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MOLECULAR CHARACTERIZATION OF PHOSPHOLAMBAN AND RENINANGIOTENSIN SYSTEM GENES MUTATIONS AND CLINICAL EPIDEMIOLOGY IN HUMAN CARDIOMYOPATHY

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ABSTRACT

Background

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.

Methods

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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.

Results

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 are We further deliberated the gene polymorphism of renin angiotensin system gene angiotensin-1-converting enzyme as an associated marker/ modifier in cardiomyopathy patients and their family members.

Conclusions

Information on epidemiological, clinical statistics, phospholamban gene mutation analysis and angiotensin-1-converting enzyme gene polymorphism 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 Angiotensin-1-converting enzyme.

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

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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 hypocontractile (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 four types of cardiomyopathies reported (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). The etiology of both HCM and DCM involve cardiac energy imbalances and the clinical expressions of them are based on the addictive factors that are involved in it.

The prevalence of the dilated cardiomyopathy has formerly been estimated at 36.5/100,000, with an incidence of 4-8/100,000 persons-year (5). An echocardiographic analysis of 4111 subjects in CARDIA study by (6) revealed hypertrophic cardiomyopathy affects 1 in 500 people, a prevalence similar to familial hypercholesterolemia. 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 (7). Most of the dilated Cardiomyopathy cases are sporadic; although 20–35% of them are familial (8). The incidence of dilated cardiomyopathy varies in men and women. However, in general, heart failure is more common in men (9). The treatment arm of the Studies on Left Ventricular Dysfunction (SOLVD), in which only 15% of the

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patients were women, reported no sex-related difference in survival in either the placebo group or the Enalapril group (10, 11). Age distribution depends on prognosis, diagnosis or onset of any underlying disease. However, advancing age is reported as an independent risk factor for mortality in several studies (12).

Phospholamban (PLN) is a small transmembrane phosphoprotein of 52 amino acids that plays an important role in cardiac contraction and relaxation. Phospholamban, expressed in the sarcoplasmic reticulum membrane controls cellular calcium levels by a mechanism that depends on its phosphorylation (13). The human ventricle and quadriceps displayed high levels of phospholamban transcripts and proteins (14), 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 (15, 16).

Susceptibility genes have a role in the development of cardiomyopathies, whereas modifier genes have a role in the evolution or prognosis of the disease. In most studies due to limited sample sizes, the role of susceptibility and modifier genes have been only suggestive (17). 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 (18). This variability in expression of these causal genes is also seen among family members sharing the same mutation. This variable expressivity, which confuses genotype/phenotype correlations, could be partially explained by both environmental influences and genetic modifiers (19). The angiotensin-1-converting enzyme (ACE) polymorphism has been commonly studied with variable results. It’s noteworthy to study

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and understand the importance of modifier genes. The genetic polymorphisms of the renin-angiotensin-aldosterone system *(*RAAS*)* are found to influence the progression to cardiac disorders (20). Angiotensin-1-converting enzyme (ACE), a modifier gene was perceived to have insertion/Insertion (I/I) genotype associated with low serum ACE activity levels, Insertion/Deletion (I/D) genotype with intermediate levels and Deletion/Deletion (D/D) high serum ACE activity levels. The effect of ACE polymorphism on survival in patients with DCM with a DD genotype had poorer prognosis than other genotypes (5-year survival rate 49 vs.72%, p=0.001) (21). The DD genotype was also associated with an increase in left ventricular mass (22).

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. We further studied the role of angiotensin-1-converting enzyme gene polymorphism as an associated marker/ modifier in cardiomyopathy patients and their family members.

MATERIALS AND METHODS

STUDY SUBJECTS

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A total of 109 unrelated index cardiomyopathy patients and their family members who were 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, were included in the present study. A panel of 100 age matched healthy control subjects with no history of heart disease was obtained. The study protocol 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.

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; dilated cardiomyopathy with Left ventricular ejection fraction (LVEF) <45% and left ventricular end diastolic diameter >27mm/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 ≤160/100mmHg. 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.

BLOOD SAMPLE COLLECTION

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

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.

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.

