Etiology of Severe Sensorineural Hearing Loss in Children: Independent Impact of Congenital Cytomegalovirus Infection and GJB2 Mutations

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Background. Sensorineural hearing loss (SNHL) is the most common congenital disease. Longitudinal studies of infants with congenital cytomegalovirus (CMV) infection have demonstrated an association between CMV and SNHL. However, because of the lack of suitable neonatally collected specimens, the proportion of CMV-associated SNHL has not been defined, nor has the relationship between CMV and the major genetic causes of SNHL, such as mutations in the GJB2 gene.

Methods. Sixty-seven children with severe SNHL were analyzed for CMV and human herpesvirus 6 (HHV-6) infections and for GJB2 mutations. DNA specimens were prepared from dried umbilical cords, which are available for everyone born in Japan. Four children with typical symptomatic infection at birth served as positive control subjects.

Results. Congenital CMV infection and GJB2 mutations were identified in 15% and 24% of the patients, respectively. HHV-6 was not detected. All children with CMV-associated cases developed SNHL before they were 2 years old. Most children with CMV-associated SNHL had no obvious clinical abnormality at birth, and their viral loads were lower than those of symptomatic children.

Conclusions. Congenital CMV infection is an important cause of severe SNHL, and it has an incidence comparable to that of GJB2-associated SNHL.

Sensorineural hearing loss (SNHL) is the most common congenital disease, with an incidence of 1–3 cases/1000 live births, or ~3 times the frequency of Down syndrome [1–4]. Universal hearing screening programs for neonates have been established in several countries and communities on the basis of research findings that early intervention in infants with hearing loss facilitates language development to a level comparable to that of their audiologically normal peers. The Joint Committee on Infant Hearing in the United States issued a statement of principles and guidelines to support the implementation of screening and intervention programs for SNHL in newborns [5].

Hereditary and environmental factors are involved in the etiology of pediatric SNHL (reviewed in [6, 7]). More than 20 genes are associated with autosomal-recessive inheritance, and 20 are associated with autosomal-dominant inheritance. Among them, mutations in the GJB2 gene, which encodes the connexin 26 protein, represent the leading hereditary cause of hearing loss. Among environmental factors, viral infection is thought to play a major role in congenital SNHL. Successful vaccination for rubella in developed countries has virtually eliminated congenital rubella infection as a cause of SNHL; however, a decrease in the seroprevalence of cytomegalovirus (CMV), which may increase the potential of congenital CMV infection, has
been observed in these same countries [8]. SNHL is detected at birth in 5%–10% of neonates with evidence of congenital CMV infection. In addition, longitudinal studies of infants with congenital CMV infection clearly have shown that many infants who are asymptomatic at birth and have normal hearing will develop SNHL during early childhood. Thus, universal newborn screening for hearing loss misses a number of children who will ultimately have late-onset SNHL that is linked to congenital CMV infection [9–13].

Although many studies have reported an association between CMV and hearing loss, the precise proportion of SNHL cases caused by congenital CMV infection has never been measured directly. In addition, to our knowledge, no previous study has compared the prevalences of CMV and any genetic mutations, such as in the GJB2 gene, in a single cohort. Discrimination of congenital infection from postnatal infection requires specimens obtained immediately after birth. Blood-spot samples routinely collected for phenylketonuria assessment have value for this purpose [14]. However, the limited retention period of the materials and issues related to privacy protection [15, 16] have hampered comprehensive and retrospective studies of the infectious etiology of SNHL. Previously, we demonstrated the feasibility of retrospective diagnosis of congenital CMV infection by using dried umbilical-cord specimens [17], which are traditionally provided by Japanese obstetric hospitals to parents as a precious keepsake. In the present study, we took advantage of this tradition to perform DNA-based assessments of the proportions of congenital CMV infection and GJB2 mutations in children with early- and late-onset SNHL.

**SUBJECTS, MATERIALS, AND METHODS**

**Study subjects.** The collection and use of human materials for the present study were approved by the Ethics Committee on Human Subjects of each institute, and informed consent was obtained from parents of all patients. We enrolled 67 children (28 boys and 39 girls) with severe SNHL who were referred to the Fukushima Rehabilitation Center for Disabled Children and the Department of Otolaryngology, Fukushima Medical University, for early speech therapy, an externally worn hearing aid, or surgical intervention, including cochlear implant. The University, for early speech therapy, an externally worn hearing aid, or surgical intervention, including cochlear implant. The University, for early speech therapy, an externally worn hearing aid, or surgical intervention, including cochlear implant. The University, for early speech therapy, an externally worn hearing aid, or surgical intervention, including cochlear implant. The University, for early speech therapy, an externally worn hearing aid, or surgical intervention, including cochlear implant.

