Clinical Genetics

Protein expression studies of desmoplakin mutations in cardiomyopathy patients reveal different molecular disease mechanisms


Mutations in the gene for desmoplakin (DSP) may cause arrhythmogenic right ventricular cardiomyopathy (ARVC) and Carvajal syndrome (CS). Desmoplakin is part of all desmosomes, which are abundantly expressed in both myocardial and epidermal tissue and serve as intercellular mechanical junctions. This study aimed to investigate protein expression in myocardial and epidermal tissue of ARVC and CS patients carrying DSP mutations in order to elucidate potential molecular disease mechanisms. Genetic investigations identified three ARVC patients carrying different heterozygous DSP mutations in addition to a homozygous DSP mutation in a CS patient. The protein expression of DSP in mutation carriers was evaluated in biopsies from myocardial and epidermal tissue by immunohistochemistry. Keratinocyte cultures were established from skin biopsies of mutation carriers and characterized by reverse transcriptase polymerase chain reaction, western blotting, and protein mass spectrometry. The results showed that the mutation carriers had abnormal DSP expression in both myocardial and epidermal tissue. The investigations revealed that the disease mechanisms varied accordingly to the specific types of DSP mutation identified and included haploinsufficiency, dominant-negative effects, or a combination hereof. Furthermore, the results suggest that the keratinocytes cultured from patients are a valuable and easily accessible resource to elucidate the effects of desmosomal gene mutations in humans.

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Arrhythmogenic right ventricular cardiomyopathy (ARVC) and dilated cardiomyopathy (DCM) are hereditary cardiac conditions with complex genetic aetiologies. Clinical and genetic investigations have revealed a considerable overlap in the disease expression of these conditions including a high risk of sudden cardiac death because of ventricular arrhythmias and heart failure (1–4).

Recent studies have identified disease-associated mutations in genes encoding desmosomal proteins in both ARVC and DCM patients (3, 5–8). Furthermore, co-inheritance of multiple desmosomal gene sequence variations has been reported to be associated with a more severe cardiac phenotype (5, 6, 9). As desmosomal proteins are expressed in both myocardium and epidermis, ARVC and DCM are occasionally associated

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with cutaneous manifestations like palmoplantar keratoderma and woolly hair, as reported in Naxos disease and Carvajal syndrome (CS) (10, 11).

Desmosomes are membrane-bound adhesive junctions that are abundantly expressed in the myocardium and stratified epithelia like the epidermis (12). They ensure mechanical integrity and provide intercellular force transmission through anchorage of the intermediate filaments to the cell membrane in adjacent cells (13). Desmoplakin is a constituent of all desmosomes and the vast majority of the protein is located in the cytoplasm while a minor part can be isolated from the cytoplasm as DSP precursor particles (14). The protein exists in two isoforms, DSP1 and DSP2, produced by alternative splicing, and DSP1 is the only isoform expressed in the myocardium, whereas both DSP1 and DSP2 are expressed in the epidermis (12).

In order to provide a better understanding of the molecular disease mechanisms leading to ARVC and DCM more knowledge about the human protein expression pattern associated with DSP mutations is needed. However, it is difficult to obtain cardiac tissue from mutation-positive individuals, which may explain why only few studies of DSP mutations have been made in cardiomyopathy patients (5, 15). So far, the effects of desmosomal gene mutations have most commonly been studied in artificial expression systems by use of transfected cell cultures and transgenic mice overexpressing mutant proteins (16). As keratinocytes express all cardiac-specific isoforms of desmosomal proteins it is likely that changes in myocardial expression of desmosomal proteins, as a result of mutations, are mirrored by similar changes in the epidermis (17).

It was the aim of the study to investigate the expression of different DSP mutations in keratinocyte cultures from affected families and to compare the results with immunohistochemistry (IHC) of desmosomal proteins in tissue samples from the myocardium and epidermis of the same individuals.

Methods

Detailed descriptions of experimental procedures are available in Appendix S1.

Study cohort

Seventy-one probands with a definite or borderline ARVC diagnosis and one patient with CS were evaluated prospectively according to the revised ARVC diagnostic criteria (18). The study was conducted in accordance with the 1964 Declaration of Helsinki and the local ethics committee approved the study (study no. 20080091). Informed consent was obtained from all participants. Relatives at risk of having inherited the condition were invited to participate in the study.

