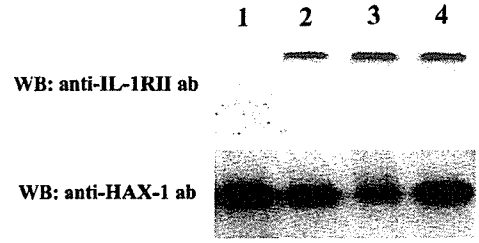


Fig. 8. Depletion of IL-1RII and HAX-1 by RNA interference. A DNA fragment that targeted the sequence in the ORFs of IL-1RII and HAX-1, and a control with a corresponding random sequence were obtained and were cloned into pSilencer 3.1H1-neo, an siRNA-expressing vector. Stable transfectants selected by G418 were cultured in DMEM plus 10% FBS for 72 h, and cell lysates were prepared by sonication. The cell lysates from various transfectants were resolved in 15% SDS/PAGE and transferred to nitrocellulose membrane. Anti-IL-1RII Ab (*Upper*) and anti-HAX-1 Ab (*Lower*) were used to perform Western blotting (WB). Lane 1, siRNA vector that contained the sequences of IL-1RII; lane 2, siRNA vector that contained random sequences for IL-1RII; lane 3, siRNA vector that contained the sequences of HAX-1; lane 4, siRNA vector that contained random sequences for HAX-1.

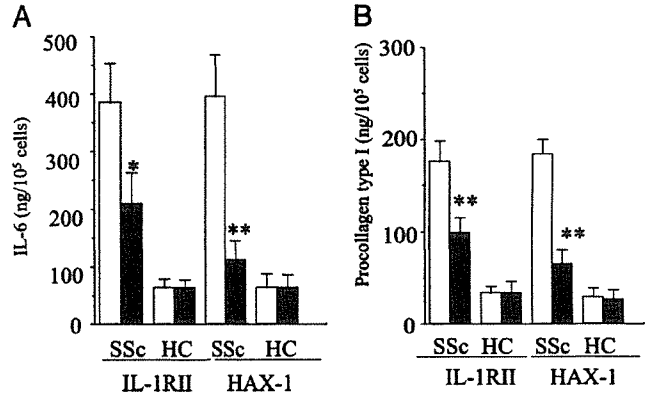


Fig. 10. IL-6 and procollagen type I C-peptide production decreases in SSc fibroblasts by the suppression of IL-1RII or HAX-1. Fibroblasts were cultured in serum-free media. After 48 h of culturing, commercial ELISA kits were used to measure IL-6 (*A*) and procollagen type I C-peptide (*B*) in culture supernatants. Open bars, random siRNA transfectants; filled bars, specific siRNA transfectants; SSc, fibroblasts derived from SSc ($n = 5$); HC, fibroblasts derived from healthy controls ($n = 3$). *, $P < 0.05$; **, $P < 0.01$ (compared with random siRNA transfectants).

involved in the transcriptional activation of several proteins. Although IL-1α was traditionally understood to exhibit biological functions such as inflammation, autoimmunity, and fibrosis through IL-1 receptors on the cell surface, the abovementioned findings and this study strongly support the theory of a nuclear site of action for IL-1α.

Another important finding is a role of IL-1RII, which binds pre-IL-1α inside human fibroblasts that are derived from SSc. McMahon *et al.* (17) and Sims *et al.* (18) reported that IL-1RII is a cell-surface receptor on B lymphocytes and neutrophils with a binding affinity for IL-1α, pre-IL-1α, and IL-1β, but it is not capable of the signal transduction of IL-1 because of the lack of the endoplasmic domain. Our current results revealed that IL-1RII combined with pre-IL-1α plays a crucial role in the biological features of pre-IL-1α within SSc fibroblasts. We also found the differential expression of IL-1RII between SSc ($n = 5$) and normal fibroblasts ($n = 3$) at the cellular mRNA and protein levels. Constitutive expression of IL-1RII, as well as pre-IL-1α, may be an important phenotype of SSc fibroblasts, although the mechanisms whereby intracellular IL-1RII was highly expressed in SSc fibroblasts remain to be clarified.

Suzuki *et al.* (19) first identified the HAX-1 protein by screening the proteins that interact with HS1 (hematopoietic lineage cell-specific protein 1). HS1 is B cell-signaling protein and is one of the major substrates of the Src and Syk/Zap-70 kinases (20). The HS1 protein mainly exists in the cytoplasm and

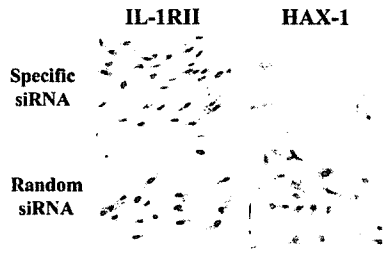


Fig. 9. Cell distribution of pre-IL-1α in SSc fibroblasts depleting IL-1RII or HAX-1. We obtained a DNA fragment targeting the sequence of IL-1RII or HAX-1, which was cloned into pSilencer 3.1 H1-neo, an siRNA-expressing vector. As a control, a scramble DNA fragment was generated that had the same number of nucleotides but did not display sequence identity with IL-1RII and HAX-1 (random siRNA). These vectors were transfected into SSc fibroblasts, and intracellular pre-IL-1α was detected by using immunocytochemistry of DAB staining.

nucleus, and, when the molecule is associated with HAX-1, it moves to the mitochondrial membrane. HAX-1 also interacts with pre-IL-1α in human chondrocytes, although the biological properties for the complex of HAX-1 and pre-IL-1α have not been fully elucidated (16). The HAX-1 protein appears to be expressed ubiquitously in various normal tissues and to constitute the domain that is responsible for binding to the pre-IL-1α, HS1, cortactin, PKD2, EBNA-LP, Bcl-2, and HIV1 Vpr proteins (21–23). The fact that HAX-1 interacts with a variety of structurally unrelated proteins suggests an essential function for HAX-1 that involves intracellular signaling and shuttling of various intracellular molecules. Our observations indicate the importance of HAX-1 for the nuclear localization of pre-IL-1α in fibroblasts. Posttranslational modifications such as phosphorylation and myristoylation of NTP-IL-1α are well recognized mechanisms that are involved in the transport of pre-IL-1α to the nucleus (24, 25). Notably, myristoylation occurs on lysine residues 82 and 83 of pre-IL-1α, located in the nuclear localization sequence (NLS). HAX-1 was associated with three segments of NTP-IL-1α, including the NLS segment (16), which suggests that the binding of HAX-1 with the NLS (KVLKKRR) of pre-IL-1α might facilitate the nuclear localization of the pre-IL-1α complex in fibroblasts. Taken together, the findings strengthen the conclusion that proteins associated with HAX-1 can shuttle between nuclear and cytoplasmic compartments.

A previous investigation looking for a nuclear target of pre-IL-1α revealed the interaction between pre-IL-1α and necdin by a yeast two-hybrid system (26). Necdin is a 47-kDa protein that functions as a cell-growth suppressor in a manner similar to that of the retinoblastoma tumor suppressor protein, Rb (27, 28). In our study, IP showed a faint band (≈47 kDa) that was subjected to N-terminal amino acid sequence analysis. However, we could not identify the molecule because of the small amount of peptide. Although we did not confirm that necdin was one of the intracellular pre-IL-1α-binding proteins, we did detect the expression of necdin in SSc and normal fibroblasts (data not shown). Moreover, the suppression of necdin with an RNAi method did not affect IL-6 and procollagen type I production in SSc fibroblasts (data not shown), which is inconsistent with the results of previous studies. This discrepancy may be explained, in part, by the different cell types used in each experiment (fibroblasts versus Saos-2 osteosarcoma cells).

Recent reports by Higgins *et al.* (29) and Kanangat *et al.* (30) demonstrated the biological functions of intracellular IL-1 receptor antagonist (icIL-1RA) in SSc fibroblasts. They indicated that icIL-1RA was overexpressed in SSc fibroblasts and that icIL-1RA was involved in the fibrogenic phenotype of SSc fibroblasts. Although we did not examine the expression of icIL-1RA in this study, icIL-1RA may have bound to intracellular IL-1RII that consisted of the pre-IL-1α complex. To determine whether icIL-1RA is the fourth component of the pre-IL-1α complex in SSc fibroblasts would have a potential role in delineating the molecular events of the fibrosis in SSc that are associated with the pre-IL-1α complex.

In conclusion, our study found the formation of the pre-IL-1α complex, which consists of pre-IL-1α, IL-1RII, and HAX-1, inside SSc fibroblasts. This complex plays a crucial role in the fibrogenic phenotype of SSc fibroblasts. Because of its nuclear localization, we believe this complex acts in the nuclei of fibroblasts; however, based on a search of the National Center for Biotechnology Information conserved domain database, these proteins do not have a DNA-binding motif. We speculate that this complex is part of a larger one. A putative pre-IL-1α complex is illustrated in Fig. 7.

Materials and Methods

Cell Culture. After providing informed consent, five female patients with SSc (median age 46) and three healthy female donors (median age 42) were enrolled in this study, which met the standards of our institutional review board. All patients were classified into diffuse cutaneous SSc according to the criteria of the American Rheumatism Association (31) and the classification of LeRoy *et al.* (32). Skin fibroblast lines were obtained from biopsied skin and explanted into tissue cultures. A murine fibroblast-like cell line, NIH 3T3, was also used in this study and was obtained from the American Tissue Culture Collection. The culture media consisted of DMEM (Sigma, St. Louis, MO) with 10% FBS (Sigma) and antibiotics (penicillin and streptomycin; Invitrogen, Carlsbad, CA) or of a serum-free medium (QBSF-51; Sigma). In this experiment, cells were used in the third through the fifth passages.

Immunocytochemical Staining. Monolayer fibroblast cultures (5×10^3 cells per well) were grown for 48 h in four-chamber slides (Lab-Tek; Nalge Nunc, Tokyo, Japan). Fibroblasts were washed twice with cold PBS and fixed with 2% paraformaldehyde plus 0.1% Triton X-100 in PBS. The primary Abs used in this experiment were monoclonal anti-human IL-1α Ab (R & D Systems, Cambridge, MA), monoclonal anti-human IL-1 receptor type II Ab (R & D Systems), and monoclonal anti-HAX Ab (BD Biosciences, San Jose, CA). Cells were incubated with the primary Ab (5 μg/ml) or as controls with preimmune mouse IgG (5 μg/ml; Dako, Kyoto, Japan) for 1 h at 4°C. The primary Ab was detected by incubation with biotinylated anti-mouse IgG Ab as the secondary Ab for 30 min at room temperature and then incubated with Avidin/Biotin-HRP Complex (ABC; Vector Laboratories, Burlingame, CA). Cells were then stained by DAB-peroxidase substrate (Sigma). Hematoxylin was used for nuclear staining. The chamber slides were dried and examined by light microscopy. Alternatively, after the treatment of the first Ab, cells were incubated with FITC- or Cy3-conjugated anti-mouse IgG Ab (Sigma). The chamber slides were washed three times and then mounted in 90% glycerol-PBS that contained 0.1% paraphenylendiamine and 1% *n*-propylgalate. A fluorescence image was obtained with fluorescence microscopy (Nikon, Tokyo, Japan).

Immunoprecipitation. SSc fibroblasts were cultured in DMEM (methionine/cystein-free) that contained 5% dialyzed FBS and 100 μCi/ml [^{35}S]methionine/cystein (1 Ci = 37 GBq; Amersham Bioscience, Buckinghamshire, U.K.) for 16 h. After a pulse, cells were harvested and suspended in 3 ml of IP procedure (IPP) buffer (10 mM Tris, pH 8.0/0.5 M NaCl/0.1% Nonidet P-40/0.1 mM PMSF/1 μg/ml leupeptin) and then sonicated on ice. Nuclear and cytosolic extracts were obtained together after centrifugation and were used for IP studies. A 40-μl volume of protein G-Sepharose was preincubated with rabbit anti-human IL-1α Ab (100 ng; Genzyme, Cambridge, MA) or control rabbit IgG (100 ng; Dako) and was added to the extracts and rotated for 3 h at 4°C. Immunoprecipitates were washed three times with IPP buffer and then fractionated by 10% sodium dodecyl (lauryl) sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) with molecular weight markers-^{14}C methylated protein (Amersham Bioscience). Radiolabeled polypeptides were visualized by autoradiography.

Peptide Sequencing. Two specific bands (65 and 35 kDa) were subjected to direct peptide sequencing. For sequencing, the proteins that were separated by SDS/PAGE were electrophoretically transferred onto poly(vinylidene difluoride) (PVDF) membrane (Bio-Rad, Richmond, CA). The PVDF membrane was stained with Coomassie brilliant blue R-250, and each band was excised and subjected to N-terminal amino acid sequence analysis (Procise 494 HT protein sequencing system; Applied Biosystems, Foster City, CA).

RT-PCR. Total RNA was extracted from cultured fibroblasts with TRIzol reagent (Invitrogen), and then 1 μg of total RNA was reverse-transcribed into cDNA with SuperScript III (Invitrogen) according to the manufacturer's instructions. Real-time RT-PCR was performed in triplicate with an ABI 7900HT system (Applied Biosystems) and a fluorescein-labeled (FAM-labeled) TaqMan gene expression assay kit (Applied Biosystems) for IL-1RII, HAX-1, and GAPDH as an endogenous control. The results were analyzed with SDS 2.1 software (Applied Biosystems). Those genes' expressions were calculated from the accurate threshold cycle (Ct), which is the PCR cycle at which an increase in fluorescein from TaqMan probes can first be detected above a baseline signal. The Ct values for GAPDH were substituted from the Ct values for IL-1RII and HAX-1 in each well to calculate ΔCt. The triplicate ΔCt values for each sample were averaged.

Construction of Expression Plasmids and Transfection. The cDNA encoding human IL-1α, pre-IL-1α, and NTP-IL-1α were all isolated by PCR and subcloned into pcDNA4-V5 (Invitrogen). The cDNA encoding human IL-1RII was isolated by PCR and subcloned into pcDNA3 (Invitrogen). For stable transfections, NIH 3T3 cells in 60-mm dishes (70% confluent) were incubated with 3 ml of Opti-MEM (Invitrogen) that contained 5 μg of DNA and 18 μl of Lipofectamine 2000 (Invitrogen). After 5 h, 3 ml of DMEM with 20% FBS was added. After 24 h, the medium was changed to DMEM with 10% FBS, followed by an additional 24 h of culture. G418 (400 μg/ml) was added to the culture medium 48 h after transfection and kept for 15 days. The G418-resistant colonies were harvested by gentle digestion with trypsin, and cells were preserved in liquid N_2 with Cellbanker (Mitsubishi Kagaku Iatron, Tokyo, Japan) until use.

