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Detection of *P53* Gene Mutations Using HRM Among Egyptian Breast Cancer Women: A Pilot Study

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Abstract

P53 is the second most frequently mutated tumor suppressor gene representing about 40-60% of all cases of breast cancer (BC) patients. Even though BC can be considered a highly curable disease when detected early. It is often diagnosed at a later stage, due to fragile economic circumstances and lack of awareness. Thereby, the aim of the present study is to establish high resolution melting (HRM) assay as a rapid and economic screening tool for identifying presence of mutations in *P53* gene among familial and non-familial BC patient hoping to assist in diagnosis and disease management. Blood samples were collected from preoperatively Egyptian BC women, and genomic DNA was extracted from 25 familial, 25 non-familial BC patients and 25 healthy volunteers. Real-time PCR amplicons for exons 5-8 in *P53* gene have been performed, followed by HRM to detect mutations. Herein, we have detected 85 mutations in exons 5, 7, and 8. Our results have revealed that presence of positive variants in *P53* exons as detected by HRM, were shown to be more frequent among BC patients than the control group (P =0.001). Moreover, percentage of BC patients who responded to hormonal therapy (HT) were found to be more among familial than non-familial group (P= 0.001). Furthermore, we have found that hormonal receptors estrogen (ER) and progestogen (PR) were more expressed among both familial and non-familial BC patients compared to expression of human epidermal receptor 2 (Her2) among the same groups. Interestingly and according to our results, patients with single mutation were found to be at lower tumor stage when compared to those with multiple exon mutations who had higher tumor stage (P=0.018). HRM was established as an economic prognostic tool for identifying mutations in the *P53* gene, which might be correlated to the risk of Egyptian BC development in women. Yet, a larger sample size needs to be studied, since all research findings underline the importance of establishing an Egyptian BC database taking into consideration clinical and pathological criteria, that can play a crucial role in drug responsiveness and disease management.

Keywords: Breast Cancer; High-Resolution Melting; *P53* gene

1. Introduction

Despite the improved understanding of breast carcinogenesisc and the fact that breast cancer (BC) can be considered a highly curable disease when detected early [1]. BC is often diagnosed at a later stage, and accordingly, it is associated with poor survival. A fact that is reflected in the mortality statistics [2, 3], accounting for 14 to 42% of all female cancers in the Arab world [4, 5]. BC mortality rates are increasing in developing countries including Egypt representing 32% of cancer cases [6] and while it is ranked as the fifth cause of death in women in

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less developed regions (324,000 deaths, 14.3% of total) it is now the second cause of cancer death in more developed regions (198,000 deaths, 15.4% of total cases) [6, 7].

Breast carcinogenesis is associated with various types of somatic and genetic alterations and there are germline mutations in high or moderate penetrance genes which in turn have a 50% risk of inheriting the genetic alteration to the offspring. Germline mutations in high penetrance genes such as the *P53*, are associated with an increased risk for BC and were recently identified to be a disease biomarker [8].

The *P53* gene is one of the most studied tumor suppressor genes (*TP53*) that encodes for the P53 protein [8], located on chromosome 17p13.1 with a gene size of 20 kb [9, 10]. *P53* is a multifunctional transcription factor also known as the guardian of the genome that plays a major role in inhibiting angiogenesis and invasion as well as cell cycle control and apoptosis depending on the type of stress and cellular context [11]. *P53* is one of the most frequently mutated genes in many types of cancers and about 90% of these mutations encode for a missense mutant protein that extends along 190 different codons localized in the DNA binding domain of the gene [12]. About 10% of the previously stated mutations were reported to have a loss in protein function either through deletion or frame shift mutations.

According to studies by Al Qasem and her colleagues (2011), *P53* mutation prevalence in Arab patients is found to be the highest in the world (40%) [13]. The majority of these mutations were found to be clustered within exons 5-8 [13]. Clinical studies showed controversial results about the predictive and prognostic values of *P53* mutations. Several methods over the past years have been offered for the screening of *P53* mutations such as immunohistochemistry (IHC) however, it has been shown to have low prognostic value compared to high-cost DNA-based sequencing techniques which give more detailed and precise prognostic information.