Protein expression study of desmoplakin mutation

Genetic investigations

Index patients underwent mutation analysis of the DSP, plakoglobin (JUP), plakophilin-2 (PKP2), desmoglein-2 (DSG2), and desmocollin-2 (DSC2) genes by dideoxy sequencing (19). The minor allele frequencies (MAFs) of detected sequence variants were assessed in 210 Danish control chromosomes of unrelated, healthy blood donors. Furthermore, the Exome Variant Server database (National Heart Lung Blood Institute Exome Sequencing Project) was searched in order to rule out the presence of detected variants in a larger number of controls with apparently normal phenotypes (20). Sequence variants were considered to be disease associated if they were predicted to introduce a premature termination codon (PTC) in the reading frame, cause abnormal splicing or deletions, or were shown to segregate with an ARVC phenotype in this or previous family studies (21). Coding variants, which did not fulfill these criteria, were considered to be of either unknown significance if present in control alleles with a MAF ≤ 0.1%, or as polymorphisms.

Protein studies in cultured primary keratinocytes

In brief, skin biopsies were obtained from DSP mutation carriers and wild type (WT) family members. Primary keratinocytes were cultured from these biopsies and western blots (WBs) of cytoskeletal and cytoplasmic protein fractions were performed using antibodies targeted against DSP, PKP2, DSG2 and beta-actin (ACTB) (22, 23). In addition, nano-liquid chromatography coupled with tandem mass spectrometry (nLC-MS) was used to estimate the expression of WT and mutant DSP protein present in the cytoskeleton of cultured keratinocytes (24).

Immunohistochemistry

Tissue sections of myocardial and epidermal tissue from mutation-positive individuals and WT controls were mounted on the same slide and underwent the same IHC staining procedures with DSP, PKP2, and DSG2 antibodies (23). Microscopy was performed by two experienced cardiovascular pathologists who independently scored immunoreactivity as normal, reduced or absent.

Desmoplakin gene expression measured by quantitative reverse transcriptase polymerase chain reaction

In family 2, DSP gene expression in cultured keratinocytes was investigated by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) using TaqMan-assays (Applied Biosystems, Naerum, Denmark) annealing to the DSP1 or both DSP transcripts. Furthermore, a specific TaqMan allelic discrimination SNP assay was used to estimate the amount of the mutant c.3805C>T DSP1 mRNA transcript (24).
Family 1 – DSP-p.S2594PfsX8

The proband, II-1, was of Turkish descent and presented at the age of 8 with congestive heart failure, woolly hair, and palmoplantar keratoderma consistent with a diagnosis of CS (Fig. 2). A transthoracic echocardiogram (TTE) showed biventricular dilation and a left ventricular ejection fraction of 15%. She gradually deteriorated and underwent cardiac transplantation at the age of 12. The explanted heart showed severe dilation of all four chambers (Fig. 2). The myocardium was thinned with extensive interstitial replacement fibrosis and hypertrophy of cardiomyocytes. However, no adipose tissue infiltrations or inflammatory changes were present (Fig. 2). Repeated clinical evaluations over a 7-year period of her parents, who were first cousins, and of her two siblings were normal.

Mutation analysis identified homozygosity for a novel deletion in the last exon of DSP, c.7780delT, which was predicted to cause insertion of a PTC in the reading frame, p.S2594PfsX8 (Fig. 1). The healthy parents and siblings were heterozygous carriers of the mutation (Fig. 3a).

Keratinocytes were cultured from skin biopsies of the proband, II-1, and her healthy mother, I-2. WB showed that the proband expressed two DSP proteins with a lower molecular weight than the corresponding WT DSP isoforms. These protein bands were not present when using an antibody binding to a C-terminal epitope in DSP consistent with the predicted truncation of both DSP isoforms (Fig. 3b). As truncated DSP protein was present in the cytoskeletal fraction it was likely to be incorporated in desmosomes. In the heterozygous mother, only minimal amounts of mutant DSP isoforms could be detected following a threefold increase in total protein load on the gel (Fig. S1a). Expression of mutant DSP in the CS patient was slightly reduced compared with DSP expression in healthy controls whereas the amount of WT DSP expressed by the heterozygous mother was decreased by almost 50% (Fig. 3b, Fig. S1b, Supporting Information). IHC using two different DSP antibodies was similar and showed normal DSP immunoreactivity in epidermal and myocardial tissue of the proband but appeared to be reduced in the epidermis of the heterozygous mother (Fig. 3c, Fig. S4).

In summary, the results indicated that the homozygous CS patient incorporated C-terminal truncated DSP in myocardial and epidermal desmosomes whereas the mutated DSP protein was almost entirely degraded in her healthy heterozygous mother. Furthermore, in the heterozygous mother, the expression of WT DSP was reduced by 50% compared to WT controls.

