Sequence Variants in *BMPR2* and Genes Involved in the Serotonin and Nitric Oxide Pathways in Idiopathic Pulmonary Arterial Hypertension and Chronic Thromboembolic Pulmonary Hypertension: Relation to Clinical Parameters and Comparison with Left Heart Disease

Silvia Ulrich^a Justyna Szamalek-Hoegel^b Martin Hersberger^a Manuel Fischler^a
Jesus Solera Garcia^b Lars C. Huber^a Ekkehard Grünig^c Bart Janssen^b
Rudolf Speich^a

^aClinic of Internal Medicine, University Hospital of Zurich, Zurich, Switzerland; ^bInstitute of Human Genetics, University of Heidelberg, and ^cThoraxklinik Heidelberg, Heidelberg, Germany

For editorial comment see p. 274

Key Words

Bone morphogenetic protein • Chronic thromboembolic pulmonary hypertension • Genetics • Idiopathic pulmonary arterial hypertension • Nitric oxide • Pulmonary hypertension • Serotonin

Abstract

Background: Idiopathic pulmonary arterial hypertension (IPAH) and chronic thromboembolic pulmonary hypertension (CTEPH) share important pathogenic and clinical features. *BMPR2* mutations are important in the pathogenesis of IPAH, but little is known about the genetic background in CTEPH. **Objective:** To search for mutations and polymorphisms in genes involved in the *BMPR2*, serotonin and nitric oxide pathways possibly associated with pulmonary and cardiac disorders in IPAH and CTEPH. **Methods:** In a cohort of Swiss patients with IPAH (n = 16) and CTEPH (n = 16), and in 24 controls with left heart disease without PH, polymorphisms in the *BMPR2*, *5-HTT*, *5-HTT-2A* and *eNOS* genes were analyzed and correlated with various clinical, functional and

hemodynamic parameters. **Results:** We found a *BMPR2* missense mutation in a patient with coronary artery disease (CAD) without PH but no *BMPR2* mutations in our collective with late-onset sporadic PH. In patients with polymorphic variants of the *BMPR2* gene, the number of blood platelets and oxygen saturation were increased. The c.600A→C synonymous variant was associated with worse exercise capacity and decreased quality of life in PH. We found no significant differences for any measured parameter according to the *eNOS*, *5-HTT-2A* and the *5-HTT* polymorphisms, although there was a higher allelic frequency of the *5-HTT* long variant in IPAH than in CTEPH and controls. **Conclusion:** Our first report of a *BMPR2* mutation in a patient with CAD without PH is interesting and warrants further investigation. Our study may reflect the clinical status and genetic background in a typical PH cohort as seen in a single tertiary care referral center.

Copyright © 2009 S. Karger AG, Basel

S.U. and J.S.-H. contributed equally to this work.

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2009 S. Karger AG, Basel
0025-7931/10/0794-0279\$26.00/0

Accessible online at:
www.karger.com/res

Silvia Ulrich, MD
Department of Medicine and Pulmonology, University Hospital of Zurich
Rämistrasse 100
CH-8091 Zurich (Switzerland)
Tel. +41 44 255 43 62, Fax +41 44 255 44 15, E-Mail silvia.ulrich@usz.ch

Introduction

Idiopathic pulmonary arterial hypertension (IPAH) and chronic thromboembolic pulmonary hypertension (CTEPH) belong to the group of pulmonary vascular disorders classified by the World Health Organization and defined by mean pulmonary artery pressure ≥ 25 mm Hg at rest [1]. Structural and functional changes in the vascular wall and thrombus formation are the main factors responsible for increased pulmonary vascular resistance in patients with pulmonary hypertension (PH) [2, 3]. The contribution of each of these factors is thought to be different among the variable underlying causes of PH [4]. Despite these presumptive distinctions, differently classified PH such as IPAH and CTEPH share important pathogenetic and clinical features. Favorable effects of vasodilator and antiproliferative agents are not only seen in IPAH but also in CTEPH [5–9]: both disorders share acute vasodilator properties [10] and a comparable pulmonary vessel histology at advanced disease [11].

Since the description of heterozygous mutations in the bone morphogenetic protein type II receptor (*BMPR2* gene) in patients with PH [12, 13], it has become widely accepted that alternations in the transforming growth factor (TGF)- β pathway, to which *BMPR2* belongs, play an important role in PH development [14–16]. Although *BMPR2* mutations are the underlying cause in $>70\%$ of familial and 11–26% of sporadic IPAH cases [12–14, 17, 18], only a minority of patients with *BMPR2* mutations develop clinical PH due to markedly reduced penetrance [14, 19, 20].

Hence, there must be other factors which, in addition to or in combination with the *BMPR2* mutations, play a pathogenetic role in the development of PH. These second or third pathogenetic ‘hits’ might be other genetic or environmental factors, or both interplaying with each other. In this study, we aimed to investigate some genetic factors possibly contributing to the pathogenesis of the two major forms of PH (IPAH and CTEPH).

Beside some rare cases of *BMPR2* mutations found in patients with congenital heart disease-associated PH [21] and pulmonary veno-occlusive disease [22, 23], little is known about *BMPR2* mutations in other forms of PH, e.g. CTEPH. A recent British study characterizing CTEPH patients did not find *BMPR2* mutations in >100 subjects [24].

