

RESEARCH ARTICLE

Novel non-HLA-susceptible regions determined by meta-analysis of four genomewide scans for ankylosing spondylitis

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Abstract

We identified novel non-HLA-susceptible regions for ankylosing spondylitis (AS) by applying the genome-search-meta-analysis (GSMA) method to combine the previous four AS genomewide scan studies including 479 families with 1175 affected individuals. Three original genomescans were mainly analysed for Caucasian families and one analysed for Han Mongolian families. Ten bins had both P_{sumrnk} and $P_{\text{ord}} < 0.05$, suggesting these bins most likely contain AS-linked loci. The 10 bins are 6.2, 16.3, 6.1, 3.3, 6.3, 16.4, 10.5, 17.1, 2.5 and 2.9. The most significant result of linkage was on chromosome 6p22.3–p21.1 (bin 6.2, $P_{\text{sumrnk}} < 0.000417$), where HLA loci are located. By addition of a genome scan of Chinese origin, our GSMA result further confirmed the HLA loci as the greatest susceptible region to AS and suggested that non-HLA loci chromosome 16q, 3p, 10q, 2p, 2q and 17p, may also contain AS-linked loci. The novel loci identified in our result give hints to further studies.

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Introduction

Ankylosing spondylitis (AS) is characterized by the inflammation in the spine and sacroiliac joints causing initial bone and joint erosion, and ultimately ankylosis and fibrosis. In patients with AS, peripheral joints and entheses are often affected, while extra-articular sites such as uvea, aorta, lungs, kidneys can also be involved. In Caucasian, the prevalence of AS is 0.2%–0.9% (Braun *et al.* 1998) and 0.2–0.4% in Mongolian race. Considering that sibling recurrence risk ratio is around 82 (Brown *et al.* 2000) and disease heritability as estimated by twin study exceeds 90% (Brown *et al.* 1997), genetic factors are highly suspected for the pathogenesis of AS. The four genome scanned previously (Brown *et al.* 1998a; Laval *et al.* 2001; Gu *et al.* 2004; Zhang *et al.* 2004) for AS studies also showed that HLA loci (including HLA-B27) is the strongest linkage region. However, only 1%–5% among those HLA-B27 positive populations developed AS, although most AS patients are HLA-B27 positive. And B27 can explain no more than 50% of the overall genetic risks (Brown *et al.* 1998b) of AS. Therefore, it is not surprising

to find increasing evidences of non-HLA genes participating in the pathogenesis of AS. But except for HLA region, other susceptible loci could not be repeated across the four studies mentioned above, either for the power of susceptible genes or the sample size applied in linkage analysis.

Due to diseases complexity, LOD scores often fail to reach the accepted levels of suggestive or significant linkage, and replication across studies occurs infrequently. But the meta-analysis can combine results of linkage analysis from different studies and accordingly gain greater statistic power. Genome-search-meta-analysis (GSMA) was developed to pool results across genomewide linkage studies to assess evidence for linkage in any region. As a rank-based gene mapping method through the whole genome that covers all disease susceptible genes, GSMA can identify susceptible loci that have not been detected due to insignificant power in individual studies. Recently, GSMA method has been regarded as a robust technology (Koziol and Feng 2005), and widely used in assessment of susceptible loci in many diseases (Fisher *et al.* 2005; Johnson *et al.* 2005). Our study assessed the reported data in four published genome scan searches of AS families by GSMA method.

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Keywords. ankylosing spondylitis; linkage analysis; genome-search-meta-analysis.

Materials

Subjects

Four AS genome scan studies were included through searching both PubMed and CNKI database (Brown *et al.* 1998a; Laval *et al.* 2001; Gu *et al.* 2004; Zhang *et al.* 2004). Three original genome scans mainly analysed for Caucasian fami-

lies and one was analysed for Han Mongolian families. There are totally 479 families with 1175 affected individuals whose demographic information is shown in table 1. We obtained the raw data from the published tables or graphs of linkage analysis resulted in each study. LOD scores were used in two genome scan studies (see table 2) (Brown *et al.* 1998a; Laval *et al.* 2001) and both LOD and NPL scores, were used in

Table 1. Population statistics of four AS genomewide scans included in GSMA.

