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作成者（著者）	Yasuko, Kojima / Masaki, Shintani / Tomotaka, Nakayama / Yoshiyuki, Furutani / Tsutomu, Saji / Toshio, Nakanishi
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Original Article

Analysis of Genetic Mutations Related to TGF-β/BMP Signaling in Children and Adults with Pulmonary Arterial Hypertension

Yasuko Kojima[1,3] Masaki Shintani[3] Tomotaka Nakayama[2]
Yoshiyuki Furutani[3] Tsutomu Saji[2] and Toshio Nakanishi[3]*

[1]Department of Pediatrics (Sakura), School of Medicine, Faculty of Medicine, Toho University
[2]Department of Pediatrics (Omori), School of Medicine, Faculty of Medicine, Toho University
[3]Department of Pediatric Cardiology, Tokyo Women's Medical University

ABSTRACT

Background: Pulmonary arterial hypertension (PAH) is a vascular disorder characterized by increased pulmonary vascular resistance and right heart failure. Mutations in the *bone morphogenic protein receptor 2* (*BMPR2*), *endoglin* (*ENG*), and *activin receptor-like kinase 1* (*ALK1*) genes, which belong to the transforming growth factor-β (TGF-β) superfamily, have been implicated in PAH pathogenesis. In a previous study, we identified 18 *BMPR2* mutations and seven *ALK1* mutations in 57 pediatric cases. In further screening of mutations in 11 genes (*ENG*, *SMAD1–8*, *ALK3*, *ALK6*) in the TGF-β superfamily, we found one mutation in *SMAD8* and two mutations in BMPR1B (*ALK6*) among PAH patients who had no mutations in *BMPR2*, *ENG*, or *ALK1*. We thus examined the correlation with TGF-β/bone morphogenic protein (BMP) signaling in the present study.

Methods: In this study, we conducted a genetic analysis of *BMPR2*, *ALK1*, *ENG*, *SMAD8*, and *ALK6* in 21 adults with PAH, then further analyzed *SMAD1–7*, *ALK2–5*, and *ALK7* in those with no mutations in *BMPR2*, *ALK1*, *ENG*, *SMAD8*, or *ALK6*. In addition to our previous study, we screened mutations of four genes (*ALK2*, *ALK4*, *ALK5*, and *ALK7*) in 29 children with PAH but no mutations in *BMPR2*, *ALK1*, *ALK3*, *ALK6*, *ENG*, or *SMAD1–8*.

Results: In this study, we identified nine *BMPR2* mutations and one *ALK1* mutation in adult PAH. We analyzed 11 adult patients with idiopathic PAH (IPAH) and no mutations in *BMPR2*, *ALK1*, *ENG*, *SMAD8*, *ALK6*, or 12 other genes (*SMAD1–7*, *ALK2–5*, *ALK7*) and 29 child patients with IPAH and no mutations in *BMPR2*, *ALK1*, *ALK3*, *ALK6*, *ENG*, *SMAD1–8*, or four other genes (*ALK2*, *ALK4*, *ALK5*, *ALK7*). No genetic mutations were found. The *ALK1* mutation was 2.6 times as frequent in children (12.3%; 7/57) as in adults (4.8%; 1/21). However, PAH symptom severity was not correlated with the presence or absence of genetic mutations.

Conclusion: In adults with IPAH, we identified mutations in *BMPR2* and *ALK1* but not in other genes belonging to the TGF-β superfamily. A few new disease-related genes were recently found in an expanded analysis; however, it is likely that as yet undiscovered mutations are involved in PAH pathogenesis.

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KEYWORDS: pulmonary arterial hypertension, TGF-β superfamily, *BMPR2*

1) 564–1 Shimoshizu, Sakura, Chiba 285–8741
2) 6–11–1 Omorinishi, Ota, Tokyo 143–8541
3) 8–1 Kawada, Shinjuku, Tokyo 162–8666
*Corresponding Author: tel: 03(3353)8112
e-mail: pnakanis@hij.twmu.ac.jp
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Idiopathic pulmonary arterial hypertension (IPAH) is a very rare progressive disease with an annual incidence of 1–2 cases per million persons and a poor prognosis. The last decade has seen considerable research in the genetic basis of heritable pulmonary artery hypertension (PAH), including the isolation of specific genes that may be related to its development.[1–7] Family history accounts for 6% of cases of PAH, and its penetrance rate is relatively low (10–20% in adults, 25% in children).[8–12]

