Significance of serum 25-hydroxyvitamin D_3 and interleukin-6 levels in immunoglobulin treatment of Kawasaki disease in children

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Abstract. The aim of the study was to investigate the significance of the level of serum 25-hydroxyvitamin D_3 [25-(OH)D_3] and interleukin (IL)-6 in serum prior to and after immunoglobulin treatment in children suffering from Kawasaki disease in order to provide a reference for the successful treatment of Kawasaki disease in children. From February, 2013 to February, 2015, 45 patients with Kawasaki disease were enrolled in the observation group. The normal control group comprised 43 healthy volunteers and the feverish control group 46 patients with respiratory infection and fever. Venous blood was collected from each case before and after immunoglobulin treatment and the level of 25-(OH)D_3 and IL-6 in the serum were measured using fluorescent quantitative PCR, enzyme-linked immunosorbent assay and western blotting. Before treatment, the level of 25-(OH)D_3 in the feverish control group was significantly lower than that of the normal control group, while the level of 25-(OH)D_3 in the observation group was significantly higher than that of the normal control group. The level of 25-(OH)D_3 in the feverish control group was lower than the IL-6 level in the normal children, but the difference was not statistically significant ($P>0.05$). The level 25-(OH)D_3 in the observation group was significantly higher than the IL-6 level in the normal control group. The serum content of 25-(OH)D_3 was significantly higher after the treatment compared to before treatment levels and after treatment IL-6 level was only slightly lower. It was observed that the 25-(OH)D_3 level in the observation group was significantly increased after immunoglobulin treatment and this was positively correlated with the effects of the treatment. The IL-6 level had no significant changes after treatment and had little correlation with the treatment effect. The results suggested that 25-(OH)D_3 may be involved in the occurrence of Kawasaki disease in children and in the aggravation of the disease to some extent.

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Introduction

Kawasaki disease (KD), also known as skin-mucosal-associated lymphoid syndrome (a vasculitis syndrome), is a rare childhood illness that affects the blood vessels (1). The disease is most common in children and can be extremely harmful to infants. Damage may occur to coronary arteries and to the heart muscle itself (1). Affected children may suffer from systemic small and middle-sized vascular inflammation which can be transformed into coronary artery sustainable expansion (2), coronary artery aneurysm and even death.

The causative factors of Kawasaki disease are unknown and the disease does not appear to be hereditary or contagious. Identifying treatment for Kawasaki disease has become the focus of investigations worldwide (3). Results obtained from prior studies (4) showed that intravenous immunoglobulin (IVIG) therapy is effective with regard to the adverse effects of Kawasaki disease, however the underlying mechanism remains to be determined. Suzuki *et al* (5) showed that serum 25-hydroxyvitamin D_3 [25-(OH)D_3] in blood ameliorates the immune system and prevents coronary artery abnormalities. Results from another related study (6) suggested that interleukin-6 (IL-6) was correlated with immunoreaction and had multiple effects such as stimulating and activating the proliferation of B cell, promoting antibody secretion, stimulating the proliferation of T cells and CTL activation (7). IL-6 affects the expression of acute phase protein in hepatic cells, which are involved in inflammatory reactions and accelerating cell development (8).

In the present study, we investigated the significance of serum 25-(OH)D_3 and IL-6 levels prior to and after immunoglobulin treatment in children suffering from Kawasaki disease. We aimed to provide some theoretical and practical references for the successful treatment of Kawasaki disease in children.

Materials and methods

General materials. From February, 2013 to February, 2015, 45 patients with Kawasaki disease, including 24 men and

21 women with an average age of 3.2±3 years, were enrolled in the present study, and constituted the observation group. The normal control group comprised 43 healthy volunteers during the same period. The normal control group comprised 22 men and 21 women with an average age of 2.9±2.7 years. The feverish control group comprised 46 patients (22 men and 24 women), with an average age of 3.1±2.9 years, and had respiratory infection and fever.

The aims of the study were established in accordance with relevant diagnostic criteria of Kawasaki disease presented by Japan KD Research Committee. The present study was approved by the ethics committee of Xuzhou Children's Hospital. Written informed consent was obtained from the patients and/or guardians.

