# UNIVERSITA' DEGLI STUDI DI PADOVA

Università degli Studi di Padova

Dipartimento di Biologia

SCUOLA DI DOTTORATO DI RICERCA IN BIOSCIENZE

INDIRIZZO: GENETICA E BIOLOGIA MOLECOLARE DELLO SVLUPPO

CICLO XX

TESI DI DOTTORATO

## ARRHYTHMOGENIC RIGHT VENTRICULAR CARDIOMYOPATHY: MUTATION SCREENING OF CANDIDATE GENES AND IN VITRO FUNCTIONAL STUDIES

**Direttore della Scuola :** Ch.mo Prof. TULLIO POZZAN

**Supervisore** :Ch.mo Prof. GIAN ANTONIO DANIELI

**Dottoranda:** MARZIA DE BORTOLI

31 gennaio 2008

# CONTENTS

# INTRODUCTION

## CLINICAL ASPECTS OF ARVC

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is an inherited heart disease which may result in arrhythmia, heart failure, and sudden cardiac death. In fact, ARVC is a major cause of sudden death in the young and athletes; its prevalence has been estimated to vary from 1:2,500 to 1:5,000. Familial occurrence of ARVC is rather common. The trait shows autosomal dominant inheritance with about 50% penetrance (Nava A. et al., 1988). The main pathologic feature is progressive loss of right ventricular myocardium, which is replaced by adipose and fibrous tissue (Thiene G. et al., 1988). These changes may be localized; in early disease they are often confined to the so-called "triangle of dysplasia": the inflow, outflow, and apical regions of the right ventricle. Aneurysm formation may occur. With the progress of the disease, diffuse myocardial involvement leads to global right ventricular dilation. Histological examination of affected myocardial tissue shows sparse myocytes interspersed among adipocytes and fibrous tissue. The process begins from epicardium and gradually extends through myocardium towards subendocardium. Fibrofatty substitution of the left ventricle is rather frequent in the advances state of the disease.

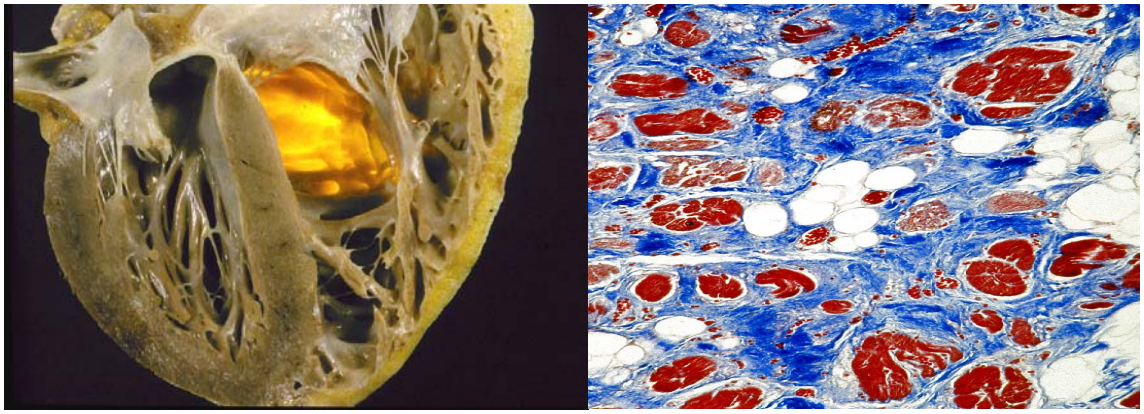

**Figure 1**: A typical case of ARVC in a 25-years old man who died suddenly at rest. Noticeable isolated fatty replacement of the right ventricular free wall and translucent infundibulum. Endomyocardial biopsy of the right ventricle free wall from another patient affected with ARVC shows rare myocytes embedded in fatty and fibrous tissues (From Nava A. et al., 1997).

Clinical manifestations of the disease occur most often between the second and fourth decade of life; they include structural and functional abnormalities of the right ventricle, electrocardiographic depolarization/repolarization changes and arrhythmias of right ventricular origin (Marcus F.I. et al., 1982; Nava A. et al., 2000).

Natural history of the disease may be subdivided into four phases, on the basis of clinical and pathological findings (Corrado D. et al., 2000). Early ARVC is often described as "concealed" owing to frequent absence of clinical findings, although minor ventricular arrhythmia and subtle structural changes are sometimes discernible. Patients tend to be asymptomatic but nonetheless they may be at risk of sudden death, notably during strong physical exercise. The "overt electrical disorder" which subsequently develops is characterized by symptomatic ventricular arrhythmia; patients typically present with palpitation, syncope, and pre-syncope. Morphological abnormalities are more obvious at this stage and usually detectable by imaging. In the third phase, further extension of disease through the right ventricular myocardium causes impaired contractility and isolated right heart failure. Left ventricular involvement with consequent biventricular failure occurs in the end-stage, which may be difficult to distinguish from dilated cardiomyopathy (DCM) (Nemec J. et al., 1999).

**GENETICS OF ARVC**

From early '90s, linkage analysis started to reveal the existence of 9 genetic loci independently involved in the determination of ARVC; however, only 4 disease genes (*RYR2*, *JUP*, *DSP* and *TGFβ3*) have been identified so far within these regions (Tab.1). Recently, genetic analysis shifted from linkage studies to candidate gene approach, thus leading to the discovery of additional genes involved in ARVC (*PKP2*, *DSG2* and *DSC2*), which escaped detection by linkage approach (Tab.1).

Until now, out of the seven genes found associated to ARVC, *RYR2* is the only one directly involved in $Ca^{2+}$ homeostasis (Tiso N. et al., 2001). *RYR2* is one of the largest human genes, including 105 exons and encoding a 565 Kda monomer, which is part of a homo-tetrameric sarcoplasmic reticulum membrane protein. The homo-tetrameric structure, known as cardiac ryanodine receptor, plays a pivotal role in intracellular calcium homeostasis and excitation-contraction coupling in cardiomyocytes (Stokes D.L. and Wagenknecht T., 2000; Missiaen L. et al., 2000). Mutations in the human *RYR2* gene have been associated with ARVC2 but also with catecholaminergic polymorphic ventricular tachycardia (CPVT; OMIM 604772) (Priori S.G. et al., 2001; Priori S.G. et al., 2002) and familial polymorphic ventricular tachycardia (FPVT; OMIM 604772) (Laitinen P.J. et al., 2001; Laitinen P.J. et al., 2003). ARVC2 is a form characterized by the presence of polymorphic, effort-induced arrhythmias, but less pronounced fibro-fatty substitution of the myocardial tissue than in classical ARVC. All

*RYR2* mutations causing ARVC2 were missense resulting in substitutions involving amino acids highly conserved in critical domains of the protein (Tiso N. et al., 2001; Bagattin A. et al., 2004). It has been proposed that *RYR2* mutations affect regulation of calcium channel. Intense adrenergic stimulation due to emotional or physical stress can lead to calcium overload, thus triggering severe arrhythmias.

| Locus | Inheritance pattern | Chromosome | Gene |
|---|---|---|---|
| ARVD1 | AD | 14q23-q24 | TGF-β3 |
| ARVD2 | AD | 1q42-q43 | RYR2 |
| ARVD3 | AD | 14q12-q22 | |
| ARVD4 | AD | 2q32.1-q32.2 | |
| ARVD5 | AD | 3p23 | |
| ARVD6 | AD | 10p12-p14 | |
| ARVD7 | AD | 10q22.3 | |
| ARVD8 | AD | 6p24 | DSP |
| ARVD9 | AD | 12p11 | PKP2 |
| ARVD10 | AD | 18q12.1 | DSG2 |
| ARVD11 | AD | 18q12.1 | DSC2 |
| ARVD12<br>Naxos Disease | AD<br>AR | 17q21 | JUP |

**Table 1**: Known ARVC loci and disease-genes.

Although ARVC is most commonly inherited as autosomal dominant trait, identification of the first disease-causing gene occurred for a recessive variant known as Naxos disease. This syndrome is characterised by arrhythmogenic right ventricular cardiomyophaty associated with palmoplantar keratoderma and peculiar woolly hair. In 1998, Coonar et al. mapped the genetic locus to 17q21 (Coonar A.S. et. al., 1998). Two years later, McKoy et al. reported a 2 base pair deletion (c.2157del2TG) in the plakoglobin gene (*JUP*) as the cause of Naxos disease. This mutation results in a frameshift and premature termination of the protein (McKoy G. et al., 2000). Interestingly, a recent paper reported a novel dominant mutation (c.118_119insGCA) in

the gene encoding Plakoglobin, causing ARVC. This dominant mutation doesn't disrupt the frame of translation but it would cause the insertion of an additional serine residue at amino acid position 39 within the N-terminal domain of plakoglobin (S39_K40insS) (Asimaki A. et al., 2007). Plakoglobin is an armadillo family member contains 12 *arm* reapts and is a major component of cell-cell adhesion complexes, which are abundant in many tissues. It is also a signaling molecule with role in desmosome assembly and development and in the regulation of gene expression (Rubenstein A. et al., 1997). In fact, Plakoglobin is found in junctions as well as in the nucleus where it may have a role in transcriptional regulation (Getsios et al., 2004). It is now accepted that plakoglobin plays a role in the Wnt/β-catenin signalling pathway and that it also could interact with Tcf/Lef protein. However it is not yet clear whether plakoglobin activates or represses Wnt/β-catenin target genes and whether it acts on distinct set of genes (Garrod D. and Chidgey M., 2007).

The first disease gene linked to autosomal dominant ARVC showing typical right ventricular phenotype was Desmoplakin (*DSP*). In 2002, a genome scan in a Italian family with ARVC detected linkage with a region of chromosome 6 short arm, which includes *DSP* gene. A missense mutation S299R was identified in exon 7 of all affected persons of this family; the resulting amino acid substitution modifies a putative phosphorylation site in the N-terminal plakoglobin binding domain (Rampazzo A. et al., 2002). Desmoplakin, together with plakoglobin, anchors to desmosomal cadherins, forming an ordered array of nontransmembrane proteins, which bind to desmin intermediate filaments in cardiomyocytes. Desmoplakin contains three main functional domains: the N-terminal which binds to the desmosome via connection with plakoglobin and plakophilin; a ROD segment which is predicted to form a dimeric coil and the C-terminal important for intermediate filaments binding (Choi H.J. et al., 2002). There are two isoforms of desmoplakin (DPI and DPII) which are generated by alternative splicing and differ only in the length of the central rod domain. Mutations in *DSP* gene have been shown to be responsible for some cases of an autosomal dominant skin disorder (striate palmoplantar keratoderma) (Armstrong D.K. et al., 1999; Whittock N.V. et al., 1999; Whittock N.V. et al., 2002); an autosomal recessive condition characterized by dilated cardiomyopathy, woolly hair, and keratoderma (so-called Carvajal syndrome) (Norgett E.E. et al., 2000), another autosomal recessive condition characterized by ARVC, woolly hair, and keratoderma (Alcalai R. et al., 2003) and a

left-sided ARVC named arrhythmogenic left ventricular cardiomyopathy (ALVC) (Norman M. et al., 2005).

The discovery of plakoglobin and desmoplakin mutations led to the idea that ARVC is due to cellular-adhesion defects, thus prompting the candidate gene approach for identifying additional genes involved in ARVC.

In 2004, Gerull et al. selected plakophilin-2 (*PKP2*) as a candidate gene for ARVC because it encodes an essential protein of cardiac desmosomes; in fact, a homozygous deletion in *PKP2* gene caused a lethal cardiac defect in mice (Grossmann K.S. et al., 2004). The authors identified 25 different heterozygous mutations in 32 of 120 unrelated ARVC patients (Gerull B. et al., 2004). Plakophilin-2 is an armadillo-related protein, located in the outer dense plaque of desmosomes. It links desmosomal cadherins to desmoplakin and the intermediate filament system (Fig.2). There are two isoforms of plakophilin-2, a shorter 'a' variant and a longer 'b' form, generated by alternative splicing. PKP2a and PKP2b differ by the insertion of 44 amino acids between armadillo repeats 2 and 3 (Mertens C. et al., 1996). Plakophilin-2 are also present in the nucleus, where it may play a role in transcriptional regulation because it has been associated with RNA polymerase III (Mertens C. et al., 2001).

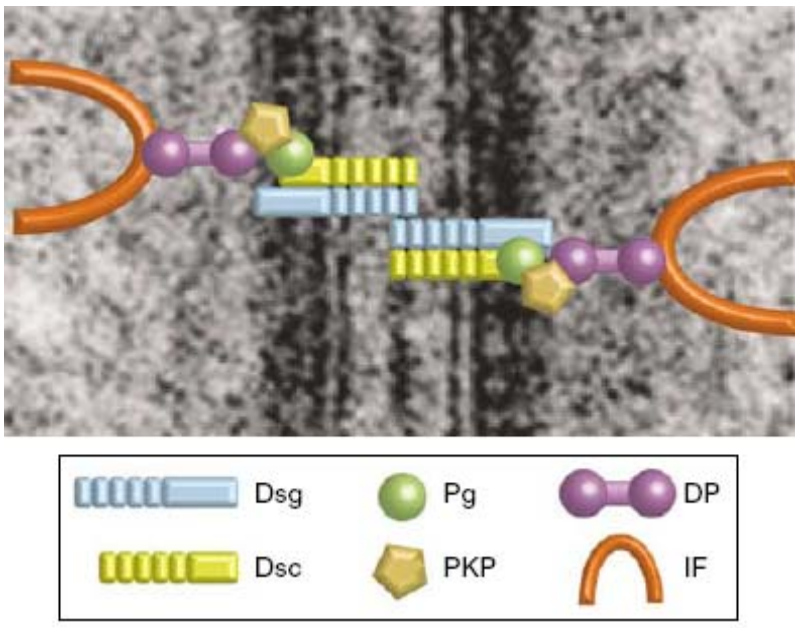


**Figure 2**: Schematic representation of relationships between desmosomal proteins. Transmembrane desmosomal cadherins, Dsg and Dsc, bind the armadillo family protein PG, which in turn anchors the plakin family member DP and PKP. The cytoplasmic plaque, which is further stabilized by lateral interactions among these proteins, anchors the IF cytoskeleton to the desmosome (From: Green K.J. and Sympson C.L., 2007).

Gerull et al. speculated that lack of plakophilin-2 or incorporation of mutant plakophilin-2 in the cardiac desmosomes might impair cell-cell contacts and might disrupt association between adjacent cardiomyocytes.

Recent studies have reported mutations in a fourth desmosomal gene, desmoglein-2 (*DSG2*), in familial cases of ARVC (Pilichou K. et al., 2006; Awad M.M. et al., 2006). After few months another desmosomal gene, desmoscollin-2 (*DSC2*) was identified as involved in ARVC (Syrris P. et al., 2006; Heuser A. et al., 2006). Desmosomal cadherins, DSGs and DSCs, are single-pass transmembrane glycoproteins, that mediate $Ca^{2+}$-dependent cell-cell adhesion (Yin T. and Green K.J., 2004), by interacting laterally and transcellularly with each other and by recruiting cytoplasmic plaque proteins which facilitate attachment of intermediate filaments. In humans there are four desmoglein isoforms (DSG1-4) and three desmocollin isoforms (DSC1-3); the corresponding genes cluster in the same region of chromosome 18 (Hunt D.M. et al., 1999). *DSG2* and *DSC2* are expressed in all desmosome-containing tissues but they are the only isoforms expressed in cardiac myocytes (Schäfer S. et al., 1994; Nuber U.A. et al., 1995). Each of the three desmocollin genes encodes a pair of proteins that are generated by alternative splicing, a longer 'a' form and a shorter 'b' form that differ only in their C-terminal tails. The desmocollin extracellular domains can divided into a number of subdomains, four cadherin-like EC domains and an extracellular anchor domain (EA). Desmoglein extracellular domains are organised in a similar fashion. Within the cell, both desmocollin 'a' and 'b' proteins possess an intracellular anchor domain (IA) but only 'a' forms have an intracellular cadherin-like sequence domain (ICS). Desmoglein cytoplasmic tails also have IA and ICS domains. Desmocollin and desmoglein ICS domains provide binding sites for other desmosomal constituents such as plakoglobin. Additional domains found in desmoglein cytoplasmic tails include the intracellular proline-rich linker domain (IPL), a repeat unit domain (RUD) made by a variable number of 29 amino acids repeats, and a glycine-rich desmoglein terminal domain (DTD) (Green K.J. and Gaudry C.A., 2000; Huber O., 2003; Garrod D. and Chidgey M., 2007).

On the other hand, different roles in the determination of ARVC have been suggested by finding regulatory mutations of *TGFβ3* gene associated with ARVC. In 1994, linkage analysis identified a genetic locus for dominant ARVC at 14q23-q24, thereafter termed ARVC1 (Rampazzo A. et al., 1994). One of the most promising candidate genes mapped to this region was transforming growth factor *β*3 (TGF*β*3). In fact a nucleotide

substitution c.-36G>A in 5'UTR of *TGFβ3* gene was detected in all affected subjects belonging to a large ARVC1 family and an additional mutation c.1723C>T was identified in 3'UTR of one patient. In vitro expression assay of constructs containing the mutations showed that mutated UTRs were two-fold more active than wild type (Beffagna G. et al., 2005). Transforming growth factor *β* family of cytokines is known to stimulate mesenchymal cells to proliferate and to produce extracellular matrix components. It is therefore conceivable that enhanced TGF*β* activity may lead to myocardial fibrosis. Myocardial fibrosis may disrupt electrical and mechanical behaviour of myocardium and extracellular matrix abnormalities may predispose re-entrant ventricular arrhythmias. Moreover, it has been shown that TGF*β*s modulate expression of genes encoding desmosomal proteins in different cell types (Kapoun A.M. et al., 2004; Yoshida M. et al., 1992). Therefore, overexpression of *TGFβ3*, caused by UTRs mutations, might affect cell to cell junctions stability, thus leading to disease expression similar to that observed in ARVC due to mutations of genes encoding desmosomal proteins.

Desmoplakin involved in ARVC8, plakophilin-2 involved in ARVC9, desmoglein-2 involved in ARVC10, desmocollin-2 involved in ARVC11 and plakoglobin involved in Naxos syndrome and in ARVC12 are all desmosomal proteins. Based on present evidence ARVC is considered a disease of the desmosome. For this reason, additional components of desmosomal complex may be targets for pathogenic mutations leading to ARVC.

## MOLECULAR PATHOGENESIS OF ARVC

While involvement of genes encoding desmosomal proteins in ARVC suggests that disruption of desmosomal integrity might be among primary molecular defects (Yang Z. et al, 2006), mechanisms leading to ARVC remain to be elucidated.

Studies reported so far point to the importance of desmosomes as intercellular adhesive organelles, required for the integrity of epithelial and cardiac tissues. However, desmosome components functions are not limited to their roles in desmosomes or in mechanical integrity of tissues, but they extend to supra-adhesive functions *in vivo*. It has been shown that mechanical forces applied to adherens junctions in ventricular cardiomyocytes activate stretch-sensitive calcium channels via cadherin' mechanical intracellular signaling, thus suggesting the importance of these channels in transduction

of mechanical forces into a cellular electrochemical signal, via increase of intracellular calcium concentration (Gannier F. et al., 1996; Tatsukawa Y. et al., 1997; Ko K. et al., 2000; Knoll R. et al., 2003). Volume overload of the right ventricle in a patient with genetically defective intercellular junctions (as in case of mutant plakoglobin, desmoplakin, plakophilin, desmoglein, desmocollin or transforming growth factor $\beta 3$) could produce unusual stretching that might affect intracellular calcium concentration and excitation-contraction coupling, thus producing arrhythmia. The existence of ARVC2 due to *RYR2* mutations supports the hypothesis of a key pathogenic role of intracellular calcium overload in the molecular pathogenesis of the disease. (Marcus F.I. et al., 2007).

