Table 1. Baseline characteristics and TNF inhibitors according to clinical response at 24 and 56 weeks. The values of continuous variables are expressed as median and 25th-75th percentile.

Characteristic	24 Weeks				56 Weeks			
	Responders, n = 149	Nonresponders, n = 39	OR (95% CI)	p	Continuation, n = 149	Discontinuation, n = 33	OR (95% CI)	p
Female/male	127/22	31/8	1.49 (0.53–3.92)	0.459	127/22	25/8	1.87 (0.67–5.23)	0.119
Age, yrs	51.5 (39–61)	52 (37.5–61.5)	NE	0.997	53 (39–61)	42 (34–60)	NE	0.113
Disease duration, yrs	7.4 (2.1–13.4)	5 (1.8–11)	NE	0.431	7.8 (2.5–14.3)	5 (1–9)	NE	0.045
MTX dose, mg/week	8 (4–8)	6 (4–8)	NE	0.143	7.5 (4–8)	8 (4–8)	NE	0.146
Steroid dose, mg/day	5 (2–7)	5 (0–7.5)	NE	0.937	5 (2–7)	5 (0–8)	NE	0.609
Previous DMARD (no/yes)	49/100	10/29	1.45 (0.62–3.60)	0.442	45/104	13/20	0.67 (0.29–1.59)	0.309
TJC	4 (3–8)	6 (1.3–12.5)	NE	0.125	5 (3–9)	6 (3–11)	NE	0.241
SJC	5 (2–8)	6 (1.3–9.8)	NE	0.661	5 (2–8)	4 (1–8)	NE	0.279
VAS global	52 (36–73.8)	53 (38.5–70)	NE	0.889	53 (36–73)	51 (46–75)	NE	0.703
mHAQ	0.5 (0.3–0.9)	0.6 (0.3–1.3)	NE	0.081	0.5 (0.25–1)	0.63 (0.4–1.2)	NE	0.106
CRP, mg/dl	1.3 (0.4–3.6)	2.7 (1.1–5.6)	NE	0.008	1.4 (0.5–3.9)	1.5 (0.4–6.3)	NE	0.677
DAS28/CRP	4.6 (3.9–5.3)	4.9 (4.2–5.6)	NE	0.147	4.7 (4.0–5.3)	4.9 (3.9–5.6)	NE	0.515
IgG, mg/dl	1360 (1192–1565)	1592 (1315–1845)	NE	0.006	1360 (1191–1600)	1583 (1280–1890)	NE	0.053
ANA (negative/positive)	9/140	5/34	0.39 (0.10–1.59)	0.147	8/141	3/30	0.57 (0.13–3.53)	0.142
Anti-dsDNA (negative/positive)	145/3	35/4	4.07 (0.72–23.02)	0.063	143/6	32/1	0.76 (0.02–6.66)	1.000
ACPA (negative/positive)	16/133	2/37	NE	0.908	16/133	7/26	NE	0.799
RF levels, IU/ml	57 (24–210)	67 (23.5–260)	NE	0.738	59.5 (25–229)	43 (12–209)	NE	0.326
Anti-Ro (negative/positive)	131/18	26/13	3.64 (1.45–9.01)	0.003	131/18	20/13	4.68 (1.82–11.99)	0.0005
Anti-La (negative/positive)	146/3	39/0	0.00 (0.00–9.40)	1.0	146/3	33/0	0.00 (0.00–11.07)	1.0
Secondary SS (no/yes)	132/17	29/10	2.68 (0.99–6.98)	0.037	132/17	23/10	3.35 (1.21–8.94)	0.012
TNF inhibitors: IFX/ETN/ADA	80/58/11	32/4/3	NE	0.0012	82/58/9	24/5/4	NE	0.016

DMARD: disease-modifying antirheumatic drugs; MTX: methotrexate; TJC: tender joint count; SJC: swollen joint count; VAS global: global health assessed visual analog scale; mHAQ: modified Health Assessment Questionnaire; CRP: C-reactive protein; DAS28: Disease Activity Score 28; ANA: antinuclear antibody; anti-dsDNA: anti-double stranded DNA antibody; ACPA: anticyclic citrullinated peptide antibody; RF: rheumatoid factor; anti-Ro: anti-Ro/SSA antibodies; anti-La: anti-La/SSB antibodies; SS: Sjögren's syndrome; TNF: tumor necrosis factor; IFX: infliximab; ETN: etanercept; ADA: adalimumab; NE: not estimated.

centage of patients who achieved a moderate or good EULAR response was lower (p = 0.006; Figure 1) and the median DAS28 score was higher in anti-Ro-positive patients (p = 0.047; Figure 2). It is noteworthy that the differences in the clinical response between anti-Ro-positive and -negative patients varied among the TNF inhibitors and was most pronounced in patients treated with IFX [9/20 (45.0%) and 70/91 (76.9%), respectively (p = 0.002; Figure 1)]. Further, there was a significant difference in the median DAS28 score between anti-Ro-positive and -negative patients at 24 weeks among IFX-treated patients (p = 0.026; Figure 2), while there was no statistical difference among patients treated with ETN (p = 0.432; Figure 2). Moreover, the median DAS28 score in the total patient population was higher at 56 weeks in anti-Ro-positive patients (p = 0.038; Figure 2).

The TNF inhibitors were discontinued at 56 weeks in 41 patients (21.6%), including 33 patients who discontinued TNF inhibitor treatment because of inefficacy, and the discontinuation rate was higher in the anti-Ro-positive patients than anti-Ro-negative patients (OR 3.74, 95% CI 1.53–9.11, p = 0.002, and OR 4.09, 95% CI 1.58–10.48, p = 0.002, respectively; Table 3). These differences were also more prominent in IFX-treated patients, compared to ETN- and ADA-treated patients (OR 3.69, 95% CI 1.27–10.33, p = 0.008; OR 1.24, 95% CI 0.02–13.10, p = 1.000; and OR 5.13, 95% CI 0.36–73.21, p = 0.133, respectively; Table 3).

Anti-Ro is an independent factor associated with a poor response to the first TNF inhibitor. To further investigate the relationship between the clinical characteristics and the response to TNF inhibitors, we used a multivariable logistic regression analysis and adjusted for all the confounding factors examined in Table 2, including anti-Ro and the type of TNF inhibitors (Table 4). This analysis revealed that the presence of anti-Ro was strongly associated with the inefficacy of TNF inhibitors at 24 weeks (OR 5.22, 95% CI 1.75–15.57, p = 0.003) and with an increased discontinuation rate at 56 weeks (OR 10.18, 95% CI 2.18–49.56, p = 0.003). IgG levels were also associated with treatment inefficacy in a multivariable logistic regression analysis, and IFX or ADA treatment was also associated with the greater discontinuation of the TNF inhibitors (OR 6.113, 95% CI 1.451–25.758, p = 0.014). The r-square values for the multivariable models at 24 and 56 weeks were 0.300 and 0.275, respectively, and they were sufficient for accurate predictions. We focused on other variables that were significantly

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Table 2. Comparison of the baseline characteristics between anti-Ro-negative and -positive patients before commencement of first TNF inhibitors. The values of continuous variables are expressed as median and 25th-75th percentile.

