EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 41, No. 5, 354-361, April 2009

Polymorphisms of *COTL1* gene identified by proteomic approach and their association with autoimmune disorders

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DOI 10.3858/emm.2009.41.5.040

Accepted 23 December 2008

Abbreviations: 2-DE, two-dimensional electrophoresis; 5LO, 5-lipoxygenase; ANA, anti-nuclear antibodies; CCP, cyclic citrullinated peptide; *COTL1*, coactosin-like 1; HRM, high resolution melting; LT, leukotrien; RA, rheumatoid arthritis; RF, rheumatoid factor; SLE, systemic lupus erythematosus; SNP, single nucleotide polymorphism

Abstract

To select candidate genes, we attempted to comparative analysis of protein levels between rheumatoid arthritis (RA) patients and healthy controls by two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF-MS). We identified 17 proteins that showed up- or down-regulated spots in RA patients. We found that coactosin-like1 (*COTL1*) were highly expressed in RA patients compared with healthy controls. We performed a case-control study to determine whether the *COTL1* gene polymorphisms were associated with RA and systemic lupus erythematosus (SLE). The genotype frequency of c.-1124G > T and the allelic frequency of c.484G > A in RA patients, and the genotype frequency of c.484G > A in SLE patients were significantly different from healthy controls (*P* = 0.009, 0.027, and 0.025, respectively). We also investigated the correlation with the levels of rheumatoid factor (RF) and anti-cyclic citrullinated peptide (CCP) antibody in RA patients, and anti-nuclear antibodies (ANA) in SLE patients. The c.484G > A polymorphism in RA patients has significant association with the levels of anti-CCP antibody (*P* = 0.03). Our findings demonstrated that c.-1124G > T and c.484G > A polymorphisms of the *COTL1* gene might be associated with the genetic susceptibility of autoimmune disorders.

Keywords: COTL1 protein, human; electrophoresis, gel, two-dimensional; polymorphism, single nucleotide; proteomics; rheumatoid arthritis; systemic lupus erythematosus

Introduction

Rheumatoid arthritis (RA) and systematic lupus erythematosus (SLE) are the complex autoimmune diseases which are caused by combination of multiple genetic, hormonal, and environmental factors (Gregersen, 1999; Kotzin, 1996). RA is accompanied by the presence of many autoantibodies such as rheumatoid factors (RFs), anti-cyclic citrullinated peptide (anti-CCP) antibody, and antibodies to immunoglobulin binding protein (BiP). RFs and anti-CCP antibody are used in clinical practice (Bläss *et al*., 1999, 2001; Schellekens *et al*., 2000). They can be detactable up to 70-80% in RA patients (Mewar *et al*., 2006; Raptopoulou *et al*., 2007). SLE is also characterized by the presence of antinuclear antibody (ANA) (Kurien *et al*., 2006).

Two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) have become a powerful method for comparative proteome analysis (O'Farrell, 1975; Wang *et al*., 1994; Mann *et al*., 1996; Lottspeich, 1999). These techniques have been used for studying RA using the samples derived from RA patients such as plasma, synovial fluid, and peripheral blood mononuclear cells. Schulz

and coworkers performed comparative proteome analysis with peripheral blood mononuclear cells (PBMCs) and found 16 proteins showed down-regulated spots in RA patients (Schulz *et al*., 2007).

Human coactosin-like 1 (COTL1) protein is similar to *Dictyostelium discoideum* coactosin (de Hostos *et al*., 1993). The *COTL1* gene is located on chromosome 16q24.1. Human *COTL1* protein is a small protein composed of 142 amino acid residues with a molecular mass of 17 kDa. Human COTL1 was also identified as a filamentous actin (F-actin) binding protein in transfected CHO and COS-7 cells (de Hostos *et al*., 1993) and as a 5-lipoxygenase (5LO) binding partner in a yeast two-hybrid screen (Provost *et al*., 2001). 5LO is the first committed enzyme of leukotrien biosynthesis. The LKKAET-like motif of COTL1 interacts with 5LO involved in leukotriene biosynthesis in the leukocytes (Provost *et al*., 1999, 2001; Samuelsson 1983).

