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Peroxisome proliferator-activated receptor γ/Pro12Ala polymorphism and peroxisome proliferator-activated receptor γ coactivator-1 alpha/Gly482Ser polymorphism in patients with sarcoidosis

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Abstract. *Background and aim of the work:.* Reduced expression and activity of the peroxisome proliferator-activated receptor γ (PPARG) have been measured in cells of bronchoalveolar lavage fluid in sarcoidosis patients. PPARG, together with its transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator-1 alpha (PPARGC1A), has important modulating effects on immune response and apoptosis. In the present study, we investigated whether the polymorphisms Pro12Ala (rs1805192) in the PPARG gene and Gly482Ser (rs8192678) in the PPARGC1A gene, which affect transcriptional activities, are associated with sarcoidosis. *Methods:* We performed an integrative "omic" approach and identified the PPARG gene as a suitable candidate. Polymerase chain reaction was performed followed by restriction fragment length polymorphism to determine PPARG/Pro12Ala and PPARGC1A/Gly482Ser genotypes of 104 sarcoidosis patients and 112 healthy control subjects. *Results:* A higher frequency of the Ala allele (p=0.0101, OR=1.84, CI 1.18-2.88), as well as a significantly higher frequency of Pro/Ala heterozygotes and Ala/Ala homozygotes at the Pro12Ala/PPARG polymorphism (p=0.0020, OR=2.45, CI 1.42-4.25) were found in patients with sarcoidosis. In addition, a higher frequency of the Ser allele (p=0.013, OR=1.69, CI 1.13-2.53) and Gly/Ser heterozygotes and Ser/Ser homozygotes (p=0.0470, OR=1.80, CI 1.04-3.10) at the Gly482Ser/PPARGC1A polymorphism were found in patients with sarcoidosis as compared to healthy control subjects. *Conclusion:* Our results indicate that the presence of the Ala allele at the PPARG/Pro12Ala polymorphism and the Ser allele at the PPARGC1A/Gly482Ser polymorphism may be a predisposing factor for sarcoidos*is. (Sarcoidosis Vasc Diffuse Lung Dis 2008; 25: 29-35)*

Key words: sarcoidosis, peroxisome proliferator-activated receptor γ, Pro12Ala polymorphism, peroxisome proliferator-activated receptor γ coactivator-1 alpha, Gly482Ser polymorphism, association study, integrative "omic" approach

Introduction

Sarcoidosis is a multi-system chronic inflammatory disorder of unknown cause characterized by multiple granulomas consisting of epithelioid macrophage cells and T-lymphocytes, resulting from a Th1 type immunological response to an unknown antigen.

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As the role of infectious agents has not been confirmed (1, 2) a genetic influence has been proposed. Familial clustering of the disease (3-5), differences in disease prevalence and disease severity among ethnic groups and association with the HLA locus on chromosome 6 (6) all speak in favor of its heritability. A complex inheritance model has been proposed and a number of association studies have been performed with the aim of identifying candidate genes (6, 7).

A literature search of genes coding for proteins potentially involved in the pathophysiology of sarcoidosis was accomplished using a literature-based biomedical discovery algorithm BITOLA (8) and a web-based literature database PubMed MEDLINE. After searching for potential gene candidates using PubMed, BITOLA was used to examine which gene candidates are involved in pathologic processes that result in sarcoidosis, but have not yet been related to sarcoidosis in previous studies and therefore signify a new discovery. To narrow the selection of candidates, the data from several transcriptomic (9-14) and proteomic studies (9, 13, 15-18) were considered.

Additionally, genomic position in the proximity of peaks of LOD score, which was previously determined in a nonparametric multipoint linkage analysis in German families with affected siblings (19), was considered.

