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Molecular Analyses Reveal Novel Mutations in Mitochondrial DNA D-loop Region of Breast Cancer Patients

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Abstract
D-Loop or displacement loop region is a triple-stranded section found in the noncoding region of the mitochondrial DNA (mtDNA) that contains important transcription and replication components of mtDNA. D-Loop is composed of 1121 base pairs which are highly venerable to mutations. This study aims to investigate D-loop mutations in females who had breast cancer and compare them with the global sequence of D-loop in healthy individuals. Forty tissue samples were taken included 21 samples were malignant cancer and 19 samples were benign cancer (control group). The mtDNA was extracted using a gSYNCTM DNA extraction kit, and a 558bp fragment of the noncoding region within D-loop section was amplified by PCR. After purification of the PCR products, amplicons were sequenced via sanger sequence. The sequence results were analyzed using Bioedite software (ver.7.7.1) to identify mutations. This study focused on extracting and analyzing the D-Loop region of mitochondrial DNA (mtDNA) from female breast cancer patients. We employed PCR and Sanger sequencing methods to explore mutations within this region. Our findings revealed a total of 18 mutations, including InsG58, G64A, C185T, T16317C, T16343C, A16352G, G16355A, A16359G, and DL/T16480, which, to our knowledge, have not been previously reported in Iraqi patients. Notably, all identified mutations were located within the hypervariable regions 1 and 2 (HV1 and HV2) of the D-Loop. Substitution mutations were the most prevalent among the mutations observed, suggesting that alterations in the D-Loop region may significantly contribute to the development of breast cancer.

Keywords: mtDNA**,** D-Loop, Mutations, breast cancer.
Abbreviations: mtDNA: mitochondrial DNA, BCL: benign cancer loop, MCL: malignant cancer loop, FFPE: formalin-fixed paraffin-embedded tissue

Introduction

Breast cancer is the most frequently diagnosed type of cancer in women. It typically begins in the cells of the lobules or the non-epithelial tissue of the breast, although there are rare cases of mixed carcinosarcomas [1, 2]. Although it affects women far more frequently than it does men, men can also get breast cancer[3, 4]. Breast cancer is a complex disease influenced by genetic and environmental factors. It can be categorized into three groups based on histological and molecular characteristics: hormone receptor-positive (ER+ or PR+), human epidermal growth factor receptor 2 positive (HER2+), and triple-negative (ER-, PR-, and HER2-) breast cancer. Therefore, Treatment should be tailored to those molecular characteristics of the cancer [5, 6].

Mitochondria are the primary energy source in all nucleated cells. They are essential for various cellular functions, including body metabolism, apoptosis, and signal transduction. Mitochondria transmit genetic information through mitochondrial DNA (mtDNA), which is prone to a high frequency of alterations [7, 8]. Margit and Sylvan Nass are the first one who isolated the mtDNA in 1963 after conducting research on special sorts of mitochondrial fibers. While examining the procedures of fixation, stabilization, and staining, their

investigations pointed out that these fibers had links to DNA [9]. Several researchers have indicated that modifications in mtDNA are critical in cancer development[10-12]. It involves the regulatory region since it contains sequences necessary for transcription and replication. According to earlier research, variations in these noncoding regions in the regulatory region may be crucial in the etiology of breast cancer [13-15].

Mitochondrial DNA is a circular, double-stranded molecule consisting of 16569 base pairs. It contains 37 genes that produce 22 tRNAs, two rRNAs (12s and 16s), and 13 essential polypeptides critical in the oxidative phosphorylation system (Figure 1) [16-18]. mtDNA is significantly exposed to damage from environmental carcinogens. It is constantly exposed to endogenous reactive oxygen species (ROS) because it lacks protective histones or non-histone proteins and possesses no introns [19]. The mitochondrial genome is distinguished by multiple DNA molecules in each cell and an elevated mutation rate. This leads to a condition called heteroplasmy, which a cell is called when it contains a mix of mutant and normal mtDNA) [20, 21]. The rest of the molecule is a non-coding sequence of 1121 base pairs, and it contains the 'control region,' a transcription promoter region and the D-loop. It is located at locations 16024-576 (gene start 567 and gene end 16024) on the mtDNA. It contains critical transcription and replication components and functions as a promoter for both the heavy and light strands. The D-loop region has two hypervariable regions (HV1 at locations 16024–16383 and HV2 at locations 57–372), a hot site for mtDNA changes [22-24]. Although the D-loop is a non-coding region of mtDNA, mutations in this region can modify transcription and change the mtDNA coding sequence, impacting the expression and functionality of 13 essential OXPHOS system proteins. Point mutations, deletions, insertions, and missense mutations are examples of somatic mtDNA mutations[25, 26].

