Multiplex PCR Technique to analysis of BRCA1/BRCA2 Mutations in Iraqi Breast Cancer Patients

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Introduction

:Breast carcinoma is the commonest malignancy in women and it has become a major health problem affecting women, worldwide. Mutations in the two breast cancer susceptibility genes, BRCA1/BRCA2 increase the risk of developing breast cancer**.Objectives:** To study the frequency of the three founder mutations; 185delAG and the 5382insC in BRCA1and the 6174delTin BRCA2 genes, using the Multiplex PCR Technique. **Methods:**In this study 150 patients with familial breast cancer diagnosed pathologically as having breast cancer collected from middle and south of Iraq from May 2017 to April 2018. In addition to control group(120 benign breast tumors) were used for dtection of BRCA1/BRCA2 mutations in the two groups.PCR amplification by using(KAPA 2G^{TM}Fast Multiplex PCR-Kit),was used for detection of mutation**.Results:** From all patients 34(22.7%) have BRCA mutations,16.7% patients with mutations were indicated to have one mutation in BRCA1"185 del AG or 5382 ins C" , 2.7% were found to have two mutations in BRCA1" 185 del AG and 5382 ins C",1.3% were verified to have one mutation "6174 del T" in BRCA2, 2.0% were indicated to have two mutations in both BRCA1 and BRCA2 "5382 ins C and6174delT",2.5%mutation were presented in control group.BRCA1"5382 ins C" mutation presented in 61.77% in ages less than 45 years,while BRCA2 "6174del T" was observed in 5.88% patients of ages higher 45 years. **Conclusion:** In Iraq, the frequency of breast cancer was presented in high percent in southern governorate than middle governorate due to They have the characteristics of significantly high frequency of family history,environmental factor high grade and advanced The BRCA1 (5382insC) mutations were percent than BRCA2.

Key words: Breast Carcinoma, BRCA1, BRCA2, PCR technique

Introduction:

Breast cancer is the most common malignancy in females worldwide, Its remained the most frequently diagnosed cancer in women and the leading cause of cancer death worldwide, with an estimated 1.7 million new cases and 521,900 deaths in 2012

compared to 1.38 million new cases and 458,000 deaths in 2008 (Ferlay *et al*.,2008; Azubuike *et al*.,2018).Breast cancer is the most common cancer and the leading cause of cancer-related deaths among Iraqi women and a major cause of morbidity and mortality (Tabarestani *et al*.,2017).Breast cancer is one of the most common diseases affecting women worldwide,it may associate ovarian and with other cancers, most cases of breast cancer are sporadic,but approximately 5-10% are due to inherited disease(Abdel-Razeq *et al*.,2018).

The risk is higher with a family history (especially mother, sister, daughter) of breast and/or ovarian cancer.Hereditary breast cancer is well-described; around 5–10% of breast cancer patients carry high risk gene mutations like BRCA1 and BRCA2 (Abdel-Razeq et al.,2018; Foulkes,2008). patients with a family history of breast cancer at young age and with many family members affected are at high risk(Litton *et al*.,2012;Girardi *et al*.,2018).Two classes of inherited susceptibility genes are considered in the etiology of breast and other common cancers.First: Genes have been identified that confer a high degree of breast cancer risk.These include BRCA1,BRCA2andTP53.Second:Variant genotypes at other loci (polygene) may confer a relatively smaller degree of cancer risk, but they carried by a larger proportion of the general population(Stegel *et al*.,2011).The germline mutations of BRCA1and BRCA2 have been associated with a significant increase in breast cancer risk and certain other cancers ,Among the most known mutations in these tumor suppressor genes are 5382insC and 185del AG in BRCA1 and 6174delT inBRCA2(Kooshyar *et al*.,2013).

In this study PCR analysis was used for the genomic detection of BRCA1 and BRCA2 specific mutations in breast cancer cases Analyzing the types and frequencies of the most common BRCA1 and BRCA2 mutations polymorphism and sporadic cases with breast cancer in Middle &Southern Iraqi familial.

