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ORIGINAL ARTICLE

Evaluation and Clinical Significance of Serum Heat Shock Protein70, 60 and 27 on Hearing Recovery in Patients With Idiopathic Unilateral Sudden Hearing Loss

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Objective: According to earlier studies, heat shock proteins (Hsps) is expressed in cochlear cells after ototoxic stimuli. In this study we investigated the possibility that Hsps could be found in circulation and observed its possible clinical role in patients with sudden sensorineural hearing loss (SSNHL).

Materials and Methods: We enrolled 61 patients with idiopathic unilateral SSNHL and 30 normal controls for this study. Their serum Hsp70, 60 and 27 levels were measured using the enzyme-linked immunosorbent assay (ELISA) test and confirmed by Western blot immunoassay. Serum Hsps levels were analyzed to find the possible clinical value in patients with SSNHL.

Results: The patients with SSNHL did not show significantly higher serum Hsp70, 60 and 27 levels than the normal controls, as measured by the ELISA test ($P > 0.05$). There were no strong bands indicating the presence of Hsp70, 60 and 27 on the Western blot assay.

Conclusion: In this study, the serum Hsp70, 60 and 27 levels in the patients with SSNHL were not elevated as compared to the normal controls. There were no clinical significance of serum Hsp 70, 60 and 27 in hearing outcome of patients with sudden hearing loss.

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Introduction

Idiopathic sudden sensorineural hearing loss (SSNHL), so-called "sudden deafness," is defined as a hearing loss of sensorineural origin that is greater than 30 dB across three contiguous frequencies and that occurs in less than three days. The incidence rates per 100,000 people in the Taiwan population were 8.85 for men, and 7.79 for women [1]. While the actual cause of this disease is still unclear, microcirculation alterations or cochlear viral infections have long been suggested. Clinical and audiologic factors known to predict the treatment response and prognosis of the disease have been reported to include the patient's age, types of initial audiogram, severity of pre-treatment hearing loss, time interval between the onset of hearing loss and the start of treatment, and the simultaneous existence of tinnitus and vertigo [2].

The presence of antibodies to a 68-kDa antigen, identified by Western blot, was demonstrated in the sera of patients with SSNHL in previous studies [3-8]. The 68-kDa antigen was noted to be heat shock protein 70 (Hsp70) [4].

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Heat shock proteins (Hsps) are essential to maintaining the vital life process of the cell. Under normal conditions, these proteins uphold the physiological function of cells. However, under stress or in extraordinary conditions, heat shock proteins are expressed in large amounts to both prevent the accumulation of abnormal proteins and repair deteriorated proteins to increase the cells' defensive ability against stimuli. Previous studies have reported that Hsps in cochlear cells are expressed after ototoxic stimuli [9]. Sugarhara et al. reported on the protection of the inner ear against intense noise by heat shock response in an animal model in 2005 [10], suggesting that an increase in Hsp may offer protection or the release of tissular components. Recent reports have suggested an association between elevated serum Hsps level and various diseases or pathologic conditions, including excessive exercise [11], atherosclerosis [12], and cochlear disorders [9].

Several studies discussed the relationship between Hsp70 and SSNHL [9, 13-17]. Some studies revealed a positive association between Hsp70 and clinical outcome [9, 15, 16], but other studies not [10, 17].

Yang et al. revealed that anti-Hsp60 and anti-Hsp70 may play a role in the pathogenesis of noise-induced hearing loss [18]. Heat shock protein 27 was noted in rat cochlea and response to acoustic overexpose in guinea pig [19, 20]. But Hsp60 and 27 in sudden hearing loss was not investigated before. This is the first study to investigate the serum levels of Hsp 60 and 27 in patients with sudden hearing loss.

We conducted a prospective study in the Department of Otolaryngology, Kaohsiung Medical University Hospital, to measure and compare the levels of serum Hsp70, 60 and 27 in patients with SSNHL to healthy subjects functioning as the normal control. We adopted the highly sensitive, enzyme-linked immunosorbent assay (ELISA) test and confirmed our results by Western blot assay. Serum Hsps level were analyzed to find the possible clinical value in patients with SSNHL.

The aim of our study was to investigate whether serum Hsp70, 60 and 27 levels increased expression in patients with SSNHL. If we can truly understand the clinical role of serum Hsps levels, perhaps we can use these levels as a serum marker to predict clinical outcome in patients with idiopathic SSNHL.

