Upregulation of Cyclooxygenase-2/Prostaglandin E_2 (COX-2/PGE_2) Pathway Member Multiple Drug Resistance-Associated Protein 4 (MRP4) and Downregulation of Prostaglandin Transporter (PGT) and 15-Prostaglandin Dehydrogenase (15-PGDH) in Triple-Negative Breast Cancer

Tyler J. Kochel[1], Olga G. Goloubeva[1,2] and Amy M. Fulton[1,3,4]

[1]Marlene and Stewart Greenebaum Cancer Center, Baltimore, MD, USA. [2]Department of Epidemiology & Public Health, University of Maryland School of Medicine, Baltimore, MD, USA. [3]Department of Pathology, University of Maryland School of Medicine, Baltimore, MD, USA. [4]Baltimore Veterans Affairs Medical Center, Baltimore, MD, USA.

ABSTRACT: Elevated levels of cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PGE_2) are indicators of a poor prognosis in breast cancer. Using several independent publicly available breast cancer gene expression databases, we investigated other members of the PGE_2 pathway. PGE_2 is produced by COX-2 and actively exported by multiple drug resistance-associated protein 4 (MRP4) into the extracellular microenvironment, where PGE_2 can bind four cognate EP receptors (EP1–EP4) and initiate diverse biological signaling pathways. Alternatively, PGE_2 is imported via the prostaglandin transporter (PGT) and metabolized by 15-prostaglandin dehydrogenase (15-PGDH/HPGD). We made the novel observation that MRP4, PGT, and 15-PGDH are differentially expressed among distinct breast cancer molecular subtypes; this finding was confirmed in independent datasets. In triple-negative breast cancer, the observed gene expression pattern (high COX-2, high MRP4, low PGT, and low 15-PGDH) would favor high levels of tumor-promoting PGE_2 in the tumor microenvironment that may contribute to the overall poor prognosis of triple-negative breast cancer.

KEYWORDS: COX-2, PGE_2, MRP4, PGT, 15-PGDH, TNBC

CITATION: Kochel et al. Upregulation of Cyclooxygenase-2/Prostaglandin E_2 (COX-2/PGE_2) Pathway Member Multiple Drug Resistance-Associated Protein 4 (MRP4) and Downregulation of Prostaglandin Transporter (PGT) and 15-Prostaglandin Dehydrogenase (15-PGDH) in Triple-Negative Breast Cancer. *Breast Cancer: Basic and Clinical Research* 2016:10 61–70 doi:10.4137/BCBCR.S38529.

TYPE: Original Research

RECEIVED: January 7, 2016. **RESUBMITTED:** March 29, 2016. **ACCEPTED FOR PUBLICATION:** March 30, 2016.

ACADEMIC EDITOR: Goberdhan P. Dimri, Editor in Chief

PEER REVIEW: Five peer reviewers contributed to the peer review report. Reviewers' reports totaled 1830 words, excluding any confidential comments to the academic editor.

FUNDING: This study was funded by the Veterans Administration Merit Award (AMF) and the Department of Health and Human Services (AMF). The authors confirm that the funder had no influence over the study design, content of the article, or selection of this journal.

COMPETING INTERESTS: Authors disclose no potential conflicts of interest.

CORRESPONDENCE: tjkochel@gmail.com

Paper subject to independent expert single-blind peer review. All editorial decisions made by independent academic editor. Upon submission manuscript was subject to anti-plagiarism scanning. Prior to publication all authors have given signed confirmation of agreement to article publication and compliance with all applicable ethical and legal requirements, including the accuracy of author and contributor information, disclosure of competing interests and funding sources, compliance with ethical requirements relating to human and animal study participants, and compliance with any copyright requirements of third parties. This journal is a member of the Committee on Publication Ethics (COPE).

Published by Libertas Academica. Learn more about this journal.

Introduction

Breast cancer is a heterogeneous disease classified into subtypes based on histopathologic (protein) or gene expression profiles, which guide treatment decisions. Breast cancers are defined histologically by estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER2) expression. More recently, gene expression profiling has been used to classify breast cancer into distinct subtypes. Each of these subtypes, defined at the protein or mRNA expression level, exhibits different biological behavior and is associated with different outcomes.

Similar to other epithelial-derived tumors, elevated levels of cyclooxygenase-2 (COX-2) protein have been detected in aggressive breast cancer. COX-2 and its inflammatory mediator, prostaglandin E_2 (PGE_2), are well-established predictors of poor prognosis in these solid tumors.[1–3] Inhibition of COX-2 for other indications has shown some tumor-preventative effect; however, the risk of cardiovascular toxicity limits the pursuit of COX-2 inhibition as a chemo-preventative regimen. Therefore, elucidating the role of other components involved in the regulation of PGE_2 could reveal new therapeutic targets.

The cyclooxygenase enzymes (COX-1 and COX-2 encoded by PTGS1 and PTGS2, respectively) catalyze the rate-limiting step in the metabolism of arachidonic acid, resulting in the formation of prostaglandins.[3] Normal, homeostatic levels of prostaglandins are maintained by the constitutive expression and activity of COX-1 in many epithelial tissues. COX-2 expression is normally induced by inflammatory stimuli, but aberrant expression of COX-2 is often found in epithelial malignancies, including breast cancer.[1–3]

PGE_2 is the major prostaglandin found in the epithelial tumor microenvironment.[3] Extracellular PGE_2 binds four cognate EP receptors (EP1–EP4) and initiates multiple intracellular signaling pathways.[4] Downstream effects of EP receptor signaling include many of the hallmarks of cancer: increased angiogenesis, antiapoptosis, proliferation, migration, invasion, immune evasion, epithelial–mesenchymal transition (EMT), and support of a cancer stem-like cell phenotype.[3,5] Elevated

EP2 and EP4 expression and/or activation is observed in breast, colon, lung, pancreas, and prostate cancers.[3,6–8]

Multiple drug resistance-associated protein 4 (MRP4/ABCC4) is the fourth member of the C subfamily of ATP-binding cassette (ABC) proteins.[9] MRP4 exports a wide range of exogenous compounds, including antiretroviral compounds, anti-HIV compounds, camptothecins, methotrexate, and ceftins.[10] MRP4 has been identified as the main efflux transporter of PGE_2, but whether this function is important to malignant behavior has not been fully elucidated.[10–14] PGE_2 is imported into cells via the prostaglandin transporter (PGT) and subsequently oxidized by the NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH). Effective internalization and oxidation of PGE_2 are required for efficient inactivation of PGE_2 and attenuation of signaling.[15] 15-Hydroxyprostaglandin dehydrogenase (15-PGDH) is a tumor suppressor in breast, colon, liver, lung, and pancreas since decreased expression of this enzyme is associated with increased tumorigenesis.[16]

The PAM50 array is a gene expression profiling array used to classify breast cancers into distinct molecular subtypes. Expression of 50 genes is evaluated in order to predict patient outcome and guide treatment decisions.[17] The subtypes of breast cancer identified by gene expression profiling are luminal A, luminal B, HER2-enriched, normal-like, and basal-like.[18,19] Luminal A and luminal B include tumors that express ER and PR, luminal B tumors being distinguished by the expression of genes associated with high cell proliferation. HER2-enriched tumors express high levels of HER2 and genes associated with uncontrolled HER2-mediated signaling. Normal-like tumors do not express ER, PR, or HER2 and also lack the expression of genes associated with high cell proliferation. Basal-like tumors usually do not express ER, PR, or HER2, but do express high levels of cell proliferation genes. Breast cancer subtypes determined from gene expression more accurately predict patterns of metastatic spread and survival after relapse than protein-based classifications.[20]

Although the roles of COX-1/COX-2 and 15-PGDH are established in breast cancer, the linkage to individual molecular subtypes is less well defined. Even less is known regarding the role of MRP4 and PGT in breast cancer. We tested the hypothesis that increased MRP4 and decreased PGT would be observed in more aggressive tumor phenotypes, ie, basal-like, HER2-enriched, and triple-negative breast cancers (TNBCs). We used publicly available datasets to determine the expression of COX-2 pathway members in breast cancer and determine if these are linked to different molecular subtypes with diverse biologies and clinical outcomes.

