

# Lack of association between leukocyte telomere length and genetic variants in two ageing-related candidate genes<sup>☆</sup>

Feng Zhang<sup>a,\*</sup>, Bernet S. Kato<sup>a</sup>, Jeffery P. Gardner<sup>b</sup>, Masayuki Kimura<sup>b</sup>,  
Tim D. Spector<sup>a</sup>, Kourosh R. Ahmadi<sup>a</sup>

<sup>a</sup>Twin Research and Genetic Epidemiology Unit, St. Thomas' Hospital, King's College London, United Kingdom

<sup>b</sup>The Centre of Human Development and Ageing, University of Medicine and Dentistry of New Jersey, United States

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## Abstract

**Background:** Leukocyte telomere length, a putative marker of ageing, is a highly variable and heritable complex trait. In order to determine the possible underlying genetic variants for leukocyte telomere length variation, we conducted an association study of leukocyte telomere length and two candidate genes for ageing-related traits, *TGFBI* and *KLOTHO*, in a female Caucasian dizygotic twin population.

**Methods and materials:** Terminal restriction fragment (TRF) length, an index of telomere length, was measured using Southern Blotting. Six and four single nucleotide polymorphisms (SNP) were genotyped in *TGFBI* and *KLOTHO* gene, respectively, and tested for association. When there is strong LD between SNPs ( $r^2 > 0.5$ ), haplotypic association was investigated using haplotype trend regression approach.

**Results:** All SNPs were in Hardy–Weinberg equilibrium ( $p > 0.05$ ). No significant association was detected for individual SNPs ( $p > 0.101$ ), or two-locus haplotypes ( $p = 0.7497$ ) with TRF variation.

**Conclusion:** We failed to find any significant association between leukocyte telomere length and 10 SNPs in two ageing-related candidate genes, *TGFBI* and *KLOTHO*. This result suggests that while we could not exclude minor effects, none of 10 SNPs in these two candidate genes showed significant association with the variation of leukocyte telomere length in our cohort. But as it is unclear whether telomere length dynamics is the cause or the effect of the ageing process, it is still possible the genes are associated with ageing via alternate mechanisms.

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**Keywords:** Leukocyte telomere length; *TGFBI*; *KLOTHO*; Ageing; Genetic association

## 1. Introduction

Telomeres are the tandem repeat of *TTAGGG* at the ends of chromosomes and undergo attrition with each division in somatic cells (Blackburn, 2000; Harley, 1991). Telomere length reflects the replicative history and proliferative potential of somatic cells, and also serves as mitotic clock at the cellular level (Harley, 1991). Particularly, leukocyte telomere dynamics (telomere length and attrition rate) *in vivo* reflects the accumulative ageing

process and provides information about the biology of human ageing above and beyond the chronological age (Aviv, 2004).

Leukocyte telomere length is a heritable but highly variable complex trait (Andrew et al., 2006; Benetos et al., 2001; Nawrot et al., 2004; Vasa-Nicotera et al., 2005). On average, it is significantly longer in women than in men (Benetos et al., 2001; Jeanclos et al., 2000; Mayer et al., 2006; Nawrot et al., 2004; Vasa-Nicotera et al., 2005). Besides the strong heritable components, accelerated leukocyte telomere attrition is also associated with environmental factors, such as smoking, obesity, life stress and social economic status (Cherkas et al., 2006; Epel et al., 2004; Valdes et al., 2005). Shorter leukocyte telomere length has been associated with ageing-related conditions, such as dementia, insulin resistance, hypertension, atherosclerosis, stroke and myocardial infarction (Benetos et al., 2001, 2004; Demissie et al., 2006; Gardner et al., 2005; Honig et al., 2006; von Zglinicki et al., 2000). Although the exact mechanism for

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\* Corresponding author at: Twin Research & Genetic Epidemiology Unit, St. Thomas' Hospital Campus, King's College London, Lambeth Palace Road, London SE1 7EH, United Kingdom. Tel.: +44 20 71886731; fax: +44 20 71886717.