Family 2 – DSP-p.R1269X

The proband, II-3, presented at the age of 49 with syncope and sustained ventricular tachycardia (VT; Table 1, Fig. 4). She had a normal electrocardiogram (ECG) whereas TTE showed a significant thinning of the mid-ventricular septum, which was accompanied by septal hypokinesia. Holter-recording revealed 2600 ventricular ectopics per 24 h (VE/24h). Fibrofatty replacement was evident in endomyocardial biopsies. She was treated with an implantable cardioverter defibrillator (ICD) and one episode of sustained VT was aborted during 9 years of follow-up.

Her 59-year-old brother, II-1, had a history of palpitations and Holter-recording documented 2000 multifocal VE/24 h while the remaining cardiac evaluation was normal. The 27-year-old son of the proband, III-2, had a significant right ventricular wall aneurysm revealed by both TTE and cardiac magnetic resonance imaging. His signal-averaged ECG (SAECG) was positive for late potentials while ECG and Holter-recordings were normal. The remaining asymptomatic family members (I-1, II-3, III-1, and III-3) had normal cardiac investigations.

Mutation analysis of the proband revealed a novel heterozygous DSP mutation, c.3805C>T, leading to a PTC in exon 23 affecting the DSP1 isoform only, p.R1269X (Fig. 1). Subsequent mutation analysis of her relatives showed that her father, brother, and son (I-1, II-1, and II-2) also carried the mutation (Fig. 4a).

The amount of the c.3805C>T DSP1 transcript was further investigated by quantitative RT-PCR. Mean DSP1/DSP1+2 expression ratio was significantly reduced by 28% in mutant cells (Fig. 4b). This finding was further validated by allelic-discriminating RT-PCR,
# Protein expression study of desmoplakin mutation

## Table 1. Clinical data on carriers of desmoplakin mutations

<table>
<thead>
<tr>
<th>Family/ID</th>
<th>Sex/age</th>
<th>Initial symptom</th>
<th>Cardiac imaging</th>
<th>Histology</th>
<th>Repolarization abnormalities</th>
<th>Depolarization/conduction abnormalities</th>
<th>Arrhythmias</th>
<th>Family history</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/II-1</td>
<td>♀/8</td>
<td>Congestive heart failure</td>
<td>Biventricular dilation, LVEF 0.15</td>
<td>FR</td>
<td>TWI in II, III, and aVF</td>
<td>LPH</td>
<td>nsVT</td>
<td>+</td>
<td>DSP- p.S2594PfsX8 (homozygous)</td>
</tr>
<tr>
<td>2/II-3</td>
<td>♀/49</td>
<td>Syncope</td>
<td>Septal thinning and hypokinesia, LVEF 0.50, RV normal</td>
<td>FFR</td>
<td>–</td>
<td>SAECG n/a</td>
<td>frequent VE (Holter n/a)</td>
<td>VT</td>
<td>+</td>
</tr>
<tr>
<td>2/III-2</td>
<td>♂/27</td>
<td>–</td>
<td>LVEDV 81 ml/m², LVET 0.65, RVEDV 94 ml/m², RVEF 0.50, RV aneurysm</td>
<td>n/a</td>
<td>–</td>
<td>LP</td>
<td>2600 VE/24 h</td>
<td>+</td>
<td>DSP- p.R1269X</td>
</tr>
<tr>
<td>2/II-1</td>
<td>♂/59</td>
<td>Palpitations</td>
<td>TTE normal</td>
<td>n/a</td>
<td>–</td>
<td>SAECG n/a</td>
<td>2000 VE/24 h</td>
<td>+</td>
<td>DSP- p.R1269X</td>
</tr>
<tr>
<td>3/II-3</td>
<td>♂/46</td>
<td>Syncope</td>
<td>LVEF 0.55, RVEDd 16 mm/m²</td>
<td>FFR</td>
<td>TWI in V1–5, II, III, aVF</td>
<td>LP</td>
<td>VT</td>
<td>+</td>
<td>DSP- p.E324–K325del PKP2- p.T526M</td>
</tr>
<tr>
<td>3/III-8</td>
<td>♂/19</td>
<td>–</td>
<td>LVEDV 85 ml/m², LVET 0.60, RVEDV 97 ml/m², RVEF 0.43, regional RV akinesia</td>
<td>n/a</td>
<td>–</td>
<td>LP</td>
<td>1100 VE/24 h</td>
<td>+</td>
<td>DSP- p.E324–K325del PKP2- p.T526M</td>
</tr>
<tr>
<td>3/II-1</td>
<td>♂/43</td>
<td>Sudden cardiac death</td>
<td>n/a</td>
<td>FR</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>+</td>
<td>DSP- p.E324–K325del PKP2- p.T526M</td>
</tr>
<tr>
<td>3/III-7</td>
<td>♀/31</td>
<td>–</td>
<td>LVEDV 75 ml/m², LVET 0.62, RVEDV 95 ml/m², RVEF 0.53, dyssynchronous RV contraction</td>
<td>n/a</td>
<td>TWI in V1–5</td>
<td>2° AVB, LP</td>
<td>300 VE/24 h</td>
<td>+</td>
<td>DSP- p.E324–K325del PKP2- p.T526M</td>
</tr>
<tr>
<td>4/II-1</td>
<td>♂/46</td>
<td>Palpitations</td>
<td>LVEF 0.50, RVEDd 24 mm/m², RV aneurysm</td>
<td>FFR</td>
<td>TWI in V1–5</td>
<td>LP</td>
<td>VT</td>
<td>–</td>
<td>DSP- p.V30M DSG2- p.R46Q</td>
</tr>
</tbody>
</table>

