Implications of Mutations of Activin Receptor-Like Kinase 1 Gene (ALK1) in Addition to Bone Morphogenetic Protein Receptor II Gene (BMPR2) in Children With Pulmonary Arterial Hypertension

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Background Mutations of the bone morphogenetic protein receptor II gene (BMPR2), and 1 mutation of the activin receptor-like kinase 1 gene (ALK1) have been reported in patients with pulmonary arterial hypertension (PAH).

Methods and Results A genomic study of ALK1 and BMPR2 was conducted in 21 PAH probands under 16 years of age to study the relationship between the clinical features of the patients and these genes. In all 4 familial aggregates of PAH, 3 ALK1 or 1 BMPR2 mutations were identified. Among 17 probands aged between 4 and 14 years with idiopathic PAH, 2 ALK1 mutations (2/17: 11.8%) and 3 BMPR2 mutations (3/17: 17.6%; 5 mutations in total: 5/17: 29.4%) were found.

Conclusion Each proband with the ALK1 mutation developed PAH, as did the probands with the BMPR2 mutation. Hence, it is proposed that ALK1 plays as notable a role as BMPR2 in the etiology of PAH. Furthermore, asymptomatic carriers with the ALK1 mutation within the serine–threonine kinase domain are at risk of developing PAH and hereditary hemorrhagic telangiectasia, so close follow-up is recommended for those individuals. (Circ J 2008; 72: 127–133)

Key Words: Activin receptor-like kinase 1 gene; Bone morphogenetic protein receptor II gene; Pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is a rare condition, with an annual incidence of 1–2 patients per 10^6 population.¹ It is characterized by sustained elevation of the mean pulmonary artery pressure of more than 25 mmHg and the presence of refractory disorder.² It has been reported that among all PAH patients, those with familial PAH (FPAH) account for at least 6%,³ with 10–20% of penetrance.⁴ Although the disease may occur at any age, PAH is usually diagnosed in the 4th decade of life, with a female-to-male ratio of 1.7:1.³ However, in children, PAH occurs almost equally in boys and girls who often present with relatively severe symptoms, such as syncope.⁵ Without therapy, the disorder progresses rapidly and leads to right heart failure. The median survival time without therapy was reported to be only 10 months for children who are diagnosed under 16 years of age,⁶ whereas it is 2.8 years for adults.⁷,⁸ The prognosis of PAH is not encouraging, although over the past decade new drugs have been being consecutively studied and Rho-kinase inhibitor is the most recent drug under evaluation.⁹

Since the year 2000, de novo and heterogeneous germ-line mutations in the bone morphogenetic protein (BMP) receptor II gene (BMPR2) on chromosome 2q33 have been reported in PAH families.¹⁰,¹¹ BMP is a member of the transforming growth factor-β (TGF-β) superfamily, and its receptor consists of the ligand-binding domain, the serine–threonine kinase domain, and the cytoplasmic tail. This ligand/receptor complex plays an important role in embryogenesis, apoptosis, organ development, cell differentiation, and cell proliferation.¹² In early reports, BMPR2 mutations were reported in Caucasians among whom 45% were FPAH patients,¹³ and 26% were idiopathic PAH (IPAH) patients.¹⁴ Machade et al demonstrated that BMPR2 mutations can occur in various domains of the receptor in PAH patients.¹⁵ Functional studies suggest that BMPR-II mutation in the extracellular and kinase domains is able to disrupt BMPR-II cell membrane localization and its signaling pathways. Mutation in the cytoplasmic domain of the receptor prevents transduction of the BMP signal.¹⁶,¹⁷ Despite vari-
HHT was also found to have the domain. Only 1 PAH patient without a family history of (HHT) in association with PAH.18,19 These missense mutations in patients with hereditary hemorrhagic telangiectasia (HHT) in association with PAH.18,19 These missense mutations of ALK1 were located in the serine–threonine kinase domain. Only 1 PAH patient without a family history of HHT was also found to have the ALK1 mutation in the serine–threonine kinase domain.20 We postulated that ALK1, as well as BMPR2, is involved in the appearance of pediatric PAH, so in our genomic analysis of IPAH and PPAH patients, mutations of both genes were investigated.

