RESEARCH ARTICLE

Role of Heat Shock Protein – 70 in the etiology of Idiopathic Pulmonary Arterial Hypertension

Chaitra KL[1], Satyanarayana ML[1], Vadapalli Shivani[1], Manchineela Sairam, Bhukya Chaitanya Kumar, Sastry BKS[2] and Nallari Pratibha[1*]

[1]Department of Genetics, Osmania University, Hyderabad – 500007
[2]Care Hospitals, Nampally, Hyderabad – 500001
*Corresponding Author: Prof. Pratibha Nallari, Department of Genetics, Osmania University, Hyderabad – 500007 | Email: prathinallari@gmail.com | Ph. No: +91-8885486499

Manuscript details:

Received: 04.10.2016
Accepted: 04.11.2016
Published : 27.01.2017

Editor: Dr. Arvind Chavhan

Cite this article as:
Chaitra KL, Satyanarayana ML, Vadapalli Shivani, Manchineela Sairam, Bhukya Chaitanya Kumar, Sastry BKS and Nallari Pratibha (2016) Role of Heat Shock Protein – 70 in the etiology of Idiopathic Pulmonary Arterial Hypertension, *International J. of Life Sciences*, 4 (4): 509-516.

Acknowledgement:
The authors would like to acknowledge funding from PVRI-GSK, and UGC-RFSMS for fellowship to one of the authors. Author contributions: KLC carried out the study and wrote the paper, SV helped in collecting samples initially, MLS designed the primers, SM and CKM helped with insilico analysis, BKSS is the cardiologist who identified the patients, PN gave the idea for the study and insights to the paper.

ABSTRACT

Idiopathic Pulmonary Arterial Hypertension is a rare disorder that affects the pulmonary artery, whereby the pressure in the pulmonary artery is increased, culminating in overload on the right ventricle and eventually heart failure. Over the years many studies have been carried out to understand the etiology and to identify the appropriate treatment. This study attempts to contribute to the overall understanding of disease in relation with certain genes that are generally expressed under stress conditions, viz., the heat shock proteins (HSPs). Of this, a prominent group of proteins, HSP70, were identified, to establish a correlation with polymorphisms in these genes and any possible effect on IPAH.

Keywords: Idiopathic Pulmonary Arterial Hypertension, Heat shock proteins, single nucleotide polymorphisms, stress, chaperones

INTRODUCTION

Idiopathic pulmonary arterial hypertension (PAH) is a rare but devastating disorder affecting 2–3 people per million each year (Gaine & Rubin, 1998). The condition is familial in 6–10% of cases. PAH is a result of extensive remodelling of the pulmonary vasculature, caused by proliferation and migration of endothelial cells, fibroblasts and smooth muscle cells. Amplified muscularization of small arteries and fibrosis of the intima leads to destruction of small pulmonary arteries (Atkinson et al., 2002; Yi et al., 2000). The resulting increase in pulmonary vascular resistance culminates in right heart failure.

Heat shock proteins (HSP) are a highly conserved family of proteins. Upon heat shock, the most prominent proteins induced belong to the HSP70 family. This family includes the constitutively expressed cytosolic heat shock cognate protein 70 (HSC70), the heat-inducible cytosolic

protein HSP70, the glucose-regulated protein 78 (GRP78) of the endoplasmic reticulum, and the mitochondrial GRP75. Chaperoning functions during synthesis, transport, assembly, renaturation, and degradation of proteins are carried out by these proteins(Gething & Sambrook, 1992). The cell attains a state of transient resistance upon secondary stress, termed thermotolerance, and this state is characterized by an increase in expression of HSP70, which may play a role in repair and protection of the cell structures in nucleolus upon stress, as translocation is observed (Angelidis, Lazaridis, & Pagoulatos, 1991; Collier & Schlesinger, 1986; Dressel & Günther, 1999; Li et al., 1991; Li & Werb, 1982). Immediate translocation of synthesized HSP70 into the cell nucleus and nucleolus occurs in several cell types and tissues, including the heart (Amrani et al., 1997). Altered HSP expression has been associated with protection in many stress conditions, such as ischemia and reperfusion damage, cardiac hypertrophy, metabolic diseases, inflammation, infection, cell and tissue trauma, aging, and cancer(Morimoto & Santoro, 1998).

