The p.A897KfsX4 frameshift variation in Desmocollin-2 is not a causative mutation in arrhythmogenic right ventricular cardiomyopathy

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Running title: DSC2 A897KfsX4 polymorphism in ARVC probands

Abstract

Mutations in genes encoding desmosomal proteins have been reported to cause arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC/D), an autosomal dominant disease characterized by progressive myocardial atrophy with fibro-fatty replacement. We screened 112 ARVC/D probands for mutations in desmocollin-2 (DSC2) gene and we detected two different amino acid substitutions (p.E102K, p.I345T) and a frameshift variation (p.A897KfsX4) in 7 (6.2%) patients. DSC2a variant p.A897KfsX4, previously reported as p.E896fsX900 mutation, was identified in five unrelated probands. Four of them were found to carry one or two mutations in different ARVC/D genes. Unexpectedly, p.A897KfsX4 variation was also found in 6 (1.5%) out of 400 control chromosomes.

In vitro functional studies demonstrated that, unlike wild-type DSC2a this C-terminal mutated protein was localised in the cytoplasm. p.A897KfsX4 variation affects the last five amino acids of the DSC2a isoform but not of DSC2b. In contrast with what we found in other human tissues, in the heart DSC2b is more expressed than DSC2a, suggesting that relative deficiency of DSC2a might be compensated by isoform b.

In conclusion, DSC2 gene mutations are not frequently involved in ARVC/D. The p.A897KfsX4 variation, identified in several Italian healthy control subjects, which affects only one of the two DSC2 isoforms, may be considered a rare variant, though possibly affecting phenotypic expression of concomitant ARVC/D mutations.

Keywords Sudden death, ARVC/D, desmosome, DSC2, polymorphism

Introduction

Arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC/D) (MIM #107970) is an inherited disorder characterised by progressive fibro-fatty replacement of the right ventricular myocardium[1]. Clinical manifestations occur most often between the second and fourth decade of life and are characterized by ventricular arrhythmias, heart failure, and sudden death. The disease shows usually autosomal dominant inheritance with reduced penetrance[2], although autosomal recessive transmission has also been reported in Naxos Syndrome[3].

Eight genes have been detected so far as being independently involved in the pathogenesis of the disease. Five of them encode major desmosomal proteins: plakoglobin [MIM *173325][3-4], desmoplakin [MIM +125647][5], plakophilin-2 [MIM *602861][6], desmoglein-2 [MIM *125671][7] and desmocollin-2 [MIM *125645][8-9]. The involvement of such genes leaded to the current idea that ARVC/D is a disorder caused mainly by defects in cell-cell adhesion.

Other three nondesmosomal genes have been associated with ARVC/D: cardiac ryanodine receptor 2 (RYR2 [MIM180902])[10], transforming growth factor beta-3 (TGFβ3 [MIM 190230])[11] and transmembrane protein 43 (TMEM43 [MIM 612048])[12].

To date, five different *DSC2* mutations have been reported: two frameshift mutations p.M477fsX480 and p. E896fsX900[8], a heterozygous splice acceptor site mutation c.631-2A>G[9] and two missense mutations p.E102K and p.I345T[13]. In vitro functional studies demonstrated that the two point mutations affect the intracellular localisation of desmocollin-2a, thus suggesting a potential pathogenic effect[13]. More recently a homozygous mutation p.S614fsX625 in *DSC2* gene

associated with autosomal recessive ARVC/D, mild palmoplantar keratoderma and woolly hair has been described[14].

In this study we investigated the frequency of DSC2 mutations in an unselected Italian ARVC/D patient cohort. In five probands, we have identified the frameshift p.A897KfsX4 variation (previously reported as p.E896fsX900[8]), which was also detected among Italian healthy control subjects. On the basis of genetic and functional data, we discuss here the pathogenic role of the p.A897KfsX4 variation.

Materials and Methods

Mutation screening

One hundred-twelve ARVC/D unrelated index cases were screened for *DSC2* mutations by denaturing high-performance liquid chromatography (DHPLC) and direct sequencing. This patient group includes 54 subjects already reported in a previous study[13]. The coding region of *DSP, PKP2* and *DSG2* genes was screened for mutations in *DSC2* mutation carriers.

A control group of 200 healthy and unrelated Italian subjects (400 alleles) was used to exclude that identified mutations could be DNA polymorphisms. All controls were matched to the probands by ancestry and underwent ordinary clinical investigations.

Mutation screening was performed in all available family members of index cases in which a *DSC2* mutation was detected.

Comparison of p.A897KfsX4 variant in patients and control subjects was assessed by One-tail Fisher's Exact test. A P value <0.05 was considered statistically significant.

Clinical study

Each patient underwent physical examination and family history, 12-lead electrocardiography (ECG), signal-averaged ECG (SAECG), 24 hour Holter ECG, two-dimensional echocardiography. A clinical diagnosis of ARVC/D was based on major and minor criteria, established by the European Society of Cardiology/International Society and Federation of Cardiology Task Force[15]. Informed consent for clinical investigations and blood sampling for DNA analysis was obtained from all participating individuals, according to the pertinent Italian legislation and in compliance with Helsinki declaration.

