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Research Article

Impact of Mutations in the D-loop Region in Ovarian Cancer in Senegalese Women

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Abstract

In Senegal, ovarian cancer is the 3rd most common cancer in women with an incidence of 5.0/100,000 women. Thirty-five cancerous tissues, twenty-seven healthy tissues were included in this study. Due to the anatomical position of the ovary, the removal of a sample of suspicious tissue from each patient involves surgery through laparotomy or laparoscopy after obtaining consent. DNA extraction, polymerase chain reaction (PCR) and sequencing were performed to obtain sequences. BioEdit version 7.0.5.3 2005, Harlequin version 3.0, DnaSP version 5.10.01, MEGA 6 were used to perform the analyses. The results show a higher percentage of transition in cancerous tissues (91.45) than in healthy tissues (75.19) in contrast to transversions which are greater in healthy tissues (24.84) than in cancerous tissues (8.54), and the mutation rate (R) is also higher in cancerous tissues (10.712) than in healthy tissues (3.079). Analysis of the polymorphism revealed high values of haplotypic diversity in both cancerous tissues (0.662±0.085) and healthy tissues (0.997±0.011), and low nucleotide diversity values in both tissues (cancerous tissues=0.00922±0.00175; healthy tissues=0.01539±0.00175), these results show us that the genetic evolution of mutations in ovarian cancer has a strong polymorphism. It was also found that the value of the genetic distance between healthy tissues (0.016) was higher than that observed between cancerous tissues (0.009). The genetic distance between healthy and cancerous tissues is 0.015 closer than that observed between healthy tissues. The value of genetic differentiation between healthy and cancerous tissues is significant; this demonstrates a much faster proliferation of cancer cells. The objective of this study is, on the one hand, to better understand the target population by clearly identifying demographic parameters and on the other hand, to evaluate the involvement of somatic mutations and mitochondrial DNA gene expression in the occurrence of ovarian cancer in women in Senegal. The specific objectives are to search for mutations of interest by sequencing mtDNA genes with quasi-maternal inheritance and the impact of these mutations in the D-loop region in healthy and diseased tissues in the patient, but also to learn about the diversity, differentiation and genetic evolution of ovarian cancer in Senegalese women.

Keywords

Cancer, Ovary, Mutations, Epidemiology, D-loop, Senegal

1. Introduction

A generic term applied to a large group of diseases that can affect any part of the body with an uncontrolled proliferation of abnormal cells, cancer is the second leading cause of death in the world with 8.8 million deaths in 2015 and nearly one in

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6 deaths worldwide is due to cancer according to the WHO. About 70% of cancer deaths occur in low- and middle-income countries, and its economic impact is significant and growing. According to the WHO, the total annual cost of cancer to the economy has been estimated at about $1160 billion. It has become a real public health problem and has become a field of research that is becoming increasingly popular.

The global annual incidence and mortality were estimated at 313,959 and 207,252 respectively. As the ovary is a very deep organ, the disease is generally only detected at an advanced stage of malignancy (stage III or stage IV / according to the FIGO classification) and the 5-year survival rarely exceeds 40% despite treatments. In Senegal, ovarian cancer is the 3rd most common cancer in women with an incidence of 5.0/100,000 women.

The family history of ovarian cancer is the main risk factor, through the transmission of susceptibility genes. This is because 10% of ovarian cancer patients inherited a genetic mutation that may have caused [1]. Mutations that occur in cells destined to become eggs are passed on from one parent to their offspring. Indeed, genomic instability is a phenomenon recognized as central to oncogenesis [2]. This also applies to the stability of the mitochondrial genome, which has its own replication and repair machinery [3].

Mitochondria are essential organelles that have their own genome, mitochondrial DNA (mtDNA), present in hundreds or even thousands of copies per cell. Regulating the abundance of this genome is an important issue for the cell, whose dysfunctions are responsible for hereditary and common pathologies, including cancer. The actors involved in mtDNA replication as well as nuclear factors involved in mtDNA biogenesis are known, but the regulators of mtDNA abundance remain unknown (the genes responsible for regulating or maintaining a normal number of mtDNA copies in cells). In fact, these mtDNA abnormalities are the cause of about 20% of mitochondrial diseases.

Subsequently, we will try to evaluate the impact of mutations in the D-loop region in healthy and cancerous individuals through certain parameters such as: genetic diversity indices, genetic distance at the intra- and inter-tissue level, degrees of differentiation (Fst), and analysis of mismatch distribution curves.

