

Endothelin-1 gene and endothelin receptor A gene polymorphisms in severe pulmonary hypertension associated with rheumatic mitral valve disease

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Abstract

Introduction: Endothelin-1 (ET-1) gene polymorphisms are implicated in pathogenesis of idiopathic pulmonary arterial hypertension.

Methods: We studied ET-1 (Lys198Asn and 3A/4A) and endothelin receptor A (ET_A) gene (His323His) polymorphisms in 123 subjects with pulmonary hypertension associated with rheumatic mitral valve disease (PH-MVD) and 123 healthy controls.

Results: The mutant homozygous Asn/Asn genotype in Lys198Asn and T/T genotype in His323His polymorphism was more prevalent in the PH-MVD group. Presence of Asn/Asn genotype was significantly associated with an increased risk (odds ratio 3.9).

Conclusions: ET-1 and ET_A gene polymorphisms are prevalent in the PH-MVD group suggesting that they may predispose to the development of PH.

Key words: pulmonary hypertension, mitral stenosis, rheumatic heart disease, endothelin-1 gene polymorphism.

Mitral stenosis (MS) is the most common valve lesion in chronic rheumatic heart disease (RHD) with about 38% of patients developing pulmonary hypertension (PH) which may progress to right heart failure [1]. Persistent PH is observed in many patients even after successful percutaneous balloon mitral valvuloplasty [2] or surgical mitral valve replacement [3] and has a high mortality rate [1]. However all patients with RHD do not develop PH implying that some patients may be genetically predisposed to develop PH.

Endothelin-1 (ET-1) is a 21-amino acid peptide produced by endothelial cells, vascular smooth muscle cells and cardiac myocytes with mitogenic, angiogenic and vasoconstrictor properties [4]. ET-1 mediates its effects through endothelin receptor type A (ET_A) present in the vascular smooth muscle cells [4]. ET-1 expression in pulmonary vascular cells is increased in idiopathic pulmonary arterial hypertension (PAH) where it contributes in the pathogenesis by promoting inflammation and fibrosis [5, 6]. The ET-1 gene polymorphisms have been shown to be associated with the development of idiopathic PAH, suggesting their role in the

pathogenesis [5, 6]. However, the significance of ET-1 in the pathogenesis of PH associated with rheumatic mitral valve disease (PH-MVD) has not been studied. We hypothesized that the development of pulmonary hypertension in RHD is influenced by genetic predisposition as only few patients develop PH despite having severe valvular heart disease. Since genetic studies are not available in such patients, we aimed to study the role of ET-1 and ET_A gene polymorphisms in PH-MVD.

Methods. In this cross-sectional study, 246 subjects, aged ≥ 18 years were enrolled from the outpatient Department of Cardiology, 123 consecutive PH-MVD subjects (Group A) and 123 age- and sex-matched healthy controls (Group B). Group A consisted of subjects having isolated chronic rheumatic mitral valve stenosis with associated PH (right ventricular systolic pressure (RVSP) ≥ 36 mm Hg). They were on rheumatic prophylaxis either with injection benzathine penicillin 1.2 million units every three weekly or tablet erythromycin 250 mg twice daily. Subjects having acute rheumatic fever in the last 1 year, other valvular lesions, and coexisting cardiac or pulmonary disease were excluded. The Institutional Ethics Committee approved the study and each participant gave written informed consent.

