Genetic background in late-onset sensorineural hearing loss patients

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Genetic testing for congenital or early-onset hearing loss patients has become a common diagnostic option in many countries. On the other hand, there are few late-onset hearing loss patients receiving genetic testing, as late-onset hearing loss is believed to be a complex disorder and the diagnostic rate for genetic testing in late-onset patients is lower than that for the congenital cases. To date, the etiology of late-onset hearing loss is largely unknown. In the present study, we recruited 48 unrelated Japanese patients with late-onset bilateral sensorineural hearing loss, and performed genetic analysis of 63 known deafness gene using massively parallel DNA sequencing. As a result, we identified 25 possibly causative variants in 29 patients (60.4%). The present results clearly indicated that various genes are involved in late-onset hearing loss and a significant portion of cases of late-onset hearing loss is due to genetic causes. In addition, we identified two interesting cases for whom we could expand the phenotypic description. One case with a novel MYO7A variant showed a milder phenotype with progressive hearing loss and late-onset retinitis pigmentosa. The other case presented with Stickler syndrome with a mild phenotype caused by a homozygous frameshift COL9A3 variant. In conclusion, comprehensive genetic testing for late-onset hearing loss patients is necessary to obtain accurate diagnosis and to provide more appropriate treatment for these patients.

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INTRODUCTION
Sensorineural hearing loss (SNHL) is one of the most common sensory disorders in humans [1, 2]. The incidence of the congenital SNHL is estimated to be 1–2 in 1000 newborns, among which at least 60% are presumed to be associated with genetic causes [3]. Sloan-Heggen et al. undertook the genetic analysis of congenital deafness patients by targeted genomic enrichment with massively parallel DNA sequencing (TGE + MPS) and identified the causative gene mutations in 44% of cases [3]. In Japan, Mori et al. reported that the diagnostic rate was 41% in congenital or early-onset (<6 year) hearing loss (HL) patients based on screening for 154 mutations in 19 deafness genes using MPS combined with Invader assay and TaqMan genotyping [4]. On the other hand, these studies also reported that the diagnostic rates in late-onset hearing loss patients were lower than those in early-onset patients (28% and 16%, respectively) [3, 4]. Late-onset SNHL is believed to be a complex disorder, associated with age-related hearing loss, idiopathic sudden SNHL, acoustic neuroma, chronic otitis media or environmental risk factors (including noise exposure and ototoxic drug exposure). However, a certain number of late-onset bilateral symmetrical HL cases are thought to involve genetic factors, particularly in those with progressive HL presenting as worse than the average hearing for age. At present, a majority of late-onset SNHL cases do not receive genetic testing; thus, the etiology of late-onset SNHL remains largely unknown.

In this study we focused on late-onset bilateral SNHL patients and aimed to show the frequency of hereditary HL as well as describe the clinical features of these cases.

MATERIALS AND METHODS
This study was conducted with the approval of the Ethics Committee of Kobe University Graduate School of Medicine (Approval number:170081). Written informed consent was obtained from all subjects. All procedures were performed in accordance with the Guidelines for Genetic Tests and Diagnoses in Medical Practice of the Japanese Association of Medical Sciences and the tenets of the Declaration of Helsinki.

Subjects
Sixty-four unrelated patients with bilateral SNHL were enrolled in this study. We defined late-onset HL as the HL with an age at onset of 6 years of age and over and, based on this definition, we excluded cases with congenital or pre-lingual onset SNHL (with an age at onset of under 6 years of age). In addition, we also excluded patients aged over 60 years at SNHL onset to remove cases of presbycusis. Finally, 48 unrelated Japanese patients with late-onset bilateral SNHL who underwent clinical genetic testing between April 2012 and April 2020 at Kobe University Graduate School of Medicine participated in this study (Table 1).

Clinical evaluations
Hearing thresholds were evaluated using pure-tone audiometry (PTA) and classified by pure-tone average over 500, 1000, 2000, and 4000 Hz. The...
severity of HL was classified into mild (21–40 dB HL), moderate (41–70 dB HL), severe (71–95 dB HL), and profound (>95 dB HL). The audiometric configurations were categorized into low-frequency, mid-frequency (U-shaped), high-frequency (gently sloping type and steeply sloping type), flat type, and deaf, as reported previously [5]. The data for age at onset of HL, the progressiveness of HL and family history were obtained from medical charts.

