ORIGINAL ARTICLE

A New Diagnostic Test for Arrhythmogenic Right Ventricular Cardiomyopathy

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ABSTRACT

BACKGROUND

The diagnosis of arrhythmogenic right ventricular cardiomyopathy (ARVC) can be challenging because the clinical presentation is highly variable and genetic penetrance is often low.

METHODS

To determine whether a change in the distribution of desmosomal proteins can be used as a sensitive and specific diagnostic test for ARVC, we performed immunohistochemical analysis of human myocardial samples.

RESULTS

We first tested myocardium from 11 subjects with ARVC; of these samples, 8 had desmosomal gene mutations. We also tested myocardium obtained at autopsy from 10 subjects with no clinical or pathological evidence of heart disease as control samples. All ARVC samples but no control samples showed a marked reduction in immunoreactive signal levels for plakoglobin (also known as γ-catenin), a protein that links adhesion molecules at the intercalated disk to the cytoskeleton. Other desmosomal proteins showed variable changes, but signal levels for the nondesmosomal adhesion molecule N-cadherin were normal in all subjects with ARVC. To determine whether a diminished plakoglobin signal level was specific for ARVC, we analyzed myocardium from 15 subjects with hypertrophic, dilated, or ischemic cardiomyopathies. In every sample, levels of N-cadherin and plakoglobin signals at junctions were indistinguishable from those in control samples. Finally, we performed blinded immunohistochemical analysis of heart-biopsy samples from the Johns Hopkins ARVC registry. We made the correct diagnosis in 10 of 11 subjects with definite ARVC on the basis of clinical criteria and correctly ruled out ARVC in 10 of 11 subjects without ARVC, for a sensitivity of 91%, a specificity of 82%, a positive predictive value of 83%, and a negative predictive value of 90%. The plakoglobin signal level was reduced diffusely in ARVC samples, including those obtained in the left ventricle and the interventricular septum.

CONCLUSIONS

Routine immunohistochemical analysis of a conventional endomyocardial-biopsy sample appears to be a highly sensitive and specific diagnostic test for ARVC.

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ARRHYTHMOGENIC RIGHT VENTRICULAR cardiomyopathy (ARVC) is associated with a high frequency of arrhythmias and sudden cardiac death.[1-3] Mutations in genes encoding desmosomal proteins (including desmoplakin, plakoglobin, plakophilin 2, desmocollin 2, and desmoglein 2) have been identified in approximately 40% of patients with ARVC.[4] However, genetic analysis remains a research tool, and in everyday practice, the diagnosis of ARVC can be challenging. The clinical presentation may be highly variable, and the genetic penetrance is often low. This is especially true in family members of an index patient in whom establishing a diagnosis of ARVC requires extensive clinical workup, often leading to equivocal results. Endomyocardial biopsy has not been consistently useful because the structural changes in ARVC tend to spare the subendocardium and do not typically involve the interventricular septum.[2] Thus, the pathological features of ARVC are often not seen in conventional endomyocardial-biopsy specimens. Moreover, these pathological features tend to be most conspicuous in patients with severe disease and are not well developed in patients with early disease. In the end, the diagnosis usually rests on fulfilling a set of clinical criteria, as defined by an international ARVC task force.[5] Although these criteria are relatively specific, they are not highly sensitive.

We had observed that the immunoreactive signal level for the desmosomal protein plakoglobin, also known as γ-catenin, was dramatically reduced at intercalated disks in patients with rare recessive forms of ARVC (Naxos disease and the Carvajal syndrome).[6,7] We have since had an opportunity to study more common forms of ARVC related to dominant mutations in a variety of desmosomal genes and have observed a reduced plakoglobin signal level.[8] On the basis of these observations, we wanted to determine whether a decreased plakoglobin signal level at the myocardial cell–cell junctions is a sensitive and specific marker for ARVC.

METHODS

We analyzed three sets of samples, all consisting of formalin-fixed, paraffin-embedded myocardium obtained on autopsy or endomyocardial biopsy. Information regarding antibodies, immunohistochemical protocols, and statistical analysis

appears in the Supplementary Appendix, available with the full text of this article at NEJM.org.