Hence, mtDNA could potentially act as a sensor for cellular DNA damage and an indicator of the onset of cancer; the D-loop, or displacement loop, controls mtDNA replication and transcription [27, 28]. One modification recently reported in the literature is the poly cytidine stretch D310. It is a region located in the D-loop between 303 and 315, characterized by cytosine repetition. This region may be the starting point for the clonal proliferation of cancerous cells, such as breast cancer cells [29, 30]. Our results showed the presence of high-frequency for globally known mutations and new mutations not mentioned in the previous literature in women with cancer in Iraq after we conducted a molecular investigation using gene sequencing to examine the connection between the clinical and pathological characteristics of breast cancer and the D-loop region in mtDNA, which might play an important role in its pathogenesis.

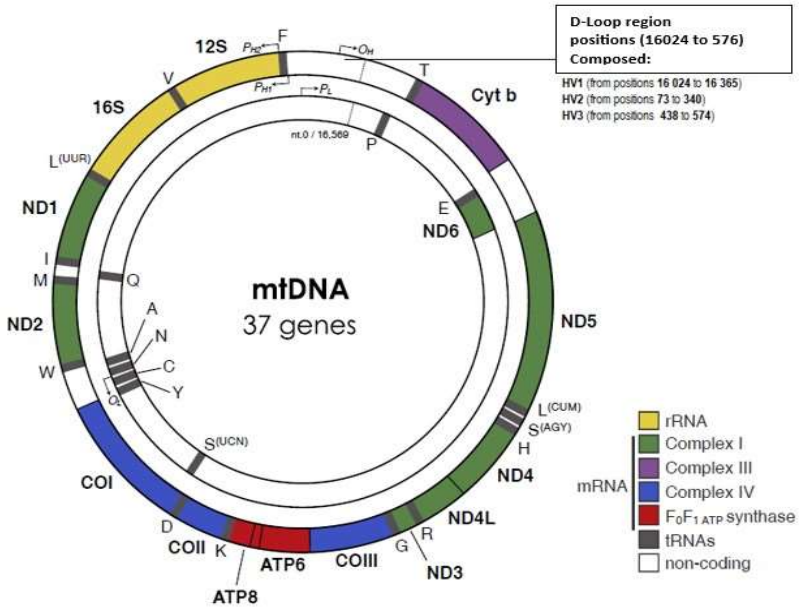


Figure 1: The human mtDNA indicates the positions of genes and regulatory areas. Adapted from [31].

Materials & Methods

Sample collection:

Breast cancer tissue samples used for this study were gathered from female patients who had been diagnosed with breast cancer from AL-Karama Hospital and a Privet Oncology Laboratory during the year 2022. The total number of samples was 40 samples, including 21 with malignant breast cancer and 19 with benign breast cancer as a control.

Mitochondrial DNA extraction:

MtDNA was extracted from FFPE tissue using the gSYNCTM DNA extraction kit (Geneaid/Cat#GS100) after melting the wax from the samples using 1 ml of xylene following the manufacturer's guidelines for extracting mtDNA from the tissue. In brief, 200 µl GST buffer and 20µl of proteinase K were added to samples and incubated overnight at 60°C. Then, the supernatant was moved to a fresh 1.5 ml microcentrifuge tube, mixed with 200 µl of GSB buffer and added to the column GS. After centrifugation, samples were washed with 400 µl of Buffer W1 to column GS. Finally, mtDNA was eluted by adding 50 µl of pre-heated elution buffer into the center of the column and centrifuged at 14000 xg for 3 minutes at room temperature. The eluted mtDNA was saved at -20C till it was used

D-loop amplification and confirmation:

The extracted mtDNA was utilized as a template for PCR amplification. To amplify D-loop region (558bp) (NCBI Reference Sequence: NC_012920.1), a standard PCR with specific primers was ran. The reaction mixture was contained 25 µL of Taq polymerase master mix (Promega cat#M7822), 19 µL nuclease-free water, 2 µL forward primer (DLP.F: 5′-AGTGGCTGTGCAGACATTCA -3′), 2 µL reverse primer, (DLP.R: 5′-ACCAACAAACCTACCCACCC -3′) and 2 µL of mtDNA. Amplification was carried out using a thermal cycler (MultiGene OptiMax Thermal Cycler) with the following protocol. The initial denaturation was at 95°C for 3 minutes, followed by 40 cycles consisting of denaturation at 95°C for 30 seconds, primer annealing at 58°C for 30 seconds, and extension at 72°C for 1 minute. The final step was at 72°C for 5 minutes. Amplicons were saved at -20C till they were used.

To analyze the PCR reaction products, 3ul of amplicons were resolved by 0.5% agarose gel, prepared with ethidium bromide. 7µL of 100bp DNA Ladder (Cat. No.is DL007, Geneaid) was loaded in the gel along with samples and run at 100 V for 40 minutes. To visualize the results, UV transillumination was used.

