

## TRANSCRIPTIONAL ACTIVITY OF INTERFERON GAMMA AND TWO SUBUNITS OF ITS RECEPTOR AS MOLECULAR MARKERS OF MYOCARDITIS

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**Abstract** : Inflammatory cytokines have an important role in the pathogenesis of myocarditis, but still little is known about the importance of interferon gamma (IFN $\gamma$ ) in this disease. The aim of the study was to evaluate the prognostic value of the initial transcriptional activity of IFN $\gamma$  and two subunits of its receptor as measured with the use of QRT-PCR and SYBRGreen chemistry in the group of 63 patients with clinically confirmed myocarditis who were treated with statin or immunosuppressive therapy. The initial values of IFN $\gamma$  and the ratio of IFN $\gamma$ Rb/IFN $\gamma$ Ra were statistically different in the analyzed group of patients. The prognostic value of IFN $\gamma$  and IFN $\gamma$ Rb/IFN $\gamma$ Ra was determined by logistic regression analysis.

**Keywords** : IFN $\gamma$ , QRT-PCR, myocarditis

Myocarditis is a rare disorder which is a continuum of three distinct disease processes; of which each possesses its own pathogenesis, diagnosis and treatment. Most cases of myocarditis are hypothesized to result from viral infection which may progress to an autoimmune phase and then to progressive cardiac dilatation (1).

The modern diagnosis of myocarditis is based on the up-regulation of major histocompatibility complex (MHC) and intercellular adhesion molecule-1 (ICAM-1) on microvessel and cardiac myocytes in biopsy specimens of heart (2, 3). Numerous studies demonstrated that TNF- $\alpha$ , IL-1, IL-6, IL-10, sTNFR1, sTNFR2 play a crucial role in the diagnosis of myocarditis (4) but the significance of IFN $\gamma$  in this issue is still unclear (5, 6). IFN $\gamma$  is a key immunoregulatory cytokine crucial for variety of distinct cellular programs. The pleiotropic effects of IFN $\gamma$  include antiviral, immunoregulatory and antineoplastic functions.

IFN $\gamma$  is involved in the regulation of the immune and inflammatory responses by up-regulating the expression of MHC molecules (class I and II), ICAM-1 and activation of macrophages. IFN $\gamma$  secreted by Th1 cells has also a cross regulatory role in controlling Th2 function and induces a production of immunoglobulin G. Cellular responses to IFN $\gamma$  are mediated by its heterodimeric cell surface receptor IFN $\gamma$ R which is composed of two chains, IFN $\gamma$ Ra binding chain and IFN $\gamma$ Rb transducing chain. IFN $\gamma$ Ra chain exhibits the high affinity ligand binding properties, while IFN $\gamma$ Rb chain is required for signaling pathway. The expression and proportion of these subunits influence IFN $\gamma$  activity (7-9). To determine the role of IFN $\gamma$  and two subunits of its receptor in the diagnosis and therapy of myocarditis, the transcriptional activity of these genes in the biopsy specimens of patients with clinically confirmed myocarditis was examined.

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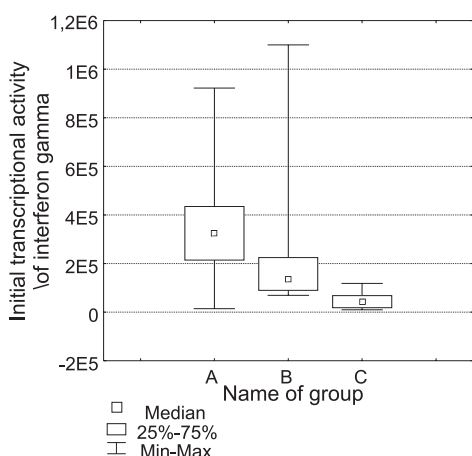


Figure 1. Initial value of transcriptional activity of IFN $\gamma$  gene expressed as the number of mRNA copies per 1 mg of total RNA. Group A- patients treated with immunosuppressive therapy, group B- patients treated with statin therapy, group C- control group ( $p = 1 \times 10^{-4}$ ).

