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RESEARCH ARTICLE

Genetic and expression changes in TNF-α as a risk factor for rheumatoid arthritis pathogenesis in northeast India

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Abstract. Antitumour necrosis factor-alpha (TNF-α) therapy is used as a clinical intervention for rheumatoid arthritis (RA) but differences exist in response to the treatment which makes the candidature of the screening of TNF-α alteration(s) at genetic and expression levels an important agenda prior to treatment. This study aims to determine the associative role of TNF-α –308G/A polymorphism and differential expression of TNF-α in the pathogenesis of RA. A case–control study where a total of 126 RA patients were enrolled based on ACR-EULAR (2010) criteria, along with 160 community matched age and sex controls over a period of three years. The differential expression level of TNF-α mRNA and protein level was studied and TNF-α –308G/A polymorphism was screened by T-ARMS PCR assay. All statistical analysis was performed using SPSS software. mRNA expression level of TNF-α was upregulated in RA cases (avg. 15.85 ± 9.52 fold) compared to control. TNF-α protein level was found to be higher in RA cases (28.62 ± 7.17 pg/mL) compared to control (23.14 ± 6.91 pg/mL). TNF-α –308 variant GA genotype was higher in RA (46.03%) than in control (25%). The presence of TNF-α –308 variant A allele was associated with increased risk of RA susceptibility (odds ratio (OR) = 2.559 at 95% confidence interval (CI), $P < 0.001$) but not severity (OR = 1.617 at 95% CI, $P = 0.571$). The presence of –308 variant genotype was associated with a higher TNF-α mRNA and protein expression. The presence of TNF-α –308A allele is associated with increased risk of RA susceptibility and differential TNF-α expression, and has prognostic significance. Association of higher TNF-α pro-inflammatory cytokine levels with northeast Indian patients makes them suitable subjects for anti-TNF-α therapy.

Keywords. autoimmunity; rheumatoid arthritis; tumour necrosis factor; proinflammatory cytokine; genetics; case–control study.

Introduction

Rheumatoid arthritis (RA) is a chronic synovial inflammatory arthropathy and has been underlined as a multisystem autoimmune disease (Nemec *et al.* 2008). RA is known to affect individuals of any age group (Alamanos and Drosos 2005) and women are reported to be approximately three times more susceptible to the disease than men. Chronic inflammation of RA is also associated with cardiovascular disease which is found to be responsible for the mortality of RA patients (Dhawan and Quyyumi 2008). RA is a global issue and affects ~0.5–1% population in developed countries (Bax *et al.* 2011). The last decade has seen an explosion in data on RA genetics, with multiple genetic linkage and association studies have confirmed the genetic basis of RA in different populations throughout the world (MacGregor *et al.* 2000; Raychaudhuri 2010) but existing data are still equivocal and inconclusive, which underlines the requirement of further scientific studies and evidences on the aetiology of RA pathogenesis.

Emerging genetic data with an emphasis on the immunogenetic links using molecular and immunological tools have been a centre stage of RA aetiological studies universally, with several immunologically important signalling pathways being screened or targeted for RA treatment (Messemaker *et al.* 2015) with variable results. Cytokines play an essential role in articular destruction in association with inflammation. Tumour necrosis factor-alpha

(TNF-α) is a potent pleiotropic proinflammatory cytokine, produced mostly by activated monocytes, macrophages and T-lymphocytes and promotes inflammatory responses that are important in the pathogenesis of RA. A high concentration of TNF-α is found in the synovial fluid of RA patients and is associated with erosions of the bone (Hassan *et al.* 2011). Positive data have been accumulated from *in vitro, in vivo* and patient-based clinical investigation studies. Gheita *et al.* (2015) have rationalized the use of anti-TNF-α antagonists such as infliximab (IFX), etanercept (ETN), adalimumab (ADA), golimumab (GOLI) and certolizumab pegol (CZP) for the treatment of RA which have definitely correlated with clinically relevant improvement in a subpopulation of RA cases. But certain patients exhibit a lack of response to anti-TNF-α therapy and clinical administration of anti-TNF-α antagonists may have adverse effects on these patients and predispose these cases to infections of multiple aetiologies which are unwanted. Therefore, studies on the importance and specificity of differential TNF-α expression in RA susceptibility and severity in specific geographical population may prove vital for efficient use of available anti-TNF-α antagonists in specific population and individuals and massive reduction in the possible adverse side effects.