Western Blotting. Confluent fibroblasts were maintained in a serum-free medium for 48 h. Cells were then trypsinized and washed with PBS. Cell lysates were prepared from fibroblasts, including PBS that contained 0.1 mM PMSF and 1 μg/ml leupeptin by sonication on ice. The cell lysates were resolved in 15% polyacrylamide gels under reducing conditions and transferred to nitrocellulose membranes (Bio-Rad). The membranes were incubated with the primary Abs for 1 h. Horseradish peroxidase-conjugated antimouse IgG Ab (Santa Cruz Biotech-

nology, Santa Cruz, CA) was applied to the membrane and incubated for 1 h. The blot was developed by the enhanced chemiluminescence system (Amersham) and exposed on x-ray film. The primary Abs used in this experiment were monoclonal anti-human IL-1α Ab (R & D Systems), monoclonal anti-human IL-1 receptor type II Ab (R & D Systems), monoclonal anti-V5 Ab (Invitrogen), and monoclonal anti-HAX Ab (BD Biosciences).

Bacterial Two-Hybrid System. Reagents and protocol were obtained from Stratagene (BacterioMatch two-hybrid system). The pre-IL-1α protein was fused briefly to the full-length bacteriophage λ repressor protein (λcI) with pBT plasmid (Stratagene), which contained the N-terminal DNA-binding domain and the C-terminal dimerization domain. The target proteins (IL-1RII and HAX-1) were fused to the N-terminal domain of the α-subunit of RNA polymerase with pTRG plasmid (Stratagene). The pre-IL-1α protein was tethered to the λ operator sequence upstream of the reporter promoter through the DNA-binding domain of λcI. When the pre-IL-1 and target proteins interact, they recruit and stabilize the binding of RNA polymerase at the promoter and activate the transcription of a reporter gene, the Amp^r gene. A second reporter gene, β-galactosidase, is expressed from the same activatable promoter, which provides an additional mechanism to validate the pre-IL-1α and target proteins' interaction. The suitable *Escherichia coli* host strain (XL1-Blue MRF' Kan) was transformed with the two plasmids. Blue colonies are positive in LB agar plates, including tetracycline, chloramphenicol, kanamycin, and X-gal.

Depletion of HAX-1 and IL-1RII by RNA Interference. The siRNA target-finder algorithm, which is available on the Ambion (Austin, TX) web site (www.ambion.com), was used to select 21 nucleotide oligomers to be tested for RNA interference. We obtained a DNA fragment targeting the sequence in the ORF and a control with a corresponding random sequence. These two DNA fragments were cloned into pSilencer 3.1 H1-neo (Ambion), an siRNA-expressing vector, according to the manufacturer's instructions. Target sequences for siRNAs of HAX-1 and IL-1RII were selected to be 5'-AACCCAAGGTTCCATAGTCCT-3' and 5'-AAGAAGAGACACGGATGTGGG-3', respectively. A random 21-nt sequence as a control was generated that had the same numbers of nucleotides but did not display sequence identity with HAX-1 and IL-1RII. Basic local alignment search tool analysis ensured that sequence identity between a random nucleotide and homosapience cDNA in the National Center for Biotechnology Information database was 15 nucleotides or fewer. The random sequences for HAX-1 and IL-1RII were 5'-AACCGCGAATCTCATAGTCCT-3' and 5'-AAGGAGAGCAGCGGATGTAAG-3', respectively. The method for stable transfections was described earlier.

Measurement of IL-6 and Procollagen Type I. Fibroblasts were cultured in 24-well culture plates with serum-free medium for 48 h, and then the supernatants were collected and preserved at -30°C until use. IL-6 and procollagen type I were measured by using commercial ELISA kits [R & D Systems and Takara Shuzo (Kyoto, Japan), respectively].

Statistical Analyses. The results of IL-6 and procollagen type I concentrations were shown as mean $\pm$ SD, and comparisons of data were performed with Student's t test. Differences were considered to be significant at $P < 0.05$.

This work was supported by a research grant from the Ministry of Health, Labor, and Welfare (to Y. Kawaguchi) in Japan.

1. Medsger TA, Jr (2001) in *Arthritis and Allied Conditions*, ed Koopman WJ (Lippincott Williams & Wilkins, Philadelphia), pp 1590–1624.
2. Varga J, Korn JH (2004) in *Systemic Sclerosis*, eds Clement PJ, Furst DE (Lippincott Williams & Wilkins, Philadelphia), pp 63–97.
3. Kawaguchi Y (1994) *Clin Exp Immunol* 97:445–450.
4. Kawaguchi Y, Hara M, Wright TM (1999) *J Clin Invest* 103:1253–1260.
5. Kawaguchi Y, McCarthy SA, Watkins SC, Wright TM (2004) *J Rheumatol* 31:1946–1954.
6. Dinarello CA (1996) *Blood* 87:2095–2147.
7. Kobayashi Y, Yamamoto K, Saido T, Kawasaki H, Oppenheim JJ, Matsushima K (1990) *Proc Natl Acad Sci USA* 87:5548–5552.
8. Wessendorf JHM, Garfinkel S, Zhan X, Brown S, Maciag T (1993) *J Biol Chem* 268:22100–22104.
9. Sims JE, March CJ, Cosman D, Widmer MB, MacDonald HR, McMahan CJ, Grubin CE, Wignall JM, Jackson JL, Call SM, *et al.* (1988) *Science* 241:585–589.
10. Greenfeder SA, Nunes P, Kwee L, Labow M, Chizzonite RA, Ju G (1995) *J Biol Chem* 270:13757–13765.
11. Lonnemann G, Engler-Blum G, Muller GA, Koch KM, Dinarello CA (1995) *Kidney Int* 47:845–854.
12. Maier JAM, Voulalas P, Roeder D, Maciag T (1990) *Science* 249:1570–1574.
13. Buryskova M, Pospisek M, Grothey A, Simmet T, Burysek L (2004) *J Biol Chem* 279:4017–4026.
14. Werman A, Werman-Venkert R, White R, Lee J-K, Werman B, Krelin Y, Voronov E, Dinarello CA, Apte RN (2004) *Proc Natl Acad Sci USA* 101:2434–2439.
15. Stevenson FT, Turck J, Locksley RM, Lovett DH (1997) *Proc Natl Acad Sci USA* 94:508–513.
16. Yin H, Morioka H, Towle CA, Vidal M, Watanabe T, Weissbach L (2001) *Cytokine* 15:122–137.
17. McMahon CJ, Slack JL, Mosley B, Cosman D, Lupton SD, Brunton LL, Grubin CE, Wignall JM, Jenkins NA, Brannan CI, *et al.* (1991) *EMBO J* 10:2821–2832.
18. Sims JE, Gayle MA, Slack JL, Alderson MR, Bird TA, Giri JG, Colotta F, Re F, Mantovani A, Shanebeck K, *et al.* (1993) *Proc Natl Acad Sci USA* 90:6155–6159.
19. Suzuki Y, Demoliere C, Kitamura D, Takeshita H, Deuschle U, Watanabe T (1997) *J Immunol* 158:2736–2744.
20. Taniuchi I, Kitamura D, Maekawa Y, Fukuda T, Kishi H, Watanabe T (1995) *EMBO J* 14:3664–3678.
21. Yedavalli VSRK, Shih H-M, Chiang Y-P, Lu C-Y, Chang L-Y, Chen M-Y, Chuang C-Y, Dayton AI, Jeang K-T, Huang L-M (2005) *J Virol* 79:13735–13746.
22. Gallagher AR, Cedzich A, Gretz N, Somlo S, Witzgall R (2000) *Proc Natl Acad Sci USA* 97:4017–4022.
23. Matsuda G, Nakajima K, Kawaguchi Y, Yamanashi Y, Hirai K (2003) *Microbiol Immunol* 47:91–99.
24. Beuscher HU, Nickells MW, Colten HR (1988) *J Biol Chem* 263:4023–4028.
25. Stevenson FT, Bursten SL, Fanton C, Locksley RM, Lovett DH (1993) *Proc Natl Acad Sci USA* 90:7245–7249.
26. Hu B, Wang S, Zhang Y, Fegahli CA, Dingman JR, Wright TM (2003) *Proc Natl Acad Sci USA* 100:10008–10013.
27. Hayashi Y, Matsuyama K, Takagi K, Sugiura H, Yoshikawa K (1995) *Biophys Res Commun* 213:317–324.
28. Tanimura H, Taniguchi N, Hara M, Yoshikawa K (1998) *J Biol Chem* 273:720–728.
29. Higgins GC, Wu Y, Postlethwaite AE (1999) *J Immunol* 163:3969–3975.
30. Kanangat S, Postlethwaite AE, Higgins GC, Hasty KA (2006) *J Invest Dermatol* 126:756–765.
31. Subcommittee for Scleroderma Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee (1980) *Arthritis Rheum* 23:581–590.
32. LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA, Jr, Rowell N, Wollheim F (1988) *J Rheumatol* 15:202–205.

Research article

Open Access

NOS2 polymorphisms associated with the susceptibility to pulmonary arterial hypertension with systemic sclerosis: contribution to the transcriptional activity

Yasushi Kawaguchi[1], Akiko Tochimoto[1], Masako Hara[1], Manabu Kawamoto[1], Tomoko Sugiura[1], Yasuhiro Katsumata[1], Jun Okada[2], Hirobumi Kondo[2], Mitsuo Okubo[3] and Naoyuki Kamatani[1]

[1]Institute of Rheumatology, Tokyo Women's Medical University, Tokyo, Japan
[2]Department of Internal Medicine, Kitasato University School of Medicine, Sagamihara, Japan
[3]Transfusion Medicine and Cell Therapy, Saitama Medical School, Kawagoe, Japan

Corresponding author: Yasushi Kawaguchi, y-kawa@ior.twmu.ac.jp

Received: 22 Feb 2006 Revisions requested: 27 Apr 2006 Revisions received: 14 May 2006 Accepted: 2 Jun 2006 Published: 3 Jul 2006

Arthritis Research & Therapy 2006, 8:R104 (doi:10.1186/ar1984)
This article is online at: http://arthritis-research.com/content/8/4/R104

Abstract

Systemic sclerosis (SSc) is a connective tissue disease characterized by tissue fibrosis. One of several complications of SSc, pulmonary arterial hypertension (PAH) can be refractory to treatment, both novel and established. In the present study we investigated the ratio of circulating nitric oxide to endothelin-1 in patients with both SSc and PAH, and determined whether polymorphisms in *NOS2* (the nitric oxide synthase 2 gene) are associated with susceptibility to PAH. Endothelin-1 in plasma and nitric oxide metabolites (nitrate and nitrite) in serum were measured. The nitric oxide/endothelin-1 ratio was significantly lower in patients with both SSc and PAH than in patients with SSc only or in healthy control individuals. We confirmed the presence of two single nucleotide polymorphisms at positions -1,026 and -277 and a pentanucleotide repeat (CCTTT) at -2.5 kilobases. There were significant differences in single nucleotide polymorphisms between patients with SSc who had PAH and those who did not, and between patients with both SSc and PAH and healthy control individuals. The CCTTT repeat was significantly shorter in patients with both SSc and PAH than in patients with SSc only or in healthy control individuals. Transcriptional activity were analyzed using the luciferase reporter assay. The transcriptional activity of *NOS2* was much greater in fibroblasts transfected by a vector with a long allele of the CCTTT repeat than in those transfected by a vector with a short allele. Polymorphisms in the *NOS2* gene are associated with transcriptional activity of the *NOS2* gene and with susceptibility to SSc-related PAH.

Introduction

Systemic sclerosis (SSc) is an autoimmune disease of unknown aetiology that is characterized by extensive fibrosis of skin and visceral organs, and dysfunction of vascular tone [1]. In its more severe forms, cardiac involvement and respiratory involvement are the most significant determinants of outcome [2]. In particular, pulmonary hypertension is a fatal complication in both diffuse and limited cutaneous SSc [3]. Pulmonary hypertension is generally divided into four major categories: pulmonary arterial hypertension (PAH), pulmonary hypertension associated with left-sided heart disease, pulmonary hypertension associated with lung disease or hypoxaemia, and pulmonary hypertension due to chronic thrombotic or embolic disease [4]. A major part of pulmonary hypertension as it pertains to SSc corresponds with the pathophysiology of PAH, a disease of the small pulmonary arteries characterized by vascular proliferation, vasoconstriction, remodelling of the pulmonary vessel wall and thrombosis in vessels.

Vasodilators such as nitric oxide (NO) and prostacyclin, along with prolonged overexpression of vasoconstrictors such as endothelin (ET)-1, not only affect vascular tone but also promote vascular remodelling, both of which have been implicated in the pathogenesis of PAH [5-12]. Previous studies

bp = base pairs; DMEM = Dulbecco's modified Eagle's medium; ET = endothelin; FBS = foetal bovine serum; NO = nitric oxide; NOS = nitric oxide synthase; PAH = pulmonary arterial hypertension; PCR = polymerase chain reaction; PPH = primary pulmonary hypertension; SNP = single nucleotide polymorphism; SSc = systemic sclerosis.

identified high levels of ET-1 in the plasma of patients with SSc, especially in those with SSc complicated by PAH [13,14]. However, reported levels of circulating NO in patients with SSc are inconsistent, with several studies [15-18] finding increased levels of NO in patients with SSc and others [19,20] finding low levels, similar to those in healthy individuals. In our previous study [21] NO levels were markedly elevated in patients with early-stage diffuse cutaneous SSc, especially when the SSc was accompanied by active alveolitis, but concentrations of NO in serum were low in late-stage limited cutaneous SSc. No patients suffered the complication of PAH in that study. Characteristic levels of NO and NO/ET-1 ratio in patients with both SSc and PAH remain to be established.

