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Age Prediction in the Chickens Using Telomere Quantity by Quantitative Fluorescence *In situ* Hybridization Technique*

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ABSTRACT : Telomeres are special structures at the ends of eukaryotic chromosomes. Vertebrate telomeres consist of tandem repeats of conserved TTAGGG sequence and associated proteins. Birds are interesting models for molecular studies on aging and cellular senescence because of their slow aging rates and longer life spans for their body size. In this longitudinal study, we explored the possibility of using telomeres as an age-marker to predict age in Single Comb White Leghorn layer chickens. We quantified the relative amount of telomeric DNA in isolated peripheral blood lymphocytes by the Quantitative Fluorescence *in situ* Hybridization technique on interphase nuclei (IQ FISH) using telomere-specific DNA probes. We found that the amount of telomeric DNA (ATD) reduced significantly with an increase in chronological age of the chicken. Especially, the telomere shortening rates are greatly increased in growing individuals compared to laying and old-aged individuals. Therefore, using the ATD values obtained by IQ FISH we established the possibility of age prediction in chickens based on the telomere theory of aging. By regression analysis of the ATD values at each age interval, we formulated an equation to predict the age of chickens. In conclusion, the telomeric DNA values by IQ FISH analyses can be used as an effective age-marker in predicting the chronological age of chickens. The study has implications in the breeding and population genetics of poultry, especially the reproductive potential. (**Key Words :** Chickens, Telomere, Aging, Age Prediction, Fluorescence *In situ* Hybridization)

INTRODUCTION

Telomeres are special end structures present in eukaryotic chromosomes comprised of tandem repeats of TTAGGG sequence, conserved in all vertebrates (Moyzis et al., 1988; Blackburn, 1991). Telomeres therefore serve to protect chromosomes and maintain genomic integrity from recombination, exonuclease degradation and end-to-end fusion (Blackburn et al., 2000; Proctor and Kirkwood, 2002). Normally, replication of eukaryotic chromosomes in somatic cells by DNA polymerase is incomplete due to inherent inability of the enzyme. The enzyme cannot fully replicate the 5′ end of the terminal DNA sequence and consequently leads to continual loss of a small amount of DNA fragments with each round of mitotic cell division (Hastie et al., 1990). Approximately, 50-100 bases of DNA fragment are lost with each round of cell division, and this loss progresses until reaching a critical state, at which cells cease dividing and may lead to death (Vaziri et al., 1993; Campisi et al., 2001). Thus, telomeres of somatic cells tend to decrease with organism age (Harley et al., 1990; Allsopp et al., 1992). Some reports have demonstrated that human telomeres significantly decreased with increasing chronological age (Friedrich et al., 2000; Tsuji et al., 2002). Studies conducted on animal species including chickens, bats, dogs, sheep, rats and worms also reached a similar conclusion that telomeres progressively shorten with increasing age (Shiels et al., 1999; Nasir et al., 2001; McKevitt et al., 2002; Haussmann et al., 2003; McKevitt et al., 2003; Hastings et al., 2004; Joeng et al., 2004; Jung et al., 2006).

In spite of higher metabolic rates, high body temperatures, and much higher blood glucose levels, birds are known for their remarkably slow aging rates and longer life spans in comparison to similar-sized, non-flying mammals (Monnier, 1990; Holmes and Austad, 1995; Holmes et al., 2003; Munshi-South and Wilkinson, 2010). Thus, avian species, particularly chickens, are an interesting model for molecular studies on aging and cellular senescence (Swanberg and Delany, 2005). Chickens have both terminal (156 telomeres) and interstitial telomeric

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sequences (Nanda and Schmid, 1994; Solovei et al., 1994; Delany et al., 2003). In spite of being only one-third in genomic size when compared with the human genome (2.5 pg vs. 6.5 pg), the chicken genome contains significantly more (10 fold) telomeric DNA sequence (Delany et al., 2003). Similarly to humans, somatic tissues of chickens exhibit telomere shortening and decreasing telomerase activity with chronological age (Taylor and Delany, 2000; Cho et al., 2005; Jung et al., 2006).