The *bone morphogenic protein receptor 2* (*BMPR2*), *endoglin* (*ENG*), and *activin receptor-like kinase1* (*ALK1*) mutations belonging to the transforming growth factor-β (TGF-β) superfamily are involved in PAH pathogenesis. *BMPR2* comprises 13 exons, which include four functional domains: the extracellular ligand binding domain (exons 2 and 3), transmembrane domain (exon 4), serine/threonine kinase domain (exons 5 to 11), and long cytoplasmic tail (exons 12 and 13). The *ALK1* gene comprises 10 exons, while *ENG* comprises 14 exons. The *BMPR2*, *ALK1*, and *ENG* coding regions, exons, and introns were extracted from each patient along with 30–100 bp of genetic information upstream and a similar amount downstream of the boundary.

In 2008, we identified three *BMPR2* mutations and two *ALK1* mutations in 21 children with PAH.[13] We further identified 18 *BMPR2* mutations and seven *ALK1* mutations among 57 children with PAH in 2012.[14] No mutations were identified in around 30% of familial PAH (FPAH) cases and 60–90% of IPAH cases. In 2012, we investigated whether other genes in the TGF-β family are involved in TGF-β/bone morphogenic protein (BMP) signaling and found mutations in children with PAH: one in *SMAD8* and two in *BMPR1B* (*ALK6*).[15, 16] These genes belong to the TGF-β superfamily and are involved in BMP/TGF-β signaling. We hypothesized that other disease genes are associated with the TGF-β superfamily.

Children with PAH and mutations tend to be older at disease onset than those without mutations.[14] We evaluated the prognosis of childhood PAH in relation to World Health Organization functional class (WHO FC) and mortality and found that the mortality rate was worst in *BMPR2* mutation carriers. The clinical features of childhood PAH in the presence or absence of *BNPR2* or *ALK1* mutations did not change significantly. In this study, we screened genes associated with the TGF-β superfamily and assessed the results in light of our previous findings.

Methods

The subjects were 21 adults (age≥16 years) with IPAH or FPAH who received treatment at several hospitals during 2001–2012. Blood samples were obtained from all subjects at either Toho University Medical Center Omori Hospital, Tokyo Women's Medical University Medical Center East, Tohoku University Hospital, University of Tsukuba Hospital, Keio University Hospital, or Nagano Chuo Hospital.

IPAH/FPAH was diagnosed on the basis of clinical symptoms as well as findings from chest X-rays, electrocardiography, echocardiography, and cardiac catheterization, in accordance with criteria for WHO FC. We obtained informed consent from all patients and/or their guardians at the relevant treatment facilities, and great care was taken to ensure that each patient's identity remained anonymous.

In previous studies, we identified 18 *BMPR2* mutations, seven *ALK1* mutations, one *SMAD8* mutation, and two *BMPR1B* (*ALK6*) mutations in 57 children with PAH (age< 16 years) who received treatment at several hospitals through March 2011.[13–16] In this study, the 21 adult patients with PAH (four with FPAH, 17 with IPAH) were screened for mutations in *BMPR2*, *ALK1*, and *ENG* (Table 1). Genomic DNA was extracted from the peripheral blood leucocytes of the patients or from their lymphoblastoid cell lines transformed by the Epstein-Barr virus as described previously.[17]

Specimens from children were analyzed as described in our previous report.[16] The results of genetic analysis of adults with PAH were compared with the reported clinical profiles of the specimens collected from the 57 children (age<16 years) with IPAH or FPAH. The amplified products were purified using QIAquick polymerase chain reaction (PCR) purification (QIAGEN, Hilden, Germany) and screened with bidirectional direct sequencing with an Applied Biosystems (ABI) 3130xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Regarding *BMPR2* and *ALK1* mutation-negative samples, multiplex ligation-dependent probe amplification (MLPA) was used to detect exonic deletions or duplications in *BMPR2*, *ALK1*, and *ENG*. MLPA was performed using 100 ng of genomic deoxyribonucleic acid (DNA) according to the manufacturer's instructions, using a SALSA MLPA HHT/PPH1 probemix (MRC-Holland, Amsterdam, Netherlands). The probe amplification products were run