Haplotype analysis

DNA samples of all frameshift variation carriers were assessed for a common haplotype, using the following microsatellite markers: D18S847, D18SH3, D18SH4 and D18S36. Markers D18SH3 and D18SH4 are new polymorphic tri- and di-nucleotides repeats that we identified starting respectively at position 26755775 and 26908386 on chromosome 18q12.1 (Human Genome Browser, http://genome.ucsc.edu/). They were amplified using primers D18SH3F 5'GTGGTGGGCATCTGTAATCC3', D18SH3R 5'GGTGCCTGCGTTTAGTAT3', D18SH4F 5'CTCCCTTATGACCCAGGAAA3' and D18SH4R 5'ACCATGTGGGAAACACCAAT3' under standard PCR conditions. D18SH4 is within the *DSC2* intron 12, whereas the other three markers are in close

proximity to the *DSC2* locus. Forward primers were fluorescently labelled. In particular, D18S847 and D18SH3 were labelled with TAMRA; D18SH4 with HEX and D18S36 with FAM. Amplification products were pooled into post-amplification panel, mixed with GeneScan ROX400 size standard. Capillary electrophoresis was carried out on an ABI PRISM 3730XL DNA sequencer and Genotyper V3.5 analysis software was used to analyze each amplicon.

Expression pattern of *DSC2* isoforms

Expression of *DSC2a* and *DSC2b* isoforms in different human tissues was examined by PCR amplification of cDNAs including heart, brain, placenta, lung, skeletal muscle, liver, kidney and pancreas (Multiple Tissue cDNA panel I, Clontech). Amplification was performed by using primers previously reported[16]. The splice form "b" resulted in a PCR fragment of 500 bp, whereas splice form "a" produced a 454 bp fragment.

Site-directed mutagenesis

PCR-based site-directed mutagenesis was performed on wild type *DSC2a*-pcDNA3.1/CT-GFP, already available to the study[13], to obtain three new constructs. Each construct differs from the others for a variation introduced in human *DSC2* coding region: an insertion c.2687_2688insGA leading to the frameshift variation p.A897KfsX4, and two nucleotide substitutions c.536A>G (p.D179G) and c.2393G>A (p.R798Q) which resulted to be common polymorphisms. The following mutagenic primers were used: DSC2D179G: 5'-tccataagaggtcctggagttg**g**ccaagaacccggaatttatttt-3'; DSC2R798Q: 5'-caccagacctcggaatcctgcc**a**gggggctggccaccatcacacc-3'; DSC2insGA: 5'-caaatttggacactagca**ga**gaagcatgcatgaagagacaag-3' and DSC2frameGA: 5'-

cactagcagagaagcatgcacaagggcaattctgcagata-3'. Two steps of mutagenesis were necessary to obtain a construct coding for p.A897KfsX4 variation, the first to introduce the GA insertion and the second one to remove the premature stop codon and the following 5 nucleotides to get the right frame with GFP sequence. Thus, the mutant construct doesn't contain additional amino acids compared to the wild-type. The coding regions of each mutant construct were fully sequenced.

Cell culture and transfection

HL-1 cells, kindly provided by Dr. W.C. Claycomb (New Orleans), were used and maintained as previously reported[17]. Cells were cultured at 37 °C in 5% CO_2. Constructs corresponding to wild-type or to a variant of human DSC2a were transiently transfected in HL-1 cells at confluence of 70-80%, using Effectene reagent (Qiagen) according to the manufacturer's instructions. Transfected cells were incubated for 48 h to allow protein expression and desmosome formation.

Immunostaining and confocal imaging

HL-1 cells were fixed with cold methanol/acetone (1:1) and immunostained for endogenous desmoglein with the murine mAb (clone DG3.10) as previously described in detail[13]. Images were acquired with a Radiance 2000 confocal microscope (BioRad) with a 60× oil objective.

Results

Mutation screening

DSC2 mutation screening was performed in a series of 112 consecutive unrelated index cases. Two different amino acid substitutions (p.E102K, p.I345T) and a frameshift (p.A897KfsX4) have been detected in 7 (6.2%) patients (Figure 1a).

We also identified two polymorphisms (c.536A>G and c.2393G>A, allele frequency 2.7 % and 4.7%, respectively) in exon 5 and in exon 15, resulting in predicted p.D179G (novel) and p.R798Q (rs61731921) amino acid substitutions.

The two missense mutations p.E102K and p.I345T, previously reported by our group[13], affect the normal localization of mutant proteins in cultured cardiomyocytes. The frameshift variation p.A897KfsX4, primarily reported as p.E896fsX900 by Syrris *et al*[8], causes a premature termination of the protein. Only the last 5 amino acids of desmocollin-2a were altered, three were changed and the last two were lost (Figure 1b). The five involved aa residues are less conserved among mammals, in contrast with the high conservation of the upstream region (Figure 1c). DSC2b is not affected by the p.A897KfsX4 variation (Figure 1a), since it has a shortened C-terminal domain caused by a stop codon in the additional exon 16.

Five unrelated ARVC/D index cases (allele frequency 2.2%) were found to carry the p.A897KfsX4 variation, as well as 6 out of 200 control subjects (allele frequency 1.5%).

