Vitamin D-binding protein (group-specific component) has decreased expression in rheumatoid arthritis

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Abstract

Objective

This study used a proteomic approach to screen the proteins with decreased expression in the synovial tissues of rheumatoid arthritis (RA) patients by comparing their expression profiles to that of osteoarthritis (OA) and ankylosing spondylitis (AS) patients. The result was complemented by a SNP analysis.

Methods

Proteins extracted from the synovial membranes (n=10 for each disease) were separated by 2-D electrophoresis. The proteins with significantly decreased expression in the RA samples were subjected to MALDI-TOF/TOF MS. The result was verified using western blotting. Tag SNPs located in the targeted gene were assessed using the Taqman assay in a cohort of 267 Chinese patients with RA, 51 patients with AS and 160 healthy controls. The genotyping result was confirmed in a large cohort of 389 patients with RA, 200 patients with AS and 371 healthy controls

Results

The proteomic approach detected significantly decreased expression of vitamin D-binding protein (VDBP) in the synovial membranes from patients with RA, which was confirmed with western blot analysis. rs2282679 was significantly associated with RA and AS (p=0.026794 and 0.007566, respectively). The result was confirmed in a large cohort of RA (OR=0.678639, 95%CI=[0.541113~0.851118], p=0.000776) and AS (OR=0.564053, 95%CI=[0.433716~0.733558], p=1.79e–005).

Conclusion

1,25-dihydroxyvitamin D3 inhibits cell proliferation, immunoglobulin production and the release of cytokines through binding to VDBP. VDBP also mediates bone resorption by activating osteoclasts. The decreased expression and the genetic effect of VDBP in RA suggest a novel pathogenic pathway that vitamin D contributes to the arthritic process of the disease.

Key words

rheumatoid arthritis, vitamin D-binding protein (VDBP) / group-specific component (Gc), 1,25-dihydroxyvitamin D3, ankylosing spondylitis, SNP

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Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterised by chronic inflammation in the joints, ultimately leading to joint cartilage destruction and permanent disability. Recently, a series of reports compared the fingerprint profiles of both the diseased and healthy states using a proteomic approach and found increased expression in RA of some RA-specific proteins including S100A9/A8, serum amyloid A, galectin and ubiquitin-proteasome pathway components (1-9). Some studies also applied a proteomic method to investigate pathological mechanism of RA. Katano *et al*. reported that increased expression of S100 calcium binding protein A8 in GM-CSF-stimulated neutrophils leads to the increased expressions of IL-8 and IL-16 (10). Sekigawa *et al*. found some protein biomarkers in patients with RA receiving anti-tumor necrosis factor-alpha antibody therapy (11). However, most of these studies were performed using patients' peripheral blood, synovial fluid or cultured synovial cells. Few studies focused on the expression profile of the entire RA synovial tissue that contained many types of synoviocytes, despite the fact that most of these cell types contribute to the inflammatory joint disorders in RA. In addition, these studies generally screened proteins with increased expression but were unable to find candidate proteins with low expression in the diseased tissues.

Previously, we performed a proteomic analysis of RA synovial tissues and compared the protein expression in patients with RA with expression profiles from synovial membranes of patients with osteoarthritis (OA) and patients with ankylosing spondylitis (AS). In that study, we found that the Ig kappa light chain C region, PRDX4, SOD2, TPI and TXNDC5 have significantly increased expression in the synovial tissues of RA patients (12). Continuing this work, proteins with significantly decreased expression in the synovial tissues of RA patients were screened in the current study by comparing their expression profiles with that of OA and AS patients. Tag SNPs of the genes encoding candidate proteins in the RA population were also genotyped to determine their genetic effect on the disease.

Methods

Patients and sample collection

Synovial tissue samples were collected during joint replacement surgery from patients with RA (n=10, 7 females; 23–68 years old, mean 49 years old), OA (n=10, 6 females; 43-71 years old, mean 53 years old) and AS (n=10, 3 females, 14-48 years old, mean 35 years old). The AS tissues were collected from the hip joints of patients, and the RA and OA tissues were collected from the knee joints of patients. The diagnosis of RA fulfilled the American College of Rheumatology criteria. The patients with RA had disease durations of 3–10 years and were classified as having erosive RA (Larsen class IV-V). They had high levels of C-reactive protein (30–100 mg/litre, mean 24 mg/litre), anti-CCP (300–3,000 U/ml) and RF (160–2,560 U/ml). The patients with RA and AS took disease-modifying anti-rheumatic drugs (DMARDs) before surgery. The patients with RA, OA and AS were also treated with non-steroidal anti-inflammatory drugs (NSAIDs), which helped to reduce the pain and swelling of the joints and decrease stiffness. The synovial samples were dissected from connective tissues and immediately stored at -80°C until they were used.

Blood was collected from patients with RA (n=267, 183 female) and AS (n=51, 10 female). The patients with RA were, on average, 51.7 years old. The patients with AS were, on average, 33.9 years old. The diagnosis of RA and AS was made according to the previous description. The patients were selected from the same population living in the Shandong area of Northern China. A total of 160 healthy people (58 females) with a mean age of 48.0 years, were blood donors. None of them had any personal or family history of other serious illnesses. Control individuals from the same geographical area were frequency-matched to the expected age distribution of the cases. The blood samples were placed in Monovette tubes containing 3.8% sodium citrate.

