


Mutations in *PIK3CA* sensitize breast cancer cells to physiologic levels of aspirin

Sanja B. Turturro¹ · Matthew S. Najor¹ · Carl E. Ruby² · Melody A. Cobleigh¹ ·
Abde M. Abukhdeir^{1,3} 

Received: 27 January 2016 / Accepted: 17 February 2016 / Published online: 25 February 2016
© The Author(s) 2016. This article is published with open access at Springerlink.com

Abstract A review of the literature finds that women diagnosed with breast cancer, who were on an aspirin regimen, experienced a decreased risk of distant metastases and death. Several recent studies have reported an improvement in overall survival in colorectal cancer patients who harbored mutations in the oncogene *PIK3CA* and received a daily aspirin regimen. Breast cancer patients on a daily aspirin regimen experienced decreased risk of distant metastases and death. *PIK3CA* is the most frequently mutated oncogene in breast cancer, occurring in up to 45 % of all breast cancers. In order to determine if mutations in *PIK3CA* sensitized breast cancers to aspirin treatment, we employed the use of isogenic cellular clones of the non-tumorigenic, breast epithelial cell line MCF-10A that harbored mutations in either *PIK3CA* or *KRAS* or both. We report that mutations in both *PIK3CA* and *KRAS* are required for the greatest aspirin sensitivity in breast cancer, and that the GSK3 β protein was hyperphosphorylated in aspirin-treated double knockin cells, but not in other clones/treatments. A more modest effect was observed with single mutant *PIK3CA*, but not *KRAS* alone.

These observations were further confirmed in a panel of breast cancer cell lines. Our findings provide the first evidence that mutations in *PIK3CA* sensitize breast cancer cells to aspirin.

Keywords Breast cancer · Aspirin · *PIK3CA* · *KRAS* · Genetics · Drug resistance

Abbreviations

CD-FBS	Charcoal-dextran treated fetal bovine serum
DKI	Cellular clones of MCF-10A carrying mutations in <i>PIK3CA</i> in combination with <i>KRAS</i>
DMEM:F12	Dulbecco's modified eagle medium
EGF	Epidermal growth factor
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
MPE	Molecular pathologic epidemiology
NSAID	Non-steroidal anti-inflammatory drug
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
RPPA	Reverse phase protein array
TRIS	Tris(hydroxymethyl)aminomethane

Electronic supplementary material The online version of this article (doi:10.1007/s10549-016-3729-8) contains supplementary material, which is available to authorized users.

✉ Abde M. Abukhdeir
abde_abukhdeir@rush.edu

¹ Department of Internal Medicine, Division of Hematology, Oncology, and Cell Therapy, Rush University Medical Center, 1725 W. Harrison St., Chicago, IL 60612, USA

² Department of Surgery, Rush University Medical Center, 1725 W. Harrison St., Chicago, IL 60612, USA

³ Department of Pharmacology, Rush University Medical Center, 1735 W. Harrison St., Chicago, IL 60612, USA

Introduction

In 2015, over 227,000 women will be newly diagnosed with breast cancer in the United States, while nearly 40,000 women who already have breast cancer will succumb to the disease [1]. The majority of women with breast cancer who succumb to their disease exhibit resistance to all available therapies. Moreover, the number of available targeted

therapies is limited by (i) the number of disease-relevant genetic alterations that have been identified and (ii) the number of therapeutic agents available for known targets.

The past decade has seen a steady surge in the use of targeted therapies in cancer. Aspirin is a common non-steroidal anti-inflammatory drug (NSAID) often used for the prevention of heart disease [2]. Several NSAIDs have been shown to inhibit cell growth and lead to apoptosis during different stages of cancer [3, 4]. Regular aspirin use has been reported to prevent several types of cancers and more recently has been shown to have anti-cancer properties [2, 5–7]. Aspirin inhibits PTGS2 (cyclooxygenase), which therefore prevents the conversion of arachidonic acid into prostaglandins. Clinically, aspirin acts as an anti-inflammatory and antiplatelet agent. Others have speculated that regular aspirin use can prevent the development of cancers [8, 9].

A review of the literature reveals that most cancer-related aspirin studies have been in the context of cancer prevention [3, 6, 7, 9]. Holmes et al. [10] reported that women diagnosed with breast cancer and taking a regular aspirin regimen experienced a decreased risk of distant recurrence of the disease and breast cancer death [10]. Similar observations have been made in other cancers [11–13]. Of note, patients diagnosed with colorectal cancers harboring mutations in *PIK3CA* and receiving aspirin treatment had increased survival [11–13].

The *PIK3CA* gene encodes the catalytic domain of the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) complex. Dysregulation of the PI3K complex leads to unabated growth signaling through the AKT and MAPK pathways and is strongly implicated in the pathogenesis of many cancers [14]. The *PIK3CA* gene is frequently mutated in both colorectal and breast cancers, occurring in up to 32 and 45 %, respectively [15, 16].

Taken together, we hypothesized that physiologic concentrations of aspirin may have an anti-proliferative effect on breast cancers harboring mutations in *PIK3CA*. To test this hypothesis, we utilized an isogenic cellular model of mutant *PIK3CA* in the human, non-tumorigenic breast epithelial cell line, MCF-10A [17, 18]. To the best of our knowledge, this is the first study to explore the mechanism of the anti-cancer properties of aspirin in the context of breast cancers harboring mutations in *PIK3CA*.

Methods

Cell culture

The non-tumorigenic human breast epithelial cell line MCF-10A (ATCC, Manassas, VA) and its derivatives were grown in a humidified atmosphere, supplemented with

5.1 % CO₂ at 37 °C in Dulbecco's modified eagle medium (DMEM:F12) devoid of phenol-red (Life Technologies, Grand Island, NY) supplemented with 5 % horse serum (Sigma, Saint Louis, MO), 1 % penicillin and streptomycin (Life Technologies, Grand Island, NY), 20 ng/mL epidermal growth factor (EGF) (Sigma, Saint Louis, MO), 25 µg/mL insulin (Sigma, Saint Louis, MO), 0.5 µg/mL hydrocortisone (Sigma, Saint Louis, MO), and 0.1 µg/mL cholera toxin (Sigma, Saint Louis, MO) (hereafter referred to as "supplemented medium") as previously described [18]. MCF-7, MDA-MB-468, and MDA-MB-436 cells (ATCC, Manassas, VA) were grown in DMEM (Life Technologies) supplemented with 5 % fetal bovine serum (FBS) (Sigma, Saint Louis, MO) and 1 % penicillin streptomycin (Life Technologies, Grand Island, NY). Cellular clones of MCF-10A carrying mutations in *PIK3CA* alone or in combination with *KRAS* (hereafter referred to as "DKI") were a generous gift from Dr. Ben Ho Park (Johns Hopkins University) and were grown in EGF-free supplemented medium (hereafter referred to as "knockin medium") [18]. All cellular assays of MCF-10A cells and its derivatives were performed in knockin medium, whereby horse serum was replaced with 1 % charcoal-dextran treated fetal bovine serum (CD-FBS) (Fisher Scientific, Pittsburg, PA) (hereafter referred to as "assay medium"). The cancer cell lines MCF-7, MDA-MB-468, and MDA-MB-436 were seeded in "cancer assay medium" which consisted of DMEM supplemented with 1 % penicillin and streptomycin, and 0.5 % CD-FBS. All cells were harvested for passaging using Tryple Express (Life Technologies, Grand Island, NY).

Cellular proliferation assays

Cells were plated in 96-well plates at a density of 2000 cells/well in assay medium. After 24 h (day 1), the medium was replaced with fresh assay medium supplemented with 0.2 ng/mL EGF and 0, 2, 3, or 4 mM aspirin (Sigma, Saint Louis, MO) and replenished on day 2. On day 4, cells were stained with either crystal violet or CellTiter-Fluor cell viability assay (Promega, Madison, WI) and counted by measuring absorbance on a SpectraMax M5 fluorescence plate reader (Molecular Devices, Sunnydale, CA), as previously described [19].

