Original Article

DOI: 10.5582/bst.2013.v7.2.89

Association of mineralization-related genes *TNAP* and *ANKH* polymorphisms with ankylosing spondylitis in the Chinese Han population

Zeying Liu[1,2], Yazhou Cui[1], Xiaoyan Zhou[1], Xiumei Zhang[1], Jinxiang Han[1,*]

[1] *Shandong Academy of Medical Sciences, Shandong Medical Biotechnological Center, Key Laboratory for Biotech Drugs of the Ministry of Health, Ji'nan, Shandong, China;*
[2] *School of Medicine and Life Sciences, University of Jinan-Shandong Academy of Medical Science, Ji'nan, Shandong, China.*

Summary **The aim of this study was to investigate two mineralization-related genes *TNAP* and *ANKH* polymorphisms associated with ankylosing spondylitis (AS) in the North Chinese Han population. We carried out a case-control study in Chinese AS cohorts involving 278 AS patients and 286 unrelated healthy controls. Five *TNAP* SNPs (rs3200254, rs1256348, rs1472563, rs1780329, rs3767155) and four *ANKH* SNPs (rs25957, rs26307, rs27356, rs28006) were genotyped by the Multiplex Snapshot method. There were significant differences in genotype (permutated $p = 0.00481$) and allele (permutated $p = 0.0126$) frequencies of the rs26307 *ANKH* SNP between AS patients and controls. Logistic regression analysis suggested an association of AS with the polymorphism in an additive model (OR = 0.640, 95%CI = 0.480-0.853, $p = 0.0023$, permutation 10,000 corrected $p = 0.0158$) and a dominant model (OR = 0.599, 95%CI = 0.423-0.846, $p = 0.0037$, permutation 10,000 corrected $p = 0.022$). Haplotype analysis identified the *ANKH* haplotype rs26307(C)/rs27356 (T) as a predisposing factor for AS (OR = 1.53, 95%CI = 1.165-2.071, $p = 0.0026$, permutation 10,000 corrected $p = 0.0103$). This study provides evidence that variation in the *ANKH* gene influences susceptibility to AS in the Northern Han Chinese population.**

Keywords: Ankylosing spondylitis, polymorphisms, *TNAP*, *ANKH*

1. Introduction

Ankylosing spondylitis (AS) is a progressive chronic disease characterized by inflammatory response and pathological mineralization. The prevalence of AS is 0.24% in the Chinese population, which is similar to the incidence in Caucasians (*1*). Twin and family studies have shown that genetic factors play an important role in the pathogenesis of AS. Most previously identified genetic risk variants for AS are related to the immune response. Until recently, in a GWAS study in the Chinese Han population, Gu *et al*. (*2*) discovered two new susceptibility loci rs4552569 and rs17095830 near genes (between *HAPLN1-EDIL3* at 5q14.3 and within *ANO*6 at 12q12) related to cartilage development and bone formation. Our previous study also identified a novel single nucleotide polymorphism (SNP) site (c.4488+74 G > A) in low-density lipoprotein receptor-related protein 5 (*LRP*5) gene was associated with AS in the Chinese Han population (*3*). These data support the hypothesis that specific polymorphisms in the bone formation genes, in particular pathological mineralization-related genes might predispose to AS.

Multiple lines of evidence indicated the potential importance of extracellular pyrophosphatase metabolism regulators tissue-nonspecific alkaline phosphatase (TNAP) and the human orthologue of mouse progressive ankylosis (ANKH) in the pathological mineralization of AS (*4*). Furthermore, several human and mouse diseases manifested by hydroxyapatite (HA) crystal deposition in the soft tissues of tendons and/or ligaments resembling

Liu ZY and Cui YZ contributed equally to this work.