Among other genetic factors, which were suggested to affect the pathogenesis of PH, there are polymorphisms in the serotonin transporter (*5-HTT*) or receptor (*5-HTR*) genes or, in analogy to left heart diseases, polymorphisms

in the nitric oxide (NO) system [2, 25, 26]. The long allelic variant in *5-HTT* has been found to be associated with PH severity in patients with chronic obstructive pulmonary disease [27] but not in IPAH [28]. *5-HTR* has been reported to play an important role in vasoconstriction and proliferation of vessel walls [29]. The c.102T $\rightarrow$ C polymorphism of the *5-HTR2A* gene has been associated systemic arterial hypertension [30–32] but has not been investigated in PH. Therefore, we decided to include this gene in our analysis. NO plays a key role in endothelial dysfunction [33], and the expression of the endothelial NO synthase (*eNOS*) is modified in PH [34]. Polymorphisms in the *eNOS* gene have been related to coronary artery disease (CAD) [35] and to high-altitude pulmonary edema in Japanese and Indian populations [36, 37] but not in Caucasians [38]. At this background, we have chosen to investigate the *4a/b* and the p.*Glu298Asp* polymorphisms of *eNOS* in our patient cohort.

The aim of the present study was to prospectively investigate genetic variants in *BMPR2* and some potentially interesting variants in *5-HTT*, *5-HTR2A* and *eNOS* in consecutive patients with IPAH and CTEPH and to look for associations with clinical, functional and hemodynamic parameters. As controls, exercise-limited patients with left heart disease not suffering from PH were included.

Patients and Methods

Study Population

Between October 2004 and May 2006, consecutive patients with IPAH and CTEPH seen at the PH clinic of the University Hospital of Zurich, Switzerland, were prospectively included upon written informed consent. PH was defined as mean pulmonary arterial pressure ≥ 25 mm Hg with a pulmonary capillary occlusion pressure ≤ 15 mm Hg by right heart catheterization. IPAH was diagnosed if a thorough evaluation by medical history, echocardiography, rheumatologic examination, antibody screening and additional tests if required according to best clinical practice (e.g. pulmonary function tests, blood gas assessment, thoracic computed tomography, coronary angiography and rheumatologic evaluation) did not reveal any other cause for elevated pulmonary pressure [1]. In every patient, a detailed family history has been obtained, and a pedigree has been constructed. CTEPH was diagnosed by both radioisotope ventilation-perfusion scan and pulmonary angiography [39].

In the control patients, PH was excluded either by heart catheterization or echocardiography, and assessment of genetics, and clinical and functional parameters was similar to the PH group. The study was approved by the local ethics committee.

Table 1. PCR and sequencing primers used for the *BMPR2* mutation analysis

BMPR2 exon	Forward primer (5' → 3')	Reverse primer (5' → 3')	Annealing temperature, °C
1	gtgatacgggcaggatcagt	atttccttgaaggcatgg	61
2	gtcattcggataagacaaa	ttaacatactcccatgtcc	54
3	tagcttacacgtactctac	cctggcttaacctgaatg	58
4	gggtacagcctttctaaagg	gatactattgaggctgggtg	61
4 (sequencing primer)	tgacattcaaaattgtttcttt		
5	gctgctaattcttctgcagc	gaatgaagtcactgttccag	61
6	cagagagctgtagcattctg	aagtgatccacctgccttag	58
7	ttgcaaattctttataaggatgc	ttcctgttgtaatttgaacc	56
7 (sequencing primer)	cgcatttttctctata		
8	gttattagaaaataatgggcaga	ttatcatttcaaagtacatcagt	56
9	agaatatgctacgttctctc	acactagatagcaatgaactaaagg	56
10	ttggtatcagaaataccctgtt	caaacaacattaaaacatattcagtc	56
11	cacatggttgacatgtacttg	tcattgaactattaggctggtt	54
12 (fragment 12-1)	aaatttgagagacagttgtca	ttcatctgggtatggcatctc	58
12 (fragment 12-2)	agcaagcacaaagctcgaatc	agtcctgctgtccagtgtgct	58
12 (fragment 12-3)	tctagcttgctttaccact	agcatgggagtttaacactgt	58
12 (fragment 12-4)	acctcatgtggtgacagtc	attggaattagttcggccac	56
12 (fragment 12-5)	attccagtcctgatgagcat	agttatttaaatggcccaa	56
13	cacctctgagacattggt	catcttctgcatgtttaatga	55

General Study Assessments

Patient's demographics (age, sex, height, weight and calculated body mass index in kg/m²), history, drug use, New York Heart Association (NYHA) functional class, 6-min walk distance (6MWD) and Borg dyspnea scale score were assessed. Quality of life (QoL) was measured by the Minnesota Living with Heart Failure Questionnaire, with higher scores representing worse QoL [40]. NT-pro brain natriuretic peptide, C-reactive protein (using a highly sensitive method, CRP_{sens}), uric acid, creatinine, albumin, bilirubin, calcium, and erythrocyte, leukocyte and thrombocyte counts were assessed.

Genetic Analysis

EDTA-supplemented blood samples were collected and DNA was extracted with the QIAamp[®] DNA Mini Kit (Qiagen, Basel, Switzerland). Primers were designed with the Oligo 4.0 software (MedProbe, Oslo, Norway) and with the Primer3 Program (<http://primer3.sourceforge.net/>), and purchased from Microsynth (Balgach, Switzerland) or from Eurofins MWG Operon (Ebersberg, Germany).

BMPR2 Mutation and Polymorphism Analysis

BMPR2 mutation analysis was performed in the Institute of Human Genetics in Heidelberg in all index patients and controls using denaturing high-performance liquid chromatography and cycle sequencing with the BigDye terminator kit (V1.1, Applied Biosystems, Darmstadt, Germany) on an ABI 3700 sequencer as described previously (table 1) [17]. Genetic investigators were blinded to the clinical data. To determine the significance of the c.818T→G (p.Met273Arg) mutation for pre-mRNA splicing, the

BMPR2 transcript was analyzed by RT-PCR as previously described [17].

