SARCOIDOSIS VASCULITIS AND DIFFUSE LUNG DISEASES 2010; 27; 131-137

TNF-alpha and TNF-beta gene polymorphisms in Polish patients with sarcoidosis. Connection with the susceptibility and prognosis

R. Kieszko[1], P. Krawczyk[1], S. Chocholska[2], A. Dmoszyńska[2], J. Milanowski[1]

[1] Department of Pneumonology, Oncology and Allergology, Medical University of Lublin, Poland; [2] Department of Hematooncology and Bone Marrow Transplantation, Medical University of Lublin, Poland.

Abstract. *Background:* Sarcoidosis is a systemic granulomatous disease of unknown aetiology, in which genetic factors, especially the genes of the highly polymorphic MHC region, seem to play an important role in the disease predisposition and course. The aim of this study was to evaluate the role of *TNF* genes polymorphism in sarcoidosis and to estimate possible association between these polymorphisms and susceptibility and prognosis of sarcoidosis. The analysis of *-308G>A TNF-α* gene (*TNFA*1* and *TNFA*2* alleles) and *252A>G TNF-β* gene polymorphisms (*TNFB*1* and *TNFB*2* alleles) were performed. *Methods:* The study comprised of 130 sarcoidosis patients (75 subjects in the radiological stage I, and 55 in the stages II/III). Löfgren syndrome (LS) was manifested in 38 patients. After at least 3-years observation, 69 patients had remission, 24 subjects manifested persistent disease and 25 patients had progression. The control group consisted of 84 healthy subjects. The genotypes were determined using PCR-RFLP assay. *Results:* The variant allele *TNFA*2* was observed significantly more frequent in patients with Löfgren syndrome when compared to control group (OR=2.301, C.I.=[1.23-4.32], χ^2=6.91, p>0.01), as well as to non-LS patients (OR=2.167, C.I.=[1.17-4.01], χ^2=6.22, p<0.05). Moreover, the variant allele *TNFA*2* was also observed significantly more frequent in patients with disease resolution than in patients with persistent disease and progression (OR=3.53, C.I.=[1.66-7.50], χ^2= 11.65, p<0.001). The variant allele *TNFA*2* was also overrepresented in patients with disease resolution after exclusion the patients with Löfgren syndrome (OR=2.4, C.I.=[1-5.772], χ^2=3.98, p<0.05). There was no significant difference in *TNF-A* allele distribution between the control group and whole sarcoidosis group. The variant allele *TNFB*1* was observed significantly more frequent in patients with disease resolution than in patients with persistent disease and progression. This difference was caused only by overrepresentation of *TNFB*1* variant allele in Löfgren group. The significant differences in the distribution of *TNFB*1* allele between the sarcoidosis and the control group was also noted (OR=1,607, C.I.=[1,033-2,5], χ^2=4.46, p<0.05), but it was limited only to patients displaying Löfgren syndrome. *Conclusion:* Two alleles *TNFB*1* and *TNFA*2* of *TNF* gene are overrepresented in polish patients with Löfgren syndrome. The *TNFA*2* allele is related with mild course of sarcoidosis in patients without LS. *(Sarcoidosis Vasc Diffuse Lung Dis 2010; 27: 131-137)*

Key words: TFN, gene polymorphism, sarcoidosis, alleles

Received: 13 August 2009
Accepted after Revision: 12 June 2010
Correspondence: Robert Kieszko, MD, PhD
Dept. of Pneumonology, Oncology and Allergology
Medical University of Lublin
Jaczewskiego 8, 20-950 Lublin, Poland
Tel. +48 81 724 4293, +48 81 724 4431
Fax +48 81 724 4823
E-mail: robert.kceszko@neostrad.pl, pulm.dept@umlub.pl
pulm.lab@umlub.pl

Introduction

Sarcoidosis is a multisystem granulomatous disease of unknown origin that predominantly affects the lung. The disease is characterised by lymphocytic alveolitis and the formation of an noncaseating epithelioid granuloma. The aetiology of sarcoidosis is not discovered yet, but it is believed that environ-

mental exposures interact with some genetic factors in determining the susceptibility, clinical manifestation and prognosis of this disease (1).

