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Transforming Growth Factor-β Receptor Mutations and Pulmonary Arterial Hypertension in Childhood

Rachel E. Harrison, MRCPCH; Rolf Berger, MD; Sheila G. Haworth, MD; Robert Tulloh, DM, FRCPCH; Christoph J. Mache, MD; Nicholas W. Morrell, MD; Micheala A. Aldred, PhD; Richard C. Trembath, FRCP

Background—Pulmonary arterial hypertension (PAH) is a potentially fatal vasculopathy that can develop at any age. Adult-onset disease has previously been associated with mutations in BMPR2 and ALK-1. Presentation in early life may be associated with congenital heart disease but frequently is idiopathic.

Methods and Results—We performed mutation analysis in genes encoding receptor members of the transforming growth factor-β cell-signaling pathway in 18 children (age at presentation <6 years) with PAH. Sixteen children were initially diagnosed with idiopathic PAH and 2 with PAH in association with congenital heart defects. Germ-line mutations were observed in 4 patients (22%) (age at disease onset, 1 month to 6 years), all of whom presented with idiopathic PAH. The BMPR2 mutations (n=2, 11%) included a partial gene deletion and a nonsense mutation, both arising de novo in the proband. Importantly, a missense mutation of ALK-1 and a branch-site mutation of endoglin were also detected. Presenting clinical features or progression of pulmonary hypertension did not distinguish between patients with mutations in the different genes or between those without mutations.

Conclusions—The cause of PAH presenting in childhood is heterogeneous in nature, with genetic defects of transforming growth factor-β receptors playing a critical role. (Circulation. 2005;111:435-441.)

Key Words: activin receptors, type I receptors, growth factor receptors, cell adhesion molecules, pulmonary heart disease, signal transduction

Characteristic features of pulmonary arterial hypertension (PAH) include sustained elevation of pulmonary arterial pressure (mean pulmonary arterial pressure >25 mm Hg), leading to right heart failure and premature death. A recently revised PAH classification distinguishes between clinical presentations related to cardiac or systemic disease, idiopathic PAH (IPAH), and familial PAH cases that demonstrate autosomal dominant inheritance. However, pathological examination of the lung consistently reveals obstruction of small pulmonary arteries caused by proliferation of both endothelial cells and vascular smooth muscle, together with active remodeling of the arterial vascular bed.

In the past 3 years, genetic studies have significantly increased our understanding of the molecular basis of PAH, demonstrating that the most frequent cause of familial PAH is mutation of receptor members of the transforming growth factor (TGF)-β superfamily. More than 50 disease-causing defects in the gene encoding the type II receptor BMPRII are currently reported, many of which have been identified in patients with no known family history of PAH because of the low penetrance of these mutations. Significant questions remain regarding the mechanisms through which BMPR2 mutations lead to the development of PAH. Analysis of the impact of disease-causing BMPR2 mutations on TGF-β signaling has provided little insight into the clinical variability of PAH, particularly in terms of disease penetrance, response to treatment, or age of onset, which may vary significantly within families.

Molecular genetic analysis of specific patient cohorts can provide another route for identifying TGF-β signaling defects central to the development of PAH. Investigation of PAH subjects in families exhibiting clinical features characteristic of hereditary hemorrhagic telangiectasia (HHT) has identified mutations of the type I receptor ALK-1 as a rare cause of PAH, although no evidence exists to date of a direct interaction between ALK-1 and BMPRII.

Raised pulmonary artery pressure associated with many of the pathophysiological features of PAH is also observed in much younger individuals, although in some of these patients, the disease is associated with cardiac anomalies or may
reflect a failure to adapt to extrauterine life.\textsuperscript{13} Uncertainty remains as to whether PAH presenting in childhood represents a single disease or distinct entities with overlapping clinical features.

We hypothesized that some cases of PAH presenting in childhood may be attributable to TGF-\(\beta\) receptor defects. We now show an important role for genetic defects encoding a range of receptor molecules within the TGF-\(\beta\) pathway in the etiology of PAH presenting in childhood. These findings have important implications for the investigation and management of families presenting with very-early-onset PAH and suggest that at least in some patients, PAH is a consequence of an inherited developmental defect of the pulmonary vasculature.