In this study, we attempted to perform proteome analysis with plasma derived from RA patients and controls to screen RA candidate genes using 2-DE and MALDI-MS techniques. We identified COTL1 protein which is highly expressed in RA patients by comparative proteome analysis, and compared the mRNA expression level of *COTL1* between RA patients and healthy controls by RT-PCR. To determine whether the SNPs of *COTL1* gene are associated with the susceptibility of autoimmune disorders such as RA and SLE, we compared the allelic and genotypic frequencies between RA or SLE patients and healthy controls without RA and SLE. We further investigated the relationship between genotypes of each polymorphism and RF or anti-CCP antibody levels in RA patients, and ANA levels in SLE patients.

Results

We performed proteome analysis using the plasma isolated from RA patients and healthy controls by 2-DE and MALDI-TOF MS technique to find RA specific proteins. More than 1500 spot images were detected on the 2-DE gel, localized in the ranges of pI 3 to 10 and Mr 10 to 200 kDa (Figure 1). The isolated spots in 2-DE were digested with trypsin and then analyzed by MALDI-TOF MS. We chose and identified 17 proteins as the candidate proteins which showed different expressions between RA patients and healthy controls (Table 1). While the two proteins out of them were down-

Table 1. Identification of proteins that exhibit significant differential expression between RA patients and healthy controls using MALDI-TOF/MS.

Spot No.	Protein identity	Accession No.	Fold change (RA/Control)	Matched peptides No.	Sequence coverage (%)	Theoretical value	
						M^r	*PI*
1	Coactosin-like 1	gi | 27695621	(+) 12.2	5/24	38	16089	5.76
2	Vitamin D-binding protein	gi | 455970	(+) 5.9	8/42	25	54513	5.34
3	Leucine-rich alpha-2-glycoprotein 1	gi | 16418467	(+) 1.6	12/71	33	38382	6.45
4	Actin, gamma 1	gi | 54696574	(+) 2.2	26/81	57	42108	5.31
5	Haptoglobin	gi | 1212947	(+) 2.4	11/68	27	38941	6.27
6	Poly (ADP-ribose) polymerase family, member 14	gi | 7023247	(+) 10.8	8/34	23	62781	6.37
7	Leucine-rich alpha-2-glycoprotein 1	gi | 16418467	(+) 1.7	8/47	25	38382	6.45
8	Zinc finger protein 582	gi | 21389593	(+) 3.8	10/53	22	61827	8.79
9	Serum amyloid A1	gi | 225986	(+) 40.8	6/67	66	11675	5.89
10	Transthyretin (prealbumin, amyloidosis type I)	gi | 3891561	(-) 3.2	9/62	92	13840	5.35
11	Transthyretin (prealbumin, amyloidosis type I)	gi | 3891561	(-) 2.7	7/59	74	13840	5.35
12	Apolipoprotein E	gi | 178853	Only control	28/66	72	36242	5.81
13	Fibrinogen alpha chain	gi | 223057	Only control	6/58	47	14443	6.16
14	Galactose-1-phosphate uridyl transferase	gi | 950381	Only RA	4/37	97	4884	6.06
15	Hemoglobin, beta	gi | 999567	Only RA	7/75	64	15866	6.70
16	S100 calcium- binding protein A9	gi | 29126810	Only RA	8/86	60	13291	5.71
17	Keratin 9	gi | 453155	Only RA	8/67	22	62178	5.14

