Polymorphism in heat-shock protein 70-1 (HSP70-1) gene promoter region and susceptibility to tuberculoid leprosy and pulmonary tuberculosis in Asian Indians

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Heat shock proteins (HSP) act as immunological target structures either by themselves because of an unusual expression pattern, or they are carrier proteins for immunogenic peptides. A three-allele polymorphism of HSP70-1 promoter region was analysed in random patients with pulmonary tuberculosis (PTB), or with tuberculoid (TT) leprosy and healthy controls from North India. HSP70-1A and HSP70-1C occurred more frequently (>60%) while HSP70-1B occurred infrequently in this population. Only HSP70-1A allele was significantly increased in TT leprosy as compared to healthy controls (91.3% vs 71.1%, P<0.03, RR = 4.58). Although a strong association of HLA-DR15 was observed with both of these patient groups in earlier studies, no correlation was found between HSP70-1 promoter alleles with any of the HLA allotypes. Amongst six possible genotype combinations of HSP70-1 promoter allele, only four (A/A, A/B, A/C, C/C) were encountered in Asian Indians. A significant increase of HSP70-1 A/C genotype was observed among DR15 negative PTB patients as compared to DR15 negative controls (87.5% vs 35.7%, X²=8.6, P<0.02) giving highest relative risk of 12.6. These findings suggest that HSP70-1 genes may play a secondary role to HLA-DR in governing susceptibility to mycobacterial infectious diseases.

A strong association has been demonstrated between HLA-DR2 and susceptibility to tuberculoid (TT) leprosy and pulmonary tuberculosis (PTB)1,2. Contributions by major histocompatibility complex (MHC)-linked non-HLA genes in governing susceptibility to various clinical diseases have been investigated in recent time. For instance, 1) alleles of complement genes (C4 and C2) in systemic lupus erythematosus3, 2) tumor necrosis factor (TNF) genes in murine autoimmune lupus nephritis4 and clinical tuberculosis5 and 3) transporter associated with antigen-processing (TAP) genes in TT leprosy and clinical PTB6 have been demonstrated as important susceptibility determinants.

Three genes (HSP70-1, HSP70-2, and HSP70-hom) encoding members of the heat-shock protein 70 (HSP70) family have been mapped to the MHC class III region7. The amino acid sequences of HSP70-1 and -2 are 100% identical to each other and 90% identical to that of HSP70-hom. However, the three genes differ in their expression pattern. HSP70-1 is expressed constitutively at a low level and at high level after thermal shock7. In contrast, HSP70-2 is expressed only following thermal shock and HSP70-hom is expressed at a low level both constitutively and after thermal shock7. The 5' upstream regulatory and untranslated regions of HSP70-1 show polymorphic variations and 3 allelic forms named HSP70-1A, -B and -C have been demonstrated on the basis of nucleotide substitutions at positions -110 and +1206 (Table 1). This restricted polymorphism has been shown to be responsible for heterogeneity in their expression. Particularly, nucleotide position -110 has been identified to be a binding site for heat shock transcription factors8. On the other hand, several heat-shock proteins of Mycobacterium leprae and M. tuberculosis have been characterized to have strong immunogenicity9,10. Thus HSPs of host and the invading mycobacterium are important candidates for immune response in leprosy and tuberculosis. In the present study, prevalence of HSP70-1 alleles have been investigated in the patients with TT leprosy and PTB from North India. In addition, the data were analysed in relation to the distribution of HLA-A, -B,
elucidate their relative role in susceptibility to these mycobacterial infectious diseases.

Materials and Methods

Patients and healthy controls — A total of 49 unrelated patients with TT leprosy (average age 36 years, 30 males and 19 females) and 55 patients with PTB (average age 36.5 years, 35 males and 20 females) were studied. All these subjects were previously studied for HLA association and the findings have been published elsewhere. Leprosy patients were diagnosed according to clinical examination and laboratory tests (skin-smear bacteriology and histopathology of the skin-lesion biopsy material). Active PTB was diagnosed by the presence of acid-fast bacilli (AFB) in direct sputum smear or in cultures, and by the standard clinical and radiological investigations. Sputum smears were examined at least thrice in each case for AFB by auramine stain fluorescent microscopy. All patients were obtained from All India Institute of Medical Sciences, Ansari Nagar, and New Delhi and from Lala Ram Sarup Institute for TB and Allied Diseases, Mehrauli, New Delhi. A team of qualified leprologists, bacteriologists and histopathologists were involved in the diagnosis.

Unrelated healthy subjects (38; average age 35.5 years; 20 males and 18 females) of the same socioeconomic status and ethnic background as the patients were included in the study as controls. None had a family history or symptoms of leprosy, tuberculosis or other related disorders. The patients and controls represented a fairly homogeneous ethnic group of North Indian Hindus from the states of Punjab, Haryana, Uttar Pradesh and Delhi. North Indians are the descendants of Aryans who migrated from the Iranian plateau during the second and third millennium.

