Retrospective analysis of estrogen receptor 1 and *N*-acetyltransferase gene expression in normal breast tissue, primary breast tumors, and established breast cancer cell lines

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Abstract. The expression levels of estrogen receptor 1 (*ESR1*), arylamine *N*-acetyltransferase 1 (*NAT1*), and arylamine *N*-acetyltransferase 2 (*NAT2*) are implicated in breast cancer; however, their co-expression profiles in normal breast tissue, primary breast tumors and established breast cancer cell lines are undefined. *NAT1* expression is widely reported to be associated with *ESR1* expression and is frequently investigated in breast cancer etiology. Furthermore, the *NAT2* phenotype has been reported to modify breast cancer risk in molecular epidemiological association studies. Understanding the relationships between the expression levels of these genes is essential to understand their role in breast cancer etiology and treatment. In the present study, *NAT1*, *NAT2* and *ESR1* expression data were accessed from repositories of RNA-Seq data covering 57 breast cancer cell lines, 1,043 primary breast tumors and 99 normal breast tissues. The relationships between gene expression, and between *NAT1* activity and RNA expression in breast cancer cell lines were evaluated using non-parametric statistical analyses. Differences in gene expression in each dataset, as well as gene expression differences in normal breast tissue compared to primary breast tumors, and stratification by estrogen receptor status were determined. *NAT1* and *NAT2* mRNA expression were detected in normal and primary breast tumor tissues; *NAT1* expression was much higher than *NAT2*. *NAT1* and *ESR1* expression were strongly associated, whereas *NAT2* and *ESR1* expression were not. Although *NAT1* and *NAT2* expression were associated, the magnitude was moderate. *NAT1*, *NAT2*, and *ESR1* expression were increased in primary breast tumor tissue compared with normal breast tissue; however, the magnitude and significance of the differences were lower for *NAT2*. Analysis of *NAT1*, *NAT2*, and *ESR1* expression in normal and primary breast tissues and breast cancer cell lines suggested that *NAT1* and *NAT2* expression are regulated by distinctive mechanisms, whereas *NAT1* and *ESR1* expression may have overlapping regulation. Defining these relationships is important for future investigations into breast cancer prevention.

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Abbreviations: NAT1, arylamine *N*-acetyltransferase 1; NAT2, arylamine *N*-acetyltransferase 2; SNPs, single nucleotide polymorphisms; ESR1, estrogen receptor 1; CCLE, Cancer Cell Line Encyclopedia; TCGA, The Cancer Genome Atlas; ER, estrogen receptor; SMZ, sulfamethazine; PABA, *p*-aminobenzoic acid; RPKM, reads per kilobase of transcript per million mapped reads; RSEM, RNA-Seq by Expectation-Maximization; ϱ, Spearman correlation coefficient

Key words: *NAT1*, *NAT2*, *ESR1*, breast cancer, TCGA, CCLE

Introduction

Human arylamine *N*-acetyltransferase 1 (NAT1) and arylamine *N*-acetyltransferase 2 (NAT2) are cytosolic phase II xenobiotic metabolizing isozymes, which catalyze the acetylation of a wide range of aromatic and heterocyclic amines via a ping-pong bi-bi reaction mechanism (1,2). This acetylation can ultimately lead to bioactivation and/or deactivation of various substrates, including breast cancer carcinogens (3-5). In addition to metabolizing xenobiotics, NAT1, but not NAT2, can catalyze the hydrolysis of acetyl-CoA using folate as a cofactor (6,7). *NAT1* and *NAT2* are encoded by two separate loci in close proximity on chromosome 8p22 (8,9) and each consist of an intronless open reading frame of 870 base pairs (10). Although *NAT1* and *NAT2* share ~87% nucleotide sequence identity and 81% deduced amino acid homology, they exhibit differing tissue localizations, and distinct but overlapping substrate specificities (11). In addition, *NAT1* and *NAT2* expression vary inter-individually from single nucleotide polymorphisms (SNPs) (2,12-17).

Although NAT1 and NAT2 catalyze *N*-acetylation, their roles in breast cancer etiology may differ. Numerous studies have investigated possible roles for NAT1 in breast cancer etiology and progression (18-24), given the association between increased expression of *NAT1* and estrogen receptor (ER)-positive breast cancers (25-31). Notably, *NAT1*

expression is not directly regulated by estrogens or dihydrotestosterone (32), thus suggesting that there may be a common regulatory element between *NAT1* and *ESR1*. Furthermore, congenic rats expressing higher NAT2 activity (orthologous to human NAT1) have been reported to exhibit greater carcinogen-induced mammary tumor susceptibility independent of carcinogen metabolism (22). In addition, SNPs in *NAT2* have been well described and have been revealed to influence acetylation rates of many known carcinogens; an association between *NAT2* genotype with breast cancer risk among smokers has been reported (33). Since *NAT1* and *NAT2* may have different roles in breast cancer, it is important to analyze relationships between the expression levels of these isozymes.

The mRNA expression levels of *NAT1* and *NAT2* have been detected by reverse transcription-polymerase chain reaction (RT-PCR) in human mammary tissue (34,35). NAT1 *N*-acetylation activity has been widely reported in normal breast tissue and breast tumor tissue (34,36-40), whereas NAT2 *N*-acetylation activity has not been observed as consistently; when NAT2 activity is observed the activity is much lower than NAT1 activity (34,38,39). In addition, since NAT1 and NAT2 have overlapping substrate specificities, activity studies of the two isozymes can be complex. For example, Deitz probed human mammary tissue samples for NAT1 and NAT2 activities with *p*-aminobenzoic acid (PABA; selective for NAT1) and sulfamethazine (SMZ; selective for NAT2), and reported that SMZ was acetylated by NAT1 at very low levels (40). By normalizing the SMZ *N*-acetylation activity to NAT1 activity, Deitz demonstrated that the SMZ *N*-acetylation activity was most likely catalyzed by NAT1 rather than NAT2. *NAT1* and *NAT2* activities have also been reported in rat mammary tissues (41).

