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ORIGINAL ARTICLE

Tristetraprolin (TTP) gene polymorphisms in patients with rheumatoid arthritis and healthy individuals

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Abstract Tristetraprolin (TTP) is an intracellular protein that modulates the production of cytokines, including TNFα, by binding to and destabilizing the mRNAs of these cytokines. Therefore, differences in TTP gene expression may affect the severity of inflammatory diseases, such as rheumatoid arthritis (RA). We searched for polymorphisms in the human TTP gene and for this purpose, we sequenced the entire TTP gene in 20 Japanese individuals (ten with RA and ten healthy volunteers) and found one single nucleotide polymorphism (SNP) in the promoter region. We analyzed this SNP (A/G) by restriction fragment length polymorphism method in 155 RA patients and 100 control subjects. While the frequency of A allele in this SNP was similar in RA patients (74.5%) and controls (76.0%), the disease duration in RA patients with genotype GG was shorter than that of patients with genotypes AA/AG and RA patients with genotype GG had a higher probability of being treated with infliximab. We studied the difference in promoter activity between the two alleles by luciferase assay and found that the promoter activity of TTP promoter region with allele A was around two-fold higher than that with allele G. We conclude that this SNP in the promoter region of the TTP gene mildly affects promoter activity, and thus, may influence the disease activity of inflammatory disorders including RA.

Keywords Tristetraprolin · ZFP36 · Single nucleotide polymorphism · TNF-α · Rheumatoid arthritis

Abbreviations

TTP	Tristetraprolin
RA	Rheumatoid arthritis
SNP	Single nucleotide polymorphism
TNFα	Tumor necrosis factor-α
PMA	Phorbol 12-myristate 13-acetate
ARE	AU-rich element
3′UTR	3′ Untranslated region
CRP	c-Reactive protein
Zfp36	Zinc finger protein 36
RFLP	Restriction fragment length polymorphism
PCR	Polymerase chain reaction
HEK293T	Human embryonic kidney 293T
ESR	Erythrocyte sedimentation rate
NCBI	National Center for Biotechnology Information
GM-CSF	Granulocyte-macrophage colony-stimulating factor
COX-2	Cyclooxygenase-2

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that mainly affects joint tissues. Tumor necrosis

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factor-α (TNFα) plays a central role in the pathogenesis of RA. The importance of TNFα in the inflammatory process of RA is proven by the dramatic improvement of symptoms observed in many of the patients treated with TNFα antagonists.

Tristetraprolin (TTP), also known as Tis11, Nup475, and G0S24, is a protein expressed widely in mammalian cells and contains two tandem repeats of a consensus amino acid sequence $CX_8CX_5CX_3H$, which is a zinc-binding motif [1, 2]. The expression of TTP is rapidly induced by serum, insulin and phorbol 12-myristate 13-acetate (PMA) [3]. The function of TTP was unknown for several years after its discovery, but it has become clear that TTP plays a major role in modulating the production of cytokines, including TNFα, granulocyte-macrophage colony-stimulating factor (GM-CSF) and cyclooxygenase-2 (COX-2) [4–7]. This is mediated by the binding of TTP to the AU-rich element (ARE) of the 3′ untranslated region (3′UTR) of the mRNAs of these cytokines, which leads to destabilization of these mRNAs. In addition, as TNFα itself induces the production of TTP, a feedback loop exists, which prevents excessive production of TNFα [8]. Therefore, TTP seems to be an important physiological regulator of TNFα production. Studies on TTP knockout mice have shown that these mice appear normal at birth but develop weight loss, severe erosive arthritis, conjunctivitis, dermatitis, splenomegaly, adenopathy, autoimmunity, and severe medullary and extramedullary myeloid hyperplasia at 1–8 weeks after birth [9]. Inflammatory symptoms can be prevented in TTP knockout mice by administration of anti-TNFα antibodies, confirming that the above symptoms are caused by TNFα overproduction. These observations prompted us to investigate the function and gene expression of TTP and define its role in the development and severity of human inflammatory disorders, RA in particular.

