Human Reproduction, Vol.32, No.1 pp. 201–207, 2017
Advanced Access publication on November 11, 2016 doi:10.1093/humrep/dew283

human reproduction

ORIGINAL ARTICLE ***Reproductive endocrinology***

Impaired telomere length and telomerase activity in peripheral blood leukocytes and granulosa cells in patients with biochemical primary ovarian insufficiency

Xiaofei Xu[1,2,3], Xinxia Chen[1,2,3], Xiruo Zhang[1,2,3], Yixun Liu[4], Zhao Wang[1,2,3], Peng Wang[1,2,3], Yanzhi Du[5], Yingying Qin[1,2,3], and Zi-Jiang Chen[1,2,3,5,6,*]

[1]Center for Reproductive Medicine, Provincial Hospital Affiliated to Shandong University, No. 324 Jingwu Road, Jinan 250021, P.R. China [2]National Research Center for Assisted Reproductive Technology and Reproductive Genetics, Jinan 250021, P.R. China [3]The Key laboratory of Reproductive Endocrinology (Shandong University) Jinan, P.R. China [4]State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, P.R. China [5]Shanghai Key Laboratory for Assisted Reproduction and Reproductive Genetics, Shanghai, P.R. China [6]Center for Reproductive Medicine, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200030, P.R. China

*Correspondence address. Center for Reproductive Medicine, Provincial Hospital Affiliated to Shandong University, Jinan, P.R. China and National Research Center for Assisted Reproductive Technology and Reproductive Genetics, Jinan, P.R. China. E-mail: chenzijiang@hotmail.com

Submitted on January 24, 2016; resubmitted on September 29, 2016; accepted on October 28, 2016

STUDY QUESTION: Are telomere length and telomerase activity associated with biochemical primary ovarian insufficiency (POI)?

SUMMARY ANSWER: Shortened telomere length and diminished telomerase activity were associated with biochemical POI.

WHAT IS KNOWN ALREADY: POI is a result of pathological reproductive aging and encompasses occult, biochemical and overt stages. Studies have indicated telomere length as a biomarker for biological aging.

STUDY DESIGN, SIZE, DURATION: A total of 120 patients with biochemical POI and 279 control women were recruited by the Center for Reproductive Medicine of Shandong University.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Telomere length in peripheral blood leukocytes (LTL) and granulosa cells (GTL) was measured using a modified Quantitative Polymerase Chain Reaction technique. The relative telomerase activity (RTA) in granulosa cells was detected using a modified quantitative-telomeric repeat amplification protocol assay.

MAIN RESULTS AND THE ROLE OF CHANCE: After adjusting for age, patients with biochemical POI ($n = 120$) exhibited significantly shorter LTLs (0.75 ± 0.09 vs 1.79 ± 0.12, $P < 0.001$; OR = 0.54, 95% CI = 0.43–0.68) and GTLs (0.78 ± 0.09 vs 1.90 ± 0.23, $P < 0.001$; OR = 0.54, 95% CI = 0.41–0.70) than the controls ($n = 279$ for LTLs; $n = 90$ for GTLs). Significantly diminished RTAs in granulosa cells were detected in patients with biochemical POI ($n = 31$) compared with the controls ($n = 38$) (1.57 ± 0.59 vs 4.63 ± 0.93, $P = 0.025$; OR = 0.84, 95% CI = 0.72–0.98).

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: The cross-sectional nature of this study might have its limit in telomere length as well as telomerase activity along with the progressing decline in ovarian function.

WIDER IMPLICATIONS OF THE FINDINGS: These findings suggest that telomere length and telomerase activity may be considered as indicators for progression of ovarian decline.

STUDY FUNDING/COMPETING INTEREST(S): This research was supported by the National Basic Research Program of China (973 Program) (2012CB944700), Science research foundation item of no-earnings health vocation (201402004) and the National Natural Science Foundation of China (31471352, 81270662, 81471509, 81300461, 81522018). The authors have no potential conflict of interest to declare.

