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（難治性疾患克服研究事業）

GJB2 変異による劣性遺伝難聴の全国的実態把握

平成２３年度総括研究報告書

平成２４年３月

研究代表者　伊藤　壽一

（京都大学大学院医学研究科）

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研究代表者　伊藤　壽一（京都大学大学院医学研究科・教授）

研究要旨

聴力の低下は生活の質の著しい低下を招くとともに、小児期の難聴は言語発達や教育にも大きな支障をきたす。内耳は生検できず、遺伝子診断が感音難聴のほぼ唯一の確定診断法である。

先天性難聴の遺伝子診断は一部の施設で行われてきたが、その共同実施が平成22年4月に先進医療として認可された。これにより、難聴の遺伝子診断が全国で行われるようになり、現在は29施設に増加している。

聴覚に関与する遺伝子は約100種存在すると考えられているが、*GJB2*遺伝子変異による劣性遺伝難聴の頻度が高いとされている。現時点の先進医療では*GJB2*遺伝子に関して、日本人難聴患者に見いだされた13変異をインベーダー法で解析している。しかし世界的には100種以上の*GJB2*遺伝子変異による難聴が報告されており、日本においても全国規模の解析を実施することにより、日本の先天性難聴に特徴的な他の変異が相当数存在する可能性がある。

本研究では遺伝子診断目的で全国から収集された難聴患者サンプルのうち同意が得られたものを用いて、*GJB2*遺伝子の全コーディング領域を直接シークエンス法で解析する。これにより難聴患者でどの種類の*GJB2*遺伝子変異がどの程度の頻度で検出されるかが明らかになる。つまり*GJB2*遺伝子の各変異による患者数等の基本的データが収集される。

一般医療では対象変異を絞ったインベーダー法を用いるのが効率よく妥当であるが、本研究により日本人の難聴の診断における変異の種類とその頻度が全国レベルで明らかとなり、より適切な対象変異の抽出により、遺伝子検査の有効性を格段に高めることができる。これはすなわち、難聴における遺伝子検査の診断基準となる。

また、*GJB2*遺伝子変異による難聴は劣性遺伝であり、健常者での保因者頻度は白人等では約3%と報告されているが、日本人での報告はない。保因者頻度は、次世代の再発率を予測する上で不可欠な情報で、遺伝カウンセリング上重要である。日本各地の地域住民の健診の際に同意を得て、健常者のゲノムサンプルを得て*GJB2*遺伝子配列を検討することで、各*GJB2*遺伝子変異の保因者頻度を確定する。

研究分担者

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A．研究目的

難聴は生活の質を著しく低下させ、また言語発達や教育にも大きな支障をきたす。内耳は生検できず、遺伝子診断が感音難聴のほぼ唯一の確定診断法である。聴覚に関与する遺伝子は多数存在するが、*GJB2*遺伝子変異による劣性遺伝難聴の頻度が高いとされている。本研究は日本人の*GJB2*遺伝子変異の種類と保因者頻度を全国レベルで確定する事を目的とする。遺伝子変異の種類を確定することで、その変異に標的を絞った効率よい遺伝子診断が可能となる。また保因者頻度は次世代の再発率を予測する上で不可欠な情報で、疫学のみならず遺伝カウンセリング上も重要である。つまり本研究により、*GJB2*遺伝子の各変異による患者数等の基本的データが収集され、難聴における遺伝子検査の診断基準が確立される。

B．研究方法

平成22年4月、複数の施設で共同実施する先天難聴の遺伝子診断が先進医療に認可された。これに伴い、全国規模で遺伝子診断が行われるようになった。この遺伝子検査のインフォームドコンセントを得る際に、全国の施設で同時に本研究の説明を行い、同意を得られたサンプルの*GJB2*遺伝子の全コーディング領域を直接シークエンス法で解析する。これにより難聴患者でどの種類の*GJB2*遺伝子変異がどの程度の頻度で検出されるかが明らかになる。

本研究の対象となる難聴患者は、厚労省より先進医療としてすでに認可されている「原因不明の感音難聴」者である。つまり音響外傷や薬物障害などの原因が明らかでなければ、進行性難聴や中途難聴者を含む。既知の*GJB2*遺伝子変異による感音難聴は軽度難聴から高度難聴まで多岐にわたり、進行性難聴例も報告されているため、患者が希望されれば軽度難聴者や中途難聴者も対象となる。本検査は先進医療としての遺伝子診断と同時に行うため、検査結果は適切なカウンセリングとともに本人へ開示する。

また、聴力が正常な各地地域住民の健診において同意を得て*GJB2*遺伝子配列を解析し、日本人での各*GJB2*遺伝子変異の保因者頻度を確定する。

（倫理面への配慮）

本研究はヘルシンキ宣言（2000年　英国エジンバラ改訂版）に基づく倫理的原則、本試験実施計画書および「ヒトゲノム・遺伝子解析研究に関する倫理指針平成16年文部科学省・厚生労働省・経済産業省告示第1号」を遵守して実施する。また、処方情報解析研究は、文科省及び厚労省が策定した「疫学研究に関する倫理指針（平成19年文部科学省・厚生労働省告示第１号）」を遵守して実施する。また本研究は予め本研究の実施計画書の内容、研究参加者等の的確性等について倫理審査委員会により審査を受け、承認を得てから実施する。研究の実施時には対象者に、同意説明文書を用いてインフォームドコンセントを行い、文書にて同意が得られた患者に研究に参加して頂く。取得したサンプルは個人識別情報の匿名化を行い、解析結果および患者医療情報はスタンドアローンのコンピューターに保存する。難聴患者には適切な遺伝子カウンセリングを行った上で、結果を開示する。本研究で対象とする*GJB2*遺伝子を含む遺伝子検査は、すでに厚労省より先進医療として認可されており、施行する上で倫理的な問題は存在しない。

C．研究結果

全国33施設から集められた日本人の両側感音難聴患者264例について、13遺伝子47変異についてインベーダー法を用い検索した。またインベーダー法でヘテロ変異の見出された症例に関しては直接シークエンス法を行い新規変異の検索を行った。

その結果、日本人難聴患者264例中46例（17.4%）に*GJB2*遺伝子変異が見出された。また、264例のうち6歳未満に難聴と診断された群141例では38例（27.0%）に*GJB2*遺伝子変異が認められた。一方、6歳以降に難聴と診断された群100例では7例(7%)に*GJB2*遺伝子変異が認められた(Usami *et al.*, *PLoS one* 2012)。

