Table 5. Double mutation cases found in simultaneous mutation screening.

Genotype	Patients Number
GJB2:p.[V37I];[V37I]; Mitochondria m.1555A>G	1 (0.4%)
GJB2:c.[235delC];p.[R143W]; *SLC26A4*:p.[M147V]	1 (0.4%)
GJB2:p.[V37I]; *SLC26A4*:p.[H723R];[H723R]	1 (0.4%)
GJB2:p.[F106Y]; *SLC26A4*:p.[H723R]; c.[1931+5G>A]	1 (0.4%)
Total	4 (1.5%)

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homozygous mutation with a mitochondrial 1555A>G mutation (Table 5).

The detection rate of mutations was 40.4% for the patients with congenital or early-onset hearing loss, i.e. in those with an awareness age of 0~6 years. The rate in congenital hearing loss patients also increased when restricting to the patients with moderate or more severe hearing loss (>50 dB; 40.7%) or severe hearing loss (>70 dB; 44.3%) (Fig. 1). In contrast, the detection rate was only 16.0% in the patients with an older age of onset/awareness (Fig. 1). Among the 13 included genes, mutations is *GJB2* and *SLC26A4* were mainly found in congenital patients or early-onset patients, in contrast with mitochondrial mutations, such as 12S rRNA m.1555A>G or tRNA(Leu(UUR)) m.3243A>G, which were predominantly found in older-onset patients (Table 4). The p.V37I mutation in the *GJB2* gene was also found in older-onset patients (data not shown).

With regard to the relationship between radiographic findings and genetic testing, the mutation detection rate was elevated when restricting to the patients with inner ear anomaly (50.0%) and EVA (63.6%) (Fig. 2).

Direct sequencing

Direct sequencing identified 9 mutations in 15 cases which were not included in the Invader assay panel and improved the mutation detection/diagnostic rate obtained by Invader assay analysis (28.0%/18.6%) to 29.5%/22.7%. (Fig. 1). Combining direct sequencing with invader screening enhanced the diagnostic rate notably but not the mutation detection rate. In detail, direct sequencing identified additional mutations in three cases with single *GJB2* mutations by Invader assay that were finally diagnosed as compound heterozygous mutations of *GJB2* (p.[T86R]; c.[511insAACG], p[T8M];[V37I] and c.[35insG];[235delC]).

In 7 cases only a single *SLC26A4* mutation was found by invader assay, and additional mutations were found by direct sequencing (two cases of p.[H723R];c[1931+5G>A] and one each cases of p.[R581S];[H723R], p.[V659L];[H723R], p.[S532I]; c.[2111insGCTGG], p.[T410M]; c.[1931+5G>A] and p.[K396E];[S532I]). Two cases carried EVA but without any mutations found in Invader assay, c[1931+5G>A]; [1931+5G>A] and p.[V659L];c[1219 delCT] compound heterozygous mutations were found by direct sequencing. With the combination of Invader assay and direct sequencing, and restriction to patients with EVA, the mutation detection rate was elevated to 17/22 cases (77.3%, Fig. 2). Fifteen of them carried homozygous or compound heterozygous *SLC26A4* mutations.

Discussion

We previously reported that simultaneous detection of common deafness gene mutations has excellent sensitivity and accuracy [2]. In this study, using samples from patients at 33 institutions nationwide from northern to southern Japan, we confirmed that the Invader assay based on the Japanese deafness gene mutation database works efficiently in the clinical base to detect the responsible gene mutations from the patients with

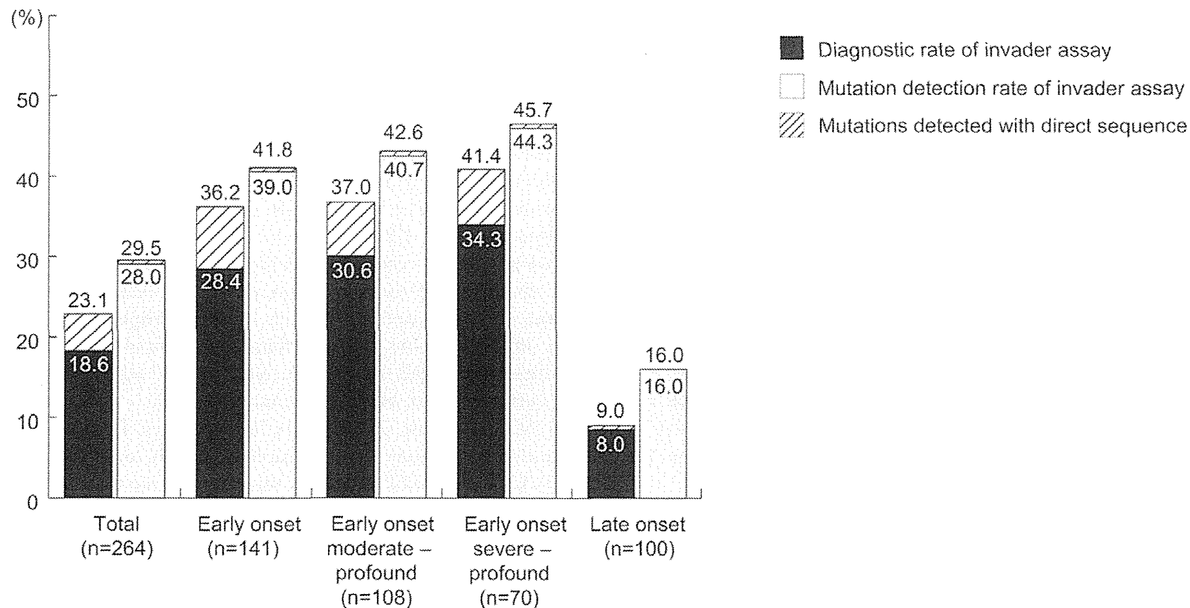

Figure 1. Detection rate by onset/awareness age and severity of hearing loss. Diagnostic rates and detection rates of this simultaneous multiple mutations screening and direct sequencing for biallelic mutations in autosominal recessive genes or mitochondrial mutations increased when restricted to congenital/early-onset hearing loss, and moderate or severe hearing loss. Combined direct sequence and invader screening enhanced the diagnostic rate but not the mutation detection rate.
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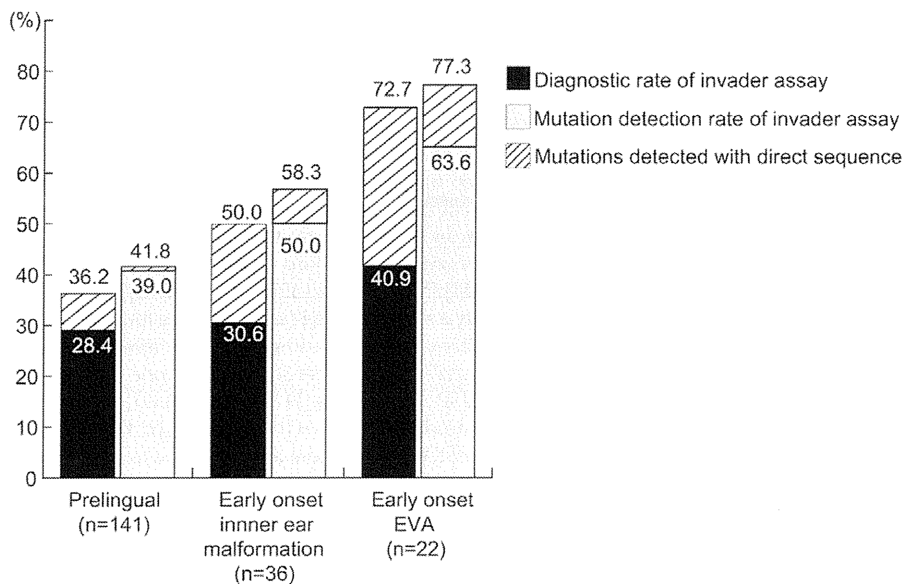

Figure 2. Radiographic findings and detection rate. Detection rate was elevated when subjects were restricted to those with inner ear anomaly or EVA. Combined direct sequence and invader screening enhanced the diagnostic rate but not the mutation detection rate.
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various onset/awareness ages. We detected mutations in 29.5% overall, and the 41.8% detection rate for congenital or early onset sensorineural hearing loss was especially remarkable. A series of epidemiological studies have demonstrated that genetic disorders are common causes of congenital deafness and it is estimated that 60–70% of the etiology may be caused by genetic factors [1]. Genetic testing is crucial to diagnose the etiology, but more than 100 genes are estimated to be involved and such genetic heterogeneicty has hampered the genetic testing for deafness as a routine clinical test. The present detection rate; i.e., 41.8%, is a strikingly good rate for a clinical application, and it is expected that clinical deafness mutation screening will greatly improve medical management and facilitate extensive genetic counseling for hearing impairment. Additional direct sequencing, as well as a new version of the screening panel which includes novel identified mutations, will likely improve the detection rate. For the older ages of onset, the detection rate was comparatively low (16.0%). Probably this is due to the panel mainly including responsible genes for congenital deafness but not the responsible genes for late onset hearing loss. An alternative explanation may be that environmental factors may be involved in this group of deafness patients.

