The human TNF-α gene (*TNFA*) is located on chromosome 6 within the MHC class III region between HLA-B and HLA-DR genes. A number of polymorphisms have been identified in the *TNFA* itself, and a polymorphic allele of the *TNFA* may correlate with a particular clinical subtype of disease. More recently, it has been reported that *TNFA*-308 polymorphisms, but not SE alleles are associated with increased radiological progression [3], although *TNFA*-308 polymorphisms are relatively rare in Japanese [4]. On the other hand, Higuchi et al. [4] identified three new polymorphisms, −1031T/C, −863C/A, and −857T/C, in the upstream 5′-flanking region of *TNFA* in Japanese. Seki et al. and Shibue et al. [5, 6] reported that the frequency of the −857T allele in RA patients is significantly higher than in controls, although *HLA-DRB1* is more strongly associated with disease susceptibility than *TNFA* polymorphisms.

In this study, we determined polymorphisms of the 5′-flanking region of *TNFA* in Japanese patients with early RA to further examine whether the polymorphisms were associated with susceptibility to and progression of RA.

## Patients and methods

The patients were derived from those reported in our previous study [7]; thus, they visited our outpatient clinic at the Institute of Rheumatology, Tokyo Women's Medical University, within 1 year of symptom onset and were enrolled in a prospective follow-up study between 1991 and 1995. For this study, we used 123 of 198 patients, who satisfied the American College of Rheumatology (ACR) 1987 criteria for RA [8]. All patients were Japanese. Twenty-five patients were males, and 98 patients were females. All patients were treated with disease-modifying antirheumatic drugs and/or nonsteroidal anti-inflammatory agents selected by rheumatologists in our outpatient clinic. No patients were treated with anti-TNF-α therapy, because this study was performed before anti-TNF-α therapy was introduced into Japan. We used the same population of patients as reported recently [9]. Controls consisted of 265 healthy Japanese volunteers with no inflammatory rheumatic diseases. One hundred forty-five were males, and 120 were females.

The radiological progression of hands/wrists and feet was evaluated every 6 months for 2 years using Larsen's score. The radiographs were read randomly by three rheumatologists. The sum of the Larsen grades for two wrists, eight finger proximal interphalangeal (PIP) joints, two thumb interphalangeal (IP) joints, ten metacarpophalangeal (MCP) joints, eight metatarsophalangeal (MTP) joints of toes 2–5, and the great toe IP joints was used to determine the overall joint damage score on a scale of 0–160. We defined the progressive score as the Δdamage score: the damage score at the first visit subtracted from the damage score at 12 or 24 months. All participants gave informed consent.

Genomic DNA was extracted from peripheral blood leukocytes using the standard phenol–chloroform extraction procedure. The *HLA-DRB1* genotype was determined using the polymerase chain reaction (PCR)-restriction fragment length polymorphism method. Alleles considered to have SE were *DRB1 *0101, *0401, *0404, *0405, *0410, and *1001*. Genotyping of *TNFA* −1031, −863, and −857 single-nucleotide polymorphisms (SNPs) was carried out using a PCR-preferential homoduplex formation assay [10]. Four haplotypes, −1031/−863/−857 TCC, TCT, CAC, and CCC, were designated as *TNFA*-U01, -U02, -U03, and -U04, respectively. The laboratory variables, ESR (mm/h, Westergren method), serum CRP (mg/dl), and rheumatoid factor (RF) were measured in each patient every 2 months for 2 years. RF status was assessed using a particle agglutination test (RAPA test; Fujizoki Pharmaceutical, Tokyo, Japan).

Statistical significance of the differences between groups was examined by Fisher's exact test or by the Mann–Whitney *U* test (Statview; Abacus Concepts Inc., Berkeley, CA). *P*-values less than 0.05 were regarded as significant, and all tests were two-tailed. As for the comparison of the Δdamage score, the Bonferroni procedure was used to control the type I error rate. We reported the unadjusted *P* values.

## Results

The characteristics of the patients at study entry are summarized in Table 1. The mean age of all patients was 49.5 years, and disease duration averaged 5.8 months. Most patients (87%) were RF positive.

As shown in Table 2, a significant positive association with RA was observed for *DRB1 *0405* ($P = 0.00012$). On

**Table 1** Characteristics of 123 patients with RA

| Variables | *n* |
|---|---|
| Female/male | 98/25 |
| Age (years) | 49.5 ± 12.3 |
| Disease duration (months) | 5.8 ± 3.2 |
| Positive RAPA | 107 (87%) |
| ESR (mm/h) | 48.8 ± 24.7 |
| CRP (mg/dl) | 2.3 ± 3.2 |

Quantitative data are shown as the mean ± SD unless otherwise noted

*ESR* erythrocyte sedimentation rate, *CRP* C-reactive protein

**Table 2** Carrier frequency of *HLA-DRB1* alleles among RA patients and controls

| *DRB1* allele | RA (%) ($n$ = 123) | Control (%) ($n$ = 265) | OR | $P$-value |
|---|---|---|---|---|
| 0101 | 19 (15.4) | 26 (9.8) | 1.68 | NS |
| 0401 | 6 (4.9) | 6 (2.3) | 2.21 | NS |
| 0403 | 5 (4.1) | 13 (4.9) | 0.82 | NS |
| 0404 | 2 (1.6) | 4 (1.5) | 1.08 | NS |
| 0405 | 54 (43.9) | 65 (24.5) | 2.41 | 0.00012* |
| 0406 | 8 (6.5) | 19 (7.2) | 0.90 | NS |
| 0410 | 3 (2.4) | 5 (1.9) | 1.30 | NS |
| 1001 | 1 (0.8) | 2 (0.8) | 1.08 | NS |
| 1101 | 5 (4.1) | 5 (1.9) | 2.20 | NS |
| 1302 | 11 (8.9) | 51 (19.2) | 0.41 | 0.0099* |
| 1405 | 5 (4.1) | 17 (6.4) | 0.62 | NS |
| 1406 | 2 (1.6) | 7 (2.6) | 0.61 | NS |
| SE (+) | 78 (63.4) | 100 (37.7) | 2.86 | $2.3 \times 10^{-6}$* |

Values are the number (%) of patients; $P$-value was determined by Chi-square analysis

*RA* rheumatoid arthritis, *NS* not significant

* Statistically significant

**Table 3** Analysis of *TNFA* promoter region haplotype in RA patients and controls

| | RA (%) ($n$ = 123) | Control (%) ($n$ = 265) | OR | $P$-value |
|---|---|---|---|---|
| Haplotype positivity | | | | |
| *TNFA*-U01 | 108 (88) | 232 (88) | 1.02 | 0.94 |
| *TNFA*-U02 | 58 (47) | 83 (31) | 1.95 | 0.0025* |
| *TNFA*-U03 | 20 (16) | 69 (26) | 0.55 | 0.033* |
| *TNFA*-U04 | 2 (2) | 14 (5) | 0.30 | 0.158 |
| Haplotype frequency | ($2n$ = 246) | ($2n$ = 530) | | |
| *TNFA*-U01 | 160 (65) | 349 (66) | 0.964 | 0.825 |
| *TNFA*-U02 | 64 (26) | 94 (18) | 1.63 | 0.008 |
| *TNFA*-U03 | 20 (8) | 73 (14) | 0.554 | 0.024 |
| *TNFA*-U04 | 2 (1) | 14 (3) | 0.302 | 0.095 |
| Allele frequency | | | | |
| −1031 T | 224 (91) | 443 (84) | | |
| C | 22 (9) | 87 (16) | | |
| −863 C | 226 (92) | 457 (86) | | |
| A | 20 (8) | 73 (14) | | |
| −857 C | 182 (74) | 436 (82) | | |
| T | 64 (26) | 94 (18) | | |

* Statistically significant

the other hand, the frequency of individuals with *DRB1 *1302* was significantly lower in patients than in controls ($P$ = 0.0099). The frequency of individuals with SE was significantly higher in patients than in controls ($P < 0.0001$) (Table 2).

The proportion of individuals with the *TNFA*-U02 allele was significantly higher in patients than in controls ($P$ = 0.0025). The proportion of individuals with U03 was significantly lower in patients than in controls ($P$ = 0.033) (Table 3).

We divided patients into four groups, depending on the positivity of U02 and SE alleles: U02+SE+, U02+SE−, U02−SE+, and U02−SE−. The frequency of the genotype combination U02+SE+ was significantly higher in patients than in controls ($P$ = 0.0013) (Table 4). The frequency of U02−SE+ was also significantly higher in RA than in controls, but the frequency of U02+SE− was not different between RA and controls.

Matsushita et al. [10] reported that *TNFA*-U02 is in linkage disequilibrium with *DRB1*0405*. Thus, we examined linkage disequilibrium between *TNFA* alleles and *HLA-DRB1* alleles using LDSUPPORT [11] and also found that *TNFA*-U02 was in strong linkage disequilibrium with *HLA-DRB1 *0405* (data not shown).

Radiographs of hands/wrists/feet were available for 72 patients after 1 year and for 73 patients after 2 years. The X-ray progression score (Δdamage score) at 12 months was slightly higher in patients with SE than in patients without SE ($P$ = 0.0444) (Fig. 1a). Although the progression score (Δdamage score) at 12 months was lower in the

**Table 4** *TNFA*-U02 haplotype and *HLA-DRB1* SE allele distribution among RA patients and controls

| | RA (%) ($n$ = 123) | Control (%) ($n$ = 265) | OR | $P$-value |
|---|---|---|---|---|
| U02 (+)/SE (+) | 41 (33) | 49 (18) | 2.2 | 0.0013* |
| U02 (+)/SE (−) | 17 (14) | 34 (13) | 1.1 | 0.788 |
| U02 (−)/SE (+) | 37 (30) | 51 (19) | 1.8 | 0.0177* |
| U02 (−)/SE (−) | 28 (23) | 131 (49) | 0.3 | <0.0001* |

Values are the number (%) of patients; $P$-value was determined by chi-square analysis