Methods

TCGA. The Cancer Genome Atlas (TCGA) is a collection of extensive datasets collected from large-scale genome sequencing efforts. Two breast cancer gene expression datasets from TCGA were examined using the University of California Santa Cruz Cancer Genome Browser (https://genome-cancer.ucsc.edu/).[21] Illumina HiSeq ($n = 1206$) and Agilent G4502A_07_3 ($n = 597$) datasets were evaluated for expression of ABCC4 (MRP4), SLCO2A1 (PGT), HPGD (15-PGDH), PTGS2 (COX-2), PTGS1 (COX-1), PTGER4 (EP4), and PTGER2 (EP2). Patient and tumor characteristics from the breast cancer Illumina HiSeq gene expression dataset are summarized in Table 1.

Table 1. Clinical characteristics of TCGA breast cancer Illumina HiSeq dataset.

	MEDIAN	RANGE
Age (years)	58	26–90
	n	%
Menopausal status		
Pre-menopausal	229	21.1%
Peri-menopausal	39	3.6%
Post-menopausal	704	64.8%
Indeterminate	34	3.1%
Not available	59	5.4%
Not evaluated	5	0.5%
Unknown	17	1.6%
Race		
White	746	68.6%
Black or African-American	180	16.6%
Asian	61	5.6%
American Indian or Alaskan native	1	0.1%
Not available	99	9.1%
Ethnicity		
Hispanic or Latino	37	3.4%
Not Hispanic or Latino	874	80.4%
Not available/unknown	176	16.2%
Tissue type		
Primary tumor	1087	
Normal tissue*	112	
Metastatic tissue*	7	
Total	1206	
Stage		
I	133	12.2%
II	441	40.6%
III	172	15.8%
IV	15	1.4%
Not determined	326	30.0%
Lymph node status		
NX	20	1.8%
N0	512	47.1%
N1	357	32.8%
N2	118	10.9%
N3	76	7.0%
Not available	4	0.4%

Table 1. (*Continued*)

	n	%
Subtype (PAM50 array)		
Luminal A	230	21.2%
Luminal B	123	11.3%
HER2-enriched	58	5.3%
Basal-like	98	9.0%
Normal-like	8	0.7%
Not determined	570	52.4%
ER status		
Positive (+)	593	54.6%
Negative (–)	179	16.5%
Not determined	315	29.0%
PR status		
Positive (+)	515	47.4%
Negative (–)	254	23.4%
Not determined	318	29.3%
HER2 status		
Positive (+)	109	10.0%
Negative (–)	649	59.7%
Not determined	329	30.3%

Note: *Matched with a primary tumor sample.

This dataset comprises 1087 primary tumor samples, 112 normal mammary tissue samples, and 7 metastatic tissue samples. The normal mammary tissue samples and metastatic tissue samples are from women with primary tumor samples in the dataset. The median age at diagnosis was 58 years with a range of 26–90 years. Approximately 20% of the tumor samples were from premenopausal women, 4% were from perimenopausal women, 65% were from postmenopausal women, and 11% were from women whose menopausal status was not determined or not available. The distribution of patient race and ethnicity reflects the general patterns of breast cancer in the U.S. The most frequent tumor stage was stage II (40.6%) and the most frequent lymph node status was N0 (node negative; 47.1%). Nearly equivalent percentages of stage I (12.2%) and stage III (15.5%) tumors were included in this dataset, and approximately 2% of the staged tumors were stage IV. We grouped all lymph node-positive tumors ($n = 551$), which were similar to the number of lymph node-negative tumors ($n = 512$). These values are within the expected distribution of women with breast cancer.[22]

Oncomine. Oncomine (www.oncomine.org) is a platform with the capability to analyze gene expression data generated from mRNA profiling arrays with respect to clinical parameters. The *Farmer Breast* dataset includes 6 apocrine, 16 basal, and 27 luminal breast carcinomas, which allows for the comparison of gene expression between molecular subtypes of breast cancer.[23] The *Karnoub Breast* dataset compares gene expression between seven normal mammary stroma samples and 15 breast cancer-associated stroma samples.[24] The *Schuetz Breast 2* dataset includes seven samples each of ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC).[25] The distinction between DCIS and IDC is very important clinically as IDC can progress rapidly while DCIS is a localized (stage 0) disease. The following gene probe IDs were used to evaluate gene expression in these datasets: ABCC4 (MRP4): 203196_at; SLCO2A1 (PGT): 204368_at; HPGD (15-PGDH): 211549_s_at; PTGS2 (COX-2): 204748_at; PTGS1 (COX-1): 205127_at; and PTGER4 (EP4): 204897_at.

Statistics. Clinical data on 1087 patients with the corresponding gene expression data from TCGA were downloaded from the UCSC Cancer Genome Browser. Exploratory gene expression data analysis was carried out using the Student's *t*-test for comparing gene distribution among distinct groups. Subjects were grouped for analysis based on their menopausal status, race and ethnicity, tumor stage, lymph node status, and molecular subtype. Pearson's correlation coefficients were estimated for associations among gene expression values. The Cox regression model was used to estimate overall survival (OS) and recurrence-free survival (RFS) and explore the relationship between the survival of a patient and several plausible risk factors. All statistical tests were done at the 0.05 level of significance. Statistical analysis was conducted using R (x64, v. 3.2.2), GraphPad Prism 5.0, and SAS (v. 9.3, SAS Inc.).

Results

We sought to investigate the expression of PGE_2 pathway members beyond COX-2, not yet described in breast cancer, and determine if expression patterns differ in breast cancer subtypes with different biologies. We examined a large database, TCGA, which contains a collection of extensive datasets generated from next-generation sequencing of a wide range of tumor types. Two independent datasets of gene expression in breast cancer (TCGA breast invasive carcinoma generated from gene expression microarray or RNAseq [AgilentG4502A_07_3 and IlluminaHiSeq, respectively]) were examined using the UCSC Cancer Genome Browser. The clinical characteristics of tissue donors are summarized in Table 1.

