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Surgical resection of breast cancers: Molecular analysis of cancer stem cells in residual disease

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A R T I C L E I N F O

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A B S T R A C T

Background: Approximately 70% of breast cancer patients have residual disease after neoadjuvant chemotherapy. This study was designed to determine whether breast cancer cells with stemlike properties are present in residual disease after neoadjuvant chemotherapy and whether they exhibit oncogenic mutations. The presence of breast cancer cells with stemlike properties with specific mutations may help explain the poor prognosis associated with residual disease.
Methods: A total of 68 breast cancer specimens were collected at the time of mastectomy or lumpectomy. A total of 44 were chemotherapy naïve and 24 were collected as residual disease after neoadjuvant chemotherapy. Tumor cells were collected by fluorescence-activated cell sorting, with breast cancer cells with stemlike properties specifically identified using breast stem cell associated antibodies. Whole tumor specimens and fluorescence-activated cell sorting breast cancer cells with stemlike properties were analyzed for genetic mutations, including PIK3CA.
Results: Breast cancer cells with stemlike properties, demonstrating EpCAM-positive, CD44-positive, CD49f$^{\pm}$, CD24$^{\pm}$ expression were present in chemotherapy-naïve tumors and residual disease. In both chemotherapy-naïve and residual disease specimens the highest frequency of PIK3CA mutations were detected in CD49f-CD24+ BCSCs (39% and 33%, respectively). PIK3CA mutations were detected in all stages of breast cancer (35%), in both chemotherapy naïve (39%) and residual disease (29%) and in both estrogen receptor positive (41%) and negative tumors (14%) (P = ns). Various PIK3CA mutations were identified in chemotherapy-naïve specimens versus residual disease specimens in both patient-paired and unpaired breast cancers.
Conclusion: Breast cancer cells with stemlike properties with mutations in PIK3CA were present in chemotherapy-naïve breast cancers and residual disease after neoadjuvant chemotherapy. These results demonstrate that neoadjuvant chemotherapy does not completely eradicate PIK3CA-defective breast cancer cells with stemlike properties. Although these findings may help explain the poor clinical outcomes in patients with residual disease, they also identify breast cancer cells with stemlike-property targets for therapies.

Introduction

Surgical treatment of breast cancer is highly effective in removing disease from the breast and regional lymph nodes. Adjuvant treatments target systemic disease and account for much of the reduction in breast cancer mortality rates since the 1990s.[1] However, women remain at high risk for distant recurrence after treatment, and disease at distant sites remains responsible for the majority of breast cancer mortality.[2] A recent trend in breast cancer treatment is the increased use of neoadjuvant chemotherapy (NAC).[3] Completing chemotherapy before breast operation provides the opportunity to increase surgical options,[4] directly examine treatment effects, and identify molecular characteristics of cells that are refractory to chemotherapy. Of women treated with NAC, 70% have residual disease (RD).[5] These women have higher recurrence and lower overall survival rates than those with complete pathologic responses.[6]

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A large body of evidence indicates that breast cancer cells with stemlike properties (BCSCs) drive breast cancer development and explain the regenerative capacity, dormancy, chemo-resistance, and metastatic ability of breast cancer.[7–11] In benign breast tissue, breast stem cells and their progenitors give rise to the normal tissues of the breast. Their nondifferentiated state and ability to self-renew make them uniquely agile and powerful cells. It is believed that when breast cells with innate or acquired stemlike abilities incur DNA damage, they transform into cancer stem cells (CSC).[12] These cells retain characteristics of normal stem cells, but acquire characteristics of malignant cells as well.[13] In vitro studies indicate that BCSCs are resistant to chemotherapy in the laboratory.[14,15] It may be the normal fluctuation between dormancy and proliferation, and the low metabolic states of benign breast stem cells, that protect their malignant counterpart, BCSCs, from chemotherapy and antiangiogenic agents.[16]

BCSCs comprise only a small proportion of the cells within a tumor.[17] Data from normal mouse mammary structures estimate stem cell frequencies of ~0.001%.[18] BCSCs can be isolated from fresh surgical specimens, using the currently established stem cell surface markers EpCAM, CD44, CD49f (or CD 29), CD24, and ALDH1.[13,17–22] These markers are responsible for cell adhesion, communications, migration, and differentiation.[23–27] In addition to cell-surface marker identification, breast cancer cells with the ability to form stem cell generated mammospheres[9–13] and to serially give rise to tumors in animal models meet the criteria for BCSC designation.[10,17] BCSCs may also harbor cancer-associated genetic abnormalities.[10]

