Clinical Score and Transcript Abundance Patterns Identify Kawasaki Disease Patients Who May Benefit From Addition of Methylprednisolone

SHOHEI OGATA, YOSHIHITO OGIHARA, KEIKO NOMOTO, KAZUMASA AKIYAMA, YAYOI NAKAHATA, KAYOKO SATO, KATSUNORI MINOURA, KENICHI KOKUBO, HIROSUKE KOBAYASHI, AND MASAHIRO ISHII

Department of Pediatrics [S.O., Y.O., K.N., K.A., Y.N., M.I.], Kitasato University School of Medicine, Sagamihara, Kanagawa 228-0855, Japan; Department of Medical Engineering and Technology [K.K., H.K.], Kitasato University School of Allied Health Sciences, Sagamihara, Kanagawa 228-0855, Japan; Department of Pediatrics [K.S., K.M.], Ebina General Hospital, Ebina, Kanagawa 243-0433, Japan

ABSTRACT: Intravenous immunoglobulin (IVIG) treatment-resistant patients are high risk of developing coronary artery lesions with Kawasaki disease. The IVIG-responsive (Group A; $n = 6$) and IVIG-resistant patients (Group B) were predicted before starting the initial treatment using the Egami scoring system and randomly allocated as a single-IVIG treatment group (group B1; $n = 6$) or as a IVIG-plus-methylprednisolone (IVMP) combined therapy group (group B2; $n = 5$). We investigated the transcript abundance in the leukocytes of those patients using a microarray analysis. Five patients in group A and one patient in group B1 responded to initial IVIG treatment. All group B2 patients responded to IVIG-plus-IVMP combined therapy. Before performing these treatments, those transcripts related to IVIG resistance and to the development of coronary artery lesions, such as IL1R, IL18R, oncostatin M, suppressor of cytokine signaling-3, S100A12 protein, carcinoembryonic antigen-related cell adhesion molecule-1, matrix metallopeptidase-9, and polycythemia rubra vera-1, were more abundant in group B patients in comparison with group A patients. Moreover, those transcripts in group B2 patients were more profoundly and broadly suppressed than group B1 patients after treatment. This study elucidated the molecular mechanism of the effectiveness of IVIG-plus-IVMP combined therapy. (*Pediatr Res* 66: 577–584, 2009)

Kawasaki disease (KD) is an acute febrile disorder characterized by systemic vasculitis that primarily affects infant and children (1). Treatment with high-dose intravenous immunoglobulin (IVIG) and aspirin results in a resolution of fever in most patients with KD while also significantly reducing the incidence of coronary artery lesions (CALs) (2). However, approximately 15 to 20% of patients with KD have IVIG resistance, even though standard high-dose IVIG therapy was appropriately initiated (3). In these patients, CALs frequently developed, despite the use of additional treatments, including further IVIG and steroids, thus indicating that current alternative treatments are ineffective after the failure of

the initial IVIG treatment (4–6). In other words, an earlier and more effective primary therapy is required for IVIG-resistant patients (7–11). Steroid therapy has been reconsidered as a therapeutic strategy to treat KD (11–15). A novel therapeutic approach may not be necessary in all cases of KD because the appropriate dose of IVIG and aspirin yields a good response in 80 to 85% of patients. Therefore, we predicted IVIG-resistant patients before starting the initial treatment using the Egami score. The Egami scoring system identifies age, days of illness, platelet count, C-reactive protein, and alanine aminotransferase (ALT) to predict IVIG-resistant patients before treatment (cutoff: 3 points; 78% sensitivity and 76% specificity) (9). The Egami score is highly sensitive and specific in Japanese patients with KD but is not highly sensitive or specific in the mixed ethnic US population (8). This study randomly devised IVIG-plus-methylprednisolone (IVMP) combined therapy or single-IVIG treatment as a primary treatment in Japanese patients with prediction of IVIG resistant in a prospective study and investigated the biologic mechanism of those treatments for prediction of IVIG-resistant patients using a microarray analysis.

PATIENTS AND METHODS

Patients and study protocol. Sixty-nine patients with KD had been admitted to Kitasato University Hospital or Ebina General Hospital from 2006 to 2008. All patients were Japanese and fulfilled the criteria for the guidelines established by the Kawasaki Disease Research Committee of Japan (http://www.kawasaki-disease.org/tebiki/tebiki.html) (16). The diagnosis and evaluation of CALs were carried out using two-dimensional echocardiography before the initial treatment, at 36 to 48 h after treatment, and at 1 mo after treatment. The presence of CALs was diagnosed according to the Japanese Ministry of Health criteria. IVIG-responsive patients were defined as those who exhibited resolution of fever (<37.5°C) within 36 to 48 h after receiving the initial treatment (13,15).

