

GJB2 and GJB6 Mutations in Children with Congenital Cytomegalovirus Infection

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ABSTRACT: Congenital cytomegalovirus (CMV) infection is a leading cause of sensorineural hearing loss (SNHL) in children. Whether connexin mutations are factors in the development of CMV-related hearing loss has not been explored. We examined gap junction protein beta-2 (GJB2) and gap junction protein beta-6 (GJB6) mutations in 149 children with congenital CMV infection and 380 uninfected neonates. Mutations in GJB2 and GJB6 were assessed by nucleotide sequencing and polymerase chain reaction (PCR) methods, respectively. The study population was predominantly African American, and 4.3% of the subjects were carriers of a connexin 26 mutation. The overall frequency of GJB2 mutations was significantly higher in the group of children with CMV infection and hearing loss (21%) compared with those with CMV infection and normal hearing (3%, $p = 0.017$) and the group of uninfected newborns (3.9%, $p = 0.016$). Eight previously reported mutations (M34T, V27I, R127H, F83L, R143W, V37I, V84L, G160S), and four novel mutations (V167M, G4D, A40T, and R160Q) were detected. None of the study children had the 342-kb deletion (delGJB6-D13S1830) in GJB6, which suggests that this mutation does not play a role in hereditary deafness in the African American population. Although GJB2 mutations were detected in children with and without CMV-related hearing loss, those with hearing loss had a higher frequency of GJB2 mutations. (*Pediatr Res* 61: 687–691, 2007)

CMV is a frequent cause of congenital infection and a leading cause of SNHL in children in the United States and northern Europe. Prospective follow-up studies have shown that approximately 5%–15% of children with asymptomatic or subclinical congenital CMV infection and 40%–75% of those with symptomatic infection develop hearing loss (1–3). However, current knowledge of the pathogenesis of hearing loss in congenitally infected children is limited. The results of a recent study have shown that the presence of petechiae and intrauterine growth retardation at birth in infants with symptomatic congenital CMV infection was predictive of hearing loss. In contrast, the presence of microcephaly and other neurologic abnormalities at birth was not predictive of

hearing loss in children with symptomatic infection (4). Urinary excretion of CMV has been correlated with hearing outcome, with SNHL and progressive SNHL associated with viral urinary excretion <4 y (5). In addition, an association between increased viral load during early infancy and hearing loss has been documented in asymptomatic children, although not all children with increased virus burden in infancy develop hearing loss (6).

Over the past decade, there has been a dramatic increase in our understanding of the importance of hereditary deafness, which is estimated to be responsible for at least 50%–60% of childhood hearing loss (7). Gap junctions of the inner ear are thought to play a role in recycling potassium in the cochlea, which is important for sensorineural hearing function. Gap junctions are composed of integral membrane proteins, called connexins, that oligomerize to form intercellular channels. Mutations in connexin genes can alter the function of the encoded protein in the inner ear, resulting in inherited SNHL (8). Although it is believed that approximately 100 genes could be responsible for hearing impairment (9), a large proportion of hereditary deafness cases are due to autosomal recessive mutations in one gene, GJB2, which encodes the protein connexin 26. However, there are racial/ethnic differences in the distribution of the various GJB2 alleles (9). More recently, a large deletion, del(GJB6-D13S1830), involving a gene close to GJB2, called GJB6 (connexin 30), has been described as a cause of deafness in the homozygous state and in heterozygosity with GJB2 mutations (10–12). To determine whether mutations in GJB2 and GJB6 are more frequent in children with CMV-related hearing loss, we compared GJB2 and GJB6 mutations in congenitally infected children with and without SNHL. In addition, because the frequency of connexin mutations in African American populations has not been well studied, we also examined the frequency of these mutations in a predominantly African American infant population born at The University of Alabama (UAB) Hospital.