Key words: primary ovarian insufficiency / telomere length / telomerase activity / granulosa cell / leukocyte

Introduction

Primary ovarian insufficiency (POI) is defined as hypergonadotropic amenorrhea due to cessation of ovarian function before the age of 40 years (Goswami and Conway, 2005). Three stages of POI have been described, including occult, biochemical and overt (formerly called premature ovarian failure) phases (Levi *et al.*, 2001; Welt, 2008). Patients with occult POI manifest reduced fecundity, regular menstruation and normal levels of FSH. Patients shifting towards biochemical POI have spontaneous menstruation, but basal FSH starts to rise (ranging from 10 to 40 IU/L). Most patients with overt POI suffer from amenorrhea with highly elevated FSH (>40 IU/L). POI results from pathological reproductive aging and it is associated with decreased fertility and infertility. Moreover, the underlying cause remains elusive in most cases and they are generally referred to as idiopathic (Nelson, 2009).

Reproductive aging is intertwined with biological aging. Previous studies have shown that the later a woman's last reproductive event (i.e. the last child) takes place, the older they will become. In other words, if a woman is still capable to conceive during her fifth decade, she will probably become very old (Laven, 2015). Telomere length is a proven biomarker for biological aging (Fossel, 1998; Baird and Kipling, 2004; Aubert and Lansdorp, 2008). Telomere attrition has been considered to be involved in age-related disorders, such as cardiovascular diseases (Brouilette *et al.*, 2003, 2007; Morgan *et al.*, 2014), neurological diseases (Tedone *et al.*, 2015; Zhan *et al.*, 2015) and cancers (Hahn, 2003; Willeit *et al.*, 2010). Indeed, telomerase-deficient mice with short telomere length presented with premature aging and reduced fecundity (Liu *et al.*, 2002).

Patients with occult POI showed shortened telomere length and low telomerase activity in granulosa cells (Butts *et al.*, 2009). In contrast, in patients with overt POI longer telomere length in peripheral blood leukocytes has been identified (Hanna *et al.*, 2009). Moreover, patients with overt POI also exhibited low telomerase activity in ovaries (Kinugawa *et al.*, 2000; Liu and Li, 2010). Compared with occult and overt POI, the biochemical stage is recognizable using specific clinical features and offers specific treatment options. Therefore, the changes of telomere length and telomerase activity in patients with biochemical POI may be more instructive; however, no data reporting on this issue have been published.

In this study, we measured relative telomere length in peripheral blood leukocytes (LTL) and granulosa cells (GTL) and relative telomerase activity (RTA) in granulosa cells of patients with biochemical POI to explore their potential roles in this disorder.

Materials and Methods

Study population

A total of 120 patients with biochemical POI undergoing IVF-embryo transfer (ET) were recruited from the Reproductive Hospital Affiliated to Shandong University. Patients met the following criteria: (i) basal FSH (on Days 2–4 of menstrual cycle) ≥10 mIU/ml; (ii) <40 years of age; (iii) with regular menstruation (23–35 days) and (iv) unilateral ovarian antral follicle count (AFC) <5. Patients with history of radio- or chemotherapy, ovarian surgery and chromosome abnormality were excluded. Two hundred and seventy-nine women treated with IVF-ET were enrolled as controls. Peripheral blood was collected from all controls, whereas granulosa cells were collected from 90 subjects of the 279 controls.

Ethical approval

This study was approved by the Institutional Medical and Ethical Review Board of Reproductive Medicine of Shandong University and written informed consent was obtained from all participants.

Granulosa cell isolation

Follicular fluid from large follicles with diameter exceeding 14 mm was pooled from each participant on the day of egg retrieval. The follicular fluid was centrifuged for 10 min at 2000 rpm, the precipitates were incubated with hyaluronidase (80 IU/ml) (Sigma, St. Louis, Mo., USA) for 30 min at 37°C, then transferred into lymphocyte separation medium (Solarbio, Beijing, China) and centrifuged at 1600 rpm for 10 min. Granulosa cells were isolated from the interface layer, then washed and resuspended in phosphate buffer saline (PBS). The cells were stored at −80°C until processed for measurement of GTL and RTA.

