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Abstract—Arrhythmogenic right ventricular dysplasia/cardioomyopathy (ARVD/C) is characterized by progressive degeneration of the right ventricular myocardium, ventricular arrhythmias, fibrous-fatty replacement, and increased risk of sudden death. Mutations in 6 genes, including 4 encoding desmosomal proteins (Junctional plakoglobin (JUP), Desmoplakin (DSP), Plakophilin 2, and Desmoglein 2), have been identified in patients with ARVD/C. Mutation analysis of 66 probands identified 4 variants in DSP; V30M, Q90R, W233X, and R2834H. To establish a cause and effect relationship between those DSP missense mutations and ARVD/C, we performed in vitro and in vivo analyses of the mutated proteins. Unlike wild-type (WT) DSP, the N-terminal mutants (V30M and Q90R) failed to localize to the cell membrane in desosome-forming cell line and failed to bind to and communoprecipitate JUP. Multiple attempts to generate N-terminal DSP (V30M and Q90R) cardiac-specific transgenes have failed: analysis of embryos revealed evidence of profound ventricular dilation, which likely resulted in embryonic lethality. We were able to develop transgenic (Tg) mice with cardiac-restricted overexpression of the C-terminal mutant (R2834H) or WT DSP. Whereas mice overexpressing WT DSP had no detectable histologic, morphological, or functional cardiac changes, the R2834H-Tg mice had increased cardiomyocyte apoptosis, cardiac fibrosis, and lipid accumulation, along with ventricular enlargement and cardiac dysfunction in both ventricles. These mice also displayed interruption of DSP-desmin interaction at intercalated discs (IDs) and marked ultra-structural changes of IDs. These data suggest DSP expression in cardiomyocytes is crucial for maintaining cardiac tissue integrity, and DSP abnormalities result in ARVD/C by cardiomyocyte death, changes in lipid metabolism, and defects in cardiac development. (Circ Res. 2006;99:646-655.)

Key Words: arrhythmogenic right ventricular dysplasia/cardioomyopathy ■ mechanical junctions ■ gene mutations

Arrhythmogenic right ventricular dysplasia (ARVD/C) is a complex myocardial disorder associated with right-sided cardiomyopathy and arrhythmia, and characterized by gradual loss of myocytes and replacement by fatty and fibrous tissue. It leads to dilation of the right ventricle (RV) and impaired cardiac function. In some patients, left ventricular (LV) involvement also occurs. The clinical course is characterized by ventricular arrhythmias (ventricular tachycardia), heart failure, syncope, and sudden death. In Italy, the prevalence has been reported to be 1:5000 population, accounting for 20% of sudden deaths in young adults and 25% of cardiac sudden deaths among athletes.

In familial cases of ARVD/C, autosomal dominant inheritance with reduced penetrance has been reported and accounts for ≈30% of cases. In the remaining sporadic cases it is believed to be due to an acquired etiology or to unidentified inheritance. To date, 10 genetic loci have been mapped for ARVD/C, and 6 genes have been identified. The first gene identified for autosomal dominant ARVD/C was the cardiac ryanodine receptor 2 (RyR2). In addition, 2 other genes responsible for Naxos Disease and Carvajal syndrome, complex autosomal recessive forms of ARVD/C with associated palmoplantar keratoderma and woolly hair have been identified as junctional plakoglobin (JUP) and desmoplakin (DSP),...
respectively. JUP and DSP are major components of desmosomes. Mutations in DSP have also been shown to cause autosomal dominant ARVD/C. More recently mutations in plakophilin 2 (PKP2), desmoglein 2 (DSG2), and the 5′ untranslated region (UTR) of transforming growth factor beta-3 (TGF-β3) have been reported in patients with ARVD/C.