METHODS

Study population. Between 1980 and 2003, 780 children were identified by newborn screening for congenital CMV infection at two hospitals in Birmingham, AL, and enrolled in a long-term prospective natural history

Abbreviations: CMV, cytomegalovirus; DBS, dried blood spot; GJB2, gap junction protein beta-2; GJB6, gap junction protein beta-6; PBL, peripheral blood lymphocyte; SNHL, sensorineural hearing loss

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study. Congenital CMV infection was identified by isolation of the virus in urine or saliva within the first 2 wk of life (13,14). Peripheral blood lymphocytes (PBLs) were available from 149 children (19 children with hearing loss and 130 children with normal hearing), and this group constituted the study population. Infants were classified as having symptomatic congenital CMV infection if they had any of the following clinical findings in the newborn period: petechiae, purpura, jaundice with conjugated hyperbilirubinemia (direct bilirubin >2 mg/dL), thrombocytopenia (<100,000/mm³), hepatosplenomegaly, microcephaly, seizures, or chorioretinitis (15). Study children were followed in an interdisciplinary clinic and were monitored with audiologic evaluations at the initial clinic visit at 3–8 wk of age, every 6 mo until 24 y of age, then annually thereafter according to a standard protocol described previously (1,16). If children were found to have hearing loss, they received audiologic examinations more frequently as determined by the audiologist. The demographic and clinical characteristics were not different between the 149 study children and the 631 children from whom peripheral blood samples were not available (data not shown). A comparison group consisted of 380 uninfected infants born at UAB Hospital between September 1, 2003, and February 29, 2004, from whom dried blood spot (DBS) specimens were collected as part of a study to screen for congenital CMV infection. The research was conducted in accordance with the guidelines for human experimentation as specified by the U.S. Department of Health and Human Services. The study was approved by the Institutional Review Board for Human Use of UAB. The archival PBL samples from children with congenital CMV infection and the DBS specimens from uninfected newborns were obtained and analyzed anonymously without identifying information; thus, the informed consent requirement was waived by the IRB.

Detection of connexin mutations. Genomic DNA was extracted from the stored PBL samples using commercial spin columns (Qiagen Inc., Chatsworth, CA). DNA was extracted from archival DBS samples (IsoCode filter paper) from three 2-mm punches according to the manufacturer's protocol (Schleicher and Schuell Bioscience, Keene, NH). The entire coding region of the GJB2 gene was amplified by PCR using the following primer set to produce a 955-bp fragment that includes 106 base pairs before the start codon 5'-TGC TTG CTT ACC CAG ACT CA-3' and 5'-TGA AAC TCC AGA TGC CAC AA-3'. Primers were designed by the authors using published GJB2 sequence (Accession #AL138688, GenBank NCBI). The fragment was amplified with HotMaster *Taq* polymerase (Eppendorf North America, Westbury, NY) using a touchdown program to improve purity and yield using the following cycling parameters: 94°C for 1 min 30 s, 10 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 30 s, and the annealing temperature was decreased by 1°C with each cycle; 30 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 7 min. The amplification was performed in a total volume of 50 μ L containing 400 ng of genomic DNA, 10 \times PCR buffer with self-adjusting Mg²⁺, 1.5 U of HotMaster *Taq* polymerase, 1.5 mM MgCl₂, and 2.6 ng of each primer. Primers for amplification of a housekeeping gene, GAPDH, were included in each PCR run to control for sample preparation. The PCR products were resolved on agarose gel electrophoresis to verify the size of the product.

GJB2 PCR products were purified using 1 μ L of exonuclease I and 1 μ L of shrimp alkaline phosphatase (USB Corporation, Cleveland, OH) per reaction. PCR products were then subjected to nucleotide sequence analysis with the forward primer described above using the ABI Prism 3100 sequencer in the sequencing core facility at UAB. Mutations were identified by comparing the nucleotide sequences of the amplified products with the published GJB2 sequence used for primer design. All nucleotide changes were confirmed by repeat sequencing with the reverse primer. Sequence analyses were carried out using the Vector NTI Version 10 software (Invitrogen Corporation, Carlsbad, CA).