RESULTS

SUBJECTS WITH DOCUMENTED ARVC

We first performed immunohistochemical analysis on cardiac tissue samples obtained either on biopsy or on autopsy from 11 subjects who were known to have ARVC. Eight of these subjects had a specific mutation in a desmosomal protein gene. Table 1 shows the clinical characteristics and the results of genetic analysis of these subjects. In every case, we found that immunoreactive signal levels for the desmosomal protein plakoglobin were reduced at intercalated disks, whereas plakoglobin signal levels were strongly positive in myocardium obtained on autopsy from 10 control subjects with no clinical or pathological evidence of heart disease (Fig. 1). Plakoglobin signal levels were reduced not only in right ventricular regions showing typical pathological changes of fibrofatty replacement but also in normal-appearing left ventricle and interventricular septum, including the subendocardium in these areas. In all ARVC samples, plakoglobin signal levels were either absent or obviously reduced in comparison with those in control samples, such that the difference was immediately apparent on inspection.

Signal levels for plakophilin 2 and desmoplakin varied in the ARVC samples (Fig. 1). The expression of plakophilin 2 at intercalated disks was indistinguishable from that in control samples, but the level of desmoplakin was clearly diminished in samples from subjects bearing a frameshift or nonsense mutation in desmoplakin (Subjects A1 and A2) and from a subject with a plakoglobin mutation (Subject A4) (Table 1). Signal levels for plakophilin 2 and desmoplakin appeared to be normal in tissue from a subject bearing a missense mutation in desmoplakin (Subject A3), whereas signal levels for both plakophilin 2 and desmoplakin were reduced in samples from subjects with mutations in plakophilin 2 or desmoglein 2 (Subjects A5, A6, A7, and A8) and in samples from subjects in whom no desmosomal gene mutations were identified (Subjects A9, A10, and A11). Myocardial samples from all 11 subjects with ARVC showed marked reductions in the amount of junctional signal for the major gap-junction protein, connexin 43, whereas a strong signal for the non-desmosomal ad-

hesion molecule N-cadherin was always present and was indistinguishable from that in control samples (Fig. 1).

OTHER FORMS OF END-STAGE HEART DISEASE

To determine whether a reduced signal level for plakoglobin at intercalated disks is specific for ARVC or is merely a marker of cardiac disease, we immunostained transmural sections of left and right ventricular free walls and interventricular septum from the native hearts of subjects who had undergone cardiac transplantation for end-stage heart disease. This set of tissue samples came from 15 subjects with end-stage heart disease: 5 subjects each with hypertrophic cardiomyopathy, dilated cardiomyopathy, or ischemic heart disease. We compared these samples with the 10 control samples used in the initial analysis

of subjects known to have ARVC. In every case, the native hearts of the subjects who had undergone cardiac transplantation showed high plakoglobin signal levels at intercalated disks, which were indistinguishable from the signal levels in control samples (Fig. 2).

Signal levels for plakophilin 2 and N-cadherin at intercalated disks also appeared to be normal, whereas all samples from subjects with end-stage heart failure had clearly diminished expression of connexin 43. The desmoplakin signal at cell–cell junctions was indistinguishable from that in control samples from subjects with hypertrophic or ischemic cardiomyopathy. However, the signal level for desmoplakin varied in samples from subjects with dilated cardiomyopathy: there was a diminished level in two subjects, a level that was similar to that in control

