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EDITORIAL

Regulatory mutations in transforming growth factor-β3 gene involved in arrhythmogenic right ventricular cardiomyopathy

AUTHOR'S RETROSPECTIVE

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This editorial refers to an article by G. Beffagna *et al.*[13] published in *Cardiovascular Research* in 2005 (see Box 1). It is accompanied by an editorial by J. Tamargo, pp. 188–190, this issue, as part of this Spotlight on Landmark Papers in *Cardiovascular Research*.

1. Introduction

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is an important cause of sudden death in young adults and athletes. This heart muscle disease is characterized by myocardial dystrophy, mostly of the right ventricle, with massive fibro-fatty infiltration accounting for ventricular electrical instability at a risk of severe arrhythmias and even cardiac arrest.[1,2] The left ventricular myocardium seems to be involved in more than half of the cases.[3] The disease is clinically heterogeneous, with inter- and intra-familial variability, ranging from benign to malignant forms with a high risk of sudden cardiac death.[3] The prevalence of the disease in the general population has been estimated to vary from 1 in 1000 to 1 in 5000 individuals,[4,5] and men are more frequently affected than women, with an approximate ratio of 3:1.[6] The disease is familial, and typically autosomal dominant with reduced penetrance in about half the cases, although autosomal recessive forms have been reported as well.[3]

Most of the pathogenic mutations have been identified in genes encoding the desmosomal proteins plakoglobin (JUP), desmoplakin (DSP), plakophilin-2 (PKP2), desmoglein-2 (DSG2), and desmocollin-2 (DSC2).[3] Although rare, mutations in some non-desmosomal proteins, such as cardiac ryanodine receptor type-2, transforming growth factor-β3 (TGFβ3), transmembrane protein 43, desmin, titin, and lamin A/C have also been associated with ARVC. Mutations in desmosomal genes have been identified in half of the patients, and a significant proportion of them were found to carry multiple mutations.[7,8]

Therefore, ARVC is actually defined as a disease of desmosomes. Desmosomes are complex cell–cell adhesion structures that provide mechanical integrity of heart tissue, affording an intercellular connection between adjacent cells. Most recently, Franke *et al.*[9] have shown that at the intercalated disks of mammalian hearts, desmosomal proteins also contribute to the assembly of extended junctions, named areae compositae. Some desmosomal proteins, such as plakoglobin, fulfil dual roles as structural proteins in cell–cell adhesion junctions and as nuclear signalling molecules. It has been proposed that mutations in desmosomal proteins implicated in ARVC may perturb the normal balance of plakoglobin in junctions and the cytosol, which, in turn, could promote dysregulated gene expression circumventing the normal controls of Wnt signalling pathways.[10]

2. Identification of regulatory mutations in the TGFβ3 gene in ARVC patients

In 1994, at the beginning of ARVC genetic studies, our group mapped for the first time the ARVD1 disease gene linked to markers on chromosome 14q42.3 in a large Italian family[4] and later confirmed locus assignment in an additional ARVC family.[11] Since then, the disease gene has been elusive. The critical interval for ARVD1 contained 40 known genes; 5 of them (*POMT2*, *KIAA0759*, *KIAA1036*, *C14orf4*, and *TAIL1*) were unsuccessfully screened for pathogenic ARVC mutations.[11] Among genes mapped to *ARVD1* critical region and expressed in myocardium, *TGFβ3* appeared a very good candidate, as it encodes a cytokine that stimulates fibrosis and modulates cell adhesion.[12] At that time, only mutations in *RYR2*, *JUP*, *DSP*, and *PKP2* genes have been associated with ARVC.

After previous analyses failed to detect any mutation in the coding region of the *TGFβ3* gene, mutation screening was extended to the promoter and untranslated regions (UTRs). A nucleotide substitution (c.-36G>A) in 5′UTR of the *TGFβ3* gene was detected in all affected subjects belonging to a large ARVD1 family[13] for which linkage

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

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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.−36G>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

Box 1 Title page including abstract from the original 2005 publication in *Cardiovascular Research*.[13] Used with permission of Oxford University Press on behalf of the European Society of Cardiology.

data demonstrated that ARVC was transmitted linked to 14q24.3 ($z = 4.41$ at $\theta = 0$ for marker D14S254). The mutation was found in all nine affected members of the kindred, and was also present in three asymptomatic relatives, in line with the incomplete penetrance of the disease (*Figure 1*).

