

CYTOMEGALOVIRUS GLYCOPROTEIN B GENE POLYMORPHISM AND ITS ASSOCIATION WITH CLINICAL PRESENTATIONS IN INFANTS

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Abstract: The clinical manifestations in cytomegalovirus infected-infants vary from asymptomatic illness to highly fatal cytomegalic inclusion disease. The influence of human cytomegalovirus (HCMV) strains on the outcome of HCMV disease is poorly explored. The present study was undertaken to explore the role of gB genotypes with clinical features in infants with clinically suspected HCMV disease. Urine samples of 71 infants (age <1 year) with clinically suspected HCMV disease were subjected to amplification of glycoprotein B (gB) gene by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism using *RsaI* and *HinfI*. HCMV DNA could be detected in 12 samples by gB gene PCR, 6 of which comprised of gB2, followed by gB1 in 5 samples and gB3 in 1 sample. Organomegaly was the most common finding (67%) followed by jaundice (50%), pneumonia (50%), seizures (42%), microcephaly (25%), low birth weight (25%) and rashes (17%). No particular genotype was significantly associated with specific clinical presentation or organ system involvement.

INTRODUCTION

Human cytomegalovirus (HCMV), a member of family Herpesviridae and sub-family β -herpesvirinae, is known for infection in immunocompromised individuals and infants. HCMV is the most frequently associated virus with congenital infection accounting for 0.3-2.5% of live births throughout the world (Demmler, 2004). Among the HCMV infected infants approximately 10% develop cytomegalic inclusion disease with a mortality rate of up to 30% (Stagno, 1990). The varied outcome of fetal

infection during pregnancy could either be due to host or viral factors. The gestational age and nature of CMV infection (primary/secondary) in mother during pregnancy are among the important host factors that have been well associated with the fetal outcome. However, the association of disease spectrum with viral factors needs to be explored.

Several genetically different strains of HCMV are known to circulate in the human population, among which glycoprotein B, H, N (gB, gH, gN) genes are commonly used for CMV genotyping (Chou and Dennison, 1991; Fries *et al*, 1994; Pignatelli *et al*, 2003). Clinical observations in different groups of patients, including bone marrow transplant recipients, allograft recipients, and HIV infected patients, have suggested that different gB and gN genotypes of HCMV strains might have some role in clinical severity and

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outcome of HCMV infection (Fries *et al*, 1994; Rasmussen *et al*, 1997). However, the association of gB and gN genotypes with disease spectrum among congenitally/perinatally infected infants with HCMV has remained controversial (Bale *et al*, 2000; Barbi *et al*, 2001). Hence, the present study was undertaken to study CMV gB gene polymorphism and its association with clinical presentations in children with clinically suspected CMV disease.

MATERIALS AND METHODS

Subjects

During the period of September, 2004 to October, 2006, 71 infants with clinically suspected HCMV disease admitted to the Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India were enrolled in the study. The criteria for HCMV disease were as described by Dobbins *et al* (1992), namely, intra-uterine growth retardation (IUGR), hepatosplenomegaly, thrombocytopenic purpura, jaundice, microcephaly, sensorineural hearing loss, mental retardation, motor deficits, seizures and chorioretinitis. The study was approved by Ethics Committee of the Institute.

Specimen collection and processing

Freshly voided 5-ml urine sample was collected in a sterile vial for PCR assay. All the samples were collected after obtaining written informed consent from the parents.

Amplification of CMV gB gene

Extraction of DNA from urine sample was conducted as described by Wang and Adler (1996). In brief, gB gene was amplified by PCR using specific primers consisting of 5' TGG AAC TGG AAC GTT TGG C 3' and 5' AAA CGC GCG GCA ATC GG 3' (gB1319 and gB 1604) (Fries *et al*, 1994). A total reaction volume of 50 μ l was prepared by adding PCR buffer containing 10 mM Tris

HCl (pH 9.6), 50 mM NaCl, 1.5 mM MgCl₂, 200 μ M dNTP's (MBI Fermentas, USA), 1.5 U *Taq* polymerase (MBI Fermentas, USA) and 50 ng of each primer (OPERON Biotechnologies, Germany) along with 5 μ l of extracted DNA. Thermal cycling consisted of initial denaturation at 95°C for 6 minutes followed by 35 cycles of denaturation at 95°C for 1.5 minutes, annealing at 55°C for 2 minutes and amplification at 72°C for 1 minute in a DNA thermocycler (Techne, UK). Positive and negative controls (reagent mixture without DNA) were conducted with each set of PCR. The amplicons were electrophoresed in 2% agarose gel (SRL Pvt Ltd), stained with 0.15% ethidium bromide and visualized under UV transilluminator (Alpha Innotech, USA).

