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Correlation between serum cystatin C, thrombomodulin and T lymphocyte subsets in children with Henoch-Schonlein purpura

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Henoch-Schönlein Purpura (HSP) is a systemic small vessel, leucocytoclastic vasculitis disease affecting children, and the abdominal pain and joint pain are its classic triad. Here, we studied the correlation between serum cystatin C (CysC), thrombomodulin (TM), and T lymphocyte subsets in HSP cases, and the diagnostic values of these indices. A total of 120 HSP children treated at The 940th Hospital of Joint Logistics Support Force of the Chinese People's Liberation Army from January 2019 to May 2020 participated in this study. Another 64 healthy children receiving routine physical examination in the same time range were enrolled as a control group. In the early morning of the next day after admission, the cubital venous blood was collected. The serum levels of CysC were measured by immunoturbidimetry, the TM was detected using ELISA, while the T lymphocyte subsets were detected by flow cytometry. Univariate and multivariate logistic regression analyses were performed to determine the susceptibility factors. The receiver operating characteristic (ROC) curves were plotted to evaluate the predictive values of CysC, TM and T lymphocyte subset alone and their combination for HSP. The serum levels of CysC, TM, and cluster of differentiation 8 ($CD8^+$) of HSP children significantly increased and their $CD3^+$, $CD4^+$ levels and $CD4^+/CD8^+$ ratio significantly decreased compared with those of control group ($P<0.05$). The serum levels of CysC and TM were significantly correlated negatively with $CD3^+$, $CD4^+$ levels and $CD4^+/CD8^+$, whereas positively with $CD8^+$ level ($P<0.05$). Diet, infection, drugs, exercise-induced tiredness, air pollution, family environment, family inheritance, age, winter and spring were the susceptibility factors for children with HSP. The diagnosis using CysC, TM and T lymphocyte subsets had an AUC of 0.901, sensitivity of 93.1%, and specificity of 90.2%. In conclusion, the combined monitoring of serum CysC, TM, and T lymphocyte subsets in children with HSP can raise the accurate diagnosis rate.

Keywords: Immunoglobulin A, Microthrombus, Nephritis, Vasculitis

Henoch-Schonlein purpura (HSP), also known as allergic purpura, frequently-occours among pre-school and school going children. It is clinically manifested as skin purpura, joint pain, abdominal pain and kidney injury. Systemic hemorrhagic vasculitis typified by the infiltration of microvascular peri-ventricular eosinophils and neutrophils is the main pathological change of this disease[1]. Bacteria, drugs, and food can all break the balance between the immunity and inflammatory responses, and may cause dysfunction of the autoimmune system leading to induction of HSP[2]. Allergic microvasculitis is an important pathological change of HSP, and one of the main features of vasculitis is vascular endothelial barrier dysfunction. Endothelial cells and extracellular matrix constitute the vascular endothelium[3].

At present, different studies have shown that oxidative stress and micro-inflammation are closely related to HSP, while long-term medication cannot eliminate free radicals but further inhibits the chain reaction in lipid oxidation[4].Vascular deposition of IgA-contained immune complexes suggest the possibility of immune-mediated mechanisms, however, actual mechanism of HSP is still unclear[5]. In most of the most cases, HSP is a self-limiting disease and treatment is supportive, however, repeated attacks of HSP can cause purpuric nephritis which further develops into renal failure[6]. As a common micro-vascular hemorrhagic disease caused by allergies in childhood, HSP not only has symptoms of rash, abdominal pain, joint pain, but also can cause different degrees of kidney damage. The CysC is a non-glycosylated basic protein with a relatively small molecular mass and a total of 122 amino acids, also known as cysteine protease inhibitor C. Almost all nucleated cells can continue to transcribe and express CysC at a constant rate; and are not histologically specific and widely present in the body[7].