**Address correspondence to:*
Dr. Jinxiang Han, Shandong Academy of Medical Sciences, Shandong Medical Biotechnological Center, Key Laboratory for Rare Disease Research of Shandong Province, and Key Laboratory for Biotech Drugs of the Ministry of Health, Shandong, China.
E-mail: samshjx@sina.com

AS have been related to defects in *TNAP* and *ANKH* genes (*5*). Some *TNAP* and *ANKH* SNPs have been variably reported to be associated with AS susceptibility in Caucasians (*6-8*). As ancestry-based heterogeneity may exist in AS susceptibility between Chinese and European populations, replication is needed to confirm the potential genetic influences of *TNAP* and *ANKH* on AS in other groups. Here we assessed the association of nine previously identified SNPs in the *TNAP* and *ANKH* genes in Chinese AS patients.

2. Patients and Materials

2.1. *Patients*

We analyzed nine SNPs in 278 unrelated Chinese Han AS patients (215 male and 63 female) and 286 healthy Chinese Han controls (206 male and 80 female) in Shandong Province, in North China. All of the case and control subjects were original local residents of Shandong Province of China. Patients with AS was diagnosed according to the modified New York criteria and were patients in Shandong Provincial Hospital and LinYi People's Hospital. The healthy control subjects were matched to the patients in sex, age and geographic location. The study was approved by the ethics committee of our institution, and written informed consent was obtained.

2.2. *Genotyping*

Nine previously reported polymorphisms of *TNAP* (rs3200254, rs1256348, rs1472563, rs1780329, rs3767155) and *ANKH* (rs25957, rs26307, rs27356, rs28006) were genotyped by the Multiplex Snapshot technique according to the manufacturer's protocol. Briefly, all nine SNPs were amplified by a Multiplex PCR kit (Qiagen, Germany). The PCR products were included in a single base extension (SBE) reaction with a SNaPshot Multiplex reagent Kit (Applied Biosystems). Snapshot products were then analyzed in the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). Genotypes were determined by GeneMapper 4.1 software (Applied Biosystems). Genotyping was tested repetitively in 10% masked random samples by different investigators and all the results were completely concordant. Detailed information on the PCR primers and SBE oligonucleotides used are shown in supplemental Table S1 (*http://www.biosciencetrends.com/docindex.php?year=2013&kanno=2*).

2.3. *Statistical analysis*

Hardy-Weinberg equilibrium was tested for each SNP on controls. Genotype and allele distributions between cases and controls were compared using χ^2 tests. The main analysis used to test for association was multiple logistic regression models, adjusted for age and gender. Multiple testing corrections were performed based upon 10,000 random permutations of the sample data. All above analyses were run using PLINK v1.07. Haplotype analyses were performed using the Haploview 4.2 program. Statistical power was estimated using STPLAN 4.3 software. The level of significance for all statistical tests was defined as a p value < 0.05.

3. Results

The alleles and genotypes frequencies of AS patients and controls in the total samples are given in Table 1. The distribution of genotypes of *TNAP* and *ANKH* polymorphisms were within the range of Hardy-Weinberg equilibrium. We estimated the power of our study material and found that our sample size had more than 80% power to detect an odds ratio (OR) of 1.50 for AS between carriers and non carriers with a significance level (alpha) of 0.05 for each SNP.

SNPs rs1256348 of *TNAP*, rs26307 and rs27356 of *ANKH* showed a significant association with AS (rs1256348 genotype p = 0.0499, allele p = 0.016; rs26307 genotype p = 0.0101, allele p = 0.00195; rs27356 genotype p = 0.0119, allele p = 0.0085, respectively), but only rs26307 remains significant after multiple testing using 10,000 permutations (genotype permutated p = 0.00481, allele permutated p = 0.0126), and rs27356 showed a marginal statistically significant level (genotype permutated p = 0.055, allele permutated p = 0.0596). The T allele of rs26307 significantly lowered the risk of developing AS (OR = 0.634, 95% CI = 0.475-0.847).

