A total of 77 IPAH patients and 100 controls were genotyped using PCR SSCP and RFLP, and appropriate statistical tests were employed to determine the significance and to interpret the results. This study has attempted to correlate promoter/gene polymorphisms of prostacyclin synthase and Peroxisome proliferator-activated receptor gamma with Idiopathic Pulmonary Arterial Hypertension. A 183-bp region in the 5'UTR contains a VNTR, which was previously reported 9-bp variable-number tandem repeat (VNTR), and appropriate statistical tests were employed to determine the significance and to interpret the results. 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 77 IPAH patients.
Molecular analyses

DNA was isolated followed by Polymerase Chain Reaction (PCR) amplification using specific primers. PCR assays were carried out in a 25 μl volume tube with 100 ng of genomic DNA, 10 μM of each primer, 2.0 mM dNTP (Merck, Germany), 1.5 mM MgCl₂ and 10x PCR buffer [50 mM KCl, 500 mMTris buffer, 160 mM (NH₄)₂SO₄, pH 8.8, and 0.1% Tween 20], 0.1% Triton X-100 and 0.5 U Tag polymerase (Invitrogen). The thermal cycling was carried out in Eppendorf Gradient Thermal cycler (Germany).

The 9 bp VNTR polymorphism in the 5'-flanking region of the promoter of the human prostacyclin synthase gene was determined by nested PCR [26]. Two sets of primer sequences were used to amplify 1530 bp fragment, encompassing the GC rich 5’-flanking region and exon 1 of the PTGIS gene by PCR with the sense primer P AF (from positions –1431 to –1410, the position of the ATG translation start site being referred to as +1) and the antisense primer P BR (from position +84 to +99). In a second step, the PCR product was used as a template to amplify a 216-bp fragment, containing the proximal promoter region and exon 1, with primers P BF (from positions -117 to –100) and P BR.

Single Stranded Confirmation Polymorphism (SSCP) was carried out for PGIS as per Orita et al [27] protocol. The PCR products were denatured at 95°C for 10 minutes, quenched in ice for 5 minutes and then loaded on 11% native polyacrylamide gels with 150 V at room temperature. The gels were visualized by silver staining. The samples exhibiting aberrant band pattern were sequenced commercially.

PCR-RFLP technique was adopted for genotyping of the PPARγ 2 P12A allele. The 154 bp amplified product was digested with HhaI restriction enzyme (New England Biolabs, USA). Digested samples were separated on 10% non-denaturing polyacrylamide gel and visualized by restriction enzyme (New England Biolabs, USA). Digested samples were then loaded on 11% native polyacrylamide gels with 150 V at room temperature for PGIS as per Orita et al [27] protocol. The PCR products were denatured at 95°C for 10 minutes, quenched in ice for 5 minutes and then loaded on 11% native polyacrylamide gels with 150 V at room temperature. The gels were visualized by silver staining. The samples exhibiting aberrant band pattern were sequenced commercially.

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Results

PGIS

The sequence immediately upstream from the translational initiation site of the human PGIS gene has GC-rich and pyrimidine-rich regions. This region consists of a number of repeats of the 9-bp nonamer sequence (CCGCCAGGCC) varying from 3 to 7 copy numbers. Screening of this region was carried out.

Table 2 gives the frequency distribution of VNTR alleles of PGIS gene. The most frequent allele observed is the R6T allele (81%), followed by the wild-type allele R4 (10.0%) and the R6 allele (6%), whereas the R5 allele was least common with a frequency of only 3.0%. In patient group the frequency of R4, R5, R6 and R6T was observed to be 12.9%, 3.8%, 5.1% and 77.9%, respectively (Table 2). Interestingly, the two rare alleles R3 and R7 were not observed either in controls or IPAH patients of Indian cohort, but reported elsewhere, pointing to the diversity of the Indian population.

