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Telomere shortening in peripheral blood leukocytes of systemic sclerosis

전신경화증의 말초혈액 백혈구에서 텔로미어 길이 분석

2012년 8월

서울대학교 대학원

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Telomere shortening in peripheral blood leukocytes of systemic sclerosis

by
Ji Ah Park

A thesis submitted to the Department of Biomedical Sciences in partial fulfillment of the requirement of the Degree of Master in Biomedical Sciences at Seoul National University College of Medicine

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Approved by Thesis Committee:

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ABSTRACT

Telomeres are critical for preserving the function of the immune system. Although telomere erosion, a marker of immunosenescence, has been well established in autoimmune diseases, such as, rheumatoid arthritis and systemic lupus erythematosus, telomere length abnormalities in systemic sclerosis (SSc) are still controversial. I investigated immunosenescence status in SSc by measuring telomere lengths in different subsets of T cells, B cells and neutrophils.

DNA of peripheral blood leukocytes (PBLs) was extracted from the whole blood of 88 SSc patients and 88 age- and sex- matched healthy controls. For subpopulation analysis, peripheral blood mononuclear cells (PBMCs) were isolated from 20 patients and 20 controls. After isolating total CD4 T, CD8 T, and B cells, subpopulations of T cells (naive, CCR7+CD45RA+; central memory, CCR7+CD45RA-; effector memory, CCR7-CD45RA-; terminal effector memory, CCR7-CD45RA+) and B cells (naive, CD19+CD27-; memory, CD19+CD27+) were further isolated using FACSAria. Neutrophils were isolated using RBC lysis buffer from granulocyte fraction. DNA was extracted from the isolated cells. Telomere lengths were measured in PBLs, different subsets of T and B cells, and in neutrophils by monochrome multiplex quantitative PCR (MMQPCR). Relative telomere to single copy gene ratios (relative T/S ratios) were used to represent telomere length.

Telomere lengths progressively shortened with aging in PBLs from SSc patients (R^2 = 0.056, p = 0.026) and controls (R^2 = 0.077, p = 0.009). For PBLs, no significant

difference in telomere lengths were found between SSc patients and controls (mean ± SD, 1.571 ± 0.439 in SSc patients vs 1.613 ± 0.400 in controls, p = 0.504 by ANCOVA). However, telomere lengths were significantly shorter in the CD4 and in the CD8 T cells of SSc patients than in those of controls (1.308 ± 0.553 *vs.* 1.956 ± 1.146, p = 0.024; 0.996 ± 0.342 *vs.* 1.389 ± 0.643, p = 0.013, respectively). When subpopulations of T cells were analyzed, telomere lengths were found to be shorter in the naive T cells (1.513 ± 0.657 *vs.* 2.320 ± 1.030, p = 0.001), central memory CD4 T cells (1.084 ± 0.369 *vs.* 1.686 ± 0.882, p = 0.007), and the effector memory CD8 T cells (0.794 ± 0.342 *vs.* 1.134 ± 0.533, p = 0.030) of SSc patients than in those of controls. For B cells, both naive and memory B cells of SSc patients had shorter telomere lengths than those of controls (1.291 ± 0.566 *vs.* 2.373 ± 1.151, p = 0.001; 1.534 ± 1.098 *vs.* 3.331 ± 2.330, p = 0.005, respectively). Unlike other cells, the neutrophils of SSc patients tended to have longer telomere lengths than those of controls (p = 0.068). I found no significant difference between telomere lengths according to clinical subsets and autoantibody status, other than that telomere lengths in effector memory CD8 T cells were shorter in patients with anti-Scl-70 antibody (p = 0.037). These findings suggest that telomere shortening and premature immunosenescence of T and B cells may contribute to the pathogenesis of SSc.

Keywords: telomere, telomere shortening, immunosenescence, systemic sclerosis

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CONTENTS

LIST OF TABLES AND FIGURES

LIST OF ABBREVIATIONS

SSc: systemic sclerosis

SLE: systemic lupus erythematosus

RA: rheumatoid arthritis

PBLs: peripheral blood leukocytes

dcSSc: diffuse cutaneous systemic sclerosis

lcSSc: limited cutaneous systemic sclerosis

TRF: terminal restriction fragment

FISH: fluorescence *in situ* hybridization

MMQPCR: monochrome multiplex quantitative polymerase chain reaction

RNP: ribonucleoprotein

ANA: antinuclear antibody

PBMCs: peripheral blood mononuclear cells

PBS: phosphate-buffered saline solution

RT: room temperature

MACS: magnetic cell sorting

CD4 T_{Naive}: naive CD4 T

CD4 T_{CM}: central memory CD4 T

CD4 T_{EM}: effector memory CD4 T

CD8 $T_{Naive:}$ naive CD8 T

CD8 T_{EM}: effector memory CD8 T

CD8 T_{TEM}: terminal effector memory CD8 T

T/S ratio: telomere to single copy gene ratio

IL-4: interleukin-4

IL-13: interleukin-13

TCR: T-cell receptor

MWM: molecular weight marker

HC: healthy controls

INTRODUCTION

Telomeres are specialized DNA capping structures that are located at the physical ends of eukaryotic linear chromosomes and consist of tandem repeats of G-rich DNA sequences (TTAGGG) (Blackburn, 1991). Telomeres are essential for the stabilization of chromosomal architecture, as they protect the ends of chromosomes from aberrant recombination and deterioration (Baird, 2008; Blackburn, 1991). Telomere sequences are not completely replicated by conventional DNA replication enzymes (DNA polymerase) during somatic cell division (Baird, 2008). In fact, in human cells, TTAGGG repeats are eroded by approximately 50–200 base pairs per cell division, and eventually reach replicative limit due to telomere shortening and then enter into senescence leading to cellular aging (Counter, 1996; Counter et al, 1992; Harley et al, 1990). Consequently, telomere lengths in various human cells reflect the proliferative history and replicative potential of cells (Allsopp et al, 1992; Kipling, 2001; Klapper et al, 2001). Telomerase, a reverse transcriptase enzyme that contains an RNA template, repeatedly synthesizes telomeric repeats for chromosome ends to compensate for the loss of telomeric sequences (Greider & Blackburn, 1985). Thus, telomerase activity provides cells with proliferative capacity by contributing to the maintenance of telomere structure and function. In immortal cells, such as, cancer cells, telomere lengths are maintained by high telomerase activity despite cell division, and thus exhibit indefinite proliferative potential (Kim et al, 1994; Shay & Bacchetti, 1997).

It has been suggested that telomeres are important for preserving the immune system and its function. Recently, the relations between immunosenescence by telomere shortening and autoimmune pathogenesis have attracted research attention (Andrews et al, 2010; Tarazona et al, 2002). Premature telomere shortening has been observed in different lymphocyte subsets in various autoimmune diseases, including systemic lupus erythematosus (SLE) (Fritsch et al, 2006; Honda et al, 2001; Kurosaka et al, 2003; Kurosaka et al, 2006; Rus & Via, 2001; Wu et al, 2007), rheumatoid arthritis (RA) (Koetz et al, 2000; Schönland et al, 2003; Steer et al, 2007), Wegener's granulomatosis (Vogt et al, 2003) and atopic dermatitis and psoriasis (Wu et al, 2000). Systemic sclerosis (SSc) is an autoimmune connective tissue disorder characterized by a triad of features, namely, autoimmune dysfunction and inflammatory processes, widespread vasculopathy, and the excessive production and deposition of collagen resulting in development of fibrosis in skin and internal organs (Abraham & Distler, 2007; Abraham & Varga, 2005; Varga & Abraham, 2007). Although etiology of SSc remains elusive, some have suggested that telomere length abnormalities in cells are involved in its pathogenesis. Artlett CM *et al*. reported that telomere lengths of the peripheral blood lymphocytes of SSc patients and of their family members were about 3kb shorter than those of controls (Artlett et al, 1996). Similarly, Paul G Shiels *et al*. found that the telomere lengths of peripheral blood leukocytes (PBLs) in patients with diffuse cutaneous SSc (dcSSc) were on average shorter than those of controls. In addition, it was also observed that telomere shortening was accelerated in the plasmacytoid dendritic cells of dcSSc patients compared to those of controls (Shiels et al, 2011). Conversely, Maclntyre A *et al*. reported that telomere

lengths in PBLs from patients aged less than 50 years with limited cutaneous SSc (lcSSc) were not significantly different from that found in age-matched controls, but that they tended to be longer in patients more than 50 years old (Maclntyre et al, 2008). Thus, the results of previous studies on telomere loss in SSc are controversial. Furthermore, data on telomere erosion in subpopulations of PBLs from SSc patients have not been previously reported.

Different methods have been devised for measuring telomere length. Southern blotting, based on terminal restriction fragment (TRF) length, is the most widely used approach to determine telomere lengths in genomic DNA (Southern, 1979). Southern blotting can be used to measure a wide range of telomere lengths from diverse sample types, but is time-consuming (3-5 days), difficult to quantify and requires large amounts of DNA (0.5-5 μg/individual). Fluorescence *in situ* hybridization (FISH) provides better options, which have higher sensitivity, specificity, and resolution. However, the FISH methods used, that is, quantitative-FISH (Poon et al, 1999) and flow-FISH (Rufer et al, 1998), require expensive, specialized equipment, and are time consuming and labor intensive. However, recently, the monochrome multiplex quantitative polymerase chain reaction (MMQPCR) method was developed by Cawthon to determine relative telomere lengths (Cawthon, 2002; Cawthon, 2009). This method is straightforward, highly accurate, and requires far less genomic DNA (2.2-20 ng/individual) and time than existing methods. Accordingly, in this study, I used the MMQPCR assay. My study hypothesized that immunosenescence of PBLs subpopulations contributes to the development of SSc. Thus, I investigated immunosenescence statuses in SSc by measuring telomere lengths in different subsets of

T cells, B cells, and neutrophils. My findings demonstrate that premature telomere shortening occurs in specific subsets of T and B cells in SSc.

MATERIALS AND METHODS

1. Patients and controls

One hundred and eight patients with SSc (mean age 49.8 years, range 15-77 years; 8 males and 100 females) diagnosed at Seoul National University Hospital between January 2006 and December 2011 were enrolled in this study. All patients fulfilled the preliminary classification criteria of the American College of Rheumatology for SSc (Masi et al, 1980). The following variables were collected at enrollment; clinical subsets, and anti-Scl-70, anti-ribonucleoprotein (RNP) antibody, and antinuclear antibody (ANA) statuses (Table 1). One hundred and eight age and sex matched controls (mean age 50.0 years, range 15-76 years; 8 males and 100 females) were also included. Telomere lengths were measured in the PBLs of 88 SSc patients and of 88 controls, and determined in subpopulations of leukocytes in 20 members of these two groups. The telomere lengths of these samples were measured by MMQPCR. To confirm a correlation between Southern blotting and MMQPCR, telomere lengths in the CD4 T cells of another 20 healthy controls (mean age 48.1 years, range 16-85 years; 2 males and 18 females) were measured by both assays. A sample of umbilical cord blood was included as standard DNA (telomere-high). This study was reviewed and approved by the Institutional Review Board of Seoul National University Hospital, and informed consent was obtained all study subjects.