Restriction fragment length polymorphism (RFLP) for HCMV gB gene genotyping

The PCR amplified products of the gB gene were digested with 2-4 units of *Hinf*I and *Rsa*I (MBI Fermentas, USA) and the digested products analyzed by 8% polyacrylamide gel electrophoresis using 50 bp molecular markers and Alpha Imager 3400 gel documentation system with Alpha-Ease FC software version 3.1.2 (Alpha Innotech, USA). Genotypes were assigned according to the scheme described by Chou and Dennison (1991). Representative strains of each genotype were subjected to nucleotide sequencing (Applied Biosystems, USA).

Statistical analysis

The association of HCMV gB gene genotypes with clinical presentations and organ system involvement was determined by contingency tables using Fisher's exact test.

RESULTS

Of a total of 71 infants with suspected CMV disease enrolled in the study, 12 (17%) were found positive by PCR for gB gene. The

Table 1
Clinical details of laboratory confirmed CMV infected infants.

Patient	Age (month)	Sex	Clinical feature	Genotype
1	6	M	HM, congenital nephrotic syndrome, hypercholesterolemia	gB2
2	3	M	HSM	gB3
3	4	M	LBW, HMD, hepatitis, pneumonia	gB2
4	2.5	M	Prematurity with HMD with sepsis and congenital pneumonia	gB1
5	1	F	HM, seizures with pneumonia	gB2
6	1.5	M	HSM, seizures	gB1
7	2	M	Multifocal seizures	gB2
8	3.5	M	HM, right focal seizures, intracranial bleeding	gB2
9	1.5	M	SFD, pneumonia with sepsis, NNJ	gB2
10	18 days	F	Sym. IUGR, microcephaly, HSM, congenital jaundice	gB1
11	1.5	M	Seizures, HSM, rashes, microcephaly	gB1
12	1	M	Pneumonia, anemia, HSM, microcephaly	gB1

HM, Hepatomegaly; HMD, Hyaline membrane disease; HSM, Hepatosplenomegaly; IUGR, Intrauterine growth retardation; LBW, Low birth weight; NNJ, Neonatal jaundice; SFD, Small for age.

mean age of CMV-infected infants (positive for CMV gB gene) was 2.3 months, with a male to female ratio of 3:1.

The 12 samples of CMV-infected infants were subjected to genotyping by RFLP (Fig 1). gB 2 genotype was found to be most common (6/12; 50%), followed by gB1 in 5 infants (42%) and gB3 in 1 infant (8%). Representative amplicon from genotype gB1, gB2 and gB3 subjected to nucleotide sequencing was 98, 96 and 98% identical, respectively, to the NCBI GenBank sequences corresponding to accession numbers M60923, M60932 and M60933, respectively.

The clinical features of laboratory confirmed CMV infected infants are shown in Table 1. Organomegaly (hepatosplenomegaly/hepatomegaly/splenomegaly) was the most common feature (67%) followed by jaundice (50%), pneumonia (50%), seizures (42%), microcephaly (25%), low birth weight (25%) and rashes (17%). No significant association ($p>0.05$) was observed between any

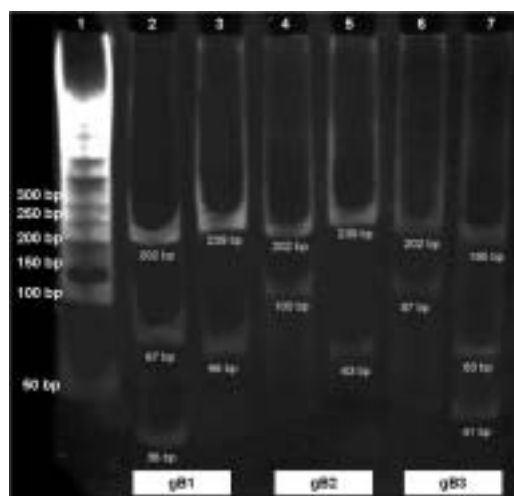
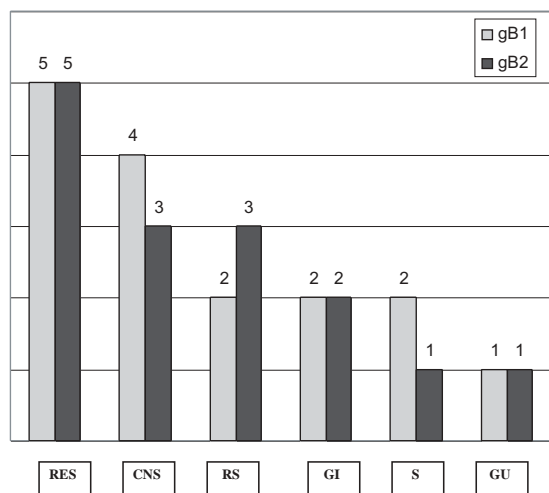


Fig 1-8% Polyacrylamide gel-electrophoresis for gB gene genotyping. Amplicons were digested with *RsaI* and *HinfI*. Lane 1, molecular marker (50 bp); lane 2, gB1 *HinfI* digested; lane 3, gB1 *RsaI* digested; lane 4, gB2 *HinfI* digested; lane 5, gB2 *RsaI* digested; lane 6, gB3 *HinfI* digested; lane 7, gB3 *RsaI* digested.