Table 1. Clinical and Genetic Characteristics of Subjects with ARVC.*			
Subject No.	Tissue Source	Clinical Characteristics	Mutation
A1	Autopsy	Sudden cardiac death at the age of 36 yr	Desmoplakin (R1113X)
A2	Autopsy	Sudden cardiac death at the age of 42 yr; history of palpitations and exertional presyncope; did not undergo medical evaluation	Desmoplakin (1218+1G→A)
A3	Autopsy	Sudden cardiac death at the age of 18 yr	Desmoplakin (S299R)
A4	Biopsy	History of syncope; QRS prolongation, T-wave inversion, and late potentials in right precordial leads on ECG; moderate global right ventricular dilatation and regional wall-motion abnormalities on echocardiography; no left ventricular involvement; sustained ventricular tachycardia associated with left bundle-branch block on electrophysiological study; currently 57 yr of age	Plakoglobin (S39_K40insS)
A5	Autopsy	Sudden cardiac death at the age of 22 yr	Plakophilin 2 (2146–1G→C)
A6	Autopsy	Sudden cardiac death at the age of 37 yr; history of right ventricular dilatation and "floppy" mitral valve	Plakophilin 2 (2506delA)
A7	Biopsy	Sustained palpitations and recurrent presyncope episodes; poor R-wave progression; T-wave inversion at V_1–V_6 on ECG; global biventricular dilatation on echocardiography; multiple right ventricular aneurysms and a single apical left ventricular aneurysm on MRI; 1400 ventricular extrasystoles recorded during a 24-hour period; extensive loss of cardiac myocytes and replacement by fibroadipose tissue on endomyocardial biopsy; currently 45 yr of age	Desmoglein 2 (C591X)
A8	Autopsy	Sudden cardiac death at the age of 15 yr	Desmoglein 2 (829_840delCTTGAAGGCATG)
A9	Autopsy	Sudden cardiac death at the age of 23 yr	No mutation identified
A10	Autopsy	Sudden cardiac death at the age of 32 yr	No mutation identified
A11	Autopsy	Sudden cardiac death at the age of 17 yr	No mutation identified

* ARVC denotes arrhythmogenic right ventricular cardiomyopathy, ECG electrocardiography, and MRI magnetic resonance imaging.
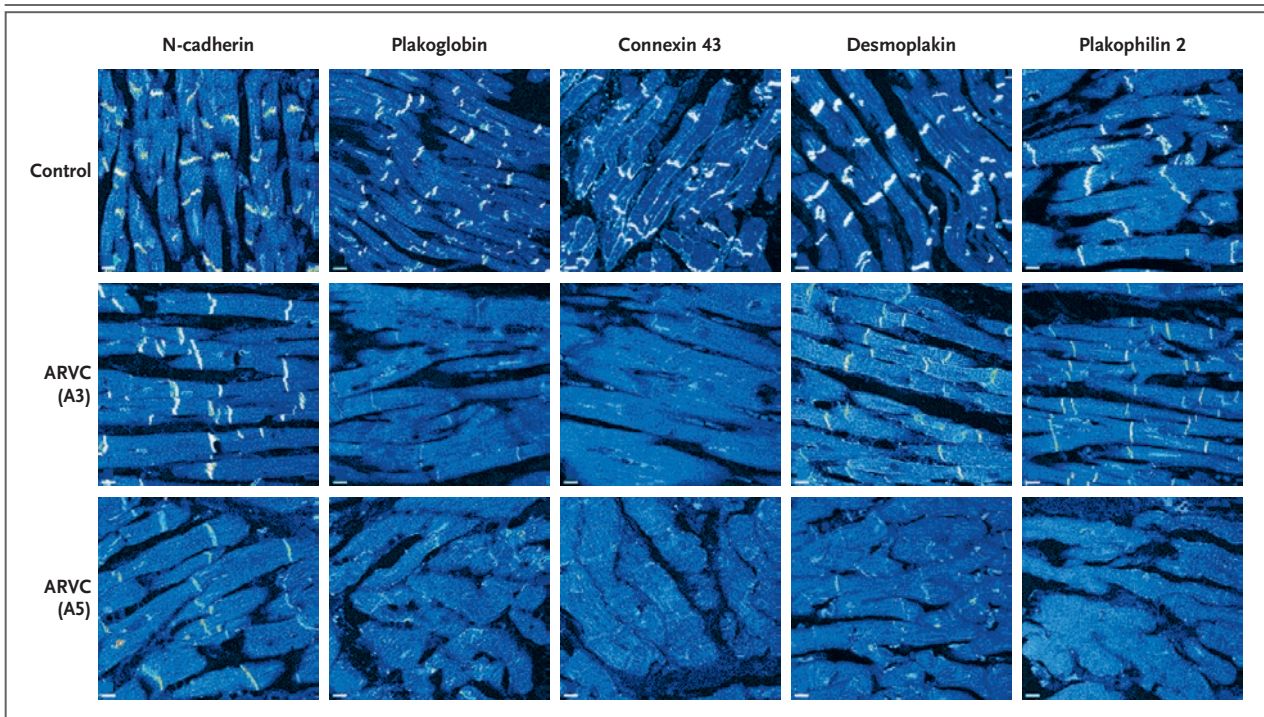
Figure 1. Immunofluorescence Images of Left Ventricular Myocardium from Two Subjects with ARVC and a Control Subject.

