Does congenital cytomegalovirus infection lead to hearing loss by inducing mutation of the *GJB2* gene?

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BACKGROUND: Congenital cytomegalovirus (CMV) infection and mutation of the gap junction β -2 (*GJB2*) gene are important causes of sensorineural hearing loss (SNHL). This study aims to determine if congenital CMV infection leads to deafness by inducing *GJB2* mutation.

METHODS: *GJB2* gene sequencing and auditory brainstem response testing were performed in 159 neonates (63 with and 96 without CMV infection) from August 2008 to August 2011. For neonates with *GJB2* mutation, their parents were further screened for *GJB2* sequence.

RESULTS: The incidence of SNHL was 12.7% in CMV-infected but 0% in uninfected children aged 1–1.5 y ($P = 0.000$). Similar mutation rates of the *GJB2* gene were observed in neonates with or without CMV infection (34.9 vs. 32.3%, respectively, $P = 0.734$). No significant difference in the mutation rate of *GJB2* was found among neonates with CMV infection and SNHL, those with CMV infection and normal hearing, and uninfected newborns with normal hearing ($P = 0.438$). Mutations 79G>A, 109G>A, 341A>G, and 608T>C were found in neonates with and without CMV infection. All of the above mutations were also found in both or one of the corresponding parents.

CONCLUSION: Congenital CMV infections may cause deafness in neonates, but this might be independent of *GJB2* gene mutation.

Congenital cytomegalovirus (CMV) infection is an important cause of sensorineural hearing loss (SNHL) in children worldwide (1,2). Up to 90% of CMV-infected neonates are asymptomatic at birth and do not present with overt clinical symptoms of CMV infection; however, they are at risk for SNHL (3,4). The pathogenesis of SNHL in children with congenital CMV infection remains not well understood, and the injury to cochlear perilymphatic epithelial cells directly induced by CMV and inflammatory response may play a role (5–7). Meanwhile, nongenetic and genetic factors were found to almost equally contribute to the etiology of hearing loss in children (8). As genetic factors, the mutations of the gap junction β -2 (*GJB2*) gene were considered an important cause of SNHL (9–12). Studies indicate that congenital CMV infection may cause chromosome aberrations, including selective

chromosome breakages, chromosome pulverization, premature chromatid condensation, and structural injury to the centrosome (13–18), e.g., genetic damages on 1q23.3 (14), 1q21, and 1q42 (17). However, whether CMV infection can induce mutations of *GJB2* on human chromosome 13q11–q12 remains unclear. Ogawa *et al.* (19) reported that congenital CMV infection and *GJB2* mutations were identified in 15 and 24%, respectively, of 67 children with severe SNHL. Ross *et al.* (20) found that the overall frequency of *GJB2* mutations in children with CMV infection and hearing loss was higher than that in children with CMV infection and normal hearing (21 vs. 3%).

However, recent studies could not determine whether congenital CMV infection induces *GJB2* mutations and increases the risk of hearing loss (19,20) because the *GJB2* sequences in corresponding parents were not detected, and the possibility that the mutation spectrum of *GJB2* in neonates was inherited from their parents could not be excluded. On the basis of existing data, the current study further examined the mutation frequency of *GJB2* in neonates with and without congenital CMV infection, as well as in their corresponding parents, to determine whether congenital CMV infection leads to hearing loss through inducing mutation of *GJB2* gene.

RESULTS

Characteristics of the Patient Population

The characteristics and clinical findings of the 63 neonates in the congenital CMV infection group and 96 neonates in the control group are shown in Table 1. No significant difference in birth weight and gestational age were found between the two groups.

