<u>Scientific Letter</u>

Cytomegalovirus Genotype Distribution among Postnatally Infected Infants: Association of Glycoprotein B, Glycoprotein N and Glycoprotein H Types with CMV-Associated Thrombocytopenia

To the editor.

Genotyping of CMV has mainly focused on gB, gN, and gH, which play a role in virus entry and may influence the infectivity or pathogenicity of CMV.[1,2] It has been hypothesized that genetic variation among CMV strains may underlie strain-specific clinical manifestations. Our previous research revealed that there might be a potential association between the genotypes of CMV and neonatal thrombocytopenia, and the detection of some specific genotypes might be indicative of severe manifestations in infants with CMV infection.[3,4] However, the study design and the criteria to define the study population (congenital and non-congenital cases) and the setting of the control group (CMV-associated thrombocytopenia and non-thrombocytopenic cases) were not clearly established. For this reason, we included patients classified on more unambiguous criteria, and the clinical data collected were complete and thoroughly detailed, which allows us to assess the association between genotypes and the outcome in the non-congenital population.

Methods

Definition. Symptomatic perinatal infection is defined as an infant presenting CMV associated symptoms and positive CMV detection in 3-12 weeks after birth. Symptomatic postnatal infection is referred to as an infant presenting CMV associated symptoms and positive CMV detection after 12 weeks of birth.[5] Altogether, in the present study, both of them referred to as CMV symptomatic postnatal infection. Moderately to severely symptomatic CMV disease is defined as multiple manifestations attributable to CMV infection. Mildly symptomatic CMV disease is characterized as by one or two isolated features of CMV infection that are mild and transient (e.g., mild hepatomegaly or a single measurement of low platelet count or raised levels of alanine aminotransferase).[6]

Patients. Thirty immunocompetent patients (median, two months; range, 25 days–11 months) with CMV-associated thrombocytopenia were analyzed, including 18 perinatal infections and 12 postnatal infections. Of these 30 patients, 20 were diagnosed with moderately to severely symptomatic CMV disease, and 10 were diagnosed with mildly symptomatic CMV disease. The clinical records of the 30 postnatally infected infants are summarized in **Table 1**. A group of 40 non-thrombocytopenic individuals, including 20 asymptomatic infants (median, two months; range, 25 days–10 months) and 20 patients (median, two months; range, 29 days–11 months) in CMV infections involving organ systems other than the hematopoietic system from the same period was also included in the study. Among 20 non-thrombocytopenic patients, respiratory symptoms including upper respiratory tract infection (20.0%, 4/20), bronchitis (25.0%, 5/20), and pneumonia (30.0%, 6/20) were the most common symptom at presentation. Other presentations were hepatitis (10.0%, 2/20), jaundice (25.0%, 5/20), and 1 case (5.0%, 1/20) had cholestasis. The baseline characteristics and clinical manifestations in these infants have been described in **Table 1** and **Table 2**.

Laboratory test for CMV infection. Patients were tested for CMV infection using serological CMV tests (IgM and IgG), viral culture, and real-time PCR for blood or urine samples. CMV IgM and CMV IgG were tested using an ELISA kit according to the manufacturer's instructions (DiaSorin S.p.A., Italy). For testing CMV in urine, urine samples were collected and cultured using the shell vial culture method (Chemicon, Temecula, CA, USA). According to the manufacturer's instructions (Daan Gene Company of Zhongshan University, China), fluorescence quantitative CMV-DNA kit was used to quantify of CMV-DNA. DNA level $> 10^3$ copies/ml indicated replication, which was considered positive

Table 1. The baseline characteristics and distribution of CMV genotypes among CMV-associated thrombocytopenia patients.

