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# INTERPLAY OF CYP1B1 POLYMORPHISMS IN IDIOPATHIC PULMONARY ARTERIAL HYPERTENSION

**KEYWORDS**

Idiopathic Pulmonary Arterial Hypertension, Xenobiotic, Estrogen metabolizing enzymes, single nucleotide polymorphisms

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**ABSTRACT** *Idiopathic Pulmonary Arterial Hypertension (IPAH) is a disorder of the pulmonary arteries, wherein, due to thickening of the pulmonary arteries, a resistance in the flow of blood is seen, resulting in increased pulmonary arterial pressure. Though the primary cause of the disease is unknown, with the advancement of science, the same may be delineated. A discordance is seen in the genders affected by the disorder. To examine whether CYP1B1, an estrogen metabolizing enzyme is involved in the pathogenesis of IPAH, this study was carried out. Two polymorphisms in CYP1B1, viz., L432V (rs1056836) and A119S (rs1056827) are considered here. Both protective and risk-conferring roles of the alleles have been established by the study, which are in accordance and substantiated by statistical analyses.*

Idiopathic Pulmonary Arterial Hypertension (IPAH) is an idiopathic form of Pulmonary Arterial Hypertension, characterized by increased pulmonary arterial pressure, and the plexiform lesion which arises from monoclonal proliferation of endothelial cells, migration and proliferation of smooth muscle cells, and the accumulation of circulating inflammatory and progenitor cells, which leads to occluding and narrowing of the pulmonary arteries in afflicted individuals (Runo & Loyd, 2003; Tuder, Groves, Badesch, & Voelkel, 1994). IPAH is known to occur with a female preponderance, and one of the underlying causes for this is assumed to be estrogen. The predominant circulating oestrogen, 17b-Oestradiol, up-regulates components of the serotonin signalling system and mediates proliferation of human pulmonary artery smooth muscle cells (hPASMCs) (Dempsie et al., 2011; White et al., 2011). Recent evidence suggests a role for oestrogen and the oestrogen-metabolising enzyme cytochrome P450 1B1 (CYP1B1) in the development of PAH.

CYP1B1 is a P450 enzyme expressed in the lung catalysing the conversion of oestrogen, predominantly to 4-hydroxyoestrogens, but also to 2-hydroxy and 16-hydroxyoestrogens (Badawi, Cavalieri, & Rogan, 2001; Hanna, Dawling, Roodi, Guengerich, & Parl, 2000). Not all oestrogen metabolites are equal in their effects, specifically, 4-OHE and 16a-OHE1 exert far greater mitogenic and genotoxic effects than 2-OHE, making the ratio of 2-OHE to 4-OHE and/or 16a-OHE1 important in the genesis of hormonal-based cancers (Belous, Hachey, Dawling, Roodi, & Parl, 2007; Eliassen, Missmer, Tworoger, & Hankinson, 2008; Kaaks, 2005; Missmer, Eliassen, Barbieri, & Hankinson, 2004; Paola Muti et al., 2002). For example, it has been hypothesised that metabolism favouring 2-OHE over the 16a- OHE1 pathway may be protective of breast cancer in at-risk subjects (P Muti et al., 2000).

Gene polymorphisms in CYP1B1 and dysregulation in CYP1B1 expression are evidenced to be associated with the risk of developing cancer of the lung, (Laroche-Clary, Morvan, Yamori, & Robert, 2010; Murray et al., 1997), systemic hypertension (Jennings et al., 2010) and primary congenital glaucoma (Stoilov, Akarsu, & Sarfarazi, 1997). West et al., identified that in female PAH patients housing a mutation in bone morphogenetic protein receptor type II (BMPRII) have dysregulated CYP1B1 gene expression (West et al., 2008). Additionally another study showed that cytoskeletal defects in pulmonary artery endothelial cells (PAECs) were observed in pulmonary hypertensive mice with a BMPR2 mutation (Johnson et al., 2012). It was recently shown that CYP1B1 expression is increased in the pulmonary vasculature from patients with PAH. Also, inhibition of CYP1B1 was shown have a protective effect against experimental PAH in mice (White et al., 2012). Serotonin may have a positive effect on the expression of CYP1B1 in hPASMCs (White et al., 2011).