Immunoblotting

Cells were seeded and treated as above, except fresh aspirin-containing medium was added 1 h before harvesting, as previously described [20]. Whole cell lysates were harvested on days 1 and 4 with and without aspirin in Laemmli Buffer (Bio Rad, Hercules, CA) and boiled for 10 min at 100 °C. Lysates were resolved using SDS-PAGE

using 4–12 % bis–tris NuPAGE gels in MES running buffer (Invitrogen, Grand Island, NY) following the manufacturer’s protocol. The proteins were transferred using Invitrogen Xcell II blotting apparatus to a PVDF membrane (Invitrogen, Grand Island, NY). Following transfer, the membranes were blocked in 5 % w/v milk in tris(hydroxymethyl)aminomethane (TRIS)-buffered saline supplemented with 0.1 % tween-20 (Sigma, Saint Louis, MO) for 1 h. Membranes were probed with either phosphorylated GSK3 β (Ser 9; 9336), total GSK3 β (9315), phosphorylated Src family (Tyr 416; 2101), total Src (ab47405, Abcam, Cambridge, MA), ACTB (β -actin) (4967), and TUBB (β -tubulin) (2146) primary antibody followed by incubation with an anti-rabbit secondary antibody conjugated to horseradish peroxidase (7074). Protein bands were visualized using enhanced chemiluminescent reagent (Perkin-Elmer, Waltham, MA). All antibodies were purchased from Cell Signaling Technology (Beverly, MA) unless otherwise noted. Densitometry was performed using Image J analysis software (NIH).

Fluorescence-activated cell sorting (FACS)

Parental MCF-10A cells and DKI cells were plated under assay conditions and treated with either 0, 2, 3, or 4 mM aspirin for up to 72 h. Cells were seeded at 50,000 cells/well on assay medium in 24-well plates. After 24 h, assay media were removed, and cells were replenished with assay medium supplemented with 0.2 ng/mL EGF and 0–4 mM of aspirin. Camptothecin (2–100 μ g/mL) (Sigma, Saint Louis, MO) served as a positive control for cell death. After 72 h, the media were removed, and cells were washed with Hank’s Balanced Salt solution (Corning, Corning, NY). Cells were harvested, and the pellets were resuspended in phosphate buffered saline and FACS buffer. Propidium iodine (Sigma, Saint Louis, MO) was added to each sample at 7 μ g/mL for 5 min prior to analysis. Harvested samples and single stain controls were run on FACSCanto (Becton–Dickinson, Franklin Lakes, NJ) and analyzed for cell cytotoxicity.

Reverse phase protein array (RPPA)

Parental MCF-10 and DKI cells were seeded on day 0 in EGF-free assay medium. The medium was replaced with assay medium supplemented or not with 4 mM aspirin the following day and cells were grown for the additional 3 days. Following harvesting, cell pellets were sent to the RPPA core facility at The MD Anderson Cancer Center for further preparation and analysis.

Cellular proteins were denatured by 1 % SDS (with β -mercaptoethanol) and diluted in five 2-fold serial dilutions in dilution buffer (lysis buffer containing 1 % SDS). Serial

diluted lysates were arrayed on nitrocellulose-coated slides (Grace Biolab) by Aushon 2470 Arrayer (Aushon BioSystems). A total of 5808 array spots were arranged on each slide including the spots corresponding to positive and negative controls prepared from mixed cell lysates or dilution buffer, respectively. Each slide was probed with a validated primary antibody plus a biotin-conjugated secondary antibody.

Statistical analyses

Statistical analysis was performed using a student’s *t* test. A *P* value of less than 0.05 was considered significant.

For analysis of our RPPA results, only antibodies with a Pearson correlation coefficient between RPPA and Western blotting of greater than 0.7 were used in reverse phase protein array study. The signal obtained was amplified using a Dako Cytomation—catalyzed system (Dako) and visualized by DAB colorimetric reaction. The slides were scanned, analyzed, and quantified using a customized-software Microvigena (VigeneTech Inc.) to generate spot intensity.

Each dilution curve was fitted with a logistic model (“Supercurve Fitting” developed by the Department of Bioinformatics and Computational Biology in MD Anderson Cancer Center, <http://bioinformatics.mdanderson.org/OOMPA>). The similarity in samples to each other was determined by Pearson correlation. The correlation values for each of the expression nodes were calculated by the RPPA core where it was determined that correlation of 0.195, coupled with more than 100° of freedom (i.e., antibodies), has a *P* value of 0.05.

Results

Mutations in oncogenes sensitize breast cancer cells to aspirin

In order to isolate the effects of physiologic levels of aspirin in the presence of specific mutations, we employed an isogenic cellular system derived from the human non-tumorigenic cell line, MCF-10A, which is devoid of any other oncogenic mutations. However, it should be noted that these cells carry a deletion in the p16/ARF locus, which is thought to contribute to their immortalization [21]. This cell line is dependent on EGF for growth, thus removing EGF from the medium results in G1 arrest. Clones carrying mutant PIK3CA are EGF independent but still respond to EGF. Therefore, cells are initially plated in the absence of EGF to synchronize cells, and then 0.2 ng/mL of EGF is added to drug containing medium 24 h later. We chose a lower dose of EGF than what is in the typical

growth medium because our previous studies have shown that high doses of EGF mask the contribution of mutant *PIK3CA* to cellular signaling [17]. We tested varying doses of aspirin for 24, 48, and 72 h and determined that the most prominent effect of aspirin was observed after 72 h. Therefore, all aspirin experiments were performed for 72 h post aspirin treatment. The half maximal inhibitory concentration (IC_{50}) value for each cellular clone was calculated and reported in Supplementary Table 1. Parental MCF-10A cells were not responsive up to 4 mM aspirin (Fig. 1). However, aspirin treatment of cells carrying mutations in *PIK3CA* at either exon 9 (E545K) or exon 20 (H1047R), resulted in a statistically significant decrease in cell number (Fig. 1). We and others have shown that individual genetic lesions may not be sufficient for tumorigenesis, but the accumulation of genetic lesions over time results in a phenotype more consistent with transformed cells [15, 18, 22]. Therefore, we tested the effects of aspirin on clones of the MCF-10A cell line that concurrently carried activating mutations in both *KRAS* (G12V) and *PIK3CA* (either exon 9 or exon 20) [18]. As expected, the combination of activated oncogenes resulted in a statistically significant decrease in cellular proliferation of up to a 40 % (Fig. 1). Interestingly, cells were not responsive to aspirin in the presence of mutant *KRAS* alone, albeit a small non-significant decrease was observed, thus indicating that mutant *PIK3CA* is required for aspirin

sensitivity (Fig. 1) and concomitant activating mutations in other genes enhance this effect. In the current study, mutations in both *PIK3CA* and *KRAS* are likely cooperating to promote more stable activation of the GSK3 β pathway. This effect is likely mediated by the RAS binding domain on *PIK3CA*, as we have previously demonstrated that inactivating mutations in this domain decreased the cooperative effects of the mutant *PIK3CA* and mutant *KRAS* [23].

Aspirin is a known inhibitor of PTGS1 and PTGS2, enzymes responsible for formation of prostanoids. In order to determine if inhibition of these enzymes resulted in the growth inhibitory effects that we observed, MCF-10A and DKI (Exon 9/*KRAS*) cells were treated with the PTGS2 inhibitor celecoxib, at a physiological dose of 10 μ M and toxic dose of 25 μ M [24]. Neither cellular clone was growth inhibited by 10 μ M celecoxib, but both clones were equally growth inhibited at 25 μ M (Fig. 2), suggesting that observed differences in cell number following aspirin treatment were not a result of PTGS2 inhibition.

Next, we used FACS to determine if the decrease in cellular proliferation was cytostatic or cytotoxic. As seen in Fig. 3, parental MCF-10A cells were unaffected by aspirin treatment. However, a dose-dependent, statistically significant increase in cytotoxicity was observed in DKI cells (relative to aspirin-free control cells), as indicated by the increase in propidium iodide staining.

