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Ovarian Tumors in Senegalese Women: Impact of D-Loop Mutations between Healthy and Cancerous Tissues

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Abstract

In Senegal in particular, ovarian cancer, which is one of the most common gynecological cancers, accounts for 2.8% of deaths. The most important risk factor is genetic, with 10% of cases occurring in a context of genetic predisposition. The sequencing of the human genome, which has led to the discovery of millions of sequence variations, makes it possible to study variations within sequences. These variations are limited to Single Nucleotide Polymorphisms (SNPs) and this common form of polymorphism occurs approximately every 1000 bases in the human genome and 1.8 million SNPs are currently listed according to [1]. The aim of this study is to gain a better understanding of the impact of mutations in the D-loop region of mtDNA on ovarian cancer in Senegalese women. This study involved searching for mutations in our study population after DNA extraction and sequencing. Mutations were found after a comparison of our sequences with the Cambridge reference sequence (NC_012920). The mutations found in the DNA studied extend from position 7 to position 16568 and most of these mutations are located in the hypervariate zones (HV1 and HV2). Heteroplasmy with three mutant alleles was also found in certain variants. Common mutations were found in both healthy and cancerous tissues, with almost identical frequencies in both types of tissue. This enabled us to understand the spread of tumor cells throughout the ovary.

Keywords

Ovarian, Cancer, Mutations, D-Loop, Heteroplasmy

1. Introduction

Human DNAmt is a double-stranded circular molecule of 16,569 pb with 37

genes, including two rRNAs, 22 tRNAs, and 13 polypeptides [2]. The number of copies of mitochondrial DNA (mtDNA) per cell varies from a few dozen to several thousand, depending on the cell type. DNAmt has a mutation rate 10 times higher than that of nuclear genomic DNA due to inefficient repair systems, exposure to mutagenic oxygen radicals resulting from oxidative phosphorylation [3] and the absence of protective histones. Links between DNAmt mutations and neurological or metabolic disorders have been reported [4]. The D-loop measures 1124 bp (pos. 16024-576) and acts as a promoter for DNAmt strands. It contains transcription and replication elements. It is also vulnerable to DNAmt alterations and includes the hypervariable (HV) regions HV1 (pos. 16024-16383) and HV2 (pos. 57-372). Sequence analysis of these two regions is used in forensic analysis and medical diagnosis [5].

Multiple DNAmt mutations have been reported in human cancers [6]. After researching variations in the D-loop of DNAmt, [7] found mutations involved in colon and stomach tumors. Other researchers have studied D-loop fluctuations in different cancers [8]. Sequencing of the human mitochondrial genome has revealed DNAmt-encoded genes and their implications for cancer [9]. Ovarian cancer is one of the most common diseases in Senegal, with a mortality rate of 2.8%, and is caused by a number of factors, including mutations in the D-loop region of mtDNA. In fact, the DNAmt gene D-loop is considered a hotspot for mutations in various types of tissues and cancer cell lines [10]. In 2001, [11] observed that DNAmt deletions were the first mutations linked to mitochondrial diseases. Numerous researchers have studied the impact of DNAmt mutations on ovarian cancers-notably [12], who determined that of 27 somatic mutations, 22 were in the non-coding region of the mitochondria known as the D-loop. These mutations in the D-loop region are associated with a number of factors, including tumors without estrogen and progesterone receptor expression. In general, mutations in the D-loop region are considered to be non-clinical markers.

2. Methods

2.1. Samples

After cleaning and aligning our forty-nine study sequences, they were analysed and a large number of mutations were identified. The tissues were preserved in 96% alcohol and brought to the laboratory where they were stored at 20˚C for subsequent molecular analysis.

2.2. Genetic Analysis

2.2.1. DNA Extraction and Amplification of the D-Loop Gene with Sequencing

Total DNA from the tissues was extracted using the Qiagen protocol, following the steps and protocol for using the kit. The sequences were amplified using primers 5'TGTTAAAAGTGCATACCGCCA3'and 5'AGCCATTTACCGTACATAGCACA3' of DNA that bind specifically to a target of the DNA polymerase enzyme that copies the target DNA with dNTPs and

Mg^{2+} ions. This process was repeated approximately 30 times to make the target more detectable. Sequencing uses a specific PCR reaction that, in addition to the usual compounds (template DNA, polymerase, primer, dNTPs and Mg^{2+}), uses modified nucleotides called ddNTPs, that are coupled to fluorochromes (ddATP-green, ddTTP-red, ddCTP-blue and ddGTP-yellow) and have an OH group at the 3' end of the deoxyribose. Sequencing involves randomly fragmenting the genome into pieces of DNA with a few thousand base pairs for easier manipulation. In this procedure, ddNTPs are incorporated by a polymerase to block elongation of the complementary DNA during copying, as the nucleotides cannot form phosphodiester bonds with each other due to the absence of a hydroxyl group on the 3' carbon.

