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Association of *hsp70* polymorphisms with risk of noise-induced hearing loss in Chinese automobile workers

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Abstract Severe noise exposure can induce heat shock proteins (Hsps), and exposure to moderate noise has been reported to confer protection against noise-induced damage to hearing. Whether there is any association of genetic variation in both constitutive and inducible *hsp70* genes with noise-induced hearing loss (NIHL) is presently unknown. Using polymerase chain reaction-restriction fragment length polymorphism, we genotyped 3 polymorphisms (+190A/B, +1267A/B, and +2437A/B) in the *hsp70-1* (rs1043618), *hsp70-2* (rs1061581), and *hsp70-hom* (rs2227956) genes, respectively, and investigated the associations of these polymorphisms with risk of developing NIHL in 194 automobile workers working in a similar noise environment as evaluated by audiological assessment. Multivariate logistic regression models were used to assess the associations with the risk genotypes, and Whap software was used to analyze their haplotypes. Our results showed that there was no statistically significant difference in the genotype and allele distributions of *hsp70-1*, *hsp70-2*, and *hsp70-hom* between the NIHL group and the normal group ($P > 0.05$) with and without adjustment for age, sex, smoking, history of explosive noise exposure, and cumulative noise exposure. However, haplotype analysis revealed that the Hap5 (ie, haplotype +190A/+1267B/+2437A) and Hap6 (ie, haplotype +190A/+1267B/+2437B) were significantly more frequent in the NIHL group than in the normal group (20/9, $P = 0.022$, and 7/0, $P = 0.005$, respectively). Compared with Hap1 (ie, +190A/+1267A/+2437A), Hap5 was associated with a nearly 3-fold increased risk of NIHL (adjusted odds ratio, 2.67; 95% confidence interval, 1.13–6.27). Seven of the NIHL patients had Hap6, but none of the controls had this haplotype. Our results suggest that some haplotypes of the *hsp70* genes may be associated with a higher susceptibility to NIHL.

INTRODUCTION

Noise-induced hearing loss (NIHL), one of the most prevalent occupational hazards in modern industries, is considered a complex disease caused by a gene-environment interaction. Thus, some individuals are more susceptible to NIHL than others (Carlsson et al 2005), and therefore it is important to explain differences in susceptibility to NIHL in order to develop methods that can predict the risk. There are still limited data about genetic polymorphisms that may be involved in susceptibility to NIHL. Animal experiments suggested that the gene coding for otocadherin 23 (*cdh23*) and plasma membrane Ca^{2+}-ATPase isoform 2 gene (PMCA2) might be involved in the susceptibility of NIHL (Kozel et al 1998; Holme et al 2004).

Heat shock proteins (Hsps), the phylogenically conserved proteins induced by numerous physical and physiological stresses, can also be induced by noise and ototoxic drugs (Lim et al 1993). Moreover, Hsps, when induced in response to moderate nontraumatic sound levels, can condition the ear to withstand effects of loud noise and protect the ear from hearing loss, although there is a noticeable individual variability (Yoshida et al

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1999; Altschuler et al 2002). Hsps function as molecular chaperones and the 70-kDa heat shock proteins (HSP70) are well known to have functions related to stress tolerance.

The human *hsp70* family consists of 3 main genes: *hsp70-1*, *hsp70-2*, and *hsp70-hom* (Milner and Campbell 1990). *Hsp70-1* and *hsp70-2* encode a similar heat-inducible protein Hsp70, but *hsp70-1* is also constitutively expressed at a low level, whereas *hsp70-hom* encodes a non-heat-inducible protein that shares high homology with the protein products of *hsp70-1/2*. These genes are polymorphic, potentially accounting for variation in their functions and susceptibility to stress tolerance (Favatier et al 1997; Wu et al 2004). Some studies reported possible associations of single nucleotide polymorphisms (SNPs) in the *hsp70* genes with Parkinson's disease (Wu et al 2004), abacavir hypersensitivity (Martin et al 2004), autoimmune diseases (Pugliese et al 1992; Favatier et al 1997; Fraile et al 1998; Vargas-Alarcon et al 2002), lung cancer (Rusin et al 2004), and acute high altitude illness (Zhou et al 2005).

We have previously observed that exposure to severe noise can induce antibodies against the inducible member of the Hsp70 family in steel industry workers (Wu et al 2001) and that the presence of anti-Hsp70 was associated with increased risk of high-frequency hearing loss and electrocardiography abnormalities in workers exposed to noise (Yang et al 2004; Yuan et al 2005). However, whether there is an association of polymorphisms of the *hsp70* genes with susceptibility to NIHL remains unknown. We therefore analyzed the *hsp70-1* +190A/B (rs1043618), *hsp70-2* +1267A/B (rs1061581), and *hsp70-hom* 2437A/B (rs2227956) polymorphisms and investigated their associations with the risk of NIHL in 194 automobile workers exposed to noise.

