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ARTICLE

A systematic variant screening in familial cases of congenital heart defects demonstrates the usefulness of molecular genetics in this field

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The etiology of congenital heart defect (CHD) combines environmental and genetic factors. So far, there were studies reporting on the screening of a single gene on unselected CHD or on familial cases selected for specific CHD types. Our goal was to systematically screen a proband of familial cases of CHD on a set of genetic tests to evaluate the prevalence of disease-causing variant identification. A systematic screening of *GATA4*, *NKX2-5*, *ZIC3* and Multiplex ligation-dependent probe amplification (MLPA) P311 Kit was setup on the proband of 154 families with at least two cases of non-syndromic CHD. Additionally, *ELN* screening was performed on families with supravalvular arterial stenosis. Twenty-two variants were found, but segregation analysis confirmed unambiguously the causality of 16 variants: *GATA4* (1×), *NKX2-5* (6×), *ZIC3* (3×), MLPA (2×) and *ELN* (4×). Therefore, this approach was able to identify the causal variant in 10.4% of familial CHD cases. This study demonstrated the existence of a *de novo* variant even in familial CHD cases and the impact of CHD variants on adult cardiac condition even in the absence of CHD. This study showed that the systematic screening of genetic factors is useful in familial CHD cases with up to 10.4% elucidated cases. When successful, it drastically improved genetic counseling by discovering unaffected variant carriers who are at risk of transmitting their variant and are also exposed to develop cardiac complications during adulthood thus prompting long-term cardiac follow-up. This study provides an important baseline at dawning of the next-generation sequencing era.

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INTRODUCTION

Congenital heart defect (CHD) is a group of structural abnormalities of the heart and great vessels with a prevalence of about 0.8% in neonates and higher in the prenatal period. The etiology of CHD is complex and combines both environmental and genetic causes.[1,2] There are already >50 genes associated with CHD in humans and even more in genetically modified mice.[3,4] According to epidemiological study, the impact of genetic factors seems to vary depending on CHD type with a much higher recurrence risk in CHD associated with heterotaxy than in the most common CHD such as atrial and ventricular septal defects (ASD and VSD, respectively).[5] Substituting a mean with an individual recurrence risk would be beneficial, because only mutation carriers are at high risk of recurrence, whereas non-carriers have a risk similar to the general population. Therefore, it is crucial to determine these genetic factors in order to improve genetic counseling by reassuring those who have no particular risk of recurrence and to give a much better recurrence risk estimation for mutation carriers. It is obviously important for psychological reason but also for an appropriate use of medical resources to focus only on those who have the higher risk of recurrence and to relieve non-carriers. In addition, medical attention on variant carriers improves early prenatal detection, which decreases morbidity. This point has a growing importance because the treatment of CHD has greatly improved during the past decades with a concomitant increase in CHD patients reaching adulthood to the point that this population is now larger than the CHD pediatric population.[6]

In other cardiovascular pathologies such as cardiomyopathies, arrhythmia and even aortopathies, the discovery of causal genes has led to the progressive implementation of molecular genetic diagnosis in the routine check-up of these pathologies. This is not yet the case in

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CHD probably because of the even higher genetic heterogeneity and the variety of inheritance modes, which together were considered insurmountable obstacles. Nevertheless, we decided in 2004 to systematically test a set of CHD genes on familial cases of CHD. These familial cases were included in a National Registry. We focused on familial cases, because we thought that genetic predisposing factors would be stronger in familial cases than in sporadic cases and also because the familial dimension would give a clue on the inheritance mode. This study demonstrated that molecular genetic diagnosis in the frame of familial CHD was feasible and led to the identification of the causal genetic factor in at least 10.4% of the cases. It gave also some information on the importance of *de novo* variants in CHD even in familial cases and on the consequences of CHD gene variants in adult hearts.

MATERIALS AND METHODS

Study design

A French National Registry was started in 2004. The Registry consists of establishing a database of phenotypic and genotypic information and to collect a tissue sample from affected individuals and their first-degree relatives. The inclusion criteria are at least two relatives with any CHD whatever the relationship between the relatives and whatever the CHD. The exclusion criteria are diagnosed syndromic conditions or exposure to chemical risk factor (rat poison, pesticide and organic solvents). No age restrictions were imposed and enrolment could include postmortem fetuses providing a tissue sample was available. Pediatric cardiologists, adult cardiologists, geneticists, fetopathologists, surgeons and other physicians were asked to identify such families and to inform patients and families in the Registry. Interested families were referred to the research team, which coordinated recruitment and data collection. The latter included signed informed consent, individual medical history, familial history, phenotyping and DNA sampling. Phenotyping was derived from an interview and from copies of medical records provided by the referring physician. A structured questionnaire included information related to pregnancy history of index cases (see Supplementary Text). It was not possible to obtain a systematic cardiac check-up for all first-degree relatives of affected individuals. Clinical data are presented on Supplementary Table S1.

Ethics

The study protocol was specifically approved by the local institutional review board (Comité de protection des personnes Sud-Est II). This procedure complies with the current laws in France (Loi Biomédecine 2004) and the last version of the Declaration of Helsinki (The World Medical Association, 2008). A signed informed consent was obtained from all participants (or parents of minors or fetuses) prior to history recording and tissue sampling. Personal information from affected individuals and relatives were entered in a database on a secured server. This customized database (FileMaker Pro version 7) can store and manage clinical, sampling and genetic data. Pedigree trees were built with *Cyrillic 2*, a pedigree drawing software. CNIL (Comité National Informatique et Liberté) approved the database.

