Milestones toward cochlear gene therapy for patients with hereditary hearing loss

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Abstract
A number of genes are reportedly responsible for hereditary hearing loss, which accounts for over 50% of all congenital hearing loss cases. Recent advances in genetic testing have enabled the identification of pathogenic variants in many cases, and systems have been developed to provide personalized treatment based on etiology. Gene therapy is expected to become an unprecedented curative treatment. Several reports have demonstrated the successful use of cochlear gene therapy to restore auditory function in mouse models of genetic deafness; however, many hurdles remain to its clinical application in humans. Herein, we focus on the frequency of deafness genes in patients with congenital and late-onset progressive hearing loss and discuss the following points regarding which genes need to be targeted to efficiently proceed with clinical application: (a) which cells’ genes are expressed within the cochlea, (b) whether gene transfer to the targeted cells is possible using vectors such as adeno-associated virus, (c) what phenotype of hearing loss in patients is exhibited, and (d) whether mouse models exist to verify the effectiveness of treatment. Moreover, at the start of clinical application, gene therapy in combination with cochlear implantation may be useful for cases of progressive hearing loss.

KEYWORDS
adeno-associated virus, cochlear implant, electric-acoustic stimulation, gene therapy, genetic deafness, hereditary hearing loss

1 INTRODUCTION

Congenital hearing loss is a relatively common disorder, occurring in 1 to 2 per 1000 newborns, among which 60%–70% of cases are hereditary.1 Due to widespread newborn hearing screening, hearing loss has been increasingly detected in the early stages after birth.1 Identifying the etiology of hearing loss has also improved due to genetic testing, imaging with CT and MRI, and testing for congenital cytomegalovirus infection following auditory testing.2 Meanwhile, hearing aids (HAs) and cochlear implants (CIs) are indicated depending on the severity of hearing loss, with CIs currently being the standard treatment for severe-to-profound hearing loss patients. However, these devices are not biological treatments. Gene therapy is expected to be developed as a treatment targeting causal genes for genetic deafness.3 Here, we report a summary of the current state of genetic testing in humans, gene therapy research in mouse models, and the areas requiring further study, as well as milestones toward the clinical application of gene therapy.
USEFULNESS OF GENETIC TESTING IN PATIENTS WITH HEREDITARY HEARING LOSS

Approximately 120 deafness genes have been identified as responsible for non-syndromic hearing loss (NSHL) (https://hereditaryhearingloss.org/), which refers to hearing loss without other signs or symptoms. Among a number of deafness genes, assumptions regarding the causative gene of NSHL from the severity of hearing loss and mode of inheritance are difficult due to genetic heterogeneity; thus, an accurate diagnosis of NSHL requires genetic testing. Over 400 different syndromes have been reported for syndromic hearing loss (SHL), which is accompanied by various symptoms besides hearing loss. Although most syndromes can be clinically diagnosed from the accompanying symptoms, many are associated with multiple genes, making accurate diagnosis difficult without genetic testing, similar to NSHL. For example, patients with Usher syndrome types 1 and 2, in which congenital hearing loss is accompanied by late-onset retinitis pigmentosa, exhibit hearing loss alone in childhood, thus appearing similar to NSHL. Patients later show vision symptoms such as night blindness, and Usher syndrome can be clinically diagnosed. Diagnosis before the appearance of vision symptoms can only be made by genetic testing; thus, in cases of SHL, genetic testing may allow early diagnosis and intervention. Identification of causal genes may enable not only accurate diagnosis, but also prediction of the accompanying symptoms, effectiveness of existing therapy, and rate of recurrence. If gene therapy based on the genetic diagnosis of deafness becomes available in the future, genetic testing will become even more clinically important.

