

Annexin A11 (ANXA11) gene polymorphisms are associated with sarcoidosis in Han Chinese

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Annexin A11 (ANXA11) gene polymorphisms are associated with sarcoidosis in

Han Chinese

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Key words: Sarcoidosis; Annexin A11; Single-nucleotide polymorphism

Word count: 2324

Abstract

Objectives: To further identify SNPs that contribute to the genetic susceptibility to sarcoidosis, we examined the potential association between sarcoidosis and fifteen SNPs of the ANXA11 gene.

Design: Case - control study.

Setting: A university hospital tuberculosist units in China.

Participants: Participants included 412 patients with sarcoidosis and 418 healthy controls.

Methods: The selected SNPs were genotyped in cases and controls by using the MALDI - TOF in the MassARRAY system (Sequenom Inc., San Diego, CA, USA). Probes and primers were designed using the Assay Design Software (Sequenom, San Diego, CA, USA).

Results: The results showed that statistically significant differences were observed in the allelic or genotypic frequencies of the rs2789679, rs1049550 and rs2819941 in the ANXA11 gene. The frequency of the T allele in rs2789679 ($\chi^2 = 12.849$, P = 0.0003, OR = 0.703, 95%CI = 0.579 - 0.852), T allele in rs1049550 ($\chi^2 = 24.420$, P = 0.0007, OR = 0.617, 95%CI= 0.503 - 0.758) and the rs2819941 C allele frequency ($\chi^2 =$ 11.803, P = 0.001, OR = 0.713, 95%CI = 0.588 - 0.865) in the patients with sarcoidosis was significantly lower than that in the controls. Furthermore, strong linkage disequilibrium (LD) was observed in two blocks (D' > 0.9). In block 2, the T -C haplotype occurred significantly less frequently (P = 0.001), whereas the C - C haplotypes occurred more frequently (P = 0.0001). Significantly more G - G - C

haplotypes (block 4) (P = 0.027) were found in the patients with sarcoidosis, but did not pass the threshold value (P = 0.025).

Conclusions: These findings point to a role for ANXA11 gene polymorphisms in sarcoidosis in the Han Chinese, and may be informative for future genetic or biological studies on sarcoidosis.

Article summary

Article focus

1. The first genome - wide association study (GWAS) in sarcoidosis conducted in a German population has revealed an association: the rs1049550 (C > T, Arg230Cys) located in exon 10 was associated with sarcoidosis.

2. More studies should be performed to demonstrate the following item: whether these SNPs modulate the risk of sarcoidosis by themselves or whether they correlate with other causative SNPs and are repeated in other populations.

3. We hypothesize that common variants in the ANXA11 gene may significantly contribute to the predisposition to develop sarcoidosis in Han Chinese.

Key messages

1. ANXA11 rs2789679 (3'UTR) was found to be associated with sarcoidosis.

2. ANXA11 rs1049550 (exon 10) was significantly associated with sarcoidosis.

3. Another significant association was observed for rs2819941 (intron 14).

Strengths and limitations of this study

1. Functional SNPs in the promoter region, 5'- and 3'-UTR, exons of ANXA11 gene were systematically screened and homogeneity of the study subjects, representing the Han Chinese, is the main strength of the current study.

2. Functional characterisation is the potential limitation of the study that could have further helped in proving the positive association observed for rs2789679 and rs1049550. The lack of correlation of serum ANXA11 levels and sample size are other limitations.

INTRODUCTION

Sarcoidosis is a systemic autoimmune disease characterized by destructive, noncaseating epithelioid granulomatous lesions, with accumulated phagocytes and activated CD4⁺ T helper type 1 lymphocytes ^{1 2}. The typical manifestations of sarcoidosis include bilateral hilar lymphadenopathy, pulmonary infiltration and ocular and skin lesions. The clinical course and prognosis of sarcoidosis is variable. The acute forms of sarcoidosis such as L \Box fgren's syndrome (LS) can resolve spontaneously in most cases. However, chronic pulmonary sarcoidosis may progress to lung fibrosis and eventually result in respiratory failure. Recent studies demonstrated that sarcoidosis occurs in a pattern of family clustering, and its racial incidence is also different, suggesting that some genetic factors may contribute to the risk and severity of sacroidosis ¹³.

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Annexin gene family is involved in the etiology of several autoimmune and chronic diseases ^{4 5}. One member of Annexin gene family, ANXA11, located on chromosome 10q22.3, is involved in calcium signaling, apoptosis, vesicle trafficking, cell growth and terminal phase of cell division ⁵⁻⁷. In this context, the development and maintenance of granulomatous inflammation in sarcoidosis have been repeatedly associated with the impaired apoptosis of activated inflammatory cells ^{8 9}. In most patients with sarcoidosis, the early stages of this disease are characterized by mononuclear cell alveolitis dominated by activated CD4⁺ T cells and macrophages. Between these cells, the uncoordinated interplay results in the formation of typical noncaseating granulomas. The mechanism by which the granulomas resolve has not

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been fully elucidated. However, it is generally assumed that the induction of apoptosis and / or by the withdrawal of inflammatory cytokines participates in the disappearance of granulomas ^{10 11}. In patients with sarcoidosis, their peripheral blood monocytes were characterized by apoptosis ⁹.

In previous studies, case - control / family 'hypothesis - driven' studies and low density linkage scans were used to identify genetic factors conferring susceptibility to sarcoidosis ¹². Notably, the first genome - wide association study (GWAS) in sarcoidosis conducted in a German population has recently revealed an association: the leading rs2789679 SNP located in the 3' - untranslated region (3'UTR) and a common nonsynonymous SNP (rs1049550, C > T, Arg230Cys) were associated with increased risk of sarcoidosis ⁴. This association has recently been supported by another report from the same population ¹³. Thus, more studies should be performed to demonstrate the following item: whether these SNPs modulate the risk of sarcoidosis by themselves or whether they correlate with other causative SNPs and are repeated in other populations. The published sarcoidosis - association studies for ANXA11 summarized in Table 1. We hypothesize that common variants in the ANXA11 gene may significantly contribute to the predisposition to develop sarcoidosis.

In this study, we investigated fifteen loci in a Chinese population from He'nan province (China) to verify the putative association between ANXA11 polymorphisms and sarcoidosis.

SUBJECTS AND METHODS

Subjects

Four - hundred and twelve patients with sarcoidosis (mean \pm SD age; 53.6 \pm 4.6 years) were recruited from our hospital between May 2005 and March 2013. Sarcoidosis was diagnosed by the evidence of non - caseating epitheloid cell granuloma in biopsy specimens and chest radiographic abnormalities. Chronic sarcoidosis was defined as a disease over at least 2 years or at least two episodes in a lifetime. Acute sarcoidosis was defined as one episode of acute sarcoidosis which had totally resolved at the date of the examination. The control group consisted of 418 unrelated healthy subjects (mean \pm SD age; 54.2 \pm 5.3 years) who underwent health examinations in the Medical Examination Center of the First Affiliated Hospital of the Xin'xiang Medical College (Xinxiang, China). None of the individuals in the control group had a history of lung diseases or showed any symptoms of the lung or other diseases by chest radiography or laboratory blood tests. All participants were from a non - genetically related Chinese Han population in He'nan Province (China). The study was performed according to the Guidelines of the Medical Ethical Committee of Xin'xiang Medical College (Xinxiang, China). Written informed consent was obtained from each participant in this study.

SNP selection

Tagging SNPs were selected from the catalogs of the International HapMap Project and the 1000 Genomes Project. The rs2789679 and rs2245168 are located in 5' - UTR, rs2236558 in intron 6, rs7067644 and rs12763624 in intron 8, rs1049550 in exon10, rs2573351 in intron 13, rs10887581, rs11201989, rs2573353, rs2789695,

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rs2573356, rs2819945, rs11202059 and rs2819941 in intron 14. Marker selection was done according to previous studies ⁴ ¹²⁻¹⁴, and preliminary analysis was performed using the HapMap data (Fig 1). We examined tagSNPs in the Haploview software v4.2, using the Chinese Han in Beijing (CHB) population and a minor allele frequency cut-off (MAF) \geq 5% (the HapMap Data Release 27). The linkage disequilibrium (LD) pattern of this gene was determined in the Chinese population using the preliminary data from the HapMap. These SNPs were further analyzed in an association study.

Genotyping

Peripheral blood was collected from a vein into a sterile tube coated with ethylenediamine tetraacetic acid (EDTA). Plasma samples were stored at -20°C. Genomic DNA was extracted from the frozen peripheral blood samples using a QIAmp Blood Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocols. The selected SNPs were genotyped in cases and controls by using the MALDI - TOF in the MassARRAY system (Sequenom Inc., San Diego, CA, USA). Probes and primers were designed using the Assay Design Software (Sequenom, San Diego, CA, USA). The completed genotyping reactions were spotted onto a 384 well spectroCHIP (Sequenom) using the MassARRAY Nanodispenser (Sequenom) and determined by the matrix - assisted laser desorption ionization time of - flight mass spectrometer. Genotype calling was performed in realtime with the MassARRAY RT software version 3.0.0.4 and analyzed using the MassARRAY Typer software version 3.4 (Sequenom).

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Statistical analysis

All statistical analyses were performed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Allele and genotype frequencies for each individual polymorphism and Hardy - Weinberg equilibrium were evaluated by Chi - square tests. Differences between the cases and controls in the frequency of the alleles, genotypes and haplotypes were evaluated by the Fisher's exact test or the Pearson Chi - square test. Unconditional logistic regression was used to calculate the odds ratio (OR) and 95% confidence interval (CI) in independent association between each locus and the presence of sarcoidosis. The Bonferroni correction was used to adjust the test level when multiple comparisons were conducted, and the *P* value was divided by the total number of loci or haplotypes. Haplotype blocks were defined according to the criteria developed by Gabriel et al. ¹⁴, as implemented in the Haploview 4.0, to examine if some SNPs significant in the single - marker association analysis also exist in the haplotype blocks. Pair - wise LD statistics (D' and r^2) and haplotype frequency were calculated, and haplotype blocks were constructed using the Haploview 4.0 ¹⁵.

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RESULTS

The genotype distribution of the fifteen polymorphisms was consistent with the Hardy - Weinberg equilibrium (P > 0.05). The analysis of strong LD in the patients with sarcoidosis and the healthy controls revealed that 2 SNPs (rs2245168, rs2236558), 2 SNPs (rs1049550, rs2573351), 2 SNPs (rs2789695, rs2573356) and 3 SNPs (rs2819945, rs11202059, rs2819941) were located in haplotype block 1, block 2, block 3 and block 4 (D' > 0.9, Fig. 2). The genotype distribution, allelic frequencies,

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and haplotypes in the patients with sarcoidosis and the healthy controls are showed in Tables 2 and Table 3.

Comparison of genotype and allele frequency distribution revealed significant differences between the patients with sarcoidosis and healthy controls for 3 SNPs: rs2789679, rs1049550 and rs2819941. The frequency of the T allele in rs2789679 (χ^2 = 12.849, *P* = 0.0003, OR = 0.703, 95% CI = 0.579 - 0.852), T allele inrs1049550 (χ^2 = 24.420, *P* = 0.0007, OR = 0.617, 95% CI = 0.503 - 0.758) and the rs2819941 C allele frequency (χ^2 = 11.803, *P* = 0.001, OR = 0.713, 95% CI = 0.588 - 0.865) in the patients with sarcoidosis was significantly lower than that in the controls, and these differences remained statistically significant after Bonferroni corrections.

Due to the positive association between 2 SNPs (rs1049550 and rs2819941), and sarcoidosis, we performed an association analysis to determine whether the haplotype was associated with the risk of sarcoidosis. Compared with the healthy controls, the T - C haplotype occurred significantly less frequently (P = 0.001) and the C - C haplotypes occurred more frequently (P = 0.0001) in block 2 in the patients with sarcoidosis. The significantly more G - G -C haplotypes (block 4) (P = 0.027) were found in the patients with sarcoidosis, but did not pass the threshold value (P = 0.025).

DISCUSSION

A key step in linkage and association studies is to identify common risk variants in different populations. To determine if common risk variants exist in distinct populations, fifteen SNPs which span approximately 0.53 Mb of the ANXA11 gene,

were genotyped in samples from the patients with sarcoidosis and healthy controls in a Chinese Han population. Recently, new sarcoidosis loci have been identified by genome - wide association studies ^{4 13}. With the fast development of genome-wide association studies, increasing number of susceptibility loci for sarcoidosis have been reported in various populations ^{4 13-14}. However, these observations should be confirmed in other genetically independent populations. In this study, we conducted the first large genetic association study of the ANXA11 gene in a Chinese Han population. The evidence of markers associated with sarcoidosis was presented, and these markers were mapped to different locations in the ANXA11 gene (81897864 -81951001). The association signals in the region were identified, and some significantly associated haplotypes also appeared this region.

Hoffman et al. reported an association between the T allele of the non synonymous SNP rs1049550 and significantly decreased risk of sarcoidosis in a German population ⁴. Similar results were also obtained in other two European populations ^{13 14}. As a part of the sarcoidosis GWAS done in Americans ¹⁶, we further confirmed this associationin a Chinese Han population. In this study, the frequency of ANXA11 rs1049550 T allele was significantly lower in the patients with sarcoidosis than that in the healthy controls. The "protective" effect of ANXA11 T allele increased with the number of its copies in the genotype, which is consistent with the result obtained in the patients with sarcoidosis in a German population ⁴, Furthermore, the T allele carriers among the patients were protected from infiltration of lung parenchyma (radiographic stages II-IV) ¹⁴. We have also demonstrated that rs1049550 BMJ Open: first published as 10.1136/bmjopen-2013-004466 on 23 July 2014. Downloaded from http://bmjopen.bmj.com/ on June 14, 2025 at Agence Bibliographique de Enseignement Superieur (ABES) . Protected by copyright, including for uses related to text and data mining, Al training, and similar technologies.

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is significant cross-ethnically at the gene level after adjustment for the single SNP association tests performed. The mechanism by which rs1049550 SNP affects the susceptibility to sarcoidosis may be that the SNP may affect the function of the ANXA11 protein rather than affect its expression. In humans, the ANXA11 protein consists of an N - terminalproline - tyrosine - glycine (PYG) - rich region, followed by four annexincore domains. The SNP rs1049550 leads to an amino - acid exchange (basic arginine to a polar cysteine) at the evolutionarily conserved position 230 (R230C) in the first annexin domain, which is responsible for Ca²⁺ - dependent trafficking of the protein inthe cell ¹⁷.

In the GWAS conducted by Hofmann et al 4, the strongest association signal was observed for SNP rs2789679. This marker is located in the 3' - UTR of the ANXA11 gene. The T allele had a frequency of 35% among the patients with sarcoidosis and 44% in controls, with 13% of the patients with sarcoidosis and 19% of controls being TT homozygous. Fine - mapping in the Black Women's Health Study (BWHS) revealed rs2819941 to be the top SNP, associated with a nonsignificant (P = 0.06) reduction in sarcoidosis risk (17% reduction per copy of the C - allele). In this case - control association study, the frequency of the T allele in rs2789679 and the rs2819941 C allele frequency in the patients with sarcoidosis was significantly lower than that in the controls. Several lines of evidence suggest that the observed associations are unlikely to be anartifact. First, both the single - SNP and the haplotype - based association analyses support the current finding. Second, population stratification is an impossible reason, because all of our samples were from the same geographical

region. Finally, consistent results were obtained from two genetically independent populations (Han Chinese and Europeans). Collectively, our results confirmed the strong association between variations in the ANXA11 gene and sarcoidosis, and suggest that ANXA11 represents a strong genetic risk factor for sarcoidosis.

We further investigated the interaction among polymorphisms and observed strong LD. The haplotype analysis revealed that significantly more C - C (block 2), and G - G - C (block 4) haplotypes and significantly fewer T - C haplotypes (block 2) were found in the patients with sarcoidosis. There were significant point - wise associations of these variants with sarcoidosis (rs2789679, rs1049550 and rs2819941). These results indicated that the patients with C - C (block 2), and G - G - C (block 4) haplotypes of the ANXA11 gene were more prone to sarcoidosis. Significantly higher frequencies of T - C haplotypes were detected in the healthy controls than in the patients with sarcoidosis, suggesting that they may show a protective effect against sarcoidosis. To some extent, this finding further supports a role of ANXA11 polymorphisms in sarcoidosis, while ethnic group difference may exist. BMJ Open: first published as 10.1136/bmjopen-2013-004466 on 23 July 2014. Downloaded from http://bmjopen.bmj.com/ on June 14, 2025 at Agence Bibliographique de Enseignement Superieur (ABES)

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In conclusion, our study suggests a potential role of the ANXA11 SNPs and their related haplotypes in the genetic susceptibility to sarcoidosis. Further studies are needed to investigate how these SNPs affect the function of ANXA11.

Contributors

LG Zhang was involved in conception and design of the study, acquisition of patient data, genotyping and drafted the paper. ZJ Xiao and GC Shi were involved in design of the study, genotyping and interpretation of results. J Huang and CX Zhang were involved in design of the study, acquisition of patient data and interpretation of result. All authors revised the draft paper.

Funding

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Competing interests

None.

Patient consent

Obtained.

Ethics approval

Ethics approval was provided by the First Hospital Affiliated to the Xinxiang

Medical College.

Provenance and peer review

Not commissioned; externally peer reviewed.

Data sharing statement

No additional data are available.

Figure Legend

Fig. 1 The structure of the human ANXA11 gene and ten SNPs located on the gene.

Fig. 2 The LD plot of the fifteen SNPs in the ANXA11 gene. Values in squares are the pair - wise calculation of r^2 (left) or D' (right). Black squares indicate $r^2 = 1$ (i.e. perfect LD between a pair of SNPs). Empty squares indicate D' = 1 (i.e. complete LD

between a pair of SNPs).

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Study	Popula	tion	Type of Study	Sample S	ize (n)	Number of	Positive SNPs
Study	Ethnic group	Country	Type of Study	Sarcoidosis	Control	SNPs typed	Fositive SINFS
Hofmann S, 2008	Caucasian	Germany	Case-control	499	490	GWAS	rs2789679, rs7091565, rs1049550
Mrazek F, 2011	Caucasian	Czech	Case-control	245	254	1	rs1049550
Cozier YC, 2012	Caucasian	America	Case-control	486	943	10p12-10q22	rs2789679, rs2819941
Li Y, 2013	Caucasian	Germany	Case-control	325	364	1	rs1049550
Levin AM, 2013	Caucasian	America	Case-control	1689	1252	25	rs1049550

Table 1 Summary of all published sarcoidosis-association studies for ANXA11

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Variable	Location	MAF	Group	(Genotype (n, %)	Allele	P ^a	P ^b	OR, 95%CI ^c	
				AA	AT	TT	А	Т			
rs2789679	3'UTR	0.388	Controls	79 (18.900)	225 (53.828)	114 (27.273)	383 (45.813)	453 (54.187)	0.0007	0.0003	1.423, 1.173-1.726
			Cases	132 (32.039)	186 (45.146)	94 (22.816)	450 (54.612)	374 (45.388)			
				CC	СТ	TT	С	Т			
rs2245168	3'UTR	0.388	Controls	156 (37.321)	200 (47.847)	62 (14.833)	512 (61.244)	324 (38.756)	0.920	0.698	1.040, 0.854-1.266
			Cases	150 (36.408)	197 (47.816)	65 (15.777)	497 (60.316)	327 (39.684)			
				TT	TG	GG	Т	G			
rs2236558	intron 6	0.488	Controls	110 (26.316)	190 (45.455)	118 (28.230)	410 (49.043)	426 (50.957)	0.172	0.917	1.010, 0.833-1.025
			Cases	94 (22.816)	214 (51.942)	104 (25.243)	402 (48.786)	422 (51.214)			
				GG	GA	AA	G	А			
rs7067644	intron 8	0.390	Controls	60 (14.354)	206 (49.282)	152 (36.364)	326 (38.995)	510 (61.005)	0.173	0.109	1.177, 0.964-1.437
			Cases	54 (13.107)	182 (44.175)	176 (42.718)	290 (35.194)	534 (64.806)			
				CC	СТ	TT	С	C			
rs12763624	intron 8	0.440	Controls	76 (18.182)	216 (51.675)	126 (30.144)	368 (44.019)	468 (55.981)	0.118	0.216	1.131, 0.931-1.374
			Cases	76 (18.447)	186 (45.146)	150 (36.408)	338 (69.547)	486 (58.981)			
				CC	CT	TT	С	Т			
rs1049550	exon 10	0.404	Controls	154 (36.842)	190 (45.455)	74 (17.703)	498 (59.569)	338 (40.431)	0.0002	0.0007	0.598, 0.488-0.734
			Cases	208 (50.485)	170 (41.262)	34 (8.252)	586 (71.117)	238 (28.883)			
				CC	СТ	TT	С	Т			
rs2573351	intron 13	0.471	Controls	96 (22.967)	202 (48.325)	120 (28.708)	394 (47.129)	442 (52.871)	0.437	0.564	0.945, 0.779-1.146

Table 2 Genotype and allele frequencies of the ANXA11 gene polymorphisms in Controls (n=418) and sarcoidosis (n=412) and their associated

			Cases	92 (22.330)	216 (52.427)	104 (25.243)	400 (48.544)	424 (51.456)			
				TT	TC	CC	Т	С			
rs10887581	intron 14	0.500	Controls	114 (27.273)	190 (45.455)	114 (27.273)	418 (50.000)	418 (50.000)	0.290	0.553	1.060, 0.874-1.28
			Cases	96 (23.301)	208 (50.485)	108 (26.214)	400 (48.544)	424 (51.456)			
				GG	GC	CC	G	С			
rs11201989	intron 14	0.425	Controls	130 (31.100)	218 (52.153)	70 (16.746)	478 (57.177)	358 (42.823)	0.144	0.293	1.110, 0.914-1.34
			Cases	128 (31.068)	194 (47.087)	90 (21.845)	450 (54.612)	374 (45.388)			
				CC	CA	AA	С	А			
rs2573353	intron 14	0.340	Controls	178 (42.584)	196 (46.890)	44 (10.526)	552 (66.029)	284 (33.971)	0.995	0.863	1.000, 0.817-1.2
			Cases	176 (42.718)	192 (46.602)	44 (10.680)	544 (66.019)	280 (33.981)			
				CC	СТ	TT	С	Т			
rs2789695	intron 14	0.349	Controls	52 (12.440)	188 (44.976)	178 (42.584)	292 (34.928)	544 (65.072)	0.918	0.685	1.043, 0.852-1.277
			Cases	48 (11.650)	184 (44.660)	180 (43.689)	280 (33.981)	544 (66.019)			
				CC	СТ	TT	С	Т			
rs2573356	intron 14	0.490	Controls	118 (28.230)	190 (45.455)	110 (26.316)	426 (50.957)	410 (49.043)	0.892	0.926	1.009, 0.833-1.2
			Cases	112 (27.184)	194 (47.087)	106 (25.728)	418 (50.728)	406 (49.272)			
				GG	GA	AA	G	А			
rs2819945	intron 14	0.452	Controls	119 (28.469)	221 (52.871)	78 (18.660)	459 (54.904)	377 (45.096)	0.101	0.827	1.022, 0.842-1.2
			Cases	130 (31.553)	188 (45.631)	94 (22.816)	448 (54.369)	376 (45.631)			
				GG	GA	AA	G	А			
rs11202059	intron 14	0.476	Controls	106 (25.359)	226 (54.067)	86 (20.574)	438 (52.392)	398 (47.608)	0.064	0.479	0.933, 0.769-1.1
			Cases	128 (31.068)	190 (46.117)	94 (22.816)	446 (54.126)	378 (45.874)			
				CC	СТ	TT	С	Т			
rs2819941	intron 14	0.457	Controls	114 (27.273)	226 (54.067)	78 (18.660)	454 (54.306)	382 (45.694)	0.0002	0.001	1.402, 1.156-1.7
			Cases	94 (22.816)	190 (46.117)	128 (31.068)	378 (45.874)	446 (54.126)			

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p values for allele frequency distribution. '\ .on and statistically significant results (*P* < 0.003). Weinberg equilibrium (p > 0.05). ^a p values for genotype frequency distribution. ^b p values for allele frequency distribution. ^cOR and 95%CI values for allele frequency distribution.

* *P* value is adjusted by Bonferroni correction and statistically significant results (P < 0.003).

All SNPs were found to be in Hardy-Weinberg equilibrium (p > 0.05).

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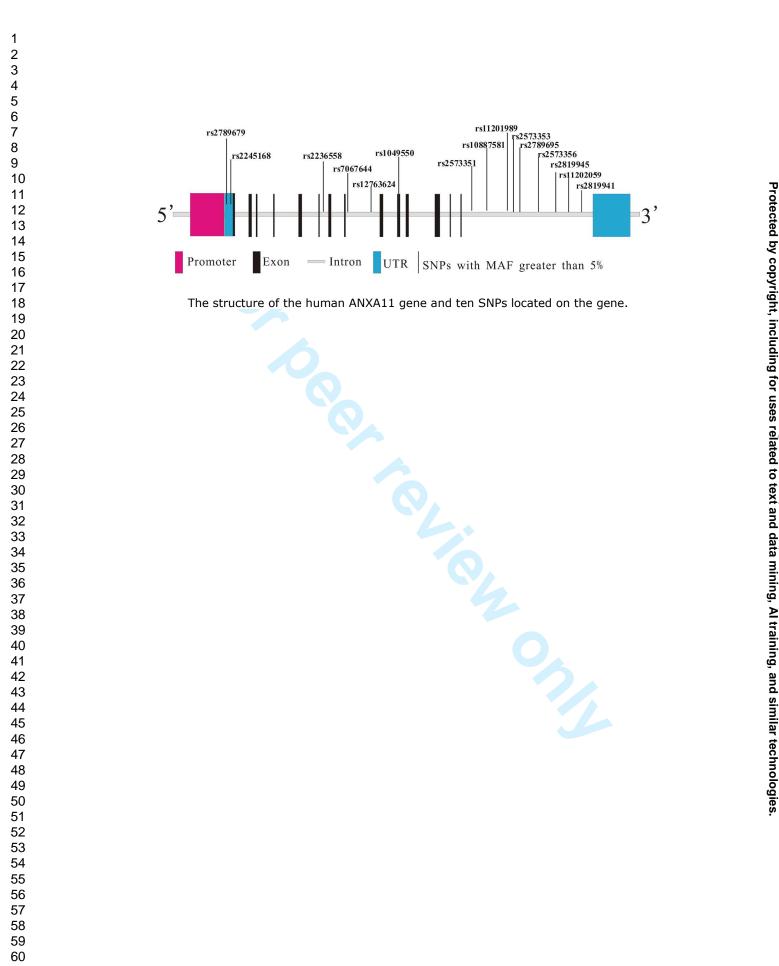
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Dlask	Hanlatura	$C_{agag}(\mathbf{r}, 0/\mathbf{)})$	Contucto(n 0/)			Statistics	
Block	Haplotype	Cases(n, %)	Controls(n, %)	χ^2	Р	OR	95%CI
	C - T	201 (48.786)	212 (50.718)	0.310	0,578	0.926	0.705~1.215
1	T - G	163 (39.563)	160 (38.278)	0.144	0.104	1.056	0.199~1.395
	C - G	47 (11.408)	48 (11.483)	0.001	0.973	0.993	0.647~1.522
	C - T	210 (50.971)	218 (52.153)	0.116	0.733	0.954	0.726~1.252
2	T - C	117 (27.725)	166 (39.713)	11.822	0.001	0.602	$0.450 {\sim} 0.805$
	C - C	82 (19.903)	31 (7.416)	27.507	0.0001	3.102	2.001~4.810
	C - T	208 (50.485)	213 (50.957)	0.018	0.892	0.981	$0.747 {\sim} 1.288$
3	T - C	139 (33.738)	146 (34.928)	0.130	0.718	0.949	0.712~1.263
	C - C	63 (15,291)	59 (14.115)	0.229	0.632	1.098	0.748~1.613
4	G - G -C	219 (53.155)	190 (45.455)	4.923	0.027	1.362	1.036~1.789
4	G - A - C	185 (44.903)	188 (44.876)	0.0001	0.997	0.758	0.712~1.311

Table 3 ANXA11 haplotype in block 1-4 frequencies and the results of their associations with risk of sarcoidosis

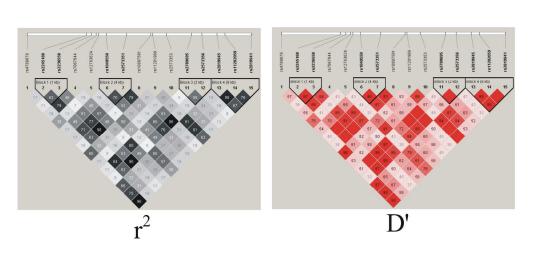
* *P* value is adjusted by Bonferroni correction and statistically significant results (P < 0.025 in block 1-3; P < 0.0167 in block 4). 011

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The LD plot of the fifteen SNPs in the ANXA11 gene. Values in squares are the pair - wise calculation of r2 (left) or D' (right). Black squares indicate r2 = 1 (i.e. perfect LD between a pair of SNPs). Empty squares indicate D' = 1 (i.e. complete LD between a pair of SNPs).

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Annexin A11 (ANXA11) gene polymorphisms are associated with sarcoidosis in a Han Chinese population - a casecontrol study

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Annexin A11 (ANXA11) gene polymorphisms are associated with sarcoidosis in a Han
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Abstract

Objectives: To further identify the single - nucleotide polymorphisms (SNPs) that contribute to the genetic susceptibility to sarcoidosis, we examined the potential association between sarcoidosis and fifteen SNPs of the ANXA11 gene.

Design: A case - control study.

Setting: A tuberculosist unit in a university hospital in China.

Participants: Participants included 412 patients with sarcoidosis and 418 healthy controls.

Methods: The selected SNPs were genotyped in cases and controls by using the MALDI - TOF in the MassARRAY system (Sequenom Inc., San Diego, CA, USA). Probes and primers were designed using the Assay Design Software (Sequenom, San Diego, CA, USA).

Results: The results showed that statistically significant differences were observed in the allelic or genotypic frequencies of the rs2789679, rs1049550 and rs2819941 in the ANXA11 gene. The frequency of the T allele in rs2789679 (P = 0.0003, OR = 0.70, 95%CI = 0.58 - 0.85), T allele in rs1049550 (P = 0.0007, OR = 0.62, 95%CI= 0.50 -0.76) and the rs2819941 C allele frequency (P = 0.001, OR = 0.71, 95%CI = 0.59 -0.87) in the patients with sarcoidosis was significantly lower than that in the controls. Comparison of genotype frequency distribution revealed significant differences between chest radiographic (CXR) stage I and stages II - IV for rs1049550. The significantly more CC genotype (P = 0.012) were found in the patients with stages I sarcoidosis. Furthermore, strong linkage disequilibrium (LD) was observed in four

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blocks (D' > 0.9). In block 2 (rs1049550-rs2573351), the T - C haplotype occurred significantly less frequently (P = 0.001), whereas the C - C haplotypes occurred more frequently (P = 0.0001).

Conclusions: These findings point to a role for ANXA11 gene polymorphisms in sarcoidosis in a Han Chinese population, and may be informative for future genetic or biological studies on sarcoidosis.

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Article summary

Article focus

1. The first genome - wide association study (GWAS) in sarcoidosis conducted in a German population has revealed that the rs1049550 (C > T, Arg230Cys) located in exon 10 were associated with sarcoidosis.

2. More studies should be performed to demonstrate the following item: whether these SNPs modulate the risk of sarcoidosis by themselves or whether they correlate with other causative SNPs and are repeated in other populations.

3. We hypothesize that common variants in the ANXA11 gene may significantly contribute to the predisposition to develop sarcoidosis in a Chinese Han population.

Key messages

1. ANXA11 rs2789679 (3'UTR) was found to be associated with sarcoidosis.

2. ANXA11 rs1049550 (exon 10) was significantly associated with sarcoidosis.

3. Another significant association was observed for rs2819941 (intron 14).

Strengths and limitations of this study

1. Functional SNPs in the promoter region, 5'- and 3'-UTR, exons of ANXA11 gene were systematically screened and homogeneity of the study subjects, representing the Han Chinese, is the main strength of the current study.

2. Functional characterization is the potential limitation of the study that could have further helped in proving the positive association observed for rs2789679 and rs1049550. The lack of correlation of serum ANXA11 levels are needed to investigate.