LTL and GTL measurement

Genomic DNA was extracted from peripheral blood leukocytes and granulosa cells using DNeasy Tissue Kit (QIAGEN, Inc., Mississauga, Ontario, Canada). Relative LTL and GTL were measured using a modified Quantitative Polymerase Chain Reaction (qPCR) technique described by Cawthon (Cawthon, 2002; Shen *et al.*, 2012; Li *et al.*, 2013). The relative telomere length was calculated as the telomere/single copy gene (T/S) ratio using β-globin as reference. For each sample, two PCRs were performed: one to amplify telomere repeats (Tel PCR, 10 μM of primer Tel-F, CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT; Tel-R, GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT), and the other to amplify the β-globin gene (S PCR, 10 μM of primer β-globin-F, CTTCTGACACAACTGTGTTCACTAGC; β-globin-R, CACCAACTTC ATCCACGTTCACC). The 2 × SYBR Green I Mix (TAKARA, Tokyo, Japan) was used for all qPCRs. Standard curves derived from serially diluted reference DNA (0.75–12 ng; 2-fold dilution; five points) for telomere and β-globin both showed good linearity ($R^2 > 0.99$, Supplemental Fig. S1).

Relative T/S ratios will reflect relative length differences in telomeric DNA only if the number of copies of S per cell that are effectively PCR-amplified is the same in all individuals being studied. To test whether PCR with the β-globin primers met this requirement, we determined, by quantitative PCR, the relative ratio of β-globin gene copies to 36B4 (officially known as ribosomal protein lateral stalk subunit P0 (RPLP0)) gene copies in patients with biochemical POI versus controls described by Cawthon (2002). The relative ratio (β-globin/36B4) was 1.0 (average = 1.0,

range = 0.93–1.08), indicating a reliable 1:1 relationship between β-globin and 36B4 as reference (Supplemental Table SI).

Terminal restriction fragment length analysis

Terminal restriction fragment (TRF) measurement by Southern blot was used to verify some samples. TRF length was measured using the Telo TTAGGG telomere length assay (Roche Diagnostics GmBH, Mannheim, Germany). Genomic DNA (1.5 μg) was digested with the restriction enzymes, RsaI and HinfI (Roche Diagnostics GmBH) for 2 h at 37°C. The DNA fragments were separated on a 0.8% agarose gel by electrophoresis, then were denatured, neutralized and transferred to a nylon membrane via capillary transfer at 15–25°C using a 20 × saline-sodium citrate (Solarbio) transfer buffer and cross-linked with UV light. Blotted DNA fragments were incubated with a telomeric probe-digoxigenin (DIG) (Roche Diagnostics GmBH) at 42°C for 3 h, followed by incubation with DIG-specific polyclonal sheep antibody (Roche Diagnostics GmBH) covalently coupled to alkaline phosphatase. Binding sites of the telomere probe were visualized using a highly sensitive chemiluminescence substrate (Roche Diagnostics GmBH) that metabolizes alkaline phosphatase. Then TRF lengths were evaluated with molecular weight markers and mean TRF lengths were estimated using Image lab software (BIO-RAD, ChemiDoc™MP, California, USA) (Supplemental Fig. SII).

Telomerase activity measurement

Telomerase activity was detected using a modified quantitative-telomeric repeat amplification protocol (Q-TRAP) assay (Herbert *et al.*, 2006; Jeon *et al.*, 2011). Granulosa cells were lysed in NP-40 lysis buffer, and incubated on ice for 30 min. The lysate was centrifuged at 12 000*g* for 30 min at 4°C, and supernatant was collected. Protein concentration was measured using the Bicinchoninic acid protein assay kit (Beyotime, Shanghai, China) according to the manufacturer's protocols. The Q-TRAP was optimized using the PCR reagent LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics GmBH), containing 2 μl extract, 12.5 μl 1 × SYBR Green Master Mix, 0.1 μg TS primer (5′-AATCCGTCGAGCAGAGTT-3′), 0.1 μg ACX primer (5′-GCGCGGCTTACCCTTACCCTTACCCTAACC-3′) and adjusted to 25 μl using sterile H_2O. The reaction plate was incubated at room temperature for 30 min. The PCR conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Each run included measurements of telomerase-positive control 293 T samples and telomerase-negative control 293 T samples inactivated by incubation for 10 min at 85°C. A standard curve derived from serially diluted immortalized 293 human embryonic kidney cells showed good linearity ($R^2 > 0.99$, Supplemental Fig. SIII). The RTA was quantified based on the standard curve and the following calculation: converted RTA of sample = $10^{[(C_t\ \text{sample}-Y_{int})/\text{slope}]}$.