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

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154 examined a comprehensive screening of Phospholamban and a key determinant/modifier
155 angiotensin-1-converting enzyme gene polymorphism.
156
157 **DNA ISOLATION and QUANTIFICATION**
158 Genomic DNA was extracted from the peripheral blood following the
159 protocol of (23). Briefly, the collected blood was mixed with an equal volume of TKM1
160 buffer (10mM Tris-HCl, 10mM KCl,10mM $MgCl_2$ and 2mM EDTA) and 100 µl Triton X.
161 The contents were centrifuged and the pellet was washed with TKM1 repeatedly until the
162 cell debris is washed out. The pellet is suspended in 800 µl of TKM2 solution (10mM Tris-
163 HCl,10mM KCl, 10mM $MgCl_2$, 2mM EDTA and 0.4M NaCl) followed by centrifugation and
164 precipitation of the supernatant in ethanol. The DNA samples were then stored at -20°C
165 for subsequent analyses. The DNA was quantified using spectrophotometer. The DNA
166 was diluted in TE to yield 50ng/µl concentration.
167
168 **PHOSPHOLAMBAN (PLN) POLYMERASE CHAIN REACTION**
169 The primers of the hotspots exons of Phospholamban (PLN) forward primer 5'-
170 tatttttctcataattaaaattcctgc-3' and reverse primer 5'-aaagtaagaattaccaaagtcagcg-3' for
171 Exon 1 and forward primer 5'-aacaatagtgctgaggaagatgaa-3' and reverse primer 5'-
172 ttgttttcctgtctgcatgg-3' for Exon 2 were used (24).
173
174 **ANGIOTENSIN-1-CONVERTING ENZYME GENOTYPING**
175 The primers of the hotspots exons of the Angiotensin-1-Converting Enzyme (ACE)
176 forward primer 5'-tatttttctcataattaaaattcctgc-3' and reverse primer 5'-

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aaagtaagaattaccaaagtcagcg-3' were used (25). Angitensin-1-converting enzyme genotyping was based on amplification of genomic DNA by PCR and the products were detected on 2% agarose gel. Amplification products 490 bp and 190 bp corresponding to the I and D alleles respectively (26).

DNA SEQUENCING

Genomic DNA from individuals with different 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.

3.3. BIOINFORMATICS

Multiple Sequence Analysis (MSA) was used for sequence comparison between generated sequences of the present study and to the corresponding reference sequences obtained from GenBank. The generated gene sequences were translated as protein sequences for future analysis (ExPASY Tool). Using Conserved Domains Database and

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Motif Finder Database alteration in the conserved domains and motifs as a consequence of mutation were identified for the generated sequences.

3.4. STATISTICAL ANALYSES

Statistical analyses were carried out using Statistical Analysis Solutions (**SAS 9.2**). 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. The Allele frequencies were computed by gene counting method. Departure from Hardy- Weinberg equilibrium was tested by chi-square test. The odds ratio was computed using SAS.

RESULTS

EPIDEMIOLOGICAL STUDY

Patients and Baseline Characteristics

A total of 109 unrelated index patients who have echocardiographically and electrocardiographically assessed for cardiomyopathy and 100 aged matched control subjects were studied. About 73 percent of the patients had dilated cardiomyopathy and 27 percent hypertrophic cardiomyopathy. Details on age, body mass index, blood pressure, blood biochemical profile, associated clinical and non-clinical features of the patients, control subjects were given in (Table 1). Males were significantly higher among

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the patients (73%). The mean age of all patients was 37.22 ± 12.43 years. Occurrence was familial in 24% of cases, but sporadic in the other 76% of patients. In patients group about 30% of them had family history of sudden cardiac death and 14% of them showed parental consanguinity. Mean value of body mass index and body surface area was same in patients and control ($p=0.085$, $p=0.515$).

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 (Table 1). Similarly, co-morbid factors such as diabetes, obesity, smoking and alcoholism did not show any influence on patient population.

CLINICAL CHARACTERISTICS

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.

