Novel mutations in arrhythmogenic right ventricular cardiomyopathy from Indian population

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BACKGROUND: Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a progressive condition with right ventricular myocardium being replaced by fibro-fatty tissue. The spectrum of the expression may range from benign palpitations to the most malignant sudden death. Most of the mutations identified for the condition are localized in desmosomal proteins although three other nondesmosomal genes (cardiac ryanodine receptor-2, TGF-β3, and TMEM43) have also been implicated in ARVC. Both desmosomal and nondesmosomal genes were screened in a set of patients from local population.
MATERIALS AND METHODS: A set of 34 patients from local population were included in this study. Diagnosis was based on the criteria proposed by task force of European Society of Cardiology/International Society and Federation of Cardiology. Polymerase chain reaction-based single-strand conformation polymorphism analysis was carried out, and samples with abnormal band pattern were commercially sequenced.
RESULTS: Screening of cardiac ryanodine receptor revealed an insertion of a base in the intronic region of exon-28 in a patient, leading to a creation of a cryptic splice site. Screening of plakohilin-2 for mutations revealed an abnormal band pattern in three patients. Two of them had similar abnormal band pattern for exon-3.1. Sequencing revealed a novel 2 base pair deletion (433_434 delCT), which would lead to premature truncation of the protein (L145EfsX8). Another patient showed abnormal band pattern for exon-3.2 and sequencing revealed a missense mutation C792T leading to amino acid change P244L, in N-terminal, and this substitution may cause disturbances in the various protein–protein interactions.
CONCLUSION: This study reports novel cardiac ryanodine receptor (RyR-2) mutations and Pkp-2 for the first time from Indian population.

Key words: Arrhythmogenic right ventricular cardiomyopathy, fibro-fatty tissue, palpitations, sudden death

Introduction

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a rarer form of cardiomyopathy characterized by progressive fibro-adipocytic replacement of myocardial cells in the right ventricle. The symptoms include ventricular tachyarrhythmia, syncope, and even sudden death as an initial manifestation of the condition, with vast clinical heterogeneity. The condition is also genetically heterogenous with 11 loci been implicated and 8 genes identified. The genes identified include desmosomal (plakophilin-2 [Pkp-2], desmoplakin [Dsp], plakoglobin [JUP], desmoglein-2 [DSG-2], and desmocollin-2 [DSC-2]) and nondesmosomal genes (TGFβ-3, RyR-2, and TMEM43).

With the discovery of five desmosomal genes harboring the mutations for ARVC, desmosomal disruption was proposed to be the major mechanism underlying the condition. But recent reports implicating the involvement of Wnt pathway and armadillo proteins in the process of fibro-fatty replacement have given a new direction in understanding the etiology of the ARVC.[1]

This study reports three novel mutations from Indian

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population in cardiac ryanodine receptor (RyR-2) and Pkp-2 genes. An intronic insertion was identified in one patient in RYR2 gene. A missense mutation and a two base pair deletion in the N-terminal region were identified in three patients. This study also describes the variability in expression of the condition in these probands.

Materials and Methods

This study included 34 patients diagnosed using the criteria proposed by Task Force of the European Society of Cardiology/International Society and Federation of Cardiology[2] and 100 control individuals selected based on the absence of history/family history of any cardiac disorder.

An informed consent was obtained from all the individuals participating in this study, and Institutional ethics committee approval from Care Hospitals Hyderabad has been obtained for clinical and genetic analyses. Diagnosis was based on 12-lead surface electrocardiograph (ECG), cardiac Doppler Echo, and history of familial condition.

For genetic analysis, DNA was isolated by rapid nonenzymatic method[3] from whole blood. All the exons of Pkp-2, DSC-2, DSG-2, and JUP genes along with regulatory and promoter regions of TGFβ-3 gene, exons coding for FKBP binding domain of RyR-2 gene, and N-terminal region of Dsp were screened for mutations.

