Novel PITX2c loss-of-function mutations associated with complex congenital heart disease

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Abstract. Congenital heart disease (CHD) is the most common form of birth defect in humans and is the leading non-infectious cause of infant mortality. Emerging evidence strongly suggests that genetic risk factors play an important role in the pathogenesis of CHD. However, CHD is of pronounced genetic heterogeneity, and the genetic defects responsible for CHD in an overwhelming majority of patients remain unclear. In this study, the entire coding region and splice junction sites of the *PITX2c* gene, which encodes a paired-like homeodomain transcription factor crucial for proper cardiovascular morphogenesis, was sequenced in 170 unrelated neonates with CHD. The available relatives of the mutation carriers and 200 unrelated ethnically matched healthy individuals were genotyped. The disease-causing potential of the *PITX2c* sequence variations was predicted by MutationTaster and PolyPhen-2. The functional effect of the mutations was characterized using a luciferase reporter assay system. As a result, 2 novel heterozygous PITX2c mutations, p.R91Q and p.T129S, were identified in 2 unrelated newborns with transposition of the great arteries and ventricular septal defect, respectively. A genetic scan of the pedigrees revealed that each mutation co-segregated with CHD transmitted in an autosomal dominant pattern with complete penetrance. The mutations, which altered the amino acids completely conserved evolutionarily, were absent in 400 normal chromosomes and were predicted to be causative. Functional analysis revealed that the PITX2c mutations were both associated with significantly diminished transcriptional activity compared with their wild-type counterpart. This study demonstrates the association between PITX2c loss-of-function mutations and the transposition of the great arteries and ventricular septal defect in humans, providing further insight into the molecular mechanisms responsible for CHD.

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Introduction

Congenital heart disease (CHD), characterized by the developmental abnormality of the heart and intrathoracic great vessels, is the most common birth defect in humans worldwide, occurring in approximately 1% of live births, and is the major non-infectious cause of infant morbidity and mortality, accounting for approximately 30% of neonatal deaths resulting from developmental malformations (1). According to the specific anatomic lesions, CHD is categorized into at least 21 clinical types, encompassing atrial septal defect, ventricular septal defect, atrioventricular septal defect, tetraology of Fallot, patent ductus arteriosus, transposition of the great arteries, right ventricular outflow tract obstruction, aortic stenosis, pulmonary atresia, coronary artery deformation, tricuspid atresia, Ebstein's anomaly of the tricuspid valve, double outlet right ventricle, hypoplastic left heart syndrome, interrupted aortic arch and total anomalous pulmonary venous connection (1). If unrepaired, these cardiovascular deformations may contribute to poor exercise tolerance, degraded life quality, delayed fetal brain development, infective endocarditis, metabolic disorders, pulmonary hypertension, congestive heart failure, thromboembolic stroke, arrhythmias and even sudden cardiac death (2-13). Despite its high prevalence and important clinical significance, the etiology for CHD in an overwhelming majority of patients remains unclear.

In mammals, the heart is the first organ to form during embryogenesis (14). Cardiogenesis is a complex and dynamic biological process that requires the orchestration of cardiac cell commitment, differentiation, proliferation and migration, and both environmental and genetic risk factors may perturb this exquisite temporal and spatial cooperation, leading to a wide variety of CHD (15-22). A growing body of evidence underscores the key role of cardiac transcription factors in

embryonic cardiovascular morphogenesis, and a long list of mutations in the genes coding for cardiac transcription factors, including the NK and GATA families, have been associated with CHD (23-41). However, CHD is a genetically heterogeneous disease and the genetic defects responsible for CHD in the majority of patients remain unknown.

Previous studies have indicated that the cardiac transcription factor, PITX2c, a member of the bicoid-like homeodomain family of transcription factors, is essential for normal cardiovascular development (42-49). The *PITX2c* gene is predominantly expressed in the embryonic and adult hearts, playing a crucial role in the embryogenesis of the left atrium, cardiac conduction system and pulmonary venous myocardium (50). In mice, targeted deletion of PITX2c has been shown to lead to embryonic lethality due to distinct types of CHD, including atrial isomerism, double-outlet right ventricle, atrial septal defect, ventricular septal defect, transposition of the great arteries, and abnormal aortic arch, as well as incomplete closure of the body wall (42). In humans, PITX2c mutations have been implicated in congenital atrial septal defect, ventricular septal defect, double outlet of the right ventricle and atrial fibrillation (51-54). These findings justify screening *PITX2c* in other cohorts of patients with CHD.

