

# Involvement of D-Loop Mutations in the Occurrence of Ovarian Carcinoma

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## Abstract

Cancer mortality is proportionally higher in Africa than elsewhere in the world. In Senegal, ovarian cancer is responsible for 2.8% of deaths and is one of the most fatal gynaecological cancers. This work is therefore being carried out in order to better understand the impact of D-Loop mutations in the evolution of ovarian cancer in Senegalese women. The genetic variability of D-Loop was studied by PCR-Sequencing, in thirty-eight (38) patients. The results revealed twenty-two (22) mutations. In this study, eleven (11) mutations (A66G, C186T, G309A, C388T, C418T, G421T, C422A, A425C, A425G, C431T and G499A) were identified as somatic mutations in ovarian cancer. All the variants are present in the Mitomap database. A variation (19.05%) in the length of the first section of the D310 area was found. The results obtained in this study show that the D310 region of the D-Loop is not the “hot spot” for mtDNA mutations in ovarian cancer in Senegalese women.

**Keywords:** Cancer, Ovary, Displacement Loop, Mutations

## Introduction

Each cell in the body contains hundreds of mitochondria; each mitochondrion contains several DNA molecules, each carrying 37 genes that participate in the production of energy. The mitochondria are organelles that function in a semi-autonomous manner, containing their own genome and are capable of replication and transcription. They are responsible for more than 90% of cellular ATP production through the process of oxidative phosphorylation [1]. Studies on mitochondria have shown that alterations in mtDNA are closely linked to hereditary diseases, ageing and cancer [2]. Indeed, the involvement of mitochondria in apoptosis and probably in tumorigenesis has raised interest in the potential role of mtDNA mutations in the development of cancers [3].

Despite the small size of the mitochondrial genome, mtDNA mutations are an important cause of hereditary diseases. Because mitochondria lack protective histones or highly efficient DNA repair mechanisms, mammalian mtDNA accumulates at a rate 10 times higher than nuclear DNA [4]. In recent years, considerable progress has been made in understanding mitochondrial genetics and identifying mutations in mtDNA acquired in cancers [5].

For example, genetic mapping and cloning of chromosomal “oncogenes” has revealed that some cancers are caused by mutations in genes of nucleus-encoded mitochondrial enzymes [6]. These alterations are particularly frequent in pre-neoplastic lesions and in human cancers, particularly in breast, ovarian, colon and rectal, stomach, liver and oesophagus cancers [7].

Cancers are among the leading causes of morbidity and mortality worldwide. More than 60% of new cancer cases occur in Africa, Asia, Central and Latin America. Cancer mortality is proportionally higher in Africa than elsewhere in the world [8]. Cancers already account for 10-20% of pathologies on the African continent [8]. Thus, ovarian cancers are among the gynecological cancers of women and have a high mortality and morbidity rate worldwide and particularly in Africa [9]. In Senegal, 260 new cases of ovarian cancer have been detected and the mortality rate has been estimated at 2.8% [10].

In addition to the degenerative diseases that dominate the health picture in our societies, cancer, or rather cancers, remains one of the most complex challenges facing medicine in terms of understanding these pathologies, diagnosis and treatment. It is in this sense

that this study aims to understand the impact of D-Loop mutations in the evolution of ovarian cancers.

## Materials and Methods

### Subjects

The study was approved by the University Cheikh Anta Diop Research Ethics committee. Informed consent was obtained from all subjects prior to enrollment. The tissues samples were derived from thirty-eight (38) patients. These tissues were obtained following surgery performed at the cancerology department of the Joliot Curie Institute of the Aristide Le Dantec Hospital in Dakar. They were preserved in 96% alcohol. Blood samples were taken from healthy control subjects (N=10).

### DNA isolation, amplification and sequencing

The extraction of DNA from the tissue and blood was performed using the Kit Qiagen Dneasy method. The PCR required a reaction volume of 50  $\mu$ l containing 34.9  $\mu$ l milliQ water, 5  $\mu$ l buffer 10X, 2  $\mu$ l dNTP, 2.5  $\mu$ l for each primer (H408 and L16340), 0.1  $\mu$ l Taq polymerase and 2  $\mu$ l cDNA. Briefly, 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 a final extension at 72°C for 10 min. Positive PCR products were purified and sequenced. Sequencing reactions were performed in an MJ Research PTC-225 Peltier thermal cycler with the ABIPRISM Kit and subjected to electrophoresis in the ABI3730 XL sequencer.

