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Research Article

Screening for Mitochondrial DNA A4977 Common Deletion Mutation as Predisposing Marker in Breast Tumors in Iraqi Patients

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Abstract: Mitochondrial DNA (mtDNA) has been proposed to be involved in carcinogenesis and ageing. The mtDNA 4977 bp deletion mutation is one of the most frequently observed mtDNA mutations in human tissues and may play a role in breast cancer. The aim of this study was to evaluate the possibility of using mtDNA 4977 bp deletion mutation as biomarker for breast tumors in Iraqi women. Mitochondrial DNA was extracted from 26 women with malignant tumors and 33 women with benign tumors. From each patients, blood sample and biopsy tissues (malignant and sub-margin) were collected. Patient´s DNA were amplified by using mtDNA 4977 deletion mutation specific-primers (HSAS8542/HSSN8416) as well as internal control-primers (ND6A/ND6B) in separated reaction mixtures. The results of agarose gel electrophoresis of amplified products of mtDNA 4977 deletion mutation showed that this mutation did not present in any of patient's sample. In conclusion, it's not believed that mtDNA mutation4977 could be act as biomarker risk factor for breast cancer in Iraqi patients.

Keywords: Breast cancer, deletion mutation, D-loop, mtDNA, PCR

INTRODUCTION

Breast cancer progression involves the accumulation of various genetic mutations, which are present in both nuclear genomes (nDNA) and mitochondrial genomes (mtDNA). Mitochondrial dysfunction is a hallmark of cancer cells in which breast cancer is one of the commonest mtDNA disorder (Ackerman and Rosai, 2004; Verma *et al*., 2007; Chatterjee *et al*., 2011; Hongying *et al*., 2013). Incidence rate of breast cancer around the world vary a great deal. In Arabic countries, the incidence rate ranged from 3.6% in Oman to 34.3% in Sudan (WHO, 2010). In Iraq, breast cancer is the commonest type of malignancy in females and there is a general trend an increase in the frequency of breast cancer as well as increase incidence in younger age group (Alwan 2010). Also, patients under 30 years old age formed about 5% of cases, where about 75% of these cases occurred in women older than 40 years (ICB, 2000; Alwan, 2014).

In regard to mtDNA, most mutations occur in the Displacement loop region (D-loop), where the origin of replication and promoter are located. Genetic variability in the D-loop region has been suggested to affect the function of the respiration chain that is responsible for high ROS levels and could contribute to cancer initiation (Suzuki *et al*., 2003; Lievre *et al*., 2005).

Several mtDNA mutations in the mtDNA D-loop have been reported in the breast cancer tissues, of these, the most common somatic mutation is deletion (mtDNA4977) which occurs between nucleotides 8,470 to 13,477 and has been reported in a wide range of tumors, stressed tissues and even in normal appearing tissues (Richard *et al*., 2000; Kamalidehghan *et al*., 2006; Hsin-Chen *et al*., 2014). It encompasses five tRNA genes and seven genes encoding sub-units of cytochrome c oxidase, complex I and ATPases. This mutation creates an mtDNA molecule that is smaller than the normal mtDNA molecule, though it is still capable of replication. Being smaller and replication competent, the mtDNA mutation 4977 molecule may accumulate with age, primarily in post-mitotic tissues at varying rates, depending on environmental and genetic factors, such as the mutation rate, the initial frequency of deletions present at conception and selective factors that affect deleted molecules (Cortopassi *et al*., 1992).

In the present study, screening for the mtDNA^{4977}deletion mutation in a samples of Iraqi women with breast tumor was done and evaluate the possibility of using this mutation as biomarker for breast tumors in Iraqi women.

MATERIALS AND METHODS

Patients: In this cross-section study, 59 female patients with breast tumor were recruited from Al-Kadymia Teaching Hospital-Breast Examination Unit and Al-

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Table 1: Sequences of primers used in PCR

Primer type	Forward primer	Reverse primer
mtDNA4977 deletion(M)	5'-TGT GGT CTT TGG AGT AGA AAC C-3'	5'-CCT TAC ACT ATT CCT CAT CAC C-3'
Internal control (IC)	5'-TTC TCC TAG ACC TAA CCT GA-3'	5'-GGA TAT ACT ACA GCG ATG GC- 3'

Dhurgham Hospital, in the period from November 2013 to September 2014.

Three samples were taken from each patient:

- Tumor tissue samples
- The adjacent normal tissue samples were obtained from the distal edge of the resection (each preserved in tube contain normal saline)
- One ml of peripheral blood (preserved in EDTA blood collection tube). All samples were frozen (-20°C) until the relevant assays were performed. Medical charts were reviewed using a standard protocol to obtain information from each patient (questionnaires) including (name, age, marital state, breast feeding, family history, ultra-sound examination, fine needle aspiration histopathological typing, stage grouping and grading of the breast cancer).

