CXCL10/IP-10 is a Biomarker and Mediator for Kawasaki Disease

Tai-Ming Ko1, Ho-Chang Kuo2,3, Jeng-Sheng Chang4,5, Shih-Ping Chen1, Yi-Min Liu1, Hui-Wen Chen1, Fuu-Jen Tsai6,7,8, Yi-Ching Lee9, Chien-Hsiun Chen1,6, Jer-Yuarn Wu1,6, Yuan-Tsong Chen1,10

1Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan; 2Department of Pediatrics and Kawasaki Disease Center, Kaohsiung Chang Gung Memorial Hospital, Taiwan; 3Chang Gung University College of Medicine, Taiwan; 4Department of Pediatric Cardiology, Children’s Hospital of China Medical University, Taichung, Taiwan; 5School of Medicine, China Medical University, Taichung, Taiwan; 6School of Chinese Medicine, China Medical University, Taichung, Taiwan; 7Department of Medical Genetics, China Medical University Hospital, Taichung, Taiwan; 8Department of Health and Nutrition Biotechnology, Asia University, Taichung, Taiwan; 9Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan, and; 10Department of Pediatrics, Duke University Medical Center, Durham, North Carolina, USA.

T-M. K., H-C. K., and J-S. C. contributed equally to this work.

Running title: IP-10 is a Biomarker and Mediator for Kawasaki Disease

Address correspondence to:

Dr. Jer-Yuarn Wu
Institute of Biomedical Sciences
Academia Sinica, 128
Academia Road, Section 2
Nankang
Taipei 11529
Taiwan
Phone: +886-2-27899075
jywui@ibms.sinica.edu.tw

Dr. Yuan-Tsong Chen
Institute of Biomedical Sciences
Academia Sinica, 128
Academia Road, Section 2
Nankang
Taipei 11529
Taiwan
Phone: +886-2-27899081
Fax: +886-2-27899085
chen0010@ibms.sinica.edu.tw

In December 2014, the average time from submission to first decision for all original research papers submitted to Circulation Research was 14.47 days.
**ABSTRACT**

**Rationale:** Kawasaki disease (KD), an acute febrile vasculitis, is the most common cause of acquired heart disease in childhood; however, diagnosing KD can be difficult.

**Objective:** To identify unique proteomic biomarkers that can be used to facilitate earlier diagnosis of KD.

**Methods and Results:** We enrolled 214 children with fever and clinical features suggestive of KD. Of those, only 100 were diagnosed with KD. Their plasma samples were globally analyzed for cytokines, chemokines, and cell adhesion molecules using an unbiased, large-scale, quantitative protein array. This study was conducted in 3 stages: discovery, replication, and blinded validation. During the discovery phase [n(KD)=37, n(control)=20], the expression of interleukin-17F, sCD40L, E-selectin, CCL23(MPIF-1), and CXCL10(IP-10) were upregulated during the acute phase in KD patients compared to that in the controls. A notable increase was observed in the IP-10 levels (KD, 3,037±226.7 pg/mL; control, 672±130.4 pg/mL; \( p =4.1 \times 10^{-11} \)). Receiver-operating characteristic analysis of the combined discovery and replication data [n(KD)=77, n(control)=77] showed that the IP-10 level had high area under the curve values (0.94 [95% confidence interval, 0.9055–0.9778]; sensitivity, 100%; and specificity, 77%). With 1,318 pg/mL as the optimal cut-off, the blinded validation study confirmed that the IP-10 levels were a good predictor of KD. With intravenous immunoglobulin treatment, the IP-10 levels returned to normal. The downstream receptor of IP-10, CXCR3, was activated in the T cells of acute KD patients.

**Conclusions:** IP-10 may be used as a biomarker to facilitate KD diagnosis, and it may provide clues about the pathogenesis of KD.

**Keywords:** Kawasaki disease, biomarker, diagnosis, vasculitis, coronary artery abnormality, IP-10.

**Nonstandard Abbreviations and Acronyms:**
- AUC: area under the curve
- CAAs: coronary artery abnormalities
- CAMs: cell adhesion molecules
- iKD: incomplete presentation of KD
- IP-10: IFN-gamma-inducible protein 10
- KD: Kawasaki disease
- MIG: monokine induced by human IFN-gamma
- MPIF-1: Myeloid progenitor inhibitory factor 1
- PBMCs: Peripheral blood mononuclear cells
- ROC: Receiver-operating characteristic
INTRODUCTION