2. Materials Et Methods

2.1. Study Population

This study was conducted in patients recruited from the Joliot Curie Institute at Aristide Le Dantec University Hospital. Patients received in this department were referred by community health centres, district hospitals, central hospitals and private clinics in Senegal but also in neighbouring countries. A standardized form was used to collect this information,(demographic characteristics and medical and family history). Regarding the family study, we identified five Senegalese families, each with at least 2 sick individuals. Informed consent was obtained for each of the affected individuals as well as the non-affected individuals who agreed to participate in the study. Due to the anatomical position of the ovary, the removal of a suspicious tissue sample requires surgery. This can be performed, depending on the case, by laparotomy (the patient's belly is open) or by laparoscopy (only small incisions are made in the patient's abdomen).

2.2. DNA Extraction, Polymerase Chain Reaction and Sequencing

First, the Qiagen DNeasy Tissue kit method was used for tissue DNA extractions. Then, we used the Puregene method (Puregene Commercial Kit from the company Gentra) where the samples are incubated at 55°C overnight with 100 µl of Cell Lysis solution and 20 µl of proteinase K. For each sample, the quality of the extracted genomic DNA was verified by electrophoretic migration on agarose gel in a 0.5X buffer (Tris-Base 2M, Acetic acid 1M, EDTA 0.05M) and a solution of Ethidium Bromide (BET: DNA intercalating agent). The gels are then placed under UV and the genomic DNA visualized by fluorescence. DNA size is approximated using a SmartLadder 200 base pair (bp) size marker. The polymerase chain reaction (PCR) is a molecular biology method that rapidly obtains, in vitro, a large number of identical DNA segments from an initial sequence. The PCR is an enzymatic amplification technique that makes to obtain a large number of identical copies of a DNA fragment. PCR required a reaction volume of 50 µL containing 32.9 µL of milliQ water, 5 µL of 10X buffer, 2 µL of dNTP, 2.5 µL for each primer (H408 and L16340), 0.1 µL of Taq polymerase, and 2 µL of cDNA. Briefly, the total DNA was subjected to the following PCR protocol: initial DNA denaturation at 95°C for 15 min, followed by 35 cycles at 95°C for 30 s, 62°C for 30 s, 72°C for 2 min, and final extension to 72°C for 10 min. Positive PCR products were purified and sequenced (See Table 1).

Table 1. *Compositions of the PCR reaction mixture.*

Amplified gene	Composants	Volume
D-loop	MilliQ water	32,9 µl
	Tampon 10X	5 µl
	dNTP	2 µl
	$MgCl_2$	3 µl
	Primers (sense and nonsense)	5 µl
	Polymerase Taq	0,1 µl
	DNA extract	2 µl

The D-loop region was amplified by PCR from tissue ex-

tracts Table 2. PCR products are controlled by electrophoretic migration on 1.5% agarose gel from 5 µl of amplifia and 3 µl of bromophenol blue. The size of the amplified gene is estimated using a SmartLadder 200bp size marker. After UV visualization, the PCR products for which the primers have snagged, are frozen in 1.5 mL Eppendorf tubes for sequencing purposes. Sequencing is the technique of determining the nucleotide sequence of a DNA fragment. It was performed by an American company based in South Korea from 30 µL of PCR products and 15 µL of primer at 10 µM for each sample.

Table 2. *Primer sequence and amplification condition for the PCR reactions.*

Gene	Primer sequence	Amplification conditions
D-loop	H408 (5'TGTTAAAAGTGCATACCGCCA3') L16340 (5'AGCCATTTACCGTACATAGCACA3')	95°C for 15mn; 35 cycles (95°C for 30s, 62°C for 30s, 72°C for 2mn) 72°C for 10mn