The primer sequences for Pkp-2, JUP, DSC2, DSG-2, and TMEM43 genes were obtained from Gerull *et al.*,[4] Whittock *et al.*,[5] Syrris *et al.*,[6] Awad *et al.*,[7] and Merner

et al.,[8] respectively. For amplification of regulatory regions of transforming growth factor β-3 and exons of RyR2 and Dsp, genes were taken from http://telethon. bio.unipd.it/ARVDnet/molgen_arvd8.html.

The amplified polymerase chain reaction (PCR) products were screened by single-strand conformation polymorphism (SSCP) for any variation in the sequence. The amplified PCR products with abnormal band pattern were purified using GeNei TMQuick PCR Purification Kit (Bangalore, India) and sequenced commercially (SolGent Co., Ltd., South Korea) using ABI 3730XL capillary DNA Sequencer (50 cm capillary).

Results

Genetic screening

Screening of the previously implicated nondesmosomal gene segments, namely, regulatory regions of TGF-β3 and exon-10 of TMEM43 did not reveal any mutations, but a polymorphism in 5′ UTR (c) region of TGF-β3 gene was identified [Table 1].

Mutational screening of exons coding for FKBP binding domain of RyR-2 which was proposed to be involved in pathogenesis of ARVC revealed an abnormal pattern in a patient and sequencing revealed an insertion of a base "G" (2487896_248797 ins G) [Figure 1] 30 bp downstream the coding region of exon-28 [Table 2]. Affect of this insertion mutation on splicing was analyzed using *in silico* splice site prediction software (http://www.fruitfly.org/cgi-bin/seq _tools /splice.pl) [Figure 2] which revealed a formation of a cryptic splice

Table 1: Single-nucleotide polymorphisms identified in arrhythmogenic right ventricular cardiomyopathy genes

| Gene	Exon	Variation	Amino acid change	Novel/known
Pkp-2	4	T1158C	L366P	Novel
Pkp-2	13/14	int 69 bps upstream G>A	-	Novel
JUP	7	C1296T	L432L	Novel
JUP	8	int 10bps downstream C>A	-	Novel
JUP	10/11	int g.3638124delG	-	Novel
RyR-2	8	int gA2298404C	-	Novel
RyR-2	15	C1479T	S453S	Known
RyR-2	28	gA2487657G	A1080A	Novel
RyR-2	28	A3377T	R1086R	Novel
DSP	7	A1192T	I305F	Known
TGF-β3	5′ UTR (c)	A57448010T	-	Novel
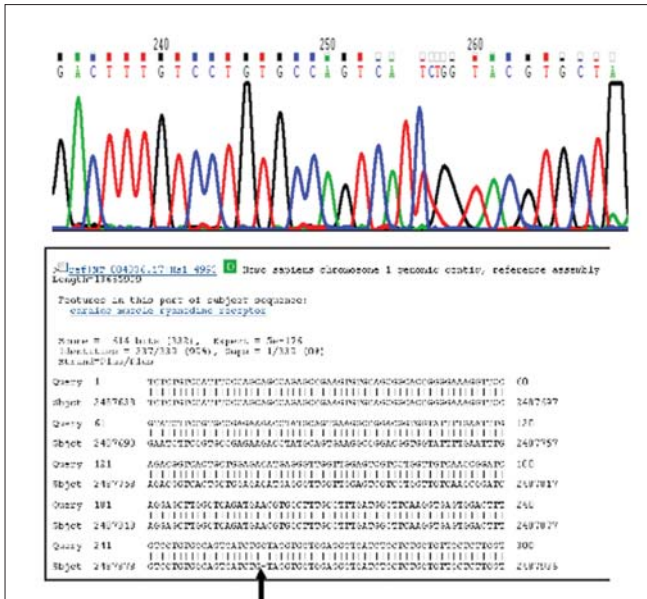
Figure 1: Alignment of nucleotide sequences shows an insertion of a base G at position 2487897 (2487896_2487897insG)
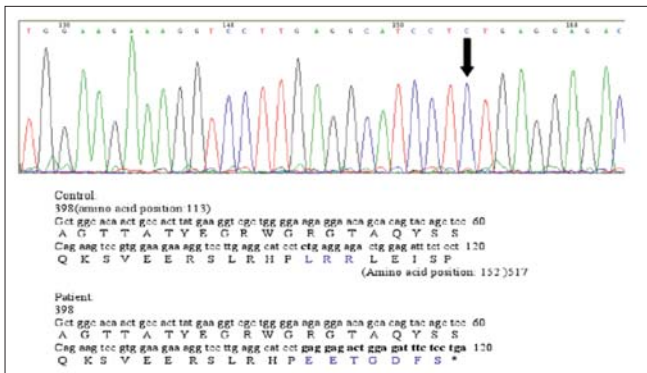