Recently, we reported that TTP gene expression is higher in the synovial tissues of RA patients than those of patients with osteoarthritis [10]. When the expression of TTP gene was compared with that of the TNFα gene, synovial tissues from patients with elevated serum C-reactive protein (CRP) tended to have a lower TTP gene expression/TNFα gene expression ratio. Thus, appropriate expression of the TTP gene may be important in reducing the severity of RA.

The TTP gene (zinc finger protein 36 *Zfp36*) is located on chromosome 19 in humans, [11] and is about 3,100 bp long, including two exons and one intron. Previous studies have shown that both the promoter and intron regions of the TTP gene are important factors that control the production of TTP mRNA [12, 13]. To date, the factors that influence interindividual differences of the TTP gene expression have not been elucidated. We hypothesized that differences such as single nucleotide polymorphisms (SNP) in the TTP gene can partially account for the interindividual differences in TTP gene expression. In addition, TTP gene polymorphisms may be related to the development or progression of RA in some other way.

In the present study, we searched for polymorphisms in the TTP gene in 20 Japanese individuals. We found one SNP in the promoter region of the TTP gene and analyzed whether this SNP is associated with the development or severity of RA in the Japanese population. Furthermore, we analyzed whether this polymorphism affects the transcription of the TTP gene.

Materials and methods

Subjects and DNA

This study was approved by the local ethics committee. All subjects gave written informed consent before biological samples were obtained. Genomic DNA from ten RA patients and ten healthy subjects randomly chosen from each subject group were subjected to sequencing of the entire TTP gene.

For restriction fragment length polymorphism (RFLP) analysis, genomic DNA purified from peripheral blood mononuclear cells of 155 RA patients (age 15–85 years, mean age at onset 43.7±14.0 years, 29 males and 126 females, disease duration 11.7±9.7 years) and 100 healthy volunteers consisting mainly of laboratory and hospital personnel were used. All RA patients fulfilled the America College of Rheumatology diagnostic criteria for RA [14] and were followed at the Division of Rheumatology, Department of Internal Medicine, University of Tsukuba Hospital. All clinicians in charge of the management of the patients were unaware of the patients' TTP genotype.

Genomic DNA were extracted from peripheral blood mononuclear cells (PBMC) of these subjects with QIAamp DNA Blood Kit (Qiagen, Hilden, Germany) or DNA Quick II DNA purification kit (Dainippon Pharmaceuticals, Osaka, Japan) according to the recommended protocols supplied by the manufacturers, and were stored at −20°C until use.

Polymerase-chain reaction (PCR) for amplification of tristetraprolin gene

To amplify the TTP gene, two sets of PCR reactions were performed under standard conditions. The first primer set TTP5-1: 5′-TTCTACAAGCCTCAGTCTCCAG-3′ and TTP3-1: 5′-TTCGCTAGGGTTGTGGATGAAG-3′ amplified the TTP gene from position 286–1,926. The second set of primers TTP5-2: 5′-ACAAGACGGAACTCTCTGTC AC-3′ and TTP3-2: 5′-CACCCAGCTTCCAAAAAGTCA-3′ amplified the gene from position 1,841 to 3,179. The amplified products were subjected to sequence analyses.

DNA sequencing

The PCR products were sequenced by a standard method. Briefly, PCR products were purified using CENTRISEP spin columns (Princeton Separations, Adelphia, NJ), and were subjected to sequencing using ABI Prism 310 Genetic Analyzer and BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), using the protocol recommended by the manufacturer. The primers used were

TTP268-5′: 5′- TTCTACAAGCCTCAGTCTCCAG-3′,
TTP650-5′: 5′- TGCCATCTACGAGGTGAGTC-3′,
TTP1000-5′: 5′-TCTCAGACGGCGTGGTTTTG-3′,
TTP1400-5′: 5′-GGTTTCTGCGGGCGGGTG-3′,
TTP1700-5′: 5′-CTGGCTTCGCACCGCTGG-3′,
TTP620-3′: 5′-GCAGTCAGATCCATGGTGTA-3′,
TTP2185-5′: 5′-ACTCCTATCAGCGTCTGGG-3′,
TTP2531-5′: 5′-ATTAACCCACTCCCCTGACC-3′,
TTP2849-5′: 5′-AAAGCCGTTGCCAAACCCCA-3′ and
TTP2274-3′: 5′-GCTGGCATATTCATCAGGGT-3′.

