIG-1 cells (left lanes, denoted as T). The names of the *PREP* genes are shown in Table 1. The RT–PCR data for *GAPDH* and the northern blot data for β-actin are also shown as loading controls. The annealing temperature and amplification cycles used in the RT–PCRs are denoted to the left of each image by a–h: a, 50°C and 35 cycles, b, 50°C and 30 cycles, c, 55°C and 35 cycles, d, 55°C and 40 cycles, e, 55°C and 45 cycles, f, 55°C and 30 cycles, and g, 50°C and 40 cycles, respectively. The northern blots are denoted as n.

human fibroblast TIG-1 cells, which are control cells that express non-PBMC transcripts. These mRNAs were biotinylated with photobiotin[7] and used to subtract the PBMC cDNA library as described previously[4] to remove the housekeeping and non-PBMC-specific genes. Briefly, after converting the PBMC cDNA library to a single-stranded form by transfection with f1 helper phage, we hybridized it with the

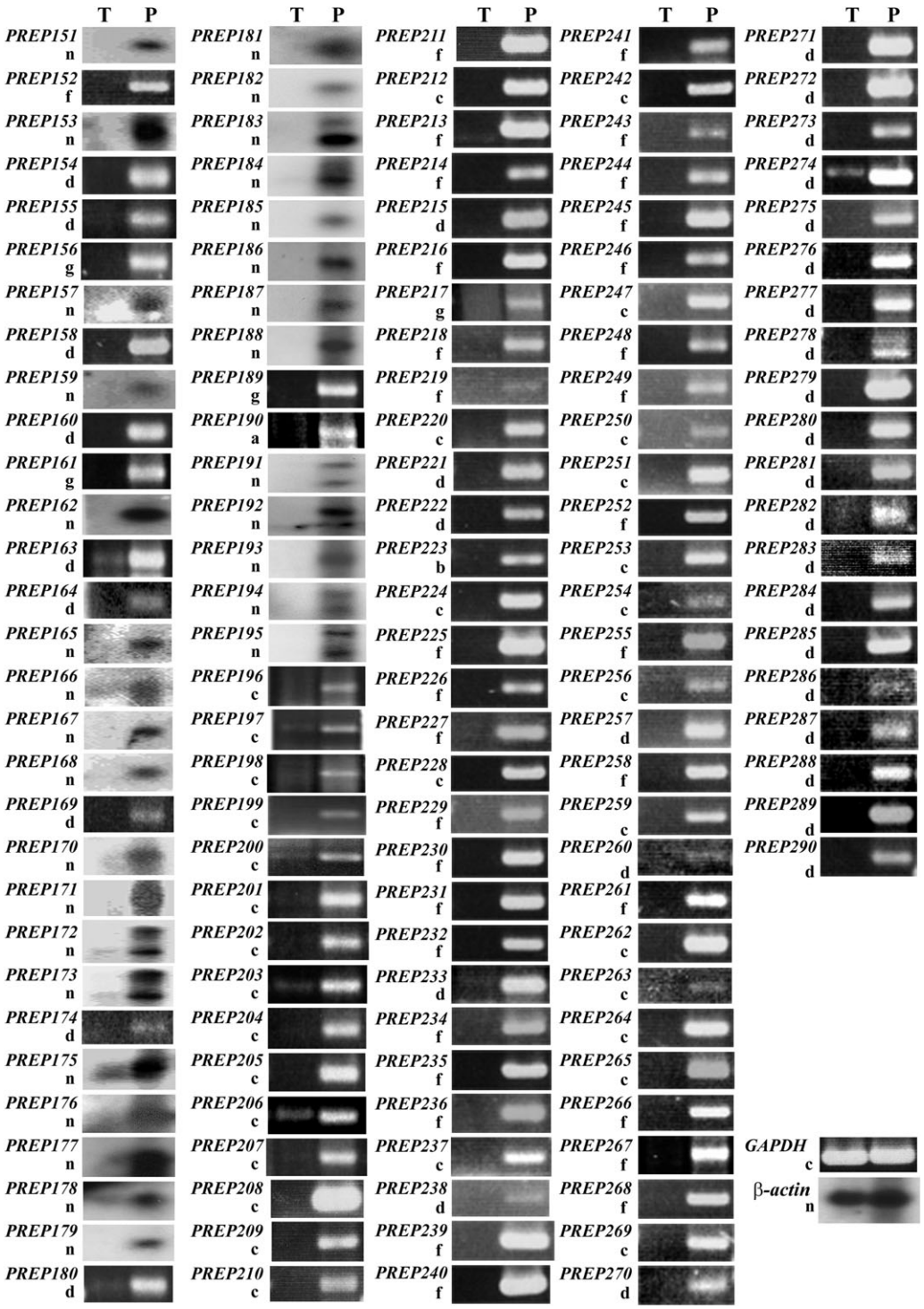

Figure 1. Continued

biotinylated TIG-1 mRNAs and subtracted it by biotin–avidin interactions. The unhybridized clones were converted to the double-stranded form, which was then used to transform competent *E. coli* cells, thereby generating a subtracted cDNA library.

To analyze this subtracted cDNA library, we prepared plasmid DNA from several hundred randomly selected and numbered cDNA clones. An aliquot of each plasmid DNA was then digested with *Eco*RI and *Not*I and their cDNA inserts were purified on 1% agarose gels and fluorescently labeled. These probes were then used in northern analysis to identify those genes whose transcript levels were much higher in PBMCs than in TIG-1 cells, namely, those genes that

showed almost a plus/minus type difference. These genes were named *PREP* after predominantly expressed in PBMC. As shown in Fig. 1, each northern sheet only included two lanes, one each for the RNAs extracted from PBMCs and TIG-1. The DNA sequences of these candidate *PREP* genes were determined from the 5′ end of the cDNA inserts by the dideoxy-chain termination reaction and were used to search the EST database by employing the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/). For