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► **To cite this version:**

Mohammed A. Aleskandarany, Emad A. Rakha, Mohamed A. H. Ahmed, Desmond G. Powe, Emma C. Paish, et al.. PIK3CA expression in invasive breast cancer: a biomarker of poor prognosis. *Breast Cancer Research and Treatment*, 2009, 122 (1), pp.45-53. 10.1007/s10549-009-0508-9 . hal-00535390

HAL Id: hal-00535390

<https://hal.science/hal-00535390>

Submitted on 11 Nov 2010

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PIK3CA expression in invasive breast cancer: a biomarker of poor prognosis

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Received: 4 August 2009 / Accepted: 6 August 2009 / Published online: 22 August 2009
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Abstract The implications of Phosphatidylinositol 3-kinase (PIK3CA) mutations and its aberrant protein expression in breast cancer (BC) different molecular subtypes and patients' outcome remain controversial. The aims of this study were to assess the prevalence and clinical significance of PIK3CA protein expression in BC and to determine its association with its different molecular classes. PIK3CA protein expression was assessed in a well-characterized series of early stage BC ($n = 1,394$) with long-term follow-up, using tissue microarrays and immunohistochemistry. Associations between PIK3CA expression and clinicopathological variables, molecular classes, and patients' outcome were investigated. Positive PIK3CA

expression was associated with poor prognostic variables including higher grade, larger size, nodal involvement, vascular invasion, and higher proliferative fraction ($P < 0.001$). Increased PIK3CA expression was associated with the basal-like breast cancer (BLBC) and HER2-positive classes as well as triple negative non-basal (TNnon-B) tumors ($P < 0.001$). The luminal class showed reduced PIK3CA expression relative to other classes. Patients with PIK3CA positive tumors had shorter BC specific and disease free survival, independent of other prognostic factors except grade. Similar associations with outcome were found when the analysis was restricted to the large luminal class of tumors. PIK3CA is an oncogenic biomarker associated with poor prognosis in BC. Although, PIK3CA over-expression was more prevalent in BLBC and HER2-positive tumors it appeared to be a marker of poor differentiation rather than of a particular subtype. Thus, targeting of PIK3CA using specific inhibitors could potentially be beneficial, particularly for patients with more aggressive poorly differentiated tumors, irrespective of their molecular subtype.

This study was approved by Nottingham Research Ethics Committee 2 under the title of "Development of a molecular genetic classification of breast cancer".

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Keywords PIK3CA · Immunohistochemistry ·
Poor prognosis · Breast cancer

Introduction

Stimulation of cells by growth factors and hormones initiates a coordinated set of events which can lead to cell growth, proliferation, cell migration, and cell survival. One of the central mechanisms for extracellular growth signaling is the phosphatidylinositol 3-kinase (PI3K) pathway [1]. PI3-Kinases are a family of lipid kinases that have attracted considerable attention in different types of

cancers owing to their extraordinarily diverse biologic functions. Many of the roles displayed by the PI3-kinase system stem from its ability to phosphorylate, and hence to activate protein kinase B (PKB, Akt) [2, 3].

The PI3-kinase is composed of an 85 kDa regulatory subunit, and a 110 kDa catalytic subunit of which four isoforms have been characterized; α , β , γ , and δ [4]. The p85 subunit localizes PI3-K activity to the plasma membrane, while the p110 subunit contains the catalytic domain of PI3-K. The gene coding for the α isoform (p110 α , PIK3CA) catalytic subunit has recently been reported to be frequently mutated or amplified in many tumor types including breast, colon, brain, stomach, liver, and ovary [5–8]. PIK3CA gene mutations have been reported to confer enhanced enzymatic activity of PIK3CA catalytic subunit [9]. Thus, PIK3CA is currently regarded as a transforming oncogene, and has been proposed as a promising therapeutic target [5, 10]. In breast cancer, the reported frequency of PIK3CA mutation ranges from 8 to 40% [11].