In order to rule out confounding in our crude association analyses, we reevaluated the polymorphism effect of rs26307 and rs27356 under 3 different models using logistic regression adjusted for age and gender. The *ANKH* rs26307 polymorphism was found to decrease the risk of AS using the additive model (CC *versus*. CT *versus*. TT: OR = 0.640, 95%CI = 0.480-0.853, p = 0.0023, permutation 10000 corrected p = 0.0158) and the dominant model (CC + CT v*ersus*. TT: OR = 0.599, 95%CI = 0.423-0.846, p = 0.0037, permutation 10000 corrected p = 0.022); and the *ANKH* rs27356 polymorphism was found to decrease the risk of AS *via* a recessive model (CC *versus*. CT + TT: OR = 0.267, 95%CI = 0.0975-0.731, p = 0.010, permutation 10,000 corrected p = 0.0414).

The linkage disequilibrium (LD) block structure revealed one haplotype block including rs26307 and rs27356 in the *ANKH* gene (Figure 1). The major haplotype combination of rs26307 (C) and rs27356 (T) is significantly associated with AS as a predisposing common haplotype (OR = 1.53, 95%CI = 1.165-2.071, p = 0.0026, permutation 10,000 corrected p = 0.0103); the minor haplotype combination of rs26307 (T) and rs27356 (C) defined a significantly protective common

Table 1. Genotype and allele frequencies and disease susceptibility

Gene	SNP	Minor Allele	Genotype (%)			*p*	Allele (%)		*p*	OR (95% CI)
TNAP	rs1256348	C	C/C	C/T	T/T		C	T		
	Cases		246 (0.885)	31 (0.112)	1 (0.004)	0.0499	523 (0.941)	33 (0.059)	0.016	2.06 (1.134-3.743)
	Controls		269 (0.941)	17 (0.059)	0 (0.000)		555 (0.970)	17 (0.030)		
TNAP	rs1472563	T	C/C	C/T	T/T		C	T		
	Cases		61 (0.219)	139 (0.500)	78 (0.281)	0.497	261 (0.469)	295 (0.531)	0.746	1.039 (0.8226-1.314)
	Controls		67 (0.234)	129 (0.451)	90 (0.315)		263 (0.460)	309 (0.540)		
TNAP	rs1780329	A	A/A	A/C	C/C		A	C		
	Cases		64 (0.230)	134 (0.482)	80 (0.288)	0.950	262 (0.471)	294 (0.529)	0.839	0.976 (0.7726-1.233)
	Controls		69 (0.241)	135 (0.472)	82 (0.287)		273 (0.477)	299 (0.523)		
TNAP	rs3200254	C	C/C	C/T	T/T		C	T		
	Cases		56 (0.201)	138 (0.496)	84 (0.302)	0.781	250 (0.450)	306 (0.550)	0.780	0.9672 (0.7649-1.223)
	Controls		60 (0.211)	141 (0.495)	84 (0.295)		261 (0.458)	309 (0.542)		
TNAP	rs3767155	T	C/C	C/T	T/T		C	T		
	Cases		125 (0.450)	123 (0.442)	30 (0.108)	0.300	373 (0.671)	183 (0.329)	0.199	1.18 (0.9167-1.518)
	Controls		147 (0.514)	110 (0.385)	29 (0.101)		404 (0.706)	168 (0.294)		
ANKH	rs25957	C	C/C	C/G	G/G		C	G		
	Cases		4 (0.014)	72 (0.259)	202 (0.727)	0.956	80 (0.144)	476 (0.856)	0.781	1.049 (0.7499-1.467)
	Controls		4 (0.014)	71 (0.248)	211 (0.738)		79 (0.138)	493 (0.862)		
ANKH	rs26307	T	C/C	C/T	T/T		C	T		
	Cases		191 (0.687)	77 (0.277)	10 (0.036)	0.0101	459 (0.826)	97 (0.174)	0.00195	0.634 (0.4746-0.8468)
	Controls		163 (0.570)	103 (0.360)	20 (0.070)		429 (0.750)	143 (0.250)		
ANKH	rs27356	C	C/C	C/T	T/T		C	T		
	Cases		5 (0.018)	80 (0.288)	193 (0.694)	0.0119	90 (0.162)	466 (0.838)	0.0085	0.6699 (0.497-0.904)
	Controls		18 (0.063)	92 (0.322)	176 (0.615)		128 (0.224)	444 (0.776)		
ANKH	rs28006	T	C/C	C/T	T/T		C	T		
	Cases		201 (0.723)	73 (0.263)	4 (0.014)	0.924	475 (0.854)	81 (0.146)	0.716	1.064 (0.7616-1.487)
	Controls		211 (0.738)	71 (0.248)	4 (0.014)		493 (0.862)	79 (0.138)		