### Identification of somatic mutations

The resulting sequences were cleaned, corrected and aligned with Bioedit software version 7.1.9 [11] using the ClustalW algorithm [12]. Alignment is indeed an important step in the data analysis, as it determines the homology of the sites. The sequences of the diseased individuals were compared with those of the controls in order to identify the different variants of D-Loop in ovarian cancer. Any variant that is present in the controls is considered a polymorphism. The variant is considered a mutation if it is present only in patients. We consulted the mitochondrial database MITOMAP ([www.mitomap.org](http://www.mitomap.org)) which provides users with detailed information on sequence, function, polymorphisms and mutations in germ and somatic lines associated with disease states [13]. Variants not found in the database have been recorded as new mutations in ovarian cancer. This test is used to verify the difference in the probability of mutation in populations with and without cancer [14]. To highlight the polymorphism of the D310 region, we performed a comparison of this region between controls and patients. Significance testing is performed by the  $\chi^2$  test with Statview software version 5.0 (Roach, 2004). For all tests performed, the level of significance is 5%.

### Polymorphism and genetic diversity

For this analysis, two groups were formed: healthy tissue and cancerous tissue. For each group, all the basic parameters of genetic variability: number of sites (N), sample size (n), number of variable sites, number of haplotypes (h), mean number of nucleotide differences (k) between sequences were estimated using DnaSP version 5.10 software [15]. However, the nucleotide frequencies of D-Loop in controls and patients, the nature of mutations (percentage of transitions and transversions), and the mutation rate (R) were calculated using MEGA7 version 7.0.14 [16].

### Genetic differentiation

The estimation of genetic differentiation between control and patient sequences generally requires two indices, genetic distance (D) and FST. Genetic distance is determined within each population (intra-population genetic distance) and between populations taken in pairs (inter-population genetic distance). Inter-population TST values were evaluated using the Harlequin program version 3.5.1.3 [17]. Intra- and inter-population genetic distances were derived using MEGA7 version 7.0.14 [16].

### Neutrality tests

In order to study the genetic evolution of tumour D-Loop mutations, the indices of haplotypic diversity (hd) and nucleotide diversity (Pi) [18] and their variance were calculated with DnaSP version 5.10 [15]. The indices of the D of Tajima [19], the D\* and F\* of Fu and Li, and the Fs of Fu [20] were used to test the deviation from the neutrality hypothesis using Arlequin software version 3.5.1.3 [17] and with DnaSP version 5.10 [15]. The Mismatch analysis combines two indices, SSD (sum of deviation squares) and Rag (irregularity index), which test the goodness of fit of the distribution. The graphs are constructed using DnaSP version 5.10 [15]. The SSD and Rag indices are estimated using Arlequin software version 3.5.1.3 [17]. The purpose of these tests is to distinguish sequences, whose evolution follows a neutral evolutionary model, and those evolving according to a non-random process.

## Results

### D-Loop somatic mutations

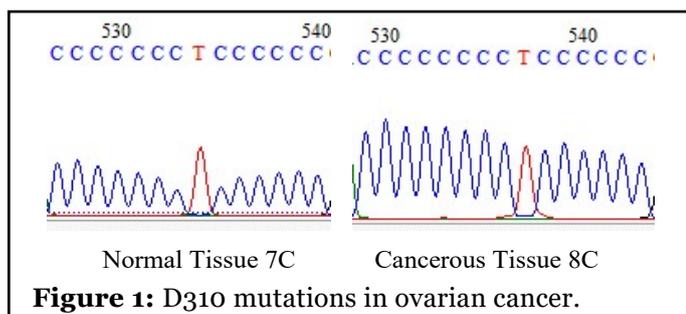
Table 1 shows the positions and frequencies of the D-Loop mutations. We first verified the reliability of the sequence using the Nucleotide Blast algorithm with the Cambridge reference sequence under accession number (NC\_012920) in order to list the mutation positions at the mitochondrial genome level in the Mitomap database. The portion of DNA studied extended from position 16390 to position 358 and the majority of mutations were found in the hypervariable zone (HV2)