The 5′ UTR of the *TGFβ3* mRNA is 1.1 kb long and contains 11 ATG codons, which mark the beginning of 11 potential upstream open reading frames. The ATG at position −142 opens a reading frame encoding a putative 88 amino acid (aa)-long peptide overlapping the sequence of the TGFβ3 first exon.[14] The G→A transition in the 5′ UTR region would lead to an Arg→His substitution (R36H) at codon 36 of the 88-aa peptide. It was demonstrated that mutations in the two ATGs closest to the TGFβ3 starting codon cause a significant increase in translation efficiency,[14] thus suggesting that these short peptides might play an inhibitory effect.

The mutation screening of the *TGFβ3* promoter and UTRs was then extended to 30 unrelated ARVC index patients, and an additional mutation (c.1723C>T) was identified in the 3′ UTR of one proband showing a typical ARVC clinical phenotype.[13]

Neither of the nucleotide changes were detected in 600 chromosomes from the same population. Moreover, these variants are actually absent from dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) and from the Exome Variant Server (http://evs.gs.washington.edu/EVS/).

Functional studies revealed that both mutations significantly increase the activity of the luciferase reporter gene (more than two-fold, $P < 0.01$) in C2C12 cells, which have been already used as an *in vitro* model in TGFβ3 expression studies. However, confirmation of *in vivo* overexpression of TGFβ3 in the mutation carriers was not available due to the lack of cardiac biopsy samples from affected patients.

Affected subjects belonging to two additional and unrelated families, in which linkage with ARVD1 was previously suspected, showed no mutations in *TGFβ3* coding sequences, UTRs, and

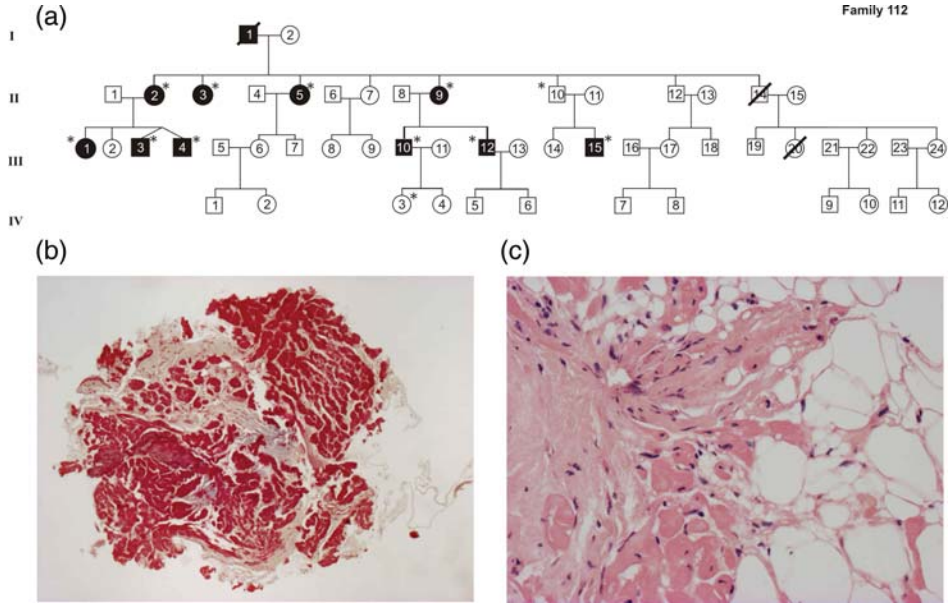

Figure 1 Identification of ARVC-associated mutation in 5′UTR of the TGFβ3 gene. (*a*) Pedigree of the large ARVD1 family showing cosegregation of the disease with the c.-36G>A mutation. Affected subjects fulfilled the clinical criteria for ARVC. Mutation carriers are indicated with an asterisk; grey-filled shapes represent subjects which have not been investigated. (*b* and *c*) Endomyocardial biopsy features. Extensive replacement-type fibrosis (Trichrome stain × 5). At higher magnification (*c*), fibrous and fatty tissue replacement is visible, in the absence of inflammatory infiltrates; surviving myocytes show myocytolysis and dysmorphic and dysmetric nuclei (Hematoxylin–Eosin stain × 40). Adapted from Ref.[13]; used with permission of Oxford University Press on behalf of the European Society of Cardiology.

promoter region. Most recently, in one of these two families, a large deletion encompassing the entire *PKP2* gene was identified (Li Mura, Rampazzo, unpublished data).

