Screening of differentially expressed genes associated with Kawasaki disease by microarray analysis

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Abstract. Kawasaki disease (KD) is an autoimmune disorder that can induce coronary artery aneurysms, particularly in the case of delayed diagnosis and/or treatment. Early diagnosis is important for treatment and reduces the risk of heart injury. The aim of the present study was to identify differentially expressed genes by comparing the levels of gene expression in human umbilical vein endothelial cells following treatment with plasma from healthy individuals and patients with acute or convalescent KD. Following comparison of the control and acute KD groups, 385 up-regulated and 537 down-regulated genes were identified in the acute KD group. In the convalescent group, 505 and 879 genes were up-regulated and down-regulated, respectively, relative to the control group. Genes involved in the immune system and cell growth factors were up-regulated, while genes functioning in methylation were down-regulated, following treatment with KD plasma. In addition, five potential candidate molecular markers of KD, C-X-C motif chemokine ligand 2 (CXCL2), interleukin (IL) 8, tripartite motif containing 58 (TRIM58), immunoglobulin superfamily member 3 (IGSF3) and runt related transcription factor 1 (RUNX1) were identified by microarray analysis and verified using quantitative polymerase chain reaction. A significant positive correlation was identified between the neutrophil polys and expression levels of four of these candidate genes, including CXCL2, IL8, TRIM58, and IGSF3 (all $P<0.01$; $R^2 \geq 0.64$). However, only CXCL2 expression was significantly positively correlated with neutrophil polys ($P=0.01$; $R^2=0.64$) and neutrophil bands ($P<0.001$; $R^2=0.73$). These results indicate that CXCL2 serves a crucial role in the injury of endothelial cells by KD plasma.

Introduction

Kawasaki disease (KD), also known as pediatrix mucocutaneous lymph node syndrome, is a leading cause of acquired heart disease in children. The major pathological feature is the presence of systemic arterial inflammatory lesions and the presence of coronary artery lesions is the most important factor affecting the prognosis of patients. It is also the primary etiology leading to the development of the secondary heart disease in children. Additionally, KD can stimulate the development of autoimmune disorders and abnormal metabolism (1). Previous studies have investigated alterations in gene expression levels in KD, particularly those associated with pathways affecting inflammation, cardiac damage and endothelial cell injury (2-4). Based on a genome-wide association study, Onouchi *et al* (5) identified three risk loci for KD. However, in order to determine the molecular basis of KD, it is essential to define changes in global gene expression. As KD is an autoimmune disease, measuring the expression of genes associated with inflammation would provide a way to determine the response to inflammatory stimuli in KD. Intravenous immunoglobulin (IVIG) is an effective agent currently used in the treatment of KD (6-8). The majority of cases of KD can be successfully treated following IVIG treatment, however the incidence of coronary aneurysm remains at 3-5% (9,10). In addition, cases of KD resistant to IVIG therapy have been reported (11,12). In Japan and the USA, 20-30% of KD cases have been reported to be IVIG-resistant (13). Therefore, a better understanding of the molecular mechanisms underlying KD is required in order to develop targeted treatments.

The development of systems biology approaches has enabled the analysis of gene expression through DNA microarrays, providing a cheap, efficient and high-throughput method to study global gene expression patterns (14,15). This technology has been widely used to screen for potential gene candidates underlying the pathogenesis of numerous diseases, including ankylosing spondylitis, which is also an autoimmune disease (16). To date, a limited number of

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transcriptomic studies of KD have been conducted. For instance, Popper et al (17) determined the importance of the activation state of neutrophils and apoptosis in the pathogenesis of KD through microarray analysis. However, the identification of genes that are predictive and prognostic of KD is required. The present study aimed to investigate gene expression patterns following treatment of human umbilical vein endothelial cells (HUVECs) with plasma obtained from patients with KD or controls. The differentially expressed genes identified may assist in the prevention of heart injury as a result of KD.

