# MOLECULAR GENETIC ANALYSIS OF NEUROPEPTIDE Y (NPY) GENE IN PATIENTS WITH CARDIAC ARRHYTHMIA

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**ABSTRACT:**

***Background:*** *The heart is powered by an electrical impulse that signals the heart to contract, each at a proper time. Cardiac arrhythmia is any group of conditions in which the electrical activity of the heart is irregular or is faster or slower than normal. In arrhythmia, the heart rate either goes beyond 100 or below 60 beats per minute and this is called tachycardia and bradycardia respectively. NPY is co release with norepinephrine during sympathetic nerve stimulation, and is extensively involved in cardiovascular regulation because it modulates heart rate, cardiac excitability, and ventricular function as well as coronary blood flow.*

***Materials and Methods:*** *In this study, we included 40 arrhythmia and 46 healthy unrelated individuals to examine the NPY gene polymorphisms. All four exons were screened using PCR and sequencing method.*

***Results:*** *Three polymorphisms (Leu7Pro, Ser50Ser and A7735G) and one novel mutation (G172T) were obtained. Association was found only between one marker (Ser50Ser) and the arrhythmia. Weak linkage disequilibrium (LD) was seen between all the pairs of Single Nucleotide Polymorphism (SNPs). The LD between Ser50Ser and A7735G was found to be significant. The distribution of haplotypes in arrhythmia and normal was not statistically significant.*

***Conclusion:*** *NPY gene Leu7Pro polymorphism, which has been reported earlier as a potential cause for many of the cardiac problems, is not associated with arrhythmia in Indian population.*

***Keywords:*** *Neuropeptide Y, Arrhythmia, SNP, Polymorphism.*

***SRJM 2009; 1(2): 8 - 13***

## INTRODUCTION:

Cardiac arrhythmia is any group of conditions in which the electrical activity of the heart is irregular or is faster or slower than normal. In a normal resting adult the heart rate should be with in 60 beats to 100 beats per minute. In arrhythmia, the heart rate either goes beyond 100 or below 60 beats per minute and this is called tachycardia and bradycardia respectively. Some of the symptoms of arrhythmia may be palpitations, feeling light headed, and oozing consciousness, shortness of breath and chest pain. Cardiac arrhythmias constitute a major cause of death and disability, with an estimated 300,000 cases of sudden cardiac death annually in the United States alone (1). The heart is powered by an electrical impulse that signals the heart's four chambers to contract, each at a proper time. This can be measured using an Electrocardiogram (ECG). The electrical activity seen is created by sequential and sometimes simultaneous activity of a number of channels in the muscle membrane of the heart tissue which allow certain ions to cross into or out of the cell. These may be sodium, calcium, potassium and some chemicals such as acetylcholine and ATP. Ion channels are pore forming transmembrane proteins that selectively conduct ions and play physiological roles in many cells such as the neurons, skeletal muscle, cardiac muscle and smooth muscle (2). Inherited mutations in genes encoding for ion channels are associated with arrhythmia, also called channelopathies. Mutations in the potassium and sodium channels encoded by SCN5A, KCNQ1, KCNH2, KCNE1, KCNE2 genes are thought to account for 50% to 75% of cases of congenital long QT syndrome (LQTS) and 15% to 30% of Brugada syndrome cases (3).

Neuropeptide Y is 36 amino acid peptide that consists of an alpha-helix folded underneath a proline helix with a tyrosine residue at the carboxy terminus. NPY gene is located on the long arm of human chromosome 7 (7q15.1) (4) and it is divided into and consists of four exons (5). The gene is highly conserved with 92% amino acid sequence identity between the cartilaginous fish Torpedo marmorata and mammals, which are separated by an evolutionary distance of more than 400 million years (6). It is co localized with norepinephrine in both central and peripheral noradrenergic neurons. Studies show that NPY can exert acute effects on post junctional cardiac ion channels (7). NPY has also shown to inhibit contractility in rat heart (8). It is suggested that a T1128C polymorphism in the signal peptide of the NPY gene results in leucine (Leu) 7 to proline (Pro) 7 substitution. Karvonen et al (9) screened the entire coding region of the NPY gene in obese, non-diabetic Finnish and Dutch subjects and found a significant and consistent association between the Leu7Pro polymorphism and high serum total cholesterol and low-density lipoprotein (LDL) levels. This substitution is likely to alter the properties of the signal peptide due to the different physicochemical

