Follistatin-related protein gene (*FRP*) is expressed in the synovial tissues of rheumatoid arthritis, but its polymorphisms are not associated with genetic susceptibility

Y. Ehara[1], D. Sakurai[1], N. Tsuchiya[1], K. Nakano[2], Y. Tanaka[2], A. Yamaguchi[3], K. Tokunaga[1]

[1]Department of Human Genetics and [3]Department of Allergy and Rheumatology, Graduate School of Medicine, The University of Tokyo, Tokyo; [2]The First Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan.

Abstract

Objective

To examine the expression level and function of follistatin-related protein gene (FRP, also referred to as FSTL1) in rheumatoid arthritis (RA), and possible association of its polymorphisms with genetic susceptibility to RA.

Methods

FRP mRNAexpression levels in the synovial tissues from 10 patients with RA and 5 patients with OA were measured using real-time RT-PCR. Effects on the growth of synovial cells were evaluated by stably introducing FRP cDNA into a rheumatoid synovial cell line, E11. Screening of genomic DNA variations was done using DNA from 12 patients with RA and 12 healthy individuals by direct sequencing. Genotypes at the detected polymorphic sites were determined in 224 patients with RA and 220 healthy individuals using PCR-single strand conformation polymorphism.

Results

FRPmRNA was overexpressed in synovial tissues from RA patients by 2.3-fold as compared with those from OA. A rheumatoid synovial cell line (E11) transfected with FRP exhibited reduced proliferation, probably mediated by secreted FRP molecule. 16 genomic variations were identified, among which 4 were polymorphisms within the promoter region and exons, and the remainder were either rare variations or intronic polymorphisms. Genotyping of 4 polymorphic sites did not reveal statistically significant association with the susceptibility to RA.

Conclusion

FRP mRNA is overexpressed in RA synovium, the product of which exerts inhibitory activity on synovial cell growth. Although new polymorphic sites were identified, they were not associated with susceptibility to RA, suggesting that overexpression of FRP is secondarily caused by synovial environment of RA.

Key words

Rheumatoid arthritis, synovium, gene expression, polymorphism, genetics.

Yukikazu Ehara, BSc; Daisuke Sakurai, PhD; Naoyuki Tsuchiya, MD, PhD; Kazuhisa Nakano, MD; Yoshiya Tanaka, MD, PhD; Akihiro Yamaguchi, MD; Katsushi Tokunaga, PhD.

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Address correspondence and reprint requests to: Dr. Naoyuki Tsuchiya, Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan 113-0033. E-mail: tsuchiya-tky@umin.ac.jp

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Introduction

Rheumatoid arthritis (RA) is characterized by abnormal proliferation of synovium and chronic inflammation in joints throughout the body that eventually result in bone and cartilage resorption. It is thought that multiple genetic factors take part in the onset of RA; however, disease-susceptibility genes other than HLA-DRB1 have not been established (1, 2).

One of the efficient approaches to find genes relevant to the pathogenesis or etiology of RA is the use of comprehensive mRNA expression profiling. Genes significantly upregulated or downregulated in the synovial tissues of RA are likely to have a functional role, and polymorphisms in the coding or regulatory sequences of such genes might be associated with susceptibility to RA. We recently analyzed mRNA expression profiles using differential display, and found some genes substantially upregulated in a synovial tissue from a patient with RA compared with that from a patient with osteoarthritis (OA), one of which was a gene coding for follistatin-related protein (*FRP*, also referred to as follistatin-like 1 [*FSTL1*]) (3).

FRP is a putative secretory protein with a molecular weight of 50-55kDa, composed of 308 amino acids. *FRP* gene was originally described as TGF-β-stimulated clone (TSC)-36, which was induced when transforming growth factor (TGF)-β1 was added to mouse osteoblast cell line MC3T3-E1 (4). It is known that the product of mouse TSC-36 gene has high homology (>92%) with the amino acid sequence of human FRP; therefore human *FRP* is likely to be a homolog of mouse TSC-36 in terms of function (5). In addition, it is reported that the amino acid sequence of TSC-36 is similar to that of follistatin, and that transcription factor AP (activator protein)-1 contributes to the expression of *FRP* gene (6).

