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BREAST CANCER AND MITOCHONDRIAL DNA MUTATIONS IN KURDISH WOMEN: A CASE-CONTROL STUDY FROM SULAYMANIYAH, IRAQ.

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Abstract

The molecular basis of breast cancer has been thoroughly investigated in the last decade. Many nuclear susceptibility genes were discovered. Moreover, research about mitochondrial DNA (mtDNA) mutations and mitochondrial haplogroup determinants (single nucleotide polymorphism; SNP) has explored their potential role in cancers in general and breast cancer in particular.

This study is to identify breast cancer-related SNPs and mtDNA haplogroups among Kurdish women living in Sulaymaniyah/Iraq.

This case-control study was conducted in Kurdistan Institute for Strategic and Scientific Research (KISSR) / Molecular Lab in Sulaymaniyah in collaboration with a specialized lab in South Korea. Twenty women with breast cancer and 20 women with benign breast diseases were enrolled. The entire mitochondrial genome of 40 breast tissue specimens was sequenced. Haplogrep 2.0 was utilized for haplogroup identification. Statistical Analysis was performed using Chi-square and Fisher's exact tests.

A total of 547 mutations (Cancer, n=344 and Control, n=203) were identified including 15 first-reported mutations. HV haplogroup in the cancer samples was a risk factor for the development of breast cancer (p=0.002) compared to H haplogroup in the control samples (p =0.006) (Odd Ratio [OR] = 28.00). Furthermore, SNP (A8860G) was an additional risk compared to other randomly selected SNPs (A750G, A1438G, and C7028T) (p <0.05 and OR >1).

In conclusions; the association of certain mtDNA haplogroups and SNPs with breast cancer risk is not new. Unlike studies performed in other populations of the world figuring out A10398G as the risky SNP, our study identified A8860G in the Kurds. Geographic and ethnic variations between human populations do exist, so an SNP that is common in one population group may be much rarer in another. Hence, more research on the molecular biology of breast cancer in our locality is warranted to clarify the situation.

Keywords: Breast tumours, Cancer genetics, Molecular Biology, Mitochondrial DNA, Mutation.

Introduction

Breast cancer is a heterogeneous, debilitating disease of a multistep carcinogenic background [1–3]. It is the most frequently diagnosed cancer in women [2–4]. The incidence of breast cancer among women in Kurdistan region, Iraq approaches the western countries and could be even higher particularly in the young [2,3,5]. "Although, major progresses have been made over the past decade in exploring the molecular basis of breast cancer and the discovery of several susceptibility nuclear-genes of high, moderate and low penetrance as predisposing factors for breast cancer, these genes hardly explain 10-15% of the cases" [2,3,6].

"Mitochondria are maternally inherited, cytoplasmic organelles acting as the main producers of energy in eukaryotic cells. They retain a small DNA genome. Mitochondrial DNA (mtDNA) is a circular molecule of 16,569 bp, coding for 2 ribosomal RNAs (12S and 16S), 22 transfer RNAs and 13 essential protein subunits of the oxidative phosphorylation system (OXPHOS)" [2,3]. "The only non-coding region is the D-loop region (displacement-region), which has been identified as a regulatory region of mtDNA replication and transcription, containing a replication origin and promoter region" [7].

"It is well known that (mtDNA) has a mutation rate several times higher than nuclear DNA. This is attributed to its limited repair mechanisms, lack of protective histones and its close proximity to the electron transport chain, which continuously generates free radicals. Furthermore, mtDNA is

organized in an economic pattern with its genes lacking introns. Otto Warburg, in 1956, observed that cancers ferment glucose in the presence of oxygen. Accordingly, Warburg proposed that abnormalities in mitochondrial respiration may be responsible for cancer production" [2,3,8].

Recently, researchers studied possible relationship of mitochondrial genome mutations with different types of cancer including breast cancer. The studies involved mitochondrial haplogroups and single nucleotide polymorphisms (SNP)'s. Mitochondrial haplogroups refer to mtDNA variations while SNPs represent the simplest form of DNA variation among individuals in which a single nucleotide at a specific position in the genome is replaced by another. Research figured out significant association between specific mtDNA haplogroups and some cancers, including breast cancer and identified specific mutations in cancer cases like T16189C, G10398A and the (4977) deletion mutation of mtDNA [2,3,9].

