

The prevalence of mtDNA⁴⁹⁷⁷ deletion in primary human endometrial carcinomas and matched control samples

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Abstract. mtDNA⁴⁹⁷⁷, the most common deletion of the mitochondrial DNA, has been detected in different types of human neoplasia. The aim of the current study was to determine the prevalence of mtDNA⁴⁹⁷⁷ deletion in primary human endometrial carcinomas (EC) as compared with matched control samples. Thirty-seven matched control tissues and EC samples were enrolled, and the 4977-bp mtDNA deletion was investigated using a PCR-based technique. Deletion of mtDNA⁴⁹⁷⁷ was detected in 32 out of 37 (84%) matched control samples and in 30 out of 37 (81%) ECs. A statistically significant correlation of mtDNA⁴⁹⁷⁷/wild-type mtDNA ratios was noted between normal and cancerous human endometrial tissues ($R=0.844$, $p<0.0001$). The intensity ratio of mtDNA bands was significantly higher in normal samples than in malignant human endometrial tissues ($p=0.021$). The mean mtDNA⁴⁹⁷⁷/wild-type mtDNA ratio was significantly higher in the control group (0.655 ± 0.379) than in the cancer group (0.570 ± 0.04 , $p=0.048$) of patients between 50 and 60 years of age. Notably, there was a relationship between clinical stage of the disease (stage II versus III) and the amount of mtDNA⁴⁹⁷⁷ in ECs ($p=0.048$). The sequence analysis of two randomly selected EC-positive cases confirmed that amplified fragments originated from mtDNA, and both encompassed deletion. In conclusion, we suggest that mitochondrial 4977-bp deletion is not specific to EC tissues. The accumulation of mtDNA⁴⁹⁷⁷ may be associated with aging processes, particularly in perimenopausal women affected by EC.

Introduction

Mitochondria are eukaryotic organelles involved in many intracellular metabolic pathways in which bio-fuels, such as fatty acids and carbohydrates, are converted into cellular ATP through the process of oxidative phosphorylation (OXPHOS) (1). The mitochondrion, in addition to the nucleus, possesses its own genome and has genetic machinery capable of producing proteins involved in bioenergetic processes (2). The mtDNA is a compactly organized, double-stranded, closed molecule of a full-length sequence weighing 16.5 kb (2). Human mtDNA encodes 13 polypeptides, all of which are the components of the respiratory chain/OXPHOS system, 2 ribosomal RNAs and 22 transfer RNAs (2,3). Moreover, human mtDNA contains a non-coding region, the D-loop (the displacement loop), which is implicated in the control of replication and transcription (4).

Since the 1930s, mitochondria have been implicated in the process of human tumorigenesis. Otto Warburg suggested that 'respiration damage' may play a pivotal role in cancer (5). Today, mitochondrial dysfunction is one of the most prominent features of cancer, as many reports have revealed a significant relationship between this phenomenon and the development and spread of human neoplasia (4,6,7). Genetic instability, point mutations and abnormal mtDNA expression have been detected in various types of human malignancies, including EC (8-14). For example, Liu and co-workers (8) sequenced the fragment of the mtDNA, encompassing the D-loop, 12S and 16S rRNA genes in 50 endometrial cancers and matched normal controls. Of the tumors, >50% carried one or more somatic changes in mtDNA and MI, in CCCCC TCCCC sequences, was found in the hyper-variable regions I and II of the D-loop and 12S rRNA (8). The frequency of the 16189T>C mitochondrial DNA variant was significantly higher in EC patients (43.1%) versus the control group (21.5%, $p=0.009$) (9). Somatic D-loop mtDNA mutations were frequently (63%) reported in type II EC-UPSC (uterine papillary serous carcinoma) (10). In another study, a total of 13 D310 mtDNA mutations were found in 56 (23%) primary malignant tumors, including one (1-bp insertion) out of six ECs (11). Notably, the haplogroup D and 5178A mitochondrial polymorphism may be significantly associated with EC risk of women from the Yunnan province in China (12). It is worth pointing out the recently published data of Wang

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Abbreviations: EC, endometrial cancer; FIGO, International Federation of Gynecology and Obstetrics; HNPCC, hereditary non-polyposis colorectal cancer syndrome; mtDNA, mitochondrial DNA; MI, microsatellite instability; OXPHOS, oxidative phosphorylation; SD, standard deviation

Key words: deletion, mitochondrial DNA, endometrial cancer

and co-investigators (14). They suggested that accumulation of mtDNA alterations in primary human endometrial adenocarcinomas occur randomly and independently in individual cells or in a small sub-group of tumor cells. However, to the best of our knowledge (Medline® database), the prevalence of mtDNA⁴⁹⁷⁷ deletion in cancerous human endometrial tissues has yet to be investigated.