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248 **MOLECULAR ASPECTS**

249 **Phospholamban Gene Mutation**

250 Phospholamban gene is located on chromosome 6q22.1. Genomic sequence of the gene
251 is 12146 bps nucleotides long which encodes 1742 bps mRNA coding region comprises
252 159 bps which encodes 2exons. The protein is a pentamer and is a major substrate for
253 cAMP-dependent protein kinase in the cardiac muscle. The protein inhibits Ca2+-ATPase
254 in unphosphorylated state. The protein is a key regulator of cardiac diastolic function.
255 Table 3 shows the detailed description of the mutations in phospholamban gene. Seven
256 mutations –two in the 5' flanking region, two in exon 1 and three in the intron1 region
257 (Figure 1 (a-f)). Transition T/G in 4887 nucleotide regions of 5' flanking region was
258 observed in two HCM probands with four affected family members and also in one dilated
259 cardiomyopathic proband with no affected relatives. 15511-15512 insertion T was
260 observed in one dilated cardiomyopathic proband with 2 affected family members and
261 one dilated cardiomyopathic proband with no affected family members.

262

263 ***Description of Familial Condition***

264 **4887(T/G) mutation:**

265 In the pedigree of the H9 proband (male, 21years) (Figure 2), the proband's father and
266 his two uncles died young of sudden cardiac arrest. The younger brother of the proband's
267 father has an affected son. The mutation was in heterozygotic state and it is not observed
268 in unaffected.

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In the pedigree of H10 proband (Figure 3), 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:

The intronic mutation in PLN gene was observed in one dilated cardiomyopathy patient and in his family members (Figure 4). 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.

Angiotensin-1-Converting Enzyme Gene Polymorphism

The present study reports on the molecular screening of a population of 109 unrelated index patients and 100 controls. Mutational screening was performed in one modifier gene Angiotensin-1-converting enzyme (ACE) i.e., zinc metallopeptidase widely distributed on the surface of endothelial and epithelial cells (27). When in demand, renin activity leads to the conversion of angiotensinogen to angiotensin I. ACE then converts angiotensin I to angiotensin II, which have been implicated in the modulation of cardiac growth (28). The human ACE gene is located on chromosome 17q23 and includes 26 exons. Polymorphism involving the presence (insertion I/I) or absence (deletion D/D) of a 287-basepair sequence of DNA in intron 16 of this modifier gene, encoding for ACE with differences in plasma ACE levels is examined in Cardiomyopathy patients (29). Table 4

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illustrates variation in the genotype and allele frequency of the patient and control. Most notably the deletion homozygotes found to be higher in the patients. Also, the percent of heterozygote varied significantly among the two groups. Similarly, the deletion allele frequency was considerably higher in patients about 60% than controls (44%). The chi square value in patients and controls was 0.109 and 1.148 respectively.

The genotypes DD vs ID, DD vs II, ID vs II, DD + ID vs II, DD vs ID + II and D vs I was taken for the odds ratio comparison. All the odds ratio values were higher than the threshold value of 1. The odds ratio was highest for the genotype DD vs II (3.82) with a $\chi 2$ value of 10.59. This was followed by DD + ID vs II and ID vs II genotypes having an odds ratio of 2.79 ($\chi 2$ =8.60) and 2.27 ($\chi 2$=4.48) respectively. The DD vs ID genotype comparison had an odds ratio of 0.60 ($\chi 2$ =1.94). It should be noted that the $\chi 2$ value is taken to evaluate the significance of the genotypes. Patients with D allele tend to be a higher risk and modify the disease pattern when compared with I allele. The odds ratio was found out to be 1.14 with a $\chi 2$ value 12.09. In Table 5 the chi square and the odds ratio values were calculated to prove the significance of the genotype of patients and reference to the control.

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 (30). Significant differences were observed for age and sex ratio between control and patients with

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cardiomyopathy. Mean age of diagnosis was higher in the patient data of the present study than in studies of HCM and DCM previously reported (31,32). 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 (33). 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 (34), Chinese (35) and Japanese (36) origin.