FREQUENCY OF GENES RESPONSIBLE FOR HEREDITARY HEARING LOSS

In recent years, due to the emergence of targeted genomic enrichment and massively parallel DNA sequencing, genetic testing has enabled comprehensive analysis of a number of genes responsible for hearing loss, which has led to a dramatic improvement in the diagnostic rate and turnaround time for diagnosis. Sloan-Heggen et al and our research team previously reported that pathogenic variants in deafness genes are identified in approximately 40% of patients with hearing loss. As stated above, over 120 deafness genes have been identified, while their frequency differs and is known to vary by ethnicity. In Japan, GJB2, SLC26A4, and CDH23 are reported to be the prevalent deafness genes (Figure 1). Sloan-Heggen et al reported that GJB2 is the most frequently identified deafness gene (21.6%), followed by STRC (16.1%), SLC26A4 (6.6%), TECTA (5.2%), MYO15A (4.8%), MYO7A (4.5%), USH2A (4.3%), CDH23 (4.1%), ADGRV1 (2.7%), TMC1 (2.3%), PCDH15 (2.0%), OTOF (2.4% in autosomal recessive NHSL patients), and TMPRSS3 (2.4% in autosomal recessive NHSL patients) in 1119 deafness patients of Caucasian, Hispanic, African American, Asian, Middle Eastern, Ashkenazi Jewish, and mixed ethnicity.

When considering which gene should be targeted for gene therapy, estimating the number of patients is important while keeping in mind the frequency of the deafness gene in patients with hearing loss.

SELECTION OF TARGET GENES FOR GENE THERAPY

As mentioned above, a preclinical study of the prevalent deafness genes in humans is needed prior to clinical application. Not only the

FIGURE 1  The frequency of genetic mutations found in 1120 Japanese patients with hearing loss (Nishio et al., 2015). Relatively prevalent deafness genes, such as GJB2, CDH23, and SLC26A4, are described.
FIGURE 2  The expression sites of causal genes of hearing loss in the cochlea (Nishio et al., 2015). The cochlea contains various types of cells that have distinct functions. The genes responsible for genetic deafness expressed in each cell type are shown.

| Protein Class | Cell Type | Expression Sites |
|---------------|-----------|------------------|
| Tectorial membrane | Light junction (CNGA4) | Extracellular matrix (COL4A1, COL4A5, COL4A6, COL4A7) |
| | Reissner’s membrane | Light junction (CNGA4) | Extracellular matrix (COL4A1, COL4A5, COL4A6, COL4A7) |
| Stria vascularis | Light junction (MTHFR) | Extracellular matrix (COL4A3, COL4A4, COL4A5) |
| Spiral ligament | Light junction (GRM6, GRIA4) | Extracellular matrix (COL4A1, COL4A5, COL4A6, COL4A7) |
| Spiral prominence | Light junction (GRM6, GRIA4) | Extracellular matrix (COL4A1, COL4A5, COL4A6, COL4A7) |
| External sulcus cell | Light junction (GRM6, GRIA4) | Extracellular matrix (COL4A1, COL4A5, COL4A6, COL4A7) |
| Claudio’s cell | Light junction (GRM6, GRIA4) | Extracellular matrix (COL4A1, COL4A5, COL4A6, COL4A7) |
| Hensen’s cell | Light junction (GRM6, GRIA4) | Extracellular matrix (COL4A1, COL4A5, COL4A6, COL4A7) |