Materials and methods

Sample collection. A total of 4 patients with KD (2 males and 2 females, aged 3-4 years old) were enrolled from The Third Xiangya Hospital of Central South University (Changsha, China) between May 2014 and June 2014 to collect plasma for HUVEC treatment. Informed consent was obtained from the patients' families. All participants exhibited a fever and showed typical KD characteristics, including rash, conjunctival injection, cervical lymphadenopathy and changes in the oral mucosa and extremities. Plasma was collected from patients prior to treatment (acute KD group) and 3 days after fever (defined as a body temperature of >38°C) was reduced by treatment with IVIG (2 g/kg/day; Shanghai RAAS Blood Products, Co., Ltd., Shanghai, China) and aspirin (30 mg/kg/day, convalescent KD group; Sigma-Aldrich; Merck kGaA, Darmstadt, Germany). In addition, plasma from 10 patients exhibiting typical KD characteristics (3.3±0.67 years-old, 5 males and 5 females, collected between September 2014 and November 2014; control group) was collected for analysis of neutrophil counts and candidate molecular markers in KD. Informed consent was provided by the families of patients and controls for analysis of correlations between gene expression and neutrophil counts. All experiments were approved by the Research Ethics Committee of Central South University (Changsha, China).

HUVEC culture and treatment. HUVECs were obtained from a newborn's umbilical cord in June 2014. Informed consent was provided by parents of the newborn. HUVEC isolation, identification and preservation were performed as previously described (18) at the Third Xiangya Hospital of Central South University. Following culture for 3 generations, cells were seeded into 42 Petri dishes at a density of 1x10⁶ cells/dish in 5 ml of Medium 199 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). There were 14 samples in total and experiments were performed in triplicate. Subsequently, cells were treated with 10% (50 μl/dish) plasma obtained from the control, acute KD or convalescent KD groups at 37°C in an atmosphere of 5% CO_2, for 24 h. The cells were then used in subsequent experiments. HUVECs were identified using cluster of differentiation (CD) 31. HUVECs were incubated with rabbit anti-CD31 polyclonal antibody (ab28364, ABCAM, Cambridge, UK, dilution 1:50) overnight at 4°C and detected using the Leica DM2000 (Leica Microsystems, Ltd., Milton Keynes, UK).

RNA isolation and analysis. Total RNA was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Subsequently,

isolated total RNA was analyzed on 1.2% agarose gels to determine the quality of the fragments. The quantity of RNA was calculated from measurements of the absorbance of samples at 260 and 280 nm using an ultraviolet spectrophotometer.

Microarray analysis. RNAs were examined using the GeneChip PrimeView Human Gene Expression Array (cat. no. 901837, Affymetrix, Inc., Santa Clara, CA, USA). Four dishes of isolated RNAs from each group were used for analysis. This process, along with quality control, was performed by the CapitalBio Corporation (Beijing, China), in accordance with the GeneChip Expression Analysis Technical Manual (http://www.affymetrix.com). Microarray data were analyzed using Significance Analysis of Microarrays software 4.01 (http://statweb.stanford.edu/~tibs/SAM/). Differential gene expression was defined by a fold change ≥2 and a q-value <5% compared with the control group. Gene annotation was performed using Molecule Annotation System Mas software (version 3.0; http://bioinfo.capitalbio.com/mas3/).

Neutrophil counts. Neutrophil counts from the plasma of ten patients were performed using an Advia 2120 Hematology System (Siemens Healthcare GmbH, Erlangen, Germany).

