ORIGINAL ARTICLE

**TBX4 mutations (small patella syndrome) are associated with childhood-onset pulmonary arterial hypertension**

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**ABSTRACT**

**Background** Childhood-onset pulmonary arterial hypertension (PAH) is rare and differs from adult-onset disease in clinical presentation, with often unexplained mental retardation and dysmorphic features (MR/DF). Mutations in the major PAH gene, BMPR2, were reported to cause PAH in only 10–16% of childhood-onset patients. We aimed to identify more genes associated with childhood-onset PAH.

**Methods** We studied 20 consecutive cases with idiopathic or heritable PAH. In patients with accompanying MR/DF (n=6) array-comparative genomic hybridisation analysis was performed, with the aim of finding common deletion regions containing candidate genes for PAH. Three patients had overlapping deletions of 17q23.2. TBX2 and TBX4 were selected from this area as candidate genes and sequenced in all 20 children. After identifying TBX4 mutations in these children, we subsequently sequenced TBX4 in a cohort of 49 adults with PAH. Because TBX4 mutations are known to cause small patella syndrome (SPS), all patients with newly detected TBX4 mutations were screened for features of SPS. We also screened a third cohort of 23 patients with SPS for PAH.

**Results** TBX4 mutations (n=3) or TBX4-containing deletions (n=3) were detected in 6 out of 20 children with PAH (30%). All living patients and two parents with TBX4 mutations appeared to have previously unrecognised SPS. In the adult PAH-cohort, one TBX4 mutation (2%) was detected. Screening in the cohort of predominantly adult) SPS patients revealed no PAH.

**Conclusions** These data indicate that TBX4 mutations are associated with childhood-onset PAH, but that the prevalence of PAH in adult TBX4 mutation carriers is low.

**INTRODUCTION**

Pulmonary arterial hypertension (PAH) is an intrinsic vascular disease of the lungs, which leads to high pulmonary vascular resistance and increased pulmonary artery pressure. This results in right ventricular hypertrophy and, eventually, death from right ventricular failure. The histological findings in PAH include medial hypertrophy, intimal fibrosis and plexiform lesions. Thrombosis, inflammation and a disrupted balance between proliferation and apoptosis have been suggested to play a role in this typical vascular remodelling process.

PAH is classified as group 1 in the five groups of the Updated Clinical Classification of Pulmonary Hypertension (PH) (Dana Point, 2008).2 PAH may be idiopathic (IPAH, subgroup 1.1) or heritable (HPAH, mutations in BMPR2, ALK1, or ENG, subgroup 1.2), associated with drugs or toxins (subgroup 1.3), connective tissue disorders or several other diseases (subgroup 1.4), or a specific disease of the newborn (persistent PH, subgroup 1.5).2

This study focuses on children in subgroups 1.1 and 1.2. Our aim was to identify genes associated with PAH in children.

PAH is a genetically heterogeneous disease, but the major gene identified for PAH is BMPR2, a gene of the TGF-β family involved in BMP-SMAD signalling.3 Mutations in two other members of the TGF-β super family (ALK1 and ENG) may also cause PAH, which in most patients is associated with hereditary haemorrhagic telangectasia (HHT).4 5 Several infrequent mutations in genes of the SMAD-family are reported to be associated with PAH,6 7 and recently a CAV1 mutation was detected by exome sequencing in a three-generation family with PAH.8 Epigenetic factors may also contribute to the disease.9

Mutations in BMP2R were reported to cause PAH in 10–16% of childhood-onset PAH patients,10–12 compared with 21–26% of adult-onset sporadic PAH patients and 69% of familial PAH patients,13 14 so the genetic aetiology of the disease may be different in childhood onset.14 15

Childhood-onset PAH is rare, with a prevalence estimated at 0.7 cases per million.16

There are, thus, no large cohorts available. It presents with more pulmonary vascular medial hypertrophy, and has a higher proportion of responders to acute pulmonary vascular dilatation tests than adult-onset PAH.17 18 PAH in children is frequently associated with mental retardation and/or dysmorphic features (MR/DF), without a syndrome diagnosis.12 This observation led to the strategy we chose for looking for PAH candidate genes. We performed array-comparative genomic hybridisation (aCGH) screening of all children with PAH and MR/DF, with the aim of detecting deletion regions harbouring candidate genes for PAH.
PATIENTS AND METHODS

Patients

Initial study population

Childhood-onset PAH patients

In the National Referral Center for Pediatric PH in The Netherlands, DNA was collected from 20 consecutive patients (10 male, 10 female), diagnosed with childhood-onset PAH (IPAH/HPAH, subgroups 1.1 and 1.2 in the 2008 Dana Point Classification)² and enrolled at the referral centre between 2003 and 2010. Patient characteristics are shown in table 1.