5 | LIMITATIONS OF MOUSE MODELS FOR HEREDITARY HEARING LOSS FOR PRECLINICAL STUDY

When considering the application of gene therapy to humans, preclinical studies using mouse models of hereditary hearing loss are essential.
| Gene (OMIM) | Length (bp) | Locus (OMIM) | Hearing loss phenotype in human | Expression profiles in the cochlea | Mouse model | Hearing loss phenotype in mice | Reference |
|-------------|-------------|--------------|-------------------------------|---------------------------------|-------------|-------------------------------|-----------|
| GJB2        | 2290        | DFNB1A       | Congenital mild to profound    | SCs                              | Conditional Gjb2 KO mouse       | Moderate          | 12:16     |
|             |             | [NM_004004.6]|                               |                                  | Tg Cx26 R75W                      | Profound          |           |
| STRC        | 5515        | DFNB16       | Congenital/childhood onset mild to moderate, nonprogressive | Stereocilia of the OHC | Strc −/−                              | Progressive        | 17,18     |
|             |             | [NM_153700.2]|                               |                                  |                          |                  |           |
| SLC26A4     | 4737        | DFNB4        | Congenital, high-frequency, fluctuating | Outer sulcus                      | Pds−/−                         | Profound          | 19:22     |
|             |             | [NM_000441.2]|                               |                                  | Sk2c6α4 insufficient            | Fluctuating        |           |
| TECTA       | 7353        | DFNA12       | Mild to moderate, mid- or high-frequency | Tectorial membrane (None) | (None)                           |                  | 23:25     |
|             |             | [NM_005422.4]|                               |                                  |                          |                  |           |
| MYO15A      | 11 811      | DFNB3        | Congenital profound or late onset moderate to severe, progressive | HCs                              | Shaker-2                         | Profound          | 26:28     |
|             |             | [NM_016239.4]|                               |                                  |                          |                  |           |
| MYO7A       | 7483        | DFNA11       | Late onset mild to severe, progressive | HCs, cuticular plate, synaptic region | Headbanger                           | Low frequency    | 29:34     |
|             |             | [NM_000260.4]|                               |                                  | Shaker-1                         |                     |           |
| USH2A       | 6372        | USH2A        | Congenital moderate to severe | Ankle link of the HCs             | Ush2a−/−                          | Moderate, nonprogressive | 35:37     |
|             |             | [NM_007123.6]|                               |                                  |                          |                  |           |
| CDH23       | 11 138      | DFNB12       | Moderate to profound, high-frequency progressive | Tip link of the HCs              | solsa, C57BL/6J                  | Progressive        | 38:43     |
|             |             | [NM_022124.6]|                               |                                  |                          |                  |           |
| ADGRV1      | 19 557      | USH2C        | High-frequency                  | Ankle link of the HCs             | CBA-2sh/USH            | Severe            | 6,44,45   |
|             |             | [NM_032119.4]|                               |                                  |                          |                  |           |
| TMC1        | 5340        | DFNA36       | Postlingual progressive         | HCs                              | Beethoven                         | Progressive        | 46:49     |
|             |             | [NM_138691.3]|                               |                                  | Baringo                          | Early-onset profound |           |
| PCDH15      | 6962        | DFNB23       | Prelingual profound             | Tip link of the HCs              | Ames waltzer                   | Profound          | 38,50,51 |
|             |             | [NM_033056.4]|                               |                                  |                          |                  |           |
| OTOF        | 7214        | DFNB9        | Congenital or prelingual severe to profound | Synaptic vesicles of the IHC     | Otof−/−                          | Profound          | 52:53     |
|             |             | [NM_194248.3]|                               |                                  |                          |                  |           |
| TMPRSS3     | 2399        | DFNB8/10     | Congenital severe or postlingual progressive, high frequency | HCs, SGNs                       | Tmprss3Y260X/Y260X              | Profound          | 54:56     |
|             |             | [NM_024022.4]|                               |                                  |                          |                  |           |

