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De novo desmin-mutation N116S is associated with arrhythmogenic right ventricular cardiomyopathy

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Arrhythmogenic right ventricular cardiomyopathy (ARVC) is an inherited heart muscle disease, frequently accompanied by sudden cardiac death and terminal heart failure. Genotyping of ARVC patients might be used for palliative treatment of the affected family. We genotyped a cohort of 22 ARVC patients referred to molecular genetic screening in our heart center for mutations in the desmosomal candidate genes *JUP*, *DSG2*, *DSC2*, *DSP* and *PKP2* known to be associated with ARVC. In 43% of the cohort, we found disease-associated sequence variants. In addition, we screened for desmin mutations and found a novel desmin-mutation p.N116S in a patient with ARVC and terminal heart failure, which is located in segment 1A of the desmin rod domain. The mutation leads to the aggresome formation in cardiac and skeletal muscle without signs of an overt clinical myopathy. Cardiac aggresomes appear to be prominent, especially in the right ventricle of the heart. Viscosimetry and atomic force microscopy of the desmin wild-type and N116S mutant isolated from recombinant *Escherichia coli* revealed severe impairment of the filament formation, which was supported by transfections in SW13 cells. Thus, the gene coding for desmin appears to be a novel ARVC gene, which should be included in molecular genetic screening of ARVC patients.

INTRODUCTION

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is an inherited heart muscle disease characterized clinically by life-threatening ventricular arrhythmias and is a major cause of sudden cardiac death (SCD) in young people and in athletes (1). In the literature, its prevalence has been estimated to vary from 2:10 000 (2) to 4:10 000 in the Veneto Region Italy (3), whereas the NCBI database specifies a prevalence of 6–44:10 000 (OMIM 602861, 609040, 125671, 125647). The exact prevalence, however, is unknown and could be higher than the estimated one because of the existence of many undiagnosed cases.

The pathology consists of a dystrophy of the right ventricular (RV) myocardium with fibro-fatty replacement, leading to RV aneurysms in some patients. The precise frequency of the familial form of ARVC is unknown and estimated to be 30–50 or 80% (4–6).

Usually, the disease is inherited as an autosomal-dominant trait with incomplete penetrance and various forms of clinical expression. The causative genes for autosomal-dominant ARVC encode proteins of the cardiac desmosomes, one of

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the two major types of cell adhesion junctions in cardiomyocytes providing mechanical attachment between cells and located at the intercalated disc. Multiple mutations were described in genes encoding for desmocollin 2 (*DSC2*), desmoglein 2 (*DSG2*), plakophilin 2 (*PKP2*), plakoglobin (*JUP*) and desmoplakin (*DSP*) (reviewed in 7). Recently, there have been repeated reports on compound and digenic heterozygosity of desmosomal gene variations contributing to ARVC (8–10). Although the association of the dominant form of ARVC with mutations in desmosomal genes is well documented, in the majority of ARVC patients, no mutations were found among these genes.

Recessive inherited forms of human ARVC associated with palmoplantar keratoderma and woolly hair are also associated with mutations in the genes coding for plakoglobin (11), desmocollin 2 (12) and desmoplakin (13, 14). Another recessive plakophilin 2 splice mutation identified in an ARVC proband with no signs of cutaneous abnormalities was described by Awad *et al.* (15).

Desmin is the typical intermediate filament (IF) protein expressed by cardiac, skeletal and smooth muscle cells. It serves as a mechanical integrator of neighboring Z-discs in the sarcomere and also as an important structural component of the intercalated disc by binding to desmosomal plaque proteins. Mutations in the desmin gene are associated with severe human diseases, including diverse forms of myofibrillar myopathies and/or dilated cardiomyopathy (16, 17). Right-sided heart failure in *desmin* gene mutation carriers has occasionally been reported in the literature (18, 19). van Tintelen *et al.* (20) recently reported that patients fulfilling ARVC task force criteria carry a mutation in the head domain of desmin. Thus, *DES* appears to be another candidate gene carrying mutations in inherited forms of ARVC.

Here we report screening of an ARVC cohort on the prevalence of mutations in desmosomal proteins and desmin. We provide further evidence on functional consequences of a novel mutation identified in the desmin 1A segment of the rod domain for the development of ARVC.

RESULTS

Mutation detection

The desmosomal genes *DSG2*, *DSC2*, *PKP2*, *JUP* and *DSP*, in addition to the *DES* gene of 22 unrelated ARVC index patients, were analyzed for gene variations by sequencing and denaturing high-pressure liquid chromatography (dHPLC). In addition, patient #5 included in the study was screened only for mutations in *PKP2* and *DES*. According to the novel task force criteria (21), 12 of these patients were classified as definite, 7 as borderline and 4 as possible ARVC. Of note, six of the seven transplanted patients were classified as definite, whereas the explanted heart of the seventh patient was not available for pathological examination (Table 1).

Overall, 16 variants in *DSG2* (4×), *DSC2* (3×), *PKP2* (6×), *DSP* (2×) and *DES* (1×) were identified in 16 patients (Table 2). The variants identified included 10 missense, 3 nonsense and 2 deletion/insertion variants predicting an amino acid frameshift. Of note, we did not find any variant relevant for ARVC in the gene *JUP*.

Non-synonymous polymorphisms found in the screened genes and already published in GenBank and/or the Ensembl SNP database (www.genecards.org, www.ensembl.org) with an allele frequency of at least 3% were not considered as disease-causing and are shown in Supplementary Material, Table S1. The human gene mutation databases [www.fsm.it/cardmoc, www.arvcdatabase.info (21) and http://grenada.lumc.nl/LOVD2/ARVC/home.php] were checked for entry of all variations/mutations found in this study.

Since there was evidence that, especially in genes coding for cardiac type II cadherins, sequence variants were misinterpreted (10, 22–24), we checked all but one (*DSG2* p.M1I) sequence variants reported here in at least 320 anonymous blood donors used as controls.

Desmoglein 2 (DSG2). We identified, by mutation screening of a 36-year-old ARVC patient (#1), who underwent heart transplantation (HTx) in the meantime, a G to A transition at codon 392 of *DSG2*, leading to the substitution of valine for isoleucine (p.V392I). This mutation was also identified in one brother who, up to now, is clinically unaffected (age 45 years). There was a history of SCD within the family. The *DSG2* p.V392I mutation was previously reported and the affected amino acid is conserved among mammals (25). From recently published data, the genotype–phenotype relationship appears to be demonstrated for this variant (9, 26).

In patient #13, we identified sequence variants c.3G>A (p.M1I) and c.1480G>A (p.D494M). The mutation p.M1I was previously found in an ARVC patient (25), whereas the variant p.D494M was classified as an undetermined variant in an ARVC database (21). Two brothers of this patient #13 were diagnosed to have ARVC and one of them was transplanted. One of them was tested only to find both mutations. M1I was not tested in blood donor controls, because this position will skip the initiation of translation at the proper position. In addition, a predicted in-frame start site at codon 179 will lead to loss of the leader sequence, which is essential for proper intracellular trafficking of the protein.