Tan*et al.*[8] found that hypertension, cystatin C, and tubular atrophy, interstitial fibrosis can serve as

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independent risk factors HSP nephritis. The immunoglobulin A (IgA), particularly IgA1 and complement C3 deposition arterioles, capillaries, and venules walls, and the immune inflammatory reaction are involved in the vascular endothelial cell injury of HSP patients[9]. Studies have displayed that dysfunction of T cells subsets leads to the pathogenesis of HSP, thus, the key factors that are involved in Th cell differentiation may act as potential novel targets for the prevention and treatment of HSP[10]. Yin & Chen[11] alsoshowed that HSP may be related to abnormal immune functions, IgA immune complex deposition, T lymphocyte dysfunction, abnormal lymphocyte activation, B interleukin (IL)-17, IL-21, IL-6,visfatin, IL-8 secretion, and intestinal mucosal barrier dysfunction.

In children with enhanced platelet aggregation and hyperfibrinogenemia, the blood is hypercoagulable and forms microthrombus damaging the vascular endothelium first[11]. Besides, the number and function of T lymphocyte subsets in the peripheral blood of children with HSP also change[13]. In this context, here we studied the changes of serum cystatin C (CysC), thrombomodulin (TM), and T lymphocyte subsets in children with HSP, aiming to explore the correlations between them and to provide clinically meaningful evidence for the HSP diagnosis.

Material and Methods

Baseline clinical data

A total of 120 children with HSP treated at The 940th Hospital of Joint Logistics Support Force of the Chinese People's Liberation Army from January 2019 to May 2020 were enrolled as the observation group, including 70 boys and 50 girls aged 3 to 18 years old, (8.93±3.16 years) on average. There were 20 cases with unclear causes for onset, 30 cases with dietary causes, 10 cases of drug allergy, 48 cases of infection, and 12 cases of exercise-induced tiredness. Forty children had only skin involvement, and the remaining 80 cases were complicated with abdominal pain, joint pain or kidney disease. Another 64 healthy children receiving routine physical examination in the same time period were enrolled as a control group, consisting of 35 boys and 29 girls aged between 3 to 18 years old, (9.08±3.14 years) on average. The two groups had comparable baseline clinical data such as age and gender ratio (P>0.05). This study was approved by the ethics committee of the Institute; and written informed consent were obtained from the guardians of all the children enrolled in this study.

Inclusion/exclusion criteria

Inclusion criteria: The clinical symptoms, signs and laboratory test results were in accordance with the diagnostic criteria for HSP in Zhu Futang Practice of Pediatrics[14]; 3 to 18 years old; first onset. *Exclusion criteria:* Complication with diseases of vital organs such as the heart, liver, and/or lung, infectious diseases or other systemic diseases; with mental disorders; use of hormone drugs or immunomodulators in the past two weeks.

Sample collection and detection

Fasting cubital venous blood (5mL) was collected, placed in an anticoagulant tube containing sodium citrate, and the sample blood was centrifuged to separate the serum. The serum level of CysC was determined by immunoturbidimetry using Olympus AU2700 Automated Biochemical Analyzer (Tokyo, Japan). The TM level was measured by ELISA kit purchased from R&D Systems (Minneapolis, MN, USA) strictly following the manufacturer's instructions. The T lymphocyte subsets, including cluster differentiation (CD)3$^+$, CD4$^+$, CD8$^+$ T lymphocytes, and CD4$^+$/CD8$^+$ ratio, were calculated with BD FACS Calibur flow cytometer (San Jose, CA, USA) and kit obtained from Beijing Zhongshan Golden Bridge Biological Technology Co. Ltd. (China).

Statistical analysis

All data were statistically analyzed by SPSS16.0 software (Chicago, IL, USA). The categorical data were expressed as mean ± SD, and inter-group comparisons were performed by the independent t test. The correlations between CysC, TM, and T lymphocyte subsets were subjected to Pearson's analysis. The predictive values of CysC, TM, and T lymphocyte subsets for pediatric HSP were assessed by plotting receiver operating characteristic (ROC) curves. Susceptibility factors were determined by stepwise multiple regression analysis. A value of P<0.05 was considered statistically significant.