Table 1 Clinical characteristics of patients

		Adult (n = 21)	Child (n = 57)
Baseline characteristics	Age at diagnosis (years)	43.3 ± 18.3	8.5 ± 3.9
	Gender (Male/Female)	5/16	26/31
	Familial PAH	4	10
Hemodynamic parameters	mPAP (mmHg)	54.2 ± 14.2 (n = 12)	64.4 ± 19.8 (n = 47)
	RAP (mmHg)	8.1 ± 4.1 (n = 16)	6.7 ± 3.4 (n = 47)
	CI (litres. min-1. m-2)	2.4 ± 0.6 (n = 13)	3.2 ± 0.9 (n = 44)
	PAWP (mmHg)	10.3 ± 3.8 (n = 16)	8.8 ± 2.5 (n = 44)

Data are expressed as mean ± SD.
mPAP: mean pulmonary artery pressure, RAP: right atrial pressure, CI: cardiac index, PAWP: pulmonary artery wedge pressure

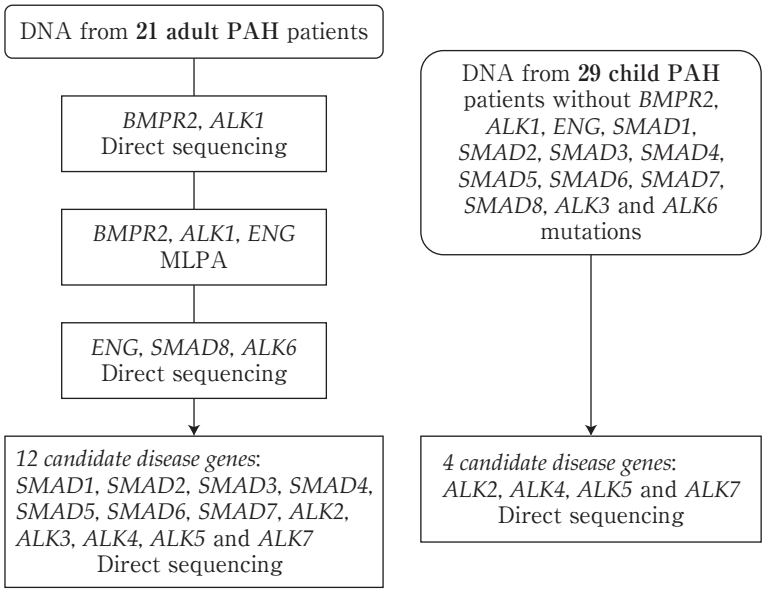

Fig. 1
Screening for *bone morphogenic protein receptor 2* (*BMPR2*), *endoglin* (*ENG*), *activin receptor-like kinase* (*ALK*) *1–7*, and *SMAD1–8* in 21 adults with pulmonary arterial hypertension (PAH), as compared with findings from 29 children with PAH.
MLPA: multiplex ligation-dependent probe amplification

on an ABI 3130xl DNA Analyzer using a GS500 size standard (Applied Biosystems). MLPA peak plots were visualized using GeneMapper® software version 4.0 (Applied Biosystems).

We screened *SMAD8* and *ALK6* mutations in 11 adult PAH patients who had no *ALK1*, *BMPR2*, or *ENG* mutations. We then screened 12 genes in the TGF-β superfamily (*SMAD1*, *SMAD2*, *SMAD3*, *SMAD4*, *SMAD5*, *SMAD6*, *SMAD7*, *ALK2*, *ALK3*, *ALK4*, *ALK5* and *ALK7*) in 11 patients with no *ALK1*, *BMPR2*, *ENG*, *SMAD8* or *ALK6* mutations.

Among the pediatric PAH cases, 18 *BMPR2* mutations and seven *ALK1* mutations were identified in our previous study.[14] We screened the 29 cases with no *BMPR2*, *ALK1*, *ALK3*, *ALK6*, *ENG* or *SMAD1–8* mutations for *ALK2*, *ALK4*, *ALK5* and *ALK7* mutations. All additional screening was performed using the same procedures utilized for DNA amplification and direct sequencing of *ALK2*, *ALK4*, *ALK5* and *ALK7* described above. When a mutation was detected, we confirmed that it was not present in 200 healthy controls by direct sequencing (Fig. 1).