The presence of the GJB6 deletion was assessed by using a PCR method that has been described previously by del Castillo *et al.* (10) and Fitzgerald (17). Primers GJB6-1R and BRK-1 were used to detect the 342-kb deletion. Primers GJB6-1R and GJB6del were used to indicate that the centromeric region was intact and amplification with BRK-1 and BRK1del indicates that the telomeric region was intact (17).

Data analysis. The frequency of connexin mutations among children with congenital CMV infection with and without hearing loss and the group of uninfected newborns was compared by constructing 2 \times 2 contingency tables, and statistical significance was calculated using the Fisher's exact test for each mutation separately. Chi-square test and Fisher's exact test were used, where appropriate, to determine whether demographic characteristics differed between the group of children with hearing loss and the group with normal hearing.

Table 1. Demographic and clinical characteristics of children with congenital CMV infection with and without hearing loss and a comparison newborn population

	Hearing loss (N = 19)	Normal hearing (N = 130)	Comparison population (N = 380)
Race			
White	16% (3/19)	14% (18/130)	25% (95/380)
Black	84% (16/19)	86% (112/130)	74% (281/380)
Other	0% (0/19)	0% (0/130)	1% (4/380)
Gender			
Male	42% (8/19)	61% (79/130)	53% (201/380)
Female	58% (11/19)	39% (51/130)	47% (179/380)
Family history of hearing loss*	11% (2/19)	0% (0/106)	N/A
Symptomatic CMV infection at birth†	68% (13/19)	17% (22/130)	—
Neurological disease‡	77% (10/13)	55% (12/22)	—
Chorioretinitis	0% (0/13)	9% (2/22)	—
Petechiae	62% (8/13)	55% (12/22)	—
Purpura	0% (0/13)	9% (2/22)	—
Hepatosplenomegaly	15% (2/13)	27% (6/22)	—
Jaundice	23% (3/13)	50% (11/22)	—
Thrombocytopenia	23% (3/13)	45% (10/22)	—
Ganciclovir therapy	0% (0/13)	<1% (1/22)	—

* $p < 0.05$.

† $p < 0.001$.

‡ Neurological disease is defined as microcephaly, seizures, and/or an abnormality on head imaging (computed tomography or ultrasonography).

RESULTS

The demographic and clinical characteristics of the group of children with congenital CMV infection are shown in Table 1. The majority of the study children were African American. There was no difference between the three groups with respect to gender. As anticipated, more children in the hearing loss group were born with symptomatic congenital CMV infection compared with the normal hearing group ($p < 0.001$). Among the 35 children born with symptomatic disease at birth, there were no differences in the incidence of neurologic abnormalities or other clinical findings between the hearing loss and normal hearing group. Only one child in the study received ganciclovir therapy, and this child had normal hearing. Family history data were available for 125 of the 149 subjects (19 in the hearing loss group and 106 in the normal hearing group). Only two children (in the hearing loss group) had a family history of hearing loss (Table 1). Among the 19 children with congenital CMV infection and hearing loss, 32% (6/19) had hearing loss within the first month of life, whereas the remaining 68% had delayed-onset loss with a median age at onset of hearing loss of 15 mo (range, 3–60 mo).

Among the group of children with congenital CMV infection, eight GJB2 mutations were identified, and all mutations were heterozygous. Although no single mutation was found to be significantly associated with hearing loss, the frequency of GJB2 mutations was higher in children with hearing loss (4/19, 21%) compared with those with normal hearing (4/130, 3%, $p = 0.017$). The types of GJB2 mutation as well as the clinical characteristics of those children with identified mutations are shown in Table 2. Among the four congenitally infected children with hearing loss with an identified GJB2

Table 2. Clinical characteristics and the type of GJB2 mutations for children with congenital CMV infection with and without SNHL

