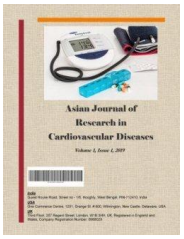

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Molecular Characterization of Phospholamban and Clinical Epidemiology in Human Cardiomyopathy

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Cardiomyopathy is commonly observed disease that may occurs due to mutations in either susceptible genes or modifier gene. People with broad age group are affected either attributable to spontaneous or inherited mutations of these genes. Various gene mutations are reported so far but only few of them were studied in detail. In the current study, we evaluated epidemiological variables like age, sex, familial status, parental consanguinity. We also described specific clinical symptoms associated with the cardiomyopathy condition in Indian population. Our studies on mutation screening of phospholamban gene revealed two transitions (4880 C/T, 4887 T/G) in 5' flanking region which might cause inherited dilated cardiomyopathy with refractory congestive heart failure. Information on epidemiological, clinical statistics and phospholamban gene mutation analysis is essential to guide the successful execution for future therapies and benefits us to identify those patients at risk for faster disease progression, congestive heart failure, and arrhythmia.

Keywords: Cardiomyopathy; hemodynamic and biochemical parameters; epidemiological and clinical parameters; phospholamban.

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1. INTRODUCTION

In the present scenario, the debilitating nature of cardiovascular disorder is alarming and needs a constant watch on the premature morbidity and mortality status. Cardiomyopathy is the heart muscle disease and most common genetic disease of the heart, characterized by heterogeneous morphologic expression and clinical condition [1]. It can manifest negligible to extreme hypertrophy, minimal to extensive fibrosis, myocyte disarray, absent to severe left ventricular outflow tract obstruction, distinct septal morphologies, or hypo contractile [2]. Cardiomyopathy can cause heart failure (HF), which in most cases leads to sudden cardiac death (SCD). Hypertrophic Cardiomyopathy (HCM), Dilated Cardiomyopathy (DCM), Restrictive Cardiomyopathy (RCM) and Arrhythmogenic Right Ventricular Cardiomyopathy (ARVC) are various types of cardiomyopathies reported so far [3]. These types can be primary myocardial disorders or at times develop as a secondary consequence of a variety of conditions *viz.,* myocardial ischemia, inflammation, viral infection, increased myocardial pressure or volume load, and toxic agents [4].

Recent study estimates prevalence of dilated and hypertrophic Cardiomyopathy as 36 cases per 100,000 people and 10–20 cases per 100,000 people respectively [5]. Most of the dilated Cardiomyopathy cases are sporadic, although 20–35% of them are familial [6]. The incidence of dilated cardiomyopathy varies in men and women. However, in general, heart failure is more common in men [7]. In most studies due to limited sample sizes, the role of susceptibility and modifier genes have been only suggestive [8]. Various genes underlying cardiomyopathy have been identified from linkage as well as candidate gene studies and include those coding for proteins involved in the cytoskeleton, the Z-disk, the nuclear envelop, ion conduction and calcium handling proteins [9].

Phospholamban gene is located on chromosome 6q22.1. Genomic sequence of the gene is 12146 bps nucleotides long which encodes 1742 bps mRNA coding region comprises 159 bps which encodes 2 exons. The protein is a pentamer and is a major substrate for cAMP-dependent protein kinase in the cardiac muscle. The protein inhibits Ca2+-ATPase in unphosphorylated state. The protein is a key regulator of cardiac diastolic function. Phospholamban (PLN) plays an important role in cardiac contraction and relaxation and is expressed in the sarcoplasmic reticulum membrane controls cellular calcium levels by a mechanism that depends on its phosphorylation [10]. The human ventricle and quadriceps displayed high levels of phospholamban transcripts and proteins [11], whereas lower expression in smooth muscles and right atrium. DCM patients with a phospholamban gene mutation have a chronically inhibited Ca2+-ATPases pump, which leads to DCM in their teenage [12,13].

