Variations of *CITED2* Are Associated with Congenital Heart Disease (CHD) in Chinese Population

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Abstract

*CITED2* was identified as a cardiac transcription factor which is essential to the heart development. *Cited2*-deficient mice showed cardiac malformations, adrenal agenesis and neural crest defects. To explore the potential impact of mutations in *CITED2* on congenital heart disease (CHD) in humans, we screened the coding region of *CITED2* in a total of 700 Chinese people with congenital heart disease and 250 healthy individuals as controls. We found five potential disease-causing mutations, p.P140S, p.S183L, p.S196G, p.Ser161delAGC and p. Ser192_Gly193delAGCGC. Two mammalian two-hybrid assays showed that the last four mutations significantly affected the interaction between *p300CH1* and *CITED2* or *HIF1A*. Further studies showed that four *CITED2* mutations recovered the promoter activity of *VEGF* by decreasing its competitiveness with *HIF1A* for binding to *p300CH1* and three mutations decreased the consociation of *TFAP2C* and *CITED2* in the transactivation of *PITX2C*. Both *VEGF* and *PITX2C* play very important roles in cardiac development. In conclusion, we demonstrated that *CITED2* has a potential causative impact on congenital heart disease.

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Introduction

Congenital heart disease (CHD) is a most common defect caused by abnormal cardiac formation in fetuses and has become the leading reason of childhood mortality with an incidence around 1%[1–3]. In the past decades, a series of CHD-causing genes have been identified such as *Mnx2*, *TBX5*, *GATA4* and *CITED2* [4–6]. It has been confirmed that their mutations can cause cardiac malformations through affecting the transcription activity of critical genes involved in heart development pathways.

*CITED2* (Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2) is one member of a new conserved family of transcriptional activators which includes four members: *CITED1* (Mg1), *CITED2* (Mg1/p35sg), *CITED3* and *CITED4* (Mg2) [7]. *CITED2* is a nuclear protein which binds closely to the CH1 region of *p300* and *CBP* by its CR2 region (including a conserved 32-amino acid sequence [8]). Meanwhile, many other transcription factors and transcription regulating factors such as *HIF1A*, *RXXz*, Nfs, Mdm2, Ets-1 and Stat2 also bind to the CH1 region of *CBP/p300* [9,10]. Thus *CITED2* may act as a pivotal transcriptional modulator to regulate the expression of some specific genes. For example, *CITED2* decreased the expression of *HIF1A* (Hypoxia Inducible Factor 1) through its competitive binding to *CBP/p300CH1*[11,12], consequently interfering the transcription of genes induced by *HIF1A* such as *VEGF* (vascular endothelial growth factor) [13]. It has been confirmed that the overexpression of *vegf* is the main factor resulting in cardiac malformation in *cited2−/−* mice [14].

Besides being a transcriptional repressor of *HIF1A*, *CITED2* acts as a transcriptional coactivator of *TEAP2* (transcription factor AP2, also called *Tfap2c*) [15]. Mutations of *TEAP2A* and *TEAP2B* result in neural tube, cranial ganglia defects and cardiac malformations [16,17]. This suggested that the coactivation of *TFAP2* with *p300* and *CITED2* and CREBBP is essential for the normal development of those structures. As a critical transcription factor, *TFAP2* can affect the transcription of many genes, including *PITX2C* (Paired-Like Homeodomain 2 C) which is critical in Nodal-*PITX2C* pathways [18]. In addition, it has been detected that *TFAP2* isoforms and *CITED2* work together on the *PITX2C* promoter1 which controls the expression of *PITX2C* in the heart of embryonic mice. The mice experiments already indicated that knocking out *pitx2c* gene can lead to valve defects, body wall dysraphism, gastrochisis, ectopia cordis and other multiple organs polymorphous defects [19].

*CITED2* gene mutation in human congenital heart disease was first reported by Sperling et al [20] in 2005. They identified 3...
mutations which alter the amino acid sequence and studied their association with HIF1A and TFAP2C. Their study confirms that CITED2 is an important transcription factor in heart development and provides new insights into the molecular mechanism of congenital heart defects. Later, Yang et al found 3 new mutations in Chinese patients with congenital heart disease (2010) [21] and Chen et al [22] demonstrated another 3 new mutations in European CHD patients. Recently, Xu et al found 3 CITED2 gene mutations, their research showed that CITED2 gene mutations and methylation may play an important role in CHD. In their study, most of these mutations were in SRJ region. The mutations in our study were identified for the first time and located in SRJ region as well. Our work aimed to determine whether the new mutations also affect HIF1A or TFAP2C and finally lead to an abnormal expression of VEGF or PITX2C which play an important role in heart development.

