Genetic variation in heat shock protein 60 gene and coronary heart disease in China: tagging-SNP haplotype analysis in a case-control study

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Abstract

Background High levels of circulating heat shock protein 60 (Hsp60) and antibody to human Hsp60 have been associated with greater risk of coronary heart disease (CHD) in several studies, but associations between poly-

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morphisms of the *hsp60* gene and CHD risk have not been investigated.

Methods By resequencing DNA from 30 unrelated Han Chinese and using HapMap Phase I Chinese data of *hsp60* gene, we selected four tagging single nucleotide polymorphisms (tagSNPs) named rs2340690, rs788016, rs2305560, and rs2565163, and determined their frequencies in 1,003 Chinese CHD patients and 1,003 age- and sex-frequency-matched controls. Furthermore, we used PHASE 2.0 software to reconstruct haplotypes and logistic regression to control for potential confounders in multivariate analyses. **Results** We found 13 SNPs in *hsp60* gene (including four novel SNPs) in Han Chinese subjects. Our results showed no significant differences in four selected SNPs in patients with CHD and controls after adjusting for other conventional risk factors and stratifying by age, sex, smoking status, past history of hypertension and DM; however, our results showed that subjects with the *GCTC* haplotype had about twofold higher risk of CHD than those with the *GTTC* haplotype (OR=1.91, 95%CI: 1.26–2.89, $P=0.002$). **Conclusions** Our results suggest that the *GCTC* haplotype in the *hsp60* gene is significantly associated with higher CHD risk in a Chinese population.

Keywords Coronary heart disease · Haplotype · *Hsp60* · Polymorphism · TagSNP

Abbreviations

BMI body mass index
CHD coronary heart disease
CIs confidence intervals
FBG fasting blood glucose
Hsp60 heat shock protein 60

LD	linkage disequilibrium
MAF	minor allele frequency
ORs	odds ratios
SNPs	single nucleotide polymorphisms
TC	total cholesterol
TG	triglyceride

Introduction

There is increasing evidence that autoimmune reactions play a pivotal role in the pathogenesis of coronary heart disease (CHD) (Hansson et al. 2002). The increased expression of heat shock protein 60 (Hsp60) in endothelial cells, macrophages, and smooth muscle cells (Kleindienst et al. 1993), may serve as an autoimmune antigen and accelerate the progression and development of atherosclerosis (Schett et al. 1995; Xu et al. 1999; Xu and Wick 1996). Human investigations have shown that high circulating Hsp60 levels (Pockley et al. 2000; Xu et al. 2000) and high levels of antibody to human Hsp60 have been associated with higher risk of CHD (Burian et al. 2001; Prohaszka et al. 2001; Veres et al. 2002; Xu et al. 1999; Zhu et al. 2001). Therefore, *hsp60* may be an important candidate gene for CHD.

The *hsp60* gene, which resides on human chromosome 2q33.1 and spans about 13.69 kb, includes 12 exons and 11 introns (Hansen et al. 2003). More than 80 single nucleotide polymorphisms (SNPs) in *hsp60* are reported in the database of National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/SNP>). To the best of our knowledge, however, only two previous studies have examined the associations between the variability in *hsp60* gene and human diseases. One French study indicated that the *G*+292*A* (dbSNP: rs41350646) polymorphism in *hsp60* was associated with hereditary spastic paraplegia (Hansen et al. 2002), and another suggested that one *MspI* polymorphism was related to sudden infant death syndrome (Rahim et al. 1996). No study has investigated the association between *hsp60* gene polymorphisms and CHD.

A haplotype is a combination of alleles of SNPs at multiple linked loci. With the completion of the International HapMap Project, it is possible to investigate disease-associated genetic polymorphisms through a whole-gene-based tagging SNP approach (Cousin 2006). In this case-control study, we resequenced the *hsp60* gene in 30 unrelated Han Chinese and used HapMap Phase I Chinese data (<http://www.hapmap.org>) on the *hsp60* gene and a linkage disequilibrium (LD)-based approach (Carlson et al. 2004). We selected four tagging polymorphisms of the *hsp60* gene to examine their associations with CHD.