3. Role of TGFβ3 in the pathogenesis of ARVC

TGFβ3 is a member of the transforming growth factor family. The TGFβ superfamily consists of pleiotropic and multifunctional peptides that exert diverse effects on different cell types; they have the ability to regulate numerous biological processes, including cell differentiation and proliferation, apoptosis, fibrous tissue deposition, and tissue remodelling during disease pathogenesis and cancer.[12]

Three structurally similar isoforms of TGFβ (TGFβ1, 2, and 3), encoded by three distinct genes, have been identified in mammalian species. They are produced in latent forms, and upon activation they usually interact with TGFβR1 and TGFβR2 receptors and induce phosphorylation of SMAD2 or SMAD3. These SMADs associate with SMAD4 and translocate to the nucleus, where they recruit coactivators or corepressors into transcriptional complexes and regulate transcription of target genes. The inhibitory SMAD6 and SMAD7 negatively regulate this canonical TGFβ signalling. TGFβ signalling also occurs through SMAD-independent pathways.[15]

Tgfβ3 is expressed in several tissues during mouse embryonic development, including heart, lung, skin, and craniofacial structures. Tgfβ3-deficient animals exhibit defective epithelial–mesenchymal interactions resulting in cleft palate and abnormal lung development, and they die soon after birth,[16] precluding any further determination of the Tgfβ3 function in adult mice. On the other hand, Tgfβ3 conditional knockout mice die at birth from the same cleft palate defect as knockout mice,[17] indicating that the conditional and knockout alleles are functionally equivalent.

Genetic polymorphisms were found in *TGFβ3* in patients with hypertension[18] and ossification of the posterior longitudinal ligament of the spine (OPLL).[19] No additional mutations associated with inherited diseases have been found thus far in the *TGFβ3* gene.

Finding *TGFβ3* mutations associated with ARVC was very interesting, as it is well established that TGFβs stimulate mesenchymal cells to proliferate and to produce extracellular matrix components.[12] In addition, TGFβ is a potent regulator of extracellular matrix deposition that suppresses the activity of matrix metalloproteinases and induces the expression of protease inhibitors, including TIMPs and PAI-1.[20] Moreover, TGFβ is able to stimulate the synthesis of connective tissue growth factor, a potent profibrotic mediator that promotes persistent fibrosis.[12] TGFβ-mediated effects on fibrous tissue deposition and myocardial hypertrophy have been supported by TGFβ overexpression in transgenic mice. TGFβ1-overexpressing mice showed significant cardiac hypertrophy which is characterized by interstitial fibrosis and hypertrophic growth of cardiomyocytes.[21] The mutations in UTRs of the *TGFβ3* gene in ARVC patients, which increases expression *in vitro*, could promote myocardial fibrosis *in vivo*. Endomyocardial biopsy in the two probands shows extensive replacement-type fibrosis (*Figure 1*), in agreement with this hypothesis.[13]

The identification of the genetic basis of ARVC in the past decade provided the opportunity to elucidate the pathogenesis of the disease, allowing the development of several transgenic mouse models recapitulating the ARVC phenotype. Most recently, a cardiomyocyte-restricted Jup knockout mouse was generated and characterized.[22]

Jup was specifically deleted in cardiomyocytes by using Cre/loxP technology. Jup mutant mice largely mimic clinical aspects of ARVC, including cardiac dysfunction, myocyte dropout, ventricular dilation and aneurysm, cardiac fibrosis, and spontaneous ventricular arrhythmias. Massive cell death due to apoptosis and necrosis contribute to the cardiomyocyte dropout in Jup mutant hearts. In contrast with recent mouse studies, in this Jup mutant model, the Wnt/β-catenin-mediated signalling was not altered. Interestingly, TGFβ-mediated signalling, whose contribution to cell death has been well documented,[23] was found to be significantly elevated in Jup mutant cardiomyocytes at the early stage of cardiomyopathy. Phospho-Smad2 was increased in Jup mutant hearts as well as TGFβI (TGFβ-induced) and Pai1 (plasminogen activator inhibitor type 1), *bona fide* TGFβ-signalling downstream effector molecules.[20] These findings suggest that the elevated TGFβ signalling is associated with the pathogenesis of Jup-related ARVC.

4. Conclusions and future perspectives

ARVC is characterized by extensive fibro-fatty replacement of the right ventricular myocardium. Approximately 40% of ARVC patients have one or more mutations in genes encoding desmosomal proteins. However, non-desmosomal protein genes have been implicated in ARVC as well. Two regulatory mutations in the *TGFβ3* gene causing its overexpression *in vitro* have been identified in two patients showing a typical ARVC phenotype. Although *TGFβ3* gene mutations seem to be extremely rare among ARVC patients, these interesting findings have potentially major significance for our understanding of the molecular pathogenesis of ARVC and of the function of TGFβ3. Moreover, it has been recently reported that the up-regulation of TGFβ signalling is likely to be associated with the pathogenesis of Jup-related ARVC. The next logical step would be to investigate whether the inhibition of TGFβ signalling can delay or prevent the right ventricular disease from becoming manifest in ARVC animal models.

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