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Evaluating intra-genetic variants within exons 3 and 4 of LMNA in Dilated Cardiomyopathy patients from North India

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ABSTRACT
Mutations in LMNA are one of the causative factors to precipitate Dilated Cardiomyopathy (DCM), although the frequency of rare mutations segregating in familial cases has not been examined till date. Clinical cardiovascular data, family histories and blood samples were collected from 191 individuals (100 DCM cases and 91 controls). DNA samples were sequenced for nucleotide alterations in exons 3 and 4 for LMNA gene. Affected male-female sex ratio was 2:1 with 64.44% DCM males. Regular smokers among DCM patients were 79%. New York Heart Association classification showed that the number of females (n=14) affected by the disease was significantly more than that of males (n=12) only in NYHA classification III group (P=0.03) whereas number of males (n=14) were higher in NYHA IV when compared to females (n=3). There was a higher intake of polyunsaturated fatty acids as 59% of DCM patients were on non-vegetarian diet. A novel transversion mutation (c.639+1 G>C) was identified in the donor splice site region of intron 3 of *LMNA* gene in a male proband aged 45 years with severe disease course. Screening of family members revealed segregation in the family with son being affected with the same mutation and manifesting similar clinical symptoms, whereas absent from other individuals; thus similar phenotype with respect to a specific mutation in *LMNA* mutation carriers are observed. Novel **exons 3 and 4 of LMNA** contributed in the affected patient by creating a new cryptic donor splice site acting as a private mutation specific to a family. Social life style, heredity (family history positive) and male sex hormones play a role in disease causation, affecting characteristics as body fat distribution, which is associated with an increased risk of heart disease. The study revealed male preponderance accompanied by smoking habits, with middle-aged men being affected preferably, while a majority of female patients depicted greater severity of disease.

Key words: Dilated Cardiomyopathy, *LMNA*, Private variant, splice-site mutation

INTRODUCTION

Dilated Cardiomyopathy (DCM) represents a heterogeneous group of inherited and acquired disorders of cardiac muscles characterized by ventricular dilation, impaired systolic function, reduced myocardial contractility and a left ventricular ejection fraction of less than 40% with a frequency of 1:250 or greater (Hershberger et al., 2013). Most of the DCM cases are sporadic but approximately 25-30% of affected individuals have a positive family history. Although more than 40 candidate genes are associated with DCM, Lamin A/C (*LMNA*), a nuclear protein which provides structural support to the nucleus and the anchoring chromatin, accounts for 6% of all the idiopathic DCM cases. Defects in *LMNA* are responsible for different diseases called Laminopathies (Prokocimer et al., 2009). Mutations in *LMNA* gene is almost always associated with arrhythmias which mostly occur before heart failure symptoms. The mechanism by which *LMNA* variants cause DCM is still not properly understood. Two hypotheses have been proposed by the investigators. First, the "mechanical stress" hypothesis which states that abnormalities in nuclear structure which result from *LMNA* mutations lead to increased susceptibility to cellular damage by physical stress. The second "gene expression" hypothesis proposes that nuclear envelop plays a role in tissue specific gene expression that can be altered by mutation in *LMNA* gene, is based primarily on observed interaction between nuclear envelope and chromatin components (Worman and Courvalin 2004). In *LMNA* associated DCM, the penetrance is very high with almost all variant carriers manifesting some aspect of the DCM phenotype by the age of 50-60 years, regardless of the type of variant (Hershberger et al., 2013).

Till date, few works has been done related to genetics of *LMNA* in Indian population. *LMNA* gene variants causing DCM in a single Indian family has been reported in Emery Dreifuss myopathy, with familial DCM (FDCM) and cardiac conduction abnormalities (Jadav et al., 2012). In another study, rare mutation S625C has been identified which leads to lowering in the level of cyclin dependent kinase inhibitor p21 causing compromised cell cycle regulation (Thanumalayan et al., 2015).

Dissecting the complex genetic basis of different phenotypes of cardiomyopathy may be the key to both better understanding and optimally managing these cardiovascular diseases. The discovery of causative mutations has important implications for diagnosis, prognosis, and intervention. India, with a population of more than a billion, has a large number of patients affected with cardiomyopathies; however, there is little information on spectrum of *LMNA* gene mutations and

their association with disease phenotype and prognosis. We therefore performed selective screening of *LMNA*, testing for the coding sequence, point mutations and describe a novel splice site variant (c.639+1 G>C) detected in Indian DCM patient defining the clinical spectrum associated with DCM.

