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# Expression of TNF-α, tristetraprolin, T-cell intracellular antigen-1 and Hu antigen R genes in synovium of patients with rheumatoid arthritis

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**Abstract.** Post-transcriptional regulation through the AU-rich element (ARE) by ARE binding proteins (ARBPs) has an important role in controlling the production of cytokines, including tumor necrosis factor (TNF)-α. Therefore, expression of ARBPs may influence, or may be influenced, by the severity of rheumatoid arthritis (RA). We measured the gene expression of ARBPs, including tristetraprolin, T-cell intracellular antigen (TIA)-1 and Hu antigen R (HuR), in synovial tissues from RA and osteoarthritis patients. cDNA was constructed from synovial tissues obtained from 21 patients with RA, and those from 12 patients with osteoarthritis. Gene expression was measured using the TaqMan PCR real-time quantification method. No significant differences were observed in the expression of tristetraprolin, TIA-1 or HuR genes between RA and osteo-arthritis synovium samples. No significant relationships between expression of tris-tetraprolin, TIA-1 or HuR genes and TNF-α gene expression serum CRP levels in samples from RA patients were observed. A significant positive relation-ship was observed between gene expression levels of TIA-1 and HuR. While HuR stabilizes TNF-α mRNA and enhances TNF-α production, TIA-1 acts as a post-transcriptional silencer, and suppresses the production of the TNF-α protein. The clear positive relationship between the expression of these two ARBPs may imply that the expression of either gene affects the expression of the other, or the mechanisms that control the expression of these genes have some factors in common.

## Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory disorder, the main lesion of inflammation being the joint synovium. In the affected synovium of patients with RA, proliferation of sinoviocytes and invasion of inflammatory cells are observed (1). Various cytokines and chemokines are produced in the proliferated synovial tissues, which further enhances inflammation and joint destruction. Although the precise mechanism that causes this vicious cycle is still unclear, it is generally accepted that the tumor necrosis factor (TNF)-α plays a central role in this inflammatory process (2,3). Thus, mechanisms that control TNF-α production may have a strong influence on the disease activity and prognosis of RA in individual patients.

The mechanisms that promote TNF-α production have been elucidated in various studies, and it has become evident that nuclear factor κB (4), and activator protein-1 (5), are key molecules that enhance TNF-α gene expression. These molecules promote the transcription of the TNF-α gene, and thus, increase the production of the TNF-α protein.

