

Original article:

**ATRIAL NATRIURETIC PEPTIDE GENE –
A POTENTIAL BIOMARKER FOR LONG QT SYNDROME**

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ABSTRACT

This study highlights the possible implication of *NPPA* (natriuretic peptide precursor A) gene in the etiology of Long QT syndrome (LQTS) by population-based as well as familial study. Three SNPs of *NPPA* - C-664G, C1363A and T1766C were examined by molecular analyses in LQTS, controls and first degree relatives (FDRs). This study revealed a possible association of 1364 C>A SNP 'C' allele with LQTS ($p = 0.0013$). All three SNPs were in tight linkage disequilibrium. The familial study highlights the association of *NPPA* SNP with cLQTS and implicating it as a potential biomarker in South Indian population.

Keywords: Atrial natriuretic peptide, Long QT syndrome, cardiogenesis, biomarker, polymorphisms

INTRODUCTION

Long QT syndrome (LQTS) includes a group of rare inherited diseases caused by cardiac ion channel gene mutations, resulting in prolonged QT due to ventricular repolarization and polymorphic ventricular tachycardia (torsade de pointes) (Kapetanopoulos et al., 2006). Congenital LQTS causes sud-

den death in young people and is mostly familial (Hunter et al., 2008). Genotype-phenotype study of LQTS emphasizes high risk for first cardiac events during adolescence (Moss, 1998).

The heart as an endocrine organ secretes the cardiac hormones atrial natriuretic peptide (ANP) and brain natriuretic peptide

(BNP) (Cameron and Ellmers, 2003). Atrial natriuretic peptide is a 28–amino acid circulating hormone with natriuretic, vasorelaxant, antihypertrophic, and antifibrotic properties (Francia et al., 2013; Bonow 1996). It is well represented in the cerebral areas involved in cardiovascular regulation (Rubattu et al., 1999).

Substantial modifications in ANP gene expression have been shown to occur during the dramatic circulatory changes that take place during the perinatal period (Ernest et al., 1998). In both human and ovine fetal ventricles, ANP mRNA is considerably higher than in adult ventricles and tends to decrease with gestational age. In addition to cell growth, ANP modulates inhibition of vascular smooth muscle proliferation, cardiac hypertrophy and induction of apoptosis in cultured cardiac myocytes (Cameron and Ellmers, 2003). The developmental natriuretic peptide precursor A (*NPPA*) gene expression pattern has served to gain an insight into the transcriptional program governing cardiac chamber development (Houweling et al., 2005). Increased ANP expression has been implicated as a marker in embryonic gene program induction during the development of ventricular hypertrophy (Rubattu et al., 1999; Cameron and Ellmers, 2003).

The *NPPA* gene, localized to 1p36.2 region, encodes the precursor of ANP, a 2 kb gene composed of 3 exons (Lynch et al., 2009). Polymorphisms of *NPPA* have been associated with cardiovascular disease, hypertension, diabetic nephropathy, atrial fibrillation and ischemic stroke (Lynch et al., 2009; Francia et al., 2013). To date, no prospective genetic-epidemiological data is available with regards to the *NPPA* polymorphisms and Long QT syndrome. Therefore, three SNPs of *NPPA* - a promoter SNP C-664G, an intronic SNP C1363A and an exonic SNP T1766C were analysed for the possible associations with LQTS in comparison to controls and first degree relatives (FDRs).

METHODOLOGY

Study subjects

Blood samples were collected for molecular and genetic analyses from confirmed 45 LQTS probands and 69 first degree relatives referred to Care Hospitals, Hyderabad, Sri Jayadeva Institute of Cardiovascular Science and Research, Bangalore, Institute of Maternal and Child Health, Calicut Medical College, Calicut and Krishna Institute of Medical Sciences, Hyderabad. The QTc of the LQTS patients and their FDRs was confirmed by electrocardiogram. LQTS patients with prolonged QTc with/without syncope and family history of sudden cardiac death were included in the study. Available first degree relatives of the LQTS probands (with/without any history of cardiovascular disease) were also included. This study has been approved by the Institutional Ethics Committee, Department of Genetics, Osmania University, Hyderabad and informed written consent was obtained from the probands and their available family members. Blood samples from 150 controls (75 M: 75 F), without any history of cardiovascular or systemic conditions, was collected from Osmania General Hospital, Hyderabad for comparative analysis.

Molecular analyses

Genomic DNA was isolated from peripheral blood samples by standard protocols in 150 controls, 45 probands and their family members. For genotyping of *NPPA* gene SNPs [C-664G, C1364A (*Hpa II*), T1766C (*Sca I*)], PCR-RFLP protocol by Kato et al (2000) was followed. The digested products were later checked on 10 % native PAGE gel followed by silver staining for genotyping of these polymorphisms.

