Evaluation of newborn screening bloodspot-based genetic testing as second tier screen for bedside newborn hearing screening

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Purpose: Bedside newborn hearing screening is highly successful in identifying deaf or hard-of-hearing infants. However, newborn hearing screening protocols have high loss to follow-up rates. We propose that bloodspot-based genetic testing for *GJB2* alleles can provide a means for rapid confirmation in a subset of infants who fail bedside newborn hearing screening. **Methods:** We performed a case-control study comparing the prevalence of common *GJB2* mutations from deidentified bloodspots designated as "refer" by newborn hearing screening and contemporaneously selected randomly chosen controls designated as "pass." Between March 2006 and December 2007, 2354 spots were analyzed for common alleles, c.35delG, c.167delT, c.235delC, and p.V37I in *GJB2* with a subset reanalyzed by conventional Sanger sequencing to search for additional alleles. **Results:** The prevalence of biallelic *GJB2* mutations in bloodspots from infants who referred by newborn hearing screening is approximately 1 in 50 (23/1177). In contrast, one bloodspot from an infant who passed newborn hearing screening was identified to harbor biallelic *GJB2* mutations. **Conclusions:** These findings show that when a newborn refers by newborn hearing screening, there is a significant chance that *GJB2*-related hearing loss is present. Bloodspot-based genetic testing for common *GJB2* alleles should be considered as second tier testing for bedside newborn hearing screening. ***Genet Med* 2011:13(12):1006–1010.**

Key Words: *genetic testing, newborn hearing screening, connexin 26, GJB2, newborn screening*

One to three per 1000 infants have sensorineural deafness/hard of hearing present at birth,[1,2] and evidence shows that identification and habilitation of deaf infants before 6 months of age improves language outcomes.[3,4] These data support the time-critical nature of newborn hearing diagnosis and treatment and formed the impetus for newborn hearing screening (NHS) programs throughout the world.

To detect congenital hearing loss, newborns are screened using one or two functional automated bedside physiologic methods. The automated auditory brainstem response detects brainstem response to sound, whereas otoacoustic emissions detects the cochlear response to sound. If a newborn passes hearing screening after one or two attempts, the infant is given the designation of "pass." If a newborn fails hearing screening after two attempts, the infant is given the designation of "refer," meaning that further screening and possibly diagnostic audiometric testing is required to determine whether that infant has permanent hearing loss (http://www.cdc.gov/ncbddd/ehdi/ehdi.htm).

Hearing loss has multiple causes, with genetic causes contributing to nearly half of all cases.[5] Autosomal recessive mutations in the Gap Junction Beta-2 gene (*GJB2*) encoding the gap junction protein connexin 26 are the most common genetic basis of hearing loss in populations worldwide[6–10] and can be identified in approximately 25–40% of North American deaf or hard-of-hearing children.[7,9,11,12] There are a small number of common *GJB2* mutations carried in the heterozygous state in various populations. The allele c.35delG is found in 3% of individuals descended from Europeans,[13,14] alleles c.235delC and p.V37I are found in 2% and 10%, respectively, of individuals descended from Asians,[15–17] and allele c.167delT is found in up to 7% of individuals descended from Ashkenazi Jews.[18] Additionally, inheritance of deletions in *GJB6,* encoding the gap junction protein connexin 30, in trans with point mutations in *GJB2* has been reported in high frequency in some European hearing loss populations,[19–21] but appears less commonly in US deaf and hard-of-hearing populations.[11]

The phenotype associated with autosomal recessive mutations in *GJB2* and *GJB6* is nonsyndromic sensorineural hearing loss with considerable genotype/phenotype correlation based on the types of mutations identified in affected individuals. Biallelic truncating mutations (i.e., frameshift and nonsense mutations such as c.35delG, c.235delC, and c.167delT) are identified more frequently in individuals with severe to profound hearing loss, whereas biallelic amino acid substitutions (missense) alleles (e.g., p.V37I) are identified more frequently in individuals with mild to moderate hearing loss.[22,23] Based on previous studies in a pediatric population,[12] where both exons of *GJB2* were sequenced in genomic DNA from 93 probands diagnosed with hearing loss in the first 2 years of life, 24.7% had bialleleic *GJB2* mutations with the majority of alleles being attributed to 35delG, V37I, 235delC, and 167delT.