Both patients and controls gave their written consent to participate in the

study and to allow their biological samples to be genetically analysed. The Ethics Committee of Shandong Academy of Medicinal Sciences approved the study.

2D electrophoresis analysis and protein identification

The samples of synovial tissue were homogenised in lysis buffer (Urea 7 M, Thiourea 2 M, CHAPS 4%, IPG buffer 2%, DTT 65 mM, PMSF 1 mM) along with Protease Inhibitor Cocktail (Sigma) on ice and then centrifuged at 14,000 x g for 30 min. The cell lysates of RA, OA and AS synovial tissues were pooled with equal protein contents, respectively. The 2D electrophoresis (2-DE) was performed as regular protocol and the procedure was described in our previous report (12).

After electrophoresis, proteins were visualised by staining with Coomassie Brilliant Blue R350 staining solution overnight. The 2-DE gels were scanned with a UMAX Powerlook 2100XL (UMAX Technologies, USA). Digitised images were analysed with ImageMaster 2D Platinum software 5.0 (GE Healthcare). The 2-DE gels loaded with samples from RA were compared to the gels run in parallel but loaded with samples from OA and AS. To compensate for non-expression-related variation in spot intensity, the volumes for each spot in a gel were normalised by the total volume of all spots, and spot intensities are expressed as the percentage of the total sum of spot volumes. Image analysis was performed by comparing the quantity of matched spots in the experimental gels *versus* control gels. Significant variation was defined as more than a three-fold change in spot density, and only those spots that had significantly low expression ($p<0.01$) were selected for further MS identification. The described experiment was repeated three times. The differentially expressed spots were excised manually from the stained gels, washed in MilliQ water, de-stained in 50 mM ammonium bicarbonate/50% acetonitrile (ACN) buffer, dehydrated in 100% ACN for 15 min and then completely dried by vacuum centrifugation. The dried pieces were suspended in trypsinisation buffer (10 ng/μl trypsin, 25 mM NH_4HCO_3, pH 8.0) for 30 min at 4°C and incubated overnight in 10 ml 25 mM NH_4HCO_3 at 37°C. Finally, peptides were extracted with 5% trifluoroacetic acid (TFA)/50% ACN (v/v) and the extraction was lyophilised and re-dissolved in 0.1% TFA/30% ACN (v/v). 0.5 ul of peptide samples were mixed with equal volume of 5 mg/ml α-cyano-4-hydroxycinamic acid matrix (α-CHCA) (Bruke, Sweden) in 0.1% TFA/50% ACN and spotted onto a standard 192-well plate (ABI). Six external standards (Mass standard kit for the 4700 proteomics analyser calibration mixture, ABI) were used to calibrate each spectrum to a mass accuracy within 50 ppm. MS spectra analysis was performed using ABI 4700 Proteomics Analyser MALDI-TOF/TOF mass spectrometer (ABI, USA) in a batch-mode acquisition method. The spectra data was analysed with the international protein index human database v3.10 (57,478 sequences; 25,254,519 residues) using GPS explorer TM software version 3.0 and MASCOT 2.0 software (Matrix Science, UK).

Western blot analysis

Western blotting was performed with synovial tissues of the RA (n=8), OA (n=8) and AS (n=9) samples that were completely independent from the samples used for 2-DE. Total protein was prepared as above description. Ten micrograms of total protein was loaded and separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), trans-blotted onto nylon membranes and probed with the rabbit monoclonal antibody against Vitamin D-binding Protein. The antibody was commercially obtained from Abcam (USA) and was diluted 4000 times in 5% nonfat dry skim milk in TBST (Tris base 0.02M, NaCl 0.137M in distilled water, pH 7.6, containing 0.1% Tween 20). Immunoreactive signals were detected with alkaline phosphatase-conjugated secondary antibodies and visualised using a Western Blotting Luminol Reagent (Amersham). Images of the western blots were acquired on a Typhoon Trio (GE Healthcare, USA). Another membrane prepared by the same protocol was probed with an anti-GADPH antibody (Santa Cruz, USA) to normalise sample loading.

Genomic DNA extraction

Genomic DNA was extracted from peripheral blood leukocytes with a routine salting out method using the DNA Blood Mini Kit from Qiagen (Germany) according to the manufacturer's guidelines. Briefly, 5 ml of blood was mixed with triton lysis buffer (0.32 M sucrose, 1% Triton X-100, 5 mM $MgCl_2$, H_2O, 10 mM Tris-HCl, pH 7.5). Leukocytes were spun down and washed with H_2O. The pellet was incubated with proteinase K at 56°C and subsequently salted out at 4°C using a substrate NaCl solution. Precipitated proteins were removed by centrifugation. The DNA in the supernatant fluid was precipitated with ethanol. The DNA pellet was dissolved in 400 μl H_2O.