5-HTT Polymorphisms

The 44-bp insertion/deletion polymorphism in 5-HTT was analyzed using 5-HTT primers and the Expand Long Template Kit from Roche Molecular Biochemicals (Rotkreuz, Switzerland). PCR resulted in a long (L) amplification product of 457 bp and in a short (S) amplification product of 414 bp for the two alleles, which were separated on agarose gels.

c.102T→C Polymorphism of HTR2A

For the detection of the c.102T→C polymorphism in the *HTR2A* gene, a LightCycler assay was developed and four oligonucleotides were added to the LightCycler DNA Master Hybridization Probes mixture (Roche Molecular Biochemicals); a melting curve analysis was performed from 40 to 85°C at a linear rate of 0.1°C/s [41].

4a/b Polymorphism of eNOS

eNOS4a is a rare short allele with 4 tandem 27-bp repeats and eNOS4b is a large allele with 5 tandem repeats. Genotyping of the 27-bp repeat in intron 4 of the *eNOS* gene (eNOS4a/b) was performed according to Wang et al. [42].

Glu298Asp Polymorphism of eNOS

For the detection of the p.Glu298Asp polymorphism in *eNOS*, a 25-μl tetraprimer PCR was developed using the Amplitaq Gold[™] System (Perkin-Elmer, Hünenberg, Switzerland) and applied as previously described [43, 44].

Table 2. Characteristics of the patients and controls

Characteristics	IPAH (n = 16)	CTEPH (n = 16)	Controls (n = 24)
<i>General</i>			
Sex, females/males	10/6	9/7	9/15
Age, years			
At study assessment	65 ± 16	65 ± 11	67 ± 12
At first PH-related symptoms	62 ± 16	62 ± 12	
NYHA functional class, II/III/IV	1/8/7	1/10/4	1/10/13
6MWD, m	348 ± 85	435 ± 64*	288 ± 93**
QoL, points			
Total	44 ± 15	36 ± 19	42 ± 21
Physical subscore	24 ± 7	20 ± 6	24 ± 10
Emotional subscore	9 ± 6	10 ± 7	6 ± 7
Pro-BNP (<88 ng/l)	2,303 ± 1,810	2,099 ± 1,576	3,617 ± 3,501
Uric acid (150–300 µmol/l)	462 ± 144	470 ± 140	488 ± 178
<i>Pulmonary hemodynamics</i>			
Mean pulmonary arterial pressure, mm Hg	48 ± 10	41 ± 10	
Pulmonary vascular resistance, dyn·s·cm ⁵	748 ± 361	708 ± 293	
Cardiac index, l/min/m ²	2.3 ± 0.7	2.0 ± 0.5	
Right atrial pressure, mm Hg	12 ± 7	11 ± 8	
Mixed venous oxygen saturation, %	58 ± 9	55 ± 8	

Means ± SD. * p < 0.05 vs. IPAH, ** p < 0.001, vs. IPAH and CTEPH. Normal range/values are shown in parentheses.

Statistical Analysis

Results are expressed as medians (interquartile ranges) or means ± SD. The Mann-Whitney U test and analysis of variance were used to compare means between groups. Fisher's exact test was applied to compare frequencies between groups. Differences of p < 0.05 were considered statistically significant.

Results

Patient Characteristics

PH Study Group. Of the 38 PH patients screened, 6 patients were excluded for reasons other than PH (2 patients with scleroderma and 4 with left heart dysfunction). We finally included 16 patients (10 females) with IPAH (age 65 ± 16 years) and 16 patients (9 females) with CTEPH (age 65 ± 11 years) in the study (table 2). All patients are Caucasians. Twenty-four patients were included at the time of their first confirmatory right heart catheterization and 8 patients at the time of a follow-up catheterization. On average, symptoms had started 15 ± 11 months before diagnosis. All patients were followed up in our PH clinic (mean follow-up time 37 ± 20 months until data analysis). Six patients died (33 ± 32 months after diagnosis), and 3 patients had successful pulmonary endar-

terectomy. All IPAH patients had a negative family history of the disease. Nine of the CTEPH patients were classified as operable by a specialized surgeon (E. Mayer, Mainz); however, 6 were not operated on due to patients' wishes or comorbidity. Most patients were in NYHA class III or IV. IPAH patients had a significantly lower 6MWD compared with CTEPH patients but comparable QoL, pro-BNP, uric acid and pulmonary hemodynamics (table 2).

Control Group and Comparison with All PH Patients. Twenty-four patients (9 females; age 67 ± 12 years) without PH (7 with valvular, 8 with ischemic and 9 patients with hypertensive heart disease) were included as controls. PH was excluded by heart catheterization or echocardiography.

Genetic Analysis

BMPR2 Sequence Variants

In the present collective, no *BMPR2* mutations were found in the 16 study patients with sporadic IPAH and in the 16 patients with CTEPH. Interestingly, a c.818T→G (p.Met273Arg) mutation was noted in a patient with CAD and impaired left ventricular pump function without PH (initial right ventricular pressure 26 mm Hg above the

Table 3. *BMPR2* sequence variants found in the Swiss collective and their frequency distribution within groups

