Relationships Between Clinical Characteristics and Decreased Plakoglobin and Connexin 43 Expressions in Myocardial Biopsies From Patients With Arrhythmogenic Right Ventricular Cardiomyopathy

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Summary

Reduced expressions of plakoglobin and connexin 43 have been reported in the myocardium of patients with arrhythmogenic right ventricular cardiomyopathy (ARVC). However, the relationships between these expression abnormalities and the clinical features of ARVC remain unknown.

The expressions of plakoglobin and connexin 43 in myocardial biopsy specimens from 10 patients with confirmed ARVC, and 13 control patients without ARVC (non-ARVC: hypertrophic cardiomyopathy, n = 7; dilated cardiomyopathy, n = 6), were examined by immunostaining to evaluate the relationships between these expressions and the clinical characteristics of ARVC. The ratios of plakoglobin/N-cadherin and of plakoglobin/connexin 43 expressions were significantly lower in the ARVC group than in the control group. Significantly more patients had decreased plakoglobin expression in the ARVC group than in the control group (9/10 versus 7/13; P = 0.0376). Sustained ventricular tachycardia occurred more frequently in patients with ARVC and with decreased expressions of both plakoglobin and connexin 43 than in those with decreased expression of plakoglobin alone (5/5 versus 1/4, P = 0.048).

Decreased expressions of both connexin 43 and plakoglobin in the myocardium might be associated with the development of arrhythmia in ARVC. (Int Heart J 2015; 56: 626-631)

Key words: Arrhythmia, Intercalated disc, Pathology

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a disorder of the heart muscle that predominantly affects the right ventricle and is associated with ventricular tachycardia, syncope, sudden death, and the characteristic pathological feature of cardiac myocytes being replaced with adipocytes and fibrosis.11 Implantation of an implantable cardioverter–defibrillator is one of the treatments of sustained ventricular arrhythmia for prevention of sudden cardiac death in ARVC patients.1 However, there is no specific biomarker or predictor of arrhythmic events in ARVC, while a number of biomarkers reflective of myocardial stress and damage have been developed,30 and P wave analysis has been reported to be useful for predicting new onset of atrial fibrillation.44 One of the reasons for a lack of specific biomarkers was because the precise mechanisms of ARVC had not been determined.

Cardiac myocytes have three different types of intercellular junctions at the cardiac intercalated disc (adherens junctions, desmosomes, and gap junctions), and the identified gene mutations of ARVC include the intracellular junction proteins, plakoglobin, desmplakin, plakophilin-2, desmoglein-2, and desmocollin-2.54

It has recently been demonstrated that the expression of plakoglobin, a component of both the adherens junctions and desmosomes, was decreased in uninvolved myocardium of ARVC with normal expression of N-cadherin by immunostaining.34 Moreover, the previous study showed that immunoreactive signals for connexin 43, a gap junction component, as well as plakoglobin, were also disturbed in around 70% of patients with ARVC.53

However, no report has examined the relationships between these protein expressions and the clinical characteristics in ARVC.

The present study compared the expressions of plakoglobin and connexin 43 between ARVC and non-ARVC patients and evaluated the relationships between changes in their expressions and the clinical features of ARVC.

References

1. Yoshida et al. Reduced expressions of plakoglobin and connexin 43 have been reported in the myocardium of patients with arrhythmogenic right ventricular cardiomyopathy (ARVC). However, the relationships between these expression abnormalities and the clinical features of ARVC remain unknown. Int Heart J 2015; 56: 626-631.

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The Ethics Committee at Nagasaki University Hospital approved the protocol for this study, which proceeded according to the Declaration of Helsinki (approval number 13052788).

Patients and clinical analysis: This retrospective analysis included data from 10 patients with ARVC diagnosed according to the revised 2011 Task Force criteria and from 13 patients without ARVC (non-ARVC) as a control group (hypertrophic cardiomyopathy, \(n = 7\); dilated cardiomyopathy, \(n = 6\)) selected from among consecutive patients at our hospital from whom diagnostic endomyocardial biopsies were obtained. Table I shows the clinical features of these patients.

Patient data were collected from a detailed medical history, a physical examination, electrocardiography (ECG), transthoracic echocardiography (TTE), coronary angiography, cardiac catheterization, and endomyocardial biopsies taken from the right and the left ventricles. The myocardial biopsy specimens were stained with hematoxylin and eosin for pathological assessment and with azan to evaluate histopathological findings, including fibrofatty replacement. At least one of magnetic resonance imaging (MRI), computed tomography (CT), or right ventriculography was performed for the diagnosis of ARVC.