Characteristic	Total, n = 190	Anti-Ro-negative, n = 158 (83.2%)	Anti-Ro-positive, n = 32 (16.8%)	OR (95% CI)	p
Female/male	160/30	130/28	30/2	0.31 (0.03–1.35)	0.119
Age, yrs	52 (38.5–61)	53 (39–62)	45 (37–58)	NE	0.152
Disease duration, yrs	6.75 (2–13.5)	7.5 (2–13.8)	6 (3.8–11.3)	NE	0.575
MTX dose, mg/wk	8 (4–8)	8 (4–8)	6 (4–8)	NE	0.154
Steroid dose, mg/day	5 (2–7)	5 (3–6.8)	4 (2–7.5)	NE	0.490
Previous DMARD (no/yes)	60/130	53/105	7/25	1.79 (0.69–5.25)	0.218
TJC	5 (3–9.8)	4.5 (3–8.8)	6 (3–12)	NE	0.212
SJC	5 (2–8)	5 (2–8)	2.5 (1–7)	NE	0.130
VAS global	52 (36–73)	52 (36–74)	50 (36.8–70.5)	NE	0.798
mHAQ	0.5 (0.1–0.9)	0.63 (0.3–1)	0.5 (0.2–1.1)	NE	0.855
CRP, mg/dl	1.5 (0.5–4)	1.5 (0.7–4.2)	1.1 (0.3–3.3)	NE	0.119
DAS28/CRP	4.68 (3.95–5.42)	4.66 (3.99–5.38)	4.79 (3.93–5.46)	NE	0.547
IgG, mg/dl	1382 (1206–1662)	1352 (1190–1610)	1553 (1352–1846)	NE	0.003
ANA-positive, %	178 (93.7)	145 (91.8)	32 (100)	Inf (0.63–Inf)	0.130
Anti-dsDNA-positive, %	7 (3.7)	2 (1.3)	5 (15.6)	9.27 (1.69–63.23)	0.004
Anti-La-positive, %	3 (1.6)	0 (0.0)	3 (9.4)	Inf (2.12–Inf)	0.004
ACPA-positive, %	167 (87.9)	136 (86.1)	31 (96.9)	NE	0.168
RF levels, IU/ml	59 (24–215)	57 (25–200)	76.5 (18.8–284.5)	NE	0.628
Secondary SS (yes, %)	28 (14.7)	8 (5.1)	20 (62.5)	30.09 (10.29–98.00)	< 0.0001
TNF inhibitors					
IFX positive, %	112	92 (82.1)	20 (17.9)	NE	NS
ETN positive, %	64	55 (85.9)	9 (14.1)	NE	NS
ADA positive, %	14	11 (78.6)	3 (21.4)	NE	NS

Anti-Ro: anti-Ro/SSA antibodies; DMARD: disease-modifying antirheumatic drugs; MTX: methotrexate; TJC: tender joint count; SJC: swollen joint count; VAS global: global health assessed on visual analog scale; mHAQ: modified Health Assessment Questionnaire; CRP: C-reactive protein; DAS28: Disease Activity Score 28; ANA: antinuclear antibody; anti-dsDNA: anti-double stranded DNA antibody; anti-La: anti-La/SSB antibodies; ACPA: anticyclic citrullinated peptide antibody; RF: rheumatoid factor; SS: Sjögren's syndrome; TNF: tumor necrosis factor; IFX: infliximab; ETN: etanercept; ADA: adalimumab; NE: not estimated; Inf: infinity; NS: not significant.

different in a univariable analysis, such as CRP and IgG at 24 weeks and disease duration at 56 weeks. However, these variables were not associated with the inefficacy of the TNF inhibitors in a multivariable analysis.

Changes in autoantibody profiles after TNF inhibitor treatment. The positive rate and titers of anti-Ro did not change at 24 and 56 weeks, while the ANA and anti-dsDNA titers were increased from baseline at 56 weeks (93.7% to 99.5% and 3.7% to 29.5%, respectively). Anti-La remained positive during the treatment period in 3 patients with secondary SS. Moreover, the positive rate of anti-dsDNA was notably increased in anti-Ro-positive patients compared to anti-Ro-negative patients (56.7% and 26.0%, respectively; p = 0.003). The TNF inhibitors were inefficacious in 8 of 14 of these patients, including 7 who were treated with IFX. None of the patients with increased anti-dsDNA titers developed clinical signs of SLE. ACPA and RF decreased frequently, with no correlation with the clinical response.

DISCUSSION

In this study, we showed that TNF inhibitors as the first biologics to treat RA were less efficacious in anti-Ro-positive patients compared to anti-Ro-negative patients. It was previously reported that RA patients with anti-Ro are more likely to have more severe disease and require more aggressive therapy[20,21]. Simmons-O'Brien, *et al* also reported that anti-Ro persisted for years in patients with RA and that these patients had chronic progressive disease[22]. In our patient population, the anti-Ro titers did not change from baseline during the treatment period with TNF inhibitors.

On the other hand, Cavazzana, *et al* reported that TNF inhibitors were equally effective in anti-Ro-positive patients[11,23]. In our study, the clinical response was lower in anti-Ro-positive patients with a sustained high DAS28 score at 24 weeks. At 56 weeks, this difference in clinical response was reduced, but this might be due to the discontinuation of TNF inhibitors in patients with inefficacy. The contrast between our results and those of the previous study[11,23] could be partially explained by differences in patient backgrounds, such as race, prevalence of secondary SS, history of other RA medications, and the assay used to detect anti-Ro. In fact, the frequency of anti-Ro in our study (16.8%) was higher than in other studies. However, Moutsopoulos, *et al* reported that 14.3% of Greek patients with RA were anti-Ro-positive by DID[24], which was similar to our results. Moreover, they reported that the prevalence of anti-Ro-pos-

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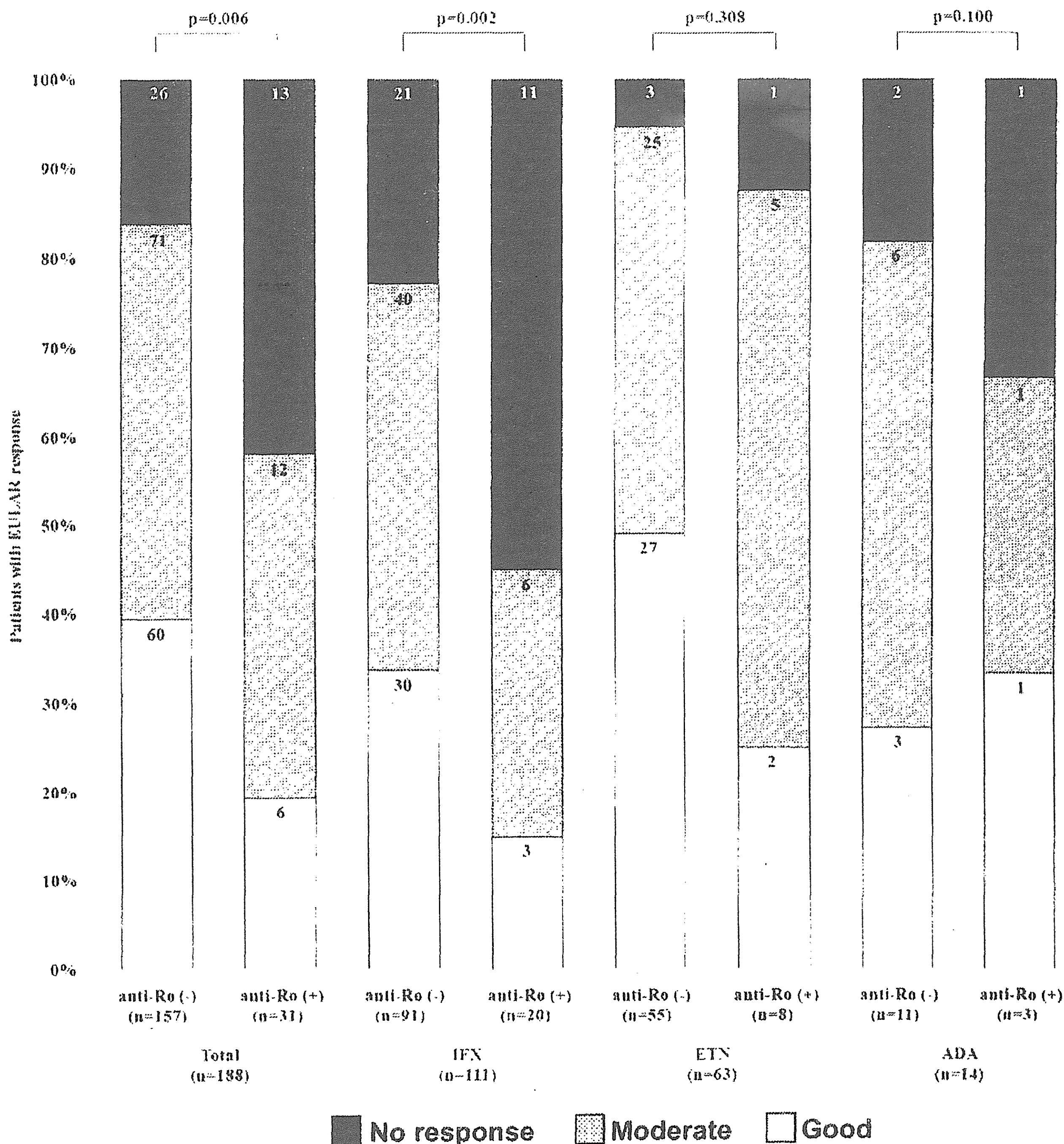

