Prevalence of *PIK3CA* mutations and the SNP rs17849079 in Arab breast cancer patients

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Carcinomas initiate and progress due to genetic and epigenetic alterations in epithelial cells. However, recently, these alterations have also been reported in stromal fibroblasts. The gain-of-function mutations in the PI3K p110 catalytic subunit (*PIK3CA*) have been identified in many cancers with a current global incidence of 26% (18–40%) in breast carcinomas. We analyzed the mutational frequency of *PIK3CA* of three hotspots (exons 1, 9, and 20) in 81 primary invasive breast cancers (BC) and 25 cultured breast cancer-associated fibroblast (CAF) samples by Sanger sequencing in Arab breast cancer patients. Associations between the incidence of any *PIK3CA* mutation and several clinicopathologic characteristics were assessed using chi-square tests for categorical or *t* test for continuous variables. Furthermore, survival curves were estimated using the Kaplan–Meier method with the log rank test to evaluate the significance of their differences. We identified a total of 21 *PIK3CA* missense mutations with a frequency of 25.9%. The majority of the mutations, 17 out of 21 (81%), were in exon 20 (p.His1047Arg, p.His1047Lys, p.Thr1025Ala, p.Gly1049Arg, p.Asp1056Asn) while the remainder, 4 out of 21 (19%) were in exon 9 (p.Glu545Lys). *PIK3CA* mutations were significantly associated with lower grade and hormone receptor positivity. Although there was a favorable trend in overall survival for patients whose tumor harbored *PIK3CA* mutations, the difference was not statistically significant (*P* = 0.10). However, we did not detect any somatic mutations in CAFs. Furthermore, we have shown a high prevalence (8.2-fold) of a silent variant (SNP, rs17849079) in the Arab breast cancer population compared with disease-free individuals.

Introduction

Breast cancer is the most common cancer type among women worldwide including in the Arabian Peninsula.[1,2] Although the highest incidence and mortalities occur in developed countries (i.e., United States and Europe), as the Western lifestyle is being adopted by increasing parts of the world, breast cancer cases continue to increase, including in the Middle East.[3] Carcinomas are known to initiate and progress due to genetic and epigenetic changes such as loss of heterozygocity (LOH), copy number variations (CNV), somatic mutations, and methylation in epithelial cells.[4-7] However, in recent years, genetic and epigenetic alterations have been also reported in stromal cells such as carcinoma-associated fibroblasts (CAFs) that may play a role in human carcinogenesis.[8,9]

The phosphoinositide 3-kinase (PI3K) signaling pathway regulates many important cellular processes, including proliferation and cell death.[10,11] Gain-of-function mutations in the PI3K p110 α catalytic subunit (*PIK3CA*) have been frequently linked to human cancer development. *PIK3CA* is considered to be one of the "mountains" in the genomes of the human cancer landscape.[12] Samuels et al. first reported the high prevalence of pathogenic mutations of *PIK3CA* in colon cancers while a lower rate was described in breast cancers, though due to the small number of breast tumor samples, the reported frequency was not reliable.[13] A subsequent study expanding the sample size of primary breast tumors along with cell lines revealed a greater and true mutation frequency.[14] Since these two initial publications, a high prevalence of *PIK3CA* mutations has been reported in many types of cancers and populations.[15] The functional significance

of these identified mutations has been studied in vitro revealing promising results for therapeutic interventions.[16-18]

The global frequency of *PIK3CA* somatic mutations in all major malignancies is about 10%, which makes *PIK3CA* one of the highest mutated oncogenes in human cancers.[15] The great majority of *PIK3CA* mutations occur in three hotspot regions namely, exon 1 (p85 binding), exon 9 (helical), and exon 20 (kinase) domains.[15] The current overall *PIK3CA* mutation frequency for breast cancers reported in the Catalogue of Somatic Mutations in Cancer database (COSMIC) ranges from 18% to 40%, depending on the studied population with an average of 26%.

