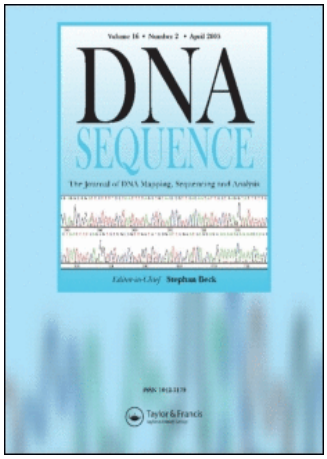

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FULL LENGTH RESEARCH ARTICLE

Sequence analysis of *HSPA1A* and *HSPA1B* in a multi-ethnic study population

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Abstract

Two copies of the Hsp70 gene, *HSPA1A* and *HSPA1B*, are located on chromosome 6p21. The coding regions of *HSPA1A* and *HSPAIB* are intronless and nearly identical, however, promoter and 3′ UTR sequences are different. The coding regions and putative promoter regions of *HSPAIA* and *HSPAIB* were re-sequenced in an affected and unaffected asthma screening panel of US Caucasians, African Americans, and US Hispanics ($n = 72$) to identify polymorphisms. *HSPAIA* and *HSPAIB* were each amplified in two separate whole-gene fragments. The polymorphisms identified were compared to those reported in dbSNP. Nine polymorphisms (one novel) were identified in *HSPAIA*, five of which are coding. Fourteen polymorphisms (five novel) were identified in *HSPAIB*, five of which are coding. One polymorphism (Asp110Glu GAG > GAC) was found in both genes. Two-thirds of the polymorphisms reported in dbSNP were not identified in our screening panel. Although similar in sequence, *HSPAIA* and *HSPAIB* do not share common patterns of polymorphisms.

Keywords: *HSPA1A, nested PCR, asthma, polymorphism, HSPA1B*

Introduction

The inducible form of heat shock protein 70 (Hsp70) is an abundant molecular chaperone that plays a key role in the assembly and activation of the glucocorticoid receptor complex. Two single exon genes (*HSPA1A* and *HSPA1B*) encode identical copies of the 641 amino acid protein Hsp70; and are located in tandem along a ~15 kb region of chromosome 6p23.1 in the class III region of the major histocompatability complex (Pratt and Toft 1997; Temple et al. 2004). The coding regions of *HSPA1A* and *HSPA1B* are identical except for six single base substitutions. The promoter and 3′ untranslated region (UTR) of the genes, however, have considerable sequence differences suggesting that regulation of *HSPA1A* transcription and translation could be distinct from *HSPA1B*.

A number of single nucleotide polymorphisms (SNPs) have been identified in *HSPA1A* and *HSPA1B*, but due to the high degree of similarity between the coding regions of each gene, many SNPs might have been assigned to both genes. Due to potential miss assignment, an accurate assessment of SNP validity and minor allele frequency (F_{ma}) is not available for either gene. Because of the location of these polymorphisms and the identical sequences flanking each *HSPA1A*/*HSPA1B* polymorphism, most SNP genotyping methods are incapable of accurately assessing in which gene the polymorphism is located. This in turn limits genotyping of these polymorphisms and thus their use in genetic association studies. In this study, we have re-sequenced *HSPA1A* and *HSPAIB* separately in a multi-ethnic asthma case and control screening panel, and for the first time were able to

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Table I. Primers utilized for sequencing.