Thereby, a sensitive and cost-effective detection tools such as high-resolution melting (HRM) technique has been recommended to be used as a preliminary screening tool prior sequencing analysis. HRM shows to be a high potential assay for detecting somatic and germline mutations. This technique is based on monitoring the change in

Thereby, a sensitive and cost-effective detection tools such as high-resolution melting (HRM) technique has been recommended to be used as a preliminary screening tool prior sequencing analysis. HRM shows to be a high potential assay for detecting somatic and germline mutations. This technique is based on monitoring the change in fluorescence as a result of the release of intercalating dye from a denatured DNA duplex as the temperature increases. This helps in detecting not only single nucleotide polymorphisms (SNPs) but silent and intronic mutations as well that can be ultimately validated by one of the sequencing techniques [14]. Thereby, the aim of our study is to adopt HRM as an economic screening tool for detecting the mutations present in *P53* gene that might be correlated to the risk of BC development among Egyptian women and can help in disease management.

2. Experimental

2.1. Patients & Methods

The present study was approved by the Institutional Review Board (IRB) of the Ministry of Health (IORG0005704/IRB0000687). A total of 50 Egyptian BC patients (25 familial and 25 non-familial) and 25 healthy volunteers were enrolled in this study for the molecular genetic testing of the *P53* gene. All participants were younger than 45 years old and BC patients did not receive chemotherapy treatment before surgery nor diagnosed with ovarian cancer. Blood samples were recruited from outpatient clinics and Radio diagnosis Department at El-Demerdash Hospital, National Cancer Institute, El Matarya Hospital, and Metalab Laboratories. All participants signed a consent form for acceptance of the publication of anonymous data.

2.2. Mutational screening for P53 gene using HRM

Blood samples were collected from preoperatively BC women (both familial and non-familial samples) and Genomic DNA was isolated according to manufacturers' instructions by GeneJET genomic DNA purification Kit (ThermoFisher Scientific, MA, USA). The concentration and quality of the genomic DNA were determined by spectrophotometric measurement using Nanodrop ND2000 Spectrophotometer (Nanodrop Technologies, USA).

Real-time PCR reactions were done to amplify the exons (5, 7, and 8) of the *P53* gene (Accession # NC_000017) using sets of primers as previously described [6], and mentioned in **Table 1**, using AriaMX System (Agilent, CA, USA). The PCR and HRM were performed in a single run on a 7500 Fast Real-Time PCR System in a reaction mix containing MeltDoctor HRM Master Mix (ThermoFisher

Scientific, MA, USA), 200 nM of each PCR primers and 20 ng of genomic DNA.

Table 1

List of primers used to amplify the selected exons of *P53* gene.

Exon	Sequence (3'-5')	Amplicon size	Annealing temperature (Ta)
Exon 5	Forward AGAGACGACAGGG CTGGTT	216bp	53.2 °C
	Reverse CTTAACCCCTCCTC CCAGAG		51.46 °C
Exon 7	Forward CTGCCTCTTGCTTC TCTTTTCC	191bp	53.04 °C
	Reverse GCTTCTTGTCCTGC TTGCTT		52.06 °C
Exon 8	Forward AAGCAAGCAGGAC AAGAAGC	225bp	52.06 °C
	Reverse CGGCATTTTGAGTG TTAGACTG		51.48 °C

The PCR reaction was run as the followings: an activation step at 95°C for 10 minutes followed by 55 cycles of 95°C for 10 seconds, a touch down of 65°C to 55°C for 10 seconds (1◦C/cycle) and 72°C for 30 seconds.
The products were heated to 95°C for 1 minute prior to the high-resolution melting step and the HRM was carried out over the range from 72°C to 95°C rising at 1°C per second with 30 acquisitions per degree [6].

The melting curves obtained from control samples (healthy women, n=25) were used for normalization and comparison with BC samples. Analysis of the obtained curves was performed using Aria MX software.

2.3. Statistical analysis

Statistical analysis was done using IBM SPSS® Statistics version 23 (IBM® Corp., Armonk, NY, USA). Qualitative data were expressed as frequency and percentage. Pearson's Chi-square test or Fisher's exact test was used to examine the relation between qualitative variables. All tests were two-tailed. A *P*-value ≤ 0.05 was considered significant.

3. Results

3.1. Demographics & Patients' clinical and pathological data of the studied groups

Clinical and pathological characterization of P53 carriers in familial and non-familial BC patients (n=50) are described in **Table 2**. Statistical analysis revealed that there were no significant differences between familial and non-familial BC patients regarding age, tumor size, tumor grade, and lymph node involvement.