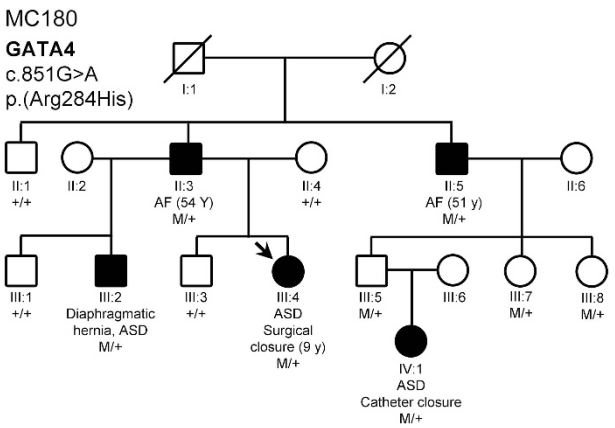

Figure 1 Pedigree tree of family MC180 with a *GATA4* mutation. Squares, males; circle, females; crossed symbols, deceased; empty symbols, unaffected; filled symbols, affected; arrow, proband. The result of variant detection is indicated below the symbol of all individuals who provided a sample (M for disease-causing variant, + for normal variant). There is no such information for those who did not provide a sample. AF, atrial fibrillation; ASD, atrial septal defect.

Genetic analysis

Blood and fetal tissue sampling were performed according to routine procedure for biobanking. DNA extraction was performed according to standard protocol. Variant screening of *GATA4* (ENST0000335135), *NKX2-5* (ENST0000329198), *ZIC3* (ENST00000370606 and ENST00000287538) and *ELN* (ENST00000358929.1) was carried out by bi-directional sequencing of PCR amplicons or by high-resolution melting (HRM). The primers and methods of screening are presented in Supplementary Table S2. Variants were confirmed on a second PCR product and on a second sample. Multiplex ligation-dependent probe amplification (MLPA) was performed according to the manufacturer's recommendation (MRC Holland, Amsterdam, The Netherlands). We used the P311_A1 Kit (MRC Holland), which amplifies the seven coding exons of *GATA4* plus 2 probes upstream and downstream of *GATA4*, the 2 exons of *NKX2-5*, 8 of the 10 exons of *TBX5*, 5 exons of *BMP4*, exons 1, 3 and 10 of the 11 exons of *CRELD1* and three probes in the 22q11 region (DiGeorge syndrome). Abnormal profiles were repeated on the same sample and on a second sample. The extent of deletions when all the MLPA probes of a gene were deleted were estimated by CGH array with the Human Genome Comparative Genomic Hybridization Microarray Kit (Agilent Technologies, Santa Clara, CA, USA). The 180 K slides were scanned on an Agilent DNA Microarray Scanner, and images were extracted with Feature Extraction Software (10.7.3.1, Agilent Technologies). Results were interpreted with Genomic Workbench software version 5.0 (Agilent Technologies). Some families of this cohort were screened for other genes or another MLPA test and are reported elsewhere.[7,8] For details on the enrolled families, see Supplementary Table S1. All variants were submitted to LOVD (www.lovd.nl/3.0/home).

RESULTS

Cohort description

From May 2004 to March 2013, we recruited 1110 subjects – including family members – in 154 families. There were 416 affected individuals and 694 unaffected relatives. We obtained a sample from 323 affected individuals (77.6%) and from 526 unaffected relatives (75.8%). Supplementary Table S1 describes the characteristics of the affected individuals.

Variant screening

A systematic variant screening was performed in at least one affected individual of each of the 154 families. The variant screening was either direct Sanger sequencing or HRM followed by PCR sequencing of abnormal profiles (see Supplementary Table S2). The *GATA4*, *NKX2.5* and *ZIC3* genes were included in this systematic screen along with the MLPA Kit P311. This MLPA kit screens for deletion or duplication of exons of the *BMP4*, *CRELD1*, *NKX2.5*, *GATA4* and *TBX5* genes and has three probes in the 22q11 deletion region. The *ELN* gene was screened only on cases with supravalvular arterial stenosis (Supplementary Tables S1 and S2).

GATA4 variant

A heterozygous missense variant was observed (c.851G>A, p.(Arg284His), variant ID 0000052423) in a girl with an ASD (patient III.4, family MC180) (arrow in Figure 1). Her brother and her mother were unaffected and did not carry the variant, which was found in the father (II.3) and a paternal uncle (II.5) who were discovered,