Figure 1. Comparison of the European League Against Rheumatism (EULAR) response rate at 24 weeks between anti-Ro/SSA antibody (anti-Ro) -negative and -positive patients treated with TNF inhibitors. For each TNF inhibitor, the percentage of patients who achieved a moderate or good EULAR response at 24 weeks was compared between the anti-Ro-positive and -negative patients. Numbers inside the bars represent the number of patients with a good, moderate, or no EULAR response. IFX: infliximab; ETN: etanercept; ADA: adalimumab.

itive individuals was higher in Greek than in British populations[25]. In terms of assays for detecting anti-Ro, an ELISA was used in the studies by Cavazzana, *et al*[11,23], while we used DID in this study. In our patients who had negative titer of anti-Ro with DID, 9 patients showed low or equivocal positive titers with ELISA and none of them had SS (data not shown). Morozzi, *et al* has also reported that low or equivocal positive titers by ELISA were obtained among patients who were anti-Ro-negative by DID, and sensitivity was different among the assays[26]. High sensitivity with

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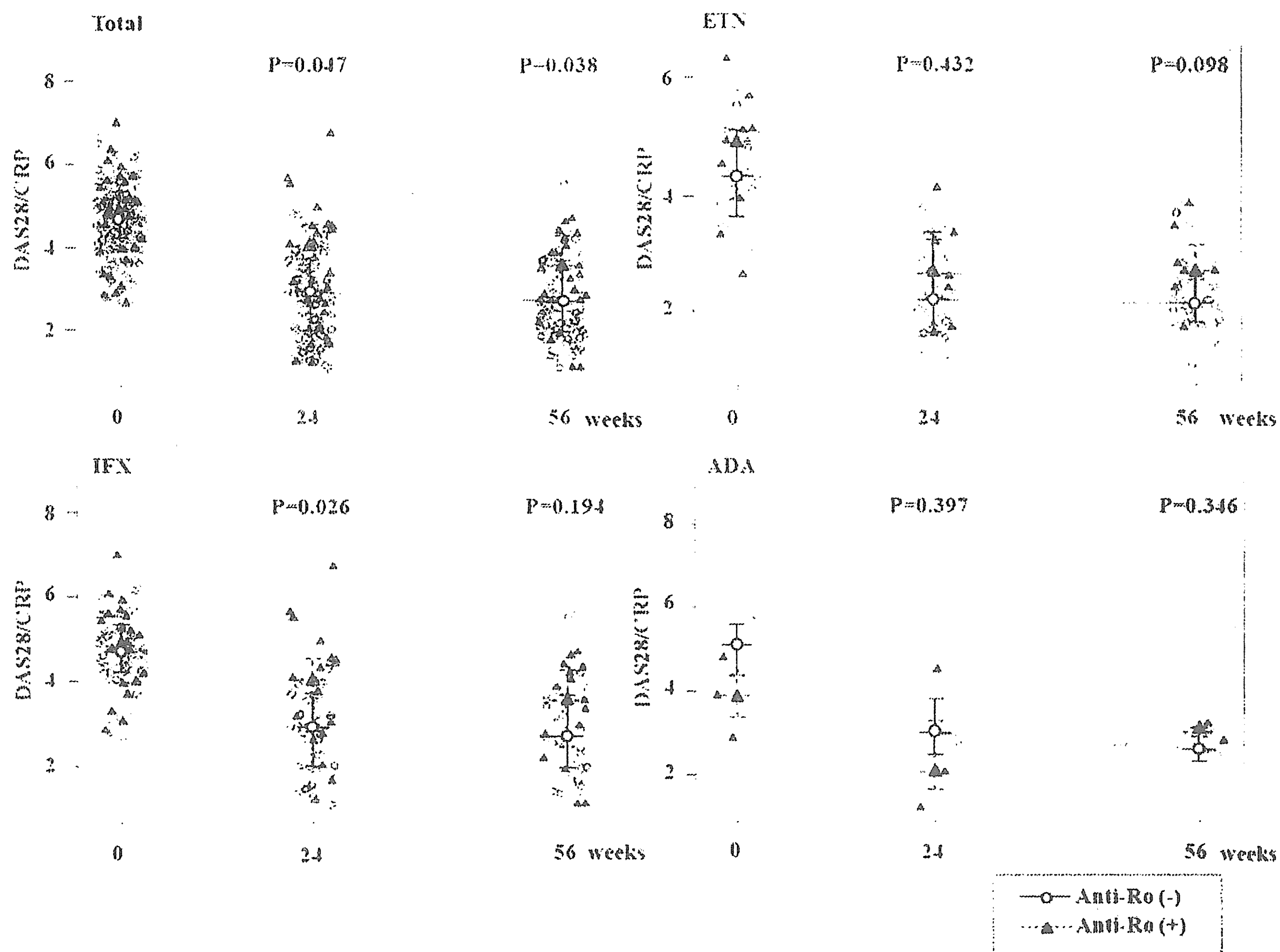

Figure 2. Comparison of disease activity at 24 and 56 weeks between anti-Ro/SSA antibody (anti-Ro) -negative and -positive patients treated with each TNF inhibitor. Values are expressed as the median, 25th–75th percentiles, and range. IFX: infliximab; ETN: etanercept; ADA: adalimumab; DAS28/CRP: Disease Activity Score 28/C-reactive protein.

ELISA might be related if low or equivocal titers were considered as a positive, and the assay might affect the association with clinical manifestations. Additionally, as noted in Materials and Methods, the positive rates of anti-Ro in healthy individuals and patients with other diseases that were determined by DID in our laboratory were comparable with previous reports.

Finally, a multivariable logistic regression analysis indicated that there was a significant association between anti-Ro and the inefficacy of IFX compared to the other TNF inhibitors in our patients. It is unclear why the presence of anti-Ro was strongly associated with the inefficacy of TNF inhibitors. Among the anti-Ro-positive patients in our study, the prevalence of secondary SS was 62.5%. It was previously reported that SS is a poor prognostic factor for RA[12], and indeed, RA patients with secondary SS were more prevalent among nonresponders. However, secondary SS was not independently associated with the inefficacy of TNF inhibitors in our study by a multivariable logistic regression analysis.

Some investigators have reported that anti-Ro-positive patients with RA have a higher incidence of DMARD-induced toxicity, and B cell activation was commonly observed in these patients[20,21,27,28,29]. TNF inhibitors are also known to induce the production of non-organ-specific autoantibodies, such as ANA, anti-dsDNA, and antiphospholipid antibodies[5,6,30,31,32]. The mechanism of ANA and anti-dsDNA might be produced after treatment with TNF inhibitors, especially IFX, because of increased release of autoantigens from apoptotic lymphocytes in the lamina propria[33,34,35,36]. Indeed, in the patients in this study the positive rate of anti-dsDNA frequently increased after they were treated with TNF inhibitors. Moreover, accelerated anti-dsDNA production was predominantly observed in patients treated with IFX, and this occurred more commonly in anti-Ro-positive patients. In addition, we examined the

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Table 3. Discontinuation of TNF inhibitor treatment at 56 weeks.

Cause of Discontinuation	Total, n = 190	Anti-Ro-negative, n = 158	Anti-Ro-positive, n = 32	OR (95% CI)	p
All causes					
Total	41	27	14	3.74 (1.53–9.11)	0.002
IFX	30	20	10	3.11 (1.145–8.13)	0.015
ETN	6	4	2	2.55 (0.22–18.73)	0.266
ADA	5	3	2	3.41 (0.27–31.14)	0.198
Inefficacy					
Total	33	20	13	4.09 (1.58–10.48)	0.002
IFX	25	15	10	3.69 (1.27–10.33)	0.008
ETN	5	4	1	1.24 (0.02–13.10)	1.000
ADA	4	2	2	5.13 (0.36–73.21)	0.133
Infusion reaction					
Total	4	4	0	0.00 (0.00–7.59)	1.000
IFX	4	4	0	0.00 (0.0–7.59)	1.000
ETN	0	0	0	0.00 (0.00–Inf)	NA
ADA	0	0	0	0.00 (0.00–Inf)	NA
Infection					
Total	3	2	1	2.50 (0.00–49.41)	0.472
IFX	2	2	0	0.00 (0.00–26.55)	1.000
ETN	1	0	1	0.00 (0.13–Inf)	0.168
ADA	0	0	0	0.00 (0.00–Inf)	NA
Malignancy					
Total	1	1	0	0.00 (0.00–191.99)	1.000
IFX	0	0	0	0.00 (0.00–Inf)	NA
ETN	0	0	0	0.00 (0.00–Inf)	NA
ADA	1	1	0	0.00 (0.00–191.99)	1.000

TNF: tumor necrosis factor; Anti-Ro: anti-Ro/SSA antibodies; IFX: infliximab; ETN: etanercept; ADA: adalimumab; Inf: infinity; NA: not available.