Figure 1 shows the study protocol. Eleven of 69 patients with KD (15.9%) were predicted IVIG-resistant patients by Egami score (group B). These patients were randomly assigned to the single-IVIG treatment group (group B1; $n = 6$) and IVIG-plus-IVMP combined therapy group (group B2; $n = 5$), using a table of random digits. The remaining 58 patients were predicted IVIG-responsive patients by Egami score. The six of 58 patients who were predicted IVIG responders by Egami score were randomly selected as group A. Groups A and B1 patients were administered single-IVIG treatment (2.0 g/kg for

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Abbreviations: CALs, coronary artery lesions; **HP,** haptoglobin; **IVIG,** intravenous immunoglobulin; **IVMP,** IVIG-plus-methylprednisolone; **KD,** Kawasaki disease; **PRV-1,** polycythemia rubra vera-1
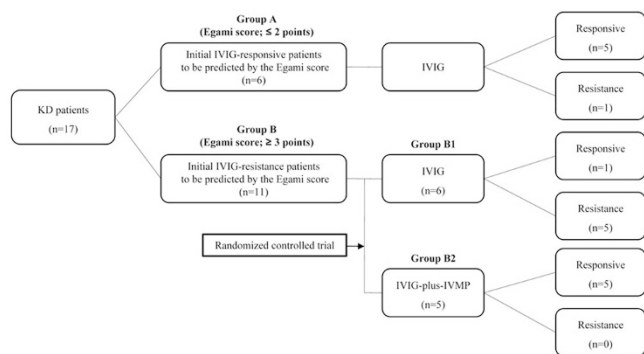
Figure 1. Study protocol and treatment results. The subjects were divided into the following groups: predicted to be IVIG-responsive patients (group A) and predicted to be IVIG-resistant patients (group B). Group A patients were administered single-IVIG (2.0 g/kg for 1 d). Group B patients were randomly assigned to receive either single-IVIG (2.0 g/kg for 1 d, group B1) or IVIG-plus-IVMP combined therapy (IVMP: 30 mg/kg for 2 h, group B2).

1 d). Group B2 patients received IVMP therapy, 30 mg/kg during 2 h before receiving IVIG treatment (2.0 g/kg for 1 d). All patients received aspirin (30 mg/kg per day) during the acute stage of illness. The dose of aspirin decreased to 5 mg/kg per day after defervescence. Venous blood was drawn from each patient before treatment and at 36–48 h after treatment. Parental informed consent was obtained for each child enrolled in this study. The Ethical Committee of Kitasato University and Ebina General Hospital approved this study.

RNA isolation, cDNA preparation, and hybridization. Total RNA was isolated from 2.5 mL of whole blood using a PAXgene tube, according to the manufacturer's recommended protocol (Paxgene Blood RNA Kit, Preana-lytiX, QIAGEN, Valencia, CA). Next, α-globin and β-globin mRNA were removed from the total RNA using the globin reduction method according to the manufacturer's recommended protocol (Affymetrix, Santa Clara, CA). The synthesis of cRNA and hybridization were performed using the one-cycle target labeling and control reagents according to the manufacturer's recommended protocol (Affymetrix) and methods described in previous reports (17,18). Briefly, 2 μg samples of total RNA from which globin mRNA had been removed were individually converted to double-stranded cDNA (ds-cDNA) using the one-cycle cDNA synthesis kit (Affymetrix). The cDNA was then subjected to *in vitro* transcription in the presence of biotinylated uridine triphosphate, using an IVT labeling kit (Affymetrix). Qualitative analyses of the isolated total RNA and synthesized cRNA were conducted by electro-pherogram (Experion; Bio-Rad Laboratories, Hercules, CA). The biotinylated cRNA was fragmented and hybridized for 16 h at 45°C with the Human Genome U133 plus 2.0 array (Affymetrix), which contains the oligonucleotide probe set for 54,675 full-length transcripts and expressed sequence tags. The arrays were washed, stained with streptavidin-phycoerythrin, and scanned using an Affymetrix Model Fluidics Station 450 and GeneChip Scanner 3000 (Affymetrix). The fluorescence intensity of each probe was quantified using the computer program GeneChip operating software, GCOS version 1.4 (Affymetrix). Each microarray was subjected to a standard quality control evaluation; the percentage of probe sets reliably detecting (present flag) was between 40 and 60%, and the 3′/5′ratio of the *GAPDH* was <3. All background intensities and noise factors were within the range of 31.37 to 55.72 and 1.3 to 2.35, respectively. The results of the GeneChip analysis can be found on GEO (http://www.ncbi.nlm.nih.gov/geo).

Microarray data analysis. The expression analysis file created from each chip (sample) was imported into GeneSpring GX version 7.3 software (Agilent Technologies, Santa Clara, CA) for further data characterization. Each array was normalized (mean centered) to the median intensity array. To minimize the influence of background noise, only probes reliably detected in at least two out of all the samples were included in the analysis. A one-way ANOVA analysis and gene ontology analysis using Fisher's exact test and the unsupervised hierarchical clustering analysis using Pearson's correlation were performed using the GeneSpring GX version 7.3 software package.

Quantitative real-time PCR. cDNA was generated from the total RNA after exclusion globin mRNA samples using a QuantiTect Reverse Transcription System kit (QIAGEN) using random primers and Oligo-dT primers. The PCR primers for CD177 (polycythemia rubra vera-1; *prv-1*), haptoglobin (*hp*), carci-noembryonic antigen-related cell adhesion molecule 1 (*ceacam1*), suppressor of cytokine signaling 3 (*socs3*), and oncostatin-m (*osm*) were designed based on the GeneBank sequence, and the PCR primers were designed using the Primer 3 software program (http://primer3.sourceforge.net/). The primer sequences were as