Quantitative polymerase chain reaction (qPCR). qPCR was performed to investigate the mRNA expression levels of C-X-C motif chemokine ligand 2 (CXCL2), interleukin-8 (IL-8), tripartite motif-containing protein 58 (TRIM58), immunoglobulin superfamily member 3 (IGSF3), runt-related transcription factor 1 (RUNX1) and GAPDH (used as the internal control). A total of 10 dishes of cells from each group were used for the analysis. In addition, to analyze the genes associated with KD in the peripheral blood, RNA from the plasma of 10 patients was isolated. cDNA was synthesized from isolated RNA using the PrimeScript RT Reagent Kit with gDNA Eraser (cat. no. RR047Q, Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. qPCR was performed using the SYBR-green kit (Takara Biotechnology Co., Ltd.) and the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR reaction mixture contained 2 μl cDNA, 0.8 μl forward primer, 0.8 μl reverse primer, 10 μl SYBR Premix Ex Taq II. The qPCR thermal cycling conditions were as follows: 94°C for 3 min, followed by 40 cycles at 95°C for 3 sec, and 60°C for 30 sec. The following primers were used for qPCR: CXCL2 forward, 5'-AAAGGG GTTCGCCGTTCTC-3', and reverse, 5'-CTGGCAGCGCAGT TCAGTG-3'; IL-8 forward, 5'-ATGACTTCCAAGCTGGCC GTGGCT-3' and reverse, 5'-TCTCAGCCCTCTTCAAAA ACTTCTC-3'; TRIM58 forward, 5'-GATTGAGACCATCCT GGCTAACA-3', and reverse, 5'-ACAACAGGCACCCAC CACC-3'; IGSF3 forward, 5'-CTGACCAGGGCGAATTCTA CT-3', and reverse, 5'-TCTTGCCTCATGGTCTGTTTTA-3'; RUNX1 forward, 5'-AACCCTCAGCCTCAAAGTCA-3', and reverse, 5'-GGGTGCACAGAAGAGGTGAT-3'; GAPDH forward, 5'-ATGCTGGCGCTGAGTACGTC-3', and reverse, 5'-CAGGGGTGCTAAGCAGTTGGT-3'. The primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China) Following amplification, the dissociation curves produced were analyzed

to identify whether amplicons had a multi-phase melt curve. Relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method (19).

Statistical analysis. Results are presented as the mean ± standard error of the mean. To confirm whether differences between groups were statistically significant, one-way analysis of variance was performed, followed by a post-hoc Tukey's least significant difference test. P<0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA).

Results

Identification of HUVECs. Cluster of differentiation 31 (CD31) was used as a biomarker to confirm the presence of HUVECs. The control, acute and convalescent groups showed marked CD31 signals, demonstrating that the cells identified were HUVECs (Fig. 1).

Screening for differentially expressed genes in KD. Total RNA from the control, acute and convalescent groups was extracted, and gene expression analysis was performed using a microarray screening for 30,000 genes. For each group, four repeats were performed and gene clustering into related branches was conducted (Fig. 2A), which identified good repeatability of the results.

By comparing the results of the control and acute KD groups (Fig. 2B), 385 genes were identified as up-regulated and 537 genes as down-regulated in acute KD. Up-regulated genes appeared to be associated with various processes, including signal transduction (such as SFRP1), ubiquitination (such as TRIM58), the immune response (including MICA, IGSF3, ITGA4, CXCL2, CXCR4, IL1A and IL-8), growth (EGR1, VEGFA, GAS1, EPGN, GDF11, IGF2R and TFRC) and transcription (E2F7, ATF3, RUNX1 and TFEC). By contrast, down-regulated genes were involved in various cellular processes, such as the cell cycle (including MAP3K5, CDKN2C, CCNF, CDKN3, CCNE2, CCNB1, CCNB2, CCNA2 and CDK1), immune responses (IL6ST and SDPR), methylation (TRDMT1, METTL7A, DNMT1 and METTL10), and transcription (TTF2, TCF19, RUNX1T1, E2F8, GABPB1, GTF3C3 and TCF7L2).

