Telomerase Activity in Normal Ovaries and Premature Ovarian Failure

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KINUGAWA, C., MURAKAMI, T., OKAMURA K. and YAJIMA, A. Telomerase Activity in Normal Ovaries and Premature Ovarian Failure. Tohoku J. Exp. Med., 2000, 190 (3), 231-238 — The average age for the onset of menopause in humans is about 51 years. On the other hand, premature ovarian failure (POF) is generally defined as the onset of menopause before the age of 40 years. Telomeres have been extensively examined as a mitotic clock. Telomeric DNA is elongated by telomerase. We analyzed the telomerase activity of 20 patients with normal ovaries and 5 patients with POF. Telomerase activity was present in the normal ovaries, however it decreased with age. Eight normal ovaries under 38 years of age showed significantly higher telomerase activity among the women with a regular menstrual cycle. Two POF patients showed high telomerase activity and 3 showed low telomerase activity. Our findings indicate that telomerase is present in the normal human ovary and that telomerase activity decreases with age. Patients with follicle dysfunction showed high telomerase activity and those with follicle depletion showed very low telomerase activity. Based on these results, we speculated that the decline of telomerase activity in the ovary is related to primordial follicle depletion with age and telomerase activity can be used as a marker of the ovarian functional age. —— aging; ovary; premature ovarian failure; primordial follicle; telomerase © 2000 Tohoku University Medical Press

There are about 5 000 000 primordial follicles in the cortex of the human ovary at 20 gestational weeks. The number of primordial follicles decreases with age and is almost lost after menopause. The average menopausal age is about 51 years (Edwards and Brody 1995; Faddy and Gosden 1996). Follicle depletion due to aging is considered to be programmed but this theory has not been confirmed.

Premature ovarian failure (POF) is generally defined as the permanent absence of menses before the age of 40. There are many causes of POF including autoimmunity, viral infections, radiation, enzyme deficiencies, iatrogenic and

Received January 17, 2000; revision accepted for publication March 6, 2000.
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chromosomal defects. However, most cases of POF are idiopathic (Devi et al. 1998). The criteria for diagnosis of POF is as follows: $\geq 4$ months of amenorrhea and 2 serum follicle stimulating hormone (FSH) values of $>40$ mIU/ml obtained $\geq 1$ month apart in a woman $<40$ years of age.

Telomeres are located in the distal ends of human chromosomes and repeat the sequence TTAGGG. They are considered to play an important role in maintaining chromosomal structure. Telomeres are called a mitotic clock because they shorten with each cell division and the telomere length shows the proliferative capacity remaining in cells. Telomeric DNA is elongated by telomerase which is a ribonucleoprotein enzyme (Rhyu 1995). Several recent studies have shown that not only cancer cells but also germline cells and some normal somatic cells express low levels of telomerase (Wright et al. 1996; Yasumoto et al. 1996).

We considered that telomerase activity was present in normal ovarian tissue because this tissue contains germ cells and telomerase activity declines with age. We analyzed the ovarian tissue of normal human ovary in various age groups and premature ovarian failure (POF) for the presence of telomerase activity.

**Materials and Methods**

*Tissue samples*

Normal ovarian tissues were obtained from 20 patients ranging from 25 to 72 years of age with benign ovarian tumors or uterine corpus carcinomas who underwent surgery between February 1997 and January 1998. Fourteen patients had a regular menstrual cycle, and 8 of these were under 38 years of age. The tissues were used as normal samples after we confirmed that no evidence of malignant tissues was present by histopathological examination.

POF tissues were obtain from 5 patients who underwent laparoscopic ovarian biopsy between September 1997 and December 1998 (Table 1). A part of the tissue in all POF samples was stained for histopathological examination with hematoxylin and eosin.

<table>
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<tr>
<th>Patient No.</th>
<th>Study age (years)</th>
<th>Age at menarche (years)</th>
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Patient No. 1 and 2 showed the follicle dysfunction type. Patient No. 3, 4 and 5 showed the follicle depletion type. All patients had normal 46, XX karyotypes and serum FSH values of $>40$ mIU/ml.
All samples were immediately frozen in liquid nitrogen after being washed in cold phosphate buffer saline and stored at $-80^\circ$C.