Although several authorities, through investigation of the effect PIK3CA gene mutation, have reported on the possible role of PIK3CA in breast cancer, most of these studies have been carried out on relatively small cohorts of primary breast tumors, or have produced conflicting results. Most of these studies demonstrated that PIK3CA mutation is a marker of poor outcome in breast cancer, particularly, for the estrogen receptor (ER) positive and HER2 negative patient subgroups [12, 13]. However, some authors [14] have observed a prognostic advantage for these mutations. In one of the largest studies so far, Saal et al. [15] demonstrated a positive association between PIK3CA mutation and nodal involvement and over-expression of HER2 and hormone receptor (HR) expression. In contrast, Bachman et al. [16] did not find an association with HR expression. Importantly, some studies have demonstrated higher frequencies of PIK3CA mutations in the HER2 positive [15], and in the basal-like [17] classes of BC. In addition, it has recently been suggested that PIK3CA inhibitor could be used in the treatment of basal-like BC [18]. Contrasting this, some authors have found an association with invasive lobular carcinoma; a type frequently occurring in the luminal-like class of BC [19], and with the resistance to hormonal therapy [20]. Thus, these predominantly small or preclinical studies have yielded a diversity of observations and conclusions.

In the current study, we studied PIK3CA protein expression in a large well-characterized series of primary operable breast carcinoma cases with long follow-up using immunohistochemistry on a TMA platform, in an attempt to elucidate its clinical and biological relevance in breast cancer with particular emphasis on different breast cancer molecular subtypes.

Materials and methods

Patients and tumors

This study was based on a well-characterized series of early stage (pT1 and pT2) primary operable invasive breast carcinoma form patients entered into the Nottingham Tenovus Primary Breast Carcinoma Series between 1987 and 1998 ($n = 1,902$). Patients were under the age of 70 years and treated in a uniform manner. Patients' clinical history and tumor characteristics, information on patients' therapy, and outcomes were available and prospectively maintained. Outcome data include survival status, survival time, cause of death, disease free interval (DFI), time to loco-regional recurrence, and/or distant metastasis. The Breast Cancer Specific Survival (BCSS) is defined as the time in months from the date of primary surgery to the date of breast cancer-related death. DFI is defined as the duration (in months) from the date of primary surgery to the appearance of loco-regional recurrence or distant metastasis. Data on a wide range of biomarkers of known clinical and biological relevance to breast cancer were also available [21]. These include, hormone receptors [estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), epidermal growth factor receptor family members (EGFR; HER1 and HER2), cytokeratins (CKs; basal CKs: CK5/6 and CK14 and luminal CKs: CK18 and 19), P53, the proliferation marker MIB1 and cadherin family members (E-cadherin and P-cadherin).

The patients had a median age of 55 years (range 18–70 years) with a median overall survival of 126 months (range 4–243 months) and the median time of event-free survival of 114 months (range 2–243 months). Distant recurrence occurred in 501 cases (28%), 454 (25%) patients died from breast cancer, while 1,024 (58%) patients were alive at the end of follow-up.

Immunohistochemistry

Breast cancer tissue microarrays (TMA) were previously prepared as described [17]. Formalin-fixed paraffin tissue sections (4 μ m) were immunohistochemically stained, employing the standard streptavidin–biotin complex method, as previously described [21]. Heat-induced epitope for retrieval of antigen was performed in citrate buffer, 1:50 concentration at pH 6.0. Primary rabbit anti-PIK3CA antibody (Prestige Antibodies powered by Atlas, Product No. HPA009985, Sigma Aldrich, UK), diluted 1:50 (optimum working dilution) in normal swine serum (NSS), was applied to each slide, and incubated for 60 min at room temperature. Strept AB complex (Dako, Denmark) diluted in 1:100 in NSS/TBS was applied and incubated for 60 min. 3,3'-Di-aminobenzidine tetrahydrochloride (Dako liquid DAB Plus,

K3468; Dako, Denmark) was used as a chromagen. The sections were counterstained with Mayer's hematoxylin.

Validation of PIK3CA antibody specificity

For validation of PIK3CA antibody specificity, Western blotting was performed on whole cell lysates of MCF-7 and MDA-MB231 human breast cancer cell lines (obtained from the American Type Culture Collection, Rockville, MD, USA) using 1:1,000 PIK3CA dilution as previously described [22].

