

Alteration of relative telomere length and telomerase reverse transcriptase expression in the granulosa cells of women during aging and assessment of *in vitro* fertilization outcomes

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Received January 27, 2023; Accepted August 11, 2023

DOI: 10.3892/mmr.2023.13093

Abstract. Telomere attrition plays a critical role in the reproductive aging process in humans. Telomere length (TL) is typically regulated by telomerase, the main component of which is telomerase reverse transcriptase (TERT). The aim of the present study was to investigate the changes of relative TL (RTL) and TERT expression in granulosa cells (GCs) during aging and its association with reproduction. Clinical data on the frozen-thawed embryo transfer cycles of older (>35 year old) and younger (≤35 year old) women from a single center over a 3-year period were retrospectively analyzed. Preimplantation genetic testing for chromosome aneuploidies in older women during the same period was also analyzed. Following the analysis of the data, several biological characteristics of senescent GCs were explored. In addition, a total of 160 women who were undergoing their first fresh cycle of *in vitro* fertilization (IVF) or intracytoplasmic sperm injection were included in the study. GCs were collected from all participants. The changes of RTL and TERT expression in GCs during aging were investigated

using quantitative PCR and western blotting. The associations of RTL and TERT with IVF outcomes were also assessed. The clinical data demonstrated that the pregnancy and live birth rates of women aged >35 years were ~20% lower than those of women aged ≤35 years, and the number of embryos with aneuploidy was 7-fold of that without euploidy in the older age group. An aging-induced change in follicle stimulating hormone receptor expression was observed. A shorter TL and increased TERT expression were detected in the older women. A significant inverse correlation between the expression levels of TERT and oocyte yield was identified. However, no association of RTL and TERT with blastocyst formation rate and the probability of clinical pregnancy was detected. It may be concluded that RTL and TERT alterations in GCs are potential determinants of ovarian aging. TERT expression in GCs appears to be a potential biomarker for the prediction of ovarian response, which provides a novel strategy for the assessment of female fertility.

Introduction

In recent decades, due to social and economic factors, an increasing number of women have postponed pregnancy until an advanced age (1), and an increased maternal age has been shown to be associated with infertility and a diminished ovarian reserve (DOR) (2). Ovarian aging has an important impact on reproductive capacity, as it alters the hormonal equilibrium, decreases the quality of oocytes and worsens reproductive outcomes (1). Hypotheses for the age-related decline in reproductive capacity and ovarian reserve involve oocyte aneuploidy (3), spindle defects (1), oxidative stress response (4), mitochondrial dysfunction (5), autophagy deficiency (6) and altered epigenetic patterns (1). Telomere attrition has also been shown to play a critical role in the reproductive

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Key words: relative telomere length, telomerase reverse transcriptase, *in vitro* fertilization, aging, granulosa cells

aging process of humans (7). Telomere shortening has been proposed as a possible mechanism leading to a reduction in the quality of oocytes as it may affect chromosome segregation and genome stability (1).

Telomeres are highly preserved nucleoprotein structures comprising 5-15 kb-long tandem repeat hexanucleotide sequence (TTAGGG) repeats that form protective caps at the ends of eukaryotic chromosomes (8,9). Telomeres play a vital role in the sequential cycles of cell division and maintenance of chromosomal stability (10). The progressive shortening of telomeres within granulosa cells (GCs) due to inadequate DNA repair and long-term oxidative stress has been suggested to be the main driving factor underlying reproductive aging (11).

Telomere length (TL) is typically regulated by telomerase, a reverse transcriptase enzyme that adds the TTAGGG sequence to telomeres, thus protecting them from progressive erosion during cell division. One of the key roles of telomerase is to delay programmed telomere shortening for the purpose of ensuring the accurate synthesis of DNA during replication (12). Telomerase consists of two fundamental elements, namely telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC). The former is the main component of telomerase and the latter serves as template for telomeric DNA synthesis. As the main component of telomerase, TERT plays an important role in the synthesis of the hexameric sequence at the end of chromosomes. The regulation of TERT expression in cells is tissue-dependent (13). TERT activity prevents telomere shortening with cell division, thereby preventing cell aging and allowing extensive self-renewal (8).

A previous study suggested that decreased expression of TERT and the telomere-binding proteins telomeric repeat-binding factor 1 (TRF1), TRF2 and protection of telomeres 1 may cause the age-related shortening of ovarian telomeres, which may be associated with the decline of female fertility (14). A study of mouse models showed that the telomere shortening of ovaries leads to elevated embryo fragmentation, increased levels of cellular arrest, degeneration and chromosomal abnormalities (15). In a study of humans in which *in vitro* matured oocytes from the germinal vesicle stage were used as experimental materials, TL was found to be a negative predictor of fragmentation in day-3 preimplantation embryos (16). Also, certain studies demonstrated that telomeres in polar bodies from women in whom a pregnancy was established after *in vitro* fertilization (IVF)-embryo transfer were longer than those from women who did not conceive (17). However, another study found no significant associations between the relative TL (RTL) of GCs and IVF outcomes (11). The exact association between TL/telomerase and IVF outcomes remains unclear and requires further investigation. Notably, the GCs surrounding the oocytes are considered markers for oocyte quality and competence (18) and play a role at the early stage of embryonic development (19).

The aim of the present study was to investigate alterations in RTL and relative TERT expression in GCs during aging. The association between RTL/TERT and ovarian/embryonic performance, and the probability of clinical pregnancy in infertile women undergoing IVF or intracytoplasmic sperm injection (ICSI) treatment was also assessed.

Materials and methods

Retrospective analysis. Clinical data from patients that had undergone frozen-thawed embryo transfer cycles and were divided into >35-year (n=4,068) and ≤35-year (n=17,505) age groups at the Reproductive Medical Center of the First Affiliated Hospital of Anhui Medical University (Hefei, China) between 2018 and 2020 were retrospectively analyzed. The age range of these patients was 20-50 years (median age, 31.00 years). Preimplantation genetic testing (PGT) of chromosome aneuploidies (PGT-A; n=53) in the older age group during the same period was also performed and the data were analyzed.

Study subjects. A total of 160 women aged between 23 and 45 years (median age, 33.50 years) who underwent their first fresh cycle of IVF or ICSI between March 2019 and December 2020 were recruited at the Reproductive Medical Center of the First Affiliated Hospital of Anhui Medical University. These included 100 women who enrolled for RTL measurement and 60 women who enrolled for TERT measurement. All 160 women underwent follicular fluid (FF) anti-Müllerian hormone (AMH) measurements.