SNPs selection and genotyping

Tag SNPs (rs2282679, rs1491710, rs705119, rs705120, rs4364228, rs10488854, rs16846994, rs222023, rs16847015, rs1155563 and rs2298849) were selected from the HapMap data (www.hapmap.org) with a pairwise r^2 ≥0.8. Minor allele frequencies (MAF) of these SNPs were over 0.05. These tag SNPs were located in the introns of the VDBP gene.

Genotyping was performed using the TaqMan SNP genotyping assays. The assays were run in a LightCyclerH 480 Instrument (Roche) and evaluated according to the manufacturer's instructions. Allele-specific probes were labeled with the fluorescent dyes VIC and FAM, respectively. The PCR reaction was carried out in a total reaction volume of 10 ul using the following amplification protocol: denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 92°C for 15 seconds and finishing with annealing and extension at 60°C for 1 minute. The genotype of each sample was determined by measuring allele-specific fluorescence using the SDS 2.3 software for allelic discrimination (Roche). Duplicate samples and negative controls were included to confirm the accuracy of genotyping.

To confirm the above genotyping result, two tag SNPs, rs2282679 and

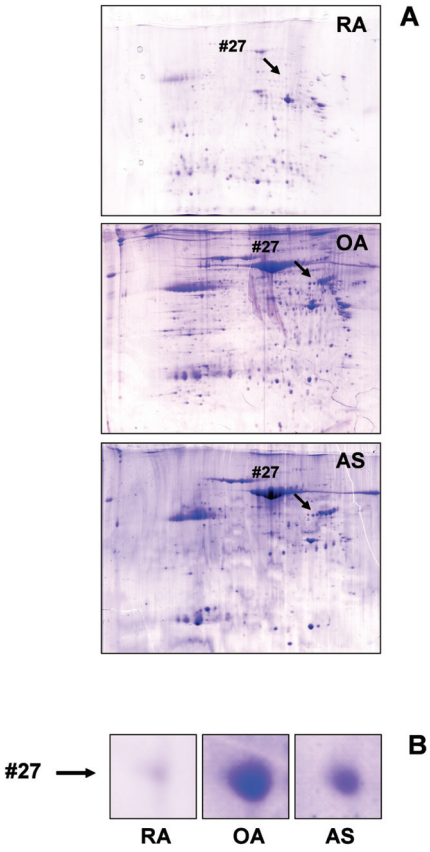

Fig. 1. 2-DE of the total proteins extracted from the synovial tissues of RA, OA and AS subjects. (**A**) The protein was visualised by staining with colloidal Coomassie Blue. The protein spot (n. 27) indicated by the arrow was identified by MALDI-TOF MS. (**B**) Comparison of an RA, OA and AS synovial protein (n. 27). The protein spot n. 27 from RA synovium, which had at least 3-fold lower expression levels than the matched spots of OA and AS synovium ($p<0.05$), was identified by MALDI-TOF MS.