Genome scan study (time)	Brown <i>et al.</i> (1998a)	Laval <i>et al.</i> (2001)	Zhang <i>et al.</i> (2004)	Gu <i>et al.</i> (2004)
Number of pedigrees	105	185	180	9
Affected individuals	253	445	424	29
Region and race	US, Caucasian	Caucasian	94% Caucasian, 6% Caucasian/American Indian or Caucasian east Asian mixed	Shanghai, China, Han Mongolian

Table 2. LOD score and NPL score of four AS genome scan linkage studies.

Brown <i>et al.</i> (1998a)		Laval <i>et al.</i> (2001)		Zhang <i>et al.</i> (2004)			Gu <i>et al.</i> (2004)		
Marker	LOD	Marker	LOD	Marker	LOD(ASM)	NPL	Marker	LOD(ASM)	NPL
D1S229	0.6	D1S199	0.5	D1S238	1.671	1.748	D6S289	0.777731	2.09796
D2S165	1.1	D1S255	2.2	D3S1300	1.645	2.079	D6S1584	0.728671	1.3345
D2S139	0.6	D1S197	0.5	D3S1566	0.885	1.584	D6S422	0.352848	1.02243
D2S160	1.3	D1S484	1.4	D4S419	1.73	2.114	D6S1691	1.571698	2.7479
D2S157	1.7	D1S2836	1.3	D5S2073	1.333	1.977	D6S276	3.882125	2.4563
D2S126	0.8	D2S391	1.3	D6S1574	1.508	2.082	D6S1618	2.005558	2.0202
D3S1300	1.4	D2S337	1.1	D6S309	1.833	2.163	D6S1568	1.0706088	1.0007
D6S276	2.1	D2S160	1.1	D6S289	3.224	3.108	D6S291	1.814634	1.54732
D6S273	3.8	D2S347	1.2	D6S422	4.897	3.376	D6S1610	1.012436	1.16344
D6S291	1.8	D2S335	0.5	D6S276	7.074	3.831	D6S1562	0.790347	0.30009
D6S281	0.6	D2S157	1.6	HLA-B	12.37	6.378	D6S1575	0.441259	1.1504
D10S220	0.8	D3S1300	0.4	DRB1	20.49	8.72	D3S1292	1.2768	0
D10S192	1.1	D3S1314	0.6	DQA1	14.88	6.793	D4S1535	1.1246	0
D10S190	1.7	D5S400	0.6	DQB1	18.37	7.623	D18S64	1.1851	0
D11S922	1.4	D6S309	0.8	DPB1	10.72	5.252			
D12S97	0.7	D6S470	2.2	D6S1610	7.232	4.377			
D13S122	0.8	D6S289	2.5	D6S257	1.155	1.633			
		D7S519	0.6						
		D8S1784	1.5						
		D8S514	1						
		D9S288	2.3						
		D9S286	1.5						
		D9S161	1.4						
		D9S283	1.8						
		D9S1682	2.3						
		D9S1826	2.8						
		D10S185	2.1						
		D10S192	1.1						
		D10S597	1.8						
		D10S190	0.8						
		D11S922	1.1						
		D11S935	0.6						
		D15S165	0.4						
		D16S3068	0						
		D16S515	1.1						

*LOD, logarithm of odds; NPL, nonparametric linkage score; AS, ankylosing spondylitis; ASM, allele-sharing model.

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Table 3. Most significant results in LOD score output of four AS genome scan by GSMA weighted analysis ($P < 0.1$).

Bin	Location	Rsumrnk	Psumrnk	Pord	Psumrnk and Pord < 0.05
6.2	6p22.3-p21.1	480	*8.33E-07	1.00E-04	+
16.3	16q12.2-q23.1	424.703	0.002223	0.023298	+
6.1	6pter-p22.3	404.117	0.004761	0.014299	+
3.3	3p22.1-p14.1	400.261	0.005149	0.0014	+
6.3	6p21.1-q15	367.495	0.01547	0.022698	+
16.4	16q23.1-qter	366.797	0.016136	0.0041	+
10.5	10q23.33-q26.13	366.467	0.016421	0.0009	+
17.1	17pter-p12	355.18	0.030281	0.011099	+
2.5	2p12-q22.1	352.388	0.034855	0.008099	+
2.9	2q34-q35	347.358	0.044087	0.011099	+
5.6	5q34-qter	342.675	0.05377	0.020898	
2.3	2p23.2-p16.2	338.968	0.061017	0.020998	
11.1	11pter-p15.1	337.134	0.064167	0.011299	

The 10 bins marked with '+' have Psumrnk and Pord value both less than 0.05; Rsumrnk, summed rank (high summed rank indicates most evidence for linkage); Psumrnk, P values for the summed rank analysis, based on simulating the by-bin LOD scores within a study; Pord, P values for the ordered rank analysis, based on number of n th position summed ranks that exceed the observed summed rank; *, Psumrnk < 0.000417.

