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Short communication

The type-2 variant of human cytomegalovirus glycoprotein N (gN-2) is not the rarest in the Chinese population of Taiwan: Influence of primer design☆

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ABSTRACT

Studies of the human cytomegalovirus (HCMV) glycoprotein N (gpUL73-gN) showed that genotypic variations exist in different geographic areas, with gN-2 unidentified in Chinese population. The purpose of this study was to determine the HCMV gN variants in the Chinese population of Taiwan. Primers were designed and a polymerase chain reaction (PCR) was carried out on the UL73 gene. The PCR products were subjected to restriction fragment length polymorphism (RFLP) analysis. The same PCR-RFLP assay was repeated using primers published previously to demonstrate the influence of primer design. Of the 48 clinical HCMV isolates, 33 were positive for PCR products by both primer sets. Fifteen were positive only by the “in-house” PCR. The distribution of gN-1, gN-2, gN-3, and gN-4 by RFLP analysis was 14:11:7:17, with one isolate positive for both gN-1 and gN-2. The published primers detected the four genotypes with the number of 14:0:2:17. The under-representation of gN-2 and gN-3 by the method published previously may be due to inappropriate primer design when re-examining the sequences. On the basis of the results of this study, gN-2 is not the rarest gN genotype in the Chinese population of Taiwan. The design of primers used for PCR-RFLP genotyping may have a great influence on the frequency distribution of HCMV genomic variants.

Human cytomegalovirus (HCMV) is a ubiquitous β-herpesvirus that is able to establish a lifelong persistent infection in immunocompetent hosts. Acute HCMV infection in immunocompetent adults may result in a mild mononucleosis-like disease that usually resolves unnoticed. However, in immunocompromised hosts such as transplant recipients and AIDS patients, HCMV infection can cause severe complications (Jacobson and Mills, 1988; Ives, 1997; Hofflin et al., 1987). Previous studies showed that clinical HCMV strains are highly polymorphic and that such polymorphism is associated with tissue tropism and severity of clinical presentations (Fries et al., 1994; Meyer-Konig et al., 1998). Among these are viral envelope glycoproteins, which are targets of strain-specific neutralizing antibodies. The genomic variants of glycoprotein B (gB) and their correlation with HCMV pathogenesis and the clinical outcome have been studied extensively, but the results are too controversial to make definite conclusions (Fries et al., 1994; Gilbert et al., 1999; Peek et al., 1998; Tarrago et al., 2003; Vilas Boas et al., 2003; Chern et al., 1998). Recent studies identified a structural glycoprotein that is encoded by HCMV UL73 (gpUL73, gN) and expressed on the surface of infected cells (Mach et al., 2000; Pignatelli et al., 2002; Dal Monte et al., 2001). In association with glycoprotein M (gM), this protein forms the envelope gCII complex (Mach et al., 2000). It was found that gN is highly polymorphic in the N-termini but conserved in the *C*-termini (Dal Monte et al., 2001; Pignatelli et al., 2001). Together with gM, the gM–gN complex is a major neutralizing antigenic target for antibody responses (Mach et al., 2000; Britt and Auger, 1985). The polymorphic N-termini of gN allowed

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the identification of four major distinct genomic variants, gN-1 to gN-4, among HCMV clinical strains (Pignatelli et al., 2001). The gN variants exist worldwide; the rarest genotype, gN-2, has not been identified in Chinese or Australian samples (Pignatelli et al., 2003a). To detect the gN genomic variants and determine their frequency distribution in the Chinese population of Taiwan, primers targeting the UL73 gene were designed and restriction fragment length polymorphism (RFLP) was carried out on the polymerase chain reaction (PCR) products. In addition, to clarify the influence of primer design for the detection of genomic variants, a PCR-RFLP assay was repeated on the same HCMV isolates using primers published previously. The results of two experiments were compared statistically.

Table 1
Prediction of RFLP patterns of the N-terminal fragments of the UL-73 gene amplified by in-house PCR using the UL-73 105725F and UL73-106122R primers[a]

gN type	PCR product size (bp)	Fragment sizes (no. of restriction sites)		
		ScaI	SalI	SacI
1	398	398 (0)	398 (0)	302, 96 (1)
2	395	395 (0)	269, 126 (1)	229, 96, 70 (2)
3	398	398 (0)	398 (0)	398 (0)
4	392	360, 32 (1)	314, 78 (1)	392 (0)
4a	392	198, 194 (1)	314, 78 (1)	296, 96 (1)

[a] Simulation of endonuclease digestion of the UL73 PCR product was performed using NEB software (http://tools.neb.com/NEBcutter2/index.php), and the restriction endonucleases ScaI, SalI, and SacI were chosen to distinguish the four gN variants and their subgroups.

