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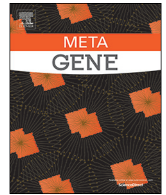

Idiopathic Premature Ovarian Failure and its association to the abnormal longitudinal changes of telomere length in a population of Iranian Infertile Women: A pilot study

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ABSTRACT

Bachground: The premature ovarian failure (POF) is a reason of infertility that affects about 1–4% of women before age 40. The importance of telomeres' length in different diseases has been explored before. This study examines the association between the relative telomere length and idiopathic POF in a group of Iranian women.
Methods: The blood genomic DNA was extracted from 40 idiopathic POF patients (case group) and 40 fertile women (control group). The relative telomere length (RTL) was evaluated by quantitative Real-Time PCR using specific telomeric primers. RTL was calculated as T (telomere)/S (single copy gene) ratio and compared between infertile and fertile groups.
Results: A strong association was considered between telomere size and idiopathic premature ovarian failure. In patients the relative telomere length showed to be significantly longer than those of control group ($P < .05$, 95% CI).
Conclusion: Our findings demonstrate a possible relationship between telomere lengthening and idiopathic POF. The reason of the observed elevated telomeres' genetic material could be explained by numerous probable mechanisms like reduced ovarian cell division rate, a sudden increase in the estrogen level before menopause and after egg depletion, and/or an autoimmune condition which could change the composition of blood cells and their consequent conversion to the cells with longer telomeres in patients. More experiments with larger population are necessary to confirm the results of present study.

1. Introduction

Premature ovarian failure (POF) (MIM—311360) is a functional insufficiency of the woman's ovaries younger than 40 years. The symptoms of POF are low hormone estrogen level, high Follicle-Stimulating Hormone (FSH) level, irregular periods and reduced fertility potential (Schoemaker et al., 1997; Lakhal et al., 2010). POF affects around 1–4% of the female population. There are significant differences in the frequency of POF among age groups in which; prevalence of POF patients is 1 in 1000 women before 30 years and is 1 in 100 women before 40 years (Blumenfeld, 2014).

Some studies reported an association between some factors and POF, including autoimmunity, viral infection, metabolic disorder, chromosomal defects, single gene disorders, idiopathic and iatrogenic (radiotherapy, chemotherapy, surgery) unhealthy lifestyles (e.g., smoking, unhealthy diet, physical inactivity, psychological or physical stress) (Panay and Fenton, 2008).

Telomeres are tandem arrays of a TTAGGG repeats which are located at the extreme end of the linear chromosomes and protected chromosome integrity (Keefe, 2016; Donate and Blasco, 2011). A six-protein complex called "shelterin," stabilize telomere. This complex includes protection of telomeres 1(POT1), telomeric repeat binding factor 1 and 2 (TRF1 andTRF2), TRF1 and TRF2 interacting nuclear protein 2 (TIN2), TPP1 and human ortholog of the yeast repressor/activator protein 1 (Rap1) (Montpetit et al., 2014). The telomere length decreases progressively with each cell division and therefore

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implicating in cellular aging. Telomere length differs significantly among people (Hastie et al., 1990; Starnino et al., 2016). Variation in telomere length among individuals may be due to diversity in telomerase activity, telomere length and rate of cell division (Keefe et al., 2006a; Olsson et al., 2017). The latest study confirms that telomeres have been linked to age-related diseases. There are few reports indicating a relationship between telomere length and POF (Kinugawa et al., 2000; Keefe et al., 2006b; Aydos et al., 2005; Xu et al., 2017).

Several different methods have been developed to measure telomere length. Terminal restriction fragment (TRF) analysis is the "gold standard" method in which, genomic DNA is digested using restriction enzymes that lack recognition sites in the telomeric and subtelomeric regions and then investigated, using agarose gel electrophoresis (Kimura et al., 2010). Another method uses fluorescent probes to quantify telomere lengths and methods which are based on polymerase chain reaction (PCR) (Montpetit et al., 2014). Several reports confirm the relationship between POF and telomere length which is due to the role of telomere in the mitotic capacity (Hanna et al., 2009a). Therefore, the present study was aimed to estimate the relationship between the relative length of telomeres and POF by quantitative Real Time-PCR (qRT-PCR) in a population of Iranian patients.