E-mail address: [feng.zhang@kcl.ac.uk](mailto:feng.zhang@kcl.ac.uk) (F. Zhang).

telomere length variation as an indicator of biological ageing is still mainly unknown, the factors implicated in the ageing-related pathways, especially inflammation and oxidative stress related pathways, have been shown to play important roles (Finch and Crimmins, 2004; Finkel and Holbrook, 2000). Transforming Growth Factor Beta-1 (*TGFBI*) and *KLOTHO* are two of the strongest candidate genes implicated in the ageing-related pathways and have been associated with ageing and its related phenotypes.

*TGFBI* gene (MIM: 190180) is located on chromosome 19q13. It consists of seven exons and spans about 23.5 kb genomic DNA in *Homo sapiens*. *TGFBI* is an important anti-inflammatory cytokine that controls cell proliferation and differentiation, and is involved in the apoptosis of different cells types (Emmanuel et al., 2002; Jackson et al., 2006; Miyazono et al., 2001; Solovyan and Keski-Oja, 2005). *TGFBI* is also linked with many ageing-related traits, such as osteoporosis, atherosclerosis, stroke, myocardial infarction, dementia and different types of cancers (Cambien et al., 1996; Langdahl et al., 2003; Lau et al., 2004; Pfeilschifter et al., 1998). Its involvement in ageing might occur through inflammation and stress response pathways, and its plasma level has been found to be increased in centenarians as compared to young people (Capri et al., 2006; Carrieri et al., 2004).

*KLOTHO* gene (MIM: 604824) is located on chromosome 13q12. It contains five exons spanning approximately 50 kb of genomic DNA. It encodes a type-I membrane protein and functions as an ageing-suppressor gene (Matsumura et al., 1998). When overexpressed in mice, it extends life span; when deleted, the mice (*klotho*  $-/-$ ) manifest a wide range of ageing-related phenotypes, such as a short lifespan, infertility, arteriosclerosis, skin atrophy, emphysema, gait disturbance, ectopic calcification, osteopenia/osteoporosis, emphysema, pituitary abnormalities and abnormal calcium metabolism (Kuro-o et al., 1997; Kurosu et al., 2005; Roush, 1997). In human population-based association studies, *KLOTHO* gene has also been associated with some of ageing phenotypes such as osteopenia/osteoporosis, arteriosclerosis and abnormal calcium (Arking et al., 2002, 2003, 2005; Kawano and Kawaguchi, 2004; Low et al., 2005; Mullin et al., 2005; Yamada et al., 2005). *KLOTHO* gene product is part of an ageing-related pathway, insulin/*IGF-1* signalling pathway, and its function of delaying the ageing might act through increasing the resistance to oxidative stress (Bartke, 2006; Kuro-o et al., 1997; Yamamoto et al., 2005). *KLOTHO* has also been reported as a cofactor to increase the affinity of *KLOTHO*-fibroblast growth factor (*FGFR*) complex that binds to *FGF23*. Mice deficient in *FGF23* (*FGF23*  $-/-$ ) exhibit a variety of ageing-like phenotypes, many of which are similar to the ones manifested in *KLOTHO* deficient mice (*klotho*  $-/-$ ) (Kuro-o, 2006; Kurosu et al., 2006; Razzaque et al., 2006).

In order to determine the possible underlying genetic variants for leukocytes telomere length variation, we undertook an association study of leukocytes telomere length variation and 10 genetic variants in two ageing candidate genes, *TGFBI* and *KLOTHO* in a large female dizygotic (DZ) twin population of Caucasian origin.

## 2. Methods and materials

### 2.1. Subjects

Subjects comprised of 982 healthy female Caucasian DZ individuals who were ascertained through the TwinsUK registry in St. Thomas' Hospital, King's College London. All participants were recruited from the general population without presence or interest in any particular disease or trait through national media campaigns in the UK. Information on medication history and demographic variables, such as age, body mass index (BMI) and smoking status, were obtained for each individual through standardised questionnaire and detailed clinical examinations.