follows: *prv-1* forward primer, 5′-GGCCCAACCTTCCAGCTT-3′; *prv-1* reverse primer, 5′-CTTCTCACGCGCAGAGAAG-3′; *hp* forward primer, 5′-CTGGAGGAGGACACCTGGTA-3′; *hp* reverse primer, 5′-TGGTCTTCT-GAACCCAGTCC-3′; *ceacam1* forward primer, 5′-ACCCACCTGCAC-AGTACTCC-3′; *ceacam1* reverse primer, 5′-TCGTCTTGACTGTGGTCCTG-3′; *socs3* forward primer, 5′-GCCACCTACTGAACCCTCCT-3′; *socs3* reverse primer, 5′-AACACCAGGGGGATCTTCTC-3′; *osm* forward primer, 5′-TACCATCGCTTCATGCACTC-3′; and *osm* reverse primer, 5′-GGAGGAGG-TAGAGGGGTCTG-3′. RT-PCR was conducted using the Chromo 4 System (Bio-Rad laboratories) in a 25-μL reaction mixture containing 12.5 μL of iQ SYBR Green SuperMix (Bio-Rad Laboratories), 1 μL of a mixture of forward and reverse primer (10 pmol/μL), and 11.5 μL of cDNA diluted in RNase-free H$_2$O. Samples were preincubated for 3 min at 95°C, and then were subjected to 40 cycles of amplification at 94°C for 15 s for denaturing and at 60°C for 30 s for annealing and 72°C for extension. The expression levels of each target cDNA relative to *GAPDH* were calculated using a comparative Ct method, and the levels were determined for each sample (19).

Statistical analysis. Analyses of clinical variables were conducted using the SPSS statistical software package, version 15 (SPSS Japan, Tokyo). Summary statistics (median, SD, and percentages) was calculated to describe the subject characteristics. For the data regarding characteristics of the study population, the laboratory and the RT-PCR data, Kruskal-Wallis test non-parametric ANOVA and Mann-Whitney U test were used to compare samples obtained from patients in each treatment group. Wilcoxon's signed rank test was used to compare the results before the initial treatment to those obtained after the initial treatment. Regarding the data of transcripts abundance, one-factor ANOVA used to compare each of the groups. The p values were corrected by use of the Bonferroni/Dunn F test as a post hoc test, if not otherwise indicated. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Characteristics and clinical outcome. Five patients in group A ($n = 6$) showed a response to IVIG treatment (83.3%). One patient in group B1 ($n = 6$) was responsive to IVIG treatment (16.7%), and all patients in group B2 ($n = 5$) responded to the IVIG-plus-IVMP combination therapy (100%; Fig. 1). Four patients exhibited transient coronary dilation in the acute phase of illness or 36 to 48 h after the initial treatment (group A: 1 patient; group B1: 3 patients). However, none of the patients developed CALs at 1 mo after onset of the disease. None of the patients experienced any side effects of IVMP therapy (*e.g.*, hypothermia, bradycardia, and hypertension). Tables 1–3 show the demographic and charac-teristics of the study population. The lymphocyte count and the aspartate aminotransferase and ALT levels were signifi-cantly different between groups A and B before the initial treatment. No significant differences between patient demo-graphics and characteristics were observed between groups B1 and B2 before initial treatment. However, significant differ-ences were observed posttreatment in the lymphocyte counts and ALT levels of group A *versus* groups B1 or B2 patients.

Profiles of transcript abundance in patients that stratified by the Egami score. Using an unsupervised hierarchical clus-tering approach, clustering analysis of transcript abundance pattern was conducted in the pretreatment samples from groups A, B1, and B2 patients (Fig. 2A). The clustering analysis used 7803 of 54,675 transcripts that showed signifi-cantly altered expression levels among the three groups (one-way ANOVA; $p < 0.05$). The transcript abundance pattern in the pretreatment samples from group B1 patients showed good similarity to that of group B2 patients ($r^2 = 0.59$). In contrast, no similarity between the transcript abundance pattern of groups A and B (group B included both groups B1 and B2) patients was observed ($r^2 = 0.15$).

Table 1. *Demographic data of the subjects before treatment*

	Group A	Group B1	Group B2	*p*
Sex (male, female)	m 3, f 3	m 4, f 2	m 3, f 2	
Age (mo)	31 ± 24.1	44.0 ± 23.9	26.0 ± 24.0	0.88
Illness days of initial treatment (d)	5.0 ± 0.9	3.5 ± 1.0	4.0 ± 1.1	0.11
WBC count (/mm^3)	14,450.0 ± 7233.2	11,000.0 ± 6343.6	12,000.0 ± 3589.6	0.58
Neutrophilic count (/mm^3)	9516.0 ± 6140.1	8202.6 ± 5933.9	8996.4 ± 1749.4	0.91
Lymphocyte count (/mm^3)	3664.0 ± 2285.6	1725.0 ± 362.0	671.5 ± 2408.9	0.07
Monocytic count (/mm^3)	609.8 ± 359.9	633.6 ± 402.9	552.0 ± 172.5	0.74
Hemoglobin (g/dL)	11.3 ± 1.0	11.5 ± 0.7	12.1 ± 1.0	0.59
Platelet count (×10^4/mm^3)	34.1 ± 11.9	27.9 ± 9.6	27.0 ± 10.6	0.45
AST (IU/L)	47.5 ± 17.0†‡	116.5 ± 324.5†	155.0 ± 108.5‡	0.02*
ALT (IU/L)	12.0 ± 12.3†‡	221.0 ± 122.2†	242.0 ± 92.8‡	0.01*
Albumin (g/dL)	3.7 ± 0.2	3.7 ± 0.4	3.9 ± 0.2	0.46
Sodium (mEq/L)	133.0 ± 2.1	131.0 ± 3.7	131.0 ± 3.7	0.38
CRP (mg/dL)	9.3 ± 7.0	9.4 ± 4.5	6.2 ± 5.0	0.33
ESR (mm/h)	83.0 ± 12.3	71.5 ± 13.1	68.0 ± 25.9	0.27
IgG (mg/dL)	598.0 ± 245.1	623.5 ± 202.5	773.0 ± 73.6	0.30

Group A: Egami score ≤2 point, group B1: ≥3 points; IVIG treatments group, group B2: ≥3 points; IVIG-plus-IVMP combined therapy group.
A value of *p* < 0.05 was considered to be statistically significant.
* *p* value calculation by Kruskal-Wallis test.
†‡ *p* value calculation by Bonferroni test.