TMA cores scoring

Stained TMAs sections were scored using high resolution digital images (NanoZoomer, Hamamatsu Photonics, Welwyn Garden City, UK), at 20 \times magnification, using a web-based interface (Distiller, Slidepath Ltd., Dublin, Ireland). Histochemical score (H-score) was used to assess the intensity of staining and the percentage of stained cells [23]. Staining intensity was scored from 0, 1, 2, or 3 for negative, weak, moderate, and strong, respectively. The percentage of positive cells at each intensity was subjectively estimated to produce a final score in the range of 0–300. All cases were scored without prior knowledge of the clinicopathologic or outcome data.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA). Cut-off values for the different biomarkers included in this study were chosen before statistical analysis. Standard cut-offs were used for established prognostic factors and were the same as previously published for this patients' series [24, 25]. Determination of the optimal PIK3CA cut-offs were performed using X-tile bioinformatics software, version 3.6.1, 2003–2005, Yale University, USA [26]. Analysis of categorical variables was performed with the appropriate statistical test. Survival curves

were analyzed by Kaplan-Meier method with significance determined by the Log Rank test. Multivariate analysis was performed by Cox hazard analysis. A *P* value <0.05 (two sided) was considered significant.

Results

PIK3CA expression was initially evaluated in 25 full-face breast excision cancer tissue sections prior to TMA staining. This showed granular cytoplasmic immunoreactivity in invasive tumor cells and in the malignant cells of associated in situ carcinomas present in some cases. The staining pattern within the studied sections was homogeneously distributed, thus validating the applicability of PIK3CA immunohistochemical detection in TMAs (Fig. 1). Western blotting analysis of MCF-7 and MDA-MB231 human breast cancer cell lines showed specific bands at the right size (110 kDa) of the PIK3CA protein confirming the specificity of the antibody used in the IHC staining (Fig. 1c).

The number of informative cases for PIK3CA was 1394/1902 TMA cores, after exclusion of non-informative and missing cores (e.g., <25% tumourous tissue per core, core folding, and cores with extensive tumor component necrosis). In the current study, the distribution of PIK3CA H-score did not display normal distribution (median = 100). The cut-off point for positivity was set using X-tile bioinformatics software. This program randomly divides the total patient cohort into two separate equal training and validation sets by producing separate lists of "censored" and "uncensored" observations, ranked by patients' follow-up time. Death from breast cancer was considered as an event (uncensored), while patients who died from other causes or were lost to follow-up during this study were censored during the analysis at the time of that event. The optimal cut-points were determined by locating the brightest pixel on the X-tile plot diagram of the training set. Statistical significance was tested by validating the obtained cut-point to the validation

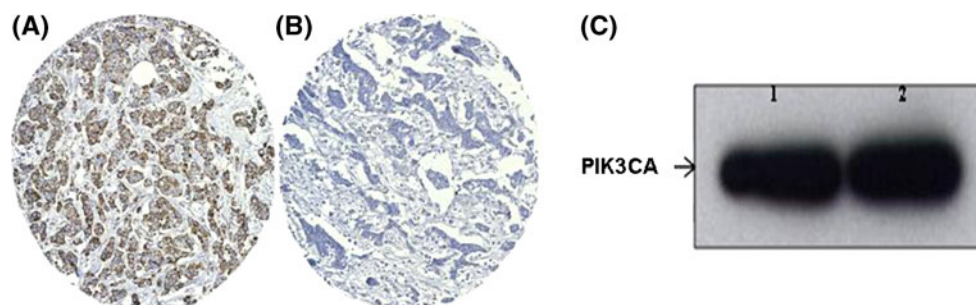


Fig. 1 PIK3CA immunohistochemical expression in invasive breast carcinoma TMA cores and Western blot of PIK3CA antibody. **a** Positive IHC expression, **b** Negative IHC expression, (both at 200 \times

magnification), and **c** Western blot analysis showing a specific band of PIK3CA protein; lane 1: MCF-7 and lane 2: MDA-MB231

Table 1 Correlation between PIK3CA expression and clinicopathologic parameters in the studied series