Based on histological sections of the pulmonary vascular tissue, some studies have have suggested that the expression of endothelial nitric oxide synthase (eNOS) and of angiotensin-converting enzyme (ACE) is modified in PAH patients.21,22 In previous reports, it was also implied that the D allele of ACE was related to pulmonary hypertension by various causes.23,24 Furthermore, polymorphism in the 894T allele of eNOS and the D allele of ACE could induce rapid progression of high-altitude pulmonary edema because of hypoxic pulmonary vasoconstriction at a high altitude.25,26 Hence, we investigated if polymorphism of eNOS or ACE in PAH patients could influence the severity of the clinical features.

### Methods

#### Subjects

Pediatric patients with PAH were recruited from Toho University and Jikei University School of Medicine. We enrolled 17 probands with sporadic PAH and 4 probands with FPAH. Written informed consent was obtained from all study subjects in accordance with the Declaration of Helsinki. If patients were under 16 years of age, the informed consent was given by their guardians. We assessed each patient by history taking, physical examination, and reviewed their medical records. All assessments were done with the approval of the ethics committees of Tokyo Women’s Medical University, Jikei University School of Medicine, and Toho University. The diagnosis of PAH was determined through clinical evaluation, chest radiography, electrocardiography, echocardiography, and cardiac catheterization based on current international consensus criteria: mean pulmonary artery pressure >25 mmHg at rest or >30 mmHg during exercise.3 PAH patients who had other diseases of the heart and lungs, pulmonary embolism or connective tissue disease because of PAH were excluded from this study by pediatric cardiologists.

#### Molecular Analysis

Genomic DNA was prepared from peripheral blood lymphocytes or lymphoblastoid cell lines transformed by the Epstein-Barr virus, as described previously.27 The ALK1 and BMPR2 coding regions and exon–intron boundaries, including regions approximately 30–100 bp upstream and downstream from the exons, were amplified from genomic DNA using primers, as described in previous reports (PRIMER information was obtained from Deng et al).28 Amplified products were purified using the QIAquick polymerase chain reaction (PCR) purification method (QIAGEN, Hilden, Germany) and screened with bi-directional direct sequencing with a BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). When a mutation was detected, we confirmed that it was not present in 200 Japanese normal chromosomes by single-strand conformational polymorphism (SSCP) analysis, restriction fragment length polymorphism (RFLP) analysis or direct sequencing.

The eNOS Glu298Asp (G894T) polymorphism in exon 7, and the eNOS T–786C polymorphism in the 5′-flanking region were identified by PCR amplification and RFLP, using primers as described previously.30 The ACE insertion/deletion (I/D) polymorphism was amplified from the genomic DNA, using a previously described primer set.31 Detection of the 287-bp I/D polymorphism was performed with PCR using primers that spanned the I/D site and yielded PCR products of 490 bp for the I allele and 190 bp for the D allele. PCR conditions of these genes were modified and the fragments were analyzed by electrophoresis, as described previously.

#### Statistical Analysis

Data on gene polymorphism were compiled according to the allele frequencies. The observed number of alleles from the genotype data in each group was compared using Fisher’s exact test. A p value <0.05 was considered to indicate statistically significant difference.

### Results

#### Clinical Characteristics

Twenty-one unrelated Japanese PAH probands, comprising 9 boys and 12 girls with clinical features, were evaluated (Table 1). In the 17 children with IPAH, the age of onset was between 4 and 14 years (median age: 9 years). Additionally, 4 familial aggregates of PAH were investigated. The clinical features of all probands became WHO functional class III within 3 years after the diagnosis of PAH. Of the 21 probands, 20 underwent cardiac catheterization soon after diagnosis; 1 patient died of a pulmonary hypertension crisis before cardiac catheterization. Their hemodynamic data showed a mean pulmonary arterial pressure of 75±15 mmHg, right atrial pressure 7.7±3.2 mmHg, cardiac index 3.1±1.0 L·min⁻¹·m⁻², and total pulmonary resistance of 27.0±10.7 Wood·Unit⁻¹·m⁻² (Table 1); 38% of the probands had syncope. It was noted that none of the study patients or their family members had clinical symptoms of HHT, such as recurrent spontaneous epistaxis, mucocutaneous telangiectasia, or documented visceral manifestations, which are the current international consensus criteria.

