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Angiotensin converting enzyme genotype affects development and course of sarcoidosis in Asian Indians

Mohammad Tahir, S.K. Sharma, Shazia Ashraf, Hemant K. Mishra
Division of Pulmonary and Critical Care Medicine, Department of Medicine, All India Institute of Medical Sciences, New Delhi, India

Abstract. *Background and Objectives:* Studies of serum angiotensin converting enzyme (SACE) activity and its association with ACE gene insertion/deletion (I/D) polymorphism in relation to sarcoidosis have yielded variable results. This has been attributed to possible ethnic differences. Present study was designed to evaluate the relationship between I/D polymorphism and susceptibility to develop sarcoidosis and its effect on SACE activity and disease course in Asian Indian patients with sarcoidosis. *Methods:* ACE genotype was performed in 72 consecutive patients with sarcoidosis and 199 controls (96 normal healthy individuals and 103 tuberculosis patients taken as disease controls). SACE activity was determined in all patients with sarcoidosis. Various parameters were compared amongst patients with different genotypes as well as between sarcoidosis and control groups. *Results:* Gene frequency of I and D in control group was 0.6 and 0.4, whereas in patients with sarcoidosis it was 0.35 and 0.65 respectively ($p < 0.001$). For individuals with D allele (DD&ID genotypes), odds ratios for developing sarcoidosis were 9.0 (95% CI: 3.4; 23.7) and 5.5 (95% CI: 2.2; 13.6) respectively considering individuals with II genotype as reference. Mean SACE activity was highest in patients with DD genotype and followed an order of DD > ID > II. Good response to initial corticosteroids was seen in 6 of 6 (100%) patients with II genotype whereas in only 32 of 37 (84%) with ID and 16 of 25 (64%) with DD ($p = 0.013$). *Interpretation and Conclusion:* In Asian Indian population 'D' allele is associated with an increased risk for development of sarcoidosis and patients with 'D' allele show poor response to corticosteroids. ***(Sarcoidosis Vasc Diffuse Lung Dis 2007; 24: 000-000)***

Key Words. Angiotensin converting enzyme (ACE). Gene Polymorphism, Insertion. Deletion. Sarcoidosis. India.

Introduction

Sarcoidosis is a multi-system disease of granulomatous inflammation. Its manifestations are essentially localized to lung and skin, but involvement of other sites such as eyes, lymph nodes, parotid glands, heart, liver, spleen, cranial nerves and central nervous system can also occur [1, 2]. The diagnosis is based on a constellation of clinical, radiological and histopathological findings [3, 4]. This granulomatous disease is common but its etiology is largely unknown [5]. Genetic factors may have a role in causation and progression. Genetic polymorphisms of transforming growth factor-beta (TGF-β), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein

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Correspondence: Dr. SK Sharma, MD, Ph.D.
Chief, Division of Pulmonary and Critical Care Medicine
Professor and Head, Department of Medicine
All India Institute of Medical Sciences,
New Delhi 11029, India
Tel.: +91-11-26593303 / +91-11-26594415,
Fax: +91-11-26589898
E-mail: sksharma@aiims.ac.in

(MIP)-1 alpha genes have not been shown to have a significant correlation with sarcoidosis [6, 7], but human leukocyte antigen(HLA)-DR allele has been found to affect the clinical course and outcome of patients with sarcoidosis [8, 9].

Angiotensin converting enzyme (ACE) is a zinc-metallopeptidase with a key function in regulation of blood volume and pressure. It is also synthesized by sarcoidosis granulomas defining this enzyme as a diagnostic and prognostic marker of sarcoidosis. SACE activity assessment has been proposed to be a marker of disease activity [10], though its sensitivity and specificity are not very good. Monitoring of SACE activity can be helpful in follow-up of patients with sarcoidosis who progress to remission either spontaneously or after corticosteroid therapy [3, 4].