The inflammatory response in sarcoidosis is characterized by the increased production of several proinflammatory cytokines, which mainly belong to tumour necrosis factor (TNF) family (2). TNF-α (cachectin) and TNF-β (lymphotoxin α, LT-α) are of main member of TNF family.

It was demonstrated that release of TNF-α is increased in the lung of patients with pulmonary sarcoidosis. TNF-α initiate the development of giant and epithelial cells and is responsible for granuloma formation in the lung (3-5). It was suggested that TNF-α is a cytokine related with persistence of lung inflammation due to persistent expression of mRNA for TNF-α in macrophages isolated from patients with chronic sarcoidosis (6).

The *TNF-α* and *TNF-β* genes are located adjacent to each other in the major histocompatibility complex class III region on chromosome 6p21.3. Several polymorphisms of the *TNF-α* gene (*TNFA*) have been identified. Among them, allele *TNFA*2* at -308 nucleotide position has been associated with higher inducible levels of gene transcription and TNF-α protein production (7).

In the first intron of the *TNF-β* gene (*TNFB*), there is a *Nco*I polymorphism consisting of the allele *TNFB*1* in the presence of the restriction site, and the allele *TNFB*2* in its absence. *TNFB*1* is the less frequent allele in Caucasian subjects and is associated with higher TNF-α and TNF-β production in healthy subjects. Although, insulin-dependent diabetes mellitus patients with the *TNF*B1* allele secreted significantly lower levels of TNF-β than those with the *TNF*B2* allele and patients carrying the *TNFB*2* allele had a higher TNF-α secretory capacity than those carrying the *TNFB*1* (8-10). On the other hand, Somoskovi et al. had shown that *TNFA* and *TNFB* polymorphisms did not determine the level of TNF-alpha production by mononuclear cells activated during sarcoid inflammation (11). These biallelic polymorphisms have been also correlated with susceptibility to fatal meningococcal disease and severe sepsis (12, 13).

The studies concerning sarcoidosis had shown the higher frequency of the *TNFA*2* and *TNFB*1* allele occurrence in patients displaying Löfgren syndrome (LS). This observation could be explained by the linkage disequilibrium (LD) between these alleles and *HLADR3* or *HLADRB1* alleles. Also, the strong linkage disequilibrium was found for the *TNFA*2* and *TNFB*1* alleles. Tight LD between *TNF* loci and *HLADR3* or *HLADRB1* make a determination of the relative roles of each gene in the immunogenesis of sarcoidosis difficult (14-17).

Several studies had shown the relationship between *TNF-α* and *TNF-β* genes polymorphism and the prognosis of sarcoidosis. However, the ethnic differences as well as different disease phenotypes in investigated groups could resulted in the discrepant results obtained by authors (Table 1).

The aim of the study

Data from the literature indicate that genetic polymorphisms play a role in regulating TNF-α level which in turn may influence the immune response

Table 1. Association of *TNF* genes polymorphisms with the prognosis of sarcoidosis.

Study	Population	Gene and polymorphism	Comments
Yamaguchi et al. (18)	110 Japanese patients and 161 control subjects	LTA_*NcoI* polymorphism	*TNFB*1* allele as the marker of prolonged clinical course
Sharma et al. (19)	96 North-Indian patients and 155 controls	LTA_*NcoI* polymorphism	The *TNFB*2* allele prevalent in the 'no treatment' group. Over-representation of he *TNFB*1* allele in patients who had frequent relapses of symptoms on tapering off the dosages of prednisolone
Mrazek et al. (16)	114 Czech patients and 425 controls	-308 *TNF-A* and LTA_*NcoI* polymorphisms	None of the polymorphisms connected with chest x-ray and need for steroid treatment
Takashige et al. (20)	26 Japanese patients with cardiac sarcoidosis and 161 control subjects	*TNFA*-308 and LTA_*NcoI* polymorphisms	A higher occurrence of the *TNFA*2* allele in the patients

of individual and finally the course and outcome of sarcoidosis. The aim of our study was to evaluate the role of *TNF*-α and *TNF*-β genes polymorphism in sarcoidosis. Moreover, we tried to examine the possible association between these polymorphisms and the susceptibility as well as the prognosis of sarcoidosis in Polish patients.

