Genetic Polymorphisms of the Serotonin Transporter, but Not the 2a Receptor or Nitric Oxide Synthetase, Are Associated with Pulmonary Hypertension in Chronic Obstructive Pulmonary Disease

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Key Words

Chronic obstructive pulmonary disease • Genetic • Polymorphism • Pulmonary hypertension • Serotonin • Serotonin transporter

Abstract

Background: Pulmonary hypertension (PH) is prognostically important in chronic obstructive pulmonary disease (COPD). Since PH only weakly correlates with hypoxemia, other factors must play a role. **Objective:** To investigate whether polymorphisms of the serotonin transporter (5HTT), serotonin-2a receptor (5HTR2a) and endothelial nitric oxide synthetase (eNOS) are related to PH in COPD. **Methods:** In 59 COPD patients who underwent right heart catheterization, 6-min walking distance, NYHA functional class, pulmonary function tests, blood gases and 5HTT, 5HTR2a and eNOS (4ab and T298C) polymorphisms were determined. **Results:** Forty-nine COPD patients in NYHA functional class III–IV were included. Ten were excluded due to comorbid causes of PH (mainly chronic thromboembolic). PH (mPAP $\geq$ 25 mm Hg) was present in 55% and usually mild, but out of proportion (mPAP $\geq$ 40 mm Hg) in 12%. Patients with PH had significantly higher frequencies of the 5HTT-L-allele (52%) com-

pared to individuals without PH (36%), and LL homozygote patients had more severe PH. In patients with out-of-proportion PH, the L-allelic frequency was even 75%. We found no association of 5HTR2a and eNOS polymorphism with PH in COPD. **Conclusions:** In this COPD cohort we confirm that PH is frequent and usually mild, but out of proportion in a subgroup. We found a significant association of the L-allelic variant of 5HTT with PH overall and especially in out-of-proportion PH. These findings may point towards a role of the serotonin system in COPD-PH and warrant further studies.

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Introduction

Chronic obstructive pulmonary disease (COPD) is one of the leading causes of morbidity in the Western world, mainly due to cigarette smoking. COPD leads to pulmonary hypertension (PH) in some subjects, but not in others [1]. The development of PH in COPD has been mainly attributed to alveolar hypoxia, which leads to pulmonary vasoconstriction, vessel remodeling and increased pulmonary vascular resistance [1]. Long-term oxygen therapy improves the prognosis of patients with

COPD and stabilizes pulmonary artery pressure, and PH is the strongest prognostic factor for COPD patients under long-term oxygen therapy, independent of the severities of airflow limitation or hypoxemia [2]. But not every hypoxemic COPD patient develops PH and the degree of PH only weakly correlates with hypoxemia; therefore, other predisposing factors must be postulated, most probably genetic ones. In most COPD patients, PH is mild [3, 4], however, a subgroup develops severe and rapidly progressive PH resembling other forms of vasoproliferative pulmonary vessel disease, such as idiopathic pulmonary arterial hypertension (PAH) [5, 6]. Recognition of a genetic susceptibility to PH in COPD patients would have relevant implications for the pathogenetic understanding, and potentially early diagnosis and treatment. Important factors contributing to the pathogenesis of PH in various diseases are nitric oxide (NO), serotonin, endothelin and prostaglandins.

Polymorphism of the serotonin transporter (5HTT) determined the severity of PH in hypoxemic French and British patients with COPD [7]. However, this association could not be found in idiopathic or familial PAH [8]. Serotonin leads to pulmonary vasoconstriction via its 2a and b receptors (5HTR2a and b). The T102C polymorphism of 5HTR2a has been associated with arterial hypertension, myocardial infarction and psychiatric disorders [9, 10], but has not been investigated in PH. NO plays a key role in systemic and pulmonary endothelial dysfunction [11]. Polymorphism in the endothelial NO synthetase (eNOS) has been associated with the development of arteriosclerosis and high altitude pulmonary edema in Japanese and Indian populations [12, 13]. However, eNOS polymorphisms were not related to pulmonary artery pressures at altitude in Caucasians [14].