those candidate *PREP* genes whose northern blot signals were weak or undetectable, we examined whether these candidate genes were indeed up-regulated by RT–PCR using oligonucleotide primers based on the DNA sequences of these genes (Supplementary Table S2) (Fig. 1). These procedures were repeated until almost all subtracted cDNAs were tested, as described previously.[4] The end result was the isolation of 197 *PREP* genes.

To reduce the possibility of missing important PBMC-specific pathogenic genes by the stepwise subtraction method, we also performed in parallel a genome-wide cDNA microarray analysis by using Agilent's Hu44K array with the same pooled RNA samples obtained from the PBMCs and TIG-1. The 399 genes that showed the greatest up-regulation in PBMCs compared with TIG-1 were then tested by RT–PCR. This resulted in the identification of 126 *PREP* genes, 33 of which were the same as those obtained by the stepwise subtraction analysis. The candidate *PREP* genes were tested by northern and/or RT–PCR analyses to confirm their PBMC-specifc expression (Fig. 1). We did not test those genes that showed lower up-regulation in PBMCs than the 399 genes because the efficiency of detecting a *PREP* was conspicuously lower in the top 151–400 microarray genes than in the top 1–150 genes. Thus, it appears that while the stepwise subtraction method failed to identify 93 *PREP* genes, the DNA microarray analysis failed to detect 164 *PREP* genes. This disparity is mainly due to the differently sized drivers or probes used for hybridization; cDNA subtraction uses kilobase-order biotinylated mRNA as the driver for subtraction, while DNA microarrays are probed by 60-base oligonucleotides. These data together indicate that we identified 290 *PREP* genes (Supplementary Table S3).

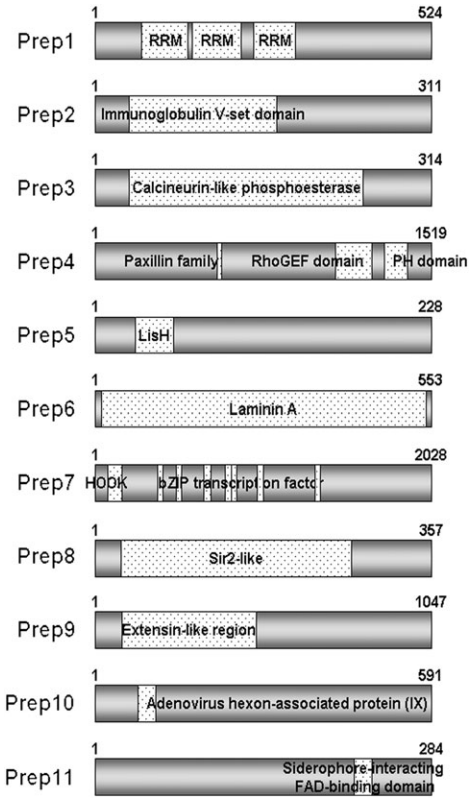

Figure 2. Schematic depiction of the uncharacterized gene products Prep1-Prep11. Numbers indicate the amino acids of the protein. RRM, RNA recognition motif; GEF, Guanine nucleotide exchange factor; PH, pleckstrin homology; LisH, LIS1 (lissencephaly) homology; bZIP, basic region/leucine zipper; Sir2, sirtuin 2; FAD, flavin adenine dinucleotide.