Clinical and audiological evaluations.** Birth weight, gestational age, any clinical manifestations, and abnormal laboratory findings were identified from medical records. Interviews with family member(s), audiological tests, computed tomography (CT) imaging, and a mental and physical developmental evaluation were performed, if necessary, when patients with SNHL attended our facilities.

Hearing levels of the patients were measured by at least 1 of the following objective audiological tests: ABR and/or auditory steady-state response using Audera (GSI) or Navigator Pro (Biologic). The patients also underwent at least 1 of the following subjective tests: play audiometry, conditioned orientation reflex audiometry, and/or pure-tone audiometry. These tests were performed by audiological experts and were repeated at least twice to confirm measurements. Hearing levels of the patients were classified into 5 categories on the basis of the severity of the worst ear: profound (>90 dB), severe (71–90 dB), moderate to severe (56–70 dB), moderate (41–55 dB), and mild (20–40 dB). The onset of SNHL was estimated from the medical records and the interview. Cerebral palsy, autism, and other disorders were clinically evaluated by appropriate specialists.

**DNA extraction.** Dried umbilical cords were cut into 5-mm squares with a disposable scalpel. Genomic DNA was isolated using the QIAamp DNA Mini Kit (Qiagen) in accordance with the manufacturer’s directions, except that specimens were incubated in the presence of proteinase K overnight. From 5-mm-square specimens, 5–10 μg of DNA was recovered. DNA specimens were coded to ensure that assay operators were blinded to clinical information.

**Analysis of the GJB2 gene.** A 0.9-kb DNA fragment containing the entire coding region of GJB2 was obtained by PCR using *Pfu* polymerase (Promega). Primer sets 5′-TCTTTTTCGAGGAACGCCG3′ and 5′-GGGCAATGGTCATTACCTTATG3′ or 5′-TCAGAGCCACCCGGC3′ and 5′-TGGCCCTATCCCTTCTCTGCTGTC3′ were used for PCR amplification. The PCR products were separated on agarose gels and purified by using a DNA extraction kit (QIAEX II; QIAGEN). The purified DNA fragments were sequenced with...
the BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems).

**Diagnostic PCR assays.** To detect CMV DNA, a 451-bp fragment derived from the conserved region of the CMV glycoprotein H gene was amplified from the DNA specimens by a nested PCR with primer sets as follows: outer primers 5′-TCTAAACAGAATCAGCAACATCTC-3′ and 5′-CCTTGGGTGTGTTGATTATCT-3′ and inner primers 5′-CAAGAATCCTACCTCATGGG-3′ and 5′-ATGATGAGGCTCTCGGCTTAC-3′. Cycle conditions for the first round were as follows: 1 cycle of 2 min at 95°C and 40 cycles of 20 s at 95°C, 1 min at 53°C, and 40 s at 72°C, followed by 1 cycle of 10 min at 72°C. Conditions for the second round were the same, except for 1 min at 48°C in place of 1 min at 53°C.

Real-time PCR was performed using TaqMan Universal PCR Master Mix (PE Applied Biosystems). Twenty-five microliters of reaction mixture contained 0.2–1.0 μg of umbilical-cord DNA, 1× TaqMan universal master mix, 0.2 mmol/L dNTP, 0.2 μmol/L each primer, and 0.25 μmol/L TaqMan probe. The PCR conditions consisted of 1 cycle of 2 min at 50°C and 10 min at 95°C followed by 50 cycles of 30 s at 95°C and 1 min at 60°C for human albumin and CMV UL83 genes and 1 min at 58°C for human herpesvirus 6 (HHV-6) UL67. The reaction and data analysis were performed using the ABI PRISM 7500 system. For human albumin gene, primers and a probe described elsewhere [18] were used. DNA extracted from a human system. For human albumin gene, primers and a probe described elsewhere [18] were used. DNA extracted from a human

**Statistical analysis.** Statistical significance was evaluated using the χ² test.

**RESULTS**

**Clinical and audiological assessment of SNHL cohort.** All cases of SNHL analyzed were nonsyndromic, and there were no familial cases. Sixty-three case patients had bilateral SNHL, and 4 had unilateral SNHL. Cases of bilateral SNHL were categorized into profound (n = 36), severe to moderately severe (n = 19), and moderate to mild (n = 8). All 4 cases of unilateral SNHL were profound or severe.