The aim of the present study was to investigate the association of 5HTT, 5HTR2a and eNOS polymorphisms with PH confirmed by right heart catheterization (RHC) in COPD patients.

Material and Methods

Study Subjects

Outpatients with COPD diagnosed according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines [15–17] who had RHC and pulmonary function testing (PFT) within 2 weeks were asked to participate upon written informed consent. Patients were scheduled for RHC according to their treating physicians' judgment (mostly physicians not involved in the study inclusion) mainly because they had severe exertional dyspnea (often more than expected from their PFT)

and/or before lung volume reduction surgery. The study was approved by the local ethics review board. Fifty-nine COPD patients (25 females) at a mean age of 68 ± 8 years were assessed. Ten patients were excluded because a thorough investigation revealed other reasons for PH: 6 patients had chronic thromboembolic PH confirmed by pulmonary angiography, 3 patients had postcapillary PH due to left heart disease and 1 patient was diagnosed with scleroderma. Thus, data from 49 COPD patients were analyzed.

Study Assessments

Patients willing to participate had their blood lymphocytes genetically analyzed. Demographics including age, sex, height, weight, body mass index, smoking history, drug use, NYHA class and 6-min walking distance were obtained, all within 2 weeks of RHC and PFT. Swan-Ganz catheters were placed by standard techniques and cardiac output was assessed by thermodilution (Baxter/Edwards, Deerfield, Ill., USA). Hemodynamic data from RHC (mean pulmonary artery pressure, mPAP; cardiac index; pulmonary vascular resistance), arterial and mixed venous blood analysis (oxygen and carbon dioxide pressures and saturation), and data from PFT (forced expiratory volume in 1 s; forced vital capacity; total lung capacity; residual volume; single breath transfer factor for carbon monoxide; all given as percent predicted) were noted. We defined mPAP according to the WHO as ≥ 25 mm Hg [18] and out-of-proportion PH in COPD as mPAP > 40 mm Hg [19].

Genetic Analysis

EDTA-supplemented blood samples were extracted with the QIAamp® DNA Mini Kit (Qiagen AG, Basel, Switzerland). Primers were designed with the OLIGO 4.0 software (Medprobe, Oslo, Norway) and purchased from Microsynth (Balgach, Switzerland).

For detection of the Glu298Asp polymorphism in eNOS, a 25- μ l tetraprimer PCR reaction was developed using the Amplitaq Gold™ System (Perkin-Elmer Corp., Hünenberg, Switzerland); 0.1 μ M of primers NOSU6749 (5'-AGGGGAGCCTCGGTGAG-ATAAAG-3') and NOSL7115 (5'-GCAGGCCCTTCTTGAGAGG-3'), as well as 0.14 μ M of primers NOSU6986G (5'-TGCA-GGCCCCAGATGAG-3') and NOSL7002T (5'-AAGGAAGAG-TTCTGGGGGA-3') were added to the standard amplification mixture with 100 ng genomic DNA and the following cycling parameters were used: 10 min at 94°C, 10 cycles (first set) of 94°C for 30 s, 60°C for 30 s, 72°C for 60 s, 25 cycles (second set) of 94°C for 30 s, 53°C for 30 s, 72°C for 60 s, and a final extension of 7 min at 72°C [20, 21].

Genotyping of the 27-bp repeat in intron 4 of the eNOS gene (eNOS4a/b) was performed according to Wang et al. [22] with minor modifications. The allele with 4 repeats was designated 4a and the longer allele with 5 repeats was designated 4b. The 44-bp insertion/deletion polymorphism in 5HTT was analyzed using 0.5 μ M of primers 5HTT U (5'-CTC TGA ATG CCA CCT AAC-3') and 5HTT L (5'-GGA TTC TGG TGC CAC CTA GAC-3') as well as the Expand Long Template Kit from Roche Molecular Biochemicals (Rotkreuz, Switzerland). PCR resulted in a long amplification product of 457 bp and in a short amplification product of 414 bp for the 2 alleles which were separated on agarose gels.