Statistical analysis

Fisher's exact test and odds risk estimate were computed for possible genotype association with LQTS. Interactive SNPs were analyzed by means of logistic regression (OR) to determine the significance of risk genotypes at 95 % confidence interval (CI)

followed by haplotype frequency computation by the EM algorithm using SNPstat software (Sole et al., 2006). Linkage disequilibrium was inferred by Haploview software (Barrett et al., 2005). Hapmap was also created to compare allelic frequencies with different populations from the International Hapmap project.

In-silico analysis

In-silico analysis was carried out to examine the influence of polymorphism in promoter region for effective transcription factor binding by “TFSEARCH” (<http://www.cbrc.jp/papia/>), mRNA secondary structure changes (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>) (Lorenz et al., 2011), splice site changes (www.cbs.dtu.dk/services/NetGene2/) (Hebsgaard et al., 1996) and possible binding site variations for SnRNP’s involved in spliceosome formation (<http://sfmap.technion.ac.il/>) (Paz et al., 2010), protein secondary structure (<http://bioinf.cs.ucl.ac.uk/psipred/>) (Buchan et al., 2013) were also elucidated.

RESULTS

Molecular analysis

PCR-RFLP analysis was carried out for *NPPA* genotyping on 150 controls, 45 LQTS patients and 69 first degree relatives (FDR) of LQTS patients. Interestingly, in case of -664 C>G and 1766 T>C SNPs, only ‘CC’ and ‘TT’ genotypes respectively were identified. Similarly, for 1364 C>A polymorphism, only CC and CA genotypes were observed.

Statistical analysis

Genotypic and allelic frequency distribution of *NPPA* polymorphisms was calculated

for controls, patients and FDRs (Table 1). ‘CC’ genotypic frequency in LQTS patients was two-fold (20 %) and three-fold in FDRs (28 %) when compared to controls (10 %). Similarly, allelic frequency was also found to be higher in LQTS patients (0.6) and FDRs (0.64) than controls (0.55) thus, indicating a possible association of ‘C’ allele with LQTS.

Odds risk estimates was computed for LQTS and FDRs in comparison to controls (Table 2). The odds ratios were found to be significant with respect to ‘CA’ genotype when FDRs were compared to controls [OR=0.29 (0.14-0.62), p=0.0013] whereas the other comparisons did not reveal any significant values.

Haplotype and linkage disequilibrium (LD) analysis

Haplotype frequencies for the various allelic combinations of the three polymorphisms (*NPPA* -664 C>G, 1364 C>A and 1766 T>C polymorphisms) were computed for their possible association with LQTS (Table 3). In continuation with the above results, the CCT haplotype frequency was higher in patient group (0.6) and FDRs (0.64) compared to controls (0.55) which indicates susceptibility of this haplotype to LQTS. Correspondingly, the ACT haplotype was found to confer protection (p = 0.0014).

A pair-wise comparison of the three polymorphisms, depicting the LD measures was carried out. Significant D’ values were observed for all the combinations i.e -664 C>G and 1364 C>A SNPs (D’= 0.96), -664C>G and 1766 T>C (D’= 0.96) and 1364 C>A and 1766 T>C (D’= 0.98), indicating a strong/tight linkage disequilibrium between the SNPs.

Table 1: Genotypic and allelic frequency distribution of the 3 polymorphisms of *NPPA* in controls, LQTS patients and FDRs

SNP	Genotype	Controls n (%)	LQTS n (%)	FDR n (%)	Allele	Controls	LQTS	FDR
C-664G	CC	150(100)	45(100)	69(100)	C	1	1	1
T1766C	TT	150(100)	45(100)	69(100)	T	1	1	1
C1364A	CC	15 (10)	9 (20)	19(28)	C	0.55	0.6	0.64
	CA	135 (90)	36 (80)	50(72)	A	0.45	0.4	0.36

Table 2: Odds risk estimates for the C1364A polymorphisms ($p < 0.05$)

	Genotype	OR (95 % CI)	p value
Ctrls vs LQTS	CC	1.00	0.08
	CA	0.44 (0.18-1.10)	
	C vs A	0.81 (0.21-3.06)	0.76
Ctrls vs FDRs	CC	1.00	0.0013
	CA	0.29 (0.14-0.62)	
	C vs A	0.68 (0.38-1.21)	0.19
FDRs vs LQTS	CC	1.00	0.36
	CA	1.52 (0.62-3.74)	
	C vs A	0.84 (0.47-1.49)	0.56

