Association of Polymorphisms of Heat Shock Protein 70 with Susceptibility to Noise-Induced Hearing Loss in the Taiwanese Population

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Key Words
Noise-induced hearing loss · Susceptibility · Heat shock protein · Polymorphism · Haplotype

Abstract
Noise-induced hearing loss (NIHL) is the major cause of adult sensorineural hearing loss. It is a complex disease caused by the interaction of environmental and genetic factors. Previous studies found that heat shock proteins (HSPs) were associated with the development of NIHL. Specifically, polymorphisms in the heat shock protein 70 (HSP70) gene family are associated with a susceptibility to NIHL. In this study, three single nucleotide polymorphisms (SNPs) of the HSP70 family (SNP1: rs2075800; SNP2: rs1043618; SNP3: rs2763979) were genotyped in 349 noise-exposed Taiwanese workers. The subjects were categorized into noise-susceptible (NS; \(n = 27\)) and general susceptibility (GS; \(n = 322\)) groups by the change of a 4K-weighted audiometric average in an interval of 5 years. The G/C genotype of SNP2 was found to be associated with NIHL susceptibility (adjusted OR = 2.634; 95% CI = 1.096–6.328). No significant association was found for SNP1 and SNP3 with NIHL susceptibility. Analysis of haplotypes composed of these three SNPs revealed a significant association between NIHL susceptibility and haplotype CCC (OR = 2.197; 95% CI = 1.110–4.370). In conclusion, the genetic polymorphisms in the HSP70 genes seem to be associated with the individual's susceptibility to NIHL in the Taiwanese population. These findings could be used as a reference in the understanding and prevention of NIHL.

Introduction

Noise-induced hearing loss (NIHL) is the major cause of adult sensorineural hearing loss. It is a complex disease caused by the interaction of environmental and genetic factors. Currently, the mechanism of NIHL is not fully understood. The possible pathogenesis may involve the direct mechanical destruction of the structures of the cochlea and inner ear cell apoptosis/necrosis caused by the metabolic products generated during signal transduction [Henderson et al., 2006; Le Prell et al., 2003, 2007]. Potential pathological pathways associated with damage from noise exposure have been proposed in previous studies [Henderson et al., 2006; Le Prell et al., 2003]. In the proposed pathways, reactive oxygen species were the major...
substances responsible for DNA and protein damage leading to apoptosis, whereas heat shock proteins (HSPs) played protective roles to prevent cell death.

Yoshida et al. [1999] found a 100- to 200-fold increase in HSP mRNA in the cochlea of mice after heat treatment, and the mice treated with heat stress were more resistant to NIHL than those without heat treatment. In Chinese automobile workers, there was a higher level of antibodies against 70-kDa heat shock proteins (HSP70s) associated with high frequency hearing loss [Yang et al., 2004]. The HSP70s are the most widely investigated HSPs in human. The human HSP70 gene family consists of HSP70-1 (HSPA1A), HSP70-2 (HSPA1B), and HSP70-Hom (HSPA1L) [Milner and Campbell, 1990]. The expression of both HSPA1A and HSPA1B is heat-inducible and these two genes encode an identical protein product of 641 amino acids. HSPA1L is not heat-inducible and encodes a protein that is highly related to HSPA1A [Milner and Campbell, 1990].

Studies have shown associations between three single nucleotide polymorphisms (SNPs) in the three HSP70 genes (rs1043618, rs1061581, and rs2227956) and NIHL [Konings et al., 2009; Yang et al., 2006]. Significant associations of these three SNPs with NIHL were found in the Swedish population, but only the SNP rs2227956 of HSPA1L was significant in the Polish population [Konings et al., 2009]. However, no significant association of each of these three SNPs with NIHL was found in a Chinese population [Yang et al., 2006]. Significant associations between NIHL and some haplotypes of these three SNPs were found, but with ethnic differences. The findings of the above studies indicated that HSP70 genes may be NIHL susceptibility genes, and could potentially be used for NIHL monitoring to help decrease the prevalence of NIHL. However, the minor allele of rs2227956 is relatively infrequent (0.100–0.189) in Asian populations and thus may limit these practical applications. In this study, we searched for more frequent SNPs in HSP70 genes in Asians, and analyzed the distribution and association of these SNPs with the susceptibility to NIHL. Ultimately, this will lead to possible applications of the use of SNPs for the prevention of NIHL.