INTRODUCTION

Sarcoidosis is a systemic autoimmune disease characterized by destructive, noncaseating epithelioid granulomatous lesions, with accumulated phagocytes and activated CD4⁺ T helper type 1 lymphocytes ^{1 2}. The typical manifestations of sarcoidosis include bilateral hilar lymphadenopathy, pulmonary infiltration and ocular and skin lesions. The clinical course and prognosis of sarcoidosis are variable. The acute forms of sarcoidosis such as L \Box fgren's syndrome (LS) can resolve spontaneously in most cases. However, chronic pulmonary sarcoidosis may progress to lung fibrosis and eventually result in respiratory failure. Recent studies have demonstrated that sarcoidosis can occur in a pattern of family clustering, and its racial incidence is also different, suggesting that some genetic factors may contribute to the risk and severity of sacroidosis ¹³. BMJ Open: first published as 10.1136/bmjopen-2013-004466 on 23 July 2014. Downloaded from http://bmjopen.bmj.com/ on June 14, 2025 at Agence Bibliographique de Enseignement Superieur (ABES)

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The Annexin gene family is involved in the etiology of several autoimmune and

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chronic diseases ^{4.5.} One member of the Annexin gene family, ANXA11, located on chromosome 10q22.3, is involved in calcium signaling, apoptosis, vesicle trafficking, cell growth and terminal phase of cell division ⁵⁻⁷. In this context, the development and maintenance of the granulomatous inflammation in sarcoidosis have been repeatedly associated with the impaired apoptosis of activated inflammatory cells ^{8.9}. In most patients with sarcoidosis, the early stages of this disease are characterized by mononuclear cell alveolitis dominated by activated CD4⁺ T cells and macrophages. Between these cells, the uncoordinated interplay results in the formation of typical non - caseating granulomas. The mechanism by which the granulomas resolve has not been fully elucidated. However, it is generally assumed that the induction of apoptosis and / or by the withdrawal of inflammatory cytokines participates in the disappearance of granulomas ^{10 11}. In the patients with sarcoidosis, their peripheral blood monocytes were characterized by apoptosis ⁹.

In previous studies, case - control / family 'hypothesis - driven' studies and low density linkage scans were used to identify genetic factors conferring the genetic susceptibility to sarcoidosis ¹². Notably, the first genome - wide association study (GWAS) in sarcoidosis conducted in a German population has recently revealed an association: the leading rs2789679 SNP (single - nucleotide polymorphism) located in the 3' - untranslated region (3' - UTR) and a common nonsynonymous SNP (rs1049550, C > T, Arg230Cys) were associated with increased risk of sarcoidosis ^{4 13}. This association has recently been supported by another report from the same population ¹⁴. Thus, more studies should be performed to demonstrate the following

item: whether these SNPs modulate the risk of sarcoidosis by themselves or whether they correlate with other causative SNPs and are repeated in other populations. The published sarcoidosis - association studies for ANXA11 are summarized in Table 1. We hypothesize that common variants in the ANXA11 gene may significantly contribute to the predisposition to develop sarcoidosis.

In this study, we investigated fifteen loci in a Chinese population from He'nan province (China) to verify the putative association between ANXA11 polymorphisms and sarcoidosis.

SUBJECTS AND METHODS

Subjects

Four hundred and twelve patients with sarcoidosis (mean \pm SD age: 53.6 \pm 4.6 years; 155 men and 257 women) were recruited from our hospital between May 2005 and March 2013. Sarcoidosis was diagnosed by the evidence of non - caseating epitheloid cell granuloma in biopsy specimens and chest radiographic abnormalities. Chronic sarcoidosis was defined as a disease over at least 2 years or at least two episodes in a lifetime. A cute sarcoidosis was defined as one episode of acute sarcoidosis which had totally resolved at the date of the examination. The control group consisted of 418 unrelated healthy subjects (mean \pm SD age: 54.2 \pm 5.3 years; 160 men and 258 women) who underwent health examinations in the Medical Examination Center of the First Affiliated Hospital of the Xinxiang Medical College (Xinxiang, China) between Oct 2009 and Sept 2013. None of the individuals in the control group had a history of lung diseases or showed any symptoms of the lung or

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other diseases by chest radiography or laboratory blood tests. Participants were excluded if they: were taking other prescribed medications that could affect the central nervous system; had a history of seizures, hematological diseases, or severe liver or kidney impairment; or were pregnant. No familial relationship was known between the study participants. The study was performed according to the Guidelines of the Medical Ethical Committee of Xinxiang Medical College (Xinxiang, China). Written informed consent was obtained from each participant in this study.

SNP selection

Tagging SNPs were selected from the catalogs of the International HapMap Project. The rs2789679 and rs2245168 are located in 5'-UTR, rs2236558 in intron 6, rs7067644 and rs12763624 in intron 8, rs1049550 in exon 10, rs2573351 in intron 13, rs10887581, rs11201989, rs2573353, rs2789695, rs2573356, rs2819945, rs11202059 and rs2819941 in intron 14 (Figure 1). Marker selection was done according to previous studies ^{4 12 14 15}, and preliminary analysis was performed using the HapMap data and the following criteria. First, we examined tagSNPs in the Haploview (v4.2), using the CHB population and a minor allele frequency cut - off (MAF) \geq 5% (HapMap Data Release 27). We found that there were a total of 29 potential tagSNPs in all. As a first screen of the most common SNPs in the sarcoidosis sample from an Eastern Chinese Han population, a MAF \geq 20% with pair - wise tagging and r² \geq 0.8 ¹⁶ was used as the cut - off when choosing tagSNPs. Second, the LD pattern of this gene was determined in the Chinese population using the preliminary data from the HapMap. Five LD blocks across the ANXA11 were defined using Haploview's

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'confidence intervals' method ^{17 18}. These SNPs were further analyzed in an association study.

Genotyping

Peripheral blood was collected from a vein into a sterile tube coated with ethylenediamine tetraacetic acid (EDTA). Plasma samples were stored at -20°C. Genomic DNA was extracted from the frozen peripheral blood samples using a QIAmp Blood Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocols. The selected SNPs were genotyped in cases and controls by using the MALDI - TOF in the MassARRAY system (Sequenom Inc., San Diego, CA, USA). Probes and primers were designed using the Assay Design Software (Sequenom, San Diego, CA, USA). The completed genotyping reactions were spotted onto a 384 well spectroCHIP (Sequenom) using the MassARRAY Nanodispenser (Sequenom) and determined by the matrix - assisted laser desorption ionization time of - flight mass spectrometer. Genotype calling was performed in realtime with the MassARRAY RT software version 3.0.0.4 and analyzed using the MassARRAY Typer software version 3.4 (Sequenom).

Statistical analysis

All data were analyzed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Hardy-Weinberg equilibrium were evaluated by Chi - square tests. Differences between the cases and controls in the frequency of the alleles, genotypes and haplotypes were evaluated by the Fisher's exact test or the Pearson Chi - square test. Unconditional logistic regression was used to calculate the odds ratio (OR) and 95%

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confidence interval (CI) in independent association between each locus and the presence of sarcoidosis. Generalized linear regression was used to evaluate the interaction effects between gene and gender or age. Gender and age of subjects were treated as covariants in binary logistic regression. P - value was calculated based on codominant, dominant for the rare allele, heterosis and recessive for the rare allele models of inheritance. The Bonferroni correction was used to adjust the test level when multiple comparisons were conducted, and the P value was divided by the total number of loci. Haplotype blocks were defined according to the criteria developed by Gabriel et al. ¹⁸. Pair - wise LD statistics (D' and r^2) and haplotype frequency were calculated, and haplotype blocks were constructed using the Haploview 4.0¹⁷. To ensure that the LD blocks most closely reflect the population level LD patterns, definition of the blocks were based on the control samples alone. The haplotype frequencies were estimated using GENECOUNTING, which computes maximum likelihood estimates of haplotype frequencies from unknown phase data by utilizing an expectation - maximization algorithm ¹⁹⁻²². The significance of any haplotypic association was evaluated using a likelihood ratio test, followed by permutation testing that compared estimated haplotype frequencies in cases and controls ¹⁹²¹.

RESULTS

The genotype distribution of the fifteen polymorphisms was consistent with the Hardy - Weinberg equilibrium (P > 0.05). The analysis of strong LD in the patients with sarcoidosis and healthy controls revealed that 2 SNPs (rs2245168, rs2236558), 2 SNPs (rs1049550, rs2573351), 2 SNPs (rs2789695, rs2573356) and 3 SNPs

(rs2819945, rs11202059, rs2819941) were located in haplotype block 1, block 2, block 3 and block 4 (D' > 0.9, Fig. 2). The genotype distribution, allelic frequencies, and haplotypes in the patients with sarcoidosis and healthy controls are showed in tables 2, table 4 and table 5.

Comparison of genotype and allele frequency distribution revealed significant differences between the patients with sarcoidosis and healthy controls for 3 SNPs: rs2789679, rs1049550 and rs2819941. The frequency of the T allele in rs2789679 (P = 0.0003, OR = 0.70, 95%CI = 0.58 - 0.85), T allele in rs1049550 (P = 0.0007, OR = 0.62, 95%CI = 0.50 - 0.76) and the rs2819941 C allele frequency (P = 0.001, OR = 0.71, 95%CI = 0.59 - 0.87) in the patients with sarcoidosis was significantly lower than that in the controls, and these differences remained statistically significant after Bonferroni corrections. There were no interactions among rs2789679, rs1049550 and rs2819941 (P > 0.05).

We performed an association analysis to determine whether the haplotype was associated with the risk of sarcoidosis (Global P < 0.05 in block 2 and block 3). Compared with the healthy controls, the T - C haplotype occurred significantly less frequently (P = 0.001) and the C - C haplotypes occurred more frequently (P = 0.001) in block 2 (rs1049550 - rs2573351) in the patients with sarcoidosis.

To assess particular disease phenotypes, the patients with sarcoidosis were divided into the subgroups according to their chest radiographic (CXR) stage. CXR stage I (isolated bilateral hilar lymphadenopathy): N = 196; CXR stages II - IV (infiltration of parenchyma): 167; CXR stage 0 (absence on pulmonary disease): N =

28; the information on CXR stage was not available for 21 patients. Comparison of genotype frequency distribution revealed significant differences between stage I and stages II - IV for rs1049550. The significantly more CC genotype (P = 0.012) were found in the patients with stage I sarcoidosis (table 3).

DISCUSSION

A key step in linkage and association studies is to identify common risk variants in different populations. To determine if common risk variants exist in distinct populations, fifteen SNPs which span approximately 0.53 Mb of the ANXA11 gene, were genotyped in samples from the patients with sarcoidosis and healthy controls in a Chinese Han population. Recently, new sarcoidosis loci have been identified by genome - wide association studies ^{4 14}. With the fast development of genome - wide association studies, increasing number of susceptibility loci for sarcoidosis have been reported in different populations ^{4 14 15}. However, these observations should be confirmed in other genetically independent populations. In this study, we conducted the first large genetic association study of the ANXA11 gene in a Chinese Han population. The evidence of markers associated with sarcoidosis was presented, and these markers were mapped to different locations in the ANXA11 gene (81897864 -81951001). The association signals in the region were identified, and some significantly associated haplotypes also appeared this region.

Hoffman et al. reported an association between the T allele of the non synonymous SNP rs1049550 and significantly decreased risk of sarcoidosis in a German population ⁴. Similar results were also obtained in other two European BMJ Open: first published as 10.1136/bmjopen-2013-004466 on 23 July 2014. Downloaded from http://bmjopen.bmj.com/ on June 14, 2025 at Agence Bibliographique de Enseignement Superieur (ABES) . Protected by copyright, including for uses related to text and data mining, Al training, and similar technologies.

populations¹⁴¹⁵. As a part of the sarcoidosis GWAS done in Americans²³, we further confirmed this association a Chinese Han population. In this study, the frequency of ANXA11 rs1049550T allele was significantly lower in the patients with sarcoidosis than that in the healthy controls. Comparison of stages II - IV, the significantly more CC genotypewas found in the patients with stages I sarcoidosis. The "protective" effect of ANXA11 T allele increased with the number of its copies in the genotype, which is consistent with the result obtained in the patients with sarcoidosis in a German population ⁴, Furthermore, the T allele carriers among the patients were protected from infiltration of lung parenchyma (radiographic stages II-IV)¹⁵. We have also demonstrated that rs1049550 is significant cross - ethnically at the gene level after adjustment for the single SNP association tests performed. The mechanism by which rs1049550 affects the susceptibility to sarcoidosis may be that the SNP may affect the function of the ANXA11 protein rather than affect its expression. In humans, the ANXA11 protein consists of an N - terminalproline - tyrosine - glycine (PYG) rich region, followed by four annexin core domains. The rs1049550 leads to an amino - acid exchange (basic arginine to a polar cysteine) at the evolutionarily conserved position 230 (R230C) in the first annexin domain, which is responsible for Ca^{2+} dependent trafficking of the protein in the cell ²⁴.

In the GWAS conducted by Hofmann et al ⁴, the strongest association signal was observed for rs2789679. This marker is located in the 3' - UTR of the ANXA11 gene. The T allele had a frequency of 35% among the patients with sarcoidosis and 44% in the healthy controls, with 13% of the patients with sarcoidosis and 19% of controls

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being TT homozygous. Fine - mapping in the Black Women's Health Study (BWHS) revealed rs2819941 to be the top SNP, associated with a nonsignificant (P = 0.06) reduction in sarcoidosis risk (17% reduction per copy of the C-allele). In this case - control association study, the frequency of the T allele in rs2789679 and the rs2819941 C allele frequency in the patients with sarcoidosis was significantly lower than that in the healthy controls. Several lines of evidence suggest that the observed associations are unlikely to be anartifact. First, both the single - SNP and the haplotype - based association analyses support the current finding. Second, population stratification is an impossible reason, because all of our samples were from the same geographical region. Finally, consistent results were obtained from two genetically independent populations (Chinese Han and Europeans). Collectively, our results confirmed the strong association between variations in the ANXA11 gene and sarcoidosis, and suggest that ANXA11 represents a strong genetic risk factor for sarcoidosis.

We further investigated the interaction among polymorphisms and observed strong LD. The haplotype analysis revealed that significantly more C - C (block 2, rs1049550 - rs2573351) and significantly fewer T - C haplotypes (block 2) were found in the patients with sarcoidosis. These results indicated that the patients with C - C (block 2) haplotypes of the ANXA11 gene were more prone to sarcoidosis. Significantly higher frequencies of T - C haplotypes were detected in the healthy controls than in the patients with sarcoidosis, suggesting that they may show a protective effect against sarcoidosis. Our sample size can detect SNP and haplotype

associations with 90% and 85% power, respectively, at a false positive rate of 5%, disease prevalence of 1‰, disease allele / haplotype frequency of 0.05 / 0.03, and a presumed odds ratio (OR) of 1.5. To some extent, this finding further supports a role of ANXA11 polymorphisms in sarcoidosis, while ethnic group difference may exist.

In conclusion, our study suggests a potential role of the ANXA11 SNPs and their related haplotypes in the genetic susceptibility to sarcoidosis. Further studies are needed to investigate how these SNPs affect the function of ANXA11.

Funding

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Contributors

LG Zhang was involved in conception and design of the study, acquisition of patient data, genotyping and drafted the paper. ZJ Xiao and GC Shi were involved in

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design of the study, genotyping and interpretation of results. J Huang and CX Zhang were involved in design of the study, acquisition of patient data and interpretation of result. All authors revised the draft paper.

Competing interests

None.

Patient consent

Obtained.

Ethics approval

Ethics approval was provided by theFirst Hospital Affiliated to the Xinxiang

Medical College.

Provenance and peer review

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Data sharing statement

No additional data are available.

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Figure Legend

Fig. 1 The structure of the human ANXA11 gene and 15 SNPs located on the gene. Fig. 2 The LD plot of the fifteen SNPs in the ANXA11 gene. Values in squares are the pair-wise calculation of r^2 (left) or D' (right). Black squares indicate $r^2 = 1$ (i.e. perfect LD between a pair of SNPs). Empty squares indicate D' = 1 (i.e. complete LD between a pair of SNPs).

Table 1 Summary of all published sarcoidosis-association studies for ANXA11

	Popul	ation		Sample S	Size (n)	Number	
Study	Ethnic group	Countr y	Type of Study	Sarcoido sis	Contr ol	of SNPs typed	Positive SNPs
Hofma nn S, 2008	Caucasi an	Germa ny	Case-cont rol	499	490	GWAS	rs27896 79, rs70915 65, rs10495 50
Mrazek F, 2011	Caucasi an	Czech	Case-cont rol	245	254	1	rs10495 50
Cozier YC, 2012	Caucasi an	Americ a	Case-cont rol	486	943	10p12-10q 22	rs27896 79, rs28199 41
Li Y, 2013	Caucasi an	Germa ny	Case-cont rol	325	364	1	rs10495 50

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Levin AM, 2013	Caucasi an	Americ a	Case-cont rol	1689	1252	25	rs10495 50
Morais A, 2013	Caucasi an	Portug al	Case-cont rol	208	197	1	rs10495 50

Table 2 Genotypic and allelic frequencies of ANXA11 polymorphisms in the controls

	Contr ols	sarc oidosis				Contr ols	sarc oidosis	
Varia ble	(n=41 8)	(n=412)	P-val ue ^a	OR, 95% CI	Varia ble	(n=41 8)	(n=412) e ^a	OR, 95% Cl
	N, %	N, %				N, %	N, %	
rs278 9679			0.00 07		rs108 8758 1		0.2 90	
AA	79, 18.9	132, 32.0	0.00 02	0.49 <i>,</i> 0.36-0.68	ТТ	114, 27.3	96, 0.1 23.3 90	1.23 <i>,</i> 0.90-1. 69
AT	225, 53.8	186 <i>,</i> 45.2	0.01 2	1.42 <i>,</i> 1.08-1.87	TC	190 <i>,</i> 45.5	208, 0.1 50.5 48	0.82, 0.62-1.

and patients with sarcoidosis

TT	114, 27.3	94 <i>,</i> 22.8	0.14 0	1.27, 0.93-1.74	сс	114, 27.3	108 <i>,</i> 26.2	0.7 29	07 1.06, 0.78-1. 44
Per T allele	383, 45.8	450 <i>,</i> 54.6	0.00 03	0.70 <i>,</i> 0.58-0.85	Per C allele	418, 50.0	424, 51.5	0.5 83	1.06,0.8 7-1.29
rs224 5168			0.92 0		rs112 0198 9			0.1 44	1.00,
СС	156, 37.3	150, 36.4	0.79 1	1.04, 0.74-1.38	GG	130, 31.1	128, 31.1	0.9 95	0.75-1. 34
СТ	200, 47.9	197, 47.8	0.95 5	0.99 <i>,</i> 0.76-1.30	GC	218, 52.2	194 <i>,</i> 47.1	0.1 41	1.23, 0.93-1. 62
TT	62, 14.8	65, 15.8	0.78 1	0.95, 0.65-1.39	CC	70, 16.8	90, 21.9	0.0 60	0.71, 0.50-1. 01
Per T allele	324, 38.8	327, 39.7	0.69 8	1.04, 0.85-1.27	Per C allele	358, 42.8	374 <i>,</i> 45.4	0.2 9	1.11, 0.91-1. 35
rs223 6558			0.17 2		rs257 3353			0.9 18	0.00
	118, 28.2	104 <i>,</i> 25.2		1.06, 0.77-1.45		178, 42.6	176 <i>,</i> 42.7		0.99, 0.75-1. 31
6558		25.2 214,	2		3353			18 0.9	0.75-1. 31 1.01, 0.77-1. 33
6558 GG	28.2 190,	25.2 214, 51.9 94,	2 0.72 0.06 0	0.77-1.45 0.77,	3353 CC	42.6 196,	42.7 192,	18 0.9 57 0.9	0.75-1. 31 1.01, 0.77-1. 33 0.98, 0.63-1. 53
6558 GG TG	28.2 190, 45.5 110,	25.2 214, 51.9 94, 22.8 402,	2 0.72 0.06 0 0.07 3	0.77-1.45 0.77, 0.59-1.01 1.33,	3353 CC CA	42.6 196, 46.9 44,	42.7 192, 46.6 44,	18 0.9 57 0.9 21 0.9	0.75-1. 31 1.01, 0.77-1. 33 0.98, 0.63-1.
6558 GG TG TT Per T	28.2 190, 45.5 110, 26.3 410,	25.2 214, 51.9 94, 22.8 402,	2 0.72 0.06 0 0.07 3 0.91	0.77-1.45 0.77, 0.59-1.01 1.33, 0.97-1.82 0.99,	3353 CC CA AA Per A	42.6 196, 46.9 44, 10.5 284,	42.7 192, 46.6 44, 10.7 280,	 18 0.9 57 0.9 21 0.9 41 0.9 	0.75-1. 31 1.01, 0.77-1. 33 0.98, 0.63-1. 53 1.00, 0.82-1. 23
6558 GG TG TT Per T allele	28.2 190, 45.5 110, 26.3 410,	25.2 214, 51.9 94, 22.8 402,	2 0.72 0.06 0 0.07 3 0.91 7 0.17 3	0.77-1.45 0.77, 0.59-1.01 1.33, 0.97-1.82 0.99,	3353 CC CA AA Per A allele rs278	42.6 196, 46.9 44, 10.5 284,	42.7 192, 46.6 44, 10.7 280,	 18 0.9 57 0.9 21 0.9 41 0.9 97 0.9 	0.75-1. 31 1.01, 0.77-1. 33 0.98, 0.63-1. 53 1.00, 0.82-1.

	49.3	44.2	7	0.94-1.68		45.0	44.7	20	0.77-1. 33
GG	60, 14.4	54, 13.1	0.59 7	1.11 <i>,</i> 0.75-1.65	CC	52, 12.4	48, 11.7	0.7 3	1.08, 0.71-1. 64
Per G allele	326, 38.0	290 <i>,</i> 35.2	0.10 9	0.85 <i>,</i> 0.70-1.04	Per C allele	292, 34.9	280, 34.0	0.6 9	0.96, 0.78-1. 17
rs127 63624			0.11 8		rs257 3356			0.8 92	
TT	126, 75.6	150, 36.4	0.00 8	0.68, 0.51-0.91	сс	118, 28.2	112, 27.2	0.7 42	1.05, 0.78-1. 43
СТ	216, 51.7	186, 45.2	0.05 9	1.30, 0.99-1.71	СТ	190 <i>,</i> 45.5	194 <i>,</i> 47.1	0.6 39	0.94, 0.71-1. 23
СС	76 <i>,</i> 18.2	76, 18.5	0.41 1	1.16, 0.81-1.67	TT	110 <i>,</i> 26.3	106, 25.7	0.8 43	1.03, 0.76-1. 41
Per C allele	368, 44.0	338, 41.0	0.21 6	0.88, 0.73-1.08	Per T allele	410 <i>,</i> 49.0	406, 49.3	0.9 26	0.99, 0.82-1. 20
rs104 9550			0.00 02		rs281 9945			0.1 01	
CC	154, 36.8	208, 50.5	0.00 07	0.57, 0.43-0.75	GG	119, 28.5	130, 31.6	0.2 94	0.85, 0.63-1.
СТ	190, 45.5	170 <i>,</i> 41.3	0.22 1	1.19, 0.90-1.56	GA	221, 52.9	188, 45.6	0.0 30	15 1.35, 1.03-1. 78
TT	74, 17.7	34, 8.3	0.00 08	2.39, 1.55-3.68	AA	78, 18.7	94, 22.8	0.1 38	0.78, 0.55-1. 09
Per T allele	338 <i>,</i> 40.4	238, 28.8	0.00 07	0.60 <i>,</i> 0.49-0.73	Per A allele	377, 45.1	376, 45.6	0.8 27	1.02, 0.84-1. 24
rs257 3351			0.43 7		rs112 0205			0.0 64	
TT	120, 28.7	104, 25.2	, 0.26 3	1.19, 0.88-1.62	9 GG	106, 25.4	128, 31.1	0.0 67	0.75 <i>,</i> 0.56-1.

									02
СТ	202 <i>,</i> 48.3	216, 52.4	0.23 8	0.85, 0.65-1.12	GA	226, 54.1	190, 46.1	0.0 21	1.38, 1.05-1. 81
СС	96, 23.0	92, 22.3	0.82 6	1.04, 0.75-1.44	AA	86, 20.6	94, 22.8	0.4 29	0.88, 0.63-1. 22
Per C	394,	400,	0.56	1.06,	Per A	398,	378,	0.4	0.93,
allele	47.1	48.5	4	0.87-1.28	allele	47.6	45.9	79	0.77-1. 13
									15
rs281 9941			0.00 02						
СС	114,	94,	0.14	1.27,					
	27.3	22.8	1	0.93-1.74					
СТ	226,	190,	0.02	1.38,					
CI	54.1	46.1	1	1.05-1.81					
тт	78,	128,	0.00	0.51,					
	18.7	31.1	04	0.37-0.70					
Per T	382,	446,	0.00	0.71,					
allele	45.7	54.1	1	0.59-0.87					
a n									

^a *P*-value was calculated by 2×3 and 2×2 chi-squared tests based on codominant, dominant for

the rare allele, heterosis and recessive for the rare allele models of inheritance.

Alpha value is adjusted by Bonferroni correction and statistically significant results (P<0.003)

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Table 3 Chest radiographic (CXR) stages of sarcoidosis patients by genotype of rs2789679, rs1049550 and rs2819941

Stages	rs2789679 (n, %)			rs	rs1049550 [*] (n, %)			rs2819941 (n, %)		
Stages -	AA	AT	TT	CC	СТ	TT	CC	СТ	TT	
stage I	59 (30.1)	92 (47.0)	45 (23.0)	101 (51.5)	82 (41.8)	13 (6.6)	47 (25.0)	88 (44.9)	61 (31.1)	
stages II - IV	46 (27.6)	75 (44.9)	46 (27.6)	72 (43.1)	68 (40.7)	27 (16.2)	41 (24.6)	73 (43.7)	53 (31.7)	
χ^2, P		1.041, 0.594			8.807, 0.012			0.052, 0.975		
* P values for g	enotype frequen	cy distribution (P	< 0.05).							

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	Haplotype		Genecounting (frequency %)				
ID	rs1049550	rs2573351	Cases	Controls	<i>P</i> -value ^a	Global P ^b	
HAP1	Т	С	27.7	39.7	0.001	0.003	
HAP2	С	С	19.9	7.4	0.0001		
НАР3	С	Т	50.5	51.0	0.892		

^a Based on 10,000 permutations.

5	^b Based on comparison of	frequency distributio	n of all haplotypes for the cor	nbination of SNPs.
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Table 5 ANXA11 haplotype in block 3 frequencies and the results of their associations with risk

3 of sarcoidosis							
_	Haplotype			Genecounting (frequency %)			b)
_	ID	rs2789695	rs2573356	Cases	Controls	<i>P</i> -value ^a	Global P ^b
_	HAP1	С	Т	33.7	34.9	0.718	0.045
	HAP2	Т	Т	15.3	14.1	0.632	
	HAP2	Т	Т	15.6	14.1	0.565	
) - -	Based of	n comparison of f	requency distrit	Sution of all		ne combinatio	n of SNPS.
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1	Annexin A11 (ANXA11) gene polymorphisms are associated with sarcoidosis in a
2	Han Chinese population
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12	Key words: Sarcoidosis; Annexin A11; Single-nucleotide polymorphism
13	Word count: 2909
14	

1	Abstract
2	Objectives: To further identify the single - nucleotide polymorphisms (SNPs) that
3	contribute to the genetic susceptibility to sarcoidosis, we examined the potential
4	association between sarcoidosis and fifteen SNPs of the ANXA11 gene.
5	Design: A case - control study.
6	Setting: A tuberculosist unit in a university hospital in China.
7	Participants: Participants included 412 patients with sarcoidosis and 418 healthy
8	controls.
9	Methods: The selected SNPs were genotyped in cases and controls by using the
10	MALDI - TOF in the MassARRAY system (Sequenom Inc., San Diego, CA, USA).
11	Probes and primers were designed using the Assay Design Software (Sequenom, San
12	Diego, CA, USA).
13	Results: The results showed that statistically significant differences were observed in
14	the allelic or genotypic frequencies of the rs2789679, rs1049550 and rs2819941 in the
15	ANXA11 gene. The frequency of the T allele in rs2789679 ($P = 0.0003$, OR = 0.70,
16	<u>95%CI = 0.58 - 0.85), T allele in rs1049550 ($P = 0.0007$, OR = 0.62, 95%CI= 0.50 -</u>
17	<u>0.76) and the rs2819941 C allele frequency ($P = 0.001$, OR = 0.71, 95%CI = 0.59 -</u>
18	0.87) in the patients with sarcoidosis was significantly lower than that in the controls.
19	The frequency of the T allele in rs2789679 ($\chi^2 = 12.849$, $P = 0.0003$, OR = 0.703,
20	95% CI = 0.579 - 0.852), T allele in rs1049550 (χ^2 = 24.420, P = 0.0007, OR = 0.617,
21	95%CI= 0.503 – 0.758) and the rs2819941 C allele frequency ($\chi^2 = 11.803$, $P = 0.001$,
22	OR = 0.713, 95%CI = 0.588 – 0.865) in the patients with sarcoidosis was significantly

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1	lower than that in the controls. Comparison of genotype frequency distribution
2	revealed significant differences between chest radiographic (CXR) stage I and stages
3	<u>II - IV for rs1049550. The significantly more CC genotype ($P = 0.012$) were found in</u>
4	the patients with stages I sarcoidosis. Furthermore, strong linkage disequilibrium (LD)
5	was observed in two-four blocks (D' > 0.9). In block 2 (rs1049550-rs2573351), the T -
6	C haplotype occurred significantly less frequently ($P = 0.001$), whereas the C - C
7	haplotypes occurred more frequently ($P = 0.0001$).
8	Conclusions: These findings point to a role for ANXA11 gene polymorphisms in
9	sarcoidosis in a Han Chinese population, and may be informative for future genetic or
10	biological studies on sarcoidosis.
11	

1	Article summary
2	Article focus
3	1. The first genome - wide association study (GWAS) in sarcoidosis conducted in a
4	German population has revealed that the rs1049550 (C > T, Arg230Cys) located in
5	exon 10 were associated with sarcoidosis.
6	2. More studies should be performed to demonstrate the following item: whether these
7	SNPs modulate the risk of sarcoidosis by themselves or whether they correlate with
8	other causative SNPs and are repeated in other populations.
9	3. We hypothesize that common variants in the ANXA11 gene may significantly
10	contribute to the predisposition to develop sarcoidosis in a Chinese Han population.
11	Key messages
12	1. ANXA11 rs2789679 (3'UTR) was found to be associated with sarcoidosis.
13	2. ANXA11 rs1049550 (exon 10) was significantly associated with sarcoidosis.
14	3. Another significant association was observed for rs2819941 (intron 14).
15	Strengths and limitations of this study
16	1. Functional SNPs in the promoter region, 5'- and 3'-UTR, exons of ANXA11 gene
17	were systematically screened and homogeneity of the study subjects, representing the
18	Han Chinese, is the main strength of the current study.
19	2. Functional characterization is the potential limitation of the study that could have
20	further helped in proving the positive association observed for rs2789679 and
21	rs1049550. The lack of correlation of serum ANXA11 levels are needed to
22	investigate.

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The lack of correlation of serum ANXA11 levels and sample size are other
limitations.
INTRODUCTION
Sarcoidosis is a systemic autoimmune disease characterized by destructive, non -
caseating epithelioid granulomatous lesions, with accumulated phagocytes and
activated CD4 ⁺ T helper type 1 lymphocytes ^{1 2} . The typical manifestations of
sarcoidosis include bilateral hilar lymphadenopathy, pulmonary infiltration and ocular
and skin lesions. The clinical course and prognosis of sarcoidosis are variable. The
acute forms of sarcoidosis such as $L\Box$ fgren's syndrome (LS) can resolve
spontaneously in most cases. However, chronic pulmonary sarcoidosis may progress
to lung fibrosis and eventually result in respiratory failure. Recent studies have
demonstrated that sarcoidosis can occur in a pattern of family clustering, and its racial
incidence is also different, suggesting that some genetic factors may contribute to the
risk and severity of sacroidosis ¹³ .
The Annexin gene family is involved in the etiology of several autoimmune and
chronic diseases ^{45.} One member of the Annexin gene family, ANXA11, located on
chromosome 10q22.3, is involved in calcium signaling, apoptosis, vesicle trafficking,
cell growth and terminal phase of cell division ⁵⁻⁷ . In this context, the development
and maintenance of the granulomatous inflammation in sarcoidosis have been
repeatedly associated with the impaired apoptosis of activated inflammatory cells ⁸⁹ .
In most patients with sarcoidosis, the early stages of this disease are characterized by
mononuclear cell alveolitis dominated by activated CD4 ⁺ T cells and macrophages.
5

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Between these cells, the uncoordinated interplay results in the formation of typical non - caseating granulomas. The mechanism by which the granulomas resolve has not been fully elucidated. However, it is generally assumed that the induction of apoptosis and / or by the withdrawal of inflammatory cytokines participates in the disappearance of granulomas ^{10 11}. In the patients with sarcoidosis, their peripheral blood monocytes were characterized by apoptosis ⁹.