Table 3: Haplotype frequencies of the 3 polymorphisms in controls and LQTS groups ($p < 0.05$)

	Haplotype	Haplotype frequency		Odds Ratio (95 % CI)	p
		Controls	LQTS		
Ctrls vs LQTS	CCT	0.55	0.6	1.00	-
	ACT	0.45	0.4	0.45 (0.18-1.11)	0.083
Ctrls vs FDRs	CCT	0.55	0.64	1.00	
	ACT	0.45	0.36	0.29 (0.14-0.61)	0.0014
FDRs vs LQTS	CCT	0.64	0.6	1.00	
	ACT	0.36	0.4	1.48 (0.6-3.65)	0.4

In-silico analysis

The *NPPA* -664 C>G promoter polymorphism leads to the creation of a new binding site for transcription regulators ADR1 [alcohol dehydrogenase (ADH) II synthesis regulator] which may influence the dysregulation of transcription resulting in a truncated protein.

Intronic SNP 1364 C>A, increases free energy from -110 Kcal/mol to -108 Kcal/mol leading to a decrease in thermodynamic stability of mRNA secondary structure. The acceptor splice site at 133rd position was also affected (confidence value changes from 0.17 to 0.07). The polymorphism seems to affect the binding sites for exonic splicing silencers – hnRNPA1, hnRNPA2B1, hnRNPF,

hnRNPH1 and exonic splicing enhancers - 9G8 and Tra2beta. These modifications further may influence the downstream signaling finally affecting ANP peptide synthesis.

Exonic 1766 T>C polymorphism located within exon 3 results in a change from a stop codon to an arginine amino acid. Protein secondary structure prediction revealed an increase in helix length and coils. The ANP protein folding maybe influenced by this SNP as a basic arginine is added to the neutral tyrosine N-terminal of ANP.

Hapmap

Hapmap created to compare allelic frequencies across populations in -664 C>G SNP shows a higher 'C' allele frequency in

all the reported populations. With respect to 1364 C>A polymorphism, the ‘A’ allele was found to be higher in the South Indian population only adding to the genetic diversity. In case of 1766 T>C SNP, ‘C’ allele frequency was found to be variable in all populations (Figure 1).

Genotype-phenotype correlation

The ‘CC’ genotype of 1364 C>A SNP was observed only in nine LQTS patients. Since, the presence of ‘C’ allele confers a risk to LQTS, the clinical features of these 9 LQTS patients expressing ‘CC’ genotype were examined to establish genotype-phenotype correlation with the important clinical features of LQTS i.e syncope,

consanguinity, family history of sudden death and deafness. Of the nine LQTS patients with ‘CC’ genotype, 77.7 % were cLQTS and predominantly females. It is also observed that 66.6 % exhibited parental consanguinity over generations, 44.4 % revealed a history of sudden deaths and 55.5 % had a history of syncope clearly establishing the susceptible allele ‘C’ in the etiopathogenesis of cLQTS in families (Table 4).

Family study

Three families revealed quite interesting results with respect to 1364 C>A SNP wherein the CC genotype was found in the proband, parents and siblings (Table 5).

