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Original Article

Phospholamban immunostaining is a highly sensitive and specific method for diagnosing phospholamban p.Arg14del cardiomyopathy

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ABSTRACT

Phospholamban (PLN) p.Arg14del cardiomyopathy is associated with an increased risk of malignant ventricular arrhythmias and severe heart failure and a poor prognosis from late adolescence. It can be diagnosed in whole heart specimens, but rarely in right ventricular biopsy specimens, by PLN immunohistochemistry showing PLN-containing aggregates concentrated in cardiomyocytes in dense perinuclear aggresomes. The purpose of this study was to determine whether PLN immunohistochemistry can be used to diagnose PLN p.Arg14del cardiomyopathy using apical left ventricular myocardial specimens harvested during left ventricular assist device (LVAD) implantation. At that stage, a genetic diagnosis, which may guide treatment and referral of family members for further investigation, is frequently not established yet. Included were myocardial specimens from 30 diverse genetic cardiomyopathy cases with known variants (9 carriers of the pathogenic PLN p.Arg14del variant, 18 cases with other pathogenic or likely pathogenic variants in cardiomyopathy-related genes, and 3 with only variants of unknown significance). Immunohistochemical analysis revealed typical dense perinuclear globular PLN-positive aggregates, representing aggresomes, in all nine PLN p.Arg14del cases. In 20 non-PLN cases, PLN-staining was absent. In one non-PLN case, one of the two independent observers misinterpreted PLN staining of heavily wrinkled nuclear membranes of cardiomyocytes as perinuclear PLN aggregates. In this genetic cardiomyopathy cohort, PLN Immunohistochemical analysis in LVAD biopsies was found to be a highly sensitive (100%) and specific (95%) method for demonstration of PLN protein aggregates in PLN p.Arg14del cardiomyopathy. In clinical practice, PLN immunohistochemical analysis of LVAD specimens can be of incremental value in the diagnostic workup of this cardiomyopathy, even more so if genetic analysis is not readily available.

Abbreviations: ACM, Arrhythmogenic cardiomyopathy; ARVC, Arrhythmogenic right ventricular cardiomyopathy; DCM, Dilated cardiomyopathy; IHC, Immunohistochemistry; LVAD, Left ventricular assist device; NGS, Next-generation sequencing; PLN, Phospholamban; RVEMB, Right ventricular endomyocardial biopsy; SERCA, Sarcoplasmic Reticulum Ca^{2+}-ATPase; VUS, Variant of Unknown Significance.

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1. Introduction

In the past decade, advances in molecular genetics, that is, the development and implementation of next-generation sequencing (NGS) techniques, have allowed identification of a still-increasing number of variants related to human cardiomyopathies like hypertrophic, dilated (DCM), and arrhythmogenic cardiomyopathy (ACM). ACM, and in particular its right dominant form arrhythmogenic right ventricular cardiomyopathy (ARVC), is morphologically characterized by myocardial atrophy due to fibrofatty replacement of primarily right ventricular myocardium and clinically by frequent and often early arrhythmogenic events [1]. In ACM, pathogenic variants in over 13 genes [*PKP2, DSP, DSC2, DSG2, JUP, TGFB3, TMEM43, LMNA, DES, TTN, PLN, RYR2, CTNNA3*] [2] have been identified so far, of which pathogenic variants in genes encoding desmosomal proteins (*PKP2, DSP, DSC2, DSG2, JUP*) comprise the major part worldwide [3].

However, it appeared that the pathogenic nondesmosomal c.40_42delAGA (p.Arg14del) variant in the phospholamban (PLN) gene is the most prevalent cardiomyopathy-related mutation in the Netherlands, being detected in 12% of patients clinically diagnosed with ARVC and in 15% of DCM cases [4]. The pathogenic PLN p.Arg14del variant was characterized as a Dutch founder mutation [5], but carriers have also been found in several other European countries, Canada, and the USA [6,7].

