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ORGINAL ARTICLE

Role of Some Estrogen Receptor and Her2 Pathway related Genes (PIK3CA, GSK3 β) in Predicting Response to Tamoxifen in Breast Cancer Patients

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ABSTRACT

Background: *PIK3CA* and *GSK-3β* have important role(s) in the resistance of estrogen receptor positive (+ER) breast cancer (BC) patients to hormonal therapy. The aim of the current study was to assess the role of *PIK3CA* and *GSK-3β* in predicting response to Tamoxifen in BC patients.

Methods: PIK3CA and GSK-3β expression levels were assessed in formalin fixed paraffin embedded tissue (FFPE) sections of 58 hormonal positive BC females, using quantitative real time PCR (RT-qPCR). The data were correlated to clinico-pathological features of the patients, response to Tamoxifen and survival outcome.

Results: The median PIK3CA expression increased significantly in non-responders to tamoxifen compared to responders [2.94 (IQR=7.5) and 0.72 (IQR=0.9); respectively, P= 0.017]. However, there was no significant difference in GSK-3β expression between responders and non-responders BC patients [1.5 (IQR=3.8) and 1.2 (IQR=2.8); respectively, P= 0.27]. PIK3CA expression associated with low ER expression, presence of distant metastasis and shorter disease free survival (DFS) rate (P=0.032, 0.026 and 0.043; respectively). GSK-3β expression increased significantly in negative LN metastasis and low-expression of progesterone receptors (P=0.01 and 0.006; respectively). There was significant correlation between the expression levels of GSK-3B and PIK3CA in BC patients (r=0.88, P<0.001), and non-responders (r=0.97, p<0.001). However, in the responders, there was a non-significant negative correlation between the two markers (r=-0.16, P=0.43). Multivariate analysis showed that tumor size and PIK3CA expression were independent prognostic factors for reduced DFS in BC patients.

Conclusion:

PIK3CA is a negative predictor for BC patients' outcome, and for poor response to Tamoxifen. PIK3CA and GSK-3β could be a potential prognostic and molecular targets for breast cancer therapy.

Key words: ESTROGEN RECEPTOR- TAMOXIFEN -BREAST CANCER- PI3Kca expression GK expression.

INTRODUCTION

Breast cancer (BC) is the most commonly diagnosed cancer among females, and still the leading cause of cancer-related death in women worldwide [1]. In the United States, it ranked the second cause of cancer mortality among women after lung cancer. Estrogen receptor-positive (ER+) breast cancer is the most commonly diagnosed BC subtype. Tamoxifen is the main therapeutic agent used for ER+ breast cancer patients, as it inhibits the ER transcription process by competing with estrogen for binding to the ER protein [3]. However, many patients still showed tamoxifen resistance, either de novo or acquired, which leads to metastasis and/or recurrence, which consequently leads to poor patients' outcomes [5]. Several mechanisms have been reported to explain this clinically established tamoxifen resistance, including estrogen receptor-alpha (ERSα) mutations, amplification of fibroblast growth factor receptor-1 (FGFR1), aberrant activation of cyclin-dependent-kinase 4/6 (CDK4/6) or PI3K/mTOR pathway. Nevertheless, some of the mechanisms of tamoxifen resistance are still unknown in many patients [6].

The phosphatidylinositol 3-kinase (*PI3K*) pathway is the most commonly altered pathway in ER+ breast cancer patients [7]. It has been reported that *PIK3CA*, which encodes the p110α subunit of the *PI3K*, is frequently mutated in 40-50% of ER+

breast cancer cases [9]. The *PI3K/Akt* pathway together with the *Wnt/b-catenin* are considered the main signal pathways involved in cell growth and proliferation, so they have important roles in the carcinogenesis of many cancers including BC [10].Indeed, there is a crosstalk between the two pathways, since in *Wnt/b-catenin* signalling pathway, glycogen synthesis kinase-3b (*GSK-3b*) can phosphorylate *b-catenin*, causing its degradation by ubiquitin-proteasome. On the other side, the activated *AKT* can inhibit *GSK-3b* activation through phosphorylation. As a result, *b-catenin* accumulates in the cells and stimulates the transcription of the downstream target genes, such as cyclinD1 and c-myc, which in turn promote cell growth. Hence, the two signaling pathways (*Wnt/b-catenin* and *PI3K/Akt)* are linked by *GSK-3b* [12].

The *GSK-3*, a serine/threonine protein kinase, has two main subtypes; 1) GSK-3α which is mainly involved in the process of glycogen metabolism, and 2) GSK-3β which is considered a potential tumor suppressor as it has the ability to inhibit target oncogenic molecules, such as *c-Jun, c-Myc, cyclin D1*, and *β-catenin* [16]. In addition, it promotes down-regulation of epithelial-mesenchymal transition and histone methyltransferase (EZH2), both of them have an important role(s) in carcinogenesis. Consequently, it is clear that *GSK-3β* suppresses breast cancer cell proliferation, migration and survival [17].

Several previously published studies demonstrated that mutations affecting *PI3K* pathway could induce sensitivity to *PI3K* inhibitors, and accordingly, combining *PI3K* inhibitors with endocrine therapy in the presence of *PI3K* mutations would be of beneficial value for those patients [21]. However, till now there is no biological biomarker with acceptable predictive value for selecting patients who will benefit from endocrine treatment [23]. Hence, the aim of the current study was to assess the role of *PIK3CA* and *GSK-3b* in predicting response to tamoxifen therapy in ER+ breast cancer patients. We suppose that, this will allow for better selection of patients who will benefit from tamoxifen therapy, and thereby, this will give a chance for appropriate management and better outcome for those patients.