Results and Discussion

Serum CysC and TM levels

The serum levels of CysC and TM of the observation group were significantly higher than those of the control group (P<0.05) (Table 1).The

Table 1 — Variations observed serum cystatin C (CysC) and thrombomodulin (TM) levels in observation and control group

Index	Observation group (n=120)	Control group (n=64)	t	P
CysC (mg/L)	1.05±0.12	0.71±0.05	21.664	<0.001
TM (μg/L)	110.43±12.44	50.25±4.38	37.442	<0.001

results were consistent with the previously reported peer-reviewed literatures[15].Wang *et al.*[16] found that the CysC level rose with increasing renal pathological grade of children with purpuric nephritis which showed that the serum levels of CysC in children with HSP were higher than those in healthy controls (P<0.05). Studies have shown that the level of CysC in plasma remains relatively stable, independent of age, gender, and diet. Therefore, CysC gene is therefore referred to as the housekeeping gene[7]. The kidney is the only organ that removes Cystatin C from the blood circulation and its excretion is almost exclusively through the glomerular filtration, and then is re-absorbed by the proximal convoluted tubules and rapidly catabolized without returning into the blood circulation. Hence, CysC is often used to assess the renal function[17]. With aggravated kidney damage, the clearance ability of CysC is weakened, thereby elevating its serum level[18].

Also, persistent raised levels of plasma TM may be closely related to the renal pathology in children with HSP, and may reflect the damage of renal blood vessels to a certain extent[19,20]. In this study, the serum levels of TM in children with HSP were significantly higher than those in the healthy controls (P<0.05). Similarly, serum thrombomodulin were raised in patients with HSP and were associated with renal histological changes in HSP patients[21]. Cayci *et al.*[22] found no significant differences (P>0.05) between patient and control groups in the levels of von Willebr and factor and thrombomodulin. TM is a glycoprotein attached to its surface, synthesized by vascular endothelial cells, and is also a co-factor required for thrombin activation of anticoagulant protein C[3]. When the vascular endothelium is damaged, TM can be detached or hydrolyzed from the surface of the endothelial cells, thereby increasing its concentration in plasma[23]. In addition, TM can also bind thrombin so that the anticoagulant protein C anticoagulation system is activated, which in turn, regulates the platelet function and blood coagulation processes[24].

Serum levels of T lymphocyte subsets

HSP usually causes immune disorders and inflammatory reactions caused by exogenous stimuli, thereby increasing the permeability of systemic small blood vessels and capillaries and causing clinical symptoms such as allergic bleeding[25]. The body's immune defense system, T lymphocytes can be divided into sub-groups with different immune functions, $CD3^+$ represents total T lymphocytes; $CD4^+$- and $CD8^+$ labeled T lymphocyte subsets. In current study, compared with the control group, the observation group had significantly lower $CD3^+$, $CD4^+$ levels and $CD4^+/CD8^+$ ratio (P<0.05). However, the level of $CD8^+$ was found to be significantly higher in the observation group than that of the control group (P<0.05) (Table 2). Similarly, another study found that proportions of $CD3^+$ and $CD4^+$ cells in patients with HSP were significantly lower than healthy children leading to a significant reduction in the ratio of $CD4^+/CD8^+$[26]. Another study also reported that during the acute phase following antigen challenge, in HSP animals and pediatric patients, compared to the controls, the frequency of $CD4^+$lymphocytes significantly decreased (P<0.05), while the frequency of $CD8^+$ T cells increased lead in to a significant decrease in the ratio of CD4/CD8[27].

Both $CD3^+$ and $CD4^+$play extremely important role(s) in the immune response[28,29]. Under physiological conditions, the two T cell subsets of $CD4^+$ and $CD8^+$ are in dynamic equilibrium and maintain the stability of the body's immune system. The proportion of T lymphocyte subsets in the serum of children with HSP is dysregulated which worsens as the disease progresses[30]. Herein, decreased serum levels of $CD3^+$, $CD4^+$, and $CD4^+/CD8^+$ in observation group, and increased $CD8^+$levels indicate that the immune function seems to be undermined upon HSP.