2. Materials and methods

2.1. Patients and controls

The study comprised 40 Iranian women with POF and 40 normal women who were randomly selected from the infertility clinics and centers in Tehran, Iran including: Day Hospital, Endocrine and Metabolism Research Center of Taleghani Hospital, Kosar infertility center and Infertility Center of Yazd between 2012 and 2014. Patients were defined as women with normal karyotype, menstrual cycle cessation before the age of 40 in the past 6 consecutive months prior to enrollment to the study, FSH > 40 IU/L for at least two measurements over 1 month apart. Control group were composed of under 40 years old women with regular menstrual cycle, normal plasma FSH levels and normal 46, XX karyotype. Also, all of them had healthy child with no previous evidence of familial history of infertility problems. Women, who showed abnormal karyotype and were affected by endometriosis or had ovarian cysts or suffered from autoimmune disease, were excluded from this study. Also, under medication, chemotherapy or radiation therapy women for ovarian failure healing were eliminated from this research.

A written consent was taken from all women who participated in this study. Moreover, the study was conducted in complete compliance with Helsinki declaration.

2.2. DNA extraction

A total of 4–5-ml peripheral blood from patients and controls was collected in tubes containing anticoagulant (EDTA). Briefly, genomic DNA was extracted from peripheral whole blood samples using a DNA extraction kit (BIONEER, Korea) according to the manufacturer's instructions.

The quality of DNA was tested using a 1% agarose gel electrophoresis. DNA concentration and purity were determined using a NanoDrop™ Spectrophotometer (Thermo Fisher Scientific).

2.3. qRT-PCR and telomere length measurement

Average relative telomere length was assayed by a quantitative Real Time- PCR (qRT- PCR) method (Cawthon, 2002). In summary the number of relative telomere sequence repeats (T) was compared to the amount of β-glubin as a single copy reference gene (S), in each genomic DNA sample and finally a T/S ratio was stimated. For each sample, qRT-PCR runs were performed in triplicate on a Rotor-Gene Q machine (QIAGEN, Germany). Primer sequences which were previously indicated for the same purpose (Cawthon, 2002) were:

Forward primer, Tel1: 5´GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT3´.

Reverse primer, Tel2: 5´TCCCGACTATCCCTATCCCTATCCCTATCCCTATCC-CTA3´.

Forward primer, FHBG1: 5´GCTTCTGACACAACTGTGTTCACTAGC3´.

Reverse primer, RHBG2: 5´CACCAACTTCATCCACGTTCACC3´.

Each PCR reaction mixture contained 12.5 μl master mix SYBER-green (TAKARA, Japan), 50–100 ng genomic DNA and primers with different final concentration in a total volume of 25 μl. Final primer concentrations were as followings: 2.5 pmol/μl Tel1, 7.5 pmol/μl Tel2 and 4 pmol/μl of forward HBG; and 4 pmol/μl reverse HBG.

Amplifications were performed in a Rotor-gene, Corbett 6000 (Corbett Research Ltd., Cambridge, UK) for Real Time- PCR assays. The PCR thermal cycles for telomere repeats included initial denaturation at 95 $^{\circ C}$ for 15 min followed by 25 cycles of 95 °C for 15 s, 54 °C for 2 min, followed by a melting curve at 65 °C − 95 °C with the rising value of 0.5 °C /S. The single copy reference gene PCR profile included initial denaturation at 95 °C for 10 min followed by 25 cycles of 95 °C for 15 s, 58 °C for 60s, followed by a melt curve 65 °C to 95 °C with the rising value of 0.5 °C /S. PCR products were electrophoresed on 1% agarose gel to confirm the size of amplified fragments. Data were analyzed by using the comparative Ct method, where Ct is defined as the cycle number in which fluorescence first crosses the threshold. ΔCt was determined by subtracting the HBG Ct values from the telomere Ct values. The result was applied to the term $2^{(-\Delta Ct)}$.

2.4. Statistical analysis

Normality test was performed by the Kolmogorov–Smirnov test. Quantitative variables were expressed as mean ± SD (standard deviation) or median (interquartile range, IQR), depending on whether the data were normally distributed.

Student's *t*-test was used to evaluate the telomere to single copy gene (T/S) ratio between women affected by POF and fertile women. All the statistical analysis was performed using SPSS version 22.0 and with 95% CI. *P* value < .05 was considered statistically significant.

3. Results

Extracted DNAs were qualitatively analyzed by 2% agarose gel electrophoresis (Fig. 1).

