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RESEARCH ARTICLE

STUDY OF NUCLEOTIDE VARIABILITY WITH THE MITOCHONDRIAL COX I GENE FROM CANCEROUS TISSUES IN SENEGALESE PATIENTS WITH CERVICAL CANCER

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Abstract

Background: Cervical cancer is the first gynecological cancer in Senegal with 1,195 new cases per year and an annual mortality of around 66% (LISCA, 2019). Mitochondrial involvement in the process of apoptosis and tumorigenesis has been analyzed previously from different cancer, and from analyses, 21 sites polymorphisms of the Cox genes (including CoxI, CoxII and CoxIII) contributed to dysfunction of mitochondrial respiratory function and have been associated with sensitivity to cancers such as prostate cancer(Green, 1998)(Cavali and Liang, 1998). These data stimulated interest in examining the potential role of mtDNA mutation of host inprogression and maintenance to cancer stades.The aim of thisstudy is to analyse the polymorphism of COXI gene from biopsies of cervical cancer in Senegalese subjects.

Methods: In this study, polymorphisms of mitochondrial CoxI gene were highlighted in 65 patients with cervical cancer women admitted to Aristide Hospital Le Dantec-Julio Curie Institute. Clinical and socidemographical data wer recorded.The total DNA of the tissues was extracted using the Standard Qiagen method (Kit QiagenDneasy Tissue), and subsequently used as template for polymerase chain reaction (PCR). At all 65 CoxI sequences were edited by Genalys PPC V.5.0.03, -Masasumi et al, 1996), BioEdit version 7.2.0 software (Hall, 1997) and ClustalW algorithm (Thomson et al., 1994).Genetic parameters were determined using DnaSP v5.10,MEGA v7.0.26, MEGA v 7.0.26, and Arlequin V 3.5.13 softwares were used to determine genetic parameters. Genetic variation variation according to epidemiological parameters were deterlined using Fst values (index of genetic differentiation and genetic structure (AMOVA) were determined using Arlequinversion 3.5.13

Results:167 variations including 163 substitutions and 4 deletions were found. Of these, 19 have already been described in other studies and deposited in the MITOMAP database. The Mitochondrial haplogroup U is the most common African haplogroup in this study. 58 transitions sites and 41 transversions sites of CoxI were recored. In this gene, it appears that the haplotypic diversity is higher ($Hd = 0.9931 \pm 0.048$) than that of the nucleotide (Pi) which has a value equal to 0.09227 ± 0.045 . Analysis of the mismatch distribution curves (Fig 1) of cancerous tissues under the assumption of an expanding population gives a multimodal appearance

Discussion and Conclusion: Genetic events leading to transformation from a normal cell to a cancer phenotype appear to be multiple. Human papilloma viruses (HPV) are probably involved in the initiation of cancer and perhaps in the maintenance of the malignant state. However, for the sake of controlling this cancer, it is necessary to identify different variants within the host cell (the woman) which could be responsible for maintaining the malignant state and the occurrence of this cancer. Our study reveals a high variability of Cox1 gene, and could be useful for an establishment of a sensitive and rapid genetic screening test.

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Introduction:-

Cervical cancer is the first gynecological cancer in Senegal with 1,195 new cases per year and an annual mortality of around 66 % (LISCA, 2019). The majority of cervical cancers appear in women under 50 years old. Cervical cancer tends to affect African women more than white women, possibly due to genetic and environmental factors in addition to the most important risk factor, namely infection with the human papillomavirus (HPV) (WHO, 2015). Tumor cells generally carry a large number of DNA changes (Stratton et al., 2009).

Until recently, research has mainly focused on alterations in nuclear DNA in various cancers. However, alterations in mtDNA in tumors have received much less attention, despite the fact that the mutation rate of mtDNA is at least 10 times higher than nuclear DNA (Wallace, 1994). It is not surprising that mtDNA is the preferred target of chemical carcinogens and that the damage caused by these compounds is persistent.

MtDNA replicates independently of nuclear DNA, throughout cell life. It is likely that single-stranded DNA is particularly sensitive to the effects of carcinogens.