The study was approved by St. Thomas' Hospital Research Ethics Committee. All participants were formally informed and consented in writing. All samples were anonymously coded. Genotyping and telomere length measurement were performed independently by people who were blind to the characteristics of samples.

### 2.2. Telomere length measurement

Terminal restriction fragment (TRF) length, an index of telomere length, was measured in DNA extracted from isolated white blood cells by Southern Blotting approach as described elsewhere (Benetos et al., 2001). Briefly, the samples were digested overnight with restriction enzymes *HinfI* (0.5 U/ $\mu$ L) and *RsaI* (0.5 U/ $\mu$ L) (Roche, Indianapolis, IN). DNA samples and DNA ladders were resolved on a 0.5% agarose gel (20 cm  $\times$  20 cm) at 50 V (GNA-200 Pharmacia Biotech). After 16 h, the DNA was depurinated for 15 min in 0.25N HCl, denatured 30 min in 0.5 mol/L NaOH per 1.5 mol/L NaCl, and neutralized for 30 min in 0.5 mol/L Tris (pH 8)/1.5 M NaCl. The DNA was transferred to a positively charged nylon membrane for 1 h (Roche) using a vacuum blotter (Applicone, Oncor, Gaithersburg, MD). The membranes were hybridized at 65 °C with the telomeric probe digoxigenin 3'-end labeled 5'-(CCCTAA)<sub>3</sub> overnight in 5 $\times$  saline sodium citrate (SSC) and 0.1% Sarkosyl, 0.02% SDS, and 1% blocking reagent and then were washed three times at room temperature in 2 $\times$  SSC, 0.1% SDS, each for 15 min, and once in 2 $\times$  SSC for 15 min. The probe was detected by the digoxigenin luminescent detection procedure and was exposed on X-ray film. TRF length was measured in duplicates for each individual (on different gels). If the difference between the duplicates was more than 5%, a third measurement was performed and the mean of two results less than 5% apart was taken. The paired samples were run together to avoid any batch effects.

### 2.3. Ascertainment of SNPs for genotyping

Six and four putatively functional single nucleotide polymorphisms (SNP) were chosen across *TGFBI* and *KLOTHO* gene, respectively. They either have been reported as functional variants, or reported as significant with ageing-related phenotypes in previous studies. The gene structure and the positions of selected SNPs are illustrated in Fig. 1 (also detailed in Table 2).

### 2.4. *TGFBI* genotyping

Genotyping was performed by restriction enzyme digestion after Polymerase Chain Reaction (PCR). The reactions were performed on Perkin Elmer 9700 thermal cycler using 50 ng genomic DNA for each PCR reaction with final volume of 20  $\mu$ L. PCR product was digested by appropriate restriction enzymes (New England BioLab) at recommended temperatures following the manufacturer's instruction (details available upon request). Digested products were run on 2.5% (w/v) agarose gel in 1 $\times$  TAE buffer containing 0.5  $\mu$ g/mL ethidium bromide. Genotypes were scored according to fragment patterns under UV transilluminator.

### 2.5. *KLOTHO* genotyping

Genotype for each sample was scored by Taqman allelic discrimination assay using ABI Prism 7200 sequence detection platform. According to manufacturer's recommendations, the concentrations of primer and probe were