The class III region of the major histocompatibility complex on human chromosome 6 encompasses two HSP70 genes – *HSP70-1* and *HSP70-2*, which code for identical polypeptides, and a third gene, *HSP70-HOM*, which encodes a testis-specific member of the HSP70 family (Fujimoto et al., 1992), all of which are intron less. While *HSP70-1* and *HSP70-2* are identical, *HSP70-HOM* shares 90% of its sequence identity with other HSP proteins(Milner & Campbell, 1992). Genetic polymorphism in *HSP70* genes may influence its immune modulatory and anti-apoptotic functions and, therefore, may have consequences on the chaperonic activity in the vascular components and in the predisposition and prognosis of the disease. This study focusses on the effect of three polymorphisms in *HSP70,* and on its activity in IPAH.

MATERIAL AND METHODS

The study included 70 cases of IPAH, from whom informed consent was obtained, and 200 randomly selected healthy subjects without history of cardiac and systemic disorders were considered as controls for comparative purposes. The study was approved by the Ethics Committee of Care Hospitals, Hyderabad. The patients included in the study were confirmed IPAH cases, referred by the cardiologist.

Molecular Analyses: DNA was isolated followed by PCR amplification using specific primers. PCR assays was carried out in a 25μl volume tube with 100 ng of genomic DNA, 10pM of each primer, 2.0mM dNTP (Merck, Germany), 1.5mM MgCl2 and 10x PCR buffer [50mM KCl, 500mM Tris buffer, 160mM (NH4)2SO4, pH 8.8, and 0.1% Tween 20], 0.1% Triton X-100 and 0.5U *Taq* polymerase (Invitrogen). The thermal cycling was carried out in Eppendorf Gradient Thermal cycler (Germany). Table 1 gives the primers, annealing temperature and the enzymes used during RFLP analysis.

Statistical Analyses:
Deviations from the Hardy-Weinberg equilibrium were tested for the polymorphisms in cases and controls by comparing observed and expected genotype frequencies based on the exact goodness of fit test. Odds ratios, with 95% confidence intervals, were calculated to compare allele and genotype frequencies. Secondary structure of the mRNA was predicted to determine any changes caused by the variation. Linkage disequilibrium and haplotype analyses were carried out to reveal any association between the variants. HAPMAP data was used to compared and correlate the frequencies obtained worldwide.

Table 1: PCR and RFLP related information for detection of polymorphisms

	HSP70-1 **(+**190G/C**)**	***HSP70-2*** **(+**1267A/G**)**	***HSP70-HOM*** **(+**2437T/C**)**
Forward Primer	CGCCATGGA GACCAACACCC	CATCGACTTC TACACGTCCA	GTCCCTGGG GCTGGAGACGG
Reverse Primer	GCGGTTCCC TGCTCTCTGTC	CAAAGTCCTT GAGTCCCAAC	GATGATAGGGT TACACATCTGCT
Size of the Amplicon	488 bp	1118 bp	627 bp
RFLP	Bsr BI; 461 þ 27	Pst-1; 934 þ 184	NcoI; 354 þ 273

RESULTS AND DISCUSSION

The patients and the control DNA samples were genotyped for the 3 polymorphic loci: *HSP70-1*:190G/C (rs1043618), *HSP70-2*:1267A/G (rs1061581), and *HSP70-HOM*: 2437T/C (rs2227956). Evaluation of implication of the respective alleles with respect to IPAH revealed changes in the genotypic frequency between control and IPAH group (Table 2).

Table 2: Allelic and genotypic frequency distribution in Controls and IPAH group

SNP ID	Gene location	Genotype	Controls (N=200)	IPAH (N=70)	Chi square (p value)
HSP70-1	+190	C/C	30 (0.15)	19 (0.27)	**8.936 (0.01)***
		G/C	107 (0.54)	24 (0.34)	
		G/G	63 (0.32)	27 (0.39)	
HSP70-2	+1267	A/A	50 (0.25)	31 (0.44)	**233.17 (0.000009)**
		A/G	77 (0.38)	34 (0.49)	
		G/G	73 (0.36)	5 (0.07)	
HSP70-HOM	+2437	C/C	89 (0.44)	42 (0.6)	**8.739 (0.01)***
		C/T	63 (0.32)	22 (0.31)	
		T/T	48 (0.24)	6 (0.09)	

