

Altered *TNFAIP3* mRNA expression in peripheral blood mononuclear cells from patients with rheumatoid arthritis

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Abstract. The tumor necrosis factor α -induced protein-3 (*TNFAIP3*) gene functions in negative immunoregulation and its single-nucleotide polymorphisms (SNPs) are associated with rheumatoid arthritis (RA) disease. However, its expression level in immune cells from RA patients remains unclear. The aim of the present study was to investigate whether the expression of *TNFAIP3* is changed in patients with RA. Reverse transcription-quantitative polymerase chain reaction analysis was used to determine *TNFAIP3* mRNA expression in peripheral blood mononuclear cells (PBMCs) from patients with RA and healthy controls. *TNFAIP3* expression was decreased in RA patients compared with the healthy controls. The expression level of the *TNFAIP3* gene negatively correlated with the RA score, anti-cyclic citrullinated peptide (CCP) antibody levels and C-reactive protein levels. Furthermore, RA patients with positive results of anti-CCP antibodies had a lower expression of *TNFAIP3* than those without anti-CCP antibodies. In conclusion, the present results suggest that the insufficient expression of the *TNFAIP3* gene in PBMCs may correlate with the diagnosis of RA.

Introduction

Rheumatoid arthritis (RA) is a common chronic inflammatory disease that predominantly affects the small joints of the hands and feet (1). The propagation of inflammation is characterized by migration of mononuclear cells into the local inflammatory

sites, leading to the hyperplasia of fibroblast-like synoviocytes (FLSs) primarily due to resistance to apoptosis and damage to cartilage and bone (2). The chronic inflammation is attributed to breakdown of immune homeostasis, which results in activation of immune cells and elevation of pro-inflammatory cytokines (3). The immune activation leads to deposition of immune complexes and mononuclear cell infiltration into susceptible organs, while the pro-inflammatory cytokines are responsible for maintenance of the immune activation (4). The major mediators of chronic inflammation in RA include tumor necrosis factor α (TNF- α), interleukin (IL)-1 β , IL-6, IL-8 and prostaglandin E2 (5). Induction of pro-inflammatory mediators requires activation of the nuclear factor (NF)- κ B inducing kinase- or inhibitor of NF- κ B (I κ B) kinase-mediated NF- κ B signal transduction pathways (6,7). The transcription factor NF- κ B has been well recognized as a key regulator of inflammation in RA (8-10).

TNF- α -induced protein-3 (*TNFAIP3*) is an important negative immunoregulatory gene. *TNFAIP3* is a dual ubiquitin-editing enzyme, whose expression is induced by a large number of stimuli in a wide variety of cells (11). Evidence from animal models indicates that *TNFAIP3* is a plausible candidate gene in RA susceptibility (11). In *TNFAIP3* knockout mice, its deficiency leads to death shortly following birth by severe inflammation and tissue damage in multiple organs (12,13). In immune cells, overexpression of *TNFAIP3* can terminate NF- κ B signaling transduced from TNF receptors, toll-like receptors, nucleotide-binding oligomerization domain containing 2 receptors or T cell receptors (14,15). The zinc-finger protein A20 is encoded by an immediate early response gene and acts as a potent I κ B signaling pathway (13). Of note, the expression of *TNFAIP3* itself is under the control of NF- κ B, suggesting that *TNFAIP3* is involved in the negative-feedback regulation of NF- κ B activation (13). *TNFAIP3*-deficient mice develop severe inflammation, which includes inflammation of the joints (16).

Although there is an association between single-nucleotide polymorphisms (SNPs) of the *TNFAIP3* gene and RA disease (17-19), the expression level of *TNFAIP3* in immune cells from RA patients is not clear. Therefore, the expression of *TNFAIP3* mRNA was compared in peripheral blood mononuclear cell (PBMC) between RA patients and healthy controls and the association between *TNFAIP3* expression level and disease activity was analyzed in order to elucidate the role of *TNFAIP3* expression in the pathogenesis of RA.