Phospholamban is an inhibitor of endogenous sarcoplasmic reticulum calcium ATPase in dephosphorylated condition. It plays a regulatory role in the calcium handling during the process of cardiac contraction/relaxation. Mutations in this gene shown to associate with elevated cytosol calcium concentration. Phospholamban is phosphorylated by protein kinase A to increase the reuptake of calcium into sarcoplasmic reticulum **(**37,38,39**).**

Besides dilated cardiomyopathy though this is the first report to show Phospholamban gene mutation associated with hypertrophic cardiomyopathy, none of the all identified mutations falls neither 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 (40, 41**)**. Alternatively, there are reports

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that show no associations **(**42,43**).** 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 (44). The increase or the decrease in phospholamban activity due to the disruption around the promoter region can lead to cardiomyopathy **(**41**).** Some carriers do not exhibit clinical conditions and mutation may be due to other genetic and environmental factors. This study concludes that PLN gene mutations are not frequent cause of hypertrophic or dilated cardiomyopathy in Indian population.

Although similar data were lacking, it was observed that Indians show a greater variation and this may be due to the polymorphism in ACE gene (modifier gene). Further, role of a dual peptide system *viz*., brain, and atrial natriuretic peptides in sodium balance and blood pressure regulation in those patients cannot be ruled out (45). Angiotensin-1-converting enzyme gene insertion/deletion (I/D) polymorphism is considered as an important modifier gene, which may influence the clinical phenotype of the cardiovascular disorders (46). The present data revealed that the frequencies of the DD genotype and D allele were significantly higher in patients compared with controls, and were associated with increased risk of HCM and DCM. Furthermore, regression analysis revealed that the genotypes DD and D allele were independent risk factors for these cardiomyopathies in Indian population. Patients with the DD genotype had the highest odds ratio of disease susceptibility and the subjects with II genotype have a lower risk of developing

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cardiomyopathies that may possibly through a cardio protective effect. The D allele compared with I allele has more than 25 percent increased risk to cardiomyopathy. On comparison, the prevalence of D allele in the present study (Indian population) was slightly higher (about 60%) than Tunisian and Turkish populations (47,48). Association of DD genotype / D allele with HCM and DCM have been reporting in many studies (49,50) and the polymorphism considered as a modifier gene marker to cardiomyopathy. The DD genotype was also found to be associated with hypertension, restenosis, diabetes and myocardial infarction (51,52). However, absences of association (53,54) or influencing the cardiac phenotype (55) were reported in few other studies. These variations were partly accounted for ethnicity of the patients and to the sampling of the patients (56, 57).

CONCLUSION:

Among the two types of cardiomyopathies; dilated cardiomyopathy (73%) was found to be most predominant, whereas hypertrophic cardiomyopathy accounts for 27%. In general, increased male predominance (73%) was observed in both the types of cardiomyopathies. Familial occurrence was in 24% of the patients, parental consanguinity was 14% and 30% had family history of sudden cardiac death. Diastolic blood pressure of the cardiomyopathy patients was significant ($p<0.05$) than the systolic blood pressure and heart rate. Also, they were characterized with higher Atrial Natriuretic Peptide and Brain Natriuretic Peptide ($p<0.001$). Contrastingly co-morbid factors and other enzymes did not show much influence between patients and control subjects. Mutation screening of phospholamban gene revealed two transitions (4880 C/T, 4887 T/G) in 5' flanking region. Among them 4887 T/G transition was inherited in a hypertrophic cardiomyopathy

patient's family. Two transitions (5073 T/G, 5076 A/T) in exon 1 and two transitions (15507 C/G, 15516 T/C) and an insertion (15512 T ins) in intron 1 were observed. Mutations located near to the promoter region have been defined as the fragment with maximal transcriptional activity. Mutations of this gene cause inherited dilated cardiomyopathy with refractory congestive heart failure. The protein is involved in blood circulation, and calcium ion transportation. No mutations occurred in the coding region of this gene so there is no change in the protein sequence consequently. Mutations located close to the promotor region have been defined as the fragment with maximal transcriptional activity. The increase or decrease in phospholamban activity due to the disruption around the promoter region can lead to cardiomyopathy. No mutations were observed in control subjects. Further, the deletion allele frequency of angiotensin-1- converting enzyme was considerably higher in cardiomyopathy patients (61.5%) than the control subjects (44%).

Availability of data and materials:

All data relevant to the findings are available in this paper. We are bound by the participant consent which precludes data deposition in repositories. Further non-identifiable data is available upon request to **Dr. N. Gnana Veera Subhashini (dr.ngvsubhashini@bcgrc.com)**

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All data generated or analyzed during this study are included in this published article.