Materials and Methods

Ethics statement

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the National Research Institute for Family Planning. Written informed consent was obtained from patients' parents or guardians.

Subjects

The study population comprised 700 patients who were diagnosed with CHD based on anthropometric measurement, physical examination for malformation and dysmorphism, and radiological evaluation. The patients with a phenotype of VSD, TOF and ASD accounted for 43.71%, 8.42% and 12% respectively, 250 unrelated healthy children were used as controls. Peripheral blood was collected from each affected individual and their parents and controls were from 6 months to 12 years old and most of them volunteered to participate in the study.

We sequenced the whole CITED2 ORF in 700 CHD patients (Table 1) and 250 healthy controls recruited from Lanzhou University, Beijing Children’s Hospital, Zhengzhou Children’s Hospital, Henan provincial Chest Hospital and Children’s Hospital of Fudan University.

Mutational analysis and bioinformatics

Genomic DNA was extracted from peripheral blood leukocytes using standard methods. The human CITED2 gene is located on 6q24.1 and is encoded by two exons. One of the exons and splice sites of CITED2 were amplified by polymerase chain reaction (PCR) using two pairs of CITED2 gene-specific primers (Table 2). PCR products were sequenced using the appropriate PCR primers and the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and run on an automated sequencer, ABI 3730XL (Applied Biosystems), to perform mutational analysis.

Site-directed mutagenesis and plasmid construction

Human CITED2 and HIF1A cDNA were obtained from OriGene True-Clone, and TFAP2C cDNA was purchased from GenoCopoeia. CITED2 mutations were constructed by using the Quick Change Lightning Site-Directed Mutagenesis kit (Strata gene, La Jolla, CA, USA). Then the introduced mutations were confirmed by DNA sequence.

The WT and mutant CITED2 were amplified by PCR from cDNA and inserted into the pEGFP-N1 vector (BD Biosciences, Palo Alto, CA, USA). The ORF of HIF1A and TFAP2C were also amplified by PCR from cDNA and inserted respectively into the pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA, USA) to create the expression plasmid pcDNA3.1-HIF1A and pcDNA3.1-TFAP2C.

A 1300-bp fragment of the p300-CH1, PITX2C promoter and an 870-bp segment of VEGF promoter amplified by PCR from Human genomic DNA were cloned respectively into the GAL4- pCMX vector and the luciferase reporter PLG3-basic vector. GAL4-HIF1A was constructed by cloning DNA fragments into GAL4-pCMX vector at the Ecorv and Nhel sites. All primers of the PCRS were list in Table 2.

The VP16-pCMX vector with the potent transactivating domain of HSV, the promoter pGL3-basic vector with 4×GAL4 DNA-binding sites and the GAL4-pCMX vector containing GAL4-DBD were provided by Dr. Ronald M. Evans (Salk Institute for Biological Studies, USA).

Cell culture and transient transfection

293T and Hela cells were maintained in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum, 100 mg/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere containing 5% CO2 at 37°C. Transfection was carried out using a standard calcium phosphate method or Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA, USA).

Table 1. Patients with congenital heart disease included in the study.

| Phenotype                                      | Total(n = 700) |
|------------------------------------------------|---------------|
| Ventricular septal defect(VSD)                 | 306           |
| Tetralogy of Fallot(TOF)                       | 59            |
| Atrial septal defect(ASD)                      | 84            |
| Patent ductus arteriosus(PDA)                  | 21            |
| Pulmonary atresia or stenosis(PS)              | 21            |
| double outlet right ventricle(DORV)            | 11            |
| Aortic coarctation(COA)                        | 4             |
| Pulmonary hypertension(PH)                     | 2             |
| Other complex cardiac malformations            | 192           |

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Subcellular localization

Hela cells were seeded in 12-well tissue culture plates 20 h prior to transfection at approximately 60% confluency. GFP-CITED2 expression constructs containing wild-type and mutant CITED2 were transfected using Lipofectamine 2000, according to the manufacturer’s instructions. The empty vector pEGFP-N1 was transfected as a control. Forty hours after transfection, the cells were fixed and permeabilised in 4% paraformaldehyde for 15 min, 0.1% Triton X-100 for 20 min and the DNA was stained with 0.5 mg/ml DAPI for 3 min at room temperature. The cells were observed by fluorescence microscopy. All steps were operated in lucifugal conditions.