2. Isolation of PBLs, PBMCs, and neutrophils from peripheral blood

To obtain PBLs, peripheral blood samples of 88 SSc patients and 88 healthy controls were incubated in cell lysis solution (Promega, Madison, WI, USA) for 10 minutes at room temperature (RT) to remove red blood cells.

Peripheral blood mononuclear cells (PBMCs) and neutrophils were isolated from heparinized blood samples (20 ml) of 20 SSc patients and 20 controls by Ficoll density gradient centrifugation. Moreover, PBMCs were also obtained from another 20 healthy controls for Southern blotting. Samples were diluted with an equal volume of sterile phosphate-buffered saline solution (PBS), and then carefully layered over 20 ml of Ficoll/Hypaque density gradient (Ficoll-Paque™ PLUS, specific gravity 1.077 g/ml, GE Healthcare Life Sciences, Uppsala, Sweden). After centrifugation at 400 x g for 20 minutes at RT, PBMCs were obtained from the plasma-Ficoll interface. Granulocyte fraction, which included neutrophils and erythrocytes, was obtained, and washed twice with PBS. Neutrophils were obtained from the granulocyte fraction after lysing erythrocytes with G-DEX II RBC lysis buffer (iNtRON Biotechnology, Seoul, South Korea).

3. Isolation of cell subsets by MACS and Flow cytometry

3.1. Magnetic cell sorting (MACS).

CD4 T cells were purified from the PBMCs of 20 healthy controls by magnetic cell sorting according to the manufacturer's instructions. In brief, PBMCs were incubated

with MicroBeads conjugated to monoclonal anti-human CD4 antibodies (MACS® ; Miltenyi Biotec, Auburn, CA, USA) for 15 minutes at 4℃ in MACS buffer (PBS, 0.5% bovine serum albumin and 2 mM EDTA, pH 7.2). The magnetically labeled cells were obtained as a positive fraction using a MACS separator. The purities of the CD4 T cells obtained were > 95% by flow cytometry (data not shown).

3.2. Flow cytometry.

Isolated PBMCs from 20 SSc patients and 20 controls were sequentially surface stained with anti-human CD27 FITC (clone M-T271; BD Pharmingen), anti-human CD8 PE-Cy7 (clone RPA-T8; BD Pharmingen), anti-human CD45RA APC (clone HI100; BD Pharmingen), anti-human CD3 APC-Cy7 (clone SK7; BD Pharmingen), anti-human CCR7 PE (clone 150503; R&D system) and anti-human CD19 PerCP-Cy5.5 (clone SJ25C1; eBioscience). Cell surface staining was performed for 45 minutes at RT in FACS buffer (PBS, 1% FBS, and 0.09% sodium azide). After washing, the stained cells were sorted using a FACSAria (BD Biosciences, San Jose, CA, USA) into the following fractions (Figure 1A): CD3 T (CD3+), CD4 T (CD3+CD8-), naive CD4 T (CD3+CD8-CCR7+CD45RA+; CD4 T_{Naive}), central memory CD4 T (CD3+CD8-CCR7+CD45RA-; CD4 T_{CM}), effector memory CD4 T (CD3+CD8-CCR7-CD45RA-; CD4 T_{EM}), CD8 T (CD3+CD8+), naive CD8 T (CD3+CD8+CCR7+CD45RA+; CD8 T_{Naive}), effector memory CD8 T (CD3+CD8+CCR7-CD45RA-; CD8 T_{EM}), terminal effector memory CD8 T (CD3+CD8+CCR7-CD45RA+; CD8 T_{TEM}), naive B (CD3-CD19+CD27-) and memory B (CD3-CD19+CD27+). Terminal effector memory CD4 T (CD3+CD8-CCR7-CD45RA+) and central memory CD8 T (CD3+CD8+CCR7+CD45RA-) fractions were

not analyzed for telomere lengths because only small numbers of cells were obtained. Phenotypic data were acquired using the FACSAria and analyzed using FlowJo 7.6 software (Treestar Inc., San Carlos, CA, USA).

4. Extraction of genomic DNA

Genomic DNA was extracted from isolated PBLs, lymphocyte subpopulations, and neutrophils using a Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). Cellular proteins of PBLs, lymphocyte subpopulations, and neutrophils were precipitated using protein precipitation solution, and nuclei were lysed using nuclei lysis solution. After centrifugation at 13,000 x g for 4 minutes, genomic DNA was concentrated by isopropanol precipitation and rehydrated using DNA rehydration solution overnight at 4℃.

5. Measurement of telomere lengths

5.1. Southern blotting.

Telomere lengths of CD4 T cells isolated by MACS were measured by Southern blotting. Using the TeloTAGGG Telomere Length Assay kit (Roche Applied Science, Mannhein, Germany), determination of mean TRF length was performed according to the manufacturer's instructions. Approximately 1ug of genomic DNA was digested in reaction mixture containing Hinf I/Rsa I enzymes (1 U/ul for each enzyme) for 2 hours at 37 ℃. Following DNA digestion, DNA fragments were separated by electrophoresis on

0.8% agarose gel in 1X TAE buffer (0.04M Tris-acetate, 0.001M EDTA, pH 8.0) and transferred to a positively charged nitrocellulose transfer membrane (Protran®, Schleicher & Schuell BioScience, Keene, NH, USA) by capillary transfer using 20X SSC buffer (3M NaCl, 0.3M sodium citrate, pH 7.0). After hybridization with a digoxigenin-labeled probe (Roche Molecular Biochemicals) specific for telomeric repeats, chemiluminescence was detected using alkaline phosphatase-conjugated anti-DIG specific antibodies (Roche Molecular Biochemicals). Exposed X-ray films were analyzed densitometrically using the gel doc XR+ system and quantity one software v4.6 (Bio-Rad Laboratories, Hercules, CA, USA).

The mean TRF length was calculated using the following formula:

$$\text{TRF} = \Sigma\,(ODi) \Big/ \Sigma\,(ODi/Li)$$

, where OD_i is the chemiluminescent signal and L_i is the length of the TRF fragment at position $_i$. This calculation accounts for higher signal intensities from larger TRF fragments due to multiple hybridizations of the telomere-specific probe.

5.2. MMQPCR.

Telomere lengths in all DNA samples in this study were measured using the MMQPCR method devised by Cawthon, with minor modification (Cawthon, 2009). Briefly, approximately 2.2 ng of each experimental DNA diluted in pure water was digested in a 15 µl master mix containing 0.75× SYBR Green I (Bioneer, Daejeon, South Korea), 1U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), 10 mM Tris–HCl pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM of each dNTP, 1 mM DTT and 1 M

betaine (Sigma–Aldrich, St Louis, MO, USA), in a final reaction volume of 25 µl. Four primers were used: the telomere primers - telg and telc (at 900 nM each), and the albumin primers as single copy gene - albu and albd (at 900 nM each) in the master mix.

The thermal cycling profile was as follows; stage 1: 95℃ for 15 min, stage 2: 2 cycles of 94℃ for 15 s and 49℃ for 15 s, and stage 3: 35 cycles of 94℃ for 15 s, 62℃ for 10 s, 74℃ for 15 s with signal acquisition for amplification of the telomere template, 84℃ for 10 s and 88℃ for 15 s with signal acquisition for amplification of the single copy gene template. As references, telomere lengths in the DNA of a newborn (telomere, high) and of a 74-year old (telomere, low) were measured for each PCR plate. All DNA samples were assayed in triplicate. After PCR, telomere (T) to single copy gene (S) ratios were calculated using the formula:

$$2^{-\Delta Ct} = \left[2^{Ct(telomere)} / 2^{Ct(albumin)}\right]^{-1}$$

The T/S ratio is proportional to the average telomere length per cell. The T/S of one sample relative to the T/S of another sample (relative T/S ratio) was used to represent telomere length according to the formula:

$$2^{-(\Delta Ct1 - \Delta Ct2)} = 2^{-\Delta\Delta Ct}$$

6. Statistical analysis

Statistical analysis was carried out with SPSS version 19 (SPSS Inc., Chicago, IL, USA). I used simple linear regression method to investigate the associations between age and mean TRF length or relative T/S ratio. ANCOVA was used to compare SSc patients and

controls in terms of telomere lengths in different cell subsets. ANCOVA and Mann-Whitney U test were used to analyze clinical data. *P*-values < 0.05 were considered statistically significant.

RESULTS

1. Correlation between mean TRF lengths and relative T/S ratios

In humans, telomere lengths are inversely correlated with age in many tissues and cell types, including peripheral blood cells and dermal fibroblasts (Harley et al, 1990; Hastie et al, 1990; Ishii et al, 2006; Iwama et al, 1998). Telomere lengths are known as a biomarker of biological aging and of prediction of life expectancy (Cawthon et al, 2003; Njajou et al, 2007). To determine methods for measuring telomere lengths, I measured the telomere lengths in DNA of CD4 T cells from 20 healthy controls, aged 16-85 years, using two methods, that is, mean TRF length was determined by Southern blotting and relative T/S ratio (relative telomere length) was determined by MMQPCR. A representative Southern blot is shown in Figure 2. Mean TRF lengths were found to be positivity correlated with relative T/S ratios (R^2 = 0.383, p = 0.004; Figure 3). As previously reported, a negative correlation was observed between age and telomere lengths, as determined using both approaches (Figure 4). I found that the MMQPCR method better suited my experimental system than Southern blotting, because it required much less DNA and time. Therefore, I used the MMQPCR assay to determine telomere lengths for all samples in this study.

2. Comparison of the telomere lengths in PBLs between SSc patients and healthy controls

I analyzed the relationship between telomere lengths and age in PBLs in 88 SSc patients and 88 controls. To compensate for age-and sex-dependent variations in telomere lengths, the two groups were age- and sex-matched. Relative T/S ratio of PBLs progressively declined with age in both groups by simple linear regression analysis (R^2 = 0.056, p = 0.026 in SSc patients, R^2 = 0.077, p = 0.009 in healthy controls) (Figure 5A). However, telomere lengths in SSc patients tended to be shorter than in healthy controls (mean ± SD, 1.571 ± 0.439 in SSc patients *vs.* 1.613 ± 0.400 in controls; p = 0.504) (Figure 5B).

3. Comparison of telomere lengths in different subpopulations of PBLs between SSc patients and controls

To investigate whether telomere lengths differ in the PBLs subpopulations of SSc patients and controls, PBLs from 20 SSc patients and 20 controls were sorted into T cells, B cells, and neutrophils.

