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RESEARCH ARTICLE

Molecular Genetic variability in the D-loop region for females with Breast Cancer and the effect of the Chemotherapy

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ABSTRACT:
The mitochondrial DNA (mtDNA) is a small circular genome placed within the mitochondria in the cytoplasm of the cell, had a smaller 1.1 kbp fragment and called the Displacement loop (D-loop). This paper aims to find the degree of variation characteristics of this fragment. The majority of the Polymorphism nucleotidewerelocated in the D-loop region,the nucleotide transition,transversion, insertion and deletion were the importantvariations in nucleotide sequencingin females who had a breast cancer. The comparison was made between females suffering from breast cancer who didn't took chemotherapy and others treated with chemotherapy. The results were compared with a local sequence (Muthana-1) in NCBI; the total number of mutations was 36 mutations (32.1%) for patients who didn't take chemotherapy, while 76 mutations (67.8%) for the patients who were given chemotherapy. The most common and frequent mutations in D-loop in females with breast cancer were the transition then transversion mutation. The total number of transition was 14 mutations (38.8%) in females without chemotherapy. On the other hand, in females who were given chemotherapy, the total number of this mutation was 33 mutations (43.4%), which was more frequent than of those who did not take chemotherapy. The total number of transversion mutations was 9 mutations (25%) in females without chemotherapy, which was less frequent and repeated comparing to the transition mutations in the same samples. In females who took chemotherapy, there were 19 mutations (25%), which was more frequent and repeated than those who didn't took chemotherapy. The total number of deletion mutations in the D-loop region was 10 mutations (27.7%) in females who didn't took chemotherapy. In contrast to the females who took chemotherapy, there were 17 mutations (22.3%). In the insertion mutations, the total number of mutations was 3 (8.3%) in females who didn't took chemotherapy, while 7 mutations (9.2%) in females who took chemotherapy. The chemotherapy makes the mtDNA in general and D-loop in particular have a high sensitivity to damage and have variations.

KEYWORDS: mtDNA, D-loop, Breast cancer, Mutations.

1. INTRODUCTION:
Mitochondria have a critical role in the production of energy and the oxidative phosphorylation that happened in the cell (Andrews et al., 1999). The development and progression of cancer may be caused by defects in the function of mitochondria (Bianchi et al., 2001; Suzuki et al., 2003).

The mitochondrial DNA (mtDNA) is divided into two regions; a large coding region, which is responsible for the production of different biological molecules involved in the energy production of the cell and a small segment (1.1 kbp), called the control region (Mao and Reddy, 2011). It does not have introns and histones and always expose to endogenous reactive oxygen species, so the mitochondrial DNA is mainly prone to damage and variations by environmental carcinogens (Suzuki et al., 2003; Lievre et al., 2005). Consequently, mitochondrial DNA can help as a possible sensor for DNA damages and a mark for cancer development. The main control site for replication and transcription in

mitochondrial DNA is the D-loop (Suzuki et al., 2003).

Genetic changeability in displacement loop was proposed to influence the function of respiratory chain reaction is which responsible for great reactive oxygen species levels and could donate to cancer origination (Gille et al., 1992; Lievre et al., 2005). Mutations of mtDNA in old age have been diagnosed with many different cancers. mtDNA mutation can have a functional influence; this may be led to change in the apoptosis and the free radical production.This paper aims to study most of this region by using the sequencing technique and found the degree of variation characteristics of this fragment.

2. MATERIALS AND METHODS:

2.1. Subjects:

SixtyIraqifemales, who suffer from breast cancer and theirage range from20 to 75 years old,were randomly enrolled in this work. The subjects were separated in two groups,50 samples of them for females took the chemotherapy with different doses, and 10 samples were collected for females didn't took the chemotherapy as control samples. Venous blood samples were collected from each person in the National AL- Amal hospital for tumors, Baghdad-Iraq during the period from 18th to 30th of January 2017.

2.2. Genotyping:

DNA from venous blood were extracted by using DNA extraction Kit (Geneaid, Taiwan). The extracted DNA was resolved on 1% agarose gel. PCR technique was used for amplification of 982bp product according to (Zhang *et al*. 2013) by using forward (F) primer 5'-CCCCATGCTTACAAGCAAGT-3' and reverse (R) primer 5'-GCTTTGAGGAGGTAAGCTAC-3'. AccuPower® PCR PreMix (Bioneer, Korea) was prepared. The primers and DNA template add to PCR PreMixtubes and the final volume for PCR reaction was made up to 25 μL. The reaction mixers placed in thermal cycler with annealing temperature 60º C.The PCR products were resolved on 1.5% agarose gels using 100 Volt for 90 min.