*RA* rheumatoid arthritis

* Statistically significant

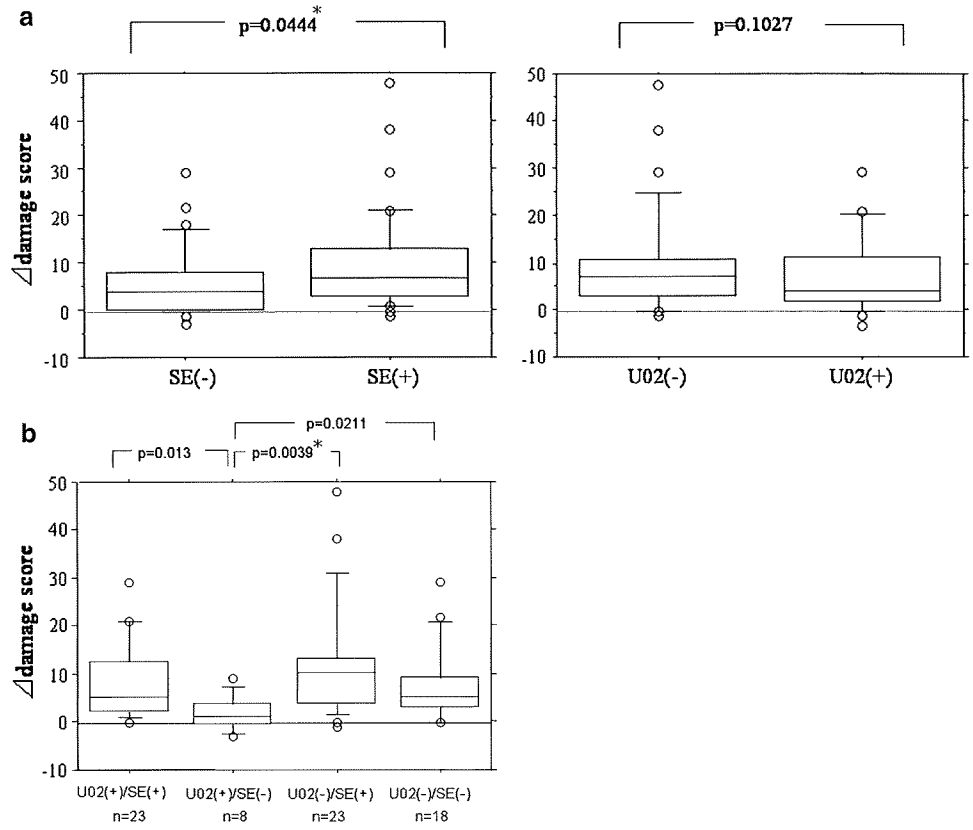


**Fig. 1** Progression of damage score (12 months). $P$ value was determined by the Mann–Whitney $U$ test. All values are the mean ± SEM, or *box plots*, in which the *upper and lower bars* show the 90th and 10th percentiles, respectively, and the *upper*, *center*, and *lower lines* of the box show 75th, 50th, and 25th percentiles, respectively. **a** *Statistically significant, **b** *statistically significant using the Bonferroni procedure

U02+ group than in the U02− group, the difference was not statistically significant ($P$ = 0.1027). To further examine the role of *TNFA* promoter haplotype in X-ray progression, *HLA-DRB1* SE genotype was analyzed in combination. There was no difference in the levels of ESR, CRP, or RF at the first visit among the U02+SE+, U02+SE−, U02−SE+, and U02−SE− groups (Table 5). As shown in Fig. 1b, patients with U02+SE− showed the lowest progression score at 12 months among the four groups and those with U02−SE+ showed the highest score. Thus, the Δdamage score at 12 months was significantly lower in the U02+SE− group than in the U02−SE+ group using the Bonferroni procedure. The difference in the Δdamage score between the U02−SE−group and U02−SE+ group was not significant. There was no difference in the percentage of MTX treatment for 2 years among the four groups, although none of the U02+SE− patients received MTX (Table 5); however, these differences were not significant at 24 months (data not shown).

## Discussion

In this study, we observed that the association of *TNFA* promoter polymorphism with RA can be explained by linkage disequilibrium between *TNFA* and *HLA-DRB1* alleles, as reported previously, even in patients in the early stages. We also demonstrated that *TNFA* alleles may have some additional effects on *HLA-DRB1* alleles, although the contribution of *TNFA* alleles alone on X-ray progression

**Table 5** Laboratory data and MTX treatment

| | ESR (mm/h) at first visit | CRP (mg/dl) at first visit | MTX at 12th month | MTX at 24th month |
|---|---|---|---|---|
| U02 (+)/SE (+) | 48.4 ± 22.2 | 2.2 ± 3.1 | 1/23 | 3/26 |
| U02 (+)/SE (−) | 55.6 ± 25.3 | 3.4 ± 3.7 | 0/8 | 0/5 |
| U02 (−)/SE (+) | 46.9 ± 28.1 | 2.6 ± 3.9 | 3/23 | 3/24 |
| U02 (−)/SE (−) | 47.8 ± 23.6 | 1.7 ± 1.7 | 1/18 | 1/18 |

Quantitative data are shown as the mean ± SD unless otherwise noted

*ESR* erythrocyte sedimentation rate, *CRP* C-reactive protein

was not significant. The progression score of U02+SE− patients was significantly lower than the score of U02−SE+. In addition, the progression score of U02+SE− patients was lowest among the four groups at 12 months, although these differences were not significant. On the other hand, the progression score of U02−SE+ was highest among the four groups; however, such tendency was not significant, partly because of the small number of patients ($n$ = 73). Further, the difference in the progression score between U02−SE− and U02−SE+ was not significant ($P$ = 0.198). We speculate that SE interference was small in the U02− group.

Udalova et al. [12] reported that the TNF-863A allele specifically reduces the binding of NF-κB p50/p50 homodimer to this part of the TNF promoter region and then increases TNF expression. In addition, they showed that the proportion of individuals with the TNF-863A allele was higher in patients undergoing joint replacement surgery for RA than controls [13]. These results support our findings because the group of patients with U02, that is, without TNF-863A, showed slower progression than the group of patients without U02 (Fig. 1a). On the other hand, the same group showed that TNF-857C homozygotes produce a higher amount of TNF [14]. In addition, Van Krugten et al. [15] showed that RA patients with −857CC genotype tended to develop erosive disease more than −857CT patients. These results support our findings that the U02+ group (without TNF-857C) had slower radiological progression than the U02− group (Fig. 1a). Recently, Kang et al. [16] reported that RA patients with the T allele at position −857 of the *TNFA* promoter responded better to etanercept therapy than C-allele homozygotes, supporting our findings. On the other hand, Higuchi et al. [4] revealed higher transcriptional promoter activities in the −1031C/−863A or −857T allele. Skoog et al. [17] reported, however, that carriers of the −863A allele had significantly lower serum TNF-α levels. The effects of these polymorphisms on TNF-α transcription and production are contradictory.

In this study, we demonstrated that SE-possessing patients were associated with more erosion than patients without SE during the first year of disease (Fig. 1a); however, the association between *HLA-DRB1* alleles and disease severity is controversial. Kaltenhauser et al. [18] examined 87 patients with RA to evaluate prognostic makers for severe joint erosion. They showed that the presence of the SE+DR4 allele influenced joint destruction. Mattey et al. [19] reported that RF-negative patients carrying an SE allele had higher Larsen scores than RF-negative patients lacking SE. On the other hand, Valenzuela-Castano et al. [20] reported no relationship between SE and the severity of structural joint damage in large joints. In 1997, we demonstrated that SE alleles failed to correlate with more severe disease [7]. In our current study, we used 123 of these 198 patients, showing a contradiction in the results. We speculate that this contradiction was derived from the different study populations; in 1997, we used patients with polyarthritis within 1 year of symptom onset, but in the current study we selected patients who satisfied the ACR 1987 criteria. In addition, different evaluation methods of disease severity were associated with the contradiction; in 1997, we used bone erosion, but in the current study we used the Δdamage score. Thus, further investigations are needed to clarify this contradiction.

Our study is very important, although it was carried out in the 1990s. Most RA patients were treated with traditional DMARDs, but not with biologics, and we therefore investigated RA patients with the classical therapy alone. Thus, it is impossible to carry out a study like ours in 2008.

In conclusion, our study suggests that the combination of the polymorphism of the *TNFA* upstream promoter region and *HLA-DRB1* allele may identify subgroups of patients with early RA related to radiological progression. Further study analyzing a larger number of patients is needed, and such a study may result in the efficient prediction of RA prognosis at an early stage of disease.

**Acknowledgments** This work was supported by a Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Science, Sports, and Culture of Japan, and a Health and Labor Sciences Research Grant from the Ministry of Health, Labor, and Welfare of Japan.

## References

1. Eigler A, Sinha B, Hartmann G, Endres S. Taming TNF: strategies to restrain this proinflammatory cytokine. Immunol Today. 1997;18:487–92.

2. Keffer J, Probert L, Cazlaris H, Georgopoulos S, Kaslaris E, Kioussis D, et al. Transgenic mise expressing human tumor necrosis factor: predictive genetic model of arthritis. EMBO J. 1991;10:4025–31.
3. Khanna D, Wu H, Park G, Gersuk V, Gold RH, Nepom GT, et al. Association of tumor necrosis factor α polymorphism, but not the shared epitope, with increased radiographic progression in a seropositive rheumatoid arthritis inception cohort. Arthritis Rheum. 2006;54:1105–16.
4. Higuchi T, Seki N, Kamizono S, Yamada A, Kimura A, Kato H, et al. Polymorphism of the 5′-flanking region of the human tumor necrosis factor (TNF)-α gene in Japanese. Tissue Antigens. 1998;51:605–12.
5. Seki N, Kamizono S, Yamada A, Higuchi T, Matsumoto H, Niiya F, et al. Polymorphisms in the 5′-flanking region of the tumor necrosis factor (TNF)-α gene in patients with rheumatoid arthritis. Tissue Antigens. 1999;54:194–7.
6. Shibue T, Tsuchiya N, Komata T, Matsushita M, Shiota M, Ohashi J, et al. Tumor necrosis factor α 5′-flanking region, tumor necrosis factor receptor II, and HLA-DRB1 polymorphisms in Japanese patients with rheumatoid arthritis. Arthritis Rheum. 2000;43:753–7.
7. Higami K, Hakoda M, Matsuda Y, Ueda H, Kashiwazaki S. Lack of association of HLA-DRB1 genotype with radiologic progression in Japanese patients with early rheumatoid arthritis. Arthritis Rheum. 1997;40:2241–7.
8. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum. 1988;31:315–24.
9. Furuya T, Hakoda M, Ichikawa N, Higami K, Nanke Y, Yago T, et al. Associations between HLA-DRB1, RANK, RANKL, OPG, and IL-17 genotypes and disease severity phenotypes in Japanese patients with early rheumatoid arthritis. Clin Rheumatol. 2007;26:2137–41.
10. Matsushita M, Tsuchiya N, Nakayama T, Ohashi J, Shibue T, Shiota M, et al. Allele typing of human TNFA 5′-flanking region using polymerase chain reaction-preferential homoduplex formation assay (PCR-PHFA): linkage disequilibrium with HLA class I and II gene in Japanese. Tissue Antigens. 1999;54:478–84.
11. Kitamura Y, Moriguchi M, Kaneko H, Morisaki H, Morisaki T, Toyama K, et al. Determination of probability distribution of diplotype configuration (diplotype distribution) for each subject from genotype data using the EM algorithm. Ann Hum Genet. 2002;66:183–93.
12. Udalova IA, Richardson A, Denys A, Smith C, Ackerman H, Foxwell B, et al. Functional consequences of a polymorphism affecting NF-κB p50–p50 binding to the TNF promoter region. Mol Cell Biol. 2000;20:9113–9.
13. Udalova IA, Richardson A, Ackerman H, Wordsworth P, Kwiatkowski D. Association of accelerated erosive rheumatoid arthritis with a polymorphism that alters NF-κB binding to the TNF promoter region. Rheumatology. 2002;41:830–1.
14. Van Heel DA, Udalova IA, De Silva AP, McGovern DP, Kinouchi Y, Hull J, et al. Inflammatory bowel disease is associated with a TNF polymorphism that affects an interaction between the OCT1 and NFκB transcription factors. Hum Mol Genet. 2002;11:1281–9.
15. Van Krugten MV, Kaijzel EL, Bakker AM, Drossaers-Bakker KW, Huizinga TWJ, Hazes JMW, et al. Definition of a TNF haplotype that is associated with protection from erosive disease in RA. Arthritis Rheum. 1999;42:S282.
16. Kang CP, Lee KW, Yoo DH, Kang C, Bae SC. The influence of a polymorphism at position -857 of the tumor necrosis factor a gene on clinical response to etanercept therapy in rheumatoid arthritis. Rheumatology. 2005;44:547–52.
17. Skoog T, van't Hooft FM, Kallin B, Jovinge S, Boquist S, Nilsson J, et al. A common functional polymorphism(C→A substitution at position -863) in the promoter region of the tumor necrosis factor-α (TNF-α) gene associated with reduced circulating levels of TNF-α. Hum Mol Genet. 1999;8:1443–9.
18. Kaltenhauser S, Wagner U, Schuster E, Wassmuth R, Arnold S, Seidel W, et al. Immunogenetic markers and seropositivity predict radiological progression in early rheumatoid arthritis independent of disease activity. J Rheumatol. 2001;28:735–44.
19. Mattey DL, Hassell AB, Dawes PT, Cheung NT, Poilton KV, Thomson W, et al. Independent association of rheumatoid factor and the HLA-DRB1 shared epitope with radiographic outcome in rheumatoid arthritis. Arthritis Rheum. 2001;44:1529–33.
20. Valenzuela-Castano A, Garcia-Lopez A, Perez-Vilches D, Rodriguez-Perez R, Gonzalez-Escribano MF, Nunez-Roldan A. The predictive value of the HLA shared epitope for severity of radiological joint damage in patients with rheumatoid arthritis. A 10 year observational prospective study. J Rheumatol. 2000;27:571–4.