The variant E713K was found in patient #15. However, we found the presence of this variant in 4 out of 86 blood donors. We consider this variant therefore as a polymorphism not associated with ARVC (22).

Desmocollin 2 (DSC2). The variant c.2687_2688insGA (p.A897KfsX901; previously published as p.E896fsX900) was identified in the *DSC2* gene of a 34-year-old man (patient #14) with positive ARVC family history and was reported previously as a mutation (27). However, in agreement with other studies (10,26,28), we classified this variant as a polymorphism as the variant was found in 12 out of 395 blood donors.

In a 62-year-old patient (patient #22), we identified the novel homozygous deletion c.1912_1917delAGAA (p.Q638LfsX647). The brother of patient #22 died suddenly at the age of 27 due to cardiac death. This deletion is predicted to skip the transmembrane domain of desmocollin 2.

Table 1. Clinical data of patient cohort

Index patient ID	Gender	Age of disease onset	Major criterion[a]	Minor criterion[a]	Task force classification[b]	Affected gene[c]	Relatives tested[d]	NYHA
1	m	30	A1, E1[e], E2	E3	DD	*DSG2*	2	4 (HTx)
2	f	15	A1, E1[e], A3	C3	DD	*DES*	4	4 (HTx)
3	f	30	A1	E4	BL	*PKP2*	n.a.	4
4	f	53	A1	E4	BL	*RYR2*	n.a.	4 (HTx)
5	m	62	A1, A3, E2		DD	*PKP2*	r.g.	2–3
6	m	33	A1, A3	D2	BL	Unknown	–	
7	m	65	A1, A3, A4		PD	Unknown	–	
8	m	18	A1, B1, C4		DD	Unknown	–	
9	f	n.a.	E1[e]	E4	BL	*DSP*	8	1
10	m	43	A1, E1[e], E2	E4	DD	*PKP2*	1	4 (HTx)
11	m	n.a.	A1, E2		DD	*PKP2*	n.a.	3–4 (HTx-L)
12	f	49	A3	A2	BL	Unknown	–	
13	f	14	A1, B1, E1, E2		DD	*DSG2*	1	2–3
14	m	n.a.	E2		PD	*DSC2*	n.a.	n.a.
15	m	37	A3	D2	BL	*DSG2*	n.a.	n.a.
16	m	38	A1, A4		PD	*DSP*	n.a.	
17	m	29	A3		PD	Unknown	–	
18	m	63	A1, E2		DD	*PKP2*	n.a.	
19	m	47	A1, E1[e]		DD	Unknown	–	4 (HTx)
20	m	19	A1, A3, A4, C1		DD	Unknown	–	n.a.
21	m	n.a.	A1, E1[e] E2		DD	*PKP2*	r.g.	4 (HTx)
22	f	59	A1[f], A4,	E4, C1	DD	*DSC2*	n.a.	3–4 (HTx)
23	m	49	A1[f],	B1	BL	Unknown	–	4 (HTx-L)

HTx, heart transplantation; HTx-L, listed or evaluated for heart transplantation; m, male; f, female; n.a., not available; r.g., rejected genotyping; NYHA, New York Heart Association functional classification of heart failure.

[a]Task force criteria according to ref. (**38**), adapted criteria indicated by[e]: (A) *structural alterations*: A1, right ventricular dilatation (RVEDD > 32 mm); A1[f], biventricular dilatation; A2, right ventricular dilatation (RVEDD 29–32 mm); A3, MRI positive; A4, angiography—regional akinesia, dyskinesia or aneurysm; (B) *tissue characterization*: B1, fibrous replacement; (C) *repolarization abnormalities*: C1, inverted T-waves V1–V3; C2, inverted T-waves V4–V5; C3, inverted T-waves V4–V6; C4, Y-waves or prolongation of QRS in V1–V3; (D) *arrhythmias*: D1, non-sustained ventricular tachycardias (VT) with negative QRS; D2, non-sustained VT or right ventricular outflow tract tachycardias with positive QRS; (E) *family history*: E1, ARVC confirmed in first degree relative; E1[e], ARVC confirmed by autopsy or pathological examination of the explanted heart in case of HTx; E2, identification of a pathogenic mutation; E3, family history of suspected ARVC; E4, SCD due to expected ARVC.

[b]Disease classification according to task force criteria (38); DD, definite diagnosis; BL, borderline diagnosis; PD, possible diagnosis of ARVC.

[c]For details on the mutation compare Table 2. Novel polymorphisms were not considered.

[d]For details see text.

In our cohort, we identified another novel polymorphism R798Q, which was found in 7% of blood donors.

Plakophilin 2 (PKP2). In our study, six ARVC patients (26%) with different *PKP2* variations/mutations were identified. The variation p.D26N was already described as an unclassified variant by van Tintelen *et al.* (29). Genotyping of 363 blood donors recognized this variation as a single-nucleotide polymorphism (SNP) as 10 individuals had the same variation.

The mutation p.Q726X (6) was found in a 64-year-old HTx candidate (patient #10) diagnosed to have ARVC. The mutation was not inherited by the patient's single daughter, who was not clinically affected by cardiomyopathy. The family declined further testing despite a family history of SCD: two brothers (aged 44 and 55) and two nephews (both aged 17) of the patient died suddenly.

Furthermore, a 62-year-old male HTx candidate (patient #5) was identified as a carrier of the mutation p.R79X (6), leading to the premature termination of translation. This mutation was also recently identified in a Scandinavian ARVC population (30), and segregation with ARVC was shown in a Dutch patient cohort (29).

Two novel *PKP2* mutations were identified in our study; the mutation p.Q220X (patient #11) creates a premature stop codon, whereas p.D601EfsX55 (patient #21) generates a premature, terminated protein exhibiting a modified C-terminus. The mutation p.R811S within exon 12 (patient #18) was recently described (28). No additional genotype–phenotype data of the affected families were available.

Desmoplakin (DSP). In patient #16, we identified the variant causing the amino acid substitution p.N1726K within the gene coding for desmoplakin. We identified this variant also in 2 out of 380 blood donors. This variant is already registered in an ARVC database (21).

In patient #9, we found the variant p.G2844V. However, this variant did not cosegregate with the disease within the family tree. Three male patients died from SCD in this family (aged 28–37 years). ARVC was confirmed by autopsy, and genomic DNA was available from two of them. The variant p.G2844V was not found in these individuals. In addition, it could not be detected in 360 blood donor controls.