Materials and Methods

A total of 61 patients with idiopathic unilateral SSNHL, admitted to the Department of Otolaryngology, Kaohsiung Medical University Hospital, along with 30 normal controls were included in this study which ran from June 2006 to September 2007. They were into the acute phase of the disease and fulfilled the following criteria for inclusion: 1) stable, unilateral SSNHL of at least 30 dB for three subsequent one octave steps in frequency occurring in less than 72 hours; 2) an interval of less than 7 days since the onset of hearing loss; 3) the absence of retrocochlear disease as confirmed by temporal bone computed tomography; 4) average hearing levels in the unaffected ear at less than 30 dB; 5) the absence of diabetes and hypertension; and, finally 6) a follow-up of more than 3 months with audiologic evaluation. The treatment protocol included an oral corticosteroid (1mg/kg) and a low molecular weight dextran infusion.

The diagnostic criteria of sudden hearing loss corresponded to the literature [21]. The severity of hearing loss was evaluated with the average hearing level at 5 frequencies (250, 500, 1000, 2000, and 4000 Hz) on the pure tone audiogram performed for the first time that sudden hearing loss was diagnosed. The improvement in hearing was assessed 3 months after the start of treatment according to the criteria proposed by Nomura [21].

The serum samples obtained from the peripheral blood of patients with SSNHL when the first day after admission and normal controls were stored at -80°C until use. Their serum Hsp70, 60 and 27 levels were measured by the enzyme-linked immunosorbent assay (ELISA) test and confirmed with Western blot immunoassay. We analyzed the correlation between the levels of serum Hsps in patients with SSNHL. Clinical variables including initial and final audiometric findings were collected.

Enzyme-linked immunosorbent assay (ELISA)

Measurement of serum Hsp 70 concentration

The antibodies to human Hsp70 in serum were assayed according to the commercial anti-human Hsp70 ELISA kit instructions (Stressgen Biotechnologies, San Diego, CA, Assay Designs, Ann Arbor, MI/USA). Briefly, the serum specimens and standards were added to the wells of a microplate pre-coated with recombinant human Hsp70 protein and incubated at room temperature for 2 hours with gentle shaking. After the removal of the unbound components by rinsing with a washing buffer, anti-human GAM-HRP conjugate was added and incubated for 1 hour. The unbound conjugate was washed off, and then a tetramethylbenzidine substrate solution was added and incubated for 15 minutes at room temperature in the dark. Reactions were stopped with 2N sulfuric acid. The absorbance was read at 450 nm. The anti-Hsp70 concentration in each specimen was calculated from the standard curve.

Measurement of serum Hsp27 and Hsp60 concentration

Hsp27 and Hsp60 in the serum were assayed using Hsp27 and Hsp60 ELISA kit, respectively (Stressgen Biotechnologies, San Diego, CA, Assay Designs, Ann Arbor, MI). Briefly, serum specimens and standards were added to the wells of a microplate pre-coated with mouse monoclonal antibody to Hsp27 or Hsp60, respectively for 1 hour at room temperature. Wells were washed with washing buffer and were then added goat anti-Hsp27 or anti-Hsp60, respectively for 1 hour. After unbound components were removed by washing the wells, HRP conjugated with anti-goat IgG was added to each well. After incubation for 30 minutes, the wells were washed, followed by the addition of tetramethylbenzidine substrate solution to develop color. The plate was incubated for 15 minutes in the dark at room temperature and absorbances of each well were read at 450nm after adding stop solution. Hsp27 and Hsp60 concentration in each specimen was calculated from the standard curve, respectively.

Western blot immunoassay

One dimensional gel was transferred to a PVDF membrane (Millipore Corporation, Billerica, MA) for 1.5 h at 400 mA using Transphor TE 62 (Hoeffer Inc., San Francisco, CA/USA). The membrane was then blocked overnight with blocking solution (50 mM Tris-HCl, pH 8.0, 0.25% gelatin, 150 mM NaCl, 5 mM EDTA, 0.05% Tween 20). The first antibody mouse anti-Hsp27, mouse anti-Hsp60 and mouse anti-Hsp70 monoclonal antibody (BioVision, Inc., Mountain View, CA/USA) with 1:3,000 dilution was added to the membrane and incubated at room temperature for 2 h. The membrane was washed three times in PBST (10 mM NaH2PO4, 130 mM NaCl, 0.05% Tween 20), and then probed with the second Ab (goat anti-mouse IgG and horseradish peroxidase conjugate, 1:5,000 in blocking solution) for 1 h. After washing with PBST for three times, the enzyme activity on the blot was visualized through chemiluminesence by adding ECL Western Blotting Reagents (Pierce Protein Research Products, div. of Thermo Fisher Scientific, Rockford, IL/USA).

