VERSITA
DOI: 10.2478/rrlm-2013-0031

Research article

The role of *eNOS* and *AGT* gene polymorphisms in secondary pulmonary arterial hypertension in Romanian children with congenital heart disease

Rolul polimorfismelor genelor *eNOS* şi *AGT* în hipertensiunea pulmonară secundară la copiii cu boală cardiacă congenitală din România

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Abstract

Background: Pulmonary arterial hypertension (PAH) is an incapacitating disease even in childhood, associated with very poor prognosis. The disease is characterised by endothelial dysfunction. Two of the key endothelial mediators involved in the PAH pathogenesis are nitric oxide (NO) and angiotensinogen (AGT). Purpose of the study: to evaluate the following gene polymorphisms: endothelial nitric oxide synthase (eNOS) G894T, eNOS 4b/4a, and angiotensinogen (AGT) M235T, as well as allele frequency and their association with PAH in children. Material and methods This study included 32 children with pulmonary arterial hypertension secondary to congenital heart disease, 46 children with congenital heart disease without pulmonary arterial hypertension referred to the Pediatric Cardiology Clinic Tg.Mures and 40 healthy controls. All patients underwent a complete physical with NYHA class evaluation, echocardiographic exam and eNOS (G894T, 4b/4a) as well as AGT M235T polymorphisms determination. Results The frequency of eNOS 894T allele ($p < 0.0001$) was significantly higher in patients with pulmonary arterial hypertension. Conclusions Our results advocate that there is a correlation between eNOS 894T allele and pulmonary arterial hypertension in children.

Keywords: *eNOS gene polymorphism, pulmonary arterial hypertension, congenital heart disease in children*

Rezumat

Introducere: Hipertensiunea arterială pulmonară este o afecțiune severă asociată cu prognostic sever la copil. Afecțiunea se caracterizează prin disfuncție endotelială. În patogeneza hipertensiunii arteriale pulmonare sunt implicați doi mediatori endoteliali importanți: oxidul nitric (NO) şi angiotensinogenul (AGT). Scopul lu-

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crării: evaluarea polimorfismelor G984T şi 4a/4b ale genei sintetazei oxidului nitric endotelial (eNOS) şi polimorfismului 235T al genei angiotensinogenului, precum şi frecvența alelelor şi asocierea lor cu hipertensiunea pulmonară în cardiopatiile congenitale la copii. Material şi metodă: In studiu au fost incluşi 32 copii cu hipertensiune arterială pulmonară secundară cardiopatiilor congenitale, 46 copii cu cardiopatii congenitale fără hipertensiune arterială pulmonară, internați în Clinica de Cardiologie Pediatrică Tg. Mureş şi 40 copii sănătoşi. Toți pacienții au fost evaluați clinic (cu specificarea clasei NYHA), ecocardiografic, şi au fost determinate polimorfismele G984T şi 4a/4b ale genei eNOS şi polimorfismul AGT M235T. Rezultate: A fost evidențiată o frecvență semnificativ mai mare a alelelor eNOS 894T ($p < 0.0001$), la pacienții cu cardiopatie congenitală şi hipertensiune arterială pulmonară secundară.. Concluzii: Rezultatele de față sugerează faptul că există o asociere între alelele eNOS 894T şi dezvoltarea hipertensiunii arteriale pulmonare.

Cuvinte cheie: *polimorfismul genei eNOS, hipertensiune arterială pulmonară secundară, copii cu cardiopatie congenitală*

Received: *27th March 2013;* ***Accepted:*** *14th August 2013;* ***Published:*** *12th September 2013.*

Introduction

The clinical definition of pulmonary arterial hypertension (PAH) is an increase in pulmonary artery pressure as follows: *at rest*, a mean pulmonary artery pressure greater than 25 mmHg; *on exercise*, greater than 30 mmHg. The cause of PAH is vasoconstriction of small vessels of the lung.

The influence of genes polymorphisms relevant for inflammatory and endothelial processes was studied in this disease.

Nitric oxide (NO) is involved in the relaxation of smooth muscle cells. L-arginine is a precursor of NO. Nitric oxide synthases (NOS) are enzymes with an important role in NO production (1, 2). Three types of NOS have been described: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (1, 2).