A set of candidate genes was identified and investigated: seven of them are involved in immunologic processes, five affect the redox state of cells, three regulate cell proliferation and apoptosis, three influence calcium metabolism, two affect cell adhesion properties and one is involved in fibrinolytic pathways. The PPARG gene was selected as a suitable candidate based on the results of the study by Culver et al. which demonstrated reduced gene expression and protein production and thus deficient PPARG activity (9).

The PPARG gene lies in the proximity of the D3S1259 polymorphism, which shows a slightly increased LOD score (19). Previously recognized polymorphisms affecting the transcriptional activity of PPARG and PARGC1A, Pro12Ala and Gly482Ser respectively, were selected to investigate our assumption, by means of an association study (20, 21).

The protein peroxisome proliferator-activated receptor γ (PPARG) is a ligand-activated transcription factor with modulating effects on immune response. Its anti-inflammatory effect is due to inactivation of the proinflammatory transcription factors NF-κB, activator protein (AP)-1 and STAT1 (22-28). A polymorphism in exon B causing the substitution of proline with alanine at amino acid position 12 (Pro12Ala, rs1805192) has been described, with consequent decreased PPARG function (20). The gene expression is significantly lower in alveolar macrophages from sarcoid lungs (9). It has also been demonstrated that production of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and other cytokines which are overproduced in sarcoidosis, is influenced by PPARG (28). Peroxisome proliferator-activated receptor γ coactivator-1α (PPARGC1A) is a transcriptional coactivator that increases the transcriptional activity of PPARG (29). A polymorphism that causes substitution of glycine with serine at position 482 (Gly482Ser, rs8192678) of the protein has been described in its coding region (21) and reported to affect its function (30).

In order to evaluate a possible involvement of PPARG and PPARGC1A in the etiology of sarcoidosis, we performed an association study of the PPARG/Pro12Ala and PPARGC1/Gly482Ser polymorphisms in Slovenian patients with sarcoidosis.

Patients and methods

Patients

Subjects diagnosed with sarcoidosis were recruited from an ongoing sarcoidosis registry begun in 2000 at the University Clinical Centre Ljubljana, Department of Pulmonary and Allergic Diseases. The diagnosis of sarcoidosis was made based on the clinical picture, radiographic presentation, bronchoalveolar lavage (BAL) and biopsy specimen from the lung, skin or lymph nodes after other granulomatous diseases were excluded. One hundred and four patients were included in the study: 36 males and 68 females, age range 21-68 years, mean age 41 years. The patients were followed from 3 to 10 years after confirmation of diagnosis. Regarding the clinical presentation at diagnosis, pulmonary lymph nodes were affected in 89 and lung interstitium in 73 patients. According to the classification system (31), 15 patients were in stage I, 55 in stage II, 16 in stage

III and 2 in stage IV at the time of the first visit. Extra-pulmonary organ involvement was found in 35 patients. Various types of skin involvement were found in 31 patients and Löfgren's syndrome was present in 19 patients. Eight patients had arthralgias. Ten patients had extrapulmonary lymph node involvement. Five patients had salivary gland involvement. In 15 patients, parenchymal organs such as liver, spleen, kidney or heart were involved and in 9 patients, various types of neural involvement were present. Regarding the clinical course, 47 patients had active disease after two years of follow up and were thus diagnosed with chronic sarcoidosis, while 57 patients who recovered within two years after diagnosis were diagnosed as acute form.

The control group consisted of 112 healthy, randomly chosen blood donors: 66 males and 46 females, age range between 39 and 65 years, mean age 52 years.

All patient and control subjects were Slovenians, unrelated to one another. All subjects participated in the study after they had given their full informed consent. The study was approved by the National Ethics Commitee.

Molecular analysis

After DNA isolation from blood leukocytes by standard procedure, polymerase chain reaction (PCR) method was performed to detect the Pro12Ala polymorphism of the PPARG gene and the Gly482Ser polymorphism of the PPARGC1A gene.