A 0.11 *P* value, falling below the 0.05 significance threshold, indicates that there was no evidence of overrepresentation of p.A897KfsX4 variant in patients compared with control subjects.

Mutations in other ARVC/D genes were identified in four of these five patients (Table 1). Proband 1 was found to carry the p.A897KfsX4 mutation and the previously reported PKP2 p.E58D common polymorphism[18]. The allele frequency of the p.E58D variant in our control population resulted to be 0.9%, in

contrast with the reported 5% in Finnish population representing a genetic isolate[18].

The additional detected mutations DSG2 p.Y87C and PKP2 p.V406SfsX3 have been previously reported as disease causing[19,20]. None of the novel identified mutations (DSG2 p.G638R; DSP p.N375I; PKP2 p.F552C) were observed in 400 control chromosomes and occurred in residues that are highly conserved among species (data not shown). Moreover, they all lead to a change in biochemical properties of the amino acid involved.

In Proband 4 it has been also identified a nucleotide substitution c.45C>T in PKP2 gene which causes a synonymous variation p.T15T. 220 control alleles are negatives for this variation. This nucleotide substitution might be a rare polymorphism or it might activate a cryptic splice site and produce an aberrant transcript.

Clinical findings

Proband 1 was a 60 yrs old woman who was diagnosed as having ARVC/D due to ECG abnormalities. Two-dimentional echocardiogram revealed a severe dilation of the right ventricle with depressed ventricular function. Moreover, frequent PVCs (premature ventricular contraction) with LBBB (left bundle-branch block) morphology were present at 24-hour Holter ECG. The genetic analysis extended to her son revealed that he carries the *DSC2* frameshift variation p.A897KfsX4 and *PKP2* polymorphism p.E58D as well. Clinical evaluation showed the presence of some criteria also in the son, even if he did not fulfilled the diagnosis (Table 2).

Proband 2 was diagnosed at the age of 45 due to palpitations. Twenty-four-hour Holter ECG demonstrated frequent episodes of non sustained ventricular arrhythmias and imaging techniques showed a severe right ventricular dilation/dysfunction. He resulted to be a double heterozygote for the *DSC2* p.A897KfsX4 variation and the *DSG2* p.Y87C mutation. His daughter, clinically unaffected, is negative for both (Table 2).

Proband 3 experienced a sustained VT episode at the age of 38. Clinical and instrumental examination revealed at that time right ventricular abnormalities that became more evident during follow-up. She carried also a missense mutation in *DSP* gene (p.N375I) and in *DSG2* gene (p.G638R). The genetic study was extended to additional family members and two subjects were double heterozygotes for *DSG2* mutation and *DSC2* variation and one subject results to carry the *DSG2* mutation (Figure 2). One of the double heterozygotes (subject 3.(II,7)) carrying the DSG2 p.G638R and the DSC2 p.A897KfsX4 fulfilled the ARVC/D diagnostic criteria, whereas the other family members were clinically unaffected (Table 2).

Proband 4 was a female patient who underwent cardiac evaluation due to several presyncopal episodes. The ECG showed negative T waves in V1-V4 and in inferior leads. Late potentials were present at 40-80 Hz. At 2D echocardiogram the right ventricle was mildly dilated with presence of kinetic alterations; the 24-holter ECG documented the presence of frequent PVCs with LBBB morphology. Cardiac magnetic resonance confirmed the presence of mild right ventricular dilatation and kinetic alterations, localised on the subtricuspid region and on the apex. The left ventricle was not involved. She was found to carry also the *PKP2*

p.V406SfsX3 mutation and the PKP2 p.T15T synonymous variation. The genetic analysis extended to her daughter revealed that she carries only p.T15T variation, in presence of negative clinical and instrumental findings.

Proband 5 underwent cardiac evaluation at the age of 45 yrs, due to the presence of PVCs detected during a pre-operatory ECG that also showed intraventricular conduction delay and negative T waves in V4-V6. SAECG documented the presence of late potentials at all filters settings. Two D echocardiogram demonstrated biventricular dimensional and kinetic abnormalities. At the age of 49 he received an ICD (implantable cardioverter defibrillator) due to recurrent VT episodes. He was found to carry additional mutations in other two ARVC/D genes: p.N375I in *DSP* and p.F552C in *PKP2*. His brother carries only the *DSP* mutation. The clinical and instrumental examination demonstrated the presence of some ECG criteria, even if he did not fulfil the diagnosis (Table 2).

Haplotype analysis

In contrast with other reported *DSC2* mutations [8,9,13], p.A897KfsX4 is the only one identified in more Italian and English ARVC/D patients. Haplotype analysis was performed using four polymorphic microsatellites, one intragenic (D18SH4) and three in close proximity to the *DSC2* gene (D18S847, D18SH3 and D18S36). Different haplotypes segregated with the p.A897KfsX4 variation in each of the five Italian patients demonstrating the absence of a founder effect for this variation (data not shown). This result confirms data reported by Syrris *et al*. for their three families, suggesting that p.A897KfsX4 is a recurrent variation.

Expression pattern of *DSC2* isoforms

Almost all analysed human cDNA samples (heart, pancreas, lung, placenta, brain, skeletal muscle, liver and kidney) showed expression of both *DSC2* splice forms (Figure 3). It is interesting to note that all of them showed a different expression level of the two isoforms. In particular, in contrast with what we found in the other tissues, in heart isoform b is more expressed than isoform a.