This study investigated the incidence of mtDNA⁴⁹⁷⁷ deletion in specimens collected from women affected by ECs and compared the results with the prevalence of this mitochondrial DNA alteration in matched normal control tissues.

Materials and methods

Patients. Forty specimens of primary human ECs and their matched normal control tissues (ovary, omentum or uterine cervix) were collected. The patients had undergone surgery at the Second Department of Gynecology, Lublin Medical University, Lublin, Poland because of EC between 2000-2006. Three patients were excluded due to insufficient data. Altogether, 37 women were enrolled. All patients gave informed consent before the specimens were collected according to institutional guidelines. The tumors were considered sporadic because none of the patients met the Amsterdam criteria for HNPCC syndrome. Neither initial chemotherapy nor hormonal or radiation therapy was performed prior to surgery. The standard surgical procedure in our Department was total abdominal hysterectomy with bilateral salpingo-oophorectomy. Lymph nodes were removed when the tissue collected at the dilatation and curettage procedure was found to be non-endometrioid, G3 (poorly-differentiated) endometrioid-type EC, or cancer infiltrating for more than a half of the myometrial wall at surgery. Clinical and pathological features of EC patients are summarized in Table I. The clinical stage of the disease was classified based on the FIGO staging system (15), whereas pathological assessment was performed at the Department of Pathology, Lublin Medical University, based on the World Health Organization classification (16).

PCR analysis. At surgery, the material collected was immediately snap-frozen in liquid nitrogen and stored at -80°C. DNA was isolated based on a standard technique using SDS-proteinase K, RNase digestion, phenol-chloroform extraction and ethanol precipitation (17). The two sets of PCR primers applied in the experiments, WT1 (5'-AATCAATTGGCGACCAATGG-3'; 7878-7897 bp) and WT2 (5'-CGCCTGGTTCTAGGAATAATGG-3'; 7979-7958 bp), amplified the D-loop segment of the mitochondrial genome (18). The second set of primers, L4 (5'-GCCCCGTATTACCCTATAGC-3'; 8251-8270 bp) and H3 (5'-GTCTAGGGCTGTTAGAAGTC-3'; 13845-13826 bp), was used to evaluate the existence of the 'common' 4977-bp deletion of mtDNA (19). The 5594-bp mtDNA fragment was flanked by the L4 and H3 set of primers, whereas despite deletion, the product had a size of 617-bp. PCR amplification was carried out by applying the Advantage 2 PCR Kit (BD Biosciences, Palo Alto, CA) in a 50 µl reaction volume with 100 ng DNA template, 1 µl of Advantage 2 Polymerase Mix, 1 µl dNTP and 1 µl (50 pmol/µl) of each primer. After denaturation at

Table I. Clinical and pathological features of EC patients.

Features	No. of cases (%)
Patient age (years)	
<50	7 (19)
50-60	17 (46)
>60	13 (35)
FIGO stage	
I	25 (67)
II	8 (22)
III	4 (11)
Histological grade	
Endometrioid	35 (94)
Non-endometrioid	2 (6)
Myometrial invasion	
None	3 (8)
≤	13 (35)
>	21 (57)
Lymph nodes metastases	
Present	2 (6)
Absent	24 (64)
Not evaluated	11 (30)
Coexistence of hyperplastic and neoplastic endometrium	
Yes	10 (27)
No	27 (73)
Metastases to the ovary/ovaries	
Positive	4 (11)
Negative	33 (89)
Infiltration of the neoplasm in the uterine cervix	
Present	11 (30)
Absent	26 (70)
Total	37 (100)

95°C for 5 min, the reaction mixture was cycled 30 times at the conditions described below: 95°C for 45 sec, 60°C for 45 sec and 72°C for 1 min, finally extended at 72°C for 5 min. 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 Kodak D1 3.6 software, and the intensity of the mtDNA bands was measured. For each positive sample, the percentage of deleted mtDNA⁴⁹⁷⁷, with respect to wild-type mtDNA, was determined by the ratio between the deleted and wild-type mtDNA band densities.

