Type 2 diabetes mellitus (T2DM) is a chronic disorder, the prevalence of which continues to increase at an alarming rate. By the year 2025, it is estimated that this disorder will affect 300 million worldwide. Developing countries, particularly India, face a major problem due to the high prevalence of diabetes. T2DM is characterized by defects in insulin secretion and insulin sensitivity.

Oxidative stress, through the production of reactive oxygen species, has been proposed as the root cause underlying the development of insulin resistance, ß-cell dysfunction, impaired glucose tolerance, and T2DM. Oxidative stress has also been implicated in the progression of long-term diabetes–associated complications. Heat-shock proteins (HSPs) are molecular chaperones synthesized under stress conditions; they are induced by denatured proteins during heat shock, ischemia, and other types of cellular stress. HSPs are important in physiological and pathological processes and are highly active within the immune system. HSPs help restore protein homoeostasis and assist cellular recovery from stress by repairing damaged proteins through refolding or by degrading them.

HSPs have also been reported to be involved in diabetes through their effect on insulin sensitivity. The HSP70 (70 kDa HSP) family is the most abundant in eukaryotic cells.

**ABSTRACT**

**Objective:** To examine 2 single nucleotide polymorphisms (SNPs) of the heat shock protein 70-2 (HSPA1B) and 70-hom (HSPA1L) genes in patients with type 2 diabetes mellitus (T2DM) in our study population.

**Methods:** A total of 104 patients with T2DM and 124 healthy control individuals were included in the study. G1538A in HSPA1B and C2437T in HSPA1L polymorphisms were determined using the polymerase chain reaction–restriction fragment length polymorphism technique.

**Results:** The frequency of the HSPA1B genotype was as follows: AA: 33 (26.6%); AG: 82 (66.2%); and GG: 9 (7.2%) in the control group and AA: 2 (2.0%), AG: 57 (54.8%), and GG: 45 (43.2%) in the T2DM group (P<.001). The frequency of the HSPA1L genotype was as follows: CC: 20 (16.1%), CT: 92 (74.2%), and TT: 12 (9.7%) in the control group and CC: 8 (7.6%), CT: 65 (62.5%), and TT: 31 (29.8%) in the T2DM group (P = .003).

**Conclusion:** Our findings indicate that HSPA1B, HSPA1L, and their functional polymorphisms may play a role in the pathogenesis of T2DM.

**Keywords:** HSP, polymorphism, single nucleotide, type 2 diabetes
cells and is essential for cell survival under stress conditions. In humans, 3 genes encoding members of the HSP70 class are mapped within the MHC class III region (6p21.3): HSP70-1 (HSPA1A) (OMIM: 140550), HSP70-2 (HSPA1B) (OMIM: 603012) and HSP70-Hom (HSPA1L) (OMIM: 140559). These genes are polymorphic, with some variants potentially accounting for a change in function and susceptibility to stress tolerance.

HSP70 polymorphisms were found to be risk factors in several human disorders. Therefore, the aim of this study was to investigate the functional single nucleotide polymorphisms (SNP) of HSPA1B and HSPA1L and to correlate the findings with the occurrence of T2DM.

### Material and Methods

#### Subjects

Peripheral blood samples were collected from 104 patients with T2DM and 124 healthy control subjects. The patient and control groups were selected from the outpatient department of M.V. Hospital for Diabetes, Chennai, India. Patients and controls were all of South Indian ethnicity. Anthropometric and certain biochemical parameters were recorded. The study protocol was approved by the institutional ethical committee of M.V. Hospital for Diabetes; written informed consent was obtained from all participating individuals.