The present study confirmed that mutations in three genes, *GJB2*, *SLC26A4*, and the mitochondrial 12 s rRNA, are so far the major known causes of hereditary hearing loss nationwide in Japanese [6], and thus much attention should be paid to these genes when performing genetic testing of hearing loss patients.

The most frequently found were mutations in the *GJB2* gene. This gene is so far the most common responsible gene for congenital deafness worldwide [7]. The detection rates (17.4% for all, 27.0% for congenital) are in accordance with our previous data of 15% in the overall deafness population and 25% in congenital deafness patients [5]. The mutation spectrum found in this study is also in accordance with our previous results [2,4,5]. In *GJB2* screening, 46 (17.4%) samples from deafness subjects had mutations of one or both alleles of the *GJB2* gene. As expected from the above reports, the c.235delC mutation was found to be the most prevalent mutation in our screening, accounting for 10.9% (29 of 264) of the hearing-impaired persons. Fourteen patients were c.235delC homozygotes and 11 were compound heterozygotes having c.235delC, confirmed by segregation analysis, and 4 patients were c.235delC heterozygotes without a second mutation. Direct sequencing identified novel mutations (p.T8M, c.35insG, p.F106Y, p.C174S and c.512insAACG) in the patients with a single mutation detected by Invader assay (Table 3).

Many benefits of *GJB2* gene genetic testing have been pointed out. There have been general rules that inactivating mutations (deletion mutations and stop mutations) show more severe phenotypes compared to those caused by non-inactivating mutations (missense mutations) [5,8,9]. As well as a highly accurate diagnosis, these genotype-phenotype correlation data could provide prognostic information to help decide the strategy of intervention with hearing, i.e., whether a child should receive cochlear implantation or hearing aids. For the patients with severe phenotypes who have *GJB2* mutations, genetic information would aid decision-making regarding cochlear implantation, because their hearing loss is of cochlear origin and they therefore are good candidates for implantation. In fact, cochlear implantation has resulted in remarkable improvement in auditory skills and development of speech production for patients with profound hearing loss associated with *GJB2* mutations [10].

In the *SLC26A4* gene, 7 cases were homozygotes, 11 cases were compound heterozygotes, and 7 cases had only one mutation (Table 4). Of the 19 *SLC26A4* mutations, 12 were not found in any samples, but the remaining 7 mutations were all confirmed in more than one patient. Especially, the p.H723R mutation was found to be in high allele frequency (4.1%). Direct sequencing identified novel mutations (c.1931+5G>A, p.S532I, p.R581S, p.V659L) in the patients with a single mutation by Invader assay and c.1219delCT mutation in a patient with EVA (Table 3).

As in our previous study [2], *SLC26A4* mutations were found only in the patients with EVA, suggesting a phenotype of hearing loss with EVA can be a diagnostic indicator of this category of disease.

Fluctuation and progressiveness of hearing loss are characteristic of hearing loss associated with EVA [11,12] and the early detection of *SLC26A4* mutations enables prediction of these clinical symptoms. Genetic testing is also useful in estimating associated abnormalities (goiter), selection of appropriate habilitation options, and better genetic counseling. In some cases, goiter is evident during the teen years [12]. In this study, 8 patients had hearing loss and goiter and 4 of them carried homozygous or compound heterozygous *SLC26A4* mutations.

In recessive mutations such as *GJB2* and *SLC26A4*, detection of two mutations in the paternal and maternal alleles is a hallmark. In the present "two step" screening method Invader assay is first performed followed by direct sequencing. As seen in Figs. 1 and 2, most of the mutations were successfully detected by the first Invader screening and the additional direct sequencing improved the "diagnostic" rate. This is very important to find the first mutation for identifying the responsible gene and the results indicate this screening is technically efficient. Difficult cases of a heterozygous state without a second mutation are also seen [4,5,13,14]. As previously reported, in a substantial proportion of patients our Invader techniques and additional direct sequencing revealed only one mutant *GJB2* or *SLC26A4* allele causing deafness by recessive pattern. We believe that there is one more occult mutation somewhere because the frequency of heterozygous patients was much higher than that of mutation frequency in the control population. Another explanation may be the high frequency of carriers in the population. But given the carrier frequency in normal controls, the number of heterozygous deafness cases was greater than would be expected. Second mutations may be present in the same gene or genes in the same chromosomal region. Recent statistical analysis has shown that one allele mutation of *GJB2* and *SLC26A4* is more likely to be a pathological status than a carrier status [15] and indeed, patients with one *SLC26A4* mutation are associated with EVA, therefore it is strongly likely that there is a second mutation within this gene. Another possibility is that mutations in the regulatory region may be involved in phenotypic expression [16].

The m.1555A>G mutation in the mitochondrial 12SrRNA gene, which was found in 5 4 subjects, was mainly found in those with older onset age. This mutation has been reported to be associated with aminoglycoside injection and found in 3% of the patients who visited the outpatient clinic [17,18]. The current findings are compatible with our previous report that this mutation is a frequently encountered cause for postlingual deafness in patients who received cochlear implantation [18]. This mutation was also found in the congenital or early onset age group as well, in line with our previous study [2]. It is likely that there is a considerably large high-risk population worldwide and a rapid screening method as well as careful counseling should be established to prevent aminoglycoside-induced hearing loss in this group.

The m.3243A>G mutation in the tRNA(Leu (UUR)) gene was found in 6 patients in the older-onset group. This mutation was first reported at a high frequently in the patients with clinical manifestations of MELAS [19], and has also been found in diabetes mellitus patients [20]. It is known to be commonly associated with hearing loss patients (especially with diabetes mellitus) [21]. The hearing loss is adult onset, symmetric high frequency involved [22]. In this study, all 6 patients with this mutation were associated with diabetes mellitus and progressive hearing loss. Five patients had maternally inherited hearing loss (the mother also had hearing loss), but one subject was a sporadic case (the mother did not have hearing loss from the anamnestic evaluation) and therefore is unlikely to be a mitochondrial candidate from clinical evaluation. The present multigene screening is also unexpectedly efficient for such atypical cases.

Heteroplasmy is one of the significant factors determining the expression of mitochondrial disease. The Invader assay is comparatively accurate at detecting the heteroplasmic rate [2], and the present two patients with the 3243 mutation showed 3% and 24% heteroplasmic rates.

In contrast to the three genes discussed above, mutations of the *COCH, KCNQ4, MYO7A, TECTA, CRYM, POU3F4 and EYA1* genes were not found in the present deaf subjects in line with our previous study [2]. This is likely due to them being very rare and usually independent mutations found in only one family. Although analysis for these mutations should be performed to identify the molecular nature of deafness as the first deafness screening step, a different strategy may be necessary for screening for them.

In conclusion, the simultaneous examination of the multiple deafness mutations by Invader assay followed by direct sequencing if necessary, will enable us to detect deafness mutations in an efficient and practical manner for clinical use. This screening strategy will facilitate more precise clinical genetic diagnosis, appropriate genetic counseling and proper medical management for auditory disorders. Against this background, since 2008 the Ministry of Health and Welfare of Japan has allowed this screening to be performed as an advanced medical technology.

A Japanese summary of this article has been provided as Supporting Information (Japanese summary S1).

Supporting Information

Japanese Summary S1 Simultaneous Screening of Multiple Mutations by Invader Assay. 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.
(PDF)

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Author Contributions

Conceived and designed the experiments: SU. Performed the experiments: SN MN SA TY. Analyzed the data: SN MN SA TY. Contributed reagents/materials/analysis tools: SN MN SA TY. Wrote the paper: SU. Collection of DNA samples and clinical data: The Deafness Gene Study Consortium.