The clinical course of the patients, including ventricular arrhythmia, heart failure, and prognosis, was also retrospectively assessed over a period of 2-25 (mean 13 ± 6) years from medical chart reviews and interviews with their family physicians. The exclusion criteria were biopsied myocardium containing few longitudinal myocardial fibers or a large fibrofatty area with little myocardium that would interfere with precise evaluations of the expressions of myocardial cell-cell adhesion proteins.

Immunohistochemical analysis: All myocardial biopsy specimens were formalin-fixed, embedded in paraffin, cut into 3-μm-thick sections, mounted on glass slides, and deparaffinized. The sections were rehydrated, heated in KN9TRS buffer (Pathology Institute, Toyama, Japan) for 40 minutes at 90°C, and then endogenous peroxidase activity was blocked. The sections were sequentially incubated with mouse monoclonal anti-plakoglobin (Sigma, St. Louis, MO, USA), mouse monoclonal anti-N-cadherin (Sigma) or anti-connexin 43 (Invitrogen; Life Technologies, Carlsbad, CA, USA) antibodies, all diluted 1:100, followed by Envision (Dako, Glostrup, Denmark) peroxidase-labeled polymer with Real DAB (Dako) as a chromogenic substrate. The tissue was then counterstained with Mayer’s hematoxylin followed by washing in PBS at pH 7.4 to blue the nuclei.

The immunoreactive distribution was semi-quantified in all areas of structurally normal myocardium in the biopsied myocardium. We counted the number of all positive-stained intercalated discs for connexin 43 in all area of samples but not only one field described in Figure 1. Thus, the total number of positive-stained intercalated discs for connexin 43 was not close to zero. In addition, we defined the reduced expression of connexin 43 as > 50% reduction in signals compared with N-cadherin. Therefore, it was possible to calculate plakoglobin/connexin 43 even in Type C.

The clinical course of these patients was compared between these two groups of patients. Statistical analysis: All values are expressed as the mean ± standard deviation (SD). Data were compared between two groups using the Mann-Whitney \(U\) test or Fischer’s exact test. \(P\) values < 0.05 were considered significant.
There were no significant differences in age, sex, and systolic and diastolic blood pressures between the two groups (Table I). Heart rate was significantly lower in the ARVC group than in the control group ($P < 0.001$), whereas blood pressure did not significantly differ between them (Table I). Significantly more patients had syncope ($P = 0.002$), sustained ventricular tachycardia ($P = 0.002$), and implantable cardioverter defibrillators (ICD) ($P = 0.024$) in the ARVC group than in the control group (Table I). All patients implanted with an ICD had syncope and sustained ventricular tachycardia (VT). Diuretics were prescribed more often in control patients than in those with ARVC ($P = 0.017$; Table I).

Cardiac catheterization revealed a significantly higher mean pulmonary arterial pressure in the control group ($P = 0.006$), although mean pulmonary capillary wedge pressure and cardiac index did not significantly differ between them (Table II). Fibrofatty replacement was evident in 5 of 10 myocardial biopsy specimens from the ARVC group, but in none from the control group ($P = 0.008$).

Among the 10 patients with ARVC, CT showed fatty infiltration of the right ventricular (RV) wall in all 4 patients examined by CT (CT was not performed in 6 of 10 patients), MRI showed late gadolinium enhancement or fatty infiltration of the RV wall in 6 of the 8 patients in whom MRI was performed (2 patients had no MRI), and right ventriculography showed hypokinesis of the RV wall in all patients, and only one patient had impaired left ventricular systolic function, with a left ventricular ejection fraction of 48% on left ventriculography. These results indicated that all of these patients had RV lesions, but there was LV involvement in one patient when ARVC was diagnosed.

The ratios of plakoglobin/N-cadherin and plakoglobin/connexin 43 were significantly lower in the ARVC group than differ significantly between the groups on echocardiography (Table II). Cardiac catheterization revealed a significantly higher mean pulmonary arterial pressure in the control group than in the ARVC group ($P = 0.006$), although mean pulmonary capillary wedge pressure and cardiac index did not significantly differ between them (Table II). Fibrofatty replacement was evident in 5 of 10 myocardial biopsy specimens from the ARVC group, but in none from the control group ($P = 0.008$).

