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RESEARCH ARTICLE

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DNA methylation in breast cancers: Differences based on estrogen receptor status and recurrence

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Abstract

DNA methylation plays a role in the etiology of primary breast cancers. We analyzed paired primary and second breast tumors to elucidate the role of methylation in recurrence. Methylation profiles from paired primary and second breast tumors of 23 women were assessed using the HumanMethylation450 BeadChip. Twelve women had estrogen receptor positive (ERpos) primary and second tumors, five had estrogen receptor negative (ERneg) primary and second tumors, and six had an ERpos primary tumor but an ERneg second tumor. Stratifying tumors by occurrence revealed that the greater methylation previously associated with ERpos tumors, is more pronounced in primary tumors than in second tumors. Further, ERneg second tumors are more methylated than ERpos second tumors among women who had ERpos primary tumors. Pathway analyses using gene lists generated from comparisons of methylation in ERpos primary tumors from the paired sets with ERpos tumors from six women without recurrences, identified differences between groups based on the ER status of the second tumor. Hypermethylated genes of significantly enriched pathways were differentially associated with survival. DNA methylation profiles of ERpos primary breast tumors support the development and use of tumor methylation profiles for stratifying women with breast cancer both for prognosis and therapy.

KEYWORDS
breast cancer, DNA methylation, estrogen receptor, HumanMethylation450 BeadChip

Decades of expression profiling have revealed the heterogeneity of breast cancers,[1] yet estrogen receptor-α (ER) status remains the primary diagnostic for treatment.[2] Seventy percent of breast cancers are ER positive (ERpos) and therefore many patients can be treated with an antiestrogen or an aromatase inhibitor, such as Tamoxifen[3] and Arimidex,[4] respectively. Despite the treatments available for ERpos breast cancers, 23% of women experience a recurrence within 5 years.[5] Similarly, 34% of women treated with chemotherapy for ER-negative (ERneg) breast cancer, experience a recurrence within 5 years.[6] A greater understanding of the biology underlying recurrence is needed to help stratify tumors and improve treatment.

DNA methylation plays a major role in the etiology of breast cancer[1] and has contributed to our understanding of breast cancer subtypes[7,8] as well as to the biology underlying recurrence.[9] Several studies have shown that ERpos tumors have greater DNA methylation overall than ERneg tumors.[10-13] Fackler and colleagues identified a set of 40 hypermethylated CpG sites specific to either ERpos or ERneg primary breast tumors.[12] We, and others have used

the HumanMethylation27 and 450 BeadChip (HM27 BC and HM450 BC) to provide insight into the breast cancer methylome,[1,12,14] and methylation profiling of primary breast tumors may provide biomarkers of metastasis.[12,13] While previous studies have identified methylation patterns associated with tumor subtype and possibly risk of metastasis, little is known regarding the DNA methylation profiles of recurrent breast tumors and the extent to which methylation patterns in these second tumors are associated with ER status.

Breast cancer literature defines recurrent tumors as those tumors occurring after or during treatment of the primary tumor.[15] Distinguishing true recurrences from new primaries remains difficult using histopathological and clinical characteristics, therefore the term "recurrence" is used broadly to indicate a second tumor that may or may not be a clonal descendant of the primary tumor.[15,16] True recurrent contralateral tumors represent locoregional metastasis and therefore, in general are associated with a less favorable diagnosis than a new primary contralateral tumor.[15,16] While genetic analysis assessing clonal relationships has proven helpful in distinguishing true recurrent and new primary tumors,[15,16] it does not provide a complete molecular profile. A greater understanding of the molecular differences between true recurrent and new primary tumors is important for providing effectively tailored treatment.

The current study was designed to assess the relationship between ER status and methylation profiles in primary and second breast tumors. Using the HM450 BC we examined methylation profiles in 23 pairs of breast tumors selected to determine the extent to which DNA methylation of primary tumors can be used to predict recurrence and ER status of the second tumor.

1 | MATERIALS AND METHODS

1.1 | Human tissue

Institutional Review Board approval was obtained from Baystate Medical Center. Samples identified in the database maintained by the Department of Surgical Pathology at Baystate Medical Center had identifiers removed and were labeled sequentially to ensure patient anonymity. Primary tumors (referred to as first tumors) were matched to a second tumor from the same woman in either the ipsilateral or contralateral breast. HM450 BC data were obtained for 46-paired tumors and assigned group labels for ease of reading as follows: 12 tumor pairs from women with ERpos first (A1) and ERpos second tumors (A2), five tumor pairs from women with ERneg first tumors (B1) and ERneg second tumors (B2), and six tumor pairs from women with ERpos first tumors (C1) and ERneg second tumors (C2). In addition, six ERpos tumors from women with no breast cancer recurrence after a 7-year follow-up period were included for methylation analysis. A summary of characteristics of the 23 subjects and the 46-paired tumors from groups A, B, and C, as well as the data on the six subjects with nonrecurrent (NR) tumors, are shown in Table 1. With the exception of two women (subjects 5 and 6; see Supporting Information Supplemental File 1) all women with ERpos primary tumors received antiestrogen treatment with Tamoxifen.

1.2 | Immunohistochemistry (IHC)

Sections (4 μm) from FFPE tissue blocks were stained for ER, PR (progesterone receptor), HER2 (human epidermal growth factor receptor 2), and Ki67 using the UltraView Universal DAB Detection Kit on the Ventana BenchMark Ultra platform. Antibodies were optimized for each protein: ER (Ventana CONFIRM antiestrogen receptor SP1 rabbit monoclonal primary antibody), PR (Ventana CONFIRM antiprogesterone receptor 1E2 rabbit monoclonal primary antibody), HER2 (Ventana PATHWAY antiHER2/neu antibody 4B5 rabbit monoclonal antibody), and Ki67 (Dako mouse monoclonal MIB-1 antibody). An anatomical pathologist (RJ) scored the slides. Allred scores were recorded for ER ranging from 0 to 8 with a score of 3 and above considered as positive. Similarly, PR status ranged from 0 to 8 with a score above 3 considered as positive. Tumors were considered HER2 positive if 30% of the cells contained 3 + membrane staining. Ki67 was scored as percent of positive cells within the area of invasive cells; with 0% being the lowest score and 100% being the highest; >15 was categorized as high, and ≤5 categorized as low.

1.3 | DNA purification

DNA was purified from tumor tissue obtained from 10 μm sections of FFPE blocks. Briefly, a pathologist (RJ) measured and outlined the breast tumor on a hematoxylin and eosin (H&E) stained slide to estimate the number of sections needed to purify a minimum of 500 ng of DNA. For samples exceeding 4 mm x 4 mm, a single section was sufficient. Tumor cells were collected from the sections by placing an unstained slide on top of the H&E stained slide and carefully removing cells within the parameters defined by the H&E slide. Cells collected from multiple sections were combined in a single tube and DNA was purified using the BiOstic FFPE tissue DNA isolation kit (Mo Bio, Carlsbad, CA).