SSCP analysis of exon 6 of PGIS revealed two types of band patterns (A and B), in both the controls and patients. The band pattern A and B was observed with a frequency of 73% and 27% in controls in comparison to 77.9% and 22.1% in IPAH patients. The electropherogram of the two band patterns are shown in Figures 1 A and B.

Table 1: Primer sequences used for PCR amplification of PGIS and PPARγ.

| Exons | Primer sequences | Annealing Temp (°C) |
|-------|------------------|--------------------|
| 1     | PAF: GGGTCCAGCGGATGAAAGTGAG P BF: AAGCAGGCTGGTGAGG G BR: GGGCCGGGAGGGAGCA | 54.6 |
| 2     | F: GACAAGTGGCCATGCTTCG R: CCGAGGGCAAGAGGAC | 58.4 |
| 3     | F: TACCTCTGGCAACTTCCAC R: GTGCATCTACAGCAGTCTC | 53.7 |
| 4     | F: ATGCTTTTGTCTTCGCTCTC R: GGGGGCTGGCAACGCTC | 51.7 |
| 5     | F: GACACATGAGTGGTCACGAG R: TGGGGCCCATGGTGC | 60.1 |
| 6     | F: TCTCTGTCTCTGGTCGTC R: CACCTGAGCATTCAACCC | 55.6 |
| 7     | F: AAGGGGCCTCTTTCCTTCG R: GAGGGCTGGAAGGCTGTC | 54.6 |
| 8     | F: CTGCACCCTGCCATGCTG R: CGGCACACAGTGCCAG | 57.6 |
| 8b    | F: GGGAGCAGGGAGAGAATC R: GACAGGGCGGGCCTGCG | 58.4 |
| 9     | F: TACCAAAAGGACCTTCTC R: CCGTCGCCCCGCAATGTC | 54.6 |
| 10    | F: CTACATGGCAGCTCTCTGTC R: AAGAAGCTGGAGCCTGCG | 57.1 |
| PPAR*P12A | F: TCTGGGAGATTCTTCTATGGCC R: CTGGAAGAACAATCAGAGG | 52.0 |

Table 2: Frequency distribution of VNTR alleles of PGIS gene.

| R4 | R5 | R6 | R6T |
|----|----|----|-----|
| N | %  | N | %  | N | %  | N | %  |
| Controls | 20 | 10 | 6 | 3 | 12 | 6 | 162 | 81 |
| IPAH | 20 | 12.9 | 6 | 3.8 | 8 | 5.2 | 120 | 77.9 |

Figure 1: A: Electropherogram of Band pattern A showing “GG” genotype; B: Electropherogram of Band pattern B showing the heterozygote “GT” genotype.
On sequencing a G>T transversion was observed in band pattern B. The sequence was then subjected to NCBI BLAST Blast of pattern- B (genotype G/T) revealed a G>T transversion at 18336700 of the exon 6 (ref sequence: NT_011362.10, chromosome 20 contig assembly). The change lies in the second base of the 275th codon (c. 824 G>T). Translation of sequence T at c.824 revealed the presence of Leucine (L) at amino acid position 275; instead of Arginine (R). This is a novel non- synonymous polymorphism (R275L). Since arginine is a basic polar amino acid and leucine is a neutral non-polar amino acid, it is likely that this change affects the structure of the protein due to polarity changes.

Probable structure of 275th amino acid region: Secondary structure prediction software using PSIPRED prediction revealed the structure shown in Figure 2. This software predicted that 270 to 278 amino acids to be part of helix. Therefore further studies are required in order to conclusively determine the role of this non-synonymous SNP in PGIS structure and function.

In comparison to rest of the known missense polymorphisms of PGIS, the frequency of R275L polymorphism was observed to be very high (27%) in controls and patients (22.5%).

A comparison of the clinical profile of IPAH patients with R275L polymorphism in comparison to IPAH patients with R275R revealed no significant difference and presentation of symptoms among the two patient groups showed an increased frequency of angina and pre-syncope in patients with R275L polymorphism.