Wakefield *et al* profiled *NAT1* expression and activity in seven breast cancer cell lines (MCF-7, T47D, ZR-75-1, Cal51, MDA-MB-231, MDA-MB-437 and MDA-MB-453) and detected *NAT1* mRNA expression and activity in all seven cell lines (28); however, *NAT2* expression and activity were not investigated. In addition, *NAT2* mRNA has been detected in MCF-7 breast cancer cells at very low levels (35); however, *NAT1* was not measured at the same time preventing a direct comparison of expression between the two isozymes. Bradshaw *et al* detected NAT1 and NAT2 by western blotting in the ER-positive breast cancer cell line MCF-7; however, the expression levels were not compared between the two proteins (42).

Limited studies have investigated the expression profiles of these isozymes together in breast tissues. Based on limited data, it has been hypothesized that *NAT2* expression is very low in breast tissue and negligible in comparison to *NAT1* expression; however, previous investigations have not addressed this hypothesis rigorously or comprehensively. To gain a better understanding of the relationship between *NAT1* and *NAT2* in breast tissues the present study evaluated the RNA expression levels of each in breast cancer cell lines, breast tumor tissue, and normal breast tissue. In addition, this study evaluated the extent to which established breast cancer cell lines reflect the *NAT* expression profile observed in primary breast tumors and normal breast tissue. Since *NAT1* and *NAT2* are so similar in terms of sequence, structure and substrates, and the association between *NAT1* and *ESR1* has been well established, the present study also evaluated the relationship between *NAT2* and *ESR1* expression in breast tissues. Since inhibition of NAT1 activity is under investigation for breast cancer prevention and treatment, understanding the relationships between *NAT1*, *NAT2* and *ESR1* is of great importance.

Materials and methods

Acquisition of publicly available data from the Cancer Cell Line Encyclopedia (CCLE) and The Cancer Genome Atlas (TCGA) data repositories. RNA expression (RNA-Seq) data for *ESR1*, *NAT1* and *NAT2* in established breast cancer cell lines were accessed on 8/11/17 (n=57) from the CCLE (43); RNA expression values were reported in reads per kilobase of transcript per million mapped reads (RPKM). A total of 15 breast cancer cell lines had no detectable *NAT2* gene expression. Data from TCGA (44) for the breast invasive carcinoma (BRCA) cohort were accessed on 2/4/18 (primary breast tumor tissue, n=1,043; normal breast tissue, n=99) via FirebrowseR (45), an R client to the Broad Institute's RESTful Firehose Pipeline; RNA expression values were reported in RNA-Seq by Expectation-Maximization (RSEM). A total of 59 of the breast tumor samples and seven of the normal tissue samples did not have gene expression data for *NAT2*.

Established breast cancer cell lines analyzed. The following breast cancer cell lines were analyzed in this study: AU565, BT-20, BT-474, BT-483, BT-549, CAL-120, CAL-148, CAL-51, CAL-85-1, CAMA-1, DU4475, EFM-19, EFM-192A, HCC1143, HCC1187, HCC1395, HCC1419, HCC1428, HCC1500, HCC1569, HCC1599, HCC1806, HCC1937, HCC1954, HCC202, HCC2157, HCC2218, HCC38, HCC70, HDQ-P1, HMC-1-8, HMEL, Hs 274.T, Hs 281.T, Hs 343.T, Hs 578.T, Hs 606.T, Hs 739.T, Hs 742.T, JIMT-1, KPL-1, MCF-7, MDA-MB-134-VI, MDA-MB-157, MDA-MB-175-VII, MDA-MB-231, MDA-MB-361, MDA-MB-415, MDA-MB-436, MDA-MB-453, MDA-MB-468, SK-BR-3, T-47D, UACC-812, UACC-893, ZR-75-1 and ZR-75-30.

Statistical analyses. Shapiro-Wilk tests were conducted to determine if the expression of the genes under study were approximately normally distributed. Significant evidence of departures from approximate normality was observed; therefore, non-parametric statistical techniques were employed for subsequent analyses. Spearman's correlation was used to evaluate the RNA expression levels between gene pairs (i.e. *ESR1* and *NAT1*, *ESR1* and *NAT2*, *NAT1* and *NAT2*). Differences in the mRNA expression levels of *NAT1* and *NAT2* in each dataset, and differences in RNA expression between primary breast tumor samples and normal breast tissue samples, were evaluated using the Wilcoxon rank-sum test; median values were compared to determine fold-differences.

RNA expression data for each gene were stratified by ER status (+ or -) as defined in the literature (46-48) for the CCLE data, or as determined by immunohistochemistry during sample collection and cataloging for TCGA data. Differences in gene expression following stratification were evaluated using Wilcoxon rank-sum tests for each gene; median values were compared to determine fold-differences. A total of 10 of the breast cancer cell lines had either conflicting or unknown ER status in the literature.

