
Proprotein convertase enzyme FURIN is upregulated in primary Sjögren's syndrome

N. Ranta^{1,2}, A. Valli¹, A. Grönholm^{1,2}, O. Silvennoinen^{1,3},
P. Isomäki^{4,5}, M. Pesu^{2,5,6}, M. Pertovaara^{4,5}

¹Faculty of Medicine and Life Sciences, University of Tampere; ²BioMediTech, Tampere; ³Fimlab Laboratories, Tampere; ⁴Department of Internal Medicine, Centre for Rheumatic Diseases, Tampere University Hospital; ⁵Faculty of Medicine and Life Sciences, University of Tampere; ⁶Department of Dermatology, Tampere University Hospital, Tampere, Finland.

Noora Ranta*, Medical student
Atte Valli*, Medical student
Anna Grönholm, PhD
Olli Silvennoinen, MD, PhD
Pia Isomäki, MD, PhD
Marko Pesu[#], MD, PhD
Marja Pertovaara[#], MD, PhD

*N. Ranta and A. Valli
contributed equally to this work.

[#]M. Pesu and M. Pertovaara
contributed equally to this work.

Please address correspondence to:
Dr Marja Pertovaara,
Department of Internal Medicine,
Centre for Rheumatic Diseases,
Tampere University Hospital,
P.O. Box 2000,
FI-33521 Tampere, Finland.

E-mail: marja.pertovaara@fimnet.fi

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ABSTRACT

Objective. The proprotein convertase enzyme FURIN is a critical regulator of the anti-inflammatory TGF β -1 cytokine and peripheral immune tolerance. In T cells, FURIN is co-regulated with IFN- γ and thus highly expressed in T helper 1 type cells. Previous studies have demonstrated that FURIN is up-regulated in inflammatory conditions, including atherosclerosis, rheumatoid arthritis and systemic lupus erythematosus. Here, we evaluated the levels of FURIN in the plasma and peripheral blood mononuclear cells (PBMCs) of patients with primary Sjögren's syndrome (pSS) and in healthy controls.

Methods. FURIN plasma levels were determined by ELISA, and the mRNA expression in PBMCs was quantitated using qPCR. FURIN levels in the plasma were correlated with the clinical and demographic characteristics of the patients.

Results. FURIN was found to be significantly upregulated at both the protein and mRNA level in pSS patients compared to healthy controls. In pSS patients, high FURIN protein levels were significantly associated with elevated IFN- γ levels in the plasma as well as a longer duration of sicca symptoms in the eyes. pSS patients with high FURIN levels in their plasma showed a trend towards lower levels of serum beta-2 microglobulin, ESR and a lower systemic disease activity index ESSDAI.

Conclusion. The proprotein convertase FURIN is significantly upregulated in pSS. Elevated FURIN levels associate with high levels of the Th1 type cytokine IFN- γ and long duration of dry eye symptoms. Patients with high FURIN levels show signs of lower disease activity suggesting that FURIN might have a protective role in pSS.

Introduction

Primary Sjögren's syndrome (pSS) is a chronic systemic autoimmune disease characterised by lymphocytic infiltration in the salivary and lacrimal glands and over-activation of B cells leading subsequently to hypergammaglobulinaemia and development of autoantibodies (1, 2). According to current knowledge, the main pathophysiological mechanisms in pSS are mediated through the interferon (IFN) I and IFN II pathways (1, 2). The type II cytokine IFN- γ has been found to be responsible for the gland dysfunctions in Ro/SSA immunised mice. *Vice versa*, a reduction in the level of IFN- γ or IFN- γ R seems to inhibit the development of pSS (2).

The proprotein convertase subtilisin/kexin enzymes (PCSKs) activate various immature proteins by catalysing their post-translational site-specific hydrolytic cleavage (3). The ubiquitously expressed PCSK enzyme FURIN catalyses the proteolysis of a large number of substrates with immunoregulatory functions including cytokines, integrins and viral envelope proteins (3-5). FURIN is upregulated in Th1 cells via the IL-12/Stat4 pathway (6). The T-cell-expressed FURIN regulates Th cell polarisation and peripheral immune tolerance by controlling the functional maturation of transforming growth factor beta (TGF β -1) (3, 6, 7). FURIN is also upregulated in chronic autoimmune inflammation as is seen in patients with rheumatoid arthritis (RA) (8) and systemic lupus erythematosus (SLE) (9).

