Compound *BMPR2* gene mutations in a malignant variant of idiopathic pulmonary arterial hypertension

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Abstract

Pulmonary arterial hypertension (PAH; MIM 600799) is frequently associated with concomitant diseases, including congenital heart disease. 6% of patients with PAH show a family history of the disease [hereditary PAH (HPAH)], with the major genetic determinants of HPAH being heterozygous germline mutations in the bone morphogenetic protein type II receptor (*BMPR2*). We present the case of a 38-year-old woman of Indian descent; initially admitted with progressive dyspnea [New York Heart Association (NYHA) class III]. The results of the proband's clinical assessments are presented here. Cardiac catheterization confirmed idiopathic PAH with severe right ventricular hypertrophy associated with pulmonary arteriopathy. Initial treatment comprised the dual endothelin receptor antagonist, bosentan, furosemide, warfarin and intravenous infusion of prostaglandin I2 (PGI2) for 3 days. Despite this, the patient died of pulmonary hemorrhagic edema and cardiogenic shock after 6 days of intensive care. After relatives' consent, *post mortem* assessments confirmed a diagnosis of PAH; the heart displayed significant right ventricular hypertrophy and it was particularly noted that the right atrial appendage had undergone extreme dilation. Pulmonary arteriopathy was characterized by medial hypertrophy, arterialization of muscular arteries and muscularization of non-muscularized distal arteries. Molecular genetic analyses revealed the presence of *cis*-mutations in the *BMPR2* gene (p.Cys123Arg and p.Arg332X). Cosegregation studies were not available.

Our findings suggest that mutations of the *BMPR2* gene gave rise to the onset of PAH in this patient and that the severity of the onset and progression could be attributed to the presence of multiple mutations in a gene-dosage manner.

Introduction

Pulmonary arterial hypertension (PAH) (Table 1),[1] is a rare, fatal disease of the pulmonary arterioles,[1,2] where vasoconstriction and vascular remodeling lead to a progressive increase in pulmonary arterial pressure resulting in right ventricular failure.[2,3] PAH has an estimated prevalence of 15-50 cases per million,[4] the majority of which arise as a result of concomitant diseases (associated PAH) which include congenital heart disease, connective tissue disorders and infection with the human immunodeficiency virus. Spontaneously-occurring, idiopathic PAH (IPAH) accounts for at least 40% of the remaining cases and typically affects around twice as many women as men with a median age at diagnosis of around 47 years, although the disease may occur at any age.[5-7] A genetic contribution to PAH has long been identified and 6% of patients with PAH have reported a family history of the condition [hereditary PAH (HPAH)].[8,9]

Heterozygous germline mutations in the bone morphogenetic protein type II receptor 2 gene (*BMPR2*) have been identified in 10 to 40% of patients with apparently sporadic IPAH[10-13] and in 58 to 74% of patients with HPAH.[14-16] BMPR2 is a member of the transforming growth factor- (TGF-) receptor superfamily; mutations in genes of several members of this family have been implicated in the onset of PAH, indicating that the pathways controlled by members of this family are important to the integrity of the pulmonary vasculature (Table 2).[17-19] Furthermore, the pore-forming subunit, Kv1.5, forms functional voltage-gated potassium channels (Kv) in the pulmonary artery smooth muscle cells and abnormal potassium channel Kv1.5 function encoded by *KCNA5* mutations alone and associated with *BMPR2* mutations have been reported in patients with IPAH.[20]

In the present case report, we describe the clinical and molecular genetic assessment of a 38-year-old woman of Indian descent who was admitted to our hospital because of progressive dyspnea [New York Heart Association (NYHA) class III]. These assessments led to a diagnosis of PAH and the identification of a double heterozygosity of the *BMPR2* gene. The patient subsequently died of pulmonary hemorrhagic edema and cardiogenic shock after 6 days of intensive care. It can be speculated that the early onset, severity of progression and fatal outcome of the disease can be explained un the light of the *two-hits* hypothesis as reported by Wang *et al*.[20]

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Materials and Methods

Clinical assessments

Physical and cardiac examinations included chest X-ray, 12-lead electrocardiogram (ECG), echocardiography, computed axial tomography (CAT) (3D reconstruction) and laboratory tests. Transthoracic echocardiography examinations were performed using an iE33 xMATRIX echocardiography system equipped with 2.5-3.5 MHz probes (Phillips Healthcare, Surrey, UK) which provided a non-invasive assessment of pulmonary artery pressure and

a useful means of monitoring the patients' condition.