Molecular screening for mtDNA4977 mutation: DNA was extracted from biopsy tissues and blood sample using DNA isolation kit (Qiagen, Germany) following the manufacturer protocol. Screening for simultaneous deletion mutation mtDNA 4977 was done using PCR according to Dani *et al*. (2004). Patient´s DNA were amplified by using mtDNA 4977 deletion mutation specific-primers (HSAS8542/HSSN8416) as well as internal control-primers (ND6A/ND6B) in separated reaction mixtures. Mutation specific primers (M) and Internal Control primers (IC) sequences were given in Table1. Briefly, two master mixes (each of 25 µL) were prepared, one for mutation detection and the second for IC as in the following: 1X PCR buffer, 200 µM of dNTPs (Promega, USA), 4 pmol of each primers (Alpha DNA, Canada), 1.5 U/reaction of Taq DNA polymerase (Promega, USA). Two microlite (equivalent to 100 ng) of DNA was added for each PCR reaction tube. No Template Control reaction tube (NTC) was prepared by adding 2 µL of double deionized distal water (instated of DNA). PCR reaction tubes were transferred to thermal cycler (Eppendroff-thermal cycler, Germany), which was programmed as following: pre-denaturation at 94°C for 4 min (X1), {95°C for 2 min, 57°C for 1 min and 72°C for1 min} (X30) and final extension at 72°C for 10 min (X1). PCR products were electrophoresed on 1.5% agarose gel. Expected results are PCR products of 127 bp in positive reaction of deletion mutation and 485 bp in reaction of internal control.

Statistic analysis: Data were analyzed using SPSS program (Statistical package for social Sciences) version 16 and Microsoft Office Excel 2007. Numeric data were expressed as mean ±SD. ANOVA test was used comparison mean more than 2 groups. Pearsons, S correlation coefficient was used to test correlation between two numerical variables. *P* less than 0.05 was consider significant.

Table 2: Classification of patients group and control group according to breast pathology

Histological subtype of lesion	No. (%)
Malignant lesions	
-Invasive ductal carcinoma.	23 (88.46)
-Invasive lobular carcinoma.	2 (7.69)
-Insitu ductal carcinoma.	1 (3.85)
Benignlesions	
-Fibro cystic disease.	23 (69.70)
-Fibroadenoma	5 (15.5)
-Duct ectasia	5 (15.5)

Table 3: The frequency of tumor type among the studied group according to the age

	Type of tumor		
Age group	Benign	Malignant	Total
< 21 years	6	0	6
%	18.2%	0.0%	10.2%
21-30 years	7	1	8
%	21.2%	3.8%	13.6%
31-40 years	6	3	9
%	18.2%	11.5%	15.3%
41-50 years	10	14	24
%	30.3%	53.8%	40.7%
> 50 years	4	8	12
%	12.1%	30.8%	20.3%
Total	33	26	59
%	100.0%	100.0%	100.0%

RESULTS

This study involved 26 Iraqi patients with breast cancer whose types and relevant patient numbers were as follows: Invasive ductal carcinoma 23, invasive lobular carcinoma 2 and In situ ductal carcinoma 1); Also, 33 patients with benign breast lesion (Fibro cystic disease 23, fibroadenoma 5 and Duct ectasia 5) as control were included (Table 2). The patients mean age was 40.63±10.83 years with a range of 13 to 71 years old.

Distribution of breast tumor types (benign and malignant) according to age group: The results showed that the presence of benign breast tumor was lower in the female older than 50 years of age group (12.1%) as compared to the other age groups but the malignant breast cancer cases were reported to be higher in the fourth decade of life (53.8%) when compared to the other age group (Table 3).

Molecular screening for mtDNA deletion 4977 mutation using PCR: The results of agarose gel electrophoresis of amplified products of mtDNA deletion 4977 mutation showed that this mutation did not present in any of patient's sample (Fig. 1).

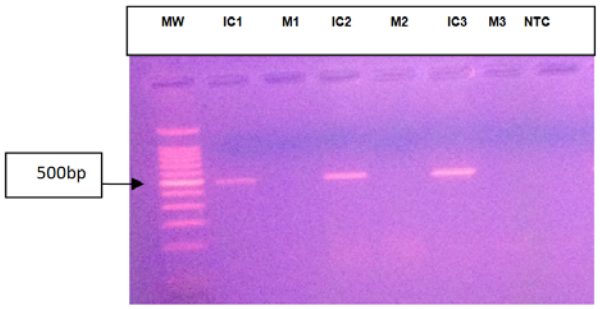

Fig. 1: Gel electrophoresis of PCR amplification of mtDNA of patient with breast cancer. Lane MW: molecular weight ladder of 100 bp.Lane IC 1,2,3:Amplicone of Internal control(485 bp) in extracted DNA from blood sample, safe margin sample and cancer tissue, respectively. Lane M 1,2,3:Amplification of deletion mutation extracted DNA from blood sample, safe margin sample and cancer tissue, respectively. NTC: no template control. Electrophoresis was done on 2% agarose using 5v/cm current for 1 h.