The severity of clinical symptoms in each patient was classified according to WHO FC and then compared with the patient's obtained genetic information. Disease sever-

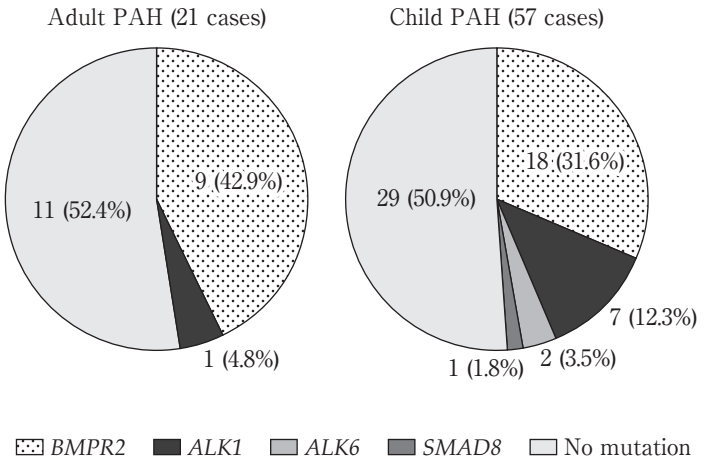

Fig. 2
Genetic mutations in adults and children with pulmonary arterial hypertension (PAH).
BMPR: bone morphogenic protein receptor, ALK: activin receptor-like kinase

Table 2 Mutation status and clinical characteristics of patients

Age at diagnosis

	Adult (n = 21)	Child (n = 57)
BMPR2 mutation carriers	38.1 ± 18.2 (n = 9)	9.1 ± 3.7 (n = 18)
ALK1 mutation carriers	34 ± 0 (n = 1)	9.3 ± 4.2 (n = 7)
ALK6 mutation carriers	NA (n = 0)	9.0 ± 3.0 (n = 2)
SMAD8 mutation carriers	NA (n = 0)	8 ± 0 (n = 1)
mutation noncarriers	50.7 ± 19.6 (n = 11)	8.0 ± 4.0 (n = 29)

Familial PAH

	Adult (n = 21)	Child (n = 57)
BMPR2 mutation carriers	4 (n = 9)	3 (n = 18)
ALK1 mutation carriers	0 (n = 1)	4 (n = 7)
ALK6 mutation carriers	NA (n = 0)	0 (n = 2)
SMAD8 mutation carriers	NA (n = 0)	0 (n = 1)
mutation noncarriers	0 (n = 11)	3 (n = 29)

BMPR: bone morphogenic protein receptor, ALK: activin receptor-like kinase

ity was classified as WHO FC I–IV on the basis of symptoms at the time of the initial diagnosis.

All identified mutations were confirmed by comparison with data obtained from 200 healthy controls using the same direct method. The wild-type genes used in this study were *BMPR2* (NM_001204), *ALK1* (NM_000020), *ENG* (NM_000118), *SMAD1* (NM_005900), *SMAD2* (NM_005901), *SMAD3* (NM_005902), *SMAD4* (NM_005359), *SMAD5* (NM_005903), *SMAD6* (NM_005585), *SMAD7* (NM_005904), *SMAD8* (NM_005905), *ALK2* (NM_001111067), *ALK3* (NM_009009), *ALK4* (NM_004302), *ALK5* (NM_004612), *ALK6* (NM_001203), and *ALK7* (NM_145259) (GenBank®).

Quantitative data (Fig. 2 and Table 2, 3) were expressed and analyzed using the chi-square test with Yates correction or the Fisher exact test and *t* test with Welch's correction, when appropriate. Statistical analysis was performed using StatMate IV for Windows (ATMS Co., Ltd., Tokyo, Japan) and Microsoft Excel 2007 (Microsoft Corp., Redmond, WA, USA). A p value less than 0.05 was considered statistically significant.

This study was approved by the ethics committee at

Table 3 Genetic mutations and World Health Organization functional class (WHO FC) at disease onset

WHO FC	Adult (n = 21)		Child (n = 57)	
	Ⅲ Ⅳ (n = 7)	Ⅰ Ⅱ (n = 9)	Ⅲ Ⅳ (n = 15)	Ⅰ Ⅱ (n = 36)
BMPR2 mutation carriers	2	3	4	9
ALK1 mutation carriers	0	0	2	5
ALK6 mutation carriers	0	0	1	1
SMAD8 mutation carriers	0	0	0	1
Mutation noncarriers	5	6	8	20