The present study focuses on the role of two polymorphisms in CYP1B1, viz. L432V (rs1056836) and A119S (rs1056827) in the progression of IPAH.

## MATERIALS AND METHODS

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. The study included 70 patients only as IPAH is a rare disorder and these samples were collected across 7 years. Randomly selected 200 healthy subjects without history of cardiac and systemic disorders were considered as controls for comparative purposes.

**Molecular Analyses:** DNA was isolated followed by PCR amplification using specific primers. PCR assays was carried out in a 25$\mu$l 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). Genotyping of both polymorphisms were carried out by means of ARMS-PCR described by Jiao et al. (Jiao et al., 2010).

**Statistical and insilico Analyses:** Deviations from the Hardy-Weinberg equilibrium were tested for the polymorphisms in cases and controls by comparing observed and expected genotype frequencies by carrying out 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. Haplotype analysis was also carried out to determine any commonly inherited haplotype in the population. 1000 genomes software was used to compare allelic frequencies across the world.

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## RESULTS

The patients and the control DNA samples were genotyped for the 2 polymorphic loci. The genotype frequencies showed that the heterozygotes in both polymorphisms in case of the controls showed increased frequencies when compared to the homozygotes, implicating heterozygote advantage. But, in case of IPAH patients, the homozygotes had a higher frequency (**Table 1b**). This was further proved by the Hardy-Weinberg test for equilibrium, wherein it was observed that both populations did not follow the rule in case of both polymorphisms (**Table 1c**).

**Table 1a: Allelic frequency distribution in Controls and IPAH group**

| | Controls | IPAH |
|---|---|---|
| **Ala119Ser** | | |
| **G** | 0.39 | 0.37 |
| **T** | 0.61 | 0.63 |
| **Val432Leu** | | |
| **C** | 0.51 | 0.74 |
| **G** | 0.49 | 0.26 |

**Table 1b: Genotypic frequency distribution in Controls and IPAH group**

| | Controls | IPAH | | |
|---|---|---|---|---|
| | **n** | **%** | **N** | **%** |
| **Ala119Ser** | | | | |
| **G/G** | 15 | 8 | 15 | 21 |
| **G/T** | 127 | 64 | 22 | 32 |
| **T/T** | 58 | 28 | 33 | 47 |
| **Val432Leu** | | | | |
| **C/C** | 26 | 13 | 47 | 67 |
| **C/G** | 152 | 76 | 9 | 13 |
| **G/G** | 22 | 11 | 14 | 20 |

**Table 1c: Test for Hardy- Weinberg Equilibrium**

| **Ala119Ser exact test for Hardy-Weinberg equilibrium** | | | | | | |
|---|---|---|---|---|---|---|
| | **TT** | **GT** | **GG** | **G** | **T** | **P-value** |
| **Controls** | 58 | 127 | 15 | 243 | 157 | **<0.0001** |
| **IPAH** | 33 | 22 | 15 | 88 | 52 | **0.0094** |

| **Val432Leu exact test for Hardy-Weinberg equilibrium** | | | | | | |
|---|---|---|---|---|---|---|
| | **CC** | **CG** | **GG** | **C** | **G** | **P-value** |
| **Controls** | 26 | 152 | 22 | 204 | 196 | **<0.0001** |
| **IPAH** | 47 | 9 | 14 | 103 | 37 | **<0.0001** |

The Odds Ratio test of association further established the theory of heterozygote advantage, by exhibiting a protective role of both the alleles (GT of Ala119Ser and GC of Val432Leu) against the disease (**Tables 2a & 2b**). (**OR: 0.30 (0.16-0.57), p<0.0001; OR=0.03(0.01-0.07), p<0.0001**). In a recessive mode of inheritance, the GG genotype despite being the wild type allele shows a detrimental effect (**OR: 3.36 (1.55-7.31), p=0.0026**). On the other hand, the homozygote GG showed a protective role in case of Val432Leu polymorphism. (**OR=0.35(0.15-0.80), p<0.0001**).

