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Running title: A novel mutation in the *DSP* gene causes ARVC

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ABSTRACT

Introduction. Arrhythmogenic right ventricular cardiomyopathy is an inherited disease characterized by a progressive myocardium fibrofatty replacement. This abnormality disrupts electrical transmission causing ventricular arrhythmias and sudden cardiac death. This genetic disease is transmitted mainly with an autosomal dominant pattern. Our aim was to identify the genetic defect responsible for the pathology in a Spanish family, and to perform its phenotype connotations. **Material and Methods.** A total of 15 individuals in a three-generation Spanish family were screened after the sudden cardiac death of one family member. All they underwent a complete physical examination, 12-lead electrocardiogram, 2-dimentional echocardiography, magnetic resonance imaging, exercise stress test, 24-hour Holter and genetic testing. **Results.** Autopsy revealed the presence of biventricular arrhythmogenic dysplasia in deceased member. Six family members showed clinical symptoms but only three of them fulfilled definite diagnostic criteria of the disease. Genetic analysis showed a novel *nonsense* genetic variation in nine family members. All family members with clinical symptoms carried the genetic variation. **Conclusions.** Genetic testing in families affected by arrhythmogenic right ventricular cardiomyopathy helps to identify the genetic cause responsible for the disease. The incomplete penetrance and variable phenotypic expression highlights the need of comprehensive genetic analysis and further phenotype implications of genetics to clarify the pathophysiology of the disease.

Keywords

Arrhythmogenic right ventricular cardiomyopathy, desmoplakin, genetic testing, sudden cardiac death

INTRODUCTION

Arrhythmogenic right ventricular cardiomyopathy (ARVC, MIM 10790) is characterized by a progressive replacement of myocytes by fibro-adipose tissue. The pathological findings are usually present in the right ventricle (RV), however up to 50% of cases may also show a left ventricular affectation (biventricular dysplasia) [1]. The presence of fibrofatty tissue in the myocardium impairs cell-to-cell electrical signalling, cause ventricular arrhythmias and even sudden cardiac death (SCD) [2]. The National Center for Biotechnology Information (NCBI) establishes its prevalence between 1:1000 and 1:5000, being more common in young men (3:1) [3]. Unfortunately, sudden death may be the first symptom in ARVC cases, being responsible for 5% of all SCD cases every year [4].

The diagnostic criteria for ARVC were recently improved, increasing the sensitivity for diagnosis in relatives of affected patients [5]. The disease has an autosomal dominant inheritance, with incomplete penetrance and a wide variety of clinical manifestations. Genetic testing can help ensuring a proper diagnosis, not only for the index case but also for family members. Up to 60% of ARVC patients are carriers of mutations, mainly in genes encoding for desmosomal proteins. Mutations in plakophilin (*PKP2)* gene are found in nearly 30-35% of ARVC patients, followed by desmoplakin (*DSP)* (10-15%), desmoglein (*DSG2)* (10%) and desmocollin (*DSC2)* (2-5%) genes [6]. In addition, non-desmosomal genes have been also identified responsible for the ARVC (*TMEM43, TGFB3, TP63* and *DES*) but with a lower incidence (<1%). Recently, mutations in titin protein (*TTN*) [7] and lamin A/C protein (*LMNA*) [8] have been described in ARVC patients although further studies are required to confirm the gene-disease relationship. Therefore, around 35% of ARVC patients remain without a genetic diagnosis [9]. In the present study we report ARVC patients of a three-generation Spanish family who carry a novel *nonsense* genetic variation in *DSP* gene.

MATERIAL

The present study was approved by the ethics committee of the Hospital Josep Trueta (Girona, Spain), and informed consent was obtained from all participants. All individuals included in our study were assessed clinically at the Arrhythmia Unit, Hospital Clinic of Barcelona (Spain), and at Sudden Death Risk Unit Hospital La Fe of Valencia (Spain). Patients were all Caucasians and natives of Spain.

The index case (III.4) was identified after suffering an episode of SCD, with a positive autopsy for biventricular arrhythmogenic dysplasia. Family members were clinically diagnosed based on the revised Task Force Criteria (TFC) of the European Society of Cardiology/International Society and Federation of Cardiology [5]. Clinical data and pedigree from the family are shown in table 1 and figure 1. All family members underwent clinical evaluation, consisting of electrocardiogram (ECG), echocardiogram (ECHO), cardiac magnetic resonance (MR), exercise test (ET) and Holter (HO) (except I.1 due to old age and I.2, dead 20 years before).

