Association of *TNFSF4* polymorphisms with susceptibility to primary Sjögren's syndrome and primary biliary cirrhosis in a Chinese Han population

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Abstract

Objectives

We aimed to evaluate the association between polymorphisms of TNFSF4 and primary Sjögren's syndrome (pSS) and primary biliary cirrhosis (PBC) in a Chinese Han population.

Methods

A total of 250 pSS patients, 221 PBC patients, and 393 healthy controls were enrolled. All individuals were ethnic Chinese Han, and each group was matched for gender ratio and age. We identified single nucleotide polymorphisms (SNPs) via the HapMap Han Chinese Beijing databank for a genetic region containing TNFSF4, and then identified haplotype tagging SNPs with the Tagger programme of Haploview. DNA samples were amplified through polymerase chain reaction (PCR) and extension products were differentiated via mass spectrometry. Association analyses were performed using PLINK software.

Results

In TNFSF4, T allele and TT genotype of rs2205960, and G allele of rs1234313, were associated with pSS ($p<0.05$); T allele of rs2205960 was correlated with PBC ($p<0.05$) as a risk factor. In the haplotype analysis, TAGG and TGGT were correlated with pSS ($p<0.05$). In genetic additive, dominant, and recessive models analysis, rs2205960 had a significant association with both pSS and PBC, and rs1234313 presented a significant association with pSS ($p<0.05$). However, no statistically significant difference was found after Bonferroni corrections.

Conclusion

Overall, no association between the allele, or genotype, or haplotype frequencies of TNFSF4 and the risk of pSS or PBC was found. TNFSF4 may have little significance as a common genetic component of pSS and PBC in the Chinese Han population.

Key words

primary Sjögren's syndrome, primary biliary cirrhosis, *TNFSF4*, single nucleotide polymorphism

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Introduction

The diseases Sjögren's syndrome (SS, also known as sicca syndrome) and primary biliary cirrhosis (PBC) are frequently seen in clinic and are often concurrent in the same individual (1, 2). Although a preponderance of evidence suggests that each is of autoimmune origin, their respective aetiologies are not definitively known (3, 4). SS is characterised by a generalised dryness, especially of the eyes (keratoconjunctivitis sicca or xerophthalmia) and mouth (xerostomia), due to lymphocytic infiltration of the lacrimal and salivary glands, but any organ or system could be affected (5). PBC is a chronic liver disease (4) that presents with fatigue, itchy skin (pruritus), and jaundice. The progressive apyogenous destruction of intrahepatic bile ducts that characterises PBC leads to interruption of bile secretion (cholestasis), fibrosis, and eventual end-stage liver disease (6, 7)

SS and PBC share common features. Both are most often encountered in women of middle-age, although any age or gender may be affected (8, 9). Both diseases share relatively high levels of secretory IgA (10, 11), and involve the destruction of epithelial tissues that is apparently immune-mediated (6). There also appears to be associated genetic and environmental risk factors (4, 12, 13), particularly in PBC, with the genetic considered the more important.

The results of several studies have strongly implied that the unique receptor-ligand pair TNFRSF4-TNFSF4 is important to the development of autoimmunity. The cytokine ligand TNFSF4 (also known as OX40L or CD252), is encoded by the gene *TNFSF4* (tumour necrosis factor ligand superfamily member 4), and is expressed on the surface of activated antigen-presenting cells and B cells. Its receptor TNFRSF4 (also known as OX40 or CD134) is expressed on cluster of differentiated $CD4^+$ and $CD8^+$ T cells. Factors that increase the expression of TNFSF4 promote TNFSF4-TNFRSF4 interactions and thus the co-stimulatory signal between antigen-presenting cells and T cells; T-cell survival is thereby promoted. TNFSF4-TNFRSF4 interactions have also been shown to be crucial in B cell differentiation and antibody production (14).

Systemic lupus erythematosus (SLE) is the first rheumatic autoimmune disorder found to be associated with single-nucleotide polymorphisms (SNPs) of *TNFSF4*, in Caucasian (15, 16) and Asian (17) populations. Recently associations have been also reported between *TNFSF4* and systemic sclerosis (SSc) (18) and primary SS (pSS) (19). In particular, the above studies find correlations between the SNP rs2205960 (in the upstream region of *TNFSF4*) and susceptibility to both SLE (15-17) and SSc (18). However, whether this site is a genetic susceptibility component of other autoimmune diseases such as pSS and PBC has not been definitively determined or if there are other SNPs of *TNFSF4* that influence the incidence of pSS or PBC in the Chinese Han population.