On the contrary, there was a significant difference between percentage of familial (44.0%) and non-familial BC patients (84.0%) who received hormonal therapy (HT) (*P*=0.001). Moreover, we have found that hormonal receptors estrogen (ER) and progestogen (PR) were more expressed among both familial and non-familial BC patients (88.0% and 80.0%) respectively, compared to the expression of human epidermal receptor 2 (Her2) among the same groups (36.0% for both groups).

Additionally, most of our familial and non-familial BC patients were premenopausal (84.0%, 96.0%) respectively, versus those with postmenopausal (16.0%, 4.0%) respectively (*P*=0.029).
Regarding the pathological diagnosis, there was a tendency for familial and non-familial BC patients to have invasive ductal carcinoma (IDC) and ductal carcinoma *in situ* DCIS (96.0%, 84.0%) respectively, compared to ILC (4.0% and 16%) for the same groups respectively (*P*=0.59).

Table 2
Clinical & pathological parameters in familial and non-familial BC patients.

Clinical & pathological parameters		*P53* gene (Exons 5, 7 and 8)		
		Familial BC N=25 (%)	Non-Familial BC N=25 (%)	*P*-value
Age	< 45 years	21 (84.0)	21 (84.0)	1.000
	= 45 years	4 (16.0)	4 (16.0)	
Tumor Size /Mean		2.8	2.9	0.764
Tumor grade	Grade I & II	23 (92.0)	22 (88.0)	1.000
	Grade III	2(8.0)	3 (12.0)	
Tumor stage	Stage I	5 (20.0)	9 (36.0)	0.214
	Stage II	17 (68.0)	15 (60.0)	
	Stage III &IV	3 (12.0)	1 (4.0)	
ER	Negative	3 (12.0)	3 (12.0)	1.000
	Positive	22 (88.0)	22 (88.0)	
PR	Negative	5(20.0)	4 (16.0)	0.585
	Positive	20 (80.0)	21 (84.0)	
Her2	Negative	16 (64.0)	16 (64.0)	1.000
	Positive	9(36.0)	9 (36.0)	
Menopausal status	Premenopausal	21 (84.0)	24 (96.0)	0.029
	Postmenopausal	4 (16.0)	1 (4.0)	
Lymph node involvement	Negative	11 (44.0)	12 (48.0)	0.548
	Positive	14 (56.0)	13 (52.0)	
Laterality	Right	12 (48.0)	10 (40.0)	0.266
	Left	13 (52.0)	15 (60.0)	
ILC IDC+DCIS	None	1 (4.0)	4 (16.0)	0.059
	Yes	24 (96.0)	21 (84.0)	

Estrogen (ER), Progestogen (PR), Human Epidermal Receptor 2 (Her2), Hormonal Therapy (HT), Invasive ductal carcinoma(IDC), Ductal carcinoma *in situ* (DCIS),Invasive Lobular Carcinoma (ILC)

3.2. Frequency of mutations in P53 gene as detected by HRM

Herein, we have identified 85 mutations in exons 5, 7, and 8, data analysis has revealed that presence of positive variants in *P53* exons as detected by HRM were shown to be significantly different from control group (P =0.001) as shown in **Table 3**. Results showed that mutant variants in exons 5,7 and 8 were present in (52.0%), (64.0%), and (60.0%) respectively, among familial BC patients compared to (52.0%), (64.0%) and (56.0%) respectively among non-familial BC patients.

Table 3
Frequency of positive mutations in the selected exons of P53 gene in BC patients as detected by HRM

P53 gene	Familial BC[a] N=25 (%)		Non-Familial BC[a] N=25 (%)		Control[b]
	Wild	Mutant	Wild	Mutant	Mutant
Exon 5	12 (48.0)	13 (52.0)	12 (48.0)	13 (52.0)	13 (52.0)
P-value	<0.001				
Exon 7	9 (36.0)	16 (64.0)	9 (36.0)	16 (64.0)	16 (64.0)
P-value	<0.001				
Exon 8	10 (40.0)	15 (60.0)	11 (44.0)	14 (56.0)	15 (60.0)
P-value	<0.001				

Groups with different letters are statistically different

3.3 Relation between presence of P53gene mutations and some clinical and pathological findings among familial and non-familial BC patients