Kawasaki disease (KD), a multisystem inflammatory condition observed in younger children, can cause acute vasculitis, most notably affecting the coronary arteries. Without treatment, approximately 20–25% of children with KD develop coronary artery abnormalities (CAAs)\(^1\). Intravenous immunoglobulin (IVIG) treatment can reduce the incidence of CAAs to approximately 5%, but early detection is necessary\(^2\)-\(^4\). KD diagnosis is difficult, especially at the early stage. Currently, KD diagnosis is based on clinical symptoms, including fever for ≥5 days, bilateral conjunctival injection without exudate, polymorphous exanthema, changes in the lips and mouth (erythema and cracking of lips, strawberry tongue, and diffuse injection of oral and pharyngeal mucosae), changes in the extremities (erythema and edema of the hands and feet), and cervical lymphadenopathy (≥1.5 cm in diameter)\(^5\),\(^6\). However, overlapping clinical features and laboratory parameters between KD and other conditions make definitive diagnosis difficult, and no specific laboratory tests are available. Therefore, identification of specific biomarkers to facilitate KD diagnosis by laboratory analysis would be valuable for preventing serious KD sequelae, especially CAAs\(^7\).

Previous studies have suggested that KD is an immune-mediated disease. This notion is also supported by our recent findings\(^8\)-\(^11\) and those of a Japanese study\(^12\) on immune-related genes (i.e., FCGR2A, IGHV, BLK, and CD40), which were identified to be responsible for KD susceptibility. Further, examination of the gene expression profile in the peripheral blood mononuclear cells (PBMCs) and urine proteomics supported the hypothesis that cytokine regulators and inflammatory molecules may be associated with KD pathogenesis\(^13\)-\(^17\). Several studies have identified cytokines and immunoregulatory molecules associated with KD. The average level of serum interleukin (IL)-6 was found to be elevated in acute KD patients\(^18\),\(^19\), the Th-1/Th-2 cytokine profile was associated with CAAs in KD patients\(^5\),\(^20\), and the genetic polymorphism in IL-18 has been shown to increase the KD risk\(^21\). Further, the increase in the meprin A and filamin C levels in the urine of acute-phase KD patients may be potential markers that could aid in KD diagnosis\(^13\). Although numerous studies have shown that the expression of multiple genes can be upregulated in acute KD, the differentiation of KD patients from those with non-KD-related fever in a simple and reliable way remains a challenge. The difficulty in KD diagnosis could be related to the shared inflammatory pathways between a KD and non-KD fever. Nonetheless, there may be unidentified plasma proteins that can act as sufficiently sensitive and specific diagnostic biomarkers for clinical use in diagnosing KD among febrile subjects.

To identify KD-specific effector molecules, we globally analyzed the profiles of cytokines, chemokines, and cell adhesion molecules (CAMs) in KD patients. This study aimed to identify a protein biomarker that can be used to facilitate the early diagnosis of KD.

METHODS

Ethical statement.

The study was approved by the Institutional Review Board and the Ethics Committee of the Institution Review Board of the China Medical University Hospital, Kaohsiung Chang Gung Memorial Hospital, and Academia Sinica in Taiwan. Written informed consent was obtained from the subjects or their parents.

Patients.

We enrolled 214 Han Chinese children with a fever and clinical features suggestive of KD. Of those, only 100 were eventually diagnosed with KD. The demographic and clinical characteristics of these children are shown in Table 1, and final diagnoses of the 114 children with non-KD are shown in Online Table 1.
The children participating in the study were recruited in Taiwan from medical centers in different geographical areas—the Chang Gung Memorial Hospital Systems including 4 hospitals in the southern and northern part of Taiwan and the China Medical University Hospital Medical Center, including three regional hospitals in the central part of Taiwan. KD was diagnosed using known clinical diagnostic criteria22, 23. Of the 100 KD patients, 37 were included in the study’s discovery phase, 40 in the replication phase, and 23 in the blinded validation phase, which included 3 patients with incomplete presentation of KD (iKD was defined as the presence of ≤4 principal symptoms of the Japanese criteria)6.

*Multiplex analysis and quantification of cytokines, chemokines, and cell adhesion molecules.*