The use of genetic testing as an adjunct to bedside NHS has been discussed,[5,24,25] but added costs, the multiplicity of genes associated with hearing loss, and confusion in nomenclature between carrier status (an individual identified with a single *GJB2* mutation or monoallelic), and affected status (an individual identified with two *GJB2* mutations or biallelic) have been limiting factors in implementation.[12,26,27]

To determine the relevance of genetic testing for newborn hearing loss, we performed a study using deidentified residual newborn screening bloodspots. We tested the hypothesis that

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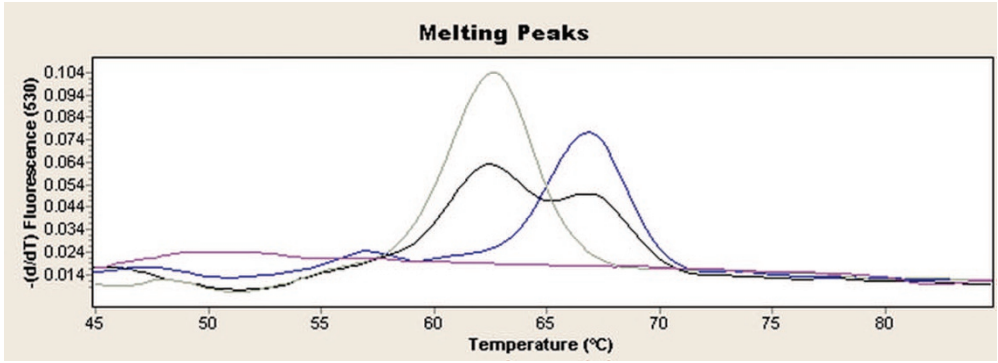

Fig. 1. Representative melt curve analysis for the c.35delG allele in *GJB2* using the Lightcycler. Using sequence complementary to the wildtype sequence surrounding the 35delG mutation, a Simple probe with a fluorescent tag was designed. Upon amplification of a 154bp fragment of *GJB2*, exon 2, amplified DNA is melted and annealed to the probe and then heated. The melt curve shows a single peak (dark blue) at approximately 67° for wildtype, but melts at a lower temperature, 62°, when the guanine is deleted at position 35 (grey) (homozygous 35delG). A heterozygote for the c.35delG allele will have two melting peaks (black). The negative control (purple) is also shown.

genetic testing would detect a higher prevalence of infants with biallelic *GJB2* mutations in bloodspots from infants who failed NHS than in those from infants who passed NHS and would also identify bloodspots from infants with biallelic *GJB2* mutations who escaped detection by NHS. We also determined whether bloodspot-based screening would be useful for detection of *GJB2* alleles in bloodspots from a diverse North American population.

METHODS

Study population

This study was performed with the approval of the Institutional Review Boards of the University of Minnesota and the Minnesota Department of Health (MDH).

From March 2006 to December 2007, 2364 deidentified bloodspots were collected from two NHS groups. The "refer" group included all possible newborn bloodspot cards submitted to the MDH from infants who "failed" NHS in both ears during that time period. The "pass" group included newborn bloodspot cards taken from contemporaneously selected random controls from infants who passed NHS in both ears during that time period. For each group, the MDH provided a deidentified 3 mm bloodspot and information regarding infant sex and ethnicity. Because of the deidentified nature of this study, data about the final hearing outcome and any subsequent diagnostic evaluations could not be obtained.

Five spots in each group did not provide adequate DNA for genotyping and were excluded from the final analysis. A total of 2354 spots were analyzed; 1177 in each of the pass and refer groups.

Genotyping

Bloodspots from the pass and refer groups were compared for the prevalence of common hearing loss associated variants in *GJB2*. DNA was isolated from each 3 mm punch using salt extraction in a 96-well plate format (5Prime, Gaithersburg, MD). We performed a rapid low-cost assay using asymmetric polymerase chain reaction and melt curve analysis and fluorescently tagged "Simple" probes for the common alleles c.35delG, c.167delT, c.235delC, and p.V37I on a Roche LightCycler 960 (Roche, Indianapolis, IN) and analyzed melt curves using LightCycler software. Melt curves for each allele could detect wild type (wt), homozygous, and heterozygous calls for each allele (Fig. 1).

