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Decreased expression of DNA repair proteins Ku70 and Mre11 is associated with aging and may contribute to the cellular senescence

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Abbreviations: DSB, double strand break; DNA-PK, DNA-dependent protein kinase; TERC, telomerase RNA component; TERT, telomerase reverse transcriptase; TRF1, telomeric-repeat binding factor 1

Abstract

The gradual loss of telomeric DNA can contribute to replicative senescence and thus, having longer telomeric DNA is generally considered to provide a longer lifespan. Maintenance and stabilization of telomeric DNA is assisted by binding of multiple DNA-binding proteins, including those involved in double strand break (DSB) repair. We reasoned that declining DSB repair capacity and increased telomere shortening in aged individuals may be associated with decreased expression of DSB repair proteins capable of telomere binding. Our data presented here show that among the DSB repair proteins tested, only the expression of Ku70 and Mre11 showed statistically significant age-dependent changes in human lymphocytes. Furthermore, we found that expressions of Ku70 and Mre11 are statistically correlated, which indicate that the function of Ku70 and Mre11 may be related. All the other DSB repair proteins tested, Sir2, TRF1 and Ku80, did not show any significant differences upon aging. In line with these data, people who live in the regional community (longevity group), which was found to have statistically longer average life span than the rest area, shows higher level of Ku70 expression than those living in the neighboring control community. Taken together, our data show, for the first time, that Ku70 and Mre11 may represent new biomarkers for aging and further suggest that maintenance of higher expression of Ku70 and Mre11 may be responsible for keeping longer life span observed in the longevity group.

Keywords: aging; Ku70; longevity; Mre11A protein; telomere binding proteins; telomere

Introduction

Telomeres are tandem repeats of the sequence T_2AG_3 located at the distal end of eukaryotic chromosomes that can function in stabilizing chromosomal end integrity (Prowse and Greider, 1995). Telomeres in somatic cells shorten with age since DNA polymerase is unable to replicate completely the 3' end of linear DNA. In culture, cells reach a replicative senescence after a certain number of cell divisions (Harley *et al*., 1990; Harley, 1991), which is thought to be attributed to telomere shortening with each cell replication cycle (Vaziri *et al*., 1994). *In vivo* studies in tissues of a variety of eukaryotes have shown a gradual decrease in telomere length related to organism's age (Harley *et al*., 1990), thus providing a pos-

sibility that telomere length can be used as a tool to estimate biological age in humans (Haussmann *et al*., 2003; Hastings *et al*., 2004; Jeon *et al*., 2005) . In addition, recent studies revealed that worms genetically engineered to have longer telomeric DNA showed longer lifespan than the corresponding wild type worms (Benard *et al*., 2001), highlighting the tight control of the lifespan by telomere length. These data suggest that telomere length can serve as a biomarker of a cell biological age.

In contrast to somatic cells, telomere length of germ cells and stem cells do not shorten over time (Bekaert *et al*., 2004). This can be possible since their telomere length is maintained by the activity of telomerase, an enzyme which adds the necessary telomeric DNA (T_2AG_3) onto the 3' ends of the telomeres (Nilsson *et al*., 1994). The telomerase holoenzyme consists of a telomerase RNA component (TERC) that serves as a template for the addition of repeats, and a protein component, telomerase reverse transcriptase (TERT). Low or absent expression of telomerase in human somatic tissues is due to the strict regulation of TERT gene transcription, whereas *TERC* gene is widely expressed. Thus, up-regulation of TERT expression in somatic cells will be essential for preventing telomere shortening and replicative senescence. Indeed, ectopic expression of human *TERT* gene was able to rescue senescence of normal human endothelial cells in culture and confer a high proliferative index similar to that of young cells (Haendeler *et al*., 2004).