Mutations	Type	Controls (%)	TC (%)	rRCS	Locus	Mitomap	Associated Diseases	Ref	P-value
A57G	Ts	10	0	16390	ATT	+	Breast cancer, ovarian tumour	Ref	0.003*
A66G	Ts	0	4.76	16399	ATT	+	Gastric carcinoma	Ref	0.0015*
C186T	Ts	0	38.09	16519	ATT	+	Ovarian tumors, prostate	Ref	0.102
G309A	Ts	0	4.76	73	HV2	+	Thyroid tumors, prostate	Ref	0.0015*
T325C	Ts	0	4.76	89	HV2	+	-	-	0.0015*
A329G	Ts	10	14.38	93	HV2	+	-	-	-
A331C	Tv	0	4.76	95	HV2	+	-	Ref	0.0015*
G379A	Ts	0	4.76	143	HV2	+	-	-	0.0015*
C382T	Ts	10	66.67	146	HV2	+	Ovarian Tumor	Ref	-
C386T	Ts	10	42.86	150	HV2	+	Thyroid tumors, lung tumors	Ref	-
C387T	Ts	0	4.76	151	HV2	+	-	-	0.0015*
C388T	Ts	0	23.81	152	HV2	+	Ovarian tumor, breast tumor	-	0.006*
C418T	Ts	0	38.09	182	HV2	+	-	Ref	0.102
G421T	Tv	0	9.52	185	HV2	+	-	Ref	0.002*
C422A	Tv	0	4.76	186	HV2	+	-	Ref	0.0015*
A425C	Tv	0	9.52	189	HV2	+	-	Ref	0.002*
A425G	Ts	0	4.76	189	HV2	+	-	Ref	0.0015*
C431T	Ts	0	33.33	195	HV2	+	Tumors of the lung, ovary	Ref	0.009*
C434T	Ts	0	28.57	198	HV2	+	-	-	0.008*
T440C	Ts	0	14.28	204	HV2	+	-	Ref	0.004*
G443A	Ts	0	4.76	207	HV2	+	Oral tumors, thyroid tumors	Ref	0.0015*
G483A	Ts	0	14.28	247	HV2	+	-	-	0.004*
G499A	Ts	0	4.76	263	HV2	+	-	Ref	0.0015*
G554A	Ts	0	4.76	317	HV2	+	Ovarian Tumor	Ref	0.0015*
C563T	Ts	0	19.05	326	HV2	+	-	-	0.005*
A595G	Ts	0	9.52	358	HV2	+	-	-	0.002*

+: indicates that the mutation is present in Mitomap, -: indicates that the mutation was not found in Mitomap, \* : indicates a significant value, Ref: indicates that the variant is referenced, Ts: Transition, Tv: Transversion

**Table 1:** Somatic mtDNA mutation in ovarian cancer.

and only the A66G and C186T mutations were located on the MT-ATT locus. Analysis of the nucleotide sequences revealed twenty-six (26) variations. However, the variant is only considered a mutation when it is present only in cancerous tissues, therefore twenty-two (22) mutations were detected with more transitions (18) than transversions (4). The A57G variant is the only one present in controls with a significant P value (0.003). In this study, eleven (11) mutations (A66G, C186T, G309A, C388T, C418T, G421T, C422A, A425C, A425G, C431T and G499A) out of twenty-two (22) or 50% of the variants were identified as somatic mutations in ovarian cancer, no new mutation was found because all the variants are present in the Mitomap database. All variants show significant differences between controls and cancerous tissues with a P value less than 0.05 except for the C186T and C418T mutations which are non-significant with a P value greater than 0.05. In cancerous tissues, there are four individuals (19.05%) with cytosine elongation in the D310 region, thus having eight (8) cytosines instead of seven (7) as observed in controls (Figure 1).



