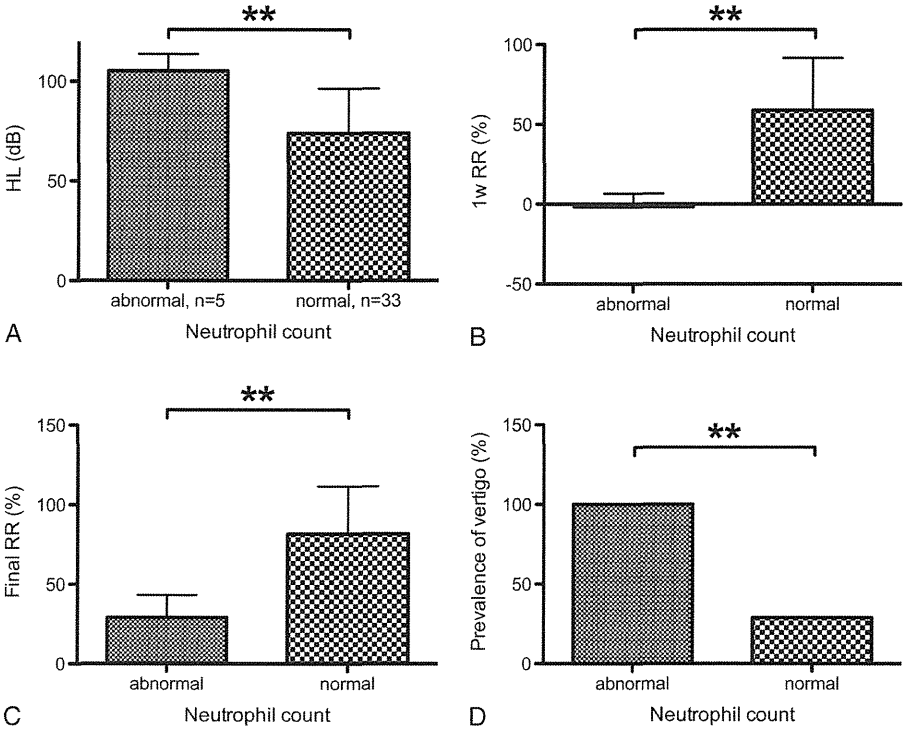

FIG. 2. Evaluation of whether a neutrophil reference range of own facility works as meaningful thresholds for ISHL patients. The patients were classified into 2 groups, above the reference range (abnormal) or within the range (normal). No patient was below the range. Patients with abnormal neutrophil counts showed significantly worse HL (*A*), 1w RR (*B*), the final RR (*C*), and higher prevalence of vertigo (*D*) than patients with normal counts. $^{**}p < 0.01$ with Mann-Whitney *U* test for (*A–C*) and chi-square test for (*D*).

We also analyzed the counts of 19 patients at the following time points: at the first visit without steroid treatment (Fig. 1D, 1st) and 1w or more after completion of treatment (2nd). The counts of patients in 1Q and 4Q showed significant change during the time course ($p < 0.05$). The average counts of 4Q changed from above the reference range into the normal range.

When the patients were classified into 2 groups, either above the reference range of the neutrophil count (Fig. 2, abnormal) or within the range (normal), patients with abnormal counts showed worse HL (A), 1w RR (B), and final RR (C) than others ($p < 0.01$ for each; Fig. 2). All the ISHL patients with abnormal counts had vertigo, whereas it was present in 29% of others (Fig. 2D, $p < 0.01$). On the other hand, all of the patients with Ménière's disease had vertigo, although their counts were normal (3730/μl ± 963).

The patients with abnormal neutrophil counts showed significantly lower NKCA (20%) than others (33%) ($p < 0.05$; Fig. 3).

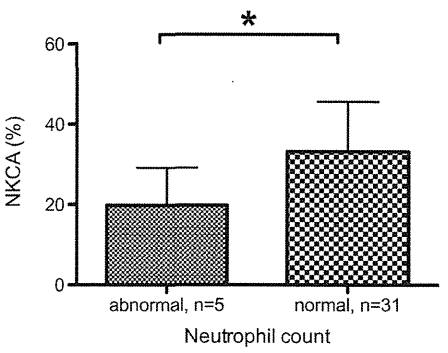

FIG. 3. The patients with abnormal neutrophil counts at the first visit showed significantly lower NKCA than patients with normal neutrophil counts. $^{*}p < 0.05$ with Mann-Whitney *U* test.

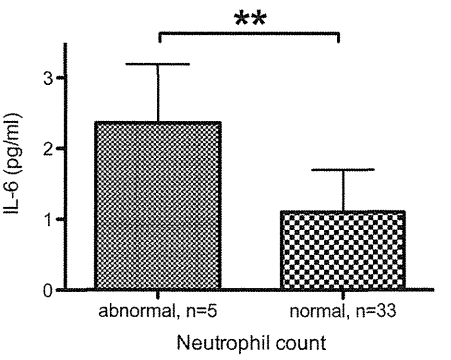

FIG. 4. Patients with abnormal neutrophil counts showed significantly higher IL-6 than patients with normal neutrophil counts at the first visit. $^{**}p < 0.01$ with Mann-Whitney *U* test.

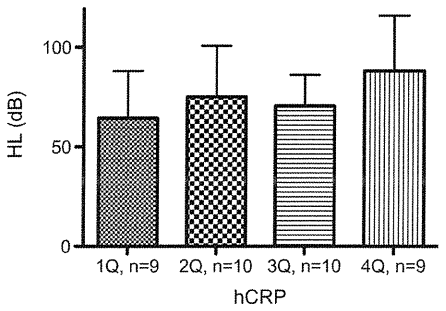

FIG. 5. There was no significant difference of HL between quartiles of hCRP with ANOVA followed by Tukey's test.

The average of the GHQ scores was above the normal limit (Table 2). However, it did not show a significant correlation with severity, prognosis of ISHL, and biomarkers.

The patients with abnormal neutrophil counts showed significantly higher IL-6 (2.36 ± 0.83 pg/ml) at the first visit than in patients with normal counts (1.1 ± 0.60 pg/ml) ($p < 0.005$; Fig. 4).

TNF did not show a significant association with the severity and prognosis of ISHL and other biomarkers.

The standard deviation of hCRP was very high (Table 2). It showed a correlation with HL at the first visit (Table 3). However, hCRP did not show statistical significance with HL when the patients were classified into 4 groups according to the hCRP quartiles (Fig. 5).

