Evaluation of desmin activity using immunohistochemical and immunofluorescent staining of myocardial biopsies in patients with chronic heart failure. Comparison of the two methods

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Abstract

Background: Desmin plays one of the key roles in cardiomyocytes. The protein protects the integration of the cell and has the following actions: mechanical, structural and regulatory. Observed abnormalities of its activity have been associated with worsening of heart failure (HF).

Aim: Evaluation of desmin activity detected with immunohistochemical (IHC) and immunofluorescent (IF) staining in cardiomyocytes in patients with chronic HF.

Methods: The study population comprised 37 patients (mean age 46.5±15.28 years, 83.8% males) with diagnosed HF of unknown aetiology, who underwent myocardial biopsy. Coronary angiography was performed to exclude presence of significant coronary artery disease. Heart failure was diagnosed based on clinical assessment and echocardiography showing left ventricular ejection fraction below 45%.

Results: The IHC and IF evaluation of cardiomyocyte desmin showed that these methods were consistent with respect to classification of 31 specimens (83.8%), while being discrepant in 6 (16.2%) cases. Desmin detection in myocardial biopsy specimens with IHC staining showed normal amounts of this protein in 11 (29.8%) cases, excess in 18 (48.6%) patients and deficiency in 8 (21.6%) cases, whereas in IF stained specimens respective values were 12 (32.4%), 15 (40.6%) and 10 (27%). No significant differences were found between all desmin groups (i.e. normal level, excessive and deficiency) evaluated with IHC and IF staining (p=0.39; p=0.25; p=0.31, respectively).

Conclusions: The IHC and IF methods allow evaluation of desmin activity in cardiomyocytes and division into three types of expression. Both methods have high consistency. The IHC, which is the more available method, seems to be a sufficient assay.

Key words: heart failure, desmin, immunohistochemistry and immunofluorescence

Kardiol Pol 2007; 65: 229-235

Introduction

Desmin (DES) is one of three true cytoskeleton proteins counted among so-called intermediate filaments. It is a myocardial fibre specific protein.

Desmin fibres in cardiomyocytes are located around the myofibrils and Z bands, where they form a densely knitted net. Desmin also connects individual structural components. Surrounding the Z band it binds them to each other as well as to the cell membrane within constamers and inserts [1, 2]. Moreover, desmin builds a fine network connecting cellular nuclei with the mitochondria and endoplasmic reticulum.

The presence of desmin is required for normal functioning of myocytes [3]. The protein protects the

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Received: 31 August 2006. **Accepted:** 04 December 2006.

Table I. Initial clinical diagnosis before myocardial biopsy

Clinical diagnosis of HF	Total population (n=37)
Dilated cardiomyopathy	22 (59%)
Myocarditis	11 (30%)
Ventricular arrhythmia	4 (11%)

integrity of the cell and has the following actions: mechanical, structural and regulatory. The latter is the most debated one and plays the following roles: contribution to myofibril genesis, regulation of gene expression and intracellular signalling. The main roles of these processes include regulation of cell components position (e.g. mitochondrial localisation) and their function [4], mechanical integration of contractile activity through the regulation of myofibril orientation with respect to cellular structures [2], regulation of shape and tone of cellular membrane and intracellular elements [5], maintenance of normal connection between cell and extracellular matrix, supporting function of two other proteins forming the cytoskeleton (tubulin and actin).

Abnormalities of desmin filaments' organisation inside cells may lead to the development of desmin--related cardiomyopathy. Inappropriate activity of desmin within the cell may follow mutation of desmin or αβ-crystalline genes. However, two other mechanisms are also known, for example abnormal protease function. Regardless of causative factor, it seems that desmin level only in cardiomyocytes may represent important information which is significant for prognosis. Therefore, in this study we attempted to determine desmin levels using immunohistochemical (IHC) and immunofluorescent (IF) staining and their clinical importance.

Cellular desmin may be detected using IHC staining in a light microscope, IF staining in a confocal or electron microscope. Also polymerase chain reaction (PCR) enables evaluation of desmin gene presence.

The aim of the present study was to evaluate desmin activity detected with IHC and IF staining in cardiomyocytes in patients with chronic HF.