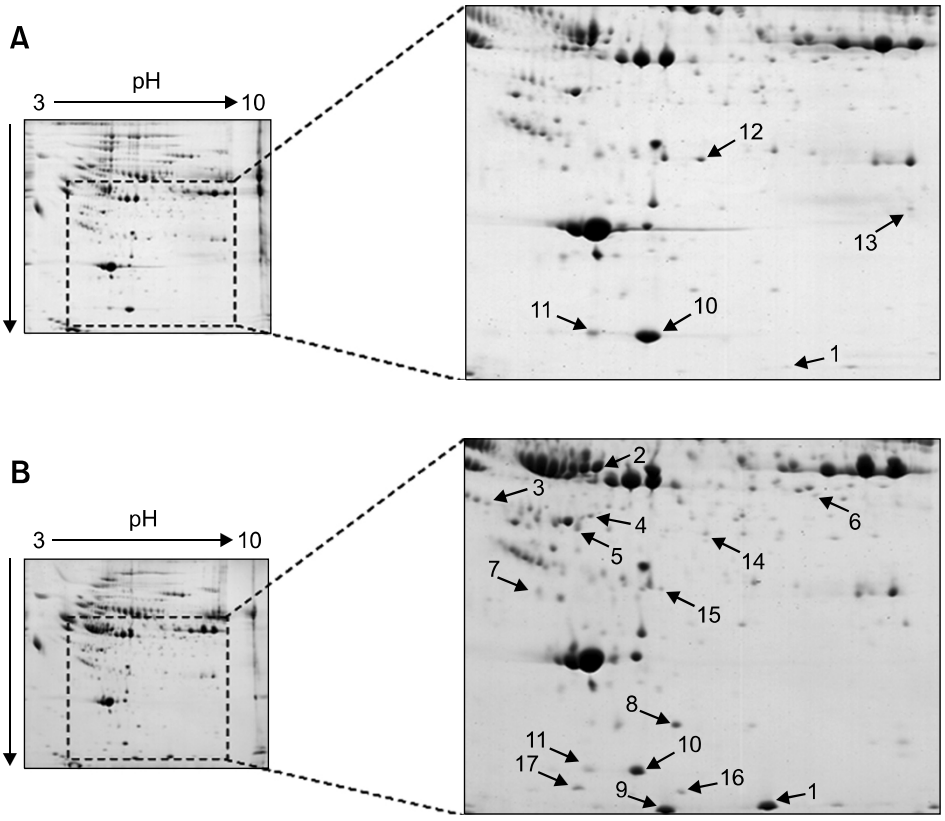

Figure 1. Comparative proteome analysis of sera from healthy controls and RA patients. (A) 2-DE gel images of plasma from healthy controls. (B) 2-DE gel images of plasma from RA patients. Each number indicates a protein that was up- or down-regulated in the expression level. Samples were separated on a nonlinear pH3 to 10 IPG strip followed by a 9 to10% linear gradient polyacrylamide gel. Each gel was stained with Coomassi blue G250. The data are representatives from five independent experiments.

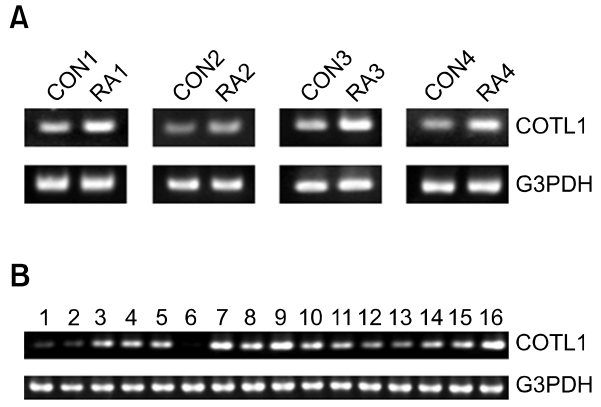

Figure 2. Expression levels and patterns of *COTL1* mRNA. (A) The expression levels of *COTL1* mRNA for 4 RA patients and 4 healthy controls. (B) The expression patterns of *COTL1* mRNA in various human tissues. Lanes: 1, heart; 2, brain; 3, placenta, 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas; 9, spleen; 10, thymus; 11, prostate; 12, testis; 13, ovary; 14, small intestine; 15, colon; and 16, peripheral leukocytes. The data are representatives from three independent experiments.