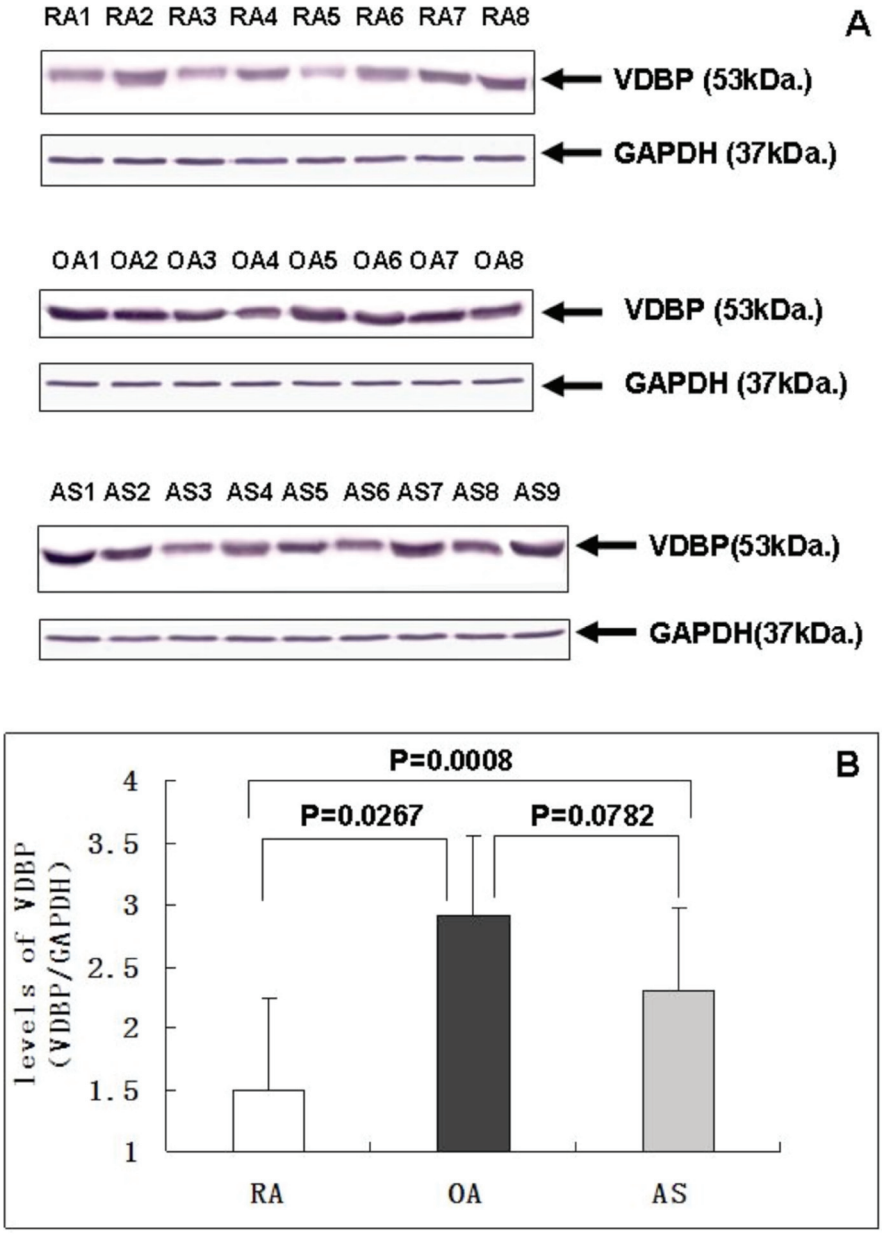

Fig. 2. Western blot analysis of the proteins with VDBP in the RA synovial membranes. (**A**) The total proteins from the synovial tissues of RA, OA and AS patients were fractioned by SDS-PAGE and probed with the monoclonal antibody against VDBP. The loading of samples was normalised using GADPH. (**B**) The VDBP expression was semi-quantitatively analysed by normalising the signal density of VDBP to that of GADPH. VDBP had significantly lower levels of expression in the synovial membranes of RA patients than in the samples from OA and AS patients.

rs4364228 were genotyped using TaqMan SNP genotyping assays in a cohort of 389 patients with RA (336 female), 200 patients (44 female) with AS and 371 healthy controls (122 female). RA patients had a mean age of 53.9 years, AS patients had a mean age of 28.8 years and the health had a mean age of 40.3 years. They were from the Shandong area of Northern China. Assays were conducted based the above protocol. These patients and health controls were completely independent from the patients and the health in the above genotyping study.

Statistical analysis

SNPs were analysed for association by comparison of the MAF in cases and controls. Associations of tag SNPs with RA and AS were evaluated using odds ratios (OR) with 95% confidence intervals (CI). Fisher's exact test was used for comparison between categorical variables. *P*-values less than 0.05 were considered statistically significant. After genotyping, SNP markers were evaluated for significant deviation from the Hardy-Weinberg equilibrium. The calculation was performed using SHEsis (http://analysis.bio-x.cn/myAnalysis.php) and haploview (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview), two powerful web-based platforms for analyses of linkage disequilibrium, haplotype construction and genetic association at polymorphism loci (13, 14).

Results

Expression of VDBP in synovial tissues

In this study, a comparative 2-DE was used to differentiate protein expression

levels in synovial membranes between RA and OA patients, and between RA and AS patients on a global level. The 2-DE gel patterns of the experiment were highly reproducible when repeated three times. Following computational analysis, approximately 300 spots were visualised on each 2-DE gel. The 2-DE gel prepared with synovial samples is shown in Figure 1A. Compared to the gels loaded with OA and AS samples, those spots from RA samples which had a three-fold or lower expression level were selected for mass spectrometric identification. Among the spots, one spot (n. 27) showed a 3-fold lower expression level in the RA sample than in the OA and AS controls (Fig. 1B). Using MALDI-TOF MS, this spot was identified as vitamin D-binding protein (VDBP). The MS together with MS/MS spectra were analysed MASCOT 2.0 software. Detailed information about this identified protein is shown as followings: molecular weight 54 kDa., isoelectric point 5.4, peptide count 17, protein score 216, intensity matched 74%, and protein species score (C.I.%) 100.
Western blot analysis was performed with a monoclonal antibody against VDBP. A band in molecular weight of 54 kDa. was detected in the total protein extracted from synovial tissues of RA, OA and AS. With GADPH (molecular weight of 37 kDa.) as a reference, VDBP had significantly lower levels of expression in the synovial membranes of RA patients than in the samples from OA patients (p=0.0008). VDBP also had significantly lower levels of expression in the synovial membranes of RA patients than in the samples from AS patients (p=0.0267). The result was shown in Figure 2.