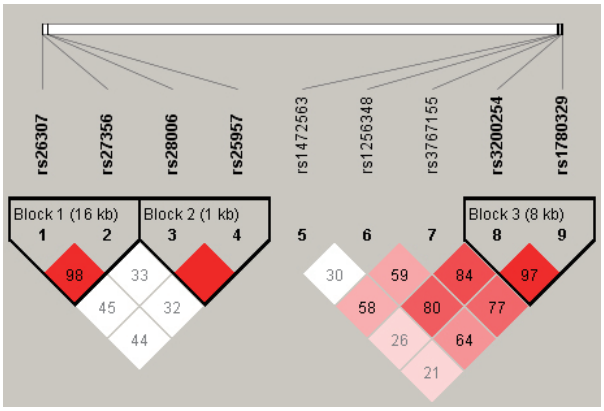

Figure 1. Linkage disequilibrium (LD) structure of the SNPs and haplotype blocks analyzed in this study.

haplotype (OR = 0.659, 95%CI = 0.487-0.890, p = 0.0064, permutation 10,000 corrected p = 0.0264) (Table 2).

4. Discussion

In order to better understand the role of *TNAP* and *ANKH* in the pathological mineralization of AS, we conducted an analysis testing the association of their polymorphism genes to AS patients in a Northern Han Chinese population.

A previous family-based association study by Tsui HW *et al*. in the Canadian population documented that a *TNAP* haplotype marker [rs3767155(G)/rs3200254(G)/rs1780329(T)] in men is significantly associated with AS in multiplex families affected (*8*). Our results revealed no association between AS and these three *TNAP* variants, either individually or by conforming haplotypes, which contradicts the results of Tsui HW *et al*., and is consistent with the results of Zhang *et al*. in a South Chinese population (*9*). We further revealed that no association exists between AS and the other two *TNAP* variants rs1256348 and rs1472563 in the Northern Chinese population, which is not consistent with the results of Tsui HW *et al*. (*8*). In agreement with the results of the previous study in the Chinese population (*9*), our data do not support the speculation that the *TNAP* gene confers susceptibility to AS in the Chinese Han population.

Four variants in *ANKH* (rs27356, rs26307, rs25957, and rs28006) have previously been genotyped in AS patients in two independent studies in Caucasian populations, but their results were inconsistent. In a study in a Portuguese population, the four markers demonstrated no significant single-locus disease associations with AS and disease severity, as measured by *BASDAI*, *BASFI*, *BASMI*, or *mSASSS*. However, in another study in a Canadian population, *ANKH* rs26307 was significantly associated with AS only in affected men; furthermore, a haplotype marker [rs26307/rs27356] at the 3' end of the gene was significantly associated with AS in men while another haplotype

Tabe 2. Haplotype analysis of *ANKH* (rs26307/rs27356) in cases and controls*

Haplotypes	Freq.	No. of Cases	No. of Controls	χ^2	p	p_c
CT	0.785	457	428	9.059	0.0026	0.0103
TC	0.191	88	127	7.428	0.0064	0.0264
TT	0.022	9	16	1.805	0.1791	0.7433

p_c: p value adjusted by 10000 permutations; *: CC haplotype were left out for its frequency < 0.01.

