Original research

Biallelic *NPR1* loss of function variants are responsible for neonatal systemic hypertension

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ABSTRACT

Background Early-onset isolated systemic hypertension is a rare condition of unknown genetic origin. Renovascular, renal parenchymal diseases or aortic coarctation are the most common causes of secondary systemic hypertension in younger children and neonates. We investigated the genetic bases of early-onset isolated systemic hypertension.
Methods Whole-exome sequencing (WES) was followed by variant filtering and Sanger sequencing for validation and familial segregation of selected variants in a large consanguineous family. mRNA expression was performed to evaluate the impact of the predicted pathogenic variant on gene expression. WES or Sanger sequencing was performed in additional unrelated affected individuals.
Results In one consanguineous family with four children presenting with isolated neonatal-onset systemic hypertension, we identified homozygous stop–gain variant in the *NPR1* gene (NM_000906.4:c.1159C>T (p.Arg387Ter)) in the affected individuals. This variant leads to a dramatic reduction of NPR1 RNA levels. *NPR1* gene analysis of additional families allowed the identification of another family with two affected children carrying homozygous frameshift variant in *NPR1* (NM_000906.4:c.175del (p.Val59TrpfsTer8)).
Conclusion We show for the first time that biallelic loss of function of *NPR1* is responsible for isolated neonatal-onset systemic hypertension in humans, which represents a new autosomal recessive genetic cause of infantile systemic hypertension or cardiogenic shock. This is consistent with studies reporting early-onset systemic hypertension and sudden death in Npr1-deficient mice. *NPR1* gene analysis should be therefore investigated in infants with early-onset systemic hypertension with or without cardiogenic shock of unknown origin.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ There are many causes of neonatal systemic hypertension (NSH) including either acquired or associated with other organ involvement. We investigated here the genetic bases of isolated NSH which remains of unknown origin.

WHAT THIS STUDY ADDS

⇒ We show for the first time that biallelic loss of function variants of *NPR1* are responsible for isolated NSH in humans in two unrelated multiplex families, which represents a new autosomal recessive genetic cause of NSH or cardiogenic shock.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ *NPR1* gene analysis should be therefore investigated in infants with isolated NSH with or without cardiogenic shock. The benefits of an accurate genetic diagnosis include more tailored management of systemic hypertension, heart involvement, improved surveillance and accurate genetic information of family members.

INTRODUCTION

Incidence of neonatal systemic hypertension (NSH) is around 1%.[1] There are many causes of NSH including either acquired or congenital causes.[2] The most common renovascular abnormality associated with systemic hypertension in neonates is thrombus formation secondary to umbilical artery catheter placement. Congenital causes of NSH are usually associated with other organ involvement with autosomal dominant or recessive inheritances. Fibromuscular dysplasia resulting in renal arterial stenosis is an important cause of renovascular NSH as well as compression of the renal arteries by tumours. Non-renal intra-abdominal tumours, including neuroblastoma or pheochromocytoma, can cause NSH either due to direct compression on renal vessels and/or ureters or due to production of vasoactive substances such as catecholamines. The common cause of NSH is congenital renal abnormalities. Both autosomal dominant and recessive polycystic kidney diseases, tuberous sclerosis and Wilms tumour may present with NSH. Broncho-pulmonary dysplasia (BPD)-associated NSH is reported with an incidence of 43% of infants with BPD.[2] Coarctation of the aorta may be detected in the neonate and has been frequently implicated as a cause of NSH. Disorders of the endocrine system, including congenital adrenal hyperplasia, hyperthyroidism, hyperaldosteronism and Williams-Beuren syndrome, may also present with NSH. Diagnostic approaches to systemic hypertension in neonates include careful clinical examination and cardiac, artery, pulmonary, kidney and endocrine investigations. However, isolated NSH remains of unknown origin in many cases. To gain further insight into the underlying cause of isolated early-onset systemic

hypertension of unknown origin, we took advantage of the added value of whole-exome sequencing (WES) to study families with such phenotype.