The eNOS activity is coded by the *eNOS* gene. There are three clinically relevant *eNOS* polymorphisms: T786C was established to be associated with myocardial infarction and angina (3); the variable number of tandem repeats (VNTR) in intron 4 associated with development of cardiovascular diseases (4), and G894T polymorphism within exon 7 was demonstrated to represent a risk factor for essential hypertension (7, 8) and coronary artery disease (5, 6).

Beside inhibition of vascular smooth cell migration (5, 9, 10) and growth (9, 11) the most important effect of eNOS is relaxation of vascular smooth muscle cells. Decreasing of NO production could be followed by elevated pulmonary vascular tone, with reducing of cyclic guanosine monophosphate (cGMP) production. In smooth muscle of the blood vessels cGMP has a relaxation effect. Therefore, NO has an important role in the pathogenesis of pulmonary hypertension. We hypothesized that variants of the eNOS gene may cause individual susceptibility to pulmonary hypertension by producing a lower amount of NO in endothelium.

On the other hand, the renin-angiotensin system is also important in pulmonary artery remodelling. The following genes responsible for the renin-angiotensin system activity were described: the angiotensinogen (*AGT)* gene, the angiotensin converting enzyme (*ACE*) gene, and the angiotensin II type 1 receptor (*ATIR*) gene. The angiotensinogen is encoded by *AGT* gene. Under the effect of renin it produces angiotensin I. Angiotensin II is produced from angiotensin I with angiotensin-converting enzyme action. Angiotensinogen II by contraction of vascular smooth muscle cells may determine vascular disease (12). Therefore, an association between the *AGT* gene polymorphism and the cardiovascular diseases, and hypertension, respectively, has been evaluated. Contradictory results have been published regarding the association with M235T variant in essential hypertension. Some studies found association (13, 14), while others did not (12, 15). The relation be-

tween *AGT* gene polymorphism and PAH in children has not been reported until now.

The purpose of this study is to evaluate the correlations between the gene polymorphisms of the endothelial nitric oxide synthase enzyme (*eNOS* G894T, *eNOS 4*b/4a), angiotensinogen (*AGT* M235T) and pulmonary hypertension in children.

Material and methods

Patients and control subjects

The present study was approved by the Ethics Committee of the University of Medicine and Pharmacy Tîrgu Mures and Institute of Cardiovascular Diseases Tîrgu Mures. Written informed consent was obtained from each subject's parent after they were informed about the nature and the purpose of the study. We evaluated 118 samples, 32 patients with PAH secondary to congenital heart disease (PAH group), 46 patients with CHD without PAH (CHD group) and 40 healthy controls. The PAH group included 32 Romanian children with pulmonary arterial hypertension: 17 boys and 15 girls, referred to the III Department of Pediatric Cardiology Clinic Tg. Mures, Romania. The patients were diagnosed with PAH associated to congenital heart disease (CHD). All patients underwent a complete physical examination (with determination of NYHA classes), echocardiographic evaluation, *eNOS* (G894T, 4b/4a) and *AGT* M235T polymorphisms determination. The CHD group comprised 24 boys and 22 girls without pulmonary arterial hypertension. Echocardiography was performed using an IE33 Philips echocardiograph. The control population included 40 healthy children, 22 boys and 18 girls, comparable for gender and age, without pulmonary arterial hypertension or congenital heart disease.

Genetic analysis

DNA was extracted from fresh blood sample using a ZymoBead Genomic DNA Kit (ZymoResearch). The *eNOS* G894T, *eNOS* 4b/4a and *AGT* M235T genotypes were determined by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis. The *eNOS* G894T polymorphism was performed using PCR-RFLP method of Colomba et al. (16). The *eNOS* 4a/4b polymorphism was examined by PCR amplification, as descrided previously (17). Genotyping of *AGT* M235T polymorphism was carried out using a protocol described by Russ et al., with minor modifications (18).

Statistical methods

Fisher's test was used to compare genotype distributions and allele frequencies between PAH patients and control groups. We used the gene counting method to estimate the allele frequencies.