SUBJECTS AND METHODS

Study subjects and environmental noise monitoring

A total of 194 autoworkers (132 men and 62 women) at the Dongfeng Motor Company (Shiyan, Hubei, China), who had been employed for at least 1 year, were included in this study. These subjects had a history of exposure only to occupational noise for at least 1 year but without exposure to other harmful factors, such as high temperature (>32°C) or known toxicants (eg, organic solvent or polycyclic aromatic hydrocarbons) in workplaces (Yang et al 2004). They had no history of fever or common infections such as influenza, diarrhea, pneumonia, or hepatitis within 1 month before medical examination. Furthermore, the subjects did not use any hearing protectors, such as earmuffs and disposable earplugs. Noise exposure levels at the selected workplaces were assessed with a sound pressure audiometer (BK-2231; Brüel & Kjaer Company, Naerum, Denmark) at 10 AM, 3 PM, and 5 PM for 3 consecutive days, twice per year, according to the Chinese national criterion for noise in the workplace (Liu and Cai 1995). To evaluate the actual noise exposure level of the worker, cumulative noise exposure (CNE) was calculated, based on the database of a 20-year noise exposure, according to monitoring data on A sound pressure level and employment time calculated as follows: $Exp_c = Leq + 16.61 \times \log_{10}(T/T_0)$, dB(A) (Talbott et al 1999), where Exp_c is the cumulative noise exposure level; LeqA, the time-weighted average exposed sound pressure level A; T, the total adjusted time worked (in years); and T_0, year 1.

Evaluation of health status

Health status was evaluated in all workers using a questionnaire and by clinical and laboratory examination. The questionnaire was used to obtain personal and family history including risk factors for hearing loss, such as age, employee years, lifestyle (smoking and drinking), history of explosive noise exposure, and history of diseases. The interview was performed by an industrial hygienist for each worker before the medical examination. The clinical laboratory examination included signs, weight, height, pulse, electrocardiogram, B-echography, blood pressure, blood routine, and hepatic function test. Venous blood was also collected in heparinized tubes to separate plasma and lymphocytes for the detection of *hsp70* gene polymorphism.

Audiological assessment and definitions of hearing loss

Pure-tone audiometry was performed for both ears at 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, and 8.0 kHz. All auditory tests were performed in a sound-attenuating booth by a trained technician. Audiometry was done using Swiss Midimate RT-150 Audiometer (Brüel & Kjaer Company) calibrated to ISO 389 (1985-E) for measurement of air conduction. Threshold value was defined as the lowest signal intensity that was detected in the subject at least 50% of the time, with a minimum of 3 tries. Masking was performed if the subject had a threshold value that differed by 40 dB or more between both ears. Otoscopic examination of the external acoustic meatus and tympanic membrane was done to exclude any ear diseases. Hearing loss can either be in the low-frequency range (0.5–2.0 kHz) or high-frequency range (4.0–8.0 kHz). We took the mean threshold of 0.5, 1.0, and 2.0 kHz (PTA512) as low-frequency hearing status and the mean threshold of 4.0, 6.0, and 8.0 kHz (PTA468) as high-frequency hearing status.

Hearing threshold worse than 25 dB in either low frequency or high frequency was defined as hearing loss.

Genotyping of *hsp70* polymorphisms

DNA was isolated from lymphocytes of 3 mL of blood using a commercial DNA extraction kit according to the manufacturer's instructions (Gentra Corp., Minneapolis, MN, USA). Genotypes for polymorphisms +190A/B (rs1043618) in the *hsp70-1* gene, +1267A/B (rs1061581) in the *hsp70-2* gene, and +2437A/B (rs2227956) in the *hsp70-hom* gene were determined by previously described polymerase chain reaction (PCR)-based assays using the primers listed in Vargas-Alarcón et al (2002). Briefly, a PCR was carried out in a 25-μL volume containing 50 ng of genomic DNA, 200 μmol/L diethylnitrophenyl thiophosphates (dNTPs), 2 mM $MgCl_2$, 1× Taq DNA polymerase buffer, 1 μmol/L each primer, and 1 unit of Taq DNA polymerase (Fermentas Inc., Hanover, MD, USA). The following PCR protocol was used for amplifying *hsp70* genes: initial amplification by incubating the PCR mixture at 94°C for 5 minutes followed by 35 cycles of incubation at 94°C and corresponding anneal temperatures (57°C for *hsp70-1*, 56°C for *hsp70-2* and *hsp70-hom*) for 1 minute each, 72°C for 1 minute, and a final incubation at 72°C for 10 minutes. For the detection of restriction fragment length polymorphism, the amplified PCR fragments of *hsp70-1*, *hsp70-2*, and *hsp70-hom* genes were digested with restriction enzymes *Bsr*BI, *Pst*I, and *Nco*I (Fermentas Inc.), respectively. Subsequently, the digested products of *hsp70-1* gene were analyzed on 12% polyacrylamide gel and those of *hsp70-2* and *hsp70-hom* genes were separated on 1.5% agarose gels. These gels were stained with ethidium bromide (0.5 μg/mL), and genotypes were determined by analyzing different bands described in Vargas-Alarcón et al (2002). All gel analyses were carried out blindly to the subject's disease status.