Desmin (DES). In our cohort, we identified a mutation in the *DES* gene in a single ARVC patient (#2) only. Sequence

Table 2. Overview on gene mutations/variations

Gene	Variation[a]	AA substitution[a,b]	Exon	Patient ID	References	Mutation/GUS/polymorphism
DSG2	1174G>A	V392I	9	1	(9,25)	M
DSG2	3G>A	M1I	1	13	(25)	M
DSC2	1912_1917delAGAA[hom]	Q638LfsX647[hom]	13	22	Novel	M
PKP2	235C>T	R79X	2	5	(6,29,30)	M
PKP2	2176C>T	Q726X	11	10	(6)	M
PKP2	658C>T	Q220X	3	11	Novel	M
PKP2	1803delC	D601EfsX655	8	21	Novel	M
DES	347A>G	N116S	1	2	Novel	M
DSG2	1480G>A	D494N	11	13	(21)	GUS
DSP	8531G>T	G2844V	24	9	Novel	GUS
PKP2	2431C>A	R811S	12	18	(28)	GUS
DSG2	2137G>A	E713K	14	15	(7,22)	P
DSC2	2687_2688insGA	A897Kfs901	17	14	(10,27,28)	P
DSC2	2393G>A	R798Q	15	19, 7	novel	P
PKP2	76G>A	D26N	1	03	(29)	P
DSP	5178C>A	N1726K	23	16	(21)	P

[a]Nomenclature of sequence and amino acid changes according the specifications of the Human Genome Variation Society (HGV), all variants were heterozygous as not otherwise mentioned; [hom] = homozygous variant; [b]amino acid substitution; M, mutation; GUS, genetic variant of unknown significance; P, polymorphism. Genes: *DSG2*, *desmoglein 2*; *DSC2*, *desmocollin 2*; *PKP2*, *plakophilin 2*; *DSP*, *desmoplakin*; *DES*, *desmin*.

analysis of *DES* revealed the missense mutation p.N116S (Fig. 1), whereas we did not find any variant among the desmosomal genes (*JUP*, *DSG2*, *DSC2*, *PKP2*, *DSP*). The patient history revealed several episodes of syncopes, and task force criteria for ARVC were fulfilled [right ventricular enddiastolic diameter (RVEDD) = 45 mm, left ventricular end-diastolic diameter = 52 mm, biopsy with fibro-fatty replacement, electrocardiography with T-wave inversion in V4, V5 and V6]. The patient was transplanted at the age of 17 due to terminal heart failure. The patient was re-transplanted at the age of 21, because the donor heart was actually rejected.

Due to the identified *DES* mutation, the patient was also examined for skeletal muscle disease. At the age of 19, the patient started to complain of exercise-induced muscle pain, fatigability and weakness when walking uphill or climbing stairs. Neurological examination at the age of 20 revealed only a slight proximal weakness of the lower extremities and that her standing up from a squatting position was slowed.

Cardiac and skeletal muscle pathology of the desmin-mutation carrier. Immunohistochemical studies of myocardial sections revealed accumulation of desmin and myotilin immunoreactive aggresomes in the RV and left ventricle (LV) of the *DES* N116S heart after HTx. Of note, the aggresome formation appeared to be more prominent in the RV compared with the LV myocardium. In contrast in myocardial sections of a non-failing control heart, an even distribution of protein without signs of the aggresome formation was observed (Fig. 2).

Skeletal muscle biopsy from the quadriceps muscle was performed at the age of 19. Light microscopic studies were unremarkable with the exception of type 1 fiber predominance and some diffusely distributed rounded atrophic fibers. There were no inflammatory infiltrations, necroses, basophilic regenerating fibers, centrally located nuclei or structural changes of muscle fibers. Morphological studies revealed a diameter of type 1 fibers between 11 and 76 μm (normal: 30–70 μm) and diameter of type 2 fibers between 14 and 67 μm, thus indicating an unspecific fiber atrophy (see Supplementary Material, Figure). Immunohistochemical stainings of skeletal muscle revealed some fibers with intracellular desmin and myotilin-positive aggresomes typically seen in patients with myofibrillar myopathies (Fig. 3).

N116 of desmin is located within the 1A segment of the rod domain in the amino acid motif 'LNDR', which is absolutely conserved in the IF protein family (31, 32). Of note, there was no history of ARVC or arrhythmias within the family, which was supported by DNA sequencing of her parents and her sister. Thus, the *DES*-variant N116S in this patient was found to be a spontaneous mutation.

Viscosimetric measurements of desmin mutant protein. Specific viscosity of the N116S mutation on desmin filament formation was investigated using, in *Escherichia coli*, expressed, purified full-length WT and mutant as well as various mixtures of desmin (75:25 and 50:50, respectively; Fig. 4). WT desmin exhibited an increase of specific viscosity over a period of 40–45 min before reaching a plateau. In contrast, the specific viscosity of N116S desmin transiently increased within the first 10 min and declined almost to a pre-assembled value (0.02–0.04). These data reveal disturbances of the filament formation of the mutant desmin *in vitro*. Intermediate kinetics were observed using mixtures of WT and N116S desmin (Fig. 4).

Transfection studies in SW13 cells. To assess the ability of N116S desmin to assemble into a *de novo* filament network, WT and mutant desmin expression vectors were transiently transfected into IF-free SW13 human adrenocortical carcinoma cells (33). In contrast to the WT, N116S desmin failed to develop an extended filamentous network as characterized by protein aggresomes throughout the cytoplasm (Fig. 5D and E). Desmin staining was not detectable in

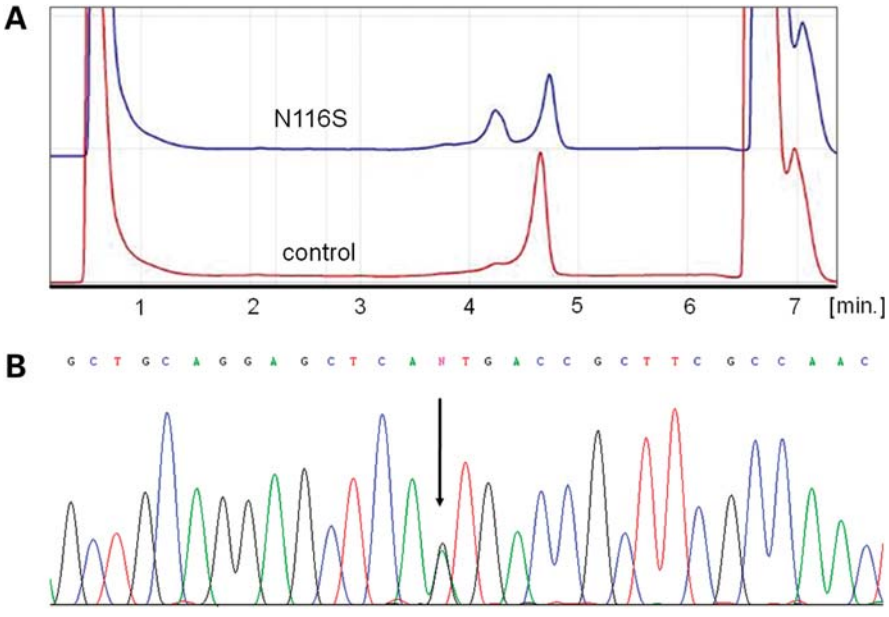

Figure 1. Identification and verification of the *DES* mutation N116S. (**A**) Temperature-modulated heteroduplex analysis showing the WT profile (nucleotide 134–366 of exon 1, control) and the variant profile (N116S) at 64.5°C. (**B**) DNA sequence showing the heterozygous A>G variant at nucleotide 347 of *DES* exon 1 (arrow) resulting in the amino acid substitution p.N116S.