2.3. Sequencing:

PCR products four subjects were sequenced in Macrogen Company (Korea). The DNA sequence data were analyzed using Mega 7 software and aligned with the Refseq, which published in NCBI databases.

2.4. Statistical Analysis:

The Statistical Analysis System- SAS (2012) software is used to study the effect of different factors in study parameters. Chi-square test was used to significant compared between percentage and T-test was used to significant compared between the means.

3. RESULTS AND DISCUSSIONS:

3.1. DNA concentration:

The extracted DNA concentration form each blood samples ranging between 150-200ng/µl and purity ranging from 1.8 to 2.0. The highest concentration of extracted DNA was found in the control samples.

3.2. The Amplification of Mitochondrial D-Loop region:

The results of gel electrophoresis revealed a single band with 982bp. The amplified band of mitochondrial D-Loop region exhibited in PCR products for all the studied samples (Figure 1 and Figure 2).

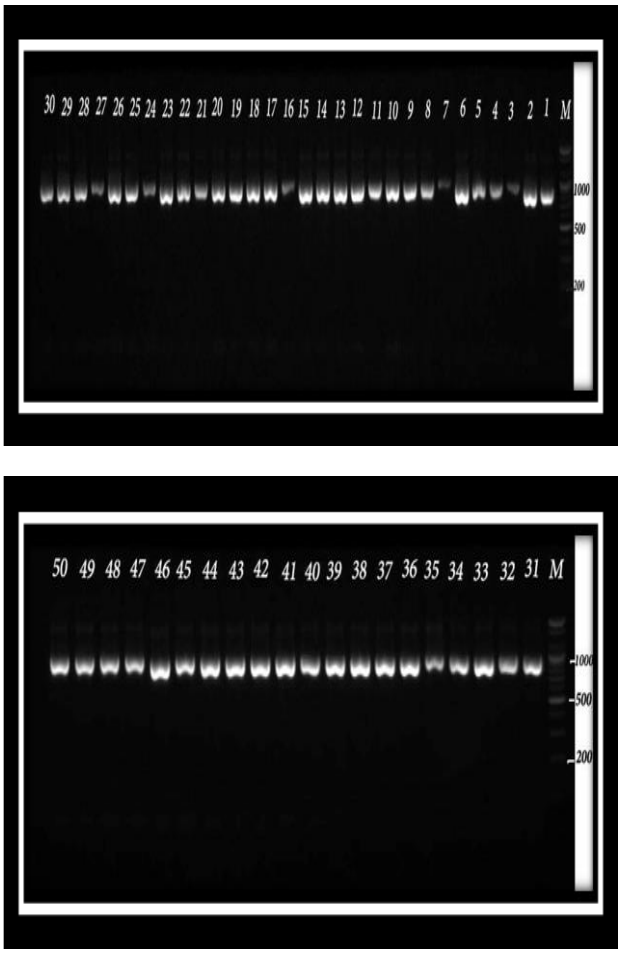

Figure 1: Agarose gel electrophoresis (1.5%, 100V/cm) for PCR product of D-Loop region for blood samples with breast cancer and taking chemotherapy.

M: 100bp DNA Ladder.

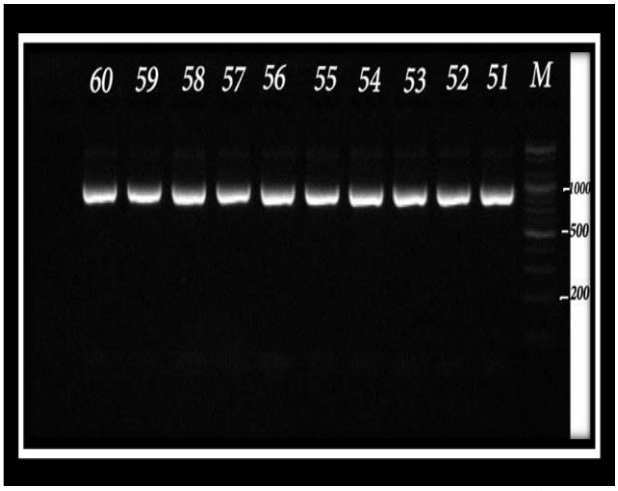

Figure 2: Agarose gel electrophoresis (1.5%, 100V/cm) for PCR product of D-loop region for blood samples with breast cancer and did not take chemotherapy (Control samples). M: 100bp DNA Ladder.

3.3. Sequencing:

Each sequence of nucleotides blasted in NCBI through Nucleotide blast in the Program(BLASTN 2.6.1+) to determine the similarity in sequences with NCBI ID sequences. These results showed that sequences belong to mtDNA and D-Loop region specifically. The results showed that all sequences had a high similarity and the color key for alignment scores appeared in red color (≥200) with the recorded samples in NCBI.