### Genetic diversity of D-Loop

Table 2 shows the different parameters of the genetic diversity of controls and cancerous tissues. It was noted that the number of variable sites is higher in cancer tissues (23) compared to controls (4). Nucleotide frequencies show that C and A bases are more frequent with 30.2% and 27.6% for controls and 29.8% and 27.6% for cancerous tissues respectively. Transitions are higher in controls (99.98%) than in cancerous tissues (87.51%). No transversion was noted in the controls in contrast to the cancerous tissues with 12.48% of transversions and the ratio of transitions to transversions was estimated to be 7.01.

### Genetic Differentiation of D-Loop

The results show that the value of the genetic distance between cancerous tissues (0.009) is greater than that observed between controls (0.001). The genetic distance between controls and cancerous tissues is 0.007 closer to that observed between cancerous tissues. The degree of genetic differentiation (0.219) is significant (P=0.023).

### Genetic evolution of D-Loop

Table 4 shows a high haplotypic diversity (Hd=0.922) and a low nucleotide diversity (Pi=0.017) for cancerous tissues. The statistical values of Tajima's D (-0.064) and Fu and Li D\* (-0.041) appear negative and non-significant while those of FU Fs (0.305) and F\* Fu and Li (0.173) are positive and also non-significant with P values greater than 0.05.

	Controls				Cancerous tissues			
Number of sites	662				662			
Number of sequences	10				21			
Variable number of sites	4				23			
Number of haplotypes	2				15			
Mean number of nucleotide differences	0.800				5.829			
Nucleotide frequencies	T	C	A	G	T	C	A	G
	25.4	30.2	27.6	16.8	25.8	29.8	27.6	16.8
Transitions (%)	99.98				87.51			
Transversions (%)	00				12.48			
R(Ts/Tv)	-				7.01			

**Table 2:** Parameters of genetic diversity of controls and cancerous tissues.

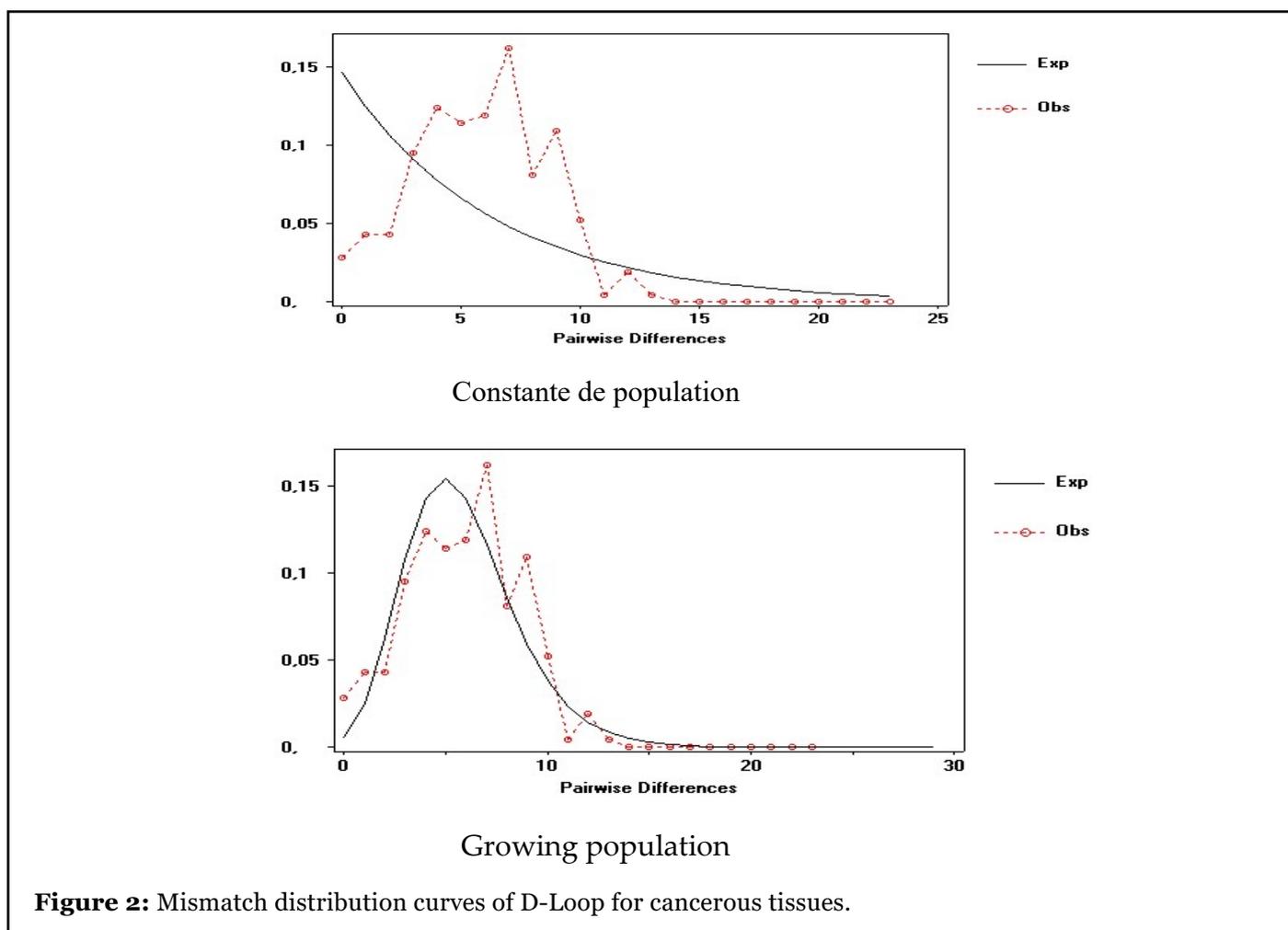
	Within-population genetic distances	Between-population genetic distances	Fst
Controls	0.001	0.007	0.219 P value=0.023
Cancerous tissues	0.009		