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Materials and methods

Human subjects. A total of 48 patients of Northern Han Chinese descent with RA that was initially diagnosed according to the criteria of the American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) (2010) were enrolled. At the same time, 41 healthy controls were recruited, who were ethnicity, gender- and age-matched with the patients and did not have any rheumatological conditions. RA score, including joint involvement, serology, duration of symptoms and acute phase reactants for diagnostic purposes, was assessed using the RA classification criteria and scoring system revised by ACR/EULAR in 2010 (20). Peripheral bloods were sampled from all the patients prior to the administration of any immunosuppressive drug to exclude the influence of the drug on *TNFAIP3* expression. All the blood samples from the patients and healthy controls were used with informed consent and approval from the Ethics Committee of Qingdao Municipal Hospital (Qingdao, Shandong, China). The characteristics of the patients and healthy subjects are shown in Table I.

Laboratory measurement. For all the RA patients, serum levels of C3, C4, immunoglobulin G (IgG), IgA, IgM, rheumatoid factor (RF) and C-reactive protein (CRP) were analyzed by an automatic nephelometric immunoassay analyzer (Siemens, Munich, Germany). Anti-keratin antibody (AKA) was analyzed by the indirect immunofluorescent assay (Euroimmun AG, Lübeck, Germany). Anti-cyclic citrullinated peptide (CCP) antibodies were detected using the quantitative enzyme-linked immunoabsorbent assay according to the instructions of the manufacturer (Euroimmun AG). For all the subjects, including the patients and healthy controls, erythrocyte sedimentation rates were determined by Westergren test (Monitor-J+ analyzer; Electa Lab Srl, Forli, Italy).

Preparation of PBMCs and extraction of RNA. Peripheral blood was sampled in sodium citrate-containing cell preparation tubes. PBMCs were separated by density gradient centrifugation from the peripheral blood anticoagulated with sodium citrate. Total RNA was extracted from PBMCs (5×10^5) using TRIzol (Takara, Dalian, China) and treated with RNase-free DNase (Sangon Biotech Inc., Shanghai, China) to remove genomic DNA contamination. RNA ($1 \mu\text{g}$) was reversely transcribed to cDNA using a reverse transcription system kit (Sangon Biotech Inc.) for each sample, and subsequently quantified by photometrical measurement.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The expression of *TNFAIP3* mRNA was evaluated by qPCR in triplicate and the level of β -actin mRNA was also detected as an internal control. qPCR was performed using the SYBR-Green I qPCR kit in accordance with the manufacturer's instructions (Takara) in an ABI PRISM® 7500 Sequence Detection System (Perkin-Elmer, Norwalk, CT, USA). Amplification conditions were as follows: 95°C for 10 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 40 sec. Primers were synthesized as described by Li *et al* (3). The primers used were as follows: *TNFAIP3* forward, 5'-CGTCCAGGTTCCAGAACACCATTC-3' and reverse, 5'-TGC

Table I. Demographic characteristics, clinical features and laboratory measurements of the studied subjects.

Characteristics	RA patients (n=48)	Healthy controls (n=41)
Demographic characteristics		
Female, no. (%)	40 (83)	33 (80)
Male, no. (%)	8 (17)	8 (20)
Age, years (range)	35 (19-50)	33 (20-51)
Clinical features		
RA standard rating	8 (6-10)	-
Laboratory measurements		
C3, g/l	1.1 (0.9-1.9)	-
C4, g/l	0.2 (0.1-0.5)	-
IgG, g/l	15.5 (7.0-35.2)	-
IgA, g/l	1.1 (0.7-4.0)	-
IgM, g/l	1.3 (0.4-2.3)	-
RF, IU/ml	352 (28-734)	-
CRP, mg/l	54 (5-198)	-
AKA, no. (%)	13 (27)	-
Anti-CCP Ab, U/ml	305 (58-1,892)	-
ESR, mm/h	58 (10-142)	8 (3-20)

Values are mean (range), unless stated. RA, rheumatoid arthritis; CCP, cyclic citrullinated peptide; AKA, anti-keratin antibody; RF rheumatoid factor; ESR, erythrocyte sedimentation rate; IgG, immunoglobulin G; Ab, antibody.