SNP ID	Gene location	Alleles	Controls	IPAH	Chi square (p value)
HSP70-1	+190	G	0.58	0.56	0.273 (0.60)
		C	0.42	0.44	
HSP70-2	+1267	A	0.44	0.69	**24.54 (<0.001)**
		G	0.56	0.31	
HSP70-HOM	+2437	T	0.4	0.24	**10.79 (0.001)**
		C	0.6	0.76	

Table 3: Relative risk estimates of the genotypes in IPAH compared to control group

SNP ID	Genotypes compared	Controls n(%)	Patients n(%)	Odds Ratio (95% CI)	P value
HSP 70-1					
	G vs C	167 (41.7%)	62 (44.2%)	1.11(0.75-1.63)	0.60
	GG vs GC	107 (53.5%)	24 (34.3%)	**0.52 (0.28-0.98)**	**0.04**
	GG vs CC	30 (15%)	19 (27.1%)	1.48 (0.71-3.07)	0.29
	GG vs GC-CC	137 (68.5%)	43 (61.4%)	0.73 (0.42-1.29)	0.28
	GG-GC vs CC	30 (15%)	19 (27.1%)	**2.11 (1.10-4.06)**	**0.22**
	GG-CC vs GC	107 (53.5%)	24 (34.3%)	**0.45 (0.26-0.80)**	**0.006**
HSP 70-2					
	A vs G	223 (55.7%)	44 (31.4%)	**0.36 (0.24-0.54)**	**<0.0001**
	AA vs AG	77 (38.5%)	34 (48.6%)	0.71 (0.39-1.30)	0.26
	AA vs GG	73 (36.5%)	5 (7.1%)	**0.11 (0.04-0.30)**	**<0.0001**
	AA vs AG-GG	150 (75%)	39 (55.7%)	**0.42 (0.24-0.74)**	**0.002**
	AA-AG vs GG	73 (36.5%)	5 (7.1%)	**0.13 (0.05-0.35)**	**<0.0001**
	AA-GG vs AG	77 (38.5%)	34 (48.6%)	1.51 (0.87-2.61)	0.14
HSP HOM					
	C vs T	159 (39.8%)	34 (25%)	**0.48 (0.31-0.75)**	**0.0012**
	CC vs CT	63 (31.5%)	22 (31.4%)	0.74 (0.40-1.36)	0.33
	CC vs TT	48 (24%)	6 (8.6%)	**0.26 (0.11-0.67)**	**0.0004**
	CC vs CT-TT	111 (55.5%)	28 (40%)	**0.53 (0.31-0.93)**	**0.02**
	CC-CT vs TT	48 (24%)	6 (8.6%)	**0.30 (0.12-0.73)**	**0.0008**
	CC-TT vs CT	63 (31.5%)	22 (31.4%)	1.00 (0.55-1.79)	0.99

GG genotype of HSP70-1 was predominant in the IPAH group (39%) when compared to the control group, which had a higher frequency of the GC genotype (54%). The most prominent change was seen in GG genotype of *HSP70-2*, where the patients showed a frequency of just 7% vs 36% in the controls. The AA and AG genotypes also deviated along similar lines (25% vs 44% for AA and 38% vs 49% for AG). A drastic change in frequencies between control and IPAH group was also observed for TT group of *HSP70-HOM* (24% vs 9%). All the variations observed were found to be statistically significant. Also, variations in the allelic frequencies of both groups in *HSP70-2* and *HOM* was also found to be statistically significant ($p \leq 0.001$).

Odds risk estimation was carried out for the three polymorphisms under study which revealed that in case of *HSP70-1*, the genotype GC conferred protection against IPAH in two models (**0.52 (0.28-0.98); P=0.012** and **0.45 (0.26-0.80)**; **P=0.0053**), whereas the genotype CC proved to be detrimental in the recessive model. In contrast the GG and TT genotype of HSP 70-2 and HSP HOM seem to play roles in a protective fashion respectively, which is further established by the protective role of the G and T alleles of *HSP70-2* and *HSP70* HOM.

Linkage disequilibrium was calculated for the 3 genotypes of HSP 70 using Haploview 4.2 software, to examine for any possible association with the disease phenotype. A significant D' value of 0.91 observed for *HSP70-1* (rs1043618) and *HSP70-2* (rs1061581) genotypes revealing a strong linkage association between the two polymorphic sites.