PLN is a transmembrane sarcoplasmic reticulum phosphoprotein that regulates calcium cycling by inhibiting the cardiac isoform of the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) in cardiac myocytes. In normal conditions, when phosphorylated, the inhibition of SERCA2a is alleviated, and calcium flux into the sarcoplasmic reticulum increases [8]. The pathogenic PLN p.Arg14del variant has been shown to cause superinhibition of SERCA2 leading to calcium overload, cardiomyocyte damage and, eventually, to myocardial fibrosis [9].

Detailed analyses, both clinical evaluation [4,10] and histopathologic examination [11,12] of whole heart specimens, revealed that the phenotype of PLN p.Arg14del cardiomyopathy is often characterized by overlapping features of both DCM and ARVC supporting the concept of ACM as a biventricular disease. Carriers are at high risk for malignant ventricular arrhythmias and end-stage heart failure with subsequent high mortality. A poor prognosis is observed from late adolescence [10].

We showed that PLN p.Arg14del cardiomyopathy can be diagnosed in explanted and autopsy hearts by PLN immunohistochemistry (IHC) which shows specific PLN-containing aggregates that are concentrated in cardiomyocytes in dense perinuclear aggresomes. Notably, aggregates could only be detected in 2 out of 25 (8%) right ventricular endomyocardial biopsies (RVEMB) cases in a second cohort [12].

The purpose of this study was to determine the sensitivity and specificity of PLN IHC to diagnose PLN p.Arg14del cardiomyopathy in the clinical setting, in a larger myocardial tissue specimen from the apex of the left ventricle (LV) harvested during left ventricular assist device (LVAD) implantation, in a cohort of genetic cardiomyopathy cases.

2. Material and methods

2.1. Source population

Thirty myocardial biopsies from the apex of the LV were collected and evaluated, obtained during LVAD implantation. These pathology specimens were provided by the local tissue bank ("DPSWeb Palga" database; coded) of the Departments of Pathology, University Medical Center Groningen and University Medical Center Utrecht, the Netherlands. Nine of these LVAD specimens were from heterozygous carriers of the pathogenic PLN p.Arg14del variant, expected to harbor PLN aggregates in cardiomyocytes. In order to establish the sensitivity and specificity of identifying PLN-positive aggregates, PLN-IHC was also performed on 21 LVAD myocardial specimens from genetic cardiomyopathy cases with other variants in cardiomyopathy-related genes. These variants, identified during standard clinical care using targeted NGS, were classified according to the American College of Medical Genetics and Genomics and the Association for Molecular Pathology recommendations [13]. In the 21 non-PLN cases, 18 pathogenic (P) or likely pathogenic (LP) variants in cardiomyopathy-related genes were found and five variants of unknown significance in the following genes: lamin A/C (*LMNA;* 7 P/LP), titin (*TTN*; 6 P/LP and 2 VUS, both VUS in 1 patient), desmoplakin (*DSP*; 1 P/LP and 1 VUS, both in 1 patient), tropomyosin 1 (*TPM1*; 2 P/LP), crystallin alpha B *(CRYAB*; 1 P/LP), myosin-binding protein C *(MYBPC3*; 1 VUS), myosin heavy chain 7 (*MYH7*; 1 VUS), and troponin T type 2 (*TNNT2*; 1 P/LP). Further details of these observed genetic variants are shown in Table 1.

2.2. Ethics statement

The study met the criteria of the code of conduct for responsible use of human tissue that is used in the Netherlands (Dutch federation of biomedical scientific societies; http://www.federa.org).

The study conformed to the principles of the Helsinki Declaration and the institutional medical ethics committees; the collection of the tissue was approved by the scientific advisory board of the biobank of the University Medical Center Utrecht, Utrecht, the Netherlands (protocol no. 12/387).