Figure 3: Alignment of cDNA sequence from 337 to 456 along with the translation product is shown. 433_434delCT causes shift in the reading frame leading to the introduction of termination codon after the addition of seven novel amino acids

Figure 2: Splice scores of normal and mutant sequence revealing an introduction of a cryptic splice in the variant due to an insertion of a base

Table 2: Novel mutations identified in RyR-2 and Pkp-2 genes

Gene	Exon	Change	Mutation type	Affected domain	Novel/ known
RyR-2	28	2487896_248797 ins G	Insertion	SPRY	Novel
Pkp-2	3.1	L145EfsX8	Deletion	N-terminal	Novel
Pkp-2	3.2	C792T	Missense	N-terminal	Novel

site due to insertion, and usage of this site would lead to introduction of 10 novel amino acids in the exon-28 before splicing. Exon-28 of RyR-2 gene is involved in the formation of SPRY domain, and although the function of the domain is unknown, it is predicted to be involved in the interdomain interactions; and defects in this domain are reported in failing hearts[9] and hence, the insertion mutation in exon-28 coding for SPRY domain might alter interdomain interactions thereby leading to heart failure, which is reported in ARVC.

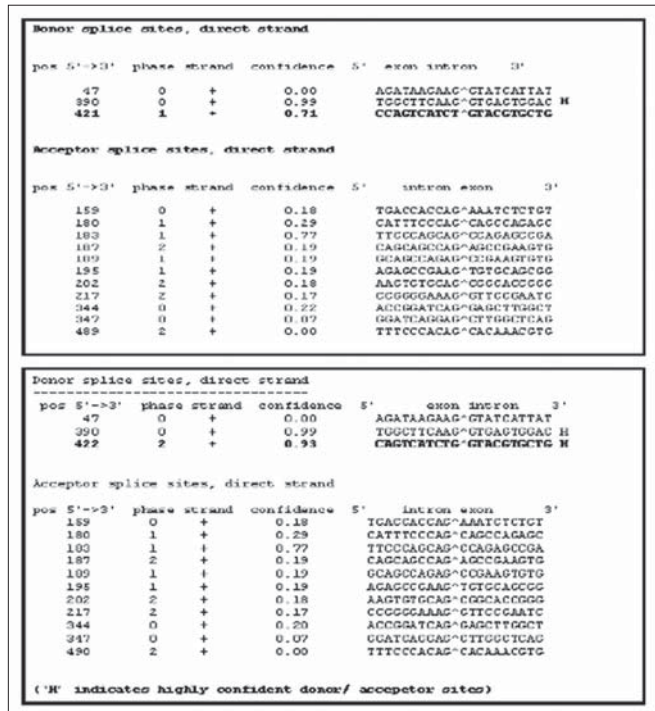Screening of desmosomal genes Dsp, JUP, desmsocollin-2, and DSG-2 do not reveal any patient-

specific pathogenic mutations, but novel and known polymorphisms were identified [Table 1].

