Identification of Evidence for Autoimmune Pathology of Bilateral Sudden Sensorineural Hearing Loss Using Proteomic Analysis

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Identification of Evidence for Autoimmune Pathology of Bilateral Sudden Sensorineural Hearing Loss Using Proteomic Analysis

Directed by Professor Sung Huhn Kim

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ABSTRACT

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Sudden sensorineural hearing loss (S-SNHL) is an inner ear disorder defined as a minimum of 30 dB hearing loss over 3 consecutive frequencies in a pure-tone audiogram occurring in less than 3 days. The pathologic mechanism of the disease remains unclear, although autoimmunity has been regarded as one of the suggested causes, especially in the bilateral form. In this study, we aimed to provide evidence for the involvement of autoimmune mechanisms in bilateral S-SNHL using proteomic approaches.

A total of 10 patients with bilateral S-SNHL and 10 normal control subjects were enrolled for this study. First, in a ProtoArray®, 20 proteins showed more than 2-fold greater signal intensity in patients with bilateral S-SNHL than in the control subjects, which suggested the presence of circulating autoantibodies in the patients' sera and their possible target antigens. Second, western blotting using the subjects' sera and mouse inner ear protein, for the assessment of the antigen-antibody reactions between circulating autoantibodies and corresponding antigens in the inner ear, showed exclusive

bands of molecular weight 63–75 kDa and 35–48 kDa between patients' sera and mouse inner ear. Third, liquid chromatography-mass spectrometry (LC-MS) / mass spectrometry (MS) using proteins extracted from the immunoprecipitation for the identification of the target antigen and autoantibody in the reaction revealed 8 overlapping proteins between the subjects' sera and mouse inner ear that can serve as candidate antigens and/or autoantibodies.

These findings provide evidence for autoimmunity as one of the pathological mechanisms for bilateral S-SNHL, and suggest candidate antigens and autoantibodies.

Key words : bilateral sudden sensorineural hearing loss, autoimmune disease, inner ear, autoantibody

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I. INTRODUCTION

Sudden sensorineural hearing loss (S-SNHL) was first described by DeKleyn in 1944, and is defined by the National Institute on Deafness and other Communication Disorders as a minimum of 30 dB hearing loss over 3 consecutive frequencies in a pure tone audiogram, occurring in less than 3 days.[1] The global incidence has been reported to be variable from 5 to 160 per 100,000 individuals.[2,3] While the disease has a wide age distribution with an average age at incidence of 50–60 years, it is equally distributed between sexes. The etiology and pathogenesis of S-SNHL remain unknown, although detailed investigations have revealed a specific cause such as viral infection, vascular occlusion, and immune system-mediated mechanisms only in about 10% of the patients.[4, 5]

Although unilateral involvement is common (~95%) in S-SNHL, bilateral involvement can occur in less than 5% of the cases.[6] Bilateral S-SNHL can be

further classified into simultaneous (i.e., the second ear is affected within 3 days of the first ear) or sequential (the second ear is affected 3 or more days after the first ear) types.[5] The 3 types of S-SNHL (unilateral, sequential bilateral, and simultaneous bilateral) were reported to have different severities of hearing loss at presentation, suspected etiologies, and prognoses.[5] While unilateral cases showed improvement in the hearing threshold after systemic steroid treatment in ~65% of the patients, simultaneous bilateral S-SNHL is associated with a more severe degree of hearing loss at presentation, and is less responsive to treatment; retrocochlear lesions are usually common during the development of this disorder.[7,8] Sequential bilateral S-SNHL and simultaneous bilateral S-SNHL without retrocochlear lesions have been reported to have variable prognoses since the incidence of the disorder is low and the etiology is not fully determined.[5,7,9,10] Although various etiologies have been suspected for S-SNHL as described above, a definite cause for S-SNHL cannot be determined in most cases. Nevertheless, autoimmunity is strongly suspected as one of the causes for S-SNHL, especially for bilateral S-SNHL without evidence of retrocochlear lesions, based on its bilaterality and responses to steroid and immune-suppressive treatment in certain cases.[5,11]

Several findings have provided evidence that autoimmunity may underlie the pathological mechanism of bilateral S-SNHL. One of the factors supporting the involvement of an autoimmune mechanism is the molecular

evidence of the presence of immune cells and mediators in the inner ear of patients with bilateral S-SNHL as well as in animal models. Tissue infiltration and free-floating lymphocytes, plasma cells, and macrophages have been observed in the inner ear of patients with proliferation of fibrous tissue and destruction of adjacent bony structures.[12] Animal models have also demonstrated similar inner ear damage with inflammatory reactions after immunization using the candidate antigens.[13] Furthermore, elevation of immune responses between animal inner ear proteins and serum samples obtained from patients with S-SNHL has been demonstrated.[14] The fact that S-SNHL frequently occurs in context of systemic immune disease and that its symptoms can be improved with immunosuppressive therapy strongly supports the involvement of autoimmunity in the pathogenesis of bilateral S-SNHL.[7,15,16]

So far, several studies have been conducted to investigate various candidate antigens and autoantibodies (autoAbs) in bilateral S-SNHL. However, evidence for autoimmunity as a pathological mechanism of bilateral S-SNHL remains insufficient, as most studies have investigated selective target proteins instead of utilizing a mass screening approach, thus limiting the probability of detecting candidate whole autoAbs and their target antigens. Consequently, it was later revealed that findings related to some of these candidate proteins were not sufficiently quantified. For example, collagen type II had been proposed as a potential target antigen, although the reported

hearing loss associated with collagen type II immunization had not been reproduced consistently.[17] In addition, heat shock protein-70 (HSP-70) was elevated in more than 35% of patients with autoimmune inner ear disease, but subsequent animal studies demonstrated no correlation between elevated anti-HSP antibody levels and pathological injury.[18]

In this study, we aimed to provide evidence for the involvement of autoimmunity in bilateral S-SNHL using proteomic approaches such as ProtoArray®, western blotting, immunoprecipitation, and liquid column mass spectrometry for the mass screening of candidate antigens and autoAbs based on the hypothesis that multiple autoAbs and target antigens must exist in order for autoimmune bilateral sudden hearing loss to develop. The results of this study may provide basic evidence necessary for the development of diagnostic biomarkers as well as the understanding of the pathological mechanisms underlying bilateral S-SNHL.

II. METHODS

1. Selection of patients and controls

Ten patients diagnosed with bilateral S-SNHL according to the criteria of the National Institute on Deafness and other Communication Disorders were enrolled as the patient group. The mean age was 45.7 ±16.0 yr, and the men:women ratio was 5:5. Four patients had simultaneous attacks in both ears, while the other six experienced sequential attacks with an average interval of 33.8 ±19.9 mo. Complete neurological examination and temporal magnetic resonance imaging (MRI) did not show any evidence of a central lesion. Detailed patient information is listed in **Table 1**.