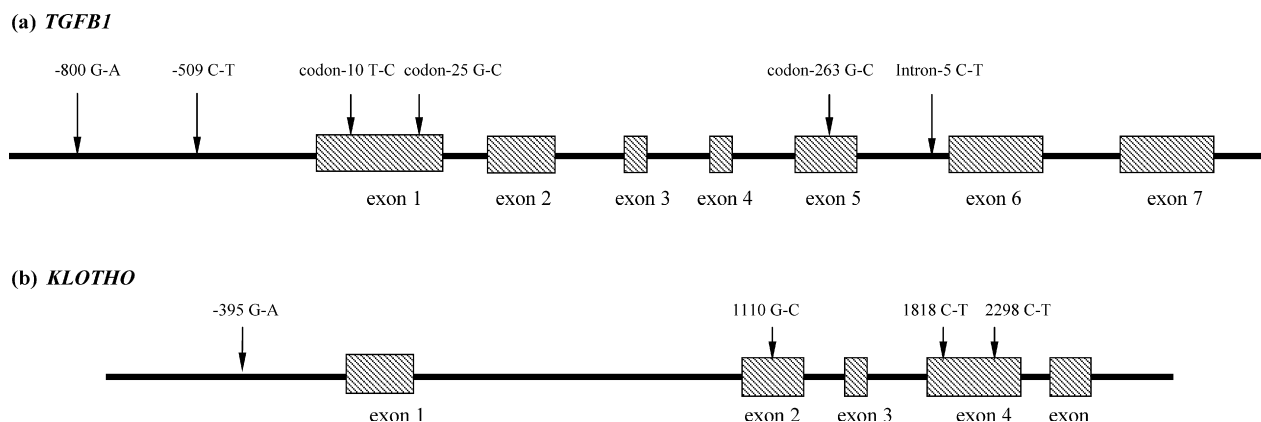


Fig. 1. Gene structure and the positions of selected polymorphisms in the *TGFBI* (a) and *KLOTHO* (b) gene.

optimised for each reaction (details available upon request). Both FAM-labelled and TET-labelled probe were used. The following thermo cycle was used during Taqman reactions: 50 °C for 2 min, 95 °C for 10 min; 40 cycles of 95 °C for 15 s followed by 60 °C for 1 min.

## 2.6. Statistical analyses

Hardy–Weinberg equilibrium tests and pair-wise LD calculations were performed using the program Haploview (Barrett et al., 2005). Both genotypic and allelic association were tested for each SNP using linear regression. Intra-family effects between twins were taken into account by using cluster function in STATA (Stata Corporation, USA) throughout the analyses, which estimates robust standard error from variance between twins.

As haplotype analysis is more powerful than single locus tests in the scenarios where there is strong LD between the variants, haplotype configuration was inferred statistically using PHASE 2.0 for each individual with their estimated probability when the LD statistics  $r^2$  is greater than 0.5 between SNPs (Stephens and Scheet, 2005; Stephens et al., 2001). In order to investigate the association between the statistically inferred haplotypes and TRF length, the estimated probability of different haplotypes for each individual was used to perform haplotype trend regression using generalised estimation equations (Zaykin et al., 2002). The analyses were performed using STATA 9 (Stata Corporation, USA). All statistical analyses were two-tailed and significance level was set at  $\alpha = 0.05$ .

## 3. Results

Table 1 provides the detailed characteristics of the study samples. TRF length was highly variable ranging from 5.15 to 9.36 kb with a mean of 7.09 kb. TRF length was negatively correlated with age and decreased at the rate of 22 base pair (bp) per year ( $p < 0.0001$ ), which is consistent with previous study (Valdes et al., 2005). As TRF length was strongly associated with age, we used age-adjusted TRF length for all analyses.

Table 1  
Characteristics of the study subjects

Characteristics	Mean (range)	Standard deviation
Age (years)	47.04 (18.51–74.62)	11.75
TRF length (kb)	7.09 (5.15–9.36)	0.68
Height (cm)	162.54 (143–191)	5.98
Weight (kg)	65.98 (40–128.3)	12.62
BMI (kg/m <sup>2</sup> )	24.99 (15.22–51.39)	4.72
% Non-smoking	53.82	–

Allele and genotype frequencies of each SNP as well as pair-wise LD ( $r^2$ ) for both genes are presented in Table 2. The allelic and genotypic frequencies of SNPs were very similar with the previous reports for both genes (Keen et al., 2001; Langdahl et al., 2003; Tzakas et al., 2005). All markers were in Hardy–Weinberg equilibrium ( $p$  value  $> 0.05$ ). Only two markers, *TGFBI*-509 C–T and codon-10 T–C, showed strong pair-wise LD with  $r^2 = 0.705$  (see Fig. 2).

