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SEQUENCES OF MITOCONDRIAL D-LOOP REGION IN IRAQI PERSONS

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ABSTRACT

Mitochondrial DNA is a circular genome placed in the mitochondria. It had 1.1 kbp fragment and named the displacement loop. The using of sequencing technique and then find the degree of variation of this fragment are the aims of study. Geneaid extraction kit was used for extracted the completely genomic DNA, and then amplified the displacement loop region by polymerase chain reaction through specific primers. The PCR products were sequenced and detected variation by using the MEGA7 program. Different polymorphisms were discovered in this region for both blood and muscle samples from Iraqi population. The accumulation of SNPs in D-loop may be associated with ageing. SNPs in D-loop region of blood and muscle samples were identified in this study, and their association with ageing was estimated. The majority of polymorphism were located in D-loop. The nucleotide transition,transversion, insertion and deletion were causes the important variations in nucleotide sequencing. The total number of mutations in blood samples of young individuals was 37 (4.3%) and 48 (5.6%) in muscle samples for same individuals while the total number of mutations in blood samples of older individuals was 667 (78%) and 93 (10.8%) in muscle samples for same individuals. There were high differences in the number of mutations in older people, specifically for blood samples incidence and frequency of mutations were greater than those of younger age groups were. The analysis of genomic single nucleotide polymorphisms in D-loop may help to detect the most important variation in both young and adult Iraqi individuals.

Key words: mtDNA, aging, mutation.

INTRODUCTION

The mitochondrial genome contained two regions; a large one is the coding region and a small segment (1.1 kbp), called the control region (Mao and Reddy, 2011). The coding regions is accountable for the creation of different molecules involved in the energy production. The control regions is contribute in pathophysiological progressions such as the degenerative disease, ageing (Kashihara *et al*., 2010; Siddique *et al.,* 2015; Karbowski and Neutzner, 2012) and cancer (Sotgia *et al*., 2011; Sumitha and Devi, 2016). The D-loop fragment does not code any protein (Penta *et al*., 2001; Chen *et al*., 2009). The higher frequency of accumulation of mutations in different mtDNA during the process of increased oxidative stress (Clayton, 2000), and somatic mutations may be occur (He *et al*., 2010). The displacement loop has the initial site of H-strand replication, HSP and LSP. Thus, it is used to regulation of replication and transcription in mtDNA (Clayton, 2000; Penta *et al*., 2001). Replication of mtDNA starts in this region as a result in the creation of a D-loop with a newly created H-strand of about 700 nt called 7S DNA (Sbisa *et al.*, 1997).

Heavy and light chains of mitochondrial DNA are wholly transcribed from the promoters (HSP and LSP) in the displacement loop. There are two small regions besides the promoter sequences known as the hypervariable regions (HV) I and II. The mutation rates in hypervariable region I and hypervariable region II are mainly higher and mutation rate vary in the regions (Jazin *et al*, 1998). The displacement loop is highly polymorphic. Some polymorphisms in D-loop are associated with aging and a diversity of tumors (Michikawa *et al*., 1999; Wang *et al*., 2001; Coskun *et al*., 2003; Zhang *et al*., 2003; Mueller *et al*., 2011).

In this work we tried to find the relationship between aging and the different types of mutations that may be occurred in D-loop region of mtDNA by using genetic analyses like polymerase chain reactions (PCR) and DNA sequences to determine the genetic polymorphism and variations in displacement loop in comparison with the data published in National Center for Biotechnology Information (NCBI).

MATERIALS AND METHODS

Subjects: Thirty aging Iraqi persons (24 male, 6 females) with aged ranging 10-75 years were randomly enrolled in this work. The subjects were separated in seven groups (Table 1). Venous blood and muscles samples were collected from each person in the department of surgery and fractures of Ghazi Al-Hariri hospital, Baghdad, Iraq during January 2017.

Extraction of the Genomic DNA: Genomic DNA extracted from blood and muscle samples by using (Geneaid Kit, Taiwan) depending on the traditional methods.