Mammalian two-hybrid assay and transcriptional assays

Mammalian two-hybrid assay plasmids including pCMX-VP16-p300, TK promoter reporter plasmid, the Renilla luciferase control plasmid pREP7-RLu, pCMX-GALA-CITED2 (wild-type or mutant), PGL3-VEGF-pro and pcDNA3.1-HIF1A or PGL3-PITX2C-pro and pcDNA3.1-TFAP2C were co-transfected into 293T cells. Thirty hours after transfection, cells were treated the same way as above.

Statistical analysis

The results represent the means of three independent experiments performed in triplicate, and the bars denote the S.D. The independent-samples t test was adopted to determine statistical significance of unpaired samples. All data were analyzed by Prism Demo 5 software.
Results

Genetic and bioinformatics analysis

From a total of 700 non-syndromic CHD patients, we identified five novel CITED2 nucleotide alterations (two amino acid deletions and three amino acid substitutions, table3). Three mutations (c.C548T, c.A586G and c.574-59delAGCGGC) were found in one, one and four patients with Ventricular septal defect (VSD) respectively. One mutation (c.C418T) was detected in one patient with Tetralogy of Fallot (TOF) and another mutation (c.481–483delAGC) was detected in one patient with Artrial septal defect (ASD).

All potential pathogenic mutations have not been reported in the NCBI dbSNP and are not included in the 1000 Genome Project database (http://browser.1000genomes.org/).

The result of sequence alignment of CITED2 proteins among several species showed that three acid substitutions were located at highly conserved regions among different species (human, chimpanzee, mice, dog, cattle, rat, chicken and zebrafish).

Table 3. Position of variations

| Coding position | Amino acid position | Phenotype of mutation carrier |
|-----------------|---------------------|------------------------------|
| c.C418T         | p. P140S, Pro-Ser   | VSD                          |
| c.C548T         | p. S183L, Ser-Leu   | VSD                          |
| c.A586G         | p. S196G, Ser-Gly   | VSD                          |
| c.481–483delAGC | p. Ser161delAGC     | ASD                          |
| c.574–579delAGCGGC | p. Ser192_Gly193delAGCGGC | VSD                        |

Figure 1. Structure of CITED2. A: Sequence alignment of CITED2 proteins among several species. The figure showed that three acid substitutions were located at highly conserved regions among many species (human, chimpanzee, mice, dog, cattle, rat, chicken and zebrafish). B: Position of mutations in the CITED2 protein identified in CHD patients. CITED2 has three conserved regions CR1-3 and serine-glycine rich junction (SRJ). All other mutations were located in SRJ except p.P140S.

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chimpanzee, mice, dog, cattle, rat, chicken and zebrafish) and two amino acid deletions were not located at highly conserved regions among these species (Figure 1).

**CITED2 mutations decrease HIF1A repression leading to up-regulation of VEGF expression**

Two mammalian two-hybrid assays were used to evaluate whether the mutation affected the interaction between every two of **CITED2**, p300CH1 and HIF1A (Figure 2). Cotransfection of both VP16- P300 and wild-type GAL4-CITED2 with the TK promoter reporter plasmid led to a nearly 10-fold increase in luciferase activity compared with VP16-P300 and empty vector of CMX-GAL4 (t test, p<0.01). The luciferase activity of p. P140S mutant was even the same as the wt-type, However, cotransfection of VP16-P300 and the four mutants (p.S183L, p.S196G, p.Ser161delAGC, p.Ser192_Gly193delAGCGGC) GAL4-CITED2 showed weakened luciferase activity (t test, p<0.05) (Figure 2A) compared with wt-type. These findings indicated that the four mutations diminished protein-protein interactions be-