In this study, we hypothesized that the amount of telomeric DNA (ATD) of peripheral blood lymphocytes in chickens is reduced with an increase in chronological age, and conversely, the quantified ATD values in lymphocytes can be used to predict the chronological age of the individual. Thus, we explored the possibility of using telomeres as an age-marker to predict chicken age. We successfully predicted chicken age by regression analysis based on telomeric DNA measurement in lymphocytes by Quantitative Fluorescence *in situ* Hybridization technique (Q FISH).

MATERIALS AND METHODS

Animals and preparation of cells

In this longitudinal study, we investigated the amount of telomeric DNA (ATD) using Quantitative Fluorescence *in situ* Hybridization technique on interphase nuclei (IQ FISH) in lymphocytes collected from chickens at different ages. The chicken flock of the present study was raised at the University farm, Jinju, Republic of Korea, comprising 178 Single Comb White Leghorn (WL) layers and was sampled at 10-week intervals starting at 10 weeks old to 80 weeks of age. Peripheral blood (3 ml) was collected from the brachial wing vein into heparin tubes using standard blood collection procedures. After collection, the lymphocytes were separated using the method described by Lovoie and Grassman (2005). Interphase nuclei of lymphocytes were prepared from the isolated lymphocyte fraction by hypotonic treatment in 0.06 M potassium chloride (KCl) solution and then the cell nuclei were fixed in 3:1 methanol:acetic acid (Carnoy's fixative) and stored at 4°C until cast onto clean, pre-cooled glass slides.

Quantitative fluorescence *in situ* hybridization on interphase nuclei using a telomere specific DNA probe

The telomeric probe containing 42 bp of telomeric sequence was simultaneously amplified and labeled with digoxigenin (dig) by polymerase chain reaction (PCR) through a dig labeling kit (Roche, Mannheim, Germany), using chicken genomic DNA as a template, and 5′-$(CCCTAA)_7$-3′ primers. The procedure for the Quantitative Fluorescence *in situ* Hybridization technique on interphase nuclei was slightly modified from that of Sohn et al. (2002, 2007). Briefly, the slide containing the interphase nuclei of the lymphocytes was incubated in 50 ml of 2xSSC (1x = 150 mM NaCl and 15 mM sodium citrate, pH 7.0) containing 1 μg of RNase A (Sigma-Aldrich, Saint Louis, MO) for 30 min and dehydrated in an increasing ethanol gradient. After dropping 100 ng of the dig-labeled probe in 10 ml of hybridization solution (40% 4x SSC, 50% formamide and 10% dextran sulfate; Roche) onto the slide, the specimen was sealed with a coverslip using rubber cement, followed by denaturation at 78°C for 10 min on a heating block and hybridization at 37°C overnight. The slide was then washed with 2x SSC at 72°C for 5 min, followed by PN buffer (7 mM sodium phosphate, pH 8.0, containing 0.1% Nonidet40; Roche) at room temperature for 2 min, and finally air dried for 10 min. The dried slide was incubated with an anti-dig-fluorescein isothiocyanate (FITC) conjugate (10 ng/ml in PN buffer containing 5% non-fat dry milk; Roche) at 37°C for 30 min. The slide was washed with PN buffer, counter-stained with propidium iodide (PI) and examined under a fluorescence microscope (Model AX-70, Olympus, Tokyo, Japan) at green (FITC) and red (PI) dual excitation wavelengths. The image was captured by a digital camera (DP-70, Olympus) and analyzed using the MetaMorph (Universal Imaging Co., PA, USA) image analysis program. At least 100 interphase nuclei in each specimen were examined for telomere specific signals in each specimen.

Statistical analysis

The measured data were expressed as the means and standard errors. The data were analyzed using the General Linear Model procedure of Statistical Analysis System Institute (SAS Institute Inc., Cary, NC, USA). Tukey's Honestly Significant Difference procedure was used to compare treatment means. Differences were considered statistically significant at $p<0.05$.