On the other hand altering desmosome function could affect β-catenin signaling. This notion is supported by the observed ability of the β-catenin binding partner PKP2 to modulate β-catenin-dependent TOP-FLASH reporter activity *in vitro* (Chen X. et al., 2002). Moreover, suppression of DP expression leads to nuclear localization of the desmosomal protein plakoglobin and a 2-fold reduction in canonical Wnt/β-catenin signaling through Tcf/Lef1 transcription factors (Garcia-Gras E. et al., 2006). Garcia-Gras et al*.,* show that heterozygous Dp-deficient mice exhibited excess adipocytes and fibrosis in the myocardium, increased myocyte apoptosis, cardiac dysfunction, and ventricular arrhythmias, thus recapitulating the phenotype of human ARVC. The pathogenesis of ARVC described in such study is based on the essential role of Wnt/β-catenin signaling in regulating the transcriptional switch between myogenesis versus adipogenesis (Ross S.E. et al., 2000; Polesskaya A. et al., 2003; Chen A.E. et al., 2005). Heart is likely to be made up of cardiac myoblasts and resident or circulating mesenchymal stems cells, which in the absence of Wnt signaling could preferentially differentiate into adipocytes (Ross S.E. et al., 2000). An alternative source of adypocytes is fibrocytes, which are considered adipocyte progenitor cells (Nishikawa T. et al. 1999). The latter possibility is supported by the predominant colocalization of adipocytes and fibrosis in the myocardium of patients with ARVC (Garcia-Gras E. et al., 2006).

Again, the question is whether ARVC is caused by defective adhesion or alterations in differentiation and morphogenesis. Impaired desmosomal adhesion could lead to cell detachment and death of cardiomyocytes, followed by inflammation and fibrofatty replacement. However alterations of desmosomal constituents can have radical effects on characteristics and behaviour of cells through alterations in intracellular signalling.

Whether all mutations involved in ARVC give rise to similar signalling defects, or indeed whether a mechanical explanation such as weakened adhesion is ultimately responsible for the phenotype in some or all of these cases remains to be seen.

**PERP: A NOVEL CANDIDATE GENE**

PERP, a tetraspan membrane protein originally identified as an apoptosis-associated target of the p53 tumor suppressor (Attardi L.D. et al., 2000), localizes specifically to desmosomes and is entirely absent from other regions of cell to cell contact (Fig.3).
Numerous structural defects in desmosomes are observed in Perp-deficient skin, suggesting a role for PERP in promoting the stable assembly of desmosomal adhesive complexes (Ihrie R.A. et al., 2005).

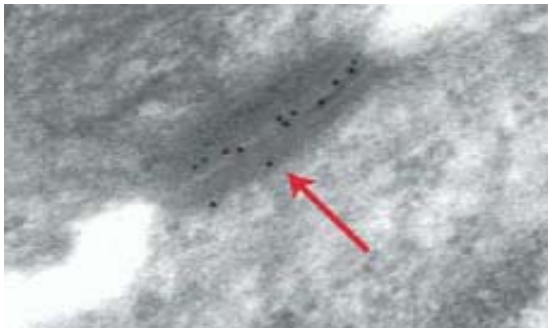

**Figure 3**: Immunogold EM using anti-Perp antibodies shows that Perp localizes specifically to desmosomes (From: Ihrie R.A. et al., 2005).

Two general models may explain how PERP might participate in desmosome assembly function: PERP's contribution to desmosomal integrity could be as core structural component or, alternately, as a chaperone facilitating the transit of other critical desmosome components to plasma membrane. Likewise transmembrane desmosomal cadherin molecules, PERP could participate either in homophilic or heterophilic interactions at plasma membrane, to provide relevant adhesive contacts. Another potential structural role for PERP is to be anchoring point for connections to intermediate filaments and cytoskeleton. As a chaperone, PERP might assist in the trafficking or assembly of desmosomal subunits (Ihrie R.A. et al., 2005). Although PERP's exact molecular function is unknown, this protein is distantly related to members of the claudin/PMP-22/EMP family of four-pass membrane proteins (Attardi L.D. et al., 2000). This multiprotein family includes stargazin, claudins, and PMP-22, which participate in a variety of cellular processes including ion channel function, receptor trafficking, tight junction formation, and myelination (Jetten A.M. and Suter U., 2000; Tsukita S. and Furuse M., 2002). As a plasma membrane protein, PERP could

act in a manner similar to any of these proteins to affect events important for tissue development or architecture. PERP may play a role in the shuttling, assembly, or stabilization of desmosomal proteins (Ihrie R.A. et al., 2005).

There are examples of tetraspan proteins acting as a molecular escorts or organizing factors for membrane proteins. For instance, stargazing is involved in delivery of the AMPA receptor to plasma membrane of cerebellar granular neurons, as well as in clustering of these receptors at the synapse (Chen L. et al., 2000). *PERP* is also a p53 target gene involved in DNA damage-induced apoptosis (Attardi L.D. et al., 2000; Ihrie R.A. et al., 2003), and during this process, the *PERP* promoter is bound not only by p53 but also by p63, indicating that *PERP* is responsive to signals from p63 (Flores E.R. et al., 2002; Ihrie R.A. et al., 2005). p63 plays a vital role in the development of stratified epithelia. While mechanism by which PERP participates in p53 mediated apoptosis is not well understood, its activities in programmed cell death and adhesion may be suspected. Alternatively, PERP may utilize distinct activities to enable the apoptotic and adhesion responses. In the future, identification of functional domains within PERP will help determine whether both activities are dependent on a common motif or different regions of this multifaceted protein (Ihrie R.A. et al., 2005).

In situ hybridization analysis indicated that during embryogenesis, *PERP* message is present in the heart (Ihrie R.A. et al., 2005). Analysis of Perp protein levels in newborn mice demonstrated that it is indeed expressed in the heart; immunohistochemistry localized Perp to intercalated discs of cardiac muscle, a site of known function for desmosomes. This suggests that PERP could play a role in myocardial cells and that its absence could cause a defect in heart function (Marques M.R. et al., 2006). These characteristics of PERP lead to the idea that *PERP* gene may be a good candidate for ARVC.

# AIM OF THIS STUDY

Since mutations causing ARVC have been identified in genes encoding desmosomal proteins, such as plakoglobin (JUP), desmoplakin (DSP), plakophilin-2 (PKP2), desmoglein-2 (DSG2) and desmocollin-2 (DSC2), arrhythmogenic right ventricular cardiomyopathy might be considered as "a disease of the desmosome". However, since no causative mutations in known ARVC genes have been detected in about 50% of index cases, additional disease-genes have to be involved in the genetic determination of the disease. Moreover identification of additional pathogenic mutations in known genes associated with ARVC remains important, because it may result in early detection of asymptomatic carriers and in increased diagnostic accuracy in the clinical evaluation of family members.

*PERP* was selected as a valid candidate gene for ARVC. During this study a protocol for DHPLC mutation screening of the human *PERP* gene was set up, and analysis by direct sequencing and DHPLC was carried out on 90 ARVC index cases.

Moreover novel *DSC2* mutations were identified by direct sequencing and DHPLC analysis and their pathogenic effects were studied, by using an in vitro functional study.

# RESULTS

## MUTATION SCREENING OF *PERP* GENE

*PERP* gene (three exons) maps on chromosome 6q24. *PERP* encodes a tetraspan membrane protein, localised to desmosomes of stratified epithelia and heart tissue (Fig. 1) (Ihrie R.A. et al., 2005). The gene encodes two different products; the isoform 2 lacks of four aminoacids (CGLA), encoded by exon1.

It is still unclear whether PERP protein is a structural constituent of desmosomes or it plays an yet unknown role in desmosome assembly (Fig.1).

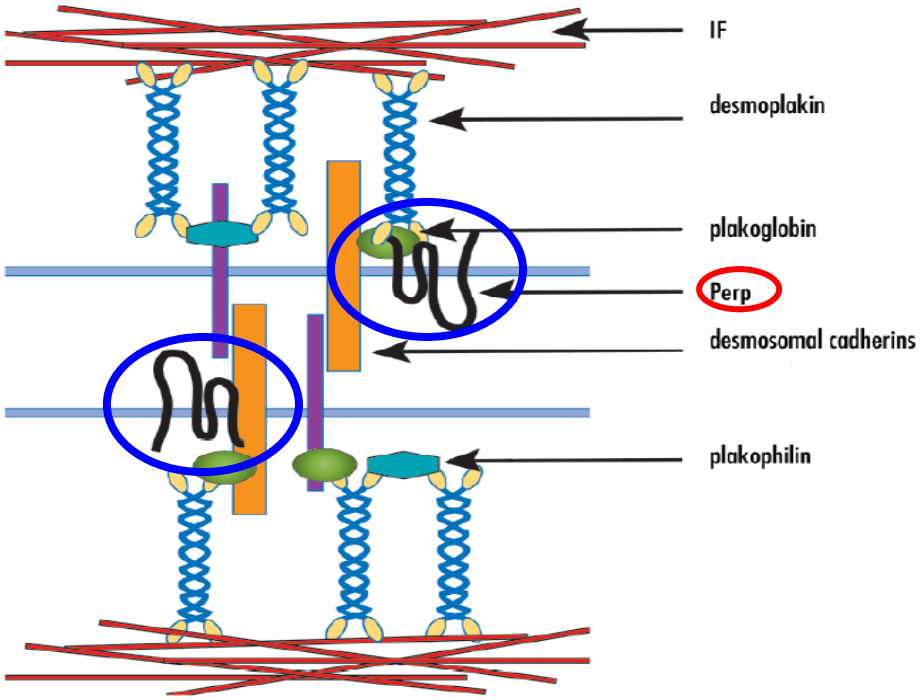


**Figure 1**: Hypothetical localization of PERP. As shown in the figure, PERP might interact directly with plakoglobin (right) or with cadherins (left), or it may play a different, but yet undefined role, in the assembly of desmosomal complex (from: Ihrie R.A. et al ., 2005).

By RT-PCR on Human Multiple cDNA Tissue panel, tissue mRNA expression of *PERP* gene was analyzed (Fig. 2). *PERP* gene appears to be expressed in skin, heart, liver and, at lowest level, in kidney.

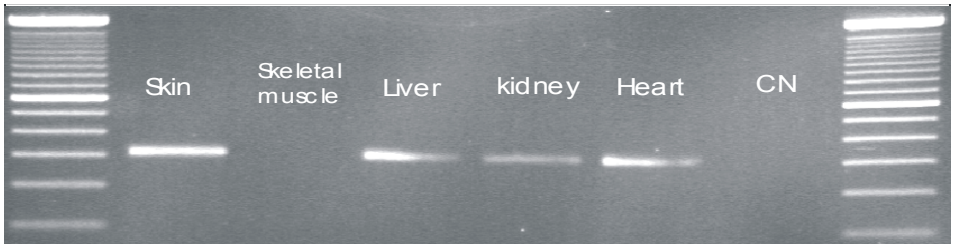


**Figure 2**: Expression pattern of *PERP* gene in some human tissues. *PERP* gene appears to be transcribed in skin, heart, liver and, though at lowest level, in kidney, whereas there is no noticeable expression in skeletal muscle.

This expression profile is compatible with the hypothesis that PERP could play a role in myocardial tissue. Therefore, *PERP* gene might be a candidate for ARVC. For this reason, *PERP* gene was screened for mutations, by using DHPLC analysis and direct sequencing of exons and UTR regions.

DNA samples from 90 unrelated Italian index cases affected with a classic form of ARVC were screened for *PERP* mutations in coding sequences and untranslated regions (UTRs). DHPLC analysis detected several abnormal elution profiles. Subsequent DNA sequencing confirmed the presence of variations within these sequences. Only two variants might be considered as putative novel mutations (G59R and c.1091C>T), whereas the remaining nucleotide changes were either known SNPs or they were detected in the control group of unrelated subjects from Italian population (Tab.1).

| Amplicon | Nucleotide Change | Amino acid Change | dbSNP rs# |
|---|---|---|---|
| Exon 2 | c.203-41T>A | - | Novel |
| | c.355+76A>G | - | rs2484067 |
| | c.355+83G>A | - | Novel |
| | c.355+172C>G | - | rs6903898 |
| | c.355+190C>T | - | rs4896313 |
| Exon 3 | c.428C>G | P143R | rs648802 |
| | c.492T>C | I164I | rs648396 |
| 3'UTR | c.870C>T | - | rs11557031 |
| | c.960C>T | - | rs481438 |
| | c.1146T>C | - | rs8085 |
| | del1431-1433AGG | - | Novel |
| | c.1444T>C | - | rs6588 |
| | c.1486G>T | - | rs11968569 |
| | c.1687C>T | - | rs8155 |

**Table 1**: SNPs detected in *PERP* gene.

**EXON 1 MUTATION: c.175G>A → G59R**

Nucleotide change c.175G>A, leading to a missense mutation Gly59Arg in exon1 (Fig.3), was detected in a ARVC patient carrying an additional mutation (S50fsX110) in *PKP2* gene. This patient showed a severe form of the disease. The *PKP2* mutation was identified as well in the DNA of the patient's son, who, on the other hand, had not inherited the variation in the *PERP* gene and resulted to be affected with a classical form of ARVC.

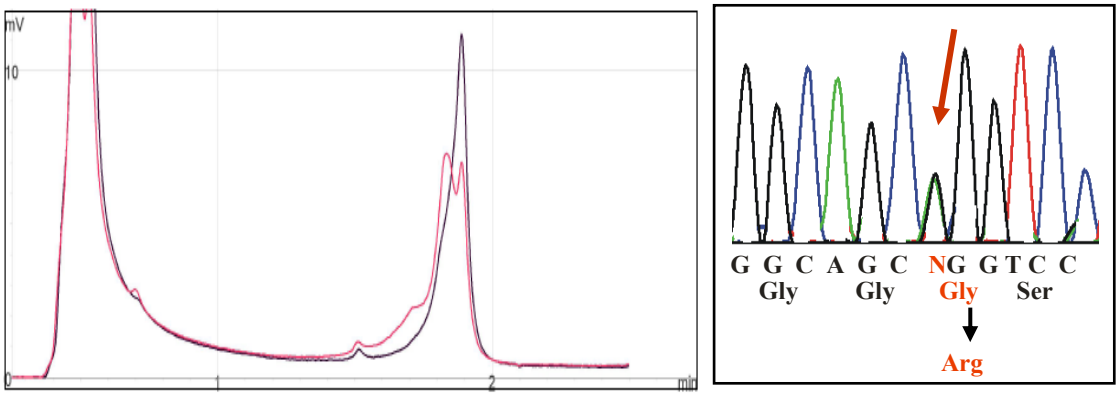


**Figure 3**: The abnormal elution profile for the amplicon of *PERP* exon 1, detected by DHPLC (left) was due to a mutation, as shown by DNA sequencing (right).

This missense mutation is not reported in the SNP database, but it was detected in 1 out of 250 control subjects (500 chromosomes) screened by DHPLC analysis and direct DNA sequencing. Accordingly, the frequency of such variant should be about 0,2%. G59R mutation modifies a highly conserved amino acid residue, localized in an highly conserved extracellular domain, for which a detailed function has not been reported yet (Fig. 4).

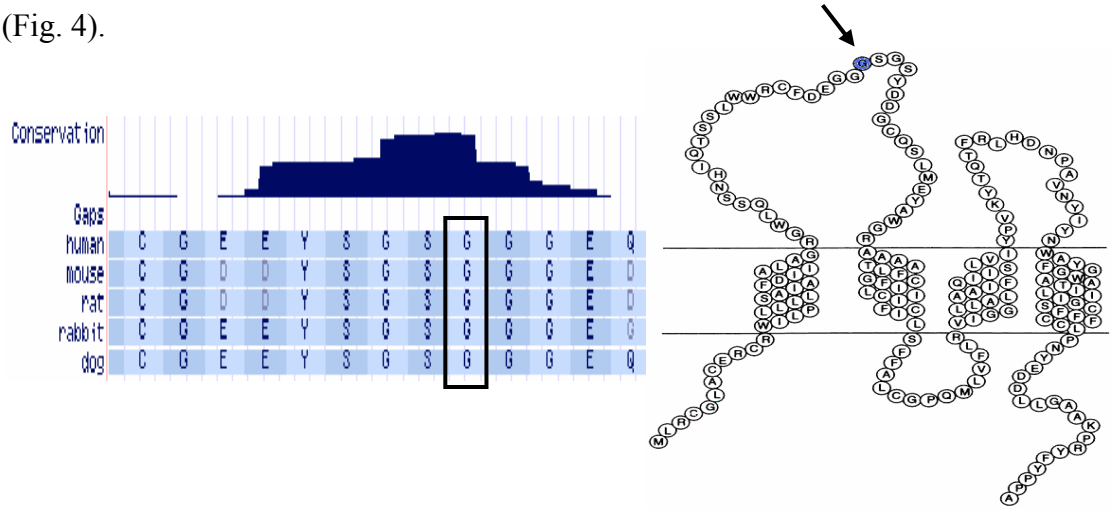


**Figure 4**: *PERP* missense mutation G59R (arrow) involves an aminoacid highly conserved among mammals. Also the entire extracellular domain appears to be highly conserved.

**3'UTR MUTATION: c.1091C>T**

In a index case affected with a classical form of ARVC, DHPLC analysis revealed an abnormal elution profile for the amplicon corresponding to one segment of the PERP 3'UTR. By direct sequencing of this amplicon, a novel nucleotide change c.1091C>T was identified (Fig.5), which is not reported in the SNP database but it was detected in 2 out of 192 control subjects.

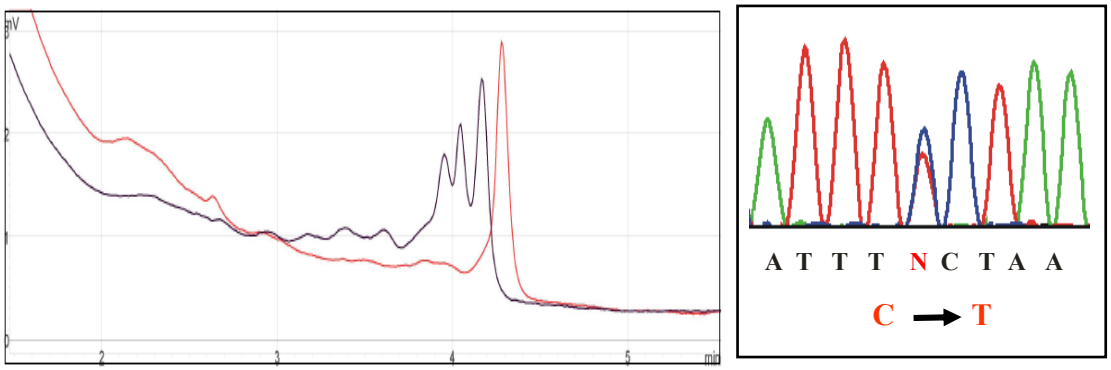


**Figure 5**: The abnormal elution profile for one segment of the *PERP* 3'UTR detected by DHPLC (left) was due to a mutation, as shown by DNA sequencing (right).

Sequence alignment of the involved 3' UTR segment of *PERP* gene with corresponding sequences of 7 different mammals shows that mutation occurred in a conserved sequence. (Fig. 6).

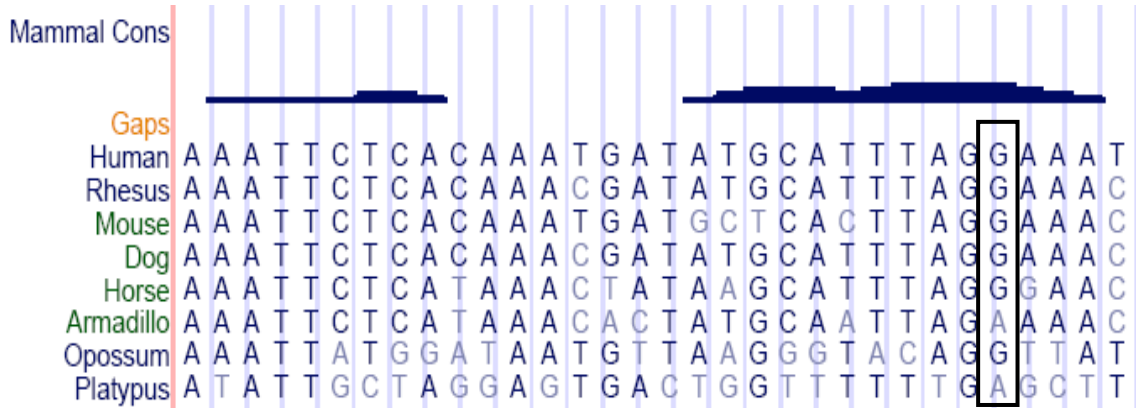


**Figure 6**: Variation c.1091C>T in 3'UTR region of *PERP* gene occurred in a conserved region among mammals.

The patient was carrier of a pathogenic mutation in *DSP* (Desmoplakin) gene (R1113X). Analysis extended to additional members of the family (Fig.7) revealed that R1113X in *DSP* gene and c.1091C>T in *PERP* gene were inherited by all the sibs. All

family members screened for such mutations resulted negative for *DSP* mutation, whereas subjects 8175, 8197 and 8176 showed to carry the *PERP* mutation.

