T A N 0 9 5 7 B | Journal Name | Manuscript No. | Dispatch: 9.10.07 | Journal: TAN | CE: T. Vijayalakshmi | Author Received: | No. of pages: 4 | ME: Senthil

Tissue Antigens ISSN 0001-2815

BRIEF COMMUNICATION

Complement receptor 1 single nucleotide polymorphisms in Czech and Dutch patients with sarcoidosis

F. Mrazek[1], M. Kvezereli[1], E. Garr[2], Z. Kubistova[1], E. Kriegova[1], R. Fillerova[1], A. Arakelyan[1], H. J. T. Ruven[3], J. Drabek[1], J. M. M. van den Bosch[3], V. Kolek[1], K. I. Welsh[2], J. C. Grutters[3], R. M. du Bois[2] & M. Petrek[1]

1 Palacky University, Olomouc, Czech Republic
2 Royal Brompton Hospital, London, UK
3 St. Antonius Hospital, Nieuwegein, The Netherlands

Key words
CR1; genetics of complex diseases; replication of a case–control study; sarcoidosis

Correspondence
Martin Petrek, MD
Department of Immunology
Faculty of Medicine
Palacky University
I. P. Pavlova Strasse 6
CZ-775 20 Olomouc
Czech Republic
Tel: +420 58 563 2770
Fax: +420 58 541 5116
e-mail: petrekm@fnol.cz

Received 8 August 2007; revised 19 September 2007; accepted 30 September 2007

doi: 10.1111/j.1399-0039.2007.00957.x

Abstract

A single nucleotide polymorphism (SNP) C5507G of the complement receptor 1 (*CR1*) gene has been associated with genetic susceptibility to sarcoidosis in an Italian population. In order to provide further data on the possible involvement of *CR1* gene polymorphisms in sarcoidosis, *CR1* SNPs C5507G and A3650G were investigated in Czech ($n = 210$) and Dutch ($n = 116$) patients with sarcoidosis with ethnically matched groups of healthy control subjects (Czech, $n = 203$; Dutch, $n = 112$). *CR1* C5507G and A3650G SNPs were not associated with susceptibility to sarcoidosis or its clinical course. Further, *CR1* messenger RNA expression in bronchoalveolar lavage cells investigated by quantitative reverse transcriptase–polymerase chain reaction did not differ between sarcoidosis patients and control subjects and was not associated with the presence of the *CR1* 5507*G allele.

Sarcoidosis is a chronic granulomatous disorder resulting from the exposure of genetically susceptible individuals to unknown environmental triggers (1–3). An interaction of unknown 'sarcoidosis antigen(s)' with specific antibodies may lead to the formation of immune complexes (ICs), potentially important in the pathogenesis of the disease (4). Complement receptor 1 (*CR1*) expressed mainly on the membrane of erythrocytes mediates transport of ICs to the phagocytes in the liver and spleen and is, therefore, involved in the clearance of ICs from the circulation (5). The hypothesis implicating ICs in the pathogenesis of sarcoidosis together with the presence of a well-documented functional polymorphism of the *CR1* gene (6, 7) led to a genetic association study performed in an Italian population in which the G allele of a single nucleotide polymorphism (SNP) at position 5507 of the *CR1* gene was found to be associated with susceptibility to sarcoidosis (4). In the present study, we aimed not only to confirm previous findings by investigating the *CR1* C5507G gene polymorphism and its closely linked nonsynonymous SNP *CR1* A3650G in two other Caucasian populations but also to address functional aspects of *CR1* gene variants by determining *CR1* messenger RNA (mRNA) expression in bronchoalveolar cells of patients with sarcoidosis and healthy control subjects.