In this study, we investigated the frequency of DSP mutations in an unselected patient cohort from the North American ARVD/C Registry, and studied the effects of those ARVD/C-associated mutations on DSP function. We have identified multiple mutations in DSP in ARVD/C patients and have demonstrated that DSP expression in cardiomyocytes is crucial for maintaining cardiac tissue integrity. In addition, we have showed that abnormalities of DSP can result in loss of function in vitro and lead to defects in cardiac development, cardiomyocyte death, changes in lipid metabolism, and ultra-structural changes in intercalated discs (IDs) when overexpressed in mice.

Materials and Methods

Clinical Diagnostic Criteria for ARVD/C
All studies on human subjects were performed in accordance with local IRB regulations after informed consent, as described in the supplement, available online at http://circres.ahajournals.org.

Mutation Detection of DSP Gene
Mutations in DSP were detected by direct DNA sequencing, as described in the online supplement.

Desmosome-Forming Cell Line and Transient Transfection
The human tongue squamous cell carcinoma cell line SCC-9 was transfected using Effectene (Qiagen). Protein expression was investigated by immunohistochemistry, as described in the online supplement.

Generation of Transgenic Mice
Cardiac-restricted DSP transgenic (Tg) mice were generated using vectors containing the mouse alpha-myosin heavy chain (α-MyHC) promoter (Dr Jeffrey Robbins, University of Cincinnati, Ohio) and full-length wild-type (WT) and mutant DSP cDNA sequences, as described in the online supplement. Histologic and functional characterizations of those Tg mice are described in the online supplement.

Statistical Analyses
Statistical significance was analyzed by the unpaired two-tailed t test using the statistical software GraphPad Prism 4 to compare data sets between any two different groups. Values are shown as mean ± SEM. Statistical significance was set at P<0.05.

Results

Mutation Analysis of the DSP Gene
Genetic variants in DSP were identified in 4 of the 66 probands (6%) enrolled in the North American ARVD/C Registry, comprising 1 nonsense and 3 missense substitutions. These substitutions included 88G>A (V30M), 269A>G (Q90R), 699G>A (W233X), and 8501G>A (R2834H) (Figure 1A), and none was identified in 200 ethnic matched control individuals (400 chromosomes).

The V30M and Q90R variations occur in the head region of the DSP protein, which is involved in binding to the linker proteins (JUP and PKPs) of desmosomes, and these abnormalities would be likely to affect these interactions and localization of DSP to desmosomes (Figure 1B). We could not detect the presence of the 699G>A (W233X) nucleotide change in the cDNA amplified from the lymphoblastoid cell line derived from patient blood. Therefore, we conclude that this nonsense substitution results in haploinsufficiency, probably due to increased decay of nonsense mRNA as previously described. R2834H would be likely to affect the DSP C terminus involved in the binding of DSP with intermediate filaments (Figure 1B). To test these hypotheses, we investi-
gated the effects of these missense substitutions in vitro and in vivo.

**Mutations in the N-Terminal DSP Result in Loss of Localization to Cell Membrane**

WT DSP NTP (N-terminal truncated DSP, first 584 aa, Flag tagged), V30M DSP NTP, Q90R DSP NTP, and W233X DSP (first 233 aa, GFP tagged) were expressed by transient transfection in the desmosome-forming cell line SSC-9 (Figure 2A). Whereas WT DSP NTP was localized to the cell membrane, both V30M and Q90R DSP NTP were mainly detected in the cytoplasm and did not accumulate at cell membranes (or cell junctions), suggesting that these two N-terminal DSP mutations affect the localization of DSP in vitro. Consistent with previous findings that demonstrated a severely truncated N-terminal DSP protein is unstable,19 transiently transfected N-terminal mutant W233X DSP formed perinuclear aggregates and was neither localized at the cell membrane nor seen diffusely in the cytoplasm (Figure 2A). In addition, we transiently transfected full length (FL) DSP (both WT and mutants including V30M, Q90R, and R2834H) in SCC-9 cells (Figure 2A). In the case of N-terminal mutants (V30M and Q90R DSP FL), no cell membrane localization was noted. In contrast, WT and R2834H DSP FL were found to be present at cell membranes, suggesting that the C-terminal mutation (R2834H) does not affect the function of DSP N terminus.

