



Heat shock protein 70 polymorphisms in Chinese patients with Graves' disease

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Genet. Mol. Res. 14 (4): 18376-18383 (2015)

Received August 14, 2015

Accepted October 29, 2015

Published December 23, 2015

DOI <http://dx.doi.org/10.4238/2015.December.23.25>

ABSTRACT. Graves' disease (GD) is a common autoimmune disease mainly affecting the thyroid. However, the correlation between the development of GD and *HSP70* alleles has not been reported in the Chinese population. Therefore, the aim of this study was to investigate the association between *HSP70* polymorphisms and GD in the Chinese population. A total of 153 patients with GD treated at the Yan'an Affiliated Hospital of Kunming Medical University between October 2010 and August 2013 were enrolled in this study; one hundred and twenty healthy volunteers were included in the control group. *HSP70* polymorphisms at positions *HSP70*-1 +190, *HSP70*-2 +1267, and *HSP70*-hom +2437 were genotyped by polymerase chain reaction-restriction fragment length polymorphism. The distribution of the *HSP70*-2 +1267 GG genotype allele frequencies among GD and control subjects differed significantly ($\chi^2 = 20.40$, $P < 0.001$; $\chi^2 = 18.18$, $P < 0.001$). The G allele of *HSP70*-2 +1267 (Odds ratio = 0.455, 95% confidence interval: 0.315-0.655) conferred a higher risk of developing GD than the A allele. We observed no significant differences in the allelic frequencies of *HSP70*-1 +190 and *HSP70*-hom

+2437. Therefore, the *HSP70-2* +1267 GG genotype and the G allele may increase the risk of GD in Chinese subjects. The results of this study may be useful in identifying patients with increased risk of GD, and offer useful reference data for targeted gene therapy of GD in the future.

Key words: Graves' disease; Heat shock protein 70; Polymorphisms; Chinese population

INTRODUCTION

Graves' disease (GD), one of the most common autoimmune diseases, mainly affects the thyroid (Zheng et al., 2015); moreover, it affects other systems, such as the cardiovascular, central nervous, and reproductive systems, in addition to the eyes and bones (Streetman and Khanderia, 2003). GD patients develop immune function disorders that compromise immune tolerance and alter recognition and regulatory functions. Such GD-induced immune function disorders also cause functional defects in antigen-specific or non-specific suppressor T lymphocytes; this prevents the body from controlling its immune response against its own tissues, and weakens helper T lymphocyte inhibition by suppressor T cells. With the assistance of specific helper T lymphocytes, B lymphocytes produce autoimmune antibodies, such as TRAb, TPOAb, and TGAAb.

The incidence of GD is mainly attributed to genetic factors; however, GD can be triggered by environmental factors, such as infection (Hammerstad et al., 2013, 2014) and psychological trauma (Vita et al., 2014). The exact cause of GD however remains unclear.

Heat shock proteins (HSPs) are chaperones for protein folding and stability. Heat shock response (HSR) occurs when cells are subjected to stress, such as ischemia, hypoxia, heat stimulation, and heavy metal poisoning, resulting in high-level expressions of heat shock proteins aimed at protecting the cells from stress hazards (Gabai et al., 1997). Depending on their molecular weight, HSPs can be divided into four subfamilies: *HSP90-110*, *HSP70*, *HSP60*, and *HSP10-30* (Jin et al., 2004). The *HSP70* subfamily contains three coding genes, *HSP70-1*, *HSP70-2*, and *HSP70-hom* (Westman et al., 1994). All *HSP70* genes have continuous open reading frames that enable their rapid transcription and translation.

HSP70 gene polymorphisms are associated with several autoimmune diseases, including systemic lupus erythematosus (Fürnrohr et al., 2010), autoimmune arthritis (Wieten et al., 2010), and Crohn's disease (Chen et al., 2013). Although *HSP70* alleles are reportedly associated with an increased risk of developing GD in Caucasians, the relationship between these alleles and the risk of GD in Chinese individuals remains unknown.

Based on this information, this study explored the association between *HSP70* polymorphisms and GD in the Chinese population. In this study, a single nucleotide polymorphism (SNP) located at position *HSP70-2*+1267 was found to be correlated with GD risk in the Chinese population.

MATERIAL AND METHODS

Subjects

A total of 153 patients with GD treated at the Yan'an Affiliated Hospital of Kunming Medical University between October 2009 and October 2014 were recruited to this study. The age of the subjects was at a range of 18 to 40 years. GD is defined as the presence of clinical and

biochemical hyperthyroidism [including suppressed thyroid-stimulating hormone (TSH), elevated free triiodothyronine (FT3), and free thyroxine (FT4)], positive titers of TPOAb, TGAb, and TRAb, and B-ultrasonographic diffuse goiter at the first diagnosis (Lu and Zhong, 2008). Patients with a history of other diseases, such as hypertension, were excluded from this study. The control group was comprised of 120 healthy participants (as determined by regular health examinations at the hospital) from the same geographic area as the GD subjects. All control subjects exhibited normal TSH, FT3, and FT4 levels, and did not express TPOAb, TGAb, and TRAb.

