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# Somatic D-loop mitochondrial DNA mutations are frequent in uterine serous carcinoma

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**Abstract**

The mitochondria plays a role in apoptosis. Its genome is also more susceptible to mutations because of high levels of reactive oxygen species and limited repair mechanisms. The D-loop of mitochondrial DNA (mtDNA) contains essential transcription and replication elements, and mutations in this region might alter the rate of DNA replication. We examined genetic alterations in the D-loop region of mtDNA in uterine serous carcinoma (USC) samples and their paired normal adjacent endometrium. DNA was extracted after laser-capture microdissection of paraffin-embedded tissues from eight patients with USC. The entire D-loop genome was amplified using nine pairs of overlapping primers. Denatured polymerase chain reaction (PCR) products were subjected to single-strand conformation polymorphism (SSCP) analysis. Somatic mtDNA alterations were detected in five tumours (63%). Our study indicates that mtDNA D-loop sequence alterations occur at a high frequency in USC suggesting that mtDNA mutations may play a role in the development of USC.


*Keywords:* Mitochondria; Mutation; Uterine cancer; DNA

## 1. Introduction

Uterine serous carcinoma (USC) is an uncommon subtype of endometrial cancer that was first described by Hendrickson and colleagues in 1982 [1]. USC commonly presents at an advanced stage (III–IV), and even when diagnosed at an early stage, it is often associated with disseminated disease and poor prognosis [2]. Although a p53-mutation-driven pathway has been suggested for carcinogenesis in USC [3], a better understanding of the molecular properties of this disease is warranted in order to identify novel biomarkers and potential targets for therapeutic intervention. In this regard, mitochondrial function and/or mutations in mitochondrial DNA (mtDNA) could play a central role at several stages in the process of carcinogenesis [4]. The important functions of mitochondria in energy metabolism, generation of reactive oxygen species (ROS), aging, and initiation of apoptosis suggest that mitochondria may contribute to the neoplastic process by serving as a major switch-point between cell death and abnormal cell growth [5].

The mtDNA genome is a highly compact, circular, 16-kb, double-stranded DNA that encodes 13 polypeptides of the mitochondrial respiratory chain, 22 transfer

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RNAs, and two ribosomal RNAs required for protein synthesis [6]. The only non-coding region is the 1.1 kb mtDNA control region, the D-loop, which contains crucial elements for mtDNA replication and transcription [7]. Lacking protective histones and many of the repair mechanisms associated with nuclear DNA, the mitochondrial genome is highly susceptible to oxidative damage [8] and hence mutation. Indeed, the mutation rate of somatic mtDNA is approximately 10–100 times higher than that of the nuclear DNA [9–11]. In most previous studies, the mtDNA genome was screened for mutations either by direct sequencing [12,13] or an oligonucleotide mismatch ligation assay [14,15]. Recently, the existence of specific mutation patterns in mtDNA has been demonstrated in colorectal [10,16], urinary bladder [12], thyroid [17], oesophageal [14], liver [18], breast [19,20], ovarian [21], gastric [22], prostate [13] and pancreatic cancers [23] and most of these mutations were found in the D-loop region. Importantly, mutations in the D-loop regulatory region might alter the rate of DNA replication by modifying the binding affinity of significant *trans*-activating factors. However, the frequency of mtDNA alterations in USC is unknown. In the present study, we examined the genetic alterations in the D-loop region of mtDNA in microdissected USC and their paired normal adjacent endometrium, in an effort to address the in role in tumourigenesis.

## 2. Materials and methods

### 2.1. Patients and tissue specimens

Formalin-fixed paraffin-embedded archival USC tumour specimens were obtained from the paraffin archive resource of the Roswell Park Cancer Institute. All tissue specimens were collected under an approved protocol from the Institutional Review Board (IRB). All pathology specimens were reviewed in our institution and tumours were classified according to standard histological criteria [1]. Surgical and pathological staging was performed according to the International Federation of Gynaecologists and Obstetricians (FIGO) recommendations. The medical records of the patients were also retrospectively reviewed. Study outcomes included objective evidence of recurrence and overall survival. The duration of survival was the interval between diagnosis and death. The observation time was the interval between diagnosis and last contact (death or last follow-up).