The obtained sequences were reanalyzed using GENETYX-MAC (version 8.0) program to compare with *ZFP36* sequence M92844 published on the PubMed website (http://www.ncbi.nlm.nih.gov/PubMed/).

Restriction fragment length polymorphism analysis

Genotypes of 1 SNP (SNP359) were analyzed by the PCR-RFLP method. Genomic DNA was amplified using primers TTP359RFLP-5′: 5′-TCCCAACCCTCTTCTCCCT-3′, and TTP359RFLP-3′: 5′-GCGCACCACTGTTTCCCAA-3′ to yield a PCR product that included SNP359 and was 1,187 bp in length. The PCR products were digested by restriction enzyme *Tsp45 I* (New England Biolabs, Ipswich, MA), and the digested DNA was electrophoresed in agarose gels to determine the genotype. Allele with adenine at position 359 (allele A) was digested by *TSP45 I,* and yielded two fragments of 828 and 359 bp, while the allele with guanine at position 359 (allele G) remained undigested (Fig. 1b). The accuracy of RFLP was confirmed by sequencing several randomly selected samples (data not shown). RFLP data were combined with data obtained by sequencing and were used for subsequent statistical analysis.

Cell culture

Human embryonic kidney 293T (HEK293T) cells and a human synovial cell line MH7A (Riken Cell Bank, Japan), isolated from the knee joint of a patient with RA, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Preliminary experiments by PCR confirmed that HEK293T and MH7A cells produce TTP mRNA.

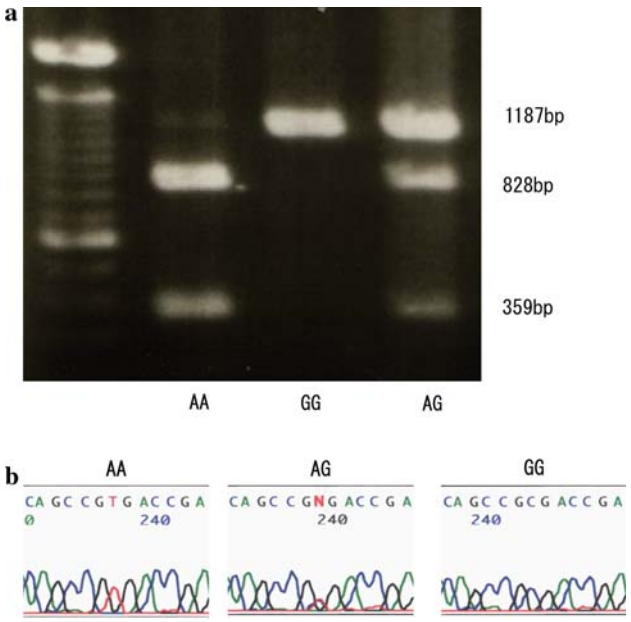

Fig. 1 Identification of a single nucleotide polymorphism at position 359 of the human tristetraprolin gene. Representative results of sequencing and restriction fragment length polymorphism (RFLP). **a** Results of sequencing. Representative results of sequencing in the 3′–5′ direction. **b** Results of RFLP

Transient transfection and luciferase assay

Luciferase vector pGL3 basic (Promega, Madison, WI) was used. We cloned position −686 to +25 of the TTP gene including either A or G allele of the SNP and prepared two sets of luciferase vector constructs. HEK293T and MH7A were transfected with luciferase vectors using the Fugene HD® reagent (Roche Diagnostics, Indianapolis, IN) and incubated for 12 h. Finally, the cells were lysed in Reporter Lysis Buffer lysis reagent (Promega), and luciferase assay was performed using the Promega luciferase assay kit with a Micro Lumat Plus luminometer (EG & G Berthold, Berthold Technologies, Bad Wildbad, Germany). Luciferase activity detected in each well was compensated by β-galactosidase activity. To analyze the effect of TNFα stimulation, we stimulated MH7A cells by TNFα (5 ng/ml) for 8 h, from 4 h after transfection.

