Role of Whole-exome Sequencing in Phenotype Classification and Clinical Treatment of Pediatric Restrictive Cardiomyopathy

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Abstract

Background: Restrictive cardiomyopathy (RCM) is the least common cardiomyopathy in which the walls are rigid and the heart is restricted from stretching and filling properly. Cardiac troponin I (cTnI) mutation-caused myofibril Ca²⁺ hypersensitivity has been shown to be associated with impaired diastolic function. This study aimed to investigate the linkage between the genotype and clinical therapy of RCM.

Methods: Five sporadic pediatric RCM patients confirmed by echocardiography were enrolled in this study. Whole-exome sequencing (WES) was performed for the cohort to find out candidate causative gene variants. Sanger sequencing confirmed the WES-identified variants.

Results: TNNI3 variants were found in all of the five patients. R192H mutation was shared in four patients while R204H mutation was found only in one patient. Structure investigation showed that the C terminus of TNNI3 was flexible and mutation on the C terminus was possible to cause the RCM. Catechins were prescribed for the five patients once genotype was confirmed. Ventricular diastolic function was improved in three patients during the follow-up.

Conclusions: Our data demonstrated that TNNI3 mutation-induced RCM1 is the most common type of pediatric RCM in this study. In addition, WES is a reliable approach to identify likely pathogenic genes of RCM and might be useful for the guidance of clinical treatment scheme.

Key words: Pediatric Restrictive Cardiomyopathy; Phenotype Classification; TNNI3; Whole-exome Sequencing

INTRODUCTION

Cardiomyopathy includes five original subtypes referred as hypertrophic, dilated, restrictive, arrhythmogenic right ventricular, and unclassified types.1 Restrictive cardiomyopathy (RCM) is the least common of cardiomyopathy characterized by impaired ventricular filling.2 RCM includes five subtypes on the basis of genotype. It is well documented that RCM1, RCM3, RCM4, and RCM5 are caused by mutations which lead to isoform of troponin I (TNNI3), troponin T, myopalladin, and filamin-C, respectively.3,4 RCM2 mapped on mutations in the gene coding region of chromosome 10q23.3.5 Besides these identified sites, RCM is suspected of having other pathogeneses as some RCM patients have no mutations among these identified gene locations.

Up to now, there is no effective treatment scheme for the patients with RCM due to its low prevalence and there is just few research with regard to RCM.6,9 In this study, we present the diagnosis and treatment of five clinically diagnosed sporadic pediatric RCM patients who had taken whole-exome sequencing (WES).

METHODS

Ethical approval

This study was approved by the Ethics Committee of Beijing Anzhen Hospital. Informed consent was obtained from parents of the cohort in this study.

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Patients
From October 2013 to May 2016, a total of 16 patients of unrelated pediatric RCM patients were admitted to Beijing Anzhen Hospital. A clinical diagnosis of idiopathic RCM was confirmed by transthoracic echocardiography (TTE) and other clinical characteristics. The inclusion criteria included pediatric patients without family history of RCM, patients who were diagnosed as idiopathic RCM during the first visit of hospital, patients without constrictive pericarditis caused by tuberculosis or other systemic diseases, patients who had no parasitic infection and eosinophilia, and patients who had taken WES with definite mutations. Finally, five patients (two males and three females) were enrolled in this study due to having been confirmed as idiopathic RCM by both clinical characteristics and WES. They were aged between 5 and 12 years old (mean age: 9 ± 3 years). Clinical data including medical history (onset time, age at treatment, and clinical symptoms), physical signs, results of the diagnostic examination, treatment strategy, and curative effect were collected. Clinical cardiac function improvement was defined as New York Heart Association (NYHA) functional classification increased by at least one level. The improvement of left ventricular diastolic function by TTE was defined as the reduction of velocity of early filling mitral flow/early diastolic tissue Doppler imaging mitral annular velocity (E/E') >10%.

Physical and diagnostic examination
Physical and diagnostic examinations were performed for all patients, including routine blood test (containing eosinophil counts and percentage), liver and kidney function, myocardial enzymes, cardiac troponin I (cTnI), brain natriuretic peptide (BNP), erythrocyte sedimentation rate, C-reactive protein, antituberculosis antibody, antinuclear antibodies, antiphospholipid antibodies, coagulation function, screening of inherited metabolic diseases, serum transferrin saturation, and antiparasite antibody. All patients underwent tuberculosis tests to rule out tuberculosis infection. In addition, TTE, electrocardiogram (ECG), chest X-ray, and cardiac magnetic resonance imaging were carried out.