Sanger sequencing:

A Gel/PCR DNA Fragments Extraction Kit (Geneaid /Cat#DF100) was used to purify D-loop band. The purified products were then sent to sequencing facilities at Macrogen corporation in Korea. Sanger sequencing approach was applied. The sequencing data were subsequently analyzed using BioEdit software ver.7.7.1 to reveal the mutations in D-loop.

Results and Discussion

Adequate amounts and cleaned mtDNA were gained from all samples (21 malignant breast cancer and 19 benign breast cancer) after using The gSYNCTM DNA extraction kit. To amplify the D-loop region, a standard PCR with specific primers was run. The PCR products were resolved by 0.5% agarose gel, and bands (D-loop amplicon 558bp) were shown (Figure 2).

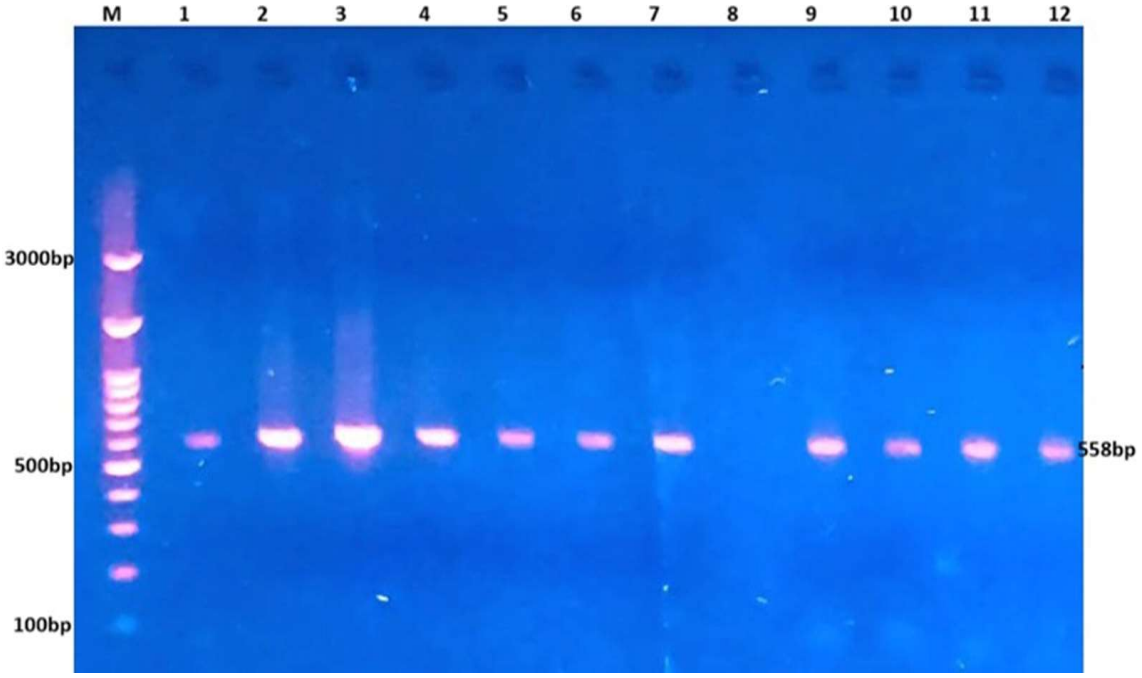


Figure 2: Agarose gel electrophoresis: 3µL of D-Loop PCR product was mixed with 1µL loading dye and loaded in the gel wells. M: A 100bp DNA marker. Lanes 1 to 12 were D-Loop positive, except lane 8, which was negative.

Even though, there are many obstacles to extract mtDNA from FFPE samples and amplify genes, our results showed that 96 % success rate. The 3% unsuccess rate could cause by the following reasons. Formalin effects on the quality of genomic materials and the cross-linked effects that the components of formalin induce between protein and nucleic acid. The Pre-extraction handling that insufficient paraffine removal can damage DNA quality[32, 33].

To investigate the mutation in D-loop region, the amplicons were cleaned with Gel/PCR DNA Fragments Extraction kit and sent to sanger sequencing facilities (Macrogen, Koria). Obtained sequences results were analyzed by Bioedite software. We identified eighteen mutations distributing with three types of mutation (insertion, deletion, and substitution) (table 1). To the best of our knowledge, this is the first study that identified nine novel mutations in Iraqi women who had breast cancer. These mutations were (Ins G58, G64A, C185T, T16317C, T16343C, A16352G, G16355A, A16359G, DL/T16480). The rest of the identified mutations were known worldwide such as T73C, A146G, G150A, G151A, A152G, A16362G, C16390T, A16519G, and G16527A which were illustrated in (Table 1).