CMV DNA Level

Of the 9,471 neonates enrolled in this study at the early stage, 3,915 were found to have CMV infection by enzyme-linked immunosorbent assay and fluorescent quantitative-PCR (FQ-PCR) assays (Figure 1). The positive rates of FQ-PCR assay were 77.27% (17 of 22) in CMV-IgM (+) neonates but only 3.47% (135 of 3,893) among CMV-IgG (+) children ($\chi^2 = 319.324$; $P = 0.000$). To compare the CMV DNA level in

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neonates with and without hearing loss, 63 patients with congenital CMV infection were analyzed. The CMV DNA level in infants with hearing loss was higher than that in the normal-hearing group (5.37 ± 1.18 ($n = 8$) vs. 3.32 ± 0.79 ($n = 55$); $t = 7.46$; $P = 0.000$). Meanwhile, the CMV DNA level in IgM (+) neonates (4.99 ± 0.26 ; $n = 17$) was higher than that in IgG (+) neonates (4.22 ± 1.4 ; $n = 135$; $t = 2.167$; $P = 0.032$).

Table 1. The characteristics of the neonates in this study

Characteristic	CMV infection group	Control group	Statistics	P
Gender (male/female)	28/35	67/29	$\chi^2 = 10.16$	0.00
Premature infant	8	9	$\chi^2 = 0.44$	0.51
Gestational age (wk, mean $\pm$ SD)	37.43 $\pm$ 1.68	37.70 $\pm$ 1.0	$t = 1.28$	0.20
Low birth weight	10	12	$\chi^2 = 0.547$	0.55
Birth weight (g, mean $\pm$ SD)	2,898.17 $\pm$ 475.97	2,951.30 $\pm$ 396.41	$t = 0.76$	0.45
Pneumonia	63	92	$\chi^2 = 2.693$	0.10
Congenital heart disease	8	5	$\chi^2 = 2.842$	0.09
Thrombocytopenia	2	0	$\chi^2 = 3.806$	0.08
Hyperbilirubinemia	22	38	$\chi^2 = 0.352$	0.55
NRDS	3	0		0.03 ^a
IVH (grades 1 and 2)	9	3		0.01 ^a

CMV, cytomegalovirus; IVH, intraventricular hemorrhage; NRDS, neonatal respiratory distress syndrome.

^aFisher's exact value.

Figure 1. The inclusion and exclusion methods of this study. ABR, auditory brainstem response; CMV, cytomegalovirus; ELISA, enzyme-linked immunosorbent assay; FQ-PCR, fluorescent quantitative PCR; GJB2, gap junction β -2.

GJB2 Gene Mutations

All the neonates positive for CMV FQ-PCR ($n = 63$) and the control neonates ($n = 96$) were tested for the mutation of GJB2. The positive rates of mutation in GJB2 gene in neonates with and without congenital CMV infection were 34.9% (22 of 63) and 32.3% (31 of 96), respectively ($\chi^2 = 0.118$; $P = 0.734$). The types of GJB2 mutations are shown in Table 2. In the 22 neonates with both congenital CMV infection and GJB2 mutation, 7 had single point mutations (79G>A and 109G>A each in 1 neonate, 608T>C in 2 neonates, and 341A>G in 3 neonates), 12 had double point mutations (79G>A+109G>A in 5 neonates; 79G>A+341A>G in 2 neonates, 109G>A+341A>G in 4 neonates, and 79G>A+608T>C in 1 neonate), and 3 had triple point mutations (all were 79G>A+109G>A+341A>G). Meanwhile, 31 neonates in the control group were also found to have GJB2 mutations. Among them, 14 had single point mutations (341A>G in 1 neonate, 608T>C in 3 neonates, and 79G>A and 109G>A each in 5 neonates), and 8 had double point mutations (79G>A+109G>A, 79G>A+341A>G, 608T>C+79G>A, and 608T>C+109G>A, each in 1 neonate, and 109G>A+341A>G in 4 neonates); 9 had triple point mutations (all were 79G>A+109G>A+341A>G). In neonates with both congenital CMV infection and GJB2 mutation, the mutation types were 13.6% (3 of 22) homozygote, 27.3% (9 of 22) compound heterozygote, and 59.1% (13 of 22) heterozygote; in the control group, the corresponding percentages were 9.7% (3/31), 25.8% (8/31), and 64.5% (20/31), respectively.