First, I analyzed telomere lengths in CD3, CD4, and CD8 T cells in both groups. Telomere lengths in the CD3 T cells of SSc patients tended to shorter than in controls (1.185 ± 0.433 *vs.* 1.366 ± 0.594; p = 0.252) (Figure 6). However, telomere lengths in the CD4 T cells of patients were significantly shorter than in controls (1.308 ± 0.553 *vs.* 1.956 ± 1.146; p = 0.024) (Figure 7A), as were the telomere lengths in the CD8 T cells of patients (0.996 ± 0.342 *vs.* 1.389 ± 0.643; p = 0.013) (Figure 7B).

To identify the cell types that influence telomere loss in the CD4 and CD8 T cells of patients, I sorted naive, central memory, effector memory, and terminal effector memory compartments from CD4 and CD8 T cells in patients and controls using a FACS sorter,

and measured the telomere lengths of the subpopulations obtained. For CD4 T cells, telomere lengths were significantly shorter in the CD4 T_{Naive} (1.513 ± 0.657 *vs.* 2.320 ± 1.030; p = 0.001) and CD4 T_{CM} (1.084 ± 0.369 *vs.* 1.686 ± 0.882; p = 0.007) cells of patients (Figure 8). Similarly, for CD8 T cells, the telomere lengths were significantly shorter in the CD8 T_{EM} cells of patients than controls (0.794 ± 0.342 *vs.* 1.134 ± 0.533; p = 0.030) (Figure 9).

It has been reported that B cells play critical roles in pathogenesis of systemic autoimmune disease, like SLE, RA, and SSc (Martin & Chan, 2004; Sato et al, 2005; Silverman, 2005). In particular, disturbed B cell homeostasis and memory B cell hyperactivity were detected in SSc patients (Sato et al, 2005). Accordingly, I examined whether telomere shortening occurs in the B cells of SSc patients. It was found that telomere lengths of naive (1.291 ± 0.566 *vs.* 2.373 ± 1.151; p = 0.001) and memory B cells (1.534 ± 1.098 *vs.* 3.331 ± 2.330, p = 0.005) were significantly shorter in SSc patients than those of controls (Figure 10).

Interestingly, in neutrophils, telomere lengths tended to be longer in SSc patients (1.354 ± 0.282 *vs.* 1.144 ±0.403; p = 0.068) (Figure 11).

4. Relations between telomere lengths and clinical subsets and autoantibody statuses in SSc patients

Finally, I examined relations between telomeric erosion in different cell subpopulations of SSc patients according to clinical subsets and the presence of autoantibodies. In PBLs, telomere lengths were not found to be dependent on clinical subsets (Table 2) or

positivity for anti-Scl-70, anti-RNP antibody, and ANA (Table 3). Furthermore, no dependence was found in different subpopulations of T, B cells, and neutrophils, with the exception of significantly shorter telomere lengths in CD8 T_{EM} cells from anti-Scl-70 antibody positive patients (0.537 ± 0.248 *vs.* 0.901 ± 0.320; $p = 0.037$) (Table 3).

Table 1. Demographic and clinical characteristics of systemic sclerosis (SSc) patients and controls.

	Controls (n = 108)	SSc (n = 108)
Demographic characteristics		
Sex, female/male (female %)	100/8 (92.6)	100/8 (92.6)
Age, mean ± SD	50.0 ± 14.0	49.8 ± 13.9
Clinical characteristics		
Clinical subsets, diffuse/limited (%)	-	54/50 (51.9/48.1)
Autoantibody status		
anti-Scl-70, n (%)	-	49 (49.5)
anti-RNP, n (%)	-	28 (36.8)
ANA, n (%)	-	83 (92.2)

RNP, ribonucleoprotein; ANA, antinuclear antibody.

Table 2. Comparison of telomere lengths in different cell subpopulations according to clinical subsets in systemic sclerosis (SSc).

	Clinical subsets		
	Diffuse SSc, relative T/S ratio mean (SD)	Limited SSc, relative T/S ratio mean (SD)	*p*-value
PBLs*	1.619 (0.448)	1.528 (0.424)	0.742
CD3 T	1.171 (0.407)	1.217 (0.528)	0.968
Total CD4	1.245 (0.529)	1.455 (0.631)	0.718
CD4 T_{Naive}	1.595 (0.675)	1.322 (0.626)	0.444
CD4 T_{CM}	0.987 (0.317)	1.311 (0.410)	0.130
CD4 T_{EM}	0.847 (0.282)	0.892 (0.167)	0.701
Total CD8	0.950 (0.231)	1.103 (0.536)	0.841
CD8 T_{Naive}	1.168 (0.634)	1.655 (0.809)	0.210
CD8 T_{EM}	0.817 (0.349)	0.733 (0.353)	0.849
CD8 T_{TEM}	0.791 (0.326)	0.936 (0.356)	0.383
Naive B	1.383 (0.609)	1.075 (0.420)	0.207
Memory B	1.429 (1.099)	1.761 (1.163)	0.467
Neutrophil	1.322 (0.245)	1.427 (0.369)	0.444

Mann-Whitney U test, * analyzed by ANCOVA.

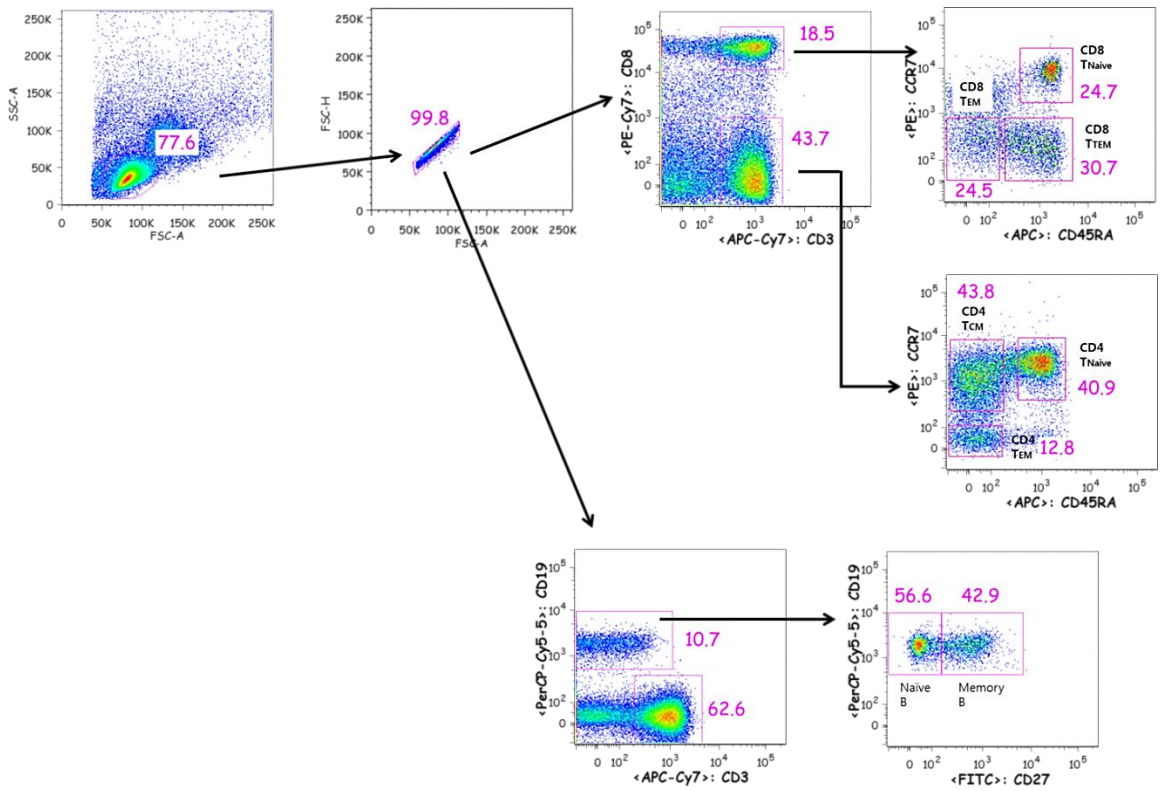
PBLs, peripheral blood leukocytes; CM, central memory; EM, effector memory; TEM, terminal effector memory; relative T/S ratio, relative telomere to single copy gene ratio.

Table 3. Telomere lengths in different cell subpopulations according to the presence of autoantibodies in systemic sclerosis.

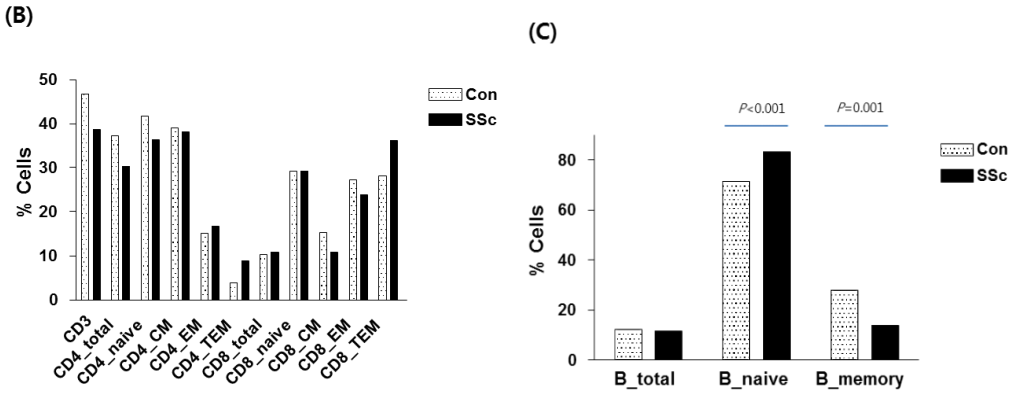
	anti-Scl-70			anti-RNP			ANA		
	positive, relative T/S ratio mean (SD)	negative, relative T/S ratio mean (SD)	*p*-value	positive, relative T/S ratio mean (SD)	negative, relative T/S ratio mean (SD)	*p*-value	positive, relative T/S ratio mean (SD)	negative, relative T/S ratio mean (SD)	*p*-value
PBLs*	1.688 (0.408)	1.481 (0.440)	0.269	1.610 (0.514)	1.600 (0.396)	0.849	1.592 (0.440)	1.411 (0.397)	0.931
CD3 T	1.227 (0.495)	1.183 (0.426)	0.967	1.268 (0.696)	1.149 (0.371)	0.959	1.229 (0.425)	1.041 (0.585)	0.487
Total CD4	1.232 (0.647)	1.374 (0.535)	0.773	1.363 (0.805)	1.307 (0.540)	0.798	1.257 (0.553)	1.665 (0.604)	0.421
CD4 T_{Naive}	1.318 (0.984)	1.613 (0.424)	0.384	1.242 (0.653)	1.562 (0.707)	0.442	1.484 (0.735)	1.615 (0.086)	0.712
CD4 T_{CM}	0.957 (0.371)	1.148 (0.380)	0.592	0.865 (0.495)	1.112 (0.340)	0.442	1.037 (0.320)	1.293 (0.658)	0.958
CD4 T_{EM}	0.835 (0.388)	0.866 (0.176)	0.494	0.789 (0.257)	0.830 (0.205)	0.871	0.836 (0.263)	0.952 (0.213)	0.301
Total CD8	0.921 (0.263)	1.037 (0.399)	0.650	1.220 (0.652)	0.909 (0.208)	0.442	1.010 (0.361)	0.908 (0.349)	0.634
CD8 T_{Naive}	1.160 (0.796)	1.421 (0.712)	0.437	1.002 (0.775)	1.408 (0.740)	0.350	1.262 (0.669)	1.693 (1.067)	0.574
CD8 T_{EM}	0.537 (0.248)	0.901 (0.320)	0.037**	0.611 (0.270)	0.818 (0.368)	0.379	0.748 (0.353)	0.956 (0.163)	0.294
CD8 T_{TEM}	0.694 (0.315)	0.890 (0.349)	0.441	0.799 (0.513)	0.812 (0.293)	0.661	0.823 (0.366)	0.869 (0.034)	0.500
Naïve B	1.171 (0.732)	1.356 (0.498)	0.837	1.342 (0.848)	1.241 (0.533)	1.000	1.258 (0.627)	1.447 (0.221)	0.712
Memory B	1.083 (0.813)	1.723 (1.227)	0.385	1.620 (1.539)	1.478 (1.050)	0.959	1.332 (1.056)	2.398 (1.234)	0.130
Neutrophil	1.324 (0.253)	1.372 (0.318)	0.650	1.470 (0.309)	1.337 (0.293)	0.505	1.325 (0.297)	1.511 (0.216)	0.359