Table 2. *Demographic data of the subjects after treatment*

	Group A	Group B1	Group B2	*p*
WBC count (/mm^3)	8100.0 ± 3042.1	11,900.0 ± 4837.1	9600.0 ± 2717.9	0.32
Neutrophic count (/mm^3)	3180.0 ± 2171.8	9302.3 ± 4896.1	3273.2 ± 1213.9	0.21
Lymphocyte count (/mm^3)	3720.0 ± 1128.0†	2044.8 ± 837.2†	4294.6 ± 2095.4	0.02*
Monocytic count (/mm^3)	569.4 ± 167.4	606.0 ± 268.6	548.8 ± 378.4	0.87
Hemoglobin (g/dL)	11.5 ± 1.6	11.0 ± 1.0	11.5 ± 0.76	0.28
Platelet count (×10^4/mm^3)	40.3 ± 15.0	34.3 ± 11.1	26.5 ± 14.3	0.52
AST (IU/L)	35.5 ± 16.8	30.5 ± 3.4	37.0 ± 14.7	0.32
ALT (IU/L)	12.5 ± 20.2†	48.5 ± 19.2	70.0 ± 38.7†	0.02*
Albumin (g/dL)	3.4 ± 0.3	2.9 ± 0.8	3.2 ± 0.22	0.29
Sodium (mEq/L)	136.0 ± 2.3	134.5 ± 4.6	136.0 ± 1.8	0.65
CRP (mg/dL)	2.9 ± 2.3	5.7 ± 5.0	1.4 ± 0.96	0.06
ESR (mm/h)	71.0 ± 20.6	70.5 ± 30.3	18.0 ± 73.0	0.96
IgG (mg/dL)	2466.0 ± 465.9	2715.5 ± 513.0	2714.0 ± 150.1	0.58

A value of *p* < 0.05 was considered to be statistically significant.
* *p* value calculation by Kruskal-Wallis test.
†‡ *p* value calculation by Bonferroni test.

We next examined to transcripts significantly differenced expression level between groups A and B patients, between IVIG-responder and IVIG-resistant patients, respectively. In pretreatment samples, 8917 of 54,675 transcripts showed significantly differenced expression levels between groups A (*n* = 6) and B (*n* = 11), as determined by a one-way ANOVA (*p* < 0.05). Moreover, 6316 of 8917 transcripts were not only groups A and B but also significantly differenced expression levels between IVIG-responsive patients (*n* = 6) and IVIG-resistant patients (*n* = 6; IVIG-resistant patient in group A: one patient, in group B1: five patients) based on a Venn diagram analysis. In a comparison of groups A and B using 6316 transcripts, 432 transcripts were more than double regulation and 179 transcripts were less than half regulation in group B. Table 4 shows the profile of the most up-regulated transcripts in group B.

Profiles of transcript abundance in single-IVIG and IVIG-plus-IVMP combined therapy. A hierarchical cluster analysis after treatments showed that the transcript abundance pattern of samples in group B2 had strong similarities to

samples from IVIG responders (r^2 = 0.66; Fig. 2*B*). The transcript abundance pattern after treatment of group B1 patients was not similar to that of group B2 patients (r^2 = 0.05).

Table 5 provides the transcripts after treatment were less abundant than those before treatment. Each group included transcripts for *PRV-1, IL18R1, SOCS3, OSM, complement component 3b/4b receptor1 (CR1), HP, IL1R2,* and *CEACAM1.* The transcripts of the group B2 patients were less abundant than those of the groups A and B1 patients.

In addition, the number of transcripts that were ≥2 SD down-regulated between pre- and posttreatments in each group were group A; 1906 ± 545.5 transcripts, group B1: 1085.7 ± 805.3 transcripts, and group B2: 2982.0 ± 471.5 transcripts. There were significantly more regulating transcripts in group B2 with a ≥2 SD than in either groups A and B1 (*p* < 0.001).

The characteristics of the down-regulated transcripts after treatment in groups B1 and B2. The distribution of those transcripts that ≥2 SD varied between pre- and posttreatment in groups B1, B2, and IVIG responders using a Venn diagram

