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WHOLE EXOME SEQUENCING IDENTIFIES VARIANTS IN THE *TNNI3* GENE IN VIETNAMESE PATIENT WITH RESTRICTIVE CARDIOMYOPATHY - A CASE REPORT

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ABSTRACT

Restrictive cardiomyopathy (RCM) is a rare heart muscle disease in which the heart wall is rigid leading to diastolic dysfunction caused by abnormal elastic properties of the myocardium and/or intercellular matrix. The prognosis is generally poor, and RCM has a high mortality rate in pediatric patients. There are no curative treatments for RCM, so cardiac transplantation is the only effective treatment. Diagnosis RCM, clinical diagnosis can be challenging because clinical presentations and imaging manifestations of RCM are similar to other cardiomyopathies so it requires other specific diagnoses. Currently, pathogenic mutations in 22 different genes have been identified in patients with RCM. Identifying mutations in these genes helps discriminate RCM from other cardiomyopathies. Besides, next-generation sequencing (including whole genome sequencing, whole exome sequencing,...) has provided an effective tool for simultaneously analyzing mutations in many different genes.

In this study, we conducted sequencing of the entire gene coding region (WES) in the patient and identified a compound heterozygote variants (c.289C>G, p.Arg97Gly and c.433C>T, p.Arg145Trp) in the *TNNI3* gene. These variants were inherited from the patient's father and mother, who were heterozygous variant carriers. These variants were also identified as the pathogenic variants in the ClinVar database (accession number VCV001331910.2 and VCV000012426.28, respectively) and were the cause of the patient's disease. Our results suggest that WES can be used to definitively diagnose the genetic variants associated with RCM and show that genetic screening is essential for families of RCM patients.

Keywords: Mutation, Restrictive cardiomyopathy (RCM), *TNNI3* gene, Vietnamese patient, Whole exome sequencing (WES).

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INTRODUCTION

Restrictive cardiomyopathy (RCM) is a rare cardiac disorder that manifests primarily as an abnormality of diastolic filling due to the reduced expansion or increased stiffness of the ventricular (Huang & Du, 2004; Muchtar et al., 2017). RCM is characterized by severely enlarged atria, and normal-sized ventricles, with increased myocardial stiffness leading to impaired ventricular filling and diastolic dysfunction (Hayashi et al., 2018). Patients present with symptoms of left and/or right ventricular heart failure with preserved ejection fraction (HFpEF), atrial fibrillation, ventricular arrhythmias, and frequent conduction disorders (Seferovic et al., 2019). RCM is a rare cardiomyopathy with an unknown prevalence (Muchtar et al., 2017) but accounts for approximately 5% of all cases of primary cardiomyopathy (Wang et al., 2017). In RCM patients, the heart muscle is stiff and unable to fully relax after each contraction. Most patients with RCM have severe symptoms such as dyspnea, fatigue, and limited exercise capacity. In children, RCM can present with developmental delays, fatigue, dyspnea, pulmonary edema, and even fainting (Chen et al., 2001; Russo & Webber, 2005). To date, there are no specific therapies for RCM patients, only medical treatments to reduce volume overload as well as anticoagulation and antiarrhythmic therapy for heart failure. The overall prognosis is poor and the 5-year survival rate of adult patients with a confirmed genetic cause was 56% (Kubo et al., 2007). Several studies have reported that 66–100% die (Bicer et al., 2015), especially in children, despite optimal medical treatment (Kucera & Fenton, 2017). Children with RCM exhibit rapid disease progression and high mortality (50% survive within the first two years after diagnosis) (Webber et al., 2012; Wittekind et al., 2019). Mogenson & Arbustini (2009) suggest that children with RCM are at high risk of ischemia (with signs of blood columns, scales, and lizards) even when there are no signs of heart failure. Heart transplantation (HTx) is the only option to prolong the patient's life (Kaski et al., 2008; DePasquale et al., 2012; Rivenes et al., 2015; Kucera & Fenton, 2017).