3.2. *Characterization of the unknown* PREP *genes*

Of the 290 *PREP* genes that we identified, *PREP1–PREP50* are uncharacterized genes. Homology and motif search of the gene products (Prep1–Prep50 proteins) using the Motif Scan algorithm (http://myhits.isb-sib.ch/cgi-bin/motif_scan) revealed the following notable motifs in *PREP1–PREP11* (Fig. 2), which may suggest their putative physiological functions. The other uncharacterized *PREP* genes did not have any notable motifs.

PREP1 encodes an RNA-binding protein because it harbors three RNA recognition motifs.[8] *PREP2* encodes a protein that harbors a V-set Ig-like domain that is responsible for binding to sialic acid.[9] A specialized subgroup of the Ig superfamily, called sialic acid-binding immunoglobulin-like lectins (Siglecs), can recognize sialylated glycoconjugates and play a role in cell–cell recognition and intracellular signaling. At least 11 related genes have been identified in the human genome, all of which encode type 1 membrane proteins that carry an N-terminal sialic acid-binding V-set Ig domain and varying numbers of C2-set Ig domains.[9] Thus, Prep2 may be a Siglec-type protein. Prep3 protein belongs to the large calcineurin-like phosphoesterase superfamily. Members of this superfamily include the polymerase-associated B-subunits and all carry an active site harboring two divalent metal ions for catalysis.[10] This suggests that Prep3 protein may participate in cell growth by regulating DNA replication. *PREP4*

encodes a novel member of the paxillin family proteins that bear the RhoGEF and PH domains.[11] Paxillin is a multi-domain adaptor that recruits associated proteins to focal adhesions, where it plays a pivotal role in transducing cell signaling, thereby eliciting changes in cell migration and gene expression.[12] Given that the RhoGEF and PH domains both are also found in these cell signaling molecules, Prep4 protein appears to function in the signal transduction of growth signals. *PREP5* encodes a protein with a LisH (LIS1 homology) motif, which is found in many signaling proteins of the WD-40 family and is believed to help regulate microtubule dynamics by mediating dimerization or binding to cytoplasmic dynein heavy chain.[13] Prep6 protein is homologous to laminin A, which is one of the 15 laminin isoforms that are the major component of basement membranes.[14] *PREP7* encodes a basic-leucine zipper (bZip) transcription factor[15] with a DNA-binding motif called AT-hook.[16] *PREP8* encodes a protein that is weakly homologous to Sir2, an NAD-dependent deacetylase that links metabolism with longevity in yeast, flies, and worms.[17] Prep9 protein harbors a region that is weakly homologous to extensin, a hydroxyproline-rich cell wall structural glycoprotein of higher plants.[18] The N-terminal region of Prep10 protein bears a domain that has homology to the adenovirus hexon-associated protein IX.[19] Prep11 protein carries in its C-terminus a domain with homology to the flavin adenine dinucleotide (FAD)-binding domain of siderophore-interacting protein which may be involved in iron chelation and iron utilization.[20]

