



Evaluation of *PIK3CA* Status in Breast Cancer and their Correlation with *ER*, *PR*, and *HER-2* expressions in Iraqi female patients

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Abstract: The present study revealed that the *PIK3CA*, catalytic subunit of phosphatidylinositol 3-kinase (PI3K), is mutated in breast cancer. This study was carried out in the laboratory of molecular biology in the Department of Biology in the College of Science – Kufa University, during the period from July 2013 through March 2014. Paraffin blocks of thirty one patients with breast carcinoma were included in this study. These samples were collected from laboratory of histopathology in Alsader Teaching Hospital in Al-Najaf governorate. Six samples with normal breast tissues were considered as control group for this study. Their ages ranged from 30 to 65 years, with a mean age of 49.9 years. Carcinoma tissue samples were evaluated previously for *ER*, *PR* and *HER-2* by Immunohistochemical analysis. The diagnosis was under the supervision of pathologist in the hospital. The clinicopathological assessment revealed that 14 (45.16%) were hormone dependent carcinoma: ER+PR+, 3 (9.67 %) and 9 (16.12%) were probably hormone dependent carcinoma: ER+PR-; ER-PR+, and 5 (29.03%) were hormone independent carcinoma: ER-PR-. *HER-2* expression was found in 7(22.58%) of cases. Mutational analysis of *PIK3CA* was done in 31 breast cancers and, finally, 11 (35.48%) mutations were identified in total. The frequency of *PIK3CA* mutations in ER-positive tumors was significantly ($p < 0.05$) higher than that in ER- negative tumors , and the frequency of *PIK3CA* mutations in PR-positive tumors tended to be higher than that in PR-negative tumors. *HER2* expression was not significantly associated with *PIK3CA* mutations.

Key words: *PIK3CA*, Breast cancer, *HER-2* ,*ER*

تقييم حالة *PIK3CA* في سرطان الثدي و علاقتها بالتعبير الجيني لجينات *ER* ، *PR* و *HER-2* في مريضات عراقيات

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الخلاصة: أظهرت الدراسة الحالية ان *PIK3CA* الوحدة الفرعية المحفزة لـ *phosphatidylinositol 3-kinase (PI3K)* تكون طافر في حالة سرطان الثدي. اجريت هذه الدراسة في مختبر البيولوجي الجزيئي بقسم علوم الحياتص 2348 في كلية العلوم ا جامعة الكوفة، خلال الفترة من تموز 2013 لغاية آذار 2014. تضمنت هذه الدراسة احدى و ثلاثون قالب شمع البارافين لسرطان الثدي. تم جمع هذه العينات من مختبر الامراض النسيجية في مستشفى الصدر التعليمي في محافظة النجف. ستة عينات انسجة ثدي طبيعية اعتبرت كمجموعة سيطرة لهذه الدراسة. تراوحت اعمارهم من 30 الى 65 سنة، بمتوسط عمري بلغ 49.9 سنة، تم تقييم النسيج المسرطن لـ *ER* ، *PR* ، و *HER-2* بواسطة التحليل المناعي النسيجي الكيميائي. تم التشخيص باشراف اخصائي علم الامراض في المستشفى. اظهر التقييم المرضي السريري ان 14(45.16%) كانت سرطان معتمد على الهرمون: *ER+PR+* ، 3(9.67%) و 9(29.03) كانت من المحتمل سرطان معتمد على الهرمون: *ER+PR-* ؛ *ER-PR+* ، و 5(16.12%) كانت سرطان غير معتمد على الهرمون: *ER-PR-* . التعبير الجيني لـ *HER-2* وجد في 7(22.58%) من الحالات. اجري تحليل طفرة *PIK3CA* لاحدى وثلاثون حالة سرطان ثدي، و اخيراً 11(35.48%) طفرة تم تشخيصها اجمالاً. كان تكرار طفرات *PIK3CA* في اورام *ER-positive* اعلى معنوياً ($p < 0.05$) من اورام *ER-negative* ، و تكرار طفرات *PIK3CA* في اورام *PR-positive* اعلى نوعاً ما من اورام *PR-negative* ، لم تكن هنالك علاقة معنوية بين الـ *HER-2* و طفرات *PIK3CA* .

Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in females worldwide, accounting for 23% (1.38 million) of the total new cancer cases and 14% (458,400) of the total cancer deaths in 2008. About half the breast cancer cases and 60% of the deaths are estimated to occur in economically developing countries (Ferlay et al, 2008; Jemal et al, 2011).

Breast cancer is in females affecting around 1.3 million women worldwide each year and causing about 460,000 deaths annually (Kloog et al, 2010). In Iraq, according to Iraqi cancer registry, breast carcinoma was the most frequent cancer among women, 63,923

Iraqi patients with various types of newly diagnosed cancer were registered by the Iraqi Ministry of Health from all Iraqi provinces with the exception of three Northern provinces (Sulaimanyia, Erbil, and Dohouk); of these patients, 31,652 (49.5%) were females, and breast cancer alone accounted for 31% of all new cancer cases among females (Al-Hasnawi and Al-Mosawi, 2009). In Basrah, breast cancer constituted one-third of all cancers in women in 2005, with an incidence rate of 13.1/ 100,000 women. The annual mortality rate of breast cancer in Basrah in 2005 was 3.2/10,000 (Al-Ali and A.L-Saad, 2008).

Breast cancer is a heterogeneous disease with distinct molecular

characteristics that have an impact on disease outcome, predict response to targeted treatment, and may influence resistance mechanisms (Jensen, 2011). Phosphatidylinositol 3-kinase (PI3K) is a family of lipid kinases which have ability to phosphorylate, and hence to activate protein kinase B (Jiang and Liu, 2008).

The PI3K can be activated by a variety of extracellular signals and involved in a number of cellular processes including cell proliferation, survival, protein synthesis, and tumor growth. The alterations of PI3K pathway such as activation of oncogenes, gene amplification, and inactivation of tumor suppressors, commonly occur in many human cancers (Jiang and Liu, 2008).

Somatic mutations in many different human cancers were discovered in the gene encoding for the phosphatidylinositol 3-kinase (PI3K) catalytic subunit, PIK3CA (Karakas et al, 2006). PIK3CA is amplified and overexpressed in breast cancer (Campbell et al., 2004; Aleskandarany et al., 2010; Firoozinia et al., 2014; Sakr et al., 2014).

PI3K activity is thus carefully regulated by growth factor–receptor interactions. In fact, the vast majority of PI3K remains inactive in the cytoplasm, far from its plasma membrane-associated substrates, and only a small percentage of PI3K becomes activated upon growth factor stimulation. Therefore, even slight modulations in receptor activity can lead to many-fold increases in PI3K activity (Yuan and Cantley, 2008)

PI3K is deregulated through a variety of mechanisms, including overexpression or activation of growth factor receptors, such as HER-2 (human epidermal growth factor receptor 2) (Pópulo et al.,2012), While other studies found that activating mutation of PIK3CA are associated with ER and PR positivity (Saal et al.,2005; Reis-Filho et al.,2006)

This study was done to determine the frequency of PIK3CA mutations in breast tumorigenesis with the polymerase chain reaction (PCR) method, through amplifying PIK3CA exon 20 gene sequences .Correlate PIK3CA mutations with clinicopathological features (age and sex) , and other prognostic parameters (steroid hormone receptor and HER-2 status in patients) was then done.

Materials and Methods

Study and Control Subjects

a) Study group: Paraffin blocks of thirty one patients with breast carcinoma were included in this study. These samples were collected from laboratory of histopathology in Alsader Teaching Hospital in Al-Najaf governorate.

b) Control group: six samples with normal breast tissues were considered as control group for this study.

DNA isolation and Polymerase Chain Reaction (PCR)

DNA from paraffin-embedded breast tumor specimens were prepared from

10µm thick sections. Genomic DNA was isolated using the protocol of Genomic DNA Mini Kit was designed specifically for purifying DNA from paraffin-embedded tissues.