It was recently reported that 44% of patients with RA possess autoantibody to FRP (7). Subsequently, it was demonstrated that FRP is induced by TGF-β in fibroblast-like synoviocytes, and that FRP downregulates production of matrix metalloproteinase (MMP)-1, MMP-3 and prostaglandin E_2 (PGE_2) in the synovial cell lines (8). Based on these observations, it has been proposed that autoantibody to FRP blocks such potentially protective activity of FRPagainst joint destruction (8).

Interestingly, the chromosomal position of *FRP* gene (3q13.33) coincides with one of the candidate loci reported from genome-wide linkage studies (9, 10), suggesting that polymorphism of *FRP* might be associated with susceptibility to RA.

In the present study, we made an attempt to examine the mRNA expression level of *FRP* in multiple patients with RAand osteoarthritis (OA), and to gain insight into its functional role by introducing *FRP* gene into a synovial cell line. We also screened for polymorphisms of human *FRP* gene, and examined whether they are associated with the susceptibility to RA.

Materials and methods

Patients and healthy individuals

Synovial tissues were obtained from 10 patients with RA as well as from 5 patients with OA, undergoing total knee replacement surgery for a therapeutic purpose. The patients are among those whose clinical characteristics were previously described (3).

Genomic DNA was obtained from 224 patients with RA(male 28, female 196, 45.2 ± 13.5 years) and 220 healthy individuals (male 131, female 89, 33.3 ± 9.6 years). RA was diagnosed according to the American College of Rheumatology criteria (11). All individuals were genetically unrelated Japanese living in Tokyo area. This study was reviewed and approved by the Research Ethics Committee of the Graduate School of Medicine, the University of Tokyo.

Transfection and proliferation assay

A rheumatoid synovial cell line, E11 (12,13), was maintained in DMEM medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), antibiotic/antimycotic solution (Sigma-Aldrich) and 2 mM L-glutamine. Full length cDNAof *FRP* was prepared by RT-PCR using primers 5'-ACCAGACCACGATGTG-

GAA-3' (forward) and 5'-GATCTC-TTTGGTGCTCACTCTC-3' (reverse), and was stably introduced to E11 using pcDNA 3.1/V5-His vector (Invitrogen, Carlsbad, CA), and Effectene Transfection Reagent (Qiagen).

The efficiency of transfection to E11 cells was estimated as follows. 0.5 x 10^6 of E11 were seeded 24 h before pSV-β-galactosidase Control Vector (Promega, Madison, WI) was introduced. After 24 h, the cells were stained with X-Gal Staining Assay Kit (Gene Therapy Systems, San Diego, USA). The transfection efficiency was estimated to be 38.6 ± 2.6% from three independent experiments.

Proliferation of E11-mock and E11-*FRP* transfectants were assayed using WST-1 cell proliferation assay (Takara Bio, Otsu, Japan), in accordance with the manufacturer's instructions.

The inhibitory activity of the culture supernatant of E11-*FRP* transfectants was examined by transfer experiments. Culture supernatant from either E11-mock or E11-*FRP* transfectants was collected after culture for 24 h, and E11 cells were cultured in each of the supernatants. Proliferation after 24 h and 48 h was measured using WST-1 assay.

RNA preparation

Synovial tissues were obtained from surgically removed knee joints, frozen with liquid nitrogen, and kept at –80°C until use. Frozen tissues were crashed and subjected to total RNA extraction using TRIZOL reagent (Life Technologies, Rockville, MD). RNA was prepared from the cultured cells using RNeasy (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Real-time RT-PCR

To compare mRNA levels of *FRP* between RA (n = 10) and OA synovia (n = 5), and between E11-*FRP* transfectant and mock transfectant, real time RT-PCR was performed with LightCycler (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The expression of *FRP* mRNA was standardized using that of β-actin mRNA. Primer sets used are the following: *FRP*: 5'-CATTCCA-GATGGCTGGTTCT-3' (forward), 5'-TGCATACGTTTCATCCTCCA-3' (reverse); β-actin: 5'-TCCTGTG-GCATCCACGAAACT-3' (forward), 5'-GAAGCATTTGCGGTGGACGAT-3'(reverse).