METHODS

Genetic studies

Blood samples were processed and genomic DNA extracted using commercial protocols (PUREGENE DNA, QIAGEN). Subsequently amplification of each gene of interest was done by polymerase chain reaction (PCR), and purified by ExoSAP-IT (ISOGEN). The analysis of the exonic and intron-exon regions was performed by direct sequencing (Genetic Analyzer 3130XL, Applied Biosystems). The mother (II.4) of our index case (III.4) underwent genetic studies of *PKP2, DSP, DSC2, DSG2, TGFB3* and *LMNA*. Plakoglobin protein (*JUP* gene) was not studied because none patient in our cohort had phenotype of Naxos disease [10]. Each sequence was compared with the reference sequence (Ensembl genome browser): *PKP2*-ENST00000070846, *DSP*-ENST00000379802, *DSC2*-ENST00000379802, *DSG2*-ENST00000261590, *TGFβ3*-ENST00000238682 and *LMNA*-ENST00000368300.

For this novel genetic variation detected, genetic analysis was realized in 206 Spanish control subjects (412 control alleles) (individuals not related to any patient and of all them with Spanish ancestors). This novel genetic variation was not previously identified as shown in consulted genetic variants database as 1000 genomes (http://browser.1000genomes.org/) and Exome sequencing project (http://evs.gs.washington.edu/EVS/). In addition, alignment between species was also performed using UniProt database (http://www.uniprot.org/).

RESULTS

Clinical

The proband (index case), a 18 year-old male (III.4), was diagnosed post-mortem after suffering an episode of SCD immediately after exercise (handball training). He was asymptomatic before the SCD event. The autopsy revealed anatomopathological changes (biventricular fibro-fatty substitution), consistent with the diagnosis of biventricular arrhythmogenic dysplasia. Other possible causes of death were ruled out, including coronary disease. The event of SCD in this young individual led to the clinical investigation of first degree family members: parents (II.4 and II.5) and two sisters (III.5 and III.6) (Table 1; Figure 1).

The mother (II.4) of our index case, a 53-year-old female, suffered several syncopes not related to physical activity. Her ECG only showed low QRS voltages in DII, DIII and aVF leads, but typical ARVC ECG abnormalities were absent. An ECHO showed normal biventricular ejection fraction and aneurisms in apex and basal segments of RV plus parasternal long-axis view of the right ventricular outflow tract (RVOT) of 33 mm, and parasternal short-axis view RVOT of 39 mm. As a result, II.4 was diagnosed with ARVC fulfilling 2 major diagnostic criteria, namely regional dysfunction and structural alterations, and family history of SCD in first-degree relative with ARVC (confirmed after autopsy). Therefore, an implantable cardiac defibrillator (ICD) was implanted because of her symptoms and received sotalol as pharmacological treatment. The index case´s father (II.5) wasn't clinically evaluated because he was apparently healthy and without previous family history of ARVC or SCD. The index case had two sisters (III.5 and III.6). The older sister (III.5), a 15 year-old girl who practiced basketball on a regular basis, showed altered ECG: low QRS voltages in limb leads, flat T-waves in DIII and aVF, inverted T-waves from V4 to V6. The ECHO showed biventricular affectation with severe decrease of left ventricular ejection fraction. An ICD was implanted and the patient was followed at the cardiology unit. The younger sister (III.6, 11 year-old girl) was asymptomatic and showed a normal ECG, ECHO and MR.

Other eleven family members related to the proband´s mother (II.4) were referred to our hospital for screening (Table 1; Figure 1). Index case´s grandfather (I.1) not was clinically evaluated due to his old age (>82 years old). Index case´s grandmother died at age of 53 years due to complications

of mitral valve replacement (I.2). The index case (III.4) had three aunts (II.2, II.3 and II.9) and two uncles (II.6 and II.7) from his mother's side. Two aunts (II.2 and II.9) and two uncles (II.6 and II.7) suffered syncopes since young age. The II.2 showed an altered ECG and ECHO; MR evaluation revealed first signs of fibrosis but not in sufficient degree to classify the alteration as minor criteria (Figure 2). She received sotalol as pharmacological treatment. The II.6, II.7 and II.9 family members showed normal ECG, ECHO and MR. Clinical history showed that II.6 suffered syncopes from vasovagal origin, and II.7 suffered several syncopes of unknown origin. One aunt (II.3) remains asymptomatic.

