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Original Article

Association of SH2 domain-containing protein 1A, immunoglobulins and T lymphocyte subsets with Epstein-Barr virus infections

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ABSTRACT

Background. We aimed to analyze the levels and associations of SH2 domain-containing protein 1A (SH2D1A), immunoglobulins and T lymphocyte (TL) subsets in children with Epstein-Barr virus (EBV) infections.

Methods. Sixty children with EBV infections admitted from January 2019 to January 2022 were selected, including 29 cases of infectious mononucleosis (IM group) and 31 cases of chronic active EBV infections (CAEBV group). Another 42 healthy children undergoing physical examination in the same period were selected as a control group. Their changes in SH2D1A, immunoglobulins and TL subsets (CD3+, CD4+ and CD8+) were compared.

Results. The levels of CD3+, CD4+ and CD8+ in the IM group were higher than those of the control group (P<0.05), while they were lower in the CAEBV group than those of the control and IM groups (P<0.05). The levels of SH2D1A, signaling lymphocyte activation molecule (SLAM) and SLAM-associated protein (SAP) were significantly higher in the IM group than those in the control and CAEBV groups (P<0.05). The CAEBV group had decreased protein expressions of SLAM and SAP compared with those of the IM group. SH2D1A was positively correlated with immunoglobulin A, immunoglobulin G and TL subsets (CD3+, CD4+ and CD8+) (P<0.05).

Conclusions. Detecting SH2D1A, immunoglobulins and TLs contributes to the diagnosis and differentiation of IM and CAEBV.

Key words: clinical diagnosis, Epstein-Barr virus, immunoglobulins, SH2 domain-containing protein 1A, signaling lymphocyte activation molecule, SLAM-associated protein, T lymphocyte subsets.

Epstein-Barr virus (EBV) was first found in the cell culture of malignant lymphoma in African children. As a double-stranded DNA virus of the human herpes virus (HHV) family, EBV is the first human B-lymphotropic virus found to be closely related to tumor occurrence and progression.[1] Humans are the only host of HHV and are generally susceptible, HHV is mainly transmitted through the oral-oral route.[2] As reported in previous studies, the positivity rate of serum anti-EBV antibodies in adults is over 95%, and most people are infected in childhood and become virus carriers for life.[3] In China, the positivity rate of anti-EBV antibody is up to 80.7% in children aged 3-5 years old, and >90% of children are seropositive by the age of 10.[4] After being infected with EBV, individuals can establish latent infections in human memory B lymphocytes. Stable immune functions can be obtained without the onset or clinical symptoms of disease in most adults, while the immune functions of children, especially infants, are still in the developmental stage. Therefore, children are more prone to deterioration and death than adults.[5]

Children with EBV infections may have no symptoms or mild respiratory symptoms. Infectious mononucleosis (IM) is the typical

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manifestation of initial EBV infections, which is an immunopathological and self-limiting disease. Most children with IM have a good prognosis and low mortality rates after symptomatic treatment, but systemic complications may occur in a few children with the progression of disease, thus causing severe IM.[6] EBV infections can also progress to lymphoma and chronic active EBV infections (CAEBV), often leading to a poor prognosis. The pathogenesis of CAEBV remains unclear, but the clonal expansion of EBV-infected T cells and NK cells has recently been implicated in the progression of CAEBV.[7,8]

Located at the X chromosome q25, SH2 domain-containing protein 1A (SH2D1A) is mainly present in activated T cells and NK cells, and its gene mutation/deletion can result in fatal EBV infections and EBV-associated hemophagocytic syndrome.[9-11] Moreover, signaling lymphocyte activation molecule (SLAM) plays an important role in T/B lymphocyte proliferation by enhancing T/B cell activation. SLAM-associated protein (SAP) encoded by SH2D1A can regulate different cells including T, NK and B lymphocytes in the immune system to exert normal immune functions.[12,13] SAP is required for NK cytotoxicity, T/B cell interaction, and T cell-dependent humoral immune responses. However, SAP deficiency affects SLAM-mediated levels of T/B lymphocytes, and leads to loss of function and excessive amplification of activation signals, ultimately causing the massive proliferation of B lymphocytes.[14,15]

Consequently, the levels of SH2D1A, immunoglobulins and T lymphocyte (TL) subsets in EBV-infected children were measured and compared with those in healthy children, aiming to clarify the pathogenesis of EBV infections and to provide references for clinical diagnosis and treatment.

