Polymorphism in the TNF-alpha gene promoter at position -1031 is associated with increased circulating levels of TNF-alpha, myeloperoxidase and nitrotyrosine in primary Sjögren’s syndrome

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Abstract
Objectives
Tumour necrosis factor-alpha (TNF-alpha) is a pro-inflammatory cytokine which is associated with the pathogenesis of many inflammatory diseases. The aim of this study was to investigate the effect of TNF-alpha -1031 gene polymorphism on circulating TNF-alpha, myeloperoxidase (MPO) and nitrotyrosine (NT) levels in primary Sjögren’s syndrome patients.

Methods
TNF-alpha-1031 T/C gene polymorphism was evaluated in 65 Sjögren’s syndrome patients and 58 age and gender matched controls via 5’ nuclease PCR analysis. Plasma TNF-alpha and NT levels were analysed by ELISA while MPO activity, total nitrate/nitrite and glutathione (GSH) levels were measured by spectral analysis.

Results
TNF-alpha -1031 C carrier genotype frequency was significantly higher (p=0.045) in Sjögren patients compared to controls (23.1 vs. 10.3%, OR=2.83, 95% CI=0.27-7.8). Plasma TNF-alpha concentration and NT levels were also significantly higher in Sjögren patients with -1031 C carrier genotype compared to patients with TT genotype. Sjögren patients showed a significant increase in plasma MPO activity which correlated with both TNF-alpha and NT levels in subjects with -1031 C carrier genotype assessed by linear regression analysis. TNF-alpha-1031 T/C gene polymorphism had no effect on plasma nitrate/nitrite and GSH levels which were significantly decreased in Sjögren’s syndrome patients compared to controls.

Conclusion
Polymorphism in the TNF-alpha gene promoter at position -1031 is associated with increased circulating levels of TNF-alpha which is correlated with increased plasma MPO activity and protein nitration in Sjögren’s syndrome.

Key words
Sjögren’s syndrome, tumour necrosis factor-alpha, myeloperoxidase, nitrotyrosine.
**Introduction**

Sjögren’s syndrome (SS) is a chronic, autoimmune disease which is characterised by lymphocytic infiltration of exocrine glands, gradually progressive lacrimal and salivary dysfunction, xerophthalmia and xerostomia as the major complaints. Fatigue, arthritis, arthralgias, Raynaud’s phenomenon, autoimmune thyroiditis, primary biliary cirrhosis, peripheral/central nervous system disease, pulmonary disease and vasculitis are some of the extraglandular features of SS. Sjögren’s syndrome patients have substantially higher risk to develop non-Hodgkin’s lymphoma than the general population (1). Primary SS is an idiopathic disease while secondary SS occurs in the presence of another autoimmune disorder such as rheumatoid arthritis or systemic lupus erythematosus (2). The estimated worldwide prevalence of SS is 0.2-3.9 % with a female to male ratio of 9:1 (3).

The pathophysiology of SS is not yet fully understood. Autoimmune epithelitis is the given name for SS to explain pathophysiology and the main target of inflammatory reactions created in the course of disease (4). Tumour necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine which has important mediatory functions in the development of autoimmune response and in destruction of salivary and lacrimal glands (5). Tumour necrosis factor-alpha is produced by monocytes, CD4+ T cells and epithelial cells (6). Expression of TNF-alpha observed in minor salivary gland duct cells in patients with SS promotes influx of mononuclear cells into salivary glands (7). Tumour necrosis factor-alpha receptors (TNFR)-p55 and -p75 are expressed at higher levels in labial salivary glands of SS patients (8). Expression of TNF inhibitor gene in lacrimal glands of rabbits with induced autoimmune dacryoadenitis has been shown to improve tear production (9). Recently published genome-wide association study on two different cohorts of primary SS, each from two different countries, have documented the association of disease with TNF superfamily (TNFSF4) gene (10).