Histology

Tissue samples collected during the *post-mortem* examination were fixed in a 10% buffered formalin solution for approximately 48 h and subsequently embedded in paraffin tissue blocks using standard histopathology methods [formalin-fixed paraffin embedded (FFPE)]. Histological sections, 3 µm in thickness, were obtained from each block and stained with hematoxylin-eosin. Selected samples were also stained using the Weigert method for elastic fibers and immunohistochemically tested using anti-smooth-muscle actin, anti-CD3 and anti-CD20 primary antibodies in order to highlight the pulmonary artery *tunica media* or the potential inflammatory infiltrate.

Mutation analysis

After informed consent of both living parents, genomic DNA of the index case was extracted from FFPE by means of the Maxwell 16™ platform (Promega). The *BMPR2*, *SMAD, TGFBR1, TGFBR2, ACVR1, ACVRL1, BMP9/GDF2, KCNK3, KCNA5, CAV1, ENG* and *SMAD2* genes screening was performed on a PGM platform (IonTorrent, LifeTechnologies) equipped with a 318 chip (www.lifetechnologies.com); sequence variants have been subsequently confirmed by conventional forward and reverse Sanger Sequencing on the ABI PRISM 3130Xl.

Modeling: potential detrimental modifications induced by the mutations on DNA and protein level were assessed by: i) PolyPhen-2 (genetics.bwh.harvard.edu/pph2/) and Sift (sift.jcvi.org/); ii) PyMol (http://pymol.sourceforge.net/) and MODELLER (Accelerys, San Diego, CA, USA) softwares.

Results

Clinical findings

The patient had been treated for respiratory disease for almost 3 years but there was no history of syncope, palpitation, chest pain or hemoptysis. Cardiac examinations showed: i) the patient had a heart systolic murmur; ii) the 12-lead ECG revealed sinus tachycardia, right axis deviation and right ventricular hypertrophy with strain; iii) the chest X-ray revealed cardiomegaly and features of pulmonary hypertension and bilateral pleural effusion; iv) chest CAT (3D reconstruction) showed a pulmonary artery trunk diameter of 39 mm and a ground glass area without pulmonary thromboembolism. Of note, an enlarged right ventricle and smaller left ventricle were observed and the septum was pushed towards the left ventricle due to very high pressure inside the right ventricle (Figure 1A and B).

Transthoracic echocardiogram revealed: i) right ventricular systolic pressure of 96 mm/Hg (the peak of gradient through a severe tricuspid regurgitation was 76+20 mmHg of right atrial pressure, by the dilatation without collapsing *vena cava*; Figure 1C and D); ii) dilatation of the right chambers (the right atrium had a diameter of 57 mm, a wall measurement of 10.5 mm and a pulmonary artery trunk measurement of up to 38 mm; Figure 1E).

Evidence for right ventricular dysfunction was shown by a low value of tricuspid annular post-systolic excursion (TAPSE 3: <10 mm, severely abnormal). The acceleration time of pulmonary outflow (ACTpo) was 40 ms. There was no evidence of left to right atrial shunt.

Laboratory investigations excluded infections and immunological diseases. Cardiac catheterization confirmed severe PAH: 115/15

Table 1. Clinical classification of pulmonary hypertension according to the European Society of Cardiology (ESC) and the European Respiratory Society (ERS) guidelines.[1]

Classification of pulmonary hypertension	
PAH	Idiopathic PAH Hereditable PAH Drug-and toxin-induced Persistent PH of the newborn Associated PAH Connective tissue disease HIV infection Portal hyptertension Congenital heart disease Schistosomiasis Chronic hemolytic anemia
PH due to left heart disease	
PH due to lung disease	Chronic obstructive pulmonary disease Interstitial lung disease
Chronic thromboembolic pulmonary hypertension	
PH with unclear multifactorial mechanisms	Sarcoidosis, etc.