PCR-SSOP genotyping of HSP70-1 promoter region — DNA was isolated from peripheral blood mononuclear cell pellets using standard salting out procedures. Alleles at HSP70-1 promoter region were determined by using the polymerase chain reaction (PCR) and hybridization with sequence specific oligonucleotide probes (SSOPs) as described previously. Promoter region (from -273 to +215) of HSP70-1 gene was PCR-amplified using appropriate primers (Table 2). Amplification was performed in 25 µl reactions using 0.625 U AmpliTaq polymerase, 2.5 µl 10x buffer (Perkin-Elmer, Norwalk, CT), and 0.2 mM dNTPs (Promega, Madison, WI) under the following conditions: 35 cycles of 95°C denaturation for 45 sec, 64°C annealing for 60 sec, and 72°C extension for 75 sec. PCR amplified product (488-bp length) was slotted onto nylon membrane (Dupont, Boston, MA) and hybridized with radiolabeled SSOPs to identify variation at position −110 and +120 (Table 2). After overnight hybridization with a SSOP at 54°C, the blots were washed under stringent conditions and autoradiographed for 30-45 min using Quanta Blue (Eastman Kodak, Rochester, NY) intensifying screens.

Table 1 — Alleles of HSP70-1 promoter region in humans

<table>
<thead>
<tr>
<th>Allele</th>
<th>Polymorphism*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP70-1A</td>
<td>aaaaCccc ttTcc</td>
</tr>
<tr>
<td>HSP70-1B</td>
<td>aaaaCccc ttTcc</td>
</tr>
<tr>
<td>HSP70-1C</td>
<td>aaaaCccc ttTcc</td>
</tr>
</tbody>
</table>

*Polymorphism at -110 and +120 positions are notified in capital letters.

Table 2 — Nucleotide sequence of primers and probes for the analysis of HSP70-1 promoter region polymorphism

<table>
<thead>
<tr>
<th>PRIMERS</th>
<th>position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward: GGGCAATGGAGACCCAACACCC</td>
<td>−110</td>
</tr>
<tr>
<td>Reverse: GCGGATCCGGGCTTCCCTGCTTC</td>
<td>+120</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PROBES</th>
<th>position</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGCAGAACCCTGGGA</td>
<td>−110</td>
</tr>
<tr>
<td>GGGAAAACCCTGGGA</td>
<td>−110</td>
</tr>
<tr>
<td>TCGAGTTCGCGGT</td>
<td>+120</td>
</tr>
<tr>
<td>TCGAGTTCGCGGT</td>
<td>+120</td>
</tr>
</tbody>
</table>

Statistical analysis — Differences in the prevalence of HSP70-1 promoter alleles in patients and healthy controls were analyzed for significance by the chi-square (X²) test. Level of significance was reported as probability (P) values. P value was corrected (Pc) by use of the Bonferroni inequality method, by multiplying the P value by the number of HSP70-1 promoter allele studied. Relative risk (RR) for each of the alleles was calculated by the standard method. Linkage disequilibrium between two gene loci (e.g. HSP70-1 and HLA-DR) was estimated by the X² method.
Results

Prevalence of three HSP70-1 promoter alleles in patients and controls—The prevalence of three HSP70-1 alleles in Asian Indian patients with TT leprosy and PTB was compared with those in healthy controls (Table 3). HSP70-1A was significantly higher in TT leprosy as compared to healthy controls (91.8% Vs 71.1%, \( P_c=0.03 \), \( RR=4.58 \)). No differences were observed in the distribution of HSP70-1B and HSP70-1C alleles among patients and controls.

Linkage disequilibrium between the alleles of HLA and HSP70-1 genes—The data were further analysed to seek association between HLA class I and II, and HSP70-1 alleles. Although DR2 was positively associated with both TT leprosy and PTB\(^1\),\(^2\) patients in North India we did not observe any significant association between HSP70-1 promoter allele and any of the HLA class I and class II alleles, including DR2, in patients or in controls.

Genotypes of HSP70-1 promoter region alleles and susceptibility—Following genotype combinations were found in Asian Indians: A/A, A/B, and A/C, C/C (Table 4). B/C and B/B genotypes were not observed in any of the study samples. Of the remaining HSP70-1 genotypes, a significant increase in prevalence of HSP70-1 A/C genotype was observed among DR15 negative PTB patients as compared to DR15 negative controls (87.5% Vs 35.7%, \( \chi^2=8.6, P_c<0.02 \)) giving a very high relative risk of 12.6 (Table 4). In addition, the genotype C/C was significantly decreased in TT leprosy patients as compared to healthy controls (6.1% Vs 28.9%, \( \chi^2=8.3, P_c<0.03, RR=0.16 \)). This decrease was more pronounced in the DR15 positive TT leprosy as compared to DR15 positive healthy controls (2.6% Vs 29.2%, \( \chi^2=9.5, P_c<0.02, RR=0.064 \)).