Table 4. Most significant results in LOD score output of four AS genome scan by GSMA weighted analysis ($P < 0.1$)

Bin	Location	Rsumrnk	Psumrnk	Pord	Psumrnk and Pord both < 0.05
6.2	6p22.3-p21.1	480	*8.33E-07	1.00E-04	+
16.3	16q12.2-q23.1	425.216	0.002178	0.023398	+
6.1	6pter-p22.3	404.117	0.004772	0.014799	+
3.3	3p22.1-p14.1	402.082	0.004973	0.0012	+
16.4	16q23.1-qter	367.31	0.015393	0.021898	+
10.5	10q23.33-q26.13	366.981	0.015683	0.004	+
6.3	6p21.1-q15	366.7	0.015856	0.0007	+
17.1	17pter-p12	355.693	0.029294	0.008799	+
2.5	2p12-q22.1	352.901	0.03386	0.006599	+
2.9	2q34-q35	347.872	0.043137	0.009499	+
5.6	5q34-qter	344.496	0.050263	0.010799	
2.3	2p23.2-p16.2	339.481	0.06034	0.019098	
11.1	11pter-p15.1	337.647	0.063549	0.010099	

The 10 bins marked with '+' have Psumrnk and Pord value both less than 0.05; Rsumrnk, summed rank (high summed rank indicates most evidence for linkage); Psumrnk, P values for the summed rank analysis, based on simulating the by-bin lod scores within a study; Pord, P values for the Ordered Rank analysis, based on number of n th position summed ranks that exceed the observed summed rank; *, Psumrnk < 0.000417.

the other two scans (Gu *et al.* 2004; Zhang *et al.* 2004) (see tables 3 and 4 respectively.)

Methods

GSMA (Pardi *et al.* 2005) is performed as described (Wise *et al.* 1999). Microsatellites of the autosomes are divided into 120, of each 30 cM bins. In Marshfield, the average width of each bin is 29.1 cM. Each microsatellite is categorized into one bin according to its location on Genethon or Marshfield map (<http://research.marshfieldclinic.org/>

genetics/GeneticResearch/compMaps.asp) For each study, each bin was assigned a rank within study (Rstudy) according to its largest linkage score within the bin, then all the ranks were arranged in descending order (rank 120 is the respondent score of the most significant result, and rank 119 is the second largest score, etc.). Tied ranks can be assigned, particularly in nonparametric linkage analysis, where many bins may have a maximum LOD score of zero. When summing all the ranks of a bin across studies, its overall rank score (Rsummk) is achieved. The weighted GSMA revised

the result according to each study's contribution, i.e., each Rsummk value was multiplied by its study's weight (the square root of the number of affected cases), divided by the mean value for the over all studies. Psumrnk and Pord are described and determined by 10,000 permutations of the weighted data set. Psumrnk is the probability of observing a bin's summed rank by chance; Pord are obtained by simulating complete replicates of the GSMA, assuming that the ranks are assigned randomly in each study, and then comparing the number of nth placed bins which obtained a higher summed rank than the observed nth placed summed rank. Pord determines whether that bin, and those with higher summed ranks, show clustering of high summed ranks. We expect 6/120 bins (5%) to have P value < 0.05 , 1% of bins to attain a P value of < 0.01 . Applying a Bonferroni correction, genomewide significance of 5% would be equivalent to a bin P value of $P_{sumrnk} = 0.000417$. We assumed the GSMA results as most likely to contain linked loci when both Psumrnk and Pord were < 0.05 and as a genomewide evidence of linkage if the GSMA Psumrnk was < 0.000417 .