Table 1: D-Loop region variants (mutations) were detected in breast cancer samples. The position in the whole D-Loop which refers to the nucleotide number (position) in the whole mtDNA while the position in the D-Loop refers to the nucleotide number (position) in the amplified region.

NO.	Sample	Mutations	Changed Nucleotide	Gene Region	Position in the whole D-Loop	Position in D-Loop	Type of Mutations
1	BCL 16 MCL 55	Ins G58	Ins G	GAAA-ATACCA	58	208-209	Insertion
2	BCL 16 MCL 40	G64A	G/A	CCCCAGACGA	64	202	Substitution

	MCL 41 MCL 55						
3	BCL 1 BCL 2 BCL 4 BCL 5 BCL 6 BCL 8 BCL 9 BCL 14 BCL 15 BCL 17 BCL 18 BCL 20 MCL 30 MCL 31 MCL 36 MCL 37 MCL 43 MCL 45 MCL 49 MCL 57 MCL 58 MCL 63	T73C	T/C	TGCATACCC	73	193	Substitution
4	BCL 11 BCL 20 MCL 35 MCL 40 MCL 63	A146G	A/G	GGATGAGGCA	146	120	Substitution
5	BCL 5 MCL 45 MCL 58	G150A	G/A	ATAGGATGAG	150	116	Substitution
6	MCL 31	G151A	G/A	AATAGGATGA	151	115	Substitution
7	BCL 1 BCL 8 BCL 9 BCL 13 BCL 19 BCL 20 MCL 31 MCL 34 MCL 35 MCL 37 MCL 49 MCL 52 MCL 54 MCL 58 MCL 61 MCL 63	A152G	A/G	TAATAGGAT	152	114	Substitution

8	MCL 45	C185T	C/T	TTCGCCTGT	185	81	Substitution
9	MCL 31	T16317C	T/C	GGCTTTATG	16317	311	Substitution
10	MCL 45	T16343C	T/C	ACTGTAATG	16343	492	Substitution
11	MCL 35	A16352G	A/G	AGGGATTTG	16352	483	Substitution
12	MCL 40	G16355A	G/A	AGAAGGGAT	16355	480	Substitution
13	BCL 17	A16359G	A/G	GACGAGAA	16359	476	Substitution
14	BCL 16 MCL 40 MCL 55	A16362G	A/G	GGGGACGAG	16362	473	Substitution
15	MCL 31 MCL 45	C16390T	C/T	GGGACCCCTAT	16390	445	Substitution
16	MCL 55	DL/T16480	DL /T	CACTTTAGCTA	16480	355	Deletion
17	BCL 2 BCL 3 BCL 4 BCL 5 BCL 8 BCL 9 BCL 13 BCL 14 BCL 15 BCL 17 MCL 31 MCL 33 MCL 34 MCL 36 MCL 45 MCL 49 MCL 54 MCL 57 MCL 58 MCL 61	A16519G	A/G	TTATGACCCT	16519	316	Substitution
18	MCL 31	G16527A	G/A	TTTAGGCTTT	16527	308	Substitution