Parameter	PIK3CA expression			Significance	
	Negative No. (%)	Moderately positive No. (%)	Strong positive No. (%)	χ^2	<i>P</i> value
Patients' age					
≤50 years	117 (23.6)	113 (26.9)	245 (49.5)	3.15	0.207
>50 years	251 (28)	229 (25.6)	416 (46.4)		
Menopausal status					
Premenopausal	126 (24.2)	142 (27.3)	252 (48.5)	2.23	0.327
Postmenopausal	242 (27.8)	220 (25.2)	409 (47)		
Grade					
1	85 (37.6)	79 (35)	62 (27.4)	204.82	<0.001
2	184 (38.3)	154 (32.1)	142 (29.6)		
3	98 (14.4)	129 (18.9)	454 (66.7)		
LN stage					
1 (Negative)	246 (28.8)	245 (28.8)	361 (42.4)	27.85	<0.001
2 (1–3 LN involved)	89 (22.2)	96 (23.9)	216 (53.9)		
3 (>3)	32 (23.9)	20 (14.9)	82 (61.2)		
Tumor size					
≤1.5 cm	139 (30.2)	148 (32.2)	173 (37.6)	27.68	<0.001
>1.5 cm	228 (24.6)	213 (23)	485 (52.4)		
Nottingham Prognostic Index					
Good (<3.4)	165 (38.8)	150 (35.3)	110 (25.9)	136.51	<0.001
Moderate (3.41–5.4)	168 (32)	176 (24.1)	387 (52.9)		
Poor (4.41–5)	34 (14.6)	36 (15.5)	163 (70)		
Vascular invasion					
Negative	234 (29.9)	219 (28)	330 (42.1)	21.48	<0.001
Definite	98 (22.4)	95 (21.7)	245 (55.9)		
Histologic tumor type					
Invasive ductal/No special type	135 (17.2)	172 (22)	476 (60.8)	138.34	<0.001
Other histologic types	227 (38.8)	184 (31.5)	174 (29.7)		
Local recurrence					
Negative	326 (27.3)	297 (24.9)	571 (47.8)	7.71	0.021
Positive	33 (20.1)	56 (34.2)	75 (45.7)		
Regional recurrence					
Negative	332 (27.5)	306 (25.4)	569 (47.1)	6.84	0.033
Positive	27 (17.9)	47 (31.1)	77 (51)		
Distant metastases					
No	280 (29.9)	246 (26.3)	410 (43.8)	21.15	<0.001
Yes	87 (19.4)	114 (25.4)	248 (55.2)		

set [26]. The optimal H-score cut-off points were 30 and 100 H-scores, resulting in classifying cases into three subgroups: negative (0–30), moderately positive (31–100), and strongly positive expression (101–300).

Association of PIK3CA with clinicopathologic parameters

Table 1 summarizes the associations between PIK3CA expression and clinico-pathological variables. PIK3CA

over-expression (=moderate and strong positive) was significantly associated with higher tumor grade, axillary nodal metastasis, larger size, tumors with a higher Nottingham Prognostic Index (NPI), and with definite vascular invasion ($P < 0.001$). Significant association was observed between PIK3CA expression and the histologic tumor type ($\chi^2 = 27.68$, $P < 0.001$). Importantly, invasive duct carcinoma no special type (NST), and medullary carcinoma showed high proportions of PIK3CA over-expression (82.9 and 88.2%, respectively). However, PIK3CA expression