Table 1 Summary of variant parameters that probably affect function

Family	*cDNA*	*Protein*	*Interspecies conservation*	*Domain*	*Grantham*	*Mutation taster*	*Polyphen2*	*EVS*
GATA4								
MC180	c.851G>A	p.(Arg284His)	10/10		0.79	Disease causing	Probably damaging	0/13005
NKX2-5								
MC096	c.488T>G	p.(Leu163Arg)	8/8	Homeobox	2.78	Disease causing	Probably damaging	0/12934
MC119	c.509A>C	p.(Gln170Pro)	4/8[a]	Homeobox	2.07	Disease causing	Possibly damaging	0/12934
MC104	c.555G>A	p.(Trp185Ter)		Homeobox				
MC146	c.709T>C	p.(Tyr237His)	7/8[b]		2.26	Disease causing	Benign	0/12934
MC146	c.720_726delCTACGGC	p.(Tyr241Trpfs*51)						
MC131	chr5.hg19:g.(171889037_171909226)_(172763588_172779302)del							
MC169	chr5.hg19:g.(172648879_172659178)_(172670032_172684386)del							
ZIC3								
MC177	c.241_242dupTA	p.(Ala82Thrfs*142)						
MC069	c.876G>C	p.(Gln292His)	4/6	Between Znf_C2H2-like	0.65	Disease causing	Probably damaging	0/10562
MC194	c.889T>G	p.(Cys297Gly)	6/6[c]	Znf_C2H2_like (Smart)	4.34	Disease causing	Probably damaging	0/10562
ELN								
MC054	c.450C>A	p.(Tyr150Ter)						
MC132	c.8delG	p.(Gly3Valfs*2)						
MC176	c.34G>T	p.(Gly12Ter)						
SPO199	c.1295_1296insG	p.(Val435Serfs*58)						

Abbreviation: EVS, exome variant server.
The nomenclature of variant description is related to NM_002052.3 and NP_002043.2 for *GATA4*; NM_004387.3 and NP_004378.1 for *NKX2-5*; NM_003413.3 and NP_003404.1 for *ZIC3*; NM_001278939.1 and NP_001265868 for *ELN*.
[a]His (Chicken, Xenopus, Zebrafish, Fugu).
[b]His (Chicken).
[c]Invertebrates included.

respectively, at the age of 54 and 51 years, with a permanent atrial fibrillation. A paternal half-brother (III.2) of the proband had neonatal surgery for a diaphragmatic hernia and a surgical ASD closure at 10 years of age (III.2 in Figure 1). He carried the *GATA4* variant contrarily to his unaffected brother (III.1). The *GATA4* variant was also found in III.5, III.7 and III.8 who are all asymptomatic and in IV.1 who had an ASD closed by catheterization.

Table 1 presents elements to evaluate the pathogenicity of this variant. This p.(Arg284His) variant (MC180) is very convincing because it is not present in public databases, interspecies highly conserved, predicted to be damaging and the familial segregation is coherent.

Three other *GATA4* heterozygous variants were observed (non-coding exon 1 deletion, c.244 A>G, p.(Thr82Ala) and c.1037C>T, p.(Ala346Val)) but their pathogenicity is dubious according to amino-acid conservation and/or phenotype/genotype familial segregation (Supplementary Text, Supplementary Figure S1 and Supplementary Table S3).

NKX2-5 variants

The index case in family MC096 (patient III.1, arrow in Figure 2) had ASD-OS (ASD ostium secundum type) and second-degree AV block that required a pacemaker when he was 19 years. He later developed atrial fibrillation. He had a heterozygous c.488 T>G, p.(Leu163Arg) (variant ID 0000052427) missense variant. The *NKX2-5* variant was inherited from the mother who had an AV block, which required a pacemaker when she was 42 years. She had two other children with another father. The youngest one, a daughter, had a patent foramen ovale, sinus bradycardia and an AV block requiring a pacemaker at the age of 9 years. No other family member had a known cardiac disease. The maternal grandparents of the proband were first-degree cousins.

The proband of family MC119 (patient IV.2, arrow in Figure 2) had a membranous VSD and a long PR interval (220 ms). A heterozygous missense variant was found (c.509 A>C, p.(Gln170Pro), variant ID 0000052425) in his DNA. Four other relatives carried the variant: his father (III.3: ASD and AV block with a pacemaker), his paternal aunt (III.2: ASD and mitral valve prolaps), his paternal grand-mother (II.2: pacemaker for AV block), and patient III:5 (ASD and VSD).

Patient II.2 of family MC104 had an ASD-OS operated when she was 5 years (arrow in Figure 2). A postsurgical complete AV block led to a permanent pacemaker. She had a heterozygous STOP-gain variant at position 185 (c.555G>A, p.(Trp185Ter) variant ID 0000052426). This variant was also found in her daughters (III.1 and III.2: ASD medically closed and VSD with spontaneous closure). The parents of the proband had no known cardiac disease.

Patient II.5 of family MC146 (arrow in Figure 2) had an ASD discovered late in life when he had an Implantable cardioverter-defibrillator (ICD) implanted. He had a hypertrophied cardiomyopathy (HCM), which evolved progressively to dilated cardiomyopathy (DCM) and arrhythmia. Two heterozygous variants were found in his DNA: a missense variant (c.709 T>C, p.(Tyr237His)) and a deletion of seven nucleotides (c.720_726delCTACGGC, p.(Tyr241Trpfs*51) variant ID 0000053073) leading to a frame shift and a premature termination codon. Both variants were transmitted to his youngest daughter (III.2) who had an AV block and a dilated left ventricle but none were transmitted to his eldest daughter. The proband (II.5) had two normal and non-carrier brothers but he had lost a sister within her first year of life. She was cyanotic. He also had lost a brother at the age of 28 years. This brother (II.3) had initially an ASD with AV block. However, after implantation of a pacemaker, he developed ventricular arrhythmia and DCM. He refused to continue his antiarrhythmic treatment and died from sudden death. The father (I.1) had no cardiac malformation, but a pacemaker was implanted when he was 48 years for an AV block. In his fifties, he developed a HCM with premature ventricular beats and ventricular tachycardia. An ICD was implanted when he was 59 years. He also carried both *NKX2-5* variants. The co-segregation of both variants across three generations demonstrated that these variants were in cis.