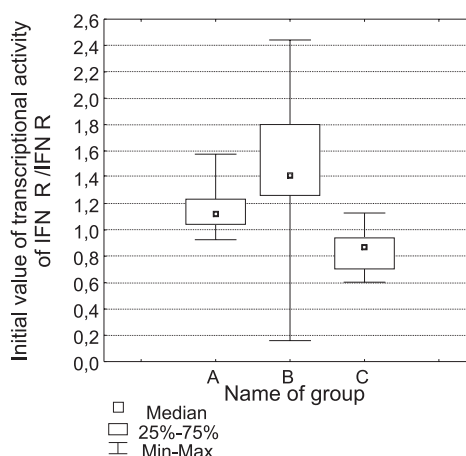


Figure 2. Initial value of transcriptional activity of IFN $\gamma$ Rb/IFN $\gamma$ Ra genes expressed as the number of mRNA copies per 1 mg of total RNA. Group A – patients treated with immunosuppressive therapy, group B – patients treated with statin therapy, group C – control group ( $p = 0.002$ ).

## EXPERIMENTAL

### Patients and therapy

The studied population consisted of 63 patients with clinically confirmed inflammatory myocarditis based on up-regulation of MHC class II and ICAM-1 in the biopsy specimens. The patients were divided into three groups: group A exhibited high expression of the analyzed molecule on cardiac myocytes, group B included the patients with medium degree of the expression of MHC class II and ICAM-1. The third group C was a control with minor expression of the analyzed adhesion molecules. An endomyocardial biopsy was performed using Cordis biopptome (Cordis corporation, Miami, FL, U.S.A). At least 5 specimens were taken from the right side of ventricular septum of each patient and they were

immediately put into liquid nitrogen and stored until tested. The experimental protocol has been approved by the local human studies committee of the Medical University of Silesia, Katowice, Poland.

All the patients (groups A, B and C) received conventional therapy including digitalis, diuretics (furosemid 40 to 80 mg/d and spironolactone 100 mg/d), ACE inhibitor (captopril 50 to 75 mg/d), beta-blocker (metoprolol 50 to 100 mg/d), nitrates and antiarrhythmic drug (amiodarone 200 to 400 mg/d) for 90 days. Patients from group A received immunosuppressive therapy with prednisone and azathioprine in addition to the conventional therapy. Prednisone was started at a dose of  $1 \text{ mg} \times \text{kg}^{-1} \times \text{d}^{-1}$ . After 12 days, the dose was tapered off every 5 days by 5 mg/d until reaching a dose of  $0.2 \text{ mg} \times \text{kg}^{-1} \times \text{d}^{-1}$ . Azathioprine was given at a dose of  $1 \text{ mg} \times \text{kg}^{-1} \times \text{d}^{-1}$  for a total of 90

Table 1. The sequences of primers used for quantification of the gene expression

Name of the primer	Sequence	Length of the PCR product
IFN $\gamma$ F	5' TGAACATCAATCAAGTATGGCTGAACTGTTCG 3'	115 bp
IFN $\gamma$ R	5'GAGCATCCCAGTAATGGTTGCTCCTGCCTG 3'	
IFN $\gamma$ Ra F	5' ATACCGAAGACAATCCAGGAAAAGTGGAACA 3'	175 bp
IFN $\gamma$ Ra R	5' GCGATGCTGCCAGTTTCAGACTGGTTACTA 3'	
IFN $\gamma$ Rb F	5' CAAGGACAGCTACCAAAGGATGACG 3'	151 bp
IFN $\gamma$ Rb R	5'CAGCTCCGATGGCTTGATCTCTTCCA 3'	

