Regulatory mutations in transforming growth factor-β3 gene cause arrhythmogenic right ventricular cardiomyopathy type 1

Giorgia Beffagna a,1, Gianluca Occhi a,1, Andrea Nava b, Libero Vitiello a, Andrea Ditadi a, Cristina Basso c, Barbara Bauce b, Gianni Carraro d, Gaetano Thiene c, Jeffrey A. Towbin e, Gian Antonio Danieli a, Alessandra Rampazzo a,*

a Department of Biology, University of Padua, Via Ugo Bassi 58/B 35131 Padua, Italy
b Department of Cardiology, University of Padua, 35131 Padua, Italy
c Institute of Pathology, University of Padua, 35131 Padua, Italy
d Department of Human Anatomy, University of Padua, 35131 Padua, Italy
e Department of Pediatrics, Section of Cardiology, Baylor College of Medicine, Houston, USA

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Abstract

Objective: Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a genetically heterogeneous disorder characterized by fibro-fatty replacement of the right ventricular myocardium, associated with high risk of sudden death. The objective of this study is to identify the gene involved in ARVD1, which has been elusive ever since its locus was mapped to chromosome 14q24.3.

Methods and results: Mutation screening of the promoter and untranslated regions (UTRs) of the transforming growth factor-beta3 (TGFβ3) gene was performed by direct sequencing of genomic DNA of one index case belonging to an ARVD1 family including 38 members in four generations. We detected a nucleotide substitution (c.303G>A) in 5′ UTR of TGFβ3 gene, invariably associated with the typical ARVC clinical phenotype in the affected family members, according to the established diagnostic criteria. Investigation extended to 30 unrelated ARVC patients, performed by denaturing high-performance liquid chromatography (DHPLC), led to the identification of an additional mutation (c.1723C>T) in the 3′ UTR of one proband. Neither nucleotide change was found in 300 control subjects. In vitro expression assays with constructs containing the mutations showed that mutated UTRs were twofold more active than wild-types.

Conclusion: We identified TGFβ3 as the disease gene involved in ARVD1. The identification of a novel ARVC gene will increase the power of the genetic screening for early diagnosis of asymptomatic carriers among relatives of ARVC patients.

Keywords: Gene expression; Cardiomyopathy; Sudden death; Ventricular arrhythmias

This article is referred to in the Editorial by S. Nattel and J.-J. Schott (pages 302–304) in this issue.

1. Introduction

Arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC [MIM 107970]) is characterized by extensive fibro-fatty replacement of the right ventricular myocardium [1]. Clinical manifestations of the disease, occurring mostly between the second and fourth decade of life, include structural and functional abnormalities of the
right ventricle, electrocardiographic depolarization/repolarization changes and arrhythmias of right ventricular origin [2,3]. Myocardial degeneration may involve left ventricle and, more rarely, interventricular septum [4]. ARVC is reported as the second most common cause of sudden death among young adults, both in Europe and in the United States [1,5]. Eight autosomal dominant forms of ARVC, all exhibiting incomplete penetrance, were identified through linkage studies [6]. ARVD2 patients showed a distinct clinical phenotype, characterized by the presence of effort-induced polymorphic ventricular tachycardia. Mutations in cardiac ryanodine receptor (RYR2) [7] and in desmoplakin (DSP) [8] were shown to cause ARVD2 or ARVD8, respectively. The recent finding of a homozygous missense mutation in DSP gene causing a syndrome and woolly hair and caused by a homozygous two-nucleotide deletion in the Plakoglobin gene [10].

ARVD1 locus was first mapped to chromosome 14q24.3 ten years ago [11]. Since then, the disease gene has been elusive. Among genes mapping to 14q24.3 (TGFβ3) appeared a very good candidate, as it encodes for a cytokine-stimulating fibrosis and modulating cell adhesion [12]. After previous analyses failed to detect any mutation in the coding region of TGFβ3 and of additional four genes within the critical region [13,14], mutation screening was extended to the promoter and untranslated regions (UTRs). Here, we report on identification, as well as extended to the promoter and untranslated regions within the critical region [13,14], mutation screening was performed on the 5' UTR of patients belonging to a large ARVD1 family, for which linkage data demonstrated that ARVC was transmitted linked to 14q24.3 (z=4.41 at θ=0 for marker D14S254) [13]; the second mutation, localized in the 3' UTR, was found in an ARVC patient, whose brother died suddenly and resulted affected with ARVC at autopsy.

