

5HTT Promoter Polymorphism in Idiopathic Pulmonary Arterial Hypertension

V. Shivani¹, K.Sujana¹, B. K. S. Sastry² and Pratibha Nallari¹

1. Department of Genetics, Osmania University, Hyderabad 500 007, Andhra Pradesh, India

2. Department of Cardiology, CARE Hospital, The Institute of Medical Sciences, Nampally, Hyderabad 500 001, Andhra Pradesh, India

KEYWORDS IPAH. Serotonin. 5HTT Promoter-Polymorphism. Frequency. Mean Pulmonary Artery Systolic Pressure. Hyperplasia

ABSTRACT A 44bp insertion/deletion in promoter of 5HTT (5Hydroxy Tryptamine Transporter) exists as a polymorphism in general population resulting in 3 genotypes LL (528 base pair), LS and SS (484 base pair). The transcriptional efficiency of the L allele is 2-3 fold higher than the S allele. The L-allelic variant of the 5-HTT gene promoter is associated with 5-HTT overexpression. This higher expression of the transporter leads to higher uptake of serotonin, resulting in activation of mitogenic pathways thereby inducing smooth muscle hyperplasia. The study focuses on the possible association of 5HTT insertion/deletion promoter polymorphism with Idiopathic Pulmonary Arterial Hypertension (IPAH) patients and distribution of the polymorphism among the control individuals from Indian population. In the present study, 65 IPAH cases and 100 controls were considered for comparative analysis. DNA samples from controls and patients were amplified using Polymerase Chain reaction and the products were genotyped on 2% agarose gel stained with ethidium bromide. Frequency of L allele was found to be much higher in IPAH (0.538) as compared to controls (0.335). The LL and LS genotypes were found to be at a higher risk for IPAH as compared to SS genotype (OR - 3.117, CI -1.293, 7.579 and OR- 5.250, CI- 2.186 - 12.776). The mPASP (mean pulmonary artery systolic pressure) was also significantly higher among the LL and LS genotypes when compared to the SS genotype in IPAH patients. The L allele could be a possible risk factor for IPAH and play a significant role in disease progression

INTRODUCTION

Idiopathic Pulmonary Arterial Hypertension (IPAH) is a rare and often fatal disease of unknown etiology, characterized by an increase in pulmonary vascular resistance and elevated pulmonary artery pressure (mPAP > 25 mm Hg at rest or > 30 mm Hg during exercise) (Rubin 1997). It is characterized by vasoconstriction, vascular remodeling and in situ thrombosis of pulmonary arteries. Progressive increase in pulmonary vascular resistance leads to right ventricular heart failure and eventual death. Pathogenesis of IPAH is complicated and multifactorial. Mutations in *BMPR-2* are considered to be the genetic basis for familial PAH and 26% of sporadic IPAH cases (Thompson et al. 2000; Machado et al. 2001). However, it has been observed that in addition to defects within *BMPR-2* gene, there is potential requirement of "multiple hits", which may be genetic, environmental or acquired factors or

a combination of all three, that act as triggers for the onset and may also contribute to the progression of the disease (Machado et al. 2005; Yu and Rubin 2005).

Serotonin and the serotonin transporter are implicated and contribute to the pathogenesis of IPAH and PAH induced by serotonergic appetite suppressant drugs (Fishman 2004). Serotonin is a vasoconstrictor as well as a mitogen, causing hyperplastic and hypertrophic changes in smooth muscles (Pakala et al. 1994). Serotonin causes vasoconstriction of pulmonary artery smooth muscle cell (PASMC) by interacting with 5-HT_{1B}, 5-HT_{2A}, and 5-HT_{2B} receptors, while the uptake of 5HT into the intracellular compartment of PASMC occurs via the serotonin transporter (5HTT), that causes smooth muscle cell proliferation and vascular remodeling. An epidemic of IPAH was reported in Europe in 1980's, that was found to be associated with the use of appetite suppressants like fenfluramine that selectively inhibit the reuptake of 5HT (Rothman et al. 1999).

