Pulmonary arterial hypertension (PAH) is a progressive, severe, potentially fatal disease with an estimated incidence of approximately 1–2 patients per million per year. In the absence of treatment, PAH leads to death with a median survival of 2.8 years for adults.

Editorial p 1329

The latest classification of PAH, the “Dana Point classification”, proposed 5 subgroups of PAH: idiopathic PAH (IPAH); heritable PAH (HPAH); drug- and toxin-induced PAH; PAH associated with other diseases such as collagen disease, HIV infection, portal hypertension, congenital heart disease, schistosomiasis and chronic hemolytic anemia; and persistent pulmonary hypertension of the newborn (PPHN). IPAH corresponds to sporadic disease in which there is neither a family history of PAH nor an identified risk factor. HPAH is inherited in an autosomal dominant fashion with 10–20% penetrance and affects females approximately twice as often as males.

Bone morphogenetic protein (BMP) receptor 2 (BMPR2), a member of the transforming growth factor (TGF)-β superfamily, was identified as a primary gene for HPAH on chromosome 2q33 in 2000. BMPR2 mutations have been identified in more than 70% of subjects with one or more affected relatives and 11–40% of IPAH. Other studies of the TGF-β superfamily revealed 2 further genes responsible for PAH. Heterozygous mutations of activin receptor-like kinase 1 gene (ALK1), located on chromosome 12q13, were demonstrated in patients with hereditary hemor-
rhagic telangiectasia (HHT) in association with PAH in 2001. Furthermore, Harrison et al demonstrated a ALK1 mutation in 1 IPAH patient without a family history of HHT in 2005. In addition, we reported 5 ALK1 mutations in children with IPAH/HPAH. More rarely, mutations in endoglin (ENG) have been identified in patients with PAH, predominantly with coexistent HHT. In 2009, we reported the first nonsense mutation of SMAD8 in an IPAH patient who had no mutations in BMPR2 or ALK1. In the same year, another group reported that pulmonary hypertension and lung tumorigenesis are promoted in Smad8 mutant mice, which reinforced our hypothesis that SMAD8 is involved in the pathogenesis of IPAH. These genetic studies have considerably increased our understanding of the molecular basis of PAH. However, almost 30% of HPAH cases and 60–90% of IPAH cases have no mutations in BMPR2, ALK1, and SMAD8.

We hypothesized that other genes that belong to the TGF-β superfamily or other signal pathways (BMP/MAP kinase p38 pathway, Toll-like pathway, Rho-kinase pathway, and so on) might be associated with the onset of IPAH/HPAH. At the beginning, we attempted to screen for 10 genes: ENG, SMAD1, SMAD2, SMAD3, SMAD4, SMAD5, SMAD6, SMAD7, BMP receptor type 1A (BMPR1A) and BMP receptor type 1B (BMPR1B), involved in the TGF-β/BMP signaling pathway in IPAH/HPAH patients who had no mutations in BMPR2, ALK1, and SMAD8.

**Methods**

**Subjects**

Seventy-four unrelated IPAH/HPAH patients were recruited from Tokyo Women’s Medical University, Toho University, Tohoku University, Kagoshima University, Sakakibara Memorial Hospital, National Hospital Organization Nagasaki Medical Center, Social Insurance Chukyo Hospital, Toyama University, Keio University, Nagano Children’s Hospital, Kyoto Second Red Cross Hospital, Tsukuba University, Hokkaido Children’s Hospital and Medical Center, Gunma Children’s Medical Center, Okinawa Prefectural Nambu Medical Center and Children’s Medical Center, Okayama University, Oita University, Shizuoka Children’s Hospital, Kitano Hospital and Beijing Anzhen Hospital (Figure 1). These subjects include 21 IPAH/HPAH patients from our previous study and 21 patients from the second cohort of another study. The diagnosis of IPAH/HPAH was made through clinical evaluation, chest radiography, electrocardiography, echocardiography and cardiac catheterization on the basis of current international consensus criteria; mean pulmonary artery pressure >25 mmHg at rest or >30 mmHg during exercise. Patients with PAH associated with another disease such as portal hypertension, congenital heart disease including small ventricular septal defect and atrial septal defect and PPHN were excluded from this study by trained cardiologists. This study was approved by an institutional review committee of Tokyo Women’s Medical University. Written informed consent was obtained from all patients or their guardians in accordance with the Declaration of Helsinki.