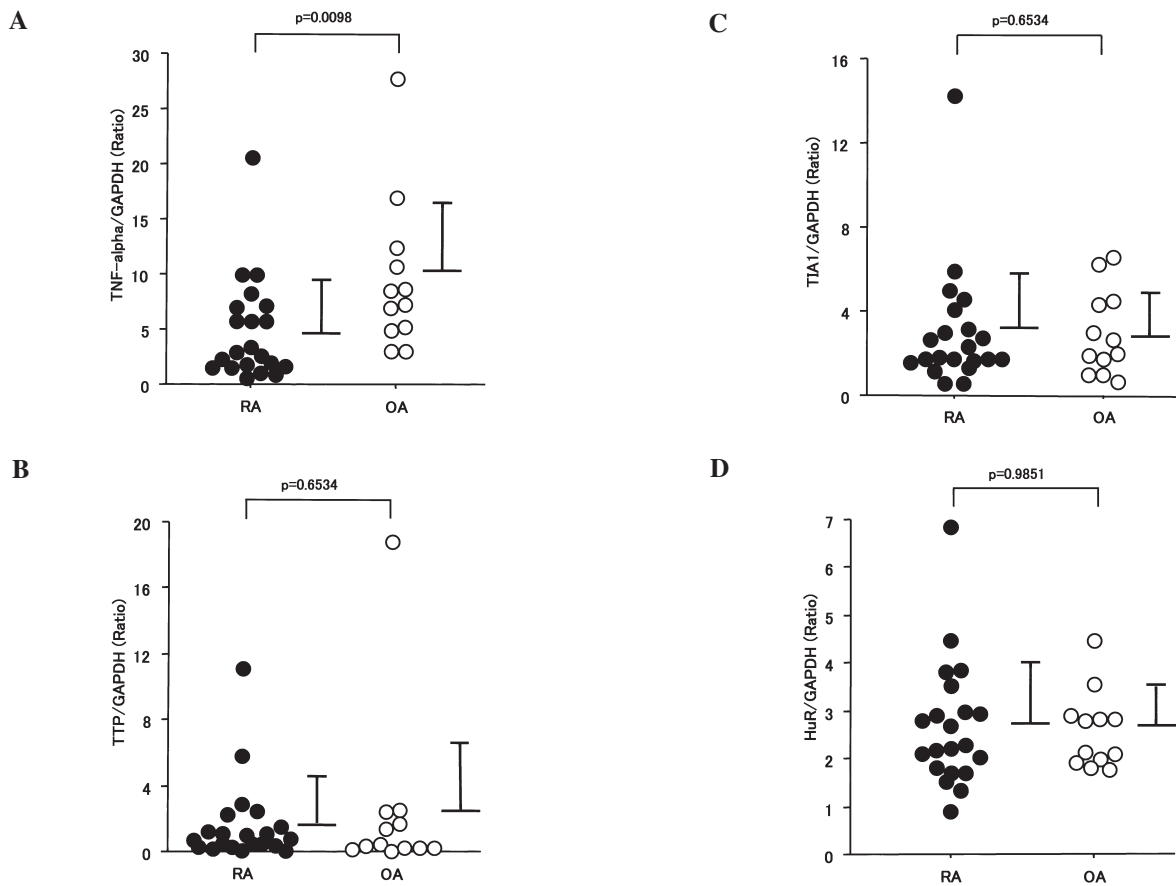In addition to these transcriptional factors, recent evidence shows that post-transcriptional regulation is also important in

Figure 1. Expression of tumor necrosis factor (TNF)-α (A), tristetraprolin (TTP) (B), T-cell intercellular antigen (TIA)-1 (C), and Hu antigen R (HuR) (D) mRNA in synovial tissues of patients with RA and OA. p-value calculated by the Mann-Whitney U test.

the regulation of cytokine production (6,7). A group of molecules termed AU-rich element (ARE) binding proteins (ARBPs) play a pivotal role in post-transcriptional regulation of inflammatory cytokine production. Among various ARBPs, tristetraprolin (TTP) is one of the most investigated. TTP is a widely distributed phosphoprotein, possesses two zinc finger domains that form the biological active site, and is an immediate early protein that responds to various stimuli (8). It has been demon-strated that TTP induces destabilization of TNF-α mRNA by directly binding to the ARE in the 3' untranslated region (3'UTR) of TNF-α mRNA, and accelerates mRNA degradation, thereby reducing the production of TNF-α protein. It has been also demonstrated that TTP knockout mice manifest erosive arthritis, dermatitis, and body weight loss, and that these symptoms could be prevented by administration of anti-TNF-α antibodies (9).

T-cell intercellular antigen (TIA)-1 and Hu antigen R (HuR) are also ARBPs that are involved in post-tran-scriptional regulation of TNF-α production. TIA-1 possess three RNA recognition motif (RRM) type RNA binding domains and binds to ARE in the 3'UTR of TNF-α mRNA (10,11). Recent studies have shown that upon binding to ARE, TIA-1 works as a translational silencer, not a transcript destabilizer (12). HuR, also called HuA (13), is a member of the embryonic lethal abnormal visual protein family, and also possesses three RRMs that bind to ARE and poly A tails of various mRNAs. HuR has been shown to stabilize ARE-containing mRNAs, upon binding to such RNAs (14,15).