Many researchers in this field consider it likely that the similarities in pathogenesis, demographic and geographic distribution, and high prevalence of comorbid autoimmune disorders indicate that they have common genetic susceptibility backgrounds (9, 20). The high concurrence of SS and PBC in particular justifies an analysis of genetic susceptibility common to both, and the underlying mechanism leading from genome to pathogenesis.

In this study, we investigated the common genetic association between *TNFSF4* polymorphisms and pSS and PBC in a Chinese Han population. We chose the *TNFSF4* SNP rs2205960 as a candidate for its strong association with both SLE and SSc, shown by the above mentioned studies, as well as 4 other tag SNPs (rs16845607, rs1234313, rs7514229, rs3861950) that may have special relevance for the Chinese Han population, found via Hapmap.

Materials and methods

Subjects

The study subjects comprised 250 pSS patients, 221 PBC patients, and 393 healthy controls. The gender ratio and age distributions were similar among the groups. All participants were un-

related Chinese Han individuals and signed written informed consent. The Ethics Committee of Peking Union Medical College Hospital (PUMCH) approved the study.

The pSS patients were recruited from PUMCH between June 2008 and March 2011. All of them conformed to the 2002 revised international classification criteria for pSS (21). And pSS patients who had other comorbid autoimmune diseases were excluded. Finally, 250 pSS patients (17 men, 233 women, 51.02±12.80 years old) were included for this study. The PBC patients were recruited from PUMCH between June 2008 and March 2011, and from Beijing You'an Hospital between May 2006 and September 2010, according to the year 2000 criteria of the American Association for the Study of Liver Diseases for PBC (22). PBC patients who had other comorbid autoimmune diseases or liver diseases were excluded. Finally, 221 PBC patients (26 men, 195 women, 53.49±10.97 years old) were included. Dry mouth, a common symptom for SS, was found in 57 PBC patients. Twenty-one of these 57 patients undertook parotid sialography and only 2 showed abnormalities. Labial gland biopsy was performed in 47 of 57 patients and no lymphocytes focus was detected. The 393 healthy control subjects (45 men, 348 women, 52.26±12.09 years old) were recruited from the medical examination centre of PUMCH between 2009 and 2011. None of the individuals in the control group had a history of autoimmune disease.

SNP selection

The SNPs of *TNFSF4* targeted by this study consisted of rs2205960, confirmed in previous studies to be related to other autoimmune diseases, and tag SNPs found via haplotypic analysis. From the HapMap Chinese Han Beijing databank (http://www.hapmap.org), we selected 4 tag SNPs (rs16845607, rs1234313, rs7514229 and rs3861950) from the promotor 5 kb upstream of the 5′ end to 5 kb downstream of the 3′ end of *TNFSF4*. For haplotyping, the likely SNPs were entered using the tag SNP selection algorithm function Tagger in Haploview, version 4.0 (http://www.broadinstitute.org/mpg/tagger/). In every linkage disequilibrium area, we chose tag SNPs (r^2>0.8) which would provide the maximum genetic information from this chromosomal region; minor allele frequency (MAF) was ≥5%. Exon SNP, which leads to non-synonymous mutation was preferred.

DNA extraction

A 2-mL blood sample, anticoagulated with ethylenediaminetetraacetic acid (EDTA), was collected from each subject. From these samples genomic DNA was extracted using purification kits (BioTeke, Beijing) and stored at -80 °C.

Genotyping

Genotyping was performed via a high-flux Sequenom MassArray system, which requires 15 ng of DNA per sample. Specific and multiplex PCR primers of all SNPs and extension primers were designed using MassArray Assay design 3.1 (Sequenom, USA) software. We conducted multiplex PCR amplification and single-base extension, and the last reaction products were transferred to 384 holes SpectorCHIP array (Sequenom, USA) after desalination. Lastly, we performed allele detection through matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) and the mass spectrum results were analysed by MassArray TYPER (Sequenom, USA) software. We randomly selected 5% of the samples to perform a repeat detection so that the results were validated and quality-controlled. The results of the repeated detection showed a genotyping error rate of <0.1%. The above processes were aided and completed by Biao Miao Biotechnology Technology (Beijing).