Amplicon resequencing and variant annotation

Amplicon libraries were prepared using an Ion AmpliSeq™ Custom Panel for 68 genes reported to cause non-syndromic hereditary HL (Thermo-Fisher Scientific, MA, USA), in accordance with the manufacturer’s instructions. The detailed protocol has been described elsewhere [6]. MPS was performed with an Ion Proton system using an Ion HiQ Chef Kit and an Ion P1 Chip (ThermoFisher Scientific). The sequence data were mapped against the human genome sequence (build GRCh37/hg19) with a Torrent Mapping Alignment Program. After sequence mapping, the DNA data were treated for (SIFT), Polymorphism Phenotyping (PolyPhen2), LRT, Mutation Taster, Mutation Assessor, REVEL, and CADD, were used through the ANNOVAR software program [7, 8]. Direct sequencing was utilized to confirm the selected variants. Copy number variation (CNV) analysis was performed by using the read depth data with our published copy number variation detection method for Ion AmpliSeq enrichment and Ion PGMTm/Proton/SS sequencing as described previously [11]. All genetic analyses were performed in Shinhoto University School of Medicine as a collaborative study.

RESULTS

Patient characteristics and identified variants

The age at onset of participants ranged from 6 to 60 years. Among them, 9 cases experienced onset in their 1st decade (6–10 years old) and the other 42 cases experienced onset in their 2nd decade or later (11–60 years old), accounting for 81.2% of all participants (Table 1).

As for the audiometric configuration, flat-type HL was the most common, being observed in 15 cases (31.3%), followed by 13 cases with gently sloping-type HL (27.1%), 10 cases with steeply sloping-type HL (20.8%), three cases with profound-type HL (6.3%), three cases with U-shaped-type HL (6.3%), and four cases with different types of HL in the left and right ears (Table 1).

We identified 25 possibly disease-causing variants from 29 probands, affording a diagnostic rate for this study of 60.4%. The most prevalent causative gene for late-onset HL in this study was a mitochondrial m.3243A>G mutation, which was observed in 6 cases, followed by four cases with COCH gene variants, three cases each with CDH23, KCNQ4 and MYO6 variants, two cases with EYA4 variants, and one case each with ACTG1, COL9A3, GJB2, MYO7A, POU4F3, STRC, USH2A, and mitochondria m.1555A>G variants (Table 2). Among the 25 identified variants, 18 had been reported previously as causative variants, and 7 were novel variants (Table 2). The novel variants consisted of two missense variants, one nonsense variant, three frameshift variants and one splicing variant. Based on the ACMG guidelines, the novel variants were categorized as pathogenic (1), likely pathogenic (7) and variants of uncertain significance variant (1) (Table 3).

Clinical features of patients with novel variants

Table 3 and Fig. 1 summarize the clinical features of the 7 individuals with novel variants.

Case 1 is a 43-year-old male. At around the age of 20, he experienced repeated episodes of dizziness and his HL gradually progressed. His mother had also experienced repeated episodes of dizziness with HL when she was in her twenties. Genetic analysis of this patient identified a heterozygous COCH gene missense variant (COCH: NM_004086:c.236A>G:p.H79R). His mother was found to carry the same variant.

Case 2 is a 61-year-old female who became aware of a loss of vision and HL in her thirties. She was diagnosed with retinitis pigmentosa through ophthalmological testing. Her HL gradually progressed. His mother also had the same type of HL. Genetic analysis of this patient identified compound heterozygous USH2A gene splicing variants. USH2A: NM_206933:c.8559–2A>G was previously reported as a pathogenic variant of Usher syndrome type 2, and the other is novel splicing variant (NM_206933:exon36:c.6806–2A>C).

Case 3 is a 37-year-old female. She visited an otorhinolaryngologist with a chief complaint of tinnitus and was diagnosed with HL when she was aged 35. Her audiometric configuration was of the gently sloping type at first, but HL progressed gradually even in the lower frequencies. She complained of dizziness about once every two years. Her father also had the same type of HL. Genetic analysis of this patient identified a heterozygous KCNQ4 frameshift variant (KCNQ4: NM_000740:c.1656dupA:p.L535Tfs*11).