As we are entering into the era of personalized medicine,[19] cancer therapeutics that specifically target a tumor's genetic and epigenetic alterations are also gaining ground. Although genomic alterations often give cancer cells a selective growth advantage during tumorigenesis,[20,21] they could also serve as targets for anti-cancer agents that can selectively kill the tumor carrying DNA alterations with relatively low toxicity. In addition, most of the past and current genomic characterizations have been done in Western populations, and whether such studies apply to all ethnic and other diverse demographics remains an open question. Therefore, it is essential to identify population-specific genomic alterations in cancer genes and determine their frequencies in order to validate existing data as therapeutic targets or biomarkers for other populations.

In this study, we assessed *PIK3CA* somatic mutation frequency in 81 primary breast tumors and 25 cultured CAF cells in Arab breast cancer patients by direct DNA sequencing. Similar to other reports, we identified a *PIK3CA* mutation frequency of 25.9% in BC tissues, with the majority located in exon 20. However, no mutations were detected in CAF cells. Intriguingly, we have found a 8.2-fold higher prevalence of a silent variant (SNP, rs17849079) in Arab breast cancer patients as compared with disease-free normal individuals. Further implications of these results are discussed below.

Results

High frequency of *PIK3CA* missense mutations in Arab breast cancer patients. To evaluate the *PIK3CA* somatic mutation frequencies in Arab BC patients, we isolated genomic DNA from 81 primary BC tissues and 189 peripheral blood samples from breast cancer free individuals, and sequenced *PIK3CA* exons 1, 9, and 20. As previously reported, about 99% of the somatic mutations identified for this gene are located in these exons.[15]

We detected 21 missense mutations in 81 BC samples (25.9%). Seventeen of these mutations (81%) were in exon 20 (kinase domain) and the remaining 4 (19%) in exon 9 (helical domain). Furthermore, 62% (13 out of 21) of the mutations were identified in the most common hotspot mutation within exon 20, His1047Arg (**Fig. 1A**; **Table 1**). However, we did not detect any alterations in exon 1, and all the mutations identified in this breast cancer population have been previously reported (**Fig. 1A**; **Table 1**). In most cases, the mutations were either A > G or G > A purine-to-purine transition mutations except in one sample
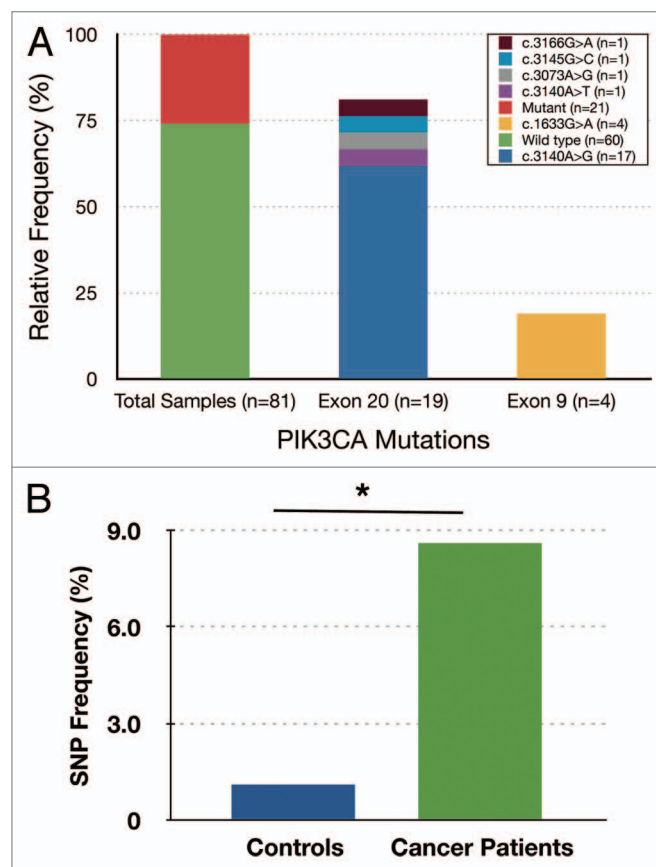