MtDNA is combined with few proteins, while nuclear DNA is protected to some extent by chromatin proteins. DNA repair systems in the mitochondria are ineffective, so that damage caused by chemical agents can persist during successive replications as long as the damaged molecules have retained their replication capacity. Mitochondrial involvement in apoptosis (Green, 1998), and also in tumorigenesis process (Cavali and Liang, 1998), stimulated interest in examining the potential role of mtDNA mutation in development and the maintenance of cancers.

The complete Human mtDNA is 16,569 bp in size, and includes 37 genes, whose the structural genes for 13 of the protein subunits of the oxidative phosphorylation system, the 12S and 16S rRNA genes and 22 tRNAs (Anderson et al., 1981). MtDNA has a high number of copies (103 to 104) per cell (Anderson et al., 1981).

Recently, somatic mutations of mtDNA have been reported in several of the female cancers, including ovarian and breast cancer. These mutations may play a role in cancer formation by increasing the production of reactive oxygen species (ROS) during mitochondrial oxidative phosphorylation. The resulting ROS are mitogenic and therefore have functional relevance in the proliferation of cancer (Diwanji and Bergmann, 2017). Mitochondrial mutations can bring about to increased production of ROS, which in turn can lead to tumorigenesis and increased tumor growth. In addition, an increase in intracellular ROS caused by mtDNA mutations can lead to tumor metastasis in vivo (Ishikawa et al., 2008). Consequently, human populations, from discrete maternal lines, harbor unique sets of mononucleotide polymorphisms (SNPs) of mtDNA which define a specific genetic history called haplogroups (Torroni et al., 1996).

SNPs in protein coding regions are common in the human genome and lead missense or silent mutations (Ng and Henikoff, 2006).

The COX genes encode three subunits of the respiratory complex IV, a key enzyme as the third and last enzyme in the electron transport chain of mitochondrial oxidative phosphorylation in aerobic metabolism. 21 sites polymorphisms of the COX genes (including COXI, COXII and COXIII) have contributed to dysfunction of mitochondrial respiratory function and have been associated with sensitivity to prostate cancer.

In this study we investigate and analysed the polymorphism of mitochondrial COXI gene. Hence we hypothesized that CoxI mutations influence the risk of progression of the cervix cancer or are simply transient. Then objectives are to screen the mutations of cox 1 sequences in cervical cancer patients and to analyze the impact of these mutations on tumor process.

Materials and Methods:-

Ethical Clearance:

Objectives and benefits of study were explained clearly to subjects using local dialect before inclusion. And written informed consent was obtained from adult or legal representatives of participants. Our protocol has been reviewed according to the rules issued by the National Committee for 188 Ethics for Health Research (CNER) of Sénégal and in accordance with the procedures 189 established by the University Cheikh Anta Diop Dakar (UCAD) for the ethical approval of any 190 research involving human participants. Based on the information provided in the protocol, 191 UCAD's Committee on Research Ethics (CER) considers that the research proposed, respects 192 the appropriate ethical standards and, as a result, approves its execution under "Protocole 193 0224/2016/CER/UCAD".

Cervical Cancer Cohort study (Tableau 1):

The study group consisted of black Senegalese women born individuals whose parents and grandparents were born in Senegal. Cervix Cancer patients were enrolled from participating and corresponding subjects hospitalized in Joliot Curie Centre at Hospital Aristide LeDantec. In brief, a total of 64 women with cervical cancer were sampled. Age, ethnicity and marital status of each patient were recorded for each patient, a sample of cancerous tissue (biopsy) is taken during surgery for Joliot Curie Institute team at Aristide LeDantec hospital. They are immediately sent to the Laboratory at the Center for Immunophysiology and Infectious Diseases of the Institut Pasteur in Dakar. The samples are stored in the refrigerator (80°C) in Nunc alcohol-free tubes.