**ARD Online First, published on September 9, 2009 as 10.1136/ard.2009.118760**

Letter to the Editor

# Replication of association between *FAM167A(C8orf13)-BLK* region and rheumatoid arthritis in a Japanese population.

[1]Ikue Ito, [1]Aya Kawasaki, [2]Satoshi Ito, [2]Yuya Kondo, [2]Makoto Sugihara, [2]Masanobu Horikoshi, [2]Taichi Hayashi, [2]Daisuke Goto, [2]Isao Matsumoto, [3]Akito Tsutsumi, [4]Yoshinari Takasaki, [5]Hiroshi Hashimoto, [6]Kunio Matsuta, [2]Takayuki Sumida, [1]Naoyuki Tsuchiya

[1]Molecular and Genetic Epidemiology Laboratory, Doctoral Program in Life System Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Japan.
[2]Division of Clinical Immunology, Doctoral Program in Clinical Sciences, Graduate School of Comprehensive Human Science, University of Tsukuba, Tsukuba, Japan.
[3]Takikawa Municipal Hospital, Takikawa, Japan.
[4]Division of Rheumatology, Department of Medicine, Juntendo University, Tokyo, Japan.
[5]Juntendo University School of Medicine, Tokyo, Japan.
[6]Matsuta Clinic, Tokyo, Japan.

Running title: Association of *BLK* with RA in Japanese

Correspondence: Dr. Naoyuki Tsuchiya, Doctoral Program in Life System Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Japan, 305-8575.
Phone/FAX: +81-29-853-3071, e-mail: tsuchiya-tky@umin.net

Key words: rheumatoid arthritis, genetics, polymorphism, BLK
Word count: 494 words

Polymorphisms in the genomic region encoding B lymphoid tyrosine kinase (*BLK*) and family with sequence similarity 167, member A (*FAM167A*, also referred to as *C8orf13)* at 8p23.1 have been associated with systemic lupus erythematosus (SLE) in Caucasian[1,2] and Asian[3,4] populations. A recent genome-wide study in a north-American population demonstrated new associations with rheumatoid arthritis (RA), among which was a single nucleotide polymorphism (SNP) rs2736340 in the intergenic region of *BLK* and *FAM167A*.[5] In the HapMap Japanese samples (http://www.hapmap.org/index.html.ja), this SNP is in absolute linkage disequilibrium ($r^2$=1) with rs13277113, previously associated with SLE.[1-4] We demonstrated that both the population frequency of the risk genotype, rs13277113A/A, and the odds ratio (OR) for SLE were substantially higher in the Japanese compared with the Caucasian populations.[3]

Thus far, the association of *FAM167A-BLK* region with RA has not been reported in non-Caucasian populations. In this study, we examined whether the association between *BLK* and RA was replicated in Japanese.

A case-control association study was performed for 603 patients and 492 healthy controls. Because the association of *FAM167A-BLK* region with SLE is already established,[1-4] RA patients complicated with SLE were excluded. All patients fulfilled the American College of Rheumatology classification criteria for RA.[6] The patients and the healthy controls were recruited at Matsuta Clinic, University of Tsukuba, the University of Tokyo and Juntendo University. This study was reviewed and approved by the Research Ethics Committees of University of Tsukuba and other participating institutes. Written informed consent was obtained from all participants, except for some participants before 2001, prior to the enforcement of the Ethics Guidelines for Human Genome/Gene Analysis Research by the Japanese government. From such participants, oral informed consent had been obtained. In accordance with the Guidelines, the latter samples were anonymized in an unlinkable fashion, and were included in this study after review and approval by the Ethics Committee of University of Tsukuba. Genotype of rs13277113 was determined using the TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA).[3] Power calculation based on the risk allele frequency in the Japanese population (0.665) showed that this sample size provides 80% power to detect susceptibility genes with an allelic OR of 1.298. Deviation from Hardy-Weinberg equilibrium was observed neither in the patients nor in the controls.

Significant association with RA was replicated in the Japanese (Table 1). Although the OR was comparable to that in the Caucasian population (1.19 for rs2736340[5]), the risk allele frequency was considerably higher in the Japanese as compared with the Caucasians (cases 0.273 and controls 0.240 for rs2736340[5]). Population

attributable risk percent was estimated to be 22.8% in the Japanese and 9.3% in the Caucasians under the dominant model. No significant difference in rs13277113 was observed between *HLA-DRB1* shared epitope positive and negative RA (data not shown).

Our observations indicated that *FAM167A-BLK* region may be a shared genetic factor for multiple autoimmune diseases in multiple populations, but the genetic contribution may be greater in the Asian populations because of the differences in the genetic background.

Competing interests

None declared.

Funding

This work was supported by Grant-in-Aid for Scientific Research (B) from Japan Society for the Promotion of Science (JSPS), Health and Labour Science Research Grants from the Ministry of Health, Labour and Welfare of Japan, Japan Rheumatism Foundation, The Naito Foundation and Mitsubishi Pharma Research Foundation.



References

1 Hom G, Graham RR, Modrek B, et al. Association of systemic lupus erythematosus with *C8orf13-BLK* and *ITGAM-ITGAX*. N Engl J Med 2008;358:900-9.

2 Harley JB, Alarcon-Riquelme ME, Criswell LA, et al. Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in *ITGAM, PXK, KIAA1542* and other loci. Nat Genet 2008;40:204-10.

3 Ito I, Kawasaki A, Ito S, et al. Replication of the association between the *C8orf13-BLK* region and systemic lupus erythematosus in a Japanese population. Arthritis Rheum 2009;60:553-8.

4 Yang W, Ng P, Zhao M, et al. Population differences in SLE susceptibility genes: *STAT4* and *BLK*, but not *PXK*, are associated with systemic lupus erythematosus in Hong Kong Chinese. Genes Immun 2009;10:219-26.

5 Gregerson PK, Amos CI, Lee AT, et al. *REL*, encoding a member of the NF-κB family of transcription factors, is a newly defined risk locus for rheumatoid arthritis. Nat Genet 2009;41:820-3.
6 Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315-24.

Table 1. Association of *BLK* rs13277113 with RA in a Japanese population

| | | Genotype frequency | | | Allele frequency | | Allelic association | |
|---|---|---|---|---|---|---|---|---|
| | n | A/A | A/G | G/G | A | G | *P* | OR (95% CI) |
| RA | 603 | 308 (0.511) | 242 (0.401) | 53 (0.088) | 858 (0.711) | 348 (0.289) | 0.018 | 1.24 (1.04-1.49) |
| Control | 492 | 218 (0.443) | 218 (0.443) | 56 (0.114) | 654 (0.665) | 330 (0.335) | | |

OR: odds ratio, 95%CI: confidence interval.

Association was tested by $\chi^2$- analysis using 2X2 contingency table.

Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 367 (2008) 311–317
BBRC
www.elsevier.com/locate/ybbrc

# Conservative sequences in 3′UTR of TCRζ mRNA regulate TCRζ in SLE T cells

Kensei Tsuzaka [a,b,*], Yuka Itami [b], Chika Kumazawa [b], Miyuki Suzuki [a], Yumiko Setoyama [a], Keiko Yoshimoto [b], Katsuya Suzuki [a], Tohru Abe [a], Tsutomu Takeuchi [a,b]

[a] *Division of Rheumatology, Department of Internal Medicine, Saitama Medical Center, Saitama Medical University, 1981 Kamoda, Kawagoe, Saitama 350-8550, Japan*

[b] *Project Research Division, Research Center for Genomic Medicine, Saitama Medical University, 1397-1 Yamane, Hidaka, Saitama 350-1241, Japan*

Received 3 December 2007
Available online 3 January 2008

**Abstract**

We have demonstrated that T-cell receptor ζ (ζ) mRNA with a 562-bp deleted alternatively spliced 3′-untranslated region (3′UTR) observed in T cells of patients with systemic lupus erythematosus (SLE) can lead to a reduction in ζ and TCR/CD3 (J. Immunol., 2003 & 2005). To determine the region in ζ mRNA 3′UTR for the regulation of ζ, ζ mRNA with 3′UTR truncations ligated into pDON-AI was used to infect murine T-cell hybridoma MA5.8 cells, which do not contain ζ. As a Western blot analysis demonstrated the importance of the regions from +871 to +950, containing conservative sequence 1 (CS1), and +1070 to +1136, containing CS2, for the production of ζ, we constructed MA5.8 mutants carrying ζ mRNA 3′UTR with deletions of these regions (ΔCS1 and ΔCS2 mutants). Western blot and FACS analyses showed significant reduction in the cell surface ζ and TCR/CD3 in both these mutants, and IL-2 production was decreased, compared with MA5.8 cells transfected with wild-type ζ mRNA. Furthermore, real-time PCR demonstrated the instability of ζ mRNA with 3′UTR deletions in these MA5.8 mutants. In conclusion, CS1 and CS2 may be responsible for the regulation of ζ and TCR/CD3 through the stability of ζ mRNA in SLE T cells.