The *TNF* gene is located on the short arm of chromosome 6 (6p21), and the promoter region of *TNF* gene is an extremely polymorphic locus which is enriched with a number of single nucleotide polymorphisms (SNPs). A transitional polymorphism from G to A nucleotide at position 308 (also known as *TNF-α* –308 or rs1800629) of the promoter region of *TNF-α* gene is associated with increased levels of TNF-α expression (Hajeer and Hutchinson 2000; Elahi *et al.* 2009). The rare A allele of *TNF-α* gene is associated with worse clinical outcomes in different diseases like sepsis and multiple trauma in comparison to the G allele (Menges *et al.* 2008; Teuffel *et al.* 2010). Recent studies have provided evidences that TNF-α polymorphism plays an important role in RA disease although there are many discrepancies for different populations (Danis *et al.* 1995; Gambhir *et al.* 2010) with no scientific studies or data available from ethnically distinct northeast Indian population. Based on the existing literature and lacunae, presented here is a novel study evaluating the importance of profile changes in TNF-α expression and genotype as risk factors of RA susceptibility and severity in northeast Indian patients.

Materials and methods

Patient enrolment and stratification

A total of 126 RA patients who were clinically diagnosed with RA were enrolled in the present study with all clinical details. The patients were recruited from the Rheumatology OPD of Gauhati Medical College and Hospital, Guwahati, India based on ACR and EULAR (2010) criteria. The RA cases were further clinically stratified as mild RA cases (ACR and EULAR (2010) criteria score: 5–7; $n = 14$) and severe RA cases (ACR and EULAR (2010) criteria score: 8–10; $n = 112$). A group of 160 age and sex matched unrelated blood donors were randomly enrolled as healthy controls. The healthy control individuals were free of any clinical evidence of autoimmune diseases or familial history of RA. All RA patients and healthy controls were from the northeast region of India and informed written consent was obtained from both the patients and the healthy donors prior to enrolment. The study was approved by the institutional ethics committee of the participating institutes and is in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Differential mRNA and protein expression analysis

Blood samples were collected from the healthy control individuals and patients in EDTA and clot vials. The serum was separated and these samples were used for enzyme-linked immunosorbent assay based study for the cytokine (Merck, EZHTNFA). Total RNA was isolated from the samples using the standard Trizol method, quantified using nanodrop spectrophotometry and was converted to c-DNA using commercially available c-DNA synthesis kit (Applied Biosystems, Foster City, USA) following the manufacturer's protocol. The c-DNA was used to determine the level of TNF-α by qPCR (7500 Fast Real-Time PCR System, Applied Biosystems) using SyBr green chemistry and *β-actin* gene as the internal normalization control. The difference in expression level of TNF-α was evaluated on the basis of fold change calculated based on $2^{-\Delta\Delta Ct}$ formulae.

TNF-α –308 genotyping analysis

Genomic DNA was extracted from collected whole blood using the standard phenol–chloroform method. The quality of extracted genomic DNA was analysed by nanodrop spectrophotometry. The *TNF-α* –308 G/A locus was amplified in a total volume of 20 μL using allele-specific PCR (amplification refractory mutation system (ARMS)-PCR) based assays (table 1) and was analysed by agarose gel electrophoresis. The interpretation of the TNF-α genotype was based on the amplification sizes as stated in table 1.

Statistical analysis

All statistical analyses were performed by the standard methods using SPSS V13.0 software (SPSS, Chicago, USA). The significance was described as Pearson *P* value.

Table 1. Primer sequences for ARMS-PCR based amplification of SNP of *TNF-α* gene (–308) with their expected product size used for amplification of *TNF-α* –308 genotype.

SNP (location)	Primer name	Primer sequence (5′ → 3′)	Product size (bp)
rs1800629 (chr6: 31575254)	Forward inner primer (G allele)	CAATAGGTTTTGAGGGGCAGGG	Product size for G allele: 198
	Reverse inner primer (A allele)	CTGGAGGCTGAACCCCGTACT	Product size for A allele: 139
	Forward outer primer	GAAGCCCCTCCCAGTTCTAGTTCTA	Product size of two outer primers: 294
	Reverse outer primer	GCACCTTCTGTCTCGGTTTCTTCT	

Table 2. Details of the enrolled subjects for the present study.