Diagnosis of restrictive cardiomyopathy is often very difficult because, in the early stages, the disease presents few symptoms. In addition, the diverse clinical and overlap with other cardiomyopathy is also a diagnostic challenge (Tariq, 2014). Family history and clinical manifestations require careful consideration and are integral to the definitive diagnosis of RCM. In 2003, Mogensen and colleagues firmly established that troponin I mutations were etiological causes of RCM (Mogensen et al., 2003). The mutations in genes encoding sarcomeric and cytoskeletal proteins, including *ACTC, ACTC1, ACTN2, BAG3, CRYAB, DCBLD2, DES, FLNC, LMNA, MYH, MYH7, MYL2, MYL3, β-MHC, MYBPC3, MYPN, TMEM87B, TNNI3, TNNT2, TNNC1, TPM1* and *TTN,* that have been reported to be etiologically linked to RCM (Muchtar et al., 2017; Cimiotti et al., 2021; Brodehl & Gerull, 2022). All known RCM genes are localized on autosomes and in most cases, the mutations are inherited as autosomal dominant mode or occur as *de novo* mutations. However, there are also some cases of a recessive inheritance pattern (Brodehl et al., 2019). Thus, it has been suggested that genetic screening of genes encoding sarcomeric proteins could be an important tool to clinically diagnose RCM (Mouton et al., 2015).

The majority of RCM genes encode sarcomere, cytoskeleton, and Z-disc proteins in the heart muscle structure such as cardiac troponin, desmin, and filamin-C. Among them the troponin complex subunits T (TNNT2 - Troponin T/cTnT) (Menon et al., 2008) and I (TNNI3 - Troponin I/cTnI) (Parvatiyar et al., 2010; Mogensen et al., 2015; Pantou et al., 2019) have been associated with hereditary RCM. The *TNNI3* gene, which is a 5966-bp gene located on chromosome 19, consisting of eight exons and encoding a 210-amino acid protein (Vallins et al., 1990; Bhavsar et al., 1996). Almost all mutations are located in the regulatory C-terminal region interacting with actin and the N-terminal domain of TNNC1. Notably, these mutations are concentrated in

specific regions of cTnI that participate in interactions thin-strand. These mutations may lead to cTnI depletion or mutations that have affected the binding of cTnI to the thin filament. In any case, reduced cTnI content can lead to alter interactions of cTn with the actin-tropomyosin complex thus leading to severe disturbances of diastolic function (Kostareva et al., 2009). Variants of *TNNI3* are now to be identified as the cause of RCM in the majority of young patients (Mogensen & Arbustini, 2009; Ding et al., 2017).

CASE PRESENTATION

Clinical presentation

A 10-year-old boy was admitted to Hanoi Heart Hospital due to difficulty breathing during vigorous exercise and swollen eyelids. Upon admission to the hospital, the child was alert but very tired, breathing rapidly, contracting respiratory muscles, heart rate 120 beats/min, blood pressure 100/50 mmHg, a 3/6 systolic murmur at the apex of the heart. The patient urinated less, eyelid swelling and dorsum of the legs, liver enlargement 3 cm below the right costal margin, hepatic jugular vein feedback (+). The child was prescribed tests and results: WBC 10 G/L, CRP 20 mg/L, Albumin 35 g/L, GOT 50 U/L, GPT 55 U/L. The echocardiography showed greatly dilated atria, non-dilated ventricles, normal systolic function, grade 3/4 mitral regurgitation, grade 2/4 tricuspid regurgitation, left ventricular diastolic failure (Figs. 1A, B). Magnetic resonance imaging of the heart and large blood vessels showed no images of pericardial thickening, no signs of pericarditis, no structural malformations of the heart and large extracardiac blood vessels, and normal coronary artery origin (Fig. 1C). Family history: the patient has an older sister who died at age 13 due to severe heart failure/cardiomyopathy and an older brother who died at age 5 due to severe heart failure of unknown cause (Fig. 2). The patient was diagnosed with restrictive cardiomyopathy and underwent WES sequencing to find the genetic cause associated with the disease.