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The ratios of plakoglobin/N-cadherin and plakoglobin/connexin 43 were significantly lower in the ARVC group than
in the control group, whereas the ratio of the expression of connexin 43/N-cadherin was not (Figure 1).

The myocardial immunostaining patterns were classified into 3 types based on N-cadherin, plakoglobin, and connexin 43 staining as follows: type A, preserved expression of all those proteins; type B, decreased expression only of plakoglobin; and type C, decreased expression of plakoglobin, and connexin 43 proteins (Figure 2). Figure 3 shows the numbers of patients with these immunostaining profiles. The number of patients with Types B or C, which included decreased plakoglobin expression, was significantly larger in the ARVC group than in the control group (9/10 versus 6/13; \( P = 0.038 \)).

Immunostaining of biopsied myocardium for plakoglobin achieved 90% sensitivity, 54% specificity, and positive and negative predictive values of 60% and 88%, respectively, for a diagnosis of ARVC.

Among patients with ARVC and decreased plakoglobin expression (Types B and C immunostaining profiles), ventricular tachycardia occurred more frequently in group C than B (5/5 versus 1/4, \( P = 0.048 \)). The frequency of fibrofatty replacement did not differ significantly between the two groups (Table III). Nine of these 10 patients did not require admission to hospital for heart failure, and one patient died of heart failure during the observation period of 24 years.

**Figure 2.** Type A immunostaining profile in myocardium. Myocardial expression of all three components is preserved. A1, N-cadherin; A2, connexin 43; A3, plakoglobin: bar, 100 \( \mu \)m. Type B immunostaining profile in myocardium. Only myocardial expression of plakoglobin is decreased. B1, N-cadherin; B2, connexin 43; B3, plakoglobin: bar, 100 \( \mu \)m. Type C immunostaining profile in myocardium. Myocardial expressions of plakoglobin and connexin 43 are decreased. C1, N-cadherin; C2, connexin 43; C3, plakoglobin: bar, 100 \( \mu \)m.

**Figure 3.** Distribution of immunostaining in ARVC and control groups. Type A, expression of all three components is preserved; type B, only plakoglobin expression is decreased; type C, expressions of both plakoglobin and connexin 43 are decreased. Types B or C, which were associated with decreased myocardial plakoglobin expression, were significantly more prevalent in the patients with than without ARVC (9/10 versus 7/13; \( P = 0.0376 \)).
Table III. Comparison of Sustained VT and Fibrofatty Replacement Between Type B and C Immunostaining Profiles in Patients With ARVC

<table>
<thead>
<tr>
<th></th>
<th>Type B (n = 4)</th>
<th>Type C (n = 5)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sustained VT</td>
<td>1 (25%)</td>
<td>5 (100%)</td>
<td>0.048</td>
</tr>
<tr>
<td>Fibrofatty replacement</td>
<td>2 (50%)</td>
<td>2 (40%)</td>
<td>0.643</td>
</tr>
</tbody>
</table>

VT indicates ventricular tachycardia.

**Discussion**

The present study showed more obvious decreased myocardial expression of plakoglobin in patients with ARVC than in control patients. It has been reported that plakoglobin redistribution is one of the candidates that play a crucial role in the final common pathway of ARVC pathogenesis, and previous studies have reported the usefulness of immunostaining for plakoglobin in the diagnosis of ARVC by endomyocardial biopsy. However, the diagnostic values for ARVC differ among these studies (sensitivity, 76-91%; specificity, 57-84%), as myocardial specimens obtained by biopsy were compared with various controls, including those from hearts with ventricular arrhythmia, dilated cardiomyopathy (DCM), other cardiac diseases, and even hearts without heart disease. Asimaki, et al. achieved the highest sensitivity (91%) and specificity (82%) in a biopsy study compared with controls including normal subjects and patients with idiopathic ventricular tachycardia. In the present study, 90% sensitivity and 54% specificity were achieved by immunostaining myocardial biopsies for plakoglobin with comparison between ARVC and non-ARVC, ie, HCM and DCM. Munkholm, et al. also studied biopsied specimens of patients with and without ARVC but with other diseases (ventricular arrhythmia, DCM, Takotsubo cardiomyopathy, and polymyositis), and their study achieved 85% sensitivity and 75% specificity, similar to the present results. Moreover, plakoglobin expression is decreased in some patients with DCM and granulomatous myocarditis, but in very few of those with lymphocytic myocarditis. These results suggest that the diagnostic values of the decreased expression of plakoglobin for ARVC may be influenced by differences in the characteristics of controls, such as the primary diseases, as well as the stage and severity of ARVC.