PAH, pulmonary arterial hypertension.

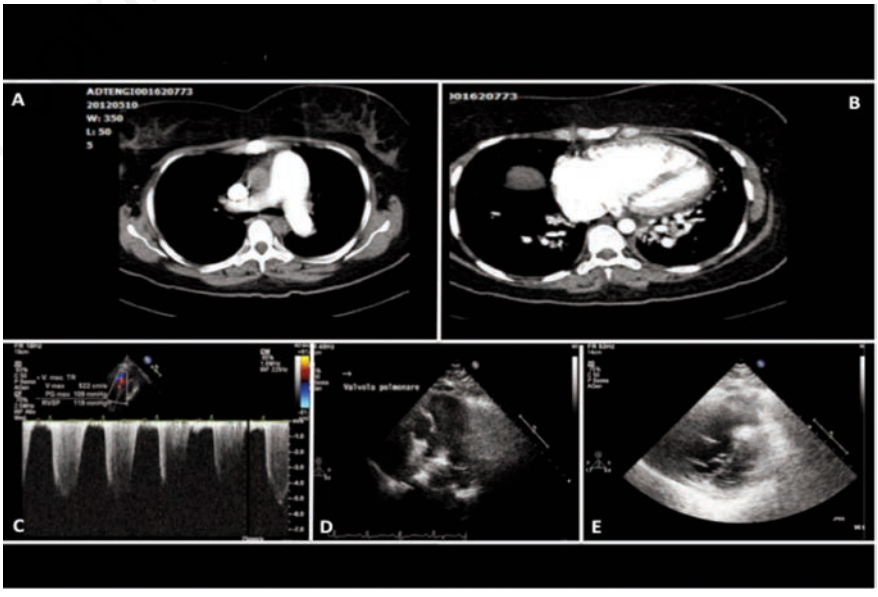

Figure 1. Cardiac imaging. A) and B) Computed tomography scans demonstrating severe idiopathic pulmonary arterial hypertension. Note the markedly enlarged pulmonary trunk with tiny branching smaller vessels. C) A transthoracic echocardiogram revealed a right ventricular systolic pressure of 96 mm/Hg. D) A pulmonary artery trunk measuring up to 38 mm was detected. E) Dilatation of right chambers: right atrium was 57 mm in diameter; right ventricle wall measurement was 10 mm.

mmHg , mPAP 65mm/Hg, PCWP 14 mm/Hg, IC: 2 L/m/mq and pulmonary vascular resistance of 26 UW.[2] O_2 saturation of 50% at the RA, 100% at the left upper pulmonary vein; 90% at the left atrium, 65% at the RV, 60% at the left pulmonary artery and 86% at the femoral artery. The aortic pressure was 92/62 (mean 44) mmHg. After adenosine 100-200-300 /kg/min PVR were unchanged. Coronary tree was normal.

Treatment for idiopathic pulmonary arterial hypertension

Following a diagnosis of severe PAH, treatment was initiated with bosentan 125 mg/day, diuretic (furosemide 125 mg), warfarin and intravenous infusion of prostaglandin I2 (PGI2) infusion for 3 days and non-invasive ventilation therapy (using a continuous positive airway pressure mask). However, no benefit was seen following these treatments and the patient subsequently died of pulmonary hemorrhagic edema and cardiogenic shock after 6 days of intensive care.

In this case a massive pulmonary edema may be due to the very high values of pulmonary pressure that led to flooding of the pulmonary alveoli and by a respiratory distress syndrome favored by the presence of an acute bronchopneumonia.

A pulmonary veno-occlusive hypertension disease was excluded from high-resolution computed tomography and capillary pulmonary wedge pressure (PCWP) was below 15 mm/Hg.