Materials and methods

Study population

The study population comprised of 130 patients (the mean age: 39.5±11.7 years; 70 female and 60 male) with newly diagnosed sarcoidosis. The patients were diagnosed in Department of Pneumonology, Oncology and Allergology, Medical University of Lublin from February 2002 to February 2005 and the observation was carried till February 2008. The diagnosis of sarcoidosis was established using defined criteria including histopathological confirmation. The diagnosis of patients with Löfgren syndrome was based on clinical, radiological and immunological (BALF CD4/CD8 lymphocyte ratio >3,5) findings. None of the patients received steroid therapy at the time of diagnosis. The assessment of the disease was based on clinical features, chest X-ray and computed tomography, lung function tests, abdomen ultrasonography, ophthalmologic investigation, bronchoscopy with BAL and routine blood tests. The severity of the disease was assessed on the basis of clinical, radiological and pulmonary function data. Löfgren syndrome was manifested in 38 patients. 75 patients (the mean age: 35.6±11.7 years) showed bilateral hilar lymphadenopathy in chest X-ray examination. In 55 patients (the mean age: 41.4±11.8 years) interstitial infiltration with or without hilar lymphadenopathy (radiological stage II or III) was demonstrated. We made a distinction between the self-limiting clinical course radiographic stage I, and the stage II or III with high probability of disease progression.

The follow-up (clinical investigation, chest X-ray and lung function tests) was performed every three months. At the end of observation, data of 118 patients were accessible. On the basis of a 3- to 6-years follow-up, the patients were divided into the group with spontaneous remission (69 patients), the group with long standing disease with no need of treatment (the stable disease group – 24 patients), and the group with disease progression which need the steroid treatment (the progression group – 25 patients). The decision to start steroid therapy was based on the presence of progressive symptomatic disease, lung function deterioration and signs of fibrosis in CT scans. Patients were judged to be in remission when symptoms, abnormalities of pulmonary function tests and radiographic disturbances had disappeared.

The research project was approved by the Bioethic Committee of the Medical University of Lublin in accordance with the Guidelines for Good Clinical Practice.

Control group

The control group consisted of 84 volunteers. None of them had any evidence of lung disease and concomitant therapy. All subjects were Caucasian of Polish origin and were not related.

Determination of the genotype of the TNF genes

All peripheral blood samples were collected in to heparinised tubes. Lymphoprep (Nycomed, Norway) gradient centrifugation was used to separate peripheral blood mononuclear cells (PBMC). Cells were collected and washed twice in PBS (Biomed, Poland). $2x10^7$ cells were suspended in newborn calf serum (Sigma, Germany) containing 5% dimethyl sulfoxide (DMSO; Sigma, Germany), placed in cryovials and stored at -80°C. Genomic DNA was purified from thawed PBMC by standard phenol/chloroform extraction procedure (21).

The *Nco*I polymorphism of -308 *TNF*-*α* promoter (*TNFA -308G>A*) and of *TNF*-*β* intron 1 (*TNFB 252A>G*, named also *LTA 252A>G*) were determined by polymerase chain reaction followed by restriction fragment length polymorphism analysis (PCR-RFLP) as previously described by Wilson et al. and Yamaguchi et al. (22, 18).

After amplification of the 107-bp fragments and *Nco*I digestion three genotypes of the -308 *TNF*-*α* promoter polymorphism could be identified:

- homozygous A/A (*TNFA*2/2*, homozygous for *TNFA*2*) lacking the *Nco*I restriction site had non-digested 107-bp band;
- homozygous G/G (*TNFA*1/1*, homozygous for *TNFA*1*) with the presence of *Nco*I restriction site had 87-bp and 20-bp fragments;
- heterozygous G/A (*TNFA*1/2*, heterozygous for *TNFA*1* and *TNFA*2*) had three band: 107-bp, 87-bp and 20-bp bands.