No.	Gender	Age	Other clinical manifestation	Severity of CMV disease	Mildly: one or two isolated manifestations / Moderately to severely: multiple manifestations	Onset of infection	Genotype		
							gB	gN	gH
1.	F	2m	-	Mildly		Perinatal	gB3	gN3	gH2
2.	M	2m	Bronchitis	Moderately to severely		Perinatal	gB1	gN4	gH1+gH2
3.	F	2m	Jaundice	Moderately to severely		Perinatal	gB1	gN1	gH2
4.	M	1m	Hepatitis	Moderately to severely		Perinatal	gB1	gN4	gH1
5.	M	1m	Pneumonia, Anemia	Moderately to severely		Perinatal	gB1	gN4	gH2
6.	M	2m	Bronchitis, Neutropenia, Anemia	Moderately to severely		Perinatal	gB1	gN4	gH1
7.	M	1m	Bronchitis, Cholestasis	Moderately to severely		Perinatal	gB3	gN4	gH1
8.	F	2m	-	Mildly		Perinatal	gB1	gN1	gH2
9.	F	1m	Upper respiratory tract infection	Moderately to severely		Perinatal	gB3	gN4	gH2
10.	M	25d	Jaundice, Neutropenia	Moderately to severely		Perinatal	gB1	gN2	gH2
11.	F	25d	Jaundice, Neutropenia	Moderately to severely		Perinatal	gB1	gN3	gH2
12.	M	2m	Neutropenia	Mildly		Perinatal	gB2	gN4	gH2
13.	M	29d	Jaundice	Moderately to severely		Perinatal	gB1	gN4	gH1
14.	M	1m	Anemia	Moderately to severely		Perinatal	gB1	gN2	gH2
15.	M	2m	Anemia	Moderately to severely		Perinatal	gB2	gN2	gH1
16.	M	1m	Anemia	Moderately to severely		Perinatal	gB1	gN3	gH1
17.	F	1m	Anemia, Hepatitis	Moderately to severely		Perinatal	gB1	gN2	gH2
18.	F	2m	Anemia,Gastrointestinal hemorrhage	Moderately to severely		Perinatal	gB1	gN2	gH1
19.	F	10m	Upper respiratory tract infection	Mildly		Postnatal	gB2	gN2	gH1
20.	M	7m	Bronchitis,Fever	Moderately to severely		Postnatal	gB1	gN4	gH2
21.	F	3m	Fever	Mildly		Postnatal	gB3	gN1	gH2
22.	M	9m	Neutropenia	Mildly		Postnatal	gB2	gN4	gH2
23.	M	8m	-	Mildly		Postnatal	gB1	gN1	gH1
24.	F	5m	-	Mildly		Postnatal	gB1+gB3	gN4	gH2
25.	M	9m	-	Mildly		Postnatal	gB2	gN3	gH2
26.	M	5m	Neutropenia	Mildly		Postnatal	gB1	gN3	gH1
27.	M	6m	Pneumonia, Anemia	Moderately to severely		Postnatal	gB1	gN4	gH1
28.	F	4m	Pneumonia,Anemia,Hepatitis, Neutropenia	Moderately to severely		Postnatal	gB1	gN4	gH1+gH2
29.	M	11m	Pneumonia, Anemia	Moderately to severely		Postnatal	gB2	gN2	gH2
30.	F	5m	Upper respiratory tract infection, Fever	Moderately to severely		Postnatal	gB1	gN2	gH2

in this study. CMV gB, gN and gH genotype analysis was done by nested PCR and restriction length polymorphism as reported.[7-9]

Statistical analyses. Statistical analysis was conducted using the SPSS ver. 21.0 software (SPSS, Inc., Chicago, IL, USA). Genotype distribution among postnatally infected patients, the relationship between the gB, gN, and gH genotypes and the severity of CMV infections were analyzed using the chi-square test for ratio comparison. Logistic regression analysis was used to assess the associated risk between particular genotypes and the variables of the study. A P-value of less than 0.05 was considered to be statistically significant.

Results

CMV Genotyping. The distribution of gB genotypes in this present study was gB1 (63.3%, 19/30), followed by gB2 (20.0%, 6/30) and gB3 (13.3%, 4/30). We also found 1 coinfection case (3.3%, 1/30) with 2 genotypes (gB1/gB3), no gB4 genotype was found. Notably, significantly higher frequency of gB1 (80.0%,16/20) was found in moderately to severely CMV infection infants compared to infants with mildly symptomatic CMV disease ($\chi2= 8.132$, $p = 0.043$) （**Figure 1**).

The overall distribution of individual genotypes in this study cohort was as follows: gN1(13.3%,4/30), gN2 (26.7%,8/30), gN3 (16.7%,5/30) and gN4 (43.3%,13/30). Comparing distribution in 20 asymptomatic infants with CMV infection, the gN1 (5.0%,1/20) was the less prevalent genomic variants in moderately to severely CMV infection patients ($\chi2=15.097$, $p = 0.002$) （**Figure 1**).