For the Pro12Ala/PPARG analysis, the sequences of primers were: forward (P12A-F) 5'-CAAGCCCAGTCCTTTCTGTG-3'; reverse (P12A-R) 5'-AGCTATGACCAGTGAAGGAATCGCTTTCCG-3'.

PCR amplification was performed on 500 ng of DNA in a 10 μl reaction mix containing: 1 μl of 10 x standard PCR buffer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 10 picomoles of each primer and 1 unit of Taq DNA polymerase (Promega, Madison,WI). The protocol of PCR amplification was as follows: initial denaturation at 94°C for 10 min, followed by 29 cycles at 94°C for 60 sec, 58°C for 90 sec and 72°C for 60 sec. and a final extension at 72°C for 10 min (Whatman Biometra Tgradient Thermocycler 2400, Germany).

After the amplification, the 237 bp large PCR products were digested with 5 units of MspI restriction enzyme (Promega, Madison, WI) at 37°C overnight and run on 2% agarose gel with ethidium bromide. The enzyme cuts the restriction site if the Pro allele is present resulting in 2 bands of 217 and 20 bp.

The Gly48Ser/PPARGC1A polymorphism analysis was performed using a forward primer (G48S-F) 5'-TAAAGATGTCTCCTCTGATT-3' and a reverse primer (G48S-R) 5'-GGAGACACATTGAACAATGAATAGGATTG-3'.

The PCR was carried out on 500 ng of DNA in a total reaction volume of 10 μl containing 1 x PCR buffer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1 picomole of each primer, 1 unit of Taq polymerase. The protocol of PCR amplification was as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 30 sec, 58°C for 30 sec and at 72°C for 60 sec, with the final extension at 72°C for 10 min.

After the amplification, the 378 bp large PCR products were digested with 5U of MspI restriction enzyme at 37°C overnight) and visualized in 2% agarose gel with ethidium bromide. The enzyme cuts the restriction site if the Gly allele is present resulting in 2 bands of 207 and 171 bp.

Statistical analyses

The statistical analysis was performed by SPSS for Windows 13.0 and MedCalc 8.2 software. The Chi-Square test was used for analysis of statistical significance, where a probability (p) value of less than 0.05 was considered statistically significant and the odds ratio (OR) value was used to compare allelic and genotype distribution in patients and control subjects. Additionally, the same tests were carried out to compare genotype distributions between the acute and the chronic patient groups. Statistical power of the two analyses was also calculated.

The calculation of statistical power of the test revealed that in both analyses, the sample size of 104 patients and 112 control subjects, would result in 80% probability to detect a 2-fold increase in the frequency of risk genotypes, assuming the percentage of risk genotype in PPARG polymorphism analysis in control subjects to be 34.8%, and the percentage of risk genotype in PPARGC1A analysis in control subjects to be 49.1% ($p < 0.05$).

Results

Results of gene candidates search

Results of PPARG and PPARGC1A genotyping

The results of the genotyping in patients with sarcoidosis and in healthy controls for the PPARG/ Pro12Ala and PPARGC1A/ Gly482Ser polymorphisms are summarized in tables 1 and 2.

An increased frequency of the Ala allele at the PPARG/ Pro12Ala polymorphism site (p=0.0101 and OR=1.84, CI 1.18-2.88) was found in patients with sarcoidosis as compared to control subjects. In addition, an increased frequency of the Pro/Ala heterozygous plus the Ala/Ala homozygous genotypes (p=0.0020, OR=2.45, CI 1.42-4.25) as opposed to the Pro/Pro homozygote genotype was found in patients with sarcoidosis. When comparing Ala/Ala and Pro/Pro genotype frequencies, between sarcoidosis patients and healthy controls, an increased frequency, although not statistically significant, of Ala/Ala homozygotes was detected in the patient group ($p^2$1, p=1?, OR=1.62, CI 0.31-8.38). A statistically significant increased frequency of Pro/Ala heterozygotes was also found, compared to Pro/Pro homozygotes ($p^2$0.01, OR=2.52, CI 1.44-4.41).