Spots from racial and ethnic groups designated Hispanic, Native American, and African American were bidirectionally sequenced for the single coding exon 2 of *GJB2* as the common alleles described earlier are not typically identified in these groups. Any sample that had a single mutant allele or an unusual melt curve in the LightCycler assay was bidirectionally sequenced. Sequencing was performed in the Biomedical Genomic Center at the University of Minnesota on a 3130 Genetic Analyzer sequencer and analyzed using CLC FreeWorkbench software (clcbio.com) followed by visual confirmation. For detailed methods, primers, and conditions see Appendix, Supplement Digital Content 1, http://links.lww.com/GIM/A191.

After the initial data analysis, 1043 samples (546 passes and 497 refers) that previously underwent allele-specific analysis and identified to have normal sequence were unidirectionally sequenced for *GJB2* exon 2 to determine whether any additional pathogenic alleles would be detected that would have been missed by our initial allele-based screening strategy.

Determination of race/ethnicity

Race and ethnicity could only be determined based on groups as represented on the MDH newborn screening bloodspot cards for the years 2006–2007. This card is used at all birthing hospitals in Minnesota and has check box designators for "White," "Black," "Asian," "Hispanic," "Native American," and "Other." The race/ethnicity was designated as "unknown" if no box was checked. In this article, samples designated "White" will be referred to as "Caucasian" and samples designated "Black" will be referred to as "African American."

Statistical analysis

Allele frequencies and characteristics in the pass and refer groups were compared using the χ^2 test. For bloodspots of Caucasian origin, the odds of newborn screening referral were computed for the *GJB2* biallelic group, using a no mutation identified group as the reference. All calculations were completed in SAS (version 9.2, SAS Institute, Inc., Cary, NC).

Table 1. Reported race/ethnicity and corresponding numbers of newborn screening bloodspots indicating NHS pass (contemporaneously selected random spots) or refer (failed NHS in both ears)

Race/ethnicity	Pass	Percentage	Refer	Percentage
Asian	41	3.5	67	5.7
African American	68	5.8	85	7.2
Hispanic	74	6.3	120	10.2
Native American	25	2.1	26	2.2
Other	57	4.8	60	5.1
Unreported	94	8.0	113	9.6
Caucasian	818	69.5	706	60.0
Total	1177		1177	

Table 2. Pass and refer groups gender composition

	Pass	Percentage	Refer	Percentage	P (χ^2 test)
Female	576	48.9	475	40.4	
Male	601	51.1	702	59.6	<0.0001
Total	1177	100	1177	100	

Table 3. Prevalence of biallelic and monoallelic mutations based on NHS pass and refer status

	Pass	Refer	P (χ^2 test)
Biallelic mutations	1	22	<0.0001
Monoallelic mutations	52	64	0.2173
No mutation identified	1124	1091	reference group
Total	1177	1177	

Table 4. Distribution of biallelic mutations by race and NHS refer or pass

Race	Biallelic genotype (no. observations)
Caucasian	Refer: c.35delG/c.35delG (6), c.35delG/p.M34T (3), p.V37I/p.L90P (1)
	Total: 10
	Pass: c.35delG/c.35delG (1)
	Total: 1
Asian	Refer: p.V37I/p.V37I (6), p.V27I,E114G/p.V27I, E114G (1), p.V37I/ p.V27I,E114G (3)
	Total: 10
Other/ unreported	Refer: p.V37I/ p.V27I,E114G (1), p.V37I/p.V37I (1), M34T/H100Y (1)[a]
	Total: 3

[a]This biallelic mutation was identified by sequencing a sample previously designated as normal. See "Methods" for details.

RESULTS

The infant race/ethnicity and gender from each individual spot reported by MDH is documented in Tables 1 and 2. Statistically significant associations between race/ethnicity and the study groups ($P < 0.0001$), and between gender and the study groups ($P < 0.0001$), were observed. Noncaucasian and male infants were more likely to be classified as refer bilaterally by NHS than Caucasians and female infants.