Patients and Methods

-Sampling and genomic DNA extraction

Fresh breast tissues and blood samples were obtained from 150 patients with familial BC collected from middle and south of Iraq.From May 2017 to April 2018.Patients were randomly selected from hospitals and private laboratories. The patients' age ranged 16-72 (mean age: 45.8±1.3) years. Breast cancer was diagnosed by histopathological examination. Fresh tissues and blood samples were obtained from a

group of 120 patients of benign breast tumors with non familial BC were also investigated as a control group individuals' age ranged 18-70 (36.4±1.2) years. Blood samples were collected in EDTA tubes, and preserved at deep freeze until the use in genomic analyses for BRCA1and BRCA2. DNA was extracted from the blood samples and Fresh breast tissues with the use of Wizard Genomic DNA purification kit, (Promega/ USA). The purified DNA was stored at -20°C or DNA was used for the desired downstream application.DNA was determine by spectrophotometer at 260nm and 280nm.DNA concentration in the solution was calculated as following:

DNAconcentration (µg/µl)= OD(260nm) x100 (dilution factor) x50 µg/ ml/1000

The ratio between the reading at 260nm and 280 nm(OD 260/OD 280) provides an estimation of the purity of nucleic acid(Sambrook *et al.*,1989). In this study, 3 founder mutations, 185delAG and 5382insC in BRCA1, and 6174delT in BRCA2, were considered. For each mutation, three primers (one common,one specific for the mutant, and one specific for the wild-type allele) were used. Agarose gel was prepared according to(Maniatis *et al.*,2001).

-Analysis of mutations

The amplified PCR products were detected according to Mehdipour et al.(2006); Fattahi *et al.*(2009) by agarose gel electrophoresis and visualized by staining with ethidium bromide. PCR amplify by using(KAPA 2G^{TM} Fast Multiplex PCR-Kit). PCR was performed in a 25 µl volume .5 µl of genomic DNA was added to12.5µl of 1X KAPA 2G Fast Multiplex Mix reaction buffer. PCR products were loaded to the agarose gel wells.The electrophoresis result was detected by using gel documentation.The positive results were based on the presence of both or the absence of any one of specific bands of amplified DNA in agarose gel (2%).The nucleotide sequences of the primer sets are listed in table(1) with their size of amplification.

Results and Discussion:

Fresh tissue specimens as well as peripheral blood samples isolated from 150 breast cancer patients were analyzed.The analysis involved a DNA extraction from all specimens of the patient and control groups as mentioned previously.The detection of gene mutations was carried out on the basis of PCR amplification by using Multiplex PCR kits and agarose gel electrophoresis.Allele–specific oligonucleotide primers were used for detection of mutations in BRCA1 and BRCA2 genes. Results of analysis of

185del AGand 5382 insC in theBRCA1gene and 6174delTin the BRCA2 gene by multiplex kit are illustrated in figure (1, 2, 3).

Results of mutation analysis revealed 34 (22.7%) out of the enrolled breast cancer patients have BRCA1 and/or BRCA2 mutations, 3(2.5%) mutation were presented in control group (Table 2).Twenty five (16.7 %) patients with mutations were indicated to have one mutation in BRCA1, i.e,three patients (2.0%) with 185 del AG and twenty two (14.7%) patients with 5382 ins C. Four patients (2.7%) were found to have two mutations in BRCA1, i.e,185 del AG and 5382 ins C.Two cases (1.3%) were verified to have one mutation (6174 del T) in BRCA2.Three cases (2.0%) were indicated to have two mutations in both BRCA1 and BRCA2 (5382 ins C and 6174 del T) (Table 3) .

The comparison of the present findings with those reported previously exhibited some controversies. Vaidyanothan et al.(2009) have identified mutations in 61 young indian patients,17 patients(28%) were found to have genetic breast cancer,15 (24.6%) have BRCA1 mutations and two (3.28%) had BRCA2 mutations.While no specific association between BRCA1 or BRCA2 mutations with cancer type was seen, the frequency of BRCA1 & BRCA2 mutations among Indian women with familial breast and Ovarian cancers is found to be 24.6% and 3.28% respectively.In a study of steffensen *et al*.(2010) who identified a Danish breast and overian cancer family with germ-line mutations in the BRCA1& BRCA2 genes.The results of analysis of BRCA gene mutations are in consistence with several previous findings. Fattahi *et al*.(2009) and Couch *et al*.(2007), showed that the most commonly detected BRCA1 mutations are a deletion of adenine and guanine [BRCA1 185delAG (exon 2)], and insertion of cytosine [BRCA1 5382insC (exon 20)], and BRCA2 mutation is the deletion of thymine [BRCA2 6174delT (exon 11)], which have a high frequency in the general population.The 5382insC and the 185delAG mutations in BRCA1 and the 6174delT in BRCA2 occur in the general Ashkenazi Jewish population with a carrier frequency of 1.09% for the 185delAG mutation,0.13% for the 5382insC mutation, and 1.52% for the 6174delT mutation.Of these,the 185delAG mutation in the BRCA1 gene has the highest prevalence and penetrance in breast cancer(Roa et al.,2010).The frequency of the BRCA1/BRCA2 mutations in the Iraqi population has been compared with other populations including those of Ashkenazi. Lahad *et al*.(2007) have studied the frequency of BRCA1/2 mutations in Ashkenazi Jews in Israel including cases of Iraqi and Iranian origin, they found that the frequency of BRCA1