TABLE 1 Patient and tumor characteristics stratified by ER status of first and second tumors

ER status of first/second tumors		Group A n = 12 pairs Pos/Pos		Group B n = 5 pairs Neg/Neg		Group C n = 6 pairs Pos/Neg		Nonrecurrent n = 6 Pos/NA
Age Mean (SD) range	At first	58.8 (15.9) 37-84		61.4 (12.9) 46-79		53 (8.2) 42-65		53.1 (8.8) 44-69
	At second	65.8 (15.6) 40-90		64.2 (13.6) 48-80		60.3 (6.1) 53-68		NA
Menopausal n (%)	At first	6 (50)		2 (40)		2 (33)		1 (16.5)
	At second	8 (66)		2 (40)		3 (50)		NA
TTR[a] (months) mean (SD) range		84.1 (72.6) 12-252		34 (33.1) 10-90		82.8 (77.7) 17-216		NA
Tumor group		**A1 n = 12**	**A2 n = 12**	**B1 n = 5**	**B2 n = 5**	**C1 n = 6**	**C2 n = 6**	**NR n = 6**
PR status n (%)	+	11 (92)	9 (75)	0 (0)	0 (0)	3 (50)	0 (0)	4 (67)
	−	1 (8)	3 (25)	5 (100)	5 (100)	3 (50)	6 (100)	2 (33)
HER2 status n (%)	+	1 (8)	2 (17)	1 (20)	1 (20)	2 (33)	3 (50)	1 (16)
	−	11 (92)	10 (83)	4 (80)	4 (80)	4 (66)	3 (50)	5 (83)
Ki67 IHC n (%)	low	9 (75)	8 (66)	2 (40)	0 (0)	6 (100)	4 (66)	5 (83)
	high	3 (25)	4 (33)	3 (60)	5 (100)	0 (0)	2 (33)	1 (16)
Tumor grade n (%)	0	1 (9)	2 (16)	0 (0)	0 (0)	2 (33)	2 (33)	1 (16)
	1	3 (27)	2 (16)	0 (0)	0 (0)	0 (0)	0 (0)	1 (16)
	2	4 (36)	3 (25)	0 (0)	0 (0)	3 (50)	0 (0)	3 (50)
	3	3 (27)	5 (41)	5 (100)	5 (100)	1 (16)	4 (66)	1 (16)
Tumor type n (%)	DCIS	1 (8)	2 (16)	0 (0)	0 (0)	2 (33)	2 (33)	1 (16)
	IDC	8 (66)	6 (50)	5 (100)	5 (100)	3 (50)	4(66)	3 (50)
	ILC	2 (16)	3 (25)	0 (0)	0 (0)	0 (0)	0 (0)	2 (33)
	IDLC	1 (8.3)	1 (8)	0(0)	0 (0)	1(16)	0 (0)	0 (0)
Tumor size n (%)	≥ 20 mm	6 (50)	7 (58)	3 (60)	3 (60)	5 (83)	4 (66)	3 (50)
	< 20 mm	6 (50)	5 (41)	2 (40)	2 (40)	1 (16)	2 (33)	3 (50)

NA = not applicable.
[a]TTR = time to recurrence.

1.4 | Illumina HumanMethylation450 BeadChip (HM450 BC)

DNA was sent to the core facility at the University of Southern California (USC) for HM450 BC (Illumina, San Diego, CA) analysis as previously described.[14,17] Data are available from the Gene Expression Omnibus (GSE 117439). A beta (β) value of 1 corresponds with complete methylation of DNA at the probe site and a β value of 0 indicates no methylation at the probe site.

1.5 | Data analysis

GenomeStudio methylation module software was used to analyze methylation data from the HM450 BC. Only those sites with a detection *P* value of ≤0.01 were included in the analysis (Illumina) to ensure that only CpGs with complete bead hybridization were used in the analysis. CpG sites where common single-nucleotide polymorphisms (SNPs) occur within 10 base pairs of the site were excluded, due to literature reports indicating SNPs in this region can affect probe binding.[18] For most analyses, a 2-fold cutoff in each direction was used to determine hypermethylated or hypomethylated CpG sites. This cutoff was selected based on previously reported literature values.[12,19,20] Where noted in the article, a 1.5-fold cutoff and a false discovery rate of 0.05 was computed in GenomeStudio for each CpG site and only those sites with a DiffScore of ≥22 in either direction were considered statistically significant. STATA (http://www.stata.com) was used to compute unpaired student *t* test and one-way analysis of variance, while Prism (GraphPad Software Inc.) was used to calculate nonparametric *t* tests. Those analyses with a *P* value of <0.05 were considered statistically significant. Gene lists were generated from the lists in GenomeStudio of differentially methylated CpG sites (dmCpGs). Five gene regions are included on the HM450 BC: promoter (TSS200 and TSS1500 regions; sites 200 and 1500 bp upstream of the

transcriptional start site respectively), 5′UTR/1st exon, body, 3′UTR, and intergenic (areas not included in the previous four regions and undefined in GenomeStudio, therefore they are not included in the pathway analyses).[20] In the case of multiple transcripts or gene overlap, a single CpG site (or MAPINFO coordinate in GenomeStudio) may represent multiple genes or gene regions.[14,21] Panther Classification System (www.pantherdb.org) was used to conduct pathway analysis from the lists of genes associated with hyper- and hypomethylated dmCpGs. Kaplan-Meier analyses (http://kmplot.com/) were conducted using genes associated with differential methylation in multiple comparison groups.

2 | RESULTS

2.1 | Patient demographics

Patient demographics and tumor characteristics from the 46-paired tumors and the six nonrecurrent tumors are shown in Table 1. Considering women with recurrent breast cancer: mean age at first and second tumor was lowest for group C (women with ERpos first tumors who had ERneg second tumors); however, neither age at first tumor nor at second tumor differed significantly among the three groups ($F = 0.58$; $P = 0.57$ for age at first; $F = 0.33$; $P = 0.73$ for age at second tumor). Time to recurrence was shortest for women with ERneg first and second tumors (group B): a mean of 34 months compared with 84.1 and 82.8 for groups A and C; but again this difference was not significant ($F = 1.044$; $P = 0.37$).

While most of the diagnostic and pathologic tumor characteristics, other than ER status, did not differ notably among groups, some interesting trends were observed. Group A had the most PR positive tumors with 92% in A1 and 75% in A2. All tumors in the ERneg B1 and B2 groups stained negative for PR as did the tumors in C2. Tumors in C1 were split evenly, with 50% of tumors staining positively for PR. Tumors in group C, had 33% and 50% positive staining for HER2 in C1 and C2, respectively, while the majority of tumors in groups A and B stained negative for HER2. Ki67 staining was high in both B groups and mostly low in the A and C groups. Tumors in the A group had an even distribution of tumor grade ranging from 0 to 3 among the samples, group B tumors were all grade 3, and group C did not contain any grade 1 samples. For all paired groups, invasive ductal carcinoma (IDC) was the most prevalent tumor type. Group C and more specifically group C1, had the greatest number of tumors larger than 20 mm (83%). The six nonrecurrent tumors occurred in women with a mean age of 53.1 years, most similar to the C1 group. All NR tumors were ERpos, with 67% scoring positive for PR and 83% scoring negative for HER2.

2.2 | ER-positive tumors exhibit greater methylation than ER-negative tumors

As shown in the box and whisker plots (Figure 1A), the overall mean β value for all CpGs is slightly greater among ERpos tumors than ERneg tumors (0.42; SD 0.27, and 0.40; SD 0.26, respectively). In addition, the number of hypermethylated CpGs (those with a fold difference ≥ 2) is greater among ERpos tumors: ERpos tumors have 2.6 times more hypermethylated CpGs as compared with ERneg tumors (2910 vs 1118) (Figure 1B,E). The marginally higher mean β value and number of hypermethylated CpGs in ERpos tumors is consistent with the literature in which mostly first tumors were examined.[10-13] Because our tissue samples consisted of an equal number of first and second tumors, we next asked whether the pattern of greater methylation in ERpos tumors was present in both first and second tumors. Restricting the comparison to first tumors revealed 8.7 times more hypermethylated CpGs in ERpos as compared with ERneg tumors (9797 vs 1120) (Figure 1C,E). In contrast, restricting the analysis to only second tumors, showed a greatly reduced difference with only 1.38 times more hypermethylated CpGs in the group of ERpos second tumors as compared with the ERneg second tumors (2507 vs 1813) (Figure 1D,E). Broadening the definition of hypermethylated CpGs, to those with a fold difference ≥ 1.5, reveals a similar trend among the three groups: ERpos tumors have more hypermethylated CpGs than ERneg tumors (Figure 1E).