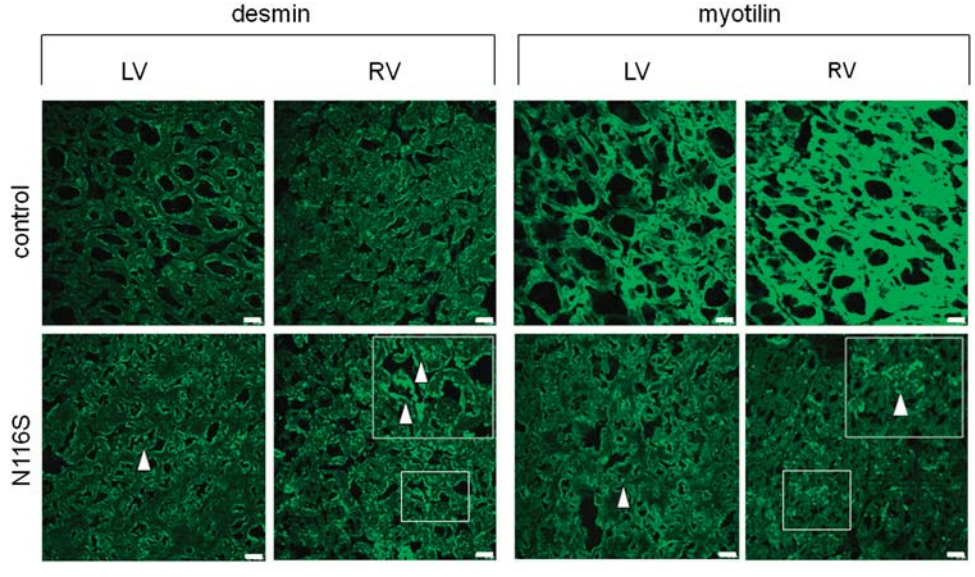

Figure 2. Immunofluorescence staining of myocardial sections using confocal microscopy. Desmin (left panel) and myotilin (right panel) staining of right (RV) and LV myocardium of a non-failing heart (control) and the N116S mutant heart. The control myocardium shows an even distribution of both proteins, whereas desmin and myotilin positive aggresomes (arrowheads) were found in the *DES* N116S heart (see insets for higher magnification of frames). Desmin and myotilin aggregation appears to be more pronounced in the right ventricle. The tissue sections were stained with antibodies against desmin or myotilin using a biotinylated secondary antibody in conjunction with Cy2-conjugated streptavidin. No fluorescence signals were detected when staining with the secondary antibody alone (dat not shown). Scale bar = 20 μm.

expression vector-transfected (Fig. 5C) and -untransfected controls (Fig. 5A).

Atomic force microscopic imaging of desmin filaments. At a relatively low surface coverage with desmin, distinct differences between the WT and the mutant desmin could be identified (Fig. 6A and B). Both desmin variants exhibited straight linear filaments that self-assemble into a protein network structure. In addition, the N116S desmin showed distinct superimposed coiled-filament morphology. At larger surface coverage, the N116S variant also exhibited larger fibrous protein aggregates that could be identified in atomic force microscopy (AFM) topography and phase images (Fig. 6C and D).

Thus, our *in vitro* AFM data are in accordance with the *in situ* histological findings and underline that the mutation

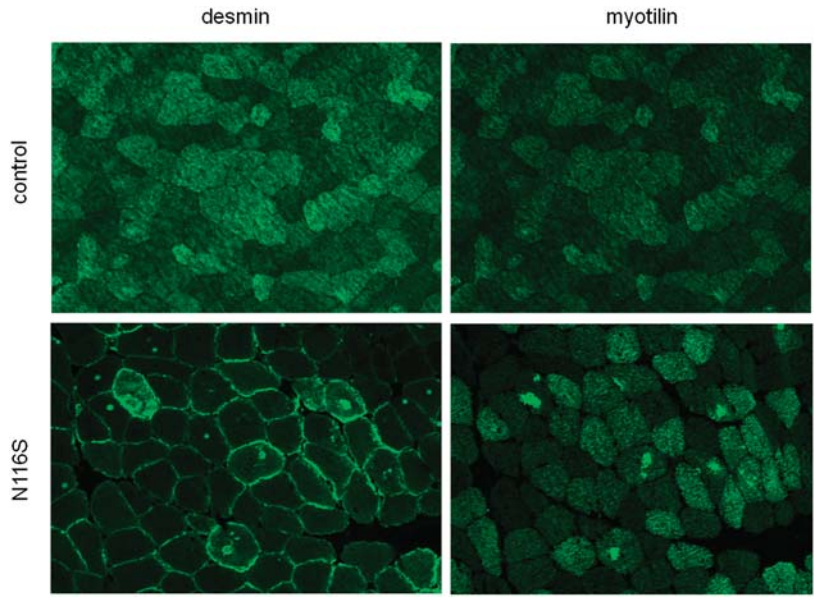

Figure 3. Immunohistology of biopsies derived from *M. quadriceps* of healthy control (upper row) and the ARVC patient (*DES* N116S). Desmin and myotilin staining of tissue slices reveals the aggresome formation in skeletal muscle fibers. Tissue slices were stained with antibodies against desmin (left) or myotilin (right) using a biotinylated secondary antibody in conjunction with Cy2-conjugated streptavidin. No fluorescence signals were detected when staining with secondary antibody alone (data not shown).

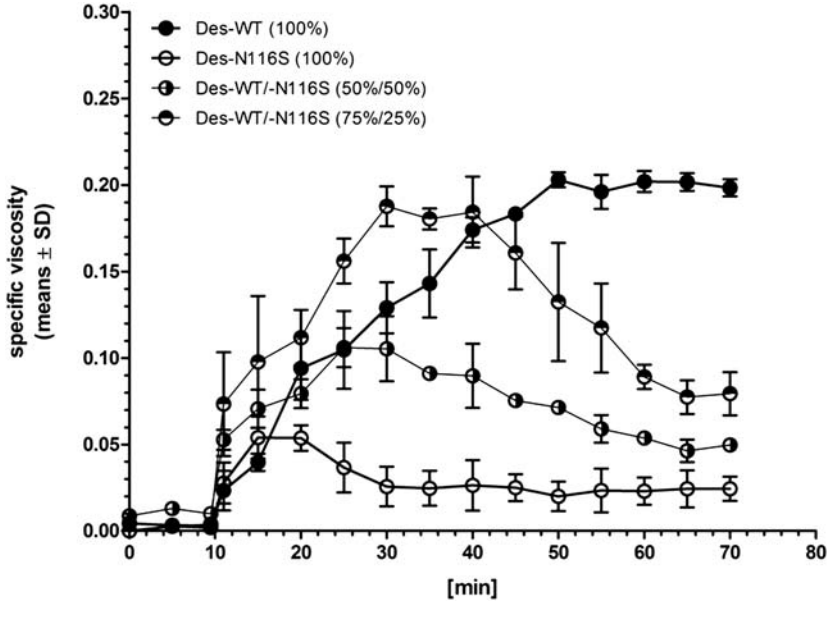

Figure 4. Viscosimetry of recombinant WT and N116S desmin. Specific viscosity was measured after 1 min and in 5 min intervals for 60 min after assembly reaction was started. The time course of the specific viscosity of WT desmin reflects the expected increase after assembly start. In contrast, specific viscosity of the mutant protein increases transiently and remains on a constant low level 30 min after the start of the assembly. Mixtures of WT and mutant desmin present an intermediate specific viscosity accordingly.