The PCR products for heart tissue were verified and confirmed by sequencing (data not shown).

Functional analysis of C-terminal mutant desmocollin-2a (p.A897KfsX4)

Site-directed mutagenesis was performed on wild type construct encoding for a human dsc2a-gfp fusion protein in order to study the functional effect of the p.A897KfsX4 variation and of two *DSC*2 polymorphisms p.D179G and p.R798Q, located in the N- and C-terminal domains of the protein, respectively.

Constructs were transfected in the desmosome-forming cell line HL-1 having a differentiated cardiomyocyte phenotype and contractile activity *in vitro.* The GFP signal revealed a predominant localization at the plasma membrane of wild type (Figure 4, panel A) and proteins carrying p.D179G and p.R798Q polymorphisms (Figure 4, panel B and C), displaying co-localization with endogenous dsg at the cell membrane to indicate well-assembled desmosomes (Figure 4, panels A", B" and C").

By contrast protein carrying frameshift variation p.A897KfsX4 was detected in the cytoplasm (Figure 4, panel D), loosing the proper desmosomal localization along cell boundaries. On the contrary, endogenous dsg is normally well distributed (Figure 4, panel D').

Discussion

Desmocollin-2 was the most recent major component of the cardiac desmosome to be implicated in ARVC/D. *DSC2* gene encodes one of desmosomal cadherins, single-pass transmembrane glycoproteins able to mediate Ca2+-dependent cell-cell adhesion, by interacting laterally and transcellularly with each other and by recruiting cytoplasmic plaque proteins which facilitate attachment of intermediate filaments[21].

We have identified two different *DSC2* mutations and one *DSC2* variation in 7 out of 112 unrelated ARVC/D index cases, two already described in our previously report[13] and p.A897KfsX4 originally referred to as a causative mutation[8]. The frameshift variation was identified in five independent patients, and four of them are carriers for one or two mutations in known ARVC/D genes. Surprisingly we found the same variation in several Italian controls with a no significant difference in the distribution of the variant between patients and control group.

On the contrary, in the original paper reporting identification of p.A897KfsX4 variation, three probands resulted to carry only this frameshift variation, which was never identified in 200 control subjects[8].

In contrast with other reported *DSC2* mutations, p.A897KfsX4 is the only one identified in several Italian and English ARVC/D patients. Haplotype analysis excludes a founder effect in our patients as well as in English cases and confirms that p.A897KfsX4 is a recurrent variation.

This variant occurs in exon 17 that encodes for ICS domain (intracellular cadherin-like sequence domain) only in the splice variant “a” of *DSC2* gene,

whereas the splice variant "b" has a different C-terminal domain. ICS domain provides binding sites for other desmosomal constituents such as plakoglobin, plakophilin and desmoplakin[22,23,24]. It has been demonstrated that the latest 37 aa in Dsc1a are fundamental for plakoglobin binding in human epithelial A-431 cells[23]. On the other hand, in vitro functional studies on HL-1 cells have demonstrated that DSC2a-GFP-A897KfsX4 protein localizes in the cytoplasm, in contrast with wild type protein and DSC2a variants carrying the polymorphism p.D179G or p.R798Q which correctly co-localize at the cell membrane with endogenous desmoglein.

However it is important to notice that the exon 17 is untranslated in DSC2b isoform. Therefore, the insertion would affect only DSC2a, leaving isoform b fully functional and possibly able to compensate in cardiac myocytes the relative deficiency of DSC2a isoform. Furthermore in contrast with what we found in the other human tissues, in heart DSC2b isoform is more expressed than DSC2a supporting the hypothesis of a possible compensation by the isoform b.

Little is known about human DSC2b and in general about all Dscb isoforms; until now only for Dsc3b it has been demonstrated a specific desmosomal interaction with PKP3[25]. Moreover, PKP1, for which strong in vitro association with the 'a' form of Dsc1 has been reported[24], was found to overlap also with the 'b' form[26]. The possibility that Dscb isoforms contain a PKP-binding domain should be further on investigated.

Four out of 5 patients carrying p.A897KfsX4 variation had also one or two additional ARVC/D mutations. On the other hand, proband 1 carrying only p.A897KfsX4 variation resulted to carry also a reported polymorphism (PKP2

p.E58D) whose pathogenic role, if any, remains to be investigated[18]. Moreover, we cannot exclude that this proband could have a large insertion, deletion or mutation in non coding regions of ARVC/D genes or also a mutation in unknown ARVC/D genes.

It may be hypothesized that p.A897KfsX4 variation could act in presence of other ARVC/D mutations as a factor able to modify their pathogenic effect. All probands showed moderate to severe forms of the disease, with biventricular involvement in some cases. However, in our study group genotype-phenotype data could not give a clear indication of the role of this variant in the disease-phenotype. Moreover, there is not a different proportion of the variant in the controls and affected subjects to provide evidence of an association between the disease and the variant. Assessment of the exact significance of the p.A897KfsX4 variation requires further studies in additional families carrying mutations in other ARVC/D genes.