Genetics

Proband´s mother (II.4) fulfilled clinical diagnostic criteria for ARVC, and after comprehensive genetic screening, revealed a novel *nonsense* genetic variation, c.2975 C>T, p.Q986X – p.Gln986STOP-) in the *DSP* gene (Figure 3). No other genetic variations with minor allele frequency (MAF) <0.01 were found in any of the studied genes. This premature stop codon causes a 986 aminoacid truncated protein in contrast to the 2871 aminoacids of wild type *DSP* gene. This truncated protein completely losses an evolutive highly conserved domains of union to intermediate filaments (central fibrous rod domain and plectin repeats) (Figure 3 and 4). In our Spanish family of 16 members, 14 were included both in the clinical and genetic study (except I.2 and III.4). The I.2 family member died 20 years before and no heart samples were collected at that time. We found that 9 family members carried the same genetic variation identified in mother's index case (II.4) (Table 1; Figure 1).

Phenotype heterogeneity

ARVC was diagnosed both in the proband (III.4 autopsy analysis revealed biventricular involvement) and in 3 other family members (II.2, II.4 and III.5). All these three family members also carry the same genetic *nonsense* variation. Genetic analysis in the index case (III.4) was not carried out due to lack of tissue sample. Two family members (II.7 and II.9) carry the same novel genetic variation and showed syncopal episodes at young age although they didn't fulfil ARVC

diagnostic criteria (Table 1). Both individuals have been instructed to limit the practice of exercise in agreement with international ARVC guidelines and are clinically followed on a regular basis because being a carrier of a causative mutation linked to ARVC is considered at risk factor for developing the disease.

Two family members who carry the genetic variation (III.3 and III.8) remain asymptomatic. One family member who carries the genetic variation (III.7) was admitted to hospital with the diagnosis of myocarditis with mild chest pain, US-TnT up to 286 ng/L, normal ECHO, normal cardiac MR and a run of ventricular tachycardia (4 ventricular ectopies). Two months thereafter she was asymptomatic without any treatment and ECHO, ECG and Holter remained within normal limits (without ventricular ectopies). It is important to note that none of the individuals without the genetic variation showed any symptoms or cardiac structural abnormalities related to ARVC (Table 1).

DISCUSSION

Our study focuses on a Spanish family affected by ARVC. In the present study, affected members fulfilled definite diagnostic criteria of ARVC as defined by the 2010 update of the 1994 consensus criteria Task Force criteria for ARVC. As usually occurs in ARVC patients, not all affected family members showed the same phenotypic severity. These phenotypic variations induce controversies among cardiologists. In our family, two patients were implanted with an ICD, both with well-defined ARVC phenotype. We only recommend ICD implantation in certain patients, depending on the severity of each case. Genetic assessment identified a novel *nonsense* heterozygous genetic variation in the *DSP* gene (p.Q986X). The nucleotide change results in a premature termination signal in an evolutive highly conserved part of the gene, leading to a loss of the desmin binding domain [11]. The pathogenicity of a new genetic variation should be consulted in *in silico* platforms. However, current bioinformatic databases do not allow consulting *nonsense* genetic variations because of *nonsense* genetic variations are always considered theoretically pathogenic. All previously described *nonsense* mutations in the *DSP* gene localized near to our genetic variation are considered highly pathogenic [12]. Therefore, in theory, *DSP*_p.Q986X could be a potential pathogenic genetic variation associated to the ARVC.

Despite all these data, two family members (II.7 and II.9) didn't fulfil ARVC diagnostic criteria but carry the novel *nonsense* genetic variation. It is plausible to assume that both family members are in the first stage of the disease, characterized by non-structural heart alterations. In this phase, sporadic electrical instability preceding structural abnormalities [13], as occurs in II.7. In addition, three of index case cousins' carried the genetic variation but remained asymptomatic after clinical assessment (III.3, III.7 and III.8). It may be due to their young age, under 18 year old age all three cases, because ARVC is a progressive structural disease and, in the early stages of the diseases it may be difficult to distinguish ARVC from a health heart. Cases without structural abnormities but with minor electrocardiographic alterations has been reported [14]. Therefore, despite hypothetical pathogenic role focus on genetic data, clinical data suggest that the novel *nonsense* genetic variation can be considered a rare variant with a possible modifier effect on the ARVC phenotype expression. Thus, in our family, asymptomatic relatives may carry the genetic variation, and this

genetic variation may only be a predisposing factor to ARVC in the presence of other genetic factors, and so its presence alone does not allow accurate prediction of ARVC phenotype. Therefore, if the genetic variation is identified in asymptomatic individual, we strongly recommend clinical follow-up in all these family members to detect the first signs of disease that may be diminished by early treatment.