The gH1, gH2 and gH1/gH2 genotypes were distributed in 36.7% (11/30), 56.7% (17/30) and 6.7%

Table 2. The baseline characteristics and distribution of CMV genotypes among non-thrombocytopenic infants.

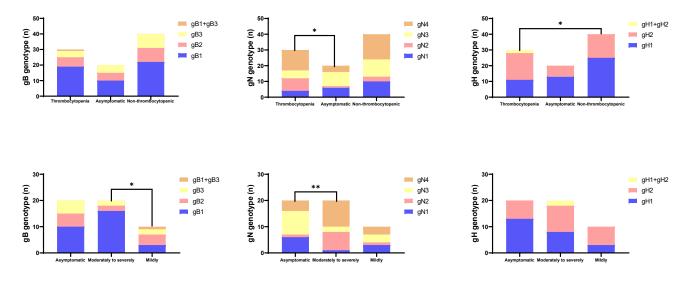| Asymptomatic CMV infection infants						Non-thrombocytopenic patients							
No.	Gender	Age	Genotype			No.	Gender	Age	Clinical manifestation	Onset of infection	Genotype		
			gB	gN	gH						gB	gN	gH
1.	F	2m	gB2	gN1	gH2	1.	F	3m	Upper respiratory tract infection	Postnatal	gB3	gN1	gH1
2.	M	1m	gB1	gN1	gH1	2.	M	2m	Bronchitis	Perinatal	gB1	gN4	gH1
3.	F	1m	gB3	gN4	gH1	3.	M	2m	Pneumonia	Perinatal	gB3	gN1	gH1
4.	F	28d	gB1	gN3	gH2	4.	F	1m	Hepatitis, Jaundice	Perinatal	gB2	gN1	gH1
5.	M	2m	gB3	gN3	gH1	5.	M	4m	Pneumonia	Postnatal	gB2	gN3	gH2
6.	M	2m	gB1	gN2	gH1	6.	F	5m	Upper respiratory tract infection	Postnatal	gB1	gN4	gH1
7.	M	1m	gB2	gN1	gH1	7.	M	2m	Jaundice	Perinatal	gB1	gN4	gH1
8.	M	2m	gB2	gN3	gH2	8.	F	2m	Pneumonia	Perinatal	gB1	gN4	gH1
9.	F	25d	gB1	gN3	gH1	9.	M	3m	Pneumonia	Postnatal	gB1	gN4	gH2
10.	M	1m	gB1	gN3	gH2	10.	F	5m	Bronchitis	Postnatal	gB1	gN3	gH2
11.	F	2m	gB3	gN1	gH1	11.	F	29d	Hepatitis, Jaundice	Perinatal	gB1	gN4	gH1
12.	F	2m	gB2	gN4	gH2	12.	F	5m	Bronchitis	Postnatal	gB2	gN4	gH1
13.	M	2m	gB2	gN4	gH1	13.	F	7m	Upper respiratory tract infection	Postnatal	gB1	gN4	gH1
14.	F	4m	gB3	gN3	gH1	14.	M	10m	Upper respiratory tract infection	Postnatal	gB1	gN4	gH2
15.	M	6m	gB1	gN3	gH2	15.	F	2m	Pneumonia	Perinatal	gB3	gN2	gH2
16.	M	10m	gB1	gN1	gH2	16.	M	29d	Jaundice	Perinatal	gB1	gN4	gH2
17.	F	3m	gB1	gN3	gH1	17.	M	1m	Jaundice, Cholestasis	Perinatal	gB1	gN1	gH1
18.	F	5m	gB1	gN4	gH1	18.	M	2m	Bronchitis	Perinatal	gB2	gN4	gH2
19.	F	5m	gB3	gN3	gH1	19.	F	2m	Bronchitis	Perinatal	gB3	gN2	gH1
20.	M	9m	gB1	gN1	gH1	20.	F	11m	Pneumonia	Postnatal	gB1	gN4	gH2

Figure 1. Distribution of CMV genotypes in different groups. *, p < 0.05; **, p < 0.01

(2/30) of the patients, respectively (**Figure 1**). Compared with the genotype distribution in non-thrombocytopenic infants, a greater frequency of gH2 in CMV-associated thrombocytopenia infants was noted with significant difference ($\chi2=6.269$, p = 0.044). No difference in the distribution of gH genotypes in symptomatic and asymptomatic patients, or in moderately to severely symptomatic CMV disease and mildly symptomatic CMV disease（**Figure 1**).