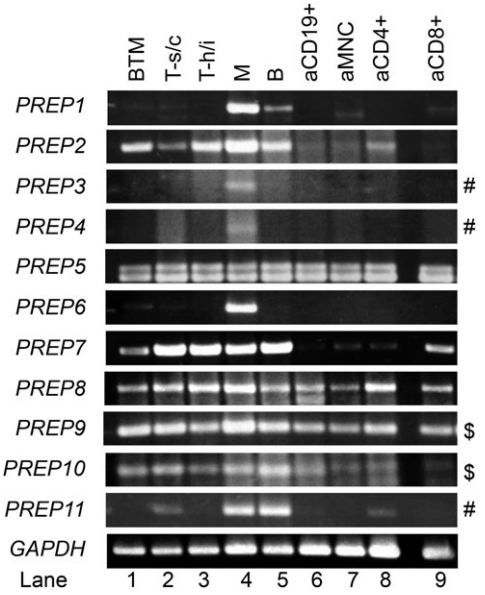

Figure 3. Determination by RT–PCR of the human blood cells that express *PREP1–PREP11*. RT–PCR was performed using a multiple tissue cDNA panel for human blood fractions (MTC, Clontech). *GAPDH* was also amplified as a loading control. PCR amplifications involved 40 cycles at 55°C except as indicated to the right of the panels: 35 cycles at 55°C (#) or 35 cycles at 58°C ($). Lane 1, mononuclear cells (B cells, T cells, and monocytes). Lane 2, resting CD8+ cells (T-suppressor/cytotoxic cells). Lane 3, resting CD4+ cells (T-helper/inducer). Lane 4, resting CD14+ cells (monocytes). Lane 5, resting CD19+ cells (B cells). Lane 6, activated CD19+ cells. Lane 7, activated mononuclear cells (aMNC). Lane 8, activated CD4+ cells. Lane 9, activated CD8+ cells.

3.3. *Expression pattern of PREP1–PREP11 and proto-oncogene PREP genes in PBMCs*

To determine whether the uncharacterized *PREP1–PREP11* genes are expressed in particular human blood cells, we performed RT–PCR on multiple tissue cDNA (MTC) panels from Clontech (Palo Alto, CA). As shown in Fig. 3, *PREP3*, *PREP4*, and *PREP6* mRNAs were only detected in resting CD14+ cells (monocytes; M), whereas *PREP1* mRNAs were predominantly found in monocytes but were also faintly expressed in resting CD19+ cells (B cells). In addition, *PREP11* showed a strong signal in monocytes and B cells and a weak signal in T-suppressor/cytotoxic cells and activated CD4+ T cells. *PREP2* was strongly detected in monocytes, T-helper/inducer, and B cells, and weakly in T-suppressor/cytotoxic and activated CD4+ cells. *PREP7* was strongly expressed in all mononuclear cells (B cells, T cells, and monocytes), weakly expressed in activated CD8+ T cells, and faintly expressed in activated CD4+, CD19+, and mononuclear cells. *PREP10* was detected strongly in all resting mononuclear cells, but only faintly in the other cell types examined. The other *PREP* genes were ubiquitously expressed at almost equal levels in all cell types examined and indeed could serve as a loading control like GAPDH.

Unexpectedly, we found six proto-oncogenes were *PREP* genes, namely, c-*ets*, c-*src*, c-*yes*, c-*fgr*, c-*fos*, and c-*vav*. We initially asked whether the augmented expression of these proto-oncogenes is due to infection-induced T-cell or macrophage stimulation in one of the PBMC donors. To test this, we examined the expression of these proto-oncogenes by RT–PCR on the MTC panel described above, since the sample RNAs used to generate the panel is not the same as our RNAs (Fig. 4). To our surprise, we found all cell types examined strongly expressed c-*ets*, c-*src*, and c-*yes*. All mononuclear cells (B cells, T cells, and monocytes) and activated CD4+ and CD19+ cells, but not activated CD8+ and mononuclear cells, also expressed c-*vav*. In contrast, c-*fgr* was only detected in T-suppressor/cytotoxic cells, while c-*fos* was only detected in resting T-suppressor/cytotoxic cells, B cells, and mononuclear cells. Thus, the expression of these proto-oncogenes is a commonly observed phenomenon.

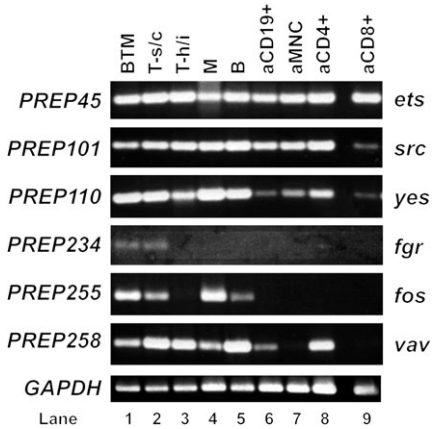

Figure 4. Expression profiles of the indicated proto-oncogenes in human blood fractions. RT–PCR was performed using the multiple tissue cDNA panel (MTC, Clontech). *GAPDH* was also amplified as a loading control. PCR amplifications involved 35 cycles at 55°C for all samples. Lane 1, mononuclear cells (B, T cells and monocytes). Lane 2, resting CD8+ cells (T-suppressor/cytotoxic cells). Lane 3, resting CD4+ cells (T-helper/inducer). Lane 4, resting CD14+ cells (monocytes). Lane 5, resting CD19+ cells (B cells). Lane 6, activated CD19+ cells. Lane 7, activated mononuclear cells (aMNC). Lane 8, activated CD4+ cells. Lane 9, activated CD8+ cells.