NKX2-5 variant parameters are depicted in Table 1.

Probands II.5 of family MC131 and III.1 of family MC169 (arrows in Figure 2) had no *NKX2-5* disease-causing variant as revealed by PCR-based strategies, but the MLPA test discovered a heterozygous deletion of both exons of this gene. A CGH array was used to confirm these deletions and establish the approximate borders. Patient II.5 (MC131) had a large deletion chr5.hg19:g.(171889037_171909226)_(172763588_172779302)del (variant ID 0000052430) of about 854 000 nucleotides, which encompassed several protein-coding genes: *NEURL1B*, RP11-779O18.1, *DUSP1*, *ERGIC1*, *RPL26L1*, *ATP6V0E1*, *CREBRF*, *BNIP1*, *NKX2-5*, and *STC2* (from centromere to telomere) (Supplementary Figure S2). The proband of family MC131 (II.5, arrow) had an ASD with a conduction block, which required a pacemaker at the age of 39 years. This man had five children. Two children had no heart defect and did not carry the deletion but two others had an AV block requiring a pacemaker during childhood. Both of the latter children have the deletion. Patient III.3 of this sibship died from meningitis. The autopsy revealed that he had an undiagnosed ASD but no details on spleen morphology were reported. The proband (II.5, Figure 2) developed a cataract at the age of 30 years and cardiac failure at the age of 53 years. The three carrier sons also had a pectus excavatum. The pedigree suggests that this large deletion appeared in the father's DNA (II.5, MC131) although we could not obtain a sample from the parents and sisters of the proband.

By contrast, patient III.1 (MC169) had a deletion removing only the *NKX2-5* gene chr5.hg19:g.(172648879_172659178)_(172670032_172684386)del (variant ID 0000052431) on the long arm of chromosome 5. The medical history of this family is remarkable, because the proband's mother had a myocardial infarction when she was pregnant. She had a medical check-up and it was discovered that she had an ASD with a long PR interval (260 ms). Presumably, she had an embolism arising from a thrombophlebitis, which reached coronary arteries through her ASD. She had no atherosclerosis of the coronary arteries. After delivery, her male newborn was diagnosed with an ASD and he had a PR interval of 160 ms. However, this conduction delay increased rapidly to 240 ms (age 3 years) and to a 2/1 AV block (age 4 years). We could test all family members demonstrating that the variant had appeared in the proband's mother.

ZIC3 variants

The DNA of a fetus who was the subject of a termination of pregnancy (case II.6 of family MC194, arrow in Figure 3) was screened for variant. He had left superior and left inferior vena cava, single atrium, complete AV canal, D-TGA and pulmonary atresia. He also had right atrial and pulmonary isomerism, intestinal malrotation and asplenia. A high bossing forehead and ante version of nostrils were noted. A hemizygous missense variant was found (c.889 T>G, p.(Cys297Gly) variant ID 0000052432), which had been inherited from the mother. She had two normal daughters from two different fathers. She had lost a male newborn (II.1) with Ivemark syndrome by premature delivery (26 weeks of amenorrhea). She had two early spontaneous abortions, an ectopic pregnancy and the most recent pregnancy (II.8) was medically terminated (22 weeks of amenorrhea) because the male fetus