また、インベーダー法により検出された*GJB2*遺伝子変異の内訳としては、c.235delC, p.V37I、p.[G45E;Y136X]、c.176-191del、p.R143W、c.299-300del、p.T86Rが検出された。このうちc.235delC変異が最も高頻度で認められ、検出された*GJB2*変異アレルのうち43/77と55.8%を占めていた。また、比較的高頻度で認められる変異として、p.[G45E;Y136X]変異が10/77(13.0%)、p.V37I変異が7/77(9.1%)、c.299-300del(6.5%)を占めることが明らかとなった。（表1、Usami *et al.*, *PLoS one* 2012)

Gene	*Exon*	*Codon location*	*Nucleotide change*	*Frequency of mutant alleles (n=528)*	*Number of patients with mutations (n=264)*
GJB2	exon 2	p.L79fs	c.235delC	43 (8.1%)	29 (10.9%)
GJB2	exon 2	p.V37I	c.109G>A	7 (1.3%)	6 (2.3%)
GJB2	exon 2	p.[G45E; Y136X]	c.[134G>A; 408C>A]	10 (1.9%)	10 (3.8%)
GJB2	exon 2	p.G59fs	c.176_191del	3 (0.6%)	3 (1.1%)
GJB2	exon 2	p.R143W	c.427C>T	4 (0.8%)	4 (1.5%)
GJB2	exon 2	p.H100fs	c.299_300del	5 (0.9%)	5 (1.9%)
GJB2	exon 2	p.T123N	c.368C>A	4 (0.8%)	4 (1.5%)
GJB2	exon 2	p.T86R	c.257C>G	1 (0.2%)	1 (0.4%)
GJB2	exon 2	p.F191L	c.570T>C	0	0
GJB2	exon 2	p.I71T	c.212T>C	0	0
GJB2	exon 2	p.A49V	c.146C>T	0	0
GJB2	exon 2	p.G12fs	c.35delG	0	0

また、*GJB2*遺伝子の直接シークエンスにより、p.T8M、c.35insG、p.F106Y、c.511insAACG、p.C174Sの5種類のインベーダー検査に含まれていない新規遺伝子変異を見出した（Usami *et al.*, *PLoS one* 2012)。

Gene	*Exon*	*Codon location*	*Nucleotide change*	*Frequency of mutant alleles (n=528)*	*Number of patients with mutations (n=264)*
GJB2	exon 2	p.T8M	c.23C>G	1 (0.2%)	1 (0.4%)
GJB2	exon 2	p.K12fs	c.35insG	1 (0.2%)	1 (0.4%)
GJB2	exon 2	p.F106Y	c.317T>A	1 (0.2%)	1 (0.4%)
GJB2	exon 2	p.A171fs	c.511insAACG	2 (0.4%)	2 (0.8%)
GJB2	exon 2	p.C174S	c.522G>C	1 (0.2%)	1 (0.4%)

*GJB2*遺伝子変異の保因者頻度に関しては、各地地域住民の健診時に同意を得て、約800例からゲノムDNAを抽出した。現時点ではそのうち151例の*GJB2*塩基配列解析が終了している。その結果を下に記載する。

Base Change	Amino Acid Change	Variants	Hetero	Homo
4G>A	D2N	unclassified	1	
23C>T	T8M	pathogenic	1	
79G>A	V27I	polymorphism	56	30
109G>A	V37I	pathogenic	3	
120A>C	silent	polymorphism	42	
235delC	L79FS	pathogenic	1	
329A>G	E110G	unclassified	1	
341A>G	E114G	polymorphism	28	3
368C>A	T123N	s/o polymorphism	1	
583A>G	M195V	pathogenic	1	
608T>C	I203T	polymorphism	18	

D．考察

全国33施設から集められた日本人の両側感音難聴患者264例についてインベーダー法および直接シークエンス法を用いた遺伝子解析を実施した結果、従来の報告と同様に約18%より変異が見出された。また、発症年齢に関しては、6歳未満に難聴と診断された群141例では38例(27.0%)に*GJB2*遺伝子変異が認められたが、6歳以降に難聴と診断された群100例では7例(7%)であった。これは、従来の報告と同様に、*GJB2*遺伝子変異による難聴は高度～重度難聴となるケースが多いことより、難聴の診断が早期になされるためであることが示唆される。また、見出される変

異の内訳に関しては、従来の報告と同様、c.235delC変異が最も高頻度で認められており、日本人難聴患者におけるコモンミューテーションと成っていることが改めて示された。

また、p.V37I変異に関しては従来の報告と同様、軽度～中等度の難聴と成るケースが多いことが示された。また、変異の頻度に関しては7/77(9.1%)で認められた。過去の報告でも、コントロール集団における保因者頻度に関しては、p.235delCよりもp.V37I変異の方が頻度が高いが、ケース群では、pV37I変異が低くなることが報告されているが、今回の結果も同様であった。これは、p.V37I変異による難聴が軽度～中等度と比較的軽度の難聴と成る事より、先進医療を実施する医療機関（主として大学病院）を受診するケースが少ない事に起因しているものと考えられる。

また、先天性の難聴と考えられる141例の中では約30%の患者より変異が見出されたことおよび*GJB2*遺伝子変異症例の多くが高度～重度の難聴となることから、難聴の早期診断のためのツールとして遺伝子診断が有用であることが改めて示された。また、遺伝子診断を実施することにより、乳児期より重症度の予測や予後の予測が可能と成る事により、治療法や療育法の選択において非常に有用な情報が得られることが再度確かめられた。

また、従来の報告と同様、欧米での変異のスペクトラムと日本人難聴患者における変異のスペクトラムは大きく異なっており、欧米におけるコモンミューテーションであるc.35delG変異は1例も認められず、民族特異性が高いため、日本人難聴患者の遺伝子変異情報に基づいた解析を行う事の重要性が改めて示された。

健診時に得られた地域住民の*GJB2*遺伝子解析からは、上記のように多数の変異が検出されたが、それらのうち病原性のない多型とされているc.79G＞A、c.120A＞C、c.341A＞G、c.368C＞A、c.608T＞Cを除くと、*GJB2*遺伝子変異の保因者は6例であった。この中でc.109G＞Aとc.235*del*Cは難聴の病因であると報告されており、少なくとも4例は確実に*GJB2*変異による劣性遺伝難聴の保因者である。c.4G＞A（p.D2N）とc.329A＞G（p.E110G）は、過去に報告がない新規の変異であり、難聴の原因となるかどうかは結論できない。よって、現時点では保因者頻度は4-6/151(2.6%～4.0%）と推定される。