PATIENTS AND METHODS

The current study is a prospective study, which included 58 female patients more than 18 years old with breast cancer who were diagnosed and treated at the Medical Oncology Departments of National Cancer Institute (NCI), Cairo University and Zagazig University during the period from April 2014 to April 2016. Written informed consent was obtained from all participants, the study was approved by the research ethical committee of Faculty of Medicine, Zagazig University and Cairo University. The study was done according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans. All patients were hormone receptor positive and all of them received tamoxifen as adjuvant hormonal treatment after surgery for early breast cancer or local recurrence (recur the disease at site of breast surgery after adjuvant treatment). The patients were prospectively enrolled into the study and then divided into two groups, responders as no development of distant metastasis or progression of the disease during the study and non-responders to hormonal therapy.

Patients were excluded from the study: 1- if they had metastatic disease from the start or had a history of comorbid illness,

2-as well as those with prior deep venous thrombosis, patients on anti-coagulant medication for two weeks following registration.

3- patients who were diagnosed with inflammatory breast cancer (IBC).

All included patients were subjected to full history taking, complete clinical exmination and laboratory investigation, as well as radiological workup.

During treatment, patients were also assessed for adverse events then the patients were checked at 8, 16 and 24 weeks of treatment for evidence of metastases or recurrence in the adjuvant setting adjuvant and after every 12 weeks until 2 years. Patients received tamoxifen 20 mg once daily as a hormonal treatment. Then the expressions of PI3K and GSK3b RNA were evaluated i continued follow up patients for 68 months to evaluated survival outcome. .

The response to tamoxifen was assessed as follows:

If the patients developed metastases or recurrence during 96 weeks they are considered as hormone resistant disease.

STATISTICAL ANALYSIS

All statistical tests were performed using R studio software (version 3.6). Normality of data was detected by Shapiro-Wilk test. Mann-Whitney was performed to compare the expression of GCK and PI3Kca between responding and non-responding breast cancer patients. Comparison between the gene expression and clinico-pathological characteristics in responding and non-responding

patients were done using Pearson's chi-square test. The correlation between GCK and PI3K was performed using Spearman correlation. Statistical significance was indicated with a two-tailed p value ≤0.05. Kaplan Meier analysis was used to estimate the disease free survival and overall survival rates. Comparisons between the different prognostic factors were done using the Log rank test. The disease free survival was defined as the length of time after treatment during which no disease is found until reappear new lesion. Patients who either progressed or lost follow up will be censored at last assessment prior to loss to follow-up.

RESULTS

Clinico-pathological Features of the Assessed Breast Cancer Patients

The present study included 58 female breast cancer patients with a median age of 44.27±7.8 years at diagnosis, and 11.7±0.76 years at menarche. The median tumor size was 4.8±1.69 cm. Nineteen out of 58 (32.8%) patients had tumor size < 4cm, and 39/58 (67.2%) had tumor size ≥ 4cm. Patients with tumor grade II represented 44/58 (76.0 %) of the cases, and those with grade III were 14/58 (24.0%). As for tumor staging, ten patients (17.2%) were stage I, 25 (43.1%) were stage II, 17 (29.3%) were stage III and 6 (10.3%) patients were stage IV. Fifty patients (86.2%) had lymph nodes (LNs) positive. Regarding the hormonal receptors (HR) status, high expressions of ER and PR were detected in 54 (93.1%) and 48 (82.8%) of the cases respectively; while low expressions of ER and PR were detected in 4 (6.9%) and 10 (17.2%) patients, respectively. Nine patients (15.6%) showed HER2 protein overexpression, and 49 cases (84.4%) were HER-2 negative **(Table 1).**

Clinico-pathological Characteristics in Relation to Patients' Response to Hormonal Therapy

Patients were classified according to their response to hormonal therapy into responder (28 cases), and non-responder (30 cases). The age of the patients was significantly younger in the non-responder group (42.2±7.02 years) compared to responders (46.5±8.1 years, **P=0.039**). The mean tumor size was significantly larger in non-responding patients (5.5±1.26 cm) compared to the responders (3.9±1.71 cm, **P<0.001**). A higher percentage of the non-responding patients showed a significantly larger tumor size (≥4cm) compared to responding patients (86.7% in the non-responders versus 46.4% in the responders, **P=0.003**). There was a trend for association between better response to therapy and early disease stage, however this did not reach a significant level (75% in responders versus 46.7% in non-responders, **P=0.053**).No other significant relation was detected between patients' response to treatment and the assessed patients' characteristics **(Table 2).**

The expression of Phosphoinositide-3kinase (*PIK3CA*) and Glycogen synthase kinase-3β (*GSK-3β*) in responding and non-responding breast cancer patients

The median *PIK3CA* fold change expression was significantly higher in non-responders compared to responders [2.94 (IQR=7.5) and 0.72 (IQR=0.9); respectively, **P= 0.017].** However, there was no significant difference in the expression levels of *GSK-3β* between responder and non-responder BC patients [1.5 (IQR=3.8) and 1.2 (IQR=2.8); respectively, **P= 0.27, Figure 1].**

The association between *GSK-3β, PIK3CA* expression and the clinico-pathological features of breast cancer patients

There was significant association between *GSK-3β* overexpression and a) low tumor grade [1.38(3.08) in grade 2 compared to 0.21(1.99) in grade 3, **P=0.048**], b) negative LN metastasis [3.73(6.86) in negative LN metastasis compared to 0.91(2.34) in positive LN metastasis, **P=0.003**], and c) progesterone receptor (PR) expression [2.88(5.43) in low PR expression, compared to 0.80(2.41) in high PR expression, **P=0.009**]. On the other hand, *PIK3CA* overexpression associated significantly with low ER expression [4.9 (8.32) in low ER expression, compared to 0.80 (1.73) in ER overexpression, **P=0.032**]. **Table 3**]