Statistical analysis

All data were entered into a computerized database. Further analysis was carried out by using the statistical analysis software SPSS package (SPSS Inc., Chicago, IL, USA). Measurements of continuous data were analyzed by univariate analysis of variance and Student's *t*-tests. Qualitative data were computed by the Pearson χ^2 contingency tables. Genotype and allele frequencies for each polymorphic site were calculated, and the differences between the patients and controls were determined by the χ^2 test. Adjusted odds ratios (ORs) with 95% confidence intervals (CIs) were computed by multivariate logistic regression analysis to test the magnitude of associations between NIHL risk and the genotypes. The haplotype analyses of the 3 *hsp70* SNP positions were performed using the program Whap (http://www.broad.mit.edu/personal/shaun/whap/) (Myers AJ et al 2005).

RESULTS

Characteristics of the subjects

Table 1 lists potential risk factors for NIHL in the workers who had been exposed to occupational noise. The 93 subjects with hearing loss were compared with 101 subjects without hearing loss (considered to have normal hearing). Those who had hearing loss were more likely to be elders (P = 0.022), men (P = 0.000), and smokers (P = 0.026), and had a higher value of CNE (P = 0.000). However, there was no difference in history of exposure to explosive noise (P = 0.186).

Distribution of the *hsp70* polymorphisms in NIHL and normal group

The distributions of the *hsp70-1*, *hsp70-2*, and *hsp70-hom* genotypes and alleles in NIHL and normal groups are listed in Table 2. There was no significant difference in the distributions of both variant genotypes (P = 0.668) and alleles (P = 0.366) of the *hsp70-1* gene between the patients and controls. For the *hsp70-2* gene, the frequency of the *hsp70-2* +1267B/B genotype was slightly higher in the NIHL group (9.7%) than in the normal group (3.0%), but the difference in the distributions of variant genotypes was not significant (P = 0.153), nor were the allele frequency distributions (P = 0.280). For the *hsp70-hom* gene, the frequencies of the *hsp70-hom* genotypes and alleles were similar in the NIHL and normal groups (P = 0.659 and P = 0.698, respectively).

Association of the genotypes with the risk of NIHL

Crude and adjusted ORs for complex NIHL were used without and with adjustment for confounding factors. As shown in Table 3, after adjustment for age, sex (0, male; 1, female), smoking (0, negative; 1, positive), history of explosive noise exposure (0, negative; 1, positive), and CNE with multiple logistic regression analysis, no significant higher risk was found in any genotype of the 3 positions or the combinations of some genotypes (P > 0.05). However, the *Hsp70-2* +1267BB genotype was associated with a borderline increased risk of NIHL (OR = 3.49; 95% CI = 0.89–13.71), compared with the *Hsp70-2* +1267A/A genotype. When we used the Whap software to analyze the haplotypes based on the observed genotypes (Table 4), we found that the frequencies of haplotypes Hap5 (ie, +190A/+1267B and +2437A) and Hap6 (ie, +190A/+1267B/+2437B) were significantly higher in the NIHL group than in the normal one (20/9, P = 0.022,

Table 1 Differences of factors in workers with NIHL and normal workers

Variables	Hearing loss (n = 93)	Normal (n = 101)	P value
Age (mean ± SD)	35.2 ± 6.9	33.0 ± 6.1	0.022[a]
Gender (N, %)			
Male	77 (82.8)	55 (54.5)	0.000[b]
Female	16 (17.2)	46 (45.5)	
Smoking (N, %)			
Yes	45 (48.4)	33 (32.7)	0.026[b]
No	48 (51.6)	68 (67.3)	
History of explosive noise exposure (N, %)			
Yes	29 (31.2)	23 (22.8)	0.186[b]
No	64 (68.8)	78 (77.2)	
CNE (mean ± SD)	93.17 ± 9.99	88.12 ± 7.67	0.000[a]

[a] Independent samples Student's t test.
[b] Two-sided χ^2 test.

