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Original Article

Endothelin-1 and endothelial nitric oxide polymorphisms in idiopathic pulmonary arterial hypertension

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Abstract: Idiopathic Pulmonary arterial hypertension (IPAH) is a debilitating disease associated with very poor prognosis. The disease is characterised by endothelial dysfunction, smooth muscle proliferation and *insitu* thrombosis in the pulmonary artery, eventually leading to right ventricular failure. Two of the key endothelial mediators implicated in the pathogenesis of IPAH are endothelin-1 (EDN1) and nitric oxide (NO). EDN1 is a potent endogenous vasoconstrictor whereas NO is a vasodilator. In the present study screening of the EDN1 gene (*EDN1*) and *NOS3* polymorphisms was taken up, to evaluate their association with IPAH. A significant association of *EDN1* 3A/4A polymorphism (+138 A; rs10478694) (OR-3.485; CI-1.254, 9.999; p=0.013) and *EDN1* Lys198Asn polymorphism (G/T, rs5370) (OR-3.378, CI-1.104, 10.582; p=0.03) with IPAH was observed. Our results indicate that *EDN1* polymorphisms in interaction with other genetic markers may play a significant role in individual's susceptibility to the disease and its clinical progression.

Keywords: IPAH, endothelial dysfunction, *EDN1*, *NOS3*, polymorphism, Linkage Disequilibrium

Introduction

Endothelial dysfunction has varied clinical implications in many diseases [1]. The endothelium is the monolayer of endothelial cells lining the vascular lumen and the endothelium derived mediators are essential to maintain vascular homeostasis. An injury to the endothelium impairs production and/or function of the vasoprotective mediators and exposes the underlying vascular smooth muscle cells to circulating mitogens, growth factors which stimulate cell proliferation, migration and extracellular matrix deposition.

Pulmonary arterial hypertension (PAH) is clinically defined as sustained elevation in mean pulmonary artery pressure to greater than 25 mm Hg at rest or greater than 30 mm Hg on exercise. PAH can occur as an idiopathic disease (IPAH) or may be associated with other clinical features (APAH). IPAH (OMIM- #178600) is characterized by progressive narrowing of the pulmonary arteries, leading to right ventricular failure and eventually death [2,3]. Both familial (FPAH) and sporadic (IPAH) forms of disease are reported, which are clinically and pathologically indistinguishable from each other. The disease commonly manifests in the 3rd and 4th decade of life, although individuals of any age can be affected [3, 4]. Mutations in the gene coding for bone morphogenic protein receptor 2 (*BMPR2*) is recognised as one of the causative factors of FPAH and 26% cases of IPAH [5]. The pulmonary vascular remodelling in IPAH is characterized by endothelial cell injury, infiltration of smooth-muscle cells into the subintima and thickening of the medial layer in proximal vessels.

In IPAH, endothelial dysfunction plays an integral role in mediating the structural changes in the pulmonary artery. NO and EDN1 are two most prominent endothelial mediators. NO is a potent endogenous vasodilator synthesized from L-arginine, catalyzed by the enzyme endothelial NO synthase, encoded by *NOS3*. EDN1 is produced as a 212 amino acid pre-

proendothelin and is processed to a relatively inactive 39 amino acids long big EDN1. The big EDN1 is converted by the membrane-bound endothelin converting enzyme-1(ECE-1) to a 21a.a. functional peptide.

Understanding the role of *EDN-1* and *NOS3* in IPAH has therapeutic significance since EDN1 receptor antagonists and NO agonists are currently few of the best possible options available in treatment of IPAH. Studies have shown that addition of sildenafil to bosentan treatment could elicit additional hemodynamic benefits in IPAH patients [6, 7].

The molecular mechanisms by which endothelial vasomediators contribute to IPAH remain obscure. Genetic variants of *EDN1* and *NOS3* are known to alter their expression. Hence the aim of the study was to screen *EDN1* and *NOS3* variants (rs2070744, rs1799983) respectively, to evaluate their role in endothelial cell dysfunction which is commonly observed in IPAH.

Materials and methods

This study was approved by the Ethical Committee of Care Hospitals, Hyderabad, India. The diagnosis of IPAH was based on WHO criteria [8] and blood samples of 77 IPAH cases (41 women and 36 men) referred by the cardiologist were included in the present study. Samples from 100 (40 females, 60 males) healthy voluntary donors selected randomly served as controls. The mean age of patients and controls was 28.6 ± 11.4yrs and 29.57 ±7.75yrs, respectively. The DNA was isolated from blood following standard protocol [9].

Single stranded conformational polymorphism (SSCP) analysis of EDN1

The 5 exons of *EDN1* along with part of 5'UTR and 3'UTR were screened using primer sets as previously described by Algovik *et al* [10]. Annealing temperatures were standardized for each primer set and PCR was carried out for 25 cycles.