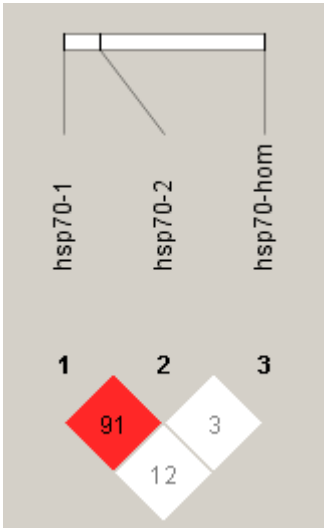
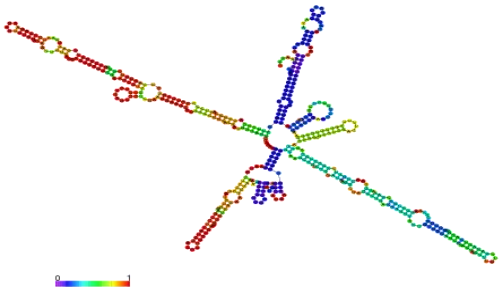
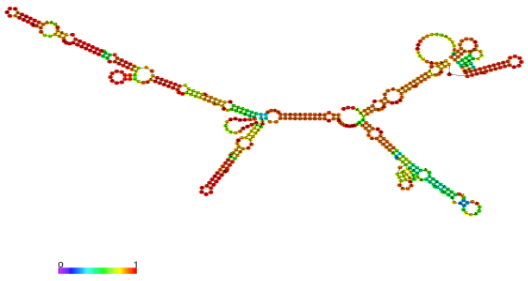


Fig 1: Linkage Disequilibrium

Secondary RNA structure prediction: Sequence variations in *HSP70* could affect the protein folding kinetics by ribosome stalling resulting in altered mRNA conformations which determines the susceptibility to the disease. Therefore, secondary structure prediction was carried out using **VIENNA RNAFOLD SERVER online tool**. Though a change in the mRNA structures is observed in the variants of *HSP70-1*, the free energy remains the same for both strucures i.e, **-195.34** kcal/mol (Fig. 2). As for the other two polymorphisms, the G/G variant of *HSP70-2* (**-453.64 kcal/mol vs. -452.93 kcal/mol** and the C/C variant of *HSP70-HOM* **(-189.61 kcal/mol vs. -188.18 kcal/mol**) seem to be more stable than their counterparts (Figs. 3 & 4).

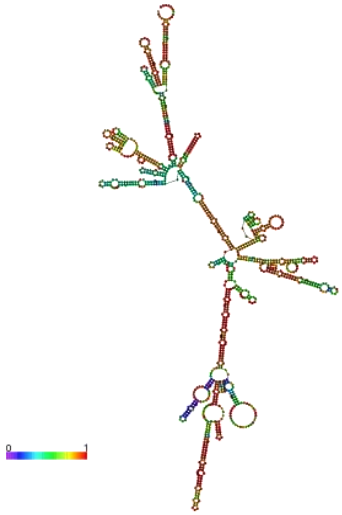


70-1 +190 G/G mRNA secondary structure with a free energy of -195.34 kcal/mol.

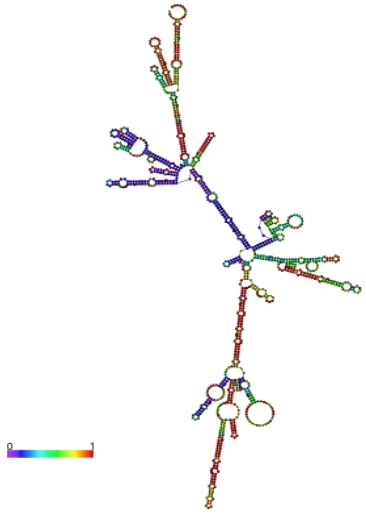


70-1 +190 C/C mRNA secondary structure with a free energy of -195.34 kcal/mol

Fig 2: *HSP70-1* secondary mRNA structures with free energies.