The ACE1 gene, located on chromosome 17, contains an insertion/deletion (I/D) polymorphism at intron 16. The ACE gene can be divided into two different alleles depending upon the presence/insertion (I) or absence/deletion (D) of a 287-bp DNA segment. The D allele of this polymorphism is linked with raised SACE activity [11]. The ACE gene I/D polymorphism has been studied in relation to sarcoidosis and various other diseases (12). Though the published reports about its association or linkage with the risk of sarcoidosis are conflicting, D allele of ACE gene may have a minor pro-inflammatory influence on disease course in these patients. Keeping with the genotype influence on SACE activity, genotype specific reference interval range for SACE activity has been proposed [13].

Hypothesis of I/D polymorphism affecting sarcoidosis disease progression and severity has been tested in various ethnic groups and has yielded variable results (14-20). These may be attributed to ethnic differences. The present study is the first one to evaluate the role of I/D polymorphism in patients with sarcoidosis in Asian Indian population. The study was designed to evaluate the variation of I/D polymorphism in patients with sarcoidosis and its comparison with normal as well as disease controls. Effect of I/D polymorphism on SACE activity as well as other clinical and biochemical parameters at presentation and follow-up was studied.

Material and Methods

Study population

Following approval of study protocol by institutional ethics committee, we enrolled consecutive patients with sarcoidosis coming to outpatient medical department and chest clinic of All India Institute of Medical Sciences (AIIMS) hospital, New Delhi from 1996 to 2001 and were followed up for a period of 5 years. For control group, we included normal healthy volunteers, acting as normal controls and confirmed pulmonary tuberculosis (PTB) patients, acting as disease controls. A total of 72 adult patients with sarcoidosis, 96 normal healthy volunteers and 103 adult patients with PTB were studied. Informed written consent was obtained from each individual included in the study.

Selection and classification of cases

Patients with sarcoidosis were classified into stage 0, I, II or III depending upon the severity of disease on chest radiographs in accordance with the American Thoracic Society guidelines.[21] Diagnosis of sarcoidosis was based on clinical features and confirmed by histopathological demonstration of non-caseating granulomas in the biopsy specimens (Biopsy proven). Biopsies were obtained from peripheral lymph nodes in 20 and skin in 21 patients. 26 patients underwent transbronchial lung biopsy and 6 patients underwent video assisted thoracoscopic surgery for intra-thoracic lymph node biopsy. Other causes of granulomatous lung disorders including inorganic or organic chemical exposure were excluded in all patients with sarcoidosis. Each enrolled patient was evaluated by detailed medical history and complete physical examination. Height and weight were measured to calculate the body mass index (BMI). Clinical characteristics were carefully recorded at initial presentation and during follow up period. The laboratory investigations included complete hemogram, serum biochemistry including liver and renal functions and tuberculin skin test with 5 tuberculin unit. Serum as well as 24-hour urinary calcium measurement was performed in all patients with sarcoidosis. Blood and urine samples for calcium measurement were collected after patients had stopped high calcium diet and any calcium supplementation for a minimum of 5 days [22]. Pulmonary functions tests (PFT) included spirometry and diffusion capacity for carbon monoxide and were done in all patients as described previously [22].

For disease controls, we included confirmed tuberculosis (TB) cases. Patients with evidence of *Mycobacterium tuberculosis* (*Mtb*) in sputum smears and/or cultures, or chest radiographs suggestive of PTB along with peripheral lymph node biopsy showing caseating granulomas and/or *Mtb*, were classified as PTB.

Serum Angiotensin Converting Enzyme (SACE) activity measurement

SACE activity was measured in 72 patients with sarcoidosis using the method described by Cushman and Cheung

as described previously [10, 23]. Briefly, production of hippuric acid from hippuryl-L-histidyl-L-leucine by ACE activity present in the serum sample was measured using spectrophotometer. Running reference ACE activity in our laboratory during study period was 12-35 U/L.