Location	Nucleotide change	Amino acid change	Frequencies, %		
			IPAHA	CTEPH	controls
Intron 3 and 4	c.420-38delT; c.529+64C→T		15	20	17
Exon 5	c.600A→C	p.L200L	20	0	4
Exon 6	c.818T→G	p.M273R	0	0	4
Exon 12	c.2324G→A	p.S775N	0	13	4
Exon 12	c.2811G→A	p.R937R	20	20	38

right atrial pressure by echocardiography). This patient underwent coronary angioplasty and stenting of his left anterior descending artery due to a non-ST elevation myocardial infarction. Two years later, he received aorto-coronary bypass surgery, and after recovery he was classified in NYHA functional class I. His echocardiographically measured right ventricular pressure was 19 mm Hg 3 months after the operation. At the last follow-up, he felt well (48 months after inclusion) without any relevant dyspnea (NYHA I). His family history was negative for PH, dyspnea, sudden deaths or syncopes. The c.818T→G (p.Met273Arg) mutation was proven in two independent blood samples. According to the *in silico* analysis performed with the use of the web resource ESEfinder, this missense mutation was predicted to alternate the sequence of exonic splicing enhancers. For verification, we have conducted RT-PCR analysis on the mRNA of the patient and found that the mutation was also present in the *BMPR2* transcript and did not influence the proper splicing. Nevertheless, the possibility remains that this methionine→arginine exchange affects the function of the *BMPR2* receptor. This exchange occurred within the kinase domain of the *BMPR2* receptor in an evolutionary highly conserved region of the gene.

Several intronic and coding polymorphisms of the *BMPR2* gene have been reported so far [23]. In our collective, we detected *BMPR2* polymorphisms in introns 3 and 4, and exons 5 and 12 (table 3). The c.600A→C (p.L200L) polymorphism in exon 5 was thereby almost exclusively found in IPAHA (3 patients), while the c.2324G→A (p.S775N) polymorphism from exon 12 was detected in CTEPH patients. The other *BMPR2* polymorphisms were found with almost equal distribution between the groups. Overall, PH patients with any *BMPR2* polymorphisms had lower blood platelet counts and higher oxy-

Fig. 1. 6MWD according to the p.L200L *BMPR2* polymorphism in patients with PH.

gen saturations than patients with the wild-type sequence (269 vs. 210·10³/μl, *p* = 0.025, and 87.5 vs. 91%, *p* = 0.028). IPAHA patients with disease onset below the age of 60 years (*n* = 4) were more likely to carry any sequence variants than patients with a later disease onset (>60 years, *n* = 12): 4/4 versus 4/12. Polymorphisms in CTEPH patients were more equally distributed for patients with an earlier (*n* = 6) versus late disease onset (*n* = 10): 2/6 versus 6/10. PH patients carrying the c.600A→C (p.L200L) sequence variant had a significantly lower 6MWD and worse physical subscores in QoL assessments (mean 6MWD 259 vs. 408 m, *p* = 0.011, and mean physical QoL score 30 vs. 21 points, *p* = 0.022; fig. 1). Two patients were acute responders to inhaled NO during heart catheterization, and both did not have *BMPR2* polymorphisms. Four of the 6 patients (67%) who died and 10 of the 26 patients (38%) who survived until the end of the study had any *BMPR2* polymorphism. Event-free survival did not differ between carriers of any *BMPR2* polymorphism and non-carriers until the end of the study.

Polymorphism of 5-*HTT*

The long allelic variant of the 5-*HTT* gene was found homozygous in 33, 6 and 20% and heterozygous in 47, 69 and 54% of patients with IPAHA, CTEPH and controls,

respectively (nonsignificant; table 4). The allelic frequency of the L variant was slightly higher in IPAH than in CTEPH and in control groups (nonsignificant). There were no significant differences in patient characteristics according to the 5-*HTT* polymorphism overall and within each group. Of the patients who succumbed, the long allelic variant was found homozygous in 1 and heterozygous in 2 patients.

c.102T→C Polymorphism of 5-*HTR2A*
The frequencies of the TT, TC and CC variants in IPAH, CTEPH and controls are shown in table 4. The allelic frequency for the C and T alleles was similar in all groups. No significant differences regarding 5-*HTR2A* variants were found for any parameter measured.

eNOSb/a
The eNOS4a allele was found heterozygous in 33, 31 and 29% of IPAH, CTEPH and controls and homozygous in 0, 6 and 4%, respectively (table 4). The allelic frequencies of the eNOS4a variant were 17, 22 and 19% in IPAH, CTEPH and controls, respectively. PH patients with the eNOS4a variant tended to have higher pulmonary vascular resistances (618, 769 and 1,105 dyn·s·m⁻⁵ for bb, ab and aa, respectively) and lower 6MWD (432, 409 and 413 m for bb, ab and aa, respectively), but the differences did not reach statistical significance in this relatively small cohort.

Endothelial NO p.Glu298Asp Polymorphism
As shown in table 4, the genotype frequencies for p.Glu298Glu, p.Glu298Asp and p.Asp298Asp were 27, 53 and 20% for IPAH, 56, 44 and 0% for CTEPH and 33, 50 and 17% for controls, respectively. We could not demonstrate any association of this polymorphism with clinical data and disease severity.

Discussion

In this cross-sectional analysis in a Swiss PH referral center, we studied the genetic variants of the *BMPR2* gene, serotonin and oxide system and their relationship to the various clinical parameters in patients with IPAH and CTEPH in comparison to a control group of similarly exercise-limited patients without PH. *BMPR2* mutations in patients with IPAH are either thought to occur in the setting of low-penetrance germline mutations or may arise de novo [23]. In our cohort, we found only one *BMPR2* mutation (p.Met273Arg), sur-

Table 4. Frequencies of the tested 5HTT, 5HTR2a and NO polymorphisms in IPAH (n = 16), CTEPH (n = 16) and control groups (n = 24)