Recent studies have revealed that stabilization of chromosome ends by telomeric DNA sequence is assisted by binding of multiple DNA-binding proteins (Wright *et al*., 2005). These proteins bind to telomeric DNA either directly or indirectly *via* binding to other proteins bound to telomeric DNA, and form a protective nucleoprotein structure that serves to “cap” the end of the chromosome (Karlseder *et al*., 2002). The human telomere-repeat binding factor 2 (TRF2) protects chromosome ends from fusion (van Steensel *et al*., 1998) and was the first telomere-associated protein involved in the maintenance of the correct terminal DNA structure necessary for proper telomere function. Other proteins known to function as DNA repair proteins, such as catalytic subunit of DNA-PK (DNA-PKcs), Ku70, Ku86, Mre11, Xrcc4, and Nbs1 were also found to be associated with telomeric DNA (Um *et al*., 2004; Chin *et al*., 2005). These proteins play essential roles in repairing double-strand breaks (DSBs). Since age-dependent accumulation of DNA damage has been reported (Kirkwood and Finch, 2002), it is presumed that aging decreases the inherent capacity of DNA in somatic cells to remain stable and reduces the efficiency of the DNA repair systems. Indeed, an age-related decline in DNA double-strand break (DSB) repair was reported in unstimulated human lymphocytes (Mayer *et al*., 1991). Likewise, age-dependent telomere shortening has been demonstrated in human CD4 T lymphocytes (Weng *et al*., 1995). Thus, the declining DSB repair capacity upon aging may underlie telomere shortening observed in human lymphocytes.

Based on these data, we reasoned that declining DSB repair capacity and increased telomere shortening in aged individuals may be associated with decreased expression of DSB repair proteins capable of telomere binding in the cell. To test this hypothesis, we obtained blood samples from various age groups of people living in Seoul, Korea and examined the level of DSB repair proteins in purified CD4+ T lymphocytes, a cell type which shows direct telomere shortening with age (Weng *et al*., 1995). Our data show that among the DSB repair proteins tested, only Ku70 and Mre11 are statistically significant age-dependent factors showing declining of cellular expression. Furthermore, Ku70 expression was significantly higher in the people living in the longevity community than those living in the control community. Collectively, our data show, for the first time, that Ku70 and Mre11 in human lymphocytes may represent new biomarkers for aging.

Materials and Methods

Purification of human CD4 lymphocytes

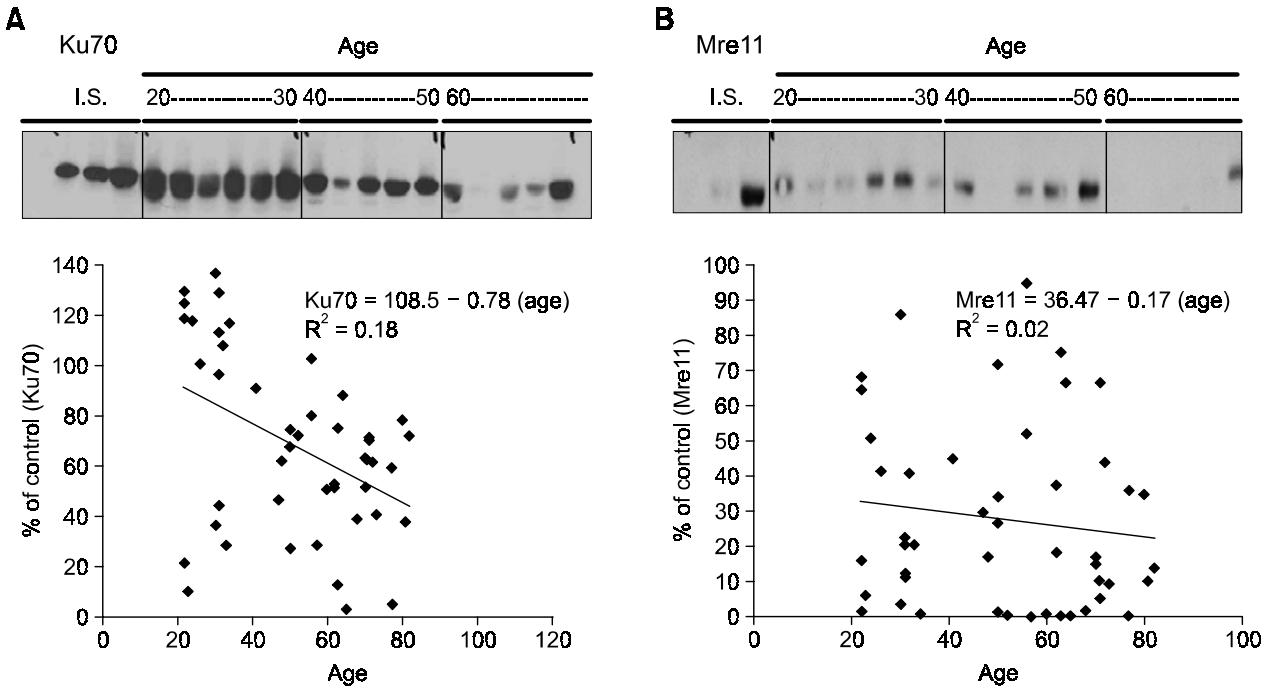
Human PBMCs (peripheral blood mononuclear cells) were obtained from 48 healthy volunteers of age between 20 and 80 yr in Seoul area and from 80 healthy volunteers from Jeju area. All individuals provided written informed consent for blood donation and fully understood the experiment procedure. All the experimental protocols were reviewed and approved by institutional review boards.