The average concentration of the extracted DNA was determined to be 250 ng/μl by measuring the absorbance at 260 nm. The purity of DNA was estimated by measuring the absorbance at 260 and 280 nm (A260/A280), and 260 over 230 nm (A260/A230) using a NanoDropTM 1000 spectrophotometer (Thermo Scientific, USA). The

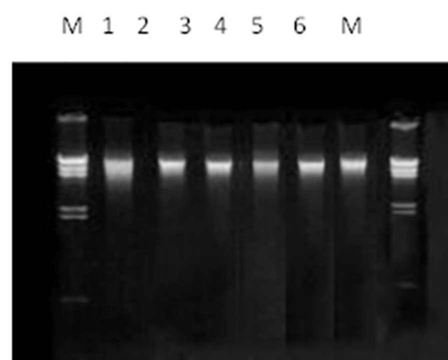

Fig. 1. Qualitative genomic DNA analysis on 2% agarose gel. M: Marker, 1–6: Extracted DNAs from blood samples.

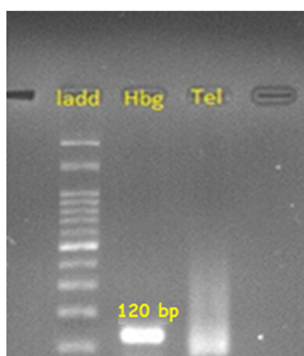

Fig. 2. Agarose gel electrophoresis following to PCR with specific primers for telomere repeat units (TEL) and for β-globin as single copy gene (HBG). PCR profile was as described in materials and methods.

A260/A280 for the DNA was 1.86 and A260/A230 was 1.96.

After PCR, as described in materials and methods, 10 µl of PCR product was loaded into each well which was latter stained by Syber Green I (Fig. 2).

We compared relative telomere length in patients with POF to those of control group by a quantitative Real Time-PCR (qRT-PCR).

Relative T/S of each sample to the T/S of another sample was calculated by t –test using SPSS version 22.0. The relative telomere length (T/S) of the POF group was significantly longer ($p < .05$) when compared to those of controls (Table 1). As our univariate analysis shows, there is a possible correlation between telomere length and idiopathic POF, respectably.

4. Discussion

Infertility is a clinical problem that afflicted many young couples these days all over the world. Age-related changes such as reducing the egg supply in the ovaries (Ovarian reserve), may lead to the development of infertility. Female fertility begins to decline after age 27 and its incline rate is accelerated after age 35 (Inhorn and Van Balen, 2002; Islam et al., 2017).

The most common cause of female infertility is ovulation defects. This may be due to a malfunction of the hypothalamus gland in stimulation of the pituitary gland. Stress, trauma, changes in body weight, lack of appetite or eating disorders and excessive exercise may be effective factors in the development of anovulation (Speroff and Fritz, 2005; Namavar Jahromi et al., 2017; Hart, 2016). Women with such problems possibly do not produce enough gonadotropin to trigger the ovaries maturation. Endocrine factors including Thyroid disease, Diabetes, Hyperprolactinemia, production of abundant adrenal androgens and liver disease may adversely effect on the functions of the hypothalamus or pituitary glands which as a result could prevent follicles maturation, ovulation (essential for sustaining pregnancy). The endocrine disorders and imbalances need to work correctly to regain control of the pregnancy (Speroff and Fritz, 2005).

Despite various causes for the disease, in many cases there may be a genetic reason to early ovarian dysfunction resulting in the egg loss (Cordts et al., 2011). Sometimes an immune disorder leads to an abnormal response of the body to its own tissues (Shah et al., 2003; Gao et al., 2017; Desai and Rajkovic, 2017).

Table 1
Comparison of length (T/S ratio) between Premature Ovarian Failure (POF) and control subjects using *t*-test.

	Number	Mean telomere length	p-value
Case/ratio c	40	0.7445	$p < .05$
Control/ratio t	40	0.5994	

Note: Significant difference was shown between groups (calculated with t-test). C: stands for Case group of Premature ovarian failure (POF); c: stands for Control group or normal women. Data are presented as mean ± SE; $p < .05$ was considered significant.

In previous report by Hanna and colleagues (2009), it was discussed that telomere length in patients' blood cells reflects less mitotic divisions in the early germ cells pool causing probable smaller follicle resource and consequent premature ovarian failure (Hanna et al., 2009a).