Methods

Population characteristics

The study population comprised 37 patients hospitalised at the Department of Invasive Cardiology of CSK MSWiA (mean age 46.5±15.28 years, 83.8% males) with confirmed HF of unknown aetiology (Table I), who underwent myocardial biopsy (MB) following compensation of clinical condition. It aimed to explain the causes of HF and applied protocol based on the commonly approved standards [6]. No additional specimens were required for IHC and IF assays.

Table II. Characteristics of population

Population characteristics	Total population (n=37)
NYHA class – mean values	1.9±0.82
NYHA I	12/32
NYHA II	16/43
NYHA III	7/18.9
NYHA IV	2/5.4
LVEF [%]	32.2±9.7
LVEDD [mm]	64.6±10.2

Abbreviations: NYHA – New York Heart Association class, LVEF – left ventricular ejection fraction, LVEDD – left ventricular end-diastolic diameter

Coronary angiography performed in all patients excluded the presence of significant stenoses in the coronary arteries. Chronic HF was diagnosed based on clinical investigation and echocardiography confirming left ventricular ejection fraction (LVEF) <45%. Table II shows characteristics of the study population with respect to HF.

On admission patients presented most commonly with the following signs and symptoms: dyspnoea (75.7%), fatigue (70.3%), leg oedema (62.2%), signs of pulmonary congestion (16.2%), and chest pain (35.1%). In-hospital treatment of the HF population was in line with the current guidelines of the European Society of Cardiology.

Myocardial biopsy protocol and sample preparation for histological and immunohistochemical evaluation

Femoral access was used for MB. In each patient 3-4 myocardial specimens were collected from the right ventricle using a Cordis bioptome. Samples for histopathological analysis were treated with 4% formaldehyde in PBS buffer (i.e. formalin). Samples for evaluation with fluorescence microscope were immersed in Tissue Tek at –70°C.

Bioptates collected and placed in formalin were then immersed in paraffin. Paraffin blocks were cut into sections of 3 μm thickness. Sections were incubated at 58°C for 30 min, and subsequently paraffin was removed and specimens were stained with haematoxylin-eosin [7]. For evaluation of connective tissue and cell necrosis, the specimens were stained with Azan [8], Masson [9] and Mallory [10] methods. Specimens were assessed for the presence of mastocytes using antibodies against mast cell tryptase. Inflammatory reaction was assessed on the basis of morphological evaluation of sections according to Dallas criteria [11] and immunohistochemical methods identifying elements of inflammatory infiltration

(lymphocytes T, granulocytes), HLA class II DPQR antigen and vascular endothelium (CD34) [12].

Immunohistochemical desmin analysis in the cells

Immunohistochemical determination of desmin involved cutting paraffin blocks into 4 µm sections placed subsequently on silane-coated slides and incubated at 58°C for 30 min and hydrated. Then sections were treated with trypsin for 30 min at 37°C (Sigma trypsin tabl. – order no. T-7168). Subsequently, they were incubated with desmin antibody (Desmin Mouse antihuman monoclonal antibody, Novocastra, order no. NCL-DES-DER1) in TBS solution (Tris Buffered Saline, DAKO) at pH of 7.8 for 60 min. The next step involved rinsing with TBS for 5 min and 30-minute incubation with Envision preparation – Daco Cytomation Dual Link System Peroxidase, order no. K4063. Visualisation reaction was performed using DAB chromogen (DAKO order no. – S3000) for 2 to 10 min. Subsequently, nuclei were stained with Meyer haematoxylin for 10 min.

The presence of desmin in preparation was semi-quantitatively evaluated in the light microscope. Desmin expression was analysed by two independent investigators. The analysis of desmin concentrations in specimens showed differences with respect to the amount of this protein; therefore, the population was divided into patients with normal, elevated and decreased desmin cellular levels. **Normal** pattern was characterised by relatively regular distribution of desmin in the specimen (low reaction intensity). **Excessive** levels were characterised by irregular, disproportionate accumulation of desmin in aggregates. **Deficiency** was found when the presence of desmin was barely visible in individual cells (Figure 1).

Immunofluorescent method of detection of desmin in cardiomyocytes

The immunofluorescence method involved cutting of frozen material on the slide, and then air drying at 20°C. Mouse antihuman antibodies against desmin were used (Dako, order no. M0760), and then antimouse antibodies labelled with rodamine (Ig fraction Polyclonal Rabbit antiMouse – sandwich method) manufactured by DAKO (order no. R0270 TRITC). Afterwards, slides were double--rinsed with PBS for 10 min. and evaluated with fluorescence microscope. Cellular desmin activity was determined based on fluorescence intensity and desmin distribution (aggregates).

