First locus for primary pulmonary vein stenosis maps to chromosome 2q

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Received 17 November 2008; revised 19 February 2009; accepted 11 June 2009; online publish-ahead-of-print 4 July 2009

Aims

Primary pulmonary vein stenosis (PVS) is a rare cardiac abnormality that exhibits a high morbidity and mortality rate. The disease is characterized by obstruction of the pulmonary venous blood flow owing to congenital hypoplasia of individual extra-pulmonary veins. We describe a consanguineous Turkish family with four affected siblings with primary PVS in association with prenatal lymphatic abnormalities. We aimed to map the first gene for primary PVS.

Methods and results

Patients had extensive cardiological examinations including electrocardiograms, echocardiograms, ventilation–perfusion scans, and cardiac catheterizations. All patients died before the age of 16 months because of severe progressive primary PVS. Chromosomal analysis revealed normal karyotypes. We performed a genome-wide linkage analysis using 250 K single nucleotide polymorphism arrays and found the first locus for primary PVS on chromosome 2q35-2q36.1 [multipoint logarithms (base 10) of odds (LOD) scores 3.6]. By fine-mapping with microsatellite markers, we confirmed the homozygous region that extended 6.6 Mb (D2S164–D2S133). Sequencing 12 (188 exons) of the 88 genes from the region revealed no disease-causing sequence variations.

Conclusion

Our findings open perspectives for the identification of the genetic cause(s) leading to PVS, which might contribute to elucidate the pathological mechanisms involved in this disorder.

Keywords

Primary pulmonary vein stenosis • Genome wide • Linkage analysis • SNP arrays

Introduction

Primary pulmonary vein stenosis (PVS) is a rare cardiac abnormality which occurs in about 0.4% of all cardiovascular malformations.1 It has a high morbidity and mortality rate. It is characterized by obstruction of the pulmonary venous blood flow owing to congenital hypoplasia of individual extrapulmonary veins or constriction of the intima at or near the venous–atrial junction.2

The condition can be an isolated anomaly but in up to 80% of patients it coexists with a variety of other congenital heart malformations such as patent ductus arteriosus and septal defects.3–5

The pulmonary venous obstruction can involve one or multiple (normally connected) veins. Symptoms present usually during the first months to years of life and are determined by the extensiveness of the PVS.4–6 The most frequent presenting symptoms are failure to thrive, dyspnoea, and recurrent pneumonias. Later, pulmonary hypertension, pulmonary oedema, haemoptysis, and congestive heart failure may develop. It is difficult to differentiate the symptoms from chronic lung disease and from other causes of pulmonary venous hypertension.7,8 Currently, most surgical therapies are unsuccessful because of restenosis and progression of the disease9 and the majority of the patients die during the first years of life.
Secondary or acquired PVS is a similar condition that has various causes at different ages. In children it can be a complication following surgery for correction of partial or total anomalous pulmonary venous return and occurs in about 5–15% of operated patients. In adults it is a well-recognized complication after radiofrequency ablation for atrial fibrillation in patients refractory to treatment with antiarrhythmic drugs.

In both the primary and secondary forms of PVS, histological findings are similar and show variable manifestations of neointimal proliferation leading to occlusion of the lumen of one or more of the pulmonary veins. The underlying mechanism for the pathological manifestations has never been elucidated.

So far only sporadic patients with PVS have been reported. We describe a consanguineous Turkish family with four affected siblings with primary PVS in association with prenatal lymphatic anomalies. We performed a genome-wide linkage analysis and identified the first locus for primary PVS.

**Methods**

**Clinical studies**

Both parents (third cousins) were healthy. Out of six pregnancies, three children were born alive and all died before the age of 16 months because of severe progressive primary PVS (Figure 1). In two children transient increased (septated) nuchal translucency and signs of fetal hydrops were present at prenatal ultrasound examinations. Furthermore, one pregnancy was terminated at 22 weeks because of severe fetal hydrops, indicating an affected foetus. Two pregnancies resulted in early spontaneous abortions.

Chromosomal analysis revealed a normal karyotype in three affected patients and the healthy parents. Detailed post-mortem macroscopic and microscopic examination of the heart and lungs of the 22-week-old foetus was performed. The parents declined autopsy in all three live born patients. Both parents had a cardiological examination that included echocardiogram and electrocardiogram. Informed consent for DNA studies was obtained from the parents.