Methods. Venous blood (4 ml) was collected from each case prior to treatment. Samples were centrifuged at 2,000 x g for 5 min at 4˚C and the upper layer of the serum was collected and transferred into a cryogenic tube (Suzhou Alpha Biotech Co., Ltd, Suzhou, China) and kept at -80˚C (1) and the content of 25-(OH)D_3 and IL-6 were verified. Antibodies used in this study were purchased from Roche Diagnostics (Basel, Switzerland), and RNA extraction kits were purchased from Takara Bio (Dalian, China). Patients were then treated with IVIG therapy (2 g/kg intravenous drip and oral aspirins, 2-5 days after the fever subsided). The serum was kept and the content of 25-(OH)D_3 and IL-6 was detected.

RT-polymerase chain reaction (PCR)

RNA extraction. Cryophylactic tissue samples (0.1 g) were extracted from the liquid nitrogen and melted on ice. Subsequently, 0.45 ml of RNA Plus was added and the tissues were ground in a precooled mortar and transferred into a 1.5-ml Eppendorf tube (Hamburg, Germany). After adding 0.45 ml of RNA Plus in the mortar, the samples were washed in centrifuge tube and 200 μl chloroform was then added. The samples were vigorously agitated using a vortex for 15 sec and kept on ice for 15 min. The samples were centrifuged at 8,000 x g for 15 min at 4˚C and the upper layer of the serum was collected in an Eppendorf tube (RNase-treated). Isopropanol (equivalent) was added to the tube and after mixing it was left on for 10 min. The samples were centrifuged again at 8,000 x g for 10 min at 4˚C and the upper layer of the serum was removed. Then, 750 μl of ethyl alcohol (75%) was added and mixed gently followed by further centrifugation at 8,000 x g at 4˚C for 10 min. The upper layer of the serum was discarded and residual ethyl alcohol was removed. The quality of extracted RNA was verified and RNA was stored to be used in reverse transcription.

Fluorescent quantitative PCR. Fluorescent quantitative PCR kits were carried out as per the manufacturer's instructions (Takara), with slight modification (Table I).

Table I. Fluorescent quantitative PCR primers.

Gene	Primer sequences (5'-3')	Fragment length (bp)
25-(OH)D_3	F: CGATCTGCATGACTTCTTCCA	164
	R: GCTAGTACGATCATCATCTAC	
IL-6	F: CGTAACGTTAGCGGCAGCTA	138
	R: CGTAGTCCAGGTACTAGCAG	
GAPDH	F: GAAGGTGAAGGTCGGAGTC	226
	R: GAAGATGGTGATGGGATTTC	

PCR, polymerase chain reaction; 25-(OH)D_3, serum 25-hydroxy-vitamin D_3; IL-6, interleukin-6; F, forward; R, reverse.

Enzyme-linked immunosorbent assay (ELISA). The standard protocol for the ELISA kit was carried out, with some improvements (9). 25-(OH)D_3 and IL-6 were diluted at a ratio of 1:40 using the assay buffer and designed the standard curve. Samples were diluted (1:100) and 80 μl of test solution and 60 μl of detection solution were added into each well (96-wells). After 1 h incubation at 25˚C, TMB chromogenic substrate was added. The light absorption value was measured at 495 nm using a microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA) and the concentration of 25-(OH)D_3 and IL-6 was calculated using the standard curve.

Western blotting. An animal cell protein extraction kit was used to extract the total protein [standard protocol carried out with some improvement (10)].

Statistical analysis. We used SPSS 20.2 software (Chicago, IL, USA) for statistical analyses. Measurement data were presented as mean ± SD, and countable data were expressed by the number of cases or the percentage. Intergroup comparison was carried out using the χ^2 test. $P<0.05$ indicated the difference was statistically significant.

Results

25-(OH)D_3 and IL-6 levels in the serum prior to treatment. We detected the 25-(OH)D_3 and IL-6 levels in the serum samples collected from the three groups prior to treatment with immunoglobulin (Fig. 1). The results showed that the content of serum 25-(OH)D_3 in the feverish control group was lower than that of the normal control group. By contrast, the level of serum 25-(OH)D_3 in the observation group was higher than that of the normal control group. The differences were statistically significant ($P<0.05$). The level of serum 25-(OH)D_3 in the feverish control group was lower than that of serum IL-6 in the normal children, but there were no statistically significant differences ($P>0.05$). The level of serum 25-(OH)D_3 in the observation group was higher than the content of serum IL-6 in the normal children, and the difference was statistically significant ($P<0.05$). The 25-(OH)D_3 and IL-6 gene expression levels in the three groups prior to treatment with immunoglobulin showed the same pattern observed in the protein expression (Fig. 2).