Statistical Analysis

For the statistical analysis of the results, SPSS for Windows (version 13.0, SPSS, Inc., Chicago, IL) was used. The differences between the means of two groups (SSNHL and normal control) were calculated by a two-sample t test and a $\chi 2$ test. Results at the $P < .05$ level were considered statistically significant.

Ethical Considerations

This study was approved by the Kaohsiung Medical University Hospital Institutional Review Board, and informed consent was obtained from all participants.

Results

A total of 61 patients with SSNHL including 34 (55.7%) men and 27 (44.3%) women were enrolled in the study. The mean age of patient was 47 (range 15–77) years and control was 30 years. The right ears were affected in 27 (44.3%) and the left ears in 34 (55.7%) patients. The mean interval between hearing loss and the beginning of treatment (serum sampling) was 3.62 days. According to Nomura's classification for

recovery from SSNHL, 19 (31.1%) patients showed cured, whereas 17 (27.9%) patients had marked recovery, 14 (23.0%) patients had slight recovery, and 11 (18.0%) patients showed unchanged (Table 1). The initial hearing loss was 69.93 dB, and the final hearing level was 37.02 dB. The Average hearing gain was 32.91 dB.

The ELISA test revealed that the mean value of the serum Hsp70, 60 and 27 level in patients with SSNHL was 124.57 ± 93.73 ng/ml, 14.64 ± 52.28 ng/ml and 4.63 ± 3.16 ng/ml while that of the normal control was 136.03 ± 102.64 ng/ml, 10.29 ± 21.40 ng/ml and 4.13 ± 1.82 ng/ml respectively (Table 2). The serum Hsps 70, 60 and 27 levels were not significantly higher in the patients with SSNHL than in the normal controls as stated by the ELISA test ($P > 0.05$), and their presence was confirmed, but not as strong bands in the Western blot assay (Figures 1-3).

Discussion

The etiology and pathogenesis of idiopathic SSNHL are controversial. Serum markers, such as heat shock protein originating from the cochlea and correlating to the clinical characteristics and prognosis of SSNHL, have not been well studied. The objective of this study was to determine the presence and relation of Hsp70, 60 and 27 and idiopathic SSNHL. The clinical significance of serum Hsp 70, 60 and 27 in patients with sudden hearing loss were studied.

Table 1. Demographic data of sudden hearing loss patients and control group.

	Patients	Control
Age (mean ± SD)(range)	47.02 ± 14.99 (15- 77)	30.93 ± 5.82
Sex		
Male	34 (55.7%)	21 (70%)
Female	27 (44.3%)	9 (30%)
Side		
Right	27 (44.3%)	
Left	34 (55.7%)	
Recovery		
Cured	19 (31.1%)	
Marked	17 (27.9%)	
Slight	14 (23.0%)	
Unchanged	11 (18.0%)	
Initial hearing loss (dB)	69.93 ± 21.50 (22 - 103)	
Final hearing level (dB)	37.02 ± 24.20 (5 -96)	
Hearing gain (dB)	32.91 ± 20.91 (0- 77)	
Contralateral hearing (dB)	20.02 ± 14.47 (7- 104)	

Table 2. Statistical comparison of the Hsp70, 60 and 27 serum levels between the patients with SSNHL and control group.

	Group		
	Patients	Control	P
Number	61	30	
Heat shock proteins			
Serum Cons. (ng/ml)			
Hsp70	124.57 ± 93.73	136.03 ± 102.64	0.528
Hsp60	14.64 ± 52.28	10.29 ± 21.40	0.192
Hsp27	4.63 ± 3.16	4.13 ± 1.82	0.065