Comparison of the gene expression in the control and convalescent KD groups (Fig. 2C) revealed 505 up-regulated and 879 down-regulated genes. The up-regulated genes included growth-associated factors (TFPI2, GAS1, E2F7, IGF2, VEGFA and TNIP1), immune system genes (CXCR4, NFATC2 and CD200) and genes associated with the cell cycle (such as G0S2). In addition, the down-regulated genes identified served a primary role in cell growth (EDIL3, IGFBP7, CDCA2, GAS6, TGFBR1, TGFB2 and BMPER), the cell cycle (CDKN3, CDKN2C, CCNB2, CCNF, CCND2 and CCNB1) and transcription (such as GTF2F2).

Identification of candidate molecular markers for KD. Besides screening for differentially expressed genes from HUVECs, these potential gene expression was determined from treatment cells. Comparison of gene expression between the control, acute and convalescent groups identified 5 genes as candidate
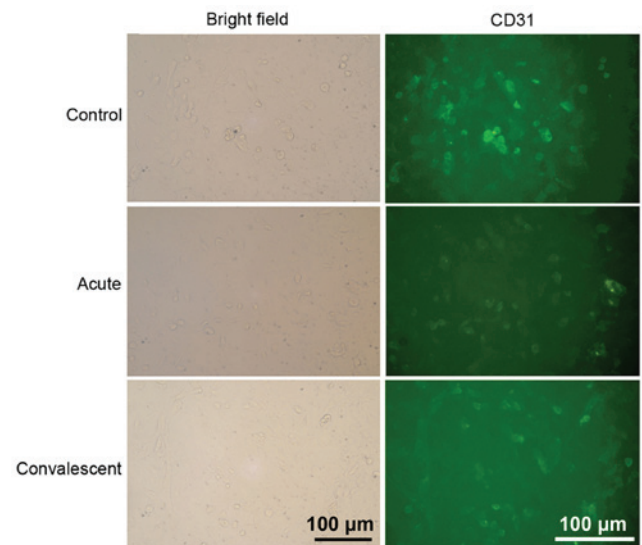

Figure 1. Morphology of cultured HUVECs following treatment with the plasma of healthy control individuals and patients with acute or convalescent KD via light and fluorescence microscopy. CD31 was used as a biomarker for HUVEC identification. Magnification, x100. KD, Kawasaki disease.
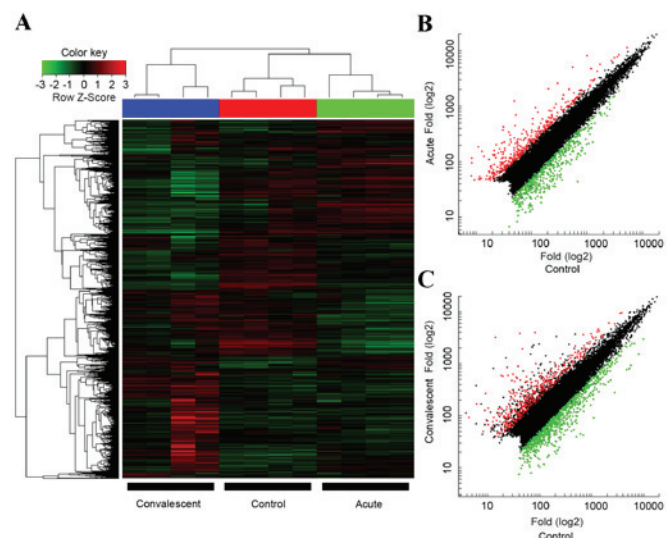

Figure 2. Patterns of gene expression in plasma samples from healthy individuals and patients with acute or convalescent KD (n=4). (A) Heatmap of gene expression in different groups by microarray analysis. Blue indicates the convalescent group, red indicated the control and green indicates the acute group. (B) Comparison between control and acute groups. (C) Comparison between control and convalescent groups. Red dots indicate the up-regulated genes and green dots indicate the down-regulated genes. KD, Kawasaki disease.