25-(OH)D_3 and IL-6 gene expression levels after immunoglobulin treatment. We measured 25-(OH)D_3 and IL-6 gene expression levels in the three groups after immunoglobulin treatment (Table II) and observed a significant increase in serum 25-(OH)D_3 levels compared with the levels prior to treatment and the differences were statistically significant

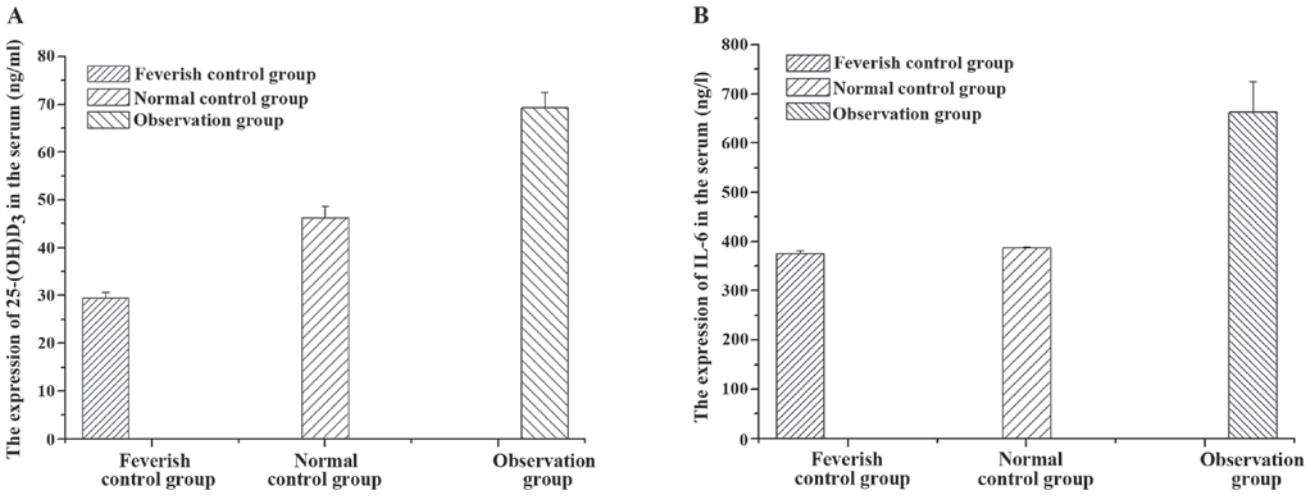

Figure 1. The levels of (A) 25-(OH)D_3 and (B) IL-6 in the serum in the normal control, feverish control and observation groups prior to treatment with immunoglobulin. 25-(OH)D_3, serum 25-hydroxyvitamin D_3; IL-6, interleukin-6.