regulated, nine proteins were up-regulated in RA patients (Table 1). Apolipoprotein E and fibrinogen alpha chain were spots only in healthy control, whereas the galactose-1-phosphate uridyl transeferase, hemoglobin-β, S100 calcium-binding protein A9, and keratin 9 were spots only in RA patient (Table 1). Fibrin(ogen) β-chain degradation products, calgranulin B (MRP14) in synovial fluids (SF), and serum amyloid A protein were identified in the plasma and SF from RA patients (Sinz *et al*. 2002). The level of serum amyloid A1 (SAA1) in RA patient was significantly higher than that of healthy control (Table 1). We focused on *COTL1* whose expression level was more than 12.2 fold higher in RA patients compared with healthy controls. For validation of 2-DE comparison results, the expression levels of *COTL1* were compared between four RA patients and four healthy controls by RT-PCR. The result showed that the expression levels of *COTL1* were much higher in RA patients (Figure 2A). We also further examined the expression pattern of *COTL1* in 15 normal human tissues and peripheral leukocytes. While the expression levels of *COTL1* in heart, brain, and skeletal muscle were only barely detected, those of *COTL1* gene in kidney, spleen, and peripheral leukocytes were highly detected (Figure 2B). Interestingly, the expression levels of *COTL1* were highest in peripheral leukocytes. This result leads us to think that the up-regulated *COTL1* expression in peripheral leukocytes might be related to the inflammatory responses of RA pathogenesis.

To determine whether the *COTL1* gene variations are associated with the susceptibility of autoimmune disorders such as RA and SLE, we chose four SNPs of *COTL1* gene, c.484G > A (rs13521), c.588C > T (rs247862), and c.1050T > A (rs1047121) in the 3' UTR, and c.-1124G > A (KN0013308) in the promoter region, from NCBI SNP database and KSNP database (http://ksnp.ngri.go.kr). We analyzed the genotypes and allele frequencies of these SNPs between healthy controls and RA or SLE patients (Table 2). The genotype frequencies of all loci were in HWE ($P > 0.05$, data not shown). The genotype frequency of c.-1124G > T and the allelic frequency of c.484G > A in RA patients were significantly different from those of control group (P = 0.009 and 0.027, respectively). The genotype frequency of c.484G > A were also significantly different between healthy controls and SLE patients (P = 0.025). These results indicate that the polymorphisms of *COTL1* may be associated with the susceptibility of autoimmune disorders such as RA and SLE.

We further attempted to analyze the possible correlation between the polymorphisms and the clinical features of RA and SLE. Although these polymorphisms in RA patients are shown to have no significant association with RF levels, the c.484G > A polymorphism of *COTL1* is significantly associated with the levels of anti-CCP antibody (P = 0.03) in RA patients (Table 3). We also calculated and compared the haplotype frequencies by c.-1124G > A, c.484G > A, c.588C > T, and c.1050T > A SNPs of *COTL1* between controls and RA or SLE patients. While five major haplotypes (more than 5%) revealing more than 86.1% of distributions are identified in controls, four major haplotypes (~88.1%) in RA patients and three major haplotypes (~88.2%) in SLE patients are identified out of sixteen possible haplotypes (Table 4). The distribution rate of the haplotype GGCA is significantly different between control group and RA or SLE patients (P = 0.021 and 0.044, respectively). Further the haplotype GGTT also shows a significant association with SLE (P = 0.038).

Discussion

RA and SLE are representative complex autoimmune diseases. RA is a common organ specific disorder with a prevalence 500 per 100,000 and SLE is a rare non organ-specific disorder with a

Table 2. Genotype and allele analysis of the SNPs in *COTL1* gene between controls and RA or SLE patients.