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properties of Leu and Pro amino acids. In general, the frequency of the Pro7 allele has been reported to be 8% among Finns and 3% among Dutchmen (9). There was no report of the presence of this allele among Japanese (10, 11,12) and Korean populations (13). Jia et al (14) reported an extremely low frequency of the Pro7 allele in China. Whereas, Bhaskar et al (15) first time documented its presence in Indian populations is with varying frequencies ranging from 0.014 to 0.233.

As NPY is co released with norepinephrine during sympathetic nerve stimulation, and is extensively involved in cardiovascular regulation because it modulates heart rate, cardiac excitability, and ventricular function as well as coronary blood flow (16). Hence the present case–control study aimed to investigate the association of genetic polymorphisms of Neuropeptide Y with arrhythmia.

## MATERIALS AND METHODS:

In the present study, we adopted ACC/AHA Guidelines for Ambulatory ECG to evaluate the symptoms of cardiac arrhythmias. Forty arrhythmia individuals who are receiving the treatment and 46 healthy and unrelated individuals were included in the present study. After the informed consent, on each study subject venipuncture was performed and a blood specimen was collected into the EDTA vaccutainer. Genomic DNA was extracted from all participants, using standard procedure (17). All single nucleotide polymorphisms (SNPs) were genotyped by polymerase chain reaction and sequencing. Nucleotide sequence of primers, annealing temperature and amplicon sizes are given in Table 1. The PCRs were performed using 40 ng of genomic DNA, PCR products were checked on 2% agarose gels, and the PCR products were directly sequenced. The protocol was carried out using ABI PRISM 3730 DNA Analyzer (Applied Biosystems, Foster city, CA) with Big Dye Terminator Cycle Sequencing Ready Reaction Kit. The allelic frequency distribution was tested for Hardy–Weinberg equilibrium by the $\div^2$ test with one degree of freedom using the HWSIM program (18). The distribution of alleles among hypertensive and normotensive individuals was tested using the Fisher's exact test. All individuals were sorted into haplogroups by means of the Arlequin 2.0 computer program (19). SNPs were examined for intermarker LD using the D' measures calculated by the Haploview Ver 4.0 software package.

## RESULTS:

The entire genotyping effort of NPY gene yielded 3 reported SNPs and one novel mutation in intron 1 (Fig. 1). These SNPs are located in the coding or untranslated regions of the gene and the names are based on the Karvonen et al.'s notation (9). The genotypic frequencies of all four SNPs are shown in Table 2 and the allele frequency is given in the fig. 2. There was no significant difference between the genotype frequencies of arrhythmia and normal samples except for Ser50Ser. The genotype frequencies for all the SNPs were found to be in Hardy Weinberg equilibrium except for Ser50Ser (HW $\chi^2 = 13.1484$, df = 1, p = 0.0045).