Table 1. Patient characteristics

	Numbers
Total number of patients	49 (100)
Females/males	18/31 (37/63)
Age, years	68 ± 8
Body mass index	24 ± 4
Smokers	44 (90)
Current/past	5/39 (11/79)
Mean pack years smoked	52 ± 30
NYHA functional class III-IV	21/28 (43/57)
6-min walking distance, m	298 ± 125
COPD severity by GOLD stage I/II/III/IV	3/12/20/14 (6/25/41/28)
Pulmonary function tests ¹	
FEV ₁ , % predicted	41 ± 21
FVC, % predicted	74 ± 25
FEV ₁ /FVC ratio	0.48 ± 0.12
Total lung capacity, % predicted	120 ± 25
Residual volume, % predicted	196 ± 74
Carbon monoxide diffusion capacity % predicted	42 ± 22
Pulmonary hemodynamics (invasive)	
mPAP, mm Hg	28 ± 11
Cardiac index, l/min/m ²	2.5 ± 0.6
Pulmonary vascular resistance, dyn·sec·m ⁻⁵	378 ± 222
Right atrial pressure, mm Hg	6.5 ± 5
Pulmonary artery occlusion pressure, mm Hg	10 ± 4
Blood gas analysis	
Arterial oxygen saturation, %	90 ± 6
Arterial oxygen partial pressure, kPa	7.9 ± 1
Arterial carbon dioxide partial pressure, kPa	5.4 ± 0.8
Mixed venous oxygen saturation, %	64 ± 8

Figures in parentheses are percentages. FEV₁ = Forced expiratory volume in 1 s; FVC = forced vital capacity.

¹ Percent predicted by the European Society of Coal and Steel.

For detection of the T102C polymorphism in the *HTR2A* gene, a LightCycler assay was developed. Four oligonucleotides were added to the LightCycler DNA Master Hybridization Probes mixture (Roche Molecular Biochemicals) at the following final concentrations: 0.5 μM of HTR2A U135 (5'-CTG GCT TAG ACA TGG ATA TT-3') and HTR2A L336 (5'-ATG AAG TAA GGA GAG ACA CG-3'), 0.4 μM of Anchor T102C (5'-LCRed640-TTA CTG TAG AGC CTG GTG TCA TCA TTT-ph-3') and 0.2 μM of Sensor T102C (5'-GTT AGC TTC TCC AGA GTT AAA GTC-FL-3'). The final magnesium concentration was 2 mmol/l, and 100 ng DNA were added in a total volume of 20 μl. The following temperature protocol was applied: 95°C for 45 s, followed by 50 cycles of 95°C for 0 s, 58°C for 10 s, and 72°C for 9 s with a ramping rate of 20°C/s. Melting curve analysis was performed from 40°C to 85°C at a linear rate of 0.1°C/s [23].

Fig. 1. Percent of COPD patients with mPAP above different thresholds (n = 49).

Statistical Analysis

Results are expressed as means ± SD. The Mann-Whitney U test and analysis of variance were used to compare means and Fisher's exact test was applied to compare frequencies between groups. Differences showing $p < 0.05$ were considered statistically significant. Statistical analysis was performed using SPSS version 15.0 software package.

Results

Patients' Characteristics and Clinical Presentation

The baseline characteristics of the 49 patients included are shown in table 1. All patients were in NYHA classes III and IV and were severely exercise limited (mean 6-min walking distance 298 ± 125 m). COPD severity according to GOLD varied, all but 3 were moderately to severely affected. Most patients had a history of cigarette smoking. PH was present in 27 (55%) of the patients. RHC was performed during a stable condition without any signs for COPD exacerbation and on room air. Overall, PH was usually mild, however, 6 (12%) patients had out-of-proportion PH (fig. 1). Patients with PH had less severe airway obstruction and lung hyperinflation compared to patients without PH (table 2). We found a weak correlation between the degrees of hypoxemia and PH (fig. 2), but an even weaker inverse correlation between the severities of airway obstruction and PH.

