SARCOIDOSIS VASCULITIS AND DIFFUSE LUNG DISEASES 2007; 24; 000-000

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The MCP-1-2518 (A to G) single nucleotide polymorphism in Czech patients with pulmonary sarcoidosis: association with Löfgren's syndrome

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Abstract. *Background and aim:* The chemokine Monocyte Chemoattractant Protein (MCP)-1/CCL2, a chemoattractant for mononuclear cells, has already been implicated in the pathogenesis of sarcoidosis. A single nucleotide polymorphism (SNP) located at the position -2518 (A to G) of the MCP-1 gene has been reported to alter production of the MCP-1 protein *in vitro* and *ex vivo*. The present study, therefore, explored a possible association between MCP-1-2518 SNP and pulmonary sarcoidosis including its clinical subtypes, especially Löfgren's syndrome (LS). Relationship between MCP-1-2518 SNP and serum MCP-1 levels was also investigated. *Methods:* MCP-1-2518 genotypes were determined using PCR with sequence specific primers in 105 sarcoidosis patients and 359 healthy control subjects. The differences in genotype and allelic frequencies between the patient and control groups were assessed by χ^2 test. MCP-1 protein concentrations in serum samples from 77 sarcoidosis patients were determined by ELISA; Mann-Whitney *U*-test was used to test for differences in protein levels. *Results:* While there was no significant difference in distribution of MCP-1-2518 alleles between sarcoidosis patients and healthy control subjects, a significantly higher proportion of the MCP-1-2518*G allele ($p = 0.01$, odds ratio (OR) = 2.3) and of the GG genotype ($p = 0.03$, OR = 3.9) was observed in the patients with LS compared to control subjects. There was also a significantly higher frequency of the MCP-1-2518*G allele in patients presenting with LS compared to the patients without LS ($p = 0.04$, OR = 2.1). MCP-1 protein in serum was not related to MCP-1-2518 gene variants. *Conclusion:* A possible interpretation of our results is, that the MCP-1-2518 SNP or a gene located nearby may modify clinical manifestation of sarcoidosis towards Löfgren's syndrome. Future investigations in other population(s) should, therefore, follow this case-control study. ***(Sarcoidosis Vasc Diffuse Lung Dis 2007; 24: 000-000)***

Key Words. Diffuse Lung Disease. Löfgren's syndrome. MCP-1. CCL2. SNP.

Introduction

Pulmonary sarcoidosis is characterized by a sustained inflammatory response largely localized to the lymphatic vessel distribution – bronchovascular bundles and interlobular septa [1]. While the nature of the inciting antigenic stimulus is unknown, genetic factors have been shown to be important determinants either of disease susceptibility or of modification of its clinical course [2]. Besides the genes within the Major Histocompatibility Complex [3], inflammation-related genes such as those coding for cytokines, chemokines and their receptors are considered to be important candidates for susceptibility or modifier genes [2].

Received: 10 July 2006
Accepted after Revision: 10 Novembre 2006
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Several expression studies have pinpointed the CC chemokine ligand (CCL) 2 as an important mediator of inflammatory reaction in the sarcoid lung [4-7]. The gene coding for this chemokine, also known as Monocyte Chemoattractant Protein (MCP)–1, is polymorphic [8] and the G variant of the SNP at the position -2518 of the MCP-1 gene has been shown to exert a regulatory effect on MCP-1 protein concentration *in vitro* [8].

Accordingly, MCP-1-2518*G allele has already been associated with increased MCP-1 protein levels in healthy subjects [9], community-based Framingham heart study offspring cohort [10] and namely in several diseases with inflammatory component, e.g. systemic sclerosis [11] or Behcet's disease [12]. By contrast, MCP-1-2518 genotype was not associated with increased MCP-1 levels in type 2 diabetes [13] and surprisingly, moderately decreased MCP-1 levels were reported in the carriers of MCP-1-2518*G allele from the LURIC (Ludwigshafen Risk and Cardiovascular Health) cohort [14].