Materials and methods

Study subjects. A cohort of 170 unrelated neonates with CHD was recruited from the Chinese Han population. The available relatives of the mutation carriers were also included. The patients were evaluated by individual and familial histories, review of the medical records, complete physical examination, 12-lead electrocardiogram and 2-dimensional transthoracic echocardiography with a color flow Doppler. Transesophageal echocardiography and cardiac catheterization were performed in some patients. Most patients underwent cardiac surgery or catheter-based repair. The patients with known chromosomal abnormalities or syndromic cardiovascular defects were excluded from the study. Clinical investigations were carried out by cardiologists who had no knowledge of the genotype.

A total of 200 unrelated, ethnically matched healthy individuals randomly enlisted from the individuals undergoing routine physical examinations were used as the control subjects. According to the reviews of medical histories and analyses of the echocardiographic records, the control individuals had no CHD. The ethnic origin of a participant was ascertained by a combination of self-reported ethnicity and a personal questionnaire asking questions regarding birthplace, language, religion and ancestry.

Peripheral venous blood specimens from patients with CHD and control individuals were prepared. The study protocol was reviewed and approved by the local institutional ethics committee and written informed consent was obtained from the parents or guardians of the participants prior to enrollment in the study.

Genetic analysis of human PITX2c. Genomic DNA was extracted from the blood lymphocytes of each participant using the Wizard Genomic DNA Purification kit (Promega Corp., Madison, WI, USA). The *PITX2c* gene was sequenced initially in 170 unrelated neonates with CHD, and the genotyping of *PITX2c* was subsequently performed in the available relatives of the mutation carriers and the 200 unrelated control individuals. The referential genomic DNA sequence of *PITX2c* was derived from GenBank (Accession no. NC_000004), which was at the National Center for Biotechnical Information (NCBI; http://www.ncbi.nlm.nih.gov/).

The primer pairs used to amplify all the coding exons and exon-intron boundaries of *PITX2c* by polymerase chain reaction (PCR) were designed as previously described (53). PCR was performed using HotStar Taq DNA Polymerase (Qiagen GmbH, Hilden, Germany) on a Veriti Thermal Cycler (Applied Biosystems, Foster, CA, USA), with standard conditions and concentrations of reagents. Amplified products were analyzed on 1% agarose gels stained with ethidium bromide and purified using the QIAquick Gel Extraction kit (Qiagen GmbH). Both strands of each PCR product were sequenced with a BigDye® Terminator v3.1 Cycle Sequencing kit under an ABI PRISM 3130 XL DNA Analyzer (both from Applied Biosystems). The sequencing primers were the same as those used for the above-mentioned specific region amplification. The DNA sequences were viewed and analyzed with DNA Sequencing Analysis Software® v5.1 (Applied Biosystems). The variant was validated by re-sequencing an independent PCR-generated amplicon from the same subject. Additionally, for an identified sequence variant, the Exome Variant Server (EVS; http://evs.gs.washington.edu/EVS) and the NCBI single nucleotide polymorphism (SNP; http://www.ncbi.nlm.nih.gov/SNP) online databases were queried to confirm its novelty.

Alignment of multiple PITX2c protein sequences among species. Multiple PITX2c protein sequences across various species were aligned using the online program, MUSCLE, version 3.6 (http://www.ncbi.nlm.nih.gov/homologene?cmd=Retrieve&dopt=MultipleAlignment&list_uids=55454).

Prediction of the pathogenic potential of a PITX2c sequence variation. The disease-causing potential of a *PITX2c* sequence variation was predicted by MutationTaster (http://www.mutationtaster.org), which automatically yielded a probability for the variation to be either a pathogenic mutation or a benign polymorphism. Notably, the P-value used here is the probability of the correct prediction rather than the probability of error as used in t-test statistics (i.e., a value close to 1 indicates high accuracy of the prediction). Besides, another online program PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) was also utilized to evaluate the causative likeliness of a variant.