Table 2. Characteristics of COPD patients with and without PH (mPAP $\geq$ 25 mm Hg)

	Without PH	With PH
Total number of patients	22 (45)	27 (55)
Females/males	5/17 (23/77)	13/14 (48/52)
Age, years	66 $\pm$ 7	70 $\pm$ 8
Body mass index	24 $\pm$ 4	24 $\pm$ 5
Smokers	21 (95)	22 (81)
Current/past	1/20 (5/95)	4/18 (18/78)
Mean pack years smoked	55 $\pm$ 28	49 $\pm$ 33
NYHA functional class III /IV	11/11 (50/50)	10/17 (37/63)
6-minute walking distance, m	268 $\pm$ 129	323 $\pm$ 118
COPD severity by GOLD stage I/II/III/IV	0/2/11/9 (0/9/50/41)	3/10/9/5 (11/40/33/16)
Pulmonary function tests ¹		
FEV ₁ , % predicted	30 $\pm$ 11	51 $\pm$ 23**
FVC, % predicted	66 $\pm$ 22	81 $\pm$ 25*
FEV ₁ /FVC ratio	0.46 $\pm$ 0.12	0.50 $\pm$ 0.11
Total lung capacity, % predicted	128 $\pm$ 23	113 $\pm$ 24*
Residual volume, % predicted	228 $\pm$ 63	167 $\pm$ 71**
Carbon monoxide diffusion capacity, % predicted	39 $\pm$ 15	44 $\pm$ 27
Pulmonary hemodynamics		
mPAP, mm Hg	19 $\pm$ 4	35 $\pm$ 10**
Cardiac index, l/min/m ²	2.5 $\pm$ 0.5	2.5 $\pm$ 0.7
Pulmonary vascular resistance, dyn $\cdot$ sec $\cdot$ m ⁻⁵	238 $\pm$ 98	473 $\pm$ 223**
Right atrial pressure, mm Hg	5 $\pm$ 4	8 $\pm$ 5
Pulmonary artery occlusion pressure, mm Hg	9 $\pm$ 4	10 $\pm$ 4
Blood gas analysis		
Arterial oxygen saturation, %	92 $\pm$ 4	88 $\pm$ 7
Arterial oxygen partial pressure, kPa	8.1 $\pm$ 1.0	7.6 $\pm$ 1.1
Arterial carbon dioxide partial pressure, kPa	5.4 $\pm$ 0.8	5.4 $\pm$ 0.8
Mixed venous oxygen saturation, %	66 $\pm$ 5	63 $\pm$ 9

Data given as numbers (%) or means $\pm$ SD. * p < 0.05; ** p < 0.01.

¹ Normal values obtained from the European Society of Coal and Steel.

Table 3. Frequencies of the serotonin transporter polymorphisms in COPD patients (overall and by the presence of PH)

COPD patients	Genotypes			Allele frequencies, %	
	SS	SL	LL	S	L
Overall (n = 49)	13 (27)	28 (57)	8 (16)	63	37
No PH (n = 22)	8 (36)	12 (55)	2 (9)	64	36
With PH (n = 27)	5 (19)	16 (59)	6 (22)	48	52*
Out-of-proportion PH (n = 6)	0	3 (50)	3 (50)	25	75**

Figures in parentheses are percentages. * p < 0.05 for COPD with versus without PH; ** p < 0.05 for out-of-proportion PH (defined as mPAP > 40 mm Hg) versus without PH and/or all others.

Fig. 2. mPAP plotted against arterial oxygen pressure (PaO₂).

Fig. 3. mPAP (mm Hg) by 5HTT allelic variants.

Table 4. Frequencies of the tested serotonin-2a receptor and NO synthetase polymorphisms in COPD patients (overall and by the presence of PH)

COPD patients	Type of polymorphism	Genotypes			Allele frequencies, %	
	5HTR2a	TT	TC	CC	T	C
Overall (n = 49)		8 (16)	30 (61)	11 (23)	44	56
No PH (n = 22)		5 (23)	12 (54)	5 (23)	50	50
With PH (n = 27)		3 (11)	18 (67)	6 (22)	48	52
	eNOS4a/b	bb	ab	aa	b	a
Overall (n = 49)		31 (63)	17 (35)	1 (2)	84	16
No PH (n = 22)		14 (64)	8 (36)	0	82	18
With PH (n = 27)		17 (63)	9 (33)	1 (4)	80	20
	eNOS298	Glu/Glu	Glu/Asp	Asp/Asp	Glu	Asp
Overall (n = 49)		25 (51)	22 (45)	2 (4)	78	22
No PH (n = 22)		11 (50)	9 (41)	2 (9)	70	30
With PH (n = 27)		14 (52)	13 (48)	0	76	24

Figures in parentheses are percentages.