The gene expression data in TCGA datasets available to investigators have been previously transformed by the generators of the data. As such, these data were mean-centered in order to more easily visualize the differences in gene expression. The mean expression value of a gene has been subtracted from each individual sample's gene expression value so that gene expression values less than the mean value are classified as negative and gene expression values higher than the mean are classified as positive. In order to interpret these data-rich figures, Figure 1 shows an enlarged version of the heat map for ABCC4 expression including detailed labels for the color scales representing breast cancer subtype determined by the gene expression-based PAM50 array, ER, PR, and HER2/ErbB2

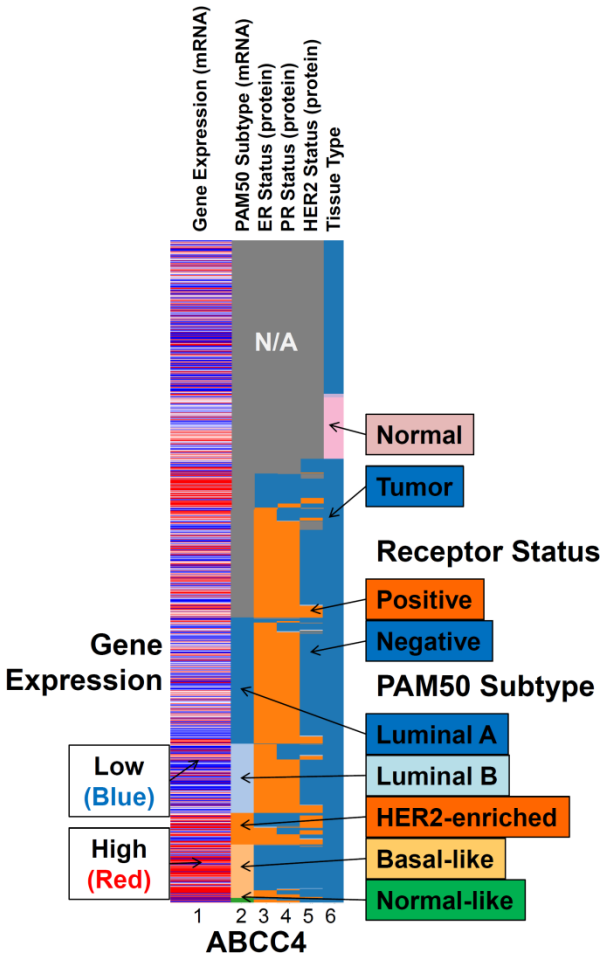

Figure 1. Detailed legend of gene expression heat maps derived from TCGA breast cancer dataset. Samples are arranged vertically so that each tissue sample is described by reading horizontally across the six columns. From left to right, the first column in the diagram indicates low (blue) or high (red) gene expression (mRNA), the second column indicates breast cancer subtype from the PAM50 gene expression array, ER, PR, and HER2 receptor status (protein) are indicated by the third, fourth, and fifth columns, respectively, and tissue sample type is indicated by the sixth column. Breast cancer subtype or receptor status was not available (N/A) for some samples indicated in the upper gray region of the diagram.

receptor status, and the sample tissue type. Each clinical sample is represented horizontally across the six multicolored columns. The first column shows the relative expression of the indicated gene, ABCC4 in this example, where red represents high gene expression (higher than the mean value for the dataset) and blue represents low gene expression. White indicates gene expression near the mean that was normalized to zero. The second column indicates the PAM50 breast cancer subtype of the sample determined by the TCGA network.[19] PAM50 is a gene expression array used to classify breast cancer into five distinct subtypes. Dark blue represents luminal A subtype tumors, light blue represents luminal B subtype tumors, orange represents HER2-enriched tumors, peach represents basal-like tumors, and green represents normal-like tumors. The next three columns represent protein expression data for ER, PR, and HER2 receptor, the three main molecular targets exploited therapeutically in breast cancer. Herein, orange indicates positive protein expression for a receptor, while dark blue indicates negative receptor status. Gray areas in the PAM50 subtype or receptor expression columns indicate that the data were not available (N/A) for that sample. Not surprisingly, most of the tumor samples in the luminal A and B groups (PAM50 mRNA expression subtypes) are also classified as having positive (orange) ER and PR protein expression. Also, most of the HER2-enriched tumors (PAM50 mRNA expression subtype) are positive for HER2 receptor protein (orange bands in the HER2 receptor column). The rightmost column indicates what tissue the sample was from, normal tissue (pink) or malignant tissue (dark blue).

We examined the expression of seven genes (ABCC4, SLCO2A1, HPGD, PTGS2, PTGS1, PTGER4, and PTGER2); heat maps of gene expression versus breast cancer subtype and receptor expression are shown (Fig. 2). We found very consistent expression patterns when comparing independent datasets generated via the Illumina HiSeq platform (Fig. 2) versus the Agilent platform (data not shown).

Elevated expression of ABCC4/MRP4 could lead to elevated PGE_2 in the tumor microenvironment and could be a contributing factor to the aggressive nature of these malignancies. We observed elevated ABCC4 expression in TNBC and basal-like as well as in HER2-enriched tumors (molecular subtypes as defined by protein or mRNA expression) (Fig. 2A, solid boxes) compared with normal tissue, luminal A or B, or ER+/PR+ tumors (dotted boxes).

SLCO2A1/PGT is responsible for the import of PGE_2 from the tumor microenvironment as the first step in PGE_2 metabolism. We observed increased SLCO2A1 expression in normal tissue and tumors classified as HER2-enriched or luminal A (Fig. 2B, solid boxes); comparatively, tumors classified as luminal B or basal had decreased SLCO2A1 expression (dotted boxes).

Following import via PGT, PGE_2 is metabolized by 15-PGDH, which terminates PGE_2-mediated actions on the cell. HPGD gene expression is high in normal breast tissue (Fig. 2C, solid box), intermediate in luminal A and B tumors (dashed box), and low in basal and HER2-enriched samples (dotted box). These observations are consistent with 15-PGDH being classified as a tumor-suppressor gene in multiple tumor types, including breast.[16]

As rate-limiting enzymes of PGE_2 synthesis, expression of COX-1 and COX-2 has been extensively studied in epithelial malignancies. COX-1 protein expression generally remains consistent from normal to malignant tissue, but COX-2 protein expression increases in aggressive tumors.[1,2,26] We examined the expression of PTGS1 and PTGS2, encoding COX-1 and COX-2, in these datasets. We observed elevated PTGS2 expression in normal breast tissue and basal subtype tumors (Fig. 2D, solid boxes). Luminal A, luminal B,

