Ficolin 1 Expression is Elevated in the Peripheral Blood Mononuclear Cells of Takayasu’s Vasculitis Patients

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Abstract

Takayasu’s arteritis (TA), a form of vasculitis (angitis), is a chronic inflammatory disease involving the large blood vessels. This study reports the identification of genes showing increased mRNA levels in peripheral blood mononuclear cells (PBMCs) from TA patients regardless of symptoms or disease activity/inactivity. Of these, mRNA for Ficolin 1 (FCN1) showed a four-fold increase in all eight TA patients examined. Increased FCN1 mRNA levels observed in cDNA microarrays were confirmed by quantitative reverse transcription polymerase chain reaction. Increased FCN1 protein expression was also observed in inflamed regions of the surgical aorta specimens. Moreover, most FCN1-positive cells were also positive for CD68, indicating the presence of monocytic cells, such as macrophages or dendritic cells, which attack infectious agents within the inflamed regions. Taken together, the results suggest that FCN1 mRNA levels in peripheral blood samples may be a diagnostic marker for TA.

Introduction

Takayasu’s arteritis (TA), a rare vasculitis presenting mainly in young females, is a chronic, idiopathic, inflammatory disease that affects the aorta and its associated branches [1]. Since the symptoms are non-specific during the acute early phase, (headaches, fever and muscle pains), the diagnosis of TA is often delayed until substantial arterial injury develops [2]. Without proper medical treatment, the disease causes inflammation of the aorta walls and those of its main branches, leading to concentric wall thickening, fibrosis, and thrombus formation. This may then progress to stenosis, occlusion, dilatation or aneurysm of the involved arteries, which are potentially life-threatening conditions [3,4].

The diagnosis of TA is based on a clinical presentation that is heterogeneous but involves two stages: an initial inflammatory process with variable systemic manifestations, and a later "pulseless phase" with occlusion and stenosis of multiple arteries [3,5]. Conventional angiography is one of the standard diagnostic procedures. Recent progress in vascular imaging allows the detection of vessel alterations at an earlier stage and better control of the course of disease. Recent progress in computed tomography (CT), magnetic resonance imaging (MRI), and fluorodeoxyglucose positron emission tomography (FDG PET) has led to their increased use in the field of oncology and in patients with inflammatory diseases [3,6]. However, these methods are still limited in their ability to accurately quantify the degree of inflammatory disease activity within the arterial walls. Thus, a simple and reliable diagnostic tool is required.

Since large-vessel biopsies are practically impossible to obtain, it is important that a diagnosis of TA is confirmed by laboratory investigations. Such investigations should be simple, as non-invasive as possible, and predict morbidity. A gene marker (that is easily accessible, e.g., from peripheral blood cells) that can specifically diagnose TA patients at an early phase of the disease, regardless of the patient’s symptoms or level of disease activity/inactivity, would be very useful. To date, few studies (apart from our own previous work) have been published regarding the genome-wide analysis of TA [7-9].

Therefore, the aim of the present study was to identify new gene markers expressed in peripheral blood mononuclear cells (PBMCs) that can distinguish TA patients (regardless of symptoms) from healthy volunteers. For this purpose, we used high-density oligonucleotide microarrays and found that the mRNA levels of a particular complement factor, Ficolin 1 (FCN1), were markedly up-regulated in PBMCs from all TA patients examined. Immunostaining revealed augmented expression of FCN1 protein within the inflamed regions of surgical specimens of aorta in 3/4 unrelated TA patients, indicating that FCN1 may serve as a marker for the diagnosis of TA.

Patients, Materials and Methods

Human subjects and Ethical permission

All TA patients used in this study were diagnosed as TA using angiography according to a previously documented proposal (the ACR criteria and the CHCC criteria) [10] by medical doctors who are rheumatologists and cardio-vascular specialists in Juntendo Hospital, Japan. The study was reviewed and approved by the Research Ethics Committee of Osaka University. Accordingly, written informed consent was obtained from all participants. Serum samples were

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consecutively obtained regardless of the patient’s symptoms and level of disease activity/inactivity.

**Preparation of RNA**

RNA was prepared from PBMCs isolated from TA patients and normal volunteers using standard methods [11]. 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. The PBMCs in the supernatant were then purified by density-gradient centrifugation on Percoll (density: 1.064 g/mL).