ただし151例の解析では推定保因者頻度の信頼区間が広く、より多くの解析が必要である。これまでに収集した約800例の解析を現在進めている。

E．結論

全国33施設から集められた日本人の両側感音難聴患者264例について、インベーダー法および直接シークエンス法を行い*GJB2*遺伝子変異の検索を行った。その結果、日本人難聴患者264例中46例（17.4%）に*GJB2*遺伝子変異が見出され、過去の報告と同様に、日本人難聴患者における主要な原因となっていることが確認された。また、予後の予測や随伴症状の予測に非常に有用であることが改めて確認された。

日本人でのGJB2保因者頻度に関しては、今回検討できた範囲では約3%前後と推定されたが、さらに解析例を増やす必要がある。

F．研究発表

1．　論文発表

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G．知的所有権の取得状況

1. 特許取得　なし
2. 実用新案登録　なし
3. その他　なし

研究成果の刊行に関する一覧表

＜論文＞

著者氏名	タイトル名	発表雑誌名	出版年・巻号・頁
Moteki H, Naito Y, Fujiwara K, Kitoh R, Nishio S Y, Oguchi K, Takumi Y, Usami SI.	Different cortical metabolic activation by visual stimuli possibly due to different time courses of hearing loss in patients with GJB2 and SLC26A4 mutations.	Acta Otolaryngol	131:1232-1236. 2011
Usami S, Nishio S, Nagano M, Abe S, Yamaguchi T, the Deafness Gene Study Consortium.	Simultaneous Screening of Multiple Mutations by Invader Assay Improves Molecular Diagnosis of Hereditary Hearing Loss: A Multicenter Study.	PLoS one	7(2):1-8. 2012
宇佐美真一	難聴の遺伝子診断	日本臨牀	69(2): 357-367. 2011
宇佐美真一	難聴の遺伝子診断	Audiology Japan	54: 44-55. 2011

研究成果の刊行物

Acta Oto-Laryngologica, 2011; 131: 1232–1236

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healthcare

SHORT COMMUNICATION

Different cortical metabolic activation by visual stimuli possibly due to different time courses of hearing loss in patients with *GJB2* and *SLC26A4* mutations

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Abstract

Conclusion. We have demonstrated differences in cortical activation with language-related visual stimuli in patients who were profoundly deafened due to genetic mutations in *GJB2* and *SLC26A4*. The differences in cortical processing patterns between these two cases may have been influenced by the differing clinical courses and pathogenesis of hearing loss due to genetic mutations. Our results suggest the importance of hearing during early childhood for the development of a normal cortical language network. *Objectives.* To investigate the cortical activation with language-related visual stimuli in patients who were profoundly deafened due to genetic mutations in *GJB2* and *SLC26A4*. *Methods:* The cortical activity of two adult patients with known genetic mutations (*GJB2, SLC26A4*) was evaluated with fluorodeoxyglucose-positron emission tomography (FDG-PET) with a visual language task and compared with that of normal-hearing controls. *Results:* A patient with a *GJB2* mutation showed activation in the right auditory association area [BA21, BA22], and the left auditory association area [BA42] even with visual language task; in contrast, a patient with an *SLC26A4* mutation showed no significant activation in the corresponding area.

Keywords: *FDG-PET, visual language task, functional brain imaging*

Introduction

Functional brain imaging is an effective method for investigating the cortical processing of language, which has provided much evidence for the plasticity of the central auditory pathway following a profound loss of hearing [1–4]. Many previous studies showed that there is a capacity of the auditory cortex for cross-modal plasticity after auditory deprivation of the brain. Cerebral glucose metabolism in the primary auditory and related cortices in individuals with prelingual deafness was shown to decrease in younger patients, but to increase as they aged and, in fact, recover fully or even exceed the normal level of activation [5–7]. Children with prelingual deafness can acquire spoken language by cochlear implantation, but its efficacy decreases with age. The development of the auditory cortex is believed to depend on the patient's auditory experience within 'critical periods' in the early lifetime. Adults who had severe congenital hearing loss in their childhood may take advantage of hearing with cochlear implants if they had exploited residual hearing with hearing aids. It has been shown that low glucose metabolism in the temporal auditory cortex predicts a good cochlear implant outcome in prelingually deafened children, which suggests that low metabolism in the auditory cortex may indicate its potential of plasticity for spoken language acquisition [7].

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Meanwhile, several etiological studies suggest that at least 60% of congenital hearing loss has genetic causes. Recent advances in molecular genetics have made genetic diagnosis possible [8]. The identification of the mutation responsible for hearing loss may provide some information as to cochlear damage, and help predict the time course and manifestations of hearing loss. Genetic testing can therefore be useful in decision-making regarding cochlear implantation and other necessary treatment.

Evaluation of brain function and diagnosing accurate etiology of hearing loss may be the keys to personalizing post-cochlear implantation habilitation programs and predicting the outcomes thereof.

In this study, we used 18 F-fluorodeoxyglucose (FDG) positron emission tomography (PET) to measure cortical glucose metabolism with a visual language task before cochlear implantation in profoundly deaf patients whose etiologies were identified by genetic testing.

Material and methods

Genetic diagnosis

Genetic screening was performed in two cases using an Invader assay to screen for 41 known hearing loss-related mutations [9] and direct sequencing for *GJB2* and *SLC26A4* mutations [10,11].

FDG-PET scanning and image analysis

FDG-PET scanning and image analysis were performed using the method described by Fujiwara et al. [12]. During the time period between the intravenous injection of 370 MBq 18 F-FDG (the dose was adjusted according to the body weight of each subject) and the PET scanning of the brain, the patients were instructed to watch a video of the face of a speaking person reading a children's book. The video lasted for 30 min, and several still illustrations taken from the book were inserted (for a few seconds each) to help the subjects to follow the story. The subjects were video-recorded to confirm that they were watching the task video. PET images were acquired with a GE ADVANCE NXi system (General Electric Medical Systems, Milwaukee, WI, USA). Spatial preprocessing and statistical analysis were performed with SPM2 (Institute of Neurology, University College of London, UK) implemented in Matlab (Mathworks, MA, USA). The cortical radioactivity of each deaf patient was compared with that of a control group of normal-hearing adults by a t test in the basic model of SPM2. The statistical significance level was set at $p < 0.001$ (uncorrected).