Normal pattern was characterised by low intensity of fluorescence, regular distribution with visible linear fluorescence within the inserts. Excessive pattern was with areas of increased fluorescence (focal desmin accumulation) and irregular distribution. Deficiency was reported when desmin fluorescence intensity with rodamin-labelled antibodies was borderline or local loss of activity was found (Figure 2).

Statistical analysis

Parametric variables are shown as means and standard deviations (SD). Non-parametric data are presented as absolute values and percentages.

Fisher's exact test was used for comparison of non-parametric data – immunohistochemical vs. immuno-fluorescent staining in individual desmin groups. Value of $p < 0.05$ was found significant. Statistical analyses were performed using Statistica 5.5 PL software (StatSoft Polska)

Results

Desmin expression using immunohistochemical and immunofluorescent staining in the entire population

Desmin expression in myocardial biopsy specimens in IHC showed normal desmin levels in 11 (29.8%) cases, elevated levels in 18 (48.6%) patients and decreased levels in 8 (21.6%) of them. The IF staining for desmin in MB revealed normal desmin levels in 12 (32.4%) patients,

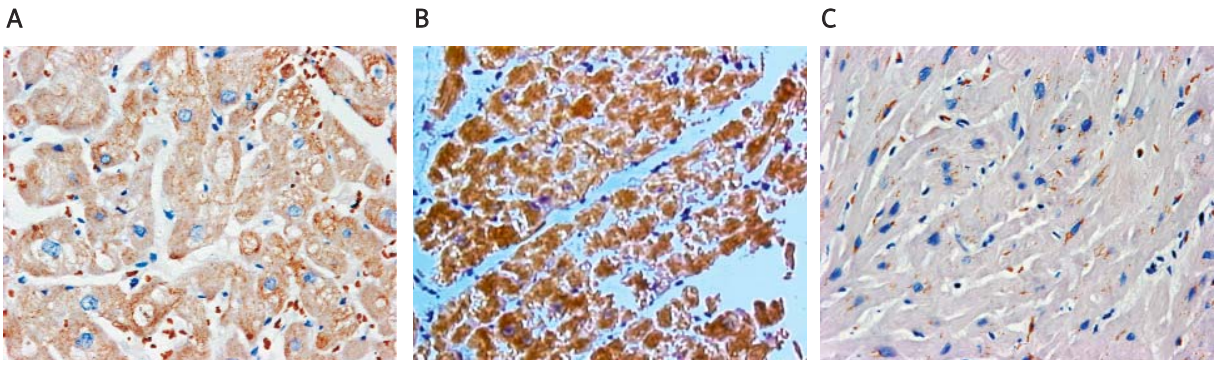

Figure 1. Immunohistochemical staining for desmin. **A** – normal 200×, **B** – excess 200×, **C** – deficiency 150×

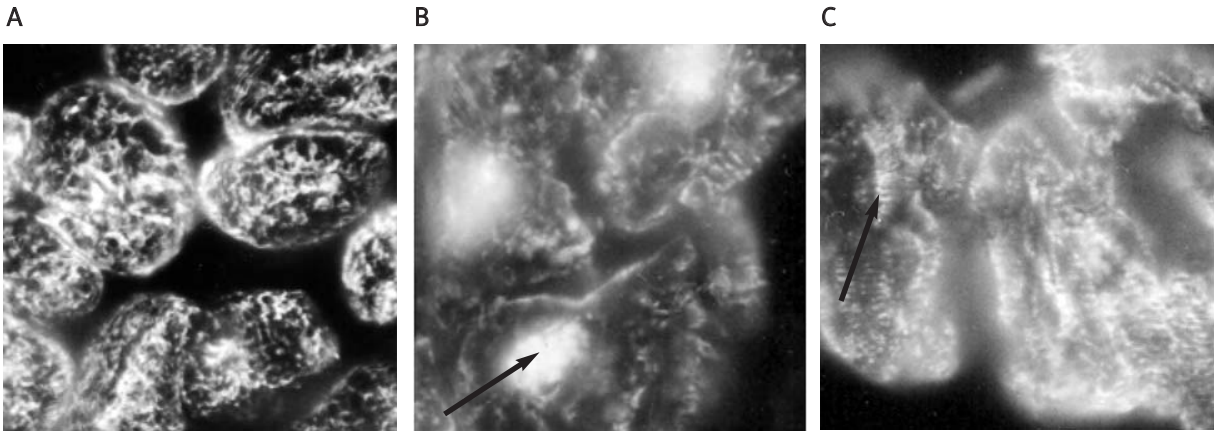

Figure 2. Immunofluorescence staining for desmin. **A** – normal, **B** – excess, **C** – deficiency (arrows)