**Molecular Analysis**

Genomic DNA was prepared from peripheral blood lymphocytes or lymphoblastoid cell lines transformed by the Epstein-Barr virus, as described previously. The BMPR2 and ALK1 coding regions and exon-intron boundaries were amplified from genomic DNA using primers, as described in previous reports (PRIMER information was obtained from Deng et
Mouse pcDNA3.0-hemagglutinin (HA)-Bmpr1b, human pcDNA3.0-hemagglutinin (HA)-Bmpr1b, constitutively active (ca) Bmpr1b was generated by mutation of genes belonging to the BMP signal pathway. After direct sequencing of BMP2R and ALK1, multiplex ligation-dependent probe amplification (MLPA) was used to detect exonic deletions/duplications of BMP2R, ALK1 and ENG in 49 patients who had no mutations in BMP2R and ALK1. MLPA was performed with 100 ng of genomic DNA according to the manufacturer’s instructions using a SALSA MLPA HHT/PH1 probe set (MRCP-Holland, Amsterdam, The Netherlands). Probe amplification products were run on an ABI 3130xl DNA Analyzer using a GS500 size standard (Applied Biosystems). MLPA peak plots were visualized using GeneMapper software v4.0 (Applied Biosystems). For each sample, peak heights of BMP2R probes were normalized against the sum of all control peaks. Patients’ samples were then normalized to the mean of 3 normal control samples. MLPA analysis revealed that 5 of 49 patients had exonic deletions in BMP2R. Some of these results were described in previous studies. 13,14 The 30 patients who had BMP2R or ALK1 mutations were excluded from this study.

Among the 44 patients with no mutations in BMP2R or ALK1, all coding exons and adjacent intronic regions for ENG, SMAD1, SMAD2, SMAD3, SMAD4, SMAD5, SMAD6 and SMAD8 were amplified using polymerase chain reaction. PCR amplified products were purified and directly sequenced like BMP2R and ALK1. SMAD8 nonsense mutation was detected in 1 patient, as described previously. 14 After the above mutational screening, we also screened SMAD7, BMPRIA and BMPRIB mutations for the remaining 43 patients by direct sequencing (Figure 1). Available data on the characteristics and hemodynamic parameters of the 43 patients with IPAH/HPAH are provided in Table S1 in the online supplement.

All sequences generated were compared with wild-type BMP2R (GenBank NM_001204), ALK1 (GenBank NM_000020), ENG (GenBank NM_000118), SMAD1 (GenBank NM_005900), SMAD2 (GenBank NM_005901), SMAD3 (GenBank NM_005902), SMAD4 (GenBank NM_005359), SMAD5 (GenBank NM_005903), SMAD6 (GenBank NM_005585), SMAD7 (GenBank NM_005905), BMPRIA (GenBank NM_000009) and BMPRIB (GenBank NM_001203).

When a mutation was detected, we confirmed that it was not present in 450 healthy controls by direct sequencing.

Preparation of Plasmid

Mouse pcDNA3.0-hemagglutinin (HA)-Bmpr1b, human pcDNA3.0-6xMyc-SMAD8, pcDNA3.0-SMAD4 and BMP-responsive promoter reporter construct 3GC2-Lux are kindly provided by Dr K. Miyazono (Tokyo, Japan). 3GC2-Lux contains 3 repeats of a GC-rich sequence derived from the proximal BMP response element in the Smad6 promoter. 21 We previously utilized the 3GC-Lux reporter gene for functional analysis of the SMAD8 mutant in PAH patients. 14 In other reports, 3GC2-Lux has also been used to assess the interaction of genes belonging to the BMP signal pathway. 22-24 Mouse constitutively active (ca) Bmpr1b was generated by mutation of Gln-203 into aspartic acid.