DISCUSSION

Correlation analysis suggests that neutrophil counts might be a clinically meaningful indicator for predicting severity and prognosis, and might be involved in the pathogenesis of ISHL. Consistent with this speculation, the neutrophil count quartiles showed significant association with those variables, and the upper most quartile decreased from above the reference range to within it during the time course. Furthermore, we demonstrated that patients with lower NKCA, abnormally high neutrophil count, and higher IL-6 showed severe hearing loss and poor prognosis as compared with those with normal neutrophil counts. These correlations were lost in the non-ISHL patients. These results suggest that these biomarkers may be specifically involved in the pathogenesis of ISHL.

Sugiura et al. (31) demonstrated that an ISHL-affected ear has a high concentration of proteins in the inner ear fluid space using fluid-attenuated inversion recovery MRI. However, the signal is scant and does not diffuse into the fluid space outside the inner ear. It seems that the inflammatory biomarkers from the inner ear are too sparse to affect the inflammatory response of the whole body or to change the circulating biomarkers. It is more likely that systemic biomarkers changed before the ISHL onset rather than the after it. In addition, although the patients in the fourth neutrophil quartile had significantly worse recovery and had been experiencing hearing loss, the counts decreased during the time course of measurement. Therefore, it seems unlikely that the count of 4Q at the first visit would be the result of the psychological stress of hearing loss. However, we could not completely exclude the possibility that biomarkers changed after the onset of ISHL.

Decreased NKCA and Elevated Neutrophils May Reflect the Systemic Stress of Severe ISHL Patients

The natural killer cell has critical roles in resistance against both viral and bacterial infections by enhancing the response of the neutrophil, shaping the immune response after infection, and regulating immune responses to autoantigens (32–36). However, NKCA is reduced by fatigue, stressful life events, inability to cope with stress, and short sleep duration (37–41). Notably, all have been suggested to be causes of ISHL (42–44). Kanzaki (45) suggested the association of depletion of NKCA followed by systemic stress with the onset of ISHL. The decrease of NKCA observed in the present study might also be induced by systemic stress in ISHL patients, although GHQ scores did not have a significant correlation with biomarkers. The decreased NKCA could result in systemic subclinical infection or dysregulation of the immune system and, thus, cause increased neutrophils. Mattox and Simmons (46) also suggested that subclinical infectious disease is associated with the onset and prognosis of ISHL based on the elevated erythrocyte sedimentation rate in ISHL patients. Considering these findings, the decreased NKCA and the abnormally high neutrophil count might result from psychological and physical stress in ISHL patients.

Elevated Neutrophils and IL-6 Induced by Systemic Stress Can Activate NF-κB in the Cochlear Lateral Wall

IL-6 was higher in patients with abnormal neutrophil counts than in patients with normal counts. It is reported that neutrophil counts and IL-6 are involved in subclinical disease, injury, and repair of the nervous system, heart, and other organs as well as cochlear injury (8,21,47–50). IL-6 induces neutrophilia, and neutrophil-derived IL-6 results in high IL-6 (49,51,52). This interaction between IL-6 and neutrophil could induce higher IL-6 in patients with abnormal neutrophil counts than in patients with normal counts.

Elevated IL-6 may induce the abnormal NF-κB activation in the cochlear lateral wall through a positive feedback loop of the NF-κB stress response system because IL-6 is not only a transcriptional target of NF-κB but also an activator of NF-κB (10,12). Local and circulating IL-6 can influence cochlear injuries via classic and trans-signaling, respectively. In trans-signaling, a complex of circulating IL-6 bound to the IL-6 receptor, which occurs naturally or by cleavage from apoptotic neutrophil, can control inflammatory response through binding with gp130, which are displayed by all cells (53).

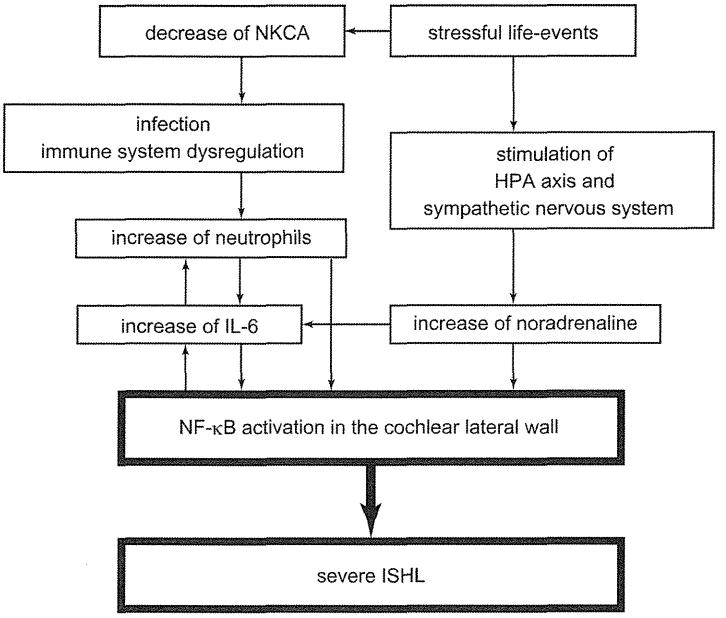

FIG. 6. The systemic stress response theory of severe ISHL based on the present study and past literatures. Different kinds of stressful life events are suggested to be a cause of ISHL, and they decrease NKCA. It induces subclinical infection and/or immune system dysregulation, and results in an acute increase of neutrophils and IL-6. Increased neutrophils and IL-6 activate NF-κB in the lateral wall through ischemic stress and IL-6 trans-signaling, respectively, forming a positive feedback loop. Systemic stress also induces and enhances noradrenaline-dependent NF-κB activation and IL-6 production. Synchronism of different kinds of NF-κB activation pathways would result in the onset of severe ISHL. HPA indicates hypothalamic-pituitary-adrenal axis.

We previously observed gp130 expression in the lateral wall as well as NF-κB activation, thus supporting lateral wall NF-κB activation via trans-signaling (7,9).

Haubner et al. (54) demonstrated that IL-6 concentration was not significantly different between 48 ISHL patients of various levels of HL and 35 control subjects, although no distinction were made between mild and severe ISHL. In the report, the IL-6 concentration not only from severe ISHL patients but also milder patients might counterbalance the increase of IL-6 in severe ISHL patients. Our findings suggest that the elevated IL-6 may be involved in the onset of severe ISHL but not in milder ISHL pathophysiology because it is accompanied by abnormal neutrophil counts in severe patients.

Elevated Neutrophils May Directly Induce the Stress Response in the Cochlea

An increase of neutrophil counts might activate the stress response in the cochlear lateral wall independent of IL-6 production. The acute or subacute change of the neutrophil counts must be associated with ISHL onset because it was not associated with chronic vascular diseases. Neutrophils have a thrombogenic profile (21) and are correlated with the risk and prognosis of myocardial infarction and stroke (17,18,21). Considering that the ISHL onset mode is like that of infarction of the heart and brain, the thrombogenic profile of the neutrophil may be involved, at least, in the onset of severe ISHL.