Ethical approval

This study was approved by the Institute of Genome Research Institutional Review Board (No: 02-2021/NCHG-HĐĐĐ), all methods were carried out in accordance with relevant guidelines and regulations. Informed consent was obtained from the parents of the pediatric patients.

Molecular investigation

Genomic DNA was extracted from peripheral blood samples using the Qiagen DNA Blood Mini kit (QIAGEN, Hilden, German) according to the manufacturer's instructions. DNA concentration and purity were analyzed using a Thermo Scientific Nanodrop spectrophotometer (Waltham, MA, USA). The library was prepared with SureSelect V7-Post kit (Agilent Technology, CA, USA) following manufacturer guidelines. Whole exome sequencing (WES) was performed with the following procedure to target region capturing by Next Generation Sequencing (Illumina, CA, USA). The paired-end reads were mapped to the reference human genome GRCh38 using BWA0.7.17 (Li & Durbin, 2009). Picard tool (http://broadinstitute.gith-ub.io/picard/) was used to process post-alignment data including creating indexes, marking, removed repeated reads on the alignment bam file. Variants calling were performed by HaplotypeCaller in the GATK package version 4.1 (van der Auwera et al., 2013). Variants in RCM-associated genes were screened to identify potentially pathogenic variants based on a minor allele frequency < 0.01.

The Sanger sequencing method was used to validate the results in the patient and the patient's family members. PCR amplification was carried out on an Eppendorf Mastercycler EP gradient (USA Scientific, Inc). PCR products were analyzed using Sanger sequencing with the ABI 3500 Genetic Analyzer machine (Thermo Fisher Scientific Inc., Waltham, MA, USA) and further analyzed using the BioEdit 7.2.5 Software.

The results of WES sequencing showed that a compound heterozygote variants, c.289C>G

(p.Arg97Gly) and c.433C>T (p.Arg145Trp) in the *TNNI3* gene (NM_000363.5), was identified in the patient. The variant c.289C>G (p.Arg97Gly) has been reported in the dsSNP databases under accession number rs730881068. This variant had a low frequency (0.00002) on the database of 1,000 Genome and was identified as a pathogenic variant in the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar) (accession number VCV001331910.2). The variant c.433C>T (p.Arg145Trp) (with a frequency of 0.00001 on the database of 1,000 Genome) has been submitted in dsSNP under accession number rs104894724 as well as identified as a pathogenic variant in the ClinVar database (https://www.ncbi.nlm.nih.gov/cl-invar) (accession number VCV000012426.28).