Site-directed mutagenesis was carried out using a site-directed mutagenesis kit (Stratagene, CA, USA). The constructed plasmids were verified by sequencing. The antibodies used were as follows: anti-HA rat antibody (Roche, Mannheim, Baden-Württemberg, Germany), anti-Myc rabbit antibody (Cell Signaling Technology, MA, USA) and anti-phospho-Smad1/5/8 and Smad8 rabbit antibody (Cell Signaling Technology). A human BMP4 enzyme-linked immunosorbent assay was from R&D Systems (Abingdon, Oxon, UK).
fade reagent with 46'-diamidino-2-phenylindole-2 HCl (DAPI) (Invitrogen). Fluorescence digital images were recorded with an LSM 5 PASCAL Laser Scanning Microscope (Carl Zeiss, New York, NY, USA).

**Statistical Analysis**

All results are expressed as mean±SD. For the statistical comparison of 2 samples, a 2-tailed Student’s t-test was used where applicable. Values of P<0.05 were considered significant. Statistical analyses were performed using JMP for Windows (version 8; SAS Institute, NC, USA).

**Results**

**Sequence Analysis**

We screened mutations in *SMAD7, BMPRIA* and *BMPR1B* genes in 43 patients with IPAH/HPAH who had no mutations in *BMPR2, ALK1* and *SMAD8* (Figure 1). In this study, no mutations were identified in *SMAD7* and *BMPR1A*.

We identified, however, 2 *BMPR1B* missense mutations in 2 independent probands with IPAH. In proband A, c.479 G>A p.S160N was identified (Figure 2A). In proband B, c.1176 C>A p.F392L was identified (Figure 2A). As depicted in Figure 2B, BMPR1B consists of an extracellular ligand-binding domain, a transmembrane domain, an amino acid stretch involved in phosphorylation called GS domain and a serine-threonine kinase domain. Mutation S160N is located outside...
of these functional domains, and mutation F392L is located in the serine-threonine kinase domain. The alignment of the BMPR1B protein between 9 distantly related species showed that these amino acids are highly conserved (Figure 2C).

**Clinical Characteristics**

**Proband A (Patient No. 11)** When the patient was 6 years old, right ventricular hypertrophy was identified by electrocardiography during a health examination and he was diagnosed with IPAH. The first symptom was mild dyspnea on exercise at 7 years of age. His hemodynamic data at 7 years of age revealed a mean pulmonary arterial pressure (mPAP) of 66 mmHg, right atrial pressure (RAP) of 12 mmHg and cardiac index (CI) of 2.0 L·min⁻¹·m⁻². His condition progressed to World Health Organization (WHO) functional class III at 7 years of age. He has been receiving epoprostenol, home oxygen therapy, a cardiotonic drug, vasodilator, anticoagulant and diuretics since the age of 7 years. His current condition is WHO functional class III at 17 years old. There is no family history of PAH (Figure 2D). His younger brother died of viral encephalitis at 2 years of age. The other family members have not been screened for BMPR1B mutations because their blood
samples were not obtainable. He has no malformation of the digits, limbs and genitalia.

Proband B (Patient No. 18) This patient’s first symptom was syncope at 12 years of age. Her hemodynamic data at 7 years of age revealed a mPAP of 111 mmHg, RAP of 11 mmHg, CI of 3.5 L·min⁻¹·m⁻² and a pulmonary artery wedge pressure of 9 mmHg. Her WHO functional class was III at 13 years of age. She has been receiving epoprostenol, home oxygen therapy, vasodilator, anticoagulant and diuretics since the age of 13 years. Her current condition is WHO functional class II at 22 years old. There is no family history of PAH, but the patient’s 39-year-old father was identified as having the same mutation (Figure 2D, S1). The patient’s mother did not have the same mutation. The other family members have not been screened for BMPR1B mutations because their blood samples were not obtainable. She has no malformation of the digits, limbs and genitalia.

