mtDNA^{4977} deletion is not a common feature in patients with premature ovarian failure and primary infertility

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Summary

The aim of the current study was to investigate the incidence of mtDNA^{4977} deletion in peripheral blood leukocytes of patients diagnosed with premature ovarian failure (POF) and primary infertility. The study group consisted of 17 patients with POF, 32 women with primary infertility, and 31 fertile women with the prevalence of the mtDNA^{4977} deletion using the reverse transcription-polymerase chain reaction (RT-PCR) based technology. None of the patients affected by POF revealed mtDNA^{4977} deletion. This deletion was detected only in one 26-year-old infertile patient. No significant difference in relation to mtDNA^{4977} deletion was reported between the groups investigated ($p > 0.05$). In conclusion, mtDNA^{4977} deletion is not a common finding in peripheral blood leukocytes of women affected by POF and primary infertility. The occurrence of mtDNA^{4977} deletion in women between 20 and 39 years of age may not increase with increasing patients' age, independently of their fertility status.

Key words: mtDNA^{4977}; Premature ovarian failure; Primary infertility.

Introduction

In recent years more and more women decide to become pregnant after the age of 35 [1]. Postponing pregnancy causes difficulties in having offspring, mainly as a result of age-related disadvantageous changes. Furthermore, the capacity of oocyte fertilization and the endometrial receptivity decrease [2].

A significant effort has been made to "stop the biological clock" and to preserve fertility in older patients. Unfortunately, the results of these attempts have not yet been satisfactory. Moreover, premature ovarian failure (POF) constitutes nowadays a problem as serious as primary infertility [3].

Mitochondria take part in cellular respiration and their function has a significant influence on the normal functioning of the gamete [4, 5]. Mitochondrial DNA (mtDNA) is a double-stranded chain, which, in humans is 16.6 kb long. Almost each cell in the human body contains around 1,000 mitochondria, and every mitochondrion has two to ten copies of mtDNA. Studies on mtDNA conducted over the last 30 years, have led to the conclusion that anomalies within mtDNA are related with fertility disorders in women [6, 7]. It is possible, that POF may be associated with a decreased oxidative phosphorylation, which is observed in the majority of cells in an aging body [8].

Over 150 types of rearrangements have been found in human mtDNA. The most common deletions are the following deletions: mtDNA^{4977}, mtDNA^{7436}, and mtDNA^{10422} [9]. The mtDNA^{4977} deletion occurs within the limits of the so-called "hot spot" in 8,468 and 13,446 nucleotide positions and is also called "common deletion" [10].

The mtDNA^{4977} deletion causes removal of the following genes: *Fo-F1-ATPase* (ATPase 6 and 8), *cytochrome oxidase* (CO III), and *oxidoreductase NADH-CoQ*, which play a pivotal role in the oxidative phosphorylation and therefore mainly results from mitochondrial function [11]. It has been observed that in women over the age of 38 years, the granulosa cells within the follicles have a lower proportion of mitochondria with normal DNA [12]. It is possible that the age-related loss of mitochondrial function results from deletion or point mutations within mtDNA. Hsieh *et al.* [11, 13] suggested that some of the mutations within the mtDNA of an oocyte may be responsible for failures in oocyte fertilization. However, to the best of the authors' knowledge, none of the researchers described the mtDNA^{4977} mutations in peripheral blood leukocytes of women suffering from POF and primary infertility.

The objective of the study was to investigate the incidence of mtDNA^{4977} deletion in peripheral blood leukocytes of patients diagnosed with POF and primary infertility.

Materials and Methods

The study subjects comprised of 17 patients with POF and 32 patients with primary infertility. The control group consisted of 31 age-matched fertile (confirmed by at least one pregnancy) individuals. All participants underwent a complete examination and history, including family diseases, at the Second Department of Gynecology of the Lublin Medical University in Lublin, (Poland). None of them mentioned fertility problems in family anamnesis. Among the fertile patients, 15 of them had one birth, seven had two births, five had three births, and four had a miscarriage. The study was approved by the Ethical Committee of the Medical University of Lublin. Informed consent was collected from all the persons enrolled.

Positive (endometrial cancer with mtDNA^{4977} deletion [14]) and negative (water instead of sample) controls were used in all experiments.

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Blood in an amount of two ml was sampled from an antecubital vein in each of the study individuals in the morning after an overnight fasting and was quickly deposited into a plastic tube containing ethylenediaminetetraacetic acid (EDTA). Leukocytes were immediately separated from plasma as the buffy coat in a Ficoll gradient and were immediately forwarded to DNA isolation.