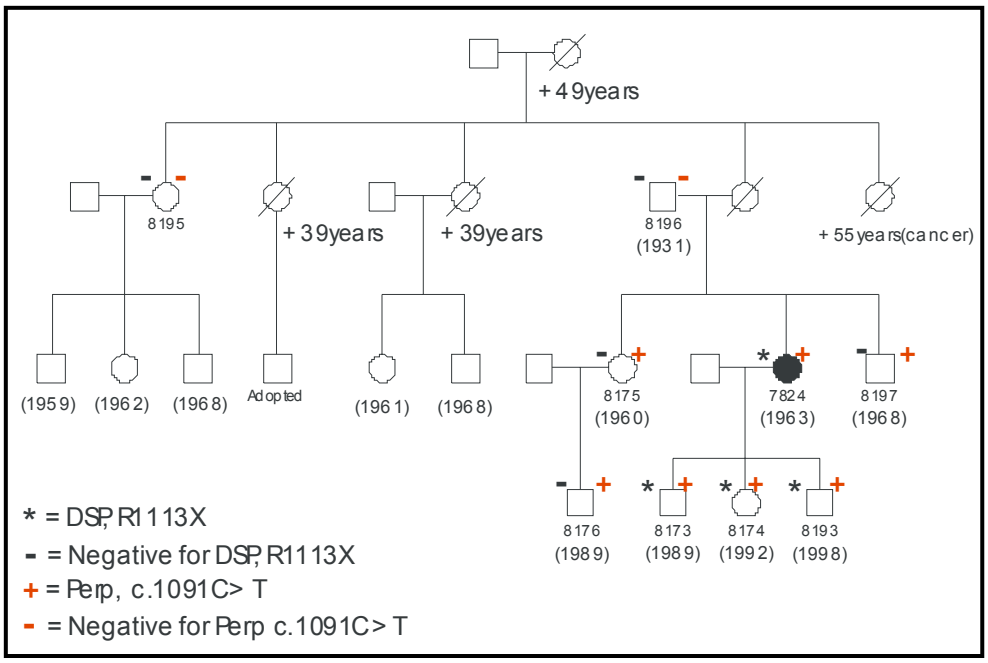


**Figure 7**: Family tree of the patient carrying *PERP* mutation (c.1091C>T) and *DSP* mutation (R1113X).

All additional family members carrying *PERP* mutation are asymptomatic and also the members carrying both mutations (in *PERP* gene and in *DSP* gene) did not fulfilled the current diagnostic criteria for ARVC.

## MUTATION SCREENING OF *DSC2* GENE

*DSC2* gene encodes one of desmosomal cadherins, single-pass transmembrane glycoproteins mediating $Ca^{2+}$-dependent cell-cell adhesion. Desmosomal cadherins, four desmogleins (*DSG*1-4) and three desmocollins (*DSC*1-3), tightly cluster on chromosome 18. DSCs occur as "a" and "b" splice variants, with the "a" variant having a slightly longer cytoplasmic domain with binding site for plakoglobin; DSCs might support desmosomal assembly (Fig.8). Through their extracellular domains in a $Ca^{2+}$-dependent manner, desmocollins bind to desmosomal cadherins on the surface of adjacent cells (Troyanovsky et al., 1993). Desmocollin-2 is almost ubiquitous in human tissues, but it is the only isoform expressed in cardiac tissue (Nuber et al., 1995). Mutations in four genes encoding major desmosomal proteins (plakoglobin, desmoplakin, plakophilin-2, and desmoglein-2) have been associated to ARVC. For such reasons *DSC2* was considered a good candidate gene for ARVC.

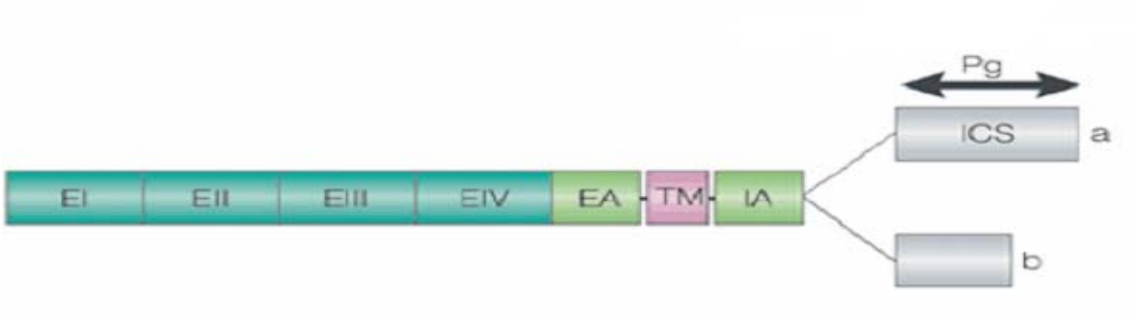


**Figure 8**: Schematic structure of desmocollin-2 (isoform 2a and 2b). EI-EIV are extracellular amino terminal domains, EA and IA are extracellular and intracellular anchor domains, TM is a short transmembrane domain and ICS is the intracellular cadherin-binding domain.

Sixty-four unrelated Italian index cases affected with ARVC were screened for *DSC2* mutations, by denaturing high-performance liquid chromatography (DHPLC) and by subsequent direct DNA sequencing.

Six *DSC2* mutations were identified (Tab.2) in seven patients.

| Amplicon | Nucleotide Change | Amino acid Change |
|---|---|---|
| 5'UTR | c.-92G>T | - |
| Exon 3 | c.304G>A | E102K |
| Exon 3 | c.348A>G | Q116Q |
| Exon 8 | c.1034C>T | I345T |
| Exon 17 | c.2687_2688insGA | E896fsX900 |
| 3'UTR | c.3241A>T | - |

**Table 2:** *DSC2* mutations detected in 7 ARVC index cases.

In two of them, two variations in UTR regions were detected: c.-92G>T in 5'UTR and c.3241A>T in 3'UTR. In a different patient, a nucleotide substitution (c.348A>G) was detected in exon 3; although this mutation corresponds to a synonymous variation (Q116Q), it might create a cryptic splice site. Two heterozygous point mutations c.304G>A (in exon 3) and c.1034C>T (in exon 8) were detected in two patients; they result in predicted p.E102K and p.I345T amino acid substitutions. In exon 17, c.2687_2688insGA was found in two patients; this variation causes a frameshift and alteration of 4 aa residues before a termination codon is prematurely introduced (E896fsX900). The screening among index cases detected nucleotide changes which

were identified as well among controls; moreover, additional variations were detected which were already reported as SNPs (Tab.3).

| Amplicon | Nucleotide Change | Amino acid Change | dbSNP rs# |
|---|---|---|---|
| Exon 2 | Ex2-155G>A<br>c.111A>G | -<br>L37L | rs28379678<br>Novel |
| Exon 5 | c.536A>G | D179G | Novel |
| Exon 9 | Ex9-40A>T | - | Novel |
| Exon 12 | c.1787C>T | A596V | Novel |
| Exon 15 | Ex15-27C>A<br>c.2326A>G<br>c.2393G>A | -<br>I776V<br>R798Q | Novel<br>rs1893963<br>Novel |
| Exon 17 | Ex17-22G>A | - | rs1790682 |

**Table 3:** Non pathogenic nucleotide variations detected in the *DSC2* gene. Three of them were already reported in the SNPs database, whereas additional six were not reported there, but they were detected among controls.

**5'UTR AND 3'UTR MUTATIONS**

By direct DNA sequencing analysis two novel variations in 5'UTR (c.-92G>T) and 3'UTR (c.3241A>T) of *DSC2* gene were identified (Fig.9) in two index cases with a classic form of ARVC. They didn't show familiar history of the disease. Both index cases were also screened for the known ARVC genes and N76S was identified in PKP2 of patient carrying the variation in 3'UTR of *DSC2* gene.

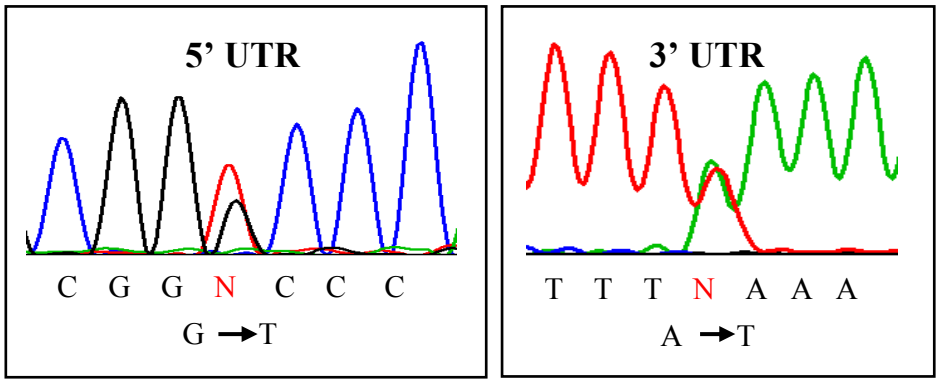


**Figure 9**: Nucleotide variations detected by DNA sequencing of PCR amplicons of *DSC2* 5'UTR and of one segment of the 3'UTR.

These changes were not identified in the SNP database and were never observed in 150 control subjects (300 chromosomes), thus suggesting that such nucleotide substitutions might be pathogenic.

**EXON 3 MUTATION: c.304G>A → E102K**

By DHPLC analysis, an abnormal pattern was observed for the amplicon of exon 3 in one patient affected with a classic form of ARVC. Direct DNA sequencing detected a novel nucleotide change c.304G>A which causes an aminoacidic substitution: E102K (Fig.10). This variation was not reported in the SNP database and it was not detected in 500 control chromosomes screened by DHPLC analysis.

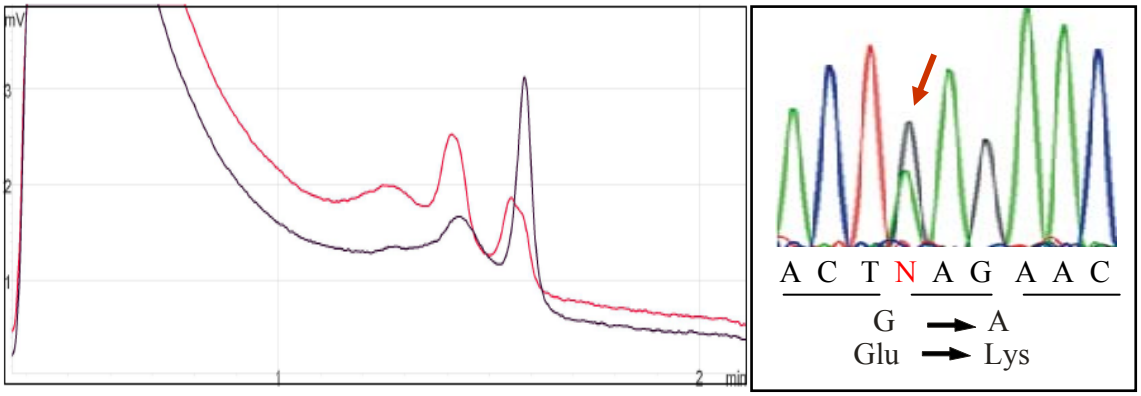


**Figure 10**: DHPLC elution profiles of the amplicon of *DSC2* exon3 of the patient (in red) and of a control (in black). DNA sequence shows a nucleotide change c.304G>A resulting in a Glu102Lys substitution (E102K).

Mutation E102K replaces a negatively-charged residue by a positively-charged one. Moreover, this mutation is located in the pro-peptide domain which is highly conserved among species (Fig. 11).

```
SFTILLSNTENQEKKKIFV   Homo_Sapiens
SFSILLSNTENQEEKKILV   Canis_familiaris
SFTILLSNTETQEEKEILV   Bos_taurus
SFTILLSNTENQEKKKIFV   Macaca_mulatta
SFTILLSNTENQEKKKIFV   Pan_troglodytes
SFTIWLFSTDSQEKREISV   Mus_musculus
SFTIWLFNTDSQEERELSV   Rattus_norvegicus
**:* * .*:.**:::: *
      E102K
```

**Figure 11**: DSC2 missense mutation p.E102K occurred in sequence highly conserved among mammals. In mouse and in rat dsc2 protein, E102 is replaced by aspartic acid (D), but glutamin (E) has physico-chemical properties similar to aspartic acid.

The genetic study was extended to additional members of the family (Fig.12).

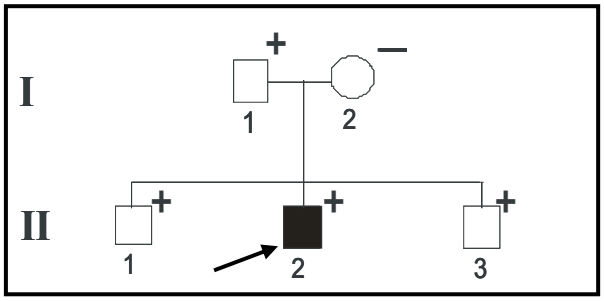


**Figure 12**: Family tree of the patient carrying E102K DSC2 mutation. Presence (+) or absence (-) of DSC2 mutation is indicated. Arrow indicates the index case.

The three additional family members carrying the same missense mutation did not fulfilled the current diagnostic criteria for ARVC.

**EXON 3 MUTATION: c.348A>G → Q116Q**

An abnormal pattern of DHPLC elution profile was observed for the amplicon of *DSC2* exon 3, obtained from DNA of one index case affected with a severe form of ARVC and carrying two *in cis DSP* mutations (K470E and A566T). Direct DNA sequencing detected a novel nucleotide change c.348A>G leading to a synonymous variation Gln116Gln (Fig 13).

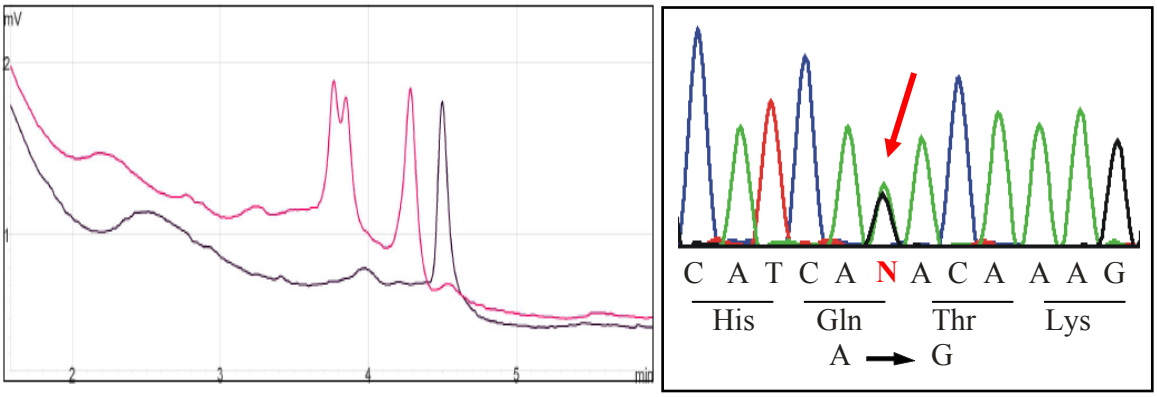


**Figure 13**: DHPLC elution profiles of *DSC2* exon 3 amplicon: three peaks were observed for the patient (in red), compared with the single peak of the control (in black). DNA sequence analysis revealed the presence of an heterozygous substitution (c.348A>G) in the individual with the abnormal peak detected by DHPLC analysis.

This nucleotide change was not detected in the SNP database and was never observed in 500 control chromosome (250 individuals) screened by DHPLC analysis. The mutation occurred in a sequence which appears highly conserved among mammals (Fig14).

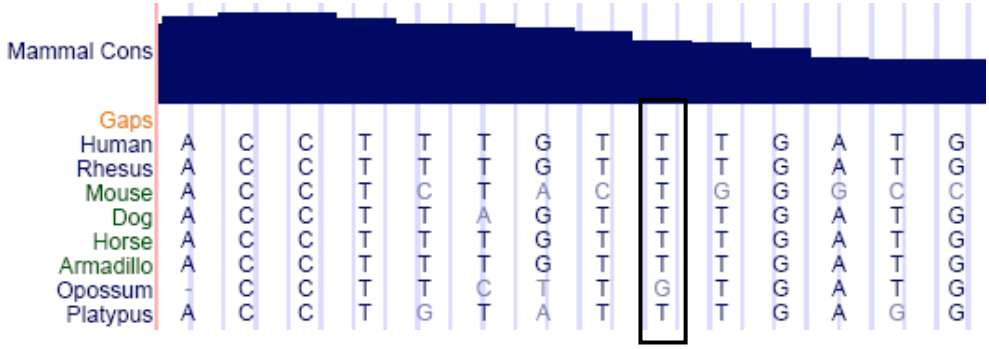


**Figure 14:** The nucleotide change c.348A>G in the exon 3 of *DSC2* gene occurred in a sequence which shows high conservation among mammals. In this figure the minus strand is considered.

In order to understand the possible effect of this nucleotide change, RNA was extracted from lymphocytes of the patient. cDNA obtained by RT-PCR was amplified with different exon primers, but agarose electrophoresis and cDNA sequencing failed to detect aberrant transcripts. RT-PCR products were cloned in pCR2.1-TOPO vector using E.Coli cells (TOP 10 OneShot) as hosts. A total of 50 clones were randomly picked up. DNA sequencing of such clones revealed 30 wild type cDNA and 20 mutant cDNA (showing the nucleotide change c.348A>G). Only two of these mutant clones (4%) showed an aberrant transcript, resulting from the skipping of 9 nucleotides just few bases downstream the mutated nucleotide (Fig. 15).

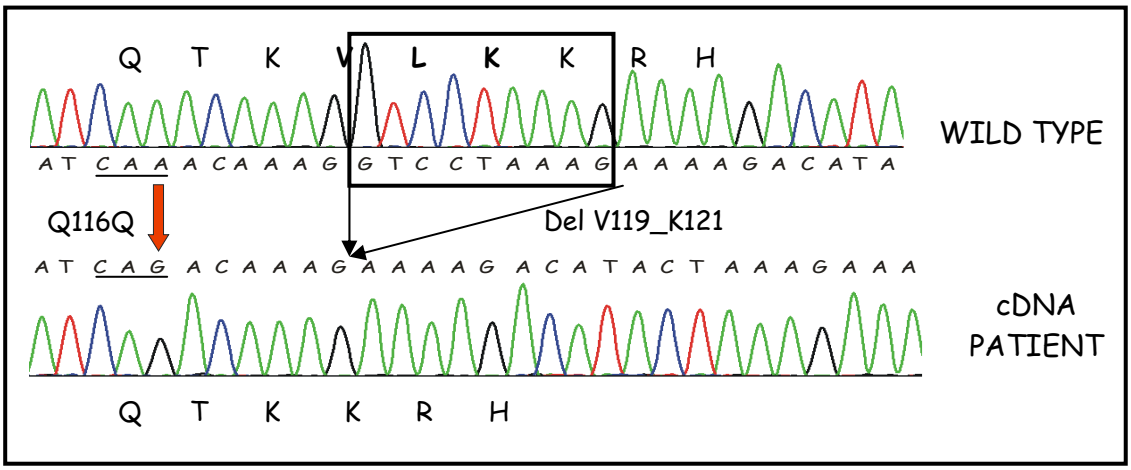


**Figure 15:** Normal and aberrant transcripts detected by DNA sequencing among cDNA clones (see the text for details).

Although the 9-nucleotide deletion of aberrant spliced mRNA doesn't alter the frame of the sequence, it leads to the loss of three aminoacids very conserved among mammals (Fig. 16).

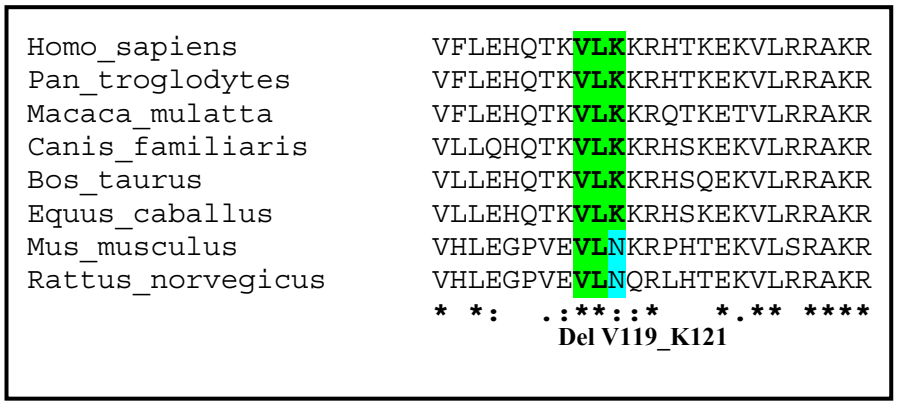


**Figure 16**: Evolutionary conservation of DSC2 aminoacids since V119 to K121 among 8 mammalian species.

In mouse and in rat dsc2 protein, K121 (lysine) is replaced by asparagine (N), but the two amino acids share similar physico-chemical properties.