A validated questionnaire concerning the age, sex, and history of other diseases was completed by each participant.

This study was approved by the Ethical Committee of the Yan'an Affiliated Hospital of Kunming Medical University. An informed consent form was signed by all participants.

Genotyping

Blood samples were collected in the morning from all subjects after fasting for 12 h. Peripheral blood (3-5 mL) was collected using an EDTA-treated vacuum tube. Genomic DNA was isolated from the leukocytes using an AxyPrep Blood Genome DNA Isolation kit (Axygen, New York, NY, USA). *HSP70-1* +190, *HSP70-2* +1267, and *HSP70-hom* +2437 expressions were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The target sequences were amplified using a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA). The reaction system (15 μ L) contained approximately 0.2 ng genomic DNA, 0.5 U Taq DNA polymerase, 0.3 μ L dNTPs (2.5 mM each), and 5 pmol primer pairs. PCR amplification was conducted using the following cycling conditions: denaturation at 95°C for 5 min; 20 cycles of denaturation at 95°C for 30 s, annealing at 68.5°C for 45 s, and extension at 72°C at 60 s; 20 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 40 s; and a final extension at 72°C for 6 min. The PCR products were subjected to agarose gel electrophoresis on a 3% agarose gel.

Statistical analysis

The data was analyzed using SPSS v.21.0 (IBM, Armonk, NY, USA). The frequency of each genotype was calculated using a χ^2 test. Odds ratios (OR) were calculated based on 95% confidence intervals (95%CI). Differences with $P < 0.05$ were considered to be significant.

RESULTS

General data

The percentage of male subjects in the GD group was 43.1%, and the percentage of males in the control group was 43.3%. No significant differences in age, gender, or alcohol drinking or smoking habits were observed between the two groups. The results are summarized in Table 1.

Genotyping

The electrophoresis bands of the analyzed alleles, *HSP70-1* +190, *HSP70-2* +1267, and *HSP70-hom* +2437 are presented in Figures 1-3.

Table 1. Clinical characteristics of the subjects.

Factors	Patients	Controls	P value
Age (years)	27.6 ± 5.4	26.7 ± 5.2	0.198
Male/female	66/87	52/68	0.974
Alcohol drinking	47	31	0.375
Smoking	42	35	0.755

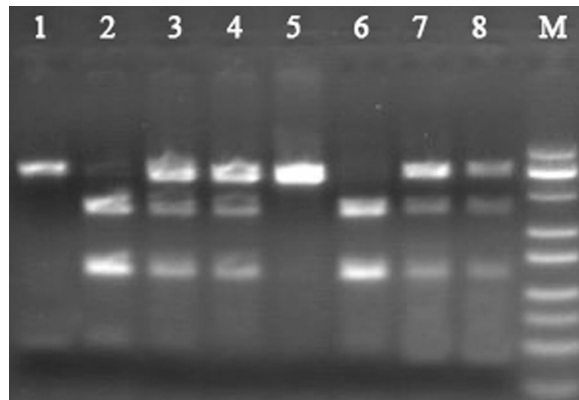


Figure 1. PCR digestion products: electrophoresis of HSP70-1 +190. Lane M contains the GM303 DNA marker; lanes 1 and 5 contained the genotype CC (359 bp); lanes 3, 4, 7, and 8 show the genotype GC (116, 243, and 359 bp, respectively); and lanes 2 and 6 contained the genotype GG (116 and 243 bp, respectively).

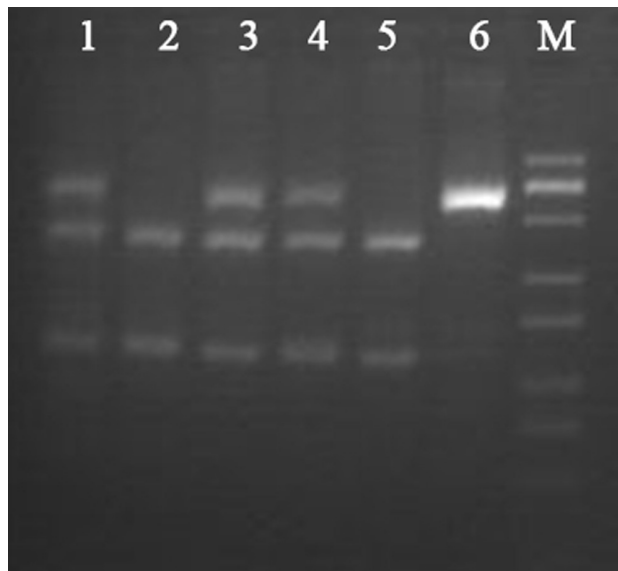


Figure 2. PCR digestion products: electrophoresis of HSP70-2 +1267. Lane M contains the GM303 DNA marker; lanes 2 and 5 contained the genotype GG (118 and 249 bp, respectively); lanes 1, 3, and 4 displayed the genotype GA (118, 249, and 367 bp, respectively); and lane 6 showed the genotype AA (367 bp).