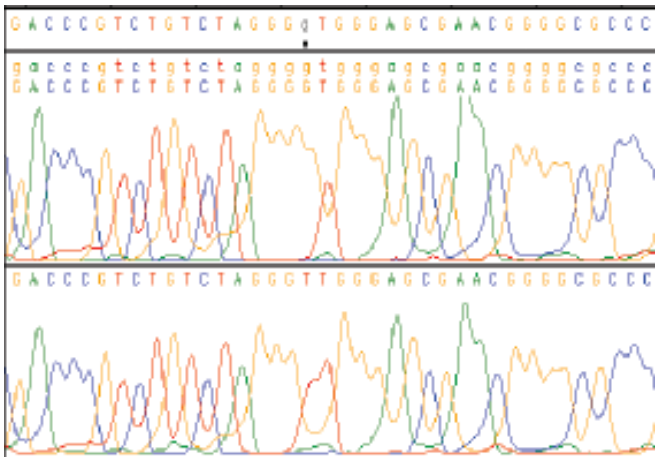

**Figure 1:** Electropherogram showing the novel base exchange in the Intron 1 of NPY gene.

A novel mutation (G to T transversion) was observed in the first intron of NPY gene only in one arrhythmia sample. Leu7Pro transition which changes the 7$^{th}$ amino acid from leucine to proline (L7P) was observed in both arrhythmia and normal samples. No homozygotes were observed in both the groups. A1258G transition which is a synonymous polymorphism (Ser50Ser) is highly polymorphic in both the groups. A to G transition in 3' UTR (A7735G)

**Table 1:** Summary of primers used in the present study

| Region | Primer sequence | Annealing temperature | Product size |
|---|---|---|---|
| Exon 1 | Np1F: 5'-CCCCGCTTCTTCAGGCAGTGC- 3' | | |
| | Np1R: 5'-TGGGGAGTGGAGCGCGATCAT- 3' | 59$^0$C | 420 bps |
| Exon 2 | NP2F: 5'-CCTGGGTTCTCTCTGCGGGACTG -3' | | |
| | NP2R: 5'-CCCATTTTGTGTAGAGTGTGCCCTGT -3' | 60$^0$C | 516 bps |
| Exon 3 | NP3F: 5'-TTCCAGATATGGAAAACGAT -3' | | |
| | NP3R: 5'-CTGCCGAAATCTCCCCTAGTCT -3' | 55$^0$C | 210 bps |
| Exon 4 | NP4F: 5'-CCCGGTCATCTTTCACTTCAG -3' | | |
| | NP4R: 5'-GCGAAACGAACCCTGAATCTG -3' | 60$^0$C | 417 bps |

**Table 2:** Genotype Frequency, Hardy-Weinberg Frequency in arrhythmia and control.

| | Genotype Number (% Frequency) | | | HW $\chi^2$ | Monte carlo simulation |
|---|---|---|---|---|---|
| **NPY Novel (G172T)** | | | | | |
| | GG | TT | GT | | |
| Arrhythmia | 39 (0.975) | 1 (0.025) | 0 (0.00) | 40 | 0.1517 |
| Control | 46 (1) | 0 (0.00) | 0 (0.00) | NA | NA |
| **NPY Leu7Pro** | | | | | |
| | TT | CC | TC | | |
| Arrhythmia | 38 (0.95) | 0 (0.00) | 2 (0.05) | 0.0263 | 0.8626 |
| Control | 45 (0.978) | 0 (0.00) | 1 (0.02) | 0.0056 | 0 |
| **NPY Ser50Ser** | | | | | |
| | AA | GG | AG | | |
| Arrhythmia | 21 (0.52) | 11 (0.27) | 8 (0.2) | 13.1484 | 0.0045 |
| Control | 8 (0.173) | 7 (0.152) | 31 (0.67) | 5.5856 | 0.0058 |
| **NPY A7735G** | | | | | |
| | AA | GG | AG | | |
| Arrhythmia | 37 (0.925) | 0 (0.00) | 3 (0.075) | 0.0607 | 0.7625 |
| Control | 45 (0.978) | 0 (0.00) | 1 (0.021) | 0.0056 | 1 |