After PCR-RFLP analysis of the 289-bp fragments the following genotypes of the *TNFB* intron 1 polymorphism could be determined:

- homozygous A/A (*TNFB*2/2*, homozygous for *TNFB*2*) lacking the *Nco*I restriction site had non-digested 289-bp band;
- homozygous G/G (*TNFB*1/1*, homozygous for *TNFB*1*) with the presence of *Nco*I restriction site was digested into 228-bp and 61-bp fragments,
- heterozygous A/G (*TNFB*1/2*, heterozygous for *TNFB*1* and *TNFB*2*) had three band: 289-bp, 228-bp and 61-bp.

Statistical Analysis

Fisher's exact test was applied to testing for Hardy-Weinberg proportions. Differences of the frequencies of the alleles between control and patients subjects as well as patients with different course of sarcoidosis were tested by Pearson χ^2 test. Odds ratios and associated 95% confidence intervals were calculated by logistic regression analysis using the estimate haplotype frequencies program (http://ihg2.helmholtz-muenchen.de). Probability value of $p<0.05$ was considered statistically significant.

Results

128 patients were genotyped for *TNFA* and 130 patients for *TNFB* gene polymorphisms. Control group consisted of 84 patients successfully genotyped for both polymorphisms. Control group and sarcoidosis patients group were in H-W equilibrium with non-significant χ^2 –values comparing the observed and expected genotype frequencies of *TNFA*. By contrast, the distribution of *TNFB* genotypes differed significantly from H-W equilibrium in control group because of the deficiency of *TNFB*1/1* homozygous and overrepresentation of *TNFB*1/2* heterozygous.

The results of *TNFA* and *TNFB* genotypes distribution in subgroups of patients with sarcoidosis and control group have been presented in Table 2. The distribution of *TNFA* allele was not significantly different between sarcoidosis and control group. We noticed the higher frequency of *TNFA*2* allele occurrence in Löfgren group than in control group (OR=2.301, C.I.=[1.23-4.32], χ^2=6.91, $p<0.01$) as well as non-LS patients (OR=2.167, C.I.=[1.17-4.01], χ^2=6.22, $p<0.05$).

The frequency of *TNFB*1* allele was higher in sarcoidosis patients when compared to control group (OR=1,607, C.I.=[1,033-2,5], χ^2=4.46, $p<0.05$). When LS patients were excluded from examination,

Table 2. The distribution of TNF gene polymorphisms within examined groups of sarcoidosis patients and in control group*.

Polymorphism	Control	Sarcoidosis	Löfgren	Non-Löfgren	Remission	Progression and Stable Disease	Non-LS patients	
							Remission	Progression and Stable Disease
TNFA (-308 promoter) Genotype	N=84	N=128	N=37	N=91	N=67	N=48	N=38	N=42
1/1	55 (65.5)	75 (58.6)	15 (40.5)	60 (65.9)	32 (47,8)	38 (79,2)	23 (60.5)	33 (78.6)
1/2	29 (34.5)	49 (38,3)	20 (54)	29 (31.9)	31 (46,3)	10 (20,8)	13 (34.2)	9 (21.4)
2/2	0 (0)	4 (3.1)	2 (5.5)	2 (2.2)	4 (5,9)	0 (0)	2 (5.3)	0 (0)
TNFB (intron 1) Genotype	N=84	N=130	N=38	N=92	N=69	N=49	N=39	N=43
2/2	46 (54.8)	55 (42.3)	8 (21.1)	47 (51.1)	23 (33,3)	29 (59.2)	18 (46.2)	26 (60.5)
2/1	37 (44)	65 (50)	24 (63.1)	41 (44.6)	38 (55,1)	18 (37.7)	19 (48.7)	15 (34.9)
1/1	1 (1.2)	10 (7.7)	6 (15.8)	4 (4.3)	8 (11,6)	2 (4.1)	2 (5.1)	2 (4.6)

*Data are presented as numbers of individuals with percentages in parenthe

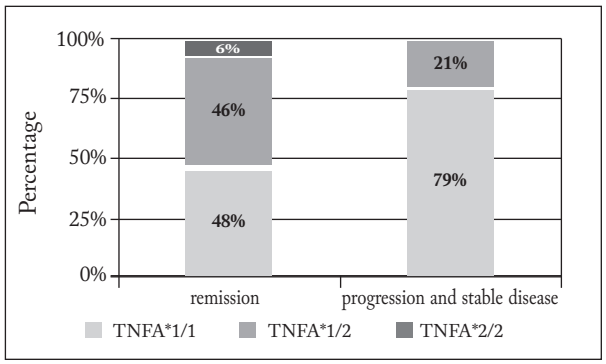

Fig. 1. The distribution of *TNFA* alleles within the group of patients with different course of sarcoidosis: remission vs progression and stable disease.