The Systemic Stress Response Theory of Severe ISHL

We suggest that an NKCA decrease, acute neutrophil increase, and an IL-6 increase may represent systemic stress in ISHL patients, and these biomarkers can be involved in NF-κB activation in the cochlear lateral wall. Kanzaki et al. (55) also hypothesized that cochlear lateral wall NF-κB activation followed by cytokine production might be a cause of hearing deterioration during maintenance therapy of steroid-dependent sudden sensorineural hearing loss. Based on the present findings, we advocate the stress response theory of severe ISHL as shown in Figure 6.

Merchant et al. (1) and Adams (6) demonstrated that systemic stress by intraperitoneal injection of LPS caused lateral wall NF-κB activation unilaterally but not bilaterally, and local (i.e., intratympanic) administration of LPS did not activate NF-κB in the lateral wall, in contrast to systemic administration. Physical and psychological stress induce and enhance the inflammatory response including NF-κB activation and IL-6 production (40,56–59). Furthermore, systemic stress activates the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system, and induces noradrenalin-dependent NF-κB activation and IL-6 production (58,60). This activation pathway can occur in the cochlear lateral wall and result in ISHL because there are several kinds of adrenergic receptors in the lateral wall (61–64). Therefore, systemic stress must enhance the lateral wall stress response more than local stress alone.

It remains unknown why systemic stress induces asymmetric hearing loss. There are presumably some predisposing factor(s) in the inner ear. For example, asymmetry of preexisting subclinical minor damage in the inner ear or asymmetry of terminal vascular structure (e.g., stenotic or not stenotic, straight or tortuous) may be the potential explanation for the asymmetric inner ear response against stimulation that activates NF-κB.

Clinical Relevance of Normal Neutrophil Counts to ISHL

Patients in the third neutrophil quartile showed statistically worse HL and prognosis than those in the first quartile, although their neutrophil counts were within the reference range and did not change significantly over the time course. Von Vietinghoff and Ley (27) reported that higher baseline counts, even within the normal range, have been shown to be an independent risk factor for cardiovascular and cancer mortality in meta-analyses and in the National Health and Nutrition Examination Survey study, respectively. Although it remains unclear what the role of the elevated numbers of circulating neutrophils is, patients with increased neutrophil counts may have a higher incidence of more severe ISHL. A large-scale survey should be performed to evaluate this relationship.

TNF and hCRP Does Not Reflect Inner Ear Injury From ISHL

Circulating TNF was not associated with affected HL and prognosis. Um et al. (65) reported the association of TNF polymorphisms with the ISHL pathogenesis. Van Wijk et al. (66) showed that intratympanic TNF inhibitor administration could improve autoimmune inner ear disease. On the other hand, Suslu et al. (67) do not recommend the use of TNF inhibitors in ISHL because of the lower TNF titers documented in 30 ISHL patients compared with 30 healthy patients, which had no response to steroid treatment. In the present study, we also could not support this treatment.

The standard deviation of hCRP was very high, and the quartiles did not show a significant association with HL. Therefore, it does not seem to be a useful biomarker for ISHL patients.

CONCLUSION

Neutrophil counts above the reference range of a facility will be a useful indicator for poor prognosis of ISHL. A systemic stress response starting from an NKCA decrease may be the pathogenesis of severe ISHL. Synchronism of different types of NF-κB activation pathways could be required to cause severe ISHL. Different kinds of pathogenesis may be involved in ISHL, and there are various degrees of severity in ISHL patients. At the present time, it is impossible to directly evaluate cellular responses in the human inner ear. However, further studies on evaluating systemic stress and inflammatory biomarkers in ISHL patients will help to prevent disease and develop new treatments for ISHL patients. In particular, an epidemiologic survey would be important for people who have experienced stressful life events.

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Cochlin-tomoprotein (CTP) detection test identified perilymph leakage preoperatively in revision stapes surgery

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ABSTRACT

Perilymphatic fistula (PLF) is defined as an abnormal leakage between perilymph from the labyrinth to the middle ear. Symptoms include hearing loss, tinnitus, and vertigo. The standard mode of PLF detection is intraoperative visualization of perilymph leakage and fistula, which ostensibly confirms the existence of PLF. Other possible methods of diagnosis include confirmation of pneumolabyrinth via diagnostic imaging. Recently, a cochlin-tomoprotein (CTP) detection test has been developed that allows definitive diagnosis of PLF-related hearing loss.

We report the case of a 45-year-old man who presented with right-sided tinnitus, hearing loss, and dizziness 30 years after stapes surgery. Middle ear lavage was performed after myringotomy. A preoperative diagnosis of PLF was reached using the CTP detection test. Intraoperative observations included a necrotic long process of the incus, displaced wire piston, and fibrous tissue in the oval window. Perilymph leakage was not evident. The oval window was closed with fascia, and vertigo disappeared within 2 weeks postoperatively. When PLF is suspected after stapes surgery, the CTP detection test can be a useful, highly sensitive, and less invasive method for preoperative diagnosis.

1. Introduction

Perilymphatic fistula (PLF) is defined as abnormal leakage between perilymph from the labyrinth to the middle ear. PLF diagnosis has been made with pneumolabyrinth in the inner ear on computed tomography (CT) and T2-weighted magnetic resonance imaging (MRI) [1]. Leakage has been confirmed during open and endoscopic surgery [2,3]. However, PLF diagnosis is clinically difficult because CT, MRI, and perioperative methods are not always able to detect the leakage.

In 2001, cochlin-tomoprotein (CTP), a novel perilymph-specific protein, was identified [4]. CTP is a protein product of *COCH*, which was originally identified from the cochlea-specific cDNA library. Later, its mutation was found to be associated with DFNA9, an autosomal dominant hereditary deafness condition. Three cochlin isoforms were identified; CTP was one of these short 16-kDa isoforms. CTP is found in the functional domain of LCCL in cochlin and is secreted to the perilymph. CTP is highly specific for perilymph. Therefore, a diagnosis of PLF can be made by detection of CTP using Western blotting in lavage of the middle ear [5].

We report a case of right-sided tinnitus, hearing loss, and dizziness manifesting 30 years after stapes surgery. PLF was diagnosed preoperatively using the CTP test in middle ear washings. PLF was not suspected based on clinical manifestations, eardrum examination, and CT. Preoperative diagnosis was possible only because of the CTP test. CTP detection test is a new, highly sensitive, less invasive, and useful method to aid in the diagnosis of PLF.