The present study investigates the association of spectrum of clinical symptoms, the epidemiological variables like age, sex, familial status, parental consanguinity and the mutations in the gene encoding phospholamban cardiac protein and to establish the genotype – phenotype correlations, to identify the modes of inheritance and the risk stratification in a group of clinically well characterized patients and their relatives associated with the cardiomyopathy condition in Indian population.

2. MATERIALS AND METHODS

2.1 Study Subjects

A total of 109 unrelated index patients were echocardiographically and electrocardiographically assessed for cardiomyopathy and 100 aged, matched healthy control subjects with no history of heart disease were studied. The mean age of all patients was 37.22 ± 12.43 years. Fig. 1 shows the detailed flowchart of the study. The present study includes cardiomyopathy patients and their family members consecutively enrolled in cardiology units of the Government General Hospital, Chennai and International Center for Cardiothoracic and Vascular Diseases (ICCTVD), Dr. K.M. Cherian Heart Foundation, A unit of Frontier lifeline, Chennai, South India.

2.2 Clinical Evaluation

The index cases were subjected to standard physical examinations, clinical evaluations, electrocardiographic and echocardiograph tests to confirm cardiomyopathy. Cardiomyopathy was diagnosed based on the presence of the following criteria: dilated cardiomyopathy with Left ventricular ejection fraction (LVEF) <45% and left ventricular end diastolic diameter >27 mm/m2. However, patients who had secondary

cause were not included in the present study. Further, hypertrophic cardiomyopathy was confirmed by LV wall thickness ≥15mm and blood pressure ≤160/100 mmHg. Demographic and clinical information (Echocardiographic and Electrocardiographic findings) have been taken from the case sheets of respective hospitals. Family history and parental consanguinity were collected from the patients. Patients were classified according to New York Heart Association (NYHA) and exercise capacity has also been taken.

2.3 Data Collection

Epidemiology parameters such as height, weight, sex, age at onset, dietary habits, addictions to smoking and alcohol were collected during personal and clinical history. Each of the subjects met the Clinical diagnostic criteria *viz.,* 12 lead electrocardiograms, echocardiogram, clinical symptoms, risk factors, medication and outcome of the disease etc.

2.4 Genetic Analysis

In the present study, the index cases are categorized into two groups *viz*., familial case showing the incidence of the disease in first and second-degree relatives and in sporadic cases lack of any familial incidence, presumably non-genetic in origin. The present study examined a comprehensive screening of phospholamban (PLN) gene polymorphism.

2.5 Blood Sample Collection

Five milliliters of peripheral blood were drawn in EDTA coated vacutainer from patients, family members and control subjects and stored at 4°C until further analysis.

2.6 Clinical Chemistry Parameters

Blood parameters like sugar, urea, creatinine, creatinine phosphokinase (CpK), atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), serum aspartate transaminase (SGOT) and Serum alanine transaminase (SGPT) were analyzed using DaytonaRM Randox auto analyzer according to the manufacturers' instructions.

2.7 DNA Isolation and Quantification

Genomic DNA was extracted from the peripheral blood following the protocol of [14]. Briefly, the collected blood was mixed with an equal volume of TKM1 buffer (10 mM Tris-HCl, 10 mM KCl, 10 mM $MgCl_2$ and 2 mM EDTA) and 100 µl Triton X. The contents were centrifuged, and the pellet was washed with TKM1 repeatedly until the cell debris is washed out. The pellet is suspended in 800 µl of TKM2 solution (10 mM Tris-HCl,10 mM KCl, 10 mM $MgCl_2$, 2 mM EDTA and 0.4M NaCl) followed by centrifugation and precipitation of the supernatant in ethanol. The DNA samples were then stored at -20°C for subsequent analyses. The DNA was quantified using spectrophotometer. The DNA was diluted in TE to yield 50ng/µl concentration.

2.8 Phospholamban (PLN) Polymerase Chain Reaction

The primers of the hotspots exons of Phospholamban (PLN) forward primer 5'-tatttttctcataattaaaattcctgc-3' and reverse primer 5'-aaagtaagaattaccaaagtcagcg-3' for Exon 1 and forward primer 5'-aacaatagtgctgaggaagatgaa-3' and reverse primer 5'-ttgttttcctgtctgcatgg-3' for Exon 2 were used [15].