Table 4. Association of anti-Ro with clinical response of TNF inhibitors.

Variable	24 Weeks* Coefficients	24 Weeks* OR	24 Weeks* (95% CI)	24 Weeks* p	56 Weeks† Coefficients	56 Weeks† OR	56 Weeks† (95% CI)	56 Weeks† p
Anti-Ro	1.65	5.22	(1.75–15.57)	0.003	2.32	10.18	(2.18–49.56)	0.003
Gender male	1.35	3.89	(1.23–12.26)	0.021	0.96	2.61	(0.74–9.22)	0.138
CRP	−1.56	0.211	(0.01–3.37)	0.271				
IgG	1.40	4.06	(1.47–11.19)	0.007				
IFX or ADA	−1.88	0.15	(0.00–29.87)	0.485	1.81	6.11	(1.45–25.76)	0.014
Duration					0.13	0.14	(0.96–1.35)	0.129
Secondary SS					1.26	3.54	(0.71–17.59)	0.123

* Association with no EULAR response at 24 weeks. † Association with discontinuation rate at 56 weeks. r-square values for 24 weeks and 56 weeks were 0.300 and 0.275, respectively. Anti-Ro: anti-Ro/SSA antibodies; TNF: tumor necrosis factor; CRP: C-reactive protein; IFX: infliximab; ADA: adalimumab; SS: Sjögren's syndrome.

immunoglobulin classes of anti-dsDNA in several patients, with the result that IgM anti-dsDNA was detected in most of the patients, while IgG or IgA anti-dsDNA was positive in few patients (data not shown).

It is possible that anti-Ro-positive patients are more likely to induce immune responses and produce autoantibodies in response to IFX treatment. We measured the trough concentration of IFX and examined human antichimeric antibodies (HACA) in several anti-Ro-positive patients who did not respond to IFX. This revealed that the IFX concentration was lower than 1 μg/ml in most patients. However, HACA was detected in only half of these patients (data not shown). Further, infusion reactions were not seen in anti-Ro-positive patients (Table 3), and the anti-Ro titers did not correlate with the clinical response (data not shown), suggesting that this correlation could not be explained simply by production of HACA.

Our data suggested that use of the anti-TNF-α antibodies IFX or ADA might be related to a lower clinical response, as shown in Table 4. We also analyzed whether each TNF

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inhibitor influenced the clinical response in a multivariable logistic regression model, but this analysis was difficult because the number of patients treated with ETN or ADA was very small. In addition, all the anti-Ro-positive patients who did not respond to IFX or ADA improved clinically when they switched to ETN or tocilizumab as the second biologic DMARD.

The presence of anti-Ro in patients with RA might be related to the inefficacy of IFX compared to the other TNF inhibitors. Further studies are needed to confirm the relationship between anti-Ro and clinical response, because of the limited number of the patients in our study treated with ETN or ADA. Although the mechanisms contributing to this association should be examined further, our results indicate that the presence of anti-Ro should be considered when choosing appropriate biologic DMARD for patients with RA.

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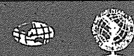

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ORIGINAL ARTICLE

Importance of serine727 phosphorylated STAT1 in IFNγ-induced signaling and apoptosis of human salivary gland cells

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Abstract

Aim: It is reported that in salivary glands of Sjögren's syndrome (SS), interferon gamma (IFNγ) and IFNγ-inducible genes containing signal transducers and activators of transcription 1 (STAT1) are upregulated and play a crucial role in the pathogenesis of SS. The aim of this study is to clarify which phosphorylation of STAT1, serine727 (Ser^{727}) or tyrosine701 (Tyr^{701}) of STAT1, is important for IFNγ signaling and IFNγ-induced apoptosis in salivary gland cells.

Methods: We established STAT1 Tyr^{701} variant (tyrosine to phenylalanine; Y701F) and STAT1 Ser^{727} variant (serine to alanine; S727A), which were transfected into human salivary gland (HSG) cells. HSG cells transfected with these mutant-STAT1 were analyzed on the expression of IFNγ-inducible genes and apoptosis after stimulation with IFNγ.

Results: In Y701F mutant-STAT1 transfected HSG cells (Ser^{727}-dominant HSG cells), IFNγ-inducible genes such as IP10, IRF1, and Fas expression were increased after stimulation with IFNγ. In Ser^{727}-dominant HSG cells, the induction of apoptosis after stimulation with IFNγ was also increased compared with S727A mutant-STAT1 transfected HSG cells (Tyr^{701}-dominant HSG cells).

Conclusion: Phosphorylation of Ser^{727} in STAT1 might be more important in IFNγ signaling and the induction of apoptosis in HSG cells than phosphorylation of Tyr^{701}. Accordingly, we propose that phosphorylation of Ser^{727} in STAT1 could be a potentially suitable new therapeutic target for SS patients to prevent the destruction of salivary glands.

Key words: apoptosis, human salivary gland cells, Interferon gamma, signal transducers and activators of transcription 1, Sjögren's syndrome.

INTRODUCTION

Sjögren's syndrome (SS) is a chronic autoimmune disease characterized pathologically by focal lymphocytic infiltration of exocrine glands, especially lachrymal and salivary glands, and clinically by dry eyes and dry mouth.[1] Inflammatory cytokines, especially Interferon

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gamma (IFNγ), are considered to play an important role in the destruction of the exocrine glands, based on the detection of IFNγ messenger RNA (mRNA) expression in the salivary glands of SS patients.[2] Increased concentrations of IFNγ contribute to the upregulation of human leukocyte antigen (HLA) class II and co-stimulatory molecules on epithelial cells and antigen-presenting cells. IFNγ also upregulates Fas and caspase-8 expression and therefore fosters apoptosis.[3] Moreover, we also reported the upregulation of several IFNγ-inducible genes containing signal transducers and activators of transcription 1 (STAT1) in the salivary glands of SS patients.[4] Thus, the IFNγ signaling seems to play a crucial role in the pathogenesis of SS.

STAT1 is known as the mediator of IFNγ signaling. Maximal activation by STAT1 of the IFNγ signaling requires phosphorylation of both tyrosine701 (Tyr^{701}) and serine727 (Ser^{727}).[5,6] We reported previously the induction of STAT1-α phosphorylation and the different localization of Tyr^{701}-phosphorylated STAT1-α and Ser^{727}-phosphorylated STAT1-α in the labial salivary glands of patients with SS.[7]

Stephanou *et al.*[8] reported that induction of apoptosis and Fas expression by ischemia/reperfusion in cardiac myocytes required Ser^{727} of STAT1 but not Tyr^{701}, suggesting that Ser^{727}-phosphorylated STAT1-α is essential for the induction of apoptosis. However, it is not clear which phosphorylation of STAT1 induces apoptosis by IFNγ in salivary gland cells. To clarify this question, we established a STAT1 Tyr^{701} variant (tyrosine to phenylalanine; Y701F) and STAT1 Ser^{727} variant (serine to alanine; S727A), which were transfected into human salivary gland (HSG) cells. HSG cells transfected with these mutant-STAT1 were analyzed on the expression of IFNγ-inducible genes and apoptosis after stimulation with IFNγ.

MATERIALS AND METHODS

Production of mutant-STAT1 and transformation of mutant-STAT1 into HSG cells

We made the Tyr^{701} mutant (tyrosine to phenylalanine; Y701F) and Ser^{727} mutant (serine to alanine; S727A) STAT1 DNA fragments (Fig. 1a). These two mutant-STAT1 DNA fragments were inserted into myc-His vectors by double digestion with BamH I and Nod I (Fig. 1b). 1.0×10^5/mL of HSG cells were pre-cultured overnight, and then culture medium was changed to medium without sera. Mutant-STAT1 Y701F and S727A were transformed into HSG cells using FuGENE6 (Roche Applied Science, Mannheim, Germany). HSG cells transfected with mutant-STAT1 were selected by zeosin. HSG cells with Y701F mutant-STAT1 were Ser^{727}-dominant HSG cells, and HSG cells with S727A mutant-STAT1 were Tyr^{701}-dominant HSG cells.