molecular markers for KD, including CXCL2, RUNX1, IL8, TRIM58 and IGSF3. These candidates were highly expressed in the acute group, but weakly expressed in the control and convalescent groups. Subsequently, qPCR was performed in order to confirm that these genes were candidate markers for KD (Fig. 3). For qPCR, cells treated with control, acute and convalescent KD plasma were collected and analyzed. The results demonstrated that levels of CXCL2, IL-8, TRIM58 and IGSF3 mRNA were significantly higher in the acute KD group compared with those in the control and convalescent
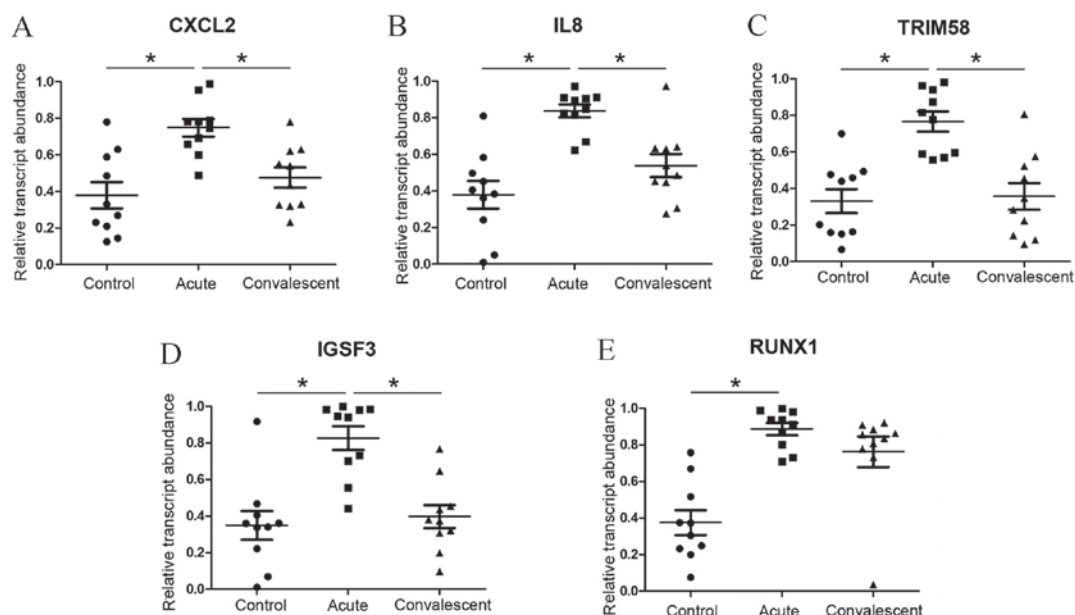
Figure 3. Quantitative polymerase chain reaction analysis of mRNA expression levels of (A) CXCL2, (B) IL-8, (C) TRIM58, (D) IGSF3 and (E) RUNX1 relative to GADPH (n=10) in the cells treated by the control, acute and convalescent plasma. *P<0.05. CXCL2, C-X-C motif chemokine ligand 2; IL-8, interleukin-8; TRIM58, tripartite motif-containing protein 58; IGSF3, immunoglobulin superfamily member 3; RUNX1, runt-related transcription factor 1; KD, Kawasaki disease.
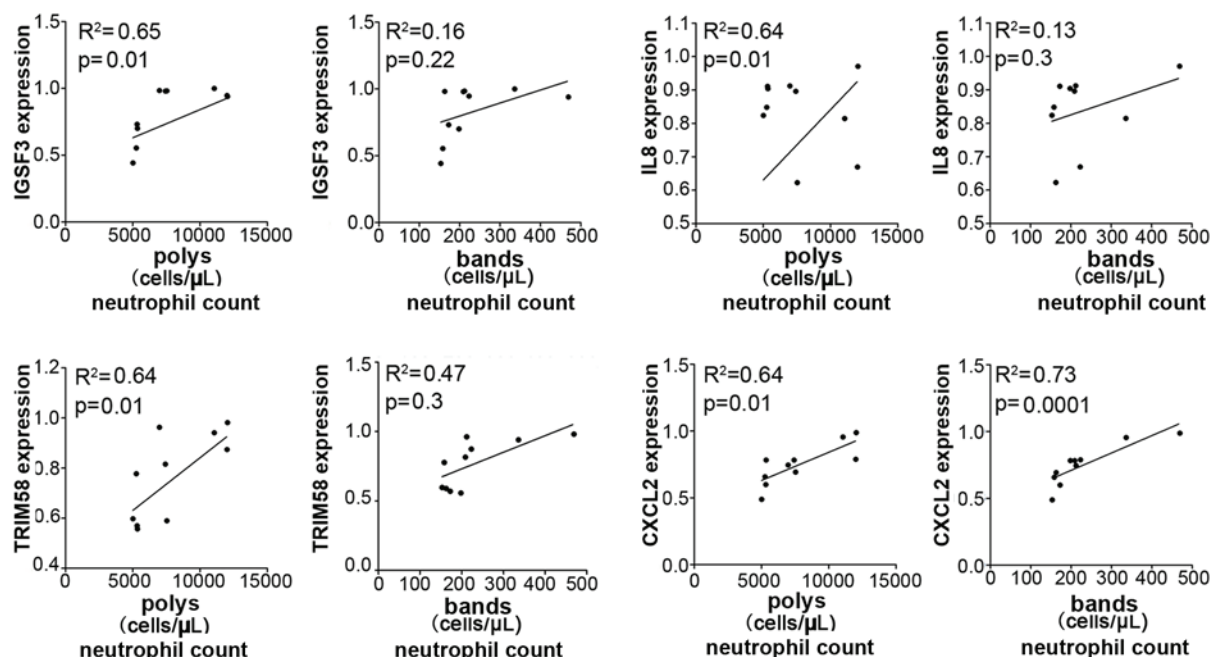