The enrolled women met the following criteria: i) Age, ≤35 years (young age group) or >35 years (older age group); and ii) had undergone their first fresh cycle of IVF or ICSI. The exclusion criteria were as follows: i) Patients with chromosomal abnormalities; ii) patients with gynecological, endocrine or autoimmune diseases, such as polycystic ovary syndrome (PCOS), hyperprolactinemia, endometriosis, adenomyosis, hyperthyroidism and systemic lupus erythematosus; iii) patients with a history of radiotherapy, chemotherapy or ovarian surgery; iv) patients diagnosed with premature ovarian failure. GCs and FF were collected from all participants. The clinical pregnancy rate was determined based on the first frozen-thawed blastocyst-stage embryo transfer cycle.

The present study was reviewed and approved by the Ethics Committee of Anhui Medical University (approval no. 20190228) and written informed consent was obtained from all participants.

Clinical sample collection. A pooled collection of FF was obtained from each patient on the day of oocyte retrieval. The first and last tubes of FF collected from each patient were not included because they contained follicle flushing fluid (cat. no. 511119; Vitrolife, Inc.). A tube containing 10-15 ml FF was obtained from each enrolled patient and then centrifuged for 15 min at 458 x g at room temperature. The supernatants were collected and stored at -80°C for future AMH measurement. Excess GCs mechanically separated from the cumulus oocyte complexes (COCs) using a 1-ml syringe needle (cat. no. 300841; Becton, Dickinson and Company) were used as experimental materials. The separated cells were washed and resuspended in PBS (cat. no. 8122153; Gibco; Thermo Fisher Scientific, Inc.) and then prepared for culture or storing at -20°C for subsequent experiments.

FF AMH level measurement. The FF AMH concentration was measured by ELISA using a sensitive diagnostic kit (cat. no. K-1401-100N; Guangzhou Kangrun Biotechnology Co.,

Table I. Primer sequences used for quantitative PCR.

Gene	OMIM accession no.	Primer (5' to 3')	Amplicon length (bp)
Telomere	-	F: GGTTTTGG GGGTGAGGGTGAGGGTGAGGGTGAGGGT R: TCCCAGACTATCCCTATCCCTATCCCTATCCCTATCCCTA	-
36B4	180510	F: CAGCAAGTGGGAAGGTG TAATCC R: CCCATTCTATCATCAACGGGTACAA	75
TERT	187270	F: CTCCC ATTTTCATCAGCAA GTTT R: CTTGGCTTTTCAGGATGGAGTAG	96
GAPDH	138400	F: GGAAGCTTGTTCATCAATGGAAATC R: TGATGACCCTTTTGGCTCCC	167

OMIM, Online Mendelian Inheritance in Man; TERT, telomerase reverse transcriptase; bp, base pairs; F, forward; R, reverse.

Ltd.) for the quantitative detection of AMH, according to the manufacturer's protocol (20). Standards covered a range of 0.06–18.0 ng/ml and the coefficient of variation was <10%. The intra- and inter-assay coefficients of variation were <10 and <15%, respectively.

RTL measurement of GCs by quantitative PCR (qPCR). Genomic DNA was extracted from GCs using a DNeasy Tissue Kit (cat. no. 69504; Qiagen, Inc.). The RTL was determined by a modified qPCR method as described previously using a LightCycler[®] 480 platform (Roche Diagnostics GmbH) (21–23). The RTL was calculated as the telomere/single copy gene (T/S) ratio.

The primer sequences used for qPCR are shown in Table I. The samples were analyzed in triplicate. Each reaction mixture contained 10 µl 2X SYBR Green qPCR Master Mix (cat. no. 43513; Jiangsu RepoDx Biotechnology Co., Ltd.), 1.0 µl each primer (5 µmol/l) and 1.0 µl genomic DNA (~200 ng/µl), and was adjusted to 20 µl using double distilled H₂O. For telomere and single copy gene (36B4 gene) PCR, pre-denaturation was performed at 94°C for 4 min followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec.

TL was determined by calculating the T/S ratio using ΔCq (Cq telomere/Cq single copy gene). The T/S ratio of each sample was normalized to the mean T/S ratio of the reference sample [$2^{-(\Delta Cq_x - \Delta Cq_r)} = 2^{-\Delta \Delta Cq}$], where x is the experimental group and r is the control group (21).

TERT expression measurement of GCs by reverse transcription-qPCR. Data on the chromosomal location and protein structure of the TERT gene were searched in the University of California Santa Cruz (UCSC) website (<http://genome.ucsc.edu>; comparative 3D structure was predicted by ModBase: <https://modbase.compbio.ucsf.edu/>) (24). For TERT gene measurement, RNA was extracted from GCs using an RNeasy Mini kit (cat. no. 74134; Qiagen AB). The ratio of the absorbance at 260 nm to that at 280 nm was used to assess the purity of the extracted RNA. The quality and concentration of the extracted RNA met the standards for subsequent experiments. Then, 2 µg RNA was reverse transcribed into cDNA (42°C for 60 min, 70°C for 5 min and 4°C hold; RevertAid RT Kit, Thermo Fisher Scientific, Inc.) followed by quantification using qPCR as described for RTL

measurement of GCs by qPCR. SYBR Green Master Mix (cat. no. 55499820; Roche Diagnostics GmbH) was used to detect the expression level of TERT. Gene expression was quantified relative to that of the reference gene GAPDH using the 2^{-ΔΔCq} method (25). The primer sequences are listed in Table I.

Cell culture. The fresh GCs were digested and dispersed in hyaluronidase solution (cat. no. 90101; FUJIFILM Irvine Scientific, Inc.). The digestion was then terminated with DMEM (cat. no. SH30022.01; HyClone; Cytiva) supplemented with 10% FBS (cat. no. 16000-044; Gibco; Thermo Fisher Scientific, Inc.). The cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in a sterile cell culture dish (cat. no. 353001; Falcon[®]; Corning Life Sciences) at 37°C with 5% CO₂ for 24 h.

Immunofluorescence (IF). Glass bottom dishes were first pre-treated with poly-lysine solution for ~40 min in a humidified 37°C incubator to coat the bottom of the dishes and then washed three times with PBS, which was removed after washing. The cells were seeded in the pre-treated glass bottom dishes as described in the cell culture section.

IF was performed to identify ovarian GCs and the intracellular localization of TERT in the GCs. The cultured cells were fixed with 4% paraformaldehyde for 20 min at room temperature followed by three washes with PBS, permeabilized for 20 min with 0.5% Triton X-100 following by rinsing with PBS three times, and then blocked for 30 min with 1% bovine serum albumin (cat. no. V900933; Sigma-Aldrich; Merck KGaA) at room temperature. The fixed samples were incubated overnight at 4°C with anti-follicle stimulating hormone (FSH) receptor (FSHR; cat. no. ab75200; dilution, 1:100; Abcam) or anti-TERT antibodies (dilution, 1:50; cat. no. ab230527; Abcam). After washing three times with PBS for 3 min per time, the samples were incubated with a goat anti-rabbit IgG secondary antibody (cat. no. A23220; dilution, 1:400; Abbkine Scientific Co., Ltd.) for 2 h at room temperature and then counterstained with DAPI (cat. no. 28718-90-3; Beyotime Institute of Biotechnology) for 15 min at room temperature followed by washing three times with PBS. Fluorescent images were captured using a laser scanning confocal microscope (Zeiss AG). GCs incubated with only a secondary antibody were used as a negative control. The mean

fluorescence intensity was measured using ImageJ version 1.8.0 software (National Institutes of Health), which included the extraction of image-color-split channels with automatic image threshold adjustment and analysis of the measurement data.