The gene encoding the human serotonin transporter (5HTT) is located at 17q11.2. A promoter polymorphism of 44-base pairs displays two allelic forms, a long (L, 528bp) and a short (S, 484bp) variant. The short variant is associ-

Correspondence Address:

Dr. Pratibha Nallari
Department of Genetics,
Osmania University,
Hyderabad 500 001,
Andhra Pradesh, India
E-mail: prathinallari@yahoo.com

ated with reduced transcriptional efficiency of the gene that results in lower serotonin uptake activity and subsequent low functional expression of the transporter (Lesch et al. 1994).

In a study, the LL genotype was found in 65% of IPAH cases (French subjects) and this genotype was associated with susceptibility to the disease (Eddahibi et al. 2001). However, another study did not find any association of 5HTT polymorphism with the risk of developing pulmonary hypertension in patients of European ancestry (Machado et al. 2006).

In view of the conflicting data, we wanted to study the distribution of insertion/deletion promoter polymorphism within the serotonin transporter gene in IPAH in Indian Population.

METHODOLOGY

The present study was approved by the Institutional Ethics Committee of Care Hospitals, Hyderabad, Andhra Pradesh, India. The patient population included 65 idiopathic pulmonary hypertension patients (32 males and 33 females), attending CARE hospital, during the period 2003-2007. The diagnosis was based on clinical features supported by Doppler echocardiography and other tests as needed to make a diagnosis of idiopathic pulmonary hypertension. The control group consisted of 100 random healthy, unrelated volunteers, without any history/family history of heart, lung and other systemic disorders. Informed written consent was obtained from all the patients and controls before drawing blood samples. Blood samples were placed in EDTA vacutainers tubes and stored at -20°C until the time of assay.

Genomic DNA was extracted from peripheral blood leukocytes using rapid non-enzymatic DNA isolation method (Lahari and Nurunberger 1991) Polymerase chain reaction (PCR)-amplification was carried out using Eppendorf Master Cycler gradient, Germany with the Forward primer 5'GGCGTTGCCGCTCTGAATGC3' and Reverse primer 5'GAGGGACTGAGC TGGACAACCAC3' (Generay Biotech, Shanghai) in a total volume of 25ul solution containing 100 ng genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM MgCl₂, 100 mM dNTPs, 1% Triton X, 0.2 mM of each primer, and 1.5 U of Taq polymerase (Sigma Aldrich, Germany) for 35 cycles (Initial denaturation 95°C 10 min, 94°C for 1 min, 60°C for 1 min,

72°C for 1 min and followed by final extension of 5mins). The PCR products were then analyzed in 2% agarose (Sigma Aldrich, Germany) gel stained with ethidium bromide.

Statistical Analysis

Allelic and genotypic frequencies were calculated to establish /examine deviations from Hardy-Weinberg equilibrium. Epidemiological and clinical variables like age at onset, Pulmonary Artery Systolic Pressure (PASP) values and Tricuspid Regurgitation (TR) velocities are expressed as the mean (SD) for intra group comparisons. Odds ratios are given with 95% confidence limits and level of significance.

RESULTS

The data analysis showed that there was a significant difference in the frequencies of 5-HTT genotypes (SS, LS, LL) between the control and patient groups (Table 1). High frequency of SS genotypes was observed in controls (57%) whereas in IPAH patients LS genotype was predominant (43.07%) followed by LL genotype (32.03%).

The L allele and S allele frequency was found to be 0.335 and 0.665 respectively in healthy subjects as compared to 0.5384 and 0.4615 in IPAH patients. (Table 1).

Table 1: Genotype and allelic frequency distribution of 5HTT genotypes in controls and IPAH group

Genotype	Controls n (%)	Patients n (%)	Allelic frequency	Controls	Patients
SS	57 (57)	16 (24.61)	S	0.665	0.461
LS	19 (19)	28 (43.07)	L	0.335	0.538
LL	24 (24)	21 (32.03)			

Table 2 gives the odds ratio of different 5 HTT genotypes in IPAH group versus the controls. An increased risk of LL genotype was found when compared to SS genotype. (OR - 3.117, CI -1.293 ,7.579).Odds Ratio of LS vs SS genotype was also highly significant (OR-5.250 , CI- 2.186 - 12.776) . This data further supports the implicated role of L allele in pathogenesis of IPAH.