CD4 positive T cells were purified from peripheral blood lymphocytes (PBL). Briefly, 50 ml of blood was collected from subjects and PBL was isolated from density gradient centrifugation using Ficoll-paque^{TM} PLUS (Amersham biosciences). CD4 positive T cells were purified using negative depletion kit (Miltenyi Biotec). The purity of CD4 T cells was examined using fluorescence activated cell sorter (FACs) analysis with CellQuest Pro Software (Becton Dickinson, NJ).

Western blot analysis

CD4 positive T cells were washed with phosphate-buffered saline (PBS) and lysed in RIPA lysis buffer (50 mM Tris-HCl pH 7.5, 1% NP-40, 150 mM sodium

chloride, 0.5% sodium deoxycholate and 0.1% SDS) with protease inhibitor cocktail (Roche). An equal amount (20 ug) of total protein was loaded onto a 10-12% SDS-PAGE. After electrophoresis, proteins were transferred to a nitrocellulose membrane. Proteins were detected by monoclonal rabbit anti-human Ku70 or monoclonal rabbit anti-human Mre11 and horseradish peroxide (HRP)-conjugated goat anti-rabbit Ig using western blotting luminal reagent (Santa Cruz Biotechnology). Blots were stripped by



C Characteristics of study subjects in Seoul area

	Seoul area
N	48
Age (years)*	51.5 ± 19.3

* Mean ± STD

D Simple regression analysis of age on ku70 as well as on Mre11 in Seoul area

	Ku70			Mre11		
Variable*	Coeff.	Std. Err.	p-value	Coeff.	Std. Err.	p-value
Intercept	108.50	13.48	< .0001	36.47	10.92	0.0017
Age	−0.78	0.25	0.0027	−0.17	0.20	0.3906

*R^2 = 0.18 (for Ku70), 0.02 (for Mre11)

Figure 1. Protein expression of Ku70 and Mre11 in CD4+ T cells obtained from 48 volunteers ranging from 20 to 80 yr old. Top panel; Total protein level of Ku70 (A) or Mre11 (B) isolated from human lymphocytes was determined by Western blots using rabbit monoclonal Abs against human Ku70 or human Mre11. Among the 48 samples tested, protein expression of 5-6 samples from each age groups (20-30, 40-50, 60 and over) was shown. I.S. stands for internal standards obtained from Ku-expressing cell line. 1, 4.5, and 20 μg of lysates obtained from A549 human lung cancer cells were loaded onto the gel. Bottom Panel; Scatter plots of Ku70 (A) or Mre11 (B) protein level on age. Percentage of Ku70 or Mre11 expression in different aged individuals was obtained by dividing protein intensity of Ku70 or Mre11 by intensity of 20 μg of internal standards shown above. (C) Characteristics of study subjects in Seoul area. (D) Result of the regression analysis of Ku70 on age, as well as that of Mre11 on age.

stripping buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM 2-mercaptoethanol). Then re-probed blots were detected by monoclonal rabbit anti-human β-actin. The density of bands was measured using Fujix-Bio-Imaging analyzer BAS2500 (Fuji Photo Film).

Measurement of telomere length

The mean telomeric restriction fragment (TRF) length was measured in human T-lymphocyte cells. Genomic DNA was extracted with DNeasy Tissue Kit (Qiagen, USA) and 5 ug of DNA was digested with *Hin*fI and *Rsa*I (Boehringer Mannheim), and then electrophoresed through 1% agarose gels in 0.5 x TBE buffer at 14°C, using a CHEF MAPPER DRIII pulsed-field gel electrophoresis apparatus (Bio-Rad). Separation conditions were a ramped pulse speed of 1-6 s at 6 V/cm for 16 h. The gel was hydrolyzed for 5-10 min in 0.25 M HCl and denatured for 1 h in 0.5 M NaOH and 1.5 M NaCl. The gel was blotted and hybridized with a [γ-^{32}P] labeled telomeric oligonucleotide probe [γ-^{32}P-$(CCCTAA)_3$]. After washing, the gel was autoradiographed on phosphor screen (Kodak) for 30 min and those lanes in which mean TRF was calculated were scanned using Personal Molecular Imager FX system (Bio-RAD). Mean TRF length was defined as: $\sum(OD_i)/\sum(OD_i/L_i)$ where OD_i is the Personal Molecular Imager FX system output and L_i is the length of the DNA at position i. The amount of telomeric DNA was calculated by integrating the volume of each smear in Quantity one software (Bio Rad).