Diagnosis	ACR and EULAR (2010) criteria score	*n*	Avg. age (years)	Male	Female
Control	NA	160	24.63 ± 10	24	136
Mild RA	5–7	14	45.96 ± 12	4	10
Severe RA	8–10	112	46.54 ± 12	20	92

Associations between *TNF-α* –308 G/A polymorphism and RA susceptibility, and RA severity were calculated as odds ratio (OR) with 95% confidence intervals (CIs); a two-tailed $P < 0.05$ was considered statistically significant.

Results

Demographical and clinical profile

The present study was based on the northeast Indian population, with clinically proven RA patients being enrolled under the supervision of a registered medical practitioner. The details of the enrolled patients are shown in table 2.

Differential mRNA and protein expression analysis in RA cases

The differential TNF-α mRNA expression data evaluated by real-time PCR showed an increased expression of TNF-α in RA cases (avg. 15.85 ± 9.52 fold) in comparison with control. However, the difference in expression pattern was not observed between mild and severe RA cases ($P = 0.970$) thus clearly indicating that transcriptional activation of TNF-α has a major role in disease susceptibility (figure 1). The typical range of serum TNF-α is in the range of 5–27 pg/mL. The TNF-α protein level data show a clear upregulation in RA cases (28.62 ± 7.17 pg/mL) as compared to controls (23.14 ± 6.91 pg/mL) ($P = 0.428$), suggesting a role in disease susceptibility. The serum TNF-α levels were nonsignificantly higher in severe RA cases compared to mild RA cases ($P = 0.879$) (figure 1), therefore underlining the importance of differential TNF-α expression in RA pathogenesis.

TNF-α –308 promoter SNP distribution analysis

A nucleotide change at position –308 (G to A) in the promoter region of *TNF-α* gene is associated with increased levels of TNF-α expression, and was thus evaluated by allele-specific PCR (ARMS-PCR) followed by agarose gel electrophoresis based analysis (figure 2). The difference in genotype distribution in the enrolled subjects is represented in tables 3 and 4.

The presence of either wild-type GG genotype or heterozygous AG genotype was observed in the enrolled subjects. The distribution of heterozygous AG genotype was significantly higher in RA cases compared to control ($P < 0.001$), and was associated with increased risk of RA susceptibility (OR = 2.559 (1.551–4.222) at 95% CI, $P < 0.001$). The presence of heterozygous variant AG genotype was also higher in severe RA cases compared to mild cases ($P = 0.413$), and was found to be associated with increased risk to RA severity (OR = 1.617 (0.510–5.129) at 95% CI, $P = 0.571$). When the difference in expression of TNF-α was analysed based on TNF-α –308 genotype distribution, the AG variant genotype was associated with significantly increased TNF-α mRNA ($P = 0.002$) and protein expression ($P = 0.009$) (figure 3); which underlines the prognostic significance of TNF-α –308 genotype in RA pathogenesis.

Discussion

RA is a complex multifactorial systemic disease, which involves chronic inflammation induced by interplay of various cytokines that contribute significantly in the development and progression of the disease (Mosaad *et al.* 2011). An imbalance between proinflammatory and anti-inflammatory cytokine activities favours the induction