Breast cancer contributes to 9.6% of global cancer related deaths. This cancer is considered an aggressive systemic disease since it is able to metastasize in its early stages with high death rate among patients who present with recurrences. Therefore, doctors need early-stage tumor markers in order to diagnose the disease as early as possible [7]. "Previous research has often focused on nuclear gene mutations, while mitochondrial gene mutations have attracted considerably less attention. In 1998, Polyak et al. reported mtDNA mutations in 7 of 10 colorectal cancer cases for the first time. Subsequently, many scholars have confirmed that detection of mtDNA mutations in tumor cells is simpler and more reliable than that of nDNA" [3].

In Iraq, a few population studies based on mitochondrial DNA haplogroups analysis were published [10–13]. In the clinical field, a master student from the University of Sulaimani has performed a research on mtDNA in 5 patients with congenital neuromuscular dysfunction (personal communication). Moreover, Ismaeel et al [14] and Dhahi & Mahdi [15] published 2 papers about association of breast cancer and specific mtDNA mutations. The current study was performed to identify potential link of certain mtDNA haplogroups and SNPs with breast cancer in Kurdish women of Sulaymaniyah, Iraq using the whole rather than selected mtDNA genome analysis.

Patients & Methods

Sample Selection: "The study was approved by the Ethical Committee of the College of Medicine/ University of Sulaimani (reference number 44, on January 30, 2017). A total of 40 subjects (20 breast cancer tissue and 20 control samples) were recruited for the study. Control samples were taken from benign breast tissue specimens (fibroadenoma and non-proliferative fibrocystic breast disease). Breast cancer tissue samples were taken from mastectomy specimens of women already diagnosed with invasive ductal carcinoma (Grade II and III) by core biopsy and with no family history of breast cancer. All samples were from unrelated Kurdish women living in the center of the city. The study was conducted in the Molecular Biology Lab of Kurdistan Institute for Strategic and Scientific Research (KISSR) in collaboration with a specialized lab in South Korea and spanned one year ending at March 1, 2018" [2,3].

DNA Extraction: "Total genomic DNA was extracted using DNA extraction kit (GeNet bio/South Korea). The extraction was performed according to the manufacturer's instruction. Purity and concentration of the extracted DNA were obtained using a Biophotometer (Eppendorf/Germany)" [2,3].

PCR Amplification and Sequencing: "The entire mitochondrial genome was amplified in the form of four overlapping PCR fragments using long Taq kit (Dongsheng Biotech/China) and the primers listed in Table 1 [https://www.medrxiv.org/content/10.1101/2021.02.12.21249541v1.full-text#T1]" [2,3]. "The amplified PCR products were purified using PCR purification kit (NORGEN biotek/Canada) and the primers listed in Table 2 [https://www.medrxiv.org/content/10.1101/2021.02.12.21249541v1.full#T2] were used for sequencing of the amplified mtDNA fragments" [3].

Data Analysis: "The algorithm implemented in the HaploGrep 2.0 was used for identification of haplogroups [16]. Chi-square and Fishers exact test were used to determine the significance of relations of breast cancer with haplogroups and SNPs. The https://www.mitomap.org/MITOMAP website which provides a comprehensive database for human mitochondrial DNA was used for allocation of mutations, identifying types of the mutations and determining amino acid substitutions" [2,3].