Table 2 Correlations between PIK3CA expression and other biomarker

Parameter	PIK3CA expression			Significance	
	Negative No. (%)	Moderately positive No. (%)	Strong positive No. (%)	χ^2	<i>P</i> value
ER					
Negative	46 (13.6)	56 (16.6)	236 (69.8)	78.8	<0.001
Positive	255 (32.2)	212 (26.6)	330 (41.4)		
AR					
Negative	67 (15.5)	96 (22.2)	270 (62.4)	58.15	<0.001
Positive	253 (32.3)	209 (26.6)	322 (41.1)		
PR					
Negative	112 (20)	103 (18.4)	345 (61.6)	69.79	<0.001
Positive	218 (30.4)	225 (31.4)	273 (38.2)		
HER2					
Negative	331 (28.6)	317 (27.4)	510 (44)	41.13	<0.001
Positive	22 (13.3)	27 (16.2)	117 (70.5)		
EGFR					
Negative	270 (39.3)	244 (26.5)	408 (44.3)	34.72	<0.001
Positive	33 (15)	42 (19.1)	145 (65.9)		
p53					
Negative	290 (30.4)	259 (27.1)	406 (42.5)	47.2	<0.001
Positive	51 (15)	74 (21.8)	214 (63.2)		
CK5/6					
Negative	307 (27.7)	297 (26.8)	504 (45.5)	19.38	<0.001
Positive	40 (18.4)	43 (19.8)	134 (61.8)		
CK14					
Negative	314 (27.5)	301 (26.3)	529 (46.2)	12.7	0.002
Positive	27 (16.6)	38 (23.3)	98 (60.1)		
CK18					
Negative	19 (15.6)	29 (23.7)	74 (60.7)	27.87	<0.001
Positive	301 (27.5)	282 (25.7)	513 (46.8)		
CK19					
Negative	12 (16.2)	20 (27)	42 (56.8)	16.72	0.01
Positive	334 (26.9)	321 (25.6)	592 (47.5)		
E-cadherin					
Negative	147 (29.3)	135 (26.8)	221 (43.9)	5.19	0.075
Positive	195 (24.7)	199 (25.2)	396 (50.1)		
P-cadherin					
Negative	157 (30.6)	161 (31.4)	195 (38)	45.36	<0.001
Positive	124 (21.7)	114 (19.9)	334 (58.4)		
MIB1*					
Low proliferative	144 (39.6)	116 (31.9)	104 (28.6)	119	<0.001
Moderately proliferative	100 (21.6)	123 (26.6)	239 (51.7)		
Highly proliferative	25 (10.2)	47 (19.3)	172 (70.5)		

was not associated with patient's age at diagnosis or menopausal status.

The association between PIK3CA expression and other biomarkers is shown in Table 2. PIK3CA over-expression was significantly associated with the negative expression of hormone receptors (ER, PR, and AR; $P < 0.001$), and

luminal CKs [CK18: $P < 0.001$ and CK19: $P = 0.01$]. Conversely, PIK3 showed a positive association with the expression of members of the epidermal growth factor receptor family (HER2 and EGFR; $P < 0.001$), basal CKs [CK5/6: $P < 0.001$ and CK14: $P = 0.002$], P-cadherin ($P < 0.001$), the cell cycle regulator p53 ($P < 0.001$) and

with increased tumor growth fraction previously assessed on full-face tissue sections using MIB1 labeling index (LI, $\chi^2 = 119$, $P < 0.001$). However, the association with E-cadherin did not reach statistical significance ($P < 0.07$).

Association of PIK3CA in different immunohistochemically defined molecular subtypes of breast cancer

A significant association was observed between PIK3CA expression and molecular breast cancer classes defined as: luminal (ER⁺ and/or PR⁺), HER2 positive (HER2⁺), BLBC (ER⁻, PR⁻, HER2⁻, and positive for CK5/6 and/or CK14, and/or EGFR), and triple negative non-basal BC (TNnon-B, ER⁻, PR⁻, HER2⁻, CK5/6⁻ and/or CK14⁻, and/or EGFR⁻), Table 3. Interestingly, not only HER2⁺ and BLBC but also TN non-B BC displayed significantly higher proportions (86.5, 88.8, and 87.9%, respectively) of PIK3CA over-expression compared with luminal tumors (67.4%; $\chi^2 = 111.46$, $P < 0.001$).

Association of PIK3CA with patients' outcome

Although PIK3CA positivity was associated with an increased likelihood to develop distant recurrence ($\chi^2 = 21.15$, $P < 0.001$), it was not significantly associated with the pattern of distant metastasis in terms of number and site of metastatic recurrence ($\chi^2 = 1.58$ and 5.17 ; $P = 0.81$ and 0.74 , respectively). However, higher proportions of cases suffered from brain or bone metastatic recurrence tended to over-express PIK3CA (85.8 and 83.6%, respectively) than those who developed metastatic recurrence elsewhere (Table 4). Importantly, the probability of distant recurrence during the first 10 years of follow-up was significantly increased in positive PIK3CA expression cases than PIK3CA negative cases (61 and 72%, respectively; $\chi^2 = 14.69$, $P = 0.001$).