Fresh heparinized blood samples that were obtained from the study subjects were centrifuged at 2,000 g for 10 min. Then the plasma samples were aliquot and were stored at -80°C for further analysis. Samples were run in duplicate using the Bio-Plex Pro™ Human Th-17 Cytokine Panel 15-Plex (Bio-Rad, Hercules, CA, USA). The complete list of cytokines (IL-1β, IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IFN-γ, sCD40L, and tumor necrosis factor [TNF]-α) was quantified in these cohorts, and their detection limits and reproducibility were provided in the product manual. Fifteen distinct sets of fluorescently dyed beads loaded with capture monoclonal antibodies specific for each cytokine were used. The signal was measured and quantified using the Bio-Plex Protein Array System (Bio-Rad). Assays were performed using Bio-Plex Protein Array System integrated with Bio-Plex Manager Software, version 3.0 (Bio-Rad). Reporter conjugate emission wavelengths were adjusted using the Bio-Plex Calibration Kit (Bio-Rad). Fluidics performance, consistent optical alignment, doublet discrimination, and identification of individual bead signatures were validated using the Bio-Plex Validation Kit, version 3.0 (Bio-Rad). For the initial screening, plasma from 6 KD patients was examined using human protein array (AAH-CYT-G8-8; Raybiotech Inc., Norcross, GA, USA), which assesses 54 chemokines and CAMs to identify proteins showing an upregulated expression in KD. The complete chemokine/CAM names are available at http://www.raybiotech.com. The identified upregulated genes, namely, IL-9, IP-10, E-selectin, and MPIF-1, were further quantified in the remaining KD patients by using enzyme-linked immunosorbent assay (ELISA). The limits of detection for the E-selectin, MPIF-1, and IP-10 ELISA were 30 pg/mL, 7 pg/mL, and 8 pg/mL, respectively. The reproducibility (intra-assay: CV <10%; inter-assay: CV <12%) and specificity of IP-10 were validated; this ELISA kit shows no cross-reactivity with any of the cytokines tested. Dilution ranged from 1:2–1:20 according to the manufacturer’s instructions (RayBiotech Inc.).

*Flow cytometry.*

The peripheral blood mononuclear cells were isolated from the heparinized blood by Ficoll-Isopaque density gradient separation (Pharmacia Fine Chemicals, Uppsala, Sweden). Immunophenotypic analyses were performed using distinct fluorochrome-conjugated monoclonal antibodies that recognize human CD3 (UCHT1; BD Biosciences, San Jose, CA, USA) or CXCR3 (1C6/CXCR3; BD Biosciences). After the PBMCs cells were incubated with dilute antibody (1:200) for 1 h at room temperature, they were examined by multicolor flow cytometry using a FACS Calibur device (BD Biosciences). Data were obtained using CellQuest acquisition software (BD Biosciences), and 0.5–2.0 × 10⁶ events were recorded for analysis in each experiment.

*Statistical analysis.*

Statistical significance was assessed using unpaired Student’s t-test and the Prism4 software (GraphPad, San Diego, CA, USA). Receiver-operating characteristic (ROC) curve analysis was performed using SAS software, version 9.3 (SAS Institute Inc., Cary, NC, USA). The ROC curve plots sensitivity and 1–specificity and provides a summary of sensitivity and specificity across a range of cut-off points for a continuous predictor. Between-group differences were determined using analysis of variance and logical regression analysis. The optimal cut-off value of each candidate biomarker was determined as the sum of its maximum sensitivity and specificity.
RESULTS

Plasma profile: The discovery study.

Using the cytokine multiplex system and protein array, 69 inflammatory cytokines were analyzed in total. In the initial screening, the plasma levels of 15 cytokines in 20 non-KD febrile controls and 37 KD patients were determined. The levels of IL-17F and sCD40L were significantly higher in the KD patients than in the febrile controls (Figure 1A–O). Only one cytokine, IL-33, was found to be downregulated (Figure 1).

For the remaining 54 inflammatory chemokines and CAMs, a proteomics approach was used to identify candidate biomarkers in a set of plasma samples obtained during the acute phase in 6 KD patients randomly selected from the discovery phase. These data were compared to those of the controls with a non-KD fever and skin rash. The average expression levels of 10 cytokines or CAMs were at least 1.3-fold higher in the KD patients than in the controls (Online Table 2). Among these 10 proteins, IL-9, IP-10, E-selectin, and MPIF-1 showed an increase in the average expression of at least 2-fold in KD patients, and this result was found in all 6 patients tested. Further, the PDGF-AA, IL-2R-α, CD14, IGF-II, and Siglec-5 genes were downregulated in the acute-phase KD patients, showing at least a 1.8-fold decrease (<60%, data not shown) compared to the controls. ELISA was then conducted with a larger sample size (20 non-KD febrile controls and 37 KD patients) to quantify candidate biomarkers (IL-9, IP-10, E-selectin, and MPIF-1). Consistent with the protein array data for the acute-phase KD patients, there were significant increases in the IP-10, MPIF-1, and E-selectin levels (Figure 1P–R). However, the increase in the IL-9 levels became insignificant when the sample size increased (data not shown). Among the 6 candidate KD biomarkers (IL-17F, IL-33, sCD40L, E-selectin, MPIF-1, and IP-10), IP-10 showed the most significant increase in KD patients (3,037 ± 226.7 pg/mL) compared to the controls (672 ± 130.4 pg/mL) (values in KD patients vs. values in non-KD febrile controls, $p$ value = $4.1 \times 10^{-11}$) (Figure 1R).