CT imaging of temporal bones identified 2 cases of Mondini malformation and 1 case of malformation of the external auditory canal and middle ear among 28 patients with SNHL for whom the test was performed. Two patients had Down syndrome, and 1 had a chromosomal abnormality. Of the 67 cases, mental retardation was diagnosed in 31, cerebral palsy in 4, and autism in 3.

**Prevalence of congenital infection with CMV and HHV-6.** All 4 umbilical-cord DNA specimens from infants with symptomatic CMV infection at birth, but none of 17 healthy infants were CMV positive both by the nested PCR and by real-time PCR, which confirmed the accuracy of PCR using umbilical-cord DNA. Ten (15%) of 67 patients with SNHL were CMV positive by both assays. All CMV-positive cases had unique CMV UL144 gene sequences (data not shown), indicating that the positive PCR results were not due to laboratory contamination. Medical records indicated that 2 of these CMV-positive patients had intrauterine growth retardation (IUGR). One of the patients with IUGR had thrombocytopenia, ventricular dilatation, and slight intracranial calcification at birth, although there was no laboratory confirmation of congenital CMV infection. Congenital CMV infection was confirmed by laboratory tests in the other patient. In addition to the patients with IUGR, 1 patient had cerebellar dysplasia, hydrocephalus, ventricular dilatation, and intracranial calcification at birth, with laboratory confirmation of CMV involvement both by PCR of blood and urine specimens and by detection of anti-CMV IgM. The remaining 7 CMV-positive patients with SNHL had no clinical manifestations at birth; therefore, no laboratory examination was conducted. HHV-6 DNA was not detected in any of the 88 umbilical-cord specimens analyzed from our collection.

**Incidences of the GJB2 defect.** Connexin 26–inactivating mutations in the GJB2 gene were present in 16 (24%) of 67
patients with SNHL. Eleven had homozygous mutations, including a 1-bp deletion at nt 235 (235delC) \( (n = 10) \) and a 16-bp deletion between nt 176 and 191 (176–191del16) \( (n = 1) \). Five patients had compound heterozygous mutations, including 235delC plus an alteration of G→A at nt 134 (G134A) \( (n = 2) \), G401A \( (n = 1) \), or 176–191delC \( (n = 1) \), and 176–191delC plus a 2-bp deletion at nt 299 and 300 \( (n = 1) \). Most of these mutations have been reported elsewhere [22]. Non-pathological polymorphic changes, including G79A, G341A, and T608C, were detected in more than one-third of patients. All CMV-positive patients had normal GJB2 genes.

**Possible etiologies other than CMV infection and GJB2 defects.** Other than CMV infection and GJB2 defects, there were 5 patients with obvious genetic problems, including Down syndrome \( (n = 2) \), Mondini malformation \( (n = 2) \), and a chromosomal abnormality \( (n = 1) \). The group with unknown etiology included infants with extremely low birth weight \((<1600 \text{ g})\) \( (n = 4) \) and those with a malformation of the external auditory canal and middle ear \( (n = 1) \), hyperbilirubinemia \( (n = 2) \), and the use of extracorporeal membrane oxygenation \( (n = 2) \). No cases were associated with rubella infection or meningitis. The group with unknown etiology may have contained patients with genetic mutations other than in GJB2, such as those in GJB6 and in mtDNA.

**Factors related to the etiology of SNHL.** SNHL developed before age 2 years in all but 1 of the CMV-positive or GJB2-deficient patients with SNHL, whereas more than one-quarter of the patients with unknown etiology developed SNHL after age 2 years (table 1; CMV, \( P < .05 \)). Importantly, at least 5 of the CMV-positive patients lost their hearing capability after age 6 months. Because there was some ambiguity of the onset age because of gaps in records and recollections, to verify the earlier onset of CMV- or GJB2-associated SNHL, we examined the ages when the patients were referred to our facilities for thorough audiological examinations and language therapy. Means and SDs of the ages at referral were 39 ± 20, 46 ± 38, and 83 ± 56 months for the CMV-positive, GJB2-deficient, and unknown etiology groups, respectively, which indicates that the patients with CMV- and GJB2-associated SNHL were referred earlier. The CMV- or GJB2-associated cases were characterized by more-severe hearing impairment than that in patients with unknown etiology (table 2; CMV and GJB2, \( P < .005 \)).