2.9 DNA Sequencing

Genomic DNA from individuals with different single-strand chain polymorphism (SSCP) patterns was amplified and sequenced with using Applied Biosystems 3730xl DNA Analyzer. The sequencing PCR was carried on 96 well micro-titer plates in a 5 µl reaction volume containing nuclease free water, the amplified template, "BigDye" (fluorescently labeled ddNTPs, dNTPs) and primers. The amplified DNA was precipitated by incubating at room temperature with 25µl of 3M-sodium acetate in ethanol (120 µl of 3M-sodium acetate in 3 ml of 100% ethanol) for 15min. The DNA was made single stranded by adding Hi-Di Form amide and sequenced on ABI3730xl automated DNA analyzer. The chromatograms obtained were analyzed on "Auto assembler" chromatogram analyzer on a Macintosh operating system. Complete sequencing work was carried out at the "Center for Cellular and Molecular Biology" Hyderabad.

2.10 Statistical Analyses

Statistical analyses were carried out using Statistical Analysis Solutions (SAS 9.**2**) & Graph pad prism. The mean and standard deviation were computed for various quantitative parameters and calculated. P-value <0.05 was

considered significant. Association and relative risk estimates were carried out using Chi square test for the qualitative parameters at 1% and 5% levels of significance.

3. RESULTS

3.1 Demographic and Baseline Characteristics

The present study shows higher prevalence of dilated cardiomyopathy (73%) over hypertrophic cardiomyopathy (27%). Males were significantly higher among the patients (Fig. 2). The occurrence of cardiomyopathy was familial in 24% of cases, but sporadic in the other 76% of patients. About 30% of patient family history showed positive for sudden cardiac death and 14% was associated with parental consanguinity. No significant variations were observed between mean values of body mass index and body surface area among patients and control ($p=0.085$, $p=0.515$).

3.2 Hemodynamic and Biochemical Parameters

Diastolic blood pressure, heart rate and systolic blood pressure of cardiomyopathy patients was extremely significant when compared with the control subjects ($p<0.05$). Cardiomyopathy patients were characterized with high atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) level ($p<0.001$). Contrastingly creatinine phosphokinase (CpK), serum aspartate transaminase (SGOT), serum alanine transaminase (SGPT), glucose, urea and creatinine levels did not show much variation between patients and control group (Fig. 3 & Table 1). Similarly, co-morbid factors such as diabetes, obesity, smoking and alcoholism did not show any influence on patient population.