2.3. Molecular Analysis

Sequences were verified, aligned-and corrected by BioEdit software version 7.0.5.3 2005 [4] which use using the Clusta W algorithm version 1.4 [5]. A comparison of healthy and cancerous tissue sequences was made with the revised Cambridge reference sequence (NC_012920) [6] in the MITOMAP database for the detection of potential D-Loop variants in ovarian cancer. For our genetic analyses which follow, two groups were formed: healthy tissue and cancerous tissue. The genetic diversity will be determined using DnaSP software version 5.10.01 [7] and MEGA 6 software [8]. The proportion of nucleotide differences per site and the average number of synonymous and non-synonymous substitutions per site using the [9] model, as well as the nature of the mutations, were performed using MEGA 6 software [8]. The number of polymorphic sites and the number of informative sites will also be determined by DnaSP version 5.10.01 [7]. FSTs and their associated probabilities were calculated using Arlequin version 3.0 [10, 11]. A significant P value ($P<0.05$) meant that there was genetic differentiation, and a non-significant P value ($P>0.05$) meant that there was no genetic differentiation. The genetic structure of the populations was apprehended by a hierarchical analysis, analysis of molecular variance (AMOVA). Neutrality tests such as the D of Tajima [12], the D* and F* of Fu and Li, the Fs of Fu [13] was used to test the deviation of the neutrality hypothesis using the Arlequin software version 3.0 [10, 11] and with DnaSP version 5.10.01 [7].

3. Results

A total of 62 sequences with a length of 559 base pairs were obtained after alignment.

Mutations D-Loop

Table 3. *D-Loop variations in ovary cancer.*

Variation	Nature	TS	TC	locus	Associated diseases
C33G	Transversion	1		ATT	
C61T	Transition	2		HVS2/ATT	
C64T	Transition	2		HVS2/ATT	
A73G	Transition	25	35	HVS2/ATT	
T89C	Transition		20	HVS2/ATT	
A93G	Transition	3	24	HVS2/ATT	
A95C	Transversion	2	2	HVS2/ATT	
G97A	Transition		20	HVS2/ATT	
C114G	Transversion	1		HVS2/OH/ATT	
G143A	Transition	3		HVS2/OH/ATT	
T146C	Transition	6	26	HVS2/OH/ATT	Absence of endometriosis

Variation	Nature	TS	TC	locus	Associated diseases
C150T	Transition	10	30	HVS2/OH/ATT	Conflicting reports longevity/cervical carcinoma/risk of HPV infection
C151T	Transition	3	1	HVS2/OH/ATT	
C151G	Transversion	1		HVS2/OH/ATT	
T152C	Transition	15	33	HVS2/OH/ATT	
C182T	Transition	7	31	HVS2/OH/ATT	
G185T	Transversion	3	2	HVS2/OH/ATT	
G185C	Transversion	2		HVS2/OH/ATT	
C186A	Transversion	1	1	HVS2/OH/ATT	
A189C	Transversion	1	1	HVS2/OH/ATT	
A189G	Transition	1		HVS2/OH/ATT	
T195C	Transition	14	32	HVS2/OH/ATT	Melanoma/MB-associated points
A197C	Transversion	1		HVS2/OH/ATT	
C198T	Transition	6	30	HVS2/OH/ATT	
T204C	Transition	4	6	HVS2/OH/ATT	
T204G	Transversion	1		HVS2/OH/ATT	
G207A	Transition	4	2	HVS2/OH/ATT	
G221C	Transversion	1		HVS2/OH/ATT	
Variation	Nature	TS	TC	Locus	Associated diseases
A232C	Transversion	1		HVS2/OH/ATT	
A240T	Transversion	1		HVS2/OH/ATT	
G247A	Transition	3	3	HVS2/OH/ATT	
C256T	Transition	1		HVS2/OH/ATT	
A263G	Transition	27	35	HVS2/OH/ATT	
T279C	Transition	1		HVS2/OH/ATT	
T292A	Transversion	1		HVS2/OH/ATT	
A297G	Transition	1		HVS2/OH/ATT	
C309T	Transition	1		HVS2/OH/ATT	
C315CC	Insertion	26	34	HVS2/OH/ATT	Melanoma patients
G316C	Transversion	1	1	HVS2/OH/ATT	
G316GA	insertion	1	1	HVS2/OH/ATT	
C325T	Transition		26	HVS2/OH/ATT	
C332T	Transition	1		HVS2/OH/ATT	
A357G	Transition	3	2	HVS2/OH/ATT	
T16386A	Transversion		2	ATT	
G16390A	Transition	7	28	ATT	POAG-association potential
C16395G	Transversion	1		ATT	
C16411A	Transversion	1		ATT	
A16421C	Transversion	1		ATT	

Variation	Nature	TS	TC	locus	Associated diseases
T16422C	Transition	1		ATT	
T16468G	Transversion	1		ATT	
T16469G	Transversion	2		ATT	
G16477A	Transition	1		ATT	
C16478A	Transversion	3		ATT	
T16479A	Transversion	1		ATT	
C16501G	Transversion	1		ATT	
C16511T	Transition	1		ATT	
T16519C	Transition	19	27	ATT	
T16522A	Transversion	1		ATT	
A16525G	Transition	1		ATT	
C16527T	Transition	2		ATT	