In previous studies, case - control / family 'hypothesis - driven' studies and low density linkage scans were used to identify genetic factors conferring the genetic susceptibility to sarcoidosis¹². Notably, the first genome - wide association study (GWAS) in sarcoidosis conducted in a German population has recently revealed an association: the leading rs2789679 SNP (single - nucleotide polymorphism) located in the 3' - untranslated region (3' - UTR) and a common nonsynonymous SNP (rs1049550, C > T, Arg230Cys) were associated with increased risk of sarcoidosis 413 . This association has recently been supported by another report from the same population¹⁴. Thus, more studies should be performed to demonstrate the following item: whether these SNPs modulate the risk of sarcoidosis by themselves or whether they correlate with other causative SNPs and are repeated in other populations. The published sarcoidosis - association studies for ANXA11 are summarized in Table 1. We hypothesize that common variants in the ANXA11 gene may significantly contribute to the predisposition to develop sarcoidosis.

In this study, we investigated fifteen loci in a Chinese population from He'nan province (China) to verify the putative association between ANXA11 polymorphisms

and sarcoidosis.

2	SUBJECTS AND METHODS
3	Subjects
4	Four hundred and twelve patients with sarcoidosis (mean \pm SD age: 53.6 \pm 4.6
5	<u>years; 155 men and 257 women) (mean \pm SD age: 53.6 \pm 4.6 years) were recruited</u>
6	from our hospital between May 2005 and March 2013. Sarcoidosis was diagnosed by
7	the evidence of non - caseating epitheloid cell granuloma in biopsy specimens and
8	chest radiographic abnormalities. Chronic sarcoidosis was defined as a disease over at
9	least 2 years or at least two episodes in a lifetime. A cute sarcoidosis was defined as
10	one episode of acute sarcoidosis which had totally resolved at the date of the
11	examination. The control group consisted of 418 unrelated healthy subjects (mean \pm
12	SD age: 54.2 ± 5.3 years; 160 men and 258 women) who underwent health
13	examinations in the Medical Examination Center of the First Affiliated Hospital of the
14	Xinxiang Medical College (Xinxiang, China) between Oct 2009 and Sept 2013. None
15	of the individuals in the control group had a history of lung diseases or showed any
16	symptoms of the lung or other diseases by chest radiography or laboratory blood tests.
17	Participants were excluded if they: were taking other prescribed medications that
18	could affect the central nervous system; had a history of seizures, hematological
19	diseases, or severe liver or kidney impairment; or were pregnant The control group
20	consisted of 418 unrelated healthy subjects (mean \pm SD age: 54.2 \pm 5.3 year) who
21	underwent health examinations in the Medical Examination Center of the First
22	Affiliated Hospital of the Xinxiang Medical College (Xinxiang, China) between Oct
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2009 and Sept 2013. No familial relationship was known between the study
 participants.All participants were from a non genetically related Chinese Han
 population in He'nan Province (China). The study was performed according to the
 Guidelines of the Medical Ethical Committee of Xinxiang Medical College (Xinxiang,
 China). Written informed consent was obtained from each participant in this study.

6 SNP selection

7 Tagging SNPs were selected from the catalogs of the International HapMap Project. The rs2789679 and rs2245168 are located in 5'-UTR, rs2236558 in intron 6, 8 rs7067644 and rs12763624 in intron 8, rs1049550 in exon 10, rs2573351 in intron 13, 9 rs10887581, rs11201989, rs2573353, rs2789695, rs2573356, rs2819945, rs11202059 10 and rs2819941 in intron 14 (Figure 1). Marker selection was done according to 11 previous studies ^{4 12 14 15}, and preliminary analysis was performed using the HapMap 12 data and the following criteria. First, we examined tagSNPs in the Haploview (v4.2), 13 using the CHB population and a minor allele frequency cut - off (MAF) \geq 5% 14 (HapMap Data Release 27). We found that there were a total of 29 potential tagSNPs 15 in all. As a first screen of the most common SNPs in the sarcoidosis sample from an 16 Eastern Chinese Han population, a MAF > 20% with pair - wise tagging and $r^2 > 0.8$ 17 ¹⁶ was used as the cut - off when choosing tagSNPs. Second, the LD pattern of this 18 gene was determined in the Chinese population using the preliminary data from the 19 HapMap. Five LD blocks across the ANXA11 were defined using Haploview's 20 'confidence intervals' method ^{17 18}. These SNPs were further analyzed in an 21 association study. 22

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1	Marker selection was done according to previous studies 4 12 14 15, and preliminary
2	analysis was performed using the HapMap data and the following criteria. We
3	examined tagSNPs in the Haploview software v4.2, using the Chinese Han in Beijing
4	(CHB) population and a minor allele frequency cut-off (MAF) \geq 5% (the HapMap
5	Data Release 27). The linkage disequilibrium (LD) pattern of this gene was
6	determined in the Chinese population using the preliminary data from the HapMap.
7	These SNPs were further analyzed in an association study.

8 Genotyping

Peripheral blood was collected from a vein into a sterile tube coated with 9 ethylenediamine tetraacetic acid (EDTA). Plasma samples were stored at -20°C. 10 Genomic DNA was extracted from the frozen peripheral blood samples using a 11 12 QIAmp Blood Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocols. The selected SNPs were genotyped in cases and controls by 13 using the MALDI - TOF in the MassARRAY system (Sequenom Inc., San Diego, CA, 14 15 USA). Probes and primers were designed using the Assay Design Software (Sequenom, San Diego, CA, USA). The completed genotyping reactions were spotted 16 onto a 384 well spectroCHIP (Sequenom) using the MassARRAY Nanodispenser 17 18 (Sequenom) and determined by the matrix - assisted laser desorption ionization time -19 of - flight mass spectrometer. Genotype calling was performed in realtime with the 20 MassARRAY RT software version 3.0.0.4 and analyzed using the MassARRAY Typer 21 software version 3.4 (Sequenom).

22 Statistical analysis

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1	All data were analyzed using the SPSS 17.0 software (SPSS Inc., Chicago, IL,
2	USA). Hardy-Weinberg equilibrium were evaluated by Chi - square tests. Differences
3	between the cases and controls in the frequency of the alleles, genotypes and
4	haplotypes were evaluated by the Fisher's exact test or the Pearson Chi - square test.
5	Unconditional logistic regression was used to calculate the odds ratio (OR) and 95%
6	confidence interval (CI) in independent association between each locus and the
7	presence of sarcoidosis. Generalized linear regression was used to evaluate the
8	interaction effects between gene and gender or age. Gender and age of subjects were
9	treated as covariants in binary logistic regression. P - value was calculated based on
10	codominant, dominant for the rare allele, heterosis and recessive for the rare allele
11	models of inheritance. The Bonferroni correction was used to adjust the test level
12	when multiple comparisons were conducted, and the P value was divided by the total
13	number of loci. Haplotype blocks were defined according to the criteria developed by
14	Gabriel et al. ¹⁸ . Pair - wise LD statistics (D' and r ²) and haplotype frequency were
15	calculated, and haplotype blocks were constructed using the Haploview 4.0 ¹⁷ . To
16	ensure that the LD blocks most closely reflect the population level LD patterns,
17	definition of the blocks were based on the control samples alone. The haplotype
18	frequencies were estimated using GENECOUNTING, which computes maximum -
19	likelihood estimates of haplotype frequencies from unknown phase data by utilizing
20	an expectation - maximization algorithm ¹⁹⁻²² . The significance of any haplotypic
21	association was evaluated using a likelihood ratio test, followed by permutation
22	testing that compared estimated haplotype frequencies in cases and controls ^{19 21} .

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1	All data were analyzed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).
2	Hardy-Weinberg equilibrium were evaluated by Chi - square tests. Differences
3	between the cases and controls in the frequency of the alleles, genotypes and
4	haplotypes were evaluated by the Fisher's exact test or the Pearson Chi – square test.
5	Unconditional logistic regression was used to calculate the odds ratio (OR) and 95%
6	confidence interval (CI) in independent association between each locus and the
7	presence of sarcoidosis. The Bonferroni correction was used to adjust the test level
8	when multiple comparisons were conducted, and the P value was divided by the total
9	number of loci. Haplotype blocks were defined according to the criteria developed by
10	Gabriel et al. ¹⁸ . Pair – wise LD statistics (D' and r^2) and haplotype frequency were
11	calculated, and haplotype blocks were constructed using the Haploview 4.0 ⁴⁷ .
12	RESULTS
13	The genotype distribution of the fifteen polymorphisms was consistent with the
14	Hardy - Weinberg equilibrium ($P > 0.05$). The analysis of strong LD in the patients
15	with sarcoidosis and healthy controls revealed that 2 SNPs (rs2245168, rs2236558), 2
16	SNPs (rs1049550, rs2573351), 2 SNPs (rs2789695, rs2573356) and 3 SNPs
17	(rs2819945, rs11202059, rs2819941) were located in haplotype block 1, block 2,
18	block 3 and block 4 (D' $>$ 0.9, Fig. 2). The genotype distribution, allelic frequencies,
19	and haplotypes in the patients with sarcoidosis and healthy controls are showed in
20	tables 2, table 4 and table 5.
21	Comparison of genotype and allele frequency distribution revealed significant

22 differences between the patients with sarcoidosis and healthy controls for 3 SNPs:

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1	rs2789679, rs1049550 and rs2819941. The frequency of the T allele in rs2789679 (P
2	= 0.0003, OR = 0.70, 95%CI = 0.58 - 0.85), T allele in rs1049550 (P = 0.0007, OR =
3	0.62, 95%CI = 0.50 - 0.76) and the rs2819941 C allele frequency ($P = 0.001$, OR =
4	0.71, 95%CI = 0.59 - 0.87) in the patients with sarcoidosis was significantly lower
5	than that in the controls, and these differences remained statistically significant after
6	Bonferroni corrections. There were no interactions among rs2789679, rs1049550 and
7	<u>rs2819941 (P > 0.05).</u>
8	We performed an association analysis to determine whether the haplotype was
9	associated with the risk of sarcoidosis (Global $P < 0.05$ in block 2 and block 3).
10	Compared with the healthy controls, the T - C haplotype occurred significantly less
11	frequently ($P = 0.001$) and the C - C haplotypes occurred more frequently ($P = 0.0001$)
12	in block 2 (rs1049550 - rs2573351) in the patients with sarcoidosis.
13	To assess particular disease phenotypes, the patients with sarcoidosis were
14	divided into the subgroups according to their chest radiographic (CXR) stage. CXR
15	stage I (isolated bilateral hilar lymphadenopathy): N = 196; CXR stages II - IV
16	(infiltration of parenchyma): 167; CXR stage 0 (absence on pulmonary disease): N =
17	28; the information on CXR stage was not available for 21 patients. Comparison of
18	genotype frequency distribution revealed significant differences between stage I and
19	stages II - IV for rs1049550. The significantly more CC genotype ($P = 0.012$) were
20	found in the patients with stage I sarcoidosis (table 3).
21	DISCUSSION
22	A key step in linkage and association studies is to identify common risk variants

1	in different populations. To determine if common risk variants exist in distinct
2	populations, fifteen SNPs which span approximately 0.53 Mb of the ANXA11 gene,
3	were genotyped in samples from the patients with sarcoidosis and healthy controls in
4	a Chinese Han population. Recently, new sarcoidosis loci have been identified by
5	genome - wide association studies ^{4 14} . With the fast development of genome - wide
6	association studies, increasing number of susceptibility loci for sarcoidosis have been
7	reported in different populations 4 14 15. However, these observations should be
8	confirmed in other genetically independent populations. In this study, we conducted
9	the first large genetic association study of the ANXA11 gene in a Chinese Han
10	population. The evidence of markers associated with sarcoidosis was presented, and
11	these markers were mapped to different locations in the ANXA11 gene (81897864 -
12	81951001). The association signals in the region were identified, and some
13	significantly associated haplotypes also appeared this region.
14	Hoffman et al. reported an association between the T allele of the non -
15	synonymous SNP rs1049550 and significantly decreased risk of sarcoidosis in a
16	German population ⁴ . Similar results were also obtained in other two European
17	populations ^{14 15} . As a part of the sarcoidosis GWAS done in Americans ²³ , we further
18	confirmed this associationin a Chinese Han population. In this study, the frequency of
19	ANXA11 rs1049550T allele was significantly lower in the patients with sarcoidosis
20	than that in the healthy controls. Comparison of stages II - IV, the significantly more
21	CC genotypewas found in the patients with stages I sarcoidosis. The "protective"
22	effect of ANXA11 T allele increased with the number of its copies in the genotype,

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1	which is consistent with the result obtained in the patients with sarcoidosis in a
2	German population ⁴ , Furthermore, the T allele carriers among the patients were
3	protected from infiltration of lung parenchyma (radiographic stages II-IV) ¹⁵ . We have
4	also demonstrated that rs1049550 is significant cross - ethnically at the gene level
5	after adjustment for the single SNP association tests performed. The mechanism by
6	which rs1049550 affects the susceptibility to sarcoidosis may be that the SNP may
7	affect the function of the ANXA11 protein rather than affect its expression. In humans,
8	the ANXA11 protein consists of an N - terminalproline - tyrosine - glycine (PYG) -
9	rich region, followed by four annexin core domains. The rs1049550 leads to an amino
10	- acid exchange (basic arginine to a polar cysteine) at the evolutionarily conserved
11	position 230 (R230C) in the first annexin domain, which is responsible for Ca^{2+} -
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12	dependent trafficking of the protein in the cell ²⁴ .
12	dependent trafficking of the protein in the cell ²⁴ .
12 13	dependent trafficking of the protein in the cell ²⁴ . In the GWAS conducted by Hofmann et al ⁴ , the strongest association signal was
12 13 14	dependent trafficking of the protein in the cell ²⁴ . In the GWAS conducted by Hofmann et al ⁴ , the strongest association signal was observed for rs2789679. This marker is located in the 3' - UTR of the ANXA11 gene.
12 13 14 15	dependent trafficking of the protein in the cell ²⁴ . In the GWAS conducted by Hofmann et al ⁴ , the strongest association signal was observed for rs2789679. This marker is located in the 3 ² - UTR of the ANXA11 gene. The T allele had a frequency of 35% among the patients with sarcoidosis and 44% in
12 13 14 15 16	 dependent trafficking of the protein in the cell²⁴. In the GWAS conducted by Hofmann et al⁴, the strongest association signal was observed for rs2789679. This marker is located in the 3' - UTR of the ANXA11 gene. The T allele had a frequency of 35% among the patients with sarcoidosis and 44% in the healthy controls, with 13% of the patients with sarcoidosis and 19% of controls
12 13 14 15 16 17	dependent trafficking of the protein in the cell ²⁴ . In the GWAS conducted by Hofmann et al ⁴ , the strongest association signal was observed for rs2789679. This marker is located in the 3' - UTR of the ANXA11 gene. The T allele had a frequency of 35% among the patients with sarcoidosis and 44% in the healthy controls, with 13% of the patients with sarcoidosis and 19% of controls being TT homozygous. Fine - mapping in the Black Women's Health Study (BWHS)
12 13 14 15 16 17 18	dependent trafficking of the protein in the cell ²⁴ . In the GWAS conducted by Hofmann et al ⁴ , the strongest association signal was observed for rs2789679. This marker is located in the 3' - UTR of the ANXA11 gene. The T allele had a frequency of 35% among the patients with sarcoidosis and 44% in the healthy controls, with 13% of the patients with sarcoidosis and 19% of controls being TT homozygous. Fine - mapping in the Black Women's Health Study (BWHS) revealed rs2819941 to be the top SNP, associated with a nonsignificant ($P = 0.06$)
12 13 14 15 16 17 18 19	dependent trafficking of the protein in the cell ²⁴ . In the GWAS conducted by Hofmann et al ⁴ , the strongest association signal was observed for rs2789679. This marker is located in the 3' - UTR of the ANXA11 gene. The T allele had a frequency of 35% among the patients with sarcoidosis and 44% in the healthy controls, with 13% of the patients with sarcoidosis and 19% of controls being TT homozygous. Fine - mapping in the Black Women's Health Study (BWHS) revealed rs2819941 to be the top SNP, associated with a nonsignificant ($P = 0.06$) reduction in sarcoidosis risk (17% reduction per copy of the C-allele). In this case -

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associations are unlikely to be anartifact. First, both the single - SNP and the haplotype - based association analyses support the current finding. Second, population stratification is an impossible reason, because all of our samples were from the same geographical region. Finally, consistent results were obtained from two genetically independent populations (Chinese Han and Europeans). Collectively, our results confirmed the strong association between variations in the ANXA11 gene and sarcoidosis, and suggest that ANXA11 represents a strong genetic risk factor for sarcoidosis. We further investigated the interaction among polymorphisms and observed strong LD. The haplotype analysis revealed that significantly more C - C (block 2, rs1049550 - rs2573351) and significantly fewer T - C haplotypes (block 2) were found in the patients with sarcoidosis. These results indicated that the patients with C - C (block 2) haplotypes of the ANXA11 gene were more prone to sarcoidosis. Significantly higher frequencies of T - C haplotypes were detected in the healthy controls than in the patients with sarcoidosis, suggesting that they may show a protective effect against sarcoidosis. Our sample size can detect SNP and haplotype associations with 90% and 85% power, respectively, at a false positive rate of 5%, disease prevalence of 1‰, disease allele / haplotype frequency of 0.05 / 0.03, and a presumed odds ratio (OR) of 1.5. To some extent, this finding further supports a role of ANXA11 polymorphisms in sarcoidosis, while ethnic group difference may exist. In conclusion, our study suggests a potential role of the ANXA11 SNPs and their related haplotypes in the genetic susceptibility to sarcoidosis. Further studies are

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1	needed to investigate how these SNPs affect the function of ANXA11.
2	Contributors
3	LG Zhang was involved in conception and design of the study, acquisition of
4	patient data, genotyping and drafted the paper. ZJ Xiao and GC Shi were involved in
5	design of the study, genotyping and interpretation of results. J Huang and CX Zhang
6	were involved in design of the study, acquisition of patient data and interpretation of
7	result. All authors revised the draft paper.
8	Funding
9	The project was supported by the Research and Development Foundation of
10	Science and Technology of Henan Province (2012k15).
11	Competing interests
12	None. Patient consent Obtained. Ethics approval
13	Patient consent
14	Obtained.
15	Ethics approval
16	Ethics approval was provided by theFirst Hospital Affiliated to the Xinxiang
17	Medical College.
18	Provenance and peer review
19	Not commissioned; externally peer reviewed.
20	Data sharing statement
21	No additional data are available.

22 Figure Legend

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	Fig. 1 The structure of the human ANXA11 gene and 15 SNPs located on the gene.
	Fig. 2 The LD plot of the fifteen SNPs in the ANXA11 gene. Values in squares are the
	pair-wise calculation of r^2 (left) or D' (right). Black squares indicate $r^2 = 1$ (i.e. perfect
	LD between a pair of SNPs). Empty squares indicate $D' = 1$ (i.e. complete LD
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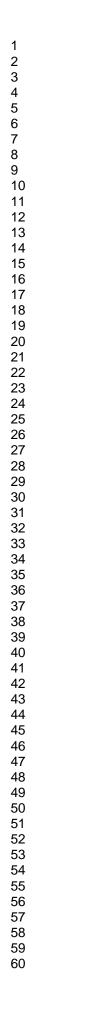
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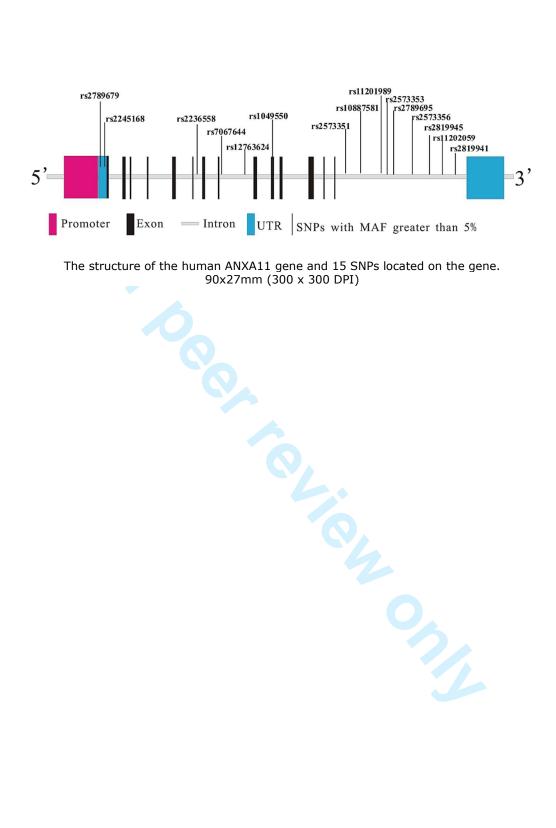
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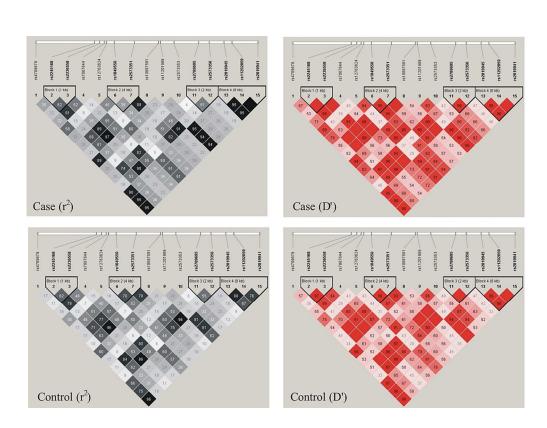
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The LD plot of the fifteen SNPs in the ANXA11 gene. Values in squares are the pair-wise calculation of r2 (left) or D' (right). Black squares indicate r2 = 1 (i.e. perfect LD between a pair of SNPs). Empty squares indicate D' = 1 (i.e. complete LD between a pair of SNPs). 90x66mm (300 x 300 DPI) BMJ Open: first published as 10.1136/bmjopen-2013-004466 on 23 July 2014. Downloaded from http://bmjopen.bmj.com/ on June 14, 2025 at Agence Bibliographique de I Enseignement Superieur (ABES) . Protected by copyright, including for uses related to text and data mining, Al training, and similar technologies.

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Annexin A11 (ANXA11) gene polymorphisms are associated with sarcoidosis in a Han Chinese population - a casecontrol study

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2 3 4 1	Annexin A11 (ANXA11) gene polymorphisms are associated with sarcoidosis in a
5 6 2	Han Chinese population - a case-control study
7	LG Zhang, ZJ Xiao [*] , GC Shi, J Huang, CX Zhang
10	
11 4 12	The Third Department of Tuberculosis, the First Hospital Affiliated to the Xinxiang
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31 12 32 33	Key words: Sarcoidosis; Annexin A11; Single-nucleotide polymorphism
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1 Abstract

Objectives: To further identify the single-nucleotide polymorphisms (SNPs) that
contribute to the genetic susceptibility to sarcoidosis, we examined the potential
association between sarcoidosis and fifteen SNPs of the ANXA11 gene.
Design: A case - control study.

Setting: A tuberculosist unit in a hospital of the university in China.

7 Participants: Participants included 412 patients with sarcoidosis and 418 healthy

8 controls.

9 Methods: The selected SNPs were genotyped using the MALDI-TOF in the
10 MassARRAY system. Probes and primers were designed using the Assay Design
11 Software (Sequenom, San Diego, CA, USA).

Results: The results showed that statistical significant differences were found in the allelic or genotypic frequencies of the rs2789679, rs1049550 and rs2819941 in the ANXA11 gene between the patients with sarcoidosis and the controls. The frequency of the T allele in rs2789679 (P = 0.0003, OR = 0.70, 95%CI = 0.58 - 0.85) and rs1049550 (P = 0.0007, OR = 0.62, 95% CI = 0.50 - 0.76), and C allele in rs2819941(P = 0.001, OR = 0.71, 95%CI = 0.59 - 0.87) in the patients with sarcoidosis was significantly lower than that in the controls. The strong linkage disequilibrium (LD) was observed in four blocks (D' > 0.9). In block 2 (rs1049550-rs2573351), the T - C haplotype occurred significantly less frequently (P = 0.001), whereas the C - C haplotypes occurred more frequently (P = 0.0001) in the patients with sarcoidosis than the controls. Furthermore, genotype frequency distribution revealed that, in

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 rs1049550, CC genotype was significantly more in the patients with chest radiographic (CXR) stage I sarcoidosis than the patients with CXR stage II-IV sarcoidosis (P = 0.012). Conclusions: These findings point to a role for the polymorphisms of ANXA11 in sarcoidosis in a Chinese Han population, and may be informative for future genetic or biological studies on sarcoidosis. 		
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<text></text>	4	Conclusions: These findings point to a role for the polymorphisms of ANXA11 in
	5	sarcoidosis in a Chinese Han population, and may be informative for future genetic
	6	or biological studies on sarcoidosis.
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1	Article summary
2	Article focus
3	1. The first genome-wide association study (GWAS) in sarcoidosis conducted in a
4	German population has revealed that the rs1049550 (C > T, Arg230Cys) located in
5	exon 10 were associated with sarcoidosis.
6	2. Whether these SNPs modulate the risk of sarcoidosis by themselves or whether
7	they correlate with other causative SNPs and are repeated in other populations remain
8	unclear.
9	3. We hypothesize that common variants in the ANXA11 gene may significantly
10	contribute to the predisposition to develop sarcoidosis in a Chinese Han population.
11	Key messages
12	1. ANXA11 rs2789679 (3'-UTR) was significantly associated with sarcoidosis.
13	2. ANXA11 rs1049550 (exon 10) was significantly associated with sarcoidosis.
14	3. Another significant association was observed for rs2819941 (intron 14).
15	Strengths and limitations of this study
16	1. A systematical screening of the functional SNPs in the promoter region, 5'- and
17	3'-UTR, exons of the ANXA11 gene, and the homogeneity of the study subjects
18	representing the Chinese Han are the main strengths of this study. Chinese Han
19	2. The lack of data proving the positive association observed for rs2789679 and
20	rs1049550 is a potential limitation of this study. Furthermore, the association of the
21	serum level of ANXA11 with sarcoidosis still needs to be investigated.

1 INTRODUCTION

Sarcoidosis is a systemic autoimmune disease characterized by destructive, non-caseating epithelioid granulomatous lesions, with accumulated phagocytes and activated CD4⁺ T helper type 1 lymphocytes ^{1 2}. The typical manifestations of sarcoidosis include bilateral hilar lymphadenopathy, pulmonary infiltration and ocular and skin lesions. The clinical course and prognosis of sarcoidosis are variable. The acute forms of sarcoidosis such as $L\Box$ fgren's syndrome (LS) can resolve spontaneously in most cases. However, chronic pulmonary sarcoidosis may progress to lung fibrosis which eventually causes respiratory failure. Recent studies have demonstrated that sarcoidosis can occur in a pattern of family clustering, and its racial incidence is also different, suggesting that some genetic factors may contribute to the risk and severity of sacroidosis ¹³.

The Annexin gene family is involved in the etiology of several autoimmune and chronic diseases ^{45.} One member of the Annexin gene family, ANXA11, located on chromosome 10q22.3, is involved in calcium signaling, apoptosis, vesicle trafficking, cell growth and terminal phase of cell division ⁵⁻⁷. In this context, the development and maintenance of the granulomatous inflammation in sarcoidosis have been repeatedly associated with the impaired apoptosis of activated inflammatory cells ⁸⁹. Most of the early-stage sarcoidosis is characterized by mononuclear cell alveolitis dominated by activated CD4⁺ T cells and macrophages. Between these cells, the uncoordinated interplay results in the formation of typical non-caseating granulomas. The mechanism by which the granulomas resolve has not been fully elucidated.

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1	However, it is generally assumed that the induction of apoptosis and/or by the
2	withdrawal of inflammatory cytokines participates in the disappearance of
3	granulomas ^{10 11} . In the patients with sarcoidosis, their peripheral blood monocytes
4	were characterized by apoptosis ⁹ .
5	In previous studies, case - control/family 'hypothesis - driven' studies and low
6	density linkage scans were used to identify genetic factors conferring the genetic
7	susceptibility to sarcoidosis ¹² . Notably, the first genome - wide association study
8	(GWAS) in sarcoidosis conducted in a German population has recently revealed an
9	association: the leading rs2789679 SNP (single - nucleotide polymorphism) located in
10	the 3'-untranslated region (3'-UTR) and a common nonsynonymous SNP (rs1049550,
11	C > T, Arg230Cys) were associated with the increased risk of sarcoidosis $^{4 13}$. This
12	association has recently been supported by another report from the same population ¹⁴ .
13	Thus, more studies should be performed to demonstrate the following items: whether
14	these SNPs modulate the risk of sarcoidosis by themselves or whether they correlate
15	with other causative SNPs and are repeated in other populations. The published
16	studies about the association of sarcoidosis and ANXA11 are summarized in
17	Supplemental table 1. We hypothesize that common variants in the ANXA11 gene
18	may significantly contribute to the predisposition to develop sarcoidosis.
19	In this study, we investigated fifteen loci in a Chinese population from He'nan
20	province (China) to verify the putative association between ANXA11 polymorphisms

21 and sarcoidosis.

22 SUBJECTS AND METHODS

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1 Subjec	ts
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2	Four hundred and twelve patients with sarcoidosis (mean \pm SD age: 53.6 \pm 4.6
3	years; 155 men and 257 women) were recruited from our hospital between May 2005
4	and March 2013. Sarcoidosis was diagnosed by the evidence of non-caseating
5	epitheloid cell granuloma in biopsy specimens and chest radiographic abnormalities.
6	Chronic sarcoidosis was defined as a disease over at least 2 years or at least two
7	episodes in a lifetime. A cute sarcoidosis was defined as one episode of acute
8	sarcoidosis which had totally resolved at the date of the examination. The control
9	group consisted of 418 unrelated healthy subjects (mean \pm SD age: 54.2 \pm 5.3 years;
10	160 men and 258 women) who underwent health examinations in the Medical
11	Examination Center of the First Affiliated Hospital of the Xinxiang Medical College
12	(Xinxiang, China) between Oct 2009 and Sept 2013. None of the individuals in the
13	control group had a history of lung diseases or showed any symptoms of the lung or
14	other diseases by chest radiography or laboratory blood tests. Participants were
15	excluded if they: were taking other prescribed medications that could affect the
16	central nervous system; had a history of seizures, hematological diseases, or severe
17	liver or kidney impairment; or were pregnant. No familial relationship was known
18	between the study participants. The study was performed according to the Guidelines
19	of the Medical Ethical Committee of Xinxiang Medical College (Xinxiang, China).
20	Written informed consent was obtained from each participant in this study.
21	SND salestion

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21 SNP selection

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1	Tagging SNPs were selected from the catalogs of the International HapMap
2	Project. The rs2789679 and rs2245168 are located in 5'-UTR, rs2236558 in intron 6,
3	rs7067644 and rs12763624 in intron 8, rs1049550 in exon 10, rs2573351 in intron 13,
4	rs10887581, rs11201989, rs2573353, rs2789695, rs2573356, rs2819945, rs11202059
5	and rs2819941 in intron 14 (Supplemental figure 1). Marker selection was based on
6	the following criteria. (a) We used the CHB data from the HapMap (release 27) to
7	select tagSNPs for ANXA11. (b) We restricted our search for tagSNPs from base pair
8	80155124 to 80205677. (c) Further, we limited the SNPs to tag to those with a minor
9	allele frequency ≥ 0.2 in the CHB. (d) Based on these restrictions, there were a total
10	of 29 SNPs. (e) Using HAPLOVIEW with an r^2 threshold ≥ 0.8 , 15 tagSNP were
11	selected and used for subsequent analyses.

12 Genotyping

Peripheral blood was collected from a vein into a sterile tube coated with 13 ethylenediamine tetraacetic acid (EDTA). Plasma samples were stored at -20°C. 14 Genomic DNA was extracted from the frozen peripheral blood samples using a 15 QIAmp Blood Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the 16 manufacturer's protocols. The selected SNPs were genotyped in cases and controls by 17 18 using the MALDI-TOF in the MassARRAY system (Sequenom Inc., San Diego, CA, 19 USA). Probes and primers were designed using the Assay Design Software (Sequenom, San Diego, CA, USA). The completed genotyping reactions were spotted 20 onto a 384 well spectroCHIP (Sequenom) using the MassARRAY Nanodispenser 21 (Sequenom) and determined by the matrix - assisted laser desorption ionization time -22

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of - flight mass spectrometer. Genotype calling was performed in realtime with the
 MassARRAY RT software version 3.0.0.4 and analyzed using the MassARRAY Typer
 software version 3.4 (Sequenom).

