Occurrence of Human Cytomegalovirus Glycoprotein B Genotypes in Immunocompetent and Immunosuppressed Taiwanese Patients

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Key Words

Human cytomegalovirus · Glycoprotein B · Genotypes · Immunocompetence · Immunosuppression · Taiwanese

Abstract

Objectives: To investigate the differences of genotypic distributions among isolates between immunosuppressed and immunocompetent patients in a Taiwanese population.

Methods: Human cytomegalovirus (HCMV) isolates from 76 patients with adequate chart data were analyzed. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to define gB genotypes which include gB1 to gB4. The clinical data of the 76 patients were retrospectively collected by chart review and classified into an immunosuppressed (n = 32) or immunocompetent (n = 44) group. **Results:** Among the 32 immunosuppressed patients, the most commonly identified HCMV genotypes were gB1 (27/32, 84.3%) and gB3 (4/32, 12.5%). 59.1% (26/44) of the immunocompetent patients were infected by gB1 while 38.6% (17/44) of them were infected by gB3. The frequency of gB1 infection in the immunosuppressed group was significantly higher than that in the immunocompetent group (p = 0.025). However, there was no statistically significant dif-

ference between gB1 and gB3 distributions by clinical diagnosis within each group. **Conclusions:** Only gB1 and gB3 genotypes were identified in this Taiwanese population. Although there is no significant difference between clinical diagnosis and gB genotyping, gB1 infection is significantly more predominant in immunosuppressed patients.

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Introduction

Human cytomegaloviruses (HCMV), members of the *Herpesviridae* family, have large (220–240 kbps) and complex viral genomes composed of double-stranded DNA [1, 2]. This important pathogen is capable of establishing HCMV diseases, ranging from asymptomatic to life-threatening.

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HCMV diseases are related to host immune status. Congenitally infected babies can be born with 'cytomegalic inclusion disease' or have asymptomatic infections, while some sequelae were noted during follow-up. Among immunocompetent hosts, HCMV infection causes asymptomatic infection, fever, infectious mononucleosis, or acute viral hepatitis. For immunosuppressed hosts, acute HCMV infection also causes a wide spectrum of disease involving specific organs or causing constitutional disturbances. In recipients of bone marrow grafts, pneumonitis is the major fatal disease of HCMV infections. HCMV retinitis, esophagitis, colitis, encephalitis, and polyradiculopathy are diseases that commonly occur among patients with AIDS [3].

The HCMV genome encodes numerous glycoproteins, of which gB, gH and gN are the most abundant. The gB, gH, and gN genes are all utilized for genotyping [1]. Many genetically different strains of HCMV exist within the human population.

Glycoprotein B is essential for both in vivo and in vitro viral replication and it also plays a role in viral-host interaction. The polymorphisms of gB interfere with HCMV pathogenesis. Experimental evidence showed that different viral strains are associated with differing abilities to replicate in endothelial and smooth muscle cells, and have differing abilities to cause immunosuppression. Nevertheless, the exact mechanisms by which these events occur remain unknown [4].

Glycoprotein B is encoded by the ORF UL55 gene, with several genetic polymorphisms. There are four major gB genotypes (gB1 to gB4) and additional non-prototypic genotypes are also described [5].

Many studies widely investigated the association between different gB genotypes and their virulence, viral cell tropism, clinical manifestations, and outcomes [6–20]. Nevertheless, previous studies pointed out inconsistent results regarding different gB subtypes and their distributions. For example, gB1 is related to acute graft rejection in transplant recipients [2]. Woo et al. [6] and Lukacs et al. [21] found a high prevalence of gB1 in congenital infections. In contrast, Yan et al. [12] found that gB3 genotype was more prevalent in congenital infections than in postnatally infected children. Additionally, mixed infections with multiple gB genotypes are associated with severe clinical manifestations [22, 23].

Although a previous study [24] indicated that only gB1 and gB3 were detected among Taiwanese patients, there are no data regarding gB genotypes and their clinical manifestations. Therefore, this study was performed to correlate gB genotypes with clinical disease presentations.

Materials and Methods

Samples

A total of 125 clinical samples from 113 patients were identified as HCMV-positive by viral culture in Taipei Veterans General Hospital from 2005 to 2006. All the isolates were stored at -70° . Among the 12 extra samples, there were 4 repeated samples from a serial follow-up of a patient, 2 from another 3 patients, and 1 from the other 2 patients.