**Table 1 Baseline Characteristics and Hemodynamic Parameters**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Median ± SD or n=21, but 1 patient died before cardiac catheterization.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>9±3.5</td>
</tr>
<tr>
<td>Gender (male)</td>
<td>9/21 (42.9%)</td>
</tr>
<tr>
<td>Familial or sporadic (familial)</td>
<td>4/21 (19.0%)</td>
</tr>
<tr>
<td>Mean pulmonary arterial pressure (mmHg)</td>
<td>75±15</td>
</tr>
<tr>
<td>Right atrial pressure (mmHg)</td>
<td>7.7±3.2</td>
</tr>
<tr>
<td>Cardiac index (L·min⁻¹·m⁻²)</td>
<td>3.1±1.0</td>
</tr>
<tr>
<td>Total pulmonary resistance (Wood·Unit⁻¹·m⁻²)</td>
<td>27.0±10.7</td>
</tr>
</tbody>
</table>

n=21, but 1 patient died before cardiac catheterization. mPAP, mean pulmonary arterial pressure; RAP, right atrial pressure; CI, cardiac index; TPR, total pulmonary resistance.
Additionally, radionuclide perfusion lung scanning, computed tomography scan, and angiography were performed in the probands to exclude the possibility of arteriovenous shunt of lung and liver as a complication of HHT.33,34

**ALK1 Mutation**

In this study, 5 different **ALK1** mutations were identified in 5 of the 21 unrelated probands with PAH, including 2 IPAH probands (11.8%) and 3 FPAH probands (75%) (Table 2, Fig 1). The 3 FPAH probands with the **ALK1** mutations included 1 child whose sister had the same mutation but was without symptoms (Proband 18). The other 2 children with FPAH (Probands 20, 21) were siblings (Table 2). Two of the **ALK1** mutations (Probands 9, 21) were new point mutations. Genomic DNA from the healthy parents of Probands 4 and 9 was not available, because they did not want to undergo gene analysis. The clinical features of the patients with the **ALK1** mutations are described in Table 2 and below.

**Proband 4** At 7 years of age, her condition progressed to WHO functional class III with episodes of syncope. An R479Q mutation was found. Another mutation of this codon 479 (R479X: 1435T → C) has been previously reported in HHT patients with PAH.35

**Proband 9** At 9 years of age, he complained of fatigue and 3 years later, his condition was WHO functional class III without episodes of syncope. A new mutation, L381P, was found.

**Proband 18** At 2 years of age, she was WHO functional class III and subsequently diagnosed with PAH without syncope. An R479Q mutation was found, which is similar to 2 different mutations of codon 484 (R484WfsX493: 1450-1451insG, R484W: 1450C → T) that have been reported previously in HHT patients with PAH.18,35 We found that her sister had the same mutation, but did not have any clinical symptoms of PAH. We confirmed that her mother did not have the same mutation, but we could not confirm the presence of a mutation in her father because the parents were divorced. However, her father was suspected to have the **ALK1** mutation.

**Proband 20** At 7 years of age, his condition progressed to WHO functional class III with episodes of syncope. A P424T mutation was found. This mutation (P424T: 1270C → A) and other mutations of codon 424 (P424S: 1270C → T, P424L: 1271C → T) has been reported previously in HHT patients without PAH.29,36,37 His elder brother was found to have PAH at 9 years of age. Their parents were investigated, and his father had the same mutation but not any symptoms of PAH or HHT.

**Proband 21** At 14 years of age, her condition progressed to WHO functional class III without episodes of syncope. A new mutation, H312Q, was found. Her elder sister with the same mutation was given a diagnosis of PAH at 12 years of age; their father had the same mutation.
Fig 1. Activin receptor-like kinase 1 gene (ALK1) mutation in pulmonary arterial hypertension. (a) DNA sequences showing 936C ▶ T, 1142T ▶ C, 1270C ▶ A, 1436G ▶ A, and 1451G ▶ A mutations in ALK1. (b) Schematic representation of wild-type ALK1 and its mutations. (c) Sequence alignment of ALK1 from different species and from different ALK members. Amino acid substitutions occur at highly conserved positions present in ALK1 orthologues.

Fig 2. Bone morphogenetic protein receptor II gene (BMPR2) mutation in pulmonary arterial hypertension. (a) DNA sequences showing 124C ▶ T, 339C ▶ G, 367T ▶ C, and 1207C ▶ T mutations in BMPR2. (b) Schematic representation of wild-type BMPR-II and its mutations. (c) Sequence alignment of BMPR-II from different species. Amino acid substitutions occur at highly conserved positions present in BMPR-II orthologues.
but no symptoms of PAH ord HHT.