Sequences of PIK3CA oncogene in exon 20 was amplified using this primer-pair:
5'- TGGGGTAAAGGGAATCAAAAG -3'
5'- CCTATGCAATCGGTCTTTG -3'

The primers for PIK3CA genes exon 20 (synthesized by AccuOligo® Bioneer Corporation .USA) were published previously (Miyake *et al.*, 2008; Zhu *et al.*, 2012).

Amplification was carried out in 20 µl tube of PCR PreMix Reaction Mixture (PCR PreMix, Bioneer Corporation, USA) containing 5 µl of template DNA (62.5 ng), 1 unit DNA polymerase, 2 µl reaction buffer, 2 µl stabilizer and loading-dye, 2 µl dNTPs, and 2µl of each primer(2 µl forward and 2 µl reverse). Distilled water was added to the final volume of 20 µl.

Amplification was performed in a thermal cycler(Cleaver scientific Ltd/UK) programmed for 35 cycles of denaturation at 94°C for 5 min, annealing at 60°C for 1 min, and extension at 68°C for 2 min, preceded by an initial denaturation of 5 min at 95°C. Final extension was for 7 min at 72°C.

Finally, the gel electrophoresis method was done according to Sambrook and Russell (2001), and 5 µl of each samples was loaded onto 1% agarose gel. Size of PCR products was 525 bp.

Statistical Analysis

Statistical analyses of all results were carried out by the help of SPSS version 17 software statistical package using chi square (P value was considered significant at level less than 0.05).

Results

During this study, 31 cases with breast carcinomas have been collected; these tissue samples were evaluated for ER, PR and HER2 by

Immunohistochemical analysis was performed by the staff of Laboratories of Alsader Teaching Hospital. The diagnosis was under the supervision of pathologist in the hospital.

The clinicopathological assessment revealed that 14 (45.16%) were hormone dependent carcinoma: ER+PR+, 3 (9.67 %) ER+PR- , and 9 (29.03%) ER-PR+ were probably hormone dependent carcinoma; and 5(16.12%) were hormone independent carcinoma: ER-PR- . HER2 expression was found in 7(22.58%) of cases (Table 1).

Table 1: Status of ER ,PR and HER2 in 31 Cases of Breast Carcinoma.

Status of ER and PR	HER2 status		Total No. (%)
	HER2 negative (0 and 1+)	HER2 positive (2+ and 3+)	
ER+/PR+	10 (71.43)	4 (28.57 %)	14 (45.16%)*
ER+/PR-	1 (3.33 %)	2 (66.66 %)	3 (9.67 %)
ER-/PR-	5 (100 %)	0	5 (16.12%)
ER-/PR+	8 (88.88 %)	1 (11.11 %)	9 (29.03%)
Total	24 (77.42)	7 (22.58%)	31 100%

*P <0.05 significant

Assessment of age presentation of patients revealed that 2 (6.45%) patients were seen in age group (30-35), 2 (6.45%) in age group (36-41), 10 (32.25%) in age group (42-47), 5 (16.12%) in age group (48-53), 5

(16.12%) in age group (54-59) , and 7 (22.58%) patients in the age group (60-65) (Figure 1). Their ages ranged from 30 to 65 years, with a mean age of 49.9 years.