Authors' contributions

GVS, MJ, BSL, EC and KMC made substantial contributions to conception and design of the experiments, or to acquisition, analysis, or interpretation of the data; GVS, EC and KMC were involved in drafting or critically revising the manuscript for important intellectual content; BSL and GVS gave final approval of the version to be published. All authors agree to be accountable for all aspects of the work, and will ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

Consent for publication

The authors declare that they have received consent from study subjects/Participants for publication.

Competing interests

The authors declare that they have no competing interests in relation to this manuscript or this study.

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593

594 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

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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

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Current ad Ex-Smokers, n (%)	64(58.72)	72	0.059
Current and Ex-Alcoholics, n (%)	54(49.54)	56	0.406

595

596

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

605

606 **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

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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

607

608

609 Data shown as Mean ± Standard deviation or number (%) LVESD- Left Ventricular End
610 systolic diameter, LVEDD-Left ventricular end diastolic diameter, LVEF- Left ventricular
611 ejection fraction, FS- Fractional shortening, IVST- Inter ventricular septal thickness, PWT-
612 Posterior wall thickness, LVMI- Left ventricular mass index, LAVI- Left atrial volume index,
613 LVOT- Left ventricular outflow tract, E/A ratio- early-to-late transmitral flow velocity, IVPG-
614 Intraventricular pressure gradient. P value is probability of chi-square with one degree of

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615 freedom of genotype frequencies in control and case datasets. *Pvalue≤ 0.05, ** Pvalue<
616 0.01, *** Pvalue< 0.001.

617

618 **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

619

620 **Table 4. Alu ACE1 Genotype and Allele frequencies in control and Patients with**
621 **cardiomyopathy**

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Subject	Genotype			Allele Frequencies		χ^2
	II	ID	DD	I	D	
Control	34	44	22	0.56	0.44	1.148
Patients	17	50	42	0.385	0.615	0.109

Table 5. Frequency distribution of Alu ACE1 Genotype and allele frequency in Control and Patients of cardiomyopathy

Case Vs Control	χ^2	P value	ODDs Ratio	95 % CI	
ID vs DD	1.9358	0.1641	0.6	0.309	1.147
DD vs II	10.589	0.0011	3.82	1.754	8.311
ID vs II	4.4768	0.0344	2.27	1.118	4.619
DD + ID vs II	8.6037	0.0034	2.79	1.437	5.408
DD vs ID + II	5.9538	0.0147	2.22	1.207	4.092
D vs I	12.088	0.0005	1.14	0.773	1.687

The present study investigates the association of spectrum of clinical symptoms and the epidemiological variables like age, sex, familial status, and parental consanguinity. The current study also evaluates Angiotensin-1-Converting Enzyme gene polymorphism and its role as a marker / modifier in Cardiomyopathy.