**Molecular studies**

**Genotyping**

Genomic DNA was isolated from peripheral blood using the Puregene DNA purification kit (Genta Systems) and from chorion villi cells (one foetus) using standard procedures. A DNA sample from another deceased patient was obtained from stored tissue material (lung biopsy, paraffin-embedded tissue). The genome-wide search was conducted using DNA from five members of the family including both parents and three patients (two live born children and one foetus).

The Affymetrix GeneChip Mapping 250 K Nsp Array containing 262,264 single nucleotide polymorphism (SNP) markers was used. Samples were processed according to the manufacturer’s instructions (Affymetrix GeneChip Mapping Assay). Affymetrix GCOS v.1.4, and GTYPE software v.4.1 were used.

**Microsatellite markers**

Microsatellite markers mapping to the identified genomic region were selected. Polymerase chain reaction (PCR) products were run on an ABI Prism 3100 genetic sequencer (Applied Biosystems) and analysed using the GeneMapper software v.3.0 (Applied Biosystems).

**Sequencing analysis**

Bidirectional sequencing of the coding region and the exon–intron boundaries of candidate genes was undertaken using PCR primers designed by Primer3 software. PCR products were purified and sequenced using BigDye Terminator chemistry v.3.1 on an ABI Prism 3130xl genetic analyzer (Applied Biosystems). Sequences were aligned and compared with consensus sequences obtained from the human genome databases using the Applied Biosystems software package SeqScape v.2.5.

**Linkage analysis and loci identification**

Genetic linkage analysis was performed to estimate whether two loci (in this case the disease gene and a set of SNPs) are in close proximity to each other on a chromosome. Logarithms (base 10) of odds (LOD) scores, a measure of the likelihood of genetic linkage between loci, were calculated. The statistical package EasyLinkage Plus v.5.08 designed to perform automated linkage analyses using large-scale SNP data was used to perform all analyses. All SNPs showing inconsistency in transmission were removed from further analyses. Allegro v.1.2c software (incorporated in the EasyLinkage Plus v.5.08 package) was used to perform fully automated single point and multipoint linkage analysis. LOD scores were obtained using a recessive model.
of inheritance, with a penetrance of 99% and a disease allele frequency of 1:10 000. Allele frequencies of genotyped SNPs were set to codominant. Map order and genetic inter-SNPs distances were taken from the Affymetrix website.

Since closely spaced SNP markers were used for the linkage analysis, the genome analyses were performed with predefined spacing of 0.2–0.4 cM, in blocks of 90 and 100 SNPs. Then, single chromosomes showing positive linkage signals were independently analysed under the same conditions and haplotypes were constructed.

Graphical visualization of haplotypes to facilitate inspection and analyses was performed with HaploPainter v.029.5, a tool for drawing pedigrees with complex haplotypes.

**Results**

**Clinical studies**

The family (Figure 1) was referred to the Department of Clinical Genetics for genetic counselling after the death of their first child because of primary PVS. During a period of 9 years, three other affected children were born.

**Patient 1**

The first child was born after an uneventful pregnancy. At the age of 10 months the boy presented with a failure to thrive. Physical examination revealed no abnormalities. However, an X-ray of the thorax showed an increased pulmonary vessel pattern suggesting pulmonary venous obstruction. Electrocardiogram (EKG) was normal but Doppler-echocardiography revealed an increased flow (2 m/s; normal <1.6 m/s) in the left pulmonary vein (LPV) and a patent foramen ovale with a minimal left–right shunt. Angiography by cardiac catheterization revealed a severe stenosis of the LPVs near the entrance of the left atrium and the right pulmonary veins (RPVs) could not be visualized. A ventilation–perfusion scintigraphy revealed no perfusion of the right lung. A magnetic resonance imaging scan revealed two normally connected LPVs which were stenotic about 2 cm from the entrance of the left atrium and an atretic remnant of the RPVs near the entrance of the left atrium (Figure 2). At the age of 13 months, the boy was operated and a veno-plasty was performed on the LPVs and RPVs. In the consecutive months, the child developed respiratory insufficiency owing to progressive PVS. The boy had recurrent haemoptysis and subsequent melena. He died at the age of 15.5 months because of respiratory insufficiency.

**Patient 2**

A prenatal ultrasound revealed an increased nuchal translucency of 11 mm and mild generalized skin oedema at a gestational age of 14 weeks (Figure 3A). At gestational ages of 19 and 31 weeks, the nuchal translucency and skin oedema were no longer observed. Advanced ultrasound examinations revealed no structural abnormalities and a normal four-chambered view. Normal connecting pulmonary veins with normal venous Doppler signals were observed. A boy was born at term with normal birth measurements. No association between the transient prenatal findings including nuchal translucency and skin oedema and PVS was suspected yet.