Expression plasmids and site-directed mutagenesis. The recombinant expression plasmid PITX2c-pcDNA4, which was constructed by Strungaru *et al* (55), was a gift from Professor Georges Christé, from Physiopathologie des troubles du rythme cardiaque, Faculté de Pharmacie de Lyon, Université Lyon 1, Lyon, France. The atrial natriuretic factor (ANF)-luciferase reporter plasmid, which contains the 2600-bp 5'-flanking region of the *ANF* gene, namely ANF(-2600)-Luc, was kindly provided by Dr Ichiro Shiojima, from the Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine (Chiba, Japan). Each of the identified variations was introduced into wild-type *PITX2c* using a QuickChange II XL Site-Directed Mutagenesis kit

(Stratagene, La Jolla, CA, USA) with a complementary pair of primers. The mutants were sequenced to confirm the desired mutations and to exclude any other sequence variations.

Luciferase reporter gene assay. Chinese hamster ovary (CHO) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, as well as 100 U/ml penicillin and 100 g/ml streptomycin. The ANF(-2600)-Luc reporter construct and an internal control reporter plasmid, pGL4.75 (hRluc/CMV; Promega), were used in transient transfection assays to explore the transactivational activity of the PITX2c mutant. The CHO cells were transfected with 2 μg of the wild-type PITX2c-pcDNA4 or mutant PITX2c-pcDNA4 (R91Q or T129S) or the empty vector pcDNA4, 2.0 μg of ANF(-2600)-Luc reporter construct, and 0.04 μg of pGL4.75 control reporter vector using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA). For co-transfection experiments, 1 μg of wild-type PITX2c-pcDNA4, 1 μg of mutant PITX2c-pcDNA4 (R91Q or T129S), 2.0 μg of ANF(-2600)-Luc, and 0.04 μg of pGL4.75 were used. The transfected cells were incubated for 24 h, then lysed and assayed for reporter activities. Firefly luciferase and *Renilla* luciferase activities were measured with the Dual-Glo luciferase assay system (Promega). The activity of the *ANF* promoter was presented as fold activation of Firefly luciferase relative to *Renilla* luciferase. Three independent experiments were conducted at minimum for wild-type or mutant PITX2c.

Statistical analysis. Experimental data are expressed as the means ± standard deviations. Continuous variables were tested for normality of distribution, and the Student's unpaired t-test was used for the comparison of numeric variables between 2 groups. A comparison of the categorical variables between 2 groups was performed using Pearson's χ^2 test or Fisher's exact test where appropriate. A two-tailed P-value <0.05 was considered to indicate a statistically significant difference.

Results

Baseline characteristics of the study population. A cohort of 170 unrelated neonates with CHD was enrolled and clinically evaluated in contrast to a total of 200 unrelated, ethnically-matched healthy individuals used as the controls. All the participants had no established environmental risk factors for CHD, such as maternal illness and drug use in the first trimester of pregnancy, parental smoking, and long-term exposure to toxicants and ionizing radiation. The baseline clinical characteristics of the 170 unrelated CHD patients are summarized in Table I.

Table I. Baseline clinical characteristics of the 170 unrelated neonates with congenital heart disease.

Parameter	No. or quantity	Percentage or range
Male	89	52.4
Age (days)	12.6±8.5	1-26
Birth weight (kg)	3.1±0.8	1.6-5.5
Positive family history	51	30
Distribution of different types of CHD		
Isolated CHD	84	49.4
VSD	21	12.4
PDA	14	8.2
ASD	10	5.9
PS	9	5.3
TGA	7	4.1
AVSD	6	3.5
COA	5	2.9
DORV	5	2.9
PTA	3	1.8
TAPVC	2	1.2
HLHS	1	0.6
PA	1	0.6
Complex CHD	86	50.6
TGA + VSD	27	15.9
TOF	15	8.8
VSD + PDA	12	7.1
ASD + VSD	9	5.3
PDA + TGA	5	2.9
VSD + DORV	5	2.9
ASD + TGA	5	2.9
ASD + PDA	4	2.4
ASD + VSD + DORV	2	1.2
IAA + VSD	1	0.6
ASD + VSD + PDA	1	0.6
Treatment		
Surgical repair	110	64.7
Follow-up	60	35.3

CHD, congenital heart disease; VSD, ventricular septal defect; ASD, atrial septal defect; PDA, patent ductus arteriosus; PS, pulmonary stenosis; TGA, transposition of the great arteries; AVSD, atrioventricular septal defect; COA, coarctation of the aorta; DORV, double outlet right ventricle; PTA, persistent truncus arteriosus; TAPVC, total abnormal pulmonary venous connection; HLHS, hypoplastic left heart syndrome; PA, pulmonary atresia; TOF, tetralogy of Fallot; IAA, interruption of the aortic arch.