Thus, HuR acts as an enhancer of TNF-α production. Presumably, these molecules act in concert to precisely control the production of the TNF-α protein. Therefore, we hypothesized that the severity of RA in individuals may be influenced, at least in part, by the dynamics of TTP, TIA-1 and HuR production.

In a recent study, we investigated the quantity of TNF-α and TTP mRNAs in synovium of RA patients, and reported that serum C-reactive protein (CRP) was significantly increased in patients whose synovium had a lower TTP/TNF-α gene expression ratio (16). These results suggested that post-transcriptional regulation of TNF-α by ARBPs is an important factor that affects the severity of RA. In this study, we further investigated the quantity of TNF-α, TTP, TIA-1, and HuR mRNA in synovium samples of RA and osteoarthritis (OA) obtained from operated joints. We compared RA with OA in the expression of the above four genes, and investigated the relationship of expression of these four genes. Results imply that although the expression of a single ARBP investigated in this study do not govern the severity of RA, expression of these ARBPs do affect each other and may contribute in determining the disease activity of RA.

**Materials and methods**

*Patients and samples*. Synovial tissues from 21 patients with RA, age (mean ± standard deviation, SD) 58.95±7.79 years, disease duration (mean ± SD) 17.28±10.71 years, CRP (mean
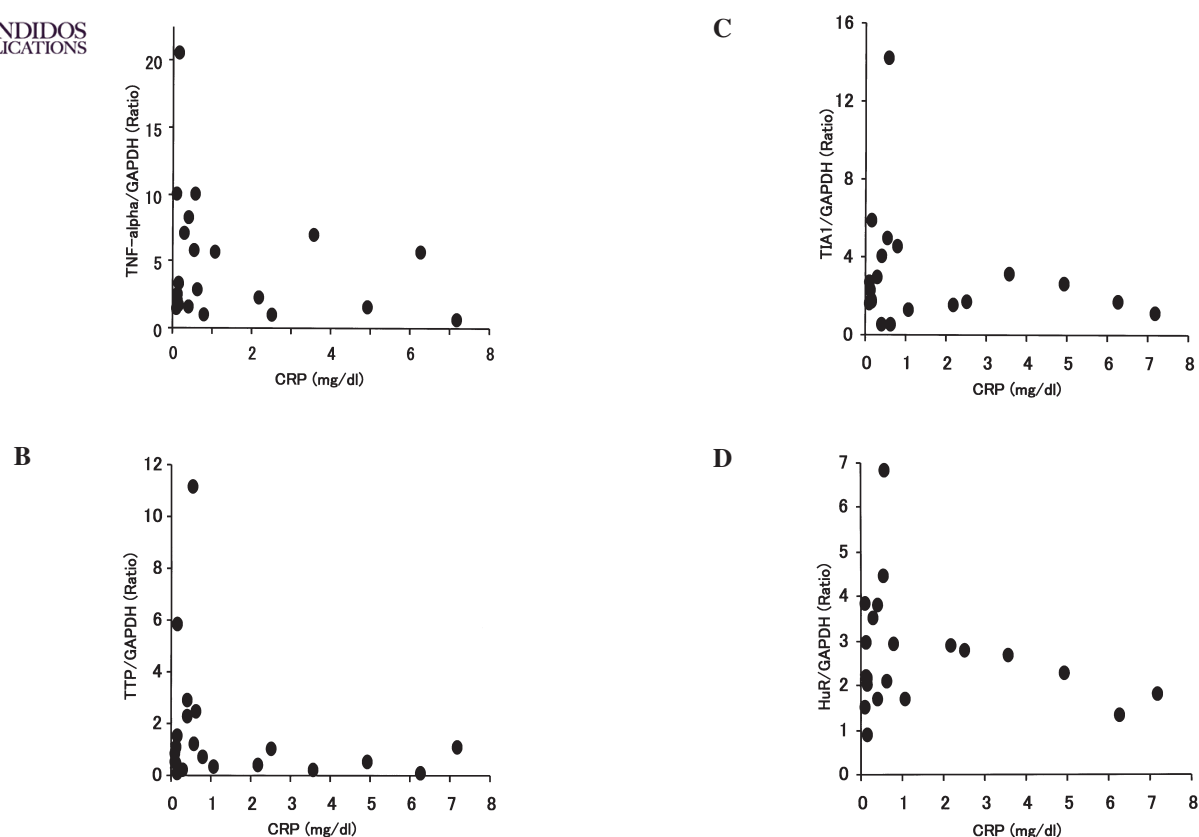
Figure 2. Relationship between C reactive protein (CRP) and gene expression. Relationship between serum CRP and expression of tumor necrosis factor (TNF)-α mRNA (A: p=0.3033, Spearman's rank correlation); tristetraprolin (TTP) (B: p=0.3213, Spearman's rank correlation); T-cell intercellular antigen (TIA)-1 (C: p=0.5088, Spearman's rank correlation); and Hu antigen R (HuR) (D: p=0.7549, Spearman's rank correlation).