**Table 1. Patients characteristics**

| Characteristic          | Number | %     |
|-------------------------|--------|-------|
| Number of subjects      | 48     | 100.0 |
| Sex                     |        |       |
| Female                  | 25     | 52.1  |
| Male                    | 23     | 47.9  |
| Family history of HL (+)| 22     | 45.8  |
| (−)                     | 26     | 54.2  |
| Age at onset            |        |       |
| 6–10 y.o.               | 9      | 18.8  |
| 11–20 y.o.              | 4      | 8.3   |
| >20 y.o.                | 35     | 72.9  |
| Severity                |        |       |
| mild/mild               | 5      | 10.4  |
| moderate/moderate       | 19     | 39.6  |
| severe/severe           | 4      | 8.3   |
| profound/profound       | 4      | 8.3   |
| normal/mild             | 1      | 2.1   |
| mild/moderate           | 3      | 6.3   |
| moderate/severe         | 7      | 14.6  |
| moderate/profound       | 3      | 6.3   |
| profound/severe         | 2      | 4.2   |
| Audiometric configuration|       |       |
| Flat                    | 15     | 31.3  |
| Gently sloping          | 13     | 27.1  |
| Steeply sloping         | 10     | 20.8  |
| Deaf                    | 3      | 6.3   |
| U-shaped                | 3      | 6.3   |
| Different types on each side | 4  | 8.3   |

y.o., years old; HL, hearing loss.
Table 2. Summary of the causative variants identified in this study

| Gene   | Nucleotide change | Amino acid change | Onset age | Age | Inheritance | Gender | Severity of HL (L/R) | Audiometric configuration | Reference          |
|--------|-------------------|-------------------|-----------|-----|-------------|--------|---------------------|---------------------------|---------------------|
| MT-TL1 | m.3243A>G         |                   | 30        | 79  | Sporadic    | M      | profound/severe     | Flat/Profound             | van den Ouweland (1992) [36] |
| MT-TL1 | m.3243A>G         |                   | 55        | 55  | Maternal    | M      | profound/moderate   | Profound/Flat             |                     |
| MT-TL1 | m.3243A>G         |                   | 55        | 45  | Maternal    | F      | moderate/moderate   | Flat                      |                     |
| MT-TL1 | m.3243A>G         |                   | 60        | 40  | Maternal    | M      | severe/moderate     | Flat                      |                     |
| MT-TL1 | m.3243A>G         |                   | 40        | 43  | Maternal    | M      | moderate/moderate   | Flat                      |                     |
| MT-TL1 | m.3243A>G         |                   | 40        | 34  | Maternal    | M      | severe/severe       | Flat                      |                     |
| COCH   | c.1115T>C         | p. I372T          | 53        | 40  | AD          | F      | severe/moderate     | Steeply sloping           | Tsukada (2015) [27]   |
| COCH   | c.1115T>C         | p. I372T          | 53        | 50  | AD          | M      | severe/moderate     | Gently sloping            | Tsukada (2015) [27]   |
| COCH   | c.1115T>C         | p. I372T          | 36        | 10  | AD          | F      | mild/mild           | Steeply sloping           | Tsukada (2015) [27]   |
| COCH   | c.2366A>G         | p.H79R            | 43        | 20  | AD          | M      | moderate/moderate   | Gently sloping            | This study            |
| CDH23  | c.719C>T/c.4762C>T| p.P240L/R1588W    | 59        | 40  | AR          | F      | moderate/moderate   | Steeply sloping           | Wagatsuma (2007) [37]  |
| CDH23  | c.719C>T/c.4762C>T| p.P240L/R1588W    | 52        | 50  | AR          | F      | severe/moderate     | Steeply sloping           | Wagatsuma (2007) [37]  |
| MYO6   | c.3496C>T         | p.R1166X          | 36        | 12  | AD          | M      | severe/severe       | U-shaped                  | Ahmed (2003) [38]     |
| MYO6   | c.1015C>T         | p. R339W          | 57        | 40  | AD          | M      | moderate/moderate   | Gently sloping            | Yang (2013) [39]      |
| MYO6   | c.2393G>A         | p. W798X          | 33        | 20  | AD          | F      | mild/moderate       | U-shaped                  | This study            |
| KCNQ4  | c.211delC         |                   | 30        | 10  | AD          | F      | moderate/moderate   | Steeply sloping           | Kamada (2006) [40]     |
| KCNQ4  | c.961G>A          |                   | 37        | 35  | AD          | F      | moderate/moderate   | Flat                      | This study            |
| KCNQ4  | c.961G>A          |                   | 37        | 35  | AD          | F      | moderate/moderate   | Flat                      | This study            |
| EYA4   | c.7777delG        |                   | 52        | 40  | AD          | F      | severe/severe       | Flat                      | Coucke (1999) [41]     |
| EYA4   | c.1886.1899del     |                   | 54        | 40  | AD          | M      | moderate/moderate   | Gently sloping            | Shinagawa (2020) [23]  |
| EB2    | c.176191del/c.235delC|                   | 51        | 10  | AR          | F      | moderate/moderate   | Gently sloping            | Abe (2000) [42] /Fuse (1999) [43] |
| MT-RNR  | m.1555A>G        |                   | 58        | 6   | Sporadic    | M      | profound/profound   | Profound/Flat             | Prezant (1993) [44]    |
| MYO7A  | c.1667GT/c.1369G>A| p.G556V/p.A457T   | 47        | 8   | AR          | M      | profound/profound   | Profound/Flat             | Bakon (2016) [13] /This study |
| COL9A3 | c.1587dupT       |                   | 58        | 20  | AR          | F      | severe/moderate     | Flat                      | This study            |
| ACTG1  | c.833C>T         | p.T278I           | 59        | 30  | AD          | F      | profound/profound   | Profound                  | van Wijk E (2003) [22] |
| POU4F3  | c.976A>T         | p.R326X           | 40        | 10  | AD          | M      | moderate/moderate   | Gently sloping            | Kiloto (2017) [45]     |
| STRC   | 2 copy loss      |                   | 21        | 20  | AR          | F      | mild/mild           | Gently sloping            | Yokota (2019) [46]     |
| USH2A  | c.8559-2A>G/c.6806-2A>C|                   | 61        | 30  | AR          | F      | moderate/profound   | Gently sloping            | Dai H (2008) [47] /This study |