Abbreviations: HC, hair cell; IHC, inner hair cell; KO, knockout; OHC, outer hair cell; SC, supporting cell; SGN, spiral ganglion neuron.
(Table 1). However, mouse models of deafness that possess similar phenotypes and mutations as in humans with hearing loss are extremely rare. For example, truncating mutations (c.235delC in Asian countries or c.35delG in the non-Asian population) in GJB2 are commonly reported in humans, whereas GJB2 knockout is known to be lethal in embryonic mice. At present, only conditional GJB2 knockout has been reported in a mouse model for the most common, autosomal recessive, GJB2-related hearing loss. A mouse model for TMC1-related hearing loss, named Beethoven mice, demonstrates a similar genetic mutation, hearing loss severity, and presence/absence of progression as in humans. Beethoven mice, generated by ENU mutagenesis, have a c.1235T>A mutation, which is orthologous to the TMC1 mutation found in patients with hearing loss. As a phenotype of hearing loss, both are known to exhibit progressive hearing loss; thus, this is one of ideal mouse models for the study of human hereditary hearing loss. Unfortunately, it remains difficult to generate mice with mutations orthologous to those in each gene responsible for human hearing loss, and that exhibit similar hearing loss to that in humans. In preclinical studies, mice with a similar hearing loss phenotype to that in humans are considered useful. However, regenerating the lost hair cells and a denatured cochlear structure is considered extremely difficult; thus, using a mouse model of hereditary hearing loss exhibiting progressive hearing loss instead of congenital profound hearing loss is considered a straightforward strategy. In addition, the therapeutic effectiveness of a model with progressive hearing loss is expected to be poor unless intervention is performed before irreversible changes occur. Mouse models for CDH23-related hearing loss, sala and C57BL/6J mice, are attractive as they exhibit similar progressive hearing loss to that in humans. For congenital hearing loss, a mouse model of hearing loss caused by OTOF, associated with neurotransmission and not cochlear architecture, reportedly provides a good target to determine therapeutic effectiveness. Furthermore, attention needs to be paid to the differences in inner ear development between mice and humans. The inner ear matures by 26 weeks' gestation in humans, whereas in mice, the inner ear does not mature until 15 days after birth. Therefore, the results of studies using neonatal mice are difficult to apply directly to humans, and experiments using adult mice are needed for clinical application in humans.

## 6 | GENE THERAPY STRATEGIES FOR HEREDITARY HEARING LOSS

HAs and CIs are effective therapeutic devices for sensorineural hearing loss. However, gene therapy is expected to be a therapeutic method that can provide curative treatment. Moreover, gene therapy may potentially suppress hearing loss progression or restore hearing function, which cannot be achieved with HAs or CIs. Gene therapy primarily includes three approaches. The first approach, “gene replacement,” is the most common method by which a functional protein is supplied by delivering a normal gene. The appropriate candidates for this strategy are inherited disorders caused by loss-of-function mutations, such as recessive diseases. The second approach, “gene silencing,” is a method to treat diseases with gain-of-function mutations by suppressing the expression of the mutated gene. The third approach, “gene editing,” enables correction of pathogenic variants by genome editing, such as the CRISPR/Cas9 system.

## 7 | SELECTION OF TARGETED GENES, VECTORS, AND DELIVERY ROUTES

In the selection of any gene therapy strategy, genetic material needs to be delivered to the inner ear by use of a vector. Some papers have already summarized delivery routes and viral vectors for cochlear gene therapy. Established routes for direct local administration have included (a) round window membrane (RWM) injection, (b) RWM injection with semi-circular canal fenestration, (c) cochleostomy, and (d) canalostomy (Figure 3). Among them, the two injection routes via the RWM enable the vector to be delivered into the perilymph, which is clinically feasible. While a number of viral or non-viral vectors have been reported, adeno-associated virus vectors (AAVs) have been used in most hereditary hearing loss studies due to their non-pathogenicity or minimal immunogenicity. There are several AAV serotypes, each of which exhibits distinct tropism. AAV cell tropism, as dictated by viral capsid proteins, is an important factor affecting transduction efficiency and specificity across cell types. The gene transfer efficacy to the inner ear by AAV vectors depends on the titer and promoter of the AAV, mouse age, delivery route, and presence/absence of an enhancer, in addition to tropism. As approximately one-third of deafness genes are expressed in the hair cells, AAV serotypes that enable efficient gene transfer to the hair cells are important. We previously reported that AAV2/2 exhibited the highest total transduction rate for both the inner and outer hair cells when AAV serotypes 2/1, 2/2, 2/8, 2/9, and 2/Anc80L65 were introduced into the adult murine cochlea under the same titer. Omichi et al summarized the tropism of AAV serotypes in the adult mouse cochlea. To date, no AAV reportedly enables robust transduction in the supporting cells, in which the most common deafness gene (GJB2) is expressed, and in the outer sulcus and the spiral prominence cells, in which SLC26A4 is expressed; thus, further investigation of appropriate vectors (eg, synthetic AAV capsid) enabling efficient gene transfer to these types of cells is warranted.