**N-Terminal DSP Mutants Can No Longer Bind JUP In Vitro**

To investigate the effects of these mutations on the interactions between DSP and JUP, PKP-1, and PKP-2, coimmuno-
precipitation studies were performed. JUP was found to be immunoprecipitated by WT DSP NTP as previously reported, but not by either V30M or Q90R DSP NTP, suggesting that V30M and Q90R disrupt the binding ability of DSP N terminus (Figure 2B). No changes were observed in the immunoprecipitation of PKP-1 or -2 (data not shown).

Overexpression of N-Terminal DSP Mutants in the Myocardium Results in Early Embryonic Lethality

We attempted to generate cardiac restricted Tg mice expressing mutant or WT full-length DSP in the myocardium under the control of the α-MyHC promoter (Figure 3A). Although lines expressing R2834H or WT DSP were successfully generated, no founders for V30M or Q90R-Tg mice were identified, despite repeated injections (supplemental Table I). We speculated that V30M and Q90R likely have adverse effects on embryonic development. Embryonic day (ED) 12.5 to 17.5 embryos were therefore isolated from newly injected pregnant mothers, and we found that there were no positive embryos after ED13.5. At ED13.5, hearts from V30M-Tg (from 4 founders) and Q90R-Tg (from 2 founders) showed severely reduced wall thickness (2 to 5 cell layers) and definitiveventricular dilation in comparison to R2834H-Tg, WT-Tg, and nontransgenic (NTG) littermates (10 to 12 cell layers; Figure 3B), suggesting cardiac development abnormalities with cardiac noncompaction and early embryonic lethality.

Mice Expressing R2834H DSP Display Cardiac Hypertrophy and Reduced Cardiac Function

We chose to compare the phenotypes of WT-Tg (from 2 founders) and R2834H-Tg lines (from 2 founders) with similar expression levels of DSP (2 to 3 times of the level of DSP in NTG littermates, determined by Western blot analysis of cardiac proteins). By 6 months of age, the R2834H-Tg mice (5.9±0.2×10^−3) had significantly increased heart weight:body weight ratios compared with NTG littermates (4.9±0.1×10^−3) and WT-Tg mice (5.0±0.1×10^−3) (n=7 per group; Figure 3C). In addition, histological analysis indicated that ventricular cardiomyocyte cross-section areas were 40% higher in R2834H-Tg mice (183.4±1.4 μm^2) compared with WT-Tg (132.4±1.5 μm^2) and NTG mice.

Figure 3. Generation of cardiac-specific WT and mutant DSP Tg mice. A, Schematic representation of construct to generate the DSP Tg mice. The α-MyHC promoter was used to drive the expression of human full-length DSP (WT or mutant) cDNA, tagged at the C terminus with Flag. B, Early embryonic lethality in Tg mice overexpressing N-terminal DSP mutants. ED13.5 embryonic hearts from V30M-Tg, Q90R-Tg, R2834H-Tg, WT-Tg, and NTG mice are shown. Note severely reduced ventricular wall thickness and ventricular dilation in V30M-Tg and Q90R-Tg mice compared with other groups. Boxed areas are shown at higher magnification, with the black bars illustrating the width of the ventricular wall thickness. L indicates the lumen of the heart. (Sections were stained with H&E; from the left to the right: 40× and 400×, respectively) C, Cardiomyocyte cross-section areas in R2834H-Tg mice (quantified using Image-Pro Plus). (**P<0.01)
(129.7±1.5 μm²; n=3 per group, >1000 determinations per animal; Figure 3D). This was accompanied by significant loss of LV function as determined by echocardiographic measurement (n=5 per group) of fractional shortening (FS), significantly increased left ventricular end systolic dimension (LVESD), and LV mass:body weight ratio. No statistical significance is observed in left ventricular end diastolic dimension (LVEDD). (*P<0.03, **P<0.01) C, Representative MRI images of 9-month-old mice. Short axis images in diastole were shown. Note the dilated and thin walled RV in R2834H-Tg mice. D, MRI measurements. R2834H-Tg mice demonstrate significantly increased end diastolic volume (EDV) of both ventricles, significantly reduced LV and RV ejection fraction (EF), and significantly reduced RV thickness and RV thickening. (*P<0.05, **P<0.01, ***P<0.001)