The odds ratio of LL vs LS and LS vs LL was not found to be statistically significant.

Table 3 gives a comparison of mean age at

Table 2: Odds test of association of 5HTT genotypes in IPAH in comparison to controls

Genotype	Controls	IPAH	Odds ratio	95%CI
LL vs SS	24 57	21 16	3.117*	1.293 - 7.579
LL vs LS	24 19	21 28	0.594	0.283 - 1.470
LL vs others	24 76	21 44	1.511	0.714 - 3.201
LS vs SS	19 57	28 16	5.250*	2.186 - 12.776
LS vs LL	19 24	28 21	1.684	0.680 - 4.191
LS vs others	19 81	28 44	2.713*	1.291 - 5.730
Others vs SS	43 57	49 16	4.060*	1.935 - 8.600

*p<0.05

onset, mean Pulmonary Artery Pressures (mPASP) and mean Tricuspid Regurgitation velocity (TR velocity) amongst the 3 genotypes. The mean age at onset for LL (23.85 ± 11.5) LS (23.64 ± 10.9) and SS (24.5 ± 13) genotypes was almost similar. A significant difference was seen with respect to the mean PASP levels in the three genotypes LL (111.05 ± 24.4), LS (97.95 ± 19) and SS (92.71 ± 27.2). No significant difference was found with respect to the TR velocities (LL, 4.7 ± 0.65 ; LS, 4.5 ± 0.558 ; SS, 4.4 ± 0.893).

Table 3: Comparison of mean age at onset, mean pulmonary artery systolic pressure and mean TR velocities in 5HTT genotypes in IPAH group

Genotype	Age at onset $X \pm SD$	mPASP $X \pm SD$	mTR velocity $X \pm SD$
LL	23.85 ± 11.5	111.05 ± 24.4	4.7 ± 0.65
LS	23.64 ± 10.9	97.36 ± 16.6	4.5 ± 0.558
SS	24.5 ± 13.0	92.71 ± 27.2	4.4 ± 0.893

DISCUSSION

The 5-HTT transporter is abundantly expressed in the lung, where it is predominantly located on PASMCs (Ramamoorthy et al. 1993). Hyperplasia of pulmonary artery smooth muscle cells (PA-SMCs) is pathological hallmark of IPAH and is an important component of pulmonary vessel remodeling that results in increased thickness of the medial muscular coat in normally muscular arteries and in extension of muscle into smaller and more peripheral arteries (Rabinovitch et al. 1979). The role of 5-HTT in pulmonary vascular remodelling was highlighted

when mice targeted with 5HTT gene disruption, developed less severe hypoxic pulmonary hypertension than wild-type controls, and the selective 5-HTT inhibitors were able to attenuate hypoxic pulmonary hypertension (Eddahibi et al. 2000). Conversely, increased 5HTT expression was associated with increased severity of hypoxic pulmonary hypertension (MacLean et al. 2004).

The 5-HTT expression is genetically controlled and the insertion /deletion promoter polymorphism affects its transcriptional activity where the long (L) promoter variant has been shown to be associated with increased 5-HTT expression as compared with the short (S) variant and results in increased 5-HTT uptake (Lesch et al. 1994).

It has been shown in bovine and rat PA-SMCs, that the mitogenic and comitogenic effects of 5-HT require internalization of indoleamine by a high-affinity and selective transporter (Lee et al. 1991). Serotonin activates the Ras or Rac or both pathways that activate NADP(H)oxidase producing a reactive oxygen species (ROS), which activates ERK and/or MAP Kinase pathway, inducing hyperplasia (Lee and Fanburg 2000).

The L variant of the 5-HTT gene polymorphism was associated with 5-HTT overexpression and was found to be more common in patients with IPAH (65%) than in controls (27%), implying that this allele may confer susceptibility to IPAH (Edahibi et al. 2001). The role for the long variant of 5HTT has also been implicated in the pathogenesis of early onset IPAH in 90% of children included in another study (Vachharajani and Saunders 2005). However, the sample size in this study was considered very small to completely support this hypothesis. In a larger cohort study involving 528 patients with familial pulmonary arterial hypertension (FPAH), Associated Pulmonary Arterial Hypertension (APAH) and Idiopathic Pulmonary Arterial Hypertension (IPAH) of European ancestry, no increased risk/susceptibility of LL genotype was found, contradicting the earlier reports (Machado et al. 2006).