NO is an endothelial-derived relaxing factor that is synthesized from L-arginine by nitric oxide synthase (NOS) [22]. Three isoforms of NOS have been identified [23]: NOS-1 (neuronal NOS), NOS-2 (inducible NOS) and NOS-3 (endothelial NOS). NOS-2 is the major source of NO production in conditions involving exposure to cytokines; this is because it is induced by a variety of cell types, including the proinflammatory cytokines interleukin-1, tumour necrosis factor-α, interferon-γ, and ET-1 [24].

Two randomized, double-blind, placebo-controlled trials [25,26] evaluated the efficacy of the ET receptor antagonist bosentan in patients with PAH that was either primary or associated with SSc. Another therapeutic strategy in PAH is to increase the activity of endogenous NO, which enhances NO-dependent cGMP-mediated pulmonary vasodilatation through inhibition of the breakdown of cGMP by phosphodiesterase type 5 [27]. Although long-term inhaled NO therapy has shown only a small benefit in patients with PAH [28], phosphodiesterase type 5 inhibitors (for example, sildenafil) have been found to improve pulmonary artery pressure in patients with PAH [29].

Because these novel therapies were developed to prolong survival and improve patients' quality of life, we speculate that an imbalance between ET-1 and NO is key to the pathogenesis of SSc complicated by PAH. Polymorphisms in the *NOS2* gene promoter are thought to regulate its transcription activity, which is reportedly associated with susceptibility to type 1 diabetes [30] and atopy [31] and with protection against malaria [32]. In the present study we determined the levels of ET-1 and NO in blood from patients with SSc with or without PAH, and we investigated the association between gene polymorphisms in *NOS2* and susceptibility to PAH.

Materials and methods

Study patients

Twenty patients with SSc complicated by PAH were recruited. All had been admitted to Aoyama Hospital of Tokyo Women's Medical University or Kitasato University Hospital. As a disease control group, 58 patients with SSc but not PAH were selected from patients admitted to Aoyama Hospital. Detailed clinical characteristics of all patients are shown in Table 1. All patients with SSc were of Japanese origin, met the criteria established by the American College of Rheumatology for SSc [33], and were classified as having either diffuse or limited cutaneous SSc according to the classification proposed by LeRoy and coworkers [34]. Ninety-five DNA samples were obtained from healthy volunteers who were unrelated individuals of Japanese origin. All DNA samples were collected, with approvals granted by the appropriate ethical committees of Tokyo Women's Medical University, Kitasato University School of Medicine, and Saitama Medical School.

We identified the presence of a complication of PAH in the following manner. All patients with SSc were first evaluated by Doppler echocardiography, and then cardiac catheterization was performed when right ventricular systolic pressure was greater than 30 mmHg, based on Doppler echocardiography. PAH was diagnosed in patients with SSc who satisfied the modified US National Institutes of Health criteria for PAH after cardiac catheterization [35], specifically mean pulmonary artery pressure above 25 mmHg at rest or 30 mmHg after exercise, with normal pulmonary artery wedge pressure. The complication of pulmonary fibrosis was identified using high-resolution computed tomography of the chest. Patients with the following complications were excluded: severe pulmonary fibrosis, with functional vital capacity below 70%; left-sided heart disease; chronic thrombotic or embolic disease; renal failure, including a history of scleroderma renal crisis; hypertension; and diabetes.

Measurement of plasma endothelin-1 and serum nitric oxide levels

Blood samples were obtained from 16 patients with both SSc and PAH and from 26 patients with SSc without PAH who were randomly selected from among patients with SSc who donated DNA samples at the time of admission to Aoyama Hospital with informed consent. No specific diet was given to patients while they were hospitalized. Twenty healthy volunteers (normal control individuals) who had no history of dieting or smoking gave informed consent to participate in the study and gave blood samples. ET-1 levels were measured in plasma using an enzyme-linked immunosorbent assay kit (R&D Systems, Cambridge, MA, USA). Because serum NO is quickly degraded into nitrite and nitrate, we measured the total levels of these NO metabolites as indicators of NO level, using a calorimetric assay kit (Cayman Chemical, Ann Arbor, MI, USA).

Sequencing the *NOS2* promoter region

Genomic DNA was extracted from the blood sample using a DNA extraction kit (Qiagen, Valencia, CA, USA). For direct sequencing, PCR was performed to amplify the promoter region of the *NOS2* gene from -100 to -1,335 bp. The forward

Table 1

Clinical characteristics of patients

Characteristic	SSc patients		Healthy controls
	With PAH	Without PAH	
Number at entry (*n* (male:female)	20 (1:19)	58 (5:53)	95 (8:87)
Age (years; mean (range)	48.6 (17–80)	47.7 (19–77)	40.9 (22–78)
Diffuse SSc:limited SSc (*n*)	6:14	33:25	
Disease duration (months; mean (range)	42.0 (6–130)	41.9 (5–120)	
Pulmonary fibrosis (*n* (%)	6 (30)	31 (53)	
Frequency of ANA (*n* (%)			
Anti-U1-snRNP antibody	11 (55)	17 (29)	0
Anti-topoisomerase I antibody	1 (5)	13 (22)	0
Anti-centromere antibody	5 (25)	12 (21)	0

ANA, antinuclear antibody; PAH, pulmonary arterial hypertension; SSc, systemic sclerosis.

Table 2

Distribution of single nucleotide polymorphisms in *NOS2* gene promoter region

Subjects	-1,026 bp			-277 bp			Haplotype			
	G/G	G/T	T/T	A/A	A/G	G/G	GA	TA	GG	TG
SSc with PAH (*n* = 20)	20 (100)	0	0	20 (100)	0	0	40	0	0	0
SSc without PAH (*n* = 58)	43 (74)	11 (19)	4 (7)	43 (74)	10 (17)	5 (9)	96	0	1	19
Healthy controls (*n* = 95)	69 (73)	23 (24)	3 (3)	73 (77)	19 (20)	3 (3)	161	0	4	25

Values indicate number (%) of genotype or number of haplotype, which consists of two SNPs at -1,026 and -277. There were significant differences between patients with SSc who did and those who did not have PAH in the distribution of two SNPs at -277 and -1,026 (both $P = 0.04$, by Fisher's exact test). Comparing the distribution of two SNPs between patients with SSc complicated by PAH and healthy control individuals, there was a significant difference at -1,026 ($P = 0.02$), but there was no difference at -277 ($P = 0.053$). With respect to haplotype, the frequency of GA was significantly higher in SSc with PAH than in SSc without PAH and in healthy control individuals ($P = 0.001$ and $P = 0.02$, respectively). PAH, pulmonary arterial hypertension; SNP, single nucleotide polymorphism; SSc, systemic sclerosis.

and reverse primers were 5'-TCATCCACACATTCACTCAAC-3' and 5'-CCAAAGGGAGTGTCCCCAGCTT-3', respectively. The sequences of the PCR products were analyzed using the ABI Prism 3100 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

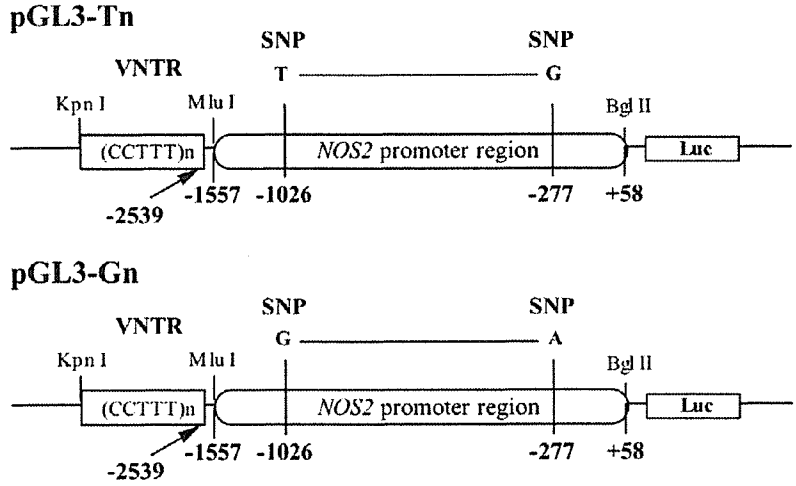
Haplotype typing in pairs of *NOS2* polymorphisms

We entered the genotype data into the PENHAPLO computer program, developed by Ito and coworkers [36], to estimate haplotype frequency in the population and to calculate the posterior probability of diplotype distribution for each study subject. This program was designed for haplotype typing using a maximum likelihood estimation method based on the expectation maximization algorithm under the assumption of Hardy-Weinberg equilibrium for the population.

Analysis of variable numbers of the CCTTT repeat polymorphism of the *NOS2* promoter region

Genomic DNA was amplified by PCR with the use of a FAM™-labelled sense primer (5'-ACCCCTGGAAGCCTACAACTGCAT-3') and an antisense primer (5'-GCCACTGCACCCTAGCCTGTCTCA-3'). The various alleles were resolved by capillary electrophoresis on an ABI Prism 3100 Genetic Analyzer System (Applied Biosystems). Allele sizes were calculated using the GeneScan Analysis computer program, with a GeneScan™-500 ROX™ size standard (Applied Biosystems) as the internal size standard.

Figure 1



A schematic construct of the promoterless pGL3-basic vector. The luciferase vector (pGL3) was inserted with variable numbers of CCTTT repeats and the 1.5 kb minimal human *NOS2* promoter region (-1,557 to +58 bp). The promoter region contained either T or G at -1026 bp (referred to as pGL3-T and pGL3-G, respectively). Each vector of pGL3-T and pGL3-G had allele G and allele A at -277 bp, respectively. The 6, 8, 10, 12, and 14 repeats of the pentanucleotide (CCTTT) region were obtained by PCR. The resulting constructs were named pGL3-T6, pGL3-G6, pGL3-T8, pGL3-G8, pGL3-T10, pGL3-G10, pGL3-T12, pGL3-G12, pGL3-T14 and pGL3-G14 and contain 6, 8, 10, 12 and 14 repeats, respectively. bp, base pairs; kb, kilobases; SNP, single nucleotide polymorphism; VNTR, variable numbers of tandem repeat.

Analysis of transcriptional activity of *NOS2* in human fibroblasts

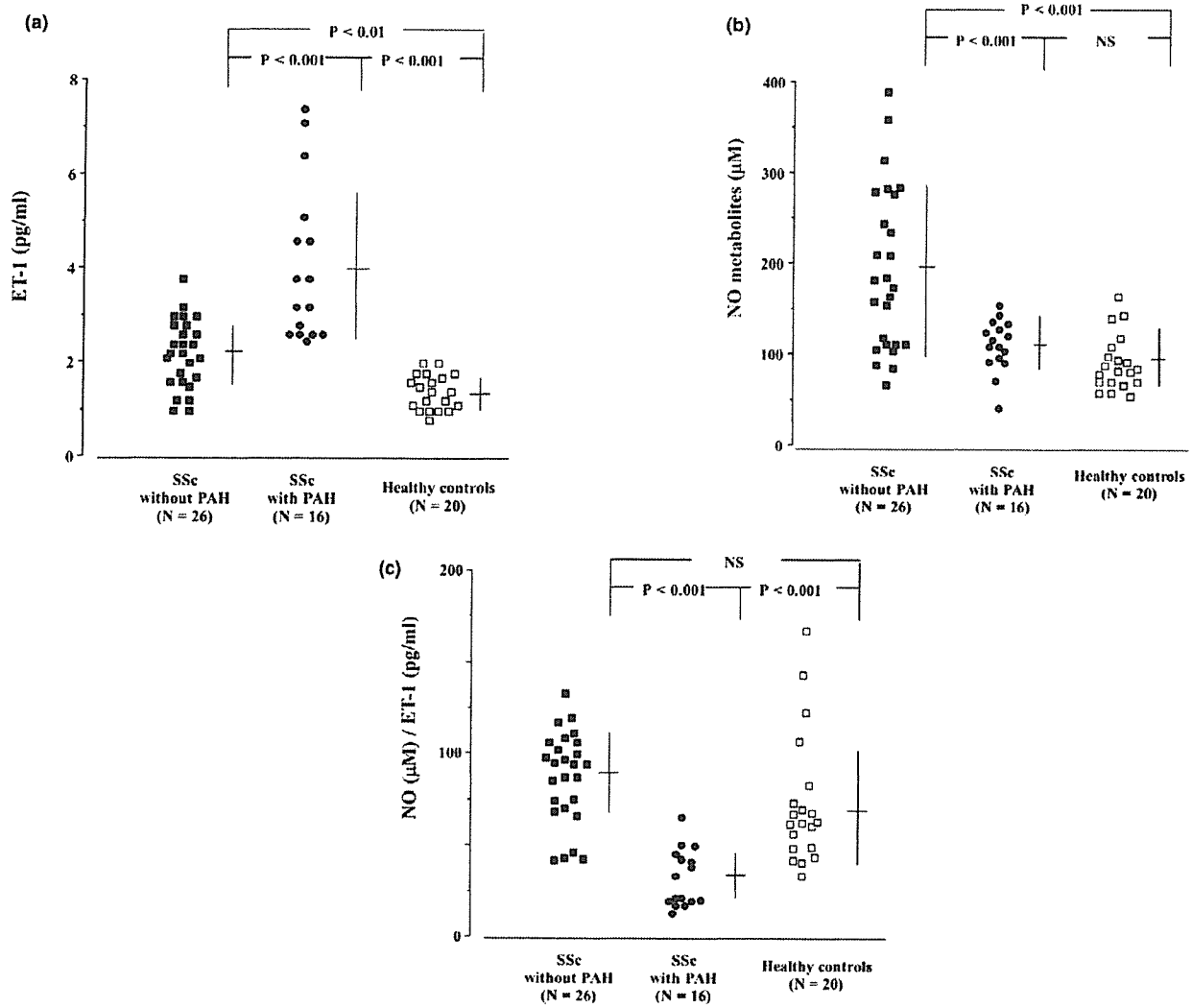
The 5' flanking region of the *NOS2* gene (-1,557 to +58) was prepared by PCR using a set of primers. The forward primer (5'-GATTCTGACTCTTTCCCTGAG-3') is located -1,557 bp from the transcription start site, and the reverse primer (5'-GGAATGAGGCTGAGTTCTCTGCGGC-3') is located +58 bp from the transcription start site. Genomic DNA containing the T/G allele at -1026 bp from the transcription start site of the *NOS2* gene was used as a PCR template. The PCR product was inserted into a pGL3-Basic Vector (Promega, Madison, WI, USA) that contained the firefly luciferase reporter element, and all constructs were sequenced using the pGL3 forward and reverse sequencing primers. The pGL3 vectors with T or G at -1,026 bp are referred to as pGL3-T and pGL3-G, respectively, as shown in Figure 1. The sequences of inserts of pGL3-T and pGL3-G were confirmed by direct sequencing. Each vector of pGL3-T and pGL3-G had allele G and allele A at -277 bp, respectively. The 6, 8, 10, 12 and 14 repeats of the pentanucleotide (CCTTT) region were obtained by PCR using forward (5'-ACCCCTGGAAGCCTACAACT-GCAT-3') and reverse (5'-GCCACTGCACCCTAGCCT-GTCTCA-3') primers. The PCR products were cloned into the upstream of the inserted *NOS2* gene promoter in pGL3-T and pGL3-G. The resulting constructs were named pGL3-T6, pGL3-G6, pGL3-T8, pGL3-G8, pGL3-T10, pGL3-G10, pGL3-T12, pGL3-G12, pGL3-T14 and pGL3-G14, and contain 6, 8, 10, 12 and 14 repeats, respectively.