Unpaired t test was used to compare clinical and echocardiographic severity parameters of PAH like NYHA classes, mean systolic pulmonary pressure, pulmonary arterial ejection time between different genotypes. Odds ratios (OR) were used as a measure of the associations between the *eNOS* G894T, *eNOS* 4b/4a or *AGT* M235T genotypes and PAH; the *eNOS* 894T allele, the 4a allele, or the *AGT* 235T allele, respectively, were considered to be dominant (TT + GT vs GG, aa vs. ba+bb and TT+MT vs. MM). A p-value of < 0.05 was considered as statistically significant. Statistical analyses were carried out with GraphPad InStat Demo software and Statistica.

Results

Genotype and allele frequencies of the e*NOS* G894T, e*NOS* 4b/4a and *AGT* M235T polymorphisms are presented in *Table 1*, *Table 2* and *Table 3*. The genotype frequency distributions in patients were in Hardy Weinberg equilibrium.

For the study of the e*NOS* G894T genotype (*Table 1*) we cumulated the homozygous TT and heterozygous GT because the prevalence of the TT genotype was very small in the study groups.

The frequency of the *eNOS* 894T allele was significantly higher in the PAH group than in the CHD and control group ($p < 0.05$). Therefore, the probability of developing PAH is higher in PAH group than in CHD or control group (odds

Table 1. Distribution of *eNOS* 894 G/T genotype and allele distribution in PAH group, CHD group and control group

	PAH group n= 32 (%)	CHD group n= 46 (%)	Control n= 40 (%)	PAH group vs CHD group, p value, OR, CI	PAH group vs control group, p value, OR, CI
Genotype					
GG	3 (9.37)	24 (52.17)	23 (57.5)	-	-
GT	25 (78.12)	17 (36.96)	10 (25)	p<0.0001, OR 11.7, 95% CI (3.05-45.3)	p<0.0001, OR 19.16, 95% CI (4.68-78.45)
TT	4 (12.5)	5 (10.87)	7 (17.5)	p= 0.049, OR 6.4, 95% CI (1.08-37.9)	p= 0.16, OR 4.38, 95% CI (0.78-24.46)
GT+TT	29 (90.62)	22 (47.82)	17 (42.5)	p<0.0001, OR 10.5, 95% CI (2.8-39.6)	p<0.0001, OR 13.07, 95% CI (3.41-50.15)
Allele frequency					
G allele	31 (48.4)	65 (70.65)	56 (70.0)	-	-
T allele	33 (51.6)	27 (29.35)	24 (30.0)	p= 0.007, OR 2.56, 95% CI 1.31-4.98	p= 0.01, OR 2.48, 95% CI (1.25-4.92)

Table 2. Distribution of *eNOS* 4a/b genotype and allele distribution in PAH group, CHD group and control group

	PAH group n= 32 (%)	CHD group n= 46 (%)	Control n= 40 (%)	PAH group vs CHD group p value, OR, CI	PAH group vs controls p value, OR, CI
Genotype					
aa	13 (40.62)	6 (13.0)	6 (15.0)	-	-
ab	5 (15.62)	20 (43.5)	15 (42.5)	p=0.0019, OR 0.11, 95% CI (0.03-0.45)	p=0.01, OR 0.15, 95% CI (0.03-0.62)
bb	14 (43.75)	20 (43.5)	19 (47.5)	p=0.08, OR 0.32, 95% CI (0.1-1.05)	p= 0.08, OR 0.34, 95% CI (0.1-1.11)
ab +bb	19 (59.37)	40 (86.9)	34 (85.0)	p=0.007, OR 0.22, 95% CI (0.07-0.66)	p=0.01, OR 0.25, 95% CI (0.08-0.78)
Allele frequency					
a allele	31 (48.4)	32 (34.8)	27 (33.75)	-	-
b allele	33 (51.6)	60 (65.2)	53 (66.25)	p=0.09, OR 0.57, 95% CI (0.29-1.09)	p= 0.08, OR 0.54, 95% CI (0.27-1.06)

ratio (OR) (TT + GT vs. GG) = 10.5; 95% confidence interval (CI), 2.8-39.6, p<0.0001, respectively (OR) (TT + GT vs. GG) = 13.07; 95% confidence interval (CI), 3.41-50.15, p<0.0001).