N116S leads to distinct disturbances of desmin filament formation and aggresome formation. N116S is therefore a pathogenic variant causing the cardiac and skeletal muscle phenotype of the ARVC patient.

DISCUSSION

ARVC is an inherited cardiomyopathy, which is believed to be caused mainly by mutations in proteins of the cardiac desmosomes. However, up to now, three other non-desmosomal genes were reported to carry mutations associated with ARVC in rare cases. One is the gene encoding the *cardiac ryanodine receptor 2* (*RYR2*) (34, 35), which mediates intracellular calcium release during excitation–contraction coupling. Another study characterized promotor mutations in the *transforming growth factor beta-3* gene associated with ARVC (36). However, the impact and significance of these mutations remain unclear. The third gene defect concerns

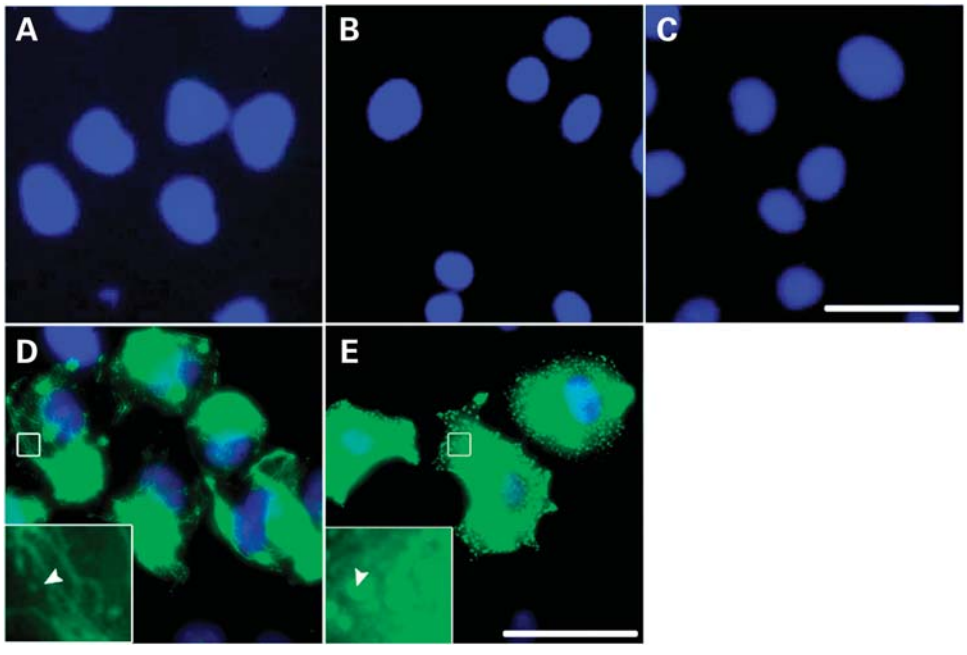

Figure 5. Immunofluorescence microscopy of desmin-transfected SW13 cells and staining by anti-desmin antibody. Desmin WT and N116S cDNA were cloned into pLPCX expression vector for transfection. The filamentous network formation was observed in SW13 cells transfected with desmin WT (**D**), whereas the aggresome formation was found in cells transfected with the mutant desmin N116S (**E**). As a control, no staining was observed in untransfected cells (**A**) and transfected with the vector pLPCX (**C**). Anti-desmin staining of untransfected SW13 is shown in (**B**). Scale bar = 50 μm.

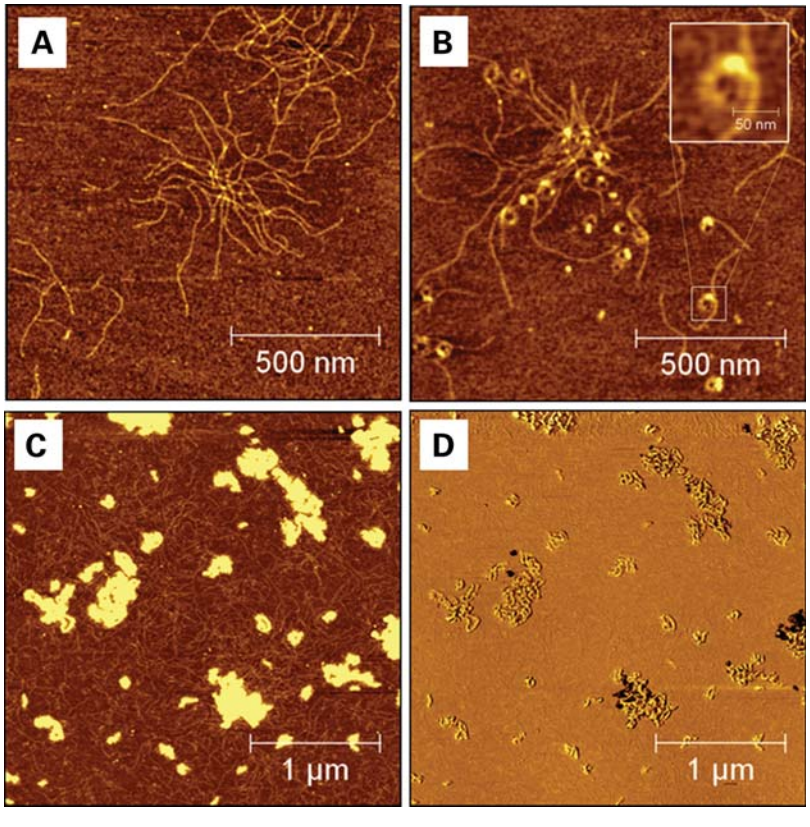

Figure 6. AFM topview images of WT and N116S mutated desmin filaments. (**A**) AFM topography image of low coverage WT desmin yielding a network of straight linear filaments. (**B**) Low coverage of N116S mutated desmin results in an analogous desmin filament network and a superimposed coiled superstructure (see inset). (**C**) AFM topographs of N116S desmin yielding a filament network structure with larger protein aggregates. (**D**) Corresponding AFM phase image of (C) representing relative material contrast of N116S desmin unveiling a filament network structure with large filamentous protein aggregates. Vertical color scale is 1 nm for all topography images.

the *transmembrane protein 43* (*TMEM43*) which is predicted to be an integral inner nuclear membrane protein with currently unknown function (37).