Statistical analyses

Whether data were distributed normally and were assessed using the Kolmogorov–Smirnov test. The relative LTLs and GTLs were ln-transformed because of a skewed distribution. Either a Student's *t*-test or the Mann–Whitney *U*-test was used to analyze different quantitative variables. The correlations between LTLs, GTLs and age were analyzed using a general linear regression model. The associations between LTLs, GTLs, RTAs and biochemical POI were analyzed using a binary logistic regression model. Odds ratios (ORs) were calculated to determine the association between LTLs, GTLs, RTAs and biochemical POI. A two-sided $P < 0.05$ was considered statistically significant. Statistical analyses were performed with Statistical Package for Social Sciences version 18.0 (SPSS 18.0; SPSS, Chicago, IL, USA).

Results

Clinical characteristics of all participants

The clinical characteristics of patients and controls were shown in Table I. Compared with controls, patients with biochemical POI were older (32.95 ± 4.27 years vs 29.98 ± 4.28 years, $P < 0.001$) and had lower levels of basal estradiol (E2) on Day 3 of the menstrual cycle (33.46 ± 13.96 pg/ml vs 39.46 ± 15.06 pg/ml, $P = 0.009$). No difference in BMI was observed between the two groups.

Associations between LTLs, GTLs and age

The age distribution of all participants was shown in Fig. 1a. Linear regression analysis revealed that the relationship between LTLs and age was determined by LTL (T/S ratios) = −0.067 × year + 3.55 [$R^2 = 0.03$; correlation coefficient (r) = −0.16; $P = 0.001$]. Moreover, on average, LTL decreased 0.067 T/S every year from age 21 up till 39 years. The relationship between GTLs and age was deduced from GTL (T/S ratios) = −0.089 × year + 4.05 ($R^2 = 0.05$; $r = -0.22$; $P = 0.001$). Similarly, GTL decreased 0.089 T/S every year from age 23 to 39 years on average (Fig. 1b).

Associations between LTLs, GTLs, RTAs and biochemical POI

Shorter LTLs, GTLs (0.75 ± 0.09 vs 1.79 ± 0.12, $P < 0.001$; 0.78 ± 0.09 vs 1.90 ± 0.23, $P < 0.001$) and diminished RTAs were established in granulosa cells (1.57 ± 0.59 vs 4.63 ± 0.93, $P = 0.025$) from POI patients compared with controls (Table II). Logistic regression analysis showed significant associations between the presence of biochemical POI and LTLs, GTLs and RTAs (LTL: adjusted for age OR = 0.54, 95% CI = 0.43–0.68, $P < 0.001$; GTL: adjusted for age OR = 0.54, 95% CI = 0.41–0.70, $P < 0.001$; RTA: OR = 0.84, 95% CI = 0.72–0.98, $P = 0.025$, Table III). Multiple linear regression analysis was performed to analyze determinants of LTLs and GTLs (Table IV). Biochemical POI was associated with 0.69T/S LTL reductions and

Table I Clinical characteristics of patients with biochemical POI and controls.

	Biochemical POI	Controls	*P*-value
Number	120	279	
Age (year)	32.95 ± 4.27	29.98 ± 4.28	**<0.001**
BMI (kg/m^2)	23.23 ± 3.70	23.60 ± 3.52	0.358
Basal E2 (pg/ml)	33.46 ± 13.96	39.46 ± 15.06	**0.009**
FSH (IU/L)	13.30 ± 3.30	6.65 ± 1.71	**<0.001**
AMH (ng/ml)	0.81 ± 0.88	4.10 ± 2.23	**<0.001**
AFC	6.82 ± 2.32	15.20 ± 3.76	**<0.001**
Peak E2 (pg/ml)	1865.26 ± 788.16	2736.88 ± 760.11	**<0.001**
Number of follicles on day of HCG (≥14 mm)	4.47 ± 1.95	9.34 ± 2.54	**<0.001**

Values were given as numbers or means ± SD. *P* values were obtained from either Student's *t*-test or Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant. POI, primary ovarian insufficiency; E2, estradiol; AMH, anti-Mullerian hormone; AFC, antral follicle count.