Representative images show that for selected junctional proteins at the intercalated disks of cardiomyocytes, immunoreactive signal levels in the subjects with arrhythmogenic right ventricular cardiomyopathy (ARVC) differ from the signal levels in the control subject. Numbers in parentheses correspond to the subject numbers shown in Table 1.

subjects in two subjects, and an apparently increased level in one subject. Taken together, these results suggest that a diminished signal level for plakoglobin is a consistent feature of ARVC but not of other forms of severe heart disease.

BLINDED ANALYSIS OF BIOPSY SAMPLES

To determine the ability of immunostaining to identify patients with ARVC, we examined 30 heart-biopsy samples from subjects who had been evaluated in the Johns Hopkins ARVC Registry. These samples were from 11 subjects who fulfilled the clinical criteria for ARVC,[5] 14 subjects who did not fulfill clinical criteria and were considered not to have ARVC, and 5 subjects who were deemed to be "borderline" regarding clinical criteria for ARVC.

Each of the subjects had been evaluated with a detailed history taking and physical examination, 12-lead electrocardiography, signal-averaged electrocardiography, Doppler echocardiography, magnetic resonance imaging (MRI), right ventricular angiography, electrophysiological testing, and conventional right ventricular endomyocar-

dial biopsy, according to protocols described previously.[9,10] Clinical and immunohistochemical diagnoses for these subjects are shown in Table 2. Representative immunofluorescence images are shown in Figure 3.

We observed reduced plakoglobin and normal N-cadherin signal levels, which we interpreted to indicate the presence of ARVC, in biopsy samples from 12 subjects. Of these subjects, 10 were considered to have ARVC, and 1 (Subject 22) was thought not to have ARVC on the basis of clinical criteria. The remaining subject (Subject 3) fell into the borderline group. Normal signal levels for plakoglobin and N-cadherin, which we used as the basis for ruling out ARVC, were seen in 13 subjects. These subjects included 10 in whom ARVC was ruled out by clinical criteria, 1 who was thought to have ARVC on the basis of clinical criteria (Subject 10), and 2 who were in the borderline category (Subjects 18 and 19). Finally, we observed reduced signal levels for both plakoglobin and N-cadherin in five subjects (Subjects 11, 12, 27, 28, and 30), which we interpreted to indicate poor tissue quality that pre-
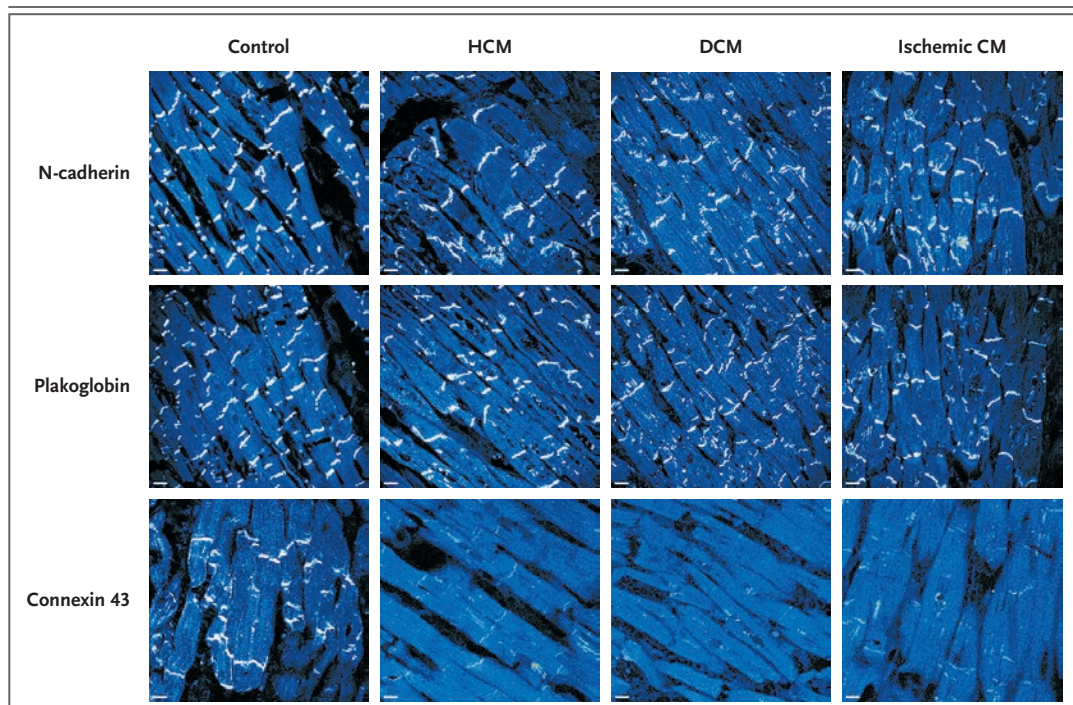
Figure 2. Immunofluorescence Images of Myocardium from a Control Subject and from Three Subjects with End-Stage Heart Disease.