### Materials and Methods

#### Subjects

The subjects of this study were factory workers who received annual health examinations performed by the Department of Preventive Medicine, Kaohsiung Medical University Hospital, over the years 2003–2008. All the subjects included in this study were exposed to noise levels exceeding 85 dBA but limited to 90 dB time-weighted average (90 dB TWA) during their working hours; thus, they were considered to have a similar dosage level of noise exposure. The health examination included routine blood work, regular blood chemistries, general physical examination, otoscopic examination and pure-tone audiometry (PTA). During the health examination, personal medical history, smoking habits, use of hearing protectors at work, and habitual use of drugs were queried. Subjects with ear diseases that may affect hearing thresholds (e.g. otitis media, cholesteatoma, ear canal stenosis, etc.) and those who used potentially ototoxic drugs (e.g. aspirin, quinolones, aminoglycosides, etc.) were excluded from this study.

### Audiological Assessment and Definitions of Noise Susceptibility

PTA was performed using the Beltone 120 audiometer (Beltone Electronics Corp., Chicago, Ill., USA) and TDH 50-P earphones (Beltone) calibrated to ISO 389 (1975). All audiometric tests were performed using standard procedures by trained technicians in sound-attenuating booths that met the requirements of the Council of Labor Affairs, Executive Yuan, Taiwan. The audiometric data were recorded at the frequencies 500, 1000, 2000, 3000, 4000, and 6000 Hz. The noise hearing level was defined with a 4K-weighted pure-tone audiometric average (4KWPTA), which is the average of the bilateral hearing levels recorded at 3000, 6000, and two-weighted 4000 Hz. Because the 4K notch is more closely related to noise than other frequencies [McBride and Williams, 2001], we believed the 4KWPTA to be more reflective of the hearing injury from noise. The individuals’ PTA data documented in 2003 were set as the baseline hearing levels. The differences in 4KWPTA between the years 2003 and 2008 were computed as a threshold shift. To analyze the effect of genetic differences on the susceptibility to noise, the cases were classified into two groups. The cases with a threshold shift <10 dB were assigned to the noise non-susceptible (general susceptibility; GS) group, while those with a threshold shift ≥10 dB were assigned to the noise-susceptible (NS) group.

### Selection and Genotyping of HSP70 Polymorphisms

The SNPs for evaluation in this study were selected as tagging SNPs (tSNPs) via the HapMap bioinformatics website (www.hapmap.org). The criteria of tSNP searching was limited to Chinese Han Beijing population with a minimal allele frequency of >0.3, and a linkage disequilibrium value of (R^2) >0.8. The results are listed in table 1.

Peripheral blood specimens were obtained with the consent of the examinees. Each specimen was collected in a heparin tube and was centrifuged (2000 g, 20 min). The buffy coat was isolated and DNA was extracted using a commercial DNA extraction kit (Gentra Corp., Minneapolis, Minn., USA). Genotypes for the selected polymorphisms were screened with the ABI TaqMan SNP genotyping assays (Applied Biosystems, Foster City, Calif., USA) by using the pre-designed commercial genotyping assays listed in table 1. The extracted DNA and genotyping assays were added to TaqMan universal PCR master mix (Roche, Branchburg, N.J., USA) according to the manufacturer’s instructions. The genotyping procedures were then performed by ABI PRISM® 7500 real-time PCR system (Applied Biosystems). The results were analyzed using ABI 7500 System sequence detection software version 1.2.3 (Applied Biosystems).
Statistical Analysis

All the data were computerized and analyzed with SPSS statistical software version 16.0 (SPSS Inc., Chicago, Ill., USA). Continuous data were analyzed by independent-sample Student’s t tests. Categorical data were computed by two-sided χ² tests or Fisher’s exact tests. Genotype and allele frequencies for each SNP were calculated. The differences in genotype and allele frequencies between GS and NS groups were compared by the χ² test. The odds ratio (OR) and 95% CI of each genotype were computed by logistic regressions to test the association of genotypes with noise susceptibility. Furthermore, adjusted ORs and 95% CIs that controlled for the affects of age, gender, smoking (yes; no), hearing protector use (yes; no), and the worker’s factory were also computed to obtain more precise associations between genotypes and noise susceptibility. The Hardy-Weinberg equilibrium and haplotype analyses of these HSP70 SNPs were performed by using the program package PLINK (http://pngu.mgh.harvard.edu/purcell/plink/) [Purcell et al., 2007]. The level of statistical significance was set at p < 0.05.