Although mutations in desmosomal genes were found in a considerable number of cases in ARVC patients, the genetic examination does not lead to the identification of relevant mutations in the majority of ARVC cases (4, 5). Here we present data of the genes *JUP*, *DSG2*, *DSC2*, *DSP* and *PKP2* in patients referred to our heart center for genetic screening due to ARVC. All patients were classified according to the novel task force criteria (Table 1) (38). In about 30% of the patients, we identified mutations in these desmosomal genes.

In *DSG2*, we identified three previously published mutations/variations. V392I located within a functional and conserved cadherin domain has already been identified earlier as a pathogenic mutation in several English (9, 25) and Dutch (26) ARVC patients. However, in contrast to Syrris *et al*. (25), Bauce *et al.* (9) and Bhuiyan *et al.* (26) who did not find the mutation in 200, 250 and 150 healthy controls, respectively, we found this mutation once in 338 blood donors (0.3%). As this frequency is comparable with the estimated prevalence of ARVC, we have classified this variant as a mutation (Table 2). However, the disease association of p.V392I should be confirmed in future studies.

In this study, the previously described mutation *DSG2* p.M1I (25) and the currently undetermined variant p.D494M (21) were identified in an 18-year-old patient (#13), with a definite diagnosis of ARVC, including a positive family history with a severe clinical phenotype. In contrast, Syrris *et al*. (25) identified the p.M1I mutation within a proband aged 14 years with features of ARVC, who stayed clinically stable during 10 years of follow-up after ICD implantation. In that study relatives identified as carriers for the family typical mutation presented variable disease expression. The mutation p.M1I is predicted to abolish translation initiation and protein export thus causing a haplo-insufficiency with unknown effects on cell function and intercellular cell contact.

Variant p.D494M affected a conserved amino acid among mammals (*Pan troglodytes*, *Canis lupus familiaris*, *Bos taurus*, *Mus musculus*, *Rattus norvegicus*, data not shown) located immediately behind the cadherin domain 4 of desmoglein 2. Because no haplotype analysis has been performed yet it is unknown whether the p.M1I and p.D494M variants affect the same *DSG2* allele. Due to the severe clinical phenotype of patient #13, carrying the M1I mutation, the additional p.D494M variant might have an impact as a modifier.

In *DSC2*, a novel homozygous deletion mutation p.Q638LfsX647 was identified in a 62-year-old female with definite diagnosis of ARVC. A recessive desmosomal mutation has already been reported in non-syndromic ARVC (15). To assess the significance of the mutation, e.g. the origin and mode of inheritance, further genotyping of family members and haplotype analysis could not be realized yet. Substitution of the amino acid glutamine by histidine at position 638 of desmocollin 2 has been reported twice in US collectives of ARVC probands. However, the authors of the studies differently assessed the significance of the mutation. Den Haan *et al.* (39) classified p.Q638H as a non-conserved desmosome protein variant, whereas Xu *et al.* (8) regarded it as a desmosomal gene mutation. Deletion mutation p.Q638LfsX647 identified in this study relates to the same gene locus but is predicted to cause a frameshift. The predicted protein missed the typical cadherin transmembrane domain because a premature stop codon shortened the protein by 256 amino acids at the C-terminus. The effect on intercellular contact, desomosome formation and possibly functional compensation by other (desmosomal) proteins remains to be determined.

Two novel *PKP2* mutations Q220X and D601EfsX55 were also identified. In agreement with the data from Gerull *et al.* (6), most of the identified *PKP2* mutations in our cohort were frameshift mutations or premature stop codons. We also found that the *PKP2* mutation Q726X did not lead to a truncated version of PKP2, because the mutated short version of PKP2 was not detectable by western blotting in myocardial extracts of the patient's explanted heart (data not shown).

The variant p.R811S was previously reported to be pathogenic (28). Although we did not identify p.R811S in 397 blood donors, we classified it as a genetic variant of unknown significance (GUS), since it is not conserved among mammals and does not affect a known functional domain of plakophilin 2.

In *DSP*, we identified the gene variation p.G2844V located within the C-terminal part of desmoplakin. The C-terminus of desmoplakin contains the amino acid repeat GSRS (40) wherein variation p.G2844V affects the glycine of the sixth repeat. Although the importance of the repeats is not yet clarified, there is evidence that the C-terminus of desmoplakin encompasses sequences critical for binding to IFs (41). Although the variant was located within a functionally relevant protein region and was not identified in controls, it was classified as a GUS, since it was not identified in two family members who died of SCD. Whether the variant is a rare polymorphism or a family-specific gene variation is currently not known.

We further analyzed in this cohort desmin as a candidate gene, which is functionally related to the intercalated disc and speculated that desmin, which is a cytoskeletal filament protein located in the myocardial z- and intercalated disc and connected to the desomosomal plaque proteins via desmoplakin (41), might also carry mutations in ARVC patients. This approach was supported by a recent report on ARVC cases in a family affected by desmin-related myopathy. However, linkage analysis of that family indicated that the genetic defect was located on chromosome 10 (42) and not related to mutations in desmin, which is coded by 2q35. Further evidence of an association of mutations in *DES* with ARVC was recently published by van Tintelen *et al.* (20). However, they found the mutation p.S13F in *DES*, which is located outside of the rod in the N-terminal head domain of desmin in a large family. The mutation was frequently associated with a myopathy and with a broad spectrum of cardiomyopathies. The desmin aggresome formation was also found within the myocardium. More than 50% of the family members presented dilated or hypertrophic cardiomyopathy with conduction delay phenotypes, whereas ARVC was found in only two cases. Patients with a predominantly LV phenotype experienced a RV failure during the course of the

disease. Interestingly, conduction delays were found to be early signs of the disease.

The same group published two other mutations in *DES* p.N342D and p.R454W, which were located in the segment 2B and in the tail domain of desmin, respectively. However, patients carrying these mutations presented *biventricular* dilatation (43). The authors found that carriers of the p.N342D allele present a predominantly RV cardiomyopathy, myopathy and a LV dilatation during the course of the disease. The mutation p.N342D was previously known to be associated with an isolated skeletal muscle phenotype (44). Biventricular dilatation was found in the carrier of p.R454W with unknown skeletal muscle involvement. This mutation was detected previously in the non-helical tail domain of desmin and was linked to a hypertrophic obstructive cardiomyopathy with later onset of a myopathy (45). Thus, the clinical phenotype of ARVC-related *DES* mutations differs considerably.

We screened 22 unrelated patients referred to our institute for genetic screening on ARVC-related mutations. We identified in *DES* in a single 17-year-old ARVC patient, who survived several syncopes and was transplanted in our center, the novel missense mutation p.N116S. Of note, the family history did not provide further evidence that the cardiomyopathy of the patient was inherited. This was supported by the results of the genetic testing in members of the family, which revealed that this mutation was indeed a spontaneous mutation not found in other relatives. Therefore, we could not prove the pathogeneity of the mutation by co-segregation analysis within the family tree.