t test, p > 0.05

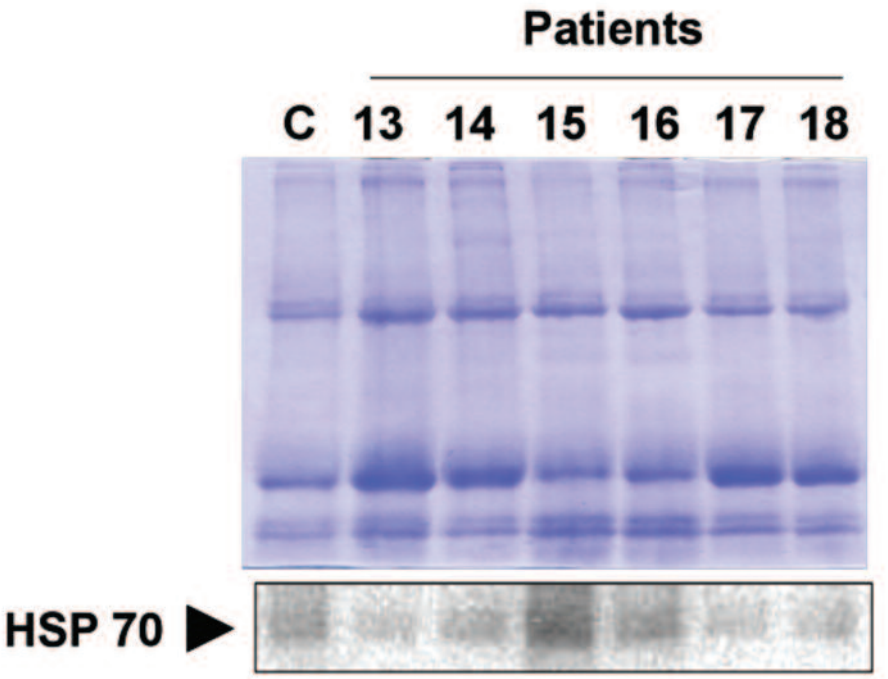

Figure 1. 1D SDS-PAGE and Hsp70 Western blot analysis of the plasma of SSNHL patients and healthy controls. Each well is loaded with 20 μg of plasma protein. C: healthy controls. 13–18 are individual patients.

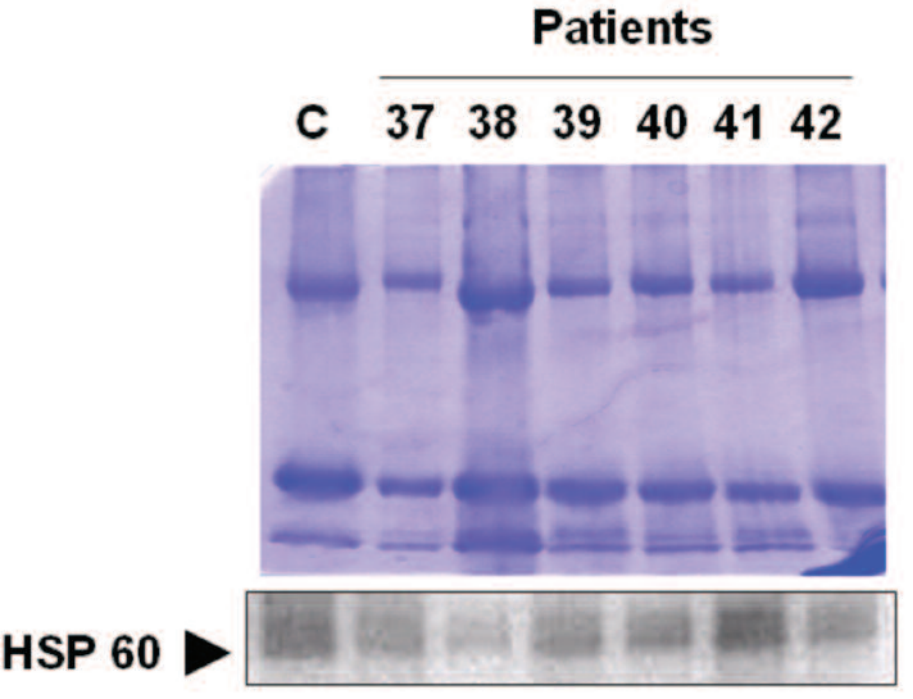

Figure 2. 1D SDS-PAGE and Hsp 60 western blot analysis of plasma of SSNHL patients and healthy people. Each well loaded with 20 ug plasma protein. C: healthy people. 37-42 are individual patients.