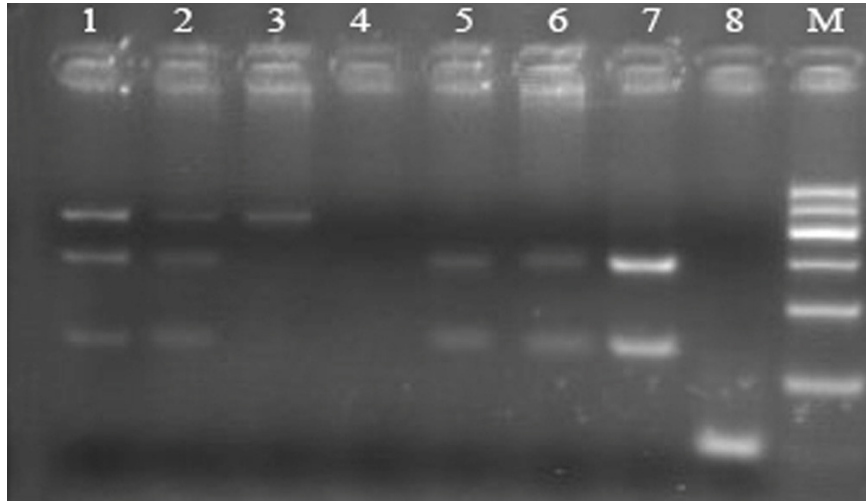


Figure 3. PCR digestion products: electrophoresis of HSP70-hom. Lane M contained the GM303 DNA marker; lanes 1 and 2 showed the genotype TC (134, 282, and 416 bp); lane 3 expressed the genotype CC (416 bp); and lanes 5, 6, and 7 contained genotype TT (134 and 282 bp).

The results of our analyses revealed that the distributions of all genotyped polymorphisms in the *HSP70* genes in the GD and control groups were consistent with the Hardy-Weinberg equilibrium (Tables 2-4). The frequencies of the *HSP70*-1 +190 G/C did not differ significantly between the GD and control groups (Table 5). Additionally, the frequency of the GG genotype and G allele at *HSP70*-2 +1267 in the GD group was significantly reduced compared to the control group (42.48 vs 31.67%, $P < 0.01$; Table 6). However, the frequencies of the *HSP70*-hom +2437 A/C polymorphisms did not differ significantly between the GD and control groups (Table 7).

Table 2. Distribution of HSP70-1 +190 alleles and Hardy-Weinberg equilibrium test data.

	Number observed	Number expected	Gene frequency	χ^2	P value
Genotype in the GD group				2.970	0.085
CC	3	7	0.020		
GG	93	96	0.608		
GC	57	50	0.372		
Genotype in the control group				2.904	0.088
CC	4.00	8	0.033		
GG	64.00	68	0.533		
GC	52.00	45	0.433		

Table 3. Distribution of HSP70-2 +1267 alleles and Hardy-Weinberg equilibrium test data.

	Number observed	Number expected	Gene frequency	χ^2	P value
Genotype in the GD group				3.354	0.067
AA	5	9	0.033		
GG	83	87	0.542		
GA	65	57	0.425		
Genotype in the control group				1.132	0.287
AA	18	21	0.150		
GG	38	41	0.317		
GA	64	58	0.533		

Table 4. Distribution of the HSP70-hom +2437 alleles and Hardy-Weinberg equilibrium test.

	Number observed	Number expected	Gene frequency	χ^2	P value
Genotype in the GD group				0.213	0.645
AA	7	8	0.046		
GG	90	91	0.588		
GA	56	54	0.366		
Genotype in the control group				0.252	0.615
AA	6	7	0.050		
GG	68	69	0.567		
GA	46	44	0.383		

Table 5. Statistical analysis of the frequencies of the HSP 70-1 +190 genotype and alleles in the GD and control groups.

	GD group	Control group	χ^2	P value
Genotype			1.766	0.414
CC	3 (1.96%)	4 (3.33%)		
GG	93 (60.78%)	64 (53.33%)		
GC	57 (37.25%)	52 (43.33%)		
Allele			1.500	0.221
C	63 (20.59%)	60 (25.00%)		
G	243 (79.41%)	180 (75.00%)		

Table 6. Statistical analysis of the frequencies of the HSP70-2 +1267 genotype and alleles in the GD and control groups.