Western blotting (WB). Proteins of the GCs were extracted using RIPA buffer (Wuhan Servicebio Technology Co., Ltd.). Protease and phosphatase inhibitors (protease and phosphatase inhibitor cocktail for general use; 50X; Beyotime Institute of Biotechnology) were added (the final concentration of protease and phosphatase inhibitors was 2%) into the RIPA buffer to prevent protein degradation. The protein concentration was determined using bicinchoninic acid (Wuhan Servicebio Technology Co., Ltd.). Proteins (10 μ g/lane) were separated by SDS-PAGE (8% separation gel) and then transferred onto 0.45- μ m PVDF membranes (MilliporeSigma). The membranes were incubated with rabbit anti-TERT (dilution, 1:1,000; Abcam) and anti- β -actin (dilution, 1:1,000; cat. no. ab179467; Abcam) primary antibodies overnight at 4°C. The latter were used to ensure equal protein loading. The membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody (cat. no. E-AB-1041; Elabscience, Inc.) at room temperature for 90 min. The immunoreactive protein bands were then developed using Pierce™ ECL Plus Western Blotting Substrate (Thermo Fisher Scientific, Inc.) and AlphaEaseFC 4.0 software (Alpha Innotech Corporation) was used to quantify band intensity.

Statistical analysis. The normality of data was assessed using the Kolmogorov-Smirnov test. The RTL was \log_{10} (LG)-transformed due to a skewed distribution. Kruskal-Wallis test with Bonferroni correction was performed for the comparison of RTL and TERT data in multiple age groups as several groups of data did not conform to the normal distribution. A binary logistic regression model was used to determine the pregnancy probability. The predictive values of TERT and RTL for clinical pregnancy were determined using receiver operating characteristic curves and the area under the curves. Mann-Whitney U tests were performed in the comparison of clinical parameters including age, body mass index, day 3 serum FSH, gonadotropin (Gn) dose and IVF outcome measures between the RTL and TERT groups; the comparison of FF AMH levels, RTL and relative TERT mRNA expression levels between the <35-year and \geq 35-year age groups; and the comparison of RTL and relative TERT mRNA expression levels between pregnant and non-pregnant patients. Independent sample t-tests were used for comparison of TERT protein expression between the <35-year and \geq 35-year age groups. A Chi-square test was performed to analyze the counts of different ovulation induction protocols in the RTL and TERT groups. Pearson's correlation test was used to analyze the correlation of normally distributed data and Spearman's rank correlation test was used for skewed data. A two-sided $P < 0.05$ was considered to indicate a statistically significant difference. Linear regression models were established when a correlation analysis had $P < 0.05$. Data are presented as the mean \pm SD. Statistical analysis was performed using SPSS 16.0 (SPSS, Inc.) and GraphPad Prism 5.0 (GraphPad Software; Dotmatics).

Results

Clinical data analysis. In the retrospective analysis, clinical pregnancy rates of 41, 40 and 34% were reported in 2020, 2019 and 2018, respectively, in the >35-year age group. The respective rates in the \leq 35-year age group were 63, 61 and 52%. In 2019 and 2018, the live birth rates of the >35-year age group were 25 and 23%, respectively, compared with 44 and 40%, respectively, in the \leq 35-year age group. With regard to PGT-A cycles, a total of 172 blastocysts from 53 patients were analyzed, of which 18 blastocysts (10.47%) were euploid, 126 blastocysts (73.26%) were aneuploid and 28 blastocysts (9.30%) were mosaic. The pregnancy and live birth rates for women aged >35 years were \sim 20% lower than those of women aged \leq 35 years, and in the older women the number of embryos with aneuploidy was 7-fold higher than that of those without euploidy (Fig. 1A-C; Table SI).

General characteristics of the study subjects. A total of 160 women aged 23-45 years met the inclusion criteria for the present study, of which 100 were enrolled for RTL measurement and 60 were enrolled for TERT measurement. All 160 women underwent FF AMH measurement. No significant difference in clinical characteristics, including age, body mass index, day-3 serum FSH, total Gn dose and IVF outcome measures was detected between the RTL and TERT groups ($P > 0.05$; Table II). Antagonist and long Gn-releasing hormone-agonist (GnRH-a) protocols were the main ovulation induction protocols in both the RTL and TERT groups, accounting for >80% of the ovulation induction protocols. In the RTL group, the percentages of antagonist, long GnRH-a and other ovulation induction protocols (milder stimulation protocols) were used in 61 (61.0%), 32 (32.0%) and 7 (7.0%) patients, respectively. In the TERT group, these protocols were used in 32 (53.3%), 21 (35.0%) and 7 (11.7%) patients, respectively. No significant difference in the frequencies of the various ovulation induction protocols was found between the RTL and TERT groups (Table III).

Measurement of FF AMH levels between the older and younger age groups. FF samples were obtained from 71 patients aged \geq 35 years and 89 patients aged <35 years. A lower FF AMH level was observed in the older age group compared with the younger age group (4.31 ± 3.79 vs. 6.41 ± 5.35 ng/ml; $P < 0.01$; Fig. 1D).

Biological characteristics of senescent GCs. Following overnight culture for 24 h, the cells grew well and were adherent. FSHR is a marker of human ovarian GCs (26). As shown by immunofluorescence staining (Fig. 2), FSHR was expressed in the GCs of women from both age groups. Although the expression of FSHR appeared to be stronger in the older age group, the number of COCs retrieved from the older age group was lower than that of the younger age group and the cell density of the COCs from the older age group was lower.

RTL association with age and ovarian/embryonic outcome. A total of 100 women were enrolled for RTL measurement, and the results are shown in Table IV (1.29 ± 1.12 and 1.86 ± 1.18 in the old and young group, respectively). Linear

Table II. Characteristics and *in vitro* fertilization outcomes of patients from whom the RTL and TERT data of granulosa cells were collected.

Patient characteristics	RTL group (n=100)	TERT group (n=60)
Age, years	33.40±6.30	32.70±6.70
BMI, kg/m ²	22.87±3.36	23.25±3.22
D3 serum FSH, mIU/ml	8.10±3.55	8.09±2.87
Total Gn dose, IU	2,268.20±784.02	2,234.8.30±752.59
Oocytes retrieved, n	11.78±7.15	13.38±10.05
MII oocytes, n	9.22±5.66	9.87±7.06
Fertilized oocytes, n	8.35±5.17	9.05±6.65
Cleaved embryos, n	8.11±4.96	8.80±6.56
Blastocysts, n	4.72±3.80	4.98±4.20

RTL, relative telomere length; TERT, telomerase reverse transcriptase; BMI, body mass index; D3, day 3; FSH, follicle stimulating hormone; Gn, gonadotropin; MII, metaphase II.