The PROC REG of SAS was used to compute the regression equation and correlation coefficient between chicken age and the amount of telomeric DNA. To predict the chicken age, a curvilinear regression was fitted using the following equation

$$\hat{Y} = aX^2+bX+c$$

where $\hat{Y}$ is predicted chicken age, X is the amount of telomeric DNA and a, b, c represent components of the curvilinear equation.

RESULTS

Amount of telomeric DNA and telomere shortening rate

The ATD values were calculated as a percentage of the fluorescence intensity of the telomeric DNA spots relative

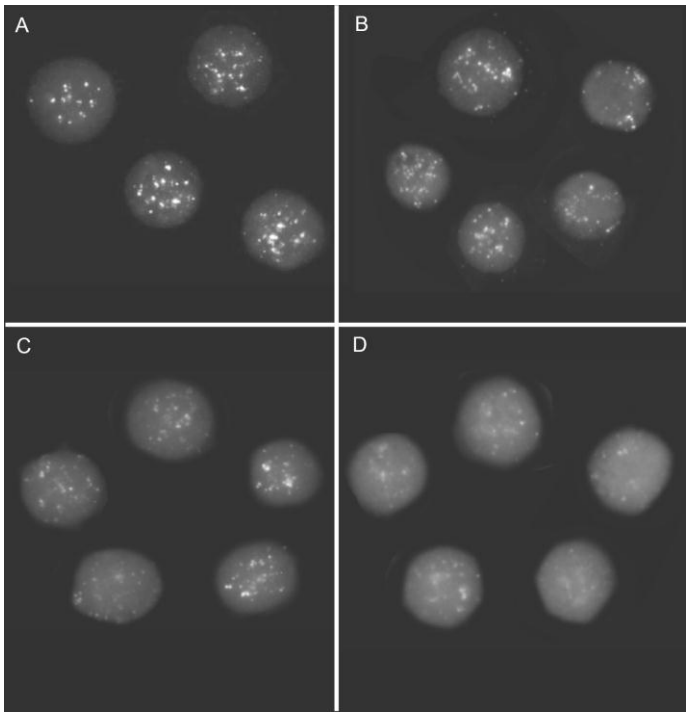

Figure 1. The distribution of telomeres on interphase nuclei of chicken lymphocytes. The telomeric DNAs were visualized as green/yellow spots due to FITC labeled telomeric DNA probe and nuclei were seen as red color due to propidium iodide. These figures show lymphocytes at (A) 10 week, (B) 30 week, (C) 60 week, (D) 80 week age, containing 2.93%, 1.99%, 1.44%, and 1.29% ATD respectively. The ATD values are calculated as percentage values of the fluorescence intensity given out by green and red color using MetaMorph (Universal Imaging Co., PA, USA), an image analysis program.

to the interphase nuclei seen as background using MetaMorph (Universal Imaging Co., PA, USA) image analysis program (Figure 1). We thus found that the ATD in peripheral blood lymphocytes of WL layers significantly ($p<0.0001$) varied with age of the chickens (Table 1; Figure 2A). The results clearly showed that the amount of telomeric DNA progressively decreased with an increase in age.

We also evaluated telomere shortening rates (TSR) based on the ATD values. TSR values were calculated by subtracting the average ATD value of the older age group from each of the raw data of the young group which was generated by the interphase nuclei using the Metamorph image analysis software. Thus, we analyzed TSRs of individual chickens in the growing stage (10 to 20 weeks age), laying stage (20 to 50 weeks age) and old age stage (50 to 80 weeks age), (Table 1, Figure 2B). The results showed that telomere shortening was maintained at a faster rate during the growing stage up until the laying period, and thereafter drastically slowed down and continued at much

Table 1. Relative amount of telomeric DNA and corresponding telomere shortening rate in the lymphocytes of white Leghorn layers at different ages