Expression of *GSK-3β* and *PIK3CA* in relation to relevant clinico-pathological characteristics among responding and non-responding patients

In responding BC patients, the median *GSK-3β* fold change expression was significantly higher in LN negative patients than in LN positive patients **(P=0.01),** as well as those with low progesterone receptor expression than those with high PR expression **(P =0.006)**. On the other hand, no significant relation was found between *GSK3β* and relevant clinico-pathological features among the non-responder BC patients **(Figure 2).**

Regarding *PIK3CA* expression, there was no significant association between median *PIK3CA* fold change expression and the assessed clinico-pathological characteristics in the responding and non-responding BC patients **(Figure 3).**

Correlation between *GSK-3β* and *PIK3CA* expression in the breast cancer patients.

There was a strong positive correlation between *GSK-3B* and *PIK3CA* expression in BC patients

(r=0.88, P<0.001). Similarly, a significant positive correlation was detected between the expression levels of *GSK-3β* and *PIK3CA* **(r=0.97, p<0.001)** in the non-responding group. However, in the responding BC patients, there was a non-significant negative correlation between the expression of *GSK-3β* and *PIK3CA* **(r=-0.16, P=0.43, Table 4).**

SURVIVAL ANALYSIS

The median follow-up period of all patients was 31 (range, 6-68) months. Disease was detected in 53.4% (31/58) of the cases, while 34.48% (20/58) of the patients died during the follow up period. The non-responders experienced a significantly poorer DFS **(P< 0.001)** and OS **(P=0.0027)** rates compared to the responding group **(Figure 4)**.

Patients were then categorized according to the median expression of *GSK-3β* into*;* high *GSK-3β* expression: ≥1.35 and low *GSK-3β* expression: <1.35. Also, according to the median expression of *PIK3CA* into high *PIK3CA* expression: ≥1.04 and low *PIK3CA* expression: <1.04.

Kaplan Meier survival analysis showed that high *PIK3CA* expression associated significantly with a shorter DFS rate of breast cancer patients **(P=0.043)**. However, there was no significant association detected between *PIK3CA* expression level and DFS rates of non-responding and responding BC patients (P= 0.88 and P=0.93; respectively). On the other hand, there was no significant association between *PIK3CA* expression and OS rates of BC patients, non-responding and responding patients (P=0.24, P=0.74, P=0.62; respectively, **Figure (5).**

Regarding GSK-3β expression, there was no significant association between GSK-3β expression level and DFS rates of all BC patients, non-responding and responding patients (P=0.84, P=1.0 and P=0.64; respectively). Similarly, no significant association was found between *GSK-3β* expression and OS of BC patients, non-responding and responding patients (P=0.38, P=0.95 and P=0.39; respectively, **Figure (6).**

Univariate and multivariate survival analysis of breast cancer patients.

A univariate survival analysis of BC cases demonstrated significant association between decreased DFS rate and large tumor size **(HR= 4.6, P= 0.004)**, late disease stage **(HR= 3.2, P=0.002)** and high PIK3CA expression **(HR= 2.1, P= 0.04)**. While, reduced OS rate was significantly associated with large tumor size only **(HR= 3.16, P= 0.04)**. Multivariate logistic regression survival analysis of BC patients adjusted for tumor size, tumor stage and *PIK3CA* expression showed that tumor size and *PIK3CA* expression were independent prognostic factors for reduced DFS in BC patients (**P=0.023** and **P=0.031**, respectively). While, large tumor size only was an independent predictor of reduced OS in BC patients **(P= 0.024, Table 5).**

Table 1: Clinico-pathological features of the assessed Breast Cancer Patients

Patient Characteristics		N (%)
Age at diagnosis (years)		44.27±7.8
Age at menarche (years)		11.7±0.76
Age (years)	<44	23(39.7)
	≥44	35(60.3)
Tumor size(cm)	<4	19(32.8)
	≥4	39(67.2)
Grade	2	44(76)
	3	14(24)
Stage	I	10(17.2)
	II	25(43.1)
	III	17(29.3)
	IV	6(10.3)
Lymph node metastasis	No	8(13.8)
	Yes	50(86.2)
No of lymph nodes	1-3	7(12.1)
	4-10	33(56.9)
	>10	18(31.0)

	Patient Characteristics	N (%)
ER receptor	low	4(6.9)
	high	54(93.1)
PR receptor	low	10(17.2)
	high	48(82.8)
HER2	Negative	49(84.4)
	Positive	9(15.6)
Adjuvant therapy	AC + Taxotere	39(67.2)
	FEC100 + Taxotere	19(32.8)
Response to hormonal therapy	Non-responding	30(51.7)
	Responding	28(48.3)

ER; Estrogen receptor, PR: Progesterone receptor, HER2; human epidermal growth factor receptor 2, AC; Adriamycin, FEC; fluorouracil, epirubicin, cyclophosphamide, RT; Radiotherapy.

Table 2: Clinico-pathological characteristics in relation to patients' response to hormonal therapy.