Statistical analyses

Disqualified SNPs were excluded using achievement ratio of genotyping (>85%), minor allele frequency (>5%) and Hardy-Weinberg equilibrium (HWE). We used Haploview 4.0 software to perform the chi-squared test with a degree of freedom =1. Samples conformed to the HWE if p>0.01. PLINK 1.07 tool-set was used to calculate and analyse all data. Gender and age, related to the incidences of pSS and PBC, were taken as covariates in analysis. For genetic model testing (additive, dominant, and recessive models), we analysed genotype frequencies using a logistic regression model that adjusted for gender and age. We used Haploview 4.0 software to perform the haplotype analyses and draw the haplotype maps. A p-value <0.05 for comparisons was considered a statistically significant difference.

Results

Comparability between groups

There was no significant difference between the pSS, PBC, and healthy controls in terms of age or gender (p>0.05).

Representation of selected SNP

The genotyping success rates were >99% for each of the 5 tested SNPs, with a minor allele frequency >5%. The information of tag SNPs were showed in Table I. The SNP rs7514229 did not meet the HWE criterion and p<0.01, therefore, rs7514229 was excluded from further analysis.

Association analyses between SNPs and pSS and PBC patients

Genotype and allele frequencies for patients and controls of the 4 SNPs in HWE were shown in Table II. We did find an association between T allele (minor) of rs2205960 of *TNFSF4* and pSS and PBC in our Chinese Han population (OR=1.309, 95%CI 1.025–1.673, p=0.031; OR=1.299, 95%CI 1.007–1.675, p=0.043, respectively), but p>0.05 was detected after Bonferroni corrections. No association with pSS and PBC was found for rs16845607 and rs3861950 of *TNFSF4* (p>0.05).

The results of the haplotype analysis showed that the haplotypes TAGG and TGGT were associated with pSS (p=0.021; p=0.020, respectively), but p>0.05 was detected after Bonferroni corrections (Table III).

Statistical analysis in genetic additive, dominant, and recessive models was shown in Table IV. The SNP rs2205960 had a significant association with both pSS (Additive model p=0.023; Domi-

Table I. Tag SNP information.

Gene	SNP ID	Genomic position (bp)	Genic position	Alleles (minor/major)	MAF[a]	HWE[b]/*p*-value	Call rate
TNFSF4	rs2205960	173191475	5 near (validate[c])	T/G	0.269	0.2802	100
	rs16845607	173173617	intron (tag[d])	A/G	0.062	0.4265	99.2
	rs1234313	173166247	intron (tag[d])	G/A	0.344	0.0453	100
	rs7514229	173186247	3 UTR (tag[d])	T/G	0.102	**0.0003**	98.7
	rs3861950	173156292	intron (tag[d])	C/T	0.089	0.3660	100

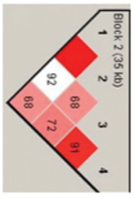
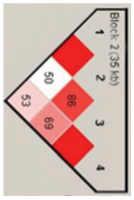
[a] MAF: minor allele frequency; [b] HWE: Hardy-Weinberg equilibrium; [c] validate: SNP confirmed in previous studies; [d] tag: tag SNP.
p-value ≤0.01 is highlighted in bold type.

Table II. Association of alleles and genotypes with pSS and PBC.

SNPs	Groups	Allele frequency (%)		OR (95%CI)	[a]*p*-value	Genotype frequency (%)			χ^2	[a]*p*-value
rs2205960		T	G			TT	TG	GG		
	Control	211 (26.9)	573 (73.0)			33 (8.4)	145 (37.0)	214 (54.6)		
	pSS	162 (32.5)	336 (67.5)	1.309 (1.025–1.673)	**0.031**	23 (9.2)	116 (46.6)	110 (44.2)	6.829	**0.033**
	PBC	143 (32.4)	299 (67.6)	1.299 (1.007–1.675)	**0.044**	26 (11.8)	91 (41.2)	104 (47.1)	3.833	0.147
rs16845607		A	G			AA	AG	GG		
	Control	48 (6.2)	730 (93.8)			0 (0.0)	48 (12.3)	341 (87.7)		
	pSS	38 (7.6)	460 (92.4)	1.256 (0.808–1.953)	0.310	1 (0.4)	36 (14.5)	212 (85.1)	2.191	–
	PBC	24 (5.4)	418 (94.6)	0.873 (0.527–1.446)	0.598	0 (0.0)	4 (10.9)	197 (89.1)	0.296	–
rs1234313		G	A			GG	GA	AA		
	Control	270 (34.4)	514 (65.6)			37 (9.4)	196 (50.0)	159 (40.6)		
	pSS	199 (40.0)	299 (60.0)	1.267 (1.065–1.598)	**0.045**	35 (14.1)	129 (51.8)	85 (34.1)	4.640	0.098
	PBC	153 (34.6)	289 (65.4)	0.992 (0.798–1.288)	0.950	23 (10.4)	107 (48.4)	91 (41.2)	0.220	0.896
rs3861950		C	T			CC	CT	TT		
	Control	70 (8.9)	714 (91.1)			5 (1.3)	60 (15.3)	327 (83.4)		
	pSS	41 (8.3)	453 (91.7)	0.923 (0.617–1.381)	0.697	2 (0.8)	37 (15.0)	208 (84.2)	0.322	0.851
	PBC	38 (8.6)	404 (91.4)	0.959 (0.635–1.451)	0.844	3 (1.4)	32 (14.5)	186 (84.2)	0.081	0.960