Age in weeks	No. of chickens	Amount of telomeric DNA	Telomere shortening rate
		---------- % ----------	
10	154	2.95±0.04^{a}	-
20	147	2.23±0.05^{b}	0.84±0.05^{a}
30	109	1.81±0.04^{c}	0.62±0.05^{b}
40	130	1.57±0.02^{d}	0.45±0.03^{c}
50	126	1.54±0.02^{de}	0.19±0.02^{d}
60	92	1.42±0.02^{ef}	0.15±0.03^{d}
70	90	1.32±0.02^{fg}	0.15±0.03^{d}
80	85	1.26±0.02^{g}	0.10±0.01^{d}
Mean		1.76±0.20	0.36±0.11

The values are means±standard error mean (SEM). Different superscripts in same column differ significantly ($p<0.0001$). The telomere shortening rate values were calculated as difference in the amount of telomeric DNA between two consecutive age groups longitudinally.

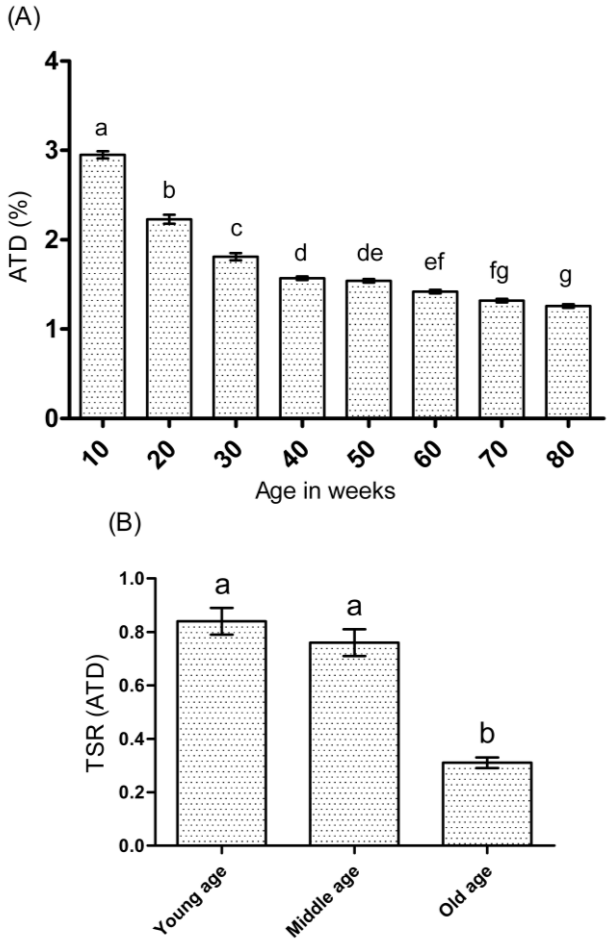

Figure 2. Trend of the amount of telomeric DNA (A) and telomere shortening rate (B) in lymphocytes of white Leghorn layers with age in weeks. (A) The values are shown as means with standard error bars. The different letters atop the error bars represent significantly different amounts of telomere across ages at $p<0.01$. (B) Telomere shortening rates were calculated by the relative amount of telomeric DNA values - a result of the Q FISH technique for white Leghorn layers. Telomere shortening rates of young-, middle- and old-aged chicken belonged to age groups of 10 to 20 weeks, 20 to 50 weeks and 50 to 80 weeks, respectively. The different letters atop the standard error mean bars represent significantly different amounts of TSR at $p<0.01$.

slower rates later in life.