HL, hearing loss.
In silico prediction scores and pathogenicity classification of novel variants identified in this study

| Case No. | Gene | Inheritance | Nucleotide change | Amino acid change | SIFT | PP2 | LRT | Mutation Taster | Mutation Assessor | ACMG |
|----------|------|-------------|------------------|------------------|------|-----|-----|------------------|------------------|-------|
| Case 1   | COCH | AD          | c.236A>G         | p.H79R           | D    | D   | D   | D               | Previously reported | Uncertain significance (PM2, PP3) |
| Case 2   | USH2A | AR          | c.8559-2A>G      | –                | –    | –   | –   | –               | –                | Previously reported |
| Case 3   | KCNQ4 | AD          | c.1656dupA       | p.L553Tfs*11     | –    | –   | –   | –               | –                | Likely pathogenic (PVS1, PM2) |
| Case 4   | EYA4  | AD          | c.1777delG       | p.G593Afs*4      | –    | –   | –   | –               | –                | Likely pathogenic (PVS1, PM2) |
| Case 5   | MYO6  | AD          | c.2393G>A        | p.W798X          | –    | –   | –   | –               | –                | Likely pathogenic (PVS1, PM2) |
| Case 6   | MYO7A | AR          | c.1369G>A        | p.A457G          | H    | P   | D   | D               | Likely pathogenic (PM2, PM3, PM5, PP3) |
| Case 7   | COL9A3 | AR        | c.1587dupT (homo) | –                | –    | –   | –   | –               | Previously reported |

**DISCUSSION**

In this study, we analyzed 48 late-onset SNHL patients, and identified 25 possibly disease-causing variants in 29 cases (60.4%). Among the 25 identified variants, 7 were novel. Genetic testing for HL, particularly in congenital or early-onset cases, is now widely available due to its clinical benefits in providing accurate diagnosis, prediction of HL severity, estimation of associated symptoms, selection of appropriate habilitation options, prevention of HL, and better genetic counseling [13]. In this study we confirmed the usefulness of genetic testing for late-onset HL cases, even though it is not commonly performed at present.

The diagnostic rate (60.4%) in this study was higher than those of previous studies (28% and 16%) [3, 4]. As our institution is a university hospital (tertiary referral hospital), and many cases in this study have multiple affected family members, it might explain the increased diagnostic ratio of this cohort. Indeed, 45.8% (22/48) of our cohort have affected family members. In a previous study, the diagnostic rate in sporadic cases was 19%, which was lower than that of autosomal recessive or autosomal dominant cases (35% and 35%, respectively) [14]. In addition, our cohort also included the patients with a maternal family history of Stickler syndrome, which may also have increased the diagnostic rate. Mitochondrial mutations (m.1555A>G or m.3243A>G) were identified in 15% (7/48) of patients in this study. As another cause of the higher diagnostic rate, our cohort included many cases with progressive HL. In a previous study, the diagnostic rate for progressive HL cases was higher than that for stable HL cases [14]. These factors may have led to the higher diagnostic rate in this study. From a diagnostic perspective, these
clinical characteristics will be useful for the selection of candidates for genetic testing to increase the diagnostic yield and efficiency.