Hearing Loss of Patients

Among the 63 patients with congenital CMV infection, 7 had abnormalities in Distortion Product Otoacoustic Emissions (DPOAE) examination in the neonatal period; 2 of them showed abnormal waveform in auditory brainstem response (ABR) testing (1 with wave I disappearance and 1 with abnormality of wave III and wave V latency). The other 56 patients displayed normal waves. Among patients in control group, 2 showed abnormality in DPOAE testing and 1 of them also displayed abnormal waveform in ABR testing (abnormality of wave I latency). For children aged 1–1.5 y, the incidence of SNHL was 12.7% (8 of 63) in patients with CMV infection (5 mild and 3 moderate) but 0% (0 of 96) in patients of the control group ($P = 0.000$). Meanwhile, the abnormality rate of waves I, III, and V in the CMV infection group was higher than that in the control group (Table 3).

To explore the relationship among congenital CMV infection, GJB2 mutation, and hearing loss, we analyzed the mutation spectrum in group 1 (8 neonates with congenital CMV infection and hearing loss), group 2 (55 neonates with congenital CMV infection and normal hearing), and group 3 (96 neonates with normal hearing but without congenital CMV infection). The overall frequency of GJB2 mutations was 50% (4 of 8) in group 1, which was not significantly different from those in group 2 (32.7%; $P = 0.434$) and group 3 (32.3%; $P = 0.438$).

Because no significant difference was found among the above-mentioned three groups of infants, it is of interest to

Table 2. The mutation of *GJB2* in neonates with and without congenital CMV infection

Nucleotide change	Consequence or amino acid change	Category	Total mutations in neonates	With both CMV infection and mutation (n = 22)	Without CMV infection but with mutation (n = 31)
79G>A	V27I	Polymorphism	28	11	17
109G>A	V37I	Polymorphism	32	13	19
341A>G	E114G	Polymorphism	27	12	15
608T>C	I203T	Polymorphism	8	3	5

CMV, cytomegalovirus; *GJB2*, gap junction β -2.

Table 3. The comparison of abnormal waveform of ABR test between CMV infection group (n = 63 infants) and noninfected group (n = 96 infants)

Group	Ears	Abnormality of waveform			Total abnormality ^a	χ^2	P
		Wave I	Wave III	Wave V			
CMV infection group	126	11	9	22	37 ^b	47.53	0.00
Noninfected group	192	1	1	3	5		

CMV, cytomegalovirus.

^aAbnormal waveform was defined as prolonged latency of wave I, III, or V, or absent response. ^bThree neonates had prolonged latency of wave I and III in bilateral ears, and two neonates had absent response (wave III and V) in a unilateral ear.

Table 4. The mutation of *GJB2* in parents of neonates who have *GJB2* mutation

Mutation spectrum	Only found in father	Only found in mother	Found in both
79G>A	6	5	1
109G>A	6	7	1
341A>G	5	4	1
608T>C	4	2	No
79G>A+109G>A	7	6	No
79G>A+341A>G	4	5	No
109G>A+341A>G	5	5	No
109G>A+608T>C	2	No	No
79G>A+109G>A+341A>G	1	No	No

GJB2, gap junction β -2; No, without this mutation spectrum.

know what exactly caused the mutation of *GJB2* in neonates with and without congenital CMV infection. To determine whether the mutation of *GJB2* in neonates was inherited from their parents, the *GJB2* gene in 53 pairs of parents whose offspring had a *GJB2* mutation (22 with and 31 without congenital CMV infection) were also sequenced (Table 4). All mutation spectra of *GJB2* in neonates were found in one or both of their parents (Table 5).