Gross and microscopic appearances

Lungs

At necropsy (Figure 2 A-D), both lungs (each 590 g) showed diffuse endoalveolar hemorrhage and marked changes in all of the pulmonary arterial branches especially in terms of wall thickening.

Heart

The heart (470 g) displayed significant right ventricular hypertrophy; the right atrial appendage had undergone extreme dilation to the extent that it was incorporated into the main right atrial chamber. Histopathology (Figure 3A-D) revealed pulmonary arteriopathy which was characterized primarily by medial hypertrophy, arterialization of muscular arteries and muscularization of non-muscularized distal arteries. Focal intimal fibrosis and mild plaques were detectable throughout the lung parenchyma. There were neither thrombotic lesions nor signs of pulmonary veno-occlusive disease.

Molecular findings

Molecular investigations on the index case identified two *BMPR2* mutations; the first was a missense mutation in exon #3 (p.Cys123Arg) and the second was a stop codon mutation in

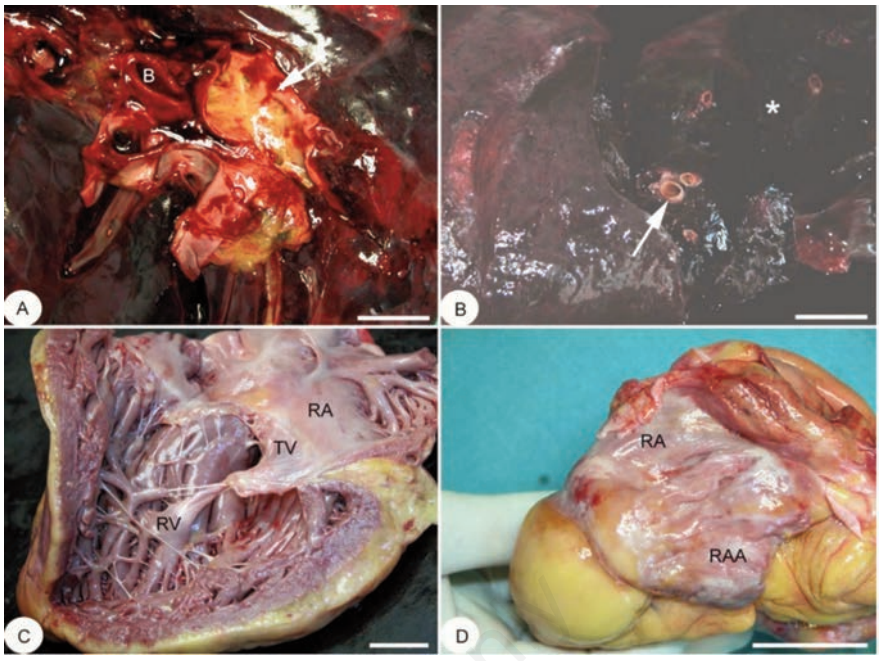

Figure 2. Autopsy images. A) The right lung hilus with an exceedingly thick pulmonary artery (arrow) which shows an aortic-like appearance. B) On cut section, the lung parenchyma (asterisk) is extremely hemorrhagic and displays rigid and thick peripheral pulmonary artery branches (arrow). C) The right ventricle is highly hypertrophic. D) The right-atrial appendage had undergone marked dilation and become part of the main right atrial chamber. Abbreviations: B, bronchus; RA, right atrium; RAA, right-atrial appendage; RV, right ventricle; TV, tricuspid valve. Size of bars A) and B): 3 cm, C) and D): 2 cm.

