Significance of Plasma Circulating Endothelial Microparticles Combined with Von Willebrand Factor in Coronary Injury of Kawasaki Disease

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Research Article

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Abstract

Background:The greatest complication of Kawasaki disease (KD) is coronary artery injury, and the requirement for early diagnosis and treatment is paramount. Thus, markers of vascular endothelial injury are of important clinical significance.

Methods:According to our diagnostic criteria, blood samples were collected from 43 patients with KD, who were then divided into coronary artery lesions (CALs) and non-CALs (NCALs) groups according to their Z-score. As the control group, an additional 26 blood samples were collected from healthy children. Flow cytometry (FCM) and enzyme-linked immunosorbent assays (ELISA) were used to detect the expression levels of plasma endothelial microparticles (EMPs) and von Willebrand factor (vWF).

Results:The expression levels of plasma CD31+/CD42b-EMPs, CD105+/CD54+EMPs, and vWF were higher in children with KD than those in the control group, and the differences were statistically significant ($P<0.05$). Also, the expression levels of CD31+/CD42b-EMPs, CD105+/CD54+EMPs and vWF in those in the CALs group at the acute and subacute stages were significantly higher than those in the NCALs group ($P<0.05$). Furthermore, receiver operating characteristic (ROC) curve analysis revealed that the area under the curve (AUC) of CD31+/CD42b-EMPs combined with vWF was 0.896, which indicates a higher diagnostic value in predicting CALs in children with KD.

Conclusions:In our study, expression levels of EMPs and vWF are expected to be used for early diagnosis, and which are associated with coronary artery injury in KD.

Introduction

Kawasaki disease (KD) is now considered to be one of the most common causes of childhood acquired heart disease in developed countries. Among the cardiovascular complications of KD, coronary artery lesions (CALs) are especially likely to result in long-term morbidity and mortality[1–2]. During KD, the cascade amplification effect of cellular factors causes the infiltration of circulating immune effector cells into blood vessels, leading to vascular endothelial injury [3–4]. The long-term effects of endothelial injury and persistent vascular response damage in children with KD is concerning, irrespective of coronary artery disease status.

The identification of KD patients with CALs is challenging, and the use of predictive biomarkers holds great promise. Circulating endothelial microparticles (EMPs) are small membranous vesicles released from endothelial cells under various stimuli. EMPs can regulate numerous pathophysiological processes, such as thrombosis, angiogenesis, endothelial function and endothelial reactivity [5–6]. During activation and apoptosis, EMPs with different phenotypes are released from endothelial cells in varying quantities. Indeed, with the exception of CD144, most of the remaining surface antigens are not specifically expressed on endothelial cells[7]. In order to improve the detection sensitivity, we ultimately selected two different groups of antigenic markers (CD31+/CD42b- and CD105+/CD54+). CD31 can be expressed on the

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hills and specific T cell subsets. Under abnormal

conditions, CD31 can induce angiogenesis and promote cellular migration[8]. CD42b is one of the primary surface glycoproteins of the platelet membrane, though it is not expressed by endothelial cells[9]. CD105 is a transmembrane glycoprotein which is abundantly expressed in the tissue vascular endothelium and is up-regulated in proliferating endothelial cells[10]. CD54 is one of the primary adhesion molecules expressed by vascular endothelial cells. It can result in the activation of several pro-inflammatory signaling cascades and promoting an inflammatory response at the blood vessel wall[11].

von Willebrand factor (vWF) is a glycoprotein polymer present in the plasma and on platelets, and is produced by endothelial cells, megakaryocytes and in the subcutaneous tissue. Under normal circumstances, circulating vWF remains in a resting state. When the body is stimulated, vWF is involved in thrombus formation via multiple different pathways[12–13]. Various studies have suggested that the level of vWF is an indirect measure of the effects of multiple different stimuli on endothelial cells, and that levels can reflect activation or apoptosis of endothelial cells [14].

Studies have also shown that children with KD have obvious endothelial cell dysfunction and a prethrombotic hypercoagulable state, and continuous development of these pathologies can ultimately lead to CALs[15]. EMPs and vWF can activate inflammatory reactions and coagulation mechanisms through different signaling pathways, resulting in endothelial injury. We speculate that EMPs and vWF play an important role in the occurrence of KD, and are involved in the formation of CALs. Therefore in the present study, we evaluated the expression levels of EMPs and vWF in KD patients, and determined their diagnostic value in KD-associated coronary artery injury.

