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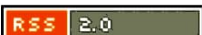
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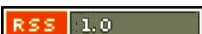
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Identification of molecular markers associated with the progression and prognosis of Cardiomyopathy

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ABSTRACT

Cardiomyopathy is one kind of Heart muscle disorder, which has a genetic cause and may lead to fatal heart strokes. Bioinformatics screening of related genes may be diagnosing tools which will help to finalize exact working out therapy. Apart from various other causes of heart muscle disorder, one most effective cause is Genetic. According to different researchers about 200 rare mutations in 20 different genes may lead to Cardiomyopathy; however its genetic etiology is largely unknown. Most common genes respective to this type of studies, specifically in focus are MYBPC3, MYH7, TNNT2 and TNNI3. In this study there is use of some bioinformatics tools to do comparative sequence analysis of lab sequenced data and data retrieved from Gene Bank, concluded considerable amount of mutations in lonely Intron 32 of MYBPC3 that is Cardiac muscle protein coding genes which may result in certain type of a Cardiomyopathy. In case if this study extended to gene sequencing or to the genome sequencing through this method, it will definitely be result in huge data, helpful for identification of molecular markers associated with prognosis and progression of not only cardiomyopathies but also other diseases too.

Keywords: Cardiomyopathy; HCM; DCM; MYH7; MYBPC3.

INTRODUCTION

Cardiomyopathy means a heart muscle disorder. The term 'Cardiomyopathy' was coined by Brigden, who described a group of uncommon, non-coronary myocardial diseases (Brigden, 1957). Cardiomyopathies due to sarcomeric mutations are a major monogenic cause for heart failure (Dhandapany *et al.*, 2009). Cardiomyopathy is a condition in which heart muscle becomes enlarged, so that it cannot pump blood properly. Cardiomyopathy can be acquired due to wrong Lifestyle or may be congenital; basically, it is an autosomal dominant inheritance pattern and has a variable age of onset and prognosis

(Baozhong *et al.*, 2007). More than 20 different genes affected by more than 200 rare disease associated mutations have been already identified (Liew & Dzau, 2004). However, its genetic etiology remains largely unknown (Reddy *et al.*, 2005).

MYBPC3 has emerged as a candidate gene for increased risk of heart failure either through Hypertrophic or Dilated Cardiomyopathies (Niimura *et al.*, 2002). Cardiac myosin binding protein C is an important constituent of the thick fibers in the C-zone of the A-band of the sarcomere. It binds with myosin, titin and actin, adds stability to the structural integrity of the sarcomere and controls heart contractility because of adrenergic stimulation (McClellan *et al.*, 2001). Mutations in *MYBPC3* gene cause disorganization of sarcomeric structure. Approximately 147 mutations have been detected in *MYBPC3* gene alone (Tanjore *et al.*, 2008). The *MYBPC3* protein has a place with fibronectinIII quality family and immunoglobulin superfamily. The *MYBPC3* gene is situated on the short (p) arm of chromosome 11 at position 11.2/11.12, ranges from base pair 47,331,405 to base pair 47,352,701, since it is around 21296 bp long. Around 35 exons (coded) with alternate introns (non-coded) comprise this gene.

MYH7 is a Second most affected and candidate gene at high risk of mutation associated with familial cardiomyopathy which may occur about 35% of total cases. It is coded for myosin heavy chain beta (MHC- β) Protein (Quiat *et al.*, 2011). It is a major protein of thick filament and play vital role by showing enzymatic activity of the ATPase in the myosin head for muscle contraction (McKillop and Geeves, 1993). MHC- β is of 223 kDa and about 1935 amino acids long (Zong *et al.* 2013). Gene *MYH7* is located on Chromosome 14 at position 14q.11, it is 22,920 bp long ranges from base pair 23,412,740 to base pair 23,435,660 (Ensembl, 2017). Cardiac troponin T is a protein which encoded by *TNNT2* gene (Gerull *et al.*, 1998). It is 35.9 kDa protein composed of 298 amino acids and activated by elevation of Calcium influx in sarcoplasm. This is one of the three subunits of troponin complex which binds to Tropomyosin; all are the components of actin (thin) filament of sarcomere and functional in contractile machinery by changing confirmations (Wei and Jin, 2011). The *TNNT2* gene location is at 1q32.1 of chromosome no. 1 it is 18756bp long ranges from bp 201,359,008 to bp 201,377,764. Mutation mostly leads to