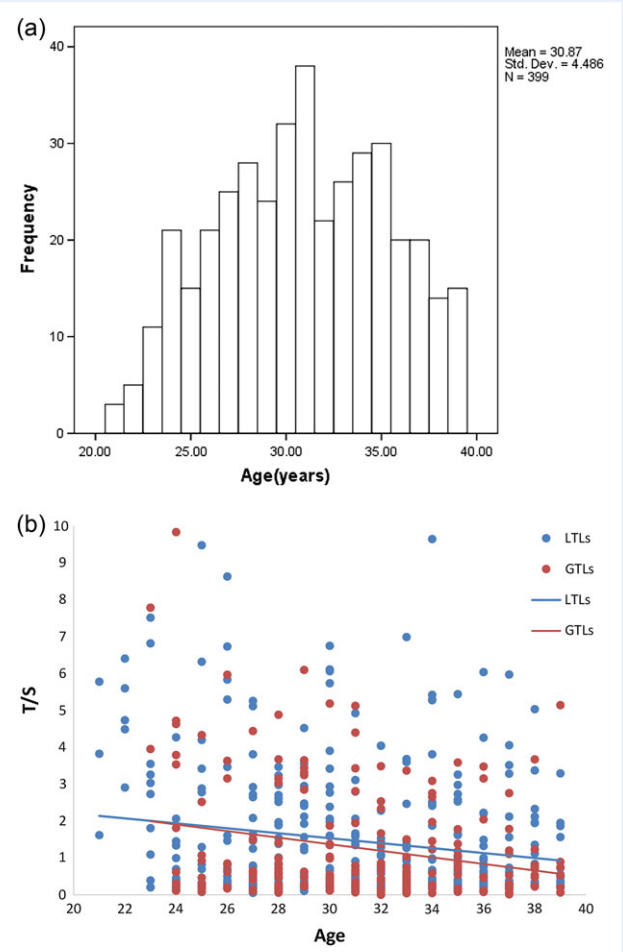

Figure 1 Telomere length shortened with advanced age in all participants. (**a**) Age distribution of all participants. (**b**) Distribution of the T/S ratio according to age. Linear regression lines were drawn for LTLs and GTLs. LTLs was shown as blue circles ($n = 399$) and GTLs as red circles ($n = 210$). LTL, telomere length in leukocyte; GTL, telomere length in granulosa cell.

0.98T/S GTL reductions on average after adjustment for age, which corresponds with ~10.30 and 11.01 years age-related reduction in LTL and GTL, respectively.

Comparison of IVF parameters between patients with biochemical POI and controls stratified by LTLs, GTLs and RTAs

As shown in Table V, patients with biochemical POI and controls were divided into two subgroups, respectively, i.e. T/S <1 group and T/S ≥1 group, RTA <1 group and RTA ≥1 group. Interestingly, the rate of two pronuclei (2PN) was significantly higher in those with higher telomerase activity compared with lower telomerase activity both in patients (56.14% vs 38.00%) and controls (63.9% vs 52.67%) ($P = 0.03$). However, no differences in rates of cleavage and high-quality embryo on D3 were observed between shorter LTLs/GTLs and longer LTLs/GTLs either in patients or in controls.

Table II Comparison of LTLs, GTLs and RTAs between patients with biochemical POI and controls.

	Biochemical POI	Controls	*P*-value
LTLs (T/S)	0.75 ± 0.09 ($n = 120$)	1.79 ± 0.12 ($n = 279$)	**<0.001**
GTLs (T/S)	0.78 ± 0.09 ($n = 120$)	1.90 ± 0.23 ($n = 90$)	**<0.001**
RTAs	1.57 ± 0.59 ($n = 31$)	4.63 ± 0.93 ($n = 38$)	**0.025**

Values are given as numbers or means ± SE. *P* values were obtained from Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant. POI, primary ovarian insufficiency; LTL, telomere length in leukocyte; GTL, telomere length in granulosa cell; RTA, relative telomerase activity.

Table III Association analysis between LTLs, GTLs, RTAs and biochemical POI.

	LTLs ln(T/S ratios)	GTLs ln(T/S ratios)	RTAs
OR			
Unadjusted	0.56	0.53	0.84
Adjusted for age	0.54	0.54	
95% CI			
Unadjusted	0.45–0.69	0.42–0.68	0.72–0.98
Adjusted for age	0.43–0.68	0.41–0.70	
P-value			
Unadjusted	<0.001	<0.001	0.025
Adjusted for age	<0.001	<0.001	

Logistic regression analysis between factor (POI = 1 or control = 0) and telomere lengths ln(T/S ratios) unadjusted or age adjusted. $P < 0.05$ was considered statistically significant. POI, primary ovarian insufficiency; LTL, telomere length in leukocyte; GTL, telomere length in granulosa cell; RTA, relative telomerase activity; OR, odd ratio.