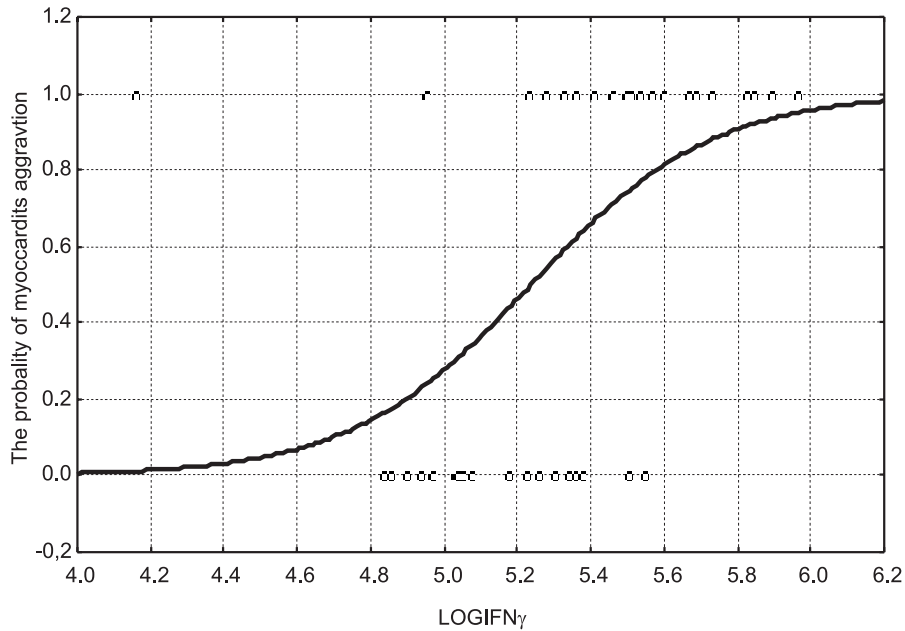


Figure 3. Logistic regression curve for IFN $\gamma$  as an independent variable

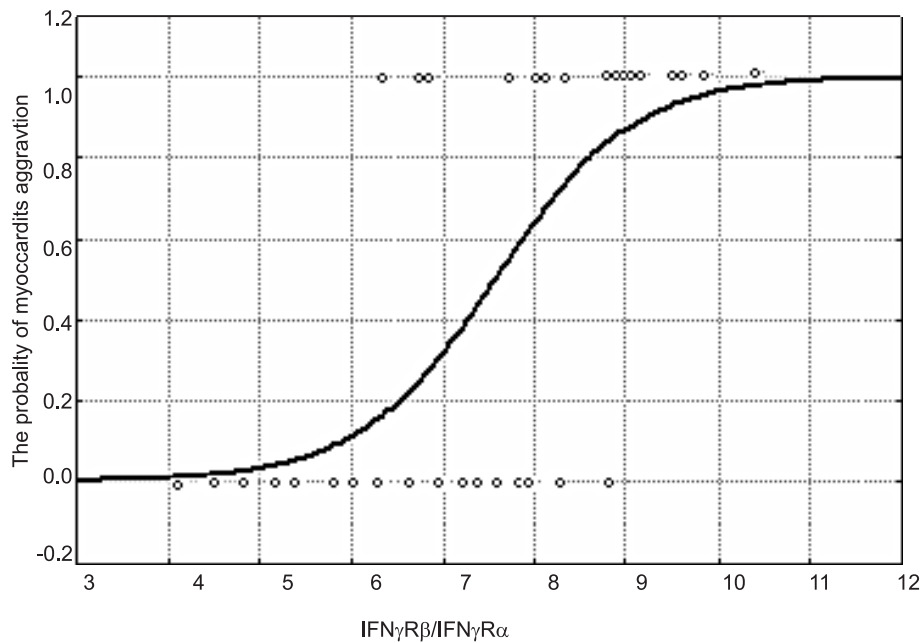


Figure 4. Logistic regression curve for IFN $\gamma$ Rb/IFN $\gamma$ Ra ratio as an independent variable

days. Patients from group B received atorvastatin at a dose of 20 mg every day.