PCR assays was carried out in a 25*µ*l volume with 100ng 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).

SSCP was carried out as per Orital *et al* 1989 .The PCR products were denatured at 94C for 10 minutes, quenched in ice for 5 minutes and then loaded on 11% native polyacrylamide gels and run at 150V at room temperature. The gels were visualized by silver staining. The samples showing aberrant band pattern were sequenced commercially.

Genotyping NOS3

The frequencies of the T-786C and G894T polymorphisms were determined by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) as described previously [12,13]. For -786 polymorphism (rs2070744) the 494bp PCR product was digested with *Msp*I (Merck, Germany). Digested samples were separated on 10% non-denaturing polyacrylamide gel and visualized by silver staining. . The genotypes were identified as TT (258 /240bp), CT (258/240/212/46bp),and CC (240/212/46bp) respectively.

For G894T polymorphism (rs1799983) the 246-bp PCR product was digested with a *Dpn*II restriction enzyme (New England Bio labs, USA) and run on 10% poly-acryl-amide gel and then the gel was silver stained. This resulted in two fragments (158 and 88 bp) when the restriction site created by the 894G-T transversion. The genotypes were identified as GG, TG, and TT. Standard samples having confirmed GT/TT genotypes were always put for digestion with each batch of PCR products, to rule out failed digestion.

Statistical Analysis

Deviations from the Hardy-Weinberg equilibrium were tested for all polymorphisms in cases and controls by comparing observed and expected genotype frequencies with an exact goodness of fit test. Odds ratios, with 95% confidence intervals were calculated to compare allele and genotype frequencies. The extent of linkage disequilibrium (LD) was expressed in terms of the maximum likelihood estimate of disequilibrium, *D'*.

Statistical analysis was carried out using

Table 1. Odds risk of estimation of EDN-1 polymorphism in IPAH

EDN-1Polymorphism	Controls	IPAH	OR	CI	P value
+138Ins A					
3A/3A	93	61			
3A/4A+ 4A/4A	6+1	14+2	3.485	(1.254 - 9.999)	0.013*
Lys198Asn					
Lys198Lys	67	34			
Lys198Asn	26	31	2.35	(1.147- 4.83)	0.018*
Asn198Asn	7	12	3.378	(1.104-10.582)	0.03*

OR- Odds Ratio, CI- Confidence Intervals, * p<0.05 considered significant

Table 2. Distribution of *EDN -1* +138 Ins A polymorphism based on gender and EDN-1 levels in two genotypes

Polymorphism	control	IPAH
3A/4A		
Males	4 (6.6 %)	11(30.5%)
Females	2 (5%)	3 (7.31%)
4A/4A		
males	–	1(2.7%)
females	1 (2.5%)	1 (2.4%)
*EDN1 Levels		
3A/3A		0.561 ± 0.127
3A/4A		0.581 ± 0.129

SNPstats software, available online. (www.bioinfo.iconcologia.net/SNPstats). Probability (*p*) values of less than 0.05 were considered as statistically significant.

Results

Two previously known polymorphisms were detected in *EDN1*; (a) an "A" insertion(I)/deletion (D) in exon1 at position +138 (rs10478694), the polymorphism is designated as 3A/3A (wild type/deletion), 3A/4A, 4A/4A (mutation/ insertion); and (b) a G/T transversion at position +5665 (rs5370) affecting the 61st nucleotide of exon 5, which substitutes Lys at 198 codon with Asn (K198N). The polymorphism is designated as – Lys198Lys, Lys198Asn, Asn198Asn. **Table 1** gives the allelic and genotypic frequencies of the two *EDN1* polymorphisms among controls and patients. No significant deviation from the Hardy Weinberg equilibrium was observed either in patients or control subjects. In rest of the exons of *EDN-1,* no genetics variations were observed.

In case of 3A/4A polymorphism, the frequencies of heterozygotes (3A/4A) were 3 times more common in IPAH (20.7%) than control subjects (6%). Also the odds ratio for the dominant model was found to be statistically significant in IPAH group (p=0.013). We have previously reported increased EDN1 levels in IPAH [14]. The EDN1 levels of 36 patients included in the present study were available. Comparison of EDN1 levels between patients with Insertion variant (n=10) and the wild-type (D) variant (n=26) is given in **Table 2**. An interesting observation was that this insertion variant was more predominant in males (33.33%) compared to female patients (9.35%). For Lys198Asn polymorphism in exon 5 , the mutant Asn198Asn and Lys198Asn genotypes were found to be significantly associated with IPAH compared to wild type homozygous Lys198Lys (**Table 1**).

The observed allele and genotype frequencies for *NOS3* - T-786C promoter and G894T exon7 polymorphisms are presented in **Table 3**. No deviations from the Hardy Weinberg equilibrium were observed either in patients or in control subjects. Similarly no significant differences in the distribution of allele and genotype frequencies were observed. The LD between the two polymorphisms was tested and observed that the T-786C and G894T loci in both patient and control groups showed, *D'* = 0.4406 (**Table 4**).