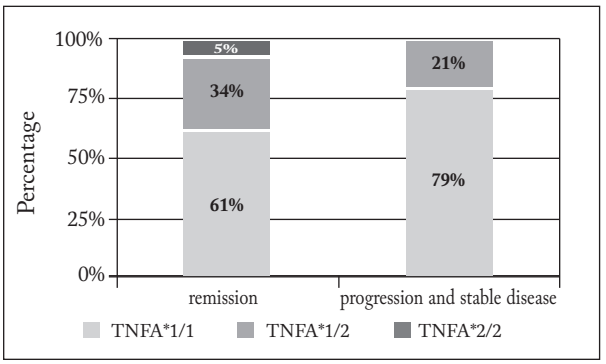

Fig. 2. The distribution of *TNFA* alleles within the group of non-LS patients with different course of sarcoidosis: remission vs progression and stable disease.

the significant differences in *TNFB*1* allele frequencies disappeared. We had speculated that the increase of *TNFB*1* allele in sarcoidosis patients was caused by overrepresentation of this allele only in LS patients. The patients carried *TNFB*1* allele had about 3-fold elevated risk of Löfgren Syndrome (OR=2,98, C.I.=[1.67-5.29], χ^2= 14.34, p<0.001).

While both *TNFB* alleles were distributed equally in groups of patients subdivided according to chest X-ray stage, the *TNFA*2* allele was overrepresented in patients with first radiological stage. When the patients displaying LS were excluded from the analysis, the frequency of *TNFA*2* allele was insignificant higher in patients with I radiologic stage than in patients with parenchymal involvement.

Significant differences in the *TNFA* and *TNFB* alleles distribution were found between the patients with remission and the patients with progression/stable disease. The frequency of *TNFA*2* (Figure 1) and *TNFB*1* alleles were higher in patients with remission when compared with the patients with progression/stable disease (OR=3.53, C.I.=[1.66-7.50], χ^2= 11.65, p<0.001 and OR=2.22, C.I.=[1.237-3.98], χ^2= 7.30, p<0.01, respectively). An increase of *TNFA*2* allele frequency in remission group was not limited to LS patients only, but was also observed in non-LS patients (Figure 2). This observation was not related with allele *TNFB*1*.

All possible combination of genotype was examined between sarcoidosis and control group. In both groups the interaction between *TNFA*2* and *TNFB*1* alleles was observed. Among the group of 29 control subjects with *TNFA*2* allele, 6 subjects carried also *TNFB*2* allele and 23 subjects carried *TNFB*1* allele (χ^2=19.9; p<0.0001). Moreover, among the group of 54 sarcoidosis patients with *TNFA*2* allele, only 1 patient carried also *TNFB*2* allele and 53 subjects carried *TNFB*1* allele (χ^2=100.15; p<0.0001).

Discussion

Biallelic polymorphisms in the promoter region of the *TNF-α* gene at -308 nucleotide position and polymorphisms in the first intron of the *TNF-β* gene have been associated with the susceptibility to sarcoidosis and with the predisposition to severe course or spontaneous remission of the disease. In presented study, we did not found the influence of *TNF-α* genotype on the risk of sarcoidosis. The overrepresentation of *TNFB*1* allele in sarcoidosis group compared to control was connected with the higher frequency of this allele in LS patients. Moreover, in our study *TNFA*2* as well as *TNFB*1* alleles were overrepresented in patients with Löfgren syndrome than in other sarcoidosis patients and in control group. The obtained results are in keeping with the study of Seitzer et al. (14). They revealed no significant differences in the distribution of *TNFA* and *TNFB* alleles between German sarcoidosis patients and the control group. However, there was a tendency in the Löfgren syndrome patients group towards a higher prevalence of the *TNFB*1* allele. In contrast, a highly significant shift to the more uncommon *TNFA*2* allele was found in the Löfgren syn-

drome patient group compared to the control group as well as to the non-LS patient group. The study of Swider et al. (21) confirmed that biallelic polymorphisms in the promoter region of the *TNF-α* gene are not connected with the susceptibility to sarcoidosis as a whole but showed higher frequency of *TNFA*2* allele in Löfgren syndrome patients from German population. The same results were obtained for the British and Dutch population (23).