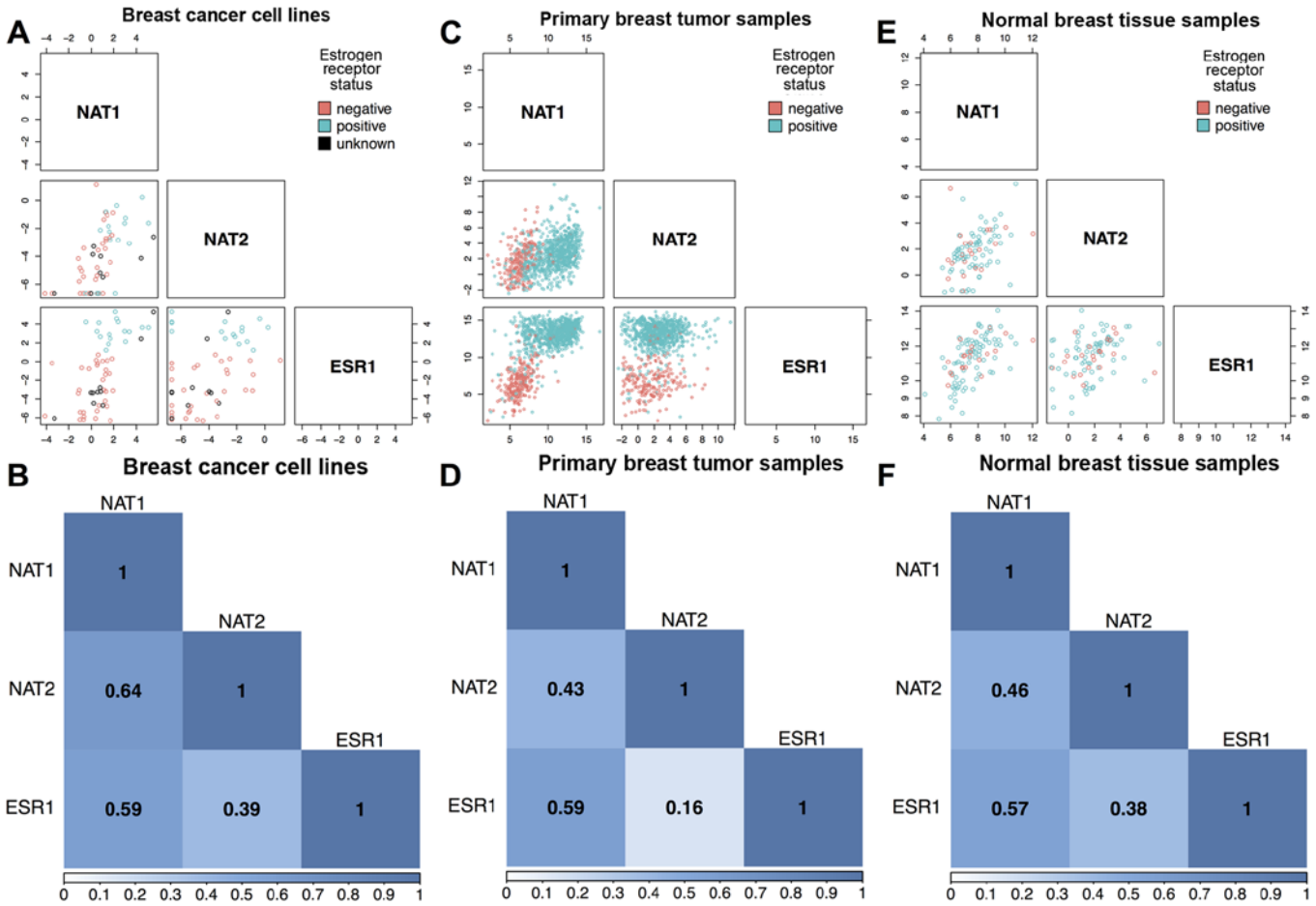

Figure 1. Scatterplot and correlation matrices for *NAT1*, *NAT2*, and *ESR1*. Associations between *NAT1*, *NAT2*, and *ESR1* RNA expression were analyzed in breast cancer cell lines, primary breast tumor tissue, and normal breast tissue using the Spearman method. In the scatterplot matrices, each open circle represents a single sample and is color-coded according to ER status; pink circles, ER^- samples; blue circles, ER^+ samples; black circles, samples with unknown ER status. In the association matrices, boxes are labeled with the Spearman correlation coefficient (ϱ) for each comparison and color reflects strength of association; dark blue represents high association, light blue represents low association, and white represents no association. (A) Scatterplot matrix of the association between *NAT1*, *NAT2* and *ESR1* RNA expression in breast cancer cell lines (n=57). (B) Correlation matrix between *NAT1*, *NAT2* and *ESR1* RNA expression in breast cancer cell lines (n=57). (C) Scatterplot matrix of the association between *NAT1*, *NAT2* and *ESR1* RNA expression in primary breast tumor samples (n=1,043 for *NAT1* vs. *ESR1*, n=984 for *NAT1* vs. *NAT2* and *NAT2* vs. *ESR1*). (D) Correlation matrix between *NAT1*, *NAT2*, and *ESR1* RNA expression in primary breast tumor samples (n=1,043 for *NAT1* vs. *ESR1*, n=984 for *NAT1* vs. *NAT2* and *NAT2* vs. *ESR1*). (E) Scatterplot matrix of the association between *NAT1*, *NAT2* and *ESR1* RNA expression in normal breast tissue samples (n=99 for *NAT1* vs. *ESR1*, n=92 for *NAT1* vs. *NAT2* and *NAT2* vs. *ESR1*). (F) Correlation matrix between *NAT1*, *NAT2* and *ESR1* RNA expression in normal breast tissue samples (n=99 for *NAT1* vs. *ESR1*, n=92 for *NAT1* vs. *NAT2* and *NAT2* vs. *ESR1*). ER, estrogen receptor; *ESR1*, estrogen receptor 1; *NAT1*, arylamine *N*-acetyltransferase 1; *NAT2*, arylamine *N*-acetyltransferase 2.

Wakefield *et al* published the NAT1 PABA *N*-acetylation activities of seven (ZR-75-1, T47D, MCF-7, MDA-MB-453, MDA-MB-436, MDA-MB-231 and CAL-51) of the 57 breast cancer cell lines included in the present study (28). The association between previously reported NAT1 activity and *NAT1* RNA expression data for the same cell lines in the CCLE repository was evaluated. All statistical analyses were performed in R: A Language and Environment for Statistical Computing, version 3.4.2 (49).