**Figure 2. Effect of CITED2 mutations on the transcriptional activation of HIF1A to its target gene VEGF.** A: Effect of mutations on CITED2-p300CH1 interactions. We cotransfected 293T cells with pCMX-VP16-p300CH1, TK promoter reporter plasmid, and the Renilla luciferase internal control plasmid, as well as empty vector pCMX-GAL4, GAL4-CITED2 wild-type, and the mutants. The significance of differences was calculated using the independent-samples t test. (*p<0.05, **p<0.01 versus wt-type, ###p<0.01 versus empty vector pCMX-GAL4.) B: Effect of mutations on HIF1A-p300CH1 interactions. Cotransfection of pCMX-VP16-p300CH1, pCMX-GAL4-HIF1A, TK promoter reporter plasmid, and the Renilla luciferase internal control plasmid, as well as empty vector pcDNA3.1 (+), pcDNA3.1 (+)-CITED2 wild-type, and the mutant. (* p<0.05, ** p<0.01 versus wt-type, # p<0.05, ### p<0.01 versus empty vector pcDNA3.1 (+)) C: Effect of wt-type on the transcriptional activation of VEGF. Transfected the VEGF reporter plasmid and the expression vector for HIF1A, CITED2 or pcDNA3.1 were transfected together in 293 T cells. The luciferase activity was normalized to Renilla activity.* p<0.05, **p<0.01 versus the untreated group (n = 3). D: Effect of CITED2 mutants on transcription activation of VEGF compared with CITED2-wt. The rest report plasmids were same as above. (*p<0.05, **p<0.01 versus wt-type, ###p<0.01 versus empty vector pcDNA3.1 (+)). The results represent the means of 3 independent experiments performed in triplicate and the significance of differences was calculated using independent-samples t test. (CITED2 = Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2, HIF1A = Hypoxia Inducible Factor 1, VEGF = vascular endothelial growth factor)

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Figure 3. Effect of CITED2 variants on the cooperation between CITED2 and TFAP2C in the transactivation of the PITX2C. A: Effect of CITED2 mutations on the transcriptional activation of PITX2C. (p<0.05, **p<0.01 versus wt-type, #p<0.05, ##p<0.01 versus empty vector pcDNA3.1(+)). B: CITED2-wt and TFAP2C working on the transcriptional activation of PITX2C. PITX2C reporter plasmid and the expression vector for TFAP2C, CITED2, or pcDNA3.1 alone were transfected respectively in 293 T cells. The luciferase activity was normalized to Renilla activity. (t test, p<0.05, **p<0.01 versus the untreated group (n = 3)).

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between p300 and CITED2, but the p.P140S mutant didn’t alter the interactions.

Another mammalian two-hybrid assay was operated and analyzed to further evaluate whether the repression of HIF1A - p300 complex was influenced by CITED2 mutation. The result showed that the luciferase activity of wt-type was only 60% of the control (t test, p<0.01) (Figure 2B). Compared with wild-type, the luciferase activity of mutants increased obviously except the p.P140S mutant. In conclusion, CITED2 mutations weaken the HIF1A repression by diminishing the protein-protein interactions between p300CH1 and CITED2 on the one hand and by enhancing the interactions between p300CH1 and HIF1A on the other hand.

As HIF1A can induce vascular endothelial growth factor (VEGF) potently, we supposed that CITED2 mutations influenced the transcription of VEGF through their effect on HIF1A. This was confirmed by our dual luciferase assay (Figure 2C). Wild-type CITED2 caused an approximately 32% decrease of activity compared with the control (t test, p<0.01). P140S showed no difference with wild-type in luciferase activity. As for the other four mutants, Ser161delAGCAGC showed an observable promotion of VEGF-promoter resulting in higher luciferase activity than wild-type (t test, p<0.01) and the rest mutants showed few differences compared with wt-type (t test, p<0.05) (Figure 2D).

CITED2 mutations impair TFAP2C coactivation resulting in abnormal transactivation of PITX2C

As a transcriptional coactivator of TFAP2, CITED2 influenced cardiac left-right patterning by regulating the left-right patterning Nodal-PITX2C pathway. PITX2C is a critical gene of the Nodal-PITX2C pathway and controls the location of heart and intestines in embryo. Our study showed that CITED2 mutations resulted in decreased luciferase activity of PITX2 by diminishing the coactivation of CITED2 and TFAP2C. The luciferase activity of three mutants were decreased obviously compared with wt. (p.P140S vs. wt-type 80% (t test, p<0.01), p.S183L vs. wt-type 85% (t test, p<0.01), p.Ser192_Gly193delAGCGGC vs. wt-type 92% (t test, p<0.01)) (Figure 3A). The rest two mutants coactivated TFAP2C to the same level as wt-type.

In addition, we designed another test to prove the TFAP2 coactivation with CITED2. The result showed that cotransfection of empty vector of pcDNA3.1 (+) with the luciferase reporter PGL3-PITX2C-pro was the lowest in all groups including pcDNA3.1-TFAP2C or wt-type pcDNA3.1-CITED2 only and both of them (Figure 3B).

In conclusion, CITED2 mutations contributed to the abnormal transactivation of PITX2C.