In our study though the frequency of LS genotype was found to be highest followed by LL genotype in IPAH cases, the frequency of L allele was much higher than that of controls, indicating that the L allele may be playing a role in susceptibility to IPAH. Also as compared to SS

genotype both LL and LS genotypes are seen at a significant increased risk for IPAH (OR - 3.117, CI -1.293 ,7.579 and OR-5.250 , CI- 2.186 - 12.776).

The PA-SMCs from patients with IPAH have been shown to grow faster than PA-SMCs from controls when stimulated by serotonin or serum (Eddahibi et al. 2001). In the same study it was shown that the uptake of 5-HT was more by PA-SMCs with the LL genotype than cells with the LS or SS genotype and the growth-stimulating effects of 5-HT or serum were more marked in LL cells than the other two types indicating that the capability of PA-SMCs to proliferate in response to serotonin or serum was directly linked to the functional polymorphism of the 5-HTT gene promoter. Also, the 5HTT activity was shown to be higher in LS than in SS cells refuting the earlier reports⁵ that suggested that the S allele is the dominant allele.

Marcos et al. (2006) have shown that PA-SMCs from patients exhibited higher 5-HTT levels as compared to controls with same genotype. There were no additional promoter sequence alterations to explain the same. Thus the over expression of 5HTT in the diseased state cannot solely be attributed to the LL genotype and other regulatory mechanisms that influence 5HTT gene expression may also be involved .

In the present study, a comparison was made between the mean PASP and TR velocity in the 3 genotypes, the mean PASP was highest in LL (111.05mmHg) followed by LS(97.36mmHg) and lowest for SS group (92.7mmHg). Since one of the main effects of 5HTT over expression is higher uptake of serotonin leading to hyperplasia, it is tempting to speculate that the differences in mean PASP levels in the 3 genotypic groups could be due to the role of the L allele in IPAH.

Few other studies have also observed the LL genotype to be associated with elevated mean pulmonary artery pressure (Eddahibi et al. 2003; Olson et al. 2007).

However, the differences in observed mean PASP, cannot be attributed only to these 5HTT genotypes as PASP is also known to be influenced by many other variables such as age, body mass index (BMI), sex, effect of treatment and right ventricular systolic function etc. (McQuillan et al. 2001).

No significant deviation was observed with respect to age at onset among the three geno-

types (LL,23.8;LS,23.64;SS,24.5). But an interesting observation in this small study is the fact that the mean age at onset in IPAH patients from India is found to be much earlier than the reported 36 yrs from western world (Ghamra and Dweik 2003) .

However, in the present study, between LL and LS genotype the odds ratio was not found to be statistically significant. Hence, among the two genotypes, which genotype is at a higher risk could not be brought out completely. It is however possible that the severity of the disease (hyperplasia) may vary with respect to the above two genotypes as indicated by the mean PASP values, which needs to be confirmed by other studies.

CONCLUSION

In the present study, the L allele for 5HTT was observed to be ~~is~~ a possible genetic risk factor for IPAH. However, it is more likely that the 5HTT genotypes (LL,LS) are not the direct cause of the disease but may play a significant role in disease progression by contributing to the vascular remodeling that is seen in IPAH or by other mechanisms. We also report a much earlier age at onset of IPAH in Indian population than that reported in literature to date.

ACKNOWLEDGEMENT

We acknowledge The Indian Council of Medical Research for the JRF grant and UGC, New Delhi for financial assistance and all the patients who participated in the study.

REFERENCES

- Eddahibi S, Hanoun N, Lanfumey L, Lesch KP, Raffestin B, Hamon M et al. 2000. Attenuated hypoxic pulmonary hypertension in mice lacking the 5-hydroxytryptamine transporter gene. *J Clin Invest*, 105: 1555-1562.
- Eddahibi S, Humbert M, Fadel E, Raffestin B, Darmon M et al. 2001. Serotonin transporter overexpression is responsible for pulmonary artery smooth muscle hyperplasia in primary pulmonary hypertension. *J Clin Invest*, 108: 1141-1150.
- Eddahibi S, Chaouat A, Morrell N, Fadel E, Fuhrman C et al. 2003. Polymorphism of the serotonin transporter gene and pulmonary hypertension in chronic obstructive pulmonary disease. *Circ*, 108(15): 1839-1844.
- Fanburg BL, Lee SL 2000. A role for the serotonin transporter in hypoxia induced pulmonary hypertension. *J Clin Invest*, 105(11): 1521-1523.