Focus on our results, our family showed an incomplete penetrance and variable expressivity, according to several published reports in ARVC families. Gender effects, genetic factors, and environmental triggers are main reasons that have been suggested to explain this phenomenon [15]. The high proportion of males among individuals affected with ARVC suggests that gender may have a significant effect although in our family, the majority of the affected patients were females. This suggests that factors other than gender are important in determining penetrance of ARVC. There may be compensatory mechanisms caused by unidentified genetic and/or environmental factor(s) in our asymptomatic family members that carry the novel genetic *nonsense* variation. In addition, some carriers of the genetic variation didn't show the phenotypic manifestations of ARVC, which raises the possibility that the combination of two or more genetic variants is required to cause ARVC. In 2010, our group reported an ARVC cohort in which several affected patients carried more than one genetic variation in one or more genes previously associated to the disease [16]. In the present study, we didn't identify any other genetic alteration, besides natural variants, in any of the other current ARVC related genes; however, genetic variations in unknown genes may be responsible for this phenotype variability. All these results supports that further comprehensive genetic studies in ARVC families must be performed, even when a single genetic variation is identified, trying to clarify the genotype-phenotype correlation in each family member. Thus, it has been recently reported a compound and digenic heterozygosity in different ARVC population [17-20], supporting both ARVC incomplete penetrance and variable expressivity [15]. Other genetic factor could be the allelic imbalance [21, 22]. This genetic process suggest that possible negative consequences caused by *nonsense* genetic variations may be reduced or even avoided to a tolerable level by translational readthrough, which enables ribosomes to ignore the stop codon and codify full proteins [23]. In addition, it was reported that in hypertrophic cardiomyopathy (HCM), mutant

mRNAs and/or truncated proteins could be unstable and degraded. Thus, *nonsense* genetic variations could act as 'null alleles', causing haploinsufficieny in heterozygous HCM patients, contributing to low levels of mutant proteins [24]. This last cellular effect could also occur in ARVC cases despite no report have been published to date.

Finally, one main limitation is the lack of experiments focus on address protein expression in the cardiac tissue. It is well reported that promoter regulation and/or post-translational modifications alter the protein expression [25, 26]. These facts modify protein levels in a specific tissue, being a potential pathogenic alteration that may induce ARVC, and could be also one of the reasons that could explain why an identical pathogenic mutation induces distinct clinical phenotypes, even among family members [27]. Family members that carry the pathogenic mutation showing less severe phenotypes are also at risk of SCD [28] and they should be followed-up regularly by cardiologists to detect early development of structural heart abnormalities and first symptoms characteristic of the disease.

In summary, the present study supports the benefits of comprehensive genetic studies in ARVC families in order to complement clinical data of patients. This combined approach is essential to increase knowledge about the link between genetic variants and the wide range of ARVC phenotypes in clinical practice.

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FIGURE LEGENDS

Figure 1. Familial pedigree. Squares represent men, circles represent woman, and slashes represent deceased family members. III.4 is the proband. Clinical affected individuals are shown as grey filled circle/squares. White filled circles/squares are no clinical affected individuals. White filled circles/squares with discontinuous line represent no clinically evaluated individuals. Black dots within the circle/square are p.Q986X carriers in *DSP*. Black dots within white circle/square represent family members carry genetic variation without clinical symptom. Note that III.4 was diagnosed after autopsy. In addition, I.1 was not evaluated due to old age.

Figure 2. Twelve-lead ECG of family member II.2. The ECG shows T negative waves.

Figure 3. *DSP* gene sequence. Note that the C (cytosine base changes to T (thymine) (c.2975 C>T), causing a *nonsense* genetic variation (p.Q986X –p.Gln986STOP-).