BMPR: bone morphogenic protein receptor, ALK: activin receptor-like kinase

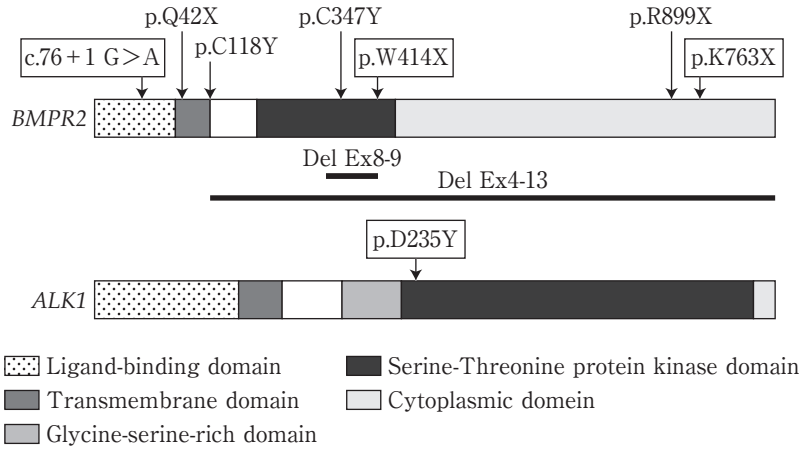

Fig. 3
Mutations discovered, by domain.
New mutations are shown in boxes
BMPR: bone morphogic protein receptor, ALK: activin receptor-like kinase

Tokyo Women's Medical University.

Results

The 11 adults with IPAH who had no *BMPR2*, *ALK1, ENG, SMAD8* or *ALK6* mutations underwent further testing of 12 other genes (*SMAD1*, *SMAD2*, *SMAD3*, *SMAD4*, *SMAD5*, *SMAD6*, *SMAD7*, *ALK2*, *ALK3*, *ALK4*, *ALK5* and *ALK7*). Children without *BMPR2*, *ALK1*, *ALK3*, *ALK6*, *ENG* or *SMAD1–8* mutations underwent further testing for *ALK2*, *ALK4*, *ALK5* and *ALK7*. Although these additional tests revealed multiple single-nucleotide polymorphisms, we found no mutations that appeared to contribute to PAH.

The average age at diagnosis of the 21 adult patients was 43.3 ± 18.3 years; 16 were female (male : female ratio, 1 : 3.2). The average age at diagnosis of the 57 pediatric patients was 8.5 ± 3.9 years; 31 were female (male : female ratio, 1 : 1.2) (Table 1).

Ten of the adult patients had genetic mutations: *BMPR2* in nine (all four adults with FPAH had this particular mutation) and *ALK1* in one. No case had more than one duplicate mutation. All mutations occurred in functional domains. There were four nonsense, two missense, one splicing, and two deletion mutations in *BMPR2*, as well as a missense mutation in *ALK1* (Fig. 3, Table 4).[1, 13, 18–20] The children had 18 mutations in *BMPR2*, seven in *ALK1*, two in *ALK6*, and one in *SMAD8*.

BMPR2 mutations were found in nine of 21 (42.9%) adults and in 18 of 57 (31.6%) children; thus, such mutations were 1.4 times as frequent in adults ($p = 0.353$). An *ALK1* mutation was found in only one of 21 (4.8%) of adults and in seven of 57 (12.3%) of children; thus, such mutations were 2.6 times as frequent in children ($p = 0.582$) (Fig. 2).

Disease onset occurred at a significantly younger age in adults with a mutation in either *BMPR2* or *ALK1* than in adults without such mutations ($p = 0.047$). The difference

Table 4 Genetic abnormalities in adult and pediatric cases[1, 13, 18-20]

No	Mutation exon	Nucleotide Change	Amino Acid Change	Mutation type	Age at diagnosis	1st report
BMPR2 mutation						
A	2	c.124 C>T	Q42X	Nonsense	17	Fujiwara M et al[13]
B	3	c.353 G>A	C118Y	Missense	34	Machado RD et al[18]
C			Del exon 8, 9	Deletion	29	Aldred MA et al[19]
D	12	c.2287 A>T	K763X	Nonsense	54	this study
E	12	c.2695 C>T	R899X	Nonsense	36	Lane KB et al[1]
F*	8	c.1040 G>A	C347Y	Missense	33	Lane KB et al[1]
G*			Del exon 4–13	Deletion	54	Pfarr N et al[20]
H*		c.76+1 G>A		Splice defect	23	this study
I*	9	c.1242 G>A	W414X	Nonsense	38	this study
ALK1 mutation						
J	6	c.703 G>T	D235Y	Missense	34	this study