In the entire cohort of 2354 bloodspots, we identified 23 spots with biallelic mutations in *GJB2* using our initial allele-specific screening strategy. As *GJB2*-related hearing loss is a recessive condition, we would expect all infants with biallelic mutations to be deaf or hard of hearing. In the refer group, we identified 22 spots of 1177 with biallelic mutations in *GJB2* (Tables 3 and 4), representing a prevalence of *GJB2*-related hearing loss in infants who fail NHS of approximately 1 in 50. In the pass group, we identified only 1 spot of 1177 with biallelic mutations in *GJB2,* showing that the frequency of having *GJB2*-related hearing loss and passing NHS is low in this population. The difference in prevalence between the two groups was statistically significant ($P < 0.0001$). The crude odds ratio for having *GJB2*-related hearing loss in Caucasian infants who were referred based on by NHS was 11.8 (95% confidence interval: 1.5–92.4).

As *GJB2*-related hearing loss is a recessive condition, we would expect that spots with a single deafness causing allele (mutation) would most likely reflect that the individual is a carrier or alternatively that a second unidentified hearing loss associated allele remains undetected. We identified single alleles in 4.5% (52/1124) of the pass group and 5.5% (64/1091) of the refer group, which was not a statistically significant difference ($P = 0.2173$) (Table 3). Single hearing loss associated alleles were distributed among all groups but were more prevalent in Caucasian and Asian groups (Tables, Supplement Digital Content 2, http://links.lww.com/GIM/A192).

The presence of a single hearing loss associated allele could be identified in 4.3% of newborn screening bloodspot cards from Caucasian infants who passed NHS. This frequency is not significantly different from the reported carrier frequency for individuals in the Midwestern United States (3.01%).[7] A number of nonpathogenic polymorphic variants were identified (Table, Supplement Digital Content 3, http://links.lww.com/GIM/A193).

Biallelic *GJB2* mutations were not distributed evenly between racial and ethnic groups. Biallelic mutations were identified only in bloodspots from Asian, Caucasian, and other/unknown ethnic groups (Table 4).

As p.V37I is typically associated with mild to moderate hearing loss, we might have expected this genotype to be missed by NHS; however, this genotype was only identified in bloodspots from infants who referred by NHS. There were no biallelic missense mutations detected among spots from the pass group, showing that NHS is quite effective at identifying potentially mild moderate hearing loss caused by missense alleles (Table 4).

To determine whether our strategy of performing allele-specific assays of c.35delG, c.167delT, c.235delC, and p.V37I would miss a significant number of spots harboring mutations, we unidirectionally sequenced 1043 samples, 546 from the pass group and 497 from the refer group. All samples in this group were found to be normal at all tested loci and did not belong to Hispanic, Native American, or African American groups. In this group of 1043 spots, an additional spot in the refer group was found to have a bialleleic mutation, p.M34T/p.H100Y, in a

sample designated as "Other." No additional biallelic mutations were found in the pass group (Table, Supplement Digital Content 4, http://links.lww.com/GIM/A194).

In this group of 1043 spots described in the preceding paragraph, we identified 24 additional single alleles, the majority being M34T. We identified six p.M34T/wt, one p.L90P/wt, and three p.V27I, E114G/wt in the pass group. We identified nine p.M34T/wt, four p.V27I, E114G/wt, and one p.H100Y/wt in the refer group. These findings suggest that our allele-specific assays were robust, but M34T should be assayed in addition to c.35delG, c.167delT, p.V37I, and c.235delC in future studies of newborn screening allele-specific panels. These alleles are not listed in Tables, Supplement Digital Content 2, http://links.lww.com/GIM/A192 as they represent only a subset of the total group.

CONCLUSION

The causes of hearing loss are heterogeneous with half of all hearing loss in children attributable to underlying genetic causes.[5] Mutations in *GJB2* are the most common genetic cause of hearing loss. To determine the relevance of genetic testing for NHS, we analyzed deidentified residual newborn screening bloodspots from infants who passed or referred in the NHS process. Based on the study design, no final hearing outcome data for any of the bloodspots could be known to the investigators. However, this time point reflects the situation when a primary care clinician is initially confronted by a newborn referred by NHS. Hence, this is a valid time point to determine the prevalence of *GJB2* mutations in bloodspots from infants who pass or refer by NHS.

We show that approximately 1 in 50 bloodspots from infants who refer by NHS will have biallelic mutations in *GJB2*. This means that there is a significant likelihood of identifying infants with *GJB2*-related hearing loss in the pool of infants who refer NHS. We calculated the odds ratio for the likelihood of having *GJB2*-related hearing loss based on pass or refer NHS as indicated by the presence of biallelic mutations in *GJB2* for bloodspots of Caucasian designation. Caucasian infants who refer by NHS are 11.8 times more likely to have *GJB2*-related hearing loss than those who pass NHS (95% confidence interval: 1.5–92.4).