A total of 641 unrelated Caucasian individuals were enrolled into this case–control study, in which 210 Czech [age, median (range): 45 (25–83); males/females: 98/112] and 116 Dutch [35(17–64); 65/51] patients with sarcoidosis were compared with 203 Czech [33 (18–54); 105/98] and 112 Dutch [47 (21–73); 58/54] healthy individuals serving as control populations. The diagnosis of sarcoidosis was made according to the criteria of ATS/ERS International Consensus Statement (8). To assess the particular disease

© 2007 The Authors
Journal compilation © 2007 Blackwell Munksgaard

phenotypes, the patients were further subdivided according to their chest radiographic stage of the disease, requirement for glucocorticosteroid treatment and the presence/absence of Löfgren's syndrome. All patients were recruited and diagnosed at single tertiary referral centre for both the Czech (Palacky University Hospital in Olomouc) and the Dutch (St. Antonius Hospital in Nieuwegein) populations. The controls were blood donors or healthy participants of the bone marrow donor registry recruited from the same region as patients. Absence of lung disease in the control subjects was checked by health questionnaire and interview emphasising family history and symptoms of respiratory disease. All subjects agreed to the anonymous usage of their biological samples [DNA or an aliquot of bronchoalveolar lavage (BAL) fluid] for the purposes of the study, which was performed with the approval of Ethical Committees of Medical Faculty in Olomouc and St. Antonius Hospital in Nieuwegein.

In order to determine the distribution of the *CR1* C5507G SNP (GenBank SNP ID: rs3811381) in patients with sarcoidosis by comparison with the control population, the Czech and Dutch Caucasian patients with sarcoidosis with ethnically matched healthy control subjects were genotyped by polymerase chain reaction with sequence-specific primers (9). Both the Czech and the Dutch populations were found in Hardy–Weinberg equilibrium with regard to distribution of the *CR1* C5507G genotypes ($P > 0.05$). The statistical power of the present study to replicate the results originally reported in the Italian study (4) was 99% for the Czech population and 93% for the Dutch population (10). In contrast to the increased frequency of the G allele of *CR1* C5507G SNP reported in Italian sarcoidosis patients (4), the frequencies observed in Czech and Dutch patients did not differ from those obtained in the relevant control groups (Table 1). Importantly, the frequencies of the G allele in normal subjects from all three Caucasian populations were similar (Table 1). However, there was a difference between G allele frequencies in Italian patients (34%) and in our two patient groups (Czech, 18%; Dutch, 23%). This difference was because of the over-representation of GG homozygotes in Italian patients: while the frequency of GG homozygotes among the Czech and Dutch patients was no more than 5%, the GG genotype was more frequent in the Italian sarcoidosis group (18%).

Although Zorzetto et al. (4) observed an increase of the proportion of *CR1* 5507 GG homozygotes especially in females with sarcoidosis, we observed no differences in *CR1* 5507 GG genotype when male and female patients were compared with controls separately. Furthermore, no relationship of the investigated *CR1* SNP with clinical course of sarcoidosis as assessed by chest radiographic stage, requirement for glucocorticosteroid treatment and the absence/presence of Löfgren's syndrome was observed ($P > 0.05$ in all cases). Because the frequencies of *CR1* 5507 GG genotypes in the Czech and Dutch sarcoidosis patients were similar and, also, not different from those observed in control subjects, we speculate that overrepresentation of GG genotype in Italian sarcoidosis female patients could

UNCORRECTED PROOF

Table 1 Genotype and allelic frequencies of the *CR1* C5507G SNP and estimated proportion of the *CR1* A3650G–C5507G haplotypes in Czech, Dutch and Italian populations (sarcoidosis patients and control subjects). Data are given as proportions of genotypes and alleles with their absolute numbers in parentheses. Distribution of *CR1* haplotypes estimated by expectation–maximisation algorithm (11) is presented as relative values[a]