The genetic study was extended also to additional members of the family (Fig. 17).

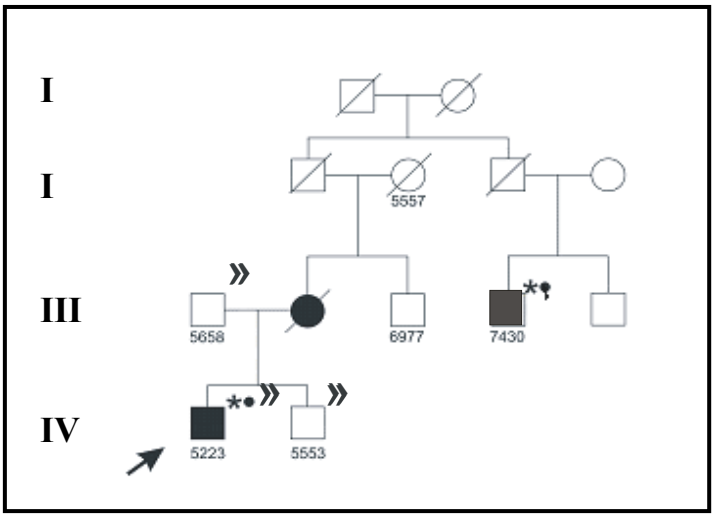


**Figure 17**: Family tree of the index case carrying Q116Q DSC2 mutation (») along with K470E (*) and A566T (●) DSP mutations.

By direct sequencing of DNA, DSC2 mutation Q116Q was detected in subjects 5658 and 5553 which are fully asymptomatic, whereas both DSP mutations (K470E and A566T) resulted only in subject 7430 which presents a classical form of ARVC.

**EXON 8 MUTATION: c.1034C>T → I345T**

The index case affected with ARVC was diagnosed at the age of 50, due to a sustained VT episode for which he received an implantable cardioverter defibrillator; he showed segmentary involvement of left ventricle. The abnormal elution profile for the amplicon

of exon 8 by DHPLC was due to a nucleotide substitution (c.1034C>T → I345T), as shown by direct DNA sequencing (Fig. 18).

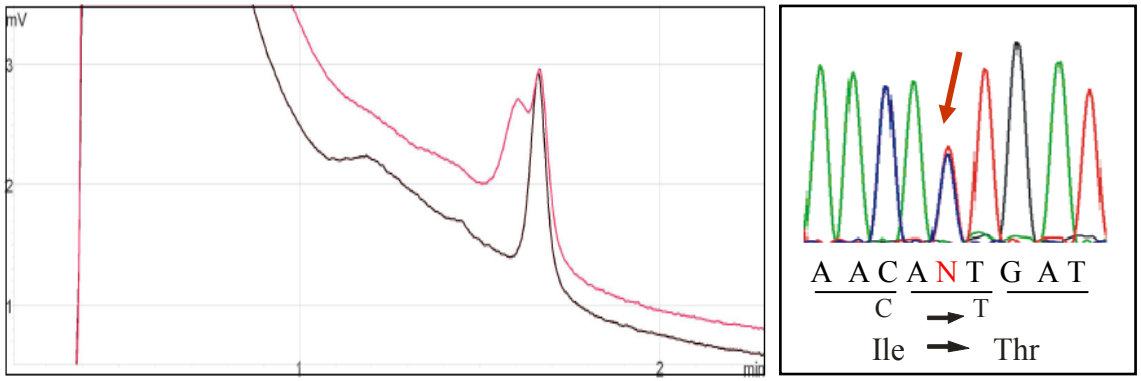


**Figure 18**: Results of the DHPLC and DNA sequencing analysis of *DSC2* exon 8 amplicon from the index case (in red) compared with a control sample (in black).

This nucleotide change is not reported in the SNP database and was never found in 250 controls (500 control chromosomes) from Italian population. Mutation p.I345T replaced a non polar hydrophobic amino acid by a polar hydrophilic amino acid. It is located in the second extracellular cadherin (EC2) domain and alters the third amino acid of the EC domain consensus sequence [L/I/V-x-L/I/V-x-D-x-N-D-N/H-x-P] (Takeichi M., 1990). Cadherin domains are important for adhesive interactions and form $Ca^{2+}$-dependent rodlike structures. Moreover this change occurred in a residue highly conserved among mammals (Fig. 19).

```
TTSTCIINIDDVNDHLPTFTR    Homo_Sapiens
TTSICIINIDDVNDNLPTFTR    Canis_familiaris
TTAICIINIEDVNDNLPTFTR    Bos_taurus
TTSTCIINIDDVNDHLPTFTR    Macaca_mulatta
TTSTCIINIDDVNDHLPTFTR    Pan_troglodytes
TTAKCIITIEDVNDNLPTFTR    Mus_musculus
TTATCVITIGDVNDNLPTFTR    Rattus_norvegicus
**: *:*.* ****:******
     I345T
```

**Figure 19**: DSC2 missense mutation I345T occurred in a sequence highly conserved among mammals.

The genetic study was extended to additional members of the family (Fig.20).

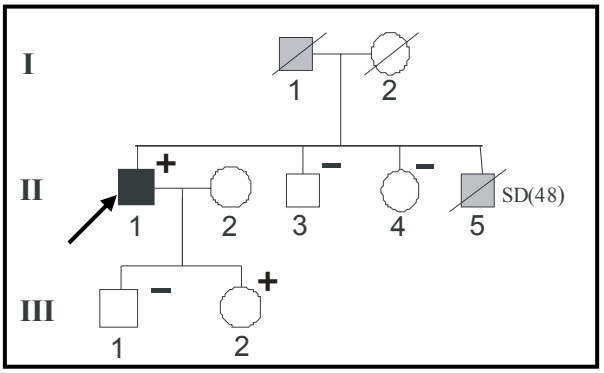


**Figure 20**: Family tree of the index case in whom I345T mutation was detected. Grey symbol represents an individual of unknown disease status. SD indicates sudden death. Presence (+) or absence (-) of the *DSC2* mutation is indicated.

The 15 years-old daughter, although fully asymptomatic, was found to carry the same *DSC2* mutation detected in her father. However, due to the young age of individual III-2, it cannot be excluded that later she could show clinical signs of the disease. All family members not carrying *DSC2* mutations were negative at clinical investigation.

**EXON 17 MUTATION: c.2687_2688insGA → E896fsX900**

Two unrelated index cases affected with a classical form of ARVC showed an abnormal pattern of the DHPLC elution profile of *DSC2* exon 17 amplicon. The DNA sequence detected the presence of a mutation (c.2687_2688insGA) resulting in a premature stop codon formation (E896fsX900) (Fig. 21).

One of the two patients is a woman carrying a mutation in the *PKP2* gene (p.E58D) while the other one is a man in whom a *DSG2* mutation (p.Y87C) was previously identified.

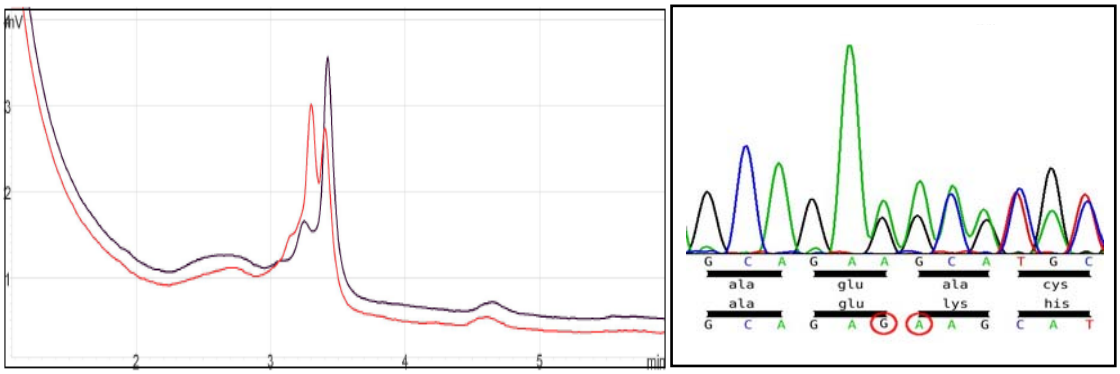


**Figure 21**: Results of the DHPLC and DNA sequencing analysis of *DSC2* exon 17 amplicon from the index case (in red) compared with a control sample (in black).

The insertion c.2687_2688insGA is not reported in the SNP database but it was observed in 6 out of 150 control subjects (300 chromosomes) screened by DHPLC analysis and direct sequencing. Accordingly, the frequency of such variant should be about 2%. This mutation would affect the C terminus of DSC2 by altering 4 aa residues before a termination codon is prematurely introduced. If compared with the wild type, only the last 5 amino acids were altered in the mutated protein, three were changed and the last two were lost (Fig. 22).

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Wild 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** | | |

**Figure 22**: Changes introduced in the amino acid sequence, caused by mutation c.2687_2688insGA.

Exon 17 encodes ICS domain in the DSC2a isoform. It is believed that the binding site to plakoglobin is located within this functionally important domain. The change occurred in five aa residues, which are non conserved among mammals, in contrast with the high conservation of the upstream region (Fig. 23).

```
Homo_sapiens            QEEDGLEFLDNLEPKFRTLAEACMKR
Macaca_mulatta          QEEDGLEFLDNLEPKFRTLAEACMKR
Pan_troglodytes         QEEDGLEFLDNLEPKFKTLAEACMKR
Bos_taurus              QEEDGLEFLDHLGPKFRTLAETCMKR
Rattus_norvegicus       QEEDGLEFLDHLEPKFRTLAEVCAKR
Mus_musculus            QEEDGLEFLDHLEPKFRTLAEVCAKR
Canis_familiaris        QEEDGLEFLDHLEPKFRTLAEACIKR
Equus_caballus          QDEDGLEFLDHLEPKFRTLAAACTTR
                        *:********:* ***:*** .* .*
```

**Figure 23**: Last five aminoacids of DSC2 protein (involved by mutation c.2687_2688insGA) show relative variance among mammalian species.

The genetic study was extended to the son of the patient, which resulted to carry the same mutations and the same phenotype of his mother, on the contrary the daughter of

the second patient resulted negative for both mutations identified in her father and she is fully asymptomatic.

**FUNCTIONAL ANALYSIS OF MUTANT DESMOCOLLINS**

To evaluate pathogenic potentials of the *DSC2* missense mutations p.E102K and p.I345T, full-length wild-type cDNA was directionally cloned in eukaryotic expression vector to obtain a fusion protein with GFP. Mutated proteins carrying p.E102K and p.I345T were obtained by site directed mutagenesis of the wild-type construct. Constructs were transfected in the desmosome-forming cell line HL-1 having a differentiated cardiomyocyte phenotype and contractile activity *in vitro*.

In transfected HL-1 cells, wild-type fusion protein was detected in the cell membrane, into cell-cell contact regions (Figure 24, panel A), and co-localised with the endogenous dsg, which was marked with monoclonal desmoglein antibody (Figure 24, panel A' and A''). This co-localisation suggests that the wild-type fusion protein has been integrated into normal-appearing desmosomes. By contrast, protein carrying the p.E102K and p.I345T mutation were predominantly localised in the cytoplasm (Figure 24, panel B and C) although a lower amount of GFP signal was detectable in membrane. Moreover immunostaining with monoclonal desmoglein antibody showed both the presence of well-assembled desmosomes in transfected HL-1 cells (Figure 24, panel B', C') and the reduced co-localisation between endogenous dsg and mutated DSC2 (Figure 24, panel B'', C'').

In addition, site-directed mutagenesis was performed on wild type construct in order to study the functional effects of two novel DSC2 polymorphisms p.D179G, p.R798Q and the putative pathogenic mutation p.E896fsX900. R798G is located in the cytoplasmic region of DSC2, where also E896fsX900 maps, whereas D179G is localized in the extracellular region of the protein, as the two missense DSC2 mutations mentioned above. HL-1 cells were then transfected with these three new constructs.

Results suggest that both polymorphisms do not have functional effect as compared with the wild type protein, whereas the frameshift mutation do have functional effect, as shown by predominant localization in the cytoplasm of DSC2, similarly to the two proteins carrying mutations p.E102K and p.I345T, respectively (Fig.25).

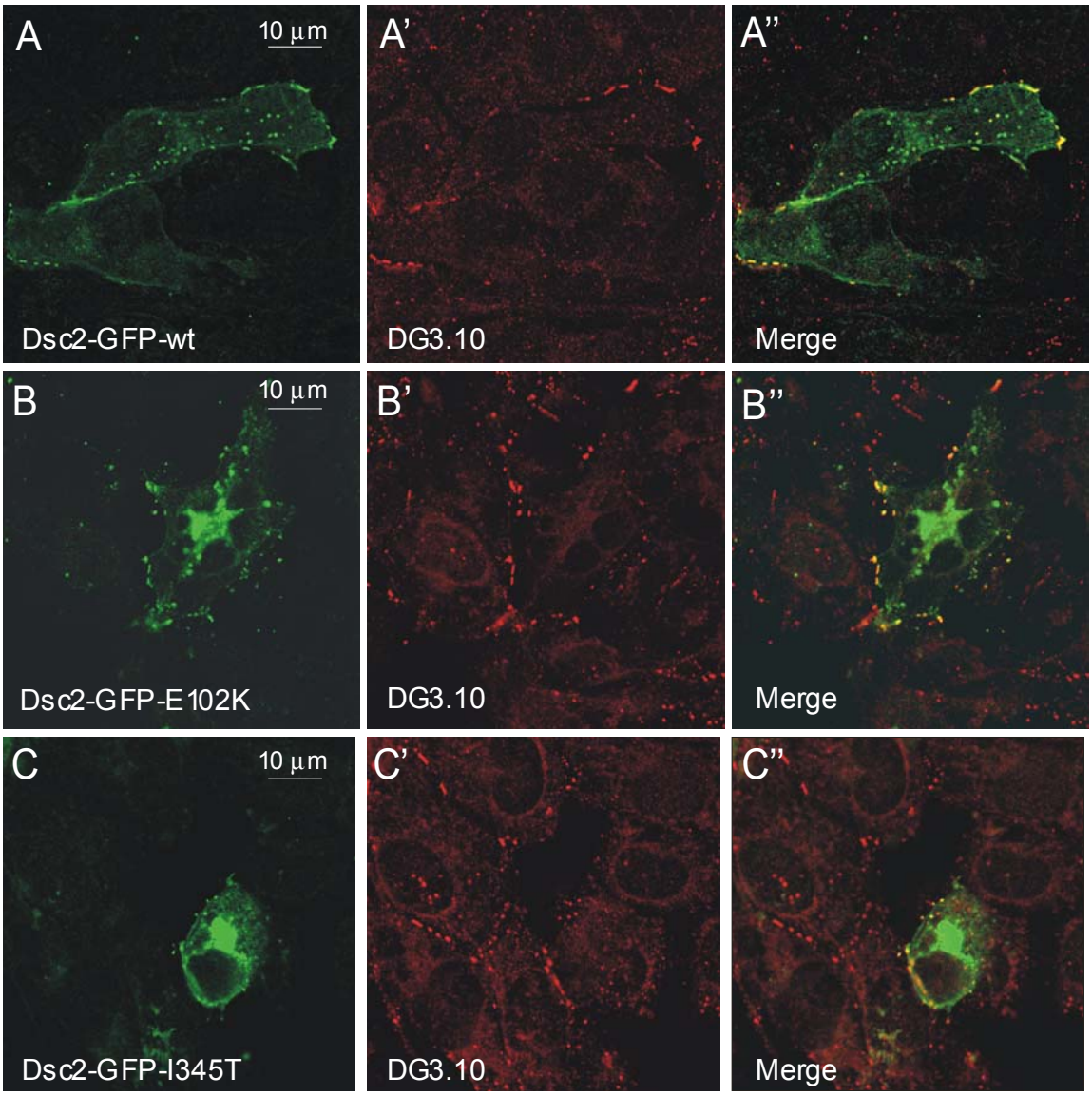


**Figure 24**: Transfection studies in HL-1 cells. Wild type DSC2 (WT-DSC2a-GFP) is localised at the cell membrane, border between two HL-1 cells (panel A), whereas E102K and I345T-DSC2a-GFP were mainly detected in the cytoplasm (panel B and C). Immunostaining with monoclonal desmoglein antibody DG3.10 showed both the presence of well-assembled desmosomes (panel A', B' and C') and the reduced co-localisation between endogenous dsg and mutated DSC2 (yellow dots in panel B'', C'').

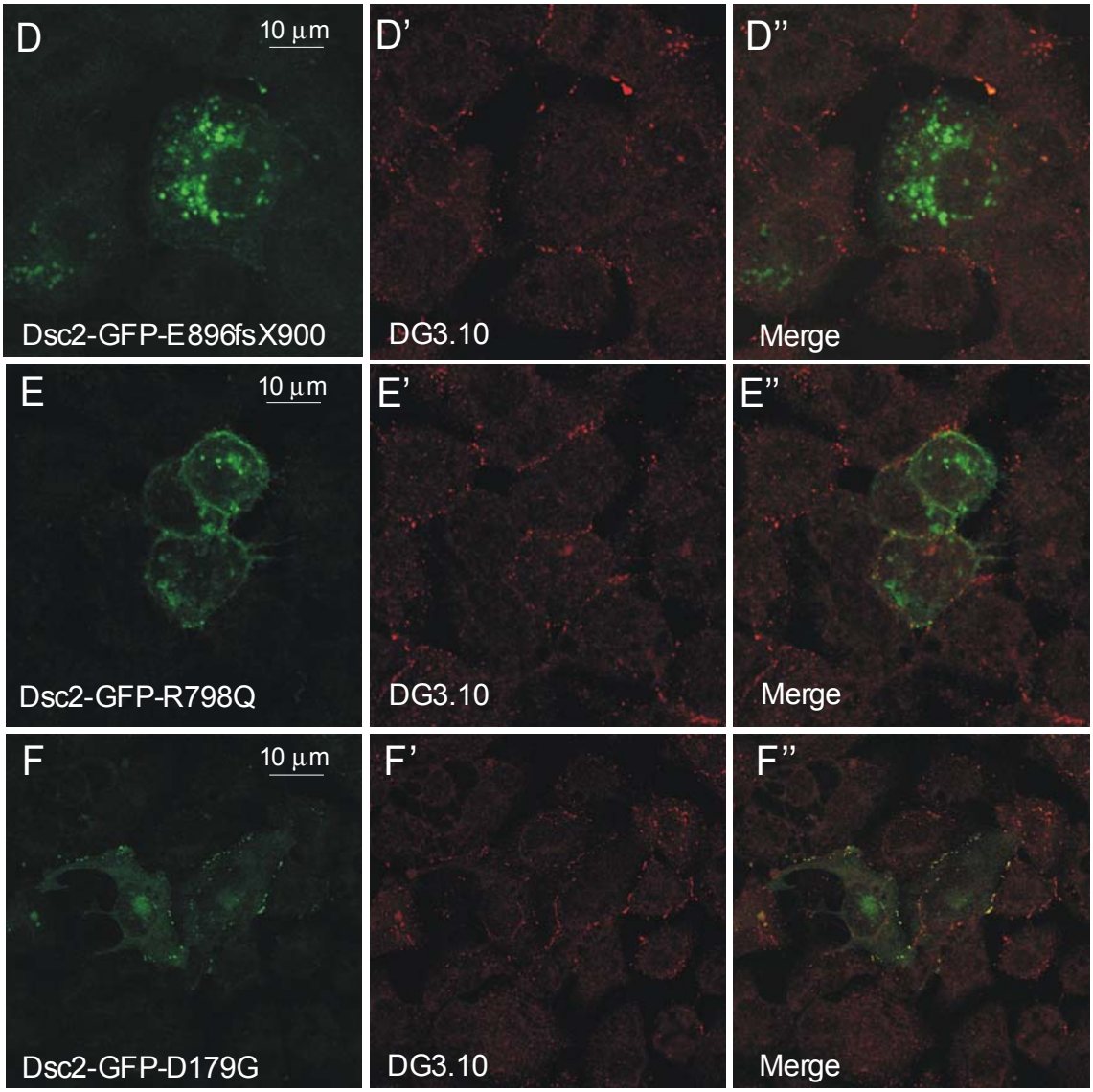


**Figure 25**: Transfection studies in HL-1 cells. Note the D179G and R798Q-DSC2a-GFP were localised at the cell membrane between two HL-1 cells (panel E and F), whereas E896fsX900-DSC2a-GFP were mainly detected in the cytoplasm (panel D). Immunostaining with monoclonal desmoglein antibody DG3.10 showed both the presence of well-assembled desmosomes (yellow dots in panel E'' and F'') and no co-localisation between endogenous dsg and DSC2a-GFP-E896fsX900 ( panel D'').