This study was approved by The Tohoku University Ethical Committee and informed consent was obtained from all patients.

*Telomeric repeat amplification protocol assay*

Frozen tissue was sliced with a surgical sterile knife and homogenized in 200 $\mu$l of ice cold lysis reagent. After 30 minutes of incubation on ice, the lysate was centrifuged at 16 000 g for 20 minutes at 4°C. The protein concentration of the supernatant was measured using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). The extracts were frozen and stored at $-80^\circ$C. Five $\mu$g of protein (standard conditions) was used for each assay. To estimate the telomerase activity, the extracts were diluted 10 and 100 fold. Telomerase activity was determined by the telomeric repeat amplification protocol (TRAP) assay (Kim et al. 1994), using a Telomerase PCR ELISA kit (Boehringer Mannheim, Mannheim, Germany). The kit was employed using a biotinylated primer for immobilization within the ELISA microtiter plate. The TRAP assay, hybridization, and ELISA were performed according to the manufacturer's protocol as described below.

Five $\mu$l of each extract was assayed in a PCR tube containing 25 $\mu$l of reaction mixture and 20 $\mu$l of sterile water. In the first step, all tubes were transferred to a thermal cycler and incubated at 25°C for 10 minutes to add telomeric repeats to the biotin labeled P1-TS primer. These elongation products were heated at 94°C for 5 minutes to inactivate telomerase. In the second step, these elongation products were amplified by PCR using P1-TS and P2 primers. Thirty cycles of PCR was performed. Each cycle was as follows: 94°C for 30 seconds; 50°C for 30 seconds; and 72°C for 90 seconds. Five $\mu$l of PCR products was assayed in reaction tubes containing 20 $\mu$l of denaturation reagent and incubated at room temperature for 10 minutes. Then, 225 $\mu$l of hybridization buffer containing a DIG-labeled detection probe complementary to the telomeric repeat sequences was added to these products. One hundred $\mu$l of these mixtures was then transferred into wells of a streptavidin coated microtiter plate (MTP). The plate was covered with a foil and incubated at 37°C on a shaker (300 rpm) for 2 hours. The MTP modules were washed 3 times with 250 $\mu$l of washing buffer per well. One hundred $\mu$l of anti-DIG-POD working solution was added to MTP, per well, and then the plate was covered with a foil. After 30 minutes of incubation at room temperature on a shaker (300 rpm), the MTP modules were washed 5 times with 250 $\mu$l of washing buffer. Finally, to each well, 100 $\mu$l of tetramethyl benzidine was added and the plate was covered with a foil. The mixture incubated for color development at room temperature for 20 minutes while being shaken at 300 rpm. After 100 $\mu$l of sulfuric acid had been added to each well to stop the color development, the absorbance of the samples at 450 nm was measured using a microtiter plate reader.
We estimated only 5 μg of protein. Telomerase activity was originally assayed with 5 μg, 0.5 μg and 0.05 μg of protein. However, the activity was weak with 0.5 and 0.05 μg.

Statistical analysis was performed by polynomial regression analysis and the Mann-Whitney U-test, using a StatView (Abacus Concepts) statistical software program on a Macintosh computer.

RESULTS

Telomerase activity in the normal human ovary

Telomerase activity decreased with age ($r=0.823$, $p<0.001$) (Fig. 1). Among the 14 subjects with a regular menstrual cycle, the telomerase activity was significantly higher in those under 38 years of age ($p<0.01$) (Fig. 2). We compared the subjects under and over 35 years, but found no significant difference ($p=0.1213$) (data not shown).

Telomerase activity in the POF ovary

The 5 patients with POF were subdivided into 2 groups by histopathological examination with hematoxylin and eosin staining: one group (two patients) with many follicles (11.8 mean primordial follicles per a medium power field) and the other (three patients) with few follicles (0.03 mean primordial follicles per a medium power field) (75 times magnitude). The former was follicle dysfunction type and the latter was follicle depletion type (Fig. 3).

The 2 POF subjects with follicle dysfunction showed high telomerase activity according to their age and the 3 POF subjects with follicle depletion showed very low telomerase activity that was not associated with their age (Fig. 4).