This study was approved by the Ethics Committee of Shinshu University School of Medicine and written consent was obtained from each participant.

Control group

The control group consisted of six normal-hearing right-handed adult subjects. The average (mean ± standard deviation) age of the normal-hearing subjects was 27.5 ± 3.8 years. The pure-tone average hearing levels were within 20 dB HL for all.

Case 1

A right-handed 22-year-old female with a *GJB2* mutation (235 delC homozygous) had hearing impairment that was noticed by her parents when she was 2 years old. She had used hearing aids ever since, but with insufficient hearing amplification. She used lip-reading and some sign language, and her speech was not intelligible to hearing people. Computed tomography (CT) findings of the middle and inner ear were normal. Her average pure-tone hearing levels were 102.5 dB for the right ear and 95 dB for the left ear (Figure 1A).

Case 2

A right-handed 26-year-old male with an *SLC26A4* mutation (H723R homozygous) had hearing impairment that was noticed by his parents when he was 2 years old, from which time he had used hearing aids bilaterally. He did not use lip-reading or sign language during the acquisition age for language. He obtained spoken language with hearing aids but had progressive hearing loss, and sometimes suffered vertigo attacks. His pronunciation was clear, and his speech was almost completely intelligible. CT findings exhibited an enlarged vestibular aqueduct on each side. His average pure-tone hearing levels were 106.2 dB for the right ear and 100 dB for left ear (Figure 1B).

Results

Figure 2 shows transaxial PET images of each participant's brain. The visual stimuli resulted in bilateral activation of the superior temporal gyrus, including Heschl's gyrus in case 1 with *GJB2* mutation (Figure 2A, white arrowhead). In contrast, in case 2 with *SLC26A4* mutation, the activation of the superior temporal gyrus was much lower than in case 1 (Figure 2B, white arrowhead).

Figure 3 shows supra-threshold clusters in each case. In case 1, activation higher than normal controls

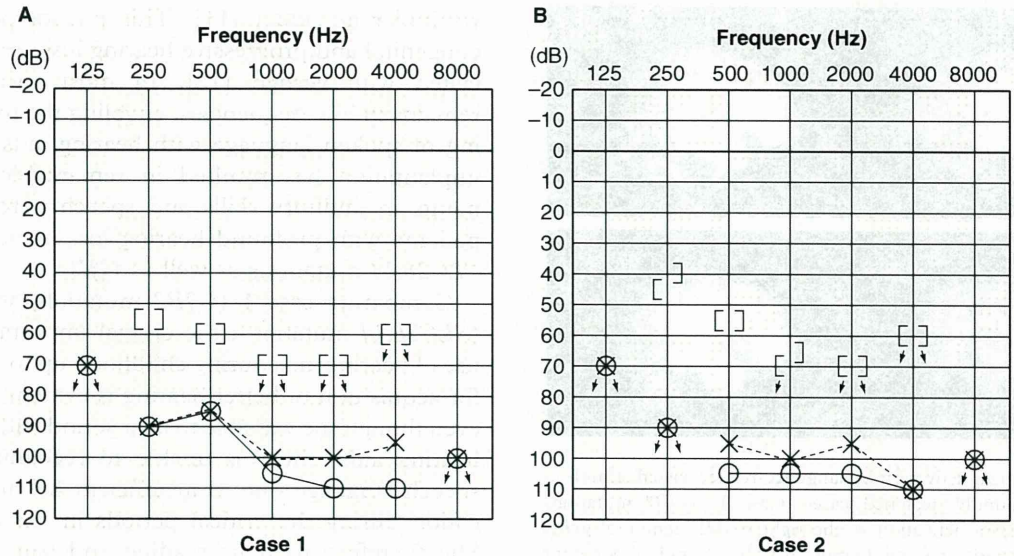

Figure 1. Pure-tone audiograms: (A) a 22-year-old female with a *GJB2* mutation; (B) a 26-year-old male with an *SLC26A4* mutation. There were no clear differences in hearing thresholds in these two cases.

was observed in the right auditory association area [BA21, BA22], and the left auditory association area [BA42] ($p < 0.001$). In case 2, the right superior frontal gyrus [BA9], and the middle temporal gyrus [BA20], showed higher activation than normal controls ($p < 0.001$).

Discussion

More than half of congenital hearing loss has been estimated to be from genetic causes, and phenotypes are affected by genetic mutations. There have been no reports of the influence of phenotype on brain function associated with hearing. This is the first report on evaluation of cortical processing of language in patients with genetic mutations as a main etiology of hearing loss. The auditory association area was activated bilaterally in case 1 (*GJB2* mutation), but not activated in case 2 (*SLC26A4* mutation). A previous study indicated that the temporal lobe is activated during speech-reading in normal subjects [13] and another study found that the temporal lobe is not activated when reading fluent speech from a talking face [14]. For the present study we used a

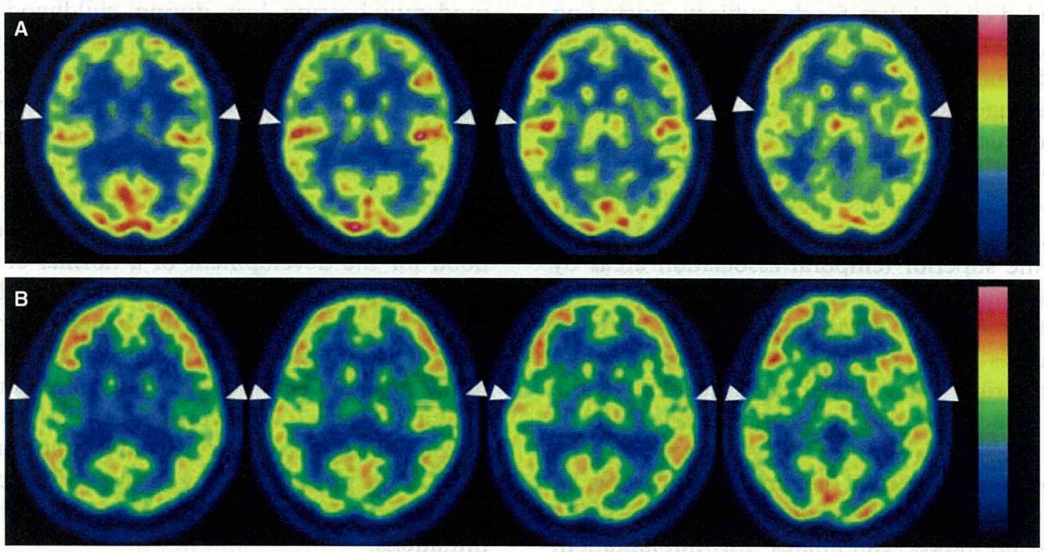

Figure 2. Transaxial PET images of each participant's brain: activation (arrowheads) of the superior temporal gyrus with visual language stimuli in each case. (A) Case 1 (*GJB2* mutation). The superior temporal gyri were strongly activated bilaterally. (B) Case 2 (*SLC26A4* mutation). The superior temporal gyri exhibited less activation than in case 1.