Fig. 1 Effect of aspirin on parental (MCF-10A), individual knockin mutations of either *PIK3CA*^{H1047R}, *PIK3CA*^{E545K}, *KRAS*^{G12V}, and double mutant knock-ins of *KRAS*^{G12V} with either *PIK3CA*^{H1047R} or *PIK3CA*^{E545K} clones of MCF-10A were treated with varying doses of aspirin for 72 h. Parental and clones of MCF-10A were seeded on day 0 in EGF-free media. Following 24 h, the media were replaced with assay media containing 0.2 ng/mL EGF and supplemented with 0–4 mM aspirin. Cells were counted after 72 h. Values for cells treated with 2, 3, and 4 mM aspirin were normalized to the respective 0 mM aspirin value for that particular cell line. Values represent mean \pm SEM (* P < 0.05, n = 4)

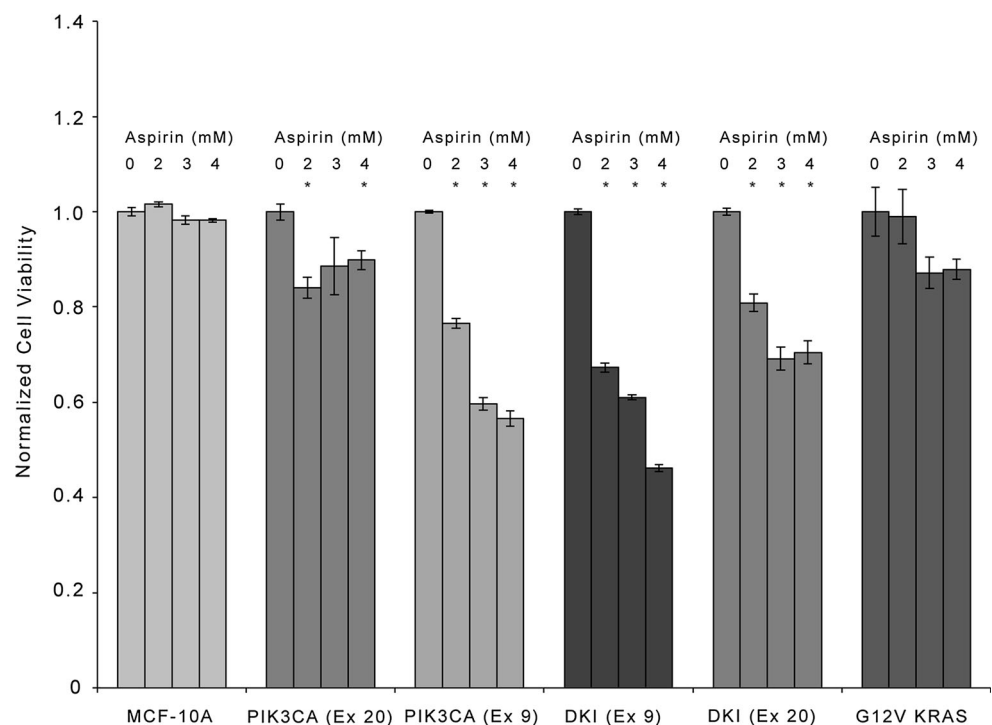


Fig. 2 Effect of celecoxib on parental (MCF-10A; *white*) and double mutant knock-ins of *KRAS*^{G12V} with *PIK3CA*^{E545K} (*black*) clones of MCF-10A were treated with various concentrations of celecoxib for 72 h. Parental MCF-10A and clone of MCF-10A were seeded on day 0 in EGF-free media. Following 24 h, the media were replaced with assay media containing 0.2 ng/mL EGF and supplemented with 0, 10 or 25 μ M celecoxib. Cells were counted after 72 h. Cell values for 10 or 25 μ M celecoxib were normalized to the respective 0 mM celecoxib value for that particular cell line. *Values* represent mean \pm SD ($*P < 0.05$, $n = 3$)

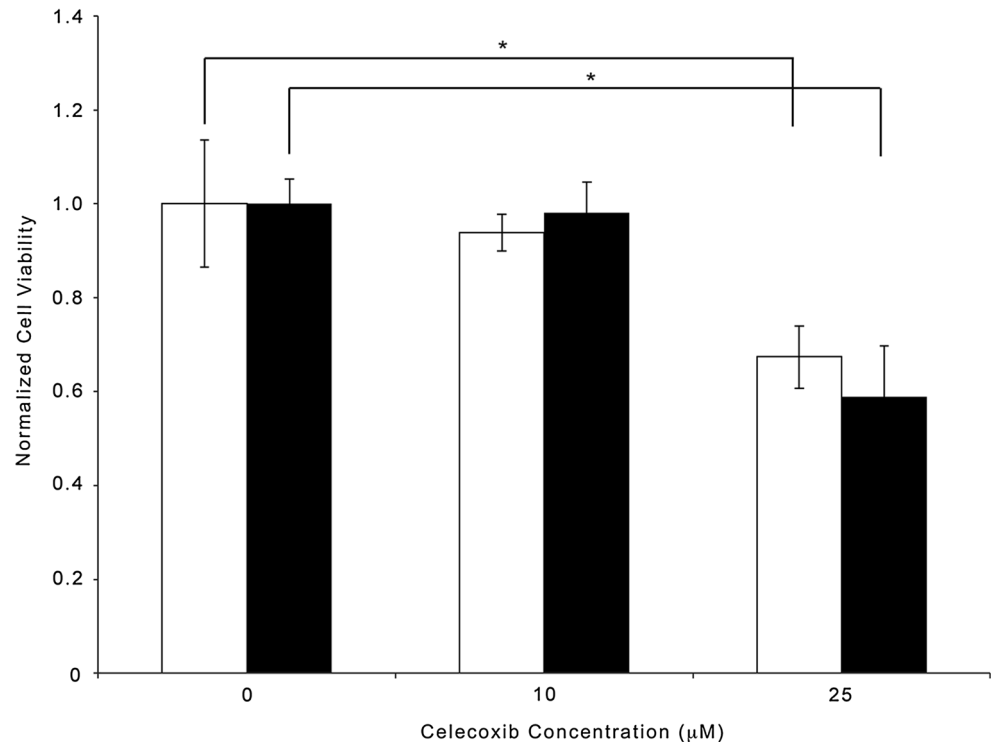
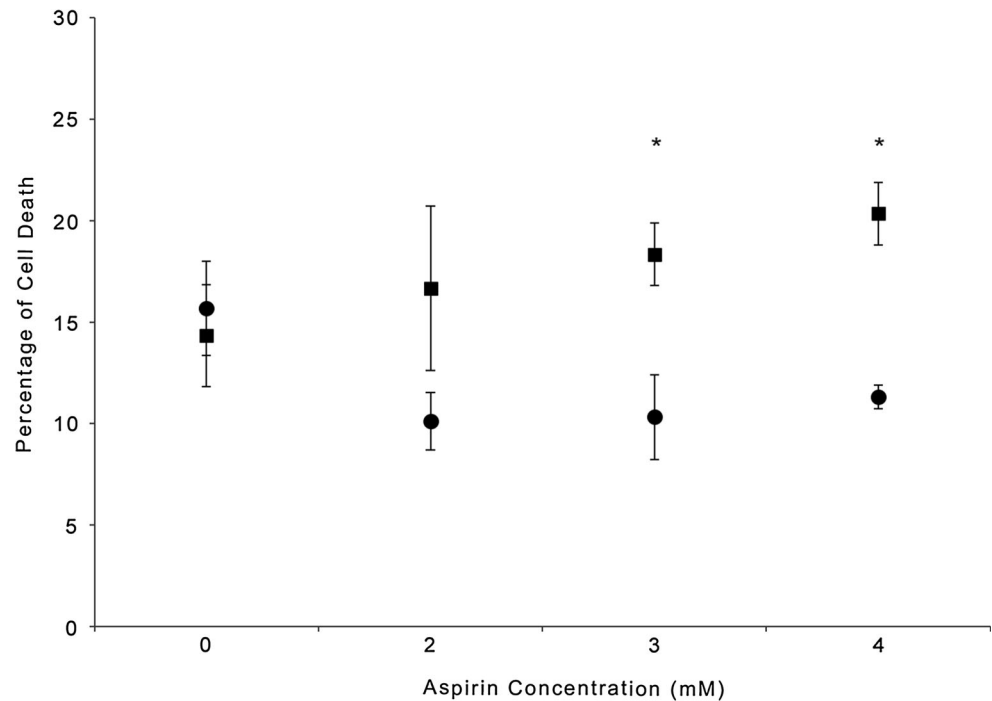