Subjects and methods

Study population

The study population was composed of 1,003 case patients and 1,003 age- and sex-frequency-matched controls. All enrolled subjects were unrelated ethnic Han Chinese. Case patients, who were from three hospitals (Tongji Hospital, Union Hospital, and Wugang Hospital) in Wuhan City, Hubei province were diagnosed as having CHD according to World Health Organization (WHO) criteria or by coronary angiography (significant coronary artery stenoses $\geq 50\%$ in at least one major coronary artery). Myocardial infarction was diagnosed by a representative set of electrocardiograph (ECG), cardiac enzyme values, and typical symptoms. Angina was defined as use of nitroglycerine, experience of typical chest pain, or ECG changes compatible with ischemic heart disease. A total of 1,078 patients diagnosed as having CHD were recruited; 1,003 of them (93.0%) consented to participate in the study and provided questionnaire information and blood samples. After cases were diagnosed with CHD, they were interviewed in person within 3 days. The control subjects, residing in the same communities as the cases, were judged to be free of CHD and peripheral atherosclerotic arterial disease by medical history, clinical examinations, and electrocardiography. The response rate for the controls was 92.4% (1,003 of 1,085). Subjects with severe liver and/or kidney disease were excluded. Medical history, sociodemographic information, family history of cardiovascular disease, medication use, home environment, and lifestyle factors were obtained through questionnaire interview.

Subjects were identified as nonsmokers, former, or current smokers. Those who had smoked less than 100 cigarettes in the past lifetime were defined as nonsmokers; otherwise, they were defined as smokers. Those smokers who stopped smoking for more than 1 year were considered former smokers. Pack-years were calculated by multiplying the number of packs of cigarettes smoked per day by the number of years the person has smoked. Habitual physical activity was classified into four groups: little, light, moderate and vigorous. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Subjects were considered to be hypertensive if their systolic blood pressure was ≥ 140 mmHg and/or diastolic pressure ≥ 90 mmHg or they were already being treated with antihypertensive drugs. All subjects gave written consent after receiving a full explanation of the study. The Ethics Committee of Tongji Medical College approved this study.

Screening for SNPs of *hsp60* gene in Chinese

Genomic DNA was amplified using *Taq* polymerase, followed by purification using the ethanol/NaAc method.

Polymerase chain reaction (PCR) products were then used as a template for sequencing reactions with the BigDye Terminator kit v3.1 (Applied Biosystems, Foster City, CA, USA). Purified sequencing reactions were run on an ABI3100 genetic analyzer. Sequence analysis, SNP detection, and genotype calling were performed using DNASTar software. The entire 1–12 exon, the boundary region between exons and introns, and upstream promoter region of *hsp60* gene were screened in 30 unrelated healthy Han Chinese. All primer sequences and reaction conditions are available on request from the corresponding author.

Selection of tagSNPs in *hsp60* gene

Using HapMap Phase I Chinese data (<http://www.hapmap.org>) acquired at the beginning of our work, according to the criteria of $r^2 \geq 0.8$ and minor allele frequency (MAF) ≥ 0.1 , we extended 2000 bp in the 5' and 3' end of *hsp60* gene and selected 3 tagSNPs named rs2565163, rs2340690, and rs788016 to be analyzed (Table 1).

Furthermore, referring to our analysis of resequencing results for the *hsp60* gene using htSNPer1.0 software, (Ding et al. 2005) we additionally selected another SNP, rs1116734, in the 5' flanking region of the *hsp60* gene, to analyze the association between *hsp60* gene polymorphisms and CHD risk (Fig. 1). Because the allelic frequency of rs1116734 could not be detected by the Taqman SNP allelic discrimination method and PCR-restriction fragment length polymorphism method, we substituted another nearby SNP, rs2305560, for this analysis.