Results

Association between NAT1 and ESR1, NAT2 and ESR1, and NAT1 and NAT2. *NAT1* RNA and *ESR1* RNA were significantly correlated (P<0.0001 for all) at moderately high magnitudes in breast cancer cell lines (Spearman rho ϱ=0.59; Fig. 1A and B), human primary breast tumors (ϱ=0.59; Fig. 1C and D) and normal breast tissue (ϱ=0.57; Fig. 1E and F). A significant (P<0.005 for all) association between *ESR1* and *NAT2* expression was observed, although the magnitude of the association was low and varied across datasets. The primary breast tumor dataset exhibited the weakest association (ϱ=0.16; Fig. 1C and D), whereas the normal breast tissue (ϱ=0.38; Fig. 1E and F) and breast cancer cell line (ϱ=0.39; Fig. 1A and B) datasets exhibited similar, albeit low, association. Strong evidence of an association (P<0.0001 for all) between *NAT1* RNA and *NAT2* RNA levels was observed in all three datasets, with moderately high magnitude in the breast cancer cell lines (ϱ=0.64; Fig. 1A and B). The primary breast tumor and normal breast tissue datasets exhibited interdependence similar to each other (ϱ=0.43 and 0.46, respectively; Figs. 1C-F); however, the association was lower than that observed in the breast cancer cell lines.

Comparison of NAT1 and NAT2 expression. *NAT1* RNA expression in breast cancer cell lines, primary breast tumors, and normal breast tissue was significantly higher compared

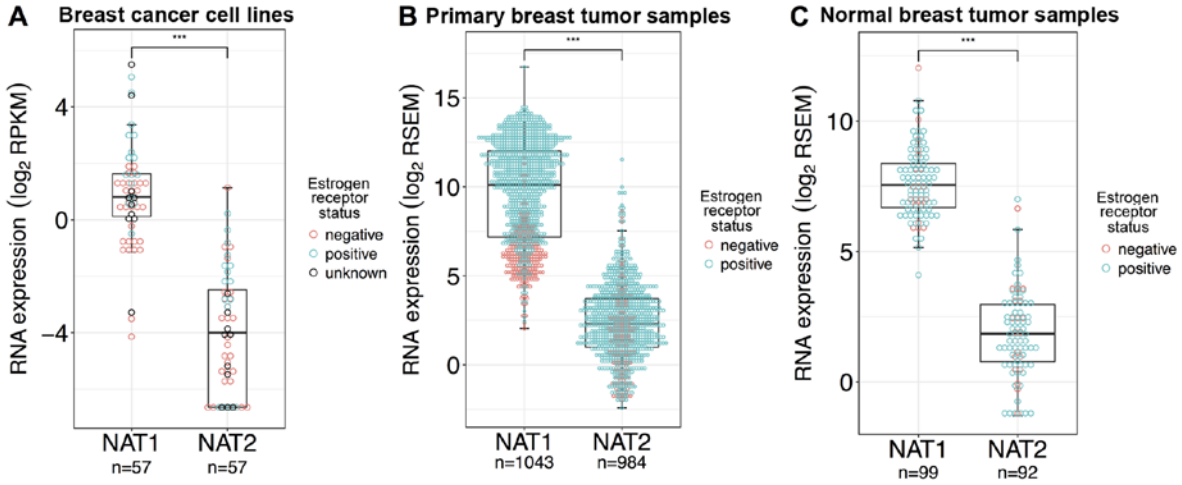

Figure 2. *NAT1* and *NAT2* RNA expression in breast cancer cell lines, primary breast tumor samples, and normal breast tissue samples. Differences in gene expression between *NAT1* and *NAT2* in breast cancer cell lines, primary breast tumor tissue and normal breast tissue were statistically evaluated by Wilcoxon rank-sum test; ***P<0.001. Each dot represents a single sample and is color-coded according to ER status; pink dots, ER$^-$ samples; blue dots, ER$^+$ samples; black dots, samples with unknown ER status. In the boxplots, the solid black line represents the median, the upper hinge represents the 75th quartile and the lower hinge represents the 25th quartile. The upper whisker represents the largest observation less than or equal to the upper hinge + 1.5 x IQR, the lower whisker represents the smallest observation greater than or equal to the lower hinge - 1.5 x IQR. (A) *NAT1* RNA expression was significantly higher than *NAT2* RNA expression in the breast cancer cell lines. (B) *NAT1* RNA expression was significantly higher than *NAT2* RNA expression in the primary breast tumor samples. (C) *NAT1* RNA expression was significantly higher than *NAT2* RNA expression in the normal breast tissue samples. ER, estrogen receptor; IQR, interquartile range; *NAT1*, arylamine *N*-acetyltransferase 1; *NAT2*, arylamine *N*-acetyltransferase 2; RPKM, reads per kilobase of transcript per million mapped reads; RSEM, RNA-Seq by Expectation-Maximization.

with *NAT2* expression by 33-, 222- and 52-fold, respectively (P<0.0001 for all; Fig. 2A-C). *NAT1* expression was higher than *NAT2* expression in all 57 breast cancer cell lines tested, with the exception of the UACC-893 cell line, which expressed the highest *NAT2* RNA of any of the breast cancer cell lines analyzed. A total of 15 of the 57 breast cancer cell lines (MDA-MB-134-IV, CAL-120, DU4475, MCF-7, JIMT-1, Hs 281.T, KPL-1, Hs 606.T, HCC70, EFM-19, CAL-148, HCC1569, HMC-1-8, HCC1599 and HCC1395) had no reported *NAT2* RNA expression, whereas all 57 reported *NAT1* RNA expression. The KPL-1 breast cancer cell line has been reported to be contaminated/misidentified and to be an MCF-7 derivative (50).

In TCGA dataset, normal breast tissue samples were collected from patients in which primary breast tumor samples were also collected (but only for 99 individuals), allowing comparison of gene expression between normal breast tissue and primary tumor breast tissue within single individuals. In the primary breast tumor samples, only nine of the 984 samples had higher *NAT2* RNA expression than *NAT1*; of those nine samples, two were ER$^+$ and seven were ER$^-$, and only one sample had a corresponding normal breast tissue sample. Notably, in that individual's normal breast tissue sample, *NAT2* RNA expression was not higher than *NAT1* RNA expression. In the normal breast tissue samples, only one of the 92 samples had higher *NAT2* RNA expression than *NAT1*; the corresponding primary breast tumor sample from the same patient had lower *NAT2* than *NAT1*.