4 Statistical analysis

5 All data were analyzed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, 6 USA). Hardy-Weinberg equilibrium was evaluated by Chi-square tests. Differences 7 between the cases and controls in the frequency of the alleles, genotypes and haplotypes were evaluated by the Fisher's exact test or the Pearson Chi-square test. 8 Unconditional logistic regression was used to calculate the odds ratio (OR) and 95% 9 confidence interval (CI) in independent association between each locus and the 10 presence of sarcoidosis. Logistic regression was used to evaluate the interaction 11 effects between gene and gender or age. Gender and age of subjects were treated as 12 covariants in binary logistic regression. P values were calculated based on 13 codominant, dominant for the rare allele, heterosis and recessive for the rare allele 14 15 models of inheritance. The Bonferroni correction was used to adjust the test level when multiple comparisons were conducted, and the P value was divided by the total 16 number of loci. Haplotype blocks were defined according to the criteria developed by 17 Gabriel et al. ¹⁵. Pair-wise LD statistics (D' and r^2) and haplotype frequency were 18 calculated, and haplotype blocks were constructed using the Haploview 4.0¹⁶. To 19 20 ensure that the LD blocks most closely reflect the population level LD patterns, 21 definition of the blocks were based on the control samples alone. The haplotype frequencies were estimated using GENECOUNTING, which computes maximum -22

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likelihood estimates of haplotype frequencies from unknown phase data by utilizing
 an expectation - maximization algorithm ¹⁷⁻²⁰. The significance of any haplotypic
 association was evaluated using a likelihood ratio test, followed by permutation
 testing that compared estimated haplotype frequencies in cases and controls ^{17 19}.

5 **RESULTS**

The genotype distribution of the fifteen polymorphisms was consistent with the 6 7 Hardy-Weinberg equilibrium (P > 0.05). The analysis of strong LD in the patients with sarcoidosis and healthy controls revealed that 2 SNPs (rs2245168, rs2236558), 2 8 SNPs (rs1049550, rs2573351), 2 SNPs (rs2789695, rs2573356) and 3 SNPs 9 (rs2819945, rs11202059, rs2819941) were located in haplotype block 1, block 2, 10 block 3 and block 4 (D' > 0.9, Fig. 1). The genotype distribution, allelic frequencies, 11 12 and haplotypes in the patients with sarcoidosis and healthy controls are showed in Tables 1-3 and Supplemental table 2 13

Comparison of genotype and allele frequency distribution revealed significant 14 differences between the patients with sarcoidosis and healthy controls for 3 SNPs: 15 rs2789679, rs1049550 and rs2819941. The frequency of the T allele in rs2789679 (P 16 = 0.0003, OR = 0.70, 95%CI = 0.58 - 0.85) and rs1049550 (P = 0.0007, OR = 0.62, 17 95%CI = 0.50 - 0.76), and C allele in rs2819941 (P = 0.001, OR = 0.71, 95%CI = 18 (0.59 - 0.87) in the patients with sarcoidosis was significantly lower than that in the 19 controls, and these differences remained statistically significant after Bonferroni 20 21 corrections. We presented a multi-SNP model of sarcoidosis risk. We screen out the most significant model to predict the risk by forward stepwise strategy in logistic 22

1	regression, and found that a model includes rs2789679 and rs1049550 is the best one
2	(Supplemental table 3), which suggests that there is a interaction between them.
3	We performed an association analysis to determine whether the haplotype was
4	associated with the risk of sarcoidosis (Global $P < 0.05$ in block 2 and block 3).
5	Compared with the controls, the T - C haplotype occurred significantly less frequently
6	(P = 0.001) but the C - C haplotypes occurred more frequently $(P = 0.0001)$ in block 2
7	(rs1049550 - rs2573351) in the patients with sarcoidosis.
8	To assess particular disease phenotypes, the patients with sarcoidosis were
9	divided into the subgroups according to their chest radiographic (CXR) stage. CXR
10	stage I (isolated bilateral hilar lymphadenopathy): $N = 196$; CXR stages II - IV
11	(infiltration of parenchyma): 167; CXR stage 0 (absence on pulmonary disease): $N =$
12	28; the information on CXR stage was not available for 21 patients. Genotype
13	frequency distribution revealed that, in rs1049550, CC genotype was significantly
14	more in the patients with chest radiographic (CXR) stage I sarcoidosis than the

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15 patients with CXR stage II-IV sarcoidosis (P = 0.012). (Table 4).

DISCUSSION

A key step in linkage and association studies is to identify common risk variants in different populations. To determine if common risk variants exist in distinct populations, fifteen SNPs which span approximately 0.53 Mb of the ANXA11 gene, were genotyped in samples from the patients with sarcoidosis and healthy controls in a Chinese Han population. Recently, new sarcoidosis loci have been identified by genome-wide association studies ^{4 14}. With the fast development of genome - wide

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1	association studies, an increasing number of susceptibility loci for sarcoidosis have
2	been found in different populations ⁴ ¹⁴ ²¹ . However, these observations should be
3	confirmed in other genetically independent populations. In this study, we conducted
4	the first large genetic association study of the ANXA11 gene in a Chinese Han
5	population. The evidence of markers associated with sarcoidosis was presented, and
6	these markers were mapped to different locations in the ANXA11 gene (81897864 -
7	81951001). The association signals in the region were identified, and some
8	significantly associated haplotypes also appeared this region.
9	Hoffman et al. reported an association between the T allele of the non -
10	synonymous SNP rs1049550 and significantly decreased risk of sarcoidosis in a
11	German population ⁴ . Similar results were also obtained in other two European
12	populations ^{14 21} . As a part of the sarcoidosis GWAS done in Americans ²² , we further
13	confirmed this association in a Chinese Han population. In this study, the frequency of
14	ANXA11 rs1049550T allele was significantly lower in the patients with sarcoidosis
15	than the healthy controls. There is interaction between rs2789679 and rs1049550.
16	Genotype frequency distribution revealed that, in rs1049550, CC genotype was
17	significantly more in the patients with stage I sarcoidosis than the patients with stage
18	II-IV sarcoidosis. The "protective" effect of ANXA11 T allele increased with the
19	number of its copies in the genotype, which is consistent with the result obtained in
20	the patients with sarcoidosis in a German population ⁴ , Furthermore, the T allele
21	carriers among the patients were protected from infiltration of lung parenchyma
22	(radiographic stages II-IV) 21 . We have also demonstrated that rs1049550 is

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significant cross - ethnically at the gene level after adjustment for the single SNP association tests performed. The mechanism by which rs1049550 affects the susceptibility to sarcoidosis is that the SNP may affect the function of the ANXA11 protein rather than affect its expression. In humans, the ANXA11 protein consists of an N - terminalproline - tyrosine - glycine (PYG) - rich region, followed by four annexin core domains. The rs1049550 leads to an amino - acid exchange (basic arginine to a polar cysteine) at the evolutionarily conserved position 230 (R230C) in the first annexin domain, which is responsible for Ca²⁺ - dependent trafficking of the protein in the cell 23 . In the GWAS conducted by Hofmann et al⁴, the strongest association signal was observed for rs2789679. This marker is located in the 3'-UTR of the ANXA11 gene. The T allele had a frequency of 35% among the patients with sarcoidosis and 44% in the healthy controls, with 13% of the patients with sarcoidosis and 19% of controls being TT homozygous. Fine-mapping in the Black Women's Health Study (BWHS) revealed rs2819941 to be the top SNP, associated with a non-significant (P = 0.06) reduction in the risk of sarcoidosis (17% reduction per copy of the C-allele). In this case-control association study, the frequency of the T allele in rs2789679 and the rs2819941 C allele frequency in the patients with sarcoidosis was significantly lower than that in the healthy controls. Several lines of evidence suggest that the observed

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21 haplotype-based association analyses support the current finding. Second, our samples

22 were from the same geographical region, excluding the . Finally, consistent results

association is unlikely to be an artifact. First, both the single-SNP and the

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1	were obtained from two genetically independent populations (Chinese Han and
2	Europeans). Collectively, our results confirmed the strong association between
3	ANXA11 polymorphisms and sarcoidosis, suggesting that ANXA11 represents a
4	strong genetic risk factor for sarcoidosis.

We further investigated the interaction among polymorphisms and observed strong LD. The haplotype analysis revealed that significantly more C - C (block 2, rs1049550 - rs2573351) and significantly fewer T - C haplotypes (block 2) were found in the patients with sarcoidosis. These results indicated that the patients with C - C (block 2) haplotypes of the ANXA11 gene were more prone to sarcoidosis. Significantly higher frequencies of T - C haplotypes were detected in the healthy controls than in the patients with sarcoidosis, suggesting that they may show protective effects against sarcoidosis. Our sample size can detect SNP and haplotype associations with 90% and 85% power, respectively, at a false positive rate of 5%, disease prevalence of 1%, disease allele / haplotype frequency of 0.05 / 0.03, and a presumed odds ratio (OR) of 1.5. To some extent, this finding further supports a role of ANXA11 polymorphisms in sarcoidosis, while ethnic group difference may exist.

In conclusion, our study suggests a potential role of the ANXA11 SNPs and their related haplotypes in the genetic susceptibility to sarcoidosis. Further studies are needed to investigate how these SNPs affect the function of ANXA11.

20 Contributors

LG Zhang was involved in conception and design of the study, acquisition of patient data, genotyping and drafted the paper. ZJ Xiao and GC Shi were involved in

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3	4	design of the study, construing and intermentation of results. I Ilyang and CV Thene
4	1	design of the study, genotyping and interpretation of results. J Huang and CX Zhang
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6	2	were involved in the design of the study, acquisition of patient data and interpretation
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8 9	3	of result. All authors revised the draft paper.
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2 The project was supported by the Research and Development Foundation of
3 Science and Technology of Henan Province (2012k15).

4 Contributorship Statement

LG Zhang was involved in conception and design of the study, acquisition of patient data, genotyping and drafted the paper. ZJ Xiao and GC Shi were involved in design of the study, genotyping and interpretation of results. J Huang and CX Zhang were involved in design of the study, acquisition of patient data and interpretation of result. All authors revised the draft paper.

10 Competing interests

- 11 None.
- 12 Data sharing statement
- 13 No additional data are available.
- **Patient consent**
- 15 Obtained.
- **Ethics approval**
- 17 Ethics approval was provided by the First Hospital Affiliated to the Xinxiang
- 18 Medical College.
- **Provenance and peer review**
- 20 Not commissioned; externally peer reviewed.

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41	10	Fig. 1 The LD glot of the fifteen SNDs in the ANYA11 error Velves in several are the
42	16	Fig. 1 The LD plot of the fifteen SNPs in the ANXA11 gene. Values in squares are the
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	17	pair-wise calculation of 1 (left) of D (light). Diack squares indicate 1 = 1 (i.e. perfect
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51	20	Supplemental Fig. 1 The structure of the human ANXA11 gene and 15 SNPs located
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2 **Table 1** Genotypic and allelic frequencies of ANXA11 polymorphisms in the controls

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and patients with sarcoidosis

	Contro	ls (n=418)	Sarcoidos	sis (n=412)	- • a			
Variable	No. %		No. % No. %		%	- P-value ^a	OR, 95% CI	
rs2789679					0.0007			
AA	79	18.9	132	32.0	0.0002	0.49, 0.36-0.6		
AT	225	53.8	186	45.2	0.012	1.42, 1.08-1.8		
TT	114	27.3	94	22.8	0.140	1.27, 0.93-1.7		
Per T allele	383	45.8	450	54.6	0.0003	0.70, 0.58-0.8		
rs1049550					0.0002			
CC	154	36.8	208	50.5	0.0007	0.57, 0.43-0.7		
СТ	190	45.5	170	41.3	0.221	1.19, 0.90-1.5		
TT	74	17.7	34	8.3	0.0008	2.39, 1.55-3.6		
Per T allele	338	40.4	238	28.8	0.0007	0.60, 0.49-0.7		
rs2819941					0.0002			
CC	114	27.3	94	22.8	0.141	1.27, 0.93-1.7		
СТ	226	54.1	190	46.1	0.021	1.38, 1.05-1.8		
TT	78	18.7	128	31.1	0.0004	0.51, 0.37-0.7		
Per T allele	382	45.7	446	54.1	0.001	0.71, 0.59-0.8		

4 ^a P-value was calculated by 2×3 and 2×2 chi-squared tests based on codominant,

5 dominant for the rare allele, heterosis and recessive for the rare allele models of

6 inheritance.

7 Alpha value is adjusted by Bonferroni correction and statistically significant results

8 (P<0.003)

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Table 2 ANXA11 haplotype in block 2 frequencies and the results of their associations with risk

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	Haplotype			Genecounting	g (frequency %	%)
ID	rs1049550	rs2573351	Cases	Controls	<i>P</i> -value ^a	Global P ^b
HAP1	Т	С	27.7	39.7	0.001	0.003
HAP2	С	С	19.9	7.4	0.0001	
HAP3	С	Т	50.5	51.0	0.892	

^a Based on 10,000 permutations.

5	^b Based on comparison of frequency distribution of all haplotypes for the combination of SNPs.
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3	Table 3 ANXA11 haplotype in block 3 frequencies and the results of their associations with risk

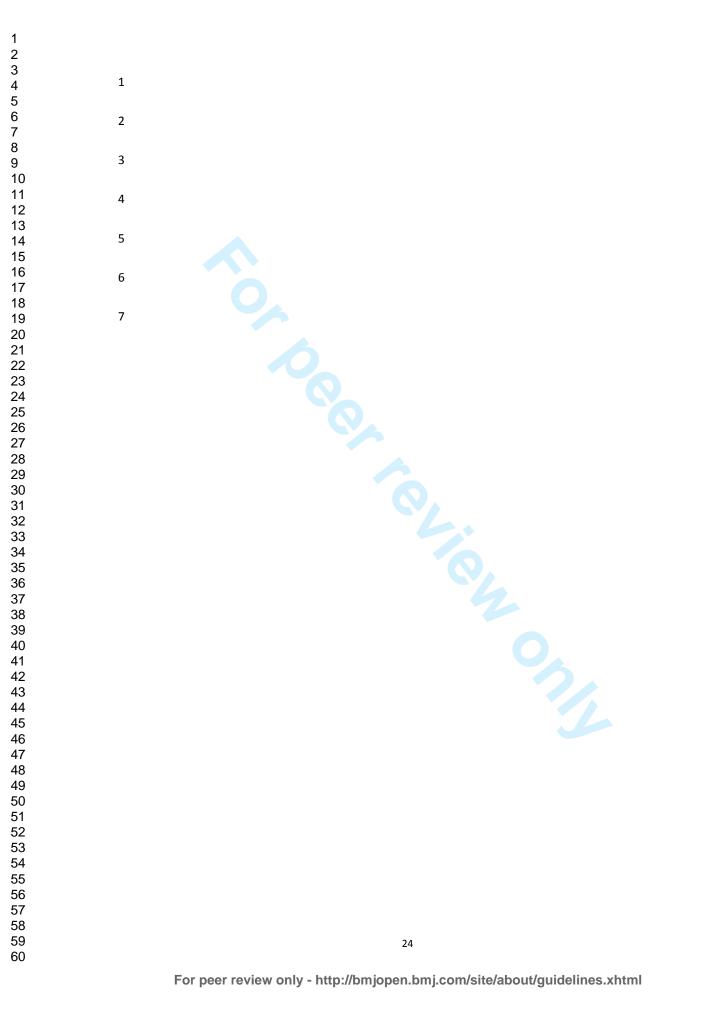
			of sarcoidosi	S		
	Haplotype			Genecounting	g (frequency %	6)
ID	rs2789695	rs2573356	Cases	Controls	<i>P</i> -value ^a	Global P ^b
HAP1	С	Т	33.7	34.9	0.718	0.045
HAP2	Т	Т	15.3	14.1	0.632	
HAP2	Т	Т	15.6	14.1	0.565	

^a Based on 10,000 permutations.

6	^b Based on comparison	of frequency	distribution of	all haplotypes	for the combination of SNPs.
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		rs2'	789679 (n	,%)	rs1(049550 [*] (n	,%)	rs28	819941 (n	,%)
	Stages	AA	AT	TT	CC	СТ	TT	CC	СТ	TT
		59	92	45	101	82	13	47	88	61
	stage I	(30.1)	(47.0)	(23.0)	(51.5)	(41.8)	(6.6)	(25.0)	(44.9)	(31.1)
	stages	46	75	46	72	68	27	41	73	53
	II - IV	(27.6)	(44.9)	(27.6)	(43.1)	(40.7)	(16.2)	(24.6)	(43.7)	(31.7)
	χ^2, P	1	1.041, 0.59	94	8	3.807, 0.01	2	0	0.052, 0.97	5
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Annexin A11 (ANXA11) gene polymorphisms are associated with sarcoidosis in a Han Chinese population - a case-control study LG Zhang, ZJ Xiao^{*}, GC Shi, J Huang, CX Zhang The Third Department of TuberculosisInternal Medicine, the First Hospital Affiliated

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Abstract

Objectives: To further identify the single—_nucleotide polymorphisms (SNPs) that contribute to the genetic susceptibility to sarcoidosis, we examined the potential association between sarcoidosis and fifteen SNPs of the ANXA11 gene.

Design: A case - control study.

Setting: A tuberculosist unit in a university hospital of the university in China.

Participants: Participants included 412 patients with sarcoidosis and 418 healthy controls.

Methods: The selected SNPs were genotyped in cases and controls by using the MALDI—_TOF in the MassARRAY system (Sequenom Inc., San Diego, CA, USA). Probes and primers were designed using the Assay Design Software (Sequenom, San Diego, CA, USA).

Results: The results showed that <u>statisticalstatistically</u> significant differences were observed_found_in the allelic or genotypic frequencies of the rs2789679, rs1049550 and rs2819941 in the ANXA11 gene_between the patients with sarcoidosis and the controls. The frequency of the T allele in rs2789679 (P = 0.0003, OR = 0.70, 95%CI = 0.58 - 0.85), T allele in_and rs1049550 (P = 0.0007, OR = 0.62, 95%CI= 0.50 - 0.76), and C allele the in rs2819941 C allele frequency (P = 0.001, OR = 0.71, 95%CI = 0.59 - 0.87) in the patients with sarcoidosis was significantly lower than that in the controls. Furthermore, The strong linkage disequilibrium (LD) was observed in four blocks (D' > 0.9). In block 2 (rs1049550-rs2573351), the T - C haplotype occurred significantly less frequently (P = 0.001), whereas the C - C haplotypes occurred more

frequently (P = 0.0001) in the patients with sarcoidosis than the controls. Furthermore, <u>e</u>-Comparison of genotype frequency distribution_-revealed that, in rs1049550, CC genotype was significantly more in the patients with chest radiographic (CXR) stage I sarcoidosis than the patients with CXR stage II-IV sarcoidosis (P = 0.012), significant differences between chest radiographic (CXR) stage I and stages II – IV for rs1049550. The significantly more CC genotype (P = 0.012) were found in the patients with stages I sarcoidosis.

-Conclusions: These findings point to a role for <u>the polymorphisms of ANXA11</u> <u>gene polymorphisms</u>-in sarcoidosis in a <u>Han ChineseChinese Han</u> population, and may be informative for future genetic or biological studies on sarcoidosis.



Article summary

Article focus

1. The first genome—_wide association study (GWAS) in sarcoidosis conducted in a German population has revealed that the rs1049550 (C > T, Arg230Cys) located in exon 10 were associated with sarcoidosis.

2. More studies should be performed to demonstrate the following item: wWhether these SNPs modulate the risk of sarcoidosis by themselves or whether they correlate with other causative SNPs and are repeated in other populations remain unclear.

3. We hypothesize that common variants in the ANXA11 gene may significantly contribute to the predisposition to develop sarcoidosis in a Chinese Han population.

Key messages

1. ANXA11 rs2789679 (3'UTR3'-UTR) was found to be associated was significantly

associated _-with sarcoidosis.

2. ANXA11 rs1049550 (exon 10) was significantly associated with sarcoidosis.

3. Another significant association was observed for rs2819941 (intron 14).

Strengths and limitations of this study

1. <u>A systematical screening of the Ff</u>unctional SNPs in the promoter region, 5'- and 3'-UTR, exons of <u>the ANXA11</u> gene, and the homogeneity of the study subjects representing the Chinese Han are the main strengths of this study.were systematically screened and homogeneity of the study subjects, representing the Han Chinese Han, is the main strength of the current study.

2. The lack of data proving the positive association observed for rs2789679 and

rs1049550 is a potential limitation of this study. Furthermore, the association of the serum level of ANXA11 with sarcoidosis still needs to be investigated. Functional characterization is the potential limitation of the study that could have further helped in proving the positive association observed for rs2789679 and rs1049550. The lack of correlation of serum ANXA11 levels are needed to investigate.

INTRODUCTION

Sarcoidosis is a systemic autoimmune disease characterized by destructive, non--caseating epithelioid granulomatous lesions, with accumulated phagocytes and activated CD4⁺ T helper type 1 lymphocytes ^{1 2}. The typical manifestations of sarcoidosis include bilateral hilar lymphadenopathy, pulmonary infiltration and ocular and skin lesions. The clinical course and prognosis of sarcoidosis are variable. The acute forms of sarcoidosis such as L fgren's syndrome (LS) can resolve spontaneously in most cases. However, chronic pulmonary sarcoidosis may progress to lung fibrosis which and eventually result incauses respiratory failure. Recent studies have demonstrated that sarcoidosis can occur in a pattern of family clustering, and its racial incidence is also different, suggesting that some genetic factors may contribute to the risk and severity of sacroidosis ¹³. BMJ Open: first published as 10.1136/bmjopen-2013-004466 on 23 July 2014. Downloaded from http://bmjopen.bmj.com/ on June 14, 2025 at Agence Bibliographique de Enseignement Superieur (ABES)

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The Annexin gene family is involved in the etiology of several autoimmune and chronic diseases ^{4 5.} One member of the Annexin gene family, ANXA11, located on chromosome 10q22.3, is involved in calcium signaling, apoptosis, vesicle trafficking, cell growth and terminal phase of cell division ⁵⁻⁷. In this context, the development and maintenance of the granulomatous inflammation in sarcoidosis have been

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repeatedly associated with the impaired apoptosis of activated inflammatory cells ⁸⁹. In most patients with sarcoidosisMost of the early-stage sarcoidosis, the early stages of this disease are <u>is</u> characterized by mononuclear cell alveolitis dominated by activated CD4⁺ T cells and macrophages. Between these cells, the uncoordinated interplay results in the formation of typical non—__caseating granulomas. The mechanism by which the granulomas resolve has not been fully elucidated. However, it is generally assumed that the induction of apoptosis and-/_/or by the withdrawal of inflammatory cytokines participates in the disappearance of granulomas ^{10 11}. In the patients with sarcoidosis, their peripheral blood monocytes were characterized by apoptosis ⁹.

In previous studies, case - control // family 'hypothesis - driven' studies and low density linkage scans were used to identify genetic factors conferring the genetic susceptibility to sarcoidosis ¹². Notably, the first genome - wide association study (GWAS) in sarcoidosis conducted in a German population has recently revealed an association: the leading rs2789679 SNP (single - nucleotide polymorphism) located in the 3'—untranslated region (3'—UTR) and a common nonsynonymous SNP (rs1049550, C > T, Arg230Cys) were associated with the increased risk of sarcoidosis ⁴ ¹³. This association has recently been supported by another report from the same population ¹⁴. Thus, more studies should be performed to demonstrate the following items: whether these SNPs modulate the risk of sarcoidosis by themselves or whether they correlate with other causative SNPs and are repeated in other populations. The published studies about the association of sarcoidosis and ANXA11—association

studies for ANXA11 are summarized in Supplemental table <u>21</u>. We hypothesize that common variants in the ANXA11 gene may significantly contribute to the predisposition to develop sarcoidosis.

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In this study, we investigated fifteen loci in a Chinese population from He'nan province (China) to verify the putative association between ANXA11 polymorphisms and sarcoidosis.

SUBJECTS AND METHODS

Subjects

Four hundred and twelve patients with sarcoidosis (mean \pm SD age: 53.6 \pm 4.6 years; 155 men and 257 women) were recruited from our hospital between May 2005 and March 2013. Sarcoidosis was diagnosed by the evidence of non—_caseating epitheloid cell granuloma in biopsy specimens and chest radiographic abnormalities. Chronic sarcoidosis was defined as a disease over at least 2 years or at least two episodes in a lifetime. A cute sarcoidosis was defined as one episode of acute sarcoidosis which had totally resolved at the date of the examination. The control group consisted of 418 unrelated healthy subjects (mean \pm SD age: 54.2 \pm 5.3 years; 160 men and 258 women) who underwent health examinations in the Medical Examination Center of the First Affiliated Hospital of the Xinxiang Medical College (Xinxiang, China) between Oct 2009 and Sept 2013. None of the individuals in the control group had a history of lung diseases or showed any symptoms of the lung or other diseases by chest radiography or laboratory blood tests. Participants were excluded if they: were taking other prescribed medications that could affect the

central nervous system; had a history of seizures, hematological diseases, or severe liver or kidney impairment; or were pregnant. No familial relationship was known between the study participants. The study was performed according to the Guidelines of the Medical Ethical Committee of Xinxiang Medical College (Xinxiang, China). Written informed consent was obtained from each participant in this study.

SNP selection

Tagging SNPs were selected from the catalogs of the International HapMap Project. The rs2789679 and rs2245168 are located in 5'-UTR, rs2236558 in intron 6, rs7067644 and rs12763624 in intron 8, rs1049550 in exon 10, rs2573351 in intron 13, rs10887581, rs11201989, rs2573353, rs2789695, rs2573356, rs2819945, rs11202059 and rs2819941 in intron 14 (Supplemental figure 1). Marker selection was based on the following criteria. (a) We used the CHB data from the HapMap (release 27) to select tagSNPs for ANXA11. (b) We restricted our search for tagSNPs from base pair 80155124 to 80205677. (c) Further, we limited the SNPs to tag to those with a minor allele frequency > 0.2 in the CHB. (d) Based on these restrictions, there were a total of 29 SNPs. (e) Using HAPLOVIEW with an r^2 threshold > 0.8, 15 tagSNP were selected and used for subsequent analyses.

Marker selection was done according to previous studies 4 12 14 15, and preliminary analysis was performed using the HapMap data and the following criteria. First, examined tagSNPs in the Haploview (v4.2), using the CHB population and a minor allele frequency cut – off (MAF) \geq 5% (HapMap Data Release 27). We found that there were a total of 29 potential tagSNPs in all. As a first screen of the most common

SNPs in the sarcoidosis sample from an Eastern Chinese Han population, a MAF $\geq 20\%$ with pair – wise tagging and $r^2 \geq 0.8^{-16}$ was used as the cut – off when choosing tagSNPs. Second, the LD pattern of this gene was determined in the Chinese population using the preliminary data from the HapMap. Five LD blocks across the ANXA11 were defined using Haploview's 'confidence intervals' method ¹⁷⁻¹⁸. These SNPs were further analyzed in an association study.

Genotyping

Peripheral blood was collected from a vein into a sterile tube coated with ethylenediamine tetraacetic acid (EDTA). Plasma samples were stored at -20°C. Genomic DNA was extracted from the frozen peripheral blood samples using a QIAmp Blood Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocols. The selected SNPs were genotyped in cases and controls by using the MALDI—_TOF in the MassARRAY system (Sequenom Inc., San Diego, CA, USA). Probes and primers were designed using the Assay Design Software (Sequenom, San Diego, CA, USA). The completed genotyping reactions were spotted onto a 384 well spectroCHIP (Sequenom) using the MassARRAY Nanodispenser (Sequenom) and determined by the matrix - assisted laser desorption ionization time - of - flight mass spectrometer. Genotype calling was performed in realtime with the MassARRAY RT software version 3.0.0.4 and analyzed using the MassARRAY Typer software version 3.4 (Sequenom).

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Statistical analysis

All data were analyzed using the SPSS 17.0 software (SPSS Inc., Chicago, IL,

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USA). Hardy-Weinberg equilibrium were was evaluated by Chi-square tests. Differences between the cases and controls in the frequency of the alleles, genotypes and haplotypes were evaluated by the Fisher's exact test or the Pearson Chi---square test. Unconditional logistic regression was used to calculate the odds ratio (OR) and 95% confidence interval (CI) in independent association between each locus and the presence of sarcoidosis. Logistic regression Generalized linear regression was used to evaluate the interaction effects between gene and gender or age. Gender and age of subjects were treated as covariants in binary logistic regression. P- values was-were calculated based on codominant, dominant for the rare allele, heterosis and recessive for the rare allele models of inheritance. The Bonferroni correction was used to adjust the test level when multiple comparisons were conducted, and the P value was divided by the total number of loci. Haplotype blocks were defined according to the criteria developed by Gabriel et al.¹⁵. Pair—wise LD statistics (D' and r²) and haplotype frequency were calculated, and haplotype blocks were constructed using the Haploview 4.0¹⁶. To ensure that the LD blocks most closely reflect the population level LD patterns, definition of the blocks were based on the control samples alone. The haplotype frequencies were estimated using GENECOUNTING, which computes maximum - likelihood estimates of haplotype frequencies from unknown phase data by utilizing an expectation - maximization algorithm 17-20. The significance of any haplotypic association was evaluated using a likelihood ratio test, followed by permutation testing that compared estimated haplotype frequencies in cases and controls 17 19.

RESULTS

The genotype distribution of the fifteen polymorphisms was consistent with the Hardy—_Weinberg equilibrium (P > 0.05). The analysis of strong LD in the patients with sarcoidosis and healthy controls revealed that 2 SNPs (rs2245168, rs2236558), 2 SNPs (rs1049550, rs2573351), 2 SNPs (rs2789695, rs2573356) and 3 SNPs (rs2819945, rs11202059, rs2819941) were located in haplotype block 1, block 2, block 3 and block 4 (D' > 0.9, Fig. <u>21</u>). The genotype distribution, allelic frequencies, and haplotypes in the patients with sarcoidosis and healthy controls are showed in tables-Tables <u>21, -3 and Supplemental table 2</u>table 4 and table <u>5</u>.

Comparison of genotype and allele frequency distribution revealed significant differences between the patients with sarcoidosis and healthy controls for 3 SNPs: rs2789679, rs1049550 and rs2819941. The frequency of the T allele in rs2789679 (P = 0.0003, OR = 0.70, 95%CI = 0.58 - 0.85), T allele and rs1049550 (P = 0.0007, OR = 0.62, 95%CI = 0.50 - 0.76), and C allele in rs2819941 the rs2819941 C allele frequency (P = 0.001, OR = 0.71, 95%CI = 0.59 - 0.87) in the patients with sarcoidosis was significantly lower than that in the controls, and these differences remained statistically significant after Bonferroni corrections. We presented a multi-SNP model of sarcoidosis risk. We screen out the most significant model to predict the risk by forward stepwise strategy in logistic regression, and found that a model includes rs2789679 and rs1049550 is the best one (Supplemental table 3), which suggests that there is a interaction between them.

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There were no interactions among rs2789679, rs1049550 and rs2819941 (P > 0.05).

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We performed an association analysis to determine whether the haplotype was associated with the risk of sarcoidosis (Global P < 0.05 in block 2 and block 3). Compared with the healthy-controls, the T - C haplotype occurred significantly less frequently (P = 0.001) and but the C - C haplotypes occurred more frequently (P = 0.001) and but the C - C haplotypes occurred more frequently (P = 0.001) in block 2 (-rs1049550 - rs2573351) in the patients with sarcoidosis.—

To assess particular disease phenotypes, the patients with sarcoidosis were divided into the subgroups according to their chest radiographic (CXR) stage. CXR stage I (isolated bilateral hilar lymphadenopathy): N = 196; CXR stages II - IV (infiltration of parenchyma): 167; CXR stage 0 (absence on pulmonary disease): N = 28; the information on CXR stage was not available for 21 patients. Genotype frequency distribution revealed that, in rs1049550, CC genotype was significantly more in the patients with chest radiographic (CXR) stage I sarcoidosis than the patients with CXR stage II-IV sarcoidosis (P = 0.012).Comparison of genotype frequency distribution revealed significant differences between stage I and stages II-IV for rs1049550. The significantly more CC genotype (P = 0.012) were found in the patients with stage I sarcoidosis (table Table 34).

DISCUSSION

A key step in linkage and association studies is to identify common risk variants in different populations. To determine if common risk variants exist in distinct populations, fifteen SNPs which span approximately 0.53 Mb of the ANXA11 gene, were genotyped in samples from the patients with sarcoidosis and healthy controls in a Chinese Han population. Recently, new sarcoidosis loci have been identified by

genome—_wide association studies ^{4 14}. With the fast development of genome - wide association studies, <u>an</u>_increasing number of susceptibility loci for sarcoidosis have been <u>reported_found_in</u> different populations ^{4 14 21}. However, these observations should be confirmed in other genetically independent populations. In this study, we conducted the first large genetic association study of the ANXA11 gene in a Chinese Han population. The evidence of markers associated with sarcoidosis was presented, and these markers were mapped to different locations in the ANXA11 gene (81897864 - 81951001). The association signals in the region were identified, and some significantly associated haplotypes also appeared this region.