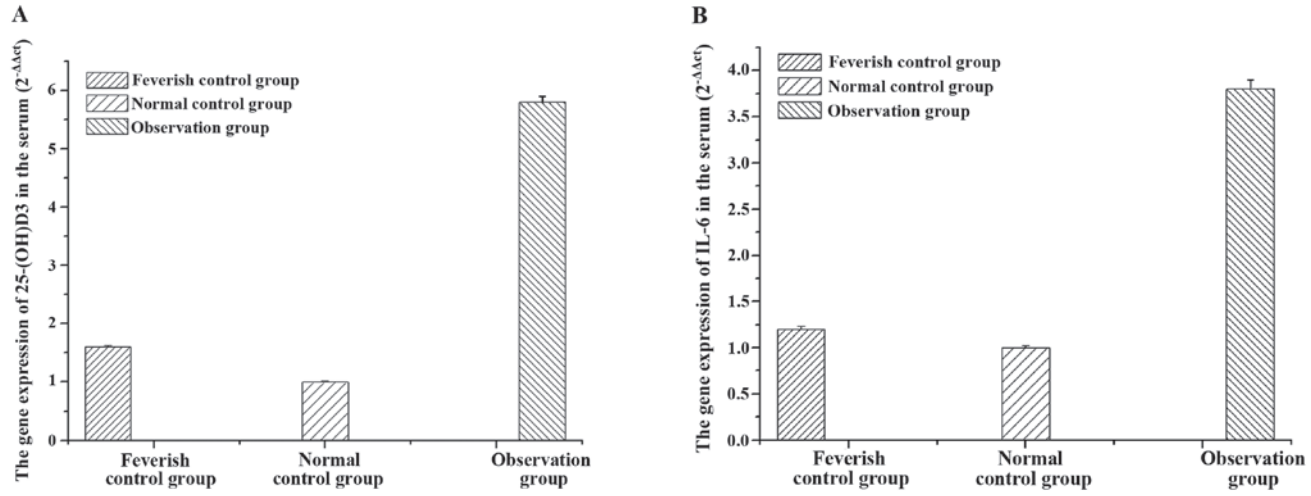

Figure 2. The (A) 25-(OH)D3 and (B) IL-6 mRNA expression level in normal control, feverish control and observation groups before immunoglobulin treatment. 25-(OH)D_3, serum 25-hydroxyvitamin D_3; IL-6, interleukin-6.

Table II. Protein expression of 25-(OH)D_3 and IL-6 in the serum of the patients in the observation group before treatment.

Gene	Before treatment	After treatment	t	P-value
25-(OH)D_3	64.3±28.4	86.3±14.2	2.74	0.012
IL-6	462.2±198.3	443.2±110.4	1.03	0.409

P<0.05, significant difference. 25-(OH)D_3, serum 25-hydroxyvitamin D_3; IL-6, interleukin-6.

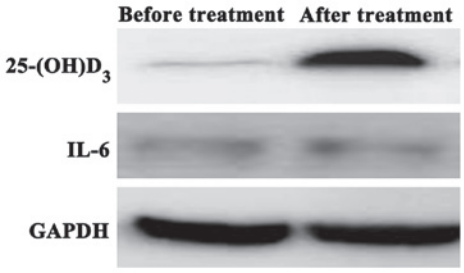

Figure 3. 25-(OH)D3 and IL-6 proteins levels in the observation group before treatment. 25-(OH)D_3, serum 25-hydroxyvitamin D_3; IL-6, interleukin-6.

(P<0.05). In the observation group, after immunoglobulin treatment, the IL-6 level in serum was lower compared with the level before treatment, albeit the difference was not statistically significant (P>0.05). The IL-6 level in the observation group after immunoglobulin treatment had no significant difference compared to that in the normal control and feverish control groups (P>0.05). The 25-(OH)D_3 and IL-6 expression levels were measured using western blotting (Fig. 3). The results were comparable to those obtained from ELISA and RT-PCR.

Correlation between 25-(OH)D_3 and IL-6 expression in the observation group prior to and after treatment. Before treatment, levels of 25-(OH)D_3 and IL-6 in the observation group were positively correlated (R^2=0.9563) (Fig. 4A). However, no