IFNγ-inducible gene expression in mutant-STAT1-transfected HSG cells after stimulation with IFNγ

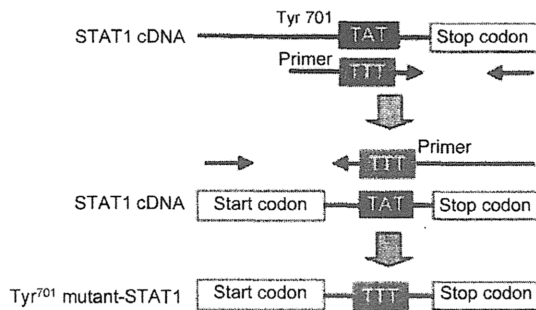
Human salivary gland cells transfected with Y701F, S727A mutant-STAT1 and empty vector were stimulated with IFNγ (2000 U/mL) for 24 h. These HSG cells were trypsinized and total RNA and cell lysate were extracted from HSG cells. Complementary DNA (cDNA) was synthesized by cDNA synthesis kit (Fermentas International, Burlington, ON, Canada). Reverse transcription–polymerase chain reaction (RT-PCR) and quantitative PCR were performed with cDNA using the human IFNγ-inducible protein 10 (IP10), interferon regulatory factor 1 (IRF1), Fas and CD40 specific primers. Human-glyceralaldehyde-3-phosphate dehydrogenase (GAPDH) was amplified to assess the cDNA yield and to analyze as the internal control.

IFNγ-inducible gene expression was examined at protein levels by Western blot analysis using the cell lysate of HSG cells. Total proteins were fractionated on sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in 100% Block-Ace (Dainippon, Osaka, Japan) for 1 h and then incubated with rabbit anti-Fas antibody (1 : 500 dilution; Cell Signaling Technologies, Beverly, MA, USA) or mouse anti-β-actin antibody (2 μg/mL; BioVision, Mountain View, CA, USA) at 4°C overnight. Secondary antibodies, anti-rabbit IgG horseradish peroxidase (HRP) linked antibody (1 : 2000 dilution; Cell Signaling Technologies) or anti-mouse IgG HRP linked antibody (1 : 2000 dilution; Dako, Tokyo, Japan), were applied at room temperature for 1 h, and then proteins were detected by enhanced chemiluminescence using an ECL Western blot detection kit (Amersham, Little Chalfont, UK).

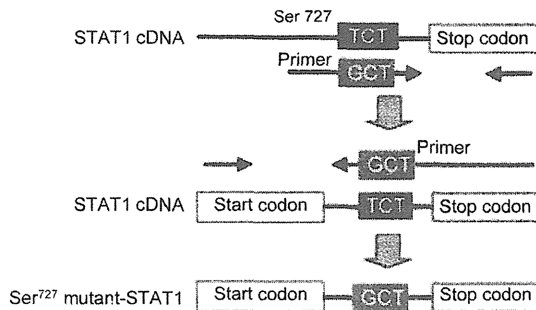
Apoptosis of mutant-STAT1-transfected HSG cells after stimulation with IFNγ

Human salivary gland cells transfected with Y701F, S727A mutant-STAT1 and empty vector were cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and peni-

(a) Tyr^{701} mutant-STAT1 (Y701F)



Ser^{727} mutant-STAT1 (S727A)



(b)

BamH I Nod I

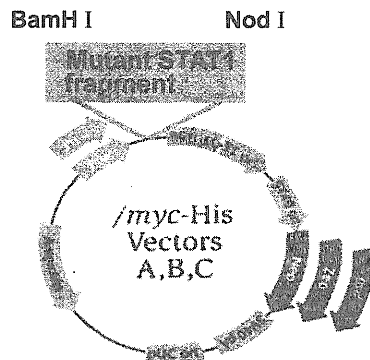


Figure 1 Production of mutant-STAT1 and transformation of mutant-STAT1 into HSG cells. (a) Production of tyrosine701 (Tyr^{701}) mutant (tyrosine to phenylalanine; Y701F) and serine727 (Ser^{727}) mutant (serine to alanine; S727A) STAT1 DNA fragments. (b) Y701F and S727A mutant-STAT1 DNA fragments were inserted into myc-His vectors by double digestion with BamH I and Nod I.

cillin/streptomycin. After stimulation with IFNγ (2000 U/mL) for 24 h, cells were trypsinized and harvested. Harvested HSG cells were washed in phosphate-buffered saline (PBS) and resuspended in binding buffer containing annexin-V-fluorescein isothiocyanate to monitor apoptosis-associated plasma membrane alteration for 20 min at room temperature. The samples were analyzed with a FACS Calibur flow cytometer (BD-Biosciences, Mountain View, CA, USA), and data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

RESULTS

IFNγ-inducible gene expression was increased in Ser^{727}-dominant HSG cells after stimulation with IFNγ

Figure 2 shows the expression of IFNγ-inducible gene mRNA in mutant-STAT1 and empty vector-transfected HSG cells after stimulation with IFNγ. In Y701F mutant-STAT1-transfected HSG cells (Ser^{727}-dominant HSG cells), IP10 and IRF1 mRNA expression were increased after stimulation with IFNγ, whereas Fas

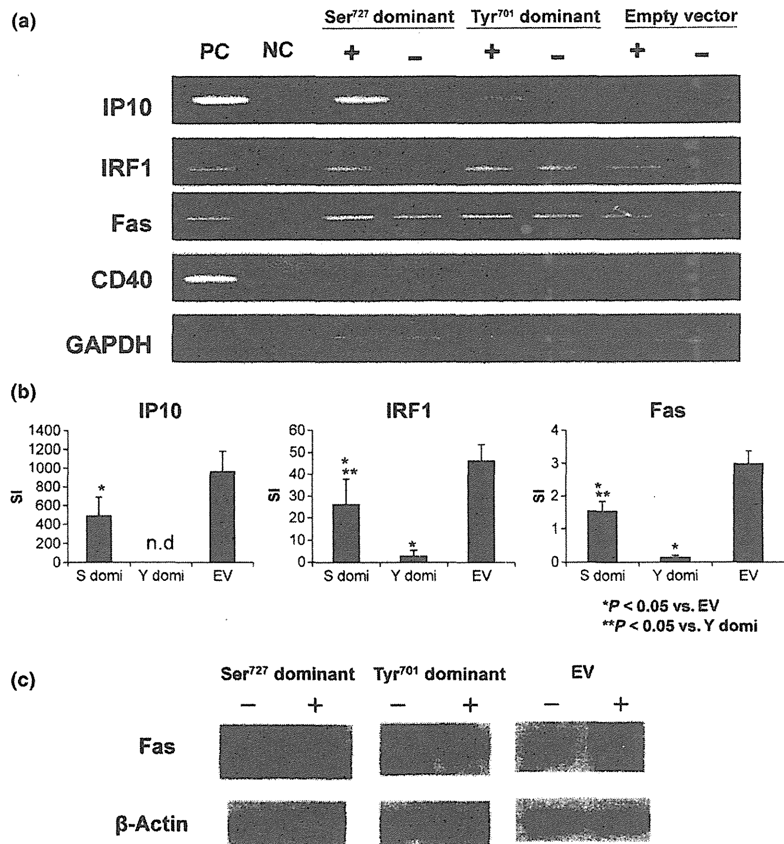


Figure 2 IFNγ-inducible gene expression in mutant-STAT1-transfected human salivary gland (HSG) cells after stimulation with IFNγ. (a) HSG cells transfected with Y701F, S727A mutant-STAT1, and empty vector were stimulated with IFNγ (2000 U/mL) for 24 h. mRNA expression of IFNγ-inducible genes (IP10, IRF1 and Fas) were analyzed by reverse transcription – polymerase chain reaction (RT-PCR). The expression of IP10, IRF1 and Fas were increased in Ser^{727}-dominant HSG cells after stimulation with IFNγ. (b) The results of quantitative PCR are shown as stimulation indexes (S.I), which were calculated by expression levels after stimulation with IFNγ/expression levels before stimulation. In Ser^{727}-dominant HSG cells, IRF1 and Fas mRNA expression levels were significantly increased after stimulation with IFNγ compared with Tyr^{701}-dominant HSG cells ($P < 0.05$, Mann–Whitney *U*-test). (c) Western blot analysis demonstrated that Fas protein expression in Ser^{727}-dominant HSG cells was increased after stimulation with IFNγ, although in Tyr^{701}-dominant HSG cells this was not increased. IP10, IFNγ-inducible protein 10; IRF1, interferon regulatory factor 1; GAPDH, human-glyceralaldehyde-3-phosphate dehydrogenase; PC, positive control; NC, negative control; +, after stimulation with IFNγ; –, before stimulation with IFNγ; EV, empty vector; S domi, Ser^{727}-dominant HSG cells; Y domi, Tyr^{701}-dominant HSG cells; n.d, not determined.