Extraction from Blood Samples: We put the sample in vortex shaker for 10 minutes. Then we take 200 µl from the samples and put it in 1.5 ml Micro-centrifuge tube. After that, we take 25 µl from proteinase k and put it over the sample. Then we put the sample in the water bath at 60 ºC for 5 min. Later on, we add 200 µl of GSB solution to the sample. In a water bath at 60 ºC for 20 min, we put the sample and we add 200 µl of absolute ethanol solution to the sample. Then we take GS-Column with tube collection (2ml) from the kit and we put the sample to the GS-Column and neglect micro-centrifuge. After that, we centrifuge the sample for 13500 rpm/min. Then we get rid of the tube collection

and used other new tubes. After that, we make a washing process and we add 400 µl from the W1 solution for each sample. We make centrifugation at 13500 rpm for 30 sec. Then we neglect the sediment from the tube collection. We add 600 µl from washing buffer solution to the sample and centrifuge it in 13500 for 30 sec. Then we neglect the washing solution and we put the sample in new micro-centrifuge at 13500 rpm for 3 minutes. After that, we neglect the tube collection, instead of we use a new micro centrifuge tube and cutting the cover. Then we add 50 µl of Elution buffer solution to the sample and we wait 3-5 min at a minimum and then Centrifuging in 13500 rpm/min to get the DNA in a micro-centrifuge tube and keep it in the freeze.

Extraction from Muscle Samples: We take 3 cm of the muscle, put it in mortar casserole and add a little quantity from liquid nitrogen. Then we take 0.5 g of the muscle and put it in the micro-centrifuge tube. After that, we add 200 µl of GST solution and 20 µl of proteinase k to the sample and vortex it. We put the sample in a water bath at 60 ºC for 1-3 hrs. To obtain a clear sample, and if it is not clear, we tolerate it overnight. Then we centrifuge the sample at 13500 rpm for 2 min. Add 200 µl of GSB buffer and then vortex it. After that, we add 200 µl of absolute ethanol solution to the sample and then continue as mentioned in the above method.

Table 1: The distribution of persons depends on their ages

Ranging age (Years)	less than 20	20-29	30-39	40-49	50-59	60-69	70-75	Total
Number of persons	5	5	5	5	3	4	3	30

Genotyping: DNA from venous blood and muscles were extracted by using DNA extraction Kit (Geneaid, Taiwan). The extracted DNA was resolved on 1.0% 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', table 2. AccuPower® PCR PreMix (Bioneer, Korea) was prepared. The primers and DNA template were added to PCR Pre-Mix tubes and the final volume for PCR reaction was made up to 25μl, see table 3. The reaction mixers placed in thermal cycler with annealing temperature 60º, see table 4.

The PCR products were resolved on 1.5% agarose gels using 100mVolt for 90 min.

Sequencing: PCR products for 30 subjects were sent to Macrogen Company (Korea) for obtaining the DNA sequence. The Mega 7 program was used for analyzed the DNA sequence data and aligned with the Refseq, which published in NCBI databases.

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

RESULTS AND DISCUSSION

DNA concentration: The extracted DNA concentration form each venous blood and muscle samples ranging between 150-200ng/µl and purity ranging from 1.8 - 2.0. The highest concentration of extracted DNA found in muscles, the mean value was 2.4 µg/µl, which is greater than the concentration of extracted DNA from the blood, this due to the muscles that contain large numbers of nuclei and mitochondria, unlike blood.

Amplification of mitochondrial D-Loop region: The results of agarose gel electrophoresis of amplified mtDNA revealed a single band with size of 982 bp. The amplified band of the displacement loop region exhibited in polymerase chain reaction products for all the studied samples (Figure 1).

Table 2: Specific Primers

Primer name	Primer sequence
D-loop Forward Primer	5' CCCCATGCTTACAAGCAAGT-3'
D-loop Reverse Primer	5'-GCTTTGAGGAGGTAAGCTAC-3'

Table 3: The components of PCR reaction

Item	Master mix	DNA	Forward Primer	Reverse Primer	Nuclease free water	Total volume
Volume	5 µl	1 µl	1 µl	1 µl	17 µl	25 µl

Table 4: The thermo cycling conditions program for D-Loop region

Steps	Time	Temperature	NO. of cycles
Initial denaturation	2 min	95 ºC	1
Denaturation	30 sec	95 ºC	35
Annealing	30 sec	60 ºC	
Extension	45 sec	72 ºC	
Final extension	5 min	72 ºC	1