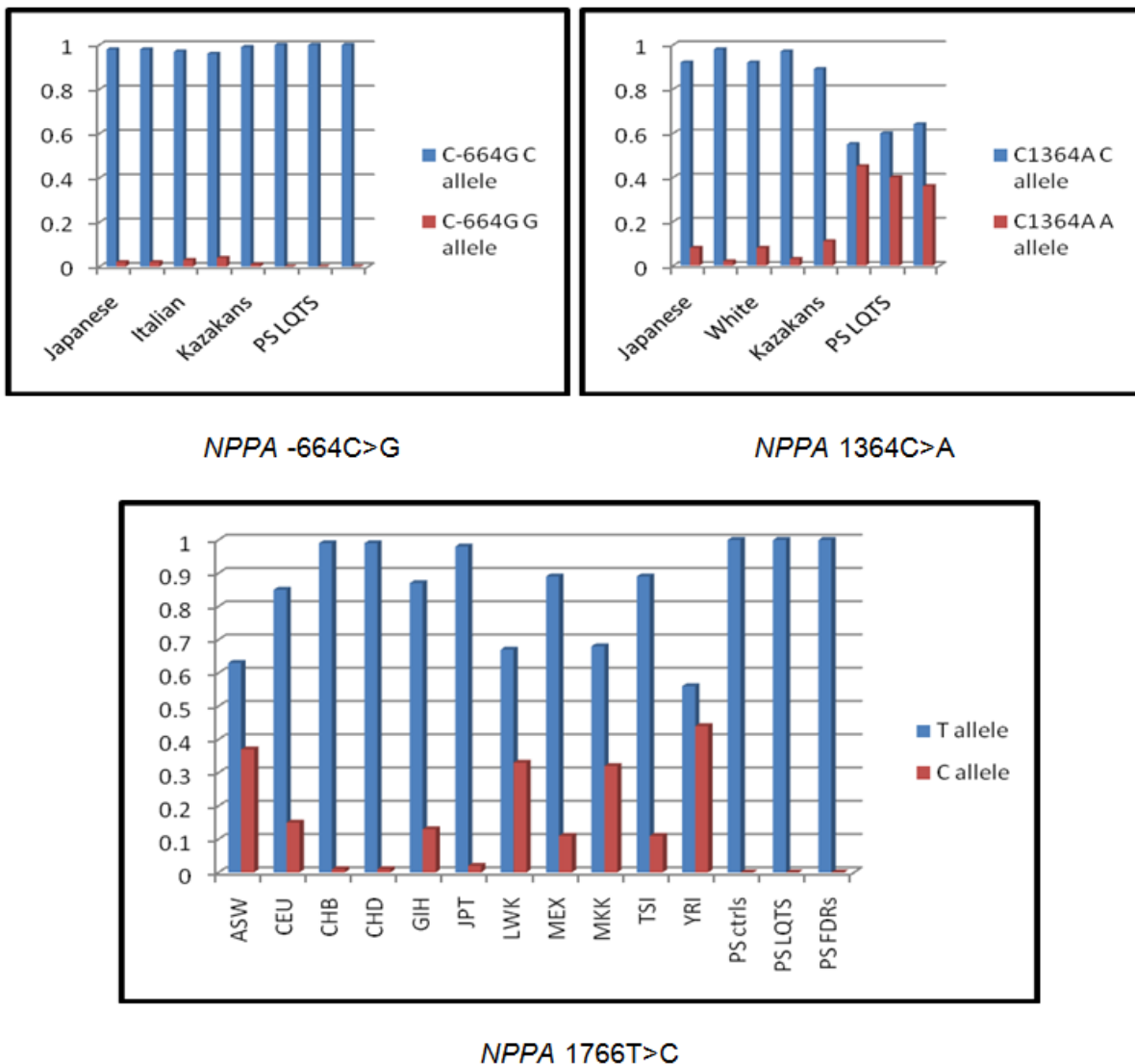


Figure 1: Hapmap of NPPA polymorphisms

Table 4: Clinical characteristics of 9 LQTS patients exhibiting CC genotype of *NPPA* 1364C>A polymorphism

cLQTS n(%)	Females n (%)	Deafness n (%)	F/h Sudden death n (%)	Consanguinity n (%)	Syncope n (%)
7 (77.7)	7 (77.7)	1 (11.1)	4 (44.4)	6 (66.6)	5 (55.5)

Table 5: Comparison of characteristics in three LQTS families with 'CC' genotype of *NPPA* 1364 C>A SNP

	Family 1	Family 2	Family 3
CC genotype subjects	Proband Mother (also cLQTS patient)	Proband (cLQTS) Father Mother Sibling	Proband Mother (also cLQTS patient) Sibling Maternal uncle (mother's sibling)
Deafness in proband	Present	-	-
Consanguinity	No	Present	No
Syncope in proband	Yes	Yes Father and sibling also exhibited H/o syncope	Yes

Both proband and his mother in family-1 exhibited the 'CC' genotype. Family-3 revealed a similar observation in the proband, his mother, sibling and maternal uncle. Family-2 presented both parents and sibling of proband with 'CC' genotype and this family had a history of syncope (most significant symptom of LQTS) in father and sibling. All the families revealed cLQTS apart from exhibiting consanguinity and syncope as common features. The presence of 1364 CC genotype in three probands and their parents (with LQTS or h/o syncope) may possibly implicate the developmental gene *NPPA* in the etiology of LQTS especially cLQTS which is also corroborated with the above findings.

DISCUSSION

This study highlights the possible implication of *NPPA* gene in the etiology of Long QT syndrome by population-based as well as family study. Three polymorphisms (-

664C>G, 1364 C>A, 1766T>C) have been considered to establish an association with LQTS.