ELISA

TGF-β concentration in the culture supernatant was measured by ELISA using Quantikine Human TGF-β1 Immunoassay (R&D, Minneapolis, MN) at 24 h and 72 h after 4 x 10^5 cells of *FRP*- and mock-transfected E11 were seeded into 6-well culture plates.

Variation screening and genotyping of genomic DNA

Genomic DNA for variation screening and genotyping were purified from peripheral blood leukocytes using a QIAamp blood kit (Qiagen).

Genomic configuration of human *FRP* gene was elucidated using genomic DNA sequence (GenBank accession No. NT_005612) and mRNA sequence (NM_007085) in combination. Variation screening of the promoter region (up to -1.6 kb) and all exons was performed on genomic DNA from 12 patients with RAand 12 healthy individuals by direct sequencing. Direct sequencing was performed by an automated sequencer (Applied Biosystems, Foster City, CA) using dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

Genotyping and association studies were performed for the detected polymorphic sites in the promoter region and in the exons using PCR–single strand conformation polymorphism (SSCP), as previously described (14).

The primers and experimental conditions used for genotyping are shown in Table I. Those used for variation screening are available upon request.

Statistical analysis

The differences in the mRNA levels between RAand OA synovia were statistically analyzed using Mann-Whitney's U test, using StatView-J4.11 for Macintosh (Abacus Concepts Inc., Berkeley, CA).

The genotype frequencies of the detected polymorphisms were compared between patients with RA and healthy individuals using the χ^2 test and Fisher's exact test. Fisher's exact test was used when one or more cells in 2 x 2

Table I. Primers and experimental conditions used for genotyping.

Primer	Position	Sequence (5' → 3')	AT (°C)	Size (bp)	Typed SNP	AA (%)	Temp (°C)	Time (min)	Gly
FRP-PRO8L	promoter	CCCTGTCAAAGAGGTAGCACA	65.2	275	-919C>G, -832G>A	12.5	10	120	+
FRP-PRO8R		TGCTGTGGGTTCTACAGTGC							
FRP-PRO14L	promoter	ACAACAGTGGGCACTCAACA	64.9	282	-1572T>A	10	20	120	-
FRP-PRO14R		CCACTGCAATCCATTGTCATA							
FRP-EX11-SSCPL	exon 11	TGCATCACGATTGAAAGAGG	63.5	301	c*.414T>C	12.5	20	120	-
FRP-EX11-SSCPR		TCCCAGAAACTCCATCCAAG							

AT: annealing temperature; Size: size of PCR products; AA: concentration of acrylamide in SSCPgel; Temp: Electrophoresis temperature; Time: duration of electrophoresis, Gly: presence or absence of 10% glycerol in SSCPgel.

contingency tables contained a value of less than 5. A *P* value < 0.05 was regarded as statistically significant.

Linkage disequilibrium (LD) analysis and estimation of haplotype frequencies were performed by the Estimation-Maximization (EM) algorithm using SNPalyze program (DYNACOM, Mobara, Japan). The extent of LD was assessed by Lewontin's *D*'and *P* values (15).

Results

Overexpression of FRP in synovial tissues of RA

To examine whether the previously reported upregulation of *FRP* in a synovial tissue from a patient with RA detected in differential display (3) is commonly observed in the majority of patients with RA, a quantitative real-time RT-PCR was performed and mRNA levels were compared between the synovial tissues from 10 patients with RA and 5 patients with OA. As shown in Figure 1, mean mRNA level of *FRP* was elevated in RA by 2.3-fold (P = 0.01), indicating that the upregulation of *FRP* is generally observed in the synovial tissues from RA.

Inhibitory activity of FRPon the growth of a rheumatoid synovial cell line E11

Next we examined for potential effects of FRP on the growth of a rheumatoid synovial cell line, E11, by stably introducing *FRP* cDNA. The expression level of FRP in the tranfectants was found to be twice as high as that of the mock transfectants in three independent experiments. As shown in Figure 2a, E11-*FRP* transfectants demonstrated significantly decreased proliferation compared with mock transfected E11 (P= 0.0003).