Figure 4 - Conservation of the altered aminoacid in the novel genetic variation. Aminoacids are represented by standard abbreviation (Q for glutamine). (*) indicates the position of the genetic variation. Species are listed in standard nomenclature.

TABLE LEGEND

Table 1. Clinical data. Results from clinical findings in all family included in the study. Major and Minor criteria indicate clinical findings based on the revised Task Force Criteria (TFC) of the European Society of Cardiology/International Society and Federation of Cardiology. Data from III.4 patient was obtained after autopsy examination. Abbreviations: ECG: Electrocardiogram. ECHO: Echocardiogram. MR: Magnetic Resonance. F: Female. M: Male. NE: No Evaluated.

TABLE

Family Member	Age	Gender	Genetic variation	ECG	ECHO	MR
I.1	82	M	p.Q986X	NE	NE	NE
I.2 -Died-	53	F	NE	NE	NE	NE
II.2	53	F	p.Q986X	Major	Minor	Normal
II.3	52	F	No	Normal	Normal	Normal
II.4	50	F	p.Q986X	Normal	Major	Normal
II.6	49	M	No	Normal	Normal	Normal
II.7	46	M	p.Q986X	Normal	Normal	Normal
II.9	42	F	p.Q986X	Normal	Normal	Normal
III.1	30	M	No	Normal	Normal	Normal
III.2	25	F	No	Normal	Normal	Normal
III.3	17	F	p.Q986X	Normal	Normal	Normal
III.4 –Proband, Died-	18	M	NE	NE	NE	NE
III.5	15	F	p.Q986X	Minor	Major	Normal
III.6	11	F	No	Normal	Normal	Normal
III.7	18	M	p.Q986X	Normal	Normal	Normal
III.8	12	F	p.Q986X	Normal	Normal	Normal

Table 1. Clinical data.

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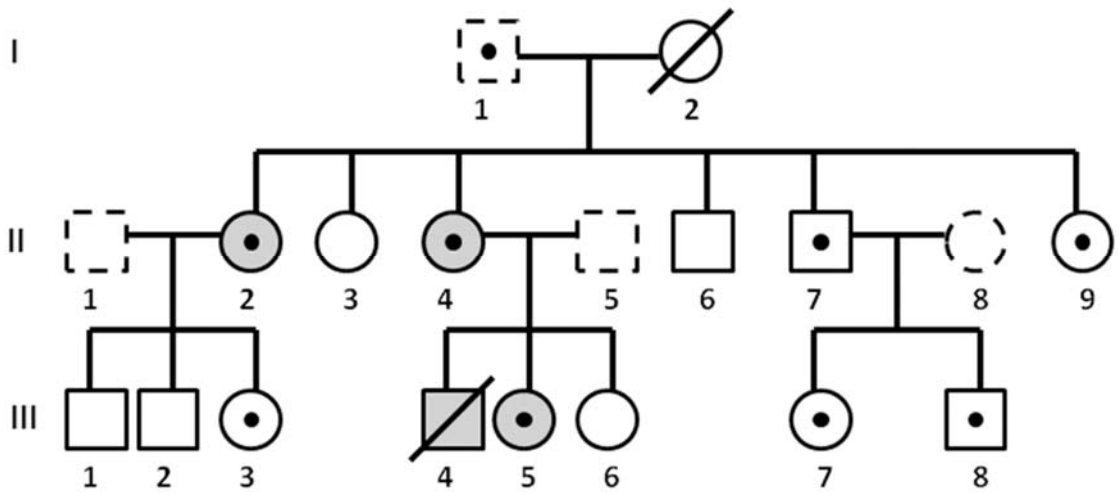