p-values ≤0.05 are highlighted in bold type. [a]*p*-values are all >0.05 after Bonferroni corrections.

Table III. Association of haplotypes with pSS and PBC.

Groups	LD MAP	Haplotypes	Frequency	Case, control ratio counts	Case, control frequencies	χ^2	[a]*p*-value
pSS		TAGG	0.53	241.3: 256.7; 431.7: 352.3	0.485, 0.551	5.313	**0.021**
		TGGT	0.23	132.3: 365.7; 164.0: 620.0	0.266, 0.209	5.453	**0.020**
		CGGG	0.08	37.7: 460.3; 61.5: 722.5	0.076, 0.078	0.032	0.859
		TAAG	0.06	31.3: 466.7; 43.9: 740.1	0.063, 0.056	0.259	0.611
		TAGT	0.05	24.7: 473.3; 38.2: 745.8	0.050, 0.049	0.005	0.941
		TGGG	0.04	20.6: 477.4; 31.7: 752.3	0.041, 0.040	0.007	0.933
PBC		TAGG	0.543	235.0: 207.0; 431.1: 352.9	0.532, 0.550	0.377	0.539
		TGGT	0.22	105.9: 336.1; 164.2: 619.8	0.240, 0.209	1.490	0.222
		CGGG	0.075	31.0: 411.0; 61.1: 722.9	0.070, 0.078	0.247	0.619
		TAAG	0.056	23.9: 418.1; 45.1: 738.9	0.054, 0.057	0.062	0.804
		TAGT	0.055	30.1: 411.9; 37.8: 746.2	0.068, 0.048	2.122	0.145
		TGGG	0.034	9.0: 433.0; 32.5: 751.5	0.020, 0.041	3.819	0.051
		CGGT	0.013	7.0: 435.0; 8.9: 775.1	0.016, 0.011	0.442	0.506

p-values ≤0.05 are highlighted in bold type. [a]*p*-values are all >0.05 after Bonferroni corrections.

nant model p=0.010) and PBC (Additive model p=0.034), and rs1234313 presented a significant association with pSS (Additive model p=0.042). However, no significant statistics difference was found after Bonferroni corrections.

Discussion

TNFR/TNF superfamily regulates various aspects of immune function. In this superfamily, one of the most important and remarkable interactions occurs between TNFSF4 and its unique receptor TNFRSF4. When combined with its receptor, TNFSF4 has been found to regulate the normal function of $CD4^+$ and $CD8^+$ T cells, regulatory T cells, natural killer cells and natural killer T cells (23, 24). An important role of TNFRSF4-

Table IV. Genetic models and statistical analysis.

SNPs	pSS ([a]*p*-value)			PBC ([a]*p*-value)		
	Additive model	Dominant model	Recessive model	Additive model	Dominant model	Recessive model
rs2205960	**0.023**	**0.010**	0.721	**0.034**	0.073	0.177
rs16845607	0.259	–	–	0.909	–	–
rs1234313	**0.042**	0.103	0.071	0.716	0.882	0.698
rs3861950	0.658	0.791	0.582	0.425	0.811	0.932

p-values ≤0.05 are highlighted in bold type. [a]*p*-values are all >0.05 after Bonferroni corrections.