ACE Genotyping

ACE genotypes were determined in 72 patients with sarcoidosis, 96 normal controls and 103 PTB patients. DNA was isolated from peripheral blood leukocytes using C1 buffer. For typing I/D polymorphism, DNA was amplified by polymerase chain reaction (PCR) as described by Rigat et al [24`. Briefly, the reactions were performed with 10 pmol of each flanking primer pair; sense oligo 5'CTGGAGACCACTCCCATCCTTTCT3' and anti-sense oligo 5'GATGTGGCCATCACATTCGTCAGAT3', in a final volume of 50 µl containing 100 ng of genomic DNA, 3 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl (pH 8.4), 0.1 mg/mL gelatine, 0.5 mM of each deoxynucleotide triphosphate (dNTP), one unit of Taq polymerase (Bangalore Genei, Bangalore, India). The DNA was amplified for 30 cycles at 94°c for 1 min, annealing at 58°c for 1 min, and extension at 72°c for 2 min in a thermal cycler (MJ Research Inc. Waltham, MA, USA). This resulted in 490 bp and 190 bp amplification products corresponding to I and D alleles. Electrophoreses of the PCR products were done in 2% agarose gel containing 5 µg/ml of ethidium bromide.

To avoid mistyping of ID due to insufficient amplification of I allele, an insertion-specific PCR was performed with primers 5'TGGGACCACAGCGCCCGCCACTAC3' and 5' TCGCCAGCCCTCCCATGCCCATAA3' under the same conditions, except that the extension temperature was 65°C (product size was 335 bp).

Follow-up

All patients were followed-up for five years for disease progression and response to treatment. Patients having erythema nodosum or ankle joint arthritis or both associated with bilateral hilar lymphadenopathy were classified as Lofgren's syndrome whereas others were classified as non-Lofgren's syndrome [25]. Patients with disease activity lasting for ≥ 2 years were classified as chronic whereas those with disease activity lasting less than 2 years were classified as non-chronic as described by Neville et al. [26]. Patients were given systemic steroids on a case to case basis depending upon clinical indication and severity of the disease. Clinical, radiological and pulmonary functions were monitored to assess the extent of improvement. Patients who had remission of disease (assessed by clinical, radiological and improvement in pulmonary function parameters) following short course of corticosteroids (8-12 weeks) were classified as having 'good response to steroids' whereas those who required systemic steroids for longer time (> 12 weeks), to go into remission, were classified as having 'poor response to treatment'.

Patients who responded to steroids but developed relapse of symptoms after stopping steroids (within 12 weeks) and when it happened more than once in a period of 1 year, they were classified as having frequent relapses. Such patients were given intermittent tapering course of steroids on a regular basis during the duration of follow-up.

Statistical Analysis

Patient data were entered electronically and doubly checked. Data analyses for statistical parameters were made using STATA 7.0 (intercooled version, Stata Corporation, Houston, TX) statistical software. To compare categorical variables chi-square test was applied. To compare normally distributed continuous variables, 'one way *ANOVA* ' was applied and for non-normally distributed variables comparison was done using 'Kruskal-Wallis H-test'. Odds ratios for development of sarcoidosis in patients having 'D' allele (DD and ID genotypes), were computed taking patients as well as control subjects without 'D' allele (II genotype) as reference. Gender based subgroup analysis was done to find any gender based differences in various parameters. Statistically significant correlation was considered at value of $p < 0.05$.

Results

Table I represents a comparison of physical characteristics of patients with sarcoidosis, normal controls and disease controls with respect to age, sex and BMI. *Table II* represents a comparison of I/D genotypes and gene frequency of 'I' and 'D' alleles amongst patients with sarcoidosis, normal controls and disease controls. Out of 72 patients with sarcoidosis, the most common genotype was ID, present in 39 (54.2%) followed by DD in 27 (37.5%) patients. The least common genotype was II, present only in 6 (8.3%) patients with sarcoidosis. Normal controls had ID as the most common genotype, present in 45 (46.9%) followed by II, present in 35 (36.4%) subjects. The least common genotype in normal controls was DD, present in 16 (16.7%) subjects. Similar pattern was also ob-

Table I
Physical profile of cases (sarcoidosis) and controls (healthy individuals and PTB patients)