### 2.2. Isolation of epithelial and stromal cells from benign endometrial tissue and from USC tissue by laser-capture microdissection

Tissue sections of 10 μm were prepared for each specimen and stained with haematoxylin and eosin (H&E). Normal tissue and malignant cells were identified and pure target cells were procured by laser-capture microdissection using the PixCell II LCM system (Arcturus, Mountain View, CA) by a board-certified pathologist. A 15 μ infrared laser beam was used to obtain populations of the cells of interest on the CapSure LCM caps (Arcturus). One thousand cells were collected from each tissue population, capturing by laser 50 cells at a time.

### 2.3. Isolation of DNA from microdissected cells from USC and adjacent normal endometrium

DNA was purified from microdissected cells by standard proteinase K treatment followed by phenol/chlorophorm/isoamyl alcohol extraction [24]. DNA was precipitated with 0.3 M sodium acetate in 70% ethanol at −20 °C overnight and resuspended in Tris–ethylene diamine tetraacetic acid (EDTA) (TE) buffer, pH 8.0. DNA quantification was performed using ethidium-bromide standards with measurements at an absorbance of 260 nm. This protocol results in the extraction of both genomic and mtDNA.

### 2.4. PCR Amplification of D-loop segment of mitochondrial DNA

mtDNA fragments containing the D-loop region (spanning nucleotide 16024 to 576) were amplified using nine pairs of primers. The primers pairs used, and the sizes of the amplified products are as follows:

Pair 1: Forward 5′-TACTTGACCACCTGTAGTAC-3′
Reverse 5′-CTGTTAAAAGTGCATACCGCCA-3′(314 bp).

Pair 2: Forward 5′-ACTTGACCACCTGTAGTAC-3′
Reverse 5′-GGGGTTTGGTGGAAATTTTTT-3′(189 bp).

Pair 3: Forward 5′-ATTATTTATCGCACCTACGT-3′
Reverse 5′-CTGTTAAAAGTGCATACCGCCA-3′(278 bp).

Pair 4: Forward 5′-ATTATTTATCGCACCTACGT-3′
Reverse 5′-GGGGTTTGGTGGAAATTTTTT-3′(153 bp).

Pair 5: Forward 5′-CTCACGGGAGCTCTCCATGC-3′
Reverse 5′-AGGGTTGATTGCTGTACTTGC-3′(172 bp).

Pair 6: Forward 5′-CTCACGGGAGCTCTCCATGC-3′
Reverse 5′-GGGGTTTGGTGGAAATTTTTT-3′(277 bp).

Pair 7: Forward 5′-CTCACGGGAGCTCTCCATGC-3′
Reverse 5′-CTGTTAAAAGTGCATACCGCCA-3′(402 bp)

Pair 8: Forward 5′-TGGATGACCCCCCTCAGATAG-3′
Reverse 5′-GCATGGAGAGCTCCCGTGAG-3′(250 bp)

Pair 9: Forward 5′-TGGATGACCCCCCTCAGA-TAG-3′
Reverse 5′-AGGGTTGATTGCTGTACTTG-C-3′(403 bp)

Sample DNA was added to each polmerase chain reaction (PCR). Thirty-microlitre reaction contained 0.2 μM each primer, 50 μM of each deoxynucleotide triphosphate (dNTP), 0.8 U AmpliTaq polymerase and 2.5 mM $MgCl_2$. Genomic DNA was subjected to the following cycling conditions: initial denaturing at 94 °C for 3 min followed by 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 15 s for 35 cycles and a final extension step at 72 °C for 5 min. One microlitre of PCR product was analysed on an ethidiumbromide-stained, 3% agarose gel (15 min at 120 V) to verify the amplification product and for quantification.

### 2.5. Single-strand conformation analysis

The amplified PCR product was subjected to single-strand conformation analysis (SSCP) analysis. Electrophoresis of the denatured PCR products was carried out in MDE™ (Mutation Detection Enhancement) gel solution and run at 180 V for at least 16 h. MDE gels were stained using Sybr-green II diluted 1:10 000 in TE for 20 min and photographed. Image capture was on an IS1000 digital imaging system (alpha-INNotech Corp., San Leandro, CA, USA) using 254 and 313 nm ultraviolet transillumination and an SG-3 filter. All cases presenting with band shifts were submitted to a second analysis performing a new PCR amplification and SSCP analysis with the initial DNA samples. Only reproducible bands were further evaluated. Only the cases with mtDNA alterations in the tumours but not in adjacent normal tissues were considered to harbour somatic mitochondrial mutations.