Ethical Concerns

The participants were free to refuse the genetic testing, and employers were not permitted to know the results. This study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital (approval No. KMUH-IRB-950281) and informed consent was obtained from each subject.

Results

Characteristics of the Subjects

A total of 349 participants from 6 similar petrochemical factories (synthetic resin, rubber and plastic factories) enrolled in this study. The participants included 336 men and 13 women with an average age of 44.13 (range 26–60) years. Of the participants, 242 (69.34%) were non-smokers, while the other 107 (30.66%) were smokers. Only 15 (4.30%) of these workers did not use hearing protection devices under noisy working circumstances. There were 27 (7.74%) noise-susceptible individuals and 322 (92.26%) noise non-susceptible subjects. The comparisons of the subjects in NS and GS groups are shown in table 2. The age, gender, smoking habits, and use of hearing protection devices were not significantly different between these 2 groups.

Distribution and Association of HSP70 Genotypes and Alleles with Noise Susceptibility

The distributions of genotypes of the HSP70 SNPs are listed in table 3. There was no significant difference in the distribution of genotypes of both SNPs rs2075800 (HSPA1L; SNP1) and rs2763979 (HSPA1B; SNP3) between GS and NS groups. The difference of the genotype distributions between the GS and NS groups for SNP rs1043618 (HSPA1A; SNP2) showed only borderline significance (p = 0.059).

For analyses of the association between HSP70 genotypes and noise susceptibility the crude and adjusted ORs were computed. As the results show in table 3, no specific genotype was related to increased noise susceptibil-
ity in SNP1 and SNP3, and the distribution of alleles between GS and NS groups was not significantly different for each SNP (p = 0.568 and 0.164, respectively). However, the heterogeneous genotype G/C of SNP2 was associated with increased noise susceptibility (p = 0.040 and 0.030, with and without adjustments for age, gender, smoking, hearing protector use, and the worker’s factory). The OR of this heterozygous genotype in comparison with the G/G genotype was about 2.5, regardless of the control of possible confounding factors. Although the G/C genotype of SNP2 was associated with increased susceptibility to NIHL, the distributions of G and C alleles were not found to be different significantly between GS and NS groups (p = 0.351).

**Association of HSP70 SNP Haplotypes with Noise Susceptibility**

Five common haplotypes were found from the analyses of the combinations of the three SNPs. The distribution of haplotypes between GS and NS groups are listed in table 4. One of these haplotypes, Haplo3 (CCC), was found to be significantly associated with increased noise susceptibility (p = 0.031). The OR for increased noise susceptibility of this haplotype was 2.197 (95% CI = 1.110–4.370) in comparison with all other haplotypes. The other haplotypes were not found to be associated with increased noise susceptibility.

**Discussion**

In this study, we analyzed three SNPs located in 3 genes of the HSP70 family in a Taiwanese population. SNP1 of the HSPA1L gene is a non-synonymous variation; lysine is substituted for glutamate. Spagnolo et al. [2007] reported a strong association between HSPA1L SNP rs2075800 and the increased risk of uveitis in patients with sarcoidosis, and suggested that the C allele of...
this SNP is a risk factor of sarcoidosis-related uveitis. They postulated that the altered expression of HSPA1L may influence the ability of the cell to withstand inflammation or alter the apoptotic threshold within the eye. In our study, a significant difference in allele distribution was not found suggesting that the influence of HSPA1L expression may be organ-dependent.