**Table 2a: Odds ratio Test of Association for Ala119Ser**

| **Model** | **Genotype** | **Controls** | **IPAH** | **OR (95% CI)** | **P-value** |
|---|---|---|---|---|---|
| Codominant | T/T | 58 (29%) | 33 (47.1%) | 1.00 | <0.0001 |
| | G/T | 127 (63.5%) | 22 (31.4%) | **0.30 (0.16-0.57)** | |
| | G/G | 15 (7.5%) | 15 (21.4%) | 1.76 (0.76-4.05) | |
| Dominant | T/T | 58 (29%) | 33 (47.1%) | 1.00 | 0.0065 |
| | G/T-G/G | 142 (71%) | 37 (52.9%) | **0.46 (0.26-0.80)** | |
| Recessive | T/T-G/T | 185 (92.5%) | 55 (78.6%) | 1.00 | 0.0026 |
| | G/G | 15 (7.5%) | 15 (21.4%) | **3.36 (1.55-7.31)** | |
| Overdominant | T/T-G/G | 73 (36.5%) | 48 (68.6%) | 1.00 | <0.0001 |
| | G/T | 127 (63.5%) | 22 (31.4%) | **0.26 (0.15-0.47)** | |

**Table 2b: Odds ratio Test of Association for Val432Leu**

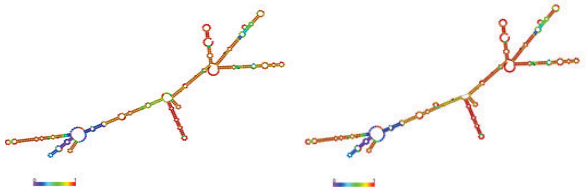
| **Model** | **Genotype** | **Controls** | **IPAH** | **OR (95% CI)** | **P-value** |
|---|---|---|---|---|---|
| Codominant | C/C | 26 (13%) | 47(67.1%) | 1.00 | <0.0001 |
| | G/C | 152 (76%) | 9 (12.9%) | **0.03 (0.01-0.07)** | |
| | G/G | 22 (11%) | 14 (20%) | **0.35 (0.15-0.80)** | |
| Dominant | C/C | 26 (13%) | 47 (67.1%) | 1.00 | <0.0001 |
| | G/C-G/G | 174 (87%) | 23 (32.9%) | **0.07 (0.04-0.14)** | |
| Recessive | C/C-G/C | 178 (89%) | 56 (80%) | 1.00 | 0.066 |
| | G/G | 22 (11%) | 14 (20%) | 2.02 (0.97-4.22) | |
| Overdominant | C/C-G/G | 48 (24%) | 61 (87.1%) | 1.00 | <0.0001 |
| | G/C | 152 (76%) | 9 (12.9%) | **0.05 (0.02-0.10)** | |

Haplotype analysis showed that the G allele of both polymorphisms may confer protection against the disease, with an Odds value of 0.48 (p=0.0058), while the G-C allele combination seems to have a detrimental effect as denoted by an odds value of >1 (**Table 3**). This may be because the G allele of Ala119Ser inherently has a property of conferring susceptibility to the disease, but, the presence of G allele of Val432Leu polymorphism has an epistatic effect, thereby rendering it useless, which is further proven by the allelic combination of G-C having a detrimental role.

**Table 3: Haplotype analysis for CYP1B1 polymorphisms**

| | **Ala119Ser** | **Val432Leu** | **Total** | **Controls** | **IPAH** | **OR (95% CI)** | **P-value** |
|---|---|---|---|---|---|---|---|
| 1 | T | C | 0.5379 | 0.51 | 0.6286 | 1.00 | --- |
| 2 | G | G | 0.3565 | 0.3925 | 0.2643 | **0.48(0.28 - 0.80)** | 0.0058 |
| 3 | T | G | 0.075 | 0.0975 | NA | 0.00 (-Inf - Inf) | 1 |
| 4 | G | C | 0.0306 | NA | 0.1071 | **>1 (>1 - >1)** | <0.0001 |

Further, since any sequence variations could affect the protein folding kinetics by ribosome stalling resulting in altered mRNA conformations which determines the susceptibility to the disease, secondary structure prediction was carried out using **VIENNA RNAFOLD SERVER online tool**. The mRNAs of both alleles of Ala119Ser showed slight differences in structure, which is further established by change in their free energies where the G allele is more stable (**-310.40 kcal/mol vs -309.00 kcal/mol**). In case of Val432Ser polymorphism, there seems to be very less difference in the free energies of both mRNAs (-271.50 kcal/mol for C allele vs -271.30 kcal/mol for G allele), despite changes in the structure of the mRNAs, which can account for protein folding variations of CYP1B1.