Western Blotting Analysis
The addition of BMP4 induced Myc-SMAD8 phosphorylation in the presence of wild-type or mutant BMPR1B. In addition, BMPR1B F392L with BMP4 increased Myc-SMAD8 phosphorylation more than wild-type BMPR1B and BMPR1B S160N (Figures 3A, B).

As shown in Figures 4A and B, additional Western blotting analysis revealed that the ca-BMPR1B F392L mutant induced Myc-SMAD8 phosphorylation more strongly than ca-BMPR1B and ca-BMPR1B S160N.

Luciferase Assay
We investigated the transcriptional activity mediated by wild-type or mutant BMPR1B and SMAD8 with or without SMAD4 to determine whether mutant BMPR1B could increase BMP-responsive promoter-reporter activity.

The first luciferase assay showed that, after stimulation with human BMP4, BMPR1B F392L and SMAD8 with or without SMAD4 induced higher activity than wild-type BMPR1B and SMAD8 with or without SMAD4 (Figure 5A).

The second luciferase assay showed that both ca-BMPR1B S160N with SMAD8 and ca-BMPR1B F392L with SMAD8 increased BMP responsive activity, and both ca-BMPR1B mutant with SMAD8 and SMAD4 induced higher activity than ca-BMPR1B with SMAD8 and SMAD4. In particular, ca-BMPR1B-F392L with SMAD8 and SMAD4 induced approximately 2-fold higher activity than ca-BMPR1B with SMAD8 and SMAD4 (Figure 5B).

Immunocytochemistry
COS1 cells transfected with the wild-type or mutant BMPR1B were subjected to immunofluorescence staining. Wild-type and 2 mutants exhibited intense and equal staining of plasma membrane and cytoplasm (Figure S2), suggesting that intracellular production and transportation of BMPR1B were not affected by these mutations.

Discussion
In this study, we first describe 2 missense mutations in BMPR1B.
BMPR1B mutations in Childhood IPAH

in IPAH patients. BMPR1B is a member of the BMP family that belongs to the TGF-β superfamilly. The TGF-β/BMP signal pathway has 2 types of receptors. There are 7 type 1 receptors (ALK1, ALK2, BMPR1A known as ALK3, ALK4, ALK5, BMPR1B known as ALK6 and ALK7) and 5 type 2 receptors (ActR2A, ActR2B, TGF-βR 2, AMHR2 and BMPR2). BMPs bind independently to both type 1 and type 2 receptors. For example, BMP4 can bind to one of the type 1 receptors, BMPR1B, and one of the type 2 receptors, BMPR2. Upon ligand binding, the type 2 receptors phosphorylate and activate the type 1 receptors. The activated type 1 receptors propagate the signal by phosphorylating a family of transcription factors, called Smads. BMPR1B activates SMAD1, SMAD5 and SMAD8 by phosphorylation. These activated Smads complex with a common partner Smad, SMAD4, and accumulate in the nucleus where they interact with transcriptional regulators for target genes. 

BMPR1B mutations are known to be associated with brachydactyly type A2 and type C/sympathalangism-like phenotype. Acromesomelic chondrodysplasia with genital anomalies is also associated with BMPR1B mutations. Four missense mutations (p.I200K, p.Q249R, p.R486Q and p.R486W) and 1 coexistence with gain-of-function because case BMPR1B mutation leads to PAH. In addition, ALK1 mutation in HHT revealed the down-regulation of SMAD1/5/8 signaling, and SMAD8 nonsense mutation that we identified in IPAH revealed a gain-of-function in our experimental condition.