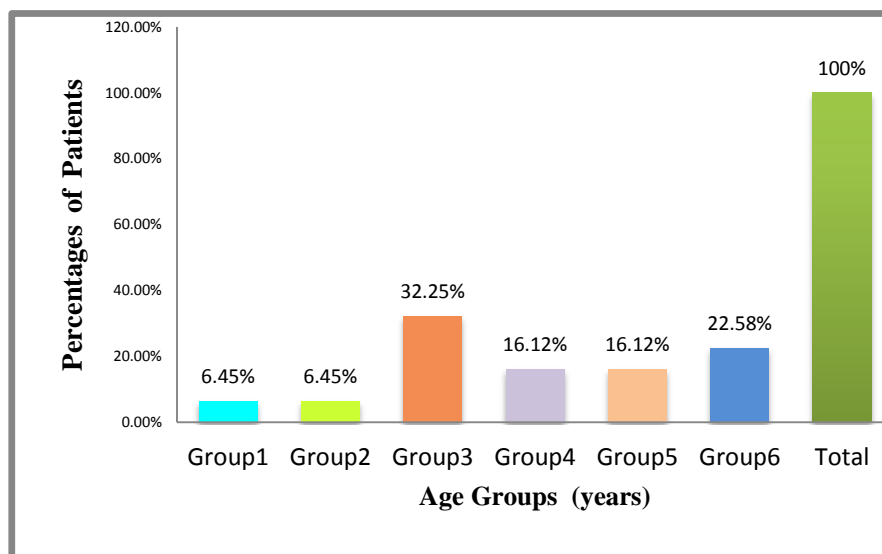


Figure 1: Age distribution of the presented breast carcinoma patients. (Group1: 30-35, Group2: 36-41, Group3: 42-47, Group4: 48-53, Group5: 54-59, and Group6: 60-65).

PIK3CA Mutational Status in Breast Carcinoma

No mutations in exon 20 of PIK3CA was found in any of the 6 normal breast samples, with a significant difference with that of neoplastic breast tissue ($p < 0.05$).

In this study, mutational analysis of PIK3CA was done in 31 breast cancers and, finally, 11 (35.48%) mutations were identified in total (Table 2; Figure 2).

Table 2: PIK3CA mutations in different types of breast tissues.

Type of Tissue	PIK3CA mutations		Total
	Positive	Negative	
Normal	0	6 100%	6 (16.21%)
Malignant	11 (35.48)	20 (64.52%)	31 (83.79%)
Total	11 (29.72%)	26 (70.28%*)	37 100%

*P < 0.05 significant

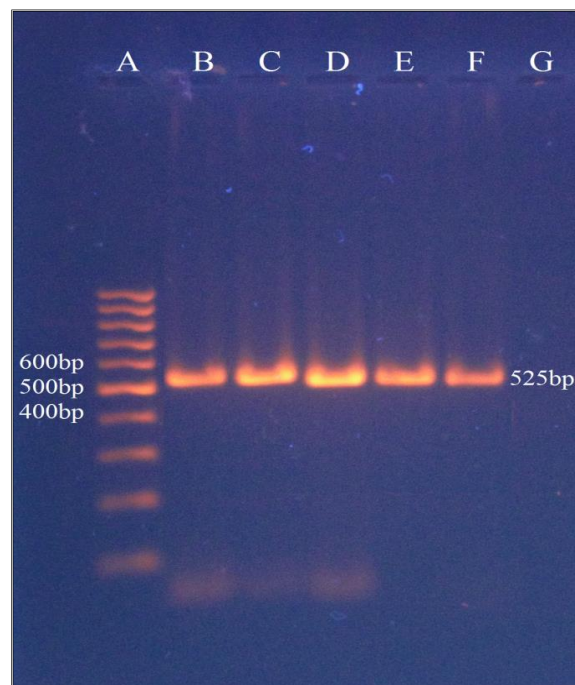


Figure 2: PCR amplified 525 bp of PIK3CA gene (exon 20), A: (100 bp) DNA ladder, from B to F: Mutation of PIK3CA gene, G: control.

PIK3CA mutations and clinico-pathologic characteristics of breast cancers

The relationship of PIK3CA mutations with hormone receptor status was shown in table 3.

The frequency of PIK3CA mutations in ER-positive tumors was significantly ($P < 0.05$) higher than that in ER-negative tumors, and the frequency of PIK3CA mutations in PR-positive tumors tended to be higher than that in PR-negative tumors.

Table 3: The Relationship of PIK3CA Mutations with Receptors.