The human TNF-alpha gene lies within the class III region of the major histocompatibility complex (MHC) and individual differences in the ability to produce TNF-alpha could be linked to the HLA genotype or to other polymorphic markers in this region (11). The TNF gene is tightly regulated at the transcriptional, post-transcriptional, translational and post-translational levels. Several single nucleotide polymorphisms (SNPs) have been described in the 5′ promoter region of the TNF-alpha gene involved in transcriptional regulation (12). The SNP occurring at position -1031 of the TNF gene is located at the 5′ promoter/enhancer region and is characterised by a cytosine to thymine substitution (11). The -1031 T/C SNP reported in the TNF-alpha gene effects transcriptional regulation. Indeed, the presence of -1031 T/C SNP has been linked to increased circulating TNF-alpha levels in several studies (11-14). Tumour necrosis factor-alpha stimulates neutrophil activation, degranulation, adhesion and chemotaxis during inflammation. Reported studies have shown a relationship between increased plasma levels of TNF-alpha and myeloperoxidase (MPO) in patients with chronic heart failure (15). Myeloperoxidase catalyses tyrosine nitration in proteins and thus nitrotyrosine (NT) is a marker of nitrosative stress that can be formed by an MPO-dependent process. Nitrotyrosine formation can also occur by nitric oxide synthase (NOS)-dependent pathways. Previous studies have reported induction of inducible NOS (NOS-2) and cytotoxic nitrogen-related oxidant formation in conjunctival epithelium of SS patients (16).

In connection with previous studies this study aimed to determine the relevance of -1031 T/C polymorphism on TNF-alpha levels in patients with SS. The impact of increased TNF-alpha levels on plasma MPO activity, protein nitration and total glutathione (GSH) levels were further analysed in TNF-alpha polymorphic genotypes in both primary SS and controls.

**Materials and methods**

**Subjects and diagnosis of primary Sjögren's syndrome**

Sixty-five patients with primary SS...
Blood samples were collected in sterile EDTA tubes and genomic DNA was extracted from venous blood by MN NucleoSpin® Blood kit according to manufacturer’s instructions (Macherey-Nagel, Düren, Germany). Each isolated DNA was tested for -1031 T/C SNP with patented wild-type and mutant type master mixes (Dr Zeydani Life Sciences, Ankara, Turkey). This system provides reagents in a ready-to-use master-mix format which has been specifically adapted for 5’ nuclelease PCR SNP analyses. The test system is designed for use with sequence specific primers and probes. The fluorescence of mutation analysis is FAM. Each master-mix also contains an internal control targeting glucose-6-phosphate dehydrogenase labelled with HEX/JOE dye. For each sample, 20.5 μl master mix and 0.3 μl hot start tag DNA polymerase was pipetted and 4.5 μl DNA (~60-100 ng) was added into respective wells. During the PCR reaction, the DNA polymerase cleaved the probe at the 5’ end and separated the reporter dye from the quencher dye only when the probe hybridised to the target DNA. This cleavage resulted in the fluorescent signal generated by the cleaved reporter dye which was monitored by real-time PCR detection system (LightCycler® 480 System, Roche Diagnostics Mannheim Germany).

Measurement of tumour necrosis factor-alpha levels
Plasma TNF-alpha levels were determined by RayBio® Human TNF-alpha ELISA kit according to manufacturer’s instructions (RayBiotech Inc. GA, USA).

Measurement of plasma myeloperoxidase activity
Myeloperoxidase activity was determined by adding an aliquot of plasma sample to 43 mM NaH₂PO₄ (pH 5.4), 1.2 mM tetramethylbenzidine (TMB; Merck, Darmstadt, Germany), and 100 μM H₂O₂ (Sigma-Aldrich Chemie, Steinheim, Germany). Absorbance kinetics was assessed spectrophotometrically at 655 nm by H₂O₂-dependent oxidation of TMB [εₗ₁ = 3.9 x 10³ per (M⁻¹ cm⁻¹)]. One unit of enzyme activity was defined as the amount of enzyme that caused the oxidation of 1 μmol TMB per minute at 25°C.

Measurement of plasma nitrotyrosine levels
Plasma nitrotyrosine content was measured via ELISA using a commercial kit (Northwest Life Science, Vancouver, WA). Antigen captured by a solid phase monoclonal antibody (nitrated keyhole limpet haemocyanin raised in mouse) was detected with a biotin-labelled goat polyclonal anti-nitrotyrosine. A streptavidin peroxidase conjugate was then added to bind the biotinylated antibody. A TMB substrate was added and the yellow product was measured at 450 nm. A standard curve of absorbance values of known nitrotyrosine standards was plotted as a function of the logarithm of nitrotyrosine standard concentrations using the GraphPad Prism Software program for windows version 5.03. (GraphPad Software Inc). Nitrotyrosine concentrations in the samples were calculated from their corresponding absorbance values via the standard curve.