**Ala119Ser G mRNA Secondary structure with a free energy of -310.40 kcal/mol.**

**Ala119Ser T mRNA secondary structure with free energy being -309 kcal/mol.**

**Figure 1a: mRNA secondary structure prediction for Ala119Ser**

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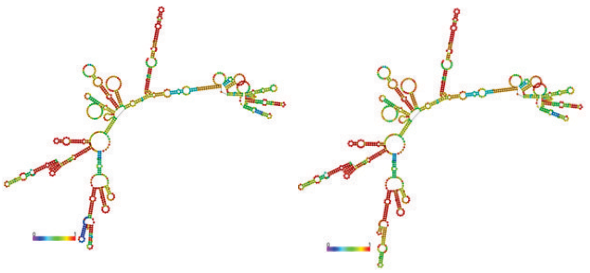

**Val432Leu C mRNA Secondary structurewith a free energy of 271.50 kcal/mol.**

**Val432Leu G mRNA secondary structure with free energy being -271.30 kcal/mol.**

**Figure 1b: mRNA secondary structure prediction for Val432Leu**

To find any discrepancies in the frequency between the population under study and the populations across the world, 1000 genomes software was used. As can be seen, in case of Ala119Ser, the allele frequencies of IPAH in our population do not follow that of any other, but a semblance is seen in that of African population. In case of Val432Leu, the allele frequencies of IPAH in our population seems to follow that of the African population, while the others seem to be quite contrary to that of the Indian population. The frequencies of the alleles in the control population of the present study are similar and not in concordance with data from any other populations. A similar trend between the Indian population and African population implies that a founder effect may have prevailed between Africa and India, at a point in time.

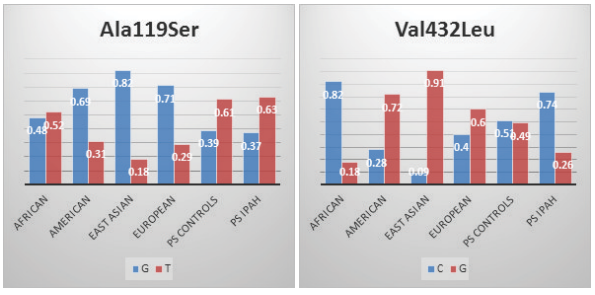


**Figure 2: Allelic frequency comparison for CYP1B1 polymorphisms**

## DISCUSSION

The CYP multigene family of enzymes metabolise many endogenous and exogenous compounds (Gonzalez, 1988). CYP1B1 is a key enzyme in oestrogen metabolism associated with several hormone-influenced cancers, such as breast, endometrial and prostate cancers. It is an active extrahepatic enzyme that is highly expressed in tissues which require oestrogen regulation, importantly, the breast, but also in the lung, implying that CYP1B1 is essential for localised metabolic control of oestrogen balance (Spivack et al., 2001). Interestingly, CYP1B1 is transcriptionally activated by oestrogens and oestrogen metabolites, via the oestrogen receptor, to hydroxylate oestrogens into 2-OHE and 4-OHE metabolites (Sissung, 2006). Thus, oestrogens have a dual role – that of substrates, and activators of CYP1B1 by direct interaction between the oestrogen receptor and oestrogen response elements on the CYP1B1 gene. Oestrogens and its metabolites are pro-proliferative. Oxidation of oestrogens also occurs by hydroxylation by other P450 enzymes at the C-16 position, and predominantly result in 16a-hydroxyoestrone (16a-OHE1) in extrahepatic tissues (Nebert, 1993; Yager & Davidson, 2006). It has been suggested that, contrary to the weak mitogen 2-OHE, 16a-OHE1 constitutively activates the oestrogen receptor and stimulates cellular. In addition to being more mitogenic than 2-OHE, 16a-OHE1 has been proven to be more genotoxic too. Thus, individuals who metabolise a larger proportion of oestrogen to 16a-OHE1 may be at increased risk to diseases that result from both the mitogenic and genotoxic effects of oestrogens, such as prostate and breast cancers (P Muti et al., 2000).