We obtained 559 base pairs of the D-Loop through the 62 sequences studied, 35 of which were of cancerous origin and 27 of healthy tissue. The study in Table 3 concerning the variations of the D-Loop in ovarian cancer shows that there are 60 variants. They are made up of 96.7% substitutions (55% transition, 41.7% transversion) and 3.3% insertion. The majority of these variations (42/60) are present in the hypervariable HVS2 region, followed by the ATT region (18/60). There are also variations present only in healthy tissues (55% (33/60) (Exp: C33G-G143A-T279C-C16411A-C16527T), which could constitute a protective action against the ovarian tumor, as well as mutations present only in cancerous tissues and which are much less numerous at 6.67% (Exp: T89C-G97A-C325T-T16386A). It should be noted that a variant is only considered a mutation when it is present only in cancerous tissues.

3.1. Indices of Genetic Diversity

The analysis of the Table 4 shows relative values of nucleotide composition with a slight predominance of T and G (cancerous tissue=57.71; healthy tissue=57.66) compared to A and C (cancerous tissue=42.29; healthy tissue=42.34) in both cancerous and healthy tissues. The percentage of transition is higher in cancerous tissues (91.45) than in healthy tissues (75.19) in contrast to transversions which are greater in healthy tissues (24.84) than in cancerous tissues (8.54), and the mutation rate (R) is also higher in cancerous tissues (10.712) than in healthy tissues (3.079). Analysis of the polymorphism revealed high values of haplotypic diversity in both cancerous tissues (0.662±0.085) and healthy tissues (0.997±0.011), and low nucleotide diversity values in both tissues (cancerous tissues=0.00922±0.00175; healthy tissues=0.01539±0.00175). The mean number of nucleotide differences (K) is greater in cancerous tissues (42.581) than in healthy tissues (11,901).

Table 4. *Parameters of genetic variability of the sequences of healthy and cancerous tissues of the D-Loop.*

Parameters	Cancerous tissues	Healthy tissues
Number of sequences	35	27
Number of haplotypic	8	26
Number of sites	559	559
Number of monomorphic sites	536	509
Number of polymorphic sites	23	50
Number of sparing information sites	19	23
Nucleotidic frequence	A G T C	A G T C

Parameters	Cancerous tissues	Healthy tissues
	24.91 30.20 27.51 17.38	24.87 30.20 27.46 17.47
% Transitions	91.45	75.19
% Transversions	8.54	24.84
Rate of Transitions/Transversions=R	10.712	3.079
Haplotypic diversity (HD±SD)	0.662±0.085	0.997±0.011
Nucleotide diversity (Pi±SD)	0.00922±0.00175	0.01539±0.00175
average number of nucleotide differences (K)	42.581	11.901

3.2. Differentiation and Genetic Structuring

The values of genetic distance at the intra- and inter-tissue level and the degree of genetic differentiation (Fst) between healthy and cancerous tissues are shown in the Table 5. The results show that the value of the genetic distance between healthy tissues (0.016) is higher than that observed between cancerous tissues (0.009). The genetic distance between healthy and cancerous tissue is 0.015. The value of genetic differentiation between healthy and cancerous tissues is highly significant.

Table 5. *Genetic distance at the intra- and inter-tissue level and degrees of differentiation (Fst).*

Tissues	Genetic distance intra	Genetic distance inter	Fst (P-value)
Cancerous	0.009±0.002	0.015±0.003	0.333 (P=0.000)
Healthy	0.016±0.003		