At the age of 2.5 months, the child had feeding problems and tachypnoea. Physical examination and cardiological examination, including EKG and two-dimensional echocardiography revealed no abnormalities. Transoesophageal Doppler-echocardiography showed an increased inflow velocity (1.8 m/s) at the entrance of the LPV and a patent foramen ovale with a haemodynamically insignificant left–right shunt. Angiography by cardiac catheterization displayed dilated pulmonary veins with severe stenotic lesions near the entrance into the left atrium. The right upper vein was not displayed. A ventilation–perfusion scintigraphy revealed complete absence of perfusion of the right upper and a partial abnormal perfusion of the left lower pulmonary lobe. At the age of 6 months, he was operated and a complete stenosis of the left lower and right upper pulmonary veins until the lung parenchyma was confirmed. A stenosis of the left upper and right lower pulmonary vein near the entrance of the left atrium was seen. A veno-plasty of both the left and right PVS was performed. One month after surgery, the boy was admitted to the hospital owing to feeding problems and progressive tachypnoea because of restenosis of the pulmonary veins. He died at the age of 7.5 months because of respiratory insufficiency.

**Patient 3**

In the fourth pregnancy, a septated nuchal translucency of 11 mm was detected at a gestational age of 10 weeks. At 19 weeks, the nuchal translucency evolved and a septated sonolucency with a thickness of 6 mm was seen, most likely representing a cystic hygroma (Figure 3B). Advanced ultrasound examinations revealed no structural abnormalities, in particular no heart abnormalities. A girl was born at a gestational age of 39 weeks with normal birth measurements. Soon after birth, she was admitted to the hospital because of haemoptysis and subsequent melena. A cardiac catheterization was performed and revealed a PVS at the left side. There was thrombocytopenia and anaemia.

At the age of 1.5 months, a cardiac catheterization was performed, which revealed a progression of the LPV stenosis and appearance of RPV stenosis. A balloon dilatation was performed...
on the left side. At the age of 2 months, the girl died because of respiratory insufficiency caused by the PVS.

Patient 4
In the fifth pregnancy, a septated nuchal translucency of 7 mm and fetal hydrops was detected at gestational age of 13 weeks. A chorionic villus sampling was performed at a gestational age of 13.5 weeks, revealing a normal male karyotype. At 19 weeks of gestation there was massive hydrops with a large nuchal cystic hygroma, bilateral hydrothorax, pericardial effusion, and ascites (Figure 3C and D). Different causes of fetal hydrops including fetomaternal blood group antagonism and maternal infections (TORCHS and Parvovirus) were excluded. Normal peak velocity of systolic blood flow in the middle cerebral artery was obtained by Doppler ultrasonography indicating no signs of foetal anaemia. Because of the poor prognosis, the pregnancy was terminated at a gestational age of 22 weeks. A severely hydropic foetus was born with a nuchal hygroma. Autopsy of the heart and lungs revealed no macroscopic abnormalities, in particular, normally connecting pulmonary veins with no signs of stenosis. Microscopic evaluation showed no structural abnormalities except for a dilatation of the lymphatic vessels in the lungs.

Genome-wide linkage analysis
The linkage analysis revealed a large homozygous region on chromosome 2q (199–232 cM) with a significant multipoint LOD score of 3.6. This locus was detected through all the analysis models applied (SNP spacing varying from 0.2 to 0.4 cM and different block sizes containing 90–100 SNPs).

We constructed haplotypes on chromosome 2q (199–232 cM) using all informative SNPs in the area. A recombination event occurring in the maternal haplotype determined the upper border of the region between rs2372938 and rs722082 (217.9 Mb), the first informative SNP that was homozygous for all three patients. A recombination occurring in patient II-6 (rs2043566, 237.51 Mb) delimited the telomeric border (data not shown).

We observed an area of approximately 1820 consecutive SNPs for which the patients were (mostly) homozygotes. We noticed that seven of the 1820 SNPs were heterozygous for at least two of the patients. However, direct DNA sequencing of the region revealed only homozygous genotypes indicating that the previously observed heterozygous SNPs (from raw array data) were likely incorrect genotype calls.