*Keywords:* Systemic lupus erythematosus; TCRζ; 3′UTR; Signal transduction; Conservative sequence; T-cell receptor; IL-2; Autoimmune disease; MA5.8 cells; mRNA stability

Systemic lupus erythematosus (SLE) is well known as a prototype systemic autoimmune disease [1]. Defects in signal transduction through the TCR/CD3 complex may cause T-cell dysfunction and autoimmunity in NOD mice as well as in MRL *lpr/lpr* mice [2–4]. Several studies, including our own, have identified a functional defect in early signaling molecules on peripheral blood T cells (PBTs) as a cause of T-cell dysfunction in human SLE [5–7]. Sakaguchi et al. [8] demonstrated that a ZAP-70 mutation causes autoimmune arthritis in the SKG mouse model, supporting the notion that functional defects in early signaling molecules can cause autoimmune diseases. On the other hand, we and other groups have reported that a reduction in tyrosine phosphorylation and the diminished expression of ζ protein play crucial roles in the pathogenesis of SLE [9–13], and that an aberrant form of the ζ mRNA 3′-untranslated region (3′UTR), which is alternatively spliced and 562-bp shorter than the wild-type 3′UTR, is predominantly expressed in SLE T cells (ζmRNA/as-3′UTR) [14,15] and leads to the up-regulation of several other proteins [16]. An *in vitro* expression analysis of the ζ protein from ζmRNA/as-3′UTR using MA5.8 cell mutants and a retrovirus system showed that the predominant expression of ζmRNA/as-3′UTR leads to the down-regulation of not only ζ, but also of other TCR/CD3 components because of the instability of these ζ mRNA splice variant forms [17]. Observations in our study

* Corresponding author. Fax: +81 49 228 3574.
*E-mail address:* kentsu@saitama-med.ac.jp (K. Tsuzaka).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.12.145

[17] and others [18] suggest that the deleted 562-bp portion of the 3′UTR in ζmRNA/as-3′UTR is critical for ζ mRNA stability. The 3′UTR region of mRNA is known to control the turnover rate of pre-synthesized mRNAs through interactions with *trans*-acting factors by altering mRNA stability and affecting the transportation and localization of mRNA [19–21]. Messenger RNA 3′UTR contains *cis*-acting elements, i.e., adenosine-uridine (AU)-rich elements (AREs), that bind to *trans*-acting proteins and participate in either the stabilization or destabilization of transcripts. Two AREs are located at positions +735 and +803 of the ζ mRNA 3′UTR, and both of these AREs are involved in the 562-bp deleted portion of the ζmRNA/as-3′UTR. Therefore, we investigated which portion, including the AREs in the ζ mRNA 3′UTR, could be responsible for the stability of ζ mRNA and the production of the TCR/CD3 complex, including ζ, using MA5.8 cell mutants and a retrovirus system. In this study, we demonstrated that two conservative sequences (CSs), rather than the AREs, are important for the production of the TCR/CD3 complex, including ζ, by influencing ζ mRNA stability.

## Materials and methods

*RT-PCR.* RT-PCR was performed according to a previously described method [13,22]. Human ζ cDNA with 3′UTR truncations (740, 871, 950, 1070, 1136, 1330, and 1457) were amplified from the PBTs of a normal healthy control subject using primers that were designed as described in Table 1 on line. The primers for amplifying the murine CD3ε and β-actin cDNA were arranged as previously reported [22].

*Cell lines and inhibition of RNA synthesis.* The MA5.8 cells (lacking endogenous ζ expression) were kindly provided by Dr. Takashi Saito and the RetroPack™PT67 (BD Biosciences Clontech, Inc., Palo Alto, CA, USA) was used as the dualtropic packaging cell line. Inhibition of RNA synthesis was performed as previously reported [17].

*Construction of MA5.8 mutants.* MA5.8 mutants were constructed using a previously described method [17,23]. To construct the MA5.8 mutants with ζmRNA 3′UTR deletions, Fragments A, B, C, and D were amplified from the PBTs of a normal healthy control subject using primers that were designed as described in Table 1 on line. Fragment A and B or Fragment C and D were ligated into SalI-cut pDON-AI and were then transfected into RetroPack™PT67 cells.

*Real-time PCR.* The primers and TaqMan probes for human ζ, murine CD3 ε, and murine β-actin were designed as previously reported [23]. Amplification and detection of specific products were performed according to a previously described amplification protocol [17]. Standard curves for the quantification of mRNA were established as previously reported [23].

*Western blot.* Western blot was performed according to a previously described method [9]. The blots were probed with a mouse anti-human ζ mAb (TIA-2) (Coulter Immunology, Hialeah, FL, USA). TIA-2 was visualized using a peroxidase-conjugated anti-mouse IgG (GE Healthcare Bio-Science Corp., Piscataway, NJ, U.S.A.). Biotinylated proteins were detected using streptavidin-peroxidase (Southern Biotechnology Associates, Birmingham, IL, USA). The densities of the specific bands were quantified as index values using the method previously reported [17].

*Flow cytometry.* The flow cytometric analysis procedure has been previously described [17].

*Antibody stimulation and IL-2 quantification.* IL-2 quantification was determined by the method described previously [23].

*Statistical analysis.* Statistical significance was calculated using the Student *t*-test for unpaired data and Statview software (version 4.5; Abacus, Berkeley, CA, USA). A value of $p < 0.05$ was considered statistically significant.

## Results

### *RT-PCR of ζ mRNA with 3′UTR truncations*

To identify the region of the ζ mRNA 3′UTR responsible for the expression of ζ and the TCR/CD3 complex, human ζ cDNA with 3′UTR truncations (740 [605 bp], 871 [736 bp], 950 [815 bp], 1070 [935 bp], 1136 [1001 bp], 1330 [1195 bp], and 1457 [1322 bp]) were amplified from the PBTs of a normal healthy control (Fig. 1 on line).

### *Construction of MA5.8 mutants with ζ mRNA 3′UTR truncations*

ζ cDNA with 3′UTR truncations (740, 871, 950, 1070, 1136, 1330, and 1457) were ligated into pDON-AI, transfected into RetroPack™PT67 cells, and used to infect MA5.8 cells to construct the MA5.8 mutants 740, 871, 950, 1070, 1136, 1330, and 1457, respectively. Also, WT and AS3′UTR mutants were constructed from full-length wild-type human ζcDNA and ζcDNA/as-3′UTR, respectively. The NEG mutant was constructed using pDON-AI without any insert DNA.

### *ζ protein expression in MA5.8 mutants with ζ mRNA 3′UTR truncations*

In a Western blot analysis using an anti-human ζ mAb (TIA-2), the production of the ζ protein by the 740 and 871 mutants was not observed (Fig. 1). The production of ζ protein by the 1136 (15.4 index), 1330 (16.7 index), and 1457 (17.2 index) mutants was almost the same as that of the WT mutant (18.5 index). However, the ζ protein expression of the 950 (11.0 index) and 1070 (10.2 index) mutants was relatively low. Therefore, we concluded that ζ protein expression gaps might exist between the 871 and 950 mutants and between the 1070 and 1136 mutants.

### *Construction of MA5.8 mutants with ζ mRNA 3′UTR deletions*

To determine whether the ζ mRNA 3′UTR regions from +871 to +950 and/or from +1070 to +1136 were responsible for the production of ζ protein, we constructed MA5.8 mutants containing ζ mRNA 3′UTR deletions of these two regions. Fragments A (736 bp, +136 to +871), B (680 bp, +952 to +1631), C (940 bp, +136 to +1075), and D (525 bp, +1107 to +1075) were amplified using RT-PCR (Fig. 2 on line). Then, Fragments A and B and Fragments C and D were ligated into pDON-AI, transfected into RetroPack™PT67 cells, and used to infect MA5.8 cells for the construction of the mutants. As the regions from +871 to +950 and +1070 to +1136 contain CS1 (5′-CCCUGCC UUGGGCCCUCUGGUUUGC-3′) and CS2 (5′-C

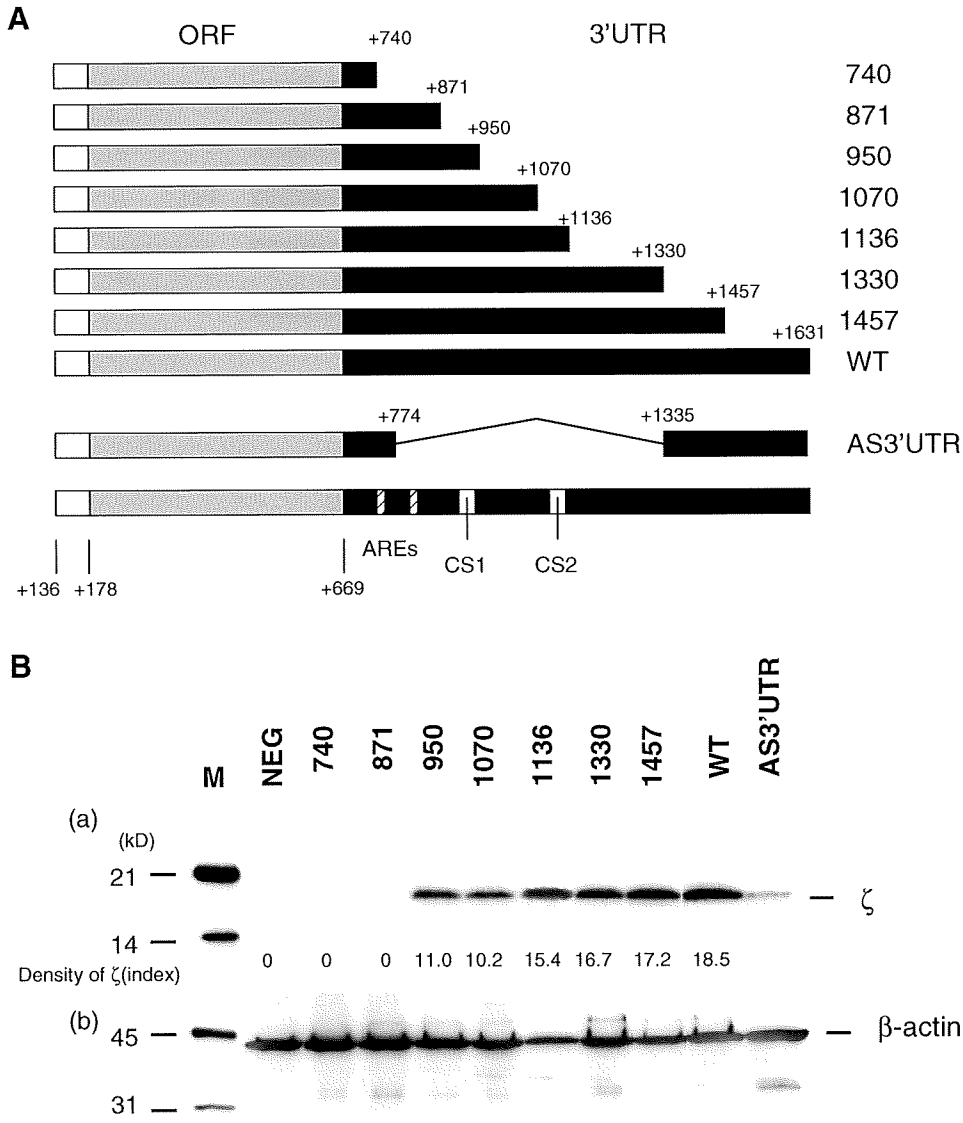


Fig. 1. (A) Scheme of human ζ cDNA with 3′UTR truncations. The shaded bars show the open reading frame (ORF), and the black bars represent the 3′UTR. The white bars show the 5′UTR. AS3′UTR refers to the ζ mRNA/as-3′UTR with a 562-bp deletion (+774 to +1335) in its 3′UTR. The wild-type ζ mRNA 3′UTR contains two A-U rich elements (AREs) (+735 and +803) and two conservative sequences (CSs: CS1, +872 to +951 and CS2, +1076 to +1106). (B) Expression of the ζ protein in MA5.8 mutants with ζ mRNA 3′UTR truncations. Cell lysates from MA5.8 and its mutants (NEG, 740, 871, 950, 1070, 1136, 1330, 1457, WT, and AS3′UTR) were electrophoresed on 15% SDS–polyacrylamide gels using a reducing method and blotted onto a PVDF membrane. The membranes were then incubated with (a) a mouse anti-human ζ mAb (TIA-2) or (b) a hamster anti-mouse β-actin mAb followed by a peroxidase-conjugated anti-mouse IgG. After treatment with chemiluminescence-enhancing reagents, the membranes were visualized on ECL X-ray films, and the densities of the 18-kDa ζ protein bands were quantified as index values.