Table III. Ovulation induction protocols in the RTL and TERT groups.

Ovulation induction protocols	RTL group, n (%)	TERT group, n (%)
Antagonist	61 (61.0)	32 (53.3)
Long GnRH-a	32 (32.0)	21 (35.0)
Other	7 (7.0)	7 (11.7)

GnRH-a, gonadotropin-releasing hormone-agonist.

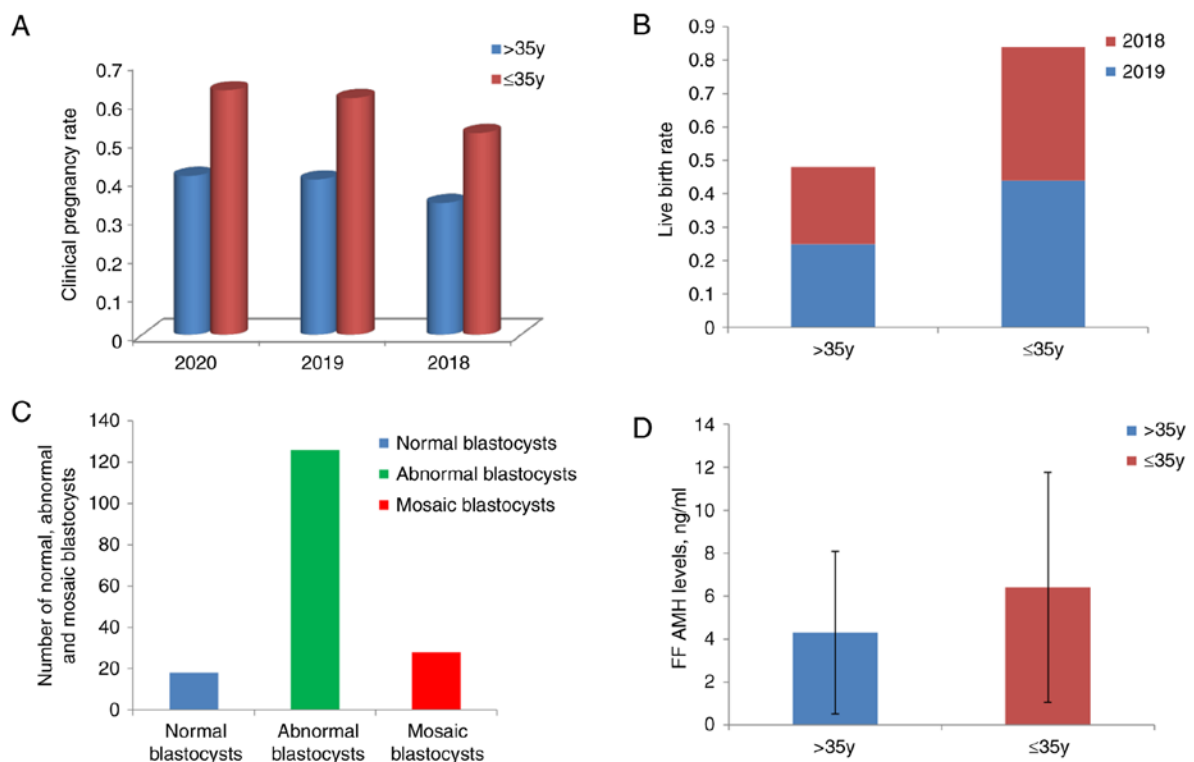


Figure 1. Clinical data analysis and FF AMH levels in patients of different ages. (A) Clinical pregnancy rates of patients aged >35 and ≤35 years between 2018 and 2020. (B) Live birth rates of patients aged ≤35 and >35 years in 2018 and 2019. (C) Results of preimplantation genetic testing for chromosome aneuploidies in the >35-year age group. Normal blastocysts are euploid blastocysts with a normal number of chromosomes, and abnormal blastocysts are aneuploid blastocysts with an abnormal number of chromosomes. (D) FF AMH levels (ng/ml) in patients aged ≤35 and >35 years. FF, follicular fluid; AMH, anti-Müllerian hormone; 35y, 35 years.

Table IV. RTL and relative TERT expression measurements by quantitative PCR in patients according to age.

Variable	>35 years		≤35 years		P-value
	n	Mean ± SD	n	Mean ± SD	
RTL, LG T/S	45	1.29±1.12	55	1.86±1.18	0.004
Relative TERT expression	26	1.02±0.87	34	0.52±0.45	0.013

RTL, relative telomere length; LG T/S, \log_{10} transformed telomere/single copy gene ratio; TERT, telomerase reverse transcriptase.

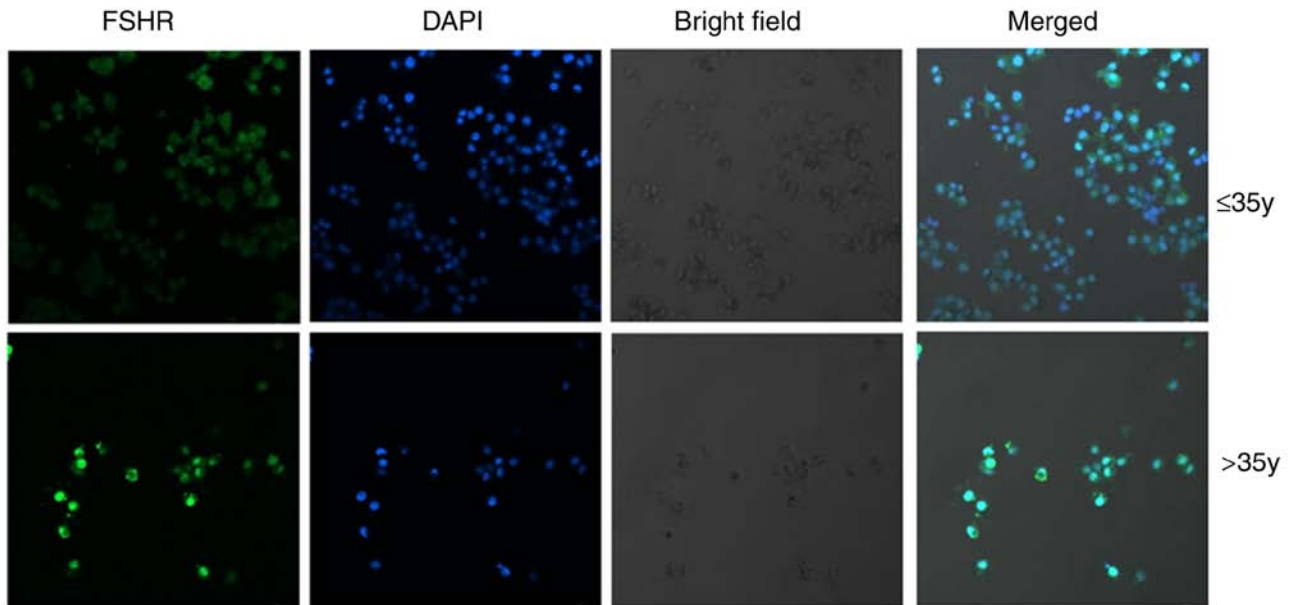


Figure 2. Immunofluorescence results showing FSHR expression in the granulosa cells of patients aged ≤35 and >35 years (magnification, x200). FSHR, follicle stimulating hormone receptor; 35y, 35 years.