2. Methods

2.1. Clinical analysis

Our population included subjects belonging to ARVD1 family 112 and 30 unrelated patients, all with a clinical diagnosis of ARVC. The study protocol included basal 12-lead electrocardiogram (ECG), signal-averaged ECG, 24-h Holter ECG and two-dimensional echocardiography. Additional invasive procedures (endomyocardial biopsies and right and left ventricular angiography with coronary arteriography) were performed only when deemed necessary.

Clinical diagnosis was established according to criteria proposed by the ESC-ISFC Task Force [15]. All patients gave informed consent to clinical and genetic studies. The investigation conformed with the principles outlined in the Declaration of Helsinki.

2.2. Mutation screening

Mutation screening of TGFβ3 promoter and UTRs was performed by direct sequencing of genomic DNA of the index case of family 112. All PCR primers were designed by PRIMER 3 and are available at our website ARVDnet (http://telethon.bio.unipd.it/ARVDnet/index.html). Each fragment was amplified from patient DNA and sequenced on an ABI 3730XL DNA sequencer.

The analysis of the TGFβ3 promoter, UTRs and coding sequences in 30 ARVD probands were performed by denaturing high-performance liquid chromatography (DHPLC, Transgenomic, NE, USA) and direct sequencing. All amplimers showing abnormal DHPLC elution profiles were sequenced. A control group of 300 healthy and unrelated subjects (600 alleles) from Venetian population was used to exclude that the detected mutations were actually DNA polymorphisms.

Mutation screening for exons deletions, performed on affected subjects belonging to two additional and unrelated ARVD1 families, was performed both by RT-PCR on lymphocytes RNA and RealTime PCR on genomic DNA. All samples analyzed by RealTime PCR were amplified in duplicate and compared to a matched control. Expression levels were obtained according to a relative quantification model [16].

2.3. In vitro expression studies

2.3.1. Plasmid construction

To test the pathogenic role of the -36G→A transition, recombinant expression vectors containing the 5'-flanking region of the TGFβ3 gene (nucleotide positions -1926 to 120) were created. PCR fragments were obtained by amplification of DNA of family 112 index case. Forward and reverse primers contained extra NcoI and HindIII sites, respectively, and the PCR product was digested and inserted upstream the luciferase reporter gene into the pGL3 Enhancer Vector (Promega). The recombinant plasmid containing the normal sequence was designated pGL3_45wt, whereas the mutant one was called pGL3_9mut.

PGL3_45wt was further engineered in order to test the effect of the 3' UTR substitution on expression levels. Specifically, the SV40 late poly (A) signal was substituted with an HpaI–SpeI fragment, generated by amplification of genomic DNA of the proband (from nucleotide 1234 to 2456) using a set of primers that contained the extra restriction sites in their 5' tails.
Plasmids containing the wild-type or the mutated sequences were designated pGL3_6wt and pGL3_Mmut, respectively.

2.3.2. Cell culture

Murine C2C12 myoblasts were cultured in growth medium (DMEM, additioned with 10% fetal bovine serum and Pen/Strep antibiotic mixture) at 37°C in an atmosphere of 5% CO2. For transfection, cells were seeded in 24-well dishes and allowed to grow until the confluence was over 80%.

2.3.3. Luciferase reporter gene assay

C2C12 myoblasts were transfected using the Lipofectamine 2000 Reagent (Invitrogen). Complexes were prepared according to manufacturer’s instructions, using 0.3 μg DNA, in a 1:3 wt/wt DNA/lipid ratio. The transfection mixture was left in contact with cells for 6–8 h in the presence of growth medium, after which cells were rinsed twice and re-fed; 24 h later, the growth medium was replaced by differentiation medium (DMEM added with 2% horse serum and Pen/Strep mixture) and cells were allowed to differentiate into myotubes for further 24 h, before analysis. Cells were lysed with a buffer provided in the Luciferase Assay Kit (Promega) and luciferase activities were compared by measuring the light emission integrated over 10 s, in a TD 20/20 luminometer (Turner Scientific). Light emission of each sample was normalized to that of a known amount of purified luciferase and to the protein concentration of each sample, measured by Bradford assay.