3.3. Genetics Demo Tests

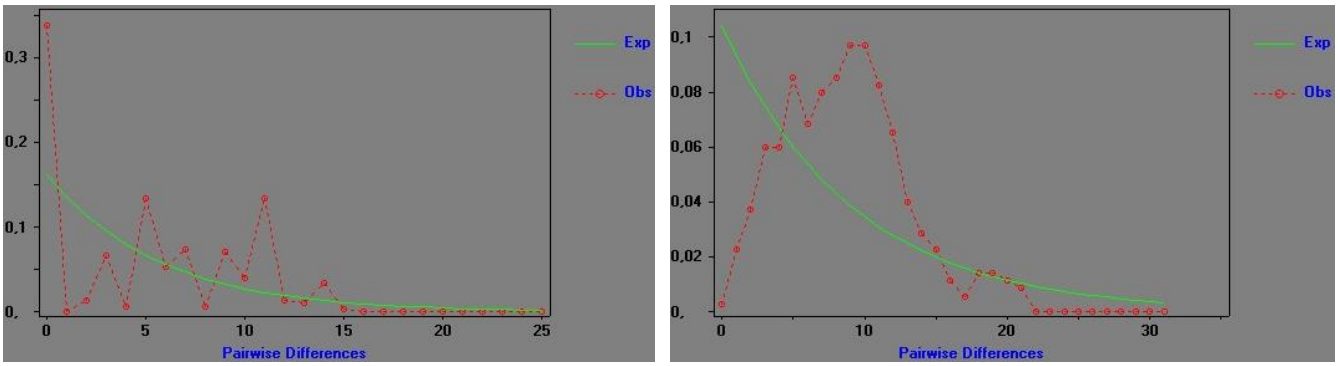
Analysis of the Table 6 shows negative and non-significant statistical values of Tajima's D for both tissues (healthy tissue=-1.278, p=0.081; cancerous tissue=-0.266, p=0.443). There were also negative and significant Fs of Fu values (-19.113, p=0.000), D* (-1.921; 0.10 > P > 0.05) and F* (-2.091; 0.10 > P > 0.05). In contrast, the H value is positive but non-significant (3.442, p = 0.326). In cancerous tissues, it can be seen that the values of Fs of Fu (2.590, p=0.856), D* (0.514 P >0.10) and F* (0.303 P >0.10) are positive and not significant, while for H (-9.400 p=0.337) it is also non-significant but positive.

Table 6. *Genetics demo tests.*

Parameters	Healthy Tissues	Cancerous Tissues
D of Tajima	-1,278	-0,266
P-value	0,081	0,443
Fs of Fu	-19,113	2,590

3.4. Mismatch Distribution Analysis

The mismatch distribution curves show a multimodal pattern for both cancerous and healthy tissue. The SSD and RI demographic indices are not significant.



A B

Figure 1. *Mismatch curve distribution of cancerous (A) and healthy (B) tissues of the D-Loop.*

SSD = 0.0846; *P*-value = 0.16 SSD = 0.0027; *P*-value 0.88
Raggedness = 0.179; *P*-value =0.06 Raggedness = 0.004; *P*-value 0.99

4. Discussion

The aim of this study was to determine the impact of D-loop mutations on ovarian cancer progression. The D-loop is a non-coding system and regulatory region of the mitochondrial genome. MtDNA is the only genetic material in the human genome not contained in the nucleus. In recent years, somatic mutations in mtDNA have been increasingly studied in ovarian cancers and the D-loop region is considered a key location for mutations in human cancer [14]. The control of the mtDNA region is highly polymorphic and contains the hypervariable zone HVS2 characterized by a high degree of polymorphism [15] and most variations (42/60 or 70%). Variations in the D-Loop consist of 91.45% transition and 8.54% transversion in cancerous tissues, and 75.19% transition and 24.84% transversion in healthy tissues. This is in line with the assertion of [16], who showed in their study that the majority of mutations in cancer cells are transitions Variations such as C33G, G143A, T279C, C16411A, and C16527T, present only in healthy tissue, account for 55%, could have a protective action against ovarian tumors. However, the mutations such as T89C, G97A, C325T, and T16386A, present only in cancerous tissue, are much less numerous (6.67%). In our study, the A73G, A93G, C315CC, and T16519C variations identified at the D-loop in 23.33% of tumors have already been described by [17] as polymorphic sites in the human population. Furthermore, according to [18], DNA polymorphism may act as a risk modifier in the late onset of diseases such as cancer. This raises the necessity of knowing whether polymorphism favors the development of mutations [19, 20]. Most somatic mtDNA mutations identified in human cancers (bladder, head and neck, lung, breast, ovary, and esophagus) are found in the hypervariable region of the promoter. Generally, it is concluded that the majority of the variants found are indeed somatically acquired [21]. Considering that polymorphic D-loop sites have a neutral function, [22] suggest that these somatic mutations could have functional consequences in cancer cells. Therefore, this also implies a possible impact of the genetic structure of D-Loop on the mode of mtDNA mutagenesis in human tumors. Some parameters (genetic diversity indices, genetic differentiation and structuring, demo-genetic tests, mismatch distribution analysis) such as the basic parameters of genetic diversity were also studied, showing variation in tumors. Indeed, tumor cells are generally characterized by faster cell proliferation, due to the presence of alterations in genes that regulate proliferation in normal cells [23]. Mutations are the ultimate source of genetic variation that causes diversity in all organisms, thus playing the role of driving evolution [23]. However, according to Moto Kimura's model known as the mutation and random drift theory, most genetic variability is neutral and polymorphisms are eliminated or fixed in individuals under the influence of the effects of genetic drift from the environment [24]. The results of the D-loop genetic distance analysis show that the value of the genetic distance between healthy tissues (0.016) is higher than that observed between cancerous tissues (0.009), which could reflect the genetic difference that may exist between tissues. This finding is further confirmed by the significant genetic differentiation between healthy and cancerous tissues. Analysis of the polymorphism revealed high values of haplotypic diversity in both cancerous tissues (0.662±0.085) and healthy tissues (0.997±0.011), and low nucleotide diversity values in both tissues (cancerous tissues=0.00922±0.00175; healthy tissues=0.01539±0.00175), suggesting rapid development of cancer cells in ovarian tumors. These results follow the Darwinian model of cancer development formulated by [25] according to which a neoplasia originates from a single cell that is the target of mutations that free it from the physiological mechanisms limiting its proliferation. Thus, a malignant tumor is a disease characterized by abnormally large cell proliferation inside a normal tissue of the body, thus threatening the survival of the latter. Tajima's D indices were negative and non-significant for both tissues (healthy tis-