Until now few *DSC2* gene mutations were reported to cause ARVC/D. Among them, the p.A897KfsX4 variation, identified in several Italian healthy control subjects and altering only one of the two DSC2 isoforms, could be considered a rare polymorphism which may affect the phenotypic expression of concomitant ARVC/D mutations.

Further clarification of co-occurrence of mutations and rare polymorphisms in different desmosomal proteins could be an important aspect in accounting for the known inter- and intra-familial variable phenotypic expression.

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References

1 Thiene G, Nava A, Corrado D, Rossi L, Pennelli N: Right ventricular cardiomyopathy and sudden death in young people. *N Engl J Med* 1988; **318**: 129-133.

2 Nava A, Thiene G, Canciani B *et al*: Familial occurrence of right ventricular dysplasia: a study involving nine families. *J Am Coll Cardiol* 1988; **12**: 1222-1228.

3 McKoy G, Protonotarios N, Crosby A *et al*: Identification of a deletion in plakoglobin in arrhythmogenic right ventricular cardiomyopathy with palmoplantar keratoderma and woolly hair (Naxos disease). *Lancet* 2000; **355**: 2119-2124.

4 Asimaki A, Syrris P, Wichter T, Matthias P, Saffitz JE, McKenna WJ: A novel dominant mutation in plakoglobin causes arrhythmogenic right ventricular cardiomyopathy. *Am J Hum Genet* 2007; **81**: 964-973.

5 Rampazzo A, Nava A, Malacrida S *et al*: Mutation in human desmoplakin domain binding to plakoglobin causes a dominant form of arrhythmogenic right ventricular cardiomyopathy. *Am J Hum Genet* 2002; **71:** 1200-1206.

6 Gerull B, Heuser A, Wichter T *et al*: Mutations in the desmosomal protein plakophilin-2 are common in arrhythmogenic right ventricular cardiomyopathy. *Nat Genet* 2004; **36**: 1162-1164.

7 Pilichou K, Nava A, Basso C *et al*: Mutations in desmoglein-2 gene are associated with arrhythmogenic right ventricular cardiomyopathy. *Circulation* 2006; **113**: 1171-1179.

8 Syrris P, Ward D, Evans A, Asimaki A, Gandjbakhch E, Sen-Chowdhry S, McKenna WJ: Arrhythmogenic right ventricular dysplasia/cardiomyopathy associated with mutations in the desmosomal gene desmocollin-2. *Am J Hum Genet* 2006; **79**: 978-984.

9 Heuser A, Plovie ER, Ellinor PT *et al*: Mutant desmocollin-2 causes arrhythmogenic right ventricular cardiomyopathy. *Am J Hum Genet* 2006; **79**: 1081-1088.

10 Tiso N, Stephan DA, Nava A *et al*: Identification of mutations in the cardiac ryanodine receptor gene in families affected with arrhythmogenic right ventricular cardiomyopathy type 2 (ARVD2). *Hum Mol Genet* 2001; **10**: 189-194.

11 Beffagna G, Occhi G, Nava A *et al*: Regulatory mutations in transforming growth factor-beta 3 gene cause arrhythmogenic right ventricular cardiomyopathy type 1. *Cardiovasc Res* 2005; **65**: 366-373.

12 Merner ND, Hodgkinson KA, Haywood AF *et al*: Arrhythmogenic right ventricular cardiomyopathy type 5 is a fully penetrant, lethal arrhythmic disorder caused by a missense mutation in the TMEM43 gene. *Am J Hum Genet* 2008; **82**: 809-821.

13 Beffagna G, De Bortoli M, Nava A *et al*: Missense mutations in Desmocollin-2 N-terminus, associated with arrhythmogenic right ventricular cardiomyopathy, affect intracellular localization of desmocollin-2 in vitro. *BMC Med Genet* 2007; **8**: 65-75.

14 Simpson MA, Mansour S, Ahnood D, *et al*: Homozygous Mutation of Desmocollin-2 in Arrhythmogenic Right Ventricular Cardiomyopathy with Mild Palmoplantar Keratoderma and Woolly Hair. *Cardiology* 2008; **113**:28-34.

15 McKenna WJ, Thiene G, Nava A *et al*: Diagnosis of arrhythmogenic right ventricular dysplasia/cardiomyopathy: Task Force of the Working Group Myocardial and Pericardial Disease of the European Society of Cardiology and of the Scientific Council on Cardiomyopathies of the International Society and Federation of Cardiology. *Br Heart J* 1994; **71**: 215-218.

16 Nuber UA, Schafer S, Schmidt A, Koch PJ, Franke WW: The widespread human desmocollin Dsc2 and tissue-specific patterns of synthesis of various desmocollin subtypes. *Eur J Cell Biol* 1995; **66**: 69-74.

17 Claycomb WC, Lanson NA, Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, Izzo NJ Jr: HL-1 cells: A cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc Natl Acad Sci USA* 1998; **95**:2979-2984.

18 Lahtinen AM, Lehtonen A, Kaartinen M *et al*: Plakophilin-2 missense mutations in arrhythmogenic right ventricular cardiomyopathy. *Int J Cardiol* 2008;**126**:92-100.

19 Pilichou K, Nava A, Basso C *et al*: Mutations in desmoglein-2 gene are associated with arrhythmogenic right ventricular cardiomyopathy. *Circulation* 2006;**113**:1171-1179.