The PI3K signaling pathway, which employs PIK3CA and other proteins, such as mTOR and AKT1, is integral to normal cell proliferation, differentiation, migration, and survival.[28,29] Cancer-associated mutations in PIK3CA occur at multiple sites in the p110 alpha catalytic subunit of the PIK3CA heterodimer molecule.[15,28–30] However, there are mutation "hotspots" located along the helical (E542 and E545) and kinase domains (H1047) of the p110 alpha subunit that produce functionally abnormal protein structures, enhanced enzymatic activity,[15] and downstream abnormal cell signaling.[31] Mutations in PIK3CA have been detected in many human cancers and in approximately 30%–40% of breast cancers.[32,33] It is important to note that they are associated with treatment resistance,[34] worse prognosis,[30–33] and increased metastatic potential. Patients who initially present with lymph node positive disease are more likely to have a PIK3CA mutation in their primary tumor BCSC's than women with node negative disease.[35]

Given the refractory behavior of CSCs to chemotherapy in vitro and their apparent role in tumor initiation and metastasis, we hypothesized that they would be present in RD and carry oncogenic mutations. Furthermore, because PIK3CA mutations are the most commonly found genetic mutation in breast cancer, and we have shown that BSCSs harbor PIK3CA mutations, we also hypothesized that BCSCs in RD would contain PIK3CA mutations. Confirmation of these results would demonstrate that, irrespective of the initial mutational load of breast cancers before NAC, NAC does not eradicate PIK3CA-defective BCSCs in all patients. Furthermore, although these findings may explain the poor clinical outcomes seen in patients with RD, they would also identify targets for personalized therapies.

Methods

Fresh primary breast tumors and residual primary tumor, >1cm in at least one dimension by imaging, were collected before and after NAC from women at the time of lumpectomy or mastectomy. No samples were collected from men because of the infrequency of male breast cancer cases during the study period. BCSCs were identified, collected, counted, and examined for oncogenic mutations. The Oregon Health & Science University Institutional Review Board (Portland) approved this study.

Isolation of BCSCs for qualitative and quantitative analysis

Breast cancer specimens were minced, digested, and incubated with breast cancer stem and progenitor cell antibodies EpCAM, CD44, CD49f, and CD24. Cells were also incubated with endothelial, lymphocytes, and hematopoietic stem cell antibodies (CD31, CD45 or CD34) so that non-epithelial cells could be identified and removed from the final collection cell pool. Using fluorescence-activated cell sorting (FACS), four distinct CD31/CD45/CD34-negative populations were collected: CD49f+CD24+, CD49f+CD24−, CD49f−CD24+, and CD49f−CD24−. CD44 and EpCAM expression were measured in each of these populations. Unsorted breast cancer tissues were also collected for whole-tumor analyses.[35] Mutation analyses of FACS BCSCs and whole-tumor samples were accomplished, as per manufacturer protocol, by quantitative RT-PCR of 2,800 mutation sites in 50 oncogenes and tumor suppressor genes on AmpliSeq Cancer Hotspot Panels (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical methods

Statistical significance of differences in demographics between groups were analyzed with χ^2 and Fisher's exact tests. Significance of differences in data between groups were analyzed by *t* test.

Results

Patient demographics and tumor characteristics of chemotherapy-naïve and RD samples

A total of 68 breast cancer specimens were obtained from 63 patients. In total, 41 patients contributed 44 chemotherapy-naïve specimens. A total of 24 residual disease specimens were obtained from 24 patients after completing NAC. Among the chemotherapy-naïve patient cohort, 6 chemotherapy-naïve tumor samples were collected before NAC treatment. Of the 6 patients, 1 patient died before completing treatment and 3 patients had insufficient RD for analysis. Two of these patients contributed paired chemotherapy-naïve and post-NAC RD specimens. Patient demographics, tumor characteristics, and NAC regimens are presented in Tables I and II.