UCCUGCUGUAAAUUUGGCUUCUGUUGUCAC-3′) (Fig. 3 on line), we defined these MA5.8 mutants as ΔCS1, and ΔCS2 mutants, respectively.

### *ζ protein expression in MA5.8 mutants with ζ mRNA 3′UTR CS deletions*

The expression of the ζ protein in MA5.8 mutants with ζ mRNA 3′UTR CS deletions was analyzed using a Western blot and TIA-2. As shown in Fig. 2, ζ production in both the ΔCS1 and ΔCS2 mutants was slightly higher than that in the AS3′UTR but was significantly lower than that in the WT mutant. From these observations, we concluded that the ζ mRNA 3′UTR regions from +871 to +950 including CS1 and from +1070 to +1136 including CS2 were important for the production of ζ protein.

### *Expression of ζ and TCR/CD3 complex on the cell surfaces of MA5.8 mutants with ζ mRNA 3′UTR CS deletions*

We investigated the expression of ζ and the TCR/CD3 complex on the cell surfaces of these MA5.8 mutants carrying ζ mRNA 3′UTR CS deletions using a FACS analysis. As shown in Fig. 3, the expression of ζ protein on the cell surfaces of both the ΔCS1 (mean channel fluorescence: 73.44) and the ΔCS2 (51.89) was much lower than that of

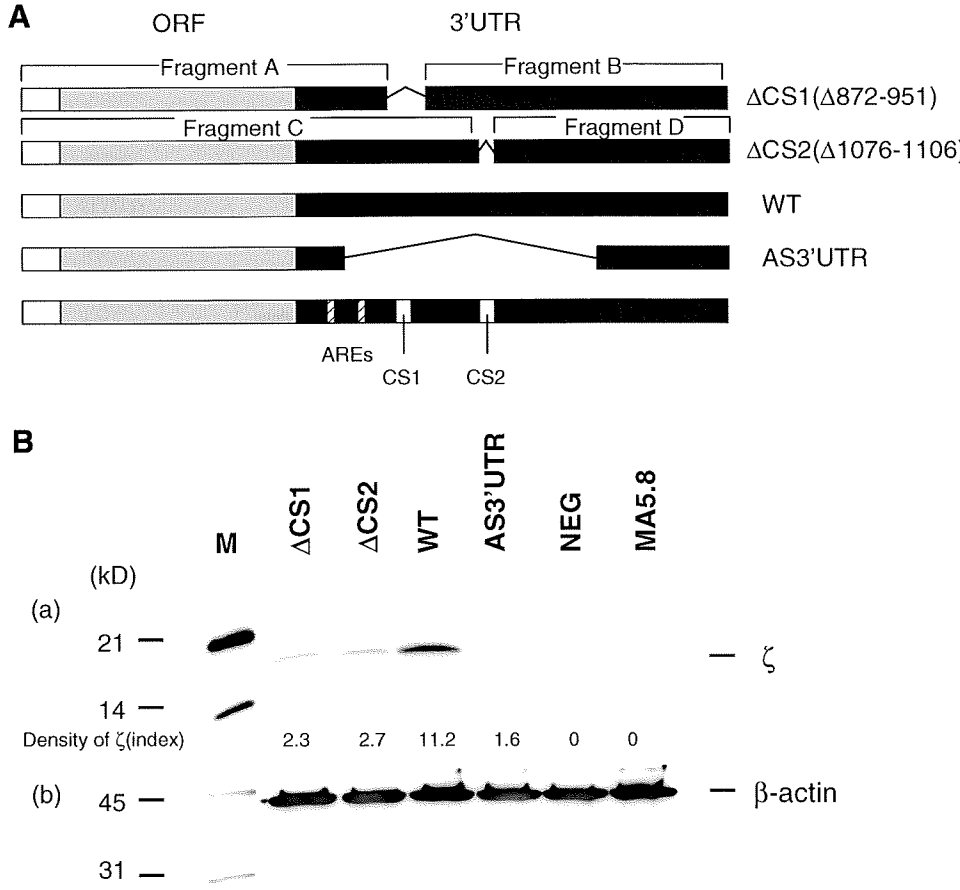


Fig. 2. (A) Scheme of the constructed ζ cDNA with 3′UTR CS deletions. Fragments A and B were ligated into SalI-cut pDON-AI to construct the ζ cDNA with the +872 to +951 deletion (ΔCS1). Fragments C and D were ligated to construct the ζ cDNA with the +1076 to +1106 deletion (ΔCS2). The shaded bars show the ORF, and the black bars represent the 3′UTR. The white bars show the 5′UTR. (B) Expression of the ζ protein in MA5.8 mutants with ζ mRNA 3′UTR CS deletions. Cell lysates from MA5.8 cells and its mutants (ΔCS1, ΔCS2, WT, AS3′UTR, NEG, MA5.8) were electrophoresed on 15% SDS–polyacrylamide gels using a reducing method and blotted onto a PVDF membrane. The membranes were then incubated with (a) a mouse anti-human ζ mAb (TIA-2) or (b) a hamster anti-mouse β-actin mAb followed by a peroxidase-conjugated anti-mouse IgG. After treatment with chemiluminescence-enhancing reagents, the membranes were visualized on ECL X-ray films, and the densities of the 18-kDa ζ protein bands were quantified as index values.

the WT mutant (138.34). Also, the production of TCR/CD3 complex on the cell surfaces of these two MA5.8 mutants with 3′UTR mRNA CS deletions, as estimated by examining CD3 ε expression, was much lower than that of the WT mutants (40.23).

*Decrease in IL-2 production in MA5.8 mutants with ζ mRNA 3′UTR CS deletions*

To evaluate the physiological effect of the ζ mRNA with 3′UTR CS deletions, MA5.8 mutants were stimulated with anti-mouse CD3 ε mAb (Fig. 4 on line). IL-2 production in the WT mutant on Day 1, 2, or 3 after stimulation was compared statistically with that in the ΔCS1, ΔCS2, or NEG mutant. IL-2 production in the ΔCS1 and ΔCS2 mutants on Day 1 to 3 was significantly ($p < 0.001$) lower than that in the WT mutants on Day 1 to 3, respectively. Consequently, IL-2 production in the MA5.8 mutants with ζ mRNA 3′UTR CS deletions seemed to be lower than usual.

*ζ mRNA stability assay*

To evaluate the relationship between the reduction in ζ protein expression and the ζ mRNA with 3′UTR CS deletions, we examined the stability of these ζ mRNA. The WT, ΔCS1, and ΔCS2 mutants were cultured and incubated with actinomycin D, and the cells were collected at 0, 6, 12, 24, and 48 h after drug exposure. ζ, CD3 ε, and β-actin cDNA in the WT, ΔCS1, or ΔCS2 mutants were quantified using real-time PCR.

As shown in Fig. 4, the relative amount of ζ mRNA in the ΔCS1 and ΔCS2 mutants rapidly decreased after treatment with actinomycin D and was significantly ($p < 0.01$) lower than that in the WT mutant over time. On the other hand, no significant decrease in the relative amount of CD3 ε mRNA, compared with ζ mRNA, was observed over time after actinomycin D treatment in both the ΔCS1 and ΔCS2 mutants. From these observations, we can conclude that the ζ mRNA with the 3′UTR CS deletions was less stable than the

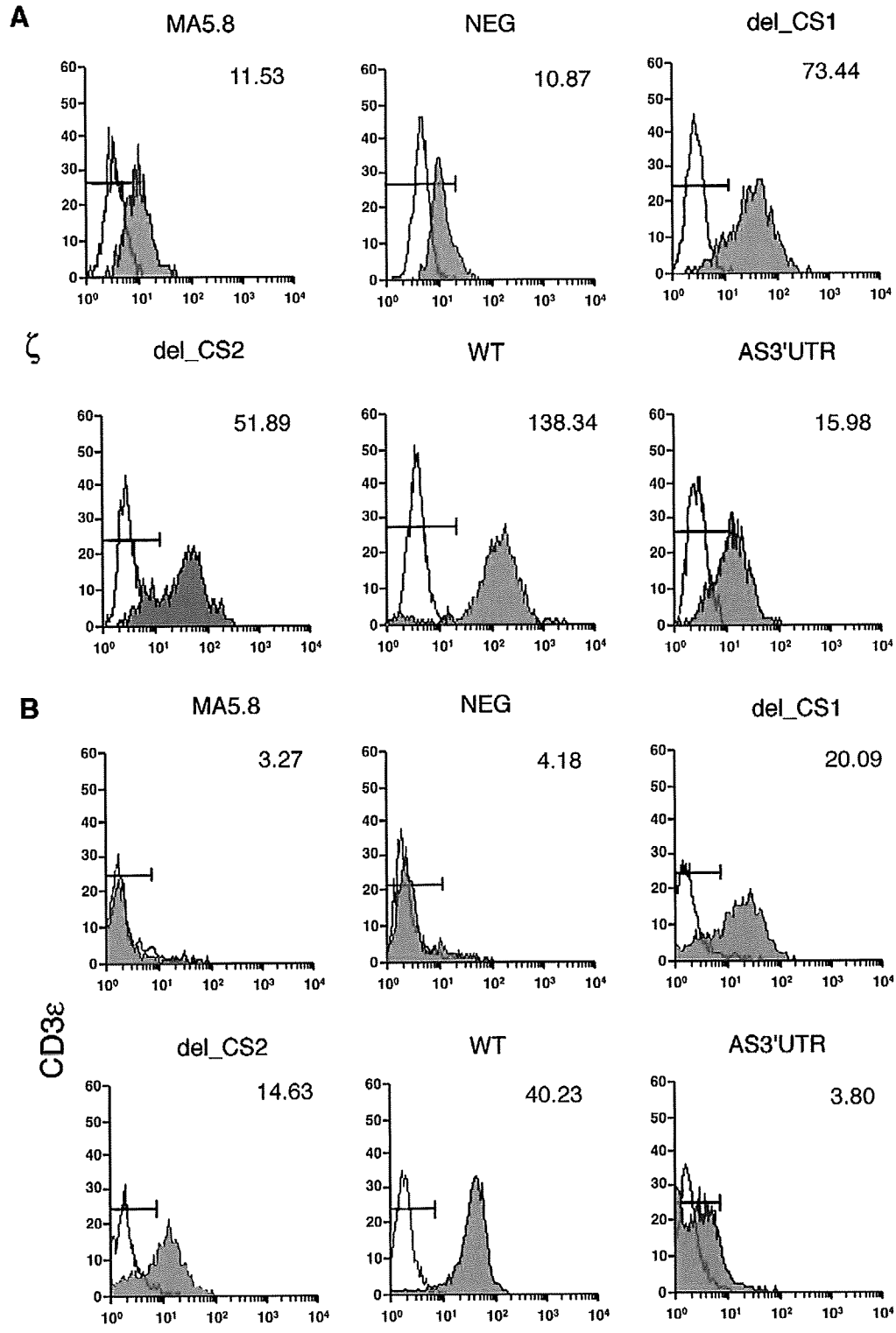


Fig. 3. Flow cytometric analysis of the MA5.8 mutants with ζ mRNA 3′UTR CS deletions. The surface expressions of (A) the ζ protein and (B) the TCR/CD3 complex on MA5.8 cells and its mutants [NEG, del_CS1(ΔCS1), del_CS2(ΔCS2), WT, AS3′UTR] were quantified using FITC-conjugated anti-human ζ mAb (TIA-2) (black profiles) and FITC-conjugated anti-mouse CD3 ε mAb (145-2C11) (black profiles), respectively. An FITC-conjugated mouse anti-human IgG (open profiles in (A)) or FITC-conjugated Armenian hamster anti-mouse IgG (open profiles in (B)) was used as the negative control. The mean channel fluorescence value is indicated within the figures at the *top right*.