- Fishman AP 2004. Primary pulmonary arterial hypertension: A look back. *J Am Coll Cardiol*, 43: 2-4.
- Ghamra, ZW, Dweik RA 2003. Primary pulmonary hypertension: An overview of epidemiology and pathogenesis. *Cleveland Clinic Journal of Medicine*, 70 Suppl 1: S2-8
- Lahari D, Nurunberger J 1991. A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. *Nucleic Acids Res*, 19: 5444.
- Lee SL, Wang WW, Moore BJ, Fanburg BL 1991. Dual effect of serotonin on growth of bovine pulmonary artery smooth muscle cells in culture. *Circ Res*, 68: 1362-1368.
- Lesch KP, Balling U, Gross J, Strauss K, Wolozin BL et al. 1994. Organization of human serotonin transporter gene. *J Neural Transm*, 95: 157- 164.
- Machado RD, James V, Southwood M, Harrison RE, Atkinson C et al. 2005. Investigation of second genetic hits at the *BMPR2* locus as a modulator of disease progression in familial pulmonary arterial hypertension. *Circ*, 111: 607-613.
- Machado RD, Koehler R, Glissmeyer E, Veal C, Suntharalingam J et al. 2006. Genetic association of the serotonin transporter in pulmonary arterial hypertension. *Am J Respir Crit Care Med*, 173: 793-797.
- MacLean MR, Deuchar GA, Hicks MN, Morecroft I, Shen S et al. 2004. Overexpression of the 5-hydroxytryptamine transporter gene: Effect on pulmonary hemodynamics and hypoxia-induced pulmonary hypertension. *Circ*, 109(17): 2150-2155.
- Marcos E, Fadel E, Sanchez O, Humbert M, Darteville P et al. 2004. Serotonin-induced smooth muscle hyperplasia in various forms of human pulmonary hypertension. *Circ Res*, 94: 1263-1270.
- McQuillan BM, Picard MH, Leavitt M, Weyman AE 2001. Clinical correlates and reference intervals for pulmonary artery systolic pressure among echocardiographically normal subjects. *Circ*, 104: 2797.
- Olson TP, Snyder EM, Frantz RP, Turner ST, Johnson BD 2007. Repeat length polymorphism of the serotonin transporter gene influences pulmonary artery pressure in heart failure. *Am Heart J*, 153(3):426-32.
- Pakala R, Willerson JT, Benedict CR 1994. Mitogenic effect of serotonin on vascular endothelial cells. *Circ*, 90: 1919-1926.
- Rabinovitch M, Gamble W, Nadas AS, Miettinen O, Reid L 1979. Rat pulmonary circulation after chronic hypoxia: Hemodynamic and structural features. *Am J Physiol*, 236: H818-H827.
- Ramamoorthy S, Bauman AL, Moore KR, Han H, Yang-Feng T et al. 1993. Antidepressant- and cocaine-sensitive human serotonin transporter: Molecular cloning expression, and chromosomal localization. *Proc Natl Acad Sci USA*, 90: 2542-2546.
- Rothman RB, Ayestas MA, Dersch CM, Baumann MH 1999. Aminorex, fenfluramine, and chlorphentermine are serotonin transporter substrates. Implications for primary pulmonary hypertension. *Circ*, 100: 869-875.
- Rubin LJ 1997. Primary pulmonary hypertension. *NEJM*, 336: 111-117.
- Vachharajani A, Saunders S 2005. Allelic variation in the serotonin transporter (5HTT) gene contributes to idiopathic pulmonary hypertension in children. *Biochem Biophys Res Commun*, 334: 376-379.
- Yuan XJ, Rubin LJ 2005. Pathogenesis of pulmonary arterial hypertension: The need for multiple hits. *Circ*, 111: 534-538.