Determination of polymorphisms at *hsp60* gene

Fasting venous blood was collected in 5-ml heparin tubes, and genomic DNA was isolated with a Puregene kit (Gentra Systems, Inc., Minneapolis, MN, USA). Genotyping of *hsp60* was performed with Taqman SNP allelic discrimination by means of an ABI 7900HT (Applied Biosystems, Foster City, CA), in 384-well format. PCR reactions were carried out in reaction volume of 5 μ l containing 5 ng

DNA, 2.5 μ l 2 $\times$ Taqman universal PCR Master MixNo AmpErase UNG (Applied Biosystems), 0.125 μ l 40 $\times$ Assay Mix. PCR conditions included 95°C for 10 min, followed by 40 cycles of 15 s at 92°C and 1 min at 60°C. Two blank controls (DNA hydration solution) and two replicate quality control samples were included in each 384-well format, and two replicate samples were genotyped with 100% concordance. The intensity of each SNP met the criteria of three clear clusters in two scales generated by SDS software (ABI).

Biological variables determination

Fasting blood glucose (FBG), total cholesterol (TC), and triglyceride (TG) were assayed using standard laboratory procedures in the department of clinical laboratory at the Union Hospital.

Statistical analysis

Continuous variables were reported as the mean value $\pm$ SD. Normal distribution of data was analyzed using the Kolmogorov–Smirnov normality test. Data with a normal distribution were compared by Student's *t* test, and those with unequal variance or without a normal distribution were analyzed by a Mann–Whitney rank sum test. Categorical values were compared by the chi-square test, which was also used to test for deviation of genotype distribution from Hardy–Weinberg equilibrium. The htSNPer1.0 software was used to select tagSNPs in *hsp60* gene (Ding et al. 2005) and PHASE 2.0 program (Stephens and Donnelly 2003) to infer haplotype frequencies based on the observed *hsp60* genotypes. The most common haplotype among controls was used as reference in the logistic regression model. The associations between variants in the *hsp60* gene and CHD risk were estimated by computing odds ratios (ORs) and 95% confidence intervals (CIs) from the multivariate logistic regression analyses. An unconditional logistic model was used to adjust for multiple cardiovascular risk factors. The probability level accepted for significance was $P < 0.05$.

Table 1 LD (r^2) between SNPs in the *hsp60* gene region in Chinese HapMap database

SNPs	rs788016	rs2565163	rs7585486	rs2340690	rs8539	rs17730989	rs2605039
rs788016	–	0.292	0.169	0.174	0.299	1.000	0.299
rs2565163		–	0.270	0.277	0.977	0.292	0.977
rs7585486			–	0.973	0.276	0.169	0.276
rs2340690				–	0.284	0.174	0.284
rs8539					–	0.299	1.000
rs17730989						–	0.299
rs2605039							–

Fig. 1 Selection of TagSNPs in *hsp60* Gene by htSNPer1.0 Software. Based on our data on the resequencing of the *hsp60* gene and the criterion of minor allele frequency >0.10, a total of 11 SNPs were analyzed by htSNPer1.0 software to select tagSNPs. The results showed that rs788016 and rs1116734 were selected for tagSNPs

All data analyses were carried out with the statistical analysis software package SPSS12.0 (SPSS Inc., Chicago, IL, USA).

Results

Hsp60 gene SNP distribution in Han Chinese

We found 13 genetic variants in the sequenced region of *hsp60*. Among them four SNPs had not been reported previously: 198189061 *a/g* (exon 2, het 0.06), 198184890 *a/g* (exon 5, het 0.04), 198184774 *ag/-* (intron 5, het 0.5), and 198180643 *ca/-* (intron 7, het 0.5) in chromosome 2; another nine SNPs reported in NCBI and European Bioinformatics Institute (EBI) are rs1116734, rs3749095, rs1050347, rs8539, rs2340690, rs3214832, rs7585486, 198180659 *a/c* (without rs ID), and rs788016 (Table 2). We found that rs1116734 and 198189061 *a/g*, and rs1116734 and rs788016 are not in LD,

but SNPs such as rs1050347, rs2340690, rs3214832, rs7585486, and 198180659 *a/c* are in perfect LD ($r^2=1$). Other SNPs such as rs8539, 198184774 *ag/-*, and 198180643 *ca/-* are also in perfect LD (data not shown).