Table 1: Sequence of the amplification Primers of four overlapping mitochondrial DNA fragments

	Primer name	Sequence
1	1F	5 -AGG TCT ATC ACC CTA TTA ACC ACT CA-3'
2	2F	5 -CAA GAG CCT TCA AAG CCC TCA GTA-3'
3	3F	5 -ACG CCA CTT ATC CAG TGA ACC ACT-3'
4	4F	5 -CCT AGC AAT AAT CCC CAT CCT CCA-3'
5	1R	5 -TGA GCA AGA GGT GGT GAG GTT GAT-3'
6	2R	5 -GGG CAC CGA TTA TTA GGG GAA CTA-3'
7	3R	5 -TAT GAG AAT GAC TGC GCC GGT GAA-3'
8	4R	5 -CGT GAT GTC TTA TTT AAG GGG AAC GT-3'

Table 2: The Sequencing primers of the four overlapping fragments of mitochondrial DNA

Primer name	Sequence	Position
5MT	5'-TGA ACT CAC TGG AAC GGG GAT GCT-3'	723-700
6MT	5'- GCA GAA GGT ATA GGG GTT AGT CCT-3'	1852-1829
7MT	5'- ATG CCT GTG TTG TGA GAG TGA-3'	2439-2416
8MT	5'-TCT TGT CCT TTC GTA CAG GGA GGA-3'	3138-3115
9MT	5'-CTG AGA CTA GTT CGG ACT CCC CTT-3'	3934-3911
10MT	5'- CGG TTG CTT GCG TGA GGA AAT ACT-3'	4665-4642
11MT	5'- GGA GTA GTG TGA TTG AGG TGG AGT-3'	5385-5362
12MT	5'-GGA GTG TGG CGA GTC AGC TAA ATA-3'	6885-6862
13MT	5'- AAG GGC ATA GAG GAC TAG GAA GCA-3'	7711-7688
14MT	5'- AGG GAG GTA GGT GGT AGT TTG TGT-3'	8477-8454
15MT	5'- GGG GTC ATG GGC TGG GTT TTA CTA-3'	9258-9235
16MT	5'- TAT AGG GTCGAA GCC GCA CTC GTA-3'	10190-10167
17MT	5'- GTG AGG GGT AGG AGT CAG GTA GTT-3'	10986-10963
18MT	5'- TAG GGA AGT CAG GGT TAG GGT GGT-3'	12381-12358
19MT	5'- AGT GCT TGA GTG GAG TAG GGC TGA-3'	13089-13066
20MT	5'- AAT CCT GCG AAT AGG CTT CCG GCT-3'	13733-13710
21MT	5'- GCT ATT GAG GAG TAT CCT GAG GCA-3'	14454-14431
22MT	5'- TGC AAG CAG GAG GAT AAT GCC GAT-3'	15112-15089
23MT	5'- GGT AGC TTA CTG GTT GTC CTC CGA-3'	15782-15759

Results

"A total of 344 mutations in the cancer samples and 203 mutations in the control samples were identified in the current study. The majority of the mutations were point mutations with only four insertion mutation regions. Based on the MITOMAP databases, single nucleotide polymorphisms (SNP) of the Kurdish ethnicity were identified and accounted for 74% of all mutations in the breast cancer samples, of which 61% were distributed in the coding region and 39% were in the non-coding region. Whereas, in the control samples, SNPs constituted 90% and the pattern of distributions were 58% in the coding region and 42% in the non-coding region" [2,3]. Full details of total SNP mutations in breast cancer and in control group samples are shown in Table 3 [https://www.medrxiv.org/content/10.1101/2021.02.12.21249541v1.full#T3] and Table 4 [https://www.medrxiv.org/content/10.1101/2021.02.12.21249541v1.full#T4].

Table 3: Total single nucleotide polymorphism (SNP) mutations in breast cancer samples