Data analyses

The clinicopathological data were collected by reviewing medical records. RFmax, IgGmax, CRPmax, and ESRmax were defined as highest value of serum rheumatoid factor, serum IgG, serum CRP and erythrocyte sedimentation rate (ESR), respectively, observed in a given patient during follow-up in our hospital. Values obtained when a patient had symptoms suggestive of infection were omitted. Radiographs of the hands taken at the nearest occasion

were evaluated by clinicians in charge of each patient, according to the criteria by Steinbrocker et al. [15], a criteria still widely used in Japan.

The relationships between SNP 359 genotypes and age at onset, IgGmax, RFmax, CRPmax, and ESRmax were examined by the Mann–Whitney *U* test. The relationship between SNP359 and usage of infliximab was compared by Fisher's direct test. Student's *t* test was used to compare the difference in promoter activities of allele A and G.

Results

Identification of polymorphisms in TTP (ZFP36) gene and validation of RFLP analysis

Apart from the two single nucleotide differences described below, several differences were observed between our sequencing data and sequence M92844 in the database. All of these differences were present in the intron region, and our results were in complete agreement with the genome sequence of the TTP gene reported on the NCBI website (http://www.ncbi.nlm.nih.gov, accession number: AC011500, from position 11,119 to 14,350).

Two single nucleotide differences at positions 359(A/G) and 503(A/C), both in the promoter region of the TTP gene, were found (Fig. 1a and not shown). We named these according to their positions as SNP359 and SNP503. The C allele of SNP503 was found in only one healthy individual among 20 healthy volunteers and 20 RA patients. The minority allele of SNP359 was more frequently found, and this SNP was subjected to further analyses. RFLP analysis yielded fragments of the predicted sizes (Fig. 1b), and the sequencing of several samples confirmed the validity of the analysis.

Allele frequencies of SNP359 in RA patients and healthy individuals

The genotype distributions and allele frequencies of SNP359 were calculated for RA patients and healthy individuals and no significant difference was observed (Table 1).

Relationship between SNP359 genotypes and clinical variables in patients with RA

The RA patients were categorized into three subgroups according to their SNP359 genotypes, and the relationships among SNP359 genotypes and RA clinical variables were analyzed (Table 2). ESRmax, CRPmax, and RFmax tended to be slightly higher in patients with GG genotype, albeit statistically insignificant (Table 3). No relationship between SNP359 genotypes and IgGmax was observed. Patients with the GG genotype tended to have younger age at onset compared to those with genotypes AA/AG, but the difference was statistically insignificant ($P = 0.0562$ by Student's *t* test, $P = 0.0655$ by Mann–Whitney's *U* test) (Table 3).

There was no significant relationship between SNP359 genotypes and radiographic staging of joint destruction (Fig. 2). However, among patients classified as stage 4 ($n = 53$), the disease duration in patients with genotype GG was shorter than that of patients with genotypes AA/AG (11.4 ± 7.2 years vs. 20.4 ± 8.9 years, $p = 0.0337$ by Student's *t* test, $p = 0.0293$ by Mann–Whitney's *U* test) (Fig. 2).