Regarding the PPARGC1A/Gly482Ser polymorphism analysis, an increase in frequency of Ser allele in the sarcoidosis patients was found as compared to the control subjects (p=0.013, OR=1.69, CI 1.13-2.53).

In the patient group, there was a higher frequency of the Gly/Ser heterozygous plus Ser/Ser homozygous genotypes (p=0.0470, OR=1.80, CI 1.04-3.10) as opposed to the Gly/Gly homozygous genoptype. A statistically significant increased frequency of homozygotes was detected in the sarcoidosis patients group as compared to controls p<0.025, OR=3.18, CI 1.25-8.12). In the patient group, a increased frequency, although not statistically significantl of Gly/Ser heterozygotes comparing to the wild type homozygote was found (p<0.20, OR=1.49, CI 0.88-2.77).

When comparing the genotype distributions within the sarcoidosis group patients, for both analy-

Table 1. Allele and genotype frequencies of PPARG/Pro12Ala polymorphism in patients with sarcoidosis and in control subjects.

Allele/Genotype		Sarcoidosis n (%)	m	f	Controls n (%)	m	f	OR (95% CI)	P
Allele	Pro	146 (70.2)			182 (81.2)			1.84 (1.18 to 2.,88)	0.01
	Ala	62 (29.8)			42 (18.8)				
	Total	208 (100)			224 (100)				
Genotype	Pro/Pro	45 (43.2)	31	14	73 (65.2)	35	38	Ala/Ala+Pro/Ala vs. Pro/Pro 2.45 (1.42 to 4.25)	0.002
	Pro/Ala	56 (53.9)	35	21	36 (32.1)	11	25		
	Ala/Ala	3 (2.9)	2	1	3 (2.7)	0	3		
	Total	104 (100)	68	36	112 (100)	46	66		

f=female, m=male

Table 2. Allele and genotype frequencies of PPARGC1A/Gly482Ser polymorphism in patients with sarcoidosis and in control subjects.

Allele/Genotype		Sarcoidosis n (%)	m	f	Controls n (%)	m	f	OR (95% CI)	P
Allele:	Gly	125 (60.1)			161 (71.9)			1.69 (1.13 to 2.53)	0.013
	Ser	83 (39.9)			63 (28.1)				
	Total	208 (100)			224 (100)				
Genotype	Gly/Gly	38 (36.5)	26	12	57 (50.9)	21	23	Ser/Ser+Gly/Ser vs. Gly/Gly 1.80 (1.04 to 3.10)	0.0470
	Gly/Ser	49 (47.1)	34	15	47 (42.0)	22	35		
	Ser/Ser	17 (16.4)	8	9	8 (7.1)	3	8		
	Total	104 (100)	68	36	112 (100)	46	66		

f=female, m=male

ses, there were no statistically significant differences between acute and chronic groups of sarcoidosis patients (data not shown).

Additionally, in order to evaluate a possible gene-gene interaction between PPARG and PPARGC1A, the correlation between the disease and the combination of the risk genotypes at both polymorphisms was examined in every sarcoidosis patient and control subject. No significant correlation was found between the combined risk genotypes and the disease (data not shown). There was no statistically significant deviation from Hardy-Weinberg equilibrium in both analyses ($p< 0.05$).

Discussion

Our study demonstrated a higher frequency of the Ala poymorphism of the PPARG gene and the Ser polymorphism of the PPARGC1A gene in sarcoidosis patients. The involvement of PPARG in the pathogenesis of sarcoidosis can be postulated on the basis of previously reported expression, proteomic and function studies.

Culver et al. (9) demonstrated that BAL specimens from patients with sarcoidosis revealed a striking reduction of PPARG activity in alveolar macrophages on the electrophoretic mobility shift assay, a decrease in PPARG nuclear protein and gene expression with immunostaining and real-time polymerase chain reaction, greatly reduced promoter binding affinity and reduced mRNA expression of PPARG as compared to healthy control subjects.

