
Seroreactivity against PTEN-induced putative kinase 1 (PINK1) in Turkish patients with Behçet's disease

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This work was supported by the Research Fund of Istanbul University (Project No. T-361/03112003), and TÜBİTAK (Project No: 105S112).

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Received on November 11, 2008; accepted in revised form on July 14, 2009.

Clin Exp Rheumatol 2009; 27 (Suppl. 53): S67-S72.

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Key words: Autoantigen, Behçet's disease, PINK1, SWAP70, ANKRDA1.

Competing interests: none declared.

ABSTRACT

Background. Behçet's disease (BD) is a multisystem inflammatory disorder characterized by recurrent oral ulcers, genital ulcers and ocular inflammation, as well as skin, joint, vascular, pulmonary, central nervous system (CNS) and gastrointestinal tract manifestations. The etiopathogenesis of BD has not yet been identified; but it has generally been accepted that several environmental factors may induce an inflammatory attack in genetically susceptible individuals. In this study, we aimed to identify antigens that could elicit high-titer IgG responses by the serological analysis of recombinant expression of cDNA libraries method (SEREX).

Methods. We screened a human testis cDNA library with pooled sera obtained from 4 BD patients by SEREX. Antigens that were identified with the initial analysis were selected for seroreactivity analysis of a larger group of BD patients (n=78) and controls (n=66) by serological immunoscreening.

Results. We observed seroreactivity against 6 antigens using the pooled sera. These included rabaptin 5 (RABPT5), PTEN-induced putative kinase 1 (PINK1), switch associated protein 70 (SWAP70), interferon-induced protein with tetratricopeptide repeats 2 (IFIT2), ankyrin repeat domain 20 family, member A1 (ANKRD20A1), and an unknown antigen. Eleven out of 82 (13.4%) BD patients were found to have antibodies elicited against PINK1 antigen, when none of the control sera showed reactivity (p=0.001). There was no significant difference in the frequency of other defined antigens between the patient and control groups. However, among BD clinical sub-groups, anti-SWAP70 antibodies were found to associate with vascular involvement.

Discussion. In this study, antibodies

against PINK1 were found to specifically associate with BD while SWAP70 antibody was associated with clinical sub-groups of BD. Although variations in both genetic background and environmental factors may affect the outcome of serological responses, our results suggest that serological screening can be used to identify antigens that elicit antibody responses associated with BD.

Introduction

Behçet's disease (BD) is a multisystem inflammatory disorder characterized by recurrent oral ulcers, genital ulcers and ocular inflammation, as well as skin, joint, vascular, pulmonary, central nervous system (CNS) and gastrointestinal tract manifestations (1). The prevalence of BD is much higher in countries along the ancient Silk Route, extending from Mediterranean countries including Turkey to Japan, than in northern Europe and the USA (1, 2). The sibling recurrence risk ratio (1s) has been reported as 11.4–52.5 in Turkey (3). The etiopathogenesis of BD has yet to be identified, but it has generally been accepted that several environmental factors may induce an inflammatory attack in genetically susceptible individuals (1, 4, 5). The HLA-B51 antigen is a strong predisposing factor for BD. Interestingly, other genes that have been suggested to play a role pathogenesis of BD such as the major histocompatibility complex (MHC) class I chain-related gene family A (MICA) and tumor necrosis factor gene family (TNF) are located within the MHC locus as well (2, 6–10) Whereas BD is associated with a strong inflammatory response, antigen specific immune responses have been also identified in BD patients. Various immunological studies show hypersensitivity to microorganisms such as

streptococci in BD patients. KTH-1 (a crude extract of *Streptococcus sanguis* SSH-83) causes increased interleukin-6 (IL-6) and interferon-gamma (IFN- γ) secretion by peripheral blood (PB) T-cells of BD patients (11). Recent studies suggest that BD patients show immune-mediated manifestations, in which autoantigen-driven, abnormal T-cell and B-cell reactions may play critical roles. Autoantibodies against alpha-enolase (12) from endothelial cells and alpha-tropomyosin (13-14), retinal S-antigen (15-16), interphotoreceptor retinoid binding protein (IRBP) (16), and heat-shock protein (HSP) 60 (17) were identified among BD patients. These autoantibodies may have potential as diagnostic markers and help in understanding the etiology of BD. SEREX is a technique designed to isolate antigens that have elicited high-titer IgG responses. This immunoscreening method is based on the construction of cDNA libraries that can be screened with patient sera to determine the antibody repertoire of any disease (18-20). Indeed, several autoantigens associated with Sjögren's syndrome, systemic lupus erythematosus (SLE), systemic sclerosis (SSc), giant cell arteritis, and polymyalgia rheumatica were identified by SEREX (21-24). These studies have demonstrated the usefulness of this immunoscreening method in the identification of autoantigens associated with autoimmune diseases. Recently, Kinectin, the carboxy-terminal subunit of the splicing factor Sip1 (Sip1 C-ter) and selenium binding protein (SBP) associated with BD were identified as candidates for serological markers by immunoscreening methods (25-27). In this study, we aimed to identify antigens against which high-titer IgG responses could be elicited in BD patient sera by SEREX and to investigate the prevalence of these autoantibodies among Turkish patients with BD and healthy controls.