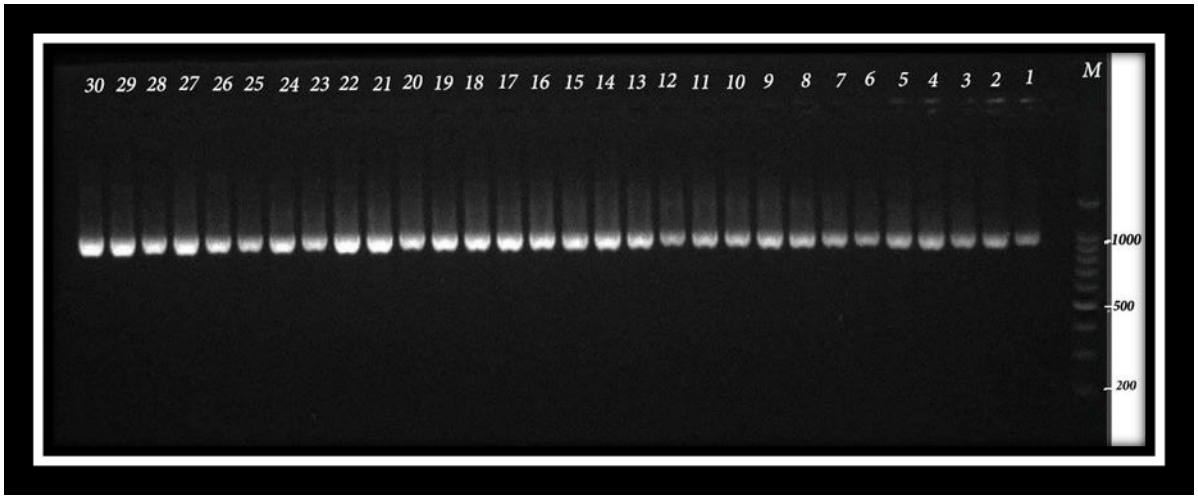

Figure 1: Amplification of mtDNA D-loop region of each sample: PCR products ≈ 982bp of samples migrated on agarose 1.5% in 100 volt.

M: DNA Ladder (100 bp**).** Lanes 1-5 for ages less than 20 years old, lanes 6-10 for 20-29 years old, lanes 11-15 for 30-39 years old, lanes 16-20 for 40-49 years old, lanes 21-23 for 50-59, lanes 24-27 for 60-69 years old and lanes 28-30 for 70-75 years old.

Sequencing: Each sequence of nucleotides blasted in NCBI through Nucleotide blast in the Program (BLA-STN 2.6.1+) to determine the similarity in sequences with NCBI ID sequences. These results showed that sequences belong to of D-Loop region. 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 all the samples, it was select the small age (under eight years old) as a control for this study and used for comparing with others to get the variation of mutations among the samples according to the age. The control sequences recorded in NCBI as an Iraqi genome, the accession number of the sequence was LC229079.1, and the figure 2 appears the nucleotide sequence of Muthana-1 (LC229079.1).

>LC229079.1 (Muthana-1) 995 bp
CGGGGTTCGTTGGCATTCACACATCACTGCAACTCCAAGCCACCCCTCAC
CCACTAGGATACCAACAAACCTACCTACCCTTAACAGTACATAGTACATA
AAGCCATTTACCGTACATAGCACATTACAGTCAAATCCCTTCTCGTCCCC
ATGGATGACCCCCCTCAGATAGGGGTCCCTTGACCACCATCCTCCGTGAA
ATCAATATCCCGCACAAGAGTGCTACTCTCCTCGCTCCGGGCCCATAACA
CTTGGGGGTAGCTAAAGTGAACTGTATCCGACATCTGGTTCCTACTTCAG
GGCCATAAAGCCTAAATAGCCCACACGTTCCCCTTAAATAAGACATCACG
ATGGATCACAGGTCTATCACCCTATTAACCACTCACGGGAGCTCTCCATG
CATTTGGTATTTTCGTCTGGGGGGTGTGCACGCGATAGCATTGCGAGACG
CTGGAGCCGGAGCACCCTATGTCGCAGTATCTGTCTTTGATTCCTGCCTC
ATCCTATTATTTATCGCACCTACGTTCAATATTACAGACGAGCATATCTA
CTAAAGCGTATTAATTAATTAATGCTTGTAGGACATAATAATAACAATTG
AATGTCTGCACAGCCGCTTTCCACACAGACATCATAACAAAAAATTTCCA
CCAAACCCCCCCCTCCCCCGGTTTCTGGCCACAGCACTTAAACACATCTT
TGCCAAACCCCAAAACAAAGAACCCTAAAACCAGCCTAACCGAATTTCAA
ATTTTAACTTTTGGGGGGTATGCACTTTTAACAGTCCCCCCCCCAACTAA
CAACTTTATTTTCCCCTCCCACCCCCCAAACAAACAAATCTCATCAATAA
AACCCCCGGCCAATCCAACCCGGCAAAAAAAAAAACCGGTTTCTAAACCCC
AAACCCCGAAACCAACCAAACCCCAAAAAACCCCCCCCCAAGTTTATGTA
CTTACTTCCCCCAAAAAAAAAAAGGTTGAGGGTTTGTGTTTTGTG

Figure 2: The nucleotide sequence of Muthana-1(LC229079.1).