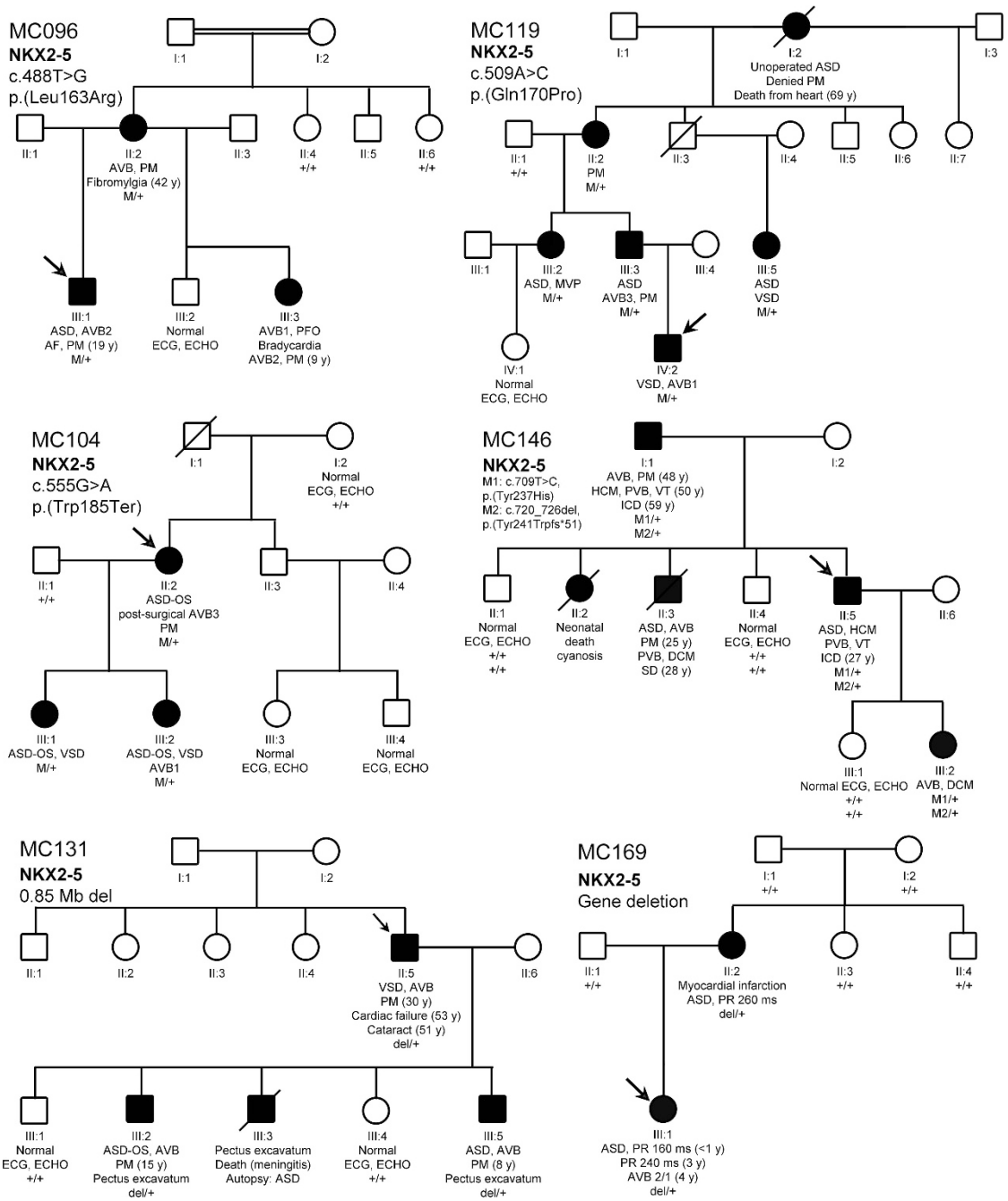

Figure 2 Pedigree tree of families with *NKX2-5* mutations. As in Figure 1, information regarding the result of variant detection is indicated below the symbol only of those who provided a sample (M for disease-causing variant, + for normal variant). AVB, atrioventricular block; ECHO, echocardiogram; MVP, mitral valve prolaps; PFO, patent foramen oval; PM, pacemaker; PVB, premature ventricular beat; s.d., sudden death; VT, ventricular tachycardia.

had single atrium, complete AV canal, double outlet right ventricle, pulmonary atresia and a right aortic arch. In addition, he had right atrial and pulmonary isomerism, abdominal situs inversus and asplenia. A hypertelorism with long and salient philtrum were noted.

A male newborn (proband III.4 of family MC069) had absent inferior vena cava, intestinal malrotation and polysplenia. At the age of 1 year, he had a liver transplantation for extra hepatic biliary atresia. A hemizygous missense variant was observed in *ZIC3* (c.876G>C, p.(Gln292His) variant ID 0000052434), which was absent in the father but present in the mother. She had right hypoacousis with speech difficulties. Members of the sibship of the mother (a male and a female) had ASD with either conduction block or atrial fibrillation but they declined giving a sample (Figure 3).

The mother of proband IV.3 (MC177) had no cardiovascular anomalies and no heterotaxy. A first pregnancy was medically terminated because of alobar holoproencephaly and cyclopia in a male fetus. With another father, she had a spontaneous abortion and a medically terminated pregnancy of another male fetus (heterotaxy with complex cardiovascular malformations). The DNA of this fetus (IV.3) was examined (arrow in Figure 3). A TA insertion was noted resulting in a frame shift (c.241_242dupTA, p.(Ala82Thrfs*142) variant ID 0000052435). This variant was absent in the father's