SNP	Gene/region	Nucleotide substitution	Amino acid Substitution	SNP	Gene/ region	Nucleotide substitution
3394	ND1	T>C	Tyr-His	16086	HV1	T>C
3834	ND1	G>A	Sync	16172	HV1	T>C
3741	ND1	C>T	Sync	16186	HV1	C>T
4011	ND1	C>T	Sync	16187	HV1	C>T
4216	ND1	T>C	Tyr-His	16189	HV1	T>C
4769	ND2	A>G	Sync	16192	HV1	C>T
4917	ND2	A>G	Asn-Asp	16193	HV1	C>T
7028	Cox1	C>T	Sync	16217	HV1	T>C
8137	Cox2	C>T	Sync	16223	HV1	C>T
8684	ATPase 6	C>T	Thr-Ile	16274	HV1	G>A
8697	ATPase 6	G>A	Sync	16209	HV1	T>C
8860	ATPase 6	A>G	Thr-Ala	16234	HV1	C>T
9755	Cox3	G>A	Sync	16249	HV1	T>C
9899	Cox3	T>C	Sync	16291	HV1	C>T
10142	ND3	C>T	Sync	16294	HV1	C>T
10586	ND4L	G>A	Sync	16309	HV1	A>G
11251	ND4	A>G	Sync	16318	HV1	A>T
11467	ND4	A>G	Sync	16362	HV1	T>C
11719	ND4	G>A	sync	16519	HV1	T>C
12372	ND5	G>A	Sync	146	HV2	T>C
12612	ND5	A>G	Sync	151	HV2	C>T
12618	ND5	G>A	Sync	152	HV2	T>C
12705	ND5	C>T	Sync	195	HV2	T>C
12879	ND5	T>C	Sync	Poly C insertion (309, 310 C	HV2	
				and 311)		
13104	ND5	C>T	Sync	263	HV2	A>G
13188	ND5	C>T	Sync	417	HV2	G.>A
13368	ND5	G>A	Sync	499	HV2	G>A
13500	ND5	T>C	Sync	462	HV3	C>T
13708	ND5	G>A	Ala-Thr	489	HV3	T>C
14139	ND5	A>G	Sync	10463	tRNA-Arg	T>C
14364	ND6	G>A	Sync			
14569	ND6	G>A	Ala-Thr			
14905	Cyt-B	G>A	Sync			
14766	Cyt-B	C>T	Thr-Ile			
15148	Cyt-B	G>A	Synch			
15326	Cyt-B	A>G	Thr-Ala			
15452	Cyt-B	C>A	Leu-Ile			
15607	Cyt-B	T>C	Sync			
12308	tRNA	A>G				
10463	tRNA	A>G				
709	rRNA	G>A				
750	rRNA	A>G				
980	rRNA	T>C				
1438	rRNA	A>G				
1811	rRNA	A>G				
1888	rRNA	G>A				
2259	rRNA	C>T				
3010	rRNA	G>A				
2706	rRNA	A>G				

Red coloured residues were identified in more than one case

"Nine Western Eurasian haplogroups and their subclasses were identified in both cancer and control samples using the Haplogrep 2.0 program. Haplogroups HV, N, R, U, J, T and H were identified in breast cancer samples, while H, HV, N, R0, J, X and W haplogroups were identified in the control samples. The most common haplogroup in the control samples was the H-haplogroup (60%)" [2,3]. while it was less frequent in breast cancer samples accounting for (5%) of cases only. In contrast, the most frequent haplogroup in breast cancer samples was HV (35%) followed by N (25%) (Table 5).

"A statistically significant association between haplogroup HV and breast cancer was identified using Chi-square and Fisher's exact tests (p-values were 0.002 and 0.006 respectively) with an odd ratio (OR) of 28. Furthermore, the homoplasmic mutation, SNP (A8860G) (Figure 1) was identified in all 20 breast cancer samples (100%), while, in control samples, it was identified in 4 samples (20%) only" [2,3].

"To identify the significance of this mutation, Chi-square, Fishers exact test were used and OR were calculated and compared with three other randomly selected SNPs (A750G, A1438G and C7028T) (Figure 2a, 2b and 2c)" [2,3].

Table 5: Frequency and percentage of the identified Haplogroups in breast cancer (A) and control (B) subjects

Breast cancer samples (A)		Control samples (B)	
Haplogroup	(n, %)	Haplogroup	(n, %)
HV	(7, 35)	H	(12, 60)
N	(5, 25)	HV	(3, 20)
U7	(2, 10)	N	(1, 5)
R0	(2, 10)	R0	(1, 5)
J	(1, 5)	J1	(1, 5)
U1	(1, 5)	X	(1, 5)
T	(1, 5)	W	(1, 5)
H	(1, 5)	-	-
Total	(20, 100)	Total	(20, 100)