In order to confirm these results, we tested 28 microsatellite markers mapping to the 2q35-2q36.1 area. Then we could include DNA samples from all four affected individuals (Figure 4). Because of a recombination event observed in patient II-5, the maximum candidate region was considerably reduced to 6.6 Mb (9.6 cM) from marker D2S164 (217.7 Mb, 214.7 cM, very close to the border indicated by the SNP analysis) until D2S133 (224.3 Mb, 224.3 cM) containing 88 genes from which 14 have been associated to distinct phenotypes (National Center for Biotechnology Information, NCBI build 36.3). The minimum region was covering 5.9 Mb (8.5 cM) between D2S295 (218 Mb,
215.8 cM) and D2S279 (223.9 Mb, 224.3 cM) after combining genotypes (SNPs and microsatellites) from all patients. We selected 12 positional candidate genes for further sequence analysis based on protein and molecular function, expression data, and animal models (Table 1). Unfortunately, no novel sequence changes were found.

We also used the SNP data to investigate possible disease-causing copy number variations (CNVs). Genome-wide analysis did not reveal any CNV co-segregating with the PVS phenotype.

Discussion

Primary PVS is a rare congenital heart malformation that has so far only been described in sporadic cases. We describe a family with PVS associated with prenatal lymphatic anomalies in four affected siblings suggesting a monogenic cause of the disease. An autosomal recessive mode of inheritance is most likely since the parents are consanguineous and have no signs or symptoms of PVS on cardiological examination.

This is the first report of prenatal lymphatic abnormalities in patients with congenital PVS. We propose that the prenatal lymphatic abnormalities are part of the PVS phenotype like it has been reported before for other syndromic and non-syndromic forms of congenital heart malformations, such as Noonan syndrome and Turner syndrome.17–19 The disease might be underdiagnosed because of the high intrauterine demise and neonatal death that is associated with hydrops fetalis and owing to the evanescent nature of the increased nuchal translucency.

Increased nuchal translucency, nuchal cystic hygroma, and hydrops fetalis have numerous causes of which chromosome abnormalities, infections and congenital malformations, in particular cardiovascular diseases, are the most frequent.17,20,21 In our
patients, both chromosomal abnormalities and infections were excluded. Two of the three patients with prenatal lymphatic abnormalities developed severe stenosis of the pulmonary veins. In the third patient, the foetus, abnormal dilatation of the lymphatic vessels in the lungs was found on microscopic examination, without gross abnormalities of the pulmonary veins. This foetus was considered affected, as the prenatal presentation (nuchal cystic hygroma and features of hydrops) was similar to the abnormalities observed in the previous affected children. The absence of pulmonary veins occlusion at autopsy was not unexpected, as symptoms or signs of PVS were absent at birth in his affected siblings but developed postnatally.

The dilatation of the lymphatic vessels might suggest that they are not secondary to venous obstruction in the lungs, but owing to abnormalities of the lymphatic vessels and/or lymphatic vessel connections itself. Pulmonary venous flow is limited during foetal life and lymphatic abnormalities or hydrops are usually not present in other conditions in which pulmonary venous obstruction occurs in prenatal life, such as partial or total anomalous pulmonary venous return. Another option is that the dilatation of the lymphatic vessels might be owing to increased drainage of fluid from the lungs by this route. The mechanism by which increased nuchal translucency and hydrops in both syndromic and non-syndromic patients with

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<td>33</td>
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<td>Angio-associated migratory cell protein (AAMP)</td>
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congenital heart malformations are produced remains unclear. A mechanistic theory is that there is a primary disorder of (lymph)angiogenesis or endothelial function, which could cause both collections of nuchal fluid and also cardiac malformations. Since the lymphatic system originates from lymphatic endothelial cells that sprout from the embryonic veins and then migrate, it might be possible that genetic defects in proteins essential for these lymphatic and venous endothelial cells lead to a disturbed venous-lymphatic phenotype.

It remains obscure why the pulmonary veins were apparently normal at birth and PVS developed only after birth. As the pulmonary veins of the aborted foetuses were normal at 22 weeks of gestation and all affected neonates had initially no clinical abnormalities suggesting PVS, the disease might have been induced by the introduction of oxygen in the pulmonary venous circulation, or haemodynamic changes in the pulmonary circulation such as increased blood flow.