Table 1. Patient demographics

	Gender /Age	Clinical features	Interval (mo)			250 Hz	500 Hz	1,000 Hz	2,000 Hz	3,000 Hz	4,000 Hz	8,000 Hz	Average
P1	M/44	Sequential	36	Pre-treatment hearing level (dB HL)	1st ear	55	55	60	40	40	60	95	53.8
					2nd ear	60	65	70	65	70	75	105	68.8
				Final hearing level (dB HL)	1st ear	50	55	60	45	50	70	90	57.5
					2nd ear	55	65	65	65	75	70	100	66.3
P2	M/28	Simultaneous	-	Pre-treatment hearing level (dB HL)	1st ear	75	90	95	85	75	70	75	85.0
					2nd ear	85	90	90	80	85	90	100	87.5
				Final hearing level (dB HL)	1st ear	65	70	70	55	30	50	50	61.3
					2nd ear	75	75	75	60	55	55	55	66.3
P3	M/30	Sequential	26	Pre-treatment hearing level (dB HL)	1st ear	25	30	40	45	40	45	20	40.0
					2nd ear	20	25	35	50	50	55	50	41.3
				Final hearing level (dB HL)	1st ear	20	25	45	45	40	40	20	38.8
					2nd ear	30	30	35	40	45	45	45	37.5
P4	F/57	Simultaneous	-	Pre-treatment hearing level (dB HL)	1st ear	70	70	80	70	85	95	105	78.8
					2nd ear	85	90	105	115	115	120	105	107.5
				Final hearing level (dB HL)	1st ear	75	80	80	75	85	90	115	81.3
					2nd ear	90	95	105	105	115	120	105	106.3

P5	F/77	Sequential	22	Pre-treatment hearing level (dB HL)	1st ear	75	75	85	85	75	75	80	80.0
					2nd ear	75	70	60	40	40	35	75	51.3
				Final hearing level (dB HL)	1st ear	75	80	90	80	80	80	95	82.5
					2nd ear	65	60	65	55	40	40	70	55.0
P6	M/60	Simultaneous	-	Pre-treatment hearing level (dB HL)	1st ear	65	55	60	35	45	55	65	51.3
					2nd ear	60	70	90	105	120	100	100	91.3
				Final hearing level (dB HL)	1st ear	35	20	15	15	20	20	35	17.5
					2nd ear	50	75	85	95	115	100	105	88.8
P7	F/36	Sequential	16	Pre-treatment hearing level (dB HL)	1st ear	40	40	25	25	25	30	30	30.0
					2nd ear	10	10	15	5	25	45	45	18.8
				Final hearing level (dB HL)	1st ear	5	5	5	5	0	10	10	6.3
					2nd ear	15	25	25	10	40	50	20	27.5
P8	F/37	Sequential	72	Pre-treatment hearing level (dB HL)	1st ear	95	105	105	95	95	85	80	97.5
					2nd ear	10	20	25	40	70	90	95	43.8
				Final hearing level (dB HL)	1st ear	90	105	105	95	100	105	95	102.5
					2nd ear	35	35	35	40	75	80	80	47.5
P9	F/55	Simultaneous	-	Pre-treatment hearing level (dB HL)	1st ear	65	70	70	105	100	100	90	86.3
					2nd ear	65	85	75	75	75	70	80	76.3
				Final hearing level (dB HL)	1st ear	60	70	80	110	105	105	90	91.3
					2nd ear	15	35	35	35	40	40	65	36.3
P10	M/33	Sequential	31	Pre-treatment hearing level (dB HL)	1st ear	30	35	30	25	15	20	25	27.5
					2nd ear	25	35	40	20	5	-5	-5	22.5
				Final hearing level (dB HL)	1st ear	15	10	5	5	5	10	10	7.5
					2nd ear	15	30	10	0	0	-5	-5	8.8

Uniform treatment was provided for all patients. All the patients underwent medical treatment within 24 hr of the onset of the hearing loss. Methylprednisolone was administered at a dose of 1 mg/kg per day for the first five days, and the dose was tapered over the next five days. After 10 days of treatment, the hearing level was evaluated by pure-tone audiometry. When the hearing level had not completely recovered (recovery of hearing to within 10 dB of the previous hearing level), intratympanic dexamethasone injection was administered 5 times every other days. The mean follow-up period was

32.0 ±26.7 mo, and none of the patients showed recurrent attacks during the follow-up period.

Ten volunteers who had no history of sensorineural hearing loss or vertigo, with audiograms showing normal hearing (average pure-tone audiometry less than 25 dB) were enrolled as the control group. None of the patients or control subjects had a history of systemic disease such as diabetes, hypertension, autoimmune disorder, and acute or chronic inflammatory disorders, and all of their laboratory parameters, including electrocardiography, chest radiography, blood cell counts (red blood cells, white blood cells, and platelets), liver and kidney function tests, and urine analysis were normal. The sex distribution and mean age of the patient and control groups did not significantly differ ($p > 0.05$ for the chi-square test and t-test).

2. Sampling of sera

Blood was sampled from every participant in each group for the experiment. Peripheral blood was sampled before the administration of any medication and within 24 hours of the development of symptoms in the bilateral S-SNHL group. In the control group, blood was sampled immediately after obtaining informed consent. The blood was stored in a tube containing ethylenediaminetetraacetic acid (EDTA), the plasma was immediately separated, and stored at -80°C until further analysis.

3. ProtoArray® analysis of serum samples (Immune Response Biomarker Profiling)

To investigate the presence of possible antigen-antibody reactions between circulating autoAbs and their target antigens, and to identify candidate target antigens for the autoimmune reaction, the ProtoArray® (Human Protein Microarray v5.0 containing 9,400 human proteins, Invitrogen Corp., Carlsbad, CA, USA) analysis was performed with sera from patients and control subjects according to the manufacturer's protocol. Briefly, after blocking the array slides with blocking buffer for 1 hr, we washed the slides with washing buffer for 5 min, after which 5 ml of diluted serum (1:500) was placed on the slides. The slides were incubated for 90 min and washed with washing buffer 4 times for 5 min each. After the slides were washed, Alexa Fluor 647 (final concentration, 1μg/ml) was added to the slides, and the slides were incubated for 90 min. The antibody was aspirated, and the slides were washed 4 times for 5 min each. These steps were performed at 4°C. The slides were dried immediately by centrifugation at 200×g for 1 min and stored in a slide box to protect them from light until scanning was performed. The dried arrays were scanned using a GenePix 4,000B microarray scanner (Molecular Devices, Sunnyvale, CA, USA). The GenePix Pro microarray data acquisition software was used to align the scanned image with the template and to acquire the pixel intensity data for each spot on the array. The reported pixel intensity was calculated as the average of duplicate signals obtained after subtracting the

background signal. The ProtoArray® Prospector software (Life Technologies, Carlsbad, CA, USA) was used to analyze the data, perform background subtraction, and normalize the signals. The normalized signal intensities obtained for controls and patients were compared using the t-test, and differences with a *p* value less than 0.05 were considered statistically.