20 van Tintelen JP, Entius MM, Bhuiyan ZA *et al*: Plakophilin-2 mutations are the major determinant of familial arrhythmogenic right ventricular dysplasia/cardiomyopathy. *Circulation* 2006;**113**:1650-1658

21 Yin T, Green KJ: Regulation of desmosome assembly and adhesion. *Semin Cell Dev Biol* 2004; **15**:665-677.

22 Troyanovsky SM, Eshkind LG, Troyanovsky RB, Leube RE, Franke WW: Contributions of cytoplasmic domains of desmosomal cadherins to desmosome assembly and intermediate filament anchorage. *Cell* 1993; **72**:561-574.

23 Troyanovsky SM, Troyanovsky RB, Eshkind LG, Leube RE, FrankeWW: Identification of amino acid sequence motifs in desmocollin, a desmosomal glycoprotein, that are required for plakoglobin binding and plaque formation. *Proc Natl Acad Sci USA* 1994; **91**:10790-10794.

24 Smith EA, Fuchs E: Defining the interactions between intermediate filaments and desmosomes. *J Cell Biol* 1998; **141**:1229-1241.

25 Bonné S, Gilbert B, Hatzfeld M, Chen X, Green KJ, van Roy F: Defining desmosomal plakophilin-3 interactions. *J Cell Biol* 2003; **161**:403-416

26 North AJ, Bardsley WG, Hyam J *et al*: Molecular map of the desmosomal plaque. *J Cell Sci* 1999; **112**:4325-4336

Titles and legends to figures

Figure 1. a: p.E102K and p.I345T mutations (black arrows) and p.A897KfsX4 variation (red arrow) identified in *DSC2* gene. p.A897KfsX4 variation affects only DSC2a isoform. **b**: Changes introduced in the amino acid sequence of desmocollin-2a, caused by the insertion c.2687_2688insGA in *DSC2* gene. **c**: Last five aminoacids of desmocollin-2a (involved by p.A897KfsX3 variation) show relative variance among species: *H. sapiens* (NP_077740), predicted *M. mulatta* (XP_001102096.1), predicted P. troglodytes (XP_512077.2), predicted *B. taurus* (XP_615164.3), *R. norvegicus* (NP_001028860.1), predicted *C. familiaris* (XP_866917.1), predicted and *E. caballus* (XP_001496356.1).

Figure 2: Pedigree of Proband 3 (marked by an arrow). Filled in symbols indicate clinically affected family members and white symbols indicate unaffected individuals. Absence (-) and presence of (#) *DSP*, (⋆) *DSG2* mutations and (•) *DSC2* variation are indicated.

Figure 3: Expression pattern of *DSC2* splice forms. Double band represents isoform b (500bp) and isoform a (454bp). Results shown are from heart (*lane 1)*, pancreas (*lane 2*), lung (*lane 3*), placenta (*lane 4*), brain (*lane 5*), skeletal muscle (*lane 6*), liver (*lane 7*) and kidney (*lane 8*).

Figure 4: Functional studies in HL-1 cells. DSC2a-GFP-WT, DSC2a-GFP-D179G and DSC2a-GFP-R798Q were localised at the cell membrane between

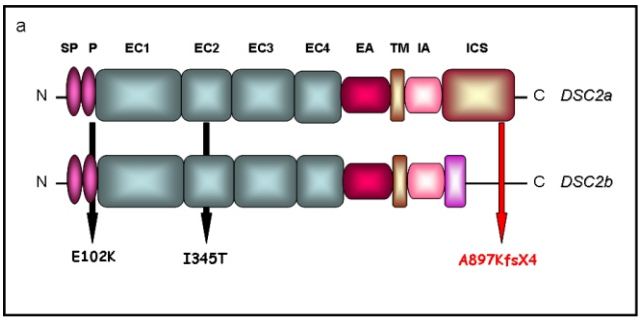
two HL-1 cells (panel A, B and C), whereas DSC2a-GFP-A897KfsX4 was detected in the cytoplasm (panel D). Immunostaining with monoclonal desmoglein antibody DG3.10 showed both the presence of well-assembled desmosomes (yellow dots in panel A", B'' and C'') and none co-localisation between endogenous dsg and DSC2a-GFP-A897KfsX4(panel D'').

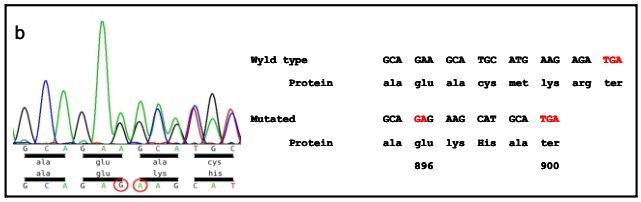
Proband	Gene	Nucleotide change	Amino acid change
1	DSC2	c.2687_2688insGA	p.A897KfsX4
2	DSC2	c.2687_2688insGA	p.A897KfsX4
	DSG2	c.260A>G	p.Y87C
3	DSC2	c.2687_2688insGA	p.A897KfsX4
	DSG2	c.1912G>A	p.G638R*
	DSP	c.1124A>T	p.N375I *
4	DSC2	c.2687_2688insGA	p.A897KfsX4
	PKP2	c.1211_1212insT	p.V406SfsX3
5	DSC2	c.2687_2688insGA	p.A897KfsX4
	DSP	c.1124A>T	p.N375I*
	PKP2	c.1655T>G	p.F552C*