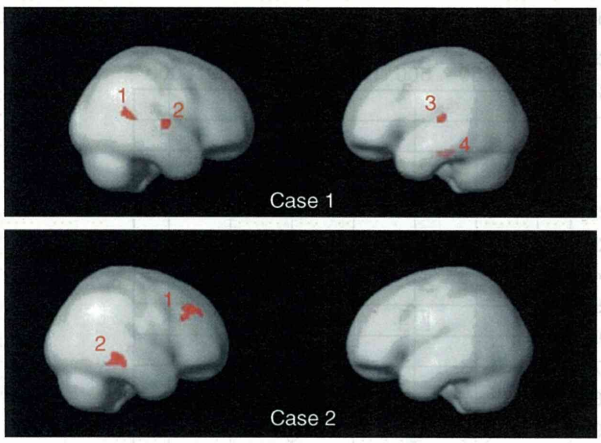

Figure 3. Cortical activation by language-related visual stimuli in the two profoundly deafened cases. Case 1 (*GJB2* mutation) showed significant activation in the right middle temporal gyrus [BA21] (1), superior temporal gyrus [BA22] (2), and left superior temporal gyrus [BA42] (3), and left cerebellum (4), while case 2 (*SLC26A4* mutation) exhibited significant activation in the right superior frontal gyrus [BA9] (1), and middle temporal gyrus [BA20] (2) (SPM2, $p < 0.001$, uncorrected).

fluent speech-reading task, similar to that described by Hall et al. [14]. Fujiwara et al. in a FDG-PET study using the same methods and task as the present study, showed that subjects with better spoken language skills had less temporal lobe activation [12].

To summarize these reports, the patients with hearing aids with better spoken language skills have less temporal lobe activation with a visual language task. Otherwise, Nishimura et al. [15] reported a sign language activation of the bilateral auditory association areas in a congenitally deafened subject. However, detailed clinical data for the subject – including his hearing levels, time course of hearing loss, and the cause of deafness – were not described. The different visual language activation patterns in the auditory cortices revealed in the current two profoundly deafened subjects with different genetic etiologies and hearing loss progressions may, thus, add further knowledge of the cross-modal plasticity brought about in the superior temporal association areas by lack of hearing.

The differences in cortical processing patterns between cases 1 and 2 – who both had hearing loss of cochlear origin – may have been influenced by the differing clinical courses of hearing loss. *GJB2* is currently known to be the most prevalent gene responsible for congenital hearing loss worldwide. Patients with severe phenotypes who have *GJB2* mutations are good candidates for implantation, because their hearing loss is of cochlear origin and non-progressive [16,17]. *SLC26A4* is known as a commonly found gene and is associated with enlarged vestibular aquaduct [11]. This phenotype includes congenital and progressive hearing loss, usually associated with vertigo [18]. In most cases hearing remains in low frequencies, enabling the understanding of spoken language with hearing aids. Cochlear implantation has resulted in remarkable improvements in auditory skills and speech perception for patients with profound hearing loss associated with *SLC26A4* mutations as well as *GJB2*.

Comparing case 1 (*GJB2* mutation) with case 2 (*SLC26A4* mutation), the crucial importance of the use of hearing aids during childhood up to age 6 years for acquisition of better hearing is evident. In case 1, even though she was able to hear sound with the use of hearing aids, she was unable to recognize enough speech language due to insufficient hearing amplification during the critical periods in her childhood. She therefore used lip-reading and some sign language in addition to hearing aids. Increased metabolism was observed by FDG-PET in the auditory association area, where no significant activation was found in the normal-hearing controls. In contrast, in case 2, a 26-year-old patient with an *SLC26A4* mutation, there was no significant activation in the corresponding area. He obtained rather hearing ability and spoken language by hearing aids with residual hearing at lower frequencies during his childhood. His hearing was supposed to be better than case 1, because 1) he did not use lip-reading or sign language during the acquisition age for language from anamnestic evaluation; 2) his pronunciation was clear, indicating better hearing (at least 40–50 dB) during the acquisition age for language; 3) from an etiological point of view, patients with *SLC26A4* mutation usually have mild to moderate hearing loss during childhood and this shows a progressive nature [18]. He had progressive hearing loss in the natural history as a phenotype of *SLC26A4* mutation. The difference in activation patterns in the cases with *GJB2* and *SLC26A4* mutations was clearly demonstrated by statistical processing with SPM, as well as in the PET scans. These results suggest the importance of hearing during early childhood for the development of a normal cortical language network, and that reorganization had occurred in the auditory cortex of the patient with a *GJB2* mutation; i.e. processing visual aspects of language in the superior temporal gyri. This implies that cross-modal plasticity as a consequence of the lack of hearing during the critical period for spoken language acquisition in early childhood was influenced by the time course of hearing loss characterized by genetic mutations.

Previous studies have suggested that auditory areas presented high accumulation of FDG with deafness of early onset, and plastic changes in auditory cortices

were strongly affected by the duration of auditory deprivation [1,5,6,19,20]. Since low activation of the auditory cortices with visual stimuli suggests the subject's lesser dependence on visual communication methods and substantial residual plasticity in his auditory cortices, case 2 with an *SLC26A4* mutation may be determined to be an appropriate candidate for cochlear implantation.

Accurate diagnosis of hearing loss and early cochlear implantation are important for successful spoken language development. The approach using PET could help those involved in the habilitation and education of prelingually deafened children to decide upon the suitable mode of communication for each individual.

