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CCN1 mutation is associated with atrial septal defect

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Abstract

The genetic basis of congenital heart disease remains unknown in most of the cases. Recently, a novel mouse model shed new light on the role of *CCN1/CYR61*, a matricellular regulatory factor, in cardiac morphogenesis. In a candidate gene approach, we analyzed a cohort of 143 patients with atrial septal defects (ASD) by sequencing the coding exons of CCN1. In addition to three frequent polymorphisms, we identified an extremely rare novel heterozygous missense mutation (*c.139C>T; p.R47W*) in one patient with severe ASD. The mutation leads to an exchange of residues with quite different properties in a highly conserved position of the N-terminal insulin-like growth factor binding protein (IGFBP) module. Further bioinformatic analysis, exclusion of known ASD disease genes as well as the exclusion of the mutation in a very high number of ethnically matched controls (more than 1000 individuals) and in public genetic databases indicates that the *p.R47W* variant is a probable disease-associated mutation. The report about ASD in mice in heterozygous Ccn1 +/- animals strongly supports this notion. Our study is the first to suggest a relationship between a probable *CCN1* mutation and ASD. Our purpose here was to draw attention to *CCN1*, a gene that we believe may be important for genetic analysis in patients with congenital heart disease.

Keywords: Atrial septal defect; ASD; CCN1; Congenital heart disease

Introduction

Congenital heart disease (CHD) is the most common birth defect in humans. The etiology for most patients is unknown. Cardiogenesis is complex and appears highly susceptible to perturbations resulting in heart malformations [1, 2]. Among the most sensitive cardiac structures are the atrial and ventricular septa. Numerous advances in understanding the molecular pathways and genes involved in septal defects have been published in the past few years [1, 2]. Single gene mutations were identified as heritable risk factors for atrial septal defects (ASD) in a number of patients (as reviewed by us in Posch et al. [3]). Various mutations in transcription factors and sarcomeric genes were described in different studies [3]. We have analyzed different genes involved in the pathogenesis of CHDs and could identify mutations in ASD patients [4, 5, 6].

The CCN protein family is a novel class of signal modulators named after three prototypical members, cysteine-rich angiogenic inducer 61 (*Cyr61; CCN1*), connective tissue growth factor (*CTGF; CCN2*), and nephroblastoma overexpressed protein (*Nov; CCN3*) [7]. Matricellular CCN proteins are regulatory factors involved in cell signalling participating in various crucial biological processes like cell differentiation, adhesion, and apoptosis [7]. A mouse model shed new light on the role of CCN proteins in cardiac valvuloseptal morphogenesis: Mo and Lau described the Ccn1-null mouse showing severe septal defects with complete penetrance [8]. Therefore, *CCN1* was considered a promising candidate gene for ASD in man.

Materials and Methods

We enrolled 143 patients (most of them of Caucasian origin) after informed consent was obtained. All of them underwent extensive non-invasive and invasive cardiovascular evaluation. The study cohort consisted of unrelated patients with atrial septal defect (ASD), nearly all sporadic cases. The vast majority (n=134; 94%) had an isolated ostium secundum atrial septal defect (ASD II). Only 9 patients (6%) had ASD II with concomitant minor CHD (including aortic coarctation, persistent ductus arteriosus, and partial anomalous venous return). The first control group of 300 unrelated Caucasians had no CHD as shown by echocardiography. The second control group - matched to the ethnicity of the identified patient - comprised 1015 samples randomly selected from a group of 5100 unrelated adults, residents of the Ashanti region in Ghana. The study complies with the 1964 Declaration of Helsinki. The research protocol has been approved by the locally appointed ethics committees in Berlin and Kumasi and informed consent has been obtained from the enrolled subjects.

We sequenced the five exons of *CCN1/CYR61* (Genbank accession number *NM_001554*) using PCR and ABI technology (Applied Biosystems, Darmstadt, Germany). After identification of variants, allele frequencies were assessed in controls using restriction endonuclease digestion (Ava II) or using HybProbe chemistry and a LightCycler (Roche, Mannheim, Germany). The significance of the identified variant *p.R47W* was assessed by considering the conservation of the affected amino acid, the nature and location of the change, its rarity assessed in own controls and population-based datasets, and the possible impact of the amino acid substitution by five different mutation prediction tools (Mutation Taster [9], MutPred [10], SIFT [11], Polyphen2 [12], and SNAP [13]). We analyzed *p.R47W* by checking two large population-based databases, dbSNP (release 138 at http://www.ncbi.nlm.nih.gov/snp) and the Exome Sequencing Project (ESP6500SI-V2 dataset) of the National Heart, Lung and Blood Institute (http://evs.gs.washington.edu/EVS/ [14]).