*Familial case

BMPR: bone morphogenic protein receptor, ALK: activin receptor-like kinase

was not significant when only *BMPR2* was considered (p = 0.055). In contrast, no significant difference was found in age at disease onset between children with and without mutations in *BMPR2*, *ALK1*, *ALK6* or *SMAD8* (p = 0.313). Additionally, there was no significant difference when *BMPR2* (p = 0.364) and *ALK1* (p = 0.478) mutations were considered separately (Table 2).

The FPAH : IPAH ratio did not differ significantly between adults (4 : 17) and children (10 : 47) (Table 2, Fig. 4).

Exertional dyspnea was the most common overall symptom in the present patients with PAH. The ratio of patients with *BMPR2* mutations to those without any mutation was 2 : 5 (40%) for those with FC III/IV PAH and 5 : 11 (45.4%) for those with FC III/IV PAH. This insignificant difference (p = 0.734) indicates that a correlation between the absence or presence of genetic mutation and disease severity is unlikely. Among children, four of 13 (30.7%) of those with *BMPR2* mutations, seven of 23 (30.4%) with any mutation (in *BMPR2*, *ALK1*, *ALK6* or *SMAD8*), and eight of 28 (28.5%) with no mutation had FC III/IV PAH. The proportion of FC III/IV PAH among patients with *BMPR2* mutation only (p = 0.822) and those with any mutation (p = 0.870) did not significantly differ from those with no mutation (Table 3).

Discussion

In an attempt to identify new disease-related genes belong to the TGF-β superfamily in adult and pediatric patients with PAH who have no *BMPR2*, *ALK1*, *SMAD8* or *ALK6* mutations, we found no other genetic mutations. *BMPR2* and *ALK1* belong to the TGF-β superfamily and are therefore related to BMP/TGF-β signaling. In 2011, a genetic analysis of 324 patients with PAH revealed one case of *SMAD1* mutation, two cases of *SMAD4* mutation, and one case of *SMAD8* mutation (all of which were also related to the TGF-β superfamily).[4] These genes appear to have a mutation rate of less than 1%.

In this and other recent studies, approximately half of patients with IPAH had no contributory genetic mutations. Thus, alternative pathways might be responsible for PAH pathogenesis, such as the effect of crosstalk in TGF-β/BMP signaling. Studies of abnormalities in other gene transfers that may not be directly related to PAH but may be involved in its development investigated genes related to crosstalk in TGF-β/BMP signaling (such as Wnt and Notch signals).[21–23] In our recent study, we found two genetic mutations in the *Notch3* gene.[24] Austin et al used next-generation sequencers for whole-exome sequencing (WES) of patients with FPAH and identified a mutation in the caveolin-1 (*CAV1*) gene, in 2012; the potassium channel subfamily K, member 3 (*KCNK3*) gene, in 2013; and the eukaryotic initiation factor 2 alpha kinase 4 (*EIF2AK4*) gene, in 2014.[12, 25–27] Their approach holds promise for identifying new PAH-related genes.

When adults with no *BMPR2* or *ALK1* mutations underwent additional analysis to detect the presence of mutations in genes related to TGF-β/BMP signaling, no such mutation in any new disease-related gene was found.