A number of syndromes, premature aging and age-related pathologies are characterized by reduced telomere length such as some tumors, Bloom syndrome, Werner syndrome, Ataxia telangiectasia, Fanconi anemia, cardiovascular diseases, diabetes mellitus, Alzheimer's, Parkinson's, recurrent pregnancy loss, gastric carcinoma, breast cancer, AIDS and infertility in men's (Kong et al., 2013). These evidences show that telomere shortening could provoke aging and may cause apoptosis leading to cell loss and tissue destruction (Zhao et al., 2014).

Given the importance of telomeres in the diagnosis of various diseases, this study examined and compared relative telomere length in patients with premature ovarian failure and normal subjects. A total of 40 Iranian women with POF as experimental group and 40 fertile women of the same origin were considered as controls. Extracted DNAs from blood samples were assessed using Real time PCR alongside with beta-globin as a single copy standard gene. Recorded Ct values showed to be significantly lower in test group (p-value $< .05$) compared to the control group. This means an elevated telomere length in POF patients where the lower Ct proves that there a higher repetitive telomere sequences exists.

Hanna and her colleagues have achieved similar results and showed telomere length in patients is greater than those in fertile women. They explained their findings as genetic material in patients must be lost in a slower song because of fewer follicles divisions (Hanna et al., 2009a). It is of our knowledge that the reduced rate in cell division during fetal life ovarian development can lead to follicular pool elimination (Kinugawa et al., 2000). Besides, decrease in ovarian cells' divisions could occur by exposure to estrogen hormones after infertility diagnosis and subsequent hormone therapy in patients. Moreover, sudden release of a large number of eggs, egg depletion, before menopause could increase estrogen level in women as another biological reason for telomere repeats elongation which is discussed elsewhere (Lee et al., 2005). As is revealed, estrogen is involved in repair process where induced DNA single-strand breaks by free radicals and subsequent telomere length shortening exist (Aviv et al., 2005; von Zglinicki, 2000). The sudden release of plenty amount of estrogen could result in the repair of the broken DNA molecules and induce telomere lengthening. This is probably due to the protective effect of estrogen against ROS (reactive oxygen species) inductions of DNA damage, estrogen induction for hTERT expression and therefore induced telomerase activity, or stimulation of nitric oxide production in VECs (vascular endothelial cells), which in turn induces the telomerase activity in these cells (Sameer et al., 2014). Increasing telomerase activity in a study conducted on the tissue of mice adrenal glands showed that over expression of telomerase TERT reverse transcriptase subunit is induced by estrogen (Bayne et al., 2008). In another study on human endometrial cell line, it was suggested that estrogen acts as a mitogen and activates the MAP / ERK pathway where activated agents in this pathway increases the activity of telomerase through influence on the TERT gene promoter (Zhou et al., 2013). Autoimmune process in POF patients as we know, most of the chosen target molecules by the immune system, is steroidal agents which are produced by different cells. Considering the fact, most ovarian cells are the main secreting cells of steroid hormones (Goswami and Conway, 2007). Previous studies demonstrated a role of telomere length in the pathogenesis of POF (Zhao et al., 2014).Hanna and et al. showed that POF patients have longer telomeres than normal people. According to the obtained data of this study, due to defects in ovarian function, follicles are divided to a lesser extent and so telomere length is less shorten (Hanna et al., 2009b).

Moreover, Kinugawa in 2000 explains the different findings related to the telomere length and its association to POF and discusses about its shortening as a sign of POF etiology. Short telomere length is less (Kinugawa et al., 2000).

In contrast, Keef et al. (2007) and Aydos et al. (2005) believe that different telomere length between people and their primary germ cells causes different initial cell pool where follicles reduction is experienced due to the shorter telomere and less mitosis (Keefe et al., 2006a; Aydos et al., 2005).

According to the obtained results there is a hope that the telomere length measurement in joint with other markers could be a useful diagnostic tool for POF. Although, these results apparently differ from the obtained results in our study, But according to the results of Hanna's and Kinugawa's studies, the difference is justifiable.

In conclusion, although the scientific debate about the association between telomere length with the etiology of the disease is still not certain, but in the future, we can reach to a solution through 1) Testing telomere length in at risk people with a family history of POF, 2) Testing a larger population to obtain more reliable results, 3) Review and investigating for the molecular mechanisms basis involved in telomere length changes in affected women with POF, 4) qualitatively analyze telomere length using telomere size markers in under study groups.

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Conflict of interest

The authors declare no conflict of interest.

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