Breast cancer stage of chemotherapy-naïve and RD samples

Breast cancers were staged according to the Prognostic American Joint Committee on Cancer 8th edition breast cancer staging criteria[36] and are presented in Table III.[19] Chemotherapy-naïve patients presented with predominately stage I breast cancers (73%), and patients with RD after NAC presented with stage I in 25% of cases and demonstrated no predominant stage at enrollment (Table III).

PIK3CA mutation status in chemotherapy-naïve and RD samples

Overall, PIK3CA mutations were found in 35% of the 68 breast cancers in this study. Among chemotherapy-naïve tumors, 39% carried PIK3CA mutations and were detected in stages I, II, and IV, but not in stage III. In the RD, 29% of tumors carried a PIK3CA mutation and were detected in stages I, II, III, and IV (Table IV). We observed no significant association of PIK3CA mutations with any given stage within either of the chemotherapy-naïve or RD cohorts.

Table I
Patient and tumor characteristics of chemotherapy-naïve breast cancer specimens

	Tumors $N = 44$ (%)
Number of patients	41
Age (years) at diagnosis, mean [range, SD]	55.7 [31–90, 12.2]
Histologic subtype	
Ductal	41 (93.2)
Lobular	3 (6.8)
Receptor status	
ER+/PR+/Her2-	30 (68.2)
ER+/PR-/Her2-	2 (4.5)
ER+/PR+/Her2+	3 (6.8)
ER-/PR-/Her2+	4 (9.1)
ER-/PR-/Her2-	5 (11.4)

SD, standard deviation; *ER*, estrogen receptor; *PR*, progesterone receptor; *Her2*, Her2/neu growth factor receptor.

Table II
Patient and tumor characteristics of breast cancer residual disease

	Tumors $N = 24$ (%)
Number of patients	22
Age (years) at diagnosis, mean [range, SD]	51 [30–72, 11.3]
Histologic subtype	
Ductal	21 (87.5)
Lobular	3 (12.5)
Receptor status	
ER+/PR+/Her2-	13 (54.2)
ER+/PR-/Her2-	3 (12.5)
ER+/PR+/Her2+	2 (8.3)
ER+/PR-/Her2+	1 (4.2)
ER-/PR-/Her2+	2 (8.3)
ER-/PR+/Her2-	1 (4.2)
ER-/PR-/Her2-	2 (8.3)
Neoadjuvant chemotherapy regimen	
Cyclophosphamide plus taxane	2 (8.3)
Doxorubicin and cyclophosphamide, then taxane	14 (58.3)
Taxane, carboplatin, trastuzumab, and pertuzumab	3 (12.5)
Pertuzumab, trastuzumab, and taxane	2 (8.3)
Doxorubicin plus cyclophosphamide then taxane plus trastuzumab	1 (4.2)
Doxorubicin plus cyclophosphamide then taxane, trastuzumab, plus pertuzumab	1 (4.2)
Aromatase inhibitor	1 (4.2)

SD, standard deviation; *ER*, estrogen receptor; *PR*, progesterone receptor; *Her2*, Her2/neu growth factor receptor.

Frequency of PIK3CA mutation in BCSC isolated from chemotherapy-naïve and RD samples

BCSCs that were EpCAM+CD44+CD49f± and CD24± were identified in both chemo- naïve and RD specimens and in all stages. Their frequency did not significantly differ by stage (data not presented). In chemo-naïve and RD tumors, BCSCs contributed to only a small proportion of the total number of tumors cells (Figure).

PIK3CA mutations were found in all four BCSC populations (Table V). In chemotherapy-naïve tumors, most PIK3CA mutations were identified in CD49f±CD24+ cells. In RD, the majority of PIK3CA mutations were found in CD49f-CD24+ cells. In two tumors, PIK3CA mutations were only detected by first collecting BCSCs by FACS and separately testing them for oncogenic mutations. These PIK3CA mutations were not detectable in unsorted whole tumor tissue.

Chemotherapy-naïve tumors and breast cancer RD demonstrated a number of variations with respect to the location and frequency of PIK3CA mutations. Although all mutations detected were located in the p110 alpha catalytic subunit of the PIK3CA protein, the molecular region, or domain, in which they occurred varied (Table VI). The mutation COSM775 (change of histidine to arginine at the 1047 amino acid position, H1047R)[37] was detected most often in both chemotherapy-naïve tumors and RD. Of the remaining seven PIK3CA mutations identified, five were found in chemotherapy-naïve tumors, but not in RD. Conversely, two of the seven were found only in RD and not in chemotherapy-naïve samples. PIK3CA mutations in RD were more restricted in location than those found in chemotherapy-naïve tumors. They occurred in the kinase and helical domains of the PIK3CA protein.