#### Biochemical Tests

Biochemical analyses were performed on a Hitachi-912 Autoanalyzer (Hitachi, Ltd., Tokyo, Japan) using kits supplied by F. Hoffman La Roche, Ltd. (Basel, Switzerland). We measured fasting plasma glucose (via the glucose oxidase-peroxidase [GOD-POD] method), serum cholesterol (via the cholesterol oxidase-phenol4-amino antipyrine peroxidase [CHOD-PAP] method), serum triglycerides (via the glycerol phosphatase oxidase-phenol4-amino antipyrine peroxidase [GPO-PAP] method), and high-density lipoprotein cholesterol (via the direct method using polyethylene glycol-pretreated enzymes). Low-density lipoprotein cholesterol was calculated using the Friedewald formula. Glycosylated hemoglobin (HbA1c) was estimated by high performance liquid chromatography using the Variant (Bio-Rad Laboratories, Inc, Hercules, CA). Serum creatinine was measured using the Jaffe method (coefficient of variation, 5.7%).

### Genotyping

Ethylenediaminetetraacetic acid (EDTA) anticoagulated venous blood samples were collected from all study subjects; the genomic DNA was isolated from whole blood by proteinase K digestion followed by phenol-chloroform extraction. Extracted DNA was aliquoted for each sample and stored at -20° C for further analysis. The HSPA1B gene polymorphism (A1538G) was genotyped using the following primers: forward, 5’-GTG CTC CGA CCT GTT CCG AAG C-3’ and reverse 5’-CGG AGT AGG TGG TGA AGA TCT G-3’ (Sigma-Aldrich Co., St. Louis, MO). The reaction mix for polymerase chain reaction contained 25-75 ng of DNA, 10 pmol of oligonucleotides, and a 2× solution of Prime Taq Premix (GeNet Bio, Nonsam, South Korea). Amplification was accomplished by initial incubation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 1 minute, followed by a final incubation at 72°C for 10 minutes. This amplified fragment of 383 base pairs (bps) contained an AG polymorphism in position 1538. Amplimers were digested by the restriction endonuclease PstI (Boehringer Ingelheim GmbH, Ingelheim am Rhein, Germany) at 37°C in 1× One Phor All buffer (Boehringer Ingelheim GmbH) for 2 hours. In the presence of PstI, site restriction digest produced 2 fragments of 244 bp and 139 bp, respectively.

The HSPA1L polymorphism (C2437T) was genotyped using the following primers: forward, 5’-GAT CCA GGT GTA TGA GGG-3’ and reverse 5’-GTA ACT TAG ATT CAG GTC TGG-3’ (Sigma-Aldrich Co). The reaction mixture for PCR contained 25-75 ng DNA, 10 pmol of oligonucleotides, and a 2× solution of Prime Taq Premix (GeNet Bio). Amplification was accomplished by initial incubation at 94°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C, and extension at 72°C for 1 minute, followed by a final incubation at 72°C for 10 minutes. This amplified fragment of 706 bp containing a CT polymorphism in position 2437. The PCR product was digested with the restriction endonuclease NcoI (Boehringer Ingelheim GmbH) at 37°C in 1× One Phor All buffer (Boehringer Ingelheim GmbH) for 2 hours. This yielded 2 fragments of lengths 550 bp and 155 bp, respectively.

The digested products were visualized by ethidium bromide staining in 1.5% agarose gel. Size was determined by comparison to a molecular weight standard 1 kb Plus (Life Technologies Corporation, Carlsbad, CA). To ensure that the genotyping was of adequate quality, we performed duplicate genotyping in 20% of the samples, randomly selected.
Statistical Analysis

Student t testing via SPSS Windows, version 10.0 (SPSS, Inc, Chicago, IL) was performed for statistical analysis. \( \chi^2 \) testing was used to compare the proportions of genotypes or alleles. \( P \) values of less than .05 were considered significant.

### Results

#### Demographic Details of Study Subjects

The demographic and laboratory characteristics of the study subjects are shown in Table 1. A statistically significant difference was noted between controls and patients in age, percentage HbA1c, fasting plasma glucose, systolic blood pressure, diastolic blood pressure, serum triglyceride levels, total cholesterol, and in the LDL cholesterol (\( P < .001 \) for all).