**Molecular study**

Total cellular RNA was extracted from the biopsy specimens according to manufacturer’s instruction (A&A Biotechnology). The purity of the

extracted RNA was confirmed by determining the ratio of the absorbance at 260/280 nm, the RNA concentration using Gene Quant II calculator (Pharmacia Biotech) and by gel electrophoresis. The initial (before therapy) transcriptional activities of IFN $\gamma$ , IFN $\gamma$ Ra and IFN $\gamma$ Rb mRNAs were evaluated by QRT-PCR on duplicate samples of RNA by

using SYBRGreen chemistry (ABIPRISM™ 7700). cDNA was synthesized from 1-2 µg of the total RNA by reverse transcription reaction at 60°C for 30 min. The cDNA products were amplified in 96-well microtiter plates with 40 cycles of PCR, with each cycle consisting of the initial denaturation at 94°C for 5 min, followed by denaturation at 94°C for 30 s, annealing at 58°C for 60 s and final extension at 72°C for 10 min. Primers were designed based on the published cDNA sequences for human IFN $\gamma$  and its receptor subunits using Primer Express version 1.0 (PE Applied Biosystems) and they are listed in Table 1. For the quantification of the genes expression, a commercially available standards of  $\beta$ -actin containing 200, 400, 1000, 2000, and 4000 copies of cDNA/mL were amplified in two repeats. A reaction mixture of a 100 µL total volume contained QuantiTect SybrGreen RT Master Mix, forward and reverse primers (10 pmol/L), QuantiTect RT Mix and template. The negative controls for contamination were run with each analysis. To confirm the absence of non-specific amplification the PCR products were analyzed by polyacrylamide gel electrophoresis, melting curve profiles and sequence analysis (ABIPRISM 377) of the final amplimers.

#### Statistical analysis

The statistical analysis was performed with the use of Statistica ver. 6.0 software. Since the analyzed data were not normally distributed (Shapiro-Wilk test), the values of the transcriptional activity of IFN $\gamma$  and its receptor are presented as median and interquartile range. Statistical significance was accepted when a value of  $p$  was  $< 0.05$ .

#### RESULTS

To evaluate the transcriptional activity of IFN $\gamma$  and two subunit of its receptor (IFN $\gamma$ R $\alpha$  and IFN $\gamma$ R $\beta$ ) in the heart biotates of all patients within immunosuppressive and control groups, series of QRT-PCR reactions were performed. The electrophoretic analysis of amplimers, melting temperature profile and sequence analysis confirmed the specificity of the performed QRT-PCR reaction. The results are expressed as the number of mRNA copies of the analyzed genes per 1 mg of total RNA. The initial values of transcriptional activity of IFN $\gamma$  gene in the heart biotates were found to be significantly different between the analyzed groups ( $p = 1 \times 10^{-4}$ ; Fig. 1). Statistical differences of the initial expression level of IFN $\gamma$ R $\alpha$  between the immunosuppressive and control group ( $p = 1.32 \times 10^{-4}$ ) and between the statin and control group ( $1.1 \times 10^{-5}$ )

were also observed. However, there was no significant difference between the statin and immunosuppressive group ( $p > 0.05$ ). Since the initial transcriptional activity of IFN $\gamma$ R $\beta$  was statistically different between the analyzed groups (control and immunosuppressive,  $p = 1.63 \times 10^{-3}$ ; statin and control,  $p = 7.8 \times 10^{-5}$ ; statin and immunosuppressive,  $p = 8.21 \times 10^{-4}$ ), the ratio of IFN $\gamma$ R $\beta$ /IFN $\gamma$ R $\alpha$  was analyzed as a molecular marker of myocarditis. The values IFN $\gamma$ R $\beta$ /IFN $\gamma$ R $\alpha$  among the analyzed groups of patients showed statistical differences ( $p = 0.002$ ; Fig. 2). Furthermore, the prognostic evaluation of IFN $\gamma$  and that of IFN $\gamma$ R $\beta$ /IFN $\gamma$ R $\alpha$  ratio in myocarditis was determined by the logistic regression analysis. For this purpose, the relationship between the initial transcriptional activity of IFN $\gamma$  or the ratio of IFN $\gamma$ R $\beta$ /IFN $\gamma$ R $\alpha$  in the heart biotates (independent variables) and the probability of myocarditis progression were analyzed. The status of severe myocarditis which requires steroid therapy, received the conventional value 1, whereas the status of the disease with mild symptoms, which requires statin therapy, received the conventional value 0 (Figs. 3 and 4). The odds ratios for IFN $\gamma$  and IFN $\gamma$ R $\beta$ /IFN $\gamma$ R $\alpha$  were 57 and 3.8, respectively.