METHODS

Whole-exome sequencing
Genomic DNA for each individual was extracted from blood or frozen tissue (lung sample in case of fetal death) with the use of a QiaAmp DNA midi or mini kit, respectively (Qiagen). WES was performed using a completed Twist Bioscience Human Core Exome (Consensus Coding Database Sequence) kit for library preparation and exome enrichment in 10 affected individuals from six unrelated families (Integragen). Sequencing was performed on a Genome Analyzer Hiseq4000 Illumina instrument in paired-end mode with a read length of 2×80 bp (Integragen). The median coverage was 80×.

Bioinformatics analysis
Reads were aligned to the human reference genome sequence (UCSC hg19, NCBI build 37.3) via the Burrows-Wheeler Aligner program.[3] Variants were selected using the SAMtools[4] then annotated using Annovar softwares.[5] Variants in coding regions (including non-synonymous and nonsense variants), intron–exon junctions (≤10 bp) or short coding insertions or deletions were selected when the minor allele frequency (MAF) was less or equal to 0.005 (using 1000G, ExAC, TopMed and GnomAD). Prediction of pathogenicity of missense variants was performed using polyphen-2 (with score ≥0.5)[6] or Sift softwares (with score ≤0.05)[7] and splice variants using Human Splicing Finder.[8]

Sanger sequencing
Selected variants identified through WES were validated by Sanger sequencing. PCR primer pairs were designed from genomic DNA to amplify and sequence each *NPR1* exon (online supplemental table S1). PCR amplification was carried out as previously described.[9] PCR products were then purified then sequenced using the forward or reverse primers (Eurofins Genomics). The obtained DNA sequences were compared with published sequences (BLAST, NCBI). Sanger sequencing was also performed to establish the genotype of each family member and to analyse the segregation of the variant within each family. The coding regions of *NPR1* were sequenced in a cohort of 11 additional affected individuals from 10 unrelated families.

Real-time PCR amplification of genomic DNA
Real time PCR amplification was conducted using genomic DNA on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Genomic deletion was defined when the ratio of tested DNA to control DNA was equal to or less than 0.5. Real-time PCR amplification of each sample was performed in duplicate using primers of each exon of *NPR1* (online supplemental table S1). *ALB* (albumin) was used as internal control (online supplemental table S1).

RT-PCR amplification
Total RNAs were extracted from lymphoblastoid cell lines of two controls (healthy individuals unrelated to affected families, C1 and C2) and two affected individuals from family 1 (F1) (II:1 and II:2) by using TRI Reagent LS method (Sigma). One hundred-nanogram RNA was used to synthesise cDNA by using random primers following the manufacturer's manual (SuperScript III Reverse Transcriptase, Invitrogen) in a final volume

of 20 µL. PCR amplification was carried out as previously described.[9] PCR amplification analysis from single-strand cDNA was performed using primers chosen in exons 3 and 5 of *NPR1* gene (online supplemental table S1). As internal control for PCR amplification, β-actin cDNA was coamplified (online supplemental table S1). RT-PCR products were separated by agarose gel electrophoresis and labelled with ethidium bromide.

Quantitative RT-PCR amplification
After RNA extraction from lymphoblastoid cell lines (see previous paragraph), reverse transcription was performed using 10 ng RNA by using IScript Reverse Transcription Supermix (Bio-Rad) in a final volume of 20 µL. Quantitative RT-PCR amplification was conducted using 1 µL of RT on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Quantitative RT-PCR amplification of each sample (control C1, patients II:1 and II:2 of F1) was performed in duplicate using primers of NPR1, β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (online supplemental table S1). A standard curve of quantitative RT-PCR was performed from the RT of control sample for the three cDNA targets. Quantitative RT-PCR amplification of β-actin and GAPDH cDNA was used as internal controls for quantification.