2.2.2. Observation of Mutations

The sequences were cleaned and aligned using Bioedit version 7.1.9 [13], which uses the Clustal W algorithm [14]. This software was used to check, correct and align the sequences, which is a crucial step in the analysis of these data. After cleaning and alignment in Bioedit, the file was imported into Geneious Prime [15] for output of the variants. Using Geneious Prime [15], it was possible to identify similarities between sequences by finding the positions of likely insertion-deletions. Sequences from healthy and diseased individuals are compared to the Cambridge reference sequence NC_012920.1; [16] [17] to identify the different variants of Dloop in the ovary, and each variant found is considered a polymorphism. The MITOMAP database provides information on germline and somatic function, polymorphisms, and mutations associated with disease [18], and variants not in the database are considered novel.

3. Results and Discussion

3.1. Nature of D-Loop Mutations

After cleaning, alignment, and elimination of certain sequences because they were not clean, 49 were analyzed and many mutations were identified. The reliability of the sequences was checked using nucleotide blasts with the Cambridge reference sequence number (NC_012920.1). On the portion of DNA studied, which extends from position 7 to position 16568, most mutations were found in the HV2 zones, with 21 variants (68T > C, 73A > T, 86A > G, 109G > A, 118C > T, 125T > A, 133T > G, 144C > G, 158C > T, 161A > GC, 167G > A, 168T > G, 170G > A, 176T > CG, 179G > T, 180C > AGT, 182T > C, 183G > A, and 212A > GC) and HV1 with 18 variants (16029G > T, 16030T > C, 16031G > A, 16032C > T, 16036A > G, 16052T > C, 16062A > G, 16063T > G, 16084T > G, 16129G > T, 16246G > A, 16277G > A, 16311G > T, 16344T > C, 16345G > A, 16347T > C, 16350A > G, and 16375G > C). Of the variants, 38 are new and these new mutations include heteroplasmic mutations. A high mutational frequency was noted in eight variants, namely 170G > A (24.15)%, 183G > A (22)%, 213G > A (22.65)%, 215G > A (24.15)%, 219A > G (20.36)%, 270T > G (20.16)%, 415G > A (22.65)%, and 16393C > T (22.53)%. For these variants with a high mutation

frequency, most already exist except for three that are new (170G > A, 213G > A and 270T > G), and all are transitions except for one (270T > G). Among the heteroplasmy type variants, two have a mutation found on three alleles instead of two (180C > AGT, and 231C > AGT), which are located in HV2. (Table 1)

Table 1. Nature and impact of D-loop mutations.

Variants	Nature	Type	Frequency%	Locus	Mitomap
7T > C	T	Homo	9.15	RC	New
32G > A	T	Homo	1.96	RC	New
39G > A	T	Homo	1.96	RC	New
48C > T	T	Homo	3.93	RC	Exists
56G > A	T	Homo	1.96	RC	New
68T > C	T	Homo	1.96	HV2	New
73A > T	V	Homo	1.96	HV2	Exists
86A > G	T	Homo	1.96	HV2	New
109G > A	T	Homo	1.96	HV2	Exists
118C > T	T	Homo	10.7	HV2-OHR	New
125T > A	V	Homo	1.96	HV2-OHR	Exists
133T > G	V	Homo	1.96	HV2-OHR	New
144C > G	V	Homo	1.96	HV2-OHR	New
158C > T	T	Homo	9.15	HV2-OHR	New
161A > GC	T	Hetero	0.23	HV2-OHR	New
167G > A	T	Homo	19.4	HV2-OHR	New
168T > G	V	Homo	1.96	HV2-OHR	New
170G > A	T	Homo	24.15	HV2-OHR	New
176T > CG	T	Hetero	0.22	HV2-OHR	New
179G > T	V	Homo	3.93	HV2-OHR	New
180C > AGT	V	Hetero	0.006	HV2	New
182T > C	T	Homo	1.96	HV2-OHR	New
183G > A	T	Homo	22	HV2-OHR	New
212A > GC	T	Hetero	0.23	HV2-OHR	New
213G > A	T	Homo	22.65	CSB1	New
214G > AC	T	Hetero	0.14	CSB1	New
215G > A	T	Homo	24.15	CSB1	New
218G > AT	T	Hetero	0.37	CSB1	New
219A > G	T	Homo	20.36	CSB1	Exists
222C > T	T	Homo	7.43	CSB1	New
227T > GC	V	Hetero	0.31	CSB1	Exists
231C > AGT	V	Hetero	0.009	HV2-TFX	New