2.3 | Second breast tumors: Methylation varies by ER status of both the first and second tumor

Data presented above along with published results show that, at least for a subset of CpGs, ERpos tumors exhibit greater hypermethylation than do ERneg tumors.[10-13] Two findings suggest that this pattern may be altered by the occurrence status (first vs second) of the tumor. First, aberrant DNA methylation was shown to increase with tumor progression,[22] and second, in our data (Figure 1D,E) the pattern of increased methylation among ERpos as compared with ERneg tumors was greatly reduced when the analysis was restricted to second tumors. Therefore, we examined DNA methylation stratifying by ER status of both the first and second tumor.

As shown in Figure 2, comparison of first and second tumors within each group reveals distinct characteristics for the three groups: 264 differentially methylated CpGs

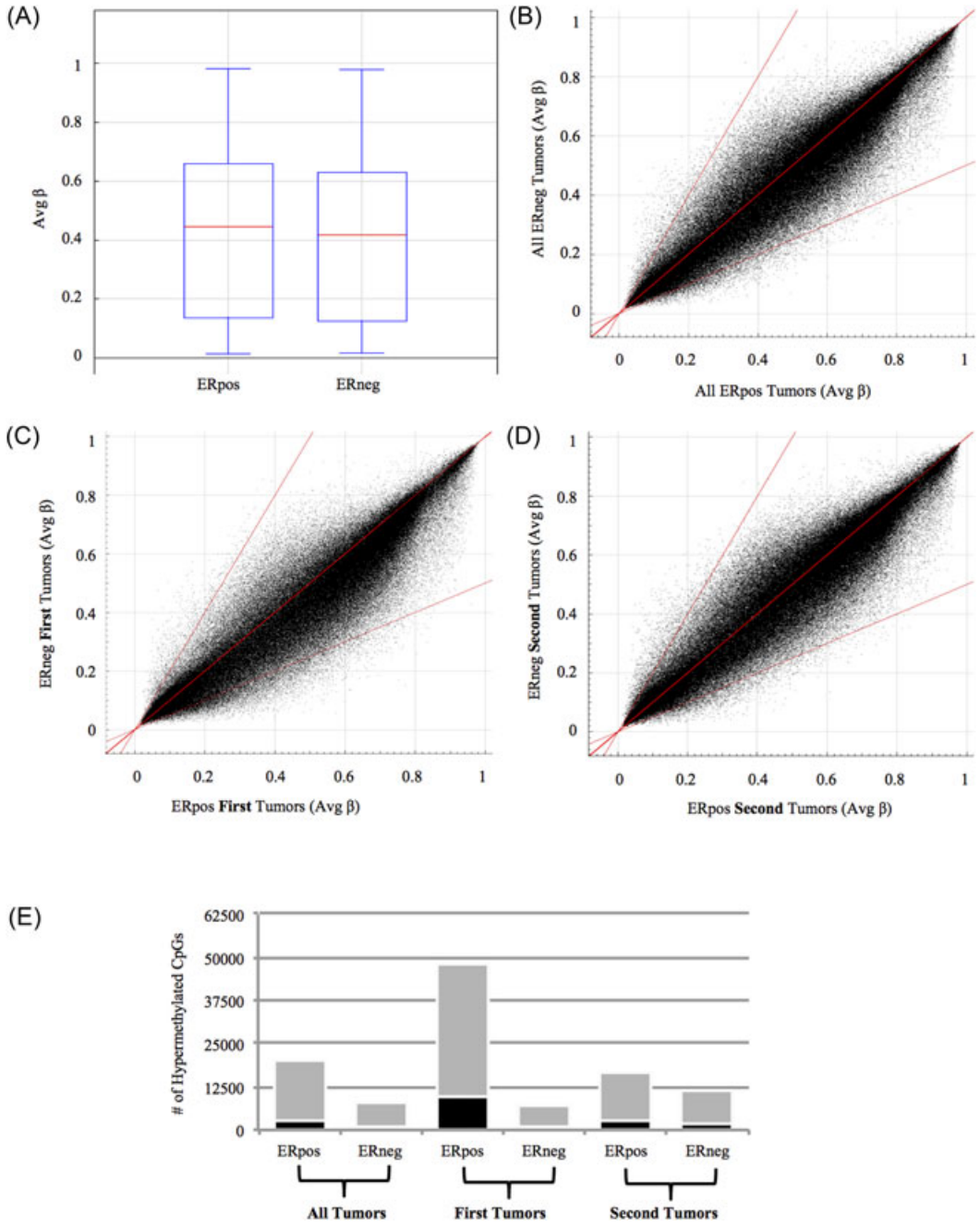

FIGURE 1 Global methylation levels in the set of paired tumors from 23 women. ERpos and ERneg tumors: all paired tumors, first tumors, and second tumors. A, Box and whisker plot shows mean, 75th and 95th percentile β values of CpGs on the Illumina HM450 BC for all 46 tumors (30 ERpos and 16 ERneg); B, Scatter plots for ERpos vs ERneg tumors. Dots in scatter plots represent mean β values of specific interrogated CpGs for all 46 tumors; C, Scatter plot restricting analysis to **first tumors** (18 ERpos and 6 ERneg); D, Scatter plot showing only **second tumors** (12 ERpos and 11 ERneg). Outer red lines mark 2.0-fold difference in β values between ERpos and ERneg breast tumors; E, Number of hypermethylated CpGs in ERpos and ERneg tumors stratified by occurrence; black bar ≥ 2.0-fold difference, full bar ≥ 1.5-fold difference

(dmCpG) in A1:A2, 2441 dmCpG in B1:B2, and 7016 dmCpG in C1:C2 (Figure 2A-C). Further, in groups A and B, the first tumors have a greater number of hypermethylated CpGs compared with second tumors, with 3.9 and 1.7 times more respectively (210 in A1 vs 54 in A2, and 1539 in B1 vs 902 in B2: 902; Figure 2A,B), and this trend was amplified when hypermethylated CpGs were defined as those with a fold difference ≥ 1.5 (Figure 2D). In contrast, this pattern was reversed within group C where *second tumors* had a slightly *greater* number of hypermethylated CpGs (3358 in C1 vs 3657 in C2; Figure 2C) and this trend was strengthened when the analysis was based on a fold difference ≥ 1.5 (Figure 2D). This reversal in pattern (greater methylation in the

second tumors of group C) was accompanied by a change in the distribution of β values. As shown in Figure 3, the distribution of hypermethylated values in group C2 differs notably from the other groups with more than half of the dmCpGs having a mean β above 0.3. In contrast, essentially all of the hypermethylated CpGs in groups A1 and A2, and 90% of the hypermethylated CpGs in groups B1, B2, and C1 had β values *below* 0.3.

Group C2 also differed with respect to the gene location of the hypermethylated CpGs: the percentage of hypermethylated CpGs was greatest in the gene body of group C2, while for all other groups, the fraction of hypermethylated CpGs was greatest in the promoter region (Figure 4). In addition to the increased methylation in the body region, C2 tumors had the highest percentage of hypermethylated CpGs in the intergenic region (25% vs 14%, 13%, 9%, 20%, and 15% for groups A1, A2, B1, B2, and C1, respectively for scored sites on the chip).