mRNA expression was increased slightly in RT-PCR analysis (Fig. 2a). On the other hand, in S727A mutant-STAT1-transfected HSG cells (Tyr^{701}-dominant HSG cells), only IP10 mRNA expression was increased slightly, whereas IRF1 and Fas mRNA expression were not increased after stimulation with IFNγ in RT-PCR analysis (Fig. 2a). The expression of CD40 mRNA was not detected in any type of HSG cell, neither before nor after stimulation with IFNγ. The results of quantitative PCR on IP10, IRF1 and Fas mRNA expression are shown as stimulation indexes (S.I.), which were calculated by expression levels after stimulation with IFNγ/expression levels before stimulation. In Ser^{727}-dominant HSG cells, IRF1 and Fas mRNA expression levels were significantly increased after stimulation with IFNγ compared with Tyr^{701}-dominant HSG cells ($P < 0.05$, Mann–Whitney *U*-test). S.I of IP10 in Tyr^{701}-dominant HSG cells was not determined, because the expression of IP10 before stimulation was not detected by quantitative PCR analysis (Fig. 2b).

Western blot analysis showed that in Ser^{727}-dominant HSG cells, Fas protein expression was also increased after stimulation with IFNγ, whereas in Tyr^{701}-dominant HSG cells, Fas protein expression was not increased (Fig. 2c).

Induction of apoptosis after stimulation with IFNγ was increased in Ser^{727}-dominant HSG cells

Figure 3 shows the induction of apoptosis after stimulation with IFNγ detected by annexin-V in mutant-STAT1 and empty vector-transfected HSG cells. The population of apoptotic cells before stimulation with IFNγ was 26.8%, 24.5% and 11.9%, and after stimulation with IFNγ, the population of apoptotic cells was changed to 34.9%, 22.8% and 12.9%, in Ser^{727}-dominant HSG cells, Tyr^{701}-dominant HSG cells, and empty vector transfected-HSG cells, respectively. Stimulation index (S.I.) with IFNγ was 1.30, 0.93 and 1.08 in Ser^{727}-dominant HSG cells, Tyr^{701}-dominant HSG cells, and empty vector transfected-HSG cells, respectively. The induction of apoptosis after stimulation with IFNγ was increased in Ser^{727}-dominant HSG cells compared with Tyr^{701}-dominant HSG cells.

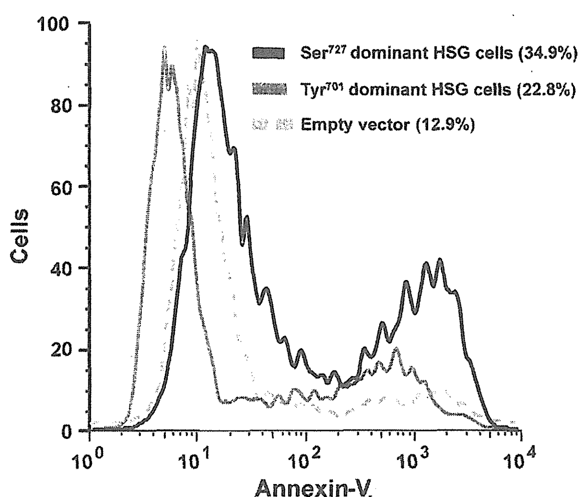


Figure 3 Apoptosis of mutant-STAT1-transfected human salivary gland (HSG) cells after stimulation with IFNγ. HSG cells transfected with Y701F, S727A mutant-STAT1, and empty vector were stimulated with IFNγ (2000 U/mL) for 24 h and then apoptotic cells were detected by annexin-V. The histogram of FLOWJO software (Tree Star, Ashland, OR, USA) showing the population of apoptotic cells after stimulation with IFNγ was 34.9%, 22.8% and 12.9%, in Ser^{727}-dominant HSG cells, Tyr^{701}-dominant HSG cells, and empty vector transfected-HSG cells, respectively. The induction of apoptosis after stimulation with IFNγ was increased in Ser^{727}-dominant HSG cells compared with Tyr^{701}-dominant HSG cells.

DISCUSSION

In Y701F mutant-STAT1-transfected HSG cells (Ser^{727}-dominant HSG cells), the expression of IFNγ-inducible genes such as IP10, IRF1 and Fas, were increased after stimulation with IFNγ. Moreover, in Ser^{727}-dominant HSG cells, the induction of apoptosis after stimulation with IFNγ was also increased compared with S727A mutant-STAT1-transfected HSG cells (Tyr^{701}-dominant HSG cells). These findings indicated that phosphorylation of Ser^{727} in STAT1 might be more important in IFNγ signaling and induction of apoptosis in HSG cells than phosphorylation of Tyr^{701}.

Previously, we reported that Tyr^{701}-phosphorylated STAT1 was localized in infiltrating lymphocytes and the adjacent ductal epithelium, while Ser^{727}-phosphorylated STAT1 was localized only in the ductal epithelium of labial salivary glands from SS patients (SS LSGs).[7] We also revealed that IP10, IRF1 and Fas genes were highly expressed in SS LSGs and colocalized with Ser^{727}-phosphorylated STAT1 but not with Tyr^{701}-phosphorylated STAT1.[7] We proposed that STAT1, especially Ser^{727}-phosphorylated STAT1, might function as a key molecule in the pathogenesis of SS, including the destruction of salivary glands.[7] Interestingly, these previous findings accord with the results in the present study.

In the present study, we showed the correlation of Ser^{727}-phosphorylated STAT1 with IFNγ signaling and the induction of apoptosis in HSG cells *in vitro*. In the pathogenesis of SS, IFNγ might induce phosphorylation of STAT1, especially Ser^{727}, in HSG cells, which cause IFNγ signaling and apoptosis. Thus, Ser^{727}-phosphorylated STAT1 might have essential roles in the destruction of salivary glands in patients with SS. On the other hand, Tyr^{701}-phosphorylated STAT1 might suppress IFNγ signaling and apoptosis. We previously showed that Tyr^{701}-phosphorylated STAT1 was localized in infiltrating lymphocytes in SS LSGs.[7] Thus, IFNγ might induce phosphorylation of Tyr^{701} of STAT1 in infiltrating lymphocytes, resulting in resistance to apoptosis.

In conclusion, phosphorylation of Ser^{727} in STAT1 might be important in IFNγ signaling and the induction of apoptosis in HSG cells. Accordingly, we propose that phosphorylation of Ser^{727} in STAT1 could be a potentially suitable new therapeutic target for SS patients to prevent the destruction of salivary glands.

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Circulating Levels of Soluble α-Klotho Are Markedly Elevated in Human Umbilical Cord Blood

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Context: Fetal serum levels of calcium and phosphate are higher than those in the maternal levels. Although α-Klotho is known to participate in calcium and phosphate metabolism in adults, its role in the perinatal period remains unknown.

Objective: This study aimed to determine the baseline levels of soluble α-Klotho in fetuses and compare them with those in neonates, mothers, and adults to clarify whether α-Klotho is involved in the fetal-specific regulation of calcium and phosphate metabolism.

Design and Setting: We conducted a cross-sectional evaluation of healthy babies (at birth and/or at 4 d after birth), their mothers, and adult volunteers at one hospital.

Participants: Twenty-one healthy mothers, their babies (23 in total, including two pairs of twins), and 25 adult volunteers participated in the study.

Main Outcome Measures: We measured the serum levels of soluble α-Klotho and fibroblast growth factor 23 (FGF23).