None of the patients had affected relatives, and six patients had unexplained MR/DF (patients 1, 2, 3, 15, 19, 20). Three patients had previously identified BMPR2 mutations (patients 7, 8 and 9; table 1), but were included because BMPR2 mutations have a low penetrance for PAH and the modifiers might be genetic.

No haemodynamic data were available for three patients because they died before cardiac catheterisation could be performed. Echocardiography and autopsy were performed in all three, and advanced pulmonary vascular lesions were present, including intima fibrosis and plexiform lesions. In one patient, histopathological features of pulmonary veno-occlusive disease (PVOD) were present, leading to the diagnosis being revised from PAH to PVOD postmortem.

Additional patient cohorts

After identifying TBX4 deletions and mutations, two additional cohorts were screened:

Adult-onset PAH patients

An additional cohort of 49 adult-onset PAH patients was tested for TBX4 mutations. The cohort included 40 IPAH and 9 HPAH patients with BMPR2 mutations; all diagnoses were confirmed with right heart catheterisation (RHC).

Small patella syndrome patients

Because TBX4 mutations are known to cause small patella syndrome (SPS), a highly variable lower limb malformation syndrome, a group of 23 patients with SPS was screened for clinical and echocardiographical signs of PH; RHC was performed if indicated by the criteria proposed in the European guidelines for PH.¹⁹ This cohort included the two carrier parents and came from 11 families. They were aged 11–67 years (one patient was 11 years old, all the others were older than 20 years). They all carried disease-causing mutations in TBX4 but did not have known symptoms of PAH. Three of the families were described in the original paper on TBX4 mutations causing SPS.²⁰

Genetic analyses

Technical details of the analyses are provided in the online supplementary data appendix S1.

aCGH analysis: screening for deletions

Of the 20 patients with childhood-onset PAH, six also had unexplained MR/DF. We performed aCGH analysis in these six patients (patients 1, 2, 3, 15, 19, 20), which is a standard diagnostic procedure in patients with MR/DF in our hospital. If a deletion or duplication was detected, their parents were also tested.

Mutation and deletion screening of TBX2 and TBX4 by direct sequencing and multiplex ligation-dependent probe amplification

From the six known genes detected by aCGH and located in overlapping 17q23.2 deletion regions, we selected TBX2 and TBX4 as candidate genes for PAH, based on their function in patients with MR/DF in our hospital. If a deletion or duplication was detected, their parents were also tested.

Table 1 Clinical characteristics and results of molecular analyses of 20 childhood-onset PAH patients

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Age m/f</th>
<th>mPAP (10–20)</th>
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<th>mPCWP (&lt;15)</th>
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<th>MR/DF</th>
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Patients 7, 17 and 20 died before right heart catheterisation could be performed, but echocardiography and autopsy confirmed a diagnosis of idiopathic PAH in all three.

Table 1 Clinical characteristics and results of molecular analyses of 20 childhood-onset PAH patients

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New disease loci

and data in the literature. T-box genes are transcription factors known to be involved in several developmental and cardiovascular diseases and are connected with the BMP/SMAD pathway.\textsuperscript{21–23} All 20 childhood-onset PAH patients, regardless of whether we had previously identified a deletion or mutation in them, were then screened for mutations in TBX2 and TBX4. For patients in whom we found a mutation in one of these genes, we also screened the parents for the same mutation. Intragenic deletion detection for the TBX2 and TBX4 genes was performed by multiplex ligation-dependent probe amplification (MLPA).

Clinical evaluation of TBX4 mutation carriers

Patients with a TBX4 mutation, or with deletions including TBX4, were physically examined by a clinical geneticist and radiographs of the pelvis, knees and feet were made to study pelvic and lower limb malformations associated with SPS.