Figure 1: "Electropherogram and sequence of the A8860G region (Point mutation site is indicated by an arrow)" [2,3]

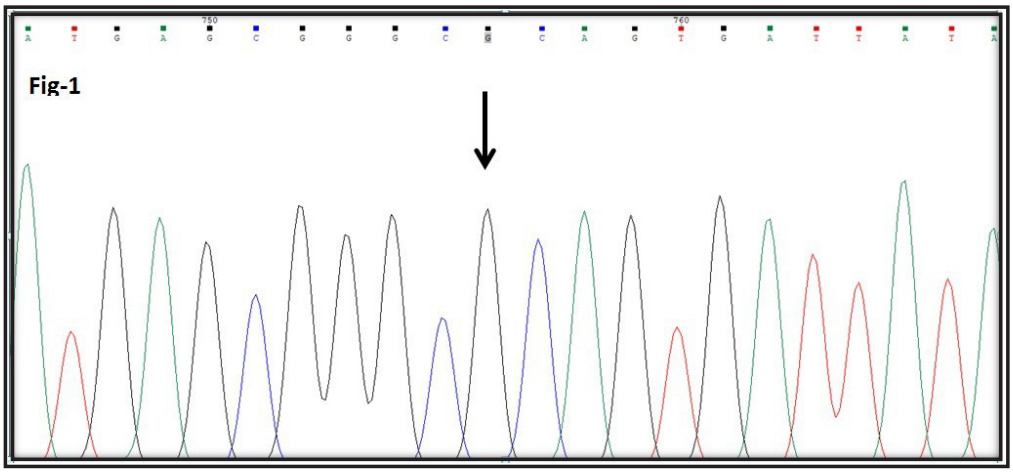


Figure 2a: "Electropherogram and sequence of the A750G region" [2,3]

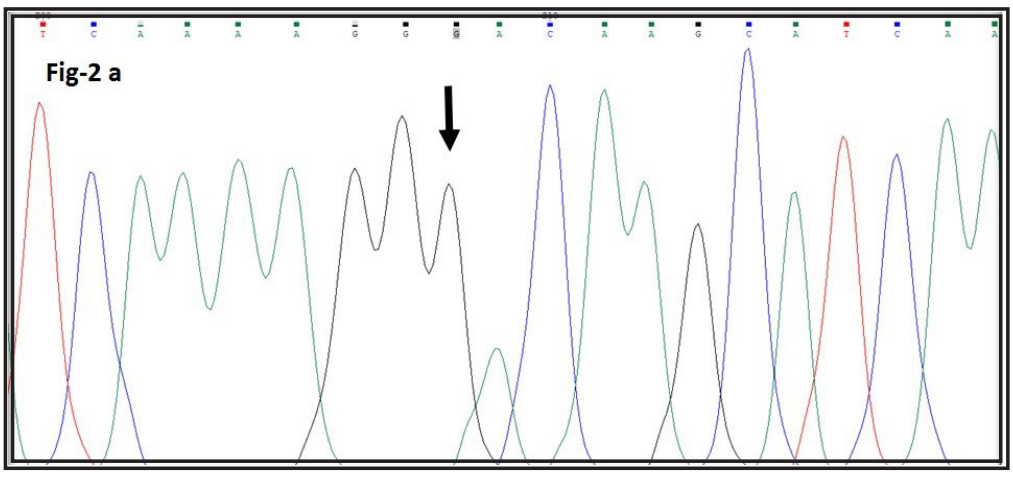


Figure 2b: "Electropherogram and sequence of the A1438G region" [2,3]