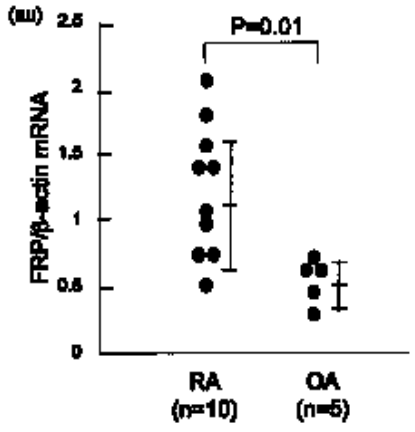

Fig. 1. mRNA levels of *FRP* in the synovia from RA as compared with OA. The mRNA concentrations of *FRP* were quantitated using real-time RT-PCR, standardized by the concentration of β-actin mRNA, and the results were expressed as arbitrary units. *FRP* mRNA level in RA synovia was 2.3-fold upregulated compared with those in OA synovia (P = 0.01, Mann-Whitney's U test).

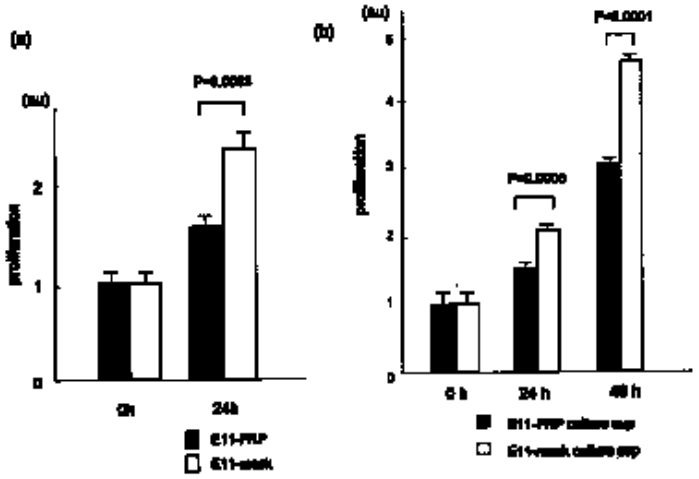

Fig. 2. (**a**) Inhibition of proliferation of a rheumatoid synovial cell line, E11, by transfection of *FRP*. E11 stably transfected with *FRP* and mock transfectants were cultured for 24 h and the cell numbers were measured using WST-1 proliferation assay. Results are shown in arbitrary units (au) calculated from absorbance. E11-*FRP* demonstrated significantly decreased proliferation as compared with mock transfectants. (**b**) Growth inhibitory activity of E11-*FRP* culture supernatants. Culture supernatant from either E11-mock or E11-*FRP* transfectants was collected after culture for 24 h, and E11 cells were cultured in each of the supernatants. Proliferation after 24 h and 48 h was measured using WST-1 assay. Significant inhibition was observed when the cells were cultured in the supernatant from E11-*FRP*.

To test whether this inhibitory effect was mediated by secreted or membrane-bound factors, proliferation was compared between E11 cells cultured in the supernatants from E11-mock and from E11-*FRP*. As shown in Figure 2b, proliferation was significantly decreased in the E11 cells cultured in the supernatants from E11-*FRP*, indicating that soluble factor(s), possibly *FRP* itself, is responsible for the inhibitory activity of E11-*FRP*.

To exclude the possibility that TGF-β was responsible for the growth inhibitory effect of the *FRP*-transfectants, TGF-β1 was measured in the culture supernatants of the *FRP*- and mock-transfectants. The concentration of TGF-β1 in *FRP* transfectants was 0.14 (24 h) and 1.30 ng/ml (72 h), which was lower than that in mock transfectants (0.22 and 1.87 ng/ml, respectively).