Table IV Determinants analysis of LTLs and GTLs.

	LTLs [ln(T/S ratios)]		GTLs [ln(T/S ratios)]	
	Estimate *B*	*P*-value	Estimate *B*	*P*-value
Age (year)	−0.028	0.033	−0.062	0.006
Factor (POI/not)	−0.690	<0.001	−0.980	<0.001

Multiple linear regression analysis of LTLs, GTLs determinants. $P < 0.05$ was considered statistically significant. POI, primary ovarian insufficiency; LTL, telomere length in leukocyte; GTL, telomere length in granulosa cell.

Discussion

This study shows that in peripheral blood leukocytes as well as in granulosa cells derived from women with biochemical POI telomere length seems to be shorter compared with healthy controls. Moreover, telomerase activity seems to be diminished in granulosa cells of patients with POI compared with healthy controls. Finally, as to be expected, with advancing age telomere length shortened.

Telomere length is positively correlated with reproductive lifespan in women (Aydos *et al.*, 2005). Diminished telomerase activity has

Table V Comparison of IVF parameters between patients with biochemical POI and controls stratified by LTLs, GTLs and RTAs.

	LTL			GTL			RTA		
	T/S < 1	T/S ≥ 1	*P*-value	T/S < 1	T/S ≥ 1	*P*-value	RTA <1	RTA ≥1	*P*-value
Biochemical POI									
Number	57	63		92	28		23	8	
Number of oocytes retrieved	298	309		459	148		100	57	
Fertilization rate	78.86% (235/298)	78.32% (242/309)	0.92	78.21% (359/459)	79.73% (118/148)	0.73	71% (71/100)	78.94% (45/57)	0.61
Rate of 2PN	55.70% (166/298)	59.22% (183/309)	0.41	56.64% (260/459)	60.14% (89/148)	0.50	38.00% (38/100)	56.14% (32/57)	**0.03**
Cleavage rate	99.31% (144/145)	98.64% (145/147)	1.00	99.06% (212/214)	98.70% (76/77)	1.00	100% (41/41)	100% (24/24)	1.00
Rate of high-quality embryos on D3	57.24% (83/145)	47.62% (70/147)	0.10	52.34% (112/214)	53.25% (41/77)	0.90	48.78% (20/41)	54.17% (13/24)	0.80
Controls									
Number	139	140		48	42		14	24	
Number of oocytes retrieved	1500	1446		520	476		150	277	
Fertilization rate	78.87% (1183/1500)	79.67% (1152/1446)	0.62	76.92% (400/520)	83.61% (398/476)	**0.01**	78.67% (118/150)	79.42% (220/277)	0.90
Rate of 2PN	64.47% (967/1500)	64.38% (931/1446)	0.97	60.19% (313/520)	62.18% (296/476)	0.56	52.67% (79/150)	63.90% (177/277)	**0.03**
Cleavage rate	99.79% (955/957)	99.68% (928/929)	0.68	99.66% (296/297)	99.66% (292/293)	1.00	98.53% (67/68)	99.43% (175/176)	0.48
Rate of high-quality embryos on D3	53.29% (510/957)	56.94% (529/929)	0.12	52.53% (156/297)	54.95% (161/293)	0.56	60.29% (41/68)	54.55% (96/176)	0.47

Peripheral blood was collected from all 279 controls, whereas granulosa cells were collected from 90 subjects of the 279 controls. *P* values were obtained from Chi-square test. $P < 0.05$ was considered statistically significant. POI, primary ovarian insufficiency; LTL, telomere length in leukocyte; GTL, telomere length in granulosa cell; RTA, relative telomerase activity. Rate of 2PN was defined as the number of 2PN divided by the total number of oocytes retrieved. The fertilization rate was the ratio between the number of fertilized eggs and number of oocytes retrieved (Barlow *et al.*, 1990).

been identified in patients with occult as well as overt POI (Butts et al., 2009; Liu and Li, 2010). Our results of shortened LTLs, GTLs and diminished RTAs in biochemical POI were consistent with most reports, which have shown that telomere attrition is associated with reproductive aging. These findings might be explained as follows. First, it might reflect an increased cellular senescence in patients with biochemical POI. A second explanation could be that most patients suffered from estrogen insufficiency due to lower levels of estradiol. Moreover, it has been reported that estrogen deficiency in mice resulted in an inhibition of telomerase activity in ovaries, which entailed shortened telomeres (Bayne et al., 2011). Last but not least, patients with POI endured a high degree of psychological and physical stress. More exposure to stress may be responsible for accelerated telomere attrition (Epel et al., 2004). In addition, the phenomena of shortened GTLs and diminished RTAs in granulosa cells verified that telomere erosion was accelerated in women with POI and their replication capacity was impaired.