Results

We applied GSMA method in the four studies with LOD score and substituted with NPL score if available. The two analyses achieved consistent results. Figure 1 (tables 3 and

4) shows the summed ranks (vertical axis) are plotted against the bin location by a single point plotted for the summed rank for each bin with chromosome numbers (horizontal axis). A total of 10 bins lie above 95% confidence level ($P = 0.05$), and four bins are above 99% confidence level ($P = 0.01$). All 10 bins ($P_{sumrnk} < 0.05$) had both Psumrnk and Pord < 0.05 , suggesting that these 10 bins most likely contain AS-linked loci; these include bins 6.2, 16.3, 6.1, 3.3, 6.3, 16.4, 10.5, 17.1, 2.5 and 2.9. The Psumrnk and Pord values are shown in table 3. The AS in GSMA method produced significant genomewide evidence for linkage on chromosome 6p22.3-6p21.1 (bin 6.2) ($P = 8.33E-07$). The bin 6.3 ($P_{sumrnk} = 0.01547$ and $P_{ord} = 0.022698$), 16.4 ($P_{sumrnk} = 0.016136$ and $P_{ord} = 0.0041$) and 10.5 ($P_{sumrnk} = 0.016421$ and $P_{ord} = 0.0009$) based on ranks under LOD calculation reported different values from those under NPL calculation, respectively [bin 6.3 ($P_{sumrnk} = 0.015856$ and $P_{ord} = 0.0007$), 16.4 ($P_{sumrnk} = 0.015393$ and $P_{ord} = 0.021898$) and 10.5 ($P_{sumrnk} = 0.015683$ and $P_{ord} = 0.004$)], but Psumrnk and Pord values under both calculation methods are not more than 0.05 for any of the three bins. Basically, we assumed that parametric linkage analysis is more reliable than nonparametric linkage analysis when LOD and NPL scores are applied. The figure of most significant results in part of NPL score combined with LOD score