Results

In total, we found one novel and three known variants in the *CCN1* gene (Fig. 1c). Three of these variants (*p.L192L, p.S316C*, and *p.R334W*) were identified as being polymorphisms because they were found in various frequencies in both cohorts, patients and Caucasian controls, and were registered in dbSNP. In contrast, we identified the novel heterozygous *c.139C>T* missense variant in exon 2 (Fig. 1b) in one patient only. The mutation leads to an exchange of the positively charged residue arginine to the aromatic hydrophobic residue tryptophane at codon 47 (*p.R47W*). We did not find this variant in dbSNP and the ESP dataset which points to the novelty and extreme rarity of *p.R47W*. It is located in module 1 of the CCN1 protein, namely the N-terminal insulin-like growth factor binding protein (IGFBP) domain, very closely to the "IGFBP motif" (Fig. 1a). The affected amino acid is highly conserved in orthologs of 14 vertebrates (ranging from chimp to zebrafish) and in paralogs of the human IGFBP isoforms 1-4 (Fig. 1a). While only PolyPhen2 predicted *p.R47W* as neutral, the other four mutation prediction tools predicted the variant as deleterious. The patient with the *p.R47W* mutation was of West African origin. Because of the high degree of genetic variability found in Africans, we chose controls with the same ethnic origin, a cohort of 1015 unrelated individuals from West Africa. All 2030 control alleles were negative for the mutation (as well as 600 alleles from the Caucasian controls without CHD). The 4406 alleles of African American origin in the ESP dataset did also not show this mutation. Further, the disease genes *GATA4* and *NKX2.5* that are known to cause non-syndromic ASD, were excluded as cause of the disease in this patient as described previously [4].

The male black patient aged 36 years was admitted to the hospital because of exertional dyspnoea. Physical examination revealed no signs of cyanosis, pulmonary congestion, or peripheral edema. The ECG confirmed sinus rhythm and showed an incomplete right bundle branch block. Furthermore, a crochetage pattern of the R wave was present in the inferior limb leads (Fig. 2a). The transthoracic echocardiography (TTE) revealed severe enlarged right atrium and ventricle. The function of both ventricles was maintained. A significant ASD (3.7 cm in diameter) with a large left-right shunt was demonstrated by TTE (Fig. 2b). Cardiac catheterization revealed a pulmonary artery blood flow relative to systemic blood flow (Qp/Qs) of 5:1. Since there was only a rudimental atrial septum, the finding was considered as a common atrium. The defect was surgically closed with a Gore-Tex patch. Unfortunately, we had no access to his family.

Discussion

To our knowledge, this report is the first to suggest a relationship between a genetic variant in *CCN1*, a growth-factor inducible immediate-early gene, and a human disease (see Online Mendelian Inheritance in Man OMIM No. *602369). Although the patient´s family was not available, there are numerous findings supporting the notion that the *p.R47W* variant is a probable disease-associated mutation rather than a rare polymorphism.

The extreme rarity of the mutation in the patient cohort, as also reported for the other ASD associated variants [3], and the exclusion of this variant in a very high number of ethnically well matched controls (our own 1015 controls and 2203 African Americans from the ESP dataset) are important points, as well as the exclusion of known ASD disease genes. The mutation leads to an exchange of residues with quite different properties in a highly conserved position of the IGFBP module harboring the important

“IGFBP motif” found in all IGFBPs and CCN proteins [15] suggesting a disturbed protein folding (strengthened by special bioinformatic tools). This motif is the central element of the “palm-like” protein structure, around which a polypeptide chain is bent, forming a disulfide ladder and assuring a proper spatial relationship between the key IGF-binding residues in that domain [16]. Interestingly, Buckway et al. showed by site-directed mutagenesis that single missense mutations in this N-terminal domain, adjacent to codon 47, were sufficient to weaken the IGF binding of IGFBP3 which shares high homology with *CCN1* [17]. Therefore, it seems highly probable that the *p.R47W* mutation also may alter the IGF binding of this domain, thus modulating IGF´s biological effects in embryonic development. Indeed, IGFs are potent cell survival signals (in combination with IGFBPs as regulators of this function) influencing apoptosis, one of the key mechanisms for sculpting embryonic tissue [18]. Furthermore, Zhu at al. reported a previously unknown function of IGFBP-4 as a cardiogenic growth factor by knockdown of the homologous IGFBP-4 in Xenopus embryos which resulted in cardiac defects [19].