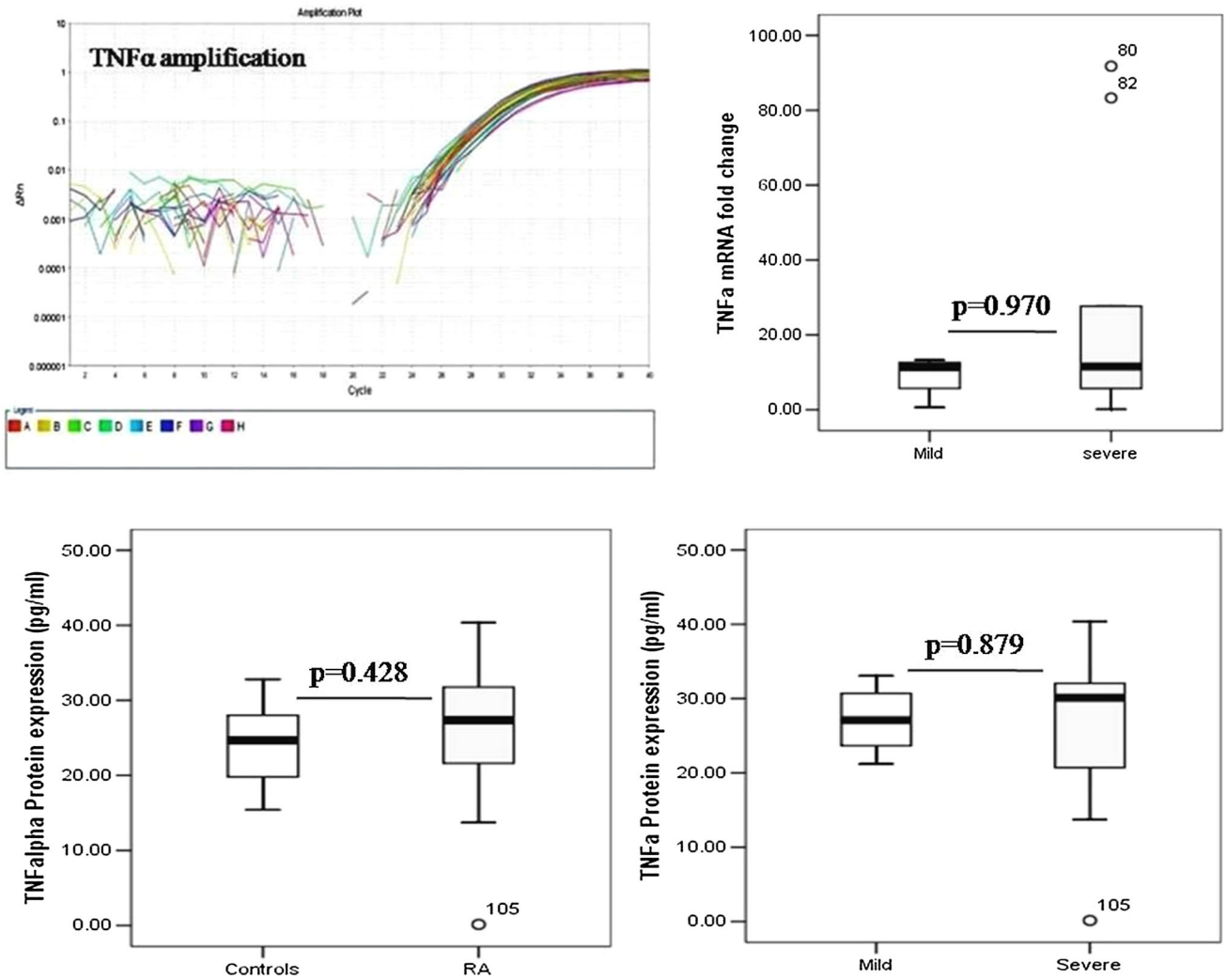

Figure 1. Top panel shows the real-time PCR based amplification for TNF-α gene c-DNA and difference in fold change in mild and severe RA cases compared to control. Bottom panel presents the difference in protein expression in control and RA cases, as well as between the mild and severe RA cases.

of auto-immunity and chronic inflammation ultimately leading to joint deformity, crippling and loss of function. TNF-α, being a proinflammatory cytokine, is considered to have a major role in disease induction. Over-production of TNF-α contributes to increased reactive oxygen species (ROS) release in RA patients, leading to tissue damage associated with inflammation (Hassan *et al.* 2011). TNF-α has been demonstrated to regulate multiple cytokines, vascular endothelial growth factor (VEGF) production, recruitment of immune and inflammatory cells into joints, angiogenesis and reduction of blood levels of matrix metalloproteinases which are known to be associated with RA pathogenesis (Feldmann and Maini 2001). Immunotherapy has markedly improved treatment outcomes in RA. TNF-α antagonists, such as infliximab (IFX), etanercept (ETN), adalimumab (ADA), golimumab (GOLI) and certolizumab pegol (CZP) have been widely used for the treatment of RA (Ma and Xu 2010). Although TNF-α antagonist based therapy has been reported to impart marked improvements in the disease conditions in RA patients, but adverse or lack of response has been documented in a subpopulation of RA patients which is a critical scenario. Therefore determining the candidature of the RA patients for anti-TNF-α therapy is a definite prerequisite. Molecular diagnostics based on differential expression of TNF-α and screening of genetic alterations in the TNF-α gene associated with differential TNF-α expression may be a useful prognostic tool for stratification of RA patients for candidature for anti-TNF α therapy in a given geographical population. A transitional polymorphism from G to A nucleotide at position 308 of the promoter region of *TNF-α* gene is associated with increased levels of TNF-α expression. The variant A allele is associated with higher levels of TNF transcript than the G allele which is considered to be the ancestral allele (Hajeer and Hutchinson 2000; Elahi *et al.* 2009). Therefore, the present study involving RA cases from northeast Indian population was undertaken to screen the