Characteristics of the subjects

In the present study, cases were more likely than controls to be smokers, nondrinkers, and to have comorbidities such as hypertension and diabetes. Age and sex were similar for cases and controls, but cases were significantly more likely than controls to have a family history of CHD. FBG levels were significantly higher in cases than in controls (6.66 ± 3.63 mmol/l vs. 5.32 ± 2.00 mmol/l, $P < 0.01$), however, TC levels were significantly lower in cases than in controls due to the use of cholesterol-lowering drugs by CHD patients (4.50 ± 1.05 vs. 5.08 ± 1.30 mmol/l, $P < 0.01$). There was no significant difference in TG levels and BMI between cases and controls (Table 3).

Table 2 Distribution of SNPs in the *hsp60* gene region by resequencing

No	rs ID	Gene pos.	Alleles	MAF	Het	Chrom. Pos
1	rs1116734	5' flanking	ccccagcg/cgtcgcc	0.45	0.50	198190549
2	rs3749095	Exon 1	gccttgcc/tgccgcc	0.33	0.44	198190024
3	New1	Exon 2	taccaca/ggtcttc	0.03	0.06	198189061
4	rs1050347	Exon 2	atctcact/ccgggct	0.23	0.35	198189010
5	rs8539	Exon 3	aatacaaa/gaacatt	0.47	0.50	198187524
6	rs2340690	Intron 3	tgttagt/ctcataat	0.23	0.35	198186392
7	New2	Exon 5	tcacagta/gaaggca	0.02	0.04	198184890
8	rs3214832	Intron 5	tttgagttatcttatgatcaaaag/-	0.23	0.35	198184834
9	New3	Intron 5	aaaaaac[ag/-]tgcctt	0.47	0.50	198184774
10	rs7585486	Intron 5	gtaattc/gcatgtt	0.23	0.35	198184533
11	—*	Intron 7	caaaaa/caaaaac	0.23	0.35	198180659
12	New4	Intron 7	aaaaaaa[ca/-]cacaaaa	0.47	0.50	198180643
13	rs788016	Intron 11	gaatac/ttttccaa	0.23	0.35	198177799

*SNP reported in Ensembl database without rs number.

Table 3 General characteristics of CHD patients and controls

Variable	Cases (n=1,003)	Controls(n=1,003)	P Value
Sex (m/f)	638/365(63.6/36.4)	637/366(63.4/36.6)	0.94
Age (mean $\pm$ SD)	59.7 $\pm$ 8.8	59.5 $\pm$ 8.5	0.31
SBP (mmHg)	134.6 $\pm$ 24.6	131.6 $\pm$ 21.1	0.004
DBP (mmHg)	82.0 $\pm$ 14.4	82.5 $\pm$ 11.1	0.45
BMI	24.46 $\pm$ 3.51	24.41 $\pm$ 3.37	0.77
FBG (mmol/L)	6.66 $\pm$ 3.63	5.32 $\pm$ 2.00	0.00
TC (mmol/L)	4.50 $\pm$ 1.05	5.08 $\pm$ 1.30	0.00
TG (mmol/L)	1.73 $\pm$ 1.24	1.69 $\pm$ 1.31	0.45
Smoking (no/past or current)	435/564 (43.5/56.5)	535/468 (53.3/46.7)	0.00
Smoking index (pack-year)			0.00
0	435 (43.5)	535 (53.3)	
0–31	281 (28.1)	273 (27.2)	
31	283 (28.4)	195 (19.5)	
Drinking (no/yes)	733/266 (73.4/26.6)	681 /316 (68.3/31.7)	0.01
Past history			
Hypertension	705 (70.4)	342 (34.9)	0.00
DM	277 (27.8)	63 (6.5)	0.00
Family history of cardiovascular disease	111 (11.1)	31 (3.1)	0.00

Hsp60 genotypes and CHD risk

As shown in Table 4, the genotype frequency of *hsp60* polymorphisms ranged from 7.6% to 58.8%. For each *hsp60* polymorphism, there was no significant difference in genotype distribution between CHD and controls, and adjustment

for the conventional risk factors such as age, sex, pack-years of smoking, drinking, activity, hypertension, DM, and family history of CHD did not appreciably alter the results (Table 4). Stratified analysis according to age (≤ 60 years and > 60 years), sex, smoking status, past history of hypertension, and diabetes yielded the same results (data not shown).