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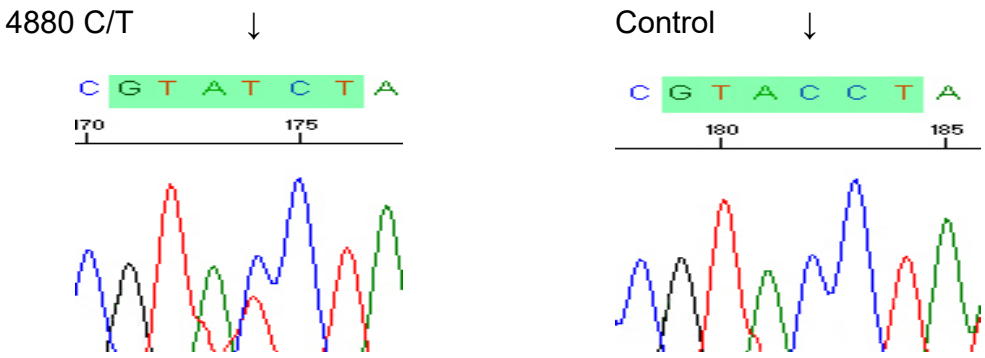
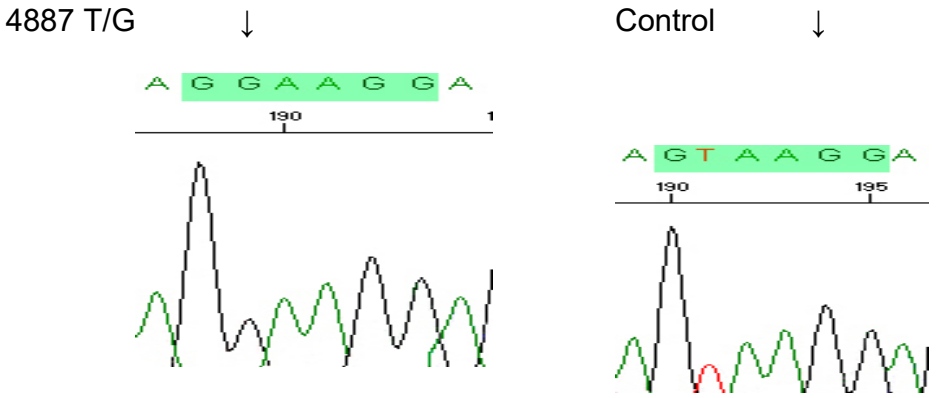
632 **Figure 1: Chromatogram of Phospholamban Gene Mutation**

633

634 (a) Representative Chromatograms showing 4880 C/T



(b) Representative Chromatograms showing 4887 T/G



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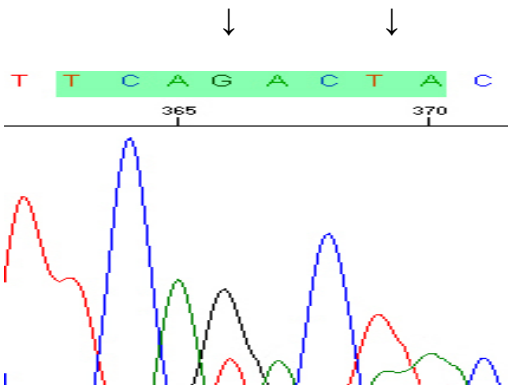
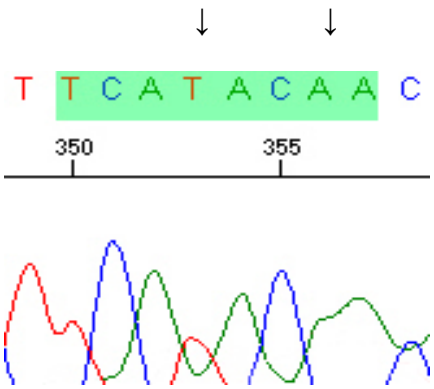
(C) Representative Chromatograms showing 5073 T/G, 5076 A/T

