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

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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 GSK3β 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 PIK3CA in combination with KRAS
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

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), 0.5 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 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
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 aspirin. Camptothecin (2–100 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 µg/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 RRPA 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 Microvigene (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 (IC50) 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 GSK3β 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 μM and toxic dose of 25 μM [24]. Neither cellular clone was growth inhibited by 10 μM celecoxib, but both clones were equally growth inhibited at 25 μM (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.
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

![Graph](image1)

Fig. 2 Effect of celecoxib on parental (MCF-10A; *white*) and double mutant knock-ins of *KRAS*<sup>G12V</sup> with *PIK3CA*<sup>E545K</sup> (*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 μM celecoxib. Cells were counted after 72 h. Cell values for 10 or 25 μM celecoxib were normalized to the respective 0 mM celecoxib value for that particular cell line. Values represent mean ± SD (*P < 0.05, n = 3)

![Graph](image2)

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 ± SD (*P < 0.05, n = 3)
respective untreated cells. Proteins were ranked based upon the normalized value. The top two proteins were phosphorylated GSK3β (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 GSK3β (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, GSK3β was significantly more phosphorylated in aspirin-treated DKI cells (Ex 9_KRAS) relative to aspirin-free control cells. However, the phosphorylation of GSK3β 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 GSK3β 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 GSK3β 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 GSK3β 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, respectively) (n = 4). Similarly, aspirin treatment of DKI (Ex 20_KRAS) cells did not result in a statistically significant change in GSK3β phosphorylation (0.93 ± 0.16, P = 0.7), indicating that aspirin’s effect on GSK3β 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 GSK3β 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
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 GSK3β in DKI cells, but not parental MCF-10A cells relative to aspirin-free controls. GSK3β has been implicated in cellular growth signaling and is a downstream target of the PI3K-AKT pathway [41, 42]. However, the role of GSK3β 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 GSK3β has been shown to support the growth of MLL leukemia cells [43]. We have previously reported that mutations in PIK3CA resulted in hyperphosphorylation of GSK3β, which sensitized cells to pharmacologic inhibition of GSK3β [17, 45]. Indeed, in the current study, we did observe an increase in basal levels of phosphorylated GSK3β in DKI cells, which was further augmented following aspirin treatment, as indicated by Western blot analysis. Moreover, this increase in phosphorylated GSK3β led to increased cytotoxicity, as reported by flow cytometry and a concomitant decrease in cell number.

The role of GSK3β in the response to aspirin remains elusive. One explanation may be increased stability of phosphorylated GSK3β. Others have reported that colorectal cancer cells treated with aspirin experienced increased phosphorylation of GSK3β 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 GSK3β 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 GSK3β [17]. Thus, mutations in PIK3CA coupled with inactivation of PP2A by aspirin may lead to even more durable phosphorylation of GSK3β. Others have reported that phosphorylation of GSK3β results in growth inhibition of cells [48]. Therefore, elevated GSK3β phosphorylation from aberrant PIK3CA signaling coupled with inhibition of the PP2A phosphatase may be leading to a
long-lasting inhibitory phosphorylation signal on GSK3β, 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 GSK3β 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.

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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.

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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, Ascensio 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
mutation as potential predictive biomarker: power and promise of molecular pathological epidemiology. Oncogene 33(23): 2949–2955. doi: 10.1038/onc.2013.244

36. Day FL, Jorissen RN, Lipton L, Mouradov D, Sakhitarian-deswaren A, Christie M, Li S, Tsui C, Tae J, Desai J, Xu ZZ, Molloy P, Whitehall V, Leggett BA, Jones IT, McLaughlin S, Ward RL, Hawkins NJ, Ruszkiewicz 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

37. Blair BG, Wu X, Zahari MS, Mohseni M, Cidado J, Wong HY, Beaver JA, Cochran RL, Zabransky DJ, Crossmann 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

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/emm.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

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

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

42. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA (1995) Inhibition of glycosynthesath kinase-3 by insulin mediated by protein kinase B. Nature 378(6559):785–789. doi:10.1038/378785a0

43. Wang Z, Smith KS, Murphy M, Piloto O, Somervaille TC, Cleary ML (2008) Glycosynthesath kinase 3 in MLL leukaemia maintenance and targeted therapy. Nature 455(7217):1205–1209. doi:10.1038/nature07284

44. Hoeftich KP, Luo J, Rubie EA, Tsao MS, Jin O, Woodgett JR (2000) Requirement for glycosynthesath kinase-3beta in cell survival and NF-kappaB activation. Nature 406(6791):86–90. doi:10.1038/35017574

45. Higgins MJ, Beaver JA, Wong HY, Gustin JP, Lauring JD, Garay JP, Konishi H, Mohsien M, Wang GM, Cidado J, Jelovac D, Cosgrove DP, Tamaki A, Abukdeir 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, Doebertz 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

48. Kunnimalaiyaan M, Vaccaro AM, Ndiaye MA, Chen H (2007) Inactivation of glycosynthesath 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

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

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

52. Vitolo MI, Weiss MB, Szmaczinski 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