SNP2 of HSPA1A is positioned in the 5′/H11541 untranslated region. In the study by Konings et al. [2009], the authors noted that the Swedish workers with G/G phenotype were more resistant to NIHL, whereas those with C/G or C/C genotypes were more susceptible to NIHL. In our study, subjects with heterogeneous G/C genotype were found to be more susceptible to NIHL than the subjects with homogeneous G/G genotype of SNP2 (table 3). This result was similar to that reported in the Swedish cohort. In contrast, although the population in our study (Taiwanese) was ethnically similar to the Chinese Han Beijing population population, the association of SNP2 (rs1043618) with noise susceptibility in this study was different from that reported in a Chinese population by Yang et al. [2006], who did not detect a significant association with NIHL. The cause of this discrepancy may be due to the different definitions of noise susceptibility between these studies. Yang et al. [2006] defined a hearing threshold worse than 25 dB in either low (i.e. mean threshold of 500, 1000, and 2000 Hz) or high frequencies (i.e. mean thresholds of 4000, 6000, and 8000 Hz) as hearing loss in their study, while we used the 4KWPTA as the basis to define the NS and GS subjects.

According to previous studies, individuals exposed to occupational noise levels of 85 dBA carried 7–9% chance of sustaining notable hearing loss after a 10-year exposure period [Alberti, 1996; Godlee, 1992]. In our study, we used the 5-year hearing threshold shift of ≥10 dB in the 4KWPTA to distinguish noise-susceptible individuals from those who were not susceptible to NIHL. The proportion of noise-susceptible subjects in this study (7.74%) corresponded to the above studies, indicating that the 4KWPTA is a good method to identify noise-susceptible individuals.

SNP3 is located 936 bp upstream of the 5′ end of the HSPA1B gene, and it is considered that it is related to and belonging to the gene HSPA1B. Although this SNP is not directly positioned within the gene HSPA1B, it was the most qualified SNP found on the HapMap website based on our selection criteria. On the other hand, since Milner and Campbell [1992] analyzed the genetic polymorphisms of HSPA1A, HSPA1B, and HSPA1L in 1992, the SNP A1267G (i.e. rs1061581) has been considered a major SNP of HSPA1B. Several studies used rs1061581 as the representative SNP of HSPA1B to analyze the associations of genetic polymorphisms with different diseases [Jalbout et al., 2003; Konings et al., 2009; Vargas-Alarcon et al., 2002; Yang et al., 2006]. Another SNP, rs539689, is also qualified to be a representative SNP of HSPA1B. This SNP meets the selection criteria described in the ‘Methods’ section. More importantly, it is located within the HSPA1B genetic area as a synonymous coding SNP. Unfortunately, because rs1061581 and rs539689 were not a part of the HapMap project, we failed to retrieve these SNPs at the initiation of this study.

The association of haplotypes with noise susceptibility was analyzed in this study. Five common haplotypes were found in our population (table 4). Among them, the haplotype CCC was found to be significantly associated with the susceptibility to NIHL. In earlier studies, Konings et al. [2009] and Yang et al. [2006] analyzed the associations of three SNPs of HSPA1L (rs2227956), HSPA1A (rs1043618), and HSPA1B (rs1061581) with NIHL in independent populations and found different haplotypes and associative significances in each population. Although

### Table 4. Distribution of haplotypes and odds ratios in the noise-susceptible and non-susceptible groups

<table>
<thead>
<tr>
<th>Haplotypes&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Susceptible group (n, %)</th>
<th>Non-susceptible group (n, %)</th>
<th>Total (n, %)</th>
<th>OR&lt;sup&gt;b&lt;/sup&gt;</th>
<th>95% CI&lt;sup&gt;b&lt;/sup&gt;</th>
<th>p value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haplo1 (CCT)</td>
<td>3 (10.63)</td>
<td>51 (15.76)</td>
<td>54 (15.47)</td>
<td>0.615</td>
<td>0.242–1.560</td>
<td>0.278</td>
</tr>
<tr>
<td>Haplo2 (CGT)</td>
<td>2 (6.04)</td>
<td>30 (9.40)</td>
<td>32 (9.17)</td>
<td>0.575</td>
<td>0.164–2.020</td>
<td>0.353</td>
</tr>
<tr>
<td>Haplo3 (CCC)</td>
<td>7 (26.41)</td>
<td>49 (15.14)</td>
<td>56 (16.05)</td>
<td>2.197</td>
<td>1.110–4.370</td>
<td>0.031&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Haplo4 (TGC)</td>
<td>9 (35.19)</td>
<td>126 (39.13)</td>
<td>135 (38.68)</td>
<td>0.830</td>
<td>0.452–1.530</td>
<td>0.547</td>
</tr>
<tr>
<td>Haplo5 (CGC)</td>
<td>6 (21.74)</td>
<td>66 (20.57)</td>
<td>72 (20.63)</td>
<td>1.077</td>
<td>0.538–2.150</td>
<td>0.835</td>
</tr>
</tbody>
</table>