Comparison of gene expression between ER$^+$ and ER$^-$ samples. *ESR1* and *NAT1* gene expression were significantly increased, 86- and 2.6-fold, respectively, in ER$^+$ breast cancer cell lines (P<0.0001 for both; Fig. 3A), whereas *NAT2* gene expression did not significantly vary between ER$^+$ and ER$^-$ breast cancer cell lines (P>0.05; Fig. 3A). Of the breast cancer cell lines with ER status defined in the literature (46-48), a connection between *ESR1* RNA expression and the reported ER status has been observed. According to the literature, samples in the dataset with *ESR1* RNA expression <1.7 RPKM were defined as ER$^-$, whereas samples with *ESR1* expression >2.3 RPKM were defined as ER$^+$. The expression levels of all three genes were significantly higher in ER$^+$ primary breast tumor samples (P<0.0001 for all; Fig. 3B); however. the fold-change between *NAT2* expression in ER$^+$ and ER$^-$ samples was smaller (1.8-fold difference) than for *NAT1* and *ESR1*. In comparison, *ESR1* and *NAT1* were ~108- and 27-fold higher, respectively. The expression levels of genes were not significantly different between ER$^+$ and ER$^-$ normal breast tissue samples (P>0.05 for all; Fig. 3C). Most of the breast cancer cell lines were ER$^-$, whereas most of the primary breast tumor and normal breast tissue samples were ER$^+$.

Comparison of NAT1, NAT2, and ESR1 gene expression between normal breast tissue and primary breast tumors. Differences in gene expression between normal breast tissue and primary breast tumor tissue were evaluated for each gene, *ESR1*, *NAT1*, and *NAT2*. More spread was observed in the primary breast tumor samples compared with the normal breast tissue samples for each gene. *ESR1* and *NAT1* gene expression were significantly elevated 2.5- and 5.9-fold, respectively, in primary breast tumor samples compared with normal breast tissue samples (P<0.0001 for both; Fig. 4). *NAT2* expression was also significantly higher in primary breast tumor samples compared with normal breast tissue samples, but at a lower significance and fold-change (1.4-fold) than *ESR1* and *NAT1* (P<0.05; Fig. 4).

Relationship between previously reported NAT1 N-acetylation activity and NAT1 RNA expression. *NAT1* *N*-acetylation

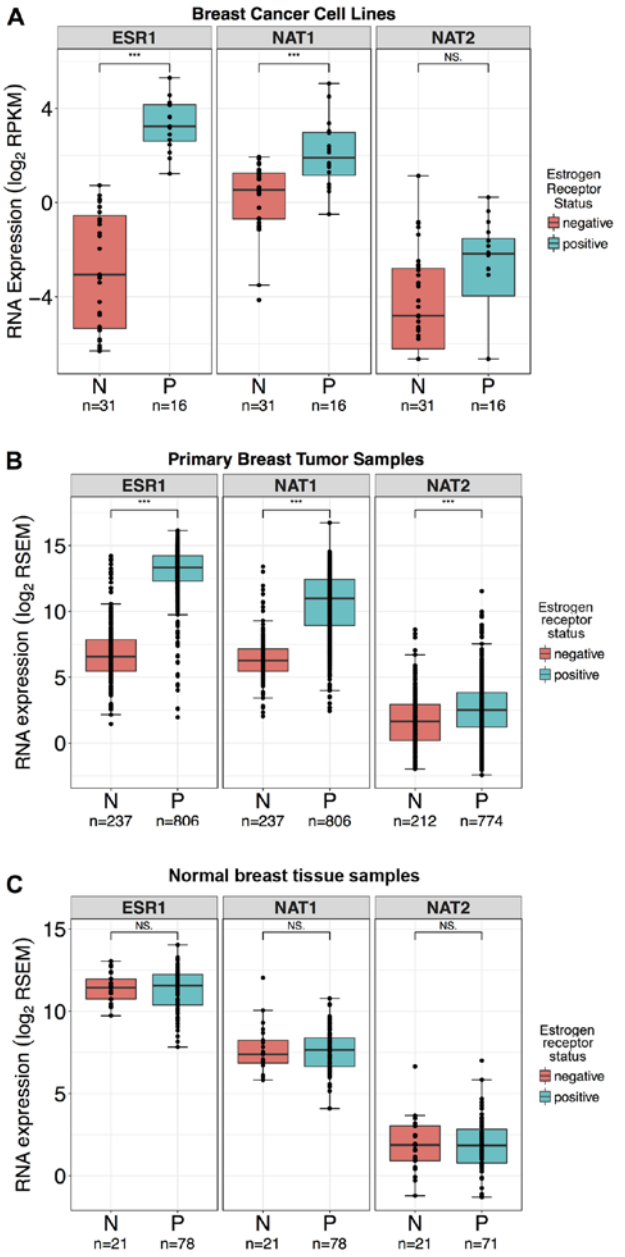

Figure 3. *ESR1*, *NAT1* and *NAT2* RNA expression in breast cancer cell lines, primary breast tumor samples, and normal breast tissue stratified by ER status. Differences in the expression levels of *ESR1*, *NAT1* and *NAT2* genes in breast cancer cell lines, primary breast tumor tissue, and normal breast tissue stratified by ER status were evaluated by Wilcoxon rank-sum test; ***P<0.001; NS, not significant. Boxplots are color-coded according to ER status; pink boxplots, ER$^-$ samples; blue boxplots, ER$^+$ samples. In the boxplots, the solid black line represents the median, the upper hinge represents the 75th quartile and the lower hinge represents the 25th quartile. The upper whisker represents the largest observation less than or equal to the upper hinge + 1.5 x IQR, the lower whisker represents the smallest observation greater than or equal to the lower hinge - 1.5 x IQR. (A) *ESR1* and *NAT1* RNA expression were significantly higher in ER$^+$ breast cancer cell lines compared with ER$^-$ breast cancer cell lines. *NAT2* RNA expression was not significantly different in ER$^+$ breast cancer cell lines compared with ER$^-$ breast cancer cell lines. A total of 10 cell lines had either conflicting reports or no available data for ER status in the literature and were excluded from the analysis. (B) *ESR1*, *NAT1* and *NAT2* RNA expression were significantly higher in ER$^+$ samples compared with ER$^-$ samples in the primary breast tumor dataset. (C) *ESR1*, *NAT1* and *NAT2* RNA expression levels were not significantly different in ER$^+$ samples compared with ER$^-$ samples in the normal breast tissue dataset. ER, estrogen receptor; IQR, interquartile range; *ESR1*, estrogen receptor 1; *NAT1*, arylamine *N*-acetyltransferase 1; *NAT2*, arylamine *N*-acetyltransferase 2; RPKM, reads per kilobase of transcript per million mapped reads; RSEM, RNA-Seq by Expectation-Maximization.