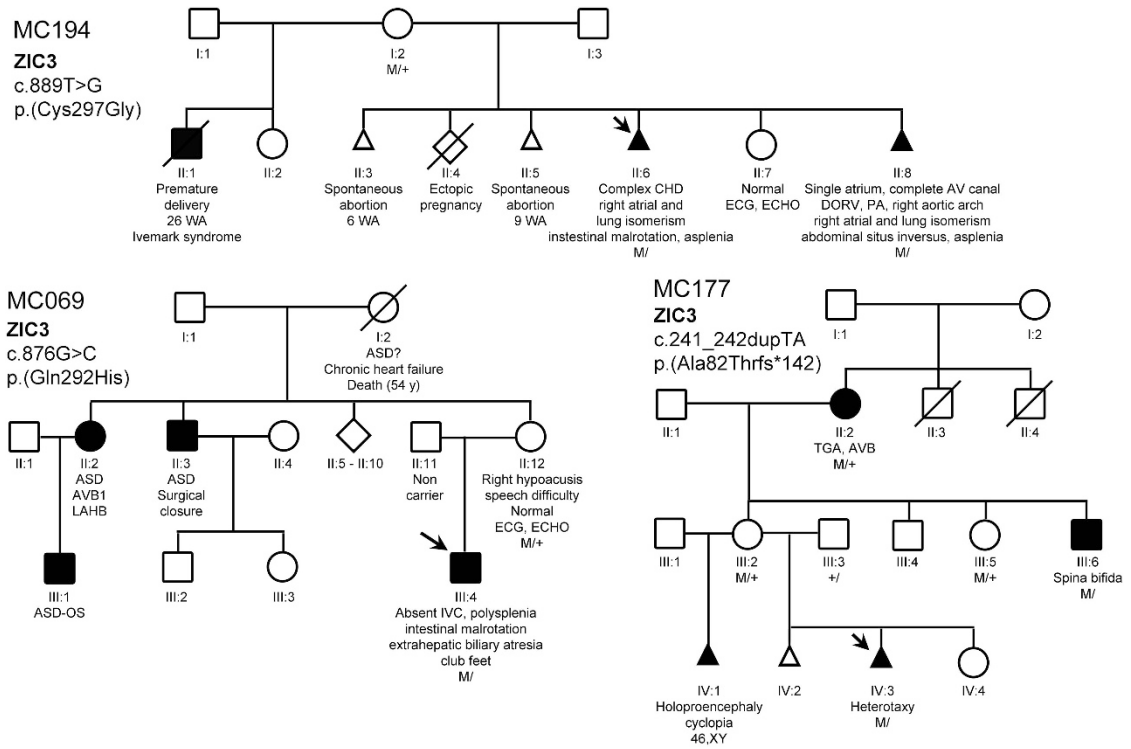

Figure 3 Pedigree tree of families with ZIC3 mutations. As in Figure 1, information regarding the result of variant detection is indicated below the symbol only of those who provided a sample (M for disease-causing variant, + for normal variant). AVB, atrioventricular block; DORV, double outlet right ventricle; IVS, inferior vena cava; LHAB, left anterior hemiblock; PA, pulmonary atresia; TGA, transposition of the great arteries; WA, weeks of amenorrhea.

DNA but found in the mother's DNA. The variant was found also in a maternal uncle and an aunt of the proband. This uncle (III.6) had a mild spina bifida but no cardiovascular malformation. The maternal grandmother who was operated during childhood for a TGA was a heterozygous carrier.

ZIC3 variant characteristics are summarized in Table 1. A hemizygous c.159_161dup, p.(Ala55dup) found in a male child with complex cardiac malformation and heterotaxy was not retained on the basis of familial segregation and population frequency (Supplementary Text, Supplementary Figure S3 and Supplementary Table S3).

ELN variants

By contrast to the three other genes, the *ELN* gene was screened only if an affected individual had a narrowing of pulmonary and/or aortic arteries because ELN was never involved in other CHD types. Accordingly, 8 families out of the 154 (5.2%) were analyzed (MC015, MC054, MC074, MC079, MC132, MC176, SPO199 and SPO212). A variant was found in four families of this subgroup (50%). A heterozygous STOP-gain codon was found in proband III.1 of family MC054 (*ELN* c.450C>A, p.(Tyr150Ter) variant ID 0000052436) (arrow in Figure 4), which was inherited from his mother. The variant was also found in a maternal aunt. All three affected family members had pulmonary supravalvar stenosis. A deletion of a single nucleotide (c.8delG, p.(Gly3Valfs*2) variant ID 0000052437) was found in the proband (III.2) of family MC132. She had aortic and pulmonary supravalvar narrowing. She had a single child, a boy, who had similar arterial anomalies and carried the maternal variant. The daughter of a first-degree cousin (IV.2) had aortic and pulmonary narrowing but did not carry the *ELN* variant. In family MC176, a heterozygous STOP-gain was found (c.34G>T, p.(Gly12Ter) variant ID 0000052438) in a mother who had peripheral pulmonary artery stenosis and in one of her sister who had identical vascular anomalies. The mother had two children who were variant carriers. Both had aortic and pulmonary stenosis. Finally, a couple composed of first-degree cousins (SPO199) had lost two children because of severe supravalvar pulmonary stenosis. The third child was also affected. He had a G insertion (c.1295_1296insG, p.(Val435-Serfs*58) variant ID 0000052439) in one copy of the *ELN* gene. This variant was inherited from the mother who had no overt cardiovascular symptoms and was absent from the father.

MLPA deletions and duplications

One deletion in the *GATA4* gene (MC143) and two deletions in the *NKX2.5* gene (MC131 and MC169) were detected by the MRC Holland MLPA P311-A1 Kit and were depicted in the Supplementary Text or above. This MLPA Kit does not probe the *ELN* and *ZIC3* genes. No anomalies were detected in this series of 154 familial CHD in the *CRELD1* and *TBX5* genes. Finally, two patients had a large duplication (3 Mb) of the 22q11 region (chr22. hg38:g.(18150191_19254103)_(20994944_21446352)dup): patient 1022 of MC074 (variant ID 0000058585) with bicuspid aortic valve and aortic coarctation and patients 1118 and 1119 of MC089 (variant ID 0000058586) with both a valvar pulmonary stenosis. In both families, the duplication was inherited from a reportedly normal parent (father in family MC074 and mother in family MC089). In addition, in family MC074 an unaffected sister was a 22q11 duplication carrier. No 22q11 duplication carriers were syndromic.