To confirm the presence of mtDNA⁴⁹⁷⁷ in the cancerous human endometria, we randomly selected two positive cases (numbers 164 and 238). PCR-products were cut from agarose

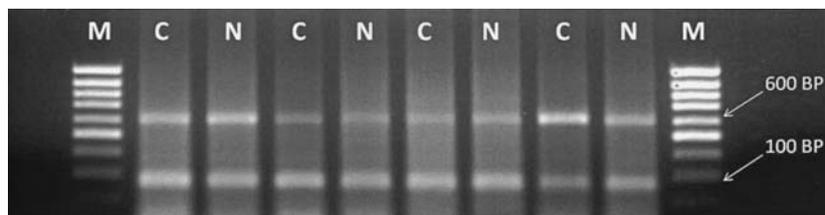


Figure 1. Detection results of 4977-bp deletion in the mtDNA of cancerous human endometrial specimens (C) and matched control samples (N). Four matched normal tissues and cancerous endometrial specimens showed mtDNA⁴⁹⁷⁷ deletion (numbers from left to right: 161/162, 164/165, 172/173 and 182/183). M, Mass Ruler™ (M) and ladder of low range (MBI Fermentas, Lithuania).

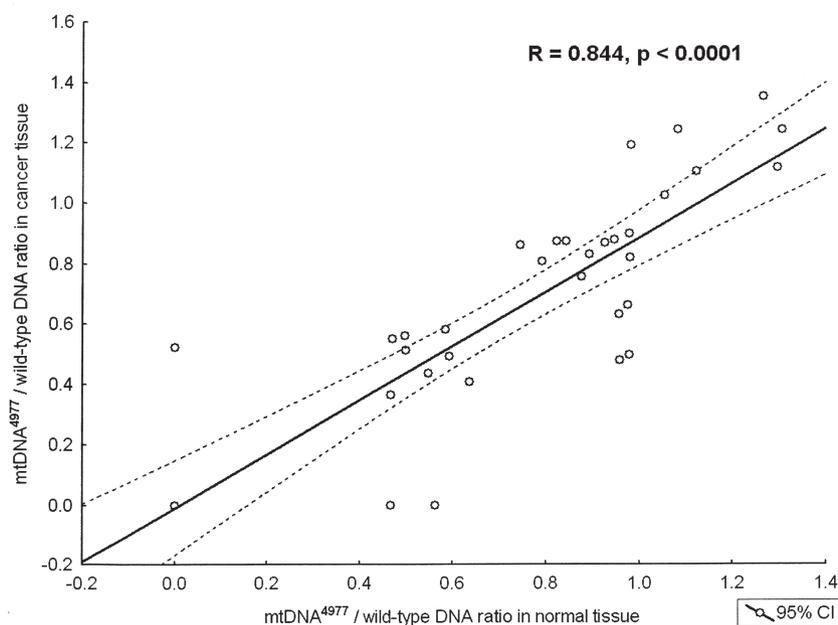


Figure 2. mtDNA⁴⁹⁷⁷/wild-type mtDNA ratios in matched control versus endometrial cancer tissue calculated by Spearman's rank correlation test.

Table II. Mean, median and SD mtDNA⁴⁹⁷⁷/wild-type mtDNA ratios in matched control and cancerous endometrial samples.

Samples	mtDNA ⁴⁹⁷⁷ /wild-type mtDNA ratio		
	Mean (range)	Median	SD
Control	0.718 (0.0-1.306)	0.374	0.062
Cancerous endometrial	0.633 (0.0-1.349)	0.398	0.066

gels and processed for direct DNA sequencing at the DNA Sequencing and Oligonucleotide Synthesis Laboratory, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland. Sequences were compared using the BioEdit Sequence Alignment Editor v. 7.0.5.5 (20).

Statistical analysis. Statistical analyses were performed using the SPSS software package v. 14.0 PL for Windows. The groups were compared by the Mann-Whitney U test or

Wilcoxon test when appropriate. mtDNA⁴⁹⁷⁷/wild-type mtDNA ratios in matched control and cancerous human endometrial samples were correlated using the Spearman rank correlation test. $P < 0.05$ was considered significant.

Results

The present study investigated 37 neoplastic endometrial and matched normal control samples for the prevalence of the mtDNA⁴⁹⁷⁷ deletion using the PCR-based methodology. The mtDNA⁴⁹⁷⁷ deletion was detected in 32 out of 37 (84%) matched control samples and in 30 out of 37 (81%) neoplastic endometrial tissues (Fig. 1). We noted a statistically significant correlation of mtDNA⁴⁹⁷⁷/wild-type mtDNA ratios between normal control and cancerous endometrial samples ($R = 0.844$, $p < 0.0001$; Fig. 2). Table II summarizes the mean, median and SD for mtDNA⁴⁹⁷⁷/wild-type mtDNA ratios. It is worth pointing out that the mean mtDNA⁴⁹⁷⁷/wild-type mtDNA ratios was significantly higher in matched controls than in EC samples ($p = 0.021$, Table II).