Table 1
Overview of observed genetic variants using targeted NGS (n=30 cases)

Case	Gene	cDNA sequence	Protein sequence	Class [13]
1	*CRYAB*	c.527A>G	p.(Ter176Trpext*19)	(L)P
2	*PLN*	c.40_42delAGA	p.(Arg14del)	P
3	*PLN*	c.40_42delAGA	p.(Arg14del)	P
4	*LMNA*	c.992G>A	p.(Arg331Gln)	P
5	*TNNT2*	c.650_652del	p.(Lys217del)	P
6	*PLN*	c.40_42delAGA	p.(Arg14del)	P
7	*TTN*	c.47756_47757del	p.(Lys15919Ser*fs**3)	LP
8	*LMNA*	c.949G>A	p.(Glu317Lys)	LP
9	*TTN*	c.32810C>T	p.(Pro10937Leu)	VUS
	TTN	c.13739A>G#	p.(His4580Arg#)	VUS
10	*TTN*	c.61121_1G>A	p.(Glu2374Gly*fs**7)	LP
11	*PLN*	c.40_42delAGA	p.(Arg14del)	P
12	*MYBPC3*	c.187C>G	p.(His63Asp)	VUS
13	*TTN*	c. 92322_92326del	p.(Ser30776His*fs**7)	LP
14	*LMNA*	c.1608+4A>G^	p.? [16]	P
15	*LMNA*	c.992G>A	p.(Arg331Gln)	P
16	*TTN*	c. 81610G>T	p.(Glu27204*)	LP
17	*TTN*	c. 69748G>T	p.(Glu23250*)	P
18	*LMNA*	c.1130G>T	p.(Arg377Leu)	P
19	*TPM1*	c.479G>A	p.(Arg160His)	LP
20	*LMNA*	c.1477C>T	p.(Gln493*)	P
21	*TPM1*	c.184G>C	p.(Glu62Gln)	P
22	*PLN*	c.40_42delAGA	p.(Arg14del)	P
23	*MYH7*	c.2573G>T	p.(Arg858His)	VUS
24	*PLN*	c.40_42delAGA	p.(Arg14del)	P
25	*PLN*	c.40_42delAGA	p.(Arg14del)	P
26	*DSP*	c.6687delA	p.(Arg2229Serfs*32)	P
		c. 273+5G>A^	p.? [17]	VUS
27	*LMNA*	c.992G>A	p.(Arg331Gln)	P
28	*PLN*	c.40_42delAGA	p.(Arg14del)	P
29	*TTN*	c.55063del	p.(Thr18355Leu*fs**3)	LP
30	*PLN*	c.40_42delAGA	p.(Arg14del)	P

Abbreviations (and used isoform for annotation, unless indicated otherwise below): *CRYAB* (NM_001885.2) = crystallin alpha B; *PLN* (NM_002667.4) = phospholamban; *LMNA* (NM_170707.3) = lamin A/C; *TNNT2* (NM_000364.3) = troponin T type 2; *TTN* (NM_133378.4) = titin; *TPM1* (NM_000366.5) = tropomyosin 1; *MYBPC3* (NM_000256.3): myosin-binding protein C; *MYH7* (NM_000257.3) = myosin heavy chain 7; *DSP* (NM_004415.3) = desmoplakin.
P = pathogenic; LP = likely pathogenic; VUS variant of unknown significance.
#NM_133379.4.
^splice site variant: sequence changes at protein level unknown.

2.3. Immunohistochemistry

IHC for PLN was performed to visualize PLN-containing protein aggregates in cardiomyocytes. The mouse monoclonal antibody 2D12 (Abcam, Cambridge, MA, USA) was used in a dilution of 1: 10,000. Applying this strong dilution, strong immunostaining of PLN aggregates was still easy to discern from the weaker background staining of PLN in the cytoplasm of the cardiomyocytes. This antibody binds specifically to PLN residues 7–13. IHC was performed on an automated immunostaining platform (Ventana Benchmark Ultra, Ventana Medical Systems, Tucson, AZ, USA) using the CC1 standard antigen retrieval protocol (Tris HCl buffer pH 9 for 1 h at 95 °C). Appropriate positive and negative controls were used throughout. Specifically, for the PLN antibody, the myocardium served as internal positive control. The PLN aggregates were examined in PLN-immunostained sections in an area of 10 mm^2 in each case, corresponding to 40 high power field (HPF; the field of the 40× lens) using an Olympus BX40 microscope. The characteristic features of these aggregates as described in our previous work [12], in particular the size, shape, and localization, were used to identify and characterize the aggresomes. All LVAD specimens were examined by two independent observers (WPtR and AJHS) of which one is an experienced cardiovascular pathologist.