Patient Characteristics	Responders (n= 28)	Non-responders (n=30)	P value
Age at diagnosis(yrs.)	46.5±8.1	42.2±7.02	**0.039***
Age at menarche(yrs.)	11.5±0.6	11.8±0.8	0.13
Age(yrs.)			0.16
<44	8(28.6)	15(50.0)	
≥44	20(71.4)	15(50.0)	
Tumor size(cm)	3.9±1.71	5.5±1.26	**<0.001**
Tumor size(cm)			**0.003**
<4	15(53.6)	4(13.3)	
≥4	13(46.4)	26(86.7)	
Grade			0.44
2	23(82.1)	21(70.0)	
3	5(18.9)	9(30.0)	
Stage			0.053
Early(I-II)	21(75.0)	14(46.7)	
Late (III-IV)	7(25.0)	16(53.3)	
LN metastasis			0.21
No	6(21.4)	2(6.7)	
Yes	22(78.6)	28(93.3)	
No. of LNs			0.34
1-3	5(17.9)	2(6.7)	
4-10	16(57.1)	17(56.7)	
>10	7(25.0)	11(3.7)	
ER receptor			1.0
Low	2(7.1)	2(6.7)	
high	26(92.8)	28(93.3)	
PR receptor			0.24
Low	7(25.0)	3(10.0)	
high	21(75.0)	27(90.0)	
HER2			0.54
Negative	25(89.3)	24(80.0)	
Positive	3(10.7)	6(20.0)	

Patient Characteristics	Responders (n= 28)	Non-responders (n=30)	P value
Adjuvant therapy			0.19
AC + Taxotere	16(57.1)	23(76.7)	
FEC100 + Taxotere	12(42.9)	7(23.3)	
Herceptin			0.54
No	25(89.3)	24(80.0)	
Yes	3(10.7)	6(20.0)	
RT			0.058
No	5(17.9)	0.0(0.0)	
Yes	23(82.1)	30(100.0)	

LN; lymph node, ER; Estrogen receptor, PR: Progesterone receptor, HER2; human epidermal growth factor receptor 2, AC Adriamycin, FEC; fluorouracil, epirubicin, cyclophosphamide, RT; Radiotherapy. *Significant at P<0.05

Table 3: Association between GSK3B, PIK3CA expression and the clinic-pathological features of breast cancer patients

	GSK3B	P value	*PIK3CA*	P value
	Median(IQR)		**Median (IQR)**	
Age				
<44	1.77(3.04)	0.43	0.838(4.05)	0.71
≥44	0.80(2.69)		0.835(1.48)	
Tumor size				
<4	1.08(3.07)	0.83	0.72(1.41)	0.26
≥4	1.32(2.67)		0.85(3.53)	
Grade				
2	1.38(3.08)	**0.048**	1.07(3.44)	0.91
3	0.21(1.99)		0.94(6.2)	
Tumor stage				
Early	1.35(2.64)	0.32	0.83(5.15)	0.92
Late	0.56(2.85)		0.98(1.85)	
LN metastasis				
No	3.73(6.86)	**0.003**	1.069(2.0)	0.78
Yes	0.91(2.34)		0.83(3.12)	
LN number				
1-3	1.32(6.39)	0.42	0.72(1.48)	0.155
4-10	0.62(1.80)		1.55(4.71)	
>10	0.44(2.33)		0.67(1.38)	
ER				
low	1.57(1.64)	0.89	4.9(8.32)	**0.032**
high	1.28(3.08)		0.80(1.73)	
PR				
low	2.88(5.43)	**0.009**	1.185(2.39)	0.67
high	0.80(2.41)		0.83(3.3)	
HER2				
Negative	1.20(2.87)	0.94	0.897(3.25)	0.63
Positive	1.68(2.65)		0.847(2.97)	

LN; lymph node, RT; radiotherapy, IQR; interquartile range. *Significant at P<0.05.

Table 4: Correlation between *GSK-3β* and *PIK3CA* in breast cancer patients, non-responding and responding group

Breast cancer patients n=58		Non-responding n=30		Responding n=28	
GSK-3β	***PIK3CA***	***GSK-3β***	***PIK3CA***	***GSK-3β***	***PIK3CA***
	r= 0.88**		r= 0.97**		r= -0.16
	P<0.001		P<0.001		P=0.43

GSK-3β: glycogen synthase kinase-3β, *PIK3CA*: phosphatidylinositol-4,5-bisphosphate-3-kinase catalytic subunit alpha, r=Pearson correlation coefficient. **Highly significant at p<0.01

Table 5: Univariate and multivariate Cox regression survival analysis of breast cancer patients.

Factor	Overall survival			Progression-free survival		
	HR	**95% CI**	**P value**	**HR**	**95% CI**	**P value**
Univariate						
Age≥ 44 vs <44	0.94	0.37-2.36	0.9	0.57	0.28-1.15	0.1
Grade III vs II	1.09	0.40-3.0	0.87	1.52	0.7-3.2	0.30
Tumor size ≥4 vs <4	3.16	1.04-9.6	**0.04***	4.6	1.6-13.3	**0.004***
Stage late vs early	2.36	0.95-5.9	0.06	3.2	1.5-6.6	**0.002***
LN metastasis Yes vs No	1.69	0.39-7.31	0.48	2.6	0.61-10.7	0.20
No of LNs >10 vs ≤10	1.3	0.64-2.64	0.46	1.4	0.79-2.50	0.25
ER high vs low	0.65	0.15-2.8	0.57	2.13	0.29-15.7	0.46
PR high vs low	2.05	0.47-8.85	0.37	1.62	0.57-4.6	0.37
HER2 +ve vs -ve	1.56	0.44-5.54	0.49	1.62	0.66-3.98	0.30
PIK3CA ≥1.04 vs <1.04	1.70	0.70-4.13	0.24	2.1	1.008-4.3	**0.04***
GSK-3β ≥1.35 vs <1.35	0.67	0.27-1.64	0.38	0.94	0.46-1.9	0.86
Multivariate						
Tumor size ≥4 vs <4				3.7	0.57-2.3	**0.023***
Stage late vs early				2.02	0.39-1.79	0.07
PIK3CA ≥1.04 vs <1.04				2.2	0.37-2.15	**0.031***

LN; lymph node, ER; estrogen receptor, PR; progesterone receptor, HER2; human epidermal growth factor receptor2, GSK-3β; glycogen synthase kinase-3β, *PIK3CA*; phosphatidylinositol-4,5-bisphosphate-3-kinase catalytic subunit alpha. *Significant at p<0.05.

