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ORIGINAL ARTICLE

Association of the *MSX2* gene polymorphisms with ankylosing spondylitis in Japanese

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Abstract Several genes have been implicated in the etiology of ankylosing spondylitis (AS); however, the significance of these genes except HLA-B27 remains to be elucidated. In this study, we examined the association of AS with novel candidate genes and previously reported genes other than HLA-B27. We examined a total of 45 single nucleotide polymorphisms (SNPs) in 15 genes by a sequential screening. We first genotyped 170 Japanese AS patients and 896 controls for the SNPs (first screen). Then, we genotyped eight SNPs with $P < 0.05$ in the first screen for 108 additional Japanese patients (second screen). We checked the replication of the association of the most significant SNP by genotyping 219 Taiwanese AS patients and 185 controls. When the first and second screens were combined, four SNPs showed nominal significance of $P < 0.05$. An intronic SNP (IVS1 + 996G > A) in *MSX2*, a novel candidate gene, showed the most significant association ($P = 0.0030$). The association was not replicated in our Taiwanese population; however, there was the same trend with the Japanese population in the allelic frequency distribution of the SNP. In the genes previously reported to have association with AS, only one synonymous SNP, c.963T > G in *ANKH*, showed a marginal association in the Japanese population ($P = 0.045$).

Keywords Ankylosing spondylitis · Association · Single-nucleotide polymorphism markers · MSX2 · ANKH

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Introduction

Ankylosing spondylitis (AS) [MIM 106300] is one of the common causes of inflammatory arthropathy, with a prevalence of 0.1–0.8% in Caucasians (Calin 1998). AS affects predominantly the axial skeleton and spinal and sacroiliac joints and eventually causes their bony ankylosis. AS frequently involves the peripheral joints and entheses and occasionally extra-articular sites such as uvea, aorta, heart, lung, and kidney.

Strong genetic factors have been implicated in the etiology of AS. A sibling risk ratio (λs) is estimated to be 82%

(Brown et al. 2002), and a heritability assessed by twin studies >90% (Brown et al. 1997). HLA-B27 is the strongest genetic factor known for AS (Brewerton et al. 1973). More than 90% of AS patients are HLA-B27 positive; however, only 1–5% of HLA-B27 carriers develop AS (Calin et al. 1983), and family and twin studies suggest that HLA-B27 contributes to <50% of the genetic risk of AS (Brown et al. 1997; Rubin et al. 1994). Consistently, genes other than HLA-B27 have also been implicated in AS, including *IL1A/B* (Maksymowych et al. 2006), *IL1RN* (van der Paardt et al. 2002), *IL10* (Goedecke et al. 2003), *CYP1A1* (Yen et al. 2003), *CYP2D6* (Beyeler et al. 1996), *ANKH* (Tsui et al. 2003), and *NPPS* (Mori et al. 2003). However, the significance of these genes remains to be elucidated. Most of these associations are not replicated in other populations, in particular those of different ethnicities.

AS patients show marked ectopic ossification in the spine, occasionally resulting in so-called "bamboo spine" (Calin 1998). Therefore, genes involved in ectopic ossification and/or calcification are good candidates for the AS susceptibility gene. Indeed, the polymorphisms of *ANKH*, which encodes the transporter of inorganic pyrophosphate (PPi), a major inhibitor of calcification, show association with AS (Tsui et al. 2003). In this study, we conducted an association study to identify novel susceptibility alleles for AS and to examine previously reported association in Japanese and Taiwanese populations. We report promising evidence for the association between *MSX2* polymorphisms and AS and replication of the association between *ANKH* polymorphism and AS.

Materials and methods

Study subjects

In the Japanese study, a 278 individuals with sporadic AS (255 males and 23 females) were recruited though four medical facilities. In the Taiwanese study, 219 affected individuals (156 males and 63 females) were recruited though two facilities. We obtained written informed consent from each patient. The study protocols were approved by the ethical committees at each institution. AS was defined by the modified New York diagnostic criteria (van der Linden et al. 1984). The mean age of Japanese and Taiwanese AS patients was 41.2 (16–78) years and 36.0 (9–89) years, respectively. Two hundred and thirty-three of 278 Japanese patients and 213 of 219 Taiwanese patients were HLA-B27 positive.