Table 5: The consolidated data for mutations observed in the mtDNA displacement loop (D-Loop) segment for study samples with Muthana-1 (LC 229079.1)

Aged		8-10 years				35-75 years			
Type of sample		Blood	%	Muscle	%	Blood	%	Muscle	%
Mutation type	Nucleotide								
Deletion	------	3	100	10	100	156	100	20	100
	Total	3	8.1	10	17.2	156	23.3	20	21.5
Transition	T→C	3	30	8	44.4	105	42.3	16	48.4
	C→T	2	20	4	22.2	74	29.8	9	27.2

	G→A	2	20	4	22.2	35	14.1	5	15.1
	A→G	3	30	2	11.1	34	13.7	3	9
	Total	10	27	18	31	248	37.1	33	35.4
Chi-Square	----	---	14.75 **	---	11.64 **	---	10.23 **	---	10.82 **
Transvertion	G→C	0	0	4	20	34	14	6	15
	A→C	1	20	3	15	101	41.7	9	22.5
	A→T	1	20	4	20	56	23.1	7	17.5
	G→T	0	0	0	0	1	0.4	1	2.5
	C→A	1	20	5	25	30	12.3	8	20
	T→A	1	20	0	0	2	0.8	6	15
	T→G	0	0	1	5	14	5.7	2	5
	C→G	1	20	3	15	4	1.6	1	2.5
	Total	5	13.5	20	34.4	242	36.2	40	43
Chi-Square	----	---	8.24 **	---	8.63 **	---	10.35 **	---	8.07 **
Insertion	A	5	26.3	0	0	10	47.6	0	0
	T	4	21	0	0	2	9.5	0	0
	G	2	10.5	0	0	2	9.5	0	0
	C	8	42.1	0	0	7	33.3	0	0
	Total	19	51.3	0	0	21	3.1	0	0
Chi-Square	----	---	2.68 **	---	0.00 NS	---	11.53 **	---	0.00 NS
Total number of mutations		37	4.3	48	5.6	667	78	93	10.8
Number of samples		1		2		18		4	

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

The D-loop appears to be the region more prone to mutations. The SNPs increased with age in this region in the striatum of aged persons (Williams *et al.*, 2013). By showing the results of the analysis sequences in the table (5), rearrangements, insertions and deletions at the displacement loop seem to accumulate in the older persons. It is still rather low, about 3% of the combined mutation load (Sevini *et al.*, 2014). The reduced efficiency of repair and the formation of replication error in the D-loop is the chief cause for the happenes of the variations (Wilson *et al*., 1993).

It was observed that the total number of mutations in D-loop for blood samples in younger individuals less than 10 years of age was 37 mutation, (4.3%) in addition; the total number of mutation in the D-loop for muscle samples of the same individuals was 48 muta-tion (5.6%), and is rather similar to mutations in the blood for the same. On the other hand, the total number of mutations for blood samples in the aging group from (35-75 year) was to 667 mutation (78.9%), and the total number of mutations in the muscle samples for the same samples was 93(11%). The results were consistent with the current study in Iraq were recorded 147 polymorphic locations occured in the displace-ment loop of the unrelated 100 mitochondrial DNA samples of Iraqi persons, the major value 79% of nucleotide transitions and low frequency of nucleotide transversion 1.7 % (Al-Rashedi *et al*., 2016).

There are significant differences in the number of mutations in older people, specifically for blood samples, where the incidence and occurrence of mutations were greater than those of younger age groups. It was also greater than the number of mutations of the muscle samples from the same individuals. It was known that the bigger polymorphic rate in the displacement loop had been admited in humans. Through the result of a study that observed that the blood was more prone to mutations and variability greater than the muscle in this region in aging. The main source of mutations looks to come from failure of the repaired mechanisms, errors in replication. The accumulation of the mutations as seen in old persons appears to hap-pen by clonal expansion and are not produced by a ROS reliant on vicious cycle (Barja, 2013).