4. Western blotting

To investigate whether circulating autoAbs in the patient serum reacted with inner ear tissue, western blotting using the patient serum and mouse inner ear tissue was performed. We used mouse inner ear tissue for ethical reasons as it would have been impossible to harvest the entire human cochlea and vestibule to use as fresh specimens for analysis. The entire temporal bone was separated from eight-week-old male C57BL/6 mice. The cochlea and vestibule were separated, and the membranous labyrinth was carefully dissected from the cochlea and vestibule. Each membranous labyrinth was lysed with 2× sample buffer (250 mM Tris-HCl [pH 6.5], 2% sodium dodecyl sulfate [SDS], 1% dithiothreitol [DTT], 0.02% bromophenol blue, and 10% glycerol). Protein levels were quantified by comparing the absorbance of the lysate with that of serially diluted bovine serum albumin (0, 0.2, 0.4, 0.6, 0.8, and 1 mg/ml) in a VersaMax enzyme-linked immunosorbent assay (ELISA) plate reader (Molecular Devices, Sunnyvale, CA, USA). Samples were heated for 5 min at 95°C in 95% glycerol. The protein levels were quantified by comparing the

absorbance of the lysate with that of serially diluted bovine serum albumin of the bands. The proteins were separated using a 10% SDS-polyacrylamide gel at 125 V for 4 hr with a running buffer (25 mM Tris-Base, 192 mM glycine, and 0.1% SDS) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) using a semi-dry transfer cell (Bio-Rad, Hercules, CA, USA) for 2 hr at 200 mA and a transfer buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20). The membranes were blocked with 5% skim milk in Tris-buffered saline (TBS; 50 mM Tris-HCl [pH 7.5] and 150 mM NaCl) for 2 h at room temperature. The blots were incubated overnight with patient serum diluted at 1:200 in 0.5% Tween-20 in TBS (TTBS). The blots were washed with TTBS, incubated with a secondary anti-human antibody (Cell Signaling Technology, Danvers, MA, USA) in TTBS for 45 min at room temperature, and visualized using enhanced chemiluminescence (Amersham Biosciences, Champaign, IL, USA).

5. Immunoprecipitation of sera

Immunoprecipitation was performed to capture antigen-autoantibody (Ag-autoAb) complexes composed of mouse inner ear antigens and circulating autoAbs from the patients. The goal of the procedure was to provide Ag-autoAb complexes for liquid chromatography mass spectrometry (LC-MS)/ mass spectrometry (MS) to identify the inner ear antigens and circulating antibodies that react with each other. Eight-week-old male

C57BL/6 mice were used, and mouse inner ear tissue was prepared as described above. The quantified protein was incubated overnight with the human antibody at 4°C. Sepharose beads (P9,424 Protein A-Sepharose human antibody; Sigma-Aldrich, St. Louis, MO, USA) were added to 1-mL tubes containing the incubated samples and the tubes with the beads were incubated at 4°C for 2 hr. After incubation, the tubes were centrifuged at 10,000 rpm for 30 sec to precipitate the beads. The supernatant was carefully removed completely with a pipette and the beads were washed 3 times with washing buffer (5 ml radioimmunoprecipitation assay buffer, 50 μ phenylmethylsulfonyl fluoride) to remove non-specific binding. For each wash, the beads were gently vortexed with washing buffer and centrifuged for precipitation, and the supernatant was discarded. Finally, 2× sample buffer was added to the sample and heated for 5 min at 99°C to elute the protein from the beads. After elution, the sample was centrifuged at 13,000 rpm for 30 sec and the supernatant was collected for LC-MS/MS.

6. One-dimensional electrophoresis (1-DE) and identification of candidate inner ear antigens for autoimmune reaction by LC-MS/MS

1-DE and LC-MS/MS were performed to analyze the inner ear antigens that react with circulating autoAbs of patients. 1-DE was performed with the samples acquired from the immunoprecipitation. The samples were lyophilized and dissolved in 15 μl of distilled water. The amount of protein in

each sample was quantified. A total of 6 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis on an 8–16% Tris-glycine gel and stained with Coomassie Brilliant Blue. Bands corresponding to the molecular weight ranges of 63-75 kDa and 35-48 kDa, where the Ag-Ab reaction was most frequent in western blotting, were excised from the 1-DE gel. After reduction with DTT and alkylation with iodoacetamide, the bands were treated with trypsin for in situ digestion. The bands were then washed with 10 mM ammonium bicarbonate and 50% acetonitrile and swollen in digestion buffer containing 50 mM ammonium bicarbonate, 5 mM CaCl2, and 1 mg of trypsin. Next, they were incubated at 37°C for 12 hr. Peptides were recovered over the course of 2 extraction cycles with 50 mM ammonium bicarbonate and 100% acetonitrile. The resulting peptide extracts were pooled, lyophilized, and stored at -20°C.

Nano LC-MS/MS analysis was performed on an Agilent 1,100 Series nano-LC and linear trap quadrupole (LTQ)-mass spectrometer (Thermo Electron, San Jose, CA, USA). LC-MS/MS was performed on the sera obtained from four patients and four controls. The capillary column used for LC-MS/MS analysis (150 mm × 60.075 mm) was obtained from Proxecon (Odense M, Denmark) and slurry-packed in-house with 5 mg, 100 Å pore size Magic C18 stationary phase (Michrom Bioresources, Auburn, CA, USA). The mobile phase A for LC separation was 0.1% formic acid in deionized water and the mobile phase B was 0.1% formic acid in acetonitrile. Chromatography

was performed using a linear gradient from 5% B to 35% B over 100 min, from 40% B to 60% B over 10 min, and from 60% B to 80% B over 20 min. The flow rate was maintained at 300 nl/min after splitting. Mass spectra were acquired using data-dependent acquisition with full mass scan (400–1800 m/z) followed by MS/MS scans. Each acquired MS/MS scan represented the average of one microscan on the LTQ. The temperature of the ion transfer tube was controlled at 200°C and the spray was 1.5–2.0 kV. The normalized collision energy was set at 35% for MS/MS.

The MASCOT and SEQUEST (BioWorks software version 3.2, Thermo Electron) search engines were used to search the UniProt human protein database (release 14.8; 82728 sequences) for tandem mass spectra. Mass tolerances of 1.2 Da and 0.6 Da were used for the precursor and fragment ions, respectively. The search included variable modification of methionine oxidation and cysteine carbamidomethylation. PeptideProphet and ProteinProphet were used to estimate the false discovery rate (FDR) for any minimum probability used as a cut-off for the MASCOT and SEQUEST search results.

1-DE and LC-MS/MS were performed in 8 subjects (4 patients and 4 controls) due to the time and cost effectiveness. Subject selection for these analyses was done based on the western blotting results. Only subjects who showed relatively higher responses were selected for these analyses.

7. Ethics statement

This study was approved by the Institutional Review Board of the Severance Hospital (approval number 4-2011-0871), and written informed consent was obtained from all participants. The Institutional Animal Care and Use Committee of the Yonsei University College of Medicine approved this study, and all the mice were treated in accordance with the guidelines for the Care and Use of Laboratory Animals of the Yonsei University College of Medicine (approval number 2011-0084).

III. RESULTS

1. Treatment response to corticosteroids in patients with bilateral S-SNHL

Among 20 ears, 7 ears responded to the treatment (4 ears fully recovered, and 3 ears partially recovered), while the other 13 ears did not respond (**Table 1**). Patient 2 had simultaneous attacks in both ears, and did not respond to initial oral medication. After 5 rounds of salvage intratympanic injection after the oral medication treatment, the hearing in both ears partially improved. Patient 6 also experienced simultaneous attacks. One ear fully recovered after the administration of oral medication, but the other ear failed to recover even after the administration of intratympanic injection in addition to the oral medication. Patient 7 had sequentially occurring sudden hearing loss and the first ear fully recovered with oral medication, however, the second ear was resistant to both oral medication and intratympanic injection. Patient 9 experienced simultaneous attacks, and only one ear partially recovered after 1 cycle of oral medication; however, the patient refused further treatment. Patient 10 had sequential bilateral sudden hearing loss; the first ear fully recovered with oral medication, and the second ear recovered after an additional intratympanic injection.