IP-10 levels: The replication study and combined studies.

To further validate the role of IP-10, a replication study involving an additional 40 KD patients and 57 non-KD febrile controls was performed. As shown in Figure 2A, this study also showed a significant increase in the IP-10 levels in KD patients compared to those in the febrile controls. When the data from the replication study were combined with those of the discovery study (combined studies), the IP-10 level was significantly elevated in 77 KD patients (3,587 ± 210.2 pg/mL) compared to the 77 non-KD febrile controls (921 ± 106.2 pg/mL) (values in KD patients vs. values in non-KD febrile controls, $p$ value = $2.8 \times 10^{-20}$) (Figure 2B).

To further confirm the role of IP-10 as a biomarker in KD diagnosis, ROC curve analyses were performed using values of IP-10 from the combined studies. IP-10 showed an extremely high area under the curve (AUC) values of 0.94 (95% confidence interval, 0.9055–0.9778) (Figure 2C) when non-KD febrile patients were used as the controls. With a plasma IP-10 level of 1,318 pg/mL as the optimal cut-off value, as defined by the sum of maximum sensitivity and specificity, IP-10 showed a high sensitivity (100%) and specificity (77%) compared to the non-KD febrile controls (Figure 2C).

Blinded validation study.

The final study phase was conducted using plasma samples from 60 children who were suspected with KD. The plasma IP-10 levels were measured in samples labeled in a blinded fashion, and the results were un-blinded and analyzed. Using a cut-off value of 1,318 pg/mL, 29 samples were IP-10 positive and 31 were IP-10 negative. KD was successfully diagnosed in 22 of the 29 IP-10 positive samples (including 2 cases...
of iKD); the remaining 7 samples were diagnosed with a non-KD fever (Figure 3). Of the 31 IP-10 negative samples, 30 were from non-KD febrile controls and 1 was from an iKD patient. Overall, the IP-10 cut-off value of 1,318 pg/mL showed good ability to distinguish between 23 KD patients and 37 non-KD febrile controls (sensitivity, 96% [22/23]; specificity, 81% [30/37]).

**Association of plasma IP-10 levels with fever duration and intravenous immunoglobulin treatment.**

To determine whether increased IP-10 levels could be detected during the early stage of KD, 37 KD samples obtained within 4 days of onset of fever (mean, 3.4 ± 0.90 days; range, 1–4 days) were examined, and the results were compared with those of 46 samples obtained at a later stage of the disease (mean, 6.0 ± 1.05 days of the onset of fever; range, 5–8 days). IP-10 levels were increased significantly in the early disease stage (3,054 ± 331.0 pg/mL) (Figure 4A). Using 1,318 pg/mL as the optimal cut-off value, 81% (30) of the 37 KD patients were identified as being in the very early stage (<4 days), while 96% (44) of the 46 KD patients were in the acute stage (>5 days).

IP-10 levels were also examined in 45 patients before and 1 wk after the initiation of IVIG treatment. High IP-10 levels before treatment returned to normal with IVIG treatment (before treatment, 3,323 ± 224.9 pg/mL; after treatment, 348 ± 64.8 pg/mL) (Figure 4B), except in 1 KD patient who was resistant to the first round of IVIG treatment and required a second course of therapy.

**Cell surface chemokine receptor cxcr3 in t cells.**

IP-10 downregulates the cell surface chemokine receptor CXCR3 in T cells. To determine the downstream effect of increased IP-10 levels in KD patients, the cell surface expression of CXCR3 in T cells of 6 KD patients was analyzed. The mean fluorescence intensity (MFI) of CD3+ T cells was measured, and there was a 3.3-fold decrease in MFIs in acute-stage KD patients compared with the MFIs of 3 healthy donors (Figure 5). In the recovery stage, the expression levels of CXCR3 were restored to normal (data not shown).