#### Frequency Distribution of HSPA1B (G1538A) and HSPA1L (C2437T) Polymorphisms

The PCR-amplified product for HSPA1B was a 383-bp amplicon. After digestion with PstI restriction enzyme, the homozygotic AA genotype was identified by the presence of a single uncut 383-bp band, the homozygotic mutant GG genotype by 244- and 139-bp bands, and the heterozygotic AG genotype by the presence of 383-, 244-, and 139-bp bands (Figure 1). The distribution of HSPA1B alleles and genotypes in T2DM and the control group from our study population is shown in Table 2. Evaluation of the HSPA1B polymorphisms by PstI restriction digestion revealed that the prevalence of AA was 2.0% in patients and 26.6% in controls. The frequency of AG was 54.8% in patients and 66.2% in controls. The frequency of GG was 43.2% in patients, and 7.2% in controls (Table 2). Statistical analysis of our data showed a significant increase in the

### Table 1. Clinical and Biochemical Characteristics of the Cohort Individuals

<table>
<thead>
<tr>
<th>N Variable</th>
<th>Healthy Controls</th>
<th>Patients with Type 2 Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Age (y)</td>
<td>40.7 (8.0)</td>
<td>49.3 (8.5)</td>
</tr>
<tr>
<td>2 Gender (male/female)</td>
<td>55/69</td>
<td>51/53</td>
</tr>
<tr>
<td>2 Body mass index (kg/m²)</td>
<td>22.1 (1.7)</td>
<td>26.7 (4.7)</td>
</tr>
<tr>
<td>4 Fasting plasma glucose (mg/dL)</td>
<td>97.4 (9.2)</td>
<td>213.4 (79.7)</td>
</tr>
<tr>
<td>5 Glycosylated hemoglobin (%)</td>
<td>5.5 (0.4)</td>
<td>9.9 (2.3)</td>
</tr>
<tr>
<td>6 Serum triglyceride (mg/dL)</td>
<td>99.9 (25.7)</td>
<td>167.8 (94.4)</td>
</tr>
<tr>
<td>7 Total cholesterol (mg/dL)</td>
<td>155.0 (25.8)</td>
<td>201.3 (54.8)</td>
</tr>
<tr>
<td>8 HDL cholesterol (mg/dL)</td>
<td>43.9 (8.2)</td>
<td>46.6 (8.7)</td>
</tr>
<tr>
<td>9 LDL cholesterol (mg/dL)</td>
<td>86.4 (9.7)</td>
<td>128.3 (44.1)</td>
</tr>
<tr>
<td>10 Systolic blood pressure (mmHg)</td>
<td>114.2 (8.7)</td>
<td>124.0 (18.5)</td>
</tr>
<tr>
<td>11 Diastolic blood pressure (mmHg)</td>
<td>76.0 (5.5)</td>
<td>81.0 (8.2)</td>
</tr>
</tbody>
</table>

HDL, high-density lipoprotein; LDL, low-density lipoprotein.

\( \text{a} n = 124; 55 \text{men, 69 women.} \)

\( \text{b} n = 104; 51 \text{men, 53 women.} \)
frequency of GG among patients and AA among controls ($P < .001$ for each). The frequency of the A allele was 29.3% and 59.7% in patients and controls, respectively. The frequency of the G allele was 70.7% and 40.3% in patients and controls, respectively (Table 2). Statistical analysis showed a significant increase in the frequency of the A allele among controls and the G allele among patients with T2DM ($P < .001$).

The PCR-amplified product for HSPA1L was a 706-bp amplicon. After digestion with NcoI restriction enzyme, the homozygotic CC genotype was identified by the presence of a single uncut 706-bp band, the homozygotic mutant TT genotype by 550- and 15-bp bands, and the heterozygotic CT genotype by the presence of 706-, 550-, and 155-bp bands (Figure 2).