	Czech patients	Czech controls	Dutch patients	Dutch controls	Italian patients	Italian controls
Genotype frequency	$n = 208$[b]	$n = 203$	$n = 116$	$n = 112$	$n = 91$	$n = 94$
CC	0.67 (140)	0.66 (133)	0.58 (67)	0.57 (64)	0.50 (46)	0.64 (60)
CG	0.30 (62)	0.33 (66)	0.39 (45)	0.38 (42)	0.32 (29)	0.30 (28)
GG	0.03 (6)	0.02 (4)	0.03 (4)	0.05 (6)	0.18 (16)	0.06 (6)
Allelic frequency	$2n = 416$	$2n = 406$	$2n = 232$	$2n = 224$	$2n = 182$	$2n = 188$
C	0.82 (342)	0.82 (332)	0.77 (179)	0.76 (170)	0.66 (121)	0.79 (148)
G	0.18 (74)[†]	0.18 (74)	0.23 (53)[‡]	0.24 (54)	0.34 (61)	0.21 (40)[§]
CR1 haplotype 3650–5507	$2n = 412$[c]	$2n = 404$[c]	$2n = 232$[c]	$2n = 116$[c]	$2n = 168$[c]	$2n = 182$[c]
AC	0.81	0.82	0.74	0.79	0.66	0.78
AG	0.01	0.00	0.00	0.00	0.01	0.01
GC	0.01	0.00	0.03	0.01	0.01	0.02
GG	0.17	0.18	0.23	0.20	0.33	0.20

[a] The data on Italian population are based on the Zorzetto et al. (4). The genotyping was performed in Czech and Dutch populations by polymerase chain reaction with sequence-specific primers (PCR-SSP). The sequences of specific primers are available on request. Polymerase chain reaction cycling conditions and composition of the PCR mixes were adopted from the 'phototyping' methodology described elsewhere (24). The specificity of PCR-SSP for both polymorphisms was verified by sequencing of the amplicons in randomly selected samples. Differences between the frequencies of CR1 gene variants in the patient and control groups were assessed by chi-squared test using Woolf–Haldane correction in cases of small numbers. Allelic frequencies compared between the groups of patients and relevant (ethnically matched) controls: [†]$P = 0.86$, [‡]$P = 0.75$, [§]$P = 0.01$.

[b] The genotypes were not determined in two patients.

[c] Only sarcoidosis patients and control subjects with known both *CR1* C5507G and A3650G genotypes were included into the analysis.

© 2007 The Authors
Journal compilation © 2007 Blackwell Munksgaard

reflect a higher proportion of a specific disease phenotype, which was rare or absent in our groups of patients. However, such a hypothetical phenotype remains unclear because subanalysis according to the clinical characteristics revealed no differences in distribution of *CR1* 5507 SNP both in the Italian and in our groups of patients.

Because we could not find any significant difference between the distribution of the *CR1* C5507G polymorphism in sarcoidosis patients and control groups in both Czech and Dutch populations, we extended our investigations to another nonsynonymous SNP of the *CR1* gene (*CR1* A3650G, rs2274567). However, there was no difference in allelic frequencies of the A3650G SNP between patients with sarcoidosis and control groups of Czech/Dutch origin. The frequencies of the *CR1* A3650G/C5507G haplotypes estimated by expectation–maximisation algorithm (software ARLEQUIN, version 3.000) (11) are listed in Table 1. Consistent with the previous results (4, 12), we detected strong linkage disequilibrium (LD) (likelihood ratio test, software ARLEQUIN) between the less common alleles of these two SNPs, i.e. allele G of the C5507G SNP and allele G of the A3650G SNP in all study groups ($D' = 0.95$ for Czech patients, and $D' = 1.00$ for Czech controls and for both Dutch patients and controls; $P < 0.00001$ for all four groups) (13).

It could be argued that an adjacent, hypothetically 'true' gene variant is in LD with the *CR1* 5507*G allele only in the Italian population but not in the Czech or Dutch populations (14). Major *CR1* haplotypes are, however, strongly conserved across ethnic groups (12). Importantly in this context, the *CR1* C5507G SNP is associated with the number of functional *CR1* molecules on the erythrocyte surface in Caucasians but not in African Americans (7). Accordingly, the number of functional *CR1* molecules on erythrocytes must, therefore, be controlled by another (further) genetic factor not present in African Americans (15). While this genetic element remains unknown (15), we cannot completely reject a hypothesis that the association observed in the Italian study was caused by LD of the *CR1* 5507*G allele with such a causative variant.