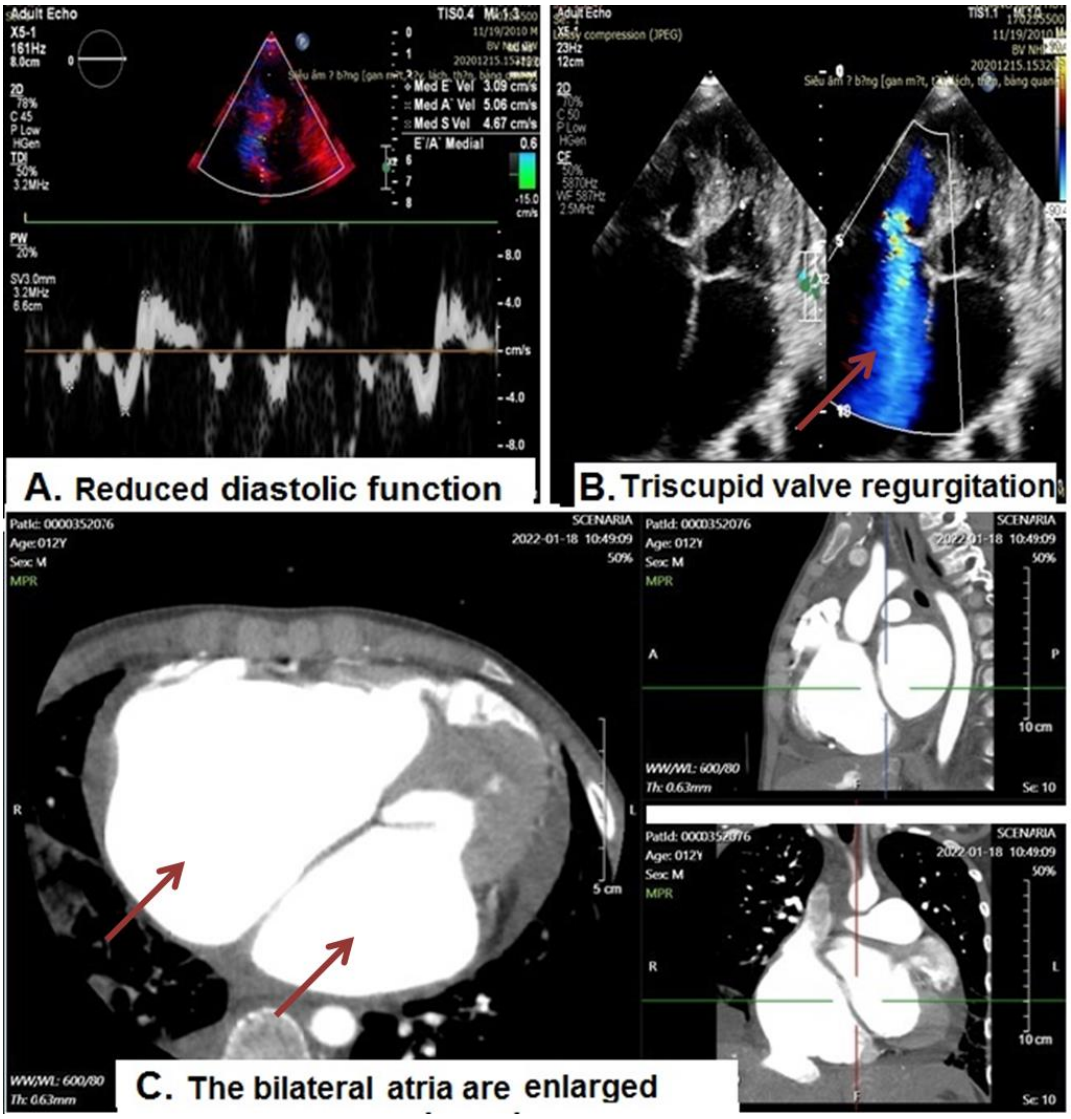

Figure 1. A, B: The echocardiography showed greatly dilated atria, non-dilated ventricles, normal systolic function, grade 3/4 mitral regurgitation, grade 2/4 tricuspid regurgitation, left ventricular diastolic failure; C: Magnetic resonance imaging of the heart and large blood vessels showed no images of pericardial thickening, no signs of pericarditis, no structural malformations of the heart and large extracardiac blood vessels, and normal coronary artery origin