Type of Receptors	PIK3CA mutations		Total
	Positive	Negative	
ER+/PR+	9 (64.29%*)	5 (35.71%)	14 (45.16%)
ER+/PR-	0	3 (100%*)	3 (9.67%)
ER-/PR-	0	5 (100%)	5 (16.12%)
ER-/PR+	2 (22.22%)	7 (77.73%*)	9 (29.03%)
Total	11 35.49	20 64.51	31 (100%)

* $P < 0.05$ significant

The relationship between PIK3CA mutations and HER2 expression was shown in Table (4).

HER2 expression was not significantly associated with PIK3CA mutations.

Table 4: The Relationship between PIK3CA Mutations and HER2 Expression

HER2 expression	PIK3CA mutations		Total
	Positive	Negative	
HER2 negative (0 and 1+)	4 (16.66%)	20 (83.33%)	24 (45.16%)
HER2 positive (2+ and 3+)	3 (42.85%)	4 (57.14%)	7 (9.67%)
Total	7 (22.85%)	24 (77.41%)	31 (100%)

* $P > 0.05$ not significant

Discussion

Recent advances in the studies of genetic variants have improved our understanding of the pathogenesis of breast cancer.

In this study a trial to investigate whether there is an association between numbers of genetic and proliferative markers abnormalities, and breast tumorigenesis or pathogenesis in a sample of Iraqi patients with breast cancer.

Recent studies have shown that PIK3CA mutations play a major role in resistance to drugs (Wang et al., 2011) or to hormonal therapy (Miller et al., 2011; Ma *et al.*, 2011) of breast carcinomas. PIK3CA mutations are mostly located within exons 9 and 20 and four hotspots (c.1624G→A, c.1633G→A, c.3140A→G and c.3140A→T) which represent more than 90% of all mutations (Harlé et al., 2013).

Barbareschi *et al.* (2007), found that exon 9 mutations have a negative prognostic value while exon 20 mutations were associated with favorable outcome, while Lai et al. (2008) reported exon 20 mutations as associated with poor prognosis.

In this study, Mutational analysis of PIK3CA was done in 31 breast cancers. Eleven mutations (35.48%) were identified in total. This study was retrospective and was based on a small number of cases and was never confirmed prospectively.

By using protein expression of PIK3CA, Aleskandarany et al. (2010) reported that PIK3CA protein expression in invasive breast cancer was associated with poor prognosis.

The spectrum of mutations found in the present study is similar to the analysis by Samuels et al. (2005), but there are some important differences. In their study, forty-three exon 9 and twenty-seven exon 20 mutations were found suggesting exon 9 mutations are the most common PIK3CA mutations in colorectal and other cancers. In

contrast, in this study 11 mutations was found within exon 20, suggesting that exon 20 mutations predominate in human breast cancers which agree with results of (Harlé *et al.*, 2013)

In this study, the frequency of PIK3CA mutations in ER-positive tumors was significantly ($P < 0.05$) higher than that in ER-negative tumors, and the frequency of PIK3CA mutations in PR-positive tumors tended to be higher than that in PR-negative tumors.

This evidence has been supported by few studies of breast cancer suggesting that PIK3CA mutations are more frequent in estrogen receptor-alpha-positive (ERa+) breast tumors (30% to 40%) than in receptor-alpha-negative (ERa-) breast tumors (10% to 20%) (Samuels et al., 2005; . Saal et al., 2005; Kalinsky et al., 2009; Hennessy et al., 2009).

Although it has been reported that PIK3CA mutations are related to HER2 status (Kalinsky et al., 2009), this issue still remains controversial since no correlation between PIK3CA mutations and HER2 status has been observed (Harlé et al., 2013) similarly to the results of the present study. Similar findings have been found concerning the correlation between PIK3CA mutations and HER2 status but again are controversial in other series (Barbareschi *et al.*, 2007; Maruyama *et al.*, 2007) and when HER2 was determined using immunohistochemistry (Perez-Tenorio *et al.*, 2007).

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