2.4 | Second breast tumors: ERneg tumors recurring after ERpos first tumors are more often new primaries

We next asked what factors might explain the difference in methylation profiles observed between C2 tumors and other groups of second tumors. Second breast tumors may be either true recurrences or new primaries (de novo tumors), and given the clonal nature of DNA methylation, it has been proposed that true recurrent tumors should share methylation profiles with their primary tumor.[15] Therefore, we asked whether C2 tumors were more likely to be new primaries and whether designation of new primary or true recurrence was related to DNA methylation profile. The 23 tumor pairs were evaluated by a pathologist (RJ), and the second tumor was scored as either a new primary or true recurrence based on histology and location[23] (see Supporting Information

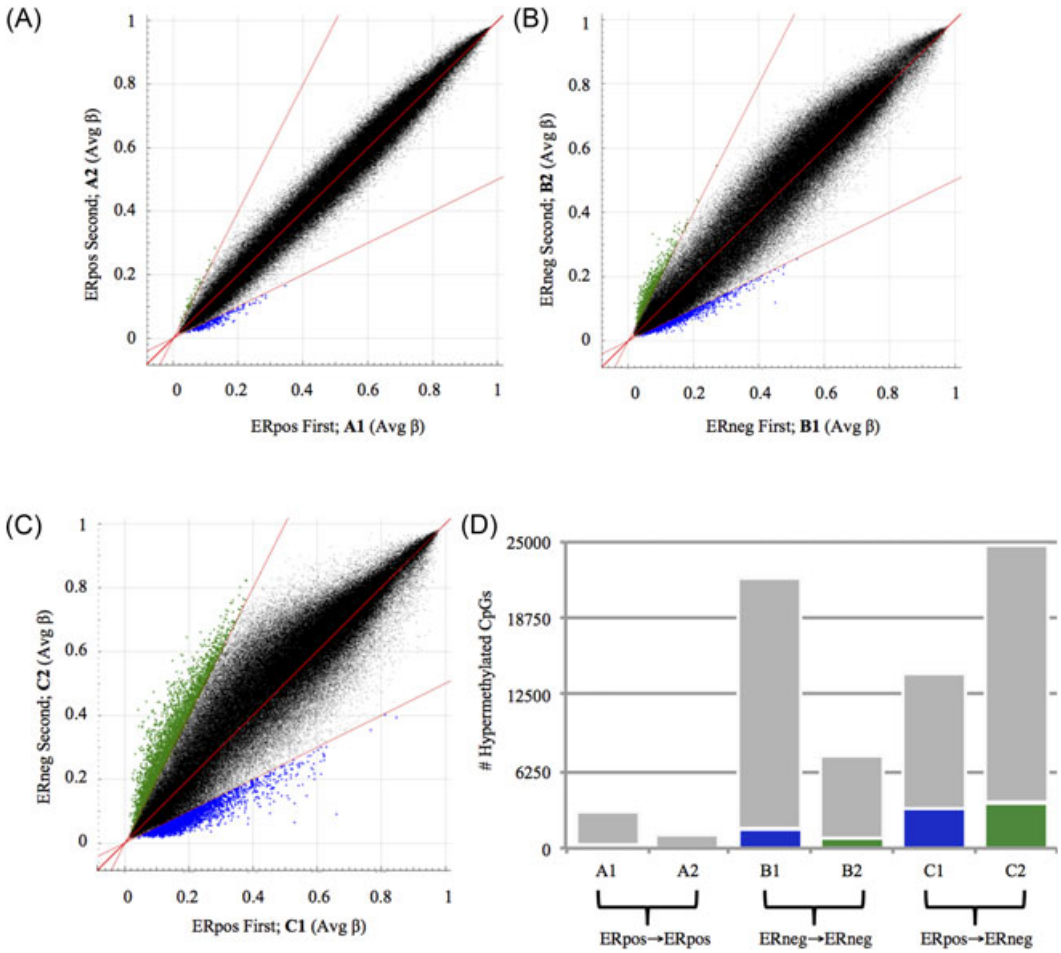

FIGURE 2 Methylation differences between first and second tumors stratified by ER. Scatterplots show average β values for tumors from three groups of women. Outer red lines mark the 2-fold difference in β values between groups. Green dots represent CpGs hypermethylated in second tumors and blue dots represent CpGs hypermethylated in first tumors. A, Both the first and second tumors were ERpos; B, Both the first and second tumors were ERneg; C, The first tumor was ERpos and the second tumor was ERneg; D, Bar graph shows the number of hypermethylated CpGs corresponding to scatterplots; blue and green solid fill ≥ 2.0-fold difference, full bar ≥ 1.5 fold difference

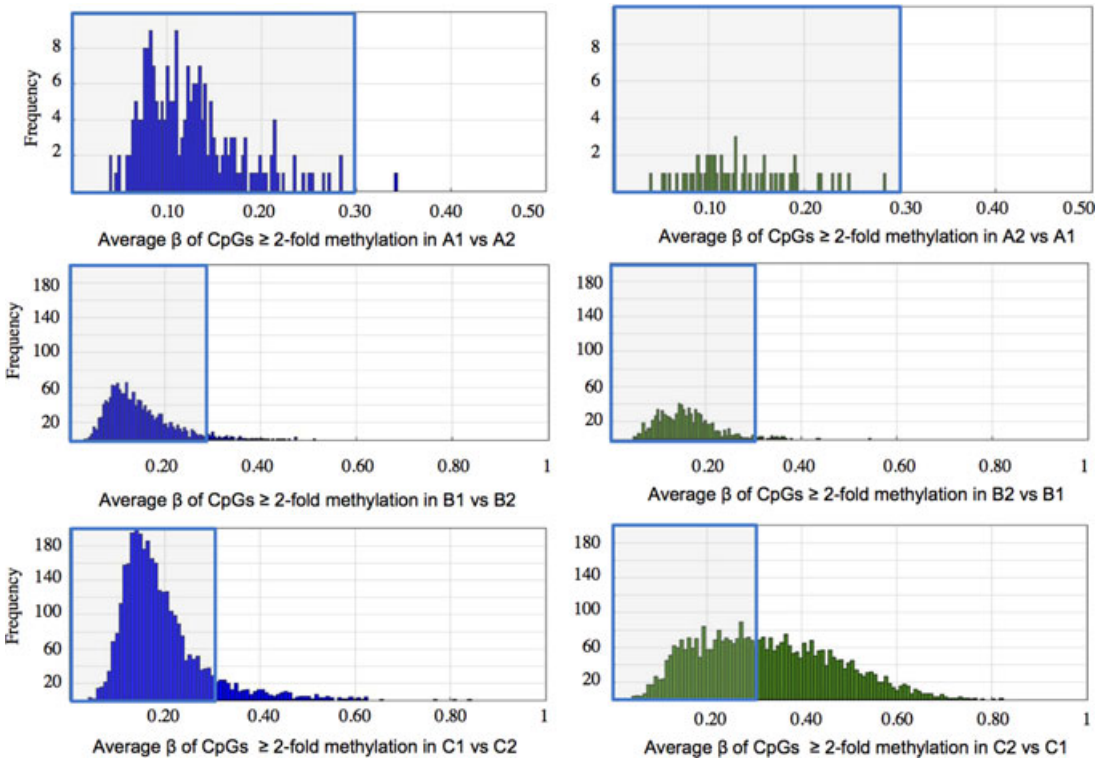

FIGURE 3 Distribution of β values of hypermethylated CpGs from comparisons of first and second tumors: stratified by ER. Within each group (A, B, and C), the frequency of hypermethylated CpGs in first tumors (blue histogram) and in second tumors (green histogram) corresponds to the hypermethylated CpGs in Figure 2A-C. The box includes β values ≤ 0.3