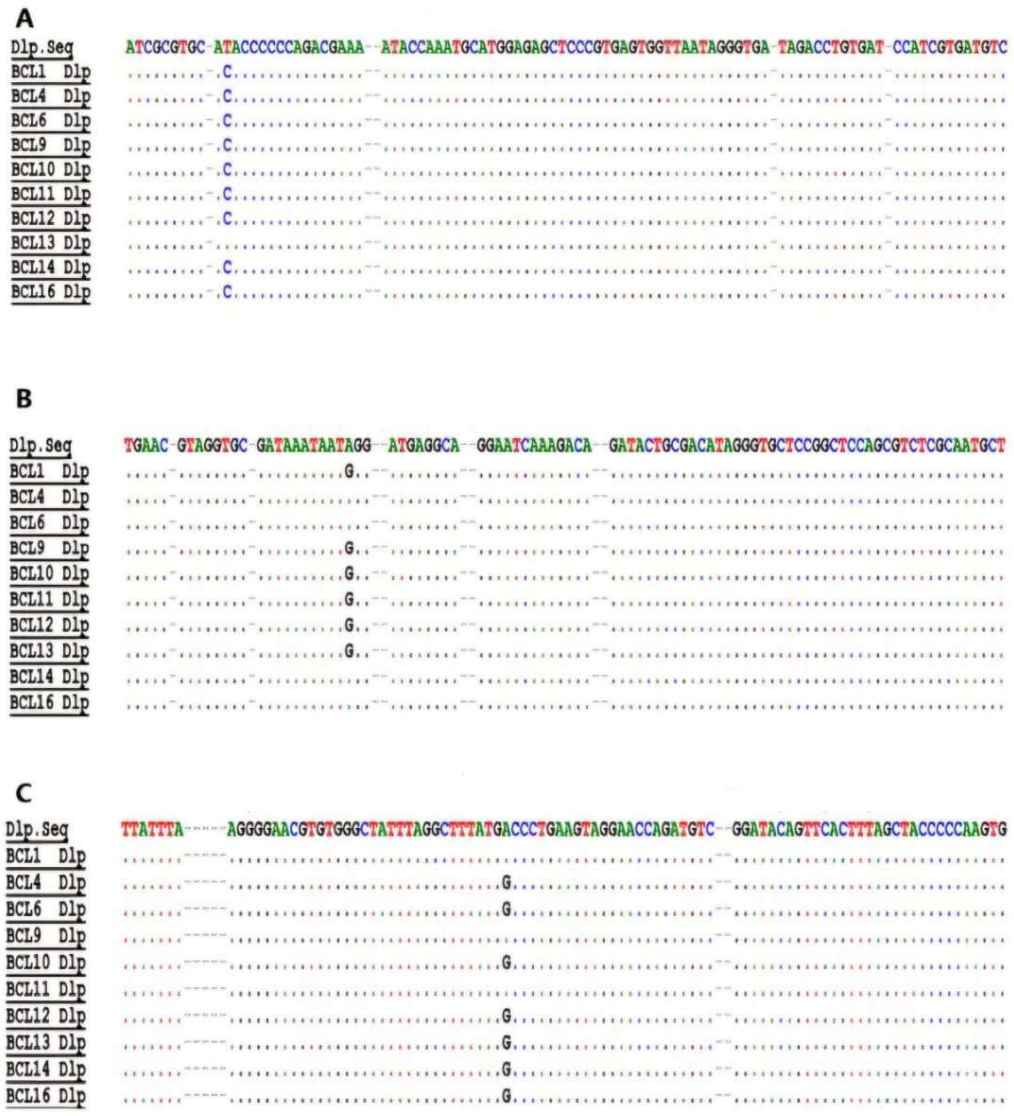
All identified mutations were within two HVs regions of D-loop. Ten of them were predominantly found in HV1 and eight in HV2, as illustrated in (Table 2).

Table 2: The table shows the percentage and frequency of D-Loop mutations in the samples along with their location within the region of the D-Loop.

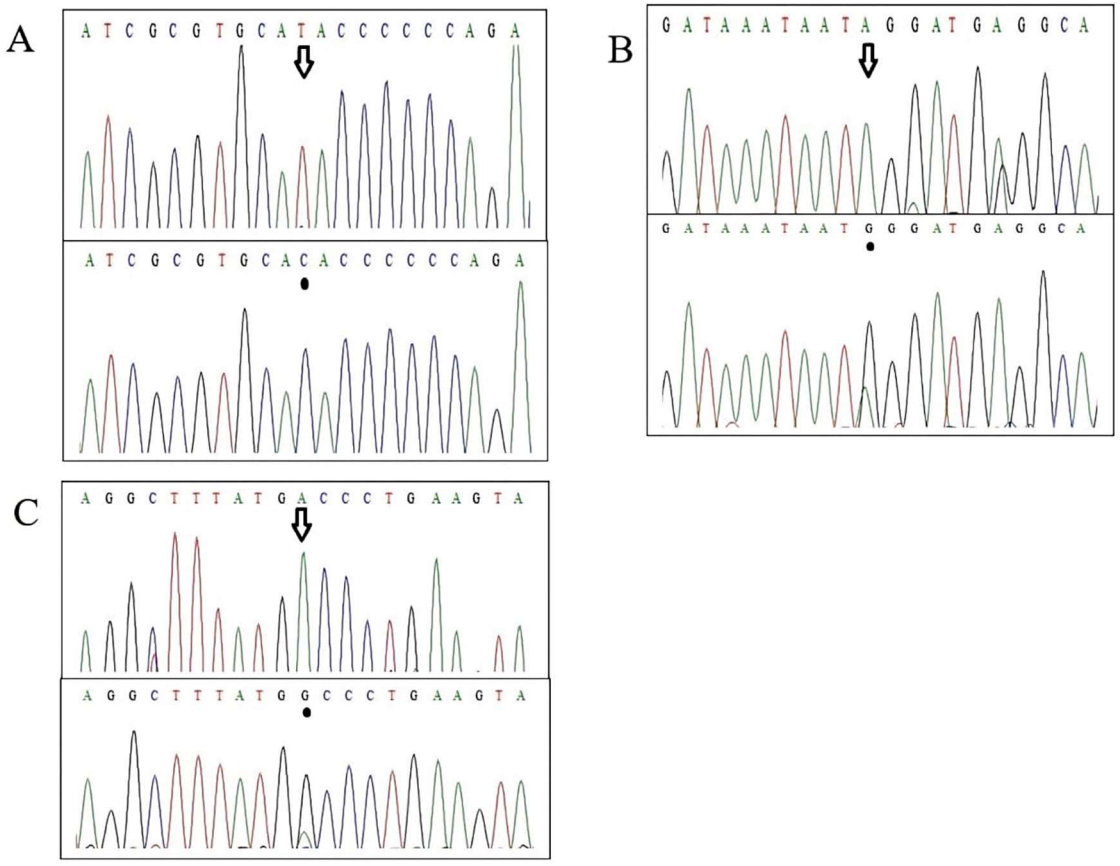
Frequency of mutations in D-Loop region				
NO.	**Mutations**	**Location in (HV1 or HV2)**	**Frequency**	**Percentage %**
1	InsG 58	HV2	2	5%
2	G64A	HV2	4	10%
3	T73C	HV2	22	55%
4	A146G	HV2	5	13%
5	G150A	HV2	3	7.50%
6	G151A	HV2	1	2.5%