Position[a]	Genotype/ Allele	Control, *n* (%)	RA, *n* (%)	SLE, *n* (%)	Odds ratio[c] (95% CI)		*P* value[b]	
					vs. RA	vs. SLE	vs. RA	vs. SLE
c.-1124G > T	GG	442 (78.0)	335 (74.6)	146 (78.1)	1.00	1.00	0.009	0.127
(KN0013308)	GT	122 (21.5)	101 (22.5)	37 (19.8)	1.09 (0.81-1.47)	0.918 (0.61-1.39)		
	TT	3 (0.5)	13 (2.9)	4 (2.1)	5.72 (1.62-20.23)	4.037 (0.89-18.25)		
	G	1006 (88.7)	771 (85.9)	329 (88.0)	1.00	1.00	0.059	0.64
	T	128 (11.3)	127 (14.1)	45 (12.0)	1.30 (1.00-1.68)	1.08 (0.75-1.54)		
c.484G > A	GG	322 (56.9)	277 (62.8)	103 (56.6)	1.00	1.00	0.097	0.025
(rs13521)	GA	197 (34.8)	139 (31.5)	74 (40.7)	0.82 (0.63-1.08)	1.17 (0.83-1.66)		
	AA	47 (8.3)	25 (5.7)	5 (2.7)	0.62 (0.37-1.03)	0.33 (0.13-0.86)		
	G	841 (74.3)	693 (78.6)	280 (76.9)	1.00	1.00	0.027	0.331
	A	291 (25.7)	189 (21.4)	84 (23.1)	0.79 (0.64-0.97)	0.87 (0.66-1.15)		
c.588C > T	CC	305 (54.3)	252 (55.4)	103 (53.9)	1.00	1.00	0.937	0.512
(rs247862)	CT	218 (38.8)	172 (37.8)	70 (36.7)	0.96 (0.74-1.24)	0.95 (0.67-1.35)		
	TT	39 (6.9)	31 (6.8)	18 (9.4)	0.96 (0.58-1.59)	1.37 (0.75-2.49)		
	C	828 (73.7)	676 (74.3)	276 (72.3)	1.00	1.00	0.76	0.593
	T	296 (26.3)	234 (25.7)	106 (27.7)	0.97 (0.79-1.18)	1.07 (0.83-1.39)		
c.1050T > A	TT	509 (89.6)	414 (91.0)	177 (93.2)	1.00	1.00	0.763	0.354
(rs1047121)	TA	59 (10.4)	41 (9.0)	13 (6.8)	0.85 (0.56-1.30)	0.63 (0.34-1.18)		
	AA	0 (0)	0 (0)	0 (0)	-	-		
	T	1077 (94.8)	869 (95.5)	367 (96.6)	1.00	1.00	0.536	0.209
	A	59 (5.2)	41 (4.5)	13 (3.4)	0.86 (0.57-1.30)	0.65 (0.35-1.19)		

[a]Calculated from the translation start site; [b]Value was determined by χ^2 test from 2 × 3 contingency table; [c]Logistic regression analysis were used to estimate odds ratio (95% confidence interval).

Table 3. Analysis of RF and anti-CCP antibody levels in RA patients and ANA levels in SLE patients among the genotypes of *COTL1* polymorphisms.

Position[a]	Genotype	RF (1U/ml)			P^b	Anti-CCP			P^b	ANA			P^b
		n	Mean	S.D.		*n*	Mean	S.D.		*n*	Mean	S.D.	
c.-1124G > T	GG	329	71.8	73.0	0.15	184	55.0	52.1	0.69	89	775.7	2.468E+03	0.54
(KN0013308)	GT	100	64.1	75.8		51	51.4	49.3		23	452.2	677	
	TT	13	105.9	94.3		5	71.7	49.2		-	-	-	
c.484G > A	GG	273	69.8	74.9	0.72	154	49.8	49.4	0.03	67	838.5	2.791E+03	0.35
(rs13521)	GA	137	73.4	73.4		73	66.6	52.9		41	415.6	786	
	AA	25	60.7	77.0		10	35.1	45.9		-	-	-	
c.588C > T	CC	247	66.7	71.1	0.29	131	55.7	51.4	0.80	63	536.2	1.364E+03	0.36
(rs247862)	CT	170	74.8	77.5		95	54.3	51.2		43	1036.7	3.179E+03	
	TT	31	85.6	80.8		18	47.0	53.6		13	209.2	76.9	
c.1050T > A	TT	408	71.6	74.3	0.65	221	54.5	50.7	0.98	109	704.8	2.244E+03	0.83
(rs1047121)	TA	40	66.0	74.9		23	54.2	58.3		7	525.7	903	
	AA	0	-	-		0	-	-		-	-	-	

[a]Calculated from the translation start site; [b]Value were analyzed by ANOVA.