Material And Methods

Patients and blood samples

According to the KD diagnostic criteria of American Heart Association (AHA) in 2017[2], we consecutively included 43 children diagnosed and hospitalized in Affiliated Hospital of Nantong University from October 2018 to August 2019. All the children did not treated with intravenous immunoglobulin (IVIG), aspirin or glucocorticoid before admission. Blood samples were obtained from patients at the acute, subacute and convalescent stages (The staging is based on the 8th edition of "Zhufutang Practical Pediatrics"). All the children underwent echocardiography, and the Z-score was calculated according to the examination results. The Z-score calculation method is based on the analysis of the database proposed by Kobayashi *et al.*[16] (<http://raise.umin.jp/zsp/>). Patients with the Z-score ≥ 2 was defined as the CALs group. The clinical information of all KD patients were collected, including gender, age, height, weight, fever duration, time to start IVIG, whether IVIG is unresponsive, whether of using glucocorticoid, white blood counts (WBC), neutrophil ratio (NE%), platelet (PLT) counts, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), alanine aminotransferase (ALT). At the same time, we included 26 age-matched (6 months $\leq$ age $\leq$ 5 years) healthy controls from outpatient department of pediatrics. The study protocol was approved by the Institution Review Board of The Affiliated Hospital of Nantong University. Informed

Blood samples were drawn into EDTA-K2 anticoagulation vacuum tube (2ml) and were centrifuged for 10 min at 160g to prepare platelet-rich plasma (PRP). The PRP was then centrifuged for 6 min at 1000g to prepare platelet-poor plasma (PPP). The 300μL upper layer of PPP and other 300 μL PPP were stored in -80°C until use.

Flow Cytometry (Fcm)

The PPP (65uL) in a polypropylene tube was incubated with 20μL of PE anti-CD31 (BD, USA), 20μL of FITC anti-CD42b (BD, USA), 20uL of APC anti-CD54 (BD, USA) and 5 uL PerCP-Cy™5.5 anti-CD105 (BD, USA) for 15 min in the dark. At the same time, we took another PPP (65uL) in a polypropylene tube incubated with equal amount of isotype control antibodies for 15min in the dark. Then 370μL of PBS was added and the sample was ready for flow cytometry on a FACSCalibur flow cytometer using CellQuest Pro software (BD, USA). EMP levels was defined as the ratio of CD31+/CD42b- EMPs and CD54+/CD105 + EMPs.

Enzyme-linked Immunosorbent Assay (Elisa)

The plasma vWF levels in children were measured using ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd.) according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were conducted with SPSS software. The measurement data are expressed as mean $\pm$ standard deviation ($\bar{x} \pm s$). Comparisons between 2 groups were carried out using t test, and those among multiple groups were carried out using one-way ANOVA. Chi-square test was used for counting data, and Spearman's rank correlation analysis was used for the correlation of each index. Independent risk factors of CALs in children with KD were predicted by binomial multivariate Logistic regression analysis. Receiver operating characteristic curve (ROC) was drawn and area under curve (AUC) was calculated to evaluate the efficacy of association index in predicting CALs. The significance level was two-sided and set at $P < 0.05$.

Results

Plasma EMP levels

The expression levels of plasma CD31+/CD42b-EMPs and CD105+/CD54 + EMPs in KD children at the acute, subacute and convalescent stages were significantly higher than those in the control group ($P < 0.05$) (Fig. 2a-b). The levels of CD31+/CD42b- and CD105+/CD54 + EMPs in CALs group patients at the acute and subacute stages were higher than those in the NCALs group, and the differences between the

two groups were statistically significant ($P < 0.05$). There were no significant differences in the levels of these indicators between the two groups in patients at the convalescent stage ($P > 0.05$) (Fig. 2c-d).

Plasma Vwf Levels

The expression levels of plasma vWF in KD children at the acute, subacute and convalescent stages were higher than those in the control group, and these results were statistically significant ($P < 0.05$) (Fig. 3a). Furthermore, plasma vWF levels of those in the CALs group at the acute and subacute stages were significantly higher than those in the NCALs group ($P < 0.05$). However, there were no significant differences at the convalescent stage ($P > 0.05$) (Fig. 3b).