The use of polymerase chain reaction (PCR) Master Mix reduced tube-to-tube difference caused by differences in the amount of enzyme. Molecular probes were used (Table 1); the probe for $mtDNA^{total}$ was labelled with reporter VIC. The probe for $mtDNA^{4977}$ was 6-carboxyfluorescein (FAM).

Total DNA from whole blood ($5x10^6$ leukocytes) was extracted. Following extraction, DNA was quantified and qualified by UV spectrophotometric analysis. Template DNA included 50-100 ng of DNA extracted from leukocytes. Reaction mixtures included 0.25µµM forward and reverse primers, 200 nM probe, and 1x PCR Universal Master Mix for a final volume of 50 µl. All experiments were performed under "multiplex" conditions. Primers and probes for both $mtDNA^{total}$ and $mtDNA^{4977}$ were present in each reaction (Table 1). The real-time PCR reactions were run on a 7300 Real-Time PCR biosystem. Cycling temperatures and times were 50°C for two minutes, 95°C for ten minutes, 95°C for 15 seconds, and 60°C for one minute.

Data were collected and analyzed using 7300 Real-Time PCR System. Data were also normalized to $mtDNA^{total}$ amplified from the cellular sample using the delta comparative threshold cycle (C_T) method. The C_T value is the parameter used for quantifying the amount of target template in the given reaction well. Delta C_T ($\varnothing C_T$) for sample was the difference between the C_T values of the $mtDNA^{4977}$ and $mtDNA^{total}$ (used as reference): $\varnothing C_T$ sample = C_T (FAM) – C_T (VIC).

The PCR-products were purified and separated on a 1.2% agarose gel at 50 V in 1X TBE buffer, and the products were visualized by ethidium bromide staining and photographed. DNA bands were analyzed densitometrically using an appropriate software, and the intensity of the mtDNA bands were measured. If the sample was positive, the percentage of deleted $mtDNA^{4977}$, with respect to wild-type mtDNA, was determined by the ratio between the deleted and wild-type mtDNA band densities.

Kruskal Wallis ANOVA, *U*-Mann-Whitney test, and Statistica ver. 9.0 PL software were applied to statistical analysis and $p < 0.05$ was considered significant. All results are showed as means ± standard deviation, medians, maximum and minimum.

Table 1. — *Primer sequences used in the experiments.*

Name	Sequence	Dye
Primer pairs for $mtDNA^{total}$		
mtDNA1307FOR	5'-GTA CCC ACG TAA AGA CGT TAG G-3'	
mtDNA1433REV	5'-TAC TGC TAA ATC CAC CTT CG-3'	
Primer pair for $mtDNA^{4977}$		
mtDNAdel49778416	5'-CCT TAC ACT ATT CCT CAT CAC C-3'	
mtDNAdel49778542	5'-TGT GGT CTT TGG AGT AGA AAC C-3'	
Molecular probe		
Pr-total14977	CCC ATG AGG TGG CAA GAA AT	VIC
Pr-del14977(T)	TGG CAG CCT AGC ATT AGC AGT	FAM
Pr-del14977(G)	TGG CAG CCT AGC ATT AGC AGG	FAM

Table 2. — *Clinical characteristics of the patients with POF, primary infertility, and the control group.*