regression analysis revealed a statistically significant negative association between the RTL of GC samples and patient age [LG RTL = $-0.03 \times \text{year} + 2.62$; $R^2=0.03$; correlation coefficient (r) = -0.20 , $P=0.04$; Fig. 3A]. To better understand the association of RTL with age, the 100 samples were divided into three subgroups (38-45, 35-37 and 23-32 years). These groupings were chosen because although women >35 years old were considered as older in the present study, an age of 35-37 years may be considered as transitional. Notably, there is a discontinuity between the two lower age groups as no GCs were obtained from patients aged 33 or 34 years. The RTL was significantly higher in patients aged 23-32 years compared with the two older age groups ($P<0.05$). However, no significant difference was detected between the patients aged 38-45 years and those aged 35-37 years (Fig. 3B).

No significant correlations were found for GC RTL with day-3 serum FSH level ($P=0.11$), the number of oocytes retrieved ($P=0.07$), the number of mature (MII) oocytes retrieved ($P=0.17$) and blastocyst formation rate ($P=0.99$) (Table V).

Relative TERT expression in relation to age and ovarian/embryonic outcome, as determined by qPCR. The 3D structure of the TERT gene located on chromosome 5p15.33

was found on the UCSC website (Fig. 4). In order to investigate the relative expression of TERT in GCs according to age, GCs from 26 older patients (36-44 years old) and 34 younger patients (23-35 years old) were obtained. Higher relative TERT expression was observed in the older age group compared with the younger age group (1.02 ± 0.87 vs. 0.52 ± 0.45 ; $P<0.05$; Table IV). Linear regression analysis revealed a significant positive association between the TERT expression of GC samples and patient age ($P<0.05$; Fig. 3C). On average, relative TERT expression increased by 0.03 every year from the age of 23 years to the age of 44 years (relative TERT gene expression = $0.03 \times \text{year} - 0.31$; $R^2=0.09$; $r=0.30$, $P=0.02$). To better understand its association with age, the 60 samples were further divided into three subgroups by age (38-44, 35-37 and 23-32 years). The level of TERT expression was lower in the 23-32-year age group than in the two older age groups ($P<0.05$). No significant difference was detected between the patients aged 38-44 years and those aged 35-37 years (Fig. 3D).

When evaluating the correlations between the relative TERT gene expression of GC samples and ovarian/embryonic outcome, a statistically significant inverse correlation was observed between relative TERT gene expression and the number of oocytes ($r=-0.48$, $P<0.001$) and MII oocytes ($r=-0.41$, $P<0.001$) retrieved. However, no statistically

Table V. Correlations of RTL and relative TERT expression in granulosa cells with ovarian/embryonic outcomes.

Ovarian/embryonic outcome	RTL		Relative TERT expression	
	P-value	Correlation coefficient	P-value	Correlation coefficient
Number of oocytes retrieved ^a	0.07	-0.18	<0.001	-0.48
Mature (MII) oocytes retrieved ^a	0.17	-0.14	0.001	-0.41
Blastocyst formation rate ^b	0.99	0.002	0.10	0.22
Day-3 serum FSH ^a	0.11	0.16	0.23	0.16

^aNormally distributed, analyzed using Pearson's correlation test; ^bskewed distribution, analyzed using Spearman's correlation test. RTL, relative telomere length; TERT, telomerase reverse transcriptase; MII, metaphase II; FSH, follicle stimulating hormone.