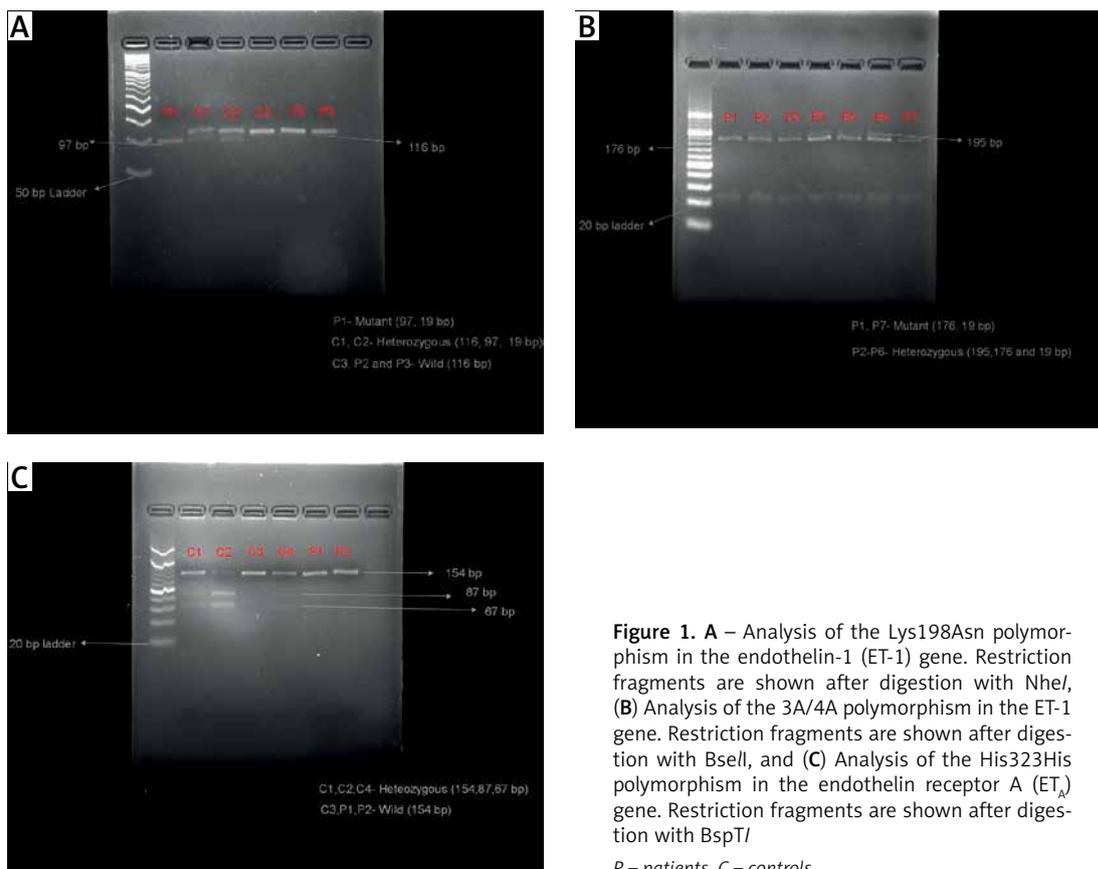
Transthoracic two-dimensional echocardiography, M-mode and Doppler examination for the assessment of valvular heart disease and ventricular function were performed in a resting, non-sedated state in the left lateral decubitus position. Mitral valve area was calculated by planimetry and pressure-half time method [7]. RVSP was calculated from tricuspid regurgitation (TR) jet obtained under color flow imaging guidance. RVSP was obtained using simplified Bernoulli formula from peak TR velocity; $RVSP = 4(V_{TR})^2 + RAP$, where V_{TR} is peak velocity of TR jet and RAP is estimated right atrial pressure. Peak end-diastolic velocity (V_{ED}) of pulmonary regurgitation jet continuous wave Doppler trace was used to calculate pulmonary artery end-diastolic pressure (PADP); $PADP = 4(V_{ED})^2 + RAP$ [1]. An average of 5 readings was taken.

Fifteen milliliters of fasting blood sample was drawn from peripheral vein for hematology, biochemistry tests and DNA extraction. Genomic DNA was extracted from peripheral blood leukocytes using DNA extraction kit (Gene Aid, India) following the manufacturer's protocol. DNA quantitation was done using Quantus fluorometer (Promega, USA) and the quality of DNA was determined by ethidium bromide stained 1% agarose gel electrophoresis. Three polymorphisms, Lys198Asn (rs5370), 3A/4A (rs10478694) on the ET-1 gene and His323His (rs5333) on the ET_A gene, were studied and analyzed by polymerase chain reaction restriction fragment length polymorphism