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Polymorphisms in Genes Involved in Inflammatory Pathways in Patients with Sudden Sensorineural Hearing Loss

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Abstract: Although the etiology of idiopathic sudden sensorineural hearing loss (SSNHL) remains unclear, the pathologically increased permeability of blood vessels, elucidated by gadolinium-enhanced magnetic resonance imaging (MRI), suggests the involvement of inflammation. Because SSNHL is considered a multifactorial disease, possibly caused by interactions between genetic factors and environmental factors, the authors investigated the associations of polymorphisms of inflammatory mediator genes with susceptibility to SSNHL. The authors compared 72 patients affected by SSNHL and 2010 adults (1010 men and 1000 women; mean age 59.2 years; range 40–79) who participated in the National Institute for Longevity Sciences Longitudinal Study of Aging. Multiple logistic regression was used to obtain odds ratios (ORs) for SSNHL in subjects with polymorphisms in the genes *IL-6* C − 572G, *IL-4R* G1902A, *IL-10* A − 592C, *TNFα* C − 863A, *TNFRSF1B* G593A, *VEGF* C936T, *VEGF* C − 2578A, and *VEGF* G − 1154A, with adjustment for age, gender, and any history of hypertension, diabetes, or dyslipidemia. The per-allele OR for the risk of SSNHL in subjects bearing *IL-6* C − 572G was 1.480 (95% confidence interval [CI], 1.037–2.111) in model 1 (no adjustment), 1.463 (CI, 1.022–2.094) in model 2 (adjusted for age and gender), and 1.460 (CI, 1.016–2.097) in model 3 (adjusted for age, gender, and a history of hypertension, diabetes, or dyslipidemia). Under the dominant model of inheritance, the ORs were 1.734 (CI, 1.080–2.783) in model 1, 1.690 (CI, 1.050–2.721) in model 2, and 1.669 (CI, 1.035–2.692) in model 3. The remaining seven polymorphisms failed to show any associations with the risk of SSNHL. These data need to be confirmed on larger series of patients. In conclusion, the *IL-6* C − 572G polymorphism is associated with a risk of SSNHL. Because permeability of blood vessels in the inner ear is frequently increased in patients with SSNHL, inflammation of the inner ear might be involved.

Keywords: case-control study, interleukin-6, polymorphism, sudden sensorineural hearing loss

INTRODUCTION

Sudden sensorineural hearing loss (SSNHL) is usually unilateral and can be associated with tinnitus and vertigo. In most cases, the cause is not identified and it is termed idiopathic SSNHL (Schreiber et al., 2010). Within 1 or 2 months following the onset of the hearing loss, complete, partial, or no hearing recovery can occur. After this critical period, hearing becomes stable and no further recovery can be expected.

The etiology of idiopathic SSNHL remains unclear. Proposed pathologies include viral infections, circulatory disturbance, and membrane breaks in the cochlea (Merchant et al., 2005). Among these, impaired inner ear perfusion and ischemic vascular damage of the cochlea are widely recognized as possible pathogenic mechanisms. Several studies have attempted to prove the vascular cause hypothesis using diagnostic imaging technology or hematologic assessment. It was reported recently that the blood-labyrinth barrier is disrupted in one-third of

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patients with SSNHL because gadolinium contrast agents injected intravenously appeared in the affected inner ear on magnetic resonance imaging (Yoshida et al., 2008). As disruption of the blood-brain barrier is associated with increased permeability of blood vessels in the inner ear, the involvement of inflammation in the etiology of SSNHL was implied through pathologically increased blood vessel permeability.

SSNHL is considered a multifactorial disease, possibly caused by interactions between genetic factors and environmental factors. Several genetic risk factors have been found for the vascular or inflammatory pathogenesis of SSNHL, including the presence of the protein kinase C-eta 1425G/A, matrix metalloproteinase-1-1607G/2G, methylenetetrahydrofolate reductase C677T, prothrombin G20210A, platelet Gly IIIaA1/A2, and factor V Leiden G1691A polymorphism (Capaccio et al., 2005a, 2005b, 2007, 2009; Uchida et al., 2010, 2011). Environmental factors related to lifestyle including alcohol abuse, truncated sleeping periods, and fatigue have been found to be risks for SSNHL (Nakashima et al., 1997; Nakamura et al., 2001). Thus, both genetic and environmental factors might increase the risk and determine the genesis of SSNHL.

Recently, we investigated the contribution of *interleukin (IL)-1A* − 889C/T and *IL-1B* − 511C/T polymorphisms to the inner ear disease risk and found a significant association of risks for both SSNHL and Ménière's disease with the *IL-1A* − 889C/T polymorphism (Furuta et al., 2011). In the present study, we aimed to assess the effects of eight other polymorphisms of genes encoding inflammatory mediators on the risk of susceptibility to SSNHL.

MATERIALS AND METHODS

Patients

Seventy-two patients (36 men, 36 women; mean age 58.2 years; range 22–82) affected by SSNHL who visited the Department of Otorhinolaryngology of Nagoya University Hospital between November 2007 and January 2010 were recruited consecutively and enrolled. Idiopathic SSNHL was defined according to the criteria established by the Sudden Deafness Research Committee of the Ministry of Health and Welfare, Japan. The detailed diagnostic criteria were as described previously (Teranishi et al., 2007). Demographic data were recorded by medical interviews or self-reporting.

Controls

The controls used in this study were derived from the National Institute for Longevity Sciences Longitudinal Study of Aging (NILS-LSA), an ongoing population-based biennial survey. Participants in the NILS-LSA were selected randomly from resident registrations, stratified by both age and gender. The NILS-LSA study area is located within 30 km of Nagoya University Hospital. Details of the NILS-LSA have been described elsewhere (Uchida et al., 2005). Of these controls, 33 participants who reported a history of SSNHL in the questionnaires were excluded. Overall, 2010 participants (1010 men and 1000 women, mean age 59.2 years; range 40–79) were selected. They completed the first wave of NILS-LSA examinations between November 1997 and April 2000, and provided samples for the eight single-nucleotide polymorphism (SNP) genotype analyses (see below).

Ethics

The study protocol was reviewed and approved by the ethics committees of Nagoya University (370-4) and the National Center for Geriatrics and Gerontology (nos. 14, 52, 74), and written informed consent was obtained from all subjects.

Genotype Analysis

Eight polymorphisms of genes encoding inflammatory mediators (*IL-6* C − 572G, also known as C − 634G, rs1800796; *IL-4R* G1902A, rs1801275; *IL-10* A − 592C, rs1800872; *tumor necrosis factor* [*TNF*]α C − 863A, rs1800630; *TNF receptor super family* [*TNFRSF*]*1B* G593A, rs1061624; *vascular endothelial growth factor* [*VEGF*] C936T, rs3025039; C − 2578A, rs699947; G − 1154A, rs1570360) were investigated. Genomic DNA was extracted from peripheral blood lymphocytes using standard procedures, and polymerase chain reaction (PCR) amplification was performed. Genotyping was carried out using allele-specific primer and intercalator-mediated fluorescence resonance energy transfer probe methods as described previously (Yamada et al., 2003, Hirota et al., 2007). Details of primer sequences and PCR conditions are listed in Table 1.

Statistical Analysis

Statistical analyses were conducted using Statistical Analysis System version 9.1.3 (SAS Institute, Cary, NC, USA) with significance accepted at $p < 0.05$. Univariate analyses of categorical variables were performed using the chi-square test or Fisher's exact test. Student's *t* test was used to assess differences in continuous variables between two groups. For multivariate analysis, multiple logistic regression was performed to obtain the odds ratios (ORs) for the risk of SSNHL in subjects with polymorphisms. Genotypes were coded as major-allele homozygotes,

Table 1. PCR conditions used in genotyping the inflammatory gene variants

Gene	rs no.	Labeled primers	Sequence (5′ → 3′)	Amplicon (F1/R)/(F2/R) (bp)	Annealing temp. (°C)	Mg (mM)
Allele-specific primer (ASP) method						
Interleukin-6	rs1800796	F1 (FITC)	GGC AGT TCT ACA ACA GCx CC	43/42	60	1.5
		F2 (Texas Red)	GCA GTT CTA CAA CAG CxG C			
		R (Biotin)	CTG TGT TCT GGC TCT CCC TG			
Interleukin-4 receptor	rs1801275	F1 (FITC)	GCC CCC ACC AGT GGC TAT xGG	49/50	67.5	2.8
		F2 (Texas Red)	GGC CCC CAC CAG TGG CTA TxA G			
		R (Biotin)	CAC CCT GCT CCA CCG CAT GTA			
Interleukin-10	rs1800872	F1 (FITC)	CAG AGA CTG GCT TCC TAC AxG A	51/53	62.5	2.5
		F2 (Texas Red)	CCA GAG ACT GGC TTC CTA CAx TA			
		R (Biotin)	GCC TGG AAC ACA TCC TGT GA			
Tumor necrosis factor-α	rs1800630	F1 (FITC)	ATG GCC CTG TCT TCG TTA AxT G	75/77	62.5	3
		F2 (Texas Red)	GGC CCT GTC TTC GTT AAx GG			
		R (Biotin)	ACA GCA ATG GGT AGG AGA ATG TC			
Vascular endothelial growth factor	rs699947	F1 (FITC)	GTC TGA TTA TCC ACC CAG ATx GT	58/57	60	5.5
		F2 (Texas Red)	TCT GAT TAT CCA CCC AGA TxT T			
		R (Biotin)	ACA GAG GCT ATG CCA GCT GTA			