Variation screening and association study

Genomic DNA variation screening revealed 4 variations in the promoter region (-1572T>A, -1427C>T, -1390G>A and -919C>G), 4 in exons (-229C>A, c.716C>T [Thr239Met], c*.411A>G and c*.414T>C) and 7 in introns (IVS1+47G>T, IVS1+103G>A, IVS1+109_111delGGG, IVS2-27G>T, IVS3+50_51insC, IVS3-73_-72insGT, IVS3-69G>A) (The sequence variations were described according to the nomenclature system proposed by den Dunnen [16]) (Fig.3). In addition, in the course of genotyping, another variation in promoter region (-832G>A) was detected.

A case-control association study was performed for 4 variations that were located in the promoter region or exons and at the same time met the definition of polymorphism (major allele frequency < 99%). Statistically significant association was not detected for any of these four single nucleotide polymorphisms (SNPs) (Table II). Others were either rare variations or intronic variations.

Haplotype frequencies and pairwise linkage disequilibrium (LD) between SNPs are demonstrated in Table III. Strong LD was observed between promoter region SNPs, while LD between

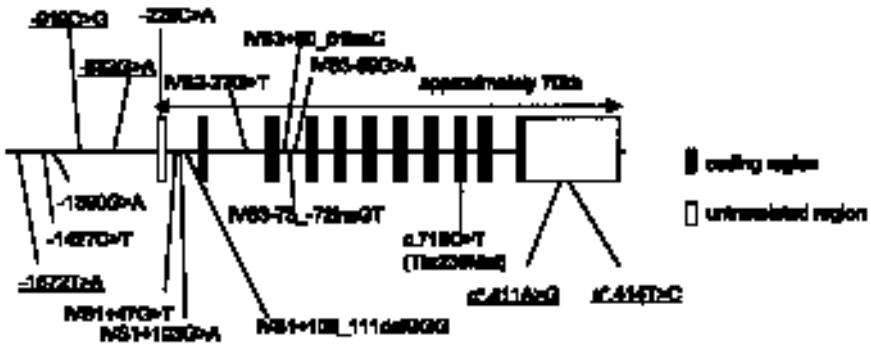

Fig. 3. Structure of the human *FRP* gene and variations detected in this study. Underlined are the SNPs within the promoter region and exons genotyped in this study. Others are either rare variations (allele frequency < 1%) or polymorphisms within introns. The designation of the variations was based on (16). The nucleotide sequences of these variations will appear in the DDBJ/EMBL/GenBank database under the accession number AB119283.

3' UTR SNP and promoter SNPs was modest. Haplotype frequencies of RA patients did not significantly differ from these of healthy controls.

Discussion

In the present study, we confirmed overexpression of *FRP* mRNA in the synovial tissues of patients with RA as compared with OA. It was previously shown in a small-scale study that *FRP* mRNA was increased in the synovial tissues of RA as compared with OA, while the concentration of *FRP* protein was not significantly different between synovial fluids from patients with RA and OA, irrespective of the presence of anti-FRP autoantibodies (7). Such a discrepancy between mRNA and protein levels might be attributable to the accelerated turnover of *FRP* in RA, as has been suggested (7).

The source of *FRP* in the synovial tissues remains unclear. A previous study demonstrated that *FRP* is rather ubiquitously expressed except for peripheral blood leukocytes (7); however, the possibility that the difference between RA and OA could be due to differences in the number of infiltrating inflammatory cells cannot be excluded. To address this issue, *FRP* mRNA levels were compared in fibroblast-like synoviocytes from each two patients with RA and OA; however, the number of the samples was too small to make any meaningful interpretation possible (data not shown). This question needs to be addressed in the future, for instance by immunohistochemistry techniques, when antibody to FRP becomes available. Since FRP is considered to be a secreted molecule, it should be possible to exert its effect even if it is expressed mainly in the infiltrating inflammatory cells, rather than the synoviocytes.