Mann-Whitney U test, * analyzed by ANCOVA, ** $p < 0.05$. PBLs, peripheral blood leukocytes; CM, central memory; EM, effector memory; TEM, terminal effector memory; RNP, ribonucleoprotein; ANA, antinuclear antibody; relative T/S ratio, relative telomere to single copy gene ratio.

(A)



(Figure 1. *continued on next page*)



(Figure 1 *continued*) FACS sorting panel (A) and phenotypical characterization of peripheral blood leukocytes (PBLs) subpopulations (B and C). (A) Cells were sorted by FACSAria into the following fractions: CD3 T (CD3+), CD4 T (CD3+CD8-), naive CD4 T (CD3+CD8-CCR7+CD45RA+; CD4 T_{Naive}), central memory CD4 T (CD3+CD8-CCR7+CD45RA-; CD4 T_{CM}), effector memory CD4 T (CD3+CD8-CCR7-CD45RA-; CD4 T_{EM}), CD8 T (CD3+CD8+), naive CD8 T (CD3+CD8+CCR7+CD45RA+; CD8 T_{Naive}), effector memory CD8 T (CD3+CD8+CCR7-CD45RA-; CD8 T_{EM}), terminal effector memory CD8 T (CD3+CD8+CCR7-CD45RA+; CD8 T_{TEM}), naive B (CD3-CD19+CD27-), and memory B (CD3-CD19+CD27+) cells. (B and C) Comparison of the phenotypical characterization of PBLs subpopulation between systemic sclerosis (SSc) and controls (Con).

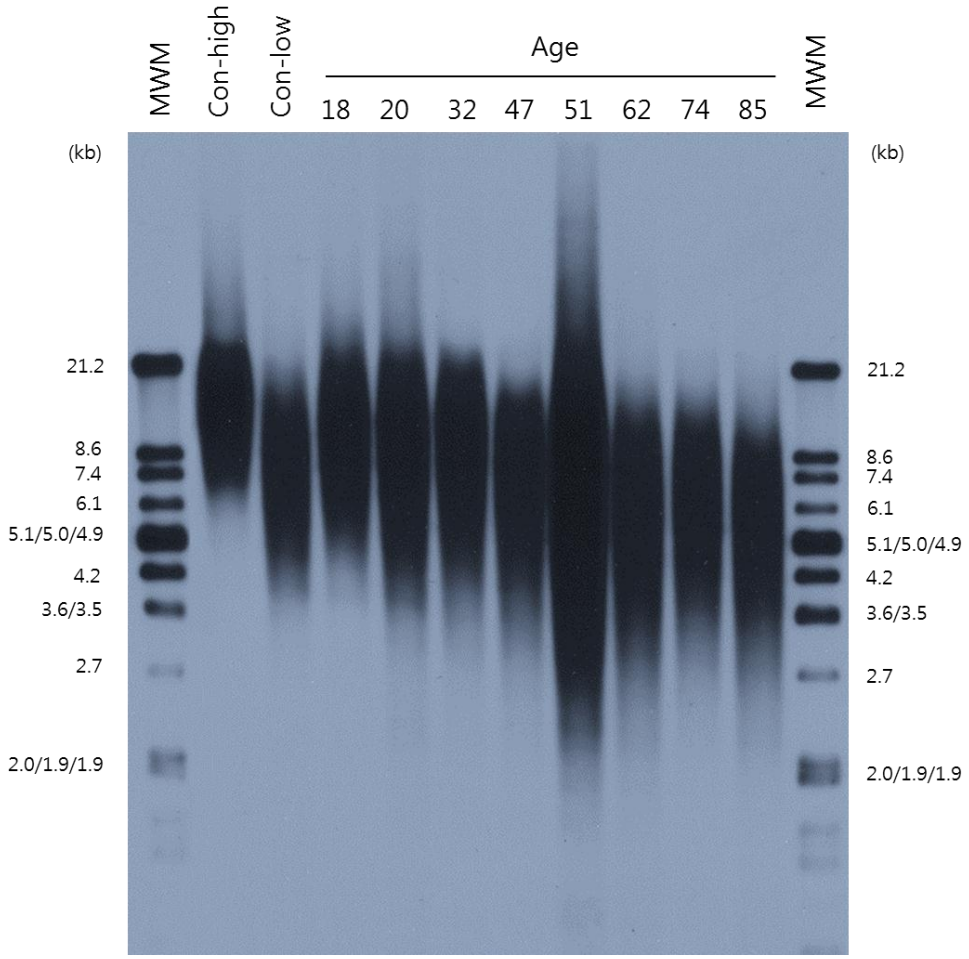


Figure 2. Representative Southern blots of mean TRF lengths in CD4 T cells from healthy controls. Telomere lengths were determined by calculating mean TRF lengths using Southern blot data and were found to decline with increasing age. TRF, terminal restriction fragment; MWM, molecular weight marker; Con-high, control DNA with long telomeres; Con-low, control DNA with short telomeres.

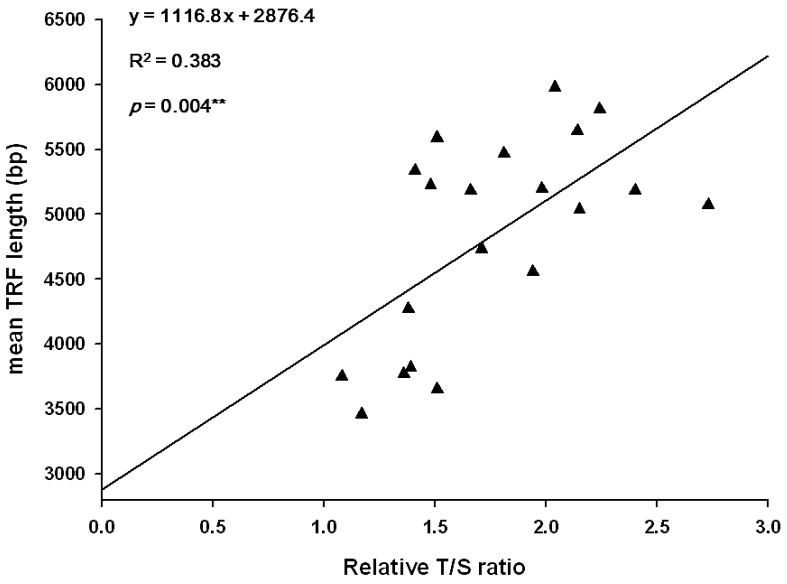


Figure 3. Correlation between relative T/S ratios (determined by MMQPCR) and mean TRF lengths (determined by Southern blotting). Telomere lengths were measured in CD4 T cells from 20 healthy controls, and results were analyzed using simple linear regression analysis. Relative T/S ratio and mean TRF length refer to telomere length. MMQPCR, monochrome multiplex quantitative polymerase chain reaction; Relative T/S ratio, relative telomere to single copy gene ratio; TRF, terminal restriction fragment.

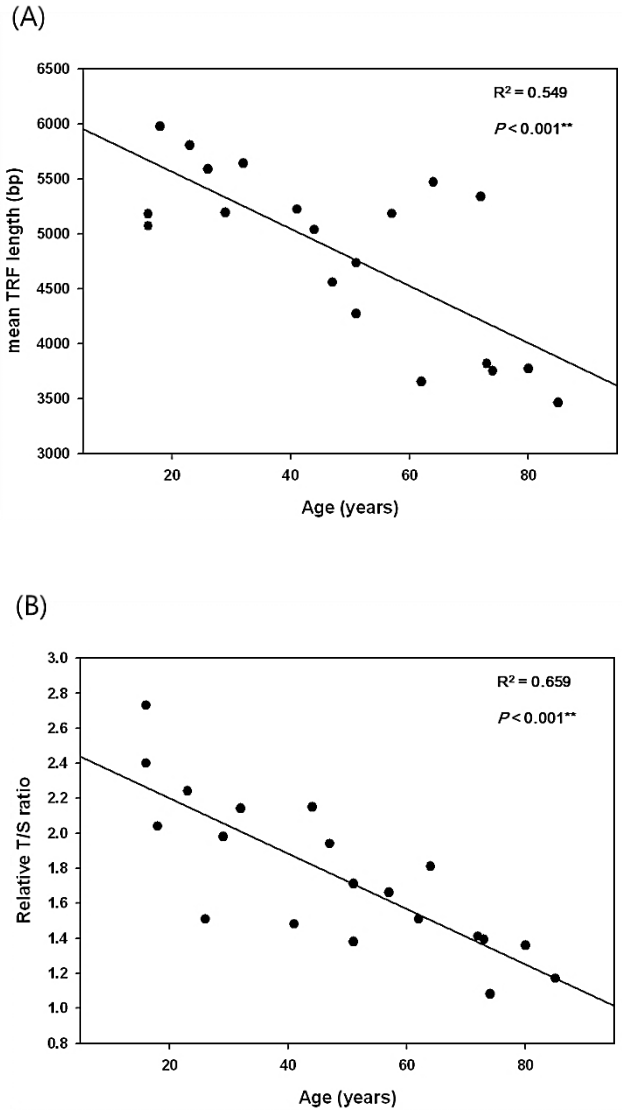


Figure 4. Correlation between age and telomere lengths as determined by Southern blotting (A) or MMQPCR (B). The healthy DNA samples were analyzed by simple linear regression analysis. MMQPCR, monochrome multiplex quantitative polymerase chain reaction; TRF, terminal restriction fragment; Relative T/S ratio, relative telomere to single copy gene ratio.