## 8 | TRENDS IN PAST GENE THERAPY RESEARCH

In 2012, Akil et al first reported the effectiveness of gene therapy in a mouse model of hereditary hearing loss using Vglut3 knockout mice. These researchers performed gene transfer by administering AAV1 containing Vglut3 cDNA to the inner ear of mouse pups via the round window and demonstrated improved hearing loss. Similarly, several successful studies in neonatal mice were reported using a gene replacement approach. As described above, hereditary hearing loss is largely classified by mode of inheritance, including cases resulting from loss-of-function mutations (autosomal recessive inheritance) and from gain-of-function mutations (autosomal dominant inheritance). The therapeutic target of gene replacement is hearing loss due to
loss-of-function mutations. Lentz et al and Shibata et al reported the possibility of using gene therapy for hearing loss due to gain-of-function mutations by gene silencing via RNA interference. In addition, Gao et al reported a study using gene editing in 2018. However, all the above studies were conducted with neonatal mice without mature inner ears (i.e., mice younger than 2 weeks of age). Therefore, we performed gene therapy via gene silencing in a mouse model of human hereditary hearing loss in 2- to 8-week-old mice, demonstrating suppression of hearing loss progression, protection of the hair cells, and suppression of their degeneration. This was the first study reporting the effectiveness of gene therapy in a mouse model of genetic deafness with fully developed inner ears. The targeted allele suppression by miRNA utilized in that study is a mutation-specific therapy that requires miRNAs designed for each targeted variant. Thus, the target patients in any clinical application include only those who have a mutation orthologous to that in mice, which is an extremely limited population. It is impossible to generate a mouse model of genetic deafness to verify the therapeutic effectiveness of manipulating a number of deafness genes as well as genetic mutations. To address this limitation, the effectiveness of gene therapy may be verified in the future using disease-specific induced pluripotent stem cells, such as the outer sulcus cells in which SLC26A4 is expressed, as this technique enables induction of differentiation to various cell types in the cochlea.

9 | PRECLINICAL STUDY REQUIRED FOR THE FUTURE DEVELOPMENT OF GENE THERAPY

Phase I/II gene therapy trials for patients with severe-to-profound hearing loss were started in the United States in 2014 (https://clinicaltrials.gov/ct2/show/NCT02132130); however, at present, gene therapy for hereditary hearing loss in humans has not been approved. The advantage of gene replacement is that it enables treatment regardless of the mutation position. However, the vector may need to be administered multiple times depending on the period of transduction to the target cells, in which immune responses to AAV in the inner ear need to be verified. In addition, the size of the cDNA that can be packaged using an AAV is approximately 4.7 kb, which may require splitting the transgene into two or three parts. Akil et al summarized the deafness genes that need to be split to avoid exceeding the packaging capacity of AAV. We previously reported the feasibility of a dual AAV vector approach, but no triple AAV vector approach has been reported and this warrants further study. In contrast, the disadvantage of gene silencing and gene editing is that miRNA and gRNA need to be designed for each targeted mutation. In gene editing using the CRISPR/Cas9 system, off-target mutations have had serious adverse effects, although gene editing using a base editor that can minimize off-target effects, in which double-stranded DNA breaks are not included, has been reported in recent years and future advancements are expected. Regardless of the method selected, achievements using prevalent deafness genes as targets in patients with hearing loss are desirable.

