Clinicoprognostical features of endometrial cancer patients with somatic mtDNA mutations

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Abstract. Somatic mitochondrial DNA (mtDNA) mutations have been found in a subset of endometrial cancers (EC) from different populations. We have investigated the relationship between mtDNA changes and clinical and pathological variables of women affected by EC. mtDNA mutations were detected both in early (3/32; 9%) and in advanced (1/8; 12%) stages of uterine tumors. However, patients carrying the mtDNA mutations or the normal mtDNA sequence had indistinguishable clinicopathological data, including age, clinical stage, histological grade and type or depth of myometrial invasion. It is noteworthy that mtDNA mutations were not detected in hyperplastic endometrial tissues or in ECs coexisting with hyperplasia, nor in a single case of endometrial stromal sarcoma. LOH at the tumor suppressor genes *RB1* and *TP53* as well as *p16^{INK4A}* alterations (LOH, gene deletion) were found in tumors carrying mtDNA mutations. These results suggest that somatic mtDNA mutations are detected in a subset of ECs, although they are unrelated to clinicopathological variables of cancer.

Introduction

Endometrial cancer is one of the most frequently occurring gynecological malignancies in the Western World, and its incidence has increased significantly during the last decade in Poland (1). In general, clinicopathological, immunohistochemical, biochemical and molecular genetic analyses have provided valuable information for dividing ECs into two different subtypes, estrogen-dependent (type I) and estrogen-independent (type II) (2-4). Factors associated with unopposed estrogenic stimulation and the presence of hyperplastic endometrial lesions (concomitant with neoplasia) are linked with the most common type of carcinoma, endometrioid-type I tumor (5-6). On the other hand, USC and clear cell carcinoma of the endometrium, which are not linked to estrogenic stimulation and are not associated with endometrial hyperplasia, are considered type II tumors (5,6). These sub-types differ not only in clinicoprognostical features but also in various molecular genetic alterations detected in oncogenes, tumor suppressor genes and mismatch-repair genes (5). For example, pRb1-cyclin D1-cdk4/6-p16^{INK4A} pathway alterations were described in endometrioid-type ECs whereas they were uncommon in USCs in humans (7).

Mitochondria play important roles in cell homeostasis, actively participating in energy metabolism, generation of reactive oxygen species, aging and initiation of programmed cell death (8,9). Mitochondrial failure has been described at all levels of structure and function, including abnormal ultrastructure, metabolic deregulation and genetic alterations (10). In general, mtDNA point mutations, gene deletions and mtMSI have been reported in human neoplasms and cell lines, but the frequency of these alterations differs in various tumors (11-13). The most well-known disorders caused by mtDNA mutations are LHON (Leber's Hereditary Optic Neuropathy), MELAS (Mitochondrial Encephalopathy, Lactic Acidosis and Stroke-like episodes), MERRF (Myoclonic Epilepsy with Ragged Red Fibers), maternally-inherited diabetes mellitus and maternally inherited cardiomyopathy (10).

Data have accumulated concerning somatic mtDNA mutations and mitochondrial microsatellite instability in various gynecologic malignancies in humans (11). Liu *et al* (14) detected a high (60%) incidence of mtDNA mutations by sequencing DNAs isolated from malignant ovarian tissues. Somatic D-loop mitochondrial DNA mutations were detected at high frequency in uterine serous carcinomas extracted from paraffin-embedded slides (15). Moreover, Wang *et al* (16) reported that about 25.4% of cervical carcinomas, 48.4%

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Abbreviations: EC, endometrial cancer; USC, uterine serous carcinoma; mt, mitochondrial; mtMSI, mitochondrial microsatellite instability; LOH, loss of heterozygosity; IHC, immunohistochemistry; PI, proliferative index

Key words: endometrial cancer, mitochondrial DNA, mutations, endometrial hyperplasia

Table I. Clinical and pathological features of EC patients studied for the somatic mtDNA mutations.