Table 3. *Comparison of samples between before and after the initial treatment*

	Group A			Group B1			Group B2		
	Pre	Post	p	Pre	Post	p	Pre	Post	p
WBC count (/mm^3)	14,450.0 ± 7233.2	8100.0 ± 3042.1	0.03*	11,000.0 ± 6343.6	11,900.0 ± 4837.1	0.35	12,000.0 ± 3589.6	9600.0 ± 2717.9	0.04*
Neutrophil count (/mm^3)	9516.0 ± 6140.1	3180.0 ± 2171.8	0.03*	8202.6 ± 5933.9	9302.3 ± 4896.1	0.35	8996.4 ± 1749.4	3273.2 ± 1213.9	0.04*
Lymphocyte count (/mm^3)	3664.0 ± 2285.6	3720.0 ± 1128.0	0.92	1725.0 ± 362.0	2044.8 ± 837.2	0.35	671.5 ± 2408.9	4294.6 ± 2095.4	0.08
Monocyte count (/mm^3)	609.8 ± 359.9	569.4 ± 167.4	0.60	633.6 ± 402.9	606.0 ± 268.6	0.35	552.0 ± 172.5	548.8 ± 378.4	0.50
Hemoglobin (g/dL)	11.3 ± 1.0	11.5 ± 1.6	0.47	11.5 ± 0.7	11.0 ± 1.0	0.05	12.1 ± 1.0	11.5 ± 0.76	0.59
Platelet count (×10^4/mm^3)	34.1 ± 11.9	40.3 ± 15.0	0.05	27.9 ± 9.6	34.3 ± 11.1	0.60	27.0 ± 10.6	26.5 ± 14.3	0.23
AST (IU/L)	47.5 ± 17.0	35.5 ± 16.8	0.89	116.5 ± 324.5	30.5 ± 3.4	0.03*	155.0 ± 108.5	37.0 ± 14.7	0.04*
ALT (IU/L)	12.0 ± 12.3	12.5 ± 20.2	0.59	221.0 ± 122.2	48.5 ± 19.2	0.03*	242.0 ± 92.8	70.0 ± 38.7	0.04*
Albumin (g/ dL)	3.7 ± 0.2	3.4 ± 0.3	0.03*	3.7 ± 0.4	2.9 ± 0.8	0.03*	3.9 ± 0.2	3.2 ± 0.22	0.04*
Sodium (mEq/L)	133.0 ± 2.1	136.0 ± 2.3	0.02*	131.0 ± 3.7	134.5 ± 4.6	0.24	131.0 ± 3.7	136.0 ± 1.8	0.04*
CRP (mg/dL)	9.3 ± 7.0	2.9 ± 2.3	0.03*	9.4 ± 4.5	5.7 ± 5.0	0.25	6.2 ± 5.0	1.4 ± 0.96	0.04*
ESR (mm/h)	83.0 ± 12.3	71.0 ± 20.6	0.35	71.5 ± 13.1	70.5 ± 30.3	0.92	68.0 ± 25.9	18.0 ± 73.0	0.59
IgG (mg/dL)	598.0 ± 245.1	2466.0 ± 465.9	0.03*	623.5 ± 202.5	2715.5 ± 513.0	0.03*	773.0 ± 73.6	2714.0 ± 150.1	0.04*

A value of $p < 0.05$ was considered to be statistically significant.

* p value calculation by Wilcoxon signed rank test.
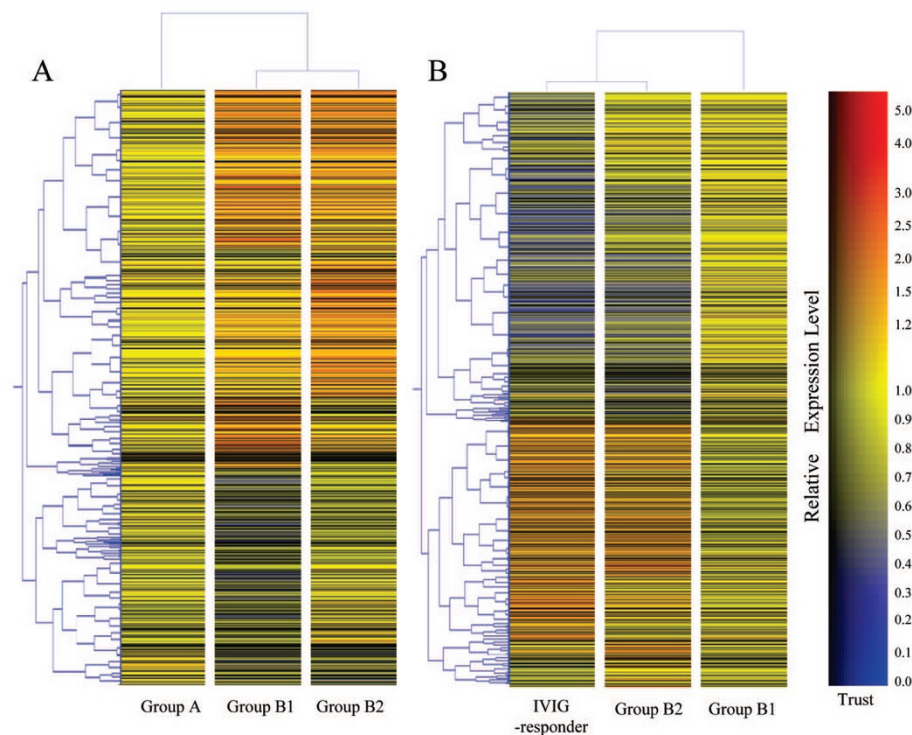
Figure 2. Unsupervised hierarchical clustergram analysis using Pearson's correlation. (*A*) An analysis of the pretreatment samples. (*B*) An analysis of the posttreatment samples. The transcript abundance pattern of group B2 was more like that of group B1 than group A in pretreatment samples (groups A *vs* B: $r^2 = 0.15$, groups B1 *vs*. B2: $r^2 = 0.56$). The transcript abundance pattern in posttreatment samples showed strong similarities between the IVIG-responder group and group B2 (IVIG responder group *vs* group B2: $r^2 = 0.66$, groups B1 *vs* B2; $r^2 = 0.05$).