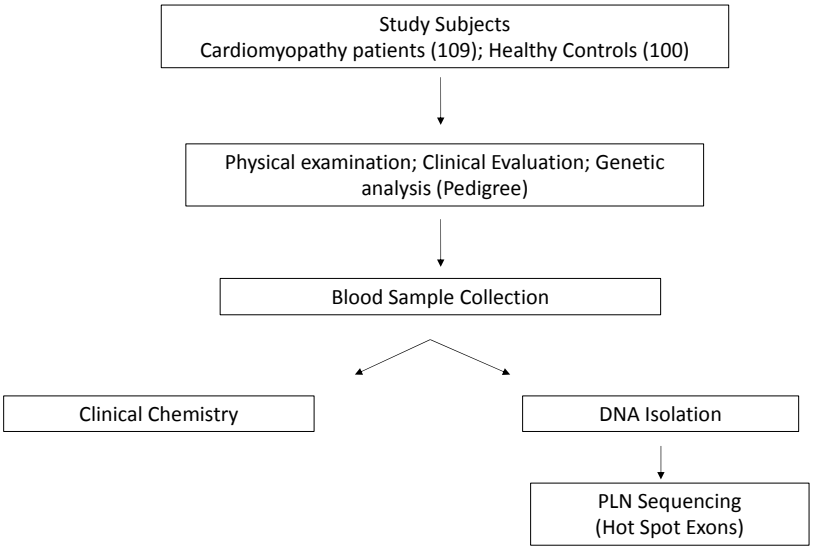

Fig. 1. Flow chart of the study

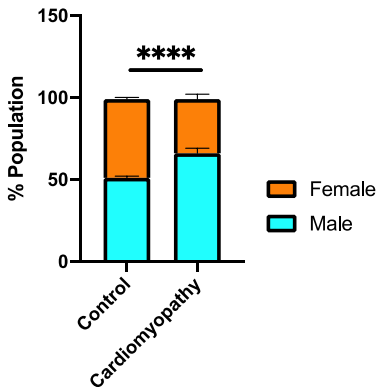

Fig. 2. Males with higher prevalence of cardiomyopathy among patients

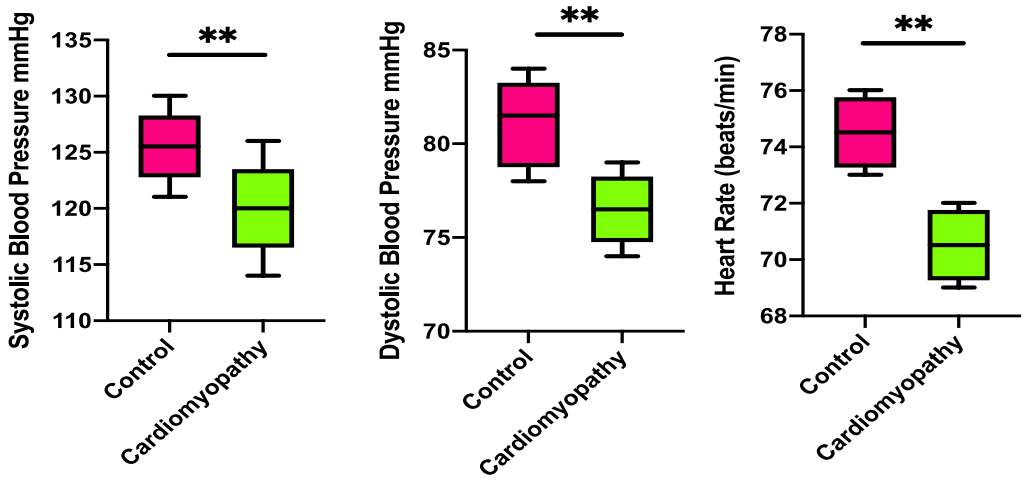

Fig. 3. Graph showing changes in heart rate, systolic blood pressure & diastolic blood pressure

3.3 Clinical Characteristics

The detailed clinical (age, body mass index, blood pressure, blood biochemical profile) and non-clinical features of the patients, control subjects were given in (Fig. 4. & Table 1). The electrocardiographic and echocardiographic characteristics of the cardiomyopathic patients and control group were given in Table 2. All comparisons were significant between cardiomyopathic patients and control ($p<0.001$) except fractional shortening, left ventricular ejection fraction, outflow tract gradient, early to late (E/A ratio) trans mitral flow velocity, and left ventricular systolic and diastolic volume.

3.4 Genotypic Characteristics

Phospholamban gene is screened for mutations at hotspots exons. Seven mutations, two in the 5' flanking region, two in exon 1 and three in the intron1 region were identified (Table 3). Electropherograms of the variants observed were represented in (Fig. 4).

Novel variants:

4887(T/G) Mutation: Transition T/G in 4887 nucleotide regions of 5' flanking region was observed in two HCM probands with four affected family members and also in one dilated cardiomyopathic proband with no affected relatives. In the pedigree of the H9 proband (male, 21years) (Fig. 5), the proband's father and his two uncles died young of sudden cardiac arrest. The younger brother of the proband's father has an affected son. The mutation was in heterozygotic state, and it is not observed in unaffected.

In the pedigree of H10 proband (Fig. 6), the T to G transition was observed in two family members The H10 proband (Male, age: 41years) had an affected offspring who was deceased subsequently to this study. The proband's parents are consanguineous and his mother died of cardiac arrest. The proband's brother had also carried this mutation and his daughter died unexpectedly at the age of 18 years.