16% of the Polymorphic nucleotide locations in the displacement loop is placed within four overlapping displacement loop regions. The variations are happend by nucleotide transition,tranversion, insertion or deletion (Al-Rashedi *et al*., 2016). Some studies in the skeletal muscle presented that the accumulation of deletion mutations happened by clonal expansion of single mutation events (Fayet *et al.*, 2002; Williams *et al.*, 2013).

The result showed, the number of deletion in the age less than 10 years were 3-10, (8.1% to 20.8%) for both blood and muscle, which are less frequent than the mutations in older individual's which have 20-156, (from 21.5% to 23.3%) for both blood and muscle. Deletion mutation includes a loss of apiece of the dis-placement loop, in that resulting in complete loss of the mitochondrial genome and makes errors in replication. Both deletion and other single nucleotide mutations have been shown to accumulate with aging in various tissues and different species, such as rhesus monkey, rodents and humans (Rossignol *et al.*, 2003). The accumulation of deletions in the mitochondrial DNA plays a main role in aged tissues of older persons and Parkinson's disease patients (Bua *et al.*, 2006 and Herbst *et al.*, 2007). The mechanism of deletion mutagenesis is not exactly recognized. The direct repeat (DR) motifs have frequently detected flanking the breakpoints of mitochondrial DNA deletions in several organisms such as human (Pak *et al.*, 2005, Chinnery *et al*., 2013).

Instead, it was noted that transition mutations were more frequent and occurred than other mutations in the displacement loop for young and old individuals. It was noted that the number of mutations for the blood and muscle samples for individuals under 10 years of age was ranged from 10- 18(27-37.5%), while in the blood samples for adults individuals, the number was to 248 mutation (37.1%), more than in the muscle samples which was 33 mutation (35.4%) in the same individuals. The distribution of mtDNA point mutation is shown nucleotide transversional changes less than transitional changes. This important difference is because of the natural structure of the nucleotides thus, the biochemical properties of complementary base pairing. The different mutational mechanisms in the mitochondrial genome regions lead to high frequ-ency of transition (Topal and Fresco, 1976).

The results showed that the higher frequency of Transition mutations types in all age groups were T→C for individuals under 10 years of age and adult individuals. The results were consistent with the current study appearances that hyper mutability of transition cytosine to thiamine and vice versa. Methylation of the base C generated the natural deamination of meth-yl-C rise to base T. In mammals, this transitional mutation happens about ten times faster than other single nucleotide mutations (Giannelli *et al.,* 1999). From results above the transition, mutations happened in blood samples from older individuals more than other included muscle samples from same individuals and both blood and muscle samples from younger individuals (Taylor *et al.*, 2003). General mutations and specific base rearrangement have been analyzed in different aged tissues (Michikawa *et al.*, 1999).

In Transversion mutations, the results showed that the number of mutations occurring from the individuals under the age of 10 years is less frequent compared with individuals over the age of 35 years, which numbers of it about 242 mutation (36.2%) in blood samples specifically. The A→C type of transversion mutations was the frequent high occurrence in both young's and older ages. The percentages of it ranged from 22.5-41.7% for both blood and muscle samples from adults. The results above came back for the fact; ROS simplify the formation of oxidative DNA base damage products as like 8OHdG.

As mentioned above in the table, the insertion mutations observed to be the least frequent among other mutations in the displacement loop for young and old individuals and for both blood and muscle samples. During the analysis of sequences, the most significant mutations occurred at the beginning of the sequence exactly (50-100bp) as well as in the last half of the sequences from site 500bp to the termination of the sequences. It was noted that significant differences observed in the locations and mutation patterns in the sequences of blood and muscle samples of older individuals specifically. The association between the reality that the mutations was increased through aging is still argumentative, and several elements must be taken. The average was estimates of the mutation frequency for human mitochondrial genome do not reflect the correct state of affairs and should be seen as basic tools for phylogenetic studies (Hasegawa *et al*., 1993). The connotation between ageing and variations in mitochondrial genome, involving rearrangements and point mutations has been deeply examining over the last time. Apoptosis and ROS suggested to play a role in age associated respiratory chain dysfunction. (Larsson, 2010). The high mutation frequency makes the D-loop as a genetic sign in human identity testing (Holland and Parsons, 1999).

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