Both of the patients received cochlear implantation after PET examination. Further follow-up of these cases may indicate that efficacy of the combination of genetic diagnosis and functional brain imaging helps to predict long-term outcomes of cochlear implantation. Examination of more cases is necessary to define the relationship of the varying cortical activation patterns with each genetic mutation.

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Simultaneous Screening of Multiple Mutations by Invader Assay Improves Molecular Diagnosis of Hereditary Hearing Loss: A Multicenter Study

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Abstract

Although etiological studies have shown genetic disorders to be a common cause of congenital/early-onset sensorineural hearing loss, there have been no detailed multicenter studies based on genetic testing. In the present report, 264 Japanese patients with bilateral sensorineural hearing loss from 33 ENT departments nationwide participated. For these patients, we first applied the Invader assay for screening 47 known mutations of 13 known deafness genes, followed by direct sequencing as necessary. A total of 78 (29.5%) subjects had at least one deafness gene mutation. Mutations were more frequently found in the patients with congenital or early-onset hearing loss, i.e., in those with an awareness age of 0–6 years, mutations were significantly higher (41.8%) than in patients with an older age of awareness (16.0%). Among the 13 genes, mutations in *GJB2* and *SLC26A4* were mainly found in congenital or early-onset patients, in contrast with mitochondrial mutations (12S rRNA m.1555A>G, tRNA(Leu(UUR)) m.3243A>G), which were predominantly found in older-onset patients. The present method of simultaneous screening of multiple deafness mutations by Invader assay followed by direct sequencing will enable us to detect deafness mutations in an efficient and practical manner for clinical use.

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Competing Interests: The authors have read the journal's policy and have the following conflicts. The authors did not receive funding from the Department of Clinical Genomics, Biomedical Laboratories, Inc. They felt that for genetic analysis of patients with hearing impairment in which many gene/gene mutations are involved, Invader Assay is the appropriate choice. However, for patent reasons, the authors cannot develop this method independently. The development of this method was therefore performed in collaboration with Biomedical Laboratories. This relationship had no influence on results and the direct sequencing results were all double checked for accuracy. Although Invader Assay is more efficient, if a method other than Invader Assay had been used, the results would have been identical.

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Introduction

From a series of etiological studies, 60–70% of childhood hearing loss has been estimated to be of genetic etiology, with the rest due to environmental causes, including newborn delivery trouble, acoustic trauma, ototoxic drug use, and prenatal/postnatal infection [1]. However, until now, there has been no multicenter study based on genetic testing. Along with early discovery of hearing loss by newborn hearing screening programs and subsequent intervention programs, much attention has been paid to the determination of the hearing loss etiology. Therefore, genetic testing has become more important for highly accurate diagnosis, prediction of severity of hearing loss, estimation of associated abnormalities, selection of appropriate habilitation options, prevention of hearing loss, and better genetic counseling. Although more than one hundred loci have been mapped and 46 genes reported to be responsible for hereditary hearing loss (Hereditary Hearing Homepage; http://webh01.ua.ac.be/hhh/), many may cause similar phenotypes without any abnormality other than hearing loss. This genetic heterogeneity has made clinical application difficult, in spite of the considerable advances in discovery of deafness genes. We have previously established a screening strategy focusing on recurrent mutations and demonstrated its benefits for clinical application [2]. We carried out the current multicenter study to determine 1) whether the simultaneous screening of the multiple deafness mutations by Invader assay is applicable for clinical use, 2) whether the genetic etiology is truly prevalent among hearing loss patients and 3) whether genetic causes differ by ages.

Materials and Methods

Subjects and clinical status

As summarized in Table 1, two hundred sixty-four Japanese patients with bilateral sensorineural hearing loss from 33 ENT departments nationwide participated in the present study. We first applied the Invader assay for screening forty-seven known mutations of 13 known deafness genes, followed by direct sequencing as necessary.

Table 1. Clinical features of subjects in this study.

	Total (n = 264)	*Early onset (n = 141)*	*Late onset (n = 100)*
Severity of HL			
normal – moderate	148	58	78
severe – profound	95	70	21
unknown	21	13	1
Inheritance			
AD or Mitochondrial	38	9	24
AR or Sporadic	119	69	42
unknown	107	63	34
Other clinical features			
inner ear malformations	52	37	10
EVA	30	22	4
goiter	8	4	3
diabetes mellitus	14	3	11

HL: Hearing loss.
AD: Autosomal dominant.
AR: Autosomal recessive.
EVA: Enlarged vestibular aqueduct.
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Hearing loss was evaluated using pure-tone audiometry (PTA) classified by a pure-tone average over 500, 1000, 2000 and 4000 Hz in the better hearing ears. For children who were unable to be tested by PTA, we used an average over 500, 1000, 2000 Hz in either auditory steady-stem response (ASSR) or conditioned oriented reflex audiometry (COR), or the response threshold (dB) from auditory brainstem response (ABR). Computed tomography (CT) scans were performed to check for congenital inner ear anomalies.

Status of hearing loss in the 264 patients was: mild (21–40 dB) in 39 patients (14.7%), moderate (41–70 dB) in 84 (31.8%), severe (71–94 dB) in 39 (14.8%) and profound (>95 dB) in 56 patients (21.2%). Twenty-four subjects were classified as having normal hearing due to a specific audiogram with hearing loss only in the high or low frequency portions. With regard to onset age (the age of awareness), 141 patients had early onset deafness (below 6 y.o.), 100 had late onset deafness, and the rest had unknown onset ages.

The inheritance composition of the subjects was as follows: 38 subjects from autosomal dominant or mitochondrial inherited families (two or more generations affected); 119 subjects from autosomal recessive families (parents with normal hearing and two or more affected siblings) or subjects with sporadic deafness (also compatible with recessive inheritance or non-genetic hearing loss). None of the patients had an X-linked pattern of inheritance. The numbers of patients with other manifestations were inner ear malformations (52), enlarged vestibular aqueduct (EVA) (30), goiter (8), and diabetes mellitus (14). None of the patients had typical clinical features of Usher syndrome or BOR syndrome.

All subjects gave prior informed consent for participation in the project and the Ethical Committee of Shinshu University as well as the relevant bodies of the participating institutions of the Deafness Gene Study Consortium approved the study.

Invader assay

Invader technology is convenient for mutation genotyping, offering a simple diagnostic platform to detect single nucleotide changes with high specificity and sensitivity from unamplified genomic DNA.