**Table 3:** Fisher's p value and odds ratio along with confidence interval of various SNPs observed.

| | Fisher's P Value | Odd's Ratio | 95% confidence Interval |
|---|---|---|---|
| **NPY G172T (Novel)** | | | |
| Control vs Arrhythmia | 0.467 | nil | 0.029 - infinity |
| **Leu7Pro** | | | |
| Control vs Arrhythmia | 0.447 | 2.368 | 0.118 - 142.779 |
| **Ser50Ser** | | | |
| Control vs Arrhythmia | 0.001 | 0.19 | 0.062 - 0.559 |
| **NPY A7735G** | | | |
| Control vs Arrhythmia | 0.257 | 3.649 | 0.276 - 195.713 |

was also seen in both the groups with less frequency. To know the association between the markers and disease we performed Fisher's Exact Test. Except, Ser50Ser none of the other markers have shown significant association with the disease [(novel: p = 0.467), (Leu7Pro: p = 0.447), (Ser50Ser: = 0.001), (A7735G: p = 0.257)] (Table 3). We found a very low frequency of Leu7Pro C-allele carriers in both the arrhythmia and normal samples (Fig. 2). No significant association of the Leu7Pro C-allele and the arrhythmia was found [p = 0.447, df = 1 and odds ratio (95 CI): 2.368 (0.118 - 142.8)]. In this study, we found an elevated frequency of Ser50Ser in both the arrhythmia and normal samples (Fig.2). The results were statistically significant [p = 0.001, df = 1 and odds ratio (95 CI): 0.19 (0.062 - 0.559)]. A7735G was the third polymorphism found in the UTR of the NPY gene. The frequency of this polymorphism was found to be very low in the arrhythmia and the normal samples (Fig. 2). Hence no association was found between A7735G polymorphism and arrhythmia [p = 0.257, df = 1 and odds ratio (95 CI): 3.649 (0.276 - 195.713)]. In this study, a novel G to T heterozygous mutation was found in one of the arrhythmia samples but none of the

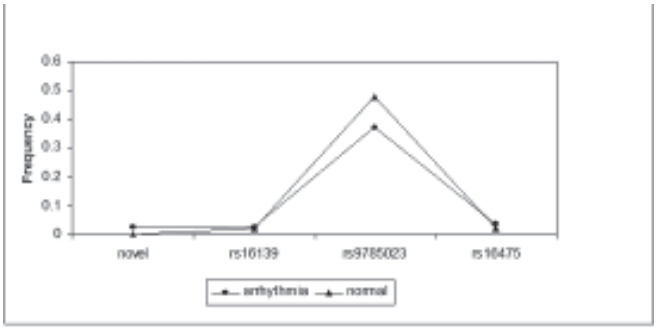


**Figure 2:** Allele frequency distribution in arrhythmia and control.

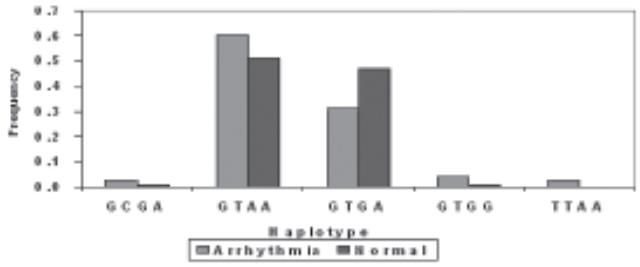


**Figure 3:** Haplotype frequency distribution in arrhythmia and control.

normal samples. It was also found that this mutation is not associated with arrhythmia [p = 0.467, df = 1 and odds ratio (95 CI): 0.0 (0.029 - infinity)]. Haplotype analysis also indicating no significant differences between the haplotypes of both arrhythmia and normal ($\chi^2$ = 0.035, df = 1 and P = 0.852) (Fig. 3). Linkage disequilibrium (LD) is the non-random association between alleles at two loci (20), and is primarily the result of a physical association. Although LD has been shown not to be uniformly distributed across the genome, limited information is available about the characteristics of LD within candidate genes at large, it is the central concept of genetic association studies. Not much significant LD was observed because of less heterozygosity in the markers of both arrhythmia and normal samples (Fig. 4).