Table III
AJCC eighth edition prognostic stages at diagnosis of breast cancer specimens, grouped by treatment status

Prognostic stage	Chemo-naïve $N = 44$ tumors N (%)	Residual disease $N = 24$ tumors N (%)	Total specimens $N = 68$ N (%)
IA	26 (59.1)	0	26 (38.2)
IB	6 (13.6)	6 (25.0)	12 (17.6)
IIA	4 (9.1)	6 (25.0)	10 (14.7)
IIB	1 (2.3)	4 (16.7)	5 (7.8)
IIIA	0	2 (8.3)	2 (2.9)
IIIB	2 (4.5)	4 (16.7)	6 (8.8)
IIIC	4 (9.1)	1 (4.2)	5 (7.4)
IV	1 (2.3)	1 (4.2)	2 (2.9)

Table IV
Percent of breast cancers with BCSC PIK3CA mutations, stratified by stage and treatment status

Prognostic stage	Chemo-naïve ($N = 44$ tumors)	Residual disease ($N = 24$ tumors)	Total specimens ($N = 68$)
I	44%	50%	25%
II	40%	20%	5.9%
III	0	12.5%	1.5%
IV	100%	100%	2.9%
Combined stages	39%	29%	35%

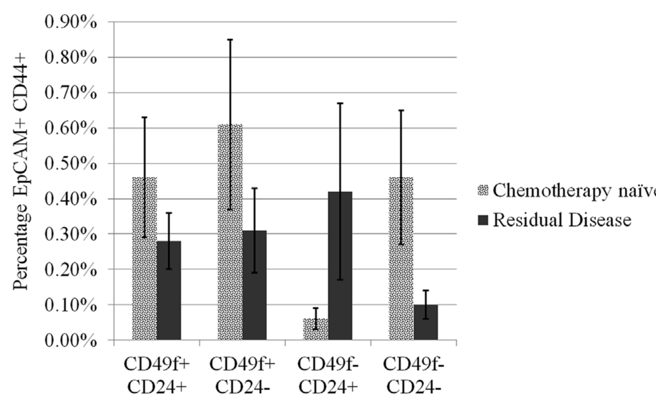

Figure. Percentage of CD49f$^{\pm}$CD24$^{\pm}$ viable cells that are EpCAM+ and CD44+, grouped by treatment status. Bars represent standard error of the mean.

Table V
Frequency of PIK3CA mutations in BCSC populations, grouped by treatment status

Population	Chemo-naïve $N = 44$ tumors (%)	Residual disease $N = 24$ tumors (%)
CD49f+CD24+	8/25 (32)	2/12 (17)
CD49f+CD24-	7/39 (18)	2/21 (10)
CD49f-CD24+	9/23 (39)	5/15 (33)
CD49f-CD24-	4/41 (10)	3/19 (16)
Whole tissue	12/43 (28)	7/24 (29)

Note. Whole tissue refers to an unsorted tumor specimen. Fractions indicate the number of positive results among specimens in which sufficient genetic materials was available for analysis.

Table VI
Identity, amino acid change, and protein location of PIK3CA mutations in breast cancer specimens, grouped by treatment status

	Mutation (amino acid change)	Chemo-naïve $N = 17$ tumors (%)	Residual disease $N = 7$ tumors (%)	Total specimens $N = 24$ (%)
Kinase	COSM775 (H1047R)	7 (41.2)	4 (57)	11 (45)
	COSM776 (H1047L)	0	3 (42)	3 (12.5)
C2	COSM754 (N345K)	2 (11.8)	0	2 (8.3)
	COSM757 (C420R)	2 (11.8)	0	2 (8.3)
Helical	COSM760 (E542K)	2 (11.8)	0	2 (8.3)
	COSM763 (E545K)	3 (17.6)	0	3 (12.5)
	COSM12459 (Q546R)	1 (5.9)	0	1 (4.2)
	COSM766 (Q546K)	0	1 (14)	1 (4.2)

Note. Kinase, C2, and helical denote the PIK3CA p110 alpha domain of each point mutation. One residual disease sample contained two PIK3CA mutations.