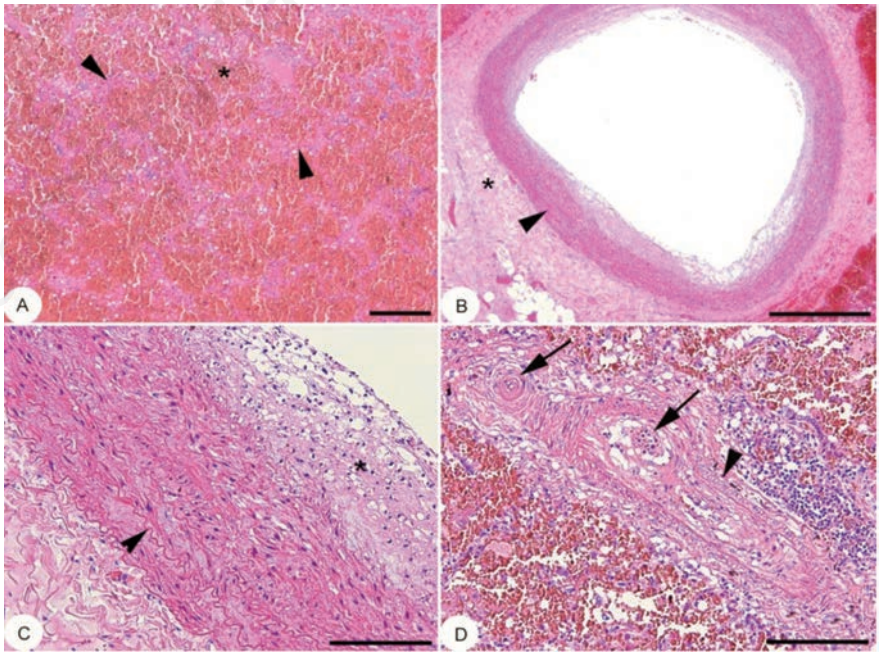

Figure 3. Histopathology. A) The lung parenchyma is diffusely modified by an endoalveolar hemorrhage (asterisk) which is delimited by the alveolar septa with exceedingly congested blood vessels (arrowheads). B) The greatest pulmonary artery roots display thickening of their wall (arrowhead) and periadventitial fibrosis (asterisk). C) In this close-up of picture B, the blood vessel wall is thickened as a result of tunica media (arrowhead) and tunica intima (asterisk) enlargement. The tunica media shows increased elastic fiber and smooth muscle cell components, thereby constituting a kind of arterialization. The tunica intima is characterized by fibrosis with edema and some mild inflammatory infiltrate. D) The peripheral pulmonary artery branches show marked thickening which is mainly secondary to smooth muscle cell proliferation. As a result, the relevant vessel lumens (arrows) are exceedingly narrowed. Hematoxylin-eosin staining. Original magnifications: A) x4 (bar 400 μm), B) x2 (bar 1 mm), C) and D) x10 (bar 200 μm); C) and D) x20 (bar 100 μm).

Table 2. Characteristics of genes currently associated with the onset of idiopathic pulmonary arterial hypertension.