**Methods**

All samples were obtained after informed consent was secured, and the study was approved by the Trent Multicenter Research Ethics Committee.

Patients diagnosed with PAH before reaching 6 years of age were ascertained from European centers specializing in the management of PAH in childhood. Pulmonary hypertension was defined by standard clinical methods, including cardiac catheterization, as described previously.\textsuperscript{2}

Patients with familial PAH (\(\geq 2\) cases of PAH in first-, second-, or third-degree relatives) or disorders known to be associated with PAH (connective tissue diseases, HIV, portal hypertension, structural pulmonary abnormalities) were excluded.

DNA was isolated by standard protocols, and the protein coding sequence for BMPR2 (exons 1 through 13, primers as previously described)\textsuperscript{14} was amplified from genomic DNA, together with intron-exon boundaries. Polymerase chain reaction (PCR) products were purified with the QIAquick purification kit (Qiagen) and cycle sequenced with ABI Big Dye terminator on an ABI PRISM 377 (Applied Biosystems). In addition, the protein coding sequence for endoglin, encoding a type III receptor, was amplified (exons 1 through 14, primers available on request) and sequenced in patient 20514.

All samples were screened for partial gene deletions or duplications by estimation of copy number of BMPR2 exons 1, 6, and 12, together with ALK-1 exons 2 through 10, primers as previously described.\textsuperscript{14} Copy number of all exons of BMPR2 was investigated in patient 7913 with multiplex ligation-dependent probe amplification (MLPA).\textsuperscript{15} Oligonucleotide probes for MLPA were designed for each exon of BMPR2, together with 3 control genes synthesized by Biolegio BV, and arranged into 3 multiplex assays of 7 or 8 probes (details available on request). MLPA was conducted on 100 ng genomic DNA with reagents kit from MRC-Holland according to the manufacturer’s protocol and analyzed on the ABI 377. For RNA isolation, leukocytes were recovered from peripheral blood through Ficoll density gradient centrifugation (Amersham Biosciences), and monocyes were isolated with the MiniMACS separation unit (Miltenyi Biotec). Monocytes were incubated in plastic tissue culture plates for 24 hours; then, cells were harvested and RNA was extracted with the QIAamp RNA blood mini kit (Qiagen). cDNA was prepared from this using (dT)\textsuperscript{10} Primer for cDNA synthesis (Roche) with AMV reverse transcriptase (Roche). Endoglin transcripts were amplified between exon 11 and the 3’ UTR by PCR (primers available on request) and size fractionated on a 1% agarose gel. Amplified products were individually excised from the agarose gel, purified with QIAquick gel purification kit (Qiagen), and cycle sequenced as above.

**Results**

Eighteen individuals (8 female, 10 male) diagnosed with PAH before reaching 6 years of age were identified (the Table). Fifteen had no additional clinical disorders known to be associated with PAH and hence were diagnosed with IPAH. One patient was diagnosed with IPAH at the age of 3 months and subsequently developed features (cutaneous telangiectasia, epistaxis) of HHT at the age of 8 years. Two patients had congenital heart defects.

Mutations were identified in 4 patients (the Table and Figures 1 and 2). Each mutation is novel and predicted to alter the expression, structure, or function of the receptor components with deleterious functional consequences. Because none of these DNA variants were detected in \(\geq 100\) normal control chromosomes, they were considered mutations that cause disease.

Heterozygous partial gene deletion of BMPR2 was identified in genomic DNA from one patient through reduced copy number of exon 6 using fluorescent dosage PCR (Figure 1A). To characterize this further, we used MLPA to assess copy number of each exon of BMPR2. These studies demonstrated genomic deletion of BMPR2 exons 5, 6, and 7, which encode part of the kinase domain of BMPRII (Figure 1B). An mRNA transcript produced from this mutant allele would be predicted to have exon 4 spliced directly to exon 8, with the loss of exons 5 through 7 (Figure 3A). This prediction was confirmed by reverse-transcriptase PCR analysis (data not shown). This deletion of 438 nucleotides does not disrupt the reading frame of the mRNA transcript, so no premature termination codon is produced, and nonsense-mediated RNA decay would not be expected.\textsuperscript{16} Translation of mutant mRNA would be expected to result in a protein with the loss of 146 amino acid residues, responsible for encoding nearly half of the kinase domain of BMPRII (Figure 3A). This would significantly impair the signaling ability of BMPRII and cause major alteration of the protein structure. This genomic deletion was seen in a 4-year-old boy with IPAH (patient 7913) who had no known family history of pulmonary hypertension. Analysis of samples from both parents confirmed that this mutation had arisen de novo.