7	A152G	HV2	16	40.00%
8	C185T	HV2	1	2.5%
9	T16317C	HV1	1	2.5%
10	T16343C	HV1	1	2.5%
11	A16352G	HV1	1	2.5%
12	G16355A	HV1	1	2.5%
13	A16359G	HV1	1	2.5%
14	A16362G	HV1	3	7.50%
15	C16390T	HV1	2	5%
16	DL/T16480	HV1	1	2.5%
17	A16519G	HV1	20	50%
18	G16527A	HV1	1	2.5%

Interestingly, substitution type of mutation was the most common mutation with the highest frequency and percentage. For instance, T nucleotide was substituted by C nucleotide with a frequency of 55% at 73 (T73C) positions, A nucleotide was substituted by G nucleotide with a frequency of 50% at position 16519 (A16519G, also known as rs3937033), and 40 % of A nucleotide was substituted with G at position 152 (A152G). (Figures 3 and 4) .



(Figure 3): The global sequence (NCBI Reference Sequence: NC_012920.1) was aligned with the most prevalent mutation of the d-loop region using bioEdit software. A: T 73 C mutation with a frequency of 55%; B: A152 G mutation with a frequency of 55%; C: A 16519 G mutation with a frequency of 50%



(Figure 4): A four-color chromatogram generated by an automated sequencing machine displays the most common mutations found in the D-Loop region (A): T 73 C (B): A152 G and (C): A 16519 G

Among these mutations, their presence has been reported in other types of cancer[34-37].

There are many mtDNA variations in the D-Loop region of breast cancer tissue. Among these changes are the MnlI restriction sites, which are between nucleotide positions 16,106 and 16,437 (13). We identified five mutations in this region which are (A16362G, T16317C, C16390T, A16352G, T16343C). The regulation of mitochondrial genome replication and expression depends on the mitochondrial D-loop, a hotspot for mtDNA changes. Consequently, Mutations in the D-loop region affect transcription, protein synthesis, and mtDNA replication, which are all directly impacted by D-loop mutations[38, 39].

D-loop controls mtDNA synthesis and transcription, which are vital for the active growth of ATP. Changes like A16519G can interfere with these processes and result in cellular energy depletion, which is important in diseases such as breast cancer. Mutations can enhance reactive oxygen species (ROS) generation, contributing to cellular damage and illness progression. For instance, A152G has been associated with increased oxidative stress, which is implicated in several diseases, including cancer. Determining certain D-loop mutations could help scientists better understand breast cancer behavior and early detection and treatment. [40, 41].

Conclusion

In this research, the D-Loop region of mtDNA was extracted and amplified from female breast cancer patients. PCR and Sanger sequencing techniques were utilized to investigate the D-loop region mutations. We found 18 mutations and to the best of our knowledge, InsG58, G64A, C185T, T16317C, T16343C, A16352G, G16355A, A16359G, DL/T16480 mutations were identified for the first time in Iraqi patients. Moreover, it has been shown that all mutations were within the HV1 and HV2 regions of the D-Loop, and substitution mutation was the most common and frequent mutation, among others. That indicates that D-loop region mutations may play an important role in the development of breast cancer.