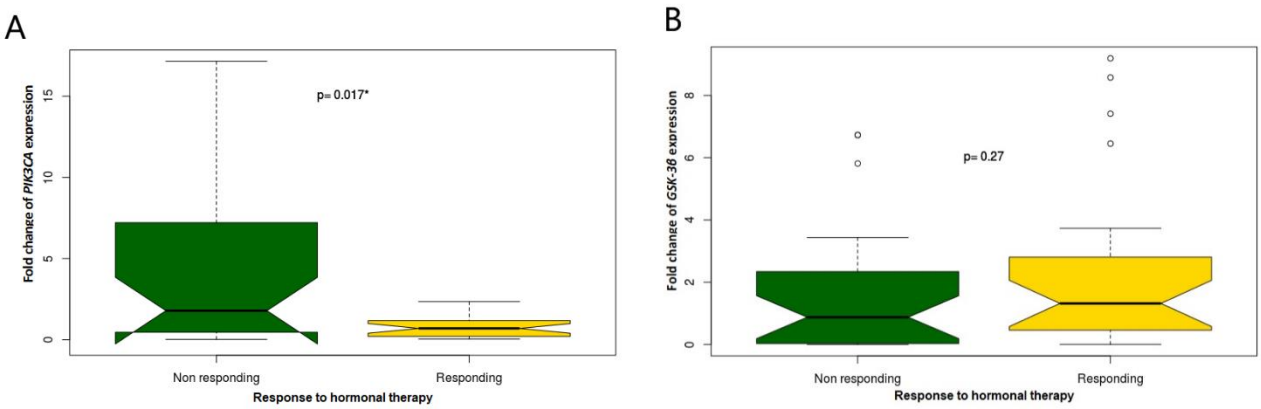

Figure 1: Fold change expression of: A) *PIK3CA* and B) *GSK-3β* rs33455 among responding and non-responding breast cancer patients. Medians are the central horizontal bars are. Notch displays confidence intervals around medians. Box represents the Inter Quartile Range (IQR) and allows to visualize the dispersion of 50% of the central data points around the median. No overlap between two boxes' notches indicates statistically significant difference between the medians

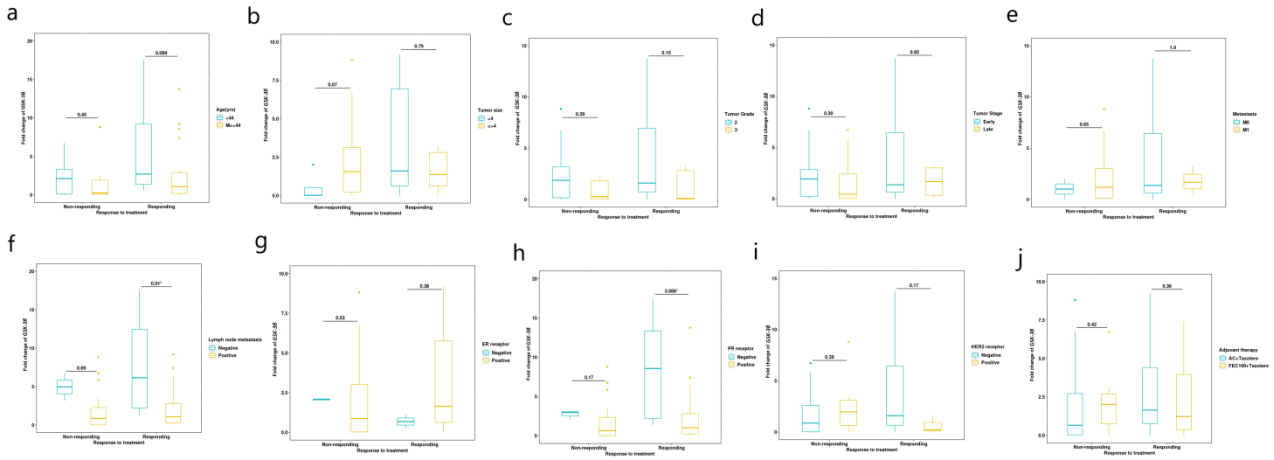

Figure 2: Association between *GSK-3β* expression and clinical features of responder and non-responder breast cancer patients.