DNA extraction, PCR and Sequencing:

The total DNA of the tissues was extracted using the Standard Qiagen method (Kit Qiagen Dneasy Tissue), and subsequently used as template for polymerase chain reaction (PCR). The mitochondrial cytochrome c oxidase subunit 1 gene (cox1) was amplified with primers: F

(CATTTTGCTGCCGGTCARCAATGTTTGTGTTTGG) and R

(CCTTTGTCGATACTGCCAAARTAATGCATDGGRAA) and previously designed used reference cox gene (Ref....) PCR was carried out in a total volume of 25 µl reaction containing 5 µl of template DNA, 12 µl of Master Mix, 4.5 µl of water and 1.5 µl of each primer. PCR amplification was performed as follows: 95 °C for 5 min, 35 cycles: 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min 30s, followed by 10min at 72°C. The sequencing was carried out, determining the nucleotide sequence of a DNA fragment. Point mutations such as SNP, insertions and deletions, were highlighted by comparing the cox 1 sequences of different individuals. The method used proposed by F. Sanger (1977), is based on the use of specific nucleotides called dideoxynucleotides which block the synthesis of DNA by DNA polymerases after their incorporation. In other words, it is a particular PCR reaction using, in addition to the usual compounds (template DNA, polymerase, primers, dNTP, Mg²⁺), modified nucleotides: dideoxynucleotides (ddNTP). These ddNTPs have the particularity of being coupled to fluorochromes: ddATP-green, ddTTP-red, ddCTP-blue and ddGTP-yellow (in black on the electropherogram). This blockage is due to the impossibility of these nucleotides to form a phosphodiester bond with another nucleotide due to the absence of the hydroxyl group on carbon 3'.

Genetic analyzes:

Genetic analysis was performed in 64 patients at all. In order to determine the polymorphisms of COX I gene, the raw sequencing data were submitted to Genalys software (Takahashi et al., 2003). This program compares the raw sequences data with genomic DNA of a reference COX1 available in NCBI. The sequences obtained and chromatograms were thoroughly double checked, cleaned, and aligned to identify homologies among sites. Using BioEdit version 8.0.5 software (Hall, 1997) and ClustalW algorithm (Thompson et al., 1994). The 64 sequences were carried out. At the end, the Cox1 sequences were submitted to the MITOMAP database in order to double check thoroughly the mutations observed at first. The sequences were submitted to a fully genetic diversity analysis. And many parameters, such as nucleotide frequencies, nature and the rate of mutations was carried out using the software MEGA 6 version 6.05 (Tamura, 2013). Other parameters such as the number of polymorphic sites, the haplotype diversity (Hd), the nucleotide diversity (Pi) vs. average number of nucleotide difference (K) (Nei, 1987)

were obtained through DnaSP version 5.10.01 (Rozas et al., 2010), Arlequin Version 3.1 (Excoffier et al., 2005) and MEGA 7.0.26 (Kumar et al., 2016) softwares. The evolution of the mutations will be done by neutrality tests which will be carried out in order to know the nature of the selection. The different parameters to be highlighted are the D of Tajima (Tajima, 1989), the D and F of Fu and Li (Fu, 1997) as well as the H of Fay and Wu are used to test the deviation from the hypothesis neutrality using DnaSP version 5.10.01 software (Rozas et al., 2010) and Arlequin software version 3.5.1.3 (Excoffier and Lischer, 2010). Values of P less than 0.05 are considered significant at a 5% confidence interval.

Results:-

Socio-demographic characteristics of study group:

Table I summarizes the demographic characteristics of 65 patients included in the study. In summary, the range of patient ages from 27 years to 82 years, with an average age of **51.33** years. The age group with the largest number of participants was the 45-55 age bracket with 48.43% enrolled patients. Regarding marital status, 87.5% of the patients were on a polygamous regime and 12.5% on a monogamous regime.

Table I:- Characteristics of study group.