Fig. 3 Cytotoxic effect of aspirin on parental MCF-10A and MCF-10A DKI (Exon 9/*KRAS*) cells. Parental (*circle*) and DKI (*square*) were seeded on day 0 in EGF-free media for 24 h. The media were then replaced with assay media containing 0.2 ng/mL EGF and supplemented with 0–4 mM aspirin. Cytotoxicity was evaluated after 72 h by measuring uptake of propidium iodide by FACS analysis. *Values* represent mean \pm SD ($*P < 0.05$, $n = 3$)



Aspirin alters cellular signaling in mutant *PIK3CA*-containing cells

Next, we attempted to define a mechanism of aspirin's action in mutant *PIK3CA*-containing cells. Therefore, we

turned to a reverse phase protein array (RPPA) to identify proteins that are differentially expressed following aspirin treatment and may account for the cytotoxicity observed in DKI cells. The values of aspirin-treated cells provided by RPPA analysis for each cell line were normalized to the

respective untreated cells. Proteins were ranked based upon the normalized value. The top two proteins were phosphorylated GSK3 β (Ser9) and Src (Tyr 416) in aspirin-treated DKI cells, relative to aspirin-free control DKI cells. However, these protein marks were not differentially expressed in parental MCF-10A cells (Supplementary Fig. 1). In order to validate the results of our RPPA analysis, we explored the levels of phosphorylated GSK3 β (Ser9) and Src (Tyr 416) proteins in parental MCF-10A and DKI cells in the presence and absence of aspirin by Western blot analysis. As shown in Fig. 4, GSK3 β was significantly more phosphorylated in aspirin-treated DKI cells (Ex 9_KRAS) relative to aspirin-free control cells. However, the phosphorylation of GSK3 β in parental MCF-10A cells was unaltered by aspirin treatment. Densitometry on three independent blots was performed for each cell line with or without treatment. The intensity of phosphorylated GSK3 β was normalized to TUBB for each sample. The ratio of aspirin-treated to untreated cells was 1.03 ± 0.066 for the parental MCF-10A cell line, while the ratio for DKI cells was 1.37 ± 0.074 . Student's *t* test revealed a statistically significant increase in phosphorylated GSK3 β in DKI cells, but not parental MCF-10A cells ($P < 0.01$ and $P < 0.5$, respectively). Interestingly, quantification of Western blots of single mutant KRAS and single mutant PIK3CA cell lines revealed that phosphorylation of GSK3 β in each of these clones were unaltered by aspirin treatment (1.1 ± 0.12 , $P = 0.2$ and 0.96 ± 0.17 , $P = 0.9$,

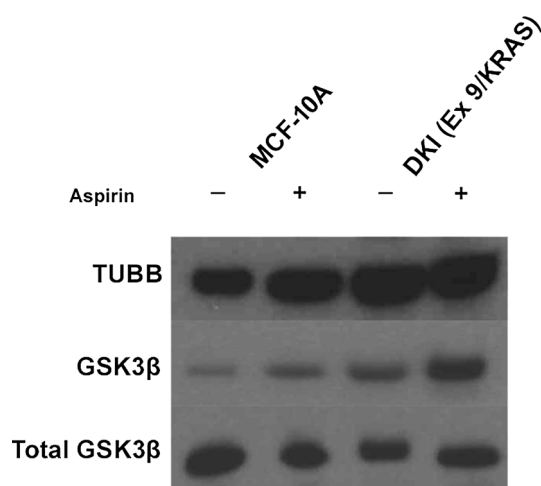


Fig. 4 Aspirin treatment results in increased phosphorylation of GSK3 β in a mutant PIK3CA-dependent manner. Western blot analysis was performed on parental MCF-10A and DKI (Ex 9_KRAS) clones following aspirin treatment against phosphorylated GSK3 β (Ser 9) and total GSK3 β . TUBB was used as a loading control. Western blot analysis revealed a statistically significant increase ($P < 0.05$) in the levels of phosphorylated GSK3 β following 4 mM aspirin treatment in DKI cells in comparison to non-treated controls and parental MCF-10A cells, regardless of treatment. Blot is representative of three independent experiments

respectively) ($n = 4$). Similarly, aspirin treatment of DKI (Ex 20_KRAS) cells did not result in a statistically significant change in GSK3 β phosphorylation (0.93 ± 0.16 , $P = 0.7$), indicating that aspirin's effect on GSK3 β is specific to cells carrying mutations in the helical domain of PIK3CA. Although RPPA analysis revealed an increase in phosphorylated Src (Tyr 416) following aspirin treatment, our Western blot analysis failed to validate this result (data not shown).

PIK3CA mutations sensitize breast cancer cells to aspirin treatment

In order to confirm that these findings were not limited to our non-tumorigenic cellular system, we looked to validate these findings in cancer cell lines. We obtained three commercially-available breast cancer cell lines that differed in the mutational status of PIK3CA. The MCF-7 cell line carries a mutation in PIK3CA (E545K), while MDA-MB-468 and MDA-MB-436 are both homozygous for wild-type PIK3CA and KRAS [25–27]. Treatment with physiologic concentrations of aspirin resulted in a statistically significant decrease in cellular proliferation in MCF-7, but not MDA-MB-468 or MDA-MB-436 cells, once again indicating the need for mutations in PIK3CA (Fig. 5). Western blot analysis revealed that aspirin treatment of MCF-7 cells, but not MDA-MB-436 or MDA-MB-468, resulted in a statistically significant increase in GSK3 β phosphorylation (Fig. 6).

Discussion

In this study, we set out to determine if mutations in PIK3CA sensitized breast cancer cells to physiologic doses of aspirin, as determined by the range of basal serum aspirin levels previously described [28, 29]. We initially observed only a modest effect of aspirin on cells carrying mutant PIK3CA alone. However, we and others have previously demonstrated that an accumulation of genetic insults are necessary to observe changes more consistent with a transformed phenotype [18, 30]. Consistent with this, we observed a more dramatic reduction in the growth of DKI cells, which carry mutations in KRAS, plus a mutation in PIK3CA in either exon 9 or exon 20, relative to clones carrying mutant PIK3CA alone following treatment with aspirin (Fig. 1). In the current study, we used aspirin concentrations as high as 4 mM. However, it is important to note that lower daily aspirin doses may exhibit a more modest effect on proliferation and apoptosis. Nonetheless, others have reported that it is possible to safely reach serum levels of aspirin as high as 10 mM, indicating that our

Fig. 5 Aspirin effect on growth of MCF-7, MDA-MB-468, and MDA-MB-436 cancer cell lines. Cell lines were seeded on day 0. Following 24 h, aspirin (4 mM; *black*) or no aspirin (*white*) was added to the assay media. Following 72 h, cells were counted. Cell values for 4 mM aspirin, for each cell line, were normalized to the 0 mM aspirin value for that particular cell line. Cell death due to addition of aspirin was observed in the MCF-7 cell line. MDA-MB-468 and MDA-MB-436 cells did not respond to aspirin treatment. Values represent mean \pm SD (* $P < 0.05$, $n = 6$)