![Fig. 1. Ovarian telomerase activity in 20 patients from 25 to 72 years of age. Telomerase activity decreased with age ($r=0.823$, $p<0.001$).]
Fig. 2. Ovarian telomerase activity in 14 patients under and over 38 years of age with regular menstrual cycles (8 patients were under and 6 patients were over 38 years). Telomerase activity was significantly higher in subjects under 38 years of age (*p < 0.01) (mean ± s.d. of under 38 years was 0.879 ± 0.245 and of over was 0.419 ± 0.128).

**DISCUSSION**

Telomerase activity was considered to exist in germline cells and cancer cells only, not in normal somatic cells (Kim et al. 1994). In the current study, telomerase activity was found in several normal somatic cells (Wright et al. 1996; Yasumoto et al. 1996). Our findings showed that telomerase is present in the normal human ovary and that telomerase activity decreases with age. Murakami et al. (1997) described that low telomerase activity was detected in the cortex of normal ovaries from premenopausal women and was likely attributable to primordial follicles. Yokoyama et al. (1998) also showed that the normal ovary had telomerase activity. Wright et al. (1996) described that the fetal ovary was positive for telomerase activity, while unfertilized adult human oocytes did not have detectable activity. They did not determine the developmental stage of the oocytes. In rat, higher telomerase activity was found in oocytes from early antral and preovulatory follicles than in mature (ovulated) oocytes (Eisenhauer et al. 1997). From these studies it is expected that the telomerase of ovary locates in primordial follicles.

There are many primordial follicles in the cortex of an ovary. The number of follicles is about 5 000 000 at 20 gestational weeks and 2 000 000 at birth. After birth, the number rapidly decreases during two periods: Soon after birth and after 37.5 years of age. About 400 000 primordial follicles are present in the
Histopathological examination with hematoxylin and eosin staining. Five patients with POF were subdivided into 2 groups as follows: 2 patients with follicle dysfunction and 3 patients with follicle depletion. (A) shows a POF patient with follicle dysfunction (patient No. 1). (B) shows a POF patient with follicle depletion (patient No. 3) (Original magnification ×75).

neonatal period, 25,000 at 37.5 years of age and 1000 at 51 years of age (Edwards and Brody 1995; Faddy and Gosden 1996). In this study, among subjects with regular menstrual cycles, telomerase activity was significantly higher in those under 38 years of age. This is understandable given the rapid depletion of primordial follicles at 38 years of age. We compared the subjects under and over 35 years, but found no significant differences. Our findings indicate that telomerase is present in the normal human ovary and that telomerase activity decreases with the primordial follicle depletion that accompanies aging.

Five patients of POF underwent laparoscopic ovarian biopsy between September 1997 and December 1998 at the Tohoku University School of Medicine. The risk of POF before age 40 is almost 1% (Coulam et al. 1986). But the number of patients who need to undergo biopsy is fewer. It is known that POF can be subdivided into two types as follows by ovarian biopsy: Follicle dysfunction type and follicle depletion type (Anasti 1998). In this study, we confirmed
that 2 patients were follicle dysfunction type and 3 patients were follicle depletion type by histopathological examination with hematoxylin and eosin staining. The former patients showed high telomerase activity according to their age and the latter subjects showed very low telomerase activity that was not associated with their age. One of the former type patients responded to the follicle induction by human menopausal gonadotropin. We could recognize the growth of follicles for this type but not for the latter type. Based on these findings, we speculated that the decline of telomerase activity in the ovary is related to primordial follicle depression.

It was found that normal endometrium expresses telomerase (Brien et al. 1997) and the activity markedly changes along with the menstrual cycle. It has been suggested that telomerase is a regulating enzyme linked to cellular proliferation and that hormone functions are involved in its regulation (Kyo et al. 1997). Several years before menopause, women show significantly higher levels of follicle stimulating hormone (FSH) (Klein et al. 1996; Reane et al. 1996). Though all 5 POF patients showed similar high levels of FSH, some patients showed high and others very low telomerase activity. Therefore, no clear relationship between ovarian telomerase activity and FSH was detected. However, telomerase is considered more useful for revealing the exact condition of the POF ovary because of the small sample volume required. Telomerase activity may be a useful marker of the ovarian functional age.
References