2. Case report

The patient was a 45-year-old man. In 1980, right stapes surgery had been performed on him and a Teflon wire piston was placed (details of the surgery were uncertain). The patient presented at our hospital with right-sided tinnitus of idiopathic origin. In December 2009, he experienced mild dizziness, but no rotatory vertigo or awareness of hearing loss was evident. In an audiometric test, deterioration of hearing by bone conduction was detected as compared with hearing level recorded during a consultation conducted 20 years previously. Therefore acute mixed hearing loss was suspected.

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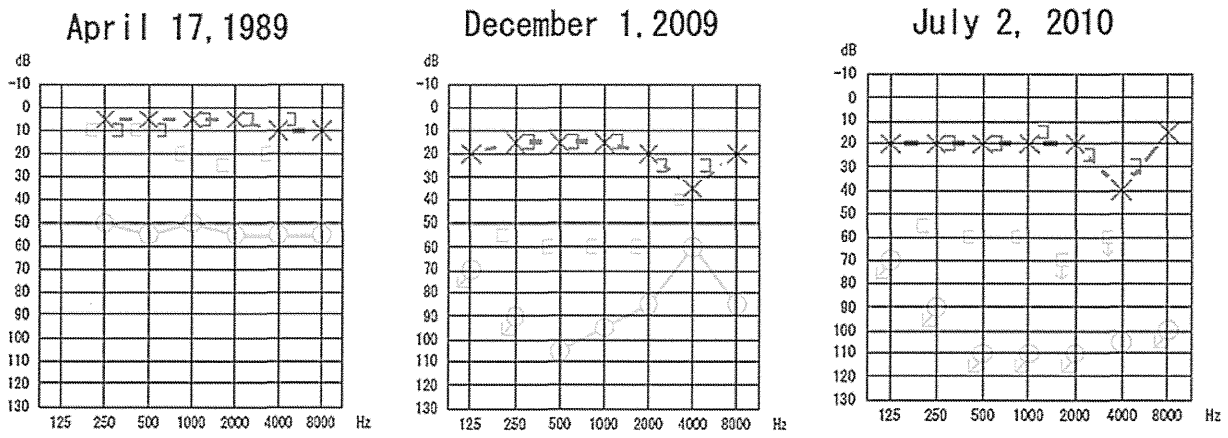

Fig. 1. Audiogram. Hearing levels in December 2009 where lower than those in 1989. In July 2010, vertigo developed, and hearing deteriorated further.

Administration of oral prednisolone (30 mg per day), ATP, and vitamin B12 was initiated. At the end of June 2010, rotatory vertigo and tinnitus appeared and hearing in the right ear deteriorated further (Fig. 1). Pure horizontal nystagmus directed to the left was observed under Frenzel glasses. On physical examination, no fluid was found in the tympanic cavity through the right tympanic membrane.

Hydrocortisone sodium succinate was administered via intravenous drip (500 mg per day) for 10 days tapering starting on July 2, 2010. Rotatory vertigo was gradually relieved, but dizziness continued. On the basis of clinical history, PLF was suspected. We obtained informed consent from him and collected middle ear washings after myringotomy under topical anesthesia and examined by the CTP detection test. The procedure of this test has been reported previously [4]. A CTP-positive signal was observed from the middle ear washings (Fig. 2), confirming the diagnosis of right PLF. After and during the test, no exacerbation of dizziness, tinnitus, or hearing loss was observed.

On November 1, 2010, surgery was performed under general anesthesia. Intraoperative observations included a necrotic long process of the incus, displaced wire piston, and fibrous tissue in the oval window. The body and short process of the incus were in the normal position. The incus and wire were transected and the wire of the piston was visible outside the oval window, but the piston was found lying deep within the vestibule. The footplate of the stapes was not found. Leakage of lymph fluid into the tympanic cavity and around the oval and round windows was not observed. Fibrous adhesions, mucosal hyperplasia, and the wire piston were removed.

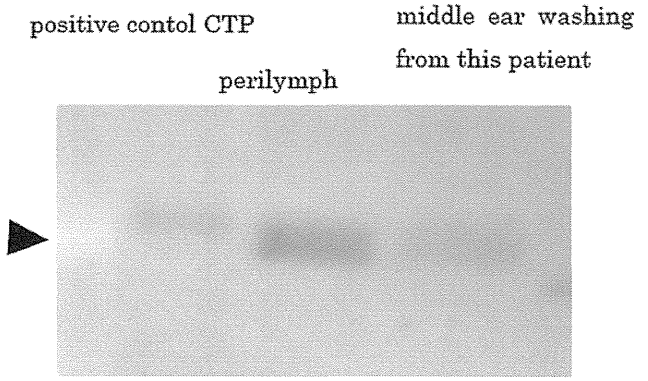

Fig. 2. Detection of cochlin-tomoprotein (CTP) in the middle ear washings. Signals represent CTP in recombinant positive control CTP (left), perilymph (middle), and middle ear washings from the patient (right) by Western blotting.

The oval and round windows were covered with the temporal fascia using fibrin glue to seal the fistula, but no prosthesis was used for the purpose of hearing improvement.

Postoperatively, mild dizziness was observed, but rotatory vertigo and nystagmus disappeared. The dizziness gradually improved and the patient was discharged 12 days after surgery.

3. Discussion

PLF causes inner ear disorders due to perilymph leakage into the tympanic cavity. PLF can be associated with a congenital anomaly, postoperative ear complications, head trauma, or barotrauma, but is most often idiopathic. PLF presents with symptoms of hearing loss, tinnitus, vestibular vertigo or dizziness, popping sounds, streaming tinnitus, and fistula signs. However, it is often indistinguishable from other inner ear diseases.

In some cases of PLF, pneumolabyrinth (air in the inner ear) and liquid leakage into the tympanic cavity can be detected by high-resolution temporal bone CT or T2-weighted MRI [1]. Although the gold standard for PLF diagnosis is intraoperative microscopic or endoscopic visualization, PLF is difficult to identify even during surgery [2,3]. Bakhos et al. [6] and Vincent et al. [7] reported that perilymphatic leakages were identified in 8% and 5.5%, respectively, of cases of revision stapes surgery. Furthermore, in their studies, PLF was suspected preoperatively in 36 cases based on clinical symptoms, but fistula was observed only in 23 cases and in 13 of them, fistula was not diagnosed due to perioperative findings [7].

Proteomic analysis of inner ear proteins identified the unique properties of CTP [4]. CTP is a protein present in perilymph, but not in other body fluids such as cerebrospinal fluid (CSF), serum, saliva, or middle ear mucosa. Therefore, CTP may be considered a specific biochemical marker for perilymph [5].