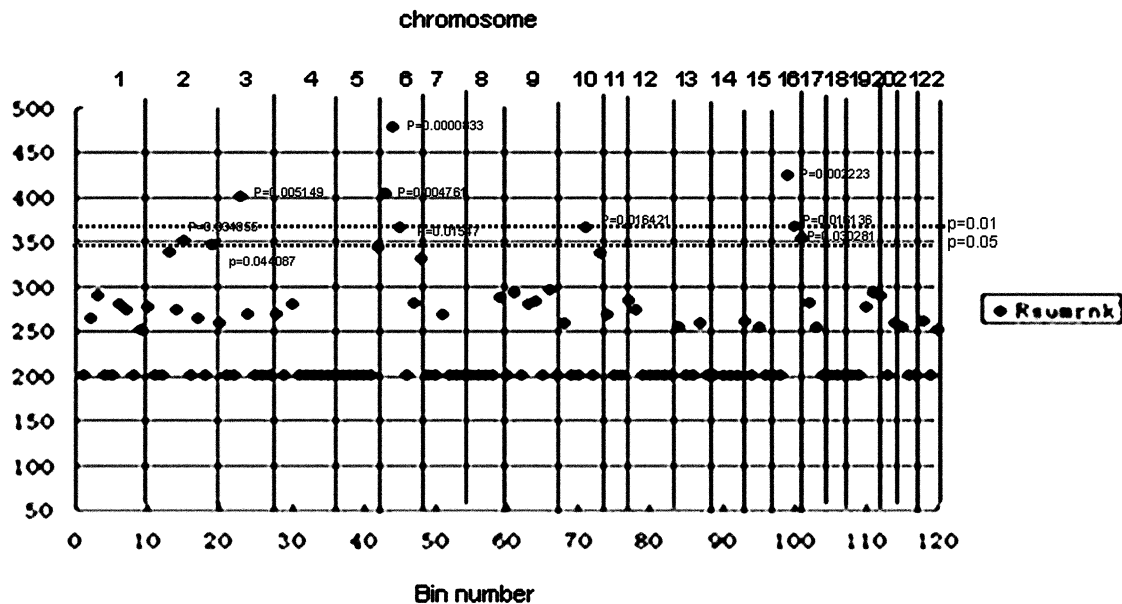


Figure 1. The summed ranks (vertical axis) are plotted against the bin location by a single point plotted for the summed rank for each bin with chromosome numbers (horizontal axis). Psumrnk and Pord are described and determined by 10,000 permutations of the weighted data set. Psumrnk is the probability of observing a bin's summed rank by chance; Pord are obtained by simulating complete replicates of the GSMA, assuming that ranks are assigned randomly in each study, and then comparing the number of nth-placed bins which obtained a higher summed rank than the observed nth-placed summed rank. Pord determines whether that bin, and those with higher summed ranks, show clustering of high summed ranks. A total ten bins lie above 95% confidence level ($P = 0.05$), and four bins are above 99% confidence level ($P = 0.01$). All 10 bins ($P_{sumrnk} < 0.05$) had both Psumrnk and Pord < 0.05 , suggesting that these 10 bins most likely contain AS-linked loci; these include bins 6.2, 16.3, 6.1, 3.3, 6.3, 16.4, 10.5, 17.1, 2.5 and 2.9.

output of four AS genome scanned by GSMA is not shown here.

Discussion

Strong genetic factors are implied in the pathogenesis of AS. HLA-B27 is presumably associated with over 90% of AS patients. But AS family studies showed that, HLA-B27 is not the only susceptible gene of AS, other genetic factors are also involved. AS susceptibility owes probably to oligogenes (Brown *et al.* 2000), which means B27 is essential but not efficient. Many genes in HLA region have been further investigated, such as HLA-DR, MICA, LMP, TAP, HSP and TNF, which may act as disease markers or related to the severity of AS either protective or reversely related based on different subtypes. HLA-A*2402 is reported to be correlated with susceptibility to AS in Basque population (de Juan *et al.* 2004). But so far no single gene can explain the pathogenesis of the disease. Previous studies of genomewide scan, including AS and spondyloarthropathy (Miceli-Richard *et al.* 2004) suggested that both HLA and non-HLA regions are linked to AS. Several candidate regions besides HLA has been identified. These include genomic regions at 1p, 2p, 2q, 3p, 9q, 10q, 11p, 16q and 19q. However, among the four studies in our research, no other locus than HLA loci could be replicated across these studies. We applied the GSMA method in our study to confirm that HLA region is the major susceptible region of AS. And non-HLA loci including 6p, 16q, 3p, 10q, 17p, 2p and 2q may also contain linkage loci. Brown *et al.* 1998 found that AS patients carrying HLA haplotype outnumbered those without this haplotype, suggesting that B27 may be recessive or codominant susceptible gene. However, 7.6% of AS patients don't carry this haplotype and MHC region can only attribute to 31% genetic susceptibility of the disease. To further understand this point, our GSMA results provide objective evidences.

GSMA method can strengthen the power of micro effect in independent studies, therefore can exclude false positive of individual studies. And it is applied to the meta-analysis of AS genomewide scan gene mapping. In our study, only those fit for the GSMA method research data are included. Further, since no evidence was found in AS linkage studies of X chromosome (Hoyle *et al.* 2000) and the gender differences in AS might be due to a higher threshold value in females caused by certain unknown reasons. GSMA method, which does not focus on analysis associated with X chromosome, is applicable in our study. Finally, we excluded AS related acute anterior uveitis (Martin *et al.* 2005) and spondyloarthropathies (Pardi *et al.* 2005) in our study, in order to make sure of the entity unity, so that our statistic results would be more reliable and stable without perturbation of confounding factors.

The sample size of pedigree and the disease diagnosis are two key factors in finding a reliable susceptible region through linkage analysis. Importantly, the reliability of GSMA method is based on the validity of the original data,

though GSMA already weightily combines different data of independently existed studies. If the linkage analysis data of some researches are incompletely published, the information lost will accordingly lead to false negative results. In other words, quality of the original data outweighs the method itself. As the cases included in the four genomewide scans were diagnosed by the well-accepted modified New York criteria (Van der *et al.* 1984) and the genotype were of strict quality control to exclude error data. The preliminary results obtained from these scans are relatively credible and therefore the GSMA method is the mature and widely used method in this case.

Moreover, the confirmation of susceptibility to AS is important as early diagnosis and effective intervention before occurrence of the joint destruction and spine dysfunction are of crucial significance to patients with AS genetic risks. And elucidation of these genes may provide targets for novel gene therapy. Especially, for individuals with high risks, proper prevention of AS onset or recurrence from provocative events is valuable. The non-MHC results in our study may provide clues for future gene screening to discover the culprit genes.