		Patients with POF	Patients with primary infertility	Control group
Number of individuals		17	32	31
Age (years)	Mean ± SD	30.14 ± 5.32	31.44 ± 3.89	31.52 ± 4.02
	Median	31	32	31
	Minimum - maximum	20-36	21-39	24-39
Menarche (years)	Mean ± SD	14.50 ± 1.09	13.85 ± 1.54	13.28 ± 1.17
	Median	15	14	13
	Minimum - maximum	13-16	10-17	11-16
Weight (kg)	Mean ± SD	60.90 ± 11.81	60.13 ± 12.79	63.61 ± 7.02
	Median	60	58	63
	Minimum - maximum	37-90	41-96	47-76
Height (cm)	Mean ± SD	163.13 ± 7.57	164.30 ± 6.36	165.57 ± 5.46
	Median	163	164	164
	Minimum - maximum	145-171	150-179	156-177
BMI (kg/m^2)	Mean ± SD	22.83 ± 4.12†	22.18 ± 4.05	19.19 ± 1.83†
	Median	22.5	21.27	19.29
	Minimum - maximum	17.60-34.29	15.99-33.22	14.24-21.51
17β-estradiol (pmol/l)[a]	Mean ± SD	32.02 ± 15.28	81.85 ± 53.95	36.31 ± 12.58
	Median	20	81.85	38.40
	Minimum - maximum	1.87-66.20	43.70-120.00	20.00-52.90
FSH (IU/l)[a]	Mean ± SD	45.98 ± 28.82*	6.59 ± 2.25*	6.39 ± 1.84*
	Median	39.9	6.16	6.51
	Minimum - maximum	14.40-107.46	3.74-11.40	3.70-9.57
LH (IU/l)	Mean ± SD	20.91 ± 18.67†#	6.36 ± 3.59†	5.99 ± 2.36#
	Median	15.10	5.04	6.10
	Minimum - maximum	1.00-77.20	3.50-12.90	2.75-9.5
FSH/LH ratio	Mean ± SD	2.80 ± 1.94#*	1.08 ± 0.45#	1.08 ± 0.58*
	Median	2.15	1.09	0.84
	Minimum - maximum	1.17-8.68	0.48-1.71	0.61-2.37

†$p < 0.05$; #$p < 0.01$; *$p < 0.001$; [a] measured at the second day of the follicular phase.
BMI = body mass index; FSH = follicle-stimulating hormone; LH = luteinizing hormone.

Results

The current study investigated peripheral blood samples collected from 17 patients with POF, 32 individuals with primary infertility, and 31 fertile women. The characteristics of the study subjects are listed at Table 2. The age matched with no other health problems for the prevalence of the $mtDNA^{4977}$ deletion using the PCR-based methodology. None of the patients affected by POF revealed $mtDNA^{4977}$ deletion. This deletion was detected only in a 26-year-old infertile patient. No significant difference between groups investigated in relation to $mtDNA^{4977}$ deletion was noted ($p > 0.05$) Figure 1 presents a graphical demonstration of real-time PCR data from 13 representative patients – 12 patients without $mtDNA^{4977}$ deletion (wells from one to 12), one patients (well 13) with deletion, and a negative control (well 14).

Discussion

POF is a frequently occurring condition. The prevalence of POF in women below 40 years of age is one to two percent and in those below 30 years of age is 0.1%. It leads to the absence of menstrual period, hypoestrogenism, and elevated levels of gonadotropins. It has been observed that POF occurs in 10%-28% of women suffering from primary amenorrhea and in 4%-18% of those with its secondary form [15, 16]. It is worth mentioning that a major component of this disorder may remain unsolved as a result of low awareness among women who do not consider a loss of menstruation before the age of 40 to be a serious medical condition requiring gynecological consultation**.** Other causes of the lack of monthly menstruation, such as pregnancy, hyperpro-