Materials and methods

Patients and controls

Sera and PBL obtained from eighty two patients with BD (mean age 35.60 ± 7.42 years, 54 males and 28 females) attending the Division of Rheumatology of

Istanbul University, Istanbul Faculty of Medicine were included in the study. All patients fulfilled the diagnostic criteria of the International Study Group for BD (28). Patients were subgrouped according to the leading involved tissues/organs during the disease course: mucocutaneous involvement (BD-M, n=26), eye involvement as posterior or panuveitis (BD-E, n=20), vascular involvement (BD-V, n= 18) and arthritis (BD-A, n=18). All serum samples were obtained from BD patients during the active phase of the disease, when they have one or more disease manifestations.

Sixty-six control sera (mean age 32.43 ± 12.72 years, 36 males and 30 females) were provided by healthy blood donors employed in the same hospital. Sera from patients with rheumatoid arthritis (RA, n=10) and systemic lupus erythematosus (SLE, n=10) during the active phase were collected as disease controls.

All individuals gave written informed consent and the study protocol was approved by the Ethics Committee of Istanbul Faculty of Medicine of Istanbul University.

Serum samples were kept frozen at -80°C . Genomic DNA was extracted from blood samples using the salting-out extraction method

For the initial screening, a sample of pooled sera obtained from 4 patients, one from each subgroup, was used. None of the patients were taking medications at the time of serum collection. Autoantigens identified by the initial screening were subsequently tested for reactivity with sera obtained from the remaining 78 BD patients as well as with the 66 healthy control sera using the isolated phage clones.

SEREX

a. Immunoscreening of the cDNA expression library

500.000 pfu's of a commercially available human fetal testis cDNA library (Stratagene, Cambridge, UK) were screened with pooled sera obtained from 4 patients with BD. SEREX were performed as previously described (18). Briefly, sera which had been preadsorbed with *E. coli*/phage lysate was

diluted 1:100 in Tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA) and 0.02% sodium azide. Membranes (Whatman® Schleicher & Schuell®) were blocked with 5% skim milk in TBS and incubated with alkaline phosphatase-conjugated goat antihuman secondary IgG (Jackson ImmunoResearch Laboratory Inc., Baltimore Pike, PA). Plaques reactive with human sera were visualized with BCIP (bromo-4-chloro-3-indolyl-phosphate)/NBT (nitroblue tetrazolium). Positive plaques were re-screened with the same pool of sera to ensure clonality.

b. Analysis of candidate clones

The reactive phage clones that were purified to monoclonality, were converted to pBluescript phagemids by *in vivo* excision using ExAssist helper phage (Stratagene, La Jolla, CA). Plasmid DNA was obtained from *E. coli* SOLR strain transformed by the phagemid. Plasmid DNA was purified using the Wizard1 Plus SV miniprep DNA purification system (Promega, UK) as described in the manufacturer's protocol. Restriction enzyme digestion of the DNA inserts with EcoRI and XhoI and electrophoresis in a standard 1.0% agarose gel was used to analyze the length of DNA insert of each candidate plasmid. The isolated cDNA inserts were sequence analyzed.

c. Serological screening according to the immunoreactivity of sera against isolated clones

To assess frequencies of antibody responses to the SEREX defined antigens in individual allogeneic sera from BD patients and controls, XL1-blue MRF' cells were transfected with approximately equal numbers of positive phage containing a cDNA insert and non-recombinant phage (control clone). Plaques were then screened with 1:100 diluted sera of patients or healthy individuals using the same strategy as the immunoscreening of cDNA expression libraries.

