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Mutations in the EGF-CFC Gene *Cryptic* Are an Infrequent Cause of Congenital Heart Disease

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Abstract *Cryptic* (*CFC1*), a member of the epidermal growth factor–Cripto/FRL-1/Cryptic (EGF–CFC) gene family, is involved in the evolutionarily conserved establishment of left–right lateral asymmetry. Inactivation of *Cfc1* in mice results in laterality defects and complex cardiac malformations. Similarly, mutations in the human *CFC1* gene have been identified in patients with heterotaxy syndrome. The cardiac defects in humans resemble those in mice lacking *Cfc1*. We postulated that some patients with isolated cardiac malformations could also have mutations in the *CFC1* gene. Our analysis of the *CFC1* gene in 167 patients with congenital heart disease revealed a novel A145T missense variant in 3 patients with type II atrial septal defect. Furthermore, we found the previously characterized R78W polymorphism in another patient with type II atrial septal defect. However, the A145T sequence alteration was also identified in 3 controls, suggesting that this variant is a polymorphism. We conclude that CFC1 variants could be a rare cause of congenital heart disease in patients without laterality defects.

Key words *Cryptic* · *CFC1* · Congenital heart disease · Atrial septal defect · Polymorphism

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Congenital heart disease (CHD) is the most common birth defect, affecting 6000 live births each year in Germany alone. Improved CHD treatments have reduced infant mortality. Even in the subgroup of severe defects requiring surgery, more than 80% of affected children survive to adulthood [7]. Despite the major advances in medical treatment, the molecular genetic basis of CHD remains poorly characterized. Much of the recent progress in our understanding of molecular mechanisms underlying CHD has been gained by genetic approaches in vertebrate model organisms, especially the zebrafish and the mouse, in which effects of gain and loss-of-function mutations on cardiac development and function can be readily assessed [11]. However, only a few Mendelian congenital heart syndromes have been identified in the past few years.

The epidermal growth factor–Cripto/FRL-1/Cryptic (EGF–CFC) gene family comprises a novel class of structurally related genes that includes mammalian *cripto* and *cryptic*, chick *cripto*, frog *frl-1*, and zebrafish *one-eyed pinhead* (*oep*). Previous studies have shown that *oep* is essential for the formation of mesoderm, endoderm, and ventral neuroectoderm [8]. The partial rescue of maternal *oep* zebrafish mutants resulted in heterotaxia with randomized direction of cardiac looping [10]. Similarly, targeted disruption of the

murine *Cfc1* gene underscored the pivotal role of EGF–CFC genes in determining the left–right (L–R) axis in vertebrates. Homozygous *Cfc1*-deficient mice showed laterality defects, including randomized spatial distribution of organs along the L–R axis, and gastric defects, hyposplenism, and pulmonary isomerism. The majority of *Cfc1* mutants died within the first postnatal week because of severe cardiac malformations, including ventricular septal defects and transposition of the great arteries [3, 10]. Mutational screening of the human *CFC1* gene in patients with heterotaxic phenotypes led to identification of loss-of-function mutations in *CFC1* [1].

Goldmuntz et al. [6] identified two mutations in *CFC1* in patients with transposition of the great arteries and double-outlet right ventricle in the absence of heterotaxy syndrome. Based on the insights gained from analysis of homozygous *Cfc1* mutant mice and the results of mutational studies in humans, we hypothesized that some patients with CHD without laterality defects would also have *CFC1* mutations, particularly patients with atrial or ventricular septal defects. To determine novel mutations in *CFC1* we screened patients with atrial or ventricular septal defects and patients with outflow tract abnormalities, including transposition of the great arteries and double-outlet right ventricle, pulmonary atresia, pulmonary stenosis, aortic isthmus stenosis, patent ductus arteriosus, Ebstein's anomaly, tetralogy of Fallot, and other complex malformations.

Materials and Methods

Patients

We investigated 167 unrelated patients with CHD and 168 healthy individuals by mutation analysis of *CFC1*. Medical history was taken, and a physical examination was performed. Patients were categorized as having CHD if the diagnosis was confirmed by echocardiography, cardiac magnetic resonance, or cardiac catheterization. We excluded laterality defects by chest radiographs or ultrasound examination.

Mutational Screening

Blood samples were obtained from patients with CHD and participants in accordance with guidelines of the local ethics committee. Genomic DNA was prepared by standard methods from human blood and lymphoblastoid cell lines. The coding region of *CFC1* was amplified by polymerase chain reaction (PCR) with six sets of primers flanking exons 1–6.

exon 1F: 5′-TGTAAAACGACGGCCAGTTCGTCCATTCTGTGTCCC-3′
exon 1R: 5′-CAGGAAACAGCTATGACCCCCCTTCTTGGCTCTAAG-3
exon 2F: 5′-TGTAAAACGACGGCCAGTGATGGCATTGTATTTTTATGTG-3′
exon 2R: 5′-CAGGAAACAGCTATGACCCTCTCTACCGCCGTTATGTT-3′
exon 3F: 5′-CAGGAAACAGCTATGACCGTCCGCAGACTGAGATGA-3
exon 3R: 5′-CAGGAAACAGCTATGACCGTCCGCAGACTGAGATGA-3′
exon 4F: 5′-ATTTTACTGCCTCCCCTGGG-3′
exon 4R: 5′-CGCCCCTCTCCTGACGCCTA-3′
exon 5F: 5′-CGGGCCACCGCATTGATG-3′
exon 5R: 5′-GCAACCGCGTGCGGGGGTGAG-3′
exon 6F: TGTAAAACGACGGCCAGTGCAGGGAGCAGGCGTTTCTA-3′
exon 6R: CAGGAAACAGCTATGACCCAAGGATCTGGAGCCAAAGG-3′