Clinical diagnosis of RCM was confirmed by two-dimensional (2D) color Doppler echocardiography. Diagnostic criteria were as follows: (1) marked enlargement of left and right atrium; (2) normal ventricular chamber size, normal or slightly thickened ventricular wall; (3) normal or less decreased ventricular systolic function; and (4) impaired ventricular diastolic function. TTE was performed according to the recommendations by American Society of Echocardiography.

Indicators for assessing diastolic function included E deceleration time (EDT), late filling mitral flow (A), ratio of E to A (E/A), E' and mitral E/E’.[10] Left ventricular diastolic dysfunction was defined as mitral EDT <150 ms, mitral E/A >2, and average mitral E/E’>13 (average of the E/E’ from septum and LV lateral wall).

Whole-exome sequencing
DNA was extracted from 2 ml of peripheral blood following the instruction of BloodGen Midi Kit (CWBI0, Beijing, China), sheared by sonication after detected by agarose gel electrophoresis, and then hybridized with NimbleGen 2.0 probe sequence capture array (Roche, Basel, Switzerland). Captured DNA was first applied for exonic DNA enrichment. The libraries were then tested for enrichment by quantitative polymerase chain reaction (PCR), and also for size distribution and concentration by the Agilent Bioanalyzer 2100 (Agilent Technologies Inc., Santa Clara, USA). The samples were thereby sequenced by Illumina Hiseq 2500 (Illumina, Santiago, USA).

Raw image files were processed by the BclToFastq (Illumina, Santiago, USA) for base calling and raw data generating. The low-quality variations were ruled out if quality score ≥20 (Q20). The sequencing reads were aligned to the National Center for Biotechnology Information (NCBI) human reference genome (hg19) using BOADICEA Web Genome Analysis Toolkit was used to analyze single-nucleotide polymorphism and insertion-deletion of the sequences.[11] The reported cardiovascular diseases-associated genes were analyzed. In addition, as mitochondria mutations could also induce cardiomyopathy, the known mitochondriopathy-related genes were assessed as well. All samples were annotated using ANNOVAR and the candidate variants were based on frequency and function.[12] A 0.5% cutoff of frequency estimated from the 1000 genomes project database was applied. Protein biological function was predicted using PROVEAN and PolyPhen-2.[13,14]

Sanger sequencing validation
Sanger sequencing was used to confirm the variants identified in both the patients and their parents. PCR primers for TNNI3 used in this study were 5'-ATAAGAAGAGAAAGAAGGAAGGAC-3' and 5'-TCAATAACACAGCCAAGAGT-3', producing a PCR product with the length of 608 bp. The PCR products were sequenced by ABI 3730XL (Thermo Fisher Scientific Inc., Waltham, MA, USA) and analyzed by DNASTAR 5.0 software (DNASTAR, Inc., Madison, WI, USA).

Medication
Once the patients were diagnosed as RCM, all of them were started with medical treatment immediately. All patients received routine treatment consisted of oral diuretics, vasodilators, and small doses of calcium antagonists. For patients with TNNI3-192 site mutation, oral administration of catechin was prescribed according to Zhang L et al.[15] The initial dose of catechin was 15 mg·kg$^{-1}$·day$^{-1}$ and was gradually added to 50 mg·kg$^{-1}$·day$^{-1}$, within 6 months if patients could tolerate.

Results
General information
To understand the general condition of the five patients, clinical data were collected. Clinical symptoms included fatigue, exercise intolerance, exertional asthma, respiratory infection,
tightness in the chest, and lower extremity edema [Table 1]. The initial onset age was from 3 to 6 years old, with an average of 5 years old. Time between onset and the first visit was 1–4 years (median: 2 years), and the follow-up time ranged from 3 to 35 months (mean: 17 ± 12 months). To date, one female patient (13 years old) has received heart transplant surgery, another 11-year-old girl died of sudden death after exercise, and others were still followed up continually.

To gain insight into the clinical changes of the cohort, conventional diagnostic examination was performed. As displayed in Table 2, two patients were with mild elevated liver enzyme whereas renal function was normal in all patients. Abnormal myocardial enzyme was found in two cases, increased cTnl was discovered in one patient, and significantly increased BNP was detected in all five patients. Other laboratory tests were normal in all the patients. As for medical device testing, no special diagnostic information for RCM occurrence was provided by ECG, X-ray, and cardiac magnetic resonance.