Only individuals with clinical abnormalities are depicted.

*Ventricular dimensions measured by TTE or ventricular volumes estimated by cardiac MRI (only available in a subset of individuals).

Twelve-lead electrocardiogram (ECG) and signal-averaged ECG (SAECG).

LVEF, left ventricular ejection fraction; LVEDV, LV end-diastolic volume; RVEF, right ventricular ejection fraction; RVEDV, RV end-diastolic volume; RVETd, RV end-diastolic diameter measured in parasternal long-axis view by TTE; VT, sustained ventricular tachycardia; nsVT, non-sustained VT; VE/24 h, ventricular ectopic beats per 24 hours; TWI, T-wave inversion; LP, late potentials; AVB, atrio-ventricular block; LPH, left posterior hemiblock; aVF, augmented left foot lead; FR, fibrous replacement; FFR, fibrofatty replacement; n/a, not available; –, none or normal findings; +, positive.
which confirmed the presence of the mutant transcript but indicated that the amount of mutant transcript was 15-fold lower than WT transcript. Subsequently, direct sequencing of PCR amplified complementary DNA (cDNA) amplicons showed only very small amounts of mutant c.3805C>T cDNA in heterozygous mutation carriers (Fig. S1). These findings indicated that the vast majority of the mutated c.3805C>T DSP1 transcript was likely to be degraded by non-sense mediated mRNA decay (25). WB of keratinocyte protein extracts showed that DSP1/DSP1+2 protein ratios were significantly lower in DSP-p.R1269X mutation carriers compared with WT individuals (Fig. 4c,d). The expected molecular weight of the truncated DSP-1-1269 protein was 150 kDa. However, WB of keratinocytes from mutation carriers did not detect any specific proteins of this size (Fig. 4d). IHC with DSP1 and DSP1+2 antibodies indicated that DSP expression was decreased in the basal epidermal cell layers in mutation carriers. Furthermore, IHC showed a decreased DSP expression in endomyocardial biopsies of the proband (Fig. 4e,f; Fig. S4). In summary, these findings were in accordance with the results of RT-PCR and suggested that the DSP-p.R1269X mutation was likely to cause DSP1 haploinsufficiency.

Family 3 – DSP-p.K324_E325del mutation

The proband, II-3, presented at the age of 46 with syncope due to sustained VT. His children (III-7 and III-8) also fulfilled ARVC diagnostic criteria but were both asymptomatic (Table 1). The father and the brother of the proband had died suddenly at the age of 36 and 42, respectively. Post-mortem examination of the brother’s (II-1) heart revealed profound abnormalities of both ventricles with fibrous scarring, myocyte hypertrophy, and inflammation. No other relatives fulfilled ARVC diagnostic criteria.

Mutation analysis of the proband identified two heterozygous sequence variants (Table 1). One in-frame deletion of two highly conserved amino acids in the N-terminal globular domain of both DSP isoforms (c.969_974delAAAAGA; p.K324_E325del; Fig. 1), and a rare missense variant in PKP2 (c.1577C>T, p.T526M), which has previously been reported in ARVC patients but also in controls with a MAF ≤0.5% (6, 20). All affected relatives (II-1, III-7, and III-8) carried both sequence variants, in addition to III-1 who was asymptomatic and non-penetrant at the age of 29. Carriers of the DSP mutation in isolation were all unaffected (Fig. 5a).