Statistical analysis

Demographic characteristics for study subjects were represented by *n* (%) or mean $\pm$ std as appropriately. Student's *t*-test was used to compare an age distribution between the longevity and control groups for subjects in Jeju area. A relation of age on Ku70, or that of Mre11, for the subjects in Seoul area was established by using a simple linear regression analysis. Effects of age and the longevity group on Ku70 were evaluated by using multiple regression analysis. Because there was no interaction effect between age and group on the expression of Ku70, main effect regression model was considered. Finally, a relationship between Ku70 and telomere length was examined by Pearson's correlation analysis. All statistical analyses were performed using SAS version 9.1 (SAS Institute Inc., Cary, NC). Reported *p*-values were two-sided, and $P < 0.05$ was considered to be statistically significant.

Results

Declining of expression of two DSB proteins, Ku70 and Mre11, is associated with increased age

Since an age-related decline in DNA double-strand break (DSB) repair (Mayer *et al*., 1991) and age-dependent telomere shortening (Weng *et al*., 1995) has been demonstrated in human lymphocytes, we examined if DSB repair proteins could show age-associated changes in their expression. For this end, we collected blood from 100 different aged volunteers ranging from 20 to 80 yr old living in Seoul, Korea. These individuals were all healthy with no visible signs of illness. CD4+ T lymphocytes were isolated from these individuals and the expression of telomere binding proteins, Ku70, Ku80, Sir2, TRF1, Mre11, were monitored by Western blot analysis using antibodies specific to each telomere binding proteins (Figure 1). Among these proteins tested, the protein level of Ku70 and Mre11 showed marked decline with respect to their age (Figure 1). As shown in Figrue 1, the protein level of Ku70 in individuals of 60 years old and over was only 50% of that in individuals of 20-30 yr old. A linear regression analysis of Ku70 (% of control) on age shows that the rate of Ku70 declining is close to -1 ($P = 0.0027$, Figure 1D), indicating that expression of Ku70 is under the tight control upon aging. Similarly, expression of Mre11 was decreased with age, but the rate of decline was much slower than that of Ku70 (Figure 1B). All the other telomere binding proteins tested, including Ku80, Sir2, TRF1, did not show any age-dependent changes in their expression level (data not shown). We next determined if the change in Ku70 expression is associated with change in Mre11 expression. As shown in Figure 2, we found that the level of Ku70 expression was directly proportional to that of Mre11 expression. These data show that upon aging, CD4 T lymphocytes gradually lose their ability to keep high level of Ku70 and Mre11, which may result in partial impairment of DSB repair and telomere shortening.

Slowed age-dependent declination of Ku70 in people living in the "longevity group"

Since the cellular level of Ku70 decreases with age, we questioned if the maintenance of Ku70 expression may be partially responsible for keeping long life span in human. To directly test this idea, we searched the database from Korean National Statistical Office for the communities that are known to have the highest average lifespan in Korea. The 46 subjects aged between 40 to 80 yr old in the longevity group were carefully selected from the families who have settled in the same community for