Because direct evidence for the involvement of *CR1* in the pathogenesis of sarcoidosis is still lacking (16–18) and because of the wide implication of *CR1* in the regulation of immune response (5), we were interested to see if sarcoid inflammation is accompanied by any change in the local *CR1* mRNA expression. *CR1* mRNA expression in BAL cells was investigated by quantitative reverse transcriptase–polymerase chain reaction (RotorGene 3000 system; Corbett Research, Sydney, Australia) in a subset of Czech sarcoidosis patients from the genetic association study in whom BAL fluid sample was available ($n = 52$) and in 14 healthy control subjects. These control individuals with no evidence of lung inflammation and with normal BAL fluid cytology were selected independently from the Czech control group used for the genetic association study. Primers and probes for *CR1* (GenBank ID: NM_000651.4) and reference housekeeping *PSMB2* (GenBank ID: NM_002794.3) genes were designed using ProbeFinder assay design tool (www.universalprobelibrary.com; Roche Applied Science, Indianapolis, IN) and their sequences are available on request. The relative *CR1* expression was calculated using second derivative method (ROTORGENE Software 6.1.71; Corbett Research) as follows: expression = average amplification$^{(\mathrm{CTt\ calibrator} - \mathrm{CTt\ target\ gene})}$/average amplification$^{(\mathrm{CTt\ calibrator} - \mathrm{CTt\ reference\ gene})}$, and transcribed human universal reference RNA (Stratagene, La Jolla, CA) was used as calibrator. No difference in the *CR1* mRNA expression was observed between sarcoidosis patients and healthy control subjects ($P = 0.69$, Mann–Whitney *U*-test). In order to analyse any possible effect of *CR1* C5507G variants on the gene transcription, *CR1* mRNA levels were compared between subjects carrying *CR1* 5507 CC ($n = 44$) and CG ($n = 20$) genotypes in the whole population as well as in groups of patients and controls separately. A presence of the less common *CR1* 5507*G allele had no effect on *CR1* mRNA expression (CC *vs* CG genotypes: $P = 0.44$).

It may be argued that the absence of data on quantitative 'systemic' levels of erythrocyte *CR1* in sarcoidosis is a major limitation of the present study. We have tried to compensate for this 'limitation', caused by the nonavailability of material from investigated subjects, by analysis at the transcript level. Our results may be interpreted as demonstrating that sarcoidosis is not accompanied by local changes of *CR1* mRNA expression at sites of ongoing granulomatous inflammation and that any genetic effect associated with *Hin*dIII restriction fragment length polymorphism/*CR1* G5507C SNP variants (H and L alleles) (6, 7) is not apparent at the *CR1* mRNA level in nucleated bronchoalveolar cells. The latter finding is in line with the previous reports connecting *CR1* H and L alleles with the *CR1* expression only on erythrocytes but not in cells of other lineages (6) and may further support a hypothesis that these alleles modify erythrocyte *CR1* density by (yet unknown) 'post-translational' mechanism (7).

In conclusion, despite an adequate methodical approach including sufficient statistical power, we could not confirm in the Czech and Dutch populations previous report associating SNP C5507G in the *CR1* gene with sarcoidosis (4). Further, our functional data do not provide support for the likelihood local *CR1* upregulation playing a role in the development of sarcoid granulomas in the lung; by contrast, our data support the concept that functional *CR1* alleles (H and L) do not affect *CR1* gene transcription. Furthermore, the present report stresses the importance of ethnicity in evaluation of particular genetic variants as disease susceptibility factors, even within subpopulations of Caucasians. In addition, it emphasises the need for replication of genetic studies across ethnic boundaries and also the

UNCORRECTED PROOF

© 2007 The Authors
Journal compilation © 2007 Blackwell Munksgaard

necessity for appropriate definition of clinical phenotype, both important prerequisites for conducting and evaluating genetic association studies, particularly in genetically complex diseases (19–23).