A heterozygous deletion was found in two families (exon 1 of NM_001202.3 in patient 1868 of family MC121 and exon 1 of NM_130851.2 in patient 1683 of family SPO117) in the BMP4 gene, but the familial segregation and the discovery of another disease-

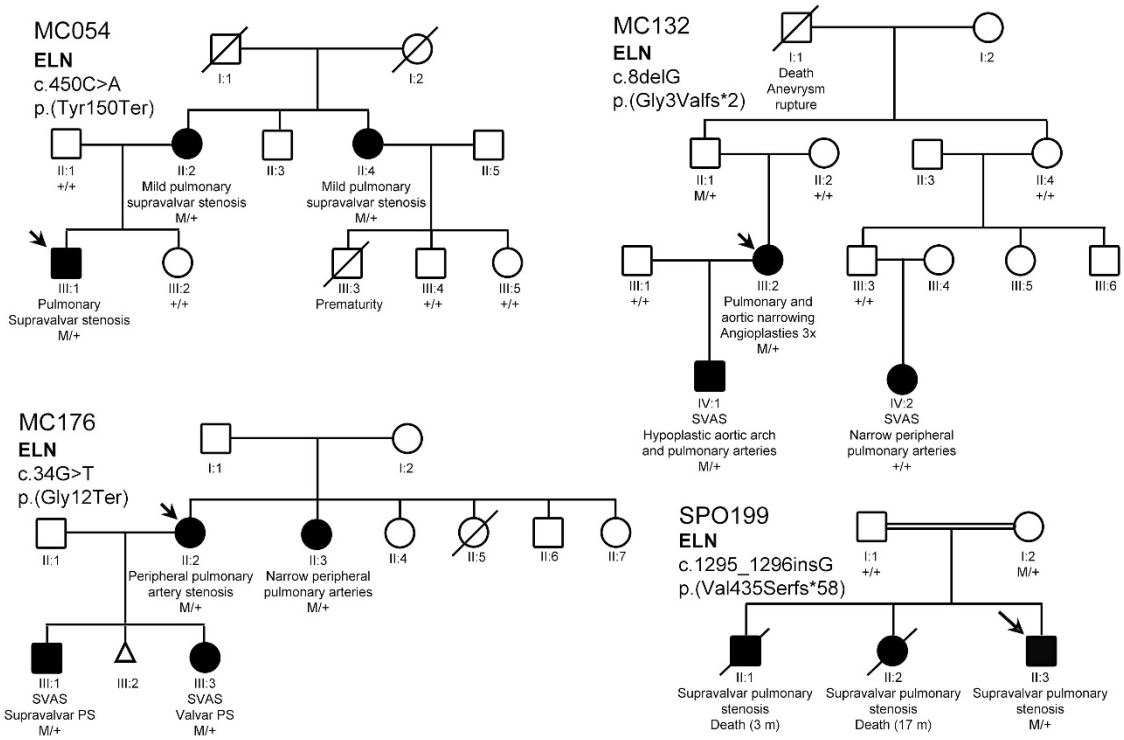

Figure 4 Pedigree tree of families with ELN mutations. As in Figure 1, information regarding the result of variant detection is indicated below the symbol only of those who provided a sample (M for disease-causing variant, + for normal variant). PS, pulmonary stenosis; SVAS, supravalvar aortic stenosis.

causing variant in family MC121 ruled out a causative role for these two exon deletions (Supplementary Text).

DISCUSSION

The purpose of this study was to evaluate the feasibility and the interest of systematically testing a set of genes in familial cases of CHD with no preselection on malformation types. By systematically testing three genes (*GATA4*, *NKX2.5*, *ZIC3*) by PCR-based methods and five genes (*BMP4*, *CRELD1*, *GATA4*, *NKX2.5* and *TBX5*) and the 22q11 chromosomal region for deletion/duplication, we were able to find disease-causing variants in 12 families out of 154 (7.8%). Because *ELN* variants are associated with specific cardiovascular anomalies (supravalvar arterial narrowing), *ELN* screening was prompted only if such CHD were present. We found four *ELN* disease-causing variants in addition to the first 12 disease-causing variants (16 total, 10.4%). All *ELN* and *NKX2.5* variants, one GATA4 variant and three ZIC3 variants were convincingly disease-causing variants on the ground of evidence on Table 1 and on familial segregation. Additional variants in these two latter genes were less convincing (Supplementary Text, Supplementary Figures S1 and S3, Supplementary Table S3). In particular, the *ZIC3* p.(Ala55dup) variant was reported earlier in a series of heterotaxy cases[9,10] and a variant with an expansion of two alanine residues was reported in a case of VACTERL association.[11]

However, the presence of an expansion of one alanine in 5% the American population casts serious doubt on its causality in any defect. We considered the classical large duplication in 22q11 as causal for the CHD in families MC074 and MC089 although with incomplete penetrance. It is well established that this duplication is pathogenic with a highly variable expressivity.[12,13] The *BMP4* exon 1 deletion in family MC121 is not causal in this family, and there is insufficient evidence to demonstrate that the *BMP4* exon 2 deletion could be causal in family SPO117 (Supplementary Text).

Families MC074 and MC089 with 22q11 duplication were included in a collaborative study concerning 92 families of this series (Supplementary Table S1) and reported elsewhere.[8] Additional MLPA probes were used and family MC061 was found to have a 2q37.3 duplication.[8]

Twenty-one families of this series were tested for a panel of 13 sarcomeric genes in a second collaborative study,[7] and disease-causing variants were found only in the alpha cardiac heavy chain myosin gene (*MYH6*), in three families (MC027 p.(Cys539Arg), MC053 p.(Lys543Arg) and MC081 p.(Arg17His)). These 21 families were selected out of the Registry because at least one affected individual had ASD (for details, see Supplementary Table S1). By adding the 2q37.3 duplication and the 3 *MYH6* disease-causing variants of these collaborative efforts to the 16 positive families of the study, we have 20 families with an identified genetic cause (12.8%).