Discussion

The results demonstrate a frequent occurrence of HSP70-1A and HSP70-1C alleles (>60%) in Asian Indians. HSP70-1B occurred infrequently in this population. This differential frequency distribution appears to be universal and have been observed in several other ethnic groups\(^1\),\(^2\),\(^3\).

Several studies of HSP70 polymorphism have suggested that these loci may be susceptibility determinants in autoimmunity including rheumatoid arthritis\(^4\), type 1 diabetes\(^5\), Celiac disease\(^6\), Graves' disease\(^7\) and systemic lupus erythematosus\(^8\). In contrast, other studies have failed to show significant relevance of HSP70 polymorphism in the susceptibility to most of these HLA-associated diseases including rheumatoid arthritis\(^9\),\(^10\),\(^11\), diabetes\(^12\), sarcoidosis\(^13\) and multiple sclerosis\(^14\). To our knowledge, this is the first report of an association

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<table>
<thead>
<tr>
<th>Allele</th>
<th>Healthy controls (n=38)</th>
<th>TT leprosy (n=49)</th>
<th>PTB (n=55)</th>
<th>( \chi^2 )</th>
<th>Pec value</th>
<th>Relative risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP70-1A</td>
<td>71.1 [27]</td>
<td>91.8 [45]</td>
<td>67.3 [37]</td>
<td>6.5*</td>
<td>0.03</td>
<td>4.58</td>
</tr>
<tr>
<td>HSP70-1C</td>
<td>71.1 [27]</td>
<td>63.3 [31]</td>
<td>83.6 [46]</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are shown in % phenotype frequency. The number of individuals positive to a particular allele is given in brackets. *TT leprosy vs controls, NS-not significant.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>TT leprosy</th>
<th>PTB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>DR15+</td>
<td>DR15-</td>
</tr>
</tbody>
</table>

Data are shown in % genotype frequency. The number of individuals positive to a particular allele is given in brackets.

\( * \) - DR15+ve control vs DR15-ve PTB, \( \chi^2=8.6, P_c<0.02, RR=12.6 \).

\( * \) - Control total ve TT leprosy total, \( \chi^2=8.3, P_c<0.03, RR=0.16 \).

\( * \) - DR15+ve control vs DR15+ve TT leprosy, \( \chi^2=9.5, P_c<0.02, RR=0.064 \).
between HSP70-1 promoter region alleles and mycobacterial infectious diseases. Several HSPs of *M. leprae* and *M. tuberculosis* have been shown to be the major targets of the immune response. Significant sequence homology has been observed among HSP proteins of *M. leprae*, *M. tuberculosis* and humans. The increased occurrence of HSP70-1A allele in TT leprosy may suggest that the *M. leprae* infection and its induced stress may elicit/enhance HSP70-1A expression on infected host cells. These molecules may mimic *M. leprae* HSP that drive to an aberrant immune response causing the disease.

The most significant result obtained in the present study is that an increase in HSP70-1 genotype A/C in HLA-DR15-ve PTB patients versus controls. Heat shock proteins control folding, binding and transport of proteins. The HSP70 anchors processed linear peptides and are expressed on the cell surface, a property which shows similarity with peptide-presenting characteristic of HLA molecules. It has been demonstrated that the peptide-binding domain of HSP70 is similar to the peptide-binding domain of HLA molecules suggesting that both of these molecules derived from a common ancestor. Recent observations that sperm receptors on the surface of sea urchin eggs have an unambiguous homology to HSP70 proteins constitutes the first example of a cell surface form of an HSP70 molecule, and gives evidence to the idea that the MHC and HSP70 might have common motifs. Further, a strong cross-reactivity has been reported between an epitope 'LQAAPALDKLÖ on *M. leprae* HSP and third hypervariable region of the HLA-DR2 molecule (amino acid 69-78 i.e., EQARAAVDTY in the alpha helix of binding groove) which is incidentally associated with TT leprosy and PTB. It is possible that HSP70-1 A/C combination may be important for presentation of disease-causing peptide(s) to immune cells in the absence of HLA-DR2 in patients with PTB, resulting in detrimental immune response.

A strong linkage has been detected between the HSP70-1C allele and HLA-DR3 in a North Italian population. The results in the present study are not agreeing with these data as we did not observe any association between HSP70-1 promoters and HLA-DR allotypes. Variation in the distribution of HSP70-1 genotypes among TT leprosy patients is not supported by higher P-value. This may be due to small sample size, and these important preliminary results must be confirmed by extensive studies. In conclusion, the findings of the present study along with data from earlier studies indicate that HSP70-1 genes may play a secondary role to HLA-DR in influencing susceptibility to mycobacterial infectious diseases.

**Acknowledgement**

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**References**