	Number of Patients	Values (%)
Stades (Figo System)		
Stade II	85	70.83%
Stade III	32	26.66%
Stade IV	3	2.5%
Year of Diagnosis		
2015	25	39.07%
2016	39	60.93%
Age (yrs)		
<45	15	23.43%
45-55	31	48.43%
>55	18	28.12%
Age Median Value	51.33	
First Sexual Rapport (yrs)		
<15	4	6.25%
15-20	31	48.43%
>20	29	45.31%
Gestivity		
<5	14	21.87%
5-10	42	65.62%
>10	8	12.5%
Marital Status		
Monogamy	8	12.5%
Polygamy	56	87.5%
Residence		
Dakar, Thies regions	28	43.75%
Center	18	28.12%
North	5	7.81%
South	13	20.31%

Socio-demographic and characteristic of 64 participants were represented. ND were undetermined and/or unknown. The number of patients showed in each stage of disease Stage II, III, IV. Age is given with median values and young women under 45 years old represent 23.43% of the cohort study. Mono

Cox1 gene polymorphisms in Senegalese patients :

Nucleotide sequences of cox1 (450 bp) were successfully sequenced for all the 65 examined patients. 30 polymorphisms were observed at all. 19 single polymorphisms (with bold asterisk) have been already described in other studies and deposited in the MITOMAP database (www.mitomap.org). 8 haplogroup including the 30

mutations were found. The frequency of these different haplogroup and their associated polymorphisms, as well as their nature were presented in Table II. The Mitochondrial haplogroup U5b is the most common African haplogroup in this study.

Table 2:- Frequencies of Cox 1 gene polymorphisms, associated Haplogroup and frequencies in cervical cancer cases.

Cox1 gene Polymorphisms: transitions, tranversion and frequencies				Cox1 gene Haplogroup	
Transition (N)	Frequency	Transversion(N)	Frequency	Haplogroup Name	Frequency
C6713T(10) C6824T(1)	0.06 0.005	-	-	N1a-H1	0.065
T6707G(2) A6806G(2)	0.012 0.012	T6965A(3)	0.02	L2b-H2	0.024
A6929G(8)	0.05	T6944A(1)	0.006	C4a-H3	0.024
C6725G(6) T6756G(2) A6759T(8) A6772C(1) C6797A(1) G6840C(1)	0.03 0.012 0.05 0.005 0.005 0.005	T6643C(1)	0.006	H2a-H4	0.13
T6827C(12)	0.07	T6956A(1) T6971del(6)	0.006 0.03	H13a-H5	0.04
		C6926A(1)	0.006	M36a-H6	0.006
T6940C(7)	0.04	T6976A(3)	0.02	A6b-H7	0.06
T6908C(2) T6956C(14) T6908C(2) A6647G(2)	0.012 0.08 0.012 0.012	C6824G(1) C6920A(5) G6727C(1) C6689A(1) A6806C(1)	0.006 0.029 0.006 0.006 0.006	U5b-H8	0.17

For each polymorphism, the frequencies and haplotype group were indicated. Polymorphisms were grouped in transition, transversions types. 17 polymorphisms indicated a transition type changes. 13 polymorphisms defined transversions type changes. The associated haplotype group frequencies were also indicated. ^dDeletion mutation type ; (N) Number of mutations observed.

Genetic diversity of Cox I gene:

The analysis of the genetic diversity of the study group is summarized in Table III. 58 transition sites and 41 transversion sites of Cox I were recorded. The ratio transitions / transversions was estimated at 1.38. In this gene, it appears that the haplotypic diversity is higher ($H_d = 0.9931 \pm 0.048$) than that of the nucleotide (P_i) which has a value equal to 0.09227 ± 0.045 . The average number of nucleotide differences (k) is 29,236 (Table 3).

Number of polymorphic sites	317		
Number of informative sites sparingly	301		
Transition rate	58.12		
Transversion rate	41.88		
Nucleotide		1st position	2nd position
%T	29.04	25.15	39.28
%C	20.73	21.92	18.25
%A	27.74	28.38	22.50
%G	22.45	24.55	19.97
Tr/Tv	1.087	0.832	1.286
No. of synonymous substitutions (S)	71		
No. of non-synonymous substitutions (N)	238		
Nucleotidediversity (Pi +/- SD) (π)	0.09227+ /- 0.045		
Haplotypicdiversity (h +/- SD) (h)	0.9931+ /- 0.048		
k :	29.236		
Genetic distance intra	0.149		

Tableau 3 : Parameters of genetic variability and diversity of Cox I sequences

Intra-specific neutrality tests for cancer tissues:

The statistical values (Table 4) of Tajima's D are significantly negative for the CoxI (D = -1.99104 P <0.05). Whereas D * and F * indices are not significant. Analysis of the mismatch distribution curves (Fig 1) of cancerous tissues under the assumption of an expanding population gives a multimodal appearance.