Each wild-type vs. mutated comparison (5' UTR and 3' UTR) was performed in at least six independent experiments, using each time 8–12 wells per plasmid. In order to rule out possible efficiency bias derived from usage of a single DNA/lipid preparation, in each experiment, two to three separate batches of complexes for each plasmid DNA were prepared. Results of each separate experiment were normalized in terms of relative luciferase activity.

2.3.4. Statistical analysis

Student’s t-test was used for statistical analysis. A P value <0.01 was consider statistically significant. All statistical calculations were performed using SPSS statistical package software.

3. Results

3.1. Mutation screening

Mutation screening of the promoter and UTRs of the TGFβ3 gene was performed by direct sequencing of genomic DNA of the index case of the ARVD1 family 112, which includes 38 members in four generations (Fig. 1). A G→A transition in 5' UTR region (c.-36G>A) (Fig. 2) was detected in all clinically affected individuals of the family and in three asymptomatic relatives (II-10, III-10 and IV-3).

Mutation screening of TGFβ3 was then performed by DHPLC in 30 ARVD probands, in which mutations in the known ARVD genes were previously excluded. A 1723C→T transition in 3' UTR of the TGFβ3 gene (Fig. 2) was
identified in a patient showing a typical ARVC clinical phenotype. Neither of the nucleotide changes were detected in 300 control subjects (600 chromosomes) from the same population. Affected subjects belonging to two additional and unrelated families (100 and 119) [11,13], in which linkage with ARVD1 was previously established, showed no mutations in TGFβ3 coding sequences, UTRs and promoter region. Deletions of entire exons were excluded both by RT-PCR on lymphocytes RNA and RealTime PCR on genomic DNA.

3.2. Clinical findings

The index case of family 112 (subject III-4) is a young male who, at the age of 11, experienced an episode of sustained ventricular tachycardia. After thorough clinical investigation, he was diagnosed as affected by ARVC. Typical ECG and echocardiographic features are shown in Fig. 3. Right and left ventricular angiography with coronary arteriography and endomyocardial biopsy were performed as well. The biopsy, taken form right ventricular free wall, revealed extensive replacement-type fibrosis and fatty infiltration. Surviving myocytes entrapped within fibrous and fatty tissue showed degenerative changes and abnormal nuclei (Fig. 3). Clinical diagnosis of ARVC was achieved in nine subjects, whose clinical features have been previously reported in part [13]. Repolarization abnormalities on 12-lead ECG were detected in six individuals, depolarization/conduction abnormalities in eight and positive late potentials in six. According to right ventricular diastolic volume obtained by echocardiographic investigation [17], five patients (II-3, III-3, III-4, III-12 and III-15) manifested a moderate form of the disease and 4 (II-2, II-5, III-1 and II-9) a mild one. Non-sustained ventricular arrhythmias with left bundle branch block morphology were recorded in three patients (II-5, III-1 and III-12). Atrial arrhythmias were never observed in the affected subjects. None of them received a defibrillator. Among family members not
carrying the mutation, none showed symptoms or signs of ARVC.

The ARVC patient carrying the TGFβ3 3′ UTR mutation is a young male who, at the age of 16, showed spontaneous sustained ventricular tachycardia with left bundle branch block morphology. The 12-lead ECG showed inverted T waves on right precordial leads V1–V4. Ecocardiographic and angiographic investigations revealed localized wall motion abnormalities of the right ventricle with enlarged right ventricular chamber. Magnetic resonance imaging revealed transmural myocardial atrophy with fatty infiltration of the right ventricular free wall. Endomyocardial biopsy showed extensive replacement by fibrous tissue [18]. His brother died suddenly at the age of 16 in early 1980s and was found affected with ARVC at autopsy.