We also analyzed the relationship between RA patients treated with infliximab, an anti-TNFα antibody, and SNP359. We assumed that patients who were placed on infliximab therapy within a short period after the introduction of this drug onto the market comprise a population of patients with more active RA, compared to those who were not. At the point when the data collection for this study was completed, among 155 RA patients included in this study, 24 had at least one infusion of infliximab. Etanercept and other biological drugs were not introduced onto the market at that time. Among nine patients with genotype GG, four received treatment with infliximab,

Table 1 Genotype distribution and allele frequencies of SNP359 polymorphism of the tristetraprolin gene in healthy individuals and patients with rheumatoid arthritis

	AA	AG	GG
A: genotypes			
Healthy (100)	60 (60.00%)	32 (32.00%)	8 (8.00%)
RA (155)	85 (54.83%)	61 (39.35%)	9 (5.81%)
	A	G	
B: allele frequencies			
Healthy (200)	152 (76.00%)	48 (24.00%)	
RA (310)	231 (74.52%)	79 (25.48%)	

Table 2 Relationship between SNP359 genotypes of the tristetraprolin gene and joint destruction in patients with rheumatoid arthritis

	SNP359 genotype		
	AA	AG	GG
Stage 1	13	8	0
Stage 2	22	14	3
Stage 3	19	16	1
Stage 4	25	20	5

Stages determined according to the criteria by Steinblocker et al. [14]

Table 3 Relationship between SNP359 genotypes of the tristetraprolin gene and disease parameters

	SNP359 genotype		
	AA	AG	GG
Age at onset (years)	45.87 ± 14.32	42.11 ± 13.17	35.11 ± 12.96
CRPmax (mg/dl)	3.14 ± 2.91	3.86 ± 3.13	4.47 ± 4.05
ESRmax (mm/h)	43.93 ± 25.04	43.93 ± 31.72	56.89 ± 39.46
RFmax (U/dl)	303.7 ± 579.5	244.4 ± 544.2	533.3 ± 731.4
IgGmax (mg/dl)	1,572.7 ± 462.9	1,449.2 ± 451.2	1,546.1 ± 565.7

Data are mean ± SD

CRPmax, *ESRmax*, *RFmax*, and *IgGmax* were defined as highest value of serum CRP, erythrocyte sedimentation rate (*ESR*), serum rheumatoid factor and serum IgG, respectively, observed in a given patient during follow-up in our hospital

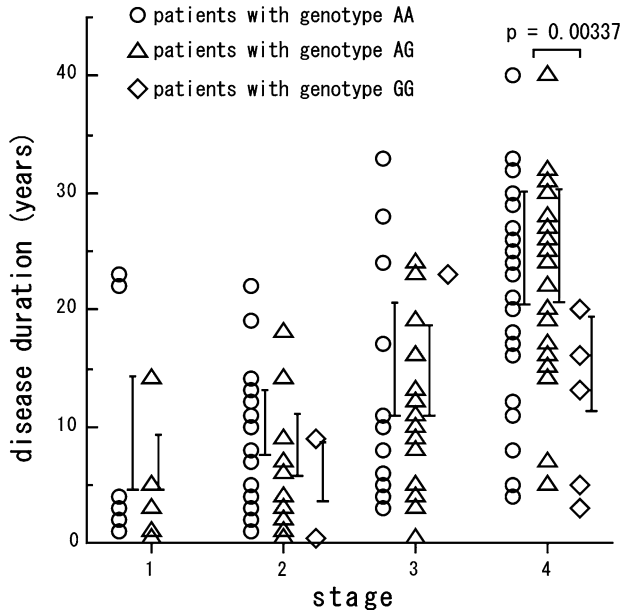

Fig. 2 Relationship between SNP359 genotypes and disease duration in patients with rheumatoid arthritis. Patients were categorized according to their SNP359 genotype status and severity of joint destruction as determined by X-ray examination of the hands. Joint destruction was defined according to Steinblocker et al. [14] *P* value calculated by two-tailed Student's *t* test

while 20 of 146 patients with genotype AA or AG were treated with infliximab. Thus, among our cohort of RA patients, patients with SNP359 genotype GG had a significantly higher probability of being selected for infliximab therapy than those with AG or GG genotype (Table 4).