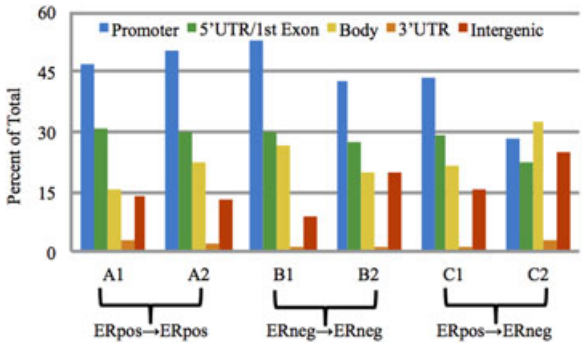

FIGURE 4 Percentage of hypermethylated CpGs across gene regions from comparisons of first and second tumors: stratified by ER. Within each of the three groups, the gene location was determined for CpGs with mean β values having a 2-fold or greater difference between the first and second tumor

Supplemental File 2). Table 2 shows the three groups of women (A, B, and C) who contributed a first (A1, B1, and C1) and second (A2, B2, and C2) breast tumor, the location of the second tumor compared with the first, and the designation as true recurrence or new primary. As noted above, within groups A and B both the first and second tumors had the same ER status (ERpos in group A and ERneg in group B), while within group C the ER status differed between the first and second tumors (ERpos and ERneg, respectively). C2 tumors were more likely to occur in the contralateral breast and to be scored as a new primary. Five of the six (83%) ERneg C2 tumors were considered new primaries (only one new primary occurred in the ipsilateral breast), while none of the five ERneg B2, and only four of the 12 (33%) ERpos A2 tumors were considered a new primary. Visual inspection of scatter plots comparing β values of the first and second tumors for each of the 23 pairs (Supporting Information Supplemental File 1), suggest that pairs with new primary have a greater number of dmCpGs as compared with pairs with true recurrences. This is especially apparent among group C in which the only pair scored as having a true recurrence (subject 18) has strikingly fewer dmCpGs. Further, statistical analysis showed pairs in which the second tumor was a new primary had a greater number of hypermethylated CpGs as compared with pairs in which the second tumor was a true recurrence (Supporting Information Supplemental File 3; Mann-Whitney $U = 27$; $P = 0.025$).

Interestingly, comparison of the group of true recurrences (n = 14; 9 ERpos and 5 ERneg) with the group of new primaries (n = 9; 3 ERpos and 6 ERneg) reveals a different pattern. True recurrences have roughly twice the number of hypermethylated CpGs (1195 vs 607), and increased promoter methylation as compared to the new primaries (Supporting Information Supplemental Files 4 and 5),

TABLE 2 Tumors stratified by ER status, occurrence, and side of second tumor

	First Tumor		Second Tumor			
	ERpos	ERneg	ERpos		ERneg	
Group			Side (#)	T/N (#)	Side (#)	T/N (#)
A	12	–	Ips (8)	T (8)	–	–
			Con (4)	N (4)	–	–
B	–	5	–	–	Ips (5)	T (5)
			–	–	Con (0)	N (0)
C	6	–	–	–	Ips (2)	T (1)
			–	–	Con (4)	N (5)

Con = contralateral; Ips = ipsilateral; N = new primary; T = true recurrence.

supporting the observation of increased methylation with tumor progression.[22]

2.5 | Comparisons between ERpos first tumors from women who had second tumors and from women who did not have second tumors

The following analyses were conducted to determine if first tumor methylation patterns could provide insight into the likelihood of an occurrence of a second tumor, the effect of hormone treatment, second tumor ER status and the biology underlying recurrence. We stratified ERpos first tumors by the ER status of the second tumor and then compared the first tumors with each other and with a group of six ERpos tumors from women who did not have a second tumor (NR). The comparison groups along with the number of dmCpGs (both hyper and hypomethylated with a > 1.5-fold cutoff in each direction) and the number of genes in which the dmCpGs occurred are shown in Table 3. Several trends are apparent from the data. First, in the comparisons between the first tumors that recurred and those that did not recur, there are more hypomethylated CpGs than hypermethylated CpGs (A1 vs NR = 6748 hypo/3301 hyper or 2.04 fold; C1 vs NR = 6903 hypo/4321 hyper or 1.60 fold). And second, there are roughly twice as many dmCpGs as there are genes with a dmCpG within both the hypomethylated and hypermethylated comparisons. We also compared the gene lists, and determined that there were twice as many hypermethylated genes shared in the C1:NR and C1:A1 lists as compared with the A1:NR and A1:C1 lists (729 vs 327, respectively).

To develop an understanding of the tumor biology underlying these dmCpG patterns, we conducted pathway analyses using Panther with the gene lists in Supporting Information Supplemental File 6. We compared ERpos first tumors (A1 and C1) with ERpos NR tumors, and highlighted the common themes by examining the shared pathways (A1:NR and C1:NR). Restricting the comparisons to the significant GO terms with fold enrichment (FE) ≥ 2 showed 21 terms (Table 4) from the lists of genes with hypermethylated CpGs, and 157 terms (Supporting Information Supplemental File 7) from the lists of genes with hypomethylated CpGs. Several of the common themes in the hypermethylated list (Table 4): morphogenesis (GO terms 8 and 12-13), neuro (GO terms 8, 11, and 16-17), and development (Go Terms 1, 4, and 14), are also common in the hypomethylated list (Supporting Information Supplemental File 7): morphogenesis (34 terms), neuro (21 terms), and development (57 terms), prompting us to compare the GO terms in the hyper- and hypomethylated lists. Eighteen of 21 GO terms derived from the hypermethylated list are also present in the hypomethylated list, leaving 139 (88.5%) GO terms unique to the hypomethylated list. Of the terms unique to the hypomethylated list, neurogenesis (GO:0022008) had the greatest significance (8E-43) and several daughter terms of neurogenesis, including generation of neurons (GO:0048699) and neuron differentiation

TABLE 3 Number of differentially methylated CpG sites and genes in group comparisons

	Hypermethylated[a]			Hypomethylated[a]		
	A1:NR	C1:NR	C1:A1	A1:NR	C1:NR	C1:A1[b]
CpGs	3301	4321	4734	6748	6903	3548
Genes	1979	2057	1956	2893	3149	1900

[a]>1.5 fold methylation difference.
[b]For simplicity in this table we present the C1:A1 hypomethylated CpGs, which could also be presented as A1:C1 hypermethylated.

TABLE 4 GO terms with ≥ 2-fold enrichment (FE) in A1 and C1 as compared to nonrecurrent (NR) tumors based on genes with significantly hypermethylated CpGs

	GO biological process complete & number of genes in term		A1 *vs.* NR			C1 *vs.* NR		
			Gene #	FE	p	Gene #	FE	p
1	axon development (GO:0061564)	377	72	2.11	6.0E-05	93	2.6	4.0E-12
2	axon guidance (GO:0007411)	218	50	2.54	5.0E-05	55	2.66	2.0E-06
3	axonogenesis (GO:0007409)	349	70	2.22	1.0E-05	90	2.72	9.0E-13
4	camera-type eye development (GO:0043010)	289	53	2.03	2.0E-02	62	2.27	6.0E-05
5	cell fate commitment (GO:0045165)	225	57	2.8	1.0E-07	62	2.91	3.0E-09
6	cell fate specification (GO:0001708)	67	22	3.63	4.0E-03	25	3.94	1.0E-04
7	cell morphogenesis involved in differentiation (GO:0000904)	499	91	2.02	6.0E-06	109	2.31	4.0E-11
8	cell morphogenesis involved in neuron differentiation (GO:0048667)	401	76	2.1	3.0E-05	96	2.53	8.0E-12
9	cell-cell adhesion (GO:0098609)	433	80	2.04	4.0E-05	83	2.02	3.0E-05
10	cell-cell adhesion via plasma-membrane adhesion molecules (GO:0098742) central nervous	217	55	2.8	3.0E-07	61	2.97	2.0E-09