For each SNP, the mean and standard deviation of TRF length in each genotype group is presented in Table 3, which also shows the association results between age-adjusted TRF length and SNPs (both genotype and allele). We found no significant association for any of the variants in either gene with the variation in TRF length.

Due to strong LD between *TGFBI*-509 C–T and codon-10 T–C ( $r^2 = 0.705$ ), haplotypes were inferred in PHASE 2.0 and the predicted haplotype frequencies with 95% confidence interval are shown in Table 4. Three common haplotypes (frequency  $> 0.05$ ) comprised 99% of the total. Only one rare haplotype was present with frequency less than 0.01. No significant haplotypic association was found in haplotype trend regression analysis ( $p = 0.7497$ ).

## 4. Discussion

As both *TGFBI* and *KLOTHO* have been previously associated with ageing process and its related phenotypes, it is logical to explore the association between these two genes and the putative ageing indicator, leukocyte telomere length. In the present study, we investigated the association between 10 SNPs in these two genes with the variation of leukocyte telomere length in a large female Caucasian twin population. The selection criteria for these SNPs are they either have been reported as functional variants, or reported as significant with ageing-related phenotypes in previous studies. Among these 10 SNPs, SNP-509 C–T in *TGFBI* gene also influences the plasma level of TGF- $\beta$ 1. Homozygous TT individuals have double the level of TGF- $\beta$ 1 in plasma when compared to homozygous CC individuals (Grainger et al., 1999). But we failed to find any significant association between leukocyte telomere length and these 10 SNPs. This suggests that while we could not exclude

Table 2  
Genetic distribution of SNPs and their pair-wise linkage disequilibrium ( $r^2$ ) in *TGFBI* (a) and *KLOTHO* (b)

(a) *TGFBI*

SNPs	Location	Amino acid change	Genotype	Number (frequency %)	Allele	Number (frequency %)	H-W <i>p</i> value	Pair-wise LD ( $r^2$ )						
								-800 G–A	-509 C–T	Codon-10 T–C	Codon-25 G–C	Codon-263 G–C	Intron-5 C–T	
-800 G–A	Promoter	–	GG	844 (85.95)	G	1821 (92.72)	1	–						
			GA	133 (13.54)	A	143 (7.28)								
			AA	5 (0.51)										
-509 C–T	Promoter	–	CC	458 (48.21)	C	1322 (69.58)	0.803	0.034	–					
			CT	406 (42.74)	T	578 (30.42)								
			TT	86 (9.05)										
Codon-10 T–C	Exon 1	Leu–Pro	TT	379 (39.69)	T	1197 (62.67)	0.249	0.048	0.705	–				
			CT	439 (45.97)	C	713 (37.33)								
			CC	137 (14.35)										
Codon-25 G–C	Exon 1	Pro–Arg	GG	802 (85.59)	G	1732 (92.42)	0.755	0.006	0.03	0.13	–			
			GC	128 (13.66)	C	142 (7.58)								
			CC	7 (0.75)										
Codon-263 G–C	Exon 5	Ile–Thr	CC	915 (97.13)	C	1857 (98.57)	1	0.001	0.034	0.024	0.001	–		
			CT	27 (2.87)	T	27 (1.43)								
Intron-5 C–T	Intron 5 <sup>a</sup>	–	CC	502 (54.45)	C	1380 (74.84)	0.139	0.007	0.027	0.035	0.007	0.004		–
			CT	376 (40.78)	T	464 (25.16)								
			TT	44 (4.77)										