The fact that we found shorter LTLs in biochemical POI is contradictory to other studies that found longer LTLs in overt POI (Hanna et al., 2009). These seemingly contradictory results might be explained as follow. First of all, long-term hormone replacement in postmenopausal women slowed down the rate of telomere attrition (Lee et al., 2005). Patients with overt POI had to take hormone replacement to maintain adequate estrogen exposure, whereas patients with biochemical POI in this study still experienced spontaneous menstrual periods. Secondly, different stages of POI may be characterized by distinct and different pathological changes specific for that stage. Last but not least, the relatively smaller sample size in the previous study ($n = 34$) may have introduced some bias (Hanna et al., 2009).

Patients with occult POI, only characterized by decreased fecundity, are hard to diagnose in a clinical setting because we lack proper diagnostic tools or markers to do so. In contrast, for patients with overt POI, in whom ovaries already have been depleted the diagnosis that is much easier to establish. Biochemical POI is the second, intermediate stage of POI in whom the primordial follicle pool has not yet been fully depleted. Impaired telomere length and telomerase activity were revealed in patients with biochemical POI. Hence this might provide an early and reliable indicator or predictor for progression of ovarian decline before the final exhaustion of oocytes takes place.

The cross-sectional nature of this study might have its limit in telomere length as well as telomerase activity along with the progressing decline in ovarian function.

In conclusion, shortened telomere length and diminished telomerase activity were associated with biochemical POI. Further longitudinal studies in a similar population at different stages of POI are needed to further substantiate the rules of telomerase and telomere length.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Acknowledgment

We are grateful to all participants involved in this study.

Authors' roles

The authors have made the following declarations about their contributions: Y.Q. and Z.-J.C. conceived, supported the study; X.X. designed, performed the experiments, analyzed the data and drafted the manuscript; X.X., X.Z. and Z.W. collected samples; X.C. and P.W. provided analysis methods and tools; Y.Q., Y.L. and Y.D. revised the article. All authors gave their final approval of the version to be published.

Funding

The National Basic Research Program of China (973 Program) (2012CB944700), Science Research Foundation item of no-earnings health vocation (201402004) and the National Natural Science Foundation of China (31471352, 81270662, 81471509, 81300461, 81522018).

Conflict of interest

None declared.

References

Aubert G, Lansdorp PM. Telomeres and aging. *Physiol Rev* 2008;**88**:557–579.

Aydos SE, Elhan AH, Tükün A. Is telomere length one of the determinants of reproductive life span? *Arch Gynecol Obstet* 2005;**272**:113–116.

Baird DM, Kipling D. The extent and significance of telomere loss with age. *Ann N Y Acad Sci* 2004;**1019**:265–268.

Barlow P, Englert Y, Puissant F, Lejeune B, Delvigne A, Van Rysselberge M, Leroy F. Fertilization failure in IVF: why and what next? *Hum Reprod* 1990;**5**:451–456.

Bayne S, Li H, Jones ME, Pinto AR, Van Sinderen M, Drummond A, Liu JP. Estrogen deficiency reversibly induces telomere shortening in mouse granulosa cells and ovarian aging in vivo. *Protein cell* 2011;**2**:333–346.

Brouilette S, Singh RK, Thompson JR, Goodall AH, Samani NJ. White cell telomere length and risk of premature myocardial infarction. *Arterioscler Thromb Vasc Biol* 2003;**23**:842–846.

Brouilette SW, Moore JS, McMahon AD, Thompson JR, Ford I, Shepherd J, West of Scotland Coronary Prevention Study Group. Telomere length, risk of coronary heart disease, and statin treatment in the West of Scotland Primary Prevention Study: a nested case-control study. *Lancet* 2007;**369**:107–114.

Butts S, Riethman H, Ratcliffe S, Shaunik A, Coutifaris C, Barnhart K. Correlation of telomere length and telomerase activity with occult ovarian insufficiency. *J Clin Endocrinol Metab* 2009;**94**:4835–4843.