**BMPR2 Mutation**

Three BMPR2 mutations were identified in 3 of the 17 patients with IPAH (17.6%), and 1 BMPR2 mutation was identified in 1 of the 4 patients with FPAH (25%) (Table 2, Fig 2). Two patients with IPAH (Probands 10, 17) had new point mutations, including 1 de novo mutation (Proband 10). Genomic DNA from the healthy parents of Probands 7 and 17 was not available, because they did not want to undergo gene analysis. The clinical features of the patients with BMPR2 mutations are described here and in Table 2.

**Proband 7** The first symptom was mild dyspnea on exercise at 9 years of age. One year later, her condition had progressed to WHO functional class III with episodes of syncope. A de novo mutation, Y113X, was found, but not with adult PAH. There are indications that polymorphism which precipitates the severity of the disease in comparison with adult PAH. There are indications that polymorphism of ACE or eNOS could be a contributing factor to syncope occurrence in PAH, so we investigated the allele frequency of eNOS 894T, eNOS –786C and ACE D. Fisher’s exact probability test was used to analyze the data because there were categories with event occurrence in less than 5 probands. The frequency of these polymorphisms in the PAH probands with syncope were determined to be eNOS 894T allele frequency (38%), eNOS –786C allele frequency (25%), and ACE D allele frequency (75%) (Table 3). Next we compared the allele frequency of these polymorphisms between the 8 probands with syncope and the 13 without syncope, but no significance difference in allele frequency was detected (Table 3). The eNOS 894T allele frequency was found in 38% of the PAH probands with syncope and in 0% of the non-syncope PAH probands, suggesting that this allele frequency could be higher in Japanese pediatric PAH probands with syncope. An increase in patient sample size would increase our understanding of this allele frequency and its correlation with pediatric PAH-associated syncope.

**Discussion**

We set out to determine whether more than 1 mutation of ALK1 could be involved in PAH without HHT. It has been reported that some HHT patients with associated PAH have an ALK1 mutation and also that 1 patient with PAH without HHT had an ALK1 mutation. Our data, with respect to the latter, showed a higher frequency of ALK1 mutations in PAH patients without HHT. The present study showed 5 ALK1 mutations, all of which were located in the serine–threonine kinase domain of highly conserved amino-acid regions. These highly conserved regions were also observed in human ALK1 to ALK7 and throughout metazoan evolution. In previous studies, ALK1 mutations in HHT probands without PAH were randomly located in ALK1 mutation19,36,40 However, ALK1 mutations in PAH patients, with or without HHT, were limited to this conserved serine–threonine kinase domain18–20,35 There have been reports of BMPR2 mutations in both adult and pediatric PAH;13,14 however, to date, the ALK1 mutation in the serine–threonine kinase domain has been observed in pediatric PAH cases only. Hence this study suggests that ALK1 mutations in the serine–threonine kinase domain may increase the risk of PAH in childhood. Mutation in the serine–threonine kinase domain of ALK1, being a TGF– type I receptor, could affect the downstream SMAD signaling pathway. Further investigation is warranted.

In addition, we investigated the clinical symptoms in the probands. In each proband with the ALK1 mutation, as seen in probands with the BMPR2 mutation, their condition was WHO functional class III with 3 years following diagnosis, without clinical symptoms of HHT. Patients with the BMPR2 mutation or ALK1 mutation have a similar frequency of syncope. It was observed that each asymptomatic carrier with the same ALK1 mutation as their child was middle-aged (father of Proband 20: 55 years of age; father

**Table 3 eNOS G894 T, and T-786 C, ACE I/D Allele Frequency in PAH Probands With and Without Syncope**

<table>
<thead>
<tr>
<th></th>
<th>Syncope (+)</th>
<th>Syncope (–)</th>
<th>p value</th>
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<tbody>
<tr>
<td>eNOS G894T polymorphism</td>
<td>3/8 (38%)</td>
<td>0/13 (0%)</td>
<td>0.08</td>
</tr>
<tr>
<td>894T allele-positive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eNOS T-786C polymorphism</td>
<td>2/8 (25%)</td>
<td>5/13 (38%)</td>
<td>0.89</td>
</tr>
<tr>
<td>–786C allele-positive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE I/D polymorphism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DD or DI (D allele-positive)</td>
<td>6/8 (75%)</td>
<td>6/13 (46%)</td>
<td>0.4</td>
</tr>
<tr>
<td>II (D allele-negative)</td>
<td>2/8 (25%)</td>
<td>7/13 (54%)</td>
<td></td>
</tr>
</tbody>
</table>