Genotype Association With CMV-associated thrombocytopenia and severity of CMV disease. In the logistic regression analysis, the gN2 [$p = 0.043$, with OR=4.598, 95%CI (1.052-20.098)] and gH2 [$p = 0.038$, with OR=2.933, 95%CI (1.060-8.117)] genotypes were associated with an elevated risk of developing thrombocytopenia. Besides, gB1 [$p = 0.022$, with OR=9.820, 95%CI (1.400-68.888)] represented the most virulent genotypes and was associated with severe manifestations in CMV-associated thrombocytopenia infants. Conversely, the gN1 [$p = 0.044$, with

OR=0.061, 95%CI (0.004-0.930)] genotype was associated with a reduced risk of severely symptomatic CMV disease.

Discussion. The gB of CMV likely plays a crucial role in viral entry into cells, the transmission of the virus from cell to cell, and the fusion of infected cells. It has been reported that the gB genotypes vary in their ability to stimulate cell-mediated or cytotoxic immune response.[10,11] Therefore, variations in gB are likely to have significant effects on the pathogenesis of CMV disease and the spectrum of host cells infected by the virus. Our previous studies also confirmed that the gB1 genotype had more virulence in infants with symptomatic CMV disease.[3,4]

But interestingly, in asymptomatic infected infants, gB1 was also the dominant genotype, and its genotype distribution was not significantly different from that of CMV-associated thrombocytopenia infants. Consequently, we speculate that CMV gB1 strains may elicit a severe immunopathological response that in some infants can control the symptoms of CMV and, in others, lead to CMV-associated thrombocytopenia with organ damage and disease manifestations. However, the virulence of gB1 in asymptomatic infants is negligible in relationship with a difference in the individual immune status.

The CMV strain with gN1 genotype may represent a less virulent virus phenotype, especially considering that the variation is a typical AD169-like glycoprotein, which is far away from CMV clinical isolates in immunology.[12-14] In our study, among CMV-associated thrombocytopenia infants (20 cases) who were classified as having moderately to severely symptomatic CMV disease, 17 had gN4 or gN2 genotypes and only one had a gN1 genotype, supporting the idea that gN1 genotype may be less virulent. In addition, compared with the genotype distribution in asymptomatic and non-thrombocytopenic infants in present study, thrombocytopenia occurred more frequently in infants infected with the CMV gN2 genotype, although the proportion of this genotype was less than that of gN4 in CMV-associated thrombocytopenia infants. The gN2 genotype was detected in 26.7% (8/30) of infants with CMV-associated thrombocytopenia and was associated with at least a 4-fold increased risk of developing thrombocytopenia. Our study is the first to demonstrate that a gN variant might be associated with a risk of CMV-associated thrombocytopenia in infants infected postnatally.

As we reported earlier, the gH2 genotype was associated with at least a 7-fold increased risk of developing CMV-associated thrombocytopenia among infants with congenital and perinatal infections[4]. After including postnatal infection and non-thrombocytopenic cases into the analysis, similar conclusions were reached.

Based on these cases, several general points can be highlighted. First, in regression analysis, the difference in the setting of the non-thrombocytopenic control group, which includes asymptomatic and symptomatic infants, may cause a discrepancy in results. Increasing the sample size and choosing an appropriate scale setting may reduce this discrepancy. Second, a specific cytomegalovirus genotype may show strong virulence in some CMV- related diseases, while in other CMV-related diseases or asymptomatic infants, it may not show corresponding characteristics of virulence. Finally, in addition to CMV gB, gN, and gH, CMV glycoprotein also includes gO, gM and gL. Six glycoproteins are essential for fibroblasts to enter CMV, and form glycoprotein complexes, gCI (gB), gCII (gM / gN), gcIII (gH / gL / gO) on the virus membrane.[15] In the study of a CMV- related disease, it is more reasonable to include all essential CMV glycoprotein genotypes into the analysis.

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