Despite the inconsistent findings concerning the effect of MCP-1-2518 polymorphism on the chemokine levels *in vivo* covering a wide spectrum of inflammatory diseases [9-14], no information has been available on relationship between this SNP and MCP-1 protein in sarcoidosis. More importantly, no data have so far been reported on plausible association between sarcoidosis and MCP-1-2518 polymorphism in Caucasians. Therefore, the present case control study in the Czech population explored a possible association between the MCP-1-2518 SNP and pulmonary sarcoidosis and further, analyzed relationship between the polymorphism and MCP-1 protein levels in serum. Additionally, possible modifying effect of the MCP-1 candidate polymorphism was investigated in subanalyses reflecting disease clinical course characterized by the Chest X-ray stage. Finally, because Löfgren's syndrome - a clearly distinguishable phenotype of sarcoidosis - has been recently assigned a partially specific genetic "make-up" [15], the patients presenting with LS were analysed separately.

Material and Methods

Study population

The study population consisted of 105 patients with sarcoidosis and 359 healthy controls (*Table I*). The criteria of diagnosis of sarcoidosis were compatible with those of the ATS/ERS/WASOG International Consensus Statement on sarcoidosis [16]. Clinical and radiographic findings were supported by histological evidence of epitheloid-cell granulomas and increased ratio of the CD4+/CD8+ T-cells in the bronchoalveolar lavage fluid (BALF). Subdivision was made according to the presence (n = 19) / absence (n = 86) of Löfgren's syndrome (LS); the acute form of sarcoidosis characterized by erythema nodosum, fever, bilateral hilar lymphadenopathy, polyarthralgia and association with favourable prognosis [16]. Serum sample for MCP-1 protein determination was available in the subset of 77 patients with sarcoidosis. No patient from this subset was treated with corticosteroids before the blood sampling.

The control subjects were blood donors or healthy participants of a bone marrow donor registry. Absence of lung disease in the control subjects was checked by a health questionnaire and by an interview emphasizing personal history and symptoms of respiratory diseases. All patients and controls were unrelated individuals of Czech origin and were recruited in one referral centre (University Hospital Olomouc, the Czech Republic).

The informed consent for the anonymous usage of their DNA and serum for the purposes of this study was obtained from all enrolled subjects. The study was performed with the approval of the Ethics committee of Medical Faculty PU & University Hospital, Olomouc.

Assessment of the MCP-1-2518 single nucleotide polymorphism

Genomic DNA was extracted from peripheral blood leukocytes by the standard salting-out method [17]. MCP-1-

Table I
Characteristics of sarcoidosis patients and the control subjects

	Sarcoidosis	*Sarcoidosis subgroups*		*Healthy controls*
		LS	*NLS*	
	(n = 105)	*(n = 19)*	*(n = 86)*	*(n = 359)*
Age (mean ± SD) (yrs)	45 ± 11	39 ± 9	46 ± 12	54 ± 8
Males / Females	43/62	5/14	38/48	240/119
Chest X-Ray Stage (I/II/III)	54/42/9	19/0/0	35/42/9	ND
Smoking (Y/N/ND)	19/84/2	6/12/1	13/72/1	ND

Smokers: current smokers and patients who ever smoked
LS: patients with Löfgren's syndrome, NLS: patients without LS, ND: data not available

Table II
Genotype, allele and phenotype (carriage rates) frequencies of the MCP-1-2518 SNP in the Czech patients with sarcoidosis and the healthy controls

Frequency	*Genotype*			*Allele*		*Phenotype*	
	AA	*AG*	*GG*	*A*	*G*	*A*	*G*
Sarcoidosis (n = 105)	52 (49)	46 (44)	7 (7)	150 (71)	60 (29)	98 (93)	53 (50)
Controls (n = 359)	206 (57)	135 (38)	18 (5)	547 (76)	171 (24)	341 (95)	153 (43)