No correlation was reported between mtDNA⁴⁹⁷⁷/wild-type mtDNA ratio and patient age ($R = 0.09$, $p = 0.71$, Spearman

Table III. Mean \pm SD mtDNA⁴⁹⁷⁷/wild-type mtDNA ratio in control samples and cancerous endometrial tissues.

	N	Mean mtDNA ⁴⁹⁷⁷ /wild-type mtDNA ratio		P ^a
		Control samples	Cancerous samples	
Patient age (years)				
50-60	17	0.655 \pm 0.379	0.570 \pm 0.404	0.048
FIGO stage				
II + III	12	0.938 \pm 0.159	0.768 \pm 0.251	0.012
Myometrial invasion				
<	13	0.788 \pm 0.389	0.705 \pm 0.367	0.033
Lymph node metastases				
Absent	24	0.769 \pm 0.369	0.629 \pm 0.439	0.009
Infiltration of the neoplasm in the uterine cervix				
Present	11	0.934 \pm 0.166	0.793 \pm 0.248	0.021
Total	37	0.718 \pm 0.062	0.633 \pm 0.066	0.021

P^a, Wilcoxon test.

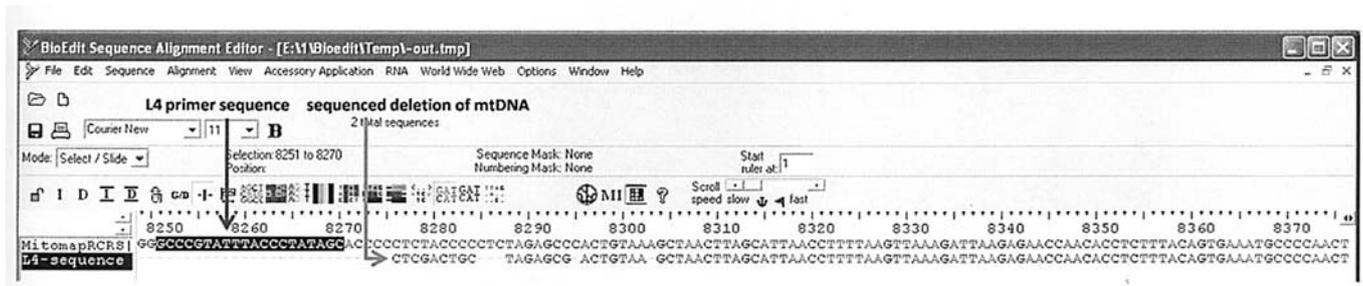


Figure 3. Sequence analysis of a 617-bp mtDNA fragment in EC sample number 238. Comparison with MITOMAP™ human mtDNA Cambridge sequence data (www.mitomap.org).

rank correlation test). Notably, there was a significant relationship between clinical stage of the disease (stage II versus stage III due to FIGO) and the amount of mtDNA⁴⁹⁷⁷ in neoplastic endometria ($p=0.048$, Mann-Whitney U test). Table III summarizes data on the association between clinico-prognostic features of EC patients and the mean mtDNA⁴⁹⁷⁷/wild-type mtDNA ratios in normal and cancerous samples. There were significant differences in the mean mtDNA⁴⁹⁷⁷/wild-type mtDNA ratios in the groups of women who were 50-60 years old, with advanced clinical stage of the disease, cancer infiltration less than half of the myometrial wall, lack of lymph node metastases and tumors infiltrating the uterine cervix (Table III).

We randomly sequenced a 617-bp mitochondrial DNA fragment flanked by the second round of PCR-primers in two EC-positive cases in order to confirm the presence of mtDNA⁴⁹⁷⁷ deletion. The sequence analysis confirmed that the amplified fragments originated from mtDNA and encompassed mtDNA⁴⁹⁷⁷ deletion (Fig. 3).

Discussion

Several studies have evaluated the existence of a number of mtDNA alterations, including point mutations, multiple gene deletions, and MI in different human cancerous tissues and cell lines (4,6,7). In general, most of the mtDNA mutations are heteroplasmic in nature, and the cells contain thousands of mutated or deleted mtDNA molecules that coexist with wild-type mitochondrial DNA (21-23). Notably, mutations of mtDNA have been reported to occur twice as much as described for nuclear DNA, and the defect phenotype associated with a particular mitochondrial alteration is generally expressed when the proportion of mutated mtDNA overcomes a threshold (23,24).