Human fibroblasts from three healthy individuals were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS; Sigma, St. Louis, MO, USA). For transient transfections, fibroblasts were cultured in six-well plates with 3 ml Opti-MEM (Invitrogen) containing 4 μg DNA (pGL3 and phRL-TK vectors) and 12 μl Lipofectamine 2000 (Invitrogen). After 4 hours, 3 ml DMEM with 20% FBS in the presence or absence of recombinant interleukin-1β (10 ng/mL; R&D Systems) was added. The medium was changed after 16 hours to DMEM with 10% FBS in the presence or absence of interleukin-1β (5 ng/mL). After an additional 24 hours of culture, the cells were washed twice using cold phosphate-buffered saline and were harvested. Firefly and *Renilla* luciferase activities were measured using the Dual-Glo Luciferase Assay System (Promega). Fibroblasts were cotransfected with a constitutively active *Renilla* luciferase vector (phRL-TK), and firefly luciferase activity was normalized by *Renilla* luciferase activity.

Statistics

Circulating ET-1 and NO concentrations are given as mean ± standard deviation, and data were compared using the Student's *t* test. We assessed the significance of the -277A/G and -1026G/T single nucleotide polymorphisms (SNPs) by the Fisher exact test. The relationship between the NO/ET-1 ratio and summed CCTTT repeat length was analyzed using linear regression analysis. An allelic distribution of the number of CCTTT repeats was compared using the Mann-Whitney *U* test. $P < 0.05$ was considered statistically significant.

Figure 2



Circulating levels of ET-1 and NO. **(a)** Plasma levels of ET-1 were measured in patients with SSc with or without PAH. **(b)** Serum levels of NO metabolites were measured. **(c)** The ratios of NO/ET-1 are shown. Data are expressed as mean ± standard deviation. ET, endothelin; NO, nitric oxide; NS, not significant; PAH, pulmonary arterial hypertension; SSc, systemic sclerosis.

Results

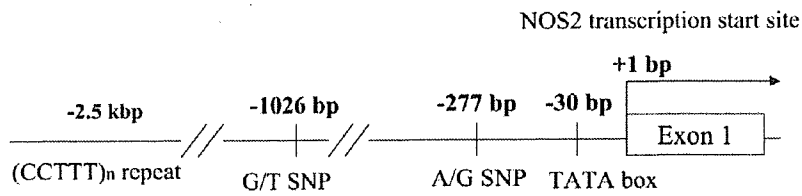
Circulating endothelin-1 and nitric oxide concentrations

Plasma ET-1 levels were significantly higher in each SSc group than in healthy control individuals (1.4 ± 0.4 pg/ml), as shown in Figure 2a. Moreover, ET-1 levels in patients with both SSc and PH were significantly higher than in patients with SSc but not PAH (4.1 ± 1.7 versus 2.2 ± 0.8 pg/ml; $P <$ 0.001). In contrast, NO levels in patients with both SSc and PAH (114 ± 28 μmol/l) were similar to those in healthy control individuals (95 ± 30 μmol/l), but NO levels in patients with SSc but not PAH (194 ± 89 μmol/l) were significantly higher than in the other two groups (Figure 2b). The NO/ET-1 ratio was significantly lower in patients with both SSc and PAH (32.6 ± 15.7; $n = 16$) than in patients with SSc but not PAH (87.8 ± 25.0; $n = 26$) and healthy control individuals (73.6 ± 35.7; $n = 20$), as shown in Figure 2c.

Determination of single nucleotide polymorphisms in the *NOS2* promoter region

We genotyped the 78 patients with SSc and the 95 control individuals for the promoter region (-100 to -1335 bp) of the *NOS2* gene by direct DNA sequencing. We confirmed the presence of two previously reported SNPs at positions -277 and -1026 (Figure 3). The distribution of genotypes is shown in Table 1. The distribution of genotypes at -1026 and -277 was significantly different between patients with SSc who had

Figure 3



Polymorphisms of the 5' flanking region of the human *NOS2* gene. TATA sequence begins at position -30 bp from the transcription start site of exon 1. bp, base pairs; SNP, single nucleotide polymorphism.

PAH and those who did not have PAH (both $P = 0.04$, by Fisher's exact test), but there was no difference between patients with SSc who did not have PAH and healthy control individuals in the distribution of genotypes at two SNPs. Between healthy control individuals and patients with both SSc and PAH, there was a significant difference in the distribution of genetypes at -1026 ($P = 0.02$); in contrast, there was no difference at -277 ($P = 0.053$).

Haplotype typing of the *NOS2* promoter region

We typed the haplotype of the gene, which consists of two SNPs at positions -1,026 and -277. The two SNPs were found to be in linkage disequilibrium. We identified three haplotypes using genes from patients with SSc and healthy control individuals: GA, GG, and TG (Table 2). The frequency of haplotype GA was significantly higher in patients with both SSc and PAH than in patients with SSc but not PAH and in healthy individuals ($P = 0.001$ and $P = 0.02$, respectively), as shown in Table 2.

Distribution of variable numbers of tandem repeat in the *NOS2* promoter region

The 15 alleles found in the present study had 6–21 repeats, and the distribution was significantly different between patients with SSc and PAH and healthy control individuals ($P < 0.0001$) and between patients with SSc with PAH and those with without PAH ($P < 0.0001$), as shown in Table 3. In contrast, there was no significant difference in distribution between patients with SSc but not PAH and healthy control individuals. If CCTTT repeat length strongly influences *NOS2* transcription, then we would expect there to be a significant correlation between CCTTT repeat length and serum NO levels or NO/ET-1 ratios. We calculated the number of summed CCTTT repeats and then analyzed the correlation between that number and serum NO levels or NO/ET-1 ratios. As shown in Figure 4, there was a significant correlation between summed repeat length and both serum NO levels ($r = 0.51$, $P < 0.01$; linear regression analysis) and NO/ET-1 ratios ($r = 0.83$, $P < 0.0001$) in all patients with SSc. However, in healthy control individuals we identified no significant correlation (data not shown).

Effects of *NOS2* polymorphisms on transcriptional activity of the gene

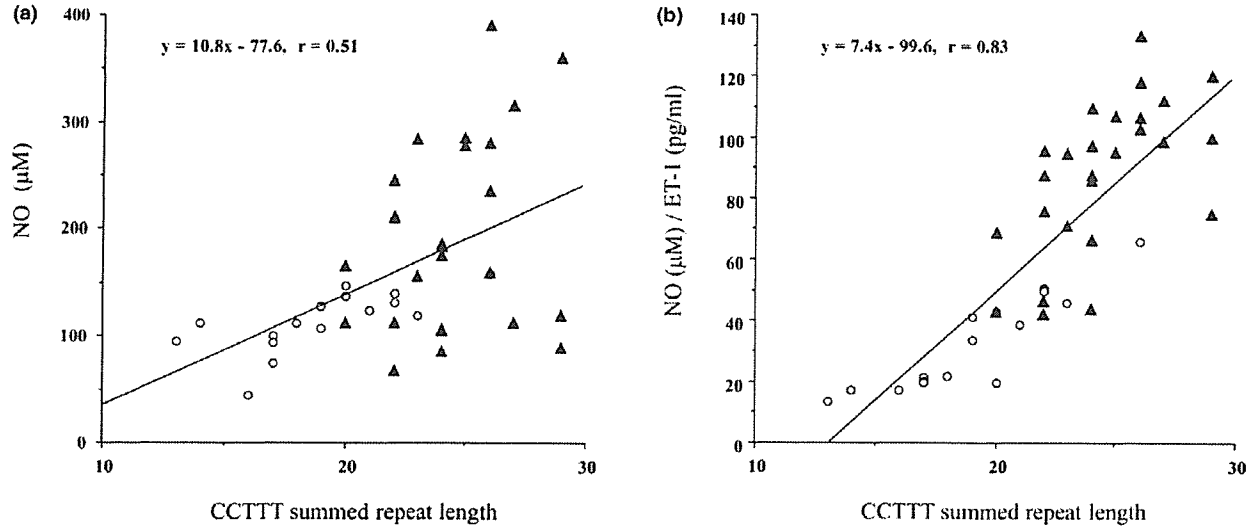
To determine whether the polymorphisms of -277 SNP and -1,026 SNP and variable numbers of tandem repeat were associated with transcription of the *NOS2* gene, we evaluated promoter activities using the series of *NOS2* promoter-luciferase constructs (as described under Materials and method, above). As shown in Figure 5, *NOS2* was almost transcriptionally silent in fibroblasts without stimuli. In contrast, transcription was induced in fibroblasts transfected with vectors, including promoter regions of the *NOS2* gene, under stimulation by interleukin-1β. The relative luciferase activities gradually increased with increasing number of CCTTT repeats in both alleles G and T at -1,026. In the case of the same number of CCTTT repeats, the relative luciferase activity was higher in vectors that included the promoter region with allele T at -1,026 than in vectors that included the promoter region with allele G. These findings indicate that transcriptional activity of the *NOS* gene that includes G at -1,026 and a small number of tandem repeats was low.

Discussion

In the present study were found that concentrations of NO metabolites were not increased in patients with both SSc and PAH, although plasma ET-1 levels were markedly elevated. Our previous report [21] indicates that serum levels of NO metabolites were significantly higher in patients with SSc than in healthy control individuals, especially in patients with a diffuse cutaneous type, active fibrosing alveolitis, or a short duration since onset. However, the population considered in that study did not include patients with PAH, which could explain why the present findings are inconsistent with those of the previous report. Although a number of reports have been published concerning concentrations of ET-1 or NO in the circulation of patients with SSc [8,13-21], this report is the first to describe an imbalance in the NO/ET-1 ratio in patients with PAH.

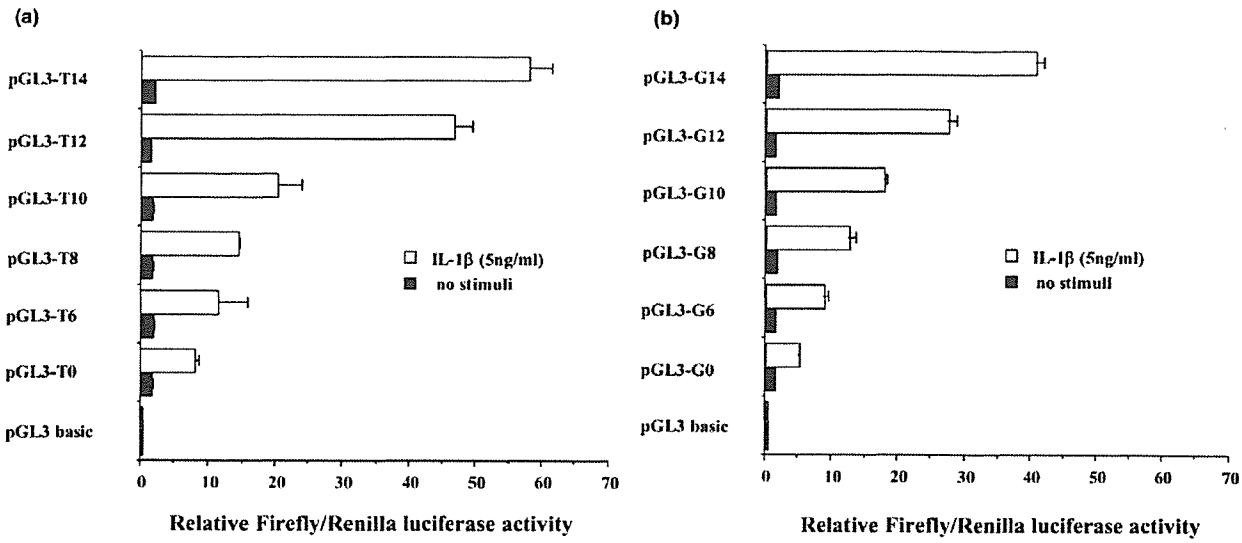
Over the past decade abnormalities in NO synthesis have been proposed as being important in the pathogenesis and development of pulmonary hypertension, especially primary pulmonary hypertension (PPH). Initially, immunohistochemical studies showed that pulmonary hypertension was associated

Figure 4



Relationship between NO levels or NO/ET-1 ratios and summed CCTTT repeat length. The association study between summed lengths of the CCTTT repeat and **(a)** serum NO levels and **(b)** NO/ET-1 ratios was performed in 16 patients with SSc with PAH (open circles) and 26 patients with SSc without PAH (black triangles). The results of linear regression analysis of the data are represented by the solid line. Serum NO levels and NO/ET-1 ratios were significantly correlated with summed CCTTT repeat lengths ($r = 0.51$, $P < 0.01$; and $r = 0.83$, $P < 0.0001$, respectively). ET, endothelin; NO, nitric oxide; PAH, pulmonary arterial hypertension; SSc, systemic sclerosis.