± SD) 1.52±2.65 mg/dl] and 12 with OA [age (mean ± SD) 73.08±2.64 years, disease duration (mean ± SD) 17±19.80 years, CRP (mean ± SD) 0.23±0.48 mg/dl] were used. All RA patients fulfilled the American College of Rheumatology criteria for classification of RA (17). All samples were taken when the patients underwent joint replacement surgery of elbow, hand, hip or knee joints, at Tsukuba University Hospital or at Takebayashi Hospital. At the time of serum sampling, RA patients were taking 0-10 mg/day prednisolone and 0-3 disease modifying antirheumatic drugs (DMARD), including methotrexate (8 mg/week maximum), salazo-sulfapyridine (1000 mg/ day maximum), and 100-200 mg/day bucillamine, a DMARD commonly used in Japan. Serum CRP, rheumatoid factor and other clinical parameters were measured 0-2 days before operation. No patient showed any signs of infection at the time of serum sampling or operation. All subjects gave written informed consent and the study was approved by the local ethics committee.

*Real-time PCR.* Expression of TTP, TIA-1, HuR, and TNF-α genes were measured using the TaqMan PCR real-time quantification method. Total RNA was extracted from synovial tissues, and cDNA was synthesized using the RevertAid first-strand cDNA synthesis kit (Fermentas, Hanover, MD). Synthe-sized cDNA samples were applied to PCR and the amount of amplified products was monitored with an ABI-7300 sequence detector (Applied Biosystems Japan, Tokyo, Japan). PCR mixture (qPCR Master mix) was purchased from Eurogentec (Seraing, Belgium); magnesium concentration was 5 mM

final, primer concentrations 200 nM final, and the probe concentration was 100 nM final. Thermal cycler conditions were 50°C for 2 min, 95°C for 10 min, then 45 cycles of 95°C for 15 sec and 60°C for 1 min. Standard curves for the gene of interest and glyceraldehydes-3 phosphate dehydrogenase (GAPDH) gene were generated from a standard sample in every assay. All measurements were done in triplicates. The level of gene expression was calculated from the standard curve, compensated with that of GAPDH gene, and was expressed as an expression ratio (expression of the gene of interest/expression of the GAPDH gene). The sequences of specific primers and probes are as follows: TNF-α forward: 5'TGGAGAAGGGTGACCGACTC3', TNF-α probe: 5'CGC TGAGATCAATCGGCCCGACTAT3', TNF-α reverse: 5'TCCTCACAGGGCAATGATCC3'. Primers and the probe for TTP, TIA-1, HuR, and GAPDH were purchased from Applied Biosystems.