The distribution of HSPA1L alleles and genotypes in T2DM and the control group from our study population is shown in Table 3. Evaluation of the HSPA1L polymorphisms by NcoI restriction digestion revealed that the prevalence of CC was 7.6% in patients and 16.1% in controls. The frequency of CT was 62.5% in patients and 74.2% in controls. The frequency of TT was 29.8%; in controls, it was 9.7% (Table 3). Statistical analysis of our data showed a significant increase of TT among patients and of CC among controls ($P = .003$). The frequency of the C allele was 38.9% and 53.2% in patients and controls, respectively. The frequency of the T allele was 61.1% and 46.8% in patients and controls, respectively (Table 3). Statistical analysis showed a significant increase in the frequency of the C allele among controls and of the T allele among patients with T2DM ($P = .003$).
Discussion

Polymorphism within HSPA1B and HSPA1L has been characterized by Milner and Campbell, who identified a polymorphic PstI site of HSPA1B (Hsp70-2) and a polymorphic NcoI site of HSPA1L (Hsp70-hom). In our study, 104 patients with T2DM were genotyped for HSPA1B (G1538A) and HSPA1L (C2437T) polymorphisms. The results show that HSPA1B polymorphism at position 1538 and HSPA1L variant at position 2437 are associated in patients with T2DM. The possible role of HSPA1B (Hsp70-2) polymorphism in various diseases has been reported previously.

Although this polymorphism does not alter the amino acid sequence of the HSP70-2 protein, a previous study showed that different genotypes of HSPA1B (Hsp70-2) were associated with different levels of mRNA expression. In our study, the GG genotypes of T2DM patients were significantly different, statistically, compared with those of healthy individuals within HSPA1B (A1538G). Also, the frequency of the G allele was significantly higher in patients compared to controls. This result is inconsistent with the findings from an earlier report in patients with chronic heart failure. The biochemical characteristics of patients with T2DM included higher total cholesterol and LDL-cholesterol levels. Thus, the observed effect of the polymorphism might be associated with an increased risk of cardiovascular disease. This finding agrees with data reported in which the author demonstrated that total cholesterol and LDL-cholesterol concentrations were significantly higher in patients with the B+ genotype (ie, the presence of a G allele) than in patients with the B− genotype with T2DM and atherosclerosis.

A study in an ethnic Polish population by Buraczynska et al investigated the association of HSP-70 polymorphism and susceptibility to diabetic nephropathy in patients with T2DM; this association was not observed for diabetes alone. In that study, the author showed a strong association of HSP70-2 polymorphism (A1267G) with the GG genotype and also an association of the G allele with diabetic nephropathy (for the G allele, odds ratio [OR], 4.77 (95% CI, 3.81-5.96). Recently, Mir et al, investigating a population similar to ours, measured HSP-70 polymorphism in patients with diabetic foot ulcers. They found that of the patients with diabetes, 70% carried the AG genotype of HSPA1B and 30% carried the GG genotype. Also, the AG genotype was significantly associated with the severity of foot ulceration, whereas in the present study, we found no significant association of the AG genotype in the patient group (P = .12). The apparent disparity between the Mir et al study and ours, although the studies were conducted in populations with similar ethnicity, could be because the healthy control group was not included in Mir’s study; further, in our study, we excluded the patients with diabetic foot ulcers. The HSP70-hom +2437 C/T polymorphism (ie, MetThr amino acid substitution at position 493) has been reported to be associated with spondyloarthropathies, sarcoidosis, and type 1 diabetes mellitus (T1DM). This substitution may be associated with variations in the peptide-binding specificity of different HSP70-hom haplotypes. In our study, the frequency of the TT genotype of HSPA1L (Hsp70-hom) was statistically different from the frequency observed in healthy individuals. Also, the frequency of the T allele in the HSPA1L gene was significantly higher in patients compared with controls. Our results are in contrast to those of 2 different studies, in which the investigators observed no statistically significant differences in the genotypic distribution between patients with T2DM-associated nephropathy and foot ulcers. The contradiction between these findings and the findings of Buraczynska et al may result from the difference in ethnic background of the study populations. With regard to the disagreement between our results and those of Mir et al, we excluded the group with diabetic foot ulcers in our study, whereas their study included those patients.

In conclusion, we observed an association of both the ‘GG genotype of HSPA1B and the TT genotype of HSPA1L with T2DM in a South Indian population. A strength of our study is that all of the patients and controls are of the same ethnic origin; furthermore, all subjects were examined in a standardized manner with well-defined diagnostic criteria. Studies in different populations and with larger sample sizes are required to validate these findings.

Acknowledgments

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References