elevated levels in 15 (40.6%) cases and its deficiency in 10 (27%) patients. No statistically significant differences were found between all desmin groups (i.e. normal level, excessive level and deficiency) evaluated with IHC and IF staining methods (p=0.39; p=0.25; p=0.31, respectively) (Figure 3).

Immunohistochemical vs. immunofluorescence reaction

The IHC and IF reactions for desmin detection in cardiomyocytes showed that both methods were consistent in 31 cases (83.8%), while discrepant results were obtained in 6 (16.2%) specimens (Figure 4).

Discrepancies (n=6) were observed mainly for excessive desmin specimens (n=4) found on IHC method which was not confirmed with IF assay. Analysis of these 4 (of 6) cases with the IF method showed decreased (2 specimens) or normal levels (2 specimens). In the remaining two specimens deficient or normal desmin levels were reported using the IHC method, whereas IF assay showed excessive and deficient levels of the protein in the cell, respectively (Figure 5).

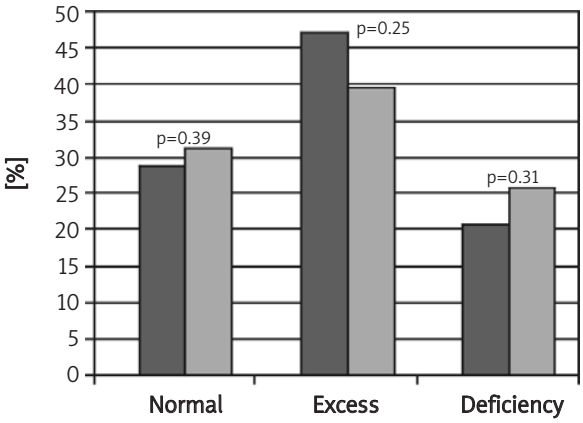

Figure 3. Comparison of desmin distribution patterns in cardiomyocytes on the basis of immunohistochemical (dark bars) and immunofluorescence (grey bars) methods

Verification of desmin activity in cardiomyocytes

Desmin expression level in cardiomyocytes was evaluated using three consecutive MB specimens collected from the same patient. All three sections were found to have comparable desmin expression activity in 51% of cases. The picture was consistent for 2 of 3 studied sections in 46% of cases. In one case (3%) desmin expression in each analysed section was various (i.e. normal, elevated, and decreased). The latter case was scored normal.

Discussion

Desmin plays an essential role in the cell and its deficiency has been associated with exacerbation of HF. In this study we aimed to evaluate desmin content, the