PITX2c mutation. All the exons and splice junction sites of the *PITX2c* gene was sequenced in the 170 unrelated neonates with CHD, and 2 heterozygous sequence variations in *PITX2c* weree identified in 2 out of the 170 patients, with a mutational prevalence of approximately 1.18% based on the patient population. Specifically, a substitution of adenine for guanine at the second nucleotide of codon 91 of the *PITX2c* gene (c.272G>A), predicting the transition of arginine into glutamine at amino acid 91 (p.R91Q), was identified in a neonate with transition of great arteries and ventricular septal defect. A change of adenine into thymine at the first nucleotide of codon 129 of the *PITX2c* gene (c.385A>T), equivalent to the transversion of threonine into serine at amino acid 129 (p.T129S), was detected in another newborn with transition of great arteries and ventricular septal defect. The sequence

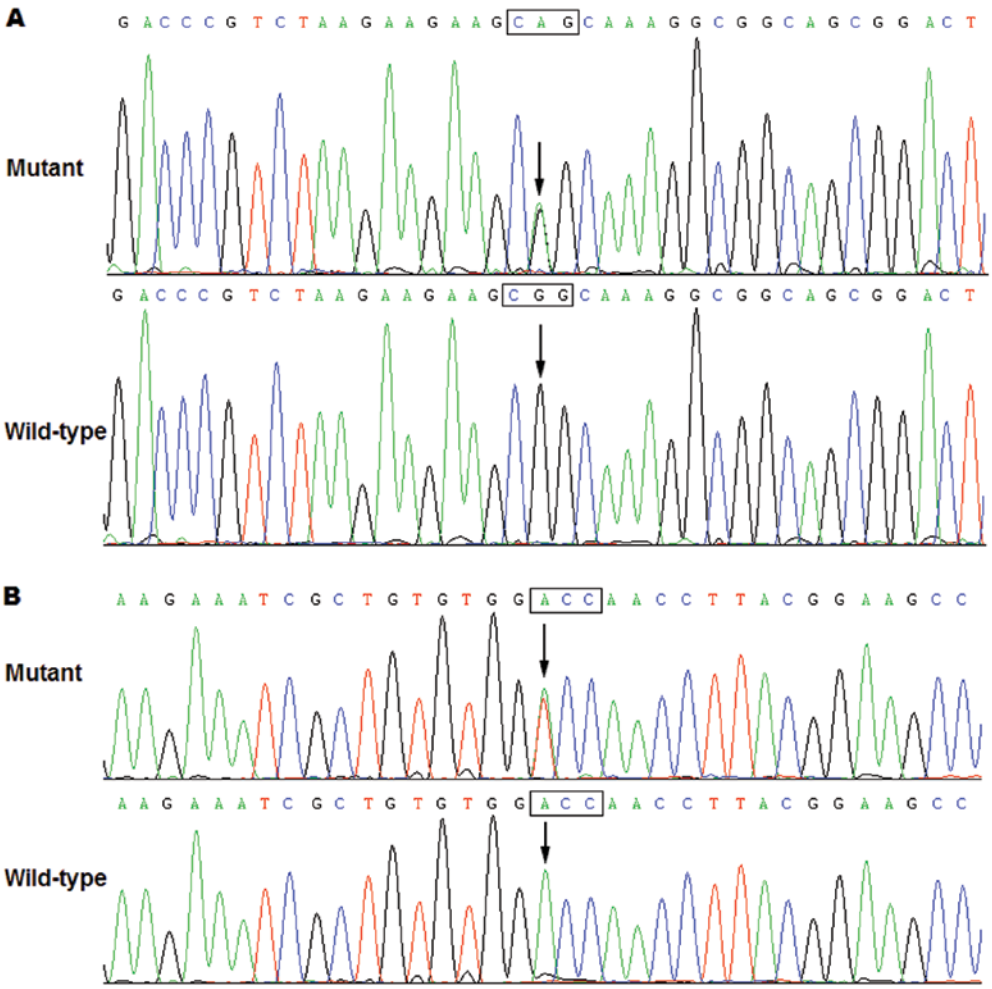

Figure 1. Sequence electropherograms of the *PITX2c* mutations in contrast to their controls. Arrows indicate the heterozygous nucleotides of G/A (A) and A/T (B) in the index patients from families 1 and 2, respectively (mutant), or the homozygous nucleotides of G/G (A) and A/A (B) in the corresponding control individuals (wild-type). The rectangle indicates the nucleotides constituting a codon of PITX2c.