We also show that passing newborn screening and having biallelic mutations in *GJB2* is a rare occurrence. We identified 1 of 1177 spots that harbored biallelic c.35delG mutations in *GJB2*. This inability of NHS to identify an infant who will most likely be deaf based on genetic testing could be due to technical issues carrying out bedside NHS or possible delayed onset of hearing loss.[12,28,29]

Both *GJB2* truncating and missense mutations were identified in the group of bloodspots from infants referred by NHS. As expected, homozygosity for the c.35delG mutation was detected by NHS. We show that alleles predisposing to mild-moderate hearing loss caused by missense mutations such as p.V37I would also be detected by NHS. We also show that using an approach where alleles 35delG, 167delT, 235delC, and V37I are screened is robust, but addition of M34T should be added for future studies. Furthermore, consideration should be given to adding assays to detect deletions in *GJB6* to any future study.[11,19,20]

On the basis of these results, are we ready to begin genetic testing for hearing loss as a second tier screen for hearing loss? Benefits would include rapid confirmation of hearing status. Under current models, most infants who fail NHS and are evaluated for hearing loss may not undergo diagnostic evaluation until 3 months. Genetic testing results performed in a state public health laboratory could be available earlier and would facilitate more rapid diagnosis and habilitation of infants who have this genetic form of hearing loss.

Current data from the Centers for Disease Control and Prevention showed that for 2008, the most recent year reported, 43% of infants who referred by NHS were lost to follow-up (www.cdc.gov/ncbddd/hearingloss/2008-data/2008_EHDI_HSFS_Summary.pdf). Awareness of the prevalence of *GJB2*-related hearing loss in infants who refer by NHS would provide impetus to be sure that they are not lost to follow-up.

Other benefits would include the identification of infants who are missed by NHS and have a false-negative result. Previous retrospective studies have shown that the rate for *GJB2*-related hearing loss in newborns who pass NHS is between 3.8% and 8% from retrospective studies of probands who already have the diagnosis of hearing loss.[12,28] The identification of bialleleic *GJB2* mutations in bloodspots from infants who pass NHS suggests that for some infants *GJB2*-related hearing loss may not be present at birth and thus escape detection using current functional NHS tools.

Limitations of genetic testing for NHS include how to handle the 4.4–5.5% of samples with a single mutant allele. Single alleles can indicate either a carrier state or a situation where the second mutant allele has not been identified. If an infant with a single mutant allele was flagged for diagnostic testing, this would vastly increase the number of infants referred to audiology providers for diagnostic testing, especially when nearly all would represent a carrier state and would greatly increase the need for genetic counseling services to communicate results.[26] However, this scenario and carrier frequency are not very different from current newborn screening strategies for successful cystic fibrosis newborn screening.[30,31] Thus, use of genetic testing to detect whether a newborn is deaf or hard of hearing could be used much as a second tier genetic testing to detect cystic fibrosis.

Genetic testing for *GJB2*-related hearing loss does not serve the entire population. Biallelic mutations in *GJB2* are found most commonly in individuals of Caucasian or Asian origin and less frequently in other groups. Further studies are required to determine the major causes of hearing loss in infants of African, Hispanic, and Native American origin.

In summary, *GJB2*-related hearing loss is the most common form of hearing loss worldwide. Caused by autosomal recessive mutations in *GJB2*, common alleles account for the majority of identified mutations, thus creating a situation in which allele-based NHS screening strategies could be potentially implemented. Bloodspot-based NHS may become an adjunct to beside-based NHS. However, before implementation, further studies are needed, including development of strategies for sharing results of single alleles, and the identification of genetic testing that would provide benefits to infants who belong to African American, Hispanic, and Native American ethnic groups.

In our study, we found that 1 in 50 bloodspots from newborns who failed NHS had biallelic mutations in *GJB2*. These findings provide strong evidence that when a newborn is found to refer by NHS, it is probable that a child will have *GJB2*-related hearing loss. This evidence provides impetus to support follow-up diagnostic audiometric evaluation and subsequent genetics evaluations for infants who refer by NHS. This study also provides a framework to consider implementation of genetic testing as a second tier test after bedside NHS.

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