Genotyping of tag SNPs located in VDBP

A case-control study was conducted using the TaqMan method to genotype the tag SNPs flanking the gene encoding VDBP. A total of 11 SNPs mapping to the VDBP gene were selected for genotyping. The allelic frequencies of these tag SNPs were compared between RA and control patients and between AS and control patients. The allelic frequency for the SNP at

Table I. Allele and genotype frequencies in a case control cohort of patients with RA and AS.

dbSNP identity	Allele /Genot.	No. of patients with RA (%)	No. of controls (%)	Fisher's p-value	Odds ratio (%95 CI)
rs2282679for RA	A	460 (0.983)	284 (1.000)	0.0268	
	C	8 (0.017)	0 (0.000)		
	AA	228 (0.974)	142 (1.000)	0.1573	
	AC	4 (0.017)	0 (0.000)		
	CC	2 (0.009)	0 (0.000)		
rs2282679 for AS	A	78 (0.975)	284 (1.000)	0.0076	
	C	2 (0.025)	0 (0.000)		
	AA	38 (0.950)	142 (1.000)	0.0074	
	AC	2 (0.050)	0 (0.000)		
rs1491710 forRA	A	343 (0.645)	220 (0.688)	0.2017	0.824916 (0.613782~1.108677)
	C	189 (0.355)	100 (0.312)		
	AA	111 (0.417)	76 (0.475)	0.4454	
	AC	121 (0.455)	68 (0.425)		
	CC	34 (0.128)	16 (0.100)		
rs1491710 for AS	A	73 (0.716)	220 (0.688)	0.5906	1.144201 (0.700345~1.869357)
	C	29 (0.284)	100 (0.312)		
	AA	27 (0.529)	76 (0.475)	0.7811	
	AC	19 (0.373)	68 (0.425)		
	CC	5 (0.098)	16 (0.100)		
rs705119 for RA	A	387 (0.736)	220 (0.692)	0.169	1.240222 (0.912405~1.685821)
	C	139 (0.264)	98 (0.308)		
	AA	141 (0.536)	76 (0.478)	0.3681	
	AC	105 (0.399)	68 (0.428)		
	CC	17 (0.065)	15 (0.094)		
rs705119 for AS	A	75 (0.735)	220 (0.692)	0.4035	1.237374 (0.750382~2.040420)
	C	27 (0.265)	98 (0.308)		
	AA	28 (0.549)	76 (0.478)	0.6755	
	AC	19 (0.373)	68 (0.428)		
	CC	4 (0.078)	15 (0.094)		
rs705120 for RA	A	253 (0.477)	148 (0.465)	0.7358	1.049127 (0.794039~1.386162)
	C	277 (0.523)	170 (0.535)		
	AA	60 (0.226)	35 (0.220)	0.926	
	AC	133 (0.502)	78 (0.491)		
	CC	72 (0.272)	46 (0.289)		
rs705120 for AS	A	52 (0.510)	148 (0.465)	0.4347	1.194595 (0.764462~1.866745)
	C	50 (0.490)	170 (0.535)		
	AA	15 (0.294)	35 (0.220)	0.5484	
	AC	22 (0.431)	78 (0.491)		
	CC	14 (0.275)	46 (0.289)		
rs4364228for RA	A	246 (0.535)	159 (0.556)	0.5727	0.918180 (0.682514~1.235219)
	G	214 (0.465)	127 (0.444)		
	AA	60 (0.261)	37 (0.259)	0.5119	
	AG	126 (0.548)	85 (0.594)		
	GG	44 (0.191)	21 (0.147)		
rs4364228 for AS	A	29 (0.392)	159 (0.556)	0.0118	0.514745 (0.305484~0.867352)
	G	45 (0.608)	127 (0.444)		
	AA	0 (0.000)	37 (0.259)	0.0024	
	AG	29 (0.784)	85 (0.594)		
	GG	8 (0.216)	21 (0.147)		
rs10488854forRA	A	101 (0.191)	56 (0.175)	0.5539	1.115089 (0.777341~1.599585)
	G	427 (0.809)	264 (0.825)		
	AA	11 (0.042)	7 (0.044)	0.7191	
	AG	79 (0.299)	42 (0.263)		
	GG	174 (0.659)	111 (0.694)		
rs10488854 for AS	A	14 (0.137)	56 (0.175)	0.3722	0.750000 (0.398085~1.413016)
	G	88 (0.863)	264 (0.825)		
	AA	2 (0.039)	7 (0.044)	0.6112	
	AG	10 (0.196)	42 (0.263)		
	GG	39 (0.765)	111 (0.694)		