The sensitivity of the CTP test is 92.3% from middle ear lavage fluid sampled after cochlear fenestration in cochlear implant surgery [8]. While its specificity is 98.2% from middle ear lavage of non-PLF cases without middle ear infections [9]. Analysis of middle ear lavage fluid sampled from patients with middle ear infections may provide false-positive results (e.g., specificity of 93.5%) because of the high protein concentration in the thick pus [9]. In this study, CTP was detected in approximately 1 μl of perilymph present in the middle ear cavity. This method may enable diagnosis of PLF from minimal amounts of leaked perilymph, which is difficult to detect by CT and MRI or perioperatively. This method is also less invasive, as lavage can be performed by myringotomy or puncture of the tympanic membrane.

Several authors have suggested identification of an endogenous perilymph marker such as beta-2 transferrin, beta trace protein, or

intrathecal fluorescein [10–12]. Although these markers are also detectable in inner ear fluid, PLF and CSF leakage can be difficult to distinguish because they are not organ specific.

In our case, the wire piston had transferred deep into the vestibule behind the long limb of the incus necrosis. Perilymph leakage occurred, leading to rotatory vertigo and deterioration of hearing. PLF was not initially suspected because 30 years had passed since stapes surgery, and typical symptoms of PLF were not present. In addition, effusion in the tympanic cavity was not detected on examination of the tympanic membrane. Thus, diagnosis of PLF was impossible by visual inspection alone or imaging techniques such as CT and MRI. The CTP detection test was the only method for detecting perilymph leakage in this case.

Our experience suggests that the CTP detection test can be a useful, highly sensitive, specific, and less invasive method to diagnose local manifestations of PLF.

Conflict of interest

The authors report no conflicts of interest.

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Polymorphisms in Genes Involved in Oxidative Stress Response in Patients with Sudden Sensorineural Hearing Loss and Ménière's Disease in a Japanese Population

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The etiologies of idiopathic sudden sensorineural hearing loss (SSNHL) and Ménière's disease remain unclear. Recently, accumulating evidence has demonstrated that oxidative stress is related to the pathology of inner ear disease. Because genetic factors may contribute partly to the etiologies of SSNHL and Ménière's disease, we investigated the associations between genetic polymorphisms located in oxidative stress response genes and susceptibility to SSNHL and Ménière's disease. We compared 84 patients affected by SSNHL, 82 patients affected by Ménière's disease, and 2107 adults (1056 men and 1051 women; mean age, 59.2 years; range, 40–79 years) who participated in the National Institute for Longevity Sciences, Longitudinal Study of Aging. Multiple logistic regression was used to calculate odds ratios for SSNHL and Ménière's disease in individuals with polymorphisms in the genes glutathione peroxidase 1 (*GPX1*) (Pro198Leu, rs1050450), paraoxonase 1 (*PON1*) (Gln192Arg, rs662; and Met55Leu, rs854560), *PON2* (Ser311Cys, rs7493), and superoxide dismutase 2 (*SOD2*) (Val16Ala, rs4880), with adjustment for age and gender. No significant differences in the distribution of the genotypes at these polymorphisms were observed among individuals with SSNHL and Ménière's disease and controls. No significant risk for SSNHL and Ménière's disease was observed in the additive genetic model, regardless of moderating variables. The C allele of *SOD2* (rs4880) was more frequent in Ménière's disease cases with a hearing level over 50 dB compared with cases with a hearing level below 50 dB, suggesting that this polymorphism is associated with progression of a hearing loss in Ménière's disease. In conclusion, no significant associations between the polymorphisms of *GPX1*, *PON1*, *PON2*, and *SOD2* and risk of SSNHL and Ménière's disease were observed in this Japanese case–control study.

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 the condition is termed idiopathic SSNHL (Schreiber *et al.*, 2010). Complete, partial, or no hearing recovery can occur within 1 or 2 months after the onset of the hearing loss. 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 breakage 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.

Ménière's disease is a syndrome characterized by repeated vertiginous spells accompanied by a fluctuating hearing loss and aural fullness. Almost simultaneously, but independently, Hallpike and Cairns (1938) and Yamakawa (1938) reported the presence of an enlarged endolymphatic space in the temporal bones of patients with Ménière's disease, demonstrating that endolymphatic hydrops is its principal underlying pathology. Thus, the clinical symptoms of SSNHL and Ménière's disease are different. However, some patients were diagnosed as having SSNHL first, and then diagnosed as having Ménière's disease because of subsequent hearing fluctuation with vertigo. SSNHL and

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Ménière's disease are the most important inner ear diseases (Hallpike and Cairns, 1938; Yamakawa, 1938).

Oxidative stress, which is defined as an excess of prooxidant species that are not counterbalanced by an adequate endogenous and exogenous antioxidant defense system, has been proposed as a risk factor for microvascular damage. It is also involved in the development of endolymphatic hydrops. In addition, cellular damage and apoptotic cell death associated with oxidative stress might contribute to the sensorineural hearing loss found in later stages of Ménière's disease (Labbe *et al.*, 2005). Furthermore, the serum concentration of reactive oxygen species (ROS; as measured using spectrophotometric methods) and a global oxidative stress index, which reflect oxidative and antioxidant counterparts, respectively, were significantly higher in patients with SSNHL than they were in controls (Capaccio *et al.*, 2011). Increased levels of protein carbonyls and 4-hydroxynonenal, which are markers of protein oxidation, have been detected in the lymphocytes of patients with Ménière's disease compared with controls, with a significant decrease in the ratio of reduced glutathione versus oxidized glutathione both in the plasma and in lymphocytes (Calabrese *et al.*, 2010). The results of these previous reports imply that the oxidative stress response is involved in the pathophysiology of both SSNHL and Ménière's disease.

Genetic factors may contribute partly to the etiologies of SSNHL and Ménière's disease. In SSNHL, significant associations have been reported mainly for polymorphisms in genes related to blood vessels, circulation, or inflammation, including protein kinase C eta type (1425G/A), matrix metalloproteinase-1 (−1607G/2G), interleukin 1A (−889C/T), interleukin 6 (−572C/G), methylenetetrahydrofolate reductase (C677T), prothrombin (G20210A), platelet Gly IIIaA1/A2, and factor V Leiden (Capaccio *et al.*, 2005a, 2005b, 2007, 2009; Uchida *et al.*, 2010, 2011; Furuta *et al.*, 2011; Nam *et al.*, 2011; Hiramatsu *et al.* 2012). In Ménière's disease, significant associations have been reported for genetic polymorphisms, such as those in the *KCNE* potassium channel genes (in the Japanese population, but not in Caucasians), adducin 1 (Gly460Trp), heat-shock protein 70-1 (190G/C), and interleukin 1A (−889C/T) (Doi *et al.*, 2005; Kawaguchi *et al.*, 2008; Teggi *et al.*, 2008; Campbell *et al.*, 2010; Furuta *et al.*, 2011). To date, there has been a limited number of papers on the relationship between genetic polymorphisms involved in oxidative stress response and SSNHL and Ménière's disease, with the exception of the two reports mentioned previously, which revealed an absence of significant associations between polymorphisms of glutathione S-transferase T1 (*GSTT1*) and *GSTM1* and SSNHL (Cadoni *et al.*, 2006; Um *et al.*, 2011).