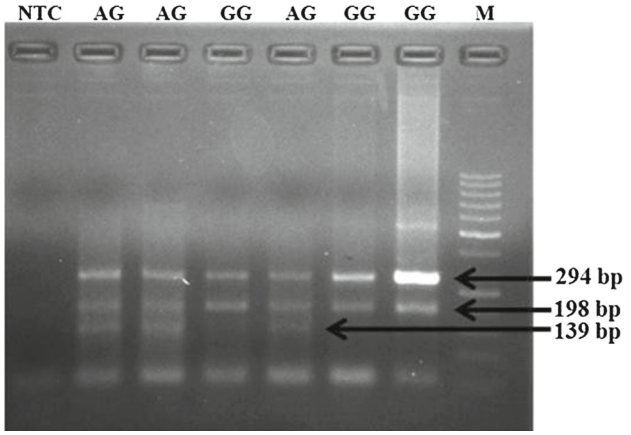

Figure 2. Representative panel of agarose gel electrophoresis results for TNF-α (–308) SNP showing the presence of different amplified band patterns indicating the presence of either wild type (198 bp) or variant (homozygous (139 bp) or heterozygous (198+139 bp)) genotypes. The outer amplified product of 294 bp was common for both the genotypes.

role of genetic alteration in TNF-α gene and associated differential TNF-α expression in the pathogenesis of RA in northeast Indian subjects, and predict the efficacy of anti-TNF-α drugs in the given population.

The ethnically distinct northeast Indian population has been found to be predisposed to RA with a prevalence rate of 0.2–0.4% (BJD India Copcord Survey 2001). A lacuna exists in terms of the role of difference in distribution of genetic alterations in TNF-α gene and linked differential TNF-α expression in RA pathogenesis from northeast India. Explorations of differential expression of TNF-α were suggestive of increased fold change at the mRNA level and serum protein levels being associated with RA susceptibility compared to controls in the northeast Indian population. A study with the north Indian population (Jahid *et al.* 2017) showed a positive correlation between circulating TNF-α levels and mRNA expression in RA patients as high as 4.5 times more than in healthy individuals. Moreover, TNF-α level was also reported to be higher in the serum of RA patients as compared to healthy controls from other groups also (Robak *et al.* 1998).

The differential TNF-α expression was comparative between mild and severe RA cases, but interestingly difference in TNF-α expression at both mRNA and serum protein levels was observed in a sub-population of cases belonging to both the mild and severe groups as well as in healthy subjects control group, which indicated that probably the underlying TNF-α genotype plays some influential role in expression status of the gene, and in turn disease pathogenesis. The –308 polymorphism is one of the most investigated variant in association with RA in different populations (Cvetkovic *et al.* 2002). However, the association of –308 polymorphism with susceptibility and severity to RA has been conflicting and equivocally documented (Brinkman *et al.* 1995; Wilson *et al.* 1997). Therefore, studying other relevant polymorphisms at the promoter region like -238 (G/A) or at the first intronic region of *TNF-α* +489 (G/A) as reported in the available literature (van Krugten *et al.* 1999; Murdaca *et al.* 2014) becomes an interesting and important agenda to validate the fact whether these polymorphisms may influence TNF-α profile changes and maybe associated with RA pathogenesis through futuristic studies. These futuristic studies on different TNF polymorphisms may crucially help for stratifying patients predisposed to RA susceptibility and severity as well as in selecting the specific anti-cytokine drug therapy for selective group of patients, thereby underlining their prognostic and biomarker significance.