## Results

*TNF-α, TTP, TIA-1 and HuR gene expression in RA or OA joint synovium.* Expression of TNF-α, TTP, TIA-1, HuR genes in synovial tissues of 21 RA and 12 OA patients were measured by TaqMan real-time PCR. Expression of TNF-α gene was significantly higher in OA synovial tissues compared to RA synovial tissues (p=0.0098, Mann-Whitney U test; Fig. 1A), Expression of TTP, TIA-1 and HuR genes were not significantly different between RA and OA synovium samples (Fig. 1B-D).
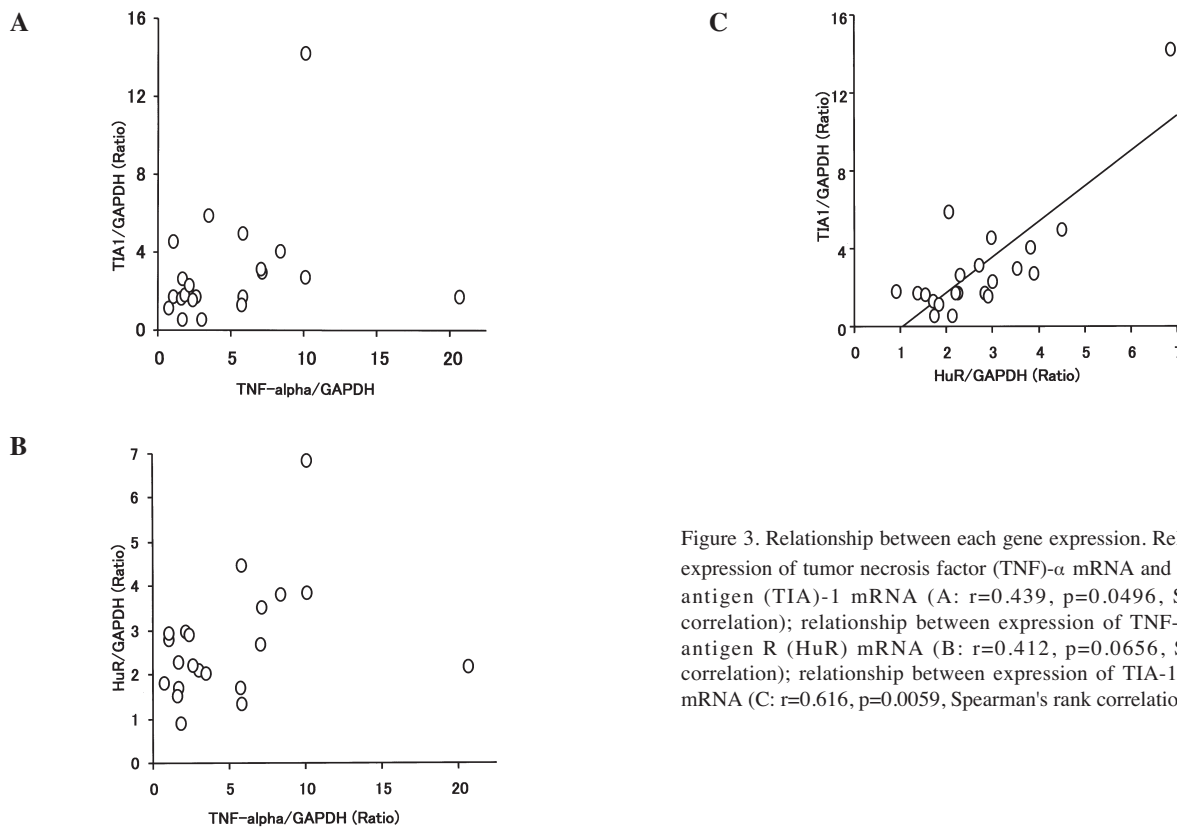
Figure 3. Relationship between each gene expression. Relationship between expression of tumor necrosis factor (TNF)-α mRNA and T cell intercellular antigen (TIA)-1 mRNA (A: r=0.439, p=0.0496, Spearman's rank correlation); relationship between expression of TNF-α mRNA and Hu antigen R (HuR) mRNA (B: r=0.412, p=0.0656, Spearman's rank correlation); relationship between expression of TIA-1 mRNA and HuR mRNA (C: r=0.616, p=0.0059, Spearman's rank correlation).