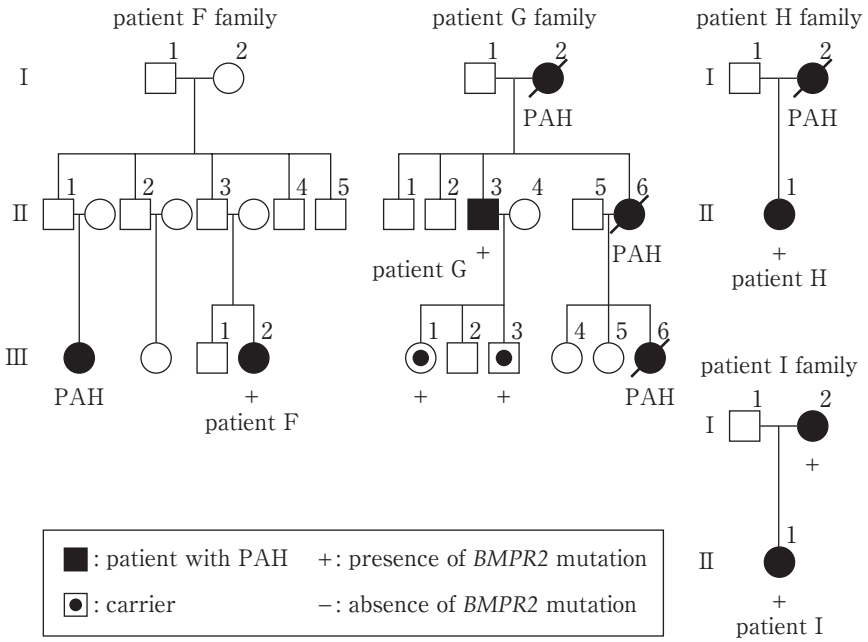

Fig. 4

Family trees of patients with familial pulmonary arterial hypertension (FPAH). *BMPR2* mutations were found in all cases.

Patient F: PAH diagnosed at age 33 years. A paternal cousin had PAH symptoms, but no further information was available.

Patient G: PAH diagnosed at age 54 years. PAH had also been diagnosed in the patient's mother, younger sister, and niece.

Patient H: PAH diagnosed at age 23 years. Her mother also had PAH, which led to sudden death.

Patient I: PAH diagnosed at age 38 years. Her 63-year-old mother has PAH symptoms.

PAH: pulmonary arterial hypertension, BMPR: bone morphogenic protein receptor

Therefore, future research should continue to search for new PAH-related genes using WES, to yield a more complete explanation of the clinical manifestations of PAH.

Despite the current findings, many issues remain unresolved, such as how these new disease-related genes lead to the clinical symptoms of PAH. Thus, additional studies with more patients and more comprehensive evaluations of the effects of each genetic phenotype are necessary.

The present study found that, in patients with *ALK1* mutations, age at disease onset tended to be younger among adults, and older among children, as compared with groups without mutations. Among adults, age at onset in our patients with an *ALK1* mutation was younger than that in other IPAH patients without mutations. Among children, patients with *ALK1* mutations were an average of 1.3 years older than those without mutations. The *ALK1* mutation was 2.6 times as common in children as in adults. Many previous studies reported *ALK1* mutations in patients with hereditary hemorrhagic telangiectasia (HHT). A few previous studies noted that pediatric cases of *ALK1* mutation-related PAH gradually developed into HHT.[28, 29] In this study, we could not investigate these outcomes.

A previous study of PAH patients with *BMPR2* mutations found that disease progression occurred an average of 10 years earlier in those with *BMPR2* mutations than in those without *BMPR2* mutations.[30] We found that children with PAH and *BMPR2* mutations died at a significantly younger age, on average, than did those with no *BMPR2* mutations.[14]

In this study, average age at disease onset among adults with PAH was younger for those with *BMPR2* mutations than for those with no mutations. However, among children, average age at disease onset was older for those with *BMPR2* mutations. In the adult group, patients with *BMPR2* mutations were an average of 12.6 years younger

than those without mutations at disease onset. In contrast, in the child group, patients with *ALK1* mutations were an average of 1.1 year older than noncarriers. These findings suggest that the disease progresses more quickly in children than in adults. However, since the present study did not investigate disease progression in relation to age, this hypothesis must be verified in future studies.

Finally, the present study has some limitations that warrant mention. The number of adulthood PAH patients was small because the main referral source was pediatric cardiologists. Therefore, the present findings cannot be generalized to the entire PAH population. Another limitation is that the study was not observational and therefore the relationship between mutations and PAH prognosis could not be evaluated. Further research with larger and more-balanced groups may yield new and important findings.

Conclusion

We performed genetic analyses of 21 adult patients with PAH and found that nine had *BMPR2* mutations and one had an *ALK1* mutation. Taken together with our previously reported data from children with PAH who underwent genetic analysis, these findings indicate that the children were 2.6 times as likely as the adults to have *ALK1* mutations. Among patients without genetic mutations, we found no new PAH disease-related genes belonging to the TGF-β superfamily. Further genetic analyses using WES and peripheral gene analysis are needed in order to detect crosstalk.

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Conflicts of interest (COI): The authors have no COI to declare.

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