Table I continues on next page

dbSNP identity	Allele /Genot.	No. of patients with RA (%)	No. of controls (%)	Fisher's *p*-value	Odds ratio (%95 CI)
rs16846994for RA	A	83 (0.156)	51 (0.159)	0.8962	0.975021 (0.666738~1.425846)
	G	449 (0.844)	269 (0.841)		
	AA	8 (0.030)	9 (0.056)	0.2654	
	AG	67 (0.252)	33 (0.206)		
	GG	191 (0.718)	118 (0.738)		
rs16846994 for AS	A	16 (0.157)	51 (0.159)	0.9518	0.981304 (0.532200~1.809391)
	G	86 (0.843)	269 (0.841)		
	AA	1 (0.020)	9 (0.056)	0.3758	
	AG	14 (0.275)	33 (0.206)		
	GG	36 (0.706)	118 (0.738)		
rs222023 for RA	A	179 (0.374)	94 (0.313)	0.082	1.311962 (0.965811~1.782175)
	G	299 (0.626)	206 (0.687)		
	AA	33 (0.138)	19 (0.127)	0.0902	
	AG	113 (0.473)	56 (0.373)		
	GG	93 (0.389)	75 (0.500)		
rs222023 for AS	A	28 (0.318)	94 (0.313)	0.9314	1.022695 (0.613737~1.704159)
	G	60 (0.682)	206 (0.687)		
	AA	6 (0.136)	19 (0.127)	0.0334	
	AG	16 (0.364)	56 (0.373)		
	GG	22 (0.500)	75 (0.500)		
rs16847015for RA	A	127 (0.238)	68 (0.212)	0.3934	1.156381 (0.828192~1.614622)
	C	407 (0.762)	252 (0.787)		
	AA	15 (0.056)	9 (0.056)	0.5546	
	AC	97 (0.363)	50 (0.312)		
	CC	155 (0.581)	101 (0.631)		
rs16847015 for AS	A	19 (0.186)	68 (0.212)	0.5686	0.848334 (0.481739~1.493904)
	C	83 (0.814)	252 (0.787)		
	AA	2 (0.039)	9 (0.056)	0.8464	
	AC	15 (0.294)	50 (0.312)		
	CC	34 (0.667)	101 (0.631)		
rs1155563 for RA	A	328 (0.617)	193 (0.603)	0.6972	1.058011 (0.796378~1.405598)
	G	204 (0.383)	127 (0.397)		
	AA	98 (0.368)	57 (0.356)	0.9074	
	AG	132 (0.496)	79 (0.494)		
	GG	36 (0.135)	24 (0.150)		
rs1155563 for AS	A	61 (0.598)	193 (0.603)	0.9272	0.979022 (0.621273~1.542774)
	G	41 (0.402)	127 (0.397)		
	AA	20 (0.392)	57 (0.356)	0.5503	
	AG	21 (0.412)	79 (0.494)		
	GG	10 (0.196)	24 (0.150)		
rs2298849 for RA	A	336 (0.651)	217 (0.678)	0.4233	0.886021 (0.658834~1.191551)
	G	180 (0.349)	103 (0.322)		
	AA	108 (0.419)	73 (0.456)	0.7191	
	AG	120 (0.465)	71 (0.444)		
	GG	30 (0.116)	16 (0.100)		
rs2298849 for AS	A	69 (0.676)	217 (0.678)	0.9752	0.992459 (0.616247~1.598344)
	G	33 (0.324)	103 (0.322)		
	AA	24 (0.471)	73 (0.456)	0.8943	
	AG	21 (0.412)	71 (0.444)		
	GG	6 (0.118)	16 (0.100)		

rs2282679 was significantly associated with RA (p=0.026794). The allelic frequency of the SNP deviated from the Hardy-Weinberg equilibrium in the RA group (p<0.05), although the p-valve was 1 for the Hardy-Weinberg equilibrium test in the controls. Each allelic frequency for the other tag SNP in the RA group and the healthy groups was nearly equal and no association was seen when the groups were compared independently. On the other hand, the tag SNPs rs2282679 and rs4364228, located in VDBP, showed statistically significant differences in allelic frequencies between the AS and controls (p=0.007566 and 0.011827, respectively). The C allele frequency at rs2282679 is 0.025 in the AS group, and was not found in the control group. Although the G allele frequency of the rs4364228 SNP was significantly higher in the AS group (0.608) than in the control group (0.444), its allelic frequency deviated from the Hardy-Weinberg equilibrium in both the AS group and the controls (p<0.05); thus, it was not considered further. Other tag SNPs of VDBP gene polymorphisms did not display significant differences in allelic frequencies between the AS and control patients. Table I show frequency distributions of SNP alleles and genotypes.

To confirm the above genotyping result, two tag SNPs, rs2282679 and rs4364228 were genotyped using TaqMan SNP genotyping assays in a cohort of 389 patients with RA, 200 patients with AS and 371 healthy controls. rs2282679 had significant differences in the allele frequency (Odds ratio=0.678639, 95% CI=[0.541113~0.851118], p=0.000776) and genotype frequency (p=0.000354) between RA cases and controls, indicating that the SNP was significantly associated with the decreased risk of RA. In AS cohort, rs2282679 also had significant differences in allele frequency (Odds ratio=0.564053, 95% CI=[0.433716~0.733558], p=1.79e-005) and genotype frequency (p=7.54e-006) between cases and controls, indicating that this SNP was significantly associated with the decreased risk of AS. Although allelic frequencies of rs2282679 and rs4364228 were in the Hardy-Weinberg equilibrium in the large of controls (p=0.928914 and 0.293146, respectively), genotyping results demonstrated that rs4364228 was not associated with RA (p=0.581697) and AS (p=0.210471). In the large cohort, 33.5% of RA patients and 37.8% of AS patients have the allele/genotype C against 66.5% of RA patients and 62.3% of AS patients with the allele/genotype A, respectively, while 25.5% of the controls have the allele/genotype C. The result was shown in Table II. The result suggests that the patients with allele C were more susceptible to develop RA than the others.