Hoffman et al. reported an association between the T allele of the non synonymous SNP rs1049550 and significantly decreased risk of sarcoidosis in a German population ⁴. Similar results were also obtained in other two European populations ¹⁴²¹. As a part of the sarcoidosis GWAS done in Americans ²², we further confirmed this association_in a Chinese Han population. In this study, the frequency of ANXA11 rs1049550T allele was significantly lower in the patients with sarcoidosis than the healthy controls. <u>There is interaction between rs2789679 and rs1049550</u>. <u>Genotype frequency distribution revealed that, in rs1049550, CC genotype was significantly more in the patients with stage I sarcoidosis than the patients with stage II-IV sarcoidosisComparison of stages II – IV, the significantly more CC genotypewas found in the patients with stages I sarcoidosis. The "protective" effect of ANXA11 T allele increased with the number of its copies in the genotype, which is consistent with the result obtained in the patients with sarcoidosis in a German population ⁴,</u> BMJ Open: first published as 10.1136/bmjopen-2013-004466 on 23 July 2014. Downloaded from http://bmjopen.bmj.com/ on June 14, 2025 at Agence Bibliographique de Enseignement Superieur (ABES) . Protected by copyright, including for uses related to text and data mining, Al training, and similar technologies.

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Furthermore, the T allele carriers among the patients were protected from infiltration of lung parenchyma (radiographic stages II-IV) ²¹. We have also demonstrated that rs1049550 is significant cross - ethnically at the gene level after adjustment for the single SNP association tests performed. The mechanism by which rs1049550 affects the susceptibility to sarcoidosis may beis that the SNP may affect the function of the ANXA11 protein rather than affect its expression. In humans, the ANXA11 protein consists of an N - terminalproline - tyrosine - glycine (PYG) - rich region, followed by four annexin core domains. The rs1049550 leads to an amino - acid exchange (basic arginine to a polar cysteine) at the evolutionarily conserved position 230 (R230C) in the first annexin domain, which is responsible for Ca^{2+} - dependent trafficking of the protein in the cell ²³.

In the GWAS conducted by Hofmann et al ⁴, the strongest association signal was observed for rs2789679. This marker is located in the 3'—_UTR of the ANXA11 gene. The T allele had a frequency of 35% among the patients with sarcoidosis and 44% in the healthy controls, with 13% of the patients with sarcoidosis and 19% of controls being TT homozygous. Fine—_mapping in the Black Women's Health Study (BWHS) revealed rs2819941 to be the top SNP, associated with a non_significant (P = 0.06) reduction in the risk of sarcoidosis risk (17% reduction per copy of the C-allele). In this case—_control association study, the frequency of the T allele in rs2789679 and the rs2819941 C allele frequency in the patients with sarcoidosis was significantly lower than that in the healthy controls. Several lines of evidence suggest that the observed associations areis unlikely to be an artifact. First, both the single—_SNP and

the haplotype—__based association analyses support the current finding. Second, <u>our</u> <u>samples were from the same geographical region</u>, <u>excluding the population</u> <u>stratification is an impossible reason</u>, because all of our samples were from the same <u>geographical region</u>. Finally, consistent results were obtained from two genetically independent populations (Chinese Han and Europeans). Collectively, our results confirmed the strong association between variations in the ANXA11 polymorphisms gene-and sarcoidosis, and-suggesting that ANXA11 represents a strong genetic risk factor for sarcoidosis.

We further investigated the interaction among polymorphisms and observed strong LD. The haplotype analysis revealed that significantly more C - C (block 2, rs1049550 - rs2573351) and significantly fewer T - C haplotypes (block 2) were found in the patients with sarcoidosis. These results indicated that the patients with C - C (block 2) haplotypes of the ANXA11 gene were more prone to sarcoidosis. Significantly higher frequencies of T - C haplotypes were detected in the healthy controls than in the patients with sarcoidosis, suggesting that they may show—a protective effects against sarcoidosis. Our sample size can detect SNP and haplotype associations with 90% and 85% power, respectively, at a false positive rate of 5%, disease prevalence of 1‰, disease allele / haplotype frequency of 0.05 / 0.03, and a presumed odds ratio (OR) of 1.5. To some extent, this finding further supports a role of ANXA11 polymorphisms in sarcoidosis, while ethnic group difference may exist. BMJ Open: first published as 10.1136/bmjopen-2013-004466 on 23 July 2014. Downloaded from http://bmjopen.bmj.com/ on June 14, 2025 at Agence Bibliographique de Enseignement Superieur (ABES)

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In conclusion, our study suggests a potential role of the ANXA11 SNPs and their related haplotypes in the genetic susceptibility to sarcoidosis. Further studies are needed to investigate how these SNPs affect the function of ANXA11.

Contributors

LG Zhang was involved in conception and design of the study, acquisition of patient data, genotyping and drafted the paper. ZJ Xiao and GC Shi were involved in design of the study, genotyping and interpretation of results. J Huang and CX Zhang were involved in the design of the study, acquisition of patient data and interpretation of result. All authors revised the draft paper.

Funding

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Competing interests

None.

Patient consent

Obtained.

Ethics approval

Ethics approval was provided by the First Hospital Affiliated to the Xinxiang

Medical College.

Provenance and peer review

Not commissioned; externally peer reviewed.

Data sharing statement

No additional data are available.

Figure Legend

Fig. 1 The structure of the human ANXA11 gene and 15 SNPs located on the gene. Fig. 2-1 The LD plot of the fifteen SNPs in the ANXA11 gene. Values in squares are the pair-wise calculation of r^2 (left) or D' (right). Black squares indicate $r^2 = 1$ (i.e.

perfect LD between a pair of SNPs). Empty squares indicate D' = 1 (i.e. complete LD between a pair of SNPs).

Supplemental Fig. 1Fig. 1 The structure of the human ANXA11 gene and 15 SNPs located on the gene.

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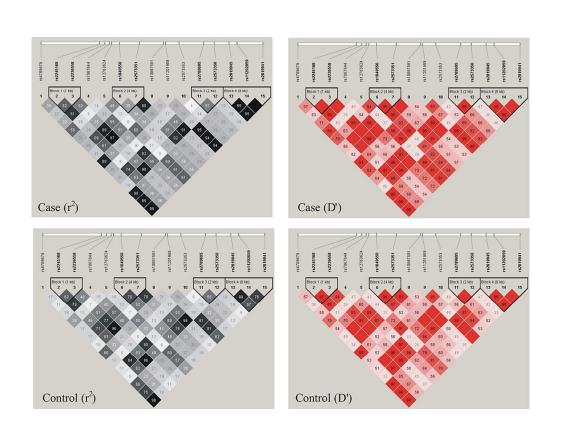
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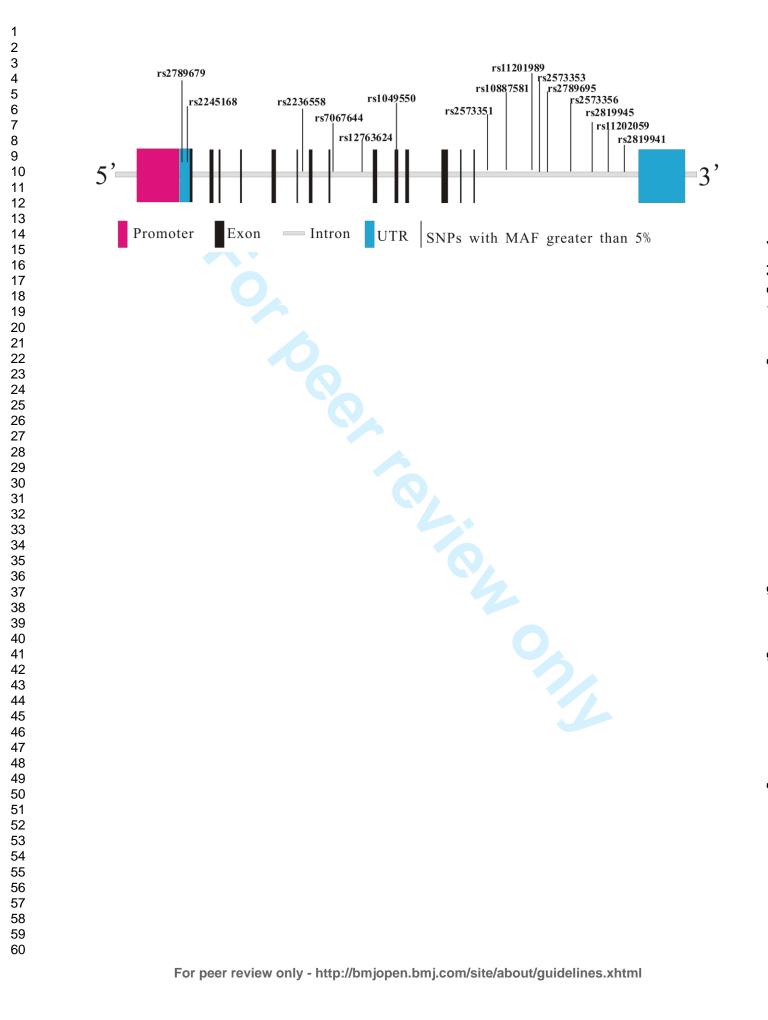
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Study	Popula	tion	Type of Study	Sample S	ize (n)	Number of	Positive SNPs
Study	Ethnic group	Country	Type of Study	Sarcoidosis	Control	SNPs typed	Fositive SINFS
Hofmann S, 2008	Caucasian	Germany	Case-control	499	490	GWAS	rs2789679, rs7091565, rs1049550
Mrazek F, 2011	Caucasian	Czech	Case-control	245	254	1	rs1049550
Cozier YC, 2012	Caucasian	America	Case-control	486	943	10p12-10q22	rs2789679, rs2819941
Li Y, 2013	Caucasian	Germany	Case-control	325	364	1	rs1049550
Levin AM, 2013	Caucasian	America	Case-control	1689	1252	25	rs1049550
Morais A, 2013	Caucasian	Portugal	Case-control	208	197	1	rs1049550

Supplemental table 1 Summary of all published sarcoidosis-association studies for ANXA11

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Variable	Controls (n=418)		Sarcoido	Sarcoidosis (n=412)		
	No.	%	No.	%	- <i>P</i> -value ^a	OR, 95% CI
rs2245168					0.920	
CC	156	37.3	150	36.4	0.791	1.04, 0.74-1.38
СТ	200	47.9	197	47.8	0.955	0.99, 0.76-1.30
TT	62	14.8	65	15.8	0.781	0.95, 0.65-1.39
Per T allele	324	38.8	327	39.7	0.698	1.04, 0.85-1.27
rs2236558					0.172	
GG	118	28.2	104	25.2	0.72	1.06, 0.77-1.45
TG	190	45.5	214	51.9	0.060	0.77, 0.59-1.01
TT	110	26.3	94	22.8	0.073	1.33, 0.97-1.82
Per T allele	410	49.0	402	48.8	0.917	0.99, 0.82-1.20
rs7067644					0.173	
AA	152	36.4	176	42.7	0.059	0.76, 0.58-1.01
GA	206	49.3	182	44.2	0.137	1.23, 0.94-1.68
GG	60	14.4	54	13.1	0.597	1.11, 0.75-1.65
Per G allele	326	38.0	290	35.2	0.109	0.85, 0.70-1.04
rs12763624					0.118	
TT	126	75.6	150	36.4	0.008	0.68, 0.51-0.91
СТ	216	51.7	186	45.2	0.059	1.30, 0.99-1.71
CC	76	18.2	76	18.5	0.411	1.16, 0.81-1.67
Per C allele	368	44.0	338	41.0	0.216	0.88, 0.73-1.08
rs2573351					0.437	

Supplemental Table 2 Genotypic and allelic frequencies of ANXA11 polymorphisms in the controls and patients with sarcoidosis

TT	120	28.7	104	25.2	0.263	1.19, 0.88-1.62
СТ	202	48.3	216	52.4	0.238	0.85, 0.65-1.12
CC	96	23.0	92	22.3	0.826	1.04, 0.75-1.44
Per C allele	394	47.1	400	48.5	0.564	1.06, 0.87-1.28
rs10887581					0.290	
TT	114	27.3	96	23.3	0.190	1.23, 0.90-1.69
TC	190	45.5	208	50.5	0.148	0.82, 0.62-1.07
CC	114	27.3	108	26.2	0.729	1.06, 0.78-1.44
Per C allele	418	50.0	424	51.5	0.583	1.06,0.87-1.29
rs11201989					0.144	
GG	130	31.1	128	31.1	0.995	1.00, 0.75-1.34
GC	218	52.2	194	47.1	0.141	1.23, 0.93-1.62
CC	70	16.8	90	21.9	0.060	0.71, 0.50-1.01
Per C allele	358	42.8	374	45.4	0.29	1.11, 0.91-1.35
rs2573353					0.918	
CC	178	42.6	176	42.7	0.957	0.99, 0.75-1.31
CA	196	46.9	192	46.6	0.921	1.01, 0.77-1.33
AA	44	10.5	44	10.7	0.941	0.98, 0.63-1.53
Per A allele	284	34.0	280	34.0	0.997	1.00, 0.82-1.23
rs2789695					0.918	
TT	178	42.6	180	34.0	0.740	0.95, 0.73-1.26
СТ	188	45.0	184	44.7	0.920	1.01, 0.77-1.33
CC	52	12.4	48	11.7	0.73	1.08, 0.71-1.64
Per C allele	292	34.9	280	34.0	0.69	0.96, 0.78-1.17
rs2573356					0.892	
CC	118	28.2	112	27.2	0.742	1.05, 0.78-1.43

СТ	190	45.5	194	47.1	0.639	0.94, 0.71-1.23
TT	110	26.3	106	25.7	0.843	1.03, 0.76-1.41
Per T allele	410	49.0	406	49.3	0.926	0.99, 0.82-1.20
rs2819945					0.101	
GG	119	28.5	130	31.6	0.294	0.85, 0.63-1.15
GA	221	52.9	188	45.6	0.030	1.35, 1.03-1.78
AA	78	18.7	94	22.8	0.138	0.78, 0.55-1.09
Per A allele	377	45.1	376	45.6	0.827	1.02, 0.84-1.24
rs11202059					0.064	
GG	106	25.4	128	31.1	0.067	0.75, 0.56-1.02
GA	226	54.1	190	46.1	0.021	1.38, 1.05-1.81
AA	86	20.6	94	22.8	0.429	0.88, 0.63-1.22
Per A allele	398	47.6	378	45.9	0.479	0.93, 0.77-1.13

^a *P*-value was calculated by 2×3 and 2×2 chi-squared tests based on codominant, dominant for the rare allele, heterosis and recessive for the rare allele models of inheritance.

Alpha value is adjusted by Bonferroni correction and statistically significant results (P<0.003) Zani resurts i

Supplemental table 3 Comparison of logistic regression models with and without

Genotypes for

rs2789679 and rs1049550

Model	Log Likelihood	Df	P valu
Constant + rs2789679	575.3	1	< 0.00
Constants+ rs2789679+rs1049550	565.8	2	0.001

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Annexin A11 (ANXA11) gene polymorphisms are associated with sarcoidosis in a Han Chinese population - a casecontrol study

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1	Annexin A11 (ANXA11) gene polymorphisms are associated with sar
2	Han Chinese population - a case-control study
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12	Key words: Sarcoidosis; Annexin A11; Single-nucleotide polymorphism
13	Word count: 3918
14	Word count: 3918

1	Abstract
2	Objectives: To further identify the single-nucleotide polymorphisms (SNPs) that
3	contribute to the genetic susceptibility to sarcoidosis, we examined the potential
4	association between sarcoidosis and fifteen SNPs of the ANXA11 gene.
5	Design: A case - control study.
6	Setting: A tuberculosist unit in a hospital of the university in China.
7	Participants: Participants included 412 patients with sarcoidosis and 418 healthy
8	controls.
9	Methods: The selected SNPs were genotyped using the MALDI-TOF in the
10	MassARRAY system. Probes and primers were designed using the Assay Design
11	Software (Sequenom, San Diego, CA, USA).
12	Results: The statistical significant differences were found in the allelic or genotypic
13	frequencies of the rs2789679, rs1049550 and rs2819941 in the ANXA11 gene
14	between the patients with sarcoidosis and the controls. The rs2789679 A allele ($P =$
15	0.00004, OR = 1.42, 95%CI = 1.17-1.73) and rs2819941 T allele ($P = 0.0006$, OR =
16	1.41, 95% CI = 1.16-1.71) were significantly more frequent in the patients with
17	sarcoidosis compared to controls. The frequency of the rs1049550 T allele ($P =$
18	0.000002, OR = 0.61, 95%CI= 0.49 - 0.74) in the patients with sarcoidosis was
19	significantly lower than that in the controls. The strong linkage disequilibrium (LD)
20	was observed in four blocks (D' > 0.9). In block 2 (rs1049550-rs2573351), the T - C
21	haplotype occurred significantly less frequently ($P = 0.001$), whereas the C - C
22	haplotypes occurred more frequently ($P = 0.0001$) in the patients with sarcoidosis

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than the controls. Furthermore, genotype frequency distribution revealed that, in rs1049550, CC genotype was significantly more in the patients with chest radiographic (CXR) stage I sarcoidosis than the patients with CXR stage II-IV sarcoidosis (P = 0.012).

Conclusions: These findings point to a role for the polymorphisms of ANXA11 in sarcoidosis in a Chinese Han population, and may be informative for future genetic Jidosis studies on sarcoidosis.

- 3 4	1	Article summary
5 6 7	2	Article focus
8 9	3	1. The first genome-wide association study (GWAS) in sarcoidosis conducted in a
10 11 12	4	German population has revealed that the rs1049550 (C > T, Arg230Cys) located in
13 14	5	exon 10 were associated with sarcoidosis.
15 16 17	6	2. Whether these SNPs modulate the risk of sarcoidosis by themselves or whether
18 19	7	they correlate with other causative SNPs and are repeated in other populations remain
20 21 22	8	unclear.
23 24	9	3. We hypothesize that common variants in the ANXA11 gene may significantly
25 26 27	10	contribute to the predisposition to develop sarcoidosis in a Chinese Han population.
28 29	11	Key messages
30 31 32	12	1. ANXA11 rs2789679 (3'-UTR) was significantly associated with sarcoidosis.
33 34	13	2. ANXA11 rs1049550 (exon 10) was significantly associated with sarcoidosis.
35 36 37	14	3. Another significant association was observed for rs2819941 (intron 14).
38 39	15	Strengths and limitations of this study
40 41 42	16	1. A systematical screening of the functional SNPs in the promoter region, 5'- and
43 44 45	17	3'-UTR, exons of the ANXA11 gene, and the homogeneity of the study subjects
46 47	18	representing the Chinese Han are the main strengths of this study. Chinese Han
48 49 50	19	2. The lack of data proving the positive association observed for rs2789679 and
50 51 52	20	rs1049550 is a potential limitation of this study. Furthermore, the association of the
53 54 55	21	serum level of ANXA11 with sarcoidosis still needs to be investigated.

INTRODUCTION

Sarcoidosis is a systemic autoimmune disease characterized by destructive, non-caseating epithelioid granulomatous lesions, with accumulated phagocytes and activated CD4⁺ T helper type 1 lymphocytes ^{1 2}. The typical manifestations of sarcoidosis include bilateral hilar lymphadenopathy, pulmonary infiltration and ocular and skin lesions. The clinical course and prognosis of sarcoidosis are variable. The acute forms of sarcoidosis such as L fgren's syndrome (LS) can resolve spontaneously in most cases. However, chronic pulmonary sarcoidosis may progress to lung fibrosis which eventually causes respiratory failure. Recent studies have demonstrated that sarcoidosis can occur in a pattern of family clustering, and its racial incidence is also different, suggesting that some genetic factors may contribute to the risk and severity of sacroidosis ¹³.

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The Annexin gene family is involved in the etiology of several autoimmune and chronic diseases ⁴⁵. One member of the Annexin gene family, ANXA11, located on chromosome 10q22.3, is involved in calcium signaling, apoptosis, vesicle trafficking, cell growth and terminal phase of cell division ⁵⁻⁷. In this context, the development and maintenance of the granulomatous inflammation in sarcoidosis have been repeatedly associated with the impaired apoptosis of activated inflammatory cells ⁸⁹. Most of the early-stage sarcoidosis is characterized by mononuclear cell alveolitis dominated by activated CD4⁺ T cells and macrophages. Between these cells, the uncoordinated interplay results in the formation of typical non-caseating granulomas. The mechanism by which the granulomas resolve has not been fully elucidated.

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1	However, it is generally assumed that the induction of apoptosis and/or by the
2	withdrawal of inflammatory cytokines participates in the disappearance of
3	granulomas ^{10 11} . In the patients with sarcoidosis, their peripheral blood monocytes
4	were characterized by apoptosis ⁹ .
5	In previous studies, case - control/family 'hypothesis - driven' studies and low
6	density linkage scans were used to identify genetic factors conferring the genetic
7	susceptibility to sarcoidosis ¹² . Notably, the first genome - wide association study
8	(GWAS) in sarcoidosis conducted in a German population has recently revealed an
9	association: the leading rs2789679 SNP (single - nucleotide polymorphism) located in
10	the 3'-untranslated region (3'-UTR) and a common nonsynonymous SNP (rs1049550,
11	C > T, Arg230Cys) were associated with the increased risk of sarcoidosis ⁴ ¹³ . This
12	association has recently been supported by another report from the same population ¹⁴ .
13	Thus, more studies should be performed to demonstrate the following items: whether
14	these SNPs modulate the risk of sarcoidosis by themselves or whether they correlate
15	with other causative SNPs and are repeated in other populations. The published
16	studies about the association of sarcoidosis and ANXA11 are summarized in
17	Supplemental table 1. We hypothesize that common variants in the ANXA11 gene
18	may significantly contribute to the predisposition to develop sarcoidosis.
19	In this study, we investigated fifteen loci in a Chinese population from He'nan
20	province (China) to verify the putative association between ANXA11 polymorphisms

21 and sarcoidosis.

22 SUBJECTS AND METHODS

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1	Subjects

2	Four hundred and twelve patients with sarcoidosis (mean \pm SD age: 53.6 \pm 4.6
3	years; 155 men and 257 women) were recruited from our hospital between May 2005
4	and March 2013. Sarcoidosis was diagnosed by the evidence of non-caseating
5	epitheloid cell granuloma in biopsy specimens and chest radiographic abnormalities.
6	Chronic sarcoidosis was defined as a disease over at least 2 years or at least two
7	episodes in a lifetime. A cute sarcoidosis was defined as one episode of acute
8	sarcoidosis which had totally resolved at the date of the examination. The control
9	group consisted of 418 unrelated healthy subjects (mean \pm SD age: 54.2 \pm 5.3 years;
10	160 men and 258 women) who underwent health examinations in the Medical
11	Examination Center of the First Affiliated Hospital of the Xinxiang Medical College
12	(Xinxiang, China) between Oct 2009 and Sept 2013. None of the individuals in the
13	control group had a history of lung diseases or showed any symptoms of the lung or
14	other diseases by chest radiography or laboratory blood tests. Participants were
15	excluded if they: were taking other prescribed medications that could affect the
16	central nervous system; had a history of seizures, hematological diseases, or severe
17	liver or kidney impairment; or were pregnant. No familial relationship was known
18	between the study participants. The study was performed according to the Guidelines
19	of the Medical Ethical Committee of Xinxiang Medical College (Xinxiang, China).
20	Written informed consent was obtained from each participant in this study.

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21 SNP selection

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1	Tagging SNPs were selected from the catalogs of the International HapMap
2	Project. The rs2789679 and rs2245168 are located in 5'-UTR, rs2236558 in intron 6,
3	rs7067644 and rs12763624 in intron 8, rs1049550 in exon 10, rs2573351 in intron 13,
4	rs10887581, rs11201989, rs2573353, rs2789695, rs2573356, rs2819945, rs11202059
5	and rs2819941 in intron 14 (Supplemental figure 1). Marker selection was based on
6	the following criteria. (a) We used the CHB data from the HapMap (release 27) to
7	select tagSNPs for ANXA11. (b) We restricted our search for tagSNPs from base pair
8	80155124 to 80205677. (c) Further, we limited the SNPs to tag to those with a minor
9	allele frequency ≥ 0.2 in the CHB. (d) Based on these restrictions, there were a total
10	of 29 SNPs. (e) Using HAPLOVIEW with an r^2 threshold ≥ 0.8 , 15 tagSNP were
11	selected and used for subsequent analyses.

12 Genotyping

Peripheral blood was collected from a vein into a sterile tube coated with 13 ethylenediamine tetraacetic acid (EDTA). Plasma samples were stored at -20°C. 14 Genomic DNA was extracted from the frozen peripheral blood samples using a 15 QIAmp Blood Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the 16 manufacturer's protocols. The selected SNPs were genotyped in cases and controls by 17 18 using the MALDI-TOF in the MassARRAY system (Sequenom Inc., San Diego, CA, 19 USA). Probes and primers were designed using the Assay Design Software (Sequenom, San Diego, CA, USA). The completed genotyping reactions were spotted 20 onto a 384 well spectroCHIP (Sequenom) using the MassARRAY Nanodispenser 21 (Sequenom) and determined by the matrix - assisted laser desorption ionization time -22

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of - flight mass spectrometer. Genotype calling was performed in realtime with the
 MassARRAY RT software version 3.0.0.4 and analyzed using the MassARRAY Typer
 software version 3.4 (Sequenom).

4 Statistical analysis

All data were analyzed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, 5 6 USA). Hardy-Weinberg equilibrium was evaluated by Chi-square tests. Differences 7 between the cases and controls in the frequency of the alleles, genotypes and haplotypes were evaluated by the Fisher's exact test or the Pearson Chi-square test. 8 Unconditional logistic regression was used to calculate the odds ratio (OR) and 95% 9 confidence interval (CI) in independent association between each locus and the 10 presence of sarcoidosis. Gender and age of subjects were treated as covariants in 11 12 binary logistic regression. P values were calculated based on codominant, dominant for the rare allele, heterosis and recessive for the rare allele models of inheritance. 13 Models of multiple logistic regression were used to test the independence of 14 individual allelic effect. In detail, the most significant SNP was chosen to be the 15 conditional SNP (covariate in the regression model) when testing other significant 16 SNPs; Meanwhile, a multi-SNP model including all significant SNPs was also 17 performed. The Bonferroni correction was used to adjust the test level when multiple 18 19 comparisons were conducted, and the P value was divided by the total number of loci. Haplotype blocks were defined according to the criteria developed by Gabriel et al.¹⁵. 20 Pair-wise LD statistics (D' and r^2) and haplotype frequency were calculated, and 21 haplotype blocks were constructed using the Haploview 4.0¹⁶. To ensure that the LD 22

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blocks most closely reflect the population level LD patterns, definition of the blocks
were based on the control samples alone. The haplotype frequencies were estimated
using GENECOUNTING, which computes maximum - likelihood estimates of
haplotype frequencies from unknown phase data by utilizing an expectation maximization algorithm ¹⁷⁻²⁰. The significance of any haplotypic association was
evaluated using a likelihood ratio test, followed by permutation testing that compared
estimated haplotype frequencies in cases and controls ^{17 19}.

RESULTS

The genotype distribution of the fifteen polymorphisms was consistent with the Hardy-Weinberg equilibrium (P > 0.05). The analysis of strong LD in the patients with sarcoidosis and healthy controls revealed that 2 SNPs (rs2245168, rs2236558), 2 SNPs (rs1049550, rs2573351), 2 SNPs (rs2789695, rs2573356) and 3 SNPs (rs2819945, rs11202059, rs2819941) were located in haplotype block 1, block 2, block 3 and block 4 (D' > 0.9, Fig. 1). The genotype distribution, allelic frequencies, and haplotypes in the patients with sarcoidosis and healthy controls are showed in Tables 1-3 and Supplemental table 2

Comparison of genotype and allele frequency distribution revealed significant differences between the patients with sarcoidosis and healthy controls for 3 SNPs: rs2789679, rs1049550 and rs2819941. The rs2789679 A allele (P = 0.00004, OR = 1.42, 95%CI = 1.17-1.73) and rs2819941 T allele (P = 0.0006, OR = 1.41, 95%CI = 1.16-1.71) were significantly more frequent in the patients with sarcoidosis compared to controls. The frequency of the rs1049550 T allele (P = 0.000002, OR = 0.61,

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1	95%CI= 0.49-0.74) in the patients with sarcoidosis was significantly lower than that
2	in the controls. These differences remained statistically significant after Bonferroni
3	corrections.
4	The multi-SNP model showed only rs1049550 present a significant effect on the
5	disease phenotype (p < 0.001). No independent effect was found for rs2789679 or
6	rs2819941 when adjusting for the effect of rs1049550. Both individual SNP and multi -
7	SNP analysis supported rs1049550: T allele was an important protective factor for
8	affecting sarcoidosis (odds ratio around 0.6). In another word, carriers of rs1049550:
9	C allele have higher susceptibility to sarcoidosis in our Chinese Han population
10	(Supplemental table 3).
11	We performed an association analysis to determine whether the haplotype was
12	associated with the risk of sarcoidosis (Global $P < 0.05$ in block 2 and block 3).
13	Compared with the controls, the T - C haplotype occurred significantly less frequently
14	(P = 0.001) but the C - C haplotypes occurred more frequently $(P = 0.0001)$ in block 2
15	(rs1049550 - rs2573351) in the patients with sarcoidosis.
16	To assess particular disease phenotypes, the patients with sarcoidosis were
17	divided into the subgroups according to their chest radiographic (CXR) stage. CXR
18	stage I (isolated bilateral hilar lymphadenopathy): N = 196; CXR stages II - IV
19	(infiltration of parenchyma): 167; CXR stage 0 (absence on pulmonary disease): N =

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frequency distribution revealed that, in rs1049550, CC genotype was significantly more in the patients with chest radiographic (CXR) stage I sarcoidosis than the

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1 patients with CXR stage II-IV sarcoidosis (P = 0.012). (Table 4).

2 DISCUSSION

A key step in linkage and association studies is to identify common risk variants in different populations. To determine if common risk variants exist in distinct populations, fifteen SNPs which span approximately 0.53 Mb of the ANXA11 gene, were genotyped in samples from the patients with sarcoidosis and healthy controls in a Chinese Han population. Recently, new sarcoidosis loci have been identified by genome-wide association studies ^{4 14}. With the fast development of genome - wide association studies, an increasing number of susceptibility loci for sarcoidosis have been found in different populations 4 14 21. However, these observations should be confirmed in other genetically independent populations. In this study, we conducted the first large genetic association study of the ANXA11 gene in a Chinese Han population. The evidence of markers associated with sarcoidosis was presented, and these markers were mapped to different locations in the ANXA11 gene (81897864 -81951001). The association signals in the region were identified, and some significantly associated haplotypes also appeared this region.

Hoffman et al. reported an association between the T allele of the non synonymous SNP rs1049550 and significantly decreased risk of sarcoidosis in a German population ⁴. Similar results were also obtained in other two European populations ^{14 21}. As a part of the sarcoidosis GWAS done in Americans ²², we further confirmed this association in a Chinese Han population. In this study, the frequency of ANXA11 rs1049550 T allele was significantly lower in the patients with sarcoidosis

than the healthy controls. Genotype frequency distribution revealed that, in rs1049550, CC genotype was significantly more in the patients with stage I sarcoidosis than the patients with stage II-IV sarcoidosis. The "protective" effect of ANXA11 T allele increased with the number of its copies in the genotype, which is consistent with the result obtained in the patients with sarcoidosis in a German population⁴, Furthermore, the T allele carriers among the patients were protected from infiltration of lung parenchyma (radiographic stages II-IV)²¹. We have also demonstrated that rs1049550 is significant cross - ethnically at the gene level after adjustment for the single SNP association tests performed. The mechanism by which rs1049550 affects the susceptibility to sarcoidosis is that the SNP may affect the function of the ANXA11 protein rather than affect its expression. In humans, the ANXA11 protein consists of an N - terminalproline - tyrosine - glycine (PYG) - rich region, followed by four annexin core domains. The rs1049550 leads to an amino - acid exchange (basic arginine to a polar cysteine) at the evolutionarily conserved position 230 (R230C) in the first annexin domain, which is responsible for Ca^{2+} - dependent trafficking of the protein in the cell ²³.

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In the GWAS conducted by Hofmann et al ⁴, the strongest association signal was observed for rs2789679. This marker is located in the 3'-UTR of the ANXA11 gene. The T allele had a frequency of 35% among the patients with sarcoidosis and 44% in the healthy controls, with 13% of the patients with sarcoidosis and 19% of controls being TT homozygous. Fine-mapping in the Black Women's Health Study (BWHS) revealed rs2819941 to be the top SNP, associated with a non-significant (P = 0.06)

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1	reduction in the risk of sarcoidosis (17% reduction per copy of the C-allele). In this
2	case-control association study, the frequency of the A allele in rs2789679 and the
3	rs2819941 T allele frequency in the patients with sarcoidosis was significantly higher
4	than that in the healthy controls. Several lines of evidence suggest that the observed
5	association is unlikely to be an artifact. First, both the single-SNP and the
6	haplotype-based association analyses support the current finding. Second, our samples
7	were from the same geographical region, excluding the. Finally, consistent results
8	were obtained from two genetically independent populations (Chinese Han and
9	Europeans). Collectively, our results confirmed the strong association between
10	ANXA11 polymorphisms and sarcoidosis, suggesting that ANXA11 represents a
11	strong genetic risk factor for sarcoidosis. Since causal SNP in ANXA11 was not
12	known, all the SNPs significantly associated with sarcoidosis were actually indirect
13	association through the real causal one. Though rs2789679 and rs2819941 were not in
14	LD with rs1049550, it may not tag the causal SNP as well as rs1049550. Hence, their
15	individual effects were eliminated when controlling for the effect of rs1049550.