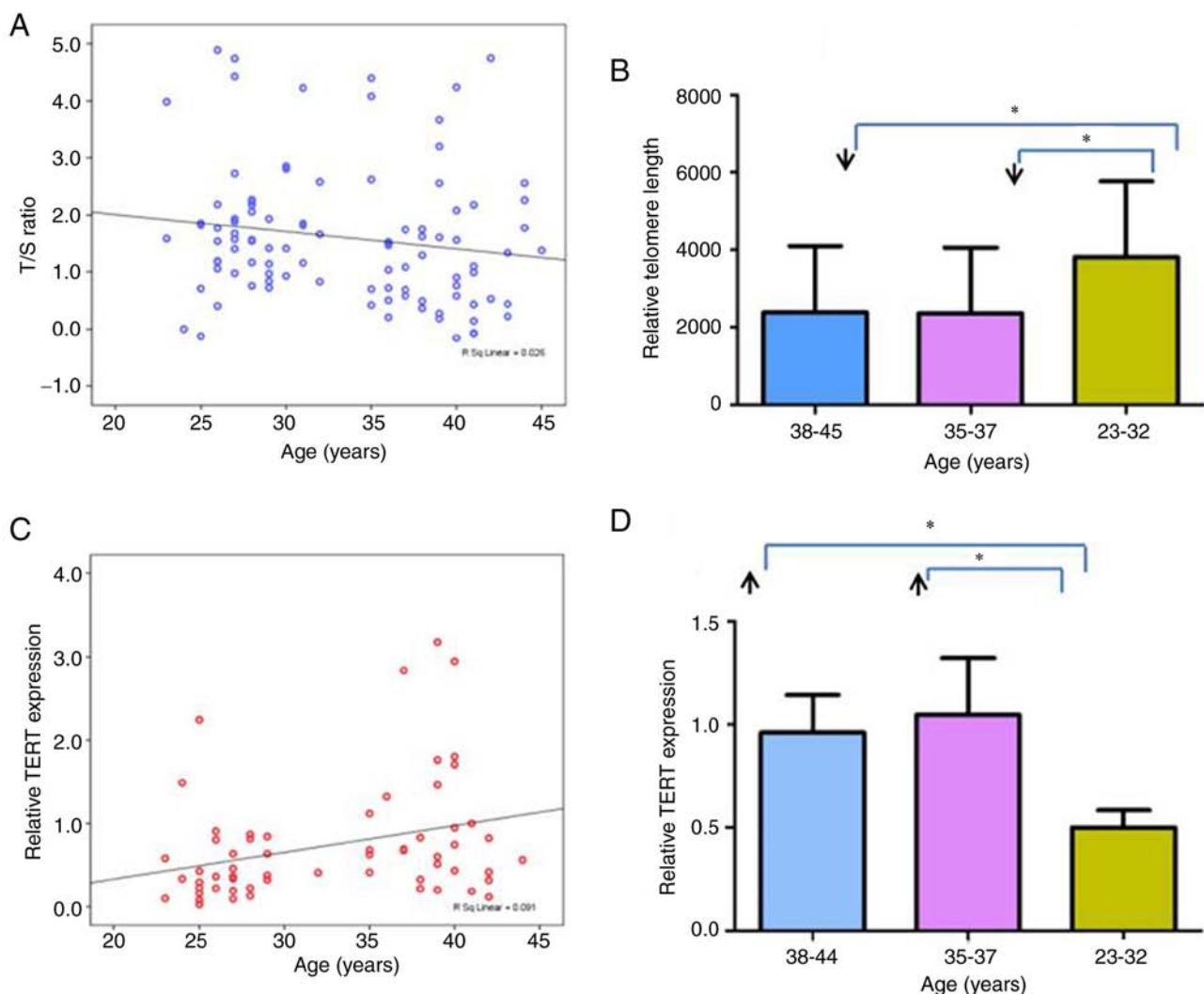


Figure 3. Analysis of relative TERT expression and relative telomere length in relation to patient age. (A) Linear regression analysis of relative telomere length (T/S ratio) and age. (B) Relative telomere length in three age subgroups. (C) Linear regression analysis of TERT expression and age. (D) TERT expression in three age subgroups. * $P < 0.05$. TERT, telomerase reverse transcriptase; T/S, telomere/single copy gene.

significant correlation was observed between relative TERT gene expression in GC samples and the serum FSH level ($P = 0.23$) and blastocyst formation rate ($P = 0.10$). The results of the correlation analysis between the TERT gene expression of GC samples and ovarian/embryonic outcomes are shown in Table V.

Associations between RTL, relative TERT expression and the probability of clinical pregnancy. Based on the clinical pregnancy outcome, all patients (>35 and ≤ 35 years) were divided into two groups, namely pregnant and non-pregnant groups. The RTL and relative TERT expression at the mRNA level were analyzed in both groups. The associations of RTL

Table VI. Associations of RTL and relative TERT expression in granulosa cells with the probability of clinical pregnancy in patients according to age.

Statistical measure	>35 years		≤35 years	
	RTL	TERT	RTL	TERT
OR	1.458	0.364	0.580	0.908
95% CI	0.748-2.842	0.093-1.427	0.333-1.012	0.184-4.487
P-value	0.268	0.147	0.055	0.906
AUC	0.630	0.267	0.360	0.390

RTL, relative telomere length; TERT, telomerase reverse transcriptase; OR, odds ratio; CI, confidence interval; AUC, area under the curve.

A



B

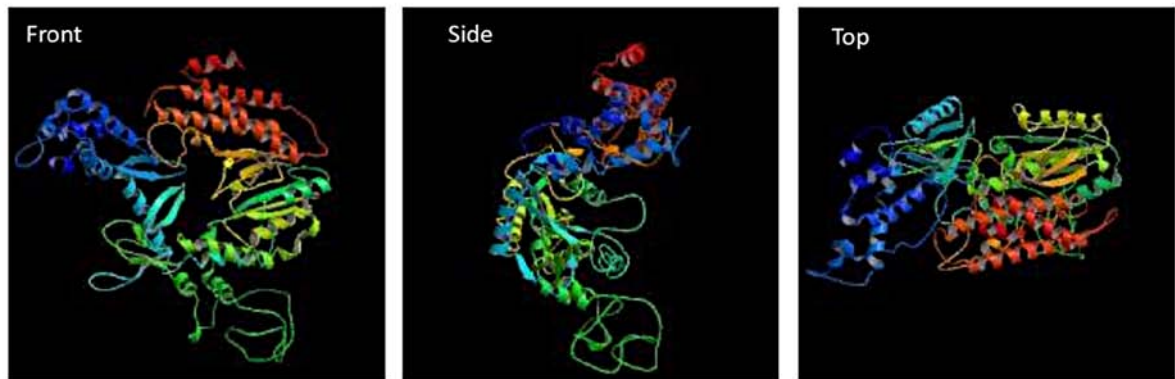


Figure 4. Data on the chromosomal location and protein structure of the TERT gene as retrieved from the University of California Santa Cruz website (<http://genome.ucsc.edu>). (A) Chromosomal location of TERT. (B) ModBase prediction of the comparative 3-dimensional structure of TERT (front/slide/top). TERT, telomerase reverse transcriptase.

and relative TERT expression with the probability of clinical pregnancy were analyzed by binary logistic regression and the results were assessed using odds ratios and 95% confidence intervals. As age is an important factor for clinical pregnancy rate, the logistic regression was performed separately in the older and younger age groups. No significant associations of RTL or relative TERT expression with the probability of clinical pregnancy were found, regardless of patient age (Table VI; Fig. 5A). The predictive values of TERT and RTL for clinical pregnancy were determined using receiver operating characteristic curves and are shown in Fig. 5B. Although the area under the curve value of 0.630 for RTL for the >35-year age group was high, TERT and RTL were determined to have no predictive value for clinical pregnancy owing to the lack of significance being found (Fig. 5B; Table VI).

Verification of TERT expression in GCs. IF and WB experiments were further performed to verify the expression of TERT in the younger and older patient age groups. The results of these assays showed that TERT was highly expressed in the patients ages >35 years, but the difference in TERT expression compared with the patients aged ≤35 years was not significant ($P>0.05$; Fig. 6).

Discussion

It is well established that a woman's fertility declines with age, leading to a lower chance of pregnancy. Both the quantity and quality of oocytes decline during aging. The clinical data in the present study were consistent with this, as they showed that the pregnancy and live birth rates for women aged >35 years were ~20% lower than those aged <35 years, and the number of embryos with aneuploidy was 7-fold that of women with euploidy in older women. Although the mechanism underlying the loss of fertility and higher risk of aneuploidies induced by aging has been widely considered, the specific mechanism is not fully understood.