dilated cardiomyopathy. *TNNI3* gene is coded for cardiac troponin I protein which is a subunit of troponin complex (Mogensen *et al.* 1998). This protein is about of 24 kDa composed of 210 amino acids. *TNNI3* gene ranges from base pair 55,151,767 to base pair 55,157,773 which is 6006 bp long located on chromosome 19 at position 19q13.4 (Jin *et al.*, 2008). More than 20 mutations in this gene may cause familial hypertrophy in about 5% of cases (Sheng and Jin, 2014). In this study there is use of some bioinformatics tools to do comparative sequence analysis of lab sequenced data and data retrieved from Gene Bank to identify molecular markers associated with Progression and prognosis of cardiomyopathies.

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MATERIALS & METHODS

This study was performed at the Molecular genetics lab of GVISH, Amravati and approved by different Government medical institute. An informed consent was obtained from all the subjects. It include step like survey for specific sample, sample collection, DNA extraction, Polymerase chain reaction, automated gene sequencing, comparative sequence analysis done by CLUSTAL X software to find out molecular markers could be associated with cardiomyopathy.

Sample Identification and Sample Collection:

In the supervision of cardiologist, cardiomyopathy patients were identified, after studying detailed medical records including ECG, Echocardiograph, and other supplementary reports. Then informed consent from subject was taken and by the help of expert pathologist 1 to 2 ml of intravenous blood sample was collected in previously

labeled and sterile Vacutainer (Potassium EDTA bottles). Each Vacutainer already had Potassium EDTA crystals in them and blood sample taken was slowly rotated in it with specific manner for at least 5 minutes, which made blood sample mixed up with EDTA to avoid cell degradation and contamination. Because EDTA is an anticoagulant better than heparin and citrate (Nicole YL, Lam et al, 2004). Samples were kept at 4°C up to DNA extraction.

DNA Extraction:

Before proceed to DNA extraction, Fractionation of blood samples had been done in centrifuge at 13000rpm for 10 minutes. Three layered sample clearly indicate, a deep red precipitate of RBC's at the bottom (45%), upper pale yellow plasma, which is 55% of the total blood and the middle, Buffy coat of leukocytes and platelets (1%), from which DNA is to be extracted. Plasma is then removed completely without disturbing middle layer by pipette. Genomic DNA was isolated from the fragmented blood using Genetix DNAsure Mini Kit as per protocol provided with it. After DNA extraction, to check the quantity of extracted DNA, quantification was done by using a UV – spectrophotometer (UV-1800, SHIMADZU Japan). DNA samples were stored at 4°C. To confirm the quality of extracted DNA, agarose gel electrophoresis was used. For separation, the DNA samples and Amplicons (after PCR) were loaded into wells on Agarose gel film, in electrophoresis unit containing 1X buffer, under the influence of an applied electric field by anode and cathode. First well was always loaded with a known size DNA marker or a Ladder to assume the position of DNA or Amplicon size.

Polymerase Chain Reaction:

Before proceed to next step PCR program was confirmed by using gradient PCR on variable annealing temperatures to determine specific annealing temperature for the given Primers. For this the temperature range of 54-65°C was used and after these bands checked out at 2% agarose gel slab and through this annealing temperature of 64.6°C was confirmed (Hingankar 2016). After determination of Primer specific annealing temperature, the step-by-step program setting (Table 1) was done by saving following cyclic conditions in PCR master-cycler each repeated for 30 cycles. In the next step PCR mix was made up in 0.2 ml PCR tube by mixing of following ingredients with given Quantity (Table 2).