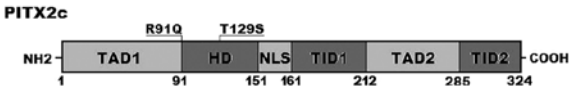

Figure 2. Schematic diagram of PITX2c protein structure with the identified mutations marked. The mutations identified in the patients with congenital heart disease are shown above the structural domains. NH2, amino-terminus; TAD1, transcriptional activation domain 1 (amino acids 1-90); HD, homeodomain (amino acids 91-151); NLS, nuclear localization signal (amino acids 145-161); TID1, transcriptional inhibitory domain 1 (amino acids 162-212); TAD2, transcriptional activation domain 2 (amino acids 213-285); TID2, transcriptional inhibitory domain 2 (amino acids 286-324); COOH, carboxyl-terminus.

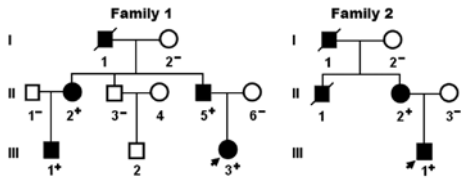

Figure 3. Pedigree structures of the families with congenital heart disease. Families are designated as family 1 and family 2, respectively. Family members are identified by generations and numbers. Square, male family member; circle, female member; closed symbol, affected member; open symbol, unaffected member; arrow, proband; symbol with a slash, deceased member; +, carrier of the heterozygous mutation; -, non-carrier.

electropherograms showing the identified heterozygous *PITX2c* variations compared with the corresponding control sequences are shown in Fig. 1. A schematic diagram of *PITX2c* showing the structural domains (56,57) and the locations of the detected mutations is presented in Fig. 2. The mutation was neither observed in 400 control chromosomes nor reported in the EVS and NCBI SNP databases, which were consulted again on January 12, 2014. A genetic scan of the available family members of the mutation carriers revealed that in each family the mutation was present in all affected family members alive, but absent in the unaffected family members examined. An analysis of the pedigrees revealed that in each family, the mutation co-segregated with CHD transmitted as an autosomal dominant trait with complete penetrance. Atrial fibrillation was confirmed by the early electrocardiograms in patients I-1 and II-1 from family 1. The pedigree structures of the families are shown in Fig. 3. The phenotypic characteristics and the results of genetic screening of the affected family members are listed in Table II.

Alignment of multiple PITX2c protein sequences. A cross-species alignment of multiple PITX2c protein sequences