Table 4. The haplotype frequencies between RA or SLE patients and controls in *COTL1* polymorphisms.

Haplotype				Frequency[a]			P^b	
c.-1124G > T	c. 484G > A	c.588C > T	c.1050T > A	Control	RA	SLE	vs. RA	vs. SLE
G	G	C	T	0.422	0.428	0.431	0.793	0.768
G	G	T	T	0.199	0.217	0.251	0.330	0.038
G	A	C	T	0.186	0.177	0.168	0.589	0.428
T	G	C	T	0.054	0.059	0.032	0.597	0.102
G	G	C	A	0.050	0.029	0.025	0.021	0.044
T	G	T	T	0.026	0.038	0.024	0.121	0.014
T	A	C	T	0.027	0.034	0.054	0.383	0.867
Others				0.036	0.018	0.015	-	-

[a]Values were constructed by the expectation maximization (EM) algorithm with genotyped SNPs; [b]Values were analyzed by the permutation test.

prevalence 10 to 50 per 100,000 (Yamada *et al*., 2005). The pathogenesis of autoimmune diseases comes from epidemiological and genetic evidence for overlapping pathogenesis. The several susceptibility loci are present for multiple autoimmune diseases. The relationship might simply be explained by increased lymphocyte proliferation increasing somatic mutations. However a similarly elevated risk is not seen in other chronic immune responses, such as allergic disease or inflammatory bowel disease. Therefore, when genetic studies are conducted, it would be better to be investigated on several diseases together than only on single disease. The reason why we have selected RA and SLE is that RA is a representative Th1 dominant disease while SLE is a Th2 skewed disease. Therefore we can speculate certain SNPs are associated with Th1 diseases or Th2 diseases. Then further studies on the other diseases that belong to each group could be conducted. We have identified several SNPs which were associated with susceptibility to RA or SLE in *Tim-3*, *Tim-1*, *Eotaxin-3*, and *FOXJ1* genes through the case-control association study in Korean population (Chae *et al*., 2004, 2005a, b; Li *et al*., 2007).

In this study, we attempted a proteomic approach such as 2-DE and MALDI-TOF MS to find the candidate molecules for RA pathogenesis. We identified the *COTL1* as a RA candidate gene that is highly expressed in peripheral leukocytes of RA patients (Figure 2). According to recent studies, Spanbroek and co-workers reported 5LO is expressed in several types of leukocytes, including dendric cells (Spanbroek *et al*., 2000). LTC_4, LTD_4 and LTE_4 are called cysteinyl leukotrienes due to their structures. Their receptors ($CysLT_1$ and

$CysLT_2$) on mast cells can mediate proinflammatory activities such as endothelial cell adhearence and production of chemokine by mast cells, and other inflammatory disorders such as asthma, RA, inflammatory bowel disease (Bailey, 1985; Sharma *et al*., 2006). Rakonjac and coworkers reported that COTL1 up-regulates Ca^{2+}-induced 5LO activity as a scaffold of 5LO in the absence of phosphatidylcholine by *in vitro* assays (Rakonjac *et al*., 2006). Mathis and co-workers demonstrated LTB_4 receptors play a critical role in the recruitment of leukocytes to the inflammatory sites in mouse model of RA (Mathis *et al*., 2007). Thus, our results suggest that up-regulation of COTL1 may affect the 5LO activity involved in LT biosynthesis, and mediate inflammation in RA.