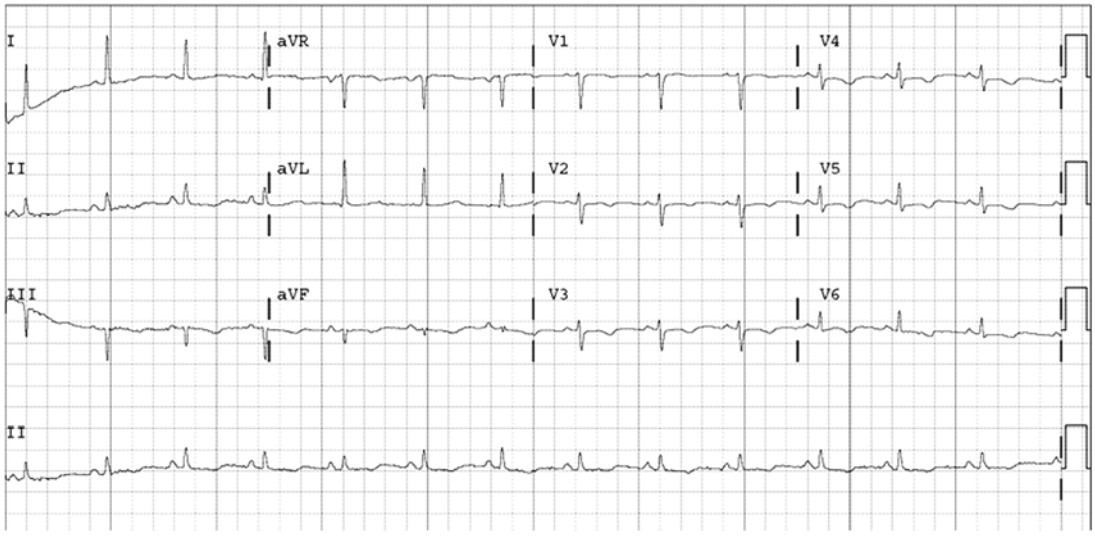

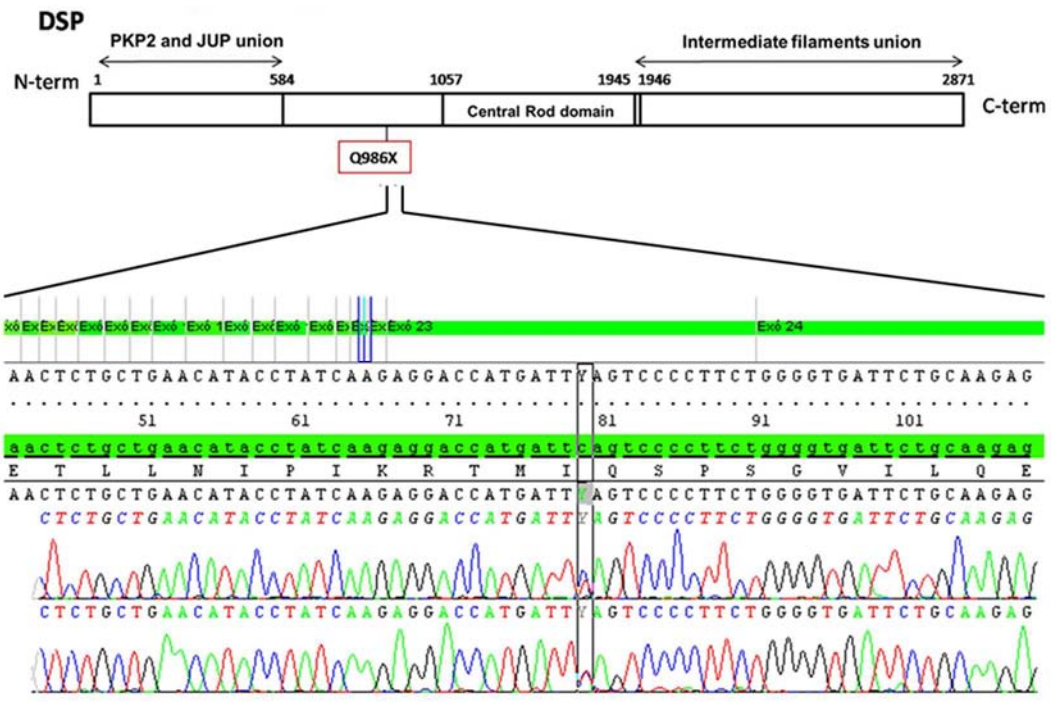

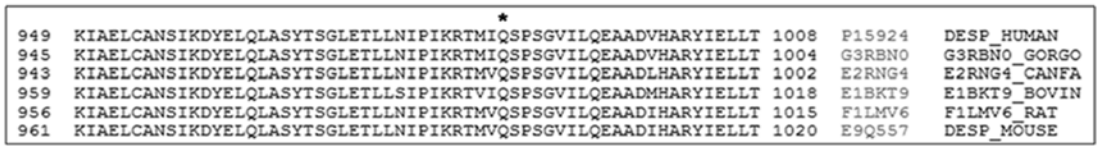

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