*Relationship between the expression of ARBP genes in RA synovial tissues and serum CRP levels*. We investigated the relation between expressions of TNF-α, TTP, TIA-1 or HuR genes in synovial tissues and serum CRP levels in RA patients. No significant correlation was observed for any of the genes studied (Fig. 2). Next, we calculated the ratio of TNF-α gene expression and TTP, TIA-1 or HuR gene expression, and compared the ratio with serum CRP. There was no significant relationship between these ratios and CRP (data not shown).

*Relationship between the expression ARBP genes in RA patients*. We questioned whether the expression of an ARBP gene or the TNF-α gene is significantly correlated to the expression of another. There were no significant correlation between the expression of TNF-α and TTP genes, TTP and TIA-1 genes, and TTP and HuR genes (data not shown). TIA-1 gene expression was positively correlated with TNF-α gene expression, but statistical significance was lost when adjusted for multiple comparison (r=0.439, p=0.0496 by Spearman's rank correlation; Fig. 3A). HuR gene expression also tended to be higher in patients with higher TNF-α gene expression (r=0.412, p=0.0656 by Spearman's rank correlation; Fig. 3B), but without statistical significance. Expression of TIA-1 and HuR genes were significantly correlated with each other, even after adjustment for multiple comparison (r=0.616, p=0.0059 by Spearman's rank correlation; Fig. 3C).

## Discussion

The importance of TNF-α, a major inflammatory cytokine, in the pathogenic process of RA is now clear (2,3). The introduction of TNF-α antagonistic biological drugs have remarkably changed the therapeutic strategies to and the

outcome of RA (18,19). However, some patients do not respond to anti-TNF-α therapies (19), and the factors that make interindividual differences in the severity of RA and responsiveness to various therapies are poorly understood. Understanding of these factors, in particular the mechanisms that control TNF-α production, may lead to development of new therapeutic strategies for refractory RA. In this study, we focused on the ARBPs that are important in the post-transcriptional regulation of TNF-α production. Differences in the expression of these molecules may affect the amount of TNF-α produced, and hence, the severity of RA in individual patients.

Rather unexpectedly, expression of the TNF-α gene was significantly higher in the synovial tissues from patients with OA than those from patients with RA. It has been shown that TNF-α plays an important role in the joint destructive process in OA, as well as in RA (20). In addition, while most OA patients were under no medication or on occasional non-steroidal anti-inflammatory drugs only, most RA patients were under more immunosuppressive therapies including DMARDs and steroids. We were unable to obtain healthy synovial tissues, which would have been preferable as controls than synovial tissues from OA patients.

In a previous study, we reported that the TTP/TNF-α gene expression ratio in the synovial tissues from RA patients is lower in patients with higher CRP (16). We considered that this finding may suggest that individual differences of TTP expression may partly account for the differences in the severity of RA. However, this finding was not reproduced in our present study, where a new set of synovial samples were used. We cannot clearly explain the reason for the discrepancy between these studies. One possible explanation is that while many of the RA samples in the previous study were from

who underwent synovectomy, samples in the study were obtained from patients who underwent total joint replacement. Therefore, the disease activity was not high in many of the RA patients included in this study, compared to the RA patients included in our previous study. Theoretically, it would be ideal to measure the expression of these genes in synovial tissues from freshly diagnosed RA patients and study whether they are related to severity, drug responsiveness and prognosis of these patients. An alternative is to measure the amount of these genes in peripheral blood mononuclear cells of RA patients, which is currently underway in our laboratory.