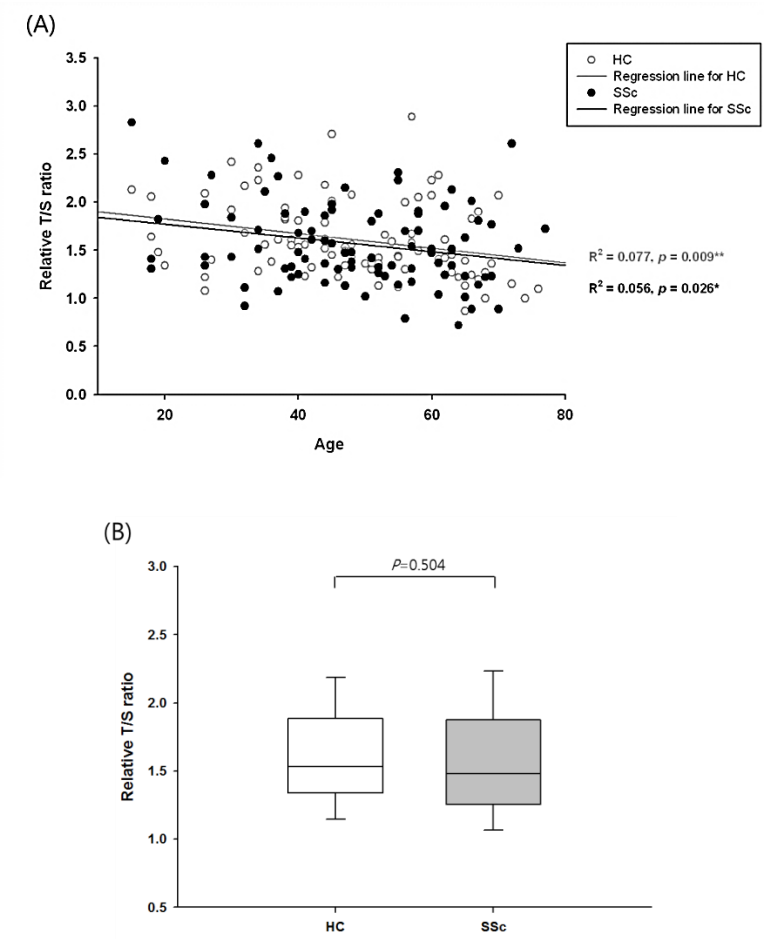


Figure 5. Telomere lengths in peripheral blood leukocytes (PBLs) in systemic sclerosis (SSc) patients and healthy controls (HC). Telomere lengths were measured in PBLs from 88 SSc patients and 88 HC, in triplicate. (A) The correlation between telomere lengths and age was analyzed by simple linear regression analysis. (B) Differences between telomere lengths in the PBLs of patients and controls were analyzed by ANCOVA. The box plots show medians, 25th and 75th percentiles as boxes, and 10th and 90th percentiles as whiskers. Relative T/S ratio = relative telomere to single copy gene ratio.

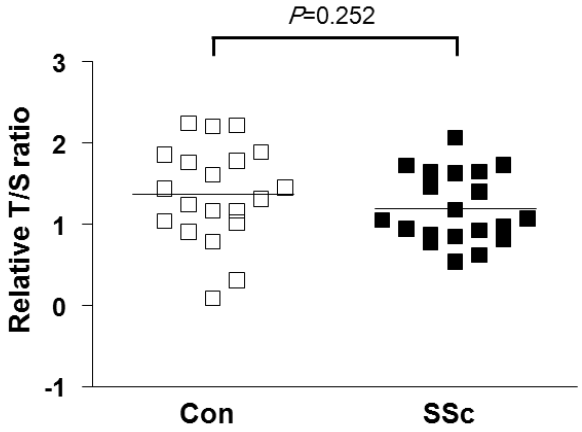


Figure 6. Telomere lengths of CD3 T cells in systemic sclerosis (SSc) patients and controls (Con). CD3 T cells were purified from 20 SSc patients and 20 controls, and relative T/S ratios were determined (p = 0.252 by ANCOVA). Horizontal lines represent means. Relative T/S ratio = relative telomere to single copy gene ratio.

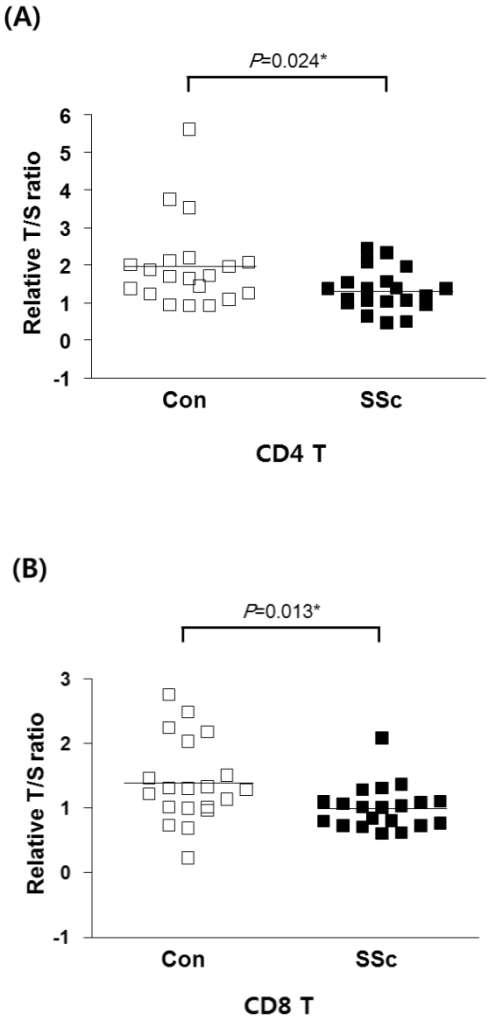


Figure 7. Telomere lengths in CD4 (A) and CD8 (B) T cells in systemic sclerosis (SSc) patients and controls (Con). Patients had significantly shorter telomeres in CD4 and CD8 T cells (p = 0.024, p = 0.013, respectively, by ANCOVA). Horizontal lines represent means. Relative T/S ratio = relative telomere to single copy gene ratio.

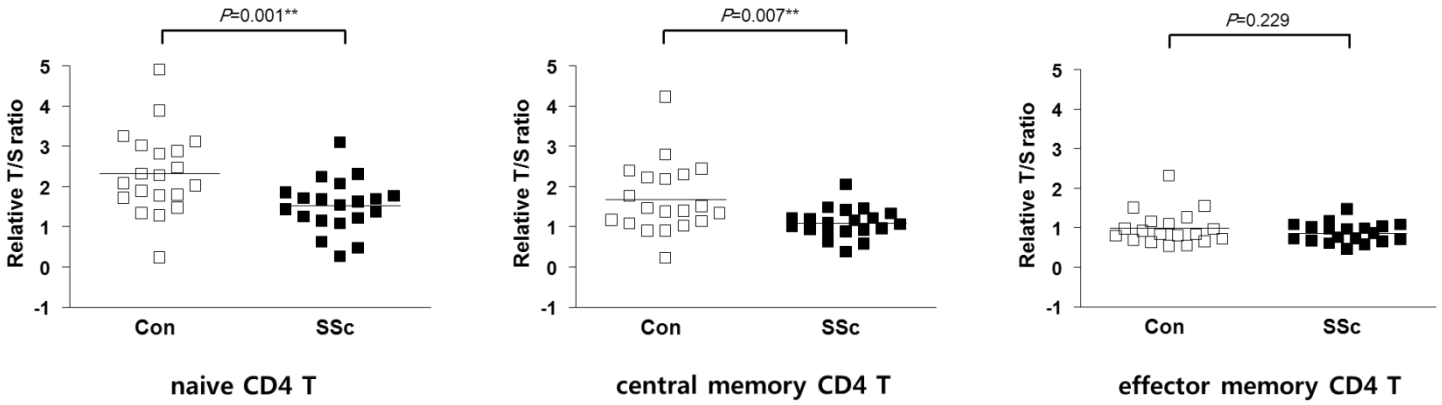


Figure 8. Telomere lengths in subpopulations of CD4 T cells in systemic sclerosis (SSc) patients and controls (Con). CD4 T cells were separated into naive, central memory, and effector memory cells. CD4 T cells from patients had significantly shorter telomeres in naive and central memory cells ($p = 0.001$, $p = 0.007$, respectively, by ANCOVA) than controls. Horizontal lines represent means. Relative T/S ratio = relative telomere to single copy gene ratio.

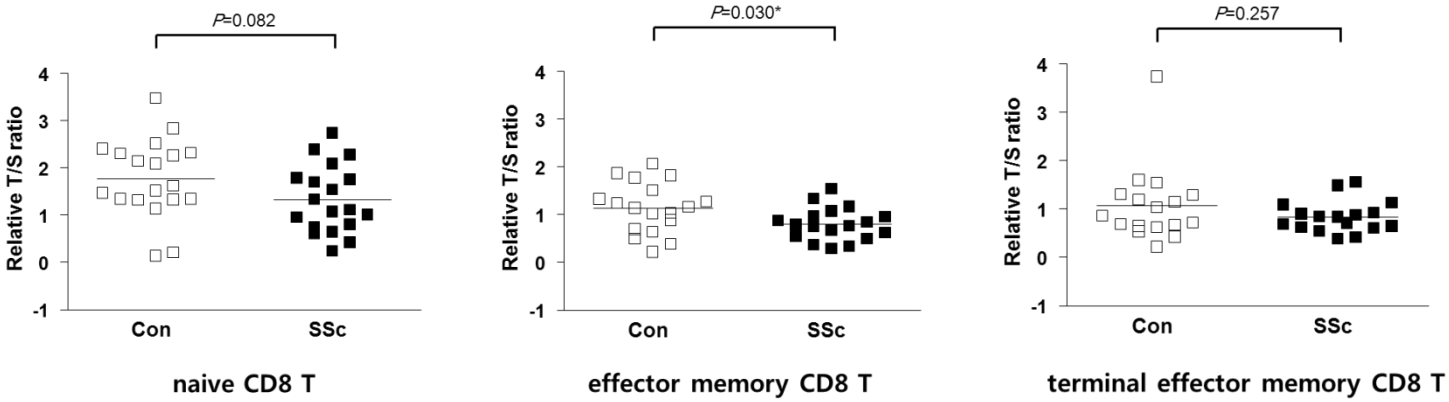


Figure 9. Telomere lengths in subpopulations of CD8 T cells in systemic sclerosis (SSc) patients and controls (Con). CD8 T cells were separated into naive, effector memory, and terminal effector memory cells. CD8 T cells from SSc patients had significantly shorter telomeres in effector memory cells (p = 0.030 by ANCOVA) than controls. Horizontal lines represent means. Relative T/S ratio = relative telomere to single copy gene ratio.