Figure 4. Correlations between gene expression and neutrophil polys, and between gene expression and neutrophil bands as measured using quantitative polymerase chain reaction (n=10). The association between the KD molecular marker genes (CXCL2, IL8, TRIM58 and IGSF3) and the neutrophil counts in plasma was also assessed (n=10). KD, Kawasaki disease; CXCL2, C-X-C motif chemokine ligand 2; IL-8, interleukin-8; TRIM58, tripartite motif-containing protein 58; IGSF3, immunoglobulin superfamily member 3.

groups (P<0.05; Fig. 3A-D). However, although RUNX1 was highly expressed in the acute group and weakly expressed in the control group, the difference in its expression between the acute and convalescent groups was not statistically significant (P>0.05; Fig. 3E).

Genes associated with KD in the peripheral blood. The correlation between the expression of these genes and neutrophil counts from the plasma of KD was analyzed. The expression of the four verified candidate KD molecular marker genes (CXCL2, IL-8, TRIM58 and IGSF3) was compared with the neutrophil counts from the plasma of 10 patients. The results identified that CXCL2 was the only gene where expression was significantly positively correlated with neutrophil polys (P=0.01; R²=0.64; Fig. 4) and neutrophil bands (P=0.001; R²=0.73; Fig. 4). No other genes were significantly correlated

with both neutrophil polys and neutrophil bands. These results indicate that CXCL2 may be a biomarker for neutrophil counts and KD in the peripheral blood.

Discussion

KD, a lymph node syndrome, has acute symptoms that are self-limited (20). Down-regulation of inflammation terminates the acute febrile (12). However, acute KD can trigger coronary artery aneurysms, particularly when there is delayed diagnosis and/or treatment (21). Therefore, rapid and efficient diagnosis is the primary issue in KD. Identification of KD biomarkers would provide a promising novel strategy for early diagnosis. In the present study, differentially expressed genes following treatment of HUVECs with KD plasma were investigated using a microarray assay. The candidate genes identified could be novel indicators and potential therapeutic targets for KD. In addition, the function of differentially expressed genes provides important information on the molecular mechanisms underlying KD.