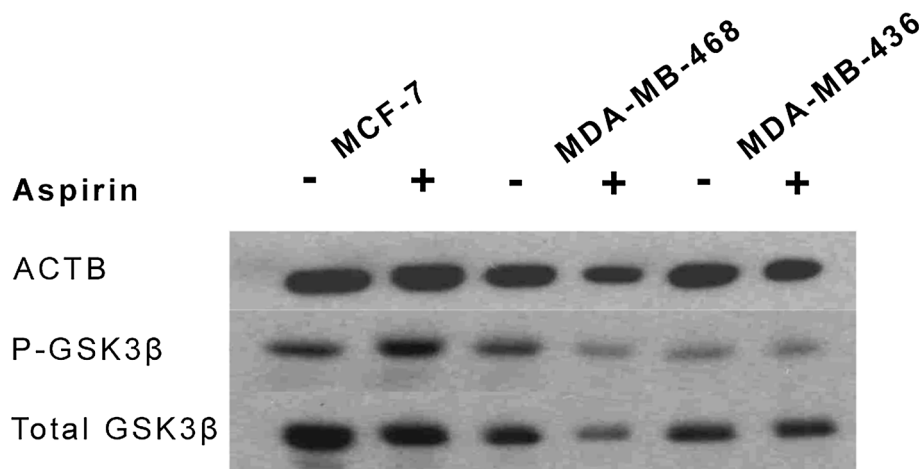
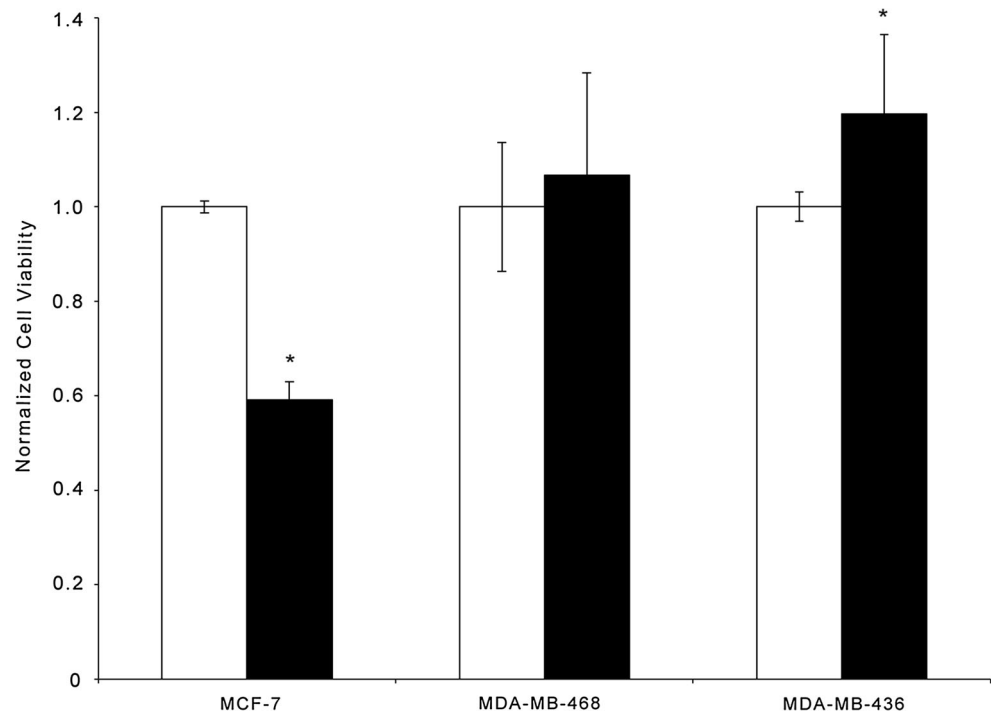


Fig. 6 Breast cancer cells expression of GSK3β via Western blot. MCF-7, MDA-MB-468, and MDA-MB-436 cells were seeded on day 0. Next day, the media were replaced, and cells were treated with 4 mM aspirin or no aspirin. After 48 h, media and aspirin were replenished. The next day, cells were harvested in Laemmli buffer, and Western blot was performed to probe for phosphorylated GSK3β

(Ser 9) and total GSK3β. ACTB was used as a loading control. Western blot analysis demonstrated that levels of phosphorylated GSK3β following aspirin (4 mM) treatment in MCF-7 cells were slightly increased in comparison to non-treated samples. *Figure* is representative of three independent experiments

findings are clinically significant, as they are likely safe and feasible [31].

PIK3CA is the most frequently mutated oncogene in breast cancer. While *KRAS* is infrequently mutated in breast cancers, *KRAS* can signal through *PIK3CA*, it is plausible that aberrant *PIK3CA* activation could occur through mutations in *KRAS* that could sensitize cells to aspirin. However, the study by Liao et al. failed to find an

association between aspirin response and mutations in *KRAS*, as only mutations in *PIK3CA* were associated with a better response to aspirin [11]. Although *KRAS* is mutated in 42 % of colorectal cancers, only 14 % of samples also carry a mutation in *PIK3CA* [32, 33]. Thus, it is possible that the sample size was insufficient to capture an association with mutations in both *PIK3CA* and *KRAS*. Another explanation may be that the requirement of mutant

KRAS toward aspirin sensitivity can be substituted with genetic alterations in other genes. Therefore, when KRAS is wild type, genetic alterations in other genes can suffice.

Molecular pathologic epidemiology (MPE) [34, 35] performed by the Liao et al. study reported a strong association in aspirin response in patients whose cancers carried mutations in PIK3CA. Similarly, we report that mutations in PIK3CA are required for aspirin sensitivity. This requirement was further validated by the breast cancer cell lines in our study, as only MCF-7 cells, which carry a mutation in PIK3CA responded to aspirin treatment. MCF-7 cells carry wild-type KRAS but do carry several other genetic alterations, one or more of which provide the oncogenic stimulus necessary to replace mutant KRAS. Taken together, we concluded that multiple oncogenic insults were required to observe the growth inhibitory effects of aspirin, but a mutation in *PIK3CA* is necessary, as cellular clones carrying mutant *KRAS* alone were not responsive to aspirin treatment.

Previous retrospective analyses investigating aspirin use in colorectal cancers in the context of mutant *PIK3CA* status did not report where *PIK3CA* was mutated [11, 12]. Thus, the potential differences in phenotype across *PIK3CA* mutations were not evaluated in these studies. Mutations in *PIK3CA* occur primarily in two hotspot regions located in either the helical (exon 9) or the kinase (exon 20) domains. In our studies, clones carrying a single mutation at exon 20 were less sensitive to aspirin than exon 9 mutants. Similarly, exon 9 DKI cells were more sensitive to aspirin than exon 20 DKI cells, with DKI cells exhibiting increased sensitivity relative to their respective single *PIK3CA* mutant clones. These mutations are reported to have similar consequences, but fundamental differences have been reported [36, 37]. The difference in aspirin sensitivity between mutant exon 9 or exon 20 clones may be attributed to differences in the aberrant activation of signaling pathways. A recent study by Blair et al. demonstrated differential phosphorylation patterns between cell lines harboring mutations in either exon 9 or exon 20 [37]. The authors reported that growth signaling in cells carrying mutations in exon 20 was largely dependent on ERBB3 phosphorylation for complete pathway activation, while cellular clones with mutations in exon 9 were not as affected by ERBB3 phosphorylation. However, a mutation in exon 9 resulted in the increased phosphorylation of a number of peptides not found to be activated in mutant exon 20 clones.

A large body of literature has reported exploring the cytotoxicity of aspirin in breast cancer cells [38–40]. However, several mechanisms have been implicated. Using MCF-7 breast cancer cells, Choi et al. [38] reported that aspirin-induced apoptosis was the result of changes in Bcl-2 protein expression [38]. A separate study using neuro-2a

cells showed that aspirin-induced apoptosis was caused by a decrease in proteasome activity [39], while Yan et al. [40] observed that aspirin-induced apoptosis in MDA-MB-453 breast cancer cells was triggered by an increase in caspase-3 expression [40]. Thus, several different mechanisms of action have been proposed. Moreover, to date, no study has explored clinical outcomes in breast cancer in the context of any specific mutational backgrounds. In our study, RPPA analysis revealed that aspirin treatment resulted in significantly increased phosphorylation of GSK3 β in DKI cells, but not parental MCF-10A cells relative to aspirin-free controls. GSK3 β has been implicated in cellular growth signaling and is a downstream target of the PI3K-AKT pathway [41, 42]. However, the role of GSK3 β in cancer has been controversial, as phosphorylation has been reported as inhibitory and activating [17, 43, 44]. Further support for the latter comes from Wang et al. [43], which demonstrated that GSK3 β has been shown to support the growth of MLL leukemia cells [43]. We have previously reported that mutations in *PIK3CA* resulted in hyperphosphorylation of GSK3 β , which sensitized cells to pharmacologic inhibition of GSK3 β [17, 45]. Indeed, in the current study, we did observe an increase in basal levels of phosphorylated GSK3 β in DKI cells, which was further augmented following aspirin treatment, as indicated by Western blot analysis. Moreover, this increase in phosphorylated GSK3 β led to increased cytotoxicity, as reported by flow cytometry and a concomitant decrease in cell number.