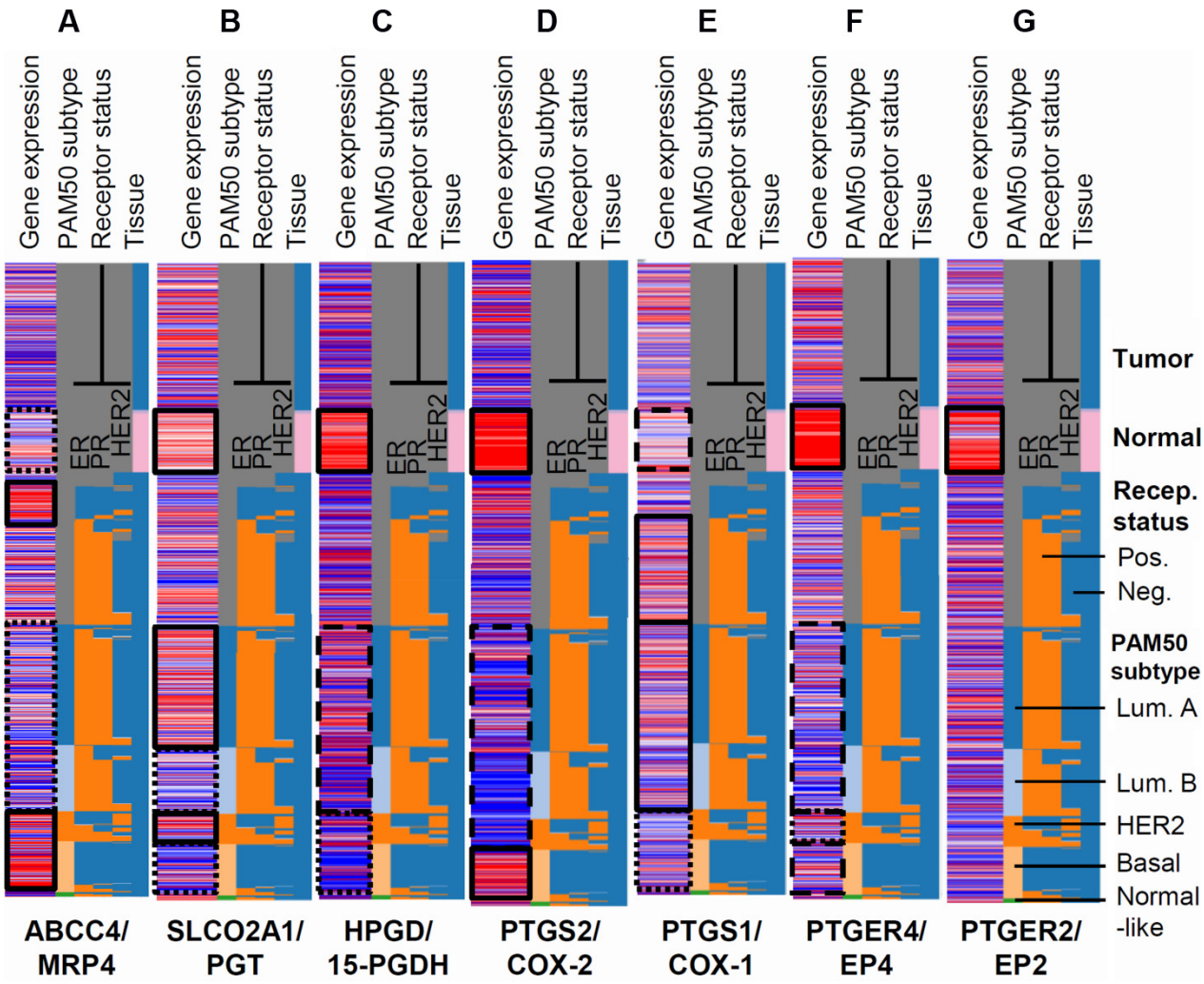

Figure 2. Gene expression heat maps from TCGA Illumina HiSeq breast cancer dataset of PGE_2 pathway genes relative to PAM50 breast cancer subtypes and receptor protein expression status. Genes evaluated were as follows: ABCC4/MRP4, SLCO2A1/PGT, HPGD/15-PGDH, PTGS2/COX-2, PTGS1/COX-1, PTGER4/EP4, and PTGER2/EP2. Boxes indicate notable regions of gene expression and are distinguished by the following borders: solid, high expression; dashed, moderate expression; and dotted, low expression.

and HER2-enriched tumors had lower expression of PTGS2 mRNA compared with basal-type breast cancer (dashed box). The differences in PTGS2 expression among the subtypes of breast cancer are quite remarkable, especially among basal subtype tumors with high PTGS2 compared with luminal or HER2-enriched tumors. Expression of PTGS2 in basal subtype tumors is greater than the mean of all samples tested and significantly greater than the expression of this gene in luminal A, luminal B, or HER2-enriched subtype tumors, all with expression less than the mean of all samples. The relationship of high PTGS2 to basal-type breast cancers was also noted by Li et al.[27]

PTGS1 expression does not vary over a wide range. We now observe that PTGS1 is highest in luminal A tumors and in samples that express both ER and PR (Fig. 2E, solid box). Normal breast tissue samples have moderate expression of PTGS1 (dashed box), whereas the three other breast cancer subtypes (luminal B, basal, and HER2-enriched) have moderate-to-low expression of PTGS1 (dotted box). Overall, PTGS1 is detected in all breast cancer subtypes.

The COX-2 product, PGE_2, mediates cell signaling through four G-protein coupled receptors (EP1–EP4). EP2 and EP4 have been implicated in malignant behavior.[4] PGE_2-mediated signaling through both EP2 and EP4 receptors regulates gene expression through activation of adenylyl cyclase and production of cAMP; however, migration and support of the breast cancer stem-like phenotype are more strongly associated with EP4 signaling.[5,8] We examined the expressions of EP4 and EP2 mRNA (PTGER4 and PTGER2, respectively). We observed elevated PTGER4 expression in normal breast tissue samples compared with malignant breast samples (Fig. 2F, solid box). Of the tumor samples, basal and HER2-enriched tumors (dashed box) showed higher PTGER4 expression than the luminal subtype tumors (dotted box). These data support our previous findings that EP4 could be a therapeutic target in more aggressive breast cancer subtypes.

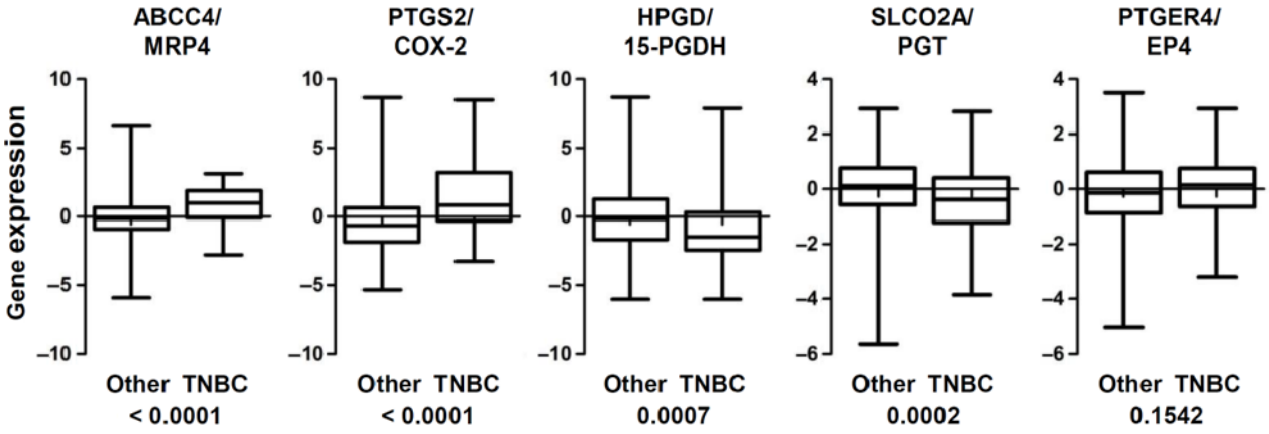

Figure 3. Expression of PGE_2 pathway genes in TNBC samples favors production and extracellular accumulation of PGE_2. Primary breast cancer samples were classified into *Other* and *TNBC* based on ER, PR, and HER2 receptor protein expression status. The distribution of each of the five PGE_2 pathway genes between these categories is shown in box-and-whisker plots. Student's *t*-test *P*-values are reported below each plot.

PTGER2 is more highly expressed in normal breast tissue compared with malignant breast tissue. There seems to be no relationship between breast cancer subtype and PTGER2 expression (Fig. 2G).