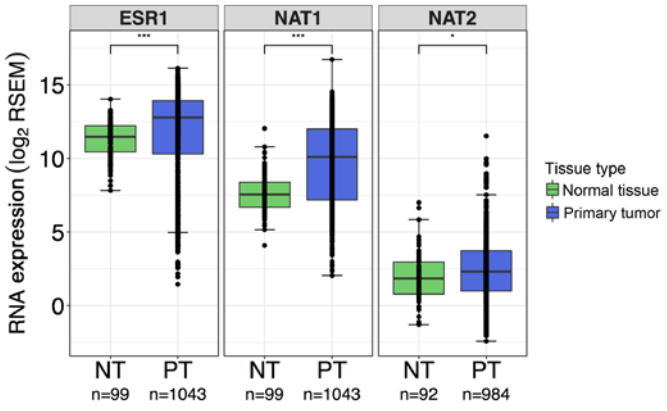

Figure 4. Comparison of *ESR1*, *NAT1* and *NAT2* RNA expression in normal breast tissue and primary breast tumor samples. Differences in gene expression of *ESR1*, *NAT1* and *NAT2* in normal breast tissue and primary breast tumor tissue were evaluated by Wilcoxon rank-sum test; ***P<0.001; *P<0.05. Boxplots are color-coded according to tissue type; green boxplots, normal breast tissue samples; blue boxplots, primary breast tumor samples. In the boxplots, the solid black line represents the median, the upper hinge represents the 75th quartile and the lower hinge represents the 25th quartile. The upper whisker represents the largest observation less than or equal to the upper hinge + 1.5 x IQR, the lower whisker represents the smallest observation greater than or equal to the lower hinge - 1.5 * IQR. For all genes, more spread was observed in data from the primary breast tumor samples compared with the normal breast tissue samples. *ESR1* and *NAT1* gene expression were significantly elevated in primary tumor tissue compared with normal breast tissue. *NAT2* expression was also significantly higher in primary tumor tissue compared with normal breast tissue, but at a lower significance than *ESR1* and *NAT1*. IQR, interquartile range; *ESR1*, estrogen receptor 1; *NAT1*, arylamine *N*-acetyltransferase 1; *NAT2*, arylamine *N*-acetyltransferase 2; RSEM, RNA-Seq by Expectation-Maximization.

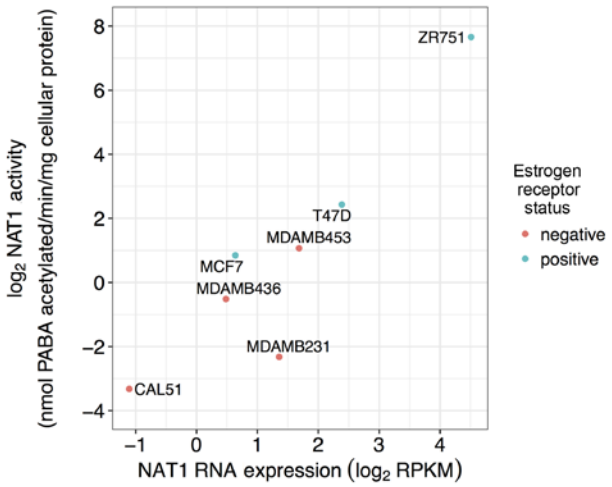

Figure 5. Association between *NAT1* RNA expression and previously reported *NAT1 N*-acetylation activity in seven established breast cancer cell lines. *NAT1* RNA expression from Cancer Cell Line Encyclopedia and previously reported *NAT1 N*-acetylation activity (28) in seven breast cancer cell lines were significantly associated (P<0.05; ϱ=0.89). Dots represent a single cell line and are color-coded according to ER status: Pink dots, ER$^-$ samples; blue dots, ER$^+$ samples. ER, estrogen receptor; *NAT1*, arylamine *N*-acetyltransferase 1; *NAT2*, arylamine *N*-acetyltransferase 2; PABA, *p*-aminobenzoic acid; RPKM, reads per kilobase of transcript per million mapped reads.

activity previously reported in the literature and *NAT1* RNA expression in seven of the 57 breast cancer cell lines were significantly associated (P<0.05) with a high magnitude (ϱ=0.89; Fig. 5).