Representative images show that immunoreactive signal levels for N-cadherin and plakoglobin at the intercalated disks of cardiomyocytes in a control subject are similar to those in subjects with hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), and ischemic cardiomyopathy (Ischemic CM), whereas signal levels for connexin 43 are reduced in subjects with severe heart disease.

cluded immunohistochemical diagnosis. Thus, we correctly diagnosed ARVC in 10 of 11 subjects with documented ARVC and correctly ruled out ARVC in 10 of 11 subjects without ARVC. We diagnosed ARVC in one of three subjects in the borderline group.

The one subject who had a reduced signal level for plakoglobin but did not have ARVC (as defined by clinical criteria) had received a diagnosis of sarcoidosis on the basis of the presence of noncaseating granulomas when the biopsy sample was initially examined. However, granulomas were not seen in the sections of this biopsy sample that were prepared for immunostaining. The one subject who had a normal level of plakoglobin but who fulfilled the clinical criteria for ARVC had a family history of ARVC, as confirmed on autopsy, but was asymptomatic himself. Clinical evaluation before gallbladder surgery revealed nonsustained ventricular tachycardia and T-wave inversions in V_1 through V_3 on electrocardiography, and MRI showed fatty infiltration of

the right ventricle with regional dyskinesis, findings that fulfilled clinical criteria for the disease. The biopsy sample showed focal interstitial fibrosis but no fat.

Table 1 in the Supplementary Appendix shows a statistical analysis of the ability of the immunohistochemical test to correctly diagnose ARVC in biopsy samples obtained from 22 subjects in whom there was a definitive diagnosis on the basis of clinical criteria and in which immunohistochemical interpretation was possible. In this data set, a reduced level of plakoglobin had a sensitivity of 91% (95% confidence interval [CI], 59 to 100), a specificity of 82% (95% CI, 48 to 98), a positive predictive value of 83% (95% CI, 52 to 98), and a negative predictive value of 90% (95% CI, 56 to 100). When data from the analysis of samples from 11 subjects with known ARVC and 10 control subjects were added to the blinded analysis of biopsy samples, the values increased to a sensitivity of 95% (95% CI, 77 to 100), a specificity of 90% (95% CI, 70 to 99), a positive

Table 2. Clinical and Immunohistochemical Diagnoses in Blinded Analysis of Cardiac-Biopsy Specimens.*