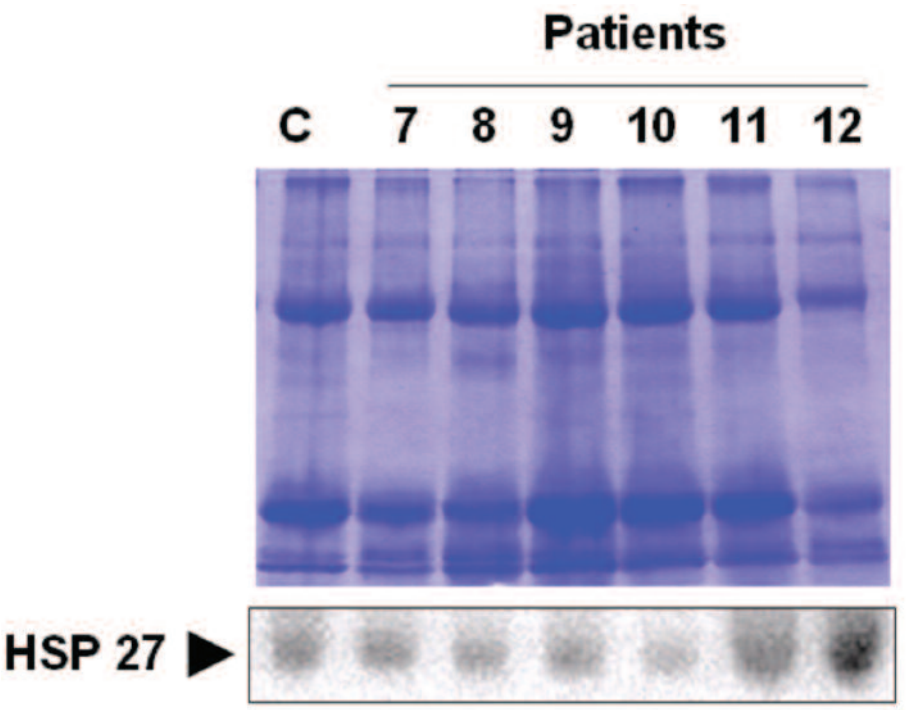

Figure 3. 1D SDS-PAGE and Hsp 27 western blot analysis of plasma of SSNHL patients and healthy people. Each well loaded with 20 ug plasma protein. C: healthy people. 7-12 are individual patients.

Heat shock proteins, which are believed to protect cells by dissolving and refolding miscoded or denatured proteins, are induced by various forms of stress including heat, ischemia, free radicals, and toxic agents [22]. Cells produce high levels of Hsps to protect themselves against these unfavorable conditions. A previous study showed that Hsp70 may be released from damaged cochlear cells into the blood during cochlear insult [9].

Several studies discussed the relationship between Hsp70 and SSNHL [9, 13-17]. Park el al. revealed that serum Hsp70 levels were significantly higher in the sera of patients with SSNHL than in normal controls 9. Patients with lower initial serum Hsp70 levels also showed better recovery from hearing loss [9]. Hsp70 levels may, consequently, have a clinical role to play in predicting the prognosis for hearing loss in patients with SSNHL.

However, some studies reported that, the Hsp70 lacks clinical utility for diagnostic screening in patients with sudden deafness [13-17]. No difference between patients and healthy controls was noted 13. Additionally, the anti-Hsp70 antibody does not offer clinically useful information in the treatment of SSNHL [17].

Gross et al. revealed a positive association between Hsp70 antibodies and a positive outcome of SSNHL [16]. Tebo et al. reported that the anti-Hsp70 antibody levels were also significantly higher in the patients as compared to the controls [15]. SSNHL patients with positive anti-Hsp70 antibodies had better responses to corticosteroid treatment [3, 8, 16]. Our study showed that no statistically significant difference was found between the Hsp70 levels in SSNHL patients and the normal controls. There was no clinical significance of serum Hsp70 level in patients with sudden hearing loss.

Yang et al. revealed that anti-Hsp60 and anti-Hsp70 may play a role in the pathogenesis of noise-induced hearing loss [18]. Heat shock protein 27 was noted in rat cochlea and response to acoustic overexpose in guinea

pig [19, 20]. But Hsp60 and 27 in sudden hearing loss was not investigated before. This is the first study investigated the serum levels of Hsp 60 and 27 in sudden hearing loss patients. But our study showed that no statistically significant difference was found between the Hsp 60 and 27 levels in SSNHL patients and the normal controls. There were no clinical significance of serum Hsp60 and 27 levels in patients with sudden hearing loss.

The limitation of this study is that we measured the systemic not the localized cochlea blood Hsps levels. One would not expect abnormalities in systemic blood levels with isolated organ dysfunction because cochlear blood flow accounts for a tiny fraction of cardiac output. It is quite possible that cochlear blood could have massive changes in Hsps levels that would not be detected. While the optimum design would be to study cochlear blood samples, this may be difficult.

Conclusion

This study showed that the serum Hsp70, 60 and 27 levels in patients with idiopathic unilateral SSNHL are not elevated as compared to the normal controls. Serum Hsp70, 60 and 27 can not provide clinical significance for hearing outcome of idiopathic unilateral SSNHL.

Acknowledgments

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