Subjects	Type of polymorphism	Genotypes %			Allele frequencies, %	
	5HTT	SS	SL	LL	S	L
IPAH		20	47	33	43	57
CTEPH		25	69	6	59	41
Controls		25	54	20	52	48
5HTR2a		TT	TC	CC	T	C
IPAH		13	67	20	47	53
CTEPH		6	56	38	34	66
Controls		25	42	33	46	54
eNOS4a/b		bb	ab	aa	b	a
IPAH		67	33	0	83	17
CTEPH		63	31	6	78	22
Controls		67	29	4	81	19
eNOS298		Glu/Glu	Glu/Asp	Asp/Asp	Glu	Asp
IPAH		27	53	20	53	47
CTEPH		56	44	0	78	22
Controls		33	50	17	58	42

prisingly not in a patient suffering from PH but from CAD. This finding is novel and remarkable, and may point towards a role of the *BMPR2* pathway in other diseases than PH. A thorough history and clinical examination did not reveal any sign for PH in this patient or his family. The same mutation was previously described in 1 IPAH case with hypothyroidism [23]. So far, functional studies providing more insight into the consequence of this missense mutation are missing. Some missense mutations, especially when located in the proximity of exon/intron boundaries, may cause aberrant pre-mRNA splicing due to alternations in sequences recognized by various splicing factors [45]. Using RT-PCR analysis, we have shown this not to be the case for the p.Met273Arg genetic variant. Further analyses have to be performed in order to elucidate the effect of this amino acid exchange on proper receptor function as well as in order to exclude a possible mosaic state of this alternation. However, due to the generally low penetrance of *BMPR2* mutations and particularly given the different pathophysiology of PH

and CAD, it is difficult to estimate whether this mutation could somehow have contributed to the patient's condition or left ventricular function. Other risk factors, mainly his smoking history with 60 pack-years until his first event, have to be considered.

The lack of any detected *BMPR2* mutations in the present IPAH cohort is unexpected but possible considering that the prevalence of *BMPR2* mutations in patients with sporadic PAH is only 11–26% [20, 23, 46] and may be even lower, as recently described by Rosenzweig et al. [47] (6.1%). It must, however, be taken into account that the screening method applied has several limitations. It has a very high detection rate for mutations in the coding regions and exon/intron boundaries, but does not allow for identification of large exonic deletions or duplications. The latter are however rare in patients with sporadic PAH, in agreement with a previous study (6 of 126 IPAH patients) [48] and based on our own observations (data not published). Furthermore, putative mutations in the untranslated regulatory and intronic sequences of the *BMPR2* gene remain undetected [49].

Another possible explanation might be the comparatively advanced age of the IPAH patients included in our cohort, with average age of disease onset being 61 ± 16 years. Based on other observations, *BMPR2* mutation carriers are suspected to develop the symptoms earlier than non-carriers [50].

Beside the rare and pathologic mutations, several polymorphic variants have also been identified in the sequence of the *BMPR2* gene [23, 51, 52]. The frequencies of some of these polymorphisms were studied in several cohorts of PAH patients and unaffected individuals [23]. The c.600A→C (p.L200L) polymorphism in exon 5 was found in 4% of our controls but in 20% of IPAH patients, and it was associated with a significantly lower 6MWD and QoL in physical domains (fig. 1). Herewith, we describe for the first time a higher prevalence of a coding *BMPR2* polymorphism in an IPAH cohort with an association to disease severity (assessed by the 6 MWD and QoL). As this single-nucleotide polymorphism does not lead to the amino acid exchange and is not predicted to alternate mRNA splicing, it may not have direct causal effects, but may be linked in some patients to another not yet identified functional alternation in the *BMPR2* gene. This novel finding may suggest that some common genetic variants of the *BMPR2* gene could play a role in the modulation of disease severity. In our small cohort, carriers of any *BMPR2* polymorphism were more likely to have a worse outcome after PH diagnosis (67% of the patients who died in our cohort were carriers of any poly-

morphism compared with 38% of the survivors); however, these results have to be verified by investigations in larger PH cohorts.

The L-allelic variant of 5-*HTT* has been found to be associated with disease severity in PH related to chronic obstructive lung disease [27, 53]; however, this association could not be found in IPAH or familial PAH [28]. In our collective, the L-allelic variant was slightly more prevalent in IPAH compared with CTEPH or controls; however, there was no association with clinical parameters or event-free survival. The base in nucleotide position 102 of the 5-*HTR2A* gene may be thymine (T) or cytosine (C), with three possible genotypes, TT, TC or CC. This sequence variant does not result in any amino-acid change, as both alleles code for a serine in codon 34, but it is supposed to be in linkage disequilibrium with functional 5-*HTR2A* gene variants. However, we found no difference in the prevalence and clinical presentation according to 5-*HTR2A* variants in PH or controls. Our findings discourage a relevant pathogenetic role of the presently investigated 5-*HTT* and 5-*HTR2A* polymorphisms in IPAH or CTEPH.

The allelic frequency of the *eNOS4a* variant in the present cohort was comparable to Japanese patients with high-altitude pulmonary edema and higher than expected from previously described healthy controls (table 4) [36], demonstrating a genetic heterogeneity in various populations of different ethnicity. A tendency towards higher pulmonary vascular resistance and lower exercise capacity assessed by 6MWD in *eNOS4a* carriers may point towards a possibly modulating role of this *eNOS* variant in PH. Concerning the p.Glu298Asp polymorphism, the Asp allelic frequency in CTEPH patients in our cohort was similar to that found in Japanese patients with high-altitude pulmonary edema [36]. However, the frequencies of the Asp allele in IPAH and controls were even higher and similar to a French population [54]. Considering the lack of any association of the p.Glu298Asp polymorphism with clinical parameters, we suggest that this polymorphism might not be significantly involved in the pathogenesis of PH.

Our single center, cross-sectional study bears several intrinsic limitations. First, the investigated cohort is small; however, PH is a rare disease and significant findings in a small cohort may help to direct future research in the field. Our control group consisted of exercise-limited patients with some form of left heart involvement without PH and not healthy controls. However, as *BMPR2* mutations are thought to be disease specific for PAH, we did not expect to find any pathologic *BMPR2* alteration

in a patient with CAD. The genetic analyses of our patients were restricted to the screening for genetic variants in *BMPR2* and in three genes of the serotonin and NO systems. However, it might be possible that other herein not investigated polymorphisms are causally involved in the pathogenesis of PH.