The role of GSK3 β in the response to aspirin remains elusive. One explanation may be increased stability of phosphorylated GSK3 β . Others have reported that colorectal cancer cells treated with aspirin experienced increased phosphorylation of GSK3 β and β -catenin but did not explore the association with the mutational status of *PIK3CA* [46]. This same group suggests that aspirin treatment may stabilize serine/threonine phosphorylations through the inhibition of a specific phosphatase but were unable to determine which phosphatase was being inhibited. Later work by Bos et al. reported that aspirin treatment resulted in increased phosphorylation of the phosphatase PP2A, which results in its inactivation [47]. PP2A has been shown to dephosphorylate both GSK3 β and β -catenin and inactivation of PP2A could result in stable phosphorylation of both proteins. Mutations in *PIK3CA* result in aberrant phosphorylation of AKT, mTOR, and GSK3 β [17]. Thus, mutations in *PIK3CA* coupled with inactivation of PP2A by aspirin may lead to even more durable phosphorylation of GSK3 β . Others have reported that phosphorylation of GSK3 β results in growth inhibition of cells [48]. Therefore, elevated GSK3 β phosphorylation from aberrant *PIK3CA* signaling coupled with inhibition of the PP2A phosphatase may be leading to a

long-lasting inhibitory phosphorylation signal on GSK3 β , which may be the cause of aspirin sensitivity in mutant PIK3CA-containing cells.

In addition to aspirin's anti-proliferative properties, other effects on tumor progression have been reported. Maity et al. shows that pretreatment of MCF-7 and MDA-MB-231 cells with aspirin caused a decrease in cell migration, which was irreversible [49]. The study by Lloyd et al. reported that aspirin suppresses cell adhesion and cell motility in the prostate cancer cell line PC-3 [50]. Lastly, aspirin treatment decreased the invasiveness of the human cervical cancer cell line Hela [51]. Although these properties are hallmarks of cancer, none of these studies show an association with mutant PIK3CA status, as all of these cell lines only carry wild-type PIK3CA, with the exception of MCF-7. However, in the study by Maity et al., both mutant PIK3CA-containing and wild-type PIK3CA-containing cells exhibited the same phenotype. Therefore, it is unlikely that mutant PIK3CA is a factor in these other aspirin-induced properties.

MDA-MB-468 cells carry wild-type PIK3CA and did not respond to aspirin. Of note, these cells harbor a mutation in the tumor suppressor gene *PTEN*. Some studies have suggested that loss of *PTEN* is equivalent to harboring a mutation in *PIK3CA*; however, we and others have shown that genetic insults to these two genes result in different phenotypes [45]. *PTEN* loss has been shown to result in aberrant AKT phosphorylation [52]. Thus, since both MDA-MB-468 and MDA-MB-436 cells do not express functional *PTEN* and are insensitive to aspirin, this implies that the sensitivity to aspirin is an AKT-independent mechanism. Our study suggests that cancer cells carrying mutations in *PTEN*, but not *PIK3CA*, will not respond to aspirin.

The current study highlights how isogenic, genetically clean, non-tumorigenic cell lines can be used as a powerful tool for elucidating drug response. Herein, we provide the first evidence that mutations in *PIK3CA* sensitize breast cancer cells to aspirin therapy. Furthermore, our results implicate hyperphosphorylation of GSK3 β as a possible mechanism and a biomarker that could predict the best responders to aspirin therapy. Taken together with the results of previous retrospective analyses of colorectal cancer specimens, these findings highlight the possibility that aspirin could be used as an adjuvant therapy or a preventive agent. Our next step will be to validate these findings in a retrospective study evaluating breast cancer specimens from patients carrying mutations in *PIK3CA* who used daily aspirin therapy.

Acknowledgments The authors would like to thank Rush University Medical Center and Rush University Cancer Center. We would like to thank Dr. Faraz Bishehsari (RUMC) for thoughtful comments

and discussions. This study was supported by the generous support of the Pink Pumpkin Foundation.

Authors' Contributions Conception and design were performed by SBT, CER, MAC, and AMA. The development of methodology was performed by SBT, MSN, and AMA. Acquisition of data was performed by SBT, MSN, CER, and AMA. Analysis and interpretation of data were performed by SBT and AMA. Writing, review, and/or revision of manuscript were performed by all authors. CER provided material support.

Compliance with ethical standards

Conflict of Interest CR is currently an employee of Sarepta Therapeutics; however, all work reported here was conducted during his faculty appointment at Rush University. No other relevant conflicts of interest to report.

Ethical Standards The authors declare that the experiments performed in the current publication comply with the current laws of the United States of America.

Open Access This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International License (<http://creativecommons.org/licenses/by-nc/4.0/>), which permits any noncommercial use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

References

1. What are the key statistics about breast cancer? (2012) <http://www.cancer.org/Cancer/BreastCancer/DetailedGuide/breast-cancer-key-statistics>
2. Fraser DM, Sullivan FM, Thompson AM, McCowan C (2014) Aspirin use and survival after the diagnosis of breast cancer: a population-based cohort study. *Br J Cancer* 111(3):623–627. doi:10.1038/bjc.2014.264
3. Agrawal A, Fentiman IS (2008) NSAIDs and breast cancer: a possible prevention and treatment strategy. *Int J Clin Pract* 62(3):444–449. doi:10.1111/j.1742-1241.2007.01668.x
4. Din FV, Valanciute A, Houde VP, Zibrova D, Green KA, Sakamoto K, Alessi DR, Dunlop MG (2012) Aspirin inhibits mTOR signaling, activates AMP-activated protein kinase, and induces autophagy in colorectal cancer cells. *Gastroenterology* 142(7):1504–1515
5. Pasche B, Wang M, Pennison M, Jimenez H (2014) Prevention and treatment of cancer with aspirin: where do we stand? *Semin Oncol* 41(3):397–401
6. Harris RE, Chlebowski RT, Jackson RD, Frid DJ, Ascenseo JL, Anderson G, Loar A, Rodabough RJ, White E, McTiernan A, Women's Health I (2003) Breast cancer and nonsteroidal anti-inflammatory drugs: prospective results from the Women's Health Initiative. *Cancer Res* 63(18):6096–6101
7. Sharpe CR, Collet JP, McNutt M, Belzile E, Boivin JF, Hanley JA (2000) Nested case-control study of the effects of non-steroidal anti-inflammatory drugs on breast cancer risk and stage. *Br J Cancer* 83(1):112–120
8. Sutcliffe P, Connock M, Gurung T, Freeman K, Johnson S, Ngianga-Bakwin K, Grove A, Gurung B, Morrow S, Stranges S, Clarke A (2013) Aspirin in primary prevention of cardiovascular