HLA-B51 genotyping

HLA-B51 genotyping was performed by amplification of genomic DNA obtained from PBLs using polymerase chain reaction (PCR) as described

previously (29). The β -globin gene was used as an internal control. PCR primer sequences used were as follows: for *HLAB51*: (forward) 5'-GGA GTA TTG GGA CCG GAA C-3', (reverse) 5'-CGT TCA GGG CGA TGT AAT CT-3', and for β -globin (forward) 5'-CAA CTT CAT CCA CGT TCA CC-3', (reverse) 5'-5'-GAA GAG CCA AGG ACA GGT AC-3'. Amplification products were visualized by EtBr stained 2% agarose gel electrophoresis.

Statistical analysis

Fisher's exact test was used to test the equality of frequency distribution among the categorical variables. All tests were two-sided, and a *p*-value less than 0.05 was defined as being statistically significant. Bonferroni correction for multiple testing was employed for subgroup-healthy control and subgroup-subgroup comparisons.

Results

A total of 500,000 pfu of the human testis cDNA library were screened using pooled sera from BD patients with different manifestations. Six reactive clones were isolated, shown in Table I. The six antigens identified by using the pooled sera were subsequently tested for their reactivity with the individual serum samples which composed the pool. All four patient sera showed seroreactivity against SWAP70. Three were seroreactive against PINK1 and two with an unknown antigen (BV16). RABPT5 and ANKRD20A1 reactive antibodies were detected in only one of the 4 patient sera (Table II).

Frequency of seroreactivity among BD patients against the six antigens was determined by extending the screening to include 78 patients with BD and 66 controls. Eleven out of 82 (13.4%) BD patient sera were seroreactive against PINK1 antigen, while none of the control sera showed reactivity (*p*=0.001). Eighteen (22%) BD patient sera were reactive against SWAP70 as opposed to only 7 (10.8%) healthy controls (*p*=0.08). Similarly, antibodies against BV16 and ANKRD20A1 were more frequently observed among patients compared to controls while anti-RBPT5 and -IFIT2 antibody frequencies

Table I. Antigens isolated after initial screening.

Clone number	Linked genes	Accession No.	Chromosomal localization	Number of clones
BV12	PTEN-induced putative kinase 1 (PINK1)	NM_032409	1p36	1
BV13	Switch-associated protein 70 (SWAP70)	NM_015055	11p15	2
BV15	RAB GTPase binding effector protein 1 (RABPT5)	NM_004703	17p13.2	1
BV16	Unknown	NW_922162.1	4	1
BV17	Interferon-induced protein with tetratricopeptide repeats 2 (IFIT2)	NM_001547	10q23-q25	1
BV20	Ankyrin repeat domain 20 family, member A1 (ANKRD20A1)	NM_001012421.1	9q12	1

Table II. Seroreactivity against the identified antigens in serum samples used for the initial screening.

	Patient 1 (with mucocutaneous involvement)	Patient 2 (with arthritis)	Patient 3 (with vascular involvement)	Patient 4 (with eye involvement)
PINK1	1*	1	1	0
SWAP70	1	1	1	1
RABPT5	0	0	1	0
BV16	1	0	1	0
IFIT2	0	0	1	0
ANKRD20A1	0	1	0	0

*0: seronegative; 1: seropositive.

Table III. Frequency of seroactivity towards isolated antigens in BD patient group and control subjects.

Linked genes	BD patients n=82 n (%)	Controls n=66 n (%)	<i>p</i> -value*
PINK1	14 (13.4)	0 (0)	0.001
SWAP70	18 (22.0)	7 (10.6)	0.08
RABPT5	9 (11.0)	7 (10.6)	1.0
BV16	6 (7.3)	1 (1.5)	0.1
IFIT2	1 (1.2)	1 (1.5)	1.0
ANKRD20A1	5 (6.1)	1 (1.5)	0.2

*Fisher's exact test.

were identical among patients and controls (Table III).

The distribution of autoantibodies against autoantigens isolated by SEREX were also analyzed for each four clinical sub-group (Table IV). These included patients with mucocutaneous involvement (BD-M), arthritis (BD-A), vascular involvement (BD-V), and eye involvement (BD-E). Anti-PINK1 antibody was significantly associated with two of the four sub-groups including BD-M and -A, patients of whom 15.4%, and 22.2% were seropositive, respectively. Anti-

PINK1 antibody was also present in 2 of the BD-V patients (11.1%) and one of the BD-E patients (5%). In contrast, anti-SWAP70 antibodies were primarily elicited by BD-V patients of whom 39% were seropositive. BD-M and BD-A patients also had anti-SWAP70 antibodies at rates higher than that of controls (23.1% and 16.7%, respectively), while only 10% of BD-E patients were seropositive for anti-SWAP70, identical to that of controls. Anti-RABPT5 antibodies were detected at twice the frequency of the control population among BD-V patients, although this

Table IV. Frequency of seroactivity towards isolated antigens in BD patients stratified according to clinical sub-groups.