RESULTS

aCGH in six childhood-onset PAH patients with unexplained MR/DF

aCGH was performed in six childhood-onset PAH patients because of associated but unexplained MR/DF (table 1). In three of these (patients 1–3), we detected a microdeletion in 17q23.2 which was not present in either of their parents and was, therefore, considered to be causative. This deletion was not reported in the Database of Genomic Variants or in our departmental reference database of 800 healthy persons, but it was reported in the literature as associated with mental retardation and congenital malformations.\textsuperscript{24 25} The smallest region of overlap in these three patients was 1 Mb and contained six genes: TBX2, TBX4, NACA2, BRIP1, INTS2 and MED13 (figure 1A).

Direct sequencing and MLPA of TBX2 and TBX4 in childhood-onset PAH patients

No mutations were detected in TBX2. Three TBX4 mutations (figure 1B) were identified in three unrelated patients with normal intelligence and without previously detected SPS features (patients 4–6; table 1). Two mutations were insertions causing a frameshift, leading to a premature stop codon, which are predicted to lead to a truncated protein, and one was a non-synonymous mutation (Tyr382Ser), which is predicted to affect the protein function (see online supplementary data appendix S2). These mutations were not present in 1000 control chromosomes in our departmental database.

The parents of two of the three patients with a TBX4 mutation were available for testing: the mutation in patient 4 was maternally derived, while that in patient 5 was paternally derived. None of the patients with a TBX4 deletion/mutation also had a BMPR2 mutation.

Results of aCGH and sequencing are summarised in table 1.

Clinical evaluation of TBX4 deletion and mutation carriers

Since TBX4 mutations are known to cause SPS, a clinical geneticist (WSK-F) physically examined the childhood-onset PAH patients with a TBX4 mutation or deletion and their carrier parents for clinical and radiological features of SPS. In all the five patients still alive, and in the two carrier parents, skeletal malformations were found characteristic for SPS, including a history of patella luxation, a hypoplastic or absent patellae, a large gap between the first and second toes, and relatively long second and
third rays of the feet. There were also absent or irregular ossification of the ischiopubic junction of the pelvis, axe-cut notches in the acetabulum, and a broad neck of the talus (figure 2A,B). The SPS features had not been recognised earlier because this syndrome is rare, is not associated with mental retardation, and the skeletal findings are only recognised after a thorough examination and x-rays of the pelvis and lower limbs.

As for all 20 patients, these six SPS-PAH patients fulfilled the Dana Point criteria for PAH groups 1.1 (idiopathic) or 1.2 (heritable). In one of our PAH patients with a TBX4 mutation, post-mortem examination of lung tissue revealed features of PVOD. This observation is comparable with what is seen in patients with BMPR2 mutations who have also been reported with PVOD, and it confirms the similarities between the genetic backgrounds of PAH and PVOD suggested previously.26 27

TBX4 mutation screening of 49 adult-onset PAH patients
In our cohort of adult-onset PAH patients, we detected one probably disease-causing mutation (c.229T>C, p.W77R), and one variant of uncertain significance (c.104C>T, p.A35V) in TBX4 (see online supplementary data). Both patients are dead, and no symptoms of SPS had been recognised. Although both patients had PAH diagnosed in adulthood, an undetected, mildly progressive childhood onset of the disease in these patients cannot be ruled out retrospectively.

Echocardiographic screening of 23 SPS patients
Echocardiographic screening of 23 SPS patients (including the two carrier parents of patients 4 and 5) from 11 families, without clinical symptoms of PAH, revealed a tricuspid regurgitation velocity of 3.3 m/s (‘PH possible’) in a 57-year-old patient (not a parent of a childhood-onset PAH patient), that could not be explained by associated conditions. RHC revealed that she did not fulfil the criteria for PAH. In two patients, both older than 60 years of age, a tricuspid regurgitation velocity of 2.8 m/s and 3.1 m/s respectively (‘PH possible’) was attributed to chronic obstructive pulmonary disease (COPD) in one patient and left ventricular dysfunction in the other. Finally, in 20 patients, a tricuspid regurgitation velocity <2.8 m/s (‘PH is unlikely’) was measured.