The fact that FURIN is an important regulator of peripheral immune tolerance and highly expressed in Th1-type lymphocytes prompted us to determine the levels of the FURIN protein and mRNA in patients with pSS compared to healthy controls.

Methods

Study population

Peripheral blood (PB) samples were obtained from 16 (14 female and 2 male) patients with pSS that were recruited from the Centre for Rheumatic Diseases at the Tampere University Hospital, Finland. The inclusion criteria were fulfilment of at least four of the revised American-European consensus group criteria for pSS (10), together with a confirmation of an active disease verified with either an ESSDAI >11 (11) or with laboratory tests: erythrocyte sedimentation rate (ESR) >20 mm/h serum immunoglobulin G (IgG) >15 mg/L, serum beta-2 microglobulin >2.2 mg/L or serum complement C4 <0.10 mg/L. The median age of the patients was 53 years (range 32–80 years) and disease duration 11 years (range 0–27 years). Three of the pSS patients used medication during sampling: one had a low-dose of prednisolone, one received prednisolone and hydroxychloroquine and the third was treated with prednisolone and azathioprine. The clinical characteristics of the pSS patients have been described in detail previously (12).

In addition, 14 control plasma samples for FURIN ELISA measurements and 10 PBMC samples for qPCR analyses were collected from anonymous healthy blood donors (Finnish Red Cross Blood Transfusion Service, Tampere, Finland). Median age of the 14 anonymous healthy blood donors (6 female, 8 male) for FURIN ELISA measurements was 50 years (range 21–63 years).

Measurement of plasma

FURIN levels

The plasma concentration of FURIN was determined using a commercial Human FURIN Enzyme-Linked Immunosorbent Assay (ELISA) Kit (Sigma-Aldrich®, St. Louis, MO, USA) according to the manufacturer's instructions. The detection limit of the ELISA assay was 123 pg/ml, and concentrations below this were considered to represent absence of the protein. Duplicate samples were analysed to ensure reliable results. The patients were further stratified into low and high plasma FURIN

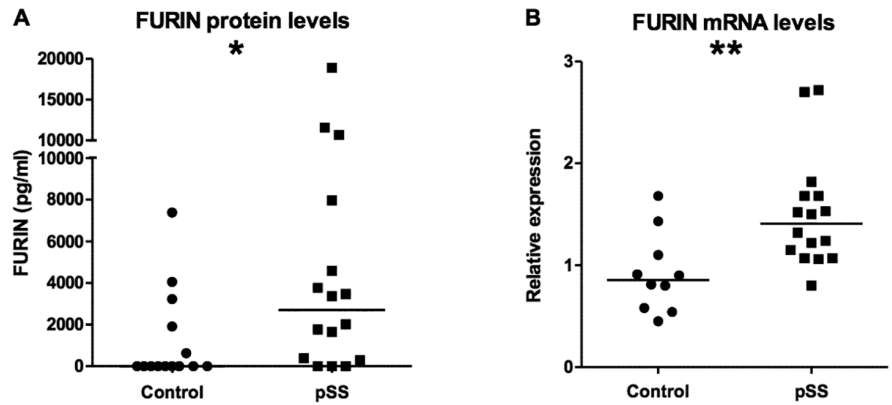


Fig. 1. FURIN levels in patients with primary Sjögren's syndrome (pSS) and in healthy controls. The horizontal lines represent the median values within the groups. **A.** Plasma FURIN levels in patients with pSS compared to healthy controls. * $p=0.0181$. **B.** FURIN gene expression in PBMCs from patients with pSS compared to healthy controls. ** $p=0.0061$.

groups by a median plasma FURIN concentration of 2690 pg/ml.

RNA isolation and qRT-PCR analysis

Total RNAs were isolated from PB mononuclear cells (PBMCs). The expression of FURIN (*FURIN*) and the TATA-binding protein (*TBP*, house-keeping) genes were quantified by real-time PCR using the CFX instrument (Bio-Rad, Hercules, CA, USA). Primers: 5'-GGCAAAGCGACGGACTAAAC-3' and 5'-CGTCCAGATGGAGACCACA-3' for *FURIN* and 5'-GAATATAATCCCAAGCGGTTTG-3' and 5'-ACTTCACATCAGCTCCCC-3' for *TBP* (12). The relative FURIN expression was calculated by dividing mean expression values of FURIN measured from triplicated samples by mean expression values obtained for the *TBP* house-keeping gene.