(Continues)

TABLE 4 (Continued)

GO biological process complete & number of genes in term			A1 *vs.* NR			C1 *vs.* NR		
			Gene #	FE	p	Gene #	FE	p
11	system neuron differentiation (GO:0021953)	174	39	2.48	4.0E-03	57	3.46	3.0E-11
12	embryonic organ morphogenesis (GO:0048562)	283	53	2.07	1.0E-02	58	2.16	8.00E-04
13	eye morphogenesis (GO:0048592)	143	32	2.48	4.0E-02	37	2.73	8.00E-04
14	forebrain development (GO:0030900)	374	69	2.04	4.0E-04	82	2.32	8.0E-08
15	homophilic cell adhesion via plasma membrane adhesion molecules (GO:0007156)	156	48	3.4	8.0E-09	49	3.32	1.0E-08
16	neuron fate commitment (GO:0048663)	69	24	3.85	4.0E-04	25	3.83	2.0E-04
17	neuron projection guidance (GO:0097485)	220	50	2.51	7.0E-05	56	2.69	8.0E-07
18	pattern specification process (GO:0007389)	404	79	2.16	4.0E-06	90	2.35	3.0E-09
19	regionalization (GO:0003002)	312	63	2.23	7.0E-05	74	2.5	2.0E-08
20	retina development in camera-type eye (GO:0060041)	135	31	2.54	4.0E-02	35	2.74	2.0E-03
21	tube morphogenesis (GO:0035239)	337	62	2.03	2.0E-03	68	2.13	1.0E-04

Pathway analyses using genes with hypermethylated CpGs was conducted using the Panther Classification System (www.pantherdb.org) released on 2017-2-28. Shading highlights the three pathways that are unique to the hypermethylated comparison (ie, not found in Supporting Information Supplemental File 7).

(GO:0030182) were also significantly hypomethylated. However, other highly significant daughter terms of neurogenesis were present in the hypermethylated list: neuron fate commitment (GO:0048663), cell fate specification (GO:0001708), and cell fate commitment (GO:0045165) (see Ancestor Chart, Supporting Information Supplemental File 8).

Given that most pathways in the hypermethylated list were also in the hypomethylated list (82%), we next asked to what extent the hyper- and hypomethylated pathways shared genes. On average, 46% of the genes in A1:NR hypermethylated GO terms were also in the A1:NR hypomethylated GO terms (range: 33% to 77%), and 33% of the genes in the C1:NR hypermethylated GO terms were also in the C1:NR hypomethylated GO terms (range: 23% to 68%). There was also considerable overlap of genes shared between hypermethylated A1:NR and C1:NR GO terms (36%; range: 29% to 59%) and between hypomethylated A1:NR and C1:NR GO terms (53%; range: 49% to 58%).

By comparing GO terms between two groups of ERpos first tumors (A1 and C1) with distinct second tumor ER status outcomes, we next asked whether biological differences were apparent in ERpos first tumors from women with ERpos versus ERneg second tumors. The full lists of significant GO terms with FE $\geq$ 2 are shown in Supporting Information Supplemental File 9 (110 GO terms generated from the list of genes with significantly hypermethylated CpGs in A1 vs C1) and Supporting Information Supplemental File 10: (189 GO terms generated from the list with of genes with significantly hypermethylated CpGs in C1 vs A1). A total of 224 different genes comprise the two lists with 75 genes (33%) shared. Table 5 shows the top 20 GO terms (based on *P* values) in comparisons of A1 versus C1 and highlights GO terms representing pathways that are over represented with hypermethylated genes in only A1 (positive regulation of cell differentiation (GO:0045597), or only C1 (nervous system development (GO:0007399), cell-cell signaling (GO:0007267), central nervous system development (GO:0007417), regulation of ion transport (GO:0043269), brain development (GO:0007420), and behavior (GO:0007610)).

To further our understanding of the biological differences between true recurrent and new primary tumors, we conducted pathway analyses using gene lists generated from the dmCpGs in the 14 true recurrent versus the nine new primary breast tumors. Significant GO terms with FE $\geq$ 2 are shown in Supporting Information Supplemental File 11. Only 14 GO terms were identified from genes hypermethylated in new primary tumors, while 60 terms were generated from the genes hypermethylated in true recurrent tumors. There was little overlap between the groups (2 pathways: cell-cell adhesion via plasma-membrane adhesion molecules (GO:0098742) and homophilic cell adhesion via plasma membrane adhesion molecules (GO:0007156)) and in general the *P* values were not highly significant.

In the final analysis, we asked to what extent the hypermethylated genes comprising the significantly enriched pathways were associated with survival. We selected pathways uniquely hypermethylated in ERpos first tumors as compared with the NR group for women with ERpos second tumors (A1:NR and A1:C1) or women with ERneg second tumors (C1:NR and C1:A1). Table 6 shows results of the Kaplan-Meier analysis based on gene expression. Positive regulation of cell differentiation (GO0051962) contained 17 genes that were hypermethylated in both A1:NR and A1:C1, of which 13 were significantly associated with survival, and for 12 of the 13 genes, high expression was related to increased survival. Cell-cell signaling (GO0007267) contained 89 genes that were hypermethylated in both C1:NR and C1:A1, of which 69 were significantly associated with survival, and for 51 of the 69, high expression was related to increased survival.

3 | DISCUSSION

The molecular heterogeneity of breast tumors contributes to a disease that, while effectively treated in most women, recurs within 5 years of treatment in 20% to 30% of women.[5] Importantly, DNA methylation is a significant component of the breast tumor molecular heterogeneity. To date, studies aimed at elucidating the impact of tumor DNA methylation on treatment outcome have compared the methylation profiles among subtypes of primary tumors,[21,24] and compared primary tumors from women who developed, and women who did not develop metastases.[12] These studies have identified specific CpGs, genes and pathways that are differentially methylated in breast tumor subtypes as well as potentially important in disease progression.[12,21,24] A handful of studies have examined DNA methylation in metastatic breast tumors,[25,26] and only one publication compared DNA methylation in pairs of primary and second breast tumors obtained from the same women; however, only 13 genes were examined.[15] A major contribution of our study is the comprehensive methylation profiling (HM450 BC) of 23 pairs of primary and second breast tumors. The inclusion of 18 tumor pairs from women with ERpos primary tumors provided the opportunity to further our understanding of DNA methylation patterns that develop under the selection pressure of antiestrogen treatment.

Several publications have reported greater methylation of ERpos tumors as compared with ERneg tumors,[10-13] although specific CpGs and regions within ERneg tumors are associated with increased methylation.