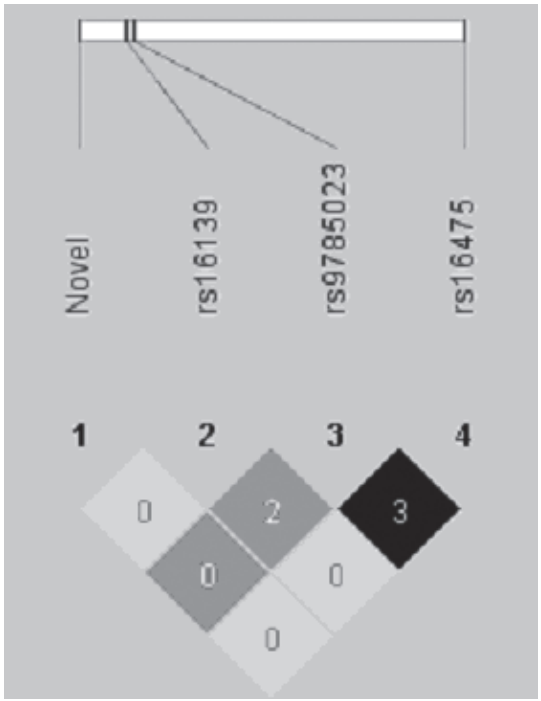


**Figure 4:** Pattern of linkage disequilibrium between the markers.

**DISCUSSION:**

Neuropeptide Y (NPY) has been shown to participate in the cardiovascular response mediated by sympathetic system (21). Ullman et al (22) reported elevated levels of Plasma neuropeptide Y-like immunoreactivity (NPY-LI) in patients with acute myocardial ischaemia and congestive heart failure (CHF) owing to increased activity of the sympathetic nervous system. However, although NPY is the most abundant peptide in the mammalian heart (23), and there is evidence of the existence of at leastY1 andY2 NPY receptors in cardiac myocytes (24), the NPY actions in heart are not fully understood (25). NPY exerts long term trophic effects that could be involved in the hypertrophic response of the heart (26). The earlier genetic and epidemiological study indicates that the NPY Leu7Pro allele is a major risk factor for obesity (27). Experimental studies indicate that the Leu7Pro polymorphism influences the heart rate level (28). Presence of the Pro7 variant is a suspected cardiovascular risk factor in Caucasians (9,28,30). There seems to be a variation of frequencies of the Leu7Pro polymorphism between ethnic groups (15). The effects of the Pro7 allele on cardiovascular regulation and diseases are not likely to be relevant in populations other than the Nordic countries because the frequency of Leu7Pro polymorphism shows a strictly geographical distribution, with most if not all positive associations found largely in Nordic countries such as Finland, Sweden and the Netherlands (9,31,32). Hence according to this study, the Leu7Pro polymorphism is not associated with arrhythmia. Contrasting results of Kallio et al (33) and Pettersson-Fernholm et al (34) it is not clear that the Leu7Pro polymorphism translates into higher or lower levels of active NPY; it is difficult to draw conclusions with respect to potential physiological or pathological roles of this polymorphism in cardiovascular control and development of diseases (35). Significant association between Ser50Ser polymorphism and arrhythmia is observed. This synonymous variant Ser50Ser was absent in the 50 obese French white compared with two non-obese controls (36). Whereas this variant is quite common in Mexican Americans (37). At haplotype level also no significant differences were observed in both arrhythmia and normal. Individuals often inherit rather long hunk of DNA from one parent or the other. The hunk is known as a haplotype, and some haplotypes themselves may also be inherited as a group. This is called linkage disequilibrium (LD). LD is the non-random association between alleles at two loci (20), and is primarily the result of a physical association. Although LD has been shown not to be uniformly distributed across the genome, limited information is available about the characteristics of LD within candidate genes at large, it is the central concept of genetic association studies. Not much significant LD was observed because of less heterozygosity in the markers of both arrhythmia and normal samples.

In conclusion, Ser50Ser is significantly associated with arrhythmia. But no association was found between and NPY gene Leu7Pro and arrhythmia. The inconsistencies between association studies may also reflect the complex interactions between multiple population-specific genetic and environmental factors. We accept as true that any association

study obtained even by case-control study should be regarded as provisional and that replication in independent population studies is critical. Relatively smaller sample size of the present study also making it difficult to extrapolate the results to the entire population.

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