# DISCUSSION

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a dominant, degenerative cardiomyopathy, frequently involved in sudden death of asymptomatic athletes and teenagers. Human genetic studies over the last few years have offered insight into the potential causes of ARVC. The involvement on ARVC of multiple desmosomal protein genes, such as plakoglobin (*JUP*), desmoplakin (*DSP*), plakophilin-2 (*PKP2*), desmoglein-2 (*DSG2*) and desmocollin-2 (*DSC2*), has led to the "desmosomal model" hypothesis. Since mutation screening in such genes failed to detect causative mutations in about 50% of patients affected with ARVC, current genetic research aims at identifying novel genes involved in such disease and novel mutations in the known genes.

## MUTATION SCREENING OF *PERP* GENE

*PERP* gene encodes for a tetraspan membrane protein which is essential for desmosomal adhesion in stratified epithelia (Ihrie R.A. et al., 2005) and localizes to the intercalated discs of cardiac muscle, a site of known function for desmosomes (Marques M.R. et al., 2006). For such reasons *PERP* was considered a good candidate gene for ARVC.

Since the major studies on *PERP* were carried out on mice, in order to check the expression of such gene in human myocardium, its expression pattern was preliminarily verified by RT-PCR from RNA of different tissues. *PERP* resulted to be expressed in human heart, therefore mutation screening of this gene was applied to a series of ninety ARVC index cases.

Two putative novel mutations, G59R in exon 1 and c.1091C>T in 3'UTR, were detected in two different ARVC index cases.

The missense mutation G59R replaced a small polar hydrophilic amino acid (glycine) by a bigger positively-charged one (arginine). This change occurred in a residue highly conserved among species and located in a conserved extracellular region for which a function is still unknown, however the G59R PERP variant was identified as well in one out of 250 controls. The index case resulted to carry as well a pathogenic mutation in *PKP2* gene (S50fsX110); the mutation was identified also in her son, who, on the contrary resulted negative for the *PERP* mutation. It is interesting to notice that patient

appears to be affected with a severe form of ARVC, whereas her son (negative for *PERP* mutation) seems to be affected with a classical form of ARVC. Therefore, the possibility that *PERP* mutation could worsen the clinical presentation should be considered.

Likewise, mutation c.1091C>T in 3'UTR changes a highly conserved nucleotide; this mutation was detected in 2 out of 192 controls. The index case carrying c.1091C>T in the *PERP* 3'UTR carries also a pathogenic mutation in *DSP* gene (R1113X). Both *DSP* and *PERP* genes map to chromosome 6, respectively on 6p24 and 6q24. The patient's father is negative for both mutation, therefore it is probable that the two variations were carried by the maternal chromosome 6. The patient and her sibs appear to have inherited the two mutations *in cis* , due or to the absence of crossing-over, or to the presence of double crossing-overs along the chromosome. A single crossing-over would lead to inheritance of only one of the two mutations, as it actually happened in the sister and in the brother of the patient (see Figure 7, on results section).

Genetic analysis of additional family members showed that individuals carrying only *PERP* variant c.1091C>T were all healthy, and also the three patient's sibs carrying both *PERP* mutation and *DSP* mutation did not fulfilled the current diagnostic criteria for ARVC, but due to their young age, it cannot be excluded that some of them could later show major clinical signs.

Although two putative mutations in *PERP* gene were identified in index cases affected with ARVC, it is impossible to establish whether these mutations might cause ARVC when they occur not associated to additional pathogenic mutations. Data suggest that a variation in Perp might worsen the clinical phenotype in patients carrying a pathogenic mutation in a different gene involved in ARVC. For such reason, *PERP* still remains a good candidate for ARVC and mutation screening should be extended to additional series of index patients affected with ARVC.

**MUTATION SCREENING OF *DSC2* GENE**

Two recent studies suggested that mutations in the gene encoding desmosomal desmocollin-2 (*DSC2*) may cause ARVC (Syrris P. et al., 2006; Heuser A. et al., 2006). In Syrris's report, two heterozygous mutations were described: a deletion (c.1430delC) and an insertion (c.2687_2688insGA). Both mutations result in frameshifts and premature truncation of the desmocollin-2 protein. On the other hand Heuser et al. identified a heterozygous splice acceptor–site mutation in *DSC2* intron 5 (c.631-2A>G),

which activates a cryptic splice-acceptor site, leading to a downstream premature termination codon.

In seven out of sixty-four ARVC unrelated Italian index cases screened in this study six different *DSC2* mutations were identified. Five of them (c.-92G>T, c.304G>A, c.348A>G, c.1034T>C and c.3241A>T) were absent among controls, thus suggesting that these genetic variants might be pathogenic. The sixth mutation (c.2687_2688insGA) was detected in two different patients and in six control subjects, suggesting the possibility of a polymorphism.

Two mutations in UTR regions were observed in two different patients: c.-92G>T in 5'UTR and c.3241A>T in 3'UTR. Although both nucleotide changes occurred in sequences showing low conservation among mammals, they were unreported in the SNP database and they were never detected among 150 Italian controls. However, to exclude that such mutations could correspond to rare polymorphisms, the size of the control group should be increased to 500 individuals. The patient carrying mutation c.3241A>T in 3'UTR carries as well a mutation (N76S) in *PKP2* gene. The missense mutation N76S changes an asparagine (N) with a serine (S) which are both small polar amino acids. This data suggests that the variation in *PKP2* gene could be non pathogenic; if so, the variation in 3'UTR of *DSC2* gene might cause ARVC. This hypothesis might be supported by the findings of a recent study (Beffagna G. et al., 2004) in which it has been demonstrated that overexpression of *TGFβ3*, due to 5'UTR and 3'UTR mutations, cause ARVC. Sequence alterations in untranslated regions of genes were reported in several hereditary human diseases, such as hereditary angioedema (Laimer M. et al., 2006) an autosomal dominant cerebellar ataxia (Ishikawa K. et al., 2005) a distinctive form of cone dystrophy (Piri N. et al., 2005) hereditary thrombophilia (Gehring N.H. et al., 2001), hereditary hyperferritinaemia-cataract (Girelli D. et al., 1997) and fragile X mental retardation syndromes (Warren S.T. and Nelson D.L., 1994). In fact 5' and 3'UTRs contain several regulatory motifs, which modify mRNA stability, localization, and degradation, thereby influencing gene expression (Mitchell P. and Tollervey D., 2000; Amack J.D. et al., 2002; Mazumder B. et al., 2003). Specific functional studies will be needed to understand the pathogenic role of mutations affecting UTRs in *DSC2*.

A novel nucleotide change c.348A>G leading to a synonymous mutation Q116Q was detected in *DSC2* gene in a patient with a severe ARVC form. This patient carried two *in cis DSP* mutations (K470E and A566T), which replace the corresponding wild type

amino acids with others with different physico-chemical properties. The nucleotide change c.348A>G was never detected among 500 control chromosomes and it occurred in a region highly conserved among species. This mutation might activate a cryptic splice site: in fact, an abberant mRNA was identified by RT-PCR in patient's RNA and subsequent cloning of PCR products. Surprisingly, the resulting aberrant spliced mRNA, showing a deletion of 9 nucleotides, was found only in 4% of screened clones carrying the variation c.348A>G. It is possible that this percentage could have been underestimated since mutant mRNA may have been degraded via nonsense-mediated mRNA decay.

According to recent studies, desmoglein/desmocollin ratio is relevant to desmosomal intercellular adhesion and must be finely regulated. L cell fibroblasts, in which the level of Dsg1 was titrated against constant amounts of Dsc1, exhibited productive adhesion only at the appropriate ratio of Dsg1 to Dsc1 (Dusek R.L. et al., 2006). This data suggest that even if mutant DSC2 is present at low concentration, this could be sufficient to alter the adhesive function of desmosomes. Moreover, although skipping of 9bp in the aberrant mRNA doesn't alter the reading frame of DNA sequence, at protein level it leads to loss of three amino acids very conserved among species and located in the N-terminal pro-sequence domain; this region is involved in the maturation of adhesive protein (Ozawa M. et al., 1990). Extension of mutation screening to additional family members detected c.348A>G *DSC2* mutation in the father and in the brother of the patient; both these subjects were asymptomatic (see Figure 17, in results section). On the contrary, one mother's cousin, showing a classical form of ARVC, carried two mutations in *DSP* gene (K470E and A566T, *in cis*). Only the index case, carrying two *DSP* mutations *in cis* and one *DSC2* mutation, resulted affected with a severe form of ARVC. Taken together, these data suggest that the *DSC2* mutation c.348A>G might lead, in presence of other pathogenic mutations, to a more severe phenotype, but that, *per se*, it would be insufficient to cause ARVC.

Two heterozygous point substitutions c.304G>A and c.1034T>C were detected in two index cases. None of the detected nucleotide changes was found in a control group of 250 healthy and unrelated subjects (500 control chromosomes). c.304G>A and c.1034T>C result in predicted p.E102K and p.I345T amino acid substitutions, respectively. Physico-chemical properties of novel amino acids strongly differ from wild type: mutation p.E102K replaced a negatively-charged residue by a positively-charged one, whereas mutation p.I345T replaced a non polar hydrophobic amino acid

by a polar hydrophilic amino acid. Both these changes occurred in a residue highly conserved among species. Only in mouse and rat E102 is replaced by aspartic acid, but the two amino acids share similar physico-chemical properties.

Cadherins are synthesized as inactive precursor proteins containing a prosequence followed by the cadherin domains. The N-terminal prosequence is proteolytically cleaved off in the late Golgi and the mature cadherin is then transported to the plasma membrane. Proteolytic removal of the prosequence results in structural rearrangements within EC1 domain with the activation of adhesive properties (Ozawa M. et al., 1990). Mutation p.E102K alters a conserved amino acid located in this propeptide region. Mutation p.I345T is located in the EC2 domain, which forms, together with EC1, the "minimal essential unit" to mediate cell adhesion, through *cis* and *trans* interactions among desmosomal cadherins (Shan W. et al., 2004). These *DSC2* mutations were detected in two ARVC index cases and in four family members who met only minor diagnostic criteria. This could be consistent with incomplete penetrance of such mutations; incomplete penetrance is rather common in ARVC and it was reported for several *DSP*, *PKP2* and *DSG2* mutations (Bauce B. et al., 2005; Syrris P. et al., 2006; Van Tintelen J.P. et al., 2006; Pilichou K. et al., 2006). However, lack of clinical manifestation could be due to the young age of most family members carrying the *DSC2* mutations.

To evaluate the pathogenic potentials of the two N-terminal *DSC2* mutations, full-length wild-type and mutated cDNAs were cloned in eukaryotic expression vectors to obtain a fusion protein with green fluorescence protein (GFP); constructs were transfected in HL-1 cells. This in vitro functional studies demonstrated that, unlike wild-type DSC2, the two N-terminal mutants predominantly localise in the cytoplasm, confirming the suspect that both mutations could have pathogenic effect.

An insertion of two bases (c.2687_2688insGA) was detected in exon 17 of *DSC2* gene in two unrelated index cases affected with ARVC; in one of them a missense mutation (E58D) in *PKP2* gene was previously detected and in the other one a missense mutation (Y87C) in *DSG2* gene was identified. These four amino acids (C=Cysteine, D=Aspartic acid, E=Glutamic acid and Y=Tyrosine) have a polar side chains; therefore the two missense mutations in *PKP2* and *DSG2* genes might be non pathogenic.

The c.2687_2688insGA *DSC2* insertion was reported in the literature as a pathogenic mutation (Syrris P. et al., 2006), since it results in a frameshift leading to a termination codon 4 aa residues downstream (E896fsX900). In Syrris report, *DSC2* E896fsX900

mutation was detected in three families, indicating either that this insertion is recurrent in patients with ARVC, or that such families may share a common ancestor. This last possibility was excluded, since analysis of microsatellite DNA markers (*D18S847, D18S49,* and *D18S457*) in close proximity to the *DSC2* locus demonstrated no allele sharing in individuals carrying E896fsX900. Therefore, E896fsX900 might be a recurrent mutation, possibly due to a "hot" mutational spot (Syrris et al., 2006).

However, in the present study the *DSC2* insertion c.2687_2688insGA was identified in 2 out of 64 patients and it was detected as well in 6 out of 150 control subjects (300 chromosomes): this would suggest that, more likely, such variant could be a polymorphism rather than a pathogenic mutation. This variant occurs in exon 17, which encodes, in DSC2a isoform, the ICS domain. Such domain shows a high degree of amino acid homology among various desmosomal and nondesmosomal cadherins. Therefore, in theory, the alteration caused by the insertion is potentially pathogenic, but in reality it affects only the last five amino acids of the protein, which are not highly conserved among species in contrast with the highly conservation of the upstream region. It is important to notice that the same region is untranslated in DSC2b isoform, being included in its 3'UTR. Therefore, the insertion would affect only DSC2 isoform a, leaving isoform b fully functional and possibly able to compensate in cardiac myocytes the relative deficiency of DSC2a isoform. In vitro functional studies on HL-1 cells demonstrated that mutant DSC2 (E896fsX900) predominantly localizes to the cytoplasm, in contrast with wild type DSC2 and DSC2 variants carrying polymorphisms D179G and R798Q. These observations conflict with the hypothesis that c.2687_2688insGA is a polymorphism; however it must be taken into account that such sequence variation could have no pathogenic effect because of the presence of a correct DSC2b isoform.

**DSC2 FUNCTIONAL STUDIES**

In vitro functional studies on HL-1 cells demonstrated that the two missense mutations in the N-terminal domain (E102K and I345T) and the frameshift (E896fsX900) in C-terminal domain affect the normal cellular localisation of DSC2. As previously reported, wild-type DSC2a-GFP fusion protein was efficiently incorporated into desmosomes and did not exert dominant-negative effect when overexpressed (Windoffer R. et al., 2002). A lower amount of GFP signal was detected in the cytoplasm, since proteins were still not fully trafficked to the membrane. Unlike wild-

type DSC2 and DSC2 variants carrying polymorphisms D179G and R798Q, mutants of DSC2 were predominantly located in the cytoplasm suggesting a potential pathogenic effect. Although all three mutations affect intracellular localization of desmocollin-2, their effect probably differ, depending on the relative position of each mutation along the protein.

Mutations p.E102K and p.I345T map to the N-terminal region, relevant to adhesive function. Cadherins have been shown to be internalised and recycled back to the plasma membrane in a constitutive manner, providing a mechanism for regulating the availability of cadherins for junction formation and for maintaining a dynamic state of cell-cell contacts (Le T.L. et al., 1999; Le T.L. et al., 2002; Kowalczyk A.P. et al., 2004; D'Souza-Schorey C., 2005). Moreover, recent studies suggest that adhesive interactions between cadherins as well as cytoskeletal associations prevent cadherin endocytosis (Izumi G. et al., 2004). Based on these findings, it can be hypothesized that in the absence of adhesive interactions, the two N-terminal mutants DSC2 might be internalised from the cell surface by endocytosis. Interestingly, in Pemphigus vulgaris (PV, MIM #169610), an autoimmune disease in which antibodies are directed against DSG3, resulting in severe mucosal erosions and epidermal blistering, PV autoantibodies trigger co-endocytosis of DSG3 and plakoglobin, leading to delivery of DSG3 to lysosomal compartments and dramatic decrease in DSG3 protein level (Calkins C.C.et al., 2006). Possibly, N-terminal *DSC2* mutations could result in alteration of the adhesion properties, leading to DSC2 internalisation.

On the other hand, mutation E896fsX900 maps to the C-terminal region, involving the ICS domain of DSC2a isoform, and could impair the binding to plakoglobin or the formation of desmosomal plaque and intermediate filaments anchorage, thus leading to disruption of desmosome structure (Syrris P. et al., 2006). As previously mentioned, *DSC2* gene encodes for two different products (DSC2a and DSC2b); a mutation in the C-terminal cytoplasmic domain could affect only the longer isoform a and compensation by the other form might be possible. Further investigation will be needed to understand whether *DSC2* mutations could induce "null alleles" potentially leading to haploinsufficiency (i.e. through cytoplasmic degradation) or mutant proteins could remain within the cells, acting in a dominant form (i.e.toxic gain−of−function).

Recently, it was shown that suppression of desmoplakin expression with use of small interfering RNA in atrial myocyte cell lines (HL-1 cells) or in heterozygous desmoplakin-deficient mice leads to nuclear localization of plakoglobin, reduction in

canonical Wnt signaling through Tcf/Lef transcription factors, and increased myocyte apoptosis (Garcia-Gras E. et al., 2006). Indeed, increased apoptosis of cardiomyocytes was reported in patients with ARVC (Yamaji K. et al., 2005). Garcia-Gras et al. have established a potential role for signaling defects in ARVC, leading to the idea that the cell adhesion proteins are not only passive players in myocardial architecture, but they might be considered as key regulators in cardiac patterning and development, in myocyte differentiation, and in the maintenance of the cellular architecture of the adult heart (MacRae C.A. et al., 2006).

# CONCLUSIONS

In this study two putative mutations in PERP gene were identified in index cases affected with ARVC, but it was impossible to establish whether these mutations might really cause ARVC. However, data suggested that a variation in PERP might produce a very severe phenotype in patients carrying a pathogenic mutation in a different gene involved in ARVC. Therefore, PERP still remains a good candidate for ARVC and mutation screening should be extended to additional series of index patients.

Novel *DSC2* mutations were detected in ARVC index cases and their pathogenic effects were investigated using a cardiomyocytes cell line. In vitro functional studies demonstrated that, unlike wild-type DSC2, the mutants are predominantly localised in the cytoplasm, affecting transmembrane localisation of DSC2 and thus suggesting the potential pathogenic effect of the reported mutations.

This method, based on transient transfection of HL-1 cell line with mutant constructs for genes encoding desmosomal proteins, could allow to study potential pathogenic effects of novel missense mutations suspected to cause ARVC.

# MATERIALS AND METHODS

## CLINICAL EVALUATION

The study involved subjects belonging to several ARVC families, sporadic cases of Italian descent, all with a clinical diagnosis of ARVC. Clinical diagnosis for ARVC was based on major and minor criteria, established by the European Society of Cardiology/International Society and Federation of Cardiology Task Force (McKenna et al., 1994). The patients were investigated at the Department of Clinical and Experimental Medicine of the University of Padua, by Prof. Nava and colleagues. Clinical investigations and blood sampling for DNA analysis were performed under informed consent, according to the pertinent Italian legislation and in compliance with Helsinki declaration. Each patients underwent 12-lead electrocardiography (ECG), signal-averaged ECG (SAECG), 24 hour Holter ECG and two-dimensional echocardiography.

## DNA EXTRACTION

Genomic Dna was extracted from blood samples by a modified salting-out procedure, evaluated at the Human Genetics Laboratory of Padua.

- Thaw blood sample (collected in K3E-EDTA tube and stored at -20°C) and transferred to a sterile 50ml tube.
- Wash with erythrocyte lysing buffer (N-N solution: 0.9% NaCl, 0.1% Nonidet P40), centrifuge for 30minutes at 4000rpm and 4°C. Wash and centrifuge are repeated twice.
- Add to the pellet 4ml of TEN solution ( 10mM TrisHcl; 2mM $Na_2EDTA$ pH 8; 400mM NaCl), and 300µl 20% SDS.
- Vortex to break up pellet
- Incubate to 80°C for 3hours with vigorous shaking. During this step the leukocytes membranes are denatured and DNA goes out in solution.
- Add 1ml of saturated NaCl solution to precipitate proteins and cells membranes and centrifuge for 10minutes at 4000rpm. Transfer the supernatant in a sterile 15ml centrifuge tube.
- Add an equal volume of chloroform and shake by hand.

- Centrifuge for 10minutes at 4000rpm. Three phases are observed, the upper of which containing the DNA. Transfer this phase to a fresh tube.
- Add an equal volume of isopropanol and invert the tube gently until the DNA has precipitated.
- Centrifuge for 20minutes at 4000rpm and discard the supernatant.
- Wash pellet twice with 70% ethanol and then centrifuge for 10minutes at 4000rpm.
- Dry the pellet and resuspend in 300-500µl of TE buffer (10mM Tris-Hcl pH 8; 1mM EDTA) and left overnight on a rotator.