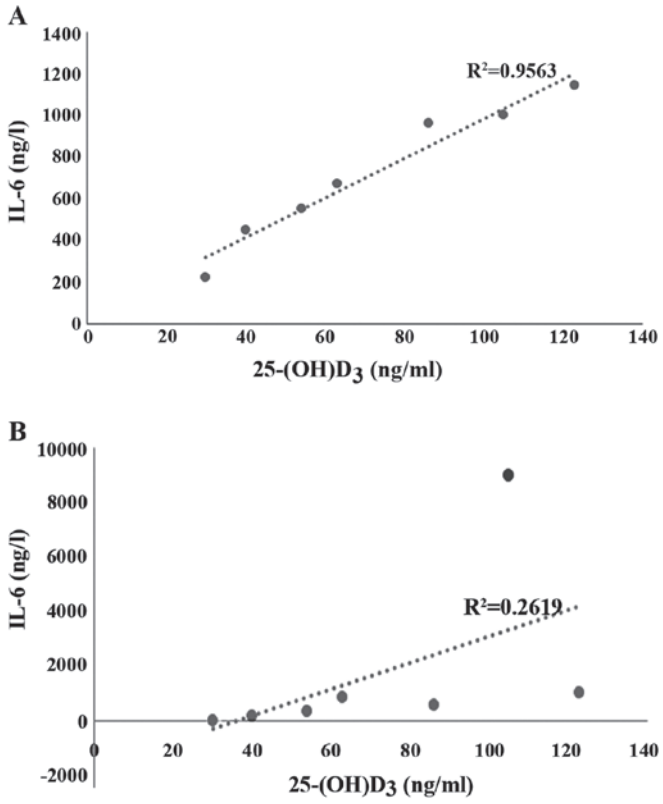

Figure 4. (A and B) Correlation between the 25-(OH)D3 gene expression and that of IL-6 in the observation group prior to and after treatment. 25-(OH)D_3, serum 25-hydroxyvitamin D_3; IL-6, interleukin-6.

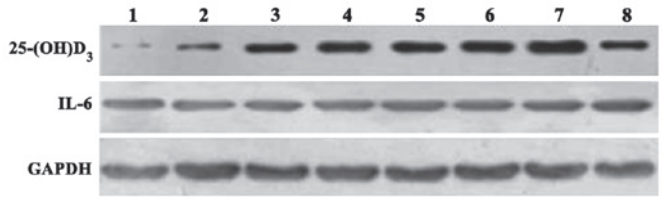

Figure 5. Correlation between the disease course and level of 25-(OH)D_3 and IL-6 in patients with Kawasaki disease. 25-(OH)D_3, serum 25-hydroxyvitamin D_3; IL-6, interleukin-6.

significant correlation was established for levels after treatment of 25-(OH)D_3 and IL-6 (Fig. 4B).

Correlation between 25-(OH)D_3 and IL-6 and the disease course in the observation group. Our results showed that the pathogenic condition of patients in the observation group was aggravated with time and the level of 25-(OH)D_3, and also increased with time whereas the content of IL-6 protein remained unchanged in the course of the disease (Fig. 5).

Discussion

Kawasaki disease is a serious threat to infant health and growth (11). Related data (12) have shown that despite advances in studies and treatment of Kawasaki disease, the exact pathogenesis of this disease remains to be determined and there is therefore no effective treatment method that can be utilized (13). It has been shown that T cells in patients with Kawasaki disease are abnormally active (14). Additionally, it has been established that T cells, to a large extent, can enhance the immunity and improve the organism's resistance against various illnesses (15). The results obtained from related studies have shown that the overactive T cell can interact with mononuclear cells and stimulate the overexpression of different cytokines and inflammatory substances that can damage the blood vessels (16,17). To shed some light on the subject of the pathogenesis of Kawasaki disease's, it is extremely important to investigate any possible connection between this disease and the level of cytokines.

Vitamin D is an important signal molecule in human body that is involved in the regulation of cellular signal substances such as calcium and phosphorus, cytomembrane elements, and immunoreaction such as inhibiting the abnormal proliferation of body T/B lymphocyte (18). Kudo *et al* (4) demonstrated that in coronary endothelial cells, 25-(OH)D_3 inhibited the release of IL-8 and the expression of cell adhesion molecule-1 induced by TNF-α. However, it could not affect the level of IL-6. It has been shown that a specific amount of 25-(OH)D_3 is needed for regulating KD coronary artery inflammation (19).

By measuring the 25-(OH)D_3 and IL-6 mRNA and protein in different groups prior to treatment, we showed that serum 25-(OH)D_3 and IL-6 levels in the feverish control group were significantly lower than those in the normal control group. The level of 25-(OH)D_3 in the serum of children in the observation group was significantly higher than the level observed in the normal control group. The level of 25-(OH)D_3 in the feverish control group was lower than the level of IL-6 in the normal children, but the difference was not statistically significant ($P>0.05$) while the content of 25-(OH)D_3 in the observation group was significantly higher than the content of serum IL-6 in the normal children. The results also revealed that before treatment 25-(OH)D_3 and IL-6 levels were positively correlated ($R^2=0.9563$). The same comparison did not reveal any significant correlation between 25-(OH)D_3 and IL-6 levels after treatment. By measuring 25-(OH)D_3 in the serum of children patients with Kawasaki disease in different courses of disease, we showed that the pathogenic condition aggravated with time and the level of 25-(OH)D_3 was also increased significantly. This finding suggested that 25-(OH)D_3 is involved in the occurrence of Kawasaki disease in children and may be involved in the aggravation of the disease to some extent.

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