GCTGGCTCGATCTCAGTTG-3'; and β -actin forward, 5'-GACTACCTCATGAAGATCCTCACC-3' and reverse, 5'-TCTCCTTAATGTCACGCACGATT-3'. Each sample was run in triplicate. The PCR products were run in an agarose gel and were in all cases confined to a single band of the expected size. A melting-curve analysis was also performed to ensure the specificity of the products. The expression of the *TNFAIP3* gene was normalized to β -actin and the relative mRNA expression of *TNFAIP3* was determined using the comparative ($2^{-\Delta\Delta C_t}$) method.

Statistical analysis. Statistical analysis was performed in the SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean \pm standard deviation. The difference in *TNFAIP3* mRNA level between subject groups was analyzed using the Student's t-test independently. Correlations analysis was performed using the Spearman's rank test. $P < 0.05$ was considered to indicate a statistically significant difference. Figs. 1-5 were generated with the GraphPad Prism software, version 5.0 (San Diego, CA, USA).

Results

Laboratory measurements of the patients with RA. The demographic characteristics, clinical manifestation and laboratory measurements in the RA patients are presented in Table I. The

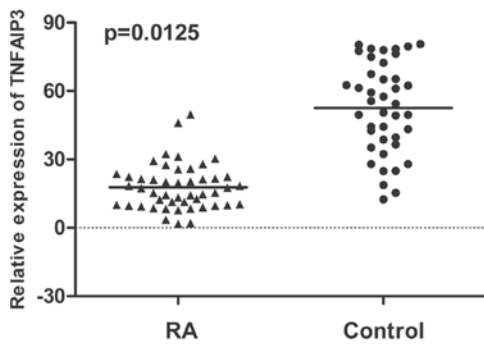


Figure 1. Tumor necrosis factor α -induced protein-3 (*TNFAIP3*) mRNA expression levels from rheumatoid arthritis (RA) patients and controls. Reverse transcription-quantitative polymerase chain reaction was performed to quantify the expression of the *TNFAIP3* gene from RA patients (n=48) and controls (n=41). Horizontal lines indicate means (21.32 in the patient group; 52.58 in the control group). There is a significant decrease in *TNFAIP3* expression in RA patients compared with that of the controls (P=0.0125).

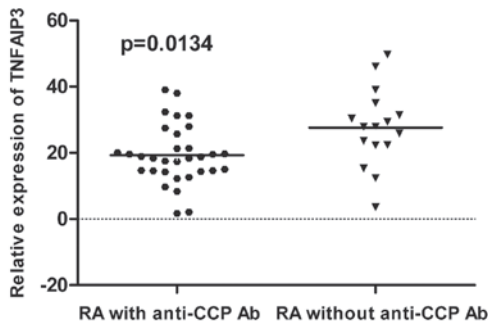


Figure 2. Tumor necrosis factor α -induced protein-3 (*TNFAIP3*) mRNA expression levels in rheumatoid arthritis (RA) patients with and without anti-cyclic citrullinated peptide (CCP) antibodies (Ab). Reverse transcription-quantitative polymerase chain reaction was performed to quantify the expression of the *TNFAIP3* gene from RA patients with (n=32) and without (n=16) anti-CCP Ab. Horizontal lines indicate means (19.44 in RA patients with anti-CCP Ab; 27.67 in RA patients without anti-CCP Ab). There is a significant decrease in *TNFAIP3* expression in RA patients with positive anti-CCP Ab compared with those without anti-CCP Ab (P=0.0134).

positive results of anti-CCP antibodies, RF and AKA in the RA patients were found in 32, 34 and 13 patients, respectively. The mean value of the RA standard rating score was 7.56 (range, 6-10). The mean value of anti-CCP antibodies for the patients was 305 U/ml (range, 58-1,892 U/ml). The mean value of CRP for the patients was 54.4 mg/l (range, 5-198 mg/l).