Overall, the present study may reflect the clinical status and genetic background in a typical PH cohort as seen in a single, tertiary care, referral center. We suggest that

the results obtained in this study require verification in a larger case-control study before any definitive conclusions can be made with respect to the current practical relevance.

Acknowledgment

We thank the Velux Foundation (Zurich, Switzerland) for financial support of the study.

References

- 1 Simonneau G, Galie N, Rubin LJ, Langleben D, Seeger W, Domenighetti G, Gibbs S, Lebrec D, Speich R, Beghetti M, Rich S, Fishman A: Clinical classification of pulmonary hypertension. *J Am Coll Cardiol* 2004;43: 5S–12S.
- 2 Humbert M, Morrell NW, Archer SL, Stenmark KR, MacLean MR, Lang IM, Christman BW, Weir EK, Eickelberg O, Voelkel NF, Rabinovitch M: Cellular and molecular pathobiology of pulmonary arterial hypertension. *J Am Coll Cardiol* 2004;43:13S–24S.
- 3 Fuster V, Steele PM, Edwards WD, Gersh BJ, McGoon MD, Frye RL: Primary pulmonary hypertension: natural history and the importance of thrombosis. *Circulation* 1984; 70:580–587.
- 4 Voelkel NF, Tuder RM: Cellular and molecular mechanisms in the pathogenesis of severe pulmonary hypertension. *Eur Respir J* 1995; 8:2129–2138.
- 5 Bonderman D, Nowotny R, Skoro-Sajer N, Jakowitsch J, Adlbrecht C, Klepetko W, Lang IM: Bosentan therapy for inoperable chronic thromboembolic pulmonary hypertension. *Chest* 2005;128:2599–2603.
- 6 Bresser P, Fedullo PF, Auger WR, Channick RN, Robbins IM, Kerr KM, Jamieson SW, Rubin LJ: Continuous intravenous epoprostenol for chronic thromboembolic pulmonary hypertension. *Eur Respir J* 2004;23: 595–600.
- 7 Hoeper MM, Kramm T, Wilkens H, Schulze C, Schafers HJ, Welte T, Mayer E: Bosentan therapy for inoperable chronic thromboembolic pulmonary hypertension. *Chest* 2005; 128:2363–2367.
- 8 Nagaya N, Sasaki N, Ando M, Ogino H, Sakamaki F, Kyotani S, Nakanishi N: Prostacyclin therapy before pulmonary thromboendarterectomy in patients with chronic thromboembolic pulmonary hypertension. *Chest* 2003;123:338–343.
- 9 Ulrich S, Speich R, Domenighetti G, Geiser T, Aubert JD, Rochat T, Huber L, Treder U, Fischler M: Bosentan therapy for chronic thromboembolic pulmonary hypertension. A national open label study assessing the effect of Bosentan on haemodynamics, exercise capacity, quality of life, safety and tolerability in patients with chronic thromboembolic pulmonary hypertension (BOCTEPH-Study). *Swiss Med Wkly* 2007; 137:573–580.
- 10 Ulrich S, Fischler M, Speich R, Popov V, Maggiorini M: Chronic thromboembolic and pulmonary arterial hypertension share acute vasoreactivity properties. *Chest* 2006; 130:841–846.
- 11 Moser KM, Bloor CM: Pulmonary vascular lesions occurring in patients with chronic major vessel thromboembolic pulmonary hypertension. *Chest* 1993;103:685–692.
- 12 Lane KB, Machado RD, Pauculo MW, Thomson JR, Phillips JA 3rd, Loyd JE, Nichols WC, Trembath RC: Heterozygous germline mutations in *BMPR2*, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. The International PPH Consortium. *Nat Genet* 2000;26:81–84.
- 13 Deng Z, Morse JH, Slager SL, Cuervo N, Moore KJ, Venetos G, Kalachikov S, Cayanis E, Fischer SG, Barst RJ, Hodge SE, Knowles JA: Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. *Am J Hum Genet* 2000;67:737–744.
- 14 Newman JH, Wheeler L, Lane KB, Loyd JE, Gaddipati R, Phillips JA 3rd, Loyd JE: Mutation in the gene for bone morphogenetic protein receptor II as a cause of primary pulmonary hypertension in a large kindred. *N Engl J Med* 2001;345:319–324.
- 15 Yang J, Davies RJ, Southwood M, Long L, Yang X, Sobolewski A, Upton PD, Trembath RC, Morrell NW: Mutations in bone morphogenetic protein type II receptor cause dysregulation of Id gene expression in pulmonary artery smooth muscle cells: implications for familial pulmonary arterial hypertension. *Circ Res* 2008;102:1212–1221.
- 16 Nasim MT, Ghouri A, Patel B, James V, Rudarakanchana N, Morrell NW, Trembath RC: Stoichiometric imbalance in the receptor complex contributes to dysfunctional *BMPR-II* mediated signalling in pulmonary arterial hypertension. *Hum Mol Genet* 2008; 17:1683–1694.
- 17 Koehler R, Grunig E, Pauculo MW, Hoeper MM, Olschewski H, Wilkens H, Halank M, Winkler J, Ewert R, Bremer H, Kreuscher S, Janssen B, Nichols WC: Low frequency of *BMPR2* mutations in a German cohort of patients with sporadic idiopathic pulmonary arterial hypertension. *J Med Genet* 2004;41: e127.
- 18 Thomson JR, Machado RD, Pauculo MW, Morgan NV, Humbert M, Elliott GC, Ward K, Yacoub M, Mikhail G, Rogers P, Newman J, Wheeler L, Higenbottam T, Gibbs JS, Egan J, Crozier A, Peacock A, Allcock R, Corris P, Loyd JE, Trembath RC, Nichols WC: Sporadic primary pulmonary hypertension is associated with germline mutations of the gene encoding *BMPR-II*, a receptor member of the TGF-beta family. *J Med Genet* 2000;37:741–745.
- 19 Newman JH, Phillips JA 3rd, Loyd JE: Narrative review: the enigma of pulmonary arterial hypertension: new insights from genetic studies. *Ann Intern Med* 2008;148:278–283.
- 20 Newman JH, Trembath RC, Morse JA, Grunig E, Loyd JE, Adnot S, Coccio F, Ventura C, Phillips JA 3rd, Knowles JA, Janssen B, Eickelberg O, Eddahibi S, Herve P, Nichols WC, Elliott G: Genetic basis of pulmonary arterial hypertension: current understanding and future directions. *J Am Coll Cardiol* 2004;43:33S–39S.
- 21 Roberts KE, McElroy JJ, Wong WP, Yen E, Widlitz A, Barst RJ, Knowles JA, Morse JH: *BMPR2* mutations in pulmonary arterial hypertension with congenital heart disease. *Eur Respir J* 2004;24:371–374.
- 22 Runo JR, Vnencak-Jones CL, Prince M, Loyd JE, Wheeler L, Robbins IM, Lane KB, Newman JH, Johnson J, Nichols WC, Phillips JA III: Pulmonary veno-occlusive disease caused by an inherited mutation in bone morphogenetic protein receptor II. *Am J Respir Crit Care Med* 2003;167:889–894.
- 23 Machado RD, Aldred MA, James V, Harrison RE, Patel B, Schwalbe EC, Gruenig E, Janssen B, Koehler R, Seeger W, Eickelberg O, Olschewski H, Elliott CG, Glissmeyer E,