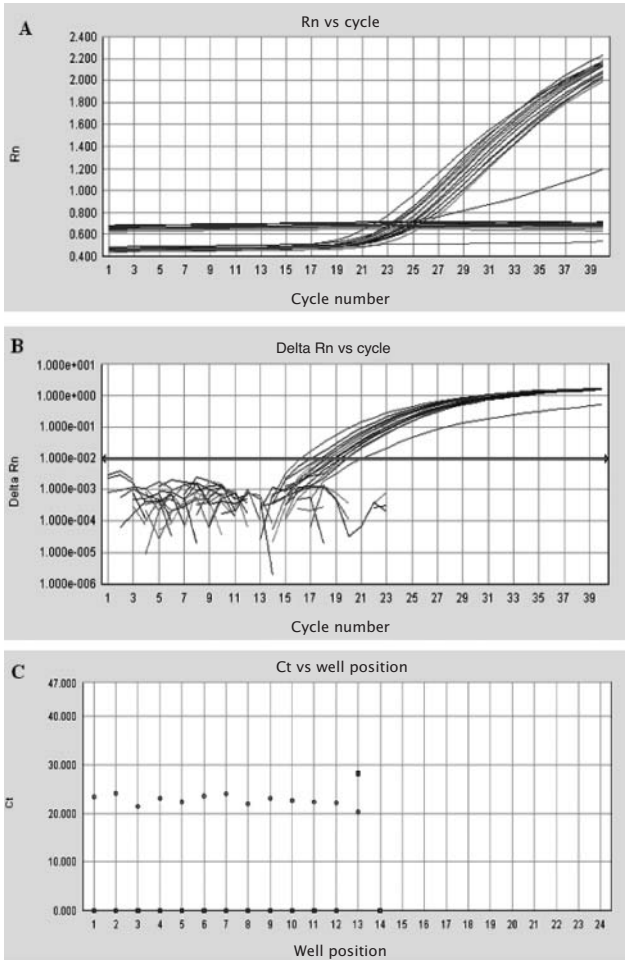

Figure 1. — Graphical demonstration of real-time PCR data from 13 representative patients [12 without mutation (wells from 1 to 12) and one patient (well 13) with mutation, well 14 – negative control]. **A:** In this view, normalized reporter (Rn) is graphed vs the cycle. **B:** ΔRn is Rn minus the baseline, graphed vs the cycle of PCR. **C:** C_T vs well position.

lactinemia occurring due to the drug-induced or spontaneous diminution of the dopaminergic hypothalamus activity, or because of adenomas of the pituitary gland, thyroid dysfunction, and POF, have to be excluded. Women should be checked for POF when amenorrhea persists for at least three to six months, and when the level of FSH exceeds 40 mIU/ml in at least two tests separated by at least a couple of months. Intermittent ovarian function must be excluded, as it gives similar symptoms, such as hypoestrogenism (less than 50 pg/ml) and high gonadotropins levels, along with the absence of follicles or loss of their function [16, 17]. The loss of the ability to conceive is mainly a result of the absence of ovarian follicles, or, less frequently, the fact that the existing follicles are unable to respond to stimulation.

The present study aimed to establish the frequency of mtDNA[4977] deletion in patients with POF and primary infertility in comparison to healthy women. Furthermore, as the normal structure of the cell membrane is lost, the damaged mitochondria may release proteins that induce apoptosis, such as cytochrome C [18]. These phenomena have been proved in observations of a mouse model. By means of microinjection, normal mitochondria were inserted into mice's oocytes, which prevented them from apoptosis [19]. Tsai *et al.* [20] presented the effects of mitochondrial DNA variations in cumulus cells upon *in vitro* fertilization and embryo transfer outcomes. Pregnancy tests were positively correlated with younger age, better-transferred embryo qualities, and lower dmtDNA-delta5Kb (mtDNA[4977] deletion) ratios in cumulus cells. These authors concluded that mtDNA[4977] status in granulosa cells might be a potential tool for oocyte evaluation and embryo selections during *in vitro* fertilization [21]. Although Keefe *et al.* [21] suggested that the common deletion may serve as a marker of oocyte senescence, others failed to conform these observations [22, 23]. Most of the previous studies have shown that the incidence of 4977bp deletion was significantly higher in older women. This observation is in line with the hypothesis that there is an age-related accumulation of mtDNA rearrangements in human oocytes. However, none of the scientists checked if deletions occur in somatic cells, such as leukocytes of infertile patients. Unfortunately, the present data definitely reported that POF and primary infertility are not associated with the presence of deletion within mtDNA[4977] in peripheral blood leukocytes. In findings among 80 patients, only one deletion revealed that the age-related effect on occurrence of the mtDNA[4977] is not apparent between the ages of 20 to 39 and may be spontaneously present.

The objective of the study of Tong *et al.* [24] was to determine if mitochondrial DNA polymerase gamma deletions were associated with spontaneous 46,XX primary ovarian insufficiency. Among 201 examined women, they found only one case of heterozygosity for a polymerase gamma, suggesting that this was not a common genetic etiology for this form of infertility [24]. The present results confirm these observations.

The authors conducted this study on peripheral blood leukocytes with the use of highly-sensitive technique. To the best of their knowledge, there are only a few studies focused on the mentioned data. A significantly higher incidence of mtDNA[4977] in peripheral blood leukocytes was observed in coronary artery disease patients with respect to healthy subjects; even the examined group was not so large as in this study (65 vs 80) [25]. Iwai *et al.* [26] examined the effect of green tea enriched with catechins on the presence of the mtDNA[4977] deletion mutation in human leukocytes obtained from ten healthy young females (median age 20.8 years, similar to this study group). They found that mutation was present in nine participants before drinking the tea and after the experiment; the mutation was noticed in none of the participants. Perhaps the dietary habits and other yet unknown predictors are more connected with mtDNA state than other conditions, including fertility. Current study was performed on the second day of the follicular phase in all participants and subsequently further research is necessary to assess a possible relationship, if it exists, between mtDNA[4977] state and menstrual cycle.

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