185delAG and 5382insC mutations was significantly higher than that of BRCA2 6174delT mutation. Analysis of these three markers in four Iranian/Iraqi BRCA gene mutation carriers revealed the same genotype that previous reports have described in Ashkenazi Jews, suggesting a common origin for the mutations in Ashkenazi and Iraqi/Iranian Jews.The present findings are in disagreement with some of those reported previously.Mehdipour *et al*.(2006) reported a low frequency of the 185delAG founder mutation in the BRCA1 gene in Iranian breast cancer patients but not 5382insC and 6174delT mutations, The total mean age of our patients was less than 45 years, as the likelihood of finding a mutation is highly age dependent. Al-Mulla *et al*.(2011) have studied different mutations in the BRCA gene in Britain population, they stated that more than half of individuals carried a mutated BRCA1 gene, and less had a mutated BRCA2 gene. Mutations in the BRCA1 gene accounted for the majority of breast cancer. It was noted that carriers of the exon 2of BRCA1 gene mutation had significantly lower cancer incidence compared with carriers of other exons mutation. These findings are consistent with the present work, suggesting to that either the 185delAG mutation is of low penetrance or, more likely, carriers of this mutation would develop cancer at an older age compared with other BRCA1 gene mutations.It is well accepted that mutation frequencies of the BRCA1 and BRCA2 genes in high-risk people vary widely among different populations,the present study is consistent with this fact.The contributions of BRCA1 and BRCA2 to breast cancer in Italian patients appear to be less significant than in patients from Iraq and other communities with these mutations. In one Italian study,Ottini *et al*.(2000) have found that BRCA1 mutations were detected in only one out of 10 cases from breast cancer families.This is a low proportion compared with other studies which suggested that mutation in BRCA1 and BRCA2 are responsible for the large majority of breast cancer families, with the greater proportion due to BRCA1. In this respect, the limits of mutation detection techniques and the small number of breast cancer cases tested should be taken into account.

The analysis of the data revealed the mean age of patients with BRCA1 and BRCA2 mutations of 34,39 and 45 year respectively.When patients were divided into two groups, those of ages less than 45 year and those of ages higher than 45 year,21 (61.77%) patients with BRCA1 exo 20 (5382 ins C) mutation were found to be of ages less than 45 year. However, the high percentage of mutations (5.88%) of BRCA2 gene (6174 del T) was observed in patients of ages after 45year.On the other hand multiple mutations BRCA1/

BRCA2(5382 and 6174) were indicated in only (8.82%) of the whole patients with mutations (Table 4).

The present investigation pointed out an agreement with some of findings reported worldwide and inconsistence with others.Identification of BRCA mutations in a substantial proportion of our Iraqi patients indicates that these genes play an important role in the incidence of breast cancer in the general population. These results were supported by Turkish study accomplished by Yazici *et al*.(2000),who demonstrated the presence of BRCA mutations in patients with a personal and family history of breast cancer below age 50.Their results suggest that BRCA1 and BRCA2 mutations are observed in a significant proportion of Turkish families with breast cancer, and those with early onset of disease.

Two other large population based studies included cases over age 45 years assessed BRCA1 alone; both found that mutation prevalence decreased with increasing age (Whittemore *et al*.,2004 ;Anton-Culver *et al*.,2000).The observed frequency is not always the same in any two countries surveyed so far. The mutation frequencies reported in the present study came in parallel with another study undertaken in the north of Iraq by Majid *et al*.(2009), who found that the age of specific annual incidence rates of breast cancer were higher among Iraqi Kurds than for Israeli Arabs and Jordanians and were similar to Egyptians.