Subjects (no.)	*Male/Female (no.)*	*Age† (years)*	*BMI† (kg/m²)*
Sarcoidosis (n=72)	36/36	44.6 ± 9.5	23.7 ± 3.6
Normal controls (n=96)	64/32	31.3 ±10.7	25.2 ± 4.3
Disease controls (n=103)	72/31	32.5 ± 5.4	23.5 ± 3.8
Total (n=271)	172/99	--	--

† Age and body mass index (BMI) are shown as mean ± standard deviation

Table II
Distribution of gene frequency 'I' and 'D' alleles and DD, ID and II genotypes amongst patients with sarcoidosis and their comparison with normal and disease controls

Subjects (no.)	*ACE Genotype*				*Gene frequency*		
	DD no. (%)	*ID no.(%)*	*II no. (%)*	*p-value*	*I*	*D*	*p-value*
Sarcoidosis cases (72)	27 (37.5)	39 (54.2)	6 (8.3)	--	0.35	0.65	--
Normal controls (a = 96)	16 (16.7)	45 (46.9)	35 (36.4)	<0.001*	0.60	0.40	<0.001†
Disease controls (b = 103)	21 (20.4)	43 (41.7)	39 (37.9)	<0.001*	0.59	0.41	<0.001†
Total controls (a + b= 199)	37 (18.6)	88 (44.2)	74 (37.2)	<0.001**	0.60	0.40	<0.001†
Odds ratio[1] (95% Confidence intervals)	9.0 (3.4-23.7)	5.5 (2.2-13.6)	1.0				

* *p-value* calculated for ACE genotype distribution in patients with sarcoidosis vs. normal controls, and disease controls (PTB patients) separately using Pearson's chi-square test statistics.
** *p-value* calculated for ACE genotype frequency distribution amongst all groups taken together using Pearson's chi-square test statistics.
† *p-value* calculated for 'I' and 'D' allelic frequency by using Pearson's chi-square test for cases vs. normal controls(a), cases vs. disease controls(b) and cases vs. total controls (a + b).
[1] Odds ratio calculated by taking cases and controls with II genotype as reference.

served amongst disease controls showing ID, II and DD genotypes in 43 (41.7%), 39 (37.9%) and 21 (20.4%) subjects respectively. Statistically significant differences were observed in genotypes distribution amongst sarcoidosis vs. normal controls ($p < 0.001$) as well as sarcoidosis vs. disease controls ($p < 0.001$). Differences in the genotypes distribution between normal and disease control subjects were not statistically significant ($p = 0.70$). Amongst patients with sarcoidosis, gene frequencies of 'I' and 'D' alleles were 0.35/0.65 respectively whereas they were 0.6/0.4 in normal controls ($p < 0.001$) and 0.59/0.41 in disease controls ($p < 0.001$) respectively. For subjects with DD and ID genotypes odds ratios of developing sarcoidosis were 9.0 (95% CI: 3.4; 23.7) and 5.5 (95% CI: 2.2; 13.6) respectively, considering subjects with II genotype as reference. Odds ratio of developing sarcoidosis for males (9.2, 95% CI: 2.4; 35.6) was marginally higher than that of females (8.3, 95% CI: 2; 34) in DD genotype subjects. Whereas, it was more for females (6.6, 95% CI: 1.7; 25.2) than for males (5.1, 95% CI: 1.4; 18.1) in ID genotype subjects.

A comparison of various clinical parameters at presentation and during follow-up amongst patients with sarcoidosis having DD, ID and II genotypes is presented in *Table III*. All patients had negative tuberculin skin test. No statistically significant differences were observed in mean duration of disease onset ($p = 0.89$) as well as type of extrapulmonary organ involvement amongst patients with sarcoidosis having DD, ID or II genotypes. Only 3 male patients (4%) presented with Lofgren's syndrome, 2 with ID and 1 with DD genotype ($p = 0.81$).