TABLE 5 Top 20 (highest *P* values) GO terms with ≥ 2-fold enrichment in A1 versus C1 or C1 versus A1 based on genes with significantly hypermethylated CpGs

GO biological process complete & number of genes in term			A1 *vs.* C1		
			Gene #	FE	p
1	neurogenesis (GO:0022008)	1521	266	2	8.0E-23
2	generation of neurons (GO:0048699)	1422	251	2.02	7.0E-22
3	neuron differentiation (GO:0030182)	940	180	2.19	4.0E-18
4	embryonic morphogenesis (GO:0048598)	564	119	2.42	6.0E-14
5	positive regulation of cell differentiation (GO:0045597)	887	160	2.07	2.0E-13
6	regulation of neurogenesis (GO:0050767)	727	138	2.17	7.0E-13
7	animal organ morphogenesis (GO:0009887)	888	158	2.04	1.0E-12
8	regulation of nervous system development (GO:0051960)	818	149	2.09	1.0E-12
9	development (GO:0048666)	749	140	2.14	1.0E-12
10	regulation of neuron differentiation (GO:0045664)	605	121	2.29	2.0E-12
11	embryonic organ development (GO:0048568)	427	96	2.58	2.0E-12
12	cell fate commitment (GO:0045165)	233	66	3.24	4.0E-12
	regulation of cell				

(Continues)

TABLE 5 (Continued)

GO biological process complete & number of genes in term			A1 *vs.* C1		
			Gene #	FE	p
13	development (GO:0060284)	841	150	2.04	5.0E-12
14	homophilic cell adhesion via plasma membrane adhesion molecules (GO:0007156)	158	52	3.77	2.0E-11
15	tube development (GO:0035295)	581	114	2.25	4.0E-11
16	cell-cell adhesion via plasma-membrane adhesion molecules (GO:0098742)	216	61	3.23	6.0E-11
17	positive regulation of nervous system development (GO:0051962)	484	97	2.3	2.0E-09
18	cell morphogenesis involved in neuron differentiation (GO:0048667)	392	84	2.45	2.0E-09
19	pattern specification process (GO:0007389)	414	87	2.41	2.0E-09
20	embryonic organ morphogenesis (GO:0048562)	290	69	2.73	3.0E-09
GO biological process complete & number of genes in term			**C1 *vs.* A1**		
			Gene #	**FE**	**p**
1	nervous system development (GO:0007399)	2260	477	2.34	3.0E-66

(Continues)

TABLE 5 (Continued)

GO biological process complete & number of genes in term			A1 *vs.* C1		
			Gene #	FE	p
2	(GO:0022008)	1521	315	2.3	7.0E-39
3	generation of neurons (GO:0048699)	1422	297	2.32	5.0E-37
4	neuron differentiation (GO:0030182)	940	211	2.49	6.0E-29
5	cell-cell signaling (GO:0007267)	1167	242	2.3	1.0E-28
6	regulation of nervous system development (GO:0051960)	818	181	2.46	1.0E-23
7	central nervous system development (GO:0007417)	931	195	2.33	8.0E-23
8	neuron development (GO:0048666)	749	167	2.48	8.0E-22
9	positive regulation of nervous system development (GO:0051962)	484	119	2.73	7.0E-18
10	regulation of neurogenesis (GO:0050767)	727	153	2.34	2.0E-17
11	regulation of ion transport (GO:0043269)	619	137	2.46	5.0E-17
12	cell morphogenesis involved in differentiation (GO:0000904)	487	117	2.67	9.0E-17

(Continues)

TABLE 5 (Continued)

GO biological process complete & number of genes in term			A1 *vs.* C1		
			Gene #	FE	p
13	cell morphogenesis involved in neuron differentiation (GO:0048667)	392	101	2.86	4.0E-16
14	neuron projection development (GO:0031175)	583	129	2.46	7.0E-16
15	regulation of cell development (GO:0060284)	841	164	2.17	1.0E-15
16	brain development (GO:0007420)	720	147	2.27	2.0E-15
17	animal organ morphogenesis (GO:0009887)	888	169	2.11	3.0E-15
18	regulation of neuron differentiation (GO:0045664)	605	130	2.39	5.0E-15
19	behavior (GO:0007610)	551	122	2.46	7.0E-15
20	neuron projection morphogenesis (GO:0048812)	429	104	2.69	7.0E-15

Pathway analyses using genes with hypermethylated CpGs was conducted using the Panther Classification System (www.pantherdb.org) released on 2016-12-28. Shading indicates unique pathways in A1:C1 hypermethylated or C1:A1 hypermethylated; note unique pathways were determined by comparing the lists of all GO terms with ≥ 2 FE (Supporting Information Supplemental File 9); not just those among the top 20.

For example, Fackler and colleagues, profiled 103 tumors using the HM27 BC and identified an "ER-classifier" set of 40 CpGs: 27 hypermethylated in ERpos and 13 hypermethylated in ERneg tumors.[12] We also observed greater methylation in the 30 ERpos tumors as compared with the 16 ERneg tumors from the 23 pairs, as well as a similar trend for the 35 of the 40 ER-classifier CpGs that are present on the HM450 BC (data not shown); however, the overall mean β was only slightly greater in the 30 ERpos tumors (Figure 1). Interestingly, the tumors profiled in Fackler et al[12] were all primary breast cancers, and when we restricted our comparisons to only primary tumors from the paired sets we observed an increase in the greater methylation of ERpos tumors. In contrast, restricting the analysis to only second tumors decreases the difference based on ER status. One interpretation of these results is that ERneg second tumors may have increased DNA methylation.

We previously reported that long-term treatment of MCF-7 cell cultures with Tamoxifen resulted in a significant increase in DNA methylation in an ERneg clone but not in an ERpos clone.[14] Therefore, we predicted that ERneg second breast tumors from women who received treatment with Tamoxifen for their ERpos

TABLE 6 Kaplan-Meier analysis of genes hypermethylated in ERpos first tumors as compared with nonrecurrent tumors. A selected pathway is shown for ERpos first tumors in women with ERpos second tumors (A1 vs NR and A1 vs C1) and for ERpos first tumors from women with ERneg second tumors (C1 vs A1 and C1 vs NR)