	GD group	Control group	χ^2	P value
Genotype			20.40	<0.001
AA	5 (3.27%)	18 (15.00%)		
GG	83 (42.48%)*	38 (31.67%)		
GA	65 (54.25%)	64 (53.33%)		
Allele			18.18	<0.001
A	75 (24.51%)	100 (41.67%)		
G	231 (75.49%)*	140 (58.33%)		

*P < 0.05.

Table 7. Statistical analysis of the frequencies of the HSP70-hom +2437 genotype and alleles in the GD and control groups.

	GD group	Control group	χ^2	P value
Genotype			0.134	0.935
AA	7 (4.58%)	6 (5%)		
GG	90 (58.82%)	68 (56.67%)		
GA	56 (36.60%)	46 (38.33%)		
Allele			0.125	0.724
A	70 (22.88%)	58 (24.17%)		
G	236 (77.12%)	182 (75.83%)		

DISCUSSION

HSP70 exerts a protective effect on the heart (Fu and Tupling, 2009) and brain tissue (Zhao et al., 2006), in addition to playing an important role in immunity. In this regard, *HSP70*

protects cells from natural killer cells and imparts anti-infective and anti-tumor immunity through its involvement in antigen processing and presentation (Gehrmann et al., 2005). Therefore, changes in *HSP70* function may contribute to the development and progression of autoimmune diseases. *HSP70* gene polymorphisms have been associated with the development of GD in other countries. However, to the best of our knowledge, such studies among the Chinese population have not been reported. Therefore, this study attempted to identify any such possible correlation in Chinese patients.

HSP70 gene polymorphisms within the HLA III region have been subject to close scrutiny, given its close association with a variety of autoimmune diseases, including systemic lupus erythematosus (Fürnrohr et al., 2010), autoimmune arthritis (Wieten et al., 2010), and Crohn's disease (Chen et al., 2013). As a multigene family, inheritance and variations in HSPs have occurred throughout the evolutionary process.

HSP polymorphisms are restricted to the *HSP70* family. The *HSP70-1* gene is polymorphic at the 110th (A/C), 120th (T/C), 190th (G/C), and 438th (C/T) loci, whereas the *HSP70-2* gene is polymorphic at the 145th (A/G), 1267th (A/G), 2074th (G/C), and 2257th loci. Among these, the *HSP70-1*+190 and *HSP70-2*+1267 loci have been correlated with multiple diseases, such as diabetic nephropathy (Buraczynska et al., 2009), gastric cancer, and gastric ulcers (Shibata et al., 2009), in addition to GD.

The frequencies of the *HSP70-2* +1267 GG genotype and G allele were noticeably increased in the GD group compared to the control group (42.48 vs 31.67%, $P < 0.01$, Table 6). Ratanachaiyavong et al. (1991) reported that the 8.5-kb *Pst*I *HSP70* allele is strongly associated with GD in the British Caucasian population. Hunt et al. (2001) reported an association between the gene polymorphisms at loci *HSP70-1* +190 and *HSP70-2* +1267 and GD in Caucasian subjects in the United Kingdom. Our results were basically consistent with the results previously reported in literature, thereby supporting the conjecture that the *HSP70-2* +1267 GG genotype is a risk factor for GD. However, the frequencies of the *HSP70-1* +190 G/C and *HSP70*-hom +2437 genotypes did not differ significantly between the GD and control groups in our study (Tables 5 and 7). This result contradicted those reported by Hunt et al. (2001); this discrepancy may be attributed to ethnical or regional differences between the subjects.

HSP70 is highly expressed in thyroid tissue (Trieb et al., 1993). The *HSP70-2* +1267 locus is located in the coding region of the gene. Pociot et al. (1993) suggested that *HSP70-2* +1267 GG homozygotes exhibit reduced *HSP70* mRNA levels. The consequent reduction in protein levels may lead to impairment in the responses to cell stress and accumulation of denatured proteins, which may eventually contribute to GD progression. Further functional analyses should be performed to elucidate the specific role of the *HSP70* gene in GD pathogenesis.

This study has some limitations. Firstly, the study was conducted in a small sample population. Therefore, the novel observations of this study must be validated in a larger population subset. In addition, functional studies are required to elucidate the involvement of *HSP70* genes in GD complications.

In conclusion, the *HSP70-2* +1267 GG genotype and G alleles may increase the risk of GD in the Chinese population. The results of this study may be useful in identifying patients with increased risk of GD, and provide useful data for targeted gene treatment of GD in the future. Based on this study, additional studies are warranted to enable further understanding of the immunoregulatory functions of *HSP70* gene products under both physiological and pathological conditions, and to elucidate the significance of the resulting associations.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the National Natural Science Foundation of China (#81460154).

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