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 arrays1 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
applications, including discovering polymorphisms and genotyping patients by identifying inheritable genetic markers. 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 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. 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. 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. 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. ExTaq 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 Biosciences 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. 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. 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 Smal and NotI 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 EcoRI and NotI; 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 (Licol 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.

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.

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-labeled cRNA of each patient was then mixed with the Cy3-labeled 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. We also prepared mRNAs from exponentially growing normal
human fibroblast TIG-1 cells, which are control cells that express non-PBMC transcripts. These mRNAs were biotinylated with photobiotin and used to subtract the PBMC cDNA library as described previously 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. 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.
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 \textit{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 \textit{EcoRI} and \textit{NotI} 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

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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. 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-specific 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).

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. PREP2 encodes a protein that harbors a V-set Ig-like domain that is responsible for binding to sialic acid. 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. 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. This suggests that Prep3 protein may participate in cell growth by regulating DNA replication. PREP4
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 panel 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 mRNA was 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 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.
3.4. 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, upper-most 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...
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 up-regulated 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
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. 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 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, 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. 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 patients, whereas the p-ANCA pattern is detected in c-ANCA pattern is detected in WG patients. Notably, ANCAs against nuclear antigens are associated with the classification of Churg-Strauss syndrome (allergic granulomatosis and angiitis), the American College of Rheumatology 1990 criteria for the classification of Churg-Strauss syndrome (allergic granulomatosis and angiitis), Arthritis Rheum., 33, 1094–1100.

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