Age prediction by regression analysis

From our results, we clearly observed a negative trend between the amount of telomeric DNA and age of the individual. We performed regression analysis on the relative amount of telomeres across age to evaluate the possibility of using telomere amount as an age-marker to predict age. We found a high negative correlation between age and the relative amount of telomeric DNA which is in agreement with the telomere theory of aging for somatic cells. The regression analysis resulted in the following age predicting equation for an amount of telomeric DNA (x) quantified by the IQ FISH technique:

$$\hat{Y} = 8.5709X^2 - 62.982X + 124.16 \quad \text{(Figure 3)}$$

where $\hat{Y}$ = predicted chronological age of chicken in weeks, X = amount of telomeric DNA of lymphocytes - a result of Q FISH analysis expressed as percentile, constants 8.5709, and -62.982 are the regression coefficients ($p< 0.001$) for the relative amount of telomeric DNA, and 124.16 is the residual value. The coefficient of determination (R^2) of this equation was calculated as 0.6085.

DISCUSSION

Actively dividing somatic cells do not effectively compensate the loss of telomeric sequences due to the DNA end replication problem (Counter et al., 1992). Aged cells have shortened telomeres. This telomere theory of aging conceived by Olovnikov (1973) and Harley (1995) is supported by numerous studies *in vitro* and *in vivo* (Harley et al., 1990; Forsyth et al., 2002). Several studies have proposed the use of telomeres in predicting the age of the remaining lifespan in humans (Cawthon et al., 2003; Aviv et

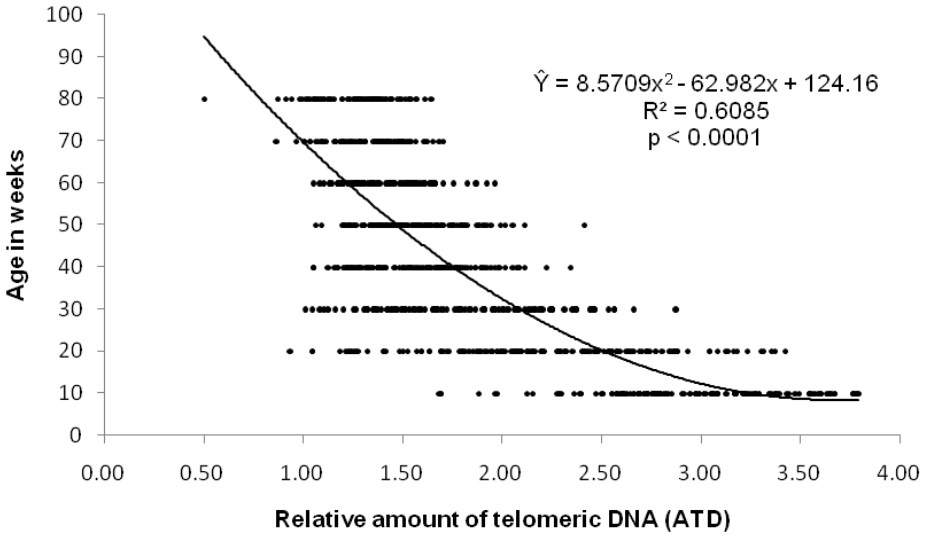

Figure 3. Regression curve and equation of chicken age in weeks on the relative amount of telomeric DNA. $\hat{Y}$ is predicted chronological age of chicken and variant x is the amount of telomeric DNA (ATD) quantified by IQ FISH. R^2 is the coefficient of determination of this regression equation. Dots(•) represent the observed ATD values in each individual.

al., 2009), tree swallows (Haussmann et al., 2005), alpine swifts (Bize et al., 2009) and nematodes (Joeng et al., 2004). Independent studies have even attempted to use telomeres as an age prediction marker in humans to aid forensic investigations (Tsuji et al., 2002; Hewakapuge et al., 2008; Ren et al., 2009). However, results of these studies are unclear, contradictory and faced by methodological problems such as inadequate sample size, cross-sectional sampling and techniques based on isolated DNA which is prone to mechanical damage. It is all the more ambiguous as there are no further studies reported to support any of these findings. These attempts, though unclear with regard to the objective of developing telomeres as age prediction markers, were certainly encouraging. Firstly, we clarified the reasons for the unclear views of earlier work and concluded that they were attributable to the cross-sectional nature of the study, which yielded biased estimates for the effects of within and between individuals. Secondly, limitations of the technique employed to quantify the amount of telomeres were noted. Thus, in order to minimize these confounding factors, we performed this longitudinal study using the same group of chicken throughout the experimental period and sampling them at a regular interval.