Among the gene expression of ARBPs in the synovial tissues of RA patients, we observed a significant relationship between the expression of HuA and TIA-1. Between the gene expression of TNF-α and the ARBPs examined, tendencies towards positive relationships were observed between TNF-α and TIA-1, and TNF-α and HuR. Although HuR, TIA-1 and TTP all bind to the 3'UTR region of TNF-α mRNA, they exert different functions. HuR acts as a stabilizer (21), TTP a destabilizer (8), and TIA-1 a translational silencer (12). These proteins act in synergy to precisely control the production of TNF-α protein (22). The mechanisms that control the production of these ARBPs are not fully understood. It has been reported that the p38 mitogen-activated protein kinase (MAPK) pathway plays an important role in post-transcriptional regulation of inflammatory genes, and that the mRNAs regulated by p38 share common ARE present in their 3'UTR (23). p38 may stabilize these mRNA by inhibiting the destabilizing action of ARBPs, or by enhancing the production or function of stabilizing ARBPs. It has recently been shown that TTP mRNA is stabilized through a p38 mediated phosphorylation pathway (24). Furthermore, TTP seem to be able to destabilize its own mRNA by binding to ARE of TTP-mRNA (24). The role of p38 in the regulation of production or function of ARBPs, and how an ARBP affect the production of another are interesting but very complicated issues that await elucidation.

The results of our present study may imply that when HuR expression is enhanced by some mechanism, TIA-1 expression also increases to prevent excess production of the TNF-α cytokine. TIA-1 and HuR are also important in the regulation of cyclooxygenase 2 (COX2) production (25). Dysregulated production or RNA-binding of TIA-1 is hypothesized to be related to enhanced COX2 production. Dysregulated TIA-1, therefore, may lead to enhanced TNF-α and COX2 expression (12,25), which in turn may lead to higher disease activity in RA patients.

No significant relationship between the expression of the TTP gene and that of HuR or TIA-1 in the synovial tissues from RA patients was observed in this study. TTP seems to be one of the most important ARBPs in the regulation of TNF-α production, considering the observation that TTP knock-out mice develop severe inflammatory symptoms that are attributable to TNF-α overproduction (9). In this study, we could not show a direct relationship between TTP gene expression and RA disease activity, TNF-α gene expression or expressions of TIA-1 or HuR. However, we believe it will be worthwhile to further investigate the expression of the TTP gene in RA or other inflammatory disorders. These studies may lead to finding a clue to explain the differences in the disease of inflammatory disorders in individual patients, and also may give a starting point to develop new methods to control these disorders.

In conclusion, by studying the expression of TTP, TIA-1 and HuR, the three major ARBPs that post-transcriptionally regulate the production of TNF-α, we found that in synovial tissues of RA patients, the expression of HuR and TIA-1 genes are significantly correlated to each other. Our results may give insight into to mechanisms that determine the disease activity of RA, and may promote further studies that elucidate the pathogenesis of RA.