Clinical specimens included throat swabs ($n = 48$), urine ($n = 18$), bronchoalveolar lavage ($n = 7$), and others ($n = 3$) including peritoneal dialysate, blood and vitreous fluid, respectively.

Because not all cultivatable viruses replicated in a single cell line, all clinical specimens sending to virology laboratory were immediately inoculated into four different cell culture media simultaneously including MRC-5 (human lung embryonal fibroblast cells), MDCK (Madin-Darby canine kidney cells), HEP-2 (continuous human epidermoid cancer cells) and RD (human rhabdomyosarcoma cells).

Review of Charts

Of the 113 patients, 85 had sufficiently complete chart data to be included in the analysis. Patient age, sex, underlying diseases, and clinical diagnosis were recorded. For further data analysis, patients were classified into two groups: the immunosuppressed group included those with hematological malignancies with or without stem cell transplantation, post-solid organ transplantation, autoimmune diseases treated with immunosuppressant agents. Immunocompetent hosts were defined as patients without underlying documented immunodeficiency or immunosuppression. Since congenital HCMV infection was considered as a different disease entity, patients <3 months of age ($n = 9$) were excluded. Thus, there were 76 patients analyzed in this study.

Ethical approval for this study was granted by the Human Investigation Review Board of the Taipei Veteran General Hospital.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

HCMV-DNA was extracted from frozen clinical isolates using the Qiaamp Blood Mini Kit (Qiagen Inc., Chatsworth, Calif., USA) according to the manufacturer's recommendations. DNA samples were resuspended in 200 μ l of diethylpyrocarbonate-treated water. The amplification reaction was performed according to the following protocol. Oligonucleotide primers, each with a concentration of 1.5 μ M/ μ l, were added to the polymerase chain reaction (PCR) mixture. The latter contained 27.75 μ l of triple distilled H_2O , 10 μ l of 5 $\times$ Go Taq buffer, 3 μ l of $MgCl_2$ (25 mM), 1 μ l of 10 mM each dNTP, and 1.25 U Taq DNA polymerase per 50 μ l of reaction mixture. The primers that we used were gB1319 (TGGAACCTGGAACGTTTGGC) and gB1604 (GAAACGCGCGGCAATCGG). The amplified PCR product was about 300 bp [25]. The reaction mixture consisted of 45- μ l aliquots and 5- μ l DNA samples. PCR was as the following: initial denaturation at 95° for 6 min, denaturation at 95° for 5 min, annealing at 55° for 2 min and extension at 72° for 1 min. A final extension was performed at 72° for 7 min. DNA from the HCMV AD169 strain was used as the positive control and normal human plasma was used as a negative control.

For the identification of gB genotypes, an aliquot of the amplified product was digested with the restriction enzymes *Hinf*I and

RsaI. The respective genotypes were determined by restriction fragment length polymorphism (RFLP) analysis. Restriction fragments were detected by electrophoresis on 2% agarose gels. The gels were stained with ethidium bromide and viewed on an ultraviolet transilluminator. The HCMV clinical isolates were then classified as gB1 to gB4 [25].

Sequencing

If RFLP failed to differentiate the genotype, the PCR products were subjected to sequencing analysis (7 isolates).

Statistical Analysis

Correlation analyses to correlate HCMV gB genotypes and patient age, sex, clinical diagnosis, and host immune status were conducted. The χ^2 method was applied, and *p* values <0.05 were considered statistically significant.

Results

Demographic Data

Among the 76 patients with sufficient clinical information, 32 patients were grouped as immunosuppressed while the other 44 were in immunocompetent group. There were 27 female and 49 male patients. As to age distribution, 97.7% (43/44) of the immunocompetent patients were <16 years. In contrast, only 18.8% (6/32) of immunosuppressed patients were <16 years. A statistically significant difference (*p* < 0.01) was noted.

Sequencing and Genotyping Results

The sequencing results showed that all the 7 isolates that could not be digested by *RsaI* belonged to gB1 genotype. Serial samples from the same patient all had the same genotyping results. Therefore, only one genotyping result for each patient was counted.

Among the 76 patients, 53 were infected by gB1 (69.7%), 21 by gB3 (27.6%), and 2 mixed gB1 and gB3 (2.6%). The most common gB genotype in the immunosuppressed group was gB1 (27/32, 84.3%), followed by gB3 (4/32, 12.5%). For the immunocompetent patients, the most common genotype was gB1 (26/44, 59.1%), followed by gB3 (17/44, 38.6%). The frequency of HCMV gB1 was significantly higher in the immunosuppressed group than in the immunocompetent group (*p* = 0.025).