Gene	rs no.	Primers	Sequence (5′ → 3′)	Amplicon (bp)	Annealing temp. (°C)	Mg (mM)	Probe (5′→3′)
Intercalator-mediated fluorescence resonance energy transfer probe (IFP) method							
Tumor necrosis factor receptor superfamily member 1B (TNFRSF1B)	rs1061624	F	CCC TCT GAC CTG CAG GCC AAG	61	65	2	AGC AGA GGC AGC GAG TTG
		R	CCA TGG CAG CAG AGG CTT TCC				
Vascular endothelial growth factor	rs3025039	F	GGT CCG GAG GGC GAG ACT C	111	65	1.5	AGA GGG ACC ATG CTG GGT
		R	GGC TCG GTG ATT TAG CAG CAA G				
Vascular endothelial growth factor	rs1570360	F	ACT GGG CGT CCG CAG AGC	62	62.5	0.7	CCG CGT GTG GAA GGG CTG AG
		R	GGG ACA GGC GAG CCT CAG C				

heterozygotes, and minor-allele homozygotes. Then, because allele frequencies are even in some SNPs, major alleles in eight SNPs were determined for practical reasons in analyses, as follows: C in *IL-6* rs1800796; A in *IL-4R* rs1801275; A in *IL-10* rs1800872; C in *TNFα* rs1800630; A in *TNFRSF1B* rs1061624; C in *VEGF* rs3025039; C in *VEGF* rs699947; G in *VEGF* rs1570360. We took different modes of inheritance in analyses as follows. For the additive per-allele model, the minor allele was compared between cases and controls by assigning scores of 0, 1, and 2 to major-allele homozygotes, heterozygotes, and minor-allele homozygotes, respectively. The dominant model was used to compare genotypes of major-allele homozygotes versus others. The recessive model compared genotype major-allele homozygotes and heterozygotes versus minor-allele homozygotes. In these analyses, we used three models with and without moderator variables. In model 1, no moderator variables were adjusted; in model 2, age and gender were taken as moderator variables; and in model 3, any history of hypertension, diabetes or dyslipidemia was adjusted in addition to age and gender.

Audiological Testing and Classification

Hearing levels were evaluated in patients with SSNHL using an audiometer (Model AA-79S; Rion, Tokyo, Japan) in a sound-insulated chamber. The initial audiograms were obtained at the first visit and the final audiograms were taken at least 2 months after the onset of deafness, except for those patients who recovered completely within this period. The average hearing level was expressed as the average score at five frequencies (250, 500, 1000, 2000, and 4000 Hz). If the patient did not respond to the maximum sound level produced by the audiometer, we defined the threshold as 5 dB added to the maximum level. The severity of hearing loss was classified based on the average hearing level at the five frequencies described above as follows: mild (21–40 dB), moderate (41–70 dB), severe (71–90 dB), and profound (more than 90 dB) (Mosnier et al., 2011).

The audiogram shape was classified as: ascending (Type A), flat (Type B), descending (Type C), U-shaped (Type D), total or subtotal (Type E), or inverted U-shaped (Type F), according to a previous report with a minor modification (Huy & Sauvaget, 2005).

The outcome of SSNHL was evaluated using the criteria of the Ministry of Health and Welfare, Japan (Nakashima et al., 1989). Recovery was ranked as follows: no change (improvement in hearing of less than 10 dB on average); slight improvement (improvement in hearing of 10 dB or more but less than 30 dB on average); remarkable improvement (improvement in hearing of 30 dB or more on average); and complete recovery (all five frequencies on the final audiogram were 20 dB or less or there was improvement to the same degree of hearing as in the contralateral ear). Complete recovery and remarkable improvement were defined as good recovery. Slight improvement and no recovery were defined as poor recovery. To analyze hearing recovery, subjects with SSNHL whose first visit to our hospital was within 1 month after onset were selected.

RESULTS

The clinical characteristics of the subjects are presented in Table 2. No significant differences in age or male-to-female

Table 2. Clinical characteristics of SSNHL patients and control group.

	SSNHL group	Control group	ALL	*p* value
Number	72	2010	2082	
Gender, % male	50.0	50.3	50.2	NS
Age, year (mean ± SD)	58.2 ± 1.3	59.2 ± 0.2	59.3 ± 0.2	NS
Hypertension, %	33.3	25.3	25.5	NS
Diabetes, %	16.7	8.1	8.4	0.01
Dyslipidemia, %	13.9	17.1	17.0	NS
Tinnitus, %	79.2	ND	ND	
Vertigo, %	34.7	ND	ND	
Grade of hearing loss, %		ND	ND	
Mild	11.1			
Moderate	41.7			
Severe	25.0			
Profound	22.2			
Type of the audiometric curve, %		ND	ND	
Type A	19.4			
Type B	40.3			
Type C	16.7			
Type D	2.8			
Type E	18.1			
Type F	2.8			

Note: ND = not determined; NS = not significant.

Table 3. Genotype distribution in SSNHL cases and controls.

SNP	Genotype distribution, count (%)						p value
	CC		CG		GG		
IL-6 − 634							
Case	32	(44.5)	34	(47.2)	6	(8.3)	0.0697
Control	1168	(58.1)	720	(35.8)	122	(6.1)	
	AA		GA		GG		
IL-4R 1902							
Case	55	(76.4)	17	(23.6)	0	(0)	0.4715
Control	1484	(73.8)	486	(24.2)	40	(2.0)	
	AA		AC		CC		
IL-10 − 592							
Case	40	(55.6)	27	(37.5)	5	(6.9)	0.1857
Control	904	(45.0)	892	(44.4)	214	(10.6)	
	CC		CA		AA		
TNFα − 863							
Case	48	(66.7)	19	(26.4)	5	(6.9)	0.4128
Control	1449	(72.1)	402	(20.0)	159	(7.9)	
	AA		AG		GG		
TNFRSF1B							
Case	26	(36.1)	33	(45.8)	13	(18.1)	0.1405
Control	524	(26.1)	1003	(49.9)	483	(24.0)	
	CC		CT		TT		
VEGF 936							
Case	39	(54.9)	28	(39.5)	4	(5.6)	0.7065
Control	1193	(59.3)	731	(36.4)	86	(4.3)	
	CC		CA		AA		
VEGF − 2578							
Case	33	(45.8)	33	(45.8)	6	(8.4)	0.8557
Control	981	(48.8)	855	(42.5)	174	(8.7)	
	GG		GA		AA		
VEGF − 1154							
Case	53	(73.6)	15	(20.8)	4	(5.6)	0.4415
Control	1530	(76.1)	421	(21.0)	59	(2.9)	

ratio were observed between the cases with SSNHL and controls. Concerning comorbid diseases, the proportion of subjects with a history of diabetes was higher in the SSNHL group than controls ($p < 0.01$). Thus, 79.2 % of the subjects with SSNHL had tinnitus and 34.7% had vertigo. In terms of grades of hearing loss, 11.1% of the subjects with SSNHL showed mild hearing loss, 41.7% showed moderate hearing loss, 25.0% showed severe hearing loss, and 22.2% showed profound hearing loss. The most common audiogram shape was flat (Type B; 40.3%), followed by ascending (Type A; 19.4%), total or subtotal (Type E; 18.1%), and descending (Type C; 16.7%). U-shaped (Type D) and inverted U-shaped (Type F) hearing losses were rare (2.8%). Genotype distributions and univariate case-control analysis in the eight SNPs are presented in Table 3. The genotype distribution of the *IL-6* rs1800796 polymorphism was close to, but failed to reach, significant difference. In addition, there was no significant difference between cases and controls in the genotype distribution of the other seven SNPs. Allelic carrier frequencies and comparisons between cases and controls for the eight SNPs are shown in Table 4. The prevalence of the G-allele in *IL-6* rs1800796 was significantly higher in subjects with SSNHL than in controls. Significance in the differences between cases and controls was marginal in terms of the prevalence of the A-allele in *IL-10* rs1800872 and the A-allele in *TNFRSF1B* rs1061624.

Table 5 shows the results of multiple logistic regression analyses for SSNHL risk according to the eight SNPs. For the *IL-6* rs1800796 polymorphism, the risk of SSNHL increased significantly with an increase in the frequency of the minor allele and the significance of the OR was

Table 4. Allele frequencies in SSNHL cases and controls.