(PCR RFLP). The primers and restriction enzymes used for ET-1 and ET_A gene polymorphisms were as previously reported [8–10] and were as follows: Lys198Asn, forward: 5'-ATGATCCCAAGCTGAAAGGCTA-3', reverse: 5'-CAGGGCTCTCCGTGGAGGCTAT-3'. The cycling conditions were: initial denaturation 93°C for 3 min, followed by 34 cycles of 93°C denaturation for 50 s, 52°C annealing for 50 s, 72°C extension for 50 s, and finally 72°C for 10 min for final extension. PCR product was a 116 base pair (bp) fragment, 3A/4A, forward: 5'-GCTGCTTTTCTCCCGTTAA-3', reverse: 5'-CAAGCCACAAACAGCAGAGA-3'. The cycling conditions were: initial denaturation 95°C for 10 min, followed by 39 cycles of 95°C, denaturation for 40 s, 57.3°C annealing for 40 s, 72°C extension for 1 min, and finally 72°C for 10 min for final extension. PCR product was a 195 bp fragment. His323His, forward: 5'-TTTCTCACTTTCCTTTAGCG-3', reverse: 5'-ACCTAAGTAATCACATCGG-3'. The cycling conditions were: initial denaturation 95°C for 3 min, followed by 33 cycles of 94°C, denaturation for 30 s, 53.2°C annealing for 30 s, 72°C extension for 30s, and finally 72°C for 10 min for final extension. PCR product was a 154 bp fragment. PCR was performed in 25 μ l of total reaction volume. The PCR amplicons on 3% agarose gel electrophoresis containing ethidium bromide showed amplified fragments. This was followed by restriction digestion of the PCR amplicons at 37°C for 15 min and heat inactivation at 65°C for 5 min with fast digest restriction enzymes NheI, BseI and BspTI (Thermo Scientific, USA) for study of Lys198Asn, 3A/4A and His323His polymorphisms respectively. The Lys and Asn alleles of Lys198Asn polymorphism correspond to 116 bp and 97 bp + 19 bp sized fragments (Figure 1 A), in 3A/4A polymorphism the fragment sizes for 3A and 4A alleles were 195 bp and 176 bp + 19 bp respectively (Figure 1 B) and in His323His (C/T) polymorphism, the alleles C and T correspond to 154 bp and 87 bp + 67 bp sized fragments respectively (Figure 1 C). The digested products were visualized on 5% agarose gel electrophoresis after staining with ethidium bromide under gel documentation system (Bio Rad, USA). Serum endothelin-1 levels were measured by ELISA using human endothelin-1 ELISA kit (Bioassay Technology Laboratory, China).

Statistical analysis. Continuous variables were tabulated as mean \pm SD, and dichotomous variables as numbers and percentages. Genotype frequencies were compared between groups by χ^2 test (2 groups) or the ANOVA test (3 groups). A significant *p*-value was < 0.05 .

Results. The baseline characteristics are given in Table I. The subjects in Group A had significantly lower body weight and body mass index ($p < 0.0001$) because of chronic rheumatic heart dis-



ease. However, hemoglobin, blood urea, serum creatinine, blood sugar levels and erythrocyte sedimentation rate (ESR) were similar in both the groups ($p = NS$). Blood anti-streptolysin O titers were < 200 IU and C-reactive protein was non-reactive in both the groups suggesting absence of acute rheumatic activity. Group A subjects had severe mitral stenosis with PH and had NYHA Class II-III dyspnea with 35% of subjects having atrial fibrillation on electrocardiogram. Echocardiography showed enlarged right atrium (RA) with markedly decreased RA fractional area change signifying RA dysfunction (Supplementary Figure S1). RV was enlarged with borderline RV systolic dysfunction (Table I). The subjects in Group B were asymptomatic healthy controls and had normal echocardiography examination.

