Remodeling of the cardiac sodium channel, connexin43, and plakoglobin at the intercalated disk in patients with arrhythmogenic cardiomyopathy

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BACKGROUND Arrhythmogenic cardiomyopathy (AC) is closely associated with desmosomal mutations in a majority of patients. Arrhythmogenesis in patients with AC is likely related to remodeling of cardiac gap junctions and increased levels of fibrosis. Recently, using experimental models, we also identified sodium channel dysfunction secondary to desmosomal dysfunction.

OBJECTIVE To assess the immunoreactive signal levels of the sodium channel protein $Na_V1.5$, as well as connexin43 (Cx43) and plakoglobin (PKG), in myocardial specimens obtained from patients with AC.

METHODS Left and right ventricular free wall postmortem material was obtained from 5 patients with AC and 5 controls matched for age and sex. Right ventricular septal biopsies were taken from another 15 patients with AC. All patients fulfilled the 2010 revised Task Force Criteria for the diagnosis of AC. Immunohistochemical analyses were performed using antibodies against Cx43, PKG, $Na_V1.5$, plakophilin-2, and N-cadherin.

RESULTS N-cadherin and desmoplakin immunoreactive signals and distribution were normal in patients with AC compared to controls. Plakophilin-2 signals were unaffected unless a plakophilin-2 mutation predicting haploinsufficiency was present. Distribution was unchanged compared to that in controls. Immunoreactive signal levels of PKG, Cx43, and $Na_V1.5$ were disturbed in 74%, 70%, and 65% of the patients, respectively.

CONCLUSIONS A reduced immunoreactive signal of PKG, Cx43, and $Na_V1.5$ at the intercalated disks can be observed in a large majority of the patients. Decreased levels of $Na_v1.5$ might contribute to arrhythmia vulnerability and, in the future, potentially could serve as a new clinically relevant tool for risk assessment strategies.

KEYWORDS AC; Arrhythmogenic cardiomyopathy; Connexin 43; $Na_V1.5$; Desmosome; Plakophilin-2; Plakoglobin; Immunohistochemistry

ABBREVIATIONS **AC** = arrhythmogenic cardiomyopathy; **Cx43** = connexin43; **ID** = intercalated disk; **N-Cad** = N-cadherin; **PKG** = plakoglobin; **PKP2** = plakophilin-2; **RVSB** = Right ventricular septal biopsy; **TFC** = Task Force Criteria

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Introduction

Arrhythmogenic cardiomyopathy (AC), previously known as arrhythmogenic right ventricular cardiomyopathy/dysplasia, is a heart muscle disease characterized by replacement of predominantly the right ventricle with fibrofatty scar tissue. In later stages of the disease, the left ventricle and the interventricular septum can also be affected. Recently, patients with predominant left ventricular involvement have been described.[1] Patients generally present with syncope, palpitations, and sudden cardiac death.[2] Mutations in genes encoding for desmosomal proteins, which are important for intercellular mechanical coupling, are associated with this disease in about 60% of the patients.[3–9]

The arrhythmogenic phenotype of AC suggests that although the mutated genes do not code for channel proteins, these gene products associate with molecules that are relevant for electrical function. In particular, several studies have identified a disturbed immunolocalization of connexin43 (Cx43), the major ventricular gap junction protein, in the AC myocardium.[10–12] In several other forms of cardiomyopathy, a heterogeneous downregulation and dephosphorylation of Cx43 are strongly associated with an increased propensity for the development of life-threatening ventricular arrhythmias.[13–16]

AC is clinically diagnosed according to the revised Task Force Criteria (TFC)[17] based on global or regional dysfunction and structural alterations, tissue characterization of the ventricular wall, re- and depolarization or conduction abnormalities, arrhythmias, genetics, and family history. Still, many cases remain un- or misdiagnosed because of the multiple facets of the clinical manifestation of the disease.

It has been described that the immunoreactivity of plakoglobin (PKG) was reduced in a high percentage of patients with AC compared with either controls or patients with other underlying cardiac diseases such as dilated or hypertrophic cardiomyopathy.[12] This early reduction in the immunoreactivity of PKG appeared present not only in the right ventricle but also in the macroscopically unaffected left ventricle and interventricular septum. Based on this finding, it was suggested that the immunoreactivity of PKG could be a tool to discriminate patients with AC from healthy subjects and patients with other forms of heart disease. However, more recent studies have shown that PKG signals are also reduced in sarcoidosis and giant cell myocarditis.[18,19]

Recently, we reported that a reduction in Cx43 protein can lead to reduced sodium channel ($Na_V1.5$) expression and function in a mouse model of severely reduced Cx43 and in isolated neonatal rat ventricular cardiomyocytes.[20] Furthermore, in vitro silencing of plakophilin-2 (PKP2), one of the desmosomal proteins that is often mutated in patients with AC, leads to a decreased sodium current.[21] In addition, PKP2-haploinsufficent mice showed a significant sodium current deficit.[22] Whether desmosomal deficiency and the AC phenotype correlate with changes in the distribution of proteins relevant to the sodium channel complex in the human heart remains to be defined.

In this study, we used immunohistochemistry to identify the immunoreactive signal levels and distribution of Cx43, PKG, and $Na_V1.5$ in patients with AC as compared to controls. Our data show that these levels and distribution of Cx43, PKG, and $Na_V1.5$ are affected in a large majority of patients with AC.

Methods

Patient samples and tissue processing

The left and right ventricular free wall myocardium (on average 2–4 cm^3) was obtained from 5 patients with AC (AC1–AC5; postmortem) and from 5 age- and sex-matched controls with no underlying heart disease (C1–C5). Right ventricular septal biopsies (RVSBs; 2–4 mm^3) were obtained from another 15 patients with AC (AC6–AC20). All patients consented to clinical evaluation according to the revised TFC. All material used in this study was flash frozen in liquid nitrogen. Frozen samples were cryosectioned at a thickness of 10 μm.

Patient screening

From 18 of 20 patients with AC, DNA was available to screen for mutations in the *PKP2*, *DSP*, *DSG2*, *DSC2*, *PKG*, *TMEM43*, and *PLN* genes by direct sequencing. In addition, using multiple ligation-dependent probe amplification, the *PKP2* gene was screened for large exon deletions. Genetic screening was performed on the patients' written consent.

Immunohistochemistry

Frozen material was serially sectioned, generating sections of 10-μm thickness that were collected on aminopropyltriethoxysilane-coated glass slides. Immunohistochemistry was performed as described previously.[23] Once the labeling was completed, analysis of the results was performed using a Nikon eclipse 80i microscope equipped for epifluorescence. An independent analysis of different patients was performed by 3 observers per experiment who were blinded to the study. First, for each and every patient, an overall conclusion was drawn and pictures supportive to the overall conclusion were captured using a Nikon digital sight DS-BMWe camera and NIS Elements BR3.0 software (using equal exposure times). Primary antibodies against N-cadherin (mouse, Sigma, 1:800), PKP2 (mouse, Progen, undiluted 1:1000), PKG (mouse, Sigma, 1:100,000), $Na_V1.5$ (rabbit, custom-made,[24] 1:100), and Cx43 (mouse, Transduction Labs, 1:200; rabbit, Zymed, 1:250) were used. Secondary labeling was performed with appropriate Texas Red (1:100) and fluorescein isothiocyanate (1:250) conjugated whole IgG antibodies (Jackson Laboratories). Blinded cross-evaluation for $Na_V1.5$ labeling of material from 9 patients was performed in Utrecht and New York.