Correlations between serum CysC, TM, and T lymphocyte subsets

The serum levels of CysC and TM were significantly correlated negatively with $CD3^+$ (r=–0.571, P<0.001; r=–0.816, P<0.001), $CD4^+$ levels (r=–0.443, P<0.001; r=–0.732, P<0.001), and $CD4^+/CD8^+$ (r=–0.542, P<0.001; r=–0.734, P<0.001), whereas positively with $CD8^+$(r=0.474, P<0.001; r=0.564, P<0.001) (Fig. 1).

Evaluation of predictive values of CysC, TM and T lymphocyte subsets for HSP by ROC curves

The ROC curve analysis showed that the diagnosis using CysC + TM + T lymphocyte subsets had an AUC of 0.901, sensitivity of 93.1%, and specificity of 90.2% (Fig. 2 & Table 3). Under normal condition, the

Table 2 — Variations observed in serum levels of T lymphocyte subsets in observation and control group

Index	Observation group (n=120)	Control group (n=64)	t	P
$CD3^+$ (%)	59.56±3.15	67.28±3.08	15.955	<0.001
$CD4^+$(%)	29.56±1.69	44.48±1.54	58.790	<0.001
$CD8^+$(%)	29.46±1.57	21.47±1.64	33.306	<0.001
$CD4^+/CD8^+$	0.96±0.18	1.94±0.18	33.163	<0.001

CD4/CD8 ratio maintains dynamic balance and keeps the body's immune function stable, however, when its ratio is higher than normal, the immune state is hyperactivity, and the ratio decreased, or even inversion indicates poor immune system. Immunological dysfunction in children with HSP nephritis manifests reduced CD3- and CD4 cells, and also as lowered immunological function of cells. These changes lead to immune deficiency and stimulate the secretion of inflammatory factors[30].

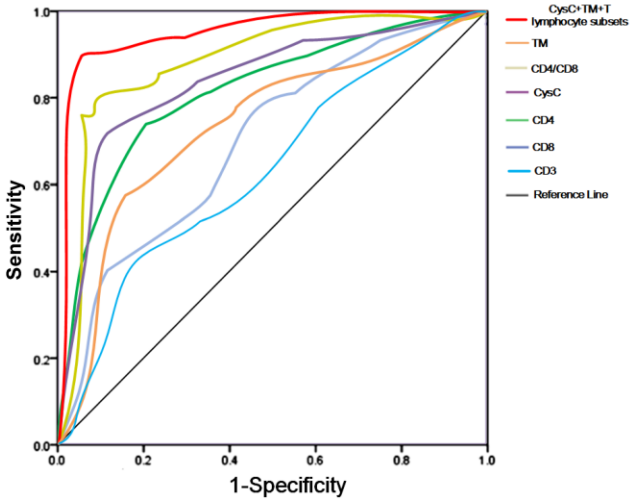

Fig. 1 — Correlations between serum cystatin C (CysC) and thrombomodulin (TM) with T lymphocyte subsets such as $CD3^+$, $CD4^+$, and their ratio $CD4^+/CD8^+$

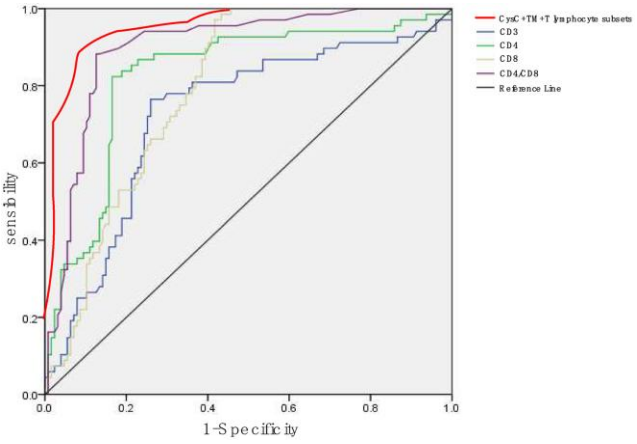

Fig. 2 — Receiver operating characteristic (ROC) curves for the predictive values of cystatin C (CysC), thrombomodulin (TM), and T lymphocyte subsets ($CD3^+$, $CD4^+$, $CD4^+/CD8^+$) for children with Henoch-Schonlein purpura (HSP)