The results of microarray analysis revealed that 385 genes were up-regulated in the acute group compared with the control group and the majority of these genes had functions in the immune system and cell growth. KD is considered to be a systemic autoimmune disease, thus increased expression of immune genes was expected. For instance, genes involved in the natural killer cell signaling pathway and toll-like receptor-signaling pathway, as well as immunoglobulin, interferon-stimulated and secretory system genes were up-regulated in KD. Polymorphisms of VEGF and its receptor are correlated with coronary artery lesions in KD, demonstrating an important association between growth factors and KD (22). With regards to cell growth genes, VEGFA was up-regulated in HUVECs following treatment with KD plasma in the current study. In addition, other growth factors, including EGR1, GAS1, EPGN, GDF11, IGF2R and TFRC, were found to be up-regulated in acute KD, suggesting that they may contribute to the development of KD.

The present study also identified that a number of immune system genes, including IL6ST and SDPR, were down-regulated in the acute KD group compared with the control group. IL6ST encodes a component of the cytokine receptor complex (23) and SDPR encodes a substrate of protein kinase C (24). However, whether these changes in expression influence the function of the cytokine receptor complex or protein kinase C requires further investigation. In addition, various genes involved in methylation were found to be down-regulated in the acute KD group. Little information has been reported on the association between KD and methylation to date. A study by Kuo *et al* (25) detected hypomethylation of FCGR2A in KD. The results of the present study demonstrated an alteration in the expression of genes involved in methylation in KD, which may contribute to hypomethylation and KD pathogenesis.

Alterations in the expression of transcription factors were identified in HUVECs following treatment with plasma from patients with acute KD in the current study. This indicates that, during the development of KD, the transcription of certain genes is promoted or blocked. Although the specific mechanisms of these genes in the pathogenesis of KD remain unclear, the results of the present study provide information

for further researches. Compared with the control group, immune system genes were up-regulated in the convalescent group, which indicated that the overexpression of immune genes observed in acute KD did not decrease rapidly following treatment. Cell cycle genes were down-regulated in the convalescent group compared with the control group, suggesting that there is an abnormal regulation of cell proliferation during the convalescent phase of KD. In addition, the expression of cell growth factors was significantly increased in the acute group compared with the control and convalescent groups, indicating that cell growth of HUVEC is controlled in the recovery stage.

Following qPCR analysis, CXCL2, IL-8, TRIM58 and IGSF3 were identified to be the major functional genes for prognosis/diagnosis in KD. These genes were highly expressed in the acute phase of KD but weakly expressed in the control and convalescent KD groups. Furthermore, a significant positive correlation was observed between these genes and neutrophil polys counts, demonstrating the reliability of these genes as key functional genes of KD. However, only CXCL2 exhibited a significant positive correlation with neutrophil bands, as well as with neutrophil polys counts. CXCL2, secreted by monocytes and macrophages (26-28), has recently been reported to be up-regulated following HIV and syphilis infection (29). Thus, CXCL2 is regarded as a novel biomarker for the presence of these pathogens. A previous study demonstrated that CXCL10 was highly expressed in the plasma of KD patients (30). Since CXCL2 and CXCL10 belong to the CXC chemokine family, the results of the present study indicate that the CXCL2 is activated in patients with KD. Therefore, CXCL2 is a potential biomarker of KD.

In conclusion, the present study compared gene expression in HUVECs following treatment with plasma from healthy individuals, as well as acute and convalescent KD patients, using microarray analysis. The majority of genes identified to be up-regulated in KD were immune system genes and cell growth factors, whereas the majority of down-regulated genes were involved in methylation. Five potential candidate KD biomarkers were identified using qPCR (CXCL2, IL-8, TRIM58, IGSF3 and RUNX1). In addition, only CXCL2 expression was significantly positively correlated with neutrophil polys and bands counts. These results indicate that CXCL2 serves a crucial role in the injury of endothelial cells by KD plasma and that CXCL2 may be used as a novel diagnostic and prognostic biomarker for KD.

Acknowledgements

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