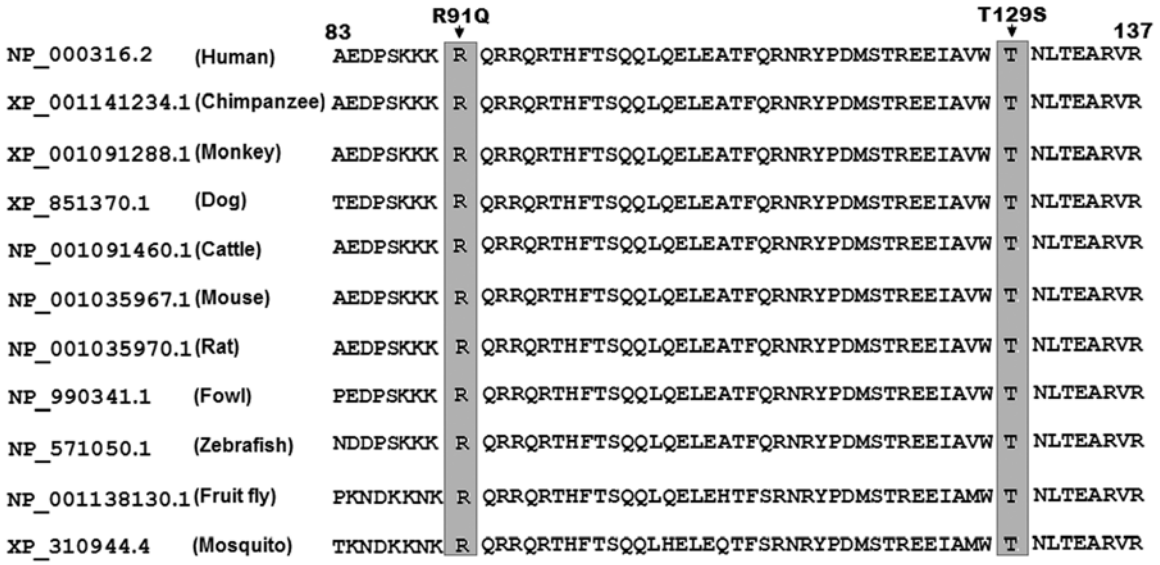

Figure 4. Multiple alignments of PITX2c protein sequences across species. The altered amino acids of p.R91 and p.T129S are completely conserved evolutionarily among a wide variety of species.

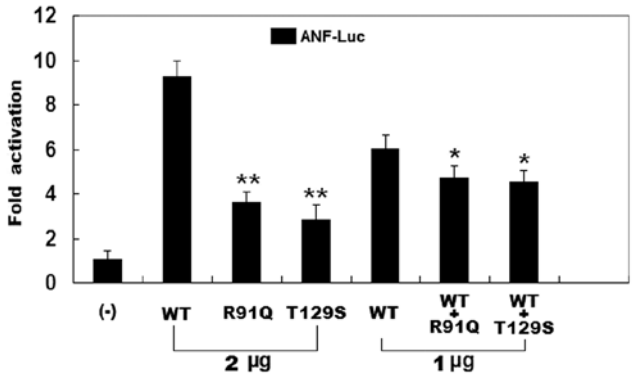

Figure 5. Functional defects associated with PITX2c mutations. Activation of atrial natriuretic factor (*ANF*) promoter driven luciferase reporter in Chinese hamster ovary (CHO) cells by wild-type PITX2c (WT) or mutant PITX2c (R91Q or T129S), alone or in combination revealed significantly decreased transcriptional activity resulted from mutant proteins. Experiments were performed in triplicate, and the mean ± standard deviations are shown. **P<0.0005 and *P<0.001 compared with the same amount (2 μg) of wild-type PITX2c.

Table II. Phenotypic characteristics and status of the PITX2c mutations in the affected family members.

Subject Information			Phenotype		Genotype (PITX2c mutation)
Identity	Gender	Age[a]	CHD	AF	
Family 1					R91Q
I-1	M	52[b]	VSD	+	NA
II-1	F	31	VSD	+	+/-
II-5	M	26	VSD	-	+/-
III-1	M	2	VSD	-	+/-
III-3	F	0	TGA, VSD	-	+/-
Family 2					T129S
I-1	M	49[b]	VSD	-	NA
II-1	M	0[b]	TGA, VSD	-	NA
II-2	F	25	VSD	-	+/-
III-1	M	0	TGA, VSD	-	+/-

F, female; M, male; CHD, congenital heart disease; VSD, ventricular septal defect; TGA, transposition of the great arteries; AF, atrial fibrillation; NA, not available or not applicable. '+' indicates the presence of mutation and '-' denotes the absence of mutation. [a]Age is presented in years; [b]age at death.

displayed that the affected amino acids were completely conserved evolutionarily (Fig. 4), indicating that the amino acids are functionally important.

Causative potential of the PITX2c variations. The *PITX2c* sequence variations of c.272G>A and c.385A>T were both predicted to be disease-causing by MutationTaster, with the same P-value of 1.000. No SNPs in the altered regions were found in the MutationTaster database. In addition, these 2 amino acid substitutions (p.R91Q and p.T129S) were also predicted to be possibly damaging by PolyPhen-2, with the same scores of 0.995 (sensitivity, 0.68; specificity, 0.97) for p.R91Q and p.T129S.

Functional defect associated with PITX2c mutations. As shown in Fig. 5, the same amounts of wild-type PITX2c (2 μg), R91Q-mutant PITX2c (2 μg) and T129S-mutant PITX2c (2 μg) activated the *ANF* promoter by approximately a 9-, 4- and 3-fold increase, respectively, when compared with the empty plasmid. When the same amount of wild-type PITX2c (1 μg) was transfected in combination with mutant PITX2c (1 μg of R91Q-mutant or 1 μg of T129S-mutant), the induced activation of the *ANF* promoter was increased by approximately 5-fold compared with the empty plasmid. These results indicate that the PITX2c mutants are associated with significantly reduced activation activity compared with their wild-type counterpart.