wild-type ζ mRNA in the WT mutants. On the other hand, the stability of the CD3 ε mRNA was similar in the MA5.8 mutants with ζ mRNA 3′UTR CS deletions and the WT mutant.

## Discussion

In this study, the down-regulation of the ζ protein in the MA5.8 mutants with ζ mRNA 3′UTR CS deletions (ΔCS1,

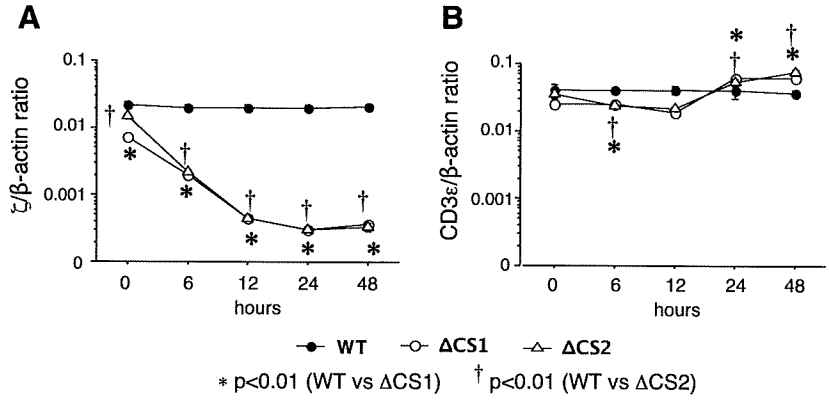


Fig. 4. Decrease in ζ mRNA stability in MA5.8 mutants with ζ mRNA 3′UTR CS deletions. MA5.8 mutants (WT, ΔCS1, ΔCS2) were cultured and incubated with 4 μg/mL of actinomycin D in the culture media. The samples were collected at various time points, and the mRNA was subsequently extracted and converted to whole cDNA. (A) ζ and (B) CD3 ε cDNA were quantified using real-time PCR and were evaluated as the ratio against β-actin cDNA. Each experiment was performed in triplicate. The bars show the mean plus or minus the SD. $^{*}p < 0.01$ for ΔCS1 (open circles) versus WT (closed circles). $^{\dagger}p < 0.01$ for ΔCS2 (open triangles) versus WT (closed circles).

ΔCS2) as confirmed using Western blot and FACS analyses, suggested that the production of the ζ protein was low in the absence of these two CSs of the ζ mRNA 3′UTR. The expression of the TCR/CD3 complex was also decreased in MA5.8 mutants without these regions, as shown by a FACS analysis.

We and other groups have previously reported that the ζ mRNA/as-3′UTR, which is predominantly expressed in SLE T cells, is less stable than the ζ mRNA/w-3′UTR and may be responsible for the reduced expression of the TCR/CD3 complex including ζ protein in SLE T cells [17,18]. Therefore, we examined the stability of ζ mRNA to investigate the reduction in ζ protein expression in the MA5.8 mutants expressing ζ mRNA 3′UTR CS deletions. From our observations, ζ mRNA with 3′UTR CS deletions appeared to be less stable and more easily degraded than ζ mRNA/w-3′UTR. Conceivably, the reduction in the stability of ζ mRNA with 3′UTR CS deletions may lead to a reduction in the expression of the intracellular ζ homodimer, leading to the absence of the expression of the TCR/CD3 complex on the cell surface. Also, the reduction in IL-2 production in these MA5.8 mutants revealed that the signal from the TCR was not transduced into the cytoplasm by anti-CD3 ε antibody stimulation in these mutants. AREs (AUUUA motifs) bind to *trans*-acting proteins and participate in either the stabilization or destabilization of the transcripts. Two AREs, which have been reported to be responsible for the stability of ζ mRNA [24], are located at positions +735 and +803 of ζ mRNA. In our study, however, both MA5.8 mutants 740 and 871 with ζ mRNA 3′UTR truncations did not express ζ protein and MA5.8 mutants ΔCS1 and ΔCS2, which contained the AREs, were easily degraded. From these observations, there might remain the possibility that the AREs at +735 and +803 are not related to the production of ζ protein or the stability of ζ mRNA. However, this possibility can be demonstrated only by using a deletion mutant lacking AREs but containing CSs. This study is now under way in our laboratory. In conclusion, the present study suggests that the regions between +872 and +951 and between +1076 and +1106 in the ζ mRNA 3′UTR are critical for ζ mRNA stability. Gramolini et al. reported that a 171-bp region in the 3′UTR of utrophin mRNA regulates utrophin mRNA stability, since the half-life of utrophin mRNA without this region is much shorter than that of the wild-type mRNA [25]. Also, Akgül et al. reported a 59-nucleotide (nt) pentobarbital-responsive element in the 3′UTR of *Drosophila* glutathione-*S*-transferase D21 (gstD21) mRNA that regulates the stability of gstD21 mRNA [26].

Interestingly, CS1 and CS2 are conserved in several mammals (Fig. 3 on line). Xie et al. reported that 106 CSs in mRNA 3′UTR are involved in post-transcriptional regulation and that half of these CSs are related to AREs, while the other half of the CSs that do not contain AREs are associated with microRNAs [27]. Since both the CS1 and CS2 in the ζ mRNA 3′UTR do not include AREs (AUUUA motifs), some miRNAs might influence the stability of the ζ mRNA by binding to the CS1 and CS2 in ζmRNA 3′UTR regions that do not contain AREs. This possibility is now being studied in our laboratory.

## Acknowledgment

This work was supported by Grants-in-Aid for Scientific Research (C) from the Ministry of Education, Science and Culture, Japan.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.12.145.

## References


[1] J.A. Mills, Systemic lupus erythematosus, N. Engl. J. Med. 30 (1994) 1871–1879.

[2] D. Zipris, A.H. Lazarus, A.R. Crow, M. Hadzija, T.L. Delovitch, Defective thymic T cell activation by concanavalin A and anti-CD3 in autoimmune nonobese diabetic mice. Evidence for thymic T cell anergy that correlates with the onset of insulitis, J. Immunol. 146 (1991) 3763–3771.

[3] M.A. Berman, C.I. Sandborg, Z. Wang, K.L. Imfeld, F. Zaldivar Jr., V. Dadufalza, B.A. Buckingham, Decreased IL-4 production in new onset type I insulin-dependent diabetes mellitus, J. Immunol. 157 (1996) 4690–4696.

[4] S.B. Wilson, S.C. Kent, K.T. Patton, T. Orban, R.A. Jackson, M. Exley, S. Porcelli, D.A. Schatz, M.A. Atkinson, S.P. Balk, J.L. Strominger, D.A. Hafler, Extreme Th1 bias of invariant Valpha24JalphaQ T cells in type 1 diabetes, Nature 39 (1998) 177–181.

[5] A.K. Dayal, G.M. Kammer, The T cell enigma in lupus, Arthritis Rheum. 39 (1996) 23–33.

[6] S. Sierakowski, E.J. Kucharz, R.W. Lightfoot, J.S. Goodwin, Impaired T-cell activation in patients with systemic lupus erythematosus, J. Clin. Immunol. 9 (1989) 469–476.

[7] T. Takeuchi, M. Pang, K. Amano, J. Koide, T. Abe, Reduced protein tyrosine phosphatase (PTPase) activity of CD45 on peripheral blood lymphocytes in patients with systemic lupus erythematosus (SLE), Clin. Exp. Immunol. 109 (1997) 20–26.

[8] N. Sakaguchi, T. Takahashi, H. Hata, T. Nomura, T. Tagami, S. Yamazaki, T. Sakihama, T. Matsutani, I. Negishi, S. Nakatsuru, S. Sakaguchi, Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice, Nature 426 (2003) 454–460.

[9] T. Takeuchi, K. Tsuzaka, M. Pang, K. Amano, J. Koide, T. Abe, TCRζ chain lacking exon 7 in two patients with systemic lupus erythematosus, Int. Immunol. 10 (1998) 911–921.

[10] S.N. Liossis, X.Z. Ding, G.J. Dennis, G.C. Tsokos, Altered pattern of TCR/CD3-mediated protein-tyrosyl phosphorylation in T cells from patients with systemic lupus erythematosus. Deficient expression of the T cell receptor ζ chain, J. Clin. Invest. 101 (1998) 1448–1457.

[11] V. Brundula, L.J. Rivas, A.M. Blasini, M. Paris, S. Salazar, I.L. Stekman, M.A. Rodriguez, Diminished levels of T cell receptor ζ chains in peripheral blood T lymphocytes from patients with systemic lupus erythematosus, Arthritis Rheum. 42 (1999) 1908–1916.

[12] M. Pang, Y. Setoyama, K. Tsuzaka, K. Yoshimoto, K. Amano, T. Abe, T. Takeuchi, Defective expression and tyrosine phosphorylation of the T cell receptor ζ chain in peripheral blood T cells from systemic lupus erythematosus patients, Clin. Exp. Immunol. 129 (2002) 160–168.

[13] K. Tsuzaka, T. Takeuchi, N. Onoda, M. Pang, T. Abe, Mutations in T cell receptor ζ chain mRNA of peripheral T cells from systemic lupus erythematosus, J. Autoimmun. 11 (1998) 381–385.

[14] K. Tsuzaka, N. Onoda, K. Yoshimoto, Y. Setoyama, K. Suzuki, M. Pang, T. Abe, T. Takeuchi, T-cell receptor ζ mRNA with alternatively spliced 3′ untranslated region is generated predominantly in the peripheral blood T cells of systemic lupus erythematosus patients, Mod. Rheumatol. 12 (2002) 167–173.