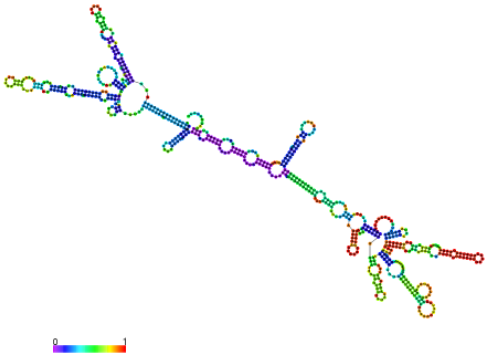


70-2 A mRNA Secondary structure with a free energy of -452.93 kcal/mol.

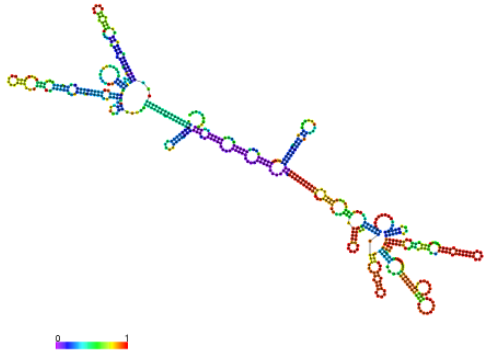


70-2 G mRNA secondary structure with a free energy being -453.64 kcal/mol.

Fig 3: *HSP70-2* secondary mRNA structures with free energies.



70-Hom T mRNA secondary structure with the free energy of the thermodynamic ensemble being 188.18 kcal/mol.



70-Hom C mRNA secondary structure with the free energy of the thermodynamic ensemble being 189.61 kcal/mol.

Fig 4: *HSP70-Hom* secondary mRNA structures with free energies.