The data from this study strongly underlined the prognostic significance of 'A' allele in RA cases, as the heterozygous AG genotype was associated with increased risk of RA susceptibility (OR = 2.559, $P < 0.001$) and severity (OR = 1.617, $P = 0.571$), and more importantly was significantly associated with increased TNF-α mRNA ($P = 0.002$) and protein expression ($P = 0.009$). These data are in agreement and concurrence with studies involving Spanish population (Edith *et al.* 2008), Czech population (Brinkman *et al.* 1995; Nemec *et al.* 2008) and Egyptian population (Gheita *et al.* 2015). But the present data are contrary to the findings on Han Chinese population (Li *et al.* 2015), Southeast Asians (Yen *et al.* 2003) and in Finnish populations (Maury *et al.* 2003) which reported a negative association of G → A polymorphism of TNF-α gene in RA pathogenesis; as well as existing data from other global pockets (Brinkman *et al.* 1995; Wilson *et al.* 1997; Lacki *et al.* 2000) or from other parts of India (Gambhir *et al.* 2010) who did not findany association of TNF-α –308 G/A polymorphism with RA pathogenesis. The aforementioned differences with respect to global or national data maybe due to ethnic differences among the populations under study, and therefore underlines the significance of studying the TNF-α genotype and differential expression for stratifying RA cases which may eventually benefit from the existing anti-TNFα therapies independently or in combinatorial therapies. This may definitely minimize the complications and side-effects of the standard disease modifying antirheumatoid drugs (DMARDs) still under use in any given population.

In conclusion, within the limitations, this first study from ethnically distinct northeast Indian population is suggestive of the role of TNF-α genotype and linked differential expression in RA disease pathogenesis. This study underlines the prognostic significance of TNF-α –308A allele in RA cases for clinical stratification and selecting drug regime. Since higher TNF-α proinflammatory cytokine levels were found to be associated with RA in northeast Indian patients, this study could be a possible solution to choose this population as suitable subjects for anti-TNF-α therapy and its benefits.

Table 3. TNF-α genotype distribution in enrolled controls and RA cases.

Sample type	Sample size (*n*)	TNF-α –308 genotype			Variant allele	*P* value	OR at 95% CI
		Wild-type	Heterozygote	Homozygote			
Control	160	120 (75.00)	40 (25.00)	0 (0.00)	40 (25.00)	Ref.	Ref.
RA	126	68 (53.97)	58 (46.03)	0 (0.00)	58 (46.03)	< 0.001	2.559 (1.551–4.222)

Cases represented as numbers (% age).

Table 4. Distribution of TNF-α genotype in mild and severe RA cases.

RA cases	Sample size (*n*)	TNF-α –308 genotype			Variant allele	*P* value	OR at 95% CI
		Wild type	Heterozygote	Homozygote			
Mild	14	9 (64.29)	5 (35.71)	0 (0.00)	5 (35.71)	Ref.	Ref.
Severe	112	59 (52.68)	53 (47.32)	0 (0.00)	53 (47.32)	0.413	1.617 (0.510–5.129)

Cases represented as numbers (% age).

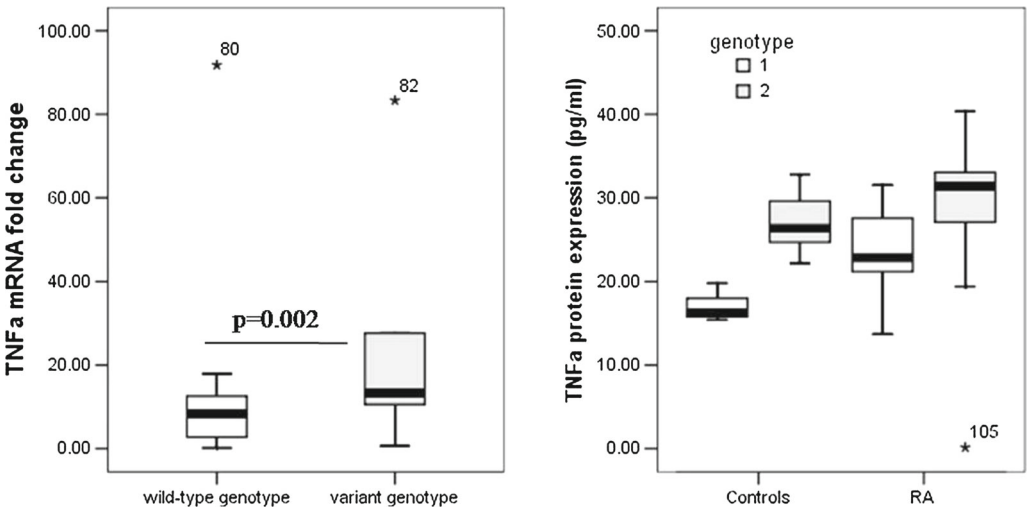

Figure 3. Box-plot analysis showing significantly increased TNF-α expression in subjects with variant genotype.

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