Our *TNF-α* and *TNF-β* genotype association results are also partly consistent with work of Mrazek et al. (16) who detected a overrepresentation of *TNFA*2* and *TNFB*1* in Löfgren syndrome patients in Czech sarcoidosis group. The study of Pandey et al. (24) concerned the *TNF-α* gene polymorphism examination in large groups of African-American and Caucasian American sarcoidosis patients. The authors has identified no *TNF-α* genotype association with sarcoidosis independently of ethnicity, but has found a higher proportion of subjects carrying *TNFA*2* allele in Caucasians Löfgren compared to control subjects. Similarities and differences in cited and presented here results may by caused by ethnic background and by using a different criteria of Löfgren syndrome definitions. The *TNF-α* polymorphism could be involved in quite different clinical presentation of sarcoidosis in different ethnic population. For instance, Takashige et al. (20) indicated that the *TNFA*2* allele is associated with cardiac sarcoidosis in Japanese patients.

Our results concerned interaction of *TNFA*2* and *TNFB*1* alleles confirmed the strong linkage disequilibrium between *TNFA*2* and *TNFB*1* alleles in healthy group (17) as well as in sarcoidosis patients (16).

It has been suggested that the polymorphism could be involved in clinical course of sarcoidosis, but these suggestions are contradictory (16, 18-20). In our polish sarcoidosis group the variant allele *TNFA*2* but not *TNFB*1* was also overrepresented in patients with disease resolution after exclusion patients with Löfgren syndrome. Our results are partly contrary to the Mrazek study results (16), despite both investigations concern similar populations. In Czech study *TNFA* and *TNFB* alleles were equally distributed in patients groups divided in terms of need for systemic steroid treatment. Possible explanations for discrepancies in results may include studies designs and fact that the indications for corticosteroid therapy remains controversial. 72% of Czech and only 21% of polish patients were treated by systemic steroids.

Yamaguchi et al. (18) had performed the first prognostic study on 110 Japanese sarcodiosis patients to assess the potential prognostic value of *TNFA* and *TNFB* polymorphisms. There was no significant difference in either allele frequency or genotype distribution between patients and control subjects. Patients with the *TNFB*1* allele had a more prolonged clinical course of disease than those without this allele (genotype *TNFB*2/2*). Investigated Japanese population consisted mainly of patients who did not require steroid treatment (only four patients were administered systemic steroid therapy during observation) and only two patients were carriers of a *TNFA*2* allele. Therefore the results are not applicable for the Caucasian population. Interestingly, the results of Yamaguchi et al. are in concordance with the latest findings of Sharma et al. (19) concerning the association of TNF alleles with sarcoidosis in patients from North India. They found that the allele A of LTA *NcoI* polymorphism (*TNFB*2)* was prevalent in the 'no treatment' group while the G allele (*TNFB*1)* was associated with frequent relapses on drug withdrawal. As in the Yamaguchi study, Sharma et al. also failed to detect any significant association for *TNFA* polymorphism with Löfgren syndrome because of this sarcoidosis phenotype is very rare in the Indian and Japanese populations.

Comparison of the results of our and Yamaguci (18), Sharma (19) and Mrazek (16) studies indicates that ethnic background is of very importance for results of *TNF* genes polymorphism and prognosis of sarcoidosis.

Conclusions

1. We confirmed that *TNFB*1* and *TNFA*2* alleles are overrepresented in polish patients displaying Löfgren syndrome.
2. *TNFA2* allele is related with mild course of sarcoidosis in both patients with and without Löfgren syndrome.

Acknowledgements

Supported by the Polish State Committee for Scientific Research; grant no. 3 PO5B 051 24.

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