It has been hypothesized that an imbalance of increased TGF-β levels and decreased BMP signals induced by BMPR2 mutation leads to PAH. In addition, ALK1 mutation in HHT revealed the down-regulation of SMAD1/5/8 signaling, and SMAD8 nonsense mutation that we identified revealed loss of BMP signals. Our findings conflict with the above hypothesis because the BMPR1B mutations caused promotion of BMP signals. BMPR1B is expressed in human pulmonary arterial smooth muscle cells (PASMCs) at higher levels than in human microvascular endothelial cells and human pulmonary artery endothelial cells. A previous report suggested that BMPR1B plays an important role in PASMC mitosis of PAH patients. Quantification using real-time PCR revealed that the BMPR1B expression in PASMCs of PAH patients was more than 10-fold higher than in PASMCs of controls. This report suggests that BMP signals through BMPR1B are promoted in PASMCs of IPAH patients. Because BMPR1B S160N and F392L, promoted BMP signals in the present study, they might be associated with the pathogenesis of PAH. Furthermore, another study reported that disruption of BMP2 led to diminished signaling by BMP2 and BMP4, and augmented signaling by BMP6 and BMP7 in PASMCs. Thus, not only inhibition but also promotion of BMP signals might be associated with the onset of PAH.

In this study, 1 BMPR1B mutant, F392L, strongly induced SMAD8 phosphorylation and increasing transcriptional activation in the presence of SMAD8 or SMAD8/SMAD4. In contrast, another mutant, S160N, did not induce them so markedly. This difference of effect might depend on the position of mutation in the BMPR1B gene. Mutation F392L is located in one of the functional domains, the serine-threonine kinase domain, but mutation S160N is located outside of the functional domains. However, mutation S160N might also cause gain-of-function because ca-BMPR1B S160N with SMAD8 and SMAD4 induced higher activity than ca-BMPR1B with SMAD8 and SMAD4 in the luciferase assay. We guess that coexistence with SMAD8 and SMAD4 is more physiological than with SMAD8 only in a living body, so these results might be appropriate. Several studies have revealed that BMP2 mutations that are located outside of the functional domains were identified in IPAH/PAH patients, PVOD patients and associated-PAH patients. There might be an unknown special function outside of the already-known functional domains in both BMPR2 and BMPR1B. Further functional analysis on mutant S160N is needed.

The age at onset of our patients with a BMPR1B mutation was younger than that of other IPAH patients. It was difficult to identify other differences in their phenotypes. The father of Proband B has the same BMPR1B mutation, but he has no clinical signs of PAH to date. This is not surprising because BMPR2 mutations have very low penetrance in familial PAH.

It is necessary to study an increased number of subjects to investigate the phenotype of PAH with BMPR1B mutation in detail. In addition, to undertake further analysis of the function of BMPR1B in the pathogenesis of PAH, further investigations using human PASMCs and/or animal models with BMPR1B mutation will be necessary.

Acknowledgments

We are grateful to the patients and their family members. We thank Dr Kohei Miyazono for providing the plasmids. We thank Dr Hisato Yagi, Dr Yoshihisa Matsuishi and Dr Maya Fujiwara for their excellent technical assistance. We also thank Dr Kumiko Matsuo for her supervision.

Funding Sources

None.

Disclosures

None.

References

to hereditary haemorrhagic telangiectasia. J Med Genet 2003; 40:
865–871.

haemorrhagic telangiectasia and dexfenfluramine associated pulmo-

NW, et al. Transforming growth factor-beta receptor mutations and
pulmonary arterial hypertension in childhood. Circulation 2005; 111:
435–441.

13. Mache CJ, Gamillscheg A, Popper HH, Haworth SG. Early-life pul-
monary arterial hypertension with subsequent development of diffuse
pulmonary arteriovenous malformations in hereditary haemorrhagic

14. Shintani M, Yagi H, Nakayama T, Saji T, Matsuoka R. A new non-
sense mutation of SMAD8 associated with pulmonary arterial hyper-

15. Huang Z, Wang D, Stansbury KI, Jones PL, Martin JF. Defective
pulmonary vascular remodeling in Smad8 mutant mice. Hum Mol

16. Herpin A, Cunningham C. Cross-talk between the bone morphoge-
etic protein pathway and other major signaling pathways results in
tightly regulated cell-specific outcomes. FEBS J 2007; 274: 2977–
2985.