Compared with GSMA result of Lee *et al.* (2005), our study included Han Mongolian in Shanghai district of China which increased genetic diversity of the background population. Moreover, studies from abroad mainly focus on nuclear families or sporadic AS patients, while in that of Gu *et al.* (2004) nine large families were included which diversified our data resources. In a single word, our result is more demographically representative as it is based on different district, different races and combined with both pedigrees and sporadic cases. Consistent results of AS susceptible region are seen in bins 6.2,16.3, 6.1, 6.3,16.4 and 17.1 between our study and Young's, though the significances are different. In contrast to the finding of a high LOD score on chromosome 19q13 only by Laval *et al.* (2001) among the four AS studies, bin 19.2 has not been identified in our study. Although Gu's genome study is enrolled, with only 29 AS affected individuals included its contributes not much to weighted analysis, which can hardly elucidate this reversal result. Further researches might explain whether this insignificance of bin19.2 is due to false positive of this STR or other confounding factors.

Transforming growth factor (TGF β 1) located at chromosome 19q13 is a multifunctional cytokine regulating inflammation, and promoting cartilage and bone formation. TGF β 1 is also a necessary factor for IgA production in B cell development. Some reported elevated IgA level in AS patients. However, no genetic polymorphism of IgA has been found to support its relation to AS susceptibility. Functional SNP TGF β 1T869C and G915C, caused leucine substituted by proline at codon 10 and arginine substituted by proline at codon 25, respectively. And these substitutions are correlated with the level of TGF β 1 production at serum level and *in vitro*. No significant difference was found in genotype, allele and haplotype frequency between AS patient and

normal controls (Van der *et al.* 2005). TGF β 1 may merely plays a down regulating role in the joint ankylosis and fibrosis of AS. The novel loci we identified are bins 3.3, 10.5, 2.5 and 2.9 that were respectively identified with a high LOD score in at least two out of four AS studies, suggesting that these regions or regions near by probably contain AS susceptible loci. Among all, chromosome 2 is especially worth our attention. At present, IL-1 gene cluster (Timms *et al.* 2004) is the hotspot for genetic susceptible loci to AS. The subtype IL-1A encodes IL-1 α , IL-1B encodes IL-1 β , while IL-1RN encodes IL-1receptor antagonist IL-1RA. Although the role of IL-1 in AS is not clear now, several individual studies have reported that IL-1RN, especially VNTR of intron 2, IL1F10, IL1A and IL1B (McGarry *et al.* 2001; Van der *et al.* 2002; Maksymowych *et al.* 2003) are closely related to the disease (Maksymowych *et al.* 2006), indicating that there must be AS susceptible loci at or around IL-1 loci whose location is at chromosome 2q13 (bin 2.5). By now, there are no evidence that matrix metalloproteinase (MMP3) (Jin *et al.* 2005), toll-like receptor 4 (TLR4) A896G or CD14-C260T (Van der *et al.* 2005) correlate with AS susceptibility and severity, which is concordant with our result. The possible reason is that their gene locations of 11q22.3, 9q32-q33 and 5q31.1 respectively, are outside the possible linkage region suggested in our study.

The GSMA result confirmed that HLA region is the susceptible region to AS, but susceptible loci correlated with AS clinical manifestation such as age of symptom onset, BASDAI and BASFI are not in HLA region (Brown *et al.* 2003). The pathophysiological mechanism of AS susceptibility and clinical manifestation are two distinct process. The detection of genes that control the progress of AS can provide theoretical basis for drug therapy aimed at postponing the process of joint ankylosis. To clarify the effect of susceptible genes on the pathogenesis of AS in animal model through a series of functional experiments and computational simulation afterwards, further investigations are necessary to investigate how genes of multi-loci interact with each other, whether different phenotypes are controlled by different genes that contribute to AS clinical manifestations or under the effect of one specific gene, other genes play a synergistic reaction. Nevertheless, the GSMA method is indispensable in guiding our way at present stage.

To sum up, GSMA further confirms that the HLA loci is the major genetic susceptible region of AS and 16q, 3p, 10q, 17p, 2p, 2q in non-HLA loci are also suggestive susceptible regions. The novel loci identified by combination of three previous genome scans and our Chinese genome scan may provide target for further fine mapping and candidate gene screening in the future.

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