SNP	Allelic carriers frequency, count (%)		*p* value
	C	G	
IL-6 − 634			
Case	98 (68.1)	46 (31.9)	0.0285
Control	3056 (76.0)	964 (24.0)	
	A	G	
IL-4R 1902			
Case	127 (88.2)	17 (11.8)	0.4397
Control	3454 (85.9)	566 (14.1)	
	A	C	
IL-10 − 592			
Case	107 (74.3)	37 (25.7)	0.0724
Control	2700 (67.2)	1320 (32.8)	
	C	A	
TNFα − 863			
Case	115 (79.9)	29 (20.1)	0.4939
Control	3300 (82.1)	720 (17.9)	
	A	G	
TNFRSF1B			
Case	85 (59.0)	59 (41.0)	0.0589
Control	2051 (51.0)	1969 (49.0)	
	C	T	
VEGF 936			
Case	106 (74.6)	36 (25.4)	0.4182
Control	3117 (77.5)	903 (22.5)	
	C	A	
VEGF − 2578			
Case	99 (68.7)	45 (31.3)	0.7332
Control	2817 (70.1)	1203 (29.9)	
	G	A	
VEGF − 1154			
Case	121 (84.0)	23 (16.0)	0.3762
Control	3481 (86.6)	539 (13.4)	

independent of moderators. The per-allele OR for the risk of SSNHL in carriers of *IL-6* rs1800796 was 1.480 (95% confidence interval [CI], 1.037–2.111) in model 1, 1.463 (95% CI, 1.022–2.094) in model 2, and 1.460 (95% CI, 1.016–2.097) in model 3. Under the dominant model of inheritance, the ORs were 1.734 (95% CI, 1.080–2.783) in model 1, 1.690 (95% CI, 1.050–2.721) in model 2, and 1.669 (95% CI, 1.035–2.692) in model 3.

For *IL-10* rs1800872 and *TNFRSF1B* rs1061624, the nonadjusted ORs under the additive and dominant models of inheritance were not significant, but this was marginal. However, this marginal significance disappeared after accounting for moderator variables. No other SNPs showed a significant association with the risk of SSNHL, regardless of moderator variables.

Table 6 shows genotype the distribution of the *IL-6* rs1800796 polymorphism and clinical information in patients showing good and poor recovery. The genotype distribution of the *IL-6* rs1800796 polymorphism was not significantly different between patients with SSNHL showing good or poor recovery. Baseline clinical characteristics, such as the initial hearing level, period from onset to first visit to our hospital, tinnitus, and vertigo, which can affect the prognosis for hearing recovery, showed no significant difference between patients with good recovery and those with poor recovery. However, the duration from onset to first visit to our hospital tended to be shorter in patients with good recovery than those with poor recovery, although this failed to reach statistical significance.

The distribution of audiogram shapes was significantly different between the two groups ($p = 0.01$). The proportion of ascending type was higher and that of descending type was lower in the good recovery group than in the poor recovery group ($p < 0.01$ and $p = 0.02$, respectively). We recently reported that a significantly higher prevalence of the *IL-1A* − 889T allele was observed in 72 patients with SSNHL of the same series as the present study compared with controls (Furuta et al., 2011) . The distributions of *IL-6* − 572C/G and *IL-1A* − 889C/T genotypes were not significantly associated with each in either the SSNHL group or controls (chi-square test, $p = 0.73$ and $p = 0.66$, respectively).

DISCUSSION

We found a significant association of SSNHL risk with the *IL-6* rs1800796 polymorphism in this case-control study. The risk increased significantly with an increase in the frequency of the minor allele of this polymorphism, and the significance of OR was independent of moderators. Thus, carriers of the *IL-6* rs1800796 minor-allele are potentially more susceptible to SSNHL than noncarriers, even after adjustment for age, gender, and three major lifestyle-related diseases. Furthermore, no associations were found in the distributions of the *IL-6* − 572C/G and *IL-1A* − 889C/T polymorphisms related to the risk of SSNHL. The genotype distribution of the *IL-6* rs1800796 polymorphism was not associated with recovery of hearing loss in patients with SSNHL.

IL-6 is an inflammatory cytokine that has been reported to play important roles in regulating hematopoiesis, immune cell activation, and inflammation. However, it is a highly versatile cytokine, with pleiotropic actions not only in immune cells, but also in other cell types, such as cells of the central nervous system. Degradation of the blood-brain barrier incurs extravasation of peripheral immune cells into the brain parenchyma and IL-6 performs a crucial role in maintaining the integrity of this barrier (Brunello et al., 2000; Spooren et al., 2011). Therefore,

Table 5. Odds ratios and 95% confidence intervals for three genetic models of polymorphisms with risk of SSNHL.

Mode of inheritance	Crude: Model 1			Adjusted: Model 2			Adjusted: Model 3		
	Odds ratio	95% CI	*p* value	Odds ratio	95% CI	*p* value	Odds ratio	95% CI	*p* value
IL-6 − 634									
Additive	1.480	1.037–2.111	0.0307	1.463	1.022–2.094	0.0376	1.460	1.016–2.097	0.0405
Dominant	1.734	1.080–2.783	0.0226	1.690	1.050–2.721	0.0307	1.669	1.035–2.692	0.0357
Recessive	1.407	0.598–3.310	0.4342	1.431	0.608–3.370	0.4118	1.460	0.617–3.451	0.3889
IL-4R 1902									
Additive	0.816	0.488–1.366	0.440	0.829	0.495–1.387	0.4749	0.842	0.502–1.413	0.515
Dominant	0.872	0.502–1.516	0.6274	0.888	0.510–1.545	0.6737	0.902	0.517–1.574	0.7172
Recessive	—	—	—	—	—	—	—	—	—
IL-10 − 592									
Additive	0.706	0.483–1.033	0.0732	0.721	0.493–1.056	0.0931	0.733	0.500–1.073	0.1102
Dominant	0.654	0.407–1.049	0.0784	0.674	0.419–1.085	0.104	0.689	0.427–1.111	0.1268
Recessive	0.626	0.250–1.571	0.3186	0.632	0.252–1.586	0.3285	0.635	0.253–1.597	0.3349
TNFα − 863									
Additive	1.116	0.778–1.600	0.5510	1.094	0.759–1.578	0.6287	1.109	0.767–1.602	0.5828
Dominant	1.292	0.784–2.129	0.3155	1.239	0.746–2.056	0.4074	1.253	0.753–2.083	0.3853
Recessive	0.869	0.345–2.187	0.7652	0.882	0.350–2.223	0.7908	0.914	0.362–2.311	0.8496
TNFRSF1B									
Additive	0.724	0.516–1.014	0.0605	0.741	0.528–1.040	0.0835	0.751	0.535–1.054	0.0976
Dominant	0.624	0.382–1.019	0.0597	0.649	0.395–1.067	0.0882	0.666	0.404–1.097	0.1105
Recessive	0.697	0.379–1.281	0.2447	0.708	0.385–1.304	0.2684	0.709	0.384–1.308	0.2712
VEGF 936									
Additive	1.181	0.796–1.753	0.4081	1.153	0.773–1.719	0.4848	1.140	0.763–1.704	0.5226
Dominant	1.198	0.744–1.928	0.4566	1.155	0.715–1.867	0.5561	1.138	0.702–1.843	0.5998
Recessive	1.337	0.476–3.749	0.5815	1.344	0.478–3.773	0.5752	1.336	0.472–3.778	0.5851
VEGF − 2578									
Additive	1.065	0.742–1.530	0.7311	1.087	0.756–1.564	0.6517	1.055	0.735–1.514	0.7727
Dominant	1.127	0.703–1.806	0.6202	1.160	0.721–1.867	0.5406	1.146	0.710–1.849	0.5768
Recessive	0.960	0.410–2.245	0.9248	0.975	0.417–2.285	0.9544	0.873	0.370–2.058	0.7564
VEGF − 1154									
Additive	1.203	0.782–1.852	0.400	1.220	0.793–1.876	0.366	1.194	0.773–1.844	0.4239
Dominant	1.143	0.670–1.949	0.6244	1.167	0.683–1.993	0.5718	1.132	0.660–1.940	0.6523
Recessive	1.946	0.687–5.510	0.2102	1.940	0.684–5.507	0.2130	1.925	0.674–5.500	0.2215

Note. Moderating variables: model 1: no variables; model 2: age and sex; model 3: histories of hypertension, dyslipidemia, and diabetes, in addition to those of model 2.

IL-6 might function as an anti-inflammatory cytokine in this instance, helping to maintain blood-brain barrier integrity in neuroinflammatory conditions.

The IL-6 receptor is localized in the spiral ganglion cells, lateral wall, and both hair cells and supporting cells in the organ of Corti in the mouse cochlea. IL-6 expression was induced in the mouse cochlea 6 h after noise exposure. Administration of an anti-IL-6 receptor antibody (MR16 - 1) ameliorated IL-6 induction and spiral ganglion losses, and improved the hearing impairment caused by noise exposure (Wakabayashi et al., 2010). Therefore, IL-6 signaling might be involved in noise-induced hearing loss.