15512 ins T Mutation: 15511-15512 insertion T was observed in one dilated cardiomyopathic proband with 2 affected family members and one dilated cardiomyopathic proband with no affected family members. The intronic mutation in PLN gene was observed in one dilated cardiomyopathy patient and in his family members (Fig. 7). The D15 proband (Male, age: 37 years) who carried this mutation has an affected father (66 years). The proband's father's brother died of sudden cardiac arrest at the age of 22 years. The mutation in their family members were in heterozygotic state and not found in unaffected.

4. DISCUSSION

Cardiomyopathies are diseases of the heart muscle and a cause of concern. They exhibit a wide spectrum in disease onset, manifestation as

well as in progression [16]. Significant differences were observed for age and sex ratio between control and patients with cardiomyopathy. Mean age of diagnosis was higher in the patient data of the present study than in studies of HCM and DCM previously reported [17,18]. This may be due to mutation carriers screened in a predictive setting represent an asymptomatic subgroup within the total population of affected. Besides age, gender was an important cofactor in the clinical manifestation of HCM and DCM [19]. The cardiomyopathy patients of the present study were characterized with significant increase in atrial natriuretic peptide and brain natriuretic peptide, which were correlated with left ventricular ejection fraction, mean pulmonary arterial pressure and pulmonary artery wedge pressure. Present data provides evidence that ANP and BNP are the best indicators for heart failure in cardiomyopathy patients. An elevated mean diastolic blood pressure (81.41 ± 4.76) and systolic blood pressure (125.0 ± 9.17) were observed in cardiomyopathy patient's data of the present study. A higher diastolic and systolic blood pressure has been observed in Caucasians [20], Chinese [21] and Japanese [22] origin.