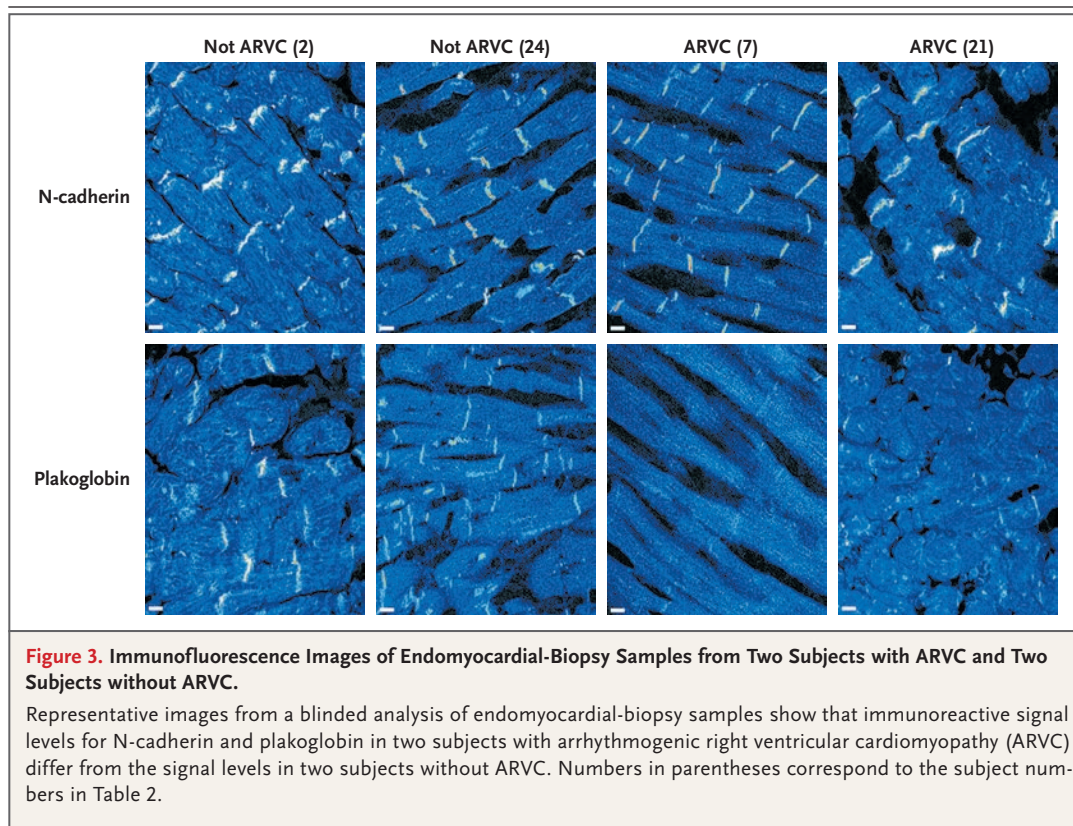| Subject No.	Clinical Diagnosis†	Immunofluorescent Signal for N-Cadherin	Immunofluorescent Signal for Plakoglobin	Immuno-histochemical Diagnosis	Comparison with Clinical Diagnosis
1	Idiopathic ventricular tachycardia	Normal	Normal	Not ARVC	Agree
2	Idiopathic ventricular tachycardia	Normal	Normal	Not ARVC	Agree
3	Borderline for ARVC	Normal	Reduced	ARVC	NA
4	ARVC	Normal	Reduced	ARVC	Agree
5	ARVC	Normal	Reduced	ARVC	Agree
6	Idiopathic ventricular tachycardia	Normal	Normal	Not ARVC	Agree
7	ARVC	Normal	Reduced	ARVC	Agree
8	Dilated cardiomyopathy	Normal	Normal	Not ARVC	Agree
9	ARVC	Normal	Reduced	ARVC	Agree
10	ARVC	Normal	Normal	Not ARVC	Disagree
11	Borderline for ARVC	Reduced (excluded from study)	Reduced	Not possible	NA
12	ARVC	Reduced (excluded from study)	Reduced	Not possible	NA
13	ARVC	Normal	Reduced	ARVC	Agree
14	Normal; family history of ARVC	Normal	Normal	Not ARVC	Agree
15	ARVC	Normal	Reduced	ARVC	Agree
16	Normal	Normal	Normal	Not ARVC	Agree
17	Idiopathic ventricular tachycardia	Normal	Normal	Not ARVC	Agree
18	Borderline for ARVC	Normal	Normal	Not ARVC	NA
19	Borderline for ARVC	Normal	Normal	Not ARVC	NA
20	Idiopathic ventricular tachycardia	Normal	Normal	Not ARVC	Agree
21	ARVC	Normal	Reduced	ARVC	Agree
22	Sarcoidosis	Normal	Reduced	ARVC	Disagree
23	ARVC	Normal	Reduced	ARVC	Agree
24	Sarcoidosis	Normal	Normal	Not ARVC	Agree
25	ARVC	Normal	Reduced	ARVC	Agree
26	Normal	Normal	Normal	Not ARVC	Agree
27	Normal	Reduced (excluded from study)	Reduced	Not possible	NA
28	Normal	Reduced (excluded from study)	Reduced	Not possible	NA
29	ARVC	Normal	Reduced	ARVC	Agree
30	Normal	Reduced (excluded from study)	Reduced	Not possible	NA

* ARVC denotes arrhythmogenic right ventricular cardiomyopathy, and NA not applicable either because the clinical diagnosis was not definitive or because tissue samples could not be evaluated.
† Clinical criteria were defined by an international ARVC task force.[5]

predictive value of 91% (95% CI, 72 to 99), and a negative predictive value of 95% (95% CI, 75 to 100).