Table 4 Association between SNPs in the *hsp60* gene and risk of CHD

Genotype	Cases		Controls		Crude OR (95%CI)	Adjusted OR (95%CI)*
	No.	(%)	No.	(%)		
Rs2340690						
TT	542	55.9	534	53.9	1.00	1.00
TC	339	35.0	371	37.4	0.90 (0.75–1.09)	0.89 (0.71–1.12)
CC	88	9.1	86	8.7	1.01 (0.73–1.39)	1.12 (0.75–1.66)
TC + CC	427	44.1	457	46.1	0.92 (0.77–1.10)	0.93 (0.75–1.16)
rs788016						
GG	497	54.2	543	55.5	1.00	1.00
AG	337	36.2	349	35.7	0.95 (0.78–1.15)	1.00 (0.78–1.27)
AA	87	9.6	86	8.8	0.91 (0.66–1.25)	1.12 (0.75–1.67)
AG + AA	424	45.8	435	44.5	0.94 (0.78–1.13)	1.02 (0.82–1.28)
rs2305560						
CC	552	58.8	543	54.9	1.00	1.00
CT	315	33.6	367	37.1	0.84 (0.70–1.02)	0.85 (0.68–1.08)
TT	71	7.6	79	8.0	0.88 (0.63–1.24)	1.00 (0.66–1.54)
CT + TT	386	41.2	446	45.1	0.85 (0.71–1.02)	0.88 (0.70–1.10)
rs2565163						
CC	310	32.3	332	33.3	1.00	1.00
CT	433	45.1	437	43.9	1.06 (0.87–1.30)	0.94 (0.74–1.21)
TT	218	22.7	227	22.8	1.03 (0.81–1.31)	1.04 (0.77–1.41)
CT + TT	651	67.8	664	66.7	1.05 (0.87–1.27)	0.98 (0.77–1.23)

*Adjusted for age, sex, pack-years of smoking, drinking, activity, hypertension, DM, and family history of CHD.

The potential associations between the polymorphisms of *hsp60* and anthropometric markers such as BMI, TC, systolic and diastolic blood pressure, and TG were also analyzed, but the results indicated that there were no significant differences in different genotype groups of four SNPs, and adjusting for conventional risk factors did not appreciably alter the results (data not shown).

Haplotype associations with CHD risk

All the pairwise LD measure D' of the four investigated SNPs in *hsp60* gene are above 0.87, whereas r^2 ranged from 0.10 to 0.90 (data not shown). A total of 13 haplotypes were estimated in the CHD and control groups by using PHASE 2.0 software to reconstruct haplotypes based on the observed genotypes (Stephens and Donnelly 2003). Among the 13 haplotypes, five were $>1.0\%$, namely *GTTC*, *ACTC*, *GCCT*, *GCTC*, and *GCCC*. The associations between the common haplotypes (covering 97.35% and 98.50% of allelic variance in CHD and controls, respectively) encompassing *hsp60* polymorphisms and CHD were also examined. Compared with the highest-frequency haplotype of *GTTC*, the *GCTC* haplotype had 91% increased risk of CHD (OR=1.91, 95% CI: 1.26–2.89, $P=0.002$) (Table 5).

Discussion

There is increasing evidence that elevated levels of Hsp60 and Hsp60 antibody are associated with CHD (Xu et al. 2000; Zhu et al. 2001). However, no previous study has examined the relationships between *hsp60* genetic variations and the risk of CHD; specifically, none has investigated the association between the *hsp60* gene and the risk of CHD using genetic markers to represent the overall variability of this gene. Therefore, the relationship between the variations in the *hsp60* gene and CHD risk still remains unclear.

In this study, we complement our resequencing SNP panel with validated markers from HapMap to provide additional coverage of the genetic variation within the *hsp60* gene. In addition, we investigated the associations between polymorphisms of *hsp60* and CHD risk in a relatively large case-control study in a Chinese population. None of the polymorphisms showed a significant association with CHD. In addition, we did not find any significant associations between *hsp60* gene variations and BMI, systolic and diastolic blood pressure, TC, and TG levels. However, using a haplotype-based approach, we found that haplotype distributions between the cases and the controls were statistically different for *GCTC*, which increased the risk of CHD by 91%.