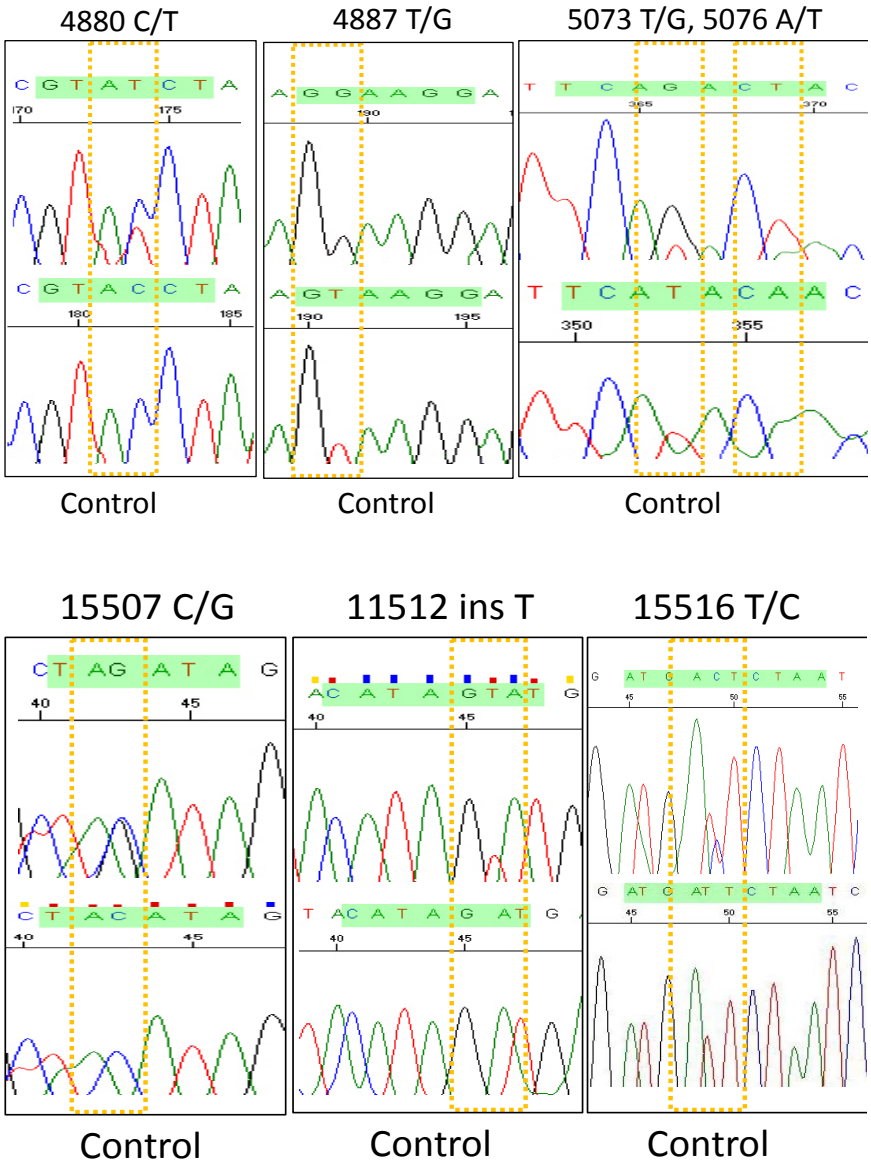

Fig. 4. Electropherograms of the variants observed

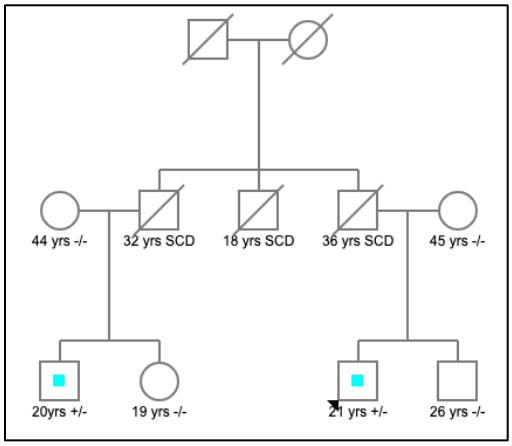

Fig. 5. Pedigree of H9 proband with 4887 T→ G mutations in 5'flanking region of PLN gene

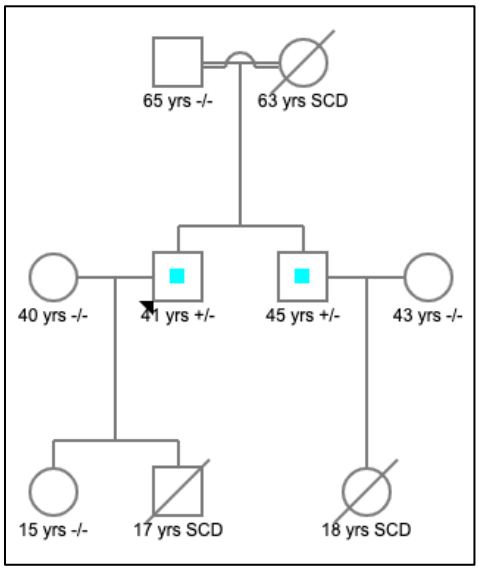

Fig. 6. Pedigree of H10 proband with 4887 T→ G mutations in 5'flanking region of PLN gene

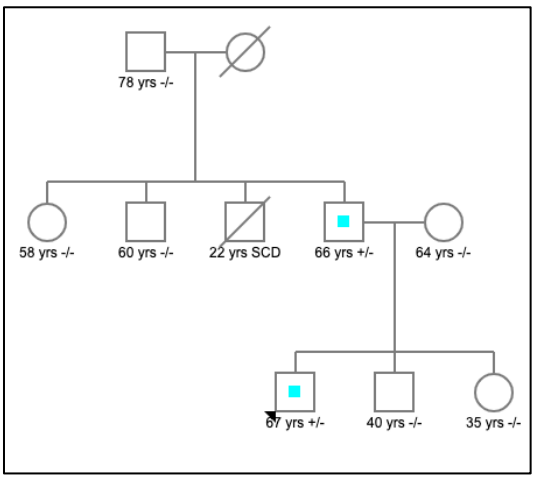

Fig. 7. Pedigree of D15 proband with 15512 ins T mutations in intron 1 of PLN gene