Quantification of *TNFAIP3* mRNA expression in PBMC from RA patients and healthy controls by RT-qPCR. The expression of *TNFAIP3* mRNA in PBMC from 48 RA patients and 41 gender- and age-matched healthy controls was examined using RT-qPCR. The mean of *TNFAIP3* mRNA expression in PBMC from RA patients (21.32) was significantly decreased compared to healthy controls (52.58) (P=0.0125) (Fig. 1). In addition, the expression of *TNFAIP3* mRNA in RA patients with the positive result of anti-CCP antibodies (19.44) was significantly lower than in those without a positive result of anti-CCP antibodies (27.67) (P=0.0134) (Fig. 2).

Analysis of the associations between *TNFAIP3* mRNA expression and the characteristics or laboratory parameters in the

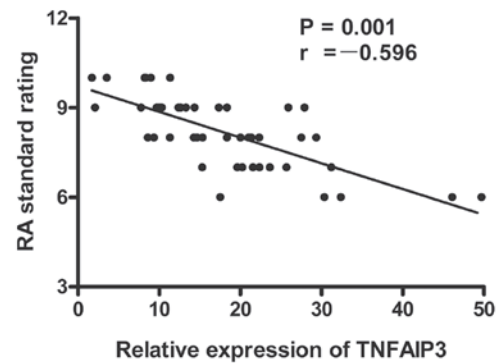


Figure 3. Negative correlation between tumor necrosis factor α -induced protein-3 (*TNFAIP3*) mRNA expression levels and rheumatoid arthritis (RA) standard rating for all the RA patients (n=48).

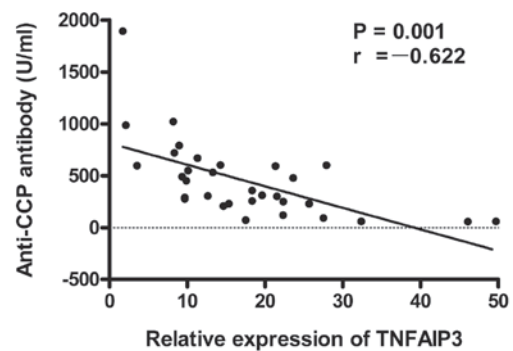


Figure 4. Negative correlation between the tumor necrosis factor α -induced protein-3 (*TNFAIP3*) mRNA expression levels and anti-cyclic citrullinated peptide (CCP) antibody levels for the patients with a positive result of anti-CCP antibody (n=32).

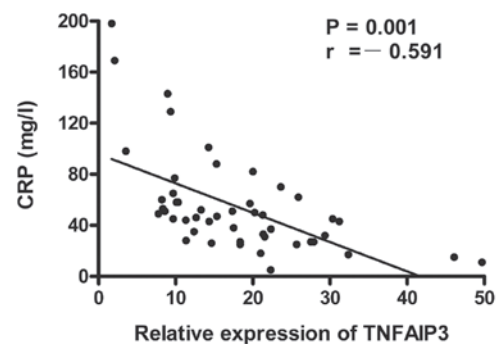


Figure 5. Negative correlation between the tumor necrosis factor α -induced protein-3 (*TNFAIP3*) mRNA expression levels and C-reactive protein (CRP) levels for all the rheumatoid arthritis patients (n=48).

patients with RA. Association of *TNFAIP3* with demographic characteristics, clinical manifestations and laboratory parameters were analyzed. The results showed that the expression level of *TNFAIP3* mRNA was negatively correlated with the RA score ($r=-0.596$, P=0.001; Fig. 3), anti-CCP antibodies ($r=-0.622$, P=0.001; Fig. 4) and CRP ($r=-0.591$, P=0.001; Fig. 5) in RA patients. No statistically significant associations were identified between *TNFAIP3* mRNA expression levels and other characteristics, clinical manifestations or laboratory parameters in the patients with RA.