It has been documented that information on haplotype blocks can increase the power by 15–50% compared with single SNP analysis (Gabriel et al. 2002; Thomas et al. 2003). It may be more reasonable to explain the relationship between genotype and phenotype, although the exact underlying molecular mechanism still remains unclear.

Hsp60 is an important chaperone protein and can be induced to protect cells from injury under stressful conditions. Most of the known risk factors for CHD, such as oxidized low-density lipoprotein, hypertension, infections, and oxidative stress, can increase the expression of Hsp60 in endothelial cells and smooth muscle cells. Extracellular Hsp60, which can induce adhesion molecules and cytokine production, may further promote local inflammation and possibly activate the innate immune system (Kol et al. 1999, 2000). On the other hand, there is 50–70% amino acid sequence homology between human and microbial Hsp60 (Wick et al. 2001). Serum antibodies against microbial Hsp60 may cross-react with human Hsp60 expressed in and released from the stressed cells of the blood vessels. These cross-reactions to human Hsp60 may play an important role in the process of CHD (Mayr et al. 1999). In addition, complement activation plays an important role in the development of CHD, and human

Table 5 Association between haplotypes of *hsp60* and CHD

Haplotype*	CHD		Controls		OR (95%CI)	P value
	No.	%	No.	%		
<i>GTTC</i>	875	43.62	883	44.02	1.00	–
<i>ACTC</i>	515	25.67	517	25.77	1.01 (0.86–1.17)	0.947
<i>GCCT</i>	460	22.93	517	25.77	0.90 (0.77–1.05)	0.178
<i>GCTC</i>	68	3.39	36	1.79	1.91 (1.26–2.89)	0.002
<i>GCCC</i>	35	1.74	23	1.15	1.54 (0.90–2.62)	0.116
Others†	53	2.65	30	1.50	1.78 (1.13–2.82)	0.013
Total	2,006	100	2,006	100	–	–

* Polymorphic bases were in 3' to 5' order, and from left to right the SNPs are rs788016, rs2565163, rs2340690, and rs2305560.

† Haplotype frequency $<1.0\%$ including *ATTG*, *GTCC*, *GCTT*, *ACCT*, *GTCT*, *ACTT*, *GTTT*, and *ACCC*.

Hsp60 may activate the complement system by complexing of antibodies to human Hsp60 (Prohaszka et al. 1999). These findings suggest that Hsp60 might play a multifunctional role in CHD development.

The present study has several strengths. This is the first study on the association between *hsp60* gene polymorphisms and CHD risk in a Chinese population. The large number of cases and controls provides sufficient power for us to detect effects of the genotypes. In addition, for the first time, we used tagging single nucleotide polymorphisms (tagSNPs) to explore the association between polymorphisms of *hsp60* and CHD risk. This made it possible to detect all the genetic variants across a specific region by using a subset of tagSNPs, which infer the allelic state of all of the common polymorphisms and cover maximum genetic variability. The use of tagSNPs in genetic association studies has been considered an efficient method to narrow association signal and localize susceptibility variants (The International HapMap Consortium 2003). Third, our populations are racially homogeneous, with all the participants being Han Chinese, thus minimizing the possibility of population stratification.

Three limitations should also be acknowledged. First, like all other case-control studies, there may have been selection bias (inclusion of patients surviving CHD). Meanwhile, some of our controls may have undiagnosed CHD, which might bias the results. Because we selected controls with normal electrocardiograms and without history of CHD, but without performing coronary angiography on them, we could not exclude the possibility that some of them were affected by CHD. Second, the haplotype of the *hsp60* gene associated with CHD in this study may be in linkage disequilibrium with polymorphisms of other nearby genes that actually contribute to the development of CHD. Finally, although we included >1,000 cases of CHD and a comparable number of age- and sex-frequency matched controls in the final analysis, the present study was limited due to exclusion of low-frequency SNPs, which might be important in CHD development.

In conclusion, this study provides evidence that the haplotypes of the *hsp60* gene are genetic susceptibility factors for CHD in Chinese, but the underlying mechanism remains to be elucidated in further studies.

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