Measurement of plasma nitrite/nitrate levels
Plasma samples were transferred to an ultrafiltration unit and centrifuged through a 10-kDa molecular mass cut-off filter (Amicon, Millipore Corporation, Bedford, MA) for 1 hr to remove proteins. Analyses were performed in duplicate via the Greiss reaction using a colorimetric assay kit (Cyanam Chemical, Ann Arbor, MI).

Measurement of plasma glutathione levels
Total GSH levels were measured by a commercially available GSH assay kit (Cyanam Chemical, Ann Arbor, MI). Plasma was deproteinated in 10% metaphosphoric acid (Sigma-Aldrich, Steinheim, Switzerland) and TEAM reagent (4 M solution of triethanolamine prepared in water) was added to deproteinated plasma samples. Each sample was concentrated by lyophilisation via Savant DNA 120 speed vac concentrator (Thermo Scientific, IL, USA) and reconstituted with MES buffer (0.4 M 2-ethanesulphonic acid, 0.1 M phosphate and 2 mM EDTA, pH 6.0) to one third of its original volume. Oxidised disulfide dimer of GSH (GSGG) was reduced to GSH by GSH reductase in the assay.

cocktail of the kit containing DTNB (5,5’-dithiobis-2-nitrobenzoic acid, Ellman’s reagent), glucose-6-phosphate dehydrogenase, GSH reductase, NADPH and glucose-6-phosphate. The sulfhydryl group of GSH reacts with DTNB to give a yellow colored 5-thio-2-nitrobenzoic acid (TNB) which was measured at an absorbance of 405 nm. The values of total GSH for each sample were calculated from their respective slopes using a GSH standard curve.

Statistical Analysis
Statistical analyses were performed by using SigmaStat statistical software version 2.0. Two-way ANOVA test was applied to calculate p-values for biochemical results, MPO activity, NT, nitrite/nitrate levels. Multiple logistic regression analysis was applied to evaluate odds ratio (ORs) and 95% confidence interval (95% CI) to estimate the relationship between polymorphism and pSS. The correlation between TNF-alpha, NT levels and MPO activity were evaluated by linear regression analysis. A p-value <0.05 was considered statistically significant.

Results
Subject characteristics
The mean age of pSS patients and control subjects were 49.3±9.6 and 47.54±9.6 years, respectively. Sjögren’s syndrome-associated antigen A was 64.5 and 33% positive in female (n=62) and male (n=3) Sjögren patients, respectively. Sjögren’s syndrome-associated antigen B was found positive in females at a ratio of 29%. Focus score ≥1 was observed in 41.9% of female Sjögren patients. Anti-nuclear antibody was 50 and 33% positive in female and male pSS patients, respectively.

Tumour necrosis factor-alpha gene polymorphism genotype
Table I shows genotypes and allele frequencies of TNF-alpha -1031 T/C gene polymorphism in SS patients and controls. TNF-alpha -1031 C carrier genotype frequency was significantly higher (p=0.045) in pSS patients compared to control group (23.1 vs. 10.3%, OR= 2.83, 95% CI=0.27–7.8). The TT genotype was observed in 49 pSS patients (75.4%) and 52 control subjects (89.7%). There was only one subject (1.5%) with CC homozygote genotype in Sjögren’s syndrome.

Biochemical analysis
Biochemical measurements performed on pSS patients and controls are shown in Table II. Two way analysis of variance followed by Tukey test was performed to evaluate statistical significance of biochemical data among pSS patients and controls. No significant difference was observed in haemoglobin, haematocrit and white blood cells count.

Fig. 1. Box plot graph data of plasma TNF-alpha levels in Sjögren’s syndrome and control group. The boundary of the box closest to zero indicates 25th percentile, the line within the box marks median and the boundary of the box farthest from zero indicates 75th percentile. Whiskers above and below the box indicate 90th and 10th percentiles. *, p=0.011 Sjögren TC vs. control TC; **, p<0.001 Sjögren TT vs. Sjögren TC.

Table I. TNF-alpha polymorphism genotype in primary Sjögren’s syndrome and controls.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Genotype</th>
<th>Sjögren’s syndrome (n=65)</th>
<th>Control (n=58)</th>
<th>OR (95% CI)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1031</td>
<td>TT</td>
<td>49 (% 75.4)</td>
<td>52 (%89.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>15 (%23.1)</td>
<td>6 (%10.3)</td>
<td>2.83 (0.27–7.8)</td>
<td>0.045</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>1 (%1.5)</td>
<td>0 (%0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table II. Biochemical results of Sjögren’s syndrome and control group.