Correlation analysis of plasma EMPs, vWF and laboratory indicators (WBC, NE%, PLT, ALT, CRP, ESR)

The results of Spearman’s rank correlation analysis(Table 1) showed that the levels of CD31+/CD42b- EMPs in KD patients at acute stage was positively correlated with the NE% ($r = 0.459$, $P = 0.024$), and the levels of CRP ($r = 0.459$, $P = 0.024$). The levels of CD105+/CD54 + EMPs were also positively correlated with the NE% ($r = 0.438$, $P = 0.032$) and the CRP levels ($r = 0.437$, $P = 0.033$), and the levels of vW were positively correlated with those of CRP ($r = 0.457$, $P = 0.025$).

Table 1
Correlation analysis of plasma EMPs, vWF and laboratory test indexes of KD patients at the acute stage

	CD31+/CD42b-EMPs(%)		CD105+/CD54 + EMPs(%)		vWF(ng/ml)	
Laboratory indexes						
	r	P-value	r	P-value	r	P-value
WBC (10 ⁹ /L)	0.360	0.084	0.391	0.059	0.383	0.065
NE (%)	0.459	0.024	0.438	0.032	0.350	0.093
PLT (10 ⁹ /L)	-0.038	0.861	-0.048	0.823	-0.332	0.113
ALT (U/L)	0.253	0.233	0.049	0.821	0.224	0.294
CRP (mg/L)	0.506	0.012	0.437	0.033	0.457	0.025
ESR (mm/h)	0.155	0.469	0.129	0.549	0.140	0.513

Risk Factors For Cals Were Analyzed Based On Z-value

KD patients were assigned to the CALs or NCALs group according to their Z-score, and the risk factors for CALs were a fever duration > 10 days, no response to IVIG and CRP ≥ 100mg/l (Table 2).

Table 2
Risk factors for KD complicated with CALs at the acute stage

Parameter	n	CAL	NCAL	χ^2	P
		(n = 11)	(n = 32)		
Sex					
male	22	7(63.6)	15(46.9)	0.92	0.337
female	21	4(36.4)	17(53.1)		
Age(years)					
<1	9	4(36.4)	5(15.6)	2.311	0.315
1~5	27	6(54.5)	21(65.6)		
>5	7	1(9.1)	6(18.8)		
Fever duratio(days)					
≤ 10	39	8(72.7)	31(96.9)	5.65	0.017
>10	4	3(27.3)	1(3.1)		
Course of disease when IVIG is enabled(days)					
≤ 10	41	10(90.9)	31(96.9)	0.657	0.418
>10	2	1(9.1)	1(3.1)		
Non-respond to IVIG					
Yes	6	4(36.4)	2(6.2)	6.183	0.013
No	37	7(63.6)	30(93.8)		
Glucocorticoid using					
Yes	1	0(0.0)	1(3.1)	0.352	0.553
No	42	11(100.0)	31(96.9)		
WBC($10^9/L$)					
<10	11	4(36.4)	7(21.9)	0.914	0.633
10~20	27	6(54.5)	21(65.6)		
>20	5	1(9.1)	4(12.5)		
NE(%)					
<80	30	6(54.5)	24(75.0)	1.624	0.203

Parameter	n	CAL	NCAL	χ^2	P
≥ 80	13	5(45.5)	8(25.0)		
PLT($10^9/L$)					
<300	20	4(36.4)	16(50.0)	0.612	0.434
≥ 300	23	7(63.6)	16(50.0)		
CRP(mg/L)					
<100	33	5(45.5)	28(87.5)	8.108	0.004
≥ 100	10	6(54.5)	4(12.5)		
ESR(mm/h)					
≤ 100	41	10(90.9)	31(96.9)	0.657	0.418
>100	2	1(9.1)	1(3.1)		
ALT(U/L)					
≤ 45	32	7(63.6)	25(78.1)	0.903	0.342
>45	11	4(36.4)	7(21.9)		

Logistic Regression Analysis

According to the above results, the plasma CD31+/CD42b- EMPs, CD105+/CD54 + EMPs and vWF values in KD patients at the acute stage were significantly different between the CALs and the NCALs groups ($P < 0.05$). The model was assessed using the Hosmer Lemeshow test ($P > 0.10$) and the model fitting effect was good. The data (Table 3) showed that the plasma CD31+/CD42b- EMPs ratio and the vWF levels were independent risk factors for CALs.

Table 3
Binomial multivariate logistic regression analysis.