Statistical analysis

Statistical analyses were performed with SPSS Statistics (IBM, v. 20). The Mann-Whitney U-test was used for comparisons of continuous variables. Correlations were calculated with Spearman's correlation coefficient. Findings were considered statistically significant at $p < 0.05$.

Ethical considerations

The study was approved by the Ethical Committee of Tampere University Hospital and conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants.

Results

First, to evaluate whether FURIN is elevated in pSS we measured protein levels in plasma using a commercial ELISA assay. Plasma FURIN levels were significantly upregulated in patients with pSS compared to healthy controls (median 2690 pg/ml vs. 0 pg/ml, $p=0.0181$, Fig. 1A). Consistent with the plasma data, also FURIN gene expression in PBMCs was significantly higher among patients with pSS compared to the controls ($p=0.0061$, Fig. 1B).

Next, we examined if the upregulated plasma FURIN associates with clinical or immunological features of patients with pSS. To this end, the pSS patients were divided into high and low-level FURIN groups with a cut-off at the median FURIN concentration 2690 pg/ml (Table I). Patients with high FURIN concentrations showed significantly elevated levels of IFN- γ compared to those with low FURIN concentrations (median 100 pg/ml vs. 27.2 pg/ml, $p=0.036$). Moreover, they had a longer duration of sicca symptoms of the eyes (median 13 years vs. 9 years, $p=0.035$). There was also a statistically significant correlation between plasma FURIN-levels and IFN- γ -levels (Spearman correlation, $r=0.631$, $p=0.009$) and with the duration of sicca symptoms of the eyes ($r=0.520$, $p=0.038$). In addition, there was a trend towards lower levels of serum beta-2 microglobulin in pSS patients with high FURIN levels compared to those with low levels

Table I. Clinical and immunological findings in 16 patients with pSS grouped by median plasma FURIN concentrations.

Variable	Plasma FURIN concentration		p-value
	<2690 pg/ml n=8	≥2690 pg/ml n=8	
Age, years	52 (40-64)	57 (43-75)	0.563
Duration of sicca symptoms of the eyes, years	7 (3-15)	19 (14-35)	0.035
Duration of sicca symptoms of the mouth, years	10 (6-22)	15 (10-21)	0.461
Disease duration, years	9 (5-17)	13 (6-19)	0.599
ESSPRI, cm	4.37 (3.17-6.33)	4.28 (2.15-5.76)	0.674
ESSDAI	6.50 (4.25-9.75)	3.00 (2.25-7.50)	0.205
Pain-VAS, cm	4.0 (0-66.0)	5.5 (0-16.0)	0.675
PGH-VAS, cm	15.0 (1.0-71.0)	14.5 (2.25-25.0)	0.523
HAQ	0.00 (0-0.38)	0.13 (0-1.10)	0.433
Haemoglobin, g/L	130 (121-136)	137 (126-142)	0.247
ESR, mm/h	29 (11-37)	11 (8-19)	0.138
C-reactive protein, g/L	0.85 (0-2.55)	0.00 (0.00-0.75)	0.277
Serum IgG, g/L	17.8 (16.5-22.3)	16.9 (14.3-20.8)	0.462
Serum IgA, g/L	2.63 (1.78-3.15)	2.12 (0.25-3.15)	0.400
Serum IgM, g/L	1.50 (0.92-2.97)	1.14 (0.61-2.21)	0.600
Serum β2m, mg/L	3.05 (2.80-3.43)	2.30 (2.08-3.08)	0.050
Serum C3, g/L	0.98 (0.84-1.21)	0.98 (0.83-1.15)	1.000
Serum C4, g/L	0.16 (0.11-0.17)	0.14 (0.12-0.18)	0.792
Anti-SSA antibody titre	240 (240-240), n=7	240 (240-240), n=6	1.00
Anti-SSB antibody titre	320 (72-320), n=7	183 (0-320), n=6	0.174
Plasma IL-1 beta, pg/mL	13.9 (1.93-33.6)	18.8 (6.14-24.6)	0.674
Plasma IL-2, pg/mL	21.5 (9.23-112)	67.0 (21.1-108)	0.345
Plasma IL-4, pg/mL	53.1 (42.6-96.7), n=7	146.3 (37.8-347), n=7	0.406
Plasma IL-6, pg/mL	8.04 (4.06-21.4), n=7	13.7 (7.65-31.0)	0.487
Plasma IL-7, pg/mL	15.2 (5.46-21.7)	22.5 (17.4-45.3)	0.115
Plasma IL-10, pg/mL	74.1 (33.7-258)	110.5 (59.0-360)	0.418
Plasma IFN-γ, pg/mL	27.2 (18.1-89.5)	100.0 (78.0-187)	0.036
Plasma TNF-α, pg/mL	7.66 (4.92-16.9)	15.2 (11.3-18.8)	0.141