**DISCUSSION**

Prompt diagnosis and IVIG treatment of KD is important, because delays can increase the incidence of CAAs and other devastating cardiac complications. Several previous studies reported increased levels of certain cytokines or cytokine regulators in association with KD, and a haptoglobin phenotype that may help in diagnosis at a late stage has been identified. Although we confirmed the elevation of IL-6 levels compared to healthy controls, this marker was not as specific or sensitive of a predictor of KD compared to IP-10 due to lack of discrimination in the suspected cases with fevers (Figure 1). While IL-1β was critical in the development of coronary lesions in a mouse model of KD, the plasma levels of IL-1β were not significantly elevated in the acute KD patients in the present study (Figure 1). Increase meprin A and filamin C levels in the urine of acute-phase KD patients might be helpful for KD diagnosis by using urine samples, however their use in the blood is not clear. In the present study, by using protein array, we found that a novel KD-specific potential biomarker IP-10 had the strongest association with KD among 69 key immune-associated molecules studied. With 1,318 pg/mL as the optimal cut-off, IP-10 was identified as an excellent biomarker for differentiating KD cases from non-KD cases including subjects who were highly suspected of KD, such as febrile cases with scarlet fever (Online Table 1). Compared to the previously reported biomarkers in the blood, IP-10 appears to be the most significant biomarker that can be used as a predictor for KD diagnosis.
Elevated chemokines levels, including IP-10, have been reported in KD compared to patients with Henoch-Schonlein purpura. Elevated IP-10 levels have also been observed in other inflammatory diseases, such as infectious diseases and some autoimmune disorders. In addition, IP-10 has been recognized as a potential biomarker for predicting the severity of some diseases, such as hepatitis C virus and rhinovirus infections. However, the levels of IP-10 in these conditions are relatively lower (<500 pg/mL) than those observed in the acute stage of KD in the present study (3,587 ± 210.2 pg/mL). Further, these diseases can easily be clinically differentiated from KD.

IP-10 is secreted by several cell types, including monocytes, endothelial cells, and fibroblasts, and can be induced by Th-17-associated cytokines. It shows potent lymphocyte chemotactic activity and binds to a common receptor, CXCR3, whose expression is upregulated on tissue-infiltrating T cells. We found that CXCR3 was downregulated in T cells during the acute stage of KD. The observation that CXCR3 needs IFN-γ for expression and is localized to sites of inflammation indicates that the IP-10-CXCR3 axis may play an important role in effector lymphocyte recruitment to inflammatory tissue. Previous studies using CXCR3-deficient or IP-10-deficient mice found reduced levels of tissue-infiltrated T cells in several disease models, including inflammation and transplantation. Additionally, in vitro studies have shown that CXCR3 ligands can promote the adhesion of lymphoblasts to human endothelial cells. IL-6–triggered STAT3 phosphorylation is an important upstream signal for IP-10 production by macrophages. Therefore, IP-10 further amplifies autocrine IL-6 production by activating B cells to sustain STAT3 signals, which may explain the high levels of IL-6 observed in the plasma samples of acute-phase KD patients. STAT3 phosphorylation is an indispensable downstream signaling event for the differentiation of macrophages and B cells to immunoglobulin A-secreting plasma cells, which may infiltrate the inflamed tissues of KD patients. Moreover, the increase in plasma levels of CXCL9 (MIG), another ligand of CXCR3, in KD patients supports the notion that activation of the CXCR3 pathway may be important for the development of KD.

In summary, the present study found a significant elevation in the plasma IP-10 levels in acute-stage KD patients. A limitation of this study was that all the enrolled subjects were Han Chinese; therefore, we were unable to determine whether the optimal IP-10 cut-off value (1,318 pg/mL) was applicable to other races. Since IP-10 functions as a chemoattractant, this increase provides a critical indicator for further investigating the pathogenesis of KD. Because of the high sensitivity and specificity of IP-10 as a potential biomarker for KD, this molecule may be useful for diagnosing KD and monitoring patients’ treatment responses.

**SOURCES OF FUNDING**
This study was supported by the Academia Sinica Genomic Medicine Multicenter Study (40-05-GMM), National Health Research Institute grant (NHRI-EX103-10341SI), National Science Council Research grant (NSC 102-2314-B-182-053-MY3), and Translational Resource Center for Genomic Medicine (TRC) (MOST103-2325-B-001-017) of National Research Program for Biopharmaceuticals (NRPB), Taiwan. The funders had no role in study design, data collection or analysis, the decision to publish or preparation of the manuscript.

**DISCLOSURES**
None
ACKNOWLEDGMENTS
We thank all affected individuals and their families who devoted their time and effort to participate in this study. We thank doctors in China Medical University Hospital in Taichung and Chang Gung Memorial Hospital in Kaohsiung, Taiwan for their contributions in recruiting KD patients. We gratefully acknowledge the members of Translational Resource Center for Genomic Medicine (TRC) (MOST103-2325-B-001-017) of National Research Program for Biopharmaceuticals (NRPB) and the National Center for Genome Medicine (NCGM) (MOST103-2319-B-001-001) of National Core Facility Program for Biotechnology (NCFPB), Ministry of Science and Technology, at Academia Sinica for their support in subject recruitment and data analysis.