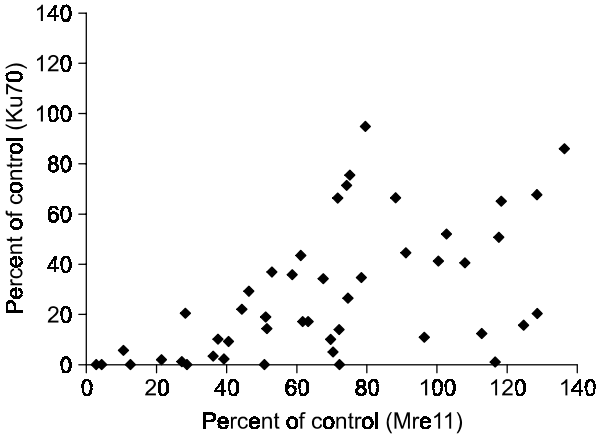
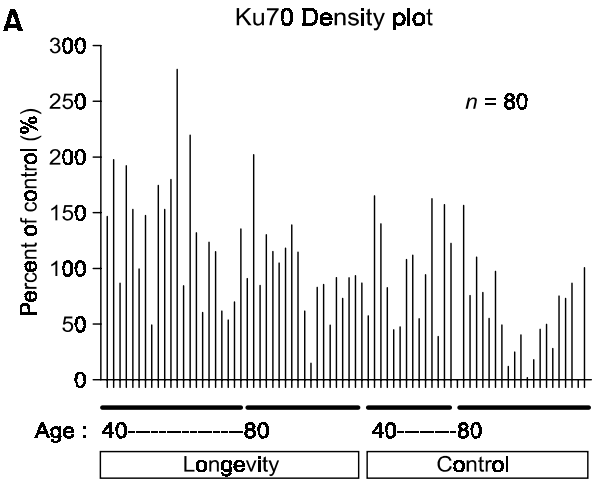
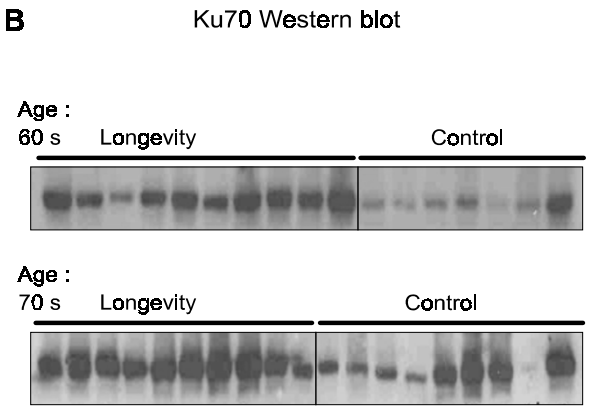


Figure 2. Correlation analysis of Ku70 and Mre11 expression in people aged between 20 and 80 yr. Scatter plots of Ku70 and Mre11 expression show significant linear relationship between Ku70 and Mre11 (Pearson's correlation coefficient, r = 0.5472, $P < .0001$). Percentage of Ku70 or Mre11 expression in different aged individuals was obtained by dividing protein intensity of Ku70 or Mre11 by intensity of 20 μg of internal standards obtained from Ku-expressing cell line, A549 human lung cancer cell lysates loaded onto the gel.

more than 100 yr. This selection minimizes the influence from other environmental and stressful exposures to the individuals in the longevity community, so that the data obtained from the longevity group can truly represent the whole longevity group. Control subjects were chosen within 100 miles of the longevity community which show similar life patterns but with much shorter average lifespan. 34 subjects from the control group were evenly distributed between 40 and 80 yr old. At the all ages tested, expression of Ku70 in the longevity group was significantly higher than that in the control group (Figure 3). Since there was no significant interaction effect between age and groups on Ku70 (P = 0.4684), the main effects regression model was used to find a relationship among age, groups and Ku70 (Figure 4). While the percentage of control in Ku70 declined significantly as age increased in both of the longevity and control groups ($P < .0001$), it was significantly higher in the longevity group than the control group (P = 0.0322). These results imply that the intrinsic amount of Ku70 in individuals of these two groups was different from the birth. Therefore, it is tempting to speculate that people who have





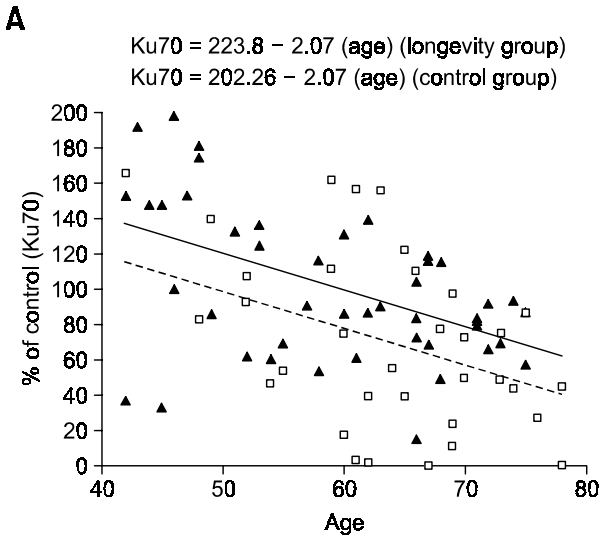
C

Characteristics of study subjects in jeju area

	Jeju area, longevity group	Jeju area, control group	*P* - value †
N (%)	46 (57.5)	34 (42.5)	
Age (years)*	59.2 ± 10.3	63.8 ± 8.9	0.0401