- disease and cancer: a systematic review of the balance of evidence from reviews of randomized trials. *PLoS One* 8(12):e81970
9. Rothwell PM, Wilson M, Price JF, Belch JFF, Meade TW, Mehta Z (2012) Effect of daily aspirin on risk of cancer metastasis: a study of incident cancers during randomised controlled trials. *Lancet* 379(9826):1591–1601. doi:[10.1016/s0140-6736\(12\)60209-8](https://doi.org/10.1016/s0140-6736(12)60209-8)
 10. Holmes MD, Chen WY, Li L, Hertzmark E, Spiegelman D, Hankinson SE (2010) Aspirin intake and survival after breast cancer. *J Clin Oncol* 28(9):1467–1472. doi:[10.1200/JCO.2009.22.7918](https://doi.org/10.1200/JCO.2009.22.7918)
 11. Liao X, Lochhead P, Nishihara R, Morikawa T, Kuchiba A, Yamauchi M, Imamura Y, Qian ZR, Baba Y, Shima K, Sun R, Nosho K, Meyerhardt JA, Giovannucci E, Fuchs CS, Chan AT, Ogino S (2012) Aspirin use, tumor PIK3CA mutation, and colorectal-cancer survival. *N Engl J Med* 367(17):1596–1606. doi:[10.1056/NEJMoa1207756](https://doi.org/10.1056/NEJMoa1207756)
 12. Domingo E, Church DN, Sieber O, Ramamoorthy R, Yanagisawa Y, Johnstone E, Davidson B, Kerr DJ, Tomlinson IP, Midgley R (2013) Evaluation of PIK3CA mutation as a predictor of benefit from nonsteroidal anti-inflammatory drug therapy in colorectal cancer. *J Clin Oncol* 31(34):4297–4305. doi:[10.1200/JCO.2013.50.0322](https://doi.org/10.1200/JCO.2013.50.0322)
 13. Fuchs CS, Ogino S (2013) Aspirin therapy for colorectal cancer with PIK3CA mutation: simply complex! *J Clin Oncol* 31(34):4358–4361. doi:[10.1200/JCO.2013.52.0080](https://doi.org/10.1200/JCO.2013.52.0080)
 14. Isakoff SJ, Engelman JA, Irie HY, Luo J, Brachmann SM, Pearlman RV, Cantley LC, Brugge JS (2005) Breast cancer-associated PIK3CA mutations are oncogenic in mammary epithelial cells. *Cancer Res* 65(23):10992–11000. doi:[10.1158/0008-5472.CAN-05-2612](https://doi.org/10.1158/0008-5472.CAN-05-2612)
 15. Beaver JA, Gustin JP, Yi KH, Rajpurohit A, Thomas M, Gilbert SF, Rosen DM, Ho Park B, Lauring J (2013) PIK3CA and AKT1 mutations have distinct effects on sensitivity to targeted pathway inhibitors in an isogenic luminal breast cancer model system. *Clin Cancer Res* 19(19):5413–5422. doi:[10.1158/1078-0432.CCR-13-0884](https://doi.org/10.1158/1078-0432.CCR-13-0884)
 16. Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, Yan H, Gazdar A, Powell SM, Riggins GJ, Willson JK, Markowitz S, Kinzler KW, Vogelstein B, Velculescu VE (2004) High frequency of mutations of the PIK3CA gene in human cancers. *Science* 304(5670):554
 17. Gustin JP, Karakas B, Weiss MB, Abukhdeir AM, Lauring J, Garay JP, Cosgrove D, Tamaki A, Konishi H, Konishi Y, Mohseni M, Wang G, Rosen DM, Denmeade SR, Higgins MJ, Vitolo MI, Bachman KE, Park BH (2009) Knockin of mutant PIK3CA activates multiple oncogenic pathways. *Proc Natl Acad Sci USA* 106(8):2835–2840
 18. Wang GM, Wong HY, Konishi H, Blair BG, Abukhdeir AM, Gustin JP, Rosen DM, Denmeade SR, Rasheed Z, Matsui W, Garay JP, Mohseni M, Higgins MJ, Cidado J, Jelovac D, Croessmann S, Cochran RL, Karnan S, Konishi Y, Ota A, Hosokawa Y, Argani P, Lauring J, Park BH (2013) Single copies of mutant KRAS and mutant PIK3CA cooperate in immortalized human epithelial cells to induce tumor formation. *Cancer Res* 73(11):3248–3261. doi:[10.1158/0008-5472.CAN-12-1578](https://doi.org/10.1158/0008-5472.CAN-12-1578)
 19. Kueng W, Silber E, Eppenberger U (1989) Quantification of cells cultured on 96-well plates. *Anal Biochem* 182(1):16–19
 20. Garay JP, Karakas B, Abukhdeir AM, Cosgrove DP, Gustin JP, Higgins MJ, Konishi H, Konishi Y, Lauring J, Mohseni M, Wang GM, Jelovac D, Weeraratna A, Sherman Baust CA, Morin PJ, Toubaji A, Meeker A, De Marzo AM, Lewis G, Subhawong A, Argani P, Park BH (2012) The growth response to androgen receptor signaling in ERalpha-negative human breast cells is dependent on p21 and mediated by MAPK activation. *Breast Cancer Res* 14(1):R27. doi:[10.1186/bcr3112](https://doi.org/10.1186/bcr3112)
 21. Imbalzano KM, Tatarkova I, Imbalzano AN, Nickerson JA (2009) Increasingly transformed MCF-10A cells have a progressively tumor-like phenotype in three-dimensional basement membrane culture. *Cancer Cell Int* 9:7. doi:[10.1186/1475-2867-9-7](https://doi.org/10.1186/1475-2867-9-7)
 22. Vogelstein B, Kinzler KW (2004) Cancer genes and the pathways they control. *Nat Med* 10(8):789–799. doi:[10.1038/nm1087](https://doi.org/10.1038/nm1087)
 23. Wang GM, Wong HY, Konishi H, Blair BG, Abukhdeir AM, Gustin JP, Rosen DM, Denmeade SR, Rasheed Z, Matsui W, Garay JP, Mohseni M, Higgins MJ, Cidado J, Jelovac D, Croessmann S, Cochran RL, Karnan S, Konishi Y, Ota A, Hosokawa Y, Argani P, Lauring J, Park BH (2013) Single copies of mutant KRAS and mutant PIK3CA cooperate in immortalized human epithelial cells to induce tumor formation. *Cancer Res* 73(11):3248–3261. doi:[10.1158/0008-5472.CAN-12-1578](https://doi.org/10.1158/0008-5472.CAN-12-1578)
 24. Lev-Ari S, Kazanov D, Liberman E, Ben-Yosef R, Arber N (2007) Down-regulation of PGE2 by physiologic levels of celecoxib is not sufficient to induce apoptosis or inhibit cell proliferation in human colon carcinoma cell lines. *Dig Dis Sci* 52(4):1128–1133. doi:[10.1007/s10620-006-9619-x](https://doi.org/10.1007/s10620-006-9619-x)
 25. Yunokawa M, Koizumi F, Kitamura Y, Katanasaka Y, Okamoto N, Kodaira M, Yonemori K, Shimizu C, Ando M, Masutomi K, Yoshida T, Fujiwara Y, Tamura K (2012) Efficacy of everolimus, a novel mTOR inhibitor, against basal-like triple-negative breast cancer cells. *Cancer Sci* 103(9):1665–1671. doi:[10.1111/j.1349-7006.2012.02359.x](https://doi.org/10.1111/j.1349-7006.2012.02359.x)
 26. Eckert LB, Repasky GA, Ulku AS, McFall A, Zhou H, Sartor CI, Der CJ (2004) Involvement of Ras activation in human breast cancer cell signaling, invasion, and anoikis. *Cancer Res* 64(13):4585–4592. doi:[10.1158/0008-5472.CAN-04-0396](https://doi.org/10.1158/0008-5472.CAN-04-0396)
 27. Kopp F, Wagner E, Roidl A (2014) The proto-oncogene KRAS is targeted by miR-200c. *Oncotarget* 5(1):185–195. doi:[10.18632/oncotarget.1427](https://doi.org/10.18632/oncotarget.1427)
 28. Pathi S, Jutooru I, Chadalapaka G, Nair V, Lee SO, Safe S (2012) Aspirin inhibits colon cancer cell and tumor growth and down-regulates specificity protein (Sp) transcription factors. *PLoS One* 7(10):e48208. doi:[10.1371/journal.pone.0048208](https://doi.org/10.1371/journal.pone.0048208)
 29. Lares-Asseff I, Flores-Perez J, Juarez-Olguin H, Ramirez-Lacayo M, Loredo-Abdala A, Carbajal-Rodriguez L (1999) Influence of nutritional status on the pharmacokinetics of acetylsalicylic acid and its metabolites in children with autoimmune disease. *Am J Clin Nutr* 69(2):318–324
 30. Fearon ER, Vogelstein B (1990) A genetic model for colorectal tumorigenesis. *Cell* 61(5):759–767
 31. Juarez Olguin H, Flores Perez J, Lares Asseff I, Loredo Abdala A, Carbajal Rodriguez L (2004) Comparative pharmacokinetics of acetyl salicylic acid and its metabolites in children suffering from autoimmune diseases. *Biopharm Drug Dispos* 25(1):1–7. doi:[10.1002/bdd.379](https://doi.org/10.1002/bdd.379)
 32. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, Antipin Y, Reva B, Goldberg AP, Sander C, Schultz N (2012) The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov* 2(5):401–404. doi:[10.1158/2159-8290.CD-12-0095](https://doi.org/10.1158/2159-8290.CD-12-0095)
 33. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, Cerami E, Sander C, Schultz N (2013) Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal* 6(269):pl1. doi:[10.1126/scisignal.2004088](https://doi.org/10.1126/scisignal.2004088)
 34. Ogino S, Chan AT, Fuchs CS, Giovannucci E (2011) Molecular pathological epidemiology of colorectal neoplasia: an emerging transdisciplinary and interdisciplinary field. *Gut* 60(3):397–411. doi:[10.1136/gut.2010.217182](https://doi.org/10.1136/gut.2010.217182)
 35. Ogino S, Lochhead P, Giovannucci E, Meyerhardt JA, Fuchs CS, Chan AT (2014) Discovery of colorectal cancer PIK3CA