#### DISCUSSION AND CONCLUSION

Since the end of the last century the exploration of molecular markers of myocarditis and dilated cardiomyopathy have intensively been studied. Many clinical reports have shown that the elevated levels of some cytokines and/or cytokine receptors could help in prediction of adverse outcomes in the patients with myocarditis and the heart failure. Nowadays, the concentration of the serum level of TNF- $\alpha$ , IL-6, sTNFR1 and sTNFR2 were considered as independent predictors of mortality and progression of disease in dilated cardiomyopathy, which was the end stage of myocarditis. Circulatory levels of these cytokines exceeding 75<sup>th</sup> percentile were associated with the worst survival (10-12).

There were small numbers of clinical results concerning the role of IFN $\gamma$  and its receptors in the pathogenesis and diagnosis of myocarditis. Transgenic mice which constitutively express IFN $\gamma$  in their livers, exhibit high circulating serum levels of this cytokine spontaneously and developed chronic active myocarditis characterized by the infiltration of not only CD4(+) and CD8(+) T cells but also macrophages and CD11c(+) dendritic cells. Echocardiographic analyses exhibited a left ventricular dilation and impaired systolic function induced

by IFN $\gamma$  over-expression. IFN $\gamma$ -mediated cardiotoxicity was associated with high level cardiac transcription of the proinflammatory cytokines such as TNF- $\alpha$  and IL-12 and the macrophage-attracting chemokines MCP1 and MIP1- $\alpha$ . Myotoxic IFN $\gamma$  effects could not be detected in smooth or striated muscle tissues, suggesting cardiomyocellular specificity of the toxic IFN $\gamma$  effect (13). In the mice model of myocarditis the usage of IL-1 antagonist decreased the expression of IFN $\gamma$ , TNF- $\alpha$  and iNOS and improved the clinical state of the animals with viral myocarditis (14). The modern diagnosis of myocarditis and the classification of patients to the given therapy group (immunosuppressive or statin) was performed on the basis of MHC expression in the biopsy specimen of the heart (2, 3). IFN $\gamma$  produced in the early state of myocarditis activated macrophage, exhibited antiviral activity and stimulated the expression of MHC and ICAM-1 molecules on cardiac myocytes (15). Moreover, TNF- $\alpha$ , which is one of the best molecular markers of dilated cardiomyopathy and myocarditis, also stimulated the expression of MHC and ICAM-1, myocyte apoptosis and left ventricular remodeling (10, 11). Therefore, because of these properties IFN $\gamma$  may be considered as a more sensitive and earlier marker of clinical outcome of patients with myocarditis than MHC expression.

In the present study we showed that the initial transcriptional activity of IFN $\gamma$  and IFN $\gamma$ R $\beta$ /IFN $\gamma$ R $\alpha$  in the biopsy specimens of cardiac myocytes was statistically different between the patients with myocarditis clinically confirmed on the basis MHC and ICAM-1 expression on cardiac myocyte. Patients who presented high expression of MHC and ICAM-1 on cardiac myocytes, demonstrated high expression of IFN $\gamma$  and IFN $\gamma$ R $\beta$ /IFN $\gamma$ R $\alpha$  and were treated with steroids. Patients with lower expression of these adhesion molecules on cardiac myocytes showed smaller transcriptional activity of IFN $\gamma$  and IFN $\gamma$ R $\beta$ /IFN $\gamma$ R $\alpha$  and were treated with statin. On the basis of the obtained regression equation, the value of the regression function for a given patient was calculated and the re-classification to the statin or steroid therapy was performed again. The 85% and 65% accordance with the classification based on the MHC and ICAM-1 expression was obtained for the steroid and statin treated group, respectively.

In conclusion, the transcriptional activity of IFN $\gamma$  and IFN $\gamma$ R $\beta$ /IFN $\gamma$ R $\alpha$  in the heart biopsates appeared to be an early and sensitive marker of inflammatory status of patients with myocarditis which may be useful in the choice of appropriate pharmacological therapy.

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