Mutation screening of desmosomal gene Pkp-2 revealed an abnormal in three patients. An abnormal but similar band pattern in two unrelated patients (P1 and P2) in exon-3.1 was observed. Sequencing of these two samples revealed a heterozygous 2 base deletion (c.433_434 delCT) in exon-3.1. This deletion would affect 145th amino acid with the addition of seven novel amino acids followed by the introduction of a premature termination codon (L145EfsX8) [Figure 3] [Table 2]. On screening the family members, both the daughters of P-1 were found to be carriers of the same mutation. Relatives of P-2 were unavailable for this study.

Because identical mutation was identified in two patients, haplotype analysis was carried out to determine

the origin of the mutation. Four repeat markers spanning 300,000 bp including the entire Pkp-2 gene were analyzed in both the patients and relatives of P-1. Primer sequences for these markers were obtained from van Tintelen *et al.*[10] The phase of P-1 was determined based on the analysis on three first-degree relatives. The chromosome containing the haplotype shared by both the carriers and patient possessed the deletion. Comparison of the haplotypes of both the patients shows that the mutation may have originated independently. Haplotype of all the typed individuals is given in [Figure 4].

In addition, one patient showed an abnormal band pattern in exon-3.2 of Pkp-2 gene and sequencing revealed a heterozygous C792T missense change leading to P>L substitution at 244th amino acid position [Table 2]. This amino acid falls in the amino terminal region preceding the armadillo repeats. It has been reported earlier by Chen *et al.*[11] that N-terminal region of Pkp-2 is essential in promoting the interactions with other desmosomal components, namely, JUP, Dsp, desmoglein, and desmocollin isoforms and more importantly this region is critical for localization of Pkp-2 to the cell borders. Further, N-terminal region has also been implicated in signaling functions through its interaction
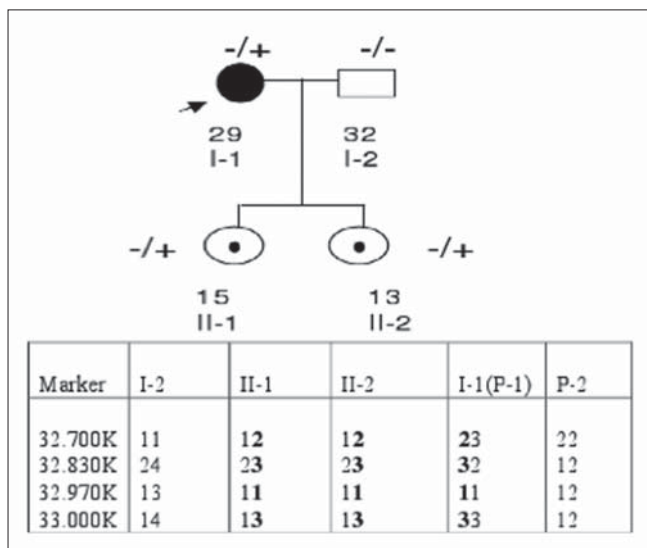

Figure 4: Te pedigree shows the available first-degree relatives of patient P-1 with their present ages; + denotes the presence of the deletion and – denotes absence of deletion. The table gives the haplotypes of these individuals. Haplotype shared by the individuals carrying the mutation in the first family of P-1 is not observed in P-2

with the β-catenin and upregulating the β-catenin/T-cell factor signaling activity.

Clinical investigations

Clinically the patient harboring RyR-2 gene mutation suffered from symptoms of dyspnea and presyncope during physical strain, an episode of syncope and swelling of limbs for the past 6–7 years and was diagnosed with ARVC at the age of 55 years. ECG evaluation revealed sinus bradycardia and low-voltage QRS complexes with QRS transition being less than/equal to V1. Repolarization abnormalities in the precordial leads with T-wave inversion beyond lead-V3 along with atrial fibrillation were also observed. 2D Doppler ECHO revealed dilatation and thinning out of right ventricular free wall with evidence of scarring on profusion imaging. This patient satisfied two major and two minor criteria [Table 3].