AUTHOR CONTRIBUTIONS
Conceived and designed the experiments: TMK, JYW, YTC. Performed the experiments: TMK, SPC. Analyzed the data: TMK, SPC, HWC, CHC. Wrote the first draft of the manuscript: TMK, YTC. Contributed to the writing of the manuscript: TMK, JSC, JYW, YTC. Agree with manuscript results and conclusions: TMK, HCK, JSC, SPC, YML, HWC, FJT, YCL, CHC, JYW, YTC. Contributed reagents/materials/analysis tools: YML, YCL, CHC, JYW. Enrolled patients: HCK, JSC, FJT.

REFERENCES

DOI: 10.1161/CIRCRESAHA.116.305834


DOI: 10.1161/CIRCRESAHA.116.305834


18. Chow YM, Lin CY, Hwang B. Serum and urinary interleukin-6 (IL-6) levels as predicting factors of kawasaki disease activity. Zhonghua Mingguo xiao er ke yi xue hui za zhi [Journal]. Zhonghua Mingguo xiao er ke yi xue hui. 1993;34:77-83


DOI: 10.1161/CIRCRESAHA.116.305834


FIGURE LEGENDS

**Figure 1.** Plasma Cytokine, Chemokine, and Cell Adhesion Molecule Levels during the Acute Phase of Kawasaki Disease (KD). A–O) Plasma cytokine levels are measured in non-KD febrile controls (n = 20) and KD patients (n = 37) using the Bio-Plex system. P–R) The levels of plasma E-selectin, MPIF-1, and IP-10 identified from the protein array are determined by enzyme-linked immunosorbent assay in the febrile controls (n = 20) and KD cases (n = 37). The p values of IL-17F, IL-33, sCD40L, E-selectin, MPIF-1, and IP-10 were 1.5 × 10^{-2}, 4.7 × 10^{-3}, 2.8 × 10^{-2}, 8.6 × 10^{-3}, 2.3 × 10^{-8}, and 4.1 × 10^{-11}, respectively. Each dot represents the average of 3 analyses with variation <5% standard deviation from a single individual. *, p < 0.05; **, p < 0.01, ***, p < 0.001; unpaired Student’s t-test.

**Figure 2.** Receiver Operating Characteristic (ROC) Curve for the Predictive Model of Kawasaki Disease with Plasma Levels of IP-10. A) Plasma IP-10 levels are determined in the febrile controls (n = 57) and KD patients (n = 40) by using enzyme-linked immunosorbent assay (ELISA). B) Those in the febrile controls (n = 77) and KD cases (n = 77) are also measured using ELISA. The combined data of the febrile controls and KD patients from the discovery and replication studies are shown. ***, p < 0.001, unpaired Student’s t-test. Each dot represents the average of 3 determinations with variation <5% (standard deviation/average) from a single individual. On the basis of the combination data, ROC curves of plasma IP-10 levels in KD patients are plotted against the febrile controls C) The area under the curve. The optimal cut-off value of the biomarker (C) was determined as the sum of its maximum sensitivity and specificity.

**Figure 3.** Plasma Levels of IP-10 in the Blinded Validation Study. A) Plasma IP-10 levels in the validation study (patients were <6 years old), which included febrile controls (C–F, n = 37), incomplete KD patients (K–I, n = 3), and KD patients (K, n = 20), are determined using enzyme-linked immunosorbent assay. Error bars indicate the standard deviation from triplicate values.

**Figure 4.** Plasma IP-10 Levels in Kawasaki Disease (KD) in Relation to the Duration of Fever (A) and Intravenous Immunoglobulin (IVIG) Treatment. A) Dot plot of IP-10 plasma levels in KD patients with blood obtained <4 days (mean, 3.4 ± 0.90 days; range, 1–4 days) from the onset of fever or >5 days (mean, 6.0 ± 1.05 days; range, 5–8 days) from the onset of fever. B) Plasma IP-10 levels before and 1 wk after IVIG treatment in 45 patients with KD. Each dot represents the average of 3 analyses with variation <5% (standard deviation/average) from a single individual.

**Figure 5.** Cell Surface Chemokine Receptor CXCR3 in T Cells of Patients with Acute Kawasaki Disease (KD). A) Open curves indicate fluorescence activated cell sorter histogram plots of CD3+ T cells stained with anti-CXCR3 antibody. Patients KD-1 to KD-6 were in the acute stage of KD. HD indicates healthy donors. B) Bar graph summarizing the mean fluorescence intensity of CXCR3 in CD3+ T cells from 3 healthy donors and 6 patients with acute KD. **, p < 0.01, unpaired Student’s t-test.
Table 1. Demographic and Clinical Characteristics of Enrolled Patients.