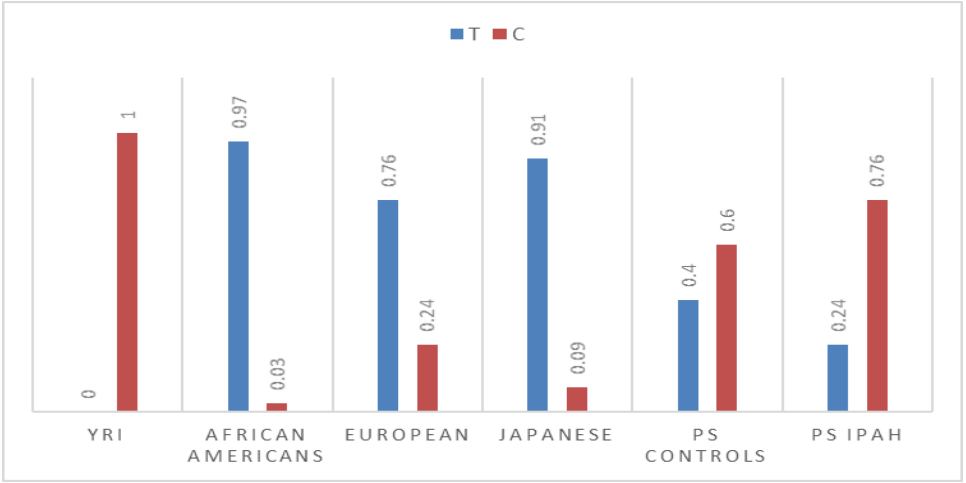


Fig. 5A: HAPMAP analysis of *HSP70-1*

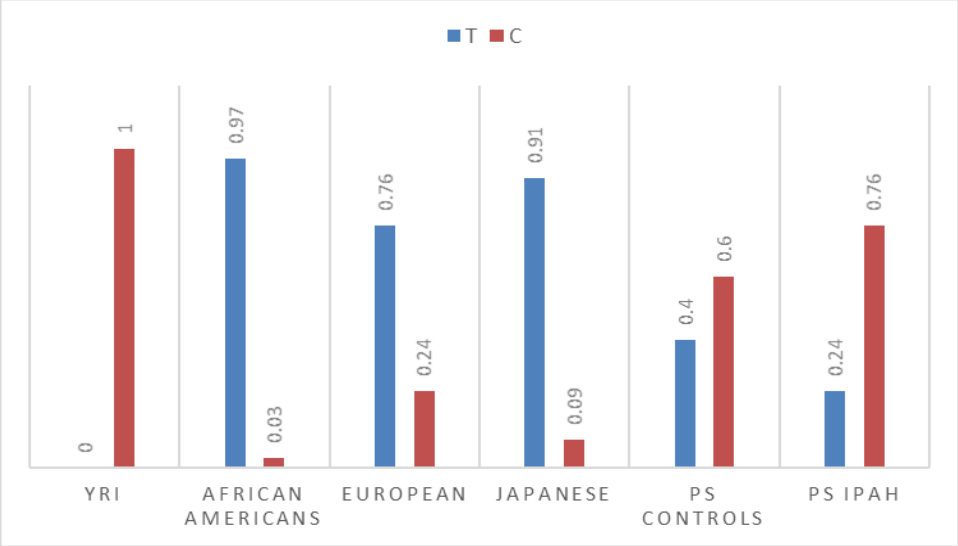


Fig 5B: HAPMAP analysis of *HSP70-2*

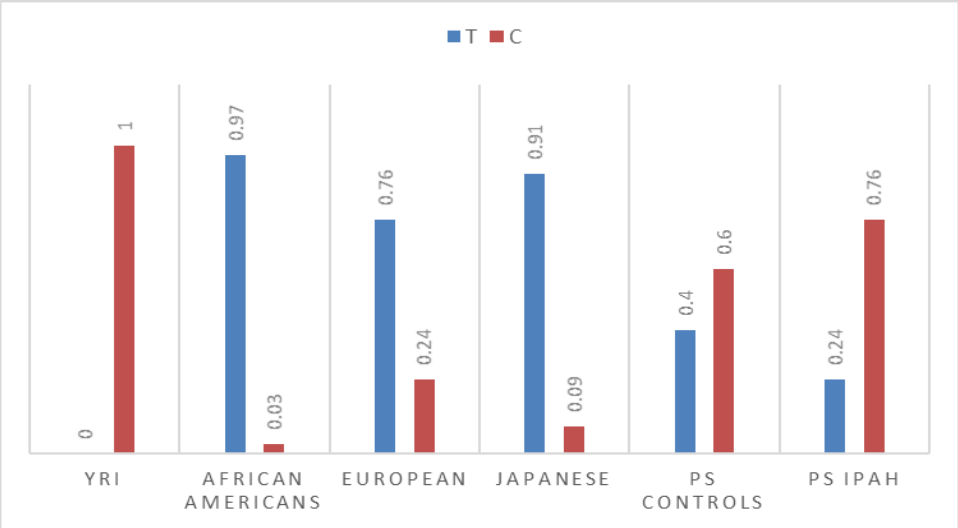


Fig 5C: HAPMAP analysis of *HSP70-Hom*

HAPMAP analysis: On obtaining HAPMAP data and upon correlating the frequencies of the present study with those across the world, *HSP70-1* seems to have a stark contrast with YRI, African Americans, European and Japanese populations. YRI has only the 'C' allele existing in its population, whereas in the other 3 populations, 'T' allele seems to be the prominent allele, in contrast to the Indian population, where the 'C' allele is predominant (Fig. 5A). The same pattern is followed both in *HSP70-2* and *HSP70-Hom*, wherein, the frequencies seen in the Indian pattern are in contrast with the frequencies across the world, clearly pinpointing the ethnic differences in the Indian subcontinent.

DISCUSSION

Idiopathic pulmonary arterial hypertension is a relatively rare and detrimental disease, with an estimated incidence of 5.9 cases per million in the general population. If left untreated, IPAH has a mean survival rate of 2.8 years from the time of diagnosis. IPAH is less common in men, with a female to male ratio of **1.7 to 1**, typically diagnosed in men in their 40s, and women in their 30s. Symptoms of IPAH include fatigue, palpitations, syncope, edema, and chest pain. Right-sided heart failure is a common occurence in the late stage of the disease. Idiopathic pulmonary arterial hypertension is characterised by increased pulmonary artery pressure and pulmonary

vascular resistance in the absence of any underlying significant cardiopulmonary or other diseases. Idiopathic PAH is a progressive disorder that generally culminates in right ventricular failure and death. Moreover, patients with idiopathic PAH are often severely limited on exertion by dyspnoea and fatigue, and thus suffer from a poor quality of life.