### 2.6. Nucleotide sequencing

Abnormal bands, as well as the corresponding normal bands, detected by SSCP were recovered from the MDE gel and submitted to PCR reamplification with the original set of primers. The re-amplified amplicons were purified by gels and columns using the Wizard PCR-Prep kit (Promega, Madison, WI, USA). Sequence analysis was then carried out using the Applied Biosystems Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin–Elmer Corp., Norwalk, CT, USA). The products of cycle sequencing were electrophoresed on a 6% Long Ranger gel (FMC Bioproducts, Rockville, MD, USA) and analysed on an Applied Biosystems Model 3700 automated DNA sequencer (Perkin–Elmer Corp.). Sequences were compared against human mtDNA sequence, GENBANK accession #J01415, as well as a comprehensive mitochondrial database, MITOMAP [5]. Sequence variants found at a particular location in both tumour and matched normal mtDNA were classified as polymorphisms. If the DNA sequence at a particular location in tumour mtDNA differed from the matched normal mtDNA, this was defined as a somatic mutation.

## 3. Results

### 3.1. Study population

A total of 16 specimens (consisting of tumour and adjacent normal tissues) were obtained by laser-capture microdissection from eight patients with USC. The characteristics of the study population are presented in Table 1. The age range of the patients was 55–86 years, and half (4/8, 50%) had stage III disease. Only one patient with stage IA disease was alive and without evidence of disease after a follow-up duration ranging from 1–34 months.

### 3.2. Mutations in the D-loop region of the mitochondrial genome

The results of the screening for D-loop somatic mutations are summarised in Table 1 and Fig. 1. In the eight tumours, we identified 13 sequence variants by SSCP. Eight of these were polymorphisms, and were not characterised further. Five of the eight patients (63%) harbored somatic D-loop mtDNA mutations, one mutation was detected in each tumour. Representative results of the sequence analysis in normal tissue and paired tumour samples are shown in Fig. 1. Four of the five mutations were transitions: A16066G (case 1), A16211G (case 2), T16166C (case 4), and A16327G (case 7). Insertion G16320 was found in case 5. All five mutations occurred in hypervariable area 1 (HV1) of mtDNA. All alterations were confirmed at least twice.

## 4. Discussion

Recently, mtDNA mutations were reported to occur in several human cancers [10,12–14,16–23]. The only gynaecological tumour evaluated for the presence of mitochondrial genome alterations is ovarian cancer [21]. Mutations of mtDNA have not been examined in any type of uterine cancer. We demonstrated here, for the first time, evidence of somatic mtDNA mutations in USC.

The D-loop region of mtDNA is highly polymorphic and contains two constitutionally hypervariable regions: HV1 (16024–16383) and HV2 (57–333) that are hot spots for acquired mutations in various tumour types [25]. In our initial analysis of mtDNA mutations in

Table 1
Clinical and pathological characteristics and mutation status of patients with serous carcinoma of the uterus

| Patient | Age (years) | Stage | Follow-up (months) | Recurrence | Status[a] | Mutation |
|---|---|---|---|---|---|---|
| 1 | 66 | IA | 34 | Abdomen | AWD | A16066G |
| 5 | 55 | IA | 31 | None | ANED | insG16320 |
| 6 | 85 | IB | 1 | – | DOOC | None |
| 4 | 80 | IC | 9 | Abdomen | DOD | T16166C |
| 8 | 79 | IIIA | 12 | Abdomen | DOD | None |
| 2 | 66 | IIIC | 24 | Abdomen | AWD | A16211G |
| 7 | 79 | IIIC | 13 | Abdomen | DOD | A16327G |
| 3 | 86 | IIIC | 10 | Abdomen | DOD | None |

[a] AWD, alive with disease; ANED, alive no evidence of disease; DOOC, dead of other causes; DOD, dead of disease.