<table>
<thead>
<tr>
<th>Variable</th>
<th>KD (N=100)</th>
<th>FC (N=114)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>1.7±1.6</td>
<td>3.6±2.9</td>
</tr>
<tr>
<td>Sex (Male %)</td>
<td>66%</td>
<td>62%</td>
</tr>
<tr>
<td>White blood cells per μL</td>
<td>13829.7±4802.7</td>
<td>10733.6±5227.5</td>
</tr>
<tr>
<td>Glutamate oxaloacetate transaminase (U/L)</td>
<td>84.7±114.8</td>
<td>35.8±12.6</td>
</tr>
<tr>
<td>Glutamate-pyruvate transaminase (U/L)</td>
<td>90.2±100.7</td>
<td>20.3±11.8</td>
</tr>
<tr>
<td>Number of principal clinical features</td>
<td>4±1</td>
<td>2±1</td>
</tr>
<tr>
<td>Duration of fever (days)</td>
<td>5±2</td>
<td>5±2</td>
</tr>
<tr>
<td>Left main coronary artery (mm)</td>
<td>2.19±0.43</td>
<td>N.A.</td>
</tr>
<tr>
<td>Right coronary artery (mm)</td>
<td>1.94±0.45</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

All variable data are expressed as mean ± standard deviation (SD). KD, Kawasaki disease; FC, febrile control.
Novelty and Significance

What Is Known?

- Kawasaki disease is the most common cause of acquired heart disease in young children.
- Delay of diagnosis can increase the incidence of CAAs and other devastating cardiac complications.
- Diagnosing KD can be difficult because of its varied clinical manifestations and lack of specific laboratory tests.

What New Information Does This Article Contribute?

- We examined the global profile of cytokines, chemokines, and cell adhesion molecules in plasma samples from a large cohort of patients with clinical features suggestive of KD.
- Plasma IP-10 levels were significantly elevated and the IP-10 downstream pathway was activated in acute-stage KD patients.
- The results identify a practicable biomarker and a clear cut-off for early KD diagnosis.

Although several studies have shown that the expression of multiple genes can be upregulated in acute KD, the differential diagnosis of KD patients from those with non-KD-related fever in a simple and reliable way remains a challenge. Hence, we sought to identify plasma proteins that could be used as sensitive and specific diagnostic biomarkers in diagnosing KD among febrile subjects. By using an unbiased, large-scale, quantitative protein array, we found that IP-10 was the most significantly increased biomarker. The increase in IP-10 was consistent across the three study phases (discovery, replication, and blinded validation) and it showed high sensitivity and specificity. In addition, activation of CXCR3 (the downstream receptor of IP-10) was detected in the T cells of acute KD patients. Thus, IP-10 could be used as a biomarker for KD, and it may also provide a novel signal pathway for further investigating KD pathogenesis.
Figure 2

A. Replication

B. Combination (discovery & replication)

C. Sensitivity = 100%
Specificity = 77%
AUC = 0.94

*** (p = 2.2 x 10^{-11})

*** (p = 2.8 x 10^{-20})
Figure 3

1318 pg/ml (cut-off value)
Figure 4

A. 

IP-10 pg/mL

< 4 days

> 5 days

1318 pg/ml (cut-off value)

fever days

B. 

IP-10 pg/mL

before IVIG

after IVIG
Figure 5
CXCL10/IP-10 is a Biomarker and Mediator for Kawasaki Disease
Tai-Ming Ko, Ho-Chang Kuo, Jeng-Sheng Chang, Shih-Ping Chen, Yi-Min Liu, Hui-Wen Chen, Fuu-Jen Tsai, Yi-Ching Lee, Chien-Hsiun Chen, Jer-Yuarn Wu and Yuan-Tsong Chen

Circ Res. published online January 20, 2015;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2015/01/20/CIRCRESAHA.116.305834

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2015/01/20/CIRCRESAHA.116.305834.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Online Table I. Final Diagnoses of 214 Pediatric Patients.