2. Evidence of circulating autoAbs and candidate target antigens for autoimmune reaction identified by the ProtoArray® analysis

We investigated the presence of circulating autoAbs in the peripheral blood and their candidate target antigens of the 10 patients with bilateral S-SNHL using the Immune Response Biomarker Profiling application on the ProtoArray®. Twenty proteins were identified to have more than 2-fold greater signal intensity in patients with bilateral S-SNHL than in controls ($p < 0.05$, **Table 2**); the signal intensity of 7 proteins was more than 5-fold higher in patients than in controls (**Table 2** and **Figure 1)**. Among these proteins, C-X-C motif chemokine 10 (CXCL10) had the highest signal intensity, and was approximately 15-fold higher in patients than in controls. These results provide evidence for the existence of circulating autoAbs and enhanced autoimmune/immune reactions with the candidate target antigens in patients with bilateral S-SNHL.

Table 2. Proteins with higher signal intensities in bilateral S-SNHL in ProtoArray® analysis

Access No.	Swissprot ID	Protein name	Fold change	Function
NM_001565.1	P02778	C-X-C motif chemokine 10	15.6	Chemoattraction, promotion of T cell adhesion to endothelial cells, antitumor activity, and inhibition of bone marrow colony formation and angiogenesis.
NM_005975.2	Q13882	PTK6 protein tyrosine kinase 6 (PTK6)	9.7	Regulation of signaling pathway that control the differrentiation and maintenance of normal epithelia
BC045667.1	Q9Y5W8	sorting nexin 13 (SNX13)	9.1	Intracellular trafficking. May play a role in endosome homeostasis. Acts as a GAP for Galphas
NM_016127.4	Q96BY9	transmembrane protein 66 (TMEM66)	6.9	Negative regulator of store-operated Ca2+ entry

				(SOCE) involved in protecting cells from Ca2+ overfilling
NM_002984.1	P13236	chemokine (C-C motif) ligand 4 (CCL4), transcript variant 1	6.9	Monokine with inflammatory and chemokinetic properties. One of the major HIV-suppressive factors produced by CD8+ T-cells.
BC032456.1	Q5VYS8	zinc finger, CCHC domain containing 6 (ZCCHC6)	6.2	Mediates the terminal uridylation of mRNAs with short (less than 25 nucleotides) poly(A) tails, hence facilitating global mRNA decay
NM_002059.3	P01242	growth hormone 2 (GH2), transcript variant 1	5.2	Growth control. It stimulates both the differentiation and proliferation of myoblasts. It also stimulates amino acid uptake and protein synthesis in muscle and other tissues.
NM_002982.1	P13500	chemokine (C-C motif) ligand 2 (CCL2)	4.3	Chemotactic factor that attracts monocytes and basophils but not neutrophils or eosinophils.
BC044218.1	Q861E6	MHC class I polypeptide-related sequence B (MICB)	4	Seems to have no role in antigen presentation. Acts as a stress-induced self-antigen that is recognized by gamma delta T cells.
BC024021.1	Q9H4A9	*dipeptidase 2 (DPEP2)	3.9	Probable metalloprotease which hydrolyzes leukotriene D4 (LTD4) into leukotriene E4 (LTE4).
BC068602.1	Q96I99	Succinyl-CoA ligase [GDP-forming] subunit beta, mitochondrial	3.6	Catalyzeds the GTP-dependent ligation of succinate and CoA to form succinyl-CoA.
NM_021644.2	P31942	*Heterogeneous Nuclear Ribonucleoprotein H3 (2H9) (HNRPH3), transcript variant 2H9A	3.3	Involved in splicing and participates in early heat shock-induced splicing arresst
NM_002925.3	O43665	Regulator of G-protein signaling 10 (RGS10), transcript variant 2	2.3	Inhibits signal transduction by increasing the GTPase activity of G protein alpha subunits.
NM_002969.2	P53778	mitogen-activated protein kinase 12 (MAPK12)	2.2	An essential component of the MAP kinase signal transduction pathway. cascades of cellular responses evoked by extracellular stimuli such as proinflammatory cytokines or physical stress
BC014667.1	P01857	Immunoglobulin heavy constant gamma 1 (G1m marker) (IGHG1)	2.1	Humoral immunity
NM_001744.2	Q16566	*calcium/calmodulin-dependent protein kinase IV (CAMK4)	2.1	calcium/calmodulin-dependent protein kinase that operates in the calcium-triggerd

				CaMKK-CaMK4 signaling cascade.
NM_024641.2	Q5SRI9	Glycoprotein endo-alpha-1,2-mannosidase	2.1	Hydrolysis of the terminal alpha-D-glucosyl-(1,3)-D-mannosyl unit from the GlcMan9(GlcNAc)2 oligosaccharide component of the glycoprotein produced in the Golgi membrane
NM_022476.1	Q9H8T0	AKT interacting protein (AKTIP), transcript variant 2	2.1	Promote vesicle trafficking and/or fusion via the homotypic vesicular protein sorting complex (the HOPS complex). Regulates apoptosis by enhancing phosphorylation and activation of AKT1. Increases release of TNFSF6 via the AKT1/GSK3B/NFATC1 signaling cascade.
BC027465.1	Q8N647	cDNA clone IMAGE:4155919, complete cds	2	unknown
NM_016009.2	Q9Y371	SH3-domain GRB2-like endophilin B1 (SH3GLB1)	2	May be required for normal outer mitochondrial membrane dynamics

Protein information was obtained from http://www.uniprot.org/.

Proteins for which the ProtoArray® signal intensity was more than 2-fold higher in the patients with bilateral S-SNHL than in the controls are listed ($p < 0.05$). *; proteins that were reported to exist in the inner ear sensory epithelium.

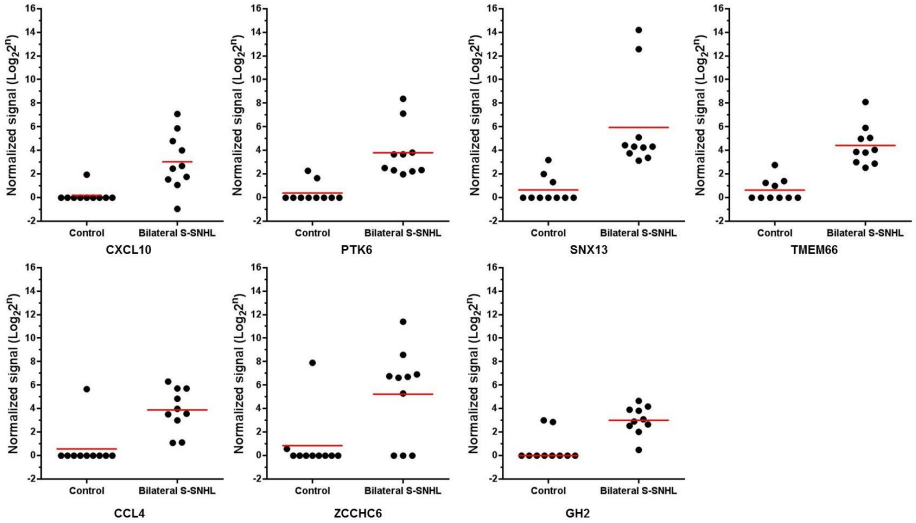

Figure 1. Difference in signal intensity of controls and patients in the ProtoArray® experiment. Normalized signal intensities of the antigens with a signal intensity more than 5-fold higher in the patients with bilateral S-SNHL than in the controls are presented. CXCL10, C-X-C motif chemokine 10; PTK6, protein tyrosine kinase 6; SNX13, sorting nexin 13; TMEM66, transmembrane protein 66; CCL4, chemokine (C-C motif) ligand 4; ZCCHC6, zinc finger; CCHC, domain containing 6; GH2, growth hormone 2.