DISCUSSION
In our cohort of patients with childhood-onset PAH, we detected a deletion of 17q23.2 harbouring the TBX4 gene or a TBX4 point mutation in 30% of the patients (6/20). Mutations in TBX4 have been reported as the cause of SPS.28 We detected features of SPS in all the living TBX4 mutation carriers. As SPS is an autosomal dominant disease with an offspring recurrence risk of 50%, we consider that SPS families should be informed about this evident association with childhood-onset PAH.

Although our study is descriptive, and we have no functional data, the finding of deletions and mutations in 30% of a childhood-onset PAH cohort indicates an association and is unlikely to be a coincidence, since these deletions and mutations are not detected in healthy controls. The small size of the cohort of patients with childhood-onset PAH is a major limitation of our study, but is a direct consequence of the rarity of this disease.

The three PAH patients with 17q23.2 deletions had mild mental retardation, all in addition to PAH and SPS, possibly caused by haploinsufficiency of other genes in the deletion region. The three patients with point mutations in TBX4 had normal intelligence. In this small patient series, we could not determine significant differences between PAH patients with or without TBX4 mutations, with regard to age at PAH diagnosis (2.2–15.4 years vs 0.3–15.8 years), or pulmonary vascular...
In addition to expression in the limbs, Tbx4 is also reported to be expressed at the position where the lung buds form in chicken, and to be expressed at high levels in the lung mesenchyme during organogenesis in the mouse. 37 47 48 Mouse models studying the development of the respiratory system indicate an important role for Tbx4 and Tbx5 in lung branching through interaction with Fgf10. 49 50 Decreased Tbx4 activity leads to a decrease in foci expressing Fgf10, a known inducer of Bmp4. In normal lung development, Fgf10 and Bmp4 expression lead to activation of the BMP pathway. 51

Although Tbx4 governs multiple processes during respiratory tract development, its role in the development of pulmonary vasculature is insufficiently known. Tbx4-null mice are embryonic lethal due to allantois failure, and since PAH is a postnatal diagnosis, these studies do not allow conclusions regarding associated PAH in these animals. However, Tbx4-mutant embryos are reported to show failure of endothelial cells to form vessels in the allantois. 41

Since it now appears that mutations in BMPR2, ALK1, ENG and Tbx4 are associated with PAH, there may be a common mechanism leading to PAH developing. BMPR2, ALK1 and ENG are involved in the BMP/TGF-β/SMAD signalling pathways. The balance between the BMP/TGF-β/SMAD signalling pathways probably influences whether a cell type is directed more towards a differentiating or a proliferating state. In particular, the BMP pathway may promote differentiation, whereas the TGF-β pathway may push the system towards a higher proliferation status. A disrupted balance between these two pathways, that is, towards a more proliferative state, may result in PAH. 1 A decreased activation of the BMP pathway through Fgf10 and Bmp4, as reported in animal models focusing on lung and limb development, might link Tbx4 mutations in humans to the BMP/TGF-β/SMAD signalling pathways involved in PAH.

More research is needed to elucidate modifying factors and downstream targets of Tbx4, which could eventually lead to the development of novel therapeutic interventions.

**CONCLUSIONS AND RECOMMENDATIONS**

We conclude that SPS is associated with childhood-onset PAH, since we detected whole-gene deletions and point mutations of Tbx4 in 6/20 patients (30%). In contrast with childhood-onset PAH, adult-onset patients showed a low frequency of Tbx4 mutations (2%). Signs of SPS should be carefully looked for and analyzed and aCGH can be considered in patients with extrapulmonary features.

Tbx4 mutations appear to have a variable expressivity, which seems age-dependent, because 23 SPS patients (22 adults, one child) with Tbx4 mutations did not show PAH at screening. Nevertheless, in young patients with SPS, we feel screening for PAH should be considered.

Tbx4 mutations might contribute to PAH through a decreased activation of the BMP pathway. Future studies on the expression of Tbx4 and on the histopathology of lung vasculature in animal models or studies in inducible pluripotent stem cells may explain the mechanism underlying this association of Tbx4 mutations and childhood-onset PAH.

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New disease loci

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New disease loci


Appendix 1

Supplementary data to manuscript “TBX4 mutations (Small Patella Syndrome) are associated with childhood-onset Pulmonary Arterial Hypertension”
by Kerstjens-Frederikse W.S. et al.