Heat-shock proteins are a group of evolutionarily highly conserved chaperone proteins (Lindquist & Craig, 1988). Diverse physiological stresses such as heat, hemodynamics, mutant proteins, and oxidative injury produce multiple changes in cells that eventually affect protein structures and function. A multitude of cells initiate a cascade of events that employ essential proteins, such as the molecular chaperones, in decisions to repair or degrade damaged proteins as a defence strategy to ensure survival. Accumulative evidence indicates that molecular chaperones such as the heat shock family of stress proteins (HSPs) actively participate in an array of cellular processes, including cytoprotection. The adaptability of the ubiquitous HSP family is further enhanced by stress-inducible regulatory networks, both at the transcriptional and posttranscriptional levels.

HSP70-1 and *HSP70-2* genes code for identical, heat-inducible proteins, and *HSP70-HOM* gene encodes a non-heat inducible protein that is highly similar to *HSP70-1*(Milner & Campbell, 1992). However, the exact function and substrate specificity of *HSP70* genes still remain unknown. A correlation between these and the polymorphisms of *HSP70* will generate a clear understanding of the association of *HSP70* with IPAH. *HSP70* gene polymorphisms were found to be risk factors in several human disorders. Heat shock proteins appear to serve a significant cardiovascular role and hence they may play an important role in susceptibility to and/or progression of IPAH.

Deviations observed in the genotype and allelic frequencies in control and IPAH group were found to be statistically relevant and were further confirmed by Odds Ratio Risk Estimation, which showed that in case of *HSP70-1*, the genotype GC conferred protection against IPAH with the difference in frequencies being statistically significant (OR = **0.52 ; P=0.012**), whereas the genotype CC was proved to be detrimental as it is significantly associated with IPAH (OR = **2.11 ; P=0.028**). In contrast the GG and TT genotypes were found to confer protection with respect to *HSP70-2* (OR= **0.11 , P<0.0001 & OR = 0.13 ; P<0.0001**) and *HSP70-HOM* respectively (OR=**0.26 ; P=0.0075 & OR=0.53 ; P=0.025 & OR=0.30 ; P=0.003**), further confirmed by the protective role conferred by the alleles.

A significant D' value of 0.91 was observed for *HSP70-1* (rs1043618) and *HSP70-2* (rs1061581) genotypes, upon linkage disequilibrium analysis, revealed the affinity of the two alleles to segregate as a unit.

As any sequence variations in the DNA of *HSP70* could affect the protein folding kinetics by ribosome stalling resulting in altered mRNA conformations, secondary structure prediction was carried out. Though a change in the mRNA structures is observed in the variants of HSP 70-1, the free energy remains the same for both strucures i.e, **-195.34** kcal/mol. As for the other two polymorphisms, the G/G variant of *HSP70-2* (**-453.64 kcal/mol vs. -452.93 kcal/mol** and the C/C variant of *HSP70-HOM* **(-189.61 kcal/mol vs. -188.18 kcal/mol**) seem to be more stable than their counterparts.

The *HSP70-1* and the *HSP70-2* polymorphisms exhibit silent changes in the coding region. The *HSP70-1* polymorphism is located in the 5'UTR of the gene and this region is involved in the control of cellular location, stability and translational efficiency of eukaryotic mRNA; thus, the polymorphism may have a major regulatory role in the expression and function of HSP70 protein. The *HSP70-HOM* polymorphic site detected by *Nco*I, corresponds to a Met>Thr amino acid substitution at position 493, which being located on the β sheets, has an important role in peptide binding, and the polymorphism may disrupt the binding specificity of *HSP70-HOM*(Milner & Campbell, 1992).

It is known that HSP70 is able to protect various organs against oxidative damage resulting from ischemia and that HSP70 may serve an autoprotective function in asthma and lung injury (Hfaiedh et al., 2005; Rajamani et al., 2006). Lung injury and heart failure by right ventricular failure are the late stage symptoms of IPAH. It may be proposed that the polymorphism of *HSP70* have a role in protecting the lung and heart from any injury in the initial stages of the disease. The implication of HSP in cardioprotection, though well established, seems to

influence the progression and severity of the diseased phenotype by altered chaperoning, protein folding and regulation of transcription factors and ultimately apoptotic mechanisms. It can be hypothesized based on the results of the study that *HSP70* has a protective role in IPAH. Thus, further studies delineating the role of HSP and its polymorphisms in IPAH is warranted.