SNP359 in the promoter region of the TTP gene affects promoter activity

Clinical analysis suggested that RA patients with genotype GG might be prone to higher disease activity than those with genotype AG/AA. Therefore, we analyzed the difference in promoter activities between the two alleles by luciferase assay. In both cell lines, the promoter activity of the TTP promoter region with allele A was 1.5–2 fold higher than that with allele G in normal condition. The difference was smaller in TNFα stimulated condition, but was still statistically significant (Fig. 3).

Table 4 Relationship between SNP359 genotypes of the tristetraprolin gene and usage of infliximab

	Usage of Infliximab			*P* value
	Yes	No	Total	
AA/AG	20	126	146	0.0331
GG	4	5	9	
Total	24	131	155	

P value by Fisher's exact test

Discussion

SNPs in the genes that encode cytokines, chemokines, and other proteins pertinent to the pathogenesis of various diseases are currently being investigated as candidate modulators of disease susceptibility or severity. In particular, SNPs in the promoter regions may affect promoter activities, thereby affecting the production of proteins. One such example was reported by Weyrich et al. [16] in which it was shown that a SNP in the promoter region of partitioning-defective protein-6α gene-affected glucose tolerance, and a difference in promoter activities was found between the two alleles.

Based on our previous study, [10] we assumed that differences in the TTP gene may affect TTP production and/or function. Among 20 Japanese individuals, we identified two single nucleotide differences at positions 359 and 503, which we named SNP359 and SNP503. However, the minority allele of SNP503 was found in only one individual among 40 RA patients and healthy

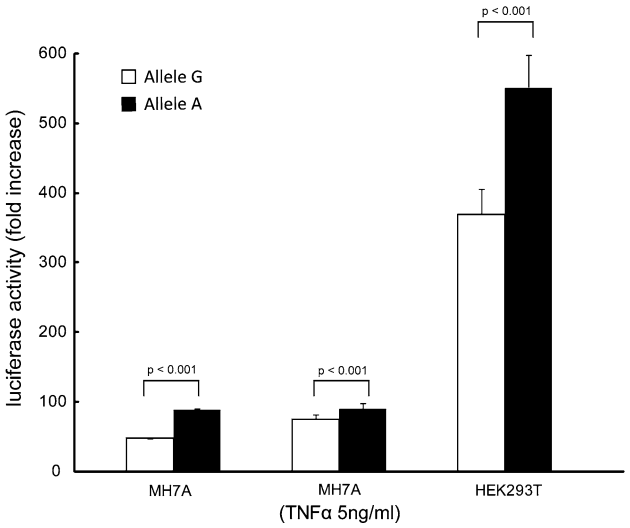

Fig. 3 Effects of SNP359 on promoter activity of the tristetraprolin gene. Luciferase vectors that carry either allele A or allele G of the promoter region were introduced into HEK293T and MH7A cells. Luciferase activities were measured after 12 h form transfection. Luciferase activity was compensated by β-galactosidase activity. See text for details. Results representative of four independent experiments. Statistical analysis was by Student's *t* test

subjects and thus was not examined further. SNP359 was recently reported as ZFP36*2 by Blackshear et al. [17]. The frequency of the G allele was 47% in their 92 subjects, compared with 25.0% in our cohort of 255 individuals. There seem to be some differences in the allele frequencies of this SNP among ethnic groups. Although previous studies showed that the intron region can affect the expression of the TTP gene, [12, 13] we did not find any polymorphisms in this region. In fact, we were unable to find the other polymorphisms described by Blackshear et al. [17] or any novel polymorphisms except for SNP503 in the present study. Our study population consisted entirely of Japanese individuals, and this may account for the small number of polymorphisms found in our study. Recently, Danielle et al. [18] reported 28 SNPs in *ZFP36* and determined the frequency of all the known *ZFP36* polymorphisms. They reported that SNP359 was not related to the development of RA in African-Americans. We also found that allele or genotype frequencies of SNP359 were not significantly different between RA patients and healthy individuals. This was not unexpected, since we assumed that genetic differences in the TTP gene may be a factor that affects disease activity of RA, but not necessarily a factor that affect disease susceptibility.