Parameter	b	β	χ^2	P-value	95%CI	
CD31+/CD42b-EMPs	0.301	0.14	4.594	0.032	1.026	1.779
CD105+/CD54 + EMPs	0.07	0.115	0.373	0.541	0.857	1.343
vWF	0.034	0.015	5.375	0.020	1.005	1.066
constant	-23.236	8.470	7.526	0.006		

ROC curve analysis

Subsequently, the CD31+/CD42b-EMPs and vWF values were further analyzed, and ROC curves were constructed for CALs diagnosis. The results (Table 4 and Fig. 4) revealed that the AUC for CD31+/CD42b-EMPs was 0.814 [95% confidence interval (CI) = 0.674 ~ 0.954; cutoff value = 27.20%], and 0.830 for vWF (95% CI = 0.685 ~ 0.975; cutoff value = 283.49 ng/ml); the sensitivity and specificity of predicting CALs were 81.8 and 75.0% respectively. The AUC for CD31+/CD42b- EMPs combined with vWF was 0.896 (95% CI = 0.819 ~ 0.994), and the sensitivity and specificity were 90.9 and 78.1% respectively. These results indicate that CD31+/CD42b- EMPs combined with vWF are of higher diagnostic value for the prediction of CALs ($P < 0.001$) than either of the two factors alone.

Table 4
ROC curve analysis

Parameter	AUC	β	P-value	95%CI	
CD31+/CD42b-EMPs	0.814	0.071	0.002	0.674	0.954
vWF	0.830	0.074	0.001	0.685	0.975
CD31+/CD42b-EMPs + vWF	0.906	0.045	<0.001	0.819	0.994

Discussion

Here, we show relatively high expression levels of EMPs and vWF in the plasma of children at different stages of KD, as well as their roles in CALs formation .

Numerous studies have linked EMPs to a variety of different vascular diseases, such as severe hypertension, acute lung injury, and acute coronary syndrome[17–19]. CD31+/CD42b- is a group of classic marker combinations used to identify EMPs. The current studies found that the levels of CD31+/CD42b-EMPs were elevated in various immune inflammatory pathologies and cardio-cerebrovascular diseases which lead to the apoptosis or endothelial cell activation[20–21]. In addition,Yu *et al.*[22] revealed that the expression levles of CD31 and CD105 by EMPs were significantly increased following apoptotic stimulation, while the levels of CD54, CD62E and CD106 were significantly increased during the activation. The similar results of this study showed that the expression levels of CD31+/CD42b- and CD105+/CD54 + EMPs in patients with acute, subacute and convalescent stage KD were found to be significantly higher than those in the control group ($P < 0.05$). The levels of EMPs were also decreased after treatment, compared with the pre-treatment values, which indicated that the activation and apoptosis of endothelial cells occurred during the development of KD.In the absence of vascular injury, vWF does not interact with circulating platelets[23]. However, during injury, vWF is able to bind to components of the subendothelial connective tissue, promoting the adhesion of platelets to collagen, and thus inducing platelet aggregation[24]. Therefore, theincreased level of vWF in the blood is considered to be an indicator of endothelial injury. Some scholars believe that the high level of vWF in patients with KD reflects a significant acute phase response[25]. In this study, the results illustrated that the levels of vWF in the acute, subacute and convalescent stages of KD was significantly higher than that

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different stages of KD; the highest levels were

observed in the acute stage, with a downward expression trend following treatment. vWF may serve as a marker of KD and reflect the level of inflammation in the acute stage .

At the same time, this study also defined CALs according to the Z-score. The Z-scores of the coronary artery which adjusted by body surface area were more suitable as a reference standard to objectively describe coronary arteries in children with KD, which may improve the early recognition rate of CALs. The comparison results of expression levels of EMPs and vWF in the CALs and NCALs groups suggested that EMPs and vWF may be involved in the formation of CALs. Spearman's rank correlation analysis were used to assess the association between plasma EMPs, vWF and several clinical laboratory indexes in the acute stage of KD. The EMPs levels were positively correlated with the NE% and the CRP value. The vWF level was also positively correlated with CRP. CRP is able to stimulate the production of EMPs by human umbilical vein endothelial cells in vitro[26], and other studies have found that an increase in vWF is positively correlated with an increase in inflammatory markers such as CRP and interleukin-6[27]. Similar results were obtained in the this study. Thus we speculated that the aforementioned factors may be involved in KD-associated inflammation in the acute phase. By means of combining the results we concluded that EMPs and vWF were associated with the development and progression of coronary artery injury in KD.