Continued

251G > C	V	Homo	1.96	TFX	Exists
268C > T	T	Homo	1.96	HV2-OHR	Exists
269G > A	T	Homo	1.96	HV2-OHR	New
270T > G	V	Homo	20.16	HV2-OHR	New
272T > C	T	Homo	1.96	HV2-OHR	New
276A > G	T	Homo	1.96	TFY	New
279G > A	T	Homo	1.96	TFY	New
287G > A	T	Homo	1.96	TFY	New
288T > A	V	Homo	1.96	TFY	New
289G > A	T	Homo	1.96	TFY	New
292C > T	T	Homo	5.72	TFY	New
299C > T	T	Homo	1.96	CSB2	Exists
301G > A	T	Homo	3.93	CSB2	New
302G > AC	T	Hetero	0.03	CSB2	New
304G > T	V	Homo	1.96	CSB2	New
309T > A	V	Homo	1.96	CSB2	New
310A > C	V	Homo	1.96	CSB2	New
311G > A	T	Homo	1.96	CSB2	New
332G > C	V	Homo	12.04	HV2-OHR	New
346G > T	V	Homo	1.96	CSB3	New
347G > C	V	Homo	1.96	CSB3	Exists
350G > A	T	Homo	1.96	CSB3	New
362C > TA	T	Hetero	0.11	CSB3	New
383A > C	V	Homo	1.96	OHR	New
386G > A	T	Homo	1.96	OHR-3H	New
390A > G	T	Homo	1.96	OHR-3H	Exists
392G > T	V	Homo	1.96	OHR-LSP	New
403T > C	T	Homo	1.96	OHR-LSP	Exists
404T > A	V	Homo	1.96	OHR-LSP	New
406G > AC	T	Hetero	0.03	OHR-LSP	New
407G > A	T	Homo	1.96	OHR-LSP	New
409T > C	T	Homo	1.96	OHR-LSP	New
415G > A	T	Homo	22.65	OHR-LSP	New
456G > TC	V	Hetero	5.72	HV3	New
457C > T	T	Homo	1.96	HV3	Exists
465A > C	V	Homo	3.93	HV3	New
16029G > T	V	Homo	0.99	HV1	New

Continued

16030T > C	T	Homo	0.99	HV1	New
16031G > A	T	Homo	0.99	HV1	New
16032C > T	T	Homo	0.99	HV1	New
16036A > G	T	Homo	0.99	HV1	New
16052T > C	T	Homo	0.99	HV1	Exists
16062A > G	T	Homo	1.96	HV1	Exists
16063T > G	V	Homo	1.96	HV1	New
16084T > G	V	Homo	0.99	HV1	Exists
16129G > T	V	Homo	1.96	HV1	Exists
16246G > A	T	Homo	0.99	HV1	Exists
16277G > A	T	Homo	0.99	HV1	New
16311G > T	V	Homo	0.99	HV1	Exists
16344T > C	T	Homo	0.99	HV1	Exists
16345G > A	T	Homo	0.99	HV1	Exists
16347T > C	T	Homo	1.96	HV1	Exists
16350A > G	T	Homo	1.96	HV1	Exists
16375G > C	V	Homo	1.96	HV1	New
16392C > T	T	Homo	0.99	7SDNA	New
16393C > T	T	Homo	22.53	7SDNA	Exists
16440A > T	V	Homo	4.83	7SDNA	Exists
16505A > T	V	Homo	0.99	7SDNA	Exists
16537G > C	V	Homo	0.99	7SDNA	New
16568G > T	V	Homo	0.99	7SDNA	New

3.2. Detection of Mutations Common to Both Types of Tissue

In order to check whether the control tissues are truly healthy, we have tried to see if there are mutations common to both types of tissues. Yielded the results listed in Table 2 below. The G183A, C180A, C222T and G39A variants are the most common in both tissue types. The G183A mutation is much more present in healthy tissues than in cancerous tissues, while the other three mutations (C180A, C222T and G39A) are present with practically the same frequency in both types of tissues.