Total RNA was extracted from the PBMC pellets by adding guanidine-thiocyanate solution and the samples were subjected to acid guanidinium-phenol-chloroform extraction followed by ethanol precipitation and DNase I treatment prior to microarray or quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses.

**DNA microarray analysis**

The quality of the RNA samples was examined using the RNA 6000 Nano LabChip Kit (p/n 5065-4476) and an Agilent 2100 Bioanalyzer (G2940BA; Agilent Technologies, Inc., Palo Alto, CA). Total RNA (500 ng) isolated from the PBMCs of each vasculitis patient was examined along with pooled RNA from the normal volunteers (Figure S1A). Each patient’s RNA and the pooled normal RNA were reverse-transcribed using oligo-dT primers containing the T7 RNA polymerase promoter sequence, and the resulting cDNA was subjected to in vitro transcription using T7 RNA polymerase so that they were labeled with Cy3 or Cy5 (CyDye, Amersham Pharmacia Biotech, Piscataway, NJ). The Cy5-labeled cRNAs from the vasculitis patients (1625 ng) were then mixed with the same amount of reverse color Cy3-labeled cRNAs from the normal volunteers. Hybridization, rinsing, scanning, and gene analysis were conducted using the Agilent Whole Human Genome Microarray (4x44K G4112F) according to the manufacturer’s protocol. The ratio of the cy5/cy3 was calculated for every probe and the results were analyzed using Subio Basic Plugin (v1.6; Subio Inc., Aichi, Japan), which allows visualization of microarray data in the form of a heat-map and a line graph. aw data have been accepted in GEO (a public repository for microarray data, aimed at storing MIAME data) with the accession number GSE33910 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=prittocsqucugxi&acc=GSE33910).

**Pathway analysis of microarray data**

The microarray data were analyzed using Ingenuity Pathways Analysis (IPA) (Ingenuity Systems, Redwood City, CA; www.ingenuity.com). Briefly, all data sets containing gene identifiers were uploaded into the application along with the corresponding expression values and overlaid onto several inflammatory related networks developed using information contained in the Ingenuity Knowledge Base.

**Quantitative real-time PCR**

We performed qRT-PCR in an ABI PRISM 7900 (PE Applied Biosystems, Foster City, CA) using the Assay-on-Demand TaqMan probe and the relevant primers according to the manufacturer’s instructions. For FCN1, an assay kit (ID Hs00157572_m1) was purchased from PE Applied Biosystems (Foster City, CA). The following oligonucleotides were used as primers and probes for the GAPDH gene: GAPDH forward primer, 5’-CCATCAATGACCCCTTCATCAG-3’; GAPDH reverse primer, 5’-TCTCGCTCTGGAAATGATTG-3’; and GAPDH TaqMan probe, 5’-VIC-ACCTCACAATCATGTTTTAC-MGBNFQ-3’. Total RNA (500 ng) was reverse-transcribed using the High Capacity cDNA Archive Kit (ABI). The cDNA was then used as a template for PCR in a 20 μL reaction using 2x Master Mix according to the manufacturer’s instructions (TaKaRa, Otsu, Japan). The PCR conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Each sample was assayed in quadruplicate and the median threshold cycle (Ct) values were used to calculate the fold change between the treated and control samples. A standard curve was generated from the amplification data for each primer using serial dilutions of PBMC RNA as the template. The fold change values were normalized to GAPDH levels using the standard curve method according to the manufacturer’s protocol.

**Histological analysis**

Surgical aorta specimens from TA patients were fixed with 10% buffered formalin, embedded in paraffin, and cut into 4 μm-thick serial sections. The first sections were stained with hematoxylin and eosin and used for pathological diagnosis of inflamed regions. The other three sections were subjected to immunohistochemistry as described previously. Briefly, deparaffinized sections were autoclaved in 0.1 M citrate buffer, blocked with bovine serum albumin (BSA), and incubated with the indicated primary antibodies diluted in PBS/2% BSA. Secondary antibody and signal enhancement reactions were performed using the Histofine Simple Stain kit (Nichirei, Tokyo, Japan), and the color was developed with aminoethylcarbazole (Impact AEC; Vector Laboratories, Burlingame, CA). Some sections were double-stained with antibodies against FCN1 and CD68 for immunofluorescence analysis. After the deparaffinized sections were autoclaved and blocked-up as described above, they were incubated with a mixture of anti-FCN1 and anti-CD68 antibodies in PBS/2% PBS. After washing three times with PBS, the sections were incubated with a mixture of Cy2- and Cy3-labeled donkey antibodies against mouse and rabbit IgG, respectively (Jackson ImmunoResearch Laboratories, PA). Sections were counterstained with hematoxylin to visualize the cell nuclei before being mounted with Ultramount Aqueous Permanent Mounting Medium (DakoCytomation, Glostrup, Denmark).