Discussion

The human *PITX2c* gene maps to chromosome 4q25, coding for a protein of 324 amino acids (58). PITX2c is predominantly expressed in the developing and adult heart and is required for normal cardiovascular development (59). In the present study, 2 novel heterozygous mutations of PITX2c, p.R91Q and p.T129S, were identified in 2 newborns with CHD. The mutant alleles were absent in the 400 reference chromosomes from an ethnically matched control population. Cross-species alignment of PITX2c protein sequences revealed that the altered amino acids were completely conserved evolutionarily. These 2 variations were predicted to be pathogenic by both MutationTaster and PolyPhen-2, and functional analysis demonstrated that the mutants were associated with a significantly reduced transcriptional activity. Therefore, it is likely that functionally compromised PITX2c predisposes to CHD in these mutation carriers.

PITX2 is a member of the paired-like homeobox transcription factor family. To date, 4 distinct PITX2 transcripts, generated by differential mRNA splicing and alternative promoter usage, have been reported, of which PITX2a, PITX2b and PITX2c differ only in their amino-termini and have been identified in humans, mice, chicks, zebrafish and *Xenopus*, whereas the 4th isoform, PITX2d, which lacks the whole amino-terminal domain and most homeodomains, has only been identified in humans. The unique amino-termini of PITX2a, PITX2b and PITX2c may have an effect on their transcriptional activity in a cell-type and promoter-dependent manner. The homeodomain may recognize and bind to specific DNA sequences (5'-TAATCC-3'), which is responsible for DNA binding and interaction with other transcription factors (60). The PITX2c mutations of p.R91Q and p.T129S identified in the present study are located in the homeodomain, and thus they may be expected to exert an effect on the transcriptional activity of PITX2c by perturbing its DNA binding.

PITX2c is an upstream regulator of multiple target genes expressed in the heart during embryogenesis, including the *ANF* gene (61). Therefore, the functional characteristics of a PITX2c mutation can be investigated by the assay of the transcriptional activity of the *ANF* promoter in cells expressing PITX2c mutant in contrast to its wild-type counterpart. In this study, the functional effect of 2 novel PITX2c mutations identified in patients with CHD was characterized by transcriptional activity analysis and the results demonstrated that the mutants were associated with a significantly decreased transcriptional activity on the downstream gene, *ANF*, suggesting that PITX2c loss-of-function mutations are potentially an alternative pathological mechanism of CHD.

The fact that dysfunctional PITX2c confers enhanced susceptibility to CHD has been substantiated in animal models. In mice, *PITX2c* is expressed specifically in the trabecular and septal myocardium with a strong expression bias in the myocardium associated with endocardial cushions of the atrioventricular canal and outflow tract, which are crucial for cardiac septation (62), and the targeted disruption of the *PITX2c* gene has been shown to result in embryonic lethality due to cardiovascular defects, including atrial isomerism, ventricular septal defect, double-outlet right ventricle, atrial septal defect and abnormal aortic arch (42). In *Xenopus*, the knockdown of PITX2c by the use of chemically modified antisense oligonucleotides has ben shown to lead to aberrant cardiac morphology, of which the most commonly observed cardiac deformity was a failure of rightward migration of the outflow tract, occurring in 23% of embryos injected with the PITX2c antisense oligonucleotides. Other cardiac deformations caused by PITX2c-targeted mRNA interference included anomalies of atrial septation, extracellular matrix restriction, relative atrial-ventricular chamber positioning and restriction of ventricular development (43). These experimental findings highlight an exquisite sensitivity of the developing cardiovascular system to the level of PITX2c.

Notably, mutant PITX2c has been causally linked to lone or familial atrial fibrillation (53,54). In this study, 2 novel PITX2c mutations were identified in 2families with ventricular septal defect, of which 2 family members also had atrial fibrillation, and 2 family members also had transition of the great arteries. Different genetic backgrounds and epigenetic modifiers may account for the pronounced phenotypic heterogeneity among these mutation carriers.

In conclusion, the current study associates PITX2c loss-of-function mutations with transition of the great arteries and ventricular septal defect in humans, which provides additional evidence supporting that the fact that PITX2c plays an important role in cardiovascular development.

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