Figure 5



Luciferase reporter assay of the *NOS2* promoter with gene polymorphisms. The **(a)** pGL3-T vectors and **(b)** pGL3-G vectors were cloned by the *NOS2* promoter region, including alleles T and G of the single nucleotide polymorphism at -1026 bp. In the series of pGL3-T or pGL3-G vectors, the 6, 8, 10, 12 and 14 repeats of the CCTTT region were cloned upstream of the inserted *NOS2* promoter in pGL3-T or pGL3-G. bp, base pairs; IL, interleukin.

with diminished expression of NOS-3 [37]. However, other studies found increase in expression of NOS-3 in patients with pulmonary hypertension and in animal models of pulmonary hypertension [38,39]. Despite these contradictory findings, it has been reported that NO levels in blood and the lungs were precisely decreased in patients with PPH and collagen disease related PAH [8-12]. Furthermore, it was determined that NOS-dependent endogenous NO synthesis was decreased in patients with PPH, which suggests that NOS activity may be diminished in patients with PPH [40]. Lung inflammation lead-

Table 3

Allele frequencies for the CCTTT-repeat polymorphism

Allele	SSc patients		Healthy controls
	With PAH	Without PAH	
6	2	0	0
7	2	0	0
8	8	0	4
9	6	4	8
10	10	11	19
11	5	16	25
12	6	29	42
13	0	22	32
14	1	14	29
15	0	7	13
16	0	4	13
17	0	4	4
18	0	2	1
19	0	2	0
20	0	0	0
21	0	1	0

$P < 0.0001$ for patients with both SSc and PAH compared with healthy control subjects by the Mann-Whitney *U* test. $P < 0.0001$ for patients with both SSc and PAH compared with patients with SSc but not PAH by the Mann-Whitney *U* test. PAH, pulmonary arterial hypertension; SSc, systemic sclerosis.

ing to increased levels of cytokines and oxidants may contribute to the development of both PPH and SSc-related PAH [41]. In the presence of increased levels of inflammatory mediators, NOS activity may be dependent on production of NOS-2, which is distinct from NOS-3 (the endothelial form of NOS) because NOS-2 is inducible by inflammatory mediators, and induced levels are much greater than levels of constitutive NOS-3 production. Peripheral mononuclear cells and lesional fibroblasts are capable of aberrant production of inflammatory cytokines in patients with SSc [42-44]. These cytokines may be involved not only in ET-1 synthesis by endothelial cells and fibroblasts but also in induction of NOS-2. Also, excessive production of ET-1 can mediate NOS-2 production through ET receptor B [45]. Although evidence based on those biological properties may promote speculation that levels of ET-1 correlate with levels of NO in the circulation, NO metabolite levels were within normal range in patients with both SSc and PAH patients whose serum contained much ET-1. We hypothesize that this discrepancy may be explained by reduced NOS-2 production resulting from polymorphisms in the *NOS2* gene.

As a result of sequencing the promoter region of the *NOS2* gene from -100 to -1,335, we were able to confirm the presence of two SNPs, consistent with previous reports [46]. In the present study, allele A at -277 SNP, allele G at -1,026 SNP and shorter forms of the CCTTT repeat were associated with susceptibility to PAH combined with SSc. The number of CCTTT repeats was previously reported to influence transcription of the *NOS2* gene [47]. However, studies of variable numbers of tandem repeat both *in vitro* and *in vivo* have yielded conflicting results [48]. To confirm whether those polymorphisms affect transcription of the *NOS2* gene in fibroblasts, we constructed a series of luciferase reporter vectors cloned by various numbers of CCTTT combined with the promoter region of the *NOS2* gene from +58 to -1,557, which included two kinds of haplotype.

Transcriptional activity was lowest in the *NOS2* gene containing the six repeats of CCTTT and haplotype GA, which suggests that transcription of the *NOS2* gene might be little induced by interleukin-1β in patients with SSc-related PAH.

Irrespective of whether patients with SSc had PAH, CCTTT repeat length was well correlated with NO/ET-1 ratio. With regard to the relationship between CCTTT repeat length and serum NO levels, we found no significant difference among SSc patients without PAH, although there were significant differences among all SSc patients and among patients with both SSc and PAH (data not shown). In the setting of aberrant production of ET-1 or cytokines, NO synthesis via NOS-2 induction may be dependent on *NOS2* gene polymorphisms. In healthy control individuals, who had no vascular damage, inflammation, or autoimmune disorders, there was no association between CCTTT repeat length and either serum NO levels or NO/ET-1 ratios (data not shown). Because NOS-2 induction is well controlled by ET-1 and cytokines, distinct from NOS-3, which is constitutively produced, it has been suggested that the CCTTT repeat length is more significantly correlated with NO/ET-1 ratios than with serum NO levels. Our observations support the concept that the *NOS2* gene polymorphism is a crucial factor in NO synthesis under conditions of vascular damage and chronic inflammation, as well as PAH.

It is not possible to determine whether SSc patients without PAH will suffer this complication in the future, and this is a limitation of the present study. The patients enrolled in the study are from a prospective cohort at our institution, and they have been observed for clinical complications, including PAH, in the follow-up clinic. None of the 58 patients with SSc but not PAH has yet been diagnosed with PAH (mean duration of observation: 45 months).

Conclusion

NO is a key factor in generating PAH complicated by SSc, and the decrease in NO synthesis might be attributable to reduced NOS-2 production, which is dependent on *NOS2* gene polymorphisms. Therapeutic options for PAH occurring as a complication of SSc are limited; however, it is not usually the first

complication, and it develops several years after SSc is diagnosed. We believe that the development of means to predict the occurrence of PAH related to SSc, and hence prevent this complication, would be a great step forward. Although prospective, longitudinal studies are needed, we propose that patients with SSc who exhibit an imbalance between NO and ET-1 production and who have a short length of CCTTT repeat of the *NOS2* gene can be treated with a phosphodiesterase type 5 inhibitor before the occurrence of PAH.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YK designed the study, recruited the patients and drafted the manuscript. AT was responsible for the recruitment and classification of the patients, and determined genotypes of *NOS2*. MH participated in coordination of the study. MK determined the phenotype of polymorphisms. TS and YK participated in coordination of the study. JO, HK and MO were responsible for the recruitment and classification of patients and healthy volunteers. NK participated in the design and coordination of the study. All authors read and approved the final manuscript.

Acknowledgements

This study was supported in part by the Japanese Ministry of Health, Labour and Welfare grant (the research for Mixed Connective Tissue Disease) and the Research for the Future Program of the Japan Society for the Promotion of Science.

References

1. Varga J, Korn JH: **Pathogenesis. Emphasis on human data.** In *Systemic Sclerosis* 2nd edition. Edited by: Clements PJ, Furst DE. Philadelphia: Williams & Wilkins; 2004:63-97.
2. Coghlan JG, Mukerjee D: **The heart and pulmonary vasculature in scleroderma: clinical features and pathobiology.** *Curr Opin Rheumatol* 2001, **13**:495-499.
3. Kawut SM, Taichman DB, Archer-Chicko CL, Palevsky HI, Kimmel SE: **Hemodynamics and survival in patients with pulmonary arterial hypertension related to systemic sclerosis.** *Chest* 2003, **123**:344-350.
4. Cooke JP: **A novel mechanism for pulmonary arterial hypertension.** *Circulation* 2003, **108**:1420-1421.
5. Stewart DJ, Levy RD, Cernacek P, Langleben D: **Increased plasma endothelin-1 in pulmonary hypertension: marker or mediator of disease?** *Ann Intern Med* 1991, **114**:464-469.
6. Giaid A, Yanagisawa M, Langleben D, Michel RP, Levy R, Shennib H, Kimura S, Masaki T, Duguid WP, Stewart DJ: **Expression of endothelin-1 in the lungs of patients with pulmonary hypertension.** *N Engl J Med* 1993, **328**:1732-1739.
7. Rubens C, Ewert R, Halank M, Wensel R, Orzechowski HD, Schultheiss HP, Hoeffken G: **Big endothelin-1 and endothelin-1 plasma levels are correlated with the severity of primary pulmonary hypertension.** *Chest* 2001, **120**:1562-1569.
8. Kharitonov SA, Cailes JB, Black CM, du Bois RM, Barnes PJ: **Decreased nitric oxide in the exhaled air of patients with systemic sclerosis with pulmonary hypertension.** *Thorax* 1997, **52**:1051-1055.
9. Rolla G, Colagrande P, Scappaticci E, Chiavassa G, Dutto L, Cannizzo S, Bucca C, Morello M, Bergerone S, Bardini D, *et al.*: **Exhaled nitric oxide in systemic sclerosis: relationships with lung involvement and pulmonary hypertension.** *J Rheumatol* 2000, **27**:1693-1698.
10. Kaneko FT, Arroliga AC, Dweik RA, Comhair SA, Laskowski D, Oppedisano R, Thomassen MJ, Erzurum SC: **Biochemical reaction products of nitric oxide as quantitative markers of primary pulmonary hypertension.** *Am J Respir Crit Care Med* 1998, **158**:917-923.
11. Cella G, Bellotto F, Tona F, Sbarai A, Mazzaro G, Motta G, Fareed J: **Plasma markers of endothelial dysfunction in pulmonary hypertension.** *Chest* 2001, **120**:1226-1230.
12. Machado RF, Londhe Nerkar MV, Dweik RA, Hammel J, Janocha A, Pyle J, Laskowski D, Jennings C, Arroliga AC, Erzurum SC: **Nitric oxide and pulmonary arterial pressures in pulmonary hypertension.** *Free Radic Biol Med* 2004, **37**:1010-1017.
13. Morelli S, Ferri C, Polettini E, Bellini C, Gualdi GF, Pittoni V, Valesini G, Santucci A: **Plasma endothelin-1 levels, pulmonary hypertension, and lung fibrosis in patients with systemic sclerosis.** *Am J Med* 1995, **99**:255-260.
14. Yamane K, Miyauchi T, Suzuki N, Yuhara T, Akama T, Suzuki H, Kashiwagi H: **Significance of plasma endothelin-1 levels in patients with systemic sclerosis.** *J Rheumatol* 1992, **19**:1566-1571.
15. Yamamoto T, Katayama I, Nishioka K: **Nitric oxide production and inducible nitric oxide synthase expression in systemic sclerosis.** *J Rheumatol* 1998, **25**:314-317.
16. Andersen GN, Caidahl K, Kazzam E, Petersson AS, Waldenstrom A, Mincheva-Nilsson L, Rantapaa-Dahlqvist S: **Correlation between increased nitric oxide production and markers of endothelial activation in systemic sclerosis: findings with the soluble adhesion molecules E-selectin, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1.** *Arthritis Rheum* 2000, **43**:1085-1093.
17. Cotton SA, Herrick AL, Jayson MI, Freemont AJ: **Endothelial expression of nitric oxide synthases and nitrotyrosine in systemic sclerosis skin.** *J Pathol* 1999, **189**:273-278.
18. Sud A, Khullar M, Wanchu A, Bambery P: **Increased nitric oxide production in patients with systemic sclerosis.** *Nitric Oxide* 2000, **4**:615-619.
19. Kahaleh BM, Pan-Sheng F, Cerinic MM, Stefanovic Racic M, Ignarro L: **Study of endothelial dependent relaxation in scleroderma.** *Arthritis Rheum* 1993, **36**:S180.
20. Allanore Y, Borderie D, Hilliquin P, Hernvann A, Levacher M, Lemarechal H, Ekindjian OG, Kahan A: **Low levels of nitric oxide (NO) in systemic sclerosis: inducible NO synthase production is decreased in cultured peripheral blood monocyte/macrophage cells.** *Rheumatology* 2001, **40**:1089-1096.
21. Takagi K, Kawaguchi Y, Hara M, Sugiura T, Harigai M, Kamatani N: **Serum nitric oxide (NO) levels in systemic sclerosis patients: correlation between NO levels and clinical features.** *Clin Exp Immunol* 2003, **134**:538-544.
22. Palmer RM, Ashton DS, Moncada S: **Vascular endothelial cells synthesize nitric oxide from L-arginine.** *Nature* 1988, **333**:664-666.
23. Forstermann U, Gath I, Schwarz P, Closs EI, Kleinert H: **Isoforms of nitric oxide synthase. Properties, cellular distribution and expressional control.** *Biochem Pharmacol* 1995, **50**:1321-1332.
24. Nathan C, Xie QW: **Regulation of biosynthesis of nitric oxide.** *J Biol Chem* 1994, **269**:13725-13728.
25. Channick RN, Simonneau G, Sitbon O, Robbins IM, Frost A, Tapson VF, Badesch DB, Roux S, Rainisio M, Bodin F, Rubin LJ: **Effects of the dual endothelin-receptor antagonist bosentan in patients with pulmonary hypertension: a randomized placebo-controlled study.** *Lancet* 2001, **358**:1119-1123.
26. Rubin LJ, Badesch DB, Barst RJ, Galie N, Black CM, Keogh AK, Pulido T, Frost A, Roux S, Leconte I, *et al.*: **Bosentan in patients with pulmonary arterial hypertension: a randomized, placebo controlled, multicenter study.** *N Engl J Med* 2002, **346**:896-903.
27. Mehta S: **Sildenafil for pulmonary arterial hypertension: exciting, but protection required.** *Chest* 2003, **123**:989-992.
28. McMullan DM, Bekker JM, Johengen MJ, Hendricks-Munoz K, Gerrets R, Black SM, Fineman JR: **Inhaled nitric oxide-induced rebound pulmonary hypertension: a role for endothelin-1.** *Am J Physiol Heart Circ Physiol* 2001, **280**:H777-H785.
29. Ghofrani HA, Pepke-Zaba J, Barbera JA, Channick R, Keogh AM, Gomez-Sanchez MA, Kneussl M, Grimminger F: **Nitric oxide pathway and phosphodiesterase inhibitors in pulmonary arterial hypertension.** *J Am Coll Cardiol* 2004, **43**:68S-72S.
30. Johannesen J, Tarnow L, Parving H-H, Nerup J, Pociot F: **CCTTT-repeat polymorphism in the human NOS2-promoter confers**