*Mean ± STD, †Student's *t*-test

Figure 3. Expression of Ku70 in CD4+ T cells obtained from the longevity group and the control group. (A), The protein level of Ku70 from 80 samples obtained from the longevity and the control group was expressed as a percentage of control (as described in Figure 1) and plotted as a bar graph against age. Characters or numbers on X coordinate are arbitrarily given subject designations. (B) Ku70 protein expression from the samples of 60 s and 70 s of old subjects was shown in anti-Ku70 immunoblots. (C), Characteristics of study subjects in Jeju area. There was no significant interaction effect between age and groups on Ku70 and the conclusion of our experiment (P = 0.4684).



B Multiple regression analysis of age and longevity group on Ku70 in jeju area

Variable*	Coeff.	Std. Err.	*P* - value
Intercept	202.26	32.24	< .0001
Age	−2.07	0.49	< .0001
Longevity†	21.54	9.88	0.0322

*R^2 = 0.27, †Reference = control group

Figure 4. Decreasing pattern of Ku70 with age for the control and the longevity group in Jeju area. (A) A scatter plot of Ku70 expression against age was shown for the longevity group and the control group, respectively. Percentage of Ku70 expression in individuals, obtained as described in Figure 1, was plotted against age. (B) A result for the multiple regression analysis with Ku70, as a dependent variable, and the age and group variables, as independent variables.

tendency to live long life span possess higher level of Ku70 protein, which is essential to maintain DSB repair activity.

No direct correlation between the level of Ku70 expression and telomere length

If the role of Ku70 is directly involved in the protection of telomeres, the length of telomere should be proportional to the cellular amount of Ku70. To test this, we measured mean telomere length from the nuclear extracts obtained from CD4+ T cells isolated from both longevity groups and the control groups and the individual telomere length was plotted against the level of Ku70. As shown in Figure 5, the telomere length measured in each individuals ranged anywhere between 5,000 to 8,000 bps. However, to our surprise, no significant correlation

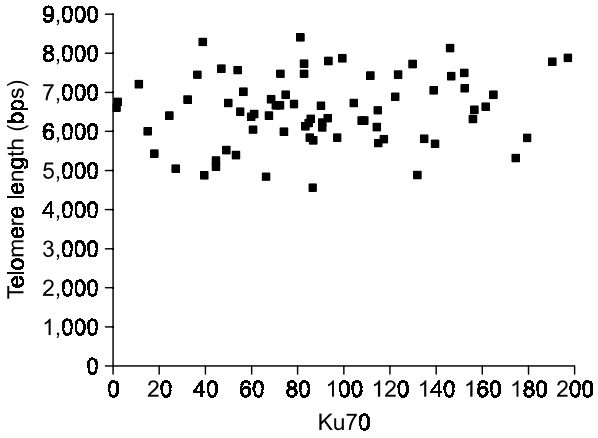


Figure 5. Scatter plot of Ku70 expression and the length of telomeres in Jeju area. Length of telomeres in CD4+ T cells was measured as described under Materials and Methods and plotted against expression of Ku70 (Pearson's correlation coefficient, r = 0.1379, *P* = 0.2350). There was no statistically significant correlation between the level of Ku70 and the length of telomere.

was observed between the length of telomere and the level of Ku70 (r = 0.138, *P* = 0.2350). It suggests that Ku70 may not directly function to regulate the length of telomere, but rather affects other aspect of repair activity within the telomere region of the chromosome. Therefore, the maintenance of Ku70 levels may be critical for keeping longer life span by affecting mechanisms other than the lengthening of telomeres.