3. Results

Immunohistochemical analysis revealed typical dense perinuclear globular PLN-positive aggregates (representing aggresomes) in all 9 PLN p.Arg14del cases, while absent in all other specimens. The observed number of these PLN aggregates per specimen was highly variable (median of 3 per 10 mm^2; range 2–89). A characteristic example of PLN aggregates in cardiomyocytes is shown in Fig. 1. PLN-containing aggregates in PLN p.Arg14del cardiomyopathy are large elongated globular cytoplasmic aggregates present perinuclearly, on one side or both sides of the nucleus. In longitudinally sectioned cardiomyocytes, these PLN-containing aggregates are most easily detected, whereas in transversely sectioned cardiomyocytes, they are much more difficult to recognize. We noted that cardiomyocytes with PLN aggregates often showed diminished cytoplasmic PLN staining (Fig. 1). In 20 genetic cardiomyopathy cases due to genetic variants other than PLN, no PLN-stained aggregates were observed. In one non-PLN case [Case 14 (Table 1): carrier of the pathogenic c.1608+4A>G splice site variant in the *LMNA* gen], one of the two observers misinterpreted PLN staining of heavily wrinkled nuclear membranes in transversely sectioned cardiomyocytes as perinuclear PLN aggregates (Fig. 2). After review, both investigators agreed that these pseudo-aggregates were condensed wrinkled nuclear membranes and not typical PLN-containing aggresomes. Overall, in this LVAD cohort, PLN immunostaining was found to have very high sensitivity (9/9: 100%) and specificity (20/21: 95%) for demonstration of PLN protein aggregates in PLN p.Arg14del cardiomyopathy.

4. Discussion

Phospholamban p.Arg14del cardiomyopathy, which clinically presents as DCM and/or ACM, is characterized by large perinuclear PLN protein aggregates, which are detectable in complete heart specimens and ultrastructurally appear to be aggresomes [12]. LVAD implantation is an established treatment option and is becoming increasingly common in selected cardiomyopathy patients with severe heart failure [14]. At the stage of LVAD implantation, a genetic diagnosis, which may guide treatment and referral of family members for further investigation, is frequently not established yet. During implantation of the device, a part of the apex of the LV becomes available for diagnostic purposes. In the present study, we observed that the application of PLN IHC on these LV myocardial tissue fragments is a highly sensitive and specific method for demonstration of PLN protein aggregates in PLN p.Arg14del cardiomyopathy. Dense perinuclear globular PLN-positive aggregates, representing aggresomes, were found in all 9 PLN p.Arg14del cases, whereas in 20 non-PLN cases, aggregated PLN immunostaining was not observed. This negative control group consisted of cases of genetic cardiomyopathy, which were related to variants in *LMNA* (7 cases), *TTN* (7 cases), *TPM1* (2 cases), and *CRYAB*, *DSP*, *MYBPC3*, *MYH7*, and *TNNT2* (1 case each), expanding earlier observations. In one non-PLN case, one of the two observers misinterpreted PLN staining of heavily wrinkled nuclear membranes in cross-sectioned cardiomyocytes as perinuclear PLN aggregates. This underscores the fact that, apart from being a specific component of the sarcoplasmic reticulum, PLN is also present in the nuclear envelope of cardiomyocytes, where it may be involved in nuclear calcium handling [15].

In our initial histopathological study [12], perinuclear PLN aggregates were found with IHC in all 20 examined whole heart specimens, in both left and right ventricular myocardium, of PLN p.Arg14del cardiomyopathy. PLN IHC was negative in four cases of genetic DCM, related to variants in the *LMNA*, desmin (*DES*), RNA-binding motif protein 20