**Comparison of CMV loads.** Viral loads of CMV-positive patients were measured and expressed as CMV DNA copy numbers per microgram of cellular DNA. As shown in figure 1, the patients with CMV-associated SNHL had substantially lower viral loads than the 4 positive control subjects—that is, children with typical symptomatic infection at birth. Because of the small number of CMV-positive patients, it was impossible to find any significant relationship between CMV copy numbers and either estimated onset age or degree of hearing impairment.

**DISCUSSION**

In the present retrospective study of the etiology of SNHL, congenital CMV infection was associated with a substantial number of severe cases of SNHL. One-fifth of cases of SNHL with onset before 2 years of age were ascribed to congenital CMV infection, a proportion similar to that attributable to hereditary mutations in GJB2.

Previously, population-based rates of SNHL caused by congenital CMV infection were indirectly estimated from the following 3 components: population-based rates of congenital infection, rates of SNHL in cohort studies of infants with congenital CMV infection, and population-based rates of SNHL. Because the population-based rates of SNHL caused by congenital infection and of overall SNHL were estimated to be 0.2–0.6 and 1–3 cases/1000 live births, respectively, simple algebra yields an estimate that congenital CMV infection may account for 10%–60% of cases of SNHL (reviewed in [23]). Barbi et al. [14] reported that dried blood spots were positive for 9 (10%) of 87 infants with SNHL who had hearing loss at >40 dB. This prevalence may have been an underestimate, because (1) the relatively small amount of DNA that was extracted from the blood spots and (2) testing was conducted only for children whose SNHL was diagnosed at age <2 months. By contrast, we used specimens that afforded greater analytic sensitivity and enrolled patients spanning a wide range of age, from birth to age 16 years. Therefore, our retrospective study is the first comprehensive study that substantiates the estimates based on population-based studies.

A survey conducted by the Japanese government in 2002

<table>
<thead>
<tr>
<th>Estimated age at onset*</th>
<th>Congenital CMV ( (n = 10) )</th>
<th>GJB2 mutation ( (n = 16) )</th>
<th>Othersb ( (n = 5) )</th>
<th>Unknown ( (n = 36) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;2 years</td>
<td>10</td>
<td>15</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>Birth</td>
<td>2</td>
<td>7</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>&lt;6 months</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>6–12 months</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>12–24 months</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Not clearc</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>After 2 years</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>11</td>
</tr>
</tbody>
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*Estimated onset age at birth.

**NOTE.** CMV, cytomegalovirus.

* Based on medical records and interviews with family members

b Mondini malformation \( (n = 2) \), Down syndrome \( (n = 2) \), or chromosomal abnormality \( (n = 1) \).

* Age at onset known to be <2 years but could not be specified.
Table 2. Severity of sensorineural hearing loss (SNHL) and etiology.

<table>
<thead>
<tr>
<th>Etiology</th>
<th>Bilateral SNHL</th>
<th>Unilateral SNHL, profound and severe</th>
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<tbody>
<tr>
<td></td>
<td>Profound (n = 36)</td>
<td>Severe and moderately severe (n = 19)</td>
</tr>
<tr>
<td>Congenital CMV infection</td>
<td>8 (22)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Genetic defects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GJB2 (n = 16)</td>
<td>14 (39)</td>
<td>2 (11)</td>
</tr>
<tr>
<td>Others (n = 5)</td>
<td>4 (11)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Unknown (n = 36)</td>
<td>10 (28)</td>
<td>16 (44)</td>
</tr>
</tbody>
</table>

NOTE. Data are no. (%) of subjects. CMV, cytomegalovirus.

reported that 15,200 children with severe (>70 dB) hearing loss were officially registered as "disabled." Because ~90% of them had SNHL, the incidence of severe SNHL was estimated to be 0.6 cases/1000 children <18 years old. Two-thirds of these children with SNHL had profound hearing loss (>90 dB). In the area with a population of 369,000 children from which our facilities have accepted referred cases, ~180 cases of SNHL have been officially registered. More than one-third of these were referred to us, and most of them were enrolled without any bias in the study. The enrolled cases had a spectrum of severities similar to that in Japan as a whole. Because only cases with hearing loss at >40 dB were referred, our study could not assess cases of mild SNHL. Further study is needed to evaluate congenital CMV infection in such cases.