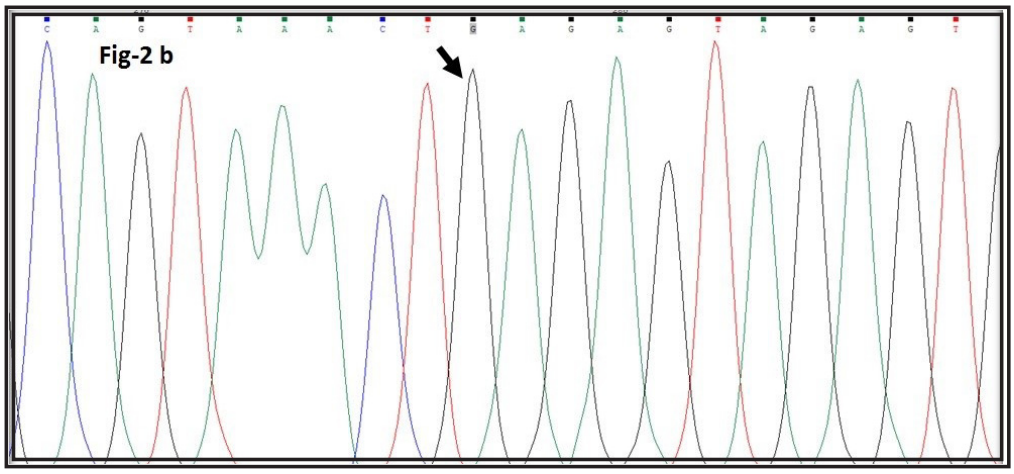
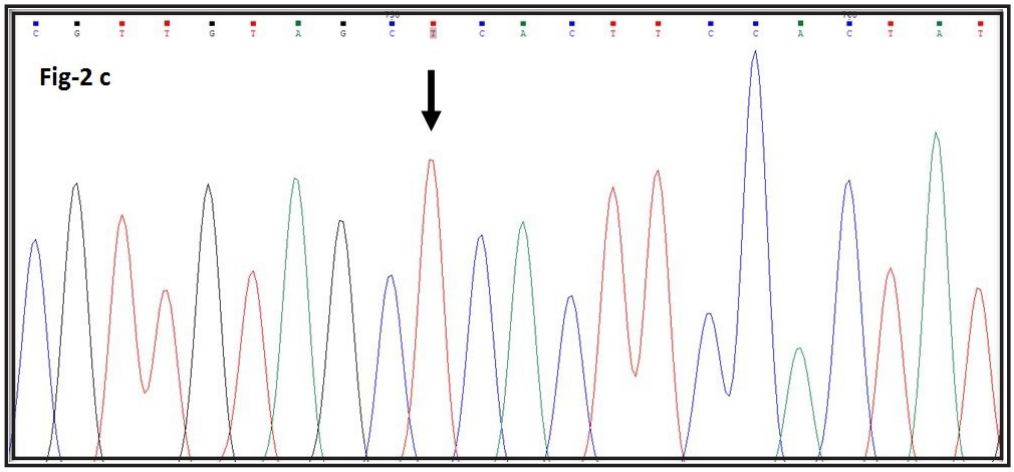


Figure 2c: "Electropherogram and sequence of the C7028T region (Point mutation site is indicated by an arrow)



As shown in (Table 6), the OR values were greater than 1 and the p-values were less than 1, indicating SN" [2,3].

Table 6: Calculated OR and p values for SNP A8860G with three randomly selected SNPs [2,3]

SNPs	Odd ratio	P value (chi-square)	P value (Fishers exact)
8860/750	4.722	0.0012	0.0014
8860/1438	5	0.009	0.013
8860/7028	5	0.011	0.021

Worthy to notice that in the current study, 15 new mutations were reported for the first time (Table 7).

Table 7: Newly recognized mtDNA mutations.

Name of Mutation	Location	Effect
C4068G	ND1	synch
C4126G	ND1	Arg-Gly
A4590G	ND2	Ile-Val
C7418G	Cox 1	Phe-Leu
C7687G	Cox 2	Ile-Met
9956-57 T insertion	Cox 3	Frame shift
T9965A	Cox 3	Tyr-stop
A10784C	ND4	Ile-Leu
CA13166 and 67GG	ND5	Thr-Stop
A13862C	ND5	Asn-Thr
A14500T	ND6	Tyr-Asn
T14868C	Cyt-B	Leu-Pro
A15414G	Cyt-B	Tyr-Cyt
C15587G	Cyt-B	Leu-Val
C15590G	Cyt-B	Arg-Gly