Gene	PCR type	Primer name	Sequence
HSPA1A	PCR	Genomic forward	CGCCTCGGTCTCCCTAAG
		Genomic reverse	CTGACCCCCAAATAAAGGAATAAA
	Nested PCR & sequence	Promoter 1 forward	TCATGGCAGACTGCTTTTTGGACA
		Promoter 1 reverse	GCCCGCCTCGGCCTCTCAA
		Promoter 2 forward	CACCGGCAGTAAATTCCAGTCAGG
		Promoter 2 reverse	GGTAAGGGGGAGGCGGGGTAACT
		Promoter 3 forward	TGGGCGACAGAGCGAGACT
		Promoter 3 reverse	AGGGTGGGGGCGTTTGTGTTGA
		Promoter 4 forward	GGGCTCCCTCAATATCAAACTGC
		Promoter 4 reverse	GGGGCTGGAAACGGAACACTGG
		Promoter 5 forward	TGTCCCAAGGCTTCCCAGAG
		Promoter 5 reverse	GCCCCCGCCCAGGTCAAAGAT
		Coding 1 forward	AGCGCCAGGCCACCAAGGATG
		Coding 1 reverse	GAGCCCCCGACCAGGACCAG
		Coding 2 forward	CTGGGTGGGGAGGACTTTGACA
		Coding 2 reverse	CCTCTCGCCCTCGTACACCTGGAT
		Coding 3 forward	CAACGGGCGCGACCTGAACAA
		Coding 3 reverse	CTCGCGCTGCACCTCGTCCTC
		Coding 4 forward	AGGGCCATGACGAAAGACAACAAT
		Coding 4 reverse	GGCCCCTAATCTACCTCCTCAATG
		Coding 5 forward	CTTGGCCGAGAAGGACGAGT
		Coding 5 reverse	AATTTCAACATTGCAAACACAGGA
HSPA1B	PCR	Genomic forward	AACCCCCTCACATTTTCCTTTCAG
		Genomic reverse	GTTTTGCAGCTGCCATCCGACTTT
	Nested PCR & sequence	Promoter 1 forward	ACCCCCTCACATTTTCCTTTCAGA
		Promoter 1 reverse	TCCCGGGTTTAAGCAATTCTCCTA
		Promoter 2 forward	AAAACAACAGGCCGGGAATGGTG
		Promoter 2 reverse	GGAGGCGCTGGTCGGGTAATG
		Promoter 3 forward	AGCTAGGAATGGGGAGGGAAATGG
		Promoter 3 reverse	TCGATGTGCCTGGGAAACTCTGAT
		Promoter 4 forward	GACCAGCGCCTCCCTTCCTC
		Promoter 4 reverse	GACCCGCCTTTCCCCTTCTG
		Promoter 5 forward	AGAGCGGGCCCTTCCTGTCAA
		Promoter 5 reverse	ATGCCGGTGCCCTGCTCTGT
		Coding 1 forward	GAAGGGGAAAGGCGGGTCTC
		Coding 1 reverse	GCCTCGGCGATCTCCTTCAT
		Coding 2 forward	GGGGATGCGGCCAAGAACCA
		Coding 2 reverse	GAGCCCCCGACCAGGACCAG
		Coding 3 forward	CTGGGTGGGGAGGACTTTGACA
		Coding 3 reverse	CACCGCCGCCCCGTAGG
		Coding 4 forward	CCACGGCCGGGGACACC
		Coding 4 reverse	AGGGCGTTCTTGGCTGACACC
		Coding 5 forward	GCGAGAGGGCCATGACGAAAGA
		Coding 5 reverse	GTCCGCTGATGATGGGGTTACACA
		Coding 6 forward	TCACGGCCACGGACAAGAGC
		Coding 6 reverse	AAAGAACAAAGGCCCCTAATCCAC
		Coding 7 forward	CAGCGAGGCGGACAAGAAGAAGG
		Coding 7 reverse	CTCCCCGCCCCAGTGAACC

accurately validate and assess the allele frequencies of DNA polymorphisms.

Materials and methods

Polymerase chain reaction and DNA sequencing

The DNA screening panel was composed of 72 subjects: 24 Caucasian, 24 African Americans, and 24 Hispanics. Each ethnic group consisted of 16 individuals with asthma and eight unaffected controls (A genome-wide search for asthma susceptibility loci in ethnically diverse populations. The Collaborative Study on the Genetics of Asthma (CSGA) 1997).

PCR primers were designed to independently amplify a ~4.5 kb region of *HSPA1A* and *HSPA1B* (Table I). Each 4.5 kb amplicon contained ~2 kb of promoter and ~500 bp of 3′ UTR in addition to the coding region. PCR was performed in 40 μl reactions using 80 ng of genomic DNA, 15 pmol of both the forward and reverse primer, 4–5 U of Taq polymerase (Life Technologies), and IX FailSafe PCR master mix (Epicentre Technologies, Madison, WI, USA). Cycling conditions were: 94°C for 5 min, followed by