Discussion

RA is a chronic destructive disease of the joints that is characterized by hyperplastic synovitis due to resistance to apoptosis, infiltration of inflammatory cells into synovial tissue and joint destruction (21). As one of the NF- κ B target genes, *TNFAIP3* has been well established for its negative-feedback mechanism to block NF- κ B activation through its ubiquitin-editing function in response to various inflammatory signaling, including TNF, IL-1 β and lipopolysaccharides (12,13,22,23). Inactivation of *TNFAIP3* by the typical two mechanisms, deletions and inactivation mutations, has been reported in marginal zone lymphomas, Hodgkin's lymphoma, primary mediastinal B cell lymphoma and activated B cell-like diffuse large B cell lymphoma, which may contribute to lymphomagenesis (24,25).

The *TNFAIP3* gene plays a critical role in the negative regulation of immunity. To the best of our knowledge, this is the first study to report the downregulation of *TNFAIP3* expression in RA patients and the negative correlation between *TNFAIP3* expression and RA score, anti-CCP antibodies, CRP. The results suggest that the decreased expression of *TNFAIP3* may be involved in the diagnosis and pathogenesis of RA.

The deficiency of *TNFAIP3* expression may contribute to the pathogenesis of RA through several mechanisms. One of the possible mechanisms is that the insufficient expression of *TNFAIP3* may cause hyperactivation of autoreactive T cell. *TNFAIP3* has ubiquitinating and deubiquitinating enzymatic activity in the TNF receptor-signaling pathway (26). *TNFAIP3* contains an N-terminal domain that belongs to the ovarian tumor superfamily of deubiquitinating cysteine proteases and deubiquitinates K63-linked polyubiquitinated receptor-interacting protein, thereby blocking TNF- α induced NF- κ B signaling (15). Since the *TNFAIP3* gene has a function in limiting the activation of T cell by deubiquitinating mucosa-associated lymphoid tissue 1 to disrupt T cell receptor signaling to NF- κ B (18), using *TNFAIP3* to block the NF- κ B pathway in rheumatoid joints reduces the inflammatory response and the tissue destruction (26). *TNFAIP3* decreased expression may induce T cell hyperactivation and the subsequent tissue damage occurring in RA patients. One of the major transcriptional circuits indicated in joint inflammation is the NF- κ B pathway. Within the joints, activation of NF- κ B leads to the expression of mediators of inflammation that include cytokines, chemokines and adhesion molecules (26). *TNFAIP3* restricts B cell survival and prevents dendritic cell activation (27,28). By contrast, *TNFAIP3* deficiency in B cells enhances B cell proliferation (29) and results in the development of the immune complex. Therefore, the decreased expression of *TNFAIP3* may lead to hyperactivation of B cells and accumulation of the immune complex.

In RA, NF- κ B regulates production of pro-inflammatory cytokines such as TNF- α , IL-1 β and IL-6, cell cycle progression, cell survival, adhesion and inhibition of apoptosis of FLS (30-32). Constitutive activation of NF- κ B in certain autoimmune diseases, including RA, has been reported (33). One possible mechanism in this case is a defector insufficient activity of physiological I κ B pathway, such as *TNFAIP3* involved in a negative-feedback loop. As a result, the decreased expression of *TNFAIP3* in RA patients may also contribute to a high

serum level of proinflammatory cytokines, including TNF- α , IL-1 β and IL-6, which is another characteristic of RA. The major source of the proinflammatory cytokines is monocytes and macrophages. Myeloid-*TNFAIP3*-deficient mice have high levels of inflammatory cytokines in their serum, consistent with a sustained NF- κ B activation and higher TNF- α production by the macrophages (34). Thus, the increased production of proinflammatory cytokines in RA may be partially ascribed to the insufficient expression of *TNFAIP3*.