Variables	No. of cases n (%)
Age	
<50	7 (17.5)
50-60	19 (47.5)
>60	14 (35)
Stage	
I	32 (80)
II	5 (12.5)
III	2 (5)
IV	1 (2.5)
Histological grade	
G1	19 (47.5)
G2	15 (37.5)
G3	6 (15)
Histological type	
Endometrioid	37 (92.5)
Non-endometrioid	3 (7.5)
Myometrial invasion	
None	5 (12.5)
<1/2	17 (42.5)
>1/2	18 (45)
Lymph node involvement[a]	
Positive	0 (0)
Negative	27 (100)
Coexistence of hyperplastic and neoplastic endometrium	
Positive	8 (20)
Negative	32 (80)
Total	40 (100)

[a]Lymph nodes were dissected in 27 cases.

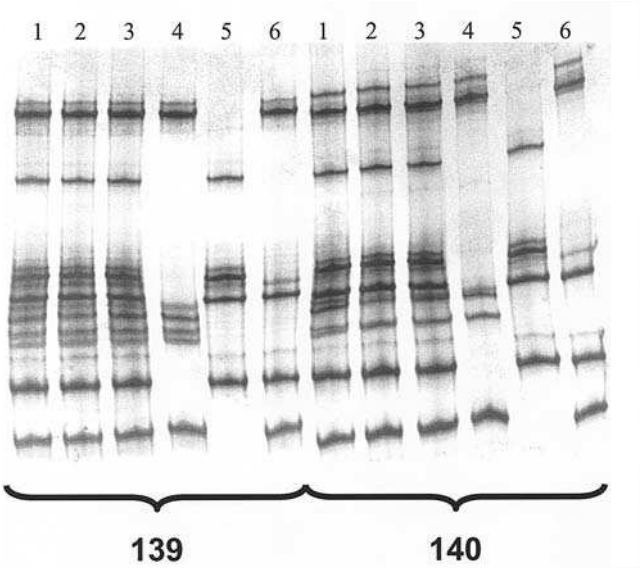

Figure 1. SSCP analysis of PCR amplified mtDNA from 2 patients - 139 and 140; in the latter mutation C12258G was detected (17). For each patient 6 lanes are analyzed, the first three represent regions 1, 4, 2 and 5 from normal, tumor and a 1:1 mixture of normal and tumor tissue, respectively. Samples 4-6 are 1:1 mixtures of normal and tumor tissue samples for regions 1 and 4, 5 and 2, and 4 and 2, respectively.

of endometrial cancers, and 21.9% of ovarian carcinomas carried one or more mtMSI. Our group has also analyzed mtDNA mutations in various tumors, including human ECs (17). However, to the best of our knowledge, there are no data assessing the relationship, if any, between somatic mtDNA mutations and clinicoprognostical features of women affected by EC.

The aim of the current study was to investigate the correlation between mtDNA somatic mutations and clinical and pathological features of EC patients. Short-time follow-up of the study group with regard to somatic mtDNA mutations has also been evaluated.

Materials and methods

Frozen tumor tissues of the studied cases and their matched normal tissues (including normal cervix, omentum or blood samples) were collected during surgery in the Second Department of Gynecology, Lublin University School of Medicine, Lublin, Poland. Altogether, 40 tissues of endometrial cancer, four cases of endometrial hyperplasia and one case of endometrial stromal sarcoma were analyzed. Clinicopathological features of EC patients enrolled are summarized at Table I. The clinical stage of the disease was classified according to the International Federation of Gynecology and Obstetrics classification (18,19). The material was assessed microscopically at the Department of Pathology, Lublin University School of Medicine, Lublin, Poland, based on the World Health Organization staging system (20).

High-molecular-weight DNA was isolated and mtDNA genetic analysis was performed as described previously (17). Briefly, a total of 1623 base-pairs (bp) of mtDNA [~10% of mtDNA, including nucleotides 135-433 (region 1), 2986-3301 (region 2), 4981-5500 (region 3), 10390-10700 (region 4) and 12005-12386 (region 5)] were separately PCR-amplified and screened for mtDNA genetic alterations by SSCP with silver staining of the gels (21) (Fig. 1). The presence of bands with variant migration patterns was confirmed by repeating PCR-SSCP analysis. Sequencing analysis was performed on an ABI PRISM 377 DNA sequencer (Pharmacia Biotech, Sweden) using PCR products obtained from the original isolates as template. All experiments with positive results were repeated in order to obtain reproducibility of the results.