Discussion

The telomeres in human somatic cells undergo progressive shortening during cell division by replication-dependent loss of sequence at DNA termini. Maintenance and stabilization of telomeric DNA is assisted by binding of multiple DNA-binding proteins, including those involved in double strand break (DSB) repairs. Because functional telomeres are essential for continuous cellular proliferation and stable genetic inheritance, loss of chromosomal end capping has consequences in both aging and cancer (Harley *et al*., 1992; Artandi *et al*., 2000; DePinho and Wong, 2003). We discovered in this study that the total protein level of Ku70 declines in human lymphocytes with respect to the age (Figure 1A). Since an age-related decline in DNA double-strand break (DSB) repair (Mayer *et al*., 1991) and age-dependent telomere shortening (Weng *et al*., 1995) was reported in human lymphocytes, decline of Ku70 expression seen in aged individuals may underlie the basis of telomere inconsistancy and cellular

senescence. Furthermore, the level of Ku70 was significantly higher in the people in the community that has higher average lifespan than those living in the control community (Figures 3 and 4), again providing a correlation between the level of Ku70 and the lifespan. Together our data are in consistent with the idea that the cellular level of Ku70 in human lymphocytes reflects the biological age, and the loss of Ku70 may be critically leading to cellular senescence.

DSBs are potentially catastrophic lesions that if not repaired will lead to loss of genetic information and mutagenesis or cell death. In mammalian cells, two major pathways exist to repair DSBs; homologous recombination (HR) and nonhomologous end-joining (NHEJ; Liang *et al*., 1998). Several processes exist in which repair of a DSB is restricted to either NHEJ or HR. Ku70, existing as the Ku70 /Ku80 heterodimer complex, is a component of DNA-dependent protein kinase (DNA-PK). Along with Ku80 and DNA-PKcs (DNA-PK catalytic subunit), Ku70 participates in DSB repair through NHEJ. Mutations in any of the component of DNA-PK result in end-to-end fusions between chromosomes and deleterious terminal rearrangements (Bailey *et al*., 1999). Similarly, telomeric fusions and dysfunction was reported when Ku70 gene was deleted in a mouse (Bailey *et al*., 1999; d'Adda di Fagagna *et al*., 2001). Thus, loss of Ku70 in aging cells as seen in our study is likely to contribute to the telomere instability and telomere shortening process. Interestingly, the level of other proteins involved in DSB repairs, Ku80, Sir2, TRF1, did not show any age-dependent changes. These data indicate that not all telomere-binding proteins undergo tight regulation of its protein expression upon aging. However, Mre11, which is shown to exist as a complex with Rad50 and Nbs1, undergoes age-dependent decline of its expression in human lymphocytes (Figure 1B). Mre11 complex was shown to influence recombinational DNA repair through promoting recombination between sister chromatids. Hypomorphic mutants of Mre11 have been shown to cause human genetic instability disorders (Stracker *et al*., 2004). Given the role of Ku70 and Mre11 in DSB repair and chromosome stability, age-dependent decline of Ku70 and Mre11 seen in our study may directly be associated with cell senescence and aging.

Aging is a process that changes healthy individuals into weak ones with decreased performance of most physiological systems and increased susceptibility of disease and death. The rate of aging and maximum lifespan varies among species, and therefore must be at least partly under genetic control (Miller *et al*., 2002; Hekimi and Guarente, 2003). It has been shown that up to 25% of the variation in human lifespan is inheritable (Herskind *et al*., 1996; Mitchell *et al*., 2001); the rest is due to environmental exposures, accidents and injuries, and chance. Very long life, to beyond age 90 years, appears to have an even stronger genetic basis (Perls *et al*., 2002), which explains why centenarians and near-centenarians tend to cluster in families. At the other extreme, the progeroid syndromes of accelerated aging and death at an early age have known genetic causes (Martin, 1985; Fossel, 2000). Although various regions of chromosomes 1, 2, 4, 7, 12 and 17 have been found to interact among themselves and with environmental factors to influence the median lifespan in 20 inbred strains (Gelman *et al*., 1988). Some of these chromosomes bear genes coding molecules with relevant functions in aging process. In this study, we are the first to show that the total protein expression of Ku70 decreases upon aging in human lymphocytes and the ability to retain Ku70, as shown in the longevity group, may partially contribute to the elongated lifespan observed in the longevity community. Decreased expression of Ku70 and Mre11 upon aging will not only impair the ability to repair DSBs, but also predisposes cells to the damage towards environmental factors including oxidative stress, ionizing radiations, toxic chemicals, etc.

Taken together, our data show, for the first time, that Ku70 and Mre11 may represent new biomarkers for aging and further suggest that maintenance of higher expression of Ku70 and Mre11 may be responsible for keeping longer life span observed in the longevity group.

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