- Carlquist J, Kim M, Torbicki A, Fijalkowska A, Szweczyk G, Parma J, Abramowicz MJ, Galie N, Morisaki H, Kyotani S, Nakanishi N, Morisaki T, Humbert M, Simonneau G, Sitbon O, Soubrier F, Coulet F, Morrell NW, Trembath RC: Mutations of the TGF-beta type II receptor BMPR2 in pulmonary arterial hypertension. *Hum Mutat* 2006;27:121–132.
- 24 Suntharalingam J, Machado RD, Sharples LD, Toshner MR, Sheares KK, Hughes RJ, Jenkins DP, Trembath RC, Morrell NW, Pepke-Zaba J: Demographic features, BMPR2 status and outcomes in distal chronic thromboembolic pulmonary hypertension. *Thorax* 2007;62:617–622.
- 25 Eddahibi S, Morrell N, d'Ortho MP, Naeije R, Adnot S: Pathobiology of pulmonary arterial hypertension. *Eur Respir J* 2002;20:1559–1572.
- 26 Eddahibi S, Raffestin B, Hamon M, Adnot S: Is the serotonin transporter involved in the pathogenesis of pulmonary hypertension? *J Lab Clin Med* 2002;139:194–201.
- 27 Eddahibi S, Chaouat A, Morrell N, Fadel E, Fuhrman C, Bugnet AS, Darteville P, Housset B, Hamon M, Weitzenblum E, Adnot S: Polymorphism of the serotonin transporter gene and pulmonary hypertension in chronic obstructive pulmonary disease. *Circulation* 2003;108:1839–1844.
- 28 Machado RD, Koehler R, Glissmeyer E, Veal C, Suntharalingam J, Kim M, Carlquist J, Town M, Elliott CG, Hoeper M, Fijalkowska A, Kurzyna M, Thomson JR, Gibbs SR, Wilkins MR, Seeger W, Morrell NW, Gruenig E, Trembath RC, Janssen B: Genetic association of the serotonin transporter in pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2006;173:793–797.
- 29 Leonard BE: Sub-types of serotonin receptors: biochemical changes and pharmacological consequences. *Int Clin Psychopharmacol* 1992;7:13–21.
- 30 Liolitsa D, Powell JF, Prince M, Lovestone S: Association study of the 5-HT(2A) receptor gene polymorphism, T102C and essential hypertension. *J Hum Hypertens* 2001;15:335–339.
- 31 Vaquero Lorenzo C, Baca-Garcia E, Diaz-Hernandez M, Botillo-Martin C, Perez-Rodriguez MM, Fernandez-Ramos C, Saiz-Gonzalez MD, Quintero-Gutierrez FJ, Saiz-Ruiz J, Fernandez Piqueras J, Gonzalez de Rivera JL, de Leon J: Association between the T102C polymorphism of the serotonin-2A receptor gene and schizophrenia. *Prog Neuropsychopharmacol Biol Psychiatry* 2006;30:1136–1138.
- 32 Yamada S, Akita H, Kanazawa K, Ishida T, Hirata K, Ito K, Kawashima S, Yokoyama M: T102C polymorphism of the serotonin (5-HT) 2A receptor gene in patients with non-fatal acute myocardial infarction. *Atherosclerosis* 2000;150:143–148.
- 33 Ghofrani HA, Pepke-Zaba J, Barbera JA, Channick R, Keogh AM, Gomez-Sanchez MA, Kneussl M, Grimminger F: Nitric oxide pathway and phosphodiesterase inhibitors in pulmonary arterial hypertension. *J Am Coll Cardiol* 2004;43:68S–72S.
- 34 Giaid A, Saleh D: Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. *N Engl J Med* 1995;333:214–221.
- 35 Casas JP, Bautista LE, Humphries SE, Hingorani AD: Endothelial nitric oxide synthase genotype and ischemic heart disease: meta-analysis of 26 studies involving 23,028 subjects. *Circulation* 2004;109:1359–1365.
- 36 Droma Y, Hanaoka M, Ota M, Katsuyama Y, Koizumi T, Fujimoto K, Kobayashi T, Kubo K: Positive association of the endothelial nitric oxide synthase gene polymorphisms with high-altitude pulmonary edema. *Circulation* 2002;106:826–830.
- 37 Ahsan A, Mohd G, Norboo T, Baig MA, Pasha MA: Heterozygotes of NOS3 polymorphisms contribute to reduced nitrogen oxides in high-altitude pulmonary edema. *Chest* 2006;130:1511–1519.
- 38 Smith EM, Baillie JK, Thompson AA, Irving JB, Porteous D, Webb DJ: Endothelial nitric oxide synthase polymorphisms do not influence pulmonary artery systolic pressure at altitude. *High Alt Med Biol* 2006;7:221–227.