Linked genes	Patients with mucocutaneous involvement n=26 n (%)	Patients with arthritis n=18 n (%)	Patients with vascular involvement n=18 n (%)	Patients with eye involvement n=20 n (%)
PINK1	4 (15.4)*	4 (22.2)*	2 (11.1)	1 (5.0)
SWAP70	6 (23.1)	3 (16.7)	7 (38.9)*	2 (10.0)
RABPT5	2 (7.7)	3 (11.1)	4 (22.2)	0 (0.0)
BV16	3 (11.5)	1 (5.6)	2 (11.1)	0 (0.0)
IFIT2	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)
ANKRD20A1	0 (0.0)	3 (16.7)	2 (11.1)	0 (0.0)

**p*-value significant after correction for multiple testing.

was not statistically significant. Seropositivity against RABPT5 among other clinical sub-groups was indistinguishable from controls, as were anti-IFIT2 antibodies. Seropositivity for anti-ANKRD20A1 were 16.7% for BD-A and 11.1% for BD-V while none of the patients in BD-M, or BD-E subgroups had antibodies against this protein.

When we compared BD sub-groups for given antigens, frequency of SWAP70 was found higher in vascular group compared to all other patients (39% vs. 16%, uncorrected $p=0.047$), however not statistically significant.

Sera from 10 patients with SLE, and 10 patients with RA were screened for seroreactivity against to PINK1. None of these patients had anti-PINK1 antibodies.

We analyzed the genotype frequencies of HLA-B51 in BD patients and controls. HLA-B51 genotype frequency was significantly higher in BD patients (60.3%) than in controls (30.2%), $p<0.01$. However, HLA-B51 frequency was not significantly different among BD clinical sub-groups, or among patients stratified according to seropositivity (data not shown). We also did not find a significant association between the presence of HLA-B51 and disease severity, gender, or age (data not shown).

Discussion

In this study, we screened a human testis cDNA expression library in search of novel antigens reactive with antibodies in BD patient sera by SEREX. Thirteen percent of Turkish BD patients investigated in this study had anti-PINK1

antibodies while none of the 66 healthy controls or the patients with SLE or RA were seropositive. Therefore, our results demonstrate that anti-PINK1 antibodies are specifically elicited by patients with BD. We also found increased anti-SWAP70 seropositivity among BD patients with vascular involvement. We also identified a novel antigen (BV16) against which a number of BD patients mounted an antibody response.

We, thus, demonstrated that antibodies against autologous antigens are frequently present sera from BD patients with mucocutaneous involvement, arthritis, or vascular involvement. Strikingly, we find patients with ocular BD have, if any, a modest increase in autologous antibody frequencies. Whether this relates to the fact that the eye is an immune-privileged tissue will need further investigation. Interestingly, BD patients with vascular involvement were found to have the highest rates of anti-SWAP70 antibodies, while PINK1 was the primary antigen for patients with arthritis and those with mucocutaneous involvement. If common pathogenic pathways underlie BD, then these observations could suggest that variable antibody responses are elicited depending on which tissues are involved in the process. Another possible suggestion is that BD sub-groups have different but possibly overlapping etiopathological mechanisms. In either case, our study clearly demonstrates that autoantibodies have potential value as candidate biomarkers for BD.

Although various autoantigens, other than those identified in this study, have

previously been associated with BD, this is the first report where PINK1 and SWAP70 have been identified as autoantigens that show significant associations with the disease. The fact that our search could identify novel autoantigens suggests that the BD immunome is not exhausted.

It is not unexpected to find different antigens at repeated screens by approaches such as SEREX. Although variations both in the genetic backgrounds and in the environmental factors in the studied populations may have an effect on the outcome of serological responses, the fact that only SWAP70 was identified twice and all other antigens only once, in a screen of 500.000 pfu, clearly reflects that the antibody repertoire of the 4 patients used for the initial screening is not fully characterized. In fact, we have tested the sensitivity of SEREX using controlled library/sera combinations and have observed that the technique will potentially miss one in every 3 to 50 proteins that are seroreactive with the serum used for initial screening (calculations not shown). Nevertheless, numerous studies have proven the strength of the technique and a vast array of tumor antigens has thus been identified (19)

To establish the sensitivity and specificity of autologous antibodies for BD, we are pursuing larger studies that will include additional cohorts of patients with other auto-inflammatory diseases, and by using methods that can generate quantitative data, such as ELISA, as well as by Western blotting.