Acknowledgments

Technical help of the staff of Immunogenetics/Immunoproteomics and Bronchology Units, University Hospital Olomouc, is gratefully acknowledged. MK was supported by the NATO Science Fellowship and awarded by the Centre for Higher Education Studies, Ministry of Schools, Youth and Sport of the Czech Republic (MSYSCR). Partial funding was obtained from the Grant Agency of the Czech Republic (310/05/2614) and from the MSYSCR (MSM6198959205).

References

1. Baughman RP, Lower EE, du Bois RM. Sarcoidosis. *Lancet* 2003: **361**: 1111–8.
2. du Bois RM, Beirne PA, Anevlavis SE. Genetics. In: Drent M, Costabel U, eds. *Sarcoidosis – European Respiratory Monograph. Sheffield: European Respiratory Journals 2005*: *64–81*. [3]
3. Iannuzzi MC. Genetics of sarcoidosis. *Semin Respir Crit Care Med* 2007: **28**: 15–21.
4. Zorzetto M, Bombieri C, Ferrarotti I et al. Complement receptor 1 gene polymorphisms in sarcoidosis. *Am J Respir Cell Mol Biol* 2002: **27**: 17–23.
5. Krych-Goldberg M, Atkinson JP. Structure-function relationships of complement receptor type 1. *Immunol Rev* 2001: **180**: 112–22.
6. Wilson JG, Murphy EE, Wong WW, Klickstein LB, Weis JH, Fearon DT. Identification of a restriction fragment length polymorphism by a CR1 cDNA that correlates with the number of CR1 on erythrocytes. *J Exp Med* 1986: **164**: 50–9.
7. Herrera AH, Xiang L, Martin SG, Lewis J, Wilson JG. Analysis of complement receptor type 1 (CR1) expression on erythrocytes and of CR1 allelic markers in Caucasian and African American populations. *Clin Immunol Immunopathol* 1998: **87**: 176–83.
8. Statement on sarcoidosis. Joint Statement of the American Thoracic Society (ATS), the European Respiratory Society (ERS) and the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) adopted by the ATS Board of Directors and by the ERS Executive Committee. *Am J Respir Crit Care Med* 1999: **160**: 736–55. [4]
9. Okayama H, Curiel DT, Brantly ML, Holmes MD, Crystal RG. Rapid, nonradioactive detection of mutations in the human genome by allele-specific amplification. *J Lab Clin Med* 1989: **114**: 105–13.
10. Lalouel JM, Rohrwasser A. Power and replication in case-control studies. *Am J Hypertens* 2002: **15**: 201–5.
11. Excoffier L, Laval G, Schneider S. Arlequin version 3.0: an integrated software package for population genetics data analysis. *Evol Bioinformatics Online* 2005: **1**: 47–50.
12. Xiang L, Rundles JR, Hamilton DR, Wilson JG. Quantitative alleles of CR1: coding sequence analysis and comparison of haplotypes in two ethnic groups. *J Immunol* 1999: **163**: 4939–45.
13. Jorde LB. Linkage disequilibrium and the search for complex disease genes. *Genome Res* 2000: **10**: 1435–44.
14. Silverman EK. Haplotype thinking in lung disease. *Proc Am Thorac Soc* 2007: **4**: 4–8.
15. Cockburn IA, Rowe JA. Erythrocyte complement receptor 1 (CR1) expression level is not associated with polymorphisms in the promoter or 3′ untranslated regions of the CR1 gene. *Int J Immunogenet* 2006: **33**: 17–20.
16. Biglino A, Albera C, Cariti G, Gioannini P. Relationship between circulating immune complexes, serum interferon and clinical features in sarcoidosis. *Respiration* 1985: **47**: 293–8.
17. Rooney DP, Finch MB, Elborn JS, Stanford CF. Circulating immune complexes in sarcoidosis, a clinical role for the Raji assay? *Eur Respir J* 1990: **3**: 760–4.
18. Schoenfeld N, Schmolke B, Schmitt M et al. Specification and quantitation of circulating immune complexes in the serum of patients with active pulmonary sarcoidosis. *Thorax* 1994: **49**: 688–91.
19. Cordell HJ, Clayton DG. Genetic association studies. *Lancet* 2005: **366**: 1121–31.
20. Ioannidis JPA, Ntzani EE, Trikalinos TA, Contopoulos-Ioannidis DG. Replication validity of genetic association studies. *Nat Genet* 2001: **29**: 306–9.
21. Iannuzzi MC, Maliarik M, Rybicki BA. Nomination of a candidate susceptibility gene in sarcoidosis. The complement receptor 1 gene. *Am J Respir Cell Mol Biol* 2000: **27**: 3–7.
22. Whitsett JA, Bachurski CJ, Barnes KC et al. Functional genomics of lung disease. *Am J Respir Cell Mol Biol* 2004: **31** (Suppl): S1–81.
23. Janssen R, Sato H, Grutters JC et al. The Clara cell10 adenine38guanine polymorphism and sarcoidosis susceptibility in Dutch and Japanese subjects. *Am J Respir Crit Care Med* 2004: **170**: 1185–7.
24. Bunce M, O'Neil CM, Barnardo MCNM et al. Phototyping: comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers (PCR-SSP). *Tissue Antigens* 1995: **46**: 355–67.