marker [rs28006/rs25957] at 5' end of the gene was significantly associated with AS in women (*6*). In the present study, we found a positive association between *ANKH* rs26307 polymorphism and AS. There was a significant difference in genotype distribution of rs26307 between AS and controls even after adjusting for age and gender. The presence of the minor T allele has a protective role for developing AS when compared with the presence of the major C allele, which can also be interpreted as a risk factor. Also, our results reflected a relationship between the *ANKH* haplotype (rs26307/rs27356) and the risk of AS, suggesting that the minor alleles [rs26307(T)/rs27356(C)] were a protective factor for AS. However, no association between the haplotype [rs26307(C)/rs27356(C)] and AS were observed in this study. Furthermore, no significant interaction between gender and rs26307 genotype and halpotype has been identified in this study, which is different from the results of Tsui *et al*. (*6*).

In conclusion, our findings suggest that genetic variant rs26307 at *ANKH* might influence susceptibility to AS in a Northern Han Chinese population but without a strong gender predilection. Further genetic association studies with a larger sample and functional analysis are needed to investigate the potential roles of *ANKH* in the mineralization pathogenesis of AS.

Acknowledgements

We thank Dr. Jintai Yu at Qingdao municipal hospital for giving valuable advice on data analysis, Dr. Qingrui Yang at Shandong Provincial Hospital and Dr. Haisheng Ji at LinYi People's Hospital in Shandong Province, China for the kind help on sample collecting. We also thank the Shanghai Genesky Bio-Tech Genetic CoreLab for their excellent technical assistance with SNP genotyping.

This work was supported by the Key Project for Drug Research and Development from the Ministry of Science and Technology of China (Grant No. 2010ZX09401-302-5-07) and the Natural Science Foundation of Shandong Province, China (ZR2010CM019).

References

1. Ng SC, Liao Z, Yu DT, Chan ES, Zhao L, Gu J. Epidemiology of spondyloarthritis in the People's Republic of China: Review of the literature and commentary. Semin Arthritis Rheum. 2007; 37:39-47.
2. Lin Z, Bei JX, Shen M, *et al*.. A genome-wide association study in Han Chinese identifies new susceptibility loci for ankylosing spondylitis. Nat Genet. 2012; 44:73-77.
3. Liu J, Zhou X, Shan Z, Yang J, Yang Q, Cui Y, Han J. The association of *LRP5* gene polymorphisms with ankylosing spondylitis in a Chinese Han population. J Rheumatol. 2011; 38:2616-2618.
4. Mebarek S, Hamade E, Thouverey C, Bandorowicz-Pikula J, Pikula S, Magne D, Buchet R. Ankylosing spondylitis, late osteoarthritis, vascular calcification, chondrocalcinosis and pseudo gout: Toward a possible drug therapy. Curr Med Chem. 2011;18:2196-2203.
5. Zhou X, Cui Y, Han J. Phosphate/pyrophosphate and MV-related proteins in mineralisation: Discoveries from mouse models. Int J Biol Sci. 2012; 8:778-790.
6. Tsui HW, Inman RD, Paterson AD, Reveille JD, Tsui FW. *ANKH* variants associated with ankylosing spondylitis: Gender differences. Arthritis Res Ther. 2005; 7:R513-525.
7. Timms AE, Zhang Y, Bradbury L, Wordsworth BP, Brown MA. Investigation of the role of *ANKH* in ankylosing spondylitis. Arthritis Rheum. 2003; 48:2898-2902.
8. Tsui HW, Inman RD, Reveille JD, Tsui FW. Association of a *TNAP* haplotype with ankylosing spondylitis. Arthritis Rheum. 2007; 56:234-243.
9. Cheng N, Cai Q, Fang M, Duan S, Lin J, Hu J, Chen R, Sun S. No significant association between genetic polymorphisms in the *TNAP* gene and ankylosing spondylitis in the Chinese Han population. Rheumatol Int. 2009; 29:305-310.

(*Received March 15, 2013; Revised April 12, 2013; Accepted April 15, 2013*)