We applied the Invader assay for screening forty-seven known mutations of 13 known deafness genes [*GJB2*(NM_004004.5), *SLC26A4*(NM_000441.1), *COCH*(NM_001135058.1), *KCNQ4*(NM_172163.2), *MYO7A*(NM_000260.3), *TECTA*(NM_005422.2), *CRYM* (NM_001888.3), *POU3F4*(NM_000307.3), *EYA1*(NM_172060.2), mitochondrial 12 s ribosomal RNA, mitochondrial tRNA(Leu), mitochondrial tRNA(Ser), and mitochondrial tRNA(Lys)] (Table 2). Mutations were selected on the basis of a mutation/gene database established in the Japanese deafness population. The detailed methodological protocol was described elsewhere [2]. In brief, 1.2 ul of primary probe/Invader oligonucleotides mixture (containing 0.5 umol/l wild type primary probes, 0.5 umol/l mutant primary probe, 0.05 umol/l Invader oligonucleotide, and 10 mmol/l MOPS) were poured into each well of 384-well plates. Fluorescent resonance energy transfer (FRET)/Cleavase mixture (Third Wave Technologies, Madison, WI) was added to the probe/Invader oligonucleotide-containing plates. Then, 3 ul of 5–100 fmol/l synthetic target oligonucleotides (positive control), 10 ug/ml yeast tRNA (no target control), and denatured genomic DNA samples (>15 ng/ul) were added. Next, 6 ul of mineral oil (Sigma, St. Louis, MO) were overlayed into all reaction wells and incubated at 63°C for 4 hour. After incubation fluorescence was measured by a Cyto Fluor 4000 fluorescent micro plate reader (Applied Biosystems, Foster CA). The heteroplasmy rate for mitochondrial mutations was quantified by detection of fluorescently labeled and digested PCR products through a fluorescence imaging system [2].

Direct sequencing

Dominant mutations and mitochondrial mutations are themselves diagnostic criteria for molecular diagnosis. But a hallmark of recessive mutations, in *GJB2* and *SLC26A4* for example, is the detection of two mutations in the paternal and maternal alleles. In this study, direct sequencing was further carried out as follows: 1) *GJB2* mutation analysis for all subjects, because the authors wanted to clarify whether the number of mutations on the invader panel are enough (saturated) or not. 2) *SLC26A4* mutation analysis for all the subjects with EVA, 3) *SLC26A4* mutation analysis for heterozygous patients for these genes. DNA fragments containing the entire coding region were sequenced as described elsewhere [3,4].

Results

The mutations found by Invader assay and direct sequencing in this study are summarized in Table 2 and 3.

Invader Assay

A total of 74 (28.0%) hearing-impaired subjects (n = 264) were found to have at least one deafness gene mutation. Among the deafness genes situated on the present diagnostic panel, mutations were most frequently found in the *GJB2* gene. Screening of *GJB2* showed mutations of one or both alleles of the gene in 43 (43/264; 16.2%) samples from the subjects, of which 13 cases had only a single mutation, and 30 cases were compound heterozygotes or homozygotes, confirmed by segregation analysis (Table 4). The most common mutation was c.235delC, accounting for nearly 67% (29/43) of all *GJB2* mutated patients. On the other hand, the *GJB2*: c.35delG mutation, which is known to be the most common mutation in Caucasian or other ethnic populations, was not found in this group. The second most common group of *GJB2* mutations consisted of p.[G45E; Y136X], p.V37I, and c.299_300del. These mutations were detected in more than 5 patients each, and their allele frequencies were relatively high. Three mutations (p.T86R, p.R143W, and c.176_191del) were observed in more than one

Table 2. Mutation list of Invader based genetic screening test.

Gene	*Exon*	*Codon location*	*Nucleotide change*	*Frequency of mutant alleles (n = 528)*	*Number of patients with mutations (n = 264)*
GJB2	exon 2	p.L79fs	c.235delC	43 (8.1%)	29 (10.9%)
GJB2	exon 2	p.V37I	c.109G>A	7 (1.3%)	6 (2.3%)
GJB2	exon 2	p.[G45E; Y136X]	c.[134G>A; 408C>A]	10 (1.9%)	10 (3.8%)
GJB2	exon 2	p.G59fs	c.176_191del	3 (0.6%)	3 (1.1%)
GJB2	exon 2	p.R143W	c.427C>T	4 (0.8%)	4 (1.5%)
GJB2	exon 2	p.H100fs	c.299_300del	5 (0.9%)	5 (1.9%)
GJB2	exon 2	p.T123N	c.368C>A	4 (0.8%)	4 (1.5%)
GJB2	exon 2	p.T86R	c.257C>G	1 (0.2%)	1 (0.4%)
GJB2	exon 2	p.F191L	c.570T>C	0	0
GJB2	exon 2	p.I71T	c.212T>C	0	0
GJB2	exon 2	p.A49V	c.146C>T	0	0
GJB2	exon 2	p.G12fs	c.35delG	0	0
SLC26A4	exon 19	p.H723R	c.2168A>G	22 (4.1%)	17 (6.4%)
SLC26A4	int 7/exon 8	splice site	c.919-2A>G	2 (0.4%)	2 (0.8%)
SLC26A4	exon 7	p.T410M	c.1229C>T	4 (0.8%)	3 (1.1%)
SLC26A4	exon 7	p.V306fs	c.917insG	0	0
SLC26A4	exon 19	p.T721M	c.2162C>T	0	0
SLC26A4	exon 8/int 8	splice site	c.1001+1G>A	0	0
SLC26A4	exon 9	p.A372V	c.1115C>T	0	0
SLC26A4	exon 5	p.M147V	c.439A>G	1 (0.2%)	1 (0.4%)
SLC26A4	int 5/exon 6	splice site	c.601-1G>A	0	0
SLC26A4	exon 9	p.K369E	c.1105A>G	1 (0.2%)	1 (0.4%)
SLC26A4	exon 15	p.S551fs	c.1652insT	1 (0.2%)	1 (0.4%)
SLC26A4	exon 15	p.C565Y	c.1693G>A	0	0
SLC26A4	exon 17	p.S666F	c.1997C>T	0	0
SLC26A4	exon 19	p.E704fs	2111ins GCTGG	1 (0.2%)	1 (0.4%)
SLC26A4	exon 4	p.L108fs	c.322delC	0	0
SLC26A4	exon 4	p.P123S	c.367C>T	0	0
SLC26A4	exon 10	p.N392Y	c.1174A>T	0	0
SLC26A4	exon 17	p.S610X	c.1829C>A	0	0
SLC26A4	exon 17	p.S657N	c.1970G>A	0	0
EYA1	exon 12	p.D396G	c.1187A>G	0	0
EYA1	exon 8	p.R264X	c.790C>T	0	0
EYA1	exon 7	p.Y193X	c.579C>G	0	0
COCH	exon 5	p.A119T	c.441G>A	0	0
KCNQ4	exon 5	p.W276S	c.827G>C	0	0
MYO7A	exon22	p.A886fs	c.2656_2664del	0	0
TECTA	exon 16	p.R1773X	c.5318C>T	0	0
TECTA	exon 20	p.R2121H	c.6063G>A	0	0
Mitochondrial 12S rRNA			m.1555A>G	-	5 (1.9%)
Mitochondrial tRNALeu			m.3243A>G	-	6 (2.3%)
Mitochondrial tRNASer			m.7445A>G	-	0
Mitochondrial tRNALys			m.8296 A>G	-	0
CRYM	exon 8	p.K314T	c.941 A>C	0	0
CRYM	exon 8	p.X315Y	c.945 A>T	0	0