Thus, in the present study, we aimed to assess the effects of five additional polymorphisms of genes involved in the oxidative stress response on the risk of susceptibility to SSNHL and Ménière's disease.

Materials and Methods

Patients

Eighty-four patients (38 men, 46 women; mean age ± standard deviation, 58.2 ± 14.3 years; range, 22–86 years) affected by SSNHL, and 82 patients (32 men, 50 women; mean age ± standard deviation, 54.4 ± 15.3 years; range, 20–79 years) affected by Ménière's disease, who visited the Department of Otorhinolaryngology of the Nagoya University Hospital between November 2007 and March 2011 were enrolled in the study. The patients were recruited consecutively. Idiopathic SSNHL was defined as a sensorineural hearing loss of sudden onset with unknown etiology accompanied by no cranial nerve symptoms other than those from the VIII nerve, according to the criteria established by the Sudden Deafness Study Group of the Ministry of Health and Welfare (1973) and revised by the Acute Severe Hearing Loss Study Group of the Ministry of Health, Labor and Welfare (2011), Japan. Among the 84 patients with SSNHL, 66 (78.6%) had tinnitus and 32 (38.1%) had vertigo. Ménière's disease was diagnosed according to the criteria of the 1995 American Academy of Otolaryngology, Head and Neck Surgery (AAO-HNS, 1995). Among the 82 patients with Ménière's disease, 41 were classified as having definite Ménière's disease, 6 were classified as having probable Ménière's disease, and 35 were classified as having possible Ménière's disease, according to AAO-HNS criteria. Demographic data were recorded via 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), which is an ongoing population-based biennial survey. Participants in the NILS-LSA were selected randomly from resident registrations and were stratified according to both age and gender. The NILS-LSA study area is located within 30 km of the Nagoya University Hospital. Details of the NILS-LSA have been described elsewhere (Uchida *et al.*, 2005). Overall, 2107 participants (1056 men and 1051 women; mean age ± standard deviation, 59.2 ± 10.9 years; range, 40–79 years) were selected. These individuals completed the first wave of NILS-LSA examinations between November 1997 and April 2000 and provided samples for the six single-nucleotide polymorphism (SNP) genotype analyses.

Ethics

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

Genotype analysis

Five polymorphisms in genes encoding mediators involved in oxidative stress response glutathione peroxidase 1 (*GPX1*; Pro198Leu, rs1050450); paraoxonase 1 (*PON1*; Gln192Arg, rs662; and Met55Leu, rs854560); *PON2* (Ser311Cys, rs7493); and superoxide dismutase 2 (*SOD2*; Val16Ala, rs4880) 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 an allele-specific primer method and an intercalator-mediated fluorescence resonance energy transfer probe method, as described previously (Yamada *et al.*, 2003; Hirota *et al.*, 2007). Details of primer sequences and PCR conditions are listed in Table 1.

Table 1. Polymerase Chain Reaction Conditions used in Genotyping the Polymorphisms of Genes involved in Oxidative Stress

Allele-specific primer (ASP) method

Gene	*rs No.*	*Labeled primers*	*Sequence (5′→3′)*	*Amplicon (F1/R)/(F2/R)(bp)*	*Annealing temperature (°C)*	*Mg (mM)*
Glutathione peroxidase 1	rs1050450	F1 (FITC) F2 (Texas Red) R (Biotin)	GCGCCCTAGGCACAGCTxAG GCGCCCTAGGCACAGCTxGG GTGTGCCCCTACGCAGGTACA	100/100	65.0	2.5
Paraoxonase 1 (Met55Leu)	rs854560	F1 (FITC) F2 (Texas Red) R (Biotin)	TCCATTAGGCAGTATCTCCXTG TCCATTAGGCAGTATCTCCXAG TTTCTGGCAGAAACTGGCTC	48/48	60.0	2.5
Paraoxonase 2	rs7493	F1 (FITC) F2 (Texas Red) R (Biotin)	CGCATCCAGAACATTCTAxGT CCGCATCCAGAACATTCTAXCT GGCATAAACTGTAGTCACTGTAGGC	51/52	60.0	1.8
Superoxide dismutase 2	rs4880	F1 (FITC) F2 (Texas Red) R (Biotin)	AGGCAGCTGGCTCCGXCT AGGCAGCTGGCTCCGXTT TTCTGCCTGGAGCCCAGATA	44/44	62.5	2.5

Intercalator-mediated fluorescence resonance energy transfer probe (IFP) method

Gene	*rs No.*	*Primers*	*Sequence (5′→3′)*	*Amplicon (bp)*	*Annealing temperature (°C)*	*Mg (mM)*	*Probe (5′→3′)*
Paraoxonase 1 (Gln192Arg)	rs662	F R	TGAGCACTTTTATGGCACAAATGATCAC CGACCACGCTAAACCCAAATACATCTC	85	65.0	2.0	TTGACCCCTACTTACGATCCTG

FITC, fluorescein isothiocyanate.

Table 2. Genotype Distributions of Oxidative Stress-Related Gene Polymorphisms in Sudden Sensorineural Hearing Loss Patients, Ménière's Disease Patients, and Controls

Genotypes/ minor-allele frequencies	*Controls* n=*2107* *No. (%)*	*SSNHL patients* n=*84* *No. (%)*	*MD patients* n=*82* *No. (%)*	p-*Value (versus controls)*
GPX1 (rs1050450)				
CC	1782 (84.6)	71 (84.5)[a]	72 (87.8)[b]	0.4351[a]
CT	318 (15.1)	12 (14.3)[a]	10 (12.2)[b]	
TT	7 (0.3)	1 (1.2)[a]	0 (0)[b]	0.6664[b]
PON1 (rs662)				
GG	928 (44.0)	34 (40.5)[a]	28 (34.2)[b]	0.3891[a]
GA	952 (45.2)	37 (44.1)[a]	44 (53.7)[b]	
AA	227 (10.8)	13 (15.5)[a]	10 (12.2)[b]	0.2056[b]
PON1 (rs854560)				
AA	1820 (86.4)	78 (92.9)[a]	69 (84.2)[b]	0.1615[a]
AT	271 (12.9)	5 (6.0)[a]	13 (15.9)[b]	
TT	16 (0.8)	1 (1.2)[a]	0 (0)[b]	0.5453[b]
PON2 (rs7493)				
CC	1375 (65.3)	59 (70.2)[a]	57 (69.5)[b]	0.4596[a]
CG	631 (30.0)	20 (23.8)[a]	21 (25.6)[b]	
GG	101 (4.8)	5 (6.0)[a]	4 (4.9)[b]	0.6983[b]
SOD2 (rs4880)				
TT	1595 (75.7)	60 (71.4)[a]	64 (78.1)[b]	0.3929[a]
TC	460 (21.8)	23 (27.4)[a]	15 (18.3)[b]	
CC	52 (2.5)	1 (1.2)[a]	3 (3.7)[b]	0.6193[b]

[a]Comparison between SSNHL patients and controls.
[b]Comparison between MD patients and controls.
SSNHL, sudden sensorineural hearing loss; MD, Ménière's disease; *GPX1*, glutathione peroxidase 1; *PON1*, paraoxonase 1; *SOD2*, superoxide dismutase 2.