Keratinocyte cultures were established from the digenic proband (II-3), a WT control (II-2), and a healthy individual carrying the DSP mutation in isolation (II-4). WB showed that carriers of the DSP mutation (II-3 and II-4) had reduced expression of both DSP isoforms in cytoskeletal and cytoplasmic protein fractions compared to WT individuals (n = 7; Fig. 5b, Fig. S1c–e). PKP2 expression appeared to be the same in WT individuals and in the carrier of the PKP2 variant (Fig. 5b).

To further investigate if the mutant DSP-K324_E325del protein was incorporated into desmosomes, cytoskeletal protein fractions of
keratinocytes cultured from mutation carriers and WT individuals were investigated by use of nLC-MS. Unfortunately, mutant DSP-K324_E325del and PKP2-T526M variant peptides were either too short or too long to be detected by nLC-MS. However, the proband also carried a common DSP-p.R1738Q variant in trans-position to the DSP-p.K324_E325del mutation (Fig. 5a). Therefore, quantification of mutant DSP-K324_E325del protein was possible because the amount of mutant protein had to be inversely proportional to the amount of expressed DSP-R1738Q variant protein.

The DSP-R1738Q variant peptide was identified by nLC-MS and two other non-polymorphic DSP peptides were used as internal standards (Fig. 5c). For each of the individuals, II-3, II-2, and II-4 with different DSP genotypes, the relative expression of the DSP-R1738Q peptide was calculated [AUC_{R1738Q}/(AUC_{ref,1}+AUC_{ref,2})]. Protein from a heterozygous DSP-p.R1738Q sample was used as a control. Analysis of the cytoskeletal protein fractions indicated that the expression of DSP-R1738Q peptide was significantly lower in the digenic proband (II-3), compared to the healthy individual carrying the DSP mutation in isolation (II-4) and the homozygous control (II-2) (Fig. S3). Based on the values in heterozygous and homozygous p.R1738Q control samples it was estimated that the amount of the DSP-R1738Q variant protein expressed by II-3, was approximately 80% of total DSP (Fig. S3). As the digenic proband (II-3) carried the DSP-K324_E325del mutation in trans-position to the DSP-p.R1738Q variant, the mutant protein represented approximately 20% of total DSP protein. In contrast, the non-affected DSP mutation carrier, II-4, did not express the mutated protein in cytoskeletal protein fractions (Fig. S3). IHC showed that DSP expression was significantly reduced in myocardial tissue of mutation carriers while PKP2 expression appeared normal. Likewise, IHC indicated a reduced DSP expression in epidermal biopsies of mutation carriers (Fig. 5d,e, Fig. S4).

In summary, expression of both DSP isoforms was reduced in keratinocytes of DSP-p.K324_E325del mutation carriers. Furthermore, the amount of mutated DSP of the digenic proband constituted about 20% of total cytoskeletal DSP protein.

**Family 4 - DSP-p.V30M and DSG2-p.R46Q**

In family 4, the proband (II-1) presented with sustained VT at the age of 46 and fulfilled ARVC diagnostic criteria while his two siblings (II-2 and II-3) had normal cardiac investigations (Table 1, Fig. 6a). Mutation analysis of the proband identified two sequence variants in DSP (c.88G>A, p.V30M) and DSG2 (c.137G>A, p.R46Q) (Table 1, Fig. 1). Both variants have previously been reported in ARVC patients (5, 26). The DSP-p.V30M variant was located in the N-terminal DSP head domain responsible for interactions with JUP...
whereas the DSG2-p.R46Q mutation was located in a highly conserved furin cleavage site in the DSG2 propeptide and believed to be disease associated (5, 6, 26). The unaffected brother, II-3, was shown to carry the DSP-p.V30M variant in isolation (Fig. 6a).

Epidermal biopsies obtained from the three siblings and WB of keratinocyte protein extracts did not reveal differences in DSP and DSG2 protein expression between mutation carriers and WT controls (Fig. 6b). nLC-MS identified both the DSP-V30M peptide and the corresponding DSP-WT peptide in keratinocyte cytoskeletal protein fractions of mutation carriers (Fig. 6c). Unfortunately, investigations of the mutated DSG2 peptide could not be performed by nLC-MS due to the presence of flanking arginine and lysine amino acids, which resulted in tryptic peptides too short for identification by this method. However, in agreement with the WB results, DSP and DSG2 immunostaining of endomyocardial biopsies of the proband in family 4 appeared normal (Fig. S4). Similar observations were made in epidermal biopsies.