Genotypic frequencies of Lys198Asn and His323His polymorphisms between Group A and B differed significantly, $p = 0.009$ and $p = 0.02$ respectively. The frequency of mutant homozygous Asn/Asn in Lys198Asn ($p = 0.01$) and T/T in His323His polymorphism ($p = 0.01$) was greater in Group A compared to Group B. The mutant Asn allele was frequent in Group A than in Group B. Further, for Lys198Asn polymorphism, mutant Asn/Asn genotype was significantly associated with an increased risk of PH-MVD with odds ratio (OR)

3.9 ($p = 0.002$) (Table II). Additionally, presence of either mutant homozygous Asn/Asn or heterozygous Lys/Asn genotypes when compared to wild type Lys/Lys was associated with a higher risk (OR = 1.91; $p = 0.03$). No significant association of 3A/4A polymorphism with the PH-MVD group was observed.

The combined analysis of homozygous variants Asn/Asn, 4A/4A and T/T showed that these variants in combination were more common in Group A compared to Group B ($p = 0.021$). There was no significant difference in serum endothelin-1 levels between the two groups.

Discussion. In this study recessive genotype Asn/Asn (Lys198Asn) of the ET-1 gene and T/T genotype (His323His) of ET_A gene polymorphisms were significantly more prevalent in the PH-MVD group compared to healthy controls. These polymorphisms have been previously reported in idiopathic PAH [5, 6], which has poor prognosis [11] and other cardiovascular disease states [8, 12, 13] suggesting a pathophysiological role of ET-1. Pousada *et al.* stated that T allele of Lys198Asn increases the susceptibility to idiopathic PAH and affects its progression [5]. As per their study, Lys198Asn polymorphism can cause alterations in both the functionality and expression of the protein favoring the appearance of disease [5]. Al-

Table I. Baseline characteristics and echocardiography parameters of subjects in both the study groups

Baseline parameters	Group A n = 123	Group B n = 123	P-value
Age [years]	34.2 ±9.9	35.4 ±9.3	0.331
Male : female (%)	43 (35%) : 80 (65%)	47 (38%) : 76 (62%)	0.69
Weight [kg]	49.0 ±2.6	59.6 ±9.3	< 0.0001*
Height [cm]	156.6 ±6.5	159.5 ±5.5	0.0002*
BMI [kg/m ²]	20.1 ±1.6	23.4 ±3.2	< 0.0001*
Heart rate [beats/min]	79.2 ±4.2	79.7 ±5.1	0.468
ECG [Sinus rhythm : Atrial fibrillation] (%)	80 (65%) : 43 (35%)	123 (100%) : 0 (0%)	< 0.001*
Systolic blood pressure [mm Hg]	127 ±8.5	127 ±7.3	0.962
Diastolic blood pressure [mm Hg]	78.1 ±4.3	78.7 ±3.1	0.176
Temperature [°F]	98.4 ±0.4	98.3 ±0.6	0.124
Hemoglobin [gm/dl]	12.6 ±1.8	12.9 ±1.8	0.222
ESR [mm/h]	20.0 ±15.0	17.2 ±14.3	0.141
Blood urea [mg/dl]	22.5 ±3.97	22.8 ±5.7	0.567
Serum creatinine [mg/dl]	0.77 ±0.10	0.75 ±0.09	0.055
Blood sugar [mg/dl]	98.5 ±23.5	94.1 ±14.7	0.078
Serum endothelin-1 levels [ng/l]	92.4 ±156	118 ±124.2	0.158
Left atrial diameter [cm]	4.91 ±0.95	3.05 ±0.47	< 0.001*
LV internal dimension in diastole [cm] (LVIDd)	4.39 ±0.79	4.42 ±0.47	0.740
LV internal dimension in systole [cm] (LVIDs)	2.99 ±0.67	2.97 ±0.46	0.744
Posterior wall thickness in diastole [cm]	0.84 ±0.15	0.84 ±0.13	0.626
IVS thickness in diastole [cm]	0.85 ±0.41	0.84 ±0.11	0.859
Right ventricular wall thickness in diastole [cm]	0.54 ±0.45	0.41 ±0.38	0.011*
Main pulmonary artery diameter [cm]	2.70 ±0.53	2.06 ±0.23	< 0.001*
LV ejection fraction (%)	56.88 ±6.04	58.29 ±4.35	0.038*
Right atrial major dimension [cm]	5.66 ±1.35	4.16 ±0.46	< 0.001*
Right atrial end-systolic area [cm ²]	17.78 ±8.56	11.92 ±2.41	< 0.001*
Right atrial end-diastolic area [cm ²]	13.19 ±8.48	6.21 ±1.71	< 0.001*
Right atrial fractional area change (FAC) (%)	29.76 ±14.94	47.90 ±9.58	< 0.001*
IVC diameter (inspiration) [cm]	0.84 ±0.42	0.54 ±0.13	< 0.001*
IVC diameter (expiration) [cm]	1.60 ±0.46	1.38 ±0.29	< 0.001*
RV basal diameter [cm]	3.93 ±0.91	3.18 ±0.45	< 0.001*
RV mid cavity diameter [cm]	2.89 ±0.77	2.22 ±0.39	< 0.001*
TAPSE [cm]	1.80 ±0.29	2.04 ±0.19	< 0.001*
RVOT VTI [cm] (average)	16.22 ±4.47	19.72 ±2.93	< 0.001*
Mitral valve area by planimetry [cm ²]	0.81 ±0.18	–	–
Mitral valve area by pressure half-time [cm ²]	0.80 ±0.22	–	–
Peak diastolic transmitral gradient [mm Hg]	19.24 ±6.53	–	–
Mean diastolic transmitral gradient [mm Hg]	11.74 ±4.71	–	–
Right ventricular systolic pressure [mm Hg]	70.26 ±24.02	–	–
Pulmonary artery end diastolic pressure [mm Hg]	25.96 ±10.29	–	–
Pulmonary artery mean pressure [mm Hg]	39 ±14.25	–	–