The IL-6 gene *IL-6* − 572C/G polymorphisms are located in the promoter site. Other investigators have reported that subjects with this allele had significantly higher plasma fibrinogen and C-reactive protein (CRP) levels than those without it. This allele is associated with a higher risk of coronary heart disease and myocardial infarction, which is related to a higher expression of IL-6 from peripheral blood mononuclear cells (Wong et al., 2007). A weakness of the present study is that plasma fibrinogen and CRP levels were not measured in these patients with SSNHL.

Other proinflammatory cytokines, such as TNFα and IL-1β are produced in the early response phase of the noise-overstimulated mouse cochlea and might initiate an inflammatory response and have some role in the mechanism of noise-induced cochlear damage (Fujioka et al., 2006). However, in the present study, no association was found between the presence of *TNFα* C − 863A and *TNFRSF1B* G593A and the risk of SSNHL.

The IL-4 receptor gene (*IL-4R*) polymorphism is well known to be associated with atopy and other inflammatory diseases. An A G transition SNP potentiates the binding specificity of the adjacent tyrosine residue. In a previous study using pyrosequencing, presence of the *IL-4R* polymorphism Q576R, also known as G1902A (rs1801275)

Table 6. Genotype distribution of *IL-6* rs1800796 polymorphism in SSNHL cases with good recovery and poor recovery.

	SSNHL cases with good recovery	SSNHL cases with poor recovery	*p* value
Number of cases	20	32	
IL-6 rs1800796			
CC, CG, GG	9, 9, 2	15, 17, 0	0.19
CC, CG/GG	9, 11	15, 17	0.90
C allele, G allele	27, 13	47, 17	0.52
Age	58.3 ± 10.9	58.8 ± 16.1	0.91
Initial hearing level on average (dB)	74.3 ± 19.5	69.8 ± 25.2	0.50
Period from onset to first visit (days)	4.3 ± 4.3	7.5 ± 8.4	0.07
Number of patients with vertigo	8	11	0.68
Number of patients with tinnitus	19	25	0.24
Audiometric curve, type (%)	A (50.0), B (25.0), C (0), D (5.0), E (15.0), F (5.0)	A (9.4), B (40.6), C (28.1), D (3.1), E (18.8), F (0_	0.01

Note. Initial hearing level on average: average hearing level of five frequencies (250, 500, 1000, 2000, 4000 Hz). Period from onset to first visit: period from onset of SSNHL to the date the patients visit our hospital for the first time. Audiometric curve, type: A = ascending; B = flat; C = descending; D = U-shaped; E = total or subtotal; F = inverted U-shaped. Values for age, initial hearing level on average and period from onset to first visit represent the average ± standard deviation.

(Schoof et al., 2009), was reported to be a risk factor for SSNHL in 97 Korean patients with sudden deafness and in 613 controls (Nam et al., 2006). However, no association between SSNHL risk and *IL-4R* G1902A polymorphism was found in the additive, dominant, or recessive model in the present study.

Polymorphisms of the gene encoding IL-10, an anti-inflammatory cytokine like IL-4, failed to show any association with risk of SSNHL. VEGF is involved in the permeability of blood vessels via inflammation. VEGF receptors have been observed in the mammalian cochlea (Michel et al., 2001; Picciotti et al., 2004, 2005). However, in the present study, no associations were found between the presence of *VEGF* C936T, *VEGF* C − 2578A, or *VEGF* G − 1154A and the risk of SSNHL.

The role of proinflammatory cytokine expression has been studied in patients with various ear disorders, including SSNHL (Quaranta et al., 2008; Suslu et al., 2009). During in vitro experiments, 24 h of hypoxia decreased the levels of mRNAs encoding IL-1β and IL-6 and increased the levels of mRNA encoding TNFα in the stria vascularis and spiral ligament compared with normoxia. The concentration of IL-6 measured in the explant tissue culture supernatants was significantly lower in hypoxic than normoxic cultures. Hypoxia can influence the expression and secretion of proinflammatory cytokines (Khan et al., 2010). Whether the activation of cytokines is the consequence of the innate immune response of the inner ear or the effect of hypoxia resulting from impaired cochlear vascular perfusion and permeability is still to be elucidated.

In clinical practice, we recommended that in dealing with subjects with the G-allele of IL-6 rs1800796, investigators should pay especial attention to comorbid diseases, including diabetes and hypertension, as well as environmental factors, such as alcohol abuse, sleep disorders and fatigue, to minimize the risk of SSNHL. Although the size of the control group in this study was adequate statistically, only 72 patients with SSNHL were analyzed. Therefore, these results need to be confirmed in larger series of patients.

In conclusion, the prevalence of the G-allele in inflammatory mediator *IL-6* rs1800796 was significantly higher among patients with SSNHL than among controls. Because the permeability of blood vessels in the inner ear is frequently increased in patients with SSNHL, inflammation of the inner ear might be involved in this order.

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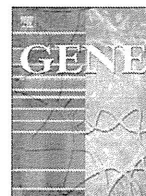

Short Communication

Contribution of complement factor H Y402H polymorphism to sudden sensorineural hearing loss risk and possible interaction with diabetes

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ABSTRACT

Sudden sensorineural hearing loss (SSNHL) is one of the most common diseases encountered by otolaryngologists; however, the etiology is unclear. The aim of this study was to assess the association between SSNHL and polymorphism of complement factor H (*CFH*) *Y402H*, which is implicated in age-related macular degeneration. We conducted a case–control study, in which the cases were 72 SSNHL patients and the controls were 2161 residents selected randomly from the resident register. The odds ratio (OR) for SSNHL risk was determined using the additive-genetic model of *CFH Y402H* polymorphism. The OR for SSNHL risk was 1.788 (95% confidence interval [CI]: 1.008–3.172) with no adjustments and 1.820 (CI: 1.025–3.232) after adjusting for age and sex. Of the three lifestyle-related diseases hypertension, dyslipidemia, and diabetes, only diabetes was significantly associated with SSNHL risk. We classified both the controls and SSNHL patients into those with or without diabetes, and the OR for SSNHL risk was 6.326 (CI: 1.885–21.225) in diabetic subjects and 1.214 (CI: 0.581–2.538) in nondiabetic subjects. We conclude that *CFH Y402H* polymorphism and SSNHL risk are significantly related, and that diabetic *CFH Y402H* minor allele carriers may be susceptible to SSNHL.
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1. Introduction

Sudden sensorineural hearing loss (SSNHL) is one of the most common diseases encountered by otolaryngologists. SSNHL involves acute or sudden onset of sensorineural hearing loss in the presence or absence of vertigo without any known causes. SSNHL has an estimated incidence between 5 and 20 per 100,000 persons per year in Western countries and 27.5 per 100,000 persons per year in 2001 in Japan. However, the actual incidence might be higher, because many who recover quickly never seek medical attention (Rauch, 2008; Teranishi et al., 2007). Although the etiology of SSNHL is unclear, the hypothesized pathology includes viral infection, vascular compromise, disruption of cochlear membranes, inner ear anomalies, and immunological diseases. SSNHL is considered to be a multifactorial disease, possibly caused by interactions between genetic factors and environmental factors.

Several recent studies have described an association between polymorphisms in the genes of some patients with SSNHL. These include polymorphisms of *methylenetetrahydrofolate reductase C677T*, *prothrombin G20210A*, and *factor V Leiden G1691A*. In SSNHL, a significant association has been reported between polymorphisms related to blood vessels and the circulation (Görür et al., 2005; Gross et al., 2006; Patscheke et al., 2001).

Complement factor H (CFH) is a glycoprotein that plays a critical role in the regulation of the complement system in plasma and in the protection of host cells and tissues from damage by complement activation. *CFH Y402H* (rs1061170) gene polymorphism is associated with coronary heart disease and age-related macular degeneration (AMD) (Edwards et al., 2005; Haines et al., 2005; Klein et al., 2005). *CFH Y402H* is located in exon 9 of the *CFH* gene on chromosome 1,

Abbreviations: aHUS, atypical hemolytic uremic syndrome; AMD, age-related macular degeneration; CFH, complement factor H; CI, confidence interval; CRP, C-reactive protein; MPGN2, membranoproliferative glomerulonephritis type II; NILS-LSA, National Institute for Longevity Sciences, Longitudinal Study of Aging; OR, odds ratio; PCR, polymerase chain reaction; SAS, Statistical Analysis System; SSNHL, sudden sensorineural hearing loss.

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region q32, and T1277C results in a substitution of histidine for tyrosine at codon 402 of the CFH protein.