In summary, WT and DSP-V30M protein were equally expressed in heterozygous carriers and DSG2 expression was not significantly reduced in the DSG2-p.R46Q mutation carrier. Furthermore, the cellular protein localization of both DSP and DSG2 appeared normal in myocardial and epidermal tissue from mutation carriers.

Discussion

Desmoplakin mutations are often associated with a biventricular ARVC phenotype and pathogenic DSP mutations have recently been identified in patients with end-stage DCM (1, 4). The majority of patients reported here had clinical abnormalities suggestive of left ventricular involvement. Histological examination revealed fibrofatty replacement changes in two index patients whereas fibrotic scarring and myocyte hypertrophy without fatty infiltrations was present in the myocardium of the CS patient and the index patient of family 3. These findings underscore the clinical, histological, and genetic overlap between ARVC and DCM phenotypes associated with DSP mutations (1, 3, 4, 6, 8).
Protein expression study of desmoplakin mutation

Fig. 5. Investigations of the DSP-p.K324_E325del and PKP2-p.T526M sequence variants in family 3. (a) Pedigree with DSP and PKP2 genotypes (for details, see Fig. 1). (b) Western blot (WB) of keratinocyte cytoplasmic and cytoskeletal protein extracts. (c) Mass spectrometry chromatogram showing the DSP-R1738Q variant peptide (*) and two DSP reference peptides identified by nano-liquid chromatography coupled with tandem mass spectrometry (nLC-MS) used for quantification of the DSP-R1738Q variant protein. (d) Immunohistochemistry (IHC) of myocardial tissue from the right ventricular (RV) septum, scale bar = 40 μm. (e) IHC of epidermal tissue, scale bar = 20 μm.

Previous investigations of patients with CS have shown that the condition is caused by homozygous or compound heterozygous DSP mutations and the heterozygous mutation carriers remain healthy without signs or symptoms of the condition (11, 27). Genetic investigations of our CS patient identified a novel homozygous frameshift mutation in the last exon of DSP. Subsequent protein investigations of keratinocytes cultured from the patient showed that the mutation introduced a C-terminal truncation of DSP and that the mutated protein was incorporated in desmosomes of the myocardium and epidermis. These results suggested that the CS phenotype may develop due to impaired interactions between the C-terminal part of DSP and the intermediate filaments. Interestingly, the heterozygous mother of our CS patient did not express significant amounts of mutated DSP protein and had a normal phenotype despite a 50% reduction of the DSP WT protein. This finding suggested that the ability to produce WT DSP in heterozygous carriers facilitate the degradation of mutated DSP protein and thereby prohibit development of the condition.

Genetic investigations of family 2 with ARVC identified a novel DSP-p.R1269X non-sense mutation, which was associated with incomplete penetrance because an 85-year-old mutation carrier had no signs of the condition. RT-PCR of mRNA from keratinocytes of mutation carriers showed that the mutant c.3805C>T DSP1 transcript was degraded which explained the decreased myocardial and epidermal expression of the DSP1 isoform. Previously, a similar mutation in the same domain of DSP (p.R1267X) has been reported in a homozygous patient with CS who had no detectable DSP1 expression (27). The patient died from heart failure at the age of 3. Three of his relatives were heterozygous mutation carriers with normal phenotypes despite DSP1 haploinsufficiency. Due to the fact that individuals with haploinsufficiency of DSP1 may remain healthy it is likely that the index patient in family 2 carrying the p.R1269X mutation inherited additional and yet unidentified genetic variations in order to develop a penetrant ARVC phenotype (5, 6, 9). The father of the proband was healthy at the age of 85 and the two other carriers of the p.R1269X mutation had only borderline signs of ARVC. This suggests that carrier status of this mutation is tolerated and the pathogenicity of the mutation is influenced by other unknown genetic factors.
Genetic investigations of family 3 with ARVC identified a novel in-frame deletion in DSP and a rare PKP2 variant (6). All digenic carriers except for one younger individual fulfilled ARVC diagnostic criteria while individuals carrying the DSP mutation or the PKP2 variant in isolation had a normal phenotype. WB of cultured keratinocytes and IHC of myocardial as well as epidermal tissue from affected individuals showed a reduction in the expression of both DSP1 and DSP2. PKP2 protein expression was the same in individuals with normal genotype and carriers of the PKP2-p.T526M variant. However, in the digenic patient carrying the DSP-p.K324_E325del mutation and the PKP2-p.T526M variant, the nLC-MS experiments showed that approximately 20% of DSP in the cytoskeleton was mutated (Fig.S3). This finding was in contrast to the healthy carrier of the DSP mutation in isolation who had reduced amounts of total DSP protein and expressed WT DSP only. Although the number of affected individuals were limited in this family it is possible that co-expression of the PKP2-p.T526M variant facilitated the incorporation of mutant DSP protein into the desmosome. This notion was further supported by the fact that PKP2 participate in the recruitment of DSP to intercellular junctions and suggests that rare sequence variants may indeed possess modifying effects on protein expression (30).