Impact of CITED2 mutations on Subcellular Localization

To further study whether the functional changes are caused by changed subcellular localization of the protein, the transfections were performed using N-terminal GFP fusion constructs of wt and mutant CITED2, followed by fluorescence microscopy. The result indicated that the effects of CITED2 mutations on VEGF and PITX2C were not caused by the incorrect localization of the protein. Whether in wt or mutant of CITED2 the proteins were discovered mainly in nucleus and a lesser degree in the cytoplasm of Hela (Figure S1).

Discussion

Previous researches of cited2-/-mice confirmed that cited2 plays a critical role in the development of heart and is essential for the normal creation of the left–right axis. Cited2-/- embryos showed a series of cardiac malformations such as VSD, ASD, outflow tract abnormalities and abnormal heart looping.

We screened the coding region and splice sites of the CITED2 gene in 700 Chinese CHD patients. Two potential pathogenic amino acid deletions (p.Ser161delAGCAGC and p.Ser192_Gly193delAGCGGC) and three potential pathogenic amino acid substitutions variants (p. P140S, p. S183L and p. S196G) were identified. These three regional highly conserved substitutions (conserved among Humans, chimpanzee, mice, dog, cattle, rat, chicken and zebrafish) were not identified in control group or the

NEW CITED2 MUTATIONS LINKS TO CHD

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variants of databases. Therefore, we supposed that these three mutations were possibly causative. Since, SRJ region is a research hot spot at present, the two potential pathogenic amino acid deletions in our study were found in SRJ region. As a result, the necessity of this study is highlight. Although the CHD phenotype was not seen in SRJ-deficient mice, as observed in mutant carrying patients, we supposed that this could be due to species differences [23,24] in the function of CITED2, or some other unidentified factors[25] might interact with CITED2 and modify its phenotype. Alternatively, it is also possible that CHD were present earlier in life but spontaneously closed at a later time in SRJ-deficient mice.

Mammalian two-hybrid analysis permits the semi-quantitative assessment of protein-protein interactions occurring within living cells. Cotransfection of wt or mutant CITED2 and p300CH1 in 293T cells, the binding between CITED2 and p300CH1 activated the TK reporter gene activation expression in vivo. The functional study greatly supported the hypothesis that the mutations are causative and might affect the formation of heart. The last four mutated proteins (p. S183L, p. S196G, p.Ser192 Gly193delAGCGGC and p.Ser192 Gly193delAGCGGC) showed significantly decreased reporter gene activation ability compared with wt-type. However, an opposite phenomenon occurred by transfecting p300CH1, HIF1A and wt or mutant CITED2 together in cells. Taken together, the results indicated that the four mutated proteins decreased the interaction between CITED2 and p300CH1 compared with wt-type, causing a weakened competitive binding to p300 CH1 of CITED2. The increased interaction between HIF1A and p300CH1 could up-regulate the promoter activity of VEGF according to our dual luciferase experiment.

Our study also showed that three mutations decreased the consociation of TFAP2C and CITED2 in the transactivation of ptx2e, an essential gene of the left–right axis establishment confirmed in mice and chick embryo. The mice experiments already indicated that knocking out ptx2e gene can lead to valve defects, body wall dysraphism, gastrochisis, ectopia cordis and other multiple organs polymorphous defects. In addition, there was no evidence that CITED2 mutations were involved in the incorrect location of the protein in the subcellular localization experiment.

In conclusion, we identified five novel human mutations among 700 CHD patients by screening the coding region and splice sites of the CITED2 gene. To confirm our hypothesis that the mutations were pathogenic, we investigated the function and mechanism of them. Our study revealed that four mutations influenced the transcription regulatory properties of VEGF and three mutations reduced costimulation capacity to promote PITX2C. Further research showed that four CITED2 mutations recovered the promoter activity of VEGF [26] caused by its decreased competitiveness with HIF1A to bind the p300CH1. Furthermore, three mutations also decreased the consociation of TFAP2C and CITED2 in the transactivation of PITX2C. Our study confirmed that CITED2 is a disease-causing gene of CHD and its mutations can result in the cardiac malformations.

Supporting Information

Figure S1 Subcellular localization of CITED2. Localization of wild-type and mutant CITED2 GFP-fusion protein in transfected Hela cells were observed by fluorescent microscope. The empty vector pEGFP-N1 was transfected as a control. All figures were drawn by fluorescence microscopy and Adobe Photoshop CS5. (TIF)

Author Contributions

Conceived and designed the experiments: XM HP YL. Performed the experiments: YL. Analyzed the data: YL XZ. Contributed reagents/materials/analysis tools: FW YW ST QW JW XZ CL. Wrote the paper: YL.

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