**Results**

Identification of TA-related genes by DNA microarray analysis

To isolate putative TA-related genes that are up- or down-regulated in the PBMCs of TA patients compared to normal volunteers, genome-wide complementary DNA microarray analysis was performed using the Agilent Hu44K array and RNA samples isolated from the PBMCs from eight individual TA patients and 17 normal volunteers, as previously described [12] (Figure S1A). TA patients were examined regardless of symptoms or disease activity/inactivity (Table 1) because we wanted to obtain a marker that distinguishes TA from normal controls even during its inactive phase.

Analysis of the microarray data revealed that 15 genes were up-regulated > 3-fold in all eight TA patients compared with the normal controls (Figure 1A, red and blue lines, and Figure S1B and C). Since TA is a disease caused by aberrant regulation of the immune system, we focused on the complement factor FCN1, whose mRNA levels were increased > 4.0-fold in all eight TA patients (Fig. 1A, blue line and font). Since two uncharacterized genes (C6orf47 and C19orf56) and another three genes (C6orf47 and C19orf56) and another three genes (C6orf47 and C19orf56) and another three genes (C6orf47 and C19orf56) and another three genes (C6orf47 and C19orf56) and another three genes (C6orf47 and C19orf56) and another three genes (C6orf47 and C19orf56)
12 other genes do not appear to be directly involved in the immune system, we decided not to study them further. We also identified 13 genes that were down-regulated in all eight TA patients; however, none appeared to be related to the immune system, so further analysis was not performed.

It is notable that mRNA levels for C5AR1, CD59 and CD46 were upregulated in patients whose disease was in the active phase. Patient #4 was followed for almost 5 years, but had not experienced any flare-ups at the time this data was collected. The data also showed that membrane cofactor protein (MCP/CD46) was up-regulated in the eight TA patients, and this may be related to the immune system, so further analysis was performed.

Examination of other complement factor genes

The complement system is regulated by more than twenty independent genes. Therefore, to examine whether other complement factor genes were also up- or down-regulated in the eight TA patients, we analyzed the data using Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com). This powerful ontology search query system allows the rapid visualization of relationships between a set of relevant genes and increases the predictive physiological significance of the enhanced FCN1 expression identified earlier. Indeed, the results showed that membrane cofactor protein (MCP/CD46) was up-regulated (Figure 2A, blue arrow) in a manner similar to that of FCN1 (yellow arrow) in patients with TA. CD59 (green arrow) and C5AR (orange arrow) were moderately up-regulated.

In addition, the heat map derived from the microarray data allowed us to compare the differential expression of the genes by showing the mRNA level of each gene in a color tile format. Each colored tile in the heat map represents a gene expression value; the largest expression value...
values are displayed in red (hot), the smallest values in blue (cool), and the intermediate values in different shades of red (pink) or blue. Gray tiles indicate no change. Indeed, the heat map showed that only FCN1 was up-regulated in all eight TA patients (Figure 2B, yellow arrow). By contrast, the mRNA levels of MCP/CD46 (blue arrow), CD59 (green arrow) and CSAR (orange arrow) in patients #4 and #5 were almost the same as those in the normal volunteers (Figure 2B). Thus, only FCN1 was identified as a promising TA-related diagnostic marker.

Expression of other immune-related genes

We next sought to examine whether the expression of other immune-related genes was enhanced in TA patients. Therefore, we performed pathway analysis and constructed a second heat map. Several genes that regulate the interferon (Figure 3) and toll-like receptor signaling pathways (Figure 4) showed increased signals in samples from TA patients.

The genes involved in the interferon signaling pathway were signal transducers and activators of transcription (STAT1; Fig. 4, turquoise arrow), proteasome (prosome, macropain subunit beta type-8 (PSMB8; pink arrow), interferon regulatory factor 1 (IRF1; gray arrow), interferon regulatory factor 9 (IRF9; ochre arrow), interferon gamma receptor 1 (IFGRI/IFGRa; black arrow) and interferon gamma receptor 2 (IFGRII/IFGRb; white arrow). Of these, STAT1, PSMB8, IRF1, IRF9 and IFGRI showed increased signals in all eight TA patients, although the signal intensity was modest in patients #3, #4 and #5. Thus, these genes may be candidate TA-related diagnostic markers. By contrast, IFGRII showed weak signals in patients #4 and #5, and so was not considered as a candidate.