The use of dried umbilical cords had 2 advantages: (1) Japanese tradition has made dried umbilical cords a specimen that is available for almost every person born in Japan, and (2) these are large specimens that provide much more DNA (5–10 μg) than can be recovered from conventional dried blood spots (~30 ng of DNA from a disc of 3 mm in diameter). The latter factor permitted us to perform a wider variety of tests for the patients with SNHL with a higher sensitivity than would have been possible using dried blood-spot specimens. As a result, we were able to compare the relative contributions of congenital CMV and HHV-6 infections and GJB2 gene defects on the SNHL disease burden in the same population.

The progressive nature of congenital CMV-associated SNHL has been carefully described [11, 24, 25]. Consistent with prior descriptions, we found that all cases of CMV-associated SNHL occurred before age 2 years, but, in 5 of 10 cases, CMV-related SNHL was not detected before 6 months of age. In another study, congenital CMV infection was detected in only 1 of 20 cases of SNHL identified through newborn hearing screening, although the rate of GJB2-related cases (30%) was equivalent to what we observed in the present study (24%) [26]. Collectively, these findings demonstrate that the identification of infants at risk for SNHL will require a combination of universal auditory screening and newborn CMV screening. Screening for congenital CMV infection might lead to new treatment options for CMV-infected infants with antiviral agents such as ganciclovir [27]. Importantly, CMV loads in patients with delayed-onset SNHL tended to be lower than those in positive control subjects (i.e., 4 children with symptomatic infection at birth). Although further study with a bigger sample size is required to confirm this finding, our finding is consistent with that of a previous report that demonstrated higher CMV loads in infants with symptomatic infection [28]. Thus, a sensitive assay for CMV detection is required for newborn CMV screening to identify asymptomatic patients who might develop SNHL later on. From this point of view, blood specimens may not be the best choice of specimens for the screening, because viral loads in blood specimens are >2 logs lower than those in urine specimens [29] (N.I. and S.K., data not shown). Because the use of dried umbilical-cord specimens is not practical for screening,

Figure 1. Comparison of cytomegalovirus (CMV) loads. White circles indicate the positive control children with symptomatic CMV infection at birth. Shaded and black circles indicate the CMV-positive children identified in the sensorineural hearing loss (SNHL) cohort with their estimated onset before and after the age of 6 months, respectively.
the development of rapid and convenient methods for the
detection of CMV in urine specimens would be essential.

It is noteworthy that congenital CMV infection and GJB2
mutations appear to be independently associated with SNHL.
However, because CMV infection is known to increase the risk
of chromosomal abnormality (reviewed in [30]), this does not
exclude possible association between CMV infection and muta-
tions in genes other than GJB2.

Like CMV, HHV-6 belongs to the betaherpesvirus subfamily,
and the viruses have a similar genomic structure. A recent study
demonstrated asymptomatic congenital infection with HHV-6
but not HHV-7 at a frequency of 1% [31]. In addition, it is
well known that HHV-6 is neurotropic and that it causes neu-
rological diseases [32]. Therefore, it would be interesting to
determine whether HHV-6 causes SNHL, as CMV does. How-
ever, we found no evidence for congenital HHV-6 infection in
any of the patients with SNHL. There are 2 possible expla-
nations: congenital HHV-6 infection rarely causes SNHL and/or
there is a lower frequency of congenital HHV-6 infection in
our general population that hampered our attempts to identify
HHV-6–associated SNHL in our sample size. Because HHV-6
DNA can be detected in maternal blood, our negative data on
HHV-6 suggest the specificity of our assay using dried umbilical
cords.

In conclusion, our retrospective analysis of the etiology of
SNHL demonstrates directly that congenital CMV infection is
responsible for a substantial proportion of early-childhood
SNHL and that almost half of the infants at risk for the de-
velopment of late-onset CMV- or GJB2-associated SNHL show
no clinical and audiological indications at birth. Our results
support the results of previous studies that advocated the need
for neonatal screening programs for both CMV and the prev-
alent genetic causes of SNHL.

Acknowledgments

We thank all the children who participated in the study and their
parents. We also thank Naoko Yamada, Kiwa Ohotomo, Yumiko Yamamoto, Yuhi
Inami, and Hitomi Komura for technical assistance; Ichiro Kurane, Phil
Pellett, and D. Scott Schmid for intellectual input; and Felicia R. Stamey
for English editing.

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