Figure 1. (**A**) PIK3CA mutation status in invasive epithelial tumors of Saudi Breast Cancer patients. The *x*-axis indicates relative frequency and *y*-axis shows mutation and change in amino acid. The total 21 missense mutations out of 81 total primary tumor samples (25.9%), 17 in exon 20 (81.0%), and 4 in exon 9 (19.0%). (**B**) The frequency of C3075T SNP (rs17849079) in disease-free control samples and in breast cancer patient population in Saudi Arabia. The frequency of SNP in breast cancer patients (7/81 or 8.6%) is significantly higher than those with disease-free controls (2/189 or 1.1%). *P* value = 0.0032.

wherein a purine-to-pyrimidine (G > C) transversion mutation was detected (**Fig. 1A**; **Table 1**).

High prevalence of the *PIK3CA* C3075T polymorphism (SNP) in Arab breast cancer patients. Next, we studied the prevalence of the rs17849079 SNP in Arab BC patients. We have found a high prevalence (7 out of 81, 8.6%) of this silent polymorphism in the analyzed 81 breast cancer tissues (**Fig. 1B**). To determine the overall frequency of this SNP in the Saudi population, we sequenced the same region using genomic DNA isolated from peripheral blood of 189 cancer-free individuals. Interestingly, the frequency of this SNP was 1.05% in healthy normal individuals compared with 8.6% in our cancer patients (**Fig. 1B**), which accounts for 8.2-fold enrichment in women with breast cancer. The frequency of this SNP in individuals with breast cancer was significantly higher than that observed in the controls (the Fisher exact test *P* = 0.0032). The estimated odds ratio of the rs17849079 SNP in affected individuals relative to disease-free individuals was OR = 8.85 (95% CI 1.8–43.6) (**Fig. 1B**).

Table 1. Summary of *PIK3CA* mutational status and their clinicopathological features in BC samples

Tumor #	Age	Grade	ER	PR	HER2	Epithelium mutation Nucleotide: codon (exon)
1	36	II	+	-	1+	WT
2	48	II	-	-	3+	WT
3	72	III	-	-	1+	WT
4	32	II	+	+	1+	c.3140A > G: p.His1047Arg (20)
5	44	II	+	-	1+	WT
6	34	III	-	-	3+	WT
7	62	III	+	+	3+	WT
8	67	III	-	-	3+	WT
9	47	III	+	+	1+	c.3145C > G: p.Gly1049Arg (20)
10	68	III	+	+	1+	WT
11	40	III	+	-	3+	WT
12	32	III	-	-	3+	WT
13	57	II	+	+	1+	WT
14	31	III	-	-	1+	WT
15	34	II	-	-	1+	WT
16	27	II	+	+	3+	c.3140A > G: p.His1047Arg (20)
17	42	II	-	-	1+	WT
18	54	III	+	-	1+	WT
19	66	III	+	+	3+	WT
20	55	III	+	+	3+	c.3075C > T: SNP (20)
21	61	II	+	+	1+	WT
22	50	II	+	+	1+	WT
23	40	I	+	-	1+	c.3140A > G: p.His1047Arg (20)
24	60	II	+	+	1+	c.3140A > G: p.His1047Arg (20)
25	57	III	+	+	1+	WT
26	42	III	-	-	3+	c.3075C > T: SNP (20) c.3140A > G: p.His1047Arg (20)
27	42	III	-	-	3+	WT
28	54	III	+	-	1+	c.3140A > G: p.His1047Arg (20)
29	38	III	-	-	3+	WT
30	32	II	+	-	1+	WT
31	51	II	+	+	3+	c.3073A > G: p.Thr1025Ala (20)
32	56	III	+	+	3+	c.3075C > T: SNP (20)
33	58	II	+	+	1+	c.3140A > G: p.His1047Arg (20)
34	61	II	+	+	1+	WT
35	45	I	+	+	1+	c.3075C > T: SNP (20)
36	40	II	-	-	1+	WT
37	39	III	-	-	3+	WT
38	36	II	+	+	3+	WT
39	55	III	-	-	1+	WT
40	51	II	+	-	3+	c.3140A > G: p.His1047Arg (20)
41	40	III	-	-	3+	c.3140A > G: p.His1047Arg (20)
42	41	III	+	+	1+	WT