SSCP analyses of exons 2,3,4,5,7,8a,8b,9 and 10 did not show any band pattern variations, indicating the conserved nature of these exons, in the study cohort.

PPAR-γ2

Following PCR amplification and restriction digestion of exon 12 of PPAR-γ2, the genotypes were identified as CC/ P12P (158 bp), CG/ P12A (154/132/ 22 bp), and GG/A12A (132/22 bp) as shown in Figure 3.

The genotype and allelic frequencies of PPAR-γ2 P12 A polymorphism is given in Table 3. The CC genotype (which codes for Proline) was the predominant genotype in both controls (69%) and IPAH group (85.7%), with the frequency being much higher in the latter group. The frequency of C/G genotype was observed to be 27% in controls and 14.2% in patients. Interestingly the G/G genotype (which codes for Alanine) was completely absent in patient group and seen only in 4% of controls. The allelic frequencies of C and G allele were observed to be 0.83 and 0.18 in controls and 0.93 and 0.07 in patients, respectively. Different ethnic populations have varied allelic frequencies for the Alanine variant. The GG genotype is in general a very rare, as evident from the Hapmap data (Figure 4).

Table 4 gives the odds risk estimates of the P12A polymorphism in IPAH patients in comparison to the controls. The CC genotype, i.e., homozygosity of Proline was found to be associated with an increased risk for IPAH (OR-2.35, CI; 1.08-5.11). The heterozygous (CG genotype), was found to be protective in nature (p-0.0074). Based on the dominant model, the combination of CG+GG genotypes were observed to be associated with reduced risk to IPAH (p-0.0072) compared to the CC genotype, further strengthening the protection conferred by CG genotype. Thus the Alanine variant was found to be a protective allele against IPAH.

Table 3: Genotype frequency and allelic frequencies of P12A polymorphism in Controls and IPAH group.

| Genotype Frequency | Controls | IPAH |
|--------------------|----------|------|
| N                  | %        | n    | %    |
| CC                 | 69       | 66   | 85.7 |
| CG                 | 27       | 11   | 14.2 |
| GG                 | 4        | 4    |      |

Table 4: Odds test of association of P12A polymorphism with IPAH.

| Genotype                  | Controls | IPAH   | OR (95% CI)  | P      |
|---------------------------|----------|--------|--------------|--------|
| CC vs. CG                 | 27       | 11     | 0.42 (0.19-0.91) | 0.0074*|
| CC vs. GG                 | 4        | 0      | 0            |        |
| CC vs. CG/GG              | 31       | 11     | 0.37 (0.17-0.79) | 0.0072*|
| CC/GG vs. GG              | 4        | 0      | 0.3          |        |

OR: Odds Ratio, CI: Confidence Intervals, * p<0.05
With respect to the WHO functional class, mean age at onset and RVSP levels, comparison among the CC and CG genotypes revealed no significant variation. But, the patients with Alanine variant showed almost a decade early age at onset (17.54 ± 6.39) of the disease than the patients homozygous for proline variant (25.52 ± 11.5).

Comparison of symptoms among patients with CC genotype and CG genotype revealed increased frequency of Palpitations (69.2% vs. 58.3%), presyncope (60% vs. 33.3%) and paroxymal nocturnal dyspnea27.7% vs. 8.3%) in the patients with CC genotypes. The frequency of PND and presyncope was three and two folds higher, respectively in patients with CC genotypes.