Clinical specimens were collected for viral culture at the Clinical Virology Laboratory of Taipei Veterans General Hospital. The specimens were inoculated onto lung fibroblasts for viral replication. Forty-eight isolates were collected. The origins of these specimens included urine, bronchial secretions, sputum, and throat swab. Viral DNA was extracted using an automated DNA extractor (COBAS AmpliPrep Instrument; Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Briefly, the DNA from 300 µl of HCMV stock was extracted by magnetic bead technology using a Total Nucleic Acid Isolation kit (Roche Diagnostics). A series of procedures including sample take-up, lyses (with proteinase K and lyses reagents), binding to magnetic particles, washing, and elution were carried out by the machine. The final DNA was dissolved in 75 µl of elution buffer (TANI EB) and stored at −80 °C for future use. A PCR was then undertaken to amplify a region in the UL73 gene that encodes the N-terminal portion of the gN. Primers were designed based on the AD169 sequence (GenBank accession no. X17403). Five microliters of HCMV DNA was added to a PCR mixture containing 10 mM Tris–HCl, 50 mM KCl, 1.5 mM Mg^{2+}, 200 µM dNTP mixture, 300 nM primer mix, and 1.25 U AmpliTaq-Gold Taq polymerase (Perkin-Elmer, Norwalk, CT) at a final volume of 100 µl. The PCR was carried out for 35 cycles (96 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min; with an initial temperature of 96 °C for 5 min to activate the AmpliTaq-Gold enzyme and then at 72 °C for 10 min for the last cycle) with the UL73-105725F (5′-ggcggtggtgtgatggag-3′; nt: 105,725–105,742) and UL73-106122R (5′-ttctggaagcagcaatgtcg-3′; nt: 106,103–106,122) primer set (Fig. 1) using the Perkin-Elmer thermal cycler (Applied Biosystem GeneAmp® PCR system 9600). The amplification products contained 392–398 base pairs (Table 1). Using restriction digestion with ScaI, SalI, and SacI (New England Biolabs, Boston, MA, USA), the PCR products were analyzed by electrophoresis in 3.5% Nusieve agarose gel (FMC BioProducts, Rockland, ME), stained with ethidium bromide and photographed under ultraviolet light. Genomic variants were interpreted according to the RFLP patterns as major gN genotypes 1–4. The same HCMV DNA samples were subjected to another round of PCR and RFLP analysis using a different set of primers: gN-up (5′-tggtgtgatggagtggaac-3′; nt: 105,730–105,748) and gN-lw (5′-tagcctttggtggtggttgc-3′; nt: 106,130–106,149) according to a previously published protocol (Pignatelli et al., 2003a).

To confirm that the PCR-RFLP analysis was able to detect accurately all four known gN genomic variants of HCMV, sequencing of the N-terminal region of the HCMV UL73 gene was performed in 10 of the isolates by automated DNA sequencing (ABI PRISM® 310 genetic analyzer) and was compared with the result of the PCR-RFLP assay. The nucleotide sequence of each UL73 region was aligned with other HCMV isolates and was edited using the BioEdit program. UL73 DNA sequences from the 10 HCMV isolates and the AD169 reference strain were used to construct the phylogenetic tree. Phylogenetic analysis was conducted with the phylip3.6 and MEGA2 programs. Neighbor joining (NJ) was used to construct the tree with 1000 bootstraps. The neighbor joining tree was built using a Kimura-2 parameter method. The Kappa test was adopted to analyze the concordance of the two tests.

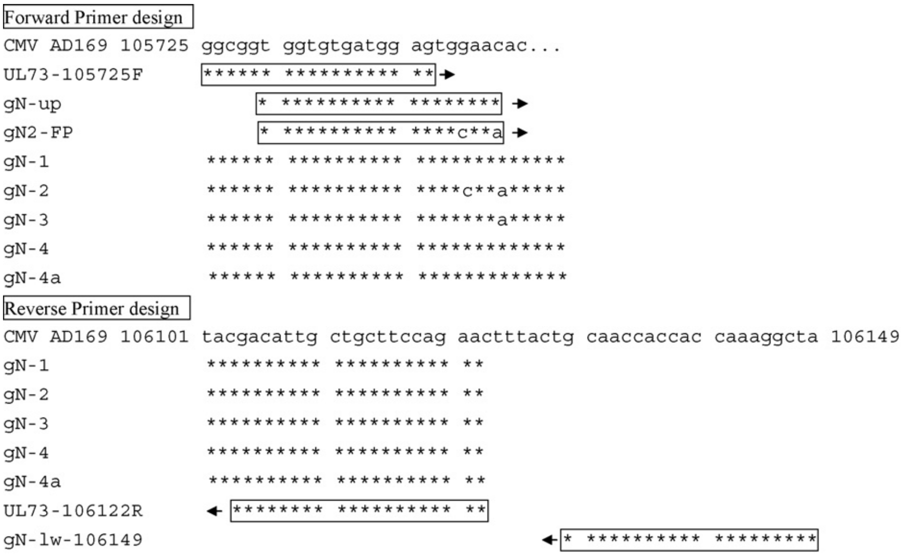

Fig. 1. Sequence comparison among different HCMV genotypes and primers. The nucleotide sequence encompassed by UL73-105725F primer is identical among different gN genotypes. The sequence of gN-up primer is identical to that of gN-1 but not gN-2 or gN-3. The gN2-FP was designed so that its sequence is identical to that of gN-2.