3.4. *Focused PREP analysis of CSS PBMCs*

We next examined whether the *PREP* genes can serve in a focused array that can be used to diagnostically. For this purpose, we used the array to determine if CSS patients can be characterized by a particular PREP mRNA expression pattern. CSS is a rare autoimmune disease (ca. 2.4 per million), namely, a small-vessel necrotizing vasculitis that is typically characterized by asthma, lung infiltrates, extravascular necrotizing granulomas, and hypereosinophilia.[21] This disease was chosen because diagnostic gene markers for this disease have not yet been reported. Moreover, it is likely that the symptoms of CSS patients will be reflected in abnormal gene expressions in their PBMCs. Indeed, we found with the aid of expression profiling analysis (GeneSpring) that, compared with normal volunteers, 33 *PREP* genes are consistently up-regulated in the PBMCs of all seven cases of CSS patients tested (Fig. 5A, uppermost panel). Of these, *PREP135*, *PREP77*, *PREP191*, *PREP234*, and *PREP136*, which are highlighted by larger arrows in Fig. 5A, showed the most conspicuous up-regulation in all CSS patients (>2.5-fold change).

PREP135 encodes the coactosin-like protein (CLP), which is a small, evolutionarily conserved F-actin-binding protein that can also bind to 5-lipoxygenase (5LO) and regulate its activity.[22] *PREP77* encodes prosaposin, the precursor of the sphingolipid activator proteins (saposin A, B, C, and D) that are required for the enzymatic hydrolysis of sphingolipids by specific lysosomal hydrolases.[23] *PREP191*, which encodes a lysosomal aspartyl peptidase cathepsin D (CTSD), is known to be involved in prosaposin