It was also found that decreased plakoglobin and connexin 43 expressions in the myocardium of patients with ARVC were associated with severe arrhythmia, although connexin 43 expression did not differ significantly between the ARVC and control groups. Asimaki, et al. also found decreased connexin 43, as well as plakoglobin, expression in patients with ARVC and in control patients with other forms of end-stage heart disease, although they did not describe a relationship between this decrease and clinical findings. On the other hand, Kwon, et al. showed that no ARVC patient had a significant decrease in connexin 43. Although they did not mention the precise clinical features of ARVC patients, the patients with severe arrhythmia similar to the present ARVC patients might not have been included in their study.

Gap junction channels in the ventricular myocardium consist mainly of connexin 43. Low levels of connexin 43 in gap junctions have been reported in the hypertrophied and ischemic human heart, and depleting gap junction plaques containing connexin 43 slow ventricular conduction velocity, leading to arrhythmia and sudden cardiac death in mice. Recently, it has been reported that down regulation of connexin 43 expression, reduction of connexin 43 phosphorylation, and increased fibrosis cause a conduction disorder that is likely to be a crucial component of arrhythmogenicity in human non-ischemic cardiomyopathy. In the present study, a decrease in expression of connexin 43 similar to that in ARVC was observed in controls, ie, HCM and DCM. However, there was no patient with sustained VT in the control group. These suggest that an isolated decrease in expression of connexin 43 may not be sufficient to induce severe arrhythmia, although Kaplan, et al. suggested that the destabilization of cell adhesion complexes might perturb the kinetics of gap junction turnover, resulting in heterogeneous conduction, which potentially contributes to arrhythmogenesis in ARVC.

The present study found that levels of plakoglobin expression in the myocardium were decreased to a greater extent than those of connexin 43 in patients with ARVC than in controls. All of the patients with decreased connexin 43 expression in ARVC also had decreased plakoglobin expression, and some patients with decreased plakoglobin expression did not have decreased connexin 43 expression in the present study. Thus, decreased connexin 43 expression in addition to decreased expression of plakoglobin as a primary abnormal feature may contribute to severe ventricular arrhythmia in ARVC. However, the precise mechanism of how these intercellular adhesion proteins function in intercalated ARVC discs is unknown. Li, et al. reported that conditional, cardiac tissue-specific plakoglobin knockout mice have decreased amounts of gap junction plaque containing connexin 43. However, conduction abnormalities were not apparent in these mice.

Desmosomes provide not only structural attachments between cells, but they also mediate intracellular signal transduction pathways via Wnt/beta-catenin signaling. Swope, et al. reported that beta-catenin associated with connexin 43 was strengthened in plakoglobin-knockout mice. They also found that conditional cardiac tissue-specific plakoglobin/beta-catenin double-knockout mice had even less gap junction plaque containing connexin 43, which was consistent with arrhythmogenicity. Moreover, a reduction of the immunoreactive signal of sodium channel Na_1.5 protein in myocardium, which might contribute to arrhythmia vulnerability, has also been reported in ARVC, in addition to the changes in plakoglobin and connexin 43.

Further study is needed to elucidate precisely how severe arrhythmia develops in patients with ARVC.

**Study limitations:** The genes encoding intercellular junction proteins in the present patients with ARVC were not analyzed, although patients with myocarditis were excluded. Thus, the relationship between myocardial levels of plakoglobin expression and plakoglobin gene abnormalities remains unknown.

Biopsied myocardium, but not heart specimens obtained at autopsy, were analyzed because the aim was to ensure that the proteins of interest remained intact for precise immunochimical evaluation. Thus, there were no normal samples as a control group in the present study. In addition, patients whose myocardial biopsy specimens included large fibrofatty areas with few myocardial cells were excluded. Therefore, differences in the clinical and pathological features between patients
with and without ARVC may have been underestimated, and it was not possible to evaluate the relationship between the severity of arrhythmia and fibrofatty replacement.

Conclusions: Decreased plakoglobin expression was more prevalent in patients with rather than without ARVC. The concomitantly decreased myocardial expressions of connexin 43 and plakoglobin might be associated with severe arrhythmia in ARVC, in addition to fibrofatty replacement.

REFERENCES