Mutations are shown as nucleotide change followed by amino acid change and the number of exon in parenthesis. Abbreviations: ER, estrogen receptor; PR, progesterone receptor; Her2, human epidermal growth factor receptor 2; WT, wild type; NA, information not available; MET, metastatic; DCIS, ductal carcinoma in situ.

Table 1. Summary of *PIK3CA* mutational status and their clinicopathological features in BC samples (continued)

Tumor #	Age	Grade	ER	PR	HER2	Epithelium mutation Nucleotide: codon (exon)
43	46	III	-	-	1+	WT
44	50	II	+	+	1+	WT
45	35	III	-	-	1+	WT
46	51	II	+	-	1+	WT
47	44	III	-	-	3+	WT
48	37	III	-	-	1+	WT
49	45	II	+	+	1+	WT
50	34	III	+	+	1+	WT
51	70	I	+	+	1+	WT
52	47	II	-	-	3+	WT
53	65	I	+	+	1+	c.3140A > G: p.His1047Arg (20)
54	36	III	-	-	3+	WT
55	39	I	+	+	3+	c.1633G > A: p.Glu545Lys (9) c.3075C > T: SNP (20)
56	60	II	+	+	1+	WT
57	63	III	+	+	1+	c.1633G > A: p.Glu545Lys (9)
58	42	I	+	+	1+	WT
59	52	II	+	+	3+	c.3075C > T: SNP (20) c.3140A > G: p.His1047Arg (20)
60	49	I	+	+	1+	WT
61	60	I	+	+	1+	c.3166G > A: p.Asp1056Asn (20)
62	48	II	+	-	1+	WT
63	56	II	+	-	3+	WT
64	53	II	+	+	1+	WT
65	28	DCIS	NA	NA	1+	c.3140A > T: p.His1047Lys (20)
66	40	II	-	-	3+	c.1633G > A: p.Glu545Lys (9)
67	45	III	-	-	3+	WT
68	44	NA	NA	NA	NA	c.1633G > A: p.Glu545Lys (9)
69	45	MET	-	-	1+	c.3075C > T: SNP (20)
70	22	II	+	+	1+	WT
71	37	III	-	-	1+	WT
72	45	III	-	-	3+	c.3140A > G: p.His1047Arg (20)
73	46	II	-	-	1+	WT
74	49	III	+	+	3+	WT
75	46	III	+	-	3+	WT
76	51	III	-	-	3+	WT
77	53	I	+	-	1+	c.3140A > G: p.His1047Arg (20)
78	48	III	+	+	1+	WT
79	39	II	+	+	3+	WT
80	40	II	-	-	3+	WT
81	36	II	-	-	3+	WT

Mutations are shown as nucleotide change followed by amino acid change and the number of exon in parenthesis. Abbreviations: ER, estrogen receptor; PR, progesterone receptor; Her2, human epidermal growth factor receptor 2; WT, wild type; NA, information not available; MET, metastatic; DCIS, ductal carcinoma in situ.

Table 2. Summary of *PIK3CA* mutational status and their clinicopathological features in CAF and their associated BC samples