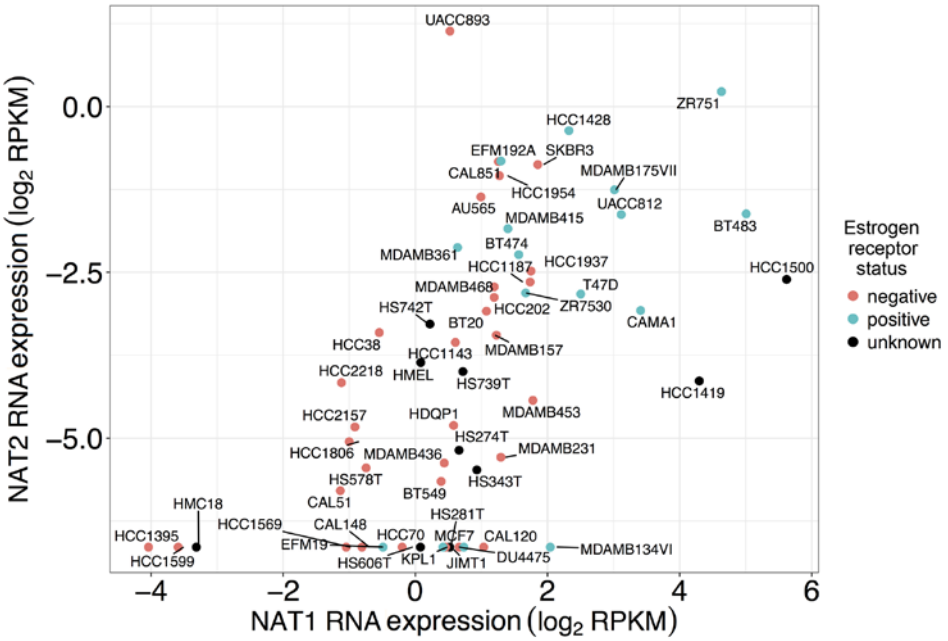

Figure 6. *NAT1* and *NAT2* association in breast cancer cell lines. Association between *NAT1* and *NAT2* RNA expression was analyzed in breast cancer cell lines (each labeled in this figure). Each dot represents a single breast cancer cell line and is color-coded according to ER status; pink dots, ER^- samples; blue dots, ER^+ samples; black dots, samples with unknown or conflicting ER status in the literature (46-48). *NAT1* and *NAT2* RNA expression was significantly associated in breast cancer cell lines ($P<0.0001$, $\varrho=0.64$).

Co-expression of NAT1 and NAT2 RNA expression in established breast cancer cell lines. Co-expression profiles of *NAT1* and *NAT2* RNA for each established breast cancer cell line included in this study are presented in Fig. 6. Of all the cell lines included in the present study, the UACC-893 cell line expressed the highest level of *NAT2* RNA, whereas the HCC1500 cell line expressed the highest level of *NAT1* RNA. The ZR-75-1 cell line expressed high levels of both *NAT1* and *NAT2* RNA, whereas the HCC1395 cell line expressed low levels of both.

Discussion

The present study analyzed established breast cancer cell lines and samples from patients with breast cancer to evaluate the extent to which breast cancer cell lines serve as appropriate models for *NAT1*, *NAT2* and *ESR1* expression in breast tumors. Overall, the present findings demonstrated a strong association between *NAT1* and *ESR1* expression, which is in agreement with previous reports that *NAT1* and *ESR1* are positively associated (25-31), and this association was observed in all three sample types at approximately the same magnitude. These findings suggested that breast cancer cell lines may accurately reflect this relationship and provide a useful model for further research into the relationship. It is well known that *ESR1* expression is frequently altered in breast cancer; therefore, the decrease in association between *NAT2* and *ESR1* in primary breast tumors compared with normal breast tissue samples and established breast cancer cell lines may be due to more dysregulation of *ESR1* than *NAT2* in primary breast tumors. The results of an analysis between *NAT2* and *ESR1* expression suggested that, while *NAT2* and *ESR1* are associated, the magnitude is low.

Interdependence between *NAT1* and *NAT2* expression was moderately high in the breast cancer cell line dataset, but substantially lower in the primary breast tumor and normal breast tissue datasets. Additionally, the strength of the association between *NAT1* and *NAT2* in the breast cancer cell line dataset was similar to the strength of the association observed between *NAT1* and *ESR1* in that dataset; however, in the primary breast tumor and normal breast tissue datasets, the association between *NAT1* and *NAT2* was lower. These findings suggested that breast cancer cell lines may over-represent the interdependence between *NAT1* and *NAT2*, and not fully replicate the relationship observed in primary breast tumors or normal breast tissue.

In the breast cancer cell line data there appears to be a cut-off (between 1.7 and 2.3 RPKM) linking *ESR1* RNA expression and the reported ER status of the breast cancer cell lines. This may provide a method to predict the ER status of breast cancer cell lines that currently have conflicting or unknown ER status in the literature. Using that method, it may be predicted that the HCC1500 and HCC1419 cell lines are ER^+, whereas the HMC-1-8, Hs 742.T, Hs 343.T, Hs 739.T, HMEL, Hs 274.T, Hs 281.T and Hs 606.T cell lines are ER^-. Notably, although 67-82% of breast cancers are ER^+ (51) and most of the primary breast tumor samples were ER^+, the majority of established breast cancer cell lines are ER^-.

NAT1 and *NAT2* RNA expression were reported in almost all samples included in the present study, which concurs with previously published results that have detected *NAT1* and *NAT2* mRNA by RT-PCR in human mammary tissue in smaller cohorts (34-36). *NAT1* RNA expression was significantly higher than *NAT2* RNA expression in the breast cancer cell lines, primary breast tumor samples and normal breast tissue. In addition, with only a few exceptions, *NAT1* RNA expression was always higher than *NAT2* RNA expression in matched samples from the breast cancer cell line, primary breast tumor sample and normal breast tissue sample datasets, thus supporting previous findings that indicated *NAT1* transcripts were 2- to 3-fold higher than *NAT2* transcripts in

human mammary tissues (52). The UACC-893 cell line, the only breast cancer cell line observed in this study to express higher *NAT2* RNA than *NAT1* RNA, is an ER^- and progesterone receptor-negative cell line that has a ~20-fold amplification of the human epidermal growth factor receptor 2/neu oncogene sequence. Further study of this cell line may aid in the identification of additional regulatory mechanisms of *NAT1* and/or *NAT2*, since it expresses a unique profile of *NAT1* and *NAT2* compared with the other breast cancer cell lines.