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Table 3. Mutation list found by direct sequencing analysis.

Gene	*Exon*	*Codon location*	*Nucleotide change*	*Frequency of mutant alleles (n = 528)*	*Number of patients with mutations (n = 264)*
GJB2	exon 2	p.T8M	c.23C>G	1 (0.2%)	1 (0.4%)
GJB2	exon 2	p.K12fs	c.35insG	1 (0.2%)	1 (0.4%)
GJB2	exon 2	p.F106Y	c.317T>A	1 (0.2%)	1 (0.4%)
GJB2	exon 2	p.A171fs	c.511insAACG	2 (0.4%)	2 (0.8%)
GJB2	exon 2	p.C174S	c.522G>C	1 (0.2%)	1 (0.4%)
SLC26A4	exon 14	p.S532I	c.1595G>T	2 (0.4%)	2 (0.8%)
SLC26A4	exon 16	p.R581S	c.1743G>C	1 (0.2%)	1 (0.4%)
SLC26A4	exon 17	p.V659L	c.1975G>C	2 (0.4%)	2 (0.8%)
SLC26A4	exon 10	p.L407fs	c.1219delCT	1 (0.2%)	1 (0.4%)
SLC26A4	exon 15/int 15	splice site	c.1931+5 G>A	5 (0.9%)	4 (1.5%)

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patient. p.F191L, p.I71T, p.A49V and c.35delG mutations were not found. One pair of p.[G45E; Y136X] mutations was detected among 10 persons in a heterozygous state. Subsequent parental DNA segregation study through direct sequencing indicated two mutations were in *cis*. The p.T123N mutation was found in 4 subjects but, based on our recent study, is not likely to be a pathologic mutation [5].

The second most frequent gene with mutations was the *SLC26A4* gene (23/264; 8.7%). Five cases were homozygotes of p.H723R, one was a homozygote of p.T410M, 3 were compound heterozygotes, and 14 had only one mutation of *SLC26A4* (Table 4). Of the 19 *SLC26A4* mutations, 12 (c.917insG, p.T721M, c.1001+1G>A, p.A372V, c.601-1G>A, p.C565Y, p.S666F, c.322delC, p.P123S, p.N392Y, p.S610X, and p.S657N) were not found in any samples, but the remaining 7 *SLC26A4* mutations were confirmed in more than one subject. Especially, the p.H723R mutation was found to be in high allele frequency (4.1%). All of the patients with *SLC26A4* mutations had EVA, which has been demonstrated to be a result of the mutations of this gene. *SLC26A4* mutations were detected by Invader assay in 63.6% of the patients with EVA.

Mitochondrial m.1555A>G mutations were found in 1.9% (5/264) of the patients and the m.3243A>G mutation was identified in 2.3% (6/264).

Mutations in nine deafness genes (*COCH*, *KCNQ4*, *MYO7A*, *TECTA*, *CRYM*, *POU3F4*, *EYA1*, mitochondrial tRNA(Lys) m.8296A>G, mitochondrial tRNA(Ser) m.7445A>G) were not identified in any patients (Table 2).

Notably, 4 subjects were found to have double gene mutations. Two cases were *SLC26A4* compound heterozygous or homozygous mutations with a *GJB2* heterozygous mutation. One case was a compound heterozygous of *GJB2* with a *SLC26A4* heterozygous mutation and the remaining case was a *GJB2*

Table 4. Diagnostic efficiency of Invader assay alone and Invader assay and direct sequencing.

	Total (n = 264)	*Early onset (n = 141)*	*Late onset (n = 100)*
Invader assay alone			
GJB2 homozygote/compound heterozygote	30 (11.4%)	29 (20.6%)	1 (1.0%)
GJB2 heterozygote	13 (4.9%)	7 (5.0%)	6 (6.0%)
SLC26A4 homozygote/compound heterozygote	9 (3.4%)	9 (6.4%)	0 (0%)
SLC26A4 heterozygote	14 (5.3%)	10 (27.1%)	2 (2.0%)
Mitochondria A1555G	5 (1.9%)	2 (1.4%)	2 (2.0%)
Mitochondria A3243G	6 (2.2%)	1 (0.7%)	5 (5.0%)
Total	74 (28.0%)*	55 (39.0%)*	16 (16.0%)
Invader assay and direct sequencing			
GJB2 homozygote/compound heterozygote	33 (12.5%)	31 (21.9%)	2 (2.0%)
GJB2 heterozygote	13 (4.9%)	7 (5.0%)	5 (5.0%)
SLC26A4 homozygote/compound heterozygote	18 (6.8%)	18 (12.7%)	0 (0%)
SLC26A4 heterozygote	7 (2.7%)	4 (2.8%)	2 (2.0%)
Mitochondria A1555G	5 (1.9%)	2 (1.4%)	2 (2.0%)
Mitochondria A3243G	6 (2.2%)	1 (0.7%)	5 (5.0%)
Total	78 (29.5%)**	59 (41.8%)**	16 (16.0%)

*Three cases carried double mutations (cases 1 to 3 in Table 5).
**Four cases carried double mutations shown in Table 5.
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