With regard to the technique employed, we must note here that absolute quantification of telomeric DNA of a given cell type is still obscure and ambiguous. Almost all of the studies are either based on telomere restriction fragment (TRF) analysis or quantitative real time polymerase chain reaction (Q PCR) based analysis. The traditional method of quantifying telomere amount by TRF assay using Southern blot analysis is less sensitive over the Q FISH technique which gives a resolution of up to 200 bp (Slijepcevic, 2001; Baird and Kipling, 2004). Telomere restriction fragment analysis dwells in the unknown realm of the flanking DNA in the sub-telomeric region and on the property and specificity of the restriction enzymes used, together with the inability to quantify the interstitial telomeric sequences. It should be noted here that the chicken genome contains ultra long telomeres with both terminal and interstitial telomeric sequences (Solovei et al., 1994; Nanda and Schmid, 1994; Delany et al., 2003). Thus, TRF analysis may be less informative in chicken species and hence would result in poor analysis qualitatively and less sensitive quantification in comparison to the Q FISH analysis. The relatively new Q PCR technique for measurement of the amount of telomeres (Cawthon, 2002, 2009) is based on relative quantification, and therefore further modifications are required for the technique to be applied to measure the absolute telomere length in chickens (O'Callaghan et al., 2008; Thomas et al., 2008). Our study used the Q FISH technique on interphase cells. Though a labor intensive and an elaborate technique needing expertise to perform, it is highly sensitive for the quantification of chicken telomeres (Slijepcevic, 2001; Baird and Kipling, 2004). Another advantage of the technique is its ability to study an individual cell or specific cell type when compared to PCR-based techniques and TRF analyses that depend highly on isolated DNA, which is prone to mechanical shear, damage, loss of DNA termini and an aggregation of genomic DNA from a group of cells. Thus, with the clear understanding of the shortcomings, we hereby established the possibility of chicken age prediction based on the telomere theory of aging.

The age predicting equation ($\hat{Y} = 8.5709X^2-62.982X+124.16$) obtained by regression analysis can predict chicken

chronological age in weeks for an amount of telomeric DNA (X) quantified by the IQ FISH technique with high accuracy (R^2 = 0.6085) at a high significance level ($p<0.0001$). Although this regression of chicken age on the telomeric quantity was highly significant and predictable, the equation was invalid over a productive life span because the regression estimates were calculated with limited data from the 10 to 80 weeks observed. It can also be seen from Figure 2B, that telomere shortening progresses at increasingly rapid rates during the intensive growth period. Telomere shortening only partially slows down when reaching middle age, and thereafter drastically slows down and continues at much slower rates further in life with advancing age. The observed results suggested a consistent replicative burst needed for rapid growth and development leading to sexual maturity, adulthood and reproductive potency in these animals. However, after attaining reproductive ability the replicative rate slows down gradually, leading to replicative senescence and quiescence which is evident as decreased TSR values. Our results are in agreement with findings in humans (Frenck et al., 1998) and also with recent findings on free-living corvids (Salomons et al., 2009). Thus, telomere biology in vertebrates, especially the loss of sequences from the ends of chromosomes in lymphocytes, occurs at different rates over time following the developmental stage-dependent model of telomere shortening.

We conclude that telomere amount obtained as ATD values by IQ FISH analyses can be used as an effective age-marker in predicting chronological age of the chicken - a vertebrate. The study has implications in breeding and population genetics of any vertebrate, especially the reproductive potential. However, it should also be noted here that several genetic, epigenetic (environmental) and stochastic factors affect the quantity of telomere in an individual. By accounting for these influences accompanied by automation and high-throughput conversion to the Q FISH technique, the accuracy of age prediction can be advanced.

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