Limitations of our study are the lack of cosegregation and of biochemical data concerning an effect of the mutation. Indeed, it is most likely that the majority of patients with ASD have a complex, multifactorial aetiology [3]. But one should also note that the cardiac phenotype most frequently seen in published mutation carriers are ASDs [3]. It may be possible that the *p.R47W* variant is unrelated to the disease. But our genetic data and pathophysiologic conclusions in combination with the *Ccn1* mouse model fit together quite well and contradict this notion. Indeed, one of the strongest arguments supporting our data is the remarkable observation by Mo and Lau that haploinsuffiency for *Ccn1* (heterozygous +/- mice) also resulted in delayed formation of the ventricular septum in the mouse embryo and persistent ASD in 20% of adult mice [8]. In these animals, *Ccn1* haploinsuffiency caused precocious apoptosis in the atrial junction of the cushion tissue and impaired gelatinase activities in the muscular component of the interventricular septum, when fusion between the endocardial cushion tissue and the atrial and ventricular septa occurred [8]. Clearly, deficiency in matrix signaling by CCN proteins could lead to autosomal dominant septal defects, identifying heterozygous *Ccn1* mice as a genetic model for ASD.

In conclusion, using a translational approach we identified the first probable mutation (*p.R47W*) in a patient with large ASD linking *CCN1*, an important factor in embryogenesis and subsequent development, to human pathology. Our results underscore close homology between the mouse model and human disease. Further studies are necessary to elucidate the mechanism of how this mutation contributes to septal defects.

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Conflict of interest: The authors declare that they have no conflict of interest.

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Figure legends:

Fig. 1 Genetic data of the *p.R47W* patient

a) Amino acid sequence alignment of residues 44-56 of the IGFBP domain of CCN1 demonstrating very high conservation. Mutated codon 47 is shaded. The "IGFBP motif" (GCGCCxxC) is boxed. Residues that are *not* conserved are shown in bold.

b) Electropherogram of the heterozygous missense mutation *c.139C>T*.

c) Localization of the detected variants (three SNPs and the *p.R47W* mutation) in the CCN1 gene/protein (shown by arrows). The upper panel shows the gene structure (exons as boxes, coding sequence shaded). The lower panel shows the protein structure (SP – signaling peptide, IGFBP – insulin-like growth factor binding protein domain, VWC – Von Willebrand factor domain, TSP-1 – thrombospondin-homology domain, CT - cysteine knot domain, AA – amino acid).

Fig. 2 Clinical vignettes of the *p.R47W* patient

a) ECG demonstrating an incomplete right bundle branch block and typical crochetage pattern of the R wave in the inferior leads II, III, and aVF.

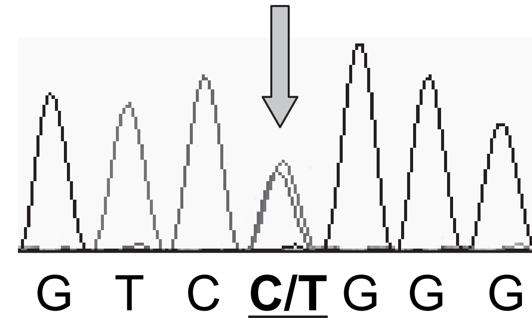
b) 2-D echocardiography revealing a large atrial septal defect (see arrowheads, modified parasternal long axis view).