Patient	Nucleotide change*	Protein change	Symptomatic CMV infection at birth	Hearing outcome
1	101T→C	M34T	Yes, petechiae, microcephaly, and intracranial calcifications	Bilateral moderate to severe loss
2	250G→C	V84L	No	Mild to moderate loss in right ear
3	478G→A	G160S	Yes, intrauterine growth retardation, microcephaly	Progressive loss in left ear
4	499G→A	V167M	Yes, petechiae, microcephaly, and intracranial calcifications	Delayed onset mild loss in left ear
5	11G→A	G4D	No	Normal
6	79G→A	V27I	No	Normal
7	250G→C	V84L	No	Normal
8	380G→A	R127H	No	Normal

* All mutations were heterozygous.

mutation, three subjects had symptomatic infection at birth. None of the four children had a family history of hearing loss. The allele frequency for each mutation in the hearing loss group was 0.026 (1/38). Among the four different mutations detected, one GJB2 mutation, V84L, has been previously reported to be associated with hearing loss (9). Another GJB2 mutation, M34T, found in a child with bilateral moderate to severe hearing loss, was thought to be a polymorphism (9). However, a recent study suggested that it could be pathologic even in the heterozygous state (18). The G160S mutation has been described by others as a polymorphism (19,20). One mutation identified in this group, V167M, has not been previously reported in the literature. This novel mutation resulted in an amino acid substitution of methionine for valine at position 167. The type of hearing loss among the children with identified GJB2 mutations varied from delayed-onset mild unilateral loss to bilateral moderate to severe loss (Table 2).

Among the four children with congenital CMV infection and normal hearing in whom GJB2 mutations were detected, all had clinically silent disease at birth and none had a family history of hearing loss. The overall allele frequency for each of the mutations was 0.0038 (1/260). Three of the four mutations found (V84L, R127H, and V27I) have been described previously (9). Two of these mutations, R127H and V27I, are thought to be benign polymorphisms (9,21,22). One novel change, G4D, which resulted in an amino acid substitution of glycine with aspartic acid at position 4, was also detected (Table 2).

Among the 380 newborns that comprised the comparison group, 15 (3.9%) had a GJB2 mutation and all were found in the heterozygous state (Table 3). The most common mutation detected was M34T, found in four subjects with an allele frequency of 0.0053 (4/760). Three GJB2 mutations (V27I, R127H, F83L), thought to be nonpathogenic polymorphisms, were each detected in two subjects (9). R143W, which has been associated with hearing loss, particularly in the African American population (23,24), was found in one subject. An additional pathogenic mutation, V37I (9), was also detected in one subject. Three novel mutations, A40T, R160Q, and G59G were detected, the latter a polymorphism.

The commonly described mutation of GJB2, 35delG, was not found in any of the study children (CMV-infected and

comparison population). In addition, none of the study children (CMV infected and comparison population) had the 342-kb deletion of GJB6.

DISCUSSION

Congenital CMV infection is an important cause of hearing loss in children, yet little is known about whether other factors are associated with hearing loss among infected children. Our study, using one of the largest cohorts of children with congenital CMV infection, is the first to explore whether GJB2 and GJB6 mutations are more frequent in children with CMV-related hearing loss. Although we did not find a single GJB2 mutation that was associated with hearing loss in the study children, we did observe an increased frequency of GJB2 mutations in children with CMV-related hearing loss compared with children with CMV infection and normal hearing and uninfected newborns. Among the 19 children with congenital CMV infection and hearing loss, 21% (4/19) had a GJB2 mutation compared with only 3% (4/130) in the group with normal hearing ($p = 0.017$) and 3.9% (15/380) in the population control group ($p = 0.016$). There are racial/ethnic differences in the distribution of various GJB2 alleles, and the frequency of GJB2 mutations in the African American population has not been well described. Our study examined the carrier rates of GJB2 mutations in a large cohort of predominantly African American children, and we found a similar

Table 3. Type and frequency of GJB2 gene mutations in 380 newborns from an urban hospital in Birmingham, AL

Nucleotide change	Protein change	No. positive*	Allele frequency†
101T→C	M34T	4	0.0053
79G→A	V27I	2	0.0026
249C→G	F83L	2	0.0026
380G→A	R127H	2	0.0026
109G→A	V37I	1	0.0013
118G→A	A40T	1	0.0013
177C→T	G59G	1	0.0013
427C→T	R143W	1	0.0013
478G→A	R160Q	1	0.0013

* All mutations were heterozygous.