sue=-1.278 p=0.081; cancerous tissue=-0.266 p=0.443); whereas those of Fu Fs have negative and significant values (-19.113 p=0.000) in healthy tissues, and positive and non-significant values (2.590 p=0.856) in cancerous tissues. Therefore, in cancerous tissues, we observe non-significant values of Tajima D and Fu Fs, which allows us to affirm that the mutations found are neutral that is, they are not responsible for a disease [26]. Indeed, for non-coding sequences such as mtDNA control region, a neutrality gap is likely explained by recent demographic changes rather than selection [23]. Analysis of the mismatch distribution curves shows Nucleotide differences between haplotypes taken in pairs leading to a multimodal distribution. This is characteristic of an expanding population.

5. Conclusion

Ovarian cancer, which occurs in the female organs that produce eggs, is the deadliest neoplasia in women worldwide. It is a malignant tumor that affects one or both ovaries. It is a morphologically and biologically heterogeneous disease that has probably contributed to the difficulty of defining the molecular alterations associated with its development and progression. It is the second most common form of cancer in the female reproductive tract and the deadliest of the gynecologic malignancies. Our objective was to evaluate the diversity and genetic evolution of D-loop in ovarian cancer in Senegal. The results of the analyses showed a strong polymorphism in ovarian cancer and that the D-loop region is a highly mutagenic location. Some genetic diversity parameters have also shown variations in tumors that are the ultimate source of genetic variation. Our study also showed a slower proliferation due to a greater genetic distance between healthy tissues than in cancerous tissues. So the study of genetic diversity parameters revealed a variation in tumors. The results also showed a genetic difference between healthy and cancerous tissue.

Studies to identify the mutations that cause ovarian cancer will make a significant contribution to a better understanding of this disease in our population and to the discovery of effective solutions for appropriate and effective treatment and prevention. Further studies with a number of variables such as larger populations, ethnicity, grade and response to chemotherapy and involving the entire mtDNA genome would be needed to better elucidate the problem of ovarian cancer, its mutations, the disease process and the survival rate of patients.

Abbreviations

DNA	DeoxyriboNucleic Acid
WHO	World Health Organization
FIGO	International Federation of Gynecology and Obstetrics
HAH	High Authority of Health
mtDNA	Mitochondria DeoxyriboNucleic Acid
FST	Degree of Genetic Differentiation or Fixation Index
D-LOOP	Displacement Loop
PCR	Polymerase Chain Reaction
DNAsp	DeoxyriboNucleic Acid Sequence Polymorphism
MEGA	Molecular Evolutionary Genetics Analysis
AMOVA	Analysis of Molecular Variance
SSD	Sum of Squares Deviation
RI	Raggedness Index
HVS	Hypervariable Segments

Conflicts of Interest

The authors declare no conflicts of interest.

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