Numerous recent reports have shown an association between *CFH Y402H* polymorphism and AMD, and the number of these reports is increasing each year. These genetic studies have contributed much to elucidating the pathogenesis of AMD. Bozkurt et al. reported recently that age-related hearing loss is more common in patients with AMD who are aged 50 years or older. AMD and age-related hearing loss share common underlying chronological and biological aging and/or disease processes, possibly because of common genetic, environmental, or lifestyle factors (e.g., exposure to oxidative stress, atherosclerosis, high cholesterol levels, or a history of heavy drinking, smoking, or occupational exposure to noise) (Bozkurt et al., 2011). It was also reported recently that gadolinium contrast agents injected intravenously appeared in the affected inner ear on magnetic resonance imaging (Yoshida et al., 2008), suggesting that disruption of the blood–labyrinth barrier or increased permeability of blood vessels in the inner ear, which is associated with the inflammatory response, is found in one-third of patients with SSNHL. Local inflammation and neovascularization in the retina are the proposed underlying pathology of AMD. Given the similarities of visual and hearing impairments, we conducted a case–control study to determine whether there is a relationship between *CFH Y402H* polymorphism and SSNHL.

2. Materials and methods

2.1. Cases

SSNHL patients (36 men and 36 women, mean age 58.3 (range: 22–82)) who visited the Department of Otorhinolaryngology of Nagoya University Hospital between November 2007 and January 2010 were enrolled. SSNHL was defined according to criteria established by the Sudden Deafness Research Committee at the Ministry of Health and Welfare, Japan. The detailed diagnostic criteria were described previously (Teranishi et al., 2007).

2.2. Controls

The control group subjects were identified from the comprehensive Longitudinal Study of Aging (NILS-LSA), an ongoing population-based study with a two-year follow up conducted by the National Institute for Longevity Sciences. Participants in the NILS-LSA were selected randomly from resident registrations and were stratified by age and sex in cooperation with the local government. The study region is located within 30 km of Nagoya University Hospital. The lifestyle of residents in this area is typical of individuals in most regions of Japan. The population in the study region is thought to share the same ethnic ancestry and to possess a homogeneous genetic background. Participants completed a series of questionnaires designed to obtain demographic characteristics and information on the presence of various medical problems. In the present analysis, 33 participants who reported a history of SSNHL in the questionnaires were excluded. The details of the NILS-LSA have been described elsewhere (Shimokata et al., 2000). Overall, 2161 participants (1089 men and 1072 women, mean age 59.2 [range: 40–79]) who completed the first-wave examination of NILS-LSA between November 1997 and April 2000 and who underwent *CFH* genotype analysis were selected as controls.

2.3. Genetic analysis

Genomic DNA was extracted from peripheral blood lymphocytes using the standard procedure. The genotype was determined by the intercalator-mediated fluorescence resonance energy-transfer probe method (Toyobo Gene Analysis, Tsuruga, Japan). The sense primer GAAAATGTTATTTTCCTTATTTGGAAAATG and antisense primer CAGGCAACGTCTATAGATTTACCCTGTAC were used. The reaction was performed in a 25 μL mixture containing 20 ng of genomic DNA, 5 pmol of each primer, 0.2 mmol/L deoxynucleoside triphosphates, 2.5 mmol/L MgCl2, and 1 U of rTaq DNA polymerase. The amplification protocol included an initial denaturation at 95 °C for 3 min; 45 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and primer extension at 72 °C for 30 s, plus a final extension at 72 °C for 2 min. The PCR products were then mixed with Texas Red-labeled probe (CTTTCTTCCATGATTTTGATTATATC) and SYBR Green I (Invitrogen, Carlsbad, CA, USA) and transferred to an ABI Prism 7900 instrument for measurement of the melting temperature (Hirota et al., 2007).

2.4. Statistical analyses

The data were analyzed using Statistical Analysis System (SAS) version 9.1.3 (SAS Institute, Cary, NC, USA). For univariate analysis, a *t* test was used to assess differences in continuous variables between two groups. Categorical variables were compared using the chi-square test or Fisher's exact test. All values are expressed as mean ± standard deviation unless otherwise specified. A p value < 0.05 was defined as significant. Age included in the analysis was defined as the age of onset of SSNHL. One patient's exact age of onset was missing because he visited our hospital long after the onset of SSNHL. For multivariate analysis, multiple logistic regression was performed to obtain the odds ratios (ORs) for SSNHL in subjects with *CFH Y402H* polymorphism.

Genotypes were coded as follows: wild-type homozygotes, TT; heterozygotes, TC; and mutant homozygotes, CC. The additive genetic model, which is the prevailing analytical model in genetic epidemiology, assumes that risk increases linearly with an increasing number of mutated alleles. The OR for SSNHL risk was determined using the additive genetic model for *CFH Y402H* polymorphism. We used two models, with and without adjustment for confounding variables as follows: no variables were adjusted in model 1, and age and sex were adjusted in model 2. We performed an interim analysis using the information about history of hypertension, diabetes, and dyslipidemia, and of these three lifestyle-related diseases, diabetes tended to contribute to SSNHL (data not shown). We applied an additional multiple logistic regression program to assess the respective effects of *CFH Y402H* polymorphism and a history of diabetes, and the interaction between the two. A GENMOD procedure Type 3 analysis was performed to compute the likelihood ratio statistics for Type III contrasts for each term in the model. In this model, *CFH Y402H* polymorphism, diabetes, and the interaction between *CFH Y402H* polymorphism and diabetes were set as independent variables after adjusting for age and sex. The ORs of the minor C allele were then calculated separately in diabetic and nondiabetic subjects to determine whether the relationship between *CFH* polymorphism and SSNHL risk differed according to the presence or absence of diabetes.

2.5. Ethics

The study protocol was reviewed and approved by the ethics committees of Nagoya University and the National Center for Geriatrics and Gerontology, and written informed consent was obtained from all subjects.

3. Results

Table 1 shows the profiles of the SSNHL cases and control group. The SSNHL patients and controls did not differ significantly in sex or age. The characteristics of the participants did not differ significantly between cases and controls except for the prevalence of diabetes. The prevalence of diabetes was significantly higher in the cases than in controls (p = 0.0098, chi-square test). Collateral analysis showed no significant association between *CFH Y402H* genotype and the

Table 1
Characteristics of cases and control groups.

	SSNHL group	Control group	*p*
	N = 72	N = 2161	
Age (year)	58.3 ± 14.0	59.2 ± 10.9	0.487
Sex (% male)	50.0	50.4	0.948
Hypertension (%)	33.3	25.4	0.129
Diabetes (%)	16.7	8.1	0.010
Dyslipidemia (%)	13.9	16.9	0.503

P value tested by *t* test or chi-square test.
SSNHL = sudden sensorineural hearing loss.

prevalence of diabetes, regardless of whether the cases were included or excluded from the analysis (data not shown).

The genotype distribution of the polymorphism is described in Table 2. The differences between SSNHL cases and controls in *CFH Y402H* genotype frequencies were marginal (p = 0.0517, Fisher's exact test). These results in both cases and controls were not significantly different from those expected based on the Hardy–Weinberg equilibrium (P > 0.05, chi-square test).

Table 3 shows the ORs of SSNHL according to *CFH Y402H* polymorphism. The OR for SSNHL risk was 1.788 (95% confidence interval [CI], 1.008–3.172) in model 1 and 1.820 (CI: 1.025–3.232) in model 2. The likelihood ratio statistics for each term for *CFH* polymorphism, diabetes, and their interaction are presented in Table 4. The likelihood ratios for *CFH* polymorphism and the interaction between *CFH* polymorphism and diabetes were significant (p = 0.01 and p = 0.035).

The ORs of the minor C allele according to the presence or absence of diabetes are shown in Table 5. *CFH* polymorphism was significantly associated with SSNHL risk in diabetic subjects but not in nondiabetic subjects. The OR for SSNHL risk was 6.326 (CI: 1.885–21.225) in diabetic subjects and 1.214 (CI: 0.581–2.538) in nondiabetic subjects.

4. Discussion

In the present study, we found a significant association between *CFH Y402H* polymorphism and SSNHL risk. The association between the *CFH* minor C allele and SSNHL varied according to diabetic status.

The strength of the present study is its inclusion of a defined control group from within the NILS-LSA cohort of inhabitants who were selected randomly from a register and stratified by both age and sex. The risk of genotypic bias in controls in a population-based study is likely to be less than in controls in a hospital-based study.

Table 2
Genotype distribution and allele frequency of *CFH Y402H* polymorphism.

		Total	SSNHL group	Control group	*p*
		N (%)	N (%)	N (%)	
Genotype	TT	1977 (88.5)	59 (81.9)	1918 (88.8)	0.052
	TC	250 (11.2)	12 (16.7)	238 (11.0)	
	CC	6 (0.3)	1 (1.4)	5 (0.2)	
		2233 (100)	72 (100)	2161 (100)	

P value tested by Fisher's exact test.

Table 3
Odds ratios of SSNHL according to *CFH Y402H* polymorphism.