According to results of our case-control association study, the genotype frequency of c.-1124G > T and the allelic frequency of c.484G > A in RA patients, and the genotype frequency of c.484G > A in SLE patients were significantly different from healthy controls ($P = 0.009$, 0.027, and 0.025, respectively). These results suggest that the c.-1124G > T polymorphism of *COTL1* gene promoter region might be one of the important genetic factors in RA susceptibility. The polymorphisms within the binding site of the promoter region might have influence on the expression level by regulation of the binding between the specific transcriptional binding site and the transcription factor. Accordingly, these polymorphisms might have some influence on susceptibility to autoimmune responses. Our results might be important in future studies to determine whether or not the c.-1124G > T polymorphism affects the levels of *COTL1* gene expression. Our result also showed that the c.484G > A polymorphism of *COTL1* is significantly associated with the levels of anti-CCP antibody ($P = 0.03$) in RA patients (Table 3). This result suggests that the polymorphisms of the *COTL1* might be associated with anti-CCP antibody levels in RA patients.

In conclusion, our results demonstrate that c.-1124G > T in the promoter region and c. 484G > A polymorphism in the 3' UTR of *COTL1* gene might be associated with susceptibility to RA and SLE in Korean population.

Methods

Plasma sample preparation for 2-DE

Subjects were selected based on the criteria approved by the outpatient clinic at Eulji University Hospital. Characteristics of RA patient and healthy control subjects are shown in (Supplemental Data Table S1) Blood was collected in tubes with appropriate concentration of lithium heparin from both healthy controls and RA patients, and centrifuged at 2,500 rpm for 15 min. Plasma was separated into 250 μl aliquots and stored at -70°C. Depletion of high-abundance proteins from plasma was performed using a Multiple Affinity Removal Column (Agilent, Wilmington, DE). The 2-DE procedure was performed as described previously (Cho *et al*., 2005). Protein samples were suspended in sample buffer (7M urea, 2M thiourea, 4.5% Chaps, 100 mM DTE, 40 mM Tris, pH 8.8).

2-DE/ Image analysis/ Trypsin digestion

2-DE, image analysis (spot detection, quantification, and matching), and in-gel trypsin digestion were performed as described (Park *et al*., 2002a, b). 2-DE gels were scanned by a Bio-Rad GS-710 densitometer (Hercules, CA) and converted into electronic files, which were then analyzed with the ImageMaster 2D Platinum Software version 5.0 (Amersham Bioscience).

Identification of proteins by MS

For 2-D gel mapping of the plasma proteome, proteins were identified by mass fingerprinting or matching with various internal 2-DE maps. Protein spots excised from 2-D gels were destained, reduced, and alkylated and then digested with trypsin (Promega, Madison, WI) as previously described (Shevchenko *et al*., 1996). Tryptic peptides were desalted and purified as described (Cho *et al*., 2002). Recovered peptides were prepared for MALDI-TOF-MS by mixing with alpha-cyano-4-hydroxy cinnamic acid, 1% formic acid in 50% acetonitrile, and droplets were allowed to dry on the MALDI sample plate. Peptide mass fingerprinting was performed using a Voyager DE-PRO MALDI-TOF MS (Applied Biosystems, Foster City, CA), operating in delayed reflector mode. Proteins were identified from the peptide mass maps using MS-FIT (http://prospector.ucsf.edu), MASCOT (http://www.matrixscience.com/search_form_select.html), and ProFound (http://129.85.19.192/profound_bin/WebProFound.exe) to search for the protein databases, SWISS_PROT (Version 44.1) and GenBank.

Subjects and DNA samples

After institutional review board approval and informed consent, blood samples and records were obtained from 568 controls (213 females, 355 males) without RA and SLE, 455 RA patients (371 females, 84 males), and 196 SLE patients (184 females, 12 males). The mean age of controls, RA patients, and SLE patients was 40.3, 53.3, and 34 years, respectively. Genomic DNA was extracted from leukocytes in peripheral blood by a standard phenol-chloroform method or by using the Genomic DNA Extraction kit (iNtRON Biotechnology, Korea) according to the manufacturer's instructions. The RA and SLE patients were recruited from the outpatient clinic at Eulji University Hospital and Chonnam national University Hospital. RA and SLE were diagnosed according to criteria of the American Rheumatism Association and the American College of Rheumatology (ACR), respectively (Arnett *et al*.,

1988; Hochberg, 1997). Anti-CCP antibody and RF levels in RA patients and ANA levels in SLE patients were determined in a routine laboratory at Eulji University Hospital and Chonnam national University Hospital. The non-RA controls were recruited from the general population and had received comprehensive medical testing at Wonkwang University Hospital. All subjects in this study were Korean.