Table 1: Cyclic conditions for Polymerase Chain Reaction

Step No.	Process	Temperature	Time
1	Pre-denaturation	95°C	3 min
2	Denaturation	95°C	45 sec
3	Annealing	64.6°C	40 sec
4	Extension	72°C	1.10 min
6	Final Extension	72°C	5 min

Table 2: PCR Mix Ingredients and Quantity

S. No.	Ingredient	Quantity (in µl)
1	Mastermix	12.5
2	MgCl ₂	1.0
3	Taq Polymerase	0.5
4	Forward Primer	1.0
5	Reverse Primer	1.0
6	Extracted DNA	1.0
7	Nuclease free Water	8.0
Total		25 µl

After PCR amplicons were stored at 4°C. To ensure amplification, amplicons run at 2% gel (Fig-1) and after proper wrapping by wax papers and labeling, PCR tubes sent for Automated Sequencing. The sequence files obtained from commercial companies was then open with MEGA-5 and converted to MEGA Format. *MYBPC3* gene (Accession no. - NM_008653) sequences were downloaded from GENE BANK. The genomic data contributed by various researchers to gene bank and sequenced data in mega format then further processed with analysis using Bioinformatics software tools like CLUSTAL X. All sequences with whole *MYBPC3* gene were edited and lined up in proper length to align them in CLUSTAL X to study all the control and test sequences comparatively for mutations to conclude results.

RESULTS & DISCUSSION

When DNA samples were checked out for quantitative estimation using spectrophotometer from range it seems that the extracted DNA was pure as absorbance ratio was 1.79. The quality of DNA band on gel electrophoresis was found to be moderate as it extracted from only 1% fraction of blood, since it was decided to use direct stock

DNA sample for PCR. After PCR, the amplicons were run on 2% gel slab and checked in Gel Doc for appearance of band patterns. When compared with DNA length marker, they were found to be in the range of 400 bp which is a range of intron 32 of *MYBPC3* gene. The successful amplicons were sequenced for both strands. With help of MEGA5, all sequences converted to FASTA format and

combined in one notepad file. File was then loaded in CLUSTAL X to study the polymorphism and mutation by comparing with control sample sequences. The aligned sequence output file (Fig-1) clearly indicates the presence or absence of deletion, also variety of mutations in samples under consideration. On the basis of observation made through alignment file the results were shown in Fig. 1.

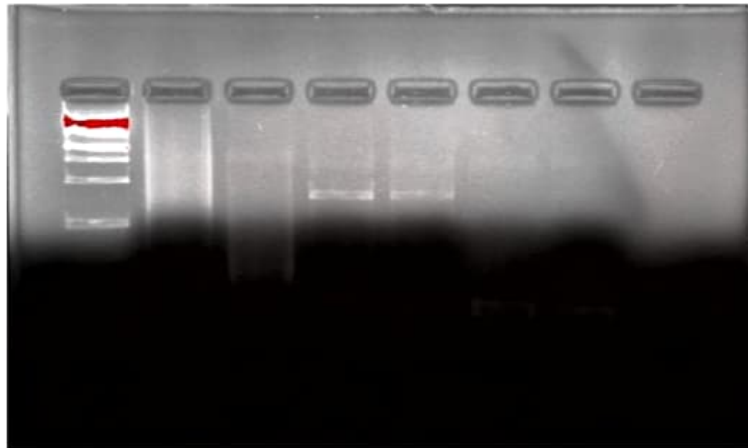


Fig-1: Amplicon bands with a 100bp marker lane on 1st well from the left side on gel Slab clicked by using Gel Doc.



Figure 1: CLUSTAL X final window showing complete aligned sequences.

CONCLUSION AND RECOMONDATION

Considerable amount of mutations in lonely Intron 32 of MYBPC3 clearly indicate that, other coded and Non-coded regions of the same gene or other related gene sequencing and mutations in it may result in certain type of a Cardiomyopathy. In case if this study extended to gene sequencing or to the genome sequencing through this method, it will definitely be result in huge data, helpful for identification of molecular markers associated with prognosis and progression of not only cardiomyopathies but also other diseases too.

Conflicts of interest: The authors stated that no conflicts of interest.

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