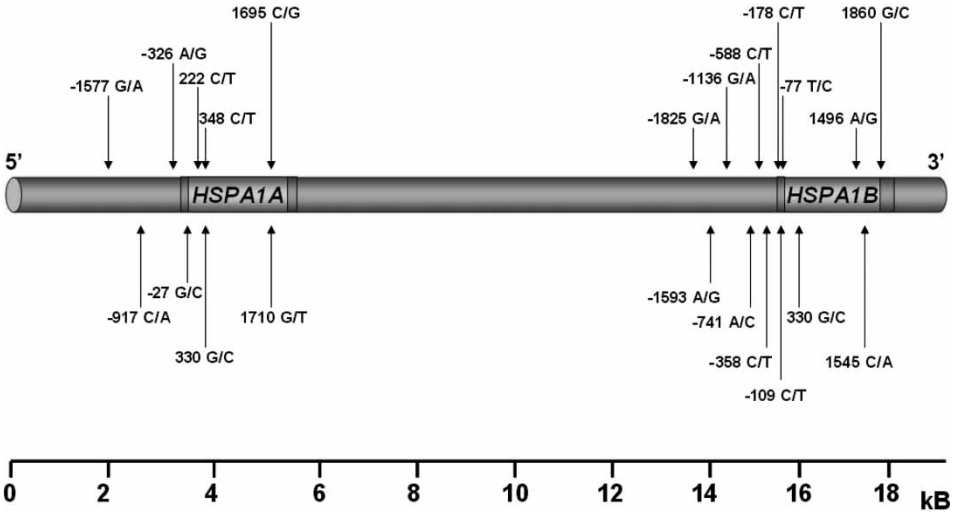

Figure 1. Location of polymorphisms identified in *HSPA1A* and *HSPA1B* genes. Regions marked *HSPA1A* and *HSPA1B* represent coding sequence. The darked regions flanking the coding region represents the 5′ and 3′ UTR sequences. The *HSPA1A* "Genomic Forward"-primer and "Genomic Reverse"-primer are located between chromosomal positions 31889307 and 31889324, and 31893708 and 31893731 respectively (Table I). The *HSPA1B* "Genomic Forward"-primer and "Genomic Reverse"-primer are located between chromosomal positions 31901566 and 31901589, and 31906221 and 31906244, respectively.

40 cycles of 94°C for 1 min, T_{anneal} (62°C for *HSPA1A*, 58°C for *HSPA1B*) for 1 min, and 72°C for 5 min, and an extension at 72°C for 7 min. The *HSPA1A* amplicon was purified via gel extraction (QIAEXII, Qiagen, Hilden, Germany). Both amplicons were purified in a final step using the Perfectprep PCR Cleanup 96 (Eppendorf, Hamburg, Germany). The purity of each amplicon was verified on a 1% agarose gel.

Nested PCR was performed on individual 4.5 kb amplicons to generate overlapping fragments (Table I). Each of the 20 μl nested PCR reactions contained 40 ng of DNA from the amplicons, 0.5 U Taq polymerase, 15 pmol of both the forward and reverse primer, and IX FailSafe PCR master mix. General cycling conditions were: 94°C for 5 min, followed by 30–35 cycles of 94°C for 1 min, T_{anneal} for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min. PCR products were verified on a 1.5% agarose gel and purified using Perfectprep PCR Cleanup 96 (Eppendorf, Hamburg, Germany) prior to DNA sequencing.

DNA sequencing reactions were performed as previously described (Hawkins et al. 2004). Sequencing products were analyzed on an ABI 3730 XL DNA Analyzer (Applied Bioystems, Inc., Foster City, CA, USA). Sequencing reactions were performed on both DNA strands.

Sequence Analysis

Sequence alignment and polymorphism identification were performed using Sequencher 4.2 (Gene Codes, Corp., Ann Arbor, MI, USA). All polymorphisms were validated through observation in two independent DNA samples or by re-sequencing independently generated PCR products. MatInspector (www.genomatix.de) was used to identify predicted transcription factor binding sites in the promoter region. A search of the region sequenced was performed in dbSNP (www.ncbi.nlm.nih.gov) to record all polymorphisms reported in the region.

Results

The database dbSNP listed 24 and 32 polymorphisms for *HSPA1A* and *HSPA1B*, respectively. We were able to validate only eight polymorphisms in *HSPA1A* and nine polymorphisms in *HSPA1B* (Figure 1, Tables II and III) in our screening panel. Only one novel polymorphism was identified in *HSPA1A* (C/T + 348, hP116P), however, five novel polymorphisms were identified in *HSPA1B* (G/A − 1825, A/G − 1593, C/T − 358, G/C + 330, D110E, and C/A + 1545, I515I). Five coding polymorphisms were identified in *HSPA1A*, only one of which was non-synonymous (G/C + 330, DlIOE). Five coding polymorphisms were identified in *HSPA1B*, two of which were non-synonymous (G/C + 330, D110E and A/G + 1496, N499S). The non-synonymous coding polymorphism G/C + 330 (D110E) was listed as being present in only *HSPA1A* (rs562047) by dbSNP and thus was considered to be novel to *HSPA1B*.