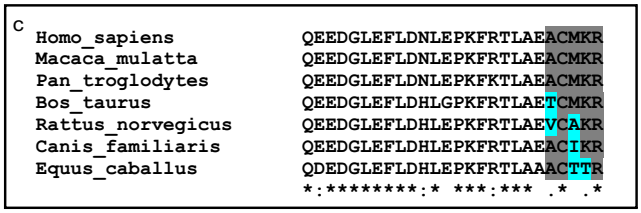
Table 1: A897KfsX4 variation carriers. * symbol indicates novel mutations.

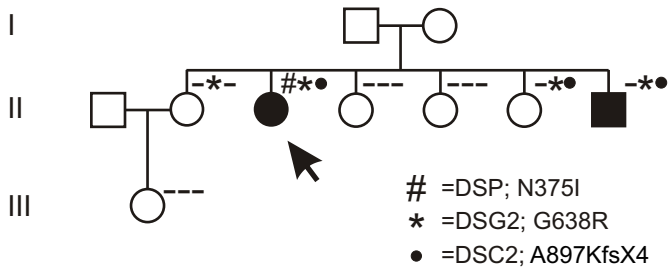
Proband #. (Family #)	Sex	Age at diagnosis/ Last follow-up	Family history		12-lead ECG				SAECG	Arrhythmias			RV size/ function		LV involv	Diagnostic criteria	Gene	Amino acid change
			Major	Minor	Negative T waves preocordial leads	Negative T waves inferior leads	Incomplete RBBB	Epsilon wave		PVCs >1000/ 24 h NSVT	LBBB SVT	VF	M	m				
1	F	60	-	+	+	-	-	-	-	+	-	-	+	-	-	1M/3m	DSC2	A897KfsX4
1.(1)	M	33	-	+	-	-	+	-	+	-	-	-	-	-	-	2m	DSC2	A897KfsX4
2	M	45	-	-	+	+	-	-	-	+	-	-	+	-	-	1M/2m	DSC2	A897KfsX4
																	DSG2	Y87C
2.(1)	F	22	-	+	-	-	-	-	-	-	-	-	-	-	-	1m	DSC2	-
																	DSG2	-
3	F	38	-	-	+	+	-	-	+	-	+	-	+	-	+	1M/3m	DSC2	A897KfsX4
																	DSG2	G638R
																	DSP	N375I
3.(II,2)	F	58	-	+	-	-	-	-	-	-	-	-	-	-	+	1m	DSC2	-
																	DSG2	G638R
																	DSP	-
3.(II,6)	F	54	-	+	-	-	-	-	-	-	-	-	-	-	-	1m	DSC2	A897KfsX4
																	DSG2	G638R
																	DSP	-
3.(II,7)	M	42	-	+	-	-	-	-	+	+	-	-	+	-	+	4m	DSC2	A897KfsX4
																	DSG2	G638R
																	DSP	-
4	F	30	-	+	+	+	-	-	np	+	-	-	-	+	-	4m	DSC2	A897KfsX4
																	PKP2	V406SfsX3
4.(1)	F	13	-	+	-	-	+*	-	-	-	-	-	-	-	-	1m	DSC2	-
																	PKP2	-
5	M	45	-	-	+	+	+	-	+	-	+	-	+	-	+	1M, 3m	DSC2	A897KfsX4
																	DSP	N375I
																	PKP2	F552C
5.(1)	M	49	-	+	-	-	+	-	+	-	-	-	-	-	-	3m	DSC2	-
																	DSP	N375I
																	PKP2	-

Table 2: Clinical and genetic findings in A897KfsX4 variation carriers. Legend: np: not performed; +*: paediatric ECG; RBBB, right bundle branch block; NSVT, nonsustained ventricular tachycardia; SVT, sustained ventricular tachycardia; VF, ventricular fibrillation; M major diagnostic criteria; m minor diagnostic criteria.


a
SP P EC1 EC2 EC3 EC4 EA TM IA ICS
N C DSC2a
N C DSC2b
E102K
I345T
A897KfsX4

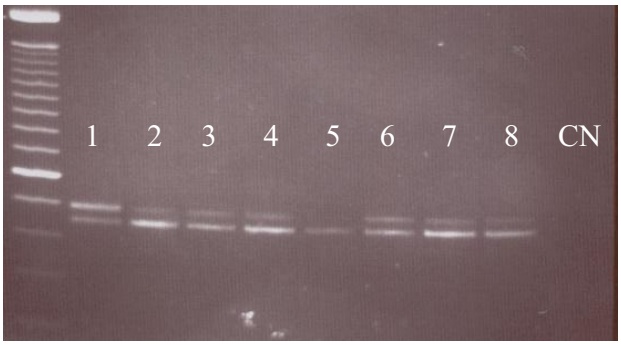

b
Wyld type GCA GAA GCA TGC ATG AAG AGA TGA
Protein ala glu ala cys met lys arg ter
Mutated GCA GAG AAG CAT GCA TGA
Protein ala glu lys His ala ter
896 900