A previous paper showed that the responsible genes differ between congenital or early-onset HL and late-onset HL [4]. In this study, we also identified many causative genes which was reported as genetic causes of late-onset and/or progressive HL, such as the KCNQ4, COCH, CDH23, EYA4, MYO7A, MYO6, ACTG1 and mitochondrial genes. As HL due to these genes is more or less

![Pedigrees and audiograms of the seven families who carried a possible pathogenic variant identified in this study. Filled symbols indicate affected individuals. Arrows indicate probands in each family. Case 6 also showed progression of hearing loss from 7 years of age](image-url)
The MYO6 gene is known to be responsible for both ADNSHL (DFNA22) and ARNLSH (DFNB37) [28]. In a previous study, patients with MYO6 variants showed late-onset mild-to-moderate progressive HL, and marked hearing deterioration occurred after the age of 40 [29]. In our study, the phenotypes of MYO6-associated HL varied as shown in Table 2.

The KCNQ4 gene is known to be one of the most frequent causative genes for ADNSHL (DFNA2) [30]. It has been shown that DFNA2 results in high-frequency-involved HL. A progressive nature is a common feature among patients with KCNQ4 mutations, regardless of the variant [31]. In this study, we identified 3 families with KCNQ4 variants, with one of them being a novel frameshift variant (c.1656dupA:p.L553fs*11). The audiometric configuration of this patient showed sensorineural hearing impairment involving all frequencies.

As a notable result, we also identified one case of phenotypically mild Stickler syndrome with a homozygous COL9A3 frameshift variant. The COL9A3 gene, first reported as a genetic cause of multiple epiphyseal dysplasia, an autosomal dominant osteochondro-dysplasia, [32] was also shown to be responsible for Stickler syndrome in two previous reports [33, 34]. Faletra et al., reported an autosomal recessive Stickler syndrome family with three affected siblings, all of whom carried a homozygous frameshift variant in the COL9A3 gene. All three individuals showed high-frequency HL and moderate-to-high myopia and amblyopia. Among the three affected siblings, the two elder brothers showed flat midface hypoplasia and a depressed nasal bridge, but the youngest sister in this family did not show these malformations. Hanson-Kahn et al., reported a patient with Stickler syndrome, who carried a homozygous COL9A3 frameshift variant and showed moderate-to-severe sensorineural HL, severe myopia, and both bilaal and femoral bowing at birth [34]. As COL9A3 variants have been reported to cause non-syndromic HL [35], COL9A3 variants may have a broad phenotypic range from mild non-syndromic HL to more severe syndromic phenotypes. Due to inter- and intra-familial phenotypic variability [34, 35], it is extremely difficult to perform diagnosis based on clinical phenotypes. The present case, the third reported to date, presenting only with HL, cataract and retinal detachment, can be classified as a very mild type of Stickler syndrome that could not be diagnosed without genetic diagnosis. At her first visit, she was diagnosed with non-syndromic HL as she had no noticeable symptoms. Later, however, as the COL9A3 mutation was identified by genetic analysis, a detailed anamnestic re-evaluation of the associated syndromes of Stickler syndrome revealed a history of cataract and retinal detachment.

In conclusion, we performed genetic analysis of 48 Japanese patients with late-onset bilateral SNHL and identified the potential genetic causes of HL in 29 cases (60.4%). The present results clearly indicated that a significant portion of cases of late-onset bilateral SNHL may involve genetic causes, and the various causative genes can possibly be identified. From an etiological perspective, we could confirm the benefits of CI in cases in which the etiology is located within the cochlea, as shown in the MYO7A- and the ACTG1-associated cases. In addition, we could expand phenotypic descriptions as seen in (1) the MYO7A-associated case with a milder phenotype of Usher syndrome and (2) the phenotypically mild Stickler syndrome caused by a homozygous frameshift variant in the COL9A3 gene. These two cases could not be diagnosed without genetic testing. Therefore, comprehensive genetic testing can be seen as a useful diagnostic tool for late-onset HL, and the accumulation of genetic findings will enable more accurate diagnosis and provide more appropriate treatment.

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COMPETING INTERESTS
The authors declare no competing interests.

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