ERpos →ERpos Positive regulation of cell differentiation (GO:0051962)			**ERpos →ERneg** Cell-cell signaling (GO:0007267)								
Gene	**HR**	**p**	**Gene**	**HR**	**p**	**Gene**	**HR**	**p**	**Gene**	**HR**	**p**
AGTR1	**0.63**	**<1e-16**	**ACHE**	**0.79**	**3.00E-05**	**FGFR2**	**0.76**	**5.70E-07**	NRXN2	0.91	0.082
BDNF	**0.67**	**2.90E-07**	**ADRA1B**	**0.79**	**3.50E-05**	**FGFR3**	**0.86**	**0.0077**	**NTF3**	**0.8**	**3.90E-05**
BMP4	0.9	0.058	ADRA2C	1.11	0.0666	FJX1	0.98	0.65	PANX2	0.99	0.86
BMP7	**0.88**	**0.023**	ADRB1	0.88	0.1	**FKBP1B**	**0.76**	**6.30E-07**	**PCDHGA11**	**0.87**	**0.011**
C2orf28	**0.96**	**0.45**	**AES**	**0.88**	**0.025**	**FRAT1**	**0.66**	**4.00E-14**	PCDHGB7	0.89	0.14
CLCF1	**0.88**	**0.018**	AMOTL1	1.07	0.36	FZD1	0.95	0.34	***PDX1***	***1.24***	***9.00E-05***
DUOXA1	0.97	0.67	**AVP**	**0.87**	**0.011**	***FZD6***	***1.26***	***3.60E-05***	***PGF***	***1.14***	***0.022***
EDN1	**0.84**	**0.027**	**CACNA1C**	**0.58**	**9.60E-12**	FZD8	0.89	0.15	**PLCG2**	**0.72**	**4.40E-05**
EPHA3	**0.81**	**0.00011**	**CACNB2**	**0.71**	**1.10E-09**	***GAL***	***1.19***	***0.0021***	**PPFIA3**	**0.9**	**0.049**
FAM5B	**0.72**	**7.40E-09**	**CAV1**	**0.87**	**0.012**	GPR120	-	-	***PSMA7***	***1.46***	***1.10E-11***
FOXP1	**0.63**	**9.80E-09**	CDK16	-	-	**GRIA2**	**0.68**	**9.20E-07**	RIMS2	1.04	0.65
HLA-G	**0.83**	**0.00063**	**CHAT**	**0.83**	**0.00089**	**GRID1**	**0.83**	**0.016**	**SCN5A**	**0.79**	**1.60E-05**
NUMA1	**0.69**	**2.60E-11**	CHRNA7	1.08	0.19	**GRID2**	**0.76**	**8.40E-07**	**SEMA3B**	**0.72**	**1.80E-09**
PRKCI	***1.32***	***5.10E-07***	CHRNB2	1.09	0.28	**GRIN2C**	**0.85**	**0.0045**	SEZ6	1.14	0.097
SYT17	**0.6**	**1.70E-10**	CPE	0.96	0.47	***HAP1***	***1.13***	***0.025***	**SLC18A3**	**0.78**	**1.30E-05**
TCF4	1.01	0.92	**CRH**	**0.77**	**2.00E-06**	HCN2	0.93	0.19	**SLC6A3**	**0.75**	**1.20E-07**
ZBTB16	**0.61**	**<1e-16**	**CTF1**	**0.8**	**8.40E-05**	**HCRTR1**	0.75	1.60E-07	***SLITRK5***	***1.14***	***0.015***
			DKK3	1.11	0.059	HRH3	0.94	0.25	**SNPH**	**0.86**	**0.0077**
			DOC2B	0.9	0.07	**HTR7**	0.77	0.001	**SYN2**	**0.73**	**7.60E-09**
			DRD4	**0.79**	**1.90E-05**	**KCNQ2**	0.72	3.70E-09	**SYN3**	**0.83**	**0.00034**
			EFNA2	***1.38***	***5.40E-05***	**KIF5A**	0.7	8.00E-11	**SYT13**	**0.83**	**0.017**
			EFNA3	1.05	0.4	**LEF1**	0.72	4.80E-09	**SYT5**	**0.9**	**0.049**
			ETV5	0.97	0.54	***LYN***	***1.15***	***0.014***	**SYT7**	**0.79**	**0.0033**
			FAM123C	-	-	**MACF1**	0.66	1.13E-13	**TOLLIP**	**0.75**	**0.00026**
			FCHSD1	**0.6**	**1.10E-19**	**MAPK8IP2**	0.86	0.0082	UNC13A	0.99	0.86
			FGF10	0.98	0.76	**MYO5A**	0.72	2.40E-05	VANGL2	0.93	0.38
			FGF12	**0.85**	**0.036**	**NKD2**	0.79	0.0031	**WNT3**	**0.6**	**1.60E-10**
			FGF20	**0.77**	**3.20E-06**	NOTUM	1	0.97	WNT3A	-	-
			FGF8	0.98	0.76	**NPTX1**	0.79	1.60E-05	**WNT7A**		**5.70E-05**
			FGFR1	**0.75**	**0.00023**	**NPY1R**	0.68	1.80E-12			

Black bold text indicates genes in which **high** expression is associated with increased survival. ***Italicized red bold text*** indicates genes in which ***low*** expression is associated with increased survival. Missing data indicate genes not present in Kaplan-Meier plotter. HR = hazard ratio, p = p-value.

primary tumors would exhibit increased methylation. Stratifying the tumor pairs by the ER status of both primary and second tumor revealed distinct methylation patterns among the three groups. As predicted, ERneg second tumors (group C2) occurring in women with ERpos first tumors (group C1) exhibit a significant increase in DNA methylation. In contrast, and also as predicted, the second ERpos tumors (group A2) occurring in women with ERpos primary tumors (group A1) did not exhibit increased methylation. Group C2 tumors also had a greater number of hypermethylated CpGs in the body and intergenic regions as compared with C1 tumors, a pattern not observed in the comparisons of primary and second tumors within the groups of all ERpos tumors (A) or all ERneg tumors (B). This finding of increased DNA methylation in ERneg second tumors as compared with ERpos primary tumors supports our results in cell culture,[14] and suggests a mechanism that likely contributes to the development of ERneg second tumors in women who received treatment with antiestrogens.

The extent to which first tumors can provide information about the probability and timing of recurrence and metastasis is an area of intense study.[15] While time to breast recurrence was not significantly different among the three groups of tumor pairs, it was substantially lower in the ERneg B group (TTR: 34 months vs 84 and 82 months in groups A and C, respectively). A shorter latency to recurrence for ERneg tumors has been demonstrated in a multivariate model in which the ERneg primary tumors were also of higher grade and larger size ($\geq$ 20 mm),[27] and interestingly, four of the five ERneg tumors in our study were $\geq$ 20 mm. However, the shorter latency to recurrence

may also be related to whether the second tumor is a new primary or a true recurrence. Haffty, et al[28] reported a significantly shorter time to recurrence among 47 true recurrences as compared to 33 new primary breast tumors. In agreement, all of the tumors in group B were scored by the pathologist as true recurrences. In contrast, only one of the six tumors in group C and eight of the 12 tumors in group A were scored a true recurrences (Table 2 and Supporting Information Supplemental File 2). Comparing all 14 true recurrences with the nine new primary second tumors shows a trend for shorter TTR among true recurrences (Mann-Whitney $U = 36$; $P = 0.095$).

While, second tumors in groups A and C differ with respect to both ER status and designation as a new primary or true recurrence (A2: 12 ERpos tumors with 8 of 12 designated true recurrence; C2: 6 ERneg tumors with 5 of 6 designated new primary), they are similar in that they developed in women who received antiestrogen treatment (except for two subjects in group A who declined treatment; see Supporting Information Supplemental File 1). Given that the comparison of C1 with C2 tumors revealed a methylation profile distinct from the comparison of A1 and A2 tumors, we asked to what extent the primary tumors in groups A1 and C1 differed from each other, and from ERpos tumors from women who did not experience a recurrence (NR). We conducted fold-enrichment analyses with gene lists generated from dmCpGs and identified pathways that were both shared and unique to the comparisons of each of the ERpos primary groups (A1 and C1) with the NR group. As expected, the majority of pathways were shared. The only pathway uniquely hypermethylated in ERpos primary tumors from women with ERpos recurrences, "positive regulation of cell differentiation" (Table 5), has been found previously to contain aberrantly methylated genes identified as biomarkers of tumor subtype classification, tailored therapy, and survival in breast cancer.[29] A novel finding was identification of six pathways uniquely hypermethylated in ERpos primary tumors from women with ERneg second tumors: "nervous system development, cell-cell signaling, central nervous system development, regulation of ion transport, brain development, and behavior" (Table 5). Further analysis of the "cell-cell signaling" pathway (Table 6) shows that 78% of the 69 genes in the pathway are hypermethylated in ERpos primary C1 tumors, and of those 69 genes, high expression is associated with increased survival. It is interesting to note that recent literature has focused on the importance of differential expression of neurogenes in breast cancer.[30] Fernández-Noguiera et al examined six neurogenes that were differentially expressed among breast cancer subtypes and had expression correlated with survival and metastasis (HRH1, NRP2, ENFB1, NGFR, APP, and STX1A). Of those genes differentially expressed, four out of six had differential methylation in our ERpos primary tumors that recurred (HRH1, NRP2, EFNB1, and NGFR). The extent to which methylation is controlling expression of these genes would need to be further investigated.

Previous studies have revealed aberrant methylation of genes involved in transcriptional regulation, cell differentiation, apoptosis, invasion, and metastasis in breast cancer.[29,31] In the current study stratification by ER status of the second tumor identified new pathways uniquely methylated in a subset of ERpos primary tumors.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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