Genetic analysis

\textit{aCGH analysis}

Array-comparative genomic hybridization (aCGH) analysis was performed using the 180K oligo array from Agilent (custom design ID: 23363; Agilent Technologies Inc., Santa Clara, CA, USA). A mixture of 40 healthy male or 40 female DNA samples was used as reference (sex-matched). Procedures were performed according to the manufacturer’s protocol. Data were extracted using Feature Extraction V.9.1 software.

\textit{Mutation analysis of TBX2 and TBX4}

Mutation analysis of all coding exons and flanking intronic sequences of the TBX4 gene was carried out using flanking intronic primers (primer sequences are available upon request). The forward primer was designed with a PT1 tail (5’-TGTAAAACGACGGCCAGT-3’) and the reverse primer was designed with a PT2 tail (5’-CAGGAAACAGCTATGACC-3’). PCR was performed in a total volume of 15 µl containing 10 µl AmpliTag Gold ®Fast PCR Master Mix (Applied Biosystems), 1.5 pmol/µl of each primer (Eurogentec, Serian, Belgium) and 2 µl genomic DNA. The samples were PCR amplified on a Perkin-Elmer (ABI) Geneamp 9700 using the following PCR program: an initial denaturation at 94°C for 1 minute, followed by 5 cycles of denaturation at 94°C for 5 seconds, annealing starting at 65°C for 30 seconds with a stepdown of 1°C every cycle, and elongation at 72°C for 1 minute, followed by 20 cycles of denaturation at 94°C for 5 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 1 minute, followed by another 15 cycles of denaturation at 94°C for 5 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 1 minute with a final step at 72°C for 5 minutes, after which the samples were cooled down to 20°C. 5 µl of the PCR products were loaded with 5 µl loading buffer and run on a 2% agarose gel with a FastRuler Low Range DNA Ladder (Fermentas) for comparison. The remainders of all PCR products were purified with ExoSAP-IT (Amersham Pharmacia Biotech, Biscataway, NY, USA) and subjected to direct sequencing on an ABI 3730 automated sequencer, using PT1 and PT2 primers.

\textit{MLPA deletion screening of TBX2 and TBX4}

For both genes, two uniquely sized probes were developed in accordance with a protocol provided by MRC Holland, The Netherlands). Ten probes were combined in one MLPA assay together with a quantity of DNA and a DNA denaturation control mix (EK-1 kit, MRC Holland). The procedure was further carried out as described by De Vries BB, Pfundt R, Leisink M et al. Diagnostic genome profiling in mental retardation. \textit{Am J Hum Genet} 2005; 77(4):606-616.
Appendix 2

Supplementary data to manuscript
“TBX4 mutations (Small Patella Syndrome) are associated with childhood-onset Pulmonary Arterial Hypertension ” by Kerstjens-Frederikse W.S. et al.

Prediction of pathogenicity of missense mutations in TBX4

1. childhood onset PAH patients
Patient 6: c.1145A>C; p.Y382S
Highly conserved nucleotide (score 1.0, [0-1]), highly conserved amino acid (considering 11 species); Grantham distance 144; Polyphen score 0.531 (possibly damaging); SIFT score 0.00 (not tolerated). Mutation is not detected in 1000 control chromosomes. Conclusion: pathogenic

2. adult onset PAH patients:
Patient A1: c.229T>C, p.W77R highly conserved nucleotide (score 1.0, [0-1]); highly conserved amino acid (considering 11 species); Grantham distance 101; Polyphen score 1.00 (probably damaging); SIFT score 0.00 (not tolerated). Mutation is not detected in 1000 control chromosomes. Conclusion: probably disease causing.
Patient A2: c.104C>T, p.A35V: weakly conserved nucleotide (score 0.31, [0-1]); moderately conserved amino acid (considering 11 species); Grantham distance 64; Polyphen score 0.480 (possibly damaging); SIFT score 0.02 (not tolerated). The mutation is not detected in 1000 control chromosomes. Conclusion: unclassified variant

-SIFT algorithm (Sorting Intolerant From Tolerant) is available at http://sift.jcvi.org
-Polyphen-2 (Polymorphism Phenotyping program version 2 is available at http://genetics.bwh.harvard.edu/pph/