Statistical analysis

Statistical analyses were conducted using Statistical Analysis System version 9.1.3 (SAS Institute, Cary, NC) with significance accepted at $p<0.05$. Univariate analyses of categorical variables were performed using the chi-squared test or the Fisher's exact test. The 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 individuals with polymorphisms. Genotypes were coded as major-allele homozygous, heterozygous, and minor-allele homozygous. The major alleles of five SNPs were determined in analyses, as follows: C in rs1050450 of *GPX1*; A in rs1695 of *GSTP1*; G in rs662 of *PON1*; T in rs3202100 of *PON1*; C in rs6954345 of *PON2*; and T in rs1799725 of *SOD2*. The additive genetic model, which is the prevailing analytical model in genetic epidemiology, assumes that there is a linear gradient in risk with an increasing number of mutated alleles. The ORs for SSNHL and Ménière's disease were determined using the additive genetic model of six polymorphisms. 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. In this analysis, we used two models with and without moderator variables. Model 1 was not adjusted for moderator variables, whereas age and gender were used as moderator variables in model 2.

Audiological testing

Hearing levels were evaluated in patients with SSNHL and Ménière's disease using an audiometer (Model AA-79S; Rion, Tokyo, Japan) in a sound-insulated chamber. 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 above the maximum level. For patients with SSNHL, the initial

Table 3. Multivariable Logistic Regression Analysis of Oxidative Stress-Related Gene Polymorphisms as Risk Factors for Sudden Sensorineural Hearing Loss and Ménière's Disease

Mode of inheritance	*Crude: model 1* *Odds ratio*	*95% CI*	p-*Value*	*Adjusted: model 2* *Odds ratio*	*95% CI*	p-*Value*
GPX1 (rs1050450)						
SSNHL	1.065	0.603–1.883	0.8272	0.916	0.498–1.683	0.7774
MD	0.752	0.389–1.454	0.3968	0.735	0.379–1.425	0.3618
PON1 (rs662)						
SSNHL	1.201	0.871–1.656	0.2630	1.216	0.879–1.681	0.2383
MD	1.285	0.930–1.777	0.1290	1.272	0.918–1.762	0.1480
PON1 (rs854560)						
SSNHL	0.572	0.268–1.218	0.1473	0.588	0.276–1.252	0.1684
MD	1.108	0.627–1.956	0.7249	1.131	0.640–1.999	0.6708
PON2 (rs7493)						
SSNHL	0.889	0.601–1.314	0.5536	0.915	0.618–1.354	0.6563
MD	0.878	0.590–1.307	0.5224	0.871	0.586–1.296	0.4971
SOD2 (rs4880)						
SSNHL	1.124	0.737–1.715	0.5868	1.105	0.719–1.700	0.6477
MD	0.953	0.606–1.499	0.8357	0.934	0.594–1.467	0.7666

Moderating variables: model 1, no variables; model 2, age and sex.
CI, confidence interval.

audiograms were obtained at the first visit and the final audiograms were taken at least 2 months after the onset of deafness, with the exception of patients who recovered completely within this period. 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 (the hearing levels in all five frequencies were 20 dB or less or there was improvement to the same degree of hearing as that found 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.

For patients with Ménière's disease, the worst hearing level at attack periods was evaluated using the average score at five frequencies described above. According to the average hearing level at the five frequencies, we divided these patients into two groups for further analysis: in one group, hearing level exceeded 50 dB, and in the other, hearing level was below 50 dB.

Results

The genotype distributions and allele frequencies of the polymorphisms of *GPX1* (Pro198Leu, rs1050450), *PON1* (Gln192Arg, rs662; and Met55Leu, rs854560), *PON2* (Ser311-Cys, rs7493), and *SOD2* (Val16Ala, rs4880) in patients with SSNHL and Ménière's disease are described in Table 2. No significant differences in the distribution of genotypes and allele frequency of the polymorphisms described above were observed among SSNHL patients, Ménière's disease patients, and controls.

Table 3 shows the results of the multivariable logistic regression analysis. No significant risk of SSNHL and Ménière's disease were observed for the five polymorphisms in the additive genetic model, regardless of adjustments for age and gender.

Table 4 shows the relationship between recovery of hearing loss and the five oxidative stress-related polymorphisms in SSNHL patients. The T allele of PON1 (rs854560) was more frequent in SSNHL cases with good recovery compared with those with poor recovery. The allele frequencies of the remaining four polymorphisms were not significantly different between patients with SSNHL exhibiting good or poor recovery. Baseline clinical characteristics, such as age, initial hearing level, period from onset to first visit to our hospital, and vertigo, which can affect the prognosis of hearing recovery, were not significantly different between patients with good recovery and those with poor recovery.

Table 5 shows the relationship between the severity of hearing loss in Ménière's disease and the five oxidative stress-related polymorphisms. The C allele of SOD2 (rs4880) was more frequent in Ménière's disease cases with a hearing level over 50 dB compared with those with a hearing level below 50 dB. The allele frequencies of the remaining four polymorphisms were not significantly different between patients with Ménière's disease with a hearing level over 50 dB or below 50 dB. The characteristics of age and duration of the disease did not differ between patients with Ménière's disease with a hearing level over 50 dB or below 50 dB.

TABLE 4. HEARING RECOVERY AND ALLELE FREQUENCY OF OXIDATIVE STRESS-RELATED POLYMORPHISMS IN SUDDEN SENSORINEURAL HEARING LOSS

	SSNHL cases with good recovery	*SSNHL cases with poor recovery*	p *Value*
Number of cases	22	39	
GPX1 (rs1050450); C allele, T allele	41, 3	76, 2	0.248
PON1 (rs662); G allele, A allele	27, 17	48, 30	0.985
PON1 (rs854560); A allele, T allele	40, 4	78, 0	0.015
PON2 (rs7493); C allele, G allele	34, 10	68, 10	0.156
SOD2 (rs4880); T allele, C allele	36, 8	66, 12	0.689
Age (years)	57.3±12.1	59.7±15.6	0.533
Average initial hearing level (dB)	74.7±19.6	67.8±25.5	0.273
Period from onset to first visit (days)	4.6±4.6	7.9±8.7	0.110
Number of patients with vertigo	8	15	0.871

Average initial hearing level: average hearing level at five frequencies (250, 500, 1000, 2000, and 4000 Hz); period from onset to first visit: period from onset of SSNHL to the date at which the patients visited our hospital for the first time.