(b) *KLOTHO*

SNPs	Location	Amino acid change	Genotype	Number (frequency %)	Allele	Number (frequency %)	H-W <i>p</i> value	Pair-wise LD ( $r^2$ )					
								-395 G–A	1110 G–A	1818 C–T	2298 C–T		
-395 G–A	Promoter	–	GG	612 (62.32)	G	1554 (79.12)	0.699	–					
			GA	330 (33.6)	A	410 (20.88)							
			AA	40 (4.07)									
1110 G–A	Exon 2	Cys–Ser	GG	688 (70.49)	G	1643 (84.17)	0.602	0.032	–				
			GC	267 (27.36)	C	309 (15.83)							
			CC	21 (2.15)									
1818 C–T	Exon 4	His–His	CC	341 (34.76)	C	1150 (58.61)	0.613	0.083	0.133	–			
			CT	468 (47.71)	T	812 (41.39)							
			TT	172 (17.53)									
2298 C–T	Exon 4	Ala–Ala	CC	752 (76.66)	C	1716 (87.46)	0.723	0.015	0.024	0.101	–		
			CT	212 (21.61)	T	246 (12.54)							
			TT	17 (1.73)									

<sup>a</sup> Located in 20 bp upstream of exon 6.

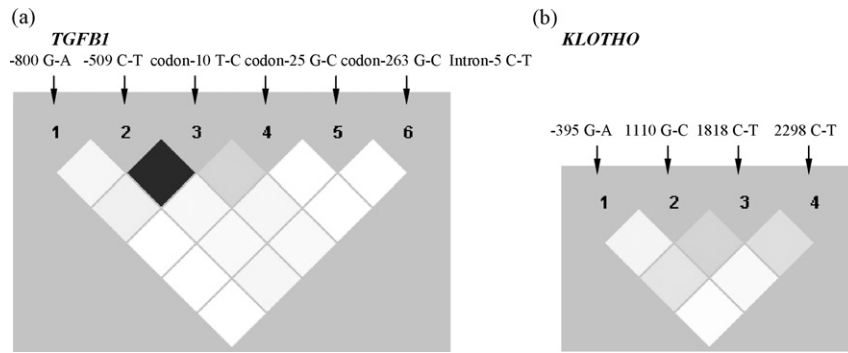


Fig. 2. The pair-wise LD ( $r^2$ ) between SNPs in the *TGFBI* (a) and *KLOTHO* (b) gene.

Table 3  
Results of genotypic and allelic association between SNPs and age-adjusted TRF

(a) <i>TGFBI</i>								
SNPs	Genotypic association				Allelic association			
	Genotype	TRFL mean (kb)	Standard deviation	<i>p</i>	Non-carrier against carrier <sup>a</sup>	TRFL mean (kb)	Standard deviation	<i>p</i>
-800 G–A	GG	7.071	0.626	0.146	GG	7.071	0.626	0.291
	GA	7.187	0.635		GA + AA	7.137	0.653	
	AA	7.471	1.094					
-509 C–T	CC	7.109	0.642	0.101	CC	7.109	0.642	0.395
	CT	7.046	0.636		CT + TT	7.071	0.622	
	TT	7.188	0.537					
codon-10 T–C	TT	7.095	0.655	0.682	TT	7.095	0.655	0.679
	CT	7.065	0.626		CT + CC	7.077	0.618	
	CC	7.117	0.59					
codon-25 G–C	GG	7.089	0.63	0.685	GG	7.089	0.63	0.712
	GC	7.057	0.65		GC + CC	7.066	0.647	
	CC	7.233	0.618					
codon-263 G–C	CC	7.083	0.594	0.329	CC	7.083	0.594	0.867
	CT	7.109	0.678		CT + TT	7.092	0.678	
Intron-5 C–T	CC	6.951	0.668	0.931	CC	7.085	0.638	0.931
	CT	7.085	0.638		CT + TT	7.075	0.565	
	TT	7.075	0.565					
(b) <i>KLOTHO</i>								
SNPs	Genotypic association				Allelic association			
	Genotype	TRFL mean (kb)	Standard deviation	<i>p</i>	Non-carrier against carrier <sup>a</sup>	TRFL mean (kb)	Standard deviation	<i>p</i>
-395 G–A	GG	7.092	0.628	0.984	GG	7.092	0.628	0.860
	GA	7.091	0.643		GA + AA	7.092	0.646	
	AA	7.108	0.671					
1110 G–A	GG	7.076	0.631	0.774	GG	7.076	0.631	0.487
	GC	7.126	0.650		GC + CC	7.126	0.645	
	CC	7.116	0.600					
1818 C–T	CC	7.126	0.628	0.921	CC	7.126	0.628	0.693
	CT	7.077	0.628		CT + TT	7.073	0.638	
	TT	7.062	0.666					
2298 C–T	CC	7.098	0.645	0.707	CC	7.098	0.645	0.408
	CT	7.076	0.609		CT + TT	7.077	0.598	
	TT	7.090	0.453					

<sup>a</sup> In terms of the rare allele.