Sample #	Age	Grade	ER	PR	HER2	Fibroblast mutation (Exon)	Tumor mutation (Exon)
1	55	II	+	-	3+	WT	NA
2	40	II	+	+	1+	WT	NA
3	54	III	+	-	3+	WT	NA
4	40	III	NA	NA	NA	WT	NA
5	47	II	+	+	1+	WT	NA
6	35	III	-	-	1+	WT	NA
7	54	II	+	-	1+	WT	NA
8	47	II	+	+	3+	WT	NA
9	32	II	+	+	1+	WT	c.3140A > G: p.His1047Arg (20)
10	52	II	+	-	1+	WT	WT
11	60	III	-	-	3+	WT	WT
12	60	II	+	+	1+	WT	NA
13	52	II	-	-	3+	WT	NA
14	40	III	+	-	3+	WT	WT
15	57	II	+	+	1+	WT	WT
16	63	III	+	-	3+	WT	NA
17	54	III	-	-	3+	WT	WT
18	42	II	+	+	3+	WT c.3075C > T: SNP (20)	c.3140A > G: p.His1047Arg (20) c.3075C > T: SNP (20)
19	54	II	+	-	1+	WT	NA
20	64	II	+	+	3+	WT	NA
21	60	I	+	+	1+	WT	NA
22	43	DCIS	NA	NA	NA	WT	NA
23	68	DCIS	NA	NA	NA	WT	NA
24	40	II	-	-	3+	WT	c.1633G > A: p.Glu545Lys (9)
25	33	III	-	-	1+	WT	NA

Mutations are shown as nucleotide change followed by amino acid change and the number of exon in parenthesis. Abbreviations: ER, estrogen receptor; PR, progesterone receptor; Her2, human epidermal growth factor receptor 2; WT, wild type; NA, information not available; DCIS, ductal carcinoma in situ.

It is noteworthy that 3 out of 7 (43%) breast tumors with this SNP also harbored a *PIK3CA* somatic mutation (**Table 1**).

Absence of *PIK3CA* mutations in CAFs. The presence of somatic mutations in CAFs remains controversial. Therefore, we decided to investigate the possible presence of *PIK3CA* mutations in 25 CAFs isolated from 25 BC tissues.

Importantly, we did not detect *PIK3CA* mutations in any of the analyzed CAF cells (**Table 2**). Additionally, in 3 tissues, tumors that harbored mutated *PIK3CA*, did not display mutant *PIK3CA* in the associated CAFs (**Table 2**).

Associations of *PIK3CA* mutations with tumor clinicopathological features and patient survival. Next, we sought to determine the association of *PIK3CA* mutations with tumor clinicopathologic features. Features significantly associated with *PIK3CA* mutations included lower grade ($P = 0.02$) and estrogen receptor (ER) positivity ($P = 0.03$), while there was a marginal association that was not statistically significant with progesterone receptor (PR) ($P = 0.07$). However, *PIK3CA* mutations did not correlate with age nor HER2 status (**Table 3**).

The presence of the exon 20 hotspot mutation, His1047Arg, was significantly associated with low-grade tumors ($P = 0.03$) and ER positivity ($P = 0.02$) (**Table 4**). However, the Glu545Lys (exon 9) mutation was not statistically associated with any of the clinicopathologic features (**Table 4**).

Moreover, we assessed the association between the presence of *PIK3CA* gain-of-function mutations and overall survival of patients. **Figure 2** shows an overall survival advantage for patients with tumors harboring *PIK3CA* mutations as compared with those tumors that do not contain such alterations. However, the difference between the two groups was not statistically significant ($P = 0.10$).

Discussion

In the era of personalized medicine, we sought to determine the frequency and nature of *PIK3CA* mutations in Arab BC patients. The obtained results are summarized in the following points: first, the overall frequency of *PIK3CA* missense

Table 3. The associations between *PIK3CA* somatic mutation status and BC samples' clinicopathologic features

Characteristic	Overall cohort	Wild type (n = 56)	SNP (n = 7)	Any PIK3CA mutation (n = 21)	P* value
Age (SD)	46.8 (10.8)	46.4 (11.1)	47.7 (6.5)	47.2 (11.0)	0.79
Grade					
I	9	3	2	5	**0.02**
II	34	25	2	9	
III	35	28	2	5	
Unknown	3			2	
ER					
Negative	29	25	1	3	**0.03**
Positive	50	31	6	16	
Unknown	2			2	
PR					
Negative	42	34	1	7	**0.07**
Positive	37	22	6	12	
Unknown	2			2	
HER2					
Negative	46	34	2	10	0.59
Positive	33	22	5	9	
Unknown	2				