Material and Methods

General data

This study has been approved by the ethics committee of our hospital, and written informed consent has been obtained from the caregivers of all children. Sixty children with EBV infections admitted from January 2019 to January 2022 were selected, including 29 cases of IM (IM group) and 31 cases of CAEBV (CAEBV group). The diagnostic criteria for IM were as follows: Fever, sore throat and hoarseness, hepatosplenomegaly, cervical lymphadenopathy >1 cm, peripheral blood atypical T cells >10, and EBV-VCA-immunoglobulin M (IgM) positivity. There were 16 boys and 13 girls aged 0.30-11.25 years old, with the mean of (4.25±1.24) years in the IM group. CAEBV was diagnosed based on the criteria of Hue et al.[16]: Persistent or recurrent IM-like symptoms such as fever, lymphadenopathy and hepatosplenomegaly, abnormal anti-EBV antibody levels (*i.e.* elevated anti-VCA/anti-early antigen [EA] antibody titers), and an increase in EBV-DNA copy number or EBV-DNA-positive tissues. There were 17 boys and 14 girls aged 0.45-11.09 years old, with a mean of (4.22±1.09) years in the CAEBV group. Additionally, healthy subjects undergoing physical examination during the same period were selected as the control group. After the cases with EBV infections and history of immunosuppressant use within 3 months were excluded, 42 children were finally enrolled, including 21 boys and 21 girls aged 0.37-11.15 years old, with a mean of (4.25±1.06) years. The gender and age compositions had no significant differences and were comparable among the three groups (P>0.05) (Table I).

Sample collection and treatment

Before antiviral therapy and/or immune therapy in the hospital, fasting blood samples were drawn from children at 7 a.m., and placed into EDTA-anticoagulated tubes. One portion of the sample was treated to generate serum for ELISA, antibody dilution test and hemagglutination (HA) test. The other portion was used to isolate peripheral blood mononuclear cells (PBMCs). Then PBMCs were added to the total RNA extraction reagent TRIzol and stored in a refrigerator at -80°C.

Table I. Baseline clinical data.

Group	n	Gender		Age (year)	Clinical symptoms at admission				
		Boy	Girl		Fever	Sore throat	Cervical lymphadenopathy	Hepatosplenomegaly	Eyelid edema
Control	42	21	21	4.25±1.06					
IM	29	16	13	4.25±1.24	23	28	29	15	23
CAEBV	31	17	14	4.22±1.09	28	26	30	17	27
P		0.883		0.241	0.233	0.102	0.329	0.809	0.419

CAEBV: Chronic active Epstein-Barr virus, IM: infectious mononucleosis.

TL subset assay

TL subsets including $CD3^+$, $CD4^+$ and $CD8^+$ were detected using the FC50 flow cytometer (BC, USA) according to the manufacturer's instructions. The supporting software CXP2.0 system was used for image analysis.

Immunoglobulin assay

The levels of IgA, IgG and IgM in the serum obtained by venous blood centrifugation were detected by immuno-scatter turbidimetry using the kits purchased from Siemens (Germany).

Quantitative RT-PCR

The primer amplification conditions were optimized in the pre-experiment. PCR amplification (a total reaction volume of 20 μL) was repeated 3 times in strict accordance with the instructions of the SsoFast EvaGreen RT-PCR kit. The relative expression of SH2D1A mRNA was calculated by ΔΔC1.