In the Japanese study, we used up to 896 subjects (508 males and 388 females) from the general population as controls in analyses of 31 single nucleotide polymorphisms (SNPs) for which genotype data were available from the Japanese Single Nucleotide Polymorphism (JSNP) database (Haga et al. 2002). The mean age of control subjects was 48.5 (3–88) years. We genotyped 376 controls (205 males and 171 females) from the general population for analyses of the 14 SNPs for which genotype data were not available in the JSNP. The mean age of this control group was 42.1 (14–92) years. In the Taiwanese study, we used 185 controls (80 males and 105 females) from the general population. The mean age of the Taiwanese control group was 46.3 (20–78) years.

Selection of candidate genes and marker SNPs

Fifteen candidate genes were selected (Table 1) according to the following criteria: (1) genes previously reported to be associated with AS and (2) genes known to be involved in ectopic ossification and/or calcification. We selected SNPs in the candidate genes that had minor allele frequencies >10% from JSNP (Haga et al. 2002) and the National Center for Biotechnology Information (NCBI) dbSNP database (http://www.ncbi.nlm.nih.gov/).

Genotyping

Genomic DNA was extracted from blood as standard procedure. SNP genotyping was performed using the Invader assay and the TaqMan assay as described previously (Ohnishi et al. 2001; Suzuki et al. 2003).

Statistical analysis

The differences in allelic distribution between cases and controls and Hardy-Weinberg equilibrium in controls were assessed by the χ^2 test. We performed the first screen using 170 Japanese AS patients and up to 896 controls. A P value <0.05 was considered statistically significant in any of the following models: genotypic, dominant, recessive, and allelic. In the second screen, 108 additional Japanese patients were genotyped and assessed by the χ^2 test with Bonferroni's correction for multiple testing. Pairwise linkage disequilibrium (LD) was estimated as described previously (Kizawa et al. 2005). Haplotype frequencies were estimated by the expectation-maximization algorithm and Arlequin softwave. We checked the replication of the association of the most significant SNP by genotyping 219 Taiwanese AS patients and 185 controls.

Results

We first screened 45 SNPs from 15 candidate genes (Table 1). Eight SNPs in six genes that cleared the cutoff point of $P < 0.05$ were subjected to the second screen.

In the combined results of the first and second screens, four SNPs from distinct genes showed the nominal *P* values <0.05 (Table 2). An intronic SNP in *MSX2* (IVS1 + 996G > A) showed the most significant association ($P = 0.0030$).

To examine LD around the *MSX2* region, we constructed a pairwise LD block by genotyping six SNPs around *MSX2* from the NCBI dbSNP for 376 controls. We found that *MSX2* was contained completely within a single LD block (Fig. 1). The level of association of promoter −3,742C > T was equivalent to that of IVS1 + 996G > A (Table 3). The haplotypes were assessed by four SNPs within the *MSX2* region. The *P* values of haplotype association were less significant than those of respective SNPs (data not shown), indicating that the presence of a hidden SNP that shows more significant association than IVS1 + 996G > A is unlikely. These associations were not affected by the stratification of AS patients according to HLA-B27 and gender status (data not shown). The significance disappeared after Bonferroni's correction for multiple testing of 45 SNPs

The association between IVS1 + 996G > A and AS was not replicated in a Taiwanese population (Table 4). However, allelic frequency distribution of IVS + 996G > A in the Taiwanese population showed the same trend observed in the Japanese population, and the odds ratio (OR) in the Taiwanese population also exceeded 1, as in the Japanese population. Power calculations indicated that the Japanese study sample had an 85% power to detect association of IVS1 + 996G > A with an OR of 1.51 and a 0.05 significance level, whereas Taiwanese study sample had only an 67% power under the same condition as the Japanese study.

Among SNPs located in genes previously reported to have association with AS, only one synonymous SNP, c.963T > G in *ANKH*, showed a marginal association ($P = 0.045$) (Table 2).