RESULTS

In F1, four affected children were born to consanguineous parents from African origin (figure 1 and table 1). In the first male fetus (F1.II.1), an increased nuchal translucency (NT) of 3.5 mm was detected at the first trimester based on ultrasound examination. Karyotype was normal. Echocardiography at 20 weeks of gestation (w.g.) was normal. Ultrasound examinations at the second and third trimesters were normal. The child was born at 39 w.g. with normal weight, size and head circumference. Before postnatal day 10, the child developed polypnea associated with heart failure and systemic hypertension (systolic blood pressure: 128 mm Hg, above the 99th percentile).[2] Echocardiography, renal ultrasound with Doppler and renal angiography were normal. Serum electrolytes, plasma active renin concentration, plasma and urine aldosterone, and thyroid hormone levels were normal. For the second female fetus (F1.II.2), an increased NT of 3.8 mm was detected at the first trimester. Ultrasound examination and echocardiography performed at 19, 22, 28 and 33 w.g. detected isolated right ventricular and ventricular septum hypertrophy. Karyotype and 22q11 FISH analysis were normal. The child was born at 39 w.g. Systemic hypertension was detected before postnatal day 5 (systolic blood pressure: 107 mm Hg, above the 99th percentile). Echocardiography was initially normal and renal ultrasound with Doppler was normal. After the age of 1 year, systolic pulmonary pressure was estimated at 40 mm Hg at ultrasonography and remained stable during follow-up. A right heart catheterisation confirmed that pulmonary arterial pressure was 47/17/31 mm Hg. For the third female fetus (F1.II.3), NT was normal (2 mm) at the first trimester as well as ultrasound examinations at 22, 25, 29 and 32 w.g. and echocardiography at 30 w.g. The child was born at 41 w.g. Before postnatal day 10, the newborn developed respiratory distress associated with cardiogenic shock and systemic hypertension (systolic blood pressure: 139 mm Hg, above the 99th percentile). Echocardiography and renal ultrasound with Doppler were normal. For the fourth female fetus (F1.II.4), an increased NT of 7.4 mm was detected at the first trimester with normal echocardiography and ultrasound examinations at 14, 16, 22, 27, 32
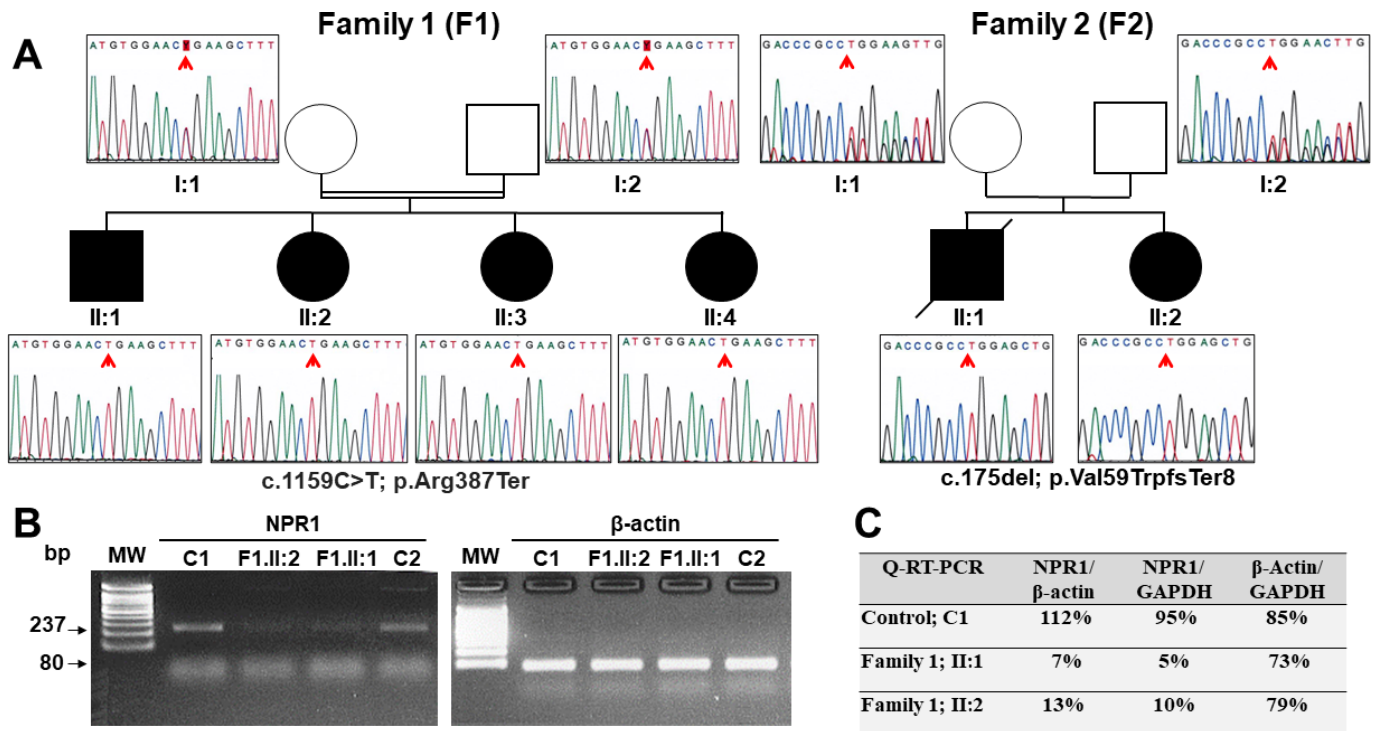
Figure 1 Homozygous loss of function variants in *NPR1* in two families with neonatal-onset systemic hypertension and transcript analysis. (A) Pedigrees with Sanger sequencing results for F1 and F2 are shown. Arrows indicate mutant nucleotide positions. The affected individuals carry homozygous *NPR1* variants. The nucleotide and amino acid changes based on NM_000906.4 and NP_000897.3 reference sequences, respectively, are indicated. Open symbols: unaffected; filled symbols: affected. (B) RT-PCR amplification was performed using NPR1-specific primers on RNAs extracted from the lymphoblastoid cells of affected individuals (F1.II:1 and F1.II:2 of family 1) and two unrelated healthy controls (C1 and C2). When compared with the β-actin control and control individuals, NPR1 RNA is markedly reduced in affected individuals. (C) Quantitative RT-PCR analysis of NPR1, β-actin and GAPDH was performed from RT of control (C1) and affected individuals (F1: II:1 and II:2). The ratio of NPR1 to β-actin or GAPDH RNA quantity is markedly reduced in both patients (≤13%) when compared with control. In contrast, the ratio of β-actin to GAPDH is similar in control and patients. F1, family 1; F2, family 2; MW, molecular weight.