3. Immune reaction of inner ear tissue antigens with patient sera

Western blotting was performed to investigate whether circulating autoAbs from patients with bilateral S-SNHL could produce an Ag-Ab reaction with antigens in the inner ear tissue. As a result, bands corresponding to molecular weights of 63–75 kDa and 35–48 kDa were commonly detected in the western

blots between the patients' sera and the inner ear tissues, whereas these bands were not generally visible in those between sera from the controls and the inner ear tissues.

Bands corresponding to a molecular weight of 63–75 kDa were detected in all of the patients and in 2 of the controls (yellow arrows in **Figure 2**). They were detected both in the cochlear and vestibular tissues in 8 of the 10 samples from patients with bilateral S-SNHL; however, one sample each showed the band only in the vestibule and cochlea, respectively.

Bands located between 35–48 kDa were detected in 7 of the patients with bilateral S-SNHL and in 2 of the controls (red arrows in **Figure 2**). They were detected both in the cochlear and vestibular tissues in 4 of the 7 patients, and the others were detected only in the vestibular tissues of 3 patients. The bands were detected only in the cochlea in 1 of the controls, and in both the cochlea and vestibule in 1 control subject. Next, we examined target antigens identified in the ProtoArray® experiment, which had molecular weights of 35–48 kDa and 63–75 kDa. As a result, following proteins had molecular weights in the range of interest: PTK6 (51.8 kDa), SUCLG2 (46.5 kDa), MICB (42.7 kDa), MAPK12 (41.9 kDa), SH3GLB1 (40.8 kDa), TMEM66 (37 kDa), HNRPH3 (36.9 kDa), and IGHG1 (36.1 kDa). These Ags may potentially be involved in inner ear autoimmune reactions.

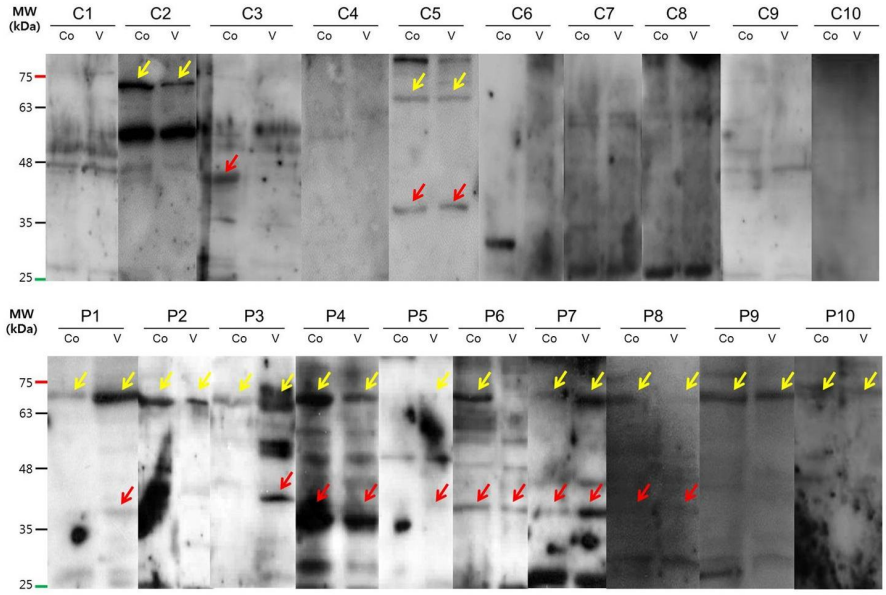

Figure 2. Western blot of the reaction of serum from controls and patients with mouse inner ear. Red and blue arrows represent the detected inner ear antigens with molecular weights of 63-75 kDa and 35-48 kDa. C, control; P, patient; Co, mouse cochlear tissue protein; V, mouse vestibular tissue protein.

4. Candidate inner ear antigens for the autoimmune reaction identified by immunoprecipitation and LC-MS/MS

LC-MS/MS was performed to identify differences in the protein composition of immune complexes in controls and patients with bilateral S-SNHL, which could provide clues to identify disease-specific autoAbs and their target inner ear antigens. It was performed with the Ag-Ab complexes acquired from immunoprecipitation followed by 1-DE. As the proteins of Ag-Ab complexes

identified by western blotting corresponded to the molecular weight of 35–48 kDa and 63–75 kDa, the bands located in those areas were excised and the proteins in those bands were concentrically analyzed (**Figure 3**). In the LC-MS/MS analysis, a total of 292 (patient 1), 849 (patient 2), 846 (control 2), and 166 (control 3) proteins were detected in the 35–48 kDa range, and a total of 560 (patient 1), 590 (patient 2), 1488 (patient 3), 1357 (patient 4), 1808 (control 1), 822 (control 2), 311 (control 3), and 1704 (control 4) proteins were detected in the 63-75 kDa range.

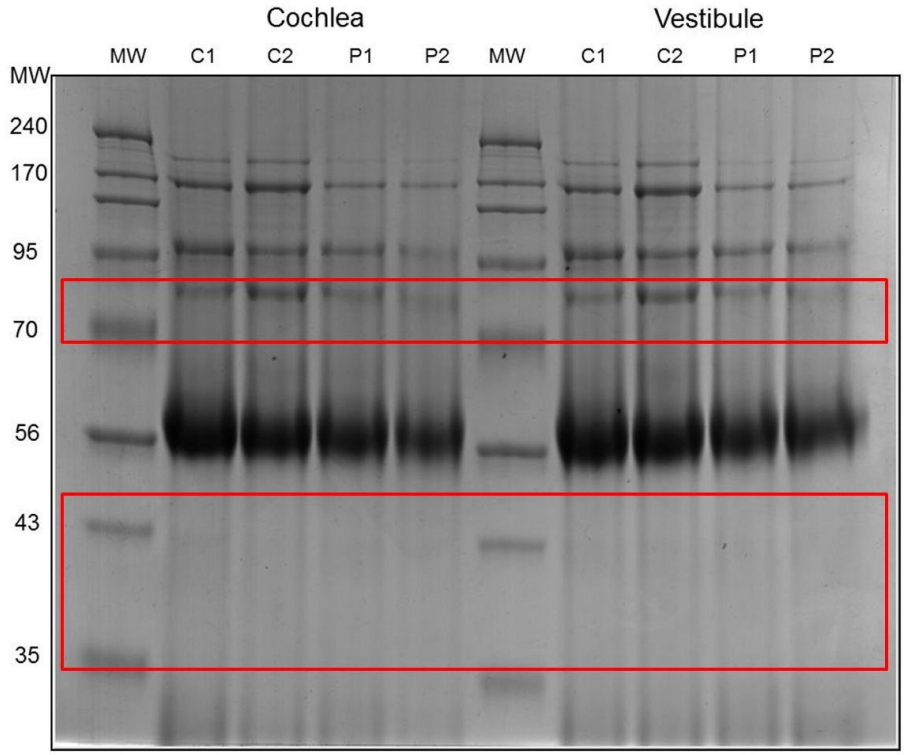

Figure 3. Representative figure of 1-DE for the acquisition of proteins of immune complexes from patients and controls. C, control; P, patient.