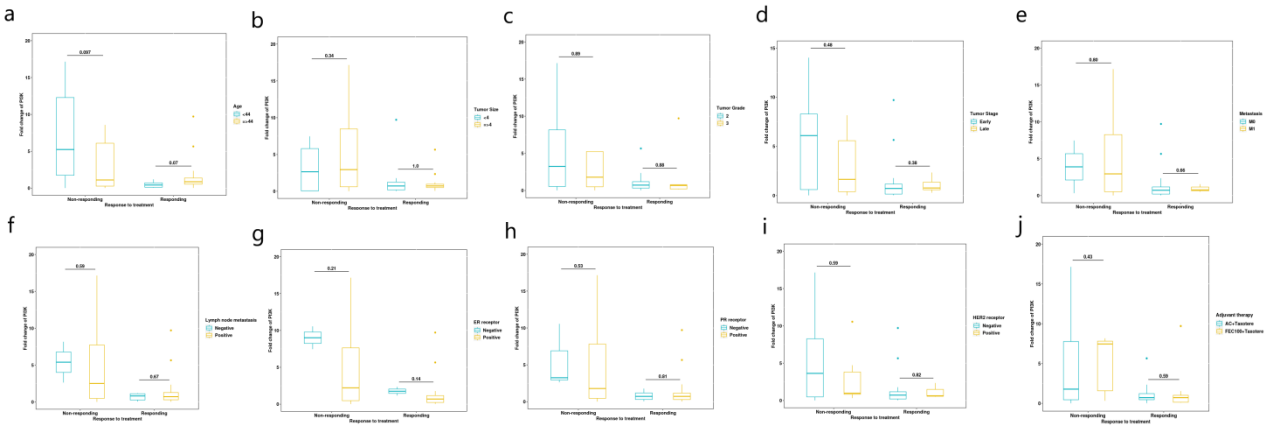

Figure 3: Association between *PIK3CA* expression and clinical features of responder and non-responder breast cancer patients.

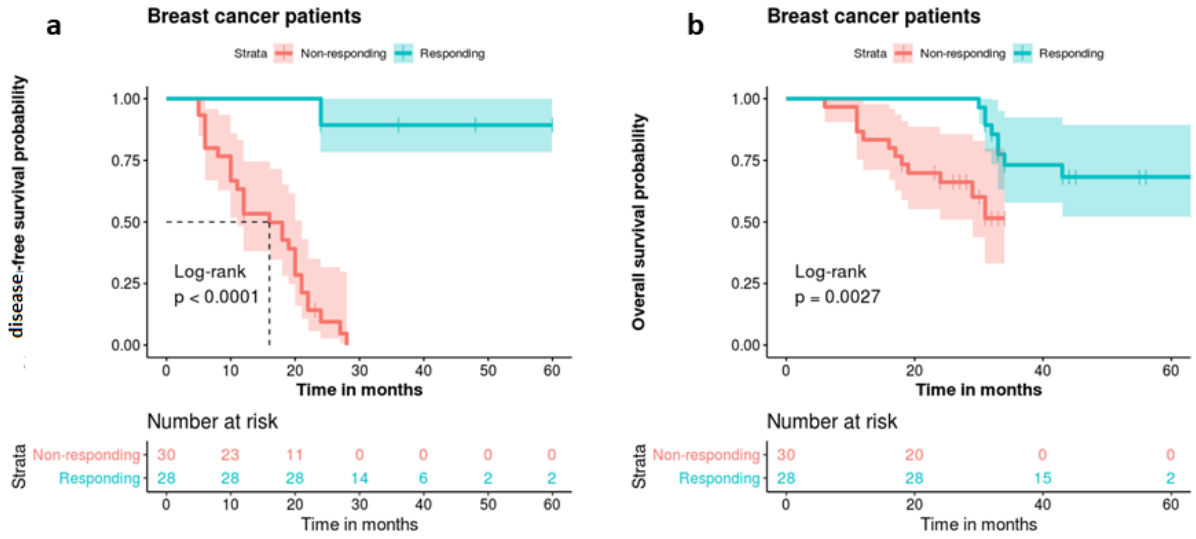

Figure 4: Kaplan Meier survival curve for a) disease-free (DFS) and b) overall survival (OS) in breast cancer patients according to the response to hormonal therapy.