**RNA EXTRACTION (PAXgene blood Rna, PreAnalytiX)**

The PAXgene Blood RNA Kit is for the purification of total RNA from 2.5ml human whole blood collected in a PAXgene Blood RNA Tube, contains a proprietary reagent composition able to protects RNA molecules from degradation by RNases and minimizes induction of gene expression for up to 3 days at 18–25°C. Purification begins with a centrifugation step to pellet nucleic acids in the PAXgene Blood RNA Tube. The pellet is washed and resuspended, and incubated in optimized buffers together with proteinase K to bring about protein digestion. An additional centrifugation through the PAXgene Shredder spin column is carried out to homogenize the cell lysate and remove residual cell debris, and the supernatant of the flow-through fraction is transferred to a fresh microcentrifuge tube. Ethanol is added to adjust binding conditions, and the lysate is applied to a PAXgene RNA spin column. During a brief centrifugation, RNA is selectively bound to the PAXgene silica membrane as contaminants pass through. Remaining contaminants are removed in several efficient wash steps. Between the first and second wash steps, the membrane is treated with DNase I to remove bound DNA. After the wash steps, RNA is eluted in Buffer BR5, denaturated at 65°C for downstream applications and stored at -80°C. Typical yields of RNA isolated from 2.5ml human blood are between 4 to 20µg. However, the yield is highly dono-dependent, and in some cases higher or lower yields may be achieved.

**DNA/RNA QUANTIFICATION**

The concentration of DNA and RNA solution is determined by measuring at 260nm against blank. The absorption of 1 OD is equivalent to approximately 50µl/ml dsDNA,

and approximately 40µl/ml RNA. The ratio $A_{260}/A_{280}$ is used to estimate the purity of nucleic acid, since protein absorb at 280nm. A ratio higher than 2.0 indicates the samples may be contaminated with chloroform or phenol. Pure DNA should have a ratio of approximately 1.8, whereas pure RNA should give a value of approximately 2.0.

## PCR PRIMERS DESIGN

All PCR primers used in this thesis were designed by PRIMER 3 (http://frodo.wi.mit.edu) and produced by SIGMA or MWG-Biotech. The primers obtained from PRIMER 3 were chosen after analysis with BLAT (http://genome.ucsc.edu) to avoid the presence of SNPs within their sequences and their annealing in additional wrong regions.

## DHPLC ANALYSIS FOR *DSC2* AND *PERP* GENES

### PCR amplifications

The analysis of the *DSC2* gene in 64 ARVC index cases and of the *PERP* gene in 90 ARVC index cases, were performed by denaturing high-performance liquid chromatography (DHPLC). Samples were amplified in a final volume of 25µl or 50µl containing approximately 50-100ng of genomic DNA, 10X PCR buffer II (Applied Biosystems), 1.5mM $MgCl_2$ (Applied Biosystems), 10pmoli of each primer (Sigma Genosys or MWG Genomics), 100µM deoxinucleotide triphosphates (Invitrogen) , and 1.25 unit of DNA TAQ GOLD polymerase. PCR conditions for all the DNA fragments were as follows: an initial denaturation cycle of 95°C for 12 minutes followed by 37 cycles composed by a denaturation step (95°C) for 30 seconds, an annealing step for 30 seconds, and an extension step (72°C) for 45 seconds. A final extension of 72°C for 10 minutes complete the amplification. The amplification product was verified using 2% agarose gel, and visualized using ethidium bromide and ultraviolet light. As the length of fragment suitable for DHPLC analysis is less than 400bp, some longer exons were splitted in shorter amplicons. All primer sequences and PCR conditions used are reported in Appendix. A and Appendix B.

### DHPLC analysis

DHPLC analysis was performed on a Transgenomic Wave DNA Fragment Analysis System (Model 3500HT; Transgenomic) with a DNASep column (Transgenomic). PCR fragments were denatured for 5min at 95°C and then left to reanneal slowly at room

temperature to promote the formation of heteroduplex. Separation was performed at a flow rate of 0.9ml/min for Mutation Detection application (8min) and if necessary at 1.5ml/min for Rapid DNA application (3min). The acetonitrile gradient was adjusted to elute the amplicon at half run, around 4min for Mutation Detection application and 1.5min for Rapid DNA application. The gradient was obtained by mixing Buffer A (0.1M TEAA, pH 7.0) and Buffer B (0.1M TEAA, pH 7.0, 25% acetonitrile). Buffer B increase was 2% per min (with flow rate 0.9ml/min) and 5% per min (with flow rate 1.5ml/min). Column temperatures were calculated by NAVIGATOR software (Transgenomic). Whenever fragments showed different melting domains, additional analyzing temperatures were used, to optimize resolution. These temperatures should give the 75-80% of double strand DNA within the fragments. DHPLC conditions used to analyse *DSC2* and *PERP* genes are listed in Appendix A and Appendix B.

**CONTROLS**

Whenever a putative mutation was detected, a search was performed on a single-nucleotide polymorphism database (dbSNP: http://www.ncbi.nlm.nih.gov/projects/SNP/). In case of negative results, DHPLC analysis on genomic DNA from unrelated control individuals was performed with Rapid DNA application, to exclude DNA polymorphisms.

**EXPRESSION ANALYSIS FOR *PERP* GENE**

Expression of *PERP* in different human tissues was assessed by RT-PCR amplification of MTC (Multiple Tissue cDNA) panel (Clontech), including heart, skeletal muscle, liver, kidney and skin. Amplification was performed by using the primers mentioned below:

| | |
|---|---|
| PERPexp0F | CAGGCCACTCTCTGCTGTC |
| PERPexp0R | CTCTGACAGCCCTCCTCGTA |

The amplification products were verified using 2% agarose gel, and visualized using ethidium bromide and ultraviolet light.

**RT-PCR**

This procedure outlines the synthesis of cDNA for subsequent amplification using PCR. Reverse transcription reaction of up to 1µg of total RNA, poly(A)+ mRNA is performed in 25µl reaction. Experimental RNA is combined with 2µl of random primer (500µg/ml,

Promega) and Rnase-free water to a final volume of 5µl. The primer/template mix is thermally denatured at 70°C for 5 minutes and chilled on ice. A reverse transcription reaction mix is assembled on ice to contain 5.6µl of nuclease-free water, 4µl of ImProm-II 5X Reaction Buffer (Promega), 1µl of ImProm-II Reverse Transcriptase (Promega), 2.4µl magnesium chloride ($MgCl_2$ 25mM, Promega), 1µl of dNTPs (10mM, Invitrogen) and 1µl of Recombinant RNasin® Ribonuclease Inhibitor (40u/µl Promega). As a final step, the template-primer combination is added to the reaction mix on ice. Following an initial annealing at 25°C for 5 minutes, the reaction is incubated at 42°C for up to one hour and then at 70°C for 15 minutes to inactivate the reverse transcriptase. The first strand of cDNA generated by reverse transcription of mRNA can be used as a template for PCR or stored at -20°C.

RT-PCR experiments were carried out in a reaction buffer (12.5µl) containing 2µl cDNA, 5pmoles oligonuclotide pimers, 100µM each of dGTP, dATP, dCTP, and dTTP, 10X PCR buffer II (Applied Biosystems), 1.5mM MgCl2 (Applied Biosystems), and 1.25unit of DNA TAQ GOLD polymerase. All polymerase chain reaction were carried out in a thermal cycler, under the following conditions: 12min 95°C and 37 cycles of denaturation (at 95°C for 1minute), annealing (at 65°C for 1minute), and extension (at 72°C for 2 minute). RT-PCR were performed by using the primers mentioned below:

| | |
|---|---|
| dsc2-2F espr | GTGATGCCTGCAAAAATGTG |
| dsc2-5R espr | ATAGTTTTGGGCCGTGTCAG |
| dsc2-4R espr | AAGGAATTGGAGCCCATCTT |

Aliquots of the applied products were separated by 2% agarose gel electrophoresis an stained by ethidium bromide.

**PURIFICATION OF PCR PRODUCTS AND DNA SEQUENCING**

Primers and dNTPs unconsumed during the PCR amplification can interfere with DNA sequencing methods. The PCR Product pre-Sequencing Kit provides two Hydrolytic enzymes, Shrimp Alkaline Phosphatase (SAP) and Exonuclease I (EXO), which remove excess of primers and dNTPs. In particular, SAP dephosphorylates dNTPs, while EXO remove primers and any extraneous single-stranded DNA produced by PCR.

- PCR amplification mixture 5µl

  EXO 0.5µl

  SAP 0.5µl

  Mix and incubate at 37°C for 15min.

- Incubate at 80°C for 15min to inactivate the enzymes.
- Add to purified PCR 1µl of primer for sequencing (10pmoli).
- Dry at 70°C for 20minutes.

PCR amplicons were sequenced at the BMR genomics, University of Padua, by an ABI 3730XL DNA sequencer (Applied Biosystems) with the BIG DYE dideoxy-terminator chemistry (Applied Biosystems). CHROMAS software (Version 1.55; Technelysium) and the LASERGENE software package (SeqMan II, DNASTAR) were used to edit, assemble, and translate sequences. Amplicons showing putative mutations were re-sequencing, by using as template the product of an new PCR reaction.

**DNA EXTRACTION FROM AGAROSE GEL (QIAEX II Gel Extraction kit, QIAGEN)**

A particular good purification of the PCR product before cloning is necessary to remove the excess of dNTPs and primers and any aspecific DNA produced by the PCR. Extraction of DNA fragments with QIAEX II kit is based on solubilization of agarose and absorption of nucleic acids to silica-gel particles in the presence of high salt. All impurities such as agarose, proteins, salts and ethidium bromide are removed during washing steps. The DNA band is excised from the agarose gel, solubilized and washed with buffers provided by the kit. The pure DNA is eluted in 20µl of water and is suitable foe subsequent applications, such as sequencing or ligation.

**PCR CLONING (TOPO TA Cloning, invitrogen)**

The cDNA fragments obtained from RT-PCR were cloned in pCR2.1-TOPO vector using E.Coli cells (TOPO10 OneShot) as hosts. TOPO TA Cloning vectors contain 3'-T overhangs that enable the direct ligation of Taq-amplified PCR products with 3'-A overhangs. Principal vector characteristics are: *Eco*RI sites flanking the PCR product insertion site for removal of inserts; kanamycin and ampicillin resistance genes for selection in E.Coli; blue/white screening of recombinant colonies; M13 forward and reverse priming sites for sequencing or PCR screening.

Ligation

| | |
|---|---|
| PCR product | 0.5 - 4 µl |
| Salt Solution | 1 µl |
| TOPO vector | 1 µl |
| Sterile water | up to 6 µl |
| Final Volume | 6 µl |

Mix reaction gently and incubate for 5 minutes at room temperature. For large products (>1Kb) increasing reaction time will yield more colonies. Place the reaction on ice and follow the chemical transformation protocol (see below).

**PLASMID CONSTRUCTIONS**

The cDNA of human *DSC2a* was kindly provided by Dr W.W. Franke (Heidelberg, Germany) and human *DSC2a* full-length coding sequence (GenBank NM_024422) was PCR-amplified with the following primers:

*DSC2a*-clonF ATTATGGAGGCAGCCCGCCC

*DSC2a*-clonR GTCTCTTCATGCATGCTTCTGCTAG

The resulting fragment was cloned into pcDNA3.1/CT-GFP-topo eukaryotic expression vector (Invitrogen) which contains cDNA coding for green fluorescent protein (GFP), and verified by sequence analysis.

**MUTAGENIC PRIMERS DESIGN**

The mutagenic oligonucleotide primers were designed using the following guidelines:

- The mutagenic primers for point substitutions or insertion must contain the desired mutation, whereas the mutagenic primers for deletions must skip the sequence corresponding to the lost oligonucleotides.
- Primers should be between 25 and 45 bases in length, with a melting temperature ($T_m$) of ≥78°C. Primers longer than 45 bases may be used, but using longer primers increase the likelihood of secondary structure formation, which may affect the efficiency of the mutagenesis reaction.

The following formula is commonly used for estimating the $T_m$ of primers:

$$T_m = 81.5 + 0.41(\%GC) - 675/N - \%mismatch$$

$N$ is the primer length in bases

Values for %GC and %mismatch are whole numbers

For calculating $T_m$ for primers intended to introduce insertions or deletions, there is a modified formula to use:

$$T_m = 81.5 + 0.41(\%GC) - 675/N$$

*N* does not include the bases which are being inserted or deleted

- The desired mutation should be in the middle of the primer with 10-15 bases of correct sequence on both sides.
- The primer optimally should have a minimum GC content of 40% and should terminate in one or more C or G bases.
- Primers for insertion or deletions must be purified either by fast polynucleotide liquid chromatography (FPLC) or by polyacrylamide gel electrophoresis (PAGE).

**ONE STEP DIRECT MUTAGENESIS**

Site-directed mutagenesis was performed on *DSC2a*-pcDNA3.1/CT-GFP, in order to reproduce two *DSC2* naturally occurring mutations p.E102K (c.304G>A), p.I345T (c.1034T>C) and three unknown polymorphisms p.D179G (c.536A>G), p.R798Q (c.2393G>A) p.E896fsX900 (c.2685_2686insAG) present in more patients (also in omozygos) or in more controls.

The following mutagenic primers and conditions were used:

```
E102K-F:   TACCATATTACTTTCCAACACTAAGAACCAAGAAAAGAAGAAAAT;
I345T-F:   CAACTTCAACTTGTATCATTAACACTGATGATGTAAATGACCACTTGC;
D179G-F:   TCCATAAGAGGTCCTGGAGTTGGCCAAGAACCTCGGAATTTATTTT;
R798Q-F:   CACCAGACCTCGGAATCCTGCCAGGGGGCTGGCCACCATCACACC;
```

For p.E896fsX900 mutation, two steps of mutagenesis were necessary. The first one to introduce the AG insertion, using the following primer:

```
DSC2mutAG:  CAAATTTGGACACTAGCAGAAGAAGCATGCATGAAGAGACAAG
```

The second one to remove the premature stop codon and to get the right frame with GFP sequence, using the following primer:

```
DSC2mutCW:  CACTAGCAGAGAAGCATGCACAAGGGCAATTCTGCAGATA
```

Set up PCR reaction:

| | |
|---|---|
| 1µl | template DNA (300ng/µl stock from Mini prep) |
| 5µl | primer F+R 1µM |
| 1µl | dNTPs 10mM |
| 2.5µl | 10X Pfu Buffer |
| 15µl | sterile water |
| 0.5µl | Pfu polymerase (Stratagene, 2.5U/µl) |

PCR conditions:

| | | |
|---|---|---|
| Denaturation: | 95°C | for 3min |
| Then 18-22 cycles of: | 95°C | for 30sec |
| | 60°C | for 1min and 30sec |
| | 68°C | for 18min (or 2 min/Kb of plasmid template) |

1µl of Dpn I (Methylation sensitive) was added to the PCR product and then was incubated for 1-2 hours at 37°C to get rid to the wild type host strand methylated. 2µl of this reaction mixture was used to transform host cells.

**ONE SHOT CHEMICAL TRANSFORMATION PROTOCOL**

The mutated constructs were cloned using Top10 chemically competent E.Coli (Invitrogen) following these steps:

- Equilibrate a water bath to 42°C
- Warm a vial of SOC medium to room temperature
- Incubate at 37°C for 30minutes two plates (LB plates containing 50µg/µl ampicillin) for each transformation
- Thaw on ice 1 vial of One Shot cells (TOP 10, invitrogen) for each transformation
- Add 2µl of reaction digested with DpnI and mix gently without pipetting
- Incubate on ice for 5 to 30 minutes
- Heat-shock the cells for 30 seconds at 42°C without shaking
- Immediately transfer the tubes to ice
- Add 250µl of room temperature SOC medium
- Cap the tube tightly and shake the tube (200rpm) at 37°C for 1 hour
- Spread 50µl and 200µl from each transformation on 2 prewarmed plates and incubate overnight at 37°C

**ANALYSING POSITIVE CLONES**

- Pick 10-20 colonies with a sterile pipet tip and wash the tips in 10µl of sterile water in a 0.5ml tubes
- Use 1.5-2µl of each colony as template in a PCR reaction
- Aliquote the PCR mix, carried out using exonic primers flanking the region with variation, and amplify for 37 cycles with DNA TAQ GOLD polymerase
- Analyze the PCR products, corresponding to each colony, by restriction analysis to confirm the presence of mutant base
- The colonies positive to the restriction analysis were cultered overnight in 5ml of liquid LB (containing 50µg/µl ampicillin), shaking the tubes horizontally (200rpm) at 37°C
- Isolate the plasmid DNA using Miniprep Kit (SIGMA)
- Each plasmids were verified for variation by sequence analysis

**PURIFICATION OF PLASMID (MINIPREP-MIDIPREP)**

**Miniprep (GenElute™ HP Plasmid Miniprep Kit, Sigma-Aldrich)**

The GenElute HP Plasmid Miniprep Kit is based on silica-binding technology, for isolating up to 25µg of high copy plasmid DNA from 1-5ml of recombinant *E.coli* coltures. An overnight colture is harvested with centrifugation and subjected to a modified alkaline-SDS lysis procedure followed by adsorption of the plasmid DNA onto silica in the presence of high salts. Contaminants are then removed by a spin wash step. Finally, the bound plasmid DNA is eluted in water or Tris-EDTA buffer. The recovered plasmid DNA, predominately in its supercoleid form, is checked in a 1% agarose gel.

**Midiprep (NucleoBond® Xtra Midi, Macherey-Nagel)**

The bacterial cells are lysed by a set of buffers based on the NaOH/SDS lysis method. After equilibration of the column together with the corresponding column filter, the entire lysate is loaded by gravity flow and simultaneously cleared by the equilibrated filter. Plasmid DNA is bound to the silica resin and after a washing step is eluted, precipitated and dissolved in a suitable buffer for further use. From 50ml of an overnight recombinant *E.coli* colture is possible to isolate more than 50µg of high copy plasmid DNA.

## SOLUTIONS AND MEDIA

### For Bacteria

| | |
|---|---|
| **SOC** | 2% Tryptone<br>0.5% Yeast Excract<br>10mM NaCl<br>2.5mM KCl<br>10mM $MgCl_2$<br>10mM $MgSO_4$<br>20mM glucose |

| | |
|---|---|
| **LB** (Luria-Bertani) Liquid Medium | 1% Tryptone<br>0.5% Yeast Extract<br>1% NaCl<br>pH 7.0 |
| Solid Medium | +1.5% Select-Agar |

### For HL-1 cell line

| | |
|---|---|
| **Supplemented Claycomb Medium** | 261ml Claycomb Medium<br>30ml Fetal bovine serum (10%)<br>3ml Penicillin/Streptomicyn (100U/ml:100µg/ml)<br>3ml Norepinephrine (0.1mM)<br>3ml L–Glutamine (2mM stock) |
| The claycomb medium bottle must be wrapped in aluminum foil since the medium is extremely light sensitive. | |

Penicillin/Streptomicyn and L–Glutamine come as a 100X solutions, and are aliquoted into working volumes and frozen at –20°C.

Norepinephrine is made up in 30mM of ascorbic acid, following this procedure:

- Add 0.59gr of ascorbic acid to 100ml of cell culture grade distilled water to have a 30mM ascorbic acid solution.
- Add 80mg of norepinephrine to 25ml of the 30mM ascorbic acid
- Filter sterilize using a 0.2µm Acrodisc syringe filter
- Aliquot in 3ml volumes into sterile microtubes and store to –20°C. This is 10mM norepinephrine. Use 3ml of this stock per 300ml for 0.1mM final concentration.

## CELL CULTERS

The HL-1 cell line was derived from AT-1 cells (mouse cardiomyocyte tumor) and is the first cell line established that can maintain the differentiated cardiomyocyte phenotype and contractile activity *in vitro*. HL-1cells were maintained as previously reported (Claycomb W.C. et al., 1998). HL-1cells require higher than normal cell densities for optimal growth and bahavior, they need a substrate (Gelatin/Fibronectin) that provides adequate anchorage during contraction and norepinephrine to maintain

contractile activity. Claycomb medium is specifically designed for the growth of HL-1 cells in a 5% $CO_2$ humid environment at 37°C.