- mutation as potential predictive biomarker: power and promise of molecular pathological epidemiology. *Oncogene* 33(23): 2949–2955. doi:[10.1038/onc.2013.244](https://doi.org/10.1038/onc.2013.244)
36. Day FL, Jorissen RN, Lipton L, Mouradov D, Sakthianandeswaren A, Christie M, Li S, Tsui C, Tie J, Desai J, Xu ZZ, Molloy P, Whitehall V, Leggett BA, Jones IT, McLaughlin S, Ward RL, Hawkins NJ, Ruzskiewicz AR, Moore J, Busam D, Zhao Q, Strausberg RL, Gibbs P, Sieber OM (2013) PIK3CA and PTEN gene and exon mutation-specific clinicopathologic and molecular associations in colorectal cancer. *Clin Cancer Res* 19(12):3285–3296. doi:[10.1158/1078-0432.CCR-12-3614](https://doi.org/10.1158/1078-0432.CCR-12-3614)
 37. Blair BG, Wu X, Zahari MS, Mohseni M, Cidado J, Wong HY, Beaver JA, Cochran RL, Zabransky DJ, Croessmann S, Chu D, Toro PV, Cravero K, Pandey A, Park BH (2015) A phosphoproteomic screen demonstrates differential dependence on HER3 for MAP kinase pathway activation by distinct PIK3CA mutations. *Proteomics* 15(2–3):318–326. doi:[10.1002/pmic.201400342](https://doi.org/10.1002/pmic.201400342)
 38. Choi BH, Chakraborty G, Baek K, Yoon HS (2013) Aspirin-induced Bcl-2 translocation and its phosphorylation in the nucleus trigger apoptosis in breast cancer cells. *Exp Mol Med* 45:e47. doi:[10.1038/emmm.2013.91](https://doi.org/10.1038/emmm.2013.91)
 39. Dikshit P, Chatterjee M, Goswami A, Mishra A, Jana NR (2006) Aspirin induces apoptosis through the inhibition of proteasome function. *J Biol Chem* 281(39):29228–29235. doi:[10.1074/jbc.M602629200](https://doi.org/10.1074/jbc.M602629200)
 40. Yan F, He Q, Hu X, Li W, Wei K, Li L, Zhong Y, Ding X, Xiang S, Zhang J (2013) Direct regulation of caspase3 by the transcription factor AP2alpha is involved in aspirin induced apoptosis in MDAMB453 breast cancer cells. *Mol Med Rep* 7(3):909–914. doi:[10.3892/mmr.2013.1257](https://doi.org/10.3892/mmr.2013.1257)
 41. Endo H, Nito C, Kamada H, Yu F, Chan PH (2006) Akt/GSK3beta survival signaling is involved in acute brain injury after subarachnoid hemorrhage in rats. *Stroke* 37(8):2140–2146. doi:[10.1161/01.STR.0000229888.55078.72](https://doi.org/10.1161/01.STR.0000229888.55078.72)
 42. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378(6559):785–789. doi:[10.1038/378785a0](https://doi.org/10.1038/378785a0)
 43. Wang Z, Smith KS, Murphy M, Piloto O, Somerville TC, Cleary ML (2008) Glycogen synthase kinase 3 in MLL leukaemia maintenance and targeted therapy. *Nature* 455(7217):1205–1209. doi:[10.1038/nature07284](https://doi.org/10.1038/nature07284)
 44. Hoefflich KP, Luo J, Rubie EA, Tsao MS, Jin O, Woodgett JR (2000) Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. *Nature* 406(6791):86–90. doi:[10.1038/35017574](https://doi.org/10.1038/35017574)
 45. Higgins MJ, Beaver JA, Wong HY, Gustin JP, Lauring JD, Garay JP, Konishi H, Mohseni M, Wang GM, Cidado J, Jelovac D, Cosgrove DP, Tamaki A, Abukheir AM, Park BH (2011) PIK3CA mutations and EGFR overexpression predict for lithium sensitivity in human breast epithelial cells. *Cancer Biol Ther* 11(3):358–367
 46. Dihlmann S, Klein S, Doeberitz Mv M (2003) Reduction of beta-catenin/T-cell transcription factor signaling by aspirin and indomethacin is caused by an increased stabilization of phosphorylated beta-catenin. *Mol Cancer Ther* 2(6):509–516
 47. Bos CL, Kodach LL, van den Brink GR, Diks SH, van Santen MM, Richel DJ, Peppelenbosch MP, Hardwick JC (2006) Effect of aspirin on the Wnt/beta-catenin pathway is mediated via protein phosphatase 2A. *Oncogene* 25(49):6447–6456. doi:[10.1038/sj.onc.1209658](https://doi.org/10.1038/sj.onc.1209658)
 48. Kunnimalaiyaan M, Vaccaro AM, Ndiaye MA, Chen H (2007) Inactivation of glycogen synthase kinase-3beta, a downstream target of the raf-1 pathway, is associated with growth suppression in medullary thyroid cancer cells. *Mol Cancer Ther* 6(3):1151–1158. doi:[10.1158/1535-7163.MCT-06-0665](https://doi.org/10.1158/1535-7163.MCT-06-0665)
 49. Maity G, De A, Das A, Banerjee S, Sarkar S, Banerjee SK (2015) Aspirin blocks growth of breast tumor cells and tumor-initiating cells and induces reprogramming factors of mesenchymal to epithelial transition. *Lab Invest* 95(7):702–717. doi:[10.1038/labinvest.2015.49](https://doi.org/10.1038/labinvest.2015.49)
 50. Lloyd FP Jr, Slivova V, Valachovicova T, Sliva D (2003) Aspirin inhibits highly invasive prostate cancer cells. *Int J Oncol* 23(5):1277–1283
 51. Qin HX, Yang J, Cui HK, Li SP, Zhang W, Ding XL, Xia YH (2013) Synergistic antitumor activity of reversine combined with aspirin in cervical carcinoma in vitro and in vivo. *Cytotechnology* 65(4):643–653. doi:[10.1007/s10616-012-9520-8](https://doi.org/10.1007/s10616-012-9520-8)
 52. Vitolo MI, Weiss MB, Szmecinski M, Tahir K, Waldman T, Park BH, Martin SS, Weber DJ, Bachman KE (2009) Deletion of PTEN promotes tumorigenic signaling, resistance to anoikis, and altered response to chemotherapeutic agents in human mammary epithelial cells. *Cancer Res* 69(21):8275–8283. doi:[10.1158/0008-5472.CAN-09-1067](https://doi.org/10.1158/0008-5472.CAN-09-1067)