Table 3 Correlations between PIK3CA expression and breast cancer subtypes

Parameter	PIK3CA expression			Significance	
	Negative No. (%)	Moderately positive No. (%)	Strong positive No. (%)	χ^2	P value
Luminal	285 (32.6)	253 (28.9)	337 (38.5)	111.46	<0.001
HER2 Positive	22 (13.3)	27 (16.3)	117 (70.5)		
Triple negative-basal	15 (11.2)	21 (15.7)	98 (73.1)		
Triple negative non-basal	8 (12.1)	14 (21.2)	44 (66.7)		

Table 4 Relationship between PIK3CA expression and pattern of distant metastasis (DM)

Distant metastatic pattern	PIK3CA expression			Significance	
	Negative No. (%)	Moderately positive No. (%)	Strong positive No. (%)	χ^2	P value
Number DM sites					
Single DM site	56 (17.9)	82 (26.2)	173 (55.9)	1.58	0.812
Two DM sites	23 (23.2)	22 (21.2)	58 (55.6)		
Disseminated disease	5 (16.7)	7 (25)	14 (58.3)		
Site of DM					
Bone	25 (16.4)	36 (23.7)	91 (59.9)	5.17	0.739
Liver	24 (21.4)	29 (25.9)	59 (52.7)		
Other LN	7 (21.3)	8 (24.2)	18 (54.5)		
Lung and pleura	13 (24.1)	16 (29.6)	25 (46.3)		
Brain	5 (14.2)	7 (20)	23 (65.8)		

Univariate survival analyses showed that patients with tumors positive for PIK3CA had shorter breast cancer specific survival [BCSS; log rank (LR) = 29.27, $P < 0.001$, Hazard ratio (HR) = 1.419, 95% CI = 1.247–1.613; Fig. 2a]. PIK3CA positive cases showed a 67% 10-year BCSS compared with 79% in the PIK3CA negative cases. Positive expression of PIK3CA also showed association with shorter distant metastasis-free survival (LR = 22.03, $P < 0.001$, HR = 1.318, 95% CI = 1.170–1.484; Fig. 2b). Moreover, when survival analysis was restricted to luminal-like class of tumors, PIK3CA expression could identify a subgroup of patients associated with a poor prognosis (LR = 16.457, $P = 0.001$, HR = 01.775, 95% CI = 01.257–2.506; Fig. 3a, b).

Cox-proportional hazards analysis revealed that PIK3CA positivity is a predictor of shorter BCSS (HR = 1.302, $P < 0.001$) and distant metastasis-free interval (HR = 1.184, $P = 0.012$) independent of tumor size, lymph node stage, molecular subtypes, adjuvant endocrine, and chemotherapy (Table 5). However, this association was rendered insignificant with inclusion of the grade into the Cox-proportional hazard analysis model for BCSS and distant metastasis-free interval ($P = 0.067$ and 0.057 , respectively).

Discussion

Breast cancer (BC) comprises an extraordinarily diverse group of diseases in terms of presentation, morphology, molecular profile, and therapeutic responses. The gene expression analysis has identified biologically and clinically distinct molecular classes of BC [27]. Of these