While *NAT1* expression was reported in all 57 breast cancer cell lines in the present study, 15 of those breast cancer cell lines had no reported *NAT2* RNA expression (Fig. 6). The cell lines with no detected *NAT2* RNA are plotted at ~-6.6 $\log_2$ RPKM NAT2. One of those 15 cell lines, MCF-7, has previously been reported to express *NAT2* RNA expression (35,53) albeit at very low levels. One reason for the difference in observation between this study and the previous studies may be that the detection threshold for *NAT2* was higher when measured by RNA-Seq for the CCLE dataset than in the previous studies. Additionally, in the previous studies that detected *NAT2* RNA in the MCF-7 breast cancer cell line, *NAT1* RNA was not measured at the same time; therefore, direct comparisons of the isozymes was not possible. To the best of our knowledge, *NAT2* RNA expression has not been investigated in any of the other 56 breast cancer cell lines until this study. The results of this study indicated that *NAT2* may be expressed in breast tissues and expression should be considered when studying *NAT1*, due to their overlapping substrate specificities and the high degree of structural similarity.

In normal breast tissue samples no significant difference in gene expression for *ESR1*, *NAT1* and *NAT2* was observed when data was stratified by ER status. However, in the primary breast tumor samples and in the breast cancer cell lines, *ESR1* and *NAT1* exhibited increased expression in the ER^+ samples compared with in the ER^- samples. *NAT2* RNA expression did not significantly vary in breast cancer cell lines when comparing ER^+ and ER^- samples, but was significantly increased in ER^+ primary breast tumor samples compared with in ER^- primary breast tumor samples, although the difference was small. This finding suggested that the dysregulation of *NAT1* and *ESR1* during tumorigenesis may share similar mechanisms; however, *NAT2* does not.

ESR1, *NAT1* and *NAT2* RNA expression were each increased in primary breast tumor samples compared with normal breast tissue samples although the significance and fold-change of *NAT2* were smaller than that of *ESR1* and *NAT1*. Additionally, for all genes, more widely spread expression was observed in the primary breast tumor samples compared with normal breast tissue. These data suggested that expression of all three genes may become modified during breast cancer tumorigenesis; however, the expression of *NAT1* and *ESR1* appear to be dysregulated to a greater extent. As recently reviewed (54), the role of NAT2 in breast cancer etiology is considered to be due to its effects on carcinogen metabolism. The present study suggested that the role of NAT2 in breast cancer is less likely a product of cell transformation, as the expression levels of *NAT2* between normal and tumor tissues exhibited smaller variance than the expression levels of *NAT1* and *ESR1*.

NAT1 N-acetylation activity has been reported in normal breast tissue and breast tumor tissue (34,36-40), whereas NAT2 *N*-acetylation activity has not been observed as consistently; when NAT2 activity is observed the activity is much lower than that of NAT1 activity (34,38,39). Wakefield *et al* profiled *NAT1* expression and activity in seven breast cancer cell lines (MCF-7, T47D, ZR-75-1, Cal51, MDA-MB-231, MDA-MB-437 and MDA-MB-453); *NAT1* mRNA and activity was observed in all seven cell lines (28); however, *NAT2* expression and activity were not co-investigated. The high degree of association between the previously reported NAT1 *N*-acetylation activity and the *NAT1* RNA expression of the same seven breast cancer cell lines suggested that *NAT1* RNA expression is highly reflective of NAT1 *N*-acetylation activity. Gene expression is not always predictive of enzyme activity, due to the numerous regulatory mechanisms that can occur between RNA expression and protein function; however, these results suggested that RNA expression of *NAT1* may serve as an appropriate predictor of NAT1 *N*-acetylation activity. Further studies with an increased number of breast cancer cell lines in which NAT1 *N*-acetylation activity has been measured are required to confirm this hypothesis. Additionally, further studies are required to determine association between *NAT2* RNA expression and NAT2 *N*-acetylation activity.

The CCLE and TCGA repositories offer a wealth of publicly available data. The present study utilized this data to analyze and annotate the previously undefined relationships between *NAT1*, *NAT2* and *ESR1* in breast cancer cell lines, primary breast tumors and normal breast tissue. The results demonstrated that *NAT1* and *NAT2* RNA were expressed in normal breast tissue and primary breast tumor tissue; however, *NAT1* RNA expression was much higher than *NAT2*. The expression of *NAT1* and *NAT2* were found to be associated; however, the magnitude was lower than that observed between *NAT1* and *ESR1* in the primary breast tumors and normal breast tissue. Additionally, although the association between *NAT1* and *NAT2* was slightly exaggerated in the breast cancer cell lines dataset, the cell lines generally reflected the *NAT1* and *NAT2* expression profiles of the primary breast tumors investigated. The present study demonstrated that while *NAT1* and *ESR1* expression were moderately associated in all datasets included in this study, *NAT2* and *ESR1* expression were associated at a lower magnitude, particularly in the primary breast tumor samples.

NAT1 and *ESR1* expression were increased in primary breast tumor samples compared with normal breast tissue samples, and were increased in ER^+ primary breast tumors compared with ER^- primary breast tumors. *NAT2* expression was slightly increased in primary breast tumor samples compared with normal breast tissue samples and in ER^+ primary breast tumors compared with ER^- primary breast tumors. Although *NAT1* and *NAT2* are both implicated in breast cancer, the majority of previous breast cancer studies have investigated each isozyme individually. The present study suggested that both isozymes should be considered in each study, since both are expressed in breast tissues. Defining the association between *NAT1*, *NAT2* and *ESR1* is of great importance, as modification of *NAT1* is currently being studied for breast cancer prevention (20,21,55,56).

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Availability of data and materials

The CCLE data have been deposited in the Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) using accession number GSE36139 and are also available at http://www.broadinstitute.org/ccle. TCGA data portal can be accessed at https://portal.gdc.cancer.gov/ or via FirebrowseR, an R client to the Broad Institute's RESTful Firehose Pipeline.

Authors' contributions

SMC designed the study, retrieved and analyzed all data, and prepared all figures in partial fulfilment of her PhD dissertation carried out under the direction of DWH. Both authors drafted the manuscript. DWH reviewed, modified and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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