Table VII
PIK3CA mutation identity of patient pairs of breast cancer specimens

Patient number	PIK3CA mutation in chemo-naïve specimen	Population in which PIK3CA mutation found	Residual disease result	Prognostic stage at diagnosis
1	COSM763	Tissue only	WT	IB
2	COSM757	CD49f-CD24+	WT	IB
3	COSM775	Tissue, CD49f-CD24-	NRD	IIA
4	WT		NRD	IIA
5	WT		NRD	IIIB
6	COSM775	CD49f-CD24+, CD49f-CD24-	Patient mortality	IV

WT, wild type; *NRD*, no residual disease.

Chemotherapy-naïve breast cancers demonstrated mutations along a broader section of the PIK3CA gene than did RD specimens, occurring in kinase and helical domains as well as the C2 domain, which may serve to target the PIK3CA protein to the cell membrane for crucial molecular interactions.[36]

Table VII presents the identity and BCSC populations in which PIK3CA mutations were found in patient-paired chemotherapy-naïve and RD samples. No mutations were identified in RD when available in this paired group. No de novo mutations were seen in RD that were not present in patient-paired chemotherapy- naïve tissues.

Hormone status of tumors carrying PIK3CA mutations

Within this study, estrogen receptor–positive (ER-positive) tumors contributed 79% of tumors and ER-negative contributed 21%. In chemotherapy-naïve tumors BCSC PIK3CA mutations were found in 43% of ER-positive tumors and 22% of ER-negative tumors ($P = .45$; Table VIII). In RD samples, for which hormone status is assigned before treatment, BCSC PIK3CA mutations were found in 37% of ER-positive tumors and 0% of ER-negative tumors and ($P = .27$). When treatment status was not considered as a factor, the incidence of BCSC PIK3CA mutations among ER-positive tumors was 43% compared with 14% in ER-negative tumors ($P = .114$).

Clinical outcomes

At the time of this writing, median follow-up time is 41 months (range 4–70 months). For patients in the chemotherapy-naïve cohort, median follow-up was 42 months (range 8–70 months). For patients with RD, median follow-up was 20 months (range 4–60 months). We observed that 6 of 63 patients have died. Of the 6 patients, 5 died of disease and 4 of them had BCSC PIK3CA mutations. We observed that 3 patients in the chemotherapy-naïve cohort have died of metastatic breast cancer. Note that 2 of these patients were diagnosed with stage IA breast cancer, and 1 with stage IV. We observed that 1 of the 2 patients with stage IA breast cancer and the patient with stage IV disease had PIK3CA COSM775 mutations. We observed that 3 patients with RD have died of metastatic breast cancer. Of these, 1 patient with stage IIB breast cancer carried a COSM776 mutation, 1 patient with stage IB carried a COSM775 and the other patient with stage IIIA breast cancer was PIK3CA wild type.

Table VIII
PIK3CA mutation frequency in breast cancer specimens by estrogen receptor status, grouped by treatment status

Estrogen receptor status	ER-negative $N = 14$ tumors (%)	ER-Positive $N = 54$ tumors (%)	P value
Chemotherapy-naïve	2 (14)	15 (28)	
Residual disease	0	7 (13)	
Total	2 (14)	22 (41)	$P = $ NS

ER, estrogen receptor.
Note. Percentages represent the proportion of each group as defined by both ER status and treatment status. Of the total 68 tumors, 54 were ER positive and 14 were ER negative. Populations are unmatched and were compared using the Fisher's exact test and χ^2 analysis. $P \leq .05$ considered significant.

Discussion

The predominant finding of this study is that PIK3CA mutated BCSCs are present in breast cancers before chemotherapy and in residual disease after treatment. BCSCs are present in all stages of breast cancer, but their frequencies vary between tumors. This is consistent with our earlier report that PIK3CA mutations may be found in all BCSC populations, but to varying degrees in each breast cancer. In both chemotherapy-naïve and RD samples, CD24+ cells were the primary source of PIK3CA mutations. Our pool of CD24+ cells likely includes those with low to moderate expression of CD24 and thus represents the same population of BCSCs reported by others as being tumorigenic and present after chemotherapy.[38] This study adds to the existing body of knowledge that BCSCs are not only present in the vast majority of RD samples but also carry PIK3CA mutations.