Comparing the same region of multiple panels allowed us to qualitatively identify the differences in gene expression among specific subtypes of cancer. To this point, our data suggested that TNBC is distinct from the other subtypes with respect to COX-2 pathway expression. These trends between breast cancer subtypes and PGE_2 pathway member gene expression patterns warranted a deeper analysis into TCGA data. The Illumina HiSeq dataset was downloaded using the UCSC Cancer Genome Browser and analyzed. TNBC samples ($n = 123$) were compared with all other primary tumor samples ($n = 964$) across the five PGE_2 pathway genes. Expression of PTGS2 (COX-2) and ABCC4 (MRP4) mRNA was significantly higher ($P < 0.0001$) in the TNBC samples compared with all other primary tumors (Fig. 3). Conversely, HPGD (15-PGDH, $P = 0.0007$) and SLCO2A1 (PGT, $P = 0.0002$) were significantly decreased in the TNBC samples compared with other primary tumor samples. PTGER4 (EP4) mRNA expression was similar in all groups. Taken together, this pattern of gene expression should lead to more PGE_2 synthesis and export to the tumor microenvironment and less import and degradation in the setting of TNBC. These results illustrate fundamental differences between TNBC and other types of breast cancer. Given these patterns, we would expect that PGE_2 levels in TNBC tumors would be elevated due to the increased production and export and decreased metabolism of PGE_2.

There were 810 patients with complete records available to estimate OS; 93 patients (11.5%) were confirmed dead at the time of data entry. OS was defined as the time from initial diagnosis to death from any cause, censored at the date of last contact. RFS was estimated using 792 cases. RFS was defined as the time from initial diagnosis to disease recurrence.

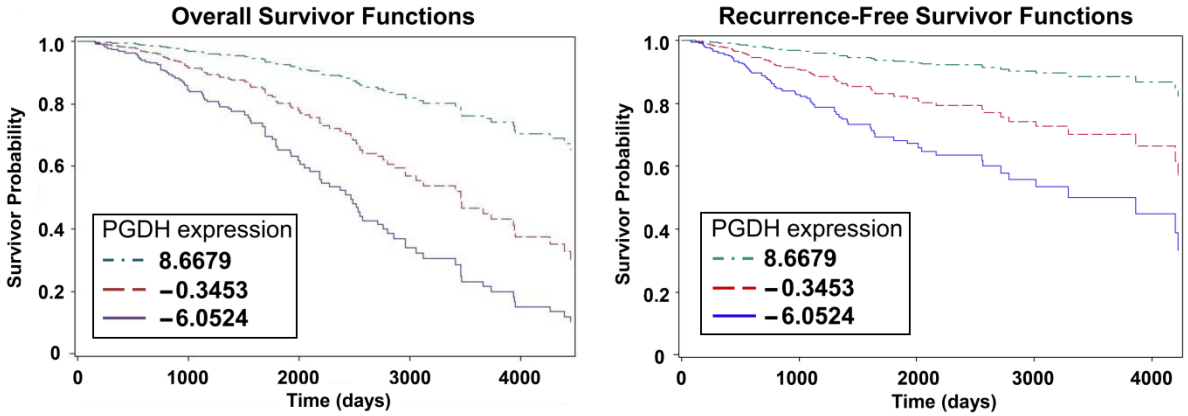

Figure 4. Cox regression of OS and RFS versus HPGD gene expression. Cox regression models at the indicated levels of HPGD expression show higher probability of survival in tumors expressing maximum observed HPGD expression (8.6679, green dash-dotted line), moderate probability of survival at median expression (−0.3453, red dashed line), and poor probability of survival at minimum observed expression (−6.0524, blue solid line).

Patients without recurrence were censored at the date of last contact, given that the patient was still disease-free. High HPGD (15-PGDH) expression was found to be an independent predictor of a better outcome, longer OS (hazard ratio = 0.89, $P = 0.01$). A gene-dosage effect was observed where maximum observed expression of HPGD was correlated with higher OS probability; minimum observed expression of HPGD was correlated with lower survival probability by Cox regression model (Fig. 4, left). This was the only gene to show a strong association with OS. RFS was estimated and compared between the following two categories of patients, namely, *new tumor* and *otherwise*, as indicators of disease progression. Elevated HPGD expression was strongly associated with prolonged RFS (hazard ratio = 0.89, $P = 0.02$). Likewise, following Cox regression, a gene-dosage effect was observed between high expression of HPGD and higher chance of RFS (Fig. 4, right). These associations were highly significant and consistent with the classification of HPGD/15-PGDH as a tumor suppressor.[16,28]

In order to investigate if these five PGE_2 pathway genes are associated with one another in their expression, we performed pairwise correlation analyses. We found no strong correlation between the pairs of PGE_2 pathway genes in primary tumors as the resulting Pearson's correlation coefficients ranged from 0.114 to 0.460 (data not shown). Other parameters compared to PGE_2 pathway gene expression included cancer stage, lymph node status, and racial background. Breast cancer stage was also not strongly associated with expression of any particular PGE_2 pathway gene and not pursued further. Of the five PGE_2 pathway genes investigated, only PTGS2 and SLCO2A1 were significantly different between lymph node-negative and lymph node-positive tumors. While these changes were significant, we did not identify strong biological associations between lymph node status and gene expression to pursue further. There is a racial disparity in breast cancers that develop in African-American (black/AA) women compared with white women.[29] Of the five COX-2 pathway genes examined in primary tumor samples from black/AA and white women, we found no strong associations between the expression of any gene and a particular racial background.

To confirm our conclusion derived from the TCGA dataset and to obtain some information regarding stromal expression of COX-2 pathway genes, we examined the expression of the same COX-2 pathway genes in three additional small breast cancer datasets. Three breast cancer subtypes (apocrine, luminal, and basal) were used to classify the samples of the Farmer Breast dataset.[23] Gene expression in breast cancer-associated stroma compared with healthy mammary stroma was evaluated using the Karnoub Breast dataset.[24] The 14 breast cancer samples in the Schuetz Breast 2 dataset are divided between DCIS and IDC classifications.[25] The distinction between DCIS and IDC is clinically relevant as IDC can progress rapidly while DCIS is a localized (stage 0) disease. Gene expression is reported as log2 median-centered intensity; the median expression signal from the entire gene array was subtracted from each gene and the resulting signal intensity was log2 transformed. A negative value for gene expression indicates that the gene is expressed, but at a level lower than the median of all genes on the microarray. Since each microarray is normalized independently, comparisons of the gene expression values between datasets are not valid. The distribution of gene expression values between categories within the same dataset was analyzed and described for each gene studied.

We observed that expression of ABCC4/MRP4 is elevated more frequently in basal breast tumors (median = 0.576) than in apocrine (median = −0.404) or luminal (median = −0.185) tumors (Fig. 5A), consistent with the pattern observed in the TCGA dataset. When gene expression in mammary stroma was examined, we observed that ABCC4 expression was elevated in breast cancer-associated stroma (median = 0.278) compared to normal mammary tissue (median = −0.471; Fig. 5B). The majority (13/15) of normal stromal tissue samples had ABCC4 expression less than zero, while six of the seven IDC samples had ABCC4 expression greater than zero. We observed a similar trend in the expression of ABCC4 in IDC tumor samples compared with DCIS samples (Fig. 5C). ABCC4 expression is elevated more frequently in IDC tumors (median = 0.789) than in DCIS tumors (median = −0.154). All the examined IDC tumor samples had expression of ABCC4 greater than zero, while the expression of ABCC4 from only two of the seven DCIS samples was greater than zero, and the maximum expression level was not as high as that seen in the IDC samples.