The data are given as absolute numbers with percentages of a particular genotype/allele/phenotype in parentheses

2518 SNP was genotyped by the polymerase chain reaction with sequence specific primers (PCR-SSP). Standard and mutant alleles were amplified in two separate reactions. The constant reverse primer (5' TGA GTG TTC ACA TAG GCT TC 3') was used for amplification with forward specific primers either for the standard allele A (5' GTG GGA GGC AGA CAG CTA 3') or for the mutant allele G (5' GTG GGA GGC AGA CAG CTG 3'). In both cases, the size of the amplicon was 175 bp. Reaction conditions and internal controls were adopted from the Phototyping methodology [18], the protocol was described elsewhere [19].

Determination of MCP-1 protein in serum

ELISA technique (R&D system, Minneapolis, USA) was used for the determination of MCP-1 protein concentration in serum obtained from 77 patients with sarcoidosis. The detailed procedure was described elsewhere [20]. Measurements of MCP-1 protein concentration were performed in duplicates in all sarcoidosis patients from whom serum samples were available (no selection was applied). The reference values of serum MCP-1 for this assay (normal, healthy volunteers) were 239 pg/ml (median), 126 (interquartile range) [20].

Statistics

Allelic and phenotype frequencies were calculated from the observed number of genotypes. χ^2-analysis was used in order to test for a deviation of the genotype distribution from Hardy-Weinberg equilibrium. The significance of differences in allelic, genotype and phenotype frequencies between all groups was determined by means of χ^2 test applying Woolf-Haldane correction for small numbers. Statistical power of the study was determined according to the protocol demonstrated elsewhere [21]. The odds ratio (OR), 95% confidence interval (CI) and p-value were calculated. Mann-Whitney U-test was used to determine the differences in serum MCP-1 levels between carriers and non-carriers of the MCP-1-2518* G allele. $p < 0.05$ was considered statistically significant.

Results

Distribution of MCP-1-2518 alleles in sarcoidosis, its clinical subtypes and the control population

To assess the relationship between the MCP-1-2518 SNP and pulmonary sarcoidosis, the presence of MCP-1-2518 alleles in patients with sarcoidosis and the healthy control population was analysed. The distribution of the MCP-1-2518 genotypes was in compliance with Hardy-Weinberg equilibrium in both patient and control groups ($p > 0.05$). No significant difference in the frequency of MCP-1-2518 alleles was observed between the patients with sarcoidosis and healthy controls ($p > 0.05$, *Table II*). Statistical power of present study was sufficient (85%) to detect odds ratio (OR) = 1.6 for the comparison between the frequency of MCP-1-2158*G allele in patients with sarcoidosis and control subjects.

To explore further the distribution of the MCP-1-2518 SNP polymorphism in particular clinical subtypes of sarcoidosis, the subgroups of patients defined according to the presence/absence of LS were firstly compared with the healthy control subjects and secondly, comparisons were made between SNP frequencies in the Chest X-ray (CXR) stages I and II. Significantly higher frequency of the G allele, an increase of the GG genotype and also an elevated proportion of the G allele carriers (*Table III*) was observed in patients with LS when compared to the healthy control

Table III
Genotype, allele and phenotype (carriage rates) frequencies of the MCP-1-2518 SNP in the Czech patients with sarcoidosis divided according to presence (LS) / absence (NLS) of Löfgren's syndrome

Frequency		*LS* *(n = 19)*	*NLS* *(n = 86)*	*Controls* *(n =3 59)*
Genotype	AA	6 (31)	46 (53)	206 (57)
AG		10 (53)	36 (42)	135 (38)
	GG	3 (16)[a]	4 (5)	18 (5)
Allele	A	22 (58)	128 (74)	547 (76)
	G	16 (42)[b]	44 (26)	171 (24)
Phenotype	A	16 (84)	82 (95)	341 (95)
	G	13 (68)[c]	40 (47)	153 (43)