*Significant. BMI – body mass index, ESR – erythrocyte sedimentation rate, IVC – inferior vena cava, IVS – interventricular septum, LV – left ventricular, n – number of study subjects, PH-MVD – pulmonary hypertension associated with rheumatic mitral valve disease, RV – right ventricular; RVOT VTI – right ventricular outflow tract velocity time integral, TAPSE – tricuspid annular plane systolic excursion.

Table II. Frequency and odds risk estimates of ET-1 and ET_A gene polymorphism genotypes in Group A and Group B subjects

Frequency of ET-1 and ET _A gene polymorphism genotypes in Group A and Group B subjects									
Subjects	Lys/Lys	Asn/Asn	Lys/Asn	Lys allele	Asn allele	Chi square, χ^2	P-value		
Group A (n = 123)	24 (19.5%)	24 (19.5%)	75 (61.0%)	0.5	0.5	9.34	0.009*		
Group B (n = 123)	39 (31.7%)	10 (8.1%)	74 (60.2%)	0.62	0.38				
Subjects	3A/3A	4A/4A	3A/4A	3A allele	4A allele	Chi square, χ^2	P-value		
Group A (n = 123)	3 (2.4%)	36 (29.3%)	84 (68.3%)	0.37	0.63	4.86	0.09		
Group B (n = 123)	11 (8.9%)	33 (26.8%)	79 (64.2%)	0.41	0.59				
Subjects	C/C	T/T	C/T	C allele	T allele	Chi square, χ^2	P-value		
Group A (n = 123)	68 (55.3%)	6 (4.9%)	49 (39.8%)	0.75	0.25	8.4	0.02*		
Group B (n = 123)	83 (67.5%)	0 (0%)	40 (32.5%)	0.84	0.16				
Odds risk estimates of ET-1 and ET _A gene polymorphism genotypes in Group A compared to Group B*									
Gene polymorphism/ SNP ID	Genotypes compared		Group A n = 123, n (%)	Group B n = 123, n (%)	Odds ratio (OR)	95% confidence interval (CI)	P-value		
Lys198Asn (rs5370)	Asn/Asn vs. Lys/Lys		24 (19.5)	10 (8.1)	3.9	1.59–9.55	0.002*		
	Lys/Asn vs. Lys/Lys		75 (61.0)	74 (60.2)	1.65	0.90–3.00	0.10		
	Asn/Asn + Lys/Asn vs. Lys/Lys		99 (80.5)	84 (68.3)	1.91	1.07–3.44	0.03*		
Polymorphism	Genotypes compared		Group A, n = 123, n (%)	Group B, n = 123, n (%)	OR	95% CI	P-value		
His323His (rs5333)	C/T vs. C/C		49 (39.8)	40 (32.5)	1.49	0.88–2.53	0.13		
	T/T + C/T vs. C/C		55 (44.7)	40 (32.5)	1.67	0.99–2.81	0.04*		