DISCUSSION

Congenital CMV infection is a leading cause of SNHL and is responsible for an estimated 8.9 (21), 14 (22), and even 20.4% (5) of all clinically significant hearing loss among young

Table 5. The mutation of *GJB2* in neonates and mode of inheritance

Nucleotide change	Mutations in neonates	Inherited from father	Inherited from mother	Unclear ^a
79G>A	28	11	11	6
109G>A	32	15	12	5
341A>G	27	14	11	2
608T>C	8	6	2	No

GJB2, gap junction β -2; No, without this mutation spectrum.

^aThe mutation type of *GJB2* in neonates was found in both father and mother.

children. In this study, 12.7% (8 of 63) of neonates with congenital CMV infection developed mild-to-moderate hearing loss between ~1 and 1.5 y old, consistent with previous studies (22,23).

The pathogenesis of SNHL in children with congenital CMV infection remains not well understood. Ross *et al.* initially found that the mutation rate of *GJB2* in children with CMV infection and hearing loss was higher than those in children with CMV infection and normal hearing (20).

On this basis, the current study further explored if congenital CMV infection causes deafness via inducing *GJB2* mutation. We detected *GJB2* mutations in neonates and identified four types of mutation in the *GJB2* gene, 79G>A, 109G>A, 341A>G, and 608T>C, which all were considered to be polymorphisms (24,25). Moreover, the *GJB2* gene sequence was also analyzed in the corresponding parents of these babies harboring *GJB2* mutation, and the results indicated that all the mutation types of *GJB2* in neonates of both the infected and uninfected groups were also found in their parents. Therefore, it can be concluded that all the *GJB2* mutations in neonates were inherited from their parents, and congenital CMV infection leads to hearing loss not through inducing mutation of *GJB2* gene. This useful information may be provided to the pregnant woman with *GJB2* mutation and/or CMV infection during pregnancy at genetic counseling that her offspring may have a mutation in *GJB2*, which does not increase the frequency of mutation even if infected by CMV during the fetal period.

There were some controversies regarding the viral load of CMV and hearing loss. Some studies suggested that peripheral blood viral load did not associate with hearing loss in children with congenital CMV infection (21,26). Meanwhile, others indicated that hearing loss associated with increased amounts of urine CMV during early infancy (27–31). The severity of

CMV infection–caused brain disorder might depend on the stage of brain development and the susceptibility of the type of embryonic cells to CMV (24). Therefore, the individual susceptibility to CMV infection as well as the instability and quantity of CMV shedding into urine lead to different amount of viral load shown in each detection, and differential follow-up time of different studies may explain the different results and the differential association between viral burden and hearing loss. In the current study, the CMV DNA level in neonates with hearing loss was higher than that in normal hearing group. Meanwhile, the CMV DNA level in neonates of the IgM-positive group was higher than that in IgG-positive neonates. Therefore, it is very important to set more frequent follow-up for neonates positive for CMV-IgM to detect the occurrence of deafness during early stages. On the other hand, IgM is known to have low sensitivity for CMV diagnosis (25). Recently, in a multicenter study, Boppana *et al.* (32) found that the sensitivity and specificity of the liquid-saliva PCR assay for CMV screening in newborns were 100 and 99.9%, respectively. Therefore, it is necessary to perform liquid-saliva PCR assay for neonates in whom CMV infection is highly suspected.

Although congenital CMV infection did not induce *GJB2* mutation in this study, some previous experiments *in vitro* indicated that CMV infection did induce genetic damage at 1q23.3, with a breakpoint situated between two hearing impairment loci, DFNA49 and DFNA7 (14). Fortunato *et al.* (17) demonstrated that infection of S-phase human fibroblast cells with CMV resulted in chromosome breaks at positions 1q21 and 1q42; these two chromosomal band locations contain loci involved in dominantly and recessively inherited hearing impairment, respectively. Therefore, it may be of interest to explore the relationship among congenital CMV infection, genetic damage on chromosome 1, and clinical hearing loss.