**Table 3:** Genetic Differentiation indices of D-Loop.

Hd	Pi	Tajima D	FU Fs	D*	F*
0.922	0.017 ± 0.008	-0.064 ± 0.552	0.305 ± 0.463	-0.041 ± 0.462	0.173 ± 0.506

**Table 4:** Neutrality tests.

Graphical representations of the genetic distances between cancerous tissues of individuals taken in pairs, assuming a constant or expanding population, show a multimodal distribution. The SSD (0.0025; P=0.072) and Rag (0.045; P=1.30) indices with non-significant P values show a growing population at the cancer tissue level.



## Discussion

Our study focuses on D-Loop, which is the non-coding and regulatory region of the mitochondrial genome. Its general objective is to determine the impact of D-Loop mutations on the evolution of ovarian cancers. The control region of the mtDNA is highly polymorphic and contains the hypervariable HV2 zone characterized by a high degree of polymorphism [21]. It is in the latter that most of the variants (88.46%) found in this study is located. 81.82% and 18.18% of the mutations are transitions and transverse respectively. This is in line with the assertion of Li and Hong [22], who showed in their study that the majority of cancer cell mutations are transition-type base substitutions. The A57G variant is the only one present in controls with a significant P value (0.003). It could therefore have a protective action against ovarian tumor as well as the C186T and C418T mutations present in TCs but not significantly. Mutations (A66G, G309A, T325C, A331C, G379A, C387T, C388T, G421T, C422A, A425C, A425G, C431T, C434T, T440C, G443A, G483A, G499A, G554A, C563T and A595G) are present only in cancerous tissues with significant P values. They may play an important role in the development of ovarian tumors. In this study eleven (11) mutations (A66G, C186T, G309A, C388T, C418T, G421T, C422A, A425C, A425G, C431T and G499A) out of twenty-two (22) or 50% of the variants were identified in Mitomap as somatic mutations in ovarian cancer. These results are in agreement with those of Bragoszewski et al. [7] who showed in their study the presence of somatic D-Loop mutations in 50% of ovarian cancer tissue samples, and most of the samples carried a single mutation. These results are consistent with previous reports describing somatic D-Loop mutations in 20-78% of human cancers. Most of the 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 actually acquired somatically. According to Chen et al. [23], assuming that the polymorphic sites of D-loop have neutral function, these somatic mutations could confer functional consequences on cancer cells. Therefore, this also implies a possible impact of the genetic structure of D-Loop on the mode of mutagenesis of mtDNA in human tumors.