To evaluate the extent of linkage disequilibrium (LD), D´ was calculated between all possible pairs of polymor-

Table II. Allele and genotype frequencies of rs2282679 and rs4364228 in a case control cohort of patients with RA and AS.

dbSNP identity	Allele/ Genot.	no. of patients with RA (%)	no. of controls (%)	Fisher's *p*-value	Odds ratio (%95 CI)
rs2282679 for RA	A	496 (0.665)	535 (0.745)	0.000776	0.678639
	C	250 (0.335)	183 (0.255)		(0.541113~0.851118)
	AA	152 (0.408)	199 (0.554)	0.000354	
	AC	192 (0.515)	137 (0.382)		
	CC	29 (0.078)	23 (0.064)		
rs2282679 for AS	A	249 (0.623)	535 (0.745)	1.79E-05	0.564053
	C	151 (0.378)	183 (0.255)		(0.433716~0.733558)
	AA	68 (0.340)	199 (0.554)	7.54E-06	
	AC	113 (0.565)	137 (0.382)		
	CC	19 (0.095)	23 (0.064)		
rs4364228 for RA	A	699 (0.925)	673 (0.917)	0.581697	1.111519
	G	57 (0.075)	61 (0.083)		(0.762967~1.619303)
	AA	322 (0.852)	307 (0.837)		
	AG	55 (0.146)	59 (0.161)		
	GG	1 (0.003)	1 (0.003)		
rs4364228 for AS	A	375 (0.938)	673 (0.917)	0.210471	1.359584
	G	2 (0.062)	61 (0.083)		(0.839372~2.202203)
	AA	175 (0.875)	307 (0.837)		
	AG	25 (0.125)	59 (0.161)		
	GG	0 (0.000)	1 (0.003)		

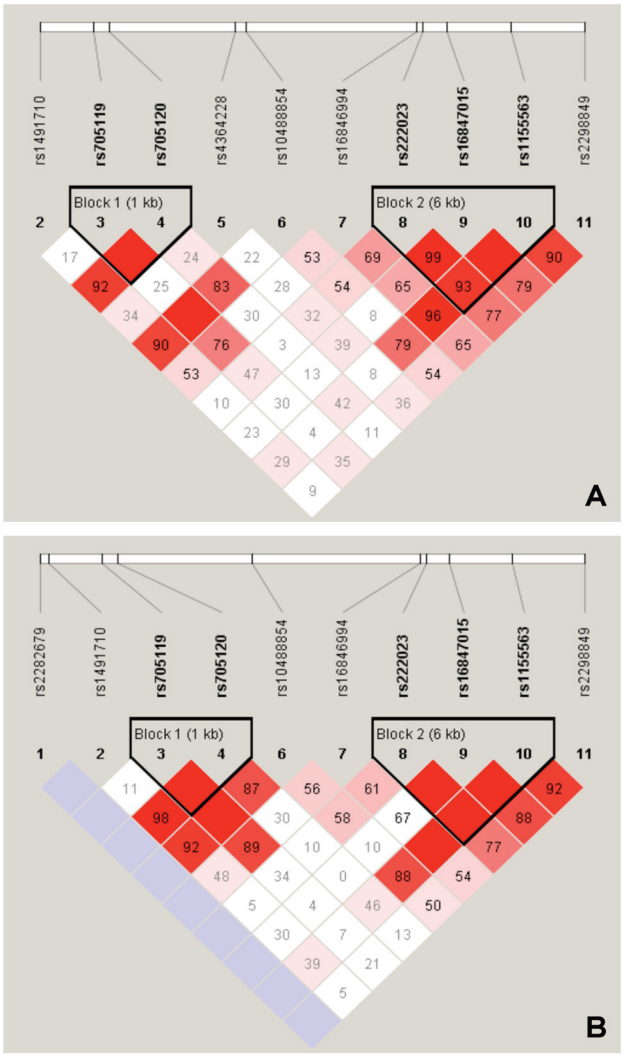

Fig. 3. Linkage disequilibrium of the genotyped SNPs in the VDBP region. (**A**) LD plot of the RA group. (**B**) LD plot of the AS group. Red areas represent higher levels of LD. Dark triangles represent haplotype blocks.

phisms. Two LD bocks were observed in the RA VDBP region. SNP rs705119 was strongly linked to rs705120. The other three nearby SNPs (rs222023, rs16847015 and rs1155563) were strongly linked with each other, and their D´ ranged from to 0.99 and 0.908. The LD distribution of genotyped SNPs in the AS cohort was similar to that in the RA group. The first block consisted of rs705119 and rs705120. The pairwise D´ values ranged from 0.99 to 0.902 among rs222023, rs16847015 and rs1155563 rs2298849, indicating that the four SNPs were strongly associated with each other. The LD distribution is shown in Figure 3.

Haplotype analysis defined six common haplotypes in the VDBP gene in the RA and AS groups. There was no significant association between these haplotypes and the risk of developing RA or AS.