Table 3. Odds risk of estimation of NOS3 polymorphism in IPAH

Genotype	Controls n (%)	IPAH n (%)	OR	CI	p
T-786C SNP					
T/T	54 (54%)	38(49.35%)			
T/C	42 (42%)	37(48.05%)	1.25	(0.68-2.30)	0.67
C/C	4 (4%)	2 (2.5%)	0.71	(0.12-4.08)	
Allele C	50 (0.25)	41 (0.27)			
G894T					
G/G	58 (58%)	39 (50.6%)			
G/T	33 (33%)	34 (44.1%)	1.53	(0.82-2.87)	0.25
T/T	9 (9%)	4 (5.1%)	0.66	(0.19-2.30)	
Allele T	51 (0.25)	42 (0.27)			

OR- Odds Ratio, CI- Confidence Intervals, p<0.05 considered significant

Table 4. Haplotype frequencies estimation of eNOS polymorphism in IPAH

Haplotype	Controls	IPAH	p
T-G	0.5369	0.4610	---
T-T	0.2131	0.2727	0.17
C-G	0.2081	0.2662	0.22
C-T	0.0419	0	----

Discussion

Intense vasoconstriction is the earliest feature in IPAH, resulting due to endothelial injury. Elevated serum EDN1 levels, correlated with pulmonary hemodynamics, have been reported in IPAH patients by several studies [14,15,16,17]. Also, circulating levels of mature EDN1 and plasma EDN1 levels are considered as a useful prognostic marker in patients with IPAH undergoing treatment with EDN1-receptor antagonist [18].

The 3A/4A polymorphism (rs10478694) of the *EDN1* has significant functional importance. Expression studies have shown, increased luciferase activity of the insertion variant (I) as compared to the wild-type (D) variant and the half-life of mRNA containing the insertion variant is prolonged [19]. In the same study, elevated EDN-1 levels were also reported in the insertion variant as compared to wild type. Significant difference in EDN1 levels between 3A/4A and 3A/3A genotypes has also been reported [20]. This *EDN1* polymorphism is also implicated in increased susceptibility to vasovagal syncope [21].

On comparison of the EDN1 levels between (3A/4A+4A/4A) and (3A/3A), the levels were found to be higher IPAH subjects with 'A' insertion (0.5805, ±0.129) than subjects with wild type genotype (0.56174, ±0.127), though this difference is not statistically significant, reflecting the small sample size. Since the 3A/4A polymorphism is associated with elevated EDN1 levels, it is possible that even a small injury may cause a significant disturbance in the vascular tone. Further this polymorphism in presence of an underlying genetic susceptibility/ mutation can trigger severe endothelial dysfunction.

The exact role of Lys198Asn in EDN1 levels still remains elusive, although it has been associated with various disorders. Expression studies have shown that there is no difference in EDN1 levels between Asn carriers and Lys carriers. It has been suggested that this polymorphism in linkage disequilibrium with other *EDN1* variants/ genes/ environmental factors may influence *EDN1* expression. For example: the 3A/3A and Lys198Lys combination was associated with lower EDN-1 levels in the high altitude natives and considered as an adaptation under hypobaric hypoxic conditions [22]. Similarly, the 4A/Asn haplotype along with another *EDN-1* polymorphism was considered as a risk factor for angina [23]. The rarer genotype Asn198Asn in association with another *EDN -1* variant (IVS-

4 G/A and Lys198Asn) was found to have the highest hazard ratios to chronic heart failure in bucindolol-treated patients [24].

With regard to the two *NOS3* polymorphisms (rs2070744, rs1799983) no significant difference was observed between IPAH and control groups. These polymorphisms are known to influence *NOS3* expression leading to reduced NO production or enhanced degradation of the enzyme [25, 26]. Our results indicate that reduce levels of NO in IPAH may not be due to down regulation of *NOS3* but may be due to NO being consumed for formation of reactive nitrogen species (RNS), as suggested previously [27].

Conclusion

There is evidence that gene-gene interactions are ubiquitous in determining the susceptibility to complex human diseases. The *EDN1* variants (rs10478694, rs5370) appear to play a significant role in IPAH. Endothelial dysfunction in the individuals with *EDN1* variants could be more pronounced, which in combinations with other gene variants, could be associated with poor prognosis. Since, it is known that EDN1 levels in the 3A/4A +4A/4A carriers are higher; IPAH patients with this polymorphism could derive more benefits with EDN1 antagonists or in combination with other treatments. We could not observe any role of *NOS3* polymorphisms for reduced NO levels in IPAH and suggest that they are unlikely to be susceptible alleles in the disease. However, as the study included only two *NOS3* variants, it is also possible that other variants of *NOS3* may be involved in IPAH.

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