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References

[1] A. Talei, M. Akrami, M. Mokhtari, and S. Tahmasebi, "Surgical and clinical pathology of breast diseases," *Histopathology-Reviews and Recent Advances,* 2012.
[2] F. Ye *et al.*, "Advancements in clinical aspects of targeted therapy and immunotherapy in breast cancer," *Molecular Cancer,* vol. 22, no. 1, p. 105, 2023/07/06 2023, doi: 10.1186/s12943-023-01805-y.
[3] L. A. Korde *et al.*, "Multidisciplinary meeting on male breast cancer: summary and research recommendations," *Journal of clinical oncology,* vol. 28, no. 12, pp. 2114-2122, 2010.
[4] S. A. Massarweh and G. L. Choi, "Special considerations in the evaluation and management of breast cancer in men," *Current problems in cancer,* vol. 40, no. 2-4, pp. 163-171, 2016.
[5] J. Xing, L. Qi, X. Liu, G. Shi, X. Sun, and Y. Yang, "Roles of mitochondrial fusion and fission in breast cancer progression: a systematic review," *World Journal of Surgical Oncology,* vol. 20, no. 1, p. 331, 2022.
[6] S. Liu *et al.*, "Metabolic reprogramming and therapeutic resistance in primary and metastatic breast cancer," *Molecular Cancer,* vol. 23, no. 1, p. 261, 2024/11/21 2024, doi: 10.1186/s12943-024-02165-x.
[7] M. M. Jahani, A. Azimi Meibody, T. Karimi, M. M. Banoei, and M. Houshmand, "An A10398G mitochondrial DNA alteration is related to increased risk of breast cancer, and associates with Her2 positive receptor," *Mitochondrial DNA Part A,* vol. 31, no. 1, pp. 11-16, 2020.
[8] Y.-c. Xu, J. Su, J.-j. Zhou, Q. Yuan, and J.-s. Han, "Roles of MT-ND1 in Cancer," *Current Medical Science,* vol. 43, no. 5, pp. 869-878, 2023.
[9] M. M. Nass and S. Nass, "Intramitochondrial fibers with DNA characteristics: I. Fixation and electron staining reactions," *The Journal of cell biology,* vol. 19, no. 3, pp. 593-611, 1963.
[10] M. Birch-Machin, "The role of mitochondria in ageing and carcinogenesis," *Clinical and experimental dermatology,* vol. 31, no. 4, pp. 548-552, 2006.
[11] T. E. Rohan, L.-J. Wong, T. Wang, J. Haines, and G. C. Kabat, "Do alterations in mitochondrial DNA play a role in breast carcinogenesis?," *Journal of oncology,* vol. 2010, no. 1, p. 604304, 2010.
[12] A. Hahn and S. Zuryn, "Mitochondrial genome (mtDNA) mutations that generate reactive oxygen species," *Antioxidants,* vol. 8, no. 9, p. 392, 2019.
[13] S. Tommasi *et al.*, "Mitochondrial DNA variants and risk of familial breast cancer: an exploratory study," *International journal of oncology,* vol. 44, no. 5, pp. 1691-1698, 2014.
[14] Y.-B. Zhao, H.-Y. Yang, X.-W. Zhang, and G.-Y. Chen, "Mutation in D-loop region of mitochondrial DNA in gastric cancer and its significance," *World Journal of Gastroenterology: WJG,* vol. 11, no. 21, p. 3304, 2005.
[15] I. Czegle, C. Huang, P. G. Soria, D. W. Purkiss, A. Shields, and E. A. Wappler-Guzzetta, "The role of genetic mutations in mitochondrial-driven cancer growth in selected tumors: breast and gynecological Malignancies," *Life,* vol. 13, no. 4, p. 996, 2023.
[16] S. Anderson *et al.*, "Sequence and organization of the human mitochondrial genome," *Nature,* vol. 290, no. 5806, pp. 457-465, 1981.
[17] R. Filograna, M. Mennuni, D. Alsina, and N. G. Larsson, "Mitochondrial DNA copy number in human disease: the more the better?," *FEBS letters,* vol. 595, no. 8, pp. 976-1002, 2021.
[18] J. S. Carew and P. Huang, "Mitochondrial defects in cancer," *Molecular cancer,* vol. 1, pp. 1-12, 2002.
[19] A. Lièvre *et al.*, "Clinical value of mitochondrial mutations in colorectal cancer," *Journal of Clinical Oncology,* vol. 23, no. 15, pp. 3517-3525, 2005.