PCR products were further analyzed by single-strand conformational polymorphism (SSCP) with a 6 or 12% polyacrylamide gel at 4–20°C (Multiphor gel apparatus, Pharmacia Biotech, Uppsala, Sweden). We visualized bands with silver staining. Aberrant conformers detected by SSCP were submitted to sequencing (ABI 3100 Avant, Applied Biosystems) as described elsewhere [1, 5]. Because of difficulties in obtaining reproducible SSCP analysis of exon 5, we subjected this exon to direct sequencing with standard protocols.

Results

A total of 167 patients with congenital heart malformations and 168 healthy individuals were tested for *CFC1* mutations. Characteristics of the cohort are summarized in Table 1. A total of two different heterozygous alterations were identified in 4 unrelated patients. The missense mutation in exon 3 leading to the amino acid change R78W, which was previously described in another study, was detected in 1 African patient with an isolated type II atrial septal defect [1]. A further sporadic heterozygous transition leading to the amino acid substitution A145T was identified in exon 5 in 2 unrelated patients with type II atrial septal defect and 1 patient with pulmonary atresia, type II atrial septal defect, and tricuspid valve defect. None of

Table 1 Cardiac malformations of the patients cohort

Cardiac finding	Total (N = 167)
Atrial septal defect	48
Ventricular septal defect	5
Double-outlet right ventricle	8
d-Transposition of the great arteries	12
Tetralogy of Fallot	13
Aortic coarctation	4
Pulmonal atresia or stenosis	20
Atrioventricular septal defect	7
Partial anomalous venous drainage	5
Other complex cardiac malformations	45

these patients showed laterality defects. Although the alanine residue in the CFC domain is conserved in the mammalian *CFC1* gene (Fig. 1), 3 control individuals out of 168 demonstrated the same substitution, giving a carrier frequency of 1.7%. There was no significant difference of genotype frequency in controls and patients (chi-square, 5.44e-05).

The R78W sequence variation was not detected in 336 chromosomes from our control population of European and Asian descent. Familial blood samples were not available for further evaluation of the mutation. Since *Cfc1*-deficient mice develop a variety of cardiac malformations, we also screened in patients displaying the corresponding heart defects. Taking into account a mutation detection sensitivity of 80–90% of the method employed in this study, we failed to identify other *CFC1* mutations [6].

Discussion

Mutations in different genes have recently been identified as a genetic cause of some atrial septal defect forms. These genes include the T-box transcription factor *TBX5*, *GATA4* and the homeodomain-containing DNA-binding protein *NKX2-5* [2, 4, 9]. Although mutations have not been identified in human patients, based on animal models other genes have also been implicated in atrial septal defect development. Mouse models in which the EGF–CFC gene *Cfc1* has been inactivated result in complex structural heart disease including atrial septal defect, suggesting this gene is a good candidate for human disease [3, 10]. Mutations in *CFC1* were initially found in patients with laterality defects displaying complex cardiac defects [1]. However, recent investigations in patients with isolated cardiac disorders, namely double-outlet right ventricle and transposition of the great arteries, suggest that *CFC1* contributes to proper development of the outflow tract [6].

We examined the importance of mutations in the EGF–CFC gene *CFC1* in patients with various cardiac malformations without heterotaxy syndrome, particularly in patients with atrial septal defects. Among 48 patients with isolated atrial septal defects screened, we found in 1 African patient the known heterozygous R78W amino acid substitution in exon 3. The R78W variant has been identified by other groups, either in patients with heterotaxy syndrome and cardiac malformations or in patients with double-outlet right ventricle. Since this mutation was also detected in control subjects of similar ethnicity, the biological significance of this mutation remains unclear [6]. We further found a missense variant (A145T) in exon 5 encoding the highly conserved CFC region in 2 patients with isolated atrial septal defect type II and 1 subject with a complex cardiac malformation including pulmonary atresia and tricuspidal valve defect. Although alanine is conserved in the mammalian *CFC1* gene, the evaluation of 336 control chromosomes revealed the presence of the same mutation in 3 probands, suggesting that the variant is a polymorphism. Since the affected subjects are not available for a more detailed reexamination, we can exclude neither a small atrial septal defect nor a spontaneous closure. To determine whether this variant is a real cause of CHD, a larger unaffected population must be analyzed. Our finding suggests that *CFC1* mutations may be a relatively infrequent cause of sporadic CHD in patients without heterotaxy syndrome.

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Fig. 1 Genomic structure and sequence alignment of the *cryptic* CFC domain. The position of the missense mutation is marked by an *asterisk*

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