Multiple polymorphisms in *TNFAIP3* are closely associated with numerous pathological conditions, including systemic lupus erythematosus (SLE), coronary artery disease in type 2 diabetes, psoriasis and RA (11,19,35,36). Several SNPs in the human *TNFAIP3* locus are associated with increased susceptibility to type 1 diabetes, SLE, celiac disease, Crohn's disease, psoriasis, multiple sclerosis and RA (12), suggesting that defects in *TNFAIP3* expression or activity could be involved in the development of specific autoimmune diseases (33). *TNFAIP3* deficiency in myeloid cells triggers erosive polyarthritis resembling RA (33). Consequently, the downregulated expression of *TNFAIP3* may give rise to RA disease, indicating a critical and cell-specific function in the etiology of RA.

The inverse correlation between *TNFAIP3* expression and RA disease severity indicated by RA score, CRP, anti-CCP antibodies or RF suggest that the insufficient expression of *TNFAIP3* may contribute to diagnosis and severity of the disease. The RA classification criteria and scoring system used is a global score, which was developed and validated for diagnostic purposes in RA. Therefore, the negative correlation between the *TNFAIP3* mRNA expression and score in the RA patients means that the low expression of *TNFAIP3* was associated with the diagnosis. Additionally, CRP, anti-CCP antibodies or RF are also indicators of the degree of inflammation and used to help diagnose disease. The level of *TNFAIP3* expression may be an index of disease diagnosis since the *TNFAIP3* mRNA expression was negatively correlated with the CRP, anti-CCP antibodies or RF in the RA patients. In addition, there was a significant difference in *TNFAIP3* expression between patients with a positive result of anti-CCP antibodies and those with a negative result of anti-CCP antibodies. Antibodies to CCPs have been described in patients with RA and these appear to be the most specific markers of the disease. The association of *TNFAIP3* expression with anti-CCP antibodies in RA patients also indicates reduction of *TNFAIP3* expression, and was involved in diagnosis of RA disease. Taken together, the analysis of the association between *TNFAIP3* expression and score, anti-CCP antibodies, RF and CRP further suggest a potential role of decreased *TNFAIP3* expression in diagnosis of RA.

As for the causes of the low expression of *TNFAIP3* in RA, there are several possible explanations. One reason may be polymorphisms of the *TNFAIP3* gene (18,19). In peripheral blood cells, no significant difference was detected in *TNFAIP3* expression according to genotype at the intergenic markers rs6920220 and rs13207033 in RA patients or controls (37). For rs5029937 and rs7749323, *TNFAIP3* expression was reduced in RA patients carrying the risk allele compared with G/G in controls (37). For rs5029937 and rs7749423, the trend demonstrated that *TNFAIP3* expression was reduced in healthy controls, suggesting a possible small allele-specific

effect on *TNFAIP3* expression (37). Therefore, the difference of *TNFAIP3* expression may be partially attributed to polymorphism of the *TNFAIP3* gene. By contrast, to identify susceptibility alleles associated with RA, a genome-wide association study was carried out to identify genetic regions with potential DNA variants determining susceptibility to RA (18,38). Two SNPs were unequivocally identified at 6q23. Although these variants are not located in a gene, they are thought to influence *TNFAIP3* as its nearest gene (~150 kb downstream of *TNFAIP3*), possibly by the presence of potential regulatory DNA elements in this region (12).

The other possible reason is promoter methylation of the *TNFAIP3* gene. *TNFAIP3* is targeted by promoter methylation in certain hematological malignancies (39). Thus, the low expression of the *TNFAIP3* gene in RA may be due in part to its aberrant methylation. This requires further investigation. The present study is limited by the sole RT-qPCR method in the measurement of *TNFAIP3* expression and lacks protein and mechanistic data. Quantification of the *TNFAIP3* protein by flow cytometry or western blot analysis, as well as demonstration of the mechanism, will be an emphasis in future studies.

In conclusion, *TNFAIP3* mRNA expression was down-regulated and inversely correlated with certain disease parameters in RA patients. The results suggest that reduction of *TNFAIP3* expression may correlate with the diagnosis of RA. The data provide the support that the *TNFAIP3* gene may be a target for gene therapy or pharmacological agents of RA disease.

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