Table 1 Ankylosing spondylitis (AS) candidate genes examined in this study

Group	Gene symbol	Gene name	Locus	Accession no.
1	*IL1RN*	Interleukin 1 receptor antagonist	2q	NM_000577
	IL10	Interleukin 10	1q	NM_000572
	ANKH	Ankylosis, progressive homolog (mouse)	5p	NM_054027
	NPPS	Nucleotide pyrophosphatase	6q	NM_006208
	CYP2D6	Cytochrome P450, family 2, subfamily D, polypeptide 6	22q	NM_000106
	NOD2	Nucleotide-binding oligomerization domain containing 2	16q	NM_022162
	HSPA1L	Heat shock 70 kDa protein 1-like	6p	NM_005527
2	*BMP4*	Bone morphogenetic protein 4	14q	NM_001202
	CSPG2	Chondroitin sulfate proteoglycan 2 (versican)	5q	NM_004385
	CST3	Cystatin C (amyloid angiopathy and cerebral hemorrhage)	20p	NM_000099
	EDN1	Endothelin 1	6p	NM_001955
	MSX2	MSH homeobox homolog 2 (drosophila)	5q	NM_002449
	SNAI1	Snail homolog 1 (drosophila)	20q	NM_005985
	SNAI2	Snail homolog 2 (drosophila)	8q	NM_003068
	ZNF145	Zinc finger protein 145	11q	NM_006006

Group 1: genes previously reported to be associated with AS, Group 2: genes known to be involved in ectopic ossification and/or calcification

Table 2 Combined results of the first and second screens in a Japanese population

Gene symbol	Sequence variation	dbSNP ID	Genotype		χ^2 test	
			Case	Control	*P* value[a]	Model
MSX2	IVS1 + 996G > A	rs4868442	7/86/185	21/144/209	0.0030	Allele
ANKH	c.963T > C	rs2288474	13/81/183	17/209/512	0.0452	CC versus others
CSPG2	IVS5 + 2599T > A	rs2292012	47/133/97	183/396/253	0.0481	Allele
EDN1	IVS4–208C > T	rs1630736	59/147/69	72/183/121	0.0493	CC versus others
IL10	IVS2 + 56A > G	rs1518111	17/125/136	72/333/341	0.0740	AA versus others
CSPG2	IVS1–463A > G	rs884571	28/103/146	96/351/392	0.0839	GG versus others
ZNF145	IVS3–5770A > G	rs2073848	32/92/153	67/296/379	0.1135	Genotype
CSPG2	IVS1–319T > C	rs1867667	55/130/92	194/399/244	0.1308	Allele

Genotype: number of homozygotes for the minor allele/heterozygotes/homozygotes for the major allele

dbSNP Database for Single Nucelotide Polymorphisms

[a] The best result according to the indicated model

Discussion

We found that *MSX2* polymorphisms are associated with AS in Japanese but not in Taiwanese. Because the power to detect the association of *MSX2* polymorphisms in Taiwanese is smaller than that in Japanese, we might have missed a causal variant in Taiwanese. Alternatively, the discrepant results may arise from population and/or environmental differences. In either case, more studies including those in different ethnic groups are needed to confirm the significance of the association between *MSX2* polymorphisms and AS.

MSX2 is a transcription factor with a homeobox domain and involved in bone development and ectopic calcification. However, its roles in these processes are still controversial. *Msx2*-deficient mice shows reduced bone formation, decreased osteoblasts, impaired chondrogenesis, and abnormal calvarial development (Satokata et al. 2000). Transgenic mice overexpressing *Msx2* shows enhanced proliferation of calvarial cells (Liu et al. 1999). A loss-of-function mutation of *MSX2* in humans, which reduces DNA binding activity, causes defects in skull ossification (Wilkie et al. 2000), whereas a gain-of-function mutation of *MSX2* results in an autosomal dominant disorder, Boston-type craniosynostosis (Jabs et al. 1993; Ma et al. 1996). *Msx2* transgenic mice under the control of the cytomegalovirus (CMV) promoter exhibits marked cardiovascular calcification because the Msx2-expressing myofibroblasts produce an osteogenic milieu (Shao et al. 2005). These findings suggest a positive role for MSX2 in bone development and ectopic calcification. In contrast, other studies have indicated that MSX2 negatively regulates bone development and ectopic calcification. MSX2 abrogates the promoter activity of osteoblast marker genes such as osteocalcin (Newberry et al. 1997). MSX2 inhibits the transcriptional activity of RUNX2, a master regulator of osteoblast differentiation (Shirakabe et al. 2001). MSX2 plays a role in preventing ligaments and tendons from calcification (Yoshizawa et al. 2004). The roles of MSX2 may vary depending on cell type and/or cell differentiation stage.