SUBJECTS AND METHODS

Collection of patient samples

This is a hospital-based study whose study participants belonged to various age and socio-economic groups. The base population recruited for this study was outpatients of the Cardiology Clinic visiting All India Institute of Medical Science (AIIMS), New Delhi, India from 1 March 2012 to 28 November 2015. After an initial screening procedure, 117 patients met the inclusion criteria. In all, 17 patients did not respond to the initial invitation, out of which 10 were not interested and 7 were nonresponsive thus 100 patients remained for the study. Clinical data and detail family history of each one of the 100 DCM patients were collected with the help of collaborating clinicians after physical and cardiological examination by two independent consultant cardiologists (SS and BV). Hospitals like AIIMS is the main referral center for cases related to cardiac disorders and are situated in the heart of New Delhi. The demographic data (name, age, address, place of inhabitation, occupation, family pedigree, educational status, monthly income, occupation/professional status) of subject during his/her life time, residence area (rural/ urban and so on) were collected by health professionals and epidemiologists. Participants were given an open-ended semi-structured interviewer–administrative questionnaire that collected information on disease history, from disease onset to the baseline evaluation from patients and controls in a face-to-face interview, and a cardiologist examined each patient and control face-to-face clinically after obtaining written informed consent. Each interview and evaluation took about 60 to 90 minutes. All the participants (100 patients with DCM and 91 healthy controls) actively took part in this study, and their good response enabled a 100% participation rate. Both participants and the accompanying family members/caregivers were allowed to answer. Before the questionnaire was formally administered, a panel consisting of cardiologists, epidemiologists and a biostatistician examined content validity. A meet on patients with DCM was organized and the participating individuals along with their family members attended the camp, and detailed follow-up visits at the outpatient department/home took place after every 1.5 years. All the 191 individuals underwent physical examination, chest X-Ray, 12-lead ECG, trans-thoracic two-dimensional echocardiography and Doppler studies. The NYHA (New York Heart

Association) classification was used to determine the severity of DCM patients. Standard views for M-mode and two-dimensional studies were obtained.

In order to achieve high diagnostic specificity as well as high sensitivity, following the history of patients, physicians would analyze meticulously for DCM and screen them, following the recommendations of AHA (Maron et al., 2006) and WHO guidelines of Cardiomyopathies diagnosis (Richardson et al., 1996).

Inclusion criteria

1. Left ventricular ejection fraction (LVEF) <40% on echocardiography
2. Absence of past history of myocardial infarction or coronary artery disease.
3. Absence of secondary cause of left ventricular dysfunction including primary valvular heart disease, ventricular outflow tract obstruction, and coronary artery anomalies.

Exclusion criteria

Patients with concomitant disease like infection, autoimmune disease, cancer, as well as patients with coronary artery disease and with advanced chronic renal failure were excluded.

None of the controls had any diagnosable cardiac disorders in their family history with similar educational levels. Ninety one non-DCM control group consisted of patients' spouses and other healthy community-based, age–sex-matched volunteers residing in the same ethnic background as the DCM patients. The experiments were conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained in accordance with the study protocol after ethical approval of the research project using human subject was issued from the Institutional Ethical Committee of collaborating hospital and institute. Both the patients and the controls were screened by renowned cardiomedicine clinical specialists (SS and BV) along with their team of trained cardiologists at collaborating hospital, New Delhi.

Collection of blood samples and genomic DNA preparation

Approximately 5ml of peripheral blood samples was collected in K2 EDTA Becton Dickinson Vacutainer (6ml) with written and informed consent from DCM patients, their family members and from normal individuals as controls making sure about adequate understanding by donors. Genomic DNA was prepared from fresh whole blood by using conventional phenol-chloroform method

Sambrook and Russel, 2001). Genomic DNA was dissolved in TE (10mM Tris-HCl, 0.1mM EDTA, pH 8.0).