† Allele frequency was calculated by dividing the number of mutations by the total number of chromosomes. The overall mutation frequency was 0.0197.

frequency of GJB2 mutations (3%) in the two control populations, the CMV-infected children with normal hearing and the uninfected newborn population. These findings are an important addition to the growing literature of the contribution of GJB2 variants to hearing loss in African American populations (19).

All mutations detected in the study children were heterozygous. A recent study by Ravecca *et al.* (25) examined 39 subjects with progressive hearing loss and found heterozygous mutations in 18% of the patients. The authors concluded that a monogenic model of inheritance could not explain the subject's hearing loss and proposed that additional mutations in other alleles might be responsible for the hearing phenotype. Alternatively, other factors, such as CMV infection or additional environmental factors, could contribute to hearing loss in this study population. Similar to the findings by Ravecca *et al.*, in the present study, 21% of children with CMV-related hearing loss carried a heterozygous mutation in connexin 26. Thus, the results of the present study as well as those of the Ravecca *et al.* study raise the question of whether GJB2 mutations (in the heterozygous or homozygous state) may serve as a modifier to increase the risk of hearing loss in children with congenital CMV infection.

There have been more than 100 GJB2 mutations that have been associated with hearing loss (9). The most common mutation among these is 35delG, which is estimated to be responsible for approximately 10% of all childhood hearing loss (26). Previous studies revealed that the 35delG mutation is less common among African Americans than other racial/ethnic groups. Morell *et al.* (27) screened 173 anonymous samples from African Americans for the 35delG mutation and found no carriers. Gasparini *et al.* (28) did not observe the 35delG mutation in 190 African American controls. In our predominantly African American cohort of 529 subjects (19 with deafness), much like earlier studies, we detected no carriers of the 35delG mutation. However, in a recent study of a large repository of deaf probands, Pandya *et al.* (24) found that seven of 50 African Americans tested were heterozygous carriers of 35delG mutation. Racial admixture among African Americans is known to vary considerably among those living in different geographic regions of the United States, which could explain the differences in the frequency of GJB2 mutations observed by Pandya *et al.* and most of the other studies of African American populations (29–31).

A large deletion in GJB6, del(GJB6-D13S1830), was found to be the second most frequent mutation causing deafness in the Spanish population (10). It has been recommended by some investigators that individuals with one recessive mutation in GJB2 should be screened for this deletion (32). However, recent studies of a predominantly white population in North America revealed that the 342-kb mutation in GJB6 occurred at a much lower frequency than that observed by the Spanish investigators (17,24). In our study, none of the 529 predominantly African American subjects were found to have a deletion in GJB6, suggesting that this large GJB6 deletion is very rare in this population and may not play a role in nonsyndromic autosomal recessive deafness in African Americans.

Although there were no significant differences between the study group and those children from whom peripheral blood or urine samples were unavailable, the selection of the study subjects based on sample availability could have introduced a bias. In addition, the number of congenitally infected children with hearing loss in our study is small. An additional limitation of the present study is the lack of hearing screening information for the newborn comparison population. Because the samples for analysis were provided anonymously without identifiers, this information was not available. Nevertheless, the results of this study show that mutations in connexin 26 are present in children with congenital CMV infection. Therefore, future studies with larger sample sizes are needed to clearly define the relationship between GJB2 mutations and congenital CMV-related hearing loss.

Our understanding of the genetic causes of nonsyndromic hearing loss is evolving, and new mutations in GJB2 as well as other connexin genes are being described on a regular basis. The findings of the present study document that connexin 26 mutations occur in children with congenital CMV infection and that these mutations are found at a higher frequency in children with CMV-related hearing loss compared with children with CMV infection and normal hearing and with an uninfected newborn population. Interpretation of these findings could change as more knowledge of the role of connexin mutations in hearing loss is acquired.

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