Chromosome location	OMIM	Gene	Inheritance	Function
2q23–q24	135100	ACVR1	AD	Activins are dimeric growth and differentiation factors which belong to the transforming growth factor-β (TGF-β) superfamily of structurally related signaling proteins. Activins signal through a heteromeric complex of receptor serine kinases which include at least two type I (I and IB) and two type II (II and IIB) receptors. These receptors are all transmembrane proteins, composed of a ligand-binding extracellular domain with cysteine-rich region, a transmembrane domain, and acytoplasmic domain with predicted serine/ threonine specificity. Type I receptors are essential for signaling; and type II receptors are required for binding ligands and for expression of type I receptors. Type I and II receptors form a stable complex after ligand binding, resulting in phosphorylation of type I receptors by type II receptors. This gene encodes activin A type I receptor which signals a particular transcriptional response in concert with activin type II receptors. Mutations in this gene are associated with fibrodysplasia ossificans progressive.
9q34.1	187300	ENG	AD	This gene encodes a homodimeric transmembrane protein which is a major glycoprotein of the vascular endothelium. This protein is a component of the transforming growth factor β receptor complex and it binds TGFB1 and TGFB3 with high affinity. Mutations in this gene cause hereditary hemorrhagic telangiectasia, also known as Osler-Rendu-Weber syndrome 1, an autosomal dominant multisystemic vascular dysplasia. Alternatively spliced transcript variants encoding different isoforms have been found for this gene.
12q11–q14	178600	ACVRL1	AD	This gene encodes a type I cell-surface receptor for the TGF-β superfamily of ligands. It shares with other type I receptors a high degree of similarity in serine-threonine kinase subdomains, a glycine- and serine-rich region (called the GS domain) precedingthe kinase domain, and a short C-terminal tail. The encoded protein, sometimes termed ALK1, shares similar domain structures with other closely related ALK or activin receptor-like kinase proteins that form a subfamily of receptor serine/threonine kinases. Mutations in this gene are associated with hemorrhagic telangectasia type 2, also known as Rendu-Osler-Weber syndrome 2.
18q21	601366	SMAD2	AD*	The protein encoded by this gene belongs to the SMAD, a family of proteins similar to the gene products of the Drosophila gene 'mothers against decapentaplegic' (Mad) and the C. elegans gene Sma. SMAD proteins are signal transducers and transcriptional modulators that mediate multiple signaling pathways. This protein mediates the signal of the transforming growth factor (TGF)-β, and thus regulates multiple cellular processes, such as cell proliferation, apoptosis, and differentiation. This protein is receptor activation (SARA) protein. In response to TGF-β signal, this protein is pho phorylated by the TGF-β receptors. The phosphorylation induces the dissociation of this protein with SARA and the association with the family member SMAD4. The asscіation with SMAD4 is important for the translocation of this protein into the nucleus, where it binds to target promoters and forms a transcription repressor complex with other cofactors. This protein can also be phosphorylated by activin type 1 receptor kinase, and mediates the signal from the activin. Alternatively spliced transcript variants have been observed for this gene.
2q33	600799	*BMPR2*	AD	This gene encodes a member of the bone morphogenetic protein (BMP) receptor family of transmembrane serine/threonine kinases. The ligands of this receptor are BMPs, which are members of the TGF-β superfamily. BMPs are involved in endochondral bone formation and embryogenesis. These proteins transduce their signals through the formation of heteromeric complexes of two different types of serine (threonine) kinase receptors: type I receptors of about 50-55 kD and type II receptors of about 70-80 kD. Type II receptors bind ligands in the absence of type I receptors, but they require their respective type I receptors for signaling, whereas type I receptors require their respective type II receptors for ligand binding. Mutations in this gene have been associated with primary pulmonary hypertension, both familial and fenfluramine-associated, and with pulmonary venoocclusive disease.
13q13.3	615343	*CAV1**	AD	The scaffolding protein encoded by this gene is the main component of the caveolae plasma membranes found in most cell types. The gene is a tumor suppressor gene candidate and a negative regulator of the Rasp42/44 mitogen-activated kinase cascade Caveolin 1 and caveolin 2 arelocated next to each other on chromosome 7 and express colocalizing proteins that form a stable hetero-oligomeric complex. Mutations in this gene have been associated with Berardinelli-Seip congenital lipodystrophy. Alternatively spliced transcripts encode α and β isoforms of caveolin 1.

Table 3. Mutations associated with the idiopathic pulmonary arterial hypertension phenotype.

Accesion number	Codon change	Amino acid change	Codon number	Disease	Reference
CM0101158	tTGT->CGT	Cys->Arg	123	Primary pulmonary hypertension	Machado *et al.*[21]
CM010163	tCGA->TGA	Arg->X	332	Primary pulmonary hypertension	Thomson *et al.*[22]