Some of the genes involved in the toll-like receptor signaling pathway also showed increased signals in all eight TA patients (Figure 4); these genes include inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma (IKBKG; navy arrow), myeloid differentiation factor 88 (MYD88; pale brown arrow) and toll-like receptor 6 (TLR6; pale green arrow). However, their signal intensities were not conspicuously high. Thus, they were not considered as candidates. Taken together, we concluded that FCN1 is the most promising TA-related diagnostic marker among the immune-related genes.

**qRT-PCR of FCN1 genes in PCMBs from TA patients**

To confirm that the microarray signals actually reflected FCN1 mRNA levels, qRT-PCR was performed using the same RNA samples used for the microarray analysis. Samples from three patients were not analyzed due to a lack of RNA. All the samples analyzed by qRT-PCR showed increased FCN1 mRNA levels compared with the normal controls (Figure 5), confirming the microarray analysis results.

**Immunohistochemistry for FCN1 in the arteries of unrelated TA patients**

To demonstrate the usefulness of FCN1 as a TA-related diagnostic marker, we examined augmented FCN1 protein expression within the inflamed regions of surgical aorta specimens from five new TA patients (#9-13) unrelated to the eight TA patients analyzed previously. Anti-C3 and anti-C4 antibodies were used as negative controls because the mRNA levels of C3 and C4 were low in all eight TA patients previously tested (Figure 2B; thin black arrows). Immunohistochemistry revealed that 4/5 aorta specimens from TA patients reacted strongly with the anti-FCN1 antibody (Figure 6A; Patients 9–12). FCN1 immunoreactive signals were detected in some inflammatory cells infiltrating the inflamed regions. When the aortic specimens were double stained with anti-FCN1 and anti-C4 antibodies, most FCN1-positive cells were also positive for anti-C4 (Figure 6B), most FCN1-positive cells were also positive for anti-C4 (Figure 6B), most FCN1-positive cells were also positive for anti-C4 (Figure 6B).
CD68 (Figure 6B; arrowheads), indicating that the cells were monocytic cells, such as macrophages or dendritic cells, which are attracted to the inflamed regions. Taken together, these results suggest that FCN1 is a useful TA-related diagnostic marker.

Discussion

The present study reports the identification of candidate genetic markers for TA from RNA isolated from PBMCs, which may be useful for diagnosing and understanding the basic pathogenesis of the disease. These genes were FCN1 (Figures 1-2), STAT1, PSMB8, IRF1, IRF9 (Figure 3), IKBKG, MYD88 and TLR6 (Fig. 4). FCN1 appears to be the most important because its mRNA levels were increased more than four-fold in PBMCs isolated from all eight TA patients compared with those in healthy volunteers (Figure 1A). Moreover, increased expression of FCN1 protein was observed in the inflamed regions of surgical aorta specimens from 80% (4/5) of unrelated TA patients (Figure 6). These results suggest that FCN1 levels in PBMCs may serve as a marker for the diagnosis of TA. Since an anti-FCN1 antibody recognized FCN1 protein overexpressed by CD68-positive monocytes (Figure 6), immunoblotting of whole leukocyte proteins could also be used to diagnose TA, if a novel, simple and easy tool such as LeukoCatch [13] is employed.

The complement system is a part of the innate immune system, which recognizes and eliminates pathogens via direct killing and/or stimulation of phagocytosis. The complement system comprises more than 30 serum and cellular proteins, including ficolins, which function as recognition molecules in the lectin complement pathway. Three ficolins have been identified in humans: FCN1, FCN2, and FCN3. FCN1, a soluble collagen-like protein primarily expressed by peripheral blood leukocytes and bone marrow cells, is involved in innate immune defenses, and its genetic polymorphism appears to be associated with...
immunodeficiency [14]. Like other ficolins (FCN2 and FCN3) and mannose-binding lectins, whose mRNA levels were low in both TA patients and healthy volunteers (with no significant difference between them; data not shown), FCN1 binds sugar structures or acetylated compounds present on microorganisms and dying host cells, thereby activating the lectin complement pathway [15]. Indeed, FCN1 forms a pathogen sensor-cum-signal transducer by docking constitutively onto a monocyte transmembrane receptor, G protein-coupled receptor 43 (GPCR43). Upon encountering microbial invaders, the FCN1-GPCR43 complex interacts with C-reactive protein (CRP) via a conformational change in FCN1, which is induced by mild acidosis at the local site of infection, thereby activating the NF-kB cascade to upregulate IL-8 production [16]. Thus, the overexpression of FCN1 observed in all TA patients may reflect microbial invasion.