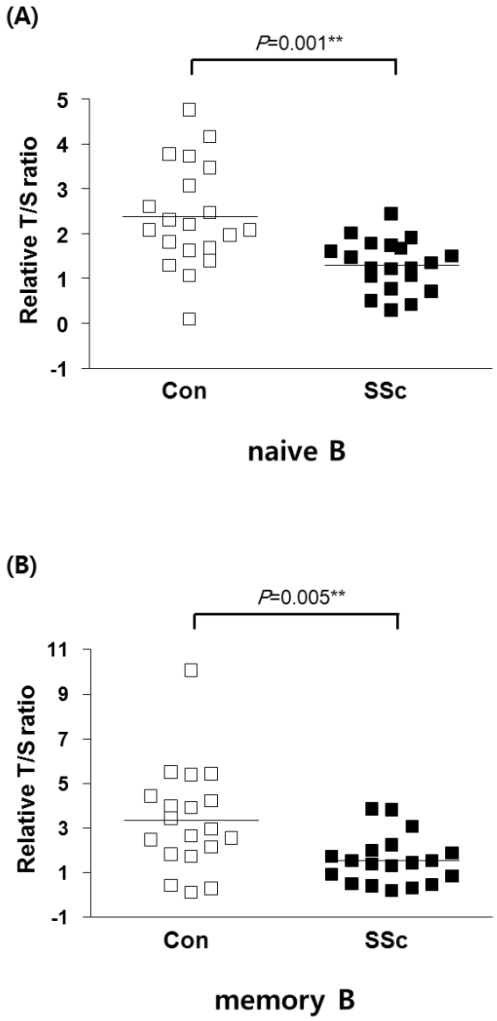


Figure 10. Telomere lengths in subpopulations of B cells in systemic sclerosis (SSc) patients and controls (Con). The telomere lengths of naive (A) and memory (B) B cells were significantly shorter in SSc patients than in controls (p = 0.001, p = 0.005, respectively, by ANCOVA). Horizontal lines represent means. Relative T/S ratio = relative telomere to single copy gene ratio.

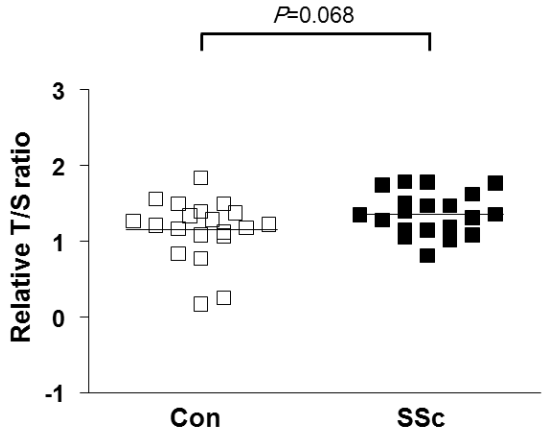


Figure 11. Telomere lengths of neutrophils in systemic sclerosis (SSc) patients and controls (Con). Telomere lengths of neutrophils in patients tended to be longer than in controls (p = 0.068 by ANCOVA). Horizontal lines represent means. Relative T/S ratio = relative telomere to single copy gene ratio.

DISCUSSION

This study describes for the first time the dynamics of telomere length in T, B cells, and their specific subpopulations as well as PBLs in SSc patients. In most studies conducted on the SSc patients, telomere lengths were measured in PBMCs or leukocytes (Artlett et al, 1996; Maclntyre et al, 2008; Shiels et al, 2011). The limitation of such studies is that they do not give a correct explanation because PBMCs and leukocytes are composed of various cell types. Therefore, my study could suggest which types of immune cells indicate the premature senescence in SSc.

I found that telomere lengths in the PBLs of SSc patients tended to be shorter than in controls. However, telomere lengths in the neutrophils of patients tended to be longer than in controls. It is possible that a longer telomere length in neutrophils of SSc patients could have effect on measured telomere lengths in PBLs, since neutrophils are the most abundant type of leukocytes. Maclntyre A *et al.* found that telomere lengths in the PBLs of lcSSc patients aged > 50 years were longer than in controls (Maclntyre et al, 2008). However, my findings showed no difference of telomere lengths in the PBLs between lcSSc patients (> 50 years) and age-matched controls (p = 0.468, data not shown). This discrepancy may be due to different ethnicities or methodologies. Maclntyre *et al* used Southern blotting, whereas I used MMQPCR to measure telomere lengths.

In total CD4 and CD8 T cells, telomere lengths were significantly shorter in SSc patients than in controls. It is known that T cells play an important role in pathogenesis of SSc

(Gu et al, 2008), in which activated CD4+ and CD8+ T cells are major components of inflammatory infiltrates in skin and involved tissues (Fleischmajer et al, 1977; Giacomelli et al, 1998; Gu et al, 2008; Roumm et al, 1984; Sakkas et al, 2002). Furthermore, infiltrating T cells are responsible for imbalance between Th1 and Th2 cytokines (Wynn, 2004). CD4+ T cells have been suggested to contribute to disease by elevating the productions of Th2 cytokines, such as, interleukin (IL)-4 (Mavalia et al, 1997). In addition, peripheral blood effector CD8+ T cells abundantly secrete the Th2 cytokine, IL-13, which is involved in the activation of fibroblasts (Fichtner-Feigl et al, 2005; Fuschiotti et al, 2009; Riccieri et al, 2003). Thus, CD4+ and CD8+ T cells, as main facilitators of Th2 polarized response, play critical roles in the development of SSc. On the other hand, the senescent T cells alter the pattern of cytokine production (Effros et al, 2005), and thus, my findings suggest that the premature senescence of CD4 and CD8 T cells may be related to the aberrant levels of specific cytokines found in SSc patients.

SSc is characterized by a number of autoantibodies, including anti-Scl-70, anti-RNP, and ANA (Ho & Reveille, 2003). In a recent study, it was found that chronic B cell activation induced skin fibrosis in a genetic mouse model of SSc via autoantibody production (Saito et al, 2002). Interestingly, I found that premature telomere shortening occurs in B cell subsets, both naive (CD27-; $p = 0.001$) and memory (CD27+; $p = 0.005$). These findings suggest that B cell senescence may induce abnormalities in B cell function. Taken together, it appears that collaboration between senescent T and B cells contributes to the development of SSc.

Telomere shortening is evidence of increased cell proliferation, and inflammation is a

major cause of accelerated telomere shortening (Georgin-Lavialle et al, 2010). SSc is an autoimmune disease, accompanied by persistent inflammation. In fact, during the pathogenesis of SSc, activated T cells are accumulated in lesional skin and organs in early-stage disease, and induce inflammatory responses. These processes allow massive clonal expansion of these cells, especially the memory compartments of T lymphocytes, and this may lead to telomere loss of the cells (Goronzy et al, 2006). In the present study, telomere attrition was observed in the CD4 T_{CM} and CD8 T_{EM} cells derived from SSc patients, and could have been due to the high proliferation rate induced by chronic inflammation. Furthermore, accelerated telomere loss was prominently observed in CD4 T_{Naive}. This suggests that impaired thymus function could cause the telomere erosion in naive T cells. In general, the immune system is under stringent homeostatic control, requiring a balance between the influx of new T cells from the thymus, their efflux by apoptosis, and self-renewal of existing T cells in the periphery (Berzins et al, 1999; Goldrath & Bevan, 1999). Thus, a lack of influx of new T cells due to thymus dysfunction imposes proliferative demands on the existing naive compartment to maintain the total size of the systems. And this increased homeostatic proliferation of naive T cells could lead to accelerated loss of telomeric sequences, which is consistent with premature cellular senescence. Furthermore, senescent naive T cells are highly prone to apoptosis.

Telomere attrition is a product of telomerase dysregulation. Telomerase activity is up-regulated in activated T and B cells to increase their lifespans and prevent cellular senescence (Hodes et al, 2002). However, as the T and B cells are continuously

stimulated, they become refractory to telomerase induction. Consequentially, telomere sequences shorten, and numbers of possible replications are reduced (Valenzuela & Effros, 2002). A previous study performed on SSc found that a specific polymorphism of the telomerase RNA component gene in skin fibroblasts (Ohtsuka et al, 2002). In another study, telomerase activity was found to be markedly lower in the PBLs of SSc patients (Tarhan et al, 2008). The present study suggests that dysregulation of the telomerase system in CD4 T_{CM} and CD8 T_{EM} triggers telomere erosion in these cells and cellular senescence.

A decline in new T cell generation due to thymus dysfunction and a high susceptibility to apoptosis due to cellular senescence, may result in loss of T cell diversity (Goronzy et al, 2007). Increased homeostatic proliferation by peripheral T cells depends on the recognition of self-antigen (Ernst et al, 1999; Viret et al, 1999), and leads to peripheral tolerance breakdown by TCR diversity contraction. As a result, the immune system may be skewed toward autoimmunity (Goronzy & Weyand, 2001). In addition, senescent CD4+ and CD8+ T cells that have lost CD28 expression have been observed in various autoimmune diseases (Markovic-Plese et al, 2001; Schirmer et al, 2001; Schmidt et al, 1996). CD28 is a pivotal co-stimulatory receptor that is required for the appropriate activation of T cells, and CD4+CD28- and CD8+CD28- T cells have been reported to express other stimulatory receptors, including killer immunoglobulin-like receptors and NKG2D (Vallejo, 2005). The expressions of these receptors are sufficient to lower the threshold of antigen-specific activation, and it may have detrimental consequences on the maintenance of tolerance. CD28- T cells also contribute to the development of

inflammatory diseases by producing high levels of proinflammatory cytokines and perforin /granzyme cytotoxicity (Goronzy & Weyand, 2003).

My results showed that telomere lengths tended to be shorter in naive, central memory, and effector memory compartment of CD4 T cells (Figure 8) and in naive, effector memory, and terminal effector memory compartment of CD8 T cells (all in descending order) (Figure 9). These findings are consistent with previous observations that the memory compartments of CD4+ and CD8+ T cells have shorter telomeres than those of naive T cells (Hodes et al, 2002; Rufer et al, 1999). Lymphocytes than other cells are more sensitive to the effects of telomere loss, because they undergo intensive proliferation. Upon recognition of a foreign antigen, naive T cells are activated, expanded, and differentiated into effector cells. After antigens have been cleared, most effector cells undergo apoptosis and some become long-lived antigen-specific memory T cells, which on encountering the same antigen rapidly expand. Accordingly, my results are consistent with the suggestion that proliferative stress is probably a major cause of telomere shortening in memory cells (Andrews et al, 2010).