- 39 Hoeper MM, Mayer E, Simonneau G, Rubin LJ: Chronic thromboembolic pulmonary hypertension. *Circulation* 2006;113:2011–2020.
- 40 Cenedese E, Speich R, Dorschner L, Ulrich S, Maggiorini M, Jenni R, Fischler M: Measurement of quality of life in pulmonary hypertension and its significance. *Eur Respir J* 2006;28:808–815.
- 41 Ruegg C, Hersberger M, Wusk B, Rentsch K, Kullak-Ublick GA, von Eckardstein A, Maly FE: Detection of the Arg702Trp, Gly908Arg and Leu1007fsinsC polymorphisms of the NOD2/CARD15 gene by real-time PCR with melting curve analysis. *Clin Chem Lab Med* 2004;42:494–498.
- 42 Wang XL, Sim AS, Badenhop RF, McCredie RM, Wilcken DE: A smoking-dependent risk of coronary artery disease associated with a polymorphism of the endothelial nitric oxide synthase gene. *Nat Med* 1996;2:41–45.
- 43 Hersberger M, Marti-Jaun J, Rentsch K, Hanseler E: Rapid detection of the CYP2D6*3, CYP2D6*4, and CYP2D6*6 alleles by tetra-primer PCR and of the CYP2D6*5 allele by multiplex long PCR. *Clin Chem* 2000;46:1072–1077.
- 44 Muntwyler J, Marti-Jaun J, Luscher TF, Hanseler E, Hersberger M: The Asp298 but not the C-786 genotype of the endothelial nitric oxide synthase is reduced with age in healthy Swiss men. *Clin Chem Lab Med* 2005;43:971–973.
- 45 Cartegni L, Chew SL, Krainer AR: Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nat Rev Genet* 2002;3:285–298.
- 46 Morse JH: Genetic studies of pulmonary arterial hypertension. *Lupus* 2003;12:209–212.
- 47 Rosenzweig EB, Morse JH, Knowles JA, Chada KK, Khan AM, Roberts KE, McElroy JJ, Juskiw NK, Mallory NC, Rich S, Diamond B, Barst RJ: Clinical implications of determining BMPR2 mutation status in a large cohort of children and adults with pulmonary arterial hypertension. *J Heart Lung Transplant* 2008;27:668–674.
- 48 Aldred MA, Vijayakrishnan J, James V, Soubrier F, Gomez-Sanchez MA, Martensson G, Galie N, Manes A, Corris P, Simonneau G, Humbert M, Morrell NW, Trembath RC: BMPR2 gene rearrangements account for a significant proportion of mutations in familial and idiopathic pulmonary arterial hypertension. *Hum Mutat* 2006;27:212–213.
- 49 Aldred MA, Machado RD, James V, Morrell NW, Trembath RC: Characterization of the BMPR2 5'-untranslated region and a novel mutation in pulmonary hypertension. *Am J Respir Crit Care Med* 2007;176:819–824.
- 50 Sztrymf B, Coulet F, Girerd B, Yaici A, Jais X, Sitbon O, Montani D, Souza R, Simonneau G, Soubrier F, Humbert M: Clinical outcomes of pulmonary arterial hypertension in carriers of BMPR2 mutation. *Am J Respir Crit Care Med* 2008;177:1377–1383.
- 51 Machado RD, Pauculo MW, Thomson JR, Lane KB, Morgan NV, Wheeler L, Phillips JA 3rd, Newman J, Williams D, Galie N, Manes A, McNeil K, Yacoub M, Mikhail G, Rogers P, Corris P, Humbert M, Donnai D, Martensson G, Tranebjaerg L, Loyd JE, Trembath RC, Nichols WC: BMPR2 haploinsufficiency as the inherited molecular mechanism for primary pulmonary hypertension. *Am J Hum Genet* 2001;68:92–102.
- 52 Morisaki H, Nakanishi N, Kyotani S, Takashima A, Tomoike H, Morisaki T: BMPR2 mutations found in Japanese patients with familial and sporadic primary pulmonary hypertension. *Hum Mutat* 2004;23:632.
- 53 Ulrich S, Hersberger M, Fischler M, Nussbaumer-Ochsner Y, Treder U, Russi EW, Speich R: Genetic polymorphisms of the serotonin transporter, but not the 2a receptor or nitric oxide synthetase, are associated with pulmonary hypertension in COPD. *Respiration* 2010;79:288–295.
- 54 Lacolley P, Gautier S, Poirier O, Pannier B, Cambien F, Benetos A: Nitric oxide synthase gene polymorphisms, blood pressure and aortic stiffness in normotensive and hypertensive subjects. *J Hypertens* 1998;16:31–35.