Statistical analysis was performed using Fisher's exact probability test or χ^2 test where appropriate. Univariate survival analysis for disease-free survival was performed using the Kaplan-Meier method (22) and the log-rank test, with the time of primary surgery serving as entry data. Data were analyzed using the software Statistica for Windows (Statsoft Inc., 1993, release 5.0). A p-value <0.05 was considered significant.

Table II. The incidence of mtDNA mutations in the study group.

Endometrial	No. of cases	
	Total n	Positive n (%)
Cancer	40	4 (10)
Hyperplasia	4	-
Stromal sarcoma	1	-
Total	45	4 (9)

Results

Study group. A total of 90 specimens (consisting of tumor and normal tissues from the same patient) were collected and investigated for the somatic mtDNA mutations. The mean age of the EC patients was 59 (range 43-82) years, and most of the cases had stage I disease (n=32; 80%) (Table I). Most of our patients (n=36; 90%) were without evidence of recurrence after a mean follow-up time of 32 months (range 8-53 months). There were two cases of simple non-atypical hyperplasia, one complex non-atypical hyperplasia and one complex atypical hyperplasia. The mean age of patients with endometrial hyperplasia was 50 (range 48-52) years, and was lower compared to the mean age of EC patients (59 years). The single patient with endometrial sarcoma stromale was 39 years old and she was classified at stage I disease according to the FIGO classification.

mtDNA mutations. The results of the screening for somatic mtDNA point mutations are summarized in Table II. In total, 4 out of 40 (10%) EC samples carried changes of mtDNA, and in one case (number 103) four different variations occurred (17). Detailed data of mtDNA-positive EC patients are shown in Table III. When clinicopathologial features of patients with uterine cancer were analyzed, somatic mtDNA mutations were reported both in early and in advanced-stage endometrioid-type ECs (Table III). Patients carrying the mtDNA mutations or the normal mtDNA sequence had indistinguishable clinicopathological data (including age, clinical stage, histological grade and type or depth of myometrial infiltration). It is noteworthy that mtDNA mutations were not detected in hyperplastic endometrial tissue nor in ECs coexisting with hyperplasia (Tables II and III). However, there was no significant relationship in the distribution of mtDNA mutations between cases with and without pre-cancerous endometrial lesions (p=0.56; Fisher exact test).

Tumors with somatic mtDNA mutations showed concomitantly nuclear genome alterations (Table III). LOH at the tumor suppressor genes *RB1* and *TP53* as well as $p16^{INK4A}$ alterations (LOH, gene deletion) were reported in tumors with mtDNA mutations. However, only in one case (number 103 from Table III) MIB-1 PI (23) was extremely high (90%). The mtDNA from this patient contained four mutations (17).

Follow-up. We also calculated the disease-free survival of EC patients regarding the presence or absence of somatic mtDNA mutations during a short-time follow-up. No significant difference in disease-free survival between patients with and without mitochondrial alterations was observed (p>0.05; log-rank test). None of the patients with mtDNA mutations developed recurrences during a short-time observation (Table III).

Discussion

Data have accumulated recently describing the significant involvement of mitochondrial genomic alterations in the process of human carcinogenesis (11-13). It is widely accepted that mitochondrial DNA is highly susceptible to genetic alterations because it is not protected by histones and chromatin structures and is continuously exposed to reactive oxygen species generated during oxidative phosphorylation (8,28). The mtDNA alterations may be part of the neoplastic transformation process or be the result of exposure to chemo-

Table III. Clinicopathological features and molecular alterations of EC patients with mtDNA mutations.