<table>
<thead>
<tr>
<th>Final diagnosis</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kawasaki disease</td>
<td>100</td>
</tr>
<tr>
<td>pneumonia</td>
<td>27</td>
</tr>
<tr>
<td>bronchiolitis</td>
<td>18</td>
</tr>
<tr>
<td>tonsillitis</td>
<td>13</td>
</tr>
<tr>
<td>sinusitis</td>
<td>9</td>
</tr>
<tr>
<td>enteritis</td>
<td>8</td>
</tr>
<tr>
<td>pharyngitis</td>
<td>5</td>
</tr>
<tr>
<td>herpangina</td>
<td>5</td>
</tr>
<tr>
<td>urinary tract infection</td>
<td>6</td>
</tr>
<tr>
<td>herpetic gingivostomatitis</td>
<td>3</td>
</tr>
<tr>
<td>viral infection (ie. Epstein–Barr virus and adenovirus)</td>
<td>3</td>
</tr>
<tr>
<td>pyelonephritis</td>
<td>2</td>
</tr>
<tr>
<td>scarlet fever</td>
<td>2</td>
</tr>
<tr>
<td>otitis media</td>
<td>1</td>
</tr>
<tr>
<td>pyuria</td>
<td>2</td>
</tr>
<tr>
<td>parotitis</td>
<td>1</td>
</tr>
<tr>
<td>suspect infectious mononucleosis</td>
<td>1</td>
</tr>
<tr>
<td>hyponatremia</td>
<td>1</td>
</tr>
<tr>
<td>fever of unknown origin</td>
<td>7</td>
</tr>
</tbody>
</table>
Online Table II. Signaling Intensities of the 10 Selected Candidate Genes Encoding Chemokines and CAMs from the Plasma of Acute-Phase Kawasaki Disease (KD) Patients.

<table>
<thead>
<tr>
<th></th>
<th>KD-1</th>
<th>KD-2</th>
<th>KD-3</th>
<th>KD-4</th>
<th>KD-5</th>
<th>KD-6</th>
<th>Ctr-1</th>
<th>Ctr-2</th>
<th>KD/Ctr fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-9</td>
<td>336</td>
<td>253</td>
<td>5,749</td>
<td>668</td>
<td>774</td>
<td>967</td>
<td>174</td>
<td>149</td>
<td>9.02</td>
</tr>
<tr>
<td>IP-10</td>
<td>4,316</td>
<td>2,562</td>
<td>2,200</td>
<td>6,595</td>
<td>3,348</td>
<td>10,314</td>
<td>596</td>
<td>913</td>
<td>6.48</td>
</tr>
<tr>
<td>E-Selectin</td>
<td>9,020</td>
<td>7,175</td>
<td>8,791</td>
<td>11,220</td>
<td>11,476</td>
<td>15,427</td>
<td>4,021</td>
<td>3,393</td>
<td>2.84</td>
</tr>
<tr>
<td>MPIF-1</td>
<td>3,072</td>
<td>882</td>
<td>668</td>
<td>1,699</td>
<td>1,631</td>
<td>3,756</td>
<td>1,060</td>
<td>532</td>
<td>2.45</td>
</tr>
<tr>
<td>SCF R</td>
<td>2,421</td>
<td>2,419</td>
<td>2,606</td>
<td>9,067</td>
<td>2,578</td>
<td>7,584</td>
<td>2,561</td>
<td>1,899</td>
<td>1.99</td>
</tr>
<tr>
<td>PDGF-AB</td>
<td>5,645</td>
<td>6,649</td>
<td>6,144</td>
<td>12,379</td>
<td>8,837</td>
<td>13,695</td>
<td>5,632</td>
<td>4,178</td>
<td>1.81</td>
</tr>
<tr>
<td>MMP-9</td>
<td>3,541</td>
<td>4,853</td>
<td>4,902</td>
<td>1,673</td>
<td>5,661</td>
<td>6,850</td>
<td>2,026</td>
<td>3,273</td>
<td>1.73</td>
</tr>
<tr>
<td>ALCAM</td>
<td>3,491</td>
<td>2,839</td>
<td>3,070</td>
<td>4,505</td>
<td>3,725</td>
<td>5,923</td>
<td>2,919</td>
<td>2,189</td>
<td>1.54</td>
</tr>
<tr>
<td>L-Selectin</td>
<td>13,597</td>
<td>14,850</td>
<td>14,208</td>
<td>20,070</td>
<td>16,264</td>
<td>25,435</td>
<td>13,621</td>
<td>11,460</td>
<td>1.39</td>
</tr>
<tr>
<td>ICAM-2</td>
<td>32,272</td>
<td>29,906</td>
<td>37,391</td>
<td>57,141</td>
<td>25,626</td>
<td>32,476</td>
<td>26,188</td>
<td>27,011</td>
<td>1.35</td>
</tr>
</tbody>
</table>

All tests were performed in duplicate. Internal negative controls were used to determine the cut-off rate for a positive signal. Six KD patients and 2 control (Ctr) subjects were screened using protein arrays. Ctr-1 was a pediatric subject with a non-KD fever. Ctr-2 was a normal healthy subject. Only the KD/Ctr ratios (the average of KD patients/average of ctr cases) of chemokines and CAMs exceeding 1.3 are shown.