17. Fukumoto Y, Shimokawa H. Recent progress in the management of

18. Rich S, Dantzker DR, Ayres SM, Bergofsky EH, Brundage BH,
Detre KM, et al. Primary pulmonary hypertension: A national pro-

K. Regional location of novel yes-related proto-oncogene, syn, on
human chromosome 6 at band q21. Jpn J Cancer Res 1986; 77:
279–282.

CE, et al. The activin receptor-like kinase 1 gene: Genomic structure
and mutations in hereditary hemorrhagic telangiectasia type 2. Am J

K, et al. Smad6 is a Smad1/5-induced smad inhibitor: Characteriza-
tion of bone morphogenetic protein-responsive element in the mouse

22. Murakami G, Watabe T, Takaoka K, Miyazono K, Imamura T. Co-
operative inhibition of bone morphogenetic protein signaling by

Synergistic effects of different bone morphogenetic protein type I
receptors on alkaline phosphatase induction. J Cell Sci 2001; 114:
1059–1061.

24. Mochizuki T, Miyazaki H, Hara T, Furuya T, Imamura T, Watabe T,
et al. Roles for the MH2 Domain of Smad7 in the specific inhibition
of transforming growth factor-β superfamily signaling. J Bio Chem

25. Laurent D, Jean-Jacques F, Sabine B. Emerging role of bone mor-
phogenetic proteins in angiogenesis. Cytokine Growth Factor Rev

26. Murakami M, Kawachi H, Ogawa K, Nishino Y, Funaba M. Recep-
tor expression modulates the specificity of transforming growth

et al. Mutations in bone morphogenetic protein receptor 1B cause
brachydactyly type A2. Proc Natl Acad Sci USA 2003; 100:
12277–12282.

et al. A novel R48EQ mutation in BMPR1B resulting in either a
brachydactyly type C/symphalangism-like phenotype or brachydac-

29. Demirhan O, Türkmen S, Schwabe GC, Soyupak S, Akgül E, Tastemir
D, et al. A homozygous BMPR1B mutation causes a new subtype of
acromesomelic chondrodysplasia with genital anomalies. J Med Genet

of pulmonary arterial hypertension: New insights from genetic stud-

analysis of mutations in the kinase domain of the TGF-beta receptor
ALK1 reveals different mechanisms for induction of hereditary hem-

32. Upton PD, Long L, Trembath RC, Morrell NW. Functional charac-
terization of bone morphogenetic protein binding sites and Smad1/5
activation in human vascular cells. Mol Pharmacol 2008; 73: 539–
552.

al. Characterization of the bone morphogenetic protein (BMP) sys-
tem in human pulmonary arterial smooth muscle cells isolated from
a sporadic case of primary pulmonary hypertension: Roles of BMP
type 1B receptor (activin receptor-like kinase 6) in the mitotic action.

34. Yu PB, Beppu H, Kawai N, Li E, Bloch KD. Bone morphogenic
protein (BMP) type II receptor deletion reveals BMP ligand-specific
gain of signaling in pulmonary artery smooth muscle cells. J Biol

outcomes of pulmonary arterial hypertension in carriers of BMPR2

al. BMPR2 germline mutations in pulmonary hypertension associ-

al. BMPR2 mutations in pulmonary arterial hypertension with con-

Supplementary Files

Table S1. Baseline Characteristics and Hemodynamic Parameters of
43 IPAH/HIPAH Patients

Figure S1. Sequence analysis of BMPR1B mutation in the family of
proband B.

Figure S2. Subcellular distribution of wild-type BMPR1B, BMPR1B
S160N and BMPR1B F392L in COS1 cells.

Please find supplemental file(s):