Table 4:- Cox I neutrality indices.

Amplifiedgene	D de Tajima	D*	F*
<i>Cox I</i>	-1.99104 P<0,05	-0.82513P>0,05	-0,50104 P>0,05

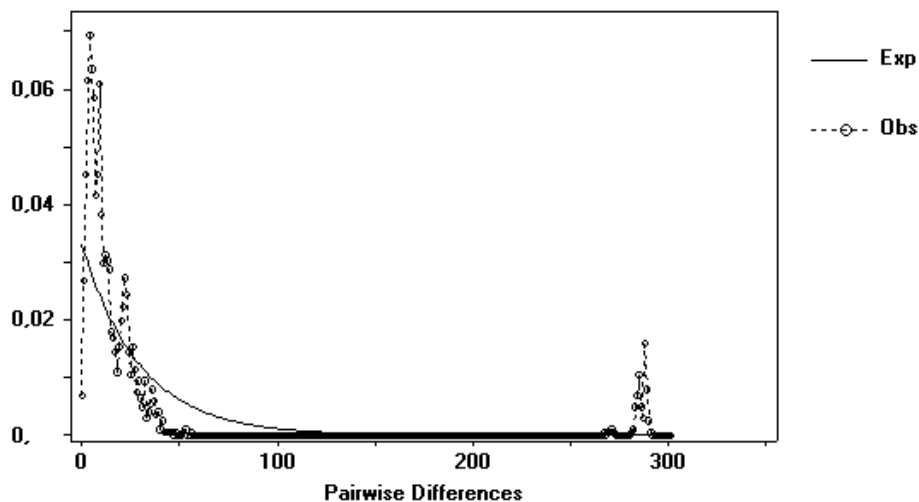


Figure I:- Cox I distribution mismatch curves.

Discussion:-

The relationships between variation in mitochondrial DNA and oncogenesis have been demonstrated in many types of tumors (Jeronimo et al., 2001 and Carew, 2002). A nucleotide variability analysis of cancer tissues was performed in Senegalese patients with cervical cancer in cytochrome oxidase I (Cox I). We used the population genetics approach applied to DNA samples taken from tumor tissue. The tissue is considered a population, exhibiting genetic diversity when cancer is present. The majority of these tissues analyzed show variations in Cox I. Among the 163 substitutions, the transition type mutations are higher with a percentage of 58.32% which confirms one of the three functions independent of the type of mutations of the MtDNA identified in tumor cells. This means that the majority of mutations are substitution of bases of the transition type (Li and Hong, 2012).

Three of our variants have already been described in other studies on ovarian and prostate cancer and in preneoplastic lesions of the gastrointestinal tract (John et al., 2005) and deposited in the MITOMAP database. These are the mutations A6929G, A6663G and T6827C.

The three majority haplogroups (H, L and U) of the African population have been found. Mitochondrial haplogroup U is the most common African haplogroup in this study. An association of haplogroup U with prostate and kidney cancer was noted by Booker et al. (2006). The legacy of the mitochondrial haplogroup U is associated with an increased risk of prostate cancer (2 times ~) and kidney cancer (2.5 times higher) in North American individuals. Therefore people with this haplotype are in a high risk group. The mitochondrial haplogroup U is found in 9.35% of the white population of the United States, (Booker et al., 2006) and in 15% of the African population (Derbeneva, 2009).