Conflicts of interest: The authors stated that no conflicts of interest.

REFERENCES

Amrani M, Latif N, Morrison K, Gray CC, Jayakumar J, Corbett J, Goodwin AT, Dunn MJ and Yacoub MH (1997) Relative induction of heat shock protein in coronary endothelial cells and cardiomyocytes: implications for myocardial protection. *J Thorac Cardiovasc Surg* 115, 200–209.

Angelidis CE, Lazaridis I & Pagoulatos GN (1991) Constitutive expression of heat-shock protein 70 in mammalian cells confers thermoresistance. *European Journal of Biochemistry / FEBS*, *199*(1), 35–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1712300

Atkinson C, Stewart S, Upton PD, Machado R, Thomson JR, Trembath RC and Morrell NW (2002) Primary pulmonary hypertension is associated with reduced pulmonary vascular expression of type II bone morphogenetic protein receptor. *Circulation*, *105*(14), 1672–1678. https://doi.org/10.1161/01.CIR.0000012754.72951.3D

Collier NC and Schlesinger MJ (1986) The dynamic state of heat shock proteins in chicken embryo fibroblasts. *The Journal of Cell Biology*, *103*(4), 1495–507. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2114322&tool=pmcentrez&rendertype=abstract

Dressel R and Günther E (1999) Heat-induced expression of MHC-linked HSP70 genes in lymphocytes varies at the single-cell level. *Journal of Cellular Biochemistry*, *72*(4), 558–569. https://doi.org/10.1002/(SICI)1097-4644(19990315)72:4<558::AID-JCB11>3.0.CO;2-X

Gaine SP and Rubin LJ (1998) Primary pulmonary hypertension. *Lancet (London, England)*, *352*(9129), 719–25. https://doi.org/10.1016/S0140-6736 (98) 02111-4

Gething MJ and Sambrook J (1992) Protein folding in the cell. *Nature*, *355*(6355), 33–45. https://doi.org/10.1038/355033a0

Hfaiedh N, Allagui MS, El Feki A, Gaubin Y, Murat JC, Soleilhavoup JP and Croute F (2005) Effects of nickel poisoning on expression pattern of the 72/73 and 94 kDa stress proteins in rat organs and in the COS-7, HepG2, and A549 cell lines. *Journal of Biochemical and Molecular Toxicology*, *19*(1), 12–18. https://doi.org/10.1002/jbt.20056

Li GC, Li LG, Liu YK, Mak JY, Chen LL and Lee WM (1991) Thermal response of rat fibroblasts stably transfected with the human 70-kDa heat shock protein-encoding gene. *Proceedings of the National Academy of Sciences of the United States of America*, *88*(5), 1681–5. https://doi.org/10.1073/pnas.88.5.1681

Li GC and Werb Z (1982) Correlation between synthesis of heat shock proteins and development of thermotolerance in Chinese hamster fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America*, *79*(10), 3218–3222. https://doi.org/10.1073/pnas.79.10.3218

Lindquist S and Craig EA (1988) The heat-shock proteins. *Annual Review of Genetics*, *22*, 631–677. https://doi.org/10.1146/annurev.ge.22.120188.003215

Milner CM and Campbell RD (1992) Polymorphic analysis of the three MHC-linked HSP70 genes. *Immunogenetics*, *36*(6), 357–362. https://doi.org/10.1007/BF00218042

Morimoto RI and Santoro MG (1998) Stress-inducible responses and heat shock proteins: new pharmacologic targets for cytoprotection. *Nature Biotechnology*, *16*(February), 833–838. https://doi.org/10.1038/nbt0998-833

Rajamani R, Muthuvel A, Senthilvelan M, Sheeladevi R (2006) Oxidative stress induced by methotrexate alone and in the presence of methanol in discrete regions of the rodent brain, retina and optic nerve. *Toxicol Lett* 165, 265-273.

Yi ES, Kim H, Ahn H, Strother J, Morris T, Masliah E, Friedman PJ (2000) Distribution of obstructive intimal lesions and their cellular phenotypes in chronic pulmonary hypertension: A morphometric and immunohisto-chemical study. *American Journal of Respiratory and Critical Care Medicine*, *162*(4 I), 1577–1586. https://doi.org/10.1164/ajrccm.162.4.9912131