**Increased Cardiac Fibrosis, Cardiomyocyte Apoptosis, and Lipid Accumulation in R2834H-Tg Mice**

Histologic studies using H&E and Masson trichrome staining from multiple sections of the heart from at least 10 mice per group revealed focal myocyte loss and replacement by fibrous tissue in both ventricles (primarily in the LV) of R2834H-Tg mice, whereas WT-Tg mice had normal cardiac histology (Figure 5A through 5B). In addition, neutral lipid accumulation was focally observed in the cardiomyocytes of R2834H-Tg mice by Oil Red O staining (Figure 5C through 5D). Analysis for apoptosis using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) indicated significantly increased cardiomyocyte apoptosis in R2834H-Tg mice with the proportion of positively stained nuclei estimated as 11.4±0.9‰ in these mice versus 1.4±0.9‰ in WT-Tg mice and 0.7±0.6‰ in NTG littermates (n=4 per group, >1000 nuclei per animal were counted; Figure 5E through 5F).

**The R2834H DSP Mutation Results in Aberrant Intermediate Filament Localization**

We hypothesized that the C-terminal mutation (R2834H) could affect the interaction between DSP and desmin filaments. In support of this hypothesis, there was less desmin detected at the IDs of R2834H-Tg mice, even though DSP and the intracellular distribution of desmin (at Z-disks) appeared normal (Figure 6A). Significantly reduced colocalization efficiency (Pearson correlation value) of DSP and desmin at IDs was noted in R2834H-Tg mice (0.23±0.02) in comparison to WT-Tg (0.69±0.01) and NTG mice.
Ultra-Structural Changes of IDs in R2834H-Tg Mice

Electron microscopic evaluation of the myocardium from 9-month-old mice was performed, mainly focusing on the cell junctions. In R2834H-Tg mice, irregularly shaped IDs with markedly widened gaps between adjacent anchoring sarcomeres were noted, affecting both the adherens junctions and desmosomes. No lipid accumulation was identified in the sections examined, and no other cardiomyopathic changes were observed. In WT-Tg mice, electron dense materials were present in the IDs but not evident in Z-lines (Figure 7).

The R2834H DSP Mutation Results in Changes of Expression and Localization of Junctional Proteins

As previously reported, large proportions of junctional proteins (desmosomes and adherens junctions) are insoluble in extraction buffer containing nondenaturing detergents such as Triton X-100, because those proteins are associated with the cytoskeleton. Therefore, we performed Western blot analysis on total lysate, the Triton-X insoluble fraction (associated with the cytoskeleton), and the soluble fraction (not associated with cytoskeleton) of proteins isolated from Tg mouse hearts to assess the relative expression level and solubility of junctional proteins (Figure 8). As expected, overexpression of Flag-tagged DSP was noted in both WT-Tg and R2834H-Tg mice. Desmin expression was also increased in both R2834H-Tg and WT-Tg mice. Notably, large amount of JUP was present in the soluble fraction of R2834H-Tg mice by comparison with WT-Tg (small amounts) and NTG mice (almost undetectable). Furthermore, the expression of PKP2 and beta-catenin were moderately upregulated in R2834H-Tg mice, especially in the soluble fraction. Although the total amount of CX43 appeared unchanged in R2834H-Tg mice, there was a redistribution of CX43 into the soluble fraction. Those data suggest disassociation of a number of junctional proteins from cell-cell junctions in R2834H Tg mice (Figure 8).