[a] GG genotype: $p = 0.03$ *versus* control subjects (Controls) and $p = 0.07$ *versus* NLS patients
[b] G allele frequency: $p = 0.01$ *versus* Controls and $p = 0.04$ *versus* NLS patients
[c] G allele carriage: $p = 0.03$ *versus* Controls and $p = 0.09$ *versus* NLS patients

group. The frequency of the G allele was also significantly higher in the sarcoid patients with LS than in the patients without LS (*Table III*). Accordingly, the individuals carrying G allele and GG genotype tended to be overrepresented among the patients with LS compared with those without LS (*Table III*). Moderate but insignificant increase of the G allele frequency was observed in sarcoid patients with the CXR-stage I by comparison with that in the CXR-stage II (p = 0.09, OR = 1.7, 95% CI = 0.91-3.31, χ^2=2.83). Because other CXR stages of sarcoidosis (stages III and IV) were infrequently represented in our cohort, these were not considered for comparisons.

MCP-1 protein in serum of patients with sarcoidosis

To investigate a plausible influence of the MCP-1-2518 SNP on the chemokine protein production in our sarcoidosis patients, MCP-1 concentrations were measured in their serum samples (n = 77). There was no significant difference in the serum MCP-1 protein level between the G allele carriers (n = 37, median, 478 pg/ml, 10th-90th percentiles, 303-782 pg/ml) compared to the non-carriers (n = 40, 419 pg/ml, 247-761 pg/ml) ($p > 0.05$); the detailed data are shown in *Fig. 1*. Further, no difference in the serum MCP-1 protein level between the patients presenting with LS (n = 10, 428 pg/ml, 386-780 pg/ml) and without LS (n = 67, 451 pg/ml, 250-766 pg/ml) was observed ($p > 0.05$).

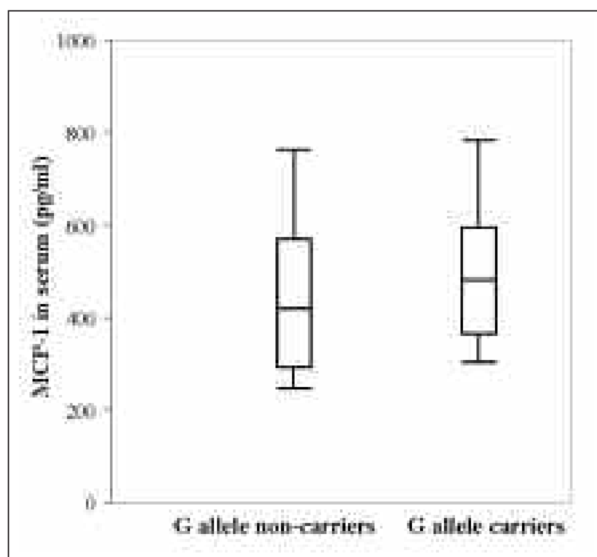

Fig. 1. The comparison of serum MCP-1 levels between MCP-1-2518*G allele carriers and non-carriers ($p > 0.05$). The data are presented as whisker box plots; the box represents the 25th to 75th percentiles, the median is indicated by a bar across the box; the whiskers on each box represent the 10th-90th percentiles

Discussion

Upregulation of the chemokine MCP-1/CCL2 in the lungs of patients with sarcoidosis has been reported previously [4-6]. Because a single nucleotide polymorphism (SNP) in the MCP-1 gene promoter (MCP-1-2518) has been assigned of functional significance *in vitro* [8], we explored the distribution of this candidate SNP in our Czech patients with pulmonary sarcoidosis and also analysed the relationship between the SNP and MCP-1 protein concentration in serum.