Note: +/- presence of mutation in heterozygote state; -/- absence of mutation; ↗ - Proband; Age below each indicates age at the time of investigation

Table 1. Baseline, hemodynamic and biochemical characteristics

Characteristics	Cases n=109	Control n=100	Cases vs Control P value
Age, Yrs	37.22 ± 12.43	40 ± 12.65	0.111
Gender			
Male, n	73	52	0.034
Female, n	36	48	
Parental Consanguinity, n (%)	15(13.76)	9	0.386
BMI (kg/m2)	24.65 ± 4.08	23.76 ± 3.33	0.085
BSA (m2)	1.79 ± 0.17	1.84 ± 0.75	0.515
Sys BP (mmHg)	125.0 ± 9.17	120.0 ± 14	0.003
Dys BP(mmHg)	81.41 ± 4.76	78.0 ± 10.0	0.002
Heart Rate (beats/min)	74.06 ± 4.08	72.3 ± 4.5	0.004
Familial status, n (%)	26(23.85)		
F/H of SCD, n (%)	33(30.28)		
NYHA			
Class I, II, n %)	37(33.94)		
Class III, IV, n (%)	72 (66.06)		
ANP (pg/ml))	130.21 ± 24.67	21.9 ±17.6	<0.001
BNP (pg/ml)	110.36 ±110.12	8.12 ± 8.08	<0.001
CpK ≥ 37U/L, n (%)	26(23.85)	21	0.74
SGOT ≥ 13U/L, n (%)	35(32.11)	20	0.059
SGPT ≥ 17U/L, n (%)	32(29.36)	30	1
Glucose (mg/dl))	96.16 ± 8.0	95 ± 7	0.265
Urea (mg/dl)	27.36 ± 2.29	27.92 ± 4.12	0.232
Creatinine (mg/dl)	0.90 ± 0.32	0.84 ± 0.19	0.098
HTN, n (%)	31(28.44)	17	0.07
DM, n (%)	39(35.78)	27	0.183
Obesity, n (%)	22(20.18)	21	1
Current ad Ex-Smokers, n (%)	64(58.72)	72	0.059
Current and Ex-Alcoholics, n (%)	54(49.54)	56	0.406

*Data shown as Mean ± Standard deviation or number or number (%) F/H of SCD- Family history of sudden cardiac death, BMI- Body mass index, BSA- Body surface area, sys BP, Dys BP systolic, diastolic blood pressure, NYHA- New York heart association functional class, ANPAtrial natriuretic peptide, BNP- Brain natriuretic peptide, CpK- Creatine phosphokinase, SGOTSerum Aspartate transaminase, SGPT- Serum alanine transaminase, HTN- Hypertension, DMDiabetes Mellitus. P value is probability of chi-square with one degree of freedom of genotype frequencies in control and case datasets. *Pvalue<0.05, ** Pvalue< 0.01, *** Pvalue< 0.001*

Phospholamban inhibits endogenous sarcoplasmic reticulum calcium ATPase in dephosphorylated condition and plays a regulatory role in the calcium handling during the process of cardiac contraction/relaxation. Mutations in this gene is shown to associate with elevated cytosol calcium concentration. Phosphomamban is phosphorylated by protein kinase A to increase the reuptake of calcium into sarcoplasmic reticulum [23,24,25]. Besides dilated cardiomyopathy though this is the first report to show Phospholamban gene mutations are associated with hypertrophic cardiomyopathy, none of the identified mutations falls within the coding region nor at conserved domain. Similar to this study there are few other studies had shown flanking regions and promoter variants of PLN associated with DCM/heart failure and HCM [26,27]. Alternatively, there are reports that show no associations [28,29]. It is possible that PLN gene mutations can express at a lower level leading to a smaller pathogenic effect during sixth decade of life in these individuals. Contrastingly two familial cases showed mutation of PLN (4887 T/G) possibly decrease the transcriptional activity of promoter and associated with hypertrophic cardiomyopathy.