is shown in Figure 3. In this distribution analysis, 707 transcripts were ≥2 SD less abundant in IVIG responders and group B2 patients, 945 transcripts in only group B2 patients, and 34 transcripts in only group B1 patients. The Gene Ontology browser was used to analyze the 707 transcripts that were less abundant in IVIG-responder group and group B2, and 945 transcripts that were less abundant in only group B2. Table 6 shows the categories of biologic processes for theses transcripts, respectively. The 707 transcripts had transcripts related to the positive regulation of the I-κB kinase/NF-κB cascade, immune response and cytokine production, neutrophil activation, TNFα synthesis, and regulation of IL1β in the category of immune response (Fisher's exact test; $p < 0.001$). The 945 transcripts had transcripts related to apoptosis, inositol phosphate dephosphorylation, and B-cell activation, natural killer cell differentiation and T-cell regulation in the category of immune response ($p < 0.001$). The 34 transcripts with ≥2 SD down-regulated in only group B1 patients included transcripts such as polycomb group ring finger-2 (*PCGF2*), platelet-derived growth factor alpha polypeptide

Table 4. *Up-regulated transcripts in the groups A patients before treatment*

Probe ID	Gene symbol	Gene name	Fold change group B/A
219975_x_at	OLAH	Oleoyl-ACP hydrolase	5.72
211372_s_at	IL1R2	Interleukin 1 receptor, type II	4.81
214637_at	OSM	Oncostatin M	4.15
205403_at	IL1R2	Interleukin 1 receptor, type II	4.11
224056_at	GNA13	Guanine nucleotide binding protein (G protein)	4.07
205110_s_at	FGF13	Fibroblast growth factor 13	4.00
209960_at	HGF	Hepatocyte growth factor (hepapoietin A; scatter factor)	3.93
206208_at	CA4	Carbonic anhydrase IV	3.89
205863_at	S100A12	S100 calcium binding protein A12	3.76
206697_s_at	HP	Haptoglobin	3.41
208470_s_at	HPR	Haptoglobin-related protein	3.24
210610_at	CEACAM1	carcinoembryonic antigen-related cell adhesion molecule 1	3.17
228824_s_at	LTB4DH	Leukotriene B4 12-hydroxydehydrogenase	3.06
206950_at	SCN9A	Sodium channel, voltage-gated, type IX, alpha subunit	3.05
215838_at	LILRA5	Leukocyte immunoglobulin-like receptor, subfamily B	3.01
217552_x_at	CR1	Complement component (3b/4b) receptor 1	2.98
206618_at	IL18R1	Interleukin 18 receptor 1	2.96
203936_s_at	MMP9	Matrix metallopeptidase 9	2.86
206359_at	SOCS3	Suppressor of cytokine signaling 3	2.73
219669_at	CD177	Polycythemia rubra vera 1 (PRV1)	2.72

Table 5. *The top 20 most down-regulated types of transcript abundance between pre- and post-treatment*

Probe ID	Gene symbol	Gene name	Fold change pre-/posttreatments		
			Group A	Group B1	Group B2
219669_at	CD177	Polycythemia rubra vera 1 (PRV1)	26.55	10.48	54.34
153829_at	MGC34824	Hypothetical protein MGC34824	16.13	6.90	32.67
216331_at	ITGA7	Integrin α 7	20.10	4.17	27.15
219975_x_at	OLAH	Oleoyl-ACP hydrolase	9.916	18.25	24.60
219295_s_at	PCOLCE2	Procollagen C-endopeptidase enhancer 2	0.52	5.58	20.98
205110_s_at	FGF13	Fibroblast growth factor 13	3.52	6.10	18.19
206618_at	IL18R1	Interleukin 18 receptor 1	6.87	5.69	18.16
206950_at	SCN9A	Sodium channel, voltage-gated, type IX, α subunit	3.02	3.62	17.75
206359_at	SOCS3	Suppressor of cytokine signaling 3	15.21	5.67	16.10
210997_at	HGF	Hepatocyte growth factor	6.60	8.43	14.18
214637_at	OSM	Oncostatin M	3.22	6.10	13.68
208470_s_at	HPR	Haptoglobin-related protein	5.44	8.94	13.62
210998_s_at	HGF	Hepatocyte growth factor	3.19	3.10	12.67
223767_at	GPR84	G protein-coupled receptor 84	7.14	7.92	12.44
217552_x_at	CR1	Complement component (3b/4b) receptor 1	8.49	4.47	12.38
206697_s_at	HP	Haptoglobin	4.45	7.99	11.84
207072_at	IL18RAP	Interleukin 18 receptor accessory protein	5.25	4.83	10.93
227697_at	SOCS3	Suppressor of cytokine signaling 3	8.42	4.05	10.63
211372_s_at	IL1R2	Interleukin 1 receptor, type II	7.60	8.51	9.93
209498_at	CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1	5.27	5.17	9.64

(*PDGFA*), adenosine A3 receptor (*ADORA3*), integrin α9 (*ITGA9*), integrin β2 (*ITGAB2*), and inositol 1. 4. 5-triphosphate 3-kinase C (*ITPKC*).