Results: In cord blood, the level of α-Klotho was markedly higher (3243 ± 1899 pg/ml) than levels in neonates at d 4 (582 ± 90 pg/ml), mothers (768 ± 261 pg/ml), and adult volunteers (681 ± 140 pg/ml) ($P < 0.001$), whereas the fetal level of FGF23 was lower than levels in the other subjects. The levels of soluble α-Klotho were negatively correlated with those of FGF23 in cord blood. Immunohistochemistry demonstrated that α-Klotho was predominantly expressed in syncytiotrophoblasts in normal term placenta.

Conclusion: Levels of soluble α-Klotho are markedly elevated in cord blood and might be useful as a biomarker for mineral metabolism in the fetus. ***(J Clin Endocrinol Metab* 96: E943–E947, 2011)**

Fetal mineral homeostasis is regulated differently from adult homeostasis. The levels of serum calcium and phosphate in the fetus are higher than the maternal levels during late gestation. PTH and PTHrP are known to be involved in calcium homeostasis (1, 2). On the other hand, the regulatory mechanism of the fetal phosphate level is poorly understood (3).

The *α-Klotho* gene encodes a single-pass transmembrane protein, which was originally identified as an aging-related gene (4). In adults, α-Klotho contributes to the

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Abbreviations: FGF23, Fibroblast growth factor 23; 25-OHD, 25-hydroxyvitamin D; TRPV, transient receptor potential vanilloid.

regulation of calcium and phosphate homeostasis. In the parathyroid, α-Klotho binds to Na^+/K^+-ATPase to regulate PTH secretion and is involved in transepithelial calcium transport (5). α-Klotho is also involved in the activation of transient receptor potential vanilloid (TRPV) 5 in the kidney (6), indicating its central role in the maintenance of calcium homeostasis. In addition, α-Klotho participates in phosphate homeostasis by cooperating with fibroblast growth factor 23 (FGF23) and the FGF receptor (7). FGF23 reduces the serum phosphate level both by suppressing phosphate reabsorption and activating vitamin D in the proximal tubules (8–10).

Although *α-Klotho* is predominantly expressed in the kidney, parathyroid, and choroid plexus, it is also expressed in other tissues including the placenta (4). Its expression in the placenta has led us to hypothesize that α-Klotho might play a role in fetal mineral homeostasis as well as in postnatal homeostasis.

In addition to the transmembrane form, α-Klotho also exists in a soluble form. The soluble form, which is produced by the shedding of the transmembrane protein, is detectable in serum, cerebrospinal fluid, and urine (6, 11). Although soluble α-Klotho is considered to be a humoral factor (12), its regulatory mechanisms and functions are largely unknown, and it is considered that FGF23 signaling requires the transmembrane form of the protein.

Recently, a sandwich ELISA for soluble α-Klotho has been established (13). In the present study, we used this assay to measure the serum levels of soluble α-Klotho in cord blood at birth and compared them to the levels in neonates, mothers, and adults. We found that high levels of soluble α-Klotho are present in cord blood and analyzed the relationship between those of soluble α-Klotho and FGF23 in cord blood. To the best of our knowledge, this is the first report on the measurement of soluble α-Klotho levels in perinatal blood samples.

Subjects and Methods

Study participants

We recruited healthy pregnant women, their babies, and adult volunteers and obtained informed consent from all participants or their legal guardians. The institutional review board of Osaka University Hospital approved this study. The inclusion criteria were an unremarkable medical history, physical examination, and screening laboratory test results for endocrine and metabolic function. The exclusion criteria were premature or postmature infant delivery (gestational age under 37 wk and over 42 wk, respectively), and the neonate being light or heavy for their delivery date (birth weight under −1.5 SD and over 1.5 SD, respectively).

Twenty-one mothers and their babies (n = 23, including two pairs of twins) were enrolled. For comparison, 25 healthy adult volunteers ranging in age from 27 to 48 yr (11 males and 14 females) were also enrolled.

Blood analyses

After the delivery of the neonate, we immediately obtained cord blood samples from the umbilical vein, before the placenta was delivered. We also collected blood from the neonates at d 4 after birth in the morning and fasting morning blood from their mothers and the adult volunteers. The maternal blood was obtained within 24 h of the delivery. The participants were under no dietary restrictions during the study.

After we had collected the blood samples, we separated the serum instantly and stored it at −80 C until analysis. We measured the levels of serum soluble α-Klotho and intact FGF23 in all samples. The serum soluble α-Klotho levels were measured using an ELISA kit provided from Kyowa Hakko-Kirin (Tokyo, Japan) (13). The intra- and interassay coefficients of variation ranged from 2.7 to 9.8% (13). The serum levels of intact FGF23 were determined using a commercial sandwich ELISA kit (Kainos Laboratories, Inc., Tokyo, Japan) (14). Serum calcium, phosphate, intact PTH, 25-hydroxyvitamin D (25-OHD), albumin, and creatinine levels were also measured in the samples except for those from neonates. We corrected the levels of calcium in the samples displaying hypoalbuminemia (albumin < 4.0 g/dl) as reported previously (15).

Immunohistochemistry

Normal human placenta (gestational age, 38 wk) was obtained from an uncomplicated pregnancy. The specimen was fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 4-μm-thick sections. Antigen retrieval was performed using 10 mM citrate buffer (pH 5.9) for 15 min at 98 C. The sections were stained using anti-α-Klotho antibody (sc-22218; Santa Cruz Biotechnology, Santa Cruz, CA) and goat ImmunoCruz Staining System (Santa Cruz Biotechnology). The slides were counterstained with hematoxylin. Normal goat IgG was used as a negative control.

Statistical analyses

The results are expressed as the mean ± SD. We compared biochemical parameters and soluble α-Klotho and FGF23 levels among the groups by ANOVA, followed by the Tukey-Kramer method. The relationship between soluble α-Klotho and FGF23 in cord blood samples was analyzed using Pearson's correlation test.

All statistical analyses were conducted using JMP software version 8.0.1 (SAS Institute Inc., Cary, NC).

Results

Levels of soluble α-Klotho in cord blood are higher than those in neonates, mothers, and adults

The biochemical findings are shown in Table 1. Serum calcium, phosphate, intact PTH, 25-OHD, albumin, and creatinine levels were within the normal range in both the mother and adult groups (16). As previously reported, serum calcium and phosphate levels were significantly higher,

TABLE 1. Biochemical parameters

	Cord blood (n = 23)	Mother (n = 21)	Adult (n = 25)
Calcium (mg/dl)[a]	10.5 ± 0.43	9.44 ± 0.34[b]	9.42 ± 0.29[b]
Phosphate (mg/dl)	4.93 ± 0.65	3.66 ± 0.59[b]	3.62 ± 0.50[b]
Intact PTH (pg/ml)	7.11 ± 5.22	26.5 ± 12.2[b,f]	36.4 ± 9.80[b]
25-OHD (ng/ml)	8.95 ± 3.47	13.3 ± 6.45[d]	15.0 ± 4.43[c]
Albumin (g/dl)	3.53 ± 0.38	3.15 ± 0.42[c,e]	4.60 ± 0.27[b]
Creatinine (mg/dl)	0.49 ± 0.09	0.48 ± 0.08[e]	0.69 ± 0.10[b]

Data are expressed as mean ± SD and were compared among the cord blood group, mother group, and adult volunteer group using the ANOVA test.

[a] The calcium values were corrected using the following formula in cases involving hypoalbuminemia (Alb < 4.0 g/dl): corrected calcium (mg/dl) = measured calcium (mg/dl) + 4 − albumin (g/dl) (15).

[b] $P < 0.0001$ *vs.* cord blood; [c] $P < 0.01$ *vs.* cord blood; [d] $P < 0.05$ *vs.* cord blood; [e] $P < 0.0001$ *vs.* adult; [f] $P < 0.01$ *vs.* adult.

whereas serum intact PTH levels were lower in the cord blood than in the sera of the mother and adult volunteers (3).

The levels of soluble α-Klotho in the cord blood were markedly higher than those in the other groups ($P <$ 0.0001) (Fig. 1A). The mean value for soluble α-Klotho in the cord blood was 3243 ± 1899 pg/ml, whereas those in the sera of the neonates at d 4, mothers, and adult volunteers were 582 ± 90, 768 ± 261, and 681 ± 140 pg/ml, respectively. There was no significant difference among the samples from neonates at d 4, mothers, and adults.