A novel nonsense mutation of BMPR2 was identified in a girl who presented with IPAH at 6 years of age (patient 21140; Figure 1C). This produces a premature termination codon in exon 1 of BMPR2 (W16X), expected to lead to nonsense- \textsuperscript{-} mediated decay of the mutant mRNA transcript.\textsuperscript{16} If any mutant transcript was translated, the resulting protein would be severely truncated, consisting of only the first 15 amino acids of the ligand-binding domain, essentially creating a null allele (Figure 3A). This girl had no known family history of PAH, and the mutation was not detected in parental samples, confirming that the mutation had occurred de novo.

In a child diagnosed with IPAH at 20 months of age (patient 7912), a novel heterozygous single-base pair substitution was identified in exon 10 of ALK-1, predicted to lead to the substitution of glutamine at amino acid residue 484 by arginine (Figure 1D). This mutation occurs within the highly conserved NANDOR box region of ALK-1, within which we have previously identified 2 independent missense mutations in patients with HHT-related PAH\textsuperscript{11,12} (R484W and K487T;
### TGF-β Alterations in Patients With PAH Presenting in Childhood

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<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Additional Features</th>
<th>Sex</th>
<th>Age at Presentation</th>
<th>MPAP, mm Hg</th>
<th>PCWP, mm Hg</th>
<th>CI, L·min⁻¹·m⁻²</th>
<th>PVR, WU·m⁻²</th>
<th>Clinical Status</th>
<th>Mutation</th>
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<td>Died, age 23 mo</td>
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<td>F</td>
<td>3 y</td>
<td>50</td>
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<td>51</td>
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<td>10.0</td>
<td>Alive, age ≥8 y</td>
<td>WT</td>
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<td>70</td>
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<td>Alive, age ≥5 y</td>
<td>WT</td>
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<tr>
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<td>41</td>
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<td>3.5</td>
<td>9.7</td>
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<td>WT</td>
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<td>IPAH</td>
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<td>1 mo</td>
<td>37</td>
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<td>3.6</td>
<td>8.6</td>
<td>Alive, age ≥8 y</td>
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<tr>
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<td>7 mo</td>
<td>79</td>
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<td>30.4</td>
<td>Died, age 12 mo</td>
<td>WT</td>
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<td>F</td>
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<td>WT</td>
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<td>F</td>
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<td>1.6</td>
<td>38.8</td>
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<tr>
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<td></td>
<td>F</td>
<td>6 y</td>
<td>66</td>
<td>8.0</td>
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<td>27.6</td>
<td>Alive, age 7 y</td>
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<td>HHT with PAVM diagnosed at 8 y of age</td>
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<td>45</td>
<td>3.0</td>
<td>2.6</td>
<td>16.1</td>
<td>Alive, age 9 y</td>
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<tr>
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<td>M</td>
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<td>PAH with CHD</td>
<td>Neonatally corrected TGA</td>
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<td>2 wk</td>
<td>53</td>
<td>7.5</td>
<td>2.8</td>
<td>16.2</td>
<td>NK</td>
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</table>

Diagnosis is the clinical classification at presentation according to the classification system proposed at the 3rd World Symposium on pulmonary arterial hypertension. MPAP indicates mean pulmonary artery pressure (normal range 10 to 20 mm Hg); PCWP, pulmonary capillary wedge pressure (normal range 4 to 12 mm Hg); CI, cardiac index (cardiac output [L/min] divided by body surface area [m²]); PVR, pulmonary vascular resistance (MPAP minus PCWP divided by CI, normal range 1.0 to 2.0 WU·m⁻²); WT, wild-type BMPR2 and ALK-1 sequence and dosage; 8 exon 5/6/7, 50% dosage reduction of BMPR2 exon 5, 6, and 7 by MLPA; ENG IVS12 −22T→C, substitution of T by C at nucleotide position −22 in intron 12 of endoglin; PAVM, pulmonary arteriovenous malformation; CHD, congenital heart disease; VSD, ventricular septal defect; TGA, transposition of the great arteries; and NK, not known.