The proteins commonly and exclusively found in LC-MS/MS of patients with bilateral S-SNHL were analyzed and compared to those found in LC-MS/MS of controls. Consequently, a total of 32 proteins in the 35–48 kDa range and 9 proteins in the 63–75 kDa range were identified **(Table 3)**; these proteins were mostly immunoglobulin and its variants (35, 85.4%), which are speculated to be candidate autoAbs. These proteins were followed by hypothetical protein (3, 7.3%), albumin (1, 2.4%), CD5 antigen-like precursor (1, 2.4%), and haptoglobin (1, 2.4%). These proteins, with molecular weights of 35–48 kDa and 63–75 kDa, were possibly found from the result of the autoimmune and/or enhanced immune reactions in the inner ear. However, there was a discrepancy between the results of the LC-MS/MS and ProtoArray® experiments; the proteins detected in the LC-MS/MS were not identified in the ProtoArray® experiment.

Table 3. Proteins identified only in the sera of bilateral S-SNHL patients by LC-MS/MS

Access No.	Description (Taxonomy)	MW(kDa)	sequence coverage (%)	Peptide score
gi|10334625	immunoglobulin heavy chain [Homo sapiens]	35-48	21	44.9
gi|10334541	immunoglobulin heavy chain [Homo sapiens]	35-48	29	44.9
gi|10334555	immunoglobulin heavy chain [Homo sapiens]	35-48	29	44.9
gi|10334587	immunoglobulin heavy chain [Homo sapiens]	35-48	29	33.4
gi|10334611	immunoglobulin heavy chain [Homo sapiens]	35-48	21	44.9
gi|10334619	immunoglobulin heavy chain [Homo sapiens]	35-48	21	44.9

gi|229536	Ig A L	35-48	23	51.4
gi|229585	Ig A1 Bur	35-48	18	45.6
gi|70058	Ig alpha-2 chain C region - human	35-48	20	68.7
gi|229601	Ig G1 H Nie	35-48	34	37.5
gi|21410208	IGL@ protein [Homo sapiens]	35-48	33	38.5
gi|587348	immunoglobulin kappa light chain variable region [Homo sapiens]	35-48	15	98.8
gi|170684534	immunoglobulin lambda 2 light chain [Homo sapiens]	35-48	34	44.9
gi|229528	protein Len,Bence-Jones	35-48	19	33.4
gi|229526	protein Rei,Bence-Jones	35-48	32	44.3
gi|384376630	7A13 anti-H3N2 influneza HA immunoglobulin lambda chain, partial [Homo sapiens]	35-48	11	57.6
gi|146387531	* Chain A, Crystal Structure Of An Autoimmune Complex Between A Human Igm Rheumatoid Factor And Igg1 Fc Reveals A Novel Fc Epitope And Evidence For Affinity Maturation	35-48	31	37.5
gi|8569502	Chain A, Fc Fragment Of Human Igg1 In Complex With An Engineered 13 Residue Peptide Dcawhlgelvwct-Nh2	35-48	49	37.5
gi|166007160	Chain C, Solution Structure Of Human Immunoglobulin M	35-48	23	37.5
gi|230581	Chain H, Dir Primaerstruktur Des Kristallisierbaren Monoklonalen Immunoglobulins Igg1 Kol. Ii. Aminosaeuresequenz Der L- Kette, Lambda-Typ, Subgruppe I (German)	35-48	26	44.9
gi|401871522	* Chain L, Crystal Structure Of A Disease-Associated Anti-Human Gm-Csf Autoantibody Mb007	35-48	36	38.5
gi|315583384	Chain L, Crystal Structure Of Anti-Il-15 Antibody In Complex With Human Il-15	35-48	37	38.5
gi|353251918	Chain L, Crystal Structure Of Broad And Potent Hiv-1 Neutralizing Antibody Pgt127 In Complex With Man9	35-48	37	38.5
gi|353251927	Chain L, Crystal Structure Of Broad And Potent Hiv-1 Neutralizing Antibody Pgt128 In Complex With A Glycosylated Engineered Gp120 Outer Domain With Miniv3 (Eodmv3)	35-48	37	38.5
gi|353251903	Chain L, Crystal Structure Of Broad And Potent Hiv-1 Neutralizing Antibody Pgt128 In Complex With Man9	35-48	37	38.5
gi|42543068	Chain L, Crystal Structure Of Human Factor Ix Gla Domain In Complex Of An Inhibitory Antibody, 10c12	35-48	37	38.5
gi|238537877	* Chain L, Crystal Structure Of The Fab Fragment Of Anti-Cd20 Antibody Ofatumumab	35-48	21	48.5
gi|442828	Chain L, Structure Of A Human Monoclonal Antibody Fab Fragment Against Gp41 Of Human Immunodeficiency Virus Type I	35-48	40	59.3

gi|5174411	CD5 antigen-like precursor [Homo sapiens]	35-48	9	77.1
gi|1620396	haptoglobin [Homo sapiens]	35-48	2	52.9
gi|16554039	unnamed protein product [Homo sapiens]: immunoglobulin like	35-48	9	37.5
gi|34527425	unnamed protein product [Homo sapiens]: immunoglobulin like	35-48	22	37.5
gi|2414494	immunoglobulin heavy chain, constant region [Homo sapiens]	63-75	19	37.9
gi|371446827	immunoglobulin G heavy chain variable region, partial [Homo sapiens]	63-75	20	6.9
gi|371447905	immunoglobulin G heavy chain variable region, partial [Homo sapiens]	63-75	40	32.5
gi|304562906	immunoglobulin gamma 1 heavy chain variable region [Homo sapiens]	63-75	26	32.5
gi|229537	Ig A H	63-75	18	47.1
gi|7439150	Ig mu chain C region - human	63-75	5	23.9
gi|119602344	hCG2038942, partial [Homo sapiens]	63-75	5	23.9
gi|28592	serum albumin [Homo sapiens]	63-75	22	19.7
gi|33451	unnamed protein product [Homo sapiens]: Ig heavy chain	63-75	24	21.1

Protein searches were conducted in MASCOT (version 2.2.04) using the NCBI database. The search parameters were as follows: 1) Enzyme specificity – Trypsin; 2) Maximum missed cleavages – 1; 3) Carbamidomethyl ©; Oxidation (M); MASCOT results were filtered using a protein probability value of less than 0.05, translating to a false discovery rate of 0.95%. MW; molecular weight. *: proteins that were reported to be involved in autoimmune disorders.

IV. DISCUSSION

Several studies have proposed a number of inner ear target antigens and corresponding autoAbs for the autoimmune pathology in S-SNHL. However, those results were not observed in the context of hearing loss that occurred bilaterally and suddenly,[19-21] and the subjects in several studies had other co-occurring systemic autoimmune diseases.[22,23] Although the number of patients enrolled in the current study was small, we found reliable evidence for the existence of autoimmune reactions and possible target antigens in patients with bilateral S-SNHL by mass screening of the target antigens and investigating the protein composition of the actual Ag-autoAb complexes from patients and controls. Most of these methods have not been adapted in previous experiments, and to our knowledge, this is the first study to investigate target proteins with mass screening. In addition, we provided a possibility for the development of diagnostic biomarkers of autoimmune inner ear disorder by the identification of candidate target antigens that showed significantly increased immune reactions with the patients' sera.