Our study population showed high haplotypic diversity ($H_d = 0.9931 \pm 0.048$) and low nucleotide diversity ($\pi = 0.09227 \pm 0.045$). This suggests that cells in the cancerous tissues of the cervix undergo rapid growth from an original, small clone. These results follow the Darwinian mode of development of cancer as it was explicitly formulated by Nowell in (1976). According to this etiological model, a neoplasia originates from a single cell which is the target of mutations which free it from physiological mechanisms limiting its proliferation. Thus, the succession of mutations conferring a selective advantage followed by periods of clonal expansion, leads to the formation of a malignant tumor (Nowell, 1976). So this result might suggest that these host mutations may have an impact on carcinogenesis of the cervix in Senegal because in the majority of cases, cancer of the cervix is due to persistent infection with a virus: the human papillomavirus (HPV). The long-term presence of this virus can sometimes affect cells in the lining of the cervix and initiate a multi-step process that can lead to the development of cancer. So these mutations can participate in this process.

Allelic frequency deviations were tested under neutrality for Cox I. The indices of D of Tajima, of D^* and F^* of Fu and Li are negative, but only the D of Tajima is Significant. This may suggest an excess of rare alleles in cancer tissue due to a sudden expansion of the mutations. This shows that the variations noted at the level of the polymorphic sites and at the level of the singleton sites are fixed under the influence of environmental effects of the random genetic drift. Indeed, Chinnery et al. (2002) showed that the random genetic drift was powerful enough to explain the fixation of rare mtDNA mutations in tumor tissue. This expansion was confirmed by the mismatch distribution curves.

Conclusion:-

The genetic events that lead to the transformation of a normal cell into a cancer cell seem to be multiple. In cervical cancer, the papilloma virus sequences are integrated into the cell genome of more than 90% of invasive cancers and are expressed in more than 80% of cases. These viruses are probably involved in the initiation of cervical cancer and perhaps in the maintenance of the malignant state. However, for the sake of controlling this cancer, it is necessary to identify different variants within the host cell (the woman) which could be responsible for maintaining the malignant state and the occurrence of this cancer for the establishment a sensitive and rapid genetic screening test. Our results showed a high variability of the Cox1 gene.

Variations	Number	variation Type	Haplogroup	Reported in MITOMAP
C6713T	10	Transition	N1a	+
C6824T	1	Transition	N1a	+

T6707G	2	Transversion	L2b	
A6806G	2	Transition	L2b	+
A6929G	8	Transition	C4a	+
T6944A	1	Transversion	C4a	
T6946d	3	Délétion	C4a	
C6725G	6	Transversion	H2a	
T6756G	2	Transversion	H2a	
A6759T	8	Transversion	H2a	
A6772C	1	Transversion	H2a	
C6797A	1	Transversion	H2a	
T6965A	3	Transversion	L2b	
T6827C	12	Transition	H13a	+
T6971d	6	Délétion	H13a	
G6917A	5	Transition	M36a	+
T6976A	3	Transversion	A6b	
G6978C	3	Transversion	A6b	
A6977G	3	Transition	A6b	+
T6643C	1	Transition	H2a	
T6956A	1	Transversion	H13a	+
A6663G	4	Transition	L2a	+
T6673C	1	Transition	A6b	
T6674C	6	Transition	A6b	+
C6686A	7	Transversion	A6b	
C6769d	1	Délétion	H2a	
C6824G	1	Transversion	U5b	+
C6920A	5	Transversion	U5b	+
T6956C	14	Transition	U5b	+
G6840C	1	Transversion	H2a	
C6686A	7	Transversion	A6b	
T6707C	6	Transition	A6b	+
G6768d	1	Délétion	L2a	
G6931T	1	Transversion	L2a	
G6727C	1	Transversion	U5b	
T6976A	3	Transversion	A6b	
T6940C	7	Transition	A6b	
T6965A	10	Transversion	L2b	
A6647G	2	Transition	U5b	+
C6689A	1	Transversion	U5b	+
A6806C	1	Transversion	U5b	+
T6908C	2	Transition	U5b	+
C6926A	1	Transversion	M36a	
T6908C	2	Transition	U5b	+

Annexe: Frequencies of Cox 1 gene polymorphisms, associated Haplogroup and frequencies in cervical cancer cases.

+ indicates a variant reference

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