Western blotting

NK cells ($CD3^-CD16^+CD56^+$) in the 6-well plate were washed once with PBS, digested with 500 μL of 0.25% trypsin-EDTA, and placed in EP tubes. After digestion was terminated by 1.5 mL of complete medium, the cells were thoroughly pipetted, and 250 μL of protein lysis buffer were added, followed by an ice bath for 30 min, during which the tube was shaken every 10 min. Following centrifugation at 12,000 r/min and 4°C for 10 min, the supernatant (total cell protein) was harvested and stored in a refrigerator at -40°C for later use. In strict accordance with the BCA protein assay kit, 10% separation gel and 5% spacer gel were prepared, and the protein sample was loaded, subjected to electrophoresis at 110 V for 100 min, and transferred onto an NC membrane. The membrane was then blocked, followed by incubation with primary antibodies at 4°C overnight, washing, incubation at room temperature for 2 h, and washing again. Finally, the image was developed with a gel imager.

Statistical analysis

SPSS 25.0 software was used for statistical analysis. The measurement data were expressed as ($\bar{x} \pm s$), and compared by the *t*-test. The count data were expressed as rate (%), and compared by the χ^2 test. Spearman's rank correlation analysis was conducted. P<0.05 was considered statistically significant.

Results

Immunoglobulin levels

There were significant differences in IgA and IgG among the three groups (P<0.05). The levels of IgA and IgG in the IM and CAEBV groups were significantly higher than those in the control group (P<0.05). The IgM level had no significant difference between the IM and CAEBV groups (P>0.05) (Table II).

Peripheral blood TL subsets

There were significant differences in TL subsets ($CD3^+$, $CD4^+$, and $CD8^+$) among the three groups (P<0.05). The levels of $CD3^+$, $CD4^+$ and $CD8^+$ in the IM group were higher than those in the

Table II. Immunoglobulin levels.

Group	n	IgA (g/L)	IgG (g/L)	IgM (g/L)
Control	42	2.59±0.67	9.69±2.15	1.31±0.41
IM	29	5.42±1.65[a]	14.27±3.98a	1.39±0.42
CAEBV	31	5.13±1.95[a]	14.17±4.47a	1.29±0.39
P		<0.001	<0.001	0.825

CAEBV: Chronic active Epstein-Barr virus, Ig: immunoglobulin, IM: infectious mononucleosis. [a]P<0.05 vs. control group.

Table III. Expressions of SH2D1A mRNA and related proteins.

Group	n	SH2D1A mRNA	SLAM	SAP
Control	42	5.65±2.67	1.88±0.26	0.26±0.12
IM	29	38.42±4.65[a]	4.38±0.31[a]	0.61±0.14[a]
CAEBV	31	5.13±3.95[a]	1.91±0.30[a]	0.25±0.13a
P		<0.001	<0.001	<0.001

CAEBV: Chronic active Epstein-Barr virus, IM: infectious mononucleosis, SAP: SLAM-associated protein, SH2D1A: SH2 domain-containing protein 1A, SLAM: signaling lymphocyte activation molecule. [a]P<0.05 vs. control group.

control group (P<0.05), while they were lower in the CAEBV group than those in the control and IM groups (P<0.05) (Fig. 1).

Expressions of SH2D1A mRNA and related proteins

Statistically significant differences were found in the levels of SH2D1A, SLAM and SAP among the three groups (P<0.05). The levels of SH2D1A, SLAM and SAP were significantly higher in the IM group than those in the control and CAEBV groups (P<0.05), while they had no significant differences between the control and CAEBV groups (P>0.05) (Table III).

Western blotting results of SH2D1A-related proteins

SH2D1A-related protein SLAM- and SAP-specific pre-stained protein bands were found at about 36, 130 and 20 kDa. The results of Western blotting showed that the expressions of SLAM and SAP were consistent with each other. Compared to the control group, the protein bands were obviously wider and most strongly stained, and the protein expression was up-regulated in the IM group. The CAEBV group had lightly stained protein bands and decreased protein expressions of SLAM and SAP compared with those of the IM group (Fig. 2).