Statistical analysis: Mann Whitney test. The values are expressed as medians (interquartile range). ESSPRI: EULAR Sjögren's syndrome patient-reported index; ESSDAI: EULAR Sjögren's syndrome disease activity index; VAS: visual analogue scale; PGH: patient's global health assessment; HAQ: Health Assessment Questionnaire; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; β2m: beta-2 microglobulin; IL: interleukin; IFN: interferon; TNF: tumour necrosis factor.

(median 2.30 mg/ml vs. 3.05 mg/ml, $p=0.050$). ESR (median 11 mm/h vs. 29 mm/h, $p=0.138$) and the systemic disease activity index ESSDAI (median 3.00 vs. 6.50, $p=0.205$) were also lower in pSS patients with high FURIN levels (Table I).

Discussion

Previous studies have shown a connection between FURIN and various conditions involving chronic inflammation such as atherosclerosis (5), rheumatoid arthritis (8) and SLE (9). In keeping with these, our recent study with 537 patients with a suspected infection indicated a strong association between high plasma FURIN levels and a history of rheumatic disease (13), yet there was no connection between plasma FURIN levels and demographic features such as age and sex (13). In the present study,

we show that both FURIN plasma levels as well as FURIN gene expression are significantly higher in patients with pSS compared to healthy controls, implying that FURIN might have a role in the pathology of pSS.

A significant association was found between high plasma levels of FURIN and elevated plasma levels of the pro-inflammatory cytokine IFN-γ. IFN-γ is generally known to mediate Th1 cell functions, macrophage activation and immunoglobulin class switching, and it has previously been widely associated with systemic autoimmunity. What is noteworthy, IFN-γ and FURIN mRNAs are simultaneously expressed by Th1 cells and they are regulated via the IL-12/Stat4 pathway (6). However, here we demonstrate for the first time that IFN-γ and FURIN also correlate at the protein level in PB.

An association was also found between high FURIN plasma levels and the duration of sicca symptoms of the eyes. In addition, there was a non-significant trend showing that pSS patients with high plasma FURIN levels had milder clinical findings, *i.e.* lower serum beta-2 microglobulin levels, ESR and ESSDAI. As has been previously shown, FURIN is essential for maintaining peripheral tolerance by regulating the maturation of TGFβ-1 (3, 6, 7). A FURIN deficiency leads to the development of less protective Treg cells and a production of overly activated effector T cells with no susceptibility to suppressive actions of wild-type Treg cells (3). A potentially protective role of systemic FURIN has previously been reported in patients with rheumatoid arthritis (8), where it seemed to reverse the Th1/Th2 balance in the joints and enhance the proportion of T regulatory (Treg) cells in the spleen. The protective, symptom-reducing effect of FURIN is additionally supported by the fact that active FURIN has been shown to restrain the production of pro-inflammatory cytokines in macrophages (14). However, the immunoregulatory role of FURIN is multifaceted. While it seems evident that FURIN exerts an important anti-inflammatory function, it can also promote pro-inflammatory cytokines including the central pSS cytokine BAFF (5).

Elevated levels of FURIN have previously been reported in salivary glands of pSS patients (15). To our knowledge, this is the first study quantitating the protein levels of FURIN in plasma and the expression of FURIN mRNA in PBMCs in patients with pSS. In conclusion, we demonstrate that both plasma FURIN levels as well as FURIN gene expression in PBMCs are higher in patients with pSS compared to healthy controls. In addition, a trend towards somewhat milder clinical findings in patients with high plasma levels of FURIN was observed. These findings are consistent with previous studies on other inflammatory diseases and might reflect a potentially protective role of FURIN in patients with pSS by preventing the autoimmune responses and overly activated immune system

orchestrated by IFN- γ . In experimental models, exogenous FURIN has been successfully used to harness autoimmunity (8). Taking into account the clinical diversity of pSS, it would be important to analyse FURIN levels in subgroups of pSS patients with various extraglandular symptoms and in patients classified by disease duration. Therefore, further studies with more pSS patients are needed to confirm the current findings and to refine our knowledge of the possible clinical feasibility of targeting FURIN in pSS.

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