3.3. Discussion

Presently, many factors linked to the study of genetic predisposition to certain diseases remain unknown. Some of these diseases are complex because they result from a number of factors, such as the environment or a person's lifestyle. Ovarian cancer is an example of pathological complexity due to several factors. This study focuses on the DNAmt D-loop gene, which is a specific region of

Table 2. Common mutations found in both types of tissue.

Tissue	Common mutations
TC-17, TC-18, TC-21, TC-27, TC-28, TC-29, TC-32, TC-34, TS-21, TS-31, TS-33, TS-43, TS-53, TS-69, TS-73, TS-7,	G183A
TC-17, TC-18, TS-21, TS-31, TS-33, TS-53, TS-69, TS-71	C180A
TC-16, TS-45, TS-47, TS-9	C222T
TC-21, TS-7	C48T
TC-27, TS-49, TS-9	T270G
TC-28, TC-32, TC-34, TS-53,	G39A
TC-32	A276G
TS-17, TS-61	A465C
TS-55	G423A
TS-55	C457T

DNA present in mitochondria, the only cellular organelles to have DNA outside the nucleus in eukaryotes. Their central role in the cell is ensured by oxidative phosphorylation and the production of oxygen radicals. Mutations in DNAmt may lead to dysfunction that can promote the development of tumors [19]. DNAmt has a double strand of 16569 base pairs and contains a non-coding region called the regulatory region that is responsible for DNAmt transcription.

In this study of genetic characterization in ovarian cancer, we analyzed 49 nucleotide sequences from 28 healthy tissues and 21 cancerous tissues with a total of 559 base pairs. Mutations were identified, mainly in the HV region. The gene studied, called D-loop, is polymorphic. Genetic analysis revealed 96 mutations, 38 of which are novel. The study by [20] supports this finding. Some experts believe that many of the mutations that cause carcinomas are found in the D-loop region of DNAmt. DNAmt is a closed-loop molecule composed of two strands that contain the D-loop region, which plays a regulatory role in replication and transcription. Numerous studies have confirmed that the HV region of the D-loop concentrates most DNAmt mutations. Mutations in the HV region can lead to tumor aggressiveness because, as stated by [21], the HV region may be responsible for the overproduction of reactive oxygen species (ROS), aberrant energy expenditure or the production of autoantigens, thus testifying to the malfunctioning of the mitochondria. This is supported by [22] who refer to the excess of ROS in mitochondria which can disrupt the multiple pathways involved in calcium homeostasis, mitochondrial permeability and cytochrome C release.

Our study confirms this finding, with 89% of mutations found in this same HV region. Our research on the D-loop region enabled us to detect 10 synonymous mutations in healthy and cancerous tissues. The C180G, G183A, C222T and T270G variants were found with high frequency in both types of tissue. This finding points to the hypothesis that breast-fed individuals are predis-

posed to ovarian cancer. Additionally, in terms of the mutations found, some are present in both types of tissue and at high frequencies, (e.g., the G183A, C180A, C222T, and G39A variants). There are mutations that appear in both tissues but are much more frequent in healthy tissues, (e.g., as G183A). This is what has enabled us to assert that the expansion of tumor cells reached most of the supposedly healthy tissues used in our study. [22] supports our claim by stating that, over time, cancer cells accumulate abnormalities and develop new properties for their growth. And these newly acquired properties can have an impact on cell meiosis, involving a disruption in multiplication and a blockage of apoptosis. As a result, within the surrounding healthy tissues, cells resulting from this meiosis begin to carry the genetic modifications that are the result of the accumulation of abnormalities. This is supported by [23] who speak of the acquisition of malignancy from normal cells as being the cumulative effect of multiple genetic modifications that accumulate during the evolution of a cell. If the damage caused by the excess cell multiplication is not limited, the mutations may reach other sites or organs. This is why the question of targeted treatment in the HV region seems to make sense. These abnormalities can progressively invade all the tissues of the organ of origin and spread to neighbouring tissues, making cancer invasive.

4. Conclusion

Genetic characterization led us to obtain mutations mostly found in the HV zone. Most of these mutations were found in both types of tissue in this study, showing that the diseased tissue had invaded most of the ovary. These factors lead to dysfunction of the D-loop gene, and the appearance of ovarian tumors.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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