The polymorphism of the D310 region was also studied in this study. The microsatellite (D310) located between positions 303-315 of the D-Loop is used as a potential marker for early cancer detection [24]. 19.05% of sick individuals have a cytosine insertion, therefore, we can hypothesize that cytosine elongation could be a risk factor for developing ovarian cancer. Indeed, the hot spot for instability has a unique sequence pattern between nucleotides 303 and 316 (CCCCCCTCCCCC), and instability has always occurred in the first polymorphic C tract, previously called D310, which is particularly rich in

somatic mutations in several types of cancer [1]. It should be noted that in a previous study, this mutation hot spot was considered a major target for mtDNA alterations in human tumors [23]. The occurrence of mutations in this single nucleotide sequence could therefore alter this regulatory function and increase the replication rate of the mtDNA, thus generating a replicative advantage at the level of the mutated mitochondria that is responsible for an accumulation of mutations during successive divisions [1].

Parameters such as the basic parameters of genetic diversity have also been studied showing tumor variation. Indeed, tumor cells are generally characterized by faster cell proliferation, due to the presence of alterations in the genes that regulate proliferation in normal cells [25]. It is in fact mutations that are the ultimate source of genetic variation that cause diversity in all organisms, thus playing the driving role in evolution [25]. However, according to Moto Kimura's model known as the theory of mutation and random drift, 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.

Analysis of the genetic differentiation of D-Loop revealed that the genetic distance between cancerous tissues (0.009) is greater than that observed between controls (0.001). The proliferation of cancer cells appears to be much faster, also indicating tumor homogeneity. In contrast, the inter-population genetic distance (0.007) between controls and cancerous tissues is closer to that of cancerous tissues. The degree of genetic differentiation ( $F_{st}$ ) has a significant P value (0.023) indicating genetic differentiation between groups.

The study of the genetic evolution of D-Loop mutations in ovarian cancer in Senegalese women is marked by high polymorphism with high haplotypic diversity ( $H_d=0.922$ ) and low nucleotide diversity ( $P_i=0.017$ ) in cancerous tissues. D-Loop mutations are therefore believed to grow rapidly in ovarian tumors. Cancer cells contain many clonal mutations, i.e. mutations that are present in most or all malignant cells in a tumor and they confer a proliferation advantage. Thus, a malignant tumor is a disease characterized by abnormally high cell proliferation within normal body tissue, such that the survival of the body is threatened. Tests of the neutrality of cancerous tissues have shown that the D of Tajima and the  $D^*$  of Fu and Li are negative and insignificant, which makes it possible to affirm that the mutations found are neutral. Indeed, for non-coding sequences such as the mtDNA control region, a discrepancy in neutrality is probably explained by recent demographic changes rather than by selection [25]. Moderate population growth in cancerous tissues is also confirmed by the multimodal distribution of mismatch curves.

## Conclusion

Ovarian cancer is a morphologically and biologically heterogeneous disease which has probably contributed to the difficulty in defining the molecular alterations associated with its development and progression. It is the second most common form of cancer in the female reproductive system and the most lethal of the gynecological malignancies. For decades, considerable efforts have been made to shed light on the molecular basis for the initiation and progression of ovarian carcinoma. Our objective was to evaluate the involvement of D-Loop mutations in ovarian cancers in Senegalese women. Our study revealed a strong polymorphism in ovarian cancer in Senegalese women. The results revealed mutations with significant P values, which could be implicated in an increased risk and play an important role in the evolution of ovarian carcinomas. With the exception of the C186T and C418T mutations present in cancerous tissues, but not significantly, they could therefore have a protective action against the ovarian tumor. In addition, the study of genetic diversity parameters revealed a variation in tumors. The results also showed a genetic difference between controls and cancerous tissues and a much more rapid proliferation can be noted in the cancerous tissues.

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