Discussion

ARVD/C is a complex disorder with clinical manifestations including ventricular arrhythmias, and specific structural changes (fibrous-fatty tissue replacement) involving RV more often than LV, leading to heart failure and cardiac sudden death. Due to the clinical features of the disease, diagnosis is difficult and treatments are limited. Therefore, understanding the underlying mechanism of the development of ARVD/C would facilitate both diagnosis and treatment of the disease.

To date, mutations in 6 genes (RYR2, JUP, DSP, PKP2, DSG2, and TGF-β3) have been identified in patients with ARVD/C. The first gene identified was RYR2 for ARVD/C type 2. However, mutations in RYR2 have been shown to be
the major cause of catecholaminergic polymorphic ventricular tachycardia (CPVT), and it has been speculated that ARVD/C type 2 is a forme fruste of CPVT. Of the remaining 5 genes, 4 encode desmosomal proteins (JUP, DSP, PKP2, and DSG2). It has been reported that TGF-β3, the only one of these genes not encoding a desmosomal protein, regulates the expression of desmosomal genes including JUP and PKP2.

Therefore, desmosomal protein dysfunction appears to be a “final common pathway” playing an important role in the development of ARVD/C.

Desmosomes are specialized cell–cell adhesion structures abundant in tissues (primarily skin and heart) undergoing high mechanical stress, connecting intermediate filaments to the cell membrane. There are 3 major groups of proteins in desmosomes: transmembrane proteins (or desmosomal cadherins) including desmocollins (DSCs) and DSGs; linker proteins (armadillo family proteins), JUP and PKPs; and DSP. JUP might serve dual roles as junctional and nuclear proteins (transcriptional cofactors of Tcf/Lef family in the canonical Wnt signaling pathway, with downstream targets involved in cell survival or adipogenesis). Deficiency of JUP, PKP2, or DSP in mice can lead to defects in heart morphogenesis and embryonic lethality due to disruption of desmosomes. DSP plays an important role in maintaining the integrity of desmosomes by binding to other desmosomal components through its N terminus and to intermediate filaments (desmin in the heart) through its C terminus.

In this study we have identified 4 novel mutations in DSP in 6% of the ARVD/C patients studied. We characterized the effects of these mutations on DSP function through a number of in vitro and in vivo studies. Our studies demonstrate that a nonsense mutation in the N terminus of DSP (W233X) can lead to haploinsufficiency, whereas two missense mutations in the N terminus of DSP (V30M and Q90R) affect the normal localization of DSP in vitro, probably due to loss of binding to JUP as shown by protein–protein interaction analysis. In addition, overexpression of the N-terminal mutants in embryonic mouse hearts can lead to embryonic lethality. The cardiac phenotypes of those embryos resemble those seen in JUP- and PKP2-deficient mice, which also have defects in cardiac morphogenesis. Based on these findings, we speculate that N-terminal mutants disrupt the normal function of desmosomes and lead to desmosomal instability. As a result, such defective desmosomes cannot sustain the constant mechanical stress seen in contracting cardiomyocytes, which in turn leads to cardiac dysfunction, cell death, and eventually embryonic lethality.

Figure 6. Immunofluorescent staining of desmosomal and gap junction proteins. A and B. The overexpression of R2834H DSP results in normal localization of DSP and aberrant desmin localization at the intercalated discs (IDs). A. Frozen cardiac sections from 6-month-old mice were stained for DSP (green) and desmin (red). Merged images are shown on the right. Arrows indicate the IDs. Bars, 10 μm. B. Colocalization efficiency of DSP and desmin at IDs was calculated by Image-Pro Plus using Pearson correlation value (ranging between –1 to 1; with –1 being no overlap; 1 being perfect registration). (**P<0.001) C. Normal localization of JUP, PKP2, and CX43 at IDs in R2834H-Tg mice. Frozen cardiac sections from 6-month-old Tg and NTG mice were stained for JUP (green), PKP2 (green), and CX43 (green). Phalloidin was used to stain actin (red). Bars, 10 μm.
The overexpression of a C-terminal DSP mutation (R2834H) leads to cardiac defects in adult mice. This R2834H DSP mutant was still able to target to desmosomes both in vitro and in vivo, suggesting that these desmosomes can function sufficiently to allow cardiac development in the embryo. However, the R2834H-Tg mice developed cardiomyocyte apoptosis, cardiac fibrosis, and cardiac dysfunction during adulthood. Most of the cardiac dysfunction and histological changes observed in these animals were present in both ventricles, which is consistent with recent findings that ARVD/C patients carrying C-terminal DSP mutations had LV involvement.9,12,30 These mice also displayed significant lipid accumulation in myocytes, suggesting alteration of lipid metabolism. However, there was no myocyte replacement by adipose tissue. We speculate that this might be due to differences between the species or other epigenetic (environmental) factors. However, this is the first model in which lipid abnormalities have been notable and could suggest that this is the precursor to fatty replacement seen in ARVD/C.