The clinical profile of two patients (P-1 and P-2) harboring same Pkp-2 gene mutation in exon-3.1 was analyzed. Patient P-1 was a 29-year-old woman, presented with symptoms such as dyspnoea, palpitations, chest pain, presyncope, and syncope with occasional swelling of limbs, while patient P-2 was a 32-year-old man, suffering from palpitations and presyncope.

Twelve-lead ECG of patient P-1 revealed normal sinus rhythm with heart rate of 60 bpm. There was a left axis deviation with left anterior hemiblock. There was also an epsilon wave in lead V1 with poor progression of R waves voltage in precordial leads. T-wave inversion was observed from leads V1 to V6 with low overall QRS voltage (in all 12 leads). ECG of patient P-2 also revealed normal sinus rhythm and heart rate of 60 bpm. There was a right axis deviation with right posterior hemiblock. There was an epsilon wave in lead V1 RBBB and upright R waves from leads V1 to V6 with small QR in lead V1. T-wave inversion was observed from leads V1 to V6 [Table 3].

Cardiac Echo Doppler of patient P-1 revealed a dilated right ventricle with hypokinetic right ventricular apex, adjoining free wall with an apical clot and RV dysfunction. The mid-segment free wall of RV was also hypokinetic with normal LV function. In contrast to P-1, RV of patient P-2 was only mildly dilated. This patient showed good RV free wall motion and normal LV function.

Table 3: Clinical profile of patients in whom mutations have been identified

Gene mutation	RyR-2	Pkp-2 (3.1) (P-1)	Pkp-2 (3.1) (P-2)	Pkp-2 (3.2)
Age at onset	55	29	32	28
Gender	F	F	M	M
Symptoms	Dyspnea, presyncope and swelling of limbs	Dyspnea, palpitations, chest pain, presyncope and syncope with occasional swelling of limbs	Palpitations and presyncope	Palpitations and presyncope
ECG	- Sinus bradycardia, - Low-voltage QRS complexes - Repolarization abnormalities in precordial leads - T-wave inversion beyond lead-V3 with atrial fibrillation	Normal sinus rhythm with heart rate of 60bpm - Left axis deviation with left anterior hemi-block	- Normal sinus rhythm with heart rate of 60 bpm - Right axis deviation with right posterior hemiblock	- Normal sinus rhythm with heart rate of 75 bpm - Left axis deviation with QRS duration 0.12 ms - Inverted T-waves beyond V2, RBBB
ECHO	- Dilatation and thinning of RV free wall - Scarring on profusion imaging	- RV grossly dilated and hypokinetic	- RV dimensions and contractility conserved	- RV morphology showed dyskinesis and right atrial enlargement
F/H of SCD	No family history of SD	Sudden death (>25 years) due to unknown cause	No family history of SD	No family history of SD
Diagnostic criteria (Max/Min)	2/2	2/4	1/2	3/1

Therefore, patient P-1 showed RV dysfunction with structural alterations and also conduction abnormalities, thereby fulfilling two major criteria for diagnosis. Further the two minor criteria of inverted T waves and family history of premature sudden death (<35 years) were also fulfilled. Patient P-2 showed depolarization abnormalities and also repolarization abnormalities along with mild RV dilation, thereby fulfilling one major and two minor criteria. Two first-degree relatives of patient P-1 (both daughters) were available for further analysis. Twelve-lead ECG and Cardiac Echo Doppler showed no abnormalities.

Clinically the patient with missense mutation in exon-3.2 of Pkp-2 gene was a 28-year-old man suffering with symptoms palpitations and presyncope with an average frequency of four episodes per week. A 12-lead ECG revealed a heart rate of 75 bpm with sinus rhythm and a QRS duration of 0.12 ms with left axis deviation. T-wave inversions in anterior leads V2-V3 and right bundle branch block morphology were also observed. 2D Doppler ECHO revealed conserved RV dimensions with normal systolic function, and RV morphology showed dyskinesis and right atrial enlargement. Hence, fulfilling three major and one minor standardized criteria [Table 3].