The mechanism by which both primary and secondary PVS develop might be similar. Histopathological examination of specimens of patients with isolated primary PVS revealed that the disease is caused by eccentric abnormal intimal proliferation of spindle-shaped cells in the pulmonary veins. The lesional cells stain positive for smooth muscle actin and muscle-specific actin and have the histological appearance of myofibroblasts. Further immunohistochemical analysis of the lesional cells in primary PVS showed expression of receptor tyrosine kinases. Defining the origin of these smooth muscle-like cells is challenging. Pathological studies of specimens of patients with secondary PVS are obviously rare but some studies revealed neointimal fibromuscular proliferation both in areas close to and remote from the site of operation or catheter ablation. The fact that the pathological findings in secondary PVS are similar to primary PVS are pointing to a comparable disease pathogenesis.

PVS should be differentiated from (intra)pulmonary veno-occlusive disease (PVOD), that is characterized by obstruction of small pulmonary veins and usually develops in adulthood. In contrast, PVS is caused by obstruction of the extrapulmonary veins at the venoatrial junctions. It might be possible that PVOD develops secondary to chronic PVS because of pulmonary hypertension as is described both in patients with primary and secondary PVS.

Heterozygous mutations in the bone morphogenetic protein receptor type II (BMPR2) have been described in patients with PVOD. Mutation analysis of BMPR2 in one of our patients revealed no pathogenic mutations.

We performed a GWLA and found a candidate interval on chromosome 2q35-2q36.1 comprising a maximum of 9.6 cM (6.6 Mb) and containing 88 genes. This interval includes some interesting candidate functional genes (Table 1). Among them, sphingosine-1-phosphate phosphatase 2 (SPP2, involved in controlling cell proliferation and migration in a variety of cell types including vascular smooth cells, cardiomyocytes, and endothelial cells) seemed particularly promising. Unfortunately, sequencing of these genes revealed no disease-causing sequence variations so far. Finding other patients with homozygosity on the chromosome 2q region may help reduce the number of genes to investigate.

Our findings open perspectives for the identification of the genetic cause(s) leading to PVS, which might contribute to elucidate the pathological mechanisms involved in this disorder. The contribution of this gene to other cases with primary or secondary PVS remains to be elucidated.

Web resources

Acknowledgements
The authors thank the parents for their cooperation. We also thank Tom De Vries-Lentsch for the photographic work, F. Petrij for support, W. Dinjens from the Department of Pathology for isolation of the DNA from the lung biopsy, and J. Gille of the DNA diagnostic laboratory of the VU Medical Center in Amsterdam for performing mutation analysis of BMPR2 gene. We acknowledge A. de Klein and B. Eussen for performing the copy number variation analysis.

Funding
This research was partially funded by the Center for Biomedical Genetics (CBG), The Netherlands.

Conflict of interest: none declared.

References
A 65-year-old male patient with a blank cardiac history was admitted to our hospital after an out-of-hospital cardiac arrest due to ventricular fibrillation. Immediate coronary angiography showed significant three-vessel disease without culprit lesions and no intervention was performed. Cardiac enzymes remained within normal limits and he made a complete recovery. Cardiovascular magnetic resonance (CMR) imaging on day 6 revealed a dilated left ventricle with low ejection fraction (26%). Late gadolinium enhancement accurately delineates reversible and irreversible myocardial injury in animal studies. In humans, scar hyperintensity was observed on T2-weighted imaging and a chronic anterior wall myocardial infarction with substantial residual viable myocardium was concluded. Unfortunately, he died 5 days later prior to a scheduled coronary artery bypass grafting operation. An autopsy was performed. Macroscopic inspection of a nitro-blue tetrazolium (NBT)-stained, mid-ventricular section of the heart (Panel B) showed scar tissue of an old anteroseptal and anterolateral wall myocardial infarction as a subendocardial rim of compact fibrosis (arrows, Panel A). In addition to these dense areas of subendocardial scarring, areas of diffuse fibrosis were visible which were less intensely staining by Sirius red, for example in the infarotateral papillary muscle (*, Panel C). This represents scar tissue of an infarct of at least several weeks old (transition phase between granulation tissue and old scar tissue). Both areas of dense and diffuse fibrosis highly correlated with in vivo LGE, albeit with different SI (* and arrows, Panel A). Small discrepancies between histology and LGE CMR are explained by slightly different slice levels, the lower spatial resolution, and partial volume effect of CMR.

Late gadolinium enhancement accurately delineates reversible and irreversible myocardial injury in animal studies. In humans, scar size determined with LGE closely correlates with positron emission tomography and can be used to predict reversible myocardial dysfunction after revascularization.

Literature reports comparing LGE CMR with histological confirmed macroscopy in humans are scarce. Our case demonstrates the ability of LGE CMR to detect both dense and diffuse fibrosis.