1. Presence of circulating autoAbs and their target antigens (ProtoArray® analysis)

Protein microarrays have previously been used for the detection of autoAbs in patients with suspected autoimmune disorders, such as inflammatory bowel disease, ovarian cancer, type I diabetes, and multiple sclerosis, and have

shown promising results.[10,24-26] In an attempt to identify the presence of autoAbs in patients with bilateral S-SNHL, we evaluated the autoimmune profiles of a limited cohort of patients and controls using a ProtoArray® containing 9,400 human proteins as target antigens. Consequently, 20 antigens were identified to which the patient group had more than 2-fold higher signal intensity than controls; among these, 7 antigens showed more than 5-fold higher signal intensity. Although the exact location or function of these proteins is not fully understood, they were assumed to play various roles such as in cell signaling, humoral and cellular immunity, growth control, and maintenance of neurotransmitter release sites in specific cell types and tissues. However, the functions of one protein, cDNA clone IMAGE:4155919, remain unknown. Among the proteins, mRNAs of DPEP2, HNRPH3, and CAMK4 have been reported to exist in the inner ear sensory epithelium and ribbon synapse,[27] strongly suggesting their potential role as candidate target antigens. The definite role of each protein has not been identified. Among the proteins, HRNPH3 was identified as an autoantigen for acute anterior uveitis.[28] This protein was identified to exist in the inner ear sensory epithelium; although its functional relevance in the inner ear remains unclear. It is reported to be involved in splicing and heat-shock-induced splicing arrest, which suggests genetic causes for autoimmune hearing loss. The other protein, MICB, was also revealed to be involved in immune reactions, although it has not been identified in the inner ear. MICB acts as a stress-induced self-antigen that is

recognized by gamma delta T cells.[29] MICB belongs to a family of "stress-induced" major histocompatibility complex (MHC) class I-like proteins, which are generally expressed in normal tissues and monocytes. It can be induced by stress, such as heat shock, oxidative stress, or viral and bacterial infections, and is regarded to play an important role in immune regulation in the pathogenesis of systemic lupus erythematous.[30] Although the existence of MICB in the inner ear has not yet been verified, it can be a target antigen for autoimmune reactions in the specific cell types of the inner ear and/or an indirect evidence for increased immune reaction by increased expression in the immune-mediating cells. IGHG1, which is involved in humoral immunity, showed a 2.1-fold higher intensity in the experiment. Interestingly, enhanced immune reaction associated with IGHG1 has been reported in patients with Meniere's disease[31]. A previous study explains the phenomenon by the presence of circulating immune complexes and an excessive autoimmune/inflammatory reaction in the inner ear. As Meniere's disease and sudden SNHL share many similarities, increased reaction with IGHG1 in this study can support the possibility of autoimmune or increased immune reaction.

The results from the ProtoArray® experiment suggest the presence of circulating autoAbs in patients' sera and candidate target antigens for bilateral S-SNHL.

2. Immune reaction between circulating autoAbs and antigens of the inner ear

In the next step, western blotting was performed to demonstrate if the circulating autoAbs could induce an immune reaction with proteins in the inner ear. The results showed exclusive presence of an immune reaction between the two proteins in the patient group. Animal inner ear antigens that have been reported to react with sera from patients with bilateral S-SNHL have molecular weights in the 42, 58, 68, and 72 kDa ranges.[32,33] Consistent with previous findings, the results of the present study showed similar reactions, especially with proteins with molecular weights in the 63–75 kDa and 35–48 kDa ranges. The greater part of candidate proteins from the ProtoArray® had molecular weights of 35-48 kDa, while the Ag-Ab reaction was most frequent for proteins in the 63–75 kDa range. This discrepancy was likely to be due to several causes. First, the ProtoArray® system consists of human amino acid sequences for target antigens; however, we used mouse inner ear proteins for the western blot experiment for ethical reasons. Although a large portion of the epitope for Ag-Ab reactions overlapped between the human and mouse inner ear, there remains a possibility that the human and mouse inner ear proteins have different epitopes. Second, since the candidate target antigens from the ProtoArray® were determined by selecting the proteins that showed more than 2-fold higher signal intensity in patients with bilateral S-SNHL than in the controls, the target antigens that showed a

smaller increase in the signal intensity could be neglected. As observed in all the screening methods such as microarray and mass spectrometry, there may be a possibility of false-positive and false-negative results in the ProtoArray® due to denatured or inactive conformations of the arrayed proteins.[34] Despite the discrepancy between the two experiments, it was strongly suspected in our results that the proteins with molecular weights in the 63-75 kDa and 35-48 kDa ranges could be the main candidates for autoimmune causes for bilateral S-SNHL.

Among the twenty candidate proteins obtained from the ProtoArray® experiment, 8 proteins weigh around 35–48 kDa: PTK6, TMEM66, MICB, SUCLA2, HNRPH3, MAPK 12, IGHG1, and SH3GLB1. In particular, as HNRPH3 is known to be expressed in the inner ear, its role as a candidate antigen is more likely. We have shown the reaction between circulating autoAbs and inner ear tissue through western blotting, and found consistent responses among the patients to common candidate antigens.

3. Identification of candidate inner ear target antigens by IP and LC-MS/MS

Finally, we attempted to analyze the protein composition of actual Ag-Ab complexes from patients and control groups to identify candidate antigens and/or autoAbs. In addition, we tried to validate if the proteins identified through LC-MS/MS matched those identified through the ProtoArray®

experiment.

As a result, the most common proteins encountered exclusively in the patient group were immunoglobulins and their variants, which were likely to be candidate autoAbs for the autoimmune reaction. The presence of increased immunoglobulins and their variants could support the evidence of increased immune reaction in the patients with bilateral S-SNHL. Among these immunoglobulins and their variants, few proteins were identified to be involved in several autoimmune disorders. The chain A from human immunoglobulin M rheumatoid factor (gi|146387531) forms an autoimmune complex in patients with rheumatoid arthritis.[35] Partial chain of anti-human granulocyte-macrophage colony-stimulating factor (GM-CSF) autoAb (gi|401871522) was shown in the experiment, and the polyclonal autoAb against human GM-CSF is known as a hallmark of pulmonary alveolar proteinosis and several other reported autoimmune diseases.[36] CD 20 is a B-lymphocyte antigen that is expressed on the surface of all B-cells during the pro-B phase. Its function is to enable optical B-cell immune response; hence, synthetic monoclonal CD20 antibodies are now used to treat certain B-cell lymphomas and autoimmune diseases.[37] A fragment of the anti-CD20 Ab (gi|238537877) was found in the experiment, and the presence of the antibody could support its possibility as an autoimmune disease in bilateral S-SNHL. However, immunoglobulin variants are the most commonly found proteins in the serum, and it is possible that some portion of the identified

immunoglobulin could be a product of contamination. The other proteins such as serum albumin and haptoglobin could also be the result of contamination, as they are one of the most abundant blood plasma proteins, and are frequently found to be non-specific ones in experiments using blood samples. Furthermore, we did not try to remove nonspecific abundant antigens such as serum albumin and immunoglobulin by using multiple affinity removal columns during the experiment because this procedure could also remove autoAbs. There were three unnamed proteins; two (gi|16554039, gi|34527425) were revealed to be immunoglobulin-like proteins, and the other (gi|33451) was revealed to be the Ig heavy chain. While these proteins could be candidate autoAbs, they could be also non-specific ones. However, we could detect several proteins for the target antigens other than immunoglobulin and their variants, albumin, and haptoglobin. The CD5 antigen-like precursor, which was exclusively found in the patient group, is mainly involved in immune system processes, cellular defense, or inflammatory responses,[38] and it could be a target antigen for the immune reaction between circulating antibodies and inner ear antigens. Consequently, the proteins detected from the patient group support the possibility of autoimmunity as a cause of increased immune and inflammatory reactions in the inner ear.