Polymerase chain reaction

PCR was carried out to amplify coding exons and adjacent flanking region of exons 3 and 4 for *LMNA* gene in a total volume of 10.0µl containing 40–100 ng genomic DNA, 0.4µM of each primer, 0.2mM of each dNTP, 0.5–1.5mM of MgCl2 (as appropriate) and 0.5 unit of Taq polymerase (Invitrogen, Carlsbad, CA, USA) in a Thermocycler (GeneAmp-9700, PE Applied Biosystems, Foster City, CA, USA). Primers for amplification reactions were designed by Primer3 software. PCR amplified DNA fragments were analyzed on 2% agarose gel and visualized by ethidium bromide staining.

Mutation and polymorphism detection

DNA sequencing: the PCR products free of contaminating bands due to nonspecific amplification were directly sequenced in forward and reverse direction using an ABI Prism 3730 DNA Analyzer (Applied Biosystems) and the Applied Biosystems BigDye Terminator Chemistry (Applied Biosystems). Nucleotide changes were detected by comparing the sequence obtained in chromatogram with the normal DCM gene sequence (GenBank Accession No.AY847595) using pair-wise BLAST and SeqScape software v2.5.

Statistical and Bioinformatic analysis

Baseline characteristics were compared between subjects with DCM and controls by unpaired Student's t-test. Data were expressed as mean (± SD). All the statistical analyses were performed using the SPSS statistical software version 16.0 (SPSS, Chicago, IL, USA) for Windows. A p-value 0.05 (two-tailed) was considered statistical significant. For in-silico analysis- Human splice finder (HSF) (Desmet et al., 2009) and Max Ent (Eng et al., 2003) were used for functional prediction of splice site mutation.

RESULTS

Clinical characteristics

Molecular screening for mutations in LMNA was performed on an Indian cohort of 100 patients recruited with a clinical diagnosis of DCM, as well as 91 healthy, unrelated and ethnically

matched controls. The mean age of controls was 50.75±7.12 years (age range: 36-72 yrs). The sequences were aligned using the LMNA gene sequence available in NCBI as the reference sequence. The sequence variants obtained were queried in the NCBI SNP database and lamin mutation databases http://www.umd.be/LMNA and http://www.dmd.nl/lmna_seqvar.html (Leiden Open Variation database) to determine whether they had been reported earlier and shown to be pathogenic (disease-causing) or not. A total of 191 study subjects were included. The *LMNA* gene was screened among 100 index patients. Mean (±SD) age of cases was 41.5±16.45 (age range: 10-66 yrs) and average age of onset was 37.24±13.12 (age range: 9-67 years; 64 males and 36 females). The demographic details of the recruited individuals are given in Table 1. Occurrence of the disease was higher among males (64%). 54.68% of the males belonged to 30-40 years age group, while 25% belonged to the 40-50 years age group. Fifty nine percent of the patients were on non-vegetarian diet whereas only 41% patients had vegetarian diet. Controls recruited were from higher age group and mean present age of the controls were higher than mean age of onset. Most of the patients belong from Bihar and Uttar Pradesh due to the close proximity of the hospital. Majority of the patients were married (78%) and educated. 94% of the patients were employed and can manage the medications and the treatment.

Majority of the patients belong to New York Heart Association Class II (56%) with a significant difference among males and female in New York Heart Association Class III (p=0.03). Among males, severity (NYHA class IV) was high compared to females with suggestive p value of 0.06 (Table 2). There was no significant difference in Electrocardiogram assessment among males and females. Left Bundle Branch Block (LBBB) was present in 69% patients. A significant difference with respect to ejection fraction among males and females (p value < 0.00) was noted. 26% patients had Moderate Mitral Valve whereas 22% presented moderate Tricuspid Regurgitation (Table 3). Severe Mitral Valve Regurgitation was present in only five percent of patients. Subjects diagnosed with DCM usually showed symptoms of respiratory distress on exertion and also on rest, fatigability and edema. Irregular pulses, narrow pulse pressure, atrial fibrillation, and elevation of jugular venous pressure were routinely observed. Cardiac examinations of the decompensated DCM patients revealed muffled heart sound with gallop rhythm (LVS3 or RVS3). The apex was down and out and there was the presence of systolic murmur at apex which was indicative of mitral regurgitation. Chest X-ray in patients revealed cardiomegaly with or without pulmonary congestion. ECG showed the following abnormalities – LBBB (left bundle branch block), ST-T and atrial ectopic, ventricular ectopic and also atrial fibrillation.