Systemic corticosteroids were given to all patients with sarcoidosis except for 4 male patients who did not require systemic steroids at all and followed a benign course ($p = 0.11$). Two of them had DD and 2 had ID genotype, the genotype related difference was statistically non-significant ($p = 0.78$).

All the 6 patients with II genotype (100%) showed good clinical as well as radiological response to systemic steroids as compared to patients with ID (32 of 37, 84%) and DD (16 of 25, 64%) genotypes and the differences observed in 'good response to treatment' were statistically significant ($p = 0.013$).

Frequent relapses were seen in 17% (1 of 6) patients with II genotype whereas it was seen in only 5% (2 of 39) patients with ID and 11% (3 of 27) patients with DD genotypes. On gender based subgroup analysis, these differences were closer to significant for males ($p = 0.053$) than for females ($p = 0.076$)

Chronic course of the disease was seen in 17% (1 of 6) patients with II, 16% (6 of 39) patients with ID and 33% (9 of 27) patients with DD genotypes, however, this difference was not significant ($p =$

Table III
Comparison of clinical features at presentation and during follow-up of patients with sarcoidosis having DD, ID and II genotypes

Clinical features	*ACE Genotype*		
	DD (n = 27)	*ID (n = 39)*	*II (n = 6)*
Age*	45.4 ± 9.2	43.3 ± 9.5	48.8 ± 10.5
Sex (male/female)	13/14	20/19	3/3
Symptoms duration in wks†	24 (6-209)	24 (1-260)	20 (8-208)
Body Mass Index* (kg/m^2)	23.3 ± 3.5	23.8 ± 3.7	26.1 ± 1.6
Lofgren's Syndrome (no.)	1	2	0
Organ involvement (no. in each group)			
Joint	10	16	1
Skin	7	14	1
Eye	9	7	1
Liver	4	5	0
Parotid gland	2	4	0
Spleen	4	2	0
Central nervous system	1	1	0
Facial nerve	0	1	0
Parameters during follow-up			
Disease Course:			
Chronic	9	6	1
Non-chronic	19	32	5
PFT Response to steroids	16	24	4
Good response to steroids[1]	16	32	6
Frequent Relapses	7	4	1

Variables shown as mean ± SD* or median (range)†
[1]Statistically significant, *p=0.013*

Table IV
Comparison of radiographic and laboratory parameters at presentation of patients with sarcoidosis having DD, ID and II genotypes

Variables	*ACE Genotype*		
	DD (n = 27)	*ID (n = 39)*	*II (n = 6)*
Serum total protein* (g/dl)	7.7 ± 0.5	7.8 ± 1.0	7.8 ± 0.4
Serum albumin* (g/dl)	4.1 ± 0.8	4.1 ± 0.6	4.6 ± 0.4
Serum alkaline phosphatase (IU/L, Normal: 80-240 IU/L)*	202.6 ± 70.7	194.9 ± 60.2	247.8 ± 38.7
Serum calcium* (mg/dl)	9.4 ± 0.9	9.5 ± 0.7	10.6 ± 1.3
24-hour urinary calcium (mg/24hr)†	155 (10-920)	140 (57.5-380)	146.5 (140-160)
Serum ACE activity(U/L)[1]* p<0.001	85.1 ± 26.1	58.3 ± 24.3	56.6 ± 17.5
Disease Radiographic stage (no. for each genotype)			
0	1	0	0
I	15	17	3
II	11	21	3
III	1	0	0

Variables shown as mean ± SD* or median (range)†
[1]Except for serum ACE activity, none of the differences were statistically significant

0.27). With regard to pulmonary function parameters, pre-treatment and post-treatment values did not differ significantly amongst the patients with three genotypes ($p = 0.76$).

Table IV represents a comparison of radiographic and various laboratory parameters amongst patients with sarcoidosis having different genotypes. Of the various laboratory parameters compared, statistically significant difference was observed in the mean SACE activity amongst the three groups ($p < 0.001$). SACE activity was highest for patients having DD genotype (85.1 ± 26.1 U/L) and followed an order of DD > ID > II. No statistically significant difference was observed in radiographic staging of disease ($p = 0.62$) amongst patients with three genotypes.