There are some limitations in this study. We showed that the mutation rates of *GJB2* were 32.7% (18 of 55) in neonates with CMV infection and normal hearing and 32.3% (31 of 96) in uninfected newborns without deafness, which were different from those (3% (4 of 130) and 3.9% (15 of 380), respectively) reported by Ross *et al.* (20). We also noticed that the study population in their reports was an African-American population, whereas our study population was a Chinese Han population. Such differences may be explained by the small sample size in this study and/or the racial difference. On the other hand, according to Dahle *et al.* (5), the incidence of SNHL was 15.47% (133 of 860) in children with congenital CMV infection. In this study, the incidence of SNHL was 12.7% (8 of 63) in children with CMV infection. Although the incidence rates of deafness were comparable, we had a much smaller sample size. These limitations may be overcome by future multicenter studies on the mutation rate of *GJB2* and the incidence of hearing loss in neonates with congenital CMV infection.

METHODS

The study was approved by the institutional review board of the Children's Hospital of Chongqing Medical University (approval

number: 2008006) and registered in the Chinese Clinical Trial Register (approval number: ChiCTR-OCC-00000489). Informed consent was obtained from all the parents of neonates who participated in this study.

In the early stage, a total of 9,741 neonates, with <14 d of life, hospitalized in the Children's Hospital of Chongqing Medical University from August 2008 to August 2011 were enrolled in this study. The final diagnosis of congenital CMV infection was based on the FQ-PCR assay. Neonates with hypoxic–ischemic encephalopathy, severe intra-ventricular hemorrhage (grades 3 to 4), purulent meningitis, bilirubin encephalopathy, external auditory canal malformation, and family history of deafness were excluded from this study. The inclusion and exclusion criteria are shown in [Figure 1](#).

FQ-PCR

Enzyme-linked immunosorbent assay for CMV infection was carried out routinely in our hospital. The urine samples of neonates positive for CMV-IgM or CMV-IgG were further tested by FQ-PCR for CMV infection. The investigators who performed the FQ-PCR were blinded to the results of the enzyme-linked immunosorbent assay assay.

The FQ-PCR detection of CMV was performed on an ABI 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) using Absolute Low ROX QPCR Mix (ABgene, Rockford, IL). The sequences of PCR primers were selected from the highly conserved region that encodes the immediate-early gene of human CMV strain AD169 (33). The forward primer was 5'-CCAGTGCCCGCAGTTTATT-3', and the reverse primer was 5'-ACCGGAGAAGAGCCCATGTC-3' with a length of 86 bp products. Fluorescent probe sequence was 6-carboxyfluorescein-5'-AACATAACGTGGGATCTCCACGCGAAT-3'-6-carboxytetramethylrhodamine. Each reaction mixture contained 43 µl of master mix and 2 µl of DNA sample. The amplification was performed in the following manner: 1 cycle at 93 °C for 2 min, followed by 40 cycles at 93 °C for 10 s and 57 °C for 45 s. If the CMV DNA amplification was negative, the PCR was repeated with the stored DNA extract. The lower limit of detection of this method was determined to be 500 copies of CMV DNA/ml of urine.

GJB2 Mutation Analysis

Blood specimens were obtained from neonates in both the CMV-infected and -uninfected groups. For neonates with a *GJB2* mutation, the blood samples from their parents were further screened for *GJB2* mutation. *GJB2* sequencing was performed in the Center of Clinical Molecular Medicine of the Children's Hospital of Chongqing Medical University. The investigators who performed the *GJB2* mutation testing were blinded to the results of the FQ-PCR for CMV infection.