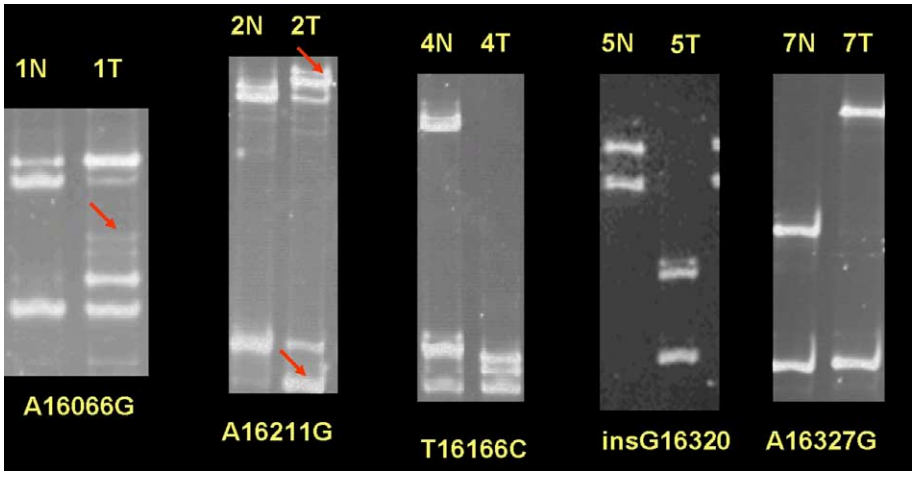


Fig. 1. Representative single-strand conformation polymorphism (SSCP) analysis gel radiographs from the five cases with somatic D-loop mitochondrial (mt) DNA mutations. The "banding" pattern was shown in paired normal (N) and tumour (T) samples. Arrows indicate band shifts. The specific mutation is indicated below the five tumour samples.

USC, we have therefore focused on the D-loop area of mtDNA. While D-loop mutations do not lead to alterations in the coding sequence of mtDNA, such alterations interfere with the sequence in the promoter region and modify the affinity for the inducers and modifiers of mtDNA replication and may change the rate of mtDNA transcription.

The frequency of mtDNA loop mutations in patients with USC is higher than that reported in a study on ovarian cancer (63% vs 36%) [21]. This observation is important since USC is frequently thought to behave, clinically and biologically like serous epithelial ovarian cancers [1]. The frequency of mtDNA mutations also differs greatly in other adenocarcinoma types. Tamura and colleagues [26] found D-loop mtDNA mutations in only 2 of 45 (4%) screened gastric carcinomas and adenomas, while Habano and colleagues [27] described mtDNA mutations in only 8% of 62 gastric carcinomas. However, the rate of detection of D-loop mutations was almost 90% in prostate cancer [28] and 68% in hepatocellular carcinoma [15]. In a recent analysis of the entire mitochondrial genome in 19 patients with breast cancer, 74% (14 of 19) displayed at least one somatic mutation. Twenty-two out of 27 (81%) total mutations were found within the D-loop region [20], while the remaining mutations were located within the coding region of mtDNA. It is important to recognise that all five mutations in our series occurred within HV1, a finding shared by many other cancer types [12,16]. However, a substantial number of somatic mutations in some other cancers were also found within the functionally more important HV2 region of mtDNA [20,28,29].

The most frequent type of point mutations in our study, as well as in previous reports, were transitions, some of them affecting the G residues, which are preferred site for oxidative DNA damage to the DNA in general, and in mtDNA in particular [30]. The five distinct somatic mutations described here are unique to USC and they have not been found in any other tumour type. Our finding of somatic mutations in the tumour and not the corresponding germline/transmitted DNA from the same individual suggests pathogenicity. Although the pathogenetic role of each mutation is not presently known, they are all located in a transcriptionally-active region of mtDNA. One might suggest that these somatic

mtDNA mutations are modifiers of tumour risk which, along with other mtDNA alterations, may augment this risk. Alternatively, it is possible that these mutations represent mtDNA damage from oxidative stress. Nevertheless, the non-random distribution of somatic mutations within HV1 raises the possibility that certain of sequences might be prone to somatic mutations [22,30].

In conclusion, this is the first study demonstrating the frequent occurrence of mutations within the D-loop region of mtDNA in patients with serous carcinoma of the uterus. It provides further evidence that oxidative damage might be an important mechanism in the development of USC. Although we were unable to observe any trend between mtDNA mutations and clinical and pathological characteristics, possibly because of our relatively small sample size (Table 1), additional evaluation of the molecular consequences of the somatic D-loop mtDNA mutations in USC and its associated precursor lesions, may elucidate the role of mitochondria in USC carcinogenesis. Further, our findings have the potential for future clinical utility for in that they may identify individuals at a higher risk of developing USC. Studies comparing the frequency of these mtDNA mutations in the peripheral blood of a large number of USC patients with those found healthy control are therefore required.

## Conflict of interest statement

None declared.

## Acknowledgement

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