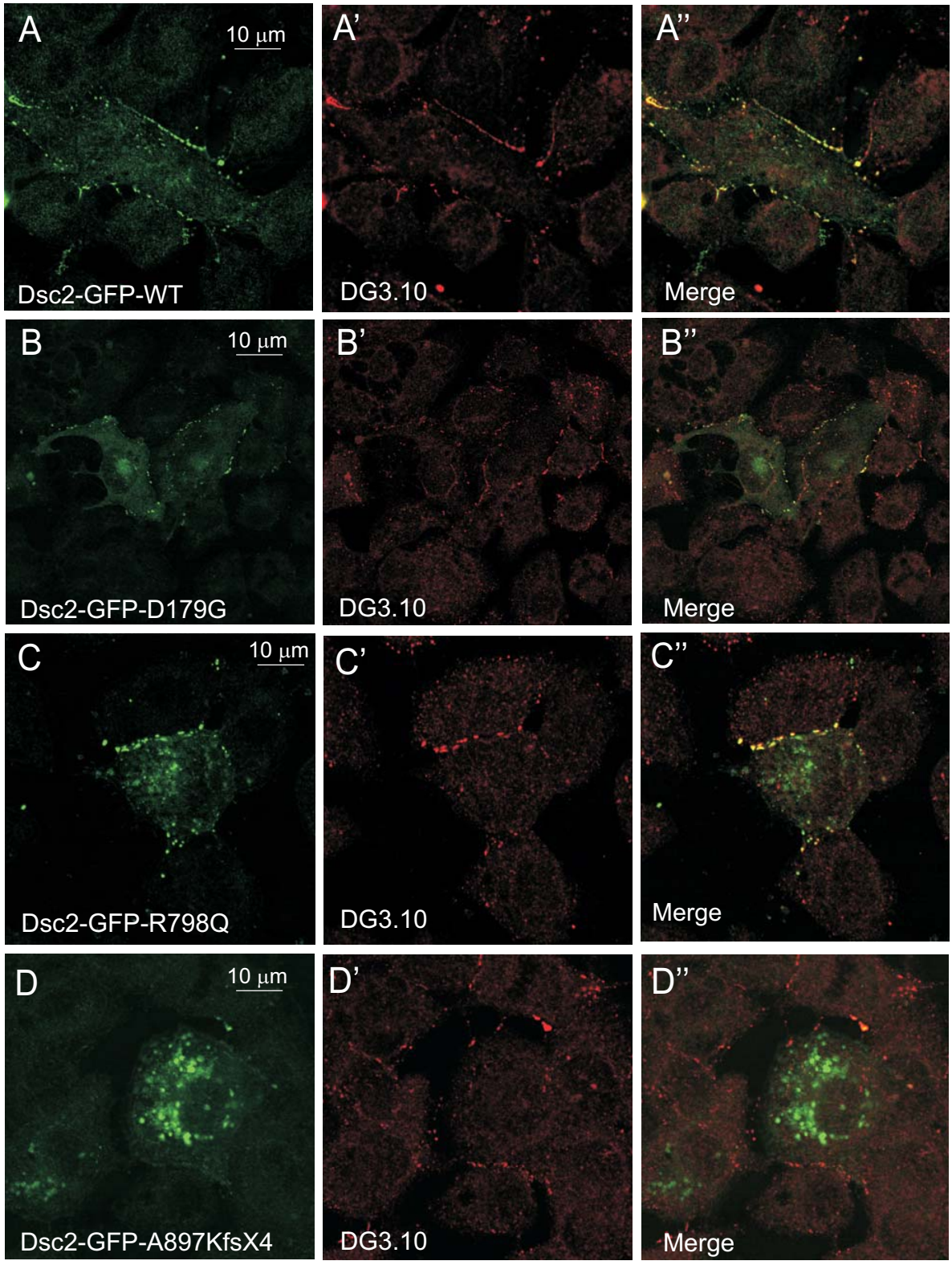

c
Homo_sapiens QEEDGLEFLDNLEPKFRTLAEACMKR
Macaca_mulatta QEEDGLEFLDNLEPKFRTLAEACMKR
Pan_troglodytes QEEDGLEFLDNLEPKFKTLAEACMKR
Bos_taurus QEEDGLEFLDHLGPKFRTLAETCMKR
Rattus_norvegicus QEEDGLEFLDHLEPKFRTLAEVCAKR
Canis_familiaris QEEDGLEFLDHLEPKFRTLAEACIKR
Equus_caballus QDEDGLEFLDHLEPKFRTLAAACTTR


I
II
III
-*-
#*●

-*●
-*●

=DSP; N375I
* =DSG2; G638R
● =DSC2; A897KfsX4


1
2
3
4
5
6
7
8
CN


A
10 μm
Dsc2-GFP-WT
A'
DG3.10
A''
Merge
B
10 μm
Dsc2-GFP-D179G
B'
DG3.10
B''
Merge
C
10 μm
Dsc2-GFP-R798Q
C'
DG3.10
C''
Merge
D
10 μm
Dsc2-GFP-A897KfsX4
D'
DG3.10
D''
Merge