**Discussion**

Prostacyclin has been recognized as a therapeutic target in treatment of IPAH culminating in improved survival via sustained clinical and haemodynamic improvement [28]. Reduced expression of PGIS, has been demonstrated in the lung tissue sample of IPAH patients [29]. The 9bp VNTR in the 5’-flanking region of PGIS, is known to significantly affect its promoter activity on IL-6 stimulation. The number of Sp1-consensus-sequence motifs in the VNTR polymorphism determines the promoter activity, with highest promoter activity observed in the R6 allele containing five Sp1-binding sites [26,30]. However, in the present study no significant difference in the distribution of VNTR alleles in IPAH group was observed, suggesting that this polymorphism may not play any role in reduced expression and levels of PGIS2 in IPAH. In contrast, Nana-Sinkam et al [9] reported that the allele containing five Sp1-binding sites has least promoter activity and reported an increased frequency of this allele in PAH patients with known BMP2R mutations suggesting a potential functional role for the promoter polymorphism in the pathogenesis of IPAH. The frequency of the VNTR promoter polymorphism is known to be influenced by ethnicity and hence could account for contrasting results obtained in Indian and Caucasian IPAH patients.

Hypermethylation of CpG dinucleotides found within the PGIS promoter is associated with reduced PGIS expression. CpG methylation provides an epigenetic mechanism for the down-regulation of PGIS expression and is implicated in lung and colorectal cancers [31]. Hence similar epigenetic mechanism may account for reduced PGIS expression and subsequently low PGIS2 levels in IPAH. It has been suggested that an individual’s PGIS allelotype is crucial in determining PGIS expression and subsequently low PGI2 levels in IPAH. It has been suggested that an individual’s PGIS allelotype is crucial in determining PGIS expression and low levels of PGI2 in IPAH. It has been suggested that an individual’s PGIS allelotype is crucial in determining PGIS expression and low levels of PGI2 in IPAH.

A novel non-synonymous polymorphism was observed in the study cohort that results in substitution of Arginine with Leucine (R275L) in exon 6 of PGIS. Difference in the presentation of symptoms of angina and presyncope were observed in patients based on this polymorphism.

The frequency of PND and presyncope was three and two folds higher, respectively in patients with CC genotypes. A reduced expression of PPARγ gene and protein has been observed in the lungs from patients with severe PAH, with complete loss of PPARγ expression in the plexiform lesions [17]. The transcription factor PPARγ and its putative target apoE are potential downstream effectors of BMPR-2 signalling. The mRNA expression of both factors and BMPR-2, is decreased in lung tissues from PAH patients [33,34]. It is also known that PPARγ activation inhibits the TGFβ signal pathway in VSMC [35].

In the present study, a high frequency of the IR risk conferring P12P genotype was observed in IPAH patients, which was found to be significantly associated with the disease. The patients with P12P genotype also had higher frequency of PND, a symptom associated with disease severity and bad prognosis but not with WHO Functional Class or RVSP levels. Thus the P12 P genotype may be a modifier allele in PAH in conjunction with BMPR-2 and apoE involved in the pathway. It is therefore possible that certain kinds of diet can be important triggers for onset of PAH as diet seems to influence PPARy2.

The P12A variants of the PPAR-γ2 were shown to cause a different drug efficacy in vitro [36]. Therefore it has been postulated that the P12A variant of the PPAR-γ gene could cause differences in the efficiency of TZD therapy in clinical application and may be useful in assessment of good responders from non-responders to TZD treatment.

PGI2 and its analogues are ligands for peroxisomal proliferator-activated receptors, and also selectively increase PPARγ activity both in non-transformed epithelial cells and in non–small-cell lung cancer. Hence, studies with larger patient cohort are necessary for determining the exact role of this polymorphism in IPAH.

Conclusion

This study has attempted to correlate promoter/gene polymorphisms of PGIS to its activity, and it can be concluded that the VNTR polymorphism and the polymorphism found in exon 6 may not have an effect on the levels of PGIS, with a possible explanation being ethnicity of the cohort studied.

Taking into account the possibility of IR being a risk factor in IPAH, the P12A polymorphism in PPARγ was studied. In the present study, the alanine variant was found to be significantly associated with a reduced risk of IPAH. This could be because of very low frequency of heterozygotes and complete absence of A12A homozygotes in the IPAH group. Hence, studies with larger patient cohort are necessary for determining the exact role of this polymorphism in IPAH.
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