low risk of diabetic nephropathy in type 1 diabetic patients. *Diabetes Care* 2000, **23**:560-562.
31. Konno S, Hizawa N, Yamaguchi E, Jinushi E, Nishimura M: **(CCTTT)n repeat polymorphism in the NOS2 gene promoter is associated with atopy.** *J Allergy* 2001, **108**:810-814.
32. Hobbs MR, Udhayakumar V, Levesque MC, Booth J, Roberts JM, Tkachuk A: **A new NOS2 promoter polymorphism associated with increased nitric oxide production and protection from severe malaria in Tanzanian and Kenyan children.** *Lancet* 2002, **360**:1468-1475.
33. Subcommittee for Scleroderma Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee: **Preliminary criteria for the classification of systemic sclerosis (scleroderma).** *Arthritis Rheum* 1980, **23**:581-590.
34. LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA Jr, Rowell N, Wollheim F: **Scleroderma (systemic sclerosis): classification subsets and pathogenesis.** *J Rheumatol* 1988, **15**:202-205.
35. Rich S, Dantzker DR, Ayres SM, Bergofsky EH, Brundage BH, Detre KM, Fishman AP, Goldring RM, Groves BM, Koerner SK, *et al.*: **Primary pulmonary hypertension. A national prospective study.** *Ann Intern Med* 1987, **107**:216-223.
36. Ito T, Inoue E, Kamatani N: **Association test algorithm between a qualitative phenotype and a haplotype or haplotype set using simultaneous estimation of haplotype frequencies, diplotype configurations and diplotype-based penetrances.** *Genetics* 2004, **168**:2339-2348.
37. Giaid A, Saleh D: **Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension.** *N Engl J Med* 1995, **333**:214-221.
38. Xue C, Johns RA: **Endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension.** *N Engl J Med* 1995, **333**:1642-1644.
39. Xue C, Johns RA: **Upregulation of nitric oxide synthase correlates temporally with onset of pulmonary vascular remodeling in the hypoxic rat.** *Hypertension* 1996, **28**:743-753.
40. Demoncheaux EAG, Higenbottam TW, Kiely DG, Wong J-M, Wharton S, Varcoe R, Siddons T, Spivey AC, Hall K, Gize AP: **Decreased whole body endogenous nitric oxide production in patients with primary pulmonary hypertension.** *J Vasc Res* 2005, **42**:133-136.
41. Humbert M, Monti G, Brenot F, Sitbon O, Portier A, Grangeot-Keros L, Duroux P, Galanaud P, Simonneau G, Emilie D: **Increased interleukin-1 and interleukin-6 serum concentrations in severe primary pulmonary hypertension.** *Am J Respir Crit Care Med* 1995, **151**:1628-1631.
42. Kadono T, Kikuchi K, Ihn H, Takehara K, Tamaki K: **Increased production of interleukin 6 and interleukin 8 in scleroderma fibroblasts.** *J Rheumatol* 1998, **25**:296-301.
43. Kawaguchi Y, Suzuki K, Hara M, Hidaka T, Ishizuka T, Kawagoe M, Nakamura H: **Increased endothelin-1 production in fibroblasts derived from patients with systemic sclerosis.** *Ann Rheum Dis* 1994, **53**:506-510.
44. Kawaguchi Y: **IL-1β gene expression and protein production by fibroblasts from patients with systemic sclerosis.** *Clin Exp Immunol* 1994, **97**:445-450.
45. Gay G, Webb DJ: **The endothelin system and its potential as a therapeutic target in cardiovascular disease.** *Pharmacol Ther* 1996, **72**:109-148.
46. Burgner D, Usen S, Rockett K, Jallow M, Ackerman H, Cervino A, Pinder M, Kwiatkowski DP: **Nucleotide and haplotypic diversity of the NOS2A promoter region and its relationship to cerebral malaria.** *Hum Genet* 2003, **112**:379-386.
47. Warpeha KM, Xu W, Liu L, Charles IG, Patterson CC, Ah-Fat F, Harding S, Hart PM, Chakravarthy U, Hughes AE: **Genotyping and functional analysis of a polymorphic (CCTTT)n repeat of NOS2A in diabetic retinopathy.** *FASEB J* 1999, **13**:1825-1832.
48. Boutlis CS, Hobbs MR, Marsh RL, Misukonis MA, Tkachuk AN, Lagog M, Booth J, Granger DL, Bockarie MJ, Mgone CS, *et al.*: **Inducible nitric oxide synthase (NOS) promoter CCTTT repeat polymorphism: relationship to in vivo nitric oxide production/NOS activity in an asymptomatic malaria-endemic population.** *Am J Trop Med Hyg* 2003, **69**:569-573.

Rheumatology Advance Access published January 25, 2007

Rheumatology 2007; 1 of 4 doi:10.1093/rheumatology/kel435

Clinical characteristics of Japanese patients with anti-OJ (anti-isoleucyl-tRNA synthetase) autoantibodies

Shinji Sato, Masataka Kuwana and Michito Hirakata

Objectives. The clinical and laboratory characteristics of seven patients with anti-aminoacyl-tRNA synthetase (ARS) autoantibodies, specifically anti-OJ (anti-isoleucyl-tRNA synthetase), were examined and compared with previously published findings.
Methods. Serum samples from 1135 Japanese patients with various autoimmune diseases and 48 normal individuals were screened for anti-OJ antibodies using RNA and protein immunoprecipitation assays. The patients whose sera contained anti-OJ antibodies were assessed regarding clinical symptoms, clinical course, laboratory findings, chest radiography and chest computed tomography.
Results. Sera from seven patients were found to contain anti-OJ antibodies. These autoantibodies were associated with interstitial lung disease (ILD) and myositis. The diagnoses of the seven patients were idiopathic interstitial pneumonias (IIPs) in three, polymyositis (PM) in three and PM-rheumatoid arthritis (RA) overlap in the remaining one. All patients had ILD, but muscle weakness and polyarthritis were seen only in four. Raynaud's phenomenon and sclerodactyly were absent in all patients.
Conclusions. These results indicate that the presence of anti-OJ autoantibodies may distinguish a subtype of anti-ARS syndrome that is more closely associated with ILD than myositis or Raynaud's phenomenon.

KEY WORDS: Interstitial lung disease (ILD), Polymyositis/dermatomyositis (PM/DM), Anti-aminoacyl-tRNA synthetase (ARS) antibodies.

Introduction

Anti-aminoacyl-tRNA synthetase (anti-ARS) autoantibodies have been found in patients with polymyositis/dermatomyositis (PM/DM) [1, 2]. Six anti-ARS autoantibodies have been described, as follows: anti-histidyl (anti-Jo-1), anti-threonyl (anti-PL-7), anti-alanyl (anti-PL-12), anti-glycyl (anti-EJ), anti-isoleucyl (anti-OJ), and anti-asparaginyl (anti-KS) tRNA synthetases [1–7]. The most common anti-ARS antibodies, anti-Jo-1, are found in approximately 20–30% of PM/DM patients. Anti-OJ antibodies are also found in PM/DM patients, although the frequency is low [1]. In previous studies, anti-OJ antibodies were found in less than 2% of all patients with PM/DM [8]. These anti-ARS antibodies have been reported to be associated with a similar syndrome characterized by myositis with a high frequency of interstitial lung disease (ILD) and arthritis, as well as increased fever, Raynaud's phenomenon, and mechanic's hands compared to the overall myositis population [9]. Although anti-ARS syndromes have common clinical symptoms, further observations have distinguished certain differences in clinical features associated with each of the different anti-ARS antibodies. It has been reported that anti-Jo-1 antibodies are closely associated with myositis [1, 3], whereas patients with anti-PL-12 and anti-KS antibodies are more likely to have ILD without clinical evidence of myositis [7]. On the other hand, we previously observed that the presence of anti-PL-7 antibodies is closely associated with PM/DM-SSc overlap as well as ILD in Japanese patients [10].

There have been two reports on the clinical significance of anti-OJ antibodies in patients in North America [11, 12], and two case reports of anti-OJ antibodies in Japan [8, 13]. However, the clinical characteristics of the Japanese patients with anti-OJ antibodies have not been examined in detail and their clinical significance remains uncertain. Here, we analyse the clinical and laboratory characteristics of Japanese patients with antibodies against anti-OJ and review published reports from elsewhere.

Department of Internal Medicine, Keio University, School of Medicine, Tokyo, Japan.

Submitted 21 June 2006; revised version accepted 8 December 2006.

Correspondence to: Shinji Sato, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail: shins@sc.itc.keio.ac.jp

Patients, materials and methods

Patients and sera

Serum samples were obtained from 1135 Japanese patients who had or were suspected of having connective tissue diseases (CTDs) seen at the Keio University Hospital and collaborating centers between 1990 and 2000. These included 120 with PM/DM, 400 with systemic lupus erythematosus, 192 with systemic sclerosis, 58 with rheumatoid arthritis (RA), 101 with overlap syndrome including mixed connective tissue disease, 114 with ILD and 150 patients with arthritis or erythema who were suspected to have CTDs. These included three patients with anti-OJ antibodies previously reported by our study group [8, 13]. We also examined 48 sera from normal individuals. Blood samples were obtained after the patients and normal controls had provided written informed consent approved by the Keio University Institutional Review Board.

Immunoprecipitation (IPP)

The IPP assay with HeLa cell extracts was performed as previously described [6]. For the analysis of RNAs, antibodies bound to protein A-Sepharose CL-4B beads were incubated with extracts of HeLa cells. They were then washed with NET-2 buffer (50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 0.05% Nonidet P-40). After ethanol precipitation, RNAs were dissolved in electrophoresis sample buffer composed of 10 M urea, 0.025% bromophenol blue, and 0.025% xylene cyanol-FF in TBE buffer (90 mM Tris-HCl, pH 8.6, 90 mM boric acid and 1 mM EDTA). The RNA samples were resolved in 7 M urea-10% polyacrylamide gels, which were then silver stained (Bio-Rad Laboratories, Hercules, CA, USA). For protein studies, antibody-coated Sepharose beads were mixed with [^{35}S] methionine-labelled HeLa extracts. After washing, the Sepharose beads were resuspended in SDS–sample buffer (2% SDS, 10% glycerol, 62.5 mM Tris-HCl, pH 6.8, 0.005% bromophenol blue). The proteins were then fractionated by 10% SDS-PAGE gels and dried. Radiolabelled protein components were analysed by autoradiography. With these assays, myositis-specific or -associated autoantibodies such as anti-ARS, anti-signal recognition particle, anti-Mi-2, anti-SSA, anti-SSB, anti-U1-RNP and anti-Ku autoantibodies are distinguishable, compared with corresponding standard sera [2]. The identification of anti-OJ antibodies was accomplished by comparing IPP patterns for both RNA and protein with standard anti-OJ serum as described previously [13].

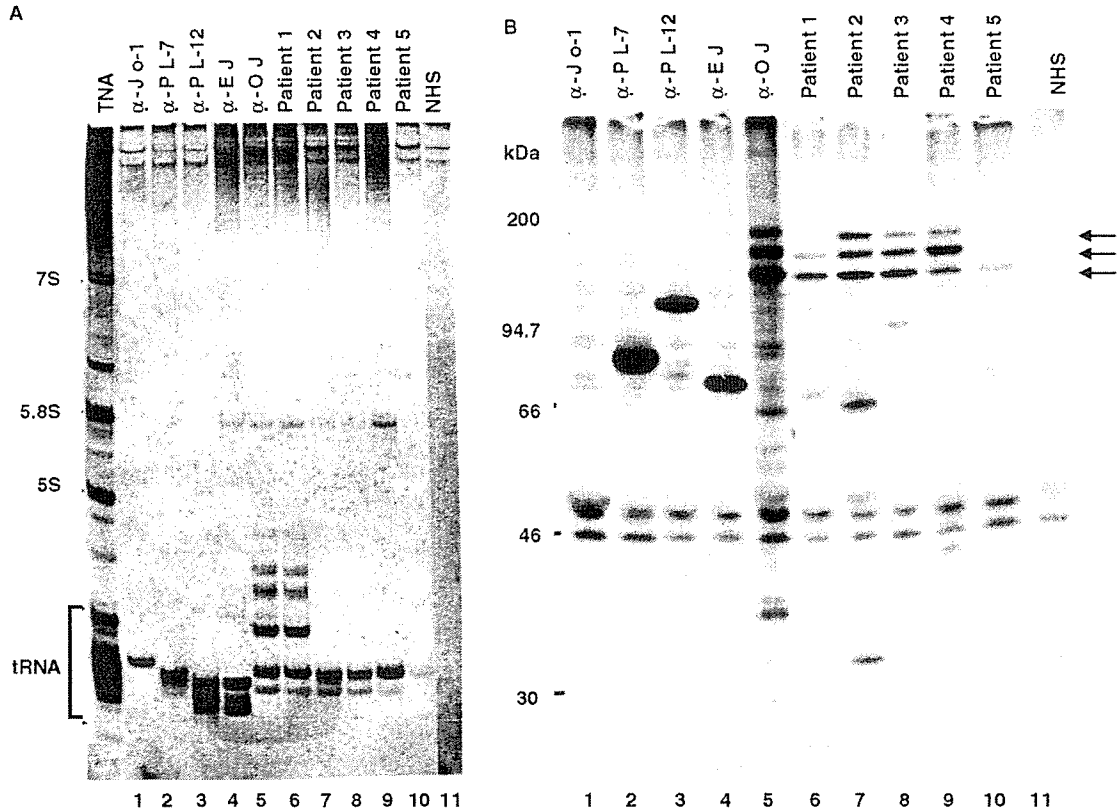


FIG. 1. (A) Immunoprecipitation (IPP) of nucleic acids with representative anti-OJ sera and controls. Urea (7M) and 10% PAGE of phenol-extracted immunoprecipitates from HeLa cell extracts were developed with silver stain. TNA, total nucleic acids, with the 5.8 and 5.0 S small ribosomal RNAs and the tRNA region indicated. Sera used for IPP include: lanes 1-5, anti-synthetase sera indicated, with antibodies to Jo-1 (histidyl-tRNA synthetase), PL-7 (threonyl-tRNA synthetase), PL-12 (alanyl-tRNA synthetase), EJ (glycyl-tRNA synthetase), OJ (isoleucyl-tRNA synthetase); lanes 6-10, anti-OJ sera as indicated; and lane 11, control serum indicated (NHS, normal human serum). The tRNA pattern with anti-OJ sera is easily distinguishable from that of the other anti-synthetases. (B) IPP of proteins with anti-OJ sera and controls. Autoradiogram of 10% SDS-PAGE of immunoprecipitates from [^{35}S] methionine-labelled HeLa cell extracts. Mr, molecular weight markers of the sizes indicated to the left (kDa). The sera used for immunoprecipitation are the same as those in Fig. 1A. The same characteristic pattern of protein bands including at least three that were thought to be glutamine, isoleucine and leucine tRNA synthetases (arrows) was seen with each of the five anti-OJ sera. And the pattern was easily different from the bands immunoprecipitated by sera containing the other anti-synthetases.