It was of particular interest to explore the molecular mechanisms responsible for the induction of such overexpression. In this study, we examined the possibility that polymorphisms within the *FRP* gene, especially those in the regulatory region, might be associated with RA. Although we identified a number of new variations, significant association with RA was not detected. There are some limitations in this study, which might possibly have led to false-negative results. The male-to-female ratio was substantially different between RA and controls; however, since the genotype frequencies were not different between male and female controls, the results were essentially identical after adjustment for gender ratio (data not shown). Although the age distribution of the controls was younger than the patients, potential misclassification caused by future development of RA among controls is less than 1% (the prevalence of RA in Japan); therefore, it is unlikely that such age difference significantly affected the results. The variations were screened in all exons, intronic regions flanking exons and the promoter region up to -1.6 kb, but the possibility that variations in other regions, for example in the middle of introns or in the further upstream promoter region, might be associated with RA cannot theoretically be excluded. In addition, the number of subjects is not sufficiently large to

Table II. Genotype and allele frequencies of SNPs in the promoter and 3'-untranslated regions of *FRP*.

SNP	Genotype, allele	RA (n=224)	(%)	Controls (n=220)	(%)
-1572T>A	T/T	200	(89.3)	197	(89.5)
	T/A	23	(10.3)	21	(9.6)
	A/A	1	(0.4)	2	(0.9)
	T	423	(94.4)	415	(94.3)
	A	25	(5.6)	25	(5.7)
-919C>G	C/C	198	(88.4)	197	(89.6)
	C/G	25	(11.2)	19	(8.6)
	G/G	1	(0.4)	4	(1.8)
	C	421	(94.0)	413	(93.9)
	G	27	(6.0)	27	(6.1)
-832G>A	G/G	215	(96.0)	215	(97.7)
	G/A	9	(4.0)	5	(2.3)
	A/A	0	(0)	0	(0)
	G	439	(98.0)	435	(98.9)
	A	9	(2.0)	5	(1.1)
c*.414T>C	T/T	96	(42.9)	100	(45.5)
	T/C	102	(45.5)	98	(44.5)
	C/C	26	(11.6)	22	(10.0)
	T	294	(65.6)	298	(67.7)
	C	154	(34.4)	142	(32.3)

Significant difference between RAand controls was not observed in any of the SNPs.

Table III. Estimated haplotype frequencies and pairwise linkage disequilibrium between SNPs.

(a) Estimated haplotype frequencies.

Haplotype	-1572	-919	-832	c*.414	Haplotype frequency (%) RA	controls
1	T	C	G	T	59.9	61.5
2	T	C	G	C	31.9	31.2
3	A	G	G	T	3.5	4.7
Others					4.7	2.6

(b) Pairwise linkage disequilibrium between SNPs

SNPs		Controls -919	Controls c*.414	RA -919	RA c*.414
-1572	*D'*	1	0.436	0.957	0.019
	P	0.000	0.177	0.000	0.797
-919	*D'*		0.495		0.054
	P		0.045		0.906

detect an association of rather weak contribution. However, at this point, it is more probable that overexpression of *FRP* is caused by inflammatory synovial environment in RA, rather than genomic polymorphisms of *FRP* gene.

Another interesting finding in this study was the inhibitory activity of FRP on the growth of rheumatoid synovial cell line, E11. Inhibitory effect of TSC-36, the homologue of FRP in mice, on the growth of human lung cancer cell has been reported (17). With respect to human synovial cells, inhibitory activity of FRPhas been demonstrated on the production of mediators of joint destruction, such as MMP-1, MMP-3 and prostaglandin E2 (8). A recent study further provided evidence that administration of FRP into mouse joints ameliorates joint destruction (18). Our finding provided further support that FRP may exert a protective effect for joint destruction on synoviocytes. Our transfer experiment suggested that secreted factor(s) other than TGF-β is responsible for the growth inhibitory effect. Owing to the lack of commercial antibodies to FRP, the possibility that secreted FRPitself is the inhibitory factor could not be addressed in this study, and needs to be confirmed in the future. Why joint damage progresses in spite of overexpression of *FRP* remains a mystery. The presence of neutralizing antibody to FRP in patients with RA may provide one explanation (7,8). Another possibility may be that FRP has unknown functions on cells other than synoviocytes, for example osteoclasts, that are agonistic for inflammation and/or joint damage. The mechanism of regulation of FRP as well as its effects on various cell lineages that constitute inflammatory joints requires further study.

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