Exons 3 and 4 of *LMNA* gene was sequenced among 191 individuals (33% familial, 67% sporadic). A single novel mutation in intron 3 was identified among 2 individuals in a familial case of DCM. Detailed demographic and clinical characters of sporadic and familial DCM cases have been previously reported (Das et al., 2015).

A novel transversion mutation (c.639+1 G>C) (Fig. 1A) was identified in the donor splice site region of intron 3 of *LMNA* gene both in patient and his son aged 20, who was asymptomatic but during his follow-up screening after three years the LVes was 68 ml which was slightly more than normal and ejection fraction was 45% but the ECG was normal. The proband aged 45 at the time of detection with a non-vegetarian diet, showed severe symptoms such as angina, dyspanea, palpitation, fatigue and pedal edema and was classified under NYHA Class III. Preliminary clinical examination of chest X-ray showed cardiac enlargement. Further ECG was characterized by atrial hypertrophy, bradycardia, left bundle branch block, bilateral enlargement and prolonged QTc. Echocardiographic evaluation demonstrated moderate mitral regurgitation (MR), left ventricular enlargement, right atrial enlargement and left enlargement with an ejection fraction of <30% (Fig. 1B). Left ventricular end-systolic and end-diastolic volumes were 79 and 134ml. The posterior wall thickness was 0.9 mm. Rest of the family members had normal echocardiography values and ECG report. As reported by the proband, his mother died suddenly due to heart failure at the age of 65 yrs and his elder sister's death was due to unknown cause at the age of 50yrs although not clinically determined (Fig. 1A).

This mutation was not found in any other family members as well as in 100 DCM, 30 HCM and 25 RCM patients. No other SNPs or variants were detected in the patient cohort. The variant was evolutionary conserved among the vertebrate orthologs (Fig. 1C). In silico analysis of c.639+1 G>C of *LMNA* gene was performed by Max Ent Scan and Human Splicing Finder (HSF). Max Ent Scan predicted a decrease in consensus value (G>C):86.81 > 75.79 and maximum entropy (G>C): 4.41 >-3.87 respectively. HSF predicted the elimination of natural donor splice site leads to the formation of a new cryptic donor site which is located 7 nucleotides up stream of native splice site with a consensus value of 80.63.

DISCUSSION

DCM is a primary myocardial disorder marked by phenotypic and genotypic heterogeneity. In our study, number of males was more than females but the severity was higher in females (Table 2) with greater frequency of left bundle branch block, greater left ventricular end diastolic and systolic

diameters, shorter exercise duration compared to men similar to Italian Multicenter Cardiomyopathy Group (De Maria *et al*., 1993). Women with heart failure had poor life satisfaction and physical function than men which are at par with findings by Riedinger *et al.*, 2001.

Analogous to another study by Ushashree *et al.*, 2014, with respect to gender, 63% and 37% constituted male and female patients respectively, with male-female sex ratio being approximately 2:1, unlike few studies which report female preponderance (De Maria *et al*., 1993). The preponderance of males could be explained on the basis of hormonal variations as androgen promotes hypertrophy (Melchert *et al*., 2001) and genetic background, apart from differential life styles. Probably male hormones confer greater vulnerability to factors altering membrane integrity and permeability (Jeanine *et al*., 2002; Olsson *et al*., 2004) as it is well established that estrogens are cardio-protective (Moolman, 2006). No parental consanguinity was detected in the studied individuals unlike studies from South India (Ushasree *et al*., 2009). 54.68% of males belonged to 30-40yrs of age group revealing that young and middle-aged men are prone to the disease, confirming the results of earlier studies from India and by American Heart Association (1980).

It has been known that smoking and alcohol predispose to heart failure (Rabinowitz *et al*., 1979; Suskin *et al*., 2008) and our data showed parallel outcomes with 79 individuals diagnosed as smokers compared to 41 controls in the smoking category; in contrast to a previous report from India (Ushasree *et al*., 2009). Diet patterns revealed that 59 DCM patients with non-vegetarian diet may be at a higher risk for the disease condition, which can be correlated to high intake of polyunsaturated fatty acid in exacerbating the disease condition whereas 41 patients took vegetarian nutritional supplements like protective antioxidant enzymes, polysaturated fats and fiber, which are essential to combat the oxidative stress, thus making them less susceptible to the already existing disorder.