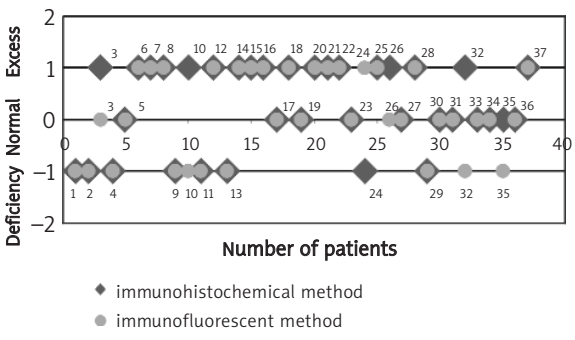

Figure 4. Comparison of desmin determination in cells using IH and IF methods

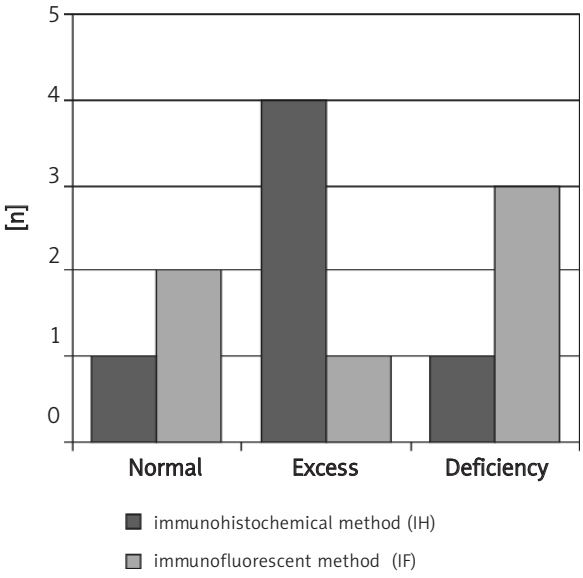

Figure 5. Discrepancies of desmin expression in cells using IH and IF methods in a group of 6 patients with HF

fundamental component of cardiomyocytes, using IHC assay, which is an easily accessible and relatively cost-effective method. Cellular desmin pattern obtained with this method was verified using IF assay.

Both methods (i.e. IHC and IF) were reported to be used in analysis of cellular desmin. However, these reports are uncommon (particularly in human-derived material) and the two methods have not been compared [13-15]. This probably results from the fact that analyses of desmin expression impairment have so far been directed towards searching for genetic factors (e.g. desmin gene mutation). Hence, the diagnostic methods used were very expensive and hardly available (electron microscopy, genetic tests). Currently, it is known that desmin accumulation in the cell only rarely results from abnormalities of the desmin gene, being related in the remaining cases to the influence of other proteins, such as the protective protein αβ-crystalline and the regulatory proteins caspase and ubiquitin. This was documented by genetic studies that failed to confirm mutation of the desmin gene, despite presence of cellular desmin aggregates in studied patients [16].

In fact, the IHC and IF methods do not allow detection of the cause of abnormal desmin distribution, but enable precise visualisation of this protein within the cell, which seems to be sufficient to predict the patient's risk [17].

In our opinion IHC remains a leading staining method due to its availability (light microscope evaluation) and reliability in terms of determination of desmin cellular levels [18]. It is also more cost-effective, more feasible and produces more permanent slides. These advantages of IHC enable a slide to be analysed by various investigators and at different times without compromising image quality. The limitation of this method is its relatively rare use which results in a low number of investigators experienced in detection of such abnormalities. In contrast, analysis of desmin levels with IF method is more expensive, requiring fluorescence microscope use (which is uncommon) and skilful fixation of images, because rodamine and fluorescein dyes have short fluorescence time. This method also requires cooperation with personnel experienced in evaluating the presence of this protein in specimens.

It should be highlighted that both deparaffination and freezing do not affect cell structure. Comparison of the two methods, i.e. IHC and IF, is now possible thanks to commercial availability of relevant reagents (antibodies) adjusted for IHC staining of deparaffinated sections. Tissue staining was not so long ago possible only for frozen sections.

Both methods enable one not only to determine desmin in cardiomyocytes but also to differentiate pictures with respect to desmin expression in the cell, i.e. normal, deficient and excessive levels. Analysis of cardiomyocyte desmin levels showed that both methods were highly consistent in 83.8% of cases. Discrepancies were present in 6 (16.2%) patients and were found primarily for excessive desmin levels as classified by the IHC method but not confirmed with IF assay. Of four cases with excessive desmin levels in the IHC method, two were found normal with IF test and another two scored deficient, which means 'severe' loss of desmin. In one case reported as IHC normal, IF test showed deficient levels. In all these cases, desmin cellular activity determined with IHC was higher than detected with IF tests; the reverse situation was noted in only one case. In this case IHC showed desmin deficiency in the specimen whereas assaying with rodamine-labeled antibodies revealed excess of desmin. Fist of all, these discrepancies may derive from the fact that specimens were evaluated by two independent investigators (and, unfortunately, still observer-dependent). Secondly, IF assaying was performed after gathering a larger number of specimens (longer collection to assaying time). This might lead to worse quality of tested material. Third, IHC and IF desmin tests were performed in different specimens collected from the same patient.

In our material equal pictures of desmin pathology in all three bioptates were observed in half of cases and in 46% of patients consistent results were obtained only in two of three studied samples. In one case all three patterns of desmin cellular activity were observed.