Agreement of quantitative real-time PCR and the results with microarray expression values for the same transcripts. mRNA levels were measured using quantitative RT-PCR in groups A ($n = 6$), B1 ($n = 6$), and B2 ($n = 5$) to confirm the microarray expression values. Five transcripts were selected that showed the largest difference in the microarray data (*PRV-1*, *HP*, *CEACAM1*, *SOCS3*, and *OSM*). The results indicated that the mRNA levels of those transcripts increased in group B in comparison with group A before treatment and most decreased in group B2 after treatment. The results for those transcripts showed markedly significant differences between groups A and B before treatment and between groups B1 and B2 after treatment (Fig. 4).

DISCUSSION

Elevated expression of mRNA related to the IVIG resistant and development CALs in pretreatments samples of group B patients. Similarities in the transcript abundance pattern of groups A, B1, and B2 were investigated before treatment using an unsupervised hierarchical clustering analysis. The results showed more strong similarities between the transcript abundance pattern of groups B1 and B2 than group A ($r^2 = 0.56$; Fig. 2A). Most of the significantly different transcripts between groups A and B also varied between the IVIG-responsive and IVIG-resistant patients (6316 transcripts). In addition, the more up-regulated transcripts before treatment in group B were related to the development CALs and *IVIG resistance* {*e.g.*, acute inflammatory proteins [*HP*, *S100A12*], adhesion molecules
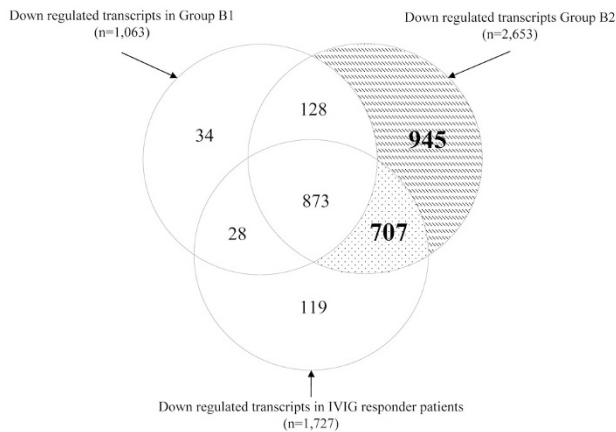
Figure 3. Distribution analysis for the down-regulated transcripts before and after treatment in groups B1 and B2, using a Venn diagram. In this distribution analysis using Venn diagram, there were 707 transcripts that were ≥2 SD down-regulated transcripts before and after treatments in IVIG-responders and group B2 patients, 945 transcripts in only Group B1, 34 transcripts in only group B1 patients (one-way ANOVA: $p < 0.05$, >2.0 × fold change).

Table 6. *Gene ontology classification of the down-regulated transcripts after treatment using GeneSpring GX 7.3*

	p
Categories of down-regulation transcripts in IVIG responsive and group B2 patients (707 transcripts)	
Positive regulation of I-κB kinase/NF-κB cascade	<0.001
Immune response	<0.001
Regulation of I-κB kinase/NF-κB cascade	<0.001
Response to pest, pathogen or parasite	<0.001
Apoptosis	<0.001
Cauterization in immune response	
Positive regulation of cytokine production	<0.001
Neutrophil activation	<0.001
Positive regulation of TNFα biosynthesis	<0.001
Regulation of cytokine production	0.0012
Positive regulation of IL1β secretion	0.0012
Categories of down-regulation transcripts in only group B2 patients (945 transcripts)	
Apoptosis	<0.001
Inositol phosphate dephosphorylation	<0.001
Ubiquiti cycle	<0.001
Protein amino acid dephosphorylation	<0.001
Glutathione metabolism	<0.001
Cauterization in immune response	
B-cell activation	<0.001
B-cell differentiation	0.0017
Natural killer cell differentiation	0.0029
Positive regulation of activated T-cell proliferation	0.028
Cytotoxic T-cell degranulation	0.044

p value calculation by Fisher's exact test.

[*CEACAM1*, matrix metallopeptidase 9 (*MMP9*)], and cell surface molecules [*IL1R2, IL18R, PRV-1, CR1*]} (20–25). Therefore, patient stratification according to the Egami score may indeed have reflected the potential responsiveness to IVIG treatment.

The transcripts after the IVIG-plus-IVMP combined therapy were less abundant in the leukocytes than those after the single-IVIG treatment. Although the hierarchical cluster analysis before treatments showed that the transcript abundance pattern of sample from group B1 patients was similar to that
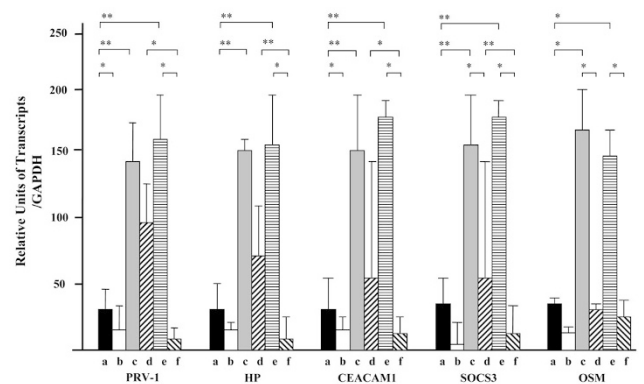
Figure 4. mRNA expression levels in patients before and after treatment in groups A, B1, and B2. The results of quantitative RT-PCR are presented as relative units of each transcript in comparison with GAPDH. a. Group A: pre-IVIG, b. group A: post-IVIG, c. group B1: pre-IVIG, d. group B1: post-IVIG, e. group B2: pre-IVIG + IVMP, f. group B2: post-IVIG + IVMP. Error bars indicate SEM. mRNA levels of those transcripts increased in group B in comparison with group A before treatment, and most decreased in group B2 after treatments. *$p < 0.05$, **$p < 0.005$ for comparison between pre- and posttreatment results (Wilcoxon signed-rank test) and among groups A, B1, and B2 (Mann-Whitney *U* test).