RES, Reticulo endothelial system; CNS, Central nervous system; RS, Respiratory system; GI, Gastrointestinal system (GI); S, Skin; GU, Genitourinary system

Fig 2–Distribution of clinical features in CMV gB1 and gB2 genotypes.

specific genotype and clinical presentations. The organ system involvement in gB 1 and 2 genotypes is shown in Fig 2.

DISCUSSION

The major scope of this study was to look for the gB gene polymorphism in children with suspected CMV disease, to ascertain its association with clinical presentations, and to analyze the situation in India, where study in this area is still inadequate. Recently, Indian experts have stressed the importance of such studies in different patient populations (Dar, 2007).

The present study encountered gB2 as the commonest genotype followed by gB1 and gB3. The predominance of CMV subtype II has been reported before (Madhavan and Priya, 2002). In contrast, studies from other countries have reported gB2 as being less prevalent than gB1 and gB3 (Bale *et al*, 2000;

Barbi *et al*, 2001; Yu *et al*, 2006). The higher rate of gB1 in our patient group has also been observed by others (Dobbins *et al*, 1992; Barbi *et al*, 2001). Interestingly the absence of gB4 in our study as also observed by other workers indicating the rarity of gB4 genotype in this subcontinent (Madhavan and Priya, 2002; Sowmya *et al*, 2007).

Diagnosis of congenital CMV infection is achieved by demonstrating the presence of virus in urine or saliva samples within 3 weeks of life. In the present study congenital infection could be ascertained definitely in only one baby (18 days old; Table 1) and 5 other infants (patient no. 3,4,9,11,12) were also congenitally infected, considering their later attendance to the Institute (after 3 weeks of life) and presence of symptoms like hyaline membrane disease, intrauterine growth retardation, low birth weight, microcephaly and neonatal jaundice (NNJ) since birth.

The critical role of CMV glycoprotein B in attachment and fusion of virus to host cell (Navarro *et al*, 1993) together with the existence of four major gB genotypes (Chou and Dennison, 1991) have made it logical to postulate the possibility of gB gene polymorphism affecting the biology of human CMV infection. We looked for the relationship between CMV gB genotypes and the clinical features of CMV disease in terms of prediction of any particular genotype being associated with specific organ-system or clinical presentation. All the three gB genotypes (gB1, gB2 and gB3) found in the present study were associated with clinical symptoms involving different organ systems. Although, no significant association was found between a particular genotype with any specific clinical presentation or involvement of organ system, the present study observed that all the three infants with severe form of neurological manifestation such as microcephaly had gB genotype1. Barbi *et al* (2001)

have reported a higher rate of CNS damage among children infected with gB1 and gB3 than in those infected with gB2 and gB4. However, no potential link of gB1 with severe neural involvement could be concluded at this stage, as this genotype was also observed with less severe form of neural involvement such as seizures and also due to low number of cases. Several other genes as potential virulence factors have also been studied, including UL144, UL146, UL147, and US28, and a possibility of association has been shown with UL144 gene and symptomatic disease in newborns (Arav Boger *et al*, 2006).

The present study used PCR-RFLP method for genotyping the CMV strains. However, recently multiplex PCR has been reported as a better alternative and this method needs to be evaluated in more studies (Sowmya *et al*, 2007).

Voided urine as a sample for detection of congenital HCMV infection was observed to serve as a better, noninvasive sample in the study, and can be used as a screening tool in these cases because of the ease of obtaining good volume of sample without any contact with blood or blood products. Collection of blood in children is painful and cause discomfort to the child, requires the involvement of a trained phlebotomist, and may also act as a source of infection. Because of this painful invasive procedure involved, many parents feel inhibited to have blood drawn from their children. On the other hand, as obtaining voided urine is simple and does not involve any such complications, parent compliance is better even if required to be repeated.

In summary, the present study has reported CMV gB gene polymorphism in infants with clinically suspected CMV disease. The potential link of gB1 with severe neural damage needs to be further substantiated with larger studies, including other aspects

such as viral load and host's immune response.

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