Results of correlation analysis of immunoglobulins, TL subsets and SH2D1A mRNA expression

The change trends of immunoglobulins, TL subsets and SH2D1A mRNA expression were similar. According to Spearman's analysis, SH2D1A expression was positively correlated with IgA, IgG and TL subsets ($CD3^+$, $CD4^+$ and $CD8^+$) (P<0.05), whereas no significant correlation was found between IgM and SH2D1A mRNA expression (P=0.0833) (Fig. 3).

Discussion

The occurrence and progression of EBV infections depend primarily on the TL-mediated cellular immune response of the human immune system. $CD3^+$, $CD4^+$ and CD8 are the surface markers of T cells, effector T cells, and cytotoxic and suppressor T cells, respectively. Therefore, EBV infections are closely related to $CD3^+$, $CD4^+$ and $CD8^+$. The immune system is normally in a balanced state in two ways. On the one hand, suppressor T cells inhibit the activation of effector B cells and T cells, weakening the immune function of the human body. On the other hand, Th cells enhance the activation of B cells and T cells by releasing cytokines, thereby strengthening the immune function. A nationwide survey in

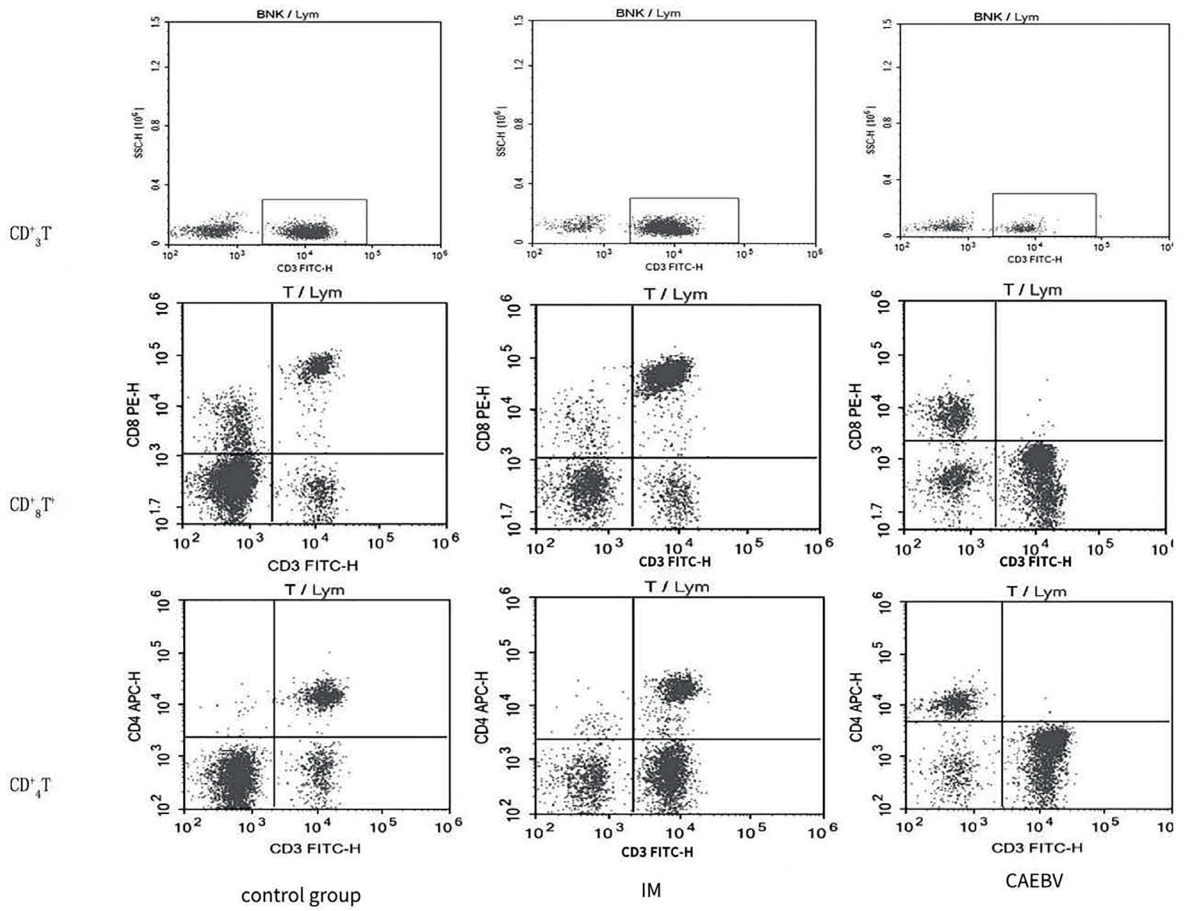