The extent to which independent observers agreed about the diagnosis of ARVC was mea-sured for two independently stained preparations of the same slides, according to the method de-scribed by Landis and Koch.[11] Interobserver agreement was high, as indicated by a kappa of 1.0 for the first set of stained slides and of 0.72

Figure 3. Immunofluorescence Images of Endomyocardial-Biopsy Samples from Two Subjects with ARVC and Two Subjects without ARVC.

Representative images from a blinded analysis of endomyocardial-biopsy samples show that immunoreactive signal levels for N-cadherin and plakoglobin in two subjects with arrhythmogenic right ventricular cardiomyopathy (ARVC) differ from the signal levels in two subjects without ARVC. Numbers in parentheses correspond to the subject numbers in Table 2.

for the second set of stained slides (Table 1 in the Supplementary Appendix). Intraobserver agreement was 0.72 for Observer 1 and 0.82 for Observer 2, indicating a high degree of reproducibility.

CONVENTIONAL IMMUNOPEROXIDASE ANALYSIS

Diagnostic immunohistochemical analysis in hospital-based clinical laboratories typically involves detection with the use of immunoperoxidase rather than immunofluorescence. To determine whether diminished plakoglobin levels in patients with ARVC can be identified with the use of immunoperoxidase methods, we had representative samples stained in the diagnostic immunohistochemistry laboratory at Beth Israel Deaconess Medical Center in Boston. The ARVC samples showed discernible junctional signal levels of plakoglobin when analyzed by immunoperoxidase at antibody dilutions used in immunofluorescence studies. This finding was presumably caused by the considerable signal amplification involved in peroxidase methods. However, extensive dilution of the primary antiplakoglobin antibody (approximately 1:50,000) resulted in a clear

distinction between ARVC and control samples (Fig. 1 in the Supplementary Appendix). These results suggest that the apparent absence of or reduction in signal in immunofluorescence preparations does not reflect a total lack of plakoglobin protein at cell junctions in samples from subjects with ARVC. Rather, plakoglobin is probably present in reduced concentrations or in different protein-binding complexes such that immunofluorescence detection is greatly decreased.

DISCUSSION

The results of this study indicate that reduced immunoreactive signal levels of plakoglobin at intercalated disks is a consistent feature in patients with ARVC and is not seen in other forms of heart-muscle disease. Our results also show that the immunoreactive level of the nondesmosomal adhesion molecule N-cadherin is normal in patients with ARVC and those with other forms of heart disease. Although a few subjects in our study had reduced N-cadherin signal levels, this finding was always associated with reduced signal for all other junctional proteins and

presumably reflected generalized, nonspecific protein degradation. Thus, assessment of N-cadherin staining provided a reliable internal control for tissue quality. Whereas the combination of a diminished level of plakoglobin and a normal level of N-cadherin indicated a high likelihood of ARVC, diminished levels of both plakoglobin and N-cadherin indicated poor tissue quality, precluding diagnostic interpretation.

Additional validation studies will be required before this new test can be used clinically. For example, we used only a single antiplakoglobin antibody, which recognizes an epitope in the N-terminal. It binds to wild-type plakoglobin and to the two known mutant forms of plakoglobin implicated in the development of ARVC. We do not know whether similar results would be obtained with the use of other antiplakoglobin antibodies or whether the observed staining abnormalities depended on the binding domain of plakoglobin or its binding partners. We also do not know whether all potential causes of ARVC result in a diminished level of plakoglobin at intercalated disks. For example, viral infection (myocarditis) has been associated with ARVC in some patients, which suggests that acquired causes may also lead to disease. Since some patients with ARVC who were analyzed in the first part of our study had no identifiable desmosomal gene mutations, they may have had an acquired cause, and each had a reduced level of plakoglobin at intercalated disks. It is also possible that some subjects from the Hopkins ARVC registry had myocarditis, but this question will require further study.