Since GCs support oocytes during follicular growth and maturation, exploration of the mechanism of GC aging provides important insights into ovarian aging. For that reason, ovarian GCs from infertile women undergoing IVF were selected as ovarian aging research models in the present study, and GCs from older patients were found to have higher FSHR expression and lower FF AMH levels than those from younger patients. Telomeres themselves do not encode any specific products (27). Telomere dysfunction is associated with increased aneuploidy, impaired mitochondrial function, decreased gluconeogenesis and

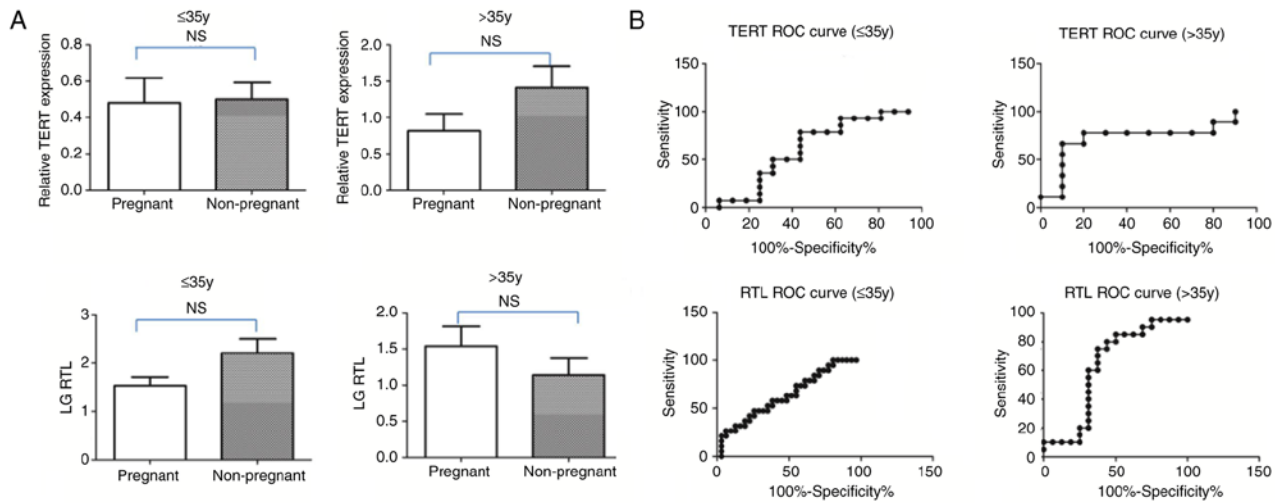


Figure 5. Associations of TERT and RTL with the probability of clinical pregnancy. (A) Comparison of RTL and TERT expression between pregnant and non-pregnant patients aged ≤ 35 and > 35 years. (B) ROC curves for TERT and RTL in patients aged ≤ 35 and > 35 years. TERT, telomerase reverse transcriptase; RTL, relative telomere length; LG, \log_{10} ; ROC, receiver operating characteristic; 35y, 35 years; NS, not significant.

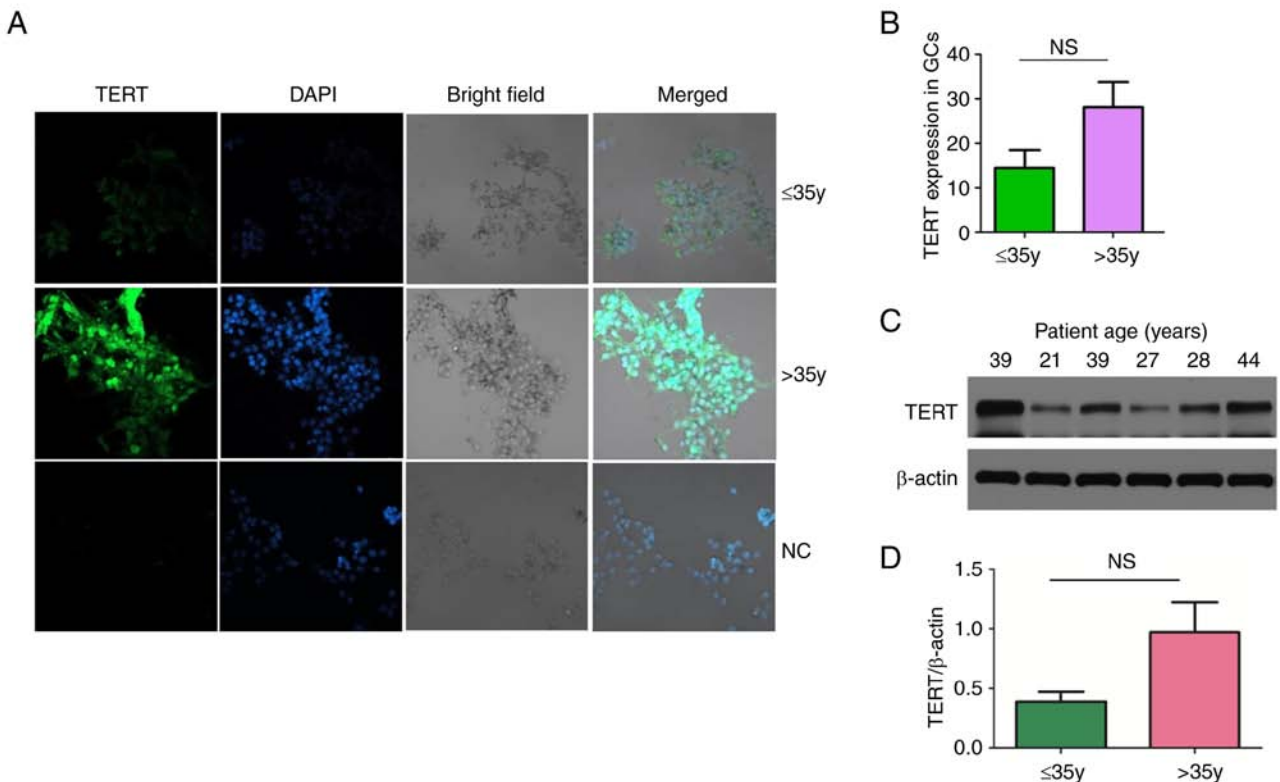


Figure 6. Verification of TERT expression at the protein level in patients of different ages by IF and WB. (A) TERT expression in patients aged ≤ 35 and > 35 years as shown by IF (magnification, $\times 200$). Granulosa cells incubated with only a secondary antibody were used as the NC. (B) Quantification of the IF staining. (C) Expression of TERT in patients of different ages as determined by WB. (D) Quantification of the WB results. TERT, telomerase reverse transcriptase; IF, immunofluorescence; WB, western blotting; NC, negative control; NS, not significant; 35y, 35 years; GCs, granulosa cells.

increased cellular reactive oxygen species levels (28). A previous study suggested that age-associated DOR may result from telomere attrition which is usually regulated by telomerase (13). Telomerase activity in GCs is important for their proliferation and differentiation capacity (29), as it plays versatile roles in various reproduction pathways. TERT encodes the catalytic subunit of telomerase, which counters telomere shortening during cell division (30). The

TERT mRNA is accompanied by another enzyme component TERC, which is an RNA molecule that encodes the catalytic component of telomerase (31). The TERT and TERC together components constitute active telomerase, which counteracts the gradual shortening of telomeres with each round of DNA replication by maintaining the telomere sequences and conferring a sustained proliferation capacity to growing cells (32).