We further investigated the interaction among polymorphisms and observed strong LD. The haplotype analysis revealed that significantly more C - C (block 2, rs1049550 - rs2573351) and significantly fewer T - C haplotypes (block 2) were found in the patients with sarcoidosis. These results indicated that the patients with C - C (block 2) haplotypes of the ANXA11 gene were more prone to sarcoidosis. Significantly higher frequencies of T - C haplotypes were detected in the healthy controls than in the patients with sarcoidosis, suggesting that they may show

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1	protective effects against sarcoidosis. Our sample size can detect SNP and haplotype
2	associations with 90% and 85% power, respectively, at a false positive rate of 5%,
3	disease prevalence of 1‰, disease allele / haplotype frequency of 0.05 / 0.03, and a
4	presumed odds ratio (OR) of 1.5. To some extent, this finding further supports a role
5	of ANXA11 polymorphisms in sarcoidosis, while ethnic group difference may exist.
6	In conclusion, our study suggests a potential role of the ANXA11 SNPs and their
7	related haplotypes in the genetic susceptibility to sarcoidosis. Further studies are
8	needed to investigate how these SNPs affect the function of ANXA11.
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Funding The project was supported by the Research and Development Foundation of Science and Technology of Henan Province (2012k15). **Contributors** LG Zhang was involved in conception and design of the study, acquisition of patient data, genotyping and drafted the paper. ZJ Xiao and GC Shi were involved in design of the study, genotyping and interpretation of results. J Huang and CX Zhang were involved in the design of the study, acquisition of patient data and interpretation of result. All authors revised the draft paper. **Competing interests** None. **Data sharing statement** No additional data are available. **Patient consent** Obtained. **Ethics** approval Ethics approval was provided by the First Hospital Affiliated to the Xinxiang Medical College. Provenance and peer review Not commissioned; externally peer reviewed.

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4	1	Figure Legends
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6	2	Fig. 1 The LD plot of the fifteen SNPs in the ANXA11 gene. Values in squares are the
7	Z	Fig. T The LD plot of the fifteen SNTS in the ANAATT gene. Values in squares are the
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9	3	pair-wise calculation of r^2 (left) or D' (right). Black squares indicate $r^2 = 1$ (i.e. perfect
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11	4	LD between a pair of SNPs). Empty squares indicate D' = 1 (i.e. complete LD
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14	5	between a pair of SNPs).
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16	6	Supplemental Fig. 1 The structure of the human ANXA11 gene and 15 SNPs located
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Table 1 Genotypic and allelic frequencies of ANXA11 polymorphisms in the controls

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and patients with sarcoidosis

	Controls (n=418)		Sarcoidos	Sarcoidosis (n=412)		
Variable -	No.	%	No.	%	- P-value ^a	OR, 95% CI
rs2789679					0.0007	
ТТ	114	27.3	94	22.8	0.140	1.27, 0.93-1.74
AT	225	53.8	186	45.2	0.012	1.42, 1.08-1.87
AA	79	18.9	132	32.0	0.0002	0.49, 0.36-0.68
Per A allele	383	45.8	450	54.6	0.00004	1.42, 1.17-1.73
rs1049550					0.0002	
CC	154	36.8	208	50.5	0.0007	0.57, 0.43-0.75
СТ	190	45.5	170	41.3	0.221	1.19, 0.90-1.56
ТТ	74	17.7	34	8.3	0.0008	2.39, 1.55-3.68
Per T allele	338	40.4	238	28.8	0.000002	0.61, 0.49-0.74
rs2819941					0.0002	
CC	114	27.3	94	22.8	0.141	1.27, 0.93-1.74
СТ	226	54.1	190	46.1	0.021	1.38, 1.05-1.81
TT	78	18.7	128	31.1	0.0004	0.51, 0.37-0.70
Per T allele	382	45.7	446	54.1	0.0006	1.41, 1.16-1.71

³ ^a *P*-value was calculated by 2×3 and 2×2 chi-squared tests based on codominant,

4 dominant for the rare allele, heterosis and recessive for the rare allele models of

5 inheritance.

6 Alpha value is adjusted by Bonferroni correction and statistically significant results

7 (P<0.003)

Table 2 ANXA11 haplotype in block 2 frequencies and the results of their associations with risk

of sarcoidosis

	Haplotype			Genecounting	g (frequency %	6)
ID	rs1049550	rs2573351	Cases	Controls	<i>P</i> -value ^a	Global P ^b
HAP1	Т	С	27.7	39.7	0.001	0.003
HAP2	С	С	19.9	7.4	0.0001	
HAP3	C	Т	50.5	51.0	0.892	

4 ^a Based on 10,000 permutations.

5 ^b Based on comparison of frequency distribution of all haplotypes for the combination of SNPs.

Table 3 ANXA11 haplotype in block 3 frequencies and the results of their associations with risk

of sarcoidosis

	Haplotype	9	4	Genecounting	(frequency %	ó)
ID	rs2789695	rs2573356	Cases	Controls	<i>P</i> -value ^a	Global P ^b
HAP1	С	Т	33.7	34.9	0.718	0.045
HAP2	Т	Т	15.3	14.1	0.632	
HAP2	Т	Т	15.6	14.1	0.565	5 9

^a Based on 10,000 permutations.

^b Based on comparison of frequency distribution of all haplotypes for the combination of SNPs.

Table 4 Chest radiographic (CXR) stages of sarcoidosis patients by genotype of

rs2789679	rs1049550	and rs2819941
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<u>S</u> 4	rs2789679 (n, %)		rs1049550 [*] (n, %)			rs2819941 (n, %)			
Stages	AA	AT	TT	CC	СТ	TT	CC	СТ	TT
stage I	59	92	45	101	82	13	47	88	61
	(30.1)	(47.0)	(23.0)	(51.5)	(41.8)	(6.6)	(25.0)	(44.9)	(31.1)
stages	46	75	46	72	68	27	41	73	53
II - IV	(27.6)	(44.9)	(27.6)	(43.1)	(40.7)	(16.2)	(24.6)	(43.7)	(31.7)
χ^2, P	1.	.041, 0.59	4	8	.807, 0.01	2	0	.052, 0.97	5

* *P* values for genotype frequency distribution (P < 0.05).

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1	Annexin A11 (ANXA11) gene polymorphisms are associated with sarcoidosis in a
2	Han Chinese population - a case-control study
3	LG Zhang, ZJ Xiao [*] , GC Shi, J Huang, CX Zhang
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11	Fax: +86 0373 -4402251
12	Key words: Sarcoidosis; Annexin A11; Single-nucleotide polymorphism
13	Word count: <u>37873918</u>
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1	Abstract
2	Objectives: To further identify the single-nucleotide polymorphisms (SNPs) that
3	contribute to the genetic susceptibility to sarcoidosis, we examined the potential
4	association between sarcoidosis and fifteen SNPs of the ANXA11 gene.
5	Design: A case - control study.
6	Setting: A tuberculosist unit in a hospital of the university in China.
7	Participants: Participants included 412 patients with sarcoidosis and 418 healthy
8	controls.
9	Methods: The selected SNPs were genotyped using the MALDI-TOF in the
10	MassARRAY system. Probes and primers were designed using the Assay Design
11	Software (Sequenom, San Diego, CA, USA).
12	Results: The statistical significant differences were found in the allelic or genotypic
13	frequencies of the rs2789679, rs1049550 and rs2819941 in the ANXA11 gene
14	between the patients with sarcoidosis and the controls. The rs2789679 A allele ($P =$
15	<u>0.00004, OR = 1.42, 95%CI = 1.17-1.73) and rs2819941 T allele ($P = 0.0006$, OR = </u>
16	<u>$1.41, 95\%$CI = 1.16-1.71) were significantly more frequent in the patients with</u>
17	sarcoidosis compared to controls. The frequency of the rs1049550 T allele ($P =$
18	0.000002, OR = 0.61, 95%CI= 0.49 - 0.74) in the patients with sarcoidosis was
19	significantly lower than that in the controls. The frequency of the T allele in
20	$r_{s}2789679 (P = 0.0003, OR = 0.70, 95\% CI = 0.58 - 0.85)$ and $r_{s}1049550 (P = 0.0007, 0.0007)$
21	OR = 0.62, 95%CI = 0.50 - 0.76, and C allele in rs2819941 (P = 0.001, OR = 0.71,
22	95%CI = 0.59 – 0.87) in the patients with sarcoidosis was significantly lower than that

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the controls. The strong linkage disequilibrium (LD) was observed in four blocks 1 (D' > 0.9). In block 2 (rs1049550-rs2573351), the T - C haplotype occurred 2 significantly less frequently (P = 0.001), whereas the C - C haplotypes occurred more 3 frequently (P = 0.0001) in the patients with sarcoidosis than the controls. Furthermore, 4 genotype frequency distribution revealed that, in rs1049550, CC genotype was 5 6 significantly more in the patients with chest radiographic (CXR) stage I sarcoidosis than the patients with CXR stage II-IV sarcoidosis (P = 0.012). 7 **Conclusions**: These findings point to a role for the polymorphisms of ANXA11 in 8

9 sarcoidosis in a Chinese Han population, and may be informative for future genetic

10 studies on sarcoidosis.

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1	Article summary
2	Article focus
3	1. The first genome-wide association study (GWAS) in sarcoidosis conducted in a
4	German population has revealed that the rs1049550 (C > T, Arg230Cys) located in
5	exon 10 were associated with sarcoidosis.
6	2. Whether these SNPs modulate the risk of sarcoidosis by themselves or whether
7	they correlate with other causative SNPs and are repeated in other populations remain
8	unclear.
9	3. We hypothesize that common variants in the ANXA11 gene may significantly
10	contribute to the predisposition to develop sarcoidosis in a Chinese Han population.
11	Key messages
12	1. ANXA11 rs2789679 (3'-UTR) was significantly associated with sarcoidosis.
13	2. ANXA11 rs1049550 (exon 10) was significantly associated with sarcoidosis.
14	3. Another significant association was observed for rs2819941 (intron 14).
15	Strengths and limitations of this study
16	1. A systematical screening of the functional SNPs in the promoter region, 5'- and
17	3'-UTR, exons of the ANXA11 gene, and the homogeneity of the study subjects
18	representing the Chinese Han are the main strengths of this study. Chinese Han
19	2. The lack of data proving the positive association observed for rs2789679 and
20	rs1049550 is a potential limitation of this study. Furthermore, the association of the
21	serum level of ANXA11 with sarcoidosis still needs to be investigated.
22	INTRODUCTION

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1	Sarcoidosis is a systemic autoimmune disease characterized by destructive,
2	non-caseating epithelioid granulomatous lesions, with accumulated phagocytes and
3	activated CD4 ⁺ T helper type 1 lymphocytes ^{1 2} . The typical manifestations of
4	sarcoidosis include bilateral hilar lymphadenopathy, pulmonary infiltration and ocular
5	and skin lesions. The clinical course and prognosis of sarcoidosis are variable. The
6	acute forms of sarcoidosis such as $L\Box$ fgren's syndrome (LS) can resolve
7	spontaneously in most cases. However, chronic pulmonary sarcoidosis may progress
8	to lung fibrosis which eventually causes respiratory failure. Recent studies have
9	demonstrated that sarcoidosis can occur in a pattern of family clustering, and its racial
10	incidence is also different, suggesting that some genetic factors may contribute to the
11	risk and severity of sacroidosis ¹³ .
12	The Annexin gene family is involved in the etiology of several autoimmune and
13	chronic diseases ^{4 5.} One member of the Annexin gene family, ANXA11, located on
14	chromosome 10q22.3, is involved in calcium signaling, apoptosis, vesicle trafficking,
15	cell growth and terminal phase of cell division ⁵⁻⁷ . In this context, the development
16	and maintenance of the granulomatous inflammation in sarcoidosis have been
17	repeatedly associated with the impaired apoptosis of activated inflammatory cells ⁸⁹ .
18	Most of the early-stage sarcoidosis is characterized by mononuclear cell alveolitis
19	dominated by activated CD4 ⁺ T cells and macrophages. Between these cells, the
20	uncoordinated interplay results in the formation of typical non-caseating granulomas.
21	The mechanism by which the granulomas resolve has not been fully elucidated.
22	However, it is generally assumed that the induction of apoptosis and/or by the

1	withdrawal of inflammatory cytokines participates in the disappearance of
2	granulomas ^{10 11} . In the patients with sarcoidosis, their peripheral blood monocytes
3	were characterized by apoptosis ⁹ .
4	In previous studies, case - control/family 'hypothesis - driven' studies and low
5	density linkage scans were used to identify genetic factors conferring the genetic
6	susceptibility to sarcoidosis ¹² . Notably, the first genome - wide association study
7	(GWAS) in sarcoidosis conducted in a German population has recently revealed an
8	association: the leading rs2789679 SNP (single - nucleotide polymorphism) located in
9	the 3'-untranslated region (3'-UTR) and a common nonsynonymous SNP (rs1049550,
10	C > T, Arg230Cys) were associated with the increased risk of sarcoidosis ⁴ ¹³ . This
11	association has recently been supported by another report from the same population ¹⁴ .
12	Thus, more studies should be performed to demonstrate the following items: whether
13	these SNPs modulate the risk of sarcoidosis by themselves or whether they correlate
14	with other causative SNPs and are repeated in other populations. The published
15	studies about the association of sarcoidosis and ANXA11 are summarized in
16	Supplemental table 1. We hypothesize that common variants in the ANXA11 gene
17	may significantly contribute to the predisposition to develop sarcoidosis.
18	In this study, we investigated fifteen loci in a Chinese population from He'nan
19	province (China) to verify the putative association between ANXA11 polymorphisms
20	and sarcoidosis.
21	SUBJECTS AND METHODS

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22 Subjects

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1	Four hundred and twelve patients with sarcoidosis (mean \pm SD age: 53.6 \pm 4.6
2	years; 155 men and 257 women) were recruited from our hospital between May 2005
3	and March 2013. Sarcoidosis was diagnosed by the evidence of non-caseating
4	epitheloid cell granuloma in biopsy specimens and chest radiographic abnormalities.
5	Chronic sarcoidosis was defined as a disease over at least 2 years or at least two
6	episodes in a lifetime. A cute sarcoidosis was defined as one episode of acute
7	sarcoidosis which had totally resolved at the date of the examination. The control
8	group consisted of 418 unrelated healthy subjects (mean \pm SD age: 54.2 \pm 5.3 years;
9	160 men and 258 women) who underwent health examinations in the Medical
10	Examination Center of the First Affiliated Hospital of the Xinxiang Medical College
11	(Xinxiang, China) between Oct 2009 and Sept 2013. None of the individuals in the
12	control group had a history of lung diseases or showed any symptoms of the lung or
13	other diseases by chest radiography or laboratory blood tests. Participants were
14	excluded if they: were taking other prescribed medications that could affect the
15	central nervous system; had a history of seizures, hematological diseases, or severe
16	liver or kidney impairment; or were pregnant. No familial relationship was known
17	between the study participants. The study was performed according to the Guidelines
18	of the Medical Ethical Committee of Xinxiang Medical College (Xinxiang, China).
19	Written informed consent was obtained from each participant in this study.
20	

20 SNP selection

Tagging SNPs were selected from the catalogs of the International HapMap Project. The rs2789679 and rs2245168 are located in 5'-UTR, rs2236558 in intron 6,

1	rs7067644 and rs12763624 in intron 8, rs1049550 in exon 10, rs2573351 in intron 13,
2	rs10887581, rs11201989, rs2573353, rs2789695, rs2573356, rs2819945, rs11202059
3	and rs2819941 in intron 14 (Supplemental figure 1). Marker selection was based on
4	the following criteria. (a) We used the CHB data from the HapMap (release 27) to
5	select tagSNPs for ANXA11. (b) We restricted our search for tagSNPs from base pair
6	80155124 to 80205677. (c) Further, we limited the SNPs to tag to those with a minor
7	allele frequency ≥ 0.2 in the CHB. (d) Based on these restrictions, there were a total
8	of 29 SNPs. (e) Using HAPLOVIEW with an r^2 threshold ≥ 0.8 , 15 tagSNP were
9	selected and used for subsequent analyses.
10	Genotyping

Peripheral blood was collected from a vein into a sterile tube coated with ethylenediamine tetraacetic acid (EDTA). Plasma samples were stored at -20°C. Genomic DNA was extracted from the frozen peripheral blood samples using a QIAmp Blood Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocols. The selected SNPs were genotyped in cases and controls by using the MALDI-TOF in the MassARRAY system (Sequenom Inc., San Diego, CA, USA). Probes and primers were designed using the Assay Design Software (Sequenom, San Diego, CA, USA). The completed genotyping reactions were spotted onto a 384 well spectroCHIP (Sequenom) using the MassARRAY Nanodispenser (Sequenom) and determined by the matrix - assisted laser desorption ionization time -of - flight mass spectrometer. Genotype calling was performed in realtime with the MassARRAY RT software version 3.0.0.4 and analyzed using the MassARRAY Typer

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1 software version 3.4 (Sequenom).

2 Statistical analysis

All data were analyzed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Hardy-Weinberg equilibrium was evaluated by Chi-square tests. Differences between the cases and controls in the frequency of the alleles, genotypes and haplotypes were evaluated by the Fisher's exact test or the Pearson Chi-square test. Unconditional logistic regression was used to calculate the odds ratio (OR) and 95% confidence interval (CI) in independent association between each locus and the presence of sarcoidosis. Logistic regression was used to evaluate the interaction effects between gene and gender or age. Gender and age of subjects were treated as covariants in binary logistic regression. P values were calculated based on codominant, dominant for the rare allele, heterosis and recessive for the rare allele models of inheritance. Models of multiple logistic regression were used to test the independence of individual allelic effect. In detail, the most significant SNP was chosen to be the conditional SNP (covariate in the regression model) when testing other significant SNPs; Meanwhile, a multi-SNP model including all significant SNPs was also performed. The Bonferroni correction was used to adjust the test level when multiple comparisons were conducted, and the P value was divided by the total number of loci. Haplotype blocks were defined according to the criteria developed by Gabriel et al. ¹⁵. Pair-wise LD statistics (D' and r^2) and haplotype frequency were calculated, and haplotype blocks were constructed using the Haploview 4.0¹⁶. To ensure that the LD blocks most closely reflect the population level LD patterns,

1	definition of the blocks were based on the control samples alone. The haplotype
2	frequencies were estimated using GENECOUNTING, which computes maximum -
3	likelihood estimates of haplotype frequencies from unknown phase data by utilizing
4	an expectation - maximization algorithm ¹⁷⁻²⁰ . The significance of any haplotypic
5	association was evaluated using a likelihood ratio test, followed by permutation
6	testing that compared estimated haplotype frequencies in cases and controls ^{17 19} .
7	RESULTS
8	The genotype distribution of the fifteen polymorphisms was consistent with the
9	Hardy-Weinberg equilibrium ($P > 0.05$). The analysis of strong LD in the patients
10	with sarcoidosis and healthy controls revealed that 2 SNPs (rs2245168, rs2236558), 2
11	SNPs (rs1049550, rs2573351), 2 SNPs (rs2789695, rs2573356) and 3 SNPs
12	(rs2819945, rs11202059, rs2819941) were located in haplotype block 1, block 2,
13	block 3 and block 4 (D' > 0.9, Fig. 1). The genotype distribution, allelic frequencies,
14	and haplotypes in the patients with sarcoidosis and healthy controls are showed in
15	Tables 1-3 and Supplemental table 2
16	Comparison of genotype and allele frequency distribution revealed significant
17	differences between the patients with sarcoidosis and healthy controls for 3 SNPs:
18	rs2789679, rs1049550 and rs2819941. The rs2789679 A allele ($P = 0.00004$, OR =
19	<u>1.42, 95%CI = 1.17-1.73</u>) and rs2819941 T allele ($P = 0.0006$, OR = 1.41, 95%CI =
20	1.16-1.71) were significantly more frequent in the patients with sarcoidosis compared
21	to controls. The frequency of the rs1049550 T allele ($P = 0.000002$, OR = 0.61,
22	95%CI= 0.49-0.74) in the patients with sarcoidosis was significantly lower than that
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1	in the controls. The frequency of the T allele in rs2789679 ($P = 0.0003$, OR = 0.70,
2	95%CI = 0.58 – 0.85) and rs1049550 (P = 0.0007, OR = 0.62, 95%CI = 0.50 – 0.76),
3	and C allele in rs2819941 ($P = 0.001$, OR = 0.71, 95%CI = 0.59 – 0.87) in the patients
4	with sarcoidosis was significantly lower than that in the controls, and \underline{T} these
5	differences remained statistically significant after Bonferroni corrections.
6	The multi-SNP model showed only rs1049550 present a significant effect on the
7	disease phenotype (p < 0.001). No independent effect was found for rs2789679 or
8	rs2819941 when adjusting for the effect of rs1049550. Both individual SNP and multi -
9	SNP analysis supported rs1049550: T allele was an important protective factor for
10	affecting sarcoidosis (odds ratio around 0.6). In another word, carriers of rs1049550:
11	C allele have higher susceptibility to sarcoidosis in our Chinese Han population
12	(Supplemental table 3).
12 13	(Supplemental table 3). We presented a multi-SNP model of sarcoidosis risk. We screen out the most
13	We presented a multi-SNP model of sarcoidosis risk. We screen out the most
13 14	We presented a multi-SNP model of sarcoidosis risk. We screen out the most significant model to predict the risk by forward stepwise strategy in logistic regression,
13 14 15	We presented a multi-SNP model of sarcoidosis risk. We screen out the most significant model to predict the risk by forward stepwise strategy in logistic regression, and found that a model includes rs2789679 and rs1049550 is the best one
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13 14 15 16 17	We presented a multi-SNP model of sarcoidosis risk. We screen out the most significant model to predict the risk by forward stepwise strategy in logistic regression, and found that a model includes rs2789679 and rs1049550 is the best one (Supplemental table 3), which suggests that there is a interaction between them. We performed an association analysis to determine whether the haplotype was
13 14 15 16 17 18	We presented a multi-SNP model of sarcoidosis risk. We screen out the most significant model to predict the risk by forward stepwise strategy in logistic regression, and found that a model includes rs2789679 and rs1049550 is the best one (Supplemental table 3), which suggests that there is a interaction between them. We performed an association analysis to determine whether the haplotype was associated with the risk of sarcoidosis (Global $P < 0.05$ in block 2 and block 3).
 13 14 15 16 17 18 19 	We presented a multi-SNP model of sarcoidosis risk. We screen out the most significant model to predict the risk by forward stepwise strategy in logistic regression, and found that a model includes rs2789679 and rs1049550 is the best one (Supplemental table 3), which suggests that there is a interaction between them. We performed an association analysis to determine whether the haplotype was associated with the risk of sarcoidosis (Global $P < 0.05$ in block 2 and block 3). Compared with the controls, the T - C haplotype occurred significantly less frequently

divided into the subgroups according to their chest radiographic (CXR) stage. CXR stage I (isolated bilateral hilar lymphadenopathy): N = 196; CXR stages II - IV (infiltration of parenchyma): 167; CXR stage 0 (absence on pulmonary disease): N = 28; the information on CXR stage was not available for 21 patients. Genotype frequency distribution revealed that, in rs1049550, CC genotype was significantly more in the patients with chest radiographic (CXR) stage I sarcoidosis than the patients with CXR stage II-IV sarcoidosis (P = 0.012). (Table 4).

DISCUSSION

A key step in linkage and association studies is to identify common risk variants in different populations. To determine if common risk variants exist in distinct populations, fifteen SNPs which span approximately 0.53 Mb of the ANXA11 gene, were genotyped in samples from the patients with sarcoidosis and healthy controls in a Chinese Han population. Recently, new sarcoidosis loci have been identified by genome-wide association studies ^{4 14}. With the fast development of genome - wide association studies, an increasing number of susceptibility loci for sarcoidosis have been found in different populations ^{4 14 21}. However, these observations should be confirmed in other genetically independent populations. In this study, we conducted the first large genetic association study of the ANXA11 gene in a Chinese Han population. The evidence of markers associated with sarcoidosis was presented, and these markers were mapped to different locations in the ANXA11 gene (81897864 -81951001). The association signals in the region were identified, and some significantly associated haplotypes also appeared this region.

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1	Hoffman et al. reported an association between the T allele of the non -
2	synonymous SNP rs1049550 and significantly decreased risk of sarcoidosis in a
3	German population ⁴ . Similar results were also obtained in other two European
4	populations ¹⁴ ²¹ . As a part of the sarcoidosis GWAS done in Americans ²² , we further
5	confirmed this association in a Chinese Han population. In this study, the frequency of
6	ANXA11 rs1049550 T allele was significantly lower in the patients with sarcoidosis
7	than the healthy controls. There is interaction between rs2789679 and rs1049550.
8	Genotype frequency distribution revealed that, in rs1049550, CC genotype was
9	significantly more in the patients with stage I sarcoidosis than the patients with stage
10	II-IV sarcoidosis. The "protective" effect of ANXA11 T allele increased with the
11	number of its copies in the genotype, which is consistent with the result obtained in
12	the patients with sarcoidosis in a German population ⁴ , Furthermore, the T allele
13	carriers among the patients were protected from infiltration of lung parenchyma
14	(radiographic stages II-IV) ²¹ . We have also demonstrated that rs1049550 is
15	significant cross - ethnically at the gene level after adjustment for the single SNP
16	association tests performed. The mechanism by which rs1049550 affects the
17	susceptibility to sarcoidosis is that the SNP may affect the function of the ANXA11
18	protein rather than affect its expression. In humans, the ANXA11 protein consists of
19	an N - terminalproline - tyrosine - glycine (PYG) - rich region, followed by four
20	annexin core domains. The rs1049550 leads to an amino - acid exchange (basic
21	arginine to a polar cysteine) at the evolutionarily conserved position 230 (R230C) in
22	the first annexin domain, which is responsible for Ca^{2+} - dependent trafficking of the

1	protein in the cell ²³ .
2	In the GWAS conducted by Hofmann et al ⁴ , the strongest association signal was
3	observed for rs2789679. This marker is located in the 3'-UTR of the ANXA11 gene.
4	The T allele had a frequency of 35% among the patients with sarcoidosis and 44% in
5	the healthy controls, with 13% of the patients with sarcoidosis and 19% of controls
6	being TT homozygous. Fine-mapping in the Black Women's Health Study (BWHS)
7	revealed rs2819941 to be the top SNP, associated with a non-significant ($P = 0.06$)
8	reduction in the risk of sarcoidosis (17% reduction per copy of the C-allele). In this
9	case-control association study, the frequency of the T_A allele in rs2789679 and the
10	rs2819941 \leftarrow <u>T</u> allele frequency in the patients with sarcoidosis was significantly
11	lower higher than that in the healthy controls. Several lines of evidence suggest that
12	the observed association is unlikely to be an artifact. First, both the single-SNP and
13	the haplotype-based association analyses support the current finding. Second, our
14	samples were from the same geographical region, excluding the. Finally, consistent
15	results were obtained from two genetically independent populations (Chinese Han and
16	Europeans). Collectively, our results confirmed the strong association between
17	ANXA11 polymorphisms and sarcoidosis, suggesting that ANXA11 represents a
18	strong genetic risk factor for sarcoidosis. Since causal SNP in ANXA11 was not
19	known, all the SNPs significantly associated with sarcoidosis were actually indirect
20	association through the real causal one. Though rs2789679 and rs2819941 were not in
21	LD with rs1049550, it may not tag the causal SNP as well as rs1049550. Hence, their
22	individual effects were eliminated when controlling for the effect of rs1049550.

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1	We further investigated the interaction among polymorphisms and observed
2	strong LD. The haplotype analysis revealed that significantly more C - C (block 2,
3	rs1049550 - rs2573351) and significantly fewer T - C haplotypes (block 2) were
4	found in the patients with sarcoidosis. These results indicated that the patients with C
5	- C (block 2) haplotypes of the ANXA11 gene were more prone to sarcoidosis.
6	Significantly higher frequencies of T - C haplotypes were detected in the healthy
7	controls than in the patients with sarcoidosis, suggesting that they may show
8	protective effects against sarcoidosis. Our sample size can detect SNP and haplotype
9	associations with 90% and 85% power, respectively, at a false positive rate of 5%,
10	disease prevalence of 1‰, disease allele / haplotype frequency of 0.05 / 0.03, and a
11	presumed odds ratio (OR) of 1.5. To some extent, this finding further supports a role
12	of ANXA11 polymorphisms in sarcoidosis, while ethnic group difference may exist.
13	In conclusion, our study suggests a potential role of the ANXA11 SNPs and their
14	related haplotypes in the genetic susceptibility to sarcoidosis. Further studies are
15	needed to investigate how these SNPs affect the function of ANXA11.
16	Contributors
17	LG Zhang was involved in conception and design of the study, acquisition of
18	patient data genotyping and drafted the paper ZI Xiao and GC Shi were involved in

- patient data, genotyping and drafted the paper. ZJ Xiao and GC Shi were involved in
 design of the study, genotyping and interpretation of results. J Huang and CX Zhang
 were involved in the design of the study, acquisition of patient data and interpretation
 of result. All authors revised the draft paper.
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4	T	The project was supported by the Research and Development Foundation of
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7	2	Science and Technology of Henan Province (2012k15).
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9	3	Competing interests
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14	5	Patient consent
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24	9	Medical College.
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26	10	Provenance and peer review
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29	11	Not commissioned; externally peer reviewed.
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31	12	Data sharing statement
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33 34	13	No additional data are available.
35	15	No additional data are available.
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37	14	Figure Legend
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39	15	Fig. 1 The LD plot of the fifteen SNPs in the ANXA11 gene. Values in squares are the
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41	16	pair-wise calculation of r^2 (left) or D' (right). Black squares indicate $r^2 = 1$ (i.e. perfect
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44	17	LD between a pair of SNPs). Empty squares indicate $D' = 1$ (i.e. complete LD
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49	19	Supplemental Fig. 1 The structure of the human ANXA11 gene and 15 SNPs located
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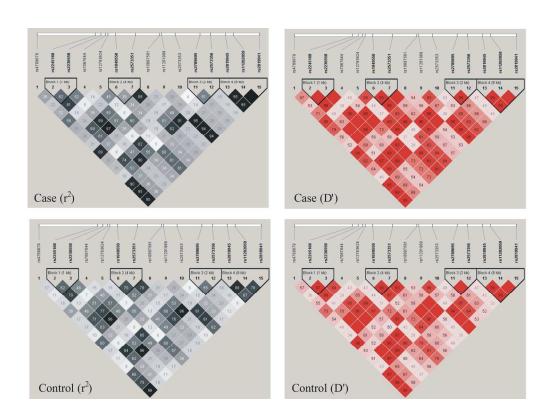
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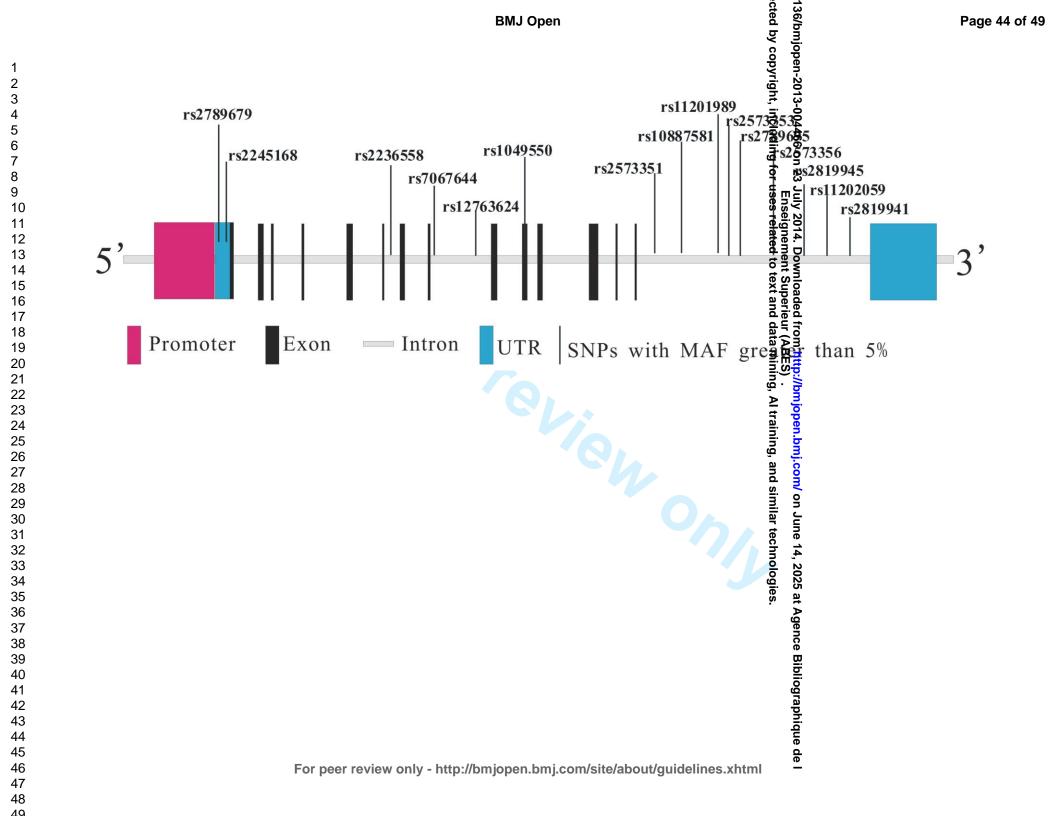
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Study	Population		Toma af Stada	Sample Size (n)		Number of	Positive SNPs	
Study	Ethnic group	Country	Type of Study	Sarcoidosis	Control	SNPs typed	rositive Sinrs	
Hofmann S, 2008	Caucasian	Germany	Case-control	499	490	GWAS	rs2789679, rs7091565, rs1049550	
Mrazek F, 2011	Caucasian	Czech	Case-control	245	254	1	rs1049550	
Cozier YC, 2012	Caucasian	America	Case-control	486	943	10p12-10q22	rs2789679, rs2819941	
Li Y, 2013	Caucasian	Germany	Case-control	325	364	1	rs1049550	
Levin AM, 2013	Caucasian	America	Case-control	1689	1252	25	rs1049550	
Morais A, 2013	Caucasian	Portugal	Case-control	208	197	1	rs1049550	