Discussion

This is the first comprehensive study on the association of ACE gene I/D polymorphism with sarcoidosis in Indian population. The diagnosis of sarcoidosis is always obscure among Indian patients particularly because of the prevailing high incidence of mycobacterial diseases such as tuberculosis and leprosy in the subcontinent [27]. However, several series in the recent years have been reported on the prevalence and disease severity of sarcoidosis in India [22, 28, 29]. Therefore, we need to have better tests for diagnosis and to follow the course of this disease. SACE has been proposed to be a cost-effective and is a relatively non-invasive test [13]. We studied its genotypes and their relationship with susceptibility to development of sarcoidosis and the disease course. ACE gene polymorphism and its association with susceptibility to develop sarcoidosis have been studied in different populations with varied results [14-20]. No significant association between ACE I/D polymorphism and risk of developing sarcoidosis has been observed in Japanese [14, 15], White European [16-18] and Spanish Caucasian populations [19]. Maliarik et al have reported an increased risk of sarcoidosis in African American population with DD genotype whereas no such relationship was found in the Caucasian population [20].

In our study of Asian Indian patient population, we found a significant difference in the distribution of 'D' and 'I' alleles amongst patients with sarcoidosis as compared to control subjects. We found a selective expression of 'D' allele in our patient population. Further, individuals with 'D' al-

lele (DD and ID genotype) were at higher risk of developing sarcoidosis as compared to those without 'D' allele (II genotype). This corroborates with the previous report by Maliarik et al, who observed an association between ACE genotype and risk of sarcoidosis development in African American population.[20] In our study, we found that both male as well as female were at higher risk of developing sarcoidosis. This contrasts with the previous report by Furuya et al, who reported only females with 'D' allele to be at a higher risk [14].

SACE activity was found to differ significantly amongst patients with sarcoidosis with different I/D alleles. Mean SACE activity was highest in patients with DD genotype and followed an order of DD>ID>II, which has been reported previously in patients with sarcoidosis as well as in normal control subjects [14-16, 19, 30]. Rigat et al reported that half of the variation in SACE activity is attributed to the difference in I/D genotype amongst healthy subjects, meaning thereby that genotype specific normal reference interval values of SACE activity should be used.[11] Though we did not measure the SACE activity for control groups to determine the genotype specific normal reference interval, the SACE activity should be analyzed using I/D genotype specific reference values.

Lofgren's syndrome at presentation was observed in only a small proportion of patients with sarcoidosis (4%) and all the patients had 'D' allele. This may be attributed to lower frequency of this form of presentation in Asian Indians [1].

In our study, we found a significant association between presence of 'D' allele and poor initial response to treatment. During the follow-up, male as well female patients with 'D' allele (DD and ID genotypes) showed a lesser tendency of frequent relapses compared to those without it (II genotype). Genotype based differences across other parameters studied such as radiographic stage of disease at presentation, improvement in pulmonary function parameters following corticosteroids, disease progression (chronic vs. non-chronic) and extrapulmonary organ involvement were not significant. Similar findings have been reported Maliarik et al. in African Americans where they found an association between presence of 'D' allele and increased susceptibility to develop sarcoidosis, and skin involvement but not with other parameters. They also reported an association between presence of 'I' allele and severe disease, we did not find such an association in our study [20]. However, we found that presence of 'D' allele was associated with poor response to corticosteroid treatment.

Therefore, we conclude that in adult Asian Indian patients with sarcoidosis, gene frequencies of 'I' and 'D' alleles were 0.35 and 0.65, respectively. SACE activity differed significantly amongst patients with sarcoidosis having different genotypes (DD, ID, II) and followed an order of DD > ID > II. Presence of 'D' allele was associated with an increased risk of developing sarcoidosis. The observed association of 'D' allele with poor response to corticosteroid treatment in patients with sarcoidosis points towards an important role of ACE gene I/D polymorphism as possible predictor of disease outcome.

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