Although myocardial degeneration and fibrofatty replacement occur preferentially in the right ventricle in patients with ARVC, we observed a diffuse reduction in the level of plakoglobin, not only in areas of the right ventricle showing typical pathological features but also in the left ventricle and interventricular septum, which otherwise appeared to be structurally normal. We also observed reduced plakoglobin signal levels in subendocardial myocytes, which are usually spared in ARVC. These observations suggest that it is not necessary to biopsy the heart in areas showing structural changes in order to diagnose ARVC. A conventional endomyocardial biopsy of the right side of the interventricular septum would reliably show this diagnostic change. It is also possible that equivalent diagnostic information could be derived from more accessible tissues containing desmosomes, such as skin, hair follicles, and buccal mucosa.

Signal levels for the major ventricular gap-junction protein, connexin 43, were clearly diminished in every subject with ARVC we examined in this study. Like plakoglobin, connexin 43 signal levels were reduced diffusely throughout the heart. These observations add to a growing body of literature indicating that the remodeling of gap junctions is a fundamental feature of ARVC.[6,7,12,13] Of course, a reduced signal level for connexin 43 is not specific for patients with ARVC. Diminished expression of connexin 43, which reflects the remodeling of gap junctions, has been well documented in ischemic and nonischemic forms of heart-muscle disease,[14,15] and we observed reduced signal levels for connexin 43 at intercalated disks in virtually all the tissue samples from subjects with end-stage hypertrophic, dilated, and ischemic cardiomyopathies. However, such reduced expression is most apparent in advanced disease, in which there has been considerable structural remodeling of the heart.[14,15] In contrast, the remodeling of gap junctions in patients with ARVC occurs diffusely in regions of the heart that show no apparent structural or functional alterations. This is a potentially important distinction, which hints at disease-specific mechanisms and suggests that gap-junction remodeling causes conduction abnormalities that promote arrhythmias in ARVC.

Our findings do not indicate that plakoglobin is absent from intercalated disks in ARVC. Using highly sensitive immunoperoxidase methods, we observed discernible junctional plakoglobin signal levels in a known ARVC specimen in which little or no signal was seen with the less sensitive method of indirect immunofluorescence. However, when the primary antiplakoglobin antibody that was used in the immunoperoxidase assay was diluted to a very low concentration (1:50,000 dilution), a clear distinction between ARVC and control tissue emerged. Thus, conditions can be defined in which conventional immunoperoxidase staining of a standard biopsy sample of right ventricular endomyocardium may be used as a diagnostic test for ARVC. Such a test could be performed in most pathology departments in which immunoperoxidase has become a standard method of tissue diagnostics.

Our results are promising in terms of devel-

oping a sensitive and specific immunohistochemical test for the diagnosis of ARVC. Previous studies of the diagnostic use of MRI,[16,17] electrocardiography,[18,19] and echocardiography[20] in patients with ARVC have generally reported high sensitivity and specificity in patients with advanced disease but much lower values in patients with earlier or less conspicuous disease. Further studies are needed to fully determine the ability of immunohistochemical testing to accurately identify ARVC before the development of extensive fibrofatty infiltration and the formation of right ventricular aneurysms, which typically produce diagnostic findings on noninvasive tests.

We do not yet know the molecular mechanism responsible for the consistent reduction in plakoglobin signal levels at intercalated disks in patients with ARVC. Nevertheless, the remarkable consistency of this reduction suggests that a shift in plakoglobin from junctional to intracellular or intranuclear pools may play a role in the pathogenesis of the disease. It should be stressed that the immunohistochemical methods used in this study were specifically designed to identify a high concentration of protein at cell–cell junctions and would not necessarily detect increased amounts of plakoglobin within cytoplasmic or nuclear sites, where the local concentration would probably be much lower than in desmosomes. If such a shift occurs in patients with ARVC, then resultant changes in signaling, perhaps through Wnt–β-catenin pathways, could contribute to myocyte injury and subsequent clinicopathological changes in ARVC. Recent studies have provided support for this mechanism.[21,22]

In conclusion, we have developed a new diagnostic test for ARVC that could prove useful if adopted in clinical practice. Further research is required to confirm the diagnostic usefulness of this approach and elucidate pathophysiological implications of altered plakoglobin signal levels at intercalated disks in patients with ARVC.

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