**TRANSFECTION OF HL-1 CELLS**

Cells were seeded in 6-well dishes and allowed to growth until the confluence was about 70%. HL-1 cells were transfected with 0.8µg of plasmid DNA per plate, 6.4 µl of enhancer reagent, and 8µl of effectene reagent (Qiagen). At 48 hours post-transfection, when a right desmosome structure is present, HL-1 cells were fixed with cold methanol/acetone (1:1) for 20 minutes at -20°C. Each set of subsequent experiments was performed in triplicate in 24 mm glass coverslips and was repeated 3 times.

**IMMUNOSTAINING AND CONFOCAL IMAGING ON HL-1 CELLS**

HL-1 cells were incubated with the murine mAb (clone DG3.10) against endogenouse dsg (PROGEN, Heidelberg, Germany), which reacts with a carboxy terminal epitope of desmoglein for 30 min at 37°C, washed three times with PBS, and incubated with TRITC conjugated antibodies (DAKO).

Slides were inspected and photographed using a Radiance 2000 confocal microscope (BioRad) with a 60× oil objective.

**Appendix A**. Conditions for PCR amplification and DHPLC analysis of *DSC2* gene.

| Amplicon | | Tm | Bp | T°C Dhplc+Time Shift | Forward 5' 3' | Reverse 5' 3' |
|---|---|---|---|---|---|---|
| 1+5'UTR | | 58 | 708 | Direct sequencing | TCAGACCTCGCTCTGTAATTGA | TATCCCCGTTCCCCTAGTTT |
| 2 | | 62 | 199 | Direct sequencing | ACACATTAAAGTTTTCTTTTTAT | GGCGTATATGTACCACAGCA |
| 3 | | 58 | 400 | 54.8/55.4 | CCCCACGTGCATACATTACT | TGGTTTTCATTCGTCTTTAAGC |
| 4 | | 58 | 269 | 55.6/58.5+3 | CCCCTACCCAGCTAATCCTC | GGAAACTATAGACTCCCACAGCA |
| 5 | | 58 | 332 | 56.5/57.2+1 | TGAAAGCTCTGCTGAAATAAAGA | GGAGTAGCCAGAGCATTGGT |
| 6 | | 60 | 266 | 54.7+0.5 | GCCAAAATGAATTTGAAGCATAC | TTGAAACACAGTTAATTTGCCATA |
| 7 | | 58 | 384 | 55+1/58+2/59.5+3 | CATAGAACATGTGAATGTTTTGGA | CAAAACCAGCATACTCCAAGG |
| 8 | | 58 | 252 | 54.3/55.6+1 | GTTGGTGCTTTCCCCCAATA | AGGCCAGAGATGTGCATATTA |
| 9 | | 60 | 324 | 52.2/53.5+0.5/55.3+1.5 | CATCGTGTTCAATTTTTGTGA | CCTTTCTTTCCATTAAATTCTAGC |
| 10 | | 58 | 374 | 56.5/57.9+2 | ACTCGTTAGCATTGCCAAAT | TAACGTAACAAAATAAGCTA |
| 11 | | 60 | 375 | 51.7/56+1.5 | CAAGAAGTAGCAGTGGCATAAGG | AACAGAGTGCATGTATCCAGCTT |
| 12 | | 58 | 345 | 57+1.5/58+2/59+3 | GTGTTCAGTGCATACTTTTGTGG | GCAGACATCCTGATGTTGAAAA |
| 13 | | 58 | 356 | 57.2+1/58+2 | TGTTCAGAAGAAATCAGTGACA | GTGTCTTGAAAGTTACTTTAAAGG |
| 14 | | 58 | 263 | 57.7+1.5 | GATTTATGTGTGTATTAACCATTG | CGCATTATAAGCGAATTCATCC |
| 15 | a | 58 | 194 | 56.2/60.6+1/61.8+2 | CATAATTTTGTGTTCCTCTCTGT | AGGATTCCGAGGTCTGGTGT |
| | b | 58 | 332 | 61/61.4+1/62.2+1.5 | GGCTTCACAACCCAAACTGT | TGAAAATTATAGTCAGAATCCAGT |
| 16 | | 58 | 226 | 53.7/55.2+1.5 | GCCACATGCGTGACTTTTAG | ACTTTCTGCCAAGGGGAAAA |
| 17+3'UTR | | 58 | 397 | 56.3+1/56.8+1 | CAATGAAAGGTAAATCAAAGCAA | AAAAACCCCCACAAATAGCA |

**Appendix B**. Conditions for PCR amplification and DHPLC analysis of *PERP* gene.

| Amplicon | Tm | Bp | T°C Dhplc+Time Shift | Forward 5' 3' | Reverse 5' 3' |
|---|---|---|---|---|---|
| 5'UTR | TD 72/62 | 372 | Direct sequencing | CAGAACCCAGCCTGTTTACC | GGTCGCTAGACTGCAACCA |
| Exon 1 | TD 72/62 | 327 | 66.8 | CAGGCCACTCTCTGCTGTC | CTCCCCCGACCCTGTGAG |
| Exon 2 | TD 72/62 | 318 | 54.6/58.3+1/60.7+1.5 | AAAAGCCCAAAACCATGTTG | GTCACGGGTCTTCTTTGTGG |
| Exon 3 | TD 72/62 | 405 | 56.8/59+1/60+1.5 | GCAGAAACTTGGTGGAAGGA | TCAAAGTCGCCTGGAGAAAC |
| 3'UTR-1 | TD 65/55 | 417 | 52.9/58.8+2 | GTGCCTTCTTCTTCTGCTG | TTGGAAACCTCATTTTTACCTT |
| 3'UTR-2 | TD 72/62 | 398 | 53/54+0.5 | TTTGTGAAGTTGTGTCTTTTCA | CGAGTCAACATGAAGCCTTA |
| 3'UTR-3 | TD 72/62 | 348 | 53.5/54.5/55+1 | CGGTCTGTTAAGGGCTAAGG | AGCATTTTCCCACACCCTAA |
| 3'UTR-4 | TD 72/62 | 406 | 54.2/56+1.5 | TCCAAACCTGTTGCCATAGT | TGTTTACCAAGAAAGACCAGGA |
| 3'UTR-5 | TD 65/55 | 383 | 53.6/55+1.5 | TCTCAGGTTTATCTGGGCTCT | ATTCCTTTCCCCAGATTTGA |

# REFERENCES


Alcalai R, Metzger S, Rosenheck S, Meiner V, Chajek-Shaul T. A recessive mutation in desmoplakin causes arrhythmogenic right ventricular dysplasia, skin disorder, and woolly hair. *J Am Coll Cardiol*. 2003;42(2):319-327.

Amack JD, Reagan SR, Mahadevan MS. Mutant DMPK 3'-UTR transcripts disrupt C2C12 myogenic differentiation by compromising MyoD. *J Cell Biol*. 2002;159(3):419-429.

Armstrong DK, McKenna KE, Purkis PE, Green KJ, Eady RA, Leigh IM, Hughes AE. Haploinsufficiency of desmoplakin causes a striate subtype of palmoplantar keratoderma. *Hum Mol Genet*. 1999;8(1):143-148.

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(5):964-973.

Attardi LD, Reczek EE, Cosmas C, Demicco EG, McCurrach ME, Lowe SW, Jacks T. PERP, an apoptosis-associated target of p53, is a novel member of the PMP-22/gas3 family. *Genes Dev*. 2000;14(6):704-718.

Awad MM, Dalal D, Cho E, Amat-Alarcon N, James C, Tichnell C, Tucker A, Russell SD, Bluemke DA, Dietz HC, Calkins H, Judge DP. DSG2 mutations contribute to arrhythmogenic right ventricular dysplasia/cardiomyopathy. *Am J Hum Genet*. 2006;79(1):136-142.

Bagattin A, Veronese C, Bauce B, Wuyts W, Settimo L, Nava A, Rampazzo A, Danieli GA. Denaturing HPLC-based approach for detecting RYR2 mutations involved in malignant arrhythmias. *Clin Chem*. 2004;50(7):1148-1155.

Bauce B, Basso C, Rampazzo A, Beffagna G, Daliento L, Frigo G, Malacrida S, Settimo L, Danieli GA, Thiene G, Nava A. Clinical profile of four families with arrhythmogenic right ventricular cardiomyopathy caused by dominant desmoplakin mutations. *Eur Heart J.* 2005;26(16):1666-1675.

Beffagna G, Occhi G, Nava A, Vitiello L, Ditadi A, Basso C, Bauce B, Carraro G, Thiene G, Towbin JA, Danieli GA, Rampazzo A. Regulatory mutations in transforming growth factor-beta 3 gene cause arrhythmogenic right ventricular cardiomyopathy type1. *Cardiovasc Res.* 2005;65(2):366-373.

Calkins CC, Setzer SV, Jennings JM, Summers S, Tsunoda K, Amagai M, Kowalczyk AP. Desmoglein endocytosis and desmosome disassembly are coordinated responses to pemphigus autoantibodies. *J Biol Chem*. 2006;281(11):7623-7634.

Chen AE, Ginty DD, Fan CM. Protein kinase A signalling via CREB controls myogenesis induced by Wnt proteins. *Nature*. 2005;433(7023):317-322.

Chen L, Chetkovich DM, Petralia RS, Sweeney NT, Kawasaki Y, Wenthold RJ, Bredt DS, Nicoll RA. Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. *Nature.* 2000;408(6815):936-943.

Chen X, Bonne S, Hatzfeld M, van Roy F, Green KJ. Protein binding and functional characterization of plakophilin 2. Evidence for its diverse roles in desmosomes and beta-catenin signaling. *J Biol Chem*. 2002;277(12):10512-10522.

Choi HJ, Park-Snyder S, Pascoe LT, Green KJ, Weis WI. Structures of two intermediate filament-binding fragments of desmoplakin reveal a unique repeat motif structure. *Nat Struct Biol*. 2002;9(8):612-620.

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(6):2979-2984.

Coonar AS, Protonotarios N, Tsatsopoulou A, Needham EW, Houlston RS, Cliff S, Otter MI, Murday VA, Mattu RK, McKenna WJ. Gene for arrhythmogenic right ventricular cardiomyopathy with diffuse nonepidermolytic palmoplantar keratoderma and woolly hair (Naxos disease) maps to 17q21. *Circulation*. 1998;97(20):2049-2058.

Corrado D, Fontaine G, Marcus FI, McKenna WJ, Nava A, Thiene G, Wichter T. Arrhythmogenic right ventricular dysplasia/cardiomyopathy: Need for an international registry. Study Group on Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy of the Working Groups on Myocardial and Pericardial Disease and Arrhythmias of the European Society of Cardiology and of the Scientific Council on Cardiomyopathies of the World Heart Federation. *Circulation*. 2000;101(11):E101-E106.

D'Souza-Schorey C. Disassembling adherens junctions: breaking up is hard to do. *Trends Cell Biol*. 2005;15(1):19-26.

Dusek RL, Getsios S, Chen F, Park JK, Amargo EV, Cryns VL, Green KJ. The differentiation-dependent desmosomal cadherin desmoglein 1 is a novel caspase-3 target that regulates apoptosis in keratinocytes. *J Biol Chem*. 2006;281(6):3614-3624.

Flores ER, Tsai KY, Crowley D, Sengupta S, Yang A, McKeon F, Jacks T. p63 and p73 are required for p53-dependent apoptosis in response to DNA damage. *Nature*. 2002;416(6880):560-564.

Gannier F, White E, Garnier D, Le Guennec JY. A possible mechanism for large stretch-induced increase in $[Ca^{2+}]i$ in isolated guinea-pig ventricular myocytes. *Cardiovasc Res*. 1996;32(1):158-167.

Garcia-Gras E, Lombardi R, Giocondo MJ, Willerson JT, Schneider MD, Khoury DS, Marian AJ. Suppression of canonical Wnt/beta-catenin signaling by nuclear plakoglobin recapitulates phenotype of arrhythmogenic right ventricular cardiomyopathy. *J Clin Invest*. 2006;116(7):2012-2021.

Garrod D, Chidgey M. Desmosomes structure, composition and function. *Biochim Biophys Acta*. 2007. Article in press.

Gehring NH, Frede U, Neu-Yilik G, Hundsdoerfer P, Vetter B, Hentze MW, Kulozik AE. Increased efficiency of mRNA 3'end formation: a new genetic mechanism contributing to hereditary thrombophilia. *Nat Genet.* 2001;28(4):389-392.

Gerull B, Heuser A, Wichter T, Paul M, Basson CT, McDermott DA, Lerman BB, Markowitz SM, Ellinor PT, MacRae CA, Peters S, Grossmann KS, Drenckhahn J, Michely B, Sasse-Klaassen S, Birchmeier W, Dietz R, Breithardt G, Schulze-Bahr E, Thierfelder L. Mutations in the desmosomal protein plakophilin-2 are common in arrhythmogenic right ventricular cardiomyopathy. *Nat Genet*. 2004;36(11):1162-1164.

Getsios S, Huen AC, Green KJ. Working out the strength and flexibility of desmosomes. *Nat Rev Mol Cell Biol*. 2004; 5(4):271-281.

Girelli D, Corrocher R, Bisceglia L, Olivieri O, Zelante L, Panozzo G, Gasparini P. Hereditary hyperferritinemia-cataract syndrome caused by a 29-base pair deletion in the iron responsive element of ferritin L-subunit gene. *Blood*. 1997;90(5):2084-2088.

Green KJ, Gaudry CA. Are desmosomes more than tethers for intermediate filaments? *Nat Rev Mol Cell Biol.* 2000;1(3):208-216.

Green KJ, Sympson CL. Desmosomes: new perspectives on a classic. *J Invest Dermatol.* 2007; 127(11):2499-2515.

Grossmann KS, Grund C, Huelsken J, Behrend M, Erdmann B, Franke WW, Birchmeier W. Requirement of plakophilin 2 for heart morphogenesis and cardiac junction formation. *J Cell Biol*. 2004;167(1):149-160.

Heuser A, Plovie ER, Ellinor PT, Grossmann KS, Shin JT, Wichter T, Basson CT, Lerman BB, Sasse-Klaassen S, Thierfelder L, MacRae CA, Gerull B. Mutant desmocollin-2 causes arrhythmogenic right ventricular cardiomyopathy. *Am J Hum Genet.* 2006;79(6):1081-1088.

Huber O. Structure and function of desmosomal proteins and their role in development and disease. *Cell Mol Life Sci.* 2003;60(9):1872-1890.

Hunt DM, Sahota VK, Taylor K, Simrak D, Hornigold N, Arnemann J, Wolfe J, Buxton RS. Clustered cadherin genes: a sequence-ready contig for the desmosomal cadherin locus on human chromosome 18. *Genomics.* 1999;62(3):445-455.

Ihrie RA, Reczek E, Horner JS, Khachatrian L, Sage J, Jacks T, Attardi LD. Perp is a mediator of p53-dependent apoptosis in diverse cell types. *Curr Biol*. 2003;13(22):1985-1990.

Ihrie RA, Marques MR, Nguyen BT, Horner JS, Papazoglu C, Bronson RT, Mills AA, Attardi LD. Perp is a p63-regulated gene essential for epithelial integrity. *Cell*. 2005;120(6):843-856.

Ishikawa K, Toru S, Tsunemi T, Li M, Kobayashi K, Yokota T, Amino T, Owada K, Fujigasaki H, Sakamoto M, Tomimitsu H, Takashima M, Kumagai J, Noguchi Y, Kawashima Y, Ohkoshi N, Ishida G, Gomyoda M, Yoshida M, Hashizume Y, Saito Y, Murayama S, Yamanouchi H, Mizutani T, Kondo I, Toda T, Mizusawa H. An autosomal dominant cerebellar ataxia linked to chromosome 16q22.1 is associated with a single-nucleotide substitution in the 5' untranslated region of the gene encoding a protein with spectrin repeat and Rho guanine-nucleotide exchange-factor domains. *Am J Hum Genet*. 2005;77(2):280-296.

Izumi G, Sakisaka T, Baba T, Tanaka S, Morimoto K, Takai Y. Endocytosis of E-cadherin regulated by Rac and Cdc42 small G proteins through IQGAP1 and actin filaments. *J Cell Biol*. 2004;166(2):237-248.

Jetten AM, Suter U. The peripheral myelin protein 22 and epithelial membrane protein family. *Prog Nucleic Acid Res Mol Biol*. 2000;64:97-129.

Kapoun AM, Liang F, O'Young G, Damm DL, Quon D, White RT, Munson K, Lam A, Schreiner GF, Protter AA. B-type natriuretic peptide exerts broad functional opposition to transforming growth factor-beta in primary human cardiac fibroblasts: fibrosis, myofibroblast conversion, proliferation and inflammation. *Circ Res*. 2004;94(4):453-461.

Knöll R, Hoshijima M, Chien K. Cardiac mechanotransduction and implications for heart disease. *J Mol Med*. 2003;81(12):750-756.

Ko K, Arora P, Lee W, McCulloch C. Biochemical and functional characterization of intercellular adhesion and gap junctions in fibroblasts. *Am J Physiol Cell Physiol*. 2000;279(1):C147-C157.

Kowalczyk AP, Reynolds AB. Protecting your tail: regulation of cadherin degradation by p120-catenin. *Curr Opin Cell Biol*. 2004;16(5):522-527.

Laimer M, Klausegger A, Aberer W, Oender K, Steinhuber M, Lanschuetzer CM, Wally V, Hintner H, Bauer JW. Haploinsufficiency due to deletion within the 3'-UTR of C1-INH-gene associated with hereditary angioedema. *Genet Med*. 2006; 8(4):249-254.

Laitinen PJ, Brown KM, Piippo K, Swan H, Devaney JM, Brahmbhatt B, Donarum EA, Marino M, Tiso N, Viitasalo M, Toivonen L, Stephan DA, Kontula K. Mutations of the cardiac ryanodine receptor (RyR2) gene in familial polymorphic ventricular tachycardia. *Circulation*. 2001;103(4):485-490.

Laitinen PJ, Swan H, Kontula K. Molecular genetics of exercise-induced polymorphic ventricular tachycardia: identification of three novel cardiac ryanodine receptor mutations and two common calsequestrin 2 amino-acid polymorphisms. *Eur J Hum Genet*. 2003;11(11):888-891.

Le TL, Joseph SR, Yap AS, Stow JL. Protein kinase C regulates endocytosis and recycling of E-cadherin. *Am J Physiol Cell Physiol*. 2002;283(2):C489-C499.

Le TL, Yap AS, Stow JL. Recycling of E-cadherin: a potential mechanism for regulating cadherin dynamics. *J Cell Biol*. 1999;146(1):219-232.

MacRae CA, Birchmeier W, Thierfelder L. Arrhytmogenic right ventricular cardiomyopathy: moving toward mechanism. *J Clin Invest*. 2006;116(7):1825-1827.

Marcus FI, Fontaine GH, Guiraudon G, Frank R, Laurenceau JL, Malergue C, Grosgogeat Y. Right ventricular dysplasia: a report of 24 adult cases. *Circulation*. 1982;65(2):384-398.

Marcus FI, Nava A, Thiene G. In: Arrhythmogenic RV cardiomyopathy/displasia. Recent advances. Springer, 2007: pp.7-12.

Marques MR, Ihrie RA, Horner JS, Attardi LD. The requirement for perp in postnatal viability and epithelial integrity reflects an intrinsic role in stratified epithelia. *J Invest Dermatol*. 2006;126(1):69-73.

Mazumder B, Seshadri V, Fox PL. Translational control by 3'UTR: the ends specify the means. *Trends Biochem Sci*. 2003;28(2):91-98.

McKenna WJ, Thiene G, Nava A, Fontaliran F, Blomstrom-Lundqvist C, Fontaine G, Camerini F. 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(3):215-218.

McKoy G, Protonotarios N, Crosby A, Tsatsopoulou A, Anastasakis A, Coonar A, Norman M, Baboonian C, Jeffery S, McKenna WJ. Identification of a deletion in plakoglobin in arrhythmogenic right ventricular cardiomyopathy with palmoplantar keratoderma and woolly hair (Naxos disease). *Lancet.* 2000;355(9221):2119-2124.

Mertens C, Kuhn C, Franke WW. Plakophilins 2a and 2b: constitutive proteins of dual location in the karyoplasm and the desmosomal plaque. *J Cell Biol*. 1996;135(4):1009-1025.

Mertens C, Hofmann I, Wang Z, Teichmann M, Sepehri Chong S, Schnölzer, Franke WW. Nuclear particles containing RNA polymerase III complexes associated with the junctional plaque protein plakophilin 2. *Proc Natl Acad Sci U S A*. 2001;98(14):7795-7800.

Missiaen L, Robberecht W, van den Bosch L, Callewaert G, Parys JB, Wuytack F, Raeymaekers L, Nilius B, Eggermont J, De Smedt H. Abnormal intracellular ca(2+)homeostasis and disease. *Cell Calcium*. 2000;28(1):1-21.