3.3. Expression of TGFβ3 UTRs–Luciferase constructs in C2C12 cells

In order to test whether the UTRs mutations could actually affect the expression levels of TGFβ3 gene, we prepared four different plasmid constructs in which the luciferase reporter gene was put under control of either wild-type or mutant regions (Fig. 4). These plasmids were then used to perform transient transfections into the murine myoblast cell line C2C12. This cell line was selected since previous studies showed that its expression levels of TGFβ3 were similar to those reported in the heart [19]. We performed twelve independent experiments: six to test the effect of the 5′ UTR mutation and six to test the effect of the 3′ UTR mutation. In each of them, half of a 24-well plate was transfected with the mutated vector and the second half received the wild-type UTRs. In both cases, luciferase reporter activity was significantly higher (about 2.5-fold) in cells transfected with the mutated constructs, when compared with those transfected with the wild-types (TSS=transcription starting site; ATG=TGFβ3 starting methionine; TGA=luciferase STOP codon; PAS=polyadenilation signal).

4. Discussion

We identified TGFβ3 as disease gene involved in ARVD1, a progressive and genetically determined myocardial disease, recognized as a common cause of sudden death among young adults. No association of TGFβ3 mutations to inherited human diseases has been reported so far.

TGFβ3 is a member of the transforming growth factor family, a large group of regulatory cytokines playing a pivotal role in development and tissue homeostasis [12].
Non-overlapping roles for TGFβs in development and in normal function of cardiovascular system were reported [20]. TGFβ1−/− mice, which has an autoimmune-like inflammatory disease [21], show no major heart abnormalities. On the contrary, TGFβ2−/− mice have clear congenital heart defects [22]. Analysis of TGFβ3−/− mice has revealed minor differences in position and curvature of the aortic arches and in ventricular wall thickness [23,24]. Null mutations in each of the three mammalian TGFβ isoforms are lethal, but the different phenotypes provide conclusive evidence for unique roles of each isoform.

The 5′ UTR of TGFβ3 mRNA contains 11 ATG codons, which marks the beginning of 11 potential upstream open reading frames (uORFs). The ATG at position −142 opens a reading frame encoding a putative 88-amino-acid peptide overlapping the sequence of TGFβ3 first exon [25]. The G→A transition we identified in the 5′ UTR of ARVD1 affected family members would lead to an Arg→His substitution (R36H) at codon 36 of such 88-aa peptide. It is important to notice that mutations in the two ATGs closest to the TGFβ3 starting codon have already been shown to cause a significant increase in translation efficiency [25], in agreement with results of our functional study. These data together suggest that these short peptides might play an inhibitory effect. Furthermore, it has been reported that in different genes uORFs affect downstream translation through mechanisms depending on amino acid sequence of the encoded peptide [26].

The presence of regulatory sequences in the 3′ UTR of TGFβ3 has never been reported so far. However our experimental evidence suggests a regulatory role of this region, in agreement with the reported involvement of 3′ TR mutations in different genetic disorders [27].

Surprisingly, no mutations were detected in TGFβ3 coding sequences, UTRs and promoter region in two additional and unrelated ARVD1 families. However, the presence of mutations in yet unidentified regulatory elements cannot be ruled out.

Finding TGFβ3 mutations associated with ARVC is very interesting, as it is well established that TGFβs stimulate mesenchymal cells to proliferate and to produce extracellular matrix components. TGFβ3 induces a fibrotic response in various tissues in vivo [28], by promoting expression of extracellular matrix genes and by suppressing the activity of genes such as matrix metalloproteinases, which are involved in extracellular matrix degradation [29,30]. It was demonstrated that overexpression of TGFβ3, which is the best characterized isoform, induces myocardial fibrosis, hypertrophy and enhanced β-adrenergic signalling [31]. In particular, atrial fibrillation diathesis has been reported in animal models with overexpression of TGFβ3 mediated by atrial fibrosis [32,33]. Conversely, heterozygous TGFβ1(−/+) deficient mice showed markedly decreased age-related fibrosis, as well as lower myocardial collagen content [34].