In family 4, the proband carried recognized sequence variants within DSP as well as DSG2. The DSG2 mutation has previously been reported in several ARVC families (6, 26). The DSP variant has only been reported in affected individuals carrying additional desmosomal gene mutations and was recently identified in healthy controls (5, 9, 20). Protein investigations of cells and tissue by IHC, WB, and nLC-MS showed that the DSP-V30M protein was co-expressed with WT DSP and incorporated in the cytoskeleton in an amount equal to WT DSP protein. The results of WB and IHC showed normal tissue expression and localization of the DSG2 protein in the digenic DSG2-p.R46Q/DSP-p.V30M mutation carrier. These results indicated that the mutant DSG2-R46Q protein was expressed and incorporated into the desmosome.

Furthermore, this finding suggested that a proportion of the expressed DSG2 remained uncleaved and retained the N-terminal pro-peptide chain that may interfere with the assembly of functional desmosomes. This may lead to impairment of interactions with other desmosomal cadherins (29).

A previous study has also investigated the DSP-p.V30M mutation in a human squamous carcinoma cell line by transient transfection (30). Immunostaining indicated that the DSP-V30M protein was reduced at cell borders, and the authors suggested that the mutation caused a mis-localization of mutant DSP. These findings are inconsistent with the results of this study in which the mutant protein was fully incorporated into the desmosome. The discrepancy may well illustrate that artificial cell model systems do not necessarily reflect in vivo regulation of gene expression, mRNA splicing, and protein quality-control mechanisms. This favours the use of human cells or tissue obtained from affected individuals to study the cellular effects of disease-associated mutations. A recent study performed IHC of myocardial tissue samples from three ARVC patients with different DSP mutations (15). The authors reported that a DSP missense mutation was associated with a normal pattern of DSP expression while the immunoreactive signal was reduced in samples from two individuals carrying DSP non-sense mutations. These findings are in agreement with our investigations and support the hypothesis that both haploinsufficiency and dominant-negative effects of mutant proteins are likely disease mechanisms in ARVC. However, in CS with recessive inheritance, family members who are heterozygous for the mutation have a normal heart although they are haploinsufficient with respect to WT DSP protein (27). Therefore, it is most likely that DSP haploinsufficiency has to be accompanied by additional genomic variations in order for the condition to develop (5, 6, 9). These circumstances may also help to explain the low

Fig. 6. Genetic and protein investigations of DSP-p.V30M and DSG2-p.R46Q mutations in family 4. (a) Pedigree, family 4 (for details, see Fig. 1); (b) Western Blot (WB) of cytoskeletal protein extracts from cultured keratinocytes. (c) Mass spectrometry chromatograms of wild type (WT) and variant DSP-V30M peptides identified by nano-liquid chromatography coupled with tandem mass spectrometry (nLC-MS) in keratinocytes of mutation-positive individuals.
penetrance (<50%) of DSP mutations observed in the family members reported here and in previous studies (7).

In conclusion, a variety of desmosomal proteins that also includes the myocardial isoforms are expressed in keratinocytes. This study has showed that protein abnormalities in cardiac tissue from patients with desmosomal cardiomyopathies are also present in their keratinocytes. Thereby it is possible to use human keratinocyte cultures obtained from affected individuals for protein expression studies and expand our knowledge about the molecular disease mechanisms. The results have illustrated that the pathogenic effects of DSP mutations include haploinsufficiency as well as dominant-negative effects, and that sequence variations in other desmosomal proteins are likely to influence incorporation of mutant DSP protein into desmosomes. Overall, the study indicated that the desmosomal cardiomyopathies are often associated with a complex mode of inheritance and a highly variable pattern of protein expression, which help to explain the heterogeneous clinical appearance of the conditions.