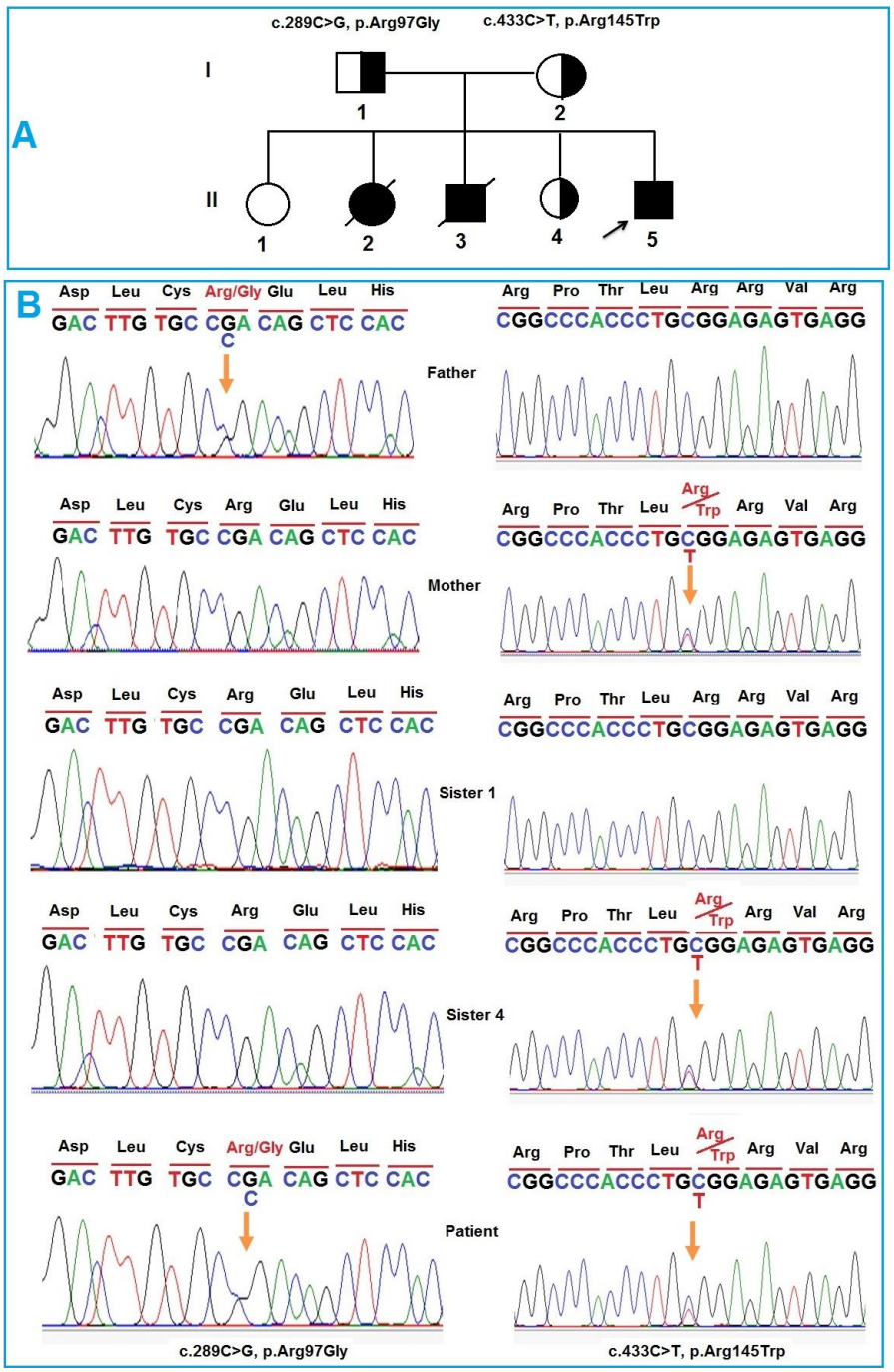

Figure 2. A: Pedigree of the patient's family. The pedigree of the patient's family including parents, an older sister who died of RCM disease in 13-years old, an older brother who died of in 5-years old, two healthy sisters, one of whom carries a heteozygous variant, and the patient. B: The Sanger sequencing results of the members in the patient's family. The results show that the patient's parents and an older sister carry a heterozygous variant in the *TNNI3* gene. The patient bears a combination of heterozygous variants c.289C>G (p.Arg97Gly) and c.433C>T (p.Arg145Trp) in the *TNNI3* gene

The Sanger sequencing results showed that the patient has inherited a compound heterozygote variants from both of the parents, and an older sister of the patient carried the variant c.433C>T (p.Arg145Trp) in heterozygous status in the *TNNI3* gene (Fig. 2). Members of the patient's family who carry only one variant in the heterozygous state do not show symptoms of the disease. This result suggests that the combination of two heterozygous variants in the *TNNI3* gene are the cause of the disease in the patient.