Clinical features

The clinical symptoms, clinical course, laboratory findings, the results of chest radiography and chest computed tomography (CT) were retrospectively assessed from medical records in all patients positive for anti-OJ antibodies. Some patients were also assessed for electromyogram (EMG), muscle biopsy, and pathological findings from video-assisted thoracoscopic surgery (VATS) at first evaluation. The assessment of muscle weakness was performed using a manual muscle test (MMT) [14].

The diagnosis of PM/DM was based on criteria proposed by Bohan and Peter [15]. ILD was defined according to the results of chest radiography, chest CT, lung function testing (percentage predicted forced vital capacity: FVC and carbon monoxide diffusing capacity: DLCO) and the diagnosis of IIPs was based on consensus classification of IIPs [16]. The resolution of myositis symptoms was defined as both improved muscle strength on a manual muscle test and normalization of the serum CK value. Pulmonary symptoms were considered improved when shown by both chest CT and pulmonary function testing.

Statistical analysis

All comparisons between the two patient groups were performed using Fisher's 2-tailed exact test or Student's *t*-test.

Results

Identification of anti-OJ antibodies

Of the 1183 sera tested, seven immunoprecipitated a characteristic identical nucleic acid band of tRNA of a size identical to anti-isoleucyl tRNA synthetase (anti-OJ). Representative examples are shown in Fig. 1A. Two bands of RNA in the tRNA size range were immunoprecipitated and are clearly distinguishable from the pattern of tRNAs precipitated by the other anti-ARS antibodies. The same sera also immunoprecipitated several protein bands corresponding to polypeptides precipitated by anti-OJ standard serum. These included at least three protein bands that were more intense than the other seven bands of the OJ complex. These proteins were easily distinguishable from those immunoprecipitated by sera reactive with the other described anti-ARS antibodies (Fig. 1B). Thus, it is concluded that they contained anti-OJ antibodies.

Clinical features in patients with anti-OJ antibodies

In this study, anti-OJ antibodies were detected in four of 120 PM/DM patients (3.3%) and three of 112 ILD patients (2.7%). However, anti-OJ antibodies were not detected in other CTDs or normal human sera.

Clinical features in the seven patients with anti-OJ autoantibodies are summarized in Table 1. Of these seven, four were female. All seven patients had ILD, although none of them

TABLE 1. Clinical features of patients with anti-OJ antibodies

Clinical findings	#1	#2	#3	#4	#5	#6	#7
Age/gender	51/female	62/male	75/female	53/male	69/female	27/male	32/female
Diagnosis	PM/RA	PM	PM	PM	IIPs	IIPs	IIPs
ILD	(+)	(+)	(+)	(+)	(+)	(−)	(+)
%VC (%)	56	88	46	n.a.	70	74	38
DLCO (ml/min[a]tor)	8.5	12.5	n.a.	n.a.	10.4	5.9	4.1
Histopathology in VATS	n.d.	n.d.	n.d.	n.d.	COP	UIP	NSIP
DM rush	(−)	(−)	(−)	(−)	(−)	(−)	(−)
Muscle weakness	(+)	(+)	(+)	(+)	(−)	(−)	(−)
Maximun CK level (IU/l)	3,297	648	672	1,682	125	113	33
EMG findings	Myogenic[a]	n.d.	Myogenic[a]	Myogenic[a]	n.d.	n.d.	n.d.
Muscle biopsy	Myositis[b]	Myositis[b]	n.d.	n.d.	n.d.	n.d.	n.d.
Arthritis	(+)	(+)	(+)	(+)	(−)	(−)	(−)
Raynaud's phenomenon	(−)	(−)	(−)	(−)	(−)	(−)	(−)
Sclerodactyly	(−)	(−)	(−)	(−)	(−)	(−)	(−)
Sjögren's syndrome	(−)	(−)	(−)	(−)	(−)	(−)	(−)
Duration of the disease (month)	57	49	42	28	43	35	63
Treatment	PSL 50 mg	PSL 60 mg, mPSL pulse, AZA 100 mg	PSL 20 mg	(−)	PSL 20 mg	PSL 60 mg	PSL 50 mg
Duration of treatment (month)	57	48	41	0	24	11	15
Effect of treatment	(+)[c]	(+)[c]	(+)[c]		(+)[d]	(−)[d]	(+)[d]

PM: polymyositis, RA: rheumatoid arthritis, DM: dermatomyositis, IIPs: idiopathic interstitial pneumonias, CK: creatine kinase, EMG: electromyogram, ILD: interstitial lung disease, VATS: video-assisted thoracoscopic surgery, COP: cryptogenic organizing pneumonia, UIP: usual interstitial pneumonia, NSIP: non-specific interstitial pneumonia, PSL: prednisolone, mPSL: methylprednisolone, AZA: azathioprine. [a] Low amplitude, resting fibrillation, positive sharpe wave (denervatrion potencials) were present. [b] Atrophy, necrosis with regeneration and infiltration of lymphocytes of muscle fibres were present. [c] Both improvement of muscle weakness on a manual muscle test and the normalization of serum CK value. [d] Improvement of chest CT and/or pulmonary function testing.

TABLE 2. Comparison of clinical features in anti-OJ-positive *vs* anti-Jo-1-positive patients

	Anti-OJ ($n=7$)	Anti-Jo-1 ($n=22$)
Age at onset, mean ± S.D. years	53 ± 18	49 ± 15
No male/no. female	3/4	7/15
Fever (%)	3 (43)	14 (64)
DM rash (%)	0 (0)	6 (27)
Arthritis (%)	4 (57)	20 (91)
Myositis (%)	4 (57)*	22 (100)*
ILD (%)	7 (100)	20 (91)
Raynaud's phenomenon (%)	0 (0)**	15 (68)**
Sclerodactyly (%)	0 (0)	1 (5)

PM: polymyositis, DM: dermatomyositis, ILD: interstitial lung disease, IPF: idiopathic pulmonary fibrosis.
*$P=0.010$, **$P=0.002$.

progressed to severe acute respiratory failure. Four patients had muscle weakness that was graded as 4/5 based on MMT and serum CK elevation. From the results of EMG and muscle biopsy, four patients were diagnosed as definite or probable PM. Two of these manifested symptoms of ILD preceding their myositis symptoms. One of the four PM patients had destructive changes in joint radiography compatible with RA. The other three patients had no muscle or skin manifestations but interstitial changes in the lung at chest high resolution CT and/or histopathlogical change in VATS resulted in a diagnosis of IIPs. VATS was done in all patients with IIPs and the results were compatible with cryptogenic organizing pneumonia (patient #5), usual interstitial pneumonia (patient #6) and non-specific interstitial pneumonia (patient #7). No Raynaud's phenomenon or sclerodactyly was present at any time in any of the seven patients. Treatment of the myositis with prednisolone and/or other immunosupressants resulted in improvement of muscle strength assessed by MMT and reduction of serum CK level in three PM patients. Three patients with IIPs had also received prednisolone that had then been gradually tapered and discontinued: improvement of chest CT and pulmonary function was achieved in two of them.

Comparison with the clinical features of patients with anti-OJ in the literature

The clinical features of patients with anti-OJ antibodies reported in the English literature were previously reviewed [11, 12]. Targoff *et al.* [11] reported on nine patients with anti-OJ and Gelpi *et al.* [12] described one patient with co-existing anti-Jo-1 and anti-OJ antibodies.

Frequencies of several clinical manifestations can be compared between the anti-OJ-positive patients reported by Targoff *et al.* and those in the present study. The frequency of myositis in our Japanese patients with anti-OJ antibodies tended to be lower than in the patients of Targoff *et al.* (57% *vs* 89%), but this difference did not reach statistical significance. The frequencies of ILD and Raynaud's phenomenon in our series were similar compared with previously reported patients [11].

Comparison with the clinical features of patients with anti-OJ and anti-Jo-1 antibodies

In the present study, anti-Jo-1 antibodies, representative of anti-ARS antibodies, were detected in 22 patients with PM/DM (18% of PM/DM in our study). We compared the frequencies of several clinical manifestations between anti-OJ- and anti-Jo-1-positive patients in our series (Table 2). It was found that the frequency of myositis and Raynaud's phenomenon in patients with anti-OJ was significantly lower than in those with anti-Jo-1 ($P=0.010$ and $P=0.002$, respectively), whereas the frequency of ILD was similar.

Discussion

Seven sera from 1135 CTD patients or suspected CTDs were found to contain anti-OJ antibodies. In the present study, these seven patients seemed not to have the typical features characteristic of the anti-ARS syndromes previously described. The most striking differences were that none of them had Raynaud's phenomenon that is common in anti-ARS syndromes in general. In fact, the frequency of Raynaud's phenomenon in our series was significantly lower than in our patients with anti-Jo-1 antibodies that are the representative anti-ARS cases. However, the sample is too small to draw a definitive conclusion in this study, but our results are similar to those in North American patients with anti-OJ antibodies [11]. Thus, the low frequency of Raynaud's phenomenon seems to be a characteristic feature of patients with anti-OJ autoantibodies compared to other anti-ARS syndromes.

Three of the anti-OJ-positive patients had no signs of myositis and were diagnosed as IIPs. This suggested that the presence of anti-OJ is more closely associated with ILD than myositis, as is the case with anti-PL-12 or anti-KS antibodies.

Although three patients received a diagnosis of IIPs at this time, the possibility remains that muscle symptoms may appear in the future, because it is known that pulmonary manifestations can appear before muscle symptoms in PM/DM patients. Another possibility is that existing myositis was underdiagnosed because of the effect of prednisolone treatment; indeed, three patients diagnosed as IIPs had been taking prednisolone for their pulmonary symptoms. Nonetheless, it is unlikely that they had myositis because the duration of prednisolone therapy was relatively short and they never had any clinical symptoms related to myositis throughout their clinical course. However, observation should be continued for identification of any future muscle symptoms.

The mechanisms responsible for these differences in clinical features associated with each of the anti-ARS autoantibodies are unknown and an accumulation of larger numbers of cases will be required to clarify this in the future.

In conclusion, we report seven Japanese patients with anti-OJ antibodies classed as suffering from IIPs or PM accompanied by ILD. These patients lacked any manifestations of Raynaud's phenomenon or sclerodactyly. Anti-OJ autoantibodies are a clinically important marker for a specific subset of anti-ARS syndrome that is more closely associated with ILD than myositis or Raynaud's phenomenon. The measurement of anti-OJ was found to be useful for diagnosis of patients with ILD with or without myositis. Further analysis of these autoantibodies may provide insights into the etiological and pathogenetic mechanisms of ILD and myositis.

Rheumatology key messages

- Anti-OJ autoantibodies may distinguish a subtype of anti-ARS syndrome that is more closely associated with ILD than myositis or Raynaud's phenomenon.
- Anti-OJ autoantibodies are closely associated with ILD.

Acknowledgements

We thank Ms Mutsuko Ishida for assisting with the RNA immunoprecipitation assay.

The authors have declared no conflict of interest.

References

1 Targoff IN. Laboratory testing in the diagnosis and management of idiopathic inflammatory myopathies. Rheum Dis Clin North Am 2004;28:859–90.
2 Hirakata M, Mimori T, Akizuki M, Craft J, Hardin JA, Homma M. Autoantibodies to small nuclear and cytoplasmic ribonucleoproteins in Japanese patients with inflammatory muscle disease. Arthritis Rheum 1992;35:449–56.
3 Yoshida S, Akizuki M, Mimori T, Yamagata H, Inada S, Homma M. The precipitating antibody to an acidic nuclear protein antigen, the Jo-1, in connective tissue diseases. A maker for a subset of polymyositis with interstitial pulmonary fibrosis. Arthritis Rheum 1983;26:604–11.
4 Mathews MB, Reichlin M, Hughes GR, Bernstein RM. Anti-threonyl-tRNA synthetase, a second myositis-related autoantibody. J Exp Med 1984;160:420–34.
5 Bunn CC, Bernstein RM, Mathews MB. Autoantibodies against alanyl tRNA synthetase and $tRNA^{Ala}$ coexist and are associated with myositis. J Exp Med 1986;163:1281–91.
6 Targoff IN. Autoantibodies to aminoacyl-transfer RNA synthetases for isoleucine and glycine. Two additional synthetases are antigenic in myositis. J Immunol 1990;144:1737–43.
7 Hirakata M, Suwa A, Nagai S *et al.* Anti-KS: Identification of autoantibodies to asparaginyl-transfer RNA synthetase associated with interstitial lung disease. J Immunol 1999;162:2315–20.
8 Suwa A, Hirakata M, Satoh S, Ezaki T, Mimori T, Inada S. A case of polymyositis with anti-OJ (isoleucyl-transfer RNA synthetase) antibodies. Clin Exp Rheumatol 1999;17:755–6.
9 Bernstein RM, Mathews MB. Jo-1 and other myositis autoantibodies. In: Brooks PM, York JR, eds. Rheumatology-85, excerpta medica international congress series. New York: Elsevier Science Publishers, 1985;273–8.
10 Sato S, Hirakata M, Kuwana M *et al.* Clinical characteristics in Japanese patients with anti-PL-7 (anit-threonyl-tRNA synthetase) autonatibodies. Clin Exp Rheumatol 2005;23:609–15.
11 Targoff IN, Trieu EP, Miller FW. Reaction of anti-OJ autoantibodies with components of the multi-enzyme complex of aminoacyl-tRNA synthetases in addition to isoleucyl-tRNA synthetase. J Clin Invest 1993;91:2556–64.
12 Gelpi C, Kanterewicz E, Gratacos J, Targoff IN, Rodriguez-Sanchez JL. Coexistence of two antisynthetases in a patient with the antisynthetase syndrone. Arthritis Rheum 1996;39:692–7.
13 Satoh S, Hirakata M, Nakamura K *et al.* Two cases of polymyositis associated with interstitial pneumonia with interstitial pneumonia with anti-OJ (isoleucyl tRNA synthetase) antibodies. Ryumachi 1998;38:534–41 (in Japanese).
14 Lane RJ, Emslie-Smith A, Mosquera IE, Hudgson P. Clinical, biochemical and histological responses to treatment in polymyositis: a prospective study. J R Soc Med. 1989;82:333–8.
15 Bohan A, Peter JB. Polymyositis and dermatomyositis. N Engl J Med 1975;292:344.
16 American Thoracic Societym European Respiratory Society.ATS/ERS International Multidisciplinary Consensus Classification of the Idiopathic Interstitial International Pneumonias Statement. Am J Respir Crit Care Med 2002;165:277–304.