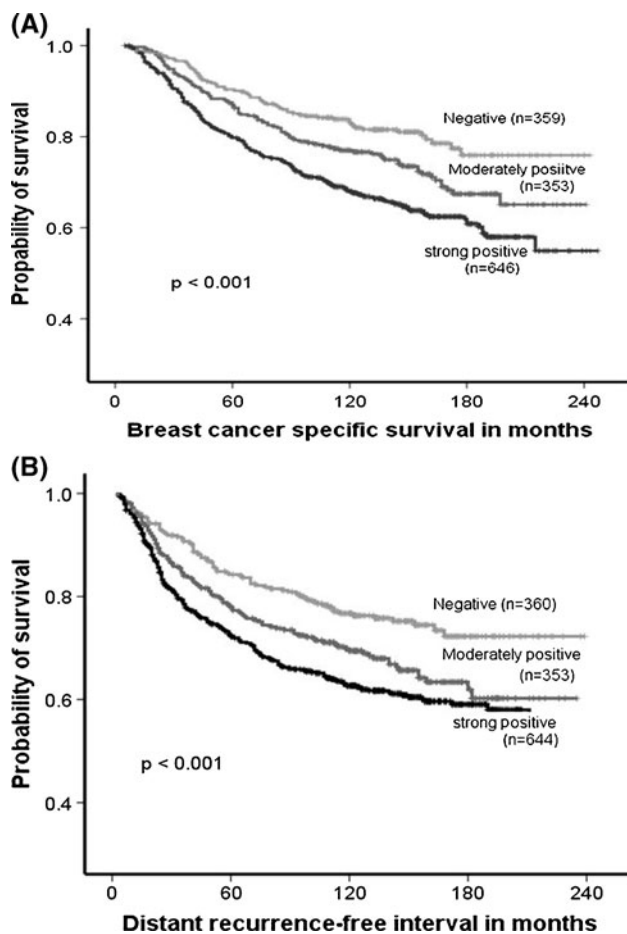


Fig. 2 Kaplan–Meier survival plot for patients’ BCSS showing the association between PIK3CA expression and breast cancer specific survival (a) and the probability of distant recurrence (b)

classes, HER2 positive and basal-like breast cancers (BLBC) are the two classes of dismal innate prognosis, with the latter currently lacking any form of specific targeted therapy. Therefore, current research is underway to unravel the signaling pathways distinct to the BLBC along with their potential for therapeutic intervention [18]. PIK3CA has been reported to be a marker that is associated with BLBC and HER2 positive classes. While most studies have assessed the role of PIK3CA gene mutation, less attention has been given to the proteomic assays of PIK3CA in invasive BC. In this study, PIK3CA protein expression was evaluated, using immunohistochemistry, in a large well-characterized series of early stage BC cases with a long-term follow-up, to explore its clinical and biological relevance in BC and its association with the specific molecular subtypes. To the best of our knowledge, this is the largest study to date pertaining to PIK3CA protein expression in BC.

In the current study, using the X-tile generated PIK3CA expression cut-off points at H-scores of 30 and 100, 26.6%

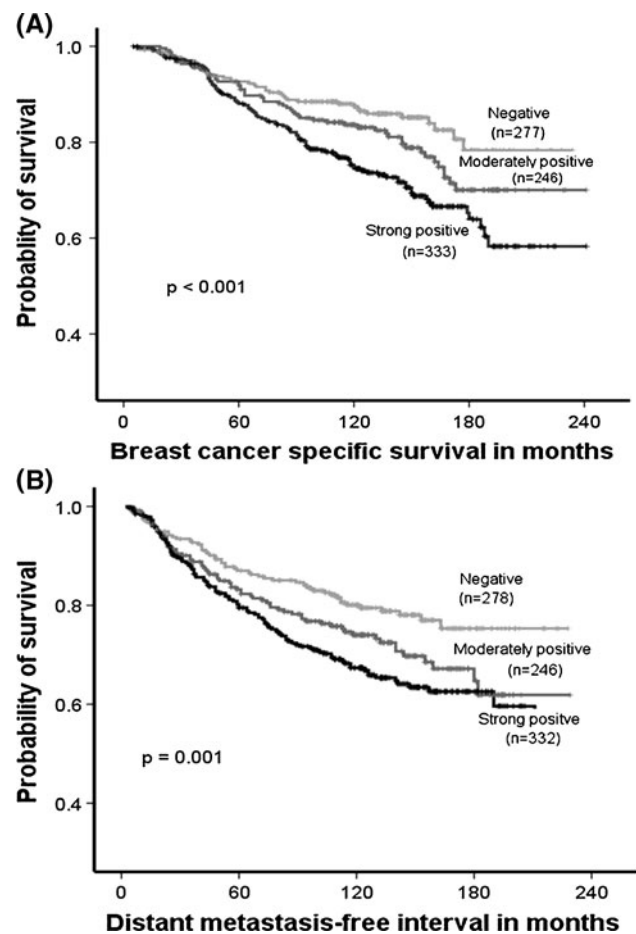


Fig. 3 Kaplan–Meier survival plot for patients’ BCSS in PIK3CA negative, moderately positive, and strong positive luminal-like BC cases (a); patients’ probability to distant recurrence in luminal-like PIK3CA negative, moderately, and strongly positive BC cases (b)

Table 5 Cox-proportional hazards analysis for predictors of overall survival and distant metastasis-free survival: effects of tumor grade, size, lymph node stage, and PIK3CA expression in patients’ series