Genotyping of -664C>G revealed only 'CC' genotype in the present study. Allele frequencies were found to vary depending on ethnic background as shown in hapmap. In accordance with our study, -664 C allele frequency was found to be higher in Japanese subjects (Kato et al., 2000). The -G664C variant has been described in association with early hypertension and left ventricular hypertrophy (Francia et al., 2013; Conen et al., 2007; Yanmin, 2006).

1766T>C polymorphisms in this study revealed only 'TT' genotype. The C allele frequency of this polymorphism; which was absent in the present study; was 0.26 in French whites, 0.11 in Mauritian Indians and 0.42 in hypertensive and 0.39 in normotensive individuals of black origin attributing to the diversity of this polymorphism (Nannipieri et al., 2001). 1766 T>C SNP had no

association in American population with atrial fibrillation and essential hypertension in Xinjiang Kazakhs (Francia et al., 2013; Conen et al., 2007, Yanmin, 2006).

In case of 1364 C>A only 'CC' and 'CA' genotypes were observed. The 'C' allele of 1364 C>A was higher in LQTS patients and FDRs indicating its possible role in LQTS etiology. 1364 C>A SNP has been shown to have an association with hypertension in black Africans (Kato et al., 2000). This study revealed a possible role of 1364 C allele in LQTS etiology. In-silico analysis of intronic SNP 1364 C>A predicted its effect on mRNA thermodynamic stability; splice site and spliceosome factor binding sites which may alter the downstream regulation of ANP peptide synthesis leading to a possible malfunction during cardiogenesis thus, playing a role in LQTS pathology.

On considering the LQTS patients with homozygous 'CC' genotype, our study established an association with cLQTS females who presented with syncope and consanguinity. Such genotype-phenotype correlations can be advantageous in subjecting patients exhibiting the above parameters to a genetic analysis of *NPPA* polymorphisms and in risk stratification of FDRs. This is the first study to the best of our knowledge to report the phenotypic/diagnostic parameters with CC genotype of 1364C>A in LQTS. Further, earlier studies have reported an association of minor allele of C-664G with increased Left Ventricular (LV) mass index, LV posterior wall thickness, LV septal thickness and relative wall thickening. Similarly, TT genotype at T1766C was correlated with risk to hypertension among those with BMI > 85th percentile (Lynch et al., 2009).

Three families each with two clinically affected/symptomatic LQTS probands exhibited CC genotype of 1364 C>A SNP. All these probands exhibited cLQTS with a history of parental consanguinity and syncope. Since, the observed probands of this study exhibited congenital LQTS, it can be deduced that 1364 C>A SNP of *NPPA* may be implicated in the etiology of cLQTS by in-

fluencing cardiogenesis regulation. Therefore, this *NPPA* polymorphism can act as a potential biomarker for cLQTS and may help in risk stratification of family members by predictive/genetic testing.

Further, the higher frequency of 'CCT' haplotype in LQTS patients may substantiate as a potential biomarker in LQTS. This was confirmed by a tight LD between -G664C, C1364A and T1766C polymorphic loci thus, implicating the developmental *NPPA* gene as a potential biomarker.

CONCLUSION

Our study hypothesizes the possible implication of *NPPA* 1364 C>A SNP in the etiology of LQTS in South Indian population. Since, all the three SNPs are in complete LD, *NPPA* can be considered a candidate gene for biomarker/diagnostics of cLQTS. The family study highlights the involvement of *NPPA* 1364 C>A SNP in the etiology of LQTS during cardiac organogenesis in the embryo. Functional and large population studies are warranted to confirm our hypothesis. This is the first family study to the best of our knowledge to report the association of LQTS with *NPPA* 1364 C>A polymorphism and the possibility to be considered a biomarker for LQTS.

AUTHOR'S CONTRIBUTIONS

Sameera Fatima Qureshi has carried out the molecular, hapmap and In-silico analysis described in this manuscript and has compiled the manuscript. Altaf Ali has helped to carry out the in-silico analysis described in this manuscript. Ananthapur Venkateshwari has interpreted the results described in this manuscript. The probands described in this manuscript have been diagnosed for LQT syndrome by M. P. Jayakrishnan, Calambur Narasimhan, Jayaprakash Shenthar and Hygriv Rao at their respective hospitals. Kumarasamy Thangaraj has been critical in the review and compilation of the manuscript. As the corresponding author, the concept,

design and compilation of this manuscript has been carried out by Pratibha Nallari.

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Competing interest

There is no conflict of interest within the authors / no declarations of interest.

Consent

Informed written consent was obtained from the probands and their family members.

Ethics Committee approval

The study has been approved by the Institutional Ethics Committee, Department of Genetics, Osmania University, INDIA obtained on 24th August 2009.

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