Table 2 *hsp70-1*, *hsp70-2*, and *hsp70-hom* genotype and allele frequencies (%) in NIHL group and normal group

Variables	*hsp70-1* +190 (rs1043618)			*hsp70-2* +1267 (rs1061581)			*hsp70-hom* +2437 (rs2227956)		
	NIHL group n (%)	Normal group n (%)	P value	NIHL group n (%)	Normal group n (%)	P value	NIHL group n (%)	Normal group n (%)	P value
Genotypes									
A/A	37 (39.8)	35 (34.7)	0.668	43 (46.2)	50 (49.5)	0.153	58 (62.4)	67 (66.3)	0.659
A/B	43 (46.2)	48 (47.5)		41 (44.1)	48 (47.5)		34 (36.6)	32 (31.7)	
B/B	13 (14.0)	18 (17.8)		9 (9.7)	3 (3.0)		1 (1.1)	2 (2.0)	
Alleles									
A	117 (62.9)	118 (58.4)	0.366	127 (68.3)	148 (73.3)	0.280	150 (80.6)	166 (82.2)	0.698
B	69 (37.1)	84 (41.6)		59 (31.7)	54 (26.7)		36 (19.4)	36 (17.8)	

and 7/0, P = 0.005, respectively). Compared with Hap1 allele (ie, +190A/+1267A and +2437A), Hap5 allele was associated with a nearly 3-fold increased risk of NIHL (adjusted OR = 2.67 and 95% CI = 1.13–6.27). Of the NIHL patients, 7 had haplotype Hap6, whereas none of the normal controls had this haplotype. The association of haplotypes 5 and 6 with NIHL is very clear.

DISCUSSION

Excessive exposure to noise results in temporary or permanent changes in hearing ability in both humans and animals (Davis et al 1950; McFadden and Plattsmier 1982; Henry 1984). NIHL, previously known as industrial deafness, remains an important problem in occupational health. An interaction between environmental and genetic predisposing factors is thought to be involved in the etiology of NIHL. Studies in animal models suggest that genetic factors may influence individual susceptibility to noise; for example, certain mouse strains with age-related hearing loss have been reported to be more susceptible to noise (Erway et al 1996; Davis et al 2001). Mice in which genes like PMCA2 (Kozel et al 2002), cadherin 23 (Holme and Steel 2004), superoxide dismutase 1 (Ohlemiller et al 1999), or glutathione peroxidase 1 (Ohlemiller et al 2000) were knocked out have been reported to be more sensitive to NIHL. However, firm evidence for the involvement of genetic factors in human NIHL is still scarce. A possible association between the absence of glutathione-*S*-transferase *M1* (GSTM1) and NIHL was reported (Rabinowitz et al 2002), but this was not confirmed in a recent study on 2 deletion polymorphisms in the *GSTM1* and *GSTT1* genes and the susceptibility to NIHL using susceptible and resistant NIHL in a Caucasian population from 3 distinct workplaces (Carlsson et al 2005). Another study suggested that *SOD2* and paraoxonase polymorphisms could predispose to NIHL (Fortunato et al 2004)

The HSP70 family proteins may be the most predominant and particularly interesting group of proteins involved in the major histocompatibility complex in disease susceptibility (Favatier et al 1997). Several studies suggest that the polymorphisms of the *hsp70* genes might be associated with many diseases; for example, individuals carrying P2/P2 genotype of the *hsp70-2* gene have an increased risk of obesity in Tunisians (Chouchane et al 2001). The *hsp70-2* gene polymorphic allele A may be a possible genetic marker of a less severe clinical phenotype

Table 3 The ORs of different *hsp70* genotypes for NIHL

Genotypes	Crude OR (CI)	*P* value	Adjusted OR (CI[a])	*P* value
hsp70-1 +190				
A/A	1.00[b]		1.00[b]	
A/B	0.85 (0.46–1.57)	0.600	0.88 (0.44–1.76)	0.712
B/B	0.68 (0.29–1.96)	0.379	0.66 (0.25–1.71)	0.390
A/B+B/B	0.80 (0.45–1.44)	0.460	0.82 (0.43–1.56)	0.539
hsp70-2 +1267				
A/A	1.00[b]		1.00[b]	
A/B	0.99 (0.55–1.80)	0.982	0.89 (0.46–1.70)	0.715
B/B	3.49 (0.89–13.71)	0.061	3.75 (0.78–18.17)	0.100
A/A+A/B	1.00		1.00	
B/B	3.50 (0.92–13.35)[c]	0.053	3.97 (0.84–18.67)[c]	0.081
hsp70-hom +2437				
A/A	1.00[b]		1.00[b]	
A/B	1.23 (0.68–2.23)	0.501	1.53 (0.77–3.04)	0.222
B/B	0.58 (0.05–6.54)	0.654	0.41 (0.02–7.25)	0.539