Variable	Hazard ratio	95% CI	P value
BCSS			
Tumor size ≥ 1.5 cm	2.268	1.693–3.039	<0.001
Lymph node stage	1.907	1.643–2.215	<0.001
Molecular phenotype	1.252	0.995–1.095	0.001
Endocrine treatment	0.941	0.826–0.027	0.071
Chemotherapy	0.927	0.716–1.200	0.566
PIK3CA expression	1.302	1.098–1.427	<0.001
Distant metastasis-free survival			
Tumor size > 1.5 cm	2.162	1.627–2.779	<0.001
Lymph node stage	1.971	1.707–2.275	<0.001
Molecular phenotype	1.162	1.022–1.321	0.022
Endocrine treatment	0.912	0.832–1	0.051
Chemotherapy	0.883	0.681–1.145	0.348
PIK3CA expression	1.184	1.038–1.349	0.012

were PIK3CA negative, 26% were moderately positive with the remaining 47.6% of the studied cases had strong PIK3CA positivity. PIK3CA over-expression was significantly associated with poor prognostic clinicopathologic parameters, including larger tumor size, higher grade, nodal involvement, higher NPI, and definite vascular invasion. PIK3CA over-expression was observed more frequently in invasive duct carcinoma NST and medullary carcinoma in contrast to other histological types known to be associated with a favorable prognosis [28, 29].

As anticipated, PIK3CA expression was directly associated with the expression of epidermal receptor growth factors family members (EGFR and HER2). Currently, it is evident that many of the transforming and pro-proliferative events mediated by over-expression of the EGFR and HER2 arise from their ability to enhance the PI3-K/Akt pathway [30]. Moreover, the inverse association of PIK3CA expression with the hormonal receptors, luminal cytokeratins (CKs: CK18 and CK19), and direct association with a higher tumor growth fraction, basal CKs, and P-cadherin underscore the poor prognostic impact of PIK3CA over-expression. These findings are consistent with the oncogenic role of PIK3CA in BC and support the results of the previous studies which demonstrated the biological relevance of PIK3CA in BC and the association between its dysfunction and variables of poor prognosis [5, 15].

Furthermore, with respect to the BC molecular classes as defined by IHC, PIK3CA over-expression was associated with not only BLBC and HER2 positive but also with the triple negative non-basal (TNnon-B) tumor group [31] and over-expression was less frequently observed in the luminal-like BC class. This is consistent with other studies that reported more frequent PIK3CA mutations in BLBC and HER2 over-expressing tumors [15, 17]. However, our results showed that there is no significant difference in its expression between BLBC, HER2 positive tumors, and TNnon-B classes of tumors. Interestingly, this association between BC molecular subtypes appeared to be grade-dependant with frequent positivity in poorly differentiated higher grade tumors, in general, rather than a direct association with a specific molecular subtype as evidenced by the results of the various univariate and multivariate analyses. Some studies have reported more frequent activation of the PI3-kinase pathway in BLBC than other BC subtypes, concluding that alteration of this pathway is peculiar to BLBC and its oncogenesis [17, 32]. However, these studies were used either smaller number of cases, or were potentially biased in terms of selecting high grade cases that would display PIK3CA deregulation irrespective of their phenotype.

Consistent with previous reports demonstrating the poor prognostic impact of PIK3CA mutations in BC [13, 33], our results showed that PIK3CA over-expressing cases had

shorter breast cancer specific survival and disease-free survival. This association was independent of all other prognostic variables included in our study, but histological grade. Moreover, higher proportions of cases suffered from brain or bone metastatic recurrence tended to overexpress PIK3CA than those who developed metastatic recurrence elsewhere.

When we restricted our analysis to luminal-like tumors, PIK3CA expression showed similar associations to that described in the whole series and identified a subclass of luminal-like tumors characterized by poor prognosis and shorter survival.

In conclusion, PIK3CA is an oncogenic biomarker whose over-expression is associated with tumor aggressiveness in BC patients inferring that PIK3CA dysregulation may have important biologic and cellular consequences in the oncogenic development pathway from transformation through progression. Although, deregulation of PIK3CA has been identified in BLBC, HER2 positive, and TNnon-B tumors, this association appears to be a reflection of the poor prognostic characteristics of these subtypes and particularly their high-grade nature. Thus, our results support the hypothesis that PIK3CA is a marker of breast cancer progression, the inhibition of which through specific targeted therapies could hopefully hamper tumor progression of BC irrespective of its molecular subtype.

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