It was found that the proteins detected through the ProtoArray® and those detected through the LC-MS/MS hardly match. This could be due to different characteristics of the two different experimental techniques for the detection

of proteins. The ProtoArray® used the Ag-Ab reaction between the patient's serum and protein sequences embedded on the chip and detects only increased immune reactions comparing the experimental and control samples. Even though the circulating autoAbs existed in the patient's serum, the results could be negative if the immune reaction between the serum and the target antigen sequence did not have high signal intensity for detection. LC-MS/MS cannot be used to identify differences in the protein expression level or protein signal intensity between the two groups. Instead, it can only identify the list of proteins that exist in the samples that are provided. Therefore, we could not compare the differences in the expression of specific proteins between the two samples, and could only identify the proteins that exclusively exist in one sample by comparing the lists of proteins in the two samples. In addition, LC-MS/MS detects proteins by analyzing the sample molecules broken into charged ions using statistical methods that correlate known masses with the identified masses through a characteristic fragmentation pattern. Therefore, there should be false negative results even though certain proteins exist in the samples. Although the *p* values in the mass spectrometry analysis were less than 0.05, only 14 proteins over 30%, and 26.8% (11 proteins) showed protein sequence coverage of under 10% in our mass spectrometry data (**Table 3**). Lower protein sequence coverage implies lesser possibility of being the candidate protein, although the possibility of false result was not high when unique amino acid sequences identified by LC-MS/MS were considered.

In summary, based on the assumption that bilateral S-SNHL is an autoimmune disease, we performed mass screening of patients with bilateral S-SNHL using several approaches. We proved the existence of circulating autoAbs and investigated several possible candidate antigens from the ProtoArray®. Western blotting confirmed the presence of an immune reaction between the circulating autoAbs and the inner ear tissues by showing consistent responses to common candidate antigens. Finally, LC-MS/MS was used to investigate a few possible candidate antigens and/or autoAbs with enhanced immune reactions. From these studies, it can be concluded that autoimmune reactions are a major pathological process underlying the disease, and multiple target antigens and autoAbs, rather than a single Ag-Ab combination, may be responsible for the autoimmune reaction associated with bilateral S-SNHL. However, in the absence of peptide sequencing, we cannot determine whether a particular protein is a specific antigen involved in an immune reaction.

4. Clinical implication and future direction

Autoimmunity as a pathological mechanism of hearing loss has been suspected in many cases, especially if the hearing loss is bilateral, progressive, and the patient has a history of autoimmune disorder or positive autoimmune profiles in the laboratory tests; however, the diagnostic method still remains unclear, which can delay proper management. In the future, definite

diagnostic method for autoimmune hearing loss should be developed for proper treatment and anticipation of patient's prognosis. Bilateral S-SNHL is a good model for investigating the evidence and pathologic mechanism of autoimmune hearing loss. In this study, we investigated the evidence of autoimmune reaction, the candidate antigens, and autoAbs and provided evidences for the autoimmunity as one of the pathologic mechanisms for bilateral S-SNHL. The results of this study can provide a basis for the diagnosis of autoimmune hearing loss. The development of highly sensitive diagnostic chips using multiple definite target antigens enables the accurate diagnosis of autoimmune hearing loss and proper management using anti-inflammatory or immune suppressive agents, which can finally delay the progression of autoimmune-induced hearing loss. In cases where autoimmune hearing loss, especially bilateral S-SNHL, several other causes such as genetic and central disorders should be ruled out before the decision of treatment methods. The multi-array diagnostic chip using biomarkers for autoimmunity enables differential diagnosis of the disease if combined with careful neurological examinations and history taking of family histories. However, to applicate these proteins as biomarkers for the bilateral S-SNHL, mass screening for multiple proteins in large population should be conducted and this effort can promise the improvement of diagnosis of autoimmune-related inner ear disorder.

V. CONCLUSION

The findings of this study suggest that autoimmunity could be one of the pathologic mechanisms underlying bilateral S-SNHL. Multiple antigens and autoAbs may be involved in the autoimmune reaction. Specific antigens identified to cause immune reactions with patient serum in ProtoArray® analysis may be candidates for diagnostic biomarkers of bilateral S-SNHL.

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ABSTRACT (IN KOREAN)

양측성 돌발성 난청 환자에서 프로테오믹스(Proteomics)를 이용한 자가 면역 반응의 규명

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돌발성 난청은 특별한 원인 없이 3일 이내에 3개 이상의 연속된 주파수에서 30dB이상의 감각신경성 청력 손실이 발생하는 것으로, 대부분 일측성으로 발생하지만 아주 드물게 양측성으로 나타나기도 한다. 현재까지 돌발성 난청의 원인에 대하여 정확하게 밝혀진 바는 없으나 다양한 가설들이 제기되고 있으며, 특히 양측성 돌발성 난청에서 자가 면역 반응이 원인일 수 있다는 보고가 있다. 이에 본 논문에서는 기존의 연구를 토대로 자가 면역 반응이 양측성 돌발성 난청의 원인일 수 있다는 가설 하에 그 근거를 찾고 원인 항원 및 항체를 밝혀, 질병의 기제를 이해하고 나아가 진단에 활용할 수 있는 생물 지표를 제공하고자 한다.

본 연구에서는 10명의 양측성 돌발성 난청 환자 및 10명의 정상 대조군에서 채취한 혈액을 이용하여 다양한 단백질체학적 접근을 통해 가설을 증명하고자 하였다. 첫째로 ProtoArray® 기법을 이용하여 상용화 된 9,400개의 인체 단백질과 혈청을 반응시켜 환자군에서 자가 항체의 존재를 확인하고, 이 항체들과 반응하는 후보 항원을 규명하였다. 이어 western blot 기법을 이용하여 환자군에 존재하는 자가 항체가 실제 내이 조직과 면역반응을 일으킴을 증명하였으며, 그 과정 중 환자군에서 공통적으로 반응을 보이는 항원이 존재함을 확인하였다. 마지막으로 고속 액체 크로마토 그래프 질량 분석법(LC-MS/MS)을 이용하여 실제 반응을

일으킨 항원-항체 면역복합체를 분리하고 분석하여 환자군에서 면역 반응이 증가되어 있음을 확인하고, 그 후보 자가 항체를 확인할 수 있었다. 세 단계의 시험 과정 중 중복되는 단백질 8개를 추려낼 수 있었으며, 이는 강력한 후보 항원 및 후보 자가 항체로 여겨진다.

본 연구를 통하여 자가 면역 반응이 양측성 돌발성 난청의 주요 원인임을 밝히고, 그 원인 항원 및 항체로 여겨지는 단백질을 검출하였다. 향후 이들을 이용하여 질병의 진단 및 경과에 대한 이해를 높일 수 있을 것으로 기대된다.

핵심되는 말: 양측성 돌발성 감각신경성 난청, 자가 면역 질환, 내이, 자가 항체

PUBLICATION LIST

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