In R2834H-tg mice, desmin failed to colocalize with DSP at the IDs by immunohistochemistry, which has also been noted in a human subject with Carvajal syndrome.31 Interruption of DSP–desmin interactions might lead to instability of desmosomes, resulting in reduced resistance to the constant mechanical stress seen in the cardiomyocyte. This was supported by ultra-structural evidence of ID remodeling (widened gaps of IDs) in R2834H-Tg mice. The ultra-structural changes seen in R2834H-Tg mice are similar to (but more severe than) that observed in human ARVD/C patients.14,32 As the fibrous tissue replacement and lipid accumulation were only detected focally in R2834H-Tg mice, we were unable to observe these changes in the electron microscopic evaluation, probably due to limitations of sampling. Although we observed ultra-structural changes (electron dense materials in the IDs) in WT-Tg mice, this was not due to the junctional proteins we examined (by immunohisto- tology and Western blot analysis) and not functionally related. We believe that changes of other desmosomal and junctional components (increased expression and redistribution of JUP, PKP2, and β-catenin), and changes of gap junction components (redistribution of CX43) in R2834H-Tg mice, are probably secondary to the interruption of DSP–desmin interactions due to the R2834H DSP mutation. The presence of excessive JUP and β-catenin in the Triton-X soluble fraction (mostly cytoplasmic) could potentially result in additional transcriptional changes important in cell survival or adipogenesis. These changes might further add to the development of the cardiac phenotypes we observed in the mutant DSP-Tg mice.

There have been several theories regarding the pathogenesis of ARVD/C.33,34 These include the “dysontogenetic theory,” which hypothesizes that ARVD/C might be a milder form of Uhl anomaly (paper thin RV with or without tricuspid valve anomalies) and that pathogenesis occurs due to the dysregulation of ventricular wall thickness either during development or postnatally.35 Another theory, the “apoptotic theory,” suggests that ARVD/C is due to increased apoptosis of cardiomyocytes.36,37 A third theory, the “transdifferentiation theory,” suggests that cardiomyocytes transdifferentiate into adipocytes in response to stress.38 Our study supports
both the dysontogenetic and apoptotic theories. In addition, our data suggest that desmosomes are not only involved in maintenance of tissue integrity, but also participate in other cellular processes including cell death and lipid metabolism.

Based on previous studies and our current findings, we believe that disruption of desmosomal integrity (due to mutations of DSP in this case) is the key factor leading to the development of ARVD/C, which results in defective mechanical linkage and in turn leads to abnormal localization of other cell–cell adhesion junction proteins (with possible subsequent transcriptional changes of related genes), and changes in gap junction components. We suggest that these defects in the desmosomal “final common pathway” might lead to the ARVD/C phenotype including: fibrosis, adipocyte infiltration, and arrhythmias.24,25 Therefore, this supports the concept that ARVD/C is a disease of the desmosome. However, detailed studies are required to further elucidate the pathogenesis of ARVD/C and provide insight into improving diagnosis and treatment of this disease.

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**Disclosures**

None.

**References**