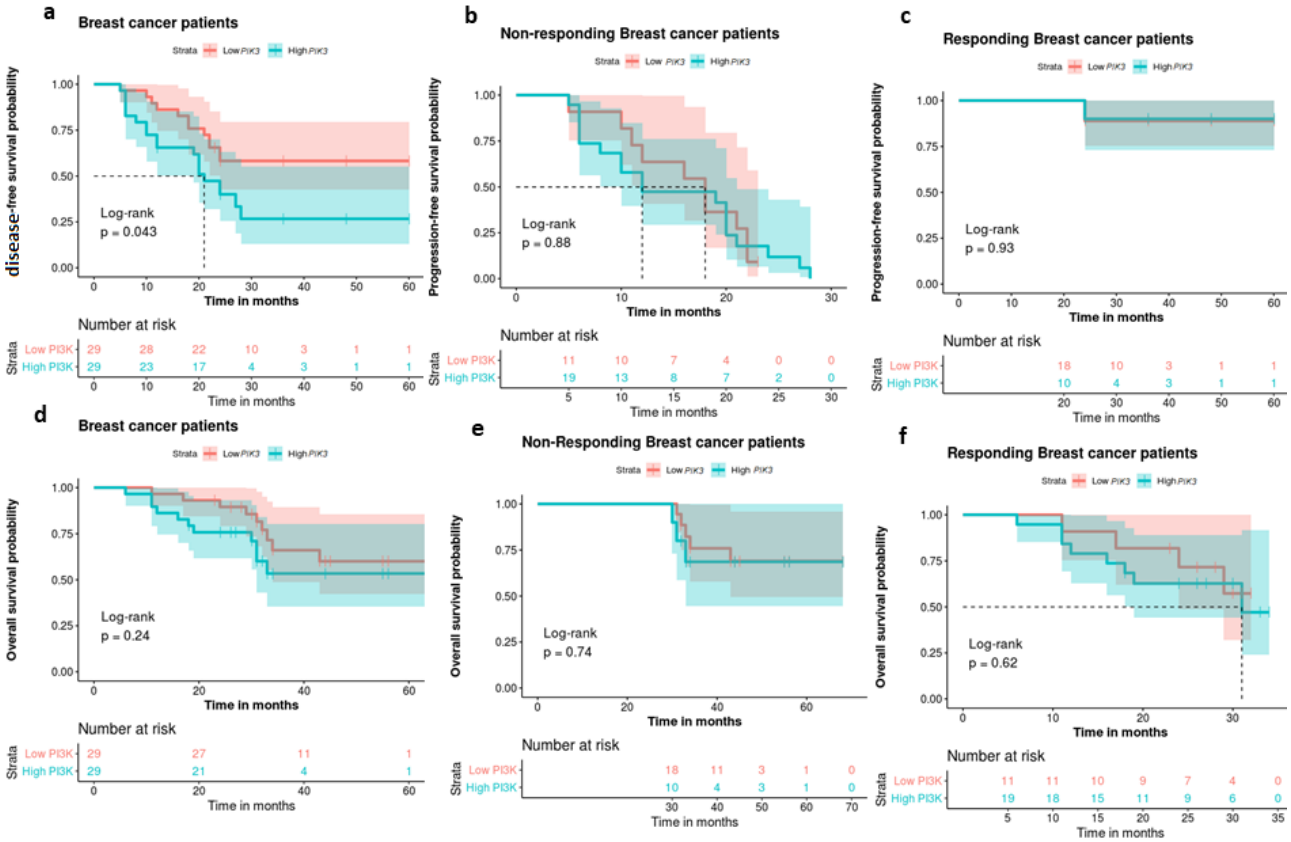

Figure 5: Kaplan Meier survival curve for a) disease-free (DFS) and b) overall survival (OS) in breast cancer patients according to *PIK3CA* expression.

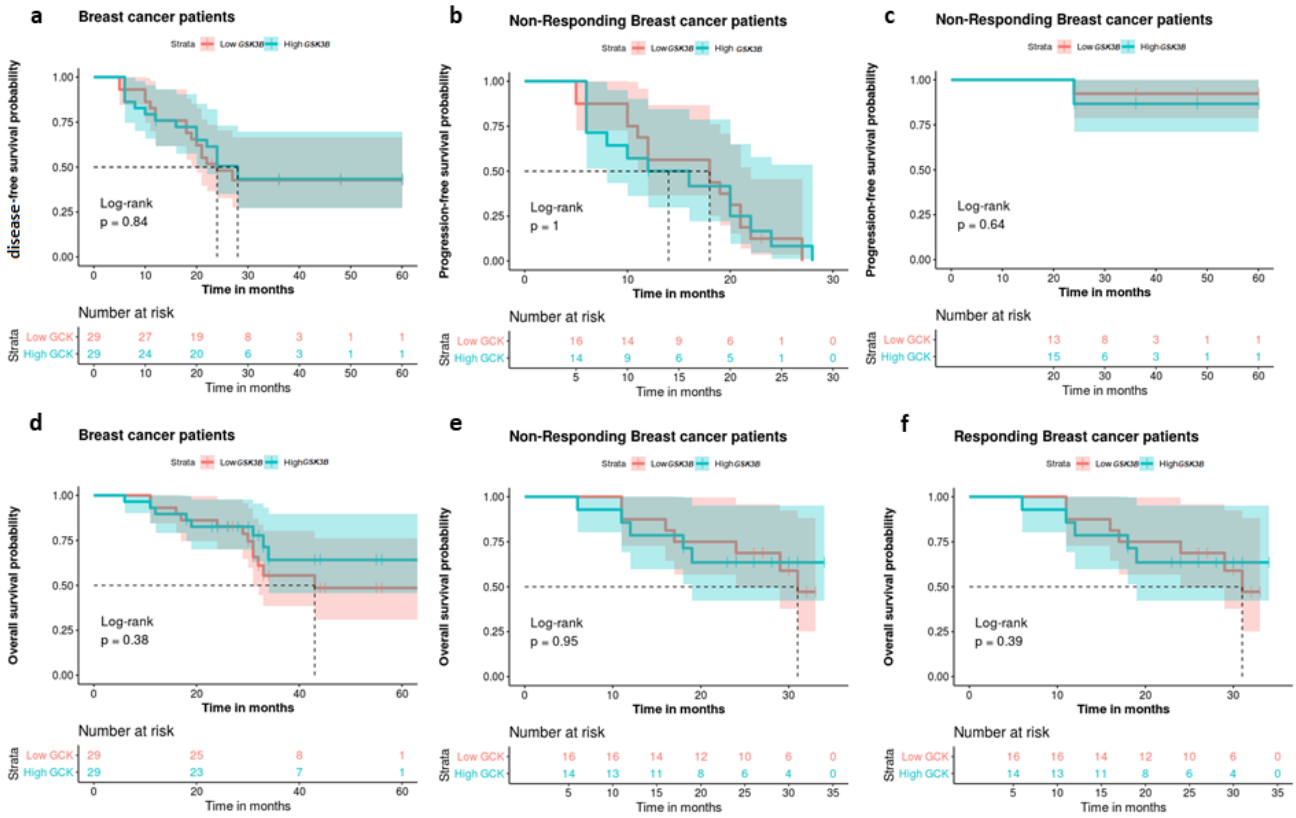

Figure 6: Kaplan Meier survival curve for a) disease-free (DFS) and b) overall survival (OS) in breast cancer patients according to *GSK-3β* expression.