and 36 w.g. Karyotype, microarray comparative genomic hybridisation (aCGH) and RASopathy-associated gene analyses were normal. The child was born at 40 w.g. and systemic hypertension was detected before postnatal day 5 (systolic blood pressure: 108 mm Hg, above the 99th percentile). Echocardiography detected a patent foramen ovale. Psychomotor development, height and weight growth were normal in the three children aged over 7 years (F1.II.1, F1.II.2 and F1.II.3) and over 1 year for the youngest (F1.II.4). The mother has no medical history, but the father is treated for systemic hypertension discovered after 30 years of age (systolic/diastolic blood pressure: 160/110 mm Hg).

WES was performed in three out of the four affected children of F1 (II:2, II:3 and II:4). Predicted deleterious variants in genes shared by the three affected individuals were selected as candidates. A homozygous variant in the *NPR1* gene was selected as the best candidate. It is a homozygous stop–gain variant located in exon 4 of the *NPR1* gene (NM_000906.4:c.1159C>T and NP_000897.3:p.Arg387Ter). This variant was annotated in dbSNP155 (rs1313788512) with a very low MAF of 0.000004 (TopMed) and 0.000007 (GnomAD). In addition, based on WES data of the three affected individuals, this variant is located within a homozygous region of 11 Mb from genomic position chr1:145 826 991–156 923 966 (GRCh38 (hg38)), indicating a large region of homozygosity by descent (data not shown, available on request). The variant was confirmed through Sanger sequencing of PCR products using primers flanking the variant (figure 1 and online supplemental table S1). The variant was found homozygous in the four affected children and heterozygous in both parents (figure 1). RT-PCR amplification products

of NPR1 cDNA from lymphoblastoid cell RNAs of two affected individuals (F1: II:1 and II:2) compared with unrelated healthy controls (C1 and C2) revealed a dramatic reduction of NPR1 RNA levels in affected individuals compared with controls and β-actin expression (figure 1 and online supplemental table S1). To quantify the effect of the homozygous *NPR1* stop–gain variant on RNA expression, quantitative RT-PCR analysis of NPR1, β-actin and GAPDH was performed from RT of control (C1) and affected individuals II:1 and II:2 of F1. Based on the standard curves from control, the ratio of NPR1 to β-actin or GAPDH RNA expression was found to be markedly reduced in both patients (≤13%), indicating a dramatic reduction of NPR1 RNA levels (figure 1). The ratio of β-actin to GAPDH was similar in control and in patients II:1 and II:2 (figure 1). These results demonstrate the deleterious effect of *NPR1* homozygous stop–gain variant on RNA stability. As the father developed systemic hypertension during adulthood, Sanger sequencing and Q-PCR of the coding regions of *NPR1* were performed on the father's DNA to determine whether an allelic variant in *NPR1* was present. No variant other than the heterozygous stop–gain variant (NM_000906.4:c.1159C>T) was identified (data not shown).