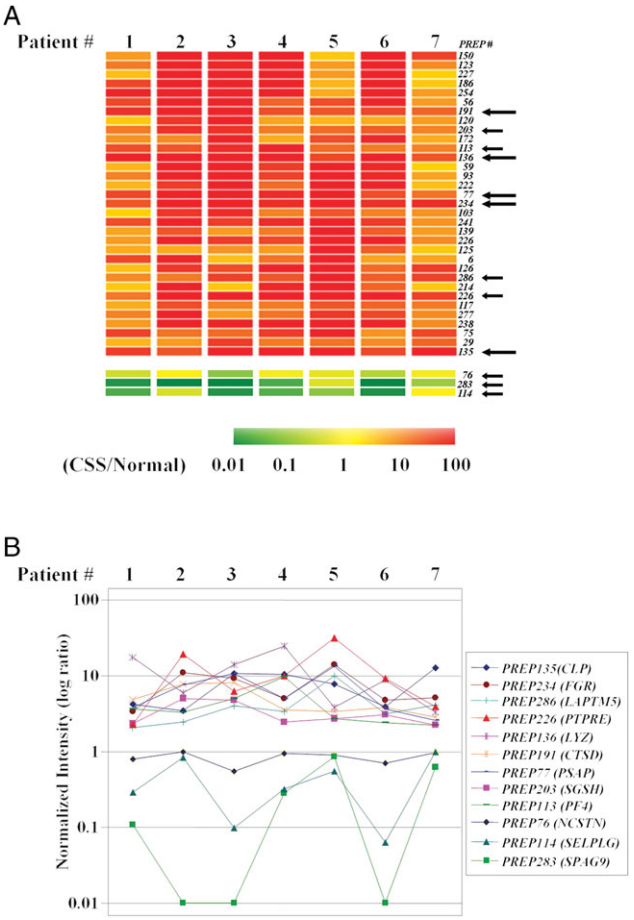

Figure 5. Expression profiles of *PREP* genes in CSS patient PBMCs. Agilent's whole human genome DNA microarray was employed for this analysis. (**A**) Presentation of mosaic tiles for the 33 and 3 *PREP* genes whose expressions are up-regulated (>1.2-fold change) or down-regulated (<1.0-fold change) in all seven CSS patients as compared with healthy volunteers. The gene names are indicated on the right of the panels. The *PREP* genes were selected by a filter and their expressions in all seven cases of CSS patients were grouped together by using GeneSpring software 7.3 (Agilent Technologies, Inc.). In other words, their raw expression values were normalized according to the manufacturer's instructions by conducting a ratio calculation of the two channels. This was followed by a per-gene normalization to ensure all ratios for each gene were normalized to 1. The nine conspicuously up-regulated *PREP* genes (>2.0-fold change) are denoted by arrows; the five large arrows signify more enhanced up-regulation (>2.5-fold change). Tile colors indicate the mean relative transcript level; green corresponds to a log10 ratio of 0.01 (down-regulation), red corresponds to a log10 ratio of 100 (up-regulation), and yellow indicates unchanged (bottom-most panel) when CSS and normal PBMCs are compared (bottom-most panel). (**B**) Graphical presentation of the nine CSS-up-regulated and three CSS-down-regulated *PREP* genes (denoted by arrows in Fig. 5A). Mean values were plotted for the genes that showed a significant ($P < 0.05$) difference in expression. The ordinate scale indicates relative log ratio values.

proteolysis.[24] Thus, the enhanced expression of *PREP77* and *PREP191* suggests sphingolipid levels are increased in CSS. This is consistent with the pivotal role sphingolipids are known to play as proinflammatory factors.[25] Thus, sphingolipids and related enzymes may not only be diagnostic marker for CSS, they may also be novel therapeutic targets in CSS (see Discussion).

PREP234 encodes Gardner–Rasheed feline sarcoma viral (*v*-fgr) oncogene homolog (*c*-fgr: FGR), which is known to play an important role in lipopolysaccharide (LPS)-induced macrophage activation.[26] Indeed, Fgr-deficient mice fail to develop lung eosinophilia in response to repeated challenge with aerosolized ovalbumin (OVA)[27]. Src-family tyrosine kinases are required to support the accumulation of polymorphonuclear leukocytes (PMN) along with adherent platelets at the site of vascular injury.[28] *PREP136* encodes lysozyme, which is one of the genes responsible for autosomal dominant hereditary amyloidosis.[29] Indeed, there are several case reports of amyloidosis associated with CSS that occurs together with a destructive inflammatory or granulomatous reaction to amyloid.[30] Several case reports have also described granulomatous angiitis in patients with sporadic, amyloid beta peptide (Abeta)-related cerebral amyloid angiopathy and Abeta-related angiitis with development of amyloid-associated inflammation.[31]

There are also four other less conspicuously CSS-up-regulated *PREP* genes (2.0–2.5-fold change). Of these, *PREP226* encodes a receptor (membrane)-type variant of the protein tyrosine phosphatase (PTPepsilonM) family protein that negatively regulates insulin receptor (IR) signaling by dephosphorylating IR, thereby suppressing the phosphorylation of IR downstream enzymes such as Akt, extracellular regulated kinase (ERK), and glycogen synthase kinase 3 (GSK3).[32] Notably, the cytosolic isoform of PTPepsilon (PTPepsilonC) selectively inhibits interleukin-6 (IL-6)- and IL-10-induced Janus kinases (JAK)-signal transducer and activator of transcription 3 (STAT3) signaling in blood cells.[33]

Another of the four less conspicuously up-regulated *PREP* genes, *PREP113*, encodes platelet factor 4 (PF4; CXC chemokine ligand 4), which is a specific marker for megakaryocyte cells and that induces the differentiation of monocytes into macrophages during the inflammatory process.[34] *PREP286* encodes lysosomal-associated multi-spanning membrane protein-5 (LAPTM5). While the function of LAPTM5 is unknown, it may participate in host defense in collaboration with other lysosome-related genes (*PREP77*, *PREP191*, and *PREP136*) in the blood cells of CSS patients. These results suggest that the regulation of cell signaling in blood cells from CSS patients is abnormal. The last of the four less conspicuously up-regulated *PREP* genes is *PREP203*, which encodes the spliceosomal protein thioredoxin-like 4B (TXNL4B). However, its physiological significance in the pathogenesis of CSS remains unclear.