Discussion

The association of certain mtDNA haplogroups and SNPs with breast cancer risk is not new. In Iraq, a few articles have been published about mtDNA mutations and breast cancer, however, the current study is the first that used the technique of whole mtDNA analysis. It is worth mentioning that 15 mtDNA mutations not reported before were identified in this study. Due to the usual late presentation of breast cancer in our locality, we used breast tissues in our study despite its relative invasiveness compared with peripheral blood sampling. Some researchers advocated to use the latter method as it is much easier to conduct, less invasive and can be beneficial in detection of early breast cancer cases [7], while other workers used both blood samples and breast tissue [15].

"Several previous studies have been conducted and showed a significant relation between specific haplogroups and cancer incidence in general" [2,3,9]. In regard to breast cancer, Chinese women of haplogroups M and subhaplogroup D5 had shown a higher incidence of this cancer [2,3,17,18], while no such a remarkable relation was identified between cancer and specific haplogroups in European and Caucasian women [2,3,19]. On the other hand, haplogroup K showed a significant association with breast cancer in European-American women [2,3,20]. The current study revealed a significant relation between haplogroup HV and breast cancer [2,3].

"Besides the haplogroups, several distinct SNPs have been previously discovered to be associated with cancers in general as T16189C, G10398A and the deletion of mtDNA 4977" [2,3,9]. "In regard to breast cancer, certain SNPs were similarly recognized as associated with increased cancer incidence. A10398G is one of the well-known SNPs in breast cancer among European-American, Malaysian and African-American women" [2,3,20–24]. " Furthermore, G9055A and T16519C were also identified as risk factors for breast cancer in Eu-

ropean-American females" [2,3,20]. " Likewise, several other germ line mutations as 2463 A-deletion, C6296A, 6298 T-deletion, A8860G, and 8460-13327deletion, were detected in Chinese women with breast cancer" [2,3,7].

Despite the fact that several SNPs were discovered in breast cancer samples in this work, but the most frequent one was SNP (A8860G) as it was observed in (n=20, 100%) of cancer cases vs. (n=4, 20%) of the controls. Our finding was similarly reported by Li et al [7]. "It is a non-synchronous mutation of the Mt-ATP 6 gene. This gene encodes ATP synthase 6 (681 amino acids), a subunit of complex V, whose mutation results in substitution of a polar uncharged amino acid (threonine) with a non-polar aliphatic amino acid (alanine), which may affect hydrophobic interactions and hence the structure of the protein. However, such a prediction of protein structure is not absolute, as these mutations may be followed by other compensatory mutations (suppressor mutations) in order to minimize the initial mutation's effect. These compensatory and suppresser mutations may explain the presence of the mutation A8860G in 20% of phenotypically healthy control samples" [2,3,25].

The observed variation in the impact of mitochondrial genome on carcinogenic process suggests that other factors such as the individual physiology, geographical location and ethnicity may have a role in shaping the final picture [26–28].

Although time-consuming and costly, whole mtDNA rather than a selected region analysis was used in this study aiming to discover more mutations. To the best of our knowledge, the use of this technique was not reported before in Iraq. Due to time constraints, this study collected relatively a small number of participants (breast cancer, n=20 and Controls, n=20) used in mitochondrial genome analysis. The study identified 15 mtDNA mutations not reported before. The details of these mutations will be the subject of a future publication. We used breast tissues in our study despite its relative invasiveness compared with peripheral blood sampling. Some researchers advocated to use blood samples as it is significantly simpler to carry out, less invasive and can be beneficial in detection of early breast cancer cases, while other workers used both blood samples and breast tissue.

Conclusions

The link between certain mtDNA haplogroups and SNPs and the breast cancer risk is not new. In Iraq, a few articles have been published about mtDNA mutations and breast cancer. However, the current study is the first that used the technique of whole mtDNA analysis. Unlike studies performed in other populations of the world figuring out A10398G as the risky SNP, our study identified A8860G in the Kurds. Geographic and ethnic variations between human populations do exist, so a SNP that is common in one population group may be much rarer in another.

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