However, the identified mutation is located in segment 1A of the rod domain of desmin. This part of the molecule is involved in elongation of the filament and the dimer formation (31, 32). Two other mutations were published in this part of desmin. The mutation p.E108K was claimed to be the first mutation ever found in segment 1A in a patient with dilated cardiomyopathy (17). The amino acid E108 is part of the heptade, which appears to be responsible for the hydrophobic seam connecting the desmin α-helices within the filament's supercoil. Another mutation of segment 1A (p.E114del) was recently identified in a Uruguayan family affected by myopathy and severe cardiomyopathy. Members of this family displayed arrhythmias, conduction block and SCD. The deletion of codon 114 was supposed to disturb the filament formation, since the heptade of the coiled-coil will be impaired (46).

The mutation p.N116S, which was identified in our study, belongs to the consensus sequence 'LNDR', which is absolutely conserved in IF proteins among eukaryotes. Even the lamin sequence of the coelenterate *Hydra vulgaris* contains this motif in its rod domain.

We cloned the full-length cDNA of desmin in *E. coli*, introduced the mutation and purified the protein for *in vitro* analysis of the protein function. We found that the mutated protein revealed impaired filament formation measured by viscosimetry. We further transfected the cDNAs of WT and mutated desmin in SW13 cells, which do not contain endogeneous desmin. SW13 transfected with the WT desmin revealed the filament formation, whereas the mutant p.N116S produced desmin aggresomes within the cytoplasm. The data found *in vitro* and in cell culture were in close correlation with the histological examinations of skeletal and cardiac muscle of the ARVC patient, which revealed that desmin deposits were present in both muscle systems. Of note, the RV myocardium of the explanted failing heart revealed an apparently higher density of desmin aggresomes compared with the LV.

Mutations recently published in association with ARVC were partly tested in *in vitro* test systems. The mutation p.R453W was analysed by viscosimetry and failed to form filaments. However, in combination with the WT protein p.R453W leads to final blockade of the capillary. This mutant also formed aggresomes in SW13 cells (45). The mutation p.N342D was not checked by viscosimetry but still revealed a lack of filament network formation in SW13 cells (44). The ARVC-related *DES* mutation p.S13F was characterized *in vitro* by transfection experiments in SW13 cells and leads to the aggresome formation, which was also found in myocardial slices of mutation carriers (20,47). We compared the profile of our viscosimetric data with that of other mutations and found that the mutant p.N116S revealed a time course comparable to the mutant p.A357P. However, this mutant was published in association with a myopathy (17,48). Therefore, the clinical phenotype of *DES* mutations is currently not predictable from *in vitro* characterizations of desmin.

As a consequence of the *in vitro* findings in desmin, the patient was examined after HTx on a skeletal myopathy as well. Although the histological examination of the skeletal muscle biopsies uncovered an aberrant desmin aggregation and unspecific fiber atrophy, clinical examination of the patient did not provide an overt myopathy. Thus, the histopathological findings of the skeletal muscle can be interpreted as subclinical alterations.

In conclusion, genotyping of variants in desmosomal proteins analyzed in this study leads to the identification of disease-causing mutations in a minority of ARVC patients. Thus, additional genes will be identified, which cause ARVC. We could further provide evidence that a novel mutation in *DES* causes the phenotype of ARVC. Mutations in *DES* cause a broad spectrum of myopathies and especially cardiomyopathies, which might be associated with rhythm disorders, conduction delays and RV failure. Of note, mutations displaying an arrhythmogenic and/or RV phenotype are found in all domains of the protein and are not located in hotspots.

Limitations of the study

We provided data on a limited cohort size from a single heart center and did not present clinical data on the control cohort of blood donors, since personal data were not available due to blinding of the DNA samples. In this study, we could also not exclude large genomic duplications or inversions. Cellular and animal models bearing variations may be helpful in future to examine the impact on cellular level to some extent.

MATERIAL AND METHODS

Patient cohort

We included 23 unrelated ARVC patients (16 males, 7 females) aged between 17 and 73 years. Nineteen patients

(about 80%) were from the Heart and Diabetes Center North Rhine Westphalia (HDZ-NRW), Germany. Of these patients, 22 were screened for all desmosomal genes causing ARVC and the gene *DES,* whereas patient #5 was screened only for mutations in *PKP2* and *DES* due to compliance problems. Patients were classified according to the revised task force criteria for ARVC (38) (Table 1). In addition, ARVC diagnosis of six patients who received HTx in our center was confirmed by pathological structural examination of the explanted heart, since we analysed a considerable number of index patients with *terminal* heart failure (Supplementary Material, Table S1). The carrier of the *DES* mutation was additionally examined for a myopathy by histology of a skeletal muscle biopsy from the *M. quadriceps*.

All members included in the study were of Caucasian origin. Written informed consent was obtained from all participants. The study was approved by the local ethics committee.

Mutation detection

Blood samples were collected from the affected individuals and DNA was extracted from white blood cells using standard techniques (Illustra^{TM} blood genomic Prep Mini Spin Kit, GE Healthcare, Buckinghamshire, UK). Mutation screening of five desmosomal genes was performed by direct sequencing. The *desmin* gene was screened by dHPLC.

The genomic sequences used to design the primers were obtained from sequences in the GenBank database on the NCBI website (www.ncbi.nih.gov/projects/genome/guide/human). Based on the published sequence of *plakophilin 2* (NM_024422), *plakoglobin* (NM_004572), *desmoplakin* (NM_004415), *desmocollin 2* (NM_024422), *desmoglein 2* (NM_001943) and *desmin* (NM_1927), amplification and sequencing of the exonic and adjacent intronic sequences were carried out following standard protocols. After amplification, PCR products were purified and labeled using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, CA, USA) and sequenced in both directions on an ABI 310 genetic analyzer. Sequencing electropherograms were inspected manually and analyzed with Variant Reporter Software v1.0 (Applied Biosystems).

DES mutation screening was done by dHPLC using a DNASep column with a WAVE DNA Fragment Analysis System (Transgenomic Inc., NE, USA) as described previously (35). The analytical temperatures for each exon are available from the authors upon request. Exons with aberrant, temperature-modulated heteroduplex profile were sequenced in both directions.

For all mutations, at least 640 chromosomes from ethnically matched, healthy individuals were genotyped using TaqMan SNP genotyping assay (Applied Biosystems) according to the manufacturer's protocol.

Tissue immunohistochemistry

Immunofluorescence studies were performed on 5 μm thick frozen serial sections of left and RV heart muscle tissue provided after HTx or of skeletal muscle tissue (M. quadriceps), respectively. The following primary antibodies were used in this study: (i) mouse monoclonal anti-desmin antibody D33 (dilution 1:500; M0760, DakoCytomation, Germany) and (ii) mouse monoclonal anti-myotilin antibody (dilution 1:20; NCL-MYOTILIN**,** Leica Microsystems, UK). As secondary antibody sheep anti-mouse Ig biotinylated antibody (RPN1001, GE Healthcare Bio-Sciences AB, Sweden) in combination with Cy2-conjugated streptavidin (both dilution 1:100) (016-220-084, Jackson ImmunoResearch, USA) was used. Immunostaining was performed according to the recommendations of the manufacturers. Staining was evaluated with a laser scanning spectral confocal microscope (Leica TCS *SP2*, Leica Microsystems, Germany).