We also identified three *PREP* genes that, compared with normal volunteers, are down-regulated in the PBMCs of all seven CSS patients (Fig. 5A, middle panel). *PREP76* encodes nicastrin;[35] its down-regulation may disturb the phagocytic response of macrophages to inflammatory cytokines since gamma-secretase catalyzes the intramembrane cleavage of its protein substrates and targets phagocytosis-related proteins of macrophages.[36] *PREP114* encodes selectin P ligand (SELPLG), which stimulates T lymphocytes, and plays a critical role in the tethering of these cells to activated platelets or endothelia expressing P-selectin.[37] *PREP283* encodes sperm-associated antigen 9 (SPAG9), which has also been identified as a serum target of the autoantibody produced in systemic sclerosis (SSc).[38] Thus, the reduced expression of these three genes could also serve as diagnostic markers of CSS. Unfortunately, we could not correlate the expression levels of these genes to the profiles of tested CSS patients (Supplementary Table S1).

3.5. *Expression pattern of CSS-up-regulated PREP genes in PBMCs*

To examine whether these 12 CSS-up- or -down-regulated *PREP* genes are expressed in particular human blood cells, we performed RT–PCR on MTC panels (Fig. 6). *PREP76*, *PREP135*, and *PREP203* were expressed by nearly all cells, and were the only genes that were expressed by activated CD19+ and activated CD8+ cells. Most of the 12 *PREP* genes were expressed by resting CD14+ cells (monocyte; M) and T-helper/inducer (T-h/i) cells, albeit with different expression levels. All genes except *PREP283* were expressed by T-suppressor/cytotoxic (T-s/c) cells. *PREP76*, *PREP113*, *PREP114*, *PREP135*, and *PREP203* were expressed at varying expression levels by activated mononuclear cells (aMNC). All genes except *PREP114*, *PREP135*, and *PREP283* were expressed by B cells, albeit comparatively weakly. Thus, many of the CSS-up-regulated or -down-regulated *PREP* genes are expressed by T cells or monocytes. Unfortunately, we could not perform the same analysis on CSS patients because of the low numbers of PBMCs available.

4. Discussion

4.1. *Isolation of genes that are predominantly expressed in human PBMCs*

Here we show for the first time (to our knowledge) that RNA diagnostics can be performed by using a

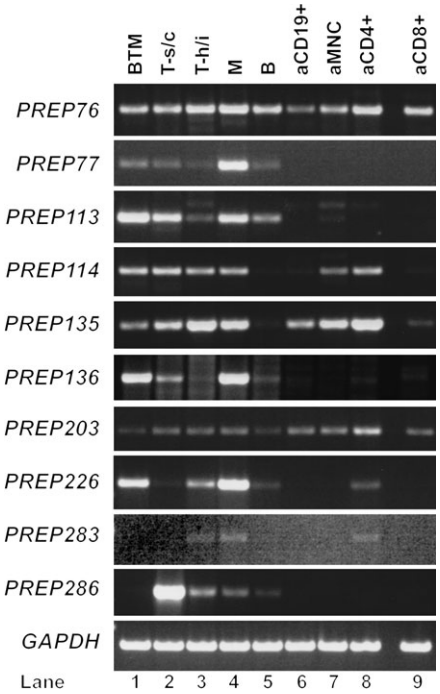

Figure 6. Expression of CSS-up-regulated or -down-regulated *PREP* genes by various human blood cells. RT–PCR was performed with the MTC. The *GAPDH* expression profile served as a loading control. All PCR amplifications involved 35 cycles at 55°C. We could not detect an amplified band for *PREP119* even though we tested three different primer sets (Supplementary Table S1). Lane 1, mononuclear cells (B, T cells and monocytes). Lane 2, resting CD8+ cells (T-suppressor/cytotoxic cells). Lane 3, resting CD4+ cells (T-helper/inducer). Lane 4, resting CD14+ cells (monocytes). Lane 5, resting CD19+ cells (B cells). Lane 6, activated CD19+ cells. Lane 7, activated mononuclear cells (aMNC). Lane 8, activated CD4+ cells. Lane 9, activated CD8+ cells.

PBMC-specific focused array. Such RNA diagnostics methods have the advantage over DNA diagnostics in that ethical problems concerning the genetic information of individuals are avoided. To select the PBMC-specific genes for use in RNA diagnostics, we utilized a technique that we developed previously, namely, stepwise subtraction.[4] In this technique, all genes expressed by a particular cell, tissue, or whole organism are used to generate a cDNA library. This library is then systematically subtracted in a stepwise manner by using the biotinylated mRNAs from a control population to isolate essentially all those genes that are up-regulated relative to the control population expression profile. This technique has proven to be useful for isolating the protein-coding genes that are specifically expressed during cancer metastasis, meiosis, and spermatogenesis, and in patients with autoimmune diseases.[4,39–41] It has also been used to identify putative non-coding RNAs that cannot be identified by microarray screening.[42] By using this technique along with high-density oligonucleotide microarray analysis, we identified 290 *PREP* genes that are predominantly expressed in human PBMCs as compared with fibroblast (TIG-1) cells (Fig. 1 and Table 1). These genes could be used to generate a PBMC-specific focused array that could be used to characterize the abnormal expression profiles in the PBMCs of patients with a particular disease. The abnormal profiles could then serve as a diagnostic tool for other patients presenting with this disease.