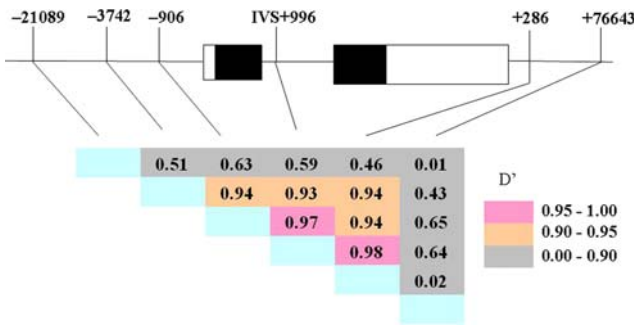

Fig. 1 The linkage disequilibrium (LD) block around *MSX2*. *Top panel*: genomic structure and sequence variations of *MSX2*. Exons are denoted by *rectangles*, and coding regions are *filled*. Nucleotide A of the translation initiation codon (ATG) is denoted as +1 to show the position in promoter. First nucleotide of 3′-flanking region is denoted as +1 to show the position in 3′-flanking region. *Bottom panel*: LD structure containing *MSX2*. LD was evaluated using D′ statistical analysis

The *ank* (progress ankylosis) is a natural mutant mouse that has a mutation in the gene encoding PPi transporter in the plasma membrane. The *ank* exhibits phenotypes similar to AS. The mutation leads to low extracellular PPi (Ho et al. 2000). Because PPi is a major inhibitor of ossification and calcification, reduced extracellular PPi levels cause ectopic ossification and/or calcification, leading to ankylosis (Ikegawa 2006). Decreased serum nucleotide pyrophosphatase (NPPS), an enzyme that produces PPi from nucleotide pyrophosphate (Okawa et al. 1998), was reported in AS patients (Mori et al. 2003). A previous study showed that the polymorphisms of *ANKH* (human homolog of *ank*) are associated with susceptibility to AS in American Caucasians (Tsui et al. 2003), whereas a large British study found no such association (Timms et al. 2003). In this study, a synonymous SNP, c.963A > G in *ANKH*, showed a marginal association. The effect of the *ANKH* polymorphisms on AS susceptibility may be small and/or intrinsically different, leading to discrepant results between populations.

Table 3 Association of single nucleotide polymorphisms (SNPs) in *MSX2* with alkankylosing spondylitis (AS)

Sequence variation	dbSNP ID	Genotype		Allelic frequency		χ^2 test		
		Case	Control	Case	Control	*P* value[a]	Model	OR (95% CI)
Promoter −3742C > T	rs6884071	9/90/178	25/149/201	0.195	0.265	0.0031	Allele	1.49 (1.14–1.94)
Promoter −906C > A	rs7447819	9/79/190	17/130/227	0.174	0.219	0.0442	CC versus others	1.40 (1.01–1.94)
IVS1 + 996G > A	rs4868442	7/86/185	21/144/209	0.180	0.249	0.0030	Allele	1.51 (1.15–1.98)
3'flanking + 286C > T	rs4647949	27/121/130	52/174/150	0.315	0.370	0.039	Allele	1.28 (1.01–1.61)

Genotype: number of homozygotes for the minor allele/heterozygotes/homozygotes for the major allele

dbSNP Database of Single Nucleotide Polymorphisms, *OR* odds ratio, *CI* confidence interval

[a] The best results according to the indicated model

Table 4 Association of *MSX2* IVS1 + 996G > A in Taiwanese ankylosing spondylitis (AS) patients

Genotype		Allelic frequency		χ^2 test		
Case	Control	Case	Control	*P* value	Model	OR (95% CI)
13/78/128	17/64/104	0.237	0.265	0.370	Allele	1.16 (0.84–1.59)

Genotype: number of homozygotes for the minor allele/heterozygotes/homozygotes for the major allele

OR odds ratio, *CI* confidence interval

This study provides promising evidence for the association between *MSX2* polymorphisms and AS and the replication of the association between *ANKH* polymorphisms and AS in the Japanese population.

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