Apart from investigations of various molecular genetic alterations during endometrial tumorigenesis (25-27), our group places emphasis on the evaluation of the prevalence of mtDNA alterations in various human malignancies, including EC (28,29). Homoplasmic MELAS A3243G mtDNA mutation



ously detected, for the first time, in a colon tumor and the additional changes in mtDNA were reported in coding regions of human ECs (28). Although somatic mtDNA mutations were reported either in early- or advanced-stage ECs, there was no significant relationship between the prevalence of mitochondrial alterations and clinicoprostnological variables of cancer (29). Data from the current study suggest that mtDNA 4977-bp deletion appears not to be specific to endometrial cancer tissues, being detected either in EC or in the matched control samples. It is of vital importance to emphasize that there was a significant correlation of mtDNA⁴⁹⁷⁷/wild-type mtDNA ratios between normal control tissues and cancerous human endometrial samples (Fig. 2).

The 4977-bp deletion is one of the most 'common' somatic mtDNA alterations, occurring between nucleotides 8,470 and 13,477 of the human mtDNA. The mtDNA⁴⁹⁷⁷ fragment encompasses genes involved in OXPHOS, including ATPase 6, ATPase 8, cytochrome oxidase III and NADH subunits (ND3, ND4, ND4L and ND5) (21). Although this deletion creates a mtDNA molecule that is smaller than the normal one, it may still replicate. It accumulates with age in post-mitotic cells, and prefers tissues with a high metabolic rate and a low proliferative index (30,31). This deletion first develops in the second and third decades of life in human muscles and liver tissues (32). It is worth pointing out that mtDNA⁴⁹⁷⁷ deletion is associated with sporadically developed human phenotypes, including Pearson syndrome, Kearns-Sayre syndrome and chronic progressive external ophthalmoplegia (1,33).

Making a comparison with adjacent tumor-free material, Dani and co-investigators (24) reported a lower amount of mtDNA⁴⁹⁷⁷ deletion in various human cancers (including breast, colorectal, gastric and head and neck tumors). Independently published reports dealing with sporadic breast (34), gastric (35) and oral (36) carcinomas revealed similar results. Although we did not find any statistically significant difference in the frequency of 'common' mtDNA⁴⁹⁷⁷ deletion between the tumoral and matched control samples studied herein, there are several explanations for this discrepancy. Firstly, the contamination of non-tumoral material (epithelial, vascular or connective tissues) in the cancerous samples collected at surgery may increase the amount of 4977-bp 'deleted' cells in neoplastic material. Moreover, the presence of mtDNA⁴⁹⁷⁷ deletion may be associated with metabolic and bioenergetic changes during the process of endometrial carcinogenesis. Therefore, the prevalence of this deletion in normal (proliferative and secretory endometria) and hyperplastic (with and without atypia) endometria should be evaluated and compared with the results presented above. These experiments are still in progress in our laboratory.

It is well-known that the frequency of 4977-bp deletion in human tissues increases with age and is associated with energy-demanding human tissues. Notably, the frequency of mtDNA⁴⁹⁷⁷ deletion in tissue samples of lung cancer patients >55 years of age was found to be significantly higher than in those <55 years of age (37). In the present study, most of the patients enrolled (30 out of 37, 81%) were >50 years of age. The mean mtDNA⁴⁹⁷⁷/wild-type mtDNA ratio was significantly higher in the control group (0.655±0.379) than in the cancerous group (0.570±0.404, p=0.048, Table III) for women

between 50 and 60 years of age (n=17). These data suggest that aging factors may play an important role in the accumulation of mtDNA deletions, particularly in peri-menopausal patients affected by EC.

It is worth citing the study conducted by Thayer *et al* (38), who identified this deletion in the leukocytes of all 71 individuals (ranging in age between 8 months to 99 years) of 21 maternal lines. All infants and children, who were free of any known mitochondrial disorders, displayed mtDNA⁴⁹⁷⁷ deletion. They also suggested the possibility of maternal transmission of 4977-bp mtDNA deletion in women without known mitochondrial disorders.

In conclusion, we suggest that mitochondrial 4977-bp deletion is not specific to EC tissues. The accumulation of mtDNA⁴⁹⁷⁷ is associated with aging processes, particularly in peri-menopausal women suffering from EC.

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