Genetic Analysis

Frequencies of the 5HTT, 5HTR2a and eNOS (4a/b and 298) genotypes are shown in table 3 and 4 for all COPD patients separated by the presence of PH. COPD patients with PH were significantly more likely to carry the LL genotypes of the 5HTT gene compared to COPD without PH ($p = 0.03$). Three of the 6 patients with PH >40 mm Hg were homozygote for the LL variant and 3 heterozygote, the allelic frequency of the L variant of the 5HTT was herewith highly overrepresented in this subset ($p < 0.001$; table 3). Overall, patients with the L-allelic variant of the 5HTT tended to have higher pulmonary artery pressure (fig. 3) and vascular resistance. With mean mPAPs of 33 ± 6 , 29 ± 2 and 23 ± 2 mm Hg (95% confidence intervals 19–26, 25–33 and 21–47 mm Hg, respectively) for the LL, LS and SS genotypes, respectively, our results were in close accordance with the study of Eddahibi et al. [7], which found mPAPs of 33 ± 6 , 29 ± 2 and 23 ± 2 mm Hg, respectively, in their French collective ($n = 67$). However, our study did not reach statistical significance due to the smaller sample size ($n = 49$). Retrospective power calculation revealed that for significance we would have needed a sample size of 48 (actually 41) and 30 (actually 21) to detect the presently found mPAP differences of 6 and 12 mm Hg, respectively, between the SS and LS and SS and LL allelic groups, respectively. If looking at the mPAP difference between the SS allele carriers versus carriers of any L allele (30 ± 5 mm Hg), the difference just failed statistical significance ($p = 0.057$). Conversely, patients homozygote for the S-allelic variant had significantly more severe airway obstruction compared with patients carrying the L allele ($p = 0.04$). There were no other significant differences of any investigated allele variant between COPD patients with and without PH, and none of the measured parameters differed according to any investigated genotype.

Discussion

In this study we confirm that even in a selected COPD collective scheduled for RHC, PH is usually relatively mild, only weakly related to hypoxemia and not related to the degree of airway obstruction [5]. However, similar to others we found that a subgroup of COPD patients (12%) presents with out-of-proportion elevated pulmonary artery pressure (>40 mm Hg) similar to the features seen in (idiopathic) PAH [1, 5, 6, 24]. In this group, we found a marked overrepresentation of the 5HTT long allelic variant (L). Hence, our data corroborate the findings

of Eddahibi et al. [7] that the homozygote LL genotype of 5HTT is associated with PH in COPD patients, suggesting a role of the serotonin system in the pathogenesis of PH in COPD.

The pathogenesis behind PH in COPD is incompletely understood. However, particularly in the development of out-of-proportion PH, genetic variants may play a role. Germline mutations in the bone morphogenetic protein receptor 2 play an important pathogenetic role in familial and sporadic, but not in other forms of PAH, such as those associated with HIV infection or scleroderma [25, 26]. However, bone morphogenetic protein receptor 2 mutations have not been studied in COPD-PH so far. Eddahibi et al. [7] showed an association of the long allelic variant of the 5HTT with the degree of PH in hypoxic COPD patients. Consistently, we found an increased frequency of the L allele in COPD patients with PH in our cohort. Strikingly, the L-allelic variant was markedly overrepresented in the 6 patients with out-of-proportion PH. In accordance, patients with the L-allelic variant had higher mPAPs and PVRs compared to patients with the S variant (table 3; fig. 3). These findings support a pathogenetic role of the serotonin system in the development of PH in COPD and indicate the need for further studies in this field. 5HTT has been shown to be a key determinant of pulmonary vessel remodeling by mediating proliferation of pulmonary artery smooth muscle cells, directly related to 5HTT gene expression [27–30]. Cells homozygous for the L-allelic variant have been shown to express 2-fold more 5HTT RNA compared to cells carrying the S-allelic variant. This difference was even more pronounced under hypoxic conditions. This may explain why the 5HTT polymorphism seems only related to PH in COPD, a disorder closely associated with hypoxemia, while there does not seem to be a relation with other forms of PH [8, 31]. In line with a previous study [7], we also found more severe airway obstruction in patients homozygous for the S-allelic variant. One speculative explanation for this difference might be that patients with the L-allelic variant become symptomatic due to higher pulmonary artery pressure earlier in the disease, when they have less airflow limitation. Given the prognostic impact of PH in COPD, early identification and especially further studies assessing therapeutic opportunities for PH in COPD are mandatory. One approach could be to investigate the effect of 5HTT inhibitors on PH in selected COPD patients, as they have been shown to inhibit the *in vitro* proliferative response of smooth muscle cells when given orally to experimental animals exposed to hypoxia [29, 32].