Discussion

Compared with samples from patients with OA and AS, RA patients' synovial tissues had low VDBP expression using the proteomic method. This decreased expression was confirmed with western blot analysis. Fairney *et al*. had detected VDBP in synovial fluid, in which VDBP and 25-OHD, a metabolite of vitamin D, maintained a serum-to-synovial fluid ratio of approximately 2:1 irrespective of the type of joint disease (15). Their research supports our detection of VDBP in the synovial tissues of patients with RA, OA and AS. VDBP, also called group-specific component (Gc), is a multifunctional glycoprotein with a molecular mass of 51-58 kDa. VDBP is a highly polymorphic serum protein with three common alleles (Gc1F, Gc1S and Gc2) and more than 120 rare variants (16). In the present study, Western blotting was conducted using a monoclonal antibody that was raised with a synthetic peptide corresponding to residues within human VDBP. It was difficult to determine the subtypes of VDBP in synovial tissues. Papiha *et al*. reported that there were 11% more individuals with the Gc2 allele in the RA patient group when compared to the control group (17).

The main function of VDBP is to bind and transport vitamin D to target tissues

in order to maintain calcium homeostasis through the vitamin D endocrine system. Recent studies have demonstrated that vitamin D_3 may have an immunoregulatory role. Through binding to specific receptors, 1,25-dihydroxyvitamin D3 (1,25-$(OH)_2$ D_3) inhibits cell proliferation, immunoglobulin production and the release of cytokines. At low levels, $1,25(OH)_2D_3$ is an endogenous immunomodulator suppressing activated T cells. Notably, cell proliferation may accelerate the arthritic process in RA (18). An increased intake of vitamin D has been associated with a lower risk of developing RA, and a significant clinical improvement was strongly correlated with the immunomodulating potential in vitamin D-treated RA patients (19). VDBP can also be converted to DBP-macrophage activating factor (DBP-MAF), which mediates bone resorption by directly activating osteoclasts (20). Bone loss, one of the characteristics of RA, is strongly associated with persistently high disease activity (21). Oelzner *et al*. investigated the relationship between disease activity and serum levels of vitamin D metabolites in RA patients. They found that high disease activity in RA was associated with an alteration in the vitamin D metabolism and increased bone resorption. The decrease in $1,25(OH)_2D_3$ levels in these patients may contribute to a negative calcium balance and inhibit bone formation (18). The present study detected low expression of VDBP in the synovial tissues of RA patients. This result corresponds to those from other reports, and suggest that the decrease in 1, $25(OH)_2D_3$ levels, VDBP or both may contribute to a negative calcium balance, inhibition of bone formation and interrupted immunomodulation that can accelerate the arthritic process in RA.

There are two known SNPs located in exon 11 (rs7041 and rs4588) that give rise to three different VDBP isoforms denominated Gc1S (most frequent in Caucasian populations), Gc1F (more frequent in African populations) and Gc2 (16, 22). Fang *et al*. genotyped two SNPs (rs7041 = Glu416Asp and rs4588 = Thr420Lys) in 6,181 elderly Caucasians. They found that the VDBP genotype was not significantly associated with osteoporosis, a bone disease leading to an increased fracture risk, in the entire study population. However, they observed the interaction between VDBP and the vitamin D receptor (VDR) haplotypes in determining fracture risk, suggesting that the genetic effect of the VDBP gene on fracture risk appears only in combination with other genetic risk factors that impair bone metabolism (23). Kahl *et al*. examined VDBP phenotypes and gene frequencies in a homogenous group of patients with RA. Although a slightly higher frequency of the Gc2 allele and a lower frequency of the Gc1S allele were observed in RA and OA patients compared to controls, they did not detect a significant association between the subtypes or deficient alleles and any of the clinical variables observed in RA. However, peri-articular bony erosions and antinuclear antibody were positively and negatively associated with the Gc 1S-2 phenotype, respectively (24). In the present study, we genotyped 11 tag SNPs using a case-control method. The results demonstrated that the tag SNP rs2282679 located in VDBP is significantly associated with RA and AS, and suggest that there is a genetic effect of the VDBP gene on the risk of developing RA and AS. Recently, Wang *et al*. undertook a genome-wide association study of 25-hydroxyvitamin D concentrations in 33,996 individuals of European descent from 15 cohorts. Their study demonstrated that rs2282679 at VDBP had a very strong association with vitamin D insufficiency (25). Ahn et al. also conducted a genome-wide association study to screen genes related to circulating vitamin D levels. With 4501 persons of European ancestry drawn from five cohorts, they found that SNP rs2282679 in VDBP was associated with 25(OH)D concentrations and was in linkage disequilibrium with rs7041, a non-synonymous SNP, and rs1155563 (26).

In summary, the present study detected a significantly decreased expression of VDBP in the synovial tissues of patients with RA. This decreased expression of VDBP may lead to a negative calcium balance, inhibited bone formation and interrupted immunomodulation to accelerate the arthritic process in RA. The study also demonstrated that the gene encoding VDBP renders affected patients susceptible to RA and AS.

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