Herein, we investigated the correlation between presence of one or more exon mutations in *P53* BC carriers and some clinical and pathological parameters. Our results have revealed that there was no significant difference in presence of single or multiple mutations in *P53* exons among familial and non-familial BC patients regarding age, tumor size, lymph node involvement. However, our results showed that presence of multiple exon mutations were more frequent among familial and non-familial BC patients who were positive for ER, PR hormonal receptors (36 % for both) compared to those with positive Her2 (12%) and showed to be more associated with higher tumor stage (24%) of BC patients than those with single exon mutation who had lower tumor stage (I) (*P*=0.018) as shown in **Table 4**.

4. Discussion

BC is becoming the most common cause of oncologic morbidity and mortality worldwide [14]. Breast carcinogenesis is associated with various types of somatic and germline genetic alterations in high or moderate penetrance genes which in turn have a 50% risk in inheriting the genetic alteration to the offspring [15].

Table 4

Relation between *P53* gene mutations with the clinical and pathological findings among Egyptian familial and non-familial BC patients.

Clinical & Pathological Parameters		*P53* gene (Exons 5, 7 and 8)				
		Non	Single	Two or more	Total	*P*-value
		N= 50 (%)	N= 50 (%)	N= 50 (%)		
Age	< 45 years	10 (20.0)	14 (28.0)	18 (36.0)	42	0.776
	= 45 years	2 (4.0)	2 (4.0)	4 (8.0)	8	
Family History	Non-familial	5 (10.0)	10 (20.0)	10 (20.0)	25	0.176
	Familial	8 (16.0)	6 (12.0)	11 (22.0)	25	
Tumor Size /Mean		26 (2.8cm)	32(2.7cm)	42(3.0cm)	-----	0.955
Menopausal Status	Premenopausal	11(22.0)	14 (28.0)	20 (40.0)	45	0.496
	Postmenopausal	2 (4.0)	1 (2.0)	3 (6.0)	5	
ER	Negative	2 (4.0)	2 (4.0)	3 (6.0)	7	1.000
	Positive	11 (22.0)	14 (28.0)	18 (36.0)	43	
PR	Negative	3 (6.0)	3 (6.0)	3 (6.0)	9	0.636
	Positive	10 (20.0)	13 (26.0)	18 (36.0)	41	
Her2	Negative	8 (16.0)	9 (18.0)	15 (30.0)	32	0.554
	Positive	5 (10.0)	7 (14.0)	6 (12.0)	28	
Laterality	Right	5 (10.0)	7 (14.0)	10 (20.0)	22	0.837
	Left	10 (20.0)	14 (28.0)	18 (36.0)	42	
	Bilateral	1 (2.0)	0 (0.0)	1 (2.0)	2	
Tumor stage	Stage I	1 (2.0)	4 (8.0)	7 (14.0)	12	0.018
	Stage II	10 (20.0)	12 (24.0)	12 (24.0)	34	
	Stage IV	2 (4.0)	1 (2.0)	1(2.0)	4	
Lymph node involvement	Negative	6 (12.0)	7 (14.0)	10 (20.0)	23	0.826
	Positive	8 (16.0)	9 (18.0)	10 (20.0)	27	
Tumor grade	Grade I	1 (2.0)	0 (0.0)	1 (2.0)	2	1.000
	Grade II	11 (22.0)	15 (30.0)	19 (38.0)	45	
	Grade III	1 (2.0)	1 (2.0)	1 (2.0)	3	
HT	None	7(14.0)	4 (8.0)	7(14.0)	18	0.075
	Yes	6 (12.0)	12 (24.0)	14 (28.0)	32	
Pathological Diagnosis	IDC+DCIS	11 (22.0)	15 (30.0)	19 (38.0)	45	0.538
	ILC, Mixed, phylloides	2(4.0)	1 (2.0)	2 (4.0)	5	
DCIS	None	0 (0.0)	1 (2.0)	3 (6.0)	4	0.110
	Yes	7 (14.0)	9 (18.0)	10 (20.0)	26	

Estrogen (ER), Progestogen (PR), Human Epidermal Receptor 2 (Her2), Hormonal Therapy (HT), Invasive ductal carcinoma(IDC), Ductal carcinoma *in situ* (DCIS),Invasive Lobular Carcinoma (ILC)

Mutations in the high penetrance BC predisposition genes such as *P53*, confers a greater relative risk of BC [16]. In Egypt, there are few studies concerned with frequency of *P53* mutations among BC women [14] this may be due to lack of economic screening diagnostic tool that can identify presence of mutations.