of samples from group B2 patients ($r^2 = 0.59$). The transcripts abundance pattern after treatments showed strong similarities between IVIG responders ($n = 6$) and group B2 patients ($r^2 = 0.66$; Fig. 2*B*). This result suggests that administering of combination IVIG-plus-IVMP therapy greatly altered the transcript abundance in the leukocytes of the group B patients. Moreover, group B2 suppressed the largest number of transcripts between the pre- and posttreatment in the three groups (Fig. 3). In our preset study, we did not examine the effects of altering the transcripts after IVMP therapy for the group A patients.

Abe *et al.* (24) reported the results of a microarray study in which the *PRV-1* and HP seemed to be good biomarker for predicting a response to IVIG among patients with KD; these transcripts were correlated with the IVIG response, and higher levels of transcripts of these transcripts were observed in IVIG-resistant patients than in IVIG-responsive patients. A microarray study by Popper *et al.* (23) showed that the *CEACAM-1* is up-regulated in IVIG-resistant patients. In this study, although transcripts such as *IL1R2, OSM, HP, CR1, IL18R1, SOCS3* and *PRV-1*, and *CEACAM1* were more abundant in group B before treatment, those transcript abundances were suppressed after treatment in each of the groups, and group B2 showed greater down-regulation of those transcripts than groups A and B1 (Tables 4 and 5). The quantitative RT-PCR and the results of the microarray expression values for *PRV-1, HP, CEACAM1, SOCS3,* and *OSM* were in agreement (Fig. 4). These results suggested that the IVIG-plus-IVMP combined therapy more profoundly and broadly down-regulated the transcripts in the leukocytes than did the single-IVIG treatment.

IVIG-plus-IVMP combination therapy suppressed a wide range of inflammatory cell types in comparison to single IVIG. The Gene Ontology browser analysis showed that the 707 transcripts with down-regulation of ≥2 SD in group B2 patients and IVIG responders had functions related to the

NF-κB cascade, neutrophil activation, TNFα synthesis, and regulation of IL1β. There were 945 transcripts with down-regulated of $\geq$2 SD in only group B2 patients had functions related to the apoptosis and to the regulation of B cells, T cells, and NK cells (Fig. 3, Table 6). Makata *et al.* (26) reported that high-dose IVIG inhibits the activation of mono-cytes/macrophages and coronary arterial endothelial cells more strongly than that of T cells, whereas dexamethasone inhibits the activation of all three cell types such as mono-cytes/macrophages, T cells, and endothelial cells. Although endothelial cells were not analyzed in this study, IVIG-plus-IVMP combination therapy also inhibited transcripts associ-ated with a wide range of inflammatory cell types (Fig. 3, Table 6). In addition, it is possible that IVIG-plus-IVMP combined therapy down-regulated the transcript abundance related to neutrophil activation, TNFα synthesis, and regula-tion of IL1β (Table 6). The 34 transcripts with down-regulation of $\geq$2 SD in only group B1 included *PDGFA* as platelet-derived growth factor, *ITGA9*, *ITGB2* as adhesion molecule, and *PCGF2*, *ITPKC* as a negative regulator of immune cell activation (27,28). The down-regulation of those transcripts may therefore be related to IVIG-resistant patients. However, there is insufficient evidence for that possibility based on the findings of this study.

This study examined the biologic mechanism of action of IVIG-plus-IVMP combined therapy and single-IVIG using a microarray analysis of samples from patients who had been predicted, according to their Egami score, to be IVIG resistant. The transcripts related to IVIG resistance and to the development of CALs were more abundant in the group B patients in comparison with those in the group A patients. Moreover, those transcripts in the IVIG-plus-IVMP com-bined therapy group were more profoundly and broadly suppressed in leukocytes than in the single-IVIG treatment group.

Study limitation. This study, used whole blood samples because it was not possible to extract mRNA from neutrophils. Therefore, any observed differences in transcript abundance before and after treatment may have been influenced by varia-tions between the leukocytes. This study investigated mRNA expression level in leukocytes, but not in endothelial cells, which therefore limits comparisons with previous studies.

The Egami score is highly sensitive and specific in Japanese patients with KD (8). Therefore, it remains unclear whether the Egami score would be equally useful for other populations. Moreover, in part due to the small number of subjects in this study, whether remains uncertain if the development of CALs is a reliable clinical endpoint.

In conclusion, IVIG-plus-IVMP combined therapy was administered as a primary treatment in Japanese patients predicted to be IVIG resistant in a prospective study and investigated the biologic mechanism of IVIG-plus-IVMP combined therapy and single-IVIG treatment for predicting IVIG-resistant patients using a microarray analysis. Pre-treatment samples from those patients with an Egami score that predicted IVIG resistance showed an up-regulation of transcripts related to vasculitis and to the development of CALs. Furthermore, the IVIG-plus-IVMP combined ther-

apy more profoundly and broadly suppressed the transcript abundance than did the single-IVIG treatment. This study pave the way for a larger clinical trial to test the hypothesis that addition of IVMP to standard therapy may benefit patients predicted to be IVIG-resistant based on the Egami score.

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