Agonists of the 5HT₂ receptors, such as fenfluramine, are associated with an increased risk for PH [33], and 5HT₂ receptor antagonists are currently investigated as treatment options in PH [34, 35].

Although usually mild, PH is the most important terminator of outcome in COPD [36]. It has been shown that PH in COPD is only moderately linked to hypoxemia and even less to the degree of airflow obstruction. Therefore, genetics, environmental and comorbid factors must play a pathogenetic role [3, 4, 36, 37]. Severe PH in COPD is rare and may indicate a clinically and pathogenetically distinct disease [6, 18]. In our collective, mild PH was present in up to 84% of patients considering a threshold mPAP ≥ 20 mm Hg (as widely used in the respiratory literature) [3, 4, 38], and in 55% if defined by the WHO (mPAP ≥ 25 mm Hg). This is in line with previously published articles [18, 39]. However, in analogy to previous reports [18], severe PH (mPAP >40 mm Hg) was found in 12% of the present COPD cohort. Whether this out-of-proportion PH in COPD reflects a pathogenetically distinct COPD subset and whether this subset might be amenable to specific PH therapy warrants further studies. It is also important to mention that other reasons for PH may be found in COPD patients. In our study we excluded 10 (27%) out of totally 37 COPD patients with PH for comorbid reasons. Many of them suffered from chronic thromboembolic PH, a potentially surgically accessible disease [40], others had diastolic left heart dysfunction and 1 patient was diagnosed with scleroderma. The diagnosis of other PH-associated comorbid conditions in COPD patients is important, as specific therapies for some PH classes may considerably improve the patients' quality of life and prognosis.

The development of PH in COPD is mainly attributed to alveolar hypoxia [4]. In line with previous studies, we

found a mild association between hypoxemia and PH severity [7, 18]. As for the degree of airway obstruction, the association with PH severity was even weakly inverse in our collective [7]. This may partly be explained by selection, as we only included COPD patients scheduled for RHC and therefore possibly had more patients with out-of-proportion PH, a subset possibly mimicking PAH [18].

The present study has several limitations. First, we studied a selected COPD population scheduled for RHC. It must therefore be assumed that PH might be overrepresented in this collective due to selection bias. It could thus be that the clinical and genetic differential characteristics of COPD patients would be different in a more general COPD collective with milder disease. However, as specific therapy for PH in COPD is currently lacking and as there is no evidence-based study demonstrating the clinical value of RHC in advanced COPD, it would be ethically difficult to search for PH using RHC in a general COPD population outside a proper study protocol. A second limitation is the relatively small sample size. However, for the above mentioned reasons, RHC is not performed very often in COPD patients. Third, we herein investigated only some genetic variants of the serotonin and NO system. It could well be true that other, herein not accounted, genetic variants play a role in COPD-associated PH.

In summary, our study confirms previous results that the long allelic variant of the serotonin transporter is associated with PH in COPD, especially in those with out-of-proportion pulmonary artery pressures. Our data support a possible role of the serotonin system in the pathogenesis of COPD-associated PH and will help to direct future research in the field.

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