PGT is responsible for the import of PGE_2 from the tumor microenvironment as the first step in PGE_2 metabolism. We observed little variation in the expression of this gene among subcategories of breast cancer. Basal breast cancer had a slightly higher maximum gene expression value compared to apocrine or luminal breast cancer, but overall, the pattern of expression was similar in apocrine, basal, and luminal subtypes (Fig. 5D). There were more basal breast cancer samples with SLCO2A1 expression around the maximum value compared to luminal samples. SLCO2A1 expression values in the stroma were greater than zero, and there was a slight increase in the median expression of SLCO2A1 in IDC-associated stroma (1.86) compared to normal stroma (0.927; Fig. 5E). We also observed a modest increase in median SLCO2A1 expression when we compared IDC (1.477) versus DCIS (1.10; Fig. 5F).

15-Prostaglandin dehydrogenase (15-PGDH/HPGD) metabolizes intracellular PGE_2 so that this ligand is unable to bind EP receptors, which results in suppression of PGE_2 signaling. When we examined three datasets for HPGD/15-PGDH expression, we observed that HPGD expression was detected in all samples, and gene expression values were generally below zero (Fig. 5G–I). This indicates that HPGD expression was less than the median expression of all genes on

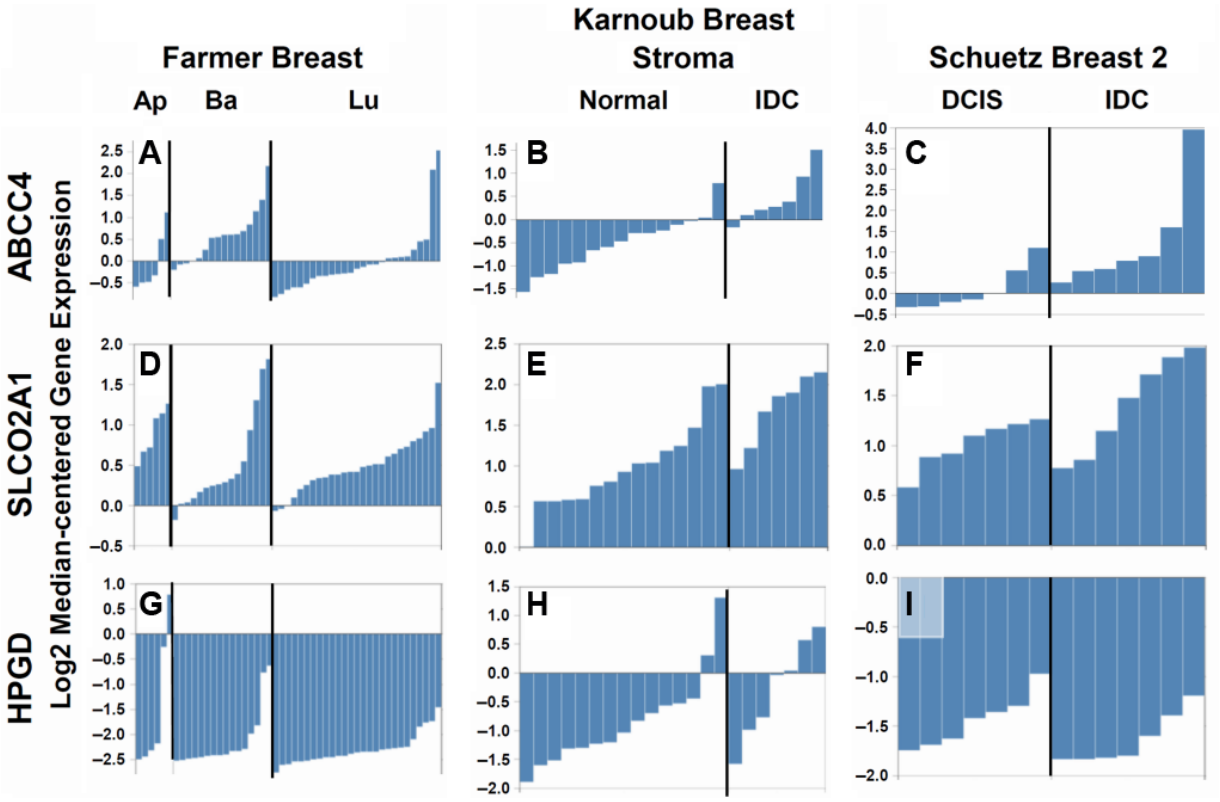

Figure 5. Expressions of ABCC4/MRP4, SLCO2A1/PGT, and HPGD/15-PGDH mRNA among breast cancer samples divided by subtype. Three breast cancer gene expression datasets were analyzed for expressions of ABCC4/MRP4, SLCO2A1/PGT, and HPGD/15-PGDH with respect to molecular or pathological subtype. (**A**, **D**, **G**) Expressions of ABCC4, SLCO2A1, and HPGD, respectively, in Farmer Breast dataset. (**B**, **E**, **H**) Expressions of ABCC4, SLCO2A1, and HPGD, respectively, in Karnoub Breast dataset. (**C**, **F**, **I**) Expressions of ABCC4, SLCO2A1, and HPGD, respectively, in Schuetz Breast 2 dataset. Gene expression is represented as log2 median-centered intensity and each bar represents one sample. Apocrine (Ap), Basal (Ba), Luminal (Lu), invasive ductal carcinoma (IDC), and ductal carcinoma in situ (DCIS).

the microarray. HPGD expression between DCIS and IDC samples was very similar (Fig. 5I).

Expressions of PTGS2 (COX-2), PTGS1 (COX-1), and PTGER4 (EP4) were also evaluated in these three datasets; however, differences between the small groups were not strong enough to draw conclusions (data not shown).

Overall, data obtained from three, smaller, independent datasets show how distinct clinical subtypes of breast cancer can exhibit different expression patterns of several members of the COX-2/PGE_2 pathway. Even in these small datasets, we observed elevated expression of ABCC4/MRP4 in breast cancer samples with the worst prognoses (basal and IDC). These findings support the trends observed in the TCGA analyses.

Discussion

Expression of COX-2 and production of PGE_2 are two indicators of poor prognosis in breast cancer. COX-1 and COX-2 enzymes are inhibited by nonsteroidal anti-inflammatory drugs (NSAIDs, eg, aspirin, ibuprofen, and indomethacin), while COX-2 is inhibited by the coxib family of molecules (celecoxib, rofecoxib, and valdecoxib), with much higher affinity for COX-2 than COX-1.[3] There is epidemiologic evidence that long-term use of NSAIDs for other indications modestly reduces breast cancer incidence.[30,31] In the cancer treatment setting, both NSAIDs and coxibs have shown some efficacy; however, chronic use of NSAIDs can lead to gastrointestinal bleeding, and prolonged use of coxibs has resulted in cardiotoxic secondary effects.[32] While the COX enzymes are responsible for the rate-limiting step in PGE_2 synthesis, other members of the PGE_2 pathway are important to consider as they may also play a role in determining the overall level of PGE_2 in a tumor. Using publicly available gene expression datasets, we have now shown that members of the PGE_2 pathway are differentially expressed among the different molecular subtypes of breast cancer. The most aggressive breast cancer subtypes (basal-like, HER2-enriched, and TNBC) have gene expression profiles that would favor accumulation of PGE_2 in the tumor microenvironment. Gene expression changes include elevated expression of ABCC4/MRP4, which would increase the export of PGE_2 from cells, decreased expression of SLCO2A1/PGT, leading to reduced clearance of PGE_2 from the microenvironment, and lowered expression of HPGD/15-PGDH, which would decrease the overall metabolism of PGE_2 in the tumor.