Supplemental table 1 Summary of all published sarcoidosis-association studies for ANXA11



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	Controls (n=418)		Sarcoidosis (n=412)		.	
Variable	No.	%	No.	%	<i>P</i> -value ^a	OR, 95% CI
rs2245168					0.920	
CC	156	37.3	150	36.4	0.791	1.04, 0.74-1.38
СТ	200	47.9	197	47.8	0.955	0.99, 0.76-1.30
TT	62	14.8	65	15.8	0.781	0.95, 0.65-1.39
Per T allele	324	38.8	327	39.7	0.698	1.04, 0.85-1.27
rs2236558					0.172	
GG	118	28.2	104	25.2	0.72	1.06, 0.77-1.45
TG	190	45.5	214	51.9	0.060	0.77, 0.59-1.01
TT	110	26.3	94	22.8	0.073	1.33, 0.97-1.82
Per T allele	410	49.0	402	48.8	0.917	0.99, 0.82-1.20
rs7067644					0.173	
AA	152	36.4	176	42.7	0.059	0.76, 0.58-1.01
GA	206	49.3	182	44.2	0.137	1.23, 0.94-1.68
GG	60	14.4	54	13.1	0.597	1.11, 0.75-1.65
Per G allele	326	38.0	290	35.2	0.109	0.85, 0.70-1.04
rs12763624					0.118	
TT	126	75.6	150	36.4	0.008	0.68, 0.51-0.91
СТ	216	51.7	186	45.2	0.059	1.30, 0.99-1.71
CC	76	18.2	76	18.5	0.411	1.16, 0.81-1.67
Per C allele	368	44.0	338	41.0	0.216	0.88, 0.73-1.08
rs2573351					0.437	

Supplemental Table	2 Genotypic and allelic	frequencies of ANXA	11 polymorphisms in the cont	rols and patients with sarcoidosis
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6	TT	120	28.7	104	25.2
7 8	СТ	202	48.3	216	52.4
9					
10	CC	96	23.0	92	22.3
11	Per C allele	394	47.1	400	48.5
12	rs10887581				
13	TT	114	27.3	96	23.3
14	TC	190	45.5	208	50.5
15	CC	114	27.3	108	26.2
16 17	Per C allele	418	50.0	424	51.5
18	rs11201989				
19	GG	130	31.1	128	31.1
20	GC	218	52.2	194	47.1
21					
22	CC	70	16.8	90	21.9
23	Per C allele	358	42.8	374	45.4
24 25	rs2573353				
26	CC	178	42.6	176	42.7
27	CA	196	46.9	192	46.6
28	AA	44	10.5	44	10.7
29	Per A allele	284	34.0	280	34.0
30	rs2789695	-			
31 32	TT	178	42.6	180	34.0
32 33					
34	CT	188	45.0	184	44.7
35	CC	52	12.4	48	11.7
36	Per C allele	292	34.9	280	34.0
37	rs2573356				
38	CC	118	28.2	112	27.2
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1.19, 0.88-1.62

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1.04, 0.75-1.44

1.06, 0.87-1.28

1.23, 0.90-1.69

0.82, 0.62-1.07

1.06, 0.78-1.44

1.06,0.87-1.29

1.00, 0.75-1.34

1.23, 0.93-1.62

0.71, 0.50-1.01

1.11, 0.91-1.35

0.99, 0.75-1.31

1.01, 0.77-1.33

0.98, 0.63-1.53

1.00, 0.82-1.23

0.95, 0.73-1.26

1.01, 0.77-1.33

1.08, 0.71-1.64

0.96, 0.78-1.17

1.05, 0.78-1.43

СТ	190	45.5	194	47.1	0.639	0.94, 0.71-1.23
TT	110	26.3	106	25.7	0.843	1.03, 0.76-1.41
Per T allele	410	49.0	406	49.3	0.926	0.99, 0.82-1.20
rs2819945					0.101	
GG	119	28.5	130	31.6	0.294	0.85, 0.63-1.15
GA	221	52.9	188	45.6	0.030	1.35, 1.03-1.78
AA	78	18.7	94	22.8	0.138	0.78, 0.55-1.09
Per A allele	377	45.1	376	45.6	0.827	1.02, 0.84-1.24
rs11202059					0.064	
GG	106	25.4	128	31.1	0.067	0.75, 0.56-1.02
GA	226	54.1	190	46.1	0.021	1.38, 1.05-1.81
AA	86	20.6	94	22.8	0.429	0.88, 0.63-1.22
Per A allele	398	47.6	378	45.9	0.479	0.93, 0.77-1.13

^a *P*-value was calculated by 2×3 and 2×2 chi-squared tests based on codominant, dominant for the rare allele, heterosis and recessive for the rare allele models of inheritance.

Alpha value is adjusted by Bonferroni correction and statistically significant results (P<0.003)

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1 2 3 4 5	Supplementary table 3 Multi-SNP analy	sis of rs2789679	, rs104955	50 and r	s2819941
6 7	Models	testing SNP	<i>P</i> -value	OR	95%CI
8 9 10	model1: rs2789679+ rs1049550	rs2789679: A	0.11	1.19	0.96-1.48
11 12	model2: rs2819941+rs1049550	rs2819941: T	0.169	1.17	0.94-1.45
13 14 15	model3: rs2789670+rs1049550+rs2819941	rs1049550: T	0.0002	0.65	0.52-0.82
14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37				0.03	0.32-0.82
37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60					

BMJ Open

Annexin A11 (ANXA11) gene polymorphisms are associated with sarcoidosis in a Han Chinese population - a casecontrol study

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Manuscript ID:	bmjopen-2013-004466.R4
Article Type:	Research
Date Submitted by the Author:	19-Jun-2014
Complete List of Authors:	Zhang, LG; the First Hospital Affiliated to the Xinxiang Medical College, The Third Department of Tuberculosis Xiao, ZJ; the First Hospital Affiliated to the Xinxiang Medical College, The Third Department of Tuberculosis Shi, GC; the First Hospital Affiliated to the Xinxiang Medical College, The Third Department of Tuberculosis J, Huang; the First Hospital Affiliated to the Xinxiang Medical College, The Third Department of Tuberculosis Zhang, CX; the First Hospital Affiliated to the Xinxiang Medical College, The Third Department of Tuberculosis
Primary Subject Heading :	Infectious diseases
Secondary Subject Heading:	Genetics and genomics
Keywords:	GENETICS, IMMUNOLOGY, INFECTIOUS DISEASES

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3 4	1	Annexin A11 (ANXA11) gene polymorphisms are associated
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8 9	3	LG Zhang, ZJ Xiao [*] , GC Shi, J Huang, CX Zh
10 11 12	4	The Third Department of Tuberculosis, the First Hospital Affi
13 14 15	5	Medical College, Xinxiang, Henan, PR China
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31 32	12	Key words: Sarcoidosis; Annexin A11; Single-nucleotide polyn
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1 Abstract

Objectives: To further identify the single-nucleotide polymorphisms (SNPs) that
contribute to the genetic susceptibility to sarcoidosis, we examined the potential
association between sarcoidosis and fifteen SNPs of the ANXA11 gene.

Design: A case - control study.

Setting: A tuberculosist unit in a hospital of the university in China.

7 Participants: Participants included 412 patients with sarcoidosis and 418 healthy

8 controls.

9 Methods: The selected SNPs were genotyped using the MALDI-TOF in the
10 MassARRAY system.

Results: The statistical significant differences were found in the allelic or genotypic frequencies of the rs2789679, rs1049550 and rs2819941 in the ANXA11 gene between the patients with sarcoidosis and the controls. The rs2789679 A allele (P =0.00004, OR = 1.42, 95%CI = 1.17-1.73) and rs2819941 T allele (P = 0.0006, OR = 1.41, 95%CI = 1.16-1.71) were significantly more frequent in the patients with sarcoidosis compared to controls. The frequency of the rs1049550 T allele (P =0.000002, OR = 0.61, 95%CI= 0.49 - 0.74) in the patients with sarcoidosis was significantly lower than that in the controls. The multi-SNP model reveals that rs1049550 is the only independent SNP association effect after accounting for the other two marginally associated SNPs. In block 2 (rs1049550-rs2573351), the T - C haplotype occurred significantly less frequently (P = 0.001), whereas the C - C haplotypes occurred more frequently (P = 0.0001) in the patients with sarcoidosis

than the controls. Furthermore, genotype frequency distribution revealed that, in rs1049550, CC genotype was significantly more in the patients with chest radiographic (CXR) stage I sarcoidosis than the patients with CXR stage II-IV sarcoidosis (P = 0.012).

Conclusions: These findings point to a role for the polymorphisms of ANXA11 in sarcoidosis in a Chinese Han population, and may be informative for future genetic Jidosis. studies on sarcoidosis.

1	Article summary
2	Article focus
3	1. The first genome-wide association study (GWAS) in sarcoidosis conducted in a
4	German population has revealed that the rs1049550 (C > T, Arg230Cys) located in
5	exon 10 were associated with sarcoidosis.
6	2. Whether these SNPs modulate the risk of sarcoidosis by themselves or whether
7	they correlate with other causative SNPs and are repeated in other populations remain
8	unclear.
9	3. We hypothesize that common variants in the ANXA11 gene may significantly
10	contribute to the predisposition to develop sarcoidosis in a Chinese Han population.
11	Key messages
12	1. ANXA11 rs2789679 (3'-UTR) was significantly associated with sarcoidosis.
13	2. ANXA11 rs1049550 (exon 10) was significantly associated with sarcoidosis.
14	3. Another significant association was observed for rs2819941 (intron 14).
15	Strengths and limitations of this study
16	1. A systematical screening of the functional SNPs in the promoter region, 5'- and
17	3'-UTR, exons of the ANXA11 gene, and the homogeneity of the study subjects
18	representing the Chinese Han are the main strengths of this study.
19	2. The lack of data proving the positive association observed for rs2789679 and
20	rs1049550 is a potential limitation of this study. Furthermore, the association of the
21	serum level of ANXA11 with sarcoidosis still needs to be investigated.

INTRODUCTION

Sarcoidosis is a systemic autoimmune disease characterized by destructive, non-caseating epithelioid granulomatous lesions, with accumulated phagocytes and activated CD4⁺ T helper type 1 lymphocytes ^{1 2}. The typical manifestations of sarcoidosis include bilateral hilar lymphadenopathy, pulmonary infiltration and ocular and skin lesions. The clinical course and prognosis of sarcoidosis are variable. The acute forms of sarcoidosis such as Löfgren's syndrome (LS) can resolve spontaneously in most cases. However, chronic pulmonary sarcoidosis may progress to lung fibrosis which eventually causes respiratory failure. Recent studies have demonstrated that sarcoidosis can occur in a pattern of family clustering, and its racial incidence is also different, suggesting that some genetic factors may contribute to the risk and severity of sacroidosis ¹³.

The Annexin gene family is involved in the etiology of several autoimmune and chronic diseases ⁴⁵. One member of the Annexin gene family, ANXA11, located on chromosome 10q22.3, is involved in calcium signaling, apoptosis, vesicle trafficking, cell growth and terminal phase of cell division ⁵⁻⁷. In this context, the development and maintenance of the granulomatous inflammation in sarcoidosis have been repeatedly associated with the impaired apoptosis of activated inflammatory cells ⁸⁹. Most of the early-stage sarcoidosis is characterized by mononuclear cell alveolitis dominated by activated CD4⁺ T cells and macrophages. Between these cells, the uncoordinated interplay results in the formation of typical non-caseating granulomas. The mechanism by which the granulomas resolve has not been fully elucidated.

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1	However, it is generally assumed that the induction of apoptosis and/or by the
2	withdrawal of inflammatory cytokines participates in the disappearance of
3	granulomas ^{10 11} . In the patients with sarcoidosis, their peripheral blood monocytes
4	were characterized by apoptosis ⁹ .
5	In previous studies, case - control/family 'hypothesis - driven' studies and low
6	density linkage scans were used to identify genetic factors conferring the genetic
7	susceptibility to sarcoidosis ¹² . Notably, the first genome - wide association study
8	(GWAS) in sarcoidosis conducted in a German population has recently revealed an
9	association: the leading rs2789679 SNP (single - nucleotide polymorphism) located in
10	the 3'-untranslated region (3'-UTR) and a common nonsynonymous SNP (rs1049550,
11	C > T, Arg230Cys) were associated with the increased risk of sarcoidosis ⁴ ¹³ . This
12	association has recently been supported by another report from the same population ¹⁴ .
13	Thus, more studies should be performed to demonstrate the following items: whether
14	these SNPs modulate the risk of sarcoidosis by themselves or whether they correlate
15	with other causative SNPs and are repeated in other populations. The published
16	studies about the association of sarcoidosis and ANXA11 are summarized in
17	Supplemental table 1. We hypothesize that common variants in the ANXA11 gene
18	may significantly contribute to the predisposition to develop sarcoidosis.
19	In this study, we investigated fifteen loci in a Chinese population from He'nan
20	province (China) to verify the putative association between ANXA11 polymorphisms

and sarcoidosis. 21

SUBJECTS AND METHODS 22

1	Subjects

2	Four hundred and twelve patients with sarcoidosis (mean \pm SD age: 53.6 \pm 4.6
3	years; 155 men and 257 women) were recruited from our hospital between May 2005
4	and March 2013. Sarcoidosis was diagnosed by the evidence of non-caseating
5	epitheloid cell granuloma in biopsy specimens and chest radiographic abnormalities.
6	Chronic sarcoidosis was defined as a disease over at least 2 years or at least two
7	episodes in a lifetime. A cute sarcoidosis was defined as one episode of acute
8	sarcoidosis which had totally resolved at the date of the examination. The control
9	group consisted of 418 unrelated healthy subjects (mean \pm SD age: 54.2 \pm 5.3 years;
10	160 men and 258 women) who underwent health examinations in the Medical
11	Examination Center of the First Affiliated Hospital of the Xinxiang Medical College
12	(Xinxiang, China) between Oct 2009 and Sept 2013. None of the individuals in the
13	control group had a history of lung diseases or showed any symptoms of the lung or
14	other diseases by chest radiography or laboratory blood tests. Participants were
15	excluded if they: were taking other prescribed medications that could affect the
16	central nervous system; had a history of seizures, hematological diseases, or severe
17	liver or kidney impairment; or were pregnant. No familial relationship was known
18	between the study participants. The study was performed according to the Guidelines
19	of the Medical Ethical Committee of Xinxiang Medical College (Xinxiang, China).
20	Written informed consent was obtained from each participant in this study.

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21 SNP selection

1	Tagging SNPs were selected from the catalogs of the International HapMap
2	Project. The rs2789679 and rs2245168 are located in 5'-UTR, rs2236558 in intron 6,
3	rs7067644 and rs12763624 in intron 8, rs1049550 in exon 10, rs2573351 in intron 13,
4	rs10887581, rs11201989, rs2573353, rs2789695, rs2573356, rs2819945, rs11202059
5	and rs2819941 in intron 14 (Supplemental figure 1). Marker selection was based on
6	the following criteria. (a) We used the CHB data from the HapMap (release 27) to
7	select tagSNPs for ANXA11. (b) We restricted our search for tagSNPs from base pair
8	80155124 to 80205677. (c) Further, we limited the SNPs to tag to those with a minor
9	allele frequency ≥ 0.2 in the CHB. (d) Based on these restrictions, there were a total
10	of 29 SNPs. (e) Using HAPLOVIEW with an r^2 threshold ≥ 0.8 , 15 tagSNP were
11	selected and used for subsequent analyses.
12	Genotyping
13	Peripheral blood was collected from a vein into a sterile tube coated with

Peripheral blood was collected from a vein into a sterile tube coated with ethylenediamine tetraacetic acid (EDTA). Plasma samples were stored at -20°C. Genomic DNA was extracted from the frozen peripheral blood samples using a QIAmp Blood Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocols. The selected SNPs were genotyped in cases and controls by using the MALDI-TOF in the MassARRAY system (Sequenom Inc., San Diego, CA, USA). Probes and primers were designed using the Assay Design Software (Sequenom, San Diego, CA, USA). The completed genotyping reactions were spotted onto a 384 well spectroCHIP (Sequenom) using the MassARRAY Nanodispenser (Sequenom) and determined by the matrix - assisted laser desorption ionization time -

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of - flight mass spectrometer. Genotype calling was performed in realtime with the
 MassARRAY RT software version 3.0.0.4 and analyzed using the MassARRAY Typer
 software version 3.4 (Sequenom).

4 Statistical analysis

All data were analyzed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, 5 6 USA). Hardy-Weinberg equilibrium was evaluated by Chi-square tests. Differences 7 between the cases and controls in the frequency of the alleles, genotypes and haplotypes were evaluated by the Fisher's exact test or the Pearson Chi-square test. 8 Unconditional logistic regression was used to calculate the odds ratio (OR) and 95% 9 confidence interval (CI) in independent association between each locus and the 10 presence of sarcoidosis. Gender and age of subjects were treated as covariants in 11 12 binary logistic regression. P values were calculated based on codominant, dominant for the rare allele, heterosis and recessive for the rare allele models of inheritance. 13 Models of multiple logistic regression were used to test the independence of 14 individual allelic effect. In detail, the most significant SNP was chosen to be the 15 conditional SNP (covariate in the regression model) when testing other significant 16 SNPs; Meanwhile, a multi-SNP model including all significant SNPs was also 17 performed. The Bonferroni correction was used to adjust the test level when multiple 18 19 comparisons were conducted, and the P value was divided by the total number of loci. Haplotype blocks were defined according to the criteria developed by Gabriel et al.¹⁵. 20 Pair-wise LD statistics (D' and r^2) and haplotype frequency were calculated, and 21 haplotype blocks were constructed using the Haploview 4.0¹⁶. To ensure that the LD 22

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> blocks most closely reflect the population level LD patterns, definition of the blocks were based on the control samples alone. The haplotype frequencies were estimated using GENECOUNTING, which computes maximum - likelihood estimates of haplotype frequencies from unknown phase data by utilizing an expectation maximization algorithm ¹⁷⁻²⁰. The significance of any haplotypic association was evaluated using a likelihood ratio test, followed by permutation testing that compared estimated haplotype frequencies in cases and controls ^{17 19}.

8 **RESULTS**

The genotype distribution of the fifteen polymorphisms was consistent with the 9 Hardy-Weinberg equilibrium (P > 0.05). The analysis of strong LD in the patients 10 with sarcoidosis and healthy controls revealed that 2 SNPs (rs2245168, rs2236558), 2 11 12 SNPs (rs1049550, rs2573351), 2 SNPs (rs2789695, rs2573356) and 3 SNPs (rs2819945, rs11202059, rs2819941) were located in haplotype block 1, block 2, 13 block 3 and block 4 (D' > 0.9, Fig. 1). The genotype distribution, allelic frequencies, 14 and haplotypes in the patients with sarcoidosis and healthy controls are showed in 15 Tables 1-3 and Supplemental table 2 16

17 Comparison of genotype and allele frequency distribution revealed significant 18 differences between the patients with sarcoidosis and healthy controls for 3 SNPs: 19 rs2789679, rs1049550 and rs2819941. The frequency of the rs1049550 T allele (P =20 0.000002, OR = 0.61, 95%CI= 0.49-0.74) in the patients with sarcoidosis was 21 significantly lower than that in the controls. These differences remained statistically 22 significant after Bonferroni corrections. The rs1049550 was the most significant of the

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three. A multi-SNP model revealed rs1049550 to be the only significant independent
 SNP association with sarcoidosis risk.

The multi-SNP model showed only rs1049550 present a significant effect on the disease phenotype (p < 0.001). No independent effect was found for rs2789679 or rs2819941 when adjusting for the effect of rs1049550. Both individual SNP and multi -SNP analysis supported rs1049550: T allele was an important protective factor for affecting sarcoidosis (odds ratio around 0.6). In another word, carriers of rs1049550: C allele have higher susceptibility to sarcoidosis in our Chinese Han population (Supplemental table 3).

We performed an association analysis to determine whether the haplotype was associated with the risk of sarcoidosis (Global P < 0.05 in block 2 and block 3). Compared with the controls, the T - C haplotype occurred significantly less frequently (P = 0.001) but the C - C haplotypes occurred more frequently (P = 0.0001) in block 2 (rs1049550 - rs2573351) in the patients with sarcoidosis. BMJ Open: first published as 10.1136/bmjopen-2013-004466 on 23 July 2014. Downloaded from http://bmjopen.bmj.com/ on June 14, 2025 at Agence Bibliographique de Enseignement Superieur (ABES)

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To assess particular disease phenotypes, the patients with sarcoidosis were 15 divided into the subgroups according to their chest radiographic (CXR) stage. CXR 16 stage I (isolated bilateral hilar lymphadenopathy): N = 196; CXR stages II - IV 17 (infiltration of parenchyma): 167; CXR stage 0 (absence on pulmonary disease): N =18 28; the information on CXR stage was not available for 21 patients. Genotype 19 frequency distribution revealed that, in rs1049550, CC genotype was significantly 20 21 more in the patients with chest radiographic (CXR) stage I sarcoidosis than the patients with CXR stage II-IV sarcoidosis (P = 0.012). (Table 4). 22

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1 DISCUSSION

A key step in linkage and association studies is to identify common risk variants in different populations. To determine if common risk variants exist in distinct populations, fifteen SNPs which span approximately 0.53 Mb of the ANXA11 gene, were genotyped in samples from the patients with sarcoidosis and healthy controls in a Chinese Han population. Recently, new sarcoidosis loci have been identified by genome-wide association studies ^{4 14}. With the fast development of genome - wide association studies, an increasing number of susceptibility loci for sarcoidosis have been found in different populations ⁴ ¹⁴ ²¹. However, these observations should be confirmed in other genetically independent populations. In this study, we conducted the first large genetic association study of the ANXA11 gene in a Chinese Han population. The evidence of markers associated with sarcoidosis was presented, and these markers were mapped to different locations in the ANXA11 gene (81897864 -81951001). The association signals in the region were identified, and some significantly associated haplotypes also appeared this region. Hoffman et al. reported an association between the T allele of the non -synonymous SNP rs1049550 and significantly decreased risk of sarcoidosis in a German population ⁴. Similar results were also obtained in other two European

populations ^{14 21}. As a part of the sarcoidosis GWAS done in Americans ²², we further
confirmed this association in a Chinese Han population. In this study, the frequency of
ANXA11 rs1049550 T allele was significantly lower in the patients with sarcoidosis
than the healthy controls. Genotype frequency distribution revealed that, in rs1049550,

CC genotype was significantly more in the patients with stage I sarcoidosis than the patients with stage II-IV sarcoidosis. The "protective" effect of ANXA11 T allele increased with the number of its copies in the genotype, which is consistent with the result obtained in the patients with sarcoidosis in a German population⁴, Furthermore, the T allele carriers among the patients were protected from infiltration of lung parenchyma (radiographic stages II-IV)²¹. We have also demonstrated that rs1049550 is significant cross - ethnically at the gene level after adjustment for the single SNP association tests performed. The mechanism by which rs1049550 affects the susceptibility to sarcoidosis is that the SNP may affect the function of the ANXA11 protein rather than affect its expression. In humans, the ANXA11 protein consists of an N - terminalproline - tyrosine - glycine (PYG) - rich region, followed by four annexin core domains. The rs1049550 leads to an amino - acid exchange (basic arginine to a polar cysteine) at the evolutionarily conserved position 230 (R230C) in the first annexin domain, which is responsible for Ca^{2+} - dependent trafficking of the protein in the cell ²³.

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In the GWAS conducted by Hofmann et al ⁴, the strongest association signal was observed for rs2789679. This marker is located in the 3'-UTR of the ANXA11 gene. The T allele had a frequency of 35% among the patients with sarcoidosis and 44% in the healthy controls, with 13% of the patients with sarcoidosis and 19% of controls being TT homozygous. Fine-mapping in the Black Women's Health Study (BWHS) revealed rs2819941 to be the top SNP, associated with a non-significant (P = 0.06) reduction in the risk of sarcoidosis (17% reduction per copy of the C-allele). In this

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1	case-control association study, the frequency of the A allele in rs2789679 and the
2	rs2819941 T allele frequency in the patients with sarcoidosis was significantly higher
3	than that in the healthy controls. Several lines of evidence suggest that the observed
4	association is unlikely to be an artifact. First, both the single-SNP and the
5	haplotype-based association analyses support the current finding. Second, our samples
6	were from the same geographical region, excluding the. Finally, consistent results
7	were obtained from two genetically independent populations (Chinese Han and
8	Europeans). Collectively, our results confirmed the strong association between
9	ANXA11 polymorphisms and sarcoidosis, suggesting that ANXA11 represents a
10	strong genetic risk factor for sarcoidosis. Since causal SNP in ANXA11 was not
11	known, all the SNPs significantly associated with sarcoidosis were actually indirect
12	association through the real causal one. Though rs2789679 and rs2819941 were not in
13	LD with rs1049550, it may not tag the causal SNP as well as rs1049550. Hence, their
14	individual effects were eliminated when controlling for the effect of rs1049550.

We further investigated the interaction among polymorphisms and observed 15 strong LD. The haplotype analysis revealed that significantly more C - C (block 2, 16 rs1049550 - rs2573351) and significantly fewer T - C haplotypes (block 2) were 17 18 found in the patients with sarcoidosis. These results indicated that the patients with C - C (block 2) haplotypes of the ANXA11 gene were more prone to sarcoidosis. 19 Significantly higher frequencies of T - C haplotypes were detected in the healthy 20 21 controls than in the patients with sarcoidosis, suggesting that they may show protective effects against sarcoidosis. Our sample size can detect SNP and haplotype 22

1	associations with 90% and 85% power, respectively, at a false positive rate of 5%,
2	disease prevalence of 1‰, disease allele / haplotype frequency of 0.05 / 0.03, and a
3	presumed odds ratio (OR) of 1.5. To some extent, this finding further supports a role
4	of ANXA11 polymorphisms in sarcoidosis, while ethnic group difference may exist.
5	In conclusion, our study suggests a potential role of the ANXA11 SNPs and their
6	related haplotypes in the genetic susceptibility to sarcoidosis. Further studies are
7	needed to investigate how these SNPs affect the function of ANXA11. A broader
8	examination of the genetic variation in ANXA11 in the Han Chinese may reveal other
9	variants associated with disease risk.
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1	Contributors
2	LG Zhang was involved in conception and design of the study, acquisition of
3	patient data, genotyping and drafted the paper. ZJ Xiao and GC Shi were involved in
4	design of the study, genotyping and interpretation of results. J Huang and CX Zhang
5	were involved in the design of the study, acquisition of patient data and interpretation
6	of result. All authors revised the draft paper.
7	Funding
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9	Science and Technology of Henan Province (2012k15).
10	Competing interests
11	None.
12	Patient consent
13	Obtained.
14	Ethics approval
15	Ethics approval was provided by the First Hospital Affiliated to the Xinxiang
16	Medical College.

- **17 Provenance and peer review**
- 18 Not commissioned; externally peer reviewed.
- 19 Data sharing statement
- 20 No additional data are available.

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1	Figure Legend
2	Fig. 1 The LD plot of the fifteen SNPs in the ANXA11 gene. Values in squares are the
3	pair-wise calculation of r^2 (left) or D' (right). Black squares indicate $r^2 = 1$ (i.e. perfect
4	LD between a pair of SNPs). Empty squares indicate D' = 1 (i.e. complete LD
5	between a pair of SNPs).
6	Supplemental Fig. 1 The structure of the human ANXA11 gene and 15 SNPs located
7	on the gene.
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Table 1 Genotypic and allelic frequencies of ANXA	11 polymorphisms in the controls
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and patients with sarcoidosis

	Controls (n=418)		Sarcoidosis (n=412)		- - a	
Variable -	No.	%	No.	%	- P-value ^a	OR, 95% CI
rs2789679					0.0007	
ТТ	114	27.3	94	22.8	0.140	1.27, 0.93-1.74
AT	225	53.8	186	45.2	0.012	1.42, 1.08-1.87
AA	79	18.9	132	32.0	0.0002	0.49, 0.36-0.68
Per A allele	383	45.8	450	54.6	0.00004	1.42, 1.17-1.73
rs1049550					0.0002	
CC	154	36.8	208	50.5	0.0007	0.57, 0.43-0.75
СТ	190	45.5	170	41.3	0.221	1.19, 0.90-1.56
TT	74	17.7	34	8.3	0.0008	2.39, 1.55-3.68
Per T allele	338	40.4	238	28.8	0.000002	0.61, 0.49-0.74
rs2819941					0.0002	
CC	114	27.3	94	22.8	0.141	1.27, 0.93-1.74
СТ	226	54.1	190	46.1	0.021	1.38, 1.05-1.81
TT	78	18.7	128	31.1	0.0004	0.51, 0.37-0.70
Per T allele	382	45.7	446	54.1	0.0006	1.41, 1.16-1.71

³ ^a *P*-value was calculated by 2×3 and 2×2 chi-squared tests based on codominant,

4 dominant for the rare allele, heterosis and recessive for the rare allele models of

5 inheritance.

6 Alpha value is adjusted by Bonferroni correction and statistically significant results

- 7 (P<0.003)

1 1	Table 2 ANXA11 haplotype in block 2 frequencies and the results of their associations with risk
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			of sarcoidos	is		
	Haplotype		Genecounting (frequency %)			
ID	rs1049550	rs2573351	Cases	Controls	<i>P</i> -value ^a	Global P ^b
HAP1	Т	С	27.7	39.7	0.001	0.003
HAP2	С	С	19.9	7.4	0.0001	
HAP3	С	Т	50.5	51.0	0.892	

^a Based on 10,000 permutations.

^b Based on comparison of frequency distribution of all haplotypes for the combination of SNPs.

of sarcoidosis

	Haplotype		Genecounting (frequency %)			
ID	rs2789695	rs2573356	Cases	Controls	<i>P</i> -value ^a	Global P ^b
HAP1	С	Т	33.7	34.9	0.718	0.045
HAP2	Т	Т	15.3	14.1	0.632	
HAP2	Т	Т	15.6	14.1	0.565	

^b Based on comparison of frequency distribution of all haplotypes for the combination of SNPs.