Several studies revealed that carriers of BRCA1 mutations have increasing risk of developing breast cancer at younger age than others,with about 20-fold higher risk of developing breast cancer than the general population(Rahman and Stratton,1998; Maksimenko *et al*.,2018).Another studies reported slightly lower incidences of 32.9% and 50.2% at ages 50 and 70 years respectively (Loman *et al*., 1998).

For BRCA2 gene mutation carriers, the estimated cumulative risk of breast cancer has been shown to be 28% by age 50 years, and 84% by age 70 years. Nevertheless, the magnitude of risk to carriers of the germ line BRCA1 and BRCA2 remains controversial and the controversy could partly have stemmed from the use of different populations and study designs,and the lack of specific mutation stratification.(Al-Mulla *et al*., 2011).Ibrahim *et al*.(2010) have illustrated that the men age of carrier mutation in BRCA1 and BRCA2 genes was 42.4 years and 34.3 years respectively and the mean age of carriers mutation in this genes were 39.8 years while the mean age of non carriers of mutation were 47.1 years.A woman with a BRCA mutation has a 20% chance of

developing breast cancer by the time she is 40 years old. However, the risk increases to 37% by the age of 50, 55% by the age of 60, and is over 70% by the age of 70.BRCA2 mutation carriers had somewhat later age of onset of breast cancer than BRCA1 carriers, these findings agreed with that of Vehmanen(2001) who stated that an early age of breast cancer onset is a clear indicator of BRCA1 as well as BRCA2 mutation carrier status in breast cancer families.The age of onset in the BRCA1 and in the BRCA2 families was significantly lower than in the mutation negative families. An older age of onset in the BRCA2 families compared with BRCA1 families has also been found in this study, and is reflected as a smaller contribution of BRCA2 to early onset breast cancer.

Conclusions:

Breast cancer is the commonest tumor in Iraq and the incidence of it presented in high percent during the period of study.Two mutations in BRCA1 and one mutations in BRCA2,the most frequent mutations among Iraq population,were 5382 ins C and185 del AG in BRCA1 gene and 6174 del T in BRCA2 gene.The 5382 ins C mutation in BRCA1 was the main mutation found in this study.BRCA1and BRCA2 genes mutations are responsible for a significant proportion of breast cancer. BRCA mutations were found to be high in individuals with family history and in young age patients, at high grades and in advanced stages .

Table 1: Primer sequences using in study and PCR amplicon

Primer	Sequence of the Primers	Size
BRCA1 185delAG		
Common forward (P1)	5’ ggttggcagcaatatgtgaa	
Wild-type reverse (P2)	5’ gctgacttaccagatgggactctc	335bp
Mutant reverse (P3)	5’ cccaaattaatacactcttgtcgtgacttaccagatgggacagta	354bp
BRCA1 5382insC		
Common reverse (P4)	5’ gacgggaatccaaattacacag	
Wild-type forward (P5)	5’ aaagcgagcaagagaatcgca	271bp
Mutant forward (P6)	5’aatcgaagaaaccaccaaagtccttagcgagcaagagaatcacc	295bp
BRCA2 6174delT		

Common reverse (P7)	5’ agctggtctgaatgttcgttact	
Wild-type forward (P8)	5’ gtgggatttttagcacagctagt	151bp
Mutant forward (P9)	5’ cagtctcatctgcaaatacttcagggatttttagcacagcatgg	171bp

Table 2:Distributions of mutations of BRCA in breast cancer patients and the control group

Type of lesion	Mutation of BRCA1& 2			
	Positive	%	Negative	%
Benign(n=120)	3	2.5	117	97.5
Malignant(n=150)	34	22.7	116	77.3

Table 3 :Frequency of BRCA gene mutations in breast and benign tumors

Mutation	Frequency			
	Breast cancer (n=150)	%	Benign tumors (n=120)	%
BRCA1				
185 del AG(exon 2)	3	2.0	1	0.8
5382 ins C(exon 20)	22	14.7	1	0.8
185 &5382(exon 2&exon 20)	4	2.7	0	0.0
BRCA2				
6174 del T(exon 11)	2	1.3	1	0.8
BRCA1&BRCA2				
5382&6174(exon20 &exon11)	3	2.0	0	0.0
Total	34	22.7	3	2.5