DISCUSSION AND CONCLUSION

Mitochondrial defects have long been suspected to play an important role in the development and progression of cancer. By virtue of the clonal nature of mitochondria and high copy number, mitochondrial mutations may provide a powerful molecular biomarker for the detection of cancer. The extent of mitochondrial DNA (mtDNA) mutations might be useful in the prognosis of cancer outcome and/or the response to certain therapies (Copeland *et al*., 2002; Nageswara *et al*., 2014).

In this study, mtDNA mutation 4977 was neither detected neither in 26 patients with breast cancer nor in (33) patient with benign breast tumor when tumor tissue, the adjacent healthy tissues and blood from each patient were screened for this mutation.

Previous studies concerning the mtDNA mutation 4977 and cancer have shown inconsistent results. Tan *et al*. (2002) were studied 22 different somatic mtDNA mutations in human breast cancer, included 4977 deletion mutation, but they did not detected it (Tan *et al*., 2002). Shu *et al*. (2008) developed a quantitative real-time PCR assay to assess the level of the mtDNA mutation 4977 in tumor tissue samples from 55 primary breast cancer patients and 21 patients with benign breast disease and they found that the differences, however, were not statistically significant (Shu *et al*., 2008). In Turkish study, the researchers applied two PCR-based methods to investigate the association of the mtDNA 9774 common deletion with 25 breast tumors and the adjacent healthy tissues in Turkish female patients but they did not found this mutation in either breast samples (Akkiprik *et al*., 2010). Also, other studies showed that there was no significant association with either breast or other types of cancers (Dani *et al*., 2004; Zhu *et al*., 2005; Ye *et al*., 2008).

In regard to the relationship between other type of mtDNA mutations and breast cancer, one Iraqi study was investigate if there is a relationship between mtDNA polymorphism (A10398G) and breast cancer in a blood sample of 21 unrelated women with malignant tumors, 22 women with benign tumors and 16 healthy Iraqi women using RFLP screening but they did not find any variation regarding this mutation between malignant tumors and reviled that A10398G polymorphism cannot be used as a biomarker for breast cancer detection in Iraqi women (Ismaeel *et al*., 2013).

In another hand, an association of the mtDNA common deletion with breast cancer has been reported. In Iranian study, mtDNA 9774 deletion mutation was studied in 34 paraffin-embedded samples and blood samples from familial breast cancer Iranian females. They detected this mutation in 5/9 of familial blood sample (Dani *et al*., 2004). In one Taiwanese study, they found that 18 of the 60 (30%) breast cancers patients displayed mtDNA4977 deletions somatic mutations in mtDNA D-loop region and the incidence of the 4977 deletion in non-tumorous breast tissues (47%) was much higher than that in breast cancers (5%) (Yin *et al*., 2006). More resent meta-analysis study suggests that the mtDNA 4977 deletion is often found in cancerous tissue and thus has the potential to be a biomarker for cancer occurrence in the tissue, but at the same time being selected against in various types of carcinoma tissues. Larger and better-designed studies are still warranted to confirm these findings (Shu *et al*., 2013).

However, in different studies, a decreased proportion of the mtDNA 4977 mutation in tumor tissue as compared with corresponding non-tumorous tissue has been observed. This phenomenon has been explained as being either the result of a dilution effect due to rapid cytoplasmic division or from the result of a selective effect *via* an unknown mechanism by which cancer cells harboring mtDNA with the large-scale deletion are eliminated because of apoptosis or selective purification during growth (Yang *et al*., 2004; Yin *et al*., 2004; Wu *et al*., 2005). Also, this inconsistency may be due to the laboratory techniques applied and intrinsic features of samples available for each study (fresh frozen tissue *versus* formalin fixed), age and ethnic background of patients. The use of non-quantitative detection methods and/or failure in quantitative PCR strategies to identify and disqualify from use, pairs of primers which will also efficiently co-amplify nuclear DNA. In base of genetic variation effect in considering such mutation as biomarker for breast cancer or not between different geographic areas and ethnic groups (Shu *et al*., 2008).

In conclusion, it's not believed that mtDNA mutation 4977 could be act as biomarker risk factor for breast cancer in Iraqi patients.

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