Table 3 ANXA11 haplotype in block 3 frequencies and the results of their associations with risk

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(31.7)

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$ stage I = \begin{cases} 59 & 92 & 45 & 101 & 82 & 13 & 47 & 88 \\ (30.1) & (47.0) & (23.0) & (51.5) & (41.8) & (6.6) & (25.0) & (44.9) & (0.5) \\ stages & 46 & 75 & 46 & 72 & 68 & 27 & 41 & 73 \\ II - IV & (27.6) & (44.9) & (27.6) & (43.1) & (40.7) & (16.2) & (24.6) & (43.7) & (0.5) \\ \hline x^2, P & 1.041, 0.594 & 8.807, 0.012 & 0.052, 0.975 \\ \hline r P values for genotype frequency distribution (P < 0.05). \end{cases} $		Stages	AA	AT	TT	CC	СТ	TT	CC	СТ	TT
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1	Annexin A11 (ANXA11) gene polymorphisms are associated with sarcoidosis in a
2	Han Chinese population - a case-control study
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12	Key words: Sarcoidosis; Annexin A11; Single-nucleotide polymorphism
13	Word count: 3918
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Abstract
Objectives: To further identify the single-nucleotide polymorphisms (SNPs) that
contribute to the genetic susceptibility to sarcoidosis, we examined the potential
association between sarcoidosis and fifteen SNPs of the ANXA11 gene.
Design: A case - control study.
Setting: A tuberculosist unit in a hospital of the university in China.
Participants: Participants included 412 patients with sarcoidosis and 418 healthy
controls.
Methods: The selected SNPs were genotyped using the MALDI-TOF in the
MassARRAY system. Probes and primers were designed using the Assay Design
Software (Sequenom, San Diego, CA, USA).
Results: The statistical significant differences were found in the allelic or genotypic
frequencies of the rs2789679, rs1049550 and rs2819941 in the ANXA11 gene
between the patients with sarcoidosis and the controls. The rs2789679 A allele ($P =$
0.00004, OR = 1.42, 95%CI = 1.17-1.73) and rs2819941 T allele ($P = 0.0006$, OR =
1.41, 95% CI = 1.16-1.71) were significantly more frequent in the patients with
sarcoidosis compared to controls. The frequency of the rs1049550 T allele ($P =$
0.000002, OR = 0.61, 95%CI= 0.49 - 0.74) in the patients with sarcoidosis was
significantly lower than that in the controls. The multi-SNP model reveals that
rs1049550 is the only independent SNP association effect after accounting for the
other two marginally associated SNPs. The strong linkage disequilibrium (LD) was
observed in four blocks (D' > 0.9). In block 2 (rs1049550-rs2573351), the T - C

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haplotype occurred significantly less frequently (P = 0.001), whereas the C - C haplotypes occurred more frequently (P = 0.0001) in the patients with sarcoidosis than the controls. Furthermore, genotype frequency distribution revealed that, in rs1049550, CC genotype was significantly more in the patients with chest radiographic (CXR) stage I sarcoidosis than the patients with CXR stage II-IV sarcoidosis (P = 0.012).

Conclusions: These findings point to a role for the polymorphisms of ANXA11 in sarcoidosis in a Chinese Han population, and may be informative for future genetic studies on sarcoidosis.

1	Article summary
2	Article focus
3	1. The first genome-wide association study (GWAS) in sarcoidosis conducted in a
4	German population has revealed that the rs1049550 (C > T, Arg230Cys) located in
5	exon 10 were associated with sarcoidosis.
6	2. Whether these SNPs modulate the risk of sarcoidosis by themselves or whether
7	they correlate with other causative SNPs and are repeated in other populations remain
8	unclear.
9	3. We hypothesize that common variants in the ANXA11 gene may significantly
10	contribute to the predisposition to develop sarcoidosis in a Chinese Han population.
11	Key messages
12	1. ANXA11 rs2789679 (3'-UTR) was significantly associated with sarcoidosis.
13	2. ANXA11 rs1049550 (exon 10) was significantly associated with sarcoidosis.
14	3. Another significant association was observed for rs2819941 (intron 14).
15	Strengths and limitations of this study
16	1. A systematical screening of the functional SNPs in the promoter region, 5'- and
17	3'-UTR, exons of the ANXA11 gene, and the homogeneity of the study subjects
18	representing the Chinese Han are the main strengths of this study.
19	2. The lack of data proving the positive association observed for rs2789679 and
20	rs1049550 is a potential limitation of this study. Furthermore, the association of the
21	serum level of ANXA11 with sarcoidosis still needs to be investigated.
22	INTRODUCTION

$\begin{array}{c}1\\2\\3\\4\\5\\6\\7\\8\\9\\10\\11\\23\\14\\15\\16\\17\\18\\9\\20\\21\\22\\324\\25\\26\\27\\28\\29\\30\\31\end{array}$		
$\begin{array}{c} 37\\ 38\\ 39\\ 40\\ 41\\ 42\\ 43\\ 44\\ 45\\ 46\\ 47\\ 48\\ 49\\ 50\\ 51\\ 52\\ 53\\ 54\\ 55\\ 56\\ 57\\ 58\\ 59\\ 60\\ \end{array}$		

1	Sarcoidosis is a systemic autoimmune disease characterized by destructive,
2	non-caseating epithelioid granulomatous lesions, with accumulated phagocytes and
3	activated CD4 ⁺ T helper type 1 lymphocytes ^{1 2} . The typical manifestations of
4	sarcoidosis include bilateral hilar lymphadenopathy, pulmonary infiltration and ocular
5	and skin lesions. The clinical course and prognosis of sarcoidosis are variable. The
6	acute forms of sarcoidosis such as Löfgren's syndrome (LS) can resolve
7	spontaneously in most cases. However, chronic pulmonary sarcoidosis may progress
8	to lung fibrosis which eventually causes respiratory failure. Recent studies have
9	demonstrated that sarcoidosis can occur in a pattern of family clustering, and its racial
10	incidence is also different, suggesting that some genetic factors may contribute to the
11	risk and severity of sacroidosis ¹³ .
12	The Annexin gene family is involved in the etiology of several autoimmune and
13	chronic diseases ^{4 5.} One member of the Annexin gene family, ANXA11, located on
14	chromosome 10q22.3, is involved in calcium signaling, apoptosis, vesicle trafficking,
15	cell growth and terminal phase of cell division ⁵⁻⁷ . In this context, the development
16	and maintenance of the granulomatous inflammation in sarcoidosis have been
17	repeatedly associated with the impaired apoptosis of activated inflammatory cells ⁸⁹ .
18	Most of the early-stage sarcoidosis is characterized by mononuclear cell alveolitis
19	dominated by activated CD4 ⁺ T cells and macrophages. Between these cells, the
20	uncoordinated interplay results in the formation of typical non-caseating granulomas.
21	The mechanism by which the granulomas resolve has not been fully elucidated.

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withdrawal of inflammatory cytokines participates in the disappearance of
 granulomas ^{10 11}. In the patients with sarcoidosis, their peripheral blood monocytes
 were characterized by apoptosis ⁹.

In previous studies, case - control/family 'hypothesis - driven' studies and low 4 density linkage scans were used to identify genetic factors conferring the genetic 5 susceptibility to sarcoidosis ¹². Notably, the first genome - wide association study 6 (GWAS) in sarcoidosis conducted in a German population has recently revealed an 7 association: the leading rs2789679 SNP (single - nucleotide polymorphism) located in 8 the 3'-untranslated region (3'-UTR) and a common nonsynonymous SNP (rs1049550, 9 C > T, Arg230Cys) were associated with the increased risk of sarcoidosis ^{4 13}. This 10 association has recently been supported by another report from the same population ¹⁴. 11 12 Thus, more studies should be performed to demonstrate the following items: whether these SNPs modulate the risk of sarcoidosis by themselves or whether they correlate 13 with other causative SNPs and are repeated in other populations. The published 14 studies about the association of sarcoidosis and ANXA11 are summarized in 15 Supplemental table 1. We hypothesize that common variants in the ANXA11 gene 16 may significantly contribute to the predisposition to develop sarcoidosis. 17

In this study, we investigated fifteen loci in a Chinese population from He'nan province (China) to verify the putative association between ANXA11 polymorphisms and sarcoidosis.

21 SUBJECTS AND METHODS

22 Subjects

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1	Four hundred and twelve patients with sarcoidosis (mean \pm SD age: 53.6 \pm 4.6
2	years; 155 men and 257 women) were recruited from our hospital between May 2005
3	and March 2013. Sarcoidosis was diagnosed by the evidence of non-caseating
4	epitheloid cell granuloma in biopsy specimens and chest radiographic abnormalities.
5	Chronic sarcoidosis was defined as a disease over at least 2 years or at least two
6	episodes in a lifetime. A cute sarcoidosis was defined as one episode of acute
7	sarcoidosis which had totally resolved at the date of the examination. The control
8	group consisted of 418 unrelated healthy subjects (mean \pm SD age: 54.2 \pm 5.3 years;
9	160 men and 258 women) who underwent health examinations in the Medical
10	Examination Center of the First Affiliated Hospital of the Xinxiang Medical College
11	(Xinxiang, China) between Oct 2009 and Sept 2013. None of the individuals in the
12	control group had a history of lung diseases or showed any symptoms of the lung or
13	other diseases by chest radiography or laboratory blood tests. Participants were
14	excluded if they: were taking other prescribed medications that could affect the
15	central nervous system; had a history of seizures, hematological diseases, or severe
16	liver or kidney impairment; or were pregnant. No familial relationship was known
17	between the study participants. The study was performed according to the Guidelines
18	of the Medical Ethical Committee of Xinxiang Medical College (Xinxiang, China).
19	Written informed consent was obtained from each participant in this study.

20 SNP selection

Tagging SNPs were selected from the catalogs of the International HapMap Project. The rs2789679 and rs2245168 are located in 5'-UTR, rs2236558 in intron 6,

7

1	rs7067644 and rs12763624 in intron 8, rs1049550 in exon 10, rs2573351 in intron 13,
2	rs10887581, rs11201989, rs2573353, rs2789695, rs2573356, rs2819945, rs11202059
3	and rs2819941 in intron 14 (Supplemental figure 1). Marker selection was based on
4	the following criteria. (a) We used the CHB data from the HapMap (release 27) to
5	select tagSNPs for ANXA11. (b) We restricted our search for tagSNPs from base pair
6	80155124 to 80205677. (c) Further, we limited the SNPs to tag to those with a minor
7	allele frequency ≥ 0.2 in the CHB. (d) Based on these restrictions, there were a total
8	of 29 SNPs. (e) Using HAPLOVIEW with an r^2 threshold ≥ 0.8 , 15 tagSNP were
9	selected and used for subsequent analyses.

Genotyping

Peripheral blood was collected from a vein into a sterile tube coated with ethylenediamine tetraacetic acid (EDTA). Plasma samples were stored at -20°C. Genomic DNA was extracted from the frozen peripheral blood samples using a QIAmp Blood Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocols. The selected SNPs were genotyped in cases and controls by using the MALDI-TOF in the MassARRAY system (Sequenom Inc., San Diego, CA, USA). Probes and primers were designed using the Assay Design Software (Sequenom, San Diego, CA, USA). The completed genotyping reactions were spotted onto a 384 well spectroCHIP (Sequenom) using the MassARRAY Nanodispenser (Sequenom) and determined by the matrix - assisted laser desorption ionization time -of - flight mass spectrometer. Genotype calling was performed in realtime with the MassARRAY RT software version 3.0.0.4 and analyzed using the MassARRAY Typer BMJ Open: first published as 10.1136/bmjopen-2013-004466 on 23 July 2014. Downloaded from http://bmjopen.bmj.com/ on June 14, 2025 at Agence Bibliographique de Enseignement Superieur (ABES) .

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1 software version 3.4 (Sequenom).

2 Statistical analysis

All data were analyzed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Hardy-Weinberg equilibrium was evaluated by Chi-square tests. Differences between the cases and controls in the frequency of the alleles, genotypes and haplotypes were evaluated by the Fisher's exact test or the Pearson Chi-square test. Unconditional logistic regression was used to calculate the odds ratio (OR) and 95% confidence interval (CI) in independent association between each locus and the presence of sarcoidosis. Gender and age of subjects were treated as covariants in binary logistic regression. P values were calculated based on codominant, dominant for the rare allele, heterosis and recessive for the rare allele models of inheritance. Models of multiple logistic regression were used to test the independence of individual allelic effect. In detail, the most significant SNP was chosen to be the conditional SNP (covariate in the regression model) when testing other significant SNPs; Meanwhile, a multi-SNP model including all significant SNPs was also performed. The Bonferroni correction was used to adjust the test level when multiple comparisons were conducted, and the *P* value was divided by the total number of loci. Haplotype blocks were defined according to the criteria developed by Gabriel et al.¹⁵. Pair-wise LD statistics (D' and r^2) and haplotype frequency were calculated, and haplotype blocks were constructed using the Haploview 4.0 16 . To ensure that the LD blocks most closely reflect the population level LD patterns, definition of the blocks were based on the control samples alone. The haplotype frequencies were estimated

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using GENECOUNTING, which computes maximum - likelihood estimates of
haplotype frequencies from unknown phase data by utilizing an expectation -
maximization algorithm ¹⁷⁻²⁰ . The significance of any haplotypic association was
evaluated using a likelihood ratio test, followed by permutation testing that compared
estimated haplotype frequencies in cases and controls ^{17 19} .
RESULTS
The genotype distribution of the fifteen polymorphisms was consistent with the
Hardy-Weinberg equilibrium ($P > 0.05$). The analysis of strong LD in the patients
with sarcoidosis and healthy controls revealed that 2 SNPs (rs2245168, rs2236558), 2
SNPs (rs1049550, rs2573351), 2 SNPs (rs2789695, rs2573356) and 3 SNPs
(rs2819945, rs11202059, rs2819941) were located in haplotype block 1, block 2,
block 3 and block 4 (D' > 0.9, Fig. 1). The genotype distribution, allelic frequencies,
and haplotypes in the patients with sarcoidosis and healthy controls are showed in
Tables 1-3 and Supplemental table 2
Comparison of genotype and allele frequency distribution revealed significant
differences between the patients with sarcoidosis and healthy controls for 3 SNPs:
rs2789679, rs1049550 and rs2819941. The rs2789679 A allele (P = 0.00004, OR =
1.42, 95%CI = $1.17-1.73$) and rs2819941 T allele (<i>P</i> = 0.0006 , OR = $1.41, 95%$ CI =
1.16-1.71) were significantly more frequent in the patients with sarcoidosis compared
to controls. The frequency of the rs1049550 T allele ($P = 0.000002$, OR = 0.61,
95%CI= 0.49-0.74) in the patients with sarcoidosis was significantly lower than that
in the controls. These differences remained statistically significant after Bonferroni
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1	corrections. The rs1049550 was the most significant of the three. A multi-SNP model
2	revealed rs1049550 to be the only significant independent SNP association with
3	sarcoidosis risk.
4	The multi-SNP model showed only rs1049550 present a significant effect on the
5	disease phenotype (p < 0.001). No independent effect was found for rs2789679 or
6	rs2819941 when adjusting for the effect of rs1049550. Both individual SNP and multi -
7	SNP analysis supported rs1049550: T allele was an important protective factor for
8	affecting sarcoidosis (odds ratio around 0.6). In another word, carriers of rs1049550:
9	C allele have higher susceptibility to sarcoidosis in our Chinese Han population
10	(Supplemental table 3).
11	We performed an association analysis to determine whether the haplotype was
12	associated with the risk of sarcoidosis (Global $P < 0.05$ in block 2 and block 3).
13	Compared with the controls, the T - C haplotype occurred significantly less frequently
14	(P = 0.001) but the C - C haplotypes occurred more frequently $(P = 0.0001)$ in block 2
15	(rs1049550 - rs2573351) in the patients with sarcoidosis.
16	To assess particular disease phenotypes, the patients with sarcoidosis were
17	divided into the subgroups according to their chest radiographic (CXR) stage. CXR
18	stage I (isolated bilateral hilar lymphadenopathy): N = 196; CXR stages II - IV

divided into the subgroups according to their chest radiographic (CXR) stage. CXR
stage I (isolated bilateral hilar lymphadenopathy): N = 196; CXR stages II - IV
(infiltration of parenchyma): 167; CXR stage 0 (absence on pulmonary disease): N =
28; the information on CXR stage was not available for 21 patients. Genotype

frequency distribution revealed that, in rs1049550, CC genotype was significantly more in the patients with chest radiographic (CXR) stage I sarcoidosis than the

1 patients with CXR stage II-IV sarcoidosis (P = 0.012). (Table 4).

2 DISCUSSION

A key step in linkage and association studies is to identify common risk variants in different populations. To determine if common risk variants exist in distinct populations, fifteen SNPs which span approximately 0.53 Mb of the ANXA11 gene, were genotyped in samples from the patients with sarcoidosis and healthy controls in a Chinese Han population. Recently, new sarcoidosis loci have been identified by genome-wide association studies ^{4 14}. With the fast development of genome - wide association studies, an increasing number of susceptibility loci for sarcoidosis have been found in different populations ⁴¹⁴²¹. However, these observations should be confirmed in other genetically independent populations. In this study, we conducted the first large genetic association study of the ANXA11 gene in a Chinese Han population. The evidence of markers associated with sarcoidosis was presented, and these markers were mapped to different locations in the ANXA11 gene (81897864 -81951001). The association signals in the region were identified, and some significantly associated haplotypes also appeared this region.

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Hoffman et al. reported an association between the T allele of the non synonymous SNP rs1049550 and significantly decreased risk of sarcoidosis in a German population ⁴. Similar results were also obtained in other two European populations ¹⁴²¹. As a part of the sarcoidosis GWAS done in Americans ²², we further confirmed this association in a Chinese Han population. In this study, the frequency of ANXA11 rs1049550 T allele was significantly lower in the patients with sarcoidosis

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1	than the healthy controls. Genotype frequency distribution revealed that, in rs1049550,
2	CC genotype was significantly more in the patients with stage I sarcoidosis than the
3	patients with stage II-IV sarcoidosis. The "protective" effect of ANXA11 T allele
4	increased with the number of its copies in the genotype, which is consistent with the
5	result obtained in the patients with sarcoidosis in a German population ⁴ , Furthermore,
6	the T allele carriers among the patients were protected from infiltration of lung
7	parenchyma (radiographic stages II-IV) ²¹ . We have also demonstrated that rs1049550
8	is significant cross - ethnically at the gene level after adjustment for the single SNP
9	association tests performed. The mechanism by which rs1049550 affects the
10	susceptibility to sarcoidosis is that the SNP may affect the function of the ANXA11
11	protein rather than affect its expression. In humans, the ANXA11 protein consists of
12	an N - terminalproline - tyrosine - glycine (PYG) - rich region, followed by four
13	annexin core domains. The rs1049550 leads to an amino - acid exchange (basic
14	arginine to a polar cysteine) at the evolutionarily conserved position 230 (R230C) in
15	the first annexin domain, which is responsible for Ca^{2+} - dependent trafficking of the
16	protein in the cell ²³ .

In the GWAS conducted by Hofmann et al ⁴, the strongest association signal was observed for rs2789679. This marker is located in the 3'-UTR of the ANXA11 gene. The T allele had a frequency of 35% among the patients with sarcoidosis and 44% in the healthy controls, with 13% of the patients with sarcoidosis and 19% of controls being TT homozygous. Fine-mapping in the Black Women's Health Study (BWHS) revealed rs2819941 to be the top SNP, associated with a non-significant (P = 0.06)

1	reduction in the risk of sarcoidosis (17% reduction per copy of the C-allele). In this
2	case-control association study, the frequency of the A allele in rs2789679 and the
3	rs2819941 T allele frequency in the patients with sarcoidosis was significantly higher
4	than that in the healthy controls. Several lines of evidence suggest that the observed
5	association is unlikely to be an artifact. First, both the single-SNP and the
6	haplotype-based association analyses support the current finding. Second, our samples
7	were from the same geographical region, excluding the. Finally, consistent results
8	were obtained from two genetically independent populations (Chinese Han and
9	Europeans). Collectively, our results confirmed the strong association between
10	ANXA11 polymorphisms and sarcoidosis, suggesting that ANXA11 represents a
11	strong genetic risk factor for sarcoidosis. Since causal SNP in ANXA11 was not
12	known, all the SNPs significantly associated with sarcoidosis were actually indirect
13	association through the real causal one. Though rs2789679 and rs2819941 were not in
14	LD with rs1049550, it may not tag the causal SNP as well as rs1049550. Hence, their
15	individual effects were eliminated when controlling for the effect of rs1049550.

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We further investigated the interaction among polymorphisms and observed strong LD. The haplotype analysis revealed that significantly more C - C (block 2, rs1049550 - rs2573351) and significantly fewer T - C haplotypes (block 2) were found in the patients with sarcoidosis. These results indicated that the patients with C - C (block 2) haplotypes of the ANXA11 gene were more prone to sarcoidosis. Significantly higher frequencies of T - C haplotypes were detected in the healthy controls than in the patients with sarcoidosis, suggesting that they may show

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1	protective effects against sarcoidosis. Our sample size can detect SNP and haplotype
2	associations with 90% and 85% power, respectively, at a false positive rate of 5%,
3	disease prevalence of 1‰, disease allele / haplotype frequency of 0.05 / 0.03, and a
4	presumed odds ratio (OR) of 1.5. To some extent, this finding further supports a role
5	of ANXA11 polymorphisms in sarcoidosis, while ethnic group difference may exist.
6	In conclusion, our study suggests a potential role of the ANXA11 SNPs and their
7	related haplotypes in the genetic susceptibility to sarcoidosis. Further studies are
8	needed to investigate how these SNPs affect the function of ANXA11. A broader
9	examination of the genetic variation in ANXA11 in the Han Chinese may reveal other
10	variants associated with disease risk.
11	
12	Contributors
13	LG Zhang was involved in conception and design of the study, acquisition of
14	patient data, genotyping and drafted the paper. ZJ Xiao and GC Shi were involved in
15	design of the study, genotyping and interpretation of results. J Huang and CX Zhang

were involved in the design of the study, acquisition of patient data and interpretation

17 of result. All authors revised the draft paper.

18 Funding

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- 21 Competing interests
 - None.

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3	1	Patient consent
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6	2	Obtained.
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19	7	Not commissioned; externally peer reviewed.
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22	8	Data sharing statement
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27	10	Figure Legend
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29	11	Fig. 1 The LD plot of the fifteen SNPs in the ANXA11 gene. Values in squares are the
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32	12	pair-wise calculation of r^2 (left) or D' (right). Black squares indicate $r^2 = 1$ (i.e. perfect
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33 34	13	LD between a pair of SNPs). Empty squares indicate D' = 1 (i.e. complete LD
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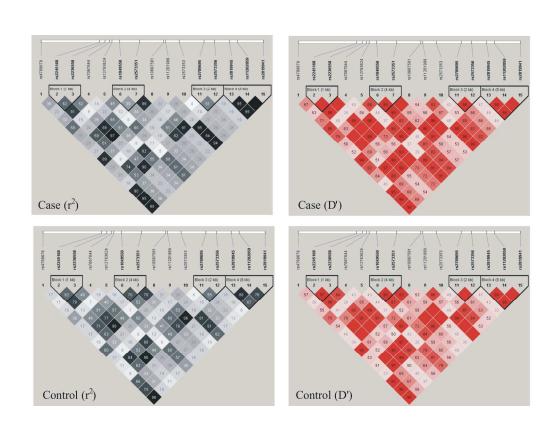
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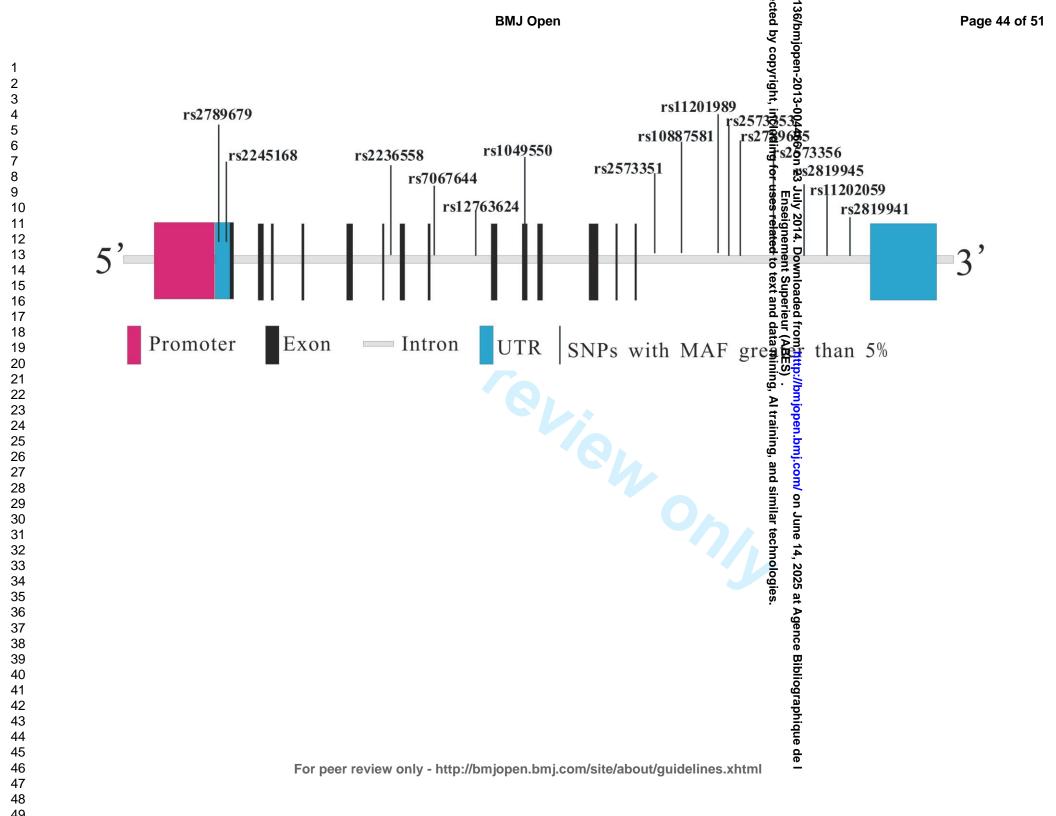
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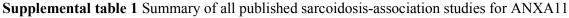


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S4m day	Population		Town of Starla	Sample Size (n)		Number of	D: 4 CNID.	
Study	Ethnic group	Country	Type of Study	Sarcoidosis	Control	SNPs typed	Positive SNPs	
Hofmann S, 2008	Caucasian	Germany	Case-control	499	490	GWAS	rs2789679, rs7091565, rs1049550	
Mrazek F, 2011	Caucasian	Czech	Case-control	245	254	1	rs1049550	
Cozier YC, 2012	Caucasian	America	Case-control	486	943	10p12-10q22	rs2789679, rs2819941	
Li Y, 2013	Caucasian	Germany	Case-control	325	364	1	rs1049550	
Levin AM, 2013	Caucasian	America	Case-control	1689	1252	25	rs1049550	
Morais A, 2013	Caucasian	Portugal	Case-control	208	197	1	rs1049550	



Case-control 200

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** • • •	Controls (n=418)		Sarcoidosis (n=412)			
Variable	No.	%	No.	%	- <i>P</i> -value ^a	OR, 95% CI
rs2245168					0.920	
CC	156	37.3	150	36.4	0.791	1.04, 0.74-1.38
СТ	200	47.9	197	47.8	0.955	0.99, 0.76-1.30
TT	62	14.8	65	15.8	0.781	0.95, 0.65-1.39
Per T allele	324	38.8	327	39.7	0.698	1.04, 0.85-1.27
rs2236558					0.172	
GG	118	28.2	104	25.2	0.72	1.06, 0.77-1.45
TG	190	45.5	214	51.9	0.060	0.77, 0.59-1.01
TT	110	26.3	94	22.8	0.073	1.33, 0.97-1.82
Per T allele	410	49.0	402	48.8	0.917	0.99, 0.82-1.20
rs7067644					0.173	
AA	152	36.4	176	42.7	0.059	0.76, 0.58-1.01
GA	206	49.3	182	44.2	0.137	1.23, 0.94-1.68
GG	60	14.4	54	13.1	0.597	1.11, 0.75-1.65
Per G allele	326	38.0	290	35.2	0.109	0.85, 0.70-1.04
rs12763624					0.118	
TT	126	75.6	150	36.4	0.008	0.68, 0.51-0.91
СТ	216	51.7	186	45.2	0.059	1.30, 0.99-1.71
CC	76	18.2	76	18.5	0.411	1.16, 0.81-1.67
Per C allele	368	44.0	338	41.0	0.216	0.88, 0.73-1.08
rs2573351					0.437	

Supplemental Table 2	2 Genotypic and alle	lic frequencies of A	ANXA11 polymorphisms in the	he controls and patients with sarcoidosis
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6	T T	100	2 0 5	104		0.0.0
7	TT	120	28.7	104	25.2	0.263
8	CT	202	48.3	216	52.4	0.238
9	CC	96	23.0	92	22.3	0.826
10 11	Per C allele	394	47.1	400	48.5	0.564
12	rs10887581					0.290
13	TT	114	27.3	96	23.3	0.190
14	TC	190	45.5	208	50.5	0.148
15	CC	114	27.3	108	26.2	0.729
16 17	Per C allele	418	50.0	424	51.5	0.583
18	rs11201989					0.144
19	GG	130	31.1	128	31.1	0.995
20	GC	218	52.2	194	47.1	0.141
21 22 23 24 25 26	CC	70	16.8	90	21.9	0.060
22						
23	Per C allele	358	42.8	374	45.4	0.29
25	rs2573353					0.918
26	CC	178	42.6	176	42.7	0.957
27	CA	196	46.9	192	46.6	0.921
28 29	AA	44	10.5	44	10.7	0.941
29	Per A allele	284	34.0	280	34.0	0.997
30 31	rs2789695					0.918
32	TT	178	42.6	180	34.0	0.740
33	СТ	188	45.0	184	44.7	0.920
34	CC	52	12.4	48	11.7	0.73
35 36	Per C allele	292	34.9	280	34.0	0.69
37	rs2573356					0.892
38	CC	118	28.2	112	27.2	0.742
39		110	20.2	112		0.7.12
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47 48 10 1.19, 0.88-1.62 0.85, 0.65-1.12 1.04, 0.75-1.44 1.06, 0.87-1.28

1.23, 0.90-1.69 0.82, 0.62-1.07 1.06, 0.78-1.44 1.06,0.87-1.29

1.00, 0.75-1.34 1.23, 0.93-1.62 0.71, 0.50-1.01 1.11, 0.91-1.35

0.99, 0.75-1.31 1.01, 0.77-1.33 0.98, 0.63-1.53 1.00, 0.82-1.23

0.95, 0.73-1.26 1.01, 0.77-1.33 1.08, 0.71-1.64 0.96, 0.78-1.17

1.05, 0.78-1.43

СТ	190	45.5	194	47.1	0.639	0.94, 0.71-1.23
TT	110	26.3	106	25.7	0.843	1.03, 0.76-1.41
Per T allele	410	49.0	406	49.3	0.926	0.99, 0.82-1.20
rs2819945					0.101	
GG	119	28.5	130	31.6	0.294	0.85, 0.63-1.15
GA	221	52.9	188	45.6	0.030	1.35, 1.03-1.78
AA	78	18.7	94	22.8	0.138	0.78, 0.55-1.09
Per A allele	377	45.1	376	45.6	0.827	1.02, 0.84-1.24
rs11202059					0.064	
GG	106	25.4	128	31.1	0.067	0.75, 0.56-1.02
GA	226	54.1	190	46.1	0.021	1.38, 1.05-1.81
AA	86	20.6	94	22.8	0.429	0.88, 0.63-1.22
Per A allele	398	47.6	378	45.9	0.479	0.93, 0.77-1.13

^a *P*-value was calculated by 2×3 and 2×2 chi-squared tests based on codominant, dominant for the rare allele, heterosis and recessive for the rare allele models of inheritance.

Alpha value is adjusted by Bonferroni correction and statistically significant results (P<0.003)

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1 2 3 4 5	Supplementary table 3 Multi-SNP analy	sis of rs2789679	, rs104955	0 and r	s2819941
5 6 7	Models	testing SNP	<i>P</i> -value	OR	95%CI
8 9 10	model1: rs2789679+ rs1049550	rs2789679: A	0.11	1.19	0.96-1.48
11 12	model2: rs2819941+rs1049550	rs2819941: T	0.169	1.17	0.94-1.45
13 14 15	model3: rs2789670+rs1049550+rs2819941	rs1049550: T	0.0002	0.65	0.52-0.82
14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43	model3: rs2789670+rs1049550+rs2819941				
44 45 46 47 48					
49 50 51 52 53 54 55 56 57 58 59 60					



CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	ltem No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	2-3
Introduction			
Background and	2a	Scientific background and explanation of rationale	5-6
objectives	2b	Specific objectives or hypotheses	6
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	7
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	7
Participants	4a	Eligibility criteria for participants	7
	4b	Settings and locations where the data were collected	7
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	8
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	7
	6b	Any changes to trial outcomes after the trial commenced, with reasons	7
Sample size	7a	How sample size was determined	7
	7b	When applicable, explanation of any interim analyses and stopping guidelines	7
Randomisation:			
Sequence	8a	Method used to generate the random allocation sequence	8
generation	8b	Type of randomisation; details of any restriction (such as blocking and block size)	8
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	7
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	7
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	7
CONSORT 2010 checklist			P

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		assessing outcomes) and how	
	11b	If relevant, description of the similarity of interventions	8
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	8-9
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	8
	120	Methous for additional analyses, such as subgroup analyses and adjusted analyses	0
Results	40-		10.11
Participant flow (a diagram is strongly	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	10-11
recommended)	13b	For each group, losses and exclusions after randomisation, together with reasons	10-11
Recruitment	14a	Dates defining the periods of recruitment and follow-up	10
	14b	Why the trial ended or was stopped	10
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	10
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	11
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	11
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	11
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	11
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	11-15
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	12-14
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	12 14
Other information			
Registration	23	Registration number and name of trial registry	13
Protocol	24	Where the full trial protocol can be accessed, if available	13
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	15
*We strongly recomment recommend reading CON	d reading NSORT 6	g this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If rele extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and ming: for those and for up to date references relevant to this checklist, see <u>www.consort-statement.org</u> .	vant, we also
CONSORT 2010 checklist			F
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