Supporting Information

The following Supporting information is available for this article:
Appendix S1. An expanded methods section.
Table S1. Desmosomal gene sequence variants in individuals with disease associated mutations in the desmoplakin gene.
Fig. S1. (a) WB of keratinocyte protein extracts from WT controls and a homozygous (II-1, Carvajal syndrome) and a heterozygous carrier (I-2) of the DSP-R1738Q variant protein. Compared to Fig. 1c in the printed manuscript, a threefold amount of protein is loaded in each lane. Small amounts of mutant C-terminal truncated DSP protein are present in the heterozygous mutation carrier. Enhanced chemiluminescence was used for sensitive detection of eventual truncated DSP proteins. (b) Quantification of both DSP isoforms. The homozygous DSP-p.S2594PfsX8 mutation carrier expressed truncated DSP protein isoforms only. Mean values from triplicate WB experiments are shown. The DSP-WT group represents DSP protein expression in seven different WT samples. Error bars = group mean ± SD. (c, d, e) Quantification of DSP1/ACTB, DSP1+2/ACTB and DSP1/DSP1+2 protein expression ratios by WB of keratinocytes from family 2, 3, and 4 and WT controls. Mean values from triplicate WB experiments are shown. Error bars indicate group mean ± SD.
Western blot including samples of family 2. Compared to Fig. 3d in the printed manuscript, a threefold amount of protein is loaded in each lane. Enhanced chemiluminescence was used for sensitive detection of eventual truncated DSP proteins.
Fig. S2 (a, b, c) qRT-PCR data from family 2. Error bars indicate mean ± SD of triplicate experiments with keratinocyte pellets from three independent cell cultures. (d) Post-read plot from a TagMan allelic discrimination SNP assay with fluorescent probes annealing to the WT DSP sequence (Y-axis) or the mutant c.3805C>T DSP sequence (Y-axis). Genomic DNA (circles) and cDNA (squares) from DSP-WT (filled symbols) and heterozygous mutation carriers (open symbols) were used in the PCR reaction. A sample without DNA (X) was included to illustrate background fluorescence. The signal from the VIC-probe was significantly reduced in cDNA samples of mutation carriers compared to the corresponding heterozygous genomic DNA samples. (e) Sequence electropherograms with DSP nucleotide sequences containing the

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3805 position. Thymidine nucleotide was only present in trace amounts in cDNA from heterozygous mutation carriers (*).

Fig. S3 (a) DSP-haplotypes of samples used for quantification of the DSP-R1738Q variant protein; p. paternal allele, m. maternal allele. (B) Mass spectrometry chromatograms of DSP peptides identified by nLC-MS in keratinocyte protein extracts from samples with different DSP genotypes. (c) Quantification of the DSP-R1738Q variant peptide. Keratinocytes of the digenic DSP-p.K324_E325del/PKP2-p.T526M proband (3.II-3) expressed significantly lower amounts of the DSP-R1738Q variant compared with the healthy DSP-p.K324_E325del carrier (3.II-4), in which the expression of DSP-R1738Q protein was similar to the expression in an individual homozygous for the DSP-p.R1738Q variant. (d) Estimation of DSPK324_E325del protein. Based on the values in (e), it was estimated that in the proband (3.II-3) approximately 20% of expressed DSP in cytoskeletal protein fraction consisted of mutant DSP-K324_E325del protein. While DSP-WT variant is likely degraded the mutant protein and expressed DSP-WT protein only; het. = heterozygous, hom. = homozygous. Error bars = mean ± SD.

Fig. S4 (a) IHC of myocardial and epidermal tissue in family 1 with a DSP1 and DSP1+2 antibodies. DSP expression appeared to be normal in the homozygous DSP-p.S2594PfsX8 mutation carrier with Carvajal syndrome whereas the heterozygous mutation carrier had reduced epidermal DSP expression compared with a wild type control. Scale bar = 40 μm. (b) IHC of myocardial and epidermal tissue in family 2 with DSP1 and DSP1+2. The DSP-p.R1269X mutation was associated with reduced epidermal and myocardial DSP expression. Scale bar = 20 μm.

Fig. S5 (b) IHC of myocardial and epidermal tissue in family 3 with a DSP1 and PKP2 antibody. Epidermal PKP2 expression was very low but ducts of the adnexal glands stained positive (bottom panel). (b) IHC of myocardial and epidermal tissue in family 4 with DSP1+2 and DSG2 antibodies. The immunoreactive pattern in mutation carriers was indistinguishable from controls.

Additional Supporting information may be found in the online version of this article.

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References