UNCORRECTED PROOF

© 2007 The Authors
Journal compilation © 2007 Blackwell Munksgaard

Author Query Form

Journal: Tissue Antigens

Article: tan_957

Dear Author,

During the copy-editing of your paper, the following queries arose. Please respond to these by marking up your proofs with the necessary changes/additions. Please write your answers on the query sheet if there is insufficient space on the page proofs. Please write clearly and follow the conventions shown on the attached corrections sheet. If returning the proof by fax do not write too close to the paper's edge. Please remember that illegible mark-ups may delay publication.
Many thanks for your assistance.

Query No.	Query	Reply
1	Please provide the department or unit name for all the affiliation addresses.	
2	Please clarify what "CTt" in the sentence "The relative *CR1* …" refers to.	
3	Please provide the volume number for reference 2.	
4	Please provide the author name (if any) for reference 8.	

UNCORRECTED PROOF

MARKED PROOF

Please correct and return this set

Please use the proof correction marks shown below for all alterations and corrections. If you wish to return your proof by fax you should ensure that all amendments are written clearly in dark ink and are made well within the page margins.

Instruction to printer	*Textual mark*	*Marginal mark*
Leave unchanged	• • • under matter to remain	Ⓘ
Insert in text the matter indicated in the margin	⋌	New matter followed by ⋌ or ⋌⊗
Delete	/ through single character, rule or underline or ⊢⊣ through all characters to be deleted	∂ or ∂⊗
Substitute character or substitute part of one or more word(s)	/ through letter or ⊢⊣ through characters	new character / or new characters /
Change to italics	— under matter to be changed	⌐
Change to capitals	≡ under matter to be changed	≡
Change to small capitals	= under matter to be changed	=
Change to bold type	~ under matter to be changed	~
Change to bold italic	≂ under matter to be changed	≂
Change to lower case	Encircle matter to be changed	≢
Change italic to upright type	(As above)	⊬
Change bold to non-bold type	(As above)	⊬
Insert 'superior' character	/ through character or ⋌ where required	Y or ⋌ under character e.g. Y or ⋌
Insert 'inferior' character	(As above)	⋌ over character e.g. ⋌
Insert full stop	(As above)	⊙
Insert comma	(As above)	,
Insert single quotation marks	(As above)	Y or ⋌ and/or Y or ⋌
Insert double quotation marks	(As above)	Y or ⋌ and/or Y or ⋌
Insert hyphen	(As above)	⊢⊣
Start new paragraph	⌐	⌐
No new paragraph	⤺	⤺
Transpose	⊔⊓	⊔⊓
Close up	linking ⁐ characters	⁐
Insert or substitute space between characters or words	/ through character or ⋌ where required	Y
Reduce space between characters or words	| between characters or words affected	⊤