Susceptibility factors for HSP

Multivariate logistic regression analysis revealed that diet, infection, drugs, exercise-induced tiredness, air pollution, family environment, family inheritance, age, winter and spring were the susceptibility factors for HSP (Table 4). Among them, most cases seem to be affected by infections, especially respiratory infection. Intake of milk, seafood, eggs, beef, and mutton were the main dietary susceptibility factors. The cases with exercise-induced tiredness were mostly attributed to sports activities[31].

An investigation into etiological factors showed that HSP occurs most frequently between the age of 6 to 7 years old, 88.3% were <10 years old, only 3 cases were <2 years old, and the mean age was 6.6 ± 1.6 years[32]. Another study also documented that the boys are affected more often than the girls[33].Some accounts had reported the trigger events including vaccinations, insect bites, and drugs have been connected with HSP[32].

Table 3 — ROC curve analysis of CysC, TM and T lymphocytes and analysis in children with HSP

Index	AUC	95%CI	Cut-off value	Sens.* (%)	Spec.# (%)
CysC (mg/L)	0.859	0.801~0.944	0.85	86.5	83.4
TM (μg/L)	0.842	0.762~0.921	93.89	87.4	85.7
$CD3^+$ (%)	0.657	0.613~0.804	60.11	75.6	71.5
$CD4^+$ (%)	0.808	0.722~0.883	30.35	82.6	80.4
$CD8^+$ (%)	0.754	0.722~0.864	29.56	80.2	76.3
$CD4^+/CD8^+$	0.878	0.804~0.938	1.06	90.8	87.5
CysC+TM+T lymphocyte subsets	0.901	0.822~0.976	15.42	93.1	90.2

[*Sensitivity; #Specificity. ROC, receiver operating characteristic; AUC, area under the curve; CI, confidence interval; CystC, cystatin C; TM, thrombomodulin; and CD, cluster differentiation]

Table 4 — Logistic regression analysis of multiple susceptibility factors for children with HSP

Factor	β	SE	Wald value	P value	OR value (95%CI)
Diet	-2.454	0.575	5.123	0.002	2.324 (1.743~2.856)
Infection	-1.653	0.818	4.667	<0.001	3.447 (2.067~4.123)
Drug	-0.509	0.535	7.713	0.001	2.211 (1.609~3.514)
Exercise-induced tiredness	-2.317	0.482	6.889	0.015	1.979 (1.391~2.046)
Air pollution	-1.765	0.754	10.013	0.024	4.954 (3.585~6.713)
Family environment	-2.146	0.645	4.645	0.020	3.013 (1.461~4.623)
Family inheritance	-0.893	0.483	7.432	0.011	1.878 (1.245~2.987)
Age	-1.742	0.924	5.228	0.005	4.954 (3.851~7.142)
Season	-2.423	0.614	8.432	0.002	2.567 (1.113~3.289)

[β, susceptibility factor; SE, standard error; OR, odds ratio; and CI, confidence interval]

Conclusion

Results of this study revealed that the serum levels of CysC and TM are significantly correlated negatively with $CD3^+$, $CD4^+$ levels and $CD4^+/CD8^+$ ratio, whereas, positively with $CD8^+$ level. The area under the curve (AUC) of $CD4^+/CD8^+$ was the largest, followed by those of CysC, TM, $CD4^+$, $CD8^+$, and $CD3^+$, respectively. The diagnosis using CysC, TM and T lymphocyte subsets had an AUC of 0.918, sensitivity of 93.5%, and specificity of 90.1%. Diverse etiological factors may have various clinical symptoms and rare complications in Henoch-Schonlein purpura (HSP) patients. Periodic monitoring of the levels all such parameters can potentially augment the optimal diagnosis rate and avoid the susceptibility factors. With early diagnosis, the risk factors for HSP patients might help in early intervention for HSP treatment.

Conflict of Interests

Authors declare no conflict of interests.

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