**Echocardiography**

2D TTE was performed to confirm the diagnosis of RCM. As illustrated in Figure 1a, dilation of left and right atriums with normal ventricular size was detected. The velocity of mitral peak E and peak A decreased significantly, E/A > 2, and the variation rate of E peak flow with suction was less than 25% [Figure 1b]. Isovolumetric relaxation time was shorter than 70 ms, and the deceleration time of peak E reduced significantly (<150 ms). Inferior vena cava broadened and the subsidence rate reduced. Echocardiography estimated average mitral E/E’ > 13.

**TNNI3 variants showed in whole-exome sequencing**

In general, the Q20 was more than 95% and quality control files demonstrated that the data were adequate for further analysis. **TNNI3** was found in all the patients. **R192H** mutation on **TNNI3** was found in four individuals whereas **R204H** mutation on **TNNI3** was found in one patient.

**De novo mutations**

Sanger sequencing was performed in the family trios using double orientation primers. The results are illustrated in Figure 2. Of note, all the five **TNNI3** mutations were **de novo** germline mutation, clarifying that their parents are healthy.

**Clinical outcome**

One patient of 13-year-old girl received heart transplant after taking catechin for 3 months due to her very serious condition. Another 11-year-old girl died of “sudden death” after taking catechin for 9 months. The other three patients survived with long-term regular follow-up. The dose of catechin was added to mean 50 mg·kg<sup>−1</sup>·day<sup>−1</sup> in half a year for the three patients. Mean follow-up range in these patients was more than 1 year, and the longest follow-up time was 27 months. The NYHA functional classification in survived patients improved from Class III to Class II [Table 3]. TTE showed that mean E/E’ decreased from 18.4 ± 4.6 to 11.2 ± 4.6, suggesting that the TTE findings were consistent with the heart function recovery [Figure 3].

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### Table 1: General clinical data of five sporadic pediatric restrictive cardiomyopathy patients

| Characteristics | Patient number |
|-----------------|---------------|
| Gender          | 1 2 3 4 5     |
| Age (years)     | Male 12 Female 8 Male 12 Female 5 Female 10 |
| Symptoms        | Fatigue + + + + + + Exercise intolerance + + + + + + Short of breath + + + + – + Signs | Edema – – – – – Jaundice – – + – – Hepatomegaly + + + + + + Ascites – – ++ – – Heart murmur – – + – – |
| +/-            | +: Positive; ++: Significant positive; –: Negative. |

### Table 2: Diagnostic examination of five sporadic pediatric restrictive cardiomyopathy patients

| Examinations | Patient number |
|--------------|---------------|
|              | 1 2 3 4 5     |
| CTnl (ng/ml) | 0.01 0.01 0.07 0.03 0.05 |
| CK-MB (mmol/L) | 17 14 16 20 13 |
| CRP (mg/L)   | 0.23 0.2 10.93 0.85 0.25 |
| BNP (ng/L)   | 1646.5 899 4869 525.3 1187 |
| ECG          | Enlargement of atrium + + + + + ST-T segment change + + – + + AF – – – – – SVT – – – – – UCG – – – – – |
| CMRI         | RCM RCM RCM RCM RCM |
| CTnl: Cardiac troponin I; CK-MB: Isoenzyme of creatine kinase; CRP: C-reaction protein; BNP: Brain natriuretic peptide; ECG: Electrocardiograph; STV: Supraventricular tachycardia; AF: Atrial fibrillation; UCG: Ultrasonic cardiogram; LVRF: Left ventricular restrictive filling; TP: Thickened pericardium; PH: Pulmonary hypertension; RCM: Restrictive cardiomyopathy. CMRI: Cardiac magnetic resonance imaging.

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**Figure 1**: Echocardiography of restrictive cardiomyopathy. (a) Bialtrial enlargement, with normal ventricular chamber size; (b) peak E wave velocity, peak A wave velocity decreased significantly, and E/A > 2. E: Velocity of early filling mitral flow; A: Velocity of late filling mitral flow.
RCM is a rare cardiomyopathy that can occur at any age during childhood.\textsuperscript{[16–19]} Progress on the treatment of the RCM was limited for the infrequent feature of the disease. A total of five sporadic RCM patients from different regions around China were enrolled in the present study. In this study, WES was performed and the results showed variants of TNNI3 in the patients. The variants could induce R192H (four patients) and R204H (one patient) mutations, which all have been reported by previous studies.\textsuperscript{[20,21]} In line with this, the mutations were considered as the pathogenesis. Four of the five sporadic patients had the same mutation site (R192H), indicating that R192H might be the main mutation pattern for patients around China.