Complement activation appears to be involved in the pathogenesis of other systemic autoimmune diseases [17]. Enhanced FCN1 mRNA levels were also detected by qRT-PCR in the PBMCs of patients with other autoimmune diseases, such as inflammatory bowel disease (IBD), although the enhanced mRNA levels were less marked than in TA [18]. We also detected slightly upregulated FCN1 mRNA levels in other forms of vasculitis (data not shown). Thus, upregulated FCN1 mRNA levels are not specific for TA, but rather represent a disease-nonspecific inflammatory process. However, considering the more than 4-fold increase in FCN1 mRNA levels detected in all eight TA patients (Figure 1) and the increased FCN1 protein levels in the inflamed regions of

Figure 4: Expression profiling of the toll-like receptor signaling pathway. Pathway analysis (A) and heat maps (B) identified several genes whose mRNA levels were augmented in the majority of TA patients. These genes are IKBKG (navy arrow), MYD88 (pale brown arrow) and TLR6 (pale green arrow). All showed enhanced signals in all eight TA patients, although their signal intensity was relatively weak.

Figure 5: FCN1 mRNA levels in TA patients. The vertical axis indicates the relative intensity of the expression level (arbitrary unit: a.u.) measured by qRT-PCR. The horizontal axis indicates the patients.

Figure 6: Immunodetection of FCN1, C3 and C4 in the aortae of patients with TA. (A) Four serial sections of aorta from Patients 9-12 were stained with H&E or immunostained with antibodies against FCN1, C3, and C4. Arrowheads indicate representative cells reactive with anti-FCN1. (B) Sections were incubated with antibodies against FCN1 and CD68 and visualized with Cy2 and Cy3, respectively. Arrowheads indicate double-positive cells. Bar= 100 µm.
surgical aorta specimens from 80% of unrelated TA patients (Figure 6), FCN1 may serve as a marker for the diagnosis of TA.

It is notable that the mRNA levels of at least five genes (STAT1, PSM8B, IRF1, IRF9 and IFGR1) that regulate the interferon signaling pathway, a hallmark of various autoimmune rheumatic diseases [19], were enhanced in all eight TA patients (Figure 4). Since interferon plays an important role in the first line of defense against viral infections, this suggests that TA patients may have generated a response to a previous acute viral infection. Moreover, increased mRNA levels for at least three of the genes (IKBKG, MYD88 and TLR6) that control the toll-like receptor signaling pathway or innate immunity [20] were also observed in all eight TA patients. Thus, it appears that all TA patients may have had previous viral infections, which activated their innate immunity.

We previously reported that the mRNA levels of at least four lysosome/autophagy-related genes were conspicuously enhanced in PBMCs from patients affected by Churg Strauss Syndrome (CSS), another rare form of vasculitis [21,22], and proposed that the mounting of an effective defense against invading microorganisms by lysosomes in blood cells may be involved in the pathogenesis of CSS [12]. In other autoimmune diseases, such as systemic lupus erythematosus and idiopathic thrombocytopenic purpura, mRNA levels for the antibacterial peptide, defensin 3, and several interferon-related genes were found to be up-regulated in the PBMCs from many patients, which also suggest an effective defense against invading viruses [23-25]. We previously identified several immune-responsive genes that were conspicuously enhanced in rheumatoid arthritis patients [26]. However, increased mRNA levels for complement factors, including FCN1, were not apparent in these autoimmune diseases. Taken together, we conclude that the up-regulation of FCN1 may serve as a relevant diagnostic marker for TA.

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Supplementary Materials

Supplementary material is available online.

Author Contributions

HN made contributions to conception and design of this study and drafted the manuscript. DO performed DNA microarray analysis. SK collected patients’ blood and performed relevant clinical research. MAS and AI contributed to pathological science and technology of Japan.

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