Our results have revealed that there was a significant difference found between number of familial (44.0%) and non-familial BC patients (84.0%) who received hormonal therapy (P=0.001). Previously, Fagerholm and his team (2018) have reported the role of family history and other genetic factors which should be taken into consideration before the beginning of hormonal therapy (HT). However, the correlation between family history and the use of HT and BC prognosis remains unclear [17, 18].

Additionally, Studies by Shah and his colleagues (2012), have also reported that frequency of recurrent *P53* mutation in BC patients was more likely to be high among BC patients with positive hormonal receptors and lymph node involvement [19]. Where, it coordinates with our results, since we have found that expression of hormonal receptors (ER, PR not Her2) was more frequent among familial and non-familial BC which are categorized as luminal-A BC patients. Our results agree with Berger (2013), Oliver and their colleagues (2006) who conducted their study on 1,794 Japanese BC patients and observed that mutations in *P53* alter the ER gene expression as result of direct protein-protein interaction between ER receptor (ERα) and *P53* or via ER binding to promotors on the *P53* gene affecting its transcription and function. Where, they have found that individuals with *P53* mutations in exons 5-8 and ER positive had a higher risk of BC specific death when compared to patients with wild type *P53* reducing their survival to 60% after 10 years [20, 21].

In accordance with studies by Ghannam and his colleagues (2011), our results disclosed that there was a tendency in familial and non-familial BC patients (96.0% and 84.0% respectively) to have IDC and DCIS more than ILC (P=0.59) [22]. This might be due to the fact that ILC is mostly diagnosed in postmenopausal women at older age (above 50) and advanced disease stage as previously reported by Du and his colleague (2018) [23].

Previous reports by AbdelHamid (2021), Minucci, (2017), Krypuy (2007) and their colleagues validated the use of HRM as a sensitive diagnostic and prognostic technique for screening mutations in BC [14, 24, 25]. The present study has adopted HRM to expose gene mutations in *P53* (exons 5,7, and 8) and proved according to Krypuy and his colleagues (2007), 94% reported mutations have been found to be present in these exons. While *P53* mutations were reported with different incidence ranges, however, more than 1,400 *P53* mutations have been identified in BC and were reported to occur more frequently among young women [25]. The majority of these mutations were found to be clustered within exon 5-8 [13].

According to the Saudi National Cancer Registry's yearly report, 26.4% of all Saudian BC women were diagnosed before the age of 40, compared to 6.5% of American BC women [26].
Our results have revealed that, positive variants among *P53* exons (5,7, and 8) as detected by HRM were shown to have a significant difference when compared to the control group (P=0.001) demonstrating the efficiency of HRM as a screening tool. These results were also supported by Krypuy and his colleagues (2007), who validated the use of HRM as method to scan mutations in exons 5-8 in *P53* gene [25].

In agreement with a study performed by Bello and his colleagues (2016) on Brazilian BC population, we did not find any correlation between presence of one or more mutations in tested exons of *P53* and age among familial and non-familial BC patients [27]. On the other hand, studies by Al-Qasem and her colleagues (2011), found that 70% of their patients harboring *P53* mutations in their tumors were younger than 50 years among Arab females [13].

Herein, we have found that familial and non-familial BC patients with *P53* single exon mutation had lower tumor stage in comparison to presence of multiple exon mutations which was more encountered in patients with higher tumor stage. This was explained previously by Ghannam and his colleagues (2011), that loss of *P53* function is usually associated with tumor survival and progression and hence advanced stage of the disease with bad prognosis [22].

5. Conclusion

The present study successfully demonstrated the application of HRM as an economic prognostic tool for identifying mutations in the *P53* gene which positively correlates with the risk of Egyptian BC development in women. Yet, a larger sample size needs to be studied, since all research findings underline the importance of establishing an Egyptian BC database taking into consideration clinical and pathological criteria, that can play a crucial role in drug responsiveness and disease management.

6. Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7. Consent for publication

Written informed consent for publication of the study results was obtained from all patients before participation.

8. Formatting of funding sources

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