Although discovered for its role in conferring resistance to chemotherapy, elevated expression of MRP4 has

been detected in drug-naïve tumors, including neuroblastoma, prostate cancer, pancreatic cancer, and acute myeloid leukemia.[33–35] We report here that ABCC4/MRP4 mRNA is expressed in breast cancer and is strongly associated with aggressive breast cancer subtypes with the worst prognoses. The strong correlation between ABCC4 expression and aggressive breast cancer supports our central hypothesis that elevated MRP4 expression is correlated with aggressiveness in breast cancer, particularly in basal-like and HER2-enriched molecular subtypes. Transcription of ABCC4 is regulated by several signaling pathways, including the aryl hydrocarbon receptor (AhR), glucocorticoid receptor (GR), nuclear factor (erythroid-derived 2)-like 2 (Nrf2), peroxisome proliferator-activated receptor alpha (PPARα), and the N-myc oncogene.[36–38] Micro-RNAs (miRNAs), miR-124a and miR-506, suppress MRP4 protein translation in a tissue-specific manner.[39] MRP4 expression is also regulated by alternative splicing and nonsense-mediated decay of a truncated mRNA transcript.[40]

The inverse relationship between PGT (PGE_2 import) and MRP4 (PGE_2 export) was first reported by Holla et al who recognized the net effect of these proteins on the regulation of PGE_2 levels in colorectal cancer.[13] In the majority of colorectal tumor samples examined, they found elevated MRP4 expression and reduced PGT expression compared with adjacent healthy tissue. We found high expression of SLCO2A1/PGT mRNA in normal breast tissue and in luminal A and HER2-enriched breast cancers; expression of SLCO2A1 was lower in luminal B and basal-like breast cancers. Lower expression of SLCO2A1/PGT would decrease the amount of PGE_2 imported for metabolism, and therefore, the remaining PGE_2 could sustain signaling through EP receptors on the cell surface.

HPGD/15-PGDH is a tumor suppressor gene in breast and other epithelial-derived cancers. Expression of 15-PGDH is necessary for metabolism and inactivation of PGE_2 in order to fully suppress PGE_2-mediated signaling even when COX-2 is inhibited.[41] We found that HPGD/15-PGDH mRNA expression was decreased in breast cancer compared with healthy breast tissue and that HPGD expression was lower in breast cancers of the most aggressive subtypes (basal-like and HER2-enriched). Expression of HPGD was also suppressed in the majority of TNBCs compared with other breast cancers. High HPGD expression was associated with improved OS and RFS. These data are consistent with a tumor-suppressor role for HPGD reported by others.[16,28]

The rate-limiting step of PGE_2 synthesis is dependent on COX-1/PTGS1 or COX-2/PTGS2, and hence the expression of these genes was also examined. PTGS1 expression did not show any striking associations with the breast cancer subtypes. PTGS2 gene expression was elevated in TNBC compared with luminal A, luminal B, or Her2-enriched subtypes. PTGS2 was also elevated in normal breast tissue. This latter finding was unexpected since there are abundant reports of elevated COX-2 protein in tumors relative to adjacent normal tissue.[1,3] This inconsistency could be due to a complex relationship between PTGS2 mRNA and COX-2 protein. PTGS2 is an immediate-early gene with a short mRNA half-life; hence, the mRNA expression level could be less stable than the protein expression level.[42] Specifically in TNBC, the regulation of PTGS2 gene expression has not been fully elucidated.

EP2 and EP4 have been implicated in malignant behavior in multiple tumor types.[4–6] PTGER4 appeared to be more closely associated with aggressive breast cancer subtypes.

Protein or lipid profiles are not available for the samples included in these datasets; however, preliminary gene and protein expression data from breast cancer cell lines is in accord with the major trends seen across the large datasets (data not shown). Luminal breast cancer cell lines express lower ABCC4/MRP4 while expressing higher SLCO2A1/PGT and HPGD/15-PGDH compared with basal-like and TNBC cell lines. PTGS2/COX-2 has been detected in most of these cell lines.[43]

Taken together, these data support our central hypothesis that the COX-2 pathway contributes to malignant behavior. Extensive literature supports a central role for COX-2 in breast and other malignancies.[1,6] Our current data support the hypothesis that other components, particularly high MRP4 and low 15-PGDH, may also contribute to a high PGE_2 environment, particularly in basal-like and TNBC breast cancers. Further investigation into the function of each of these genes and their interactions is warranted as some of the gene expression profiles shown here suggest higher PGE_2 accumulation in aggressive tumor subtypes with the worst prognoses.

Acknowledgments

The results published here are in part based on data generated by The Cancer Genome Atlas managed by the NCI and NHGRI. Information about the TCGA can be found at http://cancergenome.nih.gov

We acknowledge the University of Maryland Marlene and Stewart Greenebaum Cancer Center Biostatistics Shared Service for their assistance and analysis in this work.

Author Contributions

Conceived and designed the experiments: TJK. Analyzed the data: TJK and OGG. Wrote the first draft of the manuscript: TJK. Contributed to the writing of the manuscript: AMF. Agreed with manuscript results and conclusions: TJK, OGG, and AMF. Jointly developed the structure and arguments for the paper: TJK and AMF. Made critical revisions and approved the final version: AMF. All the authors reviewed and approved the final manuscript.

REFERENCES

1. Half E, Tang XM, Gwyn K, Sahin A, Wathen K, Sinicrope FA. Cyclooxygenase-2 expression in human breast cancers and adjacent ductal carcinoma in situ. *Cancer Res.* 2002;62(6):1676–1681.