Laminopathies are principally caused by sporadic mutations in LMNA gene in individuals. 165 *LMNA* mutations (HYPERLINK "http://www.umd.be/LMNA/" \t "externObjLink" http://www.umd.be/LMNA/) have been known to be associated with DCM till date based on studies from Europe, USA, and some South-East Asian countries (Banerjee *et al*., 2015). Abnormal *LMNA* activates mitogen-activated protein kinases MAPK cascade via G protein receptor by unknown mechanisms leading to over expression of downstream targeted genes which further aggravates over expression of sarcomeric protein (Muchir *et al*., 2007). In view of the segregation of novel c.639+1 G>C mutation in the family and severe disease course in the proband, we further applied in-silico analysis (Max ENT & HSF) for the functional prediction of the mutations. Desmet *et al.* 2009 validated HSF in 69 intronic mutations that disrupt the 5'ss or the 3'ss and resulted in exon

skipping and a group of 15 mutations that were previously reported to result in splicing defects by creating or activating cryptic splice sites. HSF was able to correctly predict the disruption of natural splice sites. Human splicing finder (HSF) predicts correct effect of mutation in almost all the cases. The HSF algorithm gives a consensus value (CV) of 5'ss and 3'ss. For *LMNA* mutation (c.639+1 G>C), HSF predicts the mutant CV value 75.79, i.e. this site will no more work as natural splice site but the position 7 base pairs upstream having a CV value of 80.63 will be recognized as new splice site.

This novel mutation c.639+1 G>C in *LMNA* gene lies in the central rod region of the Lamin protein, where Lamin B interacts with Lamin A/C for dimerization to occur. Thus, this mutation may lead to weaker Lamin leading to mechanical stress damage by muscle contraction and abnormal mechano-transduction. In this study, the single mutation found in *LMNA* gene explained DCM in about 1% of the patient cohort in North Indian population. This mutation is likely to be inherited in this family because it was not found in none of the 91 healthy controls and phenotype of the son is quite similar to father in his initial days of disease expression. This study strengthens the view of a similar phenotype with respect to a specific mutation in *LMNA* mutation carriers.

Our study has some limitations. The studied cohort is relatively small. Although common mutations within exon 3 and 4 are absent in our study, their contribution to DCM cannot be completely excluded, as *LMNA* is a large gene and mutations, other rearrangements, or upstream region polymorphisms may exist in this population. Thus, mutation screening of the entire exons of *LMNA* is important to determine the contribution of this gene to DCM in North Indian population. Although *LMNA* mutations are reported at various frequencies in populations of USA, China, Japan, Korea (Perrot et al., 2009; Van Spaendonck-Zwarts et al., 2013; Jakobs et al., 2001) this study indicates that certain specific mutation might be involved in some familial cases of DCM cutting across all castes and groups taken in our study.

We conclude that the novel c.639+1 G>C splice site mutation is contributing to cause DCM in affected patient by creating a new cryptic donor splice site acting as a private mutation specific to a family. Social life style, heredity (family history positive) and male sex hormones are known to play a role in the disease causation, affecting characteristics as body fat distribution, which are associated with an increased risk of heart disease. The study revealed male preponderance accompanied by smoking habits, with middle-aged men being affected preferably, while a majority of female patients depicted greater severity of disease.

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Conflict of interest statement

The authors declare no competing financial interests

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Table 1: Baseline Demographic and Clinical characteristics of DCM cases and controls

Variable	Cases, N (%)	Controls, N (%)	p value
Numbers of Individuals	100 (100)	91 (100)	
Sex			
Male	64 (64)	52 (57.14)	0.33
Female	36 (36)	39 (42.85)	
Present Age (yrs), Mean±SD	41.5±16.45	50.75±7.12	0.00*
Age of Onset (yrs), Mean±SD	37.24±13.12		

Significance Tests: Chi square (Present Age); .
*p-value significant at < 0.05

Table 2. Clinical Profile of DCM patients among males and females.