Phase I xenobiotic metabolizing enzymes, such as cytochrome P450, play important physiological roles in the detoxification of xenobiotics and the biosynthesis of endogenous steroid hormones (Nebert & Dalton, 2006). These enzymes metabolically activate procarcinogens to reactive oxygen species (ROS) and reactive electrophilic forms, which can damage DNA if not detoxified by phase II enzymes like catechol-O-methyltransferase (COMT). Thus, an imbalance between these enzymes may increase ROS production leading to oxidative stress. Variations at inter-individual and inter-ethnic levels in the metabolism of environmental agents and susceptibility to carcinogenicity can be influenced by genetic polymorphisms of many enzymes involved in these processes (Lai & Shields, 1999).

Predominance of adult females and a lower prevalence among pre-pubertal females compared with males suggests oestrogen as a possible disease modifier (Runo & Loyd, 2003; Yamaki & Wagenvoort, 1985). A detrimental role for oestrogens in FPAH is proven by studies which demonstrate that oestrogen is a potent mitogen of PVSMCs (Farhat, Vargas, Dingaan, & Ramwell, 1992).

Several genetic polymorphisms have been identified in the *CYP1B1* gene. This study included 2 well documented polymorphisms – Ala119Ser & Val4332Leu. The genotype frequencies showed that the heterozygotes in both polymorphisms in case of the controls showed increased frequencies when compared to the homozygotes, implicating heterozygote advantage. But, in case of IPAH patients, the homozygotes had a higher frequency. This was further proved by the Hardy-Weinberg test for equilibrium, wherein it was observed that both populations did not follow the rule in case of both polymorphisms.

The Odds Ratio Test Of Association further established the theory of heterozygote advantage, by showing a protective role of the heterozygotes (GT of Ala119Ser and GC of Val432Leu) against the disease. (**OR: 0.30 (0.16-0.57), p<0.0001; OR=0.03(0.01-0.07), p<0.0001**). Also, the homozygote GG showed a protective role in case of Val432Leu polymorphism. (**OR=0.35(0.15-0.80), p<0.0001**). But, the test also established the role of G allele of Ala119Ser polymorphism as that of conferring susceptibility to the disease (**OR: 3.36 (1.55-7.31), p=0.0026**).

Haplotype analysis showed that the G allele of both polymorphisms may confer protection against the disease, with an Odds value of 0.48 (p=0.0058). While the G allele of Ala119Ser and C allele of Val432Leu may have a detrimental effect as denoted by an odds value of >1, hinting at the epistatic effect of the second polymorphism on the first, whereby combination of G-G has a protective effect and G-C has a detrimental effect.

Further, since any sequence variations could affect the protein folding kinetics by ribosome stalling resulting in altered mRNA conformations which determines the susceptibility to the disease, secondary structure prediction was carried out which showed that the mRNAs of both alleles of Ala119Ser have slight differences in structure, which is further established by change in their free energies showing the G allele to be the more stable one (**-310.40 kcal/mol vs -309.00** kcal/mol). In case of Val432Ser polymorphism, there seems to be very less difference in the free energies of both mRNAs (-271.50 kcal/mol for C allele vs -271.30 kcal/mol for G allele), despite changes in the structure of the mRNAs. Comparison of allele frequencies between the Indian population and that of the

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world showed a small nuance between Indian and African populations, while being completely contrary with that of American, Europeans and even East Asians, thus pointing to a founder effect between the Indian and African populations.

The *L432V* polymorphism was reported to be associated with a higher catalytic activity of the enzyme (Aklillu et al., 2002; Li, Seidel, Pritchard, Wolf, & Friedberg, 2000). This may be due to changes in the tertiary or quaternary structure of the CYP1B1 protein which may be influenced by the *L432V* polymorphism which is located near a catalytically important heme-binding domain in the *CYP1B1* gene (Sissung, 2006). The A119S substitution is located in substrate recognition site one (SRS1) (Gotoh, 1992), and may affect substrate binding. Multiple functional studies report that these nonsynonymous SNPs of CYP1B1 alter enzymatic activity and catalytic specificity. Thus, it can be said that despite the statistical analysis which does not favour any one polymorphism, subtle changes may be observed in the individual harbouring the variation, which may have a detrimental effect on the onset and progression of the disease.

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