Discussion

ARVC is a rare form of cardiomyopathy, with an estimated prevalence of 1 in 5000.[12] Initial reports of ARVC are from Italian population, followed by American and now from Asian populations with mutations in similar genes underlying the condition, which underscore the significance of the pathogenic mechanism in maintaining the functional myocardium. This study, carried out for the first time in Indian population, corroborates the earlier findings of RyR-2 and Pkp-2 genes harboring mutations in individuals with ARVC.

Insertion mutation in the intronic region of exon-28 of RyR-2 gene reported in this study would lead to an induction of a cryptic splice site, usage of which would lead to the introduction of 10 novel amino acids in exon-28 before splicing which in turn may affect the SPRY domain thereby altering the interdomain interactions.

The deletion (433_434 delCT) in Pkp-2 gene observed in the present report would cause premature truncation resulting in the absence of armadillo repeats and C-terminal tail regions if the protein is translated. Nonsense-mediated decay may also operate causing haplo-insufficiency. This would result in improper assemblage of desmosomal complex releasing JUP free into cytosol, which could modulate Wnt/β-catenin signaling. On the other hand, because Pkp-2 is also an armadillo protein and earlier reports have demonstrated Pkp-2-dependent increase of endogenous β-catenin/

TCF signaling in SW480 cells through direct interactions, haplo-insufficiency may cause JUP-independent reduction in canonical Wnt//β-catenin signaling through Tcf/Lef1.[1] Although there has been some progress made in understanding the pathogenesis at molecular level, the basis of clinical expression of the condition needs to be understood.

Vast clinical heterogeneity has been observed in individuals with mutations in same genes. In this study, where both the patients harbored same mutation, patient P-1 was female who was diagnosed at the age of 29 years and patient P-2 was a male with age at diagnosis being 32 years. Comparison of the clinical expression of condition reveals severe progression of the condition in P-1 with gross RV dilation and RV hypokinesia when compared with P-2 in whom RV dimensions and contractility were highly conserved [Table 3].

Family history of P-1 had the patient's mother dying suddenly at the age of 25 years without any known cause. Although both the daughters of the patient have been detected to be carriers of the mutated allele, at the ages of 16 and 14 years, they did not show any kind of ECG or echocardiography abnormalities. Family history of P-2 did not have any sudden deaths. The fact that both the individuals with same mutation had such variable expression of the condition with one of them following two major and two minor (P-1) and the other one major and two minor (P-2) criteria proposed by Task Force of the European Society of Cardiology/International Society and Federation of Cardiology suggests the strong interaction of genetic and environmental factors in the expression of the condition. This observation is in conformation with earlier studies that have shown wide spectrum of expression even within the family members carrying the same mutation.[13] A study with monozygotic twins has also shown variability in expression implicating environmental factors as major triggers for the progression.[14] Therefore, specific genotype-based triggers need to be identified to prevent the severity of the progression.

The third variation in Pkp-2 gene, a missense mutation C792T that would result in P244L, affects N-terminal region of the protein, which is involved in regulatory activities such as desmosomal formation and β- catenin-dependant Wnt pathway modulation. So any substitution in this region causes disturbances in the various protein–protein interactions.

Conclusion

ARVC is a prevalent cardiomyopathy in Indian population. Our study identified novel genetic variants (both mutations and polymorphisms) in the already implicated genes for ARVC, hence emphasizes the high genetic diversity of our population and advocates the inclusion of our population in international population-based genotypic estimation and data base studies.

Drawbacks

Experimentally, the mutation detection was based on the band pattern variation using SSCP technique, which is not a very sensitive. Further transcript and protein expression analyses were not performed to understand the affect of the mutations identified.

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