Only one polymorphism identified in our screening panel occurred in both genes (G/C + 330, D110E). The F_{ma} of this polymorphism was higher in *HSPA1A* than in *HSPA1B*. Five *HSPA1A* polymorphisms (G/A − 1577, A/G − 326, G/C − 27, G/C + 330, D110E, and C/G + 1695, A565A) were present in all three ethnic groups. One *HSPA1A* polymorphism

Table II. *HSPA1A* Polymorphism locations and frequencies.

Position*	dbSNP accession	Map coordinates	Alleles†	Minor allele‡	Minor allele frequency			
					Caucasian American	African American	Hispanic	dbSNP
−1577	rs12213612	31889936	G/A	A	0.083	0.114	0.083	NA
−1248	**rs11414738**	**31890265**	**A/−**	–	–	–	–	**NA**
−1234	**rs6933097**	**31890279**	**A/T**	**T**	–	–	–	**NA**
−1233	**rs9279425**	**31890280**	**A/−**	–	–	–	–	**NA**
−1083	**rs12526722**	**31890430**	**A/C**	**C**	–	–	–	**NA**
−917	rs16867582	31890596	C/A	A	0.071	0.045	0	0.014
−591	**rs4713489**	**31890922**	**G/A**	**A**	–	–	–	**NA**
−340	**rs11576011**	**31891173**	**G/A**	**A**	–	–	–	**NA**
−326	rs1008438	31891187	A/G	G	0.429	0.682	0.521	0.404
−97	**rs11557922**	**31891416**	**T/C**	**C**	–	–	–	**NA**
−64	**rs11576009**	**31891449**	**G/A**	**A**	–	–	–	**NA**
−27	rs1043618	31891486	G/C	C	0.522	0.705	0.375	0.464
−7	**rs2242667**	**31891506**	**A/C**	**C**	–	–	–	**0.002**
79	**rs11557923**	**31891591**	**G/T (E27-)**	**T**	–	–	–	**NA**
222	rs1043620	31891734	C/T (1741)	T	0.104	0	0.042	NA
330	rs562047	31891842	G/C(D110E)	C	0.261	0.357	0.167	0.038
348		31891860	C/T(P116P)	T	0.109	0	0	–
635	**rs11557924**	**31892147**	**T/C (I212T)**	**C**	–	–	–	**NA**
637	**rs11557921**	**31892149**	**G/T (D213Y)**	**T**	–	–	–	**NA**
1053	**rs1061581**	**31892565**	**G/A (Q351Q)**	**A**	–	–	–	**0.333**
1695	rs506770	31893207	C/G (A565A)	G	0.063	0.455	0.104	0.983
1710	rs541340	31893222	G/T (V570V)	T	0	0.023	0	0.969
1905	**rs508603**	**31893417**	**C/T (P635P)**	**T**	–	–	–	**NA**
1920	**rs508633**	**31893432**	**A/G**	**G**	–	–	–	0.000¶

Bold, identified only in dbSNP (www.ncbi.nlm.nih.gov); NA, not Available; * Position relative to the ATG start codon, where the first base in the start codon is +1; † In parentheses is amino acid change and position; ‡ Based on allele frequency in all three populations; ¶ Frequency is too low to be reported.

Table III. *HSPA1B* Polymorphism locations and frequencies.