DISCUSSION

Previous studies show that genetically based RCM is a very rare but serious disease with high mortality, which can be caused by genetic variants of nonsarcomeric, sarcomeric and sarcomereassociated proteins. Restrictive cardiomyopathy is a form of inherited cardiomyopathy that have been identified as being caused by variants in the gene encoding the sarcomere protein. Several gene variants have been confirmed to cause RCM and *TNNI3* appears to be the most common causative gene in RCM (Menon et al., 2008; van den Wljngaard et al., 2011; Mogensen et al., 2015; Kostareva et al., 2016; Muchtar et al., 2017; Pantou et al., 2019; Wong et al., 2019; Ueno et al., 2021). To date, there is no specific treatment for RCM. In most reported cases, the prognosis of RCM is extremely poor (Xiaohui et al., 2011; Seferovic et al., 2019). The main treatment includes managing heart failure symptoms and finally, a heart transplant (Elliot et al., 2007). In patients with hereditary RCM, the 5-year survival rate is 56% (Seferovic et al., 2019).

In this study, we present a case of a male RCM patient who carrier a compound heterozygote variants (c.289C>G, p.Arg97Gly and c.433C>T, p.Arg145Trp) in the *TNNI3* gene. Cardiac contractility is governed by the thin filament regulatory proteins, cardiac troponin (cTn) and tropomyosin (Tm), and available levels of intracellular free calcium ([Ca2+]*i*). The *TNNI3* gene encodes a key component of myocardial structure, troponin I. Cardiac TnI is an inhibitory subunit that acts primarily to prevent actin and myosin from interacting in the absence of Ca2+. TnI has a unique feature which not found in other skeletal muscle isoforms in that it has an N-terminal extension of 30-amino acid containing two protein kinase A (PKA) phosphorylation sites (Ser 22 and 23). The C terminal domain of cTnI binds to actin and helps maintain the thin filament in a blocked state. The RCM mutations found in TnI were mainly located in the inhibitory peptide or in the C-terminal domain. These cTnI mutations may be destabilize leading to reduced interaction between the C-terminal domain and actin in the absence of Ca2+, thereby reducing inhibition by cTnI. This is a small region called the inhibitory peptide that is necessary for the regulatory role of cTnI in muscle contraction. In the absence of Ca2+, the inhibitory region is very important for maintaining proper off state function of the thin filament. The experimental results show that the RCM mutation in the inhibitory peptide has a stronger effect than the mutation located in the cTnI C-terminal domain. In addition, mutations located in the cTnI C-terminal domain may alter the structure and function of the inhibitory peptide (Murphy et al., 2000; Tekeda et al., 2003; Wong et al., 2019). The first report of mutations associated with RCM was done by Mogensen et al. (2003). In that study, six cTnI mutations (p.Leu144Glu, p.Arg145Trp, p.Ala171Thr, p.Lys178Glu, p.Asp190Gly, and p.Arg192His) at the C-terminal were found linked to RCM.

In our study, the p.Arg97Gly and p.Arg145Trp variants were identified to occur within the highly conserved inhibitory region (Fig. 3), those are also important functional regions of cTnI (the p.Arg97Gly variant in the TNNT binding domain and the p.Arg145Trp variant in TNNC biding domain) (Fig. 4). Cardiac troponin plays an important role in the contraction and relaxation of the heart (Perry, 1999; Lang et al., 2002). Variants occurring in function domains of cTnI leading to RCM specific diastolic dysfunction

(Kosstareva et al., 2016). Both variants (p.Arg97Gly and p.Arg145Trp) have been evaluated as pathogenic variants on the Clinvar database.