5073 T/G ,5076 A/T

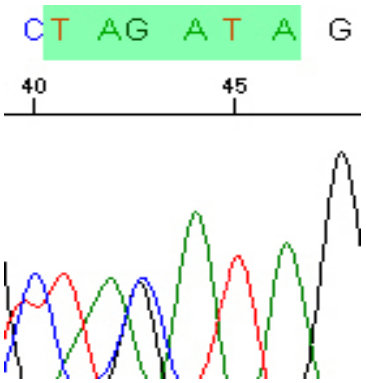


Control

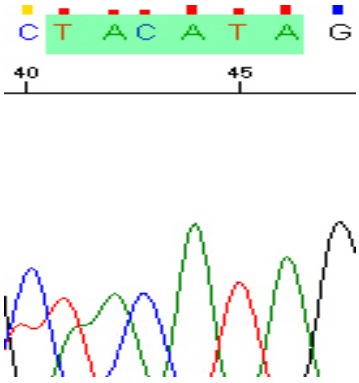


(d) Representative Chromatograms showing 15507 C/G

15507 C/G



Control



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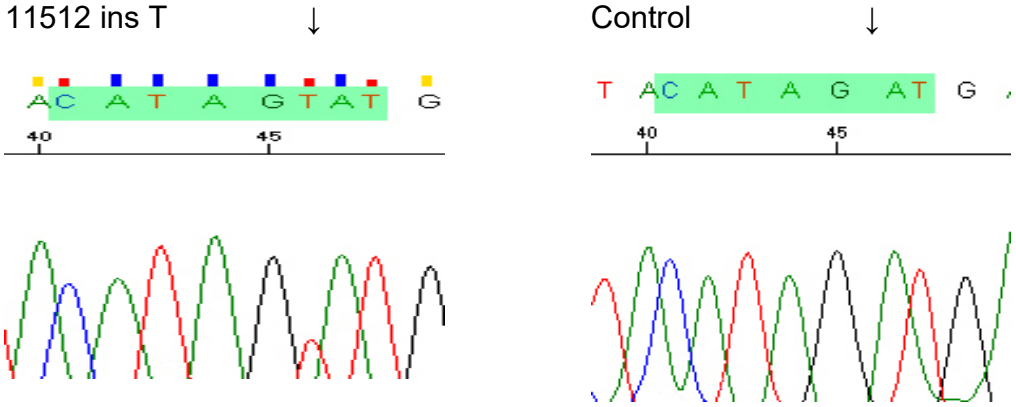
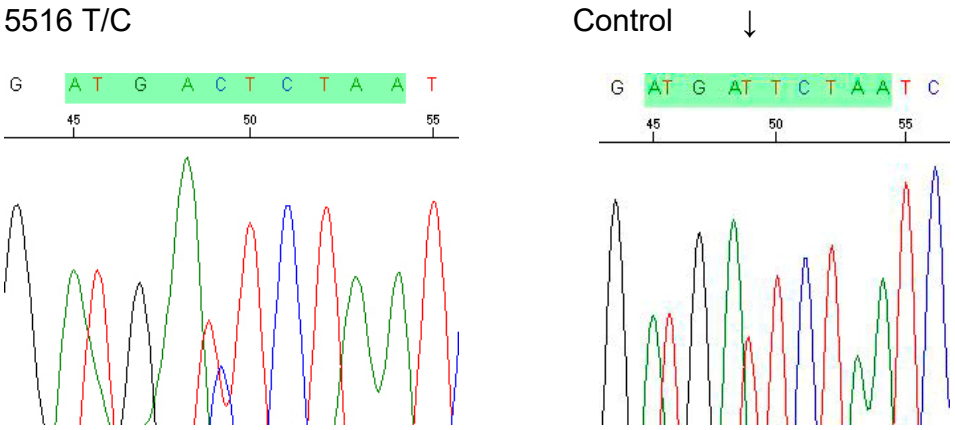
(e) Representative Chromatograms showing 15512 ins T



(f) Representative Chromatograms showing 15516 T/C



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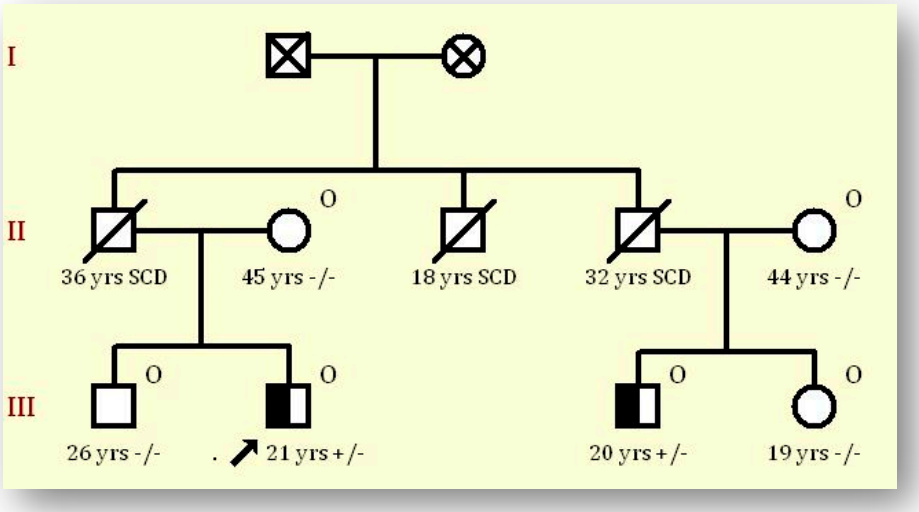
641 **Figure 2: Pedigree of H9 proband with 4887 T→ G mutations in 5'flanking region of**