DISCUSSION

Although endocrine therapy is the most commonly used regimen for the treatment of ERα positive BC patients, its effect is greatly influenced by *de novo* or acquired tamoxifen resistance in patients with BC [15]. For the past two decades, several molecules have been identified as mediators of tamoxifen resistance, or predictors of response to hormonal therapy including *PIK3CA* and *GSK-3b* [16].

In the current study, we found that *PIK3CA* was highly expressed in non-responding BC patients compared to the responders group. Our data are consistent with many recently published studies which confirmed the critical role of *PI3K/AKT* hyperactivation in patients who are resistant to endocrine therapy [17-21]. Similarly, Loibl et al., [22], reported that patients with *PI3KCA* mutations showed lower rates of pathological complete response compared to the wild type *PI3KCA* patients. In this context, Lee etal., [23], observed that PIK3C amplification promoted antiestrogen resistance through Proline rich 11 (PRR11) overexpression. In addition, Nixon et al, and Welt et al., [24, 25], reported that patients having *PIK3CA* mutations, showed a higher likelihood of clinical benefit when combining *PI3K* inhibitors with endocrine therapies in the hormonal therapy-resistant breast cancer patients. Thus, *PIK3CA* mutations could be considered as a useful sensitive biomarker for breast cancer patients who are candidate for combined *PI3K* inhibitors and endocrine therapy.

The present data also showed that patients with increased *PIK3CA* expression, have a significantly lower ER expression, increased incidence of distant metastasis and shorter DFS rate compared to those with *PIK3CA* low-expression. However, there was no significant impact on the DFS or OS rates of either the responding or the non-responding BC patients. These data are in agreement with the data of Baselga et al., [26], who provided evidence that patients who had *PIK3CA* mutations and treated with standard *HER2* therapy, showed shorter DFS rate compared to those with the wild-type *PIK3CA*.

Moreover, the multivariate logistic regression analysis of the assessed BC patients showed that a large tumor size and increased *PIK3CA* expression could be used as negative predictors of reduced DFS in BC patients. Our results are also consistent with those of Sobhani et al., [27], who performed a meta-analysis study and concluded that the presence of *PI3KCA* mutations should be

considered an independent negative prognostic factor in breast cancer patients, as well as a relative indicator of disease aggressiveness.

Regarding *GSK-3β* expression in breast cancer patients, our data revealed that there was no significant difference in the expression level of *GSK-3β* between responders and non-responders BC patients. However, it has been previously reported in several studies that *GSK-3β* is usually associated with drug resistance such as doxorubicin, anthracycline, and tamoxifen in BC patients [28,29]. This discrepancy in the results might be attributed to the small number of the patients involved in the current study, or to other factors responsible for the drug resistance associated with *GSK-3β* expression in BC patients. Our preliminary data revealed a significant association between *GSK-3β* overexpression and low tumor grade, negative LN metastasis and low PR expression in BC patients. Similarly, *GSK-3β* expression was significantly increased in the absence of LN metastasis and with low progesterone receptor expression in the responders group. While, there was no significant relation between GSK3β expression and any of the clinico-pathological features assessed in the non-responders group**.** Also, there was no significant impact on DFS or OS rates of BC patients, although many recent studies have shown that *GSK-3β* is considered a sensitive biomarker of poor clinical prognosis and outcome of breast cancer patients [30-32]. However, the exact role of *GSK-3β* in breast cancer remains unclear, and understanding the link between *GSK-3β* and BC is still a debatable and controversial issue [32, 33]. In their study, Mancinelli et al., [34], demonstrated that GSK-3β is associated with tumor disease in some cases, and associated with tumor suppression in other cases, through stabilization of different components of the β-catenin complex. As it was found to be downregulated in some tumors as cholangiocarcinoma [35], however, it was upregulated in other tumors such as colon, liver, ovarian, and pancreatic carcinomas [36-38].

Interestingly, by assessing the correlation between *GSK-3β* and *PIK3CA* expression in breast cancer patients including responder and non-responder groups, there was a non-significant negative correlation between the expression levels of *GSK-3β* and *PIK3CA* in responder breast cancer patients, as *AKT* is usually activated in human cancers, which lead to phosphorylation and inhibition of *GSK-3 at Ser21/Ser9.* This could be explained partially at least that *GSK-3B* is not completely inactivated by *PIK3CA*, and still some pools of GSK-3 remain active in cancer cells. These data are also supported by that of Ougolkov et al., [39], who found a significant increase in the expression level of *AKT* together with increased expression of *GSK-3B* in pancreatic tumor cells. Similarly, Shakoori et al, reported increased levels of active *AKT* in human colorectal carcinomas, however the level of the inactive phosphorylated *GSK-3β* Ser9 was lower than in their normal counterparts [40]. Accordingly, it could be concluded that *GSK-3B* has several regulation pathways in human cancers, and consequently, *AKT* activation and phosphorylated inhibition of *GSK-3β* are not always correlated, as it was reported by Mancinelli et al, that there was *GSK-3β* overexpression in cancer cells irrespective of *AKT* activation.

CONCLUSION

The current study provides an evidence that *PIK3CA* is considered an independent negative prognostic factor for breast cancer patients. It can also be used as a potential predictor of response to endocrine therapy. While, regarding *GSK-3β* expression, it did not associate with patients' response to hormonal therapy, or to survival rates. Meanwhile, it is associated with good prognosis of the patients including low tumor grade, negative LN metastasis and low PR expression. However, due to the relatively small number of the patients assessed, these data should be validated on another study including large number of patients, in order to confirm the exact role(s) of *GSK-3β* in breast cancer, as well as other solid and hematological tumors. These two biological markers (*PIK3CA and GSK-3β*), could be a potential molecular targeted therapy for cancer patients.

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