Statistical tests are based on available data; samples with SNP-only and unknown information are not included in *p*-value calculations.. *χ^2 test (or the Fisher exact test when an expected cell count was less than 5) used for binary/categorical variables; *t* test used for continuous variables. Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2 status; SD, standard deviation.

mutations in the Arab breast cancer population is 25.9% with a great majority of mutations being in exon 20 (**Fig. 1A**); second, the silent polymorphism (rs17849079) in the exon 20 coding region is 8.2-fold enriched in the BC population as compared with disease-free individuals (**Fig. 1B**); third, CAF cells do not harbor *PIK3CA* somatic missense mutations in the mutation hotspot regions of this gene (**Table 2**). Lastly, *PIK3CA* mutations have a slight but not significant effect in patient's overall survival (**Fig. 2**). A larger cohort may be needed to clarify this important issue.

The frequency of *PIK3CA* mutations in the Arab breast cancer population (25.9%) is similar to the global average[22] and all the identified mutations have been previously reported. The exon 20 His1047Arg alteration comprises 61.9% (13 out of 21) of total *PIK3CA* mutations, which represents a significant percentage of human breast cancers, warranting the development of targeted therapies against this particular *PIK3CA* mutation.

The functional significance of the His1047Arg and Glu545Lys mutations have been analyzed in both in vitro and in vivo model systems and reported to be gain-of-function mutations and pathogenic. The initial in vitro model systems created by Samuels et al. in 2005 and Gustin et al. in 2009 confirmed the pathogenicity of these two mutations[16,17] Gustin et al. knocked in two *PIK3CA* hotspot mutations, p.Glu545Lys (helicase domain) and p.His1047Arg (kinase domain), in the non-cancerous mammary epithelial cell line MCF-10A by somatic cell gene targeting strategies. The authors have shown that both knocked in mutants had significantly lower cell proliferation when treated with lithium

chloride as compared with their isogenic parental counterparts and importantly, unexpectedly activated signaling pathways that were not predicted based upon the known pathways of PI3 kinase activation.[17]

In a more recent study, Di Nicalantonio et al. reported that patients' tumor cells with *PIK3CA* mutations were sensitive to everolimus, an inhibitor of the mammalian target of rapamycin (mTOR). These authors also knocked in Glu545Lys and His1047Arg mutations into two immortalized human breast epithelial cell lines, hTERT-HME1 and MCF-10A. Similar to primary tumors, both cell lines with either His1047Arg or Glu545Lys knocked in mutations were more sensitive to everolimus as compared with their respective isogenic parental cell lines.[23,24]

As the PI3K/AKT/mTOR pathway is frequently activated in human cancers, there have been intense efforts in using inhibitors (i.e., everolimus, wortmannin, LY294002, PX-866) that specifically target alterations in this pathway, some of which are currently in phase II and III clinical trials.[25-28]

There is currently no consensus in the literature on the role of *PIK3CA* mutations in breast cancer patient survival. This could be due to the lack of standard treatments for breast cancer, different samples size, different patient populations or combinations thereof. The disease-free or overall survival for patients whose tumors harbor *PIK3CA* mutations have been reported to be either favorable,[29-31] unfavorable,[32,33] or not statistically significant[34-36] as compared with patients with tumors containing wild-type *PIK3CA*. Further studies are needed to provide clarity to this important issue.