Cloning and mutagenesis

The full-length cDNA of desmin wild-type (Des WT) was amplified by polymerase chain reaction (PCR) using SC319574 vector DNA (human desmin cDNA in pCMV6-AC; OriGene, USA) as a template and complementary primers. The *DES* N116S (*Des* N116S) mutation was introduced by two-step PCR [overlap extension approach (49)], with the use of oligonucleotides containing the adenine-to-guanine transition at nucleotide position 347. For expression in *E. coli* BL21 (DE3) cells (Invitrogen, USA), amplified Des WT and N116S cDNA fragments were cloned into the bacterial T7 promoter expression vector *pET100*/D-TOPO (Invitrogen), which fuses a His_6 and Xpress epitope tag to the N-terminus of *inserted fragments.* For transfection of SW13 cells, both Des WT and N116S cDNA were cloned into the eukaryotic CMV promoter expression vector pLPCX *(Clontech*, USA) using pET100/D-Des WT or pET100/D-Des N116S as a template in addition to modified sequence-specific primers to generate a C-terminal *Cla*I restriction site during PCR. Fragments were cloned first into pCRII-TOPO (Invitrogen) followed by subcloning into pLPCX using *Eco*RI and *Cla*I restriction sites. The accuracy of all clones was controlled by sequencing.

Protein expression and purification

Recombinant protein expression in *E. coli* BL21 was induced according the recommendations of the manufacturers and verified by SDS–PAGE.

Recombinant desmin WT and N116S proteins were isolated from inclusion bodies as described previously (50) with the modification that the pellet finally was dissolved in 8 M urea, 10 mM Tris–HCl (pH 8.0), 100 mM NaH_2PO_4, 15 mM β-mercaptoethanol. Purification under denaturing conditions via Ni-NTA affinity chromatography (Ni-NTA agarose; Qiagen, Germany) was performed according to the manufacturer's instructions. For further purification, proteins were dialysed against the target buffer 8 M urea, 20 mM Tris–HCl (pH 7.5) and 1 mM DTT and supplied to a HiTrap DEAE Sepharose Fast Flow column (GE Healthcare Bio-Sciences AB). Proteins were eluted by a linear salt gradient (0–0.3 mM NaCl). Fractions were analyzed by SDS–PAGE and those containing pure desmin WT or N116S were pooled. Protein concentrations were measured by the method of Bradford (51). As determined by anti-desmin immunoblot analysis,

both vectors generated a single protein of the expected 52 kDa size (data not shown).

In vitro IF assembly and viscosity measurements

Purified, recombinant desmin WT and N116S were dialyzed stepwise against two set of buffer, that is, 4 M urea, 5 mM Tris–HCl (pH 8.4) and 2 M urea and 5 mM Tris–HCl (pH 8.4), for 1 h at room temperature and finally for overnight at 4°C against 5 mM Tris–HCl (pH 8.4), 1 mM EDTA and 0.1 mM EGTA, all buffers containing 1 mM DTT. Before use, the recombinant protein samples were dialyzed for 1 h at room temperature against assembly buffer [5 mM Tris–HCl (pH 8.4), 1 mM DTT] (52). Desmin forms stable tetramers in assembly buffer, as reported (53). Viscosity measurements were performed at a protein concentration of 1 mg/ml in an Ostwald viscometer (Cannon, USA) with a sample volume of 1 ml at 22°C. Assembly studies were performed as described (52). Assembly of IFs was induced at the 10 min time point by the addition of 1/10 volume of assembly start buffer [0.2 M Tris–HCl (pH 7.0) and 0.5 M NaCl leading to the final concentration of 25 mM Tris–HCl (pH 7.5), 50 mM NaCl]. Specific viscosity (η_{sp}) was calculated by $\eta_{sp} = (t_s - t_b)/t_b$ (t_s is the flow time of the sample and t_b the flow time of the buffer) (52). The flow time was measured before (time point 0, 5 and 9 min), 1 min after assembly start and then every 5 min over a period of 1 h.

Transient transfection of cultured cells and immunofluorescene staining

Desmin- and vimentin-free human adrenocortical carcinoma cells SW13 (ATCC, USA) (33) were used for recombinant desmin cDNA transfection in a cell culture assay. Cells were cultivated in Leibovitz's L-15 medium supplemented with 10% FCS, 1% *penicillin/streptomycin* and 1% amphotericin B in T-75 flasks without filters (no air exchange) in a 5% CO_2 incubator at 37°C. For transfection, cells were grown in six-well plates on glass cover slips in growth media with 10% sodium bicarbonate added. Transfection was performed with the FuGENE6 transfection reagent (Roche Diagnostics Corp., USA) according to the manufacturer's protocol. Forty-eight hours after cDNA, transfection cells were fixed in 100% ethanol (−20°C) for 15 min, permeabilized for 15 min by 0.1% Triton X-100 and blocked for 30 min in 1% bovine serum albumin in phosphate-buffered saline (PBS). For immunostaining, the cover slips were incubated with the monoclonal anti-desmin D33 antibody (1:500 overnight at 4°C). After thoroughly rinsing in PBS, sheep anti-mouse Ig biotinylated antibody and Cy2-conjugated streptavidin, both diluted 1:100, were applied consecutively for 1 h at room temperature. Nuclear DNA was stained with 4,6-diamidino-2-phenylindole (DAPI). The cover slips were mounted on glass slides in Dako fluorescence mounting medium. For evaluation of transfected cells, fluorescence microscopy (Eclipse TE2000-U; Nikon, Japan) with Lucia G image editing software 5.30 (Nikon) was used.

Atomic force microscopic imaging

Purified desmin was analysed by AFM. Desmin filament self-assembly was done according to a recently published procedure (48). Briefly, equal volumes of assembly buffer were added to the protein solution containing 0.54 mg/μl of WT and 0.58 mg/μl of mutated desmin and heated to 37°C for 1 h. An aliquot of 1 μl was applied to APTES (3-aminopropyl-triethoxysilane; Fluka, Steinheim, Germany) functionalized mica (Plano, Germany) (54) for 10 min at room temperature. Afterwards, the samples were rinsed with deionized water (Millipore, USA) to remove weakly adsorbed proteins and finally dried under a gentle flow of nitrogen gas. AFM imaging was performed under ambient conditions in a tapping mode with a scan-line frequency of 0.5–1 Hz using non-contact aluminum-back side coated silicon cantilevers exhibiting a spring constant of 40 N/m and 300 kHz resonant frequency (Budget Sensors, Bulgaria) with a commercial AFM system (Nanoscope Multimode IIIa, Veeco Instruments, USA). Raw-data AFM images were processed by background removal (flattening), low-pass filtering and partly single scan-line error correction using the microscope manufacturer's image processing software. All AFM data are top-view represented where the vertical *z*-scale (topography) is color encoded.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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