NPR1 was therefore selected as high candidate for the early-onset systemic hypertension-causing gene. Importantly, in three out of the four affected children of F1, increased NT was observed at the first ultrasound examination between 11 and 14 w.g. For this reason, *NPR1* genetic analysis included affected individuals with either isolated increased fetal NT thickness with normal karyotype, aCGH and RASopathy-associated genes

Table 1 Main clinical features and characteristics of NPR1 variants found in patients with isolated NSH

Family	Family 1				Family 2	
	F1.II.1	F1.II.2	F1.II.3	F1.II.4	F2.II.1	F2.II.2
NPRI variant						
cDNA change (NM_000906.4)	c.1159C>T	c.1159C>T	c.1159C>T	c.1159C>T	c.175del	c.175del
Amino acid change (NP_000897.3)	p.Arg387Ter	p.Arg387Ter	p.Arg387Ter	p.Arg387Ter	p.Val59TrpfsTer8	p.Val59TrpfsTer8
Genotype	Homozygous	Homozygous	Homozygous	Homozygous	Homozygous	Homozygous
Pregnancy						
Nuchal translucency (mm)	3.5	3.8	2	7.4	nr	1.4
Echocardiography (w.g.)	N (20)	Right ventricular and ventricular septum hypertrophy (19–33)	N (30)	N (14)		
Ultrasound examinations	N	N	N	N	N	N
Birth						
Sex	Male	Female	Female	Female	Male	Female
Birth (w.g.)	39	39	41	40	38	39
Weight (g), size (cm)	3295, 49	3030, 51	2950, 46	3210, 48	3190, 51	2500, 48
Head circumference (cm)	35	33	35	34	36	33
Symptoms						
First symptoms (postnatal day)	<10	<5	<10	<5	<15	<20
Blood pressure (systolic/diastolic)	128/89	107/71	139/56	108/70	nr	116/60
Clinical description	Polypnoea and heart failure	Respiratory distress and NSH	Respiratory distress, cardiogenic shock and NSH	NSH	Cardiogenic shock and death at postnatal day 12	Cardiogenic shock and NSH
Investigations						
Postnatal echocardiography	N	N	N	Patent foramen ovale	Left ventricular hypertrophy	Left ventricular non-compaction
Renal ultrasound and Doppler	N	N	N			N
Renal angiography	N					N
Thyroid hormones	N					N
Plasma renin and aldosterone	N	N	N	N		N

Ultrasound examination: second and third trimesters, blood pressure (mm Hg).
F1, family 1; F2, family 2; N, normal; nr, not reported; NSH, neonatal systemic hypertension; w.g., week of gestation.

or early-onset isolated systemic hypertension. WES or Sanger sequencing and Q-PCR of the coding regions of *NPR1* (online supplemental table S1) were performed on the DNA samples of a cohort of 15 additional unrelated families with either increased fetal NT thickness (n=9) or childhood-onset isolated systemic hypertension (n=6) using the same criteria for variant selection. In two out of six families, systemic hypertension was detected before 1 month of age, and in four families, systemic hypertension was observed from 1 month to 7 years of age.

This approach allowed the identification of an additional family (family 2 (F2), figure 1 and table 1). In this family, two affected children were born to parents from the same geographical origin of Africa. In the first male fetus (F2.II.1), the pregnancy was reported as normal. The child was born at 38 w.g. with normal weight, size and head circumference. Before postnatal day 15, the newborn developed cardiogenic shock. Echocardiography revealed left ventricular hypertrophy. He died before postnatal day 15 right after admission in the intensive care unit. Metabolic defect or infectious diseases were excluded. For the second female fetus (F2.II.2), the pregnancy was reported as normal, including NT at the first trimester (1.4 mm). The child was born at 39 w.g. with low weight (2500 g) but normal size and head circumference. Before postnatal day 20, she developed