In this study, we collected the clinical data of these 43 KD patients, and analyzed the clinical risk factors for developing CALs. Yamashita *et al.*[28] retrospectively analyzed the data of 31380 children with KD, and concluded that being male and < 1 year old, late hospital admission, atypical cases and an IVIG non-response were significant risk factors for CALs formations. Furthermore, Flores-Montes *et al.*[29] suggested that anemia, and being male and < 2 years old were risk factors for CAL. Other previous studies have concluded that an increase in CRP and ESR, and a decrease in albumin (ALB) increase the risk of developing CALs[30–31]. In future studies, additional clinical data needs to be collected and evaluated to support our conclusions. It is interesting to note that auxiliary examinations should be conducted at the earliest possible opportunity. For children who show indicator risk factors, we should make the additional monitoring of meaningful laboratory indicators for effective evaluation which may reduce the number of missed diagnosis. At the same time, more frequent echocardiography should be performed to evaluate the coronary arteries in a timely manner.

At present, there is no specific global laboratory index for the diagnosis of KD. In the clinic, atypical manifestations frequently result in missed diagnosis and misdiagnosis, and treatment untimely results in cardiovascular complications in the later stage; therefore, early diagnosis is very important for children with KD. Binomial multivariate logistic regression analysis was used to analyze CD31+/CD42b-EMP, CD105+/CD54 + EMPs and vWF levels, which revealed that the levels of CD31+/CD42b-EMP and vWF were independent factors for predicting coronary artery injury in patients with KD. A ROC curve was generated to evaluate the diagnostic efficiency of combined and single indexes to predict the efficiency of CALs diagnosis. The results showed that the AUC value of the combined indexes was higher than that of the two individual indexes, and that the sensitivity and specificity were also higher. It can therefore be

concluded that the combined detection of CD31+/CD42b-EMPs and vWF can improve the sensitivity and specificity of KD-associated CALs diagnosis.

In summary, the results of the present study indicate that EMPs and vWF are significantly increased in the acute stage of KD, particularly in patients with CALs. The increase in EMPs and vWF may be associated with the development and progression of coronary artery injury in KD. The combined evaluation of the above two indexes has high diagnostic value, and can be used as an effective predictor of CALs formation in children with KD.

Conclusion

The present paper aims to find out the potential early diagnosis indicators in coronary artery injury process for KD. The results of the present study indicated that EMPs and vWF were significantly increased in the acute stage of KD, particularly in patients with CALs. The increasing in EMPs and vWF may be associated with the development and progression of coronary artery injury in KD. The combined evaluation of the above two indexes has high diagnostic value, and can be used as an effective predictor of CALs formation in children with KD.

Declarations

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Conflicts of interest

The authors declare that they have no potential conflict of interest.

Availability of data and material

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Code availability

Not applicable

Authors' contributions

Conception and design: Jianmei Zhao, Zhiyuan Tang. Experiment performing, data collection, data analyze and manuscript: Mingye Cheng, Tao Chen. All authors have read and approved the final version of

Ethics approval

This study has been approved by the Ethics Committee of the Affiliated Hospital of Nantong University (review number: 2018-K021).

Consent for publication

Written informed consent for publication was obtained from all participants.

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Figures

Figure 1

(a-b) isotype control of CD31/CD42b and CD105/CD54. (c-f) The lower right area shows the CD31+/CD42b-EMP in KD children at the acute, subacute and convalescent stages and control group. (g-j) The upper right area shows CD105+/CD54+EMP in KD children at the acute, subacute and convalescent stages and control group.

Figure 2

(a-b) Comparison of plasma CD31+/CD42b- and CD105+/CD54+EMPs expression levels (%) between different groups. (c-d) Comparison of plasma CD31+/CD42b- and CD105+/CD54+EMPs expression levels (%) between KD groups. */#/\$ $P \leq 0.05$, **/###/

$P \leq 0.01$, ***/###/

\$ $P \leq 0.001$; ns, $P \leq 0.05$. KD^a, the acute stage of KD; KD^s, the subacute stage of KD; KD^c, the convalescent

Figure 3

(a) Comparison of plasma vWF expression levels (ng/ml) between different groups. (b) Comparison of plasma vWF expression levels (ng/ml) between KD groups. */#/\$ $P \leq 0.05$, **/###/ $P \leq 0.01$, ***/###/ $P \leq 0.001$; ns, $P \geq 0.05$.

$P \leq 0.01$, ***/###/

$P \leq 0.001$; ns, $P \geq 0.05$.

Figure 4

Individual factors and combined factors prediction of ROC curve of CALs. The blue line represents CD31+/CD42b-EMPs; The green line represents vWF; The yellow line represents CD31+/CD42b-EMPs+vWF.