Analysis of our data suggested that patients with incorrect desmin levels (excess or deficiency) in cardiomyocytes had markedly worse prognosis compared to patients with normal levels. In the group with incorrect desmin expression resulting in its deficiency, a statistically significant increase in mortality was observed as well as higher enddiastolic left ventricular diameter and lower LVEF in comparison to patients with excess desmin in cardiomyocytes [19].

The new issue pointed out in our paper is the possibility of lower desmin cellular expression. It previously appeared that in patients with heart failure only excessive desmin levels in cells were observed [20, 21]. A dissimilar concept of cardiomyocyte desmin levels was first presented in 2004 by Italian investigators, who showed lack of desmin activity in cardiomyocytes in patients with end-stage heart failure (NYHA IV) induced by ischaemia. In their study, expression levels in cells were determined using IHC and IF staining and PCR [18].

Division of desmin expression in cardiomyocytes into normal, excessive and deficient seems to stress the natural history of HF. Evolution of the disease contributes to activation of a number of compensatory reactions in the cell aiming to maintain cellular function, and increased desmin expression remains one of these factors. When the disease cannot be stopped, cellular compensatory mechanisms become down-regulated and desmin levels decrease. This causes the presence of intermediate stages between specified desmin levels. It seems to be the cause of not uniformly homogeneous patterns of desmin abnormalities found in consecutive samples collected from the same patient.

Conclusions

1. The IHC and IF methods allow evaluation of desmin activity in cardiomyocytes and division into three patterns of expression.
2. Both methods are highly consistent.
3. The IHC, which is the more available method, seems to be sufficient for clinical evaluation.

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Ocena aktywności desminy metodą immunohistochemiczną i immunofluorescencyjną w bioptatach mięśnia serca u chorych z przewlekłą niewydolnością serca – porównanie obu metod

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Streszczenie

Wstęp: Desmina odgrywa jedną z podstawowych ról w komórce mięśniowej. Białko to stoi „na straży porządku" w komórce, pełniąc następujące funkcje: mechaniczną, strukturalną i regulatorową. Obserwowane zaburzenia aktywności tego białka wiążą się z narastaniem objawów niewydolności serca.

Cel: Ocena aktywności desminy oznaczanej barwieniem IHC i IF w kardiomiocytach chorych z przewlekłą niewydolnością serca.

Metoda: Badaną populację stanowiła grupa 37 chorych (średni wiek 46,5±15,28 lat, 83,8% mężczyzn) z rozpoznaniem niewydolności serca o nieznanej etiologii, którym wykonano biopsję mięśnia serca (BMS). Wykonana koronarografia wykluczyła obecność istotnych zwężeń w tętnicach wieńcowych. Niewydolność serca rozpoznawano na podstawie oceny klinicznej oraz badania echokardiograficznego serca wykazującego wartość frakcji wyrzutowej lewej komory <45%.

Wyniki: Immunohistochemiczna (IHC) i immunofluorescencyjna (IF) ocena desminy w komórce kardiomiocyta wykazała, że metody te są zgodne w ocenie w 31 (83,8%) przypadkach, natomiast rozbieżne w 6 (16,2%) przypadkach. Oznaczenie desminy w bioptatach mięśnia serca barwieniem IHC wykazało normalną zawartość tego białka w 11 (29,8%) przypadkach, nadmiar u 18 (48,6%) pacjentów i niedobór w 8 (21,6%) przypadkach oraz, odpowiednio, 12 (32,4%), 15 (40,6%) i 10 (27%) w barwieniu IF. Pomiędzy poszczególnymi grupami desminowymi, tj. normą, nadmiarem i niedoborem ocenianymi barwieniami IHC i IF, nie stwierdzono istotnych różnic statystycznych (odpowiednio p=0,39; p=0,25; p=0,31).

Wnioski: Barwienia IHC i IF umożliwiają ocenę aktywności desminy w kardiomiocycie oraz podział na trzy typy ekspresji. Obie metody są wysoce zgodne; IHC, jako bardziej dostępna, wydaje się być metodą wystarczającą.

Słowa kluczowe: niewydolność serca, desmina, immunohistochemia i immunofluorescencja

Kardiol Pol 2007; 65: 229-235

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Praca wpłynęła: 31.08.2006. **Zaakceptowana do druku:** 04.12.2006.