2. Ristimäki A, Sivula A, Lundin J, et al. Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. *Cancer Res*. 2002;62(3):632–635.
3. Greenhough A, Smartt HJM, Moore AE, et al. The COX-2/PGE pathway: key roles in the hallmarks of cancer and adaptation to the tumor microenvironment. *Carcinogenesis*. 2009;30(3):377–386.
4. Reader J, Holt D, Fulton A. Prostaglandin E2 EP receptors as therapeutic targets in breast cancer. *Cancer Metastasis Rev*. 2011;30(3–4):449–463.
5. Kundu N, Ma X, Kochel T, et al. Prostaglandin E receptor EP4 is a therapeutic target in breast cancer cells with stem-like properties. *Breast Cancer Res Treat*. 2013; 143(1):19–31.
6. Fulton AM, Ma X, Kundu N. Targeting prostaglandin E EP receptors to inhibit metastasis. *Cancer Res*. 2006;66(20):9794–9797.
7. Wang X, Klein RD. Prostaglandin E2 induces vascular endothelial growth factor secretion in prostate cancer cells through EP2 receptor-mediated cAMP pathway. *Mol Carcinog*. 2007;46(11):912–923.
8. Kim JI, Lakshmikanthan V, Frilot N, Daaka Y. Prostaglandin E2 promotes lung cancer cell migration via EP4-betaArrestin1-c-Src signalsome. *Mol Cancer Res*. 2010;8(4):569–577.
9. Dean M, Rzhetsky A, Allikmets R. The human ATP-binding cassette (ABC) transporter superfamily. *Genome Res*. 2001;11(7):1156–1166.
10. Russel FGM, Koenderink JB, Masereeuw R. Multidrug resistance protein 4 (MRP4/ABCC4): a versatile efflux transporter for drugs and signalling molecules. *Trends Pharmacol Sci*. 2008;29(4):200–207.
11. Menter DG, Dubois RN. Prostaglandins in cancer cell adhesion, migration, and invasion. *Int J Cell Biol*. 2012;2012:723419.
12. Fletcher JI, Haber M, Henderson MJ, Norris MD. ABC transporters in cancer: more than just drug efflux pumps. *Nat Rev Cancer*. 2010;10(2):147–156.
13. Holla VR, Backlund MG, Yang P, Newman RA, DuBois RN. Regulation of prostaglandin transporters in colorectal neoplasia. *Cancer Prev Res (Phila)*. 2008;1(2): 93–99.
14. Kochel TJ, Fulton AM. Multiple drug resistance-associated protein 4 (MRP4), prostaglandin transporter (PGT), and 15-hydroxyprostaglandin dehydrogenase (15-PGDH) as determinants of PGE2 levels in cancer. *Prostaglandins Other Lipid Mediat*. 2015;116–117:99–103.
15. Nomura T, Lu R, Pucci ML, Schuster VL. The two-step model of prostaglandin signal termination: in vitro reconstitution with the prostaglandin transporter and prostaglandin 15 dehydrogenase. *Mol Pharmacol*. 2004;65(4):973–978.
16. Tai H-H. Prostaglandin catabolic enzymes as tumor suppressors. *Cancer Metastasis Rev*. 2011;30(3–4):409–417.
17. Prat A, Parker JS, Fan C, Perou CM. PAM50 assay and the three-gene model for identifying the major and clinically relevant molecular subtypes of breast cancer. *Breast Cancer Res Treat*. 2012;135(1):301–306.
18. Sørlie T, Tibshirani R, Parker J, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci U S A*. 2003; 100(14):8418–8423.
19. Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature*. 2012;490(7418):61–70.
20. Kennecke H, Yerushalmi R, Woods R, et al. Metastatic behavior of breast cancer subtypes. *J Clin Oncol*. 2010;28(20):3271–3277.
21. Chin L, Hahn WC, Getz G, Meyerson M. Making sense of cancer genomic data. *Genes Dev*. 2011;25(6):534–555.
22. Onitilo AA, Engel JM, Greenlee RT, Mukesh BN. Breast cancer subtypes based on ER/PR and Her2 expression: comparison of clinicopathologic features and survival. *Clin Med Res*. 2009;7(1–2):4–13.
23. Farmer P, Bonnefoi H, Becette V, et al. Identification of molecular apocrine breast tumours by microarray analysis. *Oncogene*. 2005;24(29):4660–4671.
24. Karnoub AE, Dash AB, Vo AP, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature*. 2007;449(7162):557–563.
25. Schuetz CS, Bonin M, Clare SE, et al. Progression-specific genes identified by expression profiling of matched ductal carcinomas in situ and invasive breast tumors, combining laser capture microdissection and oligonucleotide microarray analysis. *Cancer Res*. 2006;66(10):5278–5286.
26. Visscher DW, Pankratz VS, Santisteban M, et al. Association between cyclooxygenase-2 expression in atypical hyperplasia and risk of breast cancer. *J Natl Cancer Inst*. 2008;100(6):421–427.
27. Li H-J, Reinhardt F, Herschman HR, Weinberg RA. Cancer-stimulated mesenchymal stem cells create a carcinoma stem cell niche via prostaglandin E2 signaling. *Cancer Discov*. 2012;2(9):840–855.
28. Wolf I, O'Kelly J, Rubinek T, et al. 15-hydroxyprostaglandin dehydrogenase is a tumor suppressor of human breast cancer. *Cancer Res*. 2006;66(15):7818–7823.
29. Rizzo M, Lund MJ, Mosunjac M, et al. Characteristics and treatment modalities for African American women diagnosed with stage III breast cancer. *Cancer*. 2009;115(13):3009–3015.
30. Brasky TM, Bonner MR, Moysich KB, et al. Non-steroidal anti-inflammatory drugs (NSAIDs) and breast cancer risk: differences by molecular subtype. *Cancer Causes Control*. 2011;22(7):965–975.
31. Agrawal A, Fentiman IS. NSAIDs and breast cancer: a possible prevention and treatment strategy. *Int J Clin Pract*. 2008;62(3):444–449.
32. Marshall SF, Bernstein L, Anton-Culver H, et al. Nonsteroidal anti-inflammatory drug use and breast cancer risk by stage and hormone receptor status. *J Natl Cancer Inst*. 2005;97(11):805–812.
33. Huynh T, Norris MD, Haber M, Henderson MJ. ABCC4/MRP4: a MYCN-regulated transporter and potential therapeutic target in neuroblastoma. *Front Oncol*. 2012;2:178.
34. Montani M, Hermanns T, Müntener M, Wild P, Sulser T, Kristiansen G. Multidrug resistance protein 4 (MRP4) expression in prostate cancer is associated with androgen signaling and decreases with tumor progression. *Virchows Arch Int J Pathol*. 2013;462(4):437–443.
35. Copsel S, Garcia C, Diez F, et al. Multidrug resistance protein 4 (MRP4/ABCC4) regulates cAMP cellular levels and controls human leukemia cell proliferation and differentiation. *J Biol Chem*. 2011;286(9):6979–6988.
36. Henderson MJ, Haber M, Porro A, et al. ABCC multidrug transporters in childhood neuroblastoma: clinical and biological effects independent of cytotoxic drug efflux. *J Natl Cancer Inst*. 2011;103(16):1236–1251.
37. Xu S, Weerachayaphorn J, Cai S-Y, Soroka CJ, Boyer JL. Aryl hydrocarbon receptor and NF-E2-related factor 2 are key regulators of human MRP4 expression. *Am J Physiol Gastrointest Liver Physiol*. 2010;299(1):G126–G135.
38. Aleksunes LM, Yeager RL, Klaassen CD. Application of multivariate statistical procedures to identify transcription factors that correlate with MRP2, 3, and 4 mRNA in adult human livers. *Xenobiotica*. 2009;39(7):514–522.
39. Markova SM, Kroetz DL. ABCC4 is regulated by microRNA-124a and microRNA-506. *Biochem Pharmacol*. 2014;87(3):515–522.
40. Lamba JK, Adachi M, Sun D, et al. Nonsense mediated decay downregulates conserved alternatively spliced ABCC4 transcripts bearing nonsense codons. *Hum Mol Genet*. 2003;12(2):99–109.
41. Yan M, Myung S-J, Fink SP, et al. 15-Hydroxyprostaglandin dehydrogenase inactivation as a mechanism of resistance to celecoxib chemoprevention of colon tumors. *Proc Natl Acad Sci U S A*. 2009;106(23):9409–9413.
42. Maier JA, Hla T, Maciag T. Cyclooxygenase is an immediate-early gene induced by interleukin-1 in human endothelial cells. *J Biol Chem*. 1990;265(19): 10805–10808.
43. Zhao X, Goswami M, Pokhriyal N, et al. Cyclooxygenase-2 expression during immortalization and breast cancer progression. *Cancer Res*. 2008;68(2):467–475.