In the present study, changes to telomeres and TERT during the natural aging of GCs were explored. Patients with endocrine diseases, such as PCOS, which may affect TL (33,34), thus confounding the results, were excluded from the study. To further explore the role of telomeres and the TERT gene in reproduction, the associations of RTL/relative TERT expression with ovarian/embryonic outcome as well as the probability of clinical pregnancy were assessed. Most studies on ovarian aging are conducted by calculating the number of non-growing follicles (NGF), which ultimately determines the reserve and function of the ovaries. The reduction in NGF counts accelerates in women aged ≥ 38 years (35). The data in the present study also showed that the number of oocytes retrieved by women aged ≥ 38 years declined sharply (data not shown), which is the theoretical basis for the allocation of older patients into two subgroups for the analysis of relative TERT expression and RTL. The results showed that the RTL of GCs gradually decreased with age, which conflicts with a previous study that found no significant change in the RTL of GCs with aging (11). However, the results of the present study also indicated that RTL was not associated with any measure of ovarian/embryonic performance or clinical pregnancy. This finding contrasts with those of previous studies which have demonstrated that variations in embryonic outcomes are associated with the shortening or elongation of TL in GCs (36,37).

In the present study, an increased TERT expression at the transcriptional level was detected in the GCs of older women. Increased TERT expression in GCs may affect telomerase activity, resulting in a shortening of TL that is associated with chromosomal and genomic instability during aging. In addition, it is worth noting that although TERT appears to be a more notable biomarker than RTL for the prediction ovarian response among infertile women, it plays no role in the prediction of embryonic development potential and clinical pregnancy, as no statistically significant correlation was identified between TERT and blastocyst formation rate. The findings of previous studies support the decline in TERT expression and activity in mouse oocytes with reproductive aging (32) and its association with telomere shortening and decline in oocyte quality (16). The data in the present study also indicate that TERT expression alters in GCs with aging, but RNA-sequencing data (data not shown) did not detect any significant changes in TERT expression between old and young human oocytes. Kosebent and Ozturk (38) suggested that TERT expression was differentially expressed at different developmental follicular stages in both young and old mice. These seemingly discordant results indicate that the regulation of TERT expression is tissue- and stage-dependent.

In addition to its established role in the opposition of cell replication-dependent telomere shortening, TERT exhibits multiple physiological activities beyond its canonical action. For instance, TERT has been shown to modulate mitochondrial and ubiquitin proteasomal function or act as a transcription co-factor (39), and thereby contribute to the regulation of gene expression, promotion of cell survival and growth and protection of cells from apoptosis (30). It has also been shown to display RNA-dependent RNA polymerase activity (39). Previous studies have also reported a pleiotropic role for TERT in the regulation of the epigenetic clock (30) and intrinsic DNA methylation age during the proliferation of

growing cells (40). In combination, all these effects of TERT are actively involved in its biological activity, which ultimately affects the aging process. The activities possessed by TERT, whether or not they are associated with telomere maintenance, may contribute to the aging process. Notably, the difference of TERT expression in GCs at the protein level between the younger and older age groups was not found to be significant. The reason for this result may be the small sample size and high heterogeneity of human samples, or other telomere regulatory mechanisms may exist at the protein level.

Hormonal regulation of telomere maintenance by telomerase in the ovary affects cell proliferation and ovarian aging. Specifically, telomerase activity in GCs is controlled by growth factors and female steroid hormones (29). The TERT gene promoter contains an estrogen response element (38). The microenvironment of GCs contains estrogens that are reported to influence telomerase activity and length (41). Mordechai *et al* (29) suggested that Gn from the serum of pregnant mares increased TERT expression at the transcriptional and translational levels in rat ovarian GCs. The present study also revealed a reduction in the AMH in the FF in the old age group compared with the young age group. Overall, these studies indicate that the changes of TERT gene expression in GCs that occur during aging may be due to changes in female hormone levels. Although the telomere theory of reproductive aging has mostly focused on the effect of shorter telomeres on meiotic errors and the elevated risk of aneuploidies (40,41), it is also possible that TL or TERT interaction with endocrine homeostasis in the ovarian microenvironment may play a role in ovarian aging. The GC and FF surrounding the oocyte together constitute the local ovarian follicle microenvironment. Reproductive aging decreases endocrinological output and compromises the homeostasis of the FF (42), with effects such as lowering the activity of antioxidant enzymes and increasing oxidative protein damage (43). Furthermore, TL is associated with the adverse effects of oxidative stress and homeostasis impairment. The TLs of GCs have been found to be shorter in patients with PCOS (34) or biochemical primary ovarian insufficiency (44) than in relevant control, and a high estradiol-17 concentration has been shown to increase the TL of GCs (45). Moreover, it has been shown that FF AMH levels can be used to determine the apoptosis rate of GCs in assisted reproductive technology cycles (46). These findings suggest an association between telomeres and the endocrine system in the ovarian microenvironment (45). Additionally, the microenvironment in ovarian follicles differs with regard to telomere biology when compared with that of certain other somatic tissues, as it has been observed that the telomeres of GCs are longer than those of leukocytes, which suggests a different mechanism of telomere maintenance in the ovarian follicle microenvironment (47). The molecular mechanism requires investigation in future studies to explore the intrinsic association between these indicators.

The present study was not without limitations. Its primary limitation is the limited sample size, which creates a certain bias and decreases the strength of the conclusions. Secondly, it would be more statistically valuable to analyze RTL and TERT in the same GC sample to assess their associations; however, it was not possible to do that due to the small number of

ovarian GCs obtained from older patients. Finally, individual differences in human samples may reduce the credibility of the conclusions. Other methods, such as telomerase activity measurements, are necessary to verify the results.

In conclusion, the findings of the present demonstrate that RTL and TERT alterations in GCs may be determinants of ovarian aging. In addition, TERT appears to be a potential biomarker for the prediction ovarian response among infertile women during IVF/ICSI treatment. The measurement of TERT in GCs provides a novel strategy for female fertility assessment and useful guidance for the research and clinical treatment of reproductive aging. Well-designed larger-scale studies are required to confirm the results of the present study and shed light on the mechanism underlying the effects of telomeres and TERT in reproductive aging.

Acknowledgements

Not applicable.

Funding

The present study was supported by National Natural Science Foundation of China (grant no. 82001631), Major Science and Technology Projects in Anhui Province (grant no. 202003a07020012) and Research Funding for Doctoral Talents of the First Affiliated Hospital of Anhui Medical University (grant no. 1465).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YXC and ZGZ designed the experiments and revised the manuscript. YH was responsible for the measurement of RTL by qPCR and relative TERT expression by RT-qPCR. YH also drafted the manuscript. MRL and JP analyzed the clinical data and verified TERT expression by IF and western blotting. ZHZ contributed to data analysis. ZZ, TTW and BY contributed to the measurement of FF AMH levels. DK was responsible for sample collection and IF to determine the biological characteristic of senescent GCs. PZ and ZLW helped to enroll the patients and designed the study. YXC and ZGZ confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The study was reviewed and approved by the Ethics Committee of Anhui Medical University (approval no. 20190228), and written informed consent was obtained from all participants.

Patient consent for publication

The patients consented to the publication of their data.

Competing interests

The authors declare they have no competing interests.

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