Table 4  
The estimated haplotype frequencies and standard deviation

-509 C–T and codon-10 T–C haplotypes	Estimated frequency (%)	Standard deviation
C–T	62.267	13.920
T–C	30.110	13.890
C–C	7.327	13.470
T–T	0.296	5.110

minor effects, none of 10 SNPs in these two candidate genes showed significant association with the variation of leukocyte telomere length in our cohort. However, we emphasize that no further conclusion should be drawn regarding to the association between these two genes and ageing *per se*. This is mainly because (1) although the studied SNPs were chosen based on their putative functional roles or as a result of having been reported as significantly associated with ageing-related traits in previous studies, our study is still only representing a fraction of the total variations in both genes under investigation; (2) it is still not unequivocally determined whether telomere length dynamics is the cause or the effect of the ageing process (Aviv, 2004). If telomere dynamics were the key determinant of ageing process, the absence of significant association between these SNPs and telomere length would suggest these two genes might not be involved in the ageing process. If telomere dynamics were only a proxy of the fundamental biological mechanism(s) governing ageing process, these two genes might still be implicated during ageing despite of the absence of significant association results in the present study. They might act through different ageing-related pathways jointly and/or independently from the one leading to the variation of telomere length dynamics. Since *TGFBI* and *KLOTHO* have been consistently associated with ageing and its related phenotypes in previous studies (Bartke, 2006; Capri et al., 2006; Carrieri et al., 2004; Kuro-o et al., 1997; Kurosu et al., 2005; Roush, 1997; Yamamoto et al., 2005), the latter is more likely to be true. But because ageing is a highly complicated biological phenomenon and its mechanism is still largely unknown, the exact molecular relationship between these two genes and ageing remains speculative.

Although our study represents the first ever association study between genetic variation in *TGFBI* and *KLOTHO* gene and leukocyte telomere length variation, there are a few potential issues, which warrant further discussion. First, our study was conducted in DZ twins, which have been previously shown to be representative of population-based samples for a wide range of common medical conditions and lifestyle characteristics (Andrew et al., 2001). Therefore, the conclusion derived from our study in twin population should be generalizable to general population.

Second, our negative finding could result from inadequate power due to our sample size. We calculated the power of our sample to detect the association between chosen SNPs and leukocyte telomere length under the assumption that the causal quantitative trait locus for telomere length is common and have strong LD with tested variants. At a significant level of 0.05, the

power of our sample ranges from 83.5 to 99% (Purcell et al., 2003), which is empirically sufficient to detect the association.

Third, as we tested the association between leukocyte telomere length and two ageing candidate genes independent of the known factors influencing leukocyte telomere length dynamics, the contributions of the known risk factors, such as BMI and smoking, have not been taken into account (Valdes et al., 2005). However, we also performed all related association tests in regression models using BMI and smoking as covariates. The results remain non-significant (data not shown), which suggests the contributions of the known risk factors, BMI and smoking, are not significant to our negative results.

Finally, our conclusions are restricted to Caucasian females. Also, similar to any candidate gene study, the results need to be replicated in other independent cohorts.

In conclusion, we failed to find any significant associations between leukocyte telomere length variation and 10 genetic variants in two ageing-related candidate genes, *TGFBI* and *KLOTHO*. But no further conclusion of the possible association between these two genes and ageing could be drawn. It is possible they are involved in the different pathway(s) leading to other ageing phenotypes rather than telomere length. But their exact mechanism in the ageing process remains speculative.

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