The detection of BCSCs with PIK3CA mutations in RD demonstrated that these cells exhibit resistance to chemotherapy, not only in vitro as already demonstrated, but in vivo as well. That BCSCs are present after NAC and contain specific PIK3CA mutations associated with poorer prognosis may explain why patients with residual disease after NAC have higher recurrence rates and poorer survival rates than patients with complete pathologic responses. Local and regional responses to NAC have been long considered a strong surrogate for the systemic response to NAC. Therefore, if BCSCs with specific PIK3CA mutations survive NAC in the primary tumor, they also may be present within distant micrometastases. Given that BCSCs are drivers of primary tumorigenesis and responsible for disease progression, it is conceivable that BCSCs that survive chemotherapy as micrometastases may lead to the further development of distant metastases. Additional follow-up time will determine whether the presence of PIK3CA mutations in BCSCs is a significant prognostic indicator.

The recent transition from the traditional anatomic staging system to the prognostic staging system has precipitated stage migration of PIK3CA-positive tumors from stage II to stage I. The status of the ER influences the prognostic staging system. It is considered a positive factor that may downstage a tumor. Therefore, PIK3CA mutations that were associated with ER-positive tumors were downstaged in the prognostic staging system. It will be interesting, with a larger cohort and longer follow-up, to determine whether the presence of BCSC PIK3CA mutations influences or stratifies the prognosis of a subset of patients with prognostic stage I tumors.

Another significant finding of this study was that chemotherapy-naïve tumors expressed a wider distribution of mutations along the PIK3CA gene compared with those found in RD. Also, the variation of PIK3CA mutations found in RD were more limited than those found in chemotherapy-naïve BCSCs. In fact, except for COSM775, the mutations found in chemotherapy-naïve tumors were completely different from those found in RD. A larger study of patient-paired tumors will help to discern whether this finding is attributable to how effective NAC is at reducing specific PIK3CA mutations. It may be that BCSC-carrying PIK3CA kinase mutations, such as COSM775 and COSM776, are the most capable of outcompeting the effects of chemotherapy by sustaining PIK3CA activity and cell proliferation, even in the presence of therapeutic cytoxicity. Therapeutics that specifically target these genetic defects in conjunction with chemotherapy may provide more complete treatment effect.

Earlier studies have demonstrated a positive association between PIK3CA mutations and ER status and activity.[39,40] In this study, a greater number of BCSCs from ER-positive tumors carried PIK3CA mutations than those collected from ER-negative tumors, a finding true in both chemotherapy-naïve and RD cohorts. However, because of the low frequency of ER-negative tumors in the study, that finding was not statistically significant. The total lack of ER-positive breast cancers in the stage III chemotherapy-naïve group may help explain the lack of PIK3CA mutations found in that cohort. What this study did demonstrate, however, was that PIK3CA mutations are not limited to ER-positive breast cancer BCSCs.

This study was limited by the availability of patient-paired specimens and, as such, the direct effect of NAC on BCSC numbers and PIK3CA mutation frequencies was not measured. Larger matched studies will be necessary to directly examine that relationship. Rather, this study was designed to determine the frequency of BCSCs in chemotherapy-naïve and RD and whether they carried PIK3CA mutations. The findings of this study demonstrated that the frequency of PIK3CA in BCSCs in both chemotherapy naïve and RD after NAC is similar to the frequency of PIK3CA mutations that has been documented in grossly examined breast cancer samples.[32,33]

In conclusion, PIK3CA mutations in BCSCs are not restricted to stage or hormone-receptor status, but may be linked to treatment status. A larger study will be required to confirm whether specific PIK3CA mutations are more resistant to chemotherapy, as our study suggests. However, the finding of BCSCs harboring PIK3CA mutations within RD provides a potential therapeutic target for improving outcomes. Oral PIK3CA inhibitors have been developed and some have undergone clinical trials in the setting of metastatic disease that has failed conventional hormonal therapy.[41] Another potential use of PIK3CA inhibitors may be in the setting of post-treatment residual disease. As this study has demonstrated, one-third of breast cancer patients harbor PIK3CA mutations in their BCSCs after NAC. The use of PIK3CA inhibitors in this setting may not only provide additional complete local clinical or pathologic responses beyond what has been achieved with NAC alone, but may also reduce distant micrometastatic disease, thereby improving distant recurrence and survival rates.

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