The TNNI3 c.433C>T (p.Arg145Trp) variant has been reported in patients with RCM (Mogensen et al., 2003; Willott et al., 2010; Hwang et al., 2017). Using actomyosin ATPase assays, Gomes et al., (2005) demonstrated that p.Arg145Trp is capable of inhibiting ATPase activity in the absence of Ca2+ and cannot produce a full relaxation in the absence of Ca2+. The p.Arg145Trp variant also resulted in an increased sensitivity to Ca2+ compared with wild type cTnI (Gomes et al., 2005). Additionally, the p.Arg145Trp variant located in the inhibitory region of cTnI resulted in an increase in the basal ATPase activity at low Ca2+ concentrations (Kobayashi & Solaro, 2006).

The results of our study showed that the variants in the *TNNI3* gene were found in the heterozygous in the patient's parents and a sister (who did not manifest the disease) and in a combined heterozygous (in the patient). These variants c.289C>G (p.Arg97Gly) and c.433C>T (p.Arg145Trp) were also identified as the pathogenic variants in the ClinVar database (https://www.ncbi.nl-m.nih.gov/clinvar) (accession number VCV001331910.2 and VCV000012426.28, respectively). These results also showed that variants in the *TNNI3* gene were the cause of the patient's disease.

Figure 3. Alignment of amino acid sequences of TNNI3 from different species such as *Homo sapiens* (X90780.1), *Bos taurus* NM_001040517.1, *Equus caballus* NM_001081904.1, *Sus scrofa* DQ641928.1, *Mus musculus NM_009406.4*, *Rattus norvegicus* NM_017144.2, *Canis familiaris* AF506750.1, *Gallus gallus* NM_213570.1. The positions of the changed amino acids (p.Arg97Gly and p.Arg145Trp) in protein TNNI3

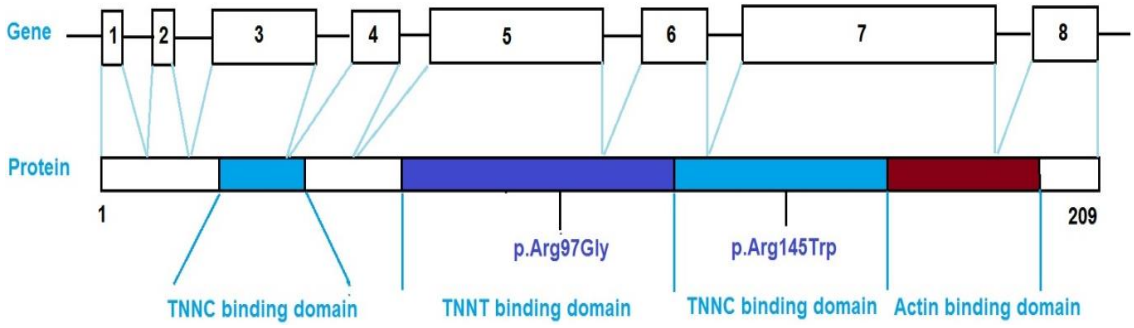

Figure 4. Schematic overview about the TNNI3 gene consisting of eight exons (NM_000363.5). Schematic domain organization and localization of the variant in the structure model of the TNNI3 protein. Modified from reference Gerhardt et al., 2022

CONCLUSION

Cardiomyopathy in children is generally rare but often has a poor prognosis. The discovery of sarcomeric protein mutations responsible for disease development in our study contributed to identifying the cause of RCM in patients and enabled screening of potential mutation carriers. Our findings support the view that genetic testing is very useful in the clinical diagnosis of RCM. WES sequencing can facilitate early diagnosis of the disease as well as proper monitoring and management of RCM patients. In patients with cardiomyopathy with a suspicious family history, early genetic testing is needed to better understand the genetic cause of this rare disease and thus be able to provide early counseling for members of affected families.

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