Table 4. The associations between *PIK3CA* mutation type and clinicopathological variables

Characteristic	Mutation type			Mutation type		
	Wild type	Exon 9 mutation	*P**-value	Wild type	Exon 20 mutation	*P**-value
Age (SD)	46.4(11.1)	46.5 (13.6)	0.99	46.4 (11.1)	47.4 (11.4)	0.77
Grade						
I	3	1	0.27	3	4	**0.03**
II	25	1		25	8	
III	28	1		28	4	
Unknown		1			1	
ER						
Negative	25	1	1	25	2	**0.02**
Positive	31	2		31	14	
Unknown		1			1	
PR						
Negative	34	1	0.56	34	6	0.09
Positive	22	2		22	10	
Unknown	1					
HER2						
Negative	34	1	0.56	34	9	0.75
Positive	22	2		22	7	
Unknown		1			1	

Statistical tests are based on available data; samples with SNP-only and unknown information are not included in *p*-value calculations. *χ² test (or the Fisher exact test when an expected cell count was less than 5) used for binary/categorical variables; *t* test used for continuous variables. Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2 status; SD, standard deviation.

The *PIK3CA* mutation status has been recently reported for colorectal and ovarian cancers in the Middle Eastern population[37,38] with frequencies of 12.4% and 3.9%, respectively. We present here the first report on the frequency and nature of *PIK3CA* mutations related to breast cancer in the Arab population.

In addition, we have shown the presence of 8.2-fold enrichment of the *PIK3CA* rs17849079 SNP among the Arab breast cancer patient population as compared with disease free controls (**Fig. 1B**). This silent polymorphism has been reported for the first time in retinoblastomas with no obvious functional consequence.[39] This SNP has also been previously classified as a silent somatic mutation in 19 samples of various cancer types worldwide.[22] Interestingly, the microRNA (miRNA), hsa-miR-4324 (accession #MI0015854), binds to the *PIK3CA* mRNA sequence at exon 20, which harbors this SNP.[40,41] This miRNA seems to bind only the wild-type allele but not the variant sequence containing this SNP. The functional significance of this could be that miRNA regulation of *PIK3CA* expression is affected by the presence of this genetic variation.

Finally, we have also shown the absence of *PIK3CA* mutations in stromal fibroblasts. There have been numerous conflicting reports regarding genetic alterations in tumor stroma including cultured CAFs.[42] The fact that CAFs maintain an activated-phenotype even after many passages in culture, has been thought to be due to somatic alterations in their genomes.[43] The initial studies supporting wide spread somatic mutations in CAFs[44,45] have been later challenged by others.[46,47] Interestingly, in our study, three tumor samples that contained *PIK3CA* mutations were not identified in their corresponding CAFs. Our results support recent studies that CAFs are not likely to harbor somatic alterations in commonly mutated oncogenes.

In conclusion, the prevalence of *PIK3CA* mutation in Arab breast cancers is similar to the global average and the presence of these mutations may favor patient survival. On the other hand there were no *PIK3CA* somatic mutations in breast CAFs that were analyzed. Furthermore, we report a high prevalence of the SNP rs17849079 among Arab breast cancer patients compared with controls, which suggests its potential use as a breast cancer susceptibility and early diagnosis molecular marker for this population.

Materials and Methods

Breast cancer tumor and blood sample collection. In this retrospective study, 81 invasive breast cancer samples were obtained from patients that underwent surgery at King Faisal Special Hospital and Research Center (KFSH&RC) in Riyadh, Saudi Arabia. All patients gave written informed consent to participate in this study, which was approved by KFSH&RC Institutional Research Ethics Committee (project #2100017). Tumor samples were either provided as frozen tissue blocks or paraffin embedded slides and were coded and kept anonymous. We also collected 189 peripheral blood samples from cancer-free individuals to be used as controls.

Generation of primary fibroblasts in tissue culture. Twenty-five CAF cells from 25 separate breast tumors were generated as

previously described.[48] Isolated CAFs were cultured in a 5% CO_2 incubator at 37 °C in Medium 199 and Ham's F12 mixed 1:1 and supplemented with 10% FCS and 1% Pen/Strep.

Genomic DNA isolation. Genomic DNA (gDNA) was isolated from cultured CAFs, frozen tumor sections and peripheral blood lymphocytes using the Qiagen Blood and Tissue Kit whereas paraffin embedded slides containing tumor cells were subjected to the QIAamp DNA FFPE Tissue Kit following the manufacturer's instructions.