No.[a]	Age (years)	Staging		Myometrial invasion	Lymph node involvement	Coexistence of hyperplastic and neoplastic endometrium	LOH[b]		$p16^{INK4A}$ alterations	IHC					mtDNA mutations	Overall survival (months)
		FIGO	WHO				*TP53*	*RB1*		$p16^{INK4A}$	pRb-1	Cyclin D1/cdk 4	p53	MIB-1 PI (%)		
103	68	Ib	G1	<1/2	-	-	+	-	-	2	2	0	1	90	Homo-plasmic	40
107	43	IIIa	G2	>1/2	-	-	-	+	+	1	2	2	2	10	Homo-plasmic	39
113	59	Ib	G2	<1/2	-	-	-	-	+	2	2	2	2	40	Homo-plasmic	34
140	60	Ic	G1	>1/2	-	-	-	-	-	2	2	2	2	40	Hetero-plasmic	28

[a]Patient numbers are the same as reported previously (17). [b]Data concerning the molecular alterations have been published (23-27).

therapeutic agents widely applied in oncology (12,29,30). In the literature, mutations in mtDNA have been identified in several human neoplasms and cell lines, although data assessing the frequency and role of mtDNA alterations in gynecological neoplasms, in ECs in particular, are limited (14-17,31-34). Liu *et al* (14), who detected somatic mitochondrial DNA mutations in 4 regions of the mitochondrial genome (16S and 12S rRNA genes, the D-loop and the cytochrome *b* gene), suggest that these regions may represent a hot-spot in ovarian tumorigenesis. A high incidence (63%) of D-loop region mtDNA mutations in type II ECs has been recently reported by Pejovic *et al* (15). Moreover, a high frequency of mitochondrial genome instability (89%; 25 out of 28) was detected in ECs by Liu *et al* (31). One out of six (16%) endometrial carcinomas showed mutations of the D310 mitochondrial mononucleotide repeat (34). In the current study, somatic mtDNA mutations were reported only in 10% (4 out of 40) of primary ECs, a frequency significantly lower compared with the data reported previously [25% by Liu *et al* (31) or 63% by Pejovic *et al* (15)]. However, only approximately 10% of mtDNA were screened for the molecular genetic alterations in human ECs, and we suggest that the frequency of mtDNA alterations would be higher when complete sequence analysis of the mtDNA genome would be performed.

Interestingly, mtDNA abnormalities were not reported in the hyperplastic endometria (n=4) nor in type I uterine malignant tumors (carcinomas concomitant with hyperplasias; n=8). To the best of our knowledge, mutations of mtDNA have not been analyzed in precancerous human endometrial lesions up to now (Medline® database). In the literature, Wang *et al* (33) reported the increase of mtDNA copy number in endometrial cancer cells compared to normal endometrial glandular cells ($p<0.001$). Studying premalignant lesions from the head and neck (n=137), Ha *et al* (35) showed a clear increase in incidence of mitochondrial C-tract alterations from benign hyperplasia (22%) to squamous carcinoma *in situ* (62%; $p<0.001$). However, only two out of 45 gastric tumors (including 15 adenomas) were positive for mtDNA mutations in the non-coding regions of the gene (36). Additional studies on a large scale need to be conducted to assess alterations of the entire mitochondrial genome in atypical and non-atypical simple/complex human endometrial hyperplasias.

It is widely accepted that the mitochondrial genome is more vulnerable to oxidative base damage compared to the nuclear genome (11,13). Due to the lack of histone protection, most mutations occur in the coding mtDNA sequences and therefore they are likely to have biological consequences. The mtDNA mutations coexist with nuclear genome alterations, and the mutation rate of mtDNA has been reported to be higher compared to the frequency of nuclear DNA alterations (37,38). Somatic mutations in mtDNA and nuclear microsatellite instability were not associated with each other in gastric carcinomas (39). Moreover, Richard *et al* (40) showed the lack of association between mitochondrial and nuclear mutations in human breast cancers, suggesting that different systems are responsible for mitochondrial and genome instability in cancer cells. Based on our own observations as well as data from the literature, the mtDNA mutations (heteroplasmic or homoplasmic) and nuclear genome alterations are present during the development and progression of cancer. However, nuclear genetic alterations have been detected in type I ECs with and without mtDNA mutations (data not shown). The interactions of mutations in the nuclear and mitochondrial genomes in ECs are not yet understood and require further analysis.

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