*Original catheter data not available. Pulmonary artery pressures estimated to be >40 mm Hg with Doppler echocardiography.

Figure 1D). This region is considered necessary for the phosphorylation of type I receptors and endocytosis of the receptor complex in TGF-β signaling. Now 5 years old, the affected child displays no manifestations of HHT, which most commonly develop in late childhood or early adulthood. Additional assessment revealed no family history of HHT, and the mutation was absent in a maternal genomic DNA sample. A paternal DNA sample was not available for analysis.

Patient 20514 was diagnosed with IPAH at 3 months of age. At 8 years of age, he developed epistaxis and cutaneous telangiectases, and contrast echocardiography demonstrated microvascular pulmonary AV malformations, leading to a revised diagnosis of HHT-related PAH. Because no mutations were identified within either ALK-1 or BMPR2, sequence analysis of endoglin was performed. A T→C substitution was identified within a putative branch-site sequence in intron 12 of endoglin, 22 bp upstream of exon 13 (Figure 2A). This might be predicted to cause skipping of exon 13 or retention of intron 12 within the endoglin transcript. To assess the consequences of the sequence variant on transcript splicing, RNA was isolated from activated monocytes, and endoglin transcript between exon 11 and the 3’ UTR was amplified and size fractionated. High levels of product were obtained for a fragment of the expected size for wild-type endoglin, and an additional fragment ∼110 bp smaller was present at much lower levels (Figure 2B). Sequencing of the smaller fragment showed juxtaposition of exons 12 and 14, indicating that exon 13 had been spliced out of this transcript (Figures 2C and 3B). The larger fragment was confirmed to be of wild-type sequence. Loss of exon 13 does not disrupt the reading frame of the endoglin transcript, so if translated, a mutant transcript would be predicted to produce a protein with deletion of amino acid residues from 582 to 618, which includes the transmembrane domain of endoglin (Figure 3B). Analysis of parental samples demonstrated that the mutation has been inherited from the patient’s father, who does not have pulmonary hypertension and currently manifests no signs or symptoms of HHT at 33 years of age. The presence of pulmonary, hepatic, and cerebral AV malformations has also been excluded in the patient’s father through bubble contrast echo, abdominal ultrasound, and cranial MRI with contrast, respectively.
Discussion

Our data indicate a critical role for diverse genetic defects of the TGF-β/H9252 pathway in the etiology of PAH presenting in childhood, with mutations identified in 4 of 18 subjects (22%). Mutations were identified in BMPR2, ALK-1, and endoglin, demonstrating significant genetic heterogeneity for the development of PAH in childhood.

We identified 2 novel mutations in BMPR2 from 18 patients developing PAH before 6 years of age (n=2, 11%), including one partial gene deletion and one nonsense mutation. This is consistent with reports that BMPR2 mutations occur in up to a third of adult patients with IPAH, but contrasts with data from a study of children developing PAH before reaching 14 years of age that detected no BMPR2 mutations in this group. The authors conclude that IPAH in childhood may have a different genetic background than in adults and proposed a recessive mode of inheritance based on pulmonary artery pressure response to exercise in relatives. Few parental samples were available for analysis in previous studies of adults with sporadic IPAH, but de novo mutation has been reported (2 of 5), in addition to inheritance of mutations from healthy fathers (3 of 5). Both BMPR2