Fig. 1. Flow cytometry results of peripheral blood TL subsets.

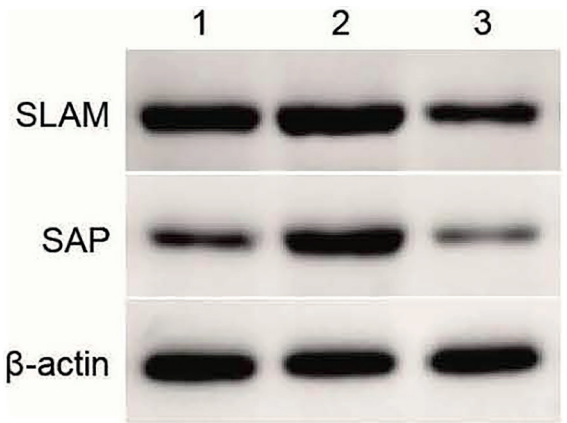

Fig. 2. Western blotting results of SH2D1A-related proteins SLAM and SAP. 1: Control group. 2: IM group. 3: CAEBV group.

Japan showed that CAEBV occurred in most of the 82 patients and its pathogenesis involved either T cells or NK cells, while B cells were involved in infections in only 2 patients.[17] After EBV infections occur, the intestinal mucosa and digestive system secrete a large number of immunoglobulins, so the body is in an infection-specific sensitization state and the immunoglobulin levels significantly increase. As a result, a series of biochemical reactions occur to prevent viral infection in the case of exposure to EBV antigens again. However, the association between humoral immunity and severity of EBV infections is unclear yet.[18,19]

In this study, the levels of $CD3^+$, $CD4^+$ and $CD8^+$ in the peripheral blood were significantly higher in IM children. Hence, strong TL reactions occur following EBV infections in IM children, thus leading to massive proliferation of TLs and abnormal levels of peripheral blood TLs. $CD8^+$ TL proliferation and activation can be found in about 80% of typical IM cases. In this