The distributions of gB genotypes in different types of specimens are shown in table 1. Most specimens were from throat swab or sputum (48/76, 63.2%). There were no statistically significant differences between the distributions of gB genotypes from different specimens.

The distribution of gB genotypes among different underlying diseases in immunosuppressed patients is shown

Table 1. Glycoprotein B1 (gB1) and B3 (gB3) distributions by specimen type

	gB1	gB3	Mixed gB1 and gB3
Urine (n = 18)	14/18	3/18	1/18
Throat swab/sputum (n = 48)	30/48	17/48	1/48
Bronchoalveolar lavage (n = 7)	6/7	1/7	–
Others ¹ (n = 3)	3/3	–	–

¹ Others: peritoneal dialysate (1), blood (1), and vitreous fluid (1).

Table 2. Glycoprotein B1 (gB1) and B3 (gB3) genotype frequencies by underlying immunosuppressive disease group (n = 32)

	gB1	gB3	gB1+gB3
Post-autologous SCT (n = 5)	5/5	–	–
Post-allogeneic SCT (n = 15)	12/15	2/15	1/15
Malignancy without SCT (n = 5)	5/5	–	–
Post-solid organ transplantation (n = 1)	–	1/1	–
Acquired immunodeficiency syndrome (n = 1)	1/1	–	–
Other immunosuppressive status ¹ (n = 5)	4/5	1/5	–
Total (n = 32)	27/32	4/32	1/32

SCT = Stem cell transplantation.

¹ Other immunosuppressive status included systemic lupus erythematosus, chronic obstructive pulmonary disease, idiopathic thrombocytopenic purpura status post-splenectomy and adult-onset Still's disease.

in table 2. The most common underlying immunosuppressive disease in this study is post-stem cell transplantation (n = 20) among which 85% (17/20) were infected by gB1.

Clinical diagnoses of both the immunosuppressed and immunocompetent groups and their gB distributions are shown in table 3. There were 2 patients who were diagnosed as CMV hepatitis, 1 in each group. Both patients were admitted due to fever, poor appetite and malaise. Mild hepatomegaly on physical examination in association with elevated hepatic enzyme levels was found, viral hepatitis was initially impressed. A presumptive diagnosis of CMV hepatitis was made later on the basis of positive CMV culture in urine specimens and a fourfold increase of CMV-specific IgG antibody titer in paired sera. The only 1 patient with CMV retinitis in our case

Table 3. Glycoprotein B1 (gB1) and B3 (gB3) distributions by clinical diagnosis within the immunosuppressed and immunocompetent groups

	gB1	gB3	Mixed gB1 and gB3
<i>Immunosuppressed group</i> (n = 32)	27/32	4/32	–
CMV pneumonitis (n = 10)	8/10	2/10	–
CMV retinitis (n = 1)	1/1	–	–
CMV hepatitis (n = 1)	1/1	–	–
Hemorrhagic cystitis (n = 1)	–	–	1/1
Other unspecified infection (n = 19)	17/19	2/19	–
<i>Immunocompetent group</i> (n = 44)	26/44	17/44	1/44
Lower respiratory tract infection	11/17	6/17	–
Upper respiratory tract infection ¹	13/18	5/18	–
CMV hepatitis	–	1/1	–
Other unspecified infection	2/7	5/7	–

¹ Among the 18 patients diagnosed with upper respiratory tract infections, 3 had hand, foot and mouth disease, and 7 had herpangina.

series was a 33-year-old female suffering from systemic lupus erythematosus for many years. She was once admitted to the ophthalmic ward due to rapidly progressive decrease of visual acuity in her right eye for 3 weeks. CMV retinitis was diagnosed on the basis of ophthalmoscopic examination showing a macular hole with retinal necrosis, and positive CMV culture from a vitreous fluid specimen. Intravenous ganciclovir was given for 2 weeks followed by one dose of intravitreal injection of ganciclovir with uneventful outcome. Among the 18 patients diagnosed as the upper respiratory tract infection, 3 had clinical diagnosis of hand, foot and mouth disease and another 7 were diagnosed as herpangina. Nevertheless, there was no statistically significant difference between gB1 and gB3 distributions by clinical diagnosis within the immunosuppressed ($p = 0.84$) and immunocompetent ($p = 0.128$) groups.