<table>
<thead>
<tr>
<th></th>
<th>Sjögren’s syndrome (TT (n=49) , TC (n=15))</th>
<th>Control group (TT (n=52) , TC (n=6))</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-Reactive protein (mg/mL)</td>
<td>7.2±7.2*  11.5±14.3*</td>
<td>4.6±4.4  4.2±1.4</td>
</tr>
<tr>
<td>Haemoglobin (mg/mL)</td>
<td>12.7±1.3  12.5±1</td>
<td>13±0.8  13±1.2</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>38.6±3.3  37.8±3</td>
<td>39.1±2.1  38.6±3.2</td>
</tr>
<tr>
<td>White blood cells (10³/mm³)</td>
<td>7.8±2.4  7.8±1.5</td>
<td>7.2±1.9  5.9±1.3</td>
</tr>
<tr>
<td>Erythrocyte sedimentation rate (mm/hour)</td>
<td>29±22*  35±18**</td>
<td>16.9±10.7  12±9.6</td>
</tr>
</tbody>
</table>

*, p=0.012 vs. control; ***, p<0.001 vs. control.
and white cell blood values between the two experimental groups.

**Plasma tumour necrosis factor-alpha levels**

Box plot graph data of plasma TNF-alpha levels in Sjögren’s syndrome and control group is shown in Figure 1. Two way analysis of variance was performed to evaluate statistical significance of TNF-alpha levels among pSS patients and controls. Plasma TNF-alpha concentration was significantly higher (p<0.001) in Sjögren patients with TT genotype compared to pSS patients. This study is also novel with respect to the analysis of plasma inflammation markers and their association with -1031 T/C promoter polymorphism in the TNF-alpha gene.

**Plasma inflammatory markers**

Plasma NT levels in pSS and control groups are shown in Figure 2A. Two way analysis of variance was performed to evaluate statistical significance of NT levels among pSS patients and controls. Nitrotyrosine levels in -1031 TC genotype pSS patients (n=15) compared to Sjögren patients with TT genotype (n=49). There was also a statistically significant difference (p=0.011) in plasma TNF-alpha levels between Sjögren patients with -1031 TC genotype compared to -1031 TC genotype controls (n=6). No significant difference was observed in TNF-alpha levels measured in control -1031 TT genotype compared to control -1031 TT genotype (n=52).

**Correlations of TNF-alpha gene polymorphism and inflammation markers**

The scatter plots and correlation coefficients between TNF-alpha levels and inflammation markers in Sjögren TC genotype are shown in Figure 3. Linear regression analysis showed a significant correlation (p<0.001) between MPO activity and TNF-alpha levels (Fig. 3A) in -1031 TC genotype. A similar significant correlation (p<0.014) was observed between MPO activity and NT levels (Fig. 3B). Tumour necrosis factor-alpha levels were also significantly correlated (p<0.025) with NT levels (Fig. 3C).

**Discussion**

To our knowledge, this is the first study evaluating polymorphism in the TNF-alpha gene promoter at position -1031 in pSS patients and respective controls. Likewise, this is the first report showing increased TNF-alpha levels in conjunction with polymorphism in the TNF-alpha gene promoter at position -1031 in pSS patients. This study is also novel with respect to the analysis of plasma inflammation markers and their association with -1031 T/C promoter polymorphism in the TNF-alpha gene.

The rapid development of research into genetic alterations in SS has resulted in recent studies evaluating the effect of gene polymorphism on inflammatory cytokines in SS (18). A recent study in 540 patients with pSS, from Sweden and Norway, has analysed a total of 1139 SNPs in 84 genes (19). In the meta-analysis of the Swedish and Norwegian cohorts, high signals were found for association between primary SS and SNPs in three gene loci, not previously associated with primary SS. These were the early B-cell factor 1 (EBF1) gene, the family with sequence similarity 167 member A-B-lymphoid tyrosine kinase (FAM167A-BLK) locus and the tumour necrosis factor superfamily gene. Primary Sjögren’s syndrome is more commonly seen in females with a fe-
and 33% of male patients tested positive for SSA autoantibodies. Similarly, 29% of female pSS patients were positive for SSB autoantibody. Another important criterion in the diagnosis of pSS is histopathologic evaluation of the minor salivary glands. A focus score ≥1 is defined as the number of lymphocytic foci containing more than 50 lymphocytes per 4 mm² of glandular tissue. The focus score is connected to the presence of autoantibodies in pSS (21). In support of this phenomenon, we have observed that 41.9% of pSS patients had a focus score ≥1.