When we studied the relationships between SNP359 genotypes and clinical parameters in RA patients, we noticed slight differences in CRPmax, ESRmax, RFmax, and age at onset among different genotype groups. However, the number of individuals with genotype GG was not large enough to draw a definite conclusion, since among our 155 RA patients, only 9 had this genotype. Another limitation of this study was that we did not follow the entire course of the disease in all of our RA patients. Thus, parameters such as CRPmax do not necessarily reflect the severity of RA in a given patient.

In our cohort of RA patients, patients with genotype GG seemed to have more RA-progressive changes than patients with other genotypes, although these changes were not statistically significant. However, interestingly, we found that in patients with stage 4 radiographic changes, those with genotype GG had significantly shorter disease duration than genotype AA/AG patients. Although the number of genotype GG patients with advanced radiographic changes is too small to draw a definitive conclusion, this observation may imply that patients with SNP359 genotype GG may have a more rapid joint destruction than other patients. In addition, we found that patients whom their caring physicians decided to use infliximab for treatment had a higher probability of having SNP359 genotype GG than patients who were not. This finding may also imply that patients with genotype GG may have higher disease activity, refractory to conventional therapies such as methotrexate.

These findings prompted us to examine whether SNP359 affects promoter activity or not. Using luciferase assay, we did find that the promoter region of the TTP gene with allele A has a higher promoter activity than that with allele G. Therefore, RA patients with genotype GG may respond better to infliximab therapy. However, in our cohort, we could not find any statistical differences in the effect of infliximab among genotypes. The number of RA patients needs to be increased in future studies.

The difference in promoter activities did not increase by stimulation with TNFα. The promoter region containing this SNP may not be in the direct downstream of TNFα stimulation.

In addition to TNFα, TTP reduces the production of GM-CSF and COX-2. GM-CSF enhances the proliferation of fibroblast-like synoviocytes from RA patients [19], and anti-GM-CSF antibodies reduces the severity of collagen-induced arthritis in mice [20].

In addition, TTP reduces the production of E2A-encoded transcription factor E47, which is a factor that promotes class switch in B cells [21]. Thus TTP may reduce the production of IgG class autoantibodies in autoimmune diseases.

The modest but significant difference in promoter activity between the two alleles is intriguing. Considering the associated changes with TTP deficiency in mice, [9] we assume that a large defect in the promoter activity of the TTP gene would almost certainly lead to the

emergence of inflammation from early stage of life. A modest change in promoter activity may have an effect after occurrence of a chronic inflammatory condition, such as RA. We speculate that in RA patients with genotype GG, a subtle defect in the promoter activity of the TTP gene may result in increased production of TNFα, and other proteins such as GM-CSF and COX2, which in turn leads to persistent inflammation and enhanced joint destruction.

Should the results described in this paper be confirmed in a larger cohort of patients, these findings might enable us to screen a subpopulation of RA patients at higher risk of joint destruction at an early stage of the disease. Patients with a higher risk of disease progression may merit intensive therapies at earlier stages of the disease. In an attempt to identify parameters that predict the efficacy of infliximab, we recently reported that gene expression of adenine/uridine-rich element binding proteins TTP, T cell intracellular antigen 1 and Hu antigen R fluctuate after initiation of infliximab therapy [22]. SNPs, including the SNP described here, may affect these changes, and hence, the efficacy of anti-TNFα biologics.

Conclusions

In summary, we have analyzed the polymorphisms of the TTP gene, and searched for the relationships among polymorphisms and disease characteristics of RA. Our results imply that SNPs in the TTP gene do not affect the susceptibility to RA. However, SNP359 in the promoter region of the TTP gene affects promoter activity, suggesting that this SNP could modulate the disease activity of RA.

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Competing interest The authors declare that they have no competing interests.

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