Discussion

This study showed that only the gB1 and gB3 genotypes were detected in this Taiwanese subpopulation. The geographic distribution of gB genotypes that we report is similar to that previously reported in Japan [9]. Nonetheless, all four major gB genotypes were detected among infants with congenital infections in mainland China [10]. In

comparison to studies in Italy, the predominant HCMV strains encountered were gB2 and gB3 [13]. In Thailand, Bhattarakosol and Chantaraarphonkun [20] found that mixed infections (35%) were high in HIV-infected patients. Glycoprotein B1 (33%) predominates in Thai patients and no gB4 was detected. In different patient populations in Costa Rica, gB2 was the most frequent gB genotype encountered (73%) [15]. In a Cuban cohort of patients with different HCMV diseases, gB2 was the most prevalent (38%), followed by gB1 (30%), and mixed HCMV infections (16%) [8]. These differences reveal variations in the geographical distributions of global HCMV strains.

Among the patients we studied, mixed infections with gB1 and gB3 only occurred in 2 patients. One of them was immunosuppressed, while the other was immunocompetent. Previous reports indicated that mixed infections were more prevalent among immunosuppressed hosts [8, 22]. However, our findings did not concur previous reports by Yan et al. [12] and Coaquette et al. [22].

This study showed no statistical difference of gB distribution within each group by clinical diagnoses. In contrast, a previous study in bone marrow transplant recipient patients showed that shedding of gB1 seemed to have a better outcome. Our patient population was more heterogeneous and the patient number in each diagnosis group was also relatively small. Nevertheless, the previous study indicated no significant predominance of a specific gB genotype even within a homogeneous population with congenital CMV disease [26].

Because more pediatric patients were included in this study (49/76, 64%), age as a confounding factor should be examined carefully. Firstly, the reason why most samples were obtained from pediatric patients is associated with clinical practice. In this hospital, only immunosuppressed adults, especially those who had stem cell or solid organ transplantations, would have been sampled (either from urine or bronchoalveolar lavage) for virus culture regularly. In contrast, virus cultures were commonly used for investigation of fevers in pediatric patients, who constituted most of the immunocompetent group. Secondly, there were no previous studies addressing the genotypic distribution among different age groups. Since a great proportion of the HCMV diseases in adults were due to reactivation, genotypic transition in adulthood was less likely. Finally, though there was no statistical significance of gB1 distribution between pediatric immunosuppressed patients (6/6, 100%) and immunocompetent children (25/43, 58.1%), the trend of greater proportion of gB1 distribution among immunosuppressed children is a consistent finding.

Most specimens among the immunocompetent group in this study were isolated from throat swabs (63.2%). There were no statistically significant differences between the distributions of gB genotypes from different specimens. Almost all of these patients had respiratory tract infections, including acute pharyngotonsillitis, acute bronchiolitis, and bronchopneumonia.

Virus isolation from throat swabs of a few patients with diagnoses of herpangina yielded HCMV. Although HCMV seldom causes acute pharyngitis, it is still among the list of the etiologic agents of pharyngitis. In immunocompetent patients, HCMV can cause a mononucleosis-like syndrome, the most common manifestations are fever, pharyngitis, lymphadenopathy, fatigue and hepatosplenomegaly, but early in its course, this disorder can be difficult to distinguish from other causes of pharyngitis [3]. Hence it was very likely that a pediatrician might occasionally mistake a HCMV-induced pharyngitis for a herpangina-like illness due to enterovirus. Mixed infection of enterovirus and HCMV is also possible [27].

Intra- and intergenotypic variations among HCMV gB genotypes were previously studied [28–30]. Although PCR-RFLP is not sufficiently sensitive to differentiate all the variants of HCMV genotypes, its combination with subsequent sequencing is still feasible due to cost-effectiveness.

We recognize several limitations to our study, including its retrospective nature and the small number of patients studied. Combining PCR for HCMV in patient blood samples, serum anti-HCMV IgM, or a fourfold elevation of anti-HCMV IgG titer supports the diagnosis of HCMV disease better than virus culture alone. Viral culture takes several days to 1 month to yield a final report of HCMV. During acute illnesses, routinely taking blood samples from every febrile patient is not feasible. A prospective, multicenter study using PCR to detect HCMV DNA in blood samples is needed to clarify the population-wide distribution of gB genotypes in the Taiwanese population.

In summary, this study showed that only gB1 and gB3 were identified in a Taiwanese population. Despite no statistical difference of gB distribution within each group by clinical diagnoses, the frequency of gB1 was significantly higher in immunosuppressed than immunocompetent patients. Since age may be a confounding factor, further prospective study regarding the implications for outcome among the different HCMV gB genotypes is needed.

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