Mitchell P, Tollervey D. mRNA stability in eukaryotes. *Curr Opin in Genet Develop*. 2000;10(2):193-198.

Nava A, Rossi L, Thiene G. In: Arrhythmogenic right ventricular cardiomyopathy/Displasia. Elsevier, 1997: pp 71-86.

Nava A, Thiene G, Canciani B, Scognamiglio R, Daliento L, Buja G, Martini B, Stritoni P, Fasoli G. Familial occurrence of right ventricular dysplasia: a study involving nine families. *J Am Coll Cardiol*. 1988;12(5):1222-1228.

Nava A, Bauce B, Basso C, Muriago M, Rampazzo A, Villanova C, Daliento L, Buja G, Corrado D, Danieli GA, Thiene G. Clinical profile and long-term follow-up of 37 families with arrhythmogenic right ventricular cardiomyopathy. *J Am Coll Cardiol*. 2000;36(7):2226-2233.

Nemec J, Edwards BS, Osborn MJ, Edwards WD. Arrhythmogenic right ventricular dysplasia masquerading as dilated cardiomyopathy. *Am J Cardiol.* 1999;84(2):237-239.

Nishikawa T, Ishiyama S, Nagata M, Sakomura Y, Nakazawa M, Momma K, Hiroe M, Kasajima T. Programmed cell death in the myocardium of arrhythmogenic right ventricular cardiomyopathy in children and adults. *Cardiovasc Pathol*. 1999;8(8):185-189.

Norgett EE, Hatsell SJ, Carvajal-Huerta L, Cabezas JC, Common J, Purkis PE, Whittock N, Leigh IM, Stevens HP, Kelsell DP. Recessive mutation in desmoplakin disrupts desmoplakin-intermediate filament interactions and causes dilated cardiomyopathy, woolly hair and keratoderma. *Hum Mol Genet*. 2000;9(18):2761-2766.

Norman M, Simpson M, Mogensen J, Shaw A, Hughes S, Syrris P, Sen-Chowdhry S, Rowland E, Crosby A, McKenna WJ. Novel mutation in desmoplakin causes arrhythmogenic left ventricular cardiomyopathy. *Circulation*. 2005;112(5):636-642.

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(1):69-74.

Ozawa M, Kemler R. Correct proteolytic cleavage is required for the cell adhesive function of uvomorulin. *J Cell Biol.* 1990;111(4):1645-1650.

Pilichou K, Nava A, Basso C, Beffagna G, Bauce B, Lorenzon A, Frigo G, Vettori A, Valente M, Towbin J, Thiene G, Danieli GA, Rampazzo A. Mutations in desmoglein-2 gene are associated with arrhythmogenic right ventricular cardiomyopathy. *Circulation*. 2006;113(9):1171-1179.

Piri N, Gao YQ, Danciger M, Mendoza E, Fishman GA, Farber DB. A substitution of G to C in the cone cGMP-phosphodiesterase gamma subunit gene found in a distinctive form of cone dystrophy. *Ophthalmology*. 2005;112(1):159-166.

Polesskaya A, Seale P, Rudnicki MA. Wnt signaling induces the myogenic specification of resident CD45+ adult stem cells during muscle regeneration. *Cell*. 2003;113(7):841-852.

Priori SG, Napolitano C, Tiso N, Memmi M, Vignati G, Bloise R, Sorrentino V, Danieli GA. Mutations in the cardiac ryanodine receptor gene (hRyR2) underlie catecholaminergic polymorphic ventricular tachycardia. *Circulation*. 2001;103(2):196-200.

Priori SG, Napolitano C, Memmi M, Colombi B, Drago F, Gasparini M, DeSimone L, Coltorti F, Bloise R, Keegan R, Cruz Filho FE, Vignati G, Benatar A, DeLogu A. Clinical and molecular characterization of patients with catecholaminergic polymorphic ventricular tachycardia. *Circulation*. 2002;106(1):69-74.

Rampazzo A, Nava A, Danieli GA, Buja G, Daliento L, Fasoli G, Scognamiglio R, Corrado D, Thiene G. The gene for arrhythmogenic right ventricular cardiomyopathy maps to chromosome 14q23-q24. *Hum Mol Genet*. 1994;3():959-962.

Rampazzo A, Nava A, Malacrida S, Beffagna G, Bauce B, Rossi V, Zimbello R, Simionati B, Basso C, Thiene G, Towbin JA, Danieli GA. Mutation in human desmoplakin domain binding to plakoglobin causes a dominant form of arrhythmogenic right ventricular cardiomyopathy. *Am J Hum Genet*. 2002;71(5):1200-1206.

Ross SE, Hemati N, Longo KA, Bennett CN, Lucas PC, Erickson RL, MacDougald OA. Inhibition of adipogenesis by Wnt signaling. *Science*. 2000;289(5481):950-953.

Rubenstein A, Merriam J, Klymkowsky MW. Localizing the adhesive and signaling functions of plakoglobin. *Dev Genet*. 1997;20(2):91-102.

Schäfer S, Koch PJ, Franke WW. Identification of the ubiquitous human desmoglein, DSG2, and the expression catalogue of the desmoglein subfamily of the desmosomal cadherins. *Exp Cell Res*. 1994;211(2):391-399.

Shan W, Yagita Y, Wang Z, Koch A, Svenningsen AF, Gruzglin E, Pedraza L, Colman DR. The minimal essential unit for cadherin-mediated intercellular adhesion comprises extracellular domains 1 and 2. J *Biol Chem*. 2004;279(53):55914-55923.

Stokes DL, Wagenknecht T. Calcium transport across the sarcoplasmic reticulum: structure and function of Ca2+-ATPase and the ryanodine receptor. *Eur J Biochem*. 2000;267(17):5274-5279.

Syrris P, Ward D, Asimaki A, Sen-Chowdhry S, Ebrahim HY, Evans A, Hitomi N, Norman M, Pantazis A, Shaw AL, Elliott PM, McKenna WJ. Clinical expression of plakophilin-2 mutations in familial arrhythmogenic right ventricular cardiomyopathy. *Circulation*. 2006;113(3):356-364.

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(5):978-984.

Takeichi M. Cadherins: a molecular family important in selective cell-cell adhesion. *Ann Rev Biochem.* 1990;59:237-252.

Tatsukawa Y, Kiyosue T, Arita M. Mechanical stretch increases intracellular calcium concentration in cultured ventricular cells from neonatal rats. *Heart Vessels.* 1997;12(3):128-135.

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(3):129-133.

Tiso N, Stephan DA, Nava A, Bagattin A, Devaney JM, Stanchi F, Larderet G, Brahmbhatt B, Brown K, Bauce B, Muriago M, Basso C, Thiene G, Danieli GA, Rampazzo A. 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(3):189-194.

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(4):561-574.

Tsukita S, Furuse M. Claudin-based barrier in simple and stratified cellular sheets. *Curr Opin Cell Biol*. 2002;14(5):531-536.

Van Tintelen JP, Entius MM, Bhuiyan ZA, Jongbloed R, Wiesfeld AC, Wilde AA, van der Smagt J, Boven LG, Mannens MM, van Langen IM, Hofstra RM, Otterspoor LC, Doevendans PA, Rodriguez LM, van Gelder IC, Hauer RN. Plakophilin-2 mutations are the major determinant of familial arrhythmogenic right ventricular dysplasia/cardiomyopathy. *Circulation.* 2006;113(13):1650-1658.

Warren ST, Nelson DL. Advances in molecular analysis of fragile X syndrome. *JAMA*. 1994;271(7):536-542.

Whittock NV, Ashton GH, Dopping-Hepenstal PJ, Gratian MJ, Keane FM, Eady RA, McGrath JA. Striate palmoplantar keratoderma resulting from desmoplakin haploinsufficiency. *J Invest Dermatol*. 1999;113(6):940-946.

Whittock NV, Wan H, Morley SM, Garzon MC, Kristal L, Hyde P, McLean WH, Pulkkinen L, Uitto J, Christiano AM, Eady RA, McGrath JA. Compound heterozygosity for non-sense and mis-sense mutations in desmoplakin underlies skin fragility/woolly hair syndrome. *J Invest Dermatol*. 2002;118(2):232-238.

Windoffer R, Borchert-Stuhlträger M, Leube RE. Desmosomes: interconnected calcium-dependent structures of remarkable stability with significant integral membrane protein turnover. *J Cell Sci.* 2002;115(8):1717-1732.

Yamaji K, Fujimoto S, Ikeda Y, Masuda K, Nakamura S, Saito Y, Yutani C. Apoptotic myocardial cell death in the setting of arrhythmogenic right ventricular cardiomyopathy. *Acta Cardiol*. 2005;60(5):465-470.

Yang Z, Bowles NE, Scherer SE, Taylor MD, Kearney DL, Ge S, Nadvoretskiy VV, DeFreitas G, Carabello B, Brandon LI, Godsel LM, Green KJ, Saffitz JE, Li H, Danieli GA, Calkins H, Marcus F, Towbin JA. Desmosomal dysfunction due to mutations in desmoplakin causes arrhythmogenic right ventricular dysplasia/cardiomyopathy. *Circ Res.* 2006;99(6):646-655.

Yin T, Green KJ. Regulation of desmosome assembly and adhesion. *Semin Cell Dev Biol*. 2004;15(6):665-677.

Yoshida M, Romberger DJ, Illig MG, Takizawa H, Sacco O, Spurzem JR, Sisson JH, Rennard SI, Beckmann JD. Transforming growth factor-beta stimulates the expression of desmosomal proteins in bronchial epithelial cells. *Am J Respir Cell Biol*. 1992;6(4): 439-455.

# SUMMARY

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a genetically determined heart muscle disorder that presents clinically with ventricular arrhythmias, heart failure, and sudden death. The pathological process consists of progressive loss of ventricular myocardium with fibro-fatty replacement. Right ventricle is mostly involved, but presentation of the disease with predominantly left ventricular involvement has been reported. ARVC is typically inherited as a dominant disease, although recessive variants exist and the involvement of family members often can only be detected by molecular genetic analysis (low penetrance of mutations). Genetic studies over the last few years have offered insight into the potential causes of ARVC. Early works demonstrated substantial genetic heterogeneity, and at least 12 independent loci and 7 disease-genes have now been identified. These findings also implicated desmosomal proteins or proteins involved in desmosomal function as candidate causes of the disorder.

In the present study *PERP* was investigated as a candidate gene for ARVC because of its possible role in cell-cell adhesion, as a structural constituent of desmosomes or as a protein playing an yet unknown role in desmosome assembly. After *PERP* human cardiac expression was tested and confirmed, 90 ARVC index cases were screened for *PERP* mutations by DHPLC analysis and direct sequencing. Two variations G59R in exon1 and c.1091C>T in 3'UTR were detected in two patients, in whom a mutation in a known ARVC gene was previously identified. The missense variation G59R was detected in 1 control out of 250 screened and the variation c.1091C>T was identified in 2 controls out of 192 screened. Moreover these two novel variants involved respectively a highly conserved amino acid and a highly conserved nucleotide. Interestingly index cases, carrying two mutations (one in *PERP* gene and one in a known ARVC gene), showed a more severe phenotype than family members carriers for only one of these variations. It is impossible to establish whether these single *PERP* mutations might lead to ARVC determination, but on the other hands, in patients carrying a pathogenic mutation in a different gene involved in ARVC, *PERP* mutations might worsen the clinical phenotype.

The idea that ARVC is due to desmosomal dysfunction was strengthened by two recent studies that reported mutations in the desmosomal desmocollin-2 (*DSC2*) gene as the cause of ARVC. During the present study six different *DSC2* mutations were identified in seven out of sixty-four ARVC unrelated Italian index cases.

Two nucleotide substitutions (c.-92G>T and c.3241A>T) in 5' and 3' UTR regions were detected in two different index cases. Neither of the nucleotide changes were found in 300 chromosomes from the same population, but to exclude that these mutations could correspond to rare polymorphisms the size of the control group should be increased to 500. Moreover in order to test whether this UTR mutations could affect the expression levels of *DSC2* gene, specific in vitro functional studies are needed.

Another nucleotide substitution (c.348A>G) absent among 500 control chromosomes, was detected in exon 3; although this mutation corresponds to a synonymous variation (Q116Q), it has been demonstrated that it creates a cryptic splice site, leading to a deletion of 9 nucleotides. Although skipping of 9bp in the mutant transcript doesn't alter the reading frame of DNA sequence, at protein level it leads to loss of three amino acids very conserved among species. This mutation mapped on a region important for maturation of the protein.

Two heterozygous point substitutions c.304G>A and c.1034T>C were detected in other two patients. Both nucleotide changes was never found in 250 unrelated controls (500 control chromosomes). Variations c.304G>A in exon 3 and c.1034T>C in exon 8 result in predicted p.E102K and p.I345T amino acid substitutions. The mutated amino acids had completely different physico-chemical properties when compared to the wild type. Both these changes occurred in a residue highly conserved among species and are located in protein regions involved on DSC2 adhesion function.

The sixth mutation c.2687_2688insGA in exon 17 was detected in two different patients and in six control subjects, suggesting the possibility of a polymorphism. This mutation would affect the C terminus of DSC2a, precisely the ICS domain, by altering 4 aa residues before a termination codon is prematurely introduced. The change occurred in the last five aa residues of the protein, which are non conserved among mammals, in contrast with the high conservation of the upstream region.

The final part of this thesis work was focused on the analysis of potential pathogenic effects of the last three *DSC2* mutations described above in cultured cardiomyocytes. Once human cDNAs coding for wild type, two polymorphic variants and mutant proteins were obtained, constructs containing also GFP protein were expressed by transient transfection of HL-1 cell line. In transfected HL-1 cells, wild type protein and the two polymorphic variants were detected in the cell membrane, into cell-cell contact regions since co-localised with the endogenous desmoglein which was marked with a monoclonal dsg antibody. In contrast the three mutant proteins were almost exclusively

distributed throughout the cytoplasm with very scarce cell membrane localisation, affecting the normal localisation of DSC2 and suggesting the potential pathogenic effect of the mutations.

# RIASSUNTO

La cardiomiopatia aritmogena del ventricolo destro (ARVC) è una malattia del muscolo cardiaco, determinata geneticamente, che si presenta a livello clinico con aritmie ventricolari, insufficienza cardiaca e morte improvvisa. Il processo patologico consiste nella perdita progressiva del miocardio ventricolare con sostituzione fibroadiposa. E' maggiormente implicato il ventricolo destro, ma sono stati riportati casi con coinvolgimento predominante del ventricolo sinistro. L'ARVC tipicamente è ereditata come malattia dominante, sebbene esistano varianti recessive e spesso la presenza della suscettibilità alla malattia in altri membri della famiglia possa essere rivelata solo da un'analisi del DNA (bassa penetranza delle mutazioni). Negli ultimi anni, gli studi di genetica molecolare hanno consentito di individuare alcuni geni implicati nella patogenesi dell'ARVC. Fin dai primi lavori è risultata evidente una forte eterogeneità genetica; finora sono stati identificati dodici loci indipendenti e per sette di questi sono stati identificati i geni in questione. Queste scoperte hanno inoltre indicato che il difetto primario spesso riguarda proteine desmosomali o proteine coinvolte nel funzionamento del desmosoma.

Nel presente studio *PERP* è stato analizzato come gene candidato per l'ARVC per il suo possibile coinvolgimento nell'adesione cellula-cellula, sia come un probabile costituente strutturale del desmosoma sia come una proteina che gioca un ruolo, non ancora noto, nell'assemblaggio del desmosoma. Dopo aver confermato che nell' uomo *PERP* è espresso anche nel miocardio, sono state cercate mutazioni nel gene *PERP* in novanta casi indice affetti da ARVC, utilizzando analisi DHPLC e sequenziamento diretto del DNA. Sono state identificate in due pazienti due variazioni, rispettivamente G59R nell'esone1 e c.1091C>T nel 3'UTR; in questi pazienti era stata tuttavia identificata in precedenza un'altra mutazione patogena in un gene diverso. La variazione missenso G59R è stata identificata in un controllo su 250 analizzati e la variazione c.1091C>T è stata identificata in due controlli su 192. Queste due nuove varianti coinvolgono rispettivamente un amino acido altamente conservato e un nucleotide altamente conservato. E' interessante notare che i casi indice, portatori di due mutazioni (una nel gene *PERP* e una in un gene noto ARVC) mostrano un fenotipo più grave rispetto ai membri della famiglia portatori di una sola di queste variazioni. Per il momento è impossibile stabilire se queste mutazioni di *PERP* da sole possano portare alla manifestazione dell'ARVC; ma mutazioni di *PERP* in pazienti già portatori di una

mutazione patogena in un diverso gene coinvolto nell'ARVC potrebbero peggiorare il fenotipo clinico.

L'idea che l'ARVC sia dovuta a disfunzioni dei desmosomi è stata rafforzata da due studi recenti che riportano mutazioni nel gene desmosomale desmocollina-2 (*DSC2*) come causa dell'ARVC. Durante il presente studio sei diverse mutazioni nel gene *DSC2* sono state identificate in sette su sessantaquattro casi indice italiani affetti da ARVC e non imparentati tra loro.

Due sostituzioni nucleotidiche, c.-92G>T e c.3241A>T nelle regioni 5' e 3' UTR, sono state identificate in due diversi casi indice. Nessuno dei due cambiamenti nucleotidici è stato trovato in 300 cromosomi della stessa popolazione, ma per escludere che queste mutazioni siano dei polimorfismi rari, la grandezza del campione di controllo dovrebbe essere aumentata almeno a 500 individui. Inoltre saranno necessari specifici studi funzionali in vitro allo scopo di testare se queste mutazioni UTR possano influenzare i livelli di espressione del gene *DSC2*.

Un'altra sostituzione nucleotidica c.348A>G, assente in 500 cromosomi di controllo, è stata identificata nell'esone 3; sebbene questa mutazione corrisponda ad una variazione sinonima (Q116Q), si è dimostrato che essa crea un sito criptico di splicing portando alla delezione di 9 nucleotidi. Anche se la perdita di nove paia di basi nel trascritto mutato non altera il frame di lettura della sequenza del DNA, a livello proteico porta alla perdita di tre amino acidi molto conservati tra le specie. Questa mutazione mappa in una regione importante per la maturazione della proteina.

Due sostituzioni puntiformi in eterozigosi c.304G>A e c.1034T>C sono state identificate in altri due pazienti. Entrambi i cambiamenti nucleotidici non sono mai stati identificati in 250 controlli non imparentati tra loro (500 cromosomi di controllo). La variazione c.304G>A nell'esone 3 e c.1034T>C nell'esone 8 risultano in due sostituzioni aminoacidiche p.E102K e p.I345T. Gli amino acidi mutati hanno proprietà fisico-chimiche completamente diverse da quelle dell' aminoacido presente nei controlli. Entrambi questi cambiamenti aminoacidici coinvolgono un residuo altamente conservato tra le specie e mappano in regioni proteiche legate alla funzione adesiva della desmocollina-2.

La sesta mutazione c.2687_2688insGA nell'esone 17 è stata identificata in due diversi pazienti e in sei soggetti di controllo, facendo sorgere il sospetto che si tratti di un polimorfismo. Questa mutazione influenzerebbe il C-terminale della desmoscollina-2a, precisamente il dominio ICS, alterando quattro residui aminoacidici prima che un

codone di terminazione sia introdotto prematuramente. Il cambiamento riguarda gli ultimi cinque aminoacidi della proteina, che non sono conservati tra i mammiferi, al contrario della forte conservazione della regione proteica a monte.

La parte finale di questo lavoro di tesi si è focalizzata sull'analisi, in colture di cardiomiociti, dei potenziali effetti patogeni delle ultime tre mutazioni sopra descritte. Una volta ottenuti i cDNA umani di controllo, due varianti polimorfe e le proteine mutate, i costrutti contenenti anche la proteina GFP, sono stati fatti esprimere mediante trasfezione transiente nella linea cellulare HL-1. Nelle cellule HL-1 trasfettate la proteina di controllo e le due varianti polimorfe venivano individuate nella membrana cellulare in regioni di contatto cellula-cellula, in quanto co-localizzavano con la desmogleina endogena (dsg), marcata da un anticorpo monoclonale contro la dsg murina. Al contrario le tre proteine mutate erano quasi esclusivamente distribuite nel citoplasma con una scarsa localizzazione nella membrana cellulare, alterando la normale localizzazione della desmocollina-2, suggerendo quindi un potenziale effetto patogeno delle mutazioni.