To detect the sequence of the *GJB2* gene, genomic DNA was extracted from each blood specimen according to the Gentra PureGene protocol (Qiagen, Valencia, CA). DNA was amplified by PCR with a pair of primers that amplify the entire open-reading frame of the *GJB2* gene. The following primer pair was used: the forward primer was 5'-TCTCCCTGTTCTGTCTAGC-3', and the reverse primer was 5'-ACTGAGCCTTGACAGCTGAG-3'. The samples were amplified according to following program (94 °C, 5 min; 94 °C, 30 s; 58 °C, 30 s; and 72 °C, 30 s for 30 cycles; and a final extension at 72 °C for 5 min). The PCR products (840 bp) of each reaction were further cleaned with 2 µl of exonuclease I (USB Corporation, Cleveland, OH) and 1 µl of shrimp alkaline phosphatase (Life Technologies, Rockville, MD), and the sequencing reactions were performed using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). The PCR samples were bidirectionally sequenced using the same forward and reverse primers. The sequences were analyzed using the Sequencher 3.0 software (Gene Codes Corporation, Ann Arbor, MI), the DNAssist2.0 software (Applied Biosystems, Foster City, CA), and the *GJB2* Genebank sequence (Genebank accession number: AY280971).

DPOAE Testing

An ILO-96 otoacoustic emission instrument (Otodynamics, Herts, UK) was used in DPOAE testing, which was performed in a quiet room by a trained examiner. When examined, the neonate was in a quiet sleeping state (without the use of sedative drugs). The external

auditory canal was conventionally cleaned. A small probe, which contained a miniature microphone to measure sound levels, was inserted into the ear canal. DPOAE testing was carried out by measuring a pair of primary tones (frequencies 1 and 2) among frequencies ranging from 1 to 8 kHz. The detection parameters were set as L1 (lower-frequency primary tone) at 65 dB sound pressure level and L2 (higher-frequency primary tone) at 55 dB sound pressure level; frequency 2/frequency 1 ratio at 1.22. Frequency 2 was presented at 2, 3, 4, and 5 kHz. An emission/noise ratio of 6 dB in three of four frequencies was considered a passing result (34).

After a test was completed, the results were displayed on the screen as "PASS," when there was DPOAE response, or "REFER," when there was no response to a stimulus. When a "REFER" result was obtained, the screening test was repeated twice to confirm the result.

ABR Testing

Testing was performed during natural sleep or under sedation by certified audiologists, according to the protocol of the institutional Speech and Hearing Center of the Children's Hospital of Chongqing Medical University. The investigators who performed the ABR testing were blinded to the results of real-time PCR for CMV infection and *GJB2* mutation testing. All the newborns with CMV infection were tested using ABR testing equipment (Navigator; Bio-logic, Mundelein, IL). The newborns without CMV infection were also subjected to ABR testing if they failed the DPOAE testing. Meanwhile, all the infants were tested by ABR testing equipment at ages from 1 to 1.5 y. The electrodes were applied to the forehead and mastoid region in sleeping infants, and the ABR using a click sound was measured. The ABR threshold was defined as the lowest level at which the wave V could be detected and replicated. The waveform was analyzed by an experienced audiologist to define the peak latencies of waves I, III, and V. Normal hearing was defined when an individual was able to hear a stimulus between 0 and 25 decibel hearing loss (dBHL). Hearing loss was defined as air conduction thresholds >25 dBHL on ABR in an infant with normal middle-ear function (35). Hearing loss was graded into mild (25–40 dBHL), moderate (41–70 dBHL), severe (71–90 dBHL), and profound (>90 dBHL) (36). In individuals with bilateral SNHL, the results with the better ear were used to grade the hearing loss (37).

Data Analysis

The demographic characteristics, newborn findings, results from FQ-PCR, and outcome data of follow-up were collected and entered into SPSS 13.0 statistical package (SPSS, Chicago, IL). To compare the levels of CMV DNA between CMV-IgM-positive and CMV-IgG-positive neonates, the CMV DNA copies per milliliter of urine were converted into continuous variables using logarithm 10. Normally distributed continuous variables were compared by a two-sample Student's *t*-test. Categorical variables were compared using the χ^2 test or Fisher's exact test wherever appropriate.

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