On further analysis of the e*NOS* 4b/4a polymorphism (*Table 2*), we found no significant difference regarding the frequency of the *eNOS* 4b allele between the PAH group and the control group (p = 0.08). The *eNOS* 4aa genotype was frequent in the PAH group (40.62%) and rare in the CHD group and controls. As a result, the probability of protection against PAH is lower in PAH group than in CHD or control group (odds ratio (OR) (ab + bb vs. aa) = 0.22; 95% CI 0.07-

Table 3. Distribution of *AGT* 235 T/M genotype and allele distribution in PAH group vs. CHD group and control group

	PAH group n= 32 (%)	CHD group n= 46 (%)	Control n= 40 (%)	PAH group vs CHD group p value, OR, CI	PAH group vs controls p value, OR, CI
Genotype					
MM	13 (40.6)	22 (47.8)	19 (47.5)	-	-
MT	11 (34.4)	16 (34.8)	14 (35.0)	p=0.79, OR=1.16 95% CI (0.41-3.25)	p=1.0, OR=1.15, 95% CI (0.39-3.31)
TT	8 (25.0)	8 (17.4)	7 (17.5)	p=0.54, OR=1.69 95% CI (0.51-5.59)	p=0.53, OR= 1.67, 95% CI (0.48-5.74)
TT + MT	19 (59.4)	24 (52.2)	21(52.5)	p=0.64, OR=1.34, 95%CI (0.53-3.33)	p=0.63, OR =1.32, 95% CI (0.51-3.38)
Allele frequency					
M allele	37 (57.8)	60 (65.2)	52 (65.0)	-	-
T allele	27 (42.2)	32 (34.8)	28 (35.0)	p=0.4, OR=1.37, 95% CI (0.71-2.63)	p=0.39, OR=1.35, 95% CI (0.69-2.66)

Table 4. Correlation between clinical and echocardiographic parameters and *eNOS* 894 genotypes

	GG (mean ± SD)	TT+GT (mean± SD)	p
NYHA	3.33±0.57	2.90±0.73	0.37
PAP_s	100 ± 34.64	108 ± 12.73	0.52

Table 5. Correlation between clinical and echocardiographic parameters and *eNOS* 4 genotypes

	aa (mean± SD)	ab+bb (mean± SD)	p
NYHA	2.88±0.78	2.90±0.56	0.97
PAP_s	107.77±13.25	101.66±19.84	0.45

0.66, p=0.007, respectively (OR) (ab+bb vs. aa) = 0.25; 95% CI, 0.08-0.78, p=0.01).

There were no significant differences between groups in terms of the *AGT* M235T genotype and allele frequencies (*Table 3*). Therefore, no significant correlation was observed between the *AGT* T allele and PAH (OR (TT +MT vs. MM) = 1.34, 95% CI 0.53-3.33).

Analyzing the relation between PAH severity, evaluated by NYHA functional class, and mean pulmonary arterial pressure (systolic) - PAP_s and gene polymorphism in PAH patients, we observed no significant differences between TT +GT genotype vs. GG genotype of e*NOS* 894 (*Table 4*), aa vs. ba+bb genotype of *eNOS* 4b/4a, respectively (*Table 5*).

Discussion

The e*NOS* has an important role in the pulmonary vascular tone. Some variants of the *eNOS* genes were described. In the current study, we analyzed the relationship between *eNOS G894T* polymorphism and PAH in children. We observed a significantly higher frequency of the *eNOS* 894 T allele and TT +GT genotype in the PAH patients compared to CHD patients and controls.

Our findings suggest that patients with T allele may have a decreased NO production, in comparison with patients with G allele. Consequently, the lower production of NO could determine elevated pulmonary vascular tone, by decreasing the cyclic guanosine monophosphate (cGMP) production. In the smooth muscle of the blood vessels cGMP has a relaxation effect. Therefore, T allele could be involved in the onset of pulmonary hypertension through decreased levels of NO production by the endothelium.

Data about association of the *eNOS* 894T allele and PAH are different. Some studies conducted on adult patients with PHT associated to chronic obstructive pulmonary disease (COPD) revealed a positive correlation between the *eNOS* 894G/T genotype and PAH (19, 20) while others did not (21, 22).