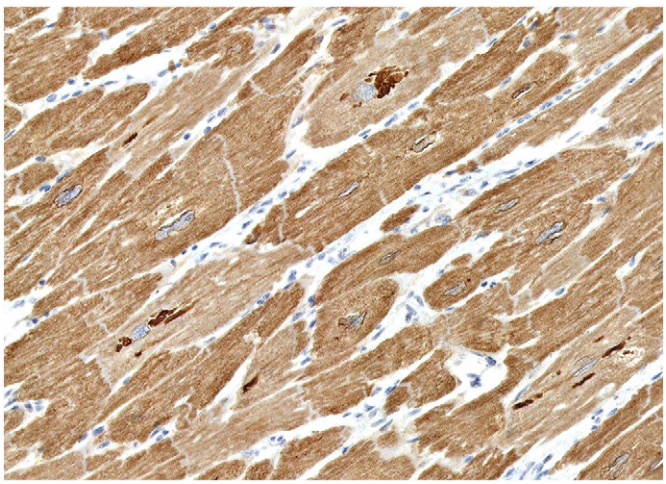

Fig. 1. Typical PLN immunostaining result in a PLN p.Arg14del mutation carrier: large globular elongated perinuclear PLN-stained aggregates in several cardiomyocytes, indicating aggresome formation, in apical LV myocardium sectioned longitudinally. Notably, the cardiomyocytes with aggregates also show diminished cytoplasmic PLN. (PLN-IHC staining, original magnification ×200).

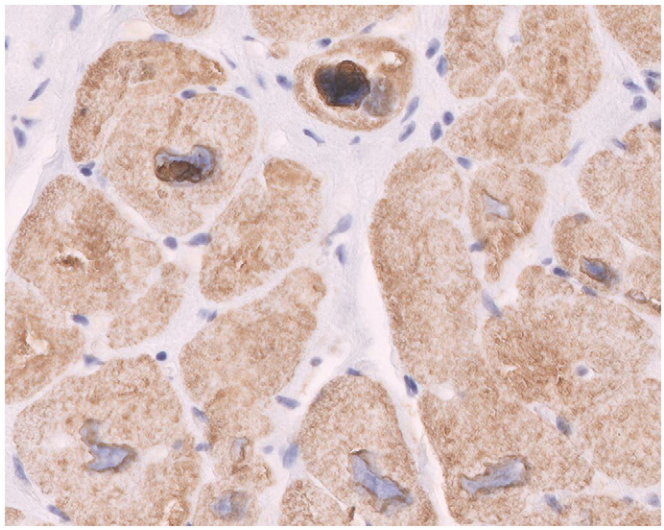

Fig. 2. PLN staining of heavily wrinkled nuclear membranes in transversely sectioned cardiomyocytes may give the false impression of perinuclear PLN-positive aggresomes in rare cases (PLN-IHC staining, original magnification ×200; Case 14, Table 1).

(*RMB20*), and dystrophin (*DMD*) genes, and seven cases of genetic ACM related to variants in desmosomal genes [4 in plakophilin-2 (*PKP2*), 1 in desmocollin-2 (*DSC2*), 1 in junctional plakoglobin (*JUP*), and 1 in desmoplakin (*DSP*)]. But when we examined RVEMB specimens of PLN p.Arg14del mutation carriers applying the same method, aggregates could only be detected in 2 out of 25 (8%) cases.

We found a striking difference between the observed number of PLN aggresomes in LVAD specimens compared to RVEMB specimens. In all nine LVAD specimens of the present study, at least two PLN-positive aggresomes were found per 10 mm^2; whereas, in our initial histopathological study, PLN-containing aggresomes were only present in 2 out of 25 (8%) of examined RVEMB cases. The larger size of the LVAD specimens may be a major factor responsible for the observed difference. In addition, two other factors can be held responsible for this much lower sensitivity of PLN IHC in RVEMB specimens in comparison to LVAD specimens. Firstly, the lower number of PLN aggregates per mm^2 and their patchy distribution in the RV wall and septum, and secondly, contraction band artifacts in cardiomyocytes of the RVEMB specimens which hampers the visualization of PLN aggregates [12].

To conclude, PLN IHC analysis of LVAD biopsies from this genetic cardiomyopathy cohort was found to be a highly sensitive and specific method for demonstration of PLN protein aggregates in PLN p.Arg14del cardiomyopathy. In clinical practice PLN IHC analysis of LVAD specimens, in contrast to RVEMB specimens, can be of incremental value in the diagnostic workup of this cardiomyopathy, even more so if genetic analysis cannot or has not been performed yet.

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