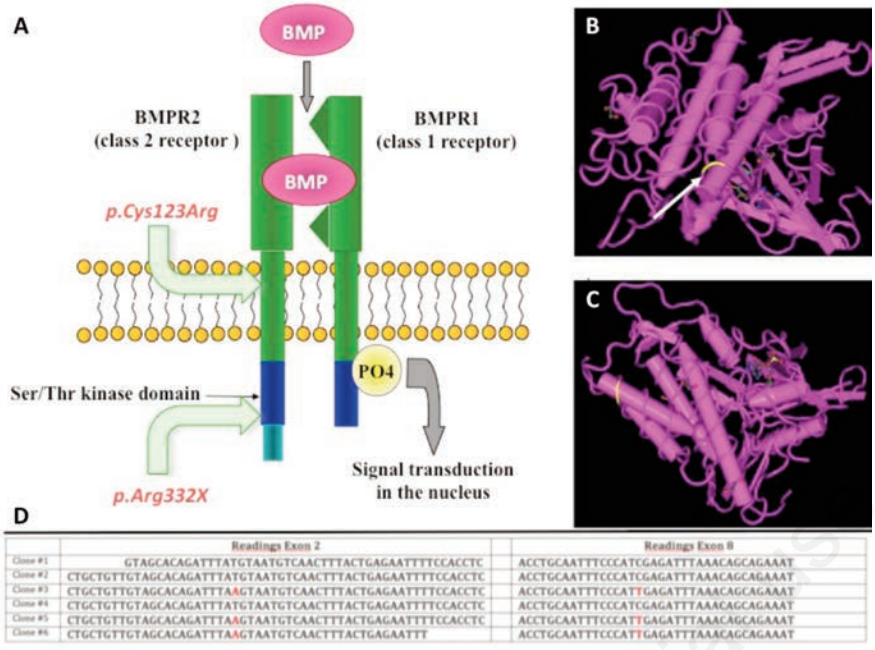

Figure 4. Molecular testing and protein modeling. A) BMPR2 receptor structure highlighting the positions of the two found mutations: the first within the transmembrane domain (p.Cys123Arg) and the second within the Ser/Thr-kinase domain (p.Arg332X). B) and C) PyMol software protein modeling highlighting in yellow the p.Cys123Arg mutation and the p.Arg332X mutation. D) Aligned clones resulting from next generation sequencing and analysis: clones always contain both mutations indicating that both mutations are on the same allele and do not segregate (cis position).

exon #8 (p.Arg332X) (Figure 4). Both mutations have previously been associated with the IPAH phenotype (Table 3)[21,22] and were absent in 250 healthy controls (500 chromosomes); *in silico* analysis PolyPhen-2/Sift) confirmed the pathogenicity of the C123R mutation (data not shown). Additionally, the phase analysis by NGS revealed that both mutations were present on the same allele (*cis*-segregation; Figure 4).

Discussion

Heterozygous germline mutations in the *BMPR2* gene have previously been implicated in the onset of PAH[8] where they have been identified in approximately 10 to 40% of patients with cases of IPAH previously thought to have arisen spontaneously[10,11] and in 58 to 74% of patients with HPAH.[14-16] In this case study of a 38-year-old woman with a history of respiratory disease who presented with severe dyspnea, we identified two mutations in the *BMPR2* gene which were in *cis* configuration. After 6 days of intensive care the patient had a severe fatal hemodynamic compromise which we feel could be attributed to the presence of the double/compound mutation of the *BMPR2* gene identified. We speculate that gene dosage could have affected the severity of the PAH phenotype, which has been previously reported for other inherited cardiovascular diseases.[23] Girolami *et al.* described the clinical outcome of four patients with triple mutations in genes coding for sarcomere proteins and concluded that the multiple mutations conferred an increased risk of end-stage progression and ventricular arrhythmia.[23]

The major limitation of the present study is the family cascade screening: we could not have access either to the medical records and genetic material of parents and siblings. The presence of cis-mutations of the *BMPR2* suggests that they might arise from one parental allele. However since both of them are living and self-reporting healthy, it can be speculated that one mutation is inherited and the second happened *de novo* in the germ line.

Carrying a single *BMPR2* mutation *per se* does not imply developing PAH since heterozygous carriers of disease-causing *BMPR2* mutations only have 20% chance of developing PAH over life times.[24] Thus multiple hits (including compound/double heterozygous mutations) are required to cause disease onset and progression in the BMPR2 mutations' carriers.

Taken together, these cardiac, genetic and pathological findings - although limited to the index case - provide further evidence of the role of the *BMPR2* gene in PAH and its potential as a target for novel therapeutic interventions. In addition, the detection of these mutations may allow preclinical diagnosis of family members of the patient we observed who are currently asymptomatic but at risk of the onset of PAH disease symptoms.

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