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

Epel ES, Blackburn EH, Lin J, Dhabhar FS, Adler NE, Morrow JD, Cawthon RM. Accelerated telomere shortening in response to life stress. *Proc Natl Acad Sci U S A* 2004;**101**:17312–17315.

Fossel M. Telomerase and the aging cell: implications for human health. *JAMA* 1998;**279**:1732–1735.

Goswami D, Conway GS. Premature ovarian failure. *Hum Reprod Update* 2005;**11**:391–410.

Hahn WC. Role of telomeres and telomerase in the pathogenesis of human cancer. *J Clin Oncol* 2003;**21**:2034–2043.

Hanna CW, Bretherick KL, Gair JL, Fluker MR, Stephenson MD, Robinson WP. Telomere length and reproductive aging. *Hum Reprod* 2009;**24**: 1206–1211.

Herbert BS, Hochreiter AE, Wright WE, Shay JW. Nonradioactive detection of telomerase activity using the telomeric repeat amplification protocol. *Nat Protoc* 2006;**1**:1583–1590.

Jeon BG, Kang EJ, Kumar BM, Maeng GH, Ock SA, Kwack DO, Rho GJ. Comparative analysis of telomere length, telomerase and reverse transcriptase activity in human dental stem cells. *Cell Transplant* 2011;**20**: 1693–1705.

Kinugawa C, Murakami T, Okamura K, Yajima A. Telomerase activity in normal ovaries and premature ovarian failure. *Tohoku J Exp Med* 2000; **190**:231–238.

Laven JS. Genetics of early and normal menopause. *Semin Reprod Med* 2015.

Lee DC, Im JA, Kim JH, Lee HR, Shim JY. Effect of long-term hormone therapy on telomere length in postmenopausal women. *Yonsei Med J* 2005;**46**:471–479.

Levi AJ, Raynault MF, Bergh PA, Drews MR, Miller BT, Scott RT. Reproductive outcome in patients with diminished ovarian reserve. *Fertil Steril* 2001;**76**:666–669.

Li Q, Du J, Feng R, Xu Y, Wang H, Sang Q, Wang L. A possible new mechanism in the pathophysiology of polycystic ovary syndrome (PCOS): the discovery that leukocyte telomere length is strongly associated with PCOS. *J Clin Endocrinol Metab* 2013;**99**:E234–E240.

Liu JP, Li H. Telomerase in the ovary. *Soc Reprod Fertil Suppl* 2010;**140**: 215–222.

Liu L, Blasco MA, Trimarchi JR, Keefe DL. An essential role for functional telomeres in mouse germ cells during fertilization and early development. *Dev Biol* 2002;**249**:74–84.

Morgan RG, Ives SJ, Walker AE, Cawthon RM, Andtbacka RH, Noyes D, Donato AJ. Role of arterial telomere dysfunction in hypertension: relative contributions of telomere shortening and telomere uncapping. *J Hypertens* 2014;**32**:1293.

Nelson LM. Primary ovarian insufficiency. *Newl Eng J Med* 2009;**360**:606–614.

Shen Q, Zhao X, Yu L, Zhang Z, Zhou D, Kan M, Zhang D, Cao L, Xing Q, Yang Y *et al*. Association of leukocyte telomere length with type 2 diabetes in mainland Chinese populations. *J Clin Endocrinol Metab* 2012; **97**:1371–1374.

Tedone E, Arosio B, Colombo F, Ferri E, Asselineau D, Piette F, Mari D. Leukocyte telomere length in Alzheimer's disease patients with a different rate of progression. *J Alzheimer's Dis* 2015;**46**:761–769.

Welt CK. Primary ovarian insufficiency: a more accurate term for premature ovarian failure. *Clin Endocrinol* 2008;**68**:499–509.

Willeit P, Willeit J, Mayr A, Weger S, Oberhollenzer F, Brandstätter A, Kiechl S. Telomere length and risk of incident cancer and cancer mortality. *JAMA* 2010;**304**:69–75.

Zhan Y, Song C, Karlsson R, Tillander A, Reynolds CA, Pedersen NL, Hägg S. Telomere length shortening and Alzheimer disease—a Mendelian Randomization Study. *JAMA Neurol* 2015;**72**:1202–1203.