Table 4: BRCA gene mutations in patients of ages before and after 45 year

Genes(Mutation)	Age(Years)					
	< 45 years		> 45 years		Total	
	No.	%	No.	%	No.	%
BRCA1						
185 del AG	1	2.94	2	5.88	3	8.82
5382 ins C	21	61.77	1	2.94	22	64.71
185 &5382	4	11.76	0	0.0	4	11.76
BRCA2						
6174 del T	0	0.0	2	5.88	2	5.88
BRCA1& BRCA2						
5382&6174	3	8.82	0	0.0	3	8.82

Total	29	85.29	5	14.71	34	100

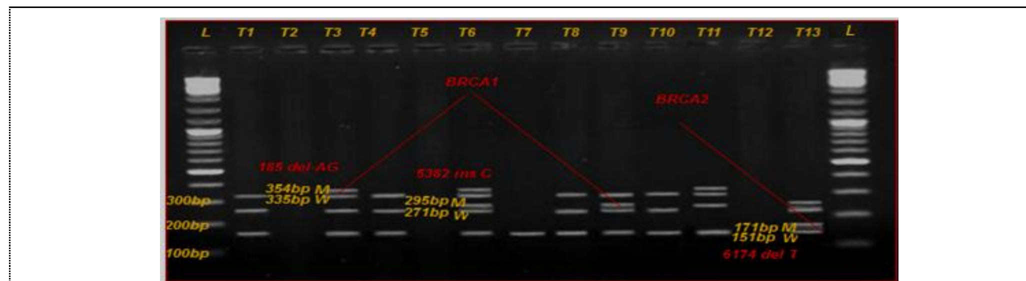

Figure1:Gel electrophoresis of Multiplex-PCRproducts(2%agarose,90 minutes,90 V)to the tissuesSamples;LaneL,DNAmolecularsizemarker1500-bpladder;lanesT1,T4,T7,T8andT10,wild-typesamples;lanesT3andT1,sampleswiththe185delAG,lanesT9,sampleswiththe5382insC;lanesT6,equalvolumesof185delAG+5382insCinBRCA1gene;lanesT13,equalolumesof5382insC+6174delTinBRCA1andBRCA2gene,respectively;lanesT2,T5,andT12,sampleswith the nagative product;M,mutant product;W,wild-type product.

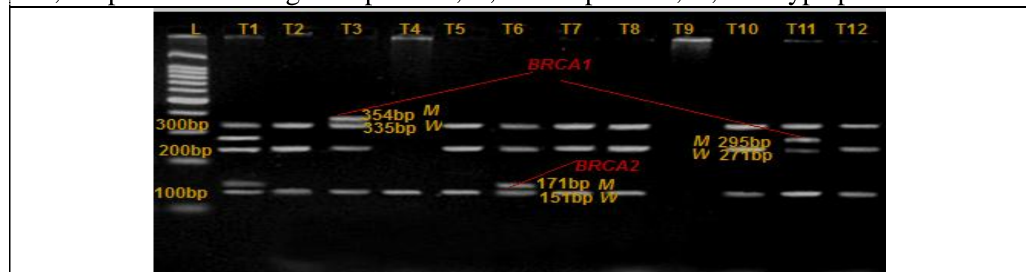

Figure2:Electrophoretogram of Multiplex-PCR productstothetissuesSample;LaneL,DNAmolecularsizemarker1500-bpladder;1500-bpladder;lanesT2,T4,T5,T7,T8,T10 and T12,wild-type samples;lanesT3,T11 andT6,patientsampleswiththe185delAG,5382insC,and6174delTmutations,respectively;lanesT1,equalvolumesof5382insC+6174delT;LanesT9 patient sample with the Negative product; M,mutant product; W, wild-type product.

Figure 3:Gel electrophoresis of Multiplex-PCR products(2% agarose,90 minutes,90 V) to the blood Samples; Lane L,DNA ladder 1500bp as a marker;lanesB1and B4 show the mutation in5382 ins C; lanesB5 show the mutation

in185 del AG;lanesB3,equal volumes of185delAG+5382insC inBRCA1gene;While,lanes B2andB6-B11 patient samples with the Negative mutations and positive wild-type product;M,mutant product;W, wild-type product.

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