<sup>a</sup> The allele order is rs2075800 (HSPA1L), rs1043618 (HSPA1A), and rs2763979 (HSPA1B) from left to right.

<sup>b</sup> Omnibus as the reference group. * p < 0.05.
the analyzed SNPs were different in our study from those of Konings et al. [2009] and Yang et al. [2006], the represented genes were the same among these 3 studies. The findings in these 3 studies supported the hypothesis that genetic polymorphisms of HSP70 family may affect the individual’s susceptibility to NIHL.

Two unexpected phenomena were observed in this study. First, the use of hearing protection devices did not lead to a significant difference between the NS and GS groups. Although the percentage of those who did not wear protective devices was higher in the NS group (7.41%) than those in the GS group (4.04%) (table 2), the statistical result showed no significant difference. This phenomenon could be explained by the small number of unprotected workers (15 persons, 4.30% of total subjects) in our population. The effect of factors that are represented by such a low percentage might be diluted or neglected during the statistical process, causing an insignificant result. The second phenomenon was that the smoking habit was not associated with the exacerbation of NIHL in several other studies, smoking was found to be associated with the exacerbation of NIHL [Agrawal et al., 2009; Ferrite and Santana, 2005; Mizoue et al., 2003; Wild et al., 2005]. In studies in which the difference in hearing thresholds had been calculated, the average hearing threshold of smokers was found to be 6–7 dB worse than that of non-smokers under the same level of noise exposure [Agrawal et al., 2009; Wild et al., 2005]. However, in our study, we categorized our participants into NS and GS groups based on the cut-off point of a 10-dB change in 4KWPTA in a period of 5 years. The higher threshold change used in our study might mask the effects of smoking on the susceptibility to NIHL, resulting in our finding of no association.

In this study, only genotype G/C of SNP2 was found significantly associated with NIHL. To explain this phenomenon, we regrouped the genotypes of SNP2 (C/C+G/C versus G/G and G/G+G/C versus C/C) and analyzed the association of these grouped genotypes with NIHL. There seemed to be a tendency for the subjects carrying the genotypes with the C allele (i.e. C/C and G/C) to be more susceptible to NIHL than those with only the G allele (OR = 2.150; 95% CI = 0.915–5.053). However, this tendency only reached a marginally significant level (p = 0.074). Such a result may be contributed by the small number of subjects in the NS group. It is reasonable to expect the tendency to be significant if the number of NS subjects is increased.

The major limitation of this study was the small number of subjects in the NS group. Someone may therefore criticize the statistical power of this study. However, comprehensive audiometric data for 5 years was required for each participant and thus limited the number of qualified subjects. In our database, there were only a few factories with greater than 50 noise-exposed workers. To increase the number of subjects in the NS group, more participants had to be recruited from other factories. However, including additional origins of the participants would decrease the similarities of the subjects and increase confounding factors, which may increase the complexity of the statistics. A multi-institutional project or seeking larger factories for investigational cooperation may be solutions for such conditions.

**Conclusions**

We analyzed three SNPs located in 3 genes of the HSP70 family in an Asian population. Our results may support the hypothesis that the genetic polymorphisms in the HSP70 genes are associated with the individual’s susceptibility to NIHL. The HSP70 family may play a key role in the development of NIHL. Further investigation of more representative SNPs of HSP70 genes and functional analyses are warranted to gather more information, which may lead to applications for the prevention of NIHL.

**References**


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