Figure 1. A, B, TGF-β sequence variation and partial gene deletions in patients with PAH presenting in childhood. A, Dosage electropherograms showing normal control (i) and heterozygous deletion of exon 6 of BMPR2 in patient 7913 (ii). Deletion was determined by assessment of dosage quotient (DQ) as described previously (exon 6 DQ <0.5 for patient 7913) and can be seen as reduced peak area of BMPR2 exon 6 compared with normal control. B, MLPA electropherograms showing normal control (i) and heterozygous deletion of exons 5, 6, and 7 of BMPR2 in patient 7913 (ii). Deletion was determined by calculation of dosage quotient as described in Methods and can be seen as reduced peak area of BMPR2 exons 5, 6, and 7 compared with normal control. Exons 4 and 8 are seen to be normal. C, D, Sequencing chromatograms demonstrating heterozygous sequence variants. C, Substitution of G by A at nucleotide position 47 in exon 1 of BMPR2, changing codon 16 from tryptophan to stop codon in patient 21140. D, Substitution of G by A at nucleotide position 1451 of ALK1 exon 10, changing codon 484 from arginine to glutamine in patient 7912. Conservation of amino acids within NANDOR box region (underlined) of ALK-1.17

Figure 2. Branch-site mutation and abnormal splicing of endoglin transcript in HHT-related PAH. A, Sequencing chromatogram demonstrating heterozygous sequence variant T→C at nucleotide –22 in intron 12 of endoglin in patient 20514 within putative branch-site sequence compared with consensus branch-site sequence. B, PCR amplification of endoglin cDNA between exon 11 and 3’ UTR with size fractionation of PCR products on 1% agarose gel demonstrates 2 fragments in patient 20514. Expected wild-type product of 384 bp is present in patient 20514 and normal control. Smaller fragment is seen in patient 20514 alone and is present at much lower levels. C, Cycle sequencing of transcripts demonstrates wild-type sequence in larger fragment and exon 12 adjacent to exon 14 in smaller fragment, indicating that exon 13 has been spliced out of transcript. Y indicates pyrimidine; R, purine; and N, any nucleotide.
mutations “private” to independent kindreds. The nonsense mutation W16X would generate the earliest premature termination codon reported thus far and effectively represents a null allele, consistent with the proposal that haploinsufficiency of BMPRII forms the molecular basis of IPAH.4

We have included a screen for partial gene deletions of BMPR2 by assessing copy number of exons 1, 6, and 12, a step not included in most PAH mutation reports to date. Detection of a large genomic deletion that extends from exons 5 through 7 suggests that future studies of PAH cohorts, particularly those exploring the molecular pathogenesis of PAH, should include a comprehensive search for small- and large-scale rearrangements at the BMPR2 locus.

Of significant interest, Roberts et al23 have also recently reported identified BMPR2 mutations in 6% of a mixed cohort of adults and children with congenital heart disease and PAH. Congenital heart disease involving significant intracardiac shunting can be considered an independent risk factor for the development of PAH through the Eisenmenger syndrome. In contrast, many congenital cardiac defects may be observed in patients with PAH but are not thought to contribute directly to the development of pulmonary hypertension. Pulmonary hypertension in association with such defects is therefore considered IPAH.13 Two recent reports have implicated BMP signaling as crucial to the normal formation of the cardiac outflow tract and cardiac septation. A homozygous mouse defective for BMPR-II receptor-mediated signaling revealed outflow tract defects, including the absence of septation of the conotruncus below the valve level.24 Further investigation of the BMP pathway in mouse cardiac development demonstrated that ablation of the BMP type I receptor ALK-3 in neural crest cells also leads to severe outflow tract defects, including failure of septation.25 Taken together, these data raise the intriguing possibility that BMPR2 mutations may lead to congenital heart defects in humans and may be a predisposition to PAH. Molecular genetic analysis of cohorts of patients with congenital heart defects is now underway to test this hypothesis.

A mutation leading to abnormal splicing of the endoglin transcript was identified in a child with HHT-related PAH. This abnormally spliced endoglin transcript was present at much lower levels than wild type, consistent with previous reports and the hypothesis that mutations in endoglin lead to HHT through haploinsufficiency.26,27 Markedly reduced levels of mutant transcript may occur despite the absence of a premature termination codon that would stimulate nonsense-mediated RNA decay through reduced splicing efficiency.28 This child developed signs of HHT at 8 years of age but had initially presented with typical features of PAH, including a high pulmonary vascular resistance, at 3 months of age. The 2 BMPR2 mutations we report are both novel, adding to the wide range of mutations already described.2–10 Remarkably few recurrent mutations have been identified, with most
Although no family history of HHT or PAH was known in this family, this mutation had been inherited from the child’s father, who remains free of manifestations of HHT at 33 years of age. These findings emphasize the considerable variation of clinical features previously noted in HHT even within a single family. Although current reports estimate that the penetrance of endoglin mutations is high, with up to 88% of patients with HHT1 reporting nosebleeds by 30 years of age, these data have been obtained by self-report in families already known to specialist HHT services, so findings may be biased toward individuals with more severe manifestations.