[20] C. J. Pérez-Amado *et al.*, "Mitochondrial DNA mutation analysis in breast cancer: shifting from germline heteroplasmy toward homoplasmy in tumors," *Frontiers in Oncology,* vol. 10, p. 572954, 2020.
[21] S. R. Lee and J. Han, "Mitochondrial nucleoid: shield and switch of the mitochondrial genome," *Oxidative medicine and cellular longevity,* vol. 2017, no. 1, p. 8060949, 2017.
[22] B. C. Levin, H. Cheng, and D. J. Reeder, "A human mitochondrial DNA standard reference material for quality control in forensic identification, medical diagnosis, and mutation detection," *Genomics,* vol. 55, no. 2, pp. 135-146, 1999.
[23] D. Syndercombe Court, "Mitochondrial DNA in forensic use," *Emerging Topics in Life Sciences,* vol. 5, no. 3, pp. 415-426, 2021.
[24] H. Sharma, A. Singh, C. Sharma, S. K. Jain, and N. Singh, "Mutations in the mitochondrial DNA D-loop region are frequent in cervical cancer," *Cancer Cell International,* vol. 5, no. 1, p. 34, 2005/12/16 2005, doi: 10.1186/1475-2867-5-34.
[25] P. Parrella *et al.*, "Detection of mitochondrial DNA mutations in primary breast cancer and fine-needle aspirates," *Cancer research,* vol. 61, no. 20, pp. 7623-7626, 2001.
[26] Y. Lin, B. Yang, Y. Huang, Y. Zhang, Y. Jiang, L. Ma, and Y.-Q. Shen, "Mitochondrial DNA-targeted therapy: A novel approach to combat cancer," *Cell Insight,* vol. 2, no. 4, p. 100113, 2023/08/01/ 2023
[27] M. Suzuki, S. Toyooka, K. Miyajima, T. Iizasa, T. Fujisawa, N. B. Bekele, and A. F. Gazdar, "Alterations in the mitochondrial displacement loop in lung cancers," *Clinical cancer research,* vol. 9, no. 15, pp. 5636-5641, 2003.
[28] A. Dhamad, M. Zampiga, E. S. Greene, F. Sirri, and S. Dridi, "Neuropeptide Y and its receptors are expressed in chicken skeletal muscle and regulate mitochondrial function," *General and Comparative Endocrinology,* vol. 310, p. 113798, 2021.
[29] M. Sanchez-Cespedes *et al.*, "Identification of a mononucleotide repeat as a major target for mitochondrial DNA alterations in human tumors," *Cancer research,* vol. 61, no. 19, pp. 7015-7019, 2001.
[30] C. Aral, H. Kaya, Ç. Ataizi-Çelikel, M. Akkiprik, Ö. Sönmez, B. M. Güllüoğlu, and A. Özer, "A novel approach for rapid screening of mitochondrial D310 polymorphism," *BMC cancer,* vol. 6, pp. 1-5, 2006.
[31] A. Amorim, T. Fernandes, and N. Taveira, "Mitochondrial DNA in human identification: a review," *PeerJ,* vol. 7, p. e7314, 2019.
[32] S. Santos, D. Sá, E. Bastos, H. Guedes-Pinto, I. Gut, F. Gärtner, and R. Chaves, "An efficient protocol for genomic DNA extraction from formalin-fixed paraffin-embedded tissues," *Research in veterinary science,* vol. 86, no. 3, pp. 421-426, 2009.
[33] P. Dedhia, S. Tarale, G. Dhongde, R. Khadapkar, and B. Das, "Evaluation of DNA extraction methods and real time PCR optimization on formalin-fixed paraffin-embedded tissues," *Asian Pacific Journal of Cancer Prevention,* vol. 8, no. 1, p. 55, 2007.
[34] V. W. Liu *et al.*, "High incidence of somatic mitochondrial DNA mutations in human ovarian carcinomas," *Cancer research,* vol. 61, no. 16, pp. 5998-6001, 2001.
[35] J. S. Penta, F. Johnson, J. T. Wachsman, and W. C. Copeland, "Mitochondrial DNA in human malignancy," *Mutation Research/Reviews in Mutation Research,* vol. 488, no. 2, pp. 119-133, 2001.
[36] E. Kirches *et al.*, "High frequency of mitochondrial DNA mutations in glioblastoma multiforme identified by direct sequence comparison to blood samples," *International journal of cancer,* vol. 93, no. 4, pp. 534-538, 2001.
[37] K. Hibi *et al.*, "Mitochondrial DNA alteration in esophageal cancer," *International journal of cancer,* vol. 92, no. 3, pp. 319-321, 2001.
[38] R.-K. Bai, S. M. Leal, D. Covarrubias, A. Liu, and L.-J. C. Wong, "Mitochondrial genetic background modifies breast cancer risk," *Cancer research,* vol. 67, no. 10, pp. 4687-4694, 2007.
[39] N. R. Tipirisetti *et al.*, "Mitochondrial control region alterations and breast cancer risk: a study in South Indian population," *PloS one,* vol. 9, no. 1, p. e85363, 2014.

[40] L. Wang, H.-X. Cheng, Y.-H. Zhou, and M. Ma, "Clinical significance of the D-loop gene mutation in mitochondrial DNA in laryngeal cancer," *OncoTargets and therapy,* pp. 3461-3466, 2021.
[41] C. Ye *et al.*, "Mutations in the mitochondrial DNA D-loop region and breast cancer risk," *Breast cancer research and treatment,* vol. 119, pp. 431-436, 2010.