Variables, N (%)	Patients			p-value
	Male (64) n (%)	**Female (36) n (%)**	**Total (100) n (%)**	
NYHA Class				
I	01 (1.56)	0	01 (01)	0.45
II	37 (57.81)	19 (52.77)	56 (56)	0.62
III	12 (18.75)	14 (38.88)	26 (26)	0.02*
IV	14 (21.875)	03 (8.33)	17 (17)	0.09
Age of onset, yrs (Mean±SD)	38.77±13.36	38.98±13.17	37.24±12.86	0.07
SYMPTOMS				
Swelling in feet	11 (17.18)	13 (36.11)	24(24)	0.03*
Shortness of breath	33 (51.56)	23 (63.88)	56 (56)	0.24
Palpitation	25 (39.06)	20 (55.55)	45 (45)	0.12
Fatigue	40 (62.50)	26 (72.22)	66 (66)	0.31
Orthopenia	21 (32.81)	19 (52.77)	40 (40)	0.04*

Significance Tests: Z test (NYHA class and symptoms); t test (Age of onset).
*p-value significant at < 0.05

Table 3. Electrocardiogram (ECG) and Echocardographic assessment of DCM patients among males and females

Variables	Patients			p-value
	Male (%)	Female (%)	Total (%)	
Number of Individuals	64 (64)	34(36)	100 (100)	
Electrocardiogram assessment				
abnormal cardiac rhythm	16 (25)	12 (33.33)	28 (28)	0.39
QRS duration ≥ 100 ms	14 (21.87)	10 (27.77)	24 (24)	0.49
LBBB/RBBB/LAHB	41 (64.06)	28 (77.77)	69 (69)	0.17
ST-T abnormalities	28 (43.75)	14 (38.88)	42 (42)	0.62
ST-T segment elevation ≥ 0.2 mV,	26 (40.62)	12 (33.33)	38 (38)	0.48
prolonged QTc interval	19 (29.68)	11 (30.55)	30 (30)	0.91
pathological Q waves	19 (29.68)	13 (36.11)	32 (32)	0.46
absence of normal Q wave	33 (51.56)	17 (47.22)	50 (50)	0.70
Giant T wave	27 (42.18)	13 (36.11)	40 (40)	0.55
2-D & M-Mode Echocardiography Measurements				
LV Ejection Fraction (%)	25.18±8.91	30.71±9.40	27.17±9.42	0.00*
LVes volume	149.27±58.36	142.65±58.58	146.83±58.18	0.58
LVed volume	210.25±78.18	204.12±71.35	208.81±74.78	0.69
Mitral Valve Regurgitation (MR)				
Absent, N (%)	12 (18.75)	06 (16.66)	18 (18)	0.58
Trivial, N (%)	15 (23.43)	09 (25)	24 (24)	
Mild, N (%)	14 (21.87)	13 (36.11)	27 (27)	
Moderate, N (%)	18 (28.12)	08 (22.22)	26 (26)	
Severe, N (%)	05 (07.81)	0	05 (05)	
Tricuspid Regurgitation (TR)				
Absent, N (%)	22 (34.37)	12 (33.33)	34 (34)	0.81
Trivial, N (%)	14 (21.87)	08 (22.22)	22 (22)	
Mild, N (%)	16 (25.00)	06 (16.66)	22 (22)	
Moderate, N (%)	12 (18.75)	10 (27.77)	22 (22)	
Severe, N (%)	0	0	0	
Aortic Regurgitation (AR)				
Absent, N (%)	49 (73.43)	31 (86.11)	80 (80)	0.25
Trivial, N (%)	08 (09.37)	05 (13.88)	13 (13)	
Mild, N (%)	07 (07.81)	0	07(07)	
Moderate, N (%)	0	0	0	
Severe, N (%)	0	0	0	

Significance Tests: t test (2-D & M-Mode Echocardiography Measurements)
Chi square test -Yates corrected (MR, TR, AR)
*p-value significant at < 0.05
Significance Test: Z test (Electrocardiogram assessment)