The finding of germ-line mutation of the ALK-1 and endoglin genes raises important points. First, and of clinical relevance, our results highlight that PAH is an uncommon but serious presentation of the autosomal dominant syndrome HHT in children and may occur as an early and initial presentation of HHT within a family. A detailed family history and careful examination of parents for subtle manifestations of HHT are required, although phenotypic variability and the possibility of de novo mutation mean that the diagnosis cannot be excluded if no abnormalities are identified within the family. In such circumstances, molecular genetic analysis provides the only means for accurate counseling of families regarding future management and recurrence risk.

Second, our data emphasize a critical role for receptor members of the TGF-β superfamily in the development and maintenance of the pulmonary vasculature. TGF-β signaling induces a transcriptional response through ligand binding to a receptor complex comprising type I and II receptor kinases, which activate intracellular Smad-dependent and -independent pathways, a process that may be further regulated by the expression of type III (eg, endoglin) receptors. Despite the lack of evidence for a direct interaction between BMPR-II and ALK-1/endoglin complexes, there is extensive cross-talk between intracellular signaling pathways activated by these receptor complexes. For example, signaling via ALK-1 and BMP receptors occurs through the same set of receptor-regulated Smad proteins, Smads 1, 5, and 8. We anticipate that elucidation of the molecular impact of these mutations on downstream pathways will point to molecular mechanisms critical to the development of PAH.

The identification of the ALK-1 mutant R484Q supplements the 2 previously reported independent missense mutations within the NANDOR box region of ALK-1 identified in HHT-related PAH (Figure 1D). ALK-1 mutant constructs containing the K487T mutation were able to localize correctly to the cell surface, in contrast to most of the ALK-1 mutant constructs investigated, and are predicted to disrupt endocytosis of the receptor complex. Interestingly, mutations in BMPR2 appear to disrupt the interaction of the BMPRRII receptor with tctex-1, a light chain of the molecular motor dynein required for normal trafficking of the receptor complex. Taken together, these data suggest that disruption of receptor complex trafficking may contribute to a common molecular mechanism underlying the pathogenesis of PAH associated with BMPR2 and ALK-1 mutations.

Clinical features at presentation did not distinguish between patients with mutations in the different genes or those without mutations. Hence, the possibility of germ-line mutation TGF-β receptor genes should be considered in all children presenting with PAH. Interestingly, substantial overlap is observed in terms of the detected mutations compared with those previously observed in subjects presenting at an older age, providing further evidence that additional genetic and/or environmental factors may influence the age of onset and presentation of PAH.

These data, obtained in a cohort with severe PAH presenting to specialist centers, do not allow estimation of the prevalence of TGF-β receptor defects in childhood-onset PAH. Genomic mutations were not identified in 14 patients in this study, including 2 children with congenital heart disease, and the cause of PAH in this group remains to be identified. It is likely that the present mutation detection methods, although comprehensive, may not identify all TGF-β receptor variants of functional significance, so further abnormalities in this pathway should be considered, in addition to investigation of other key pathways required for pulmonary vascular development, structure, and function.

In conclusion, these data demonstrate a significant contribution for genetic defects of TGF-β receptors to the etiology of PAH in childhood. This is clearly a finding of substantial importance for clinical management and genetic counseling, with the heterogeneous nature of the mutations identified representing a considerable challenge to the provision of genetic testing as a diagnostic service in IPAH. Although the molecular basis of the pathogenesis of PAH remains unclear, these findings raise important questions regarding the role of TGF-β signaling defects in the normal development of cardiopulmonary vasculature, leading to early-onset disease.

Acknowledgments

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References