Position*	dbSNP accession	Map coordinates	Alleles†	Minor allele‡	Minor allele frequency			
					Caucasian American	African American	Hispanic	dbSNP
−1825		31901882	G/A	A	0	0.021	0.021	–
−1593		31902114	A/G	G	0	0.021	0	–
−1136	rs2763979	31902571	C/T	T	0.417	0.625	0.438	0.478
−741	rs7771177	31902966	A/C	C	0	0.063	0	NA
−646	**rs5026931**	**31903061**	**G/T**	**T**	–	–	–	**NA**
−588	rs13217108	31903119	C/T	T	0.042	0.043	0	NA
−453	**rs11576017**	**31903254**	**C/T**	**T**	–	–	–	**NA**
−429	**rs11576016**	**31903278**	**C/T**	**T**	–	–	–	**NA**
−383	**rs11576015**	**31903224**	G/A	A	–	–	–	NA
−358		31903349	C/T	T	0	0.063	0	–
−338	rs11576011	**31903369**	**G/A**	**A**	–	–	–	**NA**
−192	**rs11576014**	**31903515**	**G/A**	**A**	–	–	–	**NA**
−178	rs6457452	31903529	C/T	T	0.146	0.167	0.083	0.068
−167	**rs11576013**	**31903540**	**G/C**	**C**	–	–	–	**NA**
−135	**rs2607020**	**31903572**	**G/A**	**A**	–	–	–	**NA**
−109	rs11576012	31903598	C/T	T	0.042	0	0	NA
−87	**rs2607019**	**31903620**	**T/C**	**C**	–	–	–	**NA**
−77	rs2607018	31903630	T/C	C	0	0.021	0	NA
−68	**rs2607017**	**31903639**	**G/C**	**C**	–	–	–	**NA**
79	**rs11557923**	**31903785**	**G/T (E27-)**	**T**	–	–	–	**NA**
330		31904036	G/C(D110E)	C	0.063	0.104	0.031	–
379	**rs12190359**	**31904085**	**A/G (M127V)**	**G**	–	–	–	**NA**
635	**rs11557924**	**31904341**	**T/C (I212T)**	**C**	–	–	–	**NA**
637	**rs11557921**	**31904343**	**G/T (D213Y)**	**T**	–	–	–	**NA**
1053	rs1061581	31904759	G/A(Q351Q)	A	0.542	0.292	0.438	0.333
1496	rs483638	31905202	A/G (N499S)	G	0.045	0.095	0	NA
1545		31905251	C/A(I515I)	A	0	0.094	0	–
1860	rs539689	31905566	G/C (G620G)	C	0.368	0.475	0.219	0.460
118	**rs3036297**	**31905750**	**− /AGTTA**	**AGTTA**	–	–	–	**NA**
121	**rs9281590**	**31905753**	**− /AAGTT**	**AAGTT**	–	–	–	**NA**
249	**rs1061623**	**31905881**	**A/C**	**C**	–	–	–	**0.020**
266	**rs9267570**	**31905898**	**G/T**	**T**	–	–	–	**NA**

Bold, identified only in dbSNP (www.ncbi.nlm.nih.gov); NA, not available; * Position relative to the ATG start codon, where the first base in the start codon is +1; † In parentheses is amino acid change and position; ‡ Based on allele frequency in all three populations.

(G/T + 1710, V570V) occurred only in African Americans, while one *HSPA1A* polymorphism (C/T + 348, P116P) occurred only in Caucasians. Five *HSPA1B* polymorphisms (G/A − 1136, C/T − 178, G/C + 330, D110E, G/A + 1053, Q351Q, and G/C + 1860, G620G) were present in all three ethnic groups. Five polymorphisms (A/G − 1593, A/C − 741, C/T − 358, T/C − 77, and C/A + 1545, I515I) occurred only in African Americans, while one polymorphism (C/T − 109) occurred only in Caucasians. No polymorphisms from either gene were exclusive to Hispanics.

Several significant differences were noted between our data and that of dbSNP. First, the polymorphism rs1061581 (G/A, Q351Q) was listed as being in both *HSPA1A* and *HSPA1B*, whereas this polymorphism only occurred in *HSPA1B* in our screening panel. Second, rs506770 (C/G, A565A) is listed in dbSNP as having C as the minor allele, whereas we identified G as the minor allele. Finally, rs541340 (G/T, V570V) is listed in dbSNP as having G as the minor allele, whereas we identified T as the minor allele.

The promoter sequences (2000 bp 5′ of the start codon) of *HSPA1A* and *HSPA1B* were evaluated by MatInspector. No *HSPA1A* polymorphisms altered predicted transcription factor binding sites. Two *HSPA1B* polymorphisms (−741 A/C and −588 C/T) altered predicted transcription factor binding sites. The minor allele of −741 A/C converts a predicted NMP4 (nuclear matrix protein 4)/CIZ (Cas-interacting zinc finger protein) site (ggAAA*A*aacat) to a predicted Fkh-domain factor FKHRL1 (FOXO) binding site (aagacggaAA*C*Aacatc). The minor allele of −588 C/T converts the predicted myelin transcription factor 1-like neuronal C2HC zinc finger factor 1 binding site (cgaaA*G*TTacctt) into a predicted HMGI(Y) high-mobility-group protein I (Y), architectural transcription factor organizing the framework of a nuclear protein-DNA transcriptional complex (ggtAA*T*Tttcggg).