[a] Adjusted for age,sex (0, male; 1, female); smoking (0, negative; 1, positive); history of explosive noise exposure (0, negative; 1, positive), and CNE. * $P < 0.05$, ** $P < 0.01$.
[b] Reference group.
[c] Compared with the *hsp70-2* +1267 A/A+A/B group.

TABLE 4 *hsp70* haplotype distribution and ORs in NIHL group and normal group

Haplotypes[a]	NIHL group (*N*, %)	Normal group (*N*, %)	*P* value	OR (95% CI)
Hap1 (AAA)	61 (33.0)	73 (36.0)		1.00[b]
Hap2 (BAA)	36 (19.5)	39 (19.5)	0.730	1.105 (0.627–1.946)
Hap3 (BBA)	33 (17.6)	45 (22.1)	0.650	0.878 (0.500–1.542)
Hap4 (AAB)	29 (15.8)	36 (17.8)	0.904	0.964 (0.531–1.749)
Hap5 (ABA)	20 (10.5)	9 (4.6)	0.022	2.659 (1.129–6.266)
Hap6 (ABB)	7 (3.6)	0 (0)	0.005	

[a] The allele order is *hsp70-1* +190A/B, *hsp70-2* +1267A/B, and *hsp70*-hom +2437A/B from left to right.
[b] Reference group.

associated with Crohn disease in Japanese patients (Esaki et al 1999). In ankylosing spondylitis, differences in *hsp70-1* and *hsp70-2* genotypes among ankylosing spondylitis patients and controls appear to be due to the linkage disequilibrium between HSP70 alleles and HLA-B*27 (Fraile et al 1998). We also found that individuals with *hsp70-2* +1267B/B, *hsp70-hom* +2437A/B, +2437B/B genotypes are more susceptible to high-altitude illness, whereas those with *hsp70-hom* +2437A/B genotype are tolerant to low oxygen (Zhou et al 2005).

In the present study, we did not find any statistically significant difference in the genotype and allele distributions of *hsp70-1*, *hsp70-2*, and *hsp70-hom* between the NIHL group and normal group, although it was suggestive that the *hsp70-2* +1267B/B genotype might be associated with an increased risk of NIHL. These results suggested that the polymorphisms of the 3 SNPs of the *hsp70* genes do not seem to play a major role in NIHL at least in the Chinese population, although larger studies are needed to confirm these findings. Although sometimes useful, single SNP may not be sufficiently informative in complex diseases. The haplotype-based linkage disequilibrium mapping has become a powerful and cost-effective method for performing genetic association studies, particularly in search of the genes causing complex diseases (Niu et al 2002; Salisbury et al 2003). In addition, haplotype information is useful for predicting the severity and prognosis of certain genetic diseases associated with a Mendelian inheritance (Cuppens 1998; Gambetti et al 2003).

Our results from haplotype analysis using the Whap software revealed that the frequencies of the Hap5 haplotype (ie, +190A/+1267B/+2437A) and Hap6 haplotype (ie, +190A/+1267B/+2437B) were significantly greater in the NIHL than in the normal group. The results suggest that individuals carrying Hap5 or Hap6 haplotypes are more prone to NIHL when exposed to noise. The *hsp70-1* +190 polymorphism and the *hsp70-2* +1267 polymorphism depend on silent change in the coding region; the *hsp70-hom* polymorphic site detected by *Nco*I is located on position +2437, which corresponds to a Met→Thr amino acid substitution at position 493 (Milner and Campbell 1992). Although individual SNP analysis did not show any association with NIHL, the combined information of these 3 SNPs, ie, the haplotypes, may play some role in the development of NIHL. However, the mechanisms by which these Hsp70 haplotypes are associated with the development of NIHL remain unknown but clearly warrant further investigations.

In summary, the present data suggest that genetic variation in the *hsp70* genes may contribute to the susceptibility to NIHL. Further investigations with larger populations are warranted to confirm the significance of our findings and functionality of these observed haplotypes.

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