The mutation that is located near the promoter region has been defined as the fragment with maximal transcriptional activity [30]. The increase or the decrease in phospholamban activity due to the disruption around the promoter region can lead to cardiomyopathy [27]. Some carriers do not exhibit clinical conditions and mutation may be due to other genetic and environmental factors. In conclusion, the mutations of PLN are

Table 2. Clinical characteristics of patients and control

Characteristics	DCM n=80+47=127	HCM n=29+23=52	Control n=100
LVESD(mm)	46.0 ± 6***	26 ± 7***	31 ± 5
LVEDD(mm)	71.5 ± 2***	69 ± 7***	52 ± 3
LVEF (%)	65 ± 6***	36 ± 7***	53 ± 7
FS (%)	39 ± 7**	38 ± 8	36 ± 7
IVST(mm)	18 ± 6***	11 ± 3***	8 ± 2
PWT(mm)	22 ± 5***	12 ± 2***	8 ± 2
LVMI (gm/m2)	106 ± 25***	106 ± 49***	61 ± 30
LV Volume/mass ratio (mL/gm)	0.77 ± 0.6***	0.18 ± 0.1***	0.30± 0.07
LVESV (ml)	127 ± 61***	38 ± 17*	32 ± 11
LVEDV (ml)	179 ± 7***	103 ± 37*	86 ± 24
LVOT gradient >20mmHg, n (%)	96 (75.59) **	39 (75)	57
E/A ratio	1.7 ± 1.1*	1.4 ± 0.9	1.3 ± 1
IVPG (mmHg)	1.1 ± 0.3***	1.4 ± 0.6*	1.6 ± 0.4
LAVI (ml/m2)	38 ± 14***	30 ± 9***	18 ± 7
QRS width (ms)	145 ± 11***	106 ± 14***	91 ± 16
PR (ms)	112 ± 11***	101 ± 9***	126 ± 8
Max QTc (ms)	434 ± 24***	455 ± 26***	400 ± 25

*Data shown as Mean ± Standard deviation or number (%) LVESD- Left Ventricular End systolic diameter, LVEDD-Left ventricular end diastolic diameter, LVEF- Left ventricular ejection fraction, FS- Fractional shortening, IVST- Inter ventricular septal thickness, PWT- Posterior wall thickness, LVMI- Left ventricular mass index, LAVI- Left atrial volume index, LVOT- Left ventricular outflow tract, E/A ratio- early-to-late transmitral flow velocity, IVPG- Intraventricular pressure gradient. P value is probability of chi-square with one degree of freedom of genotype frequencies in control and case datasets. *Pvalue≤ 0.05, ** Pvalue< 0.01, *** Pvalue< 0.001*

Table 3. Observed nucleotide changes in phospholamban gene

Nucleotide changes	Loci	Disease type	Familial case
4880 C/T	5' flanking region	DCM	No
4887 T/G	5' flanking region	HCM	Yes
5073 T/G	Exon1	DCM	No
5076 A/T	Exon1	DCM	No
15507 C/G	Intron1	DCM	No
15512 ins T	Intron1	DCM	Yes
15516 T/C	Intron1	DCM	No

associated with hypertrophic cardiomyopathy. Otherwise, mutations in the PLN gene are not a frequent cause of hypertrophic or dilated cardiomyopathy in our population.

5. CONCLUSION

The Present data provides evidence that atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are the best indicators for heart failure in cardiomyopathy patients, as there is significant increase which were correlated with left ventricular ejection fraction, mean pulmonary arterial pressure and pulmonary artery wedge pressure. Besides dilated cardiomyopathy, this is the first report to show Phospholamban gene mutations are associated with hypertrophic cardiomyopathy, but none of the identified mutations falls within the coding region nor at conserved domain. Otherwise, mutations in the PLN gene are not a frequent cause of hypertrophic or dilated cardiomyopathy in our population.

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ETHICAL APPROVAL AND CONSENT

The study was approved by the Institutional Review Board and a written consent was obtained from all subjects in accordance with the Institutional Ethical committee human subject guidelines.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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