10 | MILESTONES TOWARD THE CLINICAL APPLICATION OF GENE THERAPY IN HUMANS

As described above, there are many hurdles to overcome for the clinical application of gene therapy for hereditary hearing loss. However, in humans, it is important to consider how gene therapy will be
combined with present therapies. At present, the therapeutic options for sensorineural hearing loss are HAs and CIs, which are not biological treatments, but are highly useful. Even in cases of congenital severe-to-profound hearing loss, favorable language development is frequently reported with bilateral CI at an early age. Similarly, the effectiveness of HAs for mild-to-moderate hearing loss has been demonstrated. Thus, even if gene therapy potentially becomes a curative treatment, it is unlikely to immediately become an alternative therapy to CIs and HAs. In addition, even when the gene transfer method to the inner ear is via round window injection, canalostomy, or a combination, the method is invasive and patients with mild-to-moderate hearing loss are expected to prefer non-invasive therapy with HAs. Therefore, starting with a hybrid therapy based on CIs combined with gene therapy for severe-to-profound hearing loss is considered clinically feasible. Drug delivery through CIs has been studied, and administration of neurotrophins and glucocorticosteroids through CIs reportedly reduces insertion trauma, immune reaction, degeneration of spiral ganglion cells, and fibrosis and ossification in cochlear implantation. Gene transfer to the inner ear through CIs is reasonable, and is considered a useful step to confirm the safety of gene therapy delivery. Further, as the achievement successful gene therapy for patients with profound hearing loss may be extremely challenging, patients with genetic progressive hearing loss, for which CIs are applied, are considered good targets. For example, in most patients with severe-to-profound high-frequency hearing loss with only mild-to-moderate hearing loss at low frequencies, sufficient hearing could not be obtained with HAs. For such ski-slope hearing loss, electric-acoustic stimulation (EAS) was developed to perform acoustic stimulation that amplifies low-frequency residual hearing and deliver electrical stimulation (ES) through a CI so as to improves high-frequency hearing loss with a single device. Greater improvements in speech recognition in noise, music appreciation, and sound localization by EAS than by ES alone have demonstrated the significance of preserving residual hearing. Hearing at low frequencies is reportedly preserved after cochlear implantation, while most cases of low-frequency hearing loss are progressive; consequently, most patients experience hearing loss across all frequencies as part of the natural course. Therefore, preserving low-frequency residual hearing by gene therapy via CIs is clinically significant (Figure 4). This strategy is considered applicable to hereditary hearing loss cases associated with the CDH23, TMPRSS3, and ACTG1 genes. In trials of drug delivery through CIs, coating and incorporating drugs into the CI itself is common at present, while a pump will conceivably need to be developed and installed in CIs to deliver multiple drug administration.

**FIGURE 4** The schema of hybrid gene therapy based on a combination with cochlear implants. In patients with high-frequency hearing loss, cochlear implants (CIs) improve hearing ability, while the natural course of low-frequency hearing loss is observed (upper). Residual hearing can be preserved by performing cochlear gene therapy through CIs (lower).
11 | CONCLUSION

Herein, we discussed gene therapy research for hereditary hearing loss, focusing on the frequency of deafness genes, hearing loss phenotypes in patients, and mouse models of genetic deafness. If gene therapy can be provided as a precise treatment for hereditary hearing loss in the future, restored hearing function and prevention of hearing loss progression, which cannot be achieved by conventional medicine, can be realized. The number of treatable deafness genes is expected to be expanded by further study, in which research based on data from patients with hearing loss is desirable. In order to actualize gene transfer to the human inner ear, gene therapy in combination with CIs is considered a reasonable strategy for clinical application in humans.

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CONFLICT OF INTEREST

Authors declare no potential conflicts of interest.

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