In addition, two major lines of evidence that support the role of PPARG pathway in sarcoidosis are its role in nflammatory/immune response regulation (22, 23, 27, 28, 32) and in apoptotic processes (33-35).

Increased gene expression or production of TNF-α, IL-6, inducible nitric oxid synthase, interferon-γ and matrix metalloproteinases has also been demonstrated (23, 10, 36-42). These mediators are encoded by genes normally under transcriptional inhibition of PPARG (28, 43, 44).

Enhanced activity of the transcription factor NF-κB has been demonstrated in sarcoidosis (9, 45). NF-κB is normally inhibited by PPARG and modulates the expression of many pro-inflammatory genes implicated in sarcoidosis. Downstream dysregulation of the NF-κB pathway may contribute to even more altered amounts and activities of IL-6, interleukin-12, TNF-α, granulocyte/macrophage colony stimulating factor, intercellular adhesion molecule-1, macrophage chemotactic protein-1 and RANTES often found to be increased in patients with sarcoidosis (46-49).

The persistence of granulomatous inflammation in sarcoidosis might be due to reduced apoptosis of immune cells in granulomas (11, 50, 51). Various studies have suggested an important role of PPARG in regulating cell growth, differentiation, apoptosis and in pathogenesis of various tumors (28). Activation of PPARG with its specific agonists resulted in a marked increase of macrophage apoptosis even when low amounts of the agonist were used. It has been proposed that activation of PPARG may result in stimulation of TNF-α, IL-6, induced pro-apoptotic effect or in inhibition of TNF-α, IL6, induced anti-apoptotic pathway, the latter being NF-κB - dependent (33).

Polymorphisms in the PPARG gene have been shown to regulate activity of the PPARG transcription factor in vitro (20, 52). The most commonly investigated polymorphism in the PPARG gene is the Pro12Ala polymorphism (53). The presence of Ala allele causes the protein product to decrease its binding affinity to its cognate promoter element and reduce its ability to transactivate its responsive promoters (20). The Pro12Ala polymorphism has been associated with type II diabetes, changes in insulin sensitivity, changes in insulin secretion and obesity (20, 54-60).

PPARGC1A is a transcriptional coactivator which enables PPARG to attain its full transcriptional activity (29). In previous studies, the PPARGC1A/ Gly482Ser polymorphism has also been associated with type II diabetes, obesity and insulin resistance (21, 61-66). The current results suggest that the presence of Ala(12) allele in PPARG and Ser(482) allele in PPARGC1A may predispose an individual to sarcoidosis. In this context, the data show a 145% increased risk of developing sarcoidosis in homozygous and heterozygous carriers of the Ala (12)/ PPARG allele and an 80% increased risk in homozygous and heterozygous carriers of the Ser(482)/PPARGC1A allele. As, to our knowledge, there have been no studies on the role of polymorphisms in PPARG and PPARGC1A genes in

etiopathogenesis of sarcoidosis, additional studies with a larger patient population and a broader ethnic background are needed to confirm these preliminary results.

The role of PPARG in the pathogenesis of sarcoidosis may have additional Implications. PPARG activation after stimulation by prostaglandin-J2 or the thiazolidinedione rosiglitazone has been achieved with consequent anti-inflammatory effects (22-24). Such PPARG agonists ould be used in the clinical treatment of sarcoidosis. Specific agonists of PPARG protein, such as rosiglitazone or pioglitasone are used in the treatment of diabetes (22, 23, 67). The immunosuppressive effect of these drugs may also attenuate granulomatous inflammation found in sarcoidosis. In conclusion, the results of the current study suggest a role of the PPARG and PPARGC1A genes and their polymorphisms in the genetic susceptibility and pathogenesis of sarcoidosis.

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