In the present study, the incidence of GG genotype and G allele was higher in the controls and CHD cases than in patients with pulmonary hypertension. That may suggest that G allele may be protective against PAH in children.

We also analyzed the association between *eNOS* 4b/4a polymorphism and PAH. Some of the available data on *eNOS* 4a/b polymorphism suggest that the presence of the *eNOS* 4aa genotype is associated with an increased risk of acute myocardial infarction, by affecting the NO synthesis at endothelium level (23-25). Furthermore, several studies demonstrate that presence of *eNOS* 4a allele predisposes to erectile dysfunction (26) or essential hypertension (27). In the study made by Yildiz et al. on PAH patients with COPD, *eNOS* 4bb genotype has been associated with PAH in addition to hypoxemia (22).

Our study reveals no association of *eNOS* 4b/4a polymorphism with PAH. The frecuency of the "a" allele was higher in patients with PAH compared with the CHD and in the control group. In the literature, the "a" allele has been associated with lower detection of NO metabolites (4), which could explain the modified smooth muscle of the blood vessels relaxation in patients with PAH. Our findings did not support the association between the "a" allele and PAH.

In the present study, the incidence of b allele was higher in the CHD and in the control group than in patients with PAH. These finding may suggest that b allele of the *eNOS* 4b/4a gene could protect against PAH.

We did not find significant reports in the literature regarding *eNOS* 4b/4a polymorphism and PAH in children, therefore further studies are necessary.

The *eNOS* polymorphism association with PAH also has a practical implication in the therapy of PAH which involves NO synthesis in the lung. Until present, only a few studies are available (mostly animal models), with gene therapy (28, 29) or administration of L-arginine (30), with positive results.

However, large clinical trials will be necessary in order to test these therapies in patients with PAH.

We also analyzed the association between *AGT M235T* polymorphism and PAH. We found no significant difference in genotype and allele frequencies between PAH group and CHD group, respectively between PAH and controls.

Only a few data are available. Solari et al. report that *AGT* TT genotype was significantly higher in persistent pulmonary hypertension associated with congenital diaphragmatic hernia in newborns compared with controls (31). Gatti et al. (32) demonstrated the interactions among polymorphisms of the angiotensinogen (*AGT*) gene and conventional risk factors may affect the hypertension occurrence. In contrast, a recent study showed no associations between the *AGT* M235T polymorphisms and blood pressure (33). Further investigations are necessary.

To our knowledge, this is the first study that evaluates the correlation between *eNOS* G894T, *eNOS* 4b/4a *AGT* M235T gene polymorphisms and pulmonary hypertension in romanian children. Because the study group was quite small for a genetic association study, the present results

should be confirmed by future studies performed on larger number of patients with PAH.

Conclusions

In children with pulmonary artery hypertension secondary to congenital heart disease the *eNOS* 894T allele was significantly associated. We could not conclude the same in the case of *eNOS* 4a and *AGT* M235T polymorphisms in PAH. We could not find any correlation between gene polymorphism and severity of the disease. More studies will be useful to find additional polymorphism association with PAH.

Acknowledgement

The current study was carried out in the research program project MAMI no 41-042/2007 financed by the Romanian Ministry of Education, Research and Youth.

Abbreviations

ACE - angiotensine converting enzyme
AGT – angiotensinogen
ATIR - angiotensin II type 1 receptor
bp - base pairs
cGMP - cyclic guanosine monophosphate
CHD - congenital heart defects
COPD - chronic obstructive pulmonary disease
DNA - deoxyribonucleic acid
eNOS – endothelial nitric oxide synthases
Glu298Asp - acid by aspartic acid at codon 298
iNOS - inducible NOS
nNOS - neuronal NOS
NO - nitric oxide
NOS - nitric oxide synthases
NYHA - New York Heart Association functional class
OR- odds ratio
PAH - pulmonary arterial hypertension
PAET - pulmonary arterial ejection time
PCR - polymerase chain reaction
PAP_s- pulmonary arterial pressure (systolic)
RFLP - restriction fragment length polymorphism
VNTR - variable number of tandem repeats

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