Figure 1

a

	44			47									56
mutant CCN1	G	L	V	**W**	D	-	G	C	G	C	C	K	V C
human CCN1	G	L	V	R	D	-	G	C	G	C	C	K	V C
chimpanzee	G	L	V	R	D	-	G	C	G	C	C	K	V C
treeshrew	G	L	V	R	D	-	G	C	G	C	C	K	V C
microbat	G	L	V	R	D	-	G	C	G	C	C	K	V C
cow	G	L	V	R	D	-	**A**	C	G	C	C	K	V C
squirrel	G	L	V	R	D	-	G	C	G	C	C	K	V C
guinea pig	G	L	V	R	D	-	G	C	G	C	C	K	V C
tenrec	G	L	V	R	D	-	G	C	G	C	C	K	V C
shrew	G	L	V	R	D	-	G	C	**-**	C	C	K	V C
hedgehog	G	L	V	R	D	-	G	C	G	C	C	K	V C
dog	G	L	V	R	D	-	G	C	G	C	C	K	V C
mouse	G	L	V	R	D	-	G	C	G	C	C	K	V C
rabbit	G	L	V	R	D	-	G	C	**-**	C	C	K	V C
rat	G	L	V	R	D	-	G	C	G	C	C	K	V C
zebrafish	G	L	**Q**	R	D	-	**A**	C	G	C	C	**L**	V C
human IGFBP1	**E**	**V**	**T**	R	**S**	**A**	G	C	G	C	C	**P**	**M** C
human IGFBP2	**E**	L	V	R	**E**	**P**	G	C	G	C	C	**S**	V C
human IGFBP3	**E**	L	V	R	**E**	**P**	G	C	G	C	C	**L**	**T** C
human IGFBP4	**E**	L	V	R	**E**	**P**	G	C	G	C	C	**A**	**T** C

b



c

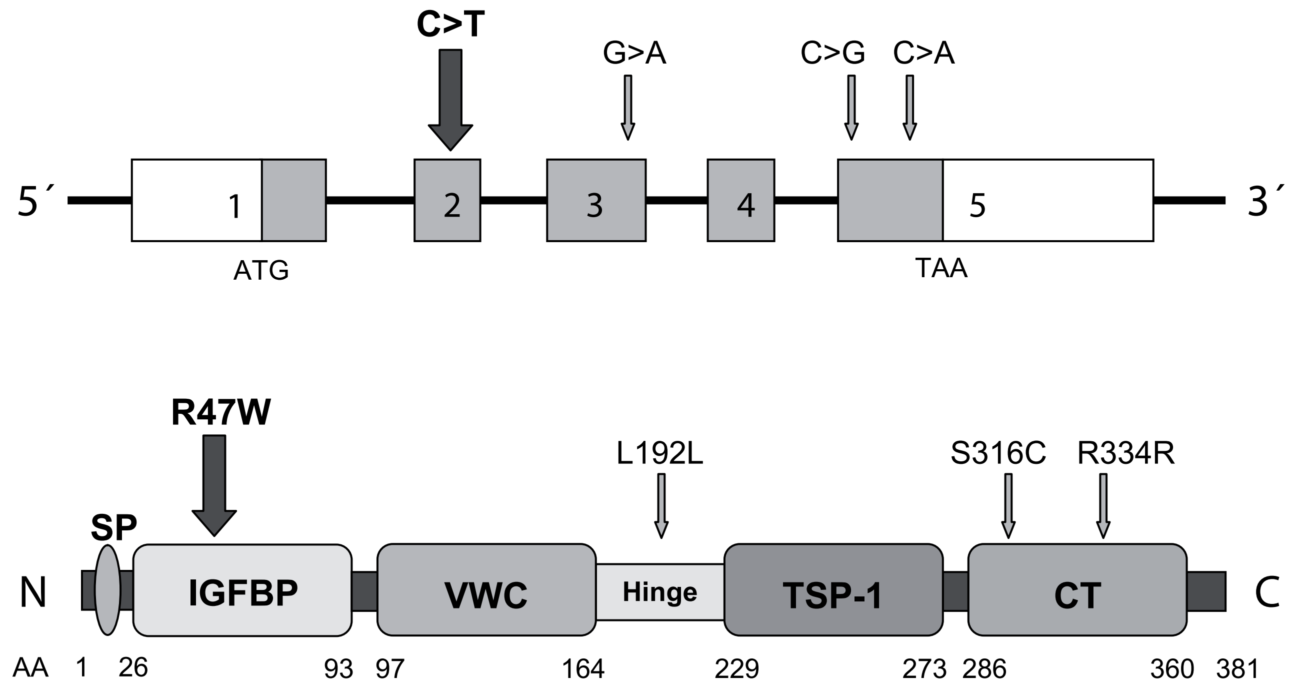


Figure 2