Discussion

This is the first detailed re-sequencing study defining genetic variations in *HSPA1A* and *HSPA1B*. While most polymorphisms identified in this study had similar F_{ma} to that reported in dbSNP, two polymorphisms (rs506770 and rs541340) were found to have significantly different F_{ma} in our screening panel, possibly due to reporting errors in dbSNP. Because past genomic builds identified only one gene encoding both proteins, any assignment of polymorphisms to the gene during these previous genomic builds would have been incorrect. A comparison of polymorphisms listed in Tables II and III with the dbSNP database indicates that approximately 66% of the *HSPA1A* and *HSPA1B* polymorphisms listed were not identified in our screening panel. There are several possible explanations for this observation. First, many of the dbSNP polymorphisms could have arisen from errors in initial sequencing of genomic DNA or errors in data interpretation. Second, many of polymorphisms in dbSNP were derived from expressed sequences, and thus could have been the result of post-transcriptional events such as cloning errors and errors in RT-PCR. In addition, polymorphisms could also be introduced by post-transcriptional events such as RNA editing. Our study also was designed to detect only the most common polymorphisms based on the number of chromosomes screened in each ethnic group. Based on the equation:

$$P = 1 - (1.0 - F_{ma})^{2N}$$

where P is the statistical power of detection, F_{ma} is the threshold minor allele frequency, and N is the number of subjects sequenced; our power to detect polymorphisms with a F_{ma} 0.05 in 24 subjects was approximately 92%, and thus we did not detect some rare polymorphisms identified in dbSNP. However, we believe that our study is comprehensive in identifying the most common variants ($F_{ma} > 0.05$) in these two genes within our study population.

Because of the nearly identical coding regions, the *HSPA1A* and *HSPA1B* loci may have been derived by a duplication event. Despite this possibility, only one polymorphism, (G/C + 330 Dl10E), was common to both *HSPA1A* and *HSPA1B*. This mutation, which produces the conservative amino acid change aspartate to glutamate in the IB domain of lobe I, lies in a region not critical to secondary structure (Zhang and Zuiderweg, 2004). Since this mutation occurs in both genes, the mutational event that introduced the polymorphism probably occurred before gene duplication, however, we can not exclude that the coding change originated independently in both genes. It is not known which gene predominantly expresses the Hsp70 protein; however, the fact that most of the coding polymorphisms are synonymous in both genes indicates that there could be selective pressure to conserve the Hsp70 protein sequence. Unlike the coding regions, however, the putative 5′ promoters and 3′ untranslated regions are dissimilar, indicating that regulation of *HSPA1A* and *HSPA1B* gene expression may be significantly different. Sequence variations in the promoter regions of *HSPA1A* and *HSPA1B* make this possibility more likely. This is supported by a recent study where the C allele of the polymorphism −178 T/C *HSPA1B* was associated with lower levels of *HSPA1B* expression (Temple et al., 2004). Our analysis of promoter sequences of *HSPA1A* and *HSPA1B* identified only two polymorphisms (*HSPA1B* −588 C/T and −741 A/C) in the promoter of either gene that alter predicted transcription factor binding sites. Reporter assays, electromobility shifts assays (EMSA), or a combination of reverse

transcription and real time PCR will need to be utilized to determine the effects of these promoter polymorphisms on gene expression/transcription.

In summary, we have identified the most common polymorphisms in *HSPA1A* and *HSPA1B* in a screening panel of asthmatics and normal controls from three ethnic groups. Our study indicates that most of the *HSPA1A* and *HSPA1B* polymorphisms identified in dbSNP are rare ($F_{ma} < 0.05$) or were derived during post transcriptional events, and probably do not exist in the genomic sequence. Our study also has revealed additional sequence variation in the 5′ and 3′ regions of each gene, which may cause each gene to have unique patterns of gene expression regulation. Therefore, the functional effects of *HSPA1A* and *HSPA1B* polymorphisms are most likely involved in regulation of mRNA and protein levels rather than altering the structure and function of the Hsp70. While our study does have value in the selection of polymorphisms for association analysis, genotyping polymorphisms in the coding regions of *HSPA1A* or *HSPA1B* will be difficult using standard genotyping techniques due to the shared sequence identity.

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