On the basis of this knowledge, we hypothesize that the herein reported mutations in UTRs of TGFβ3 gene, which in vitro increases expression, in vivo could promote myocardial fibrosis. Extensive myocardial fibrosis may disrupt electrical and mechanical behavior of the myocardium and extracellular matrix abnormalities may predispose to re-entrant ventricular arrhythmias [35]. In fact, endomyocardial biopsy in the two probands show extensive replacement-type fibrosis, in agreement with this hypothesis. Moreover, signal-averaged ECG, which has been shown to strongly correlate with the amount of fibrous tissue replacement at endomyocardial biopsy [36], revealed positive late potentials in six affected members of family 112.

It has been shown that TGFβs modulate expression of genes encoding desmosomal proteins in different cell types. cDNA microarray analysis performed on RNA from cardiac fibroblasts incubated in the presence or in the absence of exogenous TGFβ3 revealed increased expression of different genes, including Plakoglobin [37]. Yoshida et al. reported that TGFβ1 exposure of cultured airway epithelial cells increases the content of desmoplakins I and II, thus suggesting that regulation of cell–cell junctional complexes may be an important effect exerted by TGFβ3 [38]. Therefore, overexpression of TGFβ3, caused by UTRs mutations, might affect as well cell-to-cell junction stability, leading to a final outcome similar to that observed in ARVD8 [8] and in the ARVC-related Naxos syndrome [10]. This would explain as well preferential affection of the right ventricle [8]. In family 112, detection of the pathogenic mutation in three apparently healthy subjects is in agreement with reduced penetrance, observed so far in all families affected with ARVD2 and ARVD8.

It is not clear whether the origin of fatty replacement in right ventricular myocardium is a direct or a secondary consequence of TGFβ3 overexpression. Such replacement was observed in myocardium of ARVC patients, included those carrying mutations in ryanodine receptor (ARVD2), desmplakin (ARVD8) or plakoglobin (Naxos Disease). Mutations in such genes have no apparent link with fat deposition or fatty trans-differentiation, while they share in common a probable effect on intracellular calcium level [8]. Hypothetically, impairment of mitochondrial function, caused by such intracellular calcium overload, might increase intracellular concentration of free fatty acids and then trigger lipogenesis, via activation of the peroxisome proliferator-activated receptors-gamma [39]. Actually, early electron microscopy observations described many fatty droplets within cardiomyocytes in surgically excised myocardial specimens from hearts of three patients affected with ARVC [40]. Since TGFβ3, similarly to ARVD8 [8] and Naxos syndrome [10], might affect cell-to-cell junction stability, an intracellular calcium overload can be postulated also for ARVD1, thus explaining the presence of fatty replacement besides extensive fibrosis.

On the other hand, fatty replacement is observed as well in hearts of patients affected with Duchenne muscular dystrophy [41], and in seemingly normal autopsied hearts...
Additional mutation was detected in the 3' UTR of TGFβ3 as the primary step in pathogenesis of ARVD1: (1) a mutation in the 5' UTR was found to cosegregate with the clinical phenotype in a large ARVD1 family and one additional mutation was detected in the 3' UTR of an unrelated ARVC patient; (2) neither of the nucleotide changes were detected in 600 chromosomes from the same population; (3) functional studies revealed that both mutations significantly increase the activity of luciferase reporter gene in C2C12 cells, which have been already used as an in vitro model in TGFβ3 expression studies; (4) TGFβ3 pro-fibrotic activity and its possible involvement in cell–cell adhesion protein regulation may well explain both progressive myocardial degeneration and selective affection of right ventricle observed in ARVD1.

Identification of TGFβ3 as ARVD1 disease gene opens the way to understanding of molecular pathogenesis and, hopefully, to the development of more effective clinical management and drug treatment. For the time being, identification of the gene involved in ARVD1 will enable detection of asymptomatic carriers through genetic testing of at-risk family members and it could reduce risk of sudden death in juveniles through prostration of sport activity and close clinical follow-up.

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References