Fisher’s exact probability test was used for the statistical analysis. eNOS, endothelium nitric oxide synthase; ACE, angiotensin-I converting enzyme; I/D, insertion/deletion. Other abbreviation see in Table 2.
of Proband 21: 37 years of age) without any clinical symptoms of HHT or PAH. Furthermore, the penetrance of HHT is usually high and HHT develops in most patients before 30 years of age.\(^1\)\(^,\)\(^2\) Therefore, asymptomatic carriers with the \(\text{ALK1}\) mutation require close observation because they could potentially develop clinical symptoms of HHT and PAH.

In this study, 21 PAH probands under the age of 16 years were studied for \(\text{BMPR2}\) mutations as well. In 4 familial aggregates of PAH, we found 1 \(\text{BMPR2}\) mutation. We also found 3 \(\text{BMPR2}\) mutations in 17 pediatric probands with IPAH (\(\text{BMPR2}\) 17.6\%). Of the 4 \(\text{BMPR2}\) mutations identified, 3 nonsense mutations (\(\text{Q42X, Y113X, Q403X}\)) resulted in the truncation of \(\text{BMPR-II}\) and 1 missense mutation (\(\text{C123R}\)) occurring in a highly conserved ligand-binding domain was observed, as reported previously.\(^13,\)\(^14\) The presence of this conserved region has been observed throughout metazoan evolution (Fig 2c).

We analyzed the relationship between PAH patients with the \(\text{ALK1}\) or \(\text{BMPR2}\) mutation and those patients without such mutations. The clinical features, such as age at onset, disease progression and gender, and the hemodynamic parameters showed similarity between PAH patients with or without \(\text{ALK1}\) or \(\text{BMPR2}\) mutation (data not shown), indicating that \(\text{ALK1}\) and \(\text{BMPR2}\) mutations increase the risk in pediatric PAH, but do not contribute to the variation of the PAH phenotype. However, we wanted to understand the phenotypic variation among PAH patients, especially for syncope. Episodes of syncope occur mainly in pediatric rather than adult patients, so the correlation between syncope and the presence of different modifier genes, such as \(\text{eNOS}\) and \(\text{ACE}\), among PAH patients was investigated. We also studied the relationship of the allele frequency of these genes on the clinical features of PAH. All the PAH probands with the 894T allele of \(\text{eNOS}\) had episodes of syncope (Table 3), which can be interpreted as meaning that the 894T allele of \(\text{eNOS}\) might be a contributing risk factor for syncope. Although the small number of study subjects does not allow anything other than speculation, 1 potential mechanism for the 894T allele of \(\text{eNOS}\) causing syncope with PAH may involve progression of pulmonary vasoconstriction, which in turn may be related to reports implicating polymorphism of the 894T allele of \(\text{eNOS}\) with rapid progression of high-altitude pulmonary edema.\(^25\) This should be further investigated with more subjects, using similar precise procedures, to confirm this finding. Confirmation of polymorphism of \(\text{eNOS}\) as a risk for syncope in PAH patients would be a useful prognostic parameter for clinical examination.

In conclusion, in a limited series of children with PAH, mutations of \(\text{ALK1}\) were identified in 3 of 4 probands with FPAH who were given a diagnosis during childhood, and in 2 of 17 probands with IPAH. All these mutations were limited to the serine–threonine kinase domain. This is apparently the first study to detect a high number of \(\text{ALK1}\) mutations in pediatric PAH without HHT, although 1 case of \(\text{ALK1}\) mutation was reported in a PAH patient without HHT.\(^20\) That study was unable to determine if it was a sporadic mutation or was involved in PAH. Our study suggests that the \(\text{ALK1}\) mutation in the serine–threonine kinase domain, as well as \(\text{BMPR2}\) mutation, may be associated with PAH that develops in childhood. Therefore, if a carrier with the \(\text{ALK1}\) mutation is detected, close observation is necessary, because the \(\text{ALK1}\) mutation potentiates the expression of HHT and PAH. Moreover, the 894T allele of \(\text{eNOS}\) in IPAH patients is likely to be associated with syncope and may be a useful indicator.

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