N	TT (C = 0)	TC (C = 1)	CC (C = 2)	*p*
	1977	250	6	
Model 1	1	1.788 (1.008–3.172)/C-allele		0.047
Model 2	1	1.820 (1.025–3.232)/C-allele		0.041

TT = wild-type homozygotes; TC = heterozygotes; CC = mutant homozygotes.
Ranges in parenthesis indicate the 95% confidence interval.
Moderating variables.
Model 1: no adjustment.
Model 2: age and sex.

Table 4
Likelihood ratio statistics for each term among CFH polymorphism, diabetes, and the interaction.

	Degree of freedom	Chi-square value	*p*
CFH	1	6.59	0.010
Diabetes	1	1.07	0.300
*CFH**diabetes	1	4.46	0.035

Moderating variables: age and sex.
CFH*diabetes indicates the interaction between CFH polymorphism and diabetes.

No previous investigations have reported an involvement of CFH in susceptibility to SSNHL. A systematic review of the evidence for the etiology of SSNHL stated that the causes of SSNHL are speculative and probably multifactorial. Although various etiological theories have been proposed, in many patients no obvious cause is found, and SSNHL is often described as idiopathic (Chau et al., 2010). Potential etiologies include viral infection, vascular impairment, disruption of cochlear membranes, inner ear anomalies, and immunological diseases. Recent evidence on SSNHL etiology based on image diagnostic technology has been reported. Patients with SSNHL exhibit high precontrast signals in the inner ear and/or gadolinium enhancement in the affected inner ear on three-dimensional fluid-attenuated inversion recovery. The high signals in the affected inner ear may reflect minor hemorrhage or an increased concentration of protein in the inner ear that has passed through blood vessels with increased permeability (Yoshida et al., 2008). These contributing factors to epithelial permeability might be reflected in the high signals observed in the inner ear of patients with SSNHL.

AMD is one of the most common irreversible causes of severe loss of vision, including legal blindness, in the elderly population. Although its pathogenesis remains unclear, recent evidence suggests that local inflammation and neovascularization contribute to the pathogenesis of AMD. The major cause of visual loss in AMD is choroidal neovascularization, as, in this phenomenon, the vessels are weak, curled, and leaky. Several recent studies showed that *CFH Y402H* gene polymorphism is associated with AMD. Because CFH prevents uncontrolled complement activation and inflammation, its mutation is thought to increase inflammation and its consequences (Nowak, 2006).

The complement system protects against infection and attacks diseased and dysplastic cells by recognizing pathogens and unwanted host material. At the same time, healthy host cells must be protected from complement-mediated damage. Complement regulators are necessary to prevent the injudicious production of mediators derived from complement attack and potential injury to self tissue (Liszewski et al., 1996). CFH is a key regulator of the complement system of innate immunity (Wu et al., 2009). Inherited mutations in *CFH* can account for membranoproliferative glomerulonephritis type II (MPGN2), atypical hemolytic uremic syndrome (aHUS), and AMD. MPGN2 can be associated with excessive systemic complement activation because of dysfunctional CFH, and aHUS and AMD are associated with mutations affecting

Table 5
Odds ratios of SSNHL according to *CFH Y402H* polymorphism with or without diabetes.

	N		Odds ratio			*p*
	SSNHL group	Control group	TT(C = 0)	TC(C = 1)	CC(C = 2)	
Diabetes (+)	12	175	1	6.326 (1.885–21.225)/C-allele		0.003
(−)	59	1986	1	1.214 (0.581–2.538)/C-allele		0.606

Moderating variables: age and sex.

the ability of CFH to bind to anionic surfaces such as on endothelial cells and glomerular and retinal capillary walls (Alexander and Quigg, 2007).

The association between *CFH Y402H* polymorphism and diabetes and its effect on susceptibility to SSNHL may be explained by one or two of the etiologies mentioned above: vascular impairment or infection. *CFH Y402H* gene polymorphism has been reported to be associated with coronary heart disease. Some studies indicate that the C allele of the *CFH Y402H* polymorphism increases the risk of coronary heart disease, myocardial infarction, ischemic stroke, and cardiovascular mortality in the elderly population (Kardys et al., 2006; Mooijaart et al., 2007; Volcik et al., 2008). Inflammation within the vascular wall plays an important role in the pathogenesis of atherosclerosis (Oksjoki et al., 2003). It has been suggested that the complement system may also be involved in the pathogenesis of atherosclerosis (Lewis et al., 2010; Niculescu and Rus, 2004). Genetic research indicates that gene polymorphisms associated with SSNHL are involved in atherosclerosis, inflammation, and blood vessel permeability. The *CFH Y402H* polymorphism may cause excessive activation of the complement system, which causes inflammation in the inner ear. Although this idea is speculative, we consider that by affecting blood vessel permeability, chronic inflammation may be a cause of SSNHL.

Microvascular involvement (e.g., neuropathy, nephropathy, and retinopathy) and macrovascular involvement (e.g., accelerated atherosclerosis, heart attack, and stroke) are major chronic complications of diabetes. There are some reports on the relationship between type 2 diabetes and SSNHL. Rust et al. found a relationship between type 2 diabetes and inner ear damage(Rust et al., 1992). Aimoni et al. indicated that diabetes prevalence was higher in an SSNHL group (15.6%) compared with controls (8.5%) ($p = 0.03$). They concluded that the role of diabetes in the impairment of cochlear perfusion is biologically conceivable because diabetes is a well-established risk factor for microangiopathy (Aimoni et al., 2010). Weng et al. reported that the poor prognosis of sudden deafness in diabetic patients may be caused by preexisting microvascular lesions in the inner ear and that the postprandial plasma glucose level could be a risk factor indicator for cochlear dysfunction in diabetic patients (Weng et al., 2005).

On the other hand, several studies have reported the presence of a viral infection in patients with SSNHL, although the specific and direct pathogenic role of viral infections in inner ear structural damage has not been demonstrated (Pyykko and Zou, 2008; Yoshida et al., 1996). The histopathological findings in temporal bones obtained from individuals with SSNHL are similar to changes observed in individuals who lost hearing while suffering from mumps, measles, rubella, or herpes zoster (presumed viral labyrinthitis) (Schuknecht and Donovan, 1986); however, there is no proof of actual viral invasion of the ear. One could argue that deafness was not caused by true viral labyrinthitis but rather by an immunological or stress-induced response in the inner ear (Merchant et al., 2008).

Although *CFH* polymorphism has been investigated recently, the molecular mechanism responsible for this polymorphism remains unclear. C-reactive protein (CRP) is an acute-phase protein that activates complement by the classical pathway. CRP binds inhibitory complement regulators such as CFH, and the CFH–CRP complexes inhibit complement activation at the damaged host cell surfaces. The *CFH Y402H* polymorphism has been reported to alter its CRP-binding properties, leading to a markedly reduced affinity of the 402His variant for CRP (Laine et al., 2007). Because the binding of the 402His variant to CRP is much weaker than that of the Tyr402 variant, the host cells of *CFH Y402H* minor-allele carriers will be less protected by CRP and more prone to damage (Okemefuna et al., 2010).

Diabetic people are more susceptible to infections than nondiabetic individuals are (Bartelink et al., 1998; Golden et al., 1999). This increased susceptibility to infection has been attributed to defects in both cell-mediated immunity and humoral immunity (Geerlings and Hoepelman, 1999). Taken together, our results showing the association of both *CFH Y402H* polymorphism and diabetes with susceptibility to SSNHL can be explained theoretically.

There are a few study limitations. The literature on AMD shows various efforts to find biomarkers to detect AMD and to interpret the implication of *CFH* genetic variation on the disease pathogenesis. Markers of complement activation and regulation; the CRP plasma level, including high-sensitivity CRP; and a general marker of systemic inflammation have been studied in relation to AMD, although there is still controversy (Klein et al., 2008; Robman et al., 2010). The present study lacks analysis of potential markers immediately after the onset of SSNHL. Because SSNHL is not a chronic progressive condition, a prospective research design is an issue for future studies. We did not include the severity and clinical history of diabetes in our analyses, and the prevalence of diabetes was based on self-report. Among the cases, all diabetic patients had type 2 diabetes, but the type of diabetes was unclear in the controls because their data were obtained in a series of questionnaires. We plan further study of the relationships between the severity of diabetes, severity of or prognosis for SSNHL, and *CFH Y402H* polymorphism. The present case–control approach indicated an association between SSNHL and *CFH Y402H* polymorphism through an interaction with diabetes. In our study, the number of cases of SSNHL and the number of cases with the minor C allele were small. Further investigations with different cohorts are needed to replicate our results and to validate the hypothesis generated from our analyses.

5. Conclusion

We found a significant association between *CFH Y402H* polymorphism and SSNHL risk, and that the relationship between the *CFH* minor C allele and SSNHL varied according to diabetic status. Diabetic *CFH Y402H* minor allele carriers may be susceptible to SSNHL.

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