In previous studies, Lu *et al.* immunoscreened a T24 cDNA expression library and identified kinectin as an autoantigen in 23% of Chinese BD patients (25). In a more recent report, they developed an ELISA and an indirect immunofluorescent assay (IFA), both using full length kinectin and were able to demonstrate that anti-kinectin antibodies were present in 32.6% to 41.3% (IFA and ELISA, respectively) of BD patients but also at a lower rate among other autoimmune connective tissue diseases. However, the titers of anti-kinectin antibody were statistically higher in BD patients, by ELISA (41). The authors report that the discrepancy between their previous and recent study

on kinectin could be due to the different methodologies used to determine antibody levels.

Sip1 is another autoantigen identified by Delunardo *et al.* in BD by screening a cDNA library from human microvascular endothelial cells with serum IgG from two patients with BD (26). Using ELISA, the authors measured IgG, IgM and IgA specific to the carboxy-terminus of Sip1 in patients with BD, SLE, SSc, and various forms of primary vasculitis, as well as in patients with diseases that share clinical features with BD, such as inflammatory bowel disease and uveitis. IgM immunoreactivity. Anti-Sip1 antibody frequency was significantly higher in patients with BD and in patients with primary vasculitis. No seroreactivity was observed in patients with various autoimmune diseases.

Recently, Okunuki *et al.* reported SBP as a new autoantigen in Japanese BD patients (27). They compared retinal autoantigens recognized by sera from BD patients with uveitis or healthy donors using using 2-dimensional electrophoresis (2DE) followed by Western blotting (WB). They found that 20% and 16% of the BD patients with uveitis were positive for the anti-SBP antibody by western blotting and ELISA, respectively. According their results, the anti-SBP antibody-positive patient group contained a larger group of patients with ocular inflammation than the antibody-negative group. The initial patient selection is, thus, likely to influence the repertoire of antigens identified by such screening studies.

The investigation of the role of PINK1 in the pathogenesis of BD might be helpful in the clarification of complex gene-environment interactions in BD. PINK1 gene encodes a putative protein kinase with a mitochondrial targeting signal at the N-terminus. PINK1 is located on the mitochondrial membrane, intermembrane space, as well as in the cytosol (30-32). It is translated in the cytosol and transported to mitochondria by HSP90 which stabilizes its target proteins through the prevention of their degradation via the proteasome system. Interestingly, PINK1 is cleaved by proteasomes in the cytosol which is uncommon for mitochondrial proteins (33-35).

In cultured mammalian cells, overexpression of wild-type PINK1 protects cells against apoptotic stimuli, caused also by oxidative stress. It was found that PINK1 had a protective effect against oxidative-stress-induced apoptosis by phosphorylating downstream effector TNF receptor-associated protein 1 (TRAP1) that might relate to the pathogenic mechanisms of PINK1 mutations in causing Parkinson disease (31). PINK1 and TRAP1 are thought to block the mitochondrial permeability indirectly by prevention of reactive oxygen species (ROS) formation (36). In addition, PINK1 protects from cell death by activating mitochondrial protease HtrA2 by the stress activated MAP kinase p38 gamma (37). PINK1 in the cytosol activates NF- κ B through other signalling proteins and stimulates its anti-apoptotic function (38). PINK1 also blocks Bcl2-dependent pro-apoptotic intramitochondrial proteins (cytochrome C, endonuclease G) from being released into the cytosol (40).

The presence of autoantibodies against PINK1 in BD patients could possibly result in decreased PINK1 activity creating a pro-apoptotic state. Or an abnormally processed PINK1 might be presented as a self-antigen by MHC Class I molecules, subsequently activating an adaptive immune response, which might also indirectly trigger autoreactive B-cells, producing antibodies detected by SEREX.

A decrease in PINK1 activity could also lead to susceptibility of oxidative stress which induces mitochondrial autophagy (34). Induced autophagy results in increased presentation of cytosolic and nuclear antigens MHC class II molecules (40).

Defective PINK1 interaction with HSP90 could result in (i) apoptotic cell death, (ii) mitochondrial autophagy and (iii) proteosomal cleavage of PINK1. These three might also alter PINK1 presentation by MHC molecules and autoantibody production.

However the specificity and primary function of PINK1 as a potential pathogenic factor needs to be clarified.

In the last ten years, various new autoantibody or autoantigen profiles have been identified and demonstrated to

have potential clinical usefulness. The application of various technologies towards the identification of useful diagnostic and prognostic markers of many autoimmune diseases is underway. The collection of large prospective cohorts is critical for establishing the clinical value of such markers in BD.

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