RNA extraction/ RT-PCR/ PCR

For total RNA, human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats by density gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences, Sweden), washed with PBS (GIBCO BRL, Invitrogen), and lysed in TRIzol reagent (Invitrogen) according to the instructions of the manufacture. Approximately 1 μg of total RNA was used in the first-strand cDNA synthesis with a sequence specific primer using the M-MLV reverse transcriptase (Bioneer, Korea) for RT-PCR. PCR was performed using hot start Taq DNA polymerase (HS Prime *Taq*, Genet Bio, Korea), and 0.5 μM of each primer (Supplemental Data Table S2) under the following condition: 10 min at 94°C for initial denaturation, 28 cycles of PCR consisting of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, extension for 30 s at 72°C, and a final extension for 5 min at 72°C in a thermocycler (Gene Amp PCR system 9700, PE Applied Biosystems). The expression assay for several tissues was performed using multiple human tissue cDNA (Clontech).

HRM analysis

The SNPs information of *COTL1* gene derived from the NCBI SNP database and KSNP database system (http://www.ksnp.ngri.go.kr). Genotyping was performed by high resolution melting (HRM) analysis for c.484G>A, c.588C>T, and c.1050T>A. The 10 μl reaction mixture was made up using the 1× QuantiTect Probe PCR Kit (Qiagen) and consisted of 50 ng of genomic DNA, 100 nM of each primer (Supplemental Data Table S2), and 1× Evagreen solution (Biotium). PCR cycling and HRM analysis was carried out using the Rotor-Gene thermal cycler RG6000 (Corbett Research, Australia). The 138 bp, 127 bp and 133 bp amplicons were produced according to the following conditions; one cycle at 95°C for 15 min; 45 cycles at 95°C for 15 s, 68°C for 10 s and 72°C for 30 s. Optical measurements in the green channel (excitation at 470 nm and detection at 519 nm) were recorded during the extension step. After a completion of 45 cycles, melting curve data were generated by increasing the temperature from 77°C to 95°C at 0.1°C per second and recording fluorescence. HRM curve analysis was performed using the software Rotor-Gene 1.7.40 and the HRM algorithm that was provided.

Taq-Man analysis

The Taq-Man assay probes for c.-1124G>T SNP were designed by Integrated DNA Technologies (IDT). The reaction was performed in 10 μl using 225 nm each primer, 50 nM each probe (Supplemental Data Table S2), and 1× QuantiTect Probe PCR Kit (Qiagen). PCR conditions were as follows: one cycle at 95°C for 15 min; 50 cycles at 95°C for 10 s and 60°C for 45 s. The PCR was performed in the Rotor-Gene thermal cycler RG6000 (Corbett Research, Australia). The samples were read and analyzed using the software Rotor-Gene 1.7.40 (Corbett Research, Australia). The reference sequence of COTL1 was based on human chromosome 16 clone RP11-61F12.

Statistical analysis

The healthy controls and RA or SLE patients were compared by case-control association analyses. χ^2 tests were used to estimate the Hardy-Weinberg equilibrium (HWE). The haplotype frequencies of *COTL1* for multiple loci were estimated using the expectation maximization (EM) algorithm with SNPAlyze software (DYNACOM, Japan). The ANOVA was applied to analyze differences among the each genotype, and anti-CCP antibody, RF levels in RA patients or ANA levels in SLE patients. A *P* value of less than 0.05 was considered to indicate statistical significance.

Supplemental Data

Supplemental Data include two tables and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-41-5-08.pdf.

Acknowledgments

This study was supported by the grants of the Korea Health 21 R&D Project (Ministry of health, welfare and family Affairs, Republic of Korea, A010251 and A030003).

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