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## References

1. Laura PH and Barton FH: Pathology of rheumatoid arthritis and associated disorders. In: Arthritis and Allied Conditions: A Textbook of Rheumatology. Koopman WJ (ed). 13th edition. Williams & Wilkins, Baltimore, MD, pp993-1016, 1997.
2. Szekanecz Z and Koch AE: Cytokines. In: Kelley's Textbook of Rheumatology. Ruddy S, Harris ED and Sledge CB (eds). 6th edition. W. B. Sanders Com., Philadelphia, PA, pp275-290, 2001.
3. Keffer J, Probrt L, Cazlaris H, et al: Transgenic mice expressing human tumor necrosis factor: a predictive genetic model of arthritis. EMBO J 13: 4025-4031, 1991.
4. Tak PP and Firestein GS: NF-kappa B: a key role in inflammatory disease. J Clin Invest 107: 7-11, 2001.
5. Palanki MS: Inhibition of AP-1 and NF-kappa B mediated transcriptional activation: therapeutic potential in autoimmune diseases and structural diversity. Curr Med Chem 9: 219-227, 2002.
6. Clark A: Post-transcriptional regulation of pro-inflammatory gene expression. Arthritis Res 2: 172-174, 2000.
7. Anderson P, Phillip K, Stoecklin G and Kedersha N: Post-transcriptional regulation of proinflammatory proteins. J Leukoc Biol 76: 42-47, 2004.
8. Blackshear PJ: Tristetraprolin and other CCCH tandem zinc-finger proteins in the regulation of mRNA turnover. Biochem Soc Trans 30: 945-952, 2002.
9. Taylor GA, Carballo E, Lee DM, et al: A pathogenetic role for TNF alpha in the syndrome of cachexia, arthritis, and auto-immunity resulting from tristetraprolin deficiency. Immunity 4: 445-454, 1996.
10. Dember LM, Kim ND, Liu KQ and Anderson P: Individual RNA recognition motifs of TIA-1 and TIAR have different RNA binding specificities. J Biol Chem 271: 2783-2788, 1996.
11. Kedersha N and Anderson P: Stress granule: sites of mRNA triage that regulate mRNA stability and translatability. Biochem Soc Trans 30: 963-969, 2002.
12. Piecyk M, Wax S, Beck ARP, et al: TIA-1 is a translational silencer that selectively regulates the expression of TNF-alpha. EMBO J 19: 4154-4163, 2000.
13. Atasoy U, Watson J, Patel D and Keene JD: ELAV protein HuA (HuR) can redistribute between nucleus and cytoplasm and is upregulated during serum stimulation and T cell activation. J Cell Sci 111: 3145-3156, 1998.
14. Fan XC and Steitz JA: Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs. EMBO J 17: 3448-3460, 1998.
15. Peng SS, Chen CY, Xu N and Shyu AB: RNA stabilization by AU-rich element binding protein, HuR, an ELAV protein. EMBO J 17: 3460-3470, 1998.
16. Tsutsumi A, Suzuki E, Adachi Y, et al: Expression of tristetraprolin (G0S24) mRNA, a regulation of tumor necrosis factor-alpha production, in synovial tissues of patients with rheumatoid arthritis. J Rheumatol 31: 1044-1049, 2004.
17. Arnett FC, Edworthy SM, Bloch DA, et al: The American rheumatism association 1987 revise criteria for the classification of rheumatoid arthritis. Arthritis Rheum 31: 315-323, 1988.

18. Taylor PC: Anti-TNF-alpha therapy for rheumatoid arthritis: an update. Intern Med 42: 15-20, 2003.
19. Shanahan JC and St. Clair W: Short analytical review. Tumor necrosis factor alpha blockade: a novel therapy for rheumatic disease. Clin Immunol 103: 231-242, 2002.
20. Fernandes JC, Martel-Pelletier J and Pelletier JP: The role of cytokines in osteoarthritis pathphysiology. Biorheology 39: 237-246, 2002.
21. Dean JL, Wait R, Mahtani KR, Sully G, Clark AR and Saklatvala J: The 3' untranslated region of tumor necrosis factor alpha mRNA is target of the mRNA-stabilizing factor HuR. Mol Cell Biol 21: 721-730, 2001.
22. Zhang T, Kruys V, Huez G and Gueydan C: AU-rich element-mediated translation control: complexity and multiple activities of trans-activating factors. Biochem Soc Trans 30: 952-958, 2002.

23. Dean JL, Sully G, Clark AR and Saklatvala J: The involvement of AU-rich element-binding proteins in p38 mitogen-activated protein kinase pathway-mediated mRNA stabilization. Cell Signal 16: 1113-1121, 2004.
24. Tchen CR, Brook M, Saklatvala J and Clark AR: The stability of tristetraprolin mRNA is regulated by mitogen-activated rotein kinase p38 and by tristetraprolin itself. J Biol Chem 279: 32393-32400, 2004.
25. Dixon DA, Blach GC, Kedersha N, *et al*: Regulation of cyclo-oxygenase-2 expression by the translational silencer TIA-1. J Exp Med 198: 475-481, 2003.