The yield of causal gene variant identification in this series of 154 familial cases of unselected CHD is best with *NKX2.5* (6/154, 3.9%) followed by *ZIC3* (3/154, 1.9%) and *GATA4* (1/154, 0.7%). On a subgroup based on specific types of CHD, *ELN* screening identified a disease-causing variant in 4 out of the 8 familial cases (50%) with arterial narrowing, whereas *MYH6* screening found a disease-causing variant in 3 out of the 21 families with ASD (14%). The P311-A1 Kit detected at most four causal anomalies ($2\times$NXK2-5 deletions and $2\times$22q11 duplications) or 2.6% of the cohort. No *CRELD1* and *TBX5* deletions/duplications were detected in this series. The CHD phenotypes resulting from disease-causing variants discovered in this study were consistent with earlier reports: ASD, atrial fibrillation and diaphragmatic hernia in *GATA4*; ASD, AV block, cardiomyopathy and arrhythmia in *NKX2.5*; and heterotaxy and complex CHD in *ZIC3*. We screened a series of 104 cases of dilated cardiomyopathy and could not find another case with a *NKX2-5* disease-causing variant demonstrating that *NKX2-5* does not account for a high percentage of

dilated cardiomyopathy as already observed in two previous reports.[14,15] Family MC177 is interesting because the maternal grandmother had a D-TGV and carried the heterozygous *ZIC3* disease-causing variant, suggesting that this variant could be dominant or that she had an unbalanced X inactivation as already shown in two other familial cases.[16,17]

It is much easier in familial cases to infer the putative causal gene than in sporadic cases, because in familial cases the spectrum of phenotypic anomalies is broader by compiling the various anomalies of affected relatives. Despite efforts to obtain an exhaustive family history straightaway, it often occurred that family history was completed later because the first contacted family member was unaware of health problems of relatives or thought that some health problems (such as diaphragmatic hernia) were irrelevant to CHD. Furthermore, cardiac check-up prompted in asymptomatic disease-causing variant carriers may discover cardiac structural anomalies and cardiac follow-up will eventually detect age-related anomalies, such as arrhythmia (*GATA4*), cardiomyopathy or conduction blocks (*NKX2-5*).

The availability of family history and blood samples of family relatives was very important to help infer the causality of variants. Nevertheless, in two cases, the family history was misleading because we found a dominant variant (MC096 *NKX2.5* and SPO199 *ELN*) when the founders were related. *De novo* disease-causing variants account for about 10% of sporadic cases of CHD as recently shown.[18] In this study, at least 1 out of the 16 families (6%) with *bona fide* disease-causing variant was secondary to a *de novo* variant (MC169) as the variant was absent from founder's DNA (paternity was confirmed by genetic testing). A *de novo* disease-causing variant is also conceivable in three other pedigrees (MC096, MC104 and MC131), although the unavailability of founders DNA prevented any testing.

Deletions/duplications are important to detect. Out of the 154 families, the P311-A2 Kit could detect five deletions and two duplications. In the *NKX2-5* gene, out of the six causal variants, two were deletions that were initially missed by PCR/sequencing approach. The largest deletion (0.85 Mb) detected in family MC131 could explain some of the extra cardiac anomalies observed in this family as the deletion encompassed nine genes in addition to the *NKX2.5* gene. In particular, the male child III3 who died of meningitis was diagnosed at autopsy as having an ASD suggesting that he was a deletion carrier. The *DUSP1* gene – one of the deleted genes - encodes MKP-1, a MAPK phosphatase which acts as the negative regulator of MAPK. In a MKP-1-deficient mouse, it was shown that MKP-1 has a nonredundant role in negatively regulating endotoxic shock responses.[19] It is conceivable that being haploinsufficient for the *DUSP1* gene dysregulated the immune defense of this child contributing to his death.

Genome-wide[20–22] and gene-centric[23] copy number variants (CNV) were tested on a series of CHD with criteria to infer causality based either on *de novo* deletion/duplication in sporadic cases or familial segregation in multiplex cases. These studies could confirm the involvement of known CHD genes and discover new genes and genomic regions. Designing genome-wide or gene-centered screen is a strategic and financial choice, which depends on the focus on simple hospital diagnosis or inclusion also of new gene discovery. The results of the current study and of CNV detection studies suggest that combining nucleotide variant detection and CNV detection in a single screen has the potential to increase disease-causing variant detection in CHD to 15–30%.

In conclusion, screening a set of cardiac genes in the frame of familial CHD is feasible and fruitful because as many as 10.4% of cases were solved. This study provides for the first time an evaluation of what Sanger sequencing and MLPA could yield in terms of disease-causing variant identification in familial cases of CHD. This starting point will be useful to benchmark next-generation sequencing (NGS) efficiency. In this study, the elucidation of variant segregation across the family was an important asset to discriminate modifier variants from major genetic factors. Familial variant segregation will become even more instrumental in the era of NGS, which generate a large number of suspicious variants. The detection of unaffected adult variant carriers is important not only because of the high risk of recurrence for future birth but also because of cardiac alterations appearing during adulthood whether the CHD was perfectly repaired or even in the absence of CHD.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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