Values for age, average initial hearing level, and period from onset to first visit represent the average±standard deviation.

TABLE 5. HEARING LEVEL AND ALLELE FREQUENCY OF OXIDATIVE STRESS-RELATED POLYMORPHISMS IN MÉNIÈRE'S DISEASE

	MD cases with hearing level below 50 dB	*MD cases with hearing level over 50 dB*	p *Value*
Number of cases	47	35	
GPX1 (rs1050450); C allele, T allele	89, 5	65, 5	0.878
PON1 (rs662); G allele, A allele	62, 32	38, 32	0.130
PON1 (rs854560); A allele, T allele	89, 5	62, 8	0.152
PON2 (rs7493); C allele, G allele	78, 16	57, 13	0.797
SOD2 (rs4880); T allele, C allele	88, 6	55, 15	0.004
Age	51.7±15.2	58.6±16.0	0.051
Average hearing level (dB)	29.5±11.1	69.8±14.6	<0.001
Duration of the disease (years)	4.1±6.2	6.2±8.3	0.194

Average hearing level: average hearing level at five frequencies (250, 500, 1000, 2000, and 4000 Hz).

The values for age, average hearing level, and duration of the disease represent the average±standard deviation.

Discussion

In the present study, we aimed to investigate, for the first time, the association between the risk of SSNHL and Ménière's disease and five oxidative stress-related genetic polymorphisms using a case–control study. No significant differences in the distribution of genotypes and allele frequencies of the polymorphisms described above were observed among SSNHL patients, Ménière's disease patients, and controls. No significant risk of SSNHL and Ménière's disease was observed for the five polymorphisms.

Recently, it was reported that the blood–labyrinth barrier is disrupted in at least one-third of patients with SSNHL, because gadolinium contrast agents injected intravenously appeared in the affected inner ear on magnetic resonance imaging (MRI) (Yoshida *et al.*, 2008). MRI also revealed a weak blood–labyrinth barrier in ears affected by Ménière's disease compared with asymptomatic contralateral ears (Nakashima *et al.*, 2010; Tagaya *et al.*, 2011). As disruption of the blood–labyrinth barrier is associated with increased permeability of blood vessels in the inner ear and endothelium tight junctions, which leads to the disruption of the permeability of blood by ROS (Grammas *et al.*, 2011), ROS may be involved in the pathology of SSNHL and Ménière's disease.

SOD enzymes catalyze the conversion of superoxide radicals to hydrogen peroxide. The SOD2 enzyme protects against damage caused by free radicals (Fortunato *et al.*, 2004). It has been reported that Ala16Ala homozygotes may have higher SOD2 activity than Val16Val homozygotes (Sutton *et al.*, 2003). The *SOD2* Ala16Ala genotype is associated with an increased breast cancer risk, a high degree of carotid atherosclerosis, and exudative age-related macular degeneration in Japanese individuals, in whom allele Ala occurs less frequently than it does in Caucasians (Ambrosone *et al.*, 1999; Kimura *et al.*, 2000; Kakko *et al.*, 2003). Hearing loss in Ménière's disease is associated with loss of spiral ganglion neurons and hair cells. In a guinea pig model of endolymphatic hydrops, oxidative stress mediated the loss of spiral ganglion neurons (Labbe *et al.*, 2005). In the present study, Ménière's disease patients with a hearing level over 50 dB exhibited a higher minor-allele frequency compared with patients with a hearing level below 50 dB, suggesting that the *SOD2* Val16Ala (rs4880) polymorphism is associated with progression of hearing loss in Ménière's disease.

Paraoxonases exert antioxidant activity and may protect against diseases, such as atherosclerosis, diabetes, Alzheimer dementia, and Parkinson disease (Mackness *et al.*, 2000; Akhmedova *et al.*, 2001; Janka *et al.*, 2002; Fortunato *et al.*, 2003).The *PON* gene family consists of *PON1*, *PON2*, and *PON3*, which are located on chromosome 7q21–q22 (Primo-Parmo *et al.*, 1996). The *PON1* 55Leu, *PON1* 192Arg, and, more recently, *PON2* 311Cys variants have been implicated in the oxidative damage associated with the pathogenesis of neurodegenerative diseases, such as Alzheimer disease and Parkinson disease (Carmine *et al.*, 2002; Shi *et al.*, 2004). The C allele of the *PON2* Ser311Cys polymorphism is associated with a noise-induced hearing loss (Fortunato *et al.*, 2004). A higher frequency of the minor allele of the *PON1* Met55Leu polymorphism was observed in SSNHL cases with good recovery compared with those with poor recovery. However, as the frequency of the minor allele of this polymorphism is very small, that is, only four, further investigations using larger case samples are required to confirm the association between this polymorphism and hearing recovery in SSNHL.

Via its action in regulating the cellular levels of peroxide, GPX plays a critical role in minimizing the production of hydroxyl radical. Although there may be species differences, GPX appears to be expressed at comparatively high levels in the cochlea. *GPX1* knockout mice exhibited auditory brainstem response thresholds that were up to 16 dB higher before noise exposure, and up to 15 dB greater noise-induced hearing loss, depending on test frequency, compared with controls (Ohlemiller *et al.*, 2000). However, no association was found between the *GPX* Pro198Leu polymorphism and the risk of SSNHL and Ménière's disease.

Generally, statistical power depends on the sample size, the significance criterion, and the effect size (Cohen, 1992). The power level in the comparison of genotype distribution in each polymorphism in the present study varied between 0.019 and 1.0 and was below 0.8, with the exception of the cases of *PON2* Ser311Cys and *SOD2* Val16Ala. The observation that the genotype distribution for each polymorphism between controls and SSNHL or Ménière's disease was similar in this negative study may lower the effect size, leading to a low power level. This is the weakness of our study and these results need to be confirmed in larger series of patients in future studies.

Conclusion

In conclusion, no significant associations were observed between the polymorphisms of *GPX1* (Pro198Leu, rs1050450), *PON1* (Gln192Arg, rs662; and Met55Leu, rs3202100), *PON2* (Ser311Cys, rs6954345), and *SOD2* (Val16Ala, rs1799725) and the risk of SSNHL and Ménière's disease in this Japanese case–control study.

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Disclosure Statement

No competing financial interests exist.

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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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