The 290 *PREP* genes we identified include 50 uncharacterized genes, 11 of which possess notable amino acid motifs (Fig. 2). Two of these, *PREP1* and *PREP6*, were almost exclusively expressed in monocytes (Fig. 6). Of the 240 *PREP* genes that have been characterized previously, 11, eight, eight, seven, six, five, five, nine, and 36 are HLA genes, CD antigen genes, ribosomal protein genes, interferon (IFN)-related genes, S100 calcium-binding protein-related genes, interleukin (IL)-related genes, platelet-related genes, signal transduction-related genes, and immunity-related genes, respectively (Supplementary Table S4). We did not identify any other immunity-related genes as *PREP* genes by these techniques, probably because their expression levels are lower than the *PREP* genes.

4.2. *PBMC-specific array analysis with CSS patients*

To explore the usefulness of the PBMC-specific focused array analysis in RNA diagnostics, we tested the PBMCs of seven cases of CSS patients.[21] The precise pathogenetic mechanisms of CSS remain elusive, in part because the rarity of this disease. We found that compared with normal volunteers, 22 *PREP* genes were up-regulated by >1.5-fold in the PBMCs of all seven CSS patients (Fig. 5). Of these, *PREP135*, *PREP77*, *PREP191*, *PREP234*, *PREP136*, *PREP203*, and *PREP113* were the most conspicuously up-regulated in CSS patients. How these genes contribute to the pathogenesis of CSS is suggested by the known functions of their products, as follows. *PREP77* (prosaposin) and *PREP135* (CLP) may indicate the enhanced immune responses that cause vascular inflammation; *PREP203* (TXNL4B) and *PREP234* (*c*-fgr) may reflect the augmented intracellular signaling that occurs after an infection causes inflammation; and the increased expression of the lysosome-related genes *PREP77*, *PREP136*, *PREP191*, and *PREP286* may indicate the mounting of an effective defense against invading microorganisms by the lysosomes in blood cells. Thus, our *PREP* gene-focused analysis of CSS patients has helped to identify genes that may be putative diagnostic and/or therapeutic targets.

There are few molecular tools that can be used to aid the diagnosis of CSS. The main tool that is commonly employed is the detection of antineutrophil cytoplasmic autoantibodies (ANCA), which can be

detected by an immunofluorescent assay (IFA) on ethanol-fixed neutrophils. ANCA are associated with systemic necrotizing vasculitis and glomerulonephritis diseases such as CSS, Wegener's granulomatosis (WG), and microscopic polyarteritis (MPA).[43] Two different ANCA immunostaining patterns are observed, namely, a diffuse cytoplasmic staining pattern (c-ANCA) that recognizes proteinase 3, and a perinuclear/nuclear pattern (p-ANCA) that commonly recognizes myeloperoxidase (MPO), which is a neutrophil granule protein that helps generate oxygen radicals and is associated with the antimicrobial properties of neutrophils. The c-ANCA pattern is most frequently observed in WG patients, whereas the p-ANCA pattern is detected in CSS and MPA patients.[44] Notably, ANCAs against alternative antigens such as cathepsin G, which is a major protease released by activated neutrophils, are also occasionally observed.

Since antibodies against cathepsin D (*PREP191*), PTPRE (*PREP226*), FGR (*PREP234*), PF4 (*PREP113*), CLP (*PREP135*), and prosaposin (*PREP77*) are currently commercially available, it may be worthwhile to use these antibodies in IFAs on ethanol-fixed blood cell samples to determine whether these antibodies have a relationship with the ANCA-staining patterns in CSS and whether they can help confirm the diagnosis in ANCA-negative CSS patients. Unfortunately, these experiments will have to be performed in the future because the CSS blood samples we obtained were used up during RNA preparation. Moreover, as CSS occurs only very rarely (only 2.4 patients per 1 million people in Japan), it will take some time to accumulate enough patients for such a study.

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