ARTHRITIS & RHEUMATISM
Vol. 54, No. 1, January 2006, pp 312–324
DOI 10.1002/art.21539

Accuracy of Anti–Ribosomal P Protein Antibody Testing for the Diagnosis of Neuropsychiatric Systemic Lupus Erythematosus

An International Meta-Analysis

Fotini B. Karassa,[1] Antonella Afeltra,[2] Ales Ambrozic,[3] Deh-Ming Chang,[4] Filip De Keyser,[5] Andrea Doria,[6] Mauro Galeazzi,[7] Shunsei Hirohata,[8] Ilse E. A. Hoffman,[5] Murat Inanc,[9] Loreto Massardo,[10] Alessandro Mathieu,[11] Chi Chiu Mok,[12] Gabriella Morozzi,[7] Giovanni Sanna,[13] Alberto J. Spindler,[14] Athanasios G. Tzioufas,[15] Taku Yoshio,[16] and John P. A. Ioannidis[17]

Objective. **To quantitatively evaluate the diagnostic accuracy of antibodies to ribosomal P proteins (anti-P) for neuropsychiatric systemic lupus erythematosus (NPSLE) in general, for psychosis, mood disorder, or both, and for other diffuse manifestations.**

Methods. **This international meta-analysis combined standardized data from 1,537 lupus patients contributed by 14 research teams. Weighted estimation of sensitivity and specificity with fixed-effects and random-effects models, as well as summary receiver operating characteristic (SROC) curve analysis, was used to summarize test performance. The robustness of the overall estimates was examined in sensitivity analyses that included additional studies published up to November 1, 2004 in the Medline, EMBase, and Cochrane databases.**

Results. **Combining the data from the 14 teams, the weighted sensitivity and specificity estimates for the diagnosis of NPSLE were 26% (95% confidence interval [95% CI] 15–42%) and 80% (95% CI 74–85%), respectively. For psychosis, mood disorder, or both, the sensitivity and specificity were 27% (95% CI 14–47%) and 80% (95% CI 74–85%), respectively. For other diffuse manifestations, the sensitivity was 24% (95% CI 12–42%), and the specificity was 80% (95% CI 73–85%). The proportion of patients with anti-P antibodies did not vary markedly across different presentations of NPSLE. Between-study heterogeneity was substantial, but the SROC curves were consistent with the weighted estimates. In further analyses that included another 24**

Supported by a grant from the Joseph and Esther Gani Foundation (Ioannina, Greece). The Foundation had no role in the collection, analysis, or interpretation of the data or in the decision to submit the study for publication. Dr. Karassa's work was also supported by the Joseph and Esther Gani Foundation (grant 62/1516/-0001).

[1]Fotini B. Karassa, MD: University of Ioannina School of Medicine, Ioannina, and University of Thessaly School of Medicine, Larissa, Greece; [2]Antonella Afeltra, MD: University Campus Bio-Medico of Rome, Rome, Italy; [3]Ales Ambrozic, MD: University Medical Centre, Ljubljana, Slovenia; [4]Deh-Ming Chang, MD, MS, FACR: Tri-Service General Hospital, Taipei, Taiwan; [5]Filip De Keyser, MD, Ilse E. A. Hoffman, MD: Ghent University Hospital, Ghent, Belgium; [6]Andrea Doria, MD: University of Padua, Padua, Italy; [7]Mauro Galeazzi, MD, Gabriella Morozzi, MD: University of Siena, Siena, Italy; [8]Shunsei Hirohata, MD: Teikyo University School of Medicine, Tokyo, Japan; [9]Murat Inanc, MD: Istanbul University, Istanbul, Turkey; [10]Loreto Massardo, MD: Pontificia Universidad Catolica de Chile, Santiago, Chile; [11]Alessandro Mathieu, MD: University of Cagliari, Cagliari, Italy; [12]Chi Chiu Mok, MD, FRCP: Tuen Mun Hospital, Hong Kong, China; [13]Giovanni Sanna, MD: Homerton University Hospital, London, UK; [14]Alberto J. Spindler, MD: Universidad Nacional de Tucumán, Tucuman, Argentina; [15]Athanasios G. Tzioufas, MD: University of Athens Medical School, Athens, Greece; [16]Taku Yoshio, MD: Jichi Medical School, Tochigi-ken, Japan; [17]John P. A. Ioannidis, MD: Tufts University School of Medicine, Boston, Massachusetts, University of Ioannina School of Medicine, Ioannina, Greece, and Foundation for Research and Technology–Hellas, Ioannina, Greece.

Address correspondence and reprint requests to John P. A. Ioannidis, MD, Department of Hygiene and Epidemiology, University of Ioannina School of Medicine, 45110 Ioannina, Greece. E-mail: jioannid@cc.uoi.gr.

Submitted for publication April 25, 2005; accepted in revised form September 29, 2005.

published studies, only the sensitivity for psychosis and/or mood disorder was slightly improved, but it was still suboptimal (42% [95% CI 30–53%]); the specificity remained essentially the same (81% [95% CI 76–85%]).

Conclusion. **Anti-P antibody testing has limited diagnostic value for NPSLE, and it is not helpful in differentiating among various disease phenotypes.**

Neuropsychiatric manifestations occur in approximately one-half of patients with systemic lupus erythematosus (SLE) and may cause substantial impairment of quality of life as well as disability (1–3). Moreover, multiple neuropsychiatric events during the disease course are associated with adverse long-term prognosis (4,5) and may lead to death, with a mortality rate of 7–19% (2,5,6). Neuropsychiatric SLE (NPSLE) encompasses a multitude of symptoms involving the central, peripheral, and autonomic nervous systems as well as psychiatric disorders (7). Recently, an ad hoc committee of the American College of Rheumatology (ACR) proposed a standard nomenclature for 19 neuropsychiatric syndromes associated with SLE (7), yet NPSLE is difficult to diagnose and is challenging to treat. Secondary factors, such as drugs, metabolic abnormalities, or infections, can also cause neuropsychiatric disturbances in lupus patients (3,7). Manifestations reflecting diffuse cerebral involvement pose the foremost difficulty in differentiating their exact origin, since psychiatric disorders may merely be reactive psychological disturbances (2,3,7).

During the last 2 decades, several studies have explored the utility of antibodies to ribosomal P proteins (anti-P) in detecting NPSLE (6,8–35). These antibodies are directed toward 3 large-subunit ribosomal phosphoproteins, called P0 (38 kd), P1 (19 kd), and P2 (17 kd), which share a common linear determinant in the carboxyl-terminal 22–amino acid sequence (36). Early studies claimed that serum anti-P antibodies were highly accurate for the diagnosis of SLE-mediated psychosis and depression (9,26), but subsequent reports were less optimistic (11–13,18,20,25,27,31). Other studies expanded the spectrum of neuropsychiatric features that could be correlated with anti-P to include active disease, diffuse manifestations, or NPSLE overall (6,25,28,30), making even more unclear their clinical value for this entity. Methodologic shortcomings, including the criteria used to define NPSLE, the approaches adopted for detecting anti-P antibodies, and the small sample size of isolated studies, may have contributed to the uncertainty.

Because SLE is a relatively uncommon disease and NPSLE is even more uncommon, no single study can reliably assess the operating characteristics of anti-P antibodies. Yet, a rigorous appraisal of a diagnostic test may reduce the number of unwanted clinical consequences related to misleading estimates of the accuracy of that test. Ideally, one would like to assess the diagnostic accuracy of a test across a large study population and use similar, standardized, and reproducible methods. In the absence of a single very large study that could do this, an attractive alternative is to standardize data across existing cohorts of lupus patients. Therefore, the aim of this study was to evaluate the diagnostic performance of anti-P antibodies for NPSLE in general, for diffuse NPSLE manifestations, and for particular psychiatric syndromes (psychosis, mood disorder, or both) in the context of an international collaborative meta-analysis, with standardization of the data contributed by a large number of investigators.

PATIENTS AND METHODS

Eligibility criteria. The meta-analysis included lupus patients with and without NPSLE who had undergone serum anti-P antibody testing by immunoblotting, a standard enzyme-linked immunosorbent assay (ELISA), or both (37–39).

To ensure consistency, participating investigators were asked to comply with the following rules. Patients had to fulfill the ACR criteria for the classification of SLE (40) and had to be evaluated for the presence or absence of neuropsychiatric lupus syndromes according to the ACR nomenclature and case definitions (7). Patients with a neuropsychiatric syndrome during any time in the course of SLE were classified into 3 subgroups: those with psychosis, mood disorders, or both; those with other diffuse (2,6) manifestations (including acute confusional state, generalized seizures, cognitive dysfunction, anxiety disorder, and headache other than migraine or cluster headache), and those with focal (2,6) neurologic events (including cerebrovascular disease, partial seizures, migraine or cluster headache, myelopathy, demyelinating syndrome, movement disorder, aseptic meningitis, and syndromes of the peripheral nervous system) (7). When both diffuse and focal events occurred in the same patient, the designation was made according to the predominant manifestation. Severe, sustained, or progressive presentations requiring more-aggressive

treatment with cytotoxic immunosuppressive agents were considered to be predominant.

Collaborating investigators provided a clear description of the immunoassay(s) used for anti-P determination, with sufficient detail to permit replication (41). When both immunoblotting and ELISA had been used, data were reported separately for each method. Patients who had undergone testing for anti-P multiple times were considered to have this autoantibody specificity if at least 1 of the determinations yielded positive results. Investigators were also asked to specify whether immunoassays were performed without knowledge of the clinical condition of the patients and whether the diagnosis of NPSLE, as well as the assignment of neuropsychiatric syndromes, was accomplished without knowledge of the anti-P status of the participants.

Organization of the international database. Research teams who have previously published data on cohorts of SLE patients were invited to participate in this meta-analysis, provided that the study patients met the eligibility criteria defined above. Collaborating teams were identified through searches of the Medline, EMBase, and Cochrane databases conducted in January 2003, using combinations of index terms (systemic lupus erythematosus, rheumatic diseases, connective tissue disease, or autoimmune disease, as well as ribosomal, antiribosomal, anti-P, or antineuronal), cited references of eligible studies and review articles, abstracts of major rheumatology conferences, and consultation with experts in the field. We e-mailed invitations to investigators working on SLE. The meta-analysis was also announced at an autoimmune disease–related scientific meeting (42). Pertinent data were contributed on a standard reporting form. The database remained open until July 2004.

Research teams from 14 centers (8 European, 4 Asian, and 2 South American) agreed to participate. We accepted data that were already available as well as data that were prospectively generated specifically by some of the participating teams for the purposes of the collaborative project. The effort was coordinated by the Clinical and Molecular Epidemiology Unit of the Department of Hygiene and Epidemiology at the University of Ioannina School of Medicine. The coordinating center was responsible for giving instructions to participating investigators on how to standardize and summarize their individual-level databases. The contributed data sets were assessed for potential errors or inconsistencies and then assembled at the coordinating center, which was also responsible for conducting the analyses. Queries were clarified through communications with the participating investigators.

Data synthesis and statistical analysis. Measures of diagnostic performance included sensitivity and specificity of anti-P antibodies for various forms of NPSLE. The main analysis involved the following 4 comparisons: NPSLE overall and each subgroup of NPSLE (psychosis and/or mood disorder, other diffuse manifestations, and focal events) versus the non-NPSLE group; all diffuse manifestations versus focal events; and psychosis and/or mood disorder versus other diffuse manifestations. These analyses address the discriminatory ability of the test for NPSLE in general, for each disease subtype, and for different neuropsychiatric presentations. To further pursue the possibility that anti-P may be specifically associated with particular psychiatric disorders (8,9,16,22,26), we evaluated the diagnostic accuracy of anti-P antibody for patients with psychosis and/or mood disorder versus all other lupus patients.

Test performance was estimated separately from studies that used immunoblotting for the detection of anti-P antibodies and from studies that used ELISA. In the overall analysis, when both immunoblotting and ELISA data were available from the same study, the results from the ELISA were used for the calculations. Diagnostic accuracy was also evaluated for subgroups defined by race.

Summary estimates were obtained with 2 meta-analytic methods: weighted independent estimation of sensitivity and specificity, and summary receiver operating characteristic (SROC) curve analysis.

Sensitivity and specificity estimates for each comparison were independently combined across studies, using both fixed-effects (Mantel-Haenszel) and random-effects (DerSimonian-Laird) models (43,44). Fixed-effects models weigh each study by the inverse of its variance. Random-effects models also incorporate between-study variation. The random-effects approach tends to provide wider confidence intervals (CIs) and is preferable in the presence of between-study heterogeneity. Except where indicated otherwise, random-effects estimates are provided below. Between-study heterogeneity was examined with Fisher's exact test.

Because sensitivity and specificity are interdependent, independent weighting may sometimes underestimate both measures. Hence, we used SROC curve analysis to account for this mutual dependence (45,46). The method fits a curve describing the tradeoff between sensitivity and specificity across studies, with different characteristics and thresholds for an abnormal test result. The regression is calculated as follows: $D = \alpha + \beta S$, where D is the difference in the logits of the true-positive rate (sensitivity) and the false-positive rate (1 − specificity), and S is the sum of these logits. When β is not significantly different from 0, the SROC curve is symmetric around the diagonal that runs from the top left corner to the bottom right corner of the diagram. Conversely, when β is significantly different from 0, the SROC curve is not symmetric, and the overall diagnostic performance varies in different parts of the curve, with an uneven tradeoff between sensitivity and specificity across studies. This may indicate significant between-study variation in the selected test threshold, study population, or other parameters. SROC curves should not be extrapolated outside the range of observed values. Both nonweighted and weighted SROC curves were estimated (46,47); nonweighted curves consider all studies equally in the calculations, whereas weighted curves weigh each study by the variance of D.

Inclusion of other published data. Sensitivity analyses were conducted to examine whether the addition of further relevant published studies affected our summary estimates of the operating characteristics of anti-P antibodies. Only the following 2 comparisons were examined, since articles focused on these patient groups: the entire group of NPSLE