After the sequencing of all the samples, it was madea comparsion between sequences of blood samples for females with and without treatment chemotherapy with the iraqi local sequence Muthana-1 (LC229079.1) in Table 1(Hassoon et al., 2017). The results in table shows the variations and the total numbers of mutations as a result of the comparison between each sequence of blood samples of breast cancer cases with Muthana-1.

Table 1: The total numbers deletion, transition, transversion and insertions observed in the D-Loop of the mitochondrial DNA in the patients with breast cancer after comparison with Muthana-1 (LC 229079.1).

Type of sample		**Without Treatment**	**%**	**With Treatment**	**%**
Mutation type	**Nucleotide**				
Deletion	------	10	100	17	100
	Total	10	27.7	17	22.3
Chi-Square	----	---	10.52 **	---	10.48**
Transition	**T→C**	4	28.5	17	51.5
	C→T	5	35.7	4	12.1
	G→A	3	21.4	5	15.1
	A→G	2	14.2	7	21.2
	Total	14	38.8	33	43.4
Chi-Square	----	---	9.68 **	---	11.32**
Transvertion	**G→C**	1	11.1	4	21
	A→C	4	44.4	6	31.5
	A→T	2	22.2	3	15.7
	G→T	1	11.1	0	0
	C→A	1	11.1	5	26.3
	T→A	0	0	0	0
	T→G	0	0	0	0
	C→G	0	0	1	5.2
	Total	9	25	19	25
Chi-Square	----	---	10.57 **	---	9.61**
Insertion	**A**	2	66.6	6	85.7
	T	0	0	0	0
	G	0	0	0	0
	C	1	33.3	1	14.2
	Total	3	8.3	7	9.2
Chi-Square	----	---	12.74 **	---	13.61**
Total number of mutations		36	32.1	76	67.8
Number of samples		1		3	

**: High Significant (P< 0.01) NS: Non-Significant

Through the results in table 1, it was observed that the most important mutations that were found in the D-loop region was deletion, insertion and substitution including transition and transversion. The mtDNA D-loop region was amplified and then sequenced. Polymorphisms were detected in all samples. Mutations and polymorphisms in the D-loop were associated with a number of cancers; however, only a few SNPs were considered for prediction of cancer risk and outcome, and that is with a still subtle predictive value (Wang et al., 2014; Wang et al., 2017; and Guo et al., 2016).The occurrence of D-loop mutations was associated with an older onset age (50 years old), and tumors that lacked expressions of estrogen and progesterone receptors and significantly

poorer free survival. It was indicated a D-loop mutation is a significant marker independent of other clinical variables (Tseng et al., 2006).

In this study, The total number of mutation was 36 mutations (32.1%) in samples of females with breast cancer and without chemotherapy. On the other hand, it was observed in the females with breast cancer with chemotherapy that the total number of mutations was 76 mutations (67.8%). This result was more than in those who didn't took chemotherapy where there was a significant difference between them.

The most common and frequent mutations in the D-loop region were the transition mutation and then transversion mutation. The total number of transition mutations which were found was 14 mutations (38.8%) in samples of females who didn't took chemotherapy. On the other hand, it was also observed that transition mutations were the most common occurrence in D-loop in samples of females who took chemotherapy. The total number of this mutation was 33 (43.4%) which is more frequent than those without chemotherapy. Single nucleotide polymorphisms (SNPs) in this region may affect mtDNA replication and lead to alterations of the electron transport chain, which is responsible for the release of ROS and may contribute to nuclear genome damage as well as cancer initiation and progression (Bandy and Davison, 1990; Hervouet et al., 2007). These SNPs may alter mitochondrial genome transcription, thus enhancing ROS generation. The ROS mediated mechanism may subsequently promote tumor formation (Dement et al., 2007).

From the results above, the C→T type of transition mutation had the most frequent occurrence in the D-Loop region for females without chemotherapy. The total number of this mutation was 5 mutations (35.7%). As for females who took chemotherapy, it was found that the T→C type of transition mutation was the most frequent occurrence in D-Loop region. The total number of this mutation was 17 mutations (51.5%). The A→G type of transition mutation in females who didn't took chemotherapy was the lowest incidence of others types. It was 2 mutations (14.2%). Conversely, C→T type of transition mutation in females who took chemotherapy was the lowest incidence of other types, It was 4 mutations (12.1%). Our results implied that the cancer risk associated with D-loop SNPs might initiate carcinogenesis through increasing oxidative damage. Analysis of the correlation between the genetic polymorphisms in the D-Loop and 8-OHdG levels provides major new insights into the etiology of cancer.