As stated previously this is the first study showing the association between -1031 T/C gene polymorphism and pSS. The prevalence of -1031 T/C polymorphism in the TNF-alpha gene has previously been studied in a Turkish population of Behçet’s disease. An association was reported between -1031 T/C polymorphism in Behçet’s disease compared to controls. The frequency of -1031 TC genotype in Turkish Behçet’s disease and control population was reported as 23.7% vs. 14.6%, respectively (22). This is in accordance with our observation in a Turkish population of pSS patients and controls with observed TC genotype frequency of 23.1% vs. 10.3%, respectively.

The association between increased TNF-alpha levels and TNF-alpha -1031 T/C promoter polymorphism was previously reported in vivax malaria. This report has shown that -1031 TNF-alpha promoter polymorphism significantly correlated with higher TNF-alpha serum levels in vivax malaria patients (14). In the reported study higher TNF-alpha serum levels were strongly associated with clinical severity and symptoms during vivax infection. It was concluded that TNF-alpha production due to various promoter polymorphisms were consistent with the observation of high serum concentration of TNF-alpha in patients infected with vivax. The TNF-alpha -1031 C allele also contributed to higher serum TNF-alpha levels in chronic obstructive pulmonary disease (COPD) patients (13). Nevertheless, it is important to note that studies on the functional correlation of TNF-alpha -1031 genetic variant with the production of TNF-alpha show conflicting results. It has previously been reported that presence of the -1031 C allele was associated with lower serum TNF-alpha levels in patients with asthma (23). An increase or no difference in TNF-alpha production in the presence of the -1031 C allele has also been reported using various in vitro models (24, 25). Because the region spanning the TNF-alpha gene is located within the MHC, other genes residing in the MHC locus might be responsible for differential TNF-alpha production (26). Moreover, serum TNF-alpha levels may be influenced by complex interactions in inflammatory responses. Further large-scale association and functional studies are needed to clarify these issues.

Plasma MPO levels of pSS patients were previously evaluated and found to be increased compared to controls (27). Our finding of increased MPO activity confirms previous observations (27). The initiation of an inflammatory response is causally linked to activation and adherence of neutrophils. It is known that when neutrophils are activated by a pro-inflammatory signal, the granules fuse with the plasma membrane in a simultaneous manner and discharge their contents into the extracellular medium (28). This process, also called degranulation, is a way by which MPO is released into the circulation. Neutrophil-derived, haem-containing MPO catalytically consumes NO directly via the generation of radical intermediates, thereby modulating NO bioactivity and adversely impacting on vascular inflammation (29). The NO metabolite nitrite (NO₂⁻), which is also significantly decreased in the plasma of pSS patients, is a substrate for MPO and becomes oxidised to the reactive oxidising and nitrating species nitrogen dioxide (•NO₂) (29). Importantly, the avidity of MPO for endothelial and other cells results in its transcytosis into the subcellular matrix (30), which can further lead to enhancement of tissue inflammation.

Increased plasma NT levels were observed in pSS patients with -1031 TC genotype and correlated with plasma MPO activity and TNF-alpha levels.
Previous studies utilising labial biopsy specimens from patients with SS detected increased immunohistochemical staining of NT and concluded that large amounts of NT were produced in salivary duct cells of SS patients (31). Nitrotyrosine formation was also observed in conjunctival epithelium of SS patients (16). Protein nitration observed in pSS patients could be either via the formation of oxidising and nitrated species peroxynitrite (32) or by MPO catalysed oxidation of NO$_3^-$ (33).

Glutathione is critical in defending against oxidative stress induced by inflammation. Total GSH levels in plasma of pSS were significantly lower compared to healthy controls. This observation is in agreement with previous work (34) and suggest that reactive oxygen species generated during inflammation deplete GSH in pSS as seen in conditions of oxidative stress (35).

In summary, this study has shown that polymorphism in the TNF-α gene promoter at position -1031 is associated with elevated circulating levels of TNF-α which correlates with increased plasma MPO and NT in pSS patients. This finding may provide a better understanding of the link between TNF-α gene polymorphism and pSS and may ultimately help to predict response to anti-TNF-α therapy in Sjögren patients.

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