The present study has some limitations. First, the number of samples (n = 20) may have been inadequate. In fact, no correlation was found between telomere lengths and clinical profiles (Tables 2 and 3). Second, I did not investigate the causes of premature cellular senescence in SSc patients. To address this issue definitively, additional experiments would have been needed to quantify TCR rearrangement excision circles, and to measure telomerase activity, and further related functional studies would have been required. Third, the clinical features of SSc may be dependent on ethnicity (Kuwana et al, 1994; Nietert et

al, 2006; Reveille et al, 2001), and the present study was conducted on a wholly Korean cohort.

Summarizing, the present study shows that different subsets of T and B cells in SSc patients exhibit accelerated telomere shortening, as determined by MMQPCR. My findings suggest that telomere shortening and the premature senescence of immune cells contribute to the pathogenesis of SSc.

REFERENCES

Abraham D and Distler O (2007) How does endothelial cell injury start? The role of endothelin in systemic sclerosis. *Arthritis Res Ther* 9**:** 2

Abraham DJ and Varga J (2005) Scleroderma: from cell and molecular mechanisms to disease models. *Trends Immunol* 26**:** 587-595

Allsopp RC, Vaziri H, Patterson C, Goldstein S, Younglai EV, Futcher AB, Greider CW and Harley CB (1992) Telomere length predicts replicative capacity of human fibroblasts. *Proc Natl Acad Sci U S A* 89**:** 10114-10118

Andrews NP, Fujii H, Goronzy JJ and Weyand CM (2010) Telomeres and immunological diseases of aging. *Gerontology* 56**:** 390-403

Artlett CM, Black CM, Briggs DC, Stevens CO and Welsh KI (1996) Telomere reduction in scleroderma patients: a possible cause for chromosomal instability. *Br J Rheumatol* 35**:** 732-737

Baird DM (2008) Telomere dynamics in human cells. *Biochimie* 90**:** 116-121

Berzins SP, Godfrey DI, Miller JF and Boyd RL (1999) A central role for thymic emigrants in peripheral T cell homeostasis. *Proc Natl Acad Sci U S A* 96**:** 9787-9791

Blackburn EH (1991) Structure and function of telomeres. *Nature* 350**:** 569-573

Cawthon RM (2002) Telomere measurement by quantitative PCR. *Nucleic Acids Res* 30**:** e47

Cawthon RM (2009) Telomere length measurement by a novel monochrome multiplex quantitative PCR method. *Nucleic Acids Res* 37**:** e21

Cawthon RM, Smith KR, O'Brien E, Sivatchenko A and Kerber RA (2003) Association between telomere length in blood and mortality in people aged 60 years or older. *Lancet* 361**:** 393-395

Counter CM (1996) The roles of telomeres and telomerase in cell life span. *Mutat Res* 366**:** 45-63

Counter CM, Avilion AA, Lefeuvre CE, Stewart NG, Greider CW, Harley CB and Bacchetti S (1992) Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J* 11**:** 1921-1929

Effros RB, Dagarag M, Spaulding C and Man J (2005) The role of CD8+ T-cell replicative senescence in human aging. *Immunol Rev* 205**:** 147-157

Ernst B, Lee DS, Chang JM, Sprent J and Surh CD (1999) The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. *Immunity* 11**:** 173-181

Fichtner-Feigl S, Strober W, Kawakami K, Puri RK and Kitani A (2005) IL-13 signaling through the IL-13α_2 receptor is involved in induction of TGF-β_1 production and fibrosis. *Nat Med* 12**:** 99-106

Fleischmajer R, Perlish JS and Reeves JR (1977) Cellular infiltrates in scleroderma skin. *Arthritis Rheum* 20**:** 975-984

Fritsch RD, Shen X, Illei GG, Yarboro CH, Prussin C, Hathcock KS, Hodes RJ and Lipsky PE (2006) Abnormal differentiation of memory T cells in systemic lupus erythematosus. *Arthritis Rheum* 54**:** 2184-2197

Fuschiotti P, Medsger TA, Jr. and Morel PA (2009) Effector CD8+ T cells in systemic sclerosis patients produce abnormally high levels of interleukin-13 associated with increased skin fibrosis. *Arthritis Rheum* 60**:** 1119-1128

Georgin-Lavialle S, Aouba A, Mouthon L, Londono-Vallejo JA, Lepelletier Y, Gabet AS and Hermine O (2010) The telomere/telomerase system in autoimmune and systemic immune-mediated diseases. *Autoimmun Rev* 9**:** 646-651

Giacomelli R, Matucci-Cerinic M, Cipriani P, Ghersetich I, Lattanzio R, Pavan A, Pignone A, Cagnoni ML, Lotti T and Tonietti G (1998) Circulating Vdelta1+ T cells are activated and accumulate in the skin of systemic sclerosis patients. *Arthritis Rheum* 41**:** 327-334

Goldrath AW and Bevan MJ (1999) Selecting and maintaining a diverse T-cell repertoire. *Nature* 402**:** 255-262

Goronzy JJ, Fujii H and Weyand CM (2006) Telomeres, immune aging and autoimmunity. *Exp Gerontol* 41**:** 246-251

Goronzy JJ, Lee WW and Weyand CM (2007) Aging and T-cell diversity. *Exp Gerontol* 42**:** 400-406

Goronzy JJ and Weyand CM (2001) Thymic function and peripheral T-cell homeostasis in rheumatoid arthritis. *Trends in immunology* 22**:** 251-255

Goronzy JJ and Weyand CM (2003) T-cell senescence and contraction of T-cell repertoire diversity-catalysts of autoimmunity and chronic inflammation. *Arthritis Res Ther* 5**:** 225-234

Greider CW and Blackburn EH (1985) Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. *Cell* 43**:** 405-413

Gu YS, Kong J, Cheema GS, Keen CL, Wick G and Gershwin ME (2008) The immunobiology of systemic sclerosis. *Semin Arthritis Rheum* 38**:** 132-160

Harley CB, Futcher AB and Greider CW (1990) Telomeres shorten during ageing of human fibroblasts. *Nature* 345**:** 458-460

Hastie ND, Dempster M, Dunlop MG, Thompson AM, Green DK and Allshire RC (1990) Telomere reduction in human colorectal carcinoma and with ageing. *Nature* 346**:** 866-868

Ho KT and Reveille JD (2003) The clinical relevance of autoantibodies in scleroderma. *Arthritis Res Ther* 5**:** 80-93

Hodes RJ, Hathcock KS and Weng NP (2002) Telomeres in T and B cells. *Nat Rev Immunol* 2**:** 699-706

Honda M, Mengesha E, Albano S, Nichols WS, Wallace DJ, Metzger A, Klinenberg JR and Linker-Israeli M (2001) Telomere shortening and decreased replicative potential, contrasted by continued proliferation of telomerase-positive CD8+ $CD28^{lo}$ T cells in patients with systemic lupus erythematosus. *Clin Immunol* 99**:** 211-221

Ishii A, Nakamura K, Kishimoto H, Honma N, Aida J, Sawabe M, Arai T, Fujiwara M, Takeuchi F, Kato M, Oshimura M, Izumiyama N and Takubo K (2006) Telomere shortening with aging in the human pancreas. *Exp Gerontol* 41**:** 882-886

Iwama H, Ohyashiki K, Ohyashiki JH, Hayashi S, Yahata N, Ando K, Toyama K, Hoshika A, Takasaki M, Mori M and Shay JW (1998) Telomeric length and telomerase activity vary with age in peripheral blood cells obtained from normal individuals. *Hum Genet* 102**:** 397-402

Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL and Shay JW (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science* 266**:** 2011-2015

Kipling D (2001) Telomeres, replicative senescence and human ageing. *Maturitas* 38**:** 25-37

Klapper W, Parwaresch R and Krupp G (2001) Telomere biology in human aging and aging syndromes. *Mech Ageing Dev* 122**:** 695-712

Koetz K, Bryl E, Spickschen K, O'Fallon WM, Goronzy JJ and Weyand CM (2000) T cell homeostasis in patients with rheumatoid arthritis. *Proc Natl Acad Sci U S A* 97**:** 9203-9208

Kurosaka D, Yasuda J, Yoshida K, Yokoyama T, Ozawa Y, Obayashi Y, Kingetsu I, Saito S and Yamada A (2003) Telomerase activity and telomere length of peripheral blood mononuclear cells in SLE patients. *Lupus* 12**:** 591-599

Kurosaka D, Yasuda J, Yoshida K, Yoneda A, Yasuda C, Kingetsu I, Toyokawa Y, Yokoyama T, Saito S and Yamada A (2006) Abnormal telomerase activity and telomere length in T and B cells from patients with systemic lupus erythematosus. *J Rheumatol* 33**:** 1102-1107

Kuwana M, Okano Y, Kaburaki J, Tojo T and Medsger TA, Jr. (1994) Racial differences in the distribution of systemic sclerosis-related serum antinuclear antibodies. *Arthritis Rheum* 37**:** 902-906

Maclntyre A, Brouilette SW, Lamb K, Radhakrishnan K, McGlynn L, Chee MM, Parkinson EK, Freeman D, Madhok R and Shiels PG (2008) Association of increased telomere lengths in limited scleroderma, with a lack of age-related telomere erosion. *Ann Rheum Dis* 67**:** 1780-1782

Markovic-Plese S, Cortese I, Wandinger KP, McFarland HF and Martin R (2001) CD4+

CD28- costimulation-independent T cells in multiple sclerosis. *J Clin Invest* 108**:** 1185-1194

Martin F and Chan AC (2004) Pathogenic roles of B cells in human autoimmunity; insights from the clinic. *Immunity* 20**:** 517-527

Masi AT, Rodnan GP, Medsge JTA, Altman RD, D'Angelo WA, Fries JF, LeRoy EC, Kirsner AB, MacKenzie AH, McShane. D J, Myers AR and Sharp GC (1980) Preliminary criteria for the classification of systemic sclerosis (scleroderma). *Arthritis Rheum* 23**:** 581-590

Mavalia C, Scaletti C, Romagnani P, Carossino AM, Pignone A, Emmi L, Pupilli C, Pizzolo G, Maggi E and Romagnani S (1997) Type 2 helper T-cell predominance and high CD30 expression in systemic sclerosis. *Am J Pathol* 151**:** 1751-1758

Nietert PJ, Mitchell HC, Bolster MB, Shaftman SR, Tilley BC and Silver RM (2006) Racial variation in clinical and immunological manifestations of systemic sclerosis. *J Rheumatol* 33**:** 263-268