*Significant. Lys/Lys – wild-type, Asn/Asn – homozygous variant, Lys/Asn – heterozygous variant of Lys198Asn polymorphism, 3A/3A – wild-type, 4A/4A – homozygous variant, 3A/4A – heterozygous variant of 3A/4A polymorphism, C/C – wild-type, T/T – homozygous variant, C/T – heterozygous variant of His323His polymorphism, n – number of study subjects; PH-MVD – pulmonary hypertension associated with rheumatic mitral valve disease, χ^2 – Chi square, ET-1 – endothelin-1, ET_A – endothelin receptor type A. *Odds risk ratio of 3A/4A polymorphism was not estimated as the genotype frequencies between Group A and Group B were not significant. *Odds risk ratio of TT genotype could not be determined as the control group did not have representation of mutant homozygous TT genotype.

though Lys198Asn does not structurally alter the ET-1 protein, it can affect mRNA synthesis, splicing, translation and protein folding [14]. Dzholdasbekova *et al.* noted that the presence of T allele of Lys198Asn polymorphism increases the susceptibility to systemic arterial hypertension through increased production of ET-1 [8]. These studies show that Lys198Asn polymorphism in association with other ET-1 variants or environmental factors may influence ET-1 expression [15, 16].

The conflicting results have been reported regarding the significance of 3A/4A polymorphism. In an *ex vivo* study Popowski *et al.* [17] reported that adenine insertion is related to an increasing endothelin expression (measured as pre-pro-ET-1 mRNA). However, Calabro *et al.* did not find any association of 3A/4A polymorphism in idiopathic PAH [6]. In the study by Fan *et al.* no association was found between 3A/4A polymorphism and orthostatic hypotension in both hypertensive and normotensive subjects [18]. We also did not find any association between this polymorphism and PH-MVD. Vadapalli *et al.* however reported that 3A/4A polymorphism increases the predisposition to idiopathic PAH [15].

Calabro *et al.* showed a potential link between specific genotypes in the ET_A gene and susceptibility to PAH [6]. Similarly in our study, the T/T genotype of the ET_A gene was more common in the PH-MVD group. Herrmann *et al.* stated that His323His polymorphism modulates the influence of ET-1 action on the cardiovascular system [12]. The homozygous variant genotypes of all three polymorphisms together were significantly more apparent in the PH-MVD group implying that the presence of multiple small effect polymorphisms may increase the susceptibility to develop PH.

The ET-1 levels were similar in both the groups. Others studying Lys198Asn polymorphism also reported similar ET-1 levels regardless of presence of this polymorphism. Lys198Asn polymorphism is located in the coding region of the ET-1 gene [16]. Hence it may affect the processing of preproET-1 (precursor of mature ET-1) rather than modifying the gene expression suggesting that the polymorphism effects occur at the molecular level.

This is an initial study exploring ET-1 and ET_A receptor gene polymorphisms in PH-MVD patients. Larger studies are required to corroborate our findings and also in other etiologies of PH associated with left heart disease. Further *in vitro* studies investigating the functional role of these polymorphisms in the pathogenesis of PH are warranted.

In conclusion, our preliminary study for the first time found that ET-1 and ET_A gene polymorphisms were more common in PH-MVD suggesting that they may predispose to the development of PH in

patients with RHD. These polymorphisms are likely disease modifiers, and their interactions with other genes and environmental factors may influence the development of PH.

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Conflict of interest

The authors declare no conflict of interest.

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