TNFSF4 signals is suppressing the generation and function of regulatory T cells, which is supported by data shown in asthma (25). In addition, TNFRSF4-TNFSF4 interactions also influence the normal function of antigen presenting cells and diverse cell types such as mast cells, smooth muscle cells and endotheliocytes. In rheumatoid arthritis (RA) patients, $CD4^+$T cells in synovial fluid present a higher level of TNFRSF4 than that in peripheral blood T cells (26). Blocking TNFSF4 or depletion of TNFRSF4 positive cells is shown to have therapeutic effects in animal models of autoimmune and inflammatory diseases (27, 28). Researches over the past decade have shown that TNFRSF4-TNFSF4 interactions play an important role in development of autoimmune disorders.

It has been found that polymorphisms in *TNFSF4* are correlated with diverse autoimmune diseases, and also influence susceptibilities to coronary heart disease and atherosclerosis (29, 30). The SNP rs2205960, located at upstream of *TNFSF4*, is an important variant leading to susceptibility to SLE in Asians (17) and SSc in Caucasians (31). Besides, it is also related to susceptibility to SSc in individuals whose anti-centromere antibody is positive (18). A risk haplotype of SSc in *TNFSF4*, which results in elevated expression level of TNFSF4, influences the regulation of proliferation and differentiation of T cells and B cells, and leads to autoantibody increase and tissue damage (32). Compared those with the non-risk haplotype, lymphoblastoid cell lines with the risk *TNFSF4* haplotypes in SLE exhibit an increased mRNA expression and cell surface protein expression of TNFSF4 after activation (15).

Primary SS and PBC are common diseases among the systemic autoimmune diseases. The causation and pathogenesis of pSS and PBC have not been fully elucidated and both genetic and environmental factors may contribute to their development (4, 13). Several genes, such as *STAT4*, *BAK1* and *CTLA-4,* are associated with SS, but the degree of association is not replicable across different geographic regions or ethnic races. The genetic susceptibility of PBC is supported by familial clustering of PBC, high disease concordance in monozygotic twins, and increased prevalence of other autoimmune conditions in PBC patients and their family members (4). Gene sites, class II human leucocyte antigen (HLA) loci (*HLA-DRB1**08, *11 and *13), are found to be associated with PBC and the data are reliably reproducible; however, others such as *CTLA-4* and *MDR3* have been reported but the findings are not inconclusively replicated (12). Recently, genome-wide association studies have found nearly 20 predisposing gene loci for PBC, indicating that PBC is a complicated disease of multigenic inheritance (12).

The association between *TNFSF4* and pSS was first found by Nordmark *et al*. (19). In Caucasian populations, the SNPs rs1234315 and rs1234314 in the 5′ UTR of *TNFSF4* are correlated with susceptibility to the disease. No report so far describes the associations between polymorphisms in *TNFSF4* and the risk of PBC. In the present study, we investigated the potential relationship between pSS and PBC and variability of 5 SNPs in *TNFSF4*. The SNP rs7514229 did not meet the HWE criterion and was excluded. As shown in Tables II-IV, among the rest 4 SNPs (rs2205960, rs16845607, rs1234313 and rs3861950), no association with either pSS risk or PBC risk in Chinese Han populations were found after Bonferroni corrections.

SS, with a prevalence of almost 0.5%, ranks second only to RA amongst the systemic autoimmune diseases (33). And PBC affects about 1/2000 of the general population. These two diseases are often concurrent in the same individual in clinic and share common features. Liver involvement is a common complication in SS (34). In PBC patients, about 75% suffer SS symptoms of oral and ocular dryness (35) and 38% are positive for anti-SSB antibody (36). The comparison of liver histology between the PBC with pSS group and the PBC without pSS group shows no difference between two groups in pathology stages, infiltration of plasma cells in hepatic lobules and floccular inflammation (37). Many researchers assume that high prevalence of comorbid autoimmune disorders indicate the common genetic susceptibility backgrounds. However, it's still not well illuminated about the genetic susceptibility common to both pSS and PBC, and the underlying mechanism leading from genome to pathogenesis. Our present study was the first to investigate the relationship between *TNFSF4* and pSS or PBC in Chinese Han populations, and the first to study the common genetic susceptibility backgrounds of those two comorbid autoimmune disorders. Our data revealed that tag SNPs rs2205960, rs16845607, rs1234313 and rs3861950 of *TNFSF4* had no risk association with either pSS or PBC in Chinese Han populations, which reflected the complex genetic mechanisms underlying pSS and PBC. However, four SNPs we investigated may not represent all the genetic information. Further work will be needed to

assess the association of other SNPs in *TNFSF4* with susceptibility to pSS and PBC, and larger sample size will be needed to be included for the study.

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