[15] M.P. Nambiar, E.J. Enyedy, V.G. Warke, S. Krishnan, G. Dennis, M. Kammer, G.C. Tsokos, Polymorphisms/mutations of TCR ζ chain promoter and 3′ untranslated region and selective expression of TCR ζ chain with an alternatively spliced 3′ untranslated region in patients with systemic lupus erythematosus, J. Autoimmun. 16 (2001) 133–142.

[16] K. Tsuzaka, K. Nozaki, C. Kumazawa, K. Shiraishi, Y. Setoyama, K. Yoshimoto, K. Suzuki, T. Abe, T. Takeuchi, DNA microarray gene expression profile of T cells with the splice variants of TCRζ mRNA observed in SLE, J. Immunol. 176 (2006) 949–956.

[17] K. Tsuzaka, I. Fukuhara, Y. Setoyama, K. Yoshimoto, K. Suzuki, T. Abe, T. Takeuchi, TCRζ mRNA with an alternatively spliced 3′ untranslated region detected in SLE patients leads to the downregulation of TCRζ and TCR/CD3 complex, J. Immunol. 171 (2003) 2496–2503.

[18] B. Chowdhury, C.G. Tsokos, S. Krishnan, J. Robertson, C.U. Fisher, R.G. Warke, V.G. Warke, M.P. Nambiar, G.C. Tsokos, Decreased stability and translation of T cell receptor ζ mRNA with an alternatively spliced 3′-untranslated region contribute to ζ chain down-regulation in patients with systemic lupus erythematosus, J. Biol. Chem. 280 (2005) 18959–18966.

[19] G. Bassell, R.H. Singer, mRNA and cytoskeletal filaments, Curr. Opin. Cell Biol. 9 (1997) 109–115.

[20] K.C. Tsai, V.V. Cansino, D.T. Kohn, R.L. Neve, N.I. Perrone-Bizzozero, Post-transcriptional regulation of the GAP-43 gene by specific sequences in the 3′ untranslated region of the mRNA, J. Neurosci. 17 (1997) 1950–1958.

[21] J.L. Veyrune, J. Hesketh, J.M. Blanchard, 3′ Untranslated regions of c-myc and c-fos mRNAs: multifunctional elements regulating mRNA translation, degradation and subcellular localization, Prog. Mol. Subcell. Biol. 18 (1997) 35–63.

[22] J.P. Jensen, D. Hou, M. Ramsburg, A. Taylor, M. Dean, A.M. Weissman, Organization of the human T cell receptor ζ/η gene and its genetic linkage to the FcγRII-FcγRIII gene cluster, J. Immunol. 148 (1992) 2563–2571.

[23] K. Tsuzaka, Y. Setoyama, K. Yoshimoto, K. Shiraishi, K. Suzuki, T. Abe, T. Takeuchi, A splice variant of the TCR ζ mRNA lacking exon 7 leads to the down-regulation of TCR ζ, the TCR/CD3 complex, and IL-2 production in SLE T cells, J. Immunol. 174 (2005) 3518–3525.

[24] B. Chowdhury, S. Krishnan, C.G. Tsokos, J.W. Robertson, C.U. Fisher, M.P. Nambiar, G.C. Tsokos, Stability and translation of TCR ζ mRNA are regulated by the adenosine-uridine-rich elements in splice-deleted 3′ untranslated region of ζ -chain, J. Immunol. 177 (2006) 8248–8257.

[25] A.O. Gramolini, G. Belanger, B.J. Jasmin, Distinct regions in the 3' untranslated region are responsible for targeting and stabilizing utrophin transcripts in skeletal muscle cells, J. Cell Biol. 154 (2001) 1173–1183.

[26] B. Akgül, C-PD. Tu, Regulation of mRNA stability through a pentobarbital-responsive element, Arch. Biochem. Biophys. 459 (2006) 143–150.

[27] X. Xie, J. Lu, E.J. Kulbokas, T.R. Golub, V. Mootha, K. Lindblad-Toh, E.S. Lander, M. Kellis, Systematic discovery of regulatory motifs in human promoters and 3′UTRs by comparison of several mammals, Nature 434 (2005) 338–345.

*Circ J* 2008; **72:** 304–308

# Availability of Cardio-Ankle Vascular Index (CAVI) as a Screening Tool for Atherosclerosis

Koichiro Kadota, MD; Noboru Takamura, MD*; Kiyoshi Aoyagi, MD*; Hironori Yamasaki, MD‡; Toshiro Usa, MD**; Mio Nakazato, MD†; Takahiro Maeda, MD†; Mitsuhiro Wada, PhD††; Ken-ichiro Nakashima, PhD††; Koh Abe, MD; Fuminao Takeshima, MD; Yoshiyuki Ozono, MD

**Background** A novel index, the cardio-ankle vascular index (CAVI), which reflects the stiffness of the aorta, femoral artery, and tibial artery, was recently developed by measuring brachial–ankle pulse wave velocity and blood pressure.

**Methods and Results** In the present study 1,014 Japanese adults from the general population were screened to clarify the correlation between CAVI and other existing markers related to atherosclerosis, including carotid intima–media thickness (CIMT) and homocysteine (HCY). CAVI was strongly associated with age in both men and women. After adjustment for age and sex, CAVI was correlated with systolic and diastolic blood pressures. In addition, CAVI was significantly correlated with total cholesterol hemoglobin $A_{1c}$ and total HCY, as well as CIMT.

**Conclusion** CAVI is an appropriate screening tool for atherosclerosis, but further studies are needed to establish a convenient and effective screening system using it. (*Circ J* 2008; **72:** 304–308)

**Key Words:** Atherosclerosis; Cardio-ankle vascular index (CAVI); Carotid intima–media thickness (CIMT); Homocysteine (HCY)

Appropriate assessment and prevention of cardiovascular disease (CVD) is 1 of the most important medical tasks worldwide. The World Health Organization has projected that the worldwide annual number of CVD Disability-adjusted life years (DAILYs: a measure that combines years of potential life lost due to premature death with years of productive life lost due to disability) will reach 153 million by 2010, 169 million by 2020, and 187 million by 2030, and that CVD death will reach 18.1 million by 2010, 20.5 million by 2020, and 24.2 million by 2030! Because atherosclerosis is a major contributor to CVD, accounting for much of the mortality and morbidity[2] the establishment of effective and accurate strategies for screening is very important.

Recent technological advances in medical equipment have allowed noninvasive assessment of atherosclerosis in its early stages[3] High-resolution B-mode ultrasonography provides a noninvasive method of quantifying arterial wall thickening, and it has been shown that the carotid intima–media thickness (CIMT) is a strong predictor of CVD[4] In addition, pulse wave velocity (PWV) has been developed as a noninvasive clinical index of aortic stiffness[5] and reportedly predicts cardiovascular events and all-cause mortality in hypertensive patients and in the general population[6,7] However, measurement of classic PWV is technically difficult and has low reproducibility, and the data obtained vary significantly between institutions and operators because of the technical difficulty[8] In order to overcome these problems, the brachial-ankle PWV (baPWV), which is simple to measure and has high reproducibility, was developed and has been shown to predict the presence of coronary disease[9] as well as correlating with CIMT![10] However, there are problems, because this method is influenced by changes in blood pressure (BP) during the examination and by the autonomic nervous system[8,11]

A novel index, the cardio-ankle vascular index (CAVI), which reflects the stiffness of the aorta, femoral artery, and tibial artery and involves measurement of baPWV and BP, was recently developed[8,12] CAVI is essentially independent of changes in BP during examination, but shows a strong correlation with systolic BP (SBP)![12] Although there are several reports of the evaluation of CAVI in patients on hemodialysis![12] those with hyperglycemia[13] and those who had undergone heart transplantation![14] there are few on the evaluation of CAVI as a screening tool for atherosclerosis in the general population![11] and none about the correlation between CAVI and CIMT. Although we recently evaluated the availability of CAVI as a screening tool for atherosclerosis in young Japanese adult (mean age 21.4 years, range 18–31 years), no correlation was observed with other existing markers, including CIMT![15]

Because we hypothesized that CAVI might be a useful screening tool for atherosclerosis in the general adult population, in the present study we screened a representative sample to evaluate the correlation between CAVI and other existing markers related to atherosclerosis, including CIMT and homocysteine (HCY), which is considered to be an inde-

*(Received July 5, 2007; revised manuscript received October 5, 2007; accepted October 9, 2007)*

*Departments of General Medicine, *Public Health, **Endocrinology and Metabolism, Unit of Translational Medicine, †Island and Community Medicine, ††Clinical Pharmacy, Nagasaki University Graduate School of Biomedical Sciences and ‡Health Center, Nagasaki University, Nagasaki, Japan*

Mailing address: Noboru Takamura, MD, Department of Public Health, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan. E-mail: takamura@nagasaki-u.ac.jp

**Table 1 Characteristics of the Study Participants**

| | Men (n=242) | Women (n=772) | All participants (n=1,014) |
|---|---|---|---|
| Age (years) | 64.4±11.0 | 61.9±10.7 | 62.5±10.8 |
| BMI (kg/m²) | 23.4±3.0 | 22.8±3.2 | 23.0±3.1 |
| WC (cm) | 84.9±8.8 | 80.5±10.1* | 81.5±10.0 |
| SBP (mmHg) | 142±20 | 141±21 | 141±21 |
| DBP (mmHg) | 87±11 | 84±11 | 85±11 |
| TC (g/L) | 2.0±0.3 | 2.2±0.3 | 2.1±0.4 |
| TG (g/L) | 1.4±0.8 | 1.2±0.6** | 1.3±0.7 |
| HDL-C (g/L) | 0.54±0.15 | 0.62±0.15 | 0.60±0.15 |
| LDL-C (g/L) | 1.1±0.3 | 1.3±0.3 | 1.3±0.3 |
| HbA1c (%) | 5.1±0.8 | 5.0±0.6** | 5.0±0.6 |
| Creatinine (mg/L) | 10.9±2.3 | 8.4±1.6** | 9.0±2.1 |
| UA (g/L) | 64±15 | 49±12** | 53±15 |
| tHCY (μmol/L) | 10.3±4.7 | 8.3±3.8** | 8.7±4.1 |
| CIMT (mm) | 0.8±0.2 | 0.7±0.2** | 0.7±0.2 |
| CAVI | 8.5±1.4 | 8.0±1.3 | 8.1±1.3 |

*Values are mean±standard deviation. *p<0.05 and **p<0.01 vs men. BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total choles-terol; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; HbA1c, hemoglobin A1c; UA, uric acid; tHCY, total homocysteine; CIMT, carotid intima media-thickness; CAVI, cardio-ankle vascular index.*

pendent risk factor for atherosclerosis![16]

## Methods

*Subjects*

Prior to this study, ethical approval was obtained from the Special Committee of Nagasaki University (project registration number 0501120073). The study was conducted during a medical screening program for members of the general population aged over 40 years, living in Goto city (total population was 44,874 in 2007), Nagasaki Prefecture, Japan. The data were collected by the staff of Nagasaki University, in cooperation with that of Goto city. After obtaining informed consent, we enrolled 1,139 Japanese adults (285 men, 854 women); 38 participants with an apparent past or present history of cerebral infarction or hemorrhage or ischemic heart disease, and 71 participants with insufficient data were excluded from the study, as were 16 participants who showed severe hypertriglycemia (>4.0 g/L). Finally, 1,014 participants were included for further analysis (242 men, 772 women).