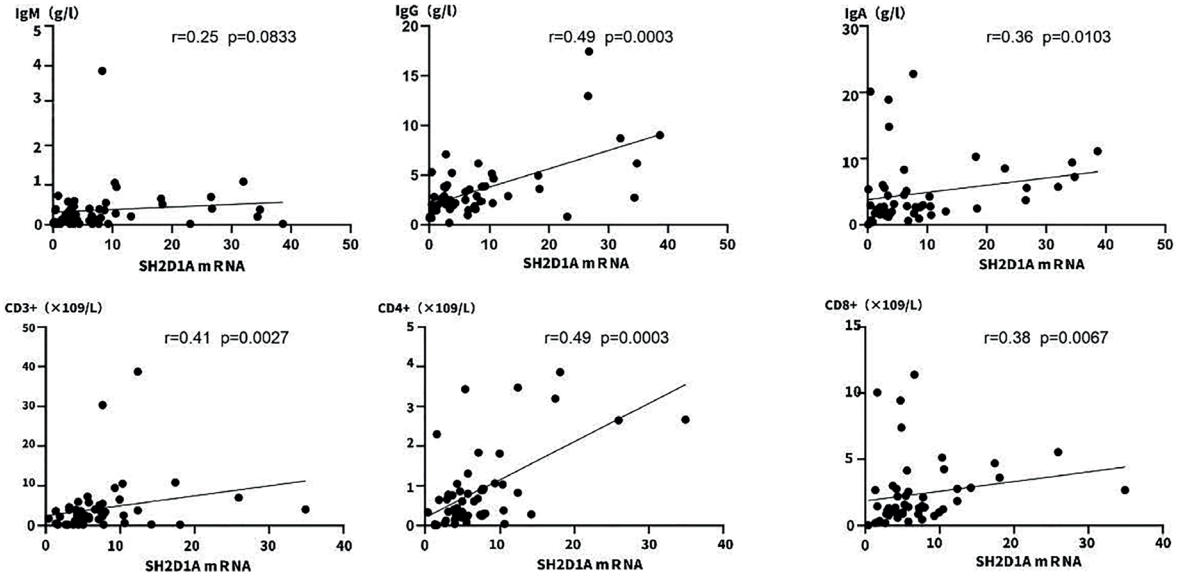

Fig. 3. Results of correlation analysis of immunoglobulins, TL subsets and SH2D1A mRNA expression.

study, the CAEBV group had lower levels of $CD3^+$, $CD4^+$ and $CD8^+$ than those of the control and IM groups ($P<0.05$). Probably, CAEBV children cannot control or eliminate EBV, and suffer from immune system disorders due to EBV immunodeficiency, which is the main mechanism of EBV infections progressing to CAEBV.

Upon stimulation by EBV, specific antibodies such as IgA, IgG and IgM antibodies are produced in lymphocytes and then involved in the immune response, resulting in associated immune expression.[20] In this study, the levels of IgA and IgG in IM and CAEBV groups were significantly higher than those in the control group ($P<0.05$), indicating that after infection with EBV, the cellular and humoral immunity became disordered, the levels of IgG and IgM increased, and immune imbalance occurred. The findings are consistent with those of Ye et al.[21]

We also found that the expressions of SH2D1A mRNA and related proteins SLAM and SAP significantly rose in IM children after EBV infections occurred, indicating severe cellular immune dysfunction. In contrast, the expressions of SH2D1A mRNA, SLAM and SAP had no obvious changes and were even at low levels in the CAEBV group. Unknown host genetic predisposition may be associated with the onset of CAEBV, and different characteristics of peripheral blood SH2D1A variants in CAEBV and IM children are of significance for early identification and diagnosis. Therefore, close attention should be paid to the possibility of delayed onset, persistent EBV infections and tumor progression in patients with significant decreases in the expressions of SH2D1A mRNA, SLAM and SAP. In addition, SH2D1A mRNA expression was positively correlated with IgA, IgG, $CD3^+$, $CD4^+$ and $CD8^+$ in children with EBV infections, suggesting that the expressions of SH2D1A and related proteins SLAM and SAP can reflect the immune function of EBV infections, as new immune-related molecular markers for the clinical diagnosis of IM and CAEBV.

In conclusion, the SH2D1A mRNA level in the peripheral blood significantly rises in children with EBV infections, which cause disorders of humoral immunity and cellular immunity. $CD8^+$ lymphocytes markedly proliferate in IM children, but lymphocytes decrease in CAEBV children. IM and CAEBV children have significantly increased levels of IgA and IgG.

The detection of SH2D1A, immunoglobulins and TLs contributes to the clinical diagnosis and differentiation of IM and CAEBV.

Ethical approval

This study has been approved by the ethic committee of our hospital, and written informed consent has been obtained from the caregivers of all children. The study has received ethical approval by Children's Hospital of Nanjing Medical University (202004024-1).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: PX, JZ, HM; data collection: PX, JZ, YX; analysis and interpretation of results: PX, JZ, YX; draft manuscript preparation: HM. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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