In addition, the total number of transversion was 9 mutations (25%) for patients who didn't took chemotherapy, which is less frequent and repeated compared to the transition substitution in the same samples. In the females who took chemotherapy, it was observed that the total number of transvesion mutations was 19 (25%), which are more frequent and repeated than those who didn't took chemotherapy. Certain polymorphisms may also play important roles in modifying cancer risk or the process of tumor-genesis for the same reasons.

The results showed that A→C type of transvertion mutations was the most frequent and common in females who didn't took chemotherapy and females who took it. We detected 4 mutations (44.4%) in females without chemotherapy, but we observed 6 mutations (31.5%) in females who took chemotherapy. In our study, the cancer risk associated with SNPs was identified in the HV segment region of the D loop with nucleotides 16293, 16298 and 16319 belonging to HV-Ⅰ, 262 to HV Ⅱand 488 to HV Ⅲ. Cancer risk and outcome associated SNPs were identified in these regions in other types of cancer as well, including esophageal squamous cell carcinoma (Zhang et al., 2010; Guo et al., 2012) and hepatocellular carcinoma (Guo et al., 2012; Wang et al., 2011). The HV segments are mutational hotspots at which germ line and tumor mtDNA mutations preferentially occur (Stoneking, 2000). The HV-Ⅲ region is more susceptible to mutations but more in cancer cells perhaps because of the increase in cell growth. Additional investigation of the biochemical consequences of mtDNA mutations in disease and various types of tumors will provide insight regarding the roles of mitochondria in the pathogenesis of neuromuscular diseases, tumorigenesis, apoptosis, and aging.

The total number of deletion mutations that occurred in the D-Loop region was 10 (27.7%) in females who didn't took chemotherapy. In contrast, it was observed the mutations increased to 17 (22.3%) in the females who took chemotherapy which is more frequent than in those who did not take chemotherapy. The specific deletions of mtDNAhave been observed in 46% of breast cancer tissues (Dani et al., 2004). Although the most common mtDNA mutations detected in breast cancer were largely single base substitutions or insertions, a large deletion of 4977bp was detected in both the malignant and paired normal breast tissues of patients with breast cancer (Sharp et al., 1992; Bianchi et al., 1995).

As for the insertion mutations, it was noted that the total number of mutations was 3 (8.3%) in females who didn't take chemotherapy, while in females who took chemotherapy, the insertion mutations was 7 (9.2%). The results showed that the base (A) was the most

frequent occurrence of the other insertion bases in the D-Loop. The total numbers of it ranged from 2 to 6 mutations (66.6-85.7%), respectively in both females who didn't took chemotherapy and the females who took it. A study on somatic mutation in the D-loop region of mtDNA has revealed that insertions or deletions at nucleotide position (np) 303-309 are the most common mutations of mtDNA in human cancers including breast cancer (Tan et al., 2002). In addition, it has also been reported that breast cancers harboring mutations in D-loop region, particularly at the polycytidine stretch or close to the replication origins of the heavy strand, had a significantly lower copy number of mtDNA than the ones without D-loop alterations (Moreno et al., 2007).

From the study, it was observed that the old age of females and the number of doses taken made a greater number of mutations which occur in D-loop region. This indicates the breakdown of the D-loop and the occurrence of many variations due to chemotherapy. The chemotherapy makes the mtDNA in general and D-loop in particular have high sensitivity to damage.The properties of mtDNA, such as high copy numbers, the high prevalence of mutations and quantitative, and qualitative alterations in cancer, encourage us to investigate the clinical relevance of mtDNA alterations in cancers. In addition, the simple structure and short length of mtDNA make the genome wide screening of mtDNA in life science easier and more cost effective than using nuclear DNA. Due to the lack of protective histone proteins, mtDNA is highly sensitive to oxidative and damage. It was highly affected by chemical treatment and other oxidizing substances. Mutations of mitochondrial DNA in old age have been diagnosed with many different cancers. These mutations include intragenic deletions (Horton et al., 1996), a missense mutation (Polyak et al., 1998) and Frame shift mutation (Habano et al., 1998). As a general principle, these mutations may interfere with neoplastic transformation by changes in cellular energy. This increases the oxidative stress of mitochondria and thus regulates the process of apoptosis (Wallace et al., 1999). Recently, a team of researchers studied mtDNA mutations for a number of cancers such as bladder, neck and lung cancers and found that there are mitochondrial mutations similar to homoplasmic in nature and this indicates that they have become prevalent in cancer cells (Fliss et al., 2000).

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