*Data Collection and Laboratory Measurements*

Height and weight were measured, and body mass index (BMI: kg/m²) was calculated as an index of obesity. SBP and diastolic BP (DBP) were recorded at rest, simultaneously with the measurement of CAVI.

Blood samples were collected from each participant after overnight fast. Serum and plasma were separated and stored at –20°C and –80°C, respectively, until assay. Serum concentrations of total cholesterol (TC), triglyceride (TG), and high-density lipoprotein-cholesterol (HDL-C) were measured by standard laboratory procedures, and low-density lipoprotein-cholesterol (LDL-C) was calculated by the Friedwald equation. In addition to fasting blood sugar and hemoglobin A1c (HbA1c), serum creatinine and uremic acid (UA) were measured by standard laboratory procedures. Plasma total HCY (tHCY) levels were measured using a high-performance liquid chromatographic method developed by Garcia and Apitz-Castro![17]

**Table 2 Simple Correlation Analysis of CAVI and Other Variables**

| | Men | Women | All participants |
|---|---|---|---|
| BMI | –0.021 | 0.003 | 0.01 |
| WC | 0.062 | 0.16** | 0.16** |
| SBP | 0.21** | 0.31** | 0.28** |
| DBP | 0.14* | 0.17** | 0.18** |
| TC | 0.039 | 0.10** | 0.042 |
| TG | –0.036 | 0.14** | 0.11** |
| HDL-C | 0.031 | –0.070 | –0.080* |
| LDL-C | 0.039 | 0.108** | 0.050 |
| HbA1c | 0.22** | 0.18** | 0.20** |
| Creatinine | 0.12 | 0.14** | 0.20** |
| UA | 0.020 | 0.088* | 0.13** |
| tHCY | 0.11 | 0.11** | 0.14** |
| CIMT | 0.32** | 0.37** | 0.37** |

*p<0.05 and **p<0.01.
Abbreviations as in Table 1.

*Measurement of CAVI and CIMT*

CAVI was recorded using a VaseraVS-1000 vascular screening system (Fukuda Denshi, Tokyo, Japan) with the participant resting in a supine position. The principles underlying CAVI have been described by Yambe et al![8] ECG electrodes are placed on both wrists, a microphone for detecting heart sounds is placed on the sternum, and cuffs are wrapped around both the arms and ankles. After automatic measurements, obtained data were analyzed using VSS-10 software (Fukuda Denshi), and the values of right and left CAVI were calculated. Averages of the right and left CAVI were used for analysis.

Measurement of CIMT by ultrasonography of the left and right carotid arteries was performed by 2 medical doctors (N.T. and M.N.), using a LOGIC Book XP with a 10-MHz linear array transducer (GE Medical Systems, Milwaukee, WI, USA). A detailed protocol has been described elsewhere![18] Averages of left and right CIMT were calculated and used in the analysis. Intra-observer variation of CIMT (N.T., n=32) was 0.91 (p<0.01), and interobserver variation (N.T. vs M.N., n=41) was 0.78 (p<0.01).

*Statistical Analysis*

Results are expressed as mean±standard deviation. Differences between women and men in the laboratory values were evaluated using the t-test. Multiple linear regression analysis was performed to evaluate CAVI and other existing parameters adjusted for confounding factors (age, sex, waist circumference (WC), SBP, TG, HDL-C, HbA1c, creatinine, UA, tHCY and CIMT, Table 1), which showed significant correlations with CAVI by simple linear regression analysis in all subjects. Although DBP also showed a significant correlation with CAVI, it was not analyzed as a confounding factor, because of intercorrelation with SBP (r=0.80, p<0.01). Because TG and tHCY levels had a skewed distribution, logarithmic transformation was performed for the simple correlation analysis and multiple linear regression analysis. Probability values less than 0.05 were considered indicative of statistical significance. All statistical analyses were performed using SPSS v11.0 software (SPSS Japan, Tokyo, Japan).

## Results

Characteristics of the study participants are shown in Table 1. The average age of the men was significantly older

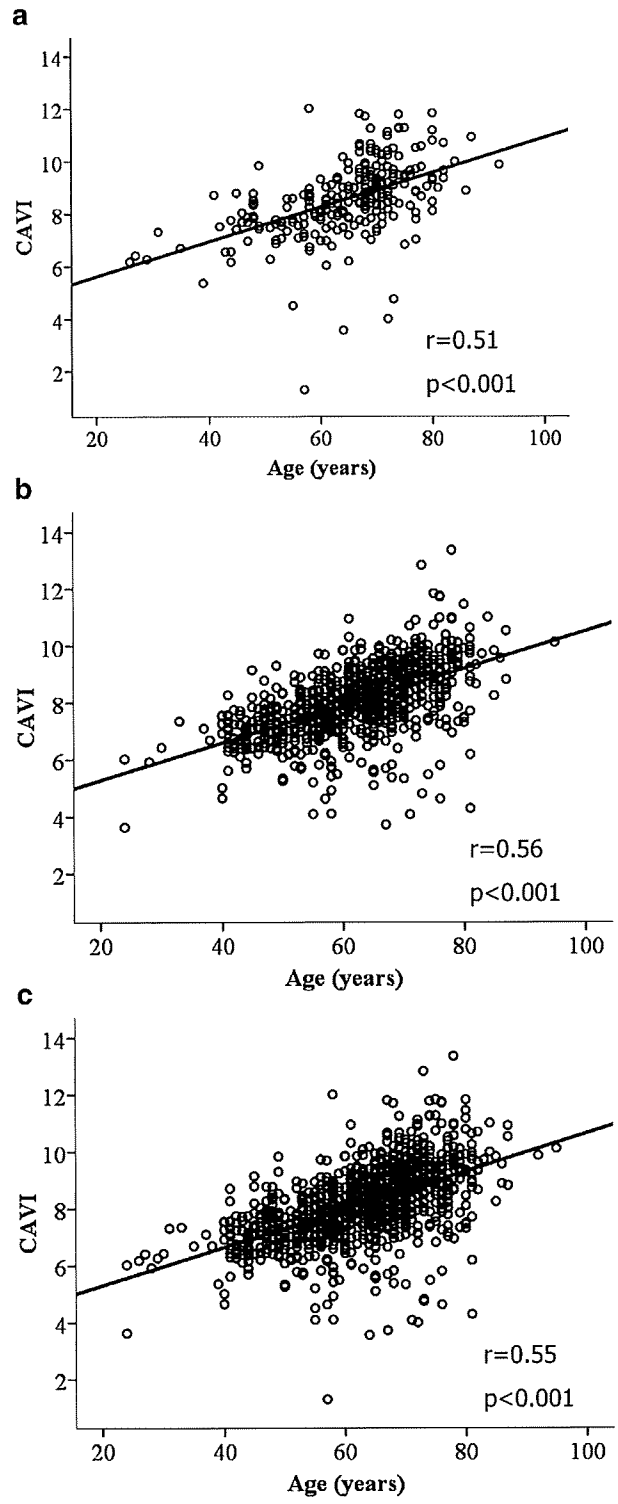


Fig 1. Relationship between cardio-ankle vascular index (CAVI) and age in (a) men (n=242), (b) women (n=772), and (c) all participants (n=1,014).

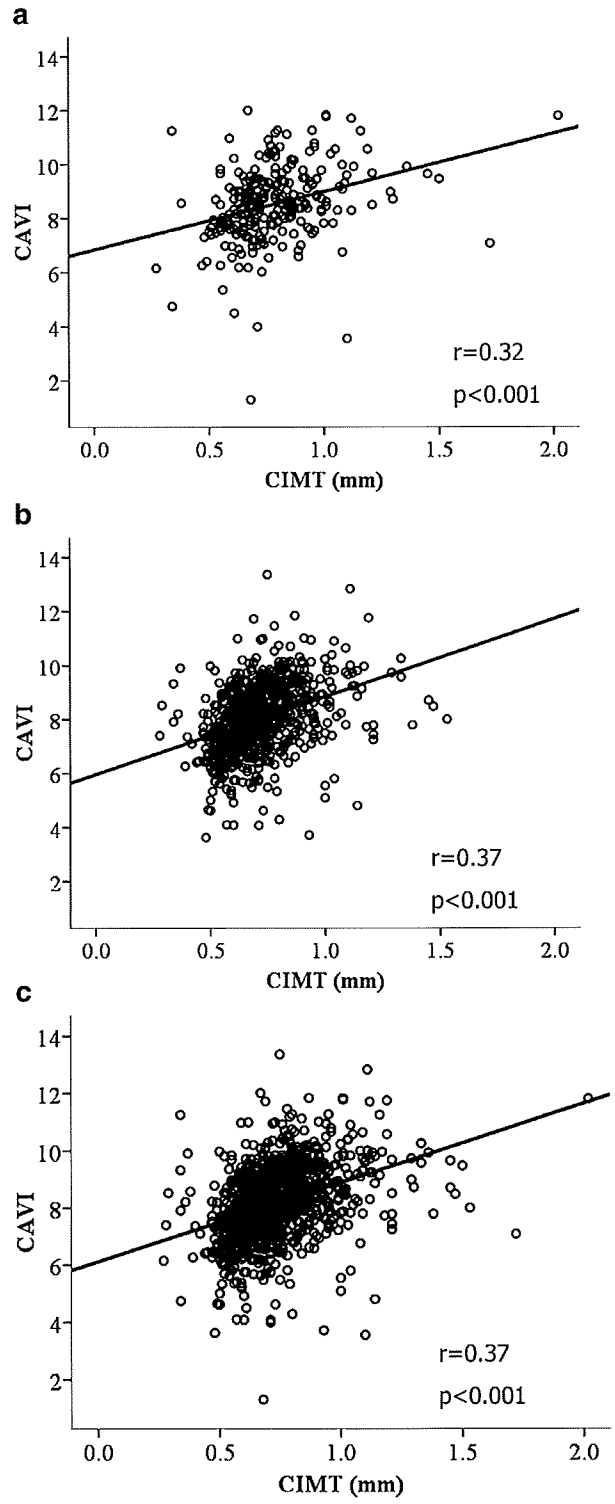


Fig 2. Relationship between cardio-ankle vascular index (CAVI) and carotid intima–media thickness (CIMT) in (a) men, (b) women, and (c) all participants.

than that of the women (64.2±11.1 years vs 61.9±10.7 years, $p<0.01$). Other than SBP and $HbA_{1c}$, all parameters showed significant differences between men and women.

By simple linear regression analysis, CAVI was significantly correlated with age in men ($r=0.51$, $p<0.001$), women ($r=0.56$, $p<0.001$), and all participants ($r=0.55$, $p<0.001$, Fig 1). It was also significantly correlated with CIMT in men ($r=0.32$, $p<0.001$) and in women ($r=0.37$, $p<0.001$) (Fig 2). Furthermore, CAVI was significantly correlated with SBP, DBP, $HbA_{1c}$ and CIMT in men and with WC, SBP, DBP, TC, TG, LDL-C, $HbA_{1c}$, creatinine, UA, tHCY, and CIMT in women. In all participants, CAVI was signifi-