On the other hand, the levels of FGF23 in cord blood were significantly lower than those in other groups ($P < 0.0001$, *vs.* neonate and adult; $P < 0.0005$, *vs.* mother) (Fig. 1B). The mean value for FGF23 in cord blood was 8.61 ± 6.48 pg/ml, whereas those in the neonates, mothers, and adult volunteers were 28.4 ± 20.5, 26.7 ± 15.1, and 34.6 ± 7.69 pg/ml, respectively.

Negative correlation between the levels of soluble α-Klotho and FGF23 in cord blood samples

Then, using only the cord blood samples (n = 23), we examined the relationship between the levels of soluble α-Klotho and FGF23. We found that soluble α-Klotho levels were inversely correlated with FGF23 levels ($R^2 = 0.20$; $P < 0.05$) (Fig. 1C).

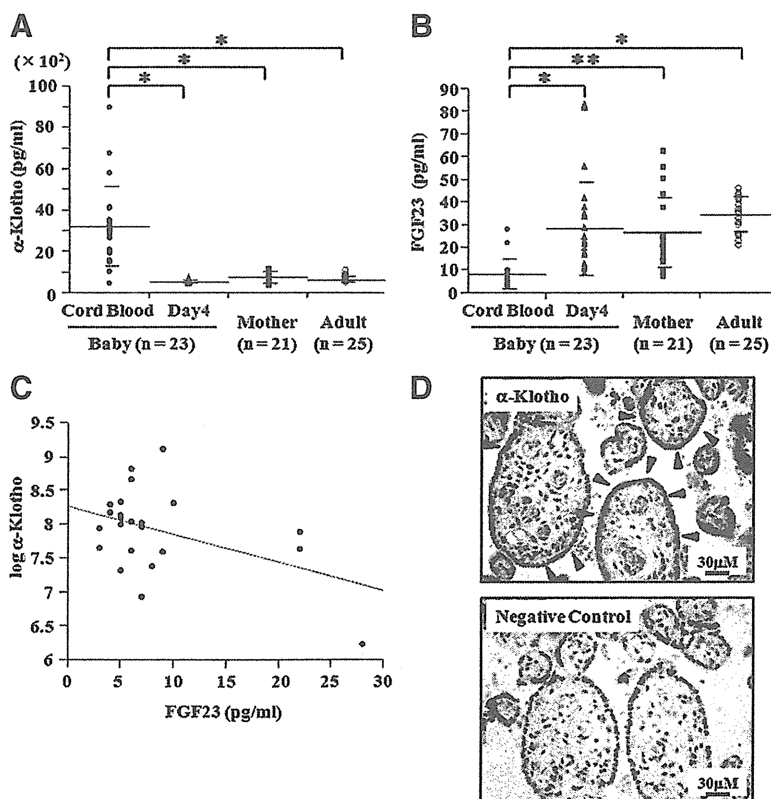

FIG. 1. A and B, Comparison of serum soluble α-Klotho (A) and FGF23 (B) levels among the cord blood, neonate at d 4, mother, and adult volunteer groups by ANOVA. *, $P < 0.0001$; **, $P < 0.0005$. *Closed circles*, *triangles*, *squares*, and *open circles* denote the values for the cord blood, neonate, mother, and adult volunteer groups, respectively. The *long and short bars* represent the mean and SD, respectively. C, Correlation of serum levels of α-Klotho with FGF23 in cord blood samples according to Pearson's correlation test. α-Klotho was log-transformed to reduce skewness. $R^2 = 0.20$; $P < 0.05$. D, Representative image of normal human term placenta stained with antibody against α-Klotho. α-Klotho was predominantly expressed in syncytiotrophoblasts (*arrowheads*). Normal goat IgG was used as a negative control.

Expression of α-Klotho in syncytiotrophoblasts in term placenta

Immunohistochemical staining demonstrated that α-Klotho was predominantly expressed in syncytiotrophoblasts with some expression in endothelium of fetal vessels and connective tissue of villi (Fig. 1D).

Discussion

In the current study, we found that the levels of soluble α-Klotho in cord vein blood that inflow to fetus after the exchange of gas and nutrients in the placenta were markedly higher than those in neonates, mothers, and adults (Fig. 1A). Immunohistochemical staining of the placenta revealed that syncytiotrophoblasts that originate from fetus predominantly expressed α-Klotho (Fig. 1D). Although we cannot exclude the possibility that other fetal tissues also contribute, it is likely that the syncytiotrophoblast is one of the major sources of the soluble α-Klotho circulating abundantly in the fetus. The lower level of soluble α-Klotho in neonates at d 4 compared with that in cord

blood supports the idea that the protein is derived from the placenta.

In contrast to the high levels of soluble α-Klotho, the levels of FGF23 in cord blood were lower than those in the other samples. This result was consistent with the findings of a previous report (17). Considering a report demonstrating that FGF23 expression in fetal rat bones was much lower than that in young adult rat bones (18), the low levels of FGF23 in cord blood might be due to the low expression of FGF23 in fetal tissues. In addition, it may suggest that the FGF23 in the mother's blood is not transferred to the fetus through the placenta.

We found a negative correlation between soluble α-Klotho and FGF23 levels in the cord blood samples. This result was also compatible with the data reported by Yamazaki *et al.* (13) in which samples from healthy children and adult volunteers were analyzed. Although the precise mechanism is unknown, the high level of soluble α-Klotho circulating in the fetus may contribute to the low level of intact FGF23 in cord blood.

We also performed multiple regression analysis and found that soluble α-Klotho was one of the determinants of the levels of phosphate (Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). Serum calcium level also might be associated with that of α-Klotho, although the P value was 0.07. It has been reported that TRPV6 is involved in maternal-fetal calcium transport in mouse models (19). Moreover, Lu *et al.* have recently reported that α-Klotho activated not only TRPV5 but also TRPV6 (20). Given these results, soluble α-Klotho may contribute to the establishment of the fetomaternal calcium gradient also. However, considering the observation that the absence of α-Klotho in mice leads to hypercalcemia and hyperphosphatemia after birth (4), it remains to be determined whether the high levels of α-Klotho is an epiphenomenon in response to the higher serum calcium and phosphate, or is causing some of the biochemical features of fetuses. Even so, we can say that the measurement of soluble α-Klotho in cord blood as a biomarker might be useful in management of some genetic neonatal conditions such as hypercalcemia and hypophosphatemia. Measurement of calcium and phosphate during the perinatal period in the α-Klotho-deficient mice and generation of syncytiotrophoblast-specific α-Klotho-knockout mice might provide further insight into the roles of Klotho in fetal mineral metabolism.

In conclusion, the levels of soluble α-Klotho in cord blood were markedly high, and syncytiotrophoblasts in placenta were likely to be one of the major sources. Soluble α-Klotho in cord blood might be useful as a biomarker for calcium and phosphate metabolism in fetus.

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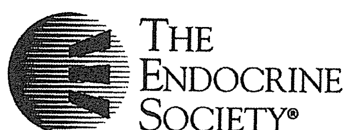

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Original Article

The QT Intervals in Infancy and Time for Infantile ECG Screening for Long QT Syndrome

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Background: Electrocardiographic and molecular studies have clarified an association between sudden infant death syndrome (SIDS) and long QT syndrome (LQTS), and few data are available for the QT interval in infancy from birth to 1 year of age. Appropriate time of electrocardiographic screening is not clarified. Medical examinations during infancy are mandatory in Japan.

Methods and Results: The study population included 1,058 infants. Electrocardiograms were collected with information of infants at birth and at examination. The QT intervals of three consecutive beats were measured in lead V_5. Statistical analysis revealed that the following formula was appropriate to minimize the effect of heart rate for infants: corrected QT interval; QTc = QT interval/RR interval$^{0.43}$. Subjects were divided into four groups as follows: 0–2, 3–6, 6–11, and 12–52 weeks of age. Tukey's multiple comparison showed that the QTc intervals were longest ($p < 0.0001$) in subjects who were 6–11 weeks of age.

Conclusions: The QTc interval showed the highest peak at 6–11 weeks of age in infancy. The peak period of occurrence of SIDS is at approximately 2 months of age. An appropriate time of electrocardiographic screening for QT prolongation will be one month of age, and follow-up studies are needed.

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Key words: Electrocardiography, Long QT syndrome, Death (sudden)

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