Njajou OT, Cawthon RM, Damcott CM, Wu SH, Ott S, Garant MJ, Blackburn EH, Mitchell BD, Shuldiner AR and Hsueh WC (2007) Telomere length is paternally inherited and is associated with parental lifespan. *Proc Natl Acad Sci U S A* 104**:** 12135

Ohtsuka T, Yamakage A and Yamazaki S (2002) The polymorphism of telomerase RNA component gene in patients with systemic sclerosis. *Br J Dermatol* 147**:** 250-254

Poon SSS, Martens UM, Ward RK and Lansdorp PM (1999) Telomere length

measurements using digital fluorescence microscopy. *Cytometry* 36**:** 267-278

Reveille JD, Fischbach M, McNearney T, Friedman AW, Aguilar MB, Lisse J, Fritzler MJ, Ahn C and Arnett FC (2001) Systemic sclerosis in 3 US ethnic groups: a comparison of clinical, sociodemographic, serologic, and immunogenetic determinants. *Semin Arthritis Rheum* 30**:** 332-346

Riccieri V, Rinaldi T, Spadaro A, Scrivo R, Ceccarelli F, Franco MD, Taccari E and Valesini G (2003) Interleukin-13 in systemic sclerosis: relationship to nailfold capillaroscopy abnormalities. *Clin Rheumatol* 22**:** 102-106

Roumm AD, Whiteside TL, Medsger TA, Jr. and Rodnan GP (1984) Lymphocytes in the skin of patients with progressive systemic sclerosis. Quantification, subtyping, and clinical correlations. *Arthritis Rheum* 27**:** 645-653

Rufer N, Brummendorf TH, Kolvraa S, Bischoff C, Christensen K, Wadsworth L, Schulzer M and Lansdorp PM (1999) Telomere fluorescence measurements in granulocytes and T lymphocyte subsets point to a high turnover of hematopoietic stem cells and memory T cells in early childhood. *J Exp Med* 190**:** 157-167

Rufer N, Dragowska W, Thornbury G, Roosnek E and Lansdorp PM (1998) Telomere length dynamics in human lymphocyte subpopulations measured by flow cytometry. *Nat Biotechnol* 16**:** 743-747

Rus V and Via CS (2001) Telomeres, telomerase, and lupus: the long and short of it. *Clin Immunol* 99**:** 195-197

Saito E, Fujimoto M, Hasegawa M, Komura K, Hamaguchi Y, Kaburagi Y, Nagaoka T, Takehara K, Tedder TF and Sato S (2002) CD19-dependent B lymphocyte signaling thresholds influence skin fibrosis and autoimmunity in the tight-skin mouse. *J Clin Invest* 109**:** 1453-1462

Sakkas LI, Xu B, Artlett CM, Lu S, Jimenez SA and Platsoucas CD (2002) Oligoclonal T cell expansion in the skin of patients with systemic sclerosis. *J Immunol* 168**:** 3649-3659

Sato S, Fujimoto M, Hasegawa M, Takehara K and Tedder TE (2005) Altered B lymphocyte function induces systemic autoimmunity in systemic sclerosis. *Mol Immunol* 42**:** 821-831

Schönland SO, Lopez C, Widmann T, Zimmer J, Bryl E, Goronzy JJ and Weyand CM (2003) Premature telomeric loss in rheumatoid arthritis is genetically determined and involves both myeloid and lymphoid cell lineages. *Proc Natl Acad Sci U S A* 100**:** 13471

Schirmer M, Goldberger C, Würzner R, Duftner C, Pfeiffer KP, Clausen J, Neumayr G and Falkenbach A (2001) Circulating cytotoxic CD8+ CD28-T cells in ankylosing spondylitis. *Arthritis Res Ther* 4**:** 71

Schmidt D, Martens P, Weyand C and Goronzy J (1996) The repertoire of CD4+ CD28-T cells in rheumatoid arthritis. *Mol Med* 2**:** 608

Shay JW and Bacchetti S (1997) A survey of telomerase activity in human cancer. *Eur J Cancer* 33**:** 787-791

Shiels PG, Broen J, McGlynn L, Thomson J, Chee M, Madhok R and Radstake T (2011) Biological ageing is a key determinant in systemic sclerosis. *J Transl Med* 9**:** 1-2

Silverman GJ (2005) Anti-CD20 therapy in systemic lupus erythematosus: a step closer to the clinic. *Arthritis Rheum* 52**:** 371-377

Southern EM (1979) Measurement of DNA length by gel electrophoresis. *Anal Biochem* 100**:** 319-323

Steer SE, Williams FM, Kato B, Gardner JP, Norman PJ, Hall MA, Kimura M, Vaughan R, Aviv A and Spector TD (2007) Reduced telomere length in rheumatoid arthritis is independent of disease activity and duration. *Ann Rheum Dis* 66**:** 476-480

Tarazona R, Solana R, Ouyang Q and Pawelec G (2002) Basic biology and clinical impact of immunosenescence. *Exp Gerontol* 37**:** 183-189

Tarhan F, Vural F, Kosova B, Aksu K, Cogulu O, Keser G, Gunduz C, Tombuloglu M, Oder G, Karaca E and Doganavsargil E (2008) Telomerase activity in connective tissue diseases: elevated in rheumatoid arthritis, but markedly decreased in systemic sclerosis. *Rheumatol Int* 28**:** 579-583

Valenzuela HF and Effros RB (2002) Divergent telomerase and CD28 expression patterns in human CD4 and CD8 T cells following repeated encounters with the same antigenic stimulus. *Clin Immunol* 105**:** 117-125

Vallejo AN (2005) CD28 extinction in human T cells: altered functions and the program of T-cell senescence. *Immunol Rev* 205**:** 158-169

Varga J and Abraham D (2007) Systemic sclerosis: a prototypic multisystem fibrotic disorder. *J Clin Invest* 117**:** 557-567

Viret C, Wong FS and Janeway CA (1999) Designing and maintaining the mature TCR repertoire: the continuum of self-peptide: self-MHC complex recognition. *Immunity* 10**:** 559-568

Vogt S, Iking-Konert C, Hug F, Andrassy K and Hansch GM (2003) Shortening of telomeres: Evidence for replicative senescence of T cells derived from patients with Wegener's granulomatosis. *Kidney Int* 63**:** 2144-2151

Wu CH, Hsieh SC, Li KJ, Lu MC and Yu CL (2007) Premature telomere shortening in polymorphonuclear neutrophils from patients with systemic lupus erythematosus is related to the lupus disease activity. *Lupus* 16**:** 265-272

Wu K, Higashi N, Hansen ER, Lund M, Bang K and Thestrup-Pedersen K (2000) Telomerase activity is increased and telomere length shortened in T cells from blood of patients with atopic dermatitis and psoriasis. *J Immunol* 165**:** 4742-4747

Wynn TA (2004) Fibrotic disease and the TH1/TH2 paradigm. *Nat Rev Immunol* 4**:** 583-594

국문 초록

텔로미어는 면역 체계의 기능을 유지하는데 중요하다. 면역노화의 마커 중 하나인 텔로미어 소실은 류마티스 관절염, 전신홍반루푸스와 같은 자가면역질환에서 잘 알려져 있지만, 전신경화증에서 텔로미어 소실에 대해서는 아직 논란의 여지가 있다. 본 연구에서는 전신경화증 환자에서 다양한 종류의 T 세포, B 세포 및 호중구의 텔로미어 길이를 측정함으로써 면역노화 상태를 알아보고자 하였다.

88명의 전신경화증 환자와 88명의 정상인의 말초혈액에서 말초혈액 백혈구의 DNA를 추출하였다. 또한, 추가로 20명의 환자와 20명의 정상인의 말초혈액에서 말초혈액 단핵구를 분리한 후, FACSAria 기계를 이용하여 전체 CD4, naive CD4, central memory CD4, effector memory CD4 T 세포, 전체 CD8, naive CD8, effector memory CD8, terminal effector memory CD8 T 세포와 naive B, memory B 세포를 각각 얻었다. 호중구는 과립구 분획에서 적혈구 용해 시약을 이용하여 얻었고, 분리된 모든 세포에서 DNA를 추출하였다. Monochrome multiplex quantitative PCR (MMQPCR) 방법을 이용하여 말초혈액 백혈구, 다양한 종류의 T 세포, B 세포 및 호중구의 텔로미어 길이를 측정하였다. 텔로미어의 길이는 특정 세포의 텔로미어 발현 대 단일카피유전자

(예: 알부민) 발현의 비율로 계산되었다 (T/S ratio).

전신경화증 환자와 정상인에서 모두, 말초혈액 백혈구의 텔로미어 길이는 나이가 증가함에 따라 점진적으로 짧아졌다 (전신경화증 환자에서 R^2 = 0.056, p = 0.026, 정상인에서 R^2 = 0.077, p = 0.009). 그러나, 전신경화증 환자와 정상인을 비교했을 때, 말초혈액 백혈구의 텔로미어 길이는 차이가 없었다 (p = 0.504). 전신경화증 환자에서 CD4와 CD8 세포의 텔로미어 길이는 정상인에 비해 상당히 짧아져 있었고 (T/S ratio mean ± SD, CD4 T 세포에서 1.308 ± 0.553 vs 1.956 ± 1.146, p = 0.024, CD8 T 세포에서 0.996 ± 0.342 vs 1.389 ± 0.643, p = 0.013), naive CD4 T 세포 (1.513 ± 0.657 vs 2.320 ± 1.030, p = 0.001), central memory CD4 T 세포 (1.084 ± 0.369 vs 1.686 ± 0.882, p = 0.007)와 effector memory CD8 T 세포 (0.794 ± 0.342 vs 1.134 ± 0.533, p = 0.030)의 텔로미어가 정상인보다 현저하게 소실되어 있는 것을 확인하였다. 또한, 전신경화증 환자의 naive B와 memory B 세포에서도 정상인보다 텔로미어의 길이가 짧아져 있었다 (naive B 세포에서 1.291 ± 0.566 vs 2.373 ± 1.151, p = 0.001, memory B 세포에서 1.534 ± 1.098 vs 3.331 ± 2.330, p = 0.005). 이러한 세포들과 달리, 전신경화증 환자의 호중구에서는 정상인에 비해 텔로미어 길이가 더 긴 경향을 보였다 (p = 0.068). Anti-Scl-70 항체 양성인 환자에서 effector memory CD8 T 세포의 텔로미어가 짧아져 있었지만 (p = 0.037) 임상적 특징에 따른 텔로미어 길이의 차이는 보이지 않았다. 본 연구

는 림프구의 텔로미어 소실이 전신경화증의 발병에 기여할 수 있음을 제시하였다.

주요어: 텔로미어, 텔로미어 소실, 면역노화, 전신경화증

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