cardiogenic shock and systemic hypertension (systolic blood pressure: 116 mm Hg, above the 99th percentile). Echocardiography revealed left ventricular myocardial dysfunction secondary to systemic hypertension. Renal ultrasound with Doppler and renal angiography were normal. aCGH and thyroid hormone, plasma renin and aldosterone levels were normal. Sanger sequencing of the coding regions of *NPR1* identified homozygous frameshift variant in exon 1 of *NPR1* in both affected children (figure 1). This variant (NM_000906.4:c.175del, p.Val59TrpfsTer8) was not annotated in all available databases and leads to a frameshift and premature stop codon. Lymphoblastoid cell lines were not available in the affected children of the second family. This frameshift variant was inherited from healthy parents who were both heterozygous for the variant. Their arterial blood pressures were normal.

DISCUSSION
We report herein the identification of biallelic *NPR1* loss of function variants in severe isolated NSH in two unrelated multiplex families, indicating that biallelic *NPR1* mutations are responsible for this condition. Our data indicate an autosomal recessive mode of inheritance of this condition. Importantly, cardiogenic

shock or heart failure was observed in four out of the six affected individuals (table 1). In the first family, we showed that the homozygous stop–gain variant in the *NPR1* gene leads to a dramatic reduction of NPR1 transcripts. NPR1 is a member of the guanylate cyclase family of receptors which produce cGMP in response to ligand binding. NPR1 elevates intracellular levels of cGMP when the atrial natriuretic peptide or brain natriuretic peptide binds to the extracellular domain of the receptor and allosterically activates its guanylate cyclase catalytic domain. This binding induces an increase in intracellular cGMP and initiates natriuresis, diuresis and vasodilation, all of which contribute to lowering blood pressure.[10 11] Disruption of the Npr1 gene in mice leads to chronic elevations of blood pressure (by 35–45 mm Hg as compared with wild-type mice), marked cardiac hypertrophy, ventricular enlargement and sudden death in homozygous mouse mutants.[12] The hearts, kidneys and vasculature of the homozygous mouse mutants (aged less than 5 months) were normal when examined by histological methods. Systemic hypertension was accompanied by marked cardiac hypertrophy and ventricular enlargement with no evidence of myocardial dysfunction.[13] In addition, Npr1 expression affects the sensitivity of blood pressure to dietary salt. Indeed, heterozygous knockout mice have salt-sensitive hypertension compared with wild-type mice.[14 15]

Increased first trimester NT during the pregnancy was observed in three of the four affected children of F1. Interestingly, a slight reduction in the expected number of homozygous Npr1 knockout mutant mice was statistically significant and is related to fetal hydrops observed in approximately 10% of homozygous embryos.[12] Therefore, increased first trimester NT is likely another consequence of biallelic *NPR1* loss of function. Even if this symptom is not constant as for other diseases associated with increased NT such as trisomy 21, Turner or Noonan syndromes, arterial pressure should be carefully followed in postnatal period.

In addition, cardiogenic shock or heart failure was observed in four out of the six affected children carrying pathogenic variants in *NPR1*. The severity of the phenotype of the affected children was also reported in homozygous Npr1 knockout mice with sudden death[12] and indicates a major role of NPR1 in regulating blood pressure in humans.

In human, genome-wide association studies, exome studies or direct sequencing of the *NPR1* gene was performed in large cohorts of patients with systemic hypertension and revealed some variants with high MAF in the untranslated region of *NPR1* associated with higher blood pressure.[16–20] However, these analyses were performed in patients with onset of systemic hypertension after 15 years of age, while in the two families reported here, systemic hypertension was detected within the first month of age.

Our data should lead to analysis of the *NPR1* gene in neonatal isolated systemic hypertension or cardiogenic shock of unknown origin. In addition, in isolated increased NT with normal karyotype, aCGH and RASopathy-associated genes, arterial pressure should be carefully followed in postnatal period, and systemic hypertension should lead to *NPR1* genetic investigation. The benefits of an accurate genetic diagnosis include more tailored management of systemic hypertension, heart involvement and improved surveillance.[2 21] A precise genetic diagnosis enables an accurate genetic information to the affected individuals and their family members.

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