Immunohistochemical analysis of tumor samples. Immunohistochemistry analysis for estrogen receptor (ER), progesterone receptor (PR), and Her2/neu were performed in the KFSH&RC diagnostic laboratory following optimized protocols. Slides were developed using an automated slide machine (BenchMark XT). Briefly, sections of formalin-fixed paraffin-embedded tumor samples were stained for ER using the mouse monoclonal antibody, ER 6F11 (Novacastra) with 1:30 dilutions, PR using the mouse monoclonal antibody, PGR 312 (Novacstra) with 1:100 dilutions, and HER2 using the rabbit monoclonal antibody, 4B5 (Ventana) and DAB Kit (Ventana). Stains were scored for HER2 as 1+ as negative and 3+ as positive. The normal tissue surrounding tumor was used as a negative control.

PCR amplification and DNA sequencing. *PIK3CA* hotspot exons (exons 1, 9, and 20) were PCR amplified in 81 BC and 25 CAF samples with specific primers for each exon including exon-intron junctions (**Table S1**). The *PIK3CA* exon 20 was also PCR amplified and sequenced in 189 normal blood samples and three tumor matching normal tissue to identify whether the silent polymorphism, rs17849079, was somatic or germline in origin.

About 20 to 100 ng of gDNA was added to the PCR reaction mix depending on the amount available for each sample. High fidelity Taq DNA Polymerase (Invitrogen, 11304-011) was used to reduce PCR artifacts during the amplification process.

PCR amplicons were sequenced by direct sequencing (Sanger sequencing) in the KFSH&RC Sequencing Core Facility. Samples with mutations were re-sequenced at least twice by repeating PCR reactions to rule out any amplification related artifacts as well as sequencing errors.

Statistical analysis. Associations between the mutation status and clinicopathologic features were assessed using the χ^2 test for categorical variables or t test for continuous variables. The Fisher exact test was used when expected cell counts were less than 5 using the Monte Carlo method as implemented in SAS. The frequency of SNPs in patients was compared with that of controls without the disease. The risk of the disease was estimated by the odds ratio (OR) and 95% confidence intervals (CIs). Survival probabilities were estimated using the
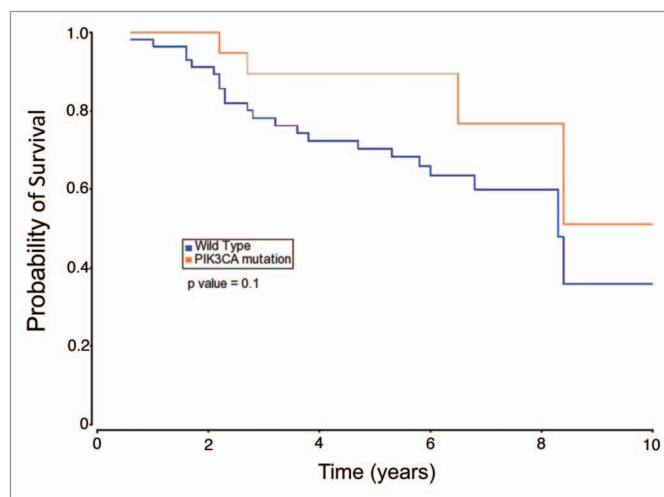

Figure 2. The Kaplan–Meier curves illustrating overall survival based on tumor *PIK3CA* mutational status.

Kaplan–Meier method and log-rank tests were used to compare survival curves between the various subgroups of patients. All statistical tests were two-tailed. A *P* value of < 0.05 was considered significant. Statistical analyses were performed with SAS version 9.2 (SAS Institute Inc.) and the MATLAB software packages (Mathworks).

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

Authors' Contributions

BK designed and performed the experiments. DC conducted statistical analysis; NK provided disease free blood samples and reagents; HG provided cultured CAF samples; AAQ and FH extracted DNA from tumor samples; MT provided some of the tumor samples and conducted the pathological analyses; TAT collected patient clinical data; BK, BHP, and AA wrote the manuscript.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cbt/articles/25945

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