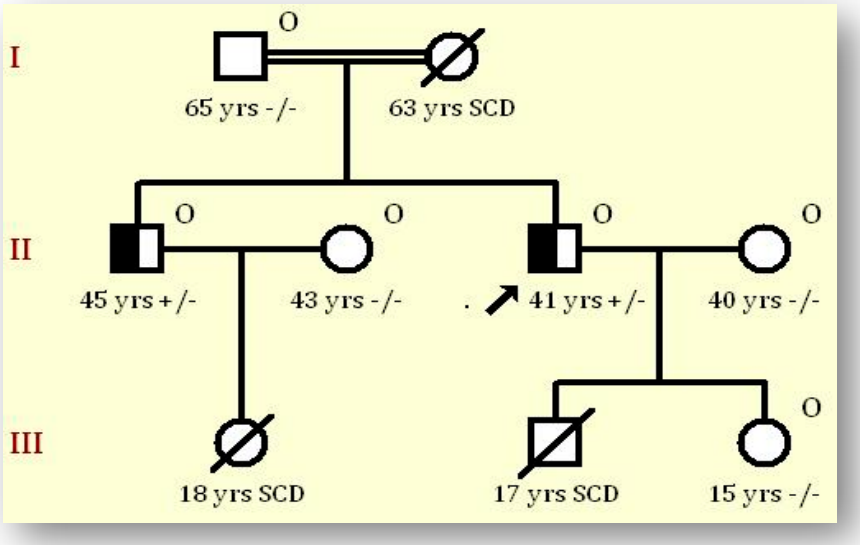
642 **PLN gene**



643

644 **Figure 3: Pedigree of H10 proband with 4887 T→ G mutations in 5'flanking region**

645 **of PLN gene**



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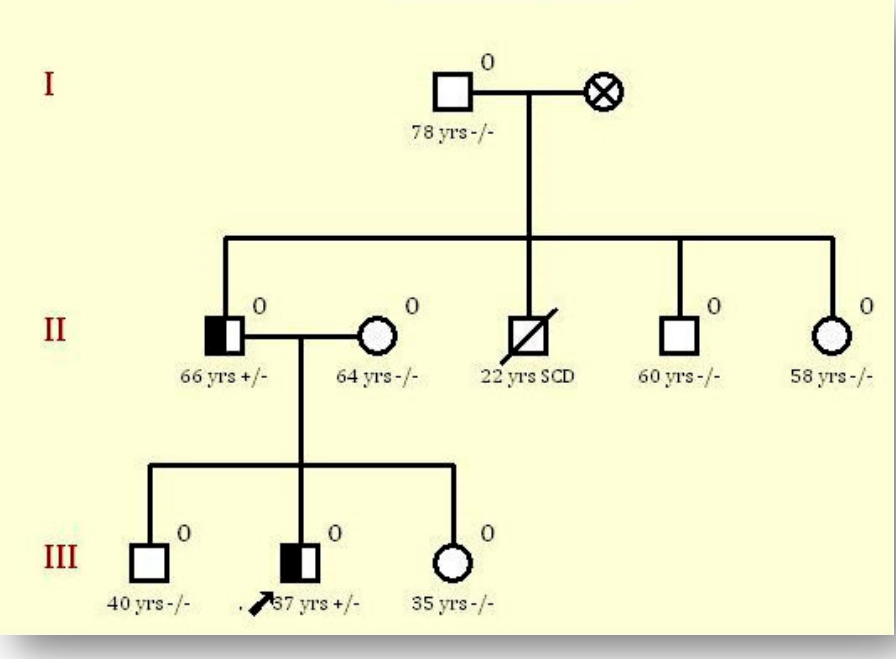
647 **Figure 4: Pedigree of D15 proband with 15512 ins T mutations in intron 1 of PLN**

648 **gene**

649

650



651

652 **Note:** +/- presence of mutation in heterozygote state; -/- absence of mutation; ↗ -

653 Proband;

654 o- Gene Sequenced; Age below each indicates age at investigation