The relationship between the MCP-1-2518 SNP and sarcoidosis has previously been addressed only once - in the Japanese population [22]. The distribution of MCP-1-2518 variants in healthy Japanese population was substantially different to that observed in Caucasians [10], but complied with distribution in geographically close, Korean population [23]. No association between MCP-1-2518 polymorphism and susceptibility to sarcoidosis or its clinical manifestation was observed in the Japanese study [22].

In line with the Japanese report [22], we did not observe any association between the MCP-1-2518 SNP and sarcoidosis in Czech population. However, when we performed further subanalysis according to the presence / absence of Löfgren's syndrome (LS), the uncommon MCP-1-2518*G allele was overrepresented in the patients with LS. The carriers of the G allele possessed almost three fold higher risk for LS in comparison with the individuals without this allele. Our data, therefore, indicate that the MCP-1-2518 SNP is not involved in the genetic predisposition to sarcoidosis, but it may be an important (direct or indirect) gene modifier of the sarcoidosis phenotype.

Although elevation of MCP-1 protein has been reported in serum and bronchoalveolar lavage fluid from sarcoidosis patients [4-7], the relationship with the MCP-1-2518 SNP has not been analysed. Lack of association between the MCP-1-2518 variants and the serum concentration of

MCP-1 in our sarcoidosis patients suggests that this SNP may not be directly involved in the regulation of MCP-1 expression in sarcoidosis. Despite MCP-1-2518 SNP has previously been found of functional relevance in healthy subjects [9] and in several diseases, e.g. in systemic sclerosis [11], others failed to associate this polymorphism with MCP-1 levels under other pathologies, e.g. in type 2 diabetes [13]. Inconsistencies in the relationship between MCP-1-2518 SNP and MCP-1 protein across a spectrum of diseases support hypothesis that effect of the investigated SNP on MCP-1 expression *in vivo* may depend on particular regulatory stimuli.

By analogy with the report on HIV infection, where MCP-1-2518 genotype was associated with chemokine protein in cerebrospinal fluid but not in circulation [24] we speculate that also in sarcoidosis effect of MCP-1-2518 genotype on MCP-1 expression may be limited only to the sites of ongoing inflammation. Indeed, if this was the case in our sarcoidosis patients, then a relationship of the MCP-1 gene variants with MCP-1 levels may not be detectable in serum, but e.g. in bronchoalveolar space. Because of restricted availability of bronchoalveolar fluid samples from our subjects we, however, could not test this hypothesis.

The most important finding of this study - implication of MPC-1-2518*G allele as a modifier towards Löfgren's syndrome – has, however some limitations. The first is that the number of the patients presenting with LS was relatively low, the second limitation is a modest statistical power of the study to detect genetic association with sarcoidosis *per se*. Despite the limitations, a possible link between MCP-1 polymorphism and LS should be explained, and a linkage disequilibrium of the G allele with an unknown "causative" allele represents most probable explanation. If this concept of MCP-1-2518*G allele being only a marker is valid, then MCP-1 serum levels should rather correlate with the "real", yet undefined allele(s) and not with the presently investigated SNP MCP-1-2518.

Further investigations should therefore follow aiming at confirmation of the hypothesis that MCP-1-2518*G allele is a marker in linkage disequilibrium with supposed "casual" allele located nearby within the polymorphic CC chemokine gene cluster on the chromosome 17 [25]. More importantly, as at this stage only Czech patients were genotyped, it is mandatory to replicate the present study in other population(s). Both the replication, and at the same time publication of the current data, are in agreement with the principles for investigations of genetic markers in complex diseases [26, 27].

Acknowledgements

This work was presented in part at the 16th European Congress of Immunology, 6-9 September 2006, Paris, France. Technical help of the staff of Immunogenetics and Bronchology Units, University Hospital Olomouc, is gratefully acknowledged. The investigations were supported by Czech governmental funding MSM6198959205, IGA MZ CR: NR9037, and GA CR: 310/05/2614.

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