To confirm the possibility that TNNI3 variants are the likely the etiology of RCM, structural investigation was performed. C terminus of TNNI3 is defined as “mobile domain” indicating that this domain is quite flexible.\textsuperscript{[22]} We hypothesized that TNNI3 might play a role in conformational changes. Therefore, to further investigate the mechanism of TNNI3 for RCM occurrence, structural investigation was performed. To date, there is no structure covering the sequence of extreme C terminus of human TNNI3; therefore, the structure of Gallus gallus was applied.\textsuperscript{[23]} As for human TNNI3 determination, human cardiac troponin in the Ca\textsuperscript{2+} saturated form (1j1e) was enrolled.\textsuperscript{[22]} Before structure investigation, sequence conservation alignment was carried out, and as illustrated in Figure 4a, the sequence of G. gallus TNNI3 is almost identical to that of human. The result argued that it is possible to use the TNNI3 structure of G. gallus to represent human TNNI3 structure. Furthermore, structure prediction showed that the secondary structure of TNNI3 is composed by two α helices connected by a loop, which is in accord with 1j1e. However, structure from G. gallus showed that the C terminus is composed by two α helices that comprising two connecting short β sheets, indicating that the C terminus has different conformations under different physiological states [Figure 4b]. Together, the result demonstrated that C terminus of TNNI3 is flexible and conformational variable, which might play an important role in the function execution of protein TNNI3.

To date, there was no specific treatment for patients with RCM. The routine treatment contains oral diuretics, vasodilators, and calcium antagonists. However, the effect is poor. Several studies\textsuperscript{[24–26]} have constructed RCM transgenic mouse lines expressing cTnI R193H mutation in the heart and showed that cTnI mutations could induce specific diastolic dysfunction. In addition, they argue that Ca\textsuperscript{2+} is highly linked to impaired relaxation in myocardial cells. Furthermore, they demonstrate that catechin is effective in reverse diastolic dysfunction and could be useful for the troponin

### Table 3: Changes of cardiac function and prognosis before and after administration of catechin

| Parameters                  | 1   | 2   | 3   | 4   | 5   |
|-----------------------------|-----|-----|-----|-----|-----|
| Weight (kg)                 | 42  | 25  | 32  | 16  | 30  |
| Catechin (mg/d)             | 1600| 1600| 800 | 725 | 400 |
| NYHA                        |     |     |     |     |     |
| Precatechin                 | III | III | VI  | III | III |
| Postcatechin                | II  | II  | I   | II  | II  |
| Follow-up (months)          | 15  | 35  | 3   | 18  | 9   |
| Prognosis                   | S   | S   | HT  | S   | SD  |

HT: Heart transplant; S: Survival; SD: Sudden death; NYHA: New York Heart Association Functional Classification.

**DISCUSSION**

RCM is a rare cardiomyopathy that can occur at any age during childhood.\textsuperscript{[16–19]} Progress on the treatment of the RCM was limited for the infrequent feature of the disease. A total of five sporadic RCM patients from different regions around China were enrolled in the present study. In this study, WES was performed and the results showed variants of TNNI3 in the patients. The variants could induce R192H (four patients) and R204H (one patient) mutations, which all have been reported by previous studies.\textsuperscript{[20,21]} In line with this, the mutations were considered as the pathogenesis. Four of the five sporadic patients had the same mutation site (R192H), indicating that R192H might be the main mutation pattern for patients around China.

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mutations-induced RCM. In this study, application of large doses of catechin (50 mg·kg⁻¹·day⁻¹) was performed and it was effective in improving diastolic function and clinical symptoms. However, catechin could neither cure RCM nor prevent sudden death. Comparing with the traditional calcium antagonists, catechin can reduce myocardial calcium hypersensitivity, which is similar to calcium antagonists. However, catechin has no obvious adverse effect such as blood pressure reduction and cardiac rhythm reduction. In line with this, we consider that catechin should be deserved promotion and application in clinic.

In conclusion, five sporadic RCM pediatric patients were enrolled and had undertaken WES. TNNI3 mutation was found in all the patients and R192H mutation was shared by four individuals. Structure investigation indicated that the C terminus of TNNI3 was flexible and the mutations were possible to induce the onset of RCM. WES played an important role in the diagnosis of genotype and clinical treatment of RCM. Catechin therapy was effective in most of the patients in our group.

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Conflicts of interest
There are no conflicts of interest.

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