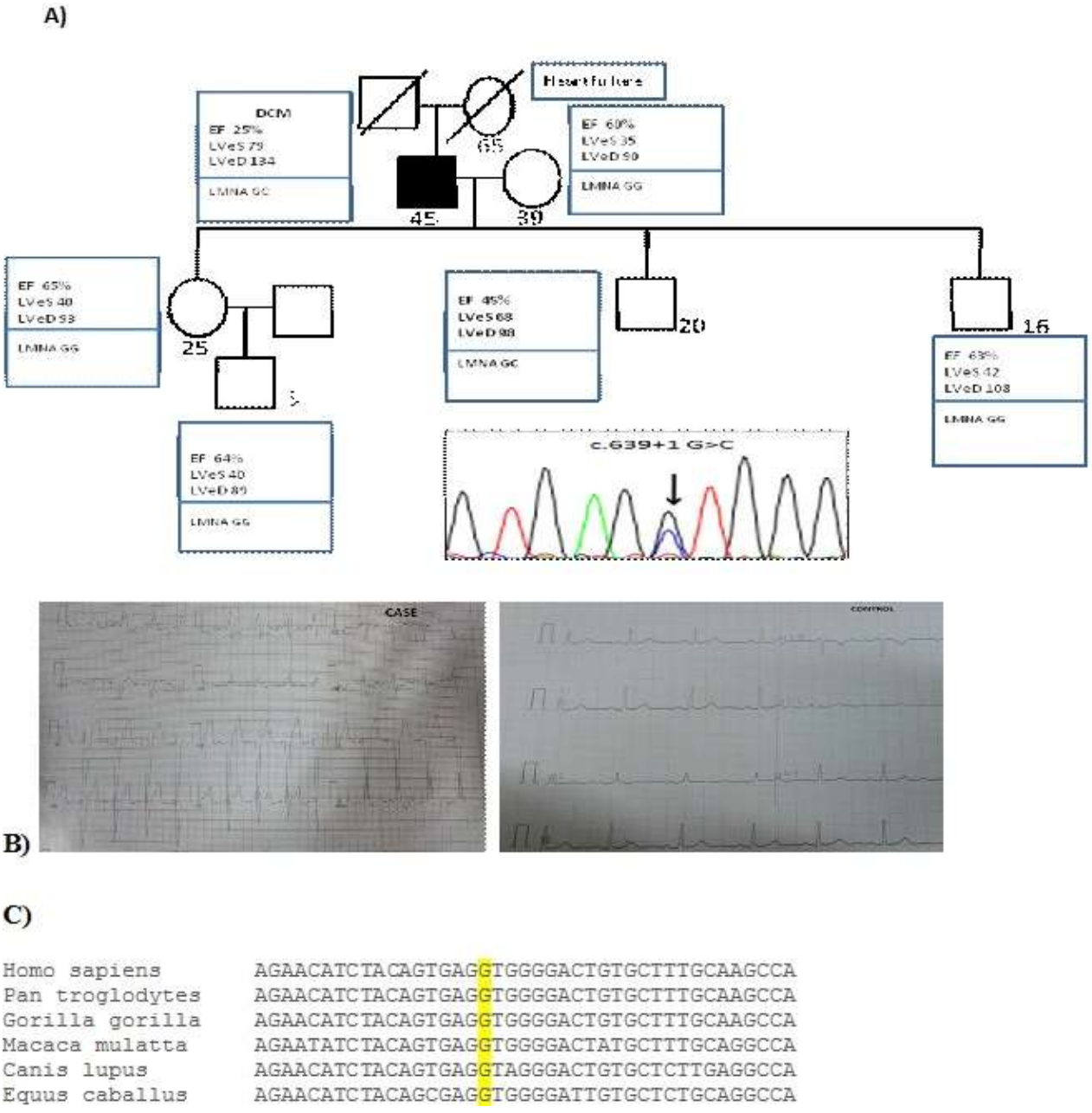

Figure 1. A) Pedigree showing Clinical and Genotypic status of patient and the family members Normal range: LVes